IBM-DLI, IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI) were stained with FITC-anti-CD45 and PE-anti-CD106 mAbs and analysed by a fluorescence activated cell sorter (FACScan) (BD Pharmingen).

Mixed leucocyte reaction

Various numbers of freshly prepared (defined as $CD45^-/CD106^+$ BM cells) or cultured BMSCs from the recipients of IBM-BMT + IBM-DLI, IBM-BMT + i.v.V-DLI or IBM-BMT alone (without DLI) were added to the culture of one-way MLR (4-day culture) where 2×10^5 responder $CD4^+$ T cells from BALB/c mice were stimulated with 12 Gy irradiated stimulator spleen cells (2×10^5 cells) from B6 mice in a 96-well flat-bottomed plate in a total volume of 0·2 ml. $CD45^+/CD106^-$ haemopoietic cells or whole BMCs served as controls for BMSCs added to the culture. The cultures were pulsed with 0·5 μCi of [^{3}H]-TdR for the last 16 h of the culture period.

Activation of T cells with concanavalin A

Splenic T cells (2×10^6 cells) from BALB/c mice were cultured with 2·5 μg/ml of concanavalin A (ConA) for 4 days. Activated T cells, thus prepared, were used as a positive control in real-time RT–PCR assay and enzyme-linked immunosorbent assay (ELISA) to detect cytokines.

Flow cytometric analyses of intracellular cytokines

CD4-enriched T cells from BALB/c mice were cultured with irradiated stimulator spleen cells from B6 mice with cultured BMSCs from the recipients of IBM-BMT + IBM-DLI, IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI) in round-bottomed plates (RPMI-1640) with 10% FCS. Cells were harvested 6 days later and stained with biotin-conjugated anti-H-$2K^d$ (visualized by streptavidin–peridinin chlorophyll-Cy5·5) and FITC-anti-CD4 mAb (BD Pharmingen) to detect responder (donor) CD4 T cells. The cells were next fixed and permeabilized with Cutofix/Cytoperm solution™ (BD Pharmingen). Intracellular cytokines were detected after the staining of cells with PE-anti-interleukin (IL)-2, -interferon (IFN)-γ or -IL-4 using an Intracellular Cytokine Staining Kit® (BD Pharmingen). Cells stained with isotype control cocktail (BD Pharmingen) served as a control. The stained cells were analysed by a FACScan® (Becton Dickinson Co., Mountain View, CA, USA).

Real-time RT–PCR assay

Cytokine messages of BMSCs were determined by real-time RT–PCR. We prepared some primers for transforming growth factor (TGF)-β (forward: TTTCGATTCAGCGCT CACTGCTCTTGTGAC, reverse: ATGTTGGACAACTGCT CCACCTTGGGCTTGC), hepatocyte growth factor (HGF) (forward: AAGAGTGGCATCAAGTGCCAG, reverse: CTG GATTGCTTGTGAAACACC), IL-2 (forward: TGGAGCA GCTGTTGATGGAC, reverse: CAATTCTGTGGCCTGCTT GG), IL-4 (forward: ACAGGAGAAGGGACGCCAT, reverse: GAAGCCCTACAGACGAGCTCA), IL-10 (forward: GGTT GCCAAGCCTTATCGGA, reverse: ACCTGCTCCACTGC CTTGCT) and IL-15 (forward: CATCCATCTCGTGCTAC TTGTGTT, reverse: CATCTATCCAGTTGGCCTCTGTTT) (Nisshinbo, Chiba, Japan).

Real-time RT–PCR was conducted on a DNA engine Opticon2 System (MJ Japan Ltd, Tokyo, Japan) by using SYBR Green I as a double-stranded DNA-specific binding dye and continuous fluorescence monitoring. The cycling conditions consisted of a denaturation step for 10 min at 95°C, 40 cycles of denaturation (94°C for 15 s), annealing (60°C for 30 s) and extension (72°C for 30 s). After amplification, melting curve analysis was performed with denaturation at 95°C, then continuous fluorescence measurement from 65°C to 95°C at 0·1°C/s. All reactions were run at least in duplicate, and included control wells without cDNA.

Detection of cytokines in MSC culture supernatant

Mesenchymal stem cell culture supernatants were collected 2 weeks later, and the amounts of IL-2, IL-4, IFN-γ and TGF-β were determined by ELISA kits.

Statistical analyses

Non-parametric analyses (Mann–Whitney *U*-test and log-rank test) were performed using StatView software (Abacus Concepts, Berkley, CA, USA). Values of $P < 0.05$ were considered statistically significant.

Results

In vitro immunosuppressive effects of BMSCs on T cell proliferation

Three days after DLI, BMCs were collected from the recipients, and non-haemopoietic BMCs (defined as $CD45^-/CD106^+$ cells) were isolated immediately as shown in Fig. 1a. The average number of these sorted cells per mouse were as follows. $CD45^-/CD106^+$ cells from the recipients of IBM-BMT + IBM-DLI: 31 033 ± 2450 cells (four mice), $CD45^-/CD106^+$ cells from the recipients of IBM-BMT + i.v.-DLI: 29 850 ± 2728 cells (four mice), $CD45^-/CD106^+$ cells from the recipients of IBM-BMT alone (without DLI): 36 630 ± 5244 cells (four mice). There were no statistical differences among these groups regarding the yields of $CD45^-/CD106^+$ cells. The sorted $CD45^-/CD106^+$ cells from these recipients were added to the culture of one-way MLR. As shown in Fig. 2, all the $CD45^-/CD106^+$ cells isolated from

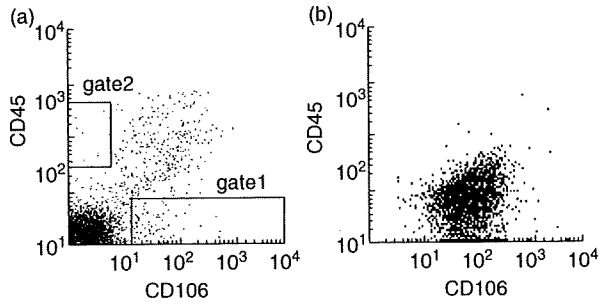

Fig. 1. Flow cytometric profiles of freshly isolated and cultured bone marrow stromal cells (BMSCs). (a) Non-haemopoietic mesenchymal stem cell-enriched cells, defined as $CD45^-/CD106^+$ cells, were sorted immediately (gate 1) from the recipient of intrabone marrow-bone marrow transplantation (IBM-BMT) + IBM-donor lymphocyte infusion (DLI) after the staining of cells with fluorescein isothiocyanate (FITC)-anti-CD45 and phycoerythrin (PE)-anti-106 monoclonal antibodies (mAbs). The dot-plot profile of $CD45^-/CD106^+$ cells from the recipients of IBM-BMT + intravenous (i.v.)-DLI or IBM-BMT alone (without DLI) was similar to (a). Haemopoietic bone marrow cell-enriched populations, sorted as $CD45^+/CD106^-$ cells (gate 2), were also prepared from the recipients, and used as controls. (b) Cultured BMSCs (for 2 weeks) obtained originally from the right tibia of the recipients of IBM-BMT + IBM-DLI were stained with FITC-anti-CD45 and PE-anti-106 mAbs, and analysed by a fluoresence activated cell sorter scan. The dot-plot profile of cultured BMSCs from the recipients of IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI) was similar to (b).

the BM of IBM-DLI, i.v.-DLI and IBM-BMT alone (without DLI) suppressed MLR only slightly, but not significantly (not statistically significant among three groups). This is the case when haemopoietic $CD45^+/CD106^-$ cells or whole BMCs were added to the culture. Thus, non-haemopoietic BMCs freshly isolated from the site of IBM-DLI could not significantly suppress T cell proliferation in MLR. This might be due to the heterogeneity of non-haemopoietic BMCs. Therefore, we next examined the inhibitory effect of cultured BMSCs after IBM-DLI.

Three days after DLI, BMCs were collected from the recipients, and cultured in DMEM with 10% FCS for 2 weeks, as shown in *Materials and methods*. The phenotypes of BMSCs, thus prepared, were negative for CD45 and CD34, but positive for CD90 and CD106 (Fig. 1b). These BMSCs were added to the culture of MLR to examine their suppressive effects.

As shown in Fig. 3a and b, the BMSCs prepared from the recipients treated with IBM-BMT + IBM-DLI significantly suppressed MLR in a dose-dependent fashion when compared with those from the recipients treated with IBM-BMT + i.v.-DLI. It is surprising that the BMSCs from the recipients of IBM-BMT + IBM-DLI still showed a suppressive effect on T cell proliferation even after long-term culture (3 months) when compared with those prepared from the recipients of IBM-BMT + i.v.-DLI (Fig. 3c), suggesting that the suppressive effects of BMSCs on the BM (IBM-DLI) are long-lasting.

The frequency of IFN-γ- and IL-4-producing T cells after coculture with BMSCs

To examine the effects of BMSCs on T cell polarization, CD4-enriched T cells from donor BALB/c mice were cultured with irradiated stimulator spleen cells from B6 mice and BMSCs cultured from the recipients of IBM-BMT + IBM-

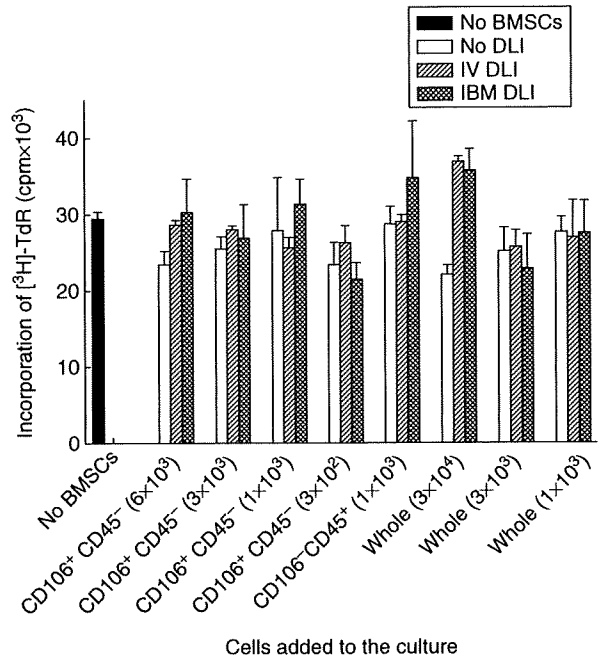

Fig. 2. Effect of freshly isolated bone marrow stromal cells (BMSCs) on T cell proliferation. Non-haemopoietic mesenchymal stem cell-enriched cells, defined as $CD45^-/CD106^+$ cells, were sorted immediately (gate 1) from the recipient of intrabone marrow-bone marrow transplantation (IBM-BMT) + IBM-donor lymphocyte infusion (DLI), IBM-BMT + intravenous (i.v.)-DLI or IBM-BMT alone (without DLI) after the staining of cells with fluorescein isothiocyanate-anti-CD45 and phycoerythrin-anti-10^6 monoclonal antibodies (mAbs). Haemopoietic cells in the bone marrow (BM), defined as $CD45^+/CD106^-$ cells, were also obtained by a cell sort (gate 2). Graded numbers of $CD45^-/CD106^+$ BMSCs (3×10^2–6×10^3), $CD45^+/CD106^-$ haemopoietic cells (1×10^3) or whole BM cells (1×10^3–3×10^4) were added to the culture of one-way mixed leucocyte reaction where 2×10^5 responder $CD4^+$T cells from BALB/c mice were stimulated with 12 Gy irradiated stimulator spleen cells (2×10^5 cells) from B6 mice in a 96-well flat-bottomed plate in a total volume of 0·2 ml and cultured for 96 h. The cultures were pulsed with 0·5 μCi of [^{3}H]-TdR for the last 16 h of the culture period. This figure shows the representative result of three experiments. The data are expressed as mean counts per minute ± standard deviation of three mice (separately cultured BMSCs obtained from the recipient).

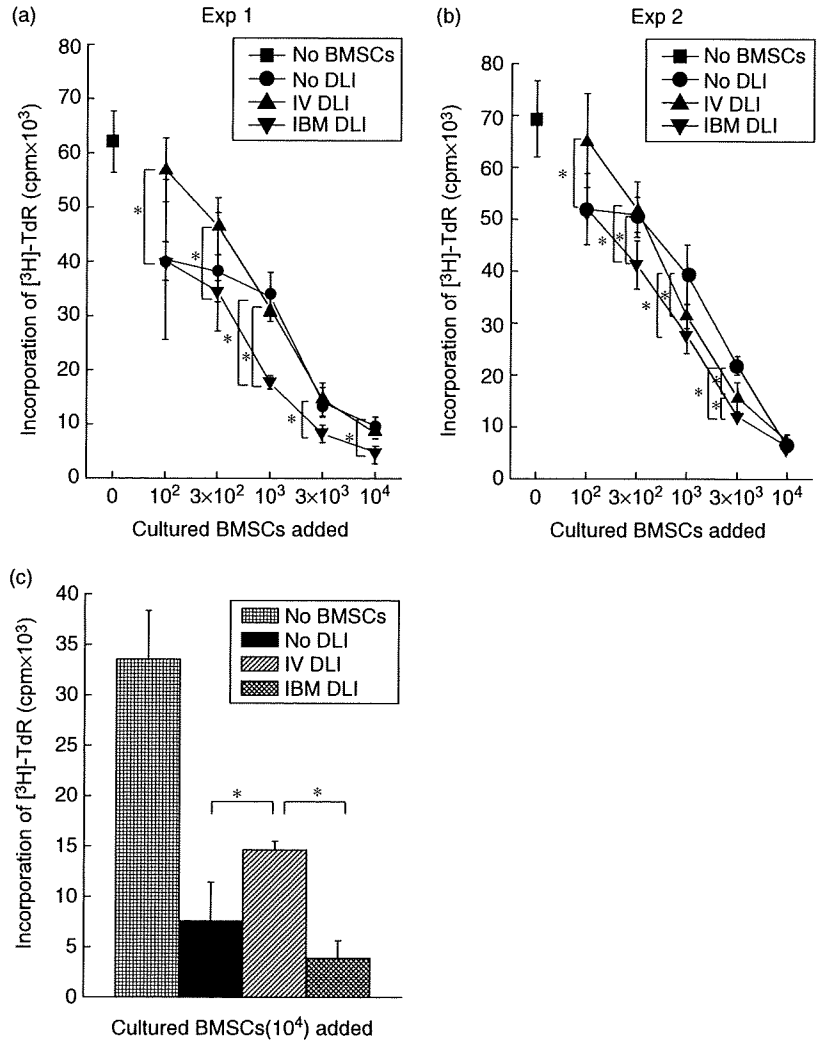

Fig. 3. Inhibitory effect of cultured bone marrow stromal cells (BMSCs) on T cell proliferation. Bone marrow cells from the right tibia were collected from the recipients of intrabone marrow-bone marrow transplantation (IBM-BMT) + IBM-donor lymphocyte infusion (DLI), IBM-BMT + intravenous (i.v.)-DLI or IBM-BMT alone (without DLI) and cultured for 2 weeks. Graded numbers of cultured BMSCs (10^2–10^4 cells) were added to the culture of one-way mixed leucocyte reaction (MLR) (a, b). BMSCs were obtained after the long-term culture (cultured for 3 months), and were added to the culture of one-way MLR (c). The data in figures are expressed as mean counts per minute ± standard deviation of three mice (separately cultured BMSCs obtained from the recipient). Symbols in the boxes represent origins of cultured BMSCs. *Statistically significant when compared with MLRs performed in the groups ($P < 0.05$).

DLI, IBM-BMT + i.v.-DLI, or IBM-BMT alone (without DLI). The development of T helper 1 (Th1) or Th2 cells was defined by intracellular staining of IFN-γ or IL-4. The frequency of IL-4-producing cells was slightly but significantly higher in the culture with BMSCs from IBM-BMT + IBM-DLI than in that with BMSCs from IBM-BMT + i.v.-DLI (Fig. 4a and b *versus* 4c and summarized in 4g). Conversely, the percentage of IFN-γ-producing cells was lower in the culture with BMSCs from IBM-BMT + IBM-DLI than in that with BMSCs from IBM-BMT + i.v.-DLI (Fig. 4d and e *versus* 4f, and summarized in 4g). Furthermore, this is the case when intracellular IL-2 was examined (data not shown). Thus, the polarization of Th2 cells is facilitated strongly after co-culture with the BMSCs from the recipients of IBM-BMT + IBM-DLI, while Th1 cells are induced dominantly by co-culture with the BMSCs from the recipients of IBM-BMT + i.v.-DLI. These findings suggest strongly that T cells injected into the BM cavity can modulate the function of BMSCs after their interaction.

Bone marrow stromal cells produce immunoregulatory cytokines: TGF-β and HGF

Previous reports have shown that BMSCs can modify T cell functions by soluble factors [18,19]. Therefore, we attempted to identify molecules involved in the immune modulation by BMSCs. First, we determined the levels for IL-2, IL-10, IFN-γ and TGF-β in the culture supernatant of BMSCs using an ELISA. The culture supernatants of enriched T cells stimulated with ConA served as a control. As shown in Table 1, IL-2, IL-10 or IFN-γ were not detected in the culture supernatants of BMSCs from the recipients of IBM-BMT + IBM-DLI, IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI), while a significant amount of TGF-β was detected in the culture supernatants of BMSCs from the recipients of IBM-BMT + IBM-DLI, but not in those from the recipients of IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI). These results indicate that TGF-β secreted from the BMSCs obtained from the recipients of IBM-BMT + IBM-DLI

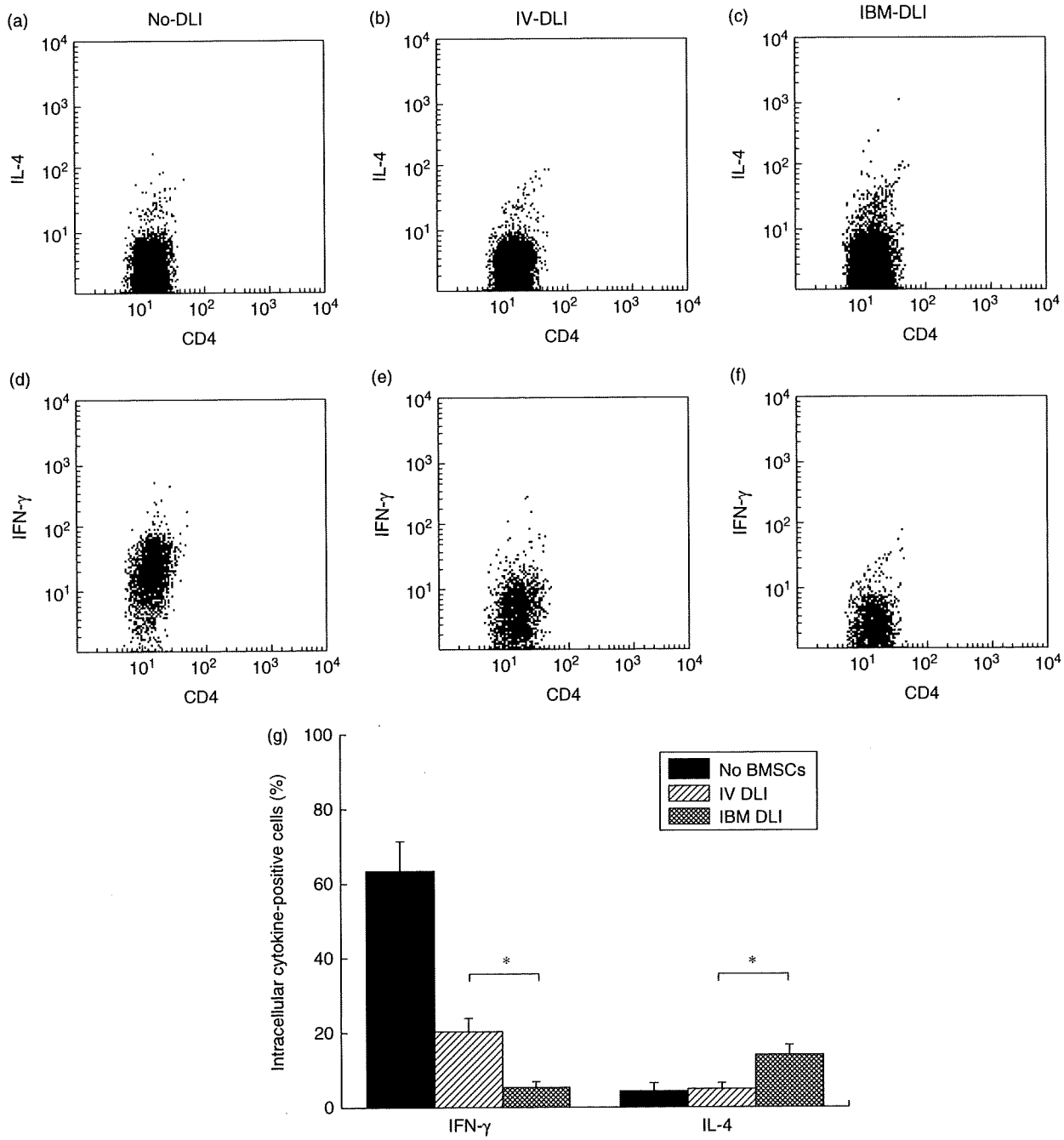

Fig. 4. Interaction of bone marrow stromal cells (BMSCs) with T cells and induction of T helper 2 cells. $CD4^+$ cell-enriched T cells from BALB/c mice were cultured with irradiated stimulator spleen cells from B6 and BMSCs cultured from the recipients of intrabone marrow-bone marrow transplantation (IBM-BMT) + IBM-donor lymphocyte infusion (DLI), IBM-BMT + intravenous (i.v.)-DLI or IBM-BMT alone (without DLI) in a round-bottomed plate (RPMI-1640) with 10% fetal calf serum. Cells were harvested 6 days later and stained with biotin-conjugated anti-H-$2K^d$ (visualized by streptavidin–peridinin chlorophyll-Cy5·5) and fluorescein isothiocyanate-anti-CD4 monoclonal antibody (mAb) to detect responder (donor) CD4 T cells. The cells were next fixed and permeabilized and intracellular cytokines were detected after the staining of cells with phycoerythrin-anti-interleukin (IL)-4 and -interferon (IFN)-γ mAbs. Representative dot-plot profiles of $CD4^+$/IL-4^+ cells (a, b, c) or $CD4^+$/IFN-γ^+ cells (d, e, f) are shown, co-cultured with BMSCs from the recipients of IBM-BMT alone (without DLI) (a, d), IBM-BMT + i.v.-DLI (b, e), or IBM-BMT + IBM-DLI (c, f). Cells in dot-plot profiles were gated positively as H-$2K^{d+}$ responder cells. Cells stained with isotype control cocktail served as a control. (g) Representative result of three experiments. Columns represent mean percentage of IFN-γ or IL-4 bearing cells ± standard deviation of three mice (separately cultured BMSCs obtained from the recipient). Symbols in the boxes represent origins of cultured BMSCs. *Statistically significant when compared with intracellular cytokines performed in the groups ($P < 0{\cdot}05$).

Table 1. Measurement of cytokines.

	No DLI†	i.v.-DLI	IBM-DLI	T cells with ConA‡
IL-2 (pg/ml)	0	0	0	87·3 ± 15·5
IFN-γ (pg/ml)	0	0	0	1418·2 ± 369·4
IL-10 (pg/ml)	0	0	0	1114·6 ± 103·1
TGF-β (ng/ml)	0	0·27 ± 0·4	14·2 ± 2·4	0·6 ± 0·5

†Bone marrow stromal cell (BMSC) culture supernatants from the recipients of intrabone marrow-bone marrow transplantation (IBM-BMT) + IBM-donor lymphocyte infusion (DLI), IBM-BMT + intravenous (i.v.)-DLI, or IBM-BMT alone (without DLI) were collected 2 weeks later. The cell supernatants were analysed for the amount of interleukin (IL)-2, IL-4, interferon (IFN)-γ and trandforming growth factor (TGF)-β by enzyme-linked immunosorbent assay. ‡Splenic T cells from BALB/c mice were activated with concanavalin A (ConA) and used as a positive control.

might be one of the candidates for attenuation of GVHD in our model system.

It has been reported that HGF also inhibits T cell proliferation or activation [18,19]. Therefore, we next determined in the culture supernatants of BMSCs whether the levels of HGF in BMSCs increased after IBM-BMT + IBM-DLI. We measured HGF (and also TGF-β) in the message level by a quantitative real-time RT–PCR because no ELISA kit is available to detect murine HGF. As shown in Fig. 5a (HGF) and 5b (TGF-β), the relative levels of both HGF and TGF-β were significantly higher in the BMSCs from the recipients of IBM-BMT + IBM-DLI than in those from the recipients of IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI). Furthermore, as summarized in Table 2, we did not detect substantial levels of IL-2, IL-4 or IL-15 mRNA in BMSCs from the recipients of IBM-BMT + IBM-DLI, IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI). However, it is noted that a slight but significant level of IL-10 message was detected only in the BMSCs from recipients of IBM-BMT + IBM-DLI, but not in those from recipients of IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI). Therefore, T cells injected directly into the BM cavity can induce the production of suppressive cytokines from BMSCs, and BMSCs might exert their inhibitory effect on T cell activation or proliferation via HGF and/or TGF-β.

Discussion

Transplantation biology has been one of the major advances in medicine during the last few decades. BMT, in particular, can cure a variety of malignancies by exploiting graft-*versus*-tumour effects exerted by the lymphocytes. In this proce-

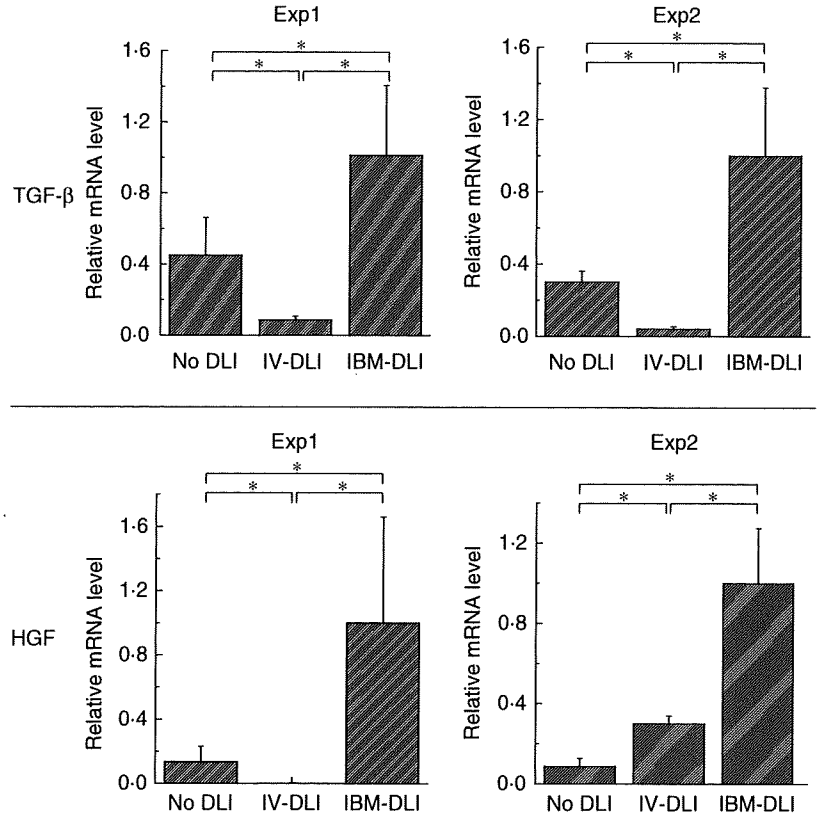

Fig. 5. Production of transforming growth factor (TGF)-β and hepatocyte growth factor (HGF) in bone marrow stromal cells (BMSCs). Culture expanded BMSCs from the recipients of intrabone marrow-bone marrow transplantation (IBM-BMT) + IBM-donor lymphocyte infusion (DLI), IBM-BMT + intravenous (i.v.)-DLI or IBM-BMT alone (without DLI) were used for analysis of cytokine messages by real-time PCR. After DNase I treatment, cDNA was synthesized, amplified using HGF or TGF-β primer, and visualized with SYBR Green by real-time reverse transcription–polymerase chain reaction. Relative intensity of HGF or TGF-β mRNA was calculated on the basis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) intensity. Columns represent relative cytokine message levels of TGF-β and HGF. Each column shows mean ± standard deviation of three mice (separately cultured BMSCs obtained from the recipient), and we performed two separate experiments. Symbols in the boxes represent origins of cultured BMSCs. *Statistically significant when compared with cytokine message performed in the groups ($P < 0{\cdot}05$).

Table 2. Analyses of cytokine messages by real-time reverse transcription–polymerase chain reaction (RT–PCR).

Cytokines examined	No DLI†	i.v.-DLI	IBM-DLI	T cells with ConA‡
IL-2	0·47 ± 0·3§	0·31 ± 0·2	0·47 ± 0·3	8·51 ± 6·1
IL-4	0	0	0·025 ± 0·03	1277·2 ± 357·4
IL-10	0	0	2·7 ± 2·3	95 000 ± 16 000
IL-15	0	0	0	n.d.

†Culture expanded bone marrow stromal cells (BMSCs) from the recipients of intrabone marrow-bone marrow transplantation (IBM-BMT) + IBM-donor lymphocyte infusion (DLI), IBM-BMT + intravenous (i.v.)-DLI, or IBM-BMT alone (without DLI) were used for analysis of cytokine messages by real-time RT–PCR. After DNase I treatment, cDNA was synthesized, amplified using interleukin (IL)-2, IL-4, IL-10 or IL-15 primer, and visualized with SYBR Green by real-time RT–PCR. ‡Splenic T cells from BALB/c mice were activated with concanavalin A (ConA) and used as a positive control.

§Relative intensities of soluble factors were calculated on the basis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Numbers in the table represent mean intensities of cytokines ± standard deviation of three mice (separately cultured BMSCs obtained from the recipient). We performed two separate experiments. n.d., not done.

dure, one of the major problems to be solved is GVHD. We have developed recently a new protocol for BMT: IBM-BMT can induce persistent allogeneic donor-specific tolerance without the use of immunosuppressants after the treatment, even when the radiation doses are reduced to sublethal levels. Therefore, we have aimed to develop a new strategy for the successful engraftment of donor-derived haematolymphoid cells without developing GVHD even in the presence of T cells in the donor inoculum. We have found that GVHD could be alleviated when BMCs containing T cells were inoculated into the BM cavity [9]. We compared the severity of GVHD induced by the intravenous injection of T cells (i.v.-DLI) with that induced by the IBM injection of T cells (IBM-DLI). Acute GVHD was observed in recipients treated with IBM-BMT + i.v.-DLI, while reduced GVHD was seen in those treated with IBM-BMT + IBM-DLI. However, the mechanisms underlying this inhibition still remain unresolved and therefore we focused on the function of BMSCs, because T cells can interact with BMSCs in the BM cavity after the IBM-DLI. The ability of MSCs to interact with immune cells and to modulate their response has important implications in the transplantation biology. We have carried out experiments in which the sorted $CD45^-/CD106^+$ cells from the recipients of IBM-BMT + IBM-DLI, IBM-BMT + i.v.-DLI or IBM-BMT alone (without DLI) were added to the culture of one-way MLR. The inhibitory ability of non-haemopoietic BMCs to activated T cells was insufficient (Fig. 2). However, cultured BMSCs from the recipients of IBM-BMT + IBM-DLI, IBM-BMT + i.v.-DLI and IBM-BMT alone (without DLI) showed an immunosuppressive effect in MLR in a dose-dependent fashion (Fig. 3). Furthermore, of interest and of importance is that the cultured BMSCs from the recipients of IBM-BMT + IBM-DLI suppressed MLR strongly even in small numbers (10^2–3×10^3) when compared with BMSCs from the recipients of IBM-BMT + i.v.-DLI.

Furthermore, the conversion of Th1 cells (defined by intracellular staining of IFN-γ) was clearly inhibited while the polarization of Th2 cells (defined by intracellular staining of IL-4) was facilitated by BMSCs from the recipients treated with IBM-DLI. In contrast to this, BMSCs from the recipients of i.v.-DLI prompted the polarization of Th1 cells (Fig. 4). These data suggest that BMSCs from the recipients of IBM-BMT + IBM-DLI interact with naive T cells to convert Th2 cells, which might be beneficial for GVHD management.

Several recent reports have described how BMSCs produce soluble factors, including TGF-β and HGF, which regulate T cell proliferation [18,21,22,32]. In our present study, BMSCs from the recipients of IBM-BMT + IBM-DLI produced significantly higher amounts of HGF and TGF-β than those from the recipients of IBM-BMT + i.v.-DLI and IBM-BMT alone (without DLI) (Fig. 5 and Table 1).

Collectively, our findings indicate clearly that BMSCs can interact with T cells that have been injected into the BM cavity as IBM-DLI, and that the function(s) of BMSCs might somehow be modulated by this interaction to produce inhibitory cytokines and to possess the ability to convert Th0 cells to Th2 cells, but not to Th1 cells. It should be noted that the modulated features of BMSCs were maintained for at least 6 weeks, thus leading to the reduction of GvH responses. We have shown, in our GVHD model, that IBM-DLI (*in vivo* injection of donor T cells into the BM cavity) (but not i.v.-DLI) can attenuate GVHD. Therefore, our present study provides the basic information that IBM-BMT is an excellent strategy to engraft donor cells efficiently along with attenuation of GVHD, even when some quantities of T cells are contaminated in BMC preparations. Thus, IBM-BMT can control GVHD easily.

T cells can recognize MHC determinants on BMSCs *in vivo*, and the BMSC recognized by T cells can modulate their functions. Therefore, we are now investigating subcellular processes after the T–BMSC interaction and identifying molecules, other than MHC, to be essential for this interaction.

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ORIGINAL ARTICLE

Transplantation of newborn thymus plus hematopoietic stem cells can rescue supralethally irradiated mice

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We attempted to rescue supralethally irradiated (SLI) mice by transplantation of hematopoietic stem cells (HSCs) plus thymus from variously aged donors (fetus, newborn and adult). Although the transplantations of these kinds of HSCs alone showed a very short survival, newborn liver cells (NLCs) (as the source of HSCs) plus newborn thymus (NT) transplantation markedly improved the survival rate. The transplantation attenuated severe damage in the small intestine, which is one of the major causes of death by SLI. In addition, the donor-derived CD4$^+$ T cells significantly increased with additional NT transplantation. The production of interleukin (IL)-7 and keratinocyte growth factor, which plays a crucial role in protection against radiation injury in the intestine, was the highest in NT. Finally, SLI mice that had received NLC plus IL-7$^{-/-}$ NT transplantation plus IL-7 injection showed improved survival, weight recovery and an elevated number of CD4$^+$ T cells compared with the mice that had received NLC plus IL-7$^{-/-}$ NT or plus IL-7 injection alone. These findings 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 with induction of CD4$^+$ T cells.

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Introduction

In recent years, bone marrow transplantation (BMT) has become a powerful strategy for the treatment of intractable diseases, such as hematological disorders (leukemia, lymphoma and aplastic anemia), congenital immunodeficiencies, metabolic disorders, autoimmune diseases and malignant tumors.[1] Using various animal models, we have found that allogeneic BMT can be used for the treatment of such diseases.[2–9] The basic theory is to replace pathogenic hematopoietic cells of hosts with normal hematopoietic stem cells (HSCs) of donors following lethal irradiation.

Exposure to supralethal irradiation (SLI) can occur, for example, in criticality accidents or in the treatment of malignant tumors.[10–13] High doses of irradiation induce severe damage not only in hematopoietic cells but also in other organs such as the gastrointestinal tract and brain,[14] leading to early death. Conventional BMT is thus ineffective for SLI recipients, because the organ damage is overwhelming. Indeed, HSC transplantation was unable to rescue a recent case of criticality accident, even though donor-derived cells were detected.[15,16] Rescue from SLI is thus extremely difficult.

The thymus is the central organ of T-cell development. We have previously reported that BMT plus thymus transplantation can accelerate hematopoietic recovery and improve survival rate, and can be used to treat autoimmune diseases in recipients such as aged or chimeric resistant hosts,[7,17] in which conventional BMT is difficult.

Interleukin (IL)-7 is produced by thymic epithelial cells, marrow stromal cells, fibroblasts and intestinal epithelia, and plays a crucial role in the early T-cell development and the functions in the thymus.[18–24] In addition, IL-7 engages in mucosal immunity, including the development of γδ T cells.[25–27] Notably, IL-7 signals have also been reported as an important factor in the regeneration of the gastrointestinal cells after irradiation.[28] Keratinocyte growth factor (KGF) is the significant cytokine for generating epithelial cells.[29] In embryogenesis, both KGF produced by thymocytes and IL-7 by thymic epithelial cells play a part in the development of the thymus.[30] Additionally, KGF is effective in treating intestine injured by irradiation and chemotherapy.[31]

In the present study, we attempted to rescue SLI mice using HSC transplantation plus thymus transplantation from variously aged donors, since the functions of the thymus greatly differ with age.[32,33] We here show that the transplantation of newborn liver cell (NLCs) plus newborn thymus (NT) can most effectively rescue SLI mice. It is likely that the high production of IL-7 by NT transplantation plays an important role in the induction of CD4$^+$ T cells.

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Materials and methods

Mice
Female 6- to 8-week-old, newborn (≤48 h after birth) and 16-day fetus C57BL/6 (B6) (H-2^b) and BALB/c (H-2^d) mice were obtained from Shimizu Laboratory Supplies (Shizuoka, Japan) and maintained until use in our animal facilities under specific pathogen-free conditions. IL-7 gene null (IL-$7^{-/-}$) mice with B6 background were kindly provided by Professor Ikuta from Kyoto University (Kyoto, Japan).[27]

HSCs and thymus transplantation
The 6- to 8-week-old female BALB/c mice received lethal irradiation (7 Gy) or SLI (9.5 Gy) 1 day before HSC transplantation. The next day, 1×10^7 B6 HSCs were injected intravenously into these mice. Bone marrow cells were collected from the femurs and tibias of 6- to 8-week-old B6 mice. Newborn and fetal livers were obtained and single-cell suspensions were created for the use of NLCs and fetal liver cells as the source of HSCs.[34,35] Adult thymus (AT), NT and fetal thymus (FT) tissues were removed from the aged mice. For thymus transplantation, one-quarter of the AT, or one NT or one FT was simultaneously transplanted under the renal capsule in some recipients with HSC transplantation. Thymus transplantation alone was also performed in other mice.

IL-7 treatment in vivo
Recombinant mouse IL-7 (Perpro Tech EC, London, UK) in PBS was injected intraperitoneally into chimeric mice for 7 days after HSC transplantation (1 μg per mice per day). Control mice were injected with PBS alone.

Reverse transcription-PCR
Reverse transcription-PCR analysis was employed for the determination of IL-7 mRNA. In brief, total RNA was extracted from each isolated thymus using RNagent (Promega, Madison, WI, USA) according to the manufacturer's instructions. Reverse transcription of 1 μg of RNA to cDNA was performed using oligo(dT) (Perkin Elmer Cetus, Norwork, CT, USA). Primer sequences of IL-7 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and PCR condition were as follows: IL-7 (forward), 5′-ACAT CATCTGAGTGCCACA-3′; IL-7 (reverse), 5′-CTCTCA GTAGTCTCTTTAG-3′ (355 bp); KGF (forward), 5′-ATC CTGCCAACTCTGCTACAGA-3′; KGF (reverse), 5′-CT TCCCTTTGACAGGAATCCCCTT3′; GAPDH (forward), 5′-ACCACAGTCCATGCCATCAC-3′; GAPDH (reverse), 5′-TCCACCACCCTGTTGCTGTA-3′ (452 bp). Each reaction was performed at 94 °C for 30 s for denaturation, then optimal annealing temperature (IL-7, 45 °C; KGF, 55 °C; GAPDH, 55 °C) for 30 s and 72 °C for 30 s for elongation (35 cycles). PCR products were analyzed by electrophoresis in 2% agarose gels and made visible by staining with ethidium bromide.

Western blotting
Each thymus tissue sample (1 mg per sample) was lysed on ice for 40 min in 20 μl of cell lysis buffer (0.5% Nonidet P-40 (Sigma, St Louis, MO, USA), 0.15 M NaCl, 5 mM EDTA, 50 mM Tris–HCl, pH 7.2) supplemented with a protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA). Following centrifugation at 8000 r.p.m. for 10 min, the lysate supernatants were normalized for protein concentration using the Bradford reagents (Pierce Chemical, Rockford, IL, USA). Samples were boiled for 5 min in SDS-reducing buffer, separately treated by SDS–PAGE (12% acryl-amide, wt/vol), and then electrophoretically transferred onto nitrocellulose membranes. Membranes were probed for invariant chain with the IN-1 Moabs. Anti-human KGF antibody (goat anti-mouse affinity-purified IgG) (R&D Systems, Minneapolis, MN, USA) and anti-mouse IL-7 antibody (goat anti-mouse affinity-purified IgG) were applied at 1:100 dilution. Binding was detected using a horseradish peroxidase-conjugated anti-goat IgG (American Pharmacia Biotech, Piscataway, NJ, USA) diluted at 1:1500 and visualized by chemiluminescence.

Analysis of surface markers and the numbers of lymphocytes by flow cytometry
Surface markers on lymphocytes from peripheral blood and spleen cells were analyzed by three-color fluorescence staining using a FACScan system (Becton Dickinson, Franklin Lakes, NJ, USA). FITC-conjugated anti-H-$2K^b$ MoAbs (Pharmingen, San Diego, CA, USA) were used to determine chimerism. FITC-, phycoerythrin- or biotin-conjugated CD4, CD8 or B220 (Becton Dickinson or Pharmingen) was used for analyses of lymphocyte subsets. Avidin-Cy5 (Dako, Kyoto, Japan) was used for the third color in the avidin/biotin system. The numbers of lymphocyte subsets in peripheral blood or in spleen cells were calculated as the total lymphocyte numbers of WBCs measured by SF-3000 with SFVU-1 unit (Sysmex, Kobe, Japan), or as the total lymphocyte numbers of spleen cells multiplied by the percentage of the lymphocyte cells.

Pathological findings
The small intestine, grafted thymus under the renal capsule and other organs from chimeric mice were fixed in 10% formaldehyde solution and embedded in paraffin. Sections 4-μm thick were prepared and stained using hematoxylin and eosin. Histology was examined under microscopy.

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

Results

Survival rates and chimerism in SLI mice receiving HSCs with or without thymus transplantation from variously aged donors
We first examined the effects on survival rates in SLI (9.5 Gy) mice that had received HSCs with or without thymus transplantation from variously aged (fetus,

newborn and adult) donors (Figure 1). In total, 80% of BALB/c mice that had been irradiated with a conventional low dose (7 Gy) survived >100 days after the transplantation of 1×10^7 bone marrow cells of B6 mice. In contrast, most of the 9.5-Gy-irradiated BALB/c mice died within 14 days after the transplantation of 1×10^7 HSCs from variously aged B6 mice (Figure 1a), since BALB/c mice are radio-sensitive and 9.5 Gy is an SLI dose. Next, we performed additional thymus transplantation in SLI mice (Figure 1b). Interestingly, NLCs with NT transplantation significantly improved the survival rate (70% survival at 100 days after transplantation), in comparison with NLC transplantation alone and all the other combinations. NT transplantation alone did not improve the survival rate. The engrafted thymus showed a normal structure under the renal capsule, and normal T-cell differentiation was observed in the thymus 8 weeks after transplantation (Figure 2).

Histology and body weight in SLI mice receiving NLC plus NT transplantation

Next, we investigated the causes of death in SLI mice. Histologically, the most damaged organ was the small intestine in the mice that had received NLC transplantation alone. In contrast to normal small intestine (Figure 3a; i), the mucosa displayed marked necrosis, and only a few cryptae were left 7 days after transplantation (Figure 3a; ii). However, with NT transplantation, severity was attenuated (Figure 3a; iii) and the mucosa with cryptae displayed good regeneration 14 days after the transplantation (Figure 3a; iv). 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 (Figure 3b). However, it was significantly recovered with additional NT transplantation. SLI mice that had received HSCs alone or HSCs with AT or FT transplantation showed short survival rates (data not shown).

Analyses of chimerism and lymphocyte subsets from SLI mice receiving NLCs with or without NT transplantation

Supralethal irradiation mice that had received NLCs plus NT transplantation showed full donor-type chimerism (H-2K^{b+}) at 2 weeks after transplantation, and it continued for more than 12 weeks (Figure 4a). However,

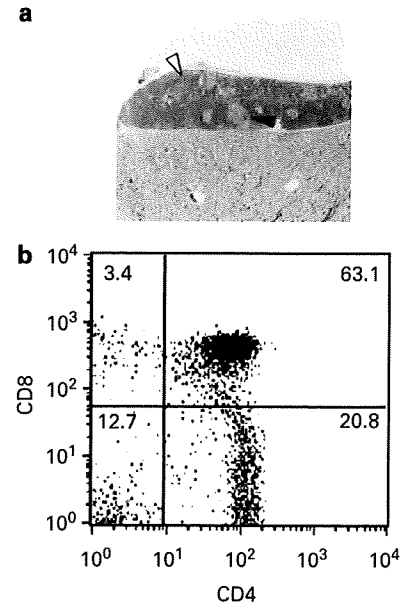

Figure 2 Histology and CD4/CD8 expression in transplanted thymus from SLI mice receiving NLCs plus NT transplantation. Histology (hematoxylin and eosin, ×200) (**a**) and percentages of CD4$^+$ and CD8$^+$ thymocytes (**b**) in engrafted NT from SLI BALB/c mice that had received 1×10^7 NLCs plus NT transplantation from B6 mice at 8 weeks after transplantation. The engrafted thymus is seen under the renal capsule, and cortical (open arrow) and medullary areas (closed arrow) were well demarcated (**a**). Cells were stained with anti-mouse CD4 and CD8 MoAbs and analyzed by flow cytometry (**b**). Representative data are shown from five independent experiments. NLCs = newborn liver cells; NT = newborn thymus; SLI = supralethal irradiation.

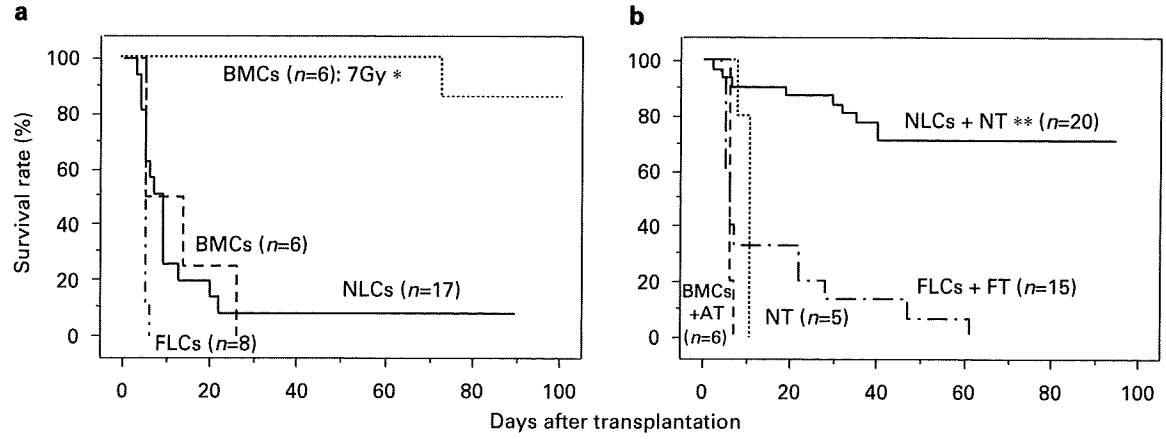

Figure 1 Survival rate in lethally irradiated or SLI mice receiving HSCs with or without thymus transplantation from variously aged donors. Survival rate for lethally irradiated mice (7 Gy) that had received 1×10^7 BMCs alone and SLI BALB/c mice (9.5 Gy) that had received 1×10^7 FLCs, NLCs or BMCs alone (**a**). Survival rate for SLI BALB/c mice (9.5 Gy) that had received 1×10^7 FLCs plus FT transplantation, NLCs plus NT transplantation, BMCs plus AT transplantation or NT transplantation alone (**b**). *$P<0.005$ compared with BMCs, NLCs or FLCs. **$P<0.005$ compared with NLCs, NT, BMCs plus AT or FLCs plus FT. AT = adult thymus; BMCs = bone marrow cells; FT = fetal thymus; HSCs = hematopoietic stem cells; FLCs = fetal liver cells; NLCs = newborn liver cells; NT = newborn thymus; SLI = supralethal irradiation.

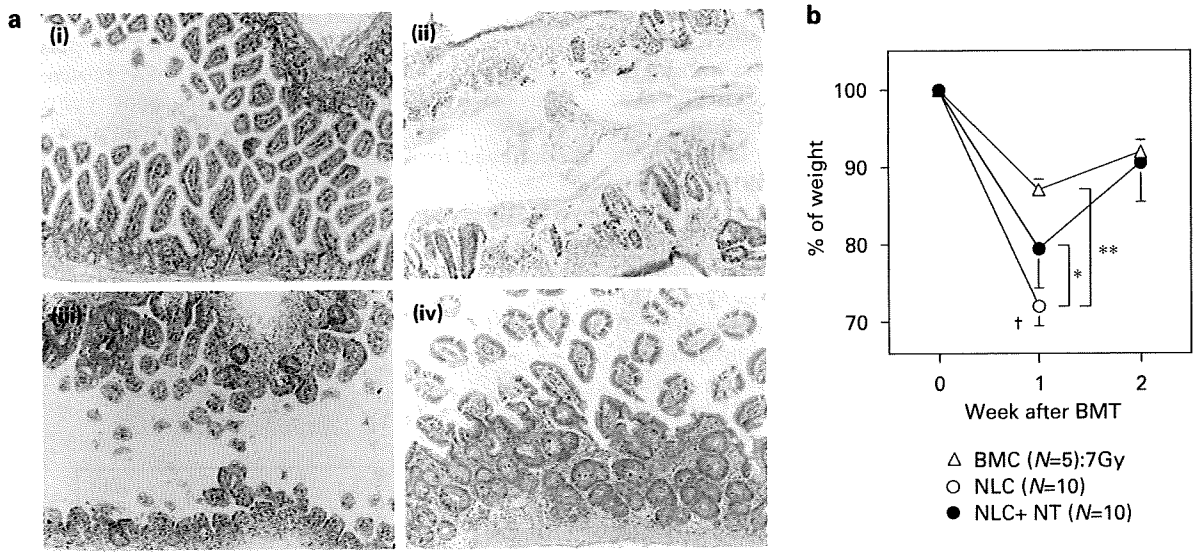

Figure 3 Small intestine histology and weight in SLI mice receiving NLCs with or without NT transplantation. Histology of the small intestine (hematoxylin and eosin, ×200) (**a**) and percentage of weight loss (**b**) in lethally irradiated (7 Gy) BALB/c mice that had received 1×10^7 BMCs or SLI BALB/c 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

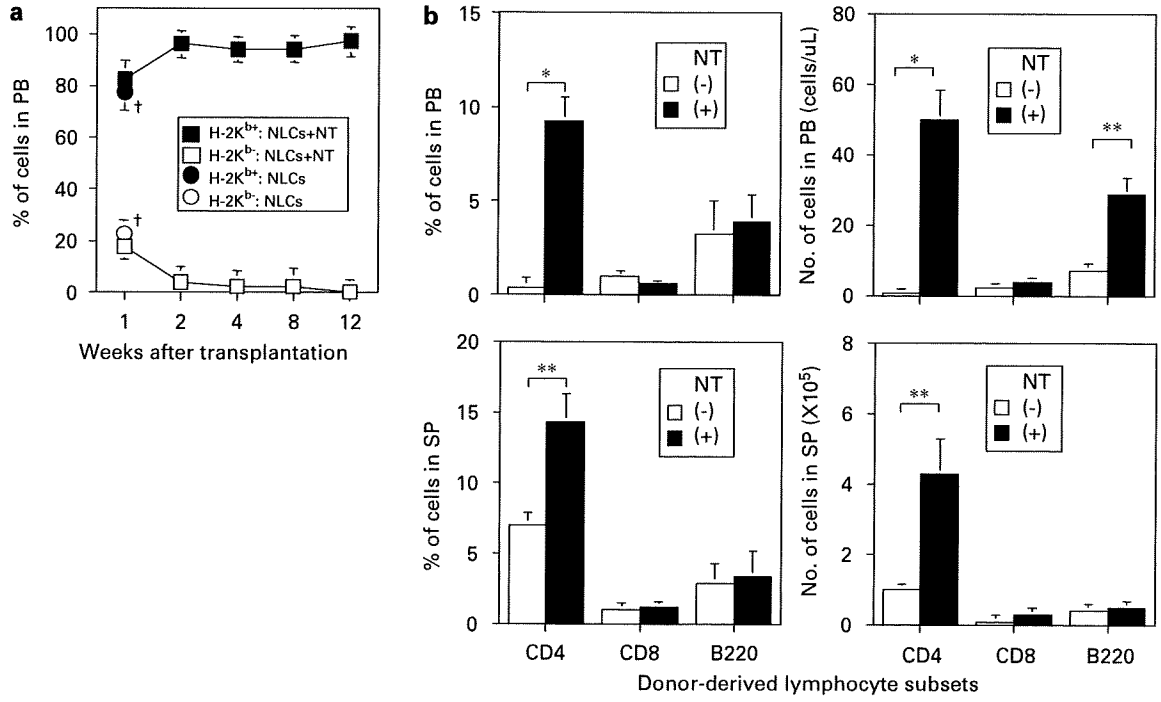

Figure 4 Analyses of chimerism and lymphocyte subsets in peripheral blood and spleen from SLI mice receiving NLCs plus NT transplantation at the early phase after transplantation. Chimerism of donor-derived cells (H-2K^{b+} cells) and host-derived cells (H-2K^{b-} cells) in the peripheral blood from 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.

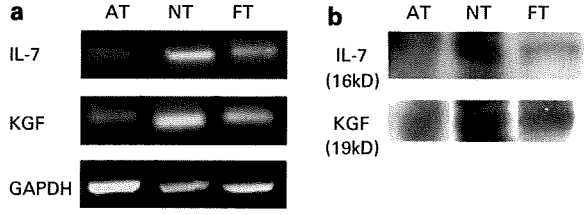

Figure 5 IL-7 and KGF levels of mRNA and protein in AT, NT and FT. The mRNA expression levels of IL-7, KGF and GAPDH according to RT-PCR (**a**) and protein levels of IL-7 and KGF by western blotting (**b**) were examined in freshly isolated AT, NT and FT from the aged donors. Representative data are 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

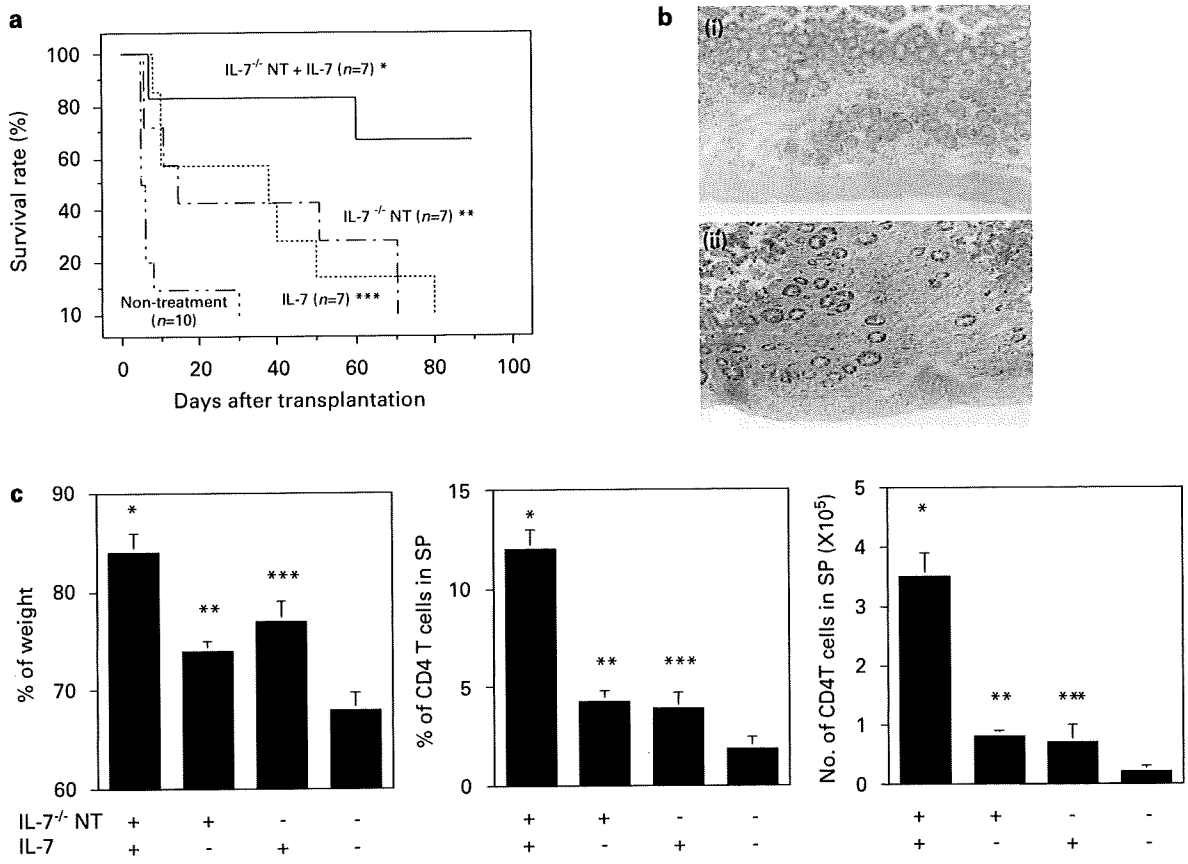

Figure 6 Analyses of survival rate, small intestinal histology, weight recovery and CD4 T-cell induction in spleen from SLI mice receiving NLCs with or without IL-7$^{-/-}$ NT transplantation in the presence or absence of IL-7 treatment. SLI BALB/c mice were transplanted with 1×10^7 NLCs with or without 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.

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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		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

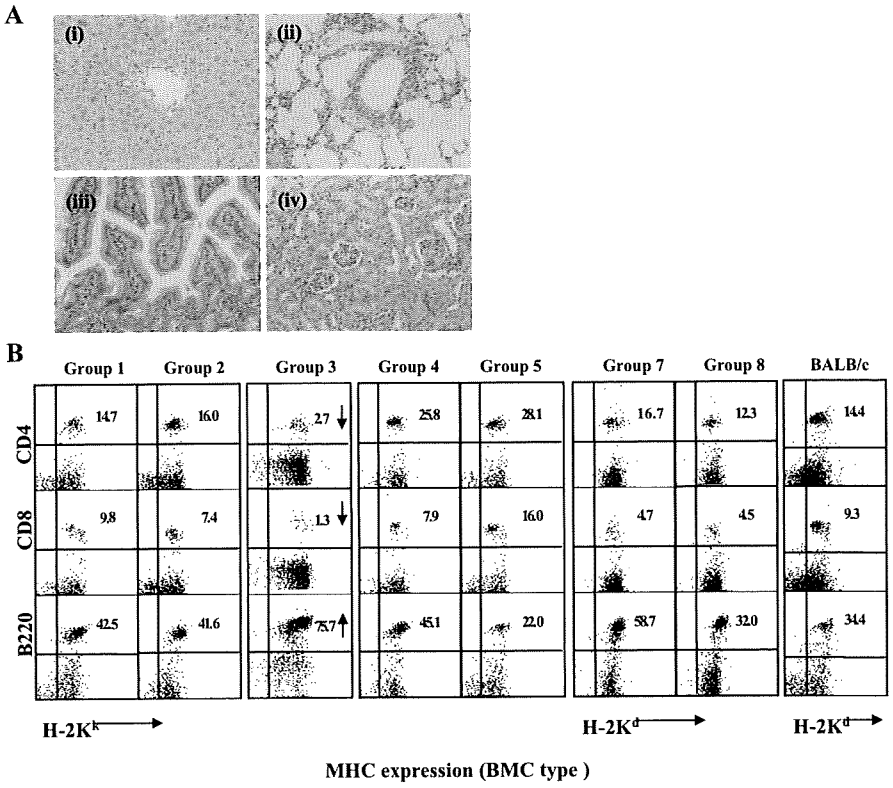

FIGURE 1. Analysis of histology and BMC-derived $CD4^+$ and $CD8^+$ T cells and B cells in spleen from experimental groups. (A) Histologic findings of liver (i), lung (ii), small intestine (iii), and kidney (iv) from group 1 (hematoxylin-eosin; magnification ×400). The mice from other groups with TT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