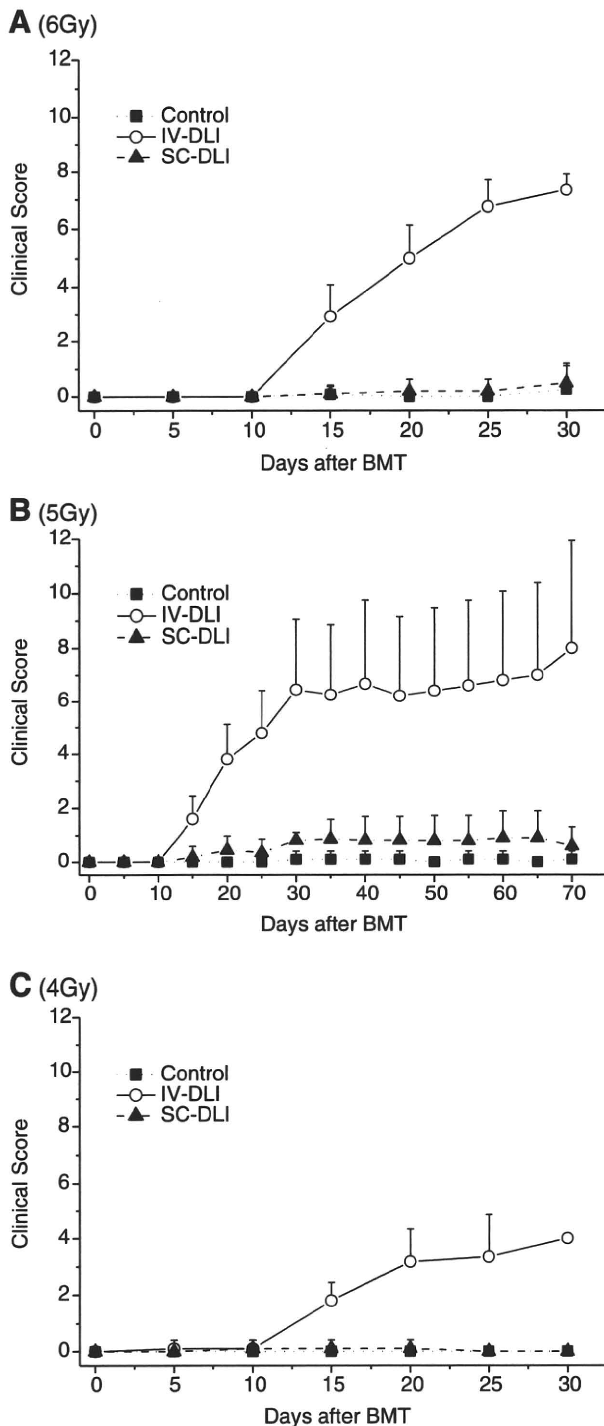

FIG. 4. SC-DLI significantly ameliorates GVHD. Recipient mice were irradiated at 6 Gy **(A)**, 5 Gy **(B)** or 4 Gy **(C)** on day −1. After BMT and DLI, the body weight of the recipients was recorded and clinical signs of GVHD were assessed every 5 days. The clinical scoring was based on 6 parameters: weight loss, posture, activity, fur texture, skin integrity, and diarrhea. $n \geq 10$. GVHD, graft-versus-host disease.

organs and tissues [27,31]. Li and colleagues claimed that the anti-CD3 treatment as precondition reduced donor T-cell infiltration of GVHD target tissues and prevented GVHD [32]. These results indicate that GVHD may be reduced by infiltration block. Moreover, the results of GVHD could be predicted by the analysis of donor T-cell infiltration to specific tissues. First, the spleens of the recipients were weighed on day 5. The means and SDs in the control group, IV-DLI group, and SC-DLI group were 50.25 ± 5.19, 93.00 ± 15.19, and 46.67 ± 5.69 mg, respectively. The spleens in the IV-DLI group were significantly heavier than in the other two groups (Fig. 6A). We also compared the percentage of donor T cells in the recipient spleen and liver at 5 days after BMT. The percentages of donor T cells in the spleen and liver of the recipients in the IV-DLI group were much higher than in the control group and the SC-DLI group (Fig. 6B, C). These results suggest that the splenocytes administered by SC inhibited donor T-cell migration into the GVHD target tissues more effectively than those injected by the conventional IV method.

Discussion

In the present study, we have shown that IV-DLI enhances the dominance of donor hematopoiesis but induces uncontrollable GVHD, followed by death due to infection. On the other hand, SC-DLI enhances the dominance of donor hematopoiesis and induces mild GVHD, which is controllable.

Adoptive immunotherapy with DLI has provided one of the most effective methods after allo-BMT as a treatment and prophylaxis of relapse in the setting of a nonmyeloablative conditioning regimen. DLI is also carried out as a combined method with BMT to convert mixed chimerism to full donor chimerism [17,18,26]. However, the donor T cells administered via the conventional IV route are associated with a major immune-mediated complication, namely GVHD. And the most severe form of GVHD has a high risk of transplant-related mortality [19,21–23,25]. Here we have demonstrated that, when a large dose of donor splenocytes (5×10^7 per mouse) is used as IV-DLI with IBM-BMT, the dominance of the donor hematopoiesis is obtained, but high mortality is induced at different irradiation doses. On the other hand, SC-DLI could enhance the dominance of the donor hematopoiesis and induce mild GVHD, which is controllable.

In the SC-DLI group, we did not find severe infiltration of lymphocytes in the main GVHD target organs, such as the liver, skin, and intestine, even at the site of injection in the skin, but we did find the mild infiltration of cells in the organs. As we found very few T cells in the organs in the control group, the T cells infiltrating the organs in the SC-DLI or IV-DLI group should be the injected donor T cells. Thus, the difference in the severity of the GVHD depended on the injection route of donor spleen cells. It has been reported that the injected T cells go into the spleen, proliferate there, and then migrate into the target organs of GVHD [33]. Therefore, the severity of GVHD could be predicted by the number of donor T cells in the recipient spleen at several days after DLI. In our experiment, we found a much greater number of donor T cells in the spleen in the IV-DLI group than in the SC-DLI or control group. In the IV-DLI group, severe GVHD was observed clinically and pathologically. Namely, many lymphocytes infiltrated the target organs, such as the skin, liver, and intestine. However, there were only a few lymphocytes in the target organs in the control group and the SC-DLI group, even at the site of injection in the skin. As shown in previous reports [27,31], the mice

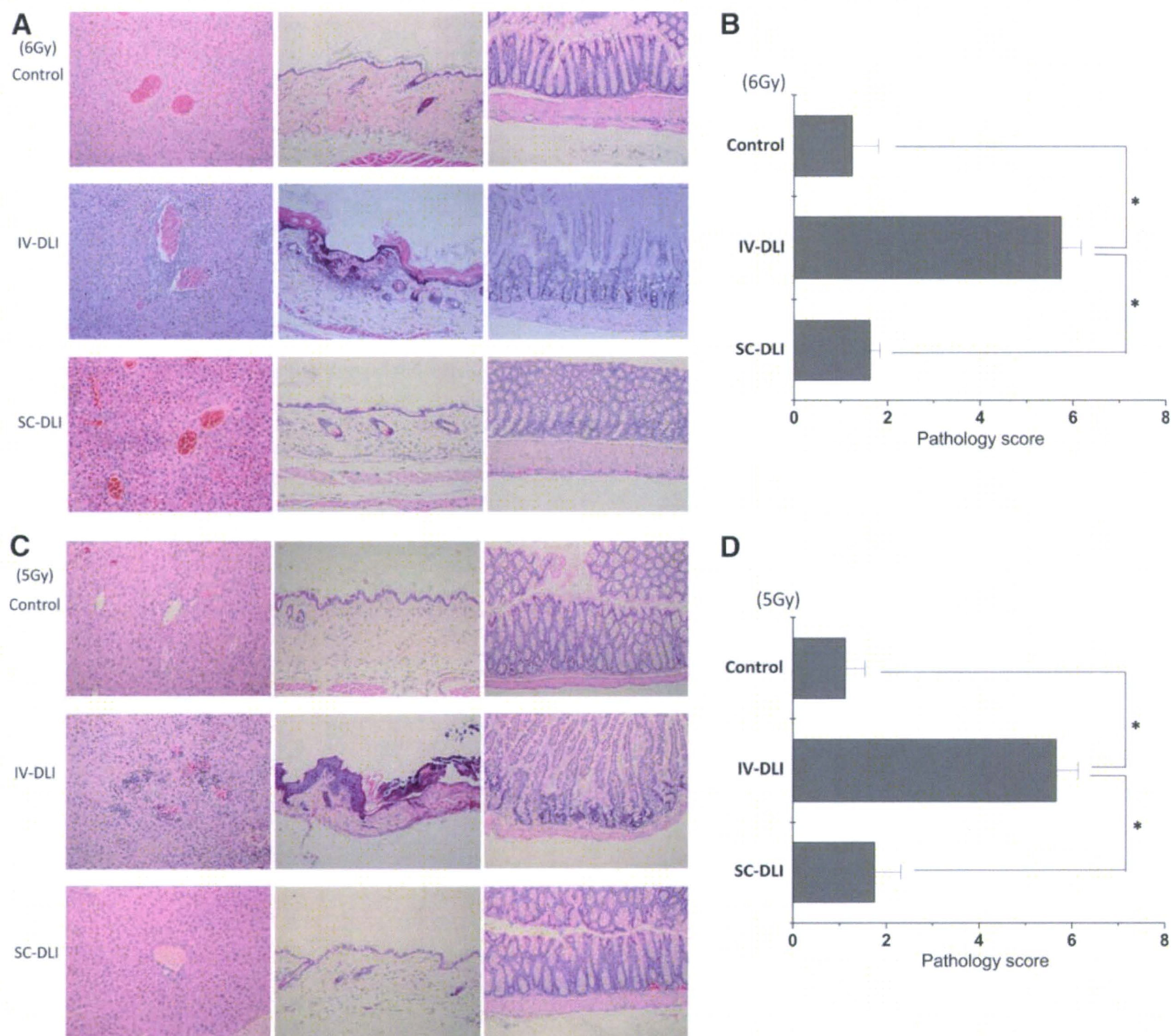

FIG. 5. Histopathological score and representative photographs of GVHD target tissues: liver, skin, and intestine. Autopsies were performed on mice that had died (IV-DLI group) or had been sacrificed at 3 months after BMT (control group and SC-DLI group). Tissues from GVHD target organs (liver, skin, and intestine) were prepared for histopathological scoring. For the SC-DLI group, the skin from the site of the injection is shown. Representative photographs of 6 Gy **(A)** and 5 Gy **(C)** irradiated mice are shown in the left panels. The right panels **(B** and **D)** show the pathological scores by means ± standard deviation: $n \geq 4$; $*P < 0.05$.

in the control or SC-DLI group, which had few donor T cells in the spleen and liver, showed no or only mild GVHD. These results suggest that the subcutaneous injection of donor T cells may disturb the infiltration of the T cells.

In the conventional IV infusion method, all the splenocytes enter blood circulation within a short time. These donor splenocytes will then migrate to lymphoid tissues within hours after the injection. And the initial location of the donor splenocytes in the peripheral lymph nodes is not dependent on recipient conditioning or allogeneic disparity [34,35]. The donor allogeneic T cells expand in an explosive manner in lymphoid tissues within 2–3 days, followed by homing and reexpanding in the GVHD target organs [27,34,35]. As a result, the explosively expanded donor T cells in the GVHD target organs induce serious GVHD.

In the SC-DLI group, we also confirmed that donor T cells played an important role in the reconstitution (data not shown); after T-cell depletion, the donor splenocytes lost the ability to reconstitute hematopoiesis with donor-derived cells. In murine allo-BMT, it is well known that donor $CD8^+$ and $CD4^+$ T cells are activated by major histocompatibility complex (MHC) I and MHC II antigen-presenting cells (APCs) (both host- and donor-derived) separately and mediate GVHD in two different ways. The host-derived APCs play a more important role in initiating GVHD than donor APCs [36]. Langerhans cells, a subtype of dendritic cells found only in the skin-draining lymph nodes, show high surface levels of MHC II [37]. Therefore, in the SC-DLI group, only the $CD4^+$ population of T cells can be effectively activated by the Langerhans cells, and the degree of the GVHD is reduced. On

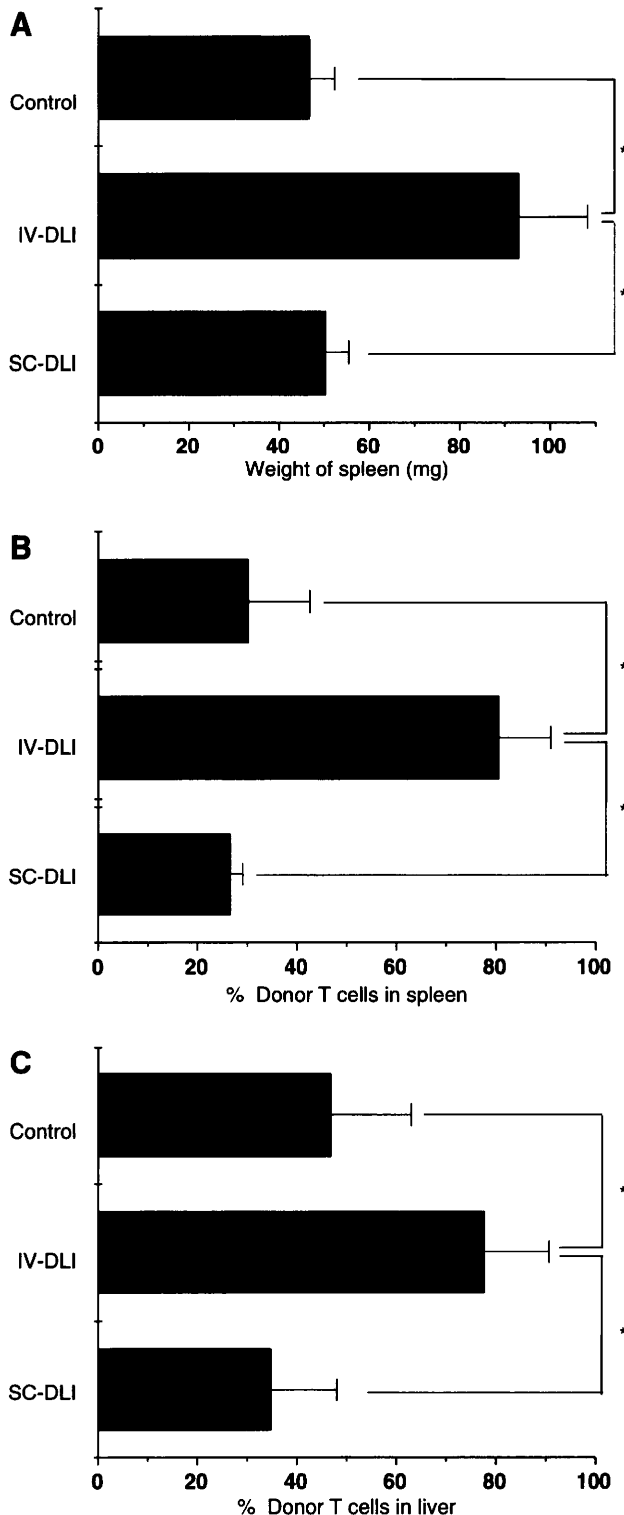

FIG. 6. SC-DLI inhibited donor T-cell infiltration of the GVHD target tissues. Five days after BMT and DLI, the weight of the spleen **(A)** and the percentage of donor T cells in the spleen **(B)** and liver **(C)** were compared between groups. Control group: $n = 3$; IV-DLI group and SC-DLI group: $n = 4$; $^{*}P < 0.05$.

the other hand, as we know, subcutaneous injection is widely used as a long-acting depot injection method, and the skin, which is a physical obstacle, can localize the large number of donor splenocytes (5×10^7/0.2 mL) in a not-so-small area (about 7 cm^2, measured after injection). The speed at which donor T cells migrate into the recipient's lymphoid tissues is limited by the physical obstacle. Therefore, the downstream of the GVHD process will be slowed down. However, donor T cells appear to migrate in a long-lasting way and the development of GVHD is thus at a low-enough level to be controllable. Moreover, the subpopulation of donor T cells and the mechanisms underlying the reduction of GVHD in the SC-DLI group should be further investigated.

In this experiment, we have shown that SC-DLI can accelerate the replacement of recipient cells and reduce the severity of GVHD. Therefore, SC-DLI is more controllable than IV-DLI in allo-BMT and should thus be an easy and safe method for performing allo-BMT.

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Author Disclosure Statement

No competing financial interests exist.

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ORIGINAL RESEARCH REPORTS

Effects of Allogeneic Hematopoietic Stem Cell Transplantation Plus Thymus Transplantation on Malignant Tumors: Comparison Between Fetal, Newborn, and Adult Mice

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We have recently shown that allogeneic intrabone marrow–bone marrow transplantation + adult thymus transplantation (TT) is effective for hosts with malignant tumors. However, since thymic and hematopoietic cell functions differ with age, the most effective age for such intervention needed to be determined. We performed hematopoietic stem cell transplantation (HSCT) using the intrabone marrow method with or without TT from fetal, newborn, and adult B6 mice ($H\text{-}2^b$) into BALB/c mice ($H\text{-}2^d$) bearing Meth-A sarcoma ($H\text{-}2^d$). The mice treated with all types of HSCT + TT showed more pronounced regression and longer survival than those treated with HSCT alone in all age groups. Those treated with HSCT + TT showed increased numbers of $CD4^+$ and $CD8^+$ T cells but decreased numbers of Gr-1/Mac-1 myeloid suppressor cells and decreased percentages of FoxP3 cells in $CD4^+$ T cells, compared with those treated with HSCT alone. In all mice, those treated with fetal liver cell (as fetal HSCs) transplantation + fetal TT or with newborn liver cell (as newborn HSCs) transplantation (NLT) + newborn TT (NTT) showed the most regression, and the latter showed the longest survival. The number of Gr-1/Mac-1 cells was the lowest, whereas the percentage of $CD62L^-CD44^+$ effector memory T cells and the production of interferon γ (IFN-γ) were highest in the mice treated with NLT + NTT. These findings indicate that, at any age, HSCT + TT is more effective against cancer than HSCT alone and that NLT + NTT is most effective.

Introduction

Allogeneic bone marrow transplantation (BMT) has been used to treat not only leukemias, immunodeficiencies, and autoimmune diseases but also solid malignant tumors [1,2], as the graft versus tumor effect induced by its alloreactivity can be anticipated in the case of malignant tumors. Although donor lymphocyte infusion is used for this purpose [3,4], graft versus host disease (GVHD), which is one of the major lethal side effects of allogeneic BMT, may occur [5,6].

We have recently developed a new BMT method, intrabone marrow (IBM)-BMT, in which bone marrow cells (BMCs) are directly injected into the bone marrow cavity [7]. IBM-BMT results in a reduced incidence of GVHD and greater engraftment of donor cells, including mesenchymal stem cells, than the conventional intravenous method [8,9].

We have also developed a BMT method in conjunction with thymus transplantation (TT). The combination of BMT and TT is effective in restoring donor-derived T cell function in aged, chimeric-resistant, tumor-bearing, supralethally irradiated, and low-dose irradiated mice and also in mice injected with a small number of BMCs [10–13]. We have further demonstrated that IBM-BMT + TT is effective for tumor regression and long-term survival [14,15].

However, hematopoietic cell and thymic functions differ with age. The proliferative activity of T cells from the fetal and newborn thymus is much higher than in those from adults [16,17], whereas the level of cytokine production increases with age [18]. In this regard, we have recently found that supralethally irradiated mice are rescued by [newborn liver cell transplantation (NLT) + newborn TT (NTT)] more efficiently than by [BMT + adult TT (ATT)] or [fetal liver cell transplantation (FLT) + fetal TT (FTT)] [12]. In the present study, we investigated the most effective donor age for [hematopoietic stem cell transplantation (HSCT) + TT] for tumor-bearing hosts.

Materials and Methods

Mice

Female 6- to 8-week-old, newborn (≤48 h after birth), and fetal day-16 C57BL/6 (B6) ($H\text{-}2^b$) and BALB/c ($H\text{-}2^d$) mice

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were obtained from Shimizu Laboratory Supplies and maintained until use in our animal facilities under specific pathogen-free conditions. All protocols for these animal experiments were performed in accordance with the Guidelines for Animal Experimentation, Kansai Medical University, and received approval from the Committee of Animal Experiments.

Cell lines

Meth A cells (H-2^d) were derived from methylcholanthrene-induced sarcomas in BALB/c mice [14]. Cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum with antibiotics.

Inoculation of tumor cells

One day before the inoculation of tumor cells, the recipients (BALB/c mice) underwent total-body irradiation (3 Gy) using a ^{137}Cs irradiator (Gammacell 40 Exactor; MDS Nordion International). The next day, 2×10^6 Meth A cells were subcutaneously inoculated into the right flank of these mice.

HSCT and TT

Recipient BALB/c mice with tumors were irradiated (8 Gy) using the ^{137}Cs irradiator 1 day before HSCT. The next day, these mice were injected with 1×10^7 B6 HSCs using the IBM-BMT method. Briefly, single-cell suspensions (1×10^7) were directly injected into the bone marrow cavity of the tibia [7]. BMCs were collected from the femurs and tibias of 6- to 8-week-old B6 mice. Newborn and fetal livers were obtained from the mice. Single-cell suspensions as newborn liver cells and fetal liver cells were prepared for use of HSCs [10]. For TT, AT, NT, and FT tissues were obtained from mice of the above ages. One quarter of the AT, or one NT or one FT, were simultaneously transplanted under the renal capsule in some recipients with HSCT. TT alone was also performed in other mice.

Histology

Histological studies were performed in the liver, intestine (for evaluation of GVHD), and engrafted thymus from the recipients 4 weeks after the BMT. The tissues were fixed in 10% formaldehyde and embedded in paraffin. Serial tissue sections (4 μm thick) were prepared and stained using hematoxylin and eosin.

Analysis of surface marker antigens and intracellular FoxP3 and cytokines by flow cytometry

Surface markers on lymphocytes from the spleen were analyzed by 3-color fluorescence staining using a FACScan system (BD Pharmingen, Franklin Lakes, NJ). Fluorescein isothiocyanate (FITC)-conjugated anti-H-2K^b (BD Pharmingen) mAbs and phycoerythrin (PE)-conjugated anti-H-2K^d mAbs were used to determine chimerism. FITC, PE, or biotin-conjugated CD4, CD8, B220, Gr-1, CD11b, CD44, or CD62L (BD Pharmingen) were used to analyze spleen cell subsets. Avidin-PE-Cy5 (Dako) was used as the third color in the avidin/biotin system. Intracytoplasmic FoxP3 staining was performed using an eBioscience FITC-anti mouse/rat FoxP3 staining set in accordance with the manufacturer's instructions (eBioscience, San Diego, CA). Intracellular cytokines [interleukin 2 (IL-2), IL-4, IL-10, IL-17, interferon γ (IFN-γ), and tumor necrosis factor] were detected using an Intracellular Cytokine Staining Kit in accordance with the manufacturer's instructions (Becton Dickinson).

Mitogen responses

To analyze lymphocyte function, mitogen responses were examined in chimeric mice 2 months after the transplantation. For mitogen response, a total of 2×10^5 splenocytes collected from chimeric mice and nontreated B6 and BALB/c mice as responders were plated in 96-well flat-bottomed plates (Corning Glass Works, Corning, NY) containing 200 μL of RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 2 μL of glutamine (Wako Pure Chemicals, Osaka, Japan), penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% heat-inactivated fetal calf serum. For mitogen responses, responder cells were incubated with 2.5 μg/mL of concanavalin A (ConA) (Calbiochem, San Diego, CA) or 25 μg/mL of lipopolysaccharide (LPS) (Difco Laboratories, Sparks, MI) for 48 or 72 h. During the last 18 h of the culture period, 20 mL of 0.5 μCi ^{3}H-thymidine (^{3}H-TdR; New England Nuclear) was introduced. Incorporation of ^{3}H-TdR was measured using Microbeta Tri-Lux (PerkinElmer, Waltham, MA). The stimulation index was calculated as the average of ^{3}H-TdR incorporation in triplicate samples of responding cells with mitogen/^{3}H-TdR incorporation of responding cells in medium alone.

Statistical analyses

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

Results

Chimerism and tumor size

To examine the effects of HSCT + TT from various ages in tumor-bearing hosts, we performed BMT ($n = 8$), BMT + ATT ($n = 10$), NLT ($n = 10$), NLT + NTT ($n = 8$), FLT ($n = 10$), or FLT + FTT ($n = 8$) in mice-bearing Meth-A sarcomas measuring >0.5 cm^2. All mice treated with HSCT showed donor BMC-derived chimerism (data not shown). In analyses of tumor size, all of the mice treated with HSCT showed significant tumor regression compared with the nontreated controls ($n = 9$) (Fig. 1A, B). Interestingly, the tumors were significantly smaller in the mice treated with HSCT + TT than in those treated with HSCT alone in all age groups (Fig. 1B). The mice treated with either NLT + NTT or FLT + FTT showed the greatest degree of tumor regression (Fig. 1A).

Survival period

We also examined the survival period (Fig. 1C). As expected, nontreated control mice bearing tumors showed the shortest survival period. Similar to tumor size, survival in the mice treated with HSCT + TT was significantly prolonged compared with those treated with HSCT alone in all age groups. However, in contrast to tumor size, mice treated with NLT + NTT showed the longest survival, followed by

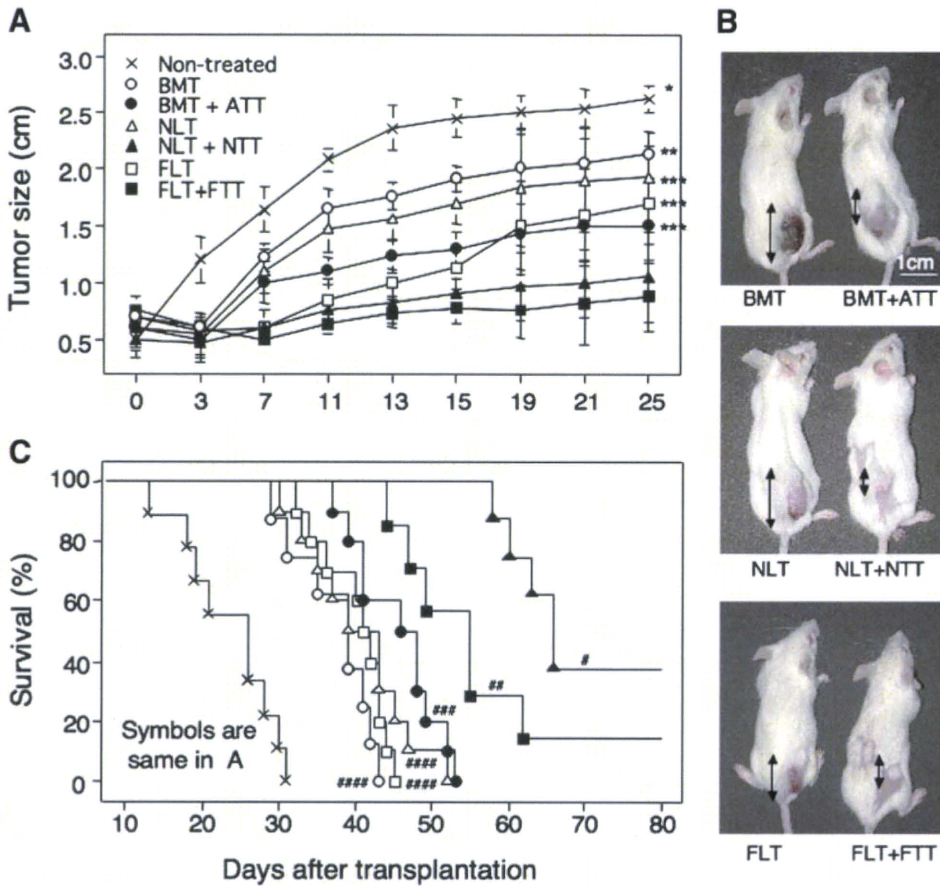

FIG. 1. Tumor size and survival rate in tumor-bearing mice treated with hematopoietic stem cell transplantation (HSCT) and thymus transplantation (TT) from various ages. Tumor size (**A**, **B**: representative data) and survival rate **(C)** are shown in tumor-bearing mice treated with bone marrow transplantation (BMT), BMT + adult TT (ATT), newborn liver cell transplantation (NLT), NLT + newborn TT (NTT), fetal liver cell transplantation (FLT), FLT + fetal TT (FTT), and nontreated controls. $^*P < 0.03$ compared with BMT, BMT + ATT, NLT, NLT NTT, FLT, and FLT + FTT. $^{**}P < 0.03$ compared with BMT + ATT, NLT + NTT, and FLT + FTT. $^{***}P < 0.03$ compared with NLT + NTT and FLT + FTT. $^{\#}P < 0.04$ compared with nontreated control, BMT, BMT + ATT, NLT, FLT, and FLT + FTT. $^{\#\#}P < 0.02$ compared with nontreated control and BMT, BMT + ATT, NLT, and FLT. $^{\#\#\#}P < 0.01$ compared with nontreated control and BMT. $^{\#\#\#\#}P < 0.001$ compared with nontreated control. Nontreated ($n = 9$), BMT ($n = 8$), BMT + ATT ($n = 10$), NLT ($n = 10$), NLT + NTT ($n = 8$), FLT ($n = 10$), FLT + FTT ($n = 8$). Data are shown as means ± standard deviation (SD). Double-headed arrows show tumor size.

those treated with FLT + FTT, and then those treated with BMT + ATT. All the mice treated with TT alone from any age died within 3 weeks after transplantation.

Analyses of TT

We next analyzed the thymus of the mice treated with HSCT + TT from various ages 4 weeks after transplantation. The size was smallest in ATT, followed by NTT, but largest in FTT (Fig. 2). Histologically, although both the cortex and medular areas were clearly shown, the ratio of cortex/medulla in TT was also smallest in ATT, followed by NTT, but largest in FTT. In analyses of thymocyte subsets, the highest percentage of $CD4^+$ or $CD8^+$ single-positive thymocytes was observed in ATT, followed by NTT, but lowest in FTT. Conversely, the percentages of $CD4^+$ and $CD8^+$

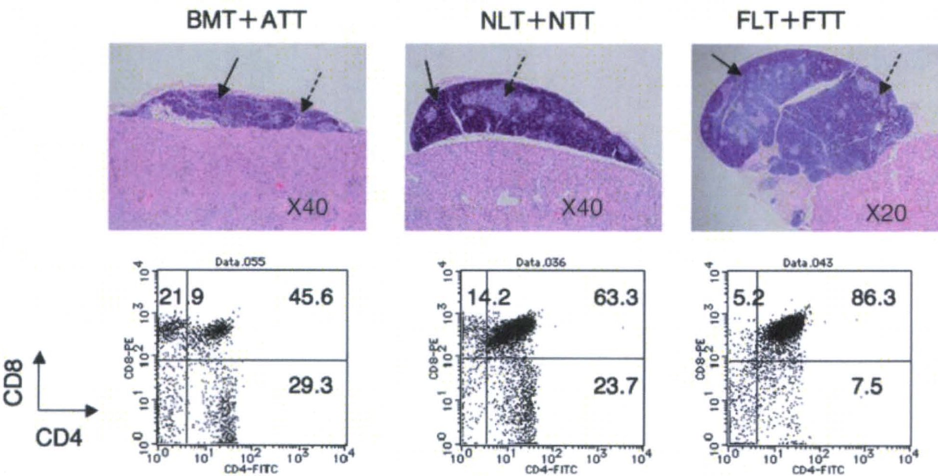

FIG. 2. Macroscopic and histological findings and FACS profiles of thymocytes in the transplanted thymus from tumor-bearing mice treated with HSCT and TT from various ages. Histological findings (*upper panels*, HE staining) and the FACS profiles of $CD4^+$ and $CD8^+$ cells in thymocytes (*lower panels*) from the transplanted thymus in tumor-bearing mice treated with BMT + ATT, NLT + NTT, or FLT + FTT (*lower panels*). *Plain arrows*, cortex; *dotted arrows*, medulla. Representative data from 4 experiments are shown.

double-positive and CD4⁻ and CD8⁻ double-negative thymocytes were lowest in ATT, followed by NTT, but highest in FTT.

Analyses of lymphocyte subsets

We investigated donor-derived lymphocyte subsets in the spleen 4 weeks after transplantation in the mice treated with HSCT and 3 weeks in the nontreated controls due to early death. The number of $CD4^+$ T cells significantly increased in the mice treated with HSCT + TT compared with those treated with HSCT alone at all ages (Fig. 3A). The numbers were highest in the mice treated with either NLT + NTT or FLT + FTT and were comparable to those of normal B6 mice. Those treated with BMT showed the lowest, although the analysis day was different from that of nontreated control. The results of $CD8^+$ T cells were similar to those of $CD4^+$ T cells except that, at all ages, they were lower than those of normal B6 mice.

The percentage of $FoxP3^+$ cells in $CD4^+$ T cells, which reflects the immunosuppressive activity [19], was the highest in the nontreated controls (Fig. 3B). The percentage of cells significantly decreased in the mice treated with HSCT + TT compared with those treated with HSCT alone in all age groups. However, the percentage was not different between the ages, and the level in the mice treated with HSCT + TT was comparable to that in normal B6 mice.

The number of Gr-1/Mac-1 myeloid suppressor cells, which are induced in hosts bearing cancer and inhibit immune function [20,21], was highest in nontreated control mice (Fig. 3D). It significantly decreased in the mice treated with HSCT + TT compared with those treated with HSCT alone, in all age groups. Interestingly, the mice treated with FLT or FLT + FTT showed the highest number of cells among the groups for HSCT or HSCT + TT. As expected, normal B6 mice showed only a few of these cells.

Analyses of proportions of effector, central memory, and naïve T cells

T cells can be functionally divided into $CD62L^-CD44^-$ naïve T cells and $CD62L^+CD44^+$ central memory (CM) and $CD62L^-CD44^+$ effector memory (EM) cells from prestimulation to terminal differentiation [22,23]. We, therefore, examined the proportion of these cells in both CD4 and CD8 subsets of T cells (Fig 4). The nontreated control mice showed a significant elevation of EM T cell number but a reduced number of CM T cells in both subsets compared with B6 mice. Interestingly, the mice treated with HSCT + TT also showed significant elevation of EM T cells but a reduction of CM T cells, compared with those treated with HSCT alone. Among all mice, those treated with NLT + NTT showed the highest % of EM T cells and the lowest % of CM T cells in both subsets.

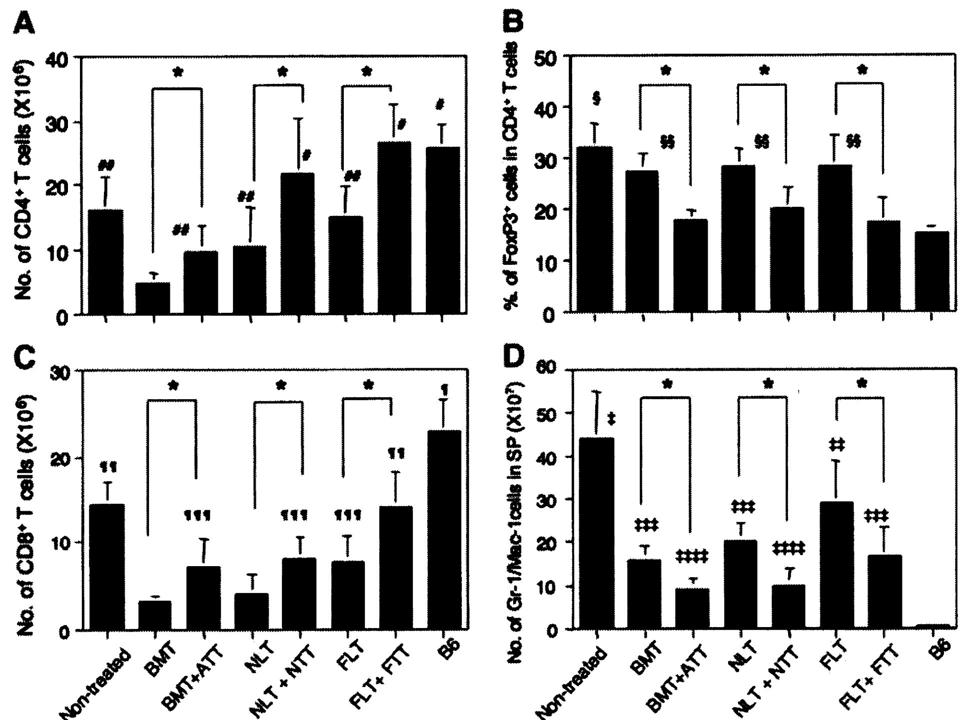

FIG. 3. Numbers of cells in the spleen from tumor-bearing mice treated with HSCT and TT from various ages. Numbers of $CD4^+$ T cells **(A)**, percentage of $FoxP3^+$ cells in $CD4^+$ T cells **(B)**, numbers of $CD8^+$ T cells **(C)**, and Gr-1/CD11b cells **(D)** in the spleen were evaluated in tumor-bearing mice treated with BMT, BMT + ATT, NLT, NLT + NTT, FLT, or FLT + FTT, nontreated controls, or B6 mice. The experiments were performed 4 weeks after transplantation in the mice treated with HSCT and 3 weeks in the nontreated controls because of early death. $^*P < 0.05$. $^\#P < 0.05$ compared with nontreated control, BMT, BMT + ATT, NLT, or FLT. $^{\#\#}P < 0.03$ compared with BMT. $^{\S}P < 0.05$ compared with nontreated control, BMT, BMT + ATT, NLT, NLT + NTT, FLT, FLT + FTT, or B6 mice. $^{\S\S}P < 0.05$ compared with BMT + ATT, NLT + NTT, FLT + FTT, or B6 mice. $^{\P}P < 0.02$ compared with nontreated control, BMT, BMT + ATT, NLT, NLT + NTT, FLT, or FLT + FTT. $^{\P\P}P < 0.05$ compared with BMT, BMT + ATT, NLT, NLT + NTT, or FLT. $^{\P\P\P}P < 0.05$ compared with BMT, or NLT. $^{\dagger}P < 0.05$ compared with BMT, BMT + ATT, NLT, NLT + NTT, FLT, FLT + FTT, or B6 mice. $^{\dagger\dagger}P < 0.05$ compared with BMT, BMT + ATT, NLT, NLT + NTT, FLT + FTT, or B6 mice. $^{\dagger\dagger\dagger}P < 0.05$ compared with BMT + ATT, NLT NTT, or B6 mice. $^{\dagger\dagger\dagger\dagger}P < 0.05$ compared with B6 mice. Nontreated ($n = 4$), BMT ($n = 4$), BMT + ATT ($n = 4$), NLT ($n = 5$), NLT + NTT ($n = 4$), FLT ($n = 5$), FLT + FTT ($n = 4$), B6 mice ($n = 4$). Data are shown as means ± SD.

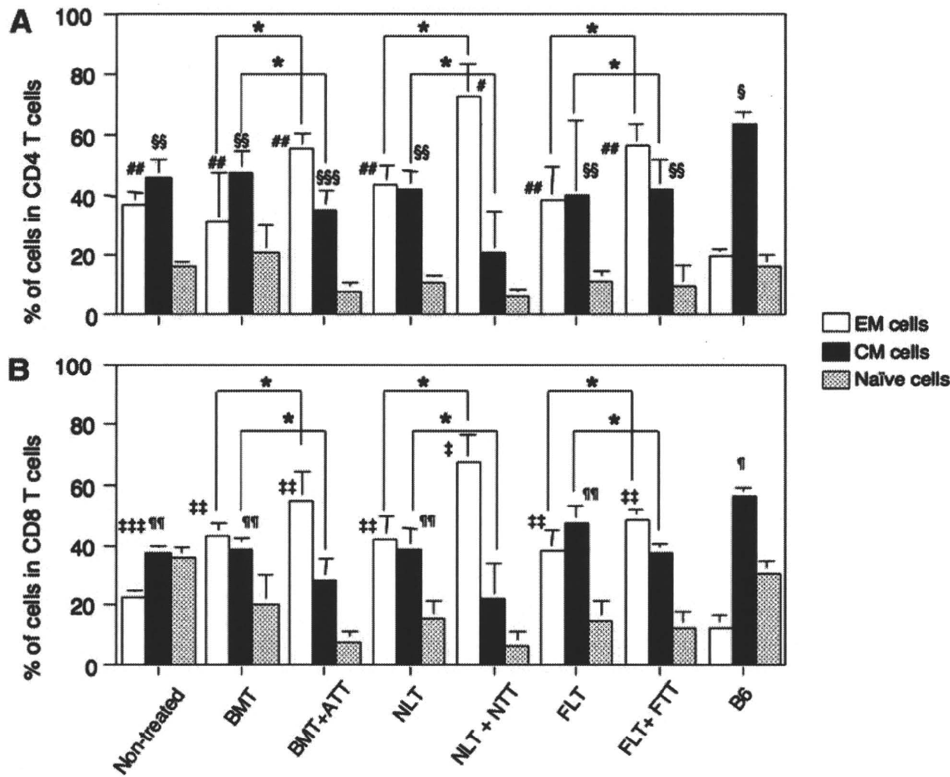

FIG. 4. Proportions of effector memory (EM), central memory (CM), and naïve T cells from tumor-bearing mice treated with HSCT and TT from various ages. Percentages of $CD62L^{-}CD44^{+}$ EM, $CD62L^{+}CD44^{+}$ CM, and $CD62L^{-}CD44^{-}$ naïve T cells in $CD4^{+}$ **(A)** and $CD8^{+}$ **(B)** subsets were analyzed in the spleens from tumor-bearing mice treated with BMT, BMT+ ATT, NLT, NLT + NTT, FLT, or FLT + FTT, or nontreated control, or B6 mice. Analyses were performed at the same time for the experiment of Fig. 3. $^{*}P < 0.05$. $^{\#}P < 0.05$ compared with nontreated control, BMT, BMT + ATT, NLT, FLT, FLT + FTT, or B6 mice. $^{\#\#}P < 0.03$ compared with B6 mice. $^{\S}P < 0.03$ compared with nontreated control, BMT, BMT + ATT, NLT, or NLT + NTT, FLT, or FLT + FTT. $^{\S\S}P < 0.03$ compared with BMT + ATT, or NLT + NTT. $^{\S\S\S}P < 0.03$ compared with NLT + NTT. $^{\dagger}P < 0.03$ compared with nontreated control, BMT, BMT + ATT, NLT, FLT, FLT + FTT, or B6 mice. $^{\dagger\dagger}P < 0.03$ compared with nontreated control and B6 mice. $^{\dagger\dagger\dagger}P < 0.03$ compared with B6 mice. $^{\P}P < 0.03$ compared with nontreated control, BMT, BMT + ATT, NLT, NLT + NTT, FLT, or FLT + FTT. $^{\P\P}P < 0.03$ compared with BMT + ATT, NLT + FTT, or FLT + FTT. Nontreated ($n = 4$), BMT ($n = 4$), BMT + ATT ($n = 4$), NLT ($n = 5$), NLT + NTT ($n = 4$), FLT ($n = 5$), FLT + FTT ($n = 4$), and B6 mice ($n = 4$). Data are shown as means ± SD.

Analyses of lymphocyte function and cytokine production

Finally, we examined lymphocyte function by monitoring mitogen response (ConA for T cells and LPS for B cells) and cytokine production. The mice treated with HSCT + TT showed a significantly elevated response to ConA but not LPS, compared with those treated with HSCT alone and with the nontreated controls, although the levels did not reach those of normal B6 mice (Fig. 5A). The stimulator index in mice treated with either FLT + FTT or NLT + NTT was significantly higher than in those treated with BMT + ATT.

The mice treated with HSCT + TT showed significantly elevated production of IL-2 and IFN-γ compared with those treated with HSCT alone (Fig. 5B). However, the production of IL-2 did not significantly differ between those treated with HSCT + TT in all age groups, and the levels were comparable to that in normal B6 mice. In contrast, the production of IFN-γ was highest in the mice treated with NLT + NTT, and the levels were comparable to that in normal B6 mice. IL-4, IL-10, IL-17, and tumor necrosis factor levels were almost undetectable and did not correlate with any clinical findings (survival and tumor regression). The above results are summarized in Table 1. Those treated with NLT + NTT showed the highest T cell numbers and functions.

Discussion

In the present study, we have examined the effects of allogeneic HSCT + TT from various ages on tumor-bearing hosts. Although the mice treated with all types of HSCT + TT showed more tumor regression with prolonged survival compared with those treated with HSCT alone, those treated with NLT + NTT or FLT + FTT showed the best regression. The mice treated with NLT + NTT showed a longer survival period than those treated with FLT + FTT. Those treated with all types of HSCT + TT showed higher numbers of both $CD4^{+}$ and $CD8^{+}$ T cells and percentage of EM cells and a lower number of $Gr\text{-}1^{+}/CD11b^{+}$ myeloid suppressor cells and percentage of $FoxP3^{+}/CD4$ T cells than those treated with HSCT alone. Interestingly, those treated with NLT + NTT showed the highest T cell numbers and lowest suppressor cell numbers. These findings indicated that HSCT + TT is effective for hosts with cancer and that the combination of NLT + NTT is best at all ages.

Although the mice treated with HSCT + TT showed greater tumor regression with more prolonged survival than those treated with HSCT alone, these results differed according to ages. In all age groups, FLT + FTT or NLT + NTT showed the best results, and the latter showed the longest survival period. Since there were no differences with HSCT alone in any age group, the transplanted thymus may also play an important role. Although the liver stem cells may

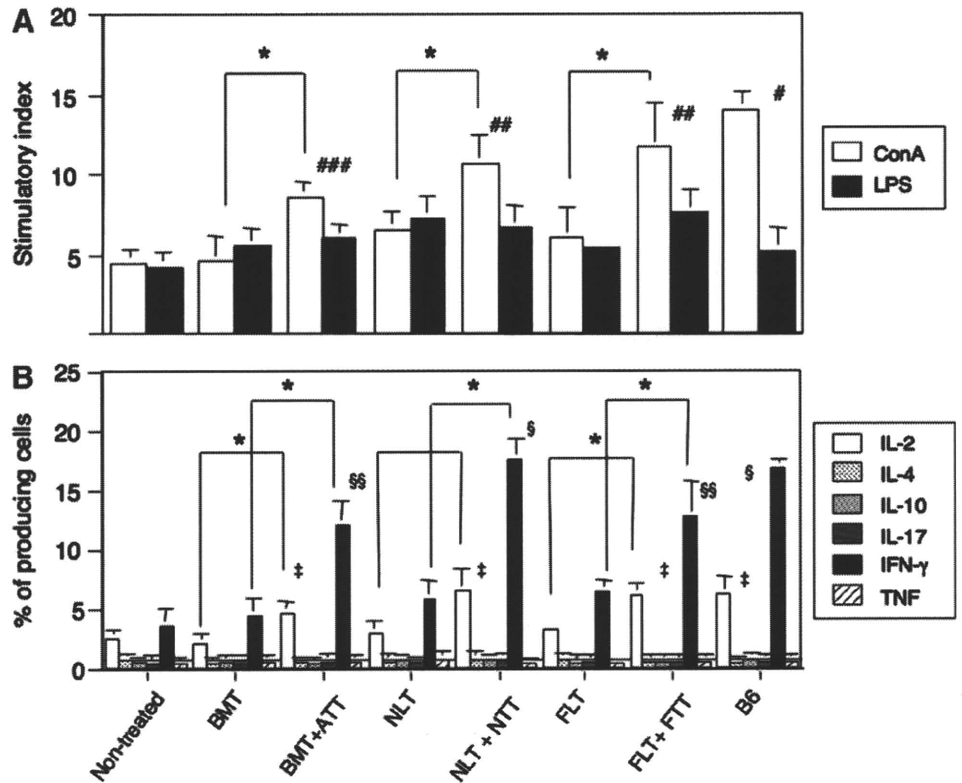

FIG. 5. Mitogen responses and percentages of cytokine-producing cells in the spleens from tumor-bearing mice treated with HSCT and TT from various ages. Mitogen responses: concanavalin A (ConA) and lipopolysaccharide (LPS) **(A)** and percentages of cytokine-producing cells **(B)** in the spleen were evaluated in the spleens from tumor-bearing mice treated with BMT, BMT ATT, NLT, NLT + NTT, FLT, or FLT + FTT, or nontreated control, or B6 mice. Analyses were performed at the same time for the experiment of Fig. 3. $^{*}P < 0.05$. $^{\#}P < 0.03$ compared with nontreated control, BMT, BMT + ATT, NLT, NLT NTT, FLT, or FLT + FTT. $^{\#\#}P < 0.03$ compared with nontreated control, BMT, BMT + ATT, NLT, or FLT. $^{\#\#\#}P < 0.03$ compared with nontreated control, BMT, NLT, or FLT. $^{\dagger}P < 0.03$ compared with nontreated control, BMT, or NLT, or FLT. $^{\S}P < 0.03$ compared with nontreated control, BMT, BMT + ATT, NLT, FLT, or FLT + FTT. $^{\S\S}P < 0.05$ compared with nontreated control, BMT, NLT, or FLT. Nontreated ($n = 4$), BMT ($n = 4$), BMT + ATT ($n = 4$), NLT ($n = 5$), NLT + NTT ($n = 4$), FLT ($n = 5$), FLT + FTT ($n = 4$), B6 mice ($n = 4$). Data are shown as means ± SD.

influence the results in FLT and/or NLT compared with BMT, the transplanted thymus plays a critical role in the further effects with the elevated T cell function.

We, therefore, analyzed the transplanted thymus. Interestingly, although ATT grafts showed some atrophic features after transplantation, FTT grafts showed marked growth, and NTT grafts showed intermediate growth. Similarly, $CD4^{+}$ and $CD8^{+}$ subsets in the thymocytes of ATT grafts shifted to being relatively mature, whereas those in FTT grafts shifted to being relatively immature, and those in NTT grafts were intermediate. These findings suggested that their characteristics of age-related proliferative activity and maturity may also reflect the transplanted thymus. This may also influence the number, pheno type and function of splenic T cells, as discussed later.

We next analyzed lymphocyte subsets in the spleen from all chimeric mice. The numbers of both $CD4^{+}$ and $CD8^{+}$ T cells significantly increased in the mice treated with HSCT

Table 1. Summary of Data in All Groups[a]

Factors	*BMT*	*BMT + ATT*	*NLT*	*NLT + NTT*	*FLT*	*FLT + FTT*
CD4 T cells	↓	→	→	↑	→	↑
CD8 T cells	↓	→	→	↑	↑	↑↑
% of FoxP3 in CD4 T cells	↓	↓↓	↓	↓↓	↓	↓↓
Gr-1/Mac-1	↓↓	↓↓↓	↓↓	↓↓↓	↓	↓↓
% of EM CD4 T cells	→	↑	→	↑↑	→	↑
% of EM CD8 T cells	↑	↑↑	↑	↑↑↑	↑	↑↑
ConA	→	↑	→	↑↑	→	↑↑
IL-2	→	↑	→	↑	→	↑
IFN-γ	→	↑	→	↑↑	→	↑

[a]Compared with nontreated controls. →, no change; ↑, mild increase, ↑↑, moderate increase; ↑↑↑, strong increase; ↓, mild decrease; ↓↓, moderate decrease; ↓↓↓, strong decrease.

ATT, adult thymus transplantation; BMT, bone marrow transplantation; ConA, concanavalin A; EM, effector memory; FLT, fetal liver cell transplantation; FTT, fetal thymus transplantation; IFN-γ, interferon γ; IL-2, interleukin 2; NLT, newborn liver cell transplantation; NTT, newborn thymus transplantation.

TT, compared with those treated with HSCT alone. The mice treated with FLT + FTT or NLT + NTT showed the highest numbers, suggesting that these T cells may play an important role in prolonging survival and tumor regression. The elevated T cell number may be related to the high proliferative activity of NT or FT. The percentage of $FoxP3^+$ cells in $CD4^+$ T cells significantly decreased in the mice treated with HSCT + TT, compared with those treated with HSCT alone, and the levels were almost the same in all age groups. These findings indicated that $FoxP3^-CD4^+$ effector cells are dominantly supplied from TT grafts compared with $FoxP3^+CD4^+$ regulatory T cells in the allo-environment [14]. However, the level was no less than that in normal mice. Since the low level of regulatory T cells was strongly associated with the induction of GVHD [19,24], the relatively elevated levels in the mice treated with HSCT + TT may lead to the prevention or inhibition of GVHD but not of graft versus tumor [25]. Therefore, we did not observe any obvious findings of GVHD in any of the mice treated with HSCT + TT in this study, although some GVHD and a related loss of $FoxP3^+CD4^+$ regulatory T cells were found in the intensive regimen of a previous study [14].

We have also found that the number of Gr-1/CD11b myeloid suppressor cells is also significantly reduced in mice treated with HSCT + TT compared with those treated with HSCT alone, as previously reported [15]. This may also contribute, at least in part, to the longer survival in the former group compared with the latter. However, the mice treated with FLT or FLT + FTT showed the highest cell numbers of myeloid suppressor cells in the HSCT or HSCT + TT groups. Since the tumor burden was the same in both NLT + NTT and FLT + FTT [26] and it induces the cells, the greater number of myeloid suppressor cells in fetal liver cells should be responsible for the difference in survival.

The percentages of EM T cells, which were derived from CM cells with terminal differentiation and had the strongest immune activity [22,23], increased in all mice with tumors, in contrast to those without tumors. In addition, the mice treated with HSCT + TT also showed a higher percentage of EM cells than those with HSCT alone. Therefore, the elevation of cell numbers may also be partially induced from TT. Interestingly, the percentage of EM cells was highest in NLT + NTT in both CD4 and CD8 T cell subsets. Although the detailed mechanism remains unknown, the T cells from NTT were more proliferative than ATT and more functional than FTT, and this may have led to the high expansion activity of these cells.

The mice treated with HSCT + TT showed significantly greater T cell function (ConA response) than those treated with HSCT alone or the nontreated controls. The mice treated with either FLT + FTT or NLT + NTT showed the greatest ConA response. However, it should be noted that IFN-γ production in those treated with NLT + NTT was highest among all the mice in the present study. This may have been because of the low numbers of Gr-1/Mac-1 cells and/or high numbers of EM T cells, in which IFN-γ is produced at the highest levels in these mice [18,22].

Thus, the results of the present study indicated that additional TT is effective in HSCT from all ages in tumor-bearing hosts and that the combination of NLT + NTT shows the greatest effect. This may have led to the highest T cell function with high levels of IFN-γ production in these mice. Although the detailed mechanism is still unknown and needs further study, the thymocytes from the day-16 FT showed a high proliferative activity with little T cell receptor expression, whereas those from the AT showed a low proliferative activity with steady T cell receptor expression [27]. Those from NT may have an intermediate character, a relative high proliferative activity with specific responses, leading to a favorable effect for the tumor regression and prolonged survival.

Although ethically and technically it may be difficult to obtain newborn human thymus tissue from various donor ages, such tissue could be obtained from patients with congenital heart disease or from aborted fetuses, as previously discussed [28,29]. In addition, we have very recently found that third-party FT can be used to induce tolerance, although it is limited in hosts with low thymic function [30]; otherwise, the grafted thymus should be rejected. In addition, a method of regenerating the thymus has also been developed [31–34]. These findings suggest that HSCT + TT will become a viable strategy for the treatment of malignant tumors in humans.

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Author Disclosure Statement

All authors state that no competing financial interests exist.

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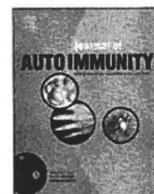

Successful modulation of type 2 diabetes in db/db mice with intra-bone marrow–bone marrow transplantation plus concurrent thymic transplantation

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ABSTRACT

There is increasing evidence that both autoimmune and autoinflammatory mechanisms are involved in the development of not only type 1 diabetes mellitus (T1 DM), but also type 2 diabetes mellitus (T2 DM). Our laboratory has focused on this concept, and in earlier efforts replaced the bone marrow cells (BMCs) of leptin receptor-deficient (db/db) mice, an animal model of T2DM, with those of normal C57BL/6 (B6) mice by IBM–BMT. However, the outcome was poor due to incomplete recovery of T cell function. Therefore, we hypothesized that intra-bone marrow–bone marrow transplantation plus thymus transplantation (IBM–BMT + TT) could be used to treat T2 DM by normalizing the T cell imbalance. Hence we addressed this issue by using such dual transplantation and demonstrate herein that seven weeks later, recipient db/db mice manifested improved body weight, reduced levels of blood glucose, and a reduction of plasma IL-6 and IL-1β. More importantly, this treatment regimen showed normal CD4/CD8 ratios, and increased plasma adiponectin levels, insulin sensitivity, and the number of insulin-producing cells. Furthermore, the expression of pancreatic pAKT, pLKB1, pAMPK and HO-1 was increased in the mice treated with IBM–BMT + TT. Our data show that IBM–BMT + TT treatment normalizes T cell subsets, cytokine imbalance and insulin sensitivity in the db/db mouse, suggesting that IBM–BMT + TT is a viable therapeutic option in the treatment of T2 DM.

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1. Introduction

There is a virtual epidemic of type 2 diabetes, and although the mechanisms for this increase are not entirely clear, it has become the focus of both genetic and environmental research [1]. Clearly, inflammation has a critical role in the development of metabolic diseases, including obesity and T2 DM [1]. Recently, it has been shown that obese adipose tissue activates CD8T cells, resulting in promoting the recruitment and activation of macrophages in the adipose tissue [2]; macrophages have been shown to infiltrate the adipose tissue in mice and humans [3]. Adipocytes regulate and mediate inflammatory cytokines such as tumor necrosis factor-α (TNFα), IL-6, matrixmetalloproteinases (MMPs), peroxisome proliferation activated receptor-r (PPAR-r) and fatty acid-binding protein –4. These cytokines inhibit or enhance each other, and their activities contribute to insulin resistance [4]. As such, both an autoinflammatory as well as an autoimmune response are involved in the pathogenesis of T2 DM.

Bone marrow transplantation (BMT) has been demonstrated to treat hematopoietic disorders, metabolic disorders and autoimmune diseases [5–19]. We have recently found that intra-bone marrow–BMT (IBM–BMT) treatment is an advantageous strategy for allogeneic BMT, compared with conventional intravenous BMT [23], since IBM–BMT can replace not only hemopoietic cells (including hemopoietic stem cells:HSCs) but also stromal cells (including mesenchymal stem cells:MSCs). In addition, we have very recently found that thymus transplantation combined with BMT (BMT + TT) is a powerful strategy to ameliorate thymic involution in recipient mice due to aging or irradiation [20–22].

Based on these findings, we carried out IBM–BMT in combination with newborn thymus transplantation (TT) in db/db mice. We here demonstrate that, after IBM–BMT + TT treatment in db/db

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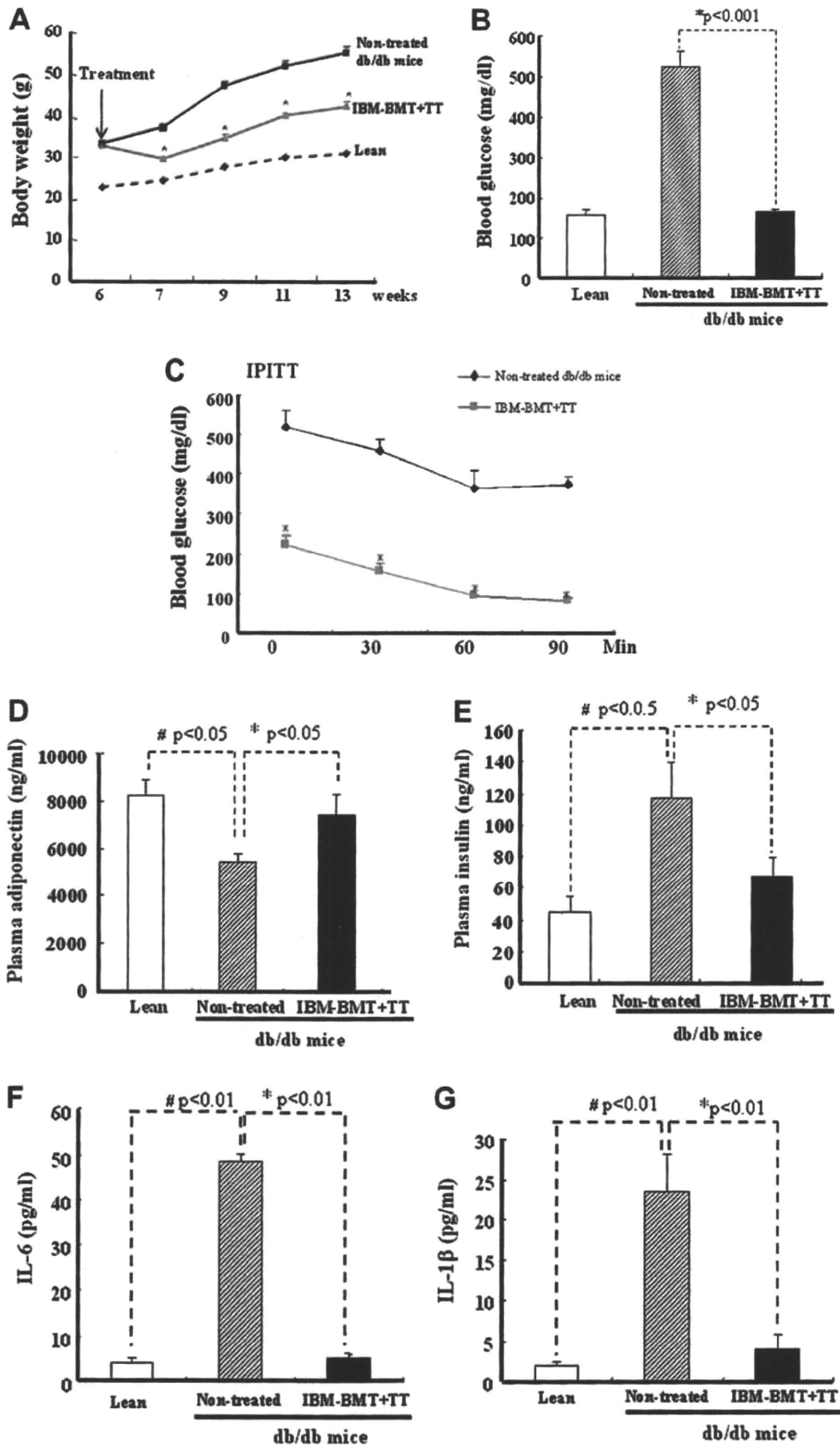

Fig. 1. Body weight, blood glucose levels, insulin sensitivity, and plasma adiponectin and insulin levels 7 weeks after the treatment (at the age of 13 weeks). (A) Body weights are shown. ($*p < 0.05$). (B) Fasting blood glucose levels ($*p < 0.001$). (C) Blood glucose levels after insulin administration ($*p < 0.05$). (D) Plasma adiponectin levels ($\#,*p < 0.05$). (E) Plasma insulin levels ($\#,*p < 0.05$). (F) Plasma IL-6 levels ($\#,*p < 0.01$). (g) Plasma IL-1b levels ($\#,*p < 0.01$). The results are mean ± SE, $n = 6$ in each group.

mice, insulin sensitivity increases and blood glucose levels decrease, resulting from the normalization of balance of lymphocyte subsets and cytokines, followed by enhanced expression of pAKT, pLKB1, pAMPK, insulin receptor phosphorylation and HO-1. This suggests that the maintenance of the balance of lymphocyte subsets and cytokine production by IBM–BMT + TT treatment is essential for the amelioration of T2 DM in db/db mice.

2. Materials and methods

2.1. Animals

Five-week-old BKS.Cg-m+Lepr^{db}/+Lepr^{db}/J (H-2k^{d}) (db/db) mice, BKS. Cg-m+/+Lepr^{db}/J(H-2k^{d}) (lean) mice and C57BL/6 (B6) (H-2k^{b}) mice were purchased from Charles River Laboratories (Yokohama, Japan) and SLC (Shizuoka, Japan) and maintained in animal facilities under specific pathogen-free conditions. All procedures were performed under protocols approved by the Institutional Animal Care and Use Committee at Kansai Medical University. Body weight and blood glucose levels were measured each week. Six-week-old mice with blood glucose levels higher than 250 mg/dl on two consecutive measurements were considered to have the onset of diabetes, and these mice were separated into three groups ($n = 6$ in each group): non-treated, treated with IBM alone, and treated with IBM–BMT + TT. All data were collected at 4 weeks and 7 weeks after treatment. The same experiment was repeated three times.

2.2. IBM–BMT + TT

The mice received fractionated irradiation twice a day (5.0 Gy × 2, 4-hour interval). One day after the irradiation, whole BMCs from B6 mice were injected into the recipient mice (1×10^7/mouse) by IBM–BMT using our previously described method [23]. Simultaneously, the newborn thymus from B6 mice was grafted under the renal capsule of the left kidneys of the recipient mice.

2.3. Flow cytometric analyses

The peripheral blood mononuclear cells were obtained from the tail vein of the recipients 30 days after transplantation. These cells were stained with antibodies against PE-H-2k^{b}, FITC-CD4, FITC-CD8a, FITC-B220 and FITC-CD11b (BD Bioscience Pharmingen, San Diego, CA) for 30 min on ice. After washing twice with 2% FCS/PBS and lysing red blood cells, the 10000 events acquired were analyzed by FACScan (BD Bioscience Pharmingen). Isotype-matched immunoglobulins were used as controls.

2.4. Insulin tolerance test

Insulin tolerance was tested at 7 weeks after treatment. After a 6-h fast, mice were injected intraperitoneally with insulin (2.0 units/kg). Blood samples were taken at various time points (0–90 min) and blood glucose levels were measured.

2.5. Cytokine and insulin measurements

Adiponectin, IL-6, IL-1β and TNF-α were determined in mouse plasma using an ELISA assay (R&D Systems, Inc. MN and Invitrogen Corporation CA). Insulin was measured using an ELISA kit (Morinaga, Yokohama, Japan).

2.6. Immunochemistry

The pancreata, adipose tissue, and livers of the recipients, lean and db mice were removed 2 months after the transplantation. After the tissues were fixed in 10% formalin for 24 h at room temperature, they were embedded in paraffin. The sections (3 μm thickness) were stained with hematoxylin and eosin. To confirm the presence of glycogen deposits, they were stained with Periodic Acid Schiff (PAS) after diastase digestion. The pancreata were stained with polyclonal guinea pig anti-swine insulin antibody (N1542, Dako Cytomation, CA). The stained sections were examined on a microscope. The size of adipocytes was randomly measured using DP2-BSW application software (Olympus, Japan).

2.7. Mitogen response

The spleen was removed from the db/db mice at 7 weeks after treatment. A total of 2×10^5 splenocytes collected from chimeric mice, and untreated B6 and db/db mice as responders, were plated in 96-well plates (Corning Glass Works, Corning, NY) containing 200 μl of RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) including 2 μl glutamine and 10% FCS. Responder cells were incubated with 2.5 μg/ml of Con A (Calbiochem, San Diego, CA) or 25 μg/ml of lipopolysaccharide (Difco Laboratories, Franklin Lakes, NJ) for 72 h. 20 μl of 0.5 μCi of ^{3}[H]-TdR (New England Nuclear, Cambridge, MA) was introduced during the last 18 h. Incorporation of ^{3}[H]-TdR was measured using Microbeta TriLux (Perkinelmer, Wellesley, MA).

2.8. Western blot analysis of pancreata pLKB1, HO-1, AMPK, pAMPK, AKT, pAKT and insulin receptor phosphorylation

At sacrifice, pancreata were dissected, pooled for each mouse and used to measure signaling molecules. Specimens were stored at −140 °C until assayed. Frozen pancreatic tissues were pulverized under liquid nitrogen and placed in a homogenization buffer (mmol/l:10 phosphate buffer, 250 sucrose, 1 EDTA, 0.1 PMSF and 0.1% v/v tergitol, pH 7.5). Homogenates were centrifuged at 27,000×g for 10 min at 4 °C, supernatant was isolated and protein levels were visualized by immunoblotting with antibodies. Antibodies against pLKB1, AMPK, pAMPK, AKT, pAKT and HO-1 were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Antibodies were prepared by dilution of HO-1, pAMPK, pAKT and insulin receptor as we described previously [24,25].

2.9. Statistical analysis

Statistical significance between experimental groups was determined by the Fisher method of analysis of multiple comparisons ($p < 0.05$ was regarded as significant). For comparison between treatment groups, the null hypothesis was tested by either a single-factor ANOVA for multiple groups or unpaired t test for two groups. Differences between experimental groups were evaluated with ANOVA with Bonferroni corrections. Statistical significance was regarded as significant at $p < 0.05$.

3. Results

3.1. Body weight, blood glucose levels, insulin sensitivity, and plasma adiponectin, insulin, IL-6 and IL-1β levels

In our preliminary experiments, we carried out IBM–BMT alone (without TT). The IBM–BMT-treated db/db mice showed decreased blood glucose levels (<150 mg/ml) one week after the treatment but rapid increases in blood glucose levels 2 weeks after the treatment; the mice became susceptible to severe infection due to a rebound

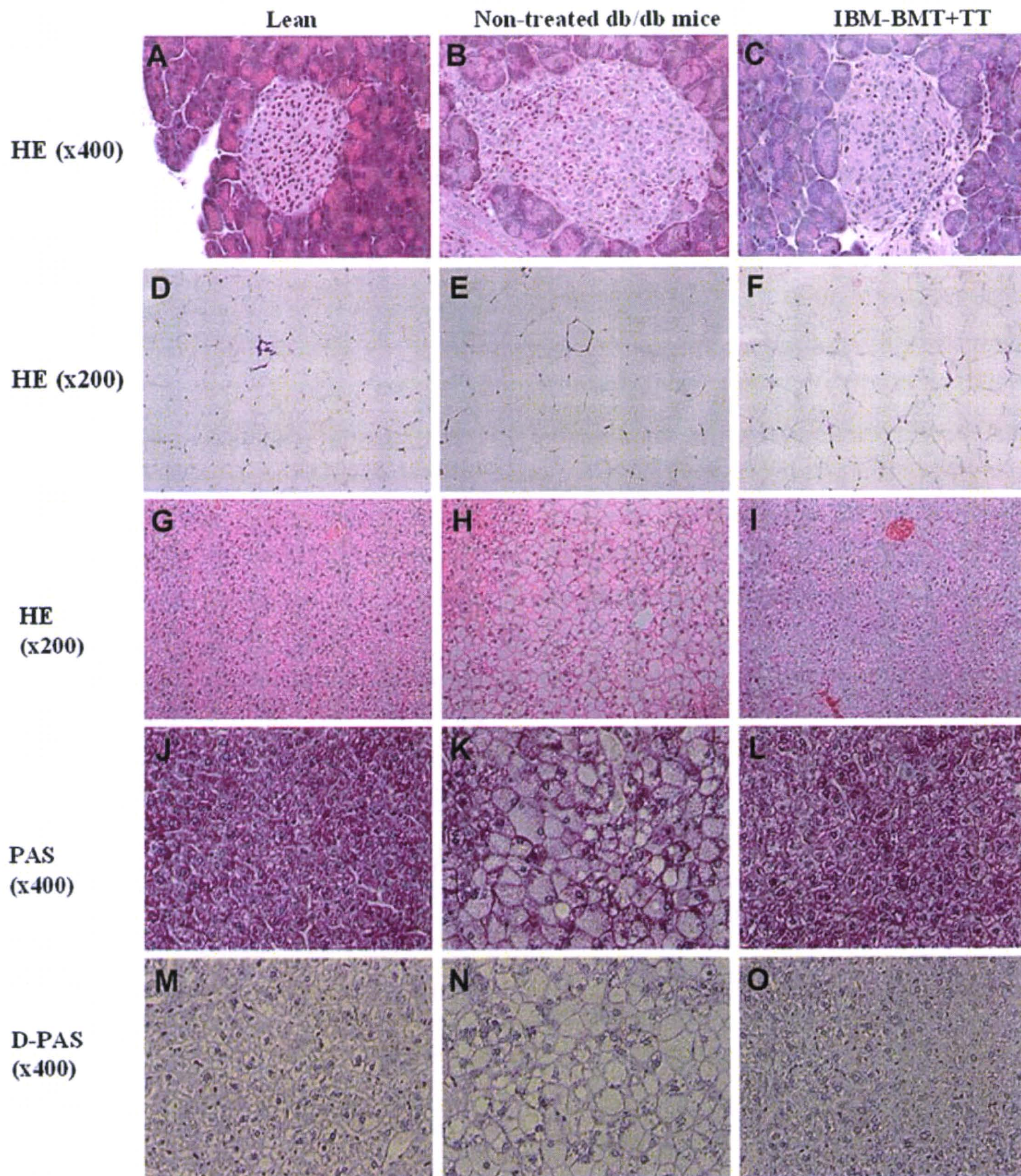

Fig. 2. The morphology of pancreas, visceral fat and liver. HE staining of the pancreas (A–C), visceral fat (E–F), and hepatocytes (G–I). Glycogen deposits in the hepatocytes (J–L) by PAS reaction, after diastase digestion (M–O).

phenomenon, and died. Therefore, in the present study, we carried out IBM–BMT + TT, and non-treated db/db mice were used as the control.

As seen in Fig. 1A, a gain in body weight was prevented in the db/db mice treated with IBM–BMT + TT at each time point after the treatment from 6 weeks to 13 weeks, in contrast to the age-matched non-treated db/db mice (*$p < 0.05$ at each time point). There was no significant difference between the two groups in food intake (5.4 ± 0.1 vs 5.6 ± 0.2 g per mouse), although food intake was only 3.3 ± 0.1 g per lean mouse. To our surprise, the fasting blood glucose levels (Fig. 1B) significantly decreased in the db/db mice treated with IBM–BMT + TT (7 weeks after the treatment), compared with non-treated db/db mice (165.3 ± 5.84 vs 522.7 ± 40.22 mg/dl, $p < 0.001$).

As shown in Fig. 1C, insulin administration to the db/db mice treated with IBM–BMT + TT produced a rapid decrease in the blood glucose levels, suggesting improved insulin sensitivity after the treatment. Blood glucose levels at all time points in the db/db mice treated with IBM–BMT + TT were lower than those in non-treated db/db mice ($p < 0.05$).

As shown in Fig. 1D, non-treated db/db mice exhibited a significant decrease in plasma adiponectin levels, compared with age-matched lean mice (db/+)(5444.04 ± 340.93 vs 8226.5 ± 674.08 ng/ml, $p < 0.05$). However, the plasma adiponectin levels significantly increased in the db/db mice treated with IBM–BMT + TT, compared with non-treated db/db mice (7437.5 ± 837.27 vs 5444.04 ± 340.93 ng/ml, $p < 0.05$) (Fig. 1D). The plasma insulin levels were higher in non-treated db/db mice than lean mice (116.7 ± 22.74 vs 45.0 ± 8.77, $p < 0.05$), and decreased significantly after IBM–BMT + TT (67.2 ± 11.9, $p < 0.05$) (Fig. 1E). In addition, the plasma IL-6 and IL-1β levels significantly decreased in the db/db mice treated with IBM–BMT + TT (5 ± 1.2 and 4.16 ± 1.7 pg/ml, $p < 0.01$), compared with non-treated db/db mice (48.6 ± 1.7 and

23.5 ± 4.7 pg/ml, $p < 0.01$) (Fig. 1F and G). However, there were no significant differences in the levels of TNFα between the treated db/db and non-treated db/db mice (data not shown).

3.2. Morphology of pancreas, visceral fat, and liver

In the HE staining, non-treated db/db mice (Fig. 2B and E) showed larger islets and larger adipocytes in the visceral adipose tissue than lean mice (Fig. 2A and D). In contrast, smaller islets were noted in the db/db mice treated with IBM–BMT + TT (Fig. 2C and F) than non-treated db/db mice: The adipocytes were significantly larger in non-treated db/db mice than in lean mice (140.4 ± 8.17 vs 87.9 ± 7.52 μm in diameter, $p < 0.01$), but smaller in the db/db mice treated with IBM–BMT + TT than in non-treated db/db mice (106.0 ± 1.29 vs 140.4 ± 8.17 μm in diameter, $p < 0.01$). In addition, enlarged hepatocytes were found in non-treated db/db mice (Fig. 2H), although the hepatocytes in the db/db mice treated with IBM–BMT + TT (Fig. 2I) were similar in size to those in lean mice (Fig. 2G). Glycogen deposits were seen in the hepatocytes in all groups (Fig. 2J–L) by PAS reaction, and disappeared after diastase digestion (Fig. 2M–O). However, the density was lower in non-treated db/db mice than lean mice and the db/db mice treated with IBM–BMT + TT (Fig. 2I and L), suggesting that the impaired glycogen synthesis resulted from impaired insulin sensitivity in the non-treated db/db mice. Thus, the glycogen synthesis was also improved after IBM–BMT + TT.

3.3. Insulin content of pancreas islet

Insulin content (brown color) was much lower in the larger islets of non-treated db/db mice (Fig. 3B) than in those of lean mice (Fig. 3A), suggesting that much more insulin was secreted into the peripheral blood in the lean mice. However, there was significantly greater insulin content in residual beta cells (arrows in Fig. 3C) in the db/db mice treated with IBM–BMT + TT than the non-treated db/db mice. This suggests that beta cell destruction due to the exhaustion could be prevented by IBM–BMT + TT.

3.3.1. Effects of IBM–BMT + TT on insulin receptor phosphorylation and its signaling pathway

Visceral fat deposits drain into the portal circulation, resulting in the elevation of free fatty acids. This has been implicated in the genesis of impaired insulin signaling and decreased phosphorylation of insulin receptors. We therefore examined the effects of IBM–BMT + TT on pancreatic tissue insulin receptor phosphorylation in the db/db mice treated with IBM–BMT + TT, non-treated db/db mice, and lean mice. The insulin receptor is a heterodimeric receptor tyrosine kinase with an extracellular alpha-chain, a transmembrane domain and an intracellular beta-chain. Additional autophosphorylation sites such as tyrosine residues 972 regulate the assembly of signal transduction complexes. Phosphorylation of insulin receptors at sites 972 was examined. As shown in Fig. 4A and B, insulin receptor phosphorylation at sites 972 significantly decreased in non-treated db/db mice, compared with lean mice. Densitometry analyses showed increases in the ratios of P-Tyr 972 in the db/db mice treated with IBM–BMT + TT, compared with non-treated db/db mice ($p < 0.03$); the levels were almost the same as those in lean mice. Similar results were observed in HO-1, HO-2, pAKT, pAMPK and pLKB1 expression (Fig. 4C–J, $p < 0.05$ vs IBM–BMT + TT).

3.4. Lymphocyte subpopulations (CD4/CD8 ratios) in thymus and in peripheral blood and donor-derived cells and lymphocyte function

Fig. 5A and B show lymphocyte populations in the thymus analyzed by FACS. The percentages of $CD4^+CD8^+$ double-positive cells decreased significantly in non-treated db/db mice, compared with lean mice ($74.6 \pm 2.04\%$ vs $84.5 \pm 0.22\%$, $p < 0.0.1$), while the percentages of $CD4^-CD8^-$ double-negative cells increased significantly ($8.0 \pm 0.87\%$ vs $4.0 \pm 0.23\%$, $p < 0.01$). The percentages of CD4-positive cells more significantly increased in non-treated db/db mice than lean mice ($12.7 \pm 1.73\%$ vs $8 \pm 0.25\%$, $p < 0.05$). The total cell numbers of the thymus decreased significantly in non-treated db/db mice, compared with lean mice (7.8 ± 0.87 vs $12 \pm 0.77 \times 10^7$ cells, $p < 0.01$) (Fig. 5C). However, the cell numbers of the thymus increased significantly in the mice treated with IBM–BMT + TT, compared with non-treated db/db mice (11.8 ± 1.65 vs $7.8 \pm 0.87 \times 10^7$cells, $p < 0.05$). Fig. 5D shows that the percentages of CD8-positive cells increased significantly in the db/db mice treated with IBM–BMT + TT, compared with non-treated db/db mice (9.5 ± 1.28 vs $4.76 \pm 0.74\%$, $p < 0.05$), while there was no significant difference in the percentages of CD4-positive cells (11.1 ± 1.95 vs $12.7 \pm 1.73\%$, ND).

Fig. 5E shows the ratios of CD4/CD8-positive cells in the peripheral blood. The ratio was significantly higher in non-treated db/db mice, compared with lean mice (2.16 ± 0.11 vs 1.3 ± 0.16, $p < 0.05$), whereas it was significantly lower in the db/db mice treated with IBM–BMT plus TT, compared with non-treated db/db mice (1.25 ± 0.08 vs 2.16 ± 0.11, $p < 0.01$). There was no significant difference in the ratio between lean mice and the db/db mice treated with IBM–BMT + TT.

Approximately, 98% of hematolymphoid cells were of donor-origin in the peripheral blood of the recipients treated with IBM–BMT + TT one month after BMT. Fig. 5F shows the results of analyses of cell surface antigens (CD4, CD8, B220 and CD11b) on donor-derived cells (15.80%, 11.90%, 42.79% and 21.29% of donor-derived cells) in the recipient mice; donor-derived cells with mature

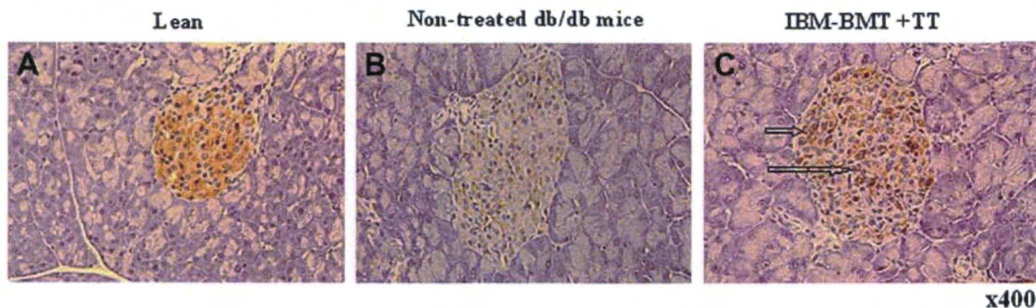

Fig. 3. Expression of insulin on the pancreata. Immunochemistry staining for insulin was performed (A–C). There was considerably more insulin content in residual beta cells (arrows in C) when compared to non-treated db/db mice.

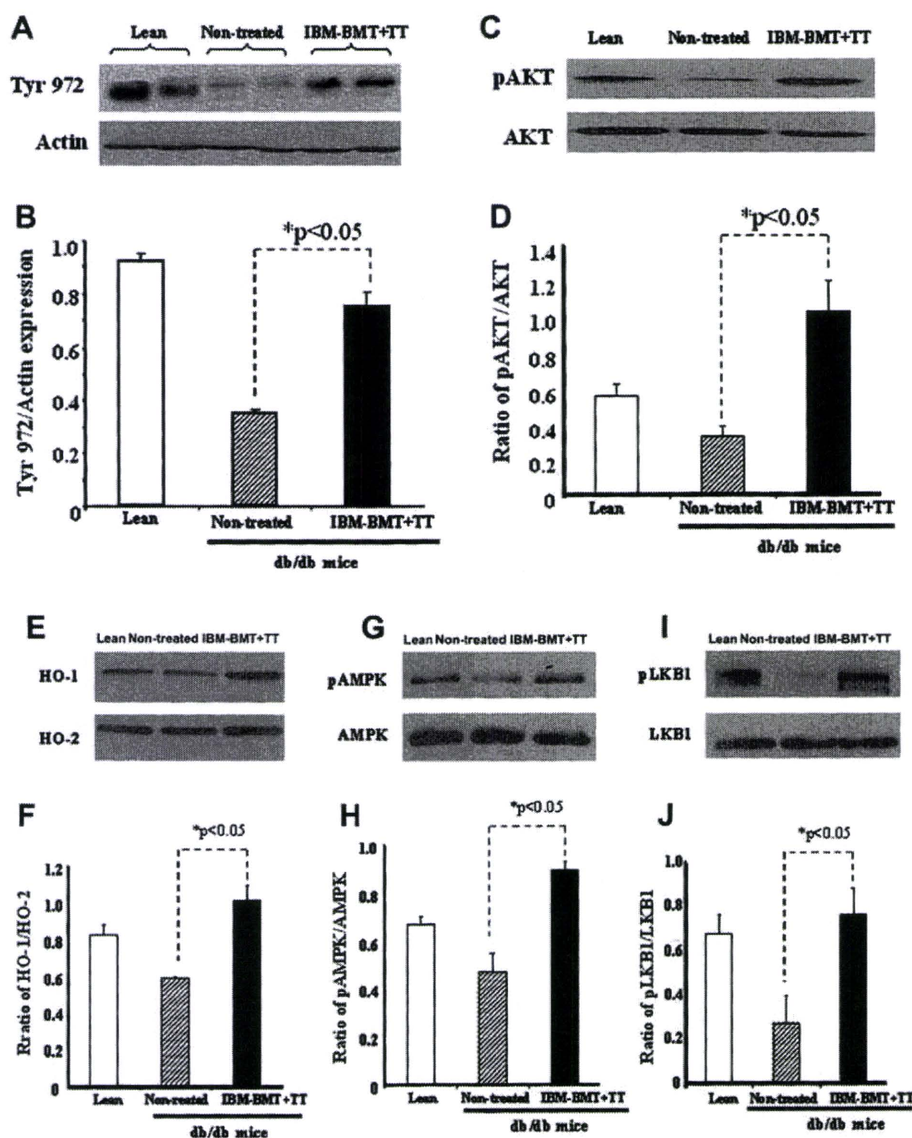

Fig. 4. Effect of IBM–BMT + TT on insulin receptor phosphorylation and its signaling pathway. (A) Western blot and densitometry analyses of insulin receptor phosphorylation (Tyr 972) and actin proteins in pancreata of lean, non-treated db/db mice and db/db mice treated with IBM–BMT + TT. (B) The ratio of quantitative densitometry evaluation of p-Tyr 972 and actin proteins was determined. Representative immunoblots are shown ($n = 3$). The expression of HO-1, HO-2, pAKT, pAMPK and pLKB1 are shown (C,E,G, I). Their ratios are shown ($p < 0.05$ vs IBM–BMT + TT) (D, F, H, J).

lineage markers were clearly observed one month after the treatment with IBM–BMT + TT.

The spleen cells of the recipients showed sufficient mitogen responses to both ConA and lipopolysaccharide (LPS) in comparison with those of non-treated db/db mice: 27254.9 ± 5558.15 vs 586.17 ± 51.85; 26416.2 ± 4164.60 vs 586.17 ± 51.85, both $p < 0.001$. These findings suggest that not only T cell but also B cell functions were restored in the db/db mice treated with IBM–BMT + TT.

4. Discussion

Leptin is an adipocyte-derived hormone that links nutritional status with neuroendocrine and immune functions. Leptin has been shown to modulate T cell proliferation, to promote Th1 responses, and to protect thymocytes from corticosteroid-induced apoptosis *in vitro* [26–29]. Leptin-deficient ob/ob mice and leptin receptor-deficient db/db mice exhibit severe hereditary obesity [30,31] and display hormonal imbalances and hematolymphoid defects [32,33]. Db/db mice exhibit a marked reduction in the size and cellularity of the thymus [34,35]. The long-signaling leptin receptor isoform is expressed in the bone marrow cells, CD34 cells, marrow stroma cells, and both CD4 and CD8T cells of normal mice [26,27,36,37]; several investigators have described the direct effects of leptin on lymphocytes. It is, however, uncertain whether high blood glucose levels result from the imbalance of lymphocyte subsets in db/db mice.

Young patients with T2 DM show evidence of islet-cell autoimmunity, with autoantibodies present in 10–75% of patients, such as islet-cell antibodies (ICA) in 5–8%, glutamic acid decarboxylase (GAD) in 8–30%, islet-autoantibodies (IA) -2 in 8–42% and insulin antibodies in 5–35% [38–42]. These patients may be the evidence of islet autoimmunity contributing to insulin deficiency [43].

We previously showed that BMT could be used to treat non-insulin-dependent-diabetes in KK-Ay mice [8]. Recently, we have found that IBM–BMT treatment leads to increased HO-1 expression, resulting in preventing the development of T2 DM in ob/ob