ATT AAA AAG-3' (6635F) and 5'-ACC CCA TAT GAT ATC ATC C-3' (6637R), and 5'-AAA GAA TCA GGA AAA TAT A-3' (6636F) and 5'-GAT ATC ATC CAT ATA TTG ATA T-3' (6638R), were used [5]. However, neither viral RNA nor proviral DNA was detected in any case (data not shown). The negative results in the detection of viral RNA or proviral DNA might be due to the elimination of the virus from the serum by host humoral immune responses or the low viral replication efficacy. In general, it is recognized that FIV is transmitted among hosts in a blood borne manner via injury, through fighting or bites. Therefore, it is conceivable that direct contact of lions with FIV-infected cats or transference from infected lions is a major possible route. However, such events do not commonly happened in Japanese zoos. Although the virus detection was unsuccessful in this study, the virus isolation from captive lions in Japanese zoos followed by genetic analyses of the viruses should clarify the infection route. The lions showing typical symptoms of FIV infection, including stomatitis, lymphadenopathy, opportunistic infections and immunodeficiency, were not observed in the present surveillance. However, FIV and other lentiviruses frequently have mutations, especially in the *env* region. Therefore, the appearance of lion-adapted and highly pathogenic strains of FIV or LLV for Lions can not be excluded. Careful monitoring of the lions with respect to FIV or LLV infection as well as CDV infection will be also required.

In the present study, we showed that captive lions in Japanese zoos possessed antibodies against CDV, FIV (LLV) or both, which are originally lethal pathogens for domestic dogs and cats. Continuous surveillance of them and studies to clarify their pathogenicity in lions and to determine the sources of infections are required.

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Competitive Repopulation Assay of Two Gene-Marked Cord Blood Units in NOD/SCID/γc^{null} Mice

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In multiunit cord blood transplantation, hematopoietic stem cells from each unrelated cord blood (UCB) unit competitively reconstitute the hematopoietic system in a recipient. To evaluate the fate of the progeny of each UCB unit and to determine the effects of graft-versus-graft reaction, we established a novel competitive repopulation assay using NOD/SCID/γc^{null} mice in which human T lymphocytes develop from $CD34^+$ cells. $CD34^+$ cells from each UCB unit were labeled with recombinant lentivirus vectors carrying genes encoding either enhanced green fluorescent protein (EGFP) or enhanced yellow fluorescent protein (EYFP). Hematopoietic chimerism composed of both $EGFP^+$ and $EYFP^+$ cells was stably maintained up to 6 months after transplantation with purified $CD34^+$ cells; the ratio of $EGFP^+$ to $EYFP^+$ cells in peripheral blood and bone marrow posttransplantation was equivalent to the ratio of these cells at transplantation. However, when mononuclear cells from two UCB units were cotransplanted with $CD34^+$ cells, engraftment was highly competitive, with cells from only one or the other of the two UCB units surviving. Further subfractionations of mononuclear cells indicate that the skewed chimerism that is often observed in clinical multiunit cord blood transplantation may be mediated by the cooperation of both $CD4^+$ and $CD8^+$ T cells. The assay established here will be a useful tool for analyzing hematopoietic reconstitution in clinical multiunit cord blood transplantation.

Key Words: gene marking, lentivirus, hematopoietic stem cell, SCID mouse-repopulating cell assay, multiunit cord blood transplantation, NOG mouse, competitive repopulation assay

INTRODUCTION

Cord blood (CB) is a potentially rich alternate source of hematopoietic stem cells (HSCs) and progenitors for clinical allogeneic transplantation [1,2]. Despite some promising outcomes with unrelated cord blood transplantation (UCBT) in pediatric recipients, the low cell content of the graft relative to recipient size may adversely affect both the time to hematopoietic recovery and survival [3–6]. Therefore, the major limitation to the widespread use of unrelated CB (UCB) as a source of HSC for transplantation, particularly in adults, is the low yield of stem cells. To overcome this, several centers have initiated multiunit UCBT (m-UCBT) in which two or more units of closely HLA-matched UCB are transplanted [7–9].

In clinical m-UCBT, HSCs from multiple UCB units competitively reconstitute the hematopoietic and immune systems, raising the possibility of a graft-versus-graft reaction or of multiple graft-versus-host reactions. Although an *in vivo* competitive repopulation assay has been widely used in mice [10–12], dogs [13], and nonhuman primates [14,15] to evaluate the potency of HSCs for hematopoietic reconstitution, there is currently no practical assay system for competitive repopulation by human HSCs, which might help to predict the results of graft-versus-graft reactions and immune reconstitution by multiple UCBs.

Xenogeneic transplantation models, in particular the severe combined immunodeficient (SCID) mouse-repopulating cell assay, have been used to evaluate *in vivo* human HSC activity, such as self-renewal and multilineage differentiation [16–19]. The nonobese diabetic (NOD)/SCID mice and related strains have

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been available for competitive repopulation assay of human HSCs, but a major shortcoming of the NOD/SCID mouse is a lack of reproducible T cell differentiation from $CD34^+$ cells [20–22]. We recently reported that the NOD/SCID/γc^{null} (NOG) mouse, which had been crossed with mice expressing a form of the IL-2Rγ chain lacking the cytoplasmic region, reproducibly develops human T cells, in addition to myeloid, NK, and B-lymphoid cells when transplanted with cord blood $CD34^+$ cells [23–25]. The repopulated T cells bear polyclonal $\alpha\beta$ TCR and respond not only to mitogenic stimuli, such as PHA and IL-2, but also to allogeneic human cells. These results indicate that functional human T lymphocytes can be reconstituted from $CD34^+$ cells in NOG mice. These animals therefore provide a new system in which it is possible to analyze *in vivo* competitive repopulation of the full lymphopoietic system using human HSCs.

Here, we report a novel competitive repopulation system, in which two UCB units were labeled with recombinant lentiviral vectors carrying either an enhanced green fluorescent protein (EGFP)- or an enhanced yellow fluorescent protein (EYFP)-encoding gene to enable easy identification of progeny cells. Using this system, we evaluated competitive repopulation by purified $CD34^+$ cells from two units of UCB with or without cotransplantation of mononuclear cells (MNC) and their purified populations.

RESULTS

Competitive Repopulation of Combined UCB $CD34^+$ Cells Transplanted into the NOG Mouse

We transplanted 11 pairs of UCB $CD34^+$ cells bearing distinct HLA alleles simultaneously into sublethally irradiated NOG mice (Table 1). To distinguish progeny derived from each UCB unit of $CD34^+$ cells, we labeled one unit by transfection with a lentivirus vector containing the EGFP gene and the other unit with a lentivirus vector containing the EYFP gene. After transplantation, we collected peripheral blood (PB) cells retro-orbitally at various intervals and analyzed them by flow cytometry for the presence of human hematopoietic cells expressing the leukocyte common antigen CD45 (Figs. 1A and 1B). Individual progeny from each unit of UCB $CD34^+$ cells were easily identified by the expression of these marker genes.

To evaluate the stability of any chimerism from two UCB donors in mice, we plotted the ratio of $EGFP^+$ to $EYFP^+$ cell number in PB over time after transplantation. Although the transduction efficiencies and numbers of $CD34^+$ cells varied between each experiment, the chimeric ratio of $EGFP^+$ to $EYFP^+$ cells observed at 3 weeks after transplantation was fairly stable up to at least 18 weeks (Fig. 1C). In some experiments, the ratio was stable up to 6 months after transplantation ($n = 3$ analyzed, data not shown).

To evaluate the relative viability of the two UCB donors in mice, we plotted the ratio of $EGFP^+$ to $EYFP^+$

TABLE 1: Characteristics of the CB units

Expt	No. cells ($\times 10^4$)	$CD34^+CD38^-$ (%)	Infection	Transduction efficiency (%)	HLA A	HLA B	HLA DR
1	5.5	10.99	GFP	12.72	2/26	46/60	8/–
	11.4	8.7	YFP	12.11	2/–	51/75	15/–
2	11	5.86	GFP	17.04	1/24	37/52	14/15
	8	4.56	YFP	16.72	2/24	7/35	1/4
3	13.8	10.21	GFP	14.84	24/–	7/61	1/9
	16	4.8	YFP	15.13	2/24	46/–	8/–
4	15.8	4.35	GFP	7.56	24/33	44/52	9/13
	10.5	5.67	YFP	8.13	1/24	37/52	10/15
5	19	6.37	GFP	18.64	2/24	46/62	4/8
	13.9	4.1	YFP	17.87	24/26	7/61	9/13
6	17.5	8.39	GFP	18.76	N.D.	N.D.	N.D.
	24	6.22	YFP	17.81	2/–	59/61	4/–
7	8.75	13.14	GFP	10.35	2/24	35/62	4/11
	4	5.24	YFP	15.84	2/33	51/–	8/9
8	9	14.58	GFP	28.16	24/26	52/62	4/15
	35	5.4	YFP	25.58	2/24	46/61	8/9
9	98	7.57	GFP	25.28	24/33	7/44	1/13
	47	6.84	YFP	26.63	24/–	52/61	9/15
10	18.75	9.24	GFP	25.20	24/26	54/61	4/9
	15.75	4.55	YFP	12.55	11/24	51/62	11/15
11	10.5	17.6	GFP	27.01	24/33	44/52	9/13
	9.5	12.53	YFP	23.84	1/24	37/52	10/15

N.D., not determined. –, blank allele.

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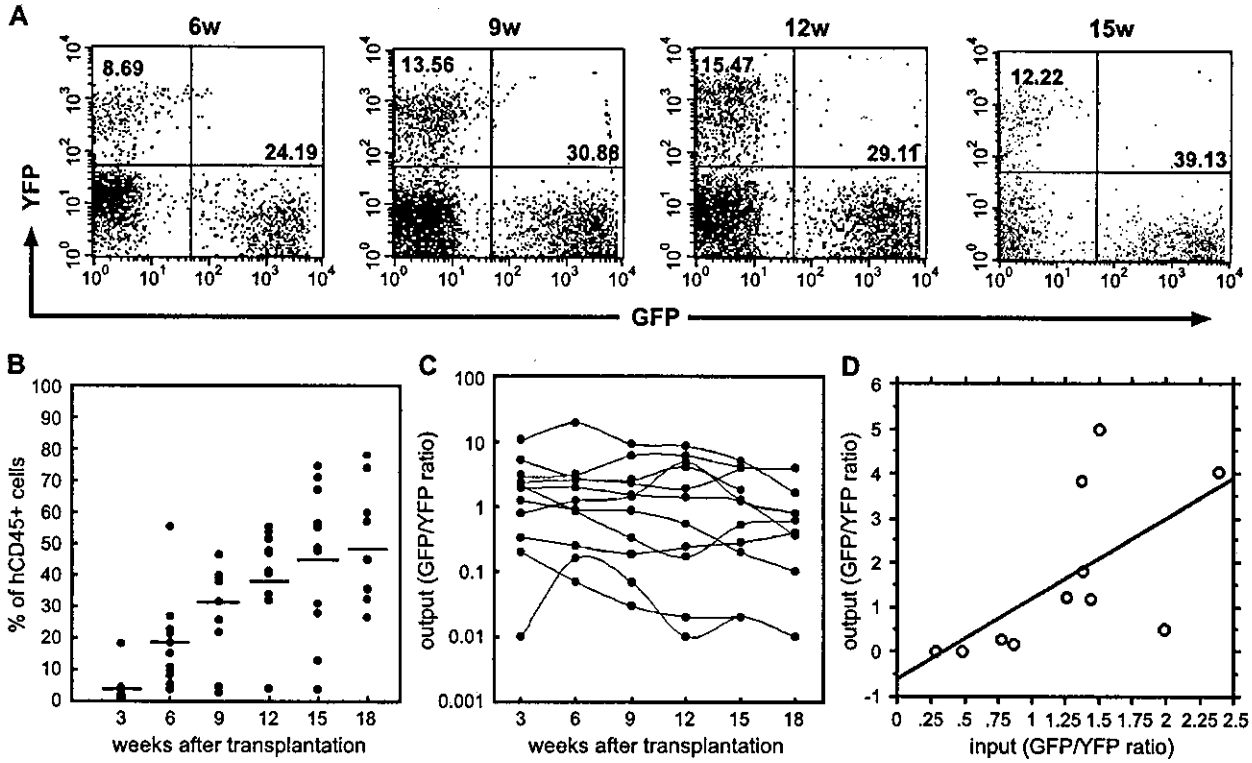

FIG. 1. (A) Representative FACS profiles of competitive repopulation of EGFP- or EYFP-transduced CB CD34$^+$ cells in an individual NOG mouse recipient. PB cells were collected at specific intervals and stained with anti-human CD45 mAb. The stained cells were then analyzed by flow cytometry gated for the expression of CD45. Each donor-derived human hematopoietic cell was distinguished by the expression of the marker EGFP or EYFP. (B) Kinetics of human hematopoietic cells in NOG mice transplanted with two units of CB CD34$^+$ cells. PB cells were collected at various intervals from transplanted NOG mice (n = 11) and stained with anti-human CD45 mAb. Each dot represents one mouse recipient, and bars indicate the average of engraftment. (C) The ratio of each donor's derived CD45$^+$ hematopoietic cells detected in the NOG mouse PB was calculated. (D) A weak linear relationship between the ratio of injected CD34$^+$ cell number (horizontal axis) and the ratio of human hematopoietic cells engrafted in the PB at 15 weeks after transplantation (vertical axis) (r = 0.623, n = 11, P = 0.039).

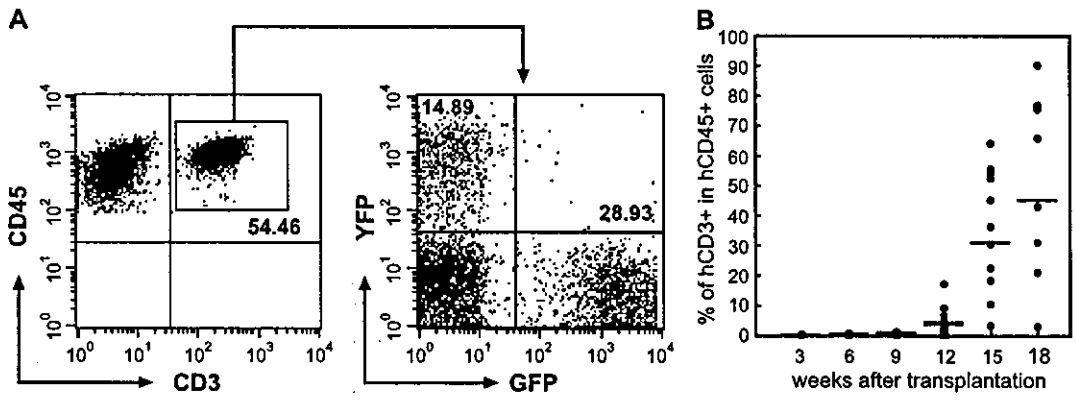

FIG. 2. (A) Representative FACS profiles of CD3$^+$ T lymphocytes in competitive repopulation of EGFP- or EYFP-transduced CB CD34$^+$ cells in an individual NOG mouse recipient. PB cells were collected at 15 weeks after transplantation. Anti-human CD3 mAb and anti-human CD45 mAb were used to detect human T lymphocytes in the PB of engrafted NOG mice. The CD3$^+$CD45$^+$ region was gated and marker gene expression was analyzed. (B) Kinetics of human CD3$^+$ T lymphocytes in NOG mice transplanted with two units of CB CD34$^+$ cells. PB cells were collected at various intervals from transplanted NOG mice (n = 11) and stained with anti-human CD3 mAb and anti-human CD45 mAb. The stained cells were then analyzed by flow cytometry gated for the expression of CD45. Each dot represents one mouse recipient, and bars indicate the average engraftment.

TABLE 2: Lineage analysis of T lymphocytes derived from two UCB units in the thymus of NOG mice

Expt	Phenotype	Lineages (%) DN	DP	$CD4^+$ SP	$CD8^+$ SP
1	$GFP^+/CD45^+$	N.D.	N.D.	N.D.	N.D.
	$YFP^+/CD45^+$	N.D.	N.D.	N.D.	N.D.
3	$GFP^+/CD45^+$	4.11	69.08	14.09	12.72
	$YFP^+/CD45^+$	3.86	66.80	16.22	13.13
4	$GFP^+/CD45^+$	8.00	50.50	26.00	15.50
	$YFP^+/CD45^+$	1.60	65.07	21.60	18.16
6	$GFP^+/CD45^+$	8.90	44.70	27.35	22.72
	$YFP^+/CD45^+$	1.96	67.99	18.22	11.83
7	$GFP^+/CD45^+$	15.17	45.07	18.16	21.6
	$YFP^+/CD45^+$	18.20	43.80	17.22	20.78
9	$GFP^+/CD45^+$	32.64	34.55	12.81	19.99
	$YFP^+/CD45^+$	22.32	20.06	16.47	41.15
10	$GFP^+/CD45^+$	2.03	35.29	28.26	34.42
	$YFP^+/CD45^+$	0.24	44.01	20.45	35.30
11	$GFP^+/CD45^+$	16.55	51.39	18.39	16.55
	$YFP^+/CD45^+$	0.29	38.07	24.08	37.56

N.D., not determined.

cell number in PB against the ratio of engrafted $CD34^+$ cells. There was a linear relationship between the $EGFP^+$ to $EYFP^+$ cell ratio observed at 15 weeks after transplantation and the $EGFP^+$ to $EYFP^+$ $CD34^+$ cell ratio at the time of transplantation ($r = 0.623$, $P = 0.039$, $n = 11$; Fig. 1D). Similarly, the $EGFP^+$ to $EYFP^+$ ratio in engrafted cells in bone marrow (BM) correlated with the $EGFP^+$ to $EYFP^+$ ratio at transplantation ($r = 0.713$, $P = 0.0458$, $n = 8$), although three mice died before the analysis.

These data demonstrate that one unit of UCB $CD34^+$ cells will engraft with the same efficiency in NOG mice regardless of whether a second, different, unit of UCB $CD34^+$ cells is simultaneously transplanted.

Multilineage Differentiation of Two Units of Cord Blood $CD34^+$ cells

Consistent with our previous results [23], we began to detect human $CD3^+$ T lymphocytes at 9 weeks posttransplantation of UCB $CD34^+$ cells and their numbers steadily increased up to 18 weeks (Fig. 2B). Both $EGFP^+$ and $EYFP^+$ $CD3^+$ T lymphocytes were detected in PB of NOG mice between 9 and 18 weeks posttransplantation (Fig. 2A).

Fifteen to 24 weeks after transplantation, we killed the mice and collected the thymus, BM, and spleen to analyze the progeny of the two units of UCB $CD34^+$ cells. In the thymus, most thymocytes in the NOG mice were human $CD3^+$ cells, which had differentiated from both $EGFP^+CD34^+$ cells and $EYFP^+CD34^+$ cells (Table 2 and Fig. 3). Thymocytes from both donors showed normal differentiation patterns, which comprised both double-positive and single-positive subsets of CD4/CD8.

In BM and spleen, we analyzed multilineage differentiation of $EGFP^+CD34^+$ cells and $EYFP^+CD34^+$ cells by gating $EGFP^+CD45^+$ and $EYFP^+CD45^+$ cells. In addition to $CD3^+$ T cells, $CD19^+$ B cells, $CD56^+$ NK cells, $CD14^+$ monocytes, $CD33^+$ myeloid cells, and primitive $CD34^+$ cells arose from both the $EGFP^+CD45^+$ and the $EYFP^+CD45^+$ units (Table 3, Fig. 4, and spleen data not shown).

These data demonstrate that the multilineage engraftment of one unit of UCB $CD34^+$ cells in NOG mice is

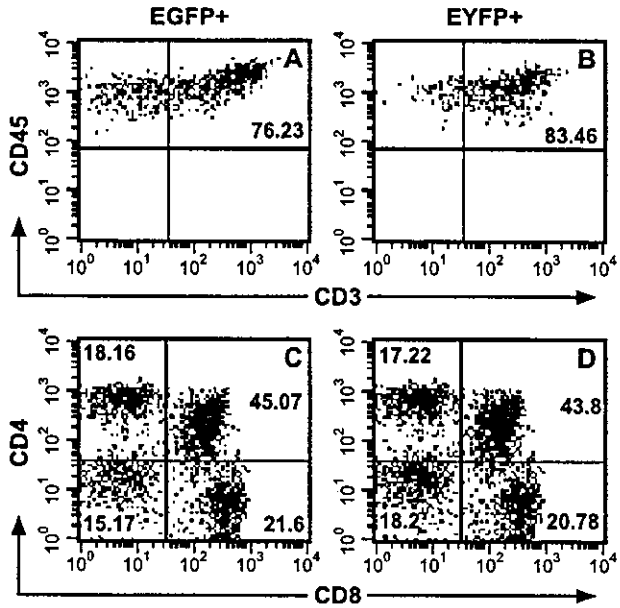

FIG. 3. Representative FACS analysis of human T cell engraftment in the thymus of a NOG mouse recipient transplanted with two combined units of CB $CD34^+$ cells. At 18 weeks after transplantation, thymocytes were collected and stained with anti-human CD45 mAb. Each (A, C) $CD45^+EGFP^+$ and (B, D) $CD45^+EYFP^+$ region was gated, and the expression of (A, B) CD3 and (C, D) CD4/CD8 was analyzed. The relative frequencies of each population are indicated.

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TABLE 3: Lineage analysis of two UCB units of $CD34^+$-derived hematopoietic cells that had differentiated in BM of NOG mice

Expt	Phenotype	Lineages (%) $CD14^+$	$CD19^+$	$CD33^+$	$CD34^+$	$CD56^+$
1	$GFP^+/CD45^+$	11.76	38.46	37.27	15.91	4.00
	$YFP^+/CD45^+$	12.22	57.47	19.54	12.05	2.33
3	$GFP^+/CD45^+$	16.28	34.34	38.26	14.01	4.35
	$YFP^+/CD45^+$	18.24	25.07	49.22	16.47	8.13
4	$GFP^+/CD45^+$	9.21	21.37	10.16	10.29	0.88
	$YFP^+/CD45^+$	10.76	29.86	12.63	10.72	1.61
6	$GFP^+/CD45^+$	24.44	11.18	37.77	3.36	4.75
	$YFP^+/CD45^+$	10.97	46.70	13.52	5.86	8.04
7	$GFP^+/CD45^+$	17.17	62.97	28.72	18.91	1.32
	$YFP^+/CD45^+$	12.85	48.52	20.42	9.49	2.55
9	$GFP^+/CD45^+$	5.25	18.37	11.13	6.44	6.17
	$YFP^+/CD45^+$	17.19	37.23	31.25	6.60	7.23
10	$GFP^+/CD45^+$	5.33	24.63	10.65	3.22	2.98
	$YFP^+/CD45^+$	3.06	18.24	3.70	3.93	2.67
11	$GFP^+/CD45^+$	10.78	52.72	13.51	3.90	2.25
	$YFP^+/CD45^+$	26.24	29.46	7.44	9.76	1.06

stable and unaffected by lymphopoietic repopulation by another UCB unit.

Effects of Cotransplantation of $CD34^-$ Mononuclear Cells on Competitive Repopulation in The NOG Mouse

Unfractionated UCB cells containing mature immune-competent cells are commonly used in clinical UCBT. These cells can induce graft-versus-host, graft-versus-tumor, and graft-versus-graft reactions. The effects of the latter reaction on engraftment in particular are poorly understood in m-UCBT. To assess these effects, we performed three sets of competitive repopulation assay by cotransplantation of pairs of $CD34^-$ MNC with $EGFP^+CD34^+$ cells and $EYFP^+CD34^+$ cells (Table 4).

Four to 6 weeks posttransplantation, we sacrificed the mice and analyzed the ratio of engrafted $EGFP^+CD45^+$ and $EYFP^+CD45^+$ cells in BM MNC by flow cytometry. When only purified $CD34^+$ cells were transplanted, both UCB units engrafted as expected. However, when $CD34^-$ MNCs were cotransplanted with $CD34^+$ cells, only $CD45^+$ cells from one or the other UCB engrafted in all mice analyzed (Fig. 5). These results indicate that $CD34^-$ MNC from one UCB somehow inhibited engraftment of cells from the other UCB.

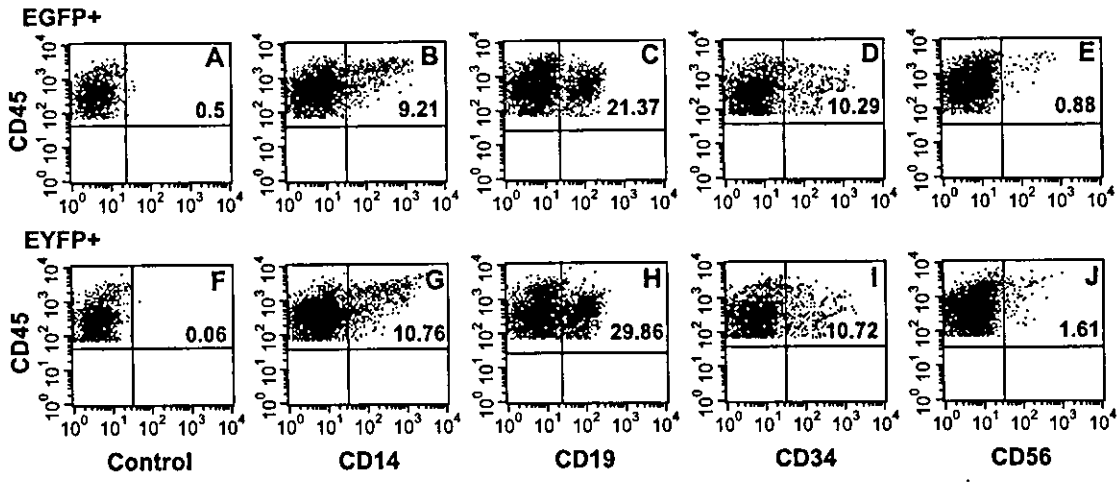

FIG. 4. Representative FACS profile of human multilineage engraftment in a NOG mouse recipient transplanted with two combined units of CB $CD34^+$ cells. At 18 weeks after transplantation, BM cells were collected and analyzed for multilineage differentiation of engrafted human hematopoietic cells. To distinguish hematopoietic cells derived from each donor, each (A–E) $CD45^+EGFP^+$ and (F–J) $CD45^+EYFP^+$ region was gated, and the percentage of human $CD45^+$ cells expressing the respective surface markers was measured. Human lineage-specific mAbs were used to detect (B, G) myeloid $CD45^+CD14^+$, (C, H) lymphoid $CD45^+CD19^+$, (E, J) $CD45^+CD56^+$, and (D, I) immature $CD34^+$ progenitor cells in the marrow of engrafted NOG mice. The relative frequencies of each population are indicated.

TABLE 4: Characteristics of purified CD34⁺ cells and MNCs in each CB unit

Expt	Infection	CD34⁺/mouse (×10⁴)	MNC/mouse (×10⁶)	HLA A	HLA B	HLA DR	HLA DRw
1	GFP	6.0	7.4	2/–	59/61	4/–	53/–
	YFP	6.0	8.8	2/24	51/67	9/12	52/53
2	GFP	12	1.0	2/24	7/51	1/9	53/–
	YFP	7.3	1.0	24/33	44/75	4/13	52/53
3	GFP	16	1.0	24/–	60/–	11/13	52/–
	YFP	11	1.0	2/24	7/61	1/9	53/–

–, blank allele.

To clarify the cell population responsible for this inhibitory effect, we cotransplanted purified CD4⁺ or CD8⁺ T lymphocytes or CD4/CD8 double-negative (DN) cells with EGFP⁺CD34⁺ cells and EYFP⁺CD34⁺ cells into NOG mice (Table 5). Although CD34⁻ MNCs eliminated either EGFP⁺CD45⁺ or EYFP⁺CD45⁺ cells as expected, there was no inhibition of engraftment of EGFP⁺CD45⁺ or EYFP⁺CD45⁺ cells by purified CD4⁺ or CD8⁺ T lymphocytes or CD4/CD8 double-negative cells in any mice (Fig. 6). These results indicate that a combination of two or more populations of CD4⁺, CD8⁺, and/or DN cells is required for this inhibitory effect.

To determine the effects of a combination of two populations on engraftment, we cotransplanted EGFP⁺CD34⁺ cells and EYFP⁺CD34⁺ cells with combined populations of CD4⁺ and CD8⁺ cells, CD4⁺ and DN cells, and CD8⁺ and DN cells into NOG mice (Table 6). Both CD4⁺ and CD8⁺ cells from one donor cooperated to inhibit engraftment of cells from the other donor, as we observed with whole CD34⁻ MNC cotransplantation (Fig. 7A). There was no inhibition of engraftment of EGFP⁺CD45⁺ or EYFP⁺CD45⁺ cells by the combination of CD4⁺ and DN cells or CD8⁺ and DN cells (Fig. 7B).

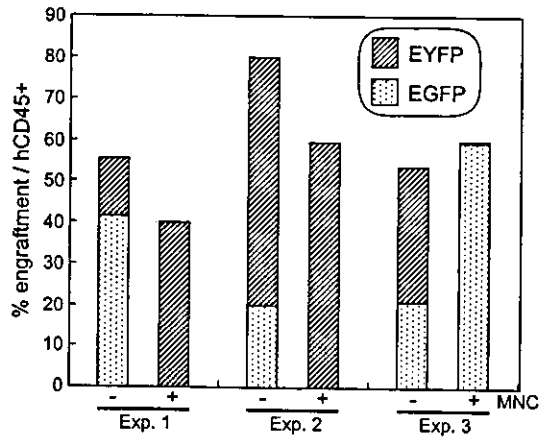

FIG. 5. The effects of CD34⁻ MNCs on competitive repopulation by CD34⁺ cells in the NOG mouse. EGFP- or EYFP-transduced CD34⁺ cells with or without a respective fraction of CD34⁻ MNCs were injected into the same NOG mouse (Table 4). At 4 to 6 weeks after transplantation, the ratio of engrafted donor-derived hematopoietic cells from each unit was measured using the expression of CD45 and the marker genes in flow cytometry. To exclude residual human T cells derived from CD34⁻ MNCs, CD3⁺ cells were gated out at FACS analysis. Data shown are the ratios of CD45⁺EGFP⁺ and CD45⁺EYFP⁺ cells in the BM MNCs of the same NOG recipient (three independent experiments).

Discussion

The possibility of competitive reconstitution of the hematopoietic system by HSCs from multiple UCB units in clinical m-UCBT raises several important questions. Do immune-competent cells developed from two unrelated HSCs affect hematopoietic engraftment or reconstitution in a host? Do mature immune-competent cells derived from two UCB units affect hematopoietic engraftment or reconstitution in a host? To address these questions, we used a novel *in vivo* competitive repopulation system using NOG mice that allows complete reconstitution of human lymphocytes, including T cells, from CD34⁺ UCB that respond to both mitogenic stimuli, such as PHA and IL-2, and allogeneic human cells [23,25].

We demonstrated here that hematopoietic cells derived from two UCB units engrafted stably, even after CD3⁺ T cells were detected in recipient PB at 12 weeks after transplantation, and that the ratio of chimerism between cells derived from the two UCB units was correlated with the ratio of cells at transplantation (Fig. 1). Recently, Kim *et al.* [26] demonstrated that two lineage-depleted UCB units could coengraft in NOD/SCID mice. However, this assay could not assess the effects of an immune system derived from HSCs on engraftment by the other, since T cells did not differentiate from CD34⁺ cells in NOD/SCID mice. Therefore, this is the first demonstration that human immune-competent cells from two immunologically distinct CD34⁺ donor stem cells can develop normally in the same host environment without inhibiting each other. In recipient NOG mouse thymus, both EGFP⁺CD3⁺ and EYFP⁺CD3⁺ cells showed a normal pattern of T cell differentiation, which comprised both double-positive and single-positive CD4/CD8 subsets (Fig. 4 and Table 2). This suggests that there may have been negative selection in the clonal deletion of cells reactive to each other. Therefore, the content of HSCs may be a good predictor of the resultant chimerism in a mouse, as shown in Fig. 1D.

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TABLE 5: Characteristics of purified CD34$^+$ cells and effector cells in each CB unit

Expt	Infection	CD34$^+$ cells[a]	MNC fraction[b]	HLA A	HLA B	HLA DR	HLA DRw
1	GFP	+	+	2/31	51/54	4/15	51/53
	YFP	+	–	2/33	39/44	9/13	52/53
2	GFP	+	–	11/24	54/67	4/8	53/–[c]
	YFP	+	+	2/24	35/62	9/12	52/53
3	GFP	+	+	2/24	35/61	4/8	53/–
	YFP	+	–	2/26	35/61	9/15	51/53

[a] EGFP- or EYFP-transduced CD34$^+$ cells were transplanted into the same NOG mouse (range of transplanted cell dose 6.8–9 × 10^4 cells).
[b] Unfractionated MNCs (range 7–10 × 10^5 cells), CD4$^+$ cells (range 3.3–5 × 10^5 cells), CD8$^+$ cells (range 1.4–2 × 10^5 cells), and CD4/CD8 double-negative fraction (range 2–3 × 10^5 cells) were prepared from one CB CD34$^-$ fraction (+) and injected into NOG mice together with CD34$^+$ cells derived from the same donor and CD34$^+$ cells derived from the second unit of CB (–).
[c] –, blank allele.

In clinical m-UCBT, the transplanted whole MNC can induce a rapid graft-versus-graft reaction. One study reported successful engraftment of only 1 of 12 randomly selected units of UCB [7]. Another study demonstrated that only one of the two UCB donors was responsible for hematopoiesis in 16 of 18 patients with sustained engraftment [27]. However, another study found that both of the transplanted UCBs contributed to stable hematopoiesis, and this success was attributed to the grafts being closely matched, with only a single mismatch between them at one HLA-DRB1 allele [8]. In the present study, we observed engraftment of either of mismatched pairs of UCB units (Tables 4 and 5), confirming the utility of this animal model to analyze engraftment and hematopoiesis in m-UCBT. We also observed that graft-versus-graft reaction was associated with the CD34$^-$ mononuclear cells in a cord blood unit. In future experiments it would be of interest to analyze the chimerism when an HLA-matched UCB pair is cotransplanted.

One possible drawback to our model is the potential antigenicity of the marker genes EGFP and EYFP used to identify the transplanted cell populations. These two proteins have very similar antigenic properties, since EYFP is simply a mutant form of EGFP [28]; therefore any specific immune reaction would be expected to have an equivalent effect on both cell populations. However, in every case shown in Figs. 5, 6, and 7 either EGFP$^+$ or EYFP$^+$ cells were eliminated, suggesting that this was due to an allospecific reaction.

To identify further the cell population responsible for graft-versus-graft reaction, we cotransplanted purified populations of CD4$^+$, CD8$^+$, or DN cells from two UCBs together with CD34$^+$ cells, but none of these cell populations alone was sufficient to generate a reaction against the other UCB unit (Fig. 6). This suggests that perhaps two populations of effector cells are needed to induce a graft-versus-graft reaction. Consistent with this, we could reproduce this inhibitory effect on engraftment by CD34$^-$ mononuclear cells only when both CD4$^+$ and CD8$^+$ cells were transplanted simultaneously (Fig. 7). These results support the hypothesis that graft-reactive CD8$^+$ CTL served as the terminal effector cell in the rejection, while CD4$^+$ cells provided the signals required for CTL development and expansion [29]. As the strongest alloreaction is provoked by major histocompatibility complex (MHC) antigens, T cells recognize the alloantigens either "directly" as foreign antigens or "indirectly" as presented by self-MHC molecules [30,31]. Our results may suggest that CD4$^+$ and CD8$^+$ T cells from one graft recognized the MHC antigens from the other directly as nonself and then eliminated hematopoietic cells of the other graft, since DN cells were not required as antigen-presenting cells to elicit the reaction. However, Kim and colleagues demonstrated that the mixed transplantation

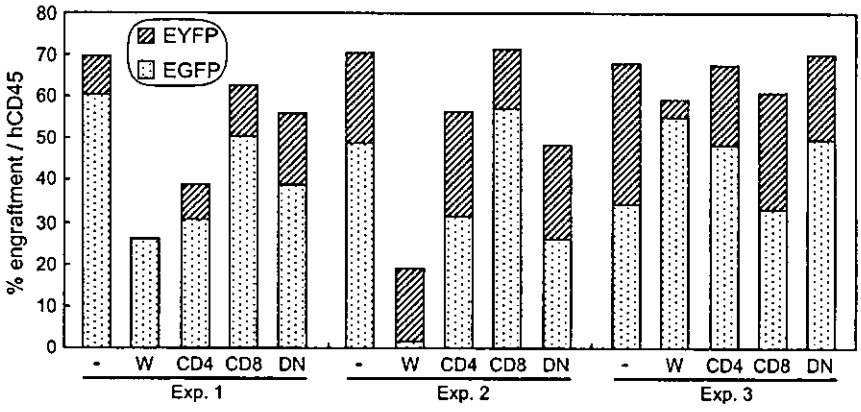

FIG. 6. The effects of fractionated effector cells contained in CD34$^-$ MNCs on competitive repopulation by CD34$^+$ cells in the NOG mouse. CD4$^+$ or CD8$^+$ T lymphocytes and the CD4/CD8 double-negative fraction were sorted from one CB CD34$^-$ fraction and injected into NOG mice together with CD34$^+$ cells derived from the same CB and CD34$^+$ cells derived from the second unit of CB (Table 5). At 3 weeks after transplantation, the ratio of engraftment was analyzed by the same method as for Fig. 5. Data shown are the ratios of CD45$^+$EGFP$^+$ and CD45$^+$EYFP$^+$ cells in the BM MNCs of the same NOG recipient (three independent experiments). (–) Transplants without CD34$^-$ MNCs; (W) transplants with whole CD34$^-$ MNC.

TABLE 6: Characteristics of purified CD34⁺ cells and effector cells in each CB unit

Expt	Infection	CD34⁺ cells[a]	MNC fraction[b]	HLA A	HLA B	HLA DR	HLA DRw
1	GFP	+	–	2/31	35/–[c]	12/15	51/52
	YFP	+	+	24/26	51/62	4/15	51/53
2	GFP	+	+	2/31	46/61	8/15	51/–
	YFP	+	–	2/24	35/55	4/9	53/–
3	GFP	+	+	24/33	44/75	8/9	53/–
	YFP	+	–	11/–	44/59	4/13	52/53

[a] EGFP- or EYFP-transduced CD34⁺ cells (5×10^4 cells) were transplanted into the same NOG mouse.
[b] Unfractionated MNCs (10×10^5 cells), CD4⁺ cells (5×10^5 cells), CD8⁺ cells (2×10^5 cells), and CD4/CD8 double-negative fraction (range $2–3 \times 10^5$ cells) were prepared from one CB CD34⁻ fraction (+). Each fraction were combined as CD4⁺ and CD8⁺ cells, CD4⁺ and DN cells, and CD8⁺ and DN cells and transplanted into NOG mice together with CD34⁺ cells derived from the same donor and CD34⁺ cells derived from the second unit of CB (–).
[c] –, blank allele.

of two allogeneic UCB grafts led to single-donor predominance independent of the degree of HLA compatibility between the two grafts [26]. Further investigation is necessary to determine how CD4⁺ and CD8⁺ cells contribute to predominant engraftment of HSCs, through either direct recognition of the MHC antigens or other nonspecific effects.

We have established a novel *in vivo* competitive repopulation assay using a new NOG mouse system that may facilitate study of the behavior of different units of stem cells after transplantation. Furthermore, we clearly demonstrated that the lentiviral gene marking strategy can easily distinguish the two distinct donor cells by flow cytometric analysis, independent of

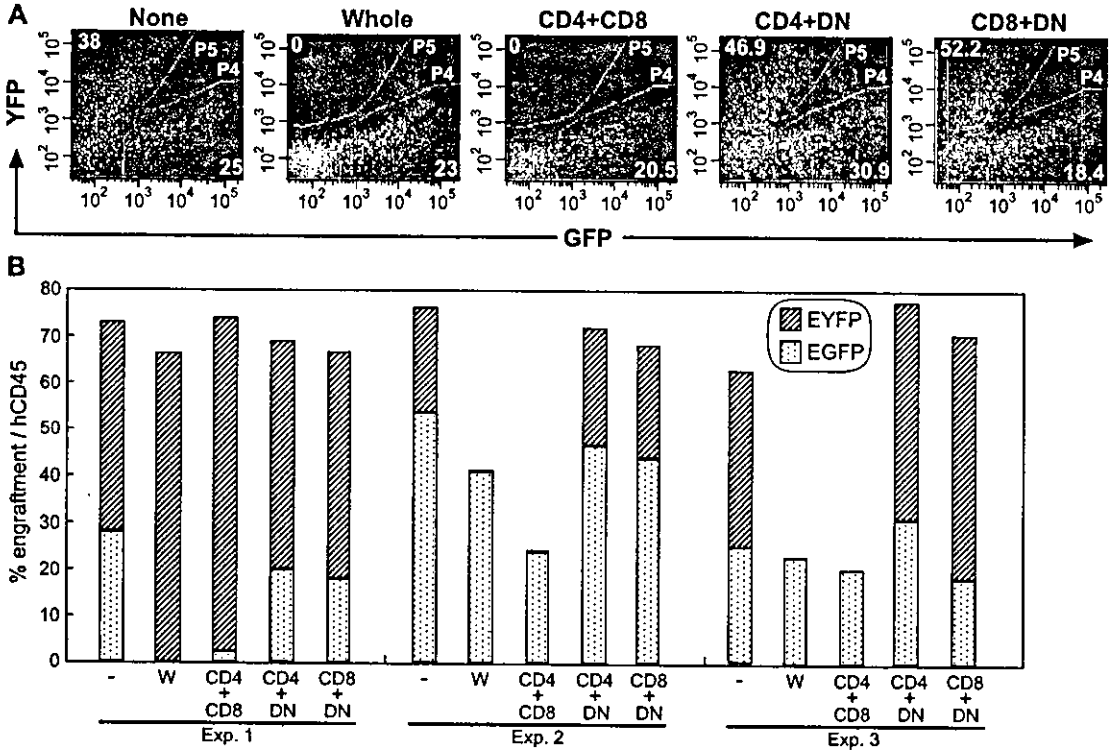

FIG. 7. The effects of combined cell populations contained in CD34⁻ MNCs on competitive repopulation by CD34⁺ cells in the NOG mouse. CD4⁺ and CD8⁺ T lymphocytes and the CD4/CD8 double-negative fraction were sorted from one CB CD34⁻ fraction. Each sorted fraction was combined to give CD4⁺ and CD8⁺ cells, CD4⁺ and DN cells, or CD8⁺ and DN cells and injected into NOG mice together with CD34⁺ cells derived from the same CB and CD34⁺ cells derived from the second unit of CB (Table 6). At 3 weeks after transplantation, the ratio of engraftment was analyzed by the same method as for Fig. 5. (A) Representative FACS profiles of competitive repopulation of EGFP- or EYFP-transduced CB CD34⁺ cells in an individual NOG mouse recipient. (B) Summary of three independent experiments. Data shown are the ratios of CD45⁺EGFP⁺ and CD45⁺EYFP⁺ cells in the BM MNCs of the same NOG recipient. (–) Transplants without CD34⁻ MNCs; (W) transplants with whole CD34⁻ MNC.

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cell surface antigens, expression of intracellular isoenzymes, or hemoglobin subtypes. This strategy is also useful for the examination of the stem cell competitive repopulation activity of variety cell sources such as BM cells and cord blood cells, *ex vivo* expanded cells, and nonexpanded cells. Gene transduction by lentiviral infection did not have any apparent untoward effects on the behavior of HSCs, such as multilineage differentiation and long-term repopulation.

We hope that the *in vivo* assay system described here will be useful, not only for examining the competitive hematopoietic repopulation from multiple UCB units, but also for predicting the effects of MNCs cotransplanted in clinical m-UCBT. To evaluate the relevance of this system as a surrogate human HSC assay, it will be important to correlate the results of engraftment in this model with engraftment in clinical m-UCBT.

Materials and Methods

Cells. CB samples were obtained from full-term deliveries following institutional guidelines approved by the Tokai University Committee on Clinical Investigation. MNCs were isolated by Ficoll–Hypaque (Lymphoprep, 1.077 ± 0.001 g/ml; Nycomed, Oslo, Norway) density gradient centrifugation. Cells were washed and suspended in phosphate-buffered saline (PBS) containing 0.1% of human serum globulin (Sigma, St. Louis, MO, USA). $CD34^+$ cell fractions were prepared from Ficoll-separated MNCs using the CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec, Sunnyvale, CA, USA) according to the manufacturer's directions. The enriched $CD34^+$ cell fraction was stained either with fluorescein isothiocyanate (FITC)-conjugated anti-CD34 mAb (581; Coulter/Immunotech, Marseille, France) or with allophycocyanin (APC)-conjugated anti-CD3 mAb (UCHT1; Coulter/Immunotech). $CD34^+CD3^-$ cells were sorted using a FACSVantage flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with HeNe and argon lasers. This resulted in a highly purified $CD34^+$ cell fraction (more than 99%) in which no $CD3^+$ cells were detected on FACS analysis. Sorted $CD34^+CD3^-$ cells were cryopreserved in liquid nitrogen until use. Column passthrough $CD34^-$ MNC fractions were also cryopreserved. On the day of transplantation, cryopreserved $CD34^-$ MNC fractions were thawed and stained with PE-conjugated anti-CD4 (SK3) and FITC-conjugated anti-CD8 (SK1) mAbs (all BD Biosciences). Stained cells were sorted using the FACSVantageSE Diva option (BD Biosciences) gated on each $CD4^+CD8^-$, $CD4^-CD8^+$, and $CD4^-CD8^-$ region. Purified populations were >98% pure. Dead cells stained with PI were excluded from the analysis. The HLA of each CB unit was determined by molecular typing using a Micro SSP HLA Classes I and II ABDR DNA Typing Tray (One Lambda, Canoga Park, CA, USA).

Lentivirus infection. Transduction of the EGFP or its yellow variant EYFP gene into $CD34^+$ cells by recombinant lentivirus infection was performed as described previously [32,33]. Briefly, cryopreserved CB $CD34^+$ cells were thawed and prestimulated by incubation in StemPro-34 medium (Invitrogen, Carlsbad, CA, USA) containing cytokines at 37°C in 5% CO_2 for 24 h. Recombinant human thrombopoietin (50 ng/ml; kindly donated by Kirin Brewery, Tokyo, Japan), stem cell factor (50 ng/ml; donated by Kirin Brewery), and Flk-2/Flt-3 ligand (50 ng/ml; R&D Systems, Minneapolis, MN, USA) were used. Prestimulated $CD34^+$ cells were cultured for 12 h under the same conditions in the presence of highly concentrated lentivirus supernatant at an m.o.i. of 50. Lentivirus-infected $CD34^+$ cells were transplanted into irradiated NOG mice. The efficiency of infection was determined by FACS analysis on the day of transplantation.

Mice. NOG mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan) and were maintained in the animal facility of the Tokai University School of Medicine in microisolator cages with autoclaved food and water. Seven- to nine-week-old NOG mice were irradiated with 250 cGy of X-rays and thereafter received acidified water containing 1.1 g/L neomycin sulfate and 131 mg/L polymyxin B sulfate (Sigma). The following day, two units of CB $CD34^+CD3^-$ cells were simultaneously injected intravenously into NOG mice. In some experiments, $CD34^-$ MNCs were cotransplanted with $CD34^+$ cells. All experiments were approved by the animal care committee of Tokai University.

Flow cytometric analysis. For kinetic analysis, mice were anesthetized with ethyl ether and PB samples were aspirated from the retro-orbital sinus. Samples were prepared as single-cell suspensions in PBS containing 0.1% human serum globulin and heparin. Human hematopoietic cells were distinguished from mouse cells by expression of human CD45. At the time of sacrifice, BM, spleen, and PB were collected for analysis of the presence of human cells by flow cytometry. BM cells were suspended in PBS using a 27-gauge needle. Spleen was teased apart, and PB was aspirated from the retro-orbital sinus. Samples were prepared as single-cell suspensions in PBS containing 0.1% human serum globulin and passed through a nylon filter to remove debris. Cells were stained with mAbs to human leukocyte differentiation antigens. APC-conjugated anti-human CD3, CD4, CD14, CD19, CD33, CD34, and CD56 mAbs (all Coulter/Immunotech) and ECD-conjugated anti-human CD8 and CD45 mAbs (all Coulter/Immunotech) were used. Four-color flow cytometric analysis was conducted using FACSVantage (BD Biosciences). Quadrants were set to include at least 97% of the isotype-negative cells. The proportion of each lineage was calculated from 20,000 events acquired using CELLQuest or FACSDiva software (BD Biosciences).

Statistical analysis. Results are expressed as individual data. Input (transplanted EGFP/EYFP ratio) and output (engrafted EGFP/EYFP ratio) were calculated by the following calculation: input (EGFP/EYFP ratio) = (EGFP transplanted cell number × infection efficiency)/(EYFP transplanted cell number × infection efficiency). Output (EGFP/EYFP ratio) = (percentage of $CD45^+EGFP^+$ cells in the recipient)/(percentage of $CD45^+EYFP^+$ cells in the recipient). The correlation of input values and output values was analyzed using StatView-J4.02 software (Abacus Concepts, Berkeley, CA, USA). P values <0.05 were considered to be significant.

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