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		Survival	% of hemopoietic cells derived from[a]		
			BMCs	Thymus		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

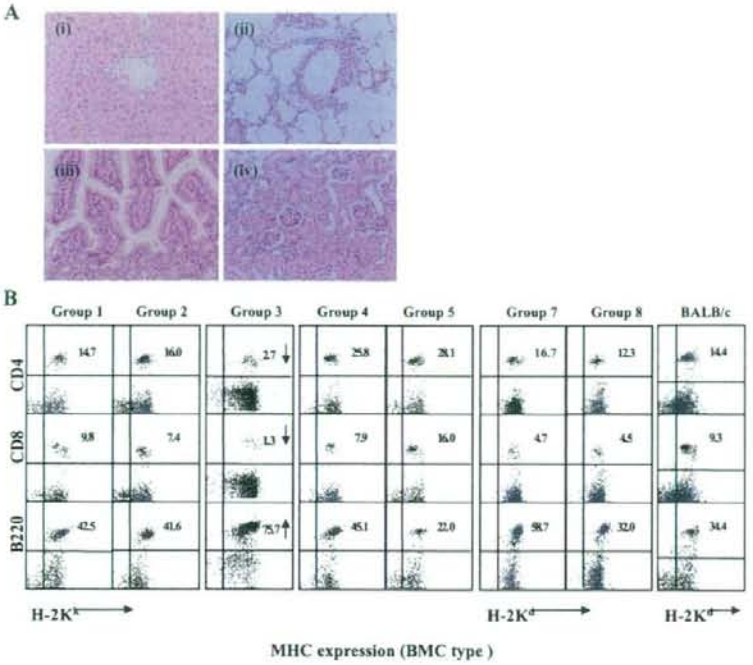

FIGURE 1. Analysis of histology and BMC-derived $CD4^+$ and $CD8^+$ T cells and B cells in spleen from experimental groups. (A) Histologic findings of liver (i), lung (ii), small intestine (iii), and kidney (iv) from group 1 (hematoxylin-eosin; magnification ×400). The mice from other groups with TT 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

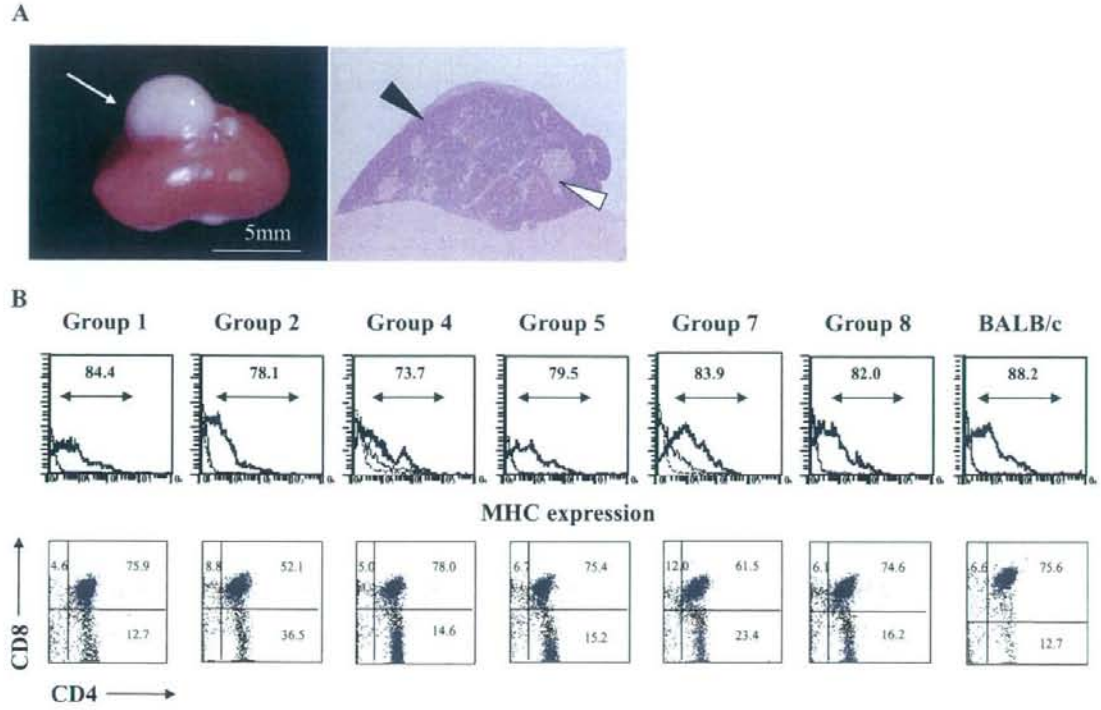

FIGURE 2. Analysis of histology, chimerism, and thymocyte subsets in transplanted thymus from experimental groups. (A) Macroscopic (*left*) and microscopic findings (*right*) in transplanted thymus from group 1 at 2 months after transplantation are shown. (B) Thymus tissue was engrafted (*arrow*), and 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

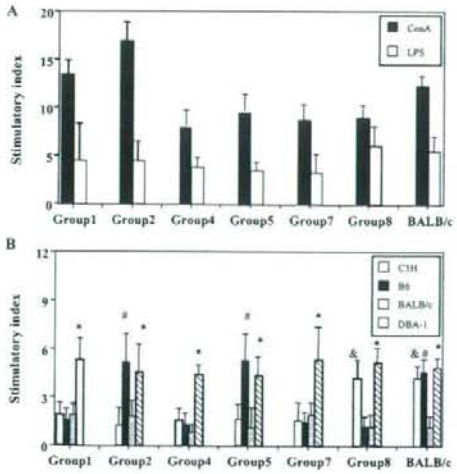

FIGURE 3. Mitogen responses and MLR in experimental groups. Mitogen responses to Con A and LPS (A) and MLR (B) in splenocytes are shown from groups 1, 4, and 7 for triple chimeras, from groups 2, 5, and 8 for MHC-matched TT, and from group 3 for absence of TT (as described in Table 1), and from untreated 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

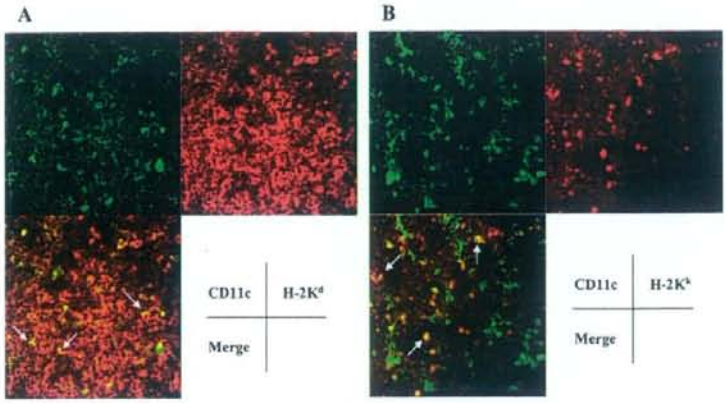

FIGURE 4. BMC- and thymus-derived dendritic cells (DCs) in transplanted thymus. The transplanted thymic lobe from group 7 at 2 months after transplantation was stained using FITC-conjugated CD11c mAb and PE-avidin/biotin-conjugated H-2K^d:BMC type (A), or H-2K^k:thymus type (B) mAb. Expressions were 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.

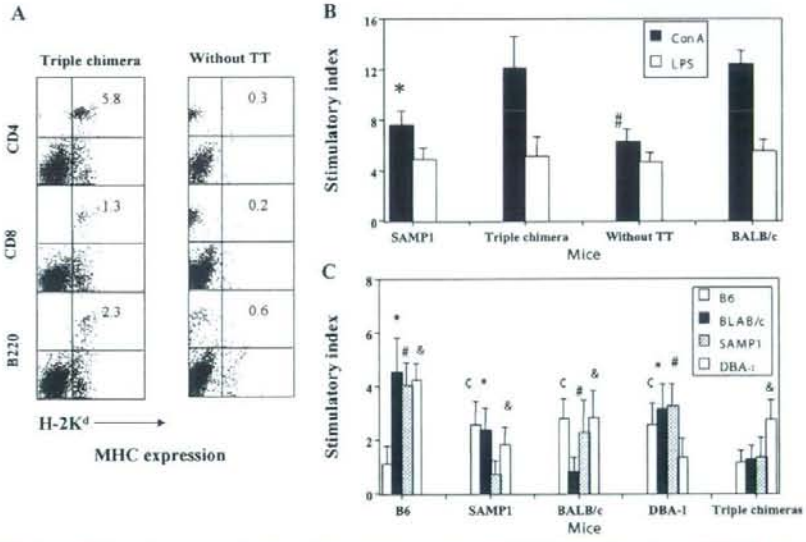

FIGURE 5. BMC-derived lymphocyte subsets, mitogen responses, and MLR in spleen from SAMP1 mice treated by triple chimeric transplantation or absence of TT. BMC-derived CD4$^+$ and CD8$^+$ T cells and B220$^+$ B cells in spleen were analyzed from triple chimeric mice or mice with absence of TT by flow 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; $^\$P<0.05$ 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 nonmyeloablative 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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REFERENCES

1. Ikehara S. Bone marrow transplantation: A new strategy for intractable disease. *Drugs Today* 2002; 38: 103.
2. Ikehara S, Ohtsuki H, Good RA, et al. Prevention of type I diabetes in nonobese diabetic mice by allogenic bone marrow transplantation. *Proc Natl Acad Sci U S A* 1985; 22: 7743.
3. Yasumizu R, Sugiura K, Iwai H, et al. Treatment of type 1 diabetes mellitus in non-obese diabetic mice by transplantation of allogeneic bone marrow and pancreatic tissue. *Proc Natl Acad Sci U S A* 1987; 84: 6555.
4. Than S, Ishida H, Inaba M, et al. Bone marrow transplantation as a strategy for treatment of non-insulin-dependent diabetes mellitus in KK-Ay mice. *J Exp Med* 1992; 176: 1233.
5. Ishida T, Inaba M, Hisha H, et al. Requirement of donor-derived stromal cells in the bone marrow for successful allogeneic bone marrow transplantation. Complete prevention of recurrence of autoimmune diseases in MRL/MP-lpr/lpr mice by transplantation of bone marrow plus bones (stromal cells) from the same donor. *J Immunol* 1994; 152: 3119.
6. Nakagawa T, Nagata N, Hosaka N, et al. Prevention of autoimmune inflammatory polyarthritis in male New Zealand black/KN mice by transplantation of bone marrow cells plus bone (stromal cells). *Arthritis Rheum* 1993; 36: 263.
7. Nishimura M, Toki J, Sugiura K, et al. Focal segmental glomerular sclerosis, a type of intractable chronic glomerulonephritis, is a stem cell disorder. *J Exp Med* 1994; 179: 1053.
8. Kaneda H, Adachi Y, Saito Y, et al. Long-term observation after simultaneous lung and intra-bone marrow-bone marrow transplantation. *J Heart Lung Transplant* 2005; 24: 1415.
9. Koike Y, Adachi Y, Suzuki Y, et al. Allogeneic intrabone marrow-bone marrow transplantation plus donor lymphocyte infusion suppresses growth of colon cancer cells implanted in skin and liver of rats. *Stem Cell* 2007; 25: 385.
10. Klingemann HG, Storb R, Fefer A, et al. Bone marrow transplantation in patients aged 45 years and older. *Blood* 1986; 67: 770.
11. Weiner RS, Bortin MM, Gale RP, et al. Interstitial pneumonitis after bone marrow transplantation. Assessment of risk factors. *Ann Intern Med* 1986; 104: 168.
12. Ringden O, Paulin T, Lonngvist B, et al. An analysis of factors predisposing to chronic graft-versus-host disease. *Exp Hematol* 1985; 13: 1062.
13. Hirokawa K, Utsuyama M, Kasai M, et al. Understanding the mechanism of the age-change of thymic function to promote T cell differentiation. *Immunol Lett* 1994; 40: 269.
14. Mackall CL, Gress RE. Pathways of T-cell regeneration in mice and humans: Implications for bone marrow transplantation and immunotherapy. *Immunol Rev* 1997; 157: 61.
15. Aspinall R. Age-associated thymic atrophy in the mouse is due to a deficiency affecting rearrangement of the TCR during intrathymic T cell development. *J Immunol* 1997; 158: 3037.
16. Hosaka N, Nose M, Kyogoku M, et al. Thymus transplantation, a critical factor for correction of autoimmune disease in aging MRL/+mice. *Proc Natl Acad Sci U S A* 1996; 93: 8558.
17. Kushida T, Inaba M, Hisha H, et al. Intra-bone marrow injection of allogeneic bone marrow cells: A powerful new strategy for treatment of intractable autoimmune diseases in MRL/lpr mice. *Blood* 2001; 97: 3292.
18. Nakamura K, Inaba M, Sugiura K, et al. Enhancement of allogeneic hematopoietic stem cell engraftment and prevention of GVHD by intra-bone marrow bone marrow transplantation plus donor lymphocyte infusion. *Stem Cells* 2004; 22: 125.
19. Ikehara S. A novel strategy for allogeneic stem cell transplantation: Perfusion method plus intra-bone marrow injection of stem cells. *Exp Hematol* 2003; 31: 1142.
20. Krampera M, Glennie S, Dyson J, et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. *Blood* 2003; 101: 3722.
21. Blanc KL, Rasmusson I, Sundberg B, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* 2004; 363: 1439.
22. Takeda T, Hosokawa M, Takeshita S, et al. A new murine model of accelerated senescence. *Mech Ageing Dev* 1981; 17: 183.
23. Odagiri Y, Uchida H, Hosokawa M, et al. Accelerated accumulation of somatic mutations in the senescence-accelerated mouse. *Nat Genet* 1998; 19: 116.
24. Hosono M, Hanada K, Toichi E, et al. Immune abnormality in relation to nonimmune diseases in SAM mice. *Exp Gerontol* 1997; 32: 181.
25. Miller JF. Studies on mouse leukaemia. Leukaemogenesis by cell-free filtrates inoculated in newborn and adult mice. *Br J Cancer* 1960; 14: 93.
26. Nagahama T, Sugiura K, Lee S, et al. A new method for tolerance induction: Busulfan administration followed by intravenous injection of neuraminidase-treated donor bone marrow. *Stem Cell* 2001; 19: 425.
27. Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. *Annu Rev Immunol* 2003; 21: 685.
28. Markert ML, Boeck A, Hale LP, et al. Transplantation of thymus tissue in complete DiGeorge syndrome. *N Engl J Med* 1999; 341: 1180.
29. Markert ML, Hicks CB, Bartlett JA, et al. Effect of highly active antiretroviral therapy and thymic transplantation on immunoreconstitution in HIV infection. *AIDS Res Hum Retroviruses* 2000; 16: 403.
30. Zhang L, Sun L, Zhao Y. Thymic epithelial progenitor cells and thymus regeneration: An update. *Cell Res* 2007; 17: 50.
31. Chen BJ, Cui X, Sempowski GD, et al. Hematopoietic stem cell dose correlates with the speed of immune reconstitution after stem cell transplantation. *Blood* 2004; 103: 4344.
32. Hashimoto F, Sugiura K, Inoue K, et al. Major histocompatibility complex restriction between hematopoietic stem cells and stromal cells in vivo. *Blood* 1997; 89: 49.
33. Li Y, Hisha H, Inaba M, et al. Evidence for migration of donor bone marrow stromal cells into recipient thymus after bone marrow transplantation plus bone grafts: A role of stromal cells in positive selection. *Exp Hematol* 2000; 28: 950.
34. Kamano C, Vagefi PA, Kumagai N, et al. Vascularized thymic lobe transplantation in miniature swine: Thymopoiesis and tolerance induction across fully MHC-mismatched barriers. *Proc Natl Acad Sci U S A* 2004; 101: 3827.
35. Nobori S, Shimizu A, Okumi M, et al. Thymic rejuvenation and the induction of tolerance by adult thymic grafts. *Proc Natl Acad Sci U S A* 2006; 103: 19081.
36. Duncan SR, Capetanakis NG, Lawson BR, et al. Thymic dendritic cells traffic to thymi of allogeneic recipients and prolong graft survival. *J Immunol* 2002; 109: 755.
37. Bartholomew A, Sturgeon C, Siatskas M, et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Exp Hematol* 2002; 30: 42.
38. Nicola MD, Carlo-Stella C, Magni M, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002; 99: 3838.
39. Lee JW, Epardaud M, Sun J, et al. Peripheral antigen display by lymph node stroma promotes T cell tolerance to intestinal self. *Nat Immunol* 2007; 8: 181.
40. Rugh R. The relation of sex, age, and weight of mice to microwave radiation sensitivity. *J Microw Power* 1976; 11: 127.
41. Hosaka N, Ryu T, Miyake T, et al. Treatment of autoimmune diseases in MRL/lpr mice by allogenic bone marrow transplantation plus adult thymus transplantation. *Clin Exp Immunol* 2007; 147: 555.
42. Hirokawa K, Utsuyama M. Combined grafting of bone marrow and thymus, and sequential multiple thymus graftings in various strains of mice. The effect on immune functions and life span. *Mech Ageing Dev* 1989; 49: 49.
43. Van den Brink MR, Alpdogan O, Boyd RL. Strategies to enhance T-cell reconstitution in immunocompromised patients. *Nat Rev Immunol* 2004; 4: 856.
44. Fulop T, Larbi A, Wikby A, et al. Dysregulation of T-cell function in the elderly: Scientific basis and clinical implications. *Drugs Aging* 2005; 22: 589.
45. Kudlacek S, Willvonseder R, Stohlawetz P, et al. Immunology and aging. *Aging Male* 2000; 3: 137.
46. Falda M, Busca A, Baldi I, et al. Nonmyeloablative allogeneic stem cell transplantation in elderly patients with hematological malignancies: Results from the GITMO (Gruppo Italiano Trapianto Midollo Osseo) multicenter prospective clinical trial. *Am J Hematol* 2007; 82: 863.

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Presence of donor-derived thymic epithelial cells in [B6→MRL/lpr] mice after allogeneic intra-bone marrow–bone marrow transplantation (IBM–BMT)☆

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ABSTRACT

We have previously shown that allogeneic intra-bone marrow–bone marrow transplantation (IBM–BMT) can be used to treat autoimmune diseases in MRL/lpr ($H\text{-}2^K$) mice with replacing not only hematolymphoid cells but also stromal cells by normal C57BL/6 (B6: $H\text{-}2^b$) mouse cells. In the present study, we examined for existence of donor-derived thymic epithelial cells (TECs) in the host thymus using green fluorescent protein (GFP)-B6 ($H\text{-}2^b$) mice. In [GFP-B6→MRL/lpr] chimeric mice, splenocytes and thymocytes were completely replaced by donor-type cells, and levels of serum autoantibodies and proteinuria were significantly – reduced to those levels of normal donors. Interestingly, GFP-expressing TECs – not only medullary TECs, which express mouse thymus stromal (MTS)-10, but also cortical TECs, which express cytokeratin 18 – were found. Also, the number of autoimmune regulator (AIRE) expressing TECs, which regulates tissue-specific antigens to delete autoreactive cells, was reduced in the chimeric mice to that of the donor, whereas the number of forkhead box N1 (FOXN1) expressing TECs, which are crucial in the terminal differentiation of TECs, remained unchanged. These findings suggest that BMCs contain the precursors of functional TECs, and that they can differentiate into TECs, thereby correcting thymic function.

1. Introduction

The thymus is the organ in which T cells develop from their precursor cells [1,2]. As these precursors develop into mature T cells, they migrate from the cortical area to the medullary area in the thymus, as they develop into mature T cells. The thymic epithelial cells (TECs) and dendritic cells (DCs) play important roles in this process. The cortical TECs (cTECs) induce major histocompatibility complex (MHC) restricted T cells by positive selection, and the medullary DCs (mDCs) delete self-antigen (Ag) reactive T cells by negative selection. Recent studies have identified functional molecules that are expressed in TECs. FOXN1 is a transcriptional factor for terminal differentiation of TECs during embryogenesis, and the lack of this gene results in the nu/nu phenotype [3,4]. AIRE is the transcriptional activator for tissue-specific antigens, and is expressed in medullary TECs (mTECs) [5]. AIRE regulates the deletion of autoreactive T cells, and its deficiency leads to autoimmune diseases [6].

cTECs and mTECs had been thought to develop from different parts of the embryo. The cTECs were believed to be derived from the ectoderm of the third pharyngeal cleft, whereas the mTECs were believed to be derived from the endoderm of the third pharyngeal pouch [7–9]. However, recent reports indicate that both cTECs and mTECs are derived from a single germ layer of endoderm [10–12]. In addition, Rossi et al. showed that a single precursor expressing MTS24 from the fetal thymus can differentiate into both cTECs and mTECs [13]. These findings suggest the existence of a common precursor for cTECs and mTECs in the embryonic thymus. However, the precursors of TECs have not been well examined in adults.

Bone marrow transplantation (BMT) has been used as a potentially curative therapy for patients with a wide variety of diseases, including hematological disorders, congenital immunodeficiencies, metabolic disorders, autoimmune diseases, and solid tumors [14–19]. To ensure fewer side effects, and/or better engraftment, a number of different approaches to BMT methods have been tried, among them being the transplantation of high doses of HSCs, and donor lymphocyte infusion (DLI) [20,21]. However, in some cases,

Abbreviations: BMCs, bone marrow cells; MCS, mesenchymal stem cell; IBM–BMT, intra-bone marrow–bone marrow transplantation; TEC, thymic epithelial cell; MTS, mouse thymus stromal; DC, dendritic cell; AIRE, autoimmune regulator; FOXN1, forkhead box N1.

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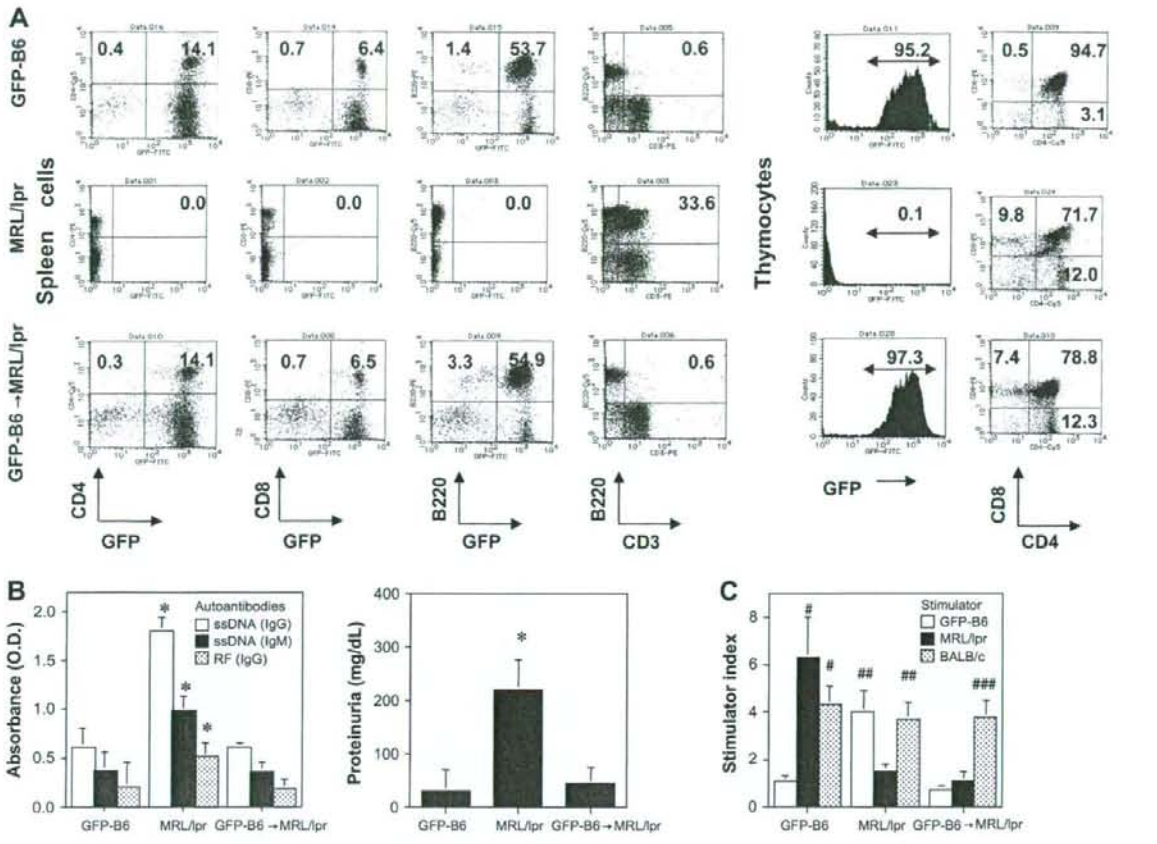

Fig. 1. FACS analyses of lymphoid subsets in the spleen and thymus of [GFP-B6→MRL/lpr] chimeric mice after IBM–BMT (A): The chimeric mice (8 weeks after IBM–BMT) show a similar pattern to that of donor GFP-B6 mice. (B) shows that autoantibody production and the degree of proteinuria are normalized 8 weeks after IBM–BMT. (C) shows that newly developed T cells are tolerant of both donor (GFP-B6)-type and host (MRL/lpr)-type MHC determinants 8 weeks after IBM–BMT. The stimulator index (SI) was calculated as the average of ^{3}H-thymidineTdR incorporation (stimulator in medium)/^{3}H-thymidineTdR incorporation (medium) in triplicate wells. Representative results are shown from three experiments (A, C). $N = 6$ in GFP-B6 mice, $N = 5$ in MRL/lpr mice, and $N = 6$ in [GFP-B6→MRL/lpr] chimeric mice (B). $^{*}P < 0.05$ compared with GFP-B6 and [GFP-B6→MRL/lpr] chimeric mice (B). $^{\#}P < 0.05$ compared with GFP-B6; $^{\#\#}P < 0.05$ compared with MRL/lpr; $^{\#\#\#}P < 0.05$ compared with GFP-B6 and MRL/lpr mice as stimulator (C). Data are shown as means ± SD.

there remains the challenge of obtaining sufficient numbers of BMCs, preventing graft failure, or preventing the induction of the lethal GVHD. The development of a new cell-based method is therefore required.

We previously developed several bone marrow transplantation (BMT) methods [22–27], including the intra-bone marrow (IBM)–BMT (IBM–BMT) method [28]: the direct injection of BMCs into the bone marrow cavity. This method facilitates the engraftment of not only donor-derived hemopoietic stem cells (HSCs) but also mesenchymal stem cells (MSCs). We have found that the IBM–BMT method can be used to treat autoimmune diseases in chimeric-resistant MRL/lpr mice by replacing normal HSCs and MSCs, although the conventional BMT method is ineffective in these mice.

BMCs are known to differentiate into several epithelial cells, such as those of the lung, liver, gastrointestinal tract, kidney, and skin after BMT [29–32].

In the present study, we used the allogeneic IBM–BMT in MRL/lpr mice to examine the origin of TECs in the recipient thymus. Interestingly, donor-derived TECs were found in both the medullary and the cortical areas. In addition, the number of AIRE-expressing cells was found to be reduced in parallel with the treatment of the autoimmune diseases. These findings suggest that BMCs contain the precursors of functional TECs, and that they can differentiate into TECs, which result in the correction of thymic function.

2. Materials and methods

2.1. Mice

Three- to four-month-old female MRL/MP-lpr/lpr mice (MRL/lpr) (H-2^{k}) [33] were used as recipients, and 6- to 8-week-old female C57BL/6(B6) (H-2^{b}) mice that expressed green fluorescent protein (GFP) were used as donors. Mice were obtained from SLC (Shizuoka, Japan) and maintained in our animal facilities under specific pathogen-free conditions.

2.2. IBM–BMT method

Three- to four-month-old MRL/lpr mice that were exhibiting the onset of autoimmune disease (proteinuria > 100 mg/dl and evident lymphadenopathy) were irradiated (5 Gy × 2, 4-h interval) with the ^{137}Cs irradiator 1 day before the BMT. BMCs were flushed from the shaft of the femora and tibiae of donor mice (B6), and single-cell

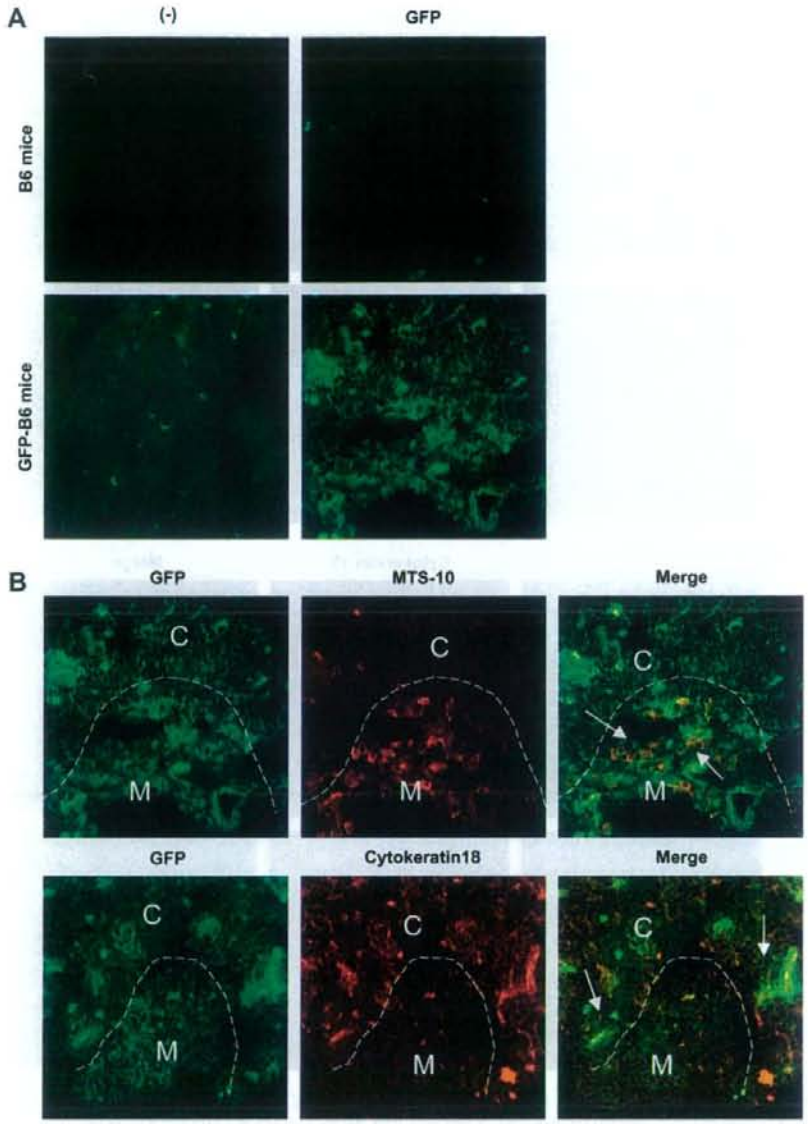

Fig. 2. GFP, MTS-10, and cytokeratin 18 expression in thymuses from B6 and GFP-B6 mice. The thymi from B6 and GFP-B6 mice were stained by anti-GFP-FITC Abs or not stained (A). The thymus from a GFP-B6 mouse was stained by anti-GFP-FITC Abs and MTS-10 Abs (upper) or anti-cytokeratin 18 Abs (lower) plus PE-conjugated second Abs (B). Arrows; GFP and MTS-10 (upper) or cytokeratin 18 (lower) double-positive cells in the stromal area of the medulla or cortex. C, Cortex; M, Medulla. ×200.

suspensions were prepared. Next, 3×10^7 GFP-B6 BMCs were directly injected into the bone marrow cavity of the tibia, as previously described for the IBM–BMT method [27].

2.3. Flow cytometric analysis of surface markers on spleen cells

Surface markers on lymphocytes from the spleen and thymocytes from the thymus were analyzed with 3-color fluorescence staining by using FACScan (Becton Dickinson, Franklin Lakes, NJ). Phycoerythrin (PE)- or biotin-conjugated CD3, CD4, CD8, or B220 (Pharmingen, San Diego, CA) were used to analyze the lymphocyte subsets. Avidin-PE-Cy5 (Dako, Kyoto, Japan) was used as the third color in the avidin/biotin system.

2.4. Immunohistochemistry

Thymus tissues were embedded in Tissue-Tek OCT compound (Sakura Finetek, Tokyo, Japan) and stored at −40 °C. Cryosections (4-mm thick) were air-dried and fixed with acetone for 10 min. Specimens were treated with 0.5% bovine serum albumin in TBS for 10 min, and then stained with FITC-conjugated anti-GFP (Invitrogen, Carlsbad, CA) and PE-conjugated anti-CD11c (Pharmingen), anti-CD8 (Pharmingen), or anti-CD4 (Pharmingen) Abs, or anti-cytokeratin 18 (Progen, Queensland, Australia), anti-autoimmune regulator (AIRE) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-FOXN1 (Santa Cruz) Abs, or MTS-10 for 1 h at room temperature in a moist chamber. After washing 3 times in TBS for 5 min with gentle

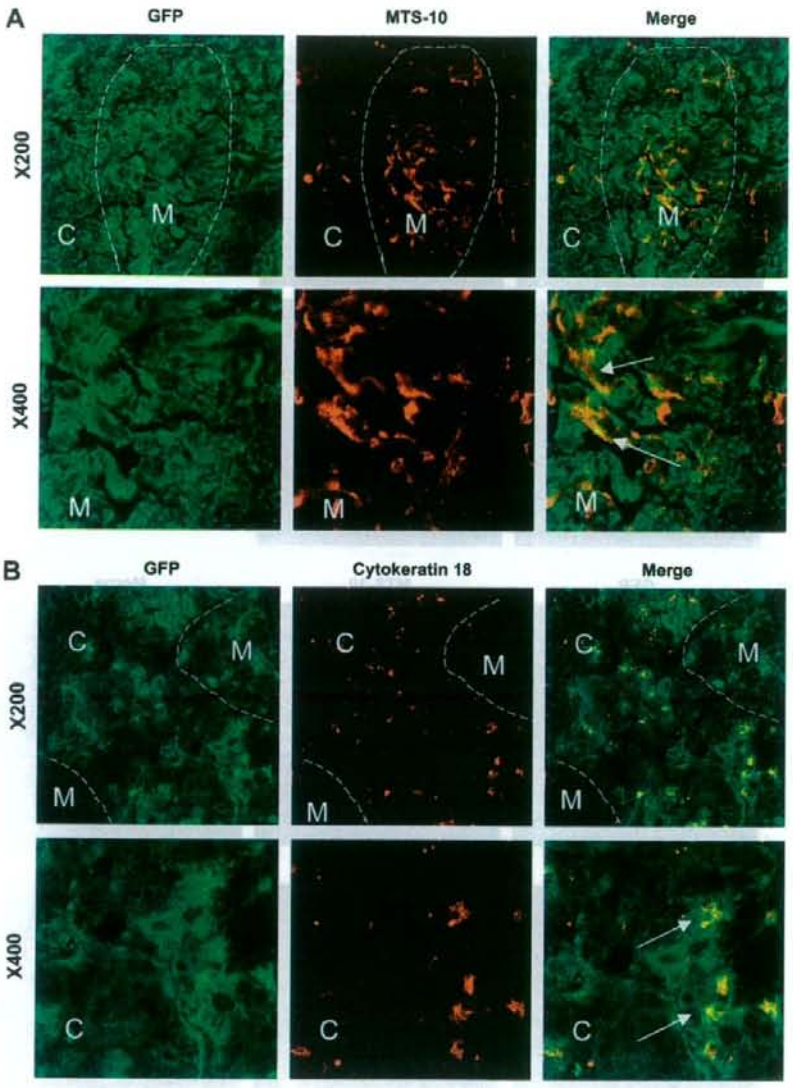

Fig. 3. The presence of donor-derived mTECs expressing GFP and MTS-10 and cTECs expressing GFP and cytokeratin 18 in the thymus from [GFP-B6→MRL/lpr] chimeric mice 8 weeks after IBM–BMT. The thymi from [GFP-B6→MRL/lpr] chimeric mice by IBM–BMT were stained with anti-GFP-FITC and MTS-10 Abs (A) or anti-cytokeratin 18 Abs (B) plus PE-conjugated second Abs. Arrows; GFP and MTS-10 (A) or cytokeratin 18 (B) double-positive cells in the stromal area of the medulla. C, Cortex; M, Medulla. Representative profiles are shown for four experiments.

shaking, a second incubation was performed with PE-conjugated anti-mouse IgG (Pharmingen) for cytokeratins, PE-conjugated anti-rat IgM Abs (Pharmingen) for MTS-10, or Texas Red (TR)-conjugated anti-goat IgG Abs (Santa Cruz) for AIRE and FOXN1. The staining was evaluated by confocal microscopy with an LSM 510 META microscope (Carl Zeiss, Minneapolis, MN). Since it has been shown that AIRE and FOXN1 are expressed in TECs as a cluster of dots [34,35], positively stained cells were counted for the in 10 high-power fields (HPF, ×400) in a blinded manner by two researchers, and the average was calculated as the number of cells per HPF [36].

2.5. Measurement of autoantibodies in sera

IgG and IgM anti-ssDNA antibodies and IgG rheumatoid factor in the sera from the chimeric and the untreated mice were measured by using a standard enzyme-linked immunosorbent assay (ELISA), as previously described [37]. The concentration of autoantibodies was measured by absorbance at 405 nm after developing the phosphatase substrate (Sigma–Aldrich, St. Louis, MO).

2.6. Mixed lymphocyte reaction (MLR)

To analyze lymphocyte function and tolerance, the MLR was performed in chimeric mice two months after transplantation. A total of 2×10^5 splenocytes collected from GFP-B6, MRL/lpr and GFP-B6 → chimeric 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

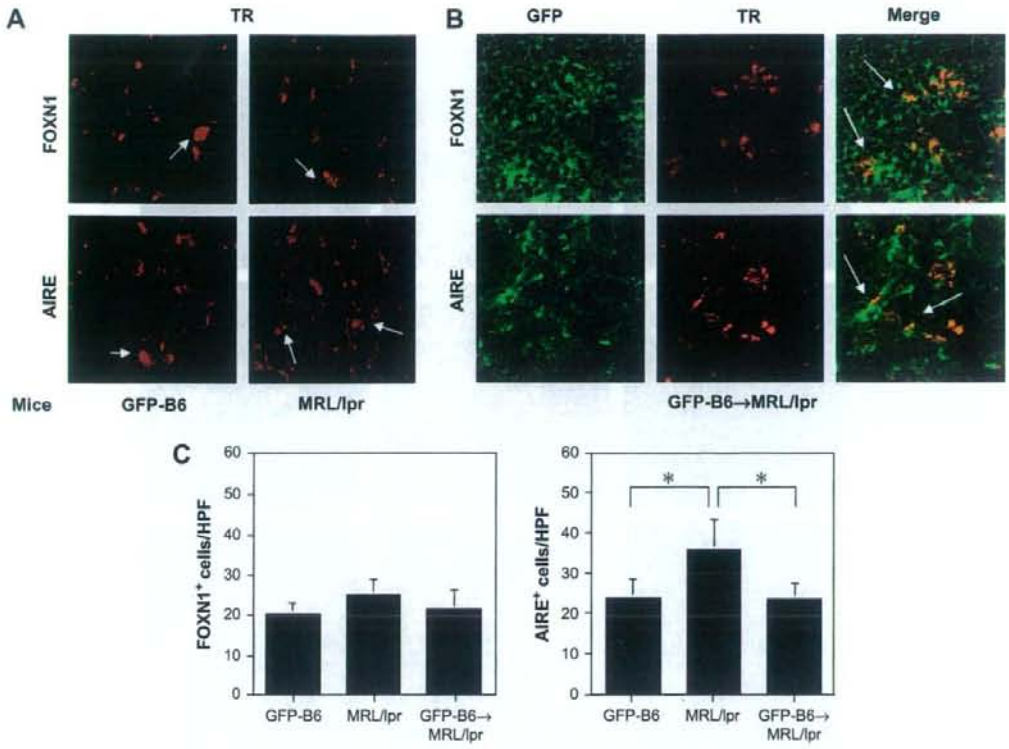

Fig. 4. The presence of donor-derived TECs expressing GFP and FOXN1 or AIRE in the thymus from GFP-B6, MRL/lpr, and [GFP-B6→MRL/lpr] chimeric mice 8 weeks after IBM–BMT. The thymi from GFP-B6, MRL/lpr were stained with anti-FOXN1 Abs (upper) or anti-AIRE Abs (lower) plus TR-conjugated second Abs in the stromal area. ×400 (A). The thymi of [GFP-B6→MRL/lpr] chimeric mice by IBM–BMT were stained by anti-GFP-FITC and anti-FOXN1 Abs (upper) or anti-AIRE Abs plus TR-conjugated second Abs (lower) in the stromal area. ×400 (B). Number of FOXN1- (left) or AIRE- (right) expressing cells/HPF in the thymus from GFP-B6, MRL/lpr, and [GFP-B6→MRL/lpr] chimeric mice after IBM–BMT. Arrows: GFP and FOXN1 (upper) or AIRE (lower) double-positive cells. Representative profiles are shown (A, B). $N = 5$ in GFP-B6 mice, $N = 5$ in MRL/lpr mice, and $N = 5$ in [B6→MRL/lpr] chimeric mice. $^*P < 0.05$.

FCS. Responders were incubated with 2×10^5 splenocytes irradiated at 15 Gy from mice including donors (B6), recipients (MRL/lpr), and a third party (BALB/c) as stimulators for 96 h. Next, 20 μl of 0.5 μCi ^{3}H-thymidine (^{3}H-TdR; New England Nuclear, Cambridge, MA) was introduced during the last 18 h of the culture period. Incorporation of ^{3}H-TdR was measured by using Microbeta TriLux (PerkinElmer, Wellesley, MA). The stimulator index (SI) was calculated as the average of ^{3}H-TdR incorporation (stimulator in medium)/^{3}H-TdR incorporation (medium) in triplicate wells.

2.7. Statistical analysis

Non-parametric analyses (Mann–Whitney *U*-test and log-rank test) were performed with StatView software (Abacus Concepts, Berkley, CA). *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Development of donor-derived hematolymphoid cells, and tolerance induction in [GFP-B6→MRL/lpr] chimeric mice after IBM–BMT

First, we performed allogeneic BMT in the autoimmune-prone MRL/lpr mice (H-2^k) by IBM–BMT from GFP-bearing B6 mice (GFP-B6) (H-2^b). Eight weeks after transplantation, all of the lymphocyte subsets (CD4$^+$ T cells, CD8$^+$ T cells, and B220$^+$ B cells) in the spleen were GFP-positive, indicating that these cells had been replaced by donor-type cells in the [GFP-B6→MRL/lpr] chimeric mice (Fig. 1A). In addition, no abnormal CD3$^+$B220$^+$ lpr T cells were found. Thymocytes had also been replaced with donor-type cells (CD4$^+$, CD8$^+$, and CD4$^+$/CD8$^+$ cells). The levels of autoantibodies in the sera and proteinuria were also reduced in the chimeric mice to the levels comparable with those in the donor GFP-B6 mice (Fig. 1B). The chimeric mice looked healthy and showed no symptoms of graft-versus-host disease (GVHD) during the observation.

Next, we analyzed the tolerance induction using spleen cells from the chimeric mice. The splenic lymphocytes showed tolerance both to the donor (B6) and the recipient (MRL/lpr) but not to third-party (BALB/c) major histocompatible complex (MHC) determinants (Fig. 1C). These results strongly indicate that the allogeneic BMT was successfully performed in the [GFP-B6→MRL/lpr] mice by IBM–BMT, as previously reported [27,38].

3.2. Presence of donor-derived TECs in medulla and cortex of [GFP-B6→MRL/lpr] chimeric mice after IBM–BMT

We next investigated whether donor-derived TECs could be found in the MRL/lpr mice treated with IBM–BMT. Since the expression of GFP was not high enough to allow adequate detection by fluorescence microscopy, we used FITC-conjugated anti-GFP antibodies (Abs) to enhance the expression [39]. GFP expression in

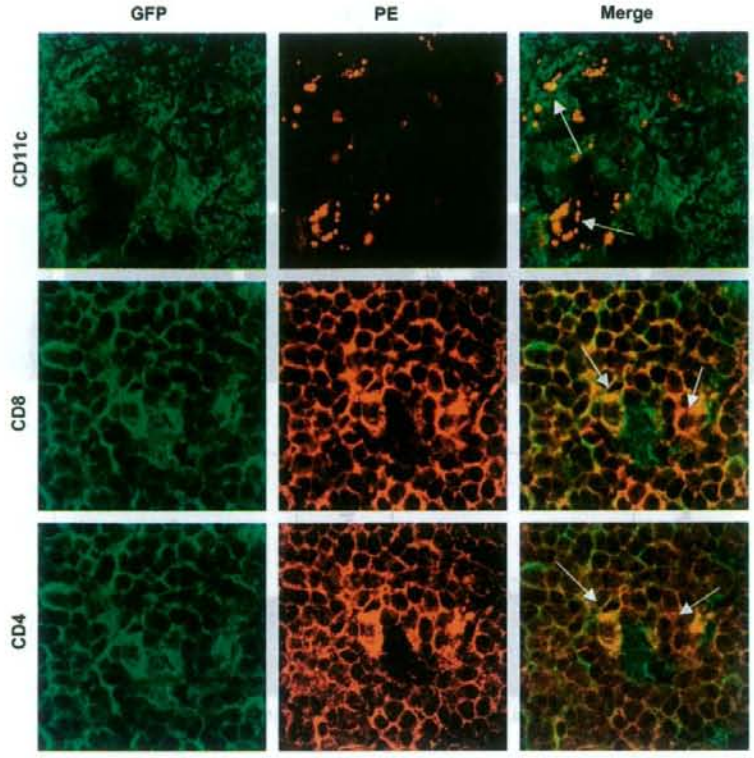

Fig. 5. GFP-expressing CD11c, CD4, or CD8 cells in the thymus from [GFP-B6→MRL/lpr] chimeric mice 8 weeks after IBM–BMT. The thymi from the [GFP-B6→MRL/lpr] chimeric mice after IBM–BMT were stained by anti-GFP-FITC and anti-CD11c-PE, anti-CD4-PE, or anti-CD8-PE Abs. ×200 in upper and ×400 in middle and lower panels. Arrows: GFP and CD11c (upper), CD4 (middle) or CD8 (lower) double-positive cells. Representative profiles are shown for four experiments.

the thymuses of B6 mice was not enhanced by the Ab staining, whereas the GFP expression in GFP-B6 mice was enhanced by the Ab staining (Fig. 2A). With or without the Ab staining, the thymuses from MRL/lpr mice showed no GFP expression (data not shown). The MTS-10 Ab reacts with mTECs, whereas cytokeratin 18 is expressed in cTECs [13,40]. We therefore confirmed, using these Abs, that the two types of TECs are also GFP-positive in GFP-B6 mice (Fig. 2B). Next, we examined the expression in the thymus of [GFP-B6→MRL/lpr] mice. Interestingly, some MTS-10$^+$ cells were also GFP$^+$ in the medulla 8 weeks after transplantation (Fig. 3A). Similarly, some of the cytokeratin 18$^+$ cells were also GFP$^+$ in the cortex (Fig. 3B).

3.3. Expression of FOXN1 and AIRE in donor-derived TECs from [GFP-B6→MRL/lpr] chimeric mice by IBM–BMT

We further examined the expression of functional molecules (FOXN1 and AIRE) in TECs of GFP-B6, MRL/lpr, and [GFP-B6→MRL/lpr] mice at that time (Fig. 4). The FOXN1$^+$ or AIRE$^+$ cells were detected in the stromal area in all the mice (Fig. 4A, B). In the [GFP-B6→MRL/lpr] chimeric mice, GFP$^+$ FOXN1- or AIRE-expressing cells were also observed (Fig. 5B). Interestingly, although there was no significant difference in the number of FOXN1-expressing cells between GFP-B6 and MRL/lpr mice, the number of AIRE-expressing cells in the MRL/lpr mice was significantly higher than in the GFP-B6 mice (Fig. 4A, C). In addition, the number of AIRE-expressing cells was significantly reduced in the chimeric mice to a level comparable to that of donor GFP-B6, although the number of the FOXN1 cells was not changed.

3.4. Analyses of donor-derived thymic DCs and thymocytes from [GFP-B6→MRL/lpr] chimeric mice by IBM–BMT

Finally, we examined the donor-derived thymic DCs and thymocytes in [GFP-B6→MRL/lpr] chimeric mice. Donor-derived GFP$^+$ CD11c$^+$ thymic DCs were observed (Fig. 5). In addition, donor-derived GFP$^+$ CD4$^+$ or CD8$^+$ thymocytes were also detected, as we expected. We also examined the host-derived cells. Only a few host-derived H-2K^{k+} CD11c$^+$ DCs, or CD4$^+$ or CD8$^+$ thymocytes were found (data not shown).

4. Discussion

In the present study, we examined the origins of TECs in chimeric mice [GFP-B6→MRL/lpr] using the IBM–BMT method. We confirmed that IBM–BMT can be used to treat autoimmune diseases in the MRL/lpr mice [27,28]. Interestingly, not only the hematopoietic cells, but also some of both the medullary and the cortical TECs were donor-derived. The donor-type TECs expressed their functional molecules, FOXN1 and AIRE. In addition, the number of AIRE-expressing cells was significantly reduced in the chimeric mice, to a level comparable to that of the donor GFP-B6 mice, which is correlated with the amelioration of autoimmune disease. These findings suggest that BMCs contain the precursors of TECs, and that donor-derived TECs are related for the induction of tolerance.

We first examined the donor-derived TECs in the [GFP-B6→MRL/lpr] mice. Interestingly, we found both donor-derived medullary and cortical types of TECs. Although TECs are relatively

radioresistant, some of them are injured by irradiation and are then regenerated [41], suggesting the presence of stem cells or the precursors of TECs in the BMCs of even adult mice; BMCs thus contain both HSCs and MSCs [42], and the donor-derived TECs seem to be likely derived from the latter, since we could not detect donor-derived TECs with unsuccessful BMT after IV-BMT (data not shown), in which donor-derived stromal cells cannot be seen in the recipients [27].

We further examined the expression of functional molecules in the TECs: FOXN1 and AIRE. These functional molecules were also expressed in GFP^+ cells, indicating that the functional TECs developed from the donor. Interestingly, the number of AIRE-expressing TECs in MRL/lpr mice was significantly reduced in the [B6→MRL/lpr] chimeric mice. In contrast, there was no change in the number of FOXN1-expressing TECs. As the dysregulation of AIRE is related to the induction of autoimmune disease [5], there should be a correlation between the downregulated expression of AIRE and the activity of autoimmune diseases. However, since Fas gene mutation is responsible for the development of autoimmune diseases in the MRL/lpr mice [33], the upregulated expression of AIRE may be a secondary compensatory phenomenon. However, the normalized expression of AIRE in the TECs may also be a significant finding in relation to the treatment of the diseases. The functional AIRE assay is necessary in order to determine the exact mechanism involved, and we are now in the process of carrying out such assay.

The TECs generally play important roles in the development of T cells. In this process, self-MHC is required for the cTECs to induce T cells that have the MHC-restricted immune response in positive selection, whereas the self-MHC is also needed in mDCs and mTECs to delete autoreactive T cells (negative selection) and to induce regulatory T cells [43]. Although we could not perform analyses of the detailed kinetics of these cells, some donor-derived TECs with some donor-derived thymocytes were found in the thymus early on (2 weeks after IBM–BMT). We also found donor-derived thymic DCs. Therefore, the existence of donor-derived TECs and thymic DCs could be necessary for successful allogeneic BMTs. It should also be noted that the immunological tolerance to the host is induced in allogeneic BMT. Although the mechanism has not been elucidated, the mixed status of donor- and host-type TECs and DCs in the thymus may play a crucial role in the induction of tolerance to both donor- and host-type MHC determinants [44].

Although it remains controversial whether the BMC-derived cells can trans-differentiate or this is the result of fusion [45,46], it should be noted that the normalized expression of AIRE by BMC-derived cells corrected the autoimmune diseases. Further study is required to elucidate not only the exact mechanism of the development of the BMC-derived TECs but also the role of the TECs in the development of autoimmune diseases.

In conclusion, we have found that i) adult BMCs (including MSCs) contain the precursors for both medullary and cortical TECs, ii) allogeneic IBM–BMT can engraft these cells, and iii) the presence of donor-derived TECs may be responsible for successful allogeneic BMT.

A recent study of TECs has mainly been focused on the fetal thymus [40]. However, if BMCs were utilized as the precursors for TECs, thymus regeneration therapy by IBM–BMT would become a useful strategy for the treatment of various intractable diseases. We are now examining the effects of IBM–BMT on rejuvenation of thymic function.

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References

[1] Jenkinson EJ, Jenkinson WE, Rossi SW, Anderson G. The thymus and T-cell commitment: the right niche for Notch? Nat Rev Immunol 2006;6:551–5.
[2] Anderson G, Jenkinson WE, Jones T, Parnell SM, Kinsella FA, White AJ, et al. Establishment and functioning of intrathymic microenvironments. Immunol Rev 2006;209:10–27.
[3] Lee D, Prowse DM, Brissette JL. Association between mouse nude gene expression and the initiation of epithelial terminal differentiation. Dev Biol 1999;208:362–74.
[4] Su DM, Navarre S, Oh WJ, Condie BG, Manley NR. A domain of Foxn1 required for crosstalk-dependent thymic epithelial cell differentiation. Nat Immunol 2003;4:1128–35.
[5] Mathis D, Benoist C. A decade of AIRE. Nat Rev Immunol 2007;7:645–50.
[6] Rizzi M, Ferrera F, Filaci G, Indiveri F. Disruption of immunological tolerance: role of *AIRE* gene in autoimmunity. Autoimmun Rev 2006;5:145–7.
[7] Boyd RL, Tucek CL, Godfrey DI, Izon DJ, Wilson TJ, Davidson NJ, et al. The thymic microenvironment. Immunol Today 1993;14:445–59.
[8] Ritter MA, Boyd RL. Development in the thymus: it takes two to tango. Immunol Today 1993;14:462–9.
[9] Nehls M, Kyewski B, Messerle M, Waldschutz R, Schuddekopf K, Smith AJ, et al. Two genetically separable steps in the differentiation of thymic epithelium. Science 1996;272:886–9.
[10] Gordon J, Wilson VA, Blair NF, Sheridan J, Farley A, Wilson L, et al. Functional evidence for a single endodermal origin for the thymic epithelium. Nat Immunol 2004;5:546–53.
[11] Rodewald HR, Paul S, Haller C, Bluethmann H, Blum C. Thymus medulla consisting of epithelial islets each derived from a single progenitor. Nature 2001;13(414):763–8.
[12] Gill J, Malin M, Hollander GA, Boyd R. Generation of a complete thymic microenvironment by MTS24(+) thymic epithelial cells. Nat Immunol 2002;3:635–42.
[13] Rossi SW, Jenkinson WE, Anderson G, Jenkinson EJ. Clonal analysis reveals a common progenitor for thymic cortical and medullary epithelium. Nature 2006;22(441):988–91.
[14] Thomas ED. Landmarks in the development of hematopoietic cell transplantation. World J Surg 2000;24:815–8.
[15] Bacigalupo A, Oneto R, Bruno B, Socié G, Passweg J, Locasciulli A, et al. Current results of bone marrow transplantation in patients with acquired severe aplastic anemia. Report of the European Group for Blood and Marrow Transplantation. On behalf of the Working Party on Severe Aplastic Anemia of the European Group for Blood and Marrow Transplantation. Acta Haematol 2000;103:19–25.
[16] Chen RL, Hou JW, Chang PY, Tsai FJ, Wang PJ. Matched unrelated bone marrow transplantation without splenectomy for a child with Gaucher disease caused by homozygosity of the L444P mutation, who also suffered from schizencephaly. J Pediatr Hematol Oncol 2007;29:57–9.
[17] Sato T, Kobayashi R, Toita N, Kaneda M, Hatano N, Iguchi A, et al. Stem cell transplantation in primary immunodeficiency disease patients. J Pediatr Hematol Oncol 2007;29:57–9.
[18] Deane S, Meyers FJ, Gershwin ME. On reversing the persistence of memory: hematopoietic stem cell transplant for autoimmune disease in the first ten years. J Autoimmun 2008;30:180–96.
[19] Ueno NT, Rizzo JD, Demirer T, Cheng YC, Hegenbart U, Zhang MJ, et al. Allogeneic hematopoietic cell transplantation for metastatic breast cancer. Bone Marrow Transplant 2008;41:537–45.
[20] Reisner Y, Martelli MF. From 'megadose' haploidentical hematopoietic stem cell transplants in acute leukemia to tolerance induction in organ transplantation. Blood Cells Mol Dis 2008;40:1–7.
[21] Or R, Hadar E, Bitan M, Resnick IB, Aker M, Ackerstein A, et al. Safety and efficacy of donor lymphocyte infusions following mismatched stem cell transplantation. Biol Blood Marrow Transplant 2006;12:1295–301.

[22] Ishida T, Inaba M, Hisha H, Sugiura K, Adachi Y, Nagata N, et al. Requirement of donor-derived stromal cells in the bone marrow for successful allogeneic bone marrow transplantation. Complete prevention of recurrence of autoimmune diseases in MRL/MP-lpr/lpr mice by transplantation of bone marrow plus bones (stromal cells) from the same donor. J Immunol 1994;152:3119–27.
[23] Hosaka N, Nose M, Kyogoku M, Nagata N, Miyashima S, Good RA, et al. Thymus transplantation, a critical factor for correction of autoimmune disease in aging MRL/+ mice. Proc Natl Acad Sci U S A 1996;93:8558–62.
[24] Hosaka N, Ryu T, Miyake T, Cui W, Nishida T, Takaki T, et al. Treatment of autoimmune diseases in MRL/lpr mice by allogeneic bone marrow transplantation plus adult thymus transplantation. Clin Exp Immunol 2007;147:555–63.
[25] Ryu T, Hosaka N, Miyake T, Cui W, Nishida T, Takaki T, et al. Transplantation of newborn thymus plus hematopoietic stem cells can rescue supralethal irradiated mice. Bone Marrow Transplant 2008;41:659–66.
[26] Kushida T, Inaba M, Hisha H, Ichioka N, Esumi T, Ogawa R, et al. Crucial role of donor-derived stromal cells in successful treatment for intractable autoimmune diseases in MRL/lpr mice by BMT via portal vein. Stem Cells 2001;19:226–35.
[27] Kushida T, Inaba M, Hisha H, Ichioka N, Esumi T, Ogawa R, et al. Intra-bone marrow injection of allogeneic bone marrow cells: a powerful new strategy for treatment of intractable autoimmune diseases in MRL/lpr mice. Blood 2001;97:3292–9.
[28] Ikehara S. A novel method of bone marrow transplantation (BMT) for intractable autoimmune diseases. J Autoimmun 2008;30:108–15.
[29] Borue X, Lee S, Grove J, Herzog EL, Harris R, Diflo T, et al. Bone marrow-derived cells contribute to epithelial engraftment during wound healing. Am J Pathol 2004;165:1767–72.
[30] Grove JE, Bruscia E, Krause DS. Plasticity of bone marrow-derived stem cells. Stem Cells 2004;22:487–500.
[31] Krause DS, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R, et al. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 2001;105:369–77.
[32] Lagasse E, Connors H, Al-Dhalimy M, Reitsma M, Dohse M, Osborne L, et al. Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nat Med 2000;6:1229–34.
[33] Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature 1992;356:314–7.
[34] Liston A, Gray DH, Lesage S, Fletcher AL, Wilson J, Webster KE, et al. Gene dosage-limiting role of AIRE in thymic expression, clonal deletion, and organ-specific autoimmunity. J Exp Med 2004;200:1015–26.
[35] Itoi M, Tsukamoto N, Amagai T. Expression of Dll4 and CCL25 in Foxn1−−negative epithelial cells in the post-natal thymus. Int Immunol 2007;19:127–32.
[36] Hosaka N, Kitajiri S, Hiraumi H, Nogaki H, Toki J, Yang G, et al. Ectopic pituitary adenoma with malignant transformation. Am J Surg Pathol 2002;26:1078–82.
[37] Miyashima S, Nagata N, Nakagawa T, Hosaka N, Takeuchi K, Ikehara S. Prevention of lpr-graft-versus-host disease and transfer of autoimmune disease in normal C57BL/6 mice by transplantation of bone marrow cells plus bones (stroma cells) from MRL/lpr mice. J Immunol 1996;156:79–84.
[38] Ikehara S. Autoimmune diseases as stem cell disorders: normal stem cell transplant for their treatment. Int J Mol Med 1998;1:5–16.
[39] Liston A, Farr AG, Chen Z, Benoist C, Mathis D, Manley NR, et al. Lack of Foxp3 function and expression in the thymic epithelium. J Exp Med 2007;204:475–80.
[40] Blackburn CC, Manley NR, Palmer DB, Boyd RL, Anderson G, Ritter MA. One for all and all for one: thymic epithelial stem cells and regeneration. Trends Immunol 2002;23:391–5.
[41] Irifune T, Tamechika M, Adachi Y, Tokuda N, Sawada T, Fukumoto T. Morphological and immunohistochemical changes to thymic epithelial cells in the irradiated and recovering rat thymus. Arch Histol Cytol 2004;67:149–58.
[42] Koide Y, Morikawa S, Mabuchi Y, Muguruma Y, Hiratsu E, Hasegawa K, et al. Two distinct stem cell lineages in murine bone marrow. Stem Cells 2007;25:1213–21.
[43] Watanabe N, Wang YH, Lee HK, Ito T, Wang YH, Cao W, et al. Hassall's corpuscles instruct dendritic cells to induce $CD4^{+}CD25^{+}$ regulatory T cells in human thymus. Nature 2005;436:1181–5.
[44] Zepp F, Cussler K, Mannhardt W, Schofer O, Schulte-Wissermann H. Intrathymic tolerance induction: determination of tolerance to class II major histocompatibility complex antigens in maturing T lymphocytes by a bone marrow-derived non-lymphoid thymus cell. Scand J Immunol 1987;26:589–601.
[45] Wang X, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al-Dhalimy M, et al. Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature 2003;422:897–901.
[46] Harris RG, Herzog EL, Bruscia EM, Grove JE, Van Arnam JS, Krause DS. Lack of a fusion requirement for development of bone marrow-derived epithelia. Science 2004;305:90–3.

Long-Term Donor-Specific Tolerance in Rat Cardiac Allografts by Intrabone Marrow Injection of Donor Bone Marrow Cells

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Background. Donor-specific central tolerance in cardiac allograft can be induced by hematopoietic chimerism via conventional intravenous bone marrow transplantation (IV-BMT). However, there are problems with IV-BMT, such as the risk of graft failure and of the toxicity from conditioning regimens.

Methods. A new method for heart transplantation is presented. This method consists of administration of fludarabine phosphate (50 mg/kg) and fractionated low-dose irradiation (3.5 Gy×2 or 4.0 Gy×2), followed by intrabone marrow injection of whole bone marrow cells (IBM-BMT) plus heterotopic heart transplantation.

Results. Cardiac allografts with IBM-BMT were accepted and survived long-term (>10 months) showing neither acute rejection nor chronic rejection including cardiac allograft vasculopathy by such conditioning regimens. In contrast, cardiac allografts with conventional IV-BMT were rejected within 1 month after the treatment with irradiation of 3.5 Gy×2 or within 3 months after the treatment with irradiation of 4.0 Gy×2. Macrochimerism (>70%) was favorably established and stably maintained by IBM-BMT but not IV-BMT. Low levels of transient mixed chimerism (<7%) were induced by IV-BMT with fludarabine plus 4.0 Gy×2, but the chimerism was lost within 1 month after the treatment.

Conclusions. These findings indicate that IBM-BMT is a feasible strategy for the induction of persistent donor-specific tolerance, enables the use of reduced radiation doses as conditioning regimens, and obviates the need for immunosuppressants.

Keywords: Tolerance induction, Heart transplantation, Intrabone marrow injection, Bone marrow transplantation.

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Despite recent advances in immunosuppressive agents, chronic rejection and side effects associated with the lifelong usage of nonspecific immunosuppressants remain a barrier to successful clinical solid organ transplantation (*1*). Mixed hematopoietic chimerism has proven its efficacy in the induction of persistent tolerance in rodents, large animals (including nonhuman primates) and recently a few renal patients by conventional intravenous (IV) bone marrow transplantation (BMT) (*2–4*). However, obstacles that hinder the clinical application of BMT as a feasible strategy for inducing tolerance in a clinical setting include the toxicity of conditioning regimens, the risk of graft failure, and the problem of graft-versus-host disease (GvHD) (*5*).

Recent studies in animal models have therefore aimed at minimizing conditioning and optimizing selective immunosuppression. Included in these approaches is the use of nonmyeloablative conditioning regimens, T-cell depletion, donor lymphocyte infusion, and blockade of stimulatory and costimulatory pathways (*6–9*). We have recently found that the injection of donor bone marrow cells (BMCs) directly into the bone marrow cavity (intrabone marrow BMT [IBM-BMT]) induces persistent donor-specific tolerance in mice even if the radiation doses are reduced to sublethal levels (*10*). IBM-BMT also enhances the rapid recovery or reconstitution of the hematolymphoid system (including bone marrow stromal cells) of donor origin, resulting in the complete amelioration of autoimmune diseases in MRL/lpr mice, in which conventional IV-BMT had been unsuccessful (*10*). It is of interest that the recipients treated with IBM-BMT have no clinical or histopathological signs of GvHD or graft failure (*10, 11*). In addition, we have more recently extended this new approach to the induction of tolerance in the transplantation of the leg (*12*), lung (*13*), and pancreatic islets (*14*) in rats. Therefore, the new BMT method (IBM-BMT) seems to have significant advantages in achieving successful allogeneic organ transplantation.

It has been reported that fludarabine eliminates normal and malignant mononuclear cells in animals and humans through the inhibition of DNA synthesis. It has also been shown to have cytotoxic effects on lymphoid cells (*15*), particularly T cells (*16*). Furthermore, the prior injection of flu-

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darabine has proven to facilitate the establishment of high levels of hematopoietic chimerism with low doses of irradiation, as shown in our previous leg (*12*) and pancreatic islet transplantation (*14*). In view of both its selective lympholytic activity (especially to eliminate donor-reactive T cells in the recipients) and relatively mild side-effect profile (*17*), we have used this immunosuppressive agent as a part of our nonmyeloablative conditioning regimens.

In the present study, we report that the combination of the injection of fludarabine, low-dose fractionated irradiation, and IBM-BMT provides a feasible clinical strategy for inducing permanent tolerance without using any immunosuppressants.

MATERIALS AND METHODS

Animals

Brown Norway (BN: $RT1^n$), Fischer 344 (F344: $RT1^l$), and PVG (PVG: $RT1^c$) rats were purchased from SLC (Shizuoka, Japan) and maintained until use in our animal facilities under specific pathogen-free conditions. BN rats at the age of 8–10 weeks were used as recipients, and F344 rats at the age of 8–10 weeks were used as donors for the transplantations of the heart, skin, and BMCs. PVG rats were used as third party stimulators in mixed leukocyte reactions (MLRs). All animal research was reviewed and approved by the Animal Experimentation Committee, Kansai Medical University.

Heterotopic Heart Transplantation

Heterotopic heart transplantation was performed, as described by Tomita Y (*18*). Briefly, after induction of anesthesia with intraperitoneal injection of sodium pentobarbital, vascularized heart transplantation was performed heterotopically into the right cervical portion of recipients using a microsurgical cuff technique. Donor hearts were procured and stored in a cold saline bath. The donor brachiocephalic artery and main pulmonary artery were anastomosed with the recipient right common carotid artery and right external jugular vein, respectively. Allograft survival was assessed by daily palpation. Graft rejection was defined as complete cessation of spontaneous ventricular contraction.

Experimental Protocol for BMT

BN rats were injected intravenously with 50 mg/kg of fludarabine phosphate, followed by fractionated whole body irradiation (3.5 Gy×2 to 5.0 Gy×2) by ^{137}Cs (Gammacell 40 Exactor; Nordion International Inc, Ontario, Canada) 1 day before the bone marrow and heart transplantation (day −1). As described previously (*11*), BMCs (3×10^7 or 10×10^7 cells/60 μL) obtained from the femurs and tibias of donor F344 rats were injected intravenously (IV-BMT) or directly into the bone marrow cavity (IBM-BMT) of the left tibia of the recipient BN rats on day 0, and the cardiac allografts from F344 rats were implanted simultaneously. In terms of the IBM-BMT technique, the knee was flexed to 90 degrees and the proximal side of the tibia was drawn to the anterior. A 26-gauge needle was inserted into the joint surface of the tibia through the patellar tendon and then inserted into the bone cavity.

Analyses for Cell Surface Antigens

Spleen cells, peripheral blood mononuclear cells (PBMNCs), and BMCs from the recipient rats were stained with fluorescein isothiocyanate (FITC)-conjugated rat anti$RT1^l$ monoclonal antibody (mAb; PharMingen, San Diego, CA) to identify the donor-derived cells, and purified mouse anti$RT1^n$ mAb (Serotec Ltd., Oxford, United Kingdom) followed by phycoerythrin (PE)-conjugated goat antimouse immunoglobulin G (Serotec Ltd.) to identify the recipient-derived cells. Donor-derived cells bearing a lineage-specific phenotype were also analyzed by FITC-anti$RT1^l$ mAb plus PE-conjugated mAb against CD45R (B220) (PharMingen), CD4, CD8, or CD11b (Caltag Laboratories, Burlingame, CA). The stained cells were analyzed by a FACScan (Becton Dickinson & Co., Mountain View, CA).

Mixed Leukocyte Reactions

MLRs were performed as described previously (*13*). Briefly, the responder spleen cells (2×10^5) were cultured with 2×10^5 irradiated (12 Gy) stimulator spleen cells from F344, BN, and PVG rats for 72 hr and pulsed with 0.5 μCi of [^{3}H]-thymidine for the last 16 hr of the culturing period.

Skin Transplantation

Full-thickness F344 donor or PVG third-party skin grafts of 2×3 cm were prepared and transplanted with 6–0 Prolene on the dorsolateral thorax of BN recipient rats. Grafts were secured with sutures and covered with sterile gauze and an elastic protective tape. The first inspection was carried out on the seventh day, followed by daily inspections for 3 weeks. The graft rejection was defined as the complete loss of viable epidermal graft tissue when more than 50% of the graft became raised, necrotic, or covered by eschar.

Histological Studies

Fresh heart necropsy tissue was fixed in 10% formalin for 48 hr and embedded in paraffin according to standard procedures. Three-micrometer-thick sections were stained with hematoxylin and eosin (H-E), Elastica-van Gieson's (EvG), and Masson's trichrome (MT). Antiα-smooth muscle actin (α-SMA) mAb (DAKO A/S, Denmark) was used for immunohistochemical staining. Furthermore, the frozen specimens were also prepared for fluorescent staining using FITC-conjugated rat anti$RT1^l$ mAb (PharMingen) and purified OX-62 mAb, reactive to rat dendritic cells (DCs) and integrin α_E chain (PharMingen), followed by PE-conjugated goat antimouse immunoglobulin G (Serotec Ltd.) to identify the donor-derived DCs in the recipient thymus by a confocal laser scanning microscope (LSM-GB200; Olympus, Tokyo, Japan). To determine GvHD, the liver, intestine, and skin were also histologically examined after H-E staining.

Statistical Analysis

Statistical analysis of the survival rate of the cardiac allografts was performed using a log-rank test. A P value of <0.05 was considered statistically significant using Student's t test.

RESULTS

Establishment of Hematopoietic Chimerism after IBM-BMT

We first determined not only the number of donor BMCs (for injection) but also the conditioning regimens for

BMT. Recipient rats were given fractionated irradiation (≥4.5 Gy×2) with prior injection of fludarabine, and BMCs (1×10^8) from donor F344 rats were injected intravenously (IV-BMT). In these conditions (1×10^8 BMCs and ≥4.5 Gy×2), macrochimerism (>90%) was established in the recipient rats regardless of IBM-BMT or IV-BMT (Table 1). Therefore, we next carried out BMT with a lower dose of BMCs (3×10^7) and a lower irradiation dose (3.5 Gy×2) to make differences in chimersim clear. PBMNCs were collected from the recipient rats every 2 weeks from the second week until the 24th week after the treatment and stained with donor-specific antirat $RT1^l$ mAb and recipient-specific antirat $RT1^n$ mAb to examine the chimerism.

As shown in Figure 1A, all the recipient rats with fludarabine plus irradiation of either 3.5 Gy×2 or 4.0 Gy×2 showed macrochimerism (>70%) after IBM-BMT. It is noted that approximately 80% of PBMNCs were of donor origin even 180 days after IBM-BMT at the lower irradiation dose (3.5 Gy×2), although the PBMNCs of the recipients treated with fludarabine plus 3.5 Gy×2, followed by IV-BMT, were of host origin (Fig. 1A). This macrochimerism achieved by IBM-BMT was maintained stably even 10 months after transplantation (data not shown). Hematolymphoid cells bearing mature lineage markers were also shown to be of donor origin when the cells in the spleen, PBMNCs, and BM were stained with antibodies against CD45R, CD4, CD8, and CD11b plus donor $RT1^l$ (Table 1 and Supplemental Table 1, available for viewing online only). It is noted that the reconstitution of donor multilineage hematolymphoid cells was similarly observed in the bone marrow of injected and noninjected sites (Supplemental Table 2, available for viewing online only). In contrast, IV-BMT after fludarabine plus 3.5 Gy×2 failed to reconstitute the recipients with donor-derived hematolymphoid cells (Table 1). Only a low level of transient donor chimerism, which lasted only about 1 month, was achieved when the rats were conditioned with fludarabine plus 4.0 Gy×2. Thus, the donor macrochimerism was stably maintained by IBM-BMT, and the low levels of chimerism seen at 2 weeks after IV-BMT (fludarabine plus 4.0 Gy×2) were almost undetectable at 4 weeks. These findings clearly indicate that IBM-BMT facilitated the rapid recovery and reconstitution of donor hematolymphoid cells even with less myelotoxic conditioning regimens (fludarabine plus 3.5 Gy×2) in comparison with conventional IV-BMT.

Survival of Cardiac Allografts

After IBM-BMT, cardiac allografts survived for more than 10 months without any signs of rejection or GvHD in the recipients conditioned with either fludarabine plus 3.5 Gy×2 or fludarabine plus 4.0 Gy×2 (Fig. 1B). In contrast, the cardiac allografts with IV-BMT were rejected within 1 month after BMT in the recipients conditioned with fludarabine plus 3.5 Gy×2 (mean survival time: 22.8±5.4

TABLE 1. Analyses of cell surface antigens on donor-derived cells in the spleen of the recipient rats

Rat	N	BMT	Radiation (Gy)	BMCs ($\times10^7$)	Cell surface antigen (%) Donor-derived CD4[a]	Donor-derived CD8	Donor-derived CD45R	Donor-derived CD11b	Donor-derived cells in chimeric rats (%)
[F344→BN]	5	IV	3.5Gy×2	3	0	0	0	0	0
					9.65±0.60	10.34±0.60	68.34±0.67	10.49±1.23	
[F344→BN]	5	IBM	3.5Gy×2	3	6.45±0.56	15.38±1.21	44.87±1.34	11.54±1.85	73.26±8.54
					10.34±0.25	21.29±0.35	62.78±0.64	16.32±0.76	
[F344→BN]	10	IV	4.0Gy×2	3	0	0.02±0.01	0.07±0.02	0	0.12±0.04
					7.67±0.58	16.66±1.25	62.04±015	8.24±0.47	
[F344→BN]	10	IBM	4.0Gy×2	3	12.21±1.42	12.46±2.43	38.25±1.81	6.88±0.88	81.36±8.86
					14.38±2.05	16.04±0.37	54.45±0.62	11.19±0.54	
[F344→BN]	10	IV	4.5Gy×2	10	10.02±1.25	17.68±2.54	56.56±2.47	9.47±1.57	94.26±7.83
					11.32±0.57	19.54±1.25	66.14±0.61	9.83±1.56	
[F344→BN]	10	IBM	4.5Gy×2	10	11.35±2.01	9.98±1.48	67.81±1.56	12.76±2.04	98.34±2.16
					12.54±0.41	10.82±0.63	68.75±0.83	14.87±0.29	
[F344→BN]	10	IV	5.0Gy×2	10	15.22±2.05	19.84±1.34	52.14±2.06	10.01±1.07	96.27±1.80
					16.11±2.57	22.14±1.72	56.13±1.05	10.04±0.21	
[F344→BN]	10	IBM	5.0Gy×2	10	23.65±1.02	11.22±1.84	52.23±2.63	8.39±0.96	99.26±0.45
					24.21±1.12	11.04±0.56	53.74±0.32	8.73±0.57	
F344	5	–	–	–	–	–	–	–	–
					21.29±0.58	26.13±0.54	59.17±0.71	9.13±0.32	
BN	5	–	–	–	–	–	–	–	–
					26.13±0.60	17.63±0.41	49.29±0.16	10.81±0.27	

The cells from the spleen were prepared from the recipient rats 12 weeks after IBM-BMT or IV-BMT, and stained with donor-specific antirat $RT1^l$ mAb and mAb against mature lineage markers (CD4, CD8, CD45R, and CD11b) to examine the reconstitution of the hematolymphoid system. The cells were analyzed using a FACScan. The results are expressed as the mean±SD of more than five rats.

[a]Donor- and recipient-derived cells.

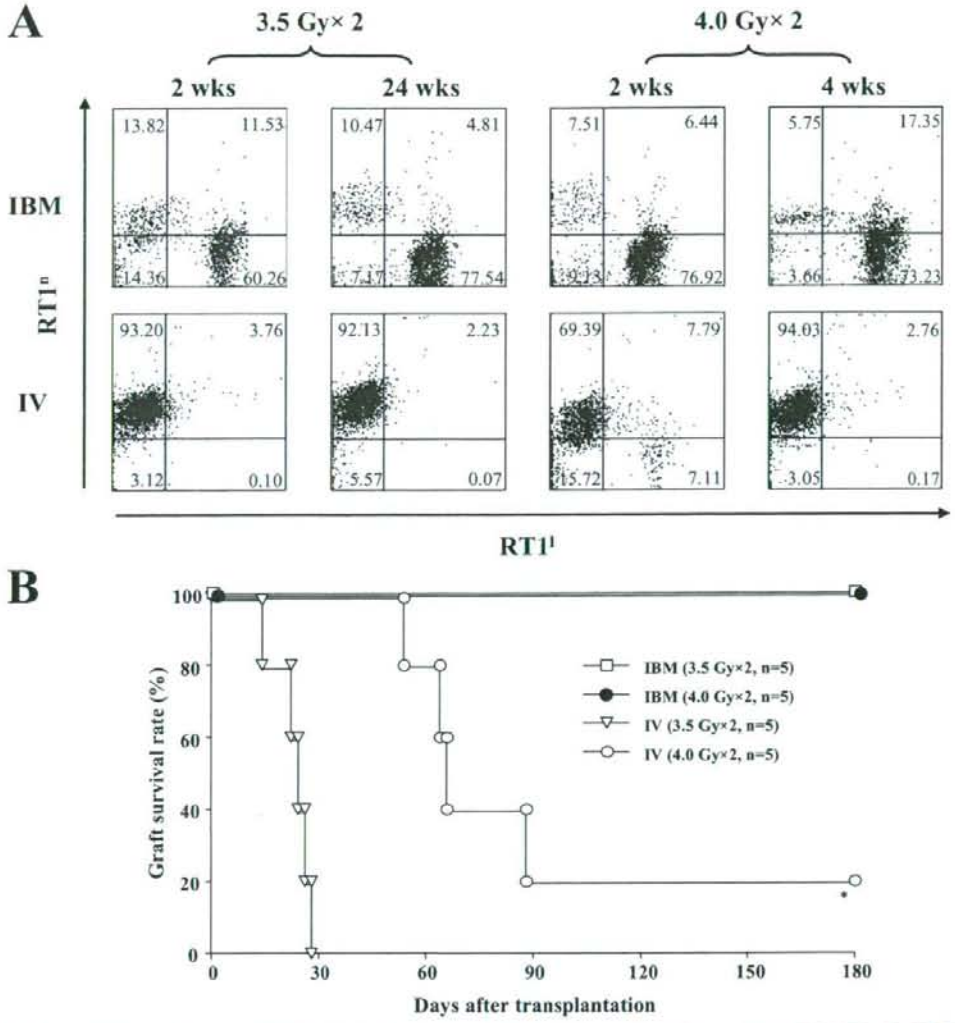

FIGURE 1. Analyses for donor-derived cells in the PBMNCs of recipients after IBM-BMT or IV-BMT. (A) PBMNCs were obtained from the recipients every 2 weeks from the 2nd to 24th week after IBM-BMT or IV-BMT plus heart transplantation, then stained with donor-specific antirat $RT1^l$ mAb (X-axis) and recipient-specific antirat $RT1^n$ mAb (Y-axis) to examine the donor cell engraftment. The stained cells were analyzed using a FACScan. FACS profiles represent representative data of five rats. Quadrants in the figures were set by the staining profile of the cells treated with isotype-matched Ig controls. Note that macrochimerism (>70%) was maintained stably after 180 days by IBM-BMT, but the transient chimerism induced by IV-BMT (fludarabine plus 4.0 Gy×2) was almost undetectable at 4 weeks. (B) Survival of cardiac allografts after IBM-BMT or IV-BMT. BN rats were injected intravenously with 50 mg/kg of fludarabine phosphate, followed by fractionated irradiation (3.5 Gy×2 or 4.0 Gy×2) 1 day before the bone marrow and heart transplantation (day −1). BMCs (3×10^7 or 10×10^7 cells/60 μL) from donor F344 rats were injected intravenously (IV-BMT) or directly into the bone marrow cavity (IBM-BMT) of the left tibia of the recipient BN rat on day 0. Vascularized heart transplantation was performed heterotopically into the right cervical portion of recipients using a microsurgical cuff technique. Allograft survival was assessed by daily palpation. Graft rejection was defined as complete cessation of spontaneous ventricular contraction. *The cardiac allograft of 1 of five rats treated with IV-BMT (4.0 Gy×2) was removed for immunohistochemical examination on day 180.

days, n=5) or within 3 months after BMT in those with fludarabine plus as much as 4.0 Gy×2 (mean survival time: 68±14.3 days, n=4) except in one instance.

Histology of Cardiac Allografts

All cardiac allografts were sectioned and stained with H-E, EvG, MT, and α-SMA mAb at the time of rejection or at

180 days after the transplantation. As already described, the cardiac allografts after IV-BMT were rejected within 1 month in the recipients conditioned with fludarabine plus 3.5 Gy×2. This was clearly confirmed by the histological findings of severe lymphocyte infiltration and extensive myocyte damage (Fig. 2D), showing evidence of acute rejection. Furthermore, despite the transient mixed chimerism induced by IV-BMT (4.0 Gy×2), severely thickened intima (cardiac allograft vasculopathy: CAV) was observed in the intramyocardial and epicardial arteries of the rejected allografts, and this was further confirmed by α-SMA staining and EvG staining (Fig. 2E). In contrast, in the allografts of the recipients conditioned with fludarabine plus 3.5 Gy×2 or 4.0 Gy×2 and treated with IBM-BMT, the arterial intima was maintained almost intact without any appearance of CAV (Fig. 2B, C).

MT staining was also performed to assess the cardiac remodeling of the allografts with respect to interstitial fibrosis during the chronic phase. While the allografts with IV-BMT (3.5 Gy×2, or 4.0 Gy×2) showed moderate to severe fibrosis in both the epicardium and intramyocardium (Fig. 2D, E and Fig. 3C, D), the prevalence of interstitial fibrosis in the allografts with IBM-BMT (3.5 Gy×2, or 4.0 Gy×2) was significantly lower than that in the allografts with IV-BMT (Fig. 2B, C and Fig. 3A, B). In addition, the coronary vessels in the allografts with IBM-BMT developed minimal myointimal thickening, compared with moderate to severe thickening with IV-BMT. Furthermore, in-

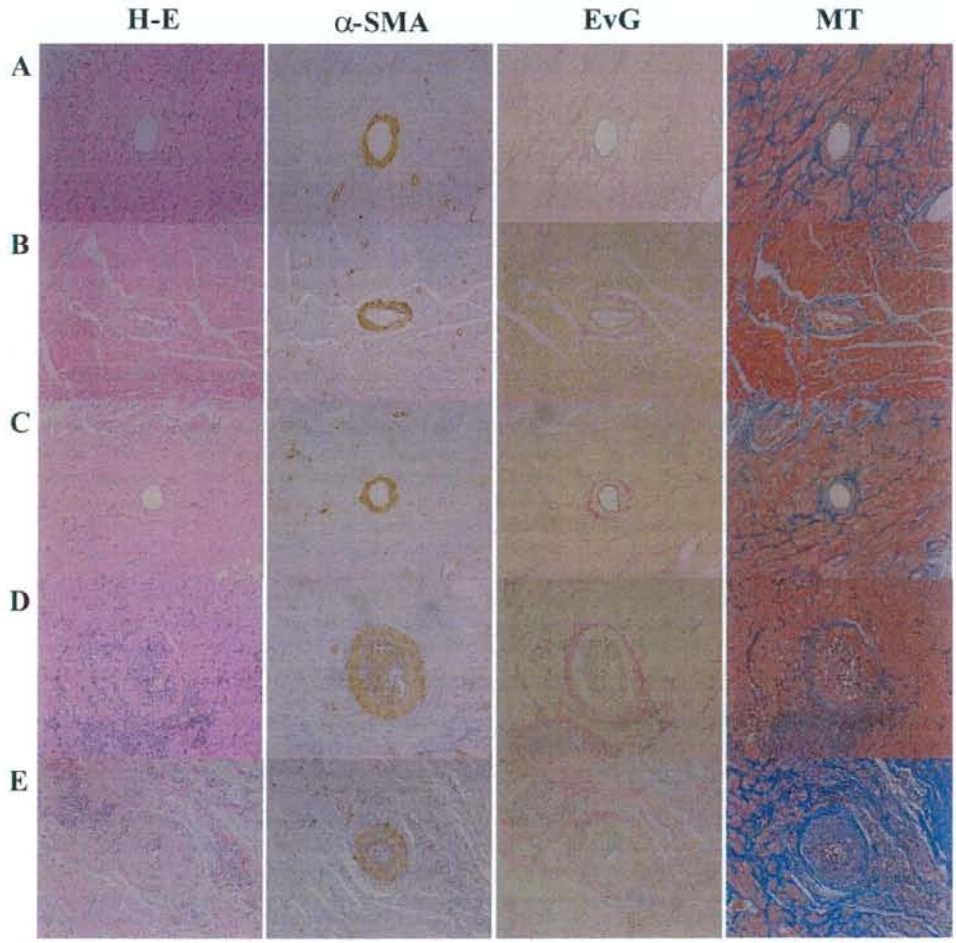

FIGURE 2. Histological findings in cardiac allografts after IBM-BMT or IV-BMT. The cardiac allografts were histologically examined at the time of rejection or 180 days after the treatment. H-E, α-SMA, EvG, or MT staining (×100) was performed to determine the rejection or the severity of CAV. (A) Intact isograft after syngeneic heart transplantation. (B) Allograft with minimal intimal thickening and sparse interstitial fibrosis at 180 days after IBM-BMT (fludarabine plus 3.5 Gy×2). (C) Allograft with well-preserved intact intima and mild interstitial fibrosis at 180 days after IBM-BMT (fludarabine plus 4.0 Gy×2). (D) Rejected allograft with severe lymphocytic infiltration and severe interstitial fibrosis at 28 days after IV-BMT (fludarabine plus 3.5 Gy×2). (E) Allograft with severe CAV and remarkable proliferation of elastic and collagenic fibers at 86 days after IV-BMT (fludarabine plus 4.0 Gy×2).