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

Shuichiro Takashima,[1] Masanori Kadowaki,[1] Kazutoshi Aoyama,[1] Motoko Koyama,[1] Takeshi Oshima,[3] Kazuma Tomizuka,[3] Koichi Akashi,[1,2] and Takanori Teshima[2]

[1]Department of Medicine and Biosystemic Science and [2]Center for Cellular and Molecular Medicine, Kyushu University Graduate School of Medical Science, Higashi-ku, Fukuoka 812-8582, Japan
[3]Innovative Drug Research Laboratories, Kyowa Hakko Kirin Co., Ltd., Machida, Tokyo 194-8533, Japan

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.

CORRESPONDENCE
Takanori Teshima:
tteshima@
cancer.med.kyushu-u.ac.jp

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

The Rockefeller University Press $30.00
J. Exp. Med. Vol. 208 No. 2 285-294
www.jem.org/cgi/doi/10.1084/jem.20101559

The Journal of Experimental Medicine

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

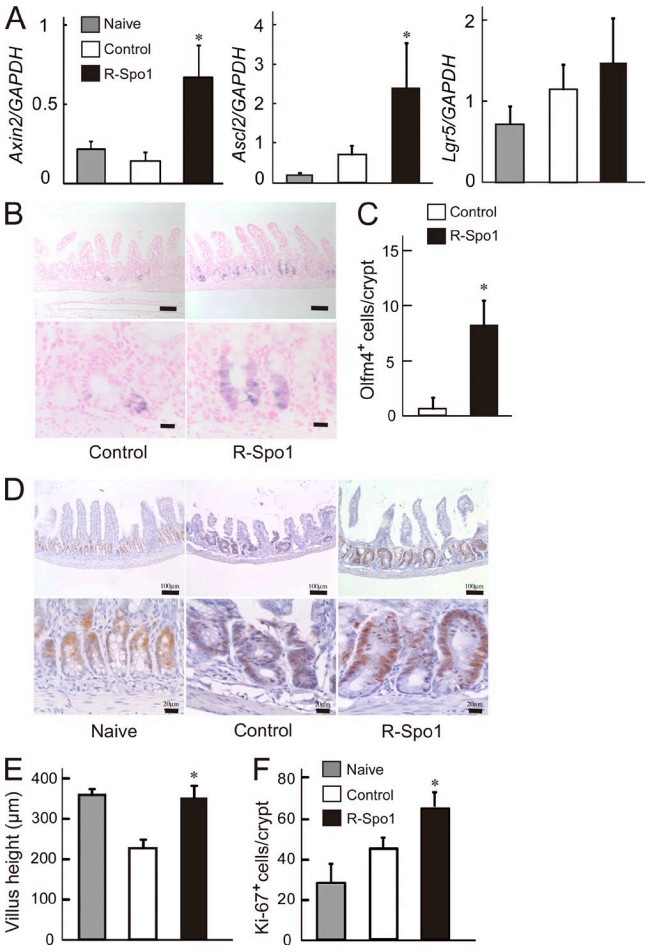

Figure 1. R-Spo1 protected against radiation-induced colitis by enhancing proliferation of ISCs via the Wnt signaling pathway. B6D2F1 mice irradiated with 15 Gy TBI on day 0 and intravenously injected with R-Spo1 (200 µg/day) or control from day −3 to −1 and day 1–3. The small intestine was harvested 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.

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

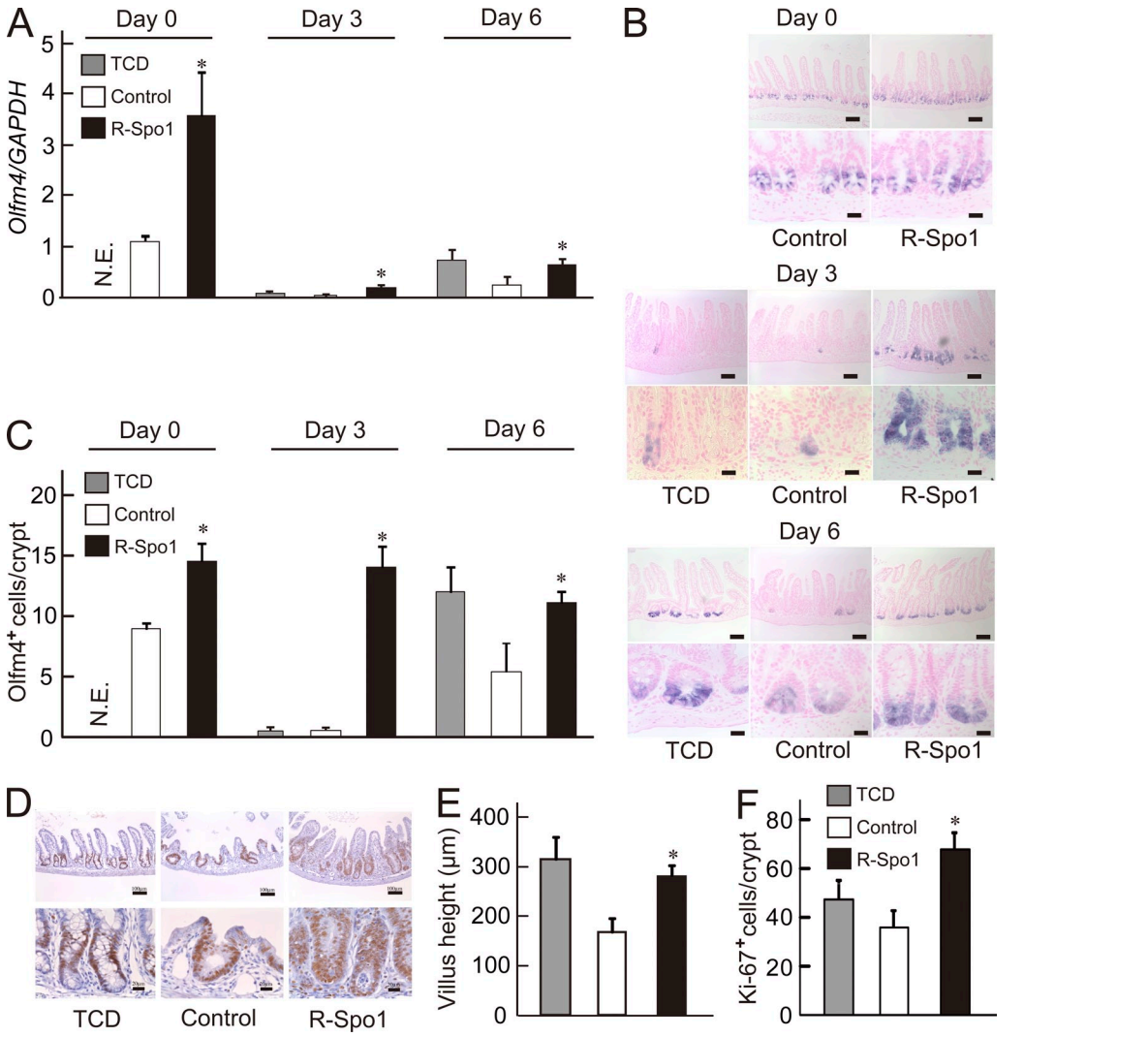

Figure 2. R-Spo1 enhanced repopulation of ISCs after allogeneic BMT. Lethally irradiated B6D2F1 mice were transplanted with 5×10^6 TCD BM with or without 2×10^6 T cells from B6 donors on day 0. R-Spo1 (200 µg/day) or control was intravenously injected from day −3 to −1 and day 1–3 after BMT. Small intestines 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.

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

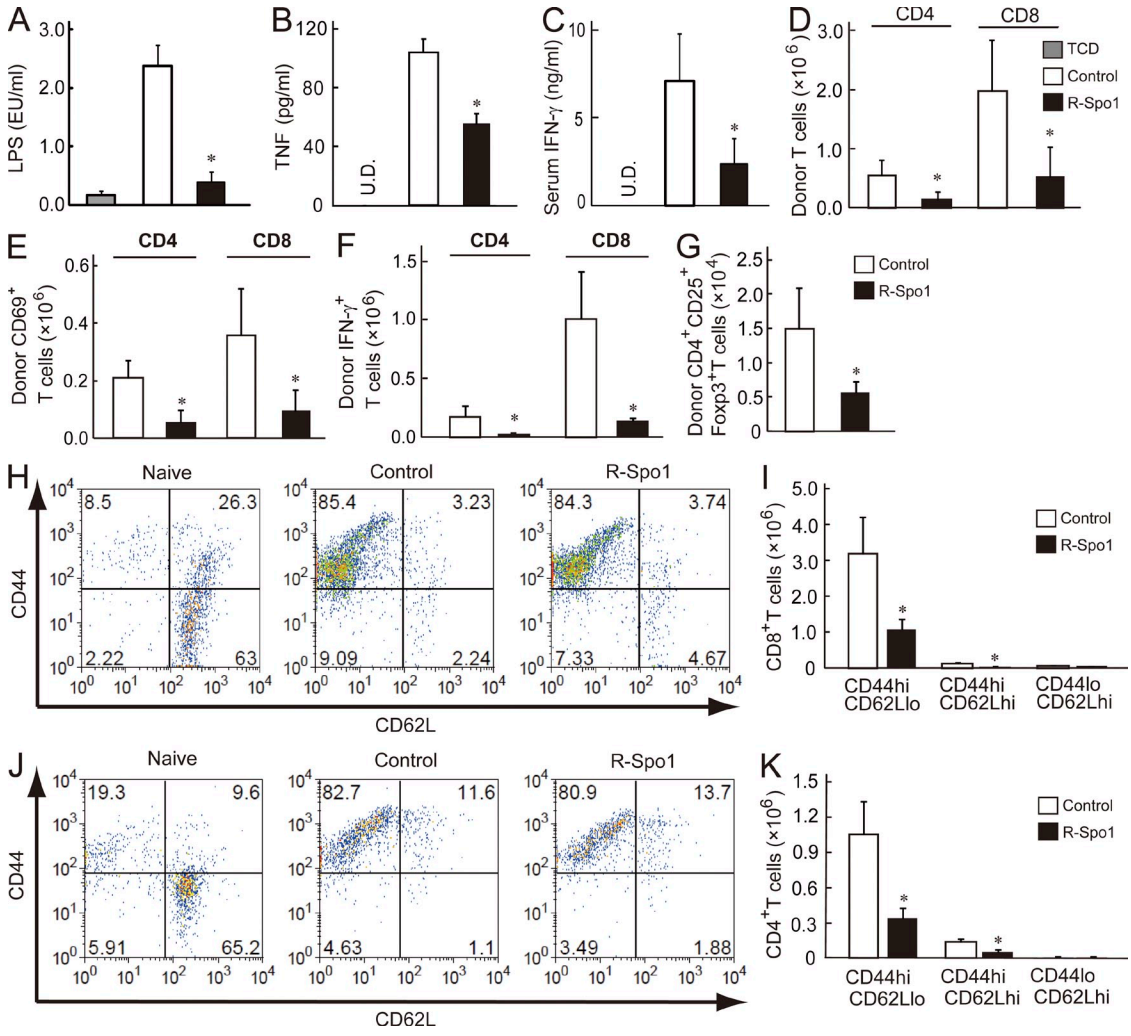

Figure 3. R-Spo1 regulated activation of inflammatory and cellular effectors in GVHD. Lethally irradiated B6D2F1 mice were transplanted with 5 × 10^6 TCD BM cells with or without 2 × 10^6 T cells from B6-Ly5.1 (CD45.1$^+$) donors on day 0. R-Spo1 (200 µg/day) or control was intravenously injected from 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.

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.

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

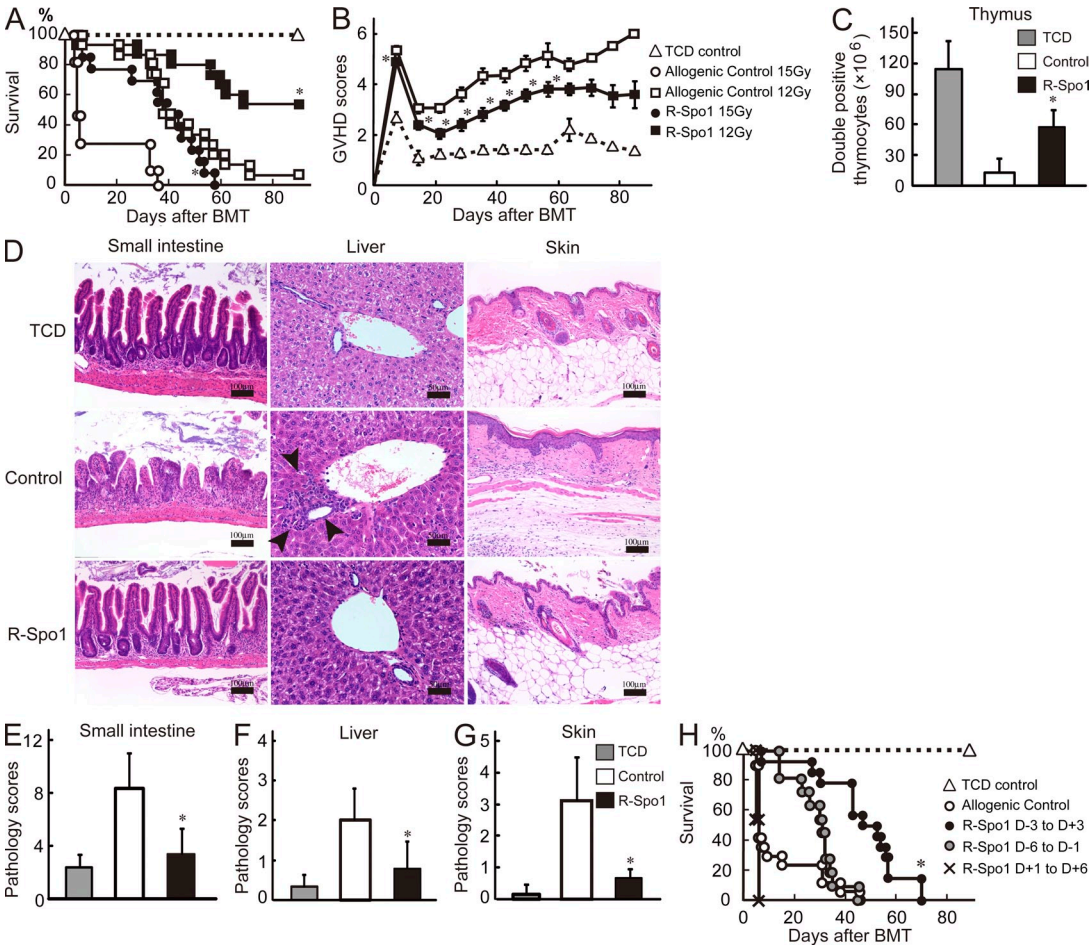

Figure 4. R-Spo1 modulated systemic GVHD. B6D2F1 mice were transplanted with 5×10^6 TCD BM cells with or without 2×10^6 T cells from B6 donors on day 0 after 15 or 12 Gy TBI. R-Spo1 (200 µg/day) or control was injected from day −3 to −1 and day 1–3 after BMT. (A and B) Survival (A) and clinical GVHD 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.

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

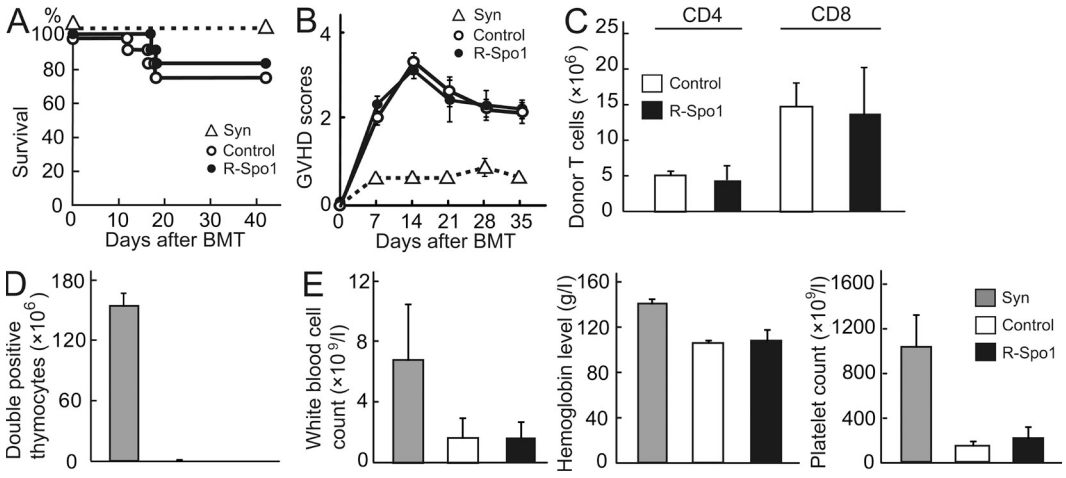

Figure 5. R-Spo1 failed to attenuate GVHD in unirradiated host. Unirradiated B6D2F1 mice were transplanted with 12 × 10^7 splenocytes from syngeneic or allogeneic B6 donors. R-Spo1 (200 µg/day) or control was administered from day −3 to −1 and day 1–3 after BMT. (A and B) Survival (A) and clinical GVHD 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.

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),

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.

This study was supported by research funds from the Ministry of Education, Culture, Sports, Science and Technology (no. 20659153 to T. Teshima), Health and Labor Science Research Grants (to T. Teshima), and a grant from the Foundation for Promotion of Cancer Research (Tokyo, Japan to T. Teshima).

The authors have no conflicting financial interests.

Submitted: 2 August 2010
Accepted: 12 January 2011

REFERENCES

Antin, J.H., S.J. Lee, D. Neuberg, E. Alyea, R.J. Soiffer, S. Sonis, and J.L. Ferrara. 2002. A phase I/II double-blind, placebo-controlled study of recombinant human interleukin-11 for mucositis and acute GVHD prevention in allogeneic stem cell transplantation. *Bone Marrow Transplant.* 29:373–377. doi:10.1038/sj.bmt.1703394

Asakura, S., D. Hashimoto, S. Takashima, H. Sugiyama, Y. Maeda, K. Akashi, M. Tanimoto, and T. Teshima. 2010. Alloantigen expression on non-hematopoietic cells reduces graft-versus-leukemia effects in mice. *J. Clin. Invest.* 120:2370–2378. doi:10.1172/JCI39165

Barker, N., J.H. van Es, J. Kuipers, P. Kujala, M. van den Born, M. Cozijnsen, A. Haegebarth, J. Korving, H. Begthel, P.J. Peters, and H. Clevers. 2007. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature.* 449:1003–1007. doi:10.1038/nature06196

Barker, N., M. van de Wetering, and H. Clevers. 2008. The intestinal stem cell. *Genes Dev.* 22:1856–1864. doi:10.1101/gad.1674008

Batlle, E., J.T. Henderson, H. Beghtel, M.M. van den Born, E. Sancho, G. Huls, J. Meeldijk, J. Robertson, M. van de Wetering, T. Pawson, and H. Clevers. 2002. Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. *Cell.* 111:251–263. doi:10.1016/S0092-8674(02)01015-2

Bhanja, P., S. Saha, R. Kabarriti, L. Liu, N. Roy-Chowdhury, J. Roy-Chowdhury, R.S. Sellers, A.A. Alfieri, and C. Guha. 2009. Protective role of R-spondin1, an intestinal stem cell growth factor, against radiation-induced gastrointestinal syndrome in mice. *PLoS One.* 4:e8014. doi:10.1371/journal.pone.0008014

Binnerts, M.E., K.A. Kim, J.M. Bright, S.M. Patel, K. Tran, M. Zhou, J.M. Leung, Y. Liu, W.E. Lomas III, M. Dixon, et al. 2007. R-Spondin1 regulates Wnt signaling by inhibiting internalization of LRP6. *Proc. Natl. Acad. Sci. USA.* 104:14700–14705. doi:10.1073/pnas.0702305104

Blazar, B.R., D.J. Weisdorf, T. Defor, A. Goldman, T. Braun, S. Silver, and J.L. Ferrara. 2006. Phase 1/2 randomized, placebo-control trial of palifermin to prevent graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation (HSCT). *Blood.* 108:3216–3222. doi:10.1182/blood-2006-04-017780

Cooke, K.R., G.R. Hill, J.M. Crawford, D. Bungard, Y.S. Brinson, J. Delmonte Jr., and J.L. Ferrara. 1998. Tumor necrosis factor- alpha production to lipopolysaccharide stimulation by donor cells predicts the severity of experimental acute graft-versus-host disease. *J. Clin. Invest.* 102:1882–1891. doi:10.1172/JCI4285

Cooke, K.R., A. Gerbitz, J.M. Crawford, T. Teshima, G.R. Hill, A. Tesolin, D.P. Rossignol, and J.L. Ferrara. 2001. LPS antagonism reduces graft-versus-host disease and preserves graft-versus-leukemia activity after experimental bone marrow transplantation. *J. Clin. Invest.* 107:1581–1589. doi:10.1172/JCI12156

Ding, Y., S. Shen, A.C. Lino, M.A. Curotto de Lafaille, and J.J. Lafaille. 2008. Beta-catenin stabilization extends regulatory T cell survival and induces anergy in nonregulatory T cells. *Nat. Med.* 14:162–169. doi:10.1038/nm1707

Epstein, R.J., G.B. McDonald, G.E. Sale, H.M. Shulman, and E.D. Thomas. 1980. The diagnostic accuracy of the rectal biopsy in acute graft-versus-host disease: a prospective study of thirteen patients. *Gastroenterology.* 78:764–771.

Farrell, C.L., J.V. Bready, K.L. Rex, J.N. Chen, C.R. DiPalma, K.L. Whitcomb, S. Yin, D.C. Hill, B. Wiemann, C.O. Starnes, et al. 1998. Keratinocyte growth factor protects mice from chemotherapy and radiation-induced gastrointestinal injury and mortality. *Cancer Res.* 58:933–939.

Ferrara, J.L., K.R. Cooke, and T. Teshima. 2003. The pathophysiology of acute graft-versus-host disease. *Int. J. Hematol.* 78:181–187. doi:10.1007/BF02983793

Gattinoni, L., X.S. Zhong, D.C. Palmer, Y. Ji, C.S. Hinrichs, Z. Yu, C. Wrzesinski, A. Boni, L. Cassard, L.M. Garvin, et al. 2009. Wnt signaling arrests effector T cell differentiation and generates CD8+ memory stem cells. *Nat. Med.* 15:808–813. doi:10.1038/nm.1982

Hill, G.R., and J.L. Ferrara. 2000. The primacy of the gastrointestinal tract as a target organ of acute graft-versus-host disease: rationale for the use of cytokine shields in allogeneic bone marrow transplantation. *Blood.* 95:2754–2759.

Hill, G.R., J.M. Crawford, K.R. Cooke, Y.S. Brinson, L. Pan, and J.L. Ferrara. 1997. Total body irradiation and acute graft-versus-host disease: the role of gastrointestinal damage and inflammatory cytokines. *Blood.* 90:3204–3213.

Hill, G.R., K.R. Cooke, T. Teshima, J.M. Crawford, J.C. Keith Jr., Y.S. Brinson, D. Bungard, and J.L. Ferrara. 1998. Interleukin-11 promotes T cell polarization and prevents acute graft-versus-host disease after allogeneic bone marrow transplantation. *J. Clin. Invest.* 102:115–123. doi:10.1172/JCI3132

Kahl, C., B.E. Storer, B.M. Sandmaier, M. Mielcarek, M.B. Maris, K.G. Blume, D. Niederwieser, T.R. Chauncey, S.J. Forman, E. Agura, et al. 2007. Relapse risk in patients with malignant diseases given allogeneic hematopoietic cell transplantation after nonmyeloablative conditioning. *Blood.* 110:2744–2748. doi:10.1182/blood-2007-03-078592

Kim, K.A., M. Kakitani, J. Zhao, T. Oshima, T. Tang, M. Binnerts, Y. Liu, B. Boyle, E. Park, P. Emtage, et al. 2005. Mitogenic influence of human R-spondin1 on the intestinal epithelium. *Science.* 309:1256–1259. doi:10.1126/science.1112521

Kim, K.A., J. Zhao, S. Andarmani, M. Kakitani, T. Oshima, M.E. Binnerts, A. Abo, K. Tomizuka, and W.D. Funk. 2006. R-Spondin proteins: a novel link to beta-catenin activation. *Cell Cycle.* 5:23–26. doi:10.4161/cc.5.1.2305

Krijanovski, O.I., G.R. Hill, K.R. Cooke, T. Teshima, J.M. Crawford, Y.S. Brinson, and J.L. Ferrara. 1999. Keratinocyte growth factor separates graft-versus-leukemia effects from graft-versus-host disease. *Blood.* 94:825–831.

Mori, T., T. Nishimura, Y. Ikeda, T. Hotta, H. Yagita, and K. Ando. 1998. Involvement of Fas-mediated apoptosis in the hematopoietic progenitor cells of graft-versus-host reaction-associated myelosuppression. *Blood.* 92:101–107.

Mowat, M., and G. Socie. 2004. Intestinal Graft-vs.-Host Disease. *In* Graft-vs.-Host Disease. Third edition. J.L. Ferrara, K.R. Cooke, and H.J. Deeg, editors. Marcel Dekker, New York. 279–327.

Nestel, F.P., K.S. Price, T.A. Seemayer, and W.S. Lapp. 1992. Macrophage priming and lipopolysaccharide-triggered release of tumor necrosis factor alpha during graft-versus-host disease. *J. Exp. Med.* 175:405–413. doi:10.1084/jem.175.2.405

Panoskaltsis-Mortari, A., D.L. Lacey, D.A. Vallera, and B.R. Blazar. 1998. Keratinocyte growth factor administered before conditioning ameliorates graft-versus-host disease after allogeneic bone marrow transplantation in mice. *Blood.* 92:3960–3967.

Pinto, D., A. Gregorieff, H. Begthel, and H. Clevers. 2003. Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. *Genes Dev.* 17:1709–1713. doi:10.1101/gad.267103

Reya, T., and H. Clevers. 2005. Wnt signalling in stem cells and cancer. *Nature.* 434:843–850. doi:10.1038/nature03319

Román-Gómez, J., L. Cordeu, X. Agirre, A. Jiménez-Velasco, E. San José-Eneriz, L. Garate, M.J. Calasanz, A. Heiniger, A. Torres, and F. Prosper. 2007. Epigenetic regulation of Wnt-signaling pathway in acute lymphoblastic leukemia. *Blood.* 109:3462–3469. doi:10.1182/blood-2006-09-047043

Sale, G.E., H.M. Shulman, G.B. McDonald, and E.D. Thomas. 1979. Gastrointestinal graft-versus-host disease in man. A clinicopathologic study of the rectal biopsy. *Am. J. Surg. Pathol.* 3:291–299. doi:10.1097/00000478-197908000-00001

Sato, T., R.G. Vries, H.J. Snippert, M. van de Wetering, N. Barker, D.E. Stange, J.H. van Es, A. Abo, P. Kujala, P.J. Peters, and H. Clevers. 2009. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature.* 459:262–265. doi:10.1038/nature07935

Teshima, T., G.R. Hill, L. Pan, Y.S. Brinson, M.R. van den Brink, K.R. Cooke, and J.L. Ferrara. 1999. IL-11 separates graft-versus-leukemia effects from graft-versus-host disease after bone marrow transplantation. *J. Clin. Invest.* 104:317–325. doi:10.1172/JCI7111

Teshima, T., R. Ordemann, P. Reddy, S. Gagin, C. Liu, K.R. Cooke, and J.L. Ferrara. 2002a. Acute graft-versus-host disease does not require alloantigen expression on host epithelium. *Nat. Med.* 8:575–581. doi:10.1038/nm0602-575

Teshima, T., P. Reddy, K.P. Lowler, M.A. KuKuruga, C. Liu, K.R. Cooke, and J.L. Ferrara. 2002b. Flt3 ligand therapy for recipients of allogeneic bone marrow transplants expands host CD8 alpha(+) dendritic cells and reduces experimental acute graft-versus-host disease. *Blood.* 99:1825–1832. doi:10.1182/blood.V99.5.1825

van der Flier, L.G., A. Haegebarth, D.E. Stange, M. van de Wetering, and H. Clevers. 2009a. OLFM4 is a robust marker for stem cells in human intestine and marks a subset of colorectal cancer cells. *Gastroenterology.* 137:15–17. doi:10.1053/j.gastro.2009.05.035

van der Flier, L.G., M.E. van Gijn, P. Hatzis, P. Kujala, A. Haegebarth, D.E. Stange, H. Begthel, M. van den Born, V. Guryev, I. Oving, et al. 2009b. Transcription factor achaete scute-like 2 controls intestinal stem cell fate. *Cell.* 136:903–912. doi:10.1016/j.cell.2009.01.031

Via, C.S., S.O. Sharrow, and G.M. Shearer. 1987. Role of cytotoxic T lymphocytes in the prevention of lupus-like disease occurring in a murine model of graft-vs-host disease. *J. Immunol.* 139:1840–1849.

Zhao, J., J. de Vera, S. Narushima, E.X. Beck, S. Palencia, P. Shinkawa, K.A. Kim, Y. Liu, M.D. Levy, D.J. Berg, et al. 2007. R-spondin1, a novel intestinotrophic mitogen, ameliorates experimental colitis in mice. *Gastroenterology.* 132:1331–1343. doi:10.1053/j.gastro.2007.02.001

Zhao, J., K.A. Kim, J. De Vera, S. Palencia, M. Wagle, and A. Abo. 2009. R-Spondin1 protects mice from chemotherapy or radiation-induced oral mucositis through the canonical Wnt/beta-catenin pathway. *Proc. Natl. Acad. Sci. USA.* 106:2331–2336. doi:10.1073/pnas.0805159106