

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

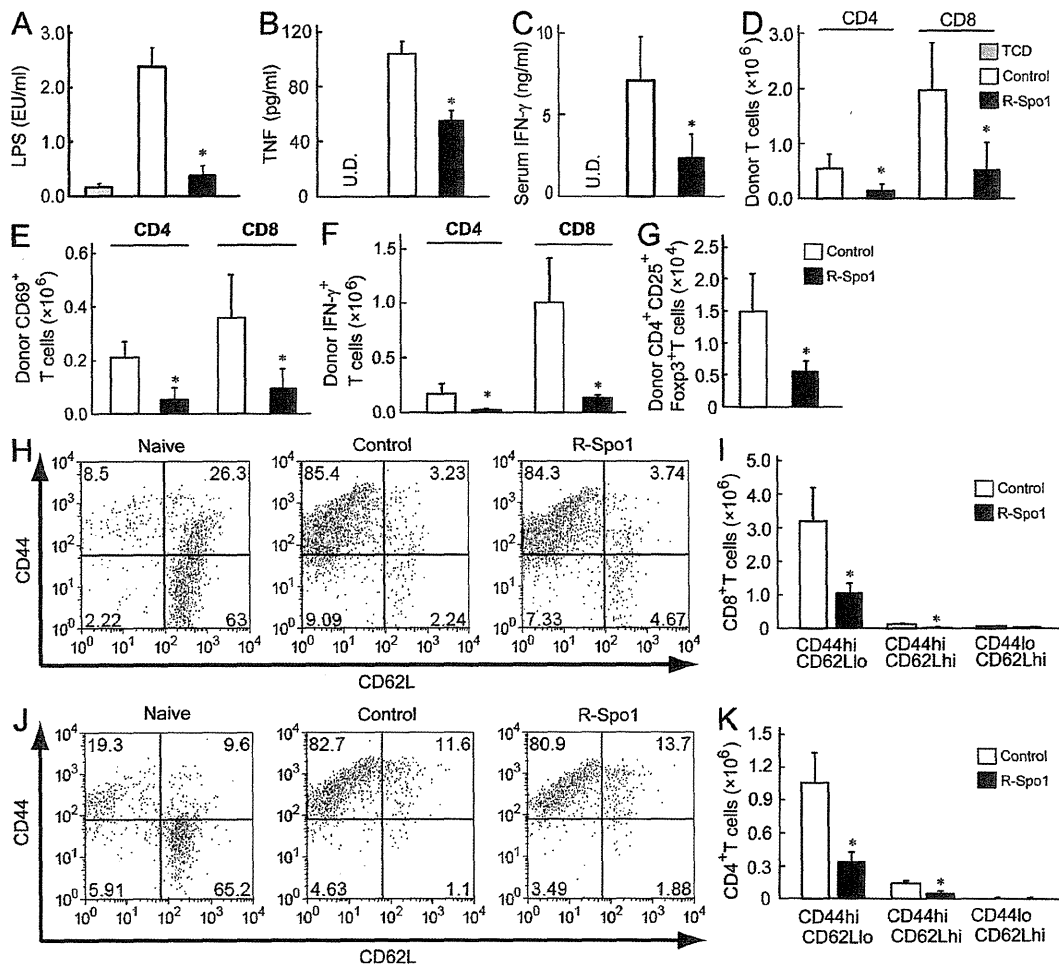


Figure 3. R-Spo1 regulated activation of inflammatory and cellular effectors in GVHD. Lethally irradiated B6D2F1 mice were transplanted with 5×10^6 TCD BM cells with or without 2×10^6 T cells from B6-Ly5.1 (CD45.1⁺) donors on day 0. R-Spo1 (200 μg/day) or control was intravenously injected from day -3 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 μ g/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 \pm 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 harvested 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 ($99.9 \pm 0.1\%$), 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 ($99.7 \pm 0.4\%$ in controls and $99.9 \pm 0.0\%$ 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). ISC's 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

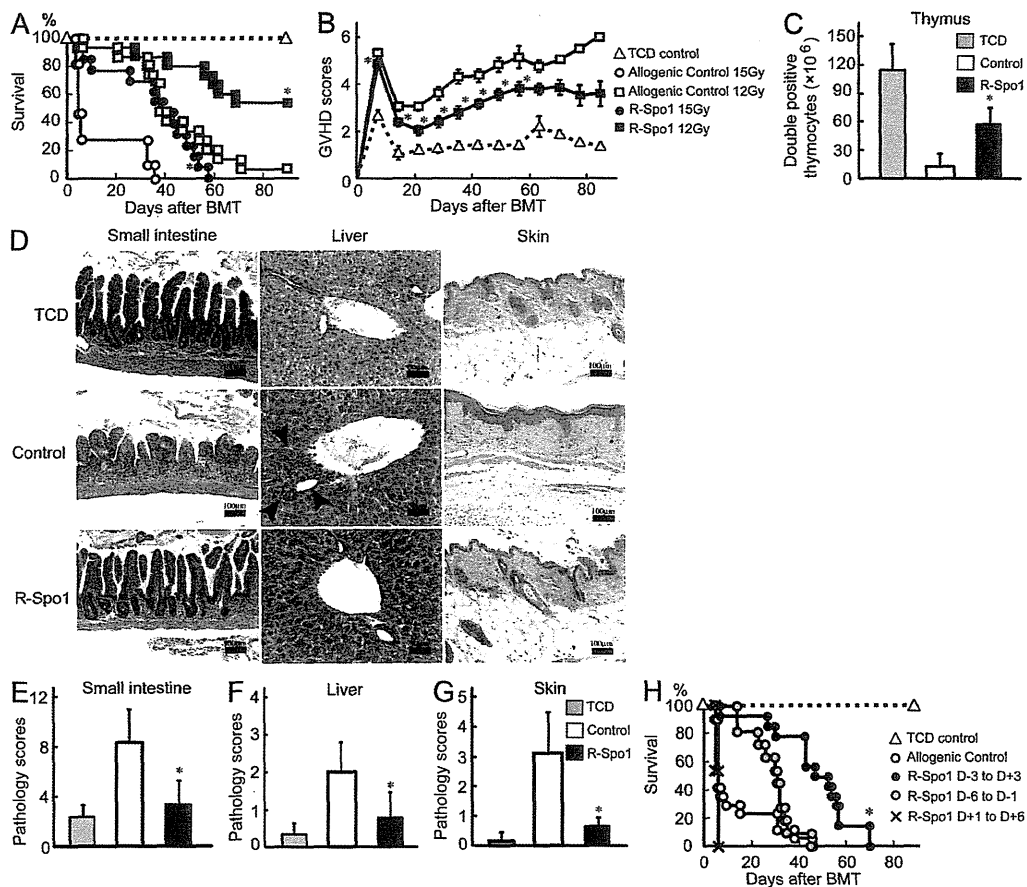


Figure 4. R-Spo1 modulated systemic GVHD. B6D2F1 mice were transplanted with 5×10^6 TCD BM cells with or without 2×10^6 T cells from B6 donors on day 0 after 15 or 12 Gy TBI. R-Spo1 (200 μ g/day) or control was injected from day -3 to -1 and day 1-3 after BMT. (A and B) Survival (A) and clinical GVHD scores (B; means \pm 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 \pm 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 μ m; (middle) 50 μ m. (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 \pm 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

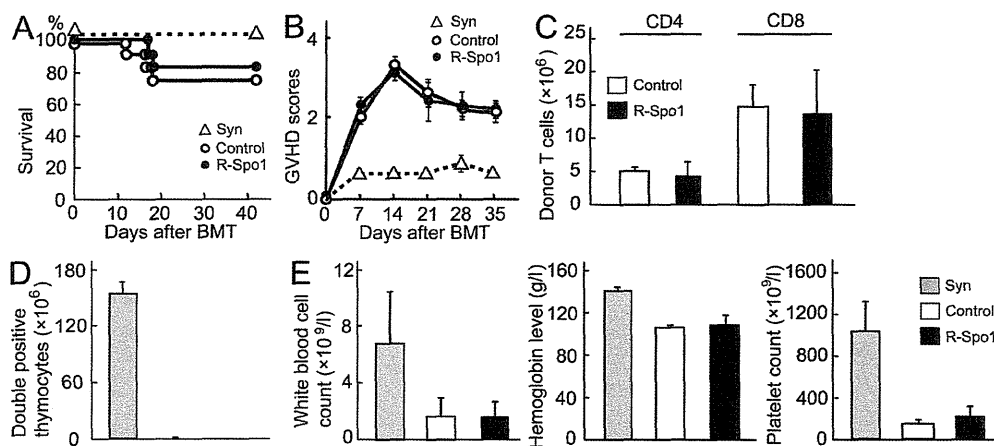


Figure 5. R-Spo1 failed to attenuate GVHD in unirradiated host. Unirradiated B6D2F1 mice were transplanted with 12×10^7 splenocytes from syngeneic or allogeneic B6 donors. R-Spo1 (200 μ g/day) or control was administered from day -3 to -1 and day 1–3 after BMT. (A and B) Survival (A) and clinical GVHD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

Panoskaltis-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-2^b, CD45.2⁺) and B6D2F1 (H-2^{b/d}, CD45.2⁺) mice were purchased from Charles River, and B6-Ly5.1 (H-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/DDJB 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 μ m. 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 μ g/ml plate-bound anti-CD3 mAbs and 2 μ g/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 amoebocyte 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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REFERENCES

- Antin, J.H., S.J. Lee, D. Neuberg, E. Alyea, R.J. Soiffer, S. Somis, 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. Haegbarth, 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
- Battle, E., J.T. Henderson, H. Begthel, 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- α 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 energy 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
- Panoskaltis-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
- Salé, 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:10.1097/0000478-197908000-00001
- 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. Gagrin, 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

Original article

Analysis of immune reconstitution after autologous CD34⁺ stem/progenitor cell transplantation for systemic sclerosis: predominant reconstitution of Th1 CD4⁺ T cells**Hiroshi Tsukamoto¹, Koji Nagafuji¹, Takahiko Horiuchi¹, Hiroki Mitoma¹, Hiroaki Niiro¹, Yojiro Arinobu², Yasushi Inoue¹, Kentaro To¹, Toshihiro Miyamoto¹, Hiromi Iwasaki², Takanori Teshima², Mine Harada^{1,3} and Koichi Akashi¹****Abstract**

Objective. The aim of this study is to evaluate the mechanism of long-term effect of autologous haematopoietic stem cell transplantation (ASCT) in treatment of SSc.

Methods. Eleven patients (three males and eight females) with SSc were enrolled. Blood mononuclear cells were harvested after mobilization treatment with CYC and G-CSF. CD34⁺ haematopoietic stem/progenitor cell fractions were purified and cryopreserved. Patients were transplanted with $>2 \times 10^6$ /kg autologous CD34⁺ cells after high-dose CYC (50 mg/kg for 4 days) conditioning. Immune reconstitution was evaluated serially by analysing lymphocyte subpopulations for 36 months.

Results. Progressive improvement of skin sclerosis has been observed for 3 years in most of the patients. The serum level of anti-Scl-70, an auto-antibody specific to SSc, was progressively decreased after ASCT. Improvement of skin sclerosis was significantly associated with the change in the serum anti-Scl-70 level after ASCT at 36 months. Serum levels of KL-6 and surfactant protein D, indicators for interstitial pneumonia activity, were also significantly decreased. The number of CD8⁺ T cells immediately recovered within a month after ASCT, while the number of CD4⁺ T cells remained low for >36 months post-transplant. The majority of CD4⁺ cells were memory but not naïve T cells, and regulatory CD4⁺ T cells were not recovered. Th1/Th2 ratio was significantly increased after ASCT.

Conclusions. ASCT with purified CD34⁺ cells was effective in controlling the disease activity of SSc. Th1/Th2 ratio was significantly increased for at least 3 years after ASCT.

Key words: Autoimmune disease, High-dose cyclophosphamide, Transplantation, Immune reconstitution, Th1/Th2 balance.

Introduction

SSc is a heterogeneous autoimmune disease (AD) characterized by predominant T-cell activation, production

of auto-antibodies and cytokine release [1, 2]. All of these contribute to diffuse microvascular injury, leading to diffuse sclerosis within the skin and internal organs. DcSSc and internal organ involvement often cause life-threatening status of patients [3].

Autologous haematopoietic stem cell transplantation (ASCT) was introduced for SSc treatment in 1996, and >100 patients with SSc have been treated [4]. Phase I–II studies demonstrated that improvement of skin sclerosis and stabilization of interstitial pneumonia (IP) were achieved [5–10]. We also reported the safety and efficacy of high-dose CYC with ASCT for SSc patients [11]. The complete or partial remission of SSc was maintained for at least 3 years in these reports [6–9].

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The working hypotheses of the efficacy of ASCT for SSc might be due to the immune reset that should include: (i) the eradication of auto-reactive lymphocytes by immunoablative conditioning; and/or (ii) the correction of dysregulated immune balance by newly developed lymphocytes derived from transplanted haematopoietic stem cells [12]. However, there remains controversy regarding whether such immune reset occurs in patients treated with ASCT. Muraro *et al.* [13] have reported that naïve CD4⁺ T cells with diverse T-cell receptor repertoire, which were generated through thymus, developed after ASCT in patients with multiple sclerosis (MS). In contrast, in SSc patients, Farge *et al.* [14] reported the sustained reduction of CD4⁺ T cells and B cells after ASCT.

Furthermore, Th1/Th2 balance might also be important in understanding the disease status of SSc. In a tight skin mouse, an animal model for SSc, the stimulation of Th1 immune responses prevents the development of scleroderma-like syndrome [15]. In human SSc, a shift from Th2 to Th1 responses is correlated with improvement of the skin fibrosis by longitudinal analysis of serum cytokine concentrations [16].

These data led us to analyse immune reconstitution in SSc patients treated with ASCT. In this study, we tracked reconstitution of T- and B-cell subpopulations after ASCT with purified CD34⁺ haematopoietic stem/progenitor cells in SSc patients, and found that despite the resolution of clinical symptoms of SSc, patients did not achieve normalization of lymphocyte compartment, even 3 years after ASCT. All patients showed sustained reduction of CD4 T cells, but Th1/Th2 ratio was increased during the 3-year observation. Thus, the clinical efficacy of ASCT might be dependent upon the skewed reconstitution of Th1 cells for a long time after ASCT.

Patients and methods

The study was approved by the ethics committee of Kyushu University Hospital. Written informed consent was obtained from all patients according to the Declaration of Helsinki.

Patients and eligibility

Eligibility of patients with SSc for ASCT was previously described [11]. Briefly, patients with SSc were eligible when they had severe diffuse SSc that had rapidly developed over the previous 4 years. They also had to have at least one of the following organ involvements: (i) pulmonary, (ii) cardiac or (iii) renal involvement. Patients with limited SSc were considered eligible when progressive and life-threatening IP was present. Patients were excluded from the study when they had uncontrolled arrhythmia, severe heart failure, pulmonary hypertension, DL_{CO} <20% of predicted and renal failure as previously described [11]. All patients were followed up for at least 36 months after transplantation for the evaluation of immune reconstitution. Blood was obtained from healthy donors (after informed consent, *n* = 10) to determine the reference values of lymphocyte subpopulations and Th1/Th2

balance, and from SSc patients (after informed consent, *n* = 13) for FoxP3 staining.

Peripheral blood stem cell collection and ASCT

Peripheral blood stem cells (PBSCs) were mobilized during haematologic recovery after relatively high-dose CYC (2 g/m²) for 2 days followed by administration of recombinant human G-CSF (filgrastim; Kirin Brewery, Tokyo, Japan) as previously described [11]. After collecting PBSCs to obtain 2 × 10⁶ CD34⁺ cells/kg or more by apheresis, CD34⁺ cells were positively selected using an immunomagnetic bead with an anti-CD34 mAb (CliniMACS; Miltenyi Biotec, Germany). For pre-transplant conditioning, high-dose CYC (50 mg/kg) was given for 4 days from Day -5 to -2 and frozen-thawed CD34⁺ cells were transplanted on Day 0. Patients 1, 2, 3, 4 and 6 received G-CSF from Day 1 [11]. Concomitantly administered prednisolone doses were kept unchanged or gradually tapered according to the individual clinical status after ASCT. The concomitantly administered immunosuppressants were stopped before the administration of CYC for mobilization.

Treatment outcome

The modified Rodnan skin score (mRSS) was used to evaluate the improvement of skin sclerosis when initial mRSS was ≥15 [11]. Serum levels of anti-Scl 70 (anti-topo I) antibody were measured by an ELISA kit (MBL, Nagoya, Japan). Serum levels of KL-6 and surfactant protein D (SP-D) were measured by ELISA kits (Sanko Junyaku Co., Tokyo and Yamasa Co., Choshi, respectively, Japan). Serum levels of immunoglobulin were measured by laser nephelometry.

Lymphocyte phenotyping

Lymphocyte immunophenotyping of samples from whole blood was performed by IF flow cytometry before mobilization, before haematopoietic stem cell transplantation (HSCT) and 1, 3, 6, 12, 18, 24, 36 months after HSCT. The following mAbs and their combinations were used: anti-CD3-FITC; anti-CD19-phycoerythrin (PE); anti-CD20-FITC; anti-CD4-FITC; anti-CD8-PE; anti-CD45RA-PE; anti-CD45RO-PE; anti-CD25-PE; anti-CD16-FITC; anti-CD56-PE, anti-CD69-FITC and anti-CD27-FITC (eBioscience, San Diego, CA, USA). The CD4⁺FoxP3⁺ cells were detected by using anti-CD4-FITC, anti-FoxP3-PE and permeabilization buffer (eBioscience) in 9 patients at 37.8 (18.0) months (one sample for each patient) after ASCT and 13 SSc controls without ASCT. Results were expressed as the absolute number of cells.

Th1/Th2 balance

Flow cytometric determination of IFN-γ and IL-4 in the cytoplasm of peripheral CD4⁺ T cells was performed by a previously described method [17]. Briefly, aliquots (500 μl) of heparinized whole blood were stimulated with a combination of 25 ng/ml phorbol myristate acetate (PMA) and 1 μg/ml ionomycin in the presence of brefeldin A (Sigma, St Louis, MO, USA) and cultured for 4 h at 37°C

in a humidified incubator containing 7% CO₂. Activated cultures were aliquoted and stained with 20 µl of peridinin chlorophyll protein-conjugated CD4-specific mAb (Becton Dickinson, San Jose, CA, USA) for 15 min at room temperature, and then treated with 2 ml of FACS lysing solution (Becton Dickinson). After a short incubation (5 min), the samples were centrifuged and combined with FACS permeabilizing solution (Becton Dickinson) for 10 min at room temperature in the dark. The sample tubes were washed twice and FITC-conjugated IFN-γ-specific mAb and PE-conjugated IL-4-specific mAb (Becton Dickinson) for 30 min at room temperature in the dark. FITC-conjugated mouse IgG2a and PE-conjugated mouse IgG1 were used as controls. After washing again, the cells were resuspended in 1% paraformaldehyde and analysed by flow cytometry. The percentage of IFN-γ or IL-4-positive cells (percentage of IFN-γ or percentage of IL-4) was counted by FACS, and Th1/Th2 balance was evaluated by a ratio of percentage of IFN-γ to percentage of IL-4 (IFN-γ/IL-4).

Cytokine and chemokine levels

Serum levels of TNF-α and TGF-β were measured with sandwich ELISA kits (Quantikine; R&D systems, Minneapolis, MN, USA), according to the manufacturer's instructions before and after ASCT. Serum levels of IL-6 and soluble receptor for IL-2 (sIL-2R) were measured with CLISA kit (Fujirebio, Tokyo, Japan) and with ELISA kit (Kyowamedix, Tokyo, Japan), respectively.

Statistical analysis

Values were expressed as mean (s.d.). Student's *t*-test was used for statistical analysis of the data. The correlations of mRSS with the level of anti-Sci-70 or Th1/Th2 balance were investigated by Pearson's correlation coefficient test. Difference with *P* < 0.05 was considered to be statistically significant.

Results

Autologous transplantation with purified CD34⁺ mobilized blood stem cells was successfully performed without treatment-related mortality

Eleven patients, eight females and three males, were enrolled in this study. Clinical results of Patients 1–6 at 12 months after ASCT were previously reported [11]. The mean (s.d.) of mRSS was 21.5 (9.0), and all patients developed clinical IP with decreased per cent vital capacity (VC) and per cent DL_{CO} [mean (s.d.), 64.9 (14.8) and 45.8 (18.0)%, respectively] (Table 1). The anti-Sci-70 antibody, an auto-antibody specific to diffuse SSc, was detected in 9 out of 11 patients. All patients were resistant to the standard treatment with CS, CYC and/or ciclosporin. Blood mononuclear cells were harvested after mobilization with CYC and G-CSF. CD34⁺ haematopoietic stem/progenitor cell fractions were purified and cryopreserved. Patients were transplanted with purified autologous CD34⁺ cells after high-dose CYC (500 mg/kg) conditioning. Consequentially, 4.7 × 10⁶/kg CD34⁺ cells with 1.1 (1.2) × 10⁴/kg CD3⁺ T-cell contaminants were transplanted. ASCT was successfully performed without transplantation-related mortality in all patients, but Patient 7 died due to the progression of IP at 20 months after ASCT. The clinical status of the remaining 10 patients was followed up for >36 months. There was a variety of post-transplant infections such as adenoviral haemorrhagic cystitis, herpes zoster and cytomegaloviral antigenaemia [18].

CD34⁺ ASCT treatment induced resolution of refractory SSc

The effect of ASCT on skin sclerosis was assessed by the change in mRSS. After ASCT, skin sclerosis was progressively resolved, and had improved by 72.0% at 36 months post-transplant (Fig. 1A). The vital capacity of the lung was increased from 64.9 to 77.8% at 36 months, although

TABLE 1 Patient profile and number of reinfused cells

Patient number	Sex	Age, years	mRSS	IP	%VC/DL _{CO}	Anti-Sci-70	Prior therapy	Follow-up, month	Number of reinfused CD34 ⁺ cells (× 10 ⁶ /kg)	Number of reinfused CD3 ⁺ cells (× 10 ⁴ /kg)
1	F	54	16	+	58/51	+	St, CYC	88	8.4	0.33
2	M	55	15	+	65/47	+	St	78	4.9	0.27
3	M	58	31	+	63/44	–	St	77	2.2	2.95
4	F	54	26	+	73/60	+	St	75	2.1	1.71
5	F	53	28	+	74/29	+	St	72	7.2	3.0
6	F	48	32	+	77/25	+	St, CYC, CsA	68	4.0	2.35
7	F	34	8	+	39/ND	–	–	20	9.1	1.03
8	F	61	26	+	90/87	+	St	52	3.0	0.32
9	F	61	32	+	56/37	+	St, CsA	47	4.2	0.13
10	F	44	15	+	45/35	+	St, CY	43	4.9	0.0
11	M	53	7	+	74/44	+	St	39	1.3	0.26
Ave. (s.d.)		52.3 (7.5)	21.5 (9.0)		64.9 (14.8)/45.8 (18.0)			60.0 (21.0)	4.7 (2.6)	1.1 (1.2)

F: female; M: male; Ave.: Average; ND: not done.

DL_{CO} was unchanged (Fig. 1B). Serum levels of KL-6 and SP-D, indicators of IP activity [19, 20], were significantly decreased (Fig. 1C and D).

Reflecting the resolution of clinical symptoms of SSc, the serum level of anti-Scl-70 progressively decreased after ASCT (Fig. 2A), independent of serum immunoglobulin levels (Fig. 2B). A significant correlation ($r=0.52$, $P<0.05$) was observed between the change in mRSS and that in the serum level of anti-Scl-70 at 36 months after ASCT.

Serum levels of pro-inflammatory or pro-fibrotic cytokines and a T-cell activation marker such as TNF- α , TGF- β , IL-6 and soluble IL-2R (sIL-2R), were also decreased after ASCT (Fig. 3A-D). These data show that ASCT with purified CD34⁺ cells is effective in controlling disease activity of SSc patients refractory to conventional immunosuppressive therapies.

The recovery of CD4⁺ T cells was particularly retarded after ASCT with purified CD34⁺ cells in SSc patients

Because the patients were infused with highly purified CD34⁺ haematopoietic stem/progenitor cells containing very low numbers of lymphocytes, we tracked the immune reconstitution after ASCT by analysing lymphocyte subpopulations for 36 months.

As shown in Fig. 4A, although the absolute number of CD8⁺ T cells returned to the normal level only a month after ASCT, the recovery of CD4⁺ T cells was apparently retarded; at 36 months post-transplant, the number of

CD4⁺ cells remained below the normal range, resulting in continuous inversion of the CD4/CD8 ratio. Previous reports have shown that the long-term remission after ASCT in MS and SLE was associated with the increased number of thymus-derived naïve CD4⁺ T cells [13, 21]. Therefore, we analysed functional subsets of CD4⁺ T cells. As shown in Fig. 4B, the number of memory CD4⁺CD45RO⁺ T cells was significantly decreased for 1–3 months after CD34⁺ ASCT, but returned to the baseline level 24 months after ASCT, although it did not reach the normal range. In contrast, naïve CD4⁺CD45RA⁺ T cells were significantly decreased after mobilization, and remained at a low level at 36 months post-ASCT. These data show that most recovered CD4⁺ T cells were memory cells but not naïve T cells. The CD4⁺CD25⁺ T cell fraction that includes activated and regulatory T cells [22] disappeared after ASCT, and remained low in number even at 36 months post-ASCT (Fig. 4C). The number of CD4⁺FoxP3⁺ cells in 9 patients at 37.8 (18.0) months after ASCT was significantly lower than that of 13 SSc controls [8.06 (7.61) vs 27.23 (13.30)/ μ l, $P<0.05$]. These results show that although the patients displayed resolution of clinical SSc after ASCT, naïve CD45RA⁺ T cells or regulatory T cells did not reconstitute well after ASCT.

SSc patients have distinct abnormalities of B-cell subpopulations, characterized by expanded naïve B cells and activated but diminished memory B cells [23]. The number of CD19⁺ B cells returned into the normal range 18 months after ASCT.

Fig. 1 Resolution of SSc after ASCT. **(A)** mRSS. The proportional change from baseline measurement was calculated for each time point. Patients with an initial mRSS of ≥ 15 were included. **(B)** VC and DL_{CO}. **(C)** KL-6. **(D)** SP-D. Data are presented as mean (s.d.). The x-axis is not drawn to scale. The data obtained before mobilization and just before transplantation (HSCT) are shown as BM and BT, respectively. * $P<0.05$ vs BM. (C and D) Dashed line shows upper normal limit.

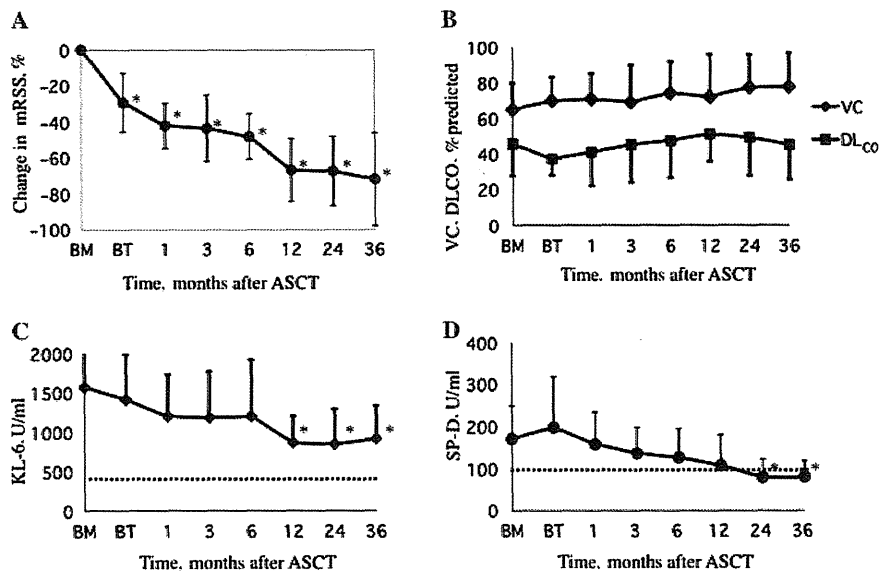


Fig. 2 Evaluation of autoantibody and immunoglobulin. (A) Change in the titre of anti-Scl-70. (B) Change in the serum levels of IgG, IgA and IgM. Data are presented as mean (s.d.). The x-axis is not drawn to scale. The data obtained before mobilization and just before transplantation (HSCT) are shown as BM and BT, respectively. **P* < 0.05 vs BM.

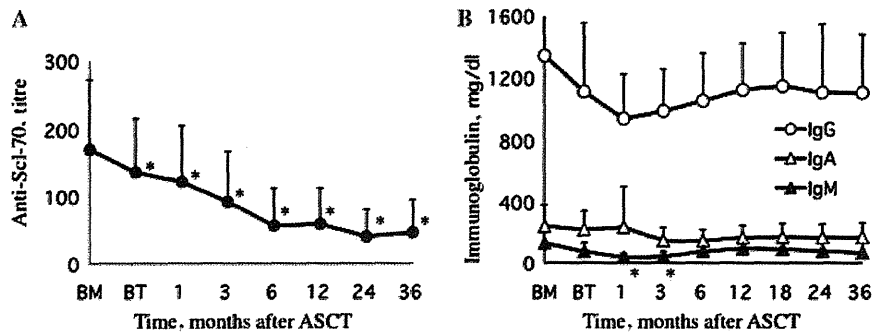
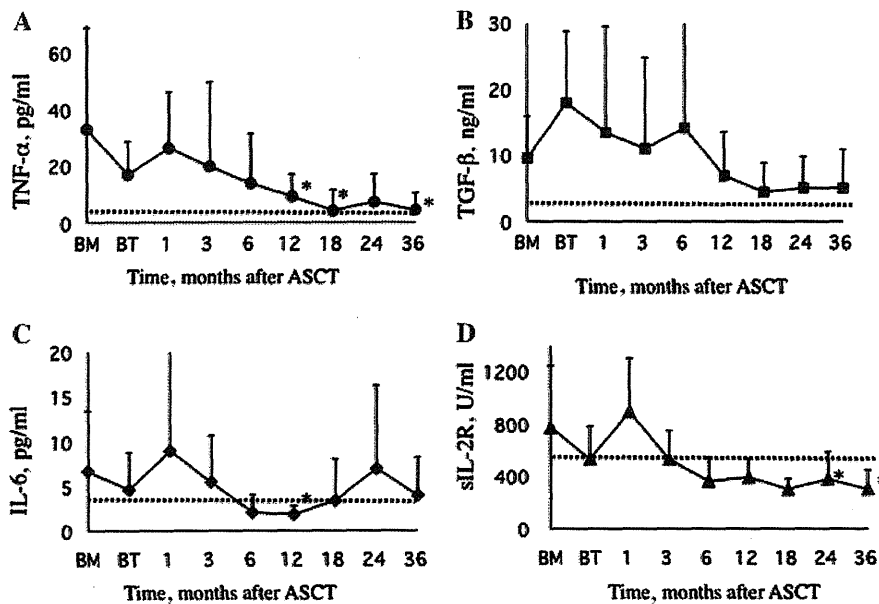


Fig. 3 Kinetics of immunological markers in SSc patients who received ASCT. Data are mean (s.d.). The x-axis is not drawn to scale. (A) TNF- α , (B) TGF- β , (C) IL-6, (D) sIL-2R. The data obtained before mobilization and just before transplantation (HSCT) are shown as BM and BT, respectively. **P* < 0.05 vs BM. Dashed line shows upper normal limit.



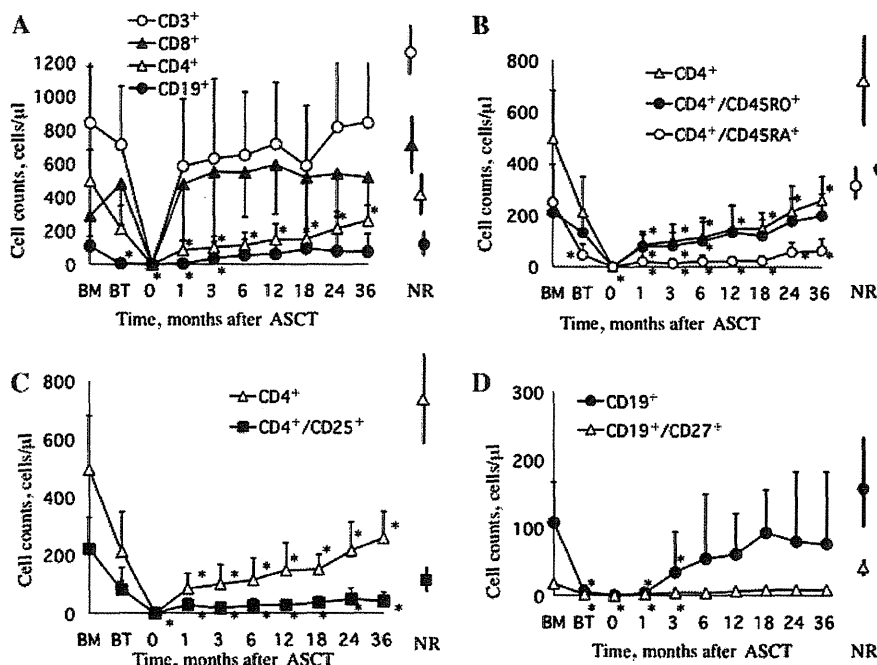
To study whether correction of abnormalities in functional subsets of B cells was associated with durable remission, we focused on the functional subset of CD19⁺ B cells. The number of CD19⁺CD27⁺ memory B cells was low at baseline, which was characteristic of SSc [23]. An increase in the number of memory B cells, however, was not observed even at 36 months after ASCT, and the vast majority of recovered B cells were naive CD19⁺CD27⁻ B cells (Fig. 4D).

Th1 CD4⁺ T cells were predominantly reconstituted after CD34⁺ ASCT

Previous data showed that Th1/Th2 balance was associated with skin sclerosis in an animal model of SSc

[15]. Therefore, we analysed Th1/Th2 balance after ASCT by measuring the ratio of intracellular IFN- γ ⁺/IL-4⁻/CD4⁺ to IFN- γ ⁻/IL-4⁺/CD4⁺ T cells (Fig. 5). In Fig. 5A, the results of Case 9 before mobilization, 3 and 24 months after HSCT were shown as representative data. IFN- γ ⁺/IL-4⁻/CD4⁺ and IFN- γ ⁻/IL-4⁺/CD4⁺ T cells were considered to be Th1 and Th2 cells, respectively. In this case, elimination of IL-4⁺/CD4⁺ (Th2) T cells as well as predominant reconstitution of IFN- γ ⁺/CD4⁺ (Th1) T cells were observed after ASCT. The ratio of IFN- γ ⁺ to IL-4⁺ CD4⁺ T cells was increased from 4.3 before mobilization to 32.8 at 3 months and 197.3 at 24 months after HSCT. When Th1/Th2 balance was analysed in all SSc patients, the ratio of IFN- γ ⁺ to IL-4⁺/CD4⁺ T cells was significantly

Fig. 4 Immune reconstitution after ASCT. Change in absolute cell counts of immune cells. **(A)** CD3⁺, CD4⁺, CD8⁺, CD19⁺ cells. **(B)** CD4⁺, CD4⁺CD45RO⁺, CD4⁺CD45RA⁺ cells. **(C)** CD4⁺, CD4⁺CD25⁺ cells. **(D)** CD19⁺, CD19⁺CD27⁺ cells. Data are presented as mean (s.d.). The x-axis is not drawn to scale. The data obtained before mobilization and just before transplantation (HSCT) are shown as BM and BT, respectively. **P* < 0.05 vs BM. Normal ranges (NR: 95% CI) are shown as right-sided vertical bars.



increased at 1 month and reached a plateau at 6 months after ASCT (Fig. 5B). The skewed reconstitution of Th1 CD4⁺ T cells was maintained for 36 months after HSCT. There were no significant correlations between the changes in mRSS and those in Th1/Th2 balance.

Discussion

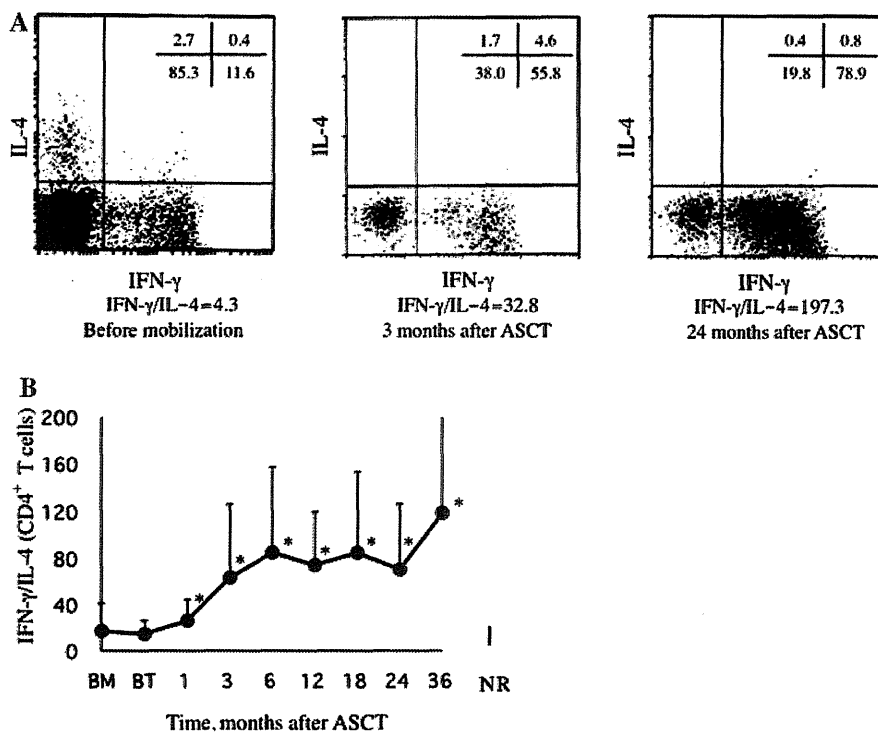
In this study, the resolution of disease was progressively obtained in SSc patients for 36 months after ASCT. This durable effect was not due to the reconstitution of naïve CD4⁺ T cells, regulatory T cells or the correction of B-cell imbalance. On the other hand, the elimination of Th2 cells by high-dose CYC as well as the predominant reconstitution of Th1 cells were observed after ASCT. Reflecting the resolution of clinical symptoms of SSc, serum levels of anti-Scl-70 progressively decreased after ASCT. Serum levels of KL-6 and SP-D, indicators for IP activity, were also significantly decreased.

In patients with SSc, production levels of type 2 cytokines such as IL-4, IL-6 and IL-13 by stimulated peripheral blood mononuclear cells and cultured CD4⁺ T cells decreased [24, 25]. Our data showed that the ratio of IFN- γ - to IL-4-producing CD4⁺ T cells was significantly increased in a month and was sustained for 36 months after ASCT. The predominant reconstitution of IFN- γ -producing cells is associated with amelioration of skin

sclerosis, probably due to an ability of IFN- γ to reduce excessive collagen synthesis by scleroderma-derived fibroblasts [26]. IL-4 increases collagen production of fibroblasts and induces the production of TGF- β in patients with SSc [27]. Therefore, the elimination of IL-4-producing T cells provides a favourable effect on SSc. The limitation of this study was small sample size and that there were not significant correlations between the changes in mRSS and those in Th1/Th2 balance. It is unclear how predominant reconstitution of Th1 CD4⁺ T cells after ASCT is induced. Polarization of CD4⁺ T cells after ASCT may depend on the local levels of cytokines such as IL-12 or IL-4 when naïve CD4⁺ T cells develop into functional T cells [28]. Predominant reconstitution of Th1 CD4⁺ T cells after ASCT may also occur in patients with other autoimmune diseases when treated by ASCT. Therefore, it is conceivable that ASCT is potentially effective for Th2-related diseases such as SSc and SLE, while its effect on Th1-related diseases such as RA is limited [4]. Macrophage activation syndrome is often observed in patients with JIA after ASCT [29]. It may be associated with a Th1 immune response after ASCT.

Our data showed that despite the resolution of clinical symptoms of SSc, patients did not achieve normalization of lymphocyte compartment, even 3 years after ASCT. The recovery of CD4⁺ T cell was delayed until 36 months after ASCT. Muraro *et al.* [13] reported that

Fig. 5 Evaluation of Th1/Th2 balance after ASCT. **(A)** Representative expression of IFN- γ and IL-4 in the cytoplasm of CD4⁺ T cells (Case 9) before mobilization, 3 months and 24 months after ASCT. IFN- γ /IL-4 was defined as the ratio of IFN- γ ⁺/IL-4⁻ to IFN- γ ⁻/IL-4⁺. Number of analysed cells was decreased after ASCT since ratio of CD4⁺ T cells in gated cells was decreased after ASCT. **(B)** Change in the ratio of intracellular IFN- γ ⁺ to IL-4⁺ CD4⁺ T cells after ASCT in patients with SSc. Data are presented as mean (s.d.). The x-axis is not drawn to scale. The data obtained before mobilization and just before transplantation (HSCT) are shown as BM and BT, respectively. * $P < 0.05$ vs BM. Normal range (NR: 95% CI) is shown as right-sided vertical bar.



naïve CD45RA⁺ T cells with diverse TCR repertoire and of thymic origin, were increased after ASCT in patients with MS, and that the increase of such cells was associated with long-term suppression of inflammatory activity of MS. In contrast, the present study revealed that the recovery of naïve CD4⁺CD45RA⁺ T cells was so severely suppressed for 36 months after HSCT and that most of the recovered CD4⁺ T cells were memory CD45RO⁺ T cells (Fig. 4B). This discrepancy of T-cell recovery after ASCT between SSc and MS, may be due to the difference of disease and/or of age at inclusion. In the study of Farge *et al.* [14], the level of naïve CD4⁺CD45RA⁺ T cells was also suppressed for 9 months after ASCT in SSc patients. In the study of Storek *et al.* [30], naïve and memory CD4⁺ T cells were equally recovered in 24 months after ASCT in patients with MS or SSc.

CD25⁺CD4⁺Foxp3⁺ regulatory T cells are a major regulator of adaptive immunity [22]. Patients with JIA showed a significant increase in thymus-derived regulatory T cells (CD25⁺CD4⁺Foxp3⁺) following ASCT [22]. However, in this study, the recovery of CD25⁺CD4⁺ T cells was severely delayed compared with that of CD25⁻CD4⁺ T cells

(Fig. 4C); the number of CD25⁺CD4⁺ T cells did not reach the lower limit of normal even at 36 months after ASCT. It is unlikely that the number of regulatory T cells was increased after ASCT, even if we considered that CD25⁺CD4⁺ T cells included activated T cells as well as regulatory T cells. When we analysed CD4⁺Foxp3⁺ T cells in nine patients with SSc, their recovery after ASCT was retarded. These results show that the expansion of regulatory T cells after ASCT was not the cause of the efficacy of ASCT on SSc.

The number of CD19⁺CD27⁺ memory B cells was low in contrast to an increased number of CD19⁺CD27⁻ naïve B cells at the baseline. Sato *et al.* [23] reported the B-cell abnormality including the expanded naïve B cells and diminished memory B cells in SSc patients. Unexpectedly, recovery of memory CD19⁺CD27⁺ B cells was severely suppressed even at 36 months after ASCT (Fig. 4D). In the study of Storek *et al.* [30], both naïve and memory B cells recovered to the normal range in 6 months after ASCT. Collectively, the resolution of clinical SSc after ASCT was not due to the reconstitution of naïve CD4⁺ T cells or to that of regulatory T cells or to the correction of B-cell imbalance.

An anti-Scl-70 antibody, a useful marker in establishing the diagnosis of SSc, predicts diffuse skin involvement and pulmonary fibrosis, and the increased level of this antibody is associated with a poor prognosis. In this study, we, for the first time, showed that the level of an anti-Scl-70 antibody was continuously decreased for 36 months after ASCT, and that the changes in anti-Scl-70 level were correlated significantly with those in mRSS. These results are consistent with a previous report that showed the correlation of serum anti-Scl-70 levels with disease activity in SSc [31], although the role of anti-Scl-70 in the pathogenesis of SSc was not clearly demonstrated in this article. It is of interest that the changes in serum anti-Scl-70 levels were independent of those in serum immunoglobulin levels, which returned to the baseline level at 12 months after ASCT (Fig. 2). In the study of Storek *et al.* [30], the level of anti-Scl-70 continued to be abnormally high throughout 24 months after ASCT. This difference might come from the difference in transplant conditioning (CYC 200 vs CYC 120 mg/kg + total body irradiation 8 Gy + anti-thymocyte globulin) or in the purity of the CD34⁺ cells.

Dysregulated cytokine production was reported in SSc patients [32, 33]. In this study, serum levels of TNF- α , TGF- β , IL-6 and sIL-2R increased before mobilization as previously reported [32], but their levels significantly decreased after ASCT (Fig. 3). Serum levels of VEGF and monocyte chemotactic protein 1 (MCP-1), however, did not decrease after ASCT (data not shown). The decreased levels of profibrotic cytokines after ASCT might reflect resolution of the disease.

Patient 7 died due to progressive IP in spite of the improvement of skin sclerosis at 20 months after ASCT. In this patient, IP was already highly advanced (per cent VC 39%) at the time of ASCT. Immune reconstitution after ASCT was similarly obtained in terms of Th1/Th2 balance and serum levels of pro-fibrotic cytokines. Therefore, the disease was fatal because of advanced and refractory IP that did not respond to the resolution of autoimmune reactions. This result suggests that patients with advanced organ involvement need to be excluded in a future study.

In conclusion, ASCT with purified CD34⁺ cells was effective in controlling the disease activity of SSc. Improvement of skin sclerosis was significantly associated with the change in serum anti-Scl-70 level after ASCT. Th1/Th2 ratio was significantly increased for at least 3 years after ASCT.

Rheumatology key messages

- ASCT causes durable remission in patients with SSc.
- Th1/Th2 ratio was significantly increased for at least 3 years after ASCT.

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References

- 1 LeRoy EC, Black CM, Fleischmajer R *et al.* Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. *J Rheumatol* 1998;15:202-5.
- 2 Yurovsky VV. The repertoire of T-cell receptors in systemic sclerosis. *Crit Rev Immunol* 1995;15:155-65.
- 3 Steen VD, Medsger TA Jr. Severe organ involvement in systemic sclerosis with diffuse scleroderma. *Arthritis Rheum* 2000;43:2437-44.
- 4 Hough RE, Snowden JA, Wulffraat NM. Haemopoietic stem cell transplantation in autoimmune diseases: a European perspective. *Br J Haematol* 2005;128:432-59.
- 5 McSweeney PA, Nash RA, Sullivan KM *et al.* High-dose immunosuppressive therapy for severe systemic sclerosis: initial outcomes. *Blood* 2002;100:1602-10.
- 6 Nash RA, McSweeney PA, Crofford LJ *et al.* High-dose immunosuppressive therapy and autologous hematopoietic cell transplantation for severe systemic sclerosis: long-term follow-up of the US multicenter pilot study. *Blood* 2007;110:1388-96.
- 7 Farge D, Passweg J, van Laar JM *et al.* Autologous stem cell transplantation in the treatment of systemic sclerosis: report from the EBMT/EULAR Registry. *Ann Rheum Dis* 2004;63:974-81.
- 8 Vonk MC, Marjanovic Z, van den Hoogen FH *et al.* Long-term follow-up results after autologous haematopoietic stem cell transplantation for severe systemic sclerosis. *Ann Rheum Dis* 2008;67:98-104.
- 9 Bohgaki T, Atsumi T, Bohgaki M *et al.* Immunological reconstitution after autologous hematopoietic stem cell transplantation in patients with systemic sclerosis: relationship between clinical benefits and intensity of immunosuppression. *J Rheumatol* 2009;36:1240-8.
- 10 Oyama Y, Barr WG, Statkute L *et al.* Autologous non-myeloablative hematopoietic stem cell transplantation in patients with systemic sclerosis. *Bone Marrow Transpl* 2007;40:549-55.
- 11 Tsukamoto H, Nagafuji K, Horiuchi T *et al.* Phase I-II trial of autologous peripheral blood stem cell transplantation in the treatment of refractory autoimmune disease. *Ann Rheum Dis* 2006;65:508-14.
- 12 Burt RK, Marmont A, Oyama Y *et al.* Randomized controlled trials of autologous hematopoietic stem cell transplantation for autoimmune diseases: the evolution from myeloablative to lymphoablative transplant regimens. *Arthritis Rheum* 2006;54:3750-60.
- 13 Muraro PA, Douek DC, Packer A *et al.* Thymic output generates a new and diverse TCR repertoire after autologous stem cell transplantation in multiple sclerosis patients. *J Exp Med* 2005;201:805-16.

- 14 Farge D, Henegar C, Carmagnat M *et al.* Analysis of immune reconstitution after autologous bone marrow transplantation in systemic sclerosis. *Arthritis Rheum* 2005;52:1555-63.
- 15 Shen Y, Ichino M, Nakazawa M, Minami M. CpG oligodeoxynucleotides prevent the development of scleroderma-like syndrome in tight-skin mice by stimulating a Th1 immune response. *J Invest Dermatol* 2005;124:1141-8.
- 16 Matsushita T, Hasegawa M, Hamaguchi Y, Takehara K, Sato S. Longitudinal analysis of serum cytokine concentrations in systemic sclerosis: association of interleukin 12 elevation with spontaneous regression of skin sclerosis. *J Rheumatol* 2006;33:275-84.
- 17 Akahoshi M, Nakashima H, Tanaka Y *et al.* Th1/Th2 balance of peripheral T helper cells in systemic lupus erythematosus. *Arthritis Rheum* 1999;42:1644-8.
- 18 Kohno K, Nagafuji K, Tsukamoto H *et al.* Infectious complications in patients receiving autologous CD34-selected hematopoietic stem cell transplantation for severe autoimmune diseases. *Transpl Infect Dis* 2009;11:318-23.
- 19 Ohnishi H, Yokoyama A, Kondo K *et al.* Comparative study of KL-6, surfactant protein-A, surfactant protein-D, and monocyte chemoattractant protein-1 as serum markers for interstitial lung diseases. *Am J Respir Crit Care Med* 2002;165:378-81.
- 20 Kuroki Y, Takahashi H, Chiba H, Akino T. Surfactant proteins A and D: disease markers. *Biochim Biophys Acta* 1998;1408:334-45.
- 21 Alexander T, Thiel A, Rosen O *et al.* Depletion of autoreactive immunologic memory followed by autologous hematopoietic stem cell transplantation in patients with refractory SLE induces long-term remission through de novo generation of a juvenile and tolerant immune system. *Blood* 2009;113:214-23.
- 22 de Kleer I, Vastert B, Klein M *et al.* Autologous stem cell transplantation for autoimmunity induces immunologic self-tolerance by reprogramming autoreactive T cells and restoring the CD4⁺CD25⁺ immune regulatory network. *Blood* 2006;107:1696-702.
- 23 Sato S, Fujimoto M, Hasegawa M, Takehara K. Altered blood B lymphocyte homeostasis in systemic sclerosis: expanded naive B cells and diminished but activated memory B cells. *Arthritis Rheum* 2004;50:1918-27.
- 24 Needleman BW, Wigley FM, Stair RW. Interleukin-1, interleukin-2, interleukin-4, interleukin-6, tumor necrosis factor alpha, and interferon-gamma levels in sera from patients with scleroderma. *Arthritis Rheum* 1992;35:67-72.
- 25 Mavalia C, Scaletti C, Romagnani P *et al.* Type 2 helper T-cell predominance and high CD30 expression in systemic sclerosis. *Am J Pathol* 1997;151:1751-8.
- 26 Serpier H, Gillery P, Salmon-Ehr V *et al.* Antagonistic effects of interferon-gamma and interleukin-4 on fibroblast cultures. *J Invest Dermatol* 1997;109:158-62.
- 27 Mueller R, Krahl T, Sarvetnick N. Tissue-specific expression of interleukin-4 induces extracellular matrix accumulation and extravasation of B cells. *Lab Invest* 1997;76:117-28.
- 28 Vieira PL, de Jong EC, Wierenga EA, Kapsenberg ML, Kaliński P. Development of Th1-inducing capacity in myeloid dendritic cells requires environmental instruction. *J Immunol* 2000;164:4507-12.
- 29 Brinkman DM, de Kleer IM, ten Cate R *et al.* Autologous stem cell transplantation in children with severe progressive systemic or polyarticular juvenile idiopathic arthritis: long-term follow-up of a prospective clinical trial. *Arthritis Rheum* 2007;56:2410-21.
- 30 Storek J, Zhao Z, Lin E *et al.* Recovery from and consequences of severe iatrogenic lymphopenia (induced to treat autoimmune diseases). *Clin Immunol* 2004;113:285-98.
- 31 Hu PQ, Fertig N, Medsger TA Jr, Wright TM. Correlation of serum anti-DNA topoisomerase I antibody levels with disease severity and activity in systemic sclerosis. *Arthritis Rheum* 2003;48:1363-73.
- 32 Sakkas LI. New developments in the pathogenesis of systemic sclerosis. *Autoimmunity* 2005;38:113-6.
- 33 Scala E, Pallotta S, Frezzolini A *et al.* Cytokine and chemokine levels in systemic sclerosis: relationship with cutaneous and internal organ involvement. *Clin Exp Immunol* 2004;138:540-6.

Self-Renewing Hematopoietic Stem Cell Is the Primary Target in Pathogenesis of Human Chronic Lymphocytic Leukemia

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SUMMARY

We report here that in chronic lymphocytic leukemia (CLL), the propensity to generate clonal B cells has been acquired already at the hematopoietic stem cell (HSC) stage. HSCs purified from patients with CLL displayed lymphoid-lineage gene priming and produced a high number of polyclonal B cell progenitors. Strikingly, their maturation into B cells was restricted always to mono- or oligo-clones with CLL-like phenotype in xenogeneic recipients. These B cell clones were independent of the original CLL clones because they had their own immunoglobulin VDJ genes. Furthermore, they used preferentially VH genes frequently used in human CLL, presumably reflecting the role of B cell receptor signaling in clonal selection. These data suggest that HSCs can be involved in leukemogenesis even in mature lymphoid tumors.

INTRODUCTION

Malignant transformation can occur through a multistep acquisition of critical somatic mutations. Therefore, the precursor of malignant stem cells should have a long life span to accumulate such mutations. In human hematopoiesis, genetic abnormalities for transformation should be accumulated in self-renewing hematopoietic stem cells (HSCs). HSCs can continuously produce a number of progenitors with the same genetic alteration, which are also potential targets for additional mutations (Rossi et al., 2008). Such HSCs or downstream progenitors finally become leukemia stem cells that possess self-renewal but lack normal differentiation activity (Huntly et al., 2004; So et al., 2003). This notion of leukemia development has been well accepted to explain acute myeloid leukemia (AML) development, and AML-initiating cells capable of reconstituting human leukemias in

xenogeneic hosts have been purified (Bonnet and Dick, 1997) as a potential therapeutic target (Jin et al., 2006, 2009; Kikushige et al., 2010; Majeti et al., 2009; Saito et al., 2010). However, in lymphoid malignancies, leukemia or lymphoma cells usually have monoclonal immunoglobulin or T cell receptor gene rearrangements, suggesting that lymphoid malignant stem cells originate after cells have committed to the lymphoid lineage. Recent studies have shown that acute lymphoid leukemia (ALL)-initiating cells upon xenogeneic transplantation are composed of multiple genetically distinct subclones (Anderson et al., 2011; Notta et al., 2011). These data clearly show that lymphoid cells can easily accumulate genetic abnormalities, presumably because they can persist longer than myeloid cells, and are capable of clonal expansion simulating self-renewal (Luckey et al., 2006). Because of such property of lymphoid cells, the involvement of HSCs in lymphoid leukemogenesis has never been underscored.

Significance

HSCs capable of self-renewal should be the main target for accumulating mutational events to develop hematological malignancies. This paper shows that HSCs play such a role also in mature lymphoid malignancies. Most human CLL cases have a precursor phase, called monoclonal B lymphocytosis (MBL), that is asymptomatic monoclonal or oligoclonal proliferation of B cells. HSCs from patients with CLL but not normal HSCs developed monoclonal or oligoclonal B cells simulating MBL after xenogeneic transplantation. Acquisition of chromosomal abnormalities appeared to be secondary events to transform MBL into clinical CLL. Thus, even in CLL, accumulation of oncogenic events starts at the HSC stage. Our xenograft model might be very useful to understand the pathogenesis of human CLL.

Chronic lymphocytic leukemia (CLL), the most common leukemia in adults in western countries, is a mature B cell malignancy (Chiorazzi et al., 2005). It is characterized by accumulation of clonal B cells in the blood, the bone marrow, and the lymphoid tissues. The consistent clonal expansion of mature B cells frequently expressing CD5 is the major phenotype of patients with CLL. Unfortunately, the development of its xenograft models by transplanting primary CLL cells into immunodeficient hosts has failed because the engraftment was extremely inefficient (Dürig et al., 2007; Hummel et al., 1996). Thus, the search for CLL-initiating cells has never been successful.

Human CLL cells have functional B cell receptors (BCRs) on their surface as a result of productive rearrangement of immunoglobulin genes (Caligaris-Cappio and Ghia, 2008; Chiorazzi et al., 2005; Stevenson and Caligaris-Cappio, 2004). CLL has been divided into two subgroups based on the presence of somatic hypermutations within the variable regions of immunoglobulin heavy-chain (IGHV) genes, which normally occurs in the germinal center during naive to memory B cell transition. The group of CLLs with mutated BCRs has a more favorable prognosis than those with unmutated BCRs (Hamblin et al., 1999). However, recent studies suggest that both types of CLLs originate from self-reactive B cell precursors and that the status of somatic hypermutations does not indicate their origin (Hervé et al., 2005; Klein et al., 2001; Rosenwald et al., 2001). Interestingly, CLL cells preferentially use the IGHV genes, such as VH1, VH3, and VH4 regions (Chiorazzi and Ferrarini, 2003; Fais et al., 1998), and express a restricted BCR repertoire including antibodies with quasi-identical complementarity-determining region 3 (CDR3) (Ghiotto et al., 2004; Messmer et al., 2004; Tobin et al., 2003, 2004; Widhopf et al., 2004), suggesting specific antigen recognition by CLL cells (Chiorazzi and Ferrarini, 2003; Stevenson and Caligaris-Cappio, 2004).

To trace the origin of genetic aberration in human CLL, it is important to note the fact that CLL cells are not always monoclonal, but more than one CLL clone is found in up to ~14% of patients with CLL (Sanchez et al., 2003). Furthermore, a recent cohort study has shown that 44 out of 45 patients with CLL have a precursor state such as monoclonal B lymphocytosis (MBL) for 6 months to 7 years (Landgren et al., 2009). MBL represents asymptomatic proliferation of clonal B cells whose numbers in circulation are below 5000/ μ l (Marti et al., 2005). Of note, human MBL is frequently (20%–70% of total cases) composed of more than one B cell clone (Dagklis et al., 2009; Lanasa et al., 2010; Nieto et al., 2009). More than a half of such MBL clones express CD5 (Scarfo et al., 2010), and patients with these CLL-like MBL clones frequently develop into clinical CLL (Rawstron et al., 2008). Furthermore, like CLL cells, CD5⁺ MBL clones use a biased set of VH genes, including VH1, 3, and 4 (Rawstron et al., 2008). The usage of such biased BCR types found in CLL and its precedent MBL clones strongly suggests that the antigenic drive contributes to clonal expansion and/or cell survival also during the transition from MBL to clinical CLL (Pleyer et al., 2009).

The question is: If progression from MBL to CLL reflects stepwise leukemogenesis, at what stage does the first oncogenic event occur. The existence of oligoclonal B cell clones in patients with CLL and with those MBL strongly suggests that the first oncogenic event could at least be traced up to the

progenitor or HSCs that have not rearranged IGH genes. These data led us to search for CLL-initiating cells within the early hematopoietic stages utilizing an efficient xenotransplantation system.

RESULTS

Clonal Selection of CLL B Cells Occurs at the Mature B Cell Stage in Human CLL

To search for the cell population with CLL-initiating activity in human CLL, we first tried to locate the developmental stage at which CLL B cell clones appear. Patients' characteristics are shown in Table S1 available online.

Figure 1A shows the FACS analysis of the bone marrow of a patient with CLL. The bone marrow contained CD34⁺CD38⁻ HSCs (Bhatia et al., 1997), and the CD34⁺CD38⁺ progenitor fraction that contains myeloid and lymphoid progenitors (Manz et al., 2002). Interestingly, percentages of CD10⁺CD19⁺ proB cells in the bone marrow of patients with CLL were high in most patients: in 12 out of 13 patients with CLL, proB cell frequency was higher than the average of 7 normal controls, and the average proB cell frequency in patients with CLL was higher than that in normal controls by ~5-fold (Figure 1B). In contrast, frequencies of the CD34⁺CD38⁻ HSC population were equal (Figure 1B). Recent reports have shown that the CD34⁺CD38⁻ HSC population can further be divided into subpopulations including CD90⁺CD45RA⁻, CD90⁻CD45RA⁻, and CD90⁻CD45RA⁺ that mainly contain long-term HSCs (LT-HSCs), multipotent progenitors (Majeti et al., 2007), and early lymphoid/myeloid progenitors (Doulatov et al., 2010; Goardon et al., 2011), respectively. We performed the HSC subpopulation analysis in six CLL cases, and found that the distribution of these HSC subpopulations did not differ in normal and CLL bone marrow, and the majority (~60%) of CD34⁺CD38⁻ cells were the most primitive CD90⁺CD45RA⁻ population (Figure 1B). Thus, we tested whether the expansion at the proB stage reflects clonal proliferation of CLL precursors by analyzing the rearrangement status of the IGH gene.

As shown in Figure 1C, the purified CD34⁺CD38⁻ HSC population in patients with CLL (CLL-HSCs) presented the germline configuration, and CD34⁺CD19⁺ CLL cells had a clonal IGH rearrangement. Of note, proB cells in CLL bone marrow exhibited polyclonal rearrangement of IGH genes, suggesting that CLL clones are selected in vivo among such expanded polyclonal B cells. These data clearly show that CD34⁺CD38⁻ CLL-HSC populations do not rearrange the IGH gene, and therefore, are not contaminated with detectable CLL clones. However, CLL-HSCs are able to develop a higher number of polyclonal B cells as compared to normal HSCs, suggesting that developmental potential of CLL-HSCs is skewed toward B cell lineage probably reflecting their cell-intrinsic abnormality.

Purified HSCs from Patients with CLL Are Able to Generate Clonal B Cells with CLL-like Phenotype after Xenogeneic Transplantation

We then tried to identify the CLL-initiating cell population by transplanting subpopulations of CLL cells into immunodeficient mice. In these experiments, NOD/SCID/IL2rg^{null} (NSG) (Ishikawa et al., 2005) newborns or NOD/RAG-1^{-/-}IL2rg^{null}

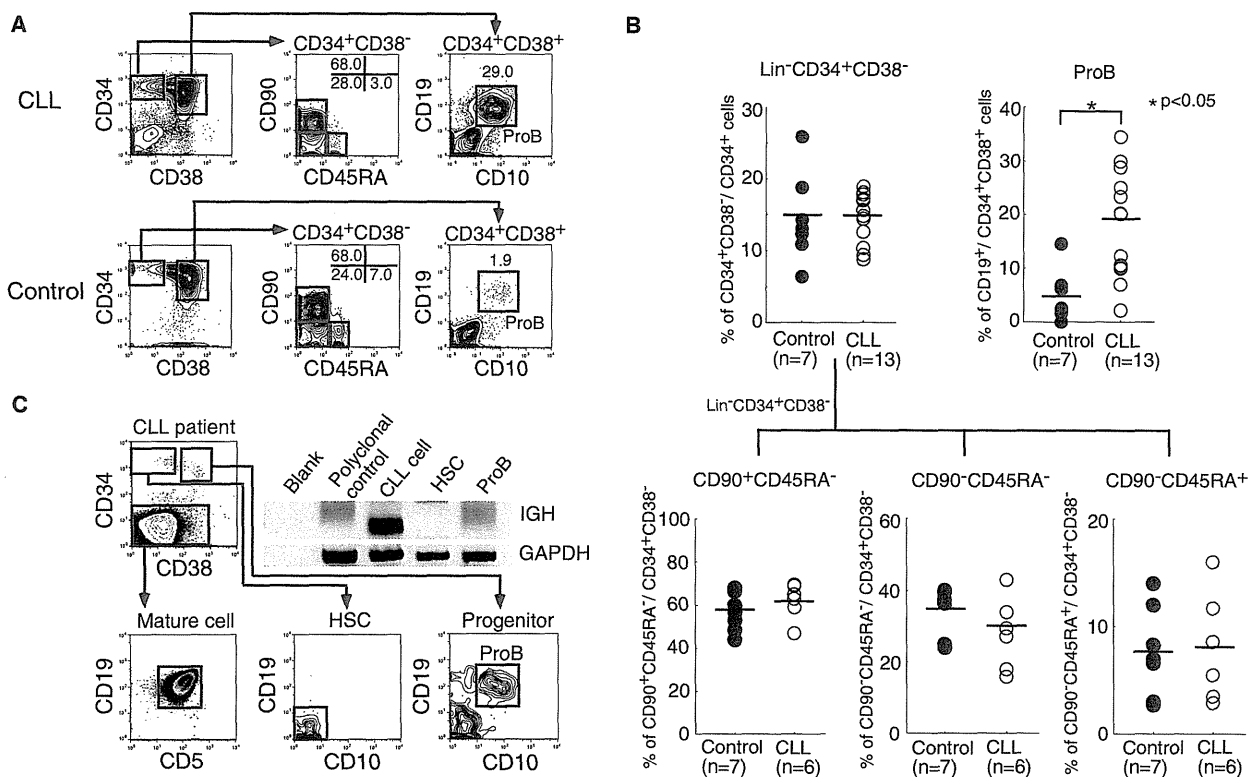


Figure 1. Hematopoietic Stem and Progenitor Cells in the Bone Marrow of Patients with CLL

(A) Stem and progenitor FACS analysis of the bone marrow of a patient with CLL and a normal control. A representative analysis is shown. (B) Frequencies of CD34⁺CD38⁻ HSCs and CD34⁺CD38⁺CD19⁺ pro B cells in 13 patients with CLL and 7 normal controls (upper panels), and frequencies of HSC subpopulations including CD34⁺CD38⁻CD90⁺CD45RA⁻, CD34⁺CD38⁻CD90⁻CD45RA⁻, and CD34⁺CD38⁻CD90⁻CD45RA⁺ fractions in 6 patients with CLL analyzed (lower panels) are shown. Note that the CLL bone marrow contains a higher number of CD34⁺CD38⁻CD10⁺CD19⁺ proB cells than the normal bone marrow does ($p < 0.05$) (see also Table S1), whereas percentages of HSC fractions do not differ in normal and CLL bone marrow. The distribution of these HSC subfractions is unchanged in patients with CLL. (C) IGH rearrangement status of HSC, proB, and B cell fractions in the bone marrow of a patient with CLL. HSCs did not rearrange IGH (germline), whereas proB cells showed polyclonal IGH rearrangement.

(NRG) (Pearson et al., 2008) adult mice were used as recipients (Table 1).

CD19⁺ CLL cells were purified from the blood or the bone marrow of patients 1–8, and 0.2 to 1×10^7 cells were transplanted. However, even until 6 months after transplantation, human CD45⁺ cells were never found in any of the 15 recipients analyzed (Figure S1). These data strongly suggest that CLL cells are incompetent for expansion to recapitulate human CLL in immunodeficient mice. We also transplanted 10^4 CD34⁺CD38⁺CD10⁺CD19⁺ proB cells in these patients, but none of ten recipients was engrafted 12 weeks after transplantation (not shown). These data led us to analyze the engraftment potential of CLL-HSCs in the xenogeneic transplantation system. Purified 3.3×10^5 to 6.5×10^4 CD34⁺CD38⁻ HSCs or 5.0×10^3 to 1×10^4 CD34⁺CD38⁻CD90⁺LT-HSCs from 16 independent patients with CLL were transplanted into 25 mice (Table 1; Table S2), and $\sim 10^4$ CD34⁺CD38⁻ cells from 11 normal controls were transplanted into 29 mice.

Previous xenogeneic transplantation studies have shown that normal HSCs are able to reconstitute multilineage hematopoietic

cells, and polyclonal B cells are normally developed in NOD-SCID or NSG mouse bone marrow and spleen (Hiramatsu et al., 2003; Ishikawa et al., 2005; Kolar et al., 2004; Matsumura et al., 2003; Rossi et al., 2001). As shown in Figures 2A and 2B, both CLL-HSCs and normal HSCs gave rise to secondary CD34⁺CD38⁻ HSCs, CD34⁺CD38⁺ progenitor cells, CD34⁺CD19⁺ B cells, and CD34⁺CD33⁺ myeloid cells in the bone marrow. Of note, the percentage of CLL-HSC-derived human proB cells was significantly higher than that of normal HSC-derived ones (Figure 2C), as we found in the bone marrow analysis of patients with CLL and normal controls (Figure 1B), suggesting again that differentiation of CLL-HSCs skews toward B cell lineage. Interestingly, CLL-HSC-derived CD19⁺ B cells in the bone marrow frequently coexpressed CD5 (Figure 3B and Table 1), which is a characteristic of de novo human CLL cells. Normal human HSCs generated mainly CD5⁻ and very rare (<1%) CD5⁺ B cells in the bone marrow in all 29 recipients. In total, 5 out of 25 mice transplanted with CLL-HSCs developed both CD5⁺ and CD5⁻ B cell clones, 9 mice developed only CD5⁺ B cell clones, and the remaining 11 mice developed only CD5⁻ B cell clones

Table 1. Results of Xenogeneic Transplantation Assays of CLL-HSCs

Patient No.	Mouse	Weeks after Transplant	Transplanted Cells	No. of Cells Transplanted ($\times 10^3$ cells)	hCD45 ⁺ Cells (%)	hCD19 ⁺ in hCD45 ⁺ (%)	hCD33 ⁺ in hCD45 ⁺ (%)	CD5 ⁻ B Cell Cells in Total B Cells (%)	No. of Clones	CD5 ⁺ B Cell Cells in Total B Cells (%)	No. of Clones
1	1-1	NRG	16	CD34 ⁺ CD38 ⁻	20	0.1	57.9	NA	1	-	-
	1-2	NRG	18	CD34 ⁺ CD38 ⁻	40	0.5	32.6	52.8	P	6.4	3
2	2	NRG	12	CD34 ⁺ CD38 ⁻	65	0.1	33.3	NA	1	-	-
3	3	NRG	16	CD34 ⁺ CD38 ⁻	14	1.6	92.3	NA	1	-	-
4	4	NSG	5	CD34 ⁺ CD38 ⁻	3.3	22.4	5.14	40.4	2	-	-
5	5	NSG	11	CD34 ⁺ CD38 ⁻	7.6	11.4	83	7.1	2	10.8	2
6	6	NSG	12	CD34 ⁺ CD38 ⁻	7.0	18.7	7.54	89	2	-	-
7	7-1	NSG	24	CD34 ⁺ CD38 ⁻	30	31.6	53.2	32.5	P	5.0	3
	7-2	NSG	24	CD34 ⁺ CD38 ⁻	7.0	1.8	17.6	58.1	2	34.3	2
	7-3	NSG	24	CD34 ⁺ CD38 ⁻	16	4.3	63.3	31.3	P	10.2	2
8	8	NSG	24	CD34 ⁺ CD38 ⁻	18	4.1	17.5	60.2	1	11	3
9	9-1	NSG	13	CD34 ⁺ CD38 ⁻	4.0	2.0	72.3	19.4	P	2.4	1
	9-2	NSG	13	CD34 ⁺ CD38 ⁻	5.0	14.0	10.2	51.8	1	-	-
10	10-1	NSG	13	CD34 ⁺ CD38 ⁻	15	18.1	88.3	2.9	1	-	-
	10-2	NSG	13	CD34 ⁺ CD38 ⁻	10	11.0	68.5	20.1	1	-	-
	10-3	NSG	30	CD34 ⁺ CD38 ⁻	5.0	18.5	63.3	24.1	3	3.1	1
11	11-1	NSG	33	CD34 ⁺ CD38 ⁻	10	0.5	50.1	NA	1	-	-
	11-2	NRG	14	CD34 ⁺ CD38 ⁻	18	0.1	28.6	50	2	-	-
12	12	NRG	12	CD34 ⁺ CD38 ⁻ CD90 ⁺	6.0	0.1	47.5	31.9	P	10.4	2
13	13-1	NRG	14	CD34 ⁺ CD38 ⁻ CD90 ⁺	8.0	0.5	87.1	2.5	P	5.3	1
	13-2	NRG	14	CD34 ⁺ CD38 ⁻ CD90 ⁺	8.0	3.7	88.1	1.1	P	3.9	1
	13-3	NRG	17	CD34 ⁺ CD38 ⁻ CD90 ⁺	6.0	1.0	86.1	5.2	P	1.0	2
14	14	NRG	9	CD34 ⁺ CD38 ⁻	60	1.0	75.1	17.1	2	0.9	2
15	15	NRG	9	CD34 ⁺ CD38 ⁻ CD90 ⁺	10	0.2	67.7	26	1	-	-
16	16	NRG	21	CD34 ⁺ CD38 ⁻ CD90 ⁺	5.0	1.8	92.1	1.9	P	1.7	1

NA, not analyzed; P, polyclonal.