

CD38 expression by hematopoietic stem cells of newborn and juvenile mice

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While investigators in a number of laboratories have documented that the hematopoietic stem cells (HSCs) of fetal and adult mice are CD38⁺, no information is available about CD38 expression by HSCs of newborn and juvenile mice. We used a murine transplantation model to examine HSC CD38 expression. First, we observed that all HSCs from newborn bone marrow are CD38⁻. Next, it was determined that the majority of HSCs in the bone marrow of 5-week-old mice are CD38⁻, with a minority being CD38⁺. These observations indicated that the CD38⁺ subpopulation of HSC appears before the age of 5 weeks and expands during adolescence. However, the majority of HSCs of 5-week-old mice became CD38⁺ following injection of 5-fluorouracil, indicating that activation of juvenile stem cells enhances CD38 expression. These observations may have implications for CD38 expression by HSCs from human umbilical cord blood and bone marrow of young children in steady state and under pathological conditions.

Leukemia (2003) 17, 171–174. doi:10.1038/sj.leu.2402785

Keywords: CD38; hematopoietic stem cells; transplantation; developmental changes

to examine CD38 expression by HSCs in the bone marrow of newborn and 5-week-old mice.

Materials and methods

Monoclonal antibodies

Fluorescein isothiocyanate (FITC)-conjugated NIM-R5 (anti-CD38; rat IgG2a) was purchased from Southern Biotechnology Associates (Birmingham, AL, USA), biotin-conjugated RAM34 (anti-CD34; rat IgG2a), FITC-conjugated A20 (anti-Ly-5.1; rat IgG2a), phycoerythrin (PE)-conjugated RB6-8C5 (anti-Gr-1; rat IgG2b), PE-conjugated 53-2.1 (anti-Thy-1.2; rat IgG2a), PE-conjugated RA3-6B2 (anti-CD45R/B220; rat IgG2a) from BD Pharmingen (San Diego, CA, USA), and PE-conjugated M1/70 (anti-Mac-1; rat IgG2b), streptavidin-conjugated PE from Caltag Laboratories (San Francisco, CA, USA).

Cell preparation

C57BL/6-Ly-5.1 mice originally obtained from Jackson Laboratories (Bar Harbor, ME, USA) and bred in the Animal Research Facility of The Veterans Affairs Medical Center were used as newborn and juvenile donors. Ten to 14-week-old C57BL/6-Ly-5.2 female mice (Charles River Laboratories, Raleigh, NC, USA) were used as irradiated recipients and as the source for radioprotective cells. In some experiments, the juvenile donor mice were treated with an intravenous injection of 5-fluorouracil (5-FU) at 100 mg/kg body weight 48 h before death. Bone marrow cells from Ly-5.1 mice were flushed from femurs and tibiae, pooled, and washed twice with phosphate-buffered saline containing 0.1% bovine serum albumin. The samples were then made into single cell suspension by repeated pipetting and filtering through 40 μ m nylon mesh. The cells with densities ranging from 1.063 to 1.077 g/ml were collected by gradient separation using Nycodenz (Accurate Chemical and Scientific Corp, Westbury, NY, USA). The resulting mononuclear cells (MNCs) were stained with FITC-conjugated anti-CD38 and biotin-conjugated anti-CD34 followed by streptavidin PE. After addition of propidium iodide at a concentration of 1 μ g/ml, the cells were washed twice, resuspended in phosphate-buffered saline containing 0.1% bovine serum albumin and kept on ice until cell sorting. Fluorescence-activated cell sorting (FACS) was performed on a FACS Vantage (Becton Dickinson, San Jose, CA, USA), with appropriate isotype-matched controls.

Hematopoietic reconstitution

Ly-5.2 mice were administered a single 950-cGy dose of total body irradiation using a 4×10^6 V linear accelerator. Sorted Ly-5.1 cells were injected into the tail vein of the irradiated Ly-5.2 mice along with 2×10^5 bone marrow cells from adult Ly-5.2 mice as radioprotective cells. In all experiments, the

Introduction

Characterization of the surface phenotypes of hematopoietic stem cells (HSCs) and development of techniques for their purification are important subjects in stem cell transplantation and gene therapy. Transplantation of purified human HSCs that are free of T lymphocytes could minimize graft-versus-host disease in allogeneic transplantation. In autologous situations, transplantation of HSCs that are free of tumor cells may reduce the risk of recurrence of malignancies.

For many years, CD34 and CD38 have been regarded as important markers for HSC. It was generally accepted that human HSCs and primitive progenitors are CD34⁺CD38^{-/low},¹ however, recent studies of murine stem cells caused controversies about surface phenotypes of HSCs. It was documented that the majority of adult murine HSCs are CD34⁻.^{2–4} In our laboratory, we observed that the developmental change of CD34 expression from positive to negative state takes place between 7 and 10 weeks of age for most of murine HSCs.⁵ We also discovered that CD34 expression by adult stem cells is influenced by the activation state of the HSCs.² Regarding stem cell CD38 expression, investigators in four different laboratories including ours reported that HSCs in yolk sac, fetal liver and adult mouse bone marrow express CD38.^{6–9} We demonstrated that activation of adult murine HSCs causes reversible changes in CD38 expression.⁶

Unknown has been the state of CD38 expression by HSCs of newborn or juvenile mice. This question has significant clinical relevance because cord blood cells recently have emerged as an important source for stem cell transplantation.¹⁰ In this paper, we used a mouse transplantation model

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Received 11 August 2002; accepted 16 September 2001

number of cells transplanted reflected the ratios of cells in the sorting windows. For analysis of engraftment, peripheral blood was obtained from the retro-orbital plexus using heparin-coated micropipets (Drummond Scientific, Broomall, PA, USA). The samples were stained with FITC-conjugated anti-Ly-5.1 and red blood cells were lysed using FACS Lysing Solution (Becton Dickinson). Analysis for donor-derived cells was carried out on a FACS Calibur (Becton Dickinson). Donor (Ly-5.1) cells in granulocyte and monocyte/macrophage, T-cell, and B-cell lineages were analyzed by staining with PE-conjugated anti-Gr-1 and PE-conjugated anti-Mac-1, PE-conjugated anti-Thy-1.2 and PE-conjugated anti-B220, respectively.

Data presentation and statistical analysis

The number of repopulating units (RU) in each test samples was calculated from the percentage donor cells engrafted, D , according to the formula: $RU = \text{competitor RU} \times D / (100 - D)$, described by Harrison *et al.*¹¹ After donor RU were calculated relative to RU of 2×10^5 bone marrow cells in each recipients, mean \pm s.d. percentages of RU in the CD38⁻ and CD38⁺ cell populations were computed. Levels of significance were determined using the Student's *t*-test.

Results

CD38 expression by HSCs of newborn mice

Earlier, we and other investigators documented that all HSCs of 5-week-old and younger mice are CD34⁺.^{5,12} We therefore tested the engraftment capabilities of CD38⁻ and CD38⁺ subpopulations of CD34⁺ bone marrow cells of newborn mice using the FACS regions shown in Figure 1. Because the ratio of the CD34⁺CD38⁻ (R1) cells to CD34⁺CD38⁺ (R2) cells was 20:1, 9.0×10^3 CD34⁺CD38⁻ or 4.5×10^2 CD34⁺CD38⁺ cells were transplanted into each recipient mouse. The levels of engraftment at 6 months after transplantation, determined by measuring the percentages of donor (Ly-5.1)-derived peripheral blood nucleated cells are shown in Figure 2. Only CD34⁺CD38⁻ cells engrafted. The evidence for multilineage engraftment in individual mice is presented in Table 1.

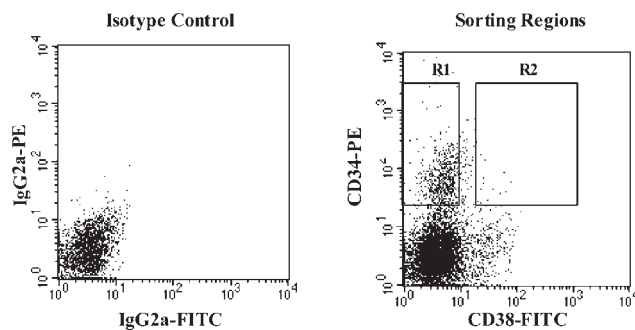


Figure 1 FACS sorting regions used for bone marrow cells of newborn mice. MNCs collected from 12 newborn mice were sorted for CD34⁺CD38⁻ and CD34⁺CD38⁺ populations using R1 and R2 electronic windows, respectively.

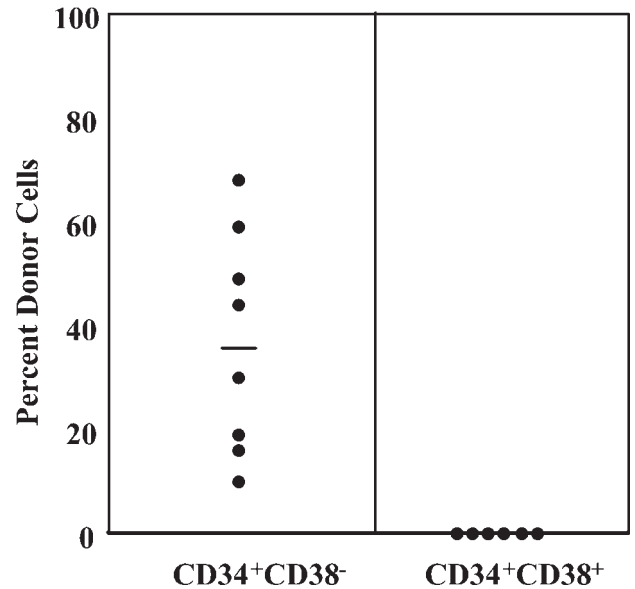


Figure 2 Percentages of donor nucleated cells in the blood of individual recipient mice at 6 months after transplantation. Mice were transplanted with CD34⁺ mononuclear marrow cells of newborn mice that were further separated on the basis of CD38 expression. Because the ratio of CD34⁺CD38⁻ (R1) and CD34⁺CD38⁺ (R2) cells was 20:1 according to Figure 1, 9.0×10^3 CD34⁺CD38⁻ cells or 4.5×10^2 CD34⁺CD38⁺ cells were injected per mouse. The groups consisted of 10 or six mice. Results shown are representative of two experiments.

Table 1 Multilineage reconstitution by CD34⁺ CD38⁻ cells of newborn mice analyzed at 6 months after transplantation

Mouse	G + M	T	B
1	12	28	7
2	68	55	41
3	9	43	30
4	58	6	10
5	44	29	9
6	70	70	70
7	52	76	57
8	77	30	50

Nucleated blood cells were stained with anti-Ly5.1 and either anti-Gr-1 and anti-Mac-1, anti-Thy-1.2 or anti-B220. Numbers indicate the percentages of cells within each lineage that expressed Ly-5.1. Results shown are representative of two experiments. G + M, granulocytes and/or macrophages; T, T- lymphocytes; B, B-lymphocytes.

CD38 expression by HSCs of 5-week-old mice

Next, we analyzed the CD38 expression by long-term engrafting cells of 5-week-old mice. Because we and other investigators documented that all bone marrow HSCs of 5-week-old and younger mice are CD34⁺,^{5,12} we set electronic sorting gates as shown in Figure 3. Since the ratio of the CD34⁺CD38⁻ (R3) cells to the CD34⁺CD38⁺ (R4) cells was 5:1, we transplanted 8.0×10^4 CD34⁺CD38⁻ cells or 1.6×10^4 CD34⁺CD38⁺ cells per mouse. The levels of engraftment are presented in Figure 4. Mice that had been transplanted with CD34⁺CD38⁻ cells showed higher engraftment levels ($18.9 \pm 12.7\%$) than mice transplanted with CD34⁺CD38⁺ cells ($2.8 \pm 2.0\%$) ($P < 0.005$). Multilineage engraftment was seen in all mice. Calculation of RU indicated that the majority

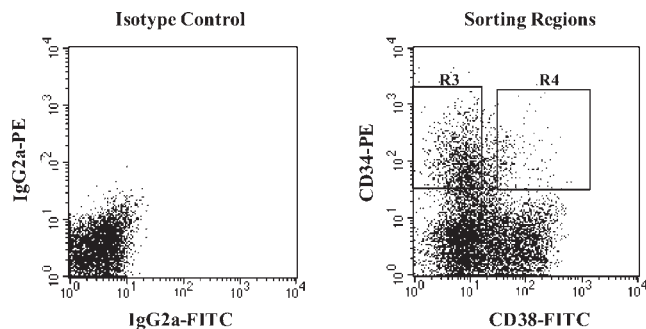


Figure 3 FACS sorting regions used for bone marrow cells of normal 5-week-old mice. MNCs collected from eight normal 5-week-old mice were sorted for CD34⁺CD38⁻ and CD34⁺CD38⁺ populations using R3 and R4 electronic windows, respectively.

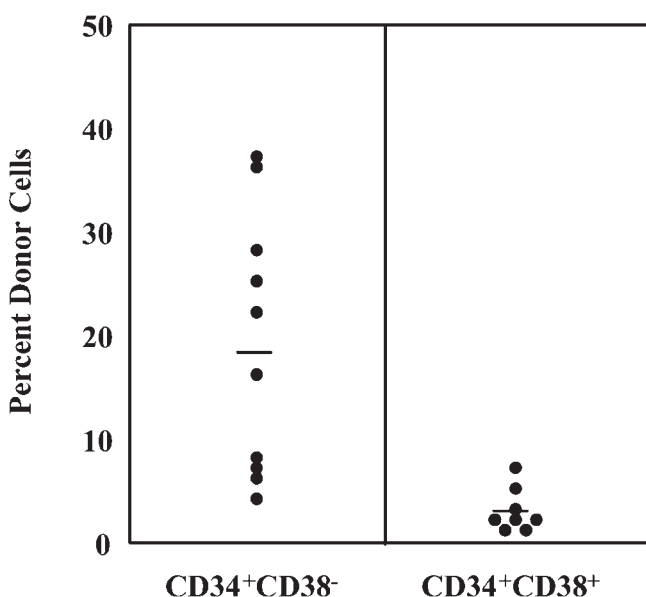


Figure 4 Percentages of donor nucleated cells in the blood of individual recipient mice at 6 months after transplantation. Mice were transplanted with CD34⁺ mononuclear marrow cells of normal 5-week-old mice that were further separated on the basis of CD38 expression. Because the ratio of CD34⁺CD38⁻ (R3) and CD34⁺CD38⁺ (R4) cells was 5:1 according to Figure 3, 8.0×10^4 CD34⁺CD38⁻ cells or 1.6×10^4 CD34⁺CD38⁺ cells were injected per mouse. The groups consisted of eight or 10 mice. The difference between the groups is significant at $P < 0.005$. Results shown are representative of two experiments.

(90%) of the long-term engrafting cells are CD38⁻ in the bone marrow of 5-week-old mice.

CD38 expression by HSCs of 5-FU-treated 5-week-old mice

We have shown in previous studies that activated adult HSCs were CD38⁻.⁶ To determine whether there are similar effects on stem cells in 5-week-old mice, we tested CD38 expression by HSCs of 5-week-old mice following 5-FU injection. Because the effects of 5-FU injection on the expression of CD34 by HSCs in 5-week-old mice are not known, we separated bone marrow cells on the basis of CD38 expression

alone. The sorting regions are presented in Figure 5. Since the ratio of the CD38⁻ (R5) to CD38⁺ (R6) cell populations in the bone marrow from 5-FU-treated mice was 1:1, we transplanted 3×10^5 cells of both populations per mouse. The average engraftment levels of the recipients transplanted with CD38⁻ cells and CD38⁺ cells were $10.4 \pm 9.0\%$ and $43.8 \pm 14.7\%$ at 6 months post-transplantation, respectively (Figure 6). These values were different at $P < 0.002$. Again, multilineage engraftment was seen in all recipient mice.

Discussion

In this report, we studied CD38 expression by HSCs in the bone marrow of newborn and 5-week-old mice. The results clearly demonstrated that all HSCs of newborn mice and the majority of HSCs in the bone marrow of 5-week-old mice are CD38⁻. We also observed that CD38⁺ marrow cells from 5-FU-treated 5-week-old mice show higher engraftment levels than CD38⁻ cells. The latter observation indicated that activation of juvenile HSCs enhances CD38 expression. This direction of changes is opposite to that of the changes seen in adult HSCs.⁶

Earlier, Randall *et al*⁷ demonstrated that only CD38^{high} population, but not CD38⁻ population, in Sca1⁺, c-kit⁺, Lin^{low/-} liver cells from 14.5 days post coitum (dpc) mice and adult mice are capable of long-term hematopoietic reconstitution. Dagher *et al*⁸ also demonstrated that only CD38⁺ population in c-kit⁺, Lin⁻ yolk sac cells derived from 9 dpc mice, as well as adult mice are capable of long-term reconstitution. Based on these observations, it was concluded that CD38 is expressed by HSCs throughout murine ontogeny.⁸ However, our results clearly indicated that all HSCs of newborn mice and the majority of HSCs in the bone marrow of 5-week-old

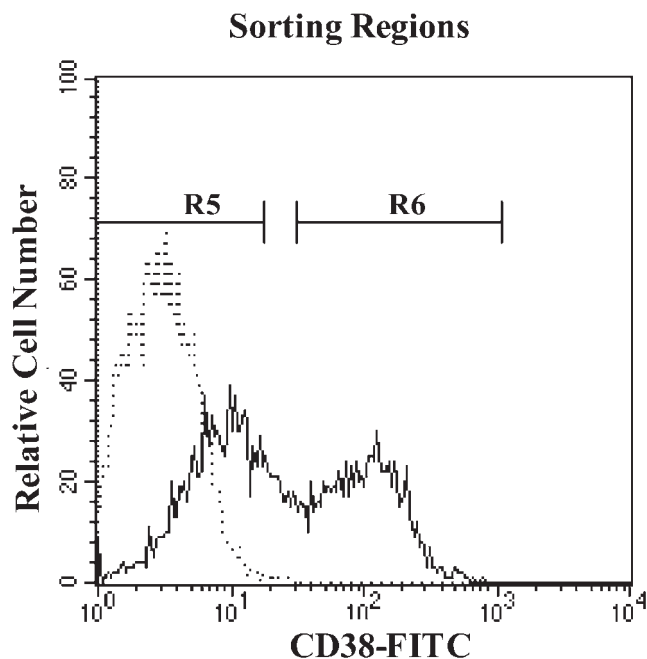


Figure 5 FACS sorting regions used for bone marrow cells of 5-FU-treated 5-week-old mice. MNCs collected from nine 5-FU-treated 5-week-old mice were sorted for CD38⁻ and CD38⁺ populations using R5 and R6 electronic windows, respectively. Dotted and solid lines represent cells stained with isotype-matched Ig and anti-CD38 antibody, respectively.

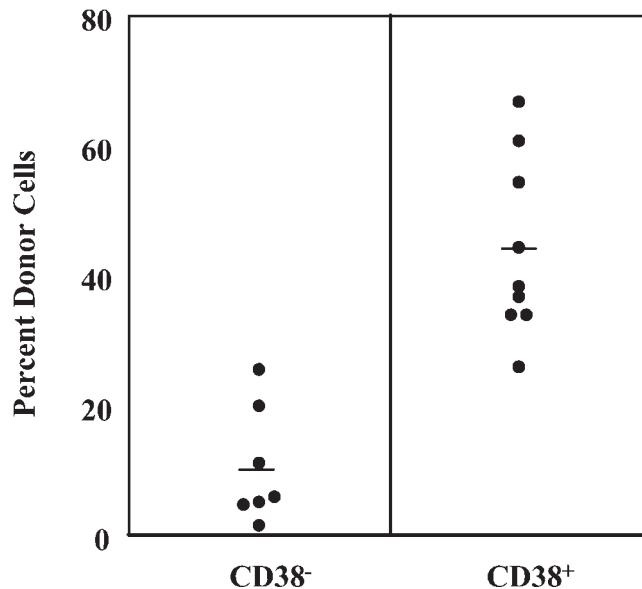


Figure 6 Percentages of donor nucleated cells in the blood of individual recipient mice at 6 months after transplantation. Mice were transplanted with either CD38⁻ or CD38⁺ mononuclear marrow cells of 5-FU-treated 5-week-old mice. Because the ratio of CD38⁻ (R5) and CD38⁺ (R6) cells was 1:1 according to Figure 5, 3.0×10^5 cells from either population were injected per mouse. Both groups consisted of 10 mice. The difference between the groups is significant at $P < 0.002$. Results shown are representative of two experiments.

mice are CD38⁻. The first ontogenetic conversion of HSC CD38 expression from CD38⁺ to CD38⁻ state must take place between 14.5 dpc and the perinatal period. Earlier, we documented that a minority of adult HSCs are CD38⁻.⁶ It appears that the second ontogenetic conversion from CD38⁻ to CD38⁺ stem cells begins before the age of 5 weeks.

In earlier reports from our laboratory,^{2,5} we described that CD34, another important surface molecule of HSCs, is also under developmental control and affected by the activation state of HSCs. HSCs in fetal and juvenile mice are CD34⁺.⁵ The change of HSC CD34 expression from the positive to negative state takes place between 7 and 10 weeks of age for the majority of murine HSCs.⁵ The physiological significance of the developmental change in stem cell CD34 expression is unclear because genetically engineered CD34-null mice show normal hematopoiesis *in vivo*.¹³

The physiological significance of the bidirectional ontogenic changes in CD38 expression of HSCs we described here is also unknown. CD38 is an ectoenzyme that utilizes NAD⁺ (nicotinamide adenine dinucleotide) and is expressed by many hematopoietic cells.¹⁴ However, CD38-null mice exhibited a normal compartment of all hematopoietic lineages,¹⁵ indicating that CD38 is dispensable for lymphohematopoiesis. It is possible that the absence of stem cell abnormalities seen in the CD38- and CD34-null mice may be due to the presence of other molecules with functional redundancy. Both CD38 and CD34 are considered to be adhesion molecules.^{13,14} The fact that they are ontologically regulated may indicate an important function that has yet to be elucidated.

Murine CD38 cDNA shows 70% sequence homology with that of human CD38.¹⁶ Thus, our observations in a mouse model may possess implications for CD38 expression by HSCs from human umbilical cord blood and the bone marrow of young children under steady-state and pathological conditions.

Acknowledgements

This work was supported by NIH grants RO1-DK54197 and PO1-CA78582 and by the Office of Research and Development, Medical Research Service, Department of Veterans Affairs. The authors thank Anne G Livingston, Karen A Rivers and Dr Tsukasa Higuchi for assistance in preparation of this manuscript, and the staff of Radiation Oncology Department of the Medical University of South Carolina for assistance in irradiation of the mice.

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