95% CI, 3.00–3.98, $P < 0.001$; platelet engraftment, HR 2.20, 95% CI, 1.89–2.57, $P < 0.001$; Supplementary Table 1). As our previous study revealed that an HLA-B mismatch had an adverse effect on OS in transplantation from an RD/1AG-MM-GVH, patients in the RD/1AG-MM-GVH group with an HLA-A, -B, or -DR mismatch were separately compared with the UCB group. We consistently observed superior neutrophil and platelet engraftment in each RD/1AG-MM-GVH group as compared with the UCB group (Supplementary Table 1).

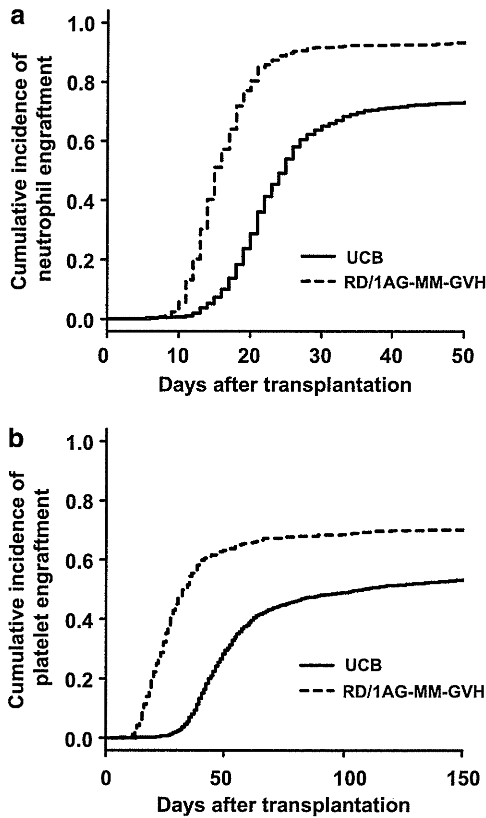

Figure 1. Neutrophil (**a**) and platelet engraftment (**b**).

Acute and chronic GVHD

The incidence of grade II–IV or grade III–IV acute GVHD in the RD/1AG-MM-GVH group was significantly higher than that in the UCB group (grade II–IV acute GVHD at day 100: UCB group, 34%, 95% CI, 32–36%; RD/1AG-MM-GVH group, 50%, 95% CI, 45–54%; Gray test, $P < 0.001$; grade III–IV acute GVHD at day 100: UCB group, 11%, 95% CI, 10–13%; RD/1AG-MM-GVH group, 21%, 95% CI, 17–24%; Gray test, $P < 0.001$; Figures 2a and b). The incidence of chronic GVHD or extensive type of chronic GVHD in the RD/1AG-MM-GVH group was also significantly higher than that in the UCB group (chronic GVHD at 3 years: UCB group, 25%, 95% CI, 23–27%; RD/1AG-MM-GVH group, 42%, 95% CI, 38–47%; Gray test, $P < 0.001$; extensive chronic GVHD at 3 years: UCB group, 11%, 95% CI, 10–13%; RD/1AG-MM-GVH group, 29%, 95% CI, 25–34%; Gray test, $P < 0.001$; Figures 2c and d). A multivariate analysis confirmed a higher risk of grade II–IV or grade III–IV acute GHVD, chronic or extensive chronic GVHD in the RD/1AG-MM-GVH group than in the UCB group (grade II–IV acute GVHD; HR 1.64, 95% CI, 1.43–1.90, grade III–IV acute GVHD; HR 2.28, 95% CI, 1.80–2.88, chronic GVHD; HR 1.47, 95% CI, 1.24–1.73, extensive chronic GVHD; HR 2.35, 95% CI, 1.90–2.91, Supplementary Table 2).

OS

The 3-year unadjusted OS rates in the UCB and RD/1AG-MM-GVH groups were 38% (36–41%) and 39% (34–43%), respectively ($P = 0.115$). The use of either UCB or RD/1AG-MM-GVH was not associated with OS rates in the multivariate analysis (UCB vs RD/1AG-MM-GVH, HR, 0.99, 95% CI, 0.87–1.12, $P = 0.833$) in all-risk patients, or either standard-risk ($P = 0.588$) or high-risk patients ($P = 0.639$; Table 2), after adjusting for the following significant risk factors: age >50 years, male recipient, acute myeloid leukemia vs MDS, high-risk disease, GVHD prophylaxis using only calcineurin inhibitor vs calcineurin inhibitor + methotrexate, and earlier year

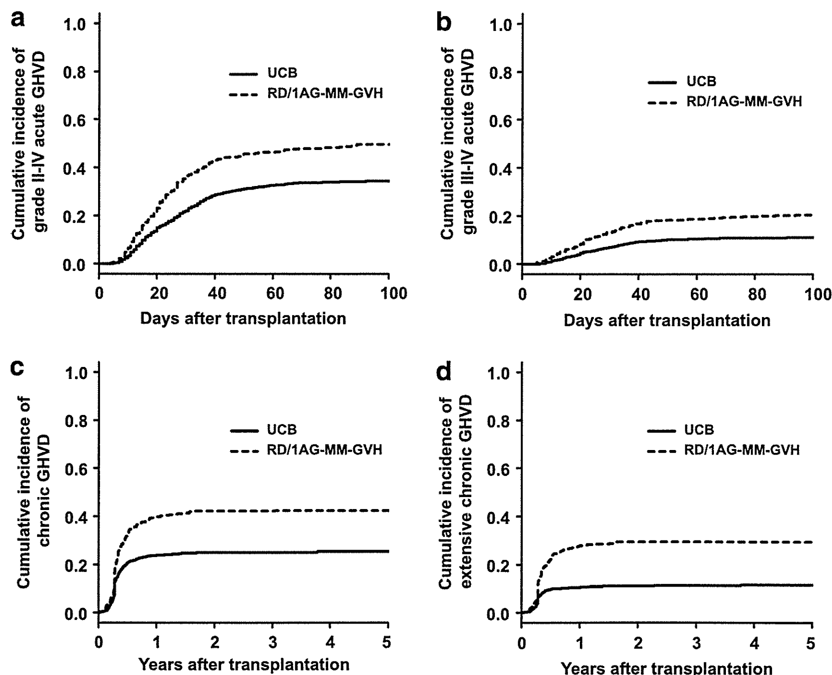

Figure 2. Acute and chronic GVHD. Cumulative incidences of grade II–IV (**a**) and grade III–IV acute GVHD (**b**) and chronic (**c**) and extensive chronic GVHD (**d**) are shown.

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Table 2. Multivariate analysis of overall mortality

Variable	*Total*[a]		*Standard risk*[b]		*High risk*[c]	
	HR (95% CI)	P *value*	*HR (95% CI)*	P *value*	*HR (95% CI)*	P *value*
(A)						
UCB	1.00	reference	1.00	reference	1.00	reference
RD/1AG-MM-GVH	0.99 (0.87–1.12)	0.833	1.06 (0.86–1.31)	0.588	0.96 (0.81–1.13)	0.639
(B)						
UCB	1.00	reference	1.00	reference	1.00	reference
RD/HLA-A-MM-GVH	0.92 (0.72–1.18)	0.519	0.99 (0.66–1.48)	0.959	0.90 (0.64–1.26)	0.551
RD/HLA-B-MM-GVH	1.20 (1.01–1.44)	0.043	1.44 (1.05–1.96)	0.023	1.12 (0.89–1.41)	0.326
RD/HLA-DR-MM-GVH	0.85 (0.70–1.02)	0.084	0.88 (0.66–1.19)	0.411	0.84 (0.65–1.08)	0.170

Abbreviations: AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CI, confidence interval; CML, chronic myelogenous leukemia; CSA, cyclosporine; HR, hazard ratio; MDS, myelodysplastic syndrome; MMF, mycophenolate mofetil; MTX, methotrexate; TAC, tacrolimus. [a]Other significant variables in model A were; patient age, 16–49 (reference, 1.00), 50–(HR, 1.50, 95% CI, 1.35–1.66, $P<0.001$); sex of recipient, female (reference, 1.00), male (HR, 1.12; 95% CI, 1.02–1.24; $P=0.023$); diagnosis, AML (reference, 1.00),ALL (HR, 1.11, 95% CI, 0.98–1.26, $P=0.112$), CML (HR, 0.90, 95% CI, 0.72–1.13, $P=0.374$), MDS (HR, 0.81, 95% CI, 0.68–0.95, $P=0.001$); disease risk, standard risk (reference, 1.00), high risk (HR, 2.24; 95% CI, 2.00–2.50; $P<0.001$), status not known, (HR, 1.59; 95% CI, 1.21–2.09; $P=0.001$); GVHD prophylaxis, CSA/TAC + MTX (reference, 1.00),CSA/TAC only (HR, 1.23; 95% CI, 1.09–1.39; $P=0.001$), CSA/TAC + steroid/MMF (HR, 1.02; 95% CI, 0.86–1.21; $P=0.820$), other/missing (HR, 1.21; 95% CI, 0.82–1.78; $P=0.342$); year of transplantation, 1998–2004 (reference, 1.00), 2005–2009 (HR, 0.89; 95% CI, 0.80–0.99; $P=0.038$). [b]Other significant variables in model A were; patient age, 16–49 (reference, 1.00), 50–(HR, 1.72, 95% CI, 1.42–2.07, $P<0.001$); GVHD prophylaxis, CSA/TAC + MTX (reference, 1.00), CSA/TAC only (HR, 1.43; 95% CI, 1.14–1.78; $P=0.002$), CSA/TAC + steroid/MMF (HR, 1.00; 95% CI, 0.73–1.37; $P=0.995$), other/missing (HR, 1.51; 95% CI, 0.67–3.39; $P=0.319$). [c]Other significant variables were; patient age, 16–49 (reference, 1.00), 50–(HR, 1.41, 95% CI, 1.23–1.61, $P<0.001$); diagnosis, AML (reference, 1.00), ALL (HR, 1.13, 95% CI, 0.95–1.34, $P=0.183$), CML (HR, 0.94, 95% CI, 0.70–1.27, $P=0.704$), MDS (HR, 0.73, 95% CI, 0.60–0.89, $P=0.002$).

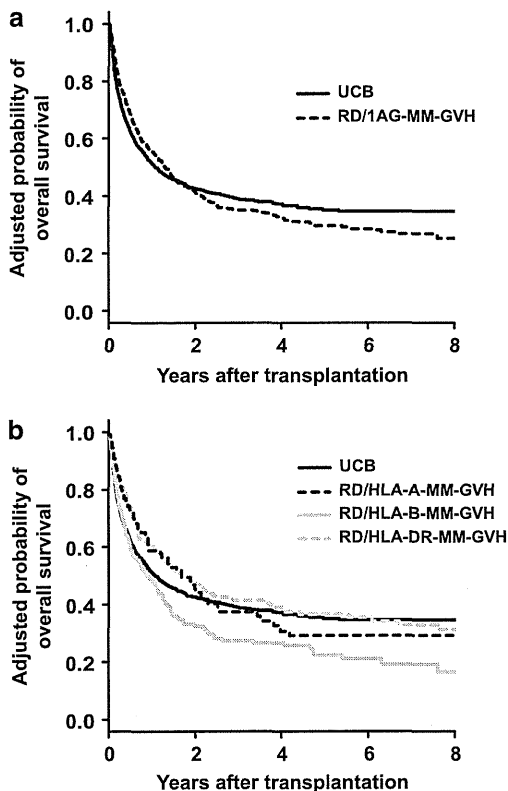

Figure 3. Overall survival. Overall survival rates in the transplantation using an unrelated cord blood vs a related donor with a 1-antigen mismatch at the HLA-A, HLA-B or HLA-DR locus in the GVH direction (**a**) or with an HLA-A, -B, or -DR antigen mismatch in the GVH direction (**b**) are shown.

of transplantation (1998–2004). Figure 3a shows the adjusted survival curves of the two groups. Next, the HLA-A, HLA-B and HLA-DR mismatched groups in transplantation from an RD/1AG-MM-GVH were compared with the UCB group. The OS rate of patients who received transplantation from an RD/1AG-MM-GVH involving an HLA-B mismatch was significantly lower than that in the UCB group ($P=0.043$; Figure 3b and Table 2), and a subgroup analysis revealed that the adverse effect of an HLA-B mismatch was significant only in standard-risk patients (standard-risk, $P=0.023$; high-risk, $P=0.326$; Table 2).

Relapse and NRM

The 3-year relapse rates in the UCB and RD/1AG-MM-GVH groups were 35% (95%CI, 33–37%) and 32% (95% CI, 28–36%), respectively (Gray test; $P=0.041$; Figure 4a), and a significant decrease in the incidence of relapse was found in the RD/1AG-MM-GVH group in the multivariate analysis (RD/1AG-MM-GVH vs UCB, HR, 0.78, 95%CI, 0.64–0.95, $P=0.012$; Table 3). The impact of reducing the incidence of relapse did not differ according to the HLA mismatch antigen in the RD/1AG-MM-GVH group (Table 3 and Figure 4b). The 3-year NRM rates in the UCB and RD/1AG-MM-GVH groups were 30% (95% CI, 28–32%) and 32% (95% CI, 28–36%), respectively (Gray test; $P=0.474$; Figure 4c), and a significant increase in the NRM rate was observed in the RD/1AG-MM-GVH group in the multivariate analysis (RD/1AG-MM-GVH vs UCB, HR, 1.24, 95% CI, 1.04–1.47, $P=0.016$; Table 3). In particular, the NRM rate of patients who received transplantation from an RD/1AG-MM-GVH with an HLA-B mismatch was significantly higher than that in the UCB group (RD/1AG-MM-GVH vs UCB, HR, 1.50, 95% CI, 1.17–1.92, $P=0.001$; Figure 4d and Table 3).

The causes of death in patients who died without relapse are shown in Supplementary Table 3. The rates of GVHD and organ failure in the RD/1AG-MM-GVH group were higher than those in the UCB group (GVHD, 18 vs 10%, organ failure, 28 vs 19%), whereas the rates of graft failure and infection were lower in the RD/1AG-MM-GVH group (graft failure, 1 vs 5%; infection, 26 vs 38%).

The impact of the use of *in vivo* T-cell depletion in the RD/1AG-MM-GVH group

Based on the fact that the leading causes of death in the RD/1AG-MM-GVH group were GVHD and organ failure, we analyzed the risk factors for the development of acute GVHD in this group.

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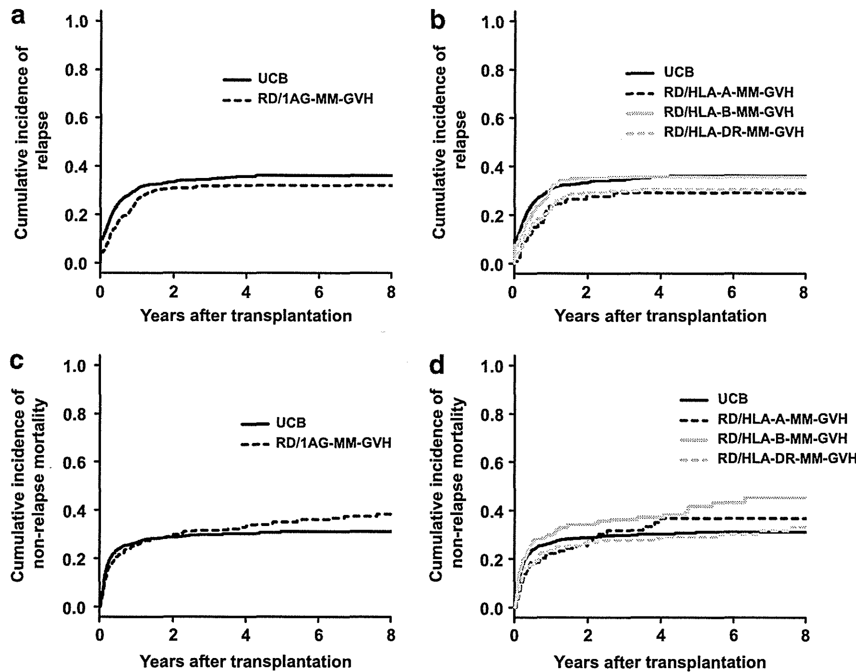

Figure 4. Relapse and non-relapse mortality. Cumulative incidence of relapse and non-relapse mortality after transplantation using an unrelated cord blood vs a related donor with a 1-antigen mismatch at the HLA-A, HLA-B or HLA-DR locus in the GVH direction (**a**, **c**) or with an HLA-A, -B, or -DR antigen mismatch in the GVH direction (**b**, **d**) are shown.

Table 3. Multivariate analysis of relapse and non-relapse mortality

Variable	*Relapse*[a]		*Non-relapse mortality*[b]	
	HR (95% CI)	P *value*	*HR (95% CI)*	P *value*
(A)				
UCB	1.00	reference	1.00	reference
RD/1AG-MM-GVH	0.78 (0.64–0.95)	0.012	1.24 (1.04–1.47)	0.016
(B)				
UCB	1.00	reference	1.00	reference
RD/HLA-A-MM-GVH	0.70 (0.49–1.00)	0.050	1.28 (0.93–1.76)	0.130
RD/HLA-B-MM-GVH	0.81 (0.62–1.07)	0.134	1.50 (1.17–1.92)	0.001
RD/HLA-DR-MM-GVH	0.80 (0.61–1.04)	0.096	1.02 (0.78–1.32)	0.901

Abbreviations: AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CI, confidence interval; CML, chronic myelogenous leukemia; CSA, cyclosporine; HR, hazard ratio; MDS, myelodysplastic syndrome; MMF, mycophenolate mofetil; MTX, methotrexate; TAC, tacrolimus. [a]Other significant variables in model A were; diagnosis, AML (reference, 1.00), ALL (HR, 1.09, 95% CI, 0.92–1.29, $P=0.336$), CML (HR, 1.39, 95% CI, 1.05–1.82, $P=0.019$), MDS (HR, 0.59, 95% CI, 0.46–0.76, $P<0.001$); time from diagnosis to transplantation, <6 months (reference, 1.00), ⩾6 months (HR, 0.80; 95% CI, 0.70–0.92; $P=0.002$); disease risk, standard risk (reference, 1.00), high risk (HR, 2.81; 95% CI, 2.41–3.27; $P<0.001$), status not known, (HR, 2.17; 95% CI, 1.45–3.23; $P<0.001$); conditioning intensity, myeloablative (reference, 1.00), reduced intensity (HR, 1.22; 95% CI, 1.04–1.44; $P=0.014$); GVHD prophylaxis, CSA/TAC + MTX (reference, 1.00), CSA/TAC only (HR, 0.65; 95% CI, 0.53–0.78; $P<0.001$), CSA/TAC + steroid/MMF (HR, 0.75; 95% CI, 0.59–0.96; $P=0.024$), other/missing (HR, 0.94; 95% CI, 0.55–1.61; $P=0.825$). [b]Other significant variables in model A were; patient age, 16–49 (reference, 1.00), 50–(HR, 1.70, 95% CI, 1.47–1.98, $P<0.001$); GVHD prophylaxis, CSA/TAC + MTX (reference, 1.00),CSA/TAC only (HR, 1.70; 95% CI, 1.44–2.01; $P<0.001$), CSA/TAC + steroid/MMF (HR, 1.18; 95% CI, 0.94–1.49; $P=0.158$), other/missing (HR, 1.47; 95% CI, 0.86–2.51; $P=0.154$); year of transplantation, 1998–2004 (reference, 1.00), 2005–2009 (HR, 0.76; 95% CI, 0.66–0.88; $P<0.001$).

In multivariate analysis, two factors were found to be significantly associated with the risk of developing grade II–IV acute GVHD in the RD/1AG-MM-GVH group: the use of *in vivo* T-cell depletion and source of stem cells (use of *in vivo* T-cell depletion, yes vs no, HR 0.40, $P=0.002$, PB vs BM, HR 1.61, $P<0.001$).

Because the use of *in vivo* T-cell depletion significantly lowered the risk of acute GVHD, we re-compared the RD/1AG-MM-GVH group and the UCB group while focusing on the use of *in vivo* T-cell depletion in the RD/1AG-MM-GVH group. The incidence of grade II–IV or grade III–IV acute GVHD or chronic or extensive chronic GVHD in the RD/1AG-MM-GVH group using *in vivo* T-cell depletion was comparable to that in the UCB group (Supplementary Figure 1 and Supplementary Table 4), whereas the incidences of neutrophil and platelet engraftment were significantly higher in the RD/1AG-MM-GVH group using *in vivo* T-cell depletion than in the UCB group (neutrophil engraftment, HR, 5.52, 95% CI, 3.36–9.05, $P<0.001$; platelet engraftment, HR 2.01, 95% CI, 1.26–3.21, $P<0.001$). Compared to the UCB group, the RD/1AG-MM-GVH group with T-cell depletion showed lower overall and NRM, albeit these differences were not significant, which suggests that the use of *in vivo* T-cell depletion may improve the outcome of transplantation from an RD/1AG-MM-GVH (Figure 5, Supplementary Table 5). It is interesting to note that the adverse impact of an HLA-B mismatch vs HLA-A or -DR

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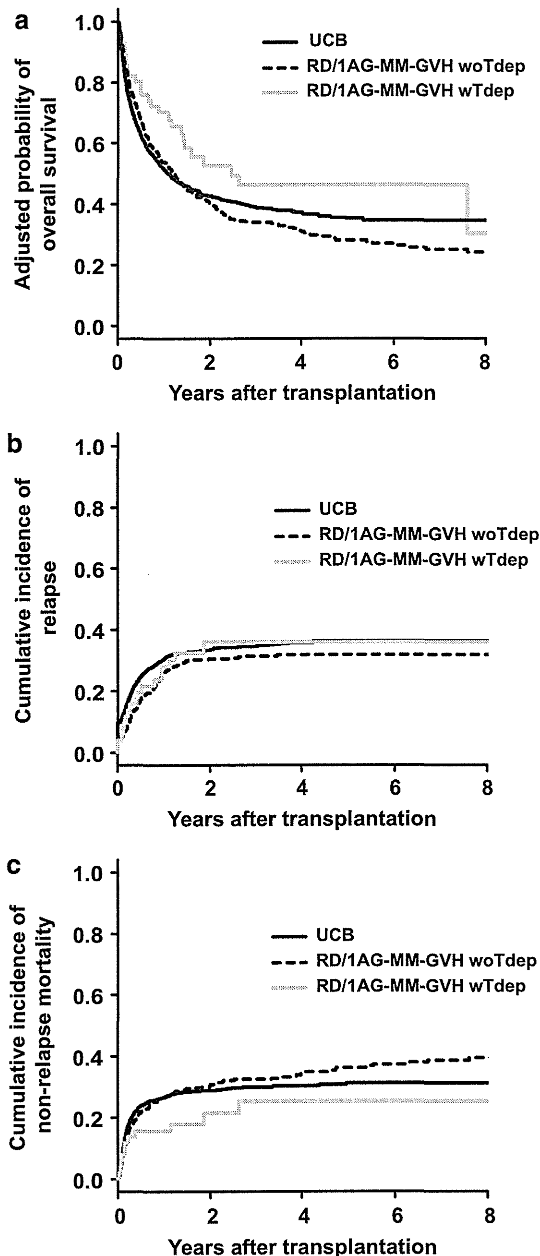

Figure 5. OS (**a**), relapse (**b**) and NRM (**c**) according to the use of *in vivo* T-cell depletion in the RD/1AG-MM-GVH group.

mismatch in the RD/1AG-MM-GVH group disappeared with the use of *in vivo* T-cell depletion (with *in vivo* T-cell depletion; HLA-B vs HLA-A/DR mismatch; HR 1.08, 95% CI, 0.45–2.62, $P=0.864$, without *in vivo* T-cell depletion; HLA-B vs HLA-A/DR mismatch; HR 1.59, 95% CI, 1.25–2.01, $P<0.001$).

With regard to the effect of stem cell source, the incidence of acute and chronic GVHD in the RD/1AG-MM-GVH group using BM was lower than that with PB but higher than that with UCB (Supplementary Figure 2). The use of PB or BM did not affect OS, relapse, or NRM (Supplementary Table 5).

DISCUSSION

In this nationwide retrospective study, we found that the survival rate in the UCB group was comparable to that in the RD/1AG-MM-GVH group regardless of the disease risk. The RD/1AG-MM-GVH group with an HLA-B mismatch showed significantly higher overall and NRM, whereas the RD/1AG-MM-GVH group with an HLA-A or HLA-DR mismatch showed an OS comparable to that in the UCB group. Neutrophil and platelet engraftment in the RD/1AG-MM-GVH group were significantly faster than those in the UCB group, whereas the incidence of acute or chronic GVHD in the RD/1AG-MM-GVH group was significantly higher. However, the incidence of acute or chronic GVHD in the RD/1AG-MM-GVH group with *in vivo* T-cell depletion was comparable to that in the UCB group, which translated into a better, but not significantly better, OS than that in the UCB group.

In Japan, unrelated BM donor coordination (from donor search to transplantation) takes a median of 4 months, whereas much less time is required for UCB or RD/1AG-MM-GVH transplantation if there is a candidate. This was reflected in the longer duration from diagnosis to transplantation in unrelated BM transplantation.[32] In contrast, UCB and RD/1AG-MM-GVH transplantation show a similar and shorter duration (Table 1 ; 7.9 months vs 7.6 months). Therefore, in cases where both UCB and RD/1AG-MM-GVH are available, donors should be chosen based on their advantages and disadvantages. Compared with UCB, the use of RD/1AG-MM-GVH has a great advantage in neutrophil and platelet engraftment, which is not inconsistent with a previous finding that engraftment in the UCB group was significantly delayed comparing with that in MUD.[33] This translated into a lower rate of death from graft failure or infection in the RD/1AG-MM-GVH group. However, these advantages were offset by a substantial increase in the incidence of acute and chronic GVHD in the RD/1AG-MM-GVH group. The risk of grade III–IV acute GVHD and extensive chronic GVHD in the RD/1AG-MM-GVH group was twice that in the UCB group. If UCB units containing adequate total nucleated cell doses (ex. $>2.5\times10^7$/kg) are available,[34] the selection of UCB would be appropriate to avoid the risk of chronic GVHD. In contrast, RD/1AG-MM-GVH would be more appropriate when early neutrophil engraftment should be prioritized, such as for a patient with an active infectious disease at transplantation.

The high incidences of GVHD and GVHD-related death in the RD/1AG-MM-GVH group indicate the need for stronger immunosuppression to improve the clinical outcome. The use of T-cell depletion, mostly by ATG, was significantly associated with a lower incidence of grade III–IV acute GVHD and extensive chronic GVHD in the RD/1AG-MM-GVH group. Although this effect was not statistically significant, the RD/1AG-MM-GVH group with *in vivo* T-cell depletion showed lower overall and treatment-related mortality, which would outweigh a possible increased risk of relapse. These findings in our cohort suggest that ATG may be effective, and the addition of ATG in the RD/1AG-MM-GVH group should be assessed in a prospective study.

As shown in our previous study,[23] overall mortality in the RD/1AG-MM-GVH group involving an HLA-B mismatch was significantly higher than that in the RD/1AG-MM-GVH group with an HLA-A or -DR mismatch, probably because of an additional HLA-C antigen mismatch as expected from linkage disequilibrium between HLA-B and HLA-C and available data on HLA-C antigen.[23,35] The incidence of grade III–IV acute GVHD in the HLA-B mismatch group was higher than that in the HLA-DR mismatch group, but was comparable to that in the HLA-A mismatch group. In addition, the incidence of death from GVHD was similar in the HLA-B and HLA-A/DR mismatch groups (data not shown). Therefore, the reason for the lower overall morality in the RD/1AG-MM-GVH group with an HLA-B mismatch remains unclear. However, the adverse effect of an HLA-B mismatch disappeared when *in vivo* T-cell depletion was used, which suggests that an immunological effect is involved in this mechanism.

This study has several limitations. First, in clinical practice in Japan, matching of HLA-DR is counted at a low resolution, as with HLA-A and HLA-B, whereas it is counted at a high resolution in the

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United States and Europe. To evaluate the impact of this difference, we divided patients in the UCB group with two antigen mismatches into two groups by using available HLA-DRB1 allele information: a group with two antigen mismatches with one additional HLA-DRB1 allele mismatch ($n = 609$) and another group with two antigen mismatches without an additional HLA-DRB1 mismatch ($n = 295$). We did not find a significant difference in OS between these two groups ($P = 0.758$), which suggests that HLA-matching using HLA-DR antigen or allele information will not affect OS in the present study. Second, the findings in the present study are based on Asian cohort who received a 'single' UCB or RD/1AG-MM-GVH transplantation. Lighter body weight in Asian population than Caucasian population may make it easy to find a suitable single UCB unit that contains adequate total nucleated cell doses. In addition, as suggested by Oh *et al.*,[36] limited heterogeneity of Japanese population may affect the outcomes of transplantation. Therefore, the findings should be externally validated in the non-Asian cohort or transplantation using double UCB units. Third, information on the dose and type of ATG was missing in two-third of the patients who received ATG. However, the available data showed that the median dose of thymoglobulin (2.5 mg/kg) or ATG-F (8 mg/kg) was equivalent to the dose that is widely used in our daily practice. Lastly, heterogeneous backgrounds may have resulted in a bias, although we tried to adjust for possible confounders by multivariate analyses. Lastly, the effect of multiple testing should be taken into account for the interpretation of secondary end points.

In conclusion, our findings suggest that both UCB and RD/1AG-MM-GVH are suitable as alternative donors for patients without an HLA-matched sibling or unrelated donor. However, the presence of an HLA-B-antigen mismatch in the GVH direction has an adverse effect on OS because of treatment-related complications. Neutrophil and platelet engraftment in the RD/1AG-MM-GVH group were significantly faster than those in the UCB group, whereas the incidence of acute and chronic GVHD in the RD/1AG-MM-GVH group was significantly higher, which translated into a high incidence of death from GVHD. Donor selection between UCB and RD/1AG-MM-GVH should be determined based on the presence of an HLA-B mismatch in RD/1AG-MM-GVH and from the risks and benefits derived from the risk of graft failure and infection in the UCB group and acute or chronic GVHD in the RD/1AG-MM-GVH group. Additional immune suppression using *in vivo* T-cell depletion may improve the clinical outcome in the RD/1AG-MM-GVH group by decreasing the incidences of GVHD and NRM and may also overcome the adverse effect of an HLA-B mismatch. This approach should be assessed in a prospective study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

JK and YK designed the research, organized the project and wrote the manuscript; JK, YA, and YK performed the statistical analysis and analyzed the data; KK and TN-I collected data from JCBBN; and all of the authors interpreted the data and reviewed and approved the final manuscript.

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ORIGINAL ARTICLE

Use of mycophenolate mofetil in patients received allogeneic hematopoietic stem cell transplantation in Japan

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Abstract We evaluated the use of mycophenolate mofetil (MMF) after hematopoietic stem cell transplantation (HSCT) in Japan from 1999 to 2008. MMF was administered to 301 patients, including 157 for the prevention of graft-versus-host disease (GVHD), 94 for the treatment of acute GVHD and 50 for the treatment of chronic GVHD. The three most common doses were 500 mg twice daily, 250 mg three times daily and 1,000 mg twice daily, given to 63, 54 and 45 patients, respectively. The incidence of grade II–IV acute GVHD was 30.0% and grade III–IV was 20.0% in the GVHD prevention group. Among treated patients, disappearance or improvement of subjective symptoms occurred in 57.0% of acute GVHD patients and in 52.0% of chronic GVHD patients. With regard to safety, the following major adverse events (grade 3 or more) were recorded: 31 infections, 31 neutropenia, 28 thrombocytopenia, 25 diarrhea and 1 renal disorder. A total of 116 patients developed grade 3 or 4 adverse events, but 79 were successfully treated with supportive treatment. Thus, our findings suggest that MMF is safe and effective for the

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prevention and treatment of GVHD in patients who have received an allogeneic stem cell transplant.

Keywords Mycophenolate mofetil (MMF) · Allogeneic stem cell transplantation · GVHD

1 Introduction

Acute and chronic graft-versus-host disease (GVHD) are important complications following allogeneic hematopoietic stem cell transplantation (HSCT) that can be prevented or treated by immunosuppressive agents such as cyclosporine, tacrolimus, steroids or other therapies [1–3]. Some patients, however, do not respond to these conventional treatments. It is well recognized that mycophenolate mofetil (MMF) is widely used in countries outside Japan, and numerous reports have documented its efficacy for prophylaxis and treatment of GVHD [4–13].

In Japan, MMF is only approved as an immunosuppressant drug for organ transplantation (e.g., renal transplantation) and has not been approved for prophylactic or therapeutic use for GVHD in the field of HSCT. As there have been several reports of experimental MMF use for HSCT in Japan [14, 15], we conducted a nationwide survey to determine the efficacy and safety of MMF in the Japanese population.

2 Patients and methods

2.1 Study design

We retrospectively collected data on MMF use after allogeneic HSCT from related donors. Questionnaires were sent to 228 institutes registered with the Japan Society for Hematopoietic Cell Transplantation (JSHCT). A total of 57 surveys were returned detailing 301 patients undergoing MMF treatment. Data regarding the purpose of treatment, dosage, length of treatment, presence or absence of subjective symptoms of GVHD, GVHD grade and stage (before and after treatment), decrease or increase in concomitant immunosuppressants, effects, adverse events and outcomes were collected. Basic information for each transplantation was extracted from the Transplant Registry Unified Management Program (TRUMP) system, which is a registry used for Japanese patient outcomes [16]. Several demographic data were not available due to the lack of patient entry into the TRUMP system. The effects of MMF with regard to subjective symptoms (none, disappearance, improvement, no change and ingravescence) and the use of steroids (none, withdrawal, dose reduction, no change and dose increase) were assessed by physicians. Adverse events were evaluated by the National Cancer Institute-Common Terminology Criteria for Adverse Events (NCI-CTCAE, ver.3). This study was approved by the ethical committees of the Japan Society of Hematopoietic Cell Transplantation and the Nagoya University School of Medicine.

2.2 Statistics

Correlations between the two subgroups were examined using the χ^2 test and Fisher's exact test. P values of less than 0.05 obtained in two-sided tests were considered statistically significant. The data were analyzed with STATA version 10 statistical software (STATA Corp, TX).

3 Results

3.1 Patient background

Patient background data are summarized in Table 1. Patient age ranged from 12 to 70 years (median 41) at the time of transplantation, and there were 173 (57.5%) male and 128 (42.5%) female patients. Among the 301 patients, 97 (32.2%) received a transplant from HLA-matched donor, and 182 (60.5%), from HLA-mismatched donors. Of the HLA-mismatched donors, 66 (36.3%) were 1 locus, 46 (25.3%) were 2 loci and 55 (30.2%) were 3 loci mismatched. There were also 22 patients (7.3%) with missing HLA data. Among the 157 patients who received MMF for GVHD prophylaxis, 119 (75.8%) received a transplant from an HLA-mismatched donor, and among the 50 patients who received MMF as a treatment for chronic GVHD, 17 (34.0%) received a transplant from an HLA-mismatched donor. The graft source was peripheral blood stem cells (PBSCs) in 176 patients, bone marrow (BM) in 101 patients and PBSCs plus BM in 2 patients. The preconditioning regimen was myeloablative in 91 patients and non-myeloablative in 166 patients. Table 1 shows that the primary disease was hematological malignancy in the majority of patients (94.4%) with aplastic anemia or other diseases accounting for the remainder of the patients. Among the patients with the hematological malignancies, 65.9% (162/246, which is clear data of disease status) were in non-complete remission at the time of transplantation.

3.2 MMF administration

The aim of MMF administration was GVHD prevention in 157 patients, acute GVHD treatment in 94 patients and chronic GVHD treatment in 50 patients (Table 1). The daily MMF dosage varied from 250 to 3,000 mg, and the number of doses per day ranged from 1 to 8. The most common dosages and frequencies of MMF administration were

Table 1 Patient characteristics

Variables	Number
Patient number	301
Median age (range)	41 (12–70)
Male/female	173/128
Disease[a]	
Acute myeloid leukemia	78 (46)
Acute lymphoblastic leukemia	66 (44)
Chronic myelogenous leukemia	15 (11)
Myelodysplastic/myeloproliferative syndrome	39 (12)
Malignant lymphoma	75 (41)
Multiple myeloma	11 (8)
Aplastic anemia	3
Other diseases	14 (11)
Purpose of MMF	
GVHD prophylaxis	157
aGVHD treatment	94
cGVHD treatment	50
Graft source[b]	
Bone marrow (BM)	101
Peripheral blood stem cell (PBSC)	176
Both BM and PBSC	2
Donor type[b]	
Matched related	97
Mismatched related	182
1 locus mismatch	66
2 loci mismatch	46
3 loci mismatch	55
Unknown	15

[a] Numbers in parenthesis indicate those of not in complete remission

[b] Twenty-two data were missing for graft source and donor type

500 mg two times per day, 250 mg three times per day and 1,000 mg two times per day given to 63 patients (20.9%), 54 patients (17.9%) and 45 patients (15.0%), respectively. Consequently, 91 patients received 1,000 mg of MMF per day, and 54 patients, 750 or 2,000 mg per day. 59 patients were treated with a daily dose higher than 2,000 mg. There was no consistent pattern between the length and purpose of treatment. MMF administration was discontinued within 30 days in 113 patients (38.4%); however, 19 patients received MMF for more than a year (Fig. 1). Most patients (289 patients, 96.0%) were given MMF concurrently with other immunosuppressants (e.g., cyclosporine, tacrolimus or steroids), and only 12 patients (4%) received MMF alone.

3.3 Adverse events

Adverse events (AEs) associated with MMF administration are listed in Table 2. The major events were neutropenia, infection, thrombocytopenia and myelosuppression. Only

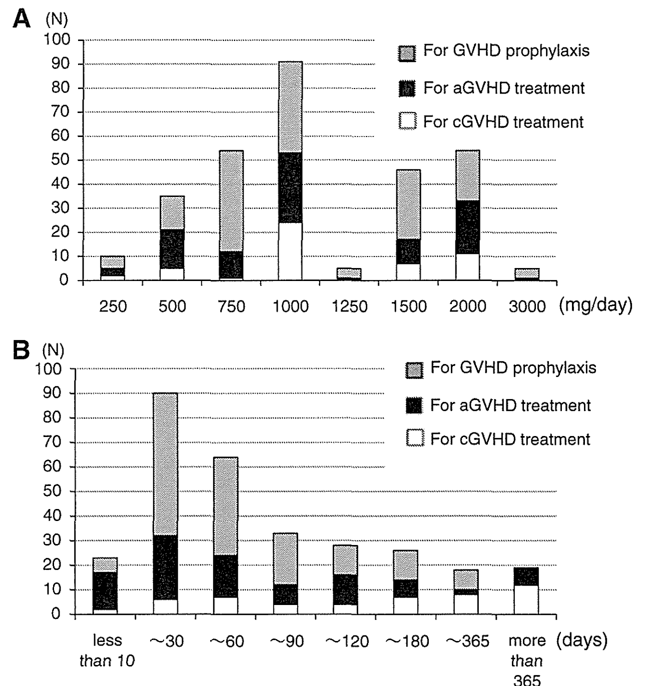

Fig. 1 **a** Initial dose of MMF. MMF was given at a variety of doses ranging from 250 mg per day to 3,000 mg per day. The most common dose was 500 mg twice a day ($N = 67$ among 91 patients taking 1,000 mg per day). **b** Dosing period of MMF. MMF was given for a variety of dosing periods (median 45 days)

three patients (1.7%) developed renal insufficiency with a grade 1, 2 or 4 increase in creatinine. Eighteen patients (6.0%) died from AEs associated with MMF (Table 3). The primary causes of death were infections in 11 patients (including 5 patients with pneumonia, 4 with sepsis and 2 with invasive *Aspergillus* infection), neutropenia in 3 patients, myelosuppression in 2 patients, 1 thrombocytopenia and 1 brain hemorrhage. There were 44 grade 4 AEs: 25 of these patients (56.8%) improved and 15 (34.1%) remained unchanged, but 4 (9.1%) eventually died. The incidence of AEs of grade 3 or higher (except infection) increased in accordance with the daily dosage of MMF (Fig. 2), but most of these AEs improved (Table 4).

3.4 Efficacy of MMF

Among the 157 patients who received MMF for GVHD prophylaxis, the incidences of grade II–IV and grade III–IV acute GVHD were 29.7% (43/145) and 20.0% (29/145), respectively. Limited and extensive chronic GVHD occurred in 21 (18.6%) and 30 (26.6%) patients, respectively ($N = 113$). No significant differences were found in the incidence of grade II–IV acute GVHD between HLA-matched and mismatched transplant patients (9/25 = 36.0 vs. 33/113 = 29.2%, $P = 0.63$), and no significant differences were noted between these two groups with regard to the

Table 2 Adverse events whose relationships to MMF were not necessarily denied

Adverse events: all (grade 3–5)	GVHD prophylaxis (N = 157)		aGVHD treatment (N = 94)		cGVHD treatment (N = 50)		Total (N = 301)	
	N	%	N	%	N	%	N	%
Infection	6 (5)	3.8 (3.2)	16 (13)	17.0 (13.8)	9 (8)	18.0 (16.0)	31 (26)	10.3 (8.6)
Diarrhea	6 (5)	3.8 (3.2)	16 (10)	17.0 (10.6)	3 (3)	6.0 (6.0)	25 (18)	8.3 (6.0)
Nausea	7 (2)	4.5 (1.3)	6 (4)	6.4 (4.3)	3 (0)	6.0 (0)	16 (6)	5.3 (2.0)
Vomiting	2 (0)	1.3 (0)	2 (0)	2.1 (0)	1 (0)	2.0 (0)	5 (0)	1.7 (0)
Neutropenia	5 (5)	3.2 (3.2)	21 (20)	22.3 (21.3)	5 (5)	10.0 (10.0)	31 (30)	10.3 (10.0)
Thrombocytopenia	5 (5)	3.2 (3.2)	18 (15)	19.1 (16.0)	5 (5)	10.0 (10.0)	28 (25)	9.3 (8.3)
Myelosuppression	7 (7)	4.5 (4.5)	10 (7)	10.6 (7.4)	4 (4)	8.0 (8.0)	21 (18)	7.0 (6.0)
Gastrointestinal bleeding	3 (2)	1.9 (1.3)	3 (3)	3.2 (3.2)	0 (0)	0 (0)	6 (5)	20.0 (1.7)
Constipation	1 (0)	0.6 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0.3 (0)
Others	5 (3)	3.2 (1.9)	7 (3)	7.4 (3.2)	3 (2)	6.0 (4.0)	15[a] (8[b])	5.0 (2.7)

Numbers in parenthesis indicate those for grade 3 or more toxicity

[a] Others: liver dysfunction (3), creatine kinase elevation (2), hair loss, hemorrhage cystitis

[b] Others: hypocalcemia, brain hemorrhage, septic shock, creatine kinase elevation, abdominal pain, TMA, diabetes mellitus, engraft failure

Table 3 Cause of death potentially associated with MMF

	Number
Infection	11
Pneumonia	5
Bacterial	2
MRSA	1
Fungal	1
CMV	1
Sepsis	4
Invasive *Aspergillus* infection	2
Neutropenia	3
Myelosuppression	2
Thrombocytopenia	1
Brain hemorrhage	1
Total	18

incidence of grade III–IV acute GVHD (6/25 = 24.0 vs. 22/113 = 19.5%, $P = 0.59$). The incidence of chronic GVHD, however, tended to be lower in the HLA-mismatched transplant group (14/23 = 60.9 vs. 35/83 = 42.2%, $P = 0.16$; Fig. 3), although this finding was not statistically significant. The incidences of grade II–IV and III–IV acute GVHD were lower in the subgroup of patients receiving 2,000 mg of MMF daily than in the subgroup receiving 1,000 mg daily (28.6 vs. 37% and 14.3 vs. 28.6% for grade II–IV and III–IV acute GVHD, respectively), although these differences were not statistically significant ($P = 0.51$ and 0.22, respectively). No dose effect was found for chronic GVHD prevention ($P = 0.72$).

Among the 94 patients in the acute GVHD treatment group, subjective symptoms disappeared in 27 (28.7%) and improved in 28 (29.8%). Symptoms remained unchanged in 17 patients (18.1%) and worsened in 22 patients (23.4%). Within this treatment group, 52 patients (55.3%) experienced improvement in their acute GVHD grade. Treatment with combined immunosuppressants was discontinued in 5 patients (5.3%) and reduced in 51 patients (54.3%). Among the 50 patients who received MMF as a treatment for chronic GVHD, the drug was effective against subjective symptoms (i.e., resulted in resolution or improvement) in 52.0% (10.0 and 42.0% experiencing resolution and improvement, respectively). Five patients (10.0%) discontinued combined immunosuppressants, and 29 (58.0%) reduced their dosage. The dosage remained unchanged in 14 patients (28.0%) and increased in only 2 patients (4%) (Fig. 4). In the acute GVHD treatment group, the effectiveness of MMF was higher among patients who had received HLA-matched transplants; however, this difference was not statistically significant for all items evaluated (58–70 vs. 32–69%, $P = 0.18$–0.60). In the chronic GVHD treatment group, the efficacy of MMF against subjective symptoms was higher in the HLA-matched subgroup than in the HLA-mismatched subgroup (17/33 = 51.5 vs. 3/9 = 33.3%, respectively, $P = 0.45$). In contrast, the rate of dosage reduction or discontinuation for combined immunosuppressants was higher in the HLA-mismatched subgroup than in the HLA-matched subgroup (7/9 = 77.8 vs. 21/33 = 63.6%, respectively, $P = 0.69$).

To assess the efficacy of MMF with regard to total daily dosage, we selected two subgroups: the most frequent dosage (1,000 mg per day) and the maximum dosage (more than 2,000 mg per day). The efficacy rate for every acute GVHD survey item was virtually identical between the 1,000 mg per day ($N = 28$) and 2,000 mg per day ($N = 23$) subgroups

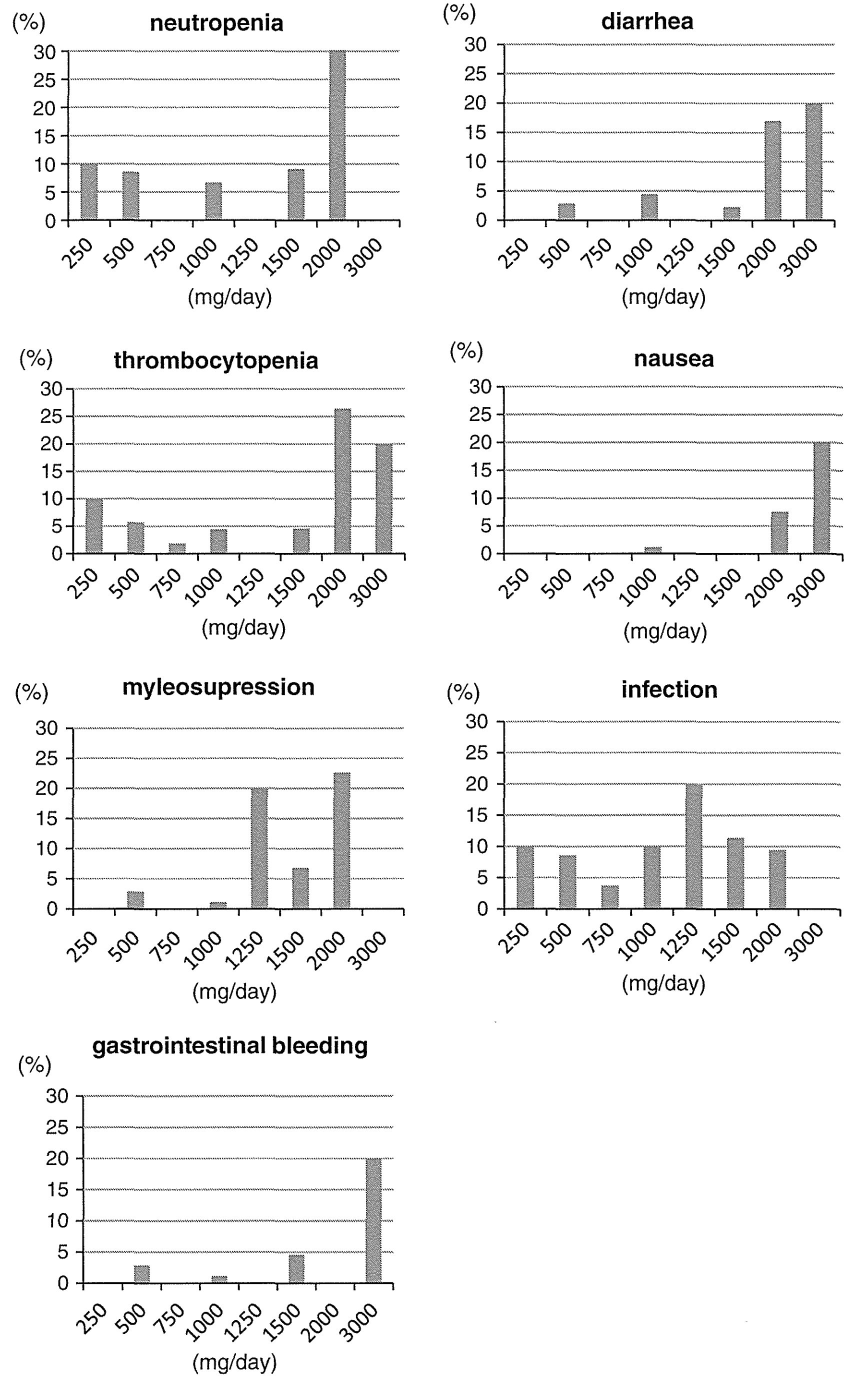

Fig. 2 Frequency of adverse events (grades 3–5) separated by total daily dose. High doses of MMF resulted in higher rates of hematological and gastrointestinal adverse events. Infections developed at all doses of MMF

(47.8–70.8 vs. 33.3–72.7%, respectively, $P = 0.06$-0.97). Among chronic GVHD patients, no difference in dose efficacy was observed between the two dosage subgroups ($N = 24$ in the 1,000 mg per day group and $N = 11$ for patients taking more than 2,000 mg per day, $P = 0.83$–0.91).

3.5 Transplantation outcome

In the GVHD prevention group, engraftment was seen in 122 of 134 evaluable patients (91.0%). Among all 301 patients, 62 (20.7%) relapsed and 169 (56.2%) died after transplantation. The overall survival rate was 41.9% at a median follow-up of 3 years. The main causes of death included disease recurrence in 33 patients (responsible for 19.5% of patient mortality), infection in 26 patients (15.4%), acute GVHD in 26 patients (15.4%) and chronic GVHD in 7 patients (4.1%). Among the 26 deaths due to acute GVHD, 18 patients were in the acute GVHD treatment group. Among the seven patients who died due to chronic GVHD, four were in the chronic GVHD treatment group.

Table 4 Rate of recovery from the adverse events in grades 3–4

	1,000 mg/day ($N = 91$)	More than 2,000 mg/day ($N = 59$)	Total ($N = 301$)
Infection	1/4 (25%)	1/2 (50%)	12/16 (75%)
Diarrhea	3/4 (75%)	7/10 (70%)	10/16 (63%)
Nausea	0/1 (0%)	4/5 (80%)	4/6 (67%)
Neutropenia	6/6 (100%)	12/15 (80%)	24/27 (89%)
Thrombocytopenia	3/4 (75%)	5/14 (36%)	11/24 (46%)
Myelosuppression	1/1 (100%)	8/11 (73%)	12/16 (75%)
Gastrointestinal bleeding	1/1 (100%)	0/1 (0%)	2/5 (40%)

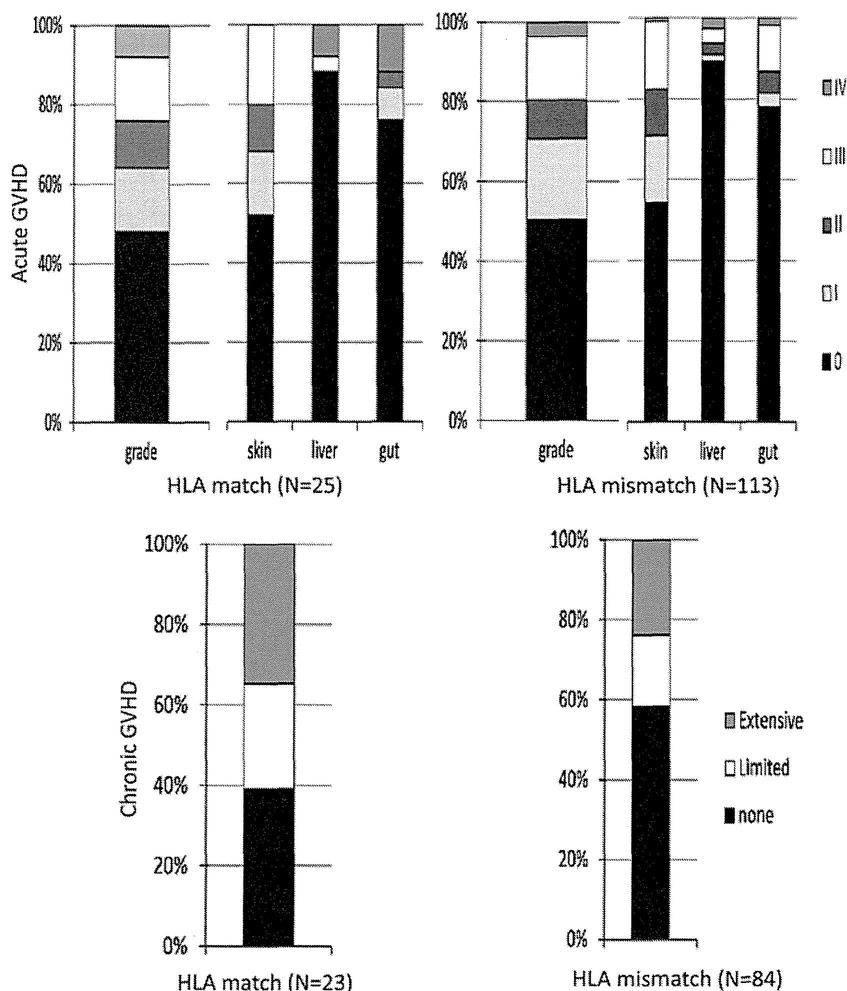

Fig. 3 Incidence of GVHD with prophylactic MMF use. The incidences of grade II–IV acute GVHD were 36.0 and 29.2% in the HLA-matched and -mismatched subgroups, respectively. In contrast, the incidence of chronic GVHD in the HLA-mismatched subgroup was lower (42.2%) than in the HLA-matched subgroup (60.9%)

4 Discussion

GVHD is one of the leading complications following allogeneic HSCT and is associated with morbidity and mortality. Calcineurin inhibitors and steroids are widely used for GVHD prevention and treatment, but several other immunosuppressive agents have been used for these purposes overseas [17–19]. Since 1997, many promising reports have compared MMF with conventional immunosuppressants [4–13]. In particular, reports focused on GVHD prevention are becoming increasing common due to the use of alternative donor sources [20]. Our current survey demonstrates that the efficacy rate of MMF is approximately 60% for the treatment of acute and chronic GVHD. Furthermore, our results also reveal that MMF is effective for the prevention of GVHD. Especially in HLA-mismatched patients, the frequency of grade III–IV acute GVHD was 20.3%, which was lower than the previous report subjected to HLA-mismatched transplants among Japanese populations [21]. As the efficacy of MMF was

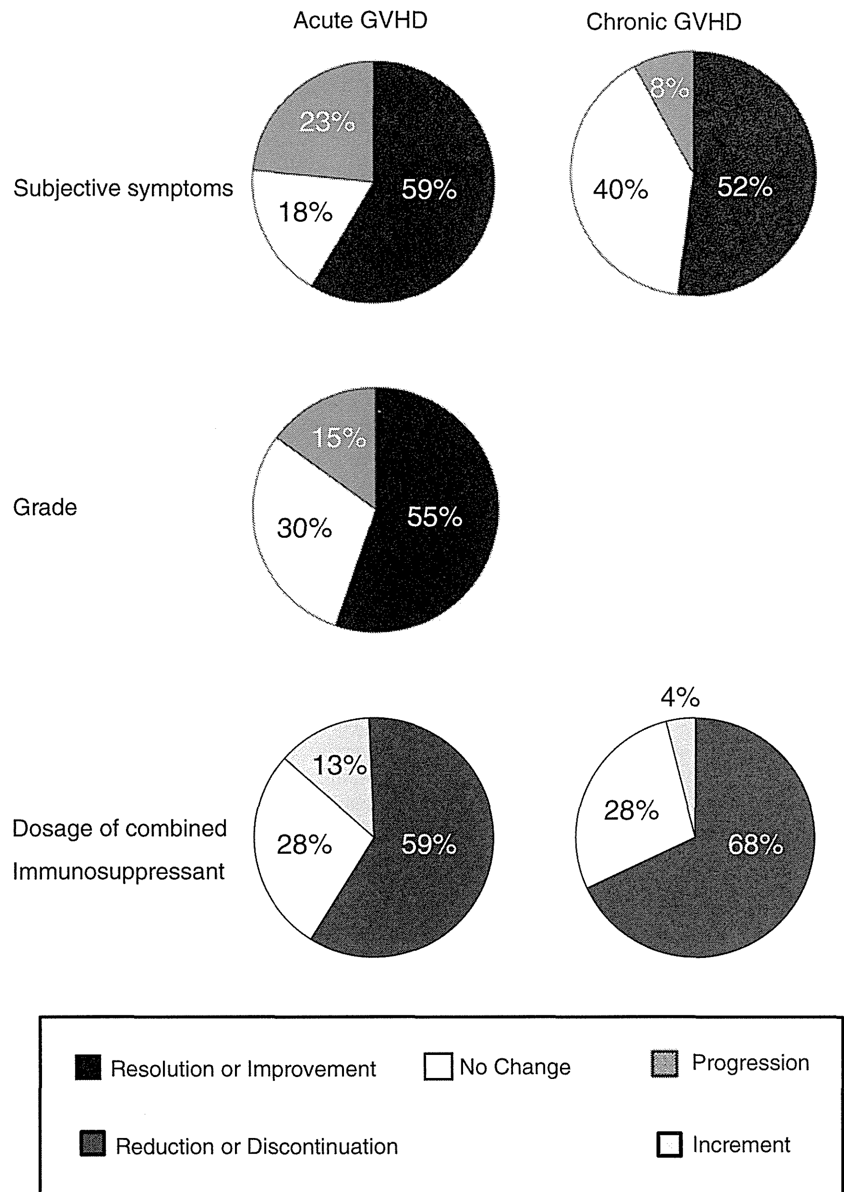

Fig. 4 Response of acute and chronic GVHD during therapeutic MMF use. Subjective symptoms of acute and chronic GVHD resolved in 59 and 52% of the cases, respectively, following the administration of MMF. In addition, 55% of the acute GVHD patients improved the grade of their disease. Finally, 60 and 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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Graft-versus-host disease disrupts intestinal microbial ecology by inhibiting Paneth cell production of α-defensins

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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′ ($H' = -\Sigma pi ln(pi)$)[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.

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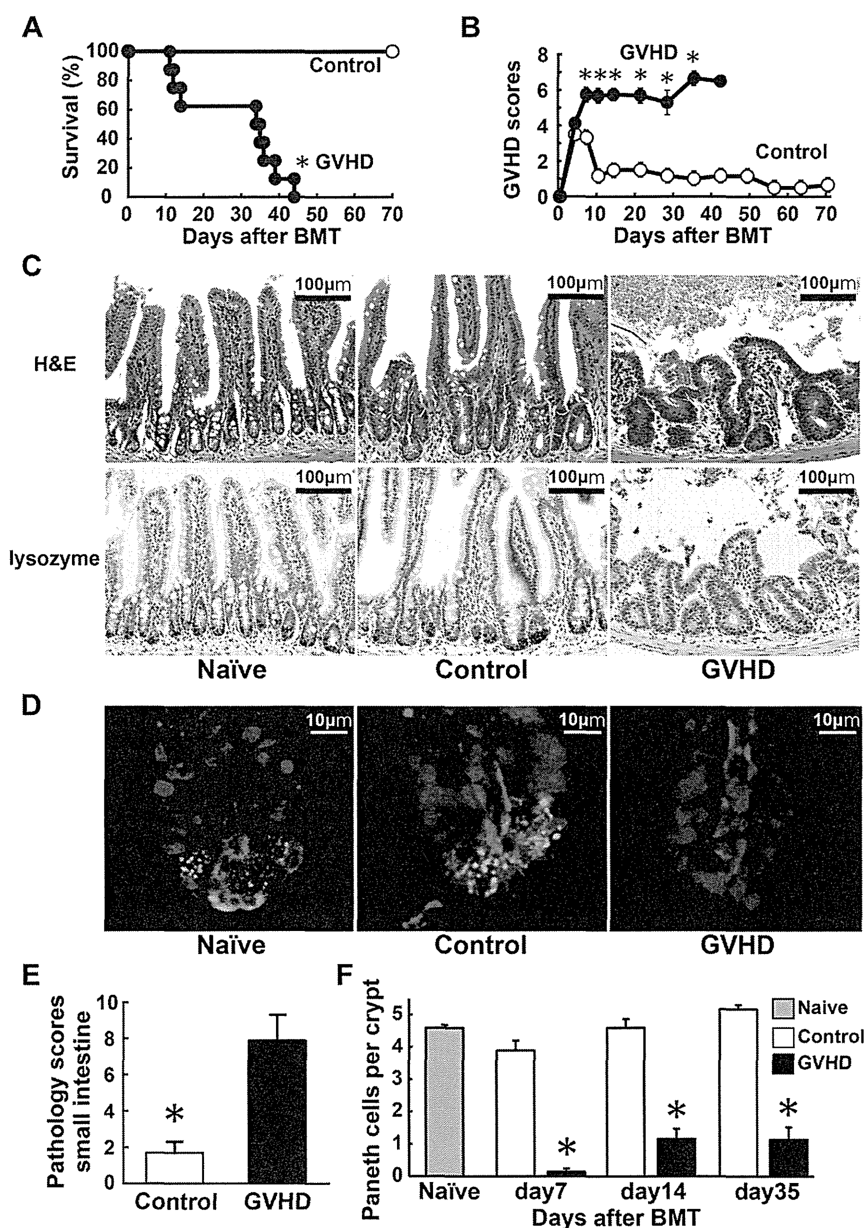

Figure 1. Paneth cell injury in GVHD. Lethally irradiated B6D2F1 mice were transplanted with 5 × 10⁶ TCD BM cells without (control group, n = 6) or with 2 × 10⁶ T cells (GVHD group, n = 12) from MHC-mismatched B6 donors on day 0. (A-B) Survival (A) and clinical GVHD scores (B) means ± SE are shown. Data 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 ($H\text{-}2^{b/d}$) 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 ($H\text{-}2^b$) 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

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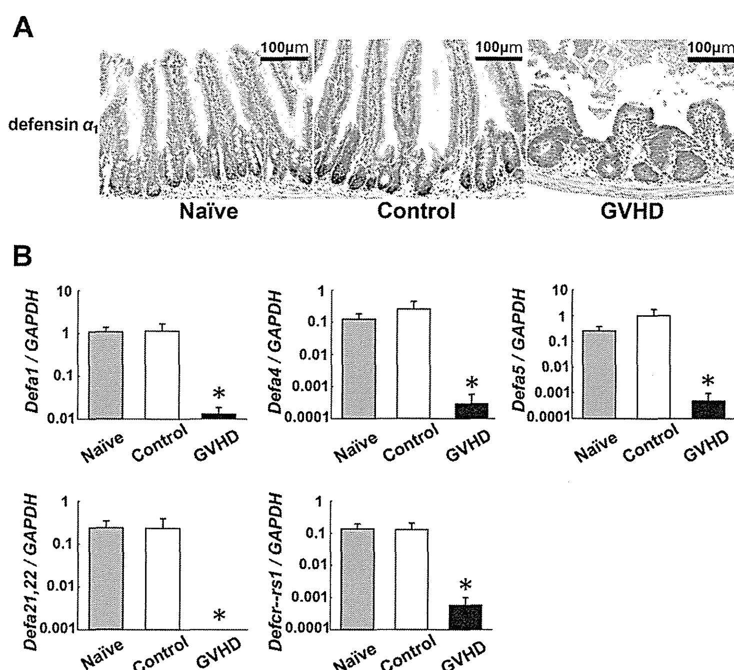

Figure 2. Decreased expression of Paneth cell–derived α-defensins in GVHD. Lethally irradiated B6D2F1 mice were transplanted with 5 × 10^{6} TCD BM without (control group) or with (GVHD group) 2 × 10^{6} T cells from B6 donors. Small intestines were isolated from mice 7 days after BMT. (A) Immunohistochemical 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-*α_1 *(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).

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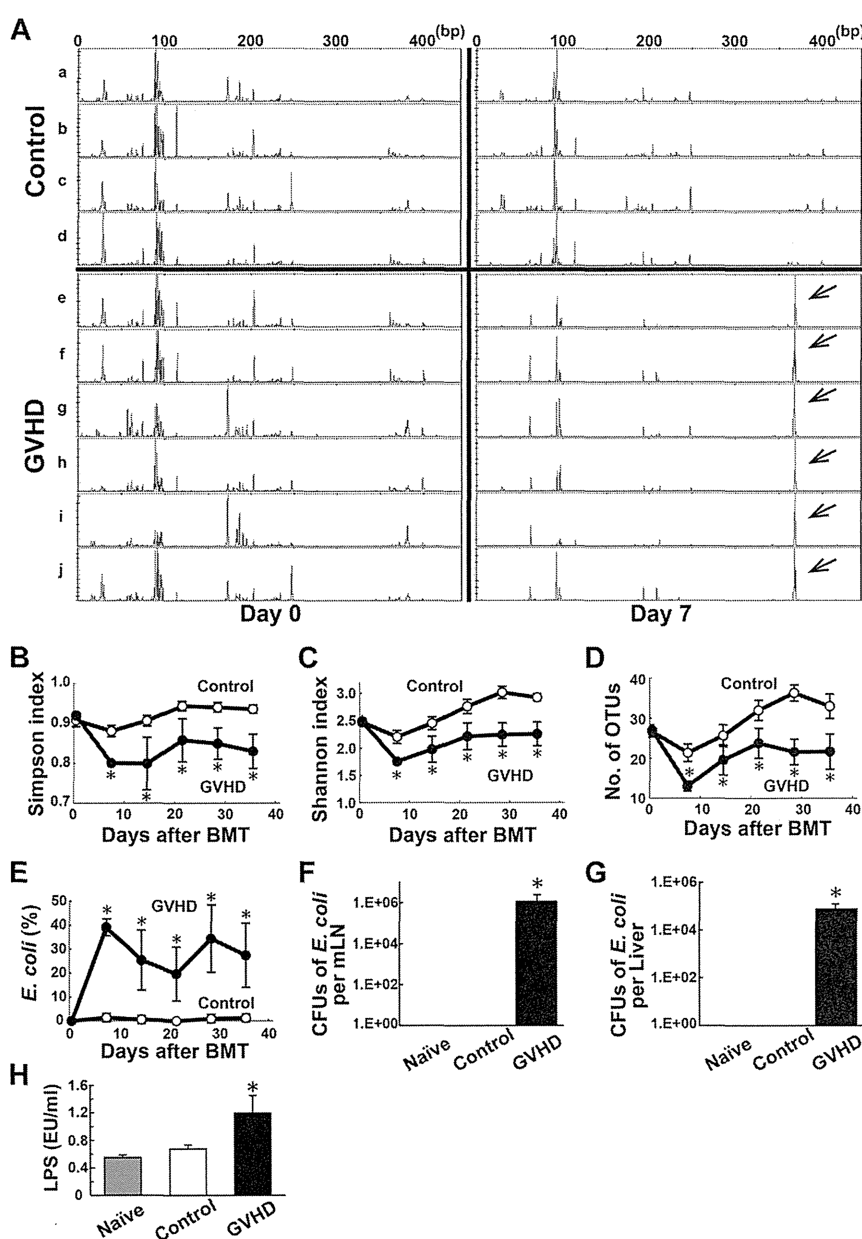

Figure 3. Perturbation of normal intestinal microbiota in GVHD. Fecal pellets were collected before and after a B6 → B6D2F1 BMT weekly and intestinal microbiota was characterized by RFLP analysis of 16S rRNA gene libraries constructed from each sample of fecal pellets and digested with *Hha*I (n = 6 / 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