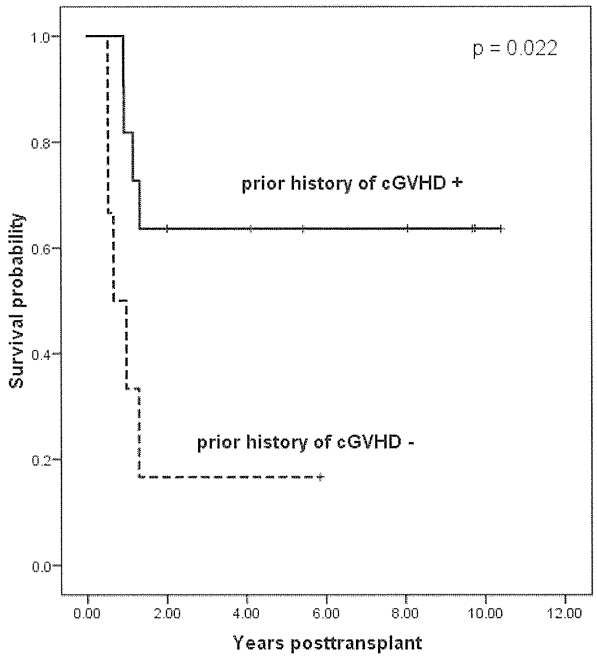

Figure 1 Kaplan-Meier estimates of overall survival based on a landmark analysis at 6 months post-transplant, grouping patients according to prior history of cGVHD (p = .022). The 5-year survival rates of patients with and without prior history of cGVHD were 64% and 17%, respectively.

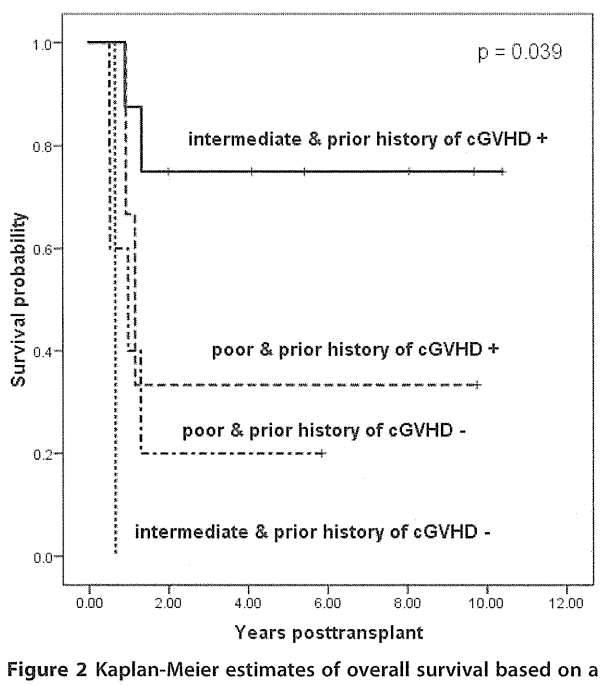

Figure 2 Kaplan-Meier estimates of overall survival based on a landmark analysis at 6 months post-transplant, grouping patients according to cytogenetics and prior history of cGVHD (p = .039). The 5-year survival rates of patients with intermediate & prior history of cGVHD +, poor & prior history of cGVHD +, and poor & prior history of cGVHD - were 75%, 33%, and 20%, respectively.

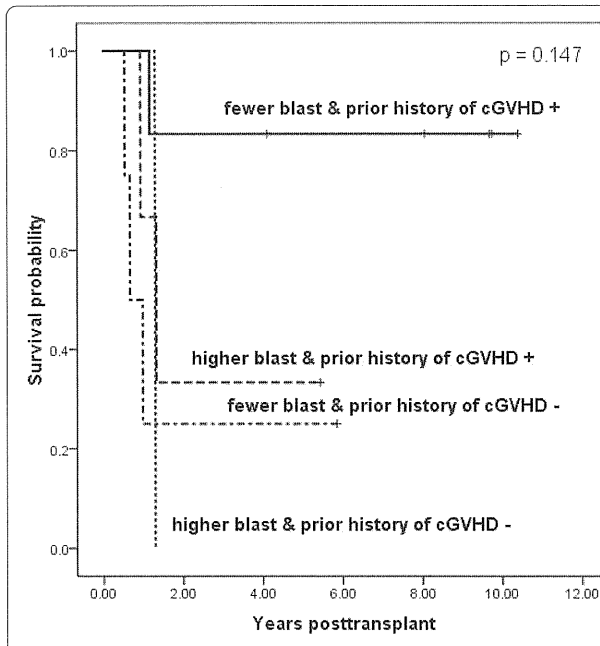

Figure 3 Kaplan-Meier estimates of overall survival based on a landmark analysis at 6 months post-transplant, grouping patients according to percent marrow blast (≤ or > 26%) at baseline and prior history of cGVHD (p = .147). Patients with CNS lesion were not included in this analysis. The 5-year survival rates of patients with fewer blast & prior history of cGVHD +, higher blast & prior history of cGVHD +, and fewer blast & prior history of cGVHD - were 83%, 33%, and 25%, respectively.

term allo-HCT outcomes in adult patients with acute leukemia not in remission [9]. However, they did not address the effect of cGVHD on survival. Baron et al. have reported that extensive cGVHD was associated with decreased risk of progression or relapse in patients with AML or MDS in complete remission at the time of nonmyeloablative HCT [16]. However, it remains unclear whether cGVHD is associated with long-term disease control in patients who have active leukemia at transplant. The results of the current study showed that GVL effects mediated by cGVHD may play a crucial role in long-term survival in or a cure of active leukemia, especially in patients without poor-risk cytogenetics. Further study on the possible relationship between cGVHD and GVL effects would be very helpful in the management of immunosuppressive treatment.

For patients who were ineligible for myeloablative conditioning due to comorbidities coupled with rapidly progressive leukemia, we administered sequential cytoreductive chemotherapy, followed by reduced-intensity conditioning for allo-HCT in order to reduce toxicity and obtain sufficient anti-leukemic efficacy. The utility of the combination of sequential cytoreductive chemotherapy and reduced-intensity conditioning for allo-HCT was previously reported [17]. Our results did not

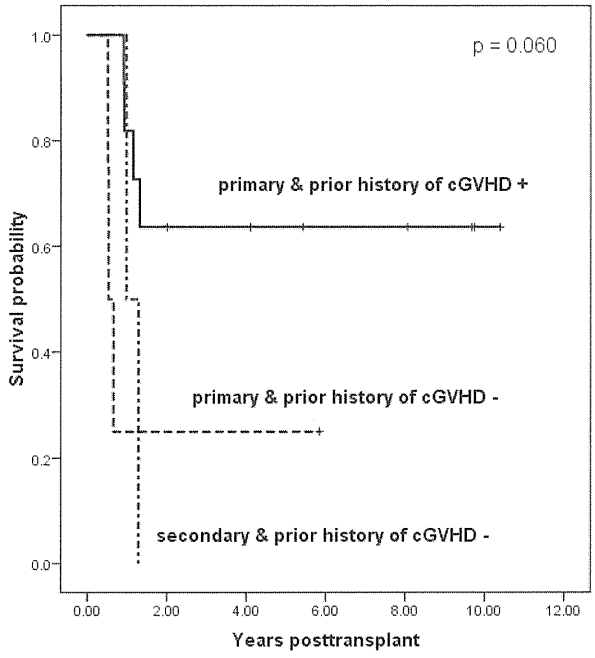

Figure 4 Kaplan-Meier estimates of overall survival based on a landmark analysis at 6 months post-transplant, grouping patients according to primary or secondary leukemia and prior history of cGVHD (p = .060). The 5-year survival rates of patients with primary & prior history of cGVHD + and primary & prior history of cGVHD - were 64% and 25%, respectively.

show that this sequential regimen had an advantage in controlling active leukemia. However, we speculated that effective tumor reduction by individual chemotherapy and/or conditioning for allo-HCT to control disease until cGVHD subsequently occurred might also be important, particularly in rapidly proliferating leukemia. In contrast, intensive conditioning did not appear to be essential in relatively indolent leukemia, even with non-remission.

Based on our results, CB might be unsuitable as a source of stem cells for treatment of active leukemia at the time of allo-HCT. However, most patients receiving CBT could not wait for an unrelated donor search because their disease tended to be aggressive compared with those in the unrelated BM group. Thus, it is difficult to arrive at any conclusions about the best stem cell source for allo-HCT in patients in non-remission status based solely on our results.

Our study has several limitations. The results might be affected by an underlying selection bias due to the nature of retrospective data. Also, our study was limited by the small number of patients, the heterogeneity of the disease, the transplant procedure and the stem cell source. However, the major strengths of our study were that the follow-up period was sufficient with more than 5 years and the impact of cGVHD as well as pre-transplant factors on long-term survival were analyzed exclusively for subjects with active leukemia.

Conclusion

These data show that allo-HCT has the potential to cure active leukemia possibly via cGVHD, particularly in patients with favorable factors even when in non-remission. Further research is warranted to explore the essential factors contributing to the success of allo-HCT such as intensity of conditioning, and GVL effects mediated through cGVHD.

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Authors' contributions

HK and HN designed the study and wrote the paper; HK analyzed results and created the figures; MH designed the research; M Nakamae and YU reviewed the patients' medical records and cleaned the data; MO reviewed the pathological specimens in this study; and KH, TN, MM, YH, M Nishimoto, AH, EI, AI, MY, MB, HO, RA, MA, YT, KK, TY reviewed the results. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

Reduced-intensity conditioning by fludarabine/busulfan without additional irradiation or T-cell depletion leads to low non-relapse mortality in unrelated bone marrow transplantation

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Abstract In reduced intensity, allogeneic stem cell transplantation from unrelated donors (u-RIST), graft-versus-host disease (GVHD), graft failure, and non-relapse mortality (NRM) are persistent problems. Although anti-thymocyte globulin, alemtuzumab, and total body irradiation (TBI) have been explored as conditioning modalities for u-RIST, the necessity for T-cell depletion or TBI to prevent GVHD or facilitate engraftment in u-RIST has not been determined. We here report the use of u-RIST with bone marrow grafting, following a simple conditioning regimen of 180 mg/m^2 fludarabine and 8 mg/kg of oral or intravenous busulfan without TBI or T-cell depletion. The study population was exclusively Japanese patients with a history of prior chemotherapy. We retrospectively analyzed 31 consecutive patients (median age 53 years). Twenty-five patients (81%) were transplanted from HLA-A, -B, and -DRB1 allele-matched donors. In all patients, neutrophil engraftment was achieved. The cumulative incidence of grade II–IV acute GVHD was 42%. However, 77% of patients with acute GVHD improved with, and could be managed by, initial, systemic, high-dose steroid treatment alone. Two-year overall and event-free survival was 62 and 53%, respectively. The NRM of 10% at 2 years was relatively low. Our results suggest that u-RIST without TBI or T-cell depletion may improve the prognosis after u-RIST in certain patient populations.

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Keywords Reduced-intensity conditioning · Bone marrow transplantation · Unrelated donor · Non-relapse mortality

1 Introduction

Although in recent times various approaches to reduced-intensity conditioning have increasingly been used in allogeneic hematopoietic stem cell transplantation, optimal conditioning for unrelated reduced intensity, allogeneic hematopoietic stem cell transplantation (u-RIST) has not yet been adequately established. In u-RIST, high rates of graft-versus-host disease (GVHD), graft failure, and non-relapse mortality (NRM) remain major problems, contributed by the elderly population and/or co-morbidity. Most of the reported conditioning regimens for u-RIST include T-cell depletion with anti-thymocyte globulin (ATG) [1–7] or alemtuzumab [5, 8–10], or low-dose total body irradiation (TBI) [11–18]. Recently, it was reported that the addition of ATG to GVHD prophylaxis resulted in a decreased incidence of acute and chronic GVHD without an increase in relapse or NRM, in a large, prospective, randomized phase 3 trial of myeloablative allogeneic hematopoietic stem cell transplantation with matched, unrelated grafts derived from peripheral blood [19]. However, it has not yet been determined whether additional TBI or T-cell depletion is necessarily required to facilitate engraftment or control GVHD in u-RIST, particularly using bone marrow-derived grafts.

The TBI may in fact evoke additional toxic effects, and induce or enhance acute GVHD via tissue damage, and thereby provoke release of inflammatory cytokines, as has been discussed [20]. Therefore, a regimen that involves TBI may cause a high incidence of NRM, particularly in

elderly patients and/or patients with co-morbidity. On the other hand, intensive T-cell depletion by ATG or alemtuzumab may cause excessive suppression of graft-versus-lymphoma or graft-versus-leukemia effects, or delay immune reconstitution, which results in an increased rate of relapse, progression, and NRM due to a high incidence of opportunistic infections [21–24].

In this study we therefore evaluated the impact on outcomes of simple conditioning, without irradiation or T-cell depletion, application being limited to u-RIST with bone marrow transplantation in Japanese patients with a history of prior chemotherapy.

2 Patients and methods

2.1 Patients

We retrospectively evaluated 31 consecutive patients with hematological malignancies, who had experienced some chemotherapy, and excluded patients without any prior chemotherapy from the analysis. The evaluable patients underwent allogeneic bone marrow transplantation at our institute between September 2002 and December 2009.

Table 1 shows the patients' characteristics and the transplantation details. The median age at transplantation was 53 years (range 22–69 years). These patients included eleven individuals with acute myeloid leukemia, nine with acute lymphoblastic leukemia (ALL) (including seven Ph-positive ALL), two with refractory anemia with excess blasts II [RAEB II, a variant of myelodysplastic syndrome (MDS)], six with non-Hodgkin's lymphoma, and three with the lymphomatous type of adult T-cell leukemia/lymphoma. All patients had experienced chemotherapy prior to transplantation with a median of six courses of prior chemotherapy (range 1–19). Reduced-intensity conditioning was chosen because of age above 50 years ($n = 17$), cardiac dysfunction ($n = 7$), prior allogeneic hematopoietic stem cell transplantation ($n = 4$), prior intense chemotherapy ($n = 2$), or the patient's choice ($n = 1$). We regarded the following patients as being at standard disease risk: acute leukemia in first or second complete remission, refractory anemia due to MDS, low-grade lymphoma of any status, or aggressive lymphoma in complete remission ($n = 20$). The other patients ($n = 11$), including those with aggressive lymphoma without remission ($n = 4$), MDS RAEB ($n = 2$), AML from chronic myelomonocytic leukemia ($n = 1$), fourth complete remission of AML ($n = 1$), AML without remission ($n = 1$), third complete remission of ALL ($n = 1$), and ALL without remission ($n = 1$), were regarded as "high risk".

As it was difficult to obtain informed consent for this retrospective study, we made the context of this study known to the public by putting up a notice at our hospital and on our website in accordance with the ethical guidelines for epidemiological research compiled by both the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labour and Welfare in Japan. This retrospective study was approved by the Institutional Review Board.

Table 1 Characteristics of patients

No. of patients, *n*	31
Median recipient age at transplantation, years (range)	53 (22–69)
Age >60/≤60	9/22
Recipient sex, *n* (male/female)	21/10
Disease, *n*	
AML	11
ALL	9 (Ph + ALL: 7)
MDS	2 (RAEB-II: 2)
NHL	9 (FL: 2, DLBCL: 2, ALCL: 1, IVLBCL: 1, ATL/L: 3)
Median prior chemotherapy, *n* (range)	6 (1–19)
CMV serostatus, *n*	
Recipient-/donor-	1
Other	30
Sex mismatch, *n*	
Female donor to male recipient	4
Other	27
ABO incompatibility, *n*	
Match	18
Mismatch	13
Relapse risk, *n*	
Standard	20
High	11
HLA matching (at HLA-A, B, DR), *n*	
Serologically 6/6 matched	31
Allele 6/6 matched	25
Allele 5/6 matched	6 (DRB1 mismatch: 5, A allele mismatch: 1)
GVHD prophylaxis, *n*	
CsA + sMTX	31
Conditioning regimen, *n*	
Oral Bu 8 mg/kg + Flu 180 mg/m^2	12
Intravenous Bu 8 mg/kg + Flu 180 mg/m^2	19

AML acute myeloid leukemia, *ALL* acute lymphocytic leukemia, *GVHD* graft-versus-host disease, *MDS* myelodysplastic syndrome, *RAEB* refractory anemia with excess blasts, *NHL* non-Hodgkin's lymphoma, *FL* follicular lymphoma, *DLBCL* diffuse large B-cell lymphoma, *ALCL* anaplastic large cell lymphoma, *IVLBCL* Intravascular large B-cell lymphoma, *ATL/L* Adult T-cell leukemia/lymphoma, *CMV* cytomegalovirus, *Flu* fludarabine, *Bu* busulfan, *sMTX* short-term methotrexate, *CsA* cyclosporine A

2.2 Donors and HLA typing

Unrelated bone marrow donors were selected through the agency of the Japan Marrow Donor Program Donor Center (JMDP). HLA serotyping and genotyping for HLA-A, -B, and -DR were performed in all the patients and donors. HLA, -A, -B, and -DR compatibility between patients and donors was determined by standard serological techniques and high-resolution DNA typing as previously reported [25]. HLA-C compatibility was not considered for donor selection because HLA-C allele typing was not routinely performed for unrelated donor selection before April 2004 in the JMDP. On HLA serotyping, all of the 31 recipient–donor pairs were serologically matched for HLA-A, B, and DR, and in addition, 25 pairs were matched for 6 of 6 HLA-A, B and DR alleles (Table 1). Of six HLA allele-mismatched pairs, five were mismatched at one DRB1 allele, and the other was mismatched at a single A allele. In 14 of the 31 pairs, the HLA-C allele was available for both the recipient and donor. Ten of 14 patients had 8/8 HLA-matches with the donor, three had a mismatched donor for a single allele (two at the C allele and one at the DRB1 allele), and one patient had a mismatched donor for two alleles (at the C and DRB1 alleles).

2.3 Conditioning regimen and GVHD prophylaxis

The conditioning regimen consisted of intravenous fludarabine (Flu) 30 mg/m^2/day for 6 days (total 180 mg/m^2), oral busulfan (Bu) 4 mg/kg/day divided into four doses per day for 2 days (total 8 mg/kg) ($n = 12$), or intravenous Bu 1.6 mg/kg/day divided into two doses per day for 5 days (total 8 mg/kg) ($n = 19$). Recently, we used 0.8 mg/kg IV Bu q12hr for 5 days because we aimed to reduce toxicity by increasing the Bu dosing interval and to ensure engraftment by increasing the total dose of Bu. In this study we regarded intravenous Bu 8 mg/kg as reduced-intensity conditioning although intravenous Bu 8 mg/kg is categorized as myeloablative conditioning according to the CIBMTR definition [26]. Non-T-cell-depleted bone marrow was infused on day 0.

As prophylaxis for GVHD, all patients received cyclosporine A (CsA) and short-term methotrexate (sMTX). CsA was started from day −1 at a dose of 3 mg/kg/day divided into two doses per day, and MTX was administered intravenously on day 1 (10 mg/m^2) and on days 3 and 5 (7 mg/m^2). The CsA dosage was adjusted to a target trough level between 150 and 300 ng/ml until day 100, except where disease progression or drug toxicity occurred, and then tapered over 3–6 months unless GVHD occurred.

Granulocyte colony-stimulating factor was administered intravenously from day 1 or day 5 in all patients until neutrophil engraftment. As antibiotic prophylaxis, polymyxin B sulfate, acyclovir, and fluconazole were used for prevention of bacterial infection, herpes viral infection, and fungal infection, respectively, after the conditioning regimen started. Sulfamethoxazole/trimethoprim was started for prophylaxis of *Pneumocystis jiroveci* pneumonia after neutrophil engraftment.

2.4 Chimerism analysis

We assessed donor–recipient chimerism by polymerase chain reaction (PCR)-based amplification of a polymorphic short tandem repeat (STR) region. Chimerism was serially evaluated in the T-cell fraction of peripheral blood samples on days 30, 60, 90 and thereafter at appropriate points after transplantation. According to the manufacturer's recommended protocol (BML, Tokyo, Japan), T-cell chimerism analysis with PCR-based STR analysis was undertaken with a commercially available assay. Briefly, peripheral blood samples were collected, and the T-cell enriched fraction was prepared by negative selection using a RosetteSep kit (Stem Cell Technologies, Vancouver, Canada) for other cell lineages (CD16, CD19, CD36, CD56, CD66b, and glycopholin A). DNA was then extracted from selected cells using a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). Mutiplex PCR was performed using a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). Separation and detection of the amplified PCR products were performed, and the area under the curve for each STR allele was automatically processed using the CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA). The range of error of chimerism was regarded as 10%.

2.5 GVHD diagnosis and treatment

Acute GVHD was diagnosed clinically, graded in accordance with the standard criteria [27] and confirmed by appropriate biopsy of the lesion. Chronic GVHD was also defined in accordance with the traditional criteria [28]. We initially treated the patients who developed acute GVHD with methylprednisolone or prednisolone at a dose of 1–2 mg/kg/day.

2.6 Statistical analysis

Neutrophil recovery was defined as the first of three consecutive days with an absolute neutrophil count (ANC) $\geq 0.5 \times 10^9$/L. Platelet recovery was defined as the first of three consecutive days with a platelet count $\geq 20 \times 10^9$/L, without platelet transfusion in the preceding 7 days. Full donor T-cell chimerism was defined as more than 90% donor T cells in the patient's peripheral blood. Primary graft failure was defined as a lack of neutrophil recovery in the absence of disease relapse or progression affecting

hematopoietic recovery. Secondary graft failure was defined as the proportion of donor T cells being less than 10% in the peripheral blood, excluding disease relapse or progression-related loss of donor T cells. Event-free survival (EFS) was defined as survival without disease relapse or progression, or death from any cause. NRM was defined as any death except for death related to relapse or progression. Cumulative incidence curves of NRM and relapse were analyzed under the assumption that they represented competing risks. Death not associated with GVHD was treated as a competing risk event in constructing the cumulative incidence curve for acute GVHD. The Kaplan–Meier method was used for overall survival (OS) and EFS. The log-rank test was used for comparing survival between high- and standard-risk groups. The Cox proportional hazard model was used to determine the significant factors that influenced OS in univariate and multivariate analyses. We defined statistical significance as a P value less than 0.05. Statistical analyses were performed using STATISTICA version 6.0 and R version 2.9.1.

3 Results

3.1 Engraftment and T-cell chimerism

All patients received bone marrow from unrelated donors. The median total number of nucleated cells in the marrow graft was 2.82 (range 1.26–4.14) $\times$ 10^8 per kg of the recipient's body weight. All 31 patients achieved neutrophil engraftment, and the median time to neutrophil engraftment was 16 days (range 12–26 days) after transplantation. Twenty-nine patients (94%) achieved platelet engraftment, and the median time to platelet engraftment was 24 days (range 18–291 days) after transplantation.

Twenty patients (65%), 26 patients (84%), and 28 patients (90%) achieved complete donor T-cell chimerism by 30, 60, and 90 days after transplant, respectively. Of three patients who did not achieve complete donor T-cell chimerism by 100 days, one patient achieved complete donor T-cell chimerism on day 236, and in the other two patients, grafts were rejected due to disease progression. Only nine of twelve patients with oral Bu conditioning achieved complete donor T-cell chimerism. On the other hand, all 19 patients who received intravenous Bu achieved complete donor T-cell chimerism by day 100 [$P = 0.049$ (Fisher's exact test)]. None had experienced graft rejection except for relapse-related graft rejection.

3.2 Regimen-related toxicities and infections

Regimen-related toxicities until 28 days after transplantation were graded in accordance with the National Cancer Institute Common Toxicity Criteria, version 3.0 and are shown in Table 2. There were 17 episodes of grade III toxicity and two episodes of grade IV toxicity. Of grade III or IV toxicities, Grade III mucositis was frequently observed (42%). One patient had a grade III hemorrhage, consisting of a subdural hematoma at 14 days after transplantation. He underwent emergency surgery to remove the hematoma and recovered. Grade IV toxicities were observed in two patients. One patient experienced grade IV hepatic toxicity due to hemophagocytic syndrome, but he recovered following a single administration of 50 mg/m^2 etoposide. The other experienced grade IV pulmonary toxicity due to engraftment syndrome and needed endotracheal intubation. Steroid administration was very effective for engraftment syndrome, and the patient could be extubated, but died from disease progression and cytomegalovirus (CMV) pneumonia. No hepatic veno-occlusive disease was documented in this study.

Table 2 Grade III and IV regimen-related toxicities up to 28 days after transplantation

	Grade, no. of episodes (% of patients)		
	I–II	III	IV
Cardiac	0 (0)	0 (0)	0 (0)
Mucositis	7 (23)	13 (42)	0 (0)
GI (nausea or diarrhea)	8 (26)	2 (6)	0 (0)
Hepatic	5 (16)	0 (0)	1 (3)
Pancreas	1 (3)	0 (0)	0 (0)
Central nervous system	0 (0)	1 (3)	0 (0)
Pulmonary	1 (3)	0 (0)	1 (3)
Renal	1 (3)	0 (0)	0 (0)
Hemorrhage	0 (0)	1 (3)	0 (0)

GI gastrointestinal

Infectious complications up until day 100 are summarized in Table 3. Culture-negative febrile neutropenia was documented in 17 patients (55%). Nine patients (29%) had bacteremia including eight Gram-positive organisms and one Gram-negative organism. Twenty-four patients (77%) became positive for CMV antigenemia: five of them developed CMV colitis and one developed CMV pneumonia. Hemorrhagic cystitis was documented in seven patients. Urinary PCR for viral DNA was performed in six of these seven patients. Adenovirus was detected in the urine of two patients, BK virus in another two, and both adenovirus and BK virus in the remaining two patients.

3.3 Graft-versus-host disease

Acute GVHD occurred in 18 patients (58%) with a median time to development of acute GVHD of 23.5 days (range 11–55 days). Five patients had grade I, eight had grade II,

Table 3 Infectious complications in the first 100 days of transplantation

	No. of episodes	No. of patients (%)
Culture-negative febrile neutropenia	18	17 (55)
Bacterial		
Bacteremia	10	9 (29)
Pneumonia	2	2 (6)
Viral		
CMV antigenemia	–	24 (77)
CMV colitis	5	5 (16)
CMV pneumonia	1	1 (3)
Hemorrhagic cystitis	7	7 (23)
VZV reactivation	2	2 (6)
HSV reactivation	1	1 (3)
Fungal		
Pneumonia	2	2 (6)
Pneumocystis pneumonia	1	1 (3)

CMV cytomegalovirus, *VZV Varicella zoster* virus, *HSV Herpes simplex* virus

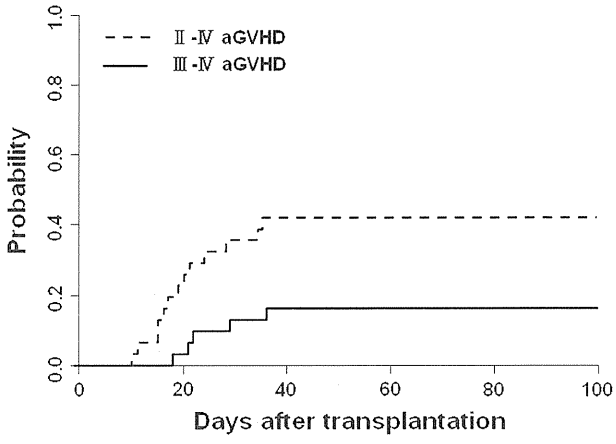

Fig. 1 Cumulative incidence of acute graft-versus-host disease (GVHD). The cumulative incidence of grades II–IV and III–IV acute GVHD are displayed

two had grade III, and three had grade IV GVHD. The cumulative incidence of grade II–IV and III–IV acute GVHD by day 100 after transplantation was 42 and 16%, respectively (Fig. 1). Systemic high-dose steroid was administered to all 13 of these patients as initial treatment of grade II–IV acute GVHD, and in 10 patients (77%) acute GVHD improved and was manageable without any additional treatment. In the other three patients (23%), acute GVHD was refractory to the steroid treatment, and they received secondary therapies including mycophenolate mofetil, pentostatin, or intra-arterial infusion of steroid for acute GVHD involving the gut. Two of them died of acute GVHD, and the third died of disease relapse although acute

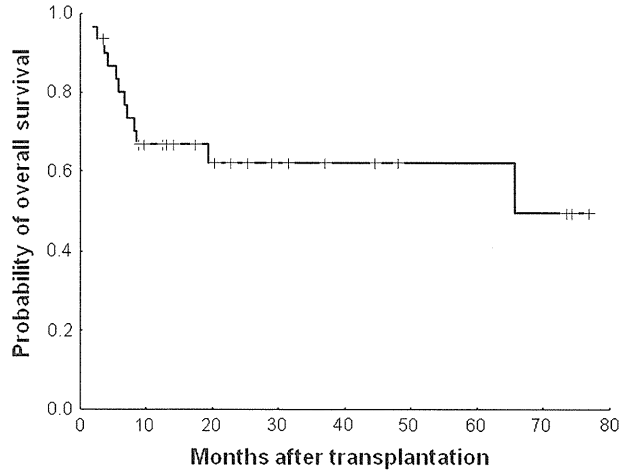

Fig. 2 Kaplan–Meier curve of overall survival. Kaplan–Meier product estimates of overall survival are presented

GVHD subsided. Chronic GVHD was observed in 15 of 25 evaluable patients (60%), and the extensive type of chronic GVHD was present in seven patients (28%).

3.4 Survival and cause of death

The median follow-up period for all 31 patients was 14.1 months (range 1.8–76.7 months). Of these patients, 19 were alive at 25.2 months of median follow-up (range 3.4–76.7 months). Of 12 deaths, nine patients died of disease relapse or progression, and three died of non-relapse-related causes including acute GVHD/thrombotic microangiopathy (day 55), acute GVHD/bacterial pneumonia (day 102), and chronic GVHD/bacterial pneumonia (day 162). Of 13 patients with disease relapse or progression, three patients underwent allogeneic hematopoietic stem cell transplantation again, and two underwent donor lymphocyte infusion, and nine died during the follow-up period.

In all patients, OS and EFS were 67 and 53% at 1 year, and 62% and 53 at 2 years, respectively (Fig. 2). In the standard disease-risk group, OS and EFS were 84 and 72% at 1 year, and 75 and 72% at 2 years. In the high disease-risk group, OS and EFS were 33 and 18% at 1 and 2 years, respectively (Log rank test: $P = 0.06$ and 0.009, respectively). The NRM at day 100 and 1 year were very low, 3 and 10%, respectively, and the rates of relapse at day 100 and 1 year were 13 and 37%, respectively.

Table 4 shows the results of univariate and multivariate analyses for factors influencing OS. On univariate analysis, elderly patients (>60) had a significantly worse outcome [HR 7.0, 95% confidence interval (CI) 20–24, $P = 0.002$], and patients with high-risk disease tended to have worse outcome (HR 2.9, 95% CI 0.9–9.4, $P = 0.07$). On multivariate analysis, older age (>60) remained significantly

Table 4 Univariate and multivariate analyses of risk factors for overall survival

	Univariate HR (95% CI)	*P* value	Multivariate HR (95% CI)	*P* value
Age (>60)	7.0 (2.0–24)	0.002	6.6 (1.8–24)	0.004
Sex (F:M)	1.7 (0.4–8.1)	0.48	–	–
HLA mismatch	0.6 (0.1–2.9)	0.56	–	
Disease status (high)	2.9 (0.9–9.4)	0.07	2.6 (0.8–8.6)	0.11
Acute GVHD (III–IV)	2.8 (0.8–11)	0.13	–	–
Intravenous busulfan	0.9 (0.3–3.3)	0.85	–	–

HR hazard ratio, *CI* confidence interval

associated with worse OS (HR 6.6, 95% CI 1.8–24, $P = 0.004$).

4 Discussion

Our simple procedure with T-cell replete, reduced-intensity conditioning without TBI, with the use of the classical combination of CsA and sMTX as GVHD prophylaxis, allowed stable engraftment and manageable GVHD, and led to very low NRM in u-RIST.

In reports of u-RIST with regimens that contained ATG [1–7] or alemtuzumab [5, 8–10], NRM rates were 10–39 and 15–30%, respectively. Although ATG or alemtuzumab-containing regimens were effective in preventing both chronic and acute GVHD [1–10] with the use of a standard dose of alemtuzumab, high relapse and infection rates due to delayed immune reconstitution are still a problem [1, 5–7, 9, 10, 22–24]. The optimal dose of ATG or alemtuzumab in u-RIST has been explored. Recent reports showed that reduced doses of ATG led to lower NRM in u-RIST [21, 29, 30].

We found five additional reports of u-RIST without use of TBI, ATG, or alemtuzumab [31–35]. NRM at 1 year was reported to be 19–32% [31–34]. In our study, the observed NRM of 3% at day 100 and 10% at 2 years is relatively low compared to that in previous reports of u-RIST. Important contributors to these favorable results might be that patients all had a history of prior chemotherapy, bone marrow alone was used as a stem cell source, and our study population was Japanese.

In RIST using ATG, alemtuzumab, or TBI, the reported incidence of acute GVHD was 19–47% [1–7], 16–36% [5, 8–10], and 3–77% [11–18], respectively. In RIST with TBI, the incidence of acute GVHD tended to be higher than in RIST with T-cell depletion. Taking differences in stem cell source, race, degree of HLA disparity, and GVHD prophylaxis into consideration, we cannot make a simple comparison among regimens. However, the observed incidence of 42% acute GVHD and of 60% chronic GVHD in our study was not higher than with RIST that involved the use of T-cell depletion. Japanese patients have a lower incidence of acute GVHD than Caucasian patients after hematopoietic stem cell transplantation from either HLA-matched siblings or unrelated donors. Although the incidence of acute GVHD was not particularly low in our study, the 77% efficacy rate of initial treatment for acute GVHD with high-dose steroids was relatively high [36], which might have contributed to the lower NRM. Furthermore, TBI was not required for conditioning to achieve rapid, complete T-cell chimerism and obtain sustained engraftment, probably because the study patients were limited to those with a history of prior chemotherapy.

A prospective, multi-institutional clinical trial of u-RIST in the Japanese population using a cladribine/Bu/TBI 4 Gy regimen showed a high NRM rate of 54% at 1 year, which largely derived from GVHD with co-existent infection [37]. This result suggests that the use of TBI 4 Gy may increase mortality associated with GVHD due to increased tissue damage, particularly in elderly patients.

Conversely, with the truly non-myeloablative conditioning developed by the Seattle Group, consisting of low-dose TBI 2 Gy with/without Flu and with CsA/MMF as post-grafting immune suppression, NRM rates were relatively low at 11–20% [11–14]. These results suggest that not only is TBI intensity important in itself, but that the intensity of conditioning combined with TBI, and post-grafting immunosuppressants are also important. However, a lower rate of sustained engraftment (10 of 18 patients, 56%) was reported when unrelated bone marrow was used [12]. Truly non-myeloablative conditioning is not likely to be sufficient to ensure sustained engraftment in unrelated bone marrow transplantation.

In our study, the achievement of complete donor T-cell chimerism by day 100 was significantly different between the oral and intravenous Bu groups. However, in the oral busulfan group, two of three cases of incomplete T-cell chimerism were caused by relapse rejection. It is therefore unknown how much the intensified busulfan dose contributed to increase lymphoablation of recipient T cells.

The current 37% cumulative incidence of relapse at 1 year appeared to be high. Predominant relapse and

progression occurred beyond 100 days after u-RIST (62% of cases relapsed or progressed). On the other hand, 38% of cases relapsed or progressed in the early phase after transplantation. For early relapse or progression of disease, a more intense conditioning regimen such as Flu/melphalan [38], Flu/Bu4 [39] or up-front allogeneic hematopoietic stem cell transplantation after cytoreductive induction therapy [40] may be effective. However, for late disease relapse/progression, a treatment strategy that enhances the graft-versus-leukemia/lymphoma effect would be needed, including the selective use of peripheral blood stem cells for chronic GVHD [41], or prophylactic DLI [42].

This study has the following limitations: (1) it was retrospective and performed at a single institution; (2) the population was small; (3) diseases were heterogeneous; and (4) HLA-mismatched donors were included. The characteristics of patients in previous reports of u-RIST were likewise heterogeneous. Ideally therefore, we should on an individual basis determine the magnitude of immunosuppression and the intensity of a conditioning regimen that is necessary and sufficient to obtain engraftment, and control GVHD and disease after taking into account numerous factors including patient age, intensity of prior chemotherapy, prior transfusion volume, residual host immune capacity, HLA compatibility, the stem cell source, ethnic background, and tumor burden.

Our regimen achieved lower NRM and better survival than those in previous reports, particularly in patients with a standard disease risk. However, our results cannot be applied to HLA-mismatched transplantation because of the small number of patients with HLA-mismatched transplantation in the present study. In addition, we cannot conclude with certainty that the low NRM was attributable to our regimen alone, based on comparison of the NRM rate in our regimen and that in other RISTs from the literature that included total-body irradiation or in vivo T-cell-depletion. To solve this problem would require a prospective study that addresses optimal conditioning for u-RIST in a cohort with as homogeneous a population as is possible.

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Conflict of interest None of the authors has a conflict of interest.

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Article

The Wnt agonist R-spondin1 regulates systemic graft-versus-host disease by protecting intestinal stem cells

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Graft-versus-host disease (GVHD) is a major complication of allogeneic bone marrow transplantation (BMT), and damage to the gastrointestinal (GI) tract plays a critical role in amplifying systemic disease. Intestinal stem cells (ISCs) play a pivotal role not only in physiological tissue renewal but also in regeneration of the intestinal epithelium after injury. In this study, we have discovered that pretransplant conditioning regimen damaged ISCs; however, the ISCs rapidly recovered and restored the normal architecture of the intestine. ISCs are targets of GVHD, and this process of ISC recovery was markedly inhibited with the development of GVHD. Injection of Wnt agonist R-spondin1 (R-Spo1) protected against ISC damage, enhanced restoration of injured intestinal epithelium, and inhibited subsequent inflammatory cytokine cascades. R-Spo1 ameliorated systemic GVHD after allogeneic BMT by a mechanism dependent on repair of conditioning-induced GI tract injury. Our results demonstrate for the first time that ISC damage plays a central role in amplifying systemic GVHD; therefore, we propose ISC protection by R-Spo1 as a novel strategy to improve the outcome of allogeneic BMT.

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Abbreviations used: BMT, BM transplantation; GI, gastrointestinal; GVHD, graft-versus-host disease; ISC, intestinal stem cell; R-Spo1, R-spondin1; SCT, stem cell transplantation; TBI, total body irradiation; TCD, T cell depleted.

An important aspect of cancer therapy is maintaining a fine balance between the use of chemoradiotherapy doses high enough to kill tumor cells and doses low enough to prevent damage to normal tissue. The gastrointestinal (GI) epithelium and BM are the most rapidly self-renewing tissues in adults and are therefore susceptible to cytotoxic exposure, showing a rapid expression of damage. Damage to these tissues is a dose-limiting and potentially lethal toxicity of chemoradiotherapy used to treat cancer patients. Allogeneic hematopoietic stem cell transplantation (SCT) is a curative therapy for hematologic malignancies that works by delivering healthy hematopoietic stem cells to replace BM destroyed by the high-dose chemoradiotherapy (pretransplant conditioning); however, this process is complicated by regimen-related toxicity against other tissues, particularly in the GI tract.

Graft-versus-host disease (GVHD), a major and devastating complication of allogeneic SCT, is a complex process involving donor T cell responses to host antigens and the dysregulation of inflammatory cytokine cascades (Hill et al., 1997; Hill and Ferrara, 2000; Teshima et al., 2002a; Ferrara et al., 2003). Increasing evidence from experimental and clinical SCT suggests that conditioning-mediated GI tract damage plays a central role in amplifying GVHD by propagating its cytokine storm characteristics (Hill et al., 1997; Hill and Ferrara, 2000; Ferrara et al., 2003). Intestinal epithelial cells are continuously regenerated from intestinal stem cells (ISCs), which are key to the regeneration of damaged intestinal epithelium (Batlle et al., 2002; Pinto et al., 2003; Barker et al., 2007, 2008). However, the dynamic process of damage and repopulation of ISCs, which play a pivotal

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role in the competitive race between tissue damage and restoration during conditioning regimens and GVHD, is not well understood.

Wnt signaling plays a critical role in the regulation of intestinal epithelial cell proliferation during their maturation or regeneration (Batlle et al., 2002; Pinto et al., 2003; Reya and Clevers, 2005; Barker et al., 2008). R-spondin1 (R-Spo1) is a potent activator of the Wnt signaling pathway. It relieves the Dickkopf-1 inhibition imposed on the Wnt signaling pathway and thereby increases levels of the Wnt pathway coreceptor low-density lipoprotein receptor–related protein-6 on cell surface (Kim et al., 2005; Binnerts et al., 2007). We have previously shown that human R-Spo1 transgenic mice had a marked thickening of the mucosa and displayed crypt epithelial hyperplasia (Kim et al., 2005). Injection of human R-Spo1 induced rapid onset of crypt cell proliferation in the intestine of normal mice through β-catenin stabilization and subsequent transcriptional activation of target genes (Kim et al., 2005). Thus, injection of R-Spo1 protected mice from chemotherapy- or radiation-induced colitis by stimulating mucosal regeneration and restoring intestinal architecture (Kim et al., 2005; Zhao et al., 2007, 2009; Bhanja et al., 2009). However, because of the lack of specific markers for ISCs, it is unclear whether this result was mediated by the direct effect of R-Spo1 on ISCs.

In this study, we investigated the dynamic process of ISC damage and repopulation during the pretransplant conditioning regimen, total body irradiation (TBI), and GVHD. The effects of R-Spo1 on this process were also examined using recently identified markers for ISCs such as *Lgr5* (*leucine-rich repeat–containing G protein–coupled receptor 5*) and *Olfm4* (*Olfactomedin-4*; Barker et al., 2007, 2008; van der Flier et al., 2009a,b). *Lgr5* and *Olfm4* mark rapidly cycling crypt base columnar cells, which can give rise to all intestinal epithelial lineages (Barker et al., 2007, 2008; van der Flier et al., 2009a,b). We then tested the hypothesis that protection of ISCs improves the outcome of allogeneic SCT by regulating systemic GVHD using a well-characterized murine model of MHC-mismatched, haploidentical BM transplantation (BMT).

RESULTS

R-Spo1 protected against radiation-induced colitis by stimulating proliferation of ISCs through the Wnt signaling pathway

We first studied the effect of R-Spo1 on the expression of Wnt target genes in the small intestine using quantitative real-time PCR. Injection of R-Spo1 (200 μg/day) over 3 d significantly up-regulated the expression of Wnt target genes, including *Axin2*, *Ascl2* (*Achaete scute-like 2*), and *Lgr5* (Fig. S1 A). We noted an elongation of villi with an increased number of *Olfm4*$^+$ ISCs in the crypts of R-Spo1–treated animals (Fig. S1, B and C). Ki-67 immunostaining also showed crypt hyperplasia paralleling an increased number of Ki-67$^+$ cycling cells in the crypts (Fig. S1, D and E).

Next, we evaluated the effect of R-Spo1 administration on the process of mucosal regeneration after TBI. According to our preliminary experiments (unpublished data), mice irradiated with 15 Gy TBI on day 0 were intravenously injected with 200 μg R-Spo1 once daily from day −3 to −1 and from day 1–3. The real-time PCR analysis of the small intestine harvested 6 h after the final administration of R-Spo1 showed up-regulated expression of *Axin2* and *Ascl2* in R-Spo1–treated mice (Fig. 1 A). The *Olfm4*$^+$ cell population was significantly greater in R-Spo1–treated mice than in controls on day 3 (Fig. 1, B and C); as a result, radiation colitis characterized

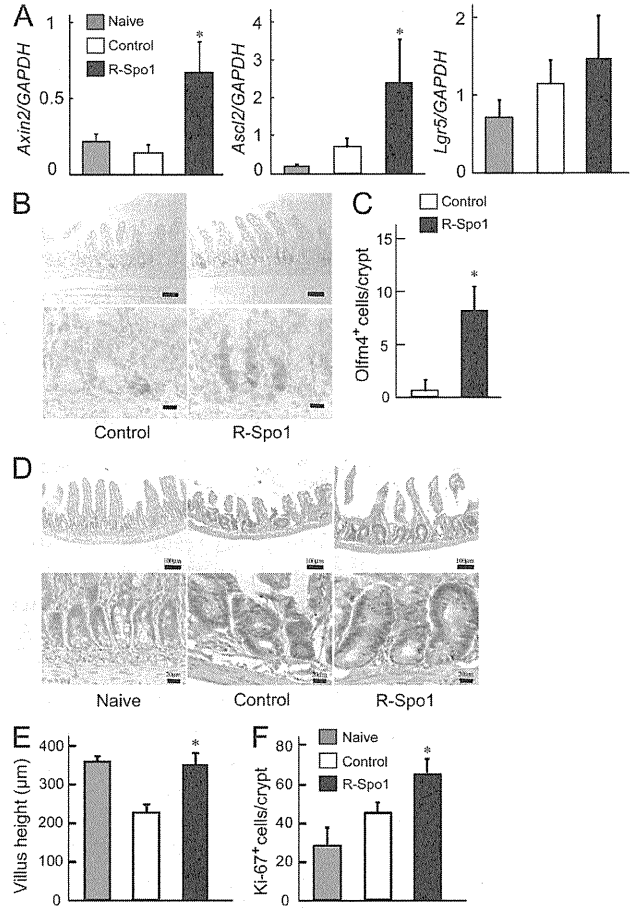

Figure 1. R-Spo1 protected against radiation-induced colitis by enhancing proliferation of ISCs via the Wnt signaling pathway. B6D2F1 mice irradiated with 15 Gy TBI on day 0 and intravenously injected with R-Spo1 (200 μg/day) or control from day −3 to −1 and day 1–3. The small intestine was harvested 6 h after the final administration of R-Spo1 for quantitative real-time PCR analysis and in situ hybridization, and 24 h later for immunohistochemistry. (A) Quantitative real-time PCR analysis of *Axin2*, *Ascl2*, and *Lgr5* transcripts normalized to those of GAPDH (naive, $n = 3$; control, $n = 4$; R-Spo1, $n = 4$). (B) In situ hybridization for *Olfm4* on representative crypts. (C) Quantification of *Olfm4*$^+$ cells per crypt ($n = 4$ per group). (D) Ki-67 staining of the terminal ileum. (E) Villus height of the terminal ileum (naive, $n = 3$; control, $n = 4$; R-Spo1, $n = 4$). (F) Quantification of Ki-67$^+$ cells per crypt (naive, $n = 3$; control, $n = 4$; R-Spo1, $n = 4$). Data are representative of two independent experiments and are shown as means ± SD. *, $P < 0.05$ compared with control. Bars: (B and D, top row) 100 μm; (B and D, bottom row) 20 μm.

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by blunting of villi was significantly reduced in R-Spo1–treated animals (Fig. 1, D and E). We also noted crypt hyperplasia and an increased number of Ki-67$^+$ cycling cells in the crypt on day 4 (Fig. 1, D and F). These results extend our previous observations regarding R-Spo1–mediated mitogenic effects on the intestinal epithelium (Kim et al., 2005) by documenting the effects of R-Spo1 on ISCs.

R-Spo1 protected against ISC damage after allogeneic BMT

GI tract damage is much more severe in allogeneic SCT than in autologous or syngeneic SCT because of the additional detrimental effects of GVHD on the GI tract. However, it remains to be elucidated whether GVHD targets ISCs that are crucial for the regeneration of damaged intestinal epithelium and also how the damage and repopulation of ISCs affects the process of mucosal injury and regeneration after allogeneic BMT. To address these issues, lethally irradiated B6D2F1 mice were transplanted with 5 × 10^6 T cell–depleted (TCD) BM cells with or without 2 × 10^6 T cells from MHC-mismatched C57BL/6 (B6) or B6-Ly5.1 donors on day 0. Small intestines were harvested from mice on day 0 before TBI and on days 3 and 6 after BMT, and quantitative real-time PCR and in situ hybridization were performed to determine the kinetics of loss and repopulation of *Olfm4*$^+$ ISCs. R-Spo1 was injected from day −3 to −1 and day 1–3 after BMT. TCD animals and control-treated allogeneic animals served as non-GVHD

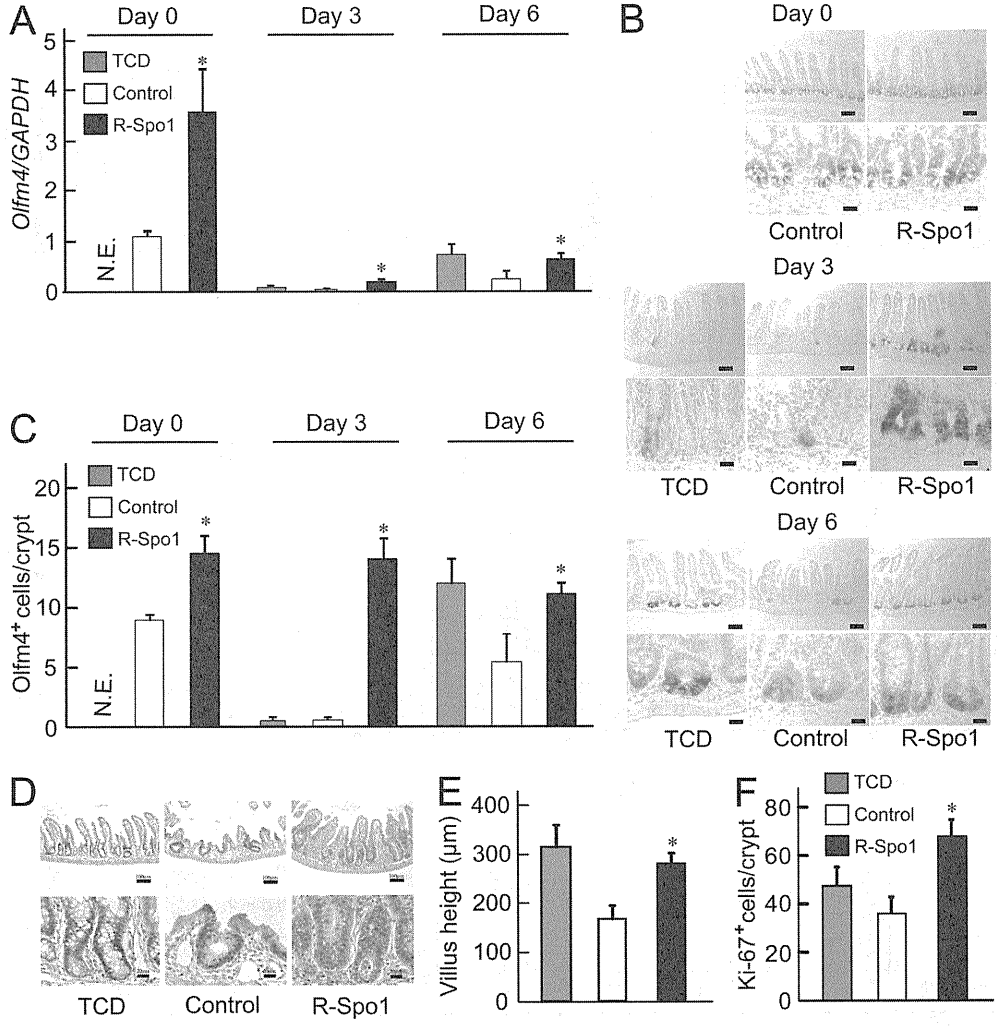

Figure 2. R-Spo1 enhanced repopulation of ISCs after allogeneic BMT. Lethally irradiated B6D2F1 mice were transplanted with 5 × 10^6 TCD BM with or without 2 × 10^6 T cells from B6 donors on day 0. R-Spo1 (200 μg/day) or control was intravenously injected from day −3 to −1 and day 1–3 after BMT. Small intestines were harvested on day 0 (before TBI) and days 3 and 6. (A) Quantitative real-time PCR analysis of *Olfm4* transcripts normalized to those of GAPDH (TCD, $n = 3$; control, $n = 4$; R-Spo1, $n = 4$ per time point). (B) In situ hybridization of *Olfm4* on representative crypts. (C) Quantification of *Olfm4*$^+$ cells per crypt (TCD, $n = 3$; control, $n = 4$; R-Spo1, $n = 4$ per time point). (D) Ki-67 staining of the terminal ileum harvested on day 6. (E) Villus height of the terminal ileum was measured as described in Fig. 1 E using the slides in D (TCD, $n = 3$; control, $n = 5$; R-Spo1, $n = 5$). (F) Quantification of Ki-67$^+$ cells per crypt (TCD, $n = 3$; control, $n = 5$; R-Spo1, $n = 5$). Data are representative of two independent experiments and are shown as means ± SD. *, $P < 0.05$ compared with control. Bars: (B and D, top rows) 100 μm; (B and D, bottom rows) 20 μm.

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controls and GVHD controls, respectively. In both groups, *Olfm4* expression levels significantly decreased in the small intestine on day 3 (Fig. 2 A). In TCD animals, *Olfm4* expression recovered to normal levels on day 6, whereas in allogeneic controls, it remained low. In contrast, *Olfm4* expression levels in R-Spo1–treated animals were significantly higher on and after day 0 in comparison with allogeneic controls (Fig. 2 A). These results were further confirmed by in situ hybridization analysis of *Olfm4* transcripts in the small intestine. The *Olfm4*$^+$ ISC population significantly decreased in both TCD and allogeneic controls on day 3 (Fig. 2, B and C). On day 6, *Olfm4*$^+$ cells were fully repopulated in TCD animals, and their numbers were significantly higher than their numbers in allogeneic controls. These results demonstrate that TBI injures ISCs and that the process of ISC repopulation is inhibited in GVHD. In R-Spo1–treated animals, the number of *Olfm4*$^+$ cells was consistently higher before and after BMT than that in allogeneic controls. Villous atrophy was severe in allogeneic controls on day 6, whereas injection of R-Spo1 resulted in crypt hyperplasia with an increased number of Ki-67$^+$ cycling cells in the crypts and dramatically ameliorated GI tract damage (Fig. 2, D–F). R-Spo1 treatment before TBI thus expanded the ISC pool and minimized intestinal damage.

R-Spo1 suppressed inflammatory cytokine cascades and donor T cell activation after allogeneic BMT

We then tested the hypothesis that protection of ISCs against TBI regulates systemic GVHD and improves the outcome of

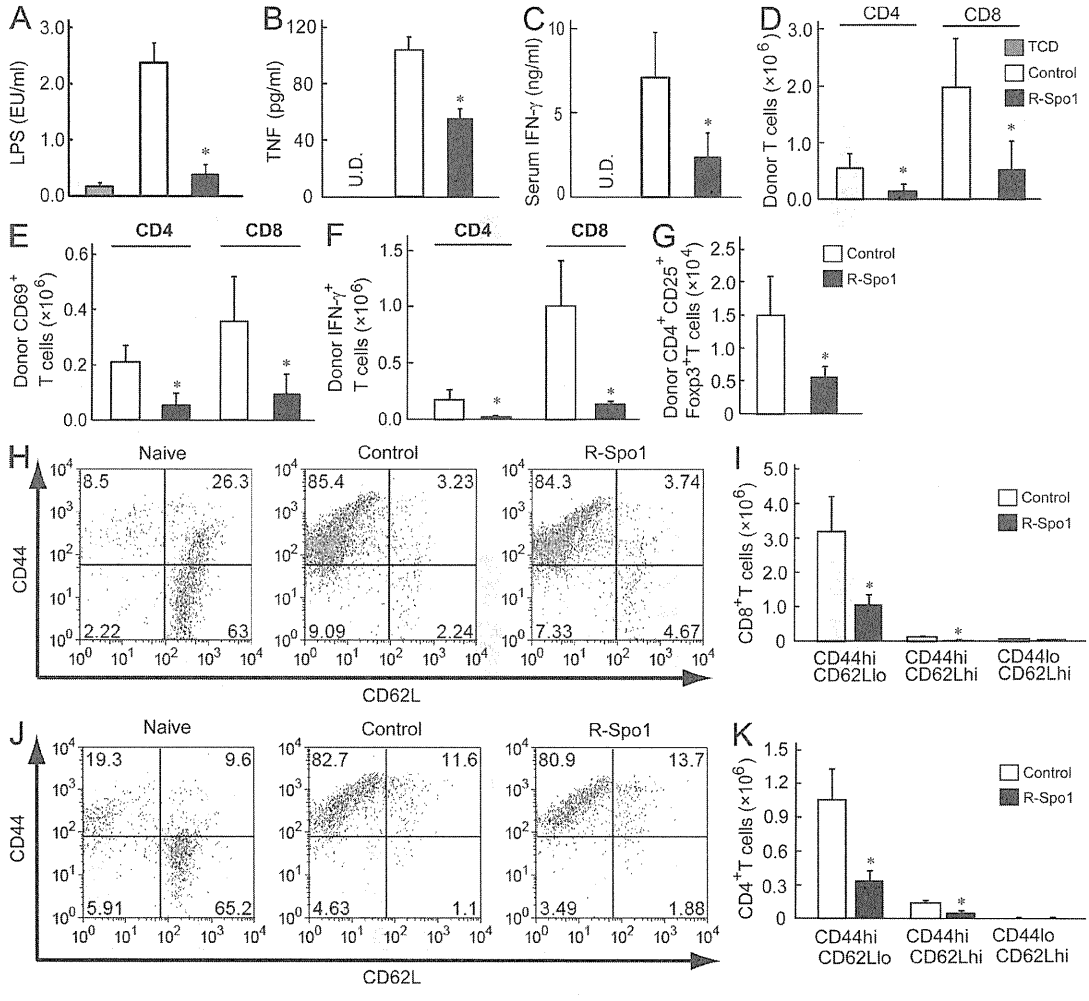

Figure 3. R-Spo1 regulated activation of inflammatory and cellular effectors in GVHD. Lethally irradiated B6D2F1 mice were transplanted with 5 × 10^6 TCD BM cells with or without 2 × 10^6 T cells from B6-Ly5.1 (CD45.1$^+$) donors on day 0. R-Spo1 (200 µg/day) or control was intravenously injected from day −3 to −1 and day 1–3 after BMT. Serum samples and splenocytes were obtained 5–7 d after BMT (TCD, n = 3; control, n = 5; R-Spo1, n = 5). (A–C) Serum levels of LPS (A), TNF (B), and IFN-γ (C) are shown. (D–G) Numbers of donor (CD45.1$^+$) T cells (D), CD69$^+$ donor T cells (E), IFN-γ$^+$ donor T cells (F), and CD4$^+$CD25$^+$Foxp3$^+$ T reg cells (G) in spleen are shown. (H–K) Flow cytometric analysis and enumeration of T cell subsets in spleen. Numbers represent the percentage of cells in the dot plot quadrants. CD8$^+$ T cells (H and I) and CD4$^+$ T cells (J and K) are shown. Data are representative of two independent experiments and are shown as means ± SD. *, P < 0.05 compared with control. U.D., undetectable.

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allogeneic SCT. Serum LPS levels are increased during GVHD and correlate with GI tract damage (Hill et al., 1997; Hill and Ferrara, 2000; Ferrara et al., 2003). LPS has been shown to stimulate production of excessive inflammatory cytokines such as TNF that are implicated in the pathogenesis of GVHD (Nestel et al., 1992; Hill et al., 1997; Hill and Ferrara, 2000; Cooke et al., 2001; Teshima et al., 2002a; Ferrara et al., 2003). LPS and TNF levels were markedly increased in allogeneic controls but were significantly reduced in R-Spo1–treated animals (Fig. 3, A and B), suggesting that the fortification of GI mucosal barrier functions by R-Spo1 suppresses subsequent inflammatory cascades in GVHD. We also investigated the effect of R-Spo1 on allogeneic donor T cell responses. Serum levels of IFN-γ, a hallmark of systemic T cell responses in GVHD, were significantly lower in R-Spo1–treated mice than in controls (Fig. 3 C). Donor T cell expansion (Fig. 3 D) and activation, as determined by CD69 expression (Fig. 3 E), and intracellular IFN-γ (Fig. 3 F) were also significantly reduced in R-Spo1–treated mice. Recent studies have shown that Wnt signaling can modulate adoptive immunity by enhancing regulatory T cell (T reg cell) survival and inducing $CD4^+$ T cell anergy, as well as by regulating effector $CD8^+$ T cell development and promoting memory $CD8^+$ T cell generation (Ding et al., 2008; Gattinoni et al., 2009). However, in our study, the number of $CD4^+CD25^+Foxp3^+$ T reg cells in the spleen was significantly less in R-Spo1–treated mice than in controls (Fig. 3 G), and the ratios of effector to memory $CD8^+$ (Fig. 3, H and I) and $CD4^+$ T cells (Fig. 3, J and K) were similar between R-Spo1–treated mice and controls.

To further confirm whether the reduction in donor T cell activation after BMT was caused by the direct effect of R-Spo1 on T cells, we investigated the effect of R-Spo1 on T cells in vivo and in vitro. Administration of R-Spo1 over 3 d had no effect on the number of T cells in naive mice (Table I),

Table I. Brief administration of R-Spo1 had no effects on immunophenotype

Immunophenotype	+Control	+R-Spo1
Spleen		
$CD4^+$	20.7 ± 4.2	17.4 ± 2.0
$CD8^+$	14.3 ± 2.5	11.9 ± 0.9
$CD4^+Foxp3^+$	2.46 ± 0.63	2.46 ± 0.34
$B220^+$	58.5 ± 10.9	57.8 ± 2.9
Mesenteric lymph node		
$CD4^+$	4.3 ± 0.7	4.9 ± 0.2
$CD8^+$	3.5 ± 0.7	4.2 ± 0.4
$CD4^+Foxp3^+$	0.65 ± 0.14	0.70 ± 0.09
Thymus		
$CD4^+CD8^+$	96.2 ± 16.9	91.6 ± 19.8
$CD4^+$	8.4 ± 1.0	8.6 ± 2.1
$CD8^+$	3.4 ± 0.3	3.6 ± 0.6

B6 mice were intravenously injected with R-Spo1 (200 µg/day) or control for 3 d, and the spleen, mesenteric lymph nodes, and thymus were harvested 6 h later. Cell numbers are shown as mean ± SD ($\times 10^6$). Data are representative of two independent experiments ($n = 4$ for +control and +R-Spo1).

and the addition of R-Spo1 to culture did not affect in vitro T cell responses to alloantigens or anti-CD3 cross-linking (Fig. S2, A and B). Furthermore, R-Spo1 addition to culture affected neither the proliferation nor the generation of effector and memory $CD8^+$ and $CD4^+$ T cells in response to anti-CD3 cross-linking in vitro (Fig. S2, C–F).

Brief administration of R-Spo1 ameliorated systemic GVHD

We studied two lethal doses of TBI, 12 and 15 Gy, for their effects on GVHD. At both TBI doses, TCD controls showed 100% survival. All allogeneic controls receiving 15 Gy TBI died by day 40, whereas those receiving 12 Gy TBI displayed 7% survival at day 90 (Fig. 4 A). The TBI dose thus significantly correlated with GVHD mortality, as has been shown previously (Hill et al., 1997). In R-Spo1–treated animals, GVHD mortality was significantly reduced in experiments with 12 Gy TBI and delayed in those with 15 Gy TBI (Fig. 4 A) and reduced GVHD severity as assessed by clinical GVHD scores (Teshima et al., 2002a) in surviving animals (Fig. 4 B). Target organs, including the small intestine, liver, skin, and thymus, were then evaluated for signs of GVHD after allogeneic BMT after 12 Gy TBI. The small intestine and liver samples were harvested 1 wk after BMT, whereas skin and thymus samples were obtained 7 wk after BMT. GVHD-mediated thymic atrophy, characterized by a reduction in the numbers of $CD4^+CD8^+$ double-positive thymocytes, was significantly restored in R-Spo1–treated mice (Fig. 4 C). Pathological analysis of the small intestine, liver, and skin showed almost normal architecture in TCD controls (Fig. 4 D). In contrast, allogeneic controls showed severe blunting of villi and inflammatory infiltration, whereas R-Spo1–treated mice showed significant restoration of the small intestinal villous architecture with little inflammatory infiltration. Liver histology of allogeneic controls revealed mononuclear cell infiltration in bile ducts and portal triads (Fig. 4 D, arrowheads), whereas these changes were less prominent in R-Spo1–treated mice. Lesser lymphocyte infiltration was observed in the skin of R-Spo1–treated mice compared with that of allogeneic controls. GVHD pathology scores in each organ were significantly lower in R-Spo1–treated mice than those in controls (Fig. 4, E–G). Flow cytometric analysis of the spleens on day 35 displayed complete donor chimerism (99.9 ± 0.1%), ruling out mixed chimerism as a cause of the reduced GVHD. These results demonstrate that brief administration of R-Spo1 modulates not only intestinal but also systemic GVHD.

Next, we studied how the scheduling of R-Spo1 administration could influence the outcome of allogeneic BMT after 15 Gy TBI. Administration of R-Spo1 from day −3 to −1 and day 1–3 significantly prolonged survival. These beneficial effects were not observed when R-Spo1 was injected only once after BMT from day 1–6 after 15 Gy TBI (Fig. 4 H). When R-Spo1 was administered only once before TBI from day −6 to −1, early GVHD mortality was reduced; however, survival was not prolonged. These results suggest that R-Spo1 injection before TBI is mandatory and that posttransplant administration of R-Spo1 results in maximum reduction of GVHD.

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R-Spo1 regulates GVHD by a mechanism dependent on repair of radiation-induced gut injury

To confirm that R-Spo1 ameliorated systemic GVHD by a mechanism dependent on repair of radiation-induced GI tract damage, the effects of R-Spo1 were evaluated in the same BMT model without conditioning, as previously described (Mori et al., 1998). Unirradiated B6D2F1 mice were intravenously injected with 12×10^7 splenocytes from MHC-mismatched B6 or B6-Ly5.1 donors on day 0. In this model, cytopenia mediated by donor T cell attack of BM is the primary cause of death in GVHD (Via et al., 1987). Injection of R-Spo1 did not impact the mortality or morbidity caused by GVHD (Fig. 5, A and B), donor T cell expansion (Fig. 5 C), thymic GVHD (Fig. 5 D), GVHD-associated cytopenia (Fig. 5 E), or donor cell engraftment (99.7 ± 0.4% in controls and 99.9 ± 0.0% in R-Spo1–treated mice on day 60).

DISCUSSION

Intestinal GVHD is characterized by severe villous atrophy and crypt degeneration. It has been suggested that crypt cell degeneration is one of the initial lesions of intestinal GVHD (Sale et al., 1979; Epstein et al., 1980; Mowat and Socie, 2004). ISCs reside in the intestinal crypts and play a pivotal role in both physiological tissue renewal and regeneration of the intestinal epithelium after injury. However, the identity of cells within the crypts (primary targets in GVHD) has been an

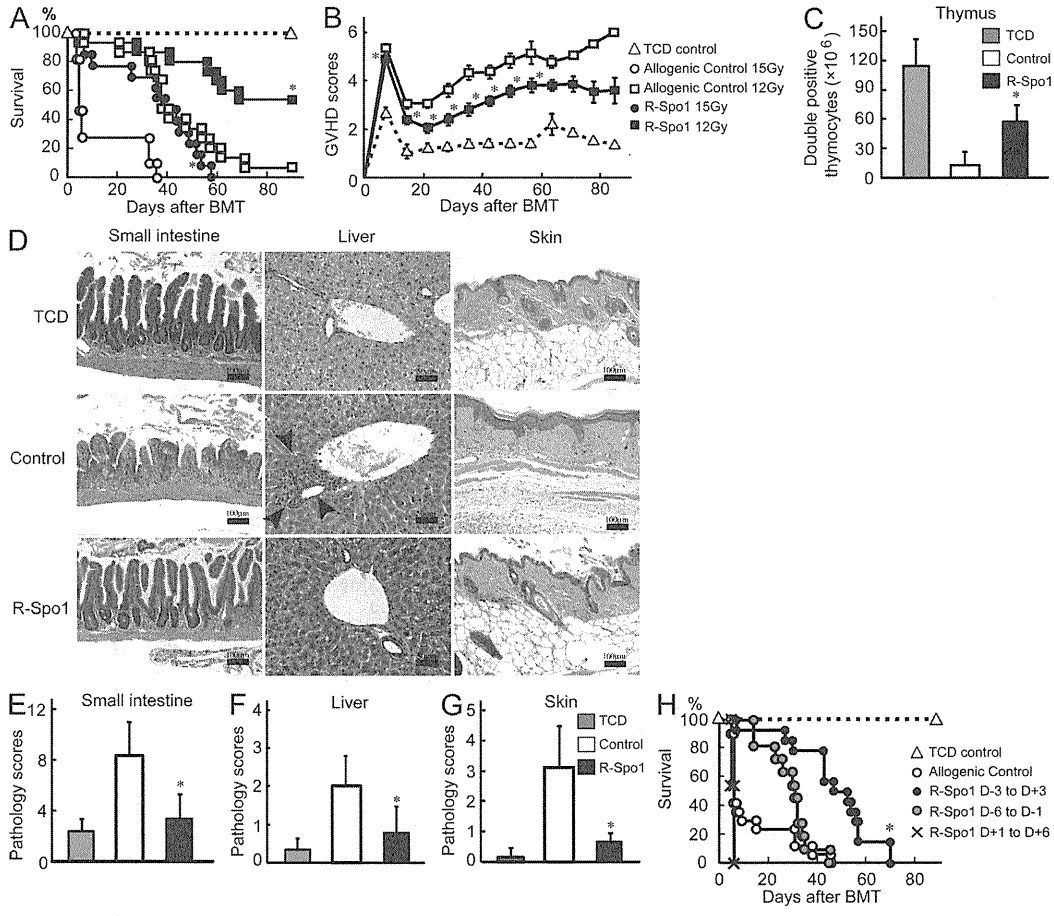

Figure 4. R-Spo1 modulated systemic GVHD. B6D2F1 mice were transplanted with 5×10^6 TCD BM cells with or without 2×10^6 T cells from B6 donors on day 0 after 15 or 12 Gy TBI. R-Spo1 (200 µg/day) or control was injected from day −3 to −1 and day 1–3 after BMT. (A and B) Survival (A) and clinical GVHD scores (B; means ± SE) are shown. TCD non-GVHD controls ($n = 6$), allogeneic controls with 15 Gy ($n = 11$) or 12 Gy TBI ($n = 15$), and R-Spo1 with 15 Gy ($n = 13$) or 12 Gy TBI ($n = 15$) are shown. Data from three independent experiments were combined. (C) Numbers of $CD4^+CD8^+$ double-positive thymocytes 7 wk after BMT (TCD, $n = 3$; control, $n = 5$; R-Spo1, $n = 5$). Data are representative of two similar experiments and are shown as means ± SD. (D) Representative histological findings of the small intestine, liver, and skin. Arrowheads indicate mononuclear cell infiltration in bile ducts and portal triads. Bars: (left and right) 100 µm; (middle) 50 µm. (E–G) Pathology scores of the small intestine (E) and liver (F) harvested on day 7 and those of the skin (G) harvested 7 wk after BMT after 12 Gy TBI (TCD, $n = 3$; control, $n = 5$; R-Spo1, $n = 5$). Data are representative of two similar experiments and are shown as means ± SD. (H) R-Spo1 was intravenously injected for six doses at different schedules after 15 Gy TBI and BMT. Survival after BMT: TCD non-GVHD controls ($n = 5$), allogeneic controls ($n = 17$), and R-Spo1 day −3 to −1 and day 1–3 (D-3 to D+3; $n = 11$), day −6 to −1 (D–6 to D–1; $n = 14$), or day 1–6 (D+1 to D+6; $n = 11$) are shown. Data from three independent experiments were combined. *, $P < 0.05$ compared with allogeneic controls.

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enigma because of the lack of specific markers. In this study, we discovered that pretransplant TBI damaged *Olfm4*$^+$ ISCs in the crypts; however, the ISCs rapidly recovered and restored the normal architecture of the small intestine within 1 wk. With development of acute GVHD, the process of ISC recovery was inhibited, and prolonged and profound intestinal damage was induced after allogeneic BMT. These observations are well in line with those from a previous study of sequential rectal biopsies from patients undergoing allogeneic BMT (Epstein et al., 1980). Severe crypt degeneration was noted in all biopsies taken soon after BMT, probably because of the conditioning regimen. These changes persisted when acute GVHD was present but disappeared in patients who did not show clinical evidence of GVHD (Epstein et al., 1980). The current study thus affirms the long-held assumption that ISCs may be targets for immune responses associated with GVHD (Sale et al., 1979; Epstein et al., 1980; Mowat and Socie, 2004).

We have previously demonstrated that R-Spo1 induces rapid onset of epithelial proliferation in the intestine by stimulating Wnt signaling and protects against chemotherapy-induced colitis (Kim et al., 2005). However, owing to the lack of specific ISC markers it was unclear whether this effect was caused by the direct effect of R-Spo1 on ISCs. The current study shows that administration of R-Spo1 up-regulates the expression of Wnt target genes such as murine *Axin2*, *Ascl2*, and *Lgr5*. *Ascl2* is a critical transcriptional factor involved in controlling the fate of ISCs in adults (van der Flier et al., 2009b), and *Lgr5* marks ISCs (Barker et al., 2007, 2008). *Olfm4* is not a Wnt target gene but a highly specific and robust marker for *Lgr5*$^+$ ISCs (van der Flier et al., 2009a,b). We found that R-Spo1 stimulated proliferation of *Olfm4*$^+$ ISCs, thus taking further the observations from our previous study (Kim et al., 2005) and confirming recent observations that R-Spo1 enhances the proliferation of cycling ISCs via the Wnt signaling pathway (Bhanja et al., 2009; Sato et al., 2009).

Administration of R-Spo1 has been shown to mediate protection against radiation colitis, which is evident from studies in mouse models with chemotherapy- or radiation-induced mucositis and gut injury (Kim et al., 2005; Zhao et al., 2007, 2009; Bhanja et al., 2009). Our results suggest that R-Spo1–mediated protection of ISCs could be primarily responsible for the protection of the GI tract from radiation, as has been suggested in a recent study (Bhanja et al., 2009). Furthermore, we have shown that brief administration of R-Spo1 suppresses systemic GVHD after allogeneic BMT by a mechanism dependent on the repair of conditioning-induced GI tract injury. Experimental and clinical studies have suggested that GI tract damage resulting from both pretransplant conditioning regimens and GVHD plays a central role in increasing GVHD severity (Hill et al., 1997; Hill and Ferrara, 2000; Ferrara et al., 2003). Disruption of the GI mucosal barrier facilitates the translocation of immunostimulatory microbial products such as LPS into the systemic circulation (Hill et al., 1997; Cooke et al., 1998, 2001; Hill and Ferrara, 2000). LPS then stimulates mononuclear cells primed by donor T cell IFN-γ to produce large amounts of inflammatory cytokines such as TNF and IL-1 and augments donor T cell activation, thereby potentiating both inflammatory and cellular effectors of GVHD (Nestel et al., 1992; Cooke et al., 1998, 2001). The administration of R-Spo1 protected against GI tract damage, leading to the fortification of GI tract mucosal barrier functions and reduction of the subsequent inflammatory milieu. An inflammatory environment would have further enhanced donor T cell activation (Nestel et al., 1992; Hill et al., 1997; Cooke et al., 1998), and R-Spo1 treatment

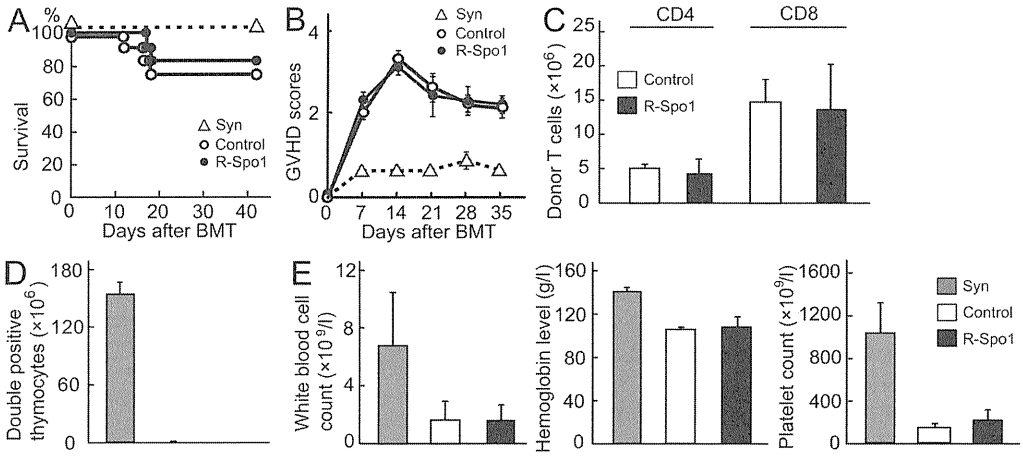

Figure 5. R-Spo1 failed to attenuate GVHD in unirradiated host. Unirradiated B6D2F1 mice were transplanted with 12 × 10^7 splenocytes from syngeneic or allogeneic B6 donors. R-Spo1 (200 µg/day) or control was administered from day −3 to −1 and day 1–3 after BMT. (A and B) Survival (A) and clinical GVHD scores (B; mean ± SE) are shown. Syngeneic controls (Syn; $n = 3$), allogeneic controls (control; $n = 12$), and R-Spo1 ($n = 12$) are shown. Data from two independent experiments were combined. (C–E) Numbers of donor CD4$^+$ and CD8$^+$ T cells in the spleen (C), CD4$^+$CD8$^+$ double-positive thymocytes (D), and a complete blood count (E) on day 14 (Syn, $n = 3$; control, $n = 4$; R-Spo1, $n = 4$) are shown. Data are representative of two independent experiments and are shown as mean ± SD.

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was also found to significantly reduce donor T cell proliferation and activation. As a result, brief administration of R-Spo1 modulates not only intestinal GVHD but also systemic GVHD. This study thus demonstrates for the first time that ISC damage plays a critical role in the exaggeration of GVHD.

The protective effects of R-Spo1 were not observed after allogeneic BMT in the absence of a conditioning regimen, thus suggesting a mechanism dependent on repair of conditioning-induced GI tract injury. In addition, R-Spo1 may act through different mechanisms before and after TBI; it protects best against systemic GVHD when administered before and after transplantation. Treatment with R-Spo1 before TBI expanded ISCs, suggesting an increased number of surviving ISCs that play a pivotal role in the regeneration of intestinal epithelium after injury. Additional administration of R-Spo1 posttransplant may further enhance proliferation and differentiation of the surviving ISCs, thereby allowing the regeneration of intestinal epithelium and fortification of mucosal barrier functions to suppress subsequent inflammatory milieu. It has been shown that a single ISC is sufficient for the reconstitution of a crypt-villus unit (Sato et al., 2009).

Reduction in the activation of donor T cells after BMT did not appear to be caused by the direct effect of R-Spo1 on T cells. A recent study has shown that β-catenin–transduced $CD4^+CD25^+$ T reg cells survive longer than control cells, whereas β-catenin–transduced $CD4^+$ T cells become anergic (Ding et al., 2008). Wnt signaling arrests effector T cell differentiation by generating $CD8^+$ memory stem cells (Gattinoni et al., 2009). However, such changes were not apparent after BMT in our study. Wnt signaling is also important for hematopoiesis (Reya and Clevers, 2005); however, brief administration of R-Spo1 did not affect hematopoietic reconstitution after TCD BMT (unpublished data). We thus believe that R-Spo1 may preferentially stimulate ISCs rather than hematopoietic and T cells, as R-Spo1 transgenic mice show intestinal epithelial hyperplasia without any effects on lymphohematopoietic development (Kim et al., 2005). Alternately, such brief administration of R-Spo1 may not be sufficient to affect both the immune system and hematopoiesis. Wnt signaling has also been implicated in the pathogenesis of various tumors such as colon cancer and leukemia (Reya and Clevers, 2005; Román-Gómez et al., 2007). However, the incidence of tumorigenesis did not increase in R-Spo1 transgenic mice (Kim et al., 2006), and long-term treatment with R-Spo1 did not promote tumor xenograft growth in immunodeficient mice inoculated with various human colorectal tumor cell lines (Zhao et al., 2009). It thus follows that although caution should be exercised, it is unlikely that brief administration of R-Spo1 enhances tumorigenesis or the growth of preexisting tumors.

In summary, we found that ISCs are targets for GVHD and that protection of ISCs by R-Spo1 significantly improved the outcome of BMT by reducing systemic GVHD severity. By documenting that ISC damage is the key to this process, these results extend previous observations that the GI tract is not only a target organ for GVHD but also a crucial amplifier of systemic GVHD severity (Hill et al., 1998; Panoskaltsis-Mortari et al., 1998; Krijanovski et al., 1999; Teshima et al., 1999; Hill and Ferrara, 2000). An intensified conditioning regimen plays a critical role in controlling leukemia, but conditioning-related toxicity, particularly of the GI tract, limits the application of this curative therapy. Reduced intensity regimens have also been developed to explore the use of this therapy in older leukemic patients; however, better control of leukemia requires intensified conditioning in high-risk patients (Kahl et al., 2007). Thus, strategies to protect the GI tract from conditioning-related toxicity may allow safer application of intensified conditioning for controlling leukemia. Such a strategy has been tested in previous studies using IL-11 or keratinocyte growth factor. However, it is unfortunate that patients receiving IL-11 displayed severe fluid retention and early mortality (Antin et al., 2002), while keratinocyte growth factor failed to reduce conditioning regimen–mediated diarrhea (Blazar et al., 2006), thus making it impossible to further test the proposed strategy. R-Spo1 use is highly promising because of its direct, specific, and potent effects on ISCs; therefore, brief treatment with R-Spo1 may be used as an effective adjunct to clinical regimens of GVHD prophylaxis. This study presents a novel combined strategy for the rescue of both hematopoietic stem cells and ISCs in clinical medicine. Such a strategy may also be useful for treatment of other solid tumors and accidentally or intentionally irradiated victims, in whom damage to BM and the GI tract is a serious problem.

MATERIALS AND METHODS

Mice and reagents. Female B6 ($H\text{-}2^b$, $CD45.2^+$) and B6D2F1 ($H\text{-}2^{b/d}$, $CD45.2^+$) mice were purchased from Charles River, and B6-Ly5.1 ($H\text{-}2^b$, $CD45.1^+$) mice were obtained from the Jackson Laboratory. Mice were maintained as previously described (Teshima et al., 2002a). All animal experiments were performed under the auspices of the Institutional Animal Care and Research Advisory Committee. Recombinant human R-Spo1 was produced in CHO cells and purified as previously described (Zhao et al., 2007).

BMT. Mice were transplanted as previously described (Teshima et al., 2002a). In brief, after lethal TBI (x ray) delivered in two doses at 4-h intervals, B6D2F1 mice were intravenously injected with 5×10^6 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 AutoMACS (Miltenyi Biotec) according to the manufacturer's instructions. In some experiments, unirradiated B6D2F1 mice were intravenously injected with 12×10^7 splenocytes.

Assessment of GVHD. Survival after BMT was monitored daily, and the degree of clinical GVHD was assessed weekly by a scoring system that sums changes in five clinical parameters: weight loss, posture, activity, fur texture, and skin integrity (maximum index = 10) as described previously (Teshima et al., 2002a). Acute GVHD was also assessed by detailed histopathological analysis using a semiquantitative scoring system (Teshima et al., 2002a). Pictures from tissue sections were taken at room temperature using a digital camera (ProgRes 3012 mF; Jenoptik) mounted on a microscope (BX51; Olympus) and analyzed using a ProgRes PlugIn for PCI software version 5.0 (Jenoptik).

Flow cytometric analysis. mAbs used were FITC-, PE-, Cy5 PE–, or allophycocyanin-conjugated or biotinylated anti–mouse TCR-β, IFN-γ, CD4, CD8, CD25, CD45.1, CD45.2, CD44, CD62L, CD69, and B220 (BD),

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and we also used Foxp3 (eBioscience). Surface marker staining and intracellular cytokine staining were performed as previously described (Teshima et al., 2002a; Asakura et al., 2010). At least 5,000 live samples were analyzed using FACSCalibur (BD) and FlowJo software (Tree Star, Inc.). The CFSE labeling of T cells was also performed as previously described (Teshima et al., 2002a).

Immunohistochemical staining and in situ hybridization. Slides were incubated at room temperature for 90 min with anti–mouse Ki-67 mAbs (Dako). We used Histofine Simple Stain MAXPO (rat) kits and subsequently diaminobenzidine solution (Nichirei) to generate brown-colored signals. Slides were then counterstained with hematoxylin. We measured villus height in 20 representative villi of the terminal ileum per slide as described previously (Farrell et al., 1998). For in situ hybridization, 1640-bp DNA fragments corresponding to nucleotide positions 17–435 of mouse *Olfm4* cDNA (GenBank/EMBL/DDBJ accession no. NM_001030294) were subcloned into pGEMT-Easy vectors (Promega) and used for generation of sense or antisense RNA probes. Digoxigenin-labeled RNA probes were prepared with DIG RNA labeling mix (Roche). Intestines were flushed, fixed in tissue fixative (Genostaff), embedded in paraffin, and sectioned at 6 µm. Sections were then dewaxed, rehydrated, and digested with proteinase K solution, refixed, treated in acetic anhydride solution, and hybridized for 16 h at 60°C with probes at concentrations of 100 ng/ml in probe diluent (Genostaff). After washing, the sections were treated with 0.5% blocking reagent (Roche) in TBST (TBS with Tween 20) for 30 min and then incubated with anti-digoxigenin alkaline phosphatase conjugate (Roche) diluted in a 1:1,000 ratio with TBST for 2 h. After washing, coloring reactions were performed with BM purple alkaline phosphatase substrate (Roche) overnight, and sections were then rewashed with PBS. Sections were then counterstained with Kernechtrot stain solution (Mutoh), dehydrated, and mounted with malinol (Mutoh).

Cell cultures. All culture media and incubation conditions have been previously described (Teshima et al., 2002b). Isolation of $CD8^+$ and $CD4^+CD25^-$ T cells was performed by AutoMACS according to the manufacturer's instructions. Methods to generate DCs were previously described (Teshima et al., 2002b). T cells were cultured at a concentration of 1×10^5 T cells/well with 2.5×10^3 irradiated DCs/well or with 5 µg/ml plate-bound anti-CD3 mAbs and 2 µg/ml anti-CD28 mAbs. Supernatants were collected for measurement of cytokine levels 96 h after the initiation of culture, and cell proliferation was determined by thymidine uptake assay.

ELISA. For measuring IFN-γ (BD) and TNF (R&D systems) levels, we performed ELISA according to the manufacturers' instructions with sensitivities of 31.25 pg/ml and 23.4 pg/ml, respectively. The Limulus amebocyte lysate assay (Lonza) was performed according to the manufacturer's instructions to determine the serum level of LPS with a sensitivity of 0.1 EU/ml. All units expressed are relative to the US 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). PCR reactions and analyses were performed with ABI PRISM 7900HT SDS 2.1 (Applied Biosystems) using TaqMan Universal PCR master mix (Applied Biosystems), primers, and labeled TaqMan probes (TaqMan Gene Expression Assays; Applied Biosystems). The relative amount of each messenger RNA was determined using the standard curve method and was normalized to the level of GAPDH in each sample.

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. $P < 0.05$ was considered statistically significant.

Online supplemental material. Fig. S1 demonstrates that R-Spo1 stimulated proliferation of ISCs through the Wnt signaling pathway. Fig. S2 shows that R-Spo1 has no effects on proliferation and effector differentiation of T cells in response to CD3 or alloantigen stimulation in vitro. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20101559/DC1.

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