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ORIGINAL ARTICLE

Risk factors affecting cardiac left-ventricular hypertrophy and systolic and diastolic function in the chronic phase of allogeneic hematopoietic cell transplantation

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Chronic impairment of cardiac function can be an important health risk and impair the quality of life, and may even be life-threatening for long-term survivors of allogeneic hematopoietic cell transplantation (HCT). However, risk factors for and/or the underlying mechanism of cardiac dysfunction in the chronic phase of HCT are still not fully understood. We retrospectively investigated factors affecting cardiac function and left-ventricular hypertrophy (LVH) in the chronic phase of HCT. Sixty-three recipients who survived for >1 year after receiving HCT were evaluated using echocardiography. Based on simple linear regression models, high-dose TBI-based conditioning was significantly associated with a decrease in left-ventricular ejection fraction and the early peak flow velocity/atrial peak flow velocity ratio, following HCT (coefficient = −5.550, $P=0.02$ and coefficient = −0.268, $P=0.02$, respectively). These associations remained significant with the use of multiple linear regression models. Additionally, the serum ferritin (s-ferritin) level before HCT was found to be a significant risk factor for LVH on multivariable logistic analysis ($P=0.03$). In conclusion, our study demonstrated that a myeloablative regimen, especially one that involved high-dose TBI, impaired cardiac function, and that a high s-ferritin level might be associated with the development of LVH in the chronic phase of HCT.

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INTRODUCTION

The long-term survival rate after allogeneic hematopoietic cell transplantation (HCT) has significantly improved over recent decades because of improvements in the HCT procedure. However, chronic complications, such as cardiovascular disease, have become increasingly evident and constitute a serious problem in long-term survivors of HCT.[1–3] It is well known that the high-dose CY used for conditioning is a major cause of acute cardiotoxicity after HCT.[4,5] However, it is still unclear as to which factors contribute to chronic cardiotoxicity after HCT. It is possible that conditioning regimens consisting of high-dose CY or high-dose TBI influence cardiac function even in the chronic phase,[6,7] a contention that has not been evaluated in adult patients. Cytokines, such as IL-2 and TNF-alpha, have the potential to affect myocardial tissue adversely,[8] while TNF-alpha is also thought to be involved in the pathogenesis of chronic GVHD.[9] Chronic GVHD may therefore contribute to cardiac tissue damage via cytokines. However, it has not yet been determined whether chronic GVHD has a detrimental effect on cardiac function after HCT.

Left-ventricular hypertrophy (LVH) is often seen in patients with hypertension (HT). One report indicates that inflammatory cytokines, including IL-2 and TNF-alpha, have a role in the development of LVH.[10] It is still not clear which factors affect LVH in HCT recipients. Here we comprehensively evaluate the factors that affect chronic LVH and cardiac systolic and diastolic function after HCT, in a retrospective cohort study that used echocardiographic assessment.

MATERIALS AND METHODS

Study design

We examined left-ventricular systolic and diastolic function and LVH in patients who were eligible for the study as they had received HCT between April 2000 and June 2010 at our institution, had undergone an echocardiographic examination within the 2 months before HCT and had survived for >1 year after HCT. All the evaluated patients visited our hospital regularly. Patients who received more than two allogeneic HCT were excluded from analysis. We also statistically investigated the factors that influenced cardiac function after HCT. Written informed consent was obtained from all the enrolled patients and this study protocol was approved by the Institutional Review Board of our institution.

Cardiac evaluation

Echocardiographic examinations were performed using a Power Vision 6000 (Toshiba, Tokyo, Japan). An electrocardiographic tracing was recorded in the left-lateral decubitus position simultaneously with the echocardiogram. Transducer frequency was 2.5 or 3.5 MHz. We recorded left-ventricular end-diastolic dimension and left-ventricular end-systolic dimension from the left-parasternal long axis view at the high papillary muscle level and calculated left-ventricular ejection fraction (LVEF) by the Teichholtz method. Trans-mitral flow-velocity patterns were measured on an apical, four-chamber view according to the standard method. We assessed LVEF as a surrogate marker of LV systolic function and the early

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peak flow velocity/atrial peak flow velocity (E/A ratio) as a surrogate marker of LV diastolic function. 'LVH after HCT' was identified when the interventricular septal wall and/or the posterior wall became >12-mm thick after HCT.

Statistical analysis

We assessed the possible factors that influence cardiac function after HCT, including intensity of the conditioning regimen (high-dose, TBI-based conditioning regimens vs high-dose busulfan (BU)-based conditioning regimens vs all others), sex, age, a history of HT, hemoglobin concentration, serum ferritin (s-ferritin) levels, chronic GVHD and cumulative dose of anthracyclines. All the high-dose TBI-based conditioning regimens included TBI 1200 cGy and CY 120 mg/kg. High-dose BU-based conditioning regimens used oral BU 16 mg or i.v. BU 12.8 mg and CY 120 mg/kg; all other regimens were defined as reduced intensity conditioning (RIC). HT was defined as a history of HT within 1 year after HCT. We also analyzed data on hemoglobin concentration determined 1 year after HCT, and s-ferritin levels taken before HCT. We defined a history of chronic GVHD as the presence of chronic GVHD within 1 year of HCT. We calculated the relative cumulative dose of anthracyclines with the use of a multiplier of 1 for doxorubicin, 0.5 for DNR, 1.6 for idarubicin, 3.4 for mitoxantrone and 0.1 for aclarubicin.[11]

All statistical tests were interpreted at the 5% significance level. All *P*-values and 95% confidence intervals were two-sided. Simple or multiple linear regression analysis was used to assess the associations of the pre-transplant or the post-transplant variables with the differences in LVEF or E/A measured before HCT and at the follow-up visit. To assess whether the correlation between the type of pre-transplant conditioning regimen and measures, including change in LVEF or E/A, varied significantly with covariates that included age, sex or chronic GVHD, first-order interactions between the variable and the covariates were inserted into a regression model that contained the interaction term and both covariates as main effects. Analysis of residuals was performed to examine model fit and adherence to regression assumptions. Multi-colinearity was assessed using a variance inflation factor. A variance inflation factor exceeding 10 is thought to indicate serious multi-colinearity, and values >4.0 may be a cause for concern.[12] Changes in LVEF or E/A before and after HCT were tested for significance with a paired *t*-test. Repeated measures analysis of variance was used to assess the differences in the degree of change in LVEF or E/A before and after HCT among the groups divided by the type of pre-transplant conditioning regimen. Univariable or multivariable logistic regression analysis was used to estimate the odds ratio for incidence of LVH. Nonlinear effects of continuous independent variables were evaluated using quadratic and log transformations. The presence of effect modification was tested by the insertion of first-order interaction terms into appropriate regression models. We calculated the 95% confidence interval for each odds ratio. All statistical analyses were performed using PASW Statistics 17.0 (SPSS, Chicago, IL, USA).

RESULTS

Study cohort

One hundred and twenty-three patients underwent allogeneic HCT at our institution between April 2000 and June 2010. In all, 25 patients died and 20 patients had moved to another hospital, leaving 78 outpatients at our institution. Of these, seven were missing pre-HCT echocardiographic data and five patients had received more than two allogeneic HCT. Sixty-six patients thus met the study criteria. However, consent for the study could not be obtained from three patients. Ultimately, a total of 63 patients were eligible for participation in the study; of these 63 patients, 56 without LVH at baseline were eligible for analysis of the incidence of LVH.

Patient characteristics

Characteristics of study subjects at baseline are shown in Table 1. The diagnoses in the 63 patients included *de novo* AML in 22 patients, ALL in 16 patients, myelodysplastic syndrome (MDS)/AML in nine patients, non-Hodgkin's lymphoma in five patients, CML in three patients, adult T-cell leukemia in three patients, aplastic anemia in three patients and one each of biphenotypic acute leukemia and chronic active EB virus infection. In most of the patients, CsA was used for prophylaxis against GVHD. Just one patient received mediastinal radiation therapy prior to HCT.

Table 1. Characteristics of study subjects at baseline

No of patients	n = *63*
Median age at HCT (range)	42 years (range, 17–69)
Gender (male/female)	30/33
Diagnosis	
De novo AML	22 (35%)
ALL	16 (25%)
MDS/AML	9 (14%)
NHL	5 (8%)
All others	11 (17%)
Conditioning regimen	
High-dose TBI-based	20 (32%)
High-dose BU-based	18 (29%)
Reduced intensity	25 (40%)
Stem cell source	
Related BM	3 (5%)
Related peripheral blood	21 (33%)
Unrelated BM	29 (46%)
Unrelated cord blood	10 (16%)
History of hypertension within 1 year of HCT	4 (6%)
Chronic GVHD within 1 year of HCT	33 (52%)
LVEF <55% before HCT	2 (3%)
Median hemoglobin level 1 year after HCT	12.7 g/dL (range, 5.6–15.7)
Median serum ferritin level before HCT	566 ng/mL (range, 30–7377)
Median cumulative anthracycline dose	90 mg/m^2 (range, 0–458)
Median follow-up time	1394 days (range, 462–3932)

Abbreviations: HCT = allogeneic hematopoietic cell transplantation; LVEF = left-ventricular ejection fraction; MDS = myelodysplastic syndrome; NHL = non-Hodgkin's lymphoma. The follow-up time indicates the time between HCT and the post-transplant echocardiographic examination.

HCT conditioning regimen

The high-dose TBI-based conditioning regimen included CY with TBI in eleven patients, CY with TBI and etoposide (60 mg/m^2) in one patient and CY with TBI and cytosine arabinoside (8 g/m^2) in eight patients. The high-dose BU-based conditioning regimen included BU and CY in 14 patients or BU and CY with TLI (750 cGy) in four patients. RIC consisted of BU (oral or i.v. 8 mg/kg) and fludarabine (180 mg/m^2) with/without TBI (400 cGy) in 22 patients, melphalan (140 mg/m^2) and fludarabine (150 mg/m^2) in one patient, oral BU (8 mg/kg) and cladribine (0.66 mg/kg) in one patient and CY (200 mg/kg) with TLI (750 cGy) in one patient.

Factors affecting LV systolic and diastolic function and LVH

Mean LVEF and E/A values pre- and post HCT in each conditioning group are shown in Table 2. The LVEF and E/A before HCT in the two myeloablative conditioning groups were higher than those in cases who underwent RIC. In the interval from pre- to post-HCT, the LVEF decreased significantly in the high-dose, TBI-based conditioning group as did the E/A with both high-dose TBI-based and high-dose BU-based conditioning (Table 2, Figures 1a and b). In the simple linear regression analysis that assessed the relationship between LVEF or E/A and other factors, high-dose TBI-based conditioning was significantly associated with a decrease in LVEF

Table 2. Characteristics of patients divided according to conditioning regimens: mean LVEF and E/A values with ranges before and after HCT

	n	*Mean age (range)*	*Pre-HCT mean LVEF (%) (range)*	*Post-HCT mean LVEF (%) (range)*	P-*value*	*Pre-HCT mean E/A (range)*	*Post-HCT mean E/A (range)*	P-*value*
High-dose TBI-based	20	34.9 (19–59)	67.0 (64–72)	59.0 (28–69)	0.001	1.51 (0.9–2.5)	1.17 (0.6–2.0)	<0.001
High-dose BU-based	18	37.6 (19–60)	64.4 (55–76)	64.7 (55–76)	0.86	1.61 (0.7–2.2)	1.24 (0.5–1.9)	0.004
RIC	25	49.8 (19–69)	62.7 (46–79)	60.3 (41–72)	0.08	1.11 (0.7–1.9)	1.04 (0.5–1.7)	0.31

Abbreviations: A = mitral late diastolic velocity; E = peak early mitral inflow velocity; E/A = the ratio of peak mitral E to A filling velocities; HCT = allogeneic hematopoietic cell transplantation; LVEF = left-ventricular ejection fraction; RIC = reduced intensity conditioning. *P*-values represent the difference between pre- and post-HCT values; $P<0.05$ is statistically significant.

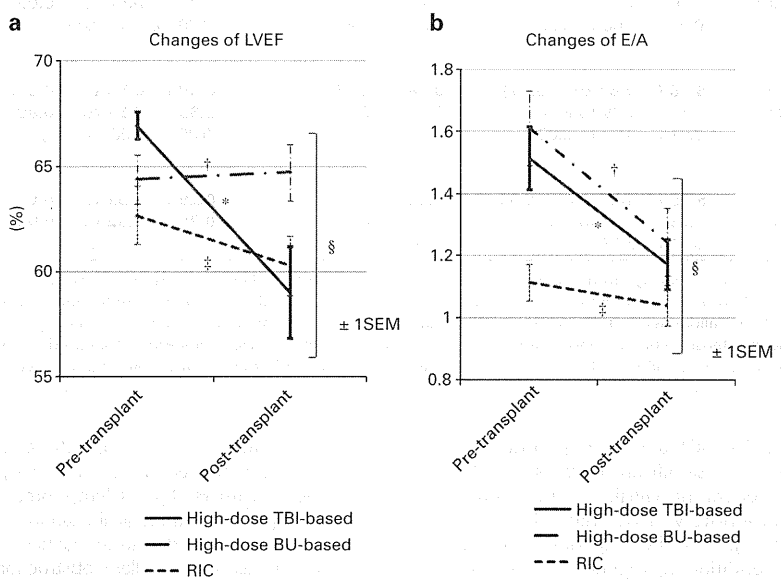

Figure 1. (**a**) Changes in LVEF from pre-transplant to post-transplant. $^{*}P=0.001$; †, ‡: not statistically significant; and §: there were statistically significant differences among the three groups ($P=0.005$). (**b**) Changes of E/A from pre-transplant to post-transplant. $^{*}P<0.001$; $^{\dagger}P=0.004$; ‡: not statistically significant; and §: there were statistically significant differences among the three groups ($P=0.02$).

Table 3. Simple linear regression analysis of changes in LVEF and E/A before and after HCT ($n=63$)

	Changes of LVEF		*Changes of E/A*	
	β (95% CI)	P-*value*	*β (95% CI)*	P-*value*
High-dose TBI-based (vs RIC)	**−5.55 (−10.16 to −0.94)**	**0.02**	**−0.27 (−0.49 to −0.04)**	**0.02**
High-dose BU-based (vs RIC)	2.73 (−2.02 to 7.49)	0.25	**−0.29 (−0.53 to −0.06)**	**0.01**
Sex (female/male)	1.43 (−2.75 to 5.62)	0.50	0.07 (−0.13 to 0.27)	0.47
Age at HCT (year)	0.05 (−0.10 to 0.21)	0.50	0.00 (−0.01 to 0.01)	0.52
HT (yes/no)	2.54 (−6.04 to 11.12)	0.56	−0.22 (−0.63 to 0.19)	0.28
Hgb level (g/dL)	0.33 (−0.75 to 1.42)	0.54	0.04 (−0.02 to 0.09)	0.16
s-ferritin level (ng/mL)	0.00 (−0.00 to 0.00)	0.57	−0.00 (−0.00 to 0.00)	0.44
Chronic GVHD (yes/no)	1.18 (−3.01 to 5.37)	0.58	−0.05 (−0.25 to 0.15)	0.62
Anthracyclines (mg/m^2)	0.01 (−0.01 to 0.02)	0.36	0.00 (−0.00 to 0.00)	0.87

Abbreviations: A = mitral late diastolic velocity; anthracyclines = cumulative dose of anthracyclines; β = regression coefficient; Chronic GVHD = the presence of chronic GVHD within 1 year of HCT; CI = confidence interval; E = peak early mitral inflow velocity; E/A = the ratio of peak mitral E to A filling velocities; HCT = allogeneic hematopoietic cell transplantation; Hgb level = hemoglobin level 1 year after HCT; HT = a history of hypertension within 1 year of HCT; LVEF = left-ventricular ejection fraction; RIC = reduced intensity conditioning; s-ferritin = serum ferritin level before HCT. $P<0.05$ is statistically significant. Bold type indicates data that achieved statistical significance.

after HCT (coefficient = −5.550, $P=0.02$). Patients who received a high-dose TBI-based or high-dose BU-based conditioning regimen had a significant decrease in E/A after HCT (coefficient = −0.268, $P=0.02$ and coefficient = −0.293, $P=0.01$, respectively) (Table 3).

Furthermore, we tested several regression models to assess the relationship between LVEF or E/A and the conditioning regimen, using multiple linear regression analyses (Table 4). The high-dose, TBI-based conditioning regimen was significantly associated with

Table 4. Multiple linear regression models of changes in LVEF and E/A before and after HCT ($n=63$)

	Changes of LVEF				*Changes of E/A*			
	β (95% CI)	*β′*	*P-value*	R^2	*β (95% CI)*	*β′*	*P-value*	R^2
Model 1								
High-dose TBI-based (vs RIC)	**−5.37 (−10.08 to −0.67)**	**−0.31**	**0.03**		**−0.29 (−0.52 to −0.06)**	**−0.34**	**0.01**	
High-dose BU-based (vs RIC)	2.92 (−1.92 to 7.77)	0.16	0.23	0.17	**−0.31 (−0.55 to −0.08)**	**−0.36**	**0.01**	0.14
Chronic GVHD	0.94 (−3.03 to 4.92)	0.06	0.64		−0.11 (−0.30 to 0.09)	−0.14	0.28	
Model 2								
High-dose TBI-based (vs RIC)	**−5.54 (−10.17 to −0.91)**	**−0.31**	**0.02**		**−0.27 (−0.50 to −0.04)**	**−0.32**	**0.02**	
High-dose BU-based (vs RIC)	2.86 (−1.92 to 7.64)	0.16	0.24	0.17	**−0.29 (−0.52 to −0.05)**	**−0.33**	**0.02**	0.13
s-ferritin level	−0.00 (−0.00 to 0.00)	−0.09	0.45		−0.00 (0.00 to 0.00)	−0.08	0.50	
Model 3								
High-dose TBI-based (vs RIC)	**−4.86 (−9.64 to −0.07)**	**−0.28**	**0.05**		**−0.30 (−0.53 to −0.06)**	**−0.35**	**0.01**	
High-dose BU-based (vs RIC)	3.68 (−1.39 to 8.74)	0.20	0.15	0.18	**−0.33 (−0.58 to −0.09)**	**−0.39**	**0.01**	0.14
Anthracyclines	0.01 (−0.00 to 0.03)	0.14	0.29		0.00 (−0.00 to 0.00)	−0.12	0.35	
Model 4								
High-dose TBI-based (vs RIC)	**−5.55 (−10.24 to −0.85)**	**−0.32**	**0.02**		**−0.29 (−0.52 to −0.06)**	**−0.34**	**0.01**	
High-dose BU-based (vs RIC)	2.73 (−2.07 to 7.53)	0.15	0.26	0.16	**−0.29 (−0.52 to −0.05)**	**−0.33**	**0.02**	0.15
HT	0.04 (−8.12 to 8.19)	0.00	0.99		−0.25 (−0.65 to 0.14)	−0.16	0.20	

Abbreviations: A = mitral late diastolic velocity; anthracyclines = cumulative dose of anthracyclines; β = regression coefficient; β′ = standardized regression; Chronic GVHD = the presence of chronic GVHD within 1 year of HCT; CI = confidence interval; E = peak early mitral inflow velocity; E/A = the ratio of peak mitral E to A filling velocities; HCT = allogeneic hematopoietic cell transplantation; HT = a history of hypertension within 1 year of HCT; LVEF = left-ventricular ejection fraction; RIC = reduced intensity conditioning; s-ferritin = serum ferritin level before HCT. High-dose TBI-based (vs RIC) and high-dose BU-based (vs RIC) regimens achieved significance in all models. $P<0.05$ is statistically significant. Bold type indicates data that achieved statistical significance.

a decrease in LVEF and E/A after HCT, and the high-dose BU-based conditioning regimen showed a significant decrease in E/A after HCT, following adjustment for chronic GVHD, s-ferritin, cumulative dose of anthracyclines and a history of HT (Table 4). In addition, after adjustment for sex, age and hemoglobin concentration, the high-dose TBI-based conditioning regimen was significantly associated with a decrease in LVEF and E/A after HCT, and the high-dose BU-based conditioning regimen showed a significant decrease in E/A after HCT (Supplementary Table 1).

Twelve patients received a new diagnosis of LVH after HCT. Several logistic regression models were tested to assess the effects of the pre- and the post-transplant variables on the incidence of LVH (Table 5). With logistic regression analysis, insertion of a quadratic transformation of s-ferritin alone improved the fit compared with the linear model. Because both the linear and the quadratic transformation of s-ferritin were significant in all models at $P<0.05$, both of these variables were retained in all models that included s-ferritin (Table 5). The s-ferritin level was identified as a significant risk factor for LVH after HCT in both the univariable and multivariable logistic analyses (Table 5). However, we could not identify a significant effect of chronic GVHD on cardiac function or LVH after HCT.

DISCUSSION

Our study shows that myeloablative conditioning, in particular regimens that included high-dose TBI, adversely affected cardiac function even in the chronic phase of HCT. Unexpectedly, we could not establish a significant correlation between chronic GVHD and cardiac function. Notably, the s-ferritin level was identified as an independent risk factor for LVH after HCT.

A long-term follow-up study of cardiac function after HCT in children showed similar results, with a report that TBI was a risk factor for cardiac dysfunction.[13] In contrast, Auner *et al.*[7] reported that high-dose TBI combined with high-dose CY was not associated with a significant decrease in cardiac function. However, these investigators evaluated cardiac function after a relatively short-term follow-up period, with a median of just 5 months. Radiotherapy-induced late cardiotoxicity has often been described in patients with Hodgkin's lymphoma who received mediastinal radiotherapy.[14–17] Pathological examination of radiotherapy-induced myocarditis showed diffuse interstitial fibrosis and microcirculatory damage, leading to capillary obstruction and extensive fibrosis,[14] with the potential to cause LV systolic and diastolic dysfunction.[16,17] In addition, CY-containing regimens without high-dose TBI significantly reduced LV diastolic function but not systolic function. Therefore, our results suggest that chronic LV systolic dysfunction after HCT might largely reflect a late, toxic effect of TBI.

There were differences in the pre-transplant LVEF and E/A in the three groups of conditioning regimens in our study—the LVEF and E/A before HCT in the two myeloablative conditioning groups were better than those in the RIC group. We thought that the results likely indicated that the patients receiving myeloablative conditioning regimens were younger and/or had fewer cardiac complications than the RIC patients.

Our data demonstrated that the s-ferritin level pre-HCT, rather than age or a history of HT, was significantly associated with development of LVH after HCT. A high s-ferritin level has been thought to reflect iron overload.[18] Although transfusion-related iron overload was reported to be causally associated with LVH,[19] the causal association between the s-ferritin level and LVH is still unclear. However, release of ferritin could be induced by IL-1 beta or TNF-alpha.[20] Thus, the relationship between the s-ferritin level and LVH might indicate that IL-1 beta or TNF-alpha was involved in the pathogenesis of myocyte hypertrophy. Further study will be required to elucidate the precise mechanism of development of LVH after HCT.

In our study, the presence of chronic GVHD did not appear to have any significant impact on cardiac function after HCT. There are some reports of cardiac dysfunction during GVHD but these are just case series.[21–24] However, a larger, retrospective cohort study showed no statistically significant difference in the incidence of congestive heart failure among patients with acute GVHD, those with a history of chronic GVHD and

Table 5. Logistic regression models of incidence of LVH in subjects without LVH at baseline ($n = 56$)

	Univariable logistic analysis		*Multivariable logistic analysis*	
	OR (95% CI)	*P-value*	*OR (95% CI)*	*P-value*
Conditioning regimen				
RIC	1.00 (reference)			
High-dose TBI-based	1.10 (0.25–4.88)	0.90		
High-dose BU-based	1.18 (0.24–5.89)	0.84		
Sex				
Male	1.00 (reference)			
Female	2.89 (0.76–11.06)	0.12		
Age at HCT	**1.08 (1.01–1.14)**	**0.02**	1.07 (1.00–1.15)	0.06
HT				
No	1.00 (reference)			
Yes	0.26 (0.02–4.42)	0.35		
Hgb level	**0.65 (0.45–0.92)**	**0.01**	0.81 (0.54–1.20)	0.29
s-ferritin level (ng/mL)				
$\leqslant 600$[a]	1.00 (reference)			
>600	3.95 (0.94–16.60)	0.06		
s-ferritin model[b]	**1.00 (1.00–1.01)**	**0.01**	**1.00 (1.00–1.01)**	**0.03**
Chronic GVHD				
No	1.00 (reference)			
Yes	1.20 (0.33–4.31)	0.78		
Anthracyclines	1.00 (1.00–1.01)	0.65		

Abbreviations: anthracyclines = cumulative dose of anthracyclines; Chronic GVHD = the presence of chronic GVHD within 1 year of HCT; CI = confidence interval; HCT = allogeneic hematopoietic cell transplantation; Hgb level = hemoglobin level 1 year after HCT; HT = a history of hypertension within 1 year of HCT; LVH = left-ventricular hypertrophy; OR = odds ratio; RIC = reduced intensity conditioning; s-ferritin = serum ferritin level before HCT. $P<0.05$ is statistically significant. Bold type indicates data that achieved statistical significance. [a]Median value of s-ferritin level. [b]s-ferritin model = (s-ferritin) $-\ \beta2/\beta1 \times$ (s-ferritin)^2; $\beta1$ and $\beta2$ denote coefficients of s-ferritin and (s-ferritin)^2, respectively; $\beta1 = 3.184\text{e-}3$ and $\beta2 = -8.050\text{e-}7$, in the univariable model; and $\beta1 = 3.591\text{e-}3$ and $\beta2 = -9.958\text{e-}7$, in the multivariable model.

those with active GVHD.[25] Uderzo *et al.*[13] report similar findings in childhood HCT.

This study was limited by its retrospective, nonrandomized design and small study population. The incidence of cardiac dysfunction might be underestimated because of survivor bias. In addition, some patients were excluded from analysis because of a change in hospital or lack of echocardiographic data pre-HCT. A further limitation was related to the assessment of cardiac diastolic dysfunction. E/A, used as a surrogate marker of cardiac diastolic function in our study, can be influenced by multiple factors that include heart rate,[26,27] loading conditions[26,28] and/or valvular regurgitation.[29] However, none of our patients had features of heart failure, such as tachycardia, dyspnea or edema. In addition, no patient had severe mitral regurgitation or stenosis.

In conclusion, our results suggest that intensive conditioning, especially regimens that involved high-dose TBI for HCT, had a significant negative effect on chronic cardiac function; a high level of s-ferritin was also found to be a crucial risk factor for LVH in the chronic phase after HCT. Long-term survivors who received an intensive conditioning regimen should therefore have their cardiac function carefully monitored, as well as the function of other organs. In the future, a larger, adequately powered, prospective study of adult HCT patients will be required to confirm our observations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

Evaluating the association between histological manifestations of cord colitis syndrome with GVHD

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Cord colitis syndrome (CCS) is a recently proposed clinical entity characterized by a persistent diarrheal illness after cord blood transplantation (CBT), which is not caused by GVHD or CMV colitis. CCS is histologically characterized by chronic active colitis with granulomatous inflammation and Paneth cell metaplasia suggesting chronicity. However, the specificity of these pathological features to CCS remains to be validated. We conducted a retrospective study of 49 patients who had diarrhea and underwent diagnostic colonoscopy with biopsy following allogeneic hematopoietic SCT. None of the patients met the clinical criteria for CCS. Chronic active colitis with granulomatous inflammation and Paneth cell metaplasia was present in 12/33 (36%) patients with biopsy-proven GVHD, 4/6 (67%) patients with CMV colitis and 2/15 (13%) patients with nonspecific colitis. In patients with GVHD and/or CMV colitis, these pathological features were present in 4/8 (50%) patients after CBT and in 11/26 (42%) patients undergoing BMT or PBSCT. These results demonstrate that chronic active colitis with granuloma and Paneth cell metaplasia is not only a specific feature of CCS but also is present in GVHD and CMV colitis, irrespective of stem cell source.

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Keywords: GVHD; cord blood transplantation; colitis

INTRODUCTION

Allogeneic hematopoietic SCT is a potentially curative therapy for various hematologic malignant and non-malignant disorders. Acute GVHD, a donor T-cell-mediated systemic inflammatory condition, remains a major obstacle for wider application of this therapy. A profuse watery diarrhea or hemorrhagic diarrhea after engraftment is a significant cause of illness in patients undergoing allogeneic SCT and is most likely due to acute GVHD and intestinal infection such as CMV colitis.[1]

Cord blood transplantation (CBT) has been increasingly performed as an alternative to BMT or PBSCT.[2–5] The major advantages of using cord blood (CB) over BM or PBSC include its rapid availability and the requirement for a less stringent criterion for HLA matching between the donor and recipient.[5,6] Recently, Herrera *et al.*[7] proposed a new clinical entity of culture-negative, antibiotic-responsive diarrhea not attributable to any known cause following CBT. This syndrome, designated as cord colitis syndrome (CCS), is histologically characterized by chronic active colitis with granulomatous inflammation and/or Paneth cell metaplasia. However, the specificity of these pathological features to CCS needs to be validated. We conducted a retrospective study of 49 patients who had diarrhea and underwent diagnostic colonoscopy with a biopsy procedure following allogeneic SCT. Chronic active colitis with granulomatous inflammation and Paneth cell metaplasia was frequently observed in acute GVHD and CMV colitis after allogeneic SCT, irrespective of stem cell source; this indicates that these were not histological hallmarks of CCS.

MATERIALS AND METHODS

Patients

A total of 224 patients underwent allogeneic SCT at Kyushu University Hospital between February 2005 and November 2011. We identified 49 consecutive patients who suffered from diarrhea and underwent diagnostic colonoscopy and biopsy procedure and reviewed their medical records and histology slides. This study was approved by the institutional review board.

Definition of CCS, GVHD and CMV colitis

CCS was defined as a persistent diarrheal illness (duration, >7 days) that occurred following CBT and was not caused by acute GVHD, bacterial (including *Clostridium difficile*) or viral infection, post-transplant lymphoproliferative disease or another identifiable cause on microbiological and histopathological examination.[7] Acute GVHD was diagnosed clinically with histological confirmation on the basis of previously published criteria.[8] Pathologically, crypt cell apoptosis or single-cell necrosis with karyorrhectic debris is the most useful marker of acute intestinal GVHD.[9,10] Intranuclear inclusion bodies and eosinophilic cytoplasmic inclusions in epithelial, endothelial and stromal cells are diagnostic for CMV colitis.[11] Acute GVHD graded according to the standard criteria was defined as moderate to severe (grades II–IV) disease.[8]

Histological analysis

For histopathological analysis, biopsy samples were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned, slide-mounted and stained with hematoxylin and eosin. CMV colitis was examined histologically and immunochemically.[12] All samples were reviewed by three pathologists. Pictures from tissue sections were recorded at room temperature using a digital camera (DP72; Olympus, Tokyo, Japan) mounted on a microscope (BX51; Olympus).

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Statistical analysis

A *P*-value <0.05 was considered statistically significant. Fisher's exact test and logistic regression analysis was performed using STATA version 8 (STATA Corp., College Station, TX, USA).

RESULTS

In all, 49 patients underwent intestinal endoscopy and biopsy procedures. The patient characteristics are listed in Table 1. Primary diseases included myelodysplastic syndrome/acute myelogenous leukemia ($n=19$), CML ($n=1$), ALL ($n=6$), malignant lymphoma ($n=11$), adult T-cell leukemia/lymphoma ($n=9$), plasma cell leukemia ($n=1$), aplastic anemia ($n=1$) and primary myelofibrosis ($n=1$). A total of 21 patients had received conventional preparative regimens comprising either TBI/Cy ($n=16$) or Bu/Cy ($n=6$). The remaining 27 cases had received purine analog-based reduced-intensity conditioning regimen comprising either fludarabine (Flu)/Cy ($n=2$), Flu/Bu ($n=12$) or Flu/melphalan ($n=13$). Low-dose TBI (2–4 Gy), antithymocyte globulin and alemutuzumab were administered in 20, 4, and 1 patient, respectively. The sources of stem cells were related PBSCs ($n=14$), related BM ($n=3$), unrelated BM ($n=22$) or unrelated CB ($n=10$). HLA-matching varied from haploidentical (3/6) to identical (6/6). In all, 17 patients received a cyclosporine-based regimen, and 32 received a tacrolimus-based regimen for GVHD prophylaxis. Moreover, 35 patients received MTX therapy, six received mycophenolate mofetil (MMF) therapy and four received antithymocyte globulin therapy.

We reviewed the medical records and confirmed that the pathological and clinical diagnoses coincided in all patients. The median day of biopsy was 50 days after HSCT (range, 15–506 days).

Neutrophilic granulomas (Figure 1a) were present in 17/28 (61%), 1/1 (100%), 4/5 (80%) and 4/15 (27%) biopsy specimens that were diagnosed as GVHD, CMV colitis, GVHD plus CMV colitis and nonspecific colitis, respectively (Table 2). Paneth cells were morphologically identified by the presence of cytoplasmic eosinophilic granules in hematoxylin- and eosin-stained sections. Paneth cell metaplasia (Figure 1b) was present in 7/28 (25%), 1/1 (100%), 3/5 (60%) and 1/15 (7%) biopsy specimens that were diagnosed as GVHD, CMV colitis, GVHD plus CMV colitis and nonspecific colitis, respectively. Chronic active colitis is histologically characterized by crypt architectural distortion and lymphoplasmacytic infiltrate deep into the crypts.[13] Chronic active colitis with neutrophilic granuloma and/or Paneth cell metaplasia, a hallmark of CCS,[7] was observed in 9/28 (32%), 1/1 (100%), 3/5 (60%) and 2/15 (13%) biopsy specimens that were diagnosed as GVHD, CMV colitis, GVHD plus CMV colitis and nonspecific colitis, respectively. There is a trend toward more frequent presence of chronic active colitis in patients with GVHD and/or CMV colitis than those with nonspecific colitis (16/39 vs 2/15, $P=0.099$).

In patients with GVHD and/or CMV colitis, these pathological features were present in 4/8 (50%) patients undergoing CBT and in 11/26 (42%) patients undergoing BMT or PBSCT (Table 3). There are no significant differences in the incidence of the chronic active colitis between stem cell sources.

Table 1. Patient characteristics

Variable	(N = *49*)
Age (years)	
Median, range	49, 17–68
Sex	
M/F	30/19
Disease	
Leukemia/lymphoma/myeloma/aplastic anemia/ primary myelofibrosis	35/11/1/1/ 1
SCT type	
CBT/BMT/PBSCT	10/25/14
Conditioning	
Myeloablative/non-myeloablative	22/27
TBI	
Yes/no	36/13
GVHD prophylaxis	
CSP-based/TAC-based	17/32

Abbreviations: CBT = cord blood transplantation; CSP = cyclosporine; TAC = tacrolimus.

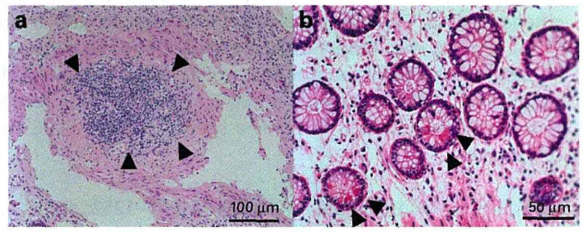

Figure 1. Neutrophilic granuloma and Paneth cell metaplasis. Granulomatous infiltration (**a**, triangle) and Paneth cell metaplasia (**b**, triangle) seen in patients with GVHD. Hematoxylin and eosin staining. Magnification: (**a**) $\times 40$ and (**b**) $\times 80$.

Table 2. Pathological findings

Pathological diagnosis	*GVHD*	*CMV colitis*	*GVHD plus CMV colitis*	*Nonspecific*
N	28	1	5	15
Crypt apoptosis	28	0	5	0
Nuclear inclusion body or pp65-positive	0	1	5	0
Neutrophilic granuloma	17	1	4	4
Paneth cell metaplasia	7	1	3	1
Chronic active colitis with neutrophilic granuloma and/or Paneth cell metaplasia	9	1	3	2

Table 3. Pathological findings in GVHD and CMV colitis by type of SCT

SCT type	*CBT (n = 10)*	*BMT (n = 25)*	*PBSCT (n = 14)*
Diagnosis			
GVHD	6	12	10
GVHD plus CMV colitis	1	2	2
CMV colitis	1	0	0
Pathological findings			
Neutrophilic granuloma	7	11	8
Paneth cell metaplasia	2	6	4
Chronic active colitis with neutrophilic granuloma and/or Paneth cell metaplasia	4	7	4

Abbreviation: CBT = cord blood transplantation.

DISCUSSION

CCS is a recently proposed diarrheal illness that affects recipients of CBT.[7] It is important to distinguish this new syndrome from other causes of diarrheal illness that have a similar presentation, in particular GVHD and CMV colitis, because treatment strategies differ a lot between these conditions. Gut biopsies have an important diagnostic role in patients with diarrhea after allogeneic SCT. The histopathological hallmark of CCS is reported to be chronic active colitis associated with neutrophilic granulomas and/or Paneth cell metaplasia, which indicates chronicity of the lesion.[13] Herrera *et al.*[7] described that these features were not observed in GVHD or other diarrheal illness after any type of allogeneic SCT. In our study, these features were present in a substantial proportion of patients with GVHD and CMV colitis.

Furthermore, Herrera *et al.*[7] also documented that only 1 patient had histological findings of CCS out of 381 patients who underwent a biopsy in recipients of BMT or PBSCT. Our study clearly confirmed that chronic active colitis with granulomas and/or Paneth cell metaplasia were frequently observed in specimens from patients with acute GVHD and CMV colitis following BMT or PBSCT.

In addition, we found that Paneth cell metaplasia was present in the colon of patients with GVHD or CMV colitis as well. Paneth cells are abundant in the ileum but are occasionally present in the colon of the elderly and some patients with inflammatory bowel diseases (Paneth cell metaplasia).[14–17] Paneth cell metaplasia was observed in affected mucosa but not in unaffected mucosa in these patients and caused by various types of inflammation, suggesting that it occurs nonspecifically in response to chronic inflammation.[16,18] Paneth cells regulate the composition of colonized microbes in the intestine and protect epithelial cells from bacterial invasion.[19,20] We recently demonstrated that Paneth cells are targeted by GVHD, resulting in a subsequent alteration in the intestinal flora.[21,22] Other groups have also demonstrated a dramatic alteration in intestinal microbiota in GVHD, both in mice and in humans.[23–25] Metaplastic Paneth cells can express α-defensins,[17,26] and therefore Paneth cell metaplasia may be a compensatory mechanism for reduced number of Paneth cells in the small intestine and subsequent alteration of the intestinal microbiota. Paneth cell metaplasia is also observed in chronic gastritis in association with *Helicobacter pylori* infection, suggesting a natural mechanism of adaptation aimed to eradicate bacteria in acid-deficient stomachs.[27,28]

MMF is an immunosuppressive agent used for prophylaxis and treatment of GVHD in allogeneic SCT. Diarrhea is a commonly seen side effect of MMF. Selbst *et al.*[29] reported that Paneth cell metaplasia is frequently observed in biopsy specimens from patients with MMF-induced colitis following solid organ transplantation and that the histological changes mimic inflammatory bowel disease or GVHD. In our study, Paneth cell metaplasia was present in three patients receiving MMF therapy and in nine patients not receiving MMF therapy.

These results must be interpreted within the context of study limitations. Our study and the study by Herrera *et al.*[7] lack healthy controls and infectious colitis other than CMV colitis. Biopsy sites may also influence the incidence of Paneth cell metaplasia. Paneth cell metaplasia is occasionally seen in elderly individuals.[30] It is more frequently observed at the ascending colon and cecum than at distal sites of elderly individuals and in affected mucosa than in healthy mucosa in patients with inflammatory bowel diseases.[16] Nevertheless, our results indicate that the presence of chronic active colitis with granuloma and/or Paneth cell metaplasia is not a specific feature of CCS. Prospective studies of the larger numbers of patients including, particularly, CBT patients are required to evaluate the incidence of chronic active colitis with granuloma and/or Paneth cell metaplasia in CCS, GVHD and other infectious colitis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Reciprocal Expression of Enteric Antimicrobial Proteins in Intestinal Graft-Versus-Host Disease

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We recently demonstrated that expression of α-defensins, the major antimicrobial peptides produced by Paneth cells, was severely suppressed in mice with graft-versus-host disease (GVHD). In this study, we found that antibacterial lectin, regenerating islet-derived IIIγ (RegIIIγ) was upregulated in villous enterocytes, thus demonstrating the reciprocal control of enteric antimicrobial proteins in GVHD. Upregulation of RegIIIγ was mediated by a mechanism independent upon radiation-induced intestinal tract damage. MyD88-mediated signaling in intestinal epithelium was required for RegIIIγ upregulation in GVHD and antibiotic therapy downregulated RegIIIγ expression. These results suggest that MyD88-mediated sensing of the intestinal microbes disregulated in GVHD induces RegIIIγ upregulation in GVHD and argue a role for RegIIIγ in the pathogenesis of GVHD.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation, a curative therapy for a number of hematologic diseases, is complicated by graft-versus-host disease (GVHD). Particularly, intestinal GVHD is critical for determining the outcome of allogeneic bone marrow transplantation (BMT) [1]. Recently, regenerating islet-derived 3α (RegIIIα) was identified as a specific biomarker for intestinal GVHD in humans using a large-scale and quantitative proteomic discovery approach [2,3]. Reg genes constitute a multigene family, which is categorized into 4 subclasses. RegIIIγ, a homologue of human RegIIIα in mice, is preferentially expressed in the small intestine. RegIIIγ have canonical C-type lectin domains that bind to the peptidoglycan, which is an essential component of the bacterial cell wall and, thus, has direct antimicrobial activity, specifically against Gram-positive bacteria and protects the epithelial barrier function of the intestinal mucosa [4].

The intestinal microbial communities are actively regulated by Paneth cells through their secretion of antimicrobial peptides. Among them, α-defensins are the most potent antimicrobial peptides that account for 70% of the bactericidal peptide activity released from Paneth cells [5,6]. We recently found that Paneth cells were targeted by GVHD, resulting in marked reduction in the expression of α-defensins [7]. Thus, it is puzzling why blood levels of RegIIIα levels are elevated, whereas α-defensins are downregulated, in GVHD. In this study, we evaluated enteric expression of RegIIIγ at the cellular level in mouse models of BMT and found that the major producers of RegIIIγ were villous enterocytes, not Paneth cells, in GVHD. Upregulation of RegIIIγ in GVHD was dependent upon MyD88-mediated sensing of the intestinal microflora.

MATERIAL AND METHODS

Mice

Female C57BL/6 (B6: H-2^b), B6D2F1 (H-2$^{b/d}$), B6-Ly5.1 (H-2^b, CD45.1$^+$), BALB.B (H-2^b), and C3H.Sw (H-2^b) mice were purchased from Charles River Japan, KBT Oriental, or Japan SLC. B6-background Myeloid differentiation factor 88 (MyD88)-deficient (MyD88$^{-/-}$) mice [8] were kindly provided by Dr. Kiyoshi Takeda at Osaka University. All animal experiments were performed under the auspices of the Institutional Animal Care and Research Advisory Committee.

BMT

Mice underwent transplantation as previously described [9]. In brief, after lethal X-ray total body irradiation 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, Tokyo, Japan) according to the manufacturer's instructions.

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In unirradiated model of BMT, B6D2F1 mice were intravenously injected with 12×10^7 splenocytes [7,10]. An antibiotic regimen consisted of polymyxin B (10^5 U/kg), metronidazole (30 mg/kg), and vancomycin (30 mg/kg) (PMV). Mice were maintained in specific pathogen-free condition and received normal chow and autoclaved hyperchlorinated water (pH 4) for the first 3 weeks after BMT and filtered water thereafter. 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 described previously [9].

Histological and Immunohistochemical Analysis

For pathological 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 [11] using rabbit antidefensinα_1 and anti- RegIIIγ (Funakoshi, Tokyo, Japan). Histofine Simple Stain MAX PO (Rat) kits and subsequently DAB solution (Nichirei Biosciences, Tokyo, Japan) 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, Tokyo, Japan) mounted on a microscope (BX51, Olympus, Tokyo, Japan).

Preparation and Analysis of Isolated Mouse Crypts

Individual crypts were isolated from the small intestine as previously described [6]. Following fixation and permeabilization, isolated crypts were incubated for 1 hour with FITC-conjugated anti-Lysozyme (10 μg/mL) (Dako, Tokyo, Japan), followed by incubation for 1 hour with Allexa Fluor 594-conjugated-phalloidin (1 U/mL) (Life Technologies Corporation, Carlsbad, CA). Tetramethyl DAPI (5 μg/mL) (Life Technologies Corporation) was used to stain the nucleus. Samples were mounted in Aqua Poly/Mount (Polysciences, Warrington, PA) and examined with a confocal laser-scanning microscope (LSM510, Carl Zeiss, Jena, Germany).

ELISA

Serum levels of RegIIIγ and IL-22 were measured by using the ELISA Kit for RegIIIγ (USCN, Wuhan, China) and the ELISA Kit for mouse IL-22 (BioLegend, San Diego, CA), respectively.

Quantitative Real-time PCR Analysis

Total RNA was purified using the RNeasy Kit (QIAGEN, Tokyo, Japan). cDNA was synthesized using a QuantiTect Reverse Transcription Kit (QIAGEN). PCR reactions and analyses were performed with ABI PRISM 7900HT SDS 2.1 (Life Technologies Corporation) using TaqMan Universal PCR master mix (Applied Biosystems), and TaqMan Gene Expression Assays (Defa1: Mm02524428_g1, RegIIIg Mm01181783_g1, and Gapdh: Mm99999915_g1, Life Technologies Corporation). The relative amount of each mRNA was determined using the standard curve method and was normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each sample.

Statistical Analysis

Mann-Whitney U tests were used to compare data. All tests were performed with the SigmaPlot Version 10.0 software. $P < .05$ was considered statistically significant.

RESULTS

Reciprocal Control of α-defensins and RegIIIγ Expression in GVHD

Lethally irradiated B6D2F1 (H-$2^{b/d}$) mice received 5×10^6 TCD-BM alone (control group) or TCD-BM 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, as previously demonstrated (data not shown) [7,10]. Pathological analysis of the small intestine 7 days after BMT showed mostly normal architecture in controls, whereas blunting of villi was observed in the GVHD group (Figure 1A). Confocal cross-sectioning of individual crypts isolated from the small intestine demonstrated Paneth cell loss in mice with GVHD, as previously shown (Figure 1B) [7].

α-Defensins are the major antimicrobial peptides produced by Paneth cells [6]. Immunohistochemical analysis showed that defensin α_1 expression was limited in Paneth cells in the crypts of naïve mice (Figure 1C). Expression of defensin α_1 was preserved in controls but was severely suppressed in mice with GVHD 7 days after BMT (Figure 1C). In contrast, RegIIIγ expression was markedly increased in villous enterocytes in the GVHD group (Figure 1D). It should be noted that major producers of RegIIIγ were not Paneth cells, and there was little expression of defensin α_1 in enterocytes in GVHD.

To confirm the differential expression of α-defensins and RegIIIγ, their expression levels in the terminal ileum were evaluated by quantitative real-time PCR analysis. In the GVHD group, expression of *defensin-α_1* (*Defa1*) was markedly reduced (Figure 1E), whereas RegIIIγ expression was significantly increased (Figure 1F).

RegIIIγ Upregulation in GVHD by a Mechanism Independent Upon Radiation-induced Intestinal Tract Damage

We evaluated if increased expression of RegIIIγ in the small intestine correlated with increased serum levels of RegIIIγ. Serum levels of RegIIIγ were not increased in the control group after BMT, whereas those were significantly and constantly elevated in the GVHD group (Figure 2A). We next addressed whether RegIIIγ upregulation could be related to radiation-induced intestinal tract damage, using an unirradiated B6 → B6D2F1 model as previously described [7,10]. Again, serum levels of RegIIIγ were significantly and constantly elevated in the GVHD group to the similar levels observed in the irradiated model (Figure 2B).

RegIIIγ Upregulation in MHC-matched Models of GVHD

We then evaluated if upregulation of RegIIIγ could be observed in clinically relevant, MHC-matched, but minor histocompatibility antigen–mismatched models of BMT. Again, serum levels of RegIIIγ were significantly elevated in the B6 → BALB.B model (Figure 2C) and the C3H.Sw → B6 model of BMT (Figure 2D).

RegIIIγ is Induced by the Stimulation of Intestinal Microbes Through MyD88-mediated Signaling in Intestinal Epithelium in GVHD

We investigated mechanisms of upregulation of enteric expression of RegIIIγ in GVHD. Because recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis and RegIIIγ expression is controlled by microorganism-associated molecular patterns that activate MyD88 pathway [12-15], we hypothesized that bacterial stimuli through MyD88 pathway could mediate the upregulation of RegIIIγ in GVHD. To test this hypothesis, wild-type (WT) or MyD88$^{-/-}$ B6 mice were used as recipients in the C3H.Sw → B6 model of BMT. Clinical GVHD scores were not significantly different between WT and MyD88$^{-/-}$ mice on days 28 and 35 after BMT (Supplemental Figure 1A). Nonetheless, serum levels of RegIIIγ were significantly lower in MyD88$^{-/-}$ mice than in WT mice on day 28 and day 35 after BMT (Figure 2E).

We and others have shown that GVHD induces dramatic alteration in the intestinal microbiota [7,16,17]. We, therefore, hypothesized that upregulated expression of RegIIIγ might be a natural mechanism of adaptation aimed to restore normal intestinal ecology. To evaluate whether modifying the enteric flora using oral antibiotics could inhibit upregulation of RegIIIγ, we treated mice with broad-spectrum antibiotic combination as described, with a slight modification [14]. PMV was administered by daily oral gavage from day 14 in the C3H.Sw → B6 model of BMT. It reduced clinical GVHD scores, as previously described (Supplemental

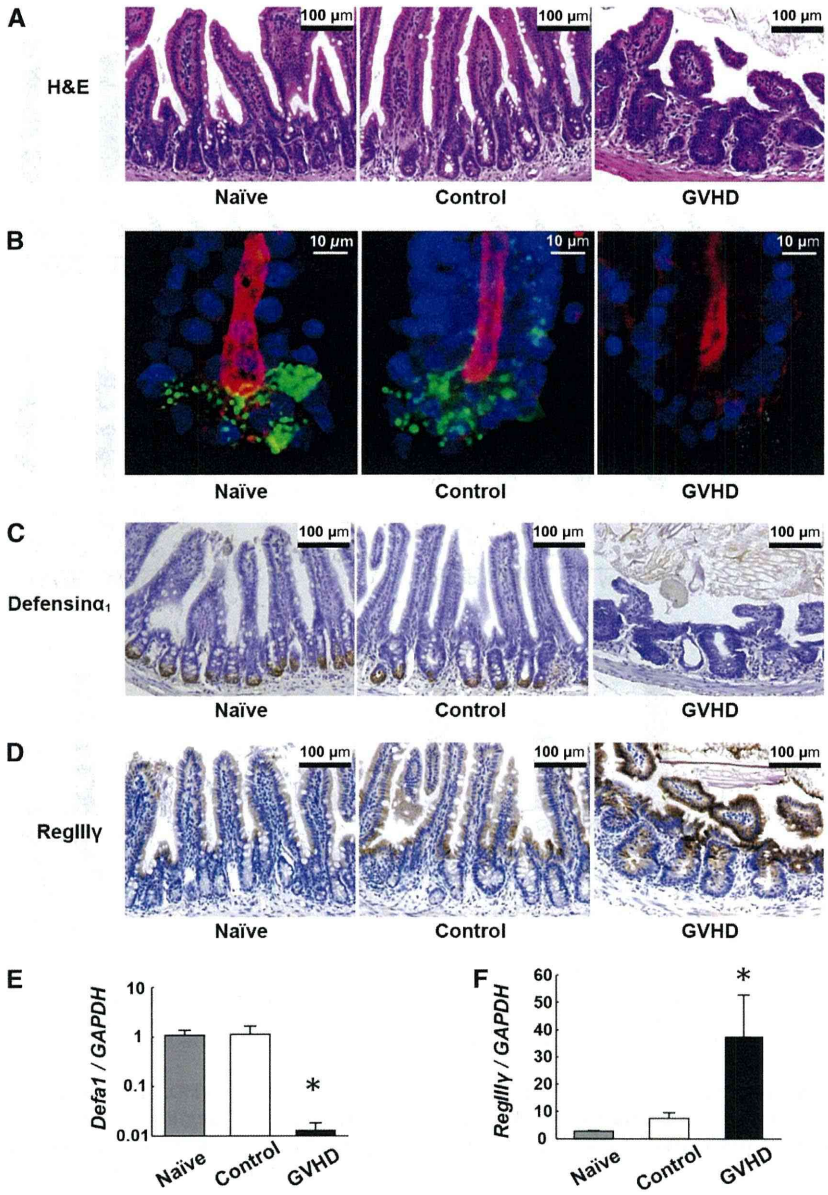

Figure 1. Reciprocal control of expression of Paneth cell-derived α-defensins and epithelial cell-derived RegIIIγ in GVHD. Lethally irradiated B6D2F1 mice underwent transplantation with T cell–deprived bone marrow (TCD-BM) cells without (control group) or with T cells (GVHD group) from MHC-mismatched B6 donors. Small intestines were isolated from mice 7 days after BMT. (A) Histology of the small intestine stained with H & E. (B) 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. Immunohistochemical staining for defensin α_1 (brown) (C) and RegIIIγ (brown) (D). Magnification: 100×. Bars, 100 μm. RNA was extracted from the small intestines on day 7 and quantitative real-time PCR analysis for *Defa1* (E) and *RegIIIγ* (F) compared to GAPDH was performed (n = 6 per group). Data are representative of 2 similar experiments and are shown as means ± SE. $^*P < .05$.

Figure 1B) [7]. Notably, the PMV regimen prevented upregulation of RegIIIγ on day 28 after BMT (Figure 2F).

Induction of RegIIIγ also requires IL-22-mediated signals from innate lymphoid cells [18,19]. However, serum levels of IL-22 were significantly lower in the GVHD group than in controls on day 28 and day 35 after BMT in the C3H.Sw → B6 model (Supplemental Figure 1C). Gut decontamination with the PMV regimen or the use of MyD88$^{-/-}$ B6 mice did not change serum levels of IL-22 (data not shown).

DISCUSSION

Epithelial antimicrobial proteins have an essential role in allowing epithelial surfaces to cope with microbial challenges. They include defensins, cathelicidins, and C-type lectins, such as the Reg family [20]. In this study, we found that enteric expression of α-defensins and RegIIIγ was reciprocally controlled in GVHD. This was due to the difference in the major producers of these molecules in GVHD. It has been shown that α-defensins are exclusively produced by Paneth cells, whereas RegIIIγ is produced by both villous enterocytes and Paneth cells in steady state [13,21]. In GVHD, enterocyte production of RegIIIγ was markedly increased, whereas Paneth cell production of α-defensins was severely suppressed.

Intestinal GVHD is characterized by severe villous atrophy and crypt degeneration. Crypt cell apoptosis is 1 of the initial

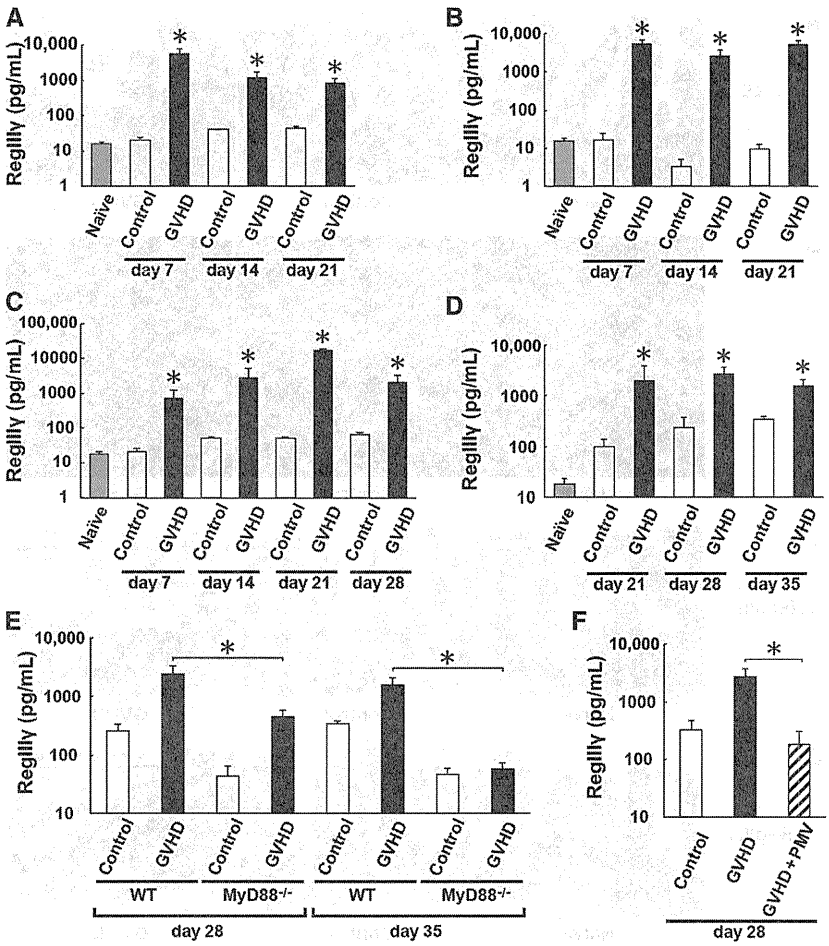

Figure 2. Increased serum levels of RegIIIγ in GVHD is inhibited by intestinal decontamination or in the absence of MyD88 signaling pathway in hosts. Serum levels of RegIIIγ were measured after BMT (n = 6 per group). Data are representative of 2 similar experiments and are shown as means ± SE. *$P < .05$. (A) Irradiated B6 → B6D2F1 model. (B) Unirradiated B6 → B6D2F1 model. (C) Irradiated B6 → BALB.B model. (D) Irradiated C3H.Sw → B6 model. (E) Irradiated C3H.Sw → B6 model. B6 mice were either WT or MyD88$^{-/-}$ B6 mice. (F) Irradiated C3H.Sw → B6 model. A cohort of mice were treated with PMV regimen by daily oral gavage from day 14 of BMT.

lesions and the cardinal features of the intestinal GVHD [22,23]. Experimental and clinical evidence favor the idea that crypt cells are the principal focus of the attack by donor T cells and inflammatory cytokines in GVHD [24]. Intestinal stem cells and their niche, Paneth cells reside in the crypts and we have shown that both intestinal stem cells and Paneth cells are targeted by GVHD [7,10]. In contrast, there is little evidence of direct damage to mature villous enterocytes in mild GVHD and damage to mature enterocyte appears only at severe stage, suggesting that these events are secondary to the alterations in crypt cell turnover [22-25].

Serum levels of RegIIIγ were increased in an unirradiated model of BMT, demonstrating a mechanism independent upon radiation-induced intestinal tract damage. These results are consistent with our recent observation that GVHD induces Paneth cell injury and subsequent dysbiosis by a mechanism independent upon conditioning [7]. A recent clinical study demonstrated that low Paneth cell numbers at onset of intestinal GVHD are associated with high risk for nonrelapse mortality [26].

We have shown that MyD88-mediated signaling in host nonhematopoietic cells is required for upregulation of RegIIIγ in villous enterocytes in GVHD. Because severity of GVHD was not altered in MyD88$^{-/-}$ mice as has been shown [27], downregulation of RegIIIγ was not secondary to amelioration of GVHD. These results suggest a potential lack of reliability of RegIIIγ as a marker for GVHD and argue against a role for it in the pathophysiology of GVHD. Administration of the broad-spectrum antibiotic combination PMV markedly decreased serum levels of RegIIIγ in GVHD. These results are consistent with previous studies demonstrating that RegIIIγ expression is controlled by microorganism-associated molecular patterns that activate MyD88 pathway in nonhematopoietic cells in steady state and in bacterial infection [12-15,17]. Sensitivity of RegIIIγ as a biomarker of intestinal GVHD may be reduced when intensive and broad antibiotics are administered.

We and others have shown that development of intestinal GVHD induced marked dysbiosis in the intestinal flora in mice and human [7,16,17,28]. Although dominantly expanded bacteria in GVHD differs at institutions [7,16,17], we found the prominent outgrowth of Gram-negative bacteria, *Escherichia coli* in the intestinal flora in GVHD [7], thus supporting the previous findings that Gram-negative bacteria, but not Gram-positive bacteria, induces RegIIIγ expression [14,29]. Thus, it is tempting to assume that enhanced production of RegIIIγ is a natural adaptation mechanism for the dysbiosis in GVHD. Paneth cell metaplasia is also observed in the colon of GVHD patients, probably as an adaptation mechanism for Paneth cell loss and the dysbiosis [30].

We have shown that Paneth cell–derived α-defensin production is markedly inhibited in GVHD that have selective bactericidal activity mostly against Gram-negative bacteria [7,31,32]. On the other hand, the antibacterial activity of RegIIIγ is likely restricted to Gram-positive bacteria because of their accessibility to peptidoglycan on the cell surface of Gram-positive bacteria [4]. Thus, bacteria belonging to different classes may battle against each other by stimulating innate antimicrobial mechanisms that selectively inactivate specific bacteria in the mammalian gut, and dramatic decrease in a ratio of α-defensins to RegIIIγ production may be in tune with overwhelming outgrowth of Gram-negative bacteria.

RegIIIγ mRNA expression also requires IL-22-mediated signals from innate lymphoid cells [18,19]. However, IL-22 was reduced in GVHD in our study. Hanash et al. recently demonstrated that IL-22–producing innate lymphoid cells were eliminated in GVHD; however, RegIIIγ was still expressed in GVHD in IL-22$^{-/-}$ mice, suggesting that RegIIIγ expression was upregulated by IL-22 independent pathways in the presence of minimum amounts of IL-22 in GVHD [33].

Recently, RegIIIα is identified as a specific biomarker for intestinal GVHD in human using a large-scale and quantitative proteomic discovery approach [2,3]. Our results support these studies and give mechanistic insights. These new insights will help to understand pathophysiology of GVHD in the context of host-microbe interaction and to establish new therapeutic strategies that can improve clinical outcome of allogeneic stem cell transplantation.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbmt.2013.07.027.

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ORIGINAL ARTICLE

Expansion of donor-reactive host T cells in primary graft failure after allogeneic hematopoietic SCT following reduced-intensity conditioning

M Koyama[1,2], D Hashimoto[3], K Nagafuji[4], T Eto[5], Y Ohno[6], K Aoyama[1], H Iwasaki[7], T Miyamoto[1], GR Hill[2], K Akashi[1,7] and T Teshima[3]

Graft rejection remains a major obstacle in allogeneic hematopoietic SCT following reduced-intensity conditioning (RIC-SCT), particularly after cord blood transplantation (CBT). In a murine MHC-mismatched model of RIC-SCT, primary graft rejection was associated with activation and expansion of donor-reactive host T cells in peripheral blood and BM early after SCT. Donor-derived dendritic cells are at least partly involved in host T-cell activation. We then evaluated if such an expansion of host T cells could be associated with graft rejection after RIC-CBT. Expansion of residual host lymphocytes was observed in 4/7 patients with graft rejection at 3 weeks after CBT, but in none of the 17 patients who achieved engraftment. These results suggest the crucial role of residual host T cells after RIC-SCT in graft rejection and expansion of host T cells could be a marker of graft rejection. Development of more efficient T cell-suppressive conditioning regimens may be necessary in the context of RIC-SCT.

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Keywords: graft rejection; cord blood transplantation; reduced intensity conditioning

INTRODUCTION

Allogeneic hematopoietic SCT is a curative therapy for various hematological malignant tumors, BM failure and congenital immune and metabolic disorders. The success of SCT is highly dependent on the suppression of the recipient's immune system to prevent graft rejection by host immunocompetent cells. Progress on SCT has minimized the rate of graft rejection by the selection of HLA-matched donors, the use of myeloablative conditioning regimens, and infusion of large numbers of hematopoietic stem cells (HSCs).[1–6] However, the incidence of graft rejection is again increasing as the wider application of allogeneic SCT with the increasing use of HLA-mismatched donors, reduced-intensity conditioning (RIC), which could retain host immune cells and HSCs compared with myeloablative conditioning, and cord blood that contains small numbers of HSCs compared with G-CSF-mobilized peripheral blood or BM.[7–9]

Graft failure or graft rejection has been defined as either a lack of initial engraftment of donor cells or loss of donor cells after initial engraftment.[10] Rejection is a major cause of graft failure and is caused by recipient T cells, natural killer (NK) cells, or Abs.[10–15] An increase in number of residual host T cells is associated with graft rejection in pediatric allogeneic transplantation.[16] HLA-C mismatch with NK epitope mismatching in the rejection direction are associated with higher rates of graft rejection after HLA-mismatched transplantation.[17,18] Positive serum crossmatch is predictive for graft failure in HLA-mismatched allogeneic SCT.[19,20]

Thus, the fate of transplanted donor HSCs, namely engraftment or rejection is determined by the competition between donor-derived and residual host-derived HSCs (stem cell competition) and by the competition between donor-derived and host-derived immune competent cells, such as T cells, NK cells and B cells (immunological competition).[11–13]

We herein investigated the process of host T cell-mediated immunological graft rejection in a mouse model of RIC-SCT using sublethal irradiation conditioning[21] and in patients who underwent cord blood transplantation (CBT) following RIC (RIC-CBT) that represents a higher risk for graft rejection than other types of allogeneic SCT.

MATERIALS AND METHODS

A mouse model of BMT

Female C57BL/6 (B6: H-2^b, CD45.2$^+$), B6D2F1 (H-2$^{b/d}$, CD45.2$^+$), DBA/2 (H-2^d, CD45.2$^+$), B6-Ly5a (H-2^b, CD45.1$^+$) and B6.FVB-Tg (Itgax-DTR/EGFP) 57Lan/J (B6.CD11c-DTR, H-2^b) mice were purchased from Charles River Japan (Yokohama, Japan), the Jackson Laboratories (Bar Harbor, ME, USA), and the Animal Resources Center (Perth, Western Australia, Australia). B6.CD11c-DTR × DBA/2F1 mice were generated by breeding B6.CD11c-DTR mice with DBA/2 mice. All experiments involving animals were performed under the auspices of the Institutional Animal Ethics Committee.

Following 6 or 10 Gy TBI (X-ray), B6 mice were injected with 5×10^6 NK cell-depleted BM cells from allogeneic B6D2F1 or syngeneic B6-Ly5a donor mice on day 0. NK-cell depletion of BM cells was performed using CD49b (DX5) microbeads and the autoMACS (Miltenyi Biotec Japan, Tokyo, Japan) according to the manufacturer's instructions. Mice were housed in sterilized microisolator cages and received autoclaved hyperchlorinated drinking water for the first 3 weeks after BMT. For donor DC depletion, all recipients were injected with 100 ng diphtheria toxin (DT) i.p. every other day from day 0 until the end of experiments.

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After transplant, complete blood counts were performed using a Celltac MEK-6358 (Nihon Kohden, Tokyo, Japan). Donor cell chimerism was evaluated weekly in the peripheral blood. mAbs used were CD4, CD8, CD45.1, CD45.2, TCRβ, H-2 K^b and H-2 K^d (BD Biosciences, San Diego, CA, USA). Donor and host cells in a BDF1 → B6 BMT were identified as H-2 K^{b+} H-2 K^{d+} and H-2 K^{b+} H-2 K^{d-}, respectively. In a B6-Ly5a → B6 syngeneic BMT, CD45.1 and CD45.2 were used as donor and host cell-specific markers, respectively. Complete donor cell engraftment, mixed donor and host chimerism, and rejection were defined as >95%, 5–95% and <5% donor chimerism of WBCs in peripheral blood, respectively. For intracellular IFN-γ staining, cells were incubated for 4 h with Leukocyte Activation Cocktail (BD Biosciences) at 37 °C. Then, the cells underwent permeabilization with BD Cytofix/cytoperm solution (BD Bioscience) and were stained with FITC-conjugated IFN-γ mAbs (BD Bioscience). Dead cells were identified as 7-amino-actinomycin D (7-AAD; BD Biosciences)-positive cells. The cells were analyzed using a FACS Calibur flow cytometer (BD Biosciences) and data were analyzed using a Flow Jo software (Tree Star, San Carlos, CA, USA).

CTL assays

Fourteen days after BMT, T cells were separated from BM of the bilateral tibias and femurs or spleen using Thy1.2 (CD90.2) microbeads and the autoMACS according to the manufacturer's instructions. The percentage of $CD8^+$ cells in this fraction was determined by flow cytometry and counts were normalized for $CD8^+$ cell numbers. CTL assays were performed as previously described.[22] Briefly, tumor targets, 2×10^6 DBA/2-derived mastocytoma cell line P815 (H-2^d) or B6-derived lymphoma cell line EL4 (H-2^b), were labeled with 100 μCi of ^{51}Cr sodium salt (Amersham Biosciences, Tokyo, Japan) for 2 h. After washing three times, the labeled targets were plated at 10^4 cells per well in U-bottom plates (Corning-Costar Corp., Cambridge, MA, USA). Allogeneic T-cell preparations, as described above, were added to quadruplicate wells at varying effector-to-target ratios and incubated for 4 h. Maximal and background release were determined by adding 1% SDS or media alone to the targets, respectively. ^{51}Cr activity in the supernatants collected 4 h later were determined using a Wallac 1470 WIZARD Gamma Counter (Wallac Oy, Turku, Finland), and lysis was expressed as a percentage of maximum: percentage of specific lysis = 100 (sample count − background count/maximum count − background count).

For CTL assay against hematopoietic cells, $CD8^+$ cells were separated from BM of the bilateral tibias and femurs using CD8 microbeads and the midiMACS 21 days after BMT. Donor and host-derived hematopoietic target cells were isolated from BM of naïve B6-Ly5a and B6D2F1 mice by depleting lineage committed cells using biotin-conjugated CD3, CD5, Ter119, Gr1, Mac1, B220 Abs and BD IMag Streptavidin Particles Plus-DM (BD Bioscience). The lineage negative targets were plated at 3×10^3 cells per well in 96 U-bottom plates with 1.5×10^5 $CD8^+$ cells and incubated for 4 h. After incubation, the cells were collected and stained with target marker (H2Dd or CD45.1), lineage, Sca-1, c-kit, Annexin V and SYTOX blue stain (Invitrogen, Palsley, UK) for FACS analysis (Fortessa LSD, BD Bioscience).[23]

Patients

We retrospectively analyzed data from 24 adult patients who underwent RIC-CBT at Kyushu University Hospital, Hamanomachi Hospital and Kitakyushu Medical Center between August 2003 and February 2007 and survived more than 28 days post transplant. Patients who received the second CBT for rejection after primary transplant were excluded. This study was approved by the institutional review board of each participating institute.

Transplantation procedure and definitions

Cord blood units were obtained from the Japan Cord Blood Bank Network. Serological typing for HLA–A, –B and –DR Ags of cord blood units and patients' blood samples was performed. HLA-mismatch in the graft-versus-host vector was defined when the recipient's Ags were not shared by the donor, while mismatch in the host-versus-graft vector was defined as when the donor's Ags were not shared by the recipient. RIC regimens were defined as previously reported.[24–26] Regimens used were fludarabine 150–180 mg/m^2 with either CY 60 mg/kg, BU 8 mg/kg or melphalan 80–140 mg/m^2 with 4 Gy TBI. The prophylaxis regimens for GVHD were CYA or tacrolimus alone, CYA plus MTX or CYA plus mycophenolate mofetil. Risk status at transplantation was categorized as either standard risk or high risk. Standard-risk diseases included acute leukemia in first CR, CML in first chronic phase and refractory anemia of myelodysplastic syndrome. Other diseases were categorized as high-risk disease. Graft failure was defined as failure of the ANC to surpass 0.5×10^9/L for 3 consecutive days before relapse, second transplantation or death.[27] Chimerism of WBCs was assessed 3 weeks after CBT by using FISH in sex-mismatched donor-recipient pairs or PCR for variable numbers of tandem repeats in sex-matched pairs at a sensitivity of 10%.[28]

Statistical analysis

Mann–Whitney *U*-tests were used to analyze cell counts and clinical scores. We defined $P < 0.05$ as statistically significant. The values are described with s.e.

RESULTS

Intensity of TBI impacts donor cell engraftment

We first examined the impact of conditioning intensity on donor cell engraftment in a murine MHC-mismatched B6D2F1 (H-$2^{b/d}$) → B6 (H-2^b) model (F1 → P) of BMT.[21] In this model, donor T cells are tolerant of the recipient MHC, but donor NK cells not expressing H-2^b-specific Ly49C/I inhibitory receptors and bearing instead H-2^d-specific Ly49A/G2 receptors can be activated to kill the recipient's targets.[29] In contrast, recipient T cells are activated by the donor-derived alloantigens, whereas recipient NK cells are not reactive to the donor's targets. Therefore, graft rejection is primarily mediated by donor-reactive host T cells in this model. Donor NK cells that can compete with host T cells were depleted from the donor inoculum. B6 mice were exposed to 6 or 10 Gy TBI and then intravenously injected with 5×10^6 NK cell-depleted BM cells from B6D2F1 mice or B6-Ly5a mice on day 0. It has been shown that conditioning with ⩽7 Gy TBI is not lethal in this strain combination.[21] After BMT, donor cell chimerism was evaluated in the peripheral blood weekly. WBC counts recovered rapidly to the normal level by 4 weeks after syngeneic BMT and allogeneic BMT following 10 Gy TBI, but not after allogeneic BMT following 6 Gy TBI (Figure 1a). Complete donor chimerism was rapidly achieved after allogeneic BMT following 10 Gy TBI, whereas donor chimerism never exceeded 10% after allogeneic BMT following 6 Gy TBI (Figure 1b). In contrast, graft failure did not develop in the syngeneic recipients with 6 Gy TBI, confirming that the graft rejection in the allogeneic recipients with 6 Gy TBI resulted from alloreactivity not insufficient numbers of stem cells.

Temporal expansion of host T cells in the peripheral blood in mice experiencing graft rejection

We enumerated donor and host T cells separately after BMT. The number of donor T cells (H-2 K^{b+} H-2 K^{d+} TCRβ$^+$) in peripheral blood increased rapidly in the allogeneic recipients with 10 Gy TBI and the syngeneic recipients with 6 Gy TBI but not in the allogeneic recipients with 6 Gy TBI (Figure 1c). Such an increase of donor T cells in these animals is likely mediated by homeostatic proliferation in lymphopenia after BMT. Residual host T cells (H-2 K^{b+} H-2 K^{d-} TCRβ$^+$) accounted for only 10% of total T cells at 6 weeks after BMT in the allogeneic recipients with 10 Gy TBI (Figure 1d). In contrast, host T cells markedly increased with a steep peak at 3 weeks after BMT in the allogeneic recipients with 6 Gy TBI. Such a surge of residual host T cells which consisted of 99% of total T cells was not seen in either of the allogeneic recipients with 10 Gy TBI or the syngeneic recipients with 6 Gy TBI. Given that those graft rejections were not caused by insufficient number of stem cells (Figure 1b), we hypothesized that the expansion of host T cells in the allogeneic recipients with 6 Gy TBI could be responsible for primary graft rejection.

Host T-cell expansion is associated with early rejection of donor BM graft

We examined whether the activated host T cells could expand not only in peripheral blood but also in hematopoietic organs where

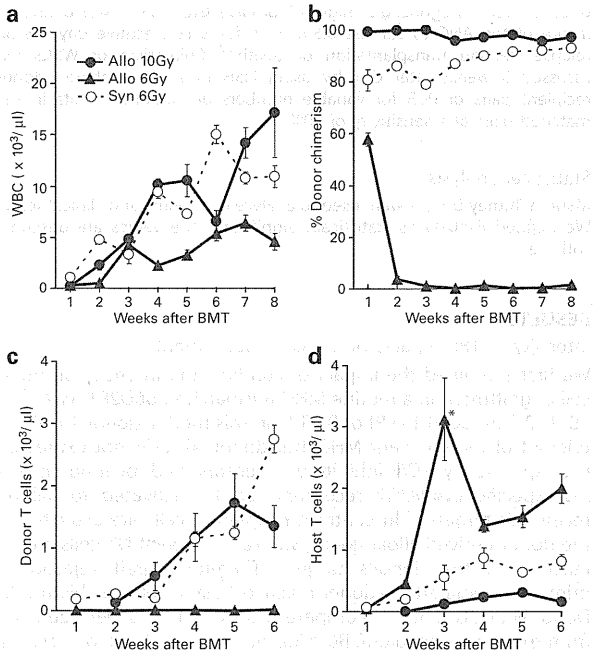

Figure 1. The impact of TBI doses on donor cell engraftment. B6 mice were exposed to 10 Gy TBI ($n=6$, closed circle with solid line) or 6 Gy TBI ($n=9$; closed triangle with solid line), and i.v. injected with 5×10^6 NK cell-depleted BM cells from B6D2F1 mice on day 0. In syngeneic controls, B6 mice were transplanted with cells from B6-Ly5a mice following 6 Gy TBI ($n=6$, open circle with broken line). Numbers **(a)** and donor chimerism **(b)** of WBCs after BMT. Numbers of donor (**c**) and host (**d**) T cells. Donor T cells were H2-K^{b+} H-2 K^{d+} TCRβ^+ cells and host T cells were H2-K^{b+} H-2 K^{d-} TCRβ^+ cells. Results from three similar experiments were combined ($n=6$–9 per group). *$P<0.01$ compared with 2 and 4 w. Data are shown as the mean ± s.e.

donor graft cells migrate. B6 recipients irradiated with 6 Gy TBI were i.v. injected with 5×10^6 NK cell-depleted BM cells from allogeneic B6D2F1 mice or syngeneic B6-Ly5a mice. Two weeks later when donor chimerism has already been lost in allogeneic recipients (Figure 1b), cells were isolated from BM and spleen to analyze chimerism and proliferation of host T cells. In allogeneic animals, host-derived cells (H2-K^{b+} H-2 K^{d-}) exceeded 99% in BM (99.6% ± 0.34%) and spleen (99.6% ± 0.30%), whereas it was 44.7% ± 22.1% in the BM and 48.4% ± 32.2% in the spleen of syngeneic recipients (data not shown). Host-derived IFN-γ^+ $CD8^+$ T cells markedly expanded in the BM and spleen from allogeneic recipients compared with those in syngeneic recipients both at 2 weeks (Figure 2a) and 3 weeks after BMT (Figure 2b). In contrast, host-derived IFN-γ^+ $CD4^+$ T cells were modestly increased in the spleen 2 weeks after BMT and in the BM 3 weeks after BMT in allogeneic recipients. Frequencies of host-derived IFN-γ^+ T cells were also increased in BM (Figure 2c). We next evaluated the cytolytic activity of those host $CD8^+$ T cells with respect to donor targets. BM T cells were isolated from allogeneic recipients treated with 6 Gy TBI 2 weeks after BMT. We confirmed with a flow cytometric analysis that more than 99% of these T cells were host-derived (data not shown). These T cells exhibited CTL activity against donor-type P815 (H-2^d) targets but not against donor-type EL4 (H-2^b) targets (Figure 2d). Similar results were obtained when splenic T cells were used (data not shown). Furthermore, the $CD8^+$ T cells isolated from BM of graft-rejected recipients targeted and induced cell death of donor-type HSCs (lineage$^-$ c-Kit^+ Sca-1^+ cells) but not host-type HSCs as Annexin V^+ $SYTOX^+$ cells (Figure 2e). These results suggest that the expansion of donor-reactive host T cells in hematopoietic tissue including BM precedes the transient increase of host T cell in peripheral blood in the allogeneic recipients who experienced primary graft rejection.

Donor DC depletion abolished the transient host T-cell expansion and improved donor chimerism partially

Next we examined if donor cell rejection could be prevented when host T-cell activation and expansion were inhibited. Donor DCs were expected as the main population to activate host T cells.[30] B6 recipients irradiated with 6 Gy TBI were transplanted with 5×10^6 NK cell-depleted BM cells from allogeneic wild-type B6D2F1 or B6.CD11c-DTR × DBA/2F1 mice. All recipients were injected with 100 ng DT i.p. every other day from day 0. We evaluated kinetics of host T-cell expansion and donor chimerism in peripheral blood every week. Host T-cell expansion was suppressed (Figure 3a) and the donor chimerism at 3 weeks after BMT was increased in donor DC-depleted recipients compared with DC-repleted controls (Figure 3b).

Host T cell surge in the patients who resulted in primary graft rejection following RI-CBT

We next investigated clinical relevance of such a "surge" of host T cells in rejection in adult patients who underwent RIC-CBT retrospectively. We focused on RIC-CBT, because primary graft rejection was rare after BMT or PBSC transplantation.[9] In 24 adult patients, who underwent RIC-CBT, 17 patients achieved engraftment at a median of 22 days (range, 13–31 days), while primary graft rejection occurred in 7 patients (Table 1). The number of infused cells, $CD34^+$ cells and HLA disparity were evenly distributed between the groups (Table 1). We clearly observed a surge of lymphocytes in 4 out of 7 patients who experienced primary graft rejection (Figure 4a), but not in patients with engraftment (Figure 4b). The chimerism analysis at 3 weeks after CBT demonstrated that expanded lymphocytes were host-derived (Figure 4c). One out of 4 patients with the transient lymphocyte increase had aplastic anemia as the original disease and the other three patients received single course of cytotoxic chemotherapy for the original malignant diseases before CBT. A flow cytometric analysis of these host lymphocytes was performed in one representative patient with rejection; 85% of the cells were $CD8^+$ T cells (data not shown).

DISCUSSION

Graft rejection is mediated by infusion of insufficient numbers of HSCs and/or humoral and cellular immunological mechanisms, involving anti-HLA Abs and donor-reactive host T cells and NK cells.[10,31] Although standard myeloablative conditioning is usually sufficient to suppress donor-reactive host T cells and permits donor cell engraftment, RIC leaves larger numbers of residual host immunocompetent cells than myeloablative conditioning. However, the impacts of RIC on the kinetics of host T cells in the context of graft rejection have not been well delineated.

We first evaluated effects of nonmyeloablative conditioning on host T cells in a mouse F1 → P model with donor NK-cell depletion, where recipient T cells can recognize donor alloantigens, while donor T cells cannot recognize recipient alloantigens. This model thus represents a simplified model that allows us to examine the effects of conditioning intensity on the ability of anti-donor host T cells to mediate graft rejection independent of the presence of donor-derived T cells and NK cells, although mechanisms of clinical rejection are more complex, involving donor T and NK-cell responses. In this model, the conditioning with 6 Gy TBI resulted in primary graft rejection, whereas syngeneic BMT with the same number of BM cells following 6 Gy TBI achieved donor-dominant chimerism, indicating the T cell-mediated graft rejection, not due

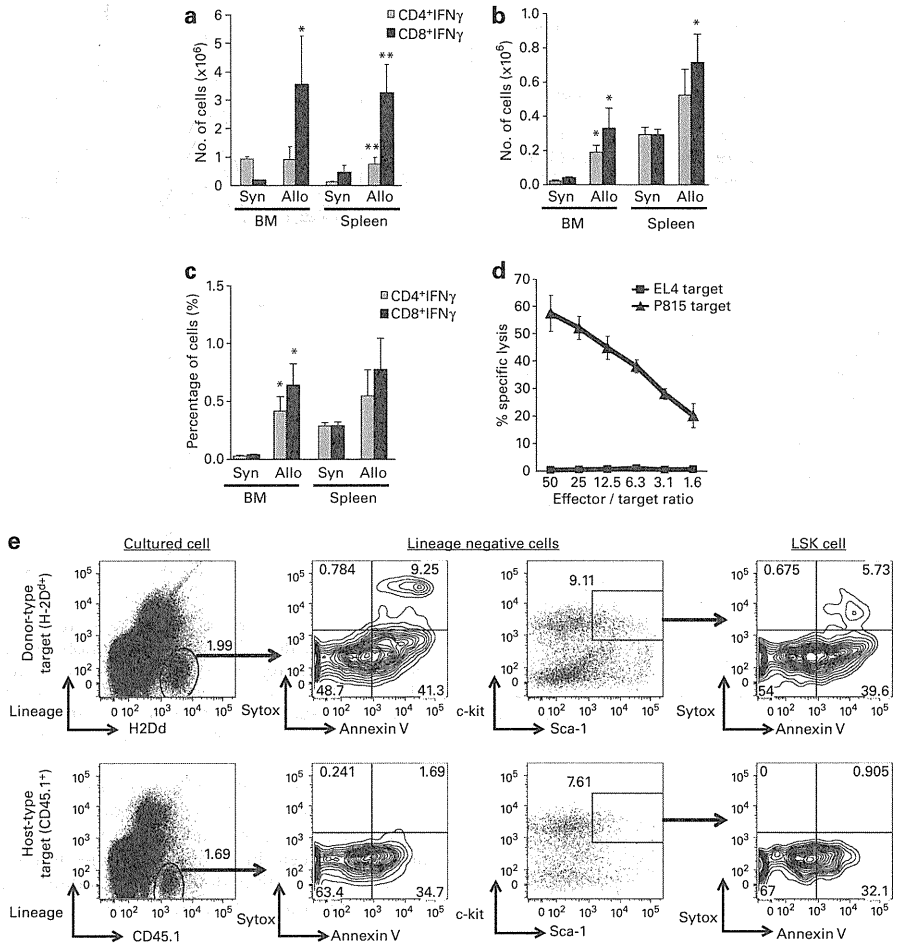

Figure 2. Expansion of anti-donor cytotoxic host T cells. B6 mice received 6 Gy TBI and then injected i.v. with 5×10^6 NK cell-depleted BM cells from B6D2F1 (allo) or B6-Ly5a mice (syn). Splenocytes and BM cells were isolated from the bilateral tibias and femurs 2 and 3 weeks after BMT. (**a**, **b**) The absolute numbers of IFNγ$^+$ CD4$^+$ cells (gray bar) and IFNγ$^+$ CD8$^+$ cells (black bar) in the BM and spleen 2 weeks (**a**) and 3 weeks after BMT (**b**). (**c**) Frequencies of IFNγ$^+$ CD4$^+$ cells (gray bar) and IFNγ$^+$ CD8$^+$ cells (black bar) in the BM and spleen 3 weeks after BMT. (**d**, **e**) BM T cells were cultured with P815 (H-2^d) targets or EL4 (H-2^b) targets at varying effector-to-target ratios (**d**), or with lineage negative BM cells from naïve donor-type B6D2F1 (H-2$^{b/d}$) mouse or host-type B6-Ly5a mice (CD45.1$^+$) (**e**) to determine cell cytotoxicity. Data shown are representative of two replicate experiments and mean ± s.e. ($n = 4$–10 per group). *$P < 0.01$, **$P < 0.0001$ compared with syn.

to an insufficient number of stem cells in the graft. The conditioning with 10 Gy TBI resulted in donor-dominant chimerism (>98% donor cells in WBC) 6 weeks after BMT, although there were some residual host-derived T cells. However, these residual T cells did not induce graft rejection probably due to their function being impaired by 10 Gy TBI.

We found graft rejection was associated with an increase in numbers of host T cells in the peripheral blood three weeks after BMT and expansion of cytotoxic CD8$^+$ T cells in the hematopoietic tissues, BM and spleen. These results correspond with the previous experimental studies that CD8-deficient recipient mice were superior for engraftment[32,33] and that preconditioning with anti-CD8 mAb enhanced allogeneic engraftment.[34]

Given that host T cells selectively targeted donor-type cells (Figure 2), these host T cells should recognize donor-type MHC and minor histocompatibility antigens directly presented on donor APCs or indirectly presented on host APCs. Although the dominant pathway of allorecognition by host T cells is difficult to be discriminated in our model, these findings lead us to the hypothesis that the inhibition of donor alloantigen presentation to host T cells could permit donor cell engraftment.[30,35] Donor DC depletion by DT administration suppressed the transient host T-cell expansion and delayed graft rejection with transient mixed chimerism, but failed to maintain sustained engraftment. This is probably due to the presence of other subsets of donor-derived APCs than CD11c$^+$ DCs, as well as insufficient DC depletion after multiple injections of DT as has been reported.[36] Nonetheless, our results are consistent with a previous study showing that donor APCs, not host APCs, particularly DCs have a critical role in eliciting BM rejection.[30] However, evidence suggesting the causative role of donor DCs in BM rejection is still limited; therefore, there is also the possibility that the other components of donor or host cells have a role in inducing rejection.[35,37] Nonetheless, our results suggest that primary graft rejection is at least partly mediated by host T cells stimulated by donor DCs.

We then evaluated the relevance of the overshoot of host T cells observed after MHC-mismatched RIC-SCT in a mouse model in patients experiencing graft rejection after HLA-mismatched RIC-CBT, as rejection is more frequent after CBT.[38] Four out of the seven patients who experienced graft rejection had the temporal