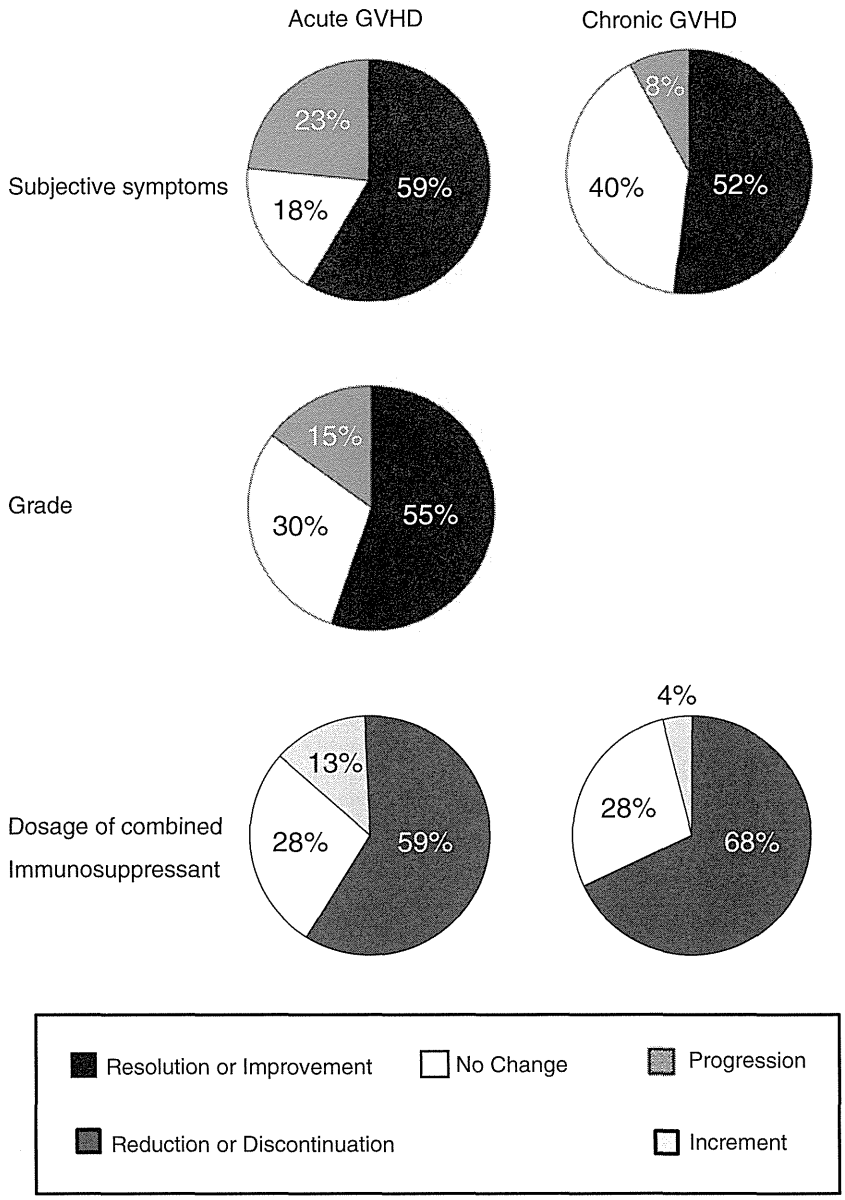

Fig. 4 Response of acute and chronic GVHD during therapeutic MMF use. Subjective symptoms of acute and chronic GVHD resolved in 59 and 52% of the cases, respectively, following the administration of MMF. In addition, 55% of the acute GVHD patients improved the grade of their disease. Finally, 60 and 68% of the acute and chronic GVHD patients, respectively, reduced or discontinued their use of combined immunosuppressant therapy

higher in patients receiving 2,000 mg per day than in those receiving 1,000 mg per day for chronic GVHD prevention, MMF doses of more than 2,000 mg per day are recommended for Japanese patients if the AEs are manageable.

Whether MMF is superior to existing immunosuppressants is a topic of continuing debate. Most previous reports on MMF have been promising, and the response rates for acute and chronic GVHD range from 47 to 71 and 26 to 76.9%, respectively, under various conditions [4, 6, 9–11, 17, 20]. On the other hand, one report suggested that MMF causes no significant improvement in the prevention of GVHD compared to cyclosporine and methotrexate (62 vs. 70%) [12]. Furthermore, another report showed that addition of MMF to an immunosuppressive regimen to control chronic GVHD had no effect (success rate of 15%) [22]. The results in this survey are not statistically different between using MMF and using cyclosporine or tacrolimus as reported in the previous report for the prevention and treatment of GVHD. We would like to emphasize, however, that the patient population in this study consisted mostly of HLA-mismatched donors and non-complete remission recipients (60.5 and 65.9%, respectively; Table 1). Even in this situation, MMF showed comparable efficacy. Therefore, we would like to conclude that MMF has a certain role for immunosuppressants.

Several reports have noted that the incidence of renal damage attributed to MMF (0–12.5%) is lower than that reported for other immunosuppressants like calcineurin inhibitors [4, 5, 11, 12, 23–25]. Our analysis revealed that the incidence of renal insufficiency (serum creatinine > 2 mg/dl)

was 1%. Serum creatinine > 2 mg/dl due to treatment with calcineurin inhibitors can be as high as 50–60 and 56–67% for cyclosporine and tacrolimus, respectively [26, 27]. Thus, MMF will be especially useful for patients with poor renal function.

In conclusion, MMF is tolerable and effective in Japanese patients who have received HSCT. Further studies are warranted to identify suitable candidates and appropriate therapeutic combinations of MMF for the prophylaxis and treatment of GVHD following allogeneic HSCT.

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ORIGINAL ARTICLE

Use of foscarnet for cytomegalovirus infection after allogeneic hematopoietic stem cell transplantation from a related donor

Maiko Asakura · Kazuhiro Ikegame · Satoshi Yoshihara · Shuichi Taniguchi · Takehiko Mori · Tetsuya Etoh · Akiyoshi Takami · Takashi Yoshida · Takahiro Fukuda · Kazuo Hatanaka · Heiwa Kanamori · Toshiaki Yujiri · Yoshiko Atsuta · Hisashi Sakamaki · Ritsuro Suzuki · Hiroyasu Ogawa

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Abstract Foscarnet is an active agent against cytomegalovirus (CMV) infection after hematopoietic stem cell transplantation (HSCT), as well as ganciclovir. We investigated the usefulness of foscarnet in patients who underwent related allogeneic HSCT. Foscarnet was used in 320 patients with a median age of 45 years (range 15–72). The purpose of administration was CMV disease in 65, preemptive use in 248 and prophylaxis in 7. Totally, 194 patients had a history of prior ganciclovir treatment. The reason for foscarnet use was insufficient therapeutic effect of prior ganciclovir in 99, and adverse event including myelosuppression in 95. The response rate in symptom was 52% for the CMV disease patients. Antigenemia disappeared in 77% of the preemptive treatment and improved in 13% of the patients. No outbreak of CMV disease was recognized. The total effectiveness of therapeutic and preemptive use was significantly higher for patients without prior ganciclovir (91 vs. 76%, $P = 0.001$). Adverse events of grade 3 or higher were recognized in 24%, including electrolyte abnormalities in 11%, neutropenia in 8%, and thrombocytopenia in 8%. Renal damage was only observed in 3% of patients. Foscarnet was concluded to be a safe and effective anti-CMV agent and to be a suitable alternative to ganciclovir.

Keywords Cytomegalovirus infection · Foscarnet · Blood and marrow transplantation · Efficacy · Adverse reaction

M. Asakura · Y. Atsuta · R. Suzuki (✉)
Department of HSCT Data Management and Biostatistics, Nagoya University School of Medicine, 1-1-20 Daiko-minami, Higashi-ku, Nagoya 461-0047, Japan
e-mail: r-suzuki@med.nagoya-u.ac.jp

K. Ikegame · S. Yoshihara · H. Ogawa
Division of Hematology, Department of Internal Medicine, Hyogo College of Medicine, Nishinomiya, Japan

S. Taniguchi
Department of Hematology, Toranomon Hospital, Tokyo, Japan

T. Mori
Division of Hematology, Department of Medicine, Keio University School of Medicine, Tokyo, Japan

T. Etoh
Department of Hematology, Hamanomachi Hospital, Fukuoka, Japan

A. Takami
Department of Cellular Transplantation Biology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan

T. Yoshida
Hematology Department, Toyama Prefectural Hospital, Toyama, Japan

T. Fukuda
Hematopoietic Stem Cell Transplantation Division, National Cancer Center Hospital, Tokyo, Japan

K. Hatanaka
Department of Internal Medicine, Rinku General Medical Center, Izumisano, Japan

H. Kanamori
Department of Hematology, Kanagawa Cancer Center, Yokohama, Japan

T. Yujiri
Third Department of Internal Medicine, Yamaguchi University School of Medicine, Ube, Japan

H. Sakamaki
Department of Hematology, Tokyo Metropolitan Komagome Hospital, Tokyo, Japan

1 Introduction

Cytomegalovirus (CMV) disease is one of the most important infectious complications after allogeneic hematopoietic stem cell transplantation (HSCT), which influences the outcome of the transplantation. The presence of graft-versus-host disease and steroid therapy are associated with the occurrence of CMV infection or reactivation. Ganciclovir is used as a first-line agent for both prophylaxis and the treatment of CMV disease [1–5]. However, approximately one-third of patients receiving ganciclovir develop drug-induced neutropenia or thrombocytopenia [6–9]. Therefore, ganciclovir is unsuitable for use in patients with poor bone marrow function. Another problem is ganciclovir resistant CMV [10–12].

For such cases, foscarnet is an important alternative agent that demonstrates anti-viral activity against all known herpes viruses including CMV [11, 13–15]. In early studies, the dose-limiting toxicities of foscarnet were found to be nephrotoxicity and neurotoxicity, which were seen in up to 50% of patients [16, 17]. Two randomized controlled trials (RCT) comparing the usefulness of preemptive foscarnet versus ganciclovir have been performed for CMV antigenemia [18, 19]. These studies revealed that the effectiveness of foscarnet was equivalent to that of ganciclovir. Adverse reactions and treatment-related mortality of foscarnet were also the same as those of ganciclovir. Renal dysfunction was only noted in 5% of the patients that received foscarnet [19].

The use of foscarnet has also been reported in cord blood transplantation, which is more complicated by viral infection [20]. These studies including the RCT only involved patients who had received foscarnet as an initial therapy. Therefore, we conducted a nationwide study in Japan of the use of foscarnet against CMV infection after related HSCT to investigate the current status, and compared its efficacy and toxicity in patients with and without prior ganciclovir use.

2 Patients and methods

2.1 Study design

This study is a retrospective survey investigating the use of foscarnet after stem cell transplantation. The subjects of this study were patients who received foscarnet after receiving allogeneic transplantation from a related donor in the period from 1998 to 2008. We performed a questionnaire at institutions carrying out allogeneic stem cell transplants in Japan. Data regarding the presence of CMV disease, CMV antigenemia, the reason for foscarnet use, the dose and duration of foscarnet, the effectiveness of therapy, and adverse events were collected. The obtained data were combined with data from the national registry of the Japan Society of Hematopoietic Cell Transplantation, which was collected by the TRUMP system [21]. This study was approved by the Ethical Committees of the Japan Society of Hematopoietic Cell Transplantation and Hyogo College of Medicine.

2.2 CMV antigenemia assay

Cytomegalovirus antigenemia was measured as described previously [22, 23]. Briefly, peripheral white blood cells were attached to slides by cytocentrifugation and stained with HRP-C7 (Teijin, Tokyo, Japan) or C10/C11 (Biotest, Dreieich, Germany) monoclonal antibodies. The number of positive cells was counted per 50,000 attached cells for HRP-C7 and per 150,000 applied cells for C10/C11. The examination was performed in duplicate, and the mean was used for further analyses.

2.3 Definition of CMV disease and infection

CMV diseases were defined as any organ infections by CMV, ideally proven by histopathologic examinations. They include gastroenteritis, pneumonia, retinitis, hepatitis, encephalitis, and cystitis. Patients who presented with interstitial pneumonia accompanied by CMV antigenemia were also diagnosed with CMV disease (pneumonia). For patients who presented with antigenemia and simultaneous diarrhea, gastrointestinal endoscopy and biopsy were recommended, but those who could not receive such diagnostic procedure were regarded as suspicious CMV disease (gastroenteritis). Both CMV antigenemia and CMV disease were regarded as CMV infection.

2.4 Type of therapy

The administration of anti-viral agents for patients without any CMV disease but accompanied by CMV antigenemia with or without febrile complications was defined as preemptive therapy in this study. Therapy of CMV disease was defined as CMV treatment. The use of anti-viral agents for those without antigenemia or CMV disease was regarded as prophylaxis.

2.5 Statistics

Pairwise comparisons were performed using the χ^2 test and Fisher's exact test for categorical variables, and the Mann–Whitney U test for continuous variables. The Kruskal–Wallis test was used to compare multiple groups. P values of <0.05 obtained in 2-sided tests were considered statistically significant. Data were analyzed with the STATA version 11 statistical software (STATA Corp, TX, USA).

3 Results

3.1 Patient characteristics

The background data of 320 patients are shown in Table 1. There were 171 males and 149 females. Their median age was 45 years, and the ages of the patients ranged from 15 to 72 years. The underlying disease of patients was acute myeloid leukemia (AML) in 110, acute lymphoblastic leukemia (ALL) in 59, chronic myelogenous leukemia (CML) in 18, myelodysplastic syndrome (MDS)/myeloproliferative disorder (MPD) in 42, chronic lymphocytic leukemia (CLL) in 2, non-Hodgkin lymphoma (NHL) in 51, Hodgkin lymphoma (HL) in 4, adult T cell lymphoma (ATL) in 16, multiple myeloma (MM) in 10, aplastic anemia (AA) in 6 and 1 each for renal cell carcinoma and virus associated hemophagocytic syndrome. Several demographic data were not available due to the lack of patient entry to the TRUMP system. CMV antibody was positive in both the patient and donor in 189 pairs (59%), in the patient only in 22 cases (7%), and in the donor only in 8 cases (3%), and it was negative in both patient and donor in 4 pairs (1%). Of 289 patients with evaluable data, 113 patients received bone marrow (BM) as a graft, 172 received peripheral blood stem cell (PBSC), and 4 received both BM and PBSC. HLA was matched in 108 of 268 patients but was mismatched in the remaining 160 (155 with serological mismatch and 5 with allele mismatch).

Table 1 Patient characteristics

Variables	Number
Patient number	320
Median age (range)	45 (15–72)
Male/female	171/149
Disease	
Acute myeloid leukemia	110
Acute lymphoblastic leukemia	59
Chronic myelogenous leukemia	18
Myelodysplastic/myeloproliferative syndrome	42
Chronic lymphocytic leukemia	2
Non-Hodgkin lymphoma	51
Hodgkin lymphoma	4
Adult T cell leukemia	16
Multiple myeloma	10
Aplastic anemia	6
Other diseases	2
CMV serology	
Donor +/Patient +	189
Donor +/Patient −	8
Donor −/Patient +	22
Donor −/Patient −	4
Graft source	
Bone marrow (BM)	113
Peripheral blood stem cell (PBSC)	172
Both BM and PBSC	4
Donor type	
Matched related	108
Mismatched related	160

3.2 CMV infection

Foscarnet was administered for CMV disease in 65 patients (20%), including 46 with gastroenteritis, 12 with pneumonia, 2 with retinitis, and one each for hepatitis, encephalitis, and cystitis. Each one other patient developed pneumonia and retinitis accompanied by simultaneous gastroenteritis. On the other hand, 248 (78%) were preemptively treated (only complicated with CMV antigenemia), and 7 (2%) were prophylactically treated. Before foscarnet administration, 194 (61%) patients had received ganciclovir, and one of the patients was treated with cidofovir after ganciclovir use. The reason for changing the anti-viral agent to foscarnet was insufficient therapeutic effect in 99 patients and adverse events due to preceding ganciclovir including myelosuppression in 95 patients. In 126 patients who had not received any anti-viral premedication, foscarnet was used because of poor bone marrow function in 116.

A total of 208 patients (67%) received steroid therapy at the time of foscarnet initiation. The rate of patients under steroid use was 58% for CMV disease, 70% for preemptive foscarnet, and 43% for prophylaxis, but the difference was not significant ($P = 0.08$).

3.3 Dosage of foscarnet

The initial dose of foscarnet ranged from 7 mg/kg to 216 mg/kg (median 88 mg/kg, Fig. 1). The dose was

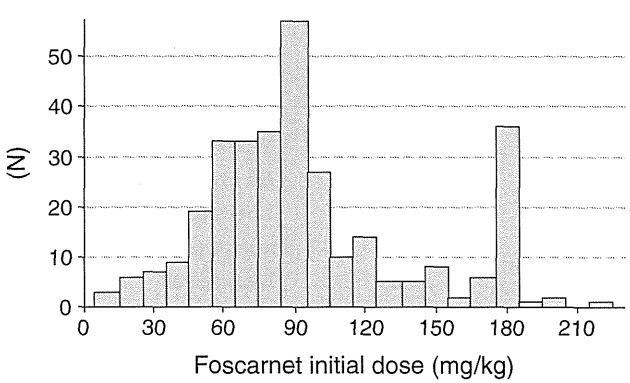

Fig. 1 Initial dose of foscarnet. Foscarnet was given at a variety of doses ranging from 7 to 216 mg/kg (median 88 mg/kg). Two peaks at 90 and 180 mg/kg were seen in the histogram

significantly higher in the patients who had received prior ganciclovir (range 10–216 mg/kg, median 91 mg/kg) than those who had not (range 7–180 mg/kg, median 72 mg/kg) ($P < 0.0001$). The median dose in the preemptive, treatment, and prophylactic groups was 89, 90, and 63 mg/kg, respectively; i.e., it was significantly lower in the prophylactic use group ($P = 0.05$). The initial dose of foscarnet did not have any correlation with creatinine clearance calculated from serum creatinine level and age by the Modification of Diet in Renal Disorder (MDRD) formula ($r = 0.21$, Fig. 2). The duration of foscarnet use ranged from 1 to 163 days (median 20 days) and was significantly shorter for patients who had received prior ganciclovir than those who had not (median 17 vs. 22 days, $P = 0.05$). As there were two peaks at 90 and 180 mg/kg in the dose of foscarnet administered, 5 dose categories (0–39, 40–79, 80–99, 100–159, and 160–220) were defined, and the efficacy and toxicity of foscarnet were estimated according to this categorization.

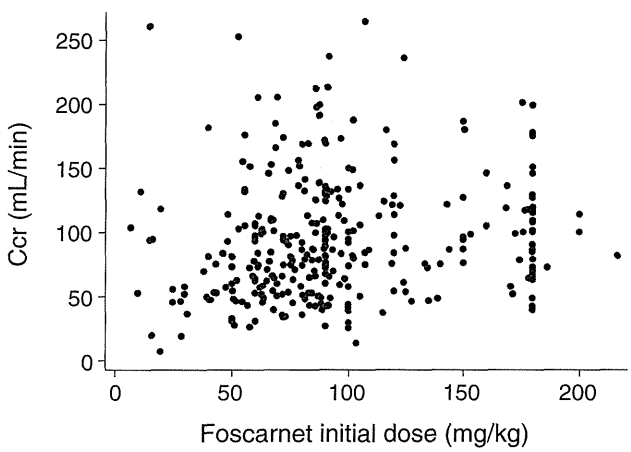

Fig. 2 Relationship between the initial dose of foscarnet and creatinine clearance. Creatinine clearance was calculated from serum creatinine level and age by the Modification of Diet in Renal Disorder (MDRD) formula [Ccr for male = $0.741 \times 175 \times (\text{age})^{(-0.203)} \times (\text{serum creatinine})^{(-1.154)}$, Ccr for female = $0.741 \times 175 \times (\text{age})^{(-0.203)} \times (\text{serum creatinine})^{(-1.154)} \times 0.742$]. No correlation was found ($r = 0.21$)

3.4 Efficacy

Among 65 patients with CMV disease, the symptoms disappeared in 5 (8%) and improved in 28 (44%), no change was seen in 20 (32%), and the symptoms worsened in 10 (16%) (Table 2). One patient was not evaluable with regards to their response, and another patient did not have any symptoms at the initiation of foscarnet because of the effect of prior ganciclovir use. The effectiveness (resolved or improved) was higher in those who did not receive ganciclovir, but the difference was not statistically significant (71 vs. 46%, $P = 0.10$). When the effectiveness in symptom was compared between HLA-matched and -mismatched transplant, the rate was almost comparable (14/25 = 56% vs. 14/29 = 48%, $P = 0.60$). Among 238 evaluable patients who received preemptive CMV therapy, antigenemia was resolved in 183 (77%) and improved in 31 (13%), but was not changed in 17 (7%) and worsened in 7 (3%). No patient developed outbreaks of CMV disease. The effectiveness was higher for those who had not received prior ganciclovir, but the difference was not significant (93/99 = 93% vs. 121/139 = 87%, $P = 0.13$).

Table 2 Response to foscarnet

	Symptoms				Antigenemia			
	Prior GCV		No prior GCV		Prior GCV		No prior GCV	
	N	%	*N*	%	*N*	%	*N*	%
CMV disease								
Disappeared	4	9	1	6	26	65	8	89
Improved/decreased	17	37	11	65	7	18	1	11
No change	18	39	2	12	4	10	0	0
Worsened/increased	7	15	3	18	3	8	0	0
No symptoms/antigenemia	1[a]	–	–	–	7	–	8	–
Unevaluable	1	–	0	–	1	–	0	–
Preemptive								
Disappeared	–	–	105	74	78	80		
Decreased	–	–	17	12	14	14		
No change	–	–	14	10	3	3		
Increased	–	–	5	4	2	2		
No antigenemia	–	–	4[a]	–	–	–		
Unevaluable	–	–	3	–	3	–		

GCV ganciclovir

[a] Symptoms/antigenemia had disappeared after prior GCV

Although the effectiveness in preemptive use was lower in HLA-matched transplant as compared with HLA-mismatched transplant, the difference was not also significant (64/75 = 85% vs. 114/123 = 93%, $P = 0.14$). Among the patients who received prior ganciclovir, the effectiveness was significantly higher in the patients in whom an insufficient effect of ganciclovir was seen compared with those who had suffered an adverse reaction to ganciclovir (64/68 = 94% vs. 57/71 = 80%, $P = 0.02$). The overall effectiveness of treatment and preemptive use was significantly higher in those who had not received prior ganciclovir (91 vs. 76%, $P = 0.001$) because of the low effectiveness in the patients of the CMV disease group who had received prior ganciclovir use. The changing courses of CMV antigenemia are box plotted in Fig. 3a for the patients who received prior ganciclovir and in Fig. 3b for those who did not. After the administration of foscarnet, the CMV antigenemia decreased in both groups ($P < 0.0001$ and $P = 0.01$, respectively).

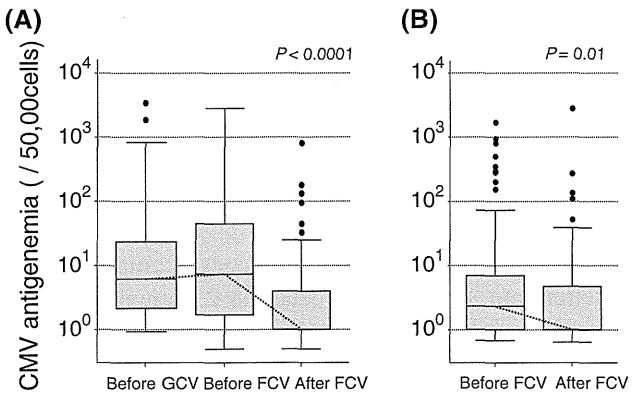

Fig. 3 Change in CMV antigenemia due to foscarnet therapy. The levels of antigenemia before ganciclovir, before foscarnet, and after foscarnet are box plotted. A significant decrease in antigenemia due to foscarnet treatment was observed both (**a**) for patients who had received prior ganciclovir treatment and (**b**) for those who had not

The responses to foscarnet according to the 5 dose categories are summarized in Fig. 4. The symptoms of CMV disease improved in around 50% of patients in every dose category. In the CMV disease patients the response rate of

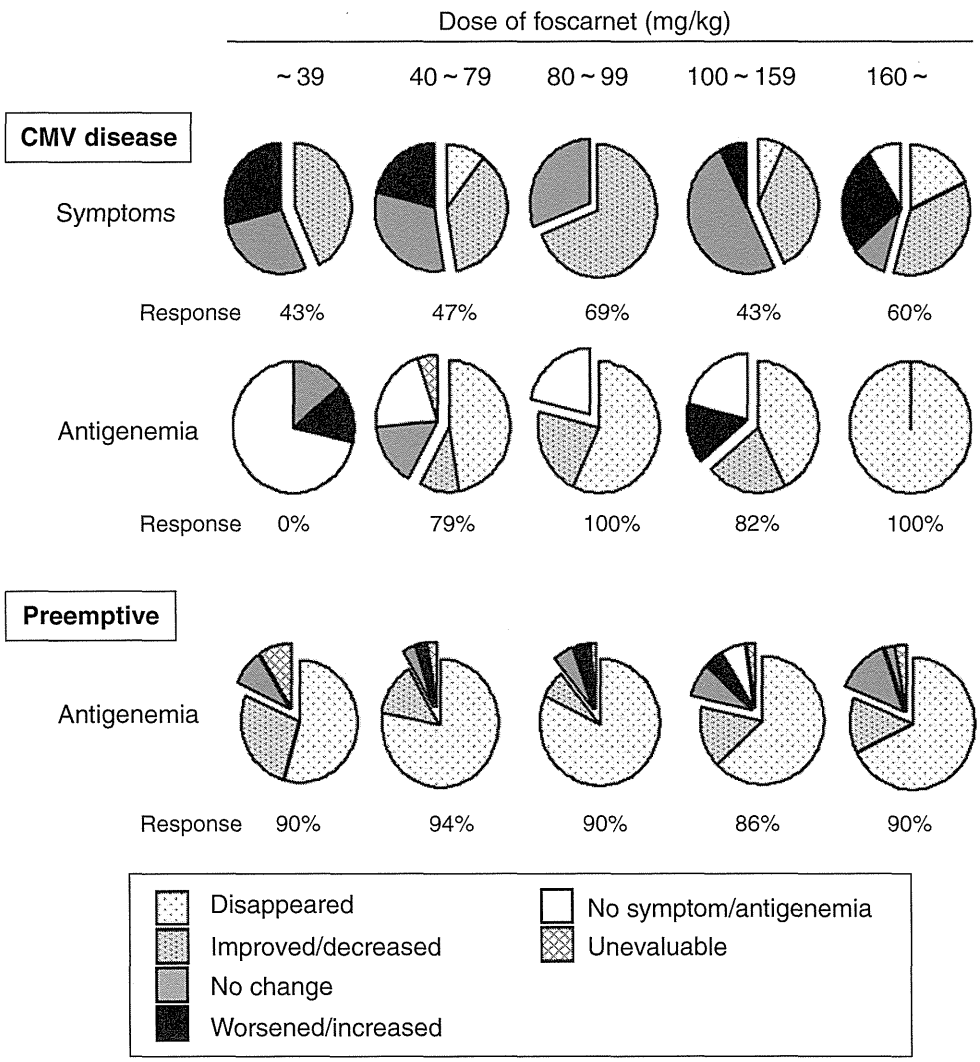

Fig. 4 Response to foscarnet according to 5 dose categories. The number of patients from the CMV disease group was 7 in the <39 mg/kg group, 19 in the 40–79 mg/kg group, 14 in the 80–99 mg/kg group, 14 in the 100–159 mg/kg group, and 11 in the 160 mg/kg or higher group, and those of the preemptive group were 11, 81, 73, 46, and 37, respectively. The response rate was around 50% for symptoms of CMV disease and was generally higher for antigenemia

antigenemia was significantly lower for those received foscarnet <40 mg/kg ($P = 0.01$).

3.5 Survival

The overall survival of all patients who received foscarnet was 34% at a median follow-up of 3 years (Fig. 5a). Patients with CMV disease showed significantly lower survival than those who received preemptive or prophylactic therapy (Fig. 5b, $P = 0.0004$). No significant difference in prognosis was found between the patients with and without preceding other anti-viral agents ($P = 0.21$). A total of 170 patients died, and the main causes of death were disease recurrence in 47, bacterial sepsis in 27, acute/chronic graft-versus-host disease in 25, and fungal infection in 10. The cumulative incidence of transplant-related mortality at 1 year was 30% (95% confidence interval 25–35%). Three patients eventually died of CMV disease, and the cumulative incidence of CMV-associated death at 1 year was 1.0% (95% confidence interval 0.3–2.6%).

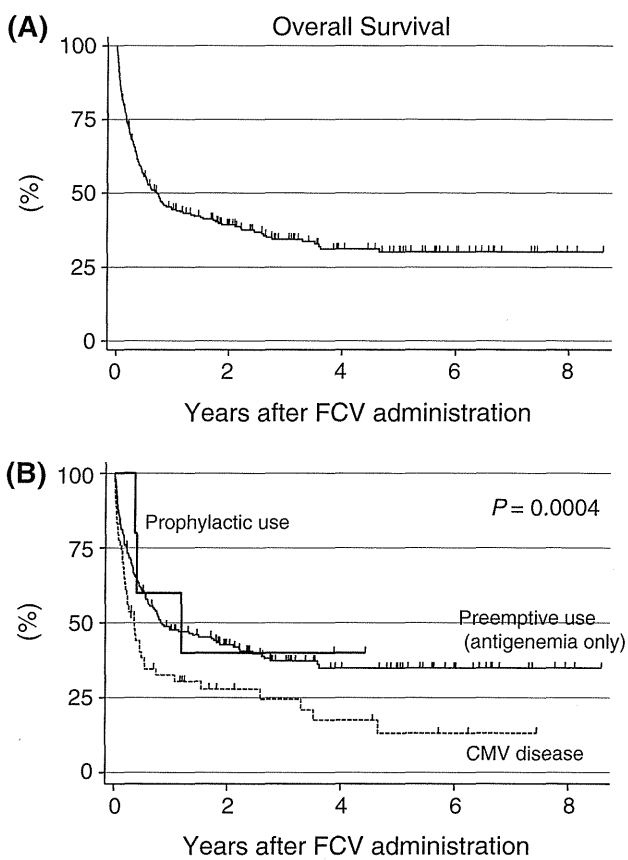

Fig. 5 Overall survival (OS) of patients who received foscarnet therapy. **a** The 3-year OS was 34%. **b** The prognosis of patients with CMV disease was significantly poorer than those of patients who had received preemptive or prophylactic use ($P = 0.0004$)

3.6 Adverse events

Adverse events (irrespective of causal association) of NCI-CTCAE grade 3 or higher are listed in Table 3. The most common adverse event was electrolyte abnormalities, which occurred in 35 patients (11%). The other major toxic events included neutropenia in 27 patients, thrombocytopenia in 26 patients, and bone marrow dysfunction in 11 patients. Renal and hepatic damage developed in 11 and 10 patients, respectively. Adverse events associated with foscarnet included neutropenia in 5 patients; electrolyte abnormalities in 4 patients; thrombocytopenia, renal dysfunction and sensory disturbance in 2 patients each; and bone marrow dysfunction in 1 patient. No patient died of an adverse reaction associated with foscarnet. The total number of patients who developed a grade 3 adverse reaction or higher was 56 (28%) in the patients who received prior ganciclovir and 21 (17%) in those who did not ($P = 0.03$). The rate of adverse events did not differ among the 5 dose categories (Table 4). The duration of foscarnet medication was not different between patients who developed adverse event of grade 3 or more (median 16 days, range 2–121) and those did not (median 20 days,

Table 3 Adverse events during foscarnet treatment

	Prior GCV $N = 198$		No prior GCV $N = 122$		Total $N = 320$	
	N	%	*N*	%	*N*	%
Graft failure	2	1.0	2	1.6	4	1.3
Neutropenia	19	9.6	8	6.6	27	8.4
Grade 3	7	3.5	2	1.6	9	2.8
Grade 4	12	6.1	6	4.9	18	5.6
Thrombocytopenia	19	9.6	7	5.7	26	8.1
Grade 3	6	3.0	0	0.0	6	1.9
Grade 4	13	6.6	7	5.7	20	6.3
BM dysfunction	7	3.5	3	2.5	10	3.1
Grade 3	4	2.0	1	0.8	5	1.6
Grade 4	3	1.5	2	1.6	5	1.6
Renal damage	6	3.0	5	4.1	11	3.4
Grade 3	4	2.0	5	4.1	9	2.8
Grade 4	2	1.0	0	0.0	2	0.6
Electrolyte abnormality	27	13.6	8	6.6	35	10.9
Grade 3	20	10.1	7	5.7	27	8.4
Grade 4	7	3.5	1	0.8	8	2.5
Neurological	3	1.5	1	0.8	4	1.3
Grade 3	3	1.5	1	0.8	4	1.3
Grade 4	0	0.0	0	0.0	0	0.0
Liver damage	9	4.5	1	0.8	10	3.1
Grade 3	7	3.5	0	0.0	7	2.2
Grade 4	2	1.0	1	0.8	3	0.9

BM bone marrow

Table 4 Adverse effects according to foscarnet dose

Dose level (mg/kg)	0–39 $N = 18$ (%)	40–79 $N = 106$ (%)	80–99 $N = 88$ (%)	100–159 $N = 60$ (%)	160– $N = 48$ (%)	Total $N = 320$ (%)
Any grade 3 or higher	33	23	17	25	35	24
Grade 3 or higher, possibly by foscarnet	28	12	13	17	17	15
Grade 3 or higher, definitely by foscarnet	0	2.8	3.4	8.3	6.3	4.4

range 1–322, $P = 0.50$). The difference was not evident for patients with possible and definite association with foscarnet ($P = 0.84$ and $P = 0.22$, respectively). When the adverse events were compared between HLA-matched and -mismatched transplant, the rates were significantly higher in the HLA-matched transplant. Any grade 3 or more toxicity was developed in 36 of 108 HLA-matched and 33 of 160 HLA-mismatched transplant ($P = 0.02$). Of these, 31 and 24, respectively, were possibly due to foscarnet use (29 vs. 15%, $P = 0.009$).

4 Discussion

The present study demonstrated that foscarnet is effective for patients with CMV infection who are not suitable for ganciclovir therapy. Sixty percent of the patients had a history of prior ganciclovir, but had demonstrated problems of ineffectiveness and/or adverse reactions. The remaining 40% had poor bone marrow function, and therefore foscarnet had been selected as the up-front use. In both situations, most of the patients were preemptively treated, and prophylactic use was seen in <2% of cases in our series.

The initial dose of foscarnet had two convergent doses, which were 90 and 180 mg/kg. The former corresponds to the maintenance dose, and the latter is the initial dose which was used in most prospective studies [18, 19]. The dose of foscarnet was significantly higher in patients with secondary therapy. This might have resulted from a higher number of more severe patients with CMV infection being present in the secondary therapy group. On the other hand, no dosage differences were found between the various purpose groups (preemptive/prophylactic/treatment). The lack of a correlation between foscarnet dose and creatinine clearance suggested that foscarnet was used irrespective of the renal function of the patient.

The most important adverse reaction of foscarnet was previously described as renal damage including electrolyte abnormalities. In that study, one-third of patients developed renal insufficiency and/or electrolyte disturbance [15]. However, a later study showed that these adverse events occurred less frequently [19]. In our series of patients, electrolyte abnormalities were recognized in 11% of patients, and renal insufficiency was found in no >3% of patients, which was consistent with the findings in the literature [24]. Thus, foscarnet seems to be a safer drug than was initially predicted.

In the preemptive use of foscarnet, >80% of patients showed CMV antigenemia disappearance in both the initial and secondary therapy groups. Foscarnet was highly effective in this setting, but its efficacy was decreased in CMV disease. The efficacy of foscarnet did not correlate with its dose, which was contradictory to a previous dose-finding study [25]. Our findings suggest a need to explore appropriate therapeutic strategies for this agent. Recently, "low-dose" administration of foscarnet at 60 mg/kg/day has been reported to be effective for CMV preemptive treatment [26, 27], which could be an option for future clinical trials. A prospective trial comparing ganciclovir alone and a combination of ganciclovir and foscarnet (half doses of both) was performed for HSCT and organ transplant patients [28]. The efficacy was equivalent for both arms, but adverse events were more frequent in the foscarnet combined arm.

In conclusion, our study shows that foscarnet is a safe and effective agent for treating CMV antigenemia after allogeneic HSCT. It remains to be determined how CMV infections should be treated, as well as how to improve the survival of affected patients.

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Graft-versus-host disease disrupts intestinal microbial ecology by inhibiting Paneth cell production of α-defensins

Yoshihiro Eriguchi,[1] Shuichiro Takashima,[1] Hideyo Oka,[1] Sonoko Shimoji,[1] Kiminori Nakamura,[2] Hidetaka Uryu,[1] Shinji Shimoda,[1] Hiromi Iwasaki,[3] Nobuyuki Shimono,[1] Tokiyoshi Ayabe,[2] Koichi Akashi,[1,3] and Takanori Teshima[3]

[1]Department of Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Science, Fukuoka, Japan; [2]Department of Cell Biological Science, Graduate School of Life Science, Faculty of Advanced Life Science, Hokkaido University, Sapporo, Japan; and [3]Center for Cellular and Molecular Medicine, Kyushu University Graduate School of Medical Science, Fukuoka, Japan

Allogeneic hematopoietic stem cell transplantation (SCT) is a curative therapy for various hematologic disorders. Graft-versus-host disease (GVHD) and infections are the major complications of SCT, and their close relationship has been suggested. In this study, we evaluated a link between 2 complications in mouse models. The intestinal microbial communities are actively regulated by Paneth cells through their secretion of antimicrobial peptides, α-defensins. We discovered that Paneth cells are targeted by GVHD, resulting in marked reduction in the expression of α-defensins, which selectively kill noncommensals, while preserving commensals. Molecular profiling of intestinal microbial communities showed loss of physiologic diversity among the microflora and the overwhelming expansion of otherwise rare bacteria *Escherichia coli*, which caused septicemia. These changes occurred only in mice with GVHD, independently on conditioning-induced intestinal injury, and there was a significant correlation between alteration in the intestinal microbiota and GVHD severity. Oral administration of polymyxin B inhibited outgrowth of *E coli* and ameliorated GVHD. These results reveal the novel mechanism responsible for shift in the gut flora from commensals toward the widespread prevalence of pathogens and the previously unrecognized association between GVHD and infection after allogeneic SCT. (*Blood*. 2012;120(1): 223-231)

Introduction

Allogeneic hematopoietic stem cell transplantation (SCT) is a curative therapy for hematologic malignant tumors, bone marrow failure, and congenital metabolic disorders. Graft-versus-host disease (GVHD) and related infections are major obstacles to SCT, and their close relationship has been indicated in clinical settings. Septicemia is the most life-threatening infection after allogeneic SCT and gram-negative rods are the most dominant pathogens of septicemia, whereas incidence of drug-resistant enterococci infection increase in neutropenic patients colonized with these bacteria in some centers.[1] GVHD is one of the major predisposing factors for the development of septicemia.[2] Since the pioneering works of van Bekkum[3] and others in the 1960s-1970s, interaction between intestinal flora and GVHD has been suggested.[3-6]

We recently demonstrated that intestinal stem cells (ISCs), which are essential to repair damaged intestinal epithelium, are targeted by GVHD.[7] Recently, Paneth cells located besides ISCs within the crypts are identified as niche for ISCs.[8] In addition, Paneth cells are essential regulators of the composition of intestinal microbiota by secreting antimicrobial peptides, α-defensins, which provide broad-spectrum antimicrobial properties by pore formation in the bacterial cell walls.[9-11] The intestine, which is the major interface between the environment and the host, is an open ecologic system that is colonized by at least 1000 distinct bacterial species, of which more than 80% are nonculturable.[12-14] Accurate identification of species in the gut microbiota requires culture-independent, molecular profiling methods. Firmicutes and Bacteroidetes make up approximately 90% of the intestinal microbiota.[12,15] These commensals are rarely pathogenic and instead make several essential contributions to human physiology and health.[12,13,15,16] In contrast, Gammaproteobacteria such as *Escherichia coli*, which have a gram-negative cell wall make up a small proportion of the microbiota.[17] A recent study showed an increase in gram-negative *Enterobacteriaceae* family members including *E coli* among the intestinal microbiota after allogeneic bone marrow transplantation (BMT) in mice.[18] It remains unclear why they are most frequent pathogens in patients with intestinal GVHD, although the role of systemic immunosuppression and use of antibiotics has been well appreciated.[19]

In this study, we focused on Paneth cells and evaluated the possible mechanistic links between GVHD and infection in mouse models of BMT. We found that GVHD targets Paneth cells and causes subsequent impairment of antimicrobial peptide secretion, leading to marked loss of diversity among the intestinal microflora. This results in shift in the gut flora from commensal microorganisms toward the widespread prevalence of gram-negative bacteria and development of bloodstream infection.

Methods

Mice

Female C57BL/6 (B6: H-2^b), B6D2F1 (H-$2^{b/d}$), B6C3F1 (H-$2^{b/k}$), B6-Ly5.1 (H-2^b, CD45.1^+), and C3H.Sw (H-2^b) mice were purchased from Charles

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River Japan, KBT Oriental, or Japan SLC. All animal experiments were performed under the auspices of the Institutional Animal Care and Research Advisory Committee.

BMT

Mice were transplanted as previously described.[20] In brief, after lethal x-ray total body irradiation (TBI) delivered in 2 doses at 4-hour intervals, mice were intravenously injected with 5×10^6 T-cell depleted bone marrow (TCD-BM) cells with or without 2×10^6 splenic T cells on day 0. Isolation of T cells and T-cell depletion were performed using the T-cell isolation kit and anti-CD90 microBeads, respectively, and the AutoMACS (Miltenyi Biotec) according to the manufacturer's instructions. In some experiments, unirradiated B6D2F1 mice were intravenously injected with 12×10^7 splenocytes.[7] Mice were maintained in specific pathogen-free conditions and received normal chow and autoclaved hyperchlorinated water (Ph 4) for the first 3 weeks after BMT and filtered water thereafter. Polymyxin B (Calbiochem) diluted in water was administered by daily oral gavage at a dose of 100 mg/kg from day −4 until day 28 after BMT. Survival after BMT was monitored daily and the degree of clinical GVHD was assessed weekly by a scoring system which sums changes in 5 clinical parameters: weight loss, posture, activity, fur texture, and skin integrity (maximum index = 10) as previously described.[20]

Histologic and immunohistochemical analysis

For pathologic analysis, samples of the small intestine were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned, slide mounted, and stained with H&E. Immunohistochemistry was performed as described[21] using rabbit anti-lysozyme (Dako) and rabbit anti-defensin1. Histofine simple stain MAX PO (Rat) kits and subsequently diaminobenzide (DAB) solution (Nichirei Biosciences) was used to generate brown-colored signals. Slides were then counterstained with hematoxylin. Pictures from tissue sections were taken at room temperature using a digital camera (DP72; Olympus) mounted on a microscope (BX51; Olympus). Acute GVHD was assessed by detailed histopathologic analysis using a semiquantitative scoring system.[22]

Preparation and analysis of isolated mouse crypts

Individual crypts were isolated from the small intestine as previously described.[23] Isolated crypts were fixed with 2% paraformaldehyde in PBS for 20 minutes and permeabilized with 0.2% Triton X-100 in PBS for 5 minutes. Crypts were incubated for 1 hour with fluorescein isothiocyanate-conjugated anti-lysozyme (10 μg/mL; Dako), washed 3 times in PBS, followed by incubation for 1 hour with Allexa Fluor 594–conjugated phalloidin (1 U/mL; Invitrogen). Tetramethyl 4,6-diamidino-2-phenylindole (DAPI; 5 μg/mL; Invitrogen) was used to stain the nucleus. Samples were mounted in aqua poly/mount (Polysciences) and examined with a confocal laser-scanning microscope (LSM510; Carl Zeiss).

Enzyme-linked immunosorbent assay

The limulus amebocyte lysate assay QCL-1000 (Lonza) was performed according to the manufacturer's instructions to determine the serum level of lipopolysaccharide (LPS) with a sensitivity of 0.1 EU/mL. All units expressed are relative to the United States reference standard EC-2.

Quantitative real-time PCR analysis

Total RNA was purified using the RNeasy Kit (QIAGEN). cDNA was synthesized using a QuantiTect reverse transcription kit (QIAGEN). Polymerase chain reactions (PCRs) and analyses were performed with ABI PRISM 7900HT SDS 2.1 (Applied Biosystems) using TaqMan universal PCR master mix (Applied Biosystems), and TaqMan gene expression assays (Defa1: Mm02524428_g1, Defa4: Mm00651736_g1, Defa5: Mm00651548_g1, Defa21/Defa22: Mm04206099_gH, Defcr-rs1: Mm00655850_m1, Lyz1: Mm00657323_m1, and Gapdh: Mm99999915_g1; Applied Biosystems). The relative amount of each mRNA was determined using the standard curve method and was normalized to the level of GAPDH in each sample.

Total fecal bacterial DNA extraction

Total DNA was isolated from fecal pellets using a QIAamp DNA stool mini kit (QIAGEN) with bead beating treatment during the cell-lysis step. Briefly, fresh fecal pellets were collected from individual mice; 0.5 g baked 0.1 mm zirconia/silica beads (Biospec Products) and ASL buffer were added to each aliquot. Fecal samples with ASL buffer were incubated at 95°C, and samples were processed for 1 minute at speed 5.5 on Fastprep system (Qbiogene).[24]

PCR amplification of 16S rRNA gene

Bacterial 16S ribosomal RNA (rRNA) genes were amplified with bacterial-universal primers, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) labeled at the 5′ end with 6-carboxyfluorescein (6-FAM) and 1492R (5′-GGTTACCTTGT TACGACTT-3′).[25] PCR amplification was performed using *EX Taq* (Takara Bio) and the following program: 3 minutes of denaturation at 95°C, 30 cycles of 0.5 minute at 95°C, 0.5 minute at 50°C, 1.5 minute at 72°C, and a final 10 minutes extension step at 72°C in a BiometraT3 thermocycler (Biometra). Amplicons were purified using a QIAquick PCR Purification kit (QIAGEN).

Restriction fragment length polymorphism (RFLP) analysis

The purified DNA products (3 μL) were digested with 10 U of either *Hha*I or *Msp*I (Takara Bio) in a total volume of 10 μL at 37°C for 3 hours. The restriction digest products (2 μL) were mixed with 10 μL deionized formamide and 0.5 μL GeneScan-1200 LIZ standard (Applied Biosystems). The samples were denatured at 95°C for 2 minutes, followed by rapid chilling on ice. The fluorescently labeled fragments (T-RFs) were separated by size on an ABI 3130 genetic analyzer (Applied Biosystems). The electropherograms were analyzed with GeneMapper Version 4.0 software (Applied Biosystems), and the fragment sizes were estimated using the Local Southern method. Each unique RFLP pattern was designated as an operational taxonomic unit (OTU). OTUs with a peak area of less than 0.5% of the total area were excluded from the analysis. Proportion of *E coli* was defined as the ratio of area of OTU for *E coli* to total areas of OTUs. Diversity of the microbial community corresponding to the RFLP banding pattern was calculated using the Simpson index of diversity 1-D ($D = \Sigma pi^2$)[26] and Shannon diversity index H′ ($H' = -\Sigma piln(pi)$)[27] and where pi is the proportion of total number of species made up of its species.

Cloning and sequencing analysis

Internal region of the 16S rRNA genes were amplified using 27F and 806R (5′-GGACTACCAGGGTATCTAAT-3′) primers, and were transformed using TOPO TA Cloning Kit with TOP10 *E coli* (Invitrogen). The nucleotide sequences of inserts were determined using the M13 forward and reverse primers. All sequences were examined for possible chimeric artifacts by the Chimera check with Bellerophon Version 3. After eliminating chimeric sequences, the partial 16S rRNA sequences were compared with the sequences in the Ribosomal Database Project and GenBank, using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Cloned sequences were identified as representing the species or phylotype of the sequence with the highest matching score. Sequences with less than 98% identity with a GenBank sequence were defined as a new phylotype. In addition, we checked whether the sequenced clones had the correct T-RFs compared with the sequence information.

Microbiologic analysis of bacterial translocation

The livers and mesenteric lymph nodes (mLNs) isolated from mice that had received transplants were removed aseptically and homogenized in 1 mL saline. Then, 500 μL of homogenate was transferred into a tube containing 4.5 mL of saline and used to perform 4 serial dilutions. From this dilution, 100 μL aliquots were cultured aerobically on blood agar and LB agar plates (Difco) for 24 hours at 37°C in room air supplemented with 10% CO_2. Colony-forming units (CFUs) were counted and adjusted per organ. Bacteria were identified by biochemical profiles.

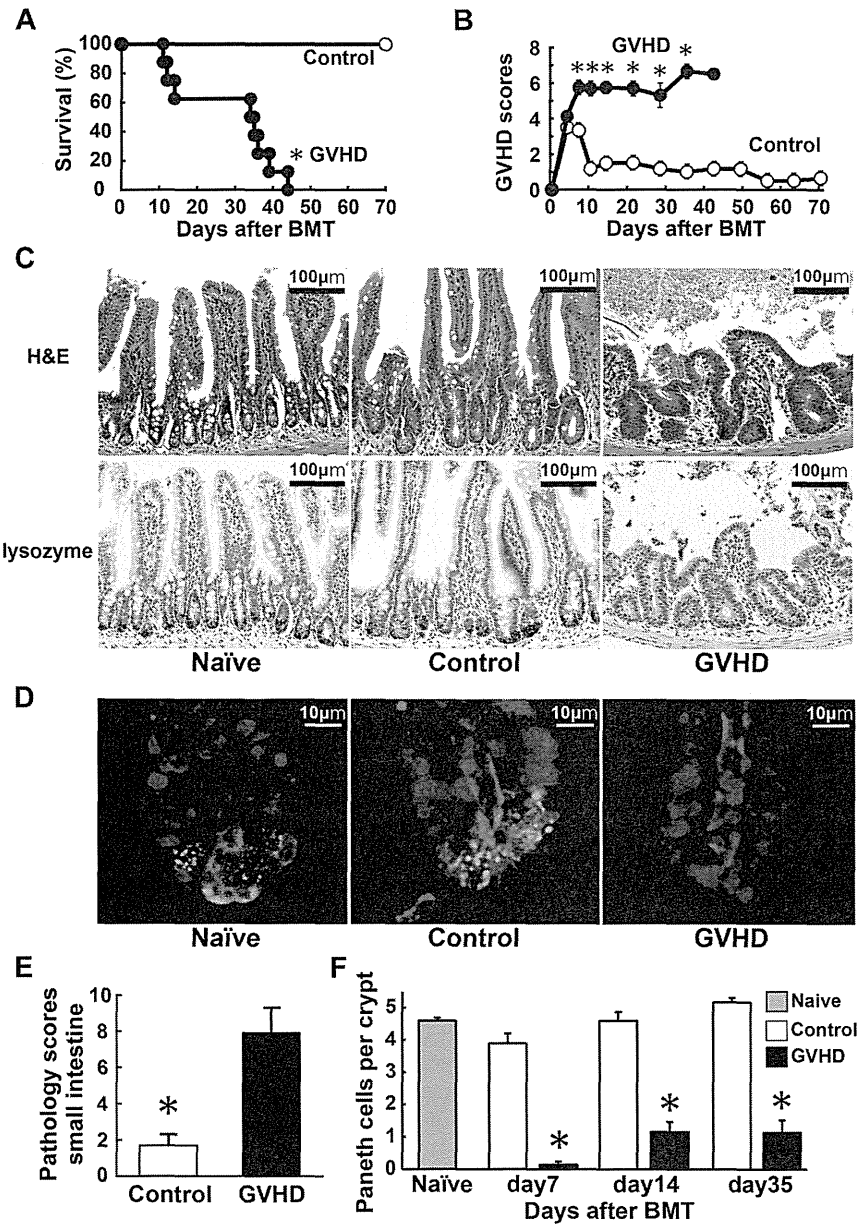

Figure 1. Paneth cell injury in GVHD. Lethally irradiated B6D2F1 mice were transplanted with 5×10^6 TCD BM cells without (control group, n = 6) or with 2×10^6 T cells (GVHD group, n = 12) from MHC-mismatched B6 donors on day 0. (A-B) Survival (A) and clinical GVHD scores (B) means ± SE are shown. Data from 2 independent experiments were combined. (C-F) Small intestines were isolated from mice 7 days after BMT. (C) Top panels: histology of the small intestine stained with H&E. Bottom panels: Lysozyme staining (brown). Magnification:100×. Bars, 100 μm. (D) Confocal cross-sectioning of the isolated small intestinal crypt. Lysozyme (green) is expressed by Paneth cells. Tetramethyl DAPI (blue) stains the nucleus and phalloidin (red) stains F-actin. Magnification: 1000×. Bars, 10 μm. (E) Pathology scores of the small intestine (mean ± SE, n = 3-6 / group). (F) Quantification of Paneth cells per crypt (mean ± SE, n = 3-6 / group; $^*P < .05$).

Statistical analysis

Mann-Whitney *U* tests were used to compare data, the Kaplan-Meier product limit method was used to obtain survival probability, and the log-rank test was applied to compare survival curves. To determine the statistically significant correlation, the Spearman rank correlation coefficient (R) was adopted. All tests were performed with SigmaPlot Version 10.0 software. $P < .05$ was considered statistically significant.

Accession numbers

Sequence data are available in the GenBank (http://www.ncbi.nih.gov/genbank) under the accession number 1509996.

Results

Paneth cell damage and decreased expression of α-defensins in GVHD

We evaluated whether Paneth cells could be damaged during GVHD. Lethally irradiated B6D2F1 (H-$2^{b/d}$) mice received 5×10^6 TCD-BM cells (control group) or these cells plus 2×10^6 T cells (GVHD group) from major histocompatibility complex (MHC)–mismatched B6 (H-2^b) donors on day 0. The allogeneic animals developed severe GVHD and all of these mice died within 50 days after BMT, whereas all TCD-BM controls survived through this period (Figure 1A). The surviving allogeneic animals showed significantly more severe signs of GVHD than controls, as assessed by clinical GVHD scores[20] (Figure 1B). Pathologic analysis of the small intestine 7 days after BMT showed mostly normal architecture in controls, whereas severe blunting of villi and inflammatory infiltration were observed in the GVHD group (Figure 1C). Paneth cells, which are typically identified microscopically by their location in the crypts and by the large granules occupying most of their cytoplasm, were hardly observed in the GVHD group. Immunohistochemical analysis for lysozyme, which indicates the presence of Paneth cells, confirmed loss of Paneth cells in the GVHD group, but not in controls (Figure 1C). Confocal cross-sectioning of individual crypts isolated from the small intestine further confirmed Paneth cell loss in these mice (Figure 1D). In mice with GVHD, GVHD pathology scores were significantly higher (Figure 1E), whereas numbers of Paneth cells

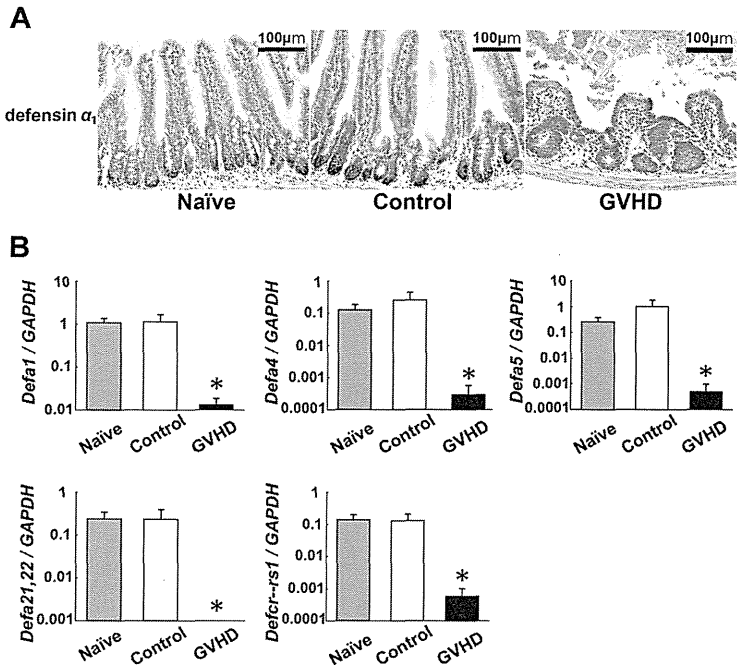

Figure 2. Decreased expression of Paneth cell–derived α-defensins in GVHD. Lethally irradiated B6D2F1 mice were transplanted with 5×10^6 TCD BM without (control group) or with (GVHD group) 2×10^6 T cells from B6 donors. Small intestines were isolated from mice 7 days after BMT. (A) Immunohistochemical staining for defensin α_1 (brown). Magnification: 100×. Bars, 100 μm. (B) RNA was extracted from samples and quantitative real-time PCR analysis for enteric defensins including *Defa1*, *Defa4*, *Defa5*, *Defa21,22*, and *Defcr-rs1* was performed (n = 6 / group). Data are representative of 2 similar experiments and are shown as mean ± SE (*$P < .05$).

were significantly and constantly lower compared with those in controls after BMT (Figure 1F).

α-Defensins are the major antimicrobial peptides produced by Paneth cells.[23] We evaluated the expression levels of enteric defensin families in the small intestines. Defensin α_1 expression was limited in Paneth cells in the crypts of naive mice (Figure 2A). Expression of defensin α_1 was preserved in controls 7 days after BMT but was severely suppressed in mice with GVHD. Quantitative real-time PCR analysis of the terminal ileum confirmed the reduced expression of *defensin-α1 (Defa1)* and other enteric defensin family members, including *Defa5*, *Defa21,22*, and *defensin α-related sequence 1 (Defa-rs1)* in the small intestine of GVHD mice (Figure 2B). These results demonstrate that GVHD targets Paneth cells and limits the expression of Paneth cell–derived defensin family members.

Perturbation of normal intestinal microbiota in GVHD

Paneth cell–derived α-defensins are essential regulators of the microbiota composition in the intestine.[11] α-defensins have selective bactericidal activity against noncommensals, whereas exhibiting minimal bactericidal activity against commensals.[28,29] We therefore hypothesized that the reduced expression of α-defensins results in dysbiosis in the intestinal microbial community. To test this hypothesis, we evaluated changes in the gut flora during the course of GVHD in a B6 → B6D2F1 murine model of BMT without administering any antibiotic or immunosuppressive drugs. Before and after BMT, fecal pellets were collected from each mouse once per week. The composition of the intestinal microflora was determined by RFLP analysis of bacteria-specific 16S rRNA genes that were constructed from each sample of fecal pellets.[30,31] Representative RFLP analysis is shown in Figure 3A. Each unique RFLP pattern is designated by an OTU that corresponds to specific species of bacteria. The peak height of each OTU indicates its relative quantity among the intestinal microflora and the number of OTUs indicates the diversity of flora. Before BMT, multiple OTUs were observed with little interindividual variation among the RFLP patterns (Figure 3A left panels). Seven days after BMT, numbers of OTUs were slightly decreased with little changes in the RFLP patterns in controls (Figure 3A right top panels); however, in the mice with GVHD, the number of OTUs decreased and the peak heights of OTUs were markedly reduced, with the exception of an aberrantly high peak at 368 bp (Figure 3A right bottom panels). Sequence analysis of subclones from a representative animal from GVHD group showed that proportions of both Firmicutes and Bacteroidetes, which are the major enteric commensals,[12,15] were decreased in mice with GVHD on day 7 compared with those before BMT (Firmicutes; 22.9% vs 52.1%, Bacteroidetes; 2.1% vs 13.5%, respectively).

These compositional changes in the intestinal microflora were consistently observed in all mice with GVHD. Diversity of the microbial community, which corresponds to the RFLP banding patterns, was significantly reduced in mice with GVHD at all time points, as assessed by the Simpson index of diversity,[26] Shannon diversity index,[27] and the number of OTUs counted (Figure 3B-D).

Overwhelming outgrowth of *E coli* in mice with GVHD

A single high peak at 368bp was noted in mice with GVHD (Figure 3A arrows). To identify the bacteria included at this OTU, plasmid DNA from the corresponding clone was purified. DNA sequencing showed a high similarity to 16S rRNA from *E coli* with a similarity rate of more than 99.5%. The proportion of *E coli* in the microbiota, which was defined as the ratio of the area of OTU for *E coli* to the total areas of all OTUs, was dramatically higher 7 days after BMT and remained higher in mice with GVHD throughout the entire observation period; however, *E coli* remained to be a small portion of the microbial population in controls (Figure 3E). Next, we evaluated whether the high levels of *E coli* in the intestine could be associated with the development of systemic infection in mice with GVHD. Seven days after BMT, mLNs and livers were harvested. *E coli* was identified from samples taken from mice with GVHD, but not the controls. The number of CFUs of *E coli* was significantly higher in the mLNs and liver of mice with GVHD than those in controls (Figure 3F-G). Serum LPS levels were also significantly higher in mice with GVHD than in controls (Figure 3H).

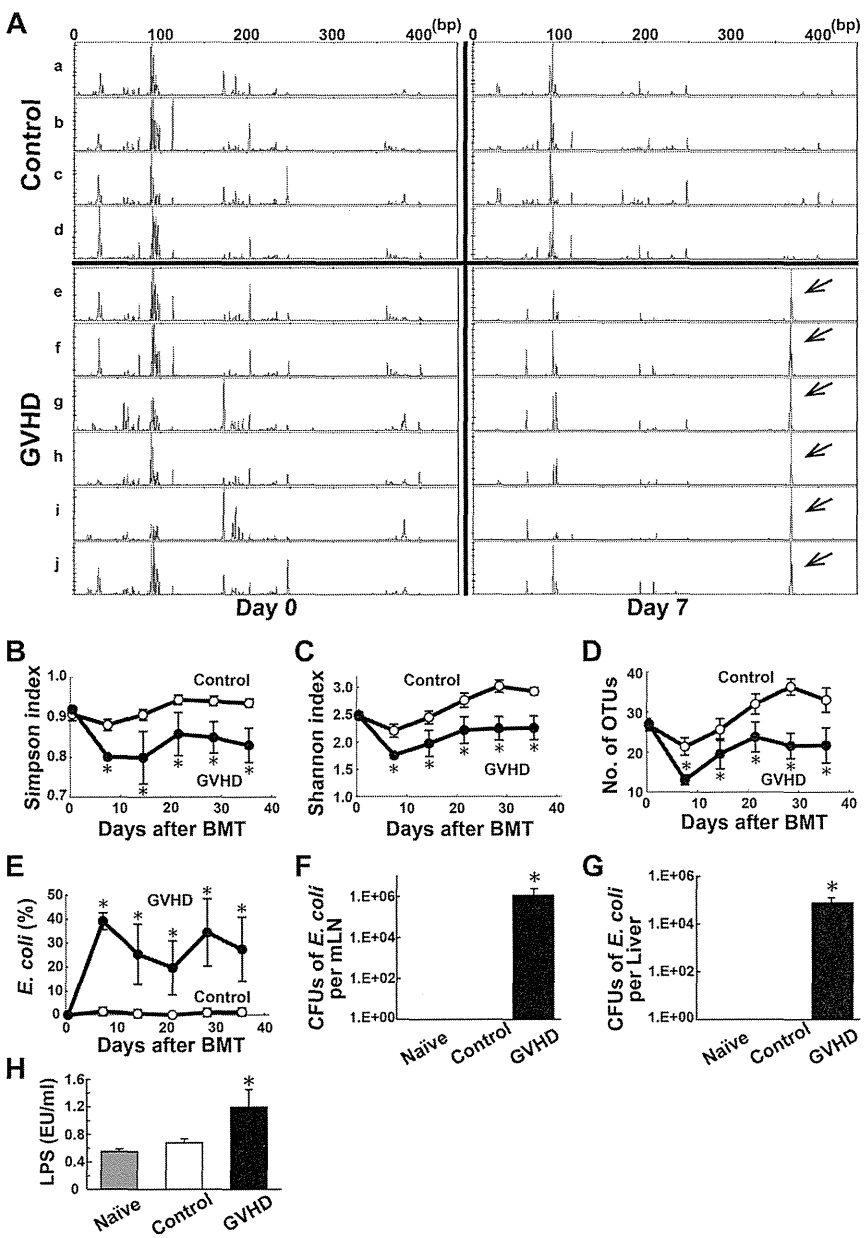

Figure 3. Perturbation of normal intestinal microbiota in GVHD. Fecal pellets were collected before and after a B6 → B6D2F1 BMT weekly and intestinal microbiota was characterized by RFLP analysis of 16S rRNA gene libraries constructed from each sample of fecal pellets and digested with *Hha*I (n = 6 / group). (A) Representative RFLP patterns are shown in control group (a-d) and GVHD group (e-j). Left panels indicate before BMT; right panels, 7 days after BMT. Arrows indicate an OTU for *Escherichia coli*. (B-D) Time course changes in flora diversity after BMT determined by using Simpson index (B), Shannon index (C), and numbers of OTUs (D). (E) Time course changes in the proportion of *E coli*. (F-G) Samples of mLNs and liver were harvested on day 7 and CFUs of *E coli* were enumerated by the culture-based and microbiologic identification method. (H) Serum LPS levels on day 7. Data are representative of 3 similar experiments and are shown as mean ± SE (*$P < .05$).

The composition of intestinal microflora in animals can differ depending on the environment and other factors.[32] Therefore, we used mice purchased from multiple vendors; however, the resulting patterns of dysbiosis were similar, regardless of the origin source of the mice. In addition, we found similar changes in the intestinal microbiota of another haplotype, the mismatched B6 → B6C3F1 (H-$2^{b/k}$) model of BMT. Diversity of intestinal flora was lost with an outgrowth of *E coli* 7 days after BMT and thereafter only in mice with GVHD (data not shown).

Association between changes in intestinal microbiota and GVHD severity

Further studies were conducted to determine whether there could be an association between the magnitude of changes observed in the intestinal flora and GVHD severity. Diversity of the flora, as determined by the Simpson index, Shannon index, and the number of OTUs was inversely correlated with GVHD severity (Figure 4A-C). On the other hand, the proportion of *E coli* in the intestinal flora was positively correlated with GVHD severity (Figure 4D).

Delayed alteration in intestinal microbial diversity after MHC-matched BMT

To further confirm that our observations were not strain or model dependent, we evaluated whether the observed changes in the intestinal flora could be observed in a clinically relevant, MHC-matched, and minor histocompatibility antigen-mismatched C3H.Sw (H-2^b) → B6 (H-2^b) model of BMT, in which GVHD developed more slowly and was less severe compared with the MHC-mismatched models of GVHD (Figure 5A-B).[33] Again, normal microbial diversity was lost in mice with GVHD and *E coli* levels were higher at 2 weeks after BMT and thereafter (Figure 5C-F). Thus, changes in the intestinal microbiota occurred more slowly in this model, at least compared with the MHC-mismatched model of GVHD; furthermore, the changes occurred in parallel with the

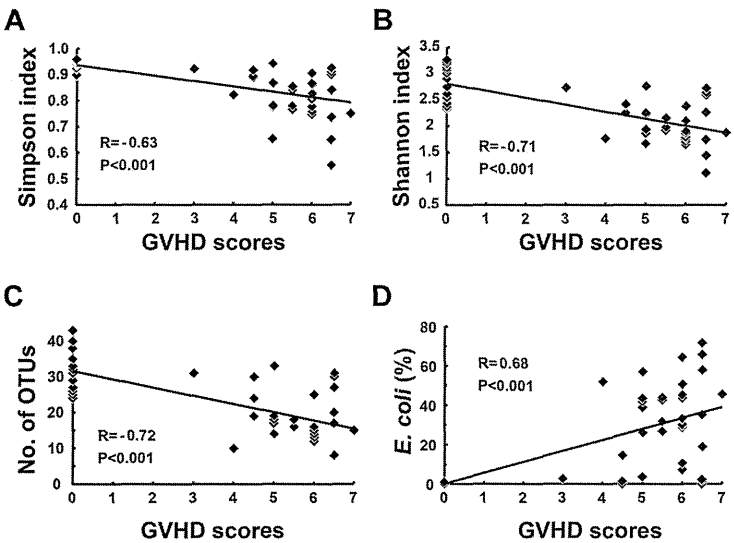

Figure 4. Correlation between the degree of flora changes and GVHD severity. Lethally irradiated B6D2F1 mice were transplanted with TCD BM with T cells from B6 donors (n = 6 / group). Fecal pellets were collected at day 0 and weekly thereafter and intestinal microbiota was characterized by RFLP analysis. Clinical GVHD scores and various parameters of the flora diversity and the proportion of *E coli* in the intestinal flora at various time points from each mice were plotted. Correlations of GVHD clinical scores and Simpson index (A), Shannon index (B), numbers of OTUs (C), and proportion of *E coli* (D). The regression line was plotted with all data. Data from 2 independent experiments were combined. R: correlation coefficient.

slower development of GVHD. It should be noted that normal flora diversity was recovered and *E coli* returned to a normally small population among the intestinal microbiota late after BMT, as GVHD severity reduced. No mortality was observed in allogeneic animals after regaining normal intestinal flora.

Loss of Paneth cells and the dysbiosis by a mechanism independent on conditioning

We addressed whether GVHD mediates Paneth cell injury and the alteration of the composition of the intestinal flora by a mechanism dependent on radiation-induced intestinal tract damage in the B6 → B6D2F1 BMT model without conditioning, as previously described.[7] Unirradiated B6D2F1 mice were intravenously injected with 12×10^7 splenocytes from syngeneic or allogeneic B6 donors on day 0. In this model, GVHD occurred early after BMT at a peak around day 20 but was spontaneously improved (Figure 6A-C). Numbers of Paneth cells were markedly reduced 2 weeks after BMT but gradually returned to normal levels thereafter in allogeneic animals (Figure 6D). The changes in the intestinal microbiota occurred in parallel with the degree of GVHD severity and Paneth cell injury; normal microbial diversity was lost with the outgrowth of *E coli*, but was gradually restored later after BMT in allogeneic animals (Figure 6E-H).

Oral administration of antibiotics inhibited the outgrowth of *E coli* and ameliorated GVHD

Finally, we evaluated whether modifying the enteric flora using oral antibiotics could ameliorate GVHD. Lethally irradiated B6D2F1 mice were transplanted with 5×10^6 TCD BM cells with or without 2×10^6 T cells from B6-Ly5.1 (CD45.1$^+$) donors. Polymyxin B (PMB), an antibiotic primarily effective against gram-negative bacteria, was administered by daily oral gavage at a dose of 100 mg/kg from day −4 until day 28 after BMT. Analysis of fecal pellets 7 days after BMT showed that the outgrowth of *E coli* was inhibited in mice treated with PMB compared with those treated with diluent (Figure 7A). PMB suppressed the outgrowth of *E coli* during PMB treatment; however, *E coli* levels increased after cessation of PMB treatment (Figure 7B). Notably, administration of PMB significantly reduced mortality and morbidity of GVHD (Figure 7C-D). Donor (CD45.1$^+$) T-cell expansion (Figure 7E) and pathology scores of the small intestine (Figure 7F) were significantly reduced in PMB-treated mice compared with those in controls.

Discussion

Intestinal GVHD is critical for determining the outcome of allogeneic BMT. Paneth cells are essential regulators of the

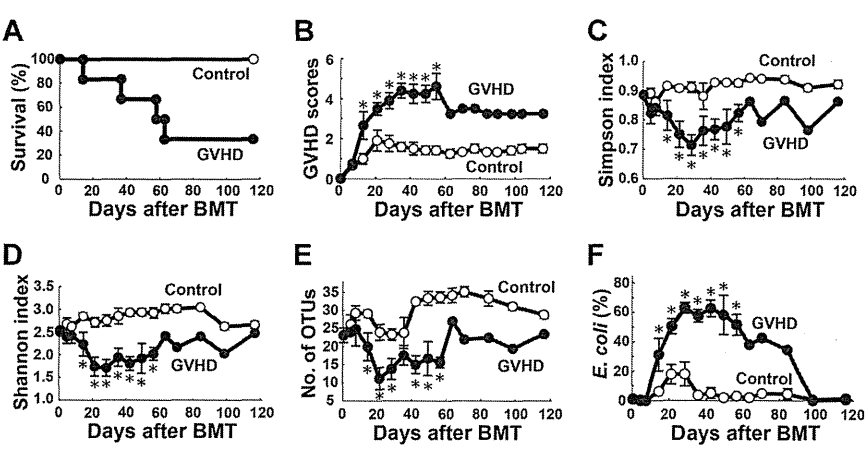

Figure 5. Delayed impairment of the intestinal ecology after MHC-matched BMT. B6 mice were transplanted with 5×10^6 TCD BM without (control group) or with (GVHD group) 2×10^6 T cells from C3H.Sw donors after 12 Gy TBI (n = 6/group). (A-B) Survival (A) and clinical GVHD scores (B, mean ± SE) in control group and GVHD group. Data are representative of 3 similar experiments. (C-F) Fecal pellets were collected once per week after BMT and intestinal microflora was characterized by RFLP analysis of 16S rRNA genes constructed from each sample of fecal pellets and digested with *Hha*I. Time course changes in flora diversity determined by Simpson index (C), Shannon index (D), and numbers of OTUs (E). (F) Time course changes in the proportion of *E coli*. Data are representative of 3 similar experiments and are shown as mean ± SE (*$P < .05$).

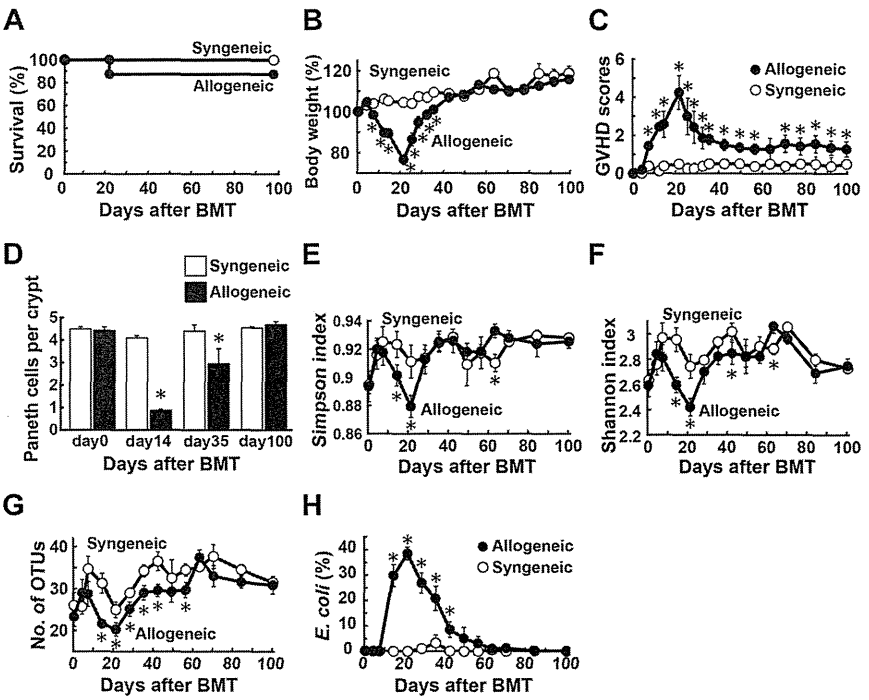

Figure 6. Paneth cell injury and the dysbiosis developed by a mechanism independent on conditioning. Unirradiated B6D2F1 mice were transplanted with 12×10^7 splenocytes from syngeneic or allogeneic MHC-mismatched B6 donors on day 0 ($n = 6$/group). Survival (A), changes in body weight (B, mean ± SE), clinical GVHD scores (C, mean ± SE), and (D) quantification of Paneth cells per crypt in the small intestine were shown. (E-H) Fecal pellets were collected before and after BMT weekly and intestinal microbiota was characterized by RFLP analysis of 16S rRNA gene libraries constructed from each sample of fecal pellets and digested with *Hha*I. Time course changes in flora diversity determined by using Simpson index (E), Shannon index (F), numbers of OTUs (G), and the proportion of *E coli* (H). Data from 2 independent experiments were combined and are shown as mean ± SE.

composition of commensal microbiota in the intestine, and they maintain the intestinal microbial environment by secreting various microbial peptides. In this study, we found that damage to Paneth cells by GVHD results in dramatically reduced expression of α-defensins in the small intestines and perturbed normal intestinal environment. These changes occurred in the absence of conditioning irradiation, thus indicating a mechanism dependent on allogeneic T-cell responses. However, Paneth cell loss occurred earlier and more prolonged in mice receiving irradiation than in unirradiated mice, suggesting that conditioning enhanced Paneth cell damage directly and indirectly by accelerating GVHD. The diversity of the intestinal microflora was lost with overwhelming expansion of specific bacteria, such as *E coli*, which are normally a very small proportion of the intestinal microbial communities. Paneth cells secret α-defensins into the intestinal lumen within minutes after sensing gram-negative and gram-positive bacteria and their components, such as LPS, through activation of pattern recognition receptors.[23,34] α-defensins are the most potent antimicrobial peptides and account for 70% of the bactericidal peptide activity released from Paneth cells.[11,23] α-Defensins are released in the small bowel lumen and persist as intact and functional forms throughout the intestinal tract.[35] Thus, they shape the composition of the microbiota in the entire intestine. Importantly, α-defensins have selective bactericidal activity against noncommensals, such as *Salmonella enterica*, *E coli*, *Klebsiella pneumoniae*, and *Staphylococcus aureus*, although exhibiting minimal bactericidal activity against commensals.[28,29] Such bacteria-dependent bactericidal activities of α-defensins are in tune with intestinal environment and may explain why the absence of α-defensins causes the alterations in the intestinal microbiota in GVHD. Because commensals have a profound influence on nutritional, physiologic, and metabolic function of the host,[14,36,37] reduction of commensals may have ill effects on the host with GVHD. In this study, *E coli* was the dominant enteric microbe in mice with GVHD among multiple strains of mice. However, the dominant species may differ between studies because of the differences in several factors, including differences in the maintenance protocols used to feed and care for the experimental animals.[32] Nonetheless, our study confirms and further extends a recent study showing the intestinal flora change, with an increase in gram-negative *Enterobacteriaceae* family members including *E coli*, after allogeneic BMT in mice.[18]

Alteration of the intestinal microbiota has been shown in experimental and clinical inflammatory bowel diseases, allergies, diabetes, and obesity.[13,38-42] This study provides several lines of evidence that suggest a close association between dysbiosis and GVHD. Mice without GVHD maintained normal microbiota after BMT, and dysbiosis only occurred in mice with GVHD, independent of the murine models used. The normal intestinal environment was never restored as long as severe GVHD persisted, but was restored when tolerance was induced after transplant. In mice with GVHD, the degree of changes to the microflora was significantly correlated to GVHD severity and MHC disparity between the donor and recipient. Furthermore, modifying enteric flora by oral administration of antibiotics inhibited the outgrowth if *E coli* and ameliorated GVHD. The flora shift toward the widespread prevalence of gram-negative bacteria increases the translocation of LPS, the major component of the outer membrane of gram-negative bacteria, into systemic circulation and further accelerates GVHD by stimulates production of inflammatory cytokines, such as TNF-α and IL-1, which are critical effector molecules that mediate GVHD.[22,43-45] Thus, GVHD and the dysbiosis can lead to a positive feedback loop that increases the translocation of LPS, thereby resulting in further cytokine production, progressive intestinal injury, and systemic GVHD acceleration. Earlier seminal studies in the 1960s-1970s suggested that GVHD is reduced in germfree mice or by treatment with poorly absorbable antibiotics.[3-6] A recent study also demonstrated that modifying the enteric flora using a probiotic microorganism reduced GVHD in mice.[46] Thus, our study again highlights an important role of oral antibiotics administration on GVHD. Our study demonstrated that dominant bacteria in the intestinal microbiota cause systemic infection. There was a microbiologic evidence of infection in mice with severe GVHD and a correlation between severity of infection and GVHD, thus suggesting that severe bacteremia, probably caused by the translocation of enteric bacteria, can also contribute to GVHD mortality, as previously suggested.[6,46] Indeed, septicemia by gram-negative rods

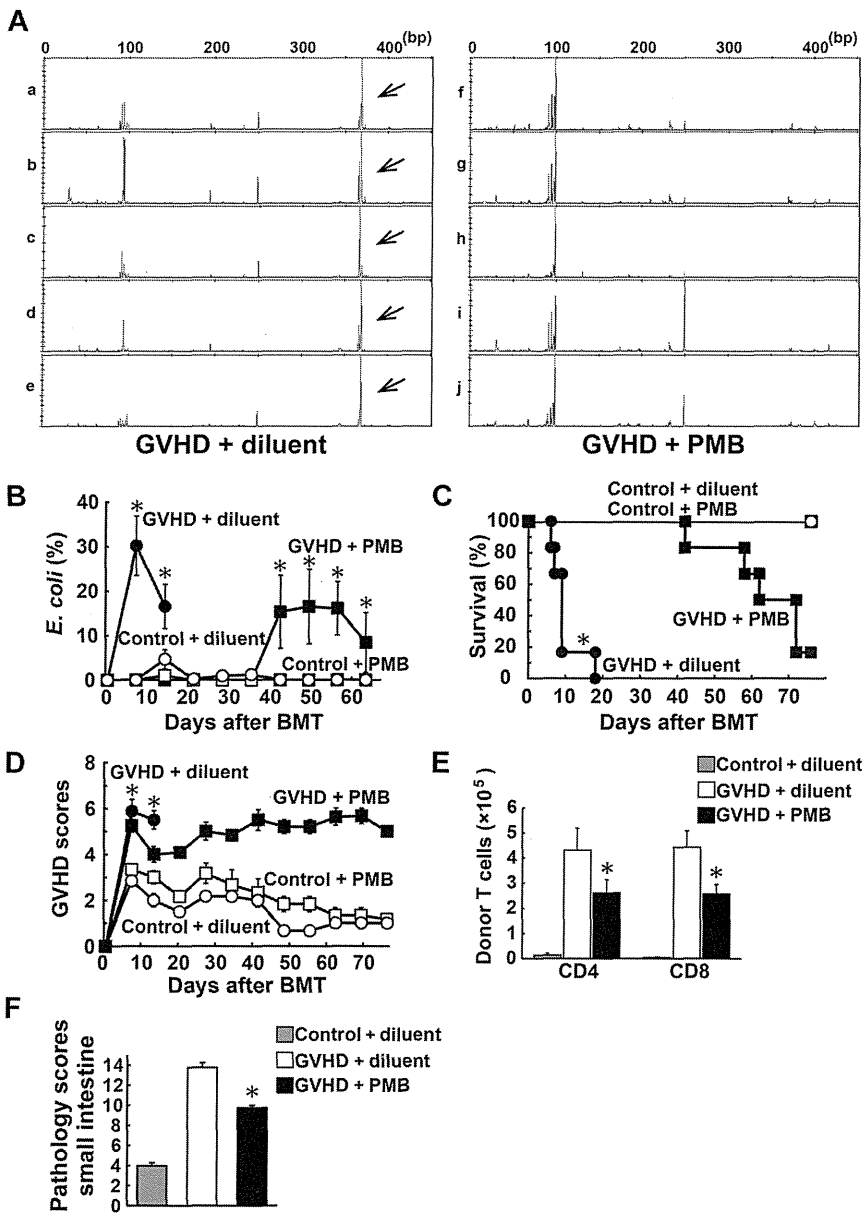

Figure 7. Oral administration of polymyxin B ameliorated GVHD. Lethally irradiated B6D2F1 mice were transplanted with 5×10^6 TCD BM without or with 2×10^6 T cells from B6 or B6-Ly5.1 (CD45.1$^+$) donors. Polymyxin B (PMB; 100 mg/kg) or diluent was administered by daily oral gavage from day −4 until day 28 after BMT. (A) Fecal pellets were collected once per week after BMT and intestinal microflora was characterized by RFLP analysis of 16S rRNA genes constructed from each sample of fecal pellets and digested with *Hha*I. Representative RFLP patterns are shown in mice with GVHD receiving diluent (a-e) and those with PMB (f-j) 7 days after BMT. Arrows indicate an OTU for *E coli*. (B) Time course changes in the proportion of *E coli* (n = 6-12 / group). Survival (C) and clinical GVHD scores (D, mean ± SE) after BMT are shown (n = 6-12/group). Data from 2 independent experiments were combined. (E) Numbers of donor (CD45.1$^+$) T cells in mLNs on day 5 (n = 20/group). (F) Pathology scores of the small intestine on day 7 (n = 20/group). Data from 3 independent experiments were combined and are shown as mean ± SE (*$P < .05$).

is one of the most frequent causes of death in patients with severe intestinal GVHD.

There are other interfaces that exist between the environment and the host, such as the skin and airways. Epithelial cells in these tissues can also release antimicrobial peptides such as β-defensins in response to bacteria and LPS.[47] GVHD-mediated epithelial cell damage of these tissues may also impair the local secretion of antimicrobial peptides, leading to aberrant overgrowth of pathogens and development of dermal infections or pneumonia, which are frequently observed in patients with GVHD. Furthermore, the development of these pathologic conditions may be associated with the unique tissue specificity of GVHD for tissues that are in contact with high microbial loads, such as the skin, liver, intestine, and lung.

Intestinal epithelial cells are continuously regenerated from ISCs, which are required to regenerate damaged sections of the intestinal epithelium.[48] Paneth cells are derived from ISCs and serve as a niche for ISCs.[8] Our previous[7] and current studies addressed intestinal GVHD at the cellular level, and demonstrated that ISCs and their niche Paneth cells could survive pretransplant conditioning and regenerate injured epithelium by conditioning in the absence of GVHD. However, both ISCs and Paneth cells are targeted by GVHD, resulting in an impairment of the physiologic repair mechanisms of injured epithelium, although it remains to be elucidated whether Paneth cell loss is induced by direct cytotoxicity to Paneth cell itself or secondary to the loss of ISCs. This phenomenon may explain the prolonged and refractory nature of clinical intestinal GVHD. These new insights will help to establish new therapeutic strategies that can be used to prevent and treat GVHD and related infections and improve the clinical outcome of allogeneic BMT.

Acknowledgments

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