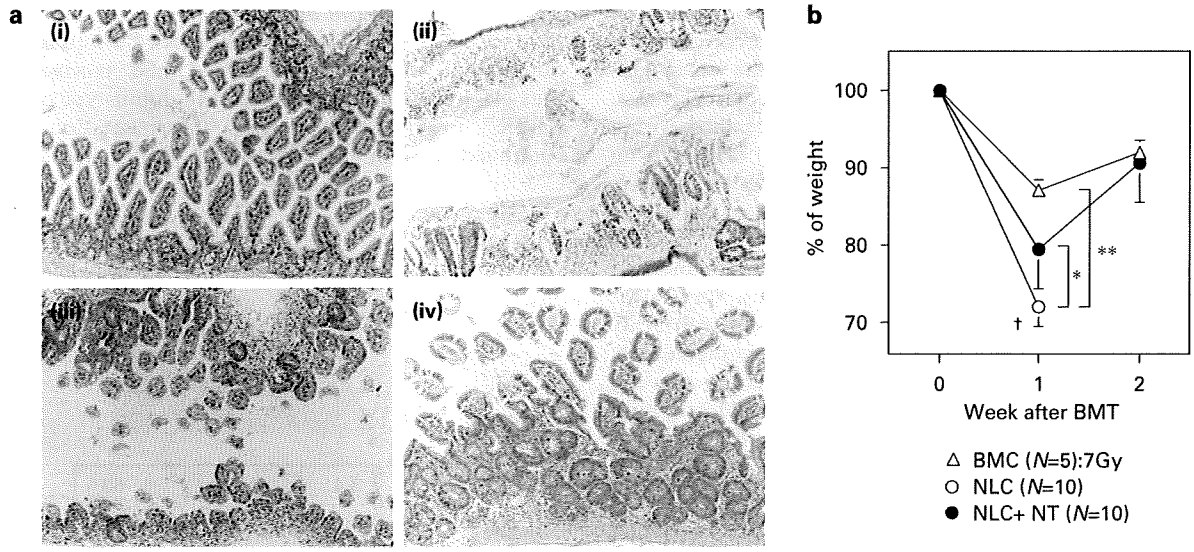

Figure 3 Small intestine histology and weight in SLI mice receiving NLCs with or without NT transplantation. Histology of the small intestine (hematoxylin and eosin, ×200) (**a**) and percentage of weight loss (**b**) in lethally irradiated (7 Gy) BALB/c mice that had received 1×10^7 BMCs or SLI BALB/c mice that had received 1×10^7 NLCs with or without NT transplantation. The small intestine from untreated BALB/c mice (i), SLI BALB/c mice transplanted with NLCs alone (ii), or with NLCs plus NT transplantation 7 days after transplantation (iii) or with NLCs plus NT transplantation 14 days after transplantation (iv). In contrast to normal small intestine (i), mucosa was largely necrotic and most cryptae were absent in SLI mice transplanted with NLCs alone. However, with addition of NT transplantation, severity was attenuated (iii) and cryptae had regenerated in 14 days (iv) (**a**). Although the body weight of SLI mice that had received NLC transplantation alone was significantly reduced compared with conventional dose (7 Gy)-irradiated mice at 7 days, it was significantly recovered with additional NT transplantation (**b**). Percentage of weight was calculated as the weight 1 or 2 weeks after HSCs with or without NT transplantation divided by the weight before transplantation, then multiplied by 100. Data shown represent mean ± s.e. $^*P<0.01$, $^{**}P<0.001$. †Most of the mice that had received NLC transplantation alone died within 2 weeks after transplantation. BMCs = bone marrow cells; HSCs = hematopoietic stem cells; NLCs = newborn liver cells; NT = newborn thymus; SLI = supralethal irradiation.

the mice that had received NLC transplantation alone also showed the same level of donor chimerism at 1 week but showed short survival. We then examined the percentage and the number of the donor-derived lymphocyte subsets in the mice at that time. Interestingly, both the percentage and the number of $CD4^+$ T cells significantly increased in the mice that had received NLCs plus NT transplantation, compared with those receiving NLC transplantation alone, in both peripheral blood and spleen (Figure 4b). In addition, the number of B cells significantly increased in peripheral blood.

Analyses of IL-7 and KGF production in thymus grafts

We next examined IL-7 and KGF production in freshly isolated thymus grafts from the donors as one of the mechanisms, since IL-7 and KGF play an important role in recovery from radiation-induced intestinal injury.[28,31] Interestingly, both mRNA and protein levels of IL-7 and KGF were the highest in NT, second highest in FT and the lowest in AT (Figure 5).

Effects of IL-7 in NT on rescue of SLI mice receiving NLCs plus NT transplantation

We finally examined the role of IL-7 produced by NT in the rescue of SLI mice, because mesenchymal cells contained in NLCs also produce IL-7. Using IL-7 null mice,[27] we carried out NLC transplantation from wild-type ($IL\text{-}7^{+/+}$) mice with or without $IL\text{-}7^{-/-}$ NT transplantation in SLI mice with or without IL-7 injections *in vivo*. The SLI mice that had received NLCs alone reached 50% mortality on the seventh day after transplantation (Figure 1a). We therefore continued the injection of IL-7 for 7 days (1 μg per day/mouse). Although the mice that had received NLC transplantation alone (non-treatment) soon died, as shown in Figure 1, either of the additional $IL\text{-}7^{-/-}$ NT transplantation or IL-7 treatments slightly improved the survival rate (Figure 6a). In contrast, the NLC + $IL\text{-}7^{-/-}$ NT transplantation plus IL-7 treatment showed a further prolonged survival. Histologically, whereas mucosa was necrotic and many cryptae were absent in SLI mice with transplantation of NLC alone (Figure 6b; ii), the pathologic findings were attenuated by addition of $IL\text{-}7^{-/-}$ NT transplantation with IL-7 treatment (Figure 6b; i). In the recovery of weight loss and the induction of both the percentage and the number of $CD4^+$ T cells in the spleen, NLCs plus $IL\text{-}7^{-/-}$ NT transplantation plus IL-7 treatment also showed the most effects, and NLCs plus $IL\text{-}7^{-/-}$ NT transplantation or plus IL-7 treatment showed a slight effect compared with NLC transplantation alone (non-treatment) (Figures 6c).

Discussion

In the present study, we investigated how to rescue SLI mice using HSCs plus thymus transplantation. Although HSC transplantation alone was ineffective, additional thymus transplantation, particularly NT thymus transplantation, significantly improved survival rates. The transplantation attenuated severe intestinal damage with weight recovery and increased the number of $CD4^+$ T cells

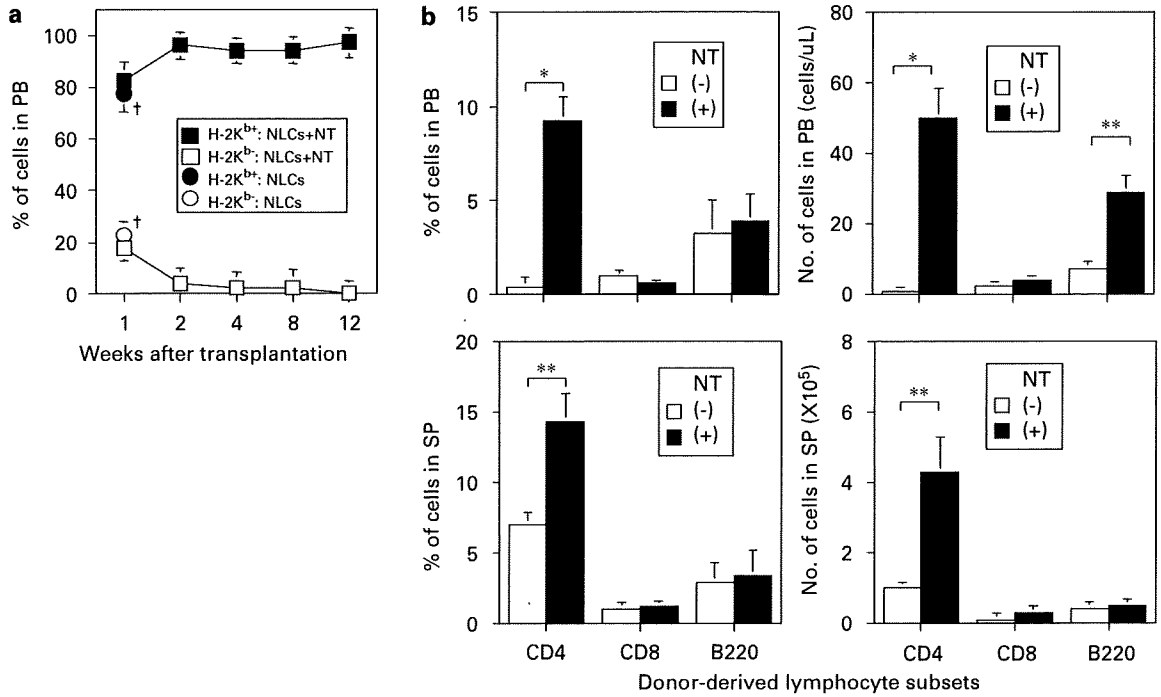

Figure 4 Analyses of chimerism and lymphocyte subsets in peripheral blood and spleen from SLI mice receiving NLCs plus NT transplantation at the early phase after transplantation. Chimerism of donor-derived cells (H-2K^{b+} cells) and host-derived cells (H-2K^{b-} cells) in the peripheral blood from SLI BALB/c mice that had received 1×10^7 NLCs with or without NT transplantation was analyzed from 1 to 12 weeks after transplantation (**a**). Percentages and numbers of donor (H-2K^{b+}) CD4$^+$, CD8$^+$ T and B220$^+$ B cells in the peripheral blood and spleen from SLI BALB/c mice that had received 1×10^7 NLCs with or without NT transplantation at 7 days after transplantation are shown (**b**). NLCs plus NT transplantation, $n = 7$; NLC transplantation alone, $n = 5$. Data represent mean ± s.e. $^*P < 0.005$, $^{**}P < 0.01$. †Most of the mice that had received NLC transplantation alone died within 2 weeks after transplantation. NLCs = newborn liver cells; NT = newborn thymus; SLI = supralethal irradiation.

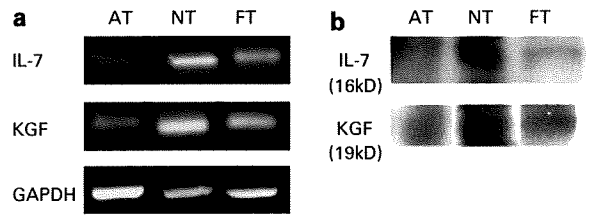

Figure 5 IL-7 and KGF levels of mRNA and protein in AT, NT and FT. The mRNA expression levels of IL-7, KGF and GAPDH according to RT-PCR (**a**) and protein levels of IL-7 and KGF by western blotting (**b**) were examined in freshly isolated AT, NT and FT from the aged donors. Representative data are shown from three independent experiments. AT = adult thymus; FT = fetal thymus; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; KGF = keratinocyte growth factor; NT = newborn thymus; RT = reverse transcription.

in the SLI-recipient mice. The production of IL-7 was elevated in NT, and NLCs plus IL-7$^{-/-}$ NT transplantation showed little effect in the rescue of SLI mice. These results suggest that NLCs plus NT transplantation can rescue SLI mice most effectively, and that high production of IL-7 in NT plays a crucial role as one of the mechanisms with induction of CD4$^+$ T cells.

First, we examined the survival effects of HSCs and thymus transplantation from variously aged donors. Although all kinds of HSC transplantation alone showed a very short survival, NLCs plus NT transplantation markedly improved the survival rate (Figure 1). In the analyses of the causes of death the SLI mice that had received NLC transplantation alone showed severe intestinal injury with significant weight loss (Figure 3). These findings are comparable with acute irradiation-induced gastrointestinal syndrome, which occurs after exposure to high-dose radiation.[14] However, additional NT transplantation attenuated intestinal damage, and the weight was recovered. These findings suggest that NLCs with NT transplantation can rescue the SLI mice with a potential protection against intestinal injury following irradiation.

We next examined chimerism and lymphocyte subsets in the mice that had received NLCs with or without NT transplantation. The donor-derived chimerism itself did not differ in the presence or absence of NT transplantation at an early phase after transplantation (Figure 4a), suggesting that SLI mice cannot be rescued by hematopoietic reconstitution alone. However, the CD4$^+$ T cells were significantly higher in the mice that had received NLCs with NT transplantation than in the mice that had received NLC transplantation alone (Figure 4b); and the number of B cells also significantly increased in peripheral blood. Some of the elevated CD4$^+$ T cells are very likely to be developed from the engrafted thymus, and the B cells are likely to be increased by the IL-7 as an inducible cytokine for early B cells from the thymus. Thus, the increased cells, especially the CD4$^+$ T cells, should play a critical role in

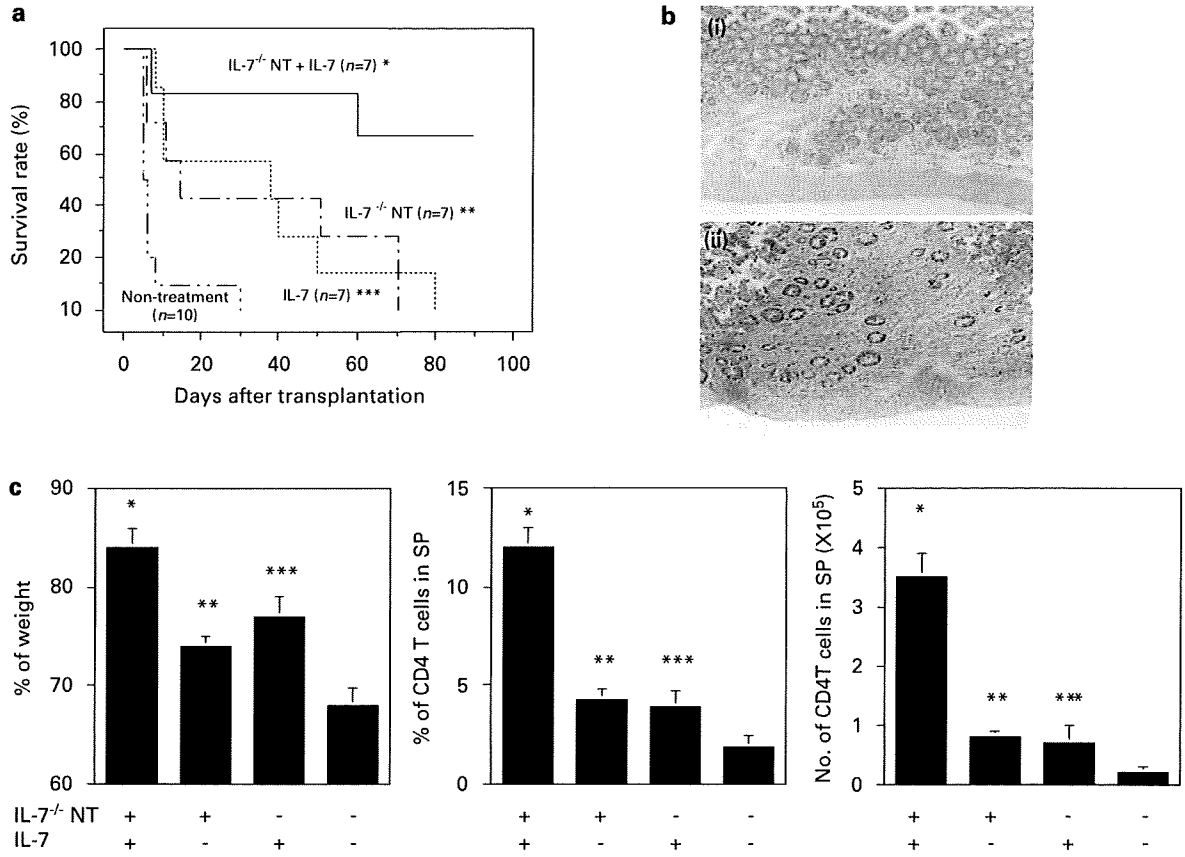

Figure 6 Analyses of survival rate, small intestinal histology, weight recovery and CD4 T-cell induction in spleen from SLI mice receiving NLCs with or without IL-7$^{-/-}$ NT transplantation in the presence or absence of IL-7 treatment. SLI BALB/c mice were transplanted with 1×10^7 NLCs with or without IL-7$^{-/-}$ NT transplantation in the presence or absence of IL-7 treatment for 7 days *in vivo* (1 μg per mouse per day). Survival rate for the mice of the four groups (**a**) and histology of the small intestine for SLI mice that had received NLCs plus IL-7$^{-/-}$ NT transplantation in the presence of IL-7 treatment (i), or NLCs alone (non-treatment) (ii) at 7 days after transplantation (**b**). Percentage of weight and percentage and number of CD4 T cells in the spleen (**c**) from the mice of the four groups at 7 days after transplantation. IL-7$^{-/-}$ NT transplantation plus IL-7 treatment, $n=5$; IL-7$^{-/-}$ NT, $n=5$; IL-7 treatment, $n=5$; non-treatment, $n=6$. Data shown represent mean ± s.e. $^*P<0.05$ compared with IL-7$^{-/-}$ NT, IL-7 or non-treatment; $^{**}P<0.001$ compared with non-treatment; $^{***}P<0.005$ compared with non-treatment (**a**). $^*P<0.05$ compared with IL-7$^{-/-}$ NT, IL-7, or non-treatment; $^{**}P<0.05$ compared with non-treatment; $^{***}P<0.01$ compared with non-treatment (**c**: left panel). $^*P<0.01$ compared with IL-7$^{-/-}$ NT, IL-7, or non-treatment; $^{**}P<0.05$ compared with non-treatment; $^{***}P<0.05$ compared with non-treatment (**c**: center panel). $^*P<0.01$ compared with IL-7$^{-/-}$ NT, IL-7, or non-treatment; $^{**}P<0.01$ compared with non-treatment; $^{***}P<0.01$ compared with non-treatment (**c**: right panel). NLCs = newborn liver cells; NT = newborn thymus; SLI = supralethal irradiation.

the rescue, although it is unknown why the number of CD8$^+$ T cells was unchanged.

We then analyzed the functions of each thymus graft. Interestingly, the production of IL-7 and KGF, which regenerate the intestinal epithelium after irradiation,[28,31] was highest in NT (Figure 5). Therefore, we finally examined the role of elevated IL-7 production by the NT transplantation in the rescue of SLI mice. We found that both IL-7$^{-/-}$ NT transplantation plus treatments of IL-7 injection are essential for survival, the recovery of weight and the induction of CD4$^+$ T cells, whereas either IL-7$^{-/-}$ NT transplantation or IL-7 injection alone showed only a slight effect (Figure 6). Although we performed IL-7 treatment for only 7 days in the SLI mice that had received NLCs and IL-7$^{-/-}$ NT, mesenchymal cells such as BM stromal cells or fibroblasts from the NLCs and/or recovered thymic epithelial cells in host thymus began to produce IL-7 later, leading to long survival. These findings suggest that although elevated IL-7 plays a significant role, the thymus graft itself is also needed for the satisfactory rescue of SLI mice.

The elevation of IL-7 and the subsequent induction of CD4$^+$ T cells by NT transplantation thus seem to be responsible for the rescue of SLI mice. Although we could not find detectable levels of IL-7 in serum by ELISA (data not shown) and no significant difference in IL-7R expression by immunohistochemistry in the intestine of the SLI mice that received NLCs in the presence or absence of NT (data not shown),[36] the signal should be one of the effective factors for the rescue of the SLI mice, given the results. Alternatively, although we did not examine the role of KGF, it may be also effective to treat the injury in the small intestine directly.[31] In this respect, the IL-7 signal itself also induces intraepithelial lymphocytes to produce KGF.[37] Concerning the induced CD4$^+$ T cells, they may be protective against infection or effective in repairing the

injured intestine.[38,39] In this respect, T cells from the NT were shown to be highly proliferative and functional for the production of various cytokines compared with AT.[32,33] This might also facilitate the rescue of SLI mice.

Although we did not examine the mechanism of the rescue directly, given the above results, the high growth activity of NT is likely to be critical in the elevation of IL-7 and KGF. In fact, although the size of the AT graft did not change or slightly decreased after reconstitution,[17] grafted NT or FT grew rapidly under the renal capsule with high proliferative acivity,[32,33] and the size became close to the grafted AT by 8 weeks after transplantation, even though their initial volume and weight was about 1/10 less than the AT (data not shown). The activity may also help regenerate or completely repair damaged organs in SLI mice. Although FT has a potential close to NT with the second highest level of IL-7 and KGF production, the levels may be insufficient for the rescue of the mice. In addition, the accompanying hormonal and cellular factors apart from IL-7, KGF and $CD4^+$ T cells might also be involved practically. Further analyses are needed for a detailed explanation of these mechanisms.

Finally, the present method might also be effective in critically accident patients or those with advanced or metastatic malignant tumors, for whom excess irradiation or chemotherapy is necessary as treatment. We have also recently found that, even if the thymus donor is different from the HSC donor, the effect is comparable to that seen with transplantation from the same donor (submitted for publication). In addition, different aged combinations of HSCs and NT, such as bone marrow cells plus NT or fetal liver cells plus NT transplantation were also effective for rescue of the SLI recipient (data not shown). Although there are ethical issues involved, an NT graft could be obtained from patients with congenital heart diseases or from aborted fetuses, as previously utilized for the graft.[40]

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Analysis of Tolerance Induction Using Triple Chimeric Mice: Major Histocompatibility Complex-Disparate Thymus, Hemopoietic Cells, and Microenvironment

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Background. Although bone marrow transplantation (BMT) has become a valuable strategy for the treatment of various intractable diseases in recent years, success rates remain low in elderly patients because of low thymic function. We have previously shown that fetal thymus transplantation (TT) with BMT is effective for elderly recipients in mice.
Methods. We performed fully major histocompatibility complex (MHC)-mismatched fetal TT from B6 (H-2^b) mice plus allogeneic BMT from C3H/HeN (H-2^k) mice by intra-bone marrow-BMT (IBM-BMT) using congenitally athymic nude (nu/nu) BALB/c (H-2^d), or BALB/c adult-thymectomized recipients to obtain triple chimeras. We next carried out the IBM-BMT+TT using senescence-accelerated mouse P1 strain (SAMP1) to examine whether this method would be applicable to aging mice.
Results. Triple chimeric mice survived for a long period with sufficient T-cell functions comparable to the mice treated with BMT plus MHC-matched TT, whereas those without TT survived for a short period with insufficient T-cell reconstitution. Almost all the hematolymphoid cells were derived from donor bone marrow cells. Interestingly, they showed tolerance to all three types of MHC determinants with donor-derived thymic dendritic cells in TT. Triple chimeric SAMP1 also survived for long periods with T-cell functions restored in contrast to non-TT SAMP1 recipients.
Conclusion. These findings suggest that third party combined TT with allogeneic IBM-BMT may be more advantageous for elderly recipients with low thymic function, than IBM-BMT alone (without TT).

Keywords: Thymus transplantation, MHC, IBM-BMT.

(*Transplantation* 2008;85: 1151–1158)

In recent years, allogeneic bone marrow transplantation (BMT) has proven to be effective in the treatment of hematologic disorders (including leukemia, lymphoma, aplastic anemia) and congenital immunodeficiencies (*1*). Using various animal models, we have found that allogeneic BMT can be used to treat autoimmune diseases such as insulin-dependent diabetes mellitus, a certain type of non–insulin-dependent diabetes mellitus, systemic lupus erythematosus, rheumatoid arthritis, chronic pancreatitis, and chronic glomerulonephritis, and also be applicable to solid cancers and organ transplantation (*2–9*). These results suggest that BMT is likely to become a powerful tool in the treatment of a wide range of diseases.

However, BMT has some problems. The success rate of allogeneic BMT is very low in elderly patients (*10–12*), who run the high risk of complications, including interstitial pneumonitis, graft-versus-host disease (GVHD), systemic infections, and relapses of primary disease. One reason is markedly reduced thymic functions because of involution, leading to insufficient or erratic T-cell development (*13–15*). We have previously demonstrated that fetal thymus transplantation (TT) with BMT from the same donor is effective in survival, reconstitution, and treatment of autoimmune diseases in aged mice (*16*). However, the thymus cannot always be obtained from the same young donor in BMT, and the induction of tolerance has not yet been studied in detail for TT.

We have recently developed intra-bone marrow (IBM)-BMT, in which bone marrow cells (BMCs) are directly injected into the BM cavity (*17*). This method allows us not only to use low-dose irradiation as a preconditioning regimen but also to effectively suppress GVHD (*18*); IBM-BMT promotes efficient proliferation of BMCs in the microenviron-

Wenhao Cui and Naoki Hosaka contributed equally to this article.

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ment (*19*), in which mesenchymal stem cells (MSCs) inhibit allo-T-cell immunity (*20, 21*). IBM-BMT is thus superior to conventional intravenous BMT.

The present study examined the effectiveness of fully major histocompatibility complex (MHC)-mismatched TT with allogeneic IBM-BMT on triple chimeric mice. The chimeric mice survived for a longer time with sufficient reconstitution and functions of T cells, the levels being comparable to MHC-matched TT. In addition, we show that this strategy is effective in the prevention of aging using the senescence-accelerated mouse P1 strain (SAMP1) (*22–24*).

MATERIALS AND METHODS

Mice

Eight-week-old female BALB/c, BALB/c nu/nu (nude) (H-2^d), C57BL/6 (B6) (H-2^b), C3H (H-2^k), DBA/1 (H-2^q), and 4-month-old SAMP1 (H-2^k) mice were purchased from Shimizu Experimental Animal Laboratory (Shizuoka, Japan), and maintained until use in our animal facilities under specific pathogen-free conditions. All animal researches were reviewed and approved by the Animal Experimentation Committee of Kansai Medical University.

Adult Thymectomy

Adult thymectomy (ATx) was performed in 8-week-old female BALB/c or B6 mice 1 week before IBM-BMT, as previously described (*25*). Briefly, the thymus was removed by suction through an incision in the neck and thoracic wall 1 week before BMT. We confirmed that no thymus tissue was left in mice at autopsy for analyses.

Intra-Bone Marrow Transplantation and Thymus Transplantation

Because radiation sensitivity differs between mouse strains, we used different radiation doses; radiosensitivity is BALB/c nude > ATxBALB/c > ATxB6 > SAMP1 mice. BALB/c nude, ATx BALB/c, and ATx B6 mice were lethally irradiated (7, 8.5, and 9.5 Gy, respectively) using a ^{137}Cs irradiator (Gammacell 40 Exactor; MDS Nordion International, Ottawa, ON, Canada) 1 day before IBM-BMT. BMCs were flushed from the shafts of donor femora and tibiae, and single-cell suspensions were prepared. Next, 1×10^7 BMCs were directly injected into the bone marrow cavity of the recipient's tibia, as previously described for the IBM-BMT method (*17*). Briefly, the knee was flexed to 90°, and the proximal side of the tibia was drawn anteriorly. A 26-gauge needle was inserted into the joint surface of the tibia through the patellar tendon and then inserted into the bone cavity. Simultaneously, a fetal-day-16 thymus was grafted under the renal capsule of the left kidney in some mice. Because the SAMP1 mice present difficulties in carrying out conventional BMT with the usual dose of irradiation and number of BMCs, we used an elevated dose of irradiation and elevated numbers of BMCs for the mice; the 4-month-old SAMP1 mice were lethally irradiated (10 Gy; 5 Gy×2 with a 4-hr interval) 1 day before BMT. As 10-Gy total body irradiation exerts strong adverse effects, mice were irradiated using a fractionated regimen, as practiced clinically. The following day, 3×10^7 BMCs from BALB/c mice were transplanted by IBM-BMT with or without simultaneous TT.

Experimental Groups for Triple Chimeric Mice

The experimental groups in this study were as follows (Table 1): group 1, BALB/c nude mice transplanted with C3H BMCs and B6 thymus; group 2, BALB/c nude mice transplanted with C3H BMCs and C3H thymus; group 3, BALB/c nude mice transplanted with C3H BMCs alone; group 4, BALB/c ATx mice transplanted with C3H BMCs and B6 thymus; group 5, BALB/c ATx mice transplanted with C3H BMCs and C3H thymus; group 6, BALB/c ATx mice transplanted with C3H BMCs alone; group 7, B6 ATx mice trans-

TABLE 1. Survival in each experimental group

Group	N	Recipient (microenvironment)	Transplantation BMCs	Transplantation Thymus	Survival	% of hemopoietic cells derived from[a] Recipient	BMCs	Thymus
1	10	BALB/c nu/nu	C3H	B6	>12wX10	0.7±0.1	93.2±1.3	0.8±0.3
2	10	BALB/c nu/nu	C3H	C3H	>12w X10	0.3±0.1	95.2±0.8	ND
3	5	BALB/c nu/nu	C3H	(−)	36, 40, 41, 47, 51d[b]	0.2±0.1	96.2±2.3	ND
4	10	BALB/c ATx	C3H	B6	>12w X10	0.5±0.1	92.1±1.9	1.1±0.3
5	10	BALB/c ATx	C3H	C3H	>12w X10	0.4±0.1	94.6±1.3	ND
6	5	BALB/c ATx	C3H	(−)	38, 41, 43, 44d, >8w[c]	0.3±0.2	93.1±1.1	ND
7	10	B6 ATx	BALB/c	C3H	>12w X10	0.5±0.3	94.2±1.9	0.9±0.3
8	10	B6 ATx	BALB/c	BALB/c	>12w X10	0.3±0.3	93.2±0.9	ND
9	5	B6 ATx	BALB/c	(−)	28, 31, 34, 43, 56d[d]	0.6±0.5	95.3±3.1	ND
10	10	SAMP1	BALB/c	B6	>12w X10	0.6±0.5	38.3±3.2	0.9±0.3
11	10	SAMP1	BALB/c	(−)	22, 24, 28X2, 30, 35, 41X2, 43, 60d[e]	67.8±0.3	0.8±0.5	0.7±0.5

[a] % of hemopoietic cells was determined by H-2 typing as chimerism in lymphocytes from the peripheral blood using flow cytometry 1 month after transplantation (n=5).
[b] $P<0.01$ compared with group 1 or 2.
[c] $P<0.05$ compared with group 4 or 5.
[d] $P<0.01$ compared with group 7 or 8.
[e] $P<0.01$ compared with group 10 (log-rank test).

planted with BALB/c BMCs and C3H thymus; group 8, B6 ATx mice transplanted with BALB/c BMCs and BALB/c thymus; group 9, B6 ATx mice transplanted with BALB/c BMCs alone; group 10, SAMP1 mice transplanted with BALB/c BMCs with B6 thymus; and group 11, SAMP1 mice transplanted with BALB/c BMCs alone.

Histologic Studies

Several organs, including the small intestine, lung, liver, kidney, and transplanted thymus, were removed from the chimeric mice, fixed in 10% formalin for 48 hr, and embedded in paraffin according to standard procedures. Sections at 4-μm thickness were stained using hematoxylin-eosin.

Flow Cytometry Analysis of Surface Markers in Lymphocytes and Thymocytes

Surface markers on lymphocytes (from peripheral blood and spleen) and thymocytes were analyzed with three-color fluorescence staining using FACScan (Becton Dickinson, Franklin Lakes, NJ). Fluorescein isothiocyanate (FITC)-conjugated anti-H-2K^b, H-2K^d, or H-2K^k mAbs (Pharmingen, San Diego, CA) were used to determine chimerism, and FITC-, phycoerythrin-, or biotin-conjugated CD4, CD8, or B220 (Pharmingen) were used to analyze lymphocyte subsets. Avidin-Cy5 (Dako, Kyoto, Japan) was used as the third color in the avidin/biotin system.

Mitogen Response and Mixed Lymphocyte Reaction

To analyze lymphocyte function and tolerance, mitogen response and mixed lymphocyte reaction (MLR) were performed in chimeric mice 2 months after transplantation. A total of 2×10^5 splenocytes collected from chimeric mice and untreated BALB/c mice as responders were plated in 96-well flat-bottomed plates (Corning Glass Works, Corning, NY) containing 200 μL of RPMI1640 medium (Nissui Seiyaku, Tokyo) supplemented with 2 μL of glutamine (Wako Pure Chemicals, Tokyo), penicillin (100 units/mL), streptomycin (100 μg/mL), and 10% heat-inactivated FCS. For mitogen responses, responder cells were incubated with 2.5 μg/mL of concanavalin A (Con A; Calbiochem, San Diego, CA) or 25 μg/mL of lipopolysaccharide (LPS; Difco Laboratories, Franklin Lakes, NJ) for 72 hr. For MLR, responders were incubated with 2×10^5 splenocytes irradiated at 15 Gy from various strains of mice, including donor, recipient, and third party (DBA-1) as stimulators for 96 hr. Next, 20 μL of 0.5 μCi ^{3}H-thymidine (^{3}H-TdR; New England Nuclear, Cambridge, MA) was introduced during the last 18 hr of the culture period. Incorporation of ^{3}H-TdR was measured using Microbeta TriLux (PerkinElmer, Wellesley, MA). Stimulation index was calculated as the average ^{3}H-TdR incorporation of triplicate samples of responding cells with either mitogen or stimulating cells/^{3}H-TdR incorporation of responding cells in medium alone.

Transplantation of Skin Grafts

For analysis of tolerance induction, skin grafts from BALB/c, B6, C3H, and DBA-1 were transplanted in triple chimeric mice from groups 1, 4, and 7 at 2 months after BMT, as previously described but with slight modifications (*26*). Briefly, full-thickness skin grafts (1×1 cm) were harvested from donor mice, and skin grafts from which the hair had been completely removed by depilatory were then kept in dishes with phosphate-buffered saline on ice before use. Next, triple chimeric mice were anesthetized, and four sections of left and right dorsal skin were gently removed. Prepared donor skin grafts were then sutured to the areas from which skin had been removed using 5-0 nylon. Grafted skins were gently covered with Vaseline gauze fixed with protective tape to prevent detachment by movement.

Immunohistochemical Staining for Transplanted Thymus

Transplanted thymic lobes in kidneys from triple chimeric mice were embedded in Tissue-Tek Optimal Cutting Temperature compound (Sakura Finetek, Tokyo, Japan) and stored at −40°C. Cryosections (4-μm thick) were air-dried and fixed with acetone for 10 min. Specimens were treated using 0.5% bovine serum albumin in Tris-buffered saline for 10 min, then stained with FITC-conjugated CD11c mAb (Pharmingen) and biotin-conjugated H-2K^b, H-2K^d, or H-2K^k mAb (Pharmingen) for 1 hr at room temperature in a moist chamber. After washing three times in Tris-buffered saline for 5 min with gentle shaking, incubation was performed with avidin-phycoerythrin (Dako) for 1 hr. Expressions were evaluated under confocal microscopy using an LSM 510 META microscope (Carl Zeiss, Minneapolis, MN).

Statistical Analysis

Nonparametric analyses (Mann-Whitney *U* and log-rank tests) were performed using StatView software (Abacus Concepts, Berkeley, CA). Values of $P<0.05$ were considered statistically significant.

RESULTS

Survival Rates and Chimerism in Triple Chimeric Mice

Table 1 shows survival rates in all 11 experimental groups in this study. All triple chimeric mice in nude (group 1) or ATx mice (groups 4 and 7) survived for a long time (>12 weeks), which was similar to the case of MHC-matched TT (groups 2, 5, and 8). In contrast, all the chimeric mice without TT (groups 3, 6, and 9) showed significantly shorter survival periods than the chimera with TT. However, hemopoietic cells were BMC-type in all the experimental groups except group 11 (described later).

Histology and Lymphocyte Reconstitution in Triple Chimeric Mice

Histologically, although a very small number of lymphocytes infiltrated organs such as the liver, lung, small intestine, and kidney, no apparent tissue damage was found in any of the groups with TT (Fig. 1A). Next, we investigated the reconstitution of donor-derived lymphocytes. Interestingly, all triple chimeric mice in groups 1, 4, and 7 showed sufficient donor BMC-derived CD4$^+$T, CD8$^+$T, and B220$^+$B cells in the spleen, which were similar to those in the chimeric mice in groups 2, 5, and 8 for MHC-matched TT and untreated BALB/c mice (Fig. 1B). However, nude mice transplanted with BMCs alone (without TT) in group 3, which survived for only a short time, showed a small percentage of T cells but a large percentage of B cells. The others transplanted with

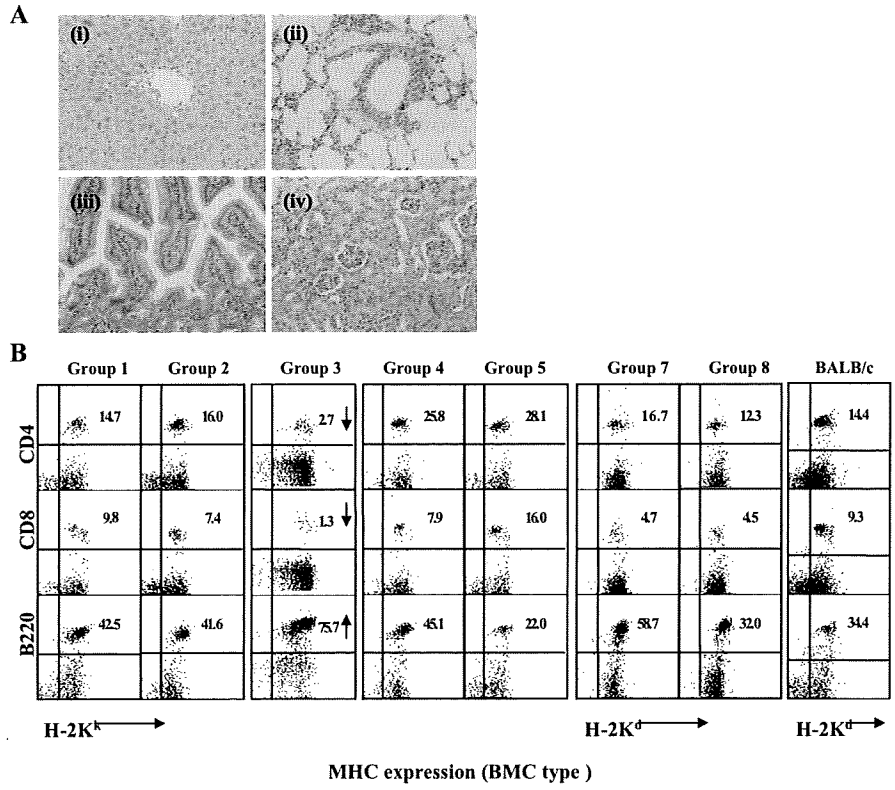

FIGURE 1. Analysis of histology and BMC-derived $CD4^+$ and $CD8^+$ T cells and B cells in spleen from experimental groups. (A) Histologic findings of liver (i), lung (ii), small intestine (iii), and kidney (iv) from group 1 (hematoxylin-eosin; magnification ×400). The mice from other groups with TT also showed the same findings (data not shown). (B) BMC-derived $CD4^+$ and $CD8^+$ T cells and $B220^+$ B cells in the spleen were analyzed from groups 1, 4, and 7 for the triple chimeras, from groups 2, 5, and 8 for MHC-matched TT, from group 3 for the absence of TT (as described in Table 1), and from untreated BALB/c mice as controls using flow cytometry. Donor BMCs were from C3H mice ($H\text{-}2^K$) in groups 1 to 5 and from BALB/c mice ($H\text{-}2^d$) in groups 7 and 8. Representative histologic findings and FACS profiles are shown from three or four experimental mice in each group. *Arrows,* small percentage of T cells but a large percentage of B cells were shown.

BMCs alone in groups 6 and 9 showed the same results (data not shown).

Histology, Chimerism, and Thymocyte Subsets of Transplanted Thymus in Triple Chimeric Mice

We confirmed that the transplanted thymus was engrafted under the renal capsule (Fig. 2A). Both the cortical and medullary areas were finely constructed. The thymocytes of the transplanted thymus showed the BMC-derived phenotype in all the groups (Fig. 2B), although the expression was lower than in mature T cells (Fig. 1). In addition, almost normal proportions of $CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+CD8^-$, and $CD4^-CD8^+$ thymocytes were observed in the triple chimeric mice from groups 1, 4, and 7, which were comparable to the mice with MHC-matched TT (groups 2, 5, and 8) and untreated BALB/c mice (Fig. 2B).

Functional Analyses and Tolerance Induction of Spleen Cells in Triple Chimeric Mice

We next examined the mitogen responsiveness of spleen cells in triple chimeric mice (Fig. 3A). Spleen cells in the triple chimeric mice from groups 1, 4, and 7 showed sufficient responsiveness to both Con A and LPS, which were comparable to the mice from groups 2, 5, and 8 (MHC-matched TT) and untreated BALB/c mice. We further investigated the induction of tolerance in the triple chimeric mice. The mice in groups 1, 4, and 7 showed tolerance to all three types of MHC determinants (BMCs, recipient, and transplanted thymus) but showed responsiveness to fourth-party (DBA/1: $H\text{-}2^q$) MHC determinants (Fig. 3B). In contrast, the mice in groups 2, 5, and 8 (MHC-matched TT) only showed tolerance for the 2-type MHC determinants (BMCs and recipient), as expected. The triple chimeric mice of groups 1, 4, and 7 also accepted the skin grafts from all the three types of MHC determinants, but the graft from the fourth party was rejected (data not shown). These skin grafts were prolonged to allow acceptance after more than 12 weeks during observation (data not shown).

Mechanisms of Tolerance Induction

We investigated the mechanisms of tolerance induction in the triple chimeric mice. It has been reported that central tolerance (negative selection) is induced by thymic dendritic cells (DCs) (*27*) We therefore examined whether donor-derived thymic DCs exist in the transplanted thymus (Fig. 4). Interestingly, both donor BMC ($H\text{-}2K^{d+}$)- and transplanted thymus ($H\text{-}2K^{k+}$)-derived $CD11c^+$ DCs were clearly present in the transplanted thymus in the triple chimeric mice (group 7; Fig. 4A,B), although few host-derived ($H\text{-}2K^{b+}$) DCs were found (data not shown). The other triple chimeric mice (groups 1 and 4) showed the same results (data not shown).

Effects of Thymus Transplantation on Aging Mice (SAMP1)

Finally, we examined the effects of TT on SAMP1 mice ($H\text{-}2^k$), which are the animal model for aging. The mice show low T-cell function with thymic involution (*22–24*). Therefore, we used the mice as a model of the elderly with low thymic function. Lethally irradiated (5 Gy×2) SAMP1, which had been transplanted with 3×10^7 BALB/c BMCs by IBM-BMT plus B6 fetal TT (as triple chimera), survived for significantly longer than the mice treated by IBM-BMT alone

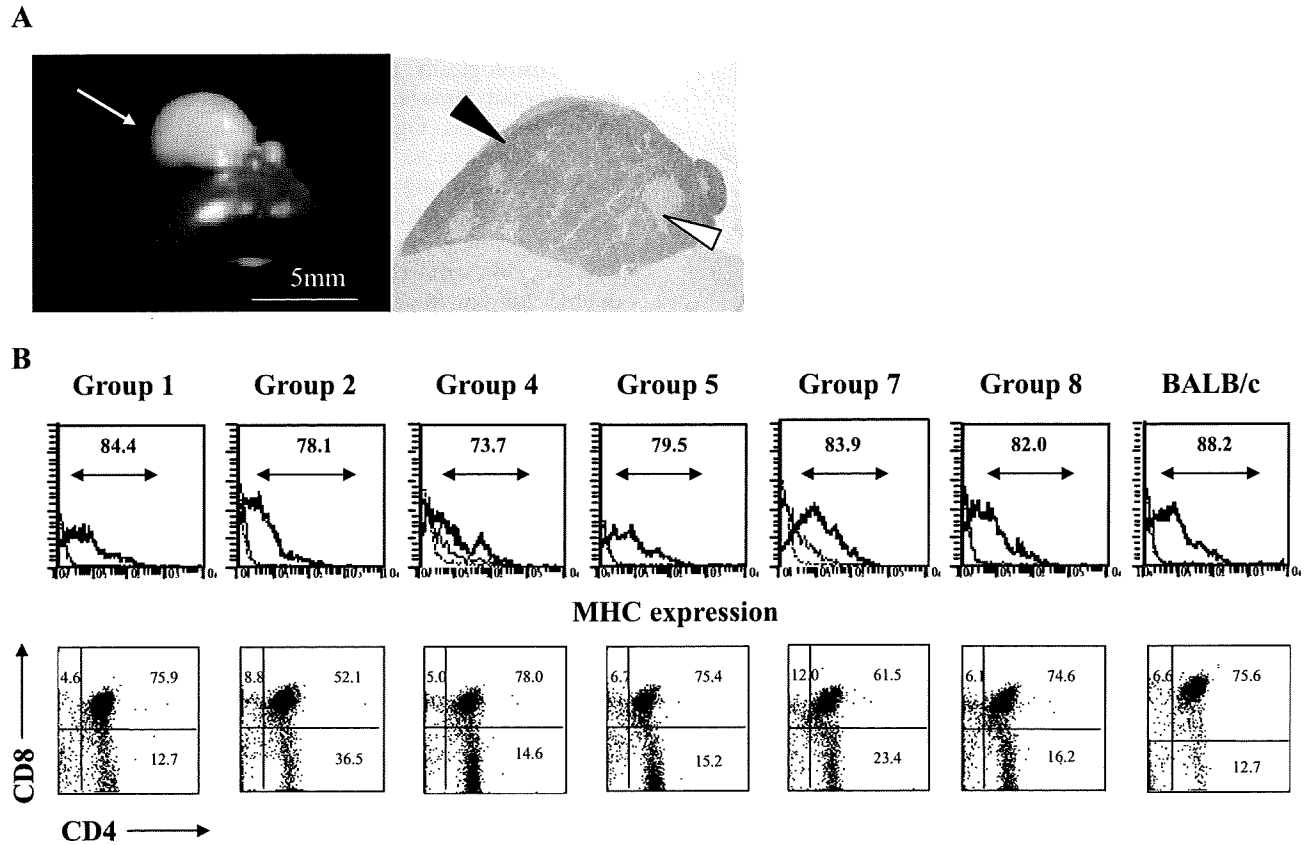

FIGURE 2. Analysis of histology, chimerism, and thymocyte subsets in transplanted thymus from experimental groups. (A) Macroscopic (*left*) and microscopic findings (*right*) in transplanted thymus from group 1 at 2 months after transplantation are shown. (B) Thymus tissue was engrafted (*arrow*), and cortical (*closed arrowhead*) and medullary (*open arrowhead*) areas displayed fine construction. FACS profile for chimerism (*upper*) and CD4- and CD8-thymocyte subsets (*lower*) are shown in transplanted thymus from groups 1, 4, and 7 for triple chimeras, from groups 2, 5, and 8 for MHC-matched TT (described in Table 1), and from untreated BALB/c as controls. Thick line, BMC type; thin line, thymus type; dotted line, recipient type in histogram by H-2K^b, H-2K^d or H-2K^k staining, as described in Table 1. CD4 and CD8 double-staining in thymocytes from transplanted thymi. Representative profiles are shown for three or four mice in each group.

(without TT; Table 1). Donor-derived CD4$^+$ and CD8$^+$ T cells as well as B220$^+$ B cells were generated well in the triple chimeric SAMP1, although very few lymphocytes were seen in the mice without TT (Fig. 5A). In functional analyses, spleen cells from the triple chimeric mice showed significant responses to both Con A and LPS, the levels being comparable to untreated BALB/c mice (Fig. 5B). In contrast, untreated SAMP1 and the mice treated by IBM-BMT alone (without TT) showed low responsiveness to Con A, but responded to LPS. In MLR assays, the triple chimeric mice also showed tolerance to all three types of MHC determinants, but responsiveness to the fourth party (DBA/1; Fig. 5C).

DISCUSSION

In the present study we have established a triple chimeric mouse model consisting of donor 1 giving MHC-disparate-thymus, donor 2 giving MHC-disparate-BMCs, and 3, the recipient MHC-disparate microenvironment (disparate to donors 1 and 2). It should be noted that we use the fresh and nontreated fetal thymus as TT. The immature and high proliferative potential may help support and reconstitute BMC-derived T cells, and the resident immature T cells may suppress alloreactivity. This beneficial effect might be adapted well for aged hosts. Although there are ethical issues involved in obtaining the thymus graft for clinical use, taking such immature thymus tissues was previously approved by patients with congenital heart diseases for treatment of DiGeorge Syndrome and human immunodeficiency virus in-fection (*28, 29*). In addition, a method of regenerating the thymus has been developed (*30*), and the thymus graft could be obtained from aborted fetuses. Thymus transplantation might be clinically applicable in the near future.

The triple chimeras showed significantly long survival with BMC-derived chimerism comparable to the mice with MHC-matched TT and BMC donors (Table 1). Both the triple chimeras and the mice with MHC-matched donor TT and BMC survived more than 6 months (data not shown). In addition, the triple chimeric mice also showed the normal T-cell reconstitution and functions, which were comparable to the mice with MHC-matched TT (Figs. 1B and 3). In contrast, the nude and ATx chimeric mice that had been treated with IBM-BMT alone showed a significantly short survival with low

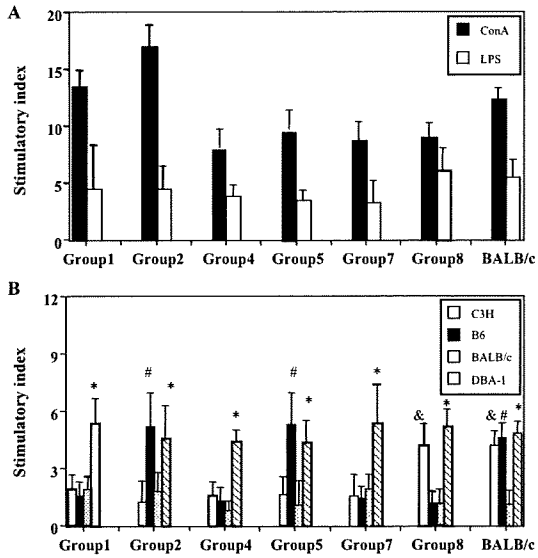

FIGURE 3. Mitogen responses and MLR in experimental groups. Mitogen responses to Con A and LPS (A) and MLR (B) in splenocytes are shown from groups 1, 4, and 7 for triple chimeras, from groups 2, 5, and 8 for MHC-matched TT, and from group 3 for absence of TT (as described in Table 1), and from untreated BALB/c mice as controls. Representative results are shown for three experiments in each group. SI was calculated as the average ^{3}H-TdR incorporation of triplicate samples of responding cells with either mitogen or stimulating cells/^{3}H-TdR incorporation of responding cells in medium alone. There were no significant statistical difference for Con A and LPS responses in triple chimeras, their MHC-matched TT (groups 1 and 2; groups 4 and 5; groups 7 and 8) and BALB/c mice (A). *$P<0.05$ compared with C3H, B6 and BALB/c in group 1, compared with C3H and BALB/c in group 2, compared with C3H, B6 and BALB/c in group 4, compared with C3H and BALB/c in group 5, compared with C3H, B6, and BALB/c in group 7, compared with B6 and BALB/c in group 8, and compared with BALB/c in BALB/c mice; $^{\#}P<0.05$ compared with C3H and BALB/c in group 2, compared with C3H and BALB/c in group 5, and compared with BALB/c in BALB/c mice; $^{\&}P<0.05$ compared with B6 and BALB/c in group 8 and compared with BALB/c in BALB/c mice. Data were shown as means±SD.

T-cell reconstitution, as expected. These results strongly suggest that, even with fully MHC-mismatched TT, hematopoietic stem cells can develop and reconstitute well in the MHC-mismatched microenvironment with long survival.

The exact mechanisms underlying the supply of sufficient T cells even with fully MHC-mismatched thymus are unknown. One possibility is that MHC type is not related to host survival with sufficient hemopoiesis and thymopoiesis, although the MHC-matched combination is better than the MHC-mismatched combination (*31*, *32*). The second possibility is that some donor BMC-derived BM and thymic stromal cells support the hemopoiesis and/or thymopoiesis as MHC-matched stromal cells. Actually, we have previously found that donor-derived bone marrow stromal cells (including MSCs) migrate into the thymus where they are engaged in positive selection and also negative selection (*33*). Further study is needed to find the origins of the stromal cells in the transplanted thymus and BM in the triple chimeric mice.

Histologically, triple chimeric mice did not show apparent organ damage, although a few lymphocytes had infiltrated the organs (Fig. 1A). In addition, triple chimeric mice showed tolerance to all three types of MHC determinants (Fig. 3B), indicating the acceptance of three types of skin grafts (data not shown). Because the skin grafts were accepted for a long time (>5 months), long-term tolerance could be induced, although we did not carry out the MLR assays using double chimeric mice at that time. These results suggest that there were at least no lethal GVHR or HVGR in the triple chimeric mice.

Regarding the mechanisms underlying tolerance induction, it is likely that thymic DCs, which can delete autoreactive T cells by negative selection (*27*), play a crucial role in our present study as well as previous reports (*34–36*). We have found both BMC-derived and thymus-derived (resident) DCs in the transplanted thymus of triple chimeric mice, although hardly any recipient-type DCs were found in the thymus (Fig. 4). It has been reported that marrow stromal cells (including MSCs) can induce tolerance in the prethymic process (*20*, *21*, *37*, *38*). In this regard, IBM-BMT would facilitate contact between donor-derived HSCs and MSCs, which results in preventing GVHD and HVGR (*17–19*). Regarding postthymic tolerance induction, a recent report shows that stromal cells of the lymph node induce tolerance in naive T cells by expressing tissue-specific antigen (*39*). They might also induce tolerance of host MHC in the microenvironment (including the lymph node). The specific mechanism for tolerance induction needs further analyses.

Finally, we have found that triple chimerism is also effective in SAMP1, which offers an animal model for senescence in humans; triple chimeric SAMP1 survived longer than SAMP1 with IBM-BMT alone (without TT; Fig. 4). The former also improved T-cell functions and showed tolerances to all three MHC determinants (Fig. 5). However, SAMP1 treated by IBM-BMT alone (without TT) showed a few donor-derived cells, indicating chimeric resistance in the absence of functional T cells. In fact, it has been reported that aged recipients show increased sensitivity to irradiation (*40*). However, in this respect, we have recently found that TT overcomes chimeric resistance in MRL/lpr mice with Fas gene defect (*41*). In addition, the triple chimeric mice in SAMP1 did not show the loss of activity, alopecia, and increased lordokyphosis, which are characteristic symptoms in aged SAMP1 (*22*, *23*).

It has also been shown that allogeneic BMT with TT elevates T-cell functions in the recipients with thymic deficiency in mice (*16*, *42*, *43*) and humans (*44*). The decline in T-cell functions with age leads to increased incidences of development of ailments such as autoimmune disease, malignancy, or infection (*11*, *45*). BMT plus TT may thus be effective not only in curing primary disease but also in preventing other diseases by improving T-cell functions.

Based on the findings, simultaneous multiple organs, tissues, or cell transplantation such as the heart, liver, kidney, islets, or dopamine-producing cells from the different donors might be applicable. In addition, the three models (nude, ATx, and SAMP1), which are primarily used for elderly patients with thymic deficiency, might also represent models for

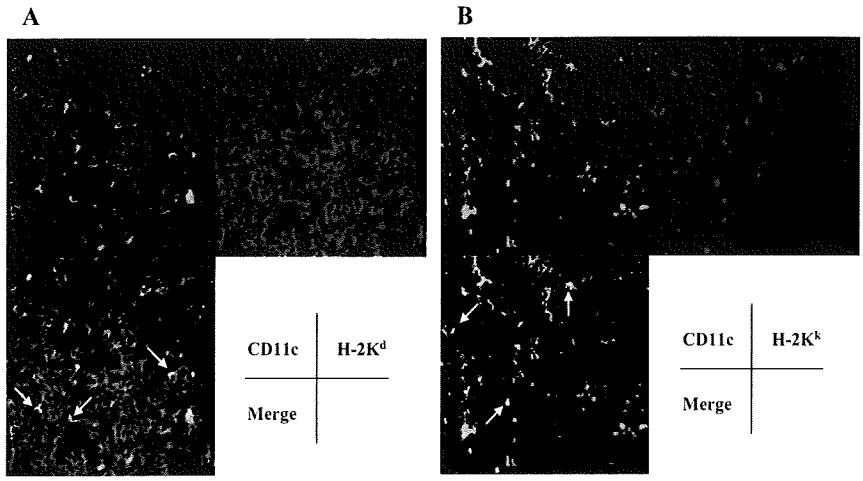

FIGURE 4. BMC- and thymus-derived dendritic cells (DCs) in transplanted thymus. The transplanted thymic lobe from group 7 at 2 months after transplantation was stained using FITC-conjugated CD11c mAb and PE-avidin/biotin-conjugated H-2K^d:BMC type (A), or H-2K^k:thymus type (B) mAb. Expressions were evaluated under confocal microscopy. Both BMC-derived (H-2K^{d+} CD11c$^+$) and thymus-derived DCs (H-2K^{k+} CD11c$^+$) were found in the transplanted thymus (*arrows*). Representative profiles are shown for three experiments.

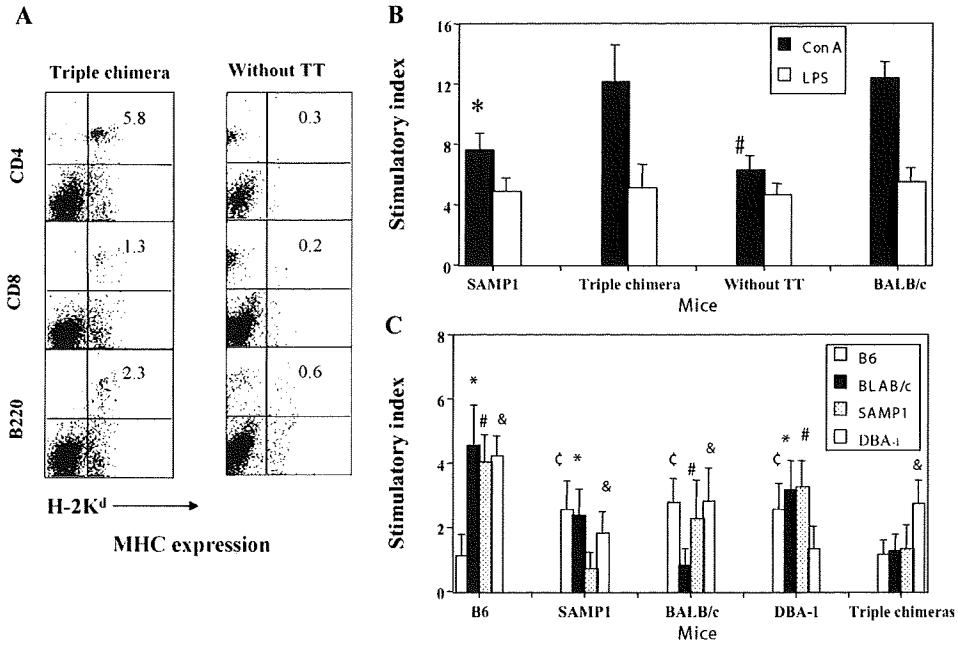

FIGURE 5. BMC-derived lymphocyte subsets, mitogen responses, and MLR in spleen from SAMP1 mice treated by triple chimeric transplantation or absence of TT. BMC-derived CD4$^+$ and CD8$^+$ T cells and B220$^+$ B cells in spleen were analyzed from triple chimeric mice or mice with absence of TT by flow cytometry. (A) Donor BMCs were from BALB/c mice (H-2^d). (B) Mitogen responses in splenocytes to Con A and LPS are shown from untreated SAMP1 mice, mice treated with triple chimeric transplantation, chimeras without TT 2 months after transplantation, and untreated BALB/c mice as controls. (C) MLR in splenocytes from untreated B6, SAMP1, BALB/c, DBA-1, and SAMP1 mice treated for triple chimeric transplantation 2 months after transplantation. Representative results are shown for three experiments. SI was calculated as the average ^{3}H-TdR incorporation of triplicate samples of responding cells with either mitogen or stimulating cells/^{3}H-TdR incorporation of responding cells in medium alone. (B) $^{*\#}P<0.05$ compared with Con A in triple chimeric mice. (C) $^*P<0.05$ compared with B6 in B6 mice, compared with SAMP1 in SAMP1 mice, and compared with DBA1 in DBA 1 mice; $^\#P<0.05$ compared with B6 in B6 mice, and compared with DBA1 in DBA1 mice; $^\&P<0.05$ compared with B6 in B6 mice, compared with SAMP1 in SAMP1 mice, compared with BALB/c in BALB/c mice, and compared with B6, BALB/c, or DBA1 in triple chimeric mice; $^\phi P<0.05$ compared with SAMP1 in SAMP1 mice, compared with BALB/c and BALB/c mice, and compared with DBA1 in DBA1 mice. Data were shown as means±SD.

DiGeorge Syndrome or patients with thymectomy after surgery for congenital heart disease. Although the recent non-myeloablative protocol for BMT induced less complication than the conventional method in older people (*46*), the addition of TT method in the present study might facilitate improved results with some rejuvenating. If some ethical issues are resolved, fetal thymus plus IBM-BMT may be a valuable strategy for the treatment of various diseases.

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ORIGINAL ARTICLE

Contribution of neural cell adhesion molecule (NCAM) to hemopoietic system in monkeys

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Abstract Neural cell adhesion molecules (CD56) are important adhesion molecules that are mainly expressed on neural cells and natural killer cells. Although freshly isolated cynomolgus monkey bone marrow cells (BMCs) contained only a few CD56-positive cells, almost all the BM adherent cells (obtained after a 2- to 3-week culture of the BMCs) were stained positively with anti-CD56 monoclonal antibody (mAb). The BM adherent cells showed uniformly fibroblastic morphology and were negative for hematolymphoid markers (CD4, CD8, CD11b, CD14, CD34, and CD45). Adipogenesis and osteogenesis were observed under inductive culture conditions. The BM adherent cells had the ability to support hemopoiesis of hemopoietic stem cells (HSCs) in vitro, and the proliferation of HSCs was significantly inhibited by the addition of anti-CD56 mAb to the coculture system. CD56 molecules were also expressed on HSCs because about 20% of an HSC-enriched population (lineage-negative and blast-gated cells) was positive for CD56. In addition, the immunostaining of monkey BM sections revealed that many stromal cells were CD56-positive, and some CD56-positive stromal cells came into direct contact with CD56-positive hemopoietic cells. These results indicate that the CD56 molecule is expressed on both HSCs and BM stromal cells (containing MSCs) in monkeys, and therefore it can be speculated that CD56 also contributes to the human hematopoietic system.

Keywords NCAM · MSCs · Bone marrow stromal cells · Cynomolgus monkey

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Introduction

The bone marrow (BM) long-term culture system, established by Dexter et al. [1] and Whitlock and Witte [2], has demonstrated the importance of direct interactions between hemopoietic cells and BM stromal cells in order to induce active proliferation and differentiation of hemopoietic progenitor–stem cells and to maintain long-term hemopoiesis. The stromal cells provide niches for hemopoietic stem cells (HSCs) by expressing adhesion molecules and secreting cell matrix molecules and growth factors. Several important adhesion molecules, such as VLA-4, VLA-5, VCAM-1, ICAM-1, and CD44, have been found and their functions have been well elucidated [3–5]. To find other

important interaction molecules for hemopoiesis, we previously established a monoclonal antibody (mAb; anti-PA6) against a mouse BM stromal cell line (PA6) [6]. The mAb inhibits pseudoemperipolesis and suppresses the proliferation of HSCs, suggesting that it reacts with molecules responsible for the interaction between HSCs and stromal cells. Affinity chromatography and mass peptide fingerprinting revealed that the PA6 protein is a neural cell adhesion molecule (NCAM, CD56) [7]. We also established a stromal cell line (FMS/PA6-P) from fetal mouse bone marrow cells (BMCs) using the anti-PA6 mAb. The cell line is highly positive for CD56 and has a higher ability to support hemopoiesis than other stromal cell lines. Moreover, the cell line has characteristics of mesenchymal stem cells (MSCs) because it can differentiate into adipocytes, osteoblasts, and vascular endothelial cells [8]. These results indicate that CD56 is expressed not only on stromal cells but also on MSCs in the murine system.

The CD56 molecule has been identified in retinal tissues of chick embryos by Thiery et al. [9] in 1977 and was shown to be an important adhesion molecule involved in the morphogenesis of neural cells in embryonic development [9, 10]. The CD56 molecule belongs to the immunoglobulin superfamily, and three isoforms (NCAM-120, NCAM-140, and NCAM-180), differing in molecular size of the intracellular domain, are known. NCAM-180 is expressed mainly on neural cells, whereas NCAM-120 and NCAM-140 are expressed on cardiac muscle, skeletal muscle, some T cell populations, and natural killer (NK) cells. However, the expression of the CD56 molecule on hematolymphoid cells, except for NK cells, has not thus far been well elucidated, although there are a few reports showing that CD56 is highly expressed on some malignant cells: plasma cells in multiple myeloma [11] and leukemic cells in acute myeloid leukemia [12]. As for BM stromal cells, it has been reported that human BM endosteal cells express CD56 [13] and that a human osteoblastic cell line U2-OS, derived from osteoblastoma, has the capacity to support hemopoiesis and is highly positive for CD56 [14].

Recently, we have established a new method for harvesting monkey BMCs (the perfusion method) [15–17]. By this method, the contamination of the collected BMCs with peripheral blood was reduced to a minimum, compared with the conventional aspiration method. To examine whether the contribution of CD56 to hemopoiesis is a feature common to all species, we attempted to investigate the expression of CD56 on monkey BM adherent cells that were obtained by the culture of BMCs collected from cynomolgus monkeys using the perfusion method. It is known that CD56 molecules regulate cell migration, homing, proliferation, and maturation by homophilic (CD56 binding to CD56) as well as heterophilic (CD56 binding to a number of proteins and extracellular matrix molecules) interactions. Therefore, we examined whether CD56 molecules were also expressed on HSC-enriched populations and whether the interaction between HSCs and stromal cells through CD56 molecules is important for the proliferation and differentiation of HSCs. Recent research has demonstrated that human and murine BMCs contain MSCs and the MSCs can be obtained easily by the culture of BMCs. In the present study, we also investigate whether the monkey BM adherent cells, expressing CD56 molecules, have characteristics of MSCs.

Materials and methods

Monkeys

Normal cynomolgus monkeys (3 to 6 years old; 3- to 6-kg body weight) were obtained from Keari (Osaka, Japan). The monkeys were free of intestinal parasites and were seronegative for tuberculosis, herpes B, hepatitis A, and hepatitis B viruses. All surgical procedures and postoperative care of animals were carried out in accordance with the guidelines of the National Institutes of Health for care and use of primates. Experiments using these monkeys were conducted in accordance with protocols approved by the university's committee for animal research.

Harvesting of monkey BMCs

Monkeys were anesthetized using Ketalar (5 mg; Sankyo Co. Ltd.; Tokyo Japan) and an analgesic agent, Pentagin (Sankyo Co. Ltd.), and BMCs were then collected from the long bones (humerus and femur) and the ilium by the perfusion methods established in our laboratory [15–17]. In brief, one BM puncture needle was inserted into the proximal side of the long bone and the other was inserted into the distal side. In the case of the ilium, one needle was inserted into the end of the iliac crest and the other into the edge of the iliac crest. A syringe containing 0.5 ml of heparin was connected to the needle and a 30-ml syringe containing 30 ml of phosphate-buffered saline (PBS) was connected to the other needle. The PBS was gently pushed from the syringe into the BM cavity, and the PBS containing BMCs was collected in the syringe containing heparin.

Adherent cell culture of monkey BMCs

Monkey whole BMCs (1×10^7) were cultured in flasks (25 cm^2, #35-3014; BD Falcon, Franklin Lakes, NJ, USA) containing IMDM (Gibco, Grand Island, NY, USA)

supplemented with 10% fetal bovine serum (FBS; lot No. 5-1064; BioSolutions International, Melbourne, Australia) at 37 °C under 5% CO_2. Every 4 or 5 days, the culture medium containing nonadherent cells was removed and fresh medium was added to the flasks. During the culture, BM adherent cells began to proliferate and reached confluence 2–3 weeks later.

Flow cytometry

Monkey BM adherent cells were washed twice with 0.02% ethylene diamine tetraacetic acid (EDTA)–PBS and the adherent cells were detached from the flasks using trypsin–EDTA treatment. The thus-prepared cells were stained with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs) against nonhuman primate CD4, CD8, CD11b, CD14, CD29, CD31, CD34, CD45, CD56, and CD62L (BD Biosciences, San Jose, CA, USA). Fresh BMCs were also stained with these mAbs. The stained cells were analyzed using a FACScan (Becton Dickinson, Mountain View, CA, USA).

Purification of monkey HSCs

Low-density (LD) BMCs ($\rho < 1.077$) were purified from fresh or frozen monkey BMCs by discontinuous density gradient centrifugation using Ficoll-Paque™ PLUS (#17-1440-02; GE Healthcare Bio-Science, Uppsala, Sweden; http://www.pnu.com). The LD cells were incubated with mAb (mouse immunoglobulin G (IgG) class) cocktails against nonhuman primate lineage markers (CD3, CD9, CD11b, CD14, CD16, and CD20) and then incubated twice with sheep antimouse IgG-conjugated immunobeads (#110.31; Dynal Inc., Oslo, Norway; http://www.dynal.no) with gentle agitation at a 3:1 bead-to-cell ratio. The immunobead-rosetted cells were removed using a magnetic particle concentrator. The remaining nonrosetted cells (lineage-negative cells) were considered as partially purified HSCs and used for culture on the BM adherent layer.

In some experiments, the partially purified HSCs were double-stained with PE-labeled anti-nonhuman primate CD34 mAb (#550619; BD Biosciences) and FITC-labeled antihuman CD56 mAb (cross-reactive with homologous monkey epitope, #0562; Exalpha, Watertown, MA, USA) and then analyzed using a FACScan. $CD34^+/56^+$ and $CD34^+/56^-$ populations included in a "blast" gate were sorted from the double-stained cells using EPICS ALTRA (Beckman Coulter, Inc., Fullerton, CA, USA). The sorted cells were morphologically analyzed by staining with May–Giemsa reagents and also functionally analyzed by clonal cell culture using methylcellulose assay.

Long-term culture of monkey HSCs

Monkey BMCs were passed through a cell strainer (70 μm mesh size, #REF352350; BD Falcon, Bedford, MA, USA) and divided into passed cells and nonpassed cells. The nonpassed cells were composed of small tissue fragments of BM and small bone pieces and therefore contained a higher percentage of adherent cells than the passed cells. These nonpassed cells were cultured in order to acquire the BM adherent cells, whereas the passed cells were frozen and stored in liquid nitrogen until BM adherent cell layers were formed.

The nonpassed cells were cultured in flasks (125 cm^2, #35-3136; BD Falcon) containing IMDM Gibco supplemented with 10% FBS (lot No. 5-1064, BioSolutions International), and a confluent adherent cell layer was formed 3 weeks later. The adherent cells were trypsinized, collected, and then subcultured in flasks (25 cm^2; 10^6 cells per flask). When the BM adherent cells had become subconfluent 2–3 days later, partially purified HSCs that were obtained from the frozen cell strainer-passed cells of the same monkey were inoculated on the BM adherent layer (5×10^5 cells per flask; triplicate). The cells were cultured in IMDM supplemented with 10% FBS (lot No. 5-1064) and a low concentration of human cytokines (IL-3, TPO, and FLT-3 ligand: 2 ng/ml, SCF: 9 ng/ml). TPO and SCF were kindly donated by the Kirin Brewery Co. Ltd. (Tokyo, Japan). Every week, half of the culture medium in the flasks (containing nonadherent cells) was removed and fresh medium was added to the flasks. The numbers of nonadherent cells per flask were counted and the nonadherent cells were then used for methylcellulose assays.

Methylcellulose assays

The colony-forming ability of the nonadherent cells obtained from the long-term culture was assessed using methylcellulose assays. Appropriate numbers of these cells were plated in 12-well plates (#3815-012; Iwaki, Chiba, Japan) in a volume of 1 ml of MethoCult GF H4434 (StemCell Technologies Inc., Vancouver, British Columbia, Canada), consisting of optimal concentrations of human cytokines (SCF, EPO, IL-3, GM-CSF, and G-CSF), 30% FBS, 1% bovine serum albumin, 2 mM L-glutamine, 100 μM 2-mercaptoethanol, and 0.9% methylcellulose (triplicate). The plates were incubated for 14 days at 37 °C under 5% CO_2, and the numbers of colonies were counted under an inverted microscope.

Whole BMCs, LD cells, and lineage-negative cells were also examined using methylcellulose assays to determine their ability to generate colonies. In addition, we investigated the colony formation of the sorted $CD34^+/56^+$ and $CD34^+/56^-$ cells.

Addition of anti-CD56 antibody in HSC proliferation assay system

To obtain BM adherent cells, cell strainer-nonpassed BMCs were cultured in flasks as described above. The BM adherent cells were trypsinized, collected, and cultured at a concentration of 3,000 cells per well in 96-well plates (#3595; Corning, Inc., NY, USA). When the BM adherent cells had become subconfluent, the partially purified HSCs prepared from the frozen BMCs of the same monkey were inoculated on the BM adherent layer (10^4 cells per well). The cells were cultured in IMDM supplemented with 10% FBS (lot No. 5-1064) and human cytokines (IL-3, TPO, and FLT-3 ligand: 2 ng/ml, SCF 9 ng/ml). In some wells, antihuman CD56 mAb (mouse IgG class, cross-reactive with homologous monkey epitope, #0561, Exalpha) was added at a concentration of 0.5 or 1 μg/ml. As a control, isotype-matched normal mouse IgG (#349040; BD Biosciences) was added at the same concentrations. Five wells were prepared for each condition. These cells were cultured for 12 days, after which ^{3}H-thymidine (^{3}H-TdR) was introduced into each well. After 24 h, the cells were harvested and the incorporation of ^{3}H-TdR was measured.

Induction of monkey BM adherent cells to adipocytes and osteoblasts

Osteogenic differentiation was induced in the subconfluent BM adherent cells by incubating them in an osteogenic medium: IMDM containing 10% FBS (lot No. A01120-494, PAA Laboratories GmbH, Linz, Austria), 0.1 μM dexamethasone, 0.2 mM L-ascorbic acid, and 10 mM β-glycerophosphate. The medium was changed every 2 or 3 days. Osteogenic differentiation was assessed by von Kossa staining 4 weeks after the initial osteogenic induction.

To induce adipogenic differentiation, the subconfluent BM adherent cells were incubated with an adipogenic medium: IMDM containing 10% FBS (lot No. A01120-494), 1 μM dexamethasone, 5 μg/ml human insulin, and 4.5 mg/ml D-glucose. The medium was changed every 3 or 4 days. Adipogenic differentiation was assessed by oil red O staining 5 weeks after the initial adipogenic induction.

Histological examinations

Monkey BM tissues were fixed with 10% neutral formalin. The sections (4 μm) were prepared and stained with anti-CD56 mAb. Briefly, the sections were deparaffinized with xylene and methanol and incubated with 3% hydrogen peroxide in methanol for 10 min to block the reactivity of endogenous peroxidase. They were washed in PBS and incubated with protein blocking agent (484360, Thermo Electron Corporation, Pittsburgh, PA, USA) for 5 min and then incubated with mouse antihuman CD56 mAb (cross-reactive with homologous monkey epitope, clone: 1B6, #413331, Nichirei Bioscience Inc., Tokyo, Japan) for 1 h. After being washed with PBS, they were incubated with peroxidase-labeled goat antimouse immunoglobulin polyclonal Ab (Dako Cytomation EnVision kit/HRP, Dako Cytomation, Glostrup, Denmark) at room temperature for 30 min. They were visualized using metal-enhanced 3,3'-diaminobenzidine.

Statistics

Statistical differences were analyzed by Student two-tailed *t* test. Each experiment was carried out three or more times. Reproducible results were obtained, and therefore representative data are shown in the figures.

Results

Immunophenotypic analyses of monkey BM adherent cells

Monkey BMCs were collected from cynomolgus monkeys by the perfusion method. After a 2- to 3-week culture, BM adherent cells showing uniform fibroblastic morphology were obtained (Fig. 1a). Surface markers of freshly isolated BMCs and the culture-expanded BM adherent cells were assessed by flow cytometry. Although the fresh BMCs contained only a few CD56-positive cells, almost all the BM adherent cells were stained positively with anti-CD56 mAb (Fig. 1b). The BM adherent cells were negative for lymphoid markers (CD4 and CD8, data not shown), an endothelial cell maker (CD31, data not shown), and hemopoietic cell markers (CD11b, CD14, CD34, and CD45; Fig. 1c) but highly positive for CD29 and weakly positive for CD62L (Fig. 1b); CD29 and CD62L are the representative cell adhesion molecules expressed on hemopoietic cells, BM stromal cells, and MSCs. The negative staining for CD11b, CD14, and CD31 indicated that the BM adherent cells did not contain macrophages, dendritic cells, or endothelial cells. It is generally accepted that CD73 and CD105 are representative markers of human MSCs. However, the mAbs reacting with monkey CD73 and CD105 homologs are not commercially available and we could not therefore confirm the expression of CD73 and CD105 molecules on the monkey BM adherent cells in the present study.

Expression of CD56 on monkey HSC-enriched population

Next, we attempted to analyze whether the CD56 molecule is also expressed on the HSC-enriched population. When whole BMCs were double-stained with anti-CD34 and anti-

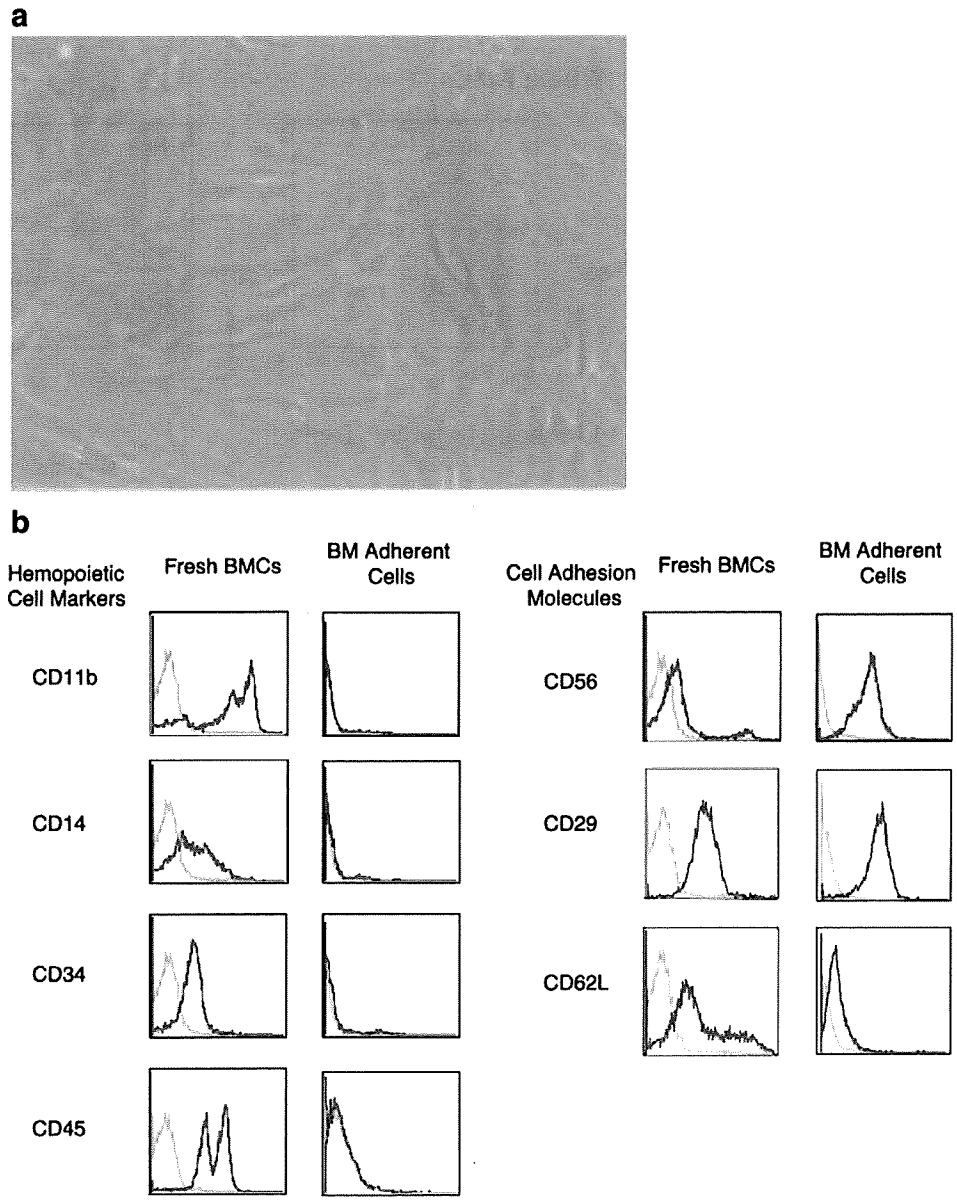

Fig. 1 Phenotypic analysis of monkey BM adherent cells. **a** Morphology of BM adherent cells. Monkey BMCs obtained by the perfusion method were cultured in flasks containing 10% FBS–IMDM, and spindle-shaped adherent cells were obtained 3 weeks later (×100). **b** Comparison of surface phenotypes between fresh BMCs and BM adherent cells. Freshly isolated BMCs and culture-expanded BM adherent cells were stained with a panel of mAbs reacting with hematolymphoid markers and cell adhesion molecules, and their phenotypes were analyzed using a FACScan. The *green lines* indicate the cells stained with isotype-matched control Abs. Representative staining patterns of five independent experiments

CD56 mAbs, only 2% were double-positive cells (Fig. 2a). We took the $CD34^-/56^+$ cells (8.8%) to be NK cells, a variety of T cell populations, and some stromal cells. After the enrichment of the HSCs, the percentage of $CD34^+/56^+$ cells increased to 9.1% in the lineage-negative population. When the cells in the "blast" window on the SSC/FSC dot plot profile were analyzed, the percentage of $CD34^+/56^+$ cells further increased and reached 20.6%. The $CD34^+/56^+$ cells and $CD34^+/56^-$ cells in the "blast" gate were sorted and stained with May–Giemsa reagents (Fig. 2b). When the $CD34^+/56^+$ cells were spun onto the cytospin slides for the May–Giemsa staining, marked cell aggregations were observed, but this was not the case with the $CD34^+/56^-$ cells. Both the $CD34^+/56^+$ and $CD34^+/56^-$ cells showed large nuclei with narrow cytoplasms, indicating that these cells have the characteristics of HSCs, although the $CD34^+/56^+$ cells are slightly larger in size. As expected, the $CD34^+/56^+$ and $CD34^+/56^-$ cells had the ability to differentiate into erythroid and myeloid lineage cells in the clonal hemopoietic colony assay using MethoCult GF H4434; burst-forming unit erythroid (BFU-E), colony-forming unit granulocyte (CFU-G), colony-forming unit-macrophage (CFU-M), and colony-forming unit granulocyte–macrophage (CFU-GM) were detected. There was no significant difference between the two populations in the cellularity of the clonal hemopoietic colonies and in the number of the colonies (Fig. 2c). None of the most primitive colony-forming cells, colony-forming unit granulocyte–erythroid–macrophage–

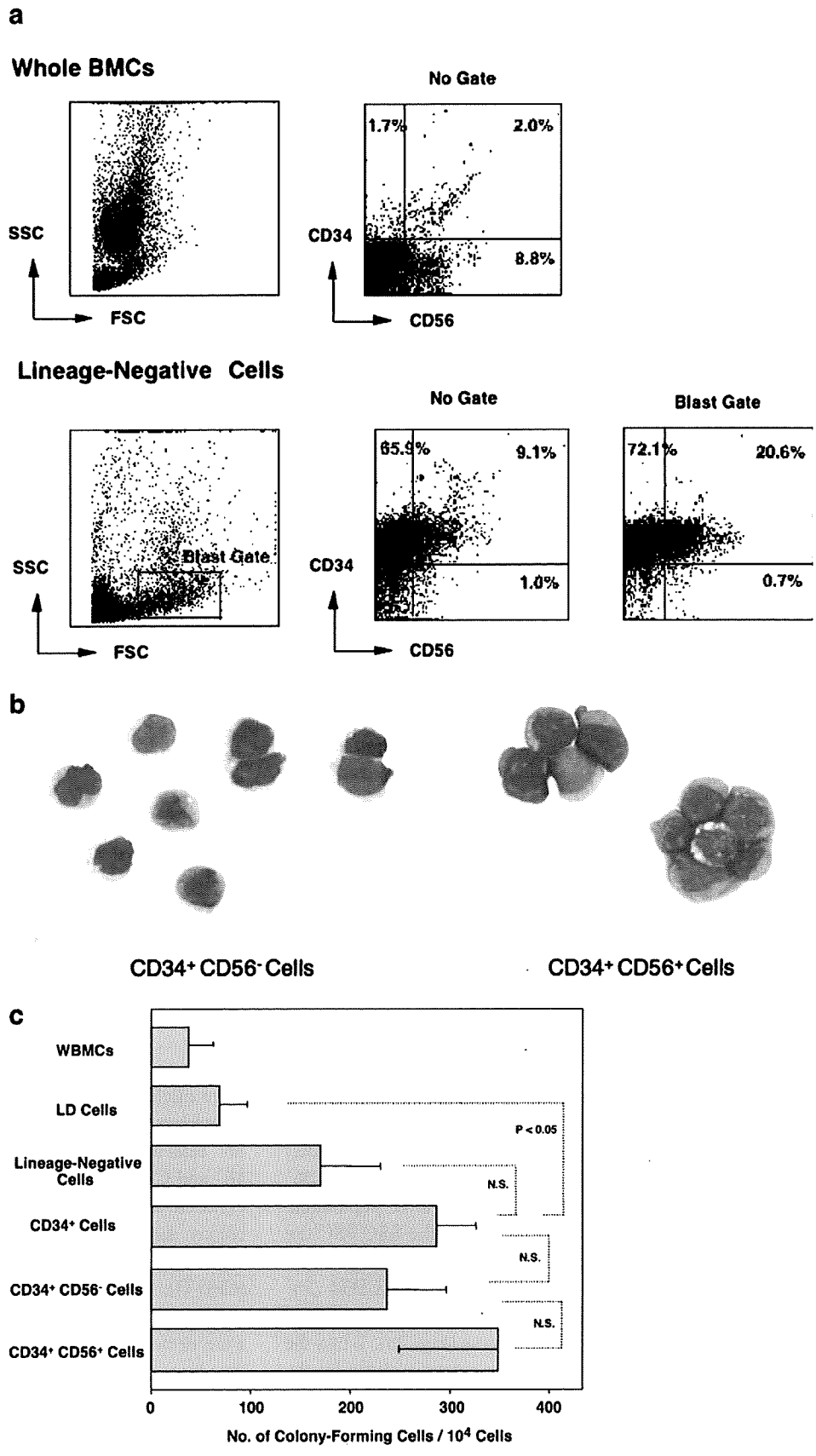

Fig. 2 Expression of CD56 molecule in monkey HSCs. **a** Increase of $CD56^+$ cells in HSC-enriched population. When whole BMCs were double-stained with anti-CD34 and CD56 mAbs, only a few $CD34^+/56^+$cells were detected. However, the percentages of the double-positive cells increased to 9.1% in the HSC-enriched population (lineage-negative cells) and a further increase in the percentages was observed in the "blast" gated population of the SSC/FSC profile. Representative staining patterns of five independent experiments. **b** Morphology of $CD34^+/56^-$ cells and $CD34^+/56^+$cells. The $CD34^+/56^-$ cells and the $CD34^+/56^+$ cells included in the "blast" window were sorted and then stained with May–Giemsa reagents. Both populations express a phenotype of HSCs (×1,000). **c** Number of hemopoietic colony-forming cells in various cell populations. Whole BMCs, LD cells, lineage-negative cells, $CD34^+$ cells, the $CD34^+/56^-$ cells, and the $CD34^+/56^+$ cells were incubated in methylcellulose containing optimal concentrations of human cytokines (MethoCult GF H4434) to obtain the number of hemopoietic colony-forming cells (triplicate). Mean±SD of five independent experiments

megakaryocytes were detected in either the $CD34^+/56^+$ or $CD34^+/56^-$ populations, since the MethoCult formulation, which we used in the present study, was designed for the induction of optimal colony formations for human BMCs.

Long-term hemopoiesis-supporting ability of monkey BM adherent cells

We have previously shown that major histocompatibility complex (MHC) restriction exists between HSCs and BM stromal cells in the murine hemopoietic system; purified HSCs can proliferate and differentiate to a higher extent in MHC-compatible stromal cells than in MHC-incompatible environments [18–20]. Therefore, in the present study, monkey partially purified HSCs were cultured on a BM stromal cell layer derived from the same monkey, and the hemopoiesis-supporting ability of the BM adherent cells was examined. Monkey BMCs were divided into cell strainer-passed cells and nonpassed cells. The nonpassed cells were cultured in order to obtain a BM adherent cell layer, whereas the passed cells were stored in liquid nitrogen until a BM adherent cell layer was formed. Partially purified HSCs were prepared from the frozen BMCs and cocultured with the BM adherent layer. As a control, the HSCs were cultured in the absence of the BM adherent layer.

When the HSCs were cocultured with the BM adherent cells, marked proliferation of the HSCs was observed from the first week and the hemopoiesis was maintained at higher levels for up to 3 weeks of culture (Fig. 3a). The nonadherent cells recovered from the culture flasks at 1 to 3 weeks of culture contained immature and mature hemopoietic cells of all lineages: myelocytes, erythroblasts, granulocytes, macrophages, and megakaryocytes (data not shown). On the other hand, the nonadherent cells recovered at and after 4 weeks of culture contained mainly granulocytes and macrophages (data not shown). The nonadherent cells were then assessed for their ability to form clonal hemopoietic colonies using MethoCult. As shown in Fig. 3b, high colony formation was observed in the nonadherent cells recovered at 2 to 3 weeks of culture. These colonies were mainly composed of granulocytes and/or macrophages (CFU-G, CFU-M, and CFU-GM), and a few erythroblasts (BFU-E) were detected. In contrast, no increase in the number of nonadherent cells was observed in the control culture without the BM adherent cells, and the cell number reached the baseline at 2 weeks of culture (Fig. 3a). These data show that the BM adherent cells have the capacity to support the hemopoiesis of HSCs at least for 6 weeks, whereas the proliferation and differentiation of HSCs cannot be induced without the BM adherent layer.

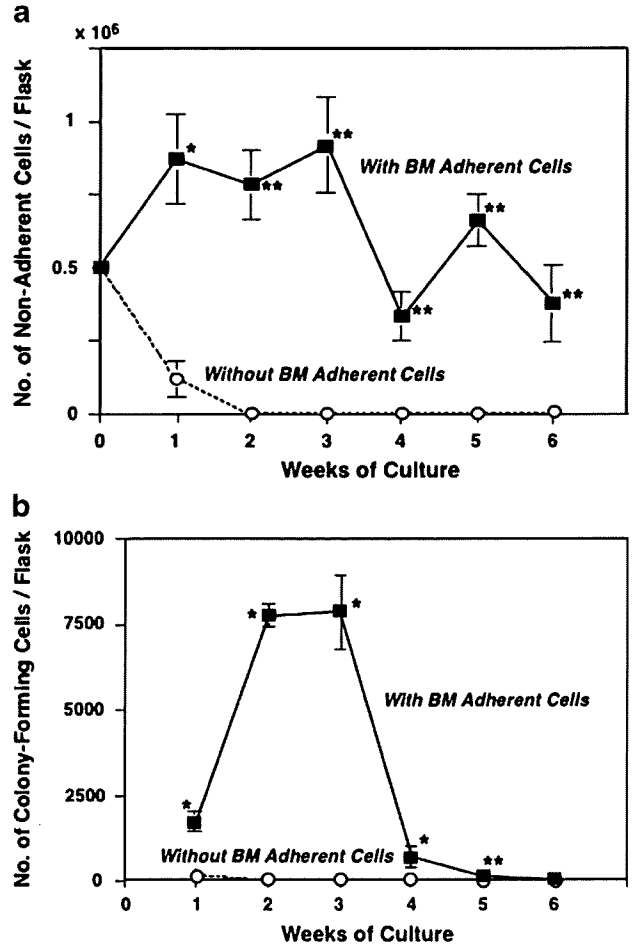

Fig. 3 Long-term hemopoiesis-supporting ability of monkey BM adherent cells. **a** Number of nonadherent cells obtained from long-term culture. Partially purified HSCs were cultured in the presence or absence of BM adherent cells (triplicate), and the number of nonadherent cells was counted every week. Mean±SD of three flasks. $*P<0.005$, $**P<0.0001$ significantly different from the control (without BM adherent cells). Representative data of five independent experiments. **b** Number of hemopoietic colony-forming cells in nonadherent cells recovered from culture flask. The nonadherent cells recovered from the long-term culture were evaluated for their colony-forming ability using methylcellulose assay (MethoCult GF H4434; triplicate). Mean±SD of three wells. $*P<0.0001$, $**P<0.005$ significantly different from the control (without BM adherent cells). Representative data of three independent experiments

Inhibitory effects of anti-CD56 antibody against proliferation of monkey HSCs

CD56 molecules were expressed on both HSCs and BM adherent–stromal cells (Figs. 1b and 2a) and the monkey BM adherent cells showed an ability to support hemopoiesis (Fig. 3). These findings suggest that the CD56 molecules play an important role in hemopoiesis in monkeys. Therefore, we next examined whether anti-CD56 mAb inhibits the proliferation of HSCs on the BM adherent cells when added to the coculture system of the partially purified HSCs and the