

Figure 3. Patients whose hematogones comprised > 5% bone marrow MNCs constitute a group with significantly improved survival, irrespective of HSC sources. (A-B) The Kaplan-Meier estimates of (A) OS and (B) RFS among patient subgroups with $\leq 1\%$ (gray line), 1%-2% (green line), 2%-3% (orange line), 3%-4% (red line), 4%-5% (purple line), $\leq 5\%$ (black bold line), or > 5% (blue bold line) hematogones in the bone marrow MNCs. Forty-three patients who developed > 5% MNC hematogones (HG⁺) showed significantly better 3-year OS and RFS, compared with any of each group ($P < .01$ and $P < .05$, respectively), as well as to 65 patients with $\leq 5\%$ MNCs hematogones (HG⁻; $P < .001$ for both). (C-D) The Kaplan-Meier estimates of (C) OS and (D) RFS in HG⁺ and HG⁻ groups that received transplants with BMT or CBT. The improved OS and RFS were seen in HG⁺ groups regardless of the source of HSC. HG⁺ indicates patients developed hematogones (> 5% of bone marrow MNCs); HG⁻ indicates patients who failed to develop hematogones ($\leq 5\%$ of bone marrow MNCs).

acute GVHD (2 patients) and viral infections (4 patients). Of the 26 patients who died of primary disease, 24 developed both acute GVHD and infections.

We analyzed the relationship between the emergence of documented hematogones in the bone marrow, and variables including sex of donor/recipient, days required for engraftment, primary diseases, times of intensive chemotherapy before HSCT, remission status, conditioning regimen, documented infectious disease, and episode of acute/chronic GVHD by using univariate and multivariate analysis. These analyses were performed in patient groups treated with BMT and CBT, respectively.

There were no correlations found between the emergence of > 5% of hematogones and clinical factors such as the day of engraftment, primary disease, times of intensive chemotherapies, and remission status, in either univariate or multivariate analyses. As shown in Table 4, in univariate analysis, a hematogone increase up to > 5% of MNCs was found more frequently in patients without viral infection (such as cytomegalovirus, human herpesvirus 6, and adenovirus; BMT: $P = .03$; CBT: $P < .01$), and those did not develop severe acute GVHD of grade II-IV (BMT: $P < .01$; CBT: $P < .01$). Time required for engraftment did not differ between patient groups with or without infections, or acute GVHD (Table 2). These data appear to be compatible with the analysis of causes of death in HG⁻ patients (Table 3). On the other hand, in multivariate analysis, severe acute GVHD of grade II-IV, but not infection was the significant risk factor for emergence of hematogones (BMT: $P = .03$; CBT: $P = .04$; Table 4). Based on

these analyses, the emergence of hematogones heralds less frequent development of severe acute GVHD.

Discussion

Hematogones are immature B-cell precursors that reside mainly in the bone marrow of every normal individual,^{1,2,27,40} and their numbers could reflect activity of normal B lymphopoiesis. Hematogones are occasionally seen in large numbers in healthy people, especially in infants and young children.^{2,4,7,8} Interestingly, recent reports have suggested that the presence of detectable numbers of hematogones at the recovery phase from myelosuppression reflects better prognosis of patients with AML treated with chemotherapy⁵ or CBT,⁶ although the underlying mechanism of this phenomenon is unclear. The increase of hematogones may reflect eradication of leukemic cells that could inhibit normal hematopoiesis,^{1,5} or rapid immune reconstitution that could suppress infection and severe acute GVHD in an allogeneic HSCT setting.⁶

In these reports, the presence of hematogones was documented when they were detectable at a low frequency: $\geq 0.01\%$ of TNCs at a recovery phase⁵ or > 0% and > 0.9% of MNCs on day 21 and 100, respectively.⁶ In contrast, the patient cohort in our study received allogeneic HSCT, and the majority (106 of 108 cases) of patients had > 0.1% of hematogones at engraftment by our multicolor flow cytometric analysis (Figure 1C). Therefore, it was critical to set an appropriate threshold value and timing of sampling

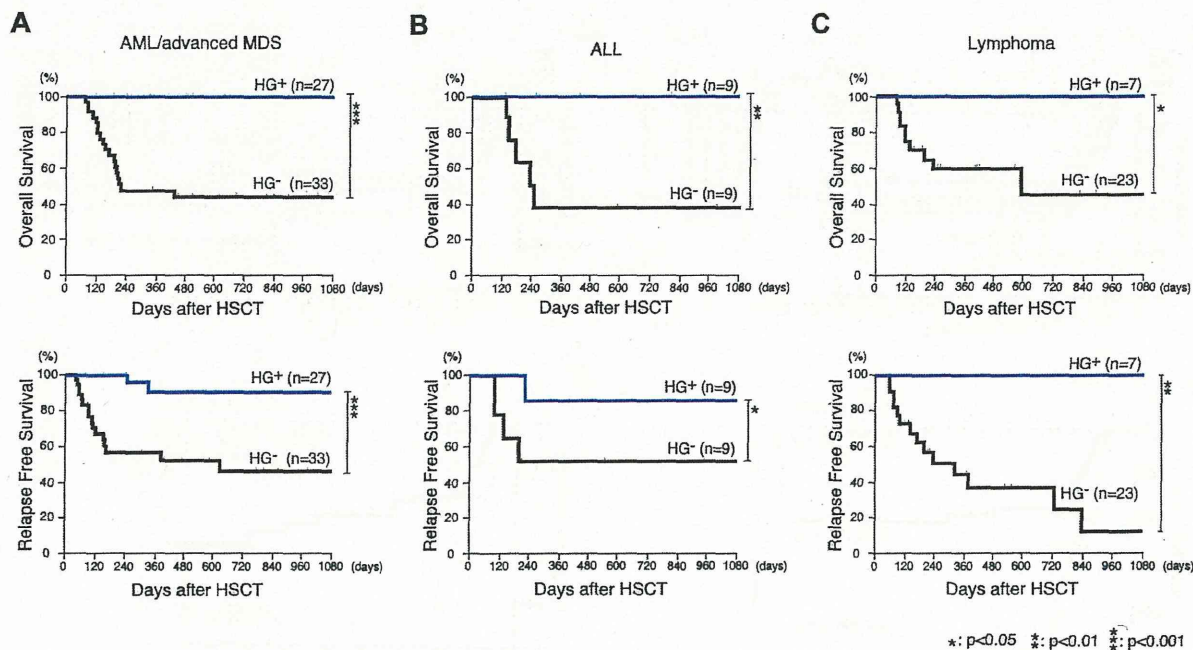


Figure 4. Patients who developed > 5% hematogones as a fraction of their MNCs constitute a group with significantly improved survival, irrespective of their primary disease. The Kaplan-Meier estimates of OS and RFS in HG⁺ and HG⁻ patients differentiated with their primary disease. In each group of patients with (A) AML or advanced MDS, (B) ALL, and (C) lymphoma, HG⁺ groups showed significantly better OS and RFS, compared with the HG⁻ group ($P < .001$ for both).

to decide a clinically meaningful increase of hematogones in an allogeneic HSCT setting. Furthermore, previous studies were performed only in patients with AML,^{5,6} but not in patients with lymphoid neoplasms, presumably because it was difficult to discriminate a small number of neoplastic lymphoid cells from hematogones.⁶

To accurately enumerate hematogones in patients with various clinical backgrounds and with different donor cell sources, we performed the analysis on the day when patients met the clinical criteria of engraftment⁹⁻¹² and displayed complete donor-type chimerism. The confirmation for donor-type chimerism allowed us to avoid miscounting neoplastic lymphoid cells as hematogones. Because these samples should be free from host-derived cells, we included patients with lymphoid malignancies in our study. We rigorously measured the frequencies of hematogones within bone marrow MNCs by 6-color flow cytometric analysis.

In our study, donor-derived hematogones were polyclonal, based on *IGH* rearrangement analysis in all cases, and therefore the presence of hematogones should be a snapshot of normal B lymphopoiesis at the recovery phase. In fact, the frequencies of hemato-

gones at engraftment were correlated with circulating B-cell numbers at least until day 90 (Figure 2). Importantly, we here show that the frequencies of hematogones were correlated with donors' age, but not with recipients' age, suggesting the age-dependent decline of B-cell potential of donor HSCs. This is compatible with previous mouse studies in which younger HSCs are capable of producing more abundant B cells.⁴¹⁻⁴³

According to our criteria, the engraftment was seen on days 25 and 32 (median) in BMT and CBT groups (Figure 1D), respectively, consistent with previous studies.^{35-39,44} Within each BMT or CBT group, the engraftment day was not significantly altered by the patients' primary disease or remission status at transplantation (Table 2). The bone marrow sampling for hematogone analysis was performed on the day of engraftment. As shown in Table 3, the timing of sampling (= the day of engraftment) was not significantly related to emergence of hematogones in univariate and multivariate analyses. Interestingly, however, our data suggest that when hematogones reach > 5% of MNCs at engraftment, it has a profound clinical impact on patients' OS and RFS (Figure 3A-B). It is of note that the emergence of documented hematogones

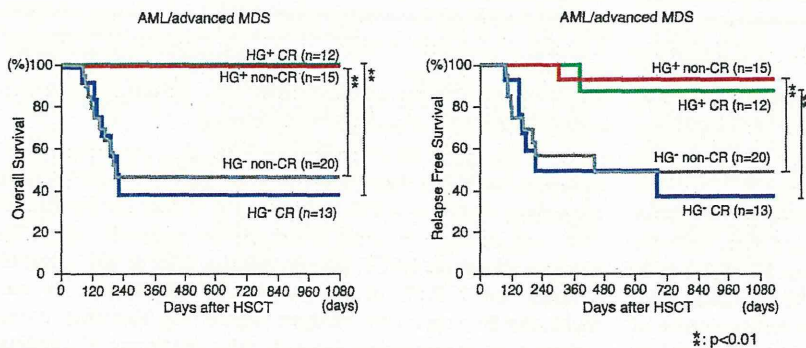


Figure 5. The presence of hematogones marks a group with good prognosis in AML/advanced MDS patients. The Kaplan-Meier estimates of OS and RFS in HG⁺ and HG⁻ patients in AML or advanced MDS differentiated with their remission status before HSCT. Significantly better OS and RFS were seen in HG⁺ groups irrespective of their remission status.

Table 3. Cause of death in patients with less than 5% hematogones

Primary disease	Cause of death, no. (%)					
	Relapse of primary disease			Transplantation-related mortality		
	Infection	Acute GVHD (Grade II-IV)	Total	Infection	Acute GVHD (Grade II-IV)	Total
AML and advanced MDS	14/33 (42.4)	14/33 (42.4)	15/33 (45.4)	2/33 (6.1)	0/33 (0)	2/33 (6.1)
ALL	3/9 (33.3)	3/9 (33.3)	3/9 (33.3)	1/9 (11.1)	1/9 (11.1)	2/9 (22.2)
Lymphoma	7/23 (30.4)	7/23 (30.4)	8/23 (34.8)	1/23 (4.3)	1/23 (4.3)	2/23 (8.7)
Total	24/65 (36.9)	24/65 (36.9)	26/65 (40)	4/65 (6.2)	2/65 (3.1)	6/65 (9.2)

ALL indicates acute lymphoblastic leukemia; AML, acute myelogenous leukemia; GVHD, graft-versus-host disease; and MDS, myelodysplastic syndrome.

was not related to times of intensive chemotherapies or remission status of patients before transplantation in both univariate and multivariate analyses (Table 3). This result suggests that the emergence of hematogones was not affected by the potential damage of host microenvironment through multiple chemotherapies.

The improvement of OS and RFS in HG⁺ patients was seen in all patient groups: those suffering from AML/advanced MDS, ALL, or Lymphoma (Figure 4). Furthermore, this effect became more evident when patients who had failed to achieve CR before transplantation were analyzed (Figure 5). In this case, the appearance of hematogones clearly marks a subgroup with favorable OS and RFS, irrespective of their primary diseases. In contrast, in patients who had achieved CR before transplantation, prolonged OS and RFS were found only in patients with AML/advanced MDS, but not in patients with ALL or lymphoma (Figure 5). A larger study including higher number of patients should be performed to clarify the impact of hematogones on HSCT results in CR patients with lymphoid malignancies.

The analyses of risk factors for the appearance of > 5% MNCs of hematogones revealed that in both BMT and CBT patients, the less frequent occurrences of severe acute GVHD and infections were significantly correlated in univariate analyses, whereas the less frequent severe acute GVHD was the only risk factor in multivariate analyses (Table 3). As shown in Table 4, all 32 deaths occurred only in HG⁻ patients, and 24 of these 32 patients developed both severe acute GVHD and infection before the relapse of the disease. In these patients, doses of immunosuppressive drugs were escalated to control acute GVHD, which might cause development of infections as well as recurrence of primary disease.^{6,45,46} It is therefore possible that less frequent development of severe acute GVHD in HG⁺ patients is one of the reasons for their better OS and RFS.

The rapid reconstitution of the immune system represented by a high number of hematogones should be able to prevent infection.^{6,45} In turn, successful prevention of acute GVHD could result in proliferation of hematogones because acute GVHD itself may suppress hematopoietic recovery by targeting the bone marrow HSC niche⁴⁷ or by attacking directly B-lymphoid cells.⁴⁶ In addition, the fact that improvement of RFS is associated with the expansion of hematogones suggests an interesting possibility that

B cells play a role in the graft-versus-leukemia effect,⁴⁸ although this is still controversial.⁴¹ Also in turn, it is possible that the successful eradication of neoplastic cells from the bone marrow by HSCT results simply in rapid expansion of hematogones.

Thus, our data suggest that the expansion of hematogones is a useful indicator to discriminate a patient group with improved OS and RFS after allogeneic HSCT. Based on rigorous evaluation of frequencies of hematogones after HSCT, we propose that 5% of MNCs is a threshold value for a clinically valuable increase of hematogones. The prognostic value of this definition should be tested by future studies in larger groups of patients.

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Authorship

Contribution: T.S. and T.M. coordinated the project, designed and performed the transplantation and experiments, analyzed the data, and wrote the manuscript; Y.K., Y.M., K. Kamezaki, K. Takenaka, H.I., K.N., T.T. and K. Kato performed the transplantation and provided technical advice; K. Takase, H.H., A.N., Y.I., T.K., and T.E. provided patient information, clinical samples, and technical advice; and K.A. designed the experiments, reviewed the data, and edited the manuscript.

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Table 4. Risk factors for development of more than 5% MNCs of hematogone based on univariate and multivariate analyses

	BMT				CBT			
	Univariate		Multivariate		Univariate		Multivariate	
	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P
Infections, Yes/No	0.19 (0.05-0.74)	.03	0.42 (0.02-1.58)	.23	0.16 (0.04-0.57)	< .01	0.37 (0.02-2.81)	.10
Acute GVHD, Grade II-IV/0-I	0.09 (0.01-0.73)	< .01	0.04 (0.00-0.85)	.03	0.12 (0.03-0.48)	< .01	0.04 (0.00-0.80)	.04

BMT indicates bone marrow transplantation; CBT, cord blood transplantation; CI, confidence interval; GVHD, graft-versus-host disease; and MNC, mononuclear cell.

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ORIGINAL ARTICLE

Higher incidence of injection site reactions after subcutaneous bortezomib administration on the thigh compared with the abdomen

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Abstract

Subcutaneous (sc) rather than intravenous administration of bortezomib (Bor) is becoming more common for treating multiple myeloma (MM) because scBor results in lower incidence and severity of peripheral neuropathy and has equivalent efficacy. Bor is an irritant cytotoxic agent when it leaks out; therefore, it is recommended that injections of scBor should be rotated among eight different sites on the abdomen and thigh. However, detailed information about injection site reaction (ISR) has not been sufficiently documented. We retrospectively analyzed the incidence and severity of ISR following scBor administration in 15 Japanese patients with MM. Grade 1 ISR occurred following 40 of 158 (25.3%) scBor injections in ten patients, whereas grade 2 ISRs occurred following seven injections (4.4%) in five patients. Five patients did not develop ISR. Of note, grade 2 ISR was documented in 6 of 65 (9.2%) thigh injections but only in 1 of 93 (1.1%) abdominal injections. These data show that grade 2 ISRs were more common in the thigh compared with the abdomen possibly because the thigh contains lesser adipose tissue than the abdomen. Grade 2 ISRs resolved without any sequela within a median of 7 d. scBor administration on the abdomen instead of the thigh should be considered, especially for emaciated patients, because ISR rapidly resolves within the interval before the next injection even if it occurs.

Key words bortezomib; subcutaneously; injection; site reaction; multiple myeloma

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The outcomes for patients with multiple myeloma (MM) have been significantly improved because the introduction of novel agents such as the proteasome inhibitor bortezomib (Bor) (1–3). Intravenous administration of Bor is a treatment barrier because it causes a significant exacerbation of peripheral neuropathy (PN) (4–6). Recent reports have revealed that the response rate in patients receiving subcutaneous Bor (scBor) is comparable to those receiving with intravenous Bor (ivBor). In contrast, the incidence and severity of PN in patients receiving scBor was significantly lower than those in patients receiving ivBor (7–9); therefore, subcutaneous (sc) injection is the preferred route of administration (6). Bor is classified as an irritant because it causes an inflammatory

reaction accompanied with aching, burning, and phlebitis when it leaks out (10). However, recent studies have revealed that injection site reaction (ISR) caused by scBor is tolerable in a vast majority of patients if the injection site is rotated among eight different sites—upper and lower quadrants of the right and left abdomen and proximal and distal sites on both thighs (7, 8, 11). In these studies, erythema was the most common ISR; only 4 of 147 patients (3%) developed ISR of grade 3 or above, and only two patients (1%) required a reduction in the Bor dose (8). However, the effects of scBor-induced ISR remain unclear (7–9).

In this study, we compared the incidence and severity of ISRs at abdominal and thigh injection sites in 15 Japanese

patients treated with scBor and found that grade 2 ISRs were more common in the thigh than the abdomen. In addition, we discuss how to prevent and manage scBor-induced ISR.

Methods

We retrospectively analyzed the incidence and severity of ISRs in 15 Japanese patients treated with scBor at a concentration of 2.5 mg/mL (3.0 mg Bor reconstituted with 1.2 mL normal saline), as described previously (8). Bor was administered subcutaneously by physicians or cancer chemotherapy nurses who had enough training to provide safe administration of chemotherapy to cancer patients. Bor was injected at eight different sites, as Moreau described previously (8). Patients were treated on day 1, 8, 15, and 22 with scBor (1.3 mg/m²) and dexamethasone (40 mg) (12) or a combination of scBor, 1.3 mg/m²; cyclophosphamide, 300 mg/m²; and dexamethasone, 40 mg as described previously (13). The tolerability of scBor at the injection site was evaluated systematically 2-hrs after injection and every day during the first treatment cycle during hospitalization. After the second cycle, ISRs were evaluated at every visit, and between visits, patients documented their own ISRs in a detailed diary. Hematologic and non-hematologic adverse events, including ISR, were graded according to National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 4.0: grade 1, tenderness with or without associated symptoms (e.g., warmth, erythema, itching); grade 2, pain, lipodystrophy, edema, or phlebitis; grade 3, ulceration, necrosis, severe tissue damage, or any indication for operative intervention; grade 4, ISRs with life-threatening consequences, requiring urgent intervention.

The frequencies of grade 0, grade 1, and grade 2 ISR in the thigh were compared with those in the abdomen using the χ^2 -test. The body mass index (BMI) and scBor dosages of patients with grade 0–1 or 2 ISRs in the thigh were compared using the Mann–Whitney U-test. The Institutional Review Board of Harasanshin Hospital approved the scBor treatment protocol.

Results

The patient characteristics are summarized in Table 1. Fifteen Japanese patients with newly diagnosed ($n = 7$) or relapsed ($n = 8$) MM were enrolled in this study. Of the eight patients with relapsed MM, five had previously received ivBor and two underwent autologous peripheral blood stem cell transplantation prior to scBor treatment. The median age of all patients was 70 yr (range 54–85 yr). No patients had a history of any allergic disease or any allergic reaction associated with ivBor has not been documented in five patients who had previously been treated with ivBor. The median dose of scBor for all patients was 10.4 (range

5.2–30.6) mg/m², and the number of scBor injection sites for each patient was 8 (range, 4–27).

A total of 158 scBor injections were administered to the 15 patients. Five patients did not develop ISR and ten developed grade 1 and/or 2 ISR; grade 1 ISR occurred after 40 injections (25.3%; ten patients), whereas grade 2 ISR occurred after seven injections (4.4%, five patients). Table 1 summarizes number of scBor injections and incidence of ISR in the abdomen or thigh in each patient. Among ten patients with grade 1 ISR, nine patients developed grade 1 ISR both in the abdomen and the thigh, whereas one patient (patient no.12) developed grade 1 ISR only in the abdomen (Table 1). Five of 10 patients with grade 1 ISR also developed grade 2 ISR after other injections. Of these five patients with grade 2 ISR, three patients developed it once in the thigh (no.5 & 8) or in the abdomen (no.15), and two patients (no.9 & 12) developed twice in the two different sites of thigh. Interestingly, grade 2 ISR occurred after 6 of 65 (9.2%) injection sites on the thighs, whereas only 1 of 93 (1.1%) injection sites on the abdomen (no.15). These results indicated a significant higher incidence of grade 2 ISR in the thigh compared with the abdomen ($P = 0.014$). In contrast, there was no significant difference between the incidence of grade 0 and 1 ISR on the thighs and abdomen ($P = 0.099$ and $P = 0.87$, respectively) (Table 1).

In our study, no treatment was required in all five patients who developed a grade 2 ISR with erythema and phlebitis, which resolved within a median of 7 d (range, 3–23 d). The median BMI was 21.6 ± 2.2 (range, 18.3–26.7) kg/m². There was no significant difference in BMI among the five patients with grade 2 ISR (20.9 ± 1.6 kg/m²) compared with ten patients with grade 0–1 ISR (21.9 ± 2.4 kg/m²) ($P = 0.62$). Patient 9, a representative case, (Fig. 1) developed grade 1 ISRs in the left upper and lower abdomen after the 1st and 2nd scBor injections (Fig. 1A,B); both ISRs resolved rapidly. Two days after the 3rd injection on the proximal left thigh erythema spread to a larger area accompanied with phlebitis (Fig. 1C), which progressed rapidly to grade 2 ISR (Fig. 1D) but resolved without therapy 24 d after the 3rd injection. The same patient developed grade 2 ISR after the 4th injection at the distal site of the left thigh, which also resolved without treatment. Besides ISR, scBor-induced adverse events such as major hematologic and non-hematologic toxicity profiles were analyzed, as shown in Table 2. Three of 15 patients (20%) developed grade 3 neutropenia and anemia; there were no signs of PN in any of the seven patients with newly diagnosed MM. In contrast, grade 2 and grade 3 PN were documented after scBor treatment in three and one patients, respectively, with relapsed/refractory MM. Significantly, all four of these patients had previously experience ivBor-induced PN prior to scBor treatment. One patient (no.7) with relapsed/refractory MM, who had previously developed grade 2 ivBor-induced PN, discontinued scBor treatment due to the exacerbation of PN

Table 1 Patient characteristics and summary of ISR after treatment with scBor

No	Age	Sex	Disease status	ivBor prior to scBor	Regimen including scBor	Total dose of scBor (mg/sqm)	Total number of scBor (abdomen/thigh)	Total number of ISR			Onset of grade 2 ISR	Maximum duration of ISR (days)	BMI at the start of scBor
								Grade 0 (abdomen/thigh)	Grade 1 (abdomen/thigh)	Grade 2 (abdomen/thigh)			
1	57	M	RR	Yes	scBD ¹ →scVCD ²	30.6	27 (14/13)	19 (8/11)	8 (6/2)	0	—	4	20.3
2	69	F	New	No	scBD	10.4	8 (3/5)	6 (2/4)	2 (1/1)	0	—	5	19.7
3	72	F	RR	Yes	scBD	21.6	25 (13/12)	13 (8/5)	12 (5/7)	0	—	3	21
4	64	M	New	No	scVCD	15.6	12 (6/6)	10 (5/5)	2 (1/1)	0	—	4	24.6
5	69	M	New	No	scVCD	5.2	4 (2/2)	1 (1/0)	2 (1/1)	1 (0/1)	3rd injection	5	20.7
6	54	M	New	No	scVCD	10.4	8 (4/4)	5 (3/2)	3 (1/2)	0	—	5	23.3
7	70	F	RR	Yes	scVCD	8	8 (4/4)	8 (4/4)	0	0	—	—	21.9
8	79	F	RR	No	scBD→scVCD	10.4	8 (5/3)	4 (3/1)	3 (2/1)	1 (0/1)	3rd injection	10	21.7
9	65	F	New	No	scVCD	10.4	8 (4/4)	3 (2/1)	3 (2/1)	2 (0/2)	3rd and 4th injections	23	21.2
10	78	M	RR	Yes	scBD	18.2	14 (12/2)	14 (12/2)	0	0	—	—	26.7
11	85	F	RR	Yes	scVCD	20.8	16 (14/2)	16 (14/2)	0	0	—	—	18.7
12	62	F	RR	No	scVCD	10.4	8 (6/2)	4 (4/0)	2 (2/0)	2 (0/2)	3rd and 4th injections	7	18.3
13	81	M	New	No	scVCD	5.2	4 (2/2)	4 (2/2)	0	0	—	—	22
14	72	M	New	No	scVCD	5.2	4 (2/2)	4 (2/2)	0	0	—	—	20.7
15	79	F	RR	No	scBD	5.2	4 (2/2)	0	3 (1/2)	1 (1/0)	3rd injection	5	22.6
Total							158 (93/65)	111 (70/41; <i>P</i> = 0.099 ³)	40 (22/18; <i>P</i> = 0.87 ³)	7 (1/6; <i>P</i> = 0.014 ³)			

ivBor, intravenous bortezomib; scBor subcutaneous bortezomib; ISR, injection site reaction; BMI, body mass index; RR, relapse/refractory; New, Newly diagnosed.

¹scBD, subcutaneous bortezomib 1.3 mg/sqm and dexamethasone 40 mg on day 1, 8, 15, and 22.

²scVCD, subcutaneous bortezomib 1.3 mg/sqm, cyclophosphamide 500 mg/sqm and dexamethasone 40 mg on day 1, 8, 15, and 22.

³Frequencies of ISR in the thigh were compared with those in the abdomen using the χ^2 -test.



Figure 1 A 65-yr-old female (patient no.9) with skin erythema, (A) classified as grade 1 injection site reaction (ISR), 2 d after the 1st subcutaneous Bor (scBor) injection on the left upper abdomen, (B) Skin erythema quickly resolved without treatment 3 d after the injection, (C) 2 d after the 3rd scBor injection on the proximal left thigh. (D) Three days after 3rd injection, skin erythema recurred and progressively increased in size with phlebitis and finally progressed to grade 2 ISR. This ISR lesion did not expand further; therefore, no treatment was required, and ISR resolved after 24 d. (E) Pigmentation persisted 43 d after the 3rd scBor injection.

Table 2 Major hematologic and non-hematologic toxicities

	n (%)
Thrombocytopenia (grade 3)	0
New grade 3 thrombocytopenia	0
Grade 3 baseline thrombocytopenia	0
Neutropenia (grade 3)	3 (20)
New grade 3 neutropenia	2 (13)
Grade 3 baseline neutropenia	1 (7)
Anemia (grade 3)	3 (20)
New grade 3 anemia	0
Grade 3 baseline anemia	3 (20)
PN (new/worse)	0/4
Grade 1/2 PN	3 (13)
Grade 3/4 PN	1 (7)
Infection (grade 3)	2 (13)
Liver dysfunction (> grade 3)	0 (0)

PN, Peripheral neuropathy.

The grading is according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 4.0

(grade 3) after eight times of scBor injection (four times each in the abdomen and the thigh), indicating no relationship between exacerbation of PN and sites of scBor injection. Two patients developed grade 3 infections, and none of the patients developed \geq grade 3 liver dysfunction.

Discussion

Moreau *et al.* demonstrated that scBor shows efficacy comparable with ivBor but is associated with a considerably lower incidence and severity of PN (7–9). In their study, severe ISR could be avoided by rotating the sc injection sites among 8 different sites on the thighs and abdomen (8). Moreover, they confirmed that there was no difference in the serum concentration of Bor after sc injections in the thigh and abdomen (8). Based on these results, the safety profile

of scBor is significantly better than that of ivBor; therefore, scBor injection is becoming increasingly widespread. We demonstrate that scBor was a safe and tolerable administration route with mild toxicity in Japanese patients, although the sample size was small.

Obeid *et al.* recently reported a case of severe grade 3 ISR following scBor injection, raising the concern of scBor-induced ISR (14). In their report, a small boil-like lesion occurred at the scBor injection site in the abdomen, which progressively increased in size and finally covered the entire abdominal wall with necrosis and induration. The ISR lesion responded well to intravenous methylprednisolone, suggesting that this was an immune-mediated phenomenon after considering the pathological diagnosis of the biopsied skin lesion (14). Our study identified grade 1 and 2 ISRs, which resolved rapidly and spontaneously, in 10/15 (66.7%) and 5/15 (33.3%) Japanese patients treated by scBor. We also previously reported that early intervention with topical steroids was effective for improving skin lesions rapidly in 1 patient with grade 2 ISR, without the need for systemic administration of methylprednisolone (15). Therefore, we consider that further investigation of ISR following scBor injection is needed to prevent and manage ISRs appropriately.

This is the first report to show that moderate-to-severe ISR was more common at the thigh than abdomen after scBor injection: \geq grade 2 ISR occurred in 6 of 65 (9.2%) sc thigh injections compared with 1 of 93 (1.1%) sc abdomen injections ($P = 0.014$). Computed tomography analysis shows that sc adipose tissue is typically much less abundant in the thigh than in abdomen (16). Therefore, local sc concentration of Bor may be higher in the patients receiving scBor on their thigh because Bor may not be thoroughly diluted in the surrounding sc tissues due to the relatively lower fat content. It is possible that a higher Bor concentration may result in the higher incidence and severity of ISR on the thigh.

Our results suggest that the severity of ISR may be associated with the amount of sc fat. However, we did not find any correlation between the severity of ISR and BMI of the individuals in our study. Moreau *et al.* did not address the relationship between the BMI and ISR, although BMI did not affect the serum concentration of Bor among the patients receiving scBor on the thigh or abdomen (11). Therefore, it is possible that scBor-induced ISRs are closely associated with the local sc fat distribution at the injection site rather than systemic fat burden reflected by BMI. Thus, scBor may frequently induce severe ISR, especially in emaciated patients with low BMI or relatively slender patients, if scBor is administered on the thigh. In our study, however, the ISRs disappeared within 10 d without sequelae in the majority of patients. In addition, the recommended schedule of Bor is generally 1.3 mg/m² on day 1, 4, 8, and 11 in 21-d cycles or day 1, 8, 15, 22 in 35-d cycles. If we choose to inject scBor at only four abdominal sites once or twice per week, each injection site will be used once every 21 or 35 d. Despite the small number of patients and samples or the absence of randomized data, our study still would suggest that four different sites at the abdomen, avoiding the thigh, would be feasible and more suitable for scBor injection because ISR may be less common and severe and rapidly disappear within the interval before the next injection. In addition, early treatment with topical steroids can be used to effectively and rapidly treat ISRs when they do occur (15). Further large, prospective studies are required to assess the safety and efficacy of scBor.

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HEMATOPOIESIS AND STEM CELLS

Polymorphic *Sirpa* is the genetic determinant for NOD-based mouse lines to achieve efficient human cell engraftment

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Key Points

- NOD-specific *Sirpa* polymorphism is the genetic determinant of highly efficient xenograft activity in NOD-based immunodeficient mouse models.

Current mouse lines efficient for human cell xenotransplantation are backcrossed into NOD mice to introduce its multiple immunodeficient phenotypes. Our positional genetic study has located the NOD-specific polymorphic *Sirpa* as a molecule responsible for its high xenograft efficiency: it recognizes human CD47 and the resultant signaling may cause NOD macrophages not to engulf human grafts. In the present study, we established C57BL/6.*Rag2*^{null}/*Il2rg*^{null} mice harboring NOD-*Sirpa* (BRGS). BRGS mice engrafted human hematopoiesis with an efficiency that was equal to or even better than that of the NOD.*Rag1*^{null}/*Il2rg*^{null} strain, one of the best xenograft models. Consequently, BRGS mice are free from other NOD-related abnormalities; for example, they

have normalized C5 function that enables the evaluation of complement-dependent cytotoxicity of antibodies against human grafts in the humanized mouse model. Our data show that efficient human cell engraftment found in NOD-based models is mounted solely by their polymorphic *Sirpa*. The simplified BRGS line should be very useful in future studies of human stem cell biology. (*Blood*. 2013;121(8):1316-1325)

Introduction

Immunodeficient mice are widely used to reconstitute human hematopoiesis by xenotransplantation of hematopoietic stem cells (HSCs).^{1,2} This “humanized” mouse model provides a powerful tool with which to evaluate the biologic properties of human HSCs and progenitors *in vivo*.^{3,4} Such xenotransplantation systems have also been used to study human cancer stem cells.⁵⁻⁸

Elimination of the lymphoid system is the first step to achieving reconstitution of human hematopoiesis. To deplete T and B cells, the *scid* mutation in the *Prkdc* gene⁹⁻¹¹ or disruption of the recombination activating gene 1 or 2 (*Rag1* and *Rag2*)^{12,13} has been introduced into various mouse strains. In addition, to deplete natural killer (NK) cells or their functions, the IL-2 receptor common γ chain subunit (*Il2rg*)¹⁴⁻¹⁶ or beta-2-microglobulin (*B2m*)¹⁷⁻¹⁹ is disrupted.

However, depletion of lymphoid cells is not sufficient and it has been shown empirically that additional strain-specific factors modulate human hematopoietic engraftment in the xenotransplantation setting. For example, within the SCID strain, the SCID with the NOD background was the gold standard for the xenotransplantation assay based on its high efficiency.¹¹ In fact, recent studies have shown that among the lymphoid-depleted mouse strains, the NOD-*scid Il2rg*^{null} (NSG/NOG)^{14,15} and NOD.*Rag1*^{null}/*Il2rg*^{null}

(NOD-RG)²⁰ strains are the most efficient; the BALB/c.*Rag2*^{null}/*Il2rg*^{null} (BALB-RG) strain is the next efficient^{21,22}; and the C57BL/6 strains with *scid*,²³ *Rag2*^{null}, *Rag2*^{null}/*B2m*^{null}, *Rag2*^{null}/*Pf4*^{null},²⁴ or *Rag2*^{null}/*Jak3*^{null}²⁵ mutations are unable to reconstitute human hematopoiesis. The NOD strain has multiple immune deficiencies, including defects of appropriate regulation of the T-lymphocyte repertoire, antigen presenting cell function, NK cell function,²⁶ and hemolytic complement (C5) and cytokine production from macrophages,²⁷ and these abnormalities are presumed to collaborate to cause the development of autoimmune diabetes and hemolytic anemia.^{26,28} To establish xenotransplantation models, lymphoid-depleted strains have been backcrossed into the NOD/ShiLt–inbred strain multiple times to introduce such numerous NOD-specific abnormalities.^{14,15} However, it was unknown whether we could select a genetic determinant(s) specially required to achieve the NOD-specific high engraftment capability for human cells.

Previously, we used positional genetics to characterize the molecular basis for this capability in the NOD strain by measuring the ability of mouse BM stromal layers to support hematopoietic long-term culture-initiating cell activity (LTC-IC) *in vitro* and identified the strain differences as the polymorphism of the *Sirpa* gene located within the insulin-dependent diabetes (*Id4-13*) locus.²⁴

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Stroma cells from the NOD BM supports LTC-IC of human cells, but those from C57BL/6 could not. Enforced expression of the NOD-type SIRPA enabled C57BL/6 stroma cells to support human LTC-IC.²⁴ This in vitro finding is also applicable to the in vivo setting, as shown by another study in which a human SIRPA BAC transgene introduced into *Rag2^{null}Il2rg^{null}* mice on a mixed 129; BALB/c background significantly improved the efficiency of human hematopoietic engraftment.²⁹

SIRPA is a transmembrane protein that contains 3 Ig-like domains within the extracellular region. It is expressed in macrophages, myeloid cells, and neurons, and interacts with its ligand CD47 through its respective IgV-like domains, where the NOD strain has specific polymorphism. CD47 is a member of the Ig superfamily that is ubiquitously expressed in hematopoietic and nonhematopoietic cells. The cytoplasmic region of SIRPA has immunoreceptor tyrosine-based inhibitory motifs, and binding cell-surface CD47 with SIRPA on macrophages provokes inhibitory signals through phosphorylation of these inhibitory motifs of SIRPA,³⁰ preventing their phagocytic activity.³¹⁻³³ A recent study also showed that transgenic expression of mouse CD47 into CD34⁺CD38⁻ human fetal liver cells significantly enhanced the human cell engraftment into BALB-RG mice.³⁴ Based on these data, the binding of NOD-SIRPA with human CD47 might produce signals for mouse macrophages not to engulf human HSCs, which presumably makes the strain permissive for human HSC engraftment.²⁴

The most important question was whether the NOD-specific highly efficient human cell engraftment in vivo could be explained solely by the NOD-*Sirpa* polymorphism. In the present study, we established a C57BL/6.*Rag2^{null}Il2rg^{null}* (C57BL/6-RG) mouse line harboring the NOD-type *Sirpa*. Our data show clearly that replacement of the C57BL/6-type *Sirpa* with the NOD-type *Sirpa* is sufficient for the C57BL/6-RG strain to be endowed with the xenotransplantation capability that is at least equal to NOD-RG mice. Therefore, we successfully segregated the genetic abnormality responsible for efficient human cell engraftment from multiple genetic abnormalities in the NOD strain. The simplified humanized mouse system established by the new C57BL/6.*Rag2^{null}Il2rg^{null}*NOD-*Sirpa* (BRGS) strain should be very useful in improving xenotransplantation strategies in future studies of human cell biology.

Methods

Mice

C57BL/6, C57BL/6.NOD-*Idd13*, NOD, NOD.CB17-*Prkdc^{scid}* (NOD-*scid*), and NOD.Cg-*Rag1^{tm1.1Mom}Il2rg^{tm1.1Wjl}/Sz* (NOD-RG) mice were purchased from the Jackson Laboratory; C57BL/6.*Rag2^{null}Il2rg^{null}* (C57BL/6-RG) mice were purchased from Taconic. All mice were bred and maintained in individual ventilated cages at the Kyushu University Animal Facility and fed with autoclaved food and water. BRGS mice were generated by breeding C57BL/6-RG and C57BL/6.NOD-*Idd13* mice and backcrossed with C57BL/6-RG mice. *Rag2* gene and *Sirpa* gene are located on chromosome 2 with 17.1 cM. First, we repeated the breeding of C57BL/6-RG and C57BL/6.NOD-*Idd13* mice, and after 10 breedings, we obtained the recombination between the *Rag2*⁻ and the *Sirpa*^{NOD} loci by chromosomal crossover. This was examined by genotyping by the microsatellite markers *D2Mit447* and *D2Mit338*, which are 0.63 cM apart on chromosome 2, during interbreeding. In addition, *Sirpa*, *Rag2*, and *Il2rg* were genetically typed by PCR and direct sequencing. In C57BL/6.NOD-*(D2Mit447-D2Mit338)* *Rag2^{null}Il2rg^{null}* mice, the region between *D2Mit447* and *D2Mit338* contains 33 genes, including *Sirpa*, but *Sirpa* is the only gene

within the *Idd13* locus that is expressed in BM stromal cells and macrophages and had coding sequence polymorphism between the NOD and other strains.²⁴ Therefore, we refer to our established mouse line as BRGS herein. Sequences of the oligonucleotide primers used are provided in supplemental Table 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). All experiments were conducted following the guidelines of the institutional animal committee of Kyushu University.

Binding affinity of mouse macrophages to human CD47-Fc

Mouse macrophages were obtained by peritoneal lavage. Cells were stained with purified anti-mouse *Sirpa* (P84; BD Biosciences) conjugated with PE and anti-mouse CD11b (3A33; Beckman Coulter) conjugated with FITC. CD11b⁺SIRPA⁺ cells were defined as mature macrophages. The binding between SIRPA and CD47 was assessed by staining with biotinylated human CD47-Fc conjugated with streptavidin-allophycocyanin (APC),³⁵ and analyzed with a FACSAria III cell sorter (BD Biosciences).

In vitro mouse macrophage phagocytosis assays for human hematopoietic stem cells

Phagocytic activity of mouse macrophages against the human CD34⁺CD38⁻ population that contains the majority of human HSCs was evaluated in vitro, as described previously.³⁶ In brief, mouse peritoneal-derived macrophages were incubated at 1.0 × 10⁴ cells in 200 μL of RPMI 1640 medium in Falcon culture tubes (2058; BD Biosciences). Cells were opsonized with CD34 antibody (sc-19621; Santa Cruz Biotechnology), incubated with mouse IFN-γ (100 ng/mL; R&D Systems) for 24 hours, and then lipopolysaccharide (0.3 μg/μL) for 1 hour. Human cord blood (CB) HSCs were then added to the tubes. Two hours after cocubation with macrophages and target cells, the phagocytic index was calculated using the following formula: phagocytic index = number of ingested cells/(number of macrophages/100). At least 200 macrophages were counted by a blinded observer.

Sensitivity of BRGS mice to irradiation

Cohorts of BRGS mice were exposed to varying doses of the whole-body irradiation from a ¹³⁷Cs γ-irradiator. The mice were examined daily and euthanized when moribund. Surviving mice were euthanized at 8 weeks after irradiation. NOG/NSG mice are highly radiosensitive because of the *scid* mutation. To examine the radiosensitivity of BRGS mice, 6- to 10-week-old BRGS mice were irradiated with 550-670 cGy, and monitored for 8 weeks. Early deaths were observed in the mouse group irradiated with more than 620 cGy, whereas those irradiated with 550-580 cGy survived at the end of 8 weeks. Based on these data, we irradiated BRGS mice at 580 cGy in all xenotransplantation experiments. The irradiation doses for experiments with NOD-RG (420 cGy) and C57BL/6-RG (670 cGy) were decided by radiosensitivity experiments.

Transplantation of human HSCs into mice

CB cells were collected during normal full-term deliveries after obtaining informed consent in accordance with the Declaration of Helsinki (provided by the Kyushu Block Red Cross Blood Center, Japan Red Cross Society). Mononuclear cells were separated by Ficoll-Hypaque density-gradient centrifugation. Lineage-depleted CB cells were obtained magnetically using a lineage cell depletion kit (Miltenyi Biotec). A total of 5 × 10³ CD34⁺CD38⁻ cells were injected intravenously into mice. Within an individual experiment, mice of each strain received CD34⁺CD38⁻ cells purified from the same mixture of CB cells from multiple donors. After transplantation, mice were given sterile water containing prophylactic enrofloxacin (Baytril; Bayer HealthCare). Mice were killed 8, 16, or 24 weeks after transplantation.

Antibodies, cell staining, and sorting

For the analyses of mouse T, B, and NK cells, mouse peripheral blood cells were stained with PE-conjugated anti-CD3 (145-2C11), FITC-conjugated

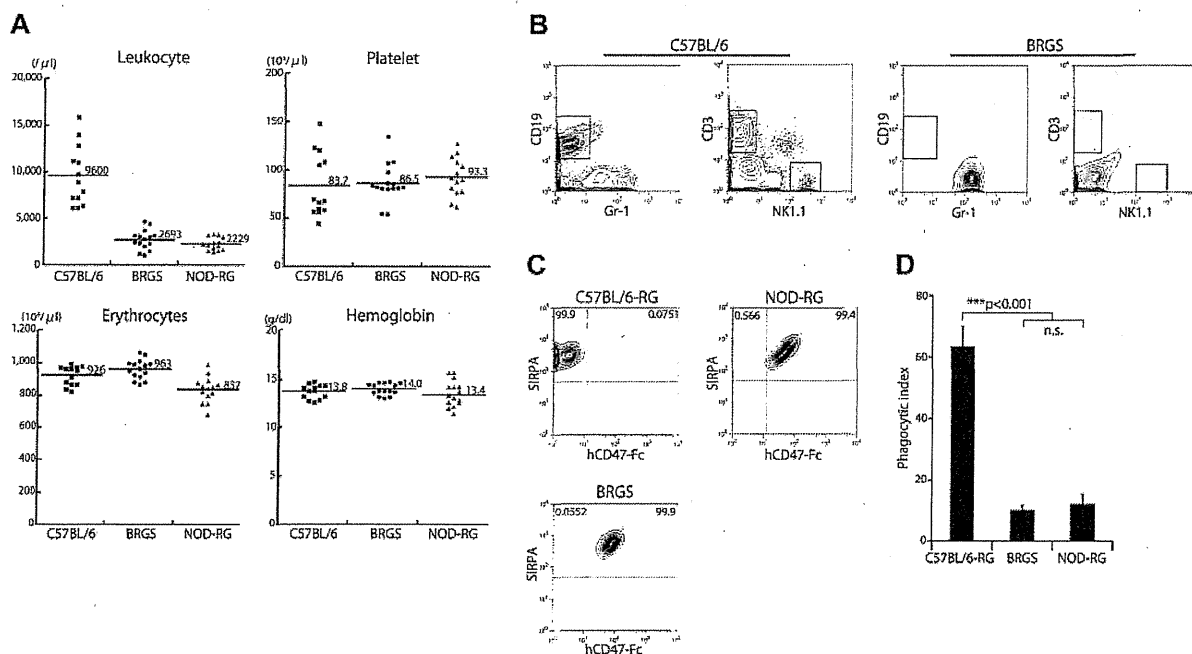


Figure 1. BRGS mice lack lymphocytes and SIRPA recognizes human CD47-Fc. (A) Frequencies of blood leukocytes, erythrocytes, hemoglobin, and platelets in BRGS mice. Leukocyte counts in BRGS ($2.69 \pm 1.01 \times 10^9/\mu\text{L}$) and NOD-RG mice ($2.23 \pm 0.7 \times 10^9/\mu\text{L}$) are significantly decreased compared with that in C57BL/6 mice ($9.6 \pm 0.32 \times 10^9/\mu\text{L}$). BRGS mice have normal erythrocyte ($9.63 \pm 0.63 \times 10^9/\mu\text{L}$), hemoglobin ($14.0 \pm 0.6 \text{ g/dL}$), and platelet ($8.7 \pm 2.0 \times 10^9/\mu\text{L}$) counts. (B) Representative FACS plots of blood in C57BL/6 and BRGS mice. BRGS mice lacked T, B, and NK cells. (C) Binding activity of human CD47-Fc to SIRPA expressed in peritoneal macrophages derived from C57BL/6-RG, BRGS, or NOD-RG mice. Macrophages from BRGS and NOD-RG mice, but not those from C57BL/6-RG mice, were stained with human CD47-Fc on FACS. (D) Phagocytosis assay of C57BL/6-RG, BRGS, or NOD-RG macrophages against human CD34⁺CD38⁻ CB HSCs ($n = 3$). The phagocytic index was determined as the number of engulfed cells per 100 macrophages. Bars indicate mean \pm SD.

anti-CD19 (1D3), APC-conjugated anti-NK1.1 (PK136; BD Biosciences), and Pacific Blue-conjugated anti-Gr-1 (RB6-8C5; BioLegend). Sorting of CD34⁺CD38⁻ subfractions was accomplished by staining lineage-depleted CB cells with FITC-conjugated anti-CD34 (581/CD34) and PE-conjugated anti-CD38 (HIT2; BD Biosciences). For analysis and sorting of human cells in the immunodeficient mice, FITC-conjugated anti-CD4 (RPA-T4), CD33 (HIM3-4), CD41a (HIP8), TCR $\alpha\beta$ (WT31), TCR $\gamma\delta$ (11F2), Ig λ L chain (JDC-12), Ig κ L chain (G20-193; BD Biosciences), anti-CD10 (SS2/36; Dako), PE-conjugated anti-CD8 (RPA-T8), CD20 (2H7), Nkp46 (9E2; BD Biosciences), CD235a (JC159; Dako), PE-Cy7-conjugated anti-CD3 (SK7; BD Biosciences), CD19 (HIB19; BioLegend), APC-conjugated anti-CD45 (J33; Beckman Coulter), and PaB-conjugated anti-mouse CD45 (30-F11; BioLegend) monoclonal antibodies were used in addition to the antibodies described in the preceding paragraph. Nonviable cells were excluded by propidium iodide staining. The cells were analyzed and sorted with a FACSAria cell sorter (BD Biosciences).

Complement-dependent hemolytic activity

To estimate the serum complement activity of mice, the peripheral blood of mice were collected in 1.5-mL tubes and allowed to stand at room temperature for 1 hour. The serum was collected after centrifugation of the blood at 200g for 15 minutes at 4°C and stored -80°C until use. The mixtures of each diluted sera of mice, 3.75×10^6 erythrocytes of sheep and 2.5 μg of zymosan (Imgenex) were incubated 10 hours at 37°C. After incubation, the absorbance of each sample at 415 nm was measured.

In vivo antibody treatment in a disseminated lymphoma xenograft model

A total of 8×10^5 Raji cells (Burkitt lymphoma cell line; American Type Culture Collection) were injected into BRGS or NOD-RG mice (6–10 weeks of age) via the tail vein. Raji cells proliferated predominantly in the BM. Ten days after injection, these mice were IP injected daily with 200 μg of

rituximab or mouse IgG2a control for 1 week and then BM cells were collected and analyzed with the FACSAria III.

Statistical analysis

Data are presented as means \pm SD. The significance of the differences between groups was determined via the Student t test. For comparison of complement-dependent hemolytic activity among the mouse strains, repeated-measures ANOVA was performed.

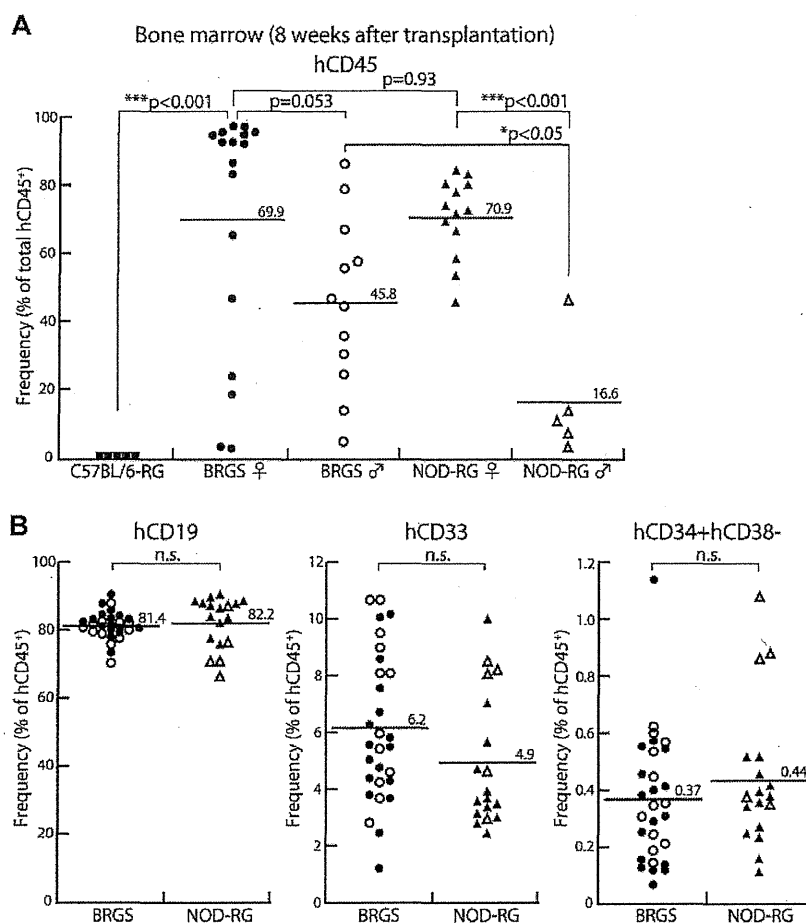
Results

Establishment of the BRGS mouse

The BRGS mouse line was established by breeding the C57BL/6-RG with the C57BL/6.NOD-*Idd13* mouse that is congenic for NOD-derived *Idd13* locus within which the *Sirpa* is the only gene that is polymorphic and is expressed in the BM stromal cells.²⁴ BRGS mice were all born healthy and displayed good fertility. They showed a median life span of 65 weeks without the development of lymphoma that usually occurs in the NOD-*scid* strain after the age of > 5 months.¹¹

As shown in Figure 1A, BRGS mice had normal levels of hemoglobin and platelets, but a low number of leukocytes. This is because of the lack of CD3⁺ T cells, CD19⁺ B cells, and NK1.1⁺ NK cells (Figure 1B). IP macrophages from either C57BL/6-RG, BRGS or NOD-RG mice were evaluated for the binding to human CD47 on FACS. CD11b⁺ peritoneal macrophages strongly expressed SIRPA in all of these strains. As shown in Figure 1C, both macrophages from the BRGS and those from the NOD-RG strain bound to the human CD47-Fc protein, whereas those from the

Figure 2. BRGS mice show efficient engraftment of human HSCs comparable to NOD-RG mice. In the BM, human HSC engraftment was examined by flow cytometric analysis 8 weeks after transplantation. C57BL/6-RG mice (■; n = 5), female BRGS mice (●; n = 17), male BRGS mice (○; n = 12), female NOD-RG mice (▲; n = 13), and male NOD-RG mice (△; n = 5) mice were analyzed. (A) Both BRGS and NOD-RG female mice showed excellent human CD45⁺ reconstitution. BRGS male mice showed significantly better engraftment compared with NOD-RG male mice. (B) Frequencies of CD19⁺ B cells, CD33⁺ myeloid cells, and CD34⁺CD38⁻ HSCs in BRGS and NOD-RG mice.



C57BL/6 strain did not, confirming that BRGS mice have the NOD-type SIRPA that can bind to human CD47. Consistent with these binding data, when macrophages of each strain were cultured with human CD34⁺CD38⁻ cells, macrophages from C57BL/6-RG mice, but not those from BRGS or NOD-RG mice, actively engulfed human CD34⁺CD38⁻ cells, as shown by the significant elevation of the phagocytic index in the C57BL/6-RG mice (Figure 1D).

BRGS mice are capable of multilineage reconstitution of human hematopoiesis with efficiency at least equal to that of NOD-RG mice

A recent study has shown that intrafemoral injection is more efficient than IV injection in the xenotransplantation setting.³⁷ We used intrafemoral injection into adult mice in the present study because our preliminary data also showed that human cell chimerisms of adult BRGS by intrafemoral injection was significantly better than those with IV injection (data not shown). We transplanted 5×10^3 CD34⁺CD38⁻ human CB cells intrafemorally into C57BL/6-RG, BRGS or NOD-RG mice at the age of 6-8 weeks. Before transplantation, C57BL/6-RG, BRGS, and NOD-RG mice were irradiated with 670, 580, and 420 cGy, respectively. Each dose was set by irradiation tolerance experiments (see the Methods).

At 8 weeks after transplantation, human CD45⁺ cells were not detectable in C57BL/6-RG mice (Figure 2A). Both BRGS and

NOD-RG showed successful reconstitution and their average frequencies of human CD45⁺ cells were 59.9% and 55.8%, respectively. Recent studies have shown that in the NSG strain,¹⁵ female recipients better support the reconstitution of human hematopoiesis, although the underlying mechanism for this remains unclear.^{38,39} As shown in Figure 2A, NOD-RG and BRGS female mice showed equally excellent human CD45⁺ reconstitution at approximately 70% chimerism. NOD-RG male mice, however, showed significantly poor reconstitution (16.6% of human cell chimerism on average) compared with NOD-RG female mice. In contrast, the percentages of human cell chimerisms in BRGS male mice (approximately 45%) were only slightly lower than those in BRGS female mice and, as a result, BRGS male mice showed significantly better engraftment compared with NOD-RG male mice.

In the BM, the percentages of CD19⁺ B cells, CD33⁺ myeloid cells, and CD34⁺CD38⁻ cells that contain the majority of human HSCs were almost equal between the BRGS and the NOD-RG strains irrespective of sex (Figure 2B). Representative FACS plots at 8 weeks after injection are shown in Figure 3A. In the spleen, small numbers of CD3⁺ T cells and CD3⁻NKp46⁺ NK cells, as well as CD41⁺ megakaryocytes and CD235a⁺ erythrocytes, were found in both BRGS and NOD-RG mice (Figure 3A) and there was no significant difference in the percentages of these cells between the 2 strains regardless of sex. The majority of human cells in the spleen were CD19⁺ B cells (Figure 3B). Although BM human

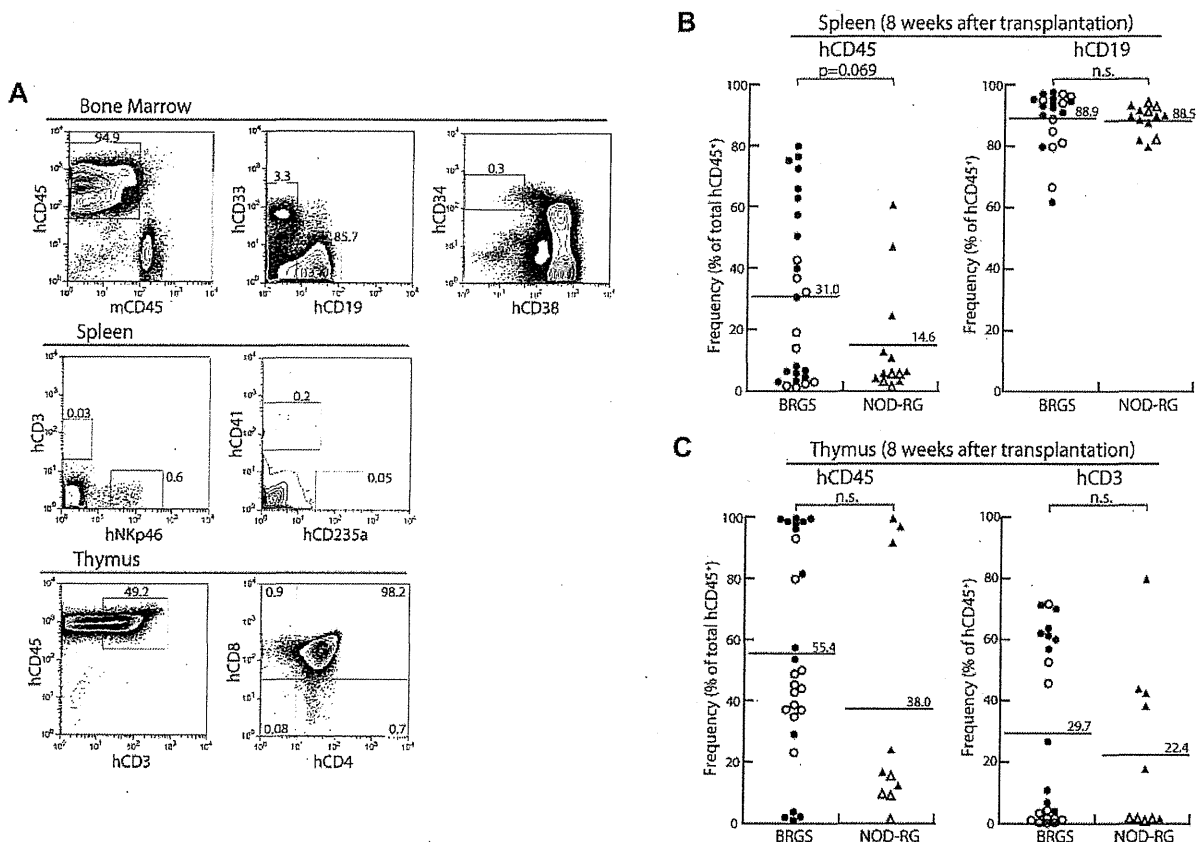


Figure 3. Multilineage human HSC reconstitution in BRGS mice. (A) Representative FACS plots at 8 weeks after transplantation in the BM, spleen, and thymus. (B) Human hematopoietic reconstitution in the spleens of BRGS and NOD-RG recipients (●: BRGS female; ○: BRGS male; ▲: NOD-RG female; △: NOD-RG male). There were no significant differences in the percentages of human CD45⁺ cells and human CD19⁺ B cells between these mice. (C) Human hematopoietic reconstitution in the thymi of BRGS and NOD-RG recipients. There were no significant differences in the percentages of human CD45⁺ cells and human CD3⁺ T cells between these mice. Symbols are as in panel B.

CD19⁺ cells were mainly CD10⁺CD20⁻ immature B cells, the majority (approximately 90%) of human spleen CD19⁺ cells were CD10⁻CD20⁺ mature B cells (data not shown). Thymic T cells were found in both the BRGS and NOD-RG strains, and the majority of human CD3⁺ T cells in the thymus were CD4⁺CD8⁺ immature T cells (Figure 3A,C).

Figure 4 shows the analysis of reconstitution of human HSCs at 16 weeks after transplantation. In this analysis, we used only female BRGS and NOD-RG mice. In the BM, both BRGS and NOD-RG mice showed sustained human cell engraftment and the frequencies of human CD45⁺ cells were 64.4% and 51.1% in average, respectively, which were comparable to their levels at 8 weeks after transplantation. The percentages of CD33⁺ myeloid cells, CD19⁺ B cells, and CD34⁺CD38⁻ HSCs were comparable to those at 8 weeks after transplantation (Figure 4A).

In the thymus, the percentage of CD3⁺ T cells was increased up to approximately 80% and approximately 60% in the BRGS and NOD-RG strains, respectively. In addition to CD4⁺CD8⁺ thymic precursors, both CD4⁺ and CD8⁺ single-positive T cells were present and expressed surface TCR- $\alpha\beta$ or TCR- $\gamma\delta$, suggesting that human T-cell maturation occurs in the BRGS thymus, as has been shown previously in the NOG, NSG, and NOD-RG mouse lines^{14-16,20} (Figure 4B). The number of CD20⁺ mature B cells in the spleen was increased and they expressed surface Ig light chain λ/κ , reflecting their normal maturation (Figure 4C).

The BRGS mouse maintains self-renewal of human HSCs in the long term

Figure 5A shows the changes in human cell chimerism in female BRGS mice in the long term. The frequency of human CD45⁺ cells was maintained at a high level at least until 24 weeks after transplantation. B-cell frequencies gradually declined, but human myeloid, T, and NK cells progressively increased after engraftment (Figure 5B). The delayed reconstitution of these lineages of human cells has also been reported in studies using NSG mice.^{40,41}

Figure 5C shows the results of the serial transplantation analysis. After confirmation of human cell engraftment at 8 weeks after the first transplantation, 1×10^6 human CD45⁺ cells were purified from primary BRGS recipients. These cells were transplanted into irradiated secondary BRGS recipients by intrafemoral injection and tested for engraftment after another 8 weeks. Four of 6 secondary BRGS recipients showed multilineage engraftment of human CD33⁺, CD19⁺, and CD3⁺ cells (Figure 5C). These data strongly suggest that BRGS mice can support long-term reconstitution and self-renewal of human HSCs.

The BRGS mouse is useful for experiments using CDC of antibodies in the xenotransplantation setting

One of the problems in NOD-based xenograft models is that the cytotoxic activities of antibodies are unable to be evaluated in vivo

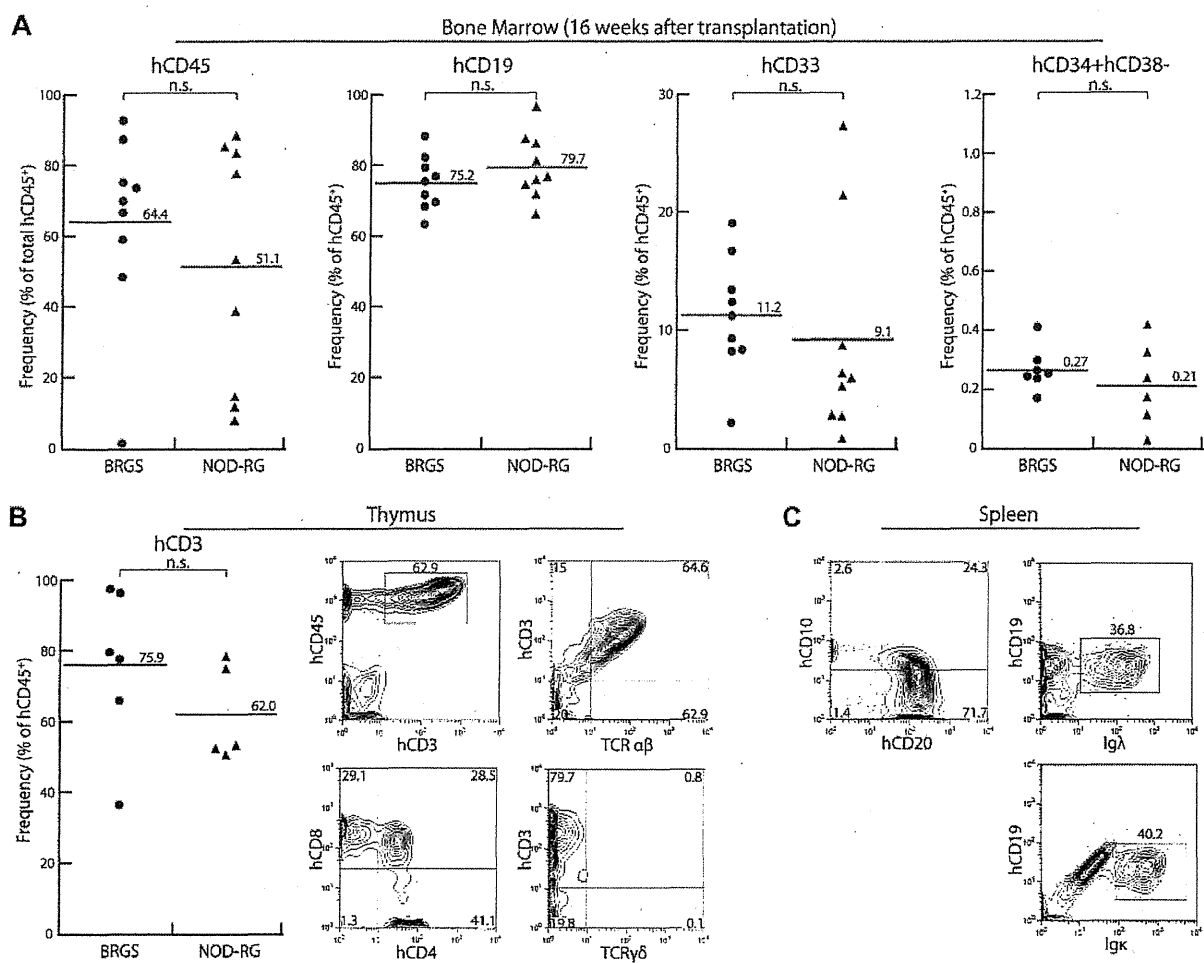


Figure 4. Human hematolymphoid reconstitution at 16 weeks after transplantation. (A) In the BM, BRGS mice showed sustained multilineage engraftment of human hematopoiesis at a level comparable to that in NOD-RG mice (●: BRGS female; ▲: NOD-RG female). (B) In the thymus, CD3⁺ T cells were developed and their frequencies were comparable in BRGS and NOD-RG mice. On FACS analysis, cells were differentiated into CD4⁺ and CD8⁺ single-positive T cells expressing the surface TCR- $\alpha\beta$ chain. (C) In the spleen, CD10⁺CD19⁺CD20⁺ mature B cells expressing surface Ig light chain λ or κ chain were present.

in humanized mice. First, antibody-dependent cell-mediated cytotoxicity (ADCC) does not operate efficiently in xenotransplantation experiments because these strains of mice are deficient in NK cells, the major player for ADCC. In addition, immunodeficient phenotypes of the NOD strain include complement-dependent hemolytic activity due to a deficiency of C5,⁴² which is essential for antibodies to exert complement-dependent cytotoxicity (CDC). All NOD-based immunodeficient strains have this abnormality, whereas the BRGS strain does not because it has a C57BL/6 background except for the NOD-type SIRPA. We tested CDC activity in C57BL/6-based strains, including the C57BL/6, C57BL/6-RG, and BRGS mice, and in NOD-based strains such as NOD, NOD-*scid*, and NOD-RG. As shown in Figure 6, sera from all of the C57BL/6-based strains, including the BRGS strain, showed CDC activities on sheep RBCs, whereas this was not found in any of NOD-based strains. There were no significant differences in CDC activities among the C57BL/6, C57BL/6-RG, and BRGS strains.

To determine whether BRGS mice had restored CDC in vivo, 8×10^5 cells of Raji, a Burkitt lymphoma cell line expressing human CD45, was injected into BRGS or NOD-RG mice. Ten days after transplantation, either rituximab, an anti-CD20 antibody that has both CDC and ADCC activities, or a control IgG2a antibody

was administered IP for 7 days (Figure 7A) and the effect of antibody injection on elimination of Raji cells was evaluated. Representative results are shown in Figure 7B. In mice injected with control IgG2a, Raji cells rapidly proliferated up to approximately 90% in the BM of both BRGS and NOD-RG mice. In contrast, by injection of rituximab, percentages of human CD45⁺ Raji cells were significantly decreased in BRGS mice (15.1%), whereas the percentages of human CD45⁺ cells in NOD-RG mice were only slightly reduced by rituximab treatment (79.2%). Representative FACS data are shown in Figure 7C. These data clearly show that the CDC activity of antibodies was able to operate in the BRGS strain.

Discussion

The NOD/ShiLt inbred mouse strain, which was originally developed by selecting cataract-prone strains,²⁷ exhibits susceptibility to the spontaneous development of autoimmune insulin-dependent diabetes mellitus (IDDM) and many other autoimmune disorders. The susceptibility to IDDM is polygenic and genetic loci associated with susceptibility to IDDM have been identified through the

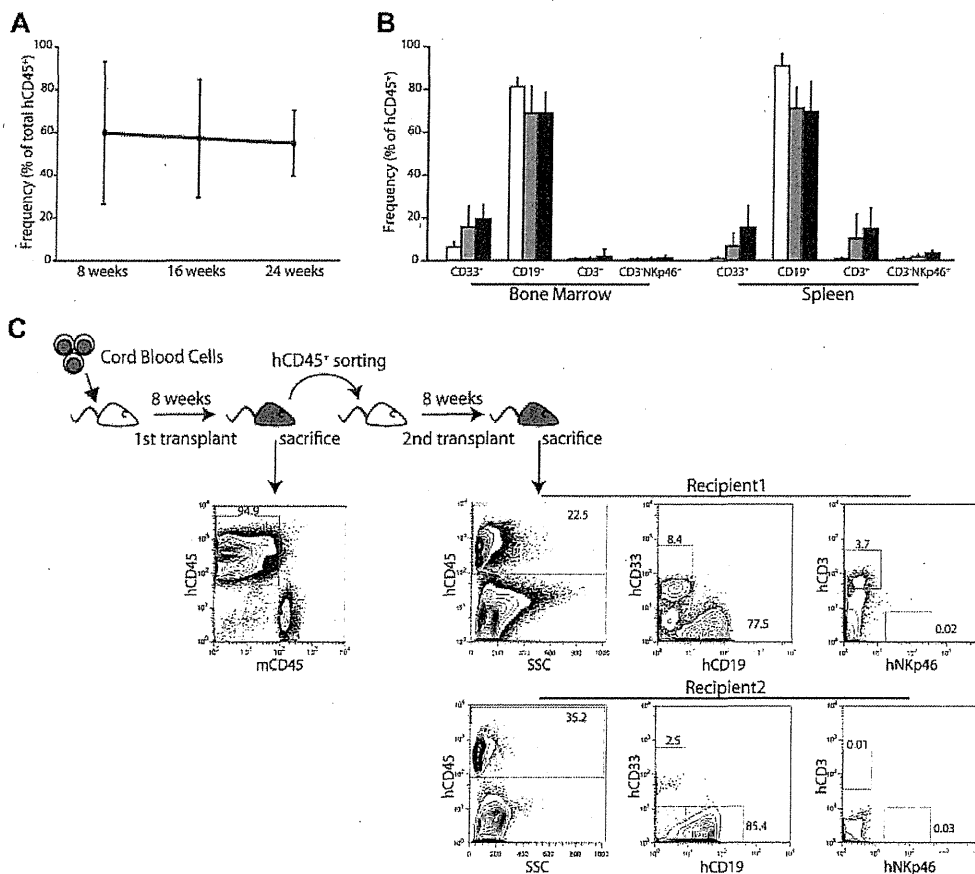


Figure 5. Evaluation of self-renewal of human HSCs in the BRGS mouse model. (A) Change in frequency of human CD45⁺ cells after transplantation. The level of human CD45⁺ cells was maintained at a high level until 24 weeks after transplantation (8 weeks, n = 29; 16 weeks, n = 17; and 24 weeks, n = 4). (B) Change in the frequency of human CD33⁺ myeloid cells, B cells, T cells, and NK cells in the BM and spleen during the 24 weeks after transplantation (□: 8 weeks; ■: 16 weeks; and ▨: 24 weeks). Note that the B-cell numbers gradually decreased and were compensated for by myeloid, T, and NK cells. (C) To test the self-renewal ability of human HSCs maintained in the first recipient mice, 1×10^6 human CD45⁺ cells were sorted from first-recipient mice and injected into second-recipient mice. Only female mice were used as recipients. After another 8 weeks, 4 of 6 BRGS secondary recipients showed multilineage engraftment of human CD33⁺, CD19⁺, and CD3⁺ cells. Representative FACS plots are shown.

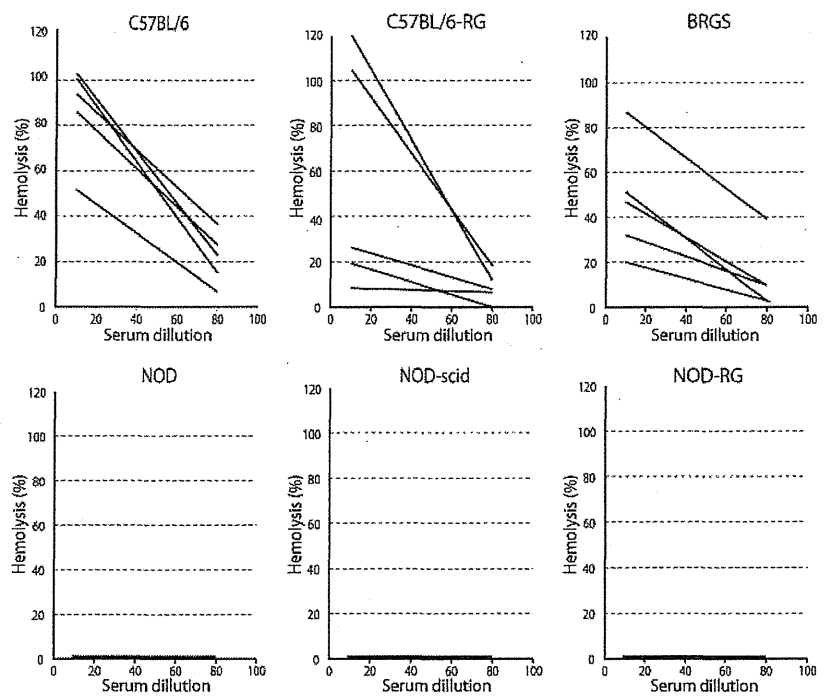
development of congenic mouse strains. More than 20 *Idd* loci have been identified. NOD mice display multiple aberrant immunophenotypes, and introduction of these abnormalities into immunodeficient mouse lines by multiple backcrossing accelerated human cell engraftment in xenotransplantation assays.^{11,14,15}

In the current study, we present formal proof that under disruption of T, B, and NK cells, NOD-specific *Sirpa* polymorphism could explain the efficient human cell engraftment in the NOD strain. We replaced the *Idd13* locus of C57BL/6-RG mice with that of B6. The NOD-*Idd13* mouse has the C57BL/6 background but is congenic for the NOD-derived 23cM segment of chromosome 2 extending from microsatellite marker *D2Mit274* through *D2Mit343*.⁴³ In a previous study, we resolved the sequence corresponding to the phenotype of support of human LTC-IC to a region of 960 kilobases, within which coding regions of 14 genes reside. *Sirpa* was the only gene within the *Idd13* locus expressed in BM stromal cells and macrophages and had coding sequence polymorphism between the NOD and other strains.²⁴ To determine whether the efficient human cell engraftment in the NOD strains was completely dependent on the NOD-type SIRPA polymorphism, we compared the engraftment efficiency of the BRGS mouse with the NOD-RG mouse as a control, because in both strains RAG and γ c genes are disrupted to disturb lymphoid cell development. The NOD-RG strain displays the excellent human

cell engraftment comparable to the NOG/NSG strain²¹ in which the SCID mutation instead of RAG-1 disruption is introduced. Our data show that the reconstitution activity of human hematopoiesis in BRGS mice is at least equal to that in NOD-RG mice in terms of engraftment levels and multilineage reconstitution. Therefore, replacement of the C57BL/6-*Sirpa* with the NOD-*Sirpa* is sufficient for the C57BL/6-RG strain to gain the human cell engraftment capability equal to the NOD-RG strain. NOD-SIRPA is able to bind human CD47, signaling of which inhibits activation of host macrophages to engulf human HSCs (Figure 1D), and therefore this signaling might be able to inhibit xenograft rejection.²⁴

The polymorphism of *Sirpa* could explain the strain-specific trend toward human cell acceptability in xenotransplantation experiments. There are 20 amino acid differences in the sequences of *Sirpa* IgV domain between the NOD and B6 strains. Among these, 5 amino acid residues are unique for NOD compared with C57BL/6, BALB/c, ICR, and C3H. By testing their binding affinity to human CD47 and their ability to support human LTC-IC, we found that the xenograft capability-related NOD-specific polymorphism can be aggregated to a single location of polymorphism (C.I., K.T., S.U., T.Y., K.I., J.K., T.M., K.A., The efficient engraftment of human hematopoiesis in the BALB/c strain is mounted by BALB/c-specific *Sirpa* polymorphism that enhances binding affinity to human CD47, manuscript in preparation). In

Figure 6. BRGS mice had CDC activity. Sera from BRGS and C57BL/6-based mice showed CDC activity, whereas none of the NOD-based strains did. Five mice were analyzed in each strain. There were no statistical differences in CDC activities among the C57BL/6-based strains.



addition, we found recently that Balb/c mice also have another polymorphism at the *Sirpa* IgV domain. Protein-binding assays show that C57BL/6-SIRPA never binds to human CD47, but Balb/c-SIRPA and NOD-SIRPA showed modest and very high binding affinity, respectively, correctly reflecting their strain-specific graft efficiencies.⁴⁴ Furthermore, a recent study has shown that the enforced expression of human SIRPA by a human BAC transgene enables the 129;Balb/c.*Rag1^{null}Il2rg^{null}* mouse to engraft

human cells as efficiently as the NSG mouse.²⁹ Therefore, in xenograft models, the degree of SIRPA-CD47 interaction decided by *Sirpa* polymorphism is one of the most critical factors to achieve efficient human cell engraftment. Further study is required to understand how the different binding affinity between these mouse polymorphic SIRPAs and human CD47 is translated into cytoplasmic signaling that leads to respective efficiency for xenotransplantation capabilities.

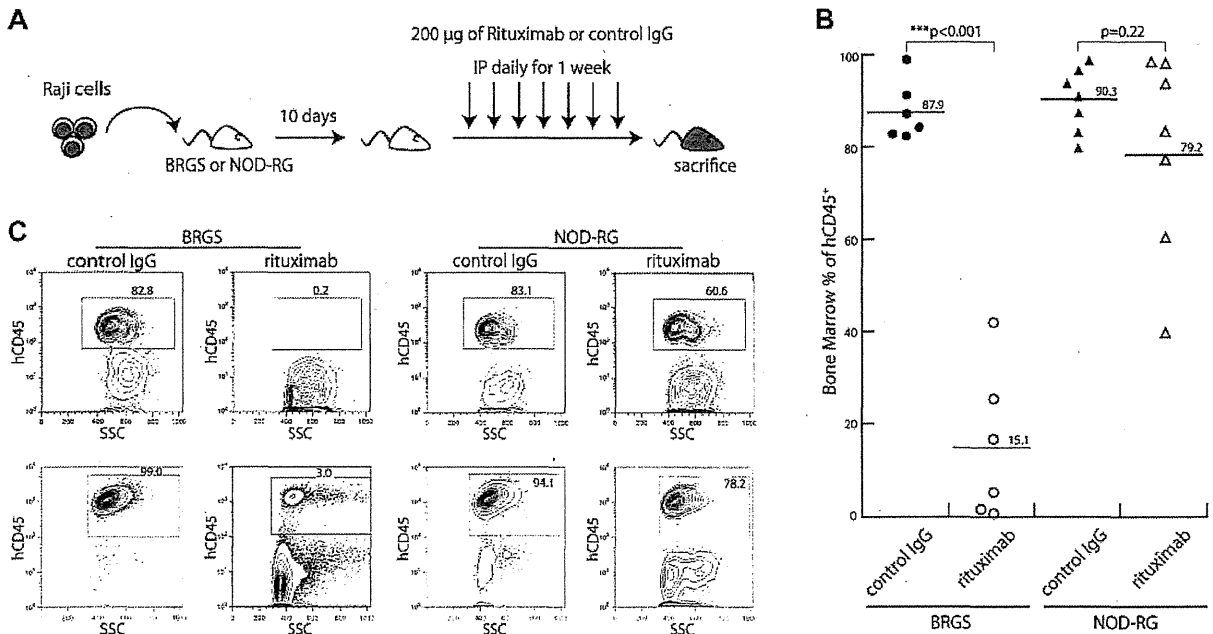


Figure 7. CDC activity of antibodies is evaluable in vivo in the BRGS xenogeneic model. (A) Experimental scheme of this experiment. Raji cells were injected into mice via the tail vein. Ten days after the injection, either rituximab or control IgG2a antibody (200 μ g each) was injected IP daily for 1 week. (B) Frequencies of human CD45⁺ Raji cells in the BM of BRGS and NOD-RG mice with or without rituximab injection. A significant reduction of Raji cells was found only in BRGS mice injected with rituximab. (C) Representative FACS plots of the BM cells of BRGS and NOD-RG mice after injection of rituximab or control IgG.

Several recent studies have shown that, in xenograft models, female mice somehow present significantly better reconstitution than do male mice.^{38,39} It remains unclear whether sex-related factors such as steroid hormones can affect the engraftment of human HSCs. In the present study, the human cell chimerism obtained in the BRGS strain was quite high, reaching > 90% in 9 of 17 BRGS female mice, but none of the 13 NOD-RG female mice achieved that level at 8 weeks after transplantation (Figure 2A). Furthermore, although BRGS male mice displayed lower levels of human chimerism (approximately 45%), NOD-RG male mice showed significantly lower levels than did NOD-RG female mice, reaching only < 20% of human cell chimerism in average on our conditions (Figure 2A). As a result, the human cell chimerism in BRGS male mice was significantly better than that in NOD-RG male mice. Therefore, the BRGS mice showed a trend toward higher levels of human chimerism in both the males and the females. These results may suggest that unknown genetic abnormalities antagonizing human cell reconstitution can exist outside of the *Idd13* locus in the NOD strain.

There remain many unknown factors that affect the efficiency of human cell reconstitution in mouse xenotransplantation models. For example, the BRGS model is capable of long-term, multilineage human hematopoietic reconstitution, but human myeloid, T, and NK cell reconstitution were significantly delayed compared with the B-cell lineage (Figure 5B). This pattern of reconstitution is commonly observed in other xenotransplantation models.^{40,41} Since the introduction of human cytokines such as thrombopoietin and membrane-bound SCF into humanized mouse models,^{45,46} myeloid reconstitution has been accelerated, so the delay could have been due to insufficient cross-reactivity of mouse cytokines with human cytokine receptors. It is also possible that the mouse hematopoietic microenvironment, including putative myeloid or lymphoid niches, is not appropriate for human HSC development. The elucidation of such unknown factors is necessary to develop further efficient xenotransplantation models for future studies.

We have also shown herein the usefulness of the BRGS line in testing the function of killing antibodies via CDC activity. Because rituximab has both ADCC and CDC activity⁴⁷ and because NK cells, the major player for ADCC, are absent in efficient xenograft models such as NOG,¹⁴ NSG,¹⁵ and NOD-RG²⁰ mice, the disappearance of Raji cells after rituximab injection in the BRGS system must have been dependent largely on its CDC activity. Selective cell depletion by killing antibodies should be very useful in xenograft experiments, for example, in targeting cancer stem cells,⁴⁸ and in removing specific human cell component(s) from reconstituted human hematology in vivo. Therefore, the BRGS humanized mouse model is applicable to future, more sophisticated xenograft experiments.

In summary, in the present study, we selected NOD-type polymorphic *Sirpa* from multiple abnormalities within the NOD

background and introduced it into the common C57BL6 mouse line together with *Rag2^{null}Il2rg^{null}* mutations. The xenograft efficiency of the BRGS line was equal to, or even better than, the NOD-RG line, which is currently one of the best xenograft models. This result formally proves that NOD-specific *Sirpa* polymorphism is the genetic determinant of highly efficient xenograft activity in NOD-based immunodeficient mouse models. Sparing other NOD-specific abnormalities in this model also resulted in normalized C5 function, which should help in future studies using CDC activity of antibodies in vivo. The use of the BRGS line should also save time in introducing other genes for further modification of the line, keeping the high efficiency corresponding to the NOD-based models without performing multiple backcrosses. Therefore, this simplified mouse model should be very useful in future xenotransplantation experiments using human cells.

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Authorship

Contribution: T.Y., K.T., and S.U. coordinated the project, designed and performed the experiments, analyzed the data, and wrote the manuscript; T.S., Y.K., T.T., and C.I. performed the experiments; M. Nishihara managed the mice; H.I., T. Miyamoto, and K.A. designed the experiments, reviewed the data, and edited the manuscript; N.H. provided the antibodies and technical advice; M. Nakao performed the experiments and provided technical advice; and T. Matozaki provided the antibodies and technical advice.

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Contribution of Bone Marrow-Derived Hematopoietic Stem/Progenitor Cells to the Generation of Donor-Marker⁺ Cardiomyocytes *In Vivo*

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Abstract

Background: Definite identification of the cell types and the mechanism relevant to cardiomyogenesis is essential for effective cardiac regenerative medicine. We aimed to identify the cell populations that can generate cardiomyocytes and to clarify whether generation of donor-marker⁺ cardiomyocytes requires cell fusion between BM-derived cells and recipient cardiomyocytes.

Methodology/Principal Findings: Purified BM stem/progenitor cells from green fluorescence protein (GFP) mice were transplanted into C57BL/6 mice or cyan fluorescence protein (CFP)-transgenic mice. Purified human hematopoietic stem cells (HSCs) from cord blood were transplanted into immune-compromised NOD/SCID/IL2r^{null} mice. GFP⁺ cells in the cardiac tissue were analyzed for the antigenicity of a cardiomyocyte by confocal microscopy following immunofluorescence staining. GFP⁺ donor-derived cells, GFP⁺CFP⁺ fused cells, and CFP⁺ recipient-derived cells were distinguished by linear unmixing analysis. Hearts of xenogeneic recipients were evaluated for the expression of human cardiomyocyte genes by real-time quantitative polymerase chain reaction. In C57BL/6 recipients, Lin^{-low}CD45⁺ hematopoietic cells generated greater number of GFP⁺ cardiomyocytes than Lin^{-low}CD45⁻ mesenchymal cells (37.0+/-23.9 vs 0.0+/-0.0 GFP⁺ cardiomyocytes per a recipient, P=0.0095). The number of transplanted purified HSCs (Lin^{-low}Sca-1⁺ or Lin^{-low}Sca-1⁺c-Kit⁺ or CD34⁻Lin⁻Sca-1⁺c-Kit⁺) showed correlation to the number of GFP⁺ cardiomyocytes (P<0.05 in each cell fraction), and the incidence of GFP⁺ cardiomyocytes per injected cell dose was greatest in CD34⁻Lin⁻Sca-1⁺c-Kit⁺ recipients. Of the hematopoietic progenitors, total myeloid progenitors generated greater number of GFP⁺ cardiomyocytes than common lymphoid progenitors (12.8+/-10.7 vs 0.67+/-1.00 GFP⁺ cardiomyocytes per a recipient, P=0.0021). In CFP recipients, all GFP⁺ cardiomyocytes examined coexpressed CFP. Human troponin C and myosin heavy chain 6 transcripts were detected in the cardiac tissue of some of the xenogeneic recipients.

Conclusions/Significance: Our results indicate that HSCs resulted in the generation of cardiomyocytes via myeloid intermediates by fusion-dependent mechanism. The use of myeloid derivatives as donor cells could potentially allow more effective cell-based therapy for cardiac repair.

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Introduction

Modification of regenerative capacity in injured heart could be potentially alternative to conventional therapy for treating patients suffering from heart failure [1–7]. Based on the promising results in rodents [3,4], clinical trials of cellular therapy using bone marrow (BM) cells for ischemic heart disease patients have been designed. In many of clinical trials for improving the function of cardiac recovery, some favorable results were obtained following

injection of BM mononuclear cells (MNCs) [2,5–7]. However, careful examination needs to be performed in basic research because cell fate and the effects of transplanted cells are not fully unveiled [8].

BM contains heterogeneous cell populations including at least two distinct stem cells, hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) [9], and various progenitors of myeloid and lymphoid lineages. Both HSCs and MSCs have been reported to acquire the phenotype of cardiomyocytes in syngeneic