

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

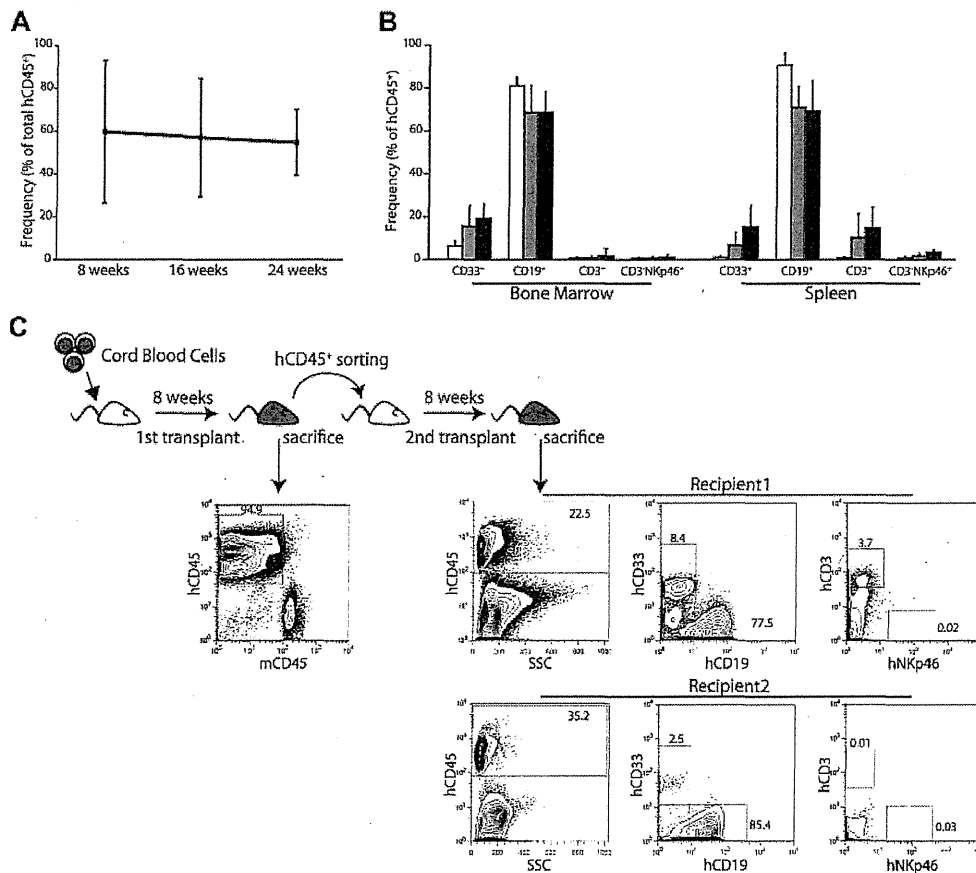


Figure 5. Evaluation of self-renewal of human HSCs in the BRGS mouse model. (A) Change in frequency of human CD45⁺ cells after transplantation. The level of human CD45⁺ cells was maintained at a high level until 24 weeks after transplantation (8 weeks, n = 29; 16 weeks, n = 17; and 24 weeks, n = 4). (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.

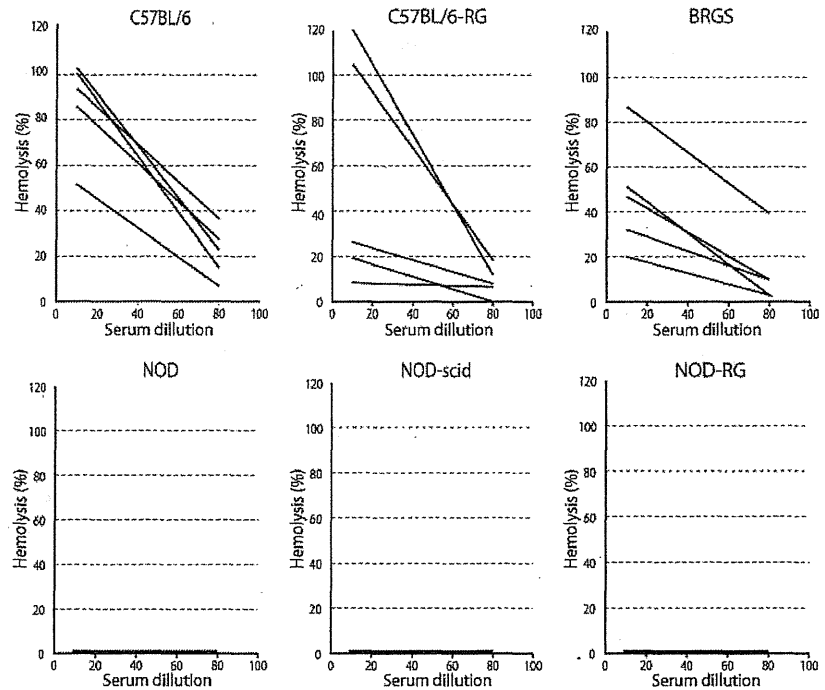
development of congenic mouse strains. More than 20 *Idd* loci have been identified. NOD mice display multiple aberrant immunophenotypes, and introduction of these abnormalities into immunodeficient mouse lines by multiple backcrossing accelerated human cell engraftment in xenotransplantation assays.^{11,14,15}

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.

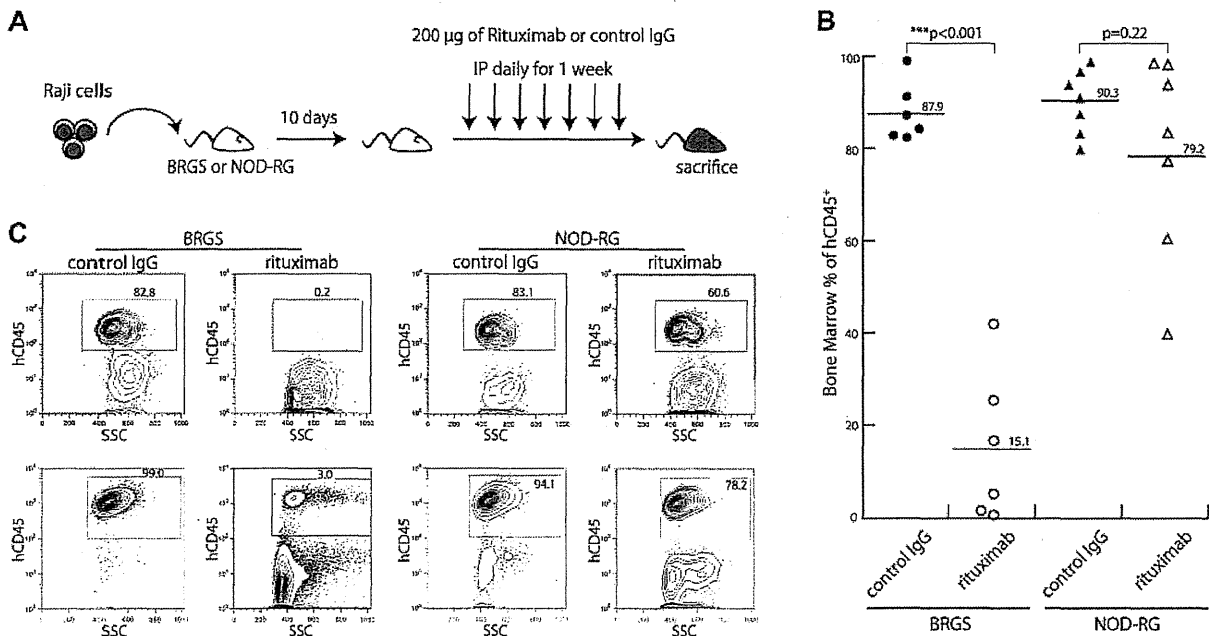


Figure 7. CDC activity of antibodies is evaluable in vivo in the BRGS xenogeneic model. (A) Experimental scheme of this experiment. Raji cells were injected into mice via the tail vein. Ten days after the injection, either rituximab or control IgG2a antibody (200 µg each) was injected IP daily for 1 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 hematolymphopoiesis *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 C57BL/6 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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TRANSPLANTATION

Quantitation of hematogones at the time of engraftment is a useful prognostic indicator in allogeneic hematopoietic stem cell transplantation

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Key Points

- Quantitation of hematogones at engraftment is useful to predict prognosis of patients treated with allogeneic stem cell transplantation.

Transient marrow expansion of normal B-cell precursors, termed hematogones, is occasionally observed after hematopoietic stem cell transplantation (HSCT). To understand the clinical significance of this phenomenon, we enumerated hematogones in 108 consecutive patients who received allogeneic HSCT for the treatment of hematologic malignancies, including acute myelogenous leukemia, advanced myelodysplastic syndromes, acute lymphoblastic leukemia, and non-Hodgkin lymphoma. Hematogone quantitation was performed at the time of complete donor engraftment (median day 25 and 32 in patients who received bone marrow and cord blood cell transplants, respectively).

Hematogones were polyclonal B cells, and their frequencies correlated positively with blood B-cell numbers, and inversely with donors' but not recipients' age, suggesting that hematogones reflect cell-intrinsic B-cell potential of donor cells. Interestingly, patients developing hematogones that comprised > 5% of bone marrow mononuclear cells constituted a group with significantly prolonged overall survival and relapse-free survival, irrespective of their primary disease or donor cell source. In addition, patients with > 5% hematogones developed severe acute graft-versus-host diseases less frequently, which may contribute toward their improved survival. We therefore conclude that the amount of hematogones at the time of engraftment may be a useful tool in predicting the prognosis of patients treated with allogeneic HSCT. (*Blood*. 2013;121(5):840-848)

Introduction

Hematogones are transient increases in lymphoblast-looking cells in the bone marrow.^{1,2} Because of the morphologic resemblance between residual leukemic clones and hematogones, expansion of hematogones during the recovery phase from chemotherapy and bone marrow transplantation occasionally causes diagnostic confusion.¹⁻³ Phenotypic analyses have demonstrated that hematogones are normal B-cell precursors, including pro-B, pre-B, and immature B cells coexpressing CD10 and CD19.^{1,2} The fact that hematogones become prominent in the recovery phase after chemotherapy or hematopoietic stem cell transplantation (HSCT)¹⁻⁶ suggests that they could reflect active B-cell reconstitution. They are also sometimes seen in steady-state hematopoiesis, especially in healthy infants and young people.^{2,4,7,8} Previous work demonstrated that in the recovery phase after chemotherapy, the percentage of hematogones in the bone marrow was inversely correlated with patients' age.¹ However, it is unclear whether the age-associated decline in hematogones frequency reflects cell-intrinsic defects of hematopoietic stem cell activity or cell-extrinsic defects such as aging of the bone marrow microenvironment.

Recent reports have shown that hematogone expansion correlates with favorable outcomes in acute myelogenous leukemia

(AML) patients treated with chemotherapy⁵ or cord blood transplantation (CBT).⁶ However, the precise number or frequency above which hematogones correlate with clinical significance has not been clarified. Previous reports^{1,5,6} have reported hematogone frequency relative to bone marrow mononuclear cells (MNCs), total nuclear cells (TNCs), and frequencies of B-cell precursors, and as a result, hematogone expansion has been described with frequencies ranging from > 0% to 0.9%.^{1,5,6}

To better understand the etiology and clinical significance of hematogones, we measured percentages of B-cell precursors in the bone marrow via flow cytometry in 108 consecutive patients with hematologic malignancies, including AML, advanced myelodysplastic syndromes (MDS), acute lymphoblastic leukemia (ALL), and lymphoma, who achieved successful engraftment after allogeneic HSCT at our institution. The analysis of hematogones was performed on the day of engraftment, defined as the day when circulating granulocytes reached $> 0.5 \times 10^9/L$ for 3 consecutive days,⁹⁻¹² and the bone marrow showed complete donor-type chimerism via polymerase chain reaction (PCR) analysis. To minimize the effect of expanding granulocytes on hematogone frequencies, bone marrow MNCs were used for flow cytometric

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Table 1. Patients' characteristics

	BMT: no. (%), [range]	CBT: no. (%), [range]	Total (%)	P
Recipient sex, male/female	35 (32)/24 (22)	26 (24)/23 (21)		NS
Recipient age, y	49.2 (mean) [20-66]	47.3 (mean) [19-67]		NS
Donor sex, male/female	37 (34)/22 (20)	24 (22)/25 (23)		NS
Donor age, y	36.7 (mean) [17-66]	0		< .001
No. of infused cell, /kg	2.80×10^8 (mean) [0.92-4.02]	0.28×10^8 (mean) [0.18-0.50]		< .001
Primary disease				NS
AML/advanced MDS				
CR	14 (13)	11 (10)	25 (23)	
non-CR	21 (19)	14 (13)	35 (32)	NS
Total	35 (32)	25 (23)	60 (56)	
ALL				
CR	3 (3)	3 (3)	6 (6)	
non-CR	2 (2)	10 (9)	12 (11)	NS
Total	5 (5)	13 (12)	18 (17)	
Lymphoma				
CR	7 (6)	3 (3)	10 (9)	
non-CR	12 (11)	8 (7)	20 (19)	NS
Total	19 (18)	11 (10)	30 (28)	
Conditioning regimen				< .01
TBI/CY	28 (26)	24 (22)		
BU/CY	14 (13)	0 (0)		
RIC	17 (16)	25 (23)		
GVHD prophylaxis				< .001
TAC + sMTX	51 (47)	6 (6)		
CSP + sMTX	8 (7)	28 (26)		
CSP + MMF	0 (0)	15 (14)		
HLA disparity				< .001
6/6	39 (36)	1 (1)		
5/6	20 (19)	7 (6)		
4/6	0 (0)	22 (20)		
3/6	0 (0)	19 (18)		
Days required for engraftment	25 (median) [15-32]	32 (median) [14-39]		< .01

ALL indicates acute lymphoblastic leukemia; AML, acute myelogenous leukemia; BMT, bone marrow transplantation; BU, busulfan; CBT, cord blood transplantation; CR, complete remission; CSP, cyclosporine; CY, cyclophosphamide; GVHD, graft-versus-host disease; MDS, myelodysplastic syndrome; MMF, mycophenolate mofetil; NS, not significant; RIC, reduced-intensity conditioning; sMTX, short-term methotrexate; TAC, tacrolimus; and TBI, total body irradiation.

analyses in all cases. Our data suggest that the number of hematogones generally reflects cell-intrinsic B-cell potential of donor hematopoietic stem cells (HSCs) and that this declines with aging. We also found that hematogone frequencies of > 5% of total MNCs is a useful cutoff line to distinguish patient groups with significantly better overall survival (OS) or with relapse-free survival (RFS), irrespective of their primary diseases or donor cell sources. We propose that the quantitation of hematogones at engraftment may be useful to predict the prognosis of patients treated with allogeneic HSCT.

Methods

Patients

From 2005 to 2010, 134 patients with high-grade hematologic malignancies were treated with allogeneic HSCT in Kyushu University Hospital. These patients included AML cases with high risk,¹³ relapsed or refractory status, advanced MDS cases with intermediate-II or high risk on International Prognostic Scoring System classification,^{14,15} ALL cases with high risk,¹⁶ relapsed or refractory status, and relapsed non-Hodgkin lymphoma cases. Within these 134 patients, grafts were rejected in 5 cases and residual malignant cells proliferated soon after HSCT in 21 cases, without achieving successful engraftment. The remaining consecutive 108 cases, in which allogeneic HSCT was successful and complete donor-type chimerism was documented, were enrolled in this study. Fifty-nine and 49 patients received bone marrow transplantation (BMT) and CBT, respectively. Patients'

characteristics are summarized in Table 1. This study was approved by the institutional review board of Kyushu University Hospital and conducted in accordance with the Declaration of Helsinki.

Evaluation of remission status before HSCT

Before HSCT, patients were intensively searched for residual malignant cells to define their pretransplantation remission status. In acute leukemia or advanced MDS cases, bone marrow samples were checked first by microscopic analysis, and were subjected to multicolor flow cytometric analysis.^{13,17} Complete remission (CR) was diagnosed when percentages of cells of leukemia phenotype were < 0.5% in the bone marrow. Furthermore, 21 patients with acute leukemia or MDS had leukemia-specific genes such as BCR-ABL, FLT3-ITD, AML1-ETO, and MLL fusions, and PCR amplification of these genes were used to detect minimal residual disease (MRD).¹³ Within these 21 patients, 17 patients were diagnosed as CR based on flow cytometric analyses. CR results for these 17 patients were also confirmed by PCR. In lymphoma patients, remission status was defined by evaluating the involvement of lymphoid organs using FDG-PET CT scan and/or MRI methods, and was also defined by evaluating the involvement of bone marrow by flow cytometry, as previously described.¹⁸

Transplantation procedures

Patients' characteristics were not statistically different between BMT and CBT recipient groups in terms of sex, age, and primary disease (Table 1). Conditioning regimen consisted of total body irradiation/cyclophosphamide (CY) for 28 BMT and 24 CBT recipients, busulfan (BU)/CY for 14 BMT recipients, and fludarabine-based reduced-intensity conditioning^{19,20} for 17 BMT and 25 CBT recipients, respectively (Table 1).

Prophylaxis for graft-versus-host disease (GVHD) was tacrolimus/short-term methotrexate (sMTX) for 51 BMT and 6 CBT recipients, cyclosporine (CSP)/sMTX for 8 BMT and 28 CBT recipients, and CSP/mycophenolate mofetil for 15 CBT recipients (Table 1). The mean number of donor cells transplanted was $2.8 \times 10^8/\text{kg}$ in BMT recipients and $0.28 \times 10^8/\text{kg}$ in CBT recipients. Bone marrow units were obtained from the Japan Marrow Donor Program or related donors, and cord blood units were obtained from the Japanese Cord Blood Bank Network.

Evaluation for engraftment

The bone marrow sampling for the analysis of hematogones was performed when patients achieved successful engraftment. The standard criterion for engraftment was used according to previous studies.⁹⁻¹² Blood neutrophil numbers were checked daily after transplantation, and the successful engraftment was defined when neutrophils exceeded $0.5 \times 10^9/\text{L}$ for 3 consecutive days. When patients met the criteria for engraftment, host/donor microchimerism analysis was performed (see the next section). If the analysis showed complete donor type chimerism, hematogones in the bone marrow were counted by multicolor flow cytometric analysis.

Chimerism analysis

To analyze donor/recipient cell chimerism, PCR amplification of polymorphic short tandem repeats (STR) was performed to confirm engraftment of donor cells. PCR using synthesized oligonucleotide templates were performed using TAKARA Taq Reagent Kits and run in the Perkin Elmer GeneAmp PCR system 9600 or 2400. The donor-cell origin and recipient-cell origin PCR product mixture was loaded onto the 373A sequencer (Applied Biosystems) with a size marker, and the data were processed using the GeneScan software (Applied Biosystems) as described previously.²¹

Flow cytometry analysis and cell sorting

The bone marrow mononuclear cells were prepared by the gradient centrifugation method as previously described.^{22,23} Cells were stained with allophycocyanin-conjugated anti-CD34 (BD Pharmingen), biotin-conjugated anti-CD38 (Caltag Laboratories), FITC-conjugated anti-CD10 (DAKO), PE-conjugated anti-CD20 (BD Biosciences), PE-Cy7-conjugated anti-CD19 (BioLegend), and Cy5-PE-conjugated lineage (Lin) mixture (anti-CD3, -CD4, -CD8 (BD Pharmingen) -CD11b (Caltag Laboratories), -CD14, and -CD56 (Beckman Coulter)).²²⁻²⁵ Streptavidin-conjugated Cy7-allophycocyanin (BD Pharmingen) was used for visualization of biotinylated antibodies. For analysis of mature B cells, peripheral blood (PB) cells were stained with FITC-conjugated anti-CD10 (DAKO), PE-conjugated anti-CD20 (BD Biosciences), PE-Cy7-conjugated anti-CD19 (BioLegend), and Cy5-PE-conjugated Lin mixture. Available PB cells at day 90 after HSCT could be obtained from 64 patients and evaluated. Cells were analyzed by using a FACSAria (BD Biosciences) or FACSCanto (BD Biosciences). Cell sorting was performed on a 5-color FACSAria (BD Biosciences). To minimize contamination, cells were collected after the second round of sorting using sorting gates identical to those used in the first-round sorting. Definition of hematogones is a series of normal B-lymphoid precursors, including $\text{CD34}^+\text{CD38}^+\text{CD10}^+\text{CD19}^+\text{Lin}^-$ pro-B cells, $\text{CD34}^{\text{low}}\text{CD38}^+\text{CD10}^+\text{CD19}^+$ pre-B cells, and $\text{CD34}^-\text{CD38}^+\text{CD10}^+\text{CD19}^+$ CD20^+ immature B cells²⁶⁻²⁸ in bone marrow MNCs. Isotype controls were used to define the cutoff of positivity of each antigen on a FACS.

PCR analysis of IGH gene rearrangement

To analyze clonality of IGH gene rearrangements status of hematogones, DNA was obtained from 10 000 double-sorted cells^{22,23,29} from all recipients presenting $> 0.1\%$ MNCs of hematogones on FACS. Then PCR amplification of DJ_H and VDJ_H gene rearrangement was performed as described previously.^{24,30}

Statistical analysis

Relationships of percentages of hematogones with age, the day of engraftment, and numbers of circulating B lymphocytes were analyzed with the

Spearman rank correlation analysis. Comparison between 2 groups or condition was tested with the Mann-Whitney *U* test. The categorical variables were analyzed with the 2-tailed χ^2 test. Survival was plotted with Kaplan-Meier curves, taking the interval from date of HSCT to death/relapse or last contact. Comparisons between each group were performed with the log-rank test and the Cox proportional hazards model. Univariate analysis was performed with logistic or exact logistic regression, and the parameters that present $P < .20$ were reevaluated by multivariate analysis.³¹ Multivariate analysis was performed with logistic regression applying Firth's bias reduction. A *P* value $< .05$ was considered to be statistically significant.

Results

Hematogones that appeared at the time of engraftment are polyclonal B-cell precursors

One hundred eight consecutive cases treated with successful allogeneic BMT or CBT were enrolled in this study. Hematogones in the bone marrow were counted on the day of engraftment by multicolor flow cytometric analysis. The successful engraftment was judged when neutrophils exceeded $> 0.5 \times 10^9/\text{L}$ for 3 consecutive days.⁹⁻¹² At this phase, it is critical to exclude residual leukemic cells or host-derived B-cell precursors from a cell fraction of hematogones. To this end, polymorphic STR was amplified to test the host/donor microchimerism, and only when patients' bone marrow consisted of 100% donor-derived cells, the analysis of hematogones was performed. The complete donor-type chimerism verifies that host-derived normal hematopoietic cells and malignant leukemic cells have been eliminated below the sensitivity of FACS,³²⁻³⁴ and therefore that phenotypically defined hematogones in these patients on FACS were donor-derived normal cells.

Hematogones were morphologically blastic cells (Figure 1A), and were identified by surface phenotype, according to the definition of pro-B, pre-B, and/or immature B cells that coexpress CD10 and CD19 on their cell surface.^{1,26-28} (Figure 1B). To minimize the effect of granulocytes on hematogone frequencies, we used MNCs instead of TNCs in our analysis. The frequencies of hematogones in MNCs are usually higher than those in TNCs (not shown), as reported previously.¹

The time median to engraftment was 25 and 32 days in patients treated with BMT and CBT, respectively (Table 1). The time required for engraftment appears to be consistent with previous reports.³⁴⁻³⁹ Percentages of B-cell precursors within the bone marrow MNCs at the time of complete donor-type engraftment were significantly higher in CBT recipients than in BMT recipients (6.37% vs 1.75%; $P < .001$; Figure 1C). There was no significant relationship between the day of engraftment (the day of sampling) and the frequency of hematogones (Figure 1D).

In 106 of 108 patients who had $> 0.1\%$ of B-cell precursors in the bone marrow MNCs, B-cell precursors were purified by a multicolor FACS and were subjected to IGH rearrangement analysis. In all of these patients, B-cell precursors were polyclonal based on the rearrangement analysis of the IGH genes (Figure 1E).

Hematogones generally represent B-cell recovery potential of the graft and their emergence is related to age of donors but not recipients

Because hematogones are normal B-cell precursors, we tested whether the presence of a high number of them could reflect the active B-cell recovery after HSCT. FACS analysis of circulating blood cells revealed that the frequency of bone marrow B-cell precursors was significantly correlated with the number of blood

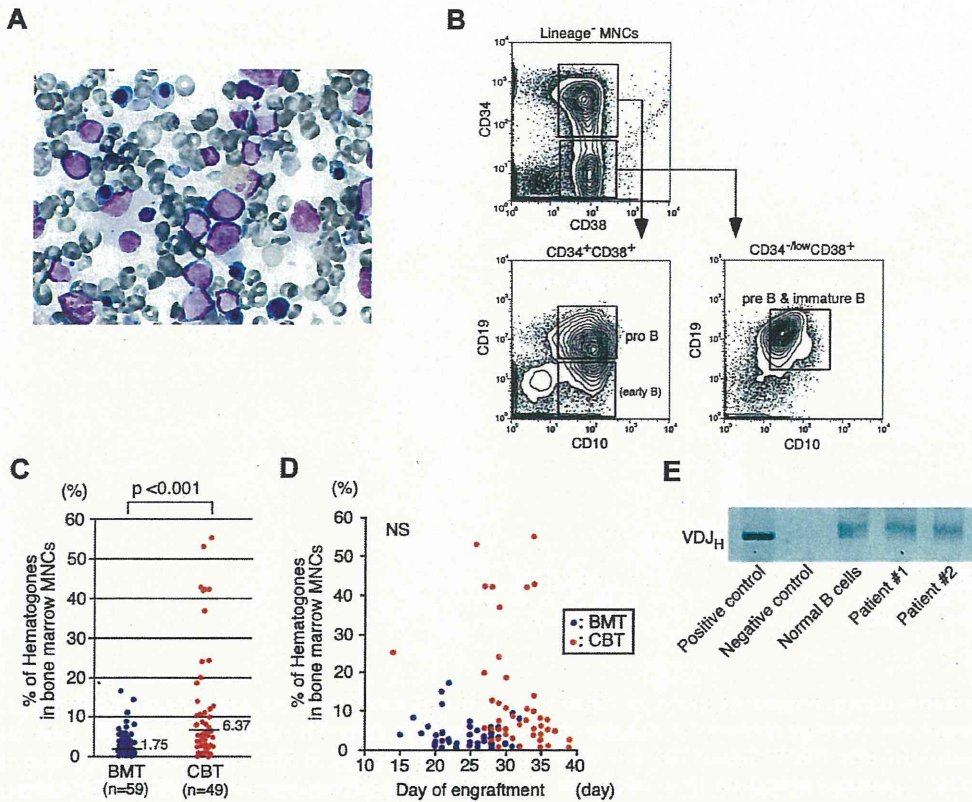


Figure 1. Detection of hematogones after allogeneic HSCT. (A) Typical appearance of hematogones in the bone marrow after HSCT (Giemsa staining $\times 1000$; OLYMPUS BH-2 microscope [Olympus]; ACT-2U imaging software [Nikon]; 27°C). (B) Evaluation of hematogones on a 5-color FACS. Hematogones are defined as MNCs coexpressing CD10 and CD19 in the bone marrow at engraftment. They include $\text{CD}34^{+}\text{CD}38^{+}\text{CD}10^{+}\text{CD}19^{+}\text{Lin}^{-}$ pro-B cells, $\text{CD}34^{-/\text{low}}\text{CD}38^{+}\text{CD}10^{+}\text{CD}19^{+}$ pre-B cells, and $\text{CD}34^{-}\text{CD}38^{+}\text{CD}10^{+}\text{CD}19^{+}\text{CD}20^{+}$ immature B cells. (C) Percentage of hematogones in the bone marrow MNCs in patients who received BMT and CBT. CBT recipients presented much higher frequency of hematogones compared with BMT recipients ($P < .001$). Solid bars indicate the median percentage of hematogones for each recipient; MNC, mononuclear cells; BMT, bone marrow transplantation; and CBT, cord blood transplantation. (D) The relationship between the day of engraftment and percentages of hematogones. There was no relationship between these parameters. (E) *IGH* rearrangement analysis of purified hematogones. B-cell precursors were polyclonal in all 106 recipients analyzed.

B cells at the time of engraftment ($R = 0.47, P < .001$; Figure 2A), and with those even on day 90 after HSCT ($R = 0.22, P < .01$; Figure 2B). These results suggest that expansion of hematogones reflects not only enhanced B-cell reconstitution potential of the graft, but also prolonged B cell–producing capability of donor HSCs.

The age of BMT donors ranged from 17 to 66 years old (median, 37 years; Table 1). Interestingly, there was a significant inverted correlation between donor age and percentage of bone marrow hematogones in patients treated with BMT ($R = 0.32, P = .02$; Figure 2C blue line). When the age of CBT donors were defined as 0-year old, the significant inverted correlation between age and hematogone numbers was also found in all patients entered in this study ($R = 0.42, P < .001$; Figure 2C black line). In contrast, recipients' age and hematogone numbers did not show any relationship (Figure 2D). Furthermore, as shown in Table 2, the time of engraftment was not affected by primary diseases of patients, or by their remission status at the time of HSCT. Thus, although the patients who fail to achieve CR are usually treated with higher total doses of chemotherapeutic drugs because of their refractory disorders, it did not affect the day of engraftment or the day of hematogone analysis for this study. These data strongly suggest that the number of hematogones after HSCT generally reflects the cell-intrinsic B-cell recovery potential of donor HSCs, which may decline by aging.

The emergence of hematogones up to > 5% of MNCs in the bone marrow represents a good prognosis for patients treated with allogeneic HSCT

It should be critical to draw a line of hematogone numbers to distinguish a group of patients with clinical significance. Therefore, we first compared the OS and RFS among patient subgroups with $\leq 1\%$, $1\% - 2\%$, $2\% - 3\%$, $3\% - 4\%$, $4\% - 5\%$, or $> 5\%$ of hematogones in our study (Figure 3A-B). Strikingly, patients who developed hematogones up to $> 5\%$ of MNCs showed significantly better 3-year OS (100%) and RFS (93.3%), compared with any other group. Patient groups with $\leq 1\%$, $1\% - 2\%$, $2\% - 3\%$, and $3\% - 4\%$ of hematogones showed similar 3-year OS and RFS that were 37%-53% and 22%-51%, respectively. Interestingly, patients with $4\% - 5\%$ hematogones appeared to show intermediate levels of OS (86%) and RFS (64%), although this is not statistically better than those in patients with $\leq 1\%$ hematogones (Figure 3A-B). Based on these results, we hypothesized that the development of $> 5\%$ of hematogones might be critical to distinguish a patient group with favorable prognosis.

According to this criteria, 43 patients developed $> 5\%$ MNCs of hematogones (HG^{+}) and the remaining 65 patients had $\leq 5\%$ MNCs of hematogones (HG^{-}). As shown in Figure 3A, in HG^{+} patients, 3-year OS was 100%, whereas in HG^{-} patients, it was 45% ($P < .001$). The favorable OS in HG^{+} groups is at least

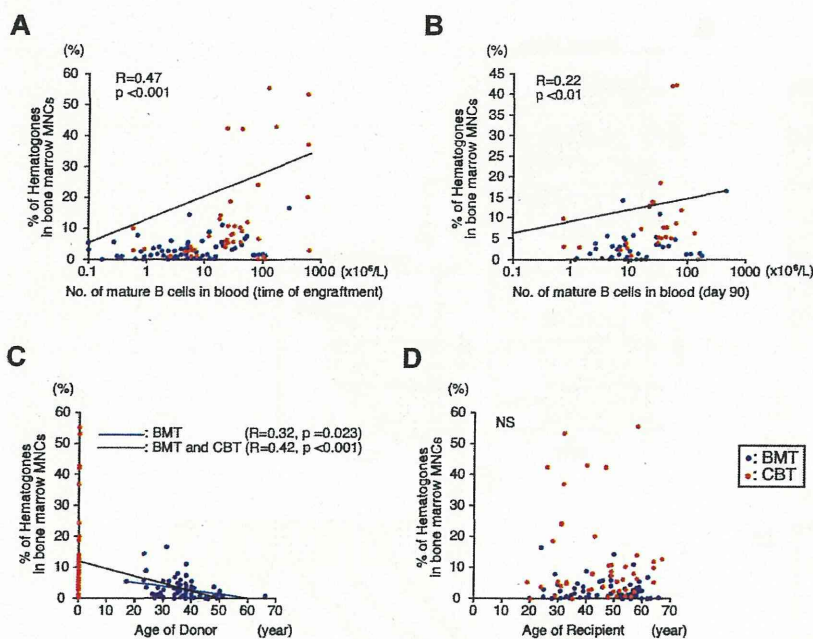


Figure 2. Analysis of hematogones, and the correlation of their frequency compared with blood B-cell numbers and age of donors. (A) A relationship between frequencies of hematogones and blood B cells at engraftment ($P < .001$). (B) A relationship between frequencies of hematogones at engraftment and blood B cells on day 90 ($P < .01$). (C) A relationship between frequency of hematogones and donor's age in patients who received BMT (blue line, $P = .023$), and in all recipients treated with either BMT or CBT (black line, $P < .001$). (D) No significant relationship was observed between frequency of hematogones and recipient age. NS indicates not significant.

because of the less frequent disease relapse. As shown in Figure 3B, significant association was observed between the presence of hematogones and 3-year RFS after HSCT: 3-year RFS was 93% and 37% in HG^+ and HG^- patients, respectively ($P < .001$). The association between the presence of $> 5\%$ hematogones and favorable OS and RFS was also seen when the analysis was performed in patient subgroups that received either BMT or CBT (Figure 3C-D). These data strongly suggest that the emergence of hematogones is a useful predictor of favorable outcomes at least in terms of OS and RFS, irrespective of donor cell source.

The emergence of hematogones ($> 5\%$ of MNCs) marks favorable outcomes for allogeneic HSCT especially in patients who failed to achieve complete remission, irrespective of primary malignant disease

We then analyzed whether the good prognosis designated by the emergence of hematogones is dependent on the primary malignant disorder. The OS and RFS were analyzed in each patient group with AML/advanced MDS, ALL, or non-Hodgkin lymphoma. As shown in Figure 4, HG^+ patients always showed significantly better OS and RFS compared with HG^- patients, in any of these patients groups suffering from different primary diseases.

It is well known that the achievement of CR at the time of transplantation favorably affects the prognosis after allogeneic HSCT.¹³ Interestingly, in AML/advanced MDS patients, the HG^+ group showed significantly prolonged OS and RFS compared with the HG^- group, irrespective of their remission status at HSCT (Figure 5). The similar analysis was performed in ALL and lymphoma patient groups (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Although each group contained only a limited number of patients, statistically significant prolonged OS and RFS were also seen in patients who did not achieve CR at HSCT in both the ALL and the lymphoma patient groups.

Thus, the appearance of hematogones might mark favorable OS and RFS regardless of their primary malignancy.

Expansion of hematogones is frequently observed in patients who did not develop infection or severe acute GVHD

In this study, all 43 HG^+ patients are currently alive, although primary diseases have relapsed in 3 patients. In contrast, 32 of 65 HG^- patients have died. The causes of death in these 32 HG^- patients are shown in Table 3. Twenty-six patients died of their refractory primary disease, and 6 patients died of TRM, including

Table 2. Time required for engraftment in patients grouped by their primary disease or complication of infection or acute GVHD

Disease	Remission status	Time required for engraftment, d					
		BMT			CBT		
		No.	Mean, d	P	No.	Mean, d	P
AML and advanced MDS	CR/non-CR	14/21	27.0/24.4	.11	11/14	31.5/31.8	.37
ALL	CR/non-CR	3/2	23.7/29.5	.35	3/10	34.3/32.1	.45
Lymphoma	CR/non-CR	7/12	26.1/22.8	.10	3/8	33.0/29.3	.44
			Overall	.10	Overall		.53
After HSCT							
Infections	Yes/No	38/21	25.3/24.8	.66	22/27	30.8/32.2	.61
Acute GVHD	Grade II-IV/Grade 0-I	26/33	24.7/25.3	.25	14/35	32.3/31.3	.47

ALL indicates acute lymphoblastic leukemia; AML, acute myelogenous leukemia; BMT, bone marrow transplantation; CBT, cord blood transplantation; CR, complete remission; GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; and MDS, myelodysplastic syndrome.