which may lead to inappropriate invasive investigation or overtreatment with antifungal agents. Previous studies have reported various risk factors for the false-positive results, including early childhood,[3] the development of chronic graft-versus-host disease (GVHD),[8] the passage of GM of food origin[9,10] and certain exoantigens from other fungal genera[11] or fungus-derived antibiotics.[12,13] However, little is known about the exact mechanism of false-positive reactions with these factors.

To clarify the cause of false-positive results, we retrospectively analysed the incidence and risk factors for false-positive GM antigenaemia in allogeneic HSCT recipients.

Patients and methods

Study population

GM ELISA became available at the University of Tokyo Hospital as a routine diagnostic test in February 2000. During a 5 year period (February 2000 to May 2005), 163 consecutive adult patients (>16 years old) underwent allogeneic HSCT at the University of Tokyo Hospital. The medical records of 157 patients who had at least two GM ELISA tests after HSCT were available for a retrospective analysis of positive GM antigenaemia. The median follow-up was 519 days (range, 15–2090 days) after HSCT. The patient characteristics are shown in Table 1. Acute leukaemia in first remission, chronic myelogenous leukaemia in first chronic phase, myelodysplastic syndrome with refractory anaemia or refractory anaemia with ringed sideroblasts, and aplastic anaemia were defined as low-risk diseases, whereas others were considered high-risk diseases. Donors other than human leucocyte antigen (HLA)-matched sibling donors were defined as alternative donors.

Transplantation procedure

The conventional preparative regimen for leukaemia/lymphoma was mainly performed with either cyclophosphamide/total body irradiation (TBI)-based regimens or busulfan/cyclophosphamide-based regimens. In cyclophosphamide/TBI-based regimens, the dose of cyclophosphamide was decreased and etoposide was added instead in patients with impaired cardiac function. Fludarabine-based regimens were used as reduced-intensity regimens for elderly or clinically infirm patients.[14] Cyclosporin A or tacrolimus was administered combined with short-term methotrexate for prophylaxis against GVHD. Alemtuzumab was added for patients who received a graft from an HLA-mismatched donor.[15] Methyl-prednisolone or prednisolone at 1 or 2 mg/kg was added for patients who developed grade II–IV acute GVHD, whereas prednisolone at 0.5 mg/kg or more was added for patients who developed extensive chronic GVHD. Prophylaxis against bacterial, herpes simplex virus and *Pneumocystis jirovecii* infections consisted of tosufloxacin, aciclovir and sulfamethoxazole/trimethoprim.

Antigen detection

GM assay was performed at least every other week after HSCT until discharge from the hospital in the majority of patients. In the outpatient setting, the monitoring of GM was continued at each visit in patients who were receiving immunosuppressive therapy, at the discretion of attending physicians. Circulating *Aspergillus* GM was detected using a sandwich immunocapture ELISA (Platelia Aspergillus, Bio-Rad, Marnes-la-Coquette, France) using a rat anti-GM monoclonal antibody.[2] The technique was performed as recommended by the manufacturer. The optical absorbance of specimens and controls was determined with a spectrophotometer set at 450 and 620 nm wavelengths. The optical density (OD) index for each sample was calculated by dividing the optical absorbance of the clinical sample by that of the threshold control. Two consecutive serum samples with an OD index of 0.6 or more were considered positive.[16]

Table 1. Patients' characteristics

Characteristic	Total patients
Sex (male/female)	105/52
Age, median (range)	41 (16–66)
Underlying disease	
acute leukaemia	70
CML	26
MDS	22
SAA	8
other	31
Graft source	
PBSC	69
BM	88
Donor type	
matched sibling	58
mismatched related	15
unrelated	84
Preparative regimen	
Cy (Etp)/TBI-based regimens	105
Bu/Cy-based regimens	15
ATG-based regimens for SAA	5
Flu-based RIC	32
GVHD prophylaxis	
CsA+MTX	115
tacrolimus+MTX	18
alemtuzumab+CsA+MTX	24
Acute GVHD	
grade 0–I	87
grade II–IV	69
Chronic GVHD	
extensive	57
limited	30
none	47

CML, chronic myelogenous leukaemia; MDS, myelodysplastic syndrome; SAA, severe aplastic anaemia; PBSC, peripheral blood stem cell; BM, bone marrow; Cy, cyclophosphamide; Etp, etoposide; TBI, total body irradiation; Bu, busulfan; ATG, antithymocyte globulin; Flu, fludarabine; RIC, reduced intensity conditioning; GVHD, graft-versus-host disease; CsA, cyclosporin A; MTX, methotrexate.

Antifungal prophylaxis and treatment for IA

As antifungal prophylaxis, fluconazole at 200 mg was principally given daily from day −14 until the end of immunosuppressive therapy. For patients with a history of IA, intravenous micafungin at 150–300 mg or oral itraconazole at 200 mg was administered instead. All patients were isolated in high-efficiency particulate air (HEPA)-filtered rooms from the start of the conditioning regimen to engraftment. Febrile neutropenia was treated with broad-spectrum antibiotics in accordance with

the published guidelines.[17] Antifungal treatment was started when febrile neutropenia persisted for at least 3–4 days or when IA was confirmed or suspected with clinical or radiological signs.

Diagnosis procedures and definitions

Diagnostic procedures included routine cultures of urine and stools, repeated cultures of blood and sputum, weekly chest X-ray, computed tomography (CT) scan of the chest and nasal sinus and, when possible, bronchoscopic examinations and open biopsy. CT scans were principally obtained for patients with (i) clinical signs and/or symptoms suggestive of IA, (ii) persistent or recurrent febrile neutropenia while on broad-spectrum antibiotic treatment, (iii) infiltrates or nodules on chest X-ray or (iv) positive GM antigenaemia. In patients with clinical suspicion of IA, bronchoscopy with bronchoalveolar lavage (BAL) and/or tissue biopsy were also performed whenever feasible. A diagnosis of IA was classified as proven or probable on the basis of the EORTC/MSG definitions.[7] True-positive GM antigenaemia was defined as two consecutive positive results with the established diagnosis of proven or probable IA. Positive GM antigenaemia in episodes that did not fulfil the diagnostic criteria for proven or probable IA was considered as inconclusive-positive if (i) sufficient examinations including chest and/or sinus CT scans were not performed despite the presence of compatible clinical signs and symptoms of IA or (ii) the possibility that the radiological abnormalities on the CT scans were due to IA could not be denied because of the use of empirical antifungal therapy or targeted antifungal therapy for other definite fungal infections at the time of positive antigenaemia. Alternatively, positive antigenaemia without sufficient evidence to diagnose proven or probable IA was considered as false-positive in any of the following: (i) no radiological abnormalities were detected on chest and/or sinus CT scans; (ii) non-specific abnormalities on CT scans improved without any antifungal treatments for IA or culture results for specimens from radiologically abnormal sites including BAL fluid or sinus aspirate were negative; or (iii) CT scans were not performed because of no evidence meeting clinical minor criteria in EORTC/MSG definitions. Positive antigenaemia recurring after the negative conversion at least 3 months apart was considered an independent episode.

Statistical analysis

Sensitivity, specificity and positive predictive value (PPV) of the GM ELISA were calculated on the basis of the clinical diagnosis of proven or probable IA. The cumulative incidences of positive GM antigenaemia and IA were evaluated using Gray's method, considering death without each event as a competing risk.[18] Probabilities in two groups were compared using Fisher's exact test. P values of less than 0.05 were considered statistically significant.

Results

Transplantation outcome

One hundred and fifty-seven allogeneic transplant recipients were included in the study. Neutrophil engraftment was obtained at a median of 17 days (9–43 days) after HSCT in 156 patients. Grade II–IV acute GVHD was observed in 69 and chronic GVHD in 87 of 134 who survived more than 100 days. Seventy patients died, the causes being haematological relapse (n = 29), infection (n = 14), non-infectious pulmonary complications (n = 15), gastrointestinal bleeding (n = 6) or other reasons (n = 6).

Diagnosis of IA

Twenty-five patients developed proven (n = 8) or probable (n = 17) IA at a median of 204 days (range 21–1527 days) after HSCT, with a 1 year cumulative incidence of 12.9% (Figure 1). Twenty-two patients (88%) had pulmonary disease, two of whom showed dissemination. The remaining three had tracheobronchitis, sinusitis and gastrointestinal involvement, respectively. IA was the direct cause of death in five patients. Positive GM antigenaemia was observed in 22 patients with proven or probable IA. In a patient-based analysis, the sensitivity and specificity of the test were 88% (22 of 25) and 79% (104 of 132), respectively.

Episodes with positive GM antigenaemia

A total of 3296 serum samples were analysed from 157 patients (mean, 21 samples/patient; range, 2–109 samples/patient). Overall, 50 patients (31.9%) developed positive GM antigenaemia at a median of 107 days (range 12–1193 days) after HSCT, with a 1 year cumulative incidence of 32.2% (Figure 1). Five patients had second positive episodes at a median interval of 358 days (range 119–1103 days) between the first and second episodes. Four positive episodes occurred in one patient.

A total of 58 positive episodes of the 50 patients were therefore analysed (Table 2). Twenty-two episodes were diagnosed true-positive based on the diagnosis of proven or probable IA. In these patients, the microbiological criterion was fulfilled with pathological findings and/or culture results in 10 and GM antigen test in 12. Seven were considered inconclusive-positive. In all the seven episodes, we could not conclude whether the abnormalities on CT scans were attributed to IA or not, because antifungal agents were administered empirically (n = 5) or for the treatment of documented candidiasis (n = 2) at the time of positive GM antigenaemia.

Twenty-nine episodes were considered false-positive, in all of which piperacillin/tazobactam or amoxicillin/clavulanate was not given at the time of positive GM antigenaemia. *Penicillium* and

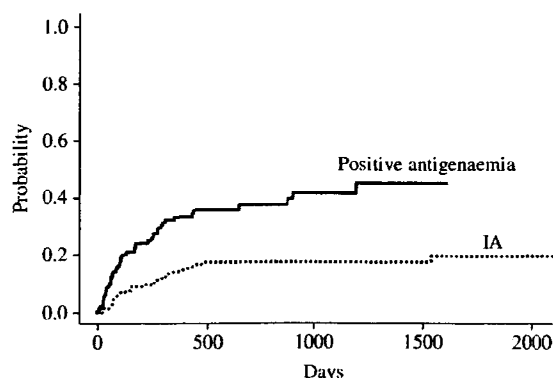

Figure 1. Cumulative incidences of IA and positive GM antigenaemia after HSCT.

Table 2. Incidence of false-positive GM antigenaemia

	Total episodes	Episodes before day 100	Episodes after day 100
True-positive	22	8	14
False-positive	29	15	14
Inconclusive-positive	7	1	6
Total	58	24	34
False-positive rate (%)	50	62.5	41.2

Paecilomyces were not detected in these false-positive episodes. At the time of false-positive antigenaemia, antifungal prophylaxis was given in 23 episodes (fluconazole, 20; itraconazole, 3), and no antifungal agents at all in the remaining 6. Empirical or targeted antifungal therapy was not performed in these episodes. CT scans were performed in 22 episodes, in which no radiological abnormalities were seen in 12, and non-specific abnormalities in the remaining 10 were caused by *P. jirovecii* infections ($n = 2$), bacterial infections ($n = 2$), pulmonary involvement of cancer ($n = 1$), heart failure ($n = 1$), bronchiolitis obliterans organizing pneumonia (BOOP) ($n = 1$) or unknown aetiology ($n = 3$). All three unexplained radiological abnormalities disappeared spontaneously.

Incidence and risk factors for false-positive GM antigenaemia

Of the 58 positive episodes, 29 satisfied the criteria of false-positive antigenaemia, with a false-positive rate of 50% (Table 2). During the first 100 days after HSCT, 15 of 24 positive episodes were considered false-positive, with a false-positive rate of 62.5% (Table 2). PPV was 33.3% or 37.5% when we included the inconclusive episode into the false-positive group or the true-positive group, respectively, in the 24 positive episodes. PPV was 55.6% or 66.7% even in nine with grade II–IV acute GVHD at the time of positive GM antigenaemia. In contrast, 14 of 34 positive episodes beyond 100 days were considered false-positive, with a rate of 41.2%, and PPV was 41.2% or 58.8%. False-positive antigenaemia occurred more frequently and therefore PPV was lower during the first 100 days.

There were no significant parameters that increased the incidence of false-positive GM antigenaemia over the entire period and during the first 100 days (Tables 3 and 4). The incidence was rather decreased in the presence of active GVHD (at any grade) and liver GVHD over the entire period, and grade II–IV GVHD, grade III–IV GVHD and liver GVHD during the first 100 days. In contrast, gastrointestinal chronic GVHD was identified as the only significant risk factor for increased false-positive GM antigenaemia beyond 100 days (Table 5). Twenty of the 30 episodes of positive GM antigenaemia without gastrointestinal chronic GVHD were true-positive, whereas all 4 positive GM antigenaemia episodes in patients with gastrointestinal chronic GVHD were false-positive (PPV 66.7% versus 0%, $P = 0.02$). Gastrointestinal chronic GVHD in these patients was associated with more than 500 mL of diarrhoea at the time of positive GM antigenaemia, the diagnosis of which was pathologically confirmed with colon biopsy.

Table 3. Risk factors for false-positive GM antigenaemia after HSCT

Factors	False-positive	Others	*P* value
Age			
>40 years	18	18	1.00
≤40 years	11	11	
Disease risk			
standard risk	7	5	0.75
high risk	22	24	
Graft source			
bone marrow	16	15	0.79
peripheral blood	13	14	
Donor type			
matched sibling donor	9	9	1.00
alternative donor	20	20	
Neutrophil count			
<500 cells/μL	2	3	1.00
≥500 cells/μL	27	26	
Active GVHD on positive GM			
yes	13	23	0.01
no	16	6	
Gastrointestinal GVHD on positive GM			
yes	6	3	0.47
no	23	26	
Liver GVHD on positive GM			
yes	5	14	0.02
no	24	15	
Skin GVHD on positive GM			
yes	137	20	0.41
no	105	50	
Prednisolone on positive GM (1)			
≥0.5 mg/kg	137	20	0.41
<0.5 mg/kg	105	50	
Prednisolone on positive GM (2)			
≥1.0 mg/kg	137	20	1.00
<1.0 mg/kg	105	50	

In thorough examinations for aspergillosis, no radiological abnormalities were seen in two patients, non-specific abnormalities on CT scan were observed but spontaneously disappeared without clinical symptoms suggestive of IA in one, and radiological findings compatible with BOOP were observed and promptly improved with systemic corticosteroids in one. There was another false-positive episode probably associated with gastrointestinal chronic GVHD, which was included in the 'no gastrointestinal chronic GVHD' group because GVHD was absent at the detection of positive GM antigenaemia, but gastrointestinal chronic GVHD developed soon thereafter. Among these five episodes, the GM levels became normal with the improvement of gastrointestinal chronic GVHD in four, whereas GM antigen monitoring was discontinued because of death from haematological relapse in the remaining one.

Table 4. Risk factors for false-positive GM antigenaemia before day 100

Factors	False-positive	Others	*P* value
Neutrophil count			
<500	1	1	1.00
≥500	14	8	
Active GVHD on positive GM			
yes	4	6	0.09
no	11	3	
Grade II–IV acute GVHD on positive GM			
yes	3	6	0.04
no	12	3	
Grade III–IV acute GVHD on positive GM			
yes	0	3	0.04
no	15	6	
Gastrointestinal GVHD on positive GM			
yes	2	3	0.33
no	13	6	
Liver GVHD on positive GM			
yes	0	5	<0.01
no	15	4	
Skin GVHD on positive GM			
yes	3	4	0.36
no	12	5	
Prednisolone on positive GM (1)			
≥0.5 mg/kg	9	5	1.00
<0.5 mg/kg	6	4	
Prednisolone on positive GM (2)			
≥1.0 mg/kg	5	4	0.68
<1.0 mg/kg	10	5	

Table 5. Risk factors for false-positive GM antigenaemia after day 100

Factors	False-positive	Others	*P* value
Active GVHD on positive GM			
yes	9	17	0.23
no	5	3	
Extensive chronic GVHD on positive GM			
yes	7	10	1.00
no	7	10	
Gastrointestinal GVHD on positive GM			
yes	4	0	0.02
no	10	20	
Liver GVHD on positive GM			
yes	5	9	0.73
no	9	11	
Skin GVHD on positive GM			
yes	5	8	1.00
no	9	12	
Oral GVHD on positive GM			
yes	3	6	0.70
no	11	14	
Prednisolone on positive GM (1)			
≥0.5 mg/kg	3	3	0.67
<0.5 mg/kg	11	17	
Prednisolone on positive GM (2)			
≥1.0 mg/kg	2	2	1.00
<1.0 mg/kg	12	18	

Discussion

This study demonstrated that the sensitivity of the GM ELISA test was 88% in patient-based analysis and PPV was 38% to 50% in episode-based analysis, which were comparable with those in previous reports.[3–6] However, false-positive GM antigenaemia frequently occurred during the first 100 days after HSCT, and PPV was lower even among patients with grade II–IV acute GVHD, in whom the pre-test probability of IA was considered to be much higher than patients without acute GVHD.

A significant correlation between the occurrence of false-positive GM antigenaemia and the presence of gastrointestinal chronic GVHD was observed in this study. GM ELISA results were false-positive in all four episodes with gastrointestinal chronic GVHD at the time of positive GM antigenaemia, and there was another false-positive episode in which GVHD was absent at the detection of positive GM antigenaemia, but gastrointestinal chronic GVHD developed soon thereafter. During these episodes, piperacillin/tazobactam or amoxicillin/clavulanate was not given, and occult infections by some fungi reacting with GM ELISA were not detected, both of which were previously reported as important risk factors for false-positive GM antigenaemia.[11–13] Meanwhile, our results were consistent with the conclusions of other studies that concurrent mucositis in HSCT recipients or immature intestinal mucosa in neonates allows the translocation of GM contained in foods, leading to frequent false-positive GM antigenaemia.[3–5,8–10] These findings suggested the possibility that passage of dietary GM into the blood from the disrupted intestinal mucosal barrier might result in false-positive antigenaemia in patients with gastrointestinal chronic GVHD.

In contrast, the development of gastrointestinal acute GVHD was not significantly associated with the occurrence of false-positive GM antigenaemia in our series. This was probably because the overall false-positive rate during the first 100 days after HSCT was higher than that beyond 100 days. Mucosal damage due to the high-dose chemotherapy or TBI in the conditioning regimen might be the cause of frequent false-positive GM antigenaemia early after HSCT.[5]

Pfeiffer *et al.*[19] recently showed the significant heterogeneity of GM test performance among patients with different prevalences of IA. They demonstrated that GM assay was more useful in immunocompromised high-risk populations such as HSCT recipients or patients with haematological malignancy than in solid-organ transplant recipients. Although emphasizing the utility of GM assay only when there is a high pre-test probability of IA, they also addressed the need for further investigations of the reasons for the heterogeneity. Prior antifungal therapy and false-positive results are possible explanations for the heterogeneity, and our findings may contribute to the effective use of the assay. However, our study is a retrospective evaluation and therefore there are some potential weaknesses. In this study,

regular screening of GM antigen was not rigorously performed, but on an on-demand basis. This is in contrast to the previous studies in which GM antigenaemia was evaluated more intensively.[3-5] This fact might have affected the diagnostic performance of this assay, but the high cost of this test precluded such intensive monitoring in daily practice. In addition, we should mention that this study might lack enough statistical power to detect the other risk factors for false-positive antigenaemia than gastrointestinal chronic GVHD because of the small number of patients with positive antigenaemia. Also, the small number of patients with positive antigenaemia precludes multivariate analysis, which might be another reason for failing to find the possible impact of the other risk factors. The other major limitation is that GM antigenaemia itself was included in the microbiological criteria, which might have precluded the evaluation of true performance of this assay. In this study, however, the number of patients diagnosed with IA falls from 22 to 10, if the GM results are excluded from the criteria, which seemed too small for the statistical analysis. Therefore, we used the original EORTC/MSG definitions that include GM antigenaemia in the microbiological criteria.

In conclusion, frequent false-positive GM antigenaemia was observed in allo-HSCT recipients during the first 100 days after transplantation or in those with gastrointestinal chronic GVHD, leading to a decreased PPV of the GM ELISA test. Therefore, GM antigenaemia results should be considered cautiously in these patients in conjunction with other diagnostic procedures including CT scans.

Acknowledgements

We thank all the clinicians who have assisted with the provision of data for this project.

Funding

This research was supported by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare.

Transparency declarations

None to declare.

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Donor-derived thymic-dependent T cells cause chronic graft-versus-host disease

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Chronic graft-versus-host disease (GVHD) is the most common cause of poor long-term outcomes after allogeneic bone marrow transplantation (BMT), but the pathophysiology of chronic GVHD still remains poorly understood. We tested the hypothesis that the impaired thymic negative selection of the recipients will permit the emergence of pathogenic T cells that cause chronic GVHD. Lethally irradiated C3H/HeN (H-2^k) recipients were reconstituted with T-cell–depleted bone marrow cells from major histocompatibility complex [MHC] class II–deficient (*H2-Ab1$^{-/-}$*) B6 (H-2^b) mice. These mice developed diseases that showed all of the clinical and histopathological features of human chronic GVHD. Thymectomy prevented chronic GVHD, thus confirming the causal association of the thymus. CD4$^+$ T cells isolated from chronic GVHD mice were primarily donor reactive, and adoptive transfer of CD4$^+$ T cells generated in these mice caused chronic GVHD in C3H/HeN mice in the presence of B6-derived antigen-presenting cells. Our results demonstrate for the first time that T cells that escape from negative thymic selection could cause chronic GVHD after allogeneic BMT. These results also suggest that self-reactivity of donor T cells plays a role in this chronic GVHD, and improvement in the thymic function may have a potential to decrease chronic GVHD. (Blood. 2007;109:1756-1764)

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Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is considered to be an effective therapy for a variety of malignant and nonmalignant diseases of the lymphohematopoietic system. Chronic graft-versus-host disease (GVHD) is a complex multiorgan disorder with features of autoimmunity and immunodeficiency.[1,2] Chronic GVHD remains the major cause of late death and morbidity after allogeneic HSCT.[3,4] Unfortunately, despite success in the prevention of acute GVHD by the introduction of cyclosporine, the incidence of chronic GVHD has not decreased.[5-10] Its incidence is increasing with the greater use of unrelated or HLA-mismatched donors, older donors and recipients, donor lymphocyte infusions, and peripheral blood stem cell transplantation. These observations suggest that chronic GVHD is not simply a continuation of acute GVHD, and therefore novel strategies are required to prevent chronic GVHD. Although progress has been made in understanding the pathophysiology of acute GVHD, the basic pathophysiology of chronic GVHD remains poorly understood.

The existing experimental models of chronic GVHD have certain limitations. For example, the most commonly used mouse model of chronic GVHD is induced by the injection of parental (P) cells into nonirradiated F1 recipients.[11] This model mimics systemic lupus erythematosus (SLE) with B-cell expansion, splenomegaly, autoantibody production, and glomerulonephritis as a result of a cognate interaction between donor CD4$^+$ T cells and host B cells.[12,13] Another murine model, B10.D2 (H-2^d) into an irradiated BALB/c (H-2^d) model, mimics human chronic GVHD with fibrosis of the skin and exocrine glands and hepatic and pulmonary involvement.[14,15] In these models, mature T cells infused are responsible for the induction of chronic GVHD. A recently developed chronic GVHD model that was made by the transplantation of DBA/2 spleen cells into sublethally irradiated BALB/c mice also leads mice to develop SLE-like features that require both donor CD4$^+$ T cells and B cells.[16] In this model, host thymus is not required for the induction of chronic GVHD.

T-cell repopulation following HSCT results from both thymic-dependent and thymic-independent pathways.[17] It is now obvious that the thymic-independent peripheral expansion of mature T cells is responsible for the development of acute GVHD because T-cell depletion (TCD) of the donor bone marrow (BM) reduces rates of acute GVHD both in mice and humans.[18,19] The infusion of mature donor CD4$^+$ T cells is also responsible for chronic GVHD in P → F1, DBA/2 → BALB/c, and B10.D2 → BALB/c models.[11,15,16] However, it has been postulated that donor-derived T cells generated from hematopoietic stem cells via the recipient's thymus could also cause chronic GVHD.[20-22] While it has been clear that ex vivo TCD from the donor BM reduces the incidence of acute GVHD,[18,19] the effect of TCD on chronic GVHD has been less well delineated. The first randomized study comparing TCD–BM transplantation (BMT) with T-cell replete BMT found that TCD of the donor BM reduces rates of acute GVHD but not chronic GVHD,[23] thus supporting the hypothesis that thymic-dependent T cells play a role in mediating chronic GVHD.

Within the thymus, T cells undergo both positive and negative selection, thus resulting in the elimination of self-reactive cells.

Submitted August 30, 2006; accepted September 29, 2006. Prepublished online as *Blood* First Edition Paper, October 10, 2006; DOI 10.1182/blood-2006-08-042853.

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Positive selection is mediated by the thymic cortical epithelium, while negative selection via clonal deletion is mediated mainly by thymic dendritic cells (DCs).[24] Recent evidence clearly demonstrates that thymopoiesis continues even in adult recipients after allogeneic BMT.[25,26] The thymus is damaged by prior chemotherapy, conditioning regimen, acute GVHD, and age-related atrophy.[22,27] Experimental data indicate that such thymic damage results in a loss of thymic negative selection.[21,28-30]

We therefore hypothesized that chronic GVHD could thus be the result of autoreactive T cells that escape negative selection in the damaged thymus. In fact, the incidence of chronic GVHD is lower in pediatric patients, whose thymic function is better than in adults.[31] We tested the hypothesis that an impaired negative selection due to the loss of major histocompatibility complex (MHC) class II expression in thymic DCs causes chronic GVHD.[32] We found that these mice developed lethal disease similar to human chronic GVHD after allogeneic BMT.

Materials and methods

Mice

Female C57BL/6 (B6: H-2^b), C3H/HeN (C3H: H-2^k), and BALB/c (H-2^d) mice were purchased from Charles River Japan (Yokohama, Japan). B6-background MHC class II–deficient *H2-Ab1*$^{-/-}$ mice (B6.129-*Abb*^{tm1} N12)[33] were from Taconic (Germantown, NY). The age range of the mice was from 8 to 16 weeks. The mice were maintained in a specific pathogen-free environment and received normal chow and hyperchlorinated drinking water for the first 3 weeks after transplantation. All experiments were performed under the auspices of the Institutional Animal Care and Research Advisory Committee at the Department of Animal Resources at Okayama University and Kyushu University.

BMT

C3H mice were exposed to 13 Gy total body irradiation (TBI: x-ray) split into 2 doses and then injected intravenously with 5×10^6 TCD-BM cells from wild-type B6 (WT) or *H2-Ab1*$^{-/-}$ B6 mice on day 0. BALB/c mice were exposed to 9.5 Gy TBI. TCD was performed using CD90 microbeads and the AutoMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). In some experiments, C3H mice were thymectomized prior to BMT as described.[32] Hybridomas secreting anti-CD8 monoclonal antibodies (mAbs) and anti-CD25 mAbs were obtained from American Type Culture Collection (Manassas, VA). For in vivo depletion of CD8$^+$ cells, mice were injected intraperitoneally with 2 mg anti-CD8 mAb on day 7 after BMT and 1 mg every week thereafter. For CD25 depletion, mice were injected intravenously with 0.5 mg anti-CD25 mAb on day 0 and every 10 days thereafter as described.[34]

Assessment of GVHD

The survival after BMT was monitored daily, and weight changes were assessed weekly. The degree of clinically acute GVHD was assessed weekly by a scoring system that sums changes in 5 clinical parameters: weight loss, posture, activity, fur texture, and skin integrity (maximum index, 10) as described.[35] Samples of skin, liver, and intestine were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned, slide mounted, and stained with hematoxylin and eosin. Histological images were captured using an Olympus BH2 microscope (Olympus, Tokyo, Japan) with a Nikon DS-5M color digital camera (Nikon, Tokyo, Japan), controlled by Nikon ATC-2U software version 1.5. An Olympus 10×/20 ocular lens and a 10×/0.17 numerical aperture (NA) (Figures 2, 5) or a 20×/0.46 NA (Figure 3F) objective lens were used. Images were cropped using Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA) and were composed using Adobe Illustrator 10. The severity of chronic GVHD of the skin was assessed by a scoring system that incorporates 5 parameters: epidermis atrophy, increased collagen density in dermis, fat atrophy, inflammation, and follicle atrophy.[36] The slides were graded from 0 to 2 for each parameter, and pathologic scores were subsequently generated by the summation of the 5 criteria scores (maximum index, 10). Chronic GVHD was defined as sclerodermatous skin change and/or one of the following pathologic features: skin sclerotic change, ductopenia/portal fibrosis in the liver, or destruction/fibrosis of the salivary glands. Acute GVHD was defined as the absence of chronic GVHD manifestations but the presence of villous atrophy with crypt cell apoptosis in the intestine.

Adoptive transfer experiments

Splenocytes were isolated from the recipient mice 6 to 11 weeks after TCD-BMT. CD4$^+$ T cells were negatively selected from splenocytes by depleting CD8$^+$, DX5$^+$, CD11b$^+$, Ter-119$^+$, and B220$^+$ cells using the AutoMACS system. Purity of the CD4$^+$ T-cell subset was more than 85%, and contamination of CD8$^+$ cells was less than 3%. A total of 1×10^7 CD4$^+$ T cells from either [WT → C3H] or [*H2-Ab1*$^{-/-}$ → C3H] mice along with 5×10^6 TCD-BM cells from B6 or C3H mice were injected intravenously into C3H mice following 13 Gy TBI.

[B6 → C3H] and [C3H → C3H] chimeras were created by reconstituting lethally irradiated C3H mice with 5×10^6 TCD-BM cells from B6 and C3H mice, respectively, as described.[37] Four months later, [WT → C3H] or [*H2-Ab1*$^{-/-}$ → C3H] CD4$^+$ T cells were adoptively transferred to these chimeras following 8 Gy TBI.

Cell culture

Thymic DCs were isolated as described.[38] BM-derived DCs were generated by culturing BM cells in the presence of 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) and 20 ng/mL interleukin 4 (IL-4) (Peprotech, London, United Kingdom) for 4 days.[39] Splenic CD4$^+$ T cells were cultured with irradiated (20 Gy) DCs. Seventy-two hours after the initiation of culture, proliferation was determined by a thymidine uptake assay as described.[40]

Flow cytometric analysis

The mAbs used were FITC-, PE-, or allophycocyanin-conjugated anti-mouse TCRβ, CD4, CD8α, CD25, H-2K^b, H2-K^k, I-A^b (BD Pharmingen, San Diego, CA), and Foxp3 (eBioscience, San Diego, CA). The cells were stained and analyzed as described.[40] Dead cells were determined as 7-amino-actinomycin D (BD Pharmingen)–positive cells. At least 5000 live events were acquired for the analysis. For carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling, CD4$^+$ T cells negatively selected from the recipient mice were stained in PBS with 1 μM CFSE purchased from Molecular Probes (Eugene, OR).[37] These CFSE-labeled cells were stimulated with irradiated splenocytes in culture. Four days later, cell division was assessed as dilution of CFSE in CD4$^+$ T cells.

Statistical analysis

The survival curves were plotted using Kaplan-Meier estimates. The Mann-Whitney *U* test was used for the statistical analysis of the in vitro data and the pathology scores, while the Mantel-Cox log-rank test was used to compare survival curves. *P* values less than .05 were considered statistically significant.

Results

Allogeneic TCD-BMT from MHC class II–deficient donors caused chronic GVHD

Allogeneic transplantation of TCD-BM does not induce acute or chronic GVHD in mice. This may be due to a near normal thymic function after TBI in mice. We first examined whether impaired thymic negative selection could allow the emergence of donor- or

host-reactive T cells after allogeneic TCD-BMT. Lethally irradiated C3H (H-2^k) mice were injected with 5×10^6 TCD-BM cells from either WT or *H2-Abl*$^{-/-}$ B6 (H-2^b) donors. After TCD-BMT from *H2-Abl*$^{-/-}$ B6 donors, MHC class II molecules were expressed on the radioresistant thymic epithelium that supports positive selection but not on the radiosensitive hematopoietic elements responsible for negative selection.[32] A flow cytometric analysis of the thymus 4 weeks after allogeneic BMT from *H2-Abl*$^{-/-}$ B6 donors confirmed the replacement of host thymic DCs by donor-derived DCs as previously shown[32,41]; more than 98% of DCs were MHC class II negative. This analysis also demonstrated the emergence of CD4 single-positive thymocytes in both [*H2-Abl*$^{-/-}$ → C3H] mice and [WT → C3H] mice (data not shown), thus confirming the preservation of the ability of the thymus to perform efficient positive selection after TCD-BMT as previously shown.[32,41] An analysis of the donor cell chimerism in the spleen 6 weeks after transplantation showed that 97.9% ± 0.3% and 96.4% ± 0.3% were donor derived in [*H2-Abl*$^{-/-}$ → C3H] mice and [WT → C3H] mice, respectively, thus confirming complete donor cell engraftment.

Interestingly, the [*H2-Abl*$^{-/-}$ → C3H] mice began to lose weight 6 weeks after BMT. Weight loss was significantly greater in the [*H2-Abl*$^{-/-}$ → C3H] mice than in the [WT → C3H] mice at 7 weeks after BMT and thereafter (Figure 1A). This systemic illness was lethal so that only 16% survived on day 100 after BMT (Figure 1B, $P < .001$). Similar results were obtained when BALB/c

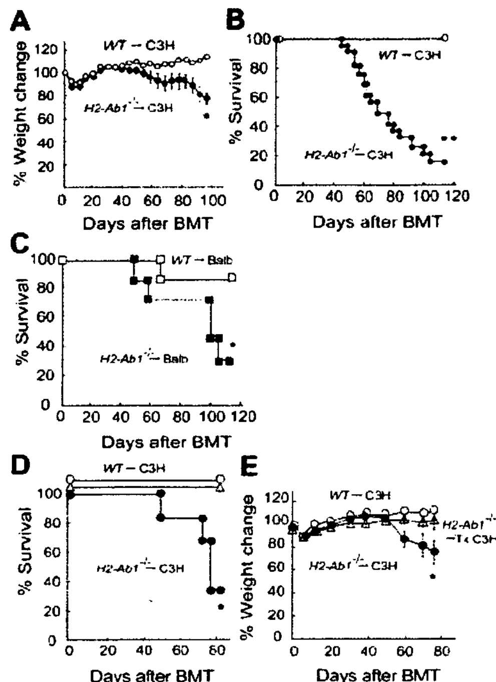

Figure 1. Thymic-dependent GVHD after allogeneic TCD-BMT from MHC class II–deficient donors. C3H mice were irradiated and underwent transplantation with TCD-BM from either control WT or *H2-Ab1*$^{-/-}$ B6 donors. Weight changes as the mean ± SE (A) and survivals (B) after BMT are shown. Data from 3 similar experiments are combined. ○, [WT → C3H], n = 22; ●, [*H2-Ab1*$^{-/-}$ → C3H], n = 27. (C) Lethally irradiated BALB/c mice underwent transplantation with TCD-BM from either control WT or *H2-Ab1*$^{-/-}$ B6 donors. The survivals after BMT are shown. □, [WT → BALB/c], n = 6; ■, [*H2-Ab1*$^{-/-}$ → BALB/c], n = 7. (D-E) C3H mice were thymectomized (Tx) and underwent transplantation with TCD-BM from *H2-Ab1*$^{-/-}$ B6 donors following 13 Gy TBI (△, n = 4). Nonthymectomized C3H mice also underwent transplantation with TCD-BM from WT (○, n = 4) and *H2-Ab1*$^{-/-}$ (●, n = 6) B6 donors following TBI. Survivals (D) and weight changes as the mean ± SE (E) are shown. *$P < .05$, **$P < .001$.

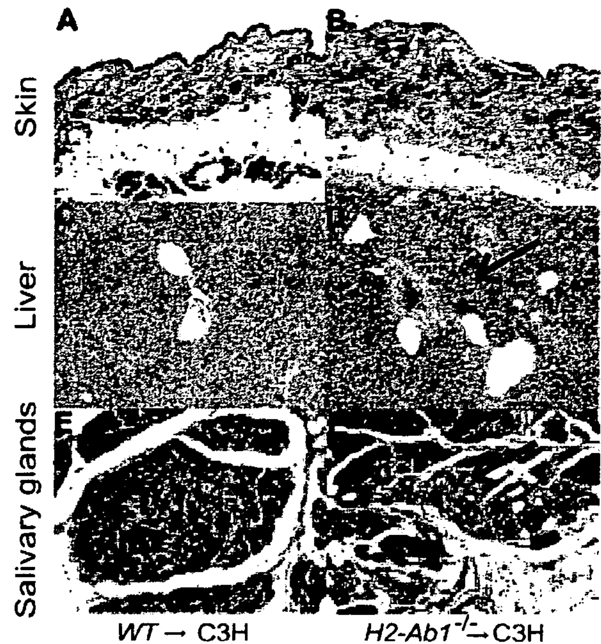

Figure 2. Histologic analysis of [*H2-Ab1*$^{-/-}$ → C3H] mice showed pathologic features similar to human chronic GVHD. The histologic findings of the skin (A-B), liver (C-D), and salivary glands (E-F) from [WT → C3H] mice and [*H2-Ab1*$^{-/-}$ → C3H] mice are shown. Sclerodermatous skin changes, such as epidermal atrophy, fat loss, follicular dropout, and dermal thickness (B); bile duct loss and fibrosis in the portal area and mild periportal mononuclear infiltrates in the liver (D, arrow); and lymphocyte inflammation, fibrosis, and atrophy of acinar tissue in the salivary glands (F, arrow) were observed in [*H2-Ab1*$^{-/-}$ → C3H] mice (original magnification, × 100).

(H-2^d) mice underwent transplantation with TCD-BM from *H2-Abl*$^{-/-}$ B6 donors. Seventy-one percent of the [*H2-Abl*$^{-/-}$ → BALB/c] mice died from GVHD by day 100 after BMT (Figure 1C).

We then determined whether the thymus played a causative role in the development of GVHD. C3H mice were thymectomized prior to BMT and then underwent transplantation with TCD-BM cells from *H2-Abl*$^{-/-}$ B6 donors. Control, unthymectomized C3H recipients of TCD-BMT from *H2-Abl*$^{-/-}$ B6 donors again developed severe and lethal GVHD with only a 33% survival on day 80 after BMT, whereas all thymectomized recipients survived this period (Figure 1D) without any significant weight loss (Figure 1E). We thus confirmed the causal association of the thymus with the development of this disease and ruled out the possibility that the peripheral expansion of mature T cells contaminated in TCD-BM may have caused GVHD.

As expected, a histologic examination of the skin, liver, and salivary glands 10 weeks after BMT in the [WT → C3H] mice showed no signs of GVHD (Figure 2A,C,E). In contrast, the [*H2-Abl*$^{-/-}$ → C3H] mice showed standard pathologic features of chronic GVHD in the skin (Figure 2B), liver (Figure 2D), and salivary glands (Figure 2F). The skin pathology showed epidermal atrophy, follicular dropout, fat loss, and dermal fibrosis with scarce cell infiltration (Figure 2B). The liver pathology showed mononuclear cell infiltrates, bile duct loss, and fibrosis in the portal area (Figure 2D). Dry mouth is one of diagnostic features of chronic GVHD. We found lymphocytic inflammation, fibrosis, and atrophy of acinar tissue in the salivary glands (Figure 2F). In addition, we observed the interstitial and alveolar infiltrate of lymphocytes and macrophages in the lungs (data not shown). Pathology scores of the skin[36] were significantly higher in the [*H2-Abl*$^{-/-}$ → C3H] mice than in the controls (Table 1). Liver injury was also demonstrated

Table 1. Development of skin GVHD in [*H2-Ab1*$^{-/-}$ → C3H] mice

Pathology	Score
Thickened dermis	1.6 ± 0.5*
Fat loss	1.9 ± 0.2*
Epidermis atrophy	0.8 ± 0.3*
Follicle loss	0.9 ± 0.5*
Inflammation	0.0 ± 0.0
Total	5.3 ± 1.0*

The skin was analyzed using the histopathologic scoring system described in "Assessment of GVHD" 10 weeks after BMT. For [*H2-Ab1*$^{-/-}$ → C3H] mice, n = 16. The data are expressed as the mean ± SD. Scores were 0.0 ± 0.0 for all 15 [WT → C3H] mice.

*$P < .01$.

by the elevation of the serum alanine transferase (ALT), asparate aminotransferase (AST), alkaline phosphatase (ALP), and bilirubin levels at 10 week after BMT (Table 2). [*H2-Abl*$^{-/-}$ → C3H] mice also developed pancytopenia at 10 weeks after BMT (Table 2). Similar pathologic changes were also observed in the [*H2-Abl*$^{-/-}$ → BALB/c] mice (data not shown).

To examine whether CD8$^+$ cells are involved in the effector mechanisms, [*H2-Abl*$^{-/-}$ → C3H] chimeras were depleted of CD8$^+$ cells after BMT by chronic administration of anti-CD8 mAb. Flow cytometric analysis of the spleen 6 weeks after BMT confirmed the effective elimination of CD8$^+$ cells (less than 0.2%). However, GVHD did develop in CD8-depleted chimeras as severe as control [*H2-Abl*$^{-/-}$ → C3H] chimeras (data not shown), suggesting that CD4$^+$ cells alone are sufficient for the development of GVHD.

Adoptive transfer of the pathogenic CD4$^+$ T cells caused acute GVHD in B6 recipients

To determine the emergence of donor- or host-reactive CD4$^+$ T cells in these mice, CD4$^+$ T cells were isolated from spleens 6 weeks after BMT. These cells were stimulated with irradiated DCs either from B6 or C3H mice to determine the proliferative responses. As expected, CD4$^+$ T cells isolated from [WT → C3H] mice did not respond to B6 (donor) or C3H (recipient) stimulators (Figure 3A). In contrast, [*H2-Abl*$^{-/-}$ → C3H] CD4$^+$ T cells proliferated vigorously in response to B6 stimulators. Similarly, [*H2-Abl*$^{-/-}$ → BALB/c] CD4$^+$ T cells were also B6 reactive (Figure 3B). Cell division of CFSE-labeled donor T cells was also analyzed in culture. [*H2-Abl*$^{-/-}$ → C3H] CD4$^+$ T cells divided in response to B6 stimulators and CD3 stimulation but not to C3H stimulators (Figure 3C). Thus, these results demonstrate the emergence of primarily donor-reactive CD4$^+$ T cells.

[*H2-Abl*$^{-/-}$ → C3H] chimeras lack MHC class II expression on antigen-presenting cells (APCs) in the periphery and thus are not relevant to clinical BMT. Also, it is not clear whether GVHD in these mice is due to the impaired thymic negative selection, due to the lack of MHC class II in the periphery, or both. We therefore tested whether [*H2-Abl*$^{-/-}$ → C3H] CD4$^+$ T cells could cause GVHD after adoptive transfer to B6 or C3H mice. A total of 1×10^7 [WT → C3H] or [*H2-Abl*$^{-/-}$ → C3H] CD4$^+$ T cells were transferred to lethally irradiated B6 or C3H mice together with host-type TCD-BM. In the control, the transfer of [WT → C3H] CD4$^+$ T cells did not cause GVHD in B6 or C3H mice, as expected (Figure 3D-E). In contrast, the transfer of [*H2-Abl*$^{-/-}$ → C3H] CD4$^+$ T cells caused severe and lethal disease in B6 recipients (Figure 3D-E). A pathologic analysis of the liver and intestine showed standard pathologic features of acute GVHD. In the intestine villous atrophy, crypt cell apoptosis, and lymphocytic infiltrates were noted (Figure 3F, left panel). In the liver, mononuclear cells densely infiltrated the bile duct epithelium and the portal area with necrotic hepatocytes (Figure 3F, right panel). These mice did not meet the criteria for chronic GVHD (Table 3). In contrast, the transfer of [*H2-Abl*$^{-/-}$ → C3H] CD4$^+$ T cells did not cause GVHD in C3H recipients (Figure 3D-E). Thus, both in vitro and in vivo experiments demonstrated that pathogenic CD4$^+$ T cells that developed in [*H2-Abl*$^{-/-}$ → C3H] mice were primarily B6 reactive.

Adoptive transfer of the pathogenic CD4$^+$ T cells caused chronic GVHD in C3H recipients in the presence of B6 APCs

We then investigated whether these pathogenic T cells that escaped from thymic negative selection could cause GVHD in C3H mice in the presence of B6-derived APCs. CD4$^+$ T cells isolated from the [*H2-Abl*$^{-/-}$ → C3H] mice at 6 weeks after transplantation were transferred to lethally irradiated C3H mice together with either B6 TCD-BM or C3H TCD-BM. Again, the transfer of [*H2-Abl*$^{-/-}$ → C3H] CD4$^+$ T cells did not cause GVHD in C3H recipients when transferred with C3H TCD-BM (Figure 4A). Interestingly, these cells transmitted lethal GVHD in secondary C3H recipients when transferred with B6 TCD-BM. Recipients of [*H2-Abl*$^{-/-}$ → C3H] CD4$^+$ T cells and B6 TCD-BM began to lose weight 4 weeks after transfer. Weight loss was significantly greater in these mice than in the recipients of [*H2-Abl*$^{-/-}$ → C3H] CD4$^+$ T cells and C3H TCD-BM at 6 weeks after transfer and thereafter (Figure 4B). Recipients of [*H2-Abl*$^{-/-}$ → C3H] CD4$^+$ T cells and C3H TCD-BM looked healthy (Figure 4C, left panel), but those of [*H2-Abl*$^{-/-}$ → C3H] cells and B6 TCD-BM were hunched and displayed sclerodermatous skin changes (Figure 4C, right panel). This systemic illness was lethal so that only 16% survived on day 80 after transfer (Figure 4A, $P < .001$).

A histologic examination of the skin, liver, and salivary glands 6 weeks after transfer of the [WT → C3H] CD4$^+$ T cells and B6 TCD-BM showed no signs of GVHD (Figure 5A,C,E). In contrast, the transfer of [*H2-Abl*$^{-/-}$ → C3H] CD4$^+$ T cells and B6 TCD-BM produced similar chronic GVHD to the primary [*H2-Abl*$^{-/-}$ → C3H] in the skin, liver, and salivary glands (Figure 5B,D,F). In these recipients, the skin pathology scores were significantly elevated (Figure 4D). Furthermore, these mice showed profound

Table 2. Development of liver injury and pancytopenia in the [*H2-Ab1*$^{-/-}$ → C3H] mice

Parameter	Naive	[WT → C3H]	[*H2-Ab1*$^{-/-}$ → C3H]
No. of mice	5	4	5
Liver			
AST level, IU/L	72.2 ± 11.3	94.3 ± 26.9	364.5 ± 17.7*
ALT level, IU/L	27.0 ± 5.4	43.3 ± 15.8	138.0 ± 68.0*
ALP level, IU/L	312.0 ± 126.0	446.5 ± 22.7	634.6 ± 132.7
Bilirubin level, mg/dL	0.08 ± 0.03	0.08 ± 0.01	0.18 ± 0.06*
CBCs			
White blood cell count, × 10^9/L	10.6 ± 3.8	13.0 ± 3.1	4.2 ± 2.3†
Red blood cell count, × 10^{12}/L	8.9 ± 0.3	7.9 ± 8.3	5.2 ± 1.6*
Hemoglobin level, g/L	132.0 ± 5.0	129.6 ± 14.2	95.2 ± 15.0*
Platelet count, × 10^9/L	731.3 ± 83.0	798.0 ± 17.7	31.4 ± 22.3†

The complete blood counts (CBCs) and serum indices of liver damage were measured 10 weeks after BMT. The data are expressed as the mean ± SD.

To convert bilirubin level from milligrams per deciliter to micromoles per liter, multiply milligrams per deciliter by 17.1.

*$P < .05$, [WT → C3H] versus [*H2-Ab1*$^{-/-}$ → C3H].

†$P < .01$, [WT → C3H] versus [*H2-Ab1*$^{-/-}$ → C3H].

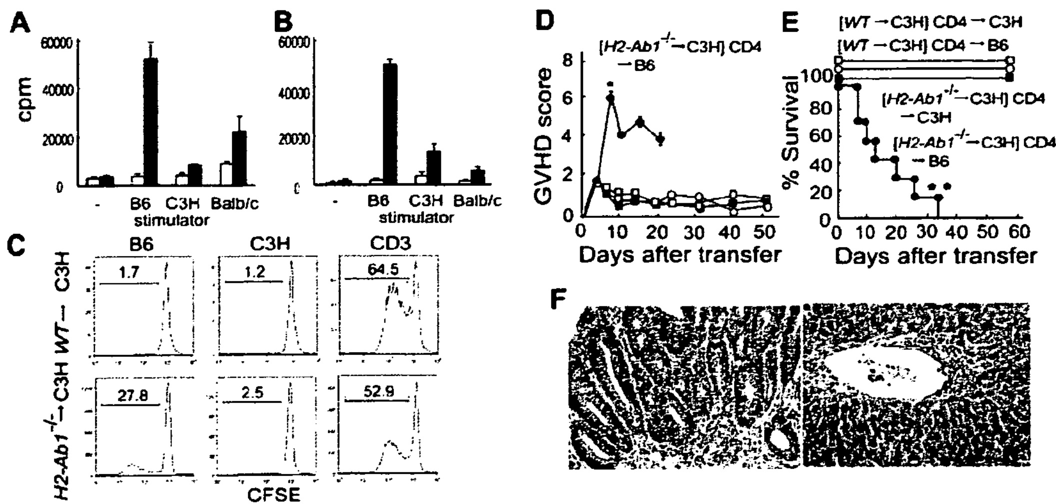

Figure 3. CD4⁺ T cells from [*H2-Ab1*$^{-/-}$ → C3H] mice were predominantly B6 reactive. (A) C3H mice were irradiated and underwent transplantation with TCD-BM from either control WT or *H2-AB1*$^{-/-}$ B6 donors. Six weeks after BMT, splenic CD4⁺ T cells isolated from [WT → C3H] (□) or [*H2-Ab1*$^{-/-}$ → C3H] (■) mice were stimulated with irradiated DCs from B6, C3H, and BALB/c mice. Seventy-two hours later, proliferation was determined by a thymidine uptake assay. Data are shown as the mean ± SD. (B) Similarly, BALB/c mice underwent transplantation, and [WT → BALB/c] (□) or [*H2-Ab1*$^{-/-}$ → BALB/c] (■) CD4⁺ T cells were stimulated with irradiated DCs. Proliferation was determined by a thymidine uptake assay after 72 hours. Data are shown as the mean ± SD. (C) CFSE-labeled [WT → C3H] and [*H2-Ab1*$^{-/-}$ → C3H] CD4⁺ T cells were stimulated with irradiated splenocytes in culture. Cell division determined by dilution of CFSE is shown for CD4⁺ cells. (D-F) A total of 1 × 10^7 [WT → C3H] (open symbols) or [*H2-Ab1*$^{-/-}$ → C3H] (closed symbols) CD4⁺ T cells were transferred to lethally irradiated C3H (squares) or B6 (circles) mice together with host-type TCD-BM. Clinically acute GVHD scores as the mean ± SE (D) and survivals (E) of mice after transfer are shown; n = 3 to 6 per group. The histologic findings of the small intestine (F, left panel) and liver (F, right panel) from B6 recipients of [*H2-Ab1*$^{-/-}$ → C3H] CD4⁺ T cells are shown (original magnification, × 200). *P < .05, **P < .001.

leukocytopenia and thrombocytopenia at 7 weeks after transfer (Figure 4E). These mice did not meet the criteria for acute GVHD (Table 3). These results suggest that [*H2-Ab1*$^{-/-}$ → C3H] CD4⁺ T cells cause acute GVHD when target epithelium expresses B6 antigens but chronic GVHD when B6 antigens are provided by hematopoietic cells in the absence of B6 antigen expression on target epithelium.

To further confirm the requirement of B6-derived APCs for the disease, we created [B6 → C3H] and [C3H → C3H] chimeras by reconstituting lethally irradiated C3H mice with TCD-BM from B6 and C3H mice, respectively. Four months later, a flow cytometric analysis of splenic DCs isolated from these animals showed complete replacement by donor-derived DCs (data not shown) as previously described.[37] Next, these chimeric mice were sublethally irradiated and injected with 1 × 10^7 CD4⁺ T cells isolated either from the [WT → C3H] or [*H2-Ab1*$^{-/-}$ → C3H] mice alone without TCD-BM. In the control, the transfer of the [WT → C3H] CD4⁺ T cells did not cause GVHD in either the [B6 → C3H] or [C3H → C3H] chimeras (Figure 6). However, the transfer of [*H2-Ab1*$^{-/-}$ → C3H] CD4⁺ T cells did cause a significant weight loss and lethal GVHD in the [B6 → C3H] chimeras but not in the [C3H → C3H] chimeras, thus confirming the requirement of B6-derived APCs for the transmission of the disease to secondary recipients.

We finally evaluated whether the impaired MHC class II–mediated thymic negative selection affects development of CD4⁺ CD25⁺ regulatory T (Treg) cells. Cells were isolated from the

Table 3. Number of mice that fall into either category of acute or chronic GVHD

	Acute GVHD, no. of mice	Chronic GVHD, no. of mice
[*H2-Ab1*$^{-/-}$ → C3H] CD4 + B6 TCD-BM → B6	3	0
[*H2-Ab1*$^{-/-}$ → C3H] CD4 + B6 TCD-BM → C3H	0	6

A total of 1 × 10^7 [*H2-Ab1*$^{-/-}$ → C3H] CD4⁺ T cells were transferred to lethally irradiated B6 or C3H mice together with TCD-BM from B6 mice.

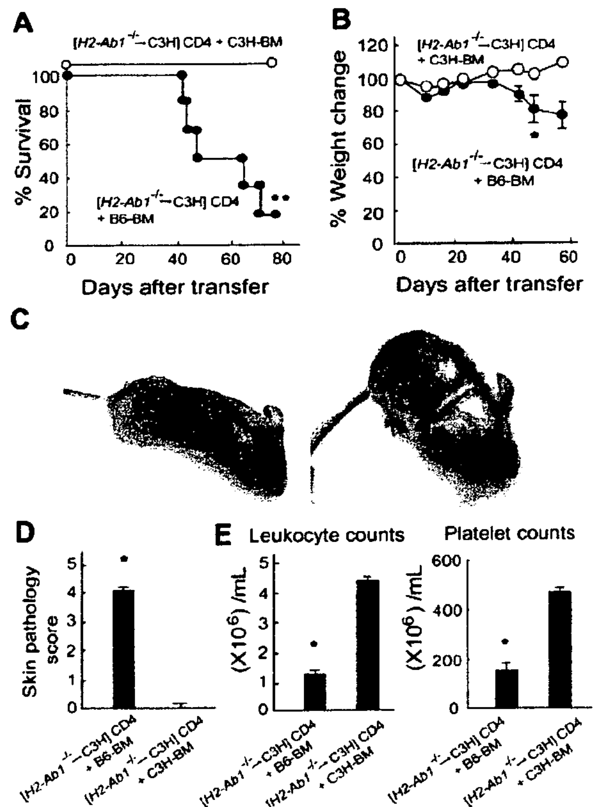

Figure 4. Transfer of CD4⁺ T cells from [*H2-Ab1*$^{-/-}$ → C3H] mice transmitted chronic GVHD in the secondary recipients when reconstituted with B6 TCD-BM but not C3H TCD-BM. A total of 1 × 10^7 [*H2-Ab1*$^{-/-}$ → C3H] CD4⁺ T cells were transferred to lethally irradiated C3H mice together with TCD-BM from C3H (○) or B6 (●) mice. The survivals (A) and weight changes as the mean ± SE (B) after transfer are shown; n = 3 to 6 per group. (C) Appearances of C3H recipients of pathogenic CD4⁺ T cells with C3H TCD-BM (left) and those with B6 TCD-BM (right) 7 weeks after transfer are shown. (D) Skin pathologic scores at 10 weeks after transplantation are shown as the mean ± SD. (E) CBCs were measured 7 weeks after transfer. Data are expressed as the mean ± SD. *P < .05, **P < .001.

spleen and thymus 5 and 8 weeks after BMT. A flow cytometric analysis showed that the numbers of CD4⁺ Foxp3⁺ T cells in the spleen and thymus were comparable between [WT → C3H] and [$H2\text{-}Ab1^{-/-}$ → C3H] mice (Figure 7A-B). To examine whether these Treg cells are functional in [$H2\text{-}Ab1^{-/-}$ → C3H] mice, these mice were depleted of $CD25^+$ cells after TCD-BMT by chronic administration of anti-CD25 mAb. GVHD was more severe in CD25-depleted mice than in controls (Figure 7C), suggesting that Treg cells are functional in these mice but not sufficient to inhibit GVHD.

Discussion

Thymic-dependent GVHD in our model is similar to human chronic GVHD in terms of its clinical manifestations and pathology. Multiple organs are involved, such as the skin, liver, lung, salivary glands, and hematopoietic system. In particular, skin sclerodermatous change and destruction of salivary glands are frequent and specific manifestations of chronic GVHD in humans.[42] Hepatic disease is characterized by bile duct mononuclear cell infiltrates followed by bile duct loss and fibrosis. Such a "vanishing bile duct" is also one of the key histologic features of hepatic chronic GVHD. Other features include lung injury, immunodeficiency, and pancytopenia; these are also consistent with human chronic GVHD.[42,43] Thymopoiesis declines with age; however, it is now clear that a substantial output is maintained late into adulthood following HSCT as demonstrated by the observation that the number of naive or T-cell–receptor excision circle–containing ($TREC^+$) $CD4^+$ T cells usually increases between 3 and 12 months after grafting.[25,26] In mice, the thymic-dependent $CD4^+$ T cells significantly repopulate recipients by 6 weeks after transplantation.[44]

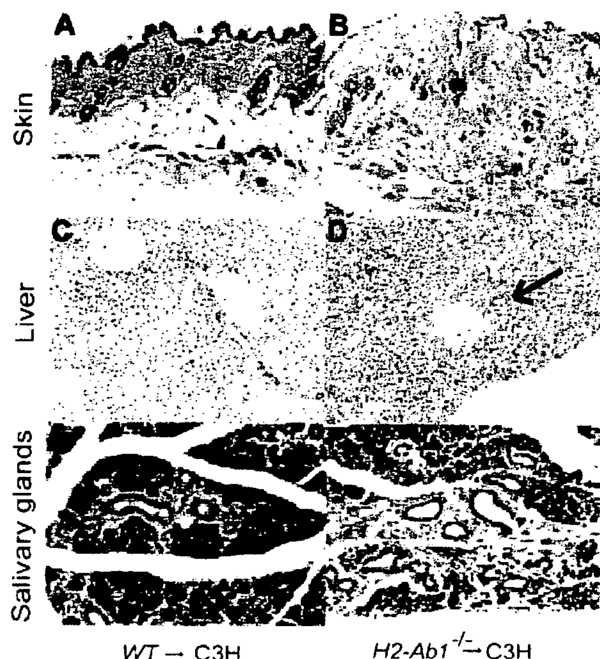

Figure 5. Histologic analysis of recipients of [$H2\text{-}Ab1^{-/-}$ → C3H] $CD4^+$ T cells and B6 TCD-BM showed pathologic features similar to human chronic GVHD. The histologic findings of the skin (A-B), liver (C-D), and salivary glands (E-F) from recipients of [WT → C3H] $CD4^+$ T cells with B6 TCD-BM and those of [$H2\text{-}Ab1^{-/-}$ → C3H] $CD4^+$ T cells with B6 TCD-BM are shown. Sclerodermatous skin changes, such as epidermal atrophy, fat loss, follicular dropout, and dermal thickness (B); bile duct loss and fibrosis in the portal area and mild periportal mononuclear infiltrates in the liver (D, arrow); and lymphocyte inflammation, fibrosis, and atrophy of acinar tissue in the salivary glands (F, arrow) were observed (original magnification, × 100).

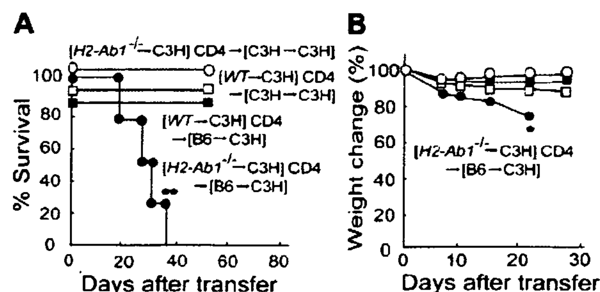

Figure 6. Transfer of $CD4^+$ T cells from [$H2\text{-}Ab1^{-/-}$ → C3H] mice transmitted GVHD in [B6 → C3H] chimeras. The [B6 → C3H] and [C3H → C3H] chimeric mice were sublethally irradiated and injected with splenic $CD4^+$ T cells (1×10^7) isolated either from [WT → C3H] or [$H2\text{-}Ab1^{-/-}$ → C3H] mice, respectively. The survivals after transfer (A) and weight change (B) are shown (n = 3 to 5 per group). [C3H → C3H] chimeric mice were transferred with [WT → C3H] $CD4^+$ T cells (□) or [$H2\text{-}Ab1^{-/-}$ → C3H] $CD4^+$ T cells (○). [B6 → C3H] chimeras underwent transplantation with [WT → C3H] $CD4^+$ T cells (■) or [$H2\text{-}Ab1^{-/-}$ → C3H] $CD4^+$ T cells (●). *$P < .05$, **$P < .001$.

Experimental studies and clinical observations have convincingly shown that GVHD is initiated by donor T cells. The donor T-cell repopulation after HSCT results from both the peripheral expansion of mature donor T cells and the thymic-dependent generation of donor T cells from donor hematopoietic stem cells. It has been shown that the former pathway plays an important role in both acute and chronic GVHD in murine models.[11,16,19,45] The role of thymic-dependent T cells in causing GVHD is still not clear, although it has been postulated that the thymic damage by conditioning, acute GVHD, and age-related atrophy disrupt thymic education of T cells could cause chronic GVHD.[20-22,27,46] Infusion of mature donor T cells causes acute GVHD, which disrupts thymic negative selection and leads to the emergence of thymic-dependent autoreactive T cells.[21,28-30] Nonetheless, the in vivo pathogenic role of these T cells was not addressed in these studies. On the other hand, it has been shown that thymic-dependent T cells could also induce syngeneic GVHD, which has features similar to acute GVHD after allogeneic HSCT.[32,47] We herein demonstrate, to our knowledge, for the first time that the thymic-dependent T cells that escape from negative selection have the ability to cause lethal disease similar to human chronic GVHD after allogeneic BMT. Thymectomy of the recipients prior to BMT prevented the development of the disease, thus confirming the causative role of the thymus in its development. In contrast to our results, Zhang et al[16] reported that host thymus was not required for chronic GVHD in a sublethally irradiated DBA/2 into BALB/c model. Taken in that context, our data would suggest that both the peripheral expansion of mature donor T cells and thymic-dependent generation of donor T cells from donor hematopoietic stem cells may play a role in the induction of chronic GVHD.

A disrupted peripheral regulatory mechanism is also responsible for the development of GVHD as well as autoimmune diseases.[48,49] It is thus possible that dysfunction of peripheral regulatory mechanisms may be involved in this disease process. However, we found that the numbers of $CD4^+CD25^+$ $Foxp3^+$ Treg cells in [$H2\text{-}Ab1^{-/-}$ → C3H] mice were equivalent to those in controls, and these cells were functional in these mice. Thus, functional Treg cells normally developed in these mice, as they did in mice in which the MHC class II expression was limited on thymic epithelial cells but not on the hematopoietically derived element.[50,51] Also, it has been shown that MHC class II–deficient Treg

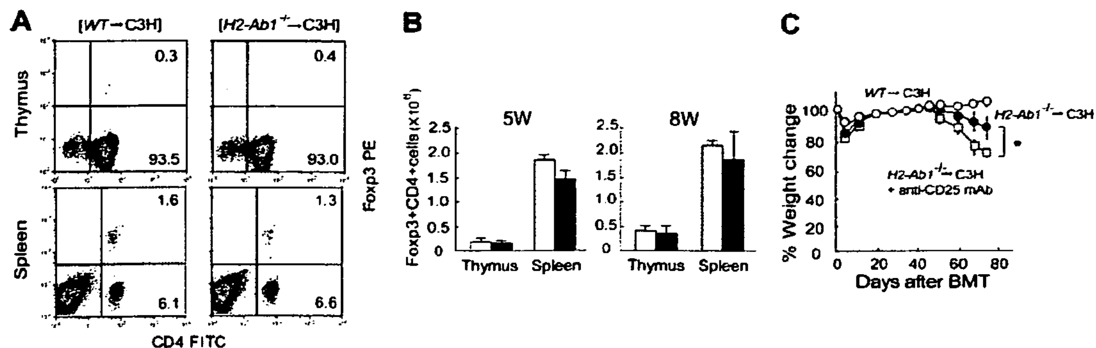

Figure 7. The numbers of CD4+ Foxp3+ T cells were comparable between [WT → C3H] and [*H2-Ab1*$^{-/-}$ → C3H] mice. Cells isolated from the thymus and spleen from [WT → C3H] and [*H2-Ab1*$^{-/-}$ → C3H] mice were stained for CD4 and Foxp3 mAbs. (A) The dot plots show the 2-color staining pattern for CD4-FITC and Foxp3-PE on CD4 single-positive thymocytes and splenocytes 5 weeks after BMT. (B) The numbers of CD4+ Foxp3+ T cells on the CD4 single-positive thymocytes and splenocytes from [WT → C3H] or [*H2-Ab1*$^{-/-}$ → C3H] mice were enumerated 5 and 8 weeks after BMT (n = 3 per group). (C) C3H mice were irradiated and underwent transplantation with TCD-BM from either control WT or *H2-Ab1*$^{-/-}$ B6 donors. After BMT, mice were injected with anti-CD25 mAbs or control Abs. Weight changes as the mean ± SE after BMT are shown. ○, [WT → C3H], n = 4; ●, control [*H2-Ab1*$^{-/-}$ → C3H], n = 6; □, [*H2-Ab1*$^{-/-}$ → C3H] treated with anti-CD25 mAbs, n = 6. *$P < .05$.

cells are as suppressive as MHC class II–bearing Treg cells.[51] Nonetheless, the role of non-CD4+CD25+ Foxp3+ Treg cells will be explored in the future.

In vitro experiments and adoptive transfer experiments suggest that [*H2-Abl*$^{-/-}$ → C3H] CD4+ T cells are reactive to self-antigens. The transfer of these CD4+ T cells caused GVHD in B6 recipients but not in C3H recipients. Thus, there seems to be some degree of partial tolerization to C3H antigens. Negative selection can be also mediated by thymic medullary epithelial cells (MECs)[52] and, thus, C3H-derived MECs may be sufficient to eliminate most C3H-reactive T cells. Nonetheless, these cells can transmit GVHD to C3H mice when a repopulation of B6-derived hematopoiesis has occurred. Stimulation of B6-reactive T cells with B6 antigens may enhance the ability of these cells to perpetuate the disease. Similar to our study, several previous studies suggested that donor-reactive T cells, particularly CD4+ T cells, are associated with GVHD. CD4+ T cells play a critical role in a B10.D2 → BALB/c BMT model of chronic GVHD.[53] Tivol and colleagues[54] showed that acute GVHD in a B6 → BALB/c BMT model results in the emergence of B6-reactive donor T cells that can cause severe autoimmune colitis in B6 recipients but not in BALB/c recipients after transfer. Similar to our findings, transfer of the GVHD T cells could induce severe colitis in BALB/c recipients only when B6-derived APCs were present.[54] It is possible that impaired negative selection in these animals might have occurred due to destruction of donor-derived APCs in the thymus. However, the role of thymus was not addressed in this B6 → BALB/c model. Conditioning damages the thymus and donor T cells invade the thymus, and the thymic structure is destroyed.[21,22] Therefore, both donor- and host-reactive T cells could be generated.

Given the requirement of B6 APCs for the transmission of chronic GVHD to C3H mice, it is puzzling that chronic GVHD developed in [*H2-Abl*$^{-/-}$ → C3H] chimeras in the absence of MHC class II expression on B6-derived APCs. We considered the involvement of CD8+ T cells stimulated by abnormal CD4+ T cells, but depletion of CD8+ T cells did not abrogate GVHD in [*H2-Abl*$^{-/-}$ → C3H] chimeras. MHC class II expression on APCs may influence the reactivity of postthymic T cells, referred to as "tuning."[55] Mature CD4+ T cells become hyperreactive against both syngeneic and allogeneic skin grafts upon transfer into MHC class II–deficient hosts.[55] Therefore, it is tempting to speculate that absence of MHC class II expression on APCs in [*H2-Abl*$^{-/-}$ → C3H] chimeras dynamically reduces the activation threshold of CD4+ T cells and caused chronic GVHD in these mice. In transfer experiments, MHC class II–positive B6-derived APCs directly stimulate B6-reactive CD4+ T cells to cause GVHD, whereas C3H-derived APCs may "tune" the pathogenic CD4+ T cells in the absence of B6-derived APCs.

The transfer of [*H2-Abl*$^{-/-}$ → C3H] CD4+ T cells caused acute, not chronic, GVHD in B6 mice. This result is consistent with our previous observations that the transfer of CD4+ T cells from [*H2-Abl*$^{-/-}$ → B6] mice caused acute GVHD in syngeneic B6 mice.[32] Notably, however, these are not relevant to clinical BMT, because there is no clinical situation in which donor T cells infuse back to donors. On the other hand, it is intriguing that [*H2-Abl*$^{-/-}$ → C3H] and [*H2-Abl*$^{-/-}$ → BALB/c] CD4+ T cells caused chronic GVHD in allogeneic C3H and BALB/c mice, respectively, after transfer with B6 TCD-BM in the current study. Although the precise mechanisms of how these cells differentially cause acute and chronic GVHD remain to be elucidated, expression of B6 antigens on target epithelium may cause a more inflammatory form of GVHD, acute GVHD, through the direct attack on target epithelial cells by B6-reactive T cells, whereas absence of B6 antigens on target epithelium may cause less inflammatory chronic GVHD in the presence of B6-derived APCs. In this scenario, it is possible that B6-reactive T cells attack the target epithelium in C3H recipients through the secretion of fibrosing and inflammatory cytokines such as transforming growth factor-β,[56-59] promotion of B-cell activation and autoantibody production,[2,16,60] or nonspecific bystander lysis.[61]

We have shown that alloreactive donor T cells could damage epithelial cells that lack alloantigen expression when alloantigens are expressed on APCs in MHC-mismatched mouse models of BMT.[37] In these experiments, alloantigen expression on APCs is sufficient for the migration of donor T cells into target tissues.[37] Thus, APCs that reside in GVHD target tissues may recruit and stimulate alloreactive donor T cells to cause bystander injury of the surrounding epithelial cells.[37,62] Alternately, it is still possible that B6-reactive CD4+ T cells cross-react on C3H or BALB/c sufficiently to cause chronic GVHD, although we are not able to demonstrate such cross-reactivity in vitro and adoptive transfer experiments. There are now accumulating evidences of cross-reactivity between self-antigens and alloantigens. In K14 mice expressing MHC class II only on thymic cortical epithelium, negative selection was impaired and autoreactive CD4+ T cells generated.[63] Surprisingly, more than half of the T-cell hybrids established from the K14 CD4+ T cells reacted to both B6-derived APCs and allogeneic APCs.[64] Thus, stimulation of B6-reactive T cells with B6 antigens may enhance reactivity to C3H antigens sufficiently to cause GVHD. Thus, the reactivity and/or frequency of host-reactive T cells may determine the development of acute or chronic GVHD.

It remains to be investigated whether chronic GVHD is a later manifestation of acute GVHD or has a different pathogenesis involving different effector cells. Both could be true, but previous studies[16,54,65] and the current study favor the latter hypothesis. Several attempts to decrease acute GVHD does not result in a reduction of the chronic GVHD rates.[5-10] Current study suggests that the pathogenesis of acute GVHD and chronic GVHD is different, and our model will be useful to study the pathogenesis and pathophysiology of chronic GVHD. Our results also suggest that an improvement in the thymic function may have a potential to decrease chronic GVHD.

Acknowledgments

This work was supported by research funds from Ministry of Education, Culture, Sports, Science, and Technology grant 17659293 (T.T.), the Health and Labor Science Research Grants for Clinical Research for Evidence Based Medicine (T.T.), and Takeda Science Foundation (T.T.).

Authorship

Contribution: Y.S. performed research and wrote the paper; D.H., S.A., K.T., M.H., and M.T. performed research; and T.T. designed and organized the research.

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

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FTY720 enhances the activation-induced apoptosis of donor T cells and modulates graft-*versus*-host disease

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FTY720 is a novel immunosuppressant that improves the outcomes after solid organ and bone marrow transplantation (BMT) due to the sequestration of T cells into LN. We tested the hypothesis that the sequestration of donor T cells in LN by FTY720 would enhance their interaction with host APC, thus causing a greater degree of activation-induced apoptosis of alloreactive T cells, and thereby resulting in a reduction of graft-*vs.*-host disease (GVHD). The short-term administration of FTY720 improved the recipient survival after allogeneic BMT. FTY720 treatment facilitated a rapid contraction of the donor T cell pool in association with an increased degree of apoptosis of donor T cells. The donor T cell reactivity to host alloantigens was diminished in host's LN and adoptive transfer of donor T cells isolated from LN of FTY720-treated recipients of allogeneic BMT induced less severe GVHD in secondary recipients than the transfer from controls. Caspase-dependent apoptosis was involved in this mechanism because FTY720-induced protection was abrogated when a pan-caspase inhibitor was administered. These findings thus demonstrate the presence of a novel mechanism by which FTY720 modulates the allogeneic T cell responses: namely, by the induction of activation-induced apoptosis of alloreactive T cells in LN.

Received 1/4/06
Revised 17/9/06
Accepted 12/10/06

[DOI 10.1002/eji.200636123]

Key words:
Animal Models · Apoptosis · Transplantation

Introduction

Graft-*vs.*-host disease (GVHD) remains a major obstacle for the wider application of allogeneic hematopoietic stem cell transplantation (HSCT). It is a complex process involving dysregulated inflammatory cytokine cascades and CTL responses against host alloantigens [1, 2]. The interaction of donor T cells and host APC in secondary lymphoid organs (SLO) is critical for initiating GVHD after allogeneic HSCT [1, 3]. Alloreactive donor T cells activated in SLO release Th1 cytokines such as IL-2 and IFN-γ which induce T cell expansion, inflammatory cytokine cascades, and donor-derived CTL responses [4].

Activation-induced cell death (AICD) represents a critical physiologic pathway to control the expansion of antigen-activated T cells at the down phase of an immune response [5, 6]. Alloreactive donor T cells vigorously proliferate early after HSCT, but subsequently contract rapidly [7–9]. AICD has been suggested to be a chief mechanism of clonal deletion, which is largely responsible for the rapid contraction of allo-

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Abbreviations: **7-AAD:** 7-amino-actinomycin D · **AICD:** activation-induced cell death · **B6:** C57BL/6 · **BMT:** bone marrow transplantation · **GVHD:** graft-*vs.*-host disease · **HSCT:** hematopoietic stem cell transplantation · **MLN:** mesenteric lymph node · **S1P:** sphingosine-1-phosphate receptor · **SLO:** secondary lymphoid organ · **TCD:** T cell-depleted · **ZVAD-fmk:** carbobenzoxy-valyl-alanyl-aspartyl-(β-o-methyl)-fluoromethylketon

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reactive donor T cells following an initial massive expansion after allogeneic HSCT [7, 9]. AICD can be enhanced by the prolonged interaction between antigen-specific T cells and antigen-loaded APC *in vitro* [10–13].

FTY720 is a chemical derivative of myriocin (ISP-1), a metabolite of the ascomycete *Isaria sinclairii* [14]. FTY720 is rapidly phosphorylated after administration; thereafter it binds four of the five sphingosine-1-phoshate receptors (S1P1, S1P3, S1P4, S1P5), down-regulates S1P1 on lymphocytes, and inhibits the egression of lymphocytes from the thymus and SLO such as LN and Peyer's patches into the peripheral blood and lymph [15–18]. FTY720 produces lymphocytopenia and prolongs allograft survival in experimental solid organ transplantation [19, 20].

Recently, Kim *et al.* [21] showed that prolonged administration of FTY720 could reduce GVHD in a mouse BM transplantation (BMT) model. Although the mechanism by which FTY720 inhibits GVHD has not yet been fully studied, it most likely alters lymphocyte trafficking. We hypothesized that the sequestration of donor T cells in SLO by FTY720 would therefore enhance the interaction of donor T cells and host APC, thus resulting in an enhanced AICD of alloreactive donor T cells and a reduction in the severity of GVHD. Using well-defined mouse models of allogeneic BMT, we therefore uncovered a novel mechanism by which FTY720 modulates the allogeneic T cell responses: namely, the modulation of the alloreactivity of donor T cells in host's LN.

Results

Administration of FTY720 reduces GVHD after allogeneic BMT

A previous study showed that the high-dose and long-term administration of FTY720 (1–3 mg/kg) reduced acute GVHD whereas low-dose FTY720 at a dose of 0.1 mg/kg had only a minor protective effect on experimental GVHD [21]. We first determined whether the low-dose and short-term administration of FTY720 (0.3 mg/kg) could modulate GVHD. B6D2F1 mice were lethally irradiated (15 Gy) and injected i.v. with 4×10^6 purified T cells plus 4×10^6 T cell-depleted (TCD) BM cells from allogeneic C57BL/6 (B6) or syngeneic B6D2F1 donors. Total body irradiation of 15 Gy, split into two doses separated by 3 h to minimize the degree of gastrointestinal toxicity, was often used in this strain combination to induce GVHD mortality [22]. FTY720 at a dose of 0.3 mg/kg or distilled water was administered by daily oral gavage from day 0 until day 10 after BMT.

GVHD was severe in the allogeneic controls and all recipients died by day 10 (Fig. 1A). The administration of FTY720 significantly improved the survival to 50% at day 50. In another set of experiments with a reduced T cell dose (1×10^6), FTY720 again significantly improved both the survival rate (Fig. 1A) and the clinical GVHD scores (6.0±0.5 *vs.* 4.1±0.3 at day 14, $p<0.05$) compared to the controls. IFN-γ and TNF-α are crucial cytokines that can be implicated in the efferent and afferent phase of the pathophysiology of acute GVHD [23]. In FTY720-treated mice, the serum levels of both IFN-γ (Fig. 1B) and TNF-α (Fig. 1C) on day 7 significantly decreased in comparison to the controls. Similar results were obtained using a different strain combination, B6 (H-2^b)→B6C3F1 (H-$2^{b/k}$); FTY720 significantly improved the survival rate (Fig. 1D) and clinical GVHD scores (Fig. 1E).

An analysis of the thymus 50 days after BMT showed that numbers of $CD4^+CD8^+$ double-positive thymocytes were higher in FTY720-treated recipients than in allogeneic controls, thus indicating a reduction of the thymic GVHD in the FTY720-treated animals (Fig. 1F). An analysis of the donor cell engraftment on day 50 after allogeneic BMT in the spleens showed complete donor engraftment (>99%) in both FTY720- and control-treated recipients, thus ruling out the rejection or mixed chimerism as a potential cause of GVHD suppression.

FTY720 does not impair the initial donor T cell activation

Since FTY720 can suppress the activation of alloreactive T cells at a high concentration *in vitro* [24], we next examined whether FTY720 directly impaired the activation and proliferation of donor T cells *in vivo.* B6D2F1 ($CD45.2^+$) mice were lethally irradiated and reconstituted with 4×10^6 CFSE-labeled T cells from B6-Ly5a ($CD45.1^+$) mice plus 4×10^6 unlabeled TCD BM cells from B6 ($CD45.2^+$) mice. The cells were isolated from mesenteric LN (MLN) on day 4 post-transplantation, and the division of donor T cells was determined by CFSE dilution of $CD45.1^+CD45.2^-DAPI^-$ cells. The proliferation of donor T cells in MLN from FTY720-treated recipients was at least as vigorous as that from the controls (Fig. 2A). The donor T cells harvested from FTY720-treated recipients on day 4 post-transplantation proliferated more vigorously than those from the controls in response to host alloantigens *in vitro* (Fig. 2B).

We then examined whether FTY720 affects the speed of the host DC disappearance in LN after BMT. The lethally irradiated B6D2F1 mice were transplanted with cells from B6 donors, followed by the daily administration of FTY720 (0.3 mg/kg) or distilled water. There-

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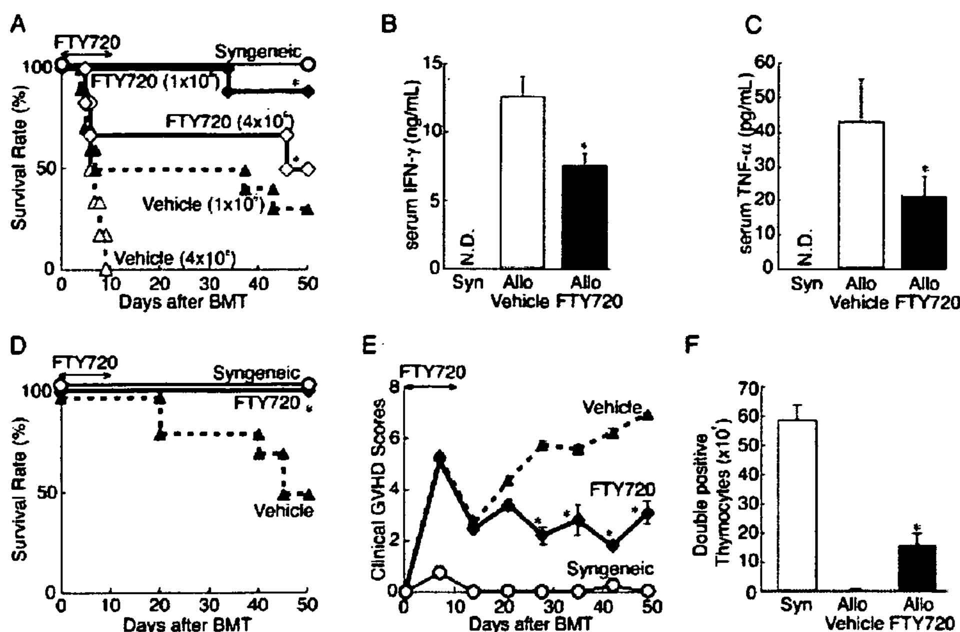

Figure 1. Administration of FTY720 reduces GVHD after allogeneic BMT. (A–C) Lethally irradiated (15 Gy) B6D2F1 mice were transplanted with 1×10^6 or 4×10^6 T cells and 4×10^6 TCD BM cells isolated from B6 (allogeneic) or B6D2F1 (syngeneic) mice. The mice were administered p.o. with FTY720 or diluent from day 0 to 10. (A) The survivals from three consecutive experiments are shown (*n*=9–12/group). The serum levels of IFN-γ (B) and TNF-α (C) were measured 7 days after BMT (*n*=3–4/group). Data are representative of three similar experiments and are shown as the mean ± SD. (D–F) The lethally irradiated B6C3F1 mice were similarly transplanted with cells from B6C3F1 or B6 mice and given FTY720 or diluent on days 0–10. Survival curves (D) and clinical GVHD scores (E) are shown (*n*=9–12/group). The clinical scores are shown as the mean ± SE. (F) Numbers of double-positive thymocytes at 50 days after BMT are shown as the mean ± SD. The results of two consecutive experiments were combined; ND, not detected; Syn, syngeneic; Allo, allogeneic; $^*p<0.05$ *vs.* allogeneic controls.

after, at 6 h, 24 h, and 5 days later, the numbers of host DC (H-2^{d+}CD11c^+) in LN were enumerated. The kinetics of host DC disappearance in LN were comparable between the FTY720-treated and control mice (Fig. 2C).

FTY720 induces a rapid contraction of donor T cell pool

The administration of FTY720 causes lymphocyte sequestration in the LN and lymphopenia in the peripheral blood after experimental allogeneic BMT [21, 25]. We next examined the fate of donor T cells sequestered in SLO. B6D2F1 mice were lethally irradiated and reconstituted with 4×10^6 B6-Ly5a T cells and 4×10^6 B6 TCD BM. Syngeneic control mice (B6) were similarly transplanted with 1×10^7 B6-Ly5a T cells and 4×10^6 B6 TCD BM cells. A flow cytometric analysis showed that >95% of MLN T cells from FTY720- and control-treated recipients were donor-derived, when assessed by CD45.1 positivity among the TCRβ$^+$ cells on days 4–7 after BMT. After allogeneic BMT, the numbers of CD45.1$^+$ donor T cells in the MLN were significantly greater on day 4 but surprisingly less on days 6 and 7 in the FTY720-treated recipients than in the controls (Fig. 3A). In contrast, the number of donor T cells was significantly higher in the FTY720-treated syngeneic recipients compared to diluent-treated controls ($0.98\pm0.35\times10^6$ *vs.* $1.78\pm0.11\times10^6$ on day 6, $p<0.05$), as previously shown [26].

To evaluate whether this earlier contraction of a donor T cell pool in the FTY720-treated allogeneic recipients was related to donor T cell apoptosis, the frequencies of the apoptotic donor T cells were assessed by Annexin-V$^+$ cells among donor T cells in MLN. The rates of apoptotic cells were significantly higher in FTY720-treated recipients than in the controls on day 5 after BMT (Fig. 3B, C). Early-phase apoptosis identified as Annexin-V$^+$ and 7-amino-actinomycin D (7-AAD)$^-$ cells was also augmented in FTY720-treated recipients (24.9±1.9% *vs.* 32.6±1.1%, $p<0.05$). FTY720 did not enhance the apoptosis of donor T cells after syngeneic BMT (data not shown), thus ruling out any direct cytotoxic effects of FTY720 on T cells.

FTY720 predominantly induces the deletion of host-reactive donor T cells

We next examined whether FTY720 induces the deletion of host-reactive T cells. B6D2F1 mice were lethally

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irradiated and reconstituted with 4×10^6 CFSE-labeled T cells from B6-Ly5a mice plus 4×10^6 unlabeled TCD BM cells from B6 mice. MLN were harvested on day 4, and apoptosis was assessed by Annexin-V positivities on non-divided (CFSE-non-diluted) and dividing (CFSE-diluted) cells. Annexin-V positivities were significantly higher in dividing cells than in non-dividing cells (Fig. 4A, B). FTY720 enhanced the apoptosis of the

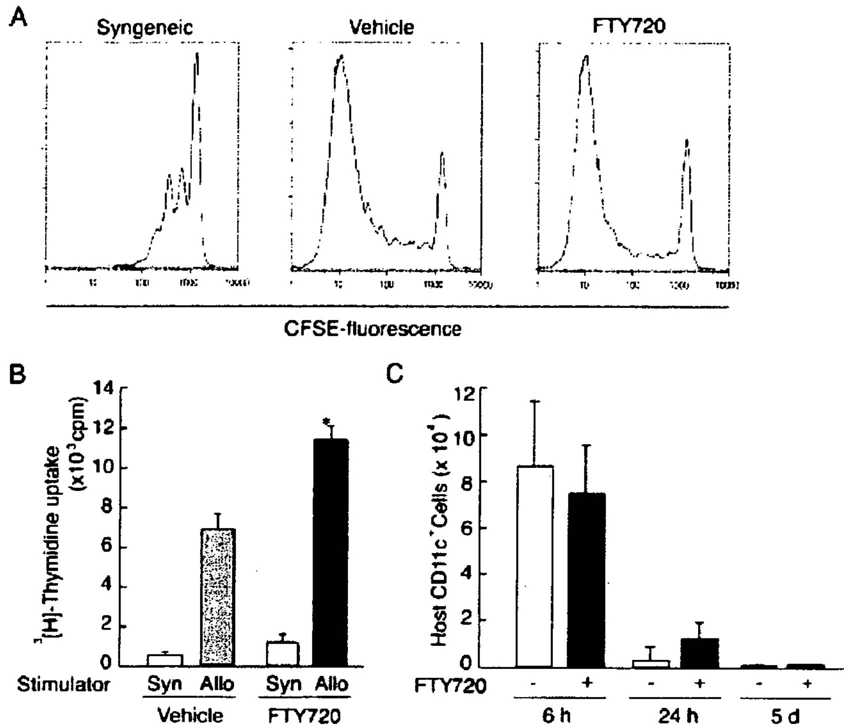

Figure 2. Administration of FTY720 does not inhibit the initial activation and proliferation of donor T cells after allogeneic BMT. Lethally irradiated B6D2F1 (allogeneic) and B6 (syngeneic) mice were transplanted with 4×10^6 CFSE-labeled T cells from B6-Ly5a plus 4×10^6 unlabeled TCD BM cells from B6 mice. FTY720 and diluent were administered as Fig. 1. Four days after BMT, MLN from each group were harvested and combined. (A) The division of donor T cells is shown as CFSE dilution in $CD45.1^+CD45.2^-DAPI^-$ cells. (B) The numbers of cells were normalized for T cells (1×10^5/well) and then were re-stimulated by B6D2F1 (allogeneic) and B6 (syngeneic) derived stimulators. Forty-eight hours later, the cells were pulsed with [^{3}H]thymidine. The [^{3}H]thymidine uptakes are shown as the mean ± SD. Data are representative of three similar experiments. (C) MLN were harvested 6 h, 24 h, and 5 days after BMT. The numbers of host DC (H-2^{d+}CD11c$^+$) are shown as the mean ± SD (n=3–4/group).

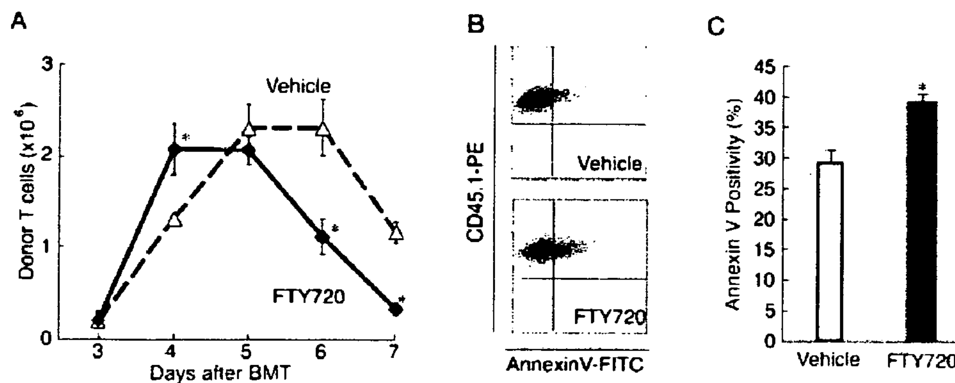

Figure 3. FTY720 induces a rapid contraction of donor T cell pool after allogeneic BMT. Lethally irradiated B6D2F1 mice were transplanted with 4×10^6 T cells from B6-Ly5a plus 4×10^6 TCD BM cells from B6 mice, and FTY720 or diluent was administered from day 0. The cells were isolated from MLN 3–7 days after BMT. (A) The numbers of donor T cells ($CD45.1^+CD4^+$ plus $CD45.1^+CD8^+$) in MLN are shown (n=4–6/group). (B–C) Five days after BMT, the frequencies of Annexin-V$^+$ cells in CD45.1$^+$ donor T cells were determined by flow cytometry. The dot plots of Annexin-V-FITC and CD45.1-PE staining from representative recipients (B) and frequencies of Annexin-V$^+$ cells among CD45.1$^+$ donor T cells (C) are shown (n=6/group). Data are representative of three similar experiments, and are shown as the mean ± SE; $^*p<0.05$.

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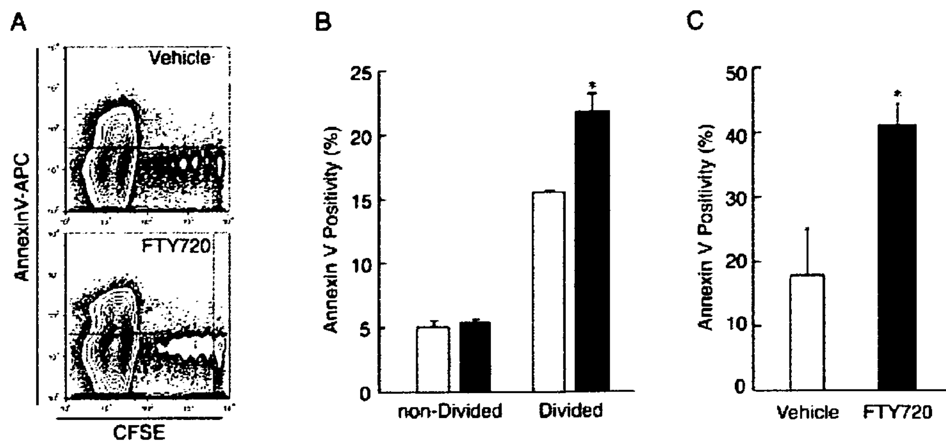

Figure 4. FTY720 predominantly enhances the apoptosis of host-reactive donor T cells. (A, B) BMT was performed as in Fig. 2 and FTY720 was daily administered. Four days after BMT, the MLN from each group were harvested and combined. CFSE dilution associated with Annexin-V expression was observed in CD45.1$^+$CD45.2$^-$DAPI$^-$ cells. The dot plots from representative recipients (A) and frequencies of Annexin-V$^+$ cells among non-divided and dividing donor T cells (B) are shown; APC, allophycocyanin. (C) B6D2F1 mice were transplanted with 2×10^6 T cells from B6-2C TCR-Tg mice plus 4×10^6 TCD BM cells from B6 mice, and FTY720 or diluent was administered from day 0. Annexin-V positivities in 1B2$^+$ T cells are shown as mean ± SE. The data are representative of two similar experiments; $^*p<0.05$.

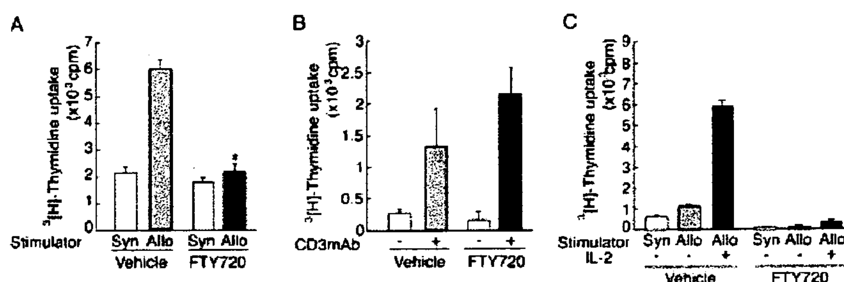

Figure 5. The alloreactivities of donor T cells from FTY720-treated recipients are reduced. BMT was performed as shown in Fig. 3 and FTY720 was administered on days 0–10. LN T cells isolated 11 days after BMT were re-stimulated with B6D2F1 (allogeneic) and B6 (syngeneic) stimulators (1×10^5 T cells/well) (A) and anti-CD3 mAb plus anti-CD28 mAb (1.5×10^4 T cells/well) (B). IL-2 was added to culture at a concentration of 10 U/mL (C). Cell proliferation as determined by the [^{3}H]thymidine uptakes are shown as the mean ± SD; Syn, syngeneic; Allo, allogeneic. The data are representative of two similar experiments; $^*p<0.05$.

dividing cells, but not of the non-dividing cells. To confirm the pro-apoptotic effect of FTY720 on alloreactive T cells, B6D2F1 mice were lethally irradiated and reconstituted with 2×10^6 T cells from host-MHC specific TCR-Tg (B6-2C TCR-Tg) mice plus 4×10^6 TCD BM cells from B6 mice. FTY720 again significantly increased Annexin-V positivities on 2C TCR-Tg T cells (Fig. 4C).

T cells isolated from MLN of B6D2F1 recipients of B6 donors 11 days after allogeneic BMT were cultured with host alloantigens *in vitro*. Alloantigen-induced proliferation was dramatically diminished in T cells from FTY720-treated recipients (Fig. 5A), but the non-specific proliferation to CD3 stimulation was not impaired (Fig. 5B). The addition of IL-2 in culture did not restore the allo-specific proliferation of donor T cells (Fig. 5C), thus ruling out anergy as the mechanism of the reduced alloresponse.

Adoptively-transferred T cells from FTY720-treated recipients induce a weak GVHD

We next examined whether the administration of FTY720 could lead to a reduction in the alloreactivity of donor T cells *in vivo*. Donor T cells (1×10^6) isolated from MLN of the recipients 6 days after allogeneic BMT were then transferred into lethally irradiated B6D2F1 recipients together with naive BM cells. In the secondary recipients of T cells from diluent-treated allogeneic recipients, severe GVHD developed and all died from GVHD (Fig. 6A). In contrast, the survivals of the secondary recipients of FTY720-treated T cells were significantly prolonged. The transfer of T cells isolated from MLN of FTY720-treated, non-transplanted naive B6 mice did not show an improved survival (Fig. 6B), thus indicating that the FTY720-

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induced reduction of alloreactivity occurs only after allogeneic BMT.

Caspase-dependent apoptosis is involved in the FTY720-induced modulation of GVHD

We next performed experiments to determine whether the caspase-dependent apoptosis of donor T cells is involved in the FTY720-induced reduction of alloreactivity of donor T cells, using a pan-caspase inhibitor, carbobenzoxy - valyl - alanyl - aspartyl - (β-o-methyl)-fluoromethylketon (ZVAD-fmk). B6D2F1 transplanted with cells from B6 donors received FTY720 at a dose of 0.3 mg/kg on days 0–5 and ZVAD-fmk at a dose of 10 mg/kg s.c. on days 1–5 post-transplantation. We delayed the administration of ZVAD-fmk by 24 h after BMT to avoid any suppressive effect on donor T cell activation by ZVAD-fmk according to the findings of a previous report [27].

The ZVAD-fmk treatment abrogated the protective effects of FTY720 for severity of GVHD as judged by changes in body weight and clinical scores on day 6 after BMT (Table 1). Adoptive transfer of 1×10^6 LN T cells isolated from FTY720-treated, ZVAD-fmk-untreated recipients again induced less severe GVHD in the secondary recipients than the transfer from the diluent-treated controls. These FTY720-induced protective effects in the secondary recipients were abrogated when ZVAD-fmk was administered to the primary recipients (Table 1). These data suggested that caspase-dependent apoptosis was involved in FTY720-induced protection against GVHD early after primary BMT and reduction of alloreactivities of donor T cells within host's LN.

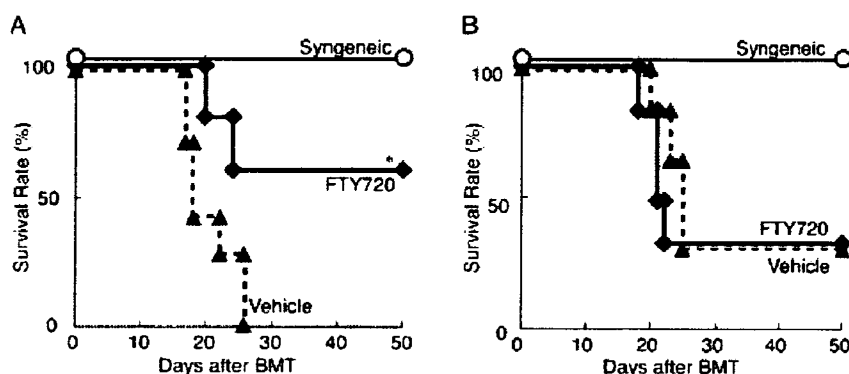

Figure 6. Adoptive transfer of donor T cells from FTY720-treated recipients transmits less severe GVHD in the secondary recipients. (A) 1×10^6 MLN T cells harvested from recipients 6 days after BMT were transferred into lethally irradiated secondary B6D2F1 recipients together with 2×10^6 naive TCD BM cells. The survival rates of the secondary recipients are shown (n=5–7/group). For syngeneic controls, MLN T cells and TCD BM cells isolated from naive B6D2F1 mice were transferred to lethally irradiated B6D2F1 mice. (B) Naive B6 mice were given FTY720 or diluent for 6 days. MLN T cells (1×10^6) were isolated from these mice and adoptively transferred as above (n=6/group). The data are representative of three experiments; $^*p<0.05$.

Table 1. ZVAD-fmk abrogates protective effects of FTY720[a)]

BMT					Adoptive Transfer		
BMT	FTY720	ZVAD	Day 6 BW (%)	Day 6 GVHD Scores	Survivors		
					Day 14	Day 28	Day 50
Syn	–	–	93.8±1.8	1.8±0.4	3/3[b)]	3/3[b)]	3/3[b)]
Allo	–	–	75.4±1.5	5.7±0.1	0/6	0/6	0/6
Allo	+	–	80.3±0.3[c)]	4.5±0.0[c)]	6/6[c)]	2/6[c)]	2/6[c)]
Allo	–	+	78.3±1.1	5.1±0.1	0/7	0/7	0/7
Allo	+	+	77.9±0.5	5.0±0.0	2/7	0/7	0/7

a) BMT was performed as in Fig. 3. FTY720 was administered on days 0–5 and ZVAD-fmk was injected s.c. at a dose of 10 mg/kg on days 1–5. Six days after BMT, GVHD severity was assessed by body weight (BW) loss and clinical GVHD scores, and LN T cells (1×10^6) harvested from recipients were transferred into secondary B6D2F1 recipients as in Fig. 6. The data from a representative experiment of three consecutive experiments are shown; Syn, syngeneic; Allo, allogeneic.

b) These controls were transferred with MLN T cells and TCD BM from naive B6D2F1 mice.

c) $p<0.05$ vs. FTY720-untreated, ZVAD-fmk-untreated allogeneic controls.

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