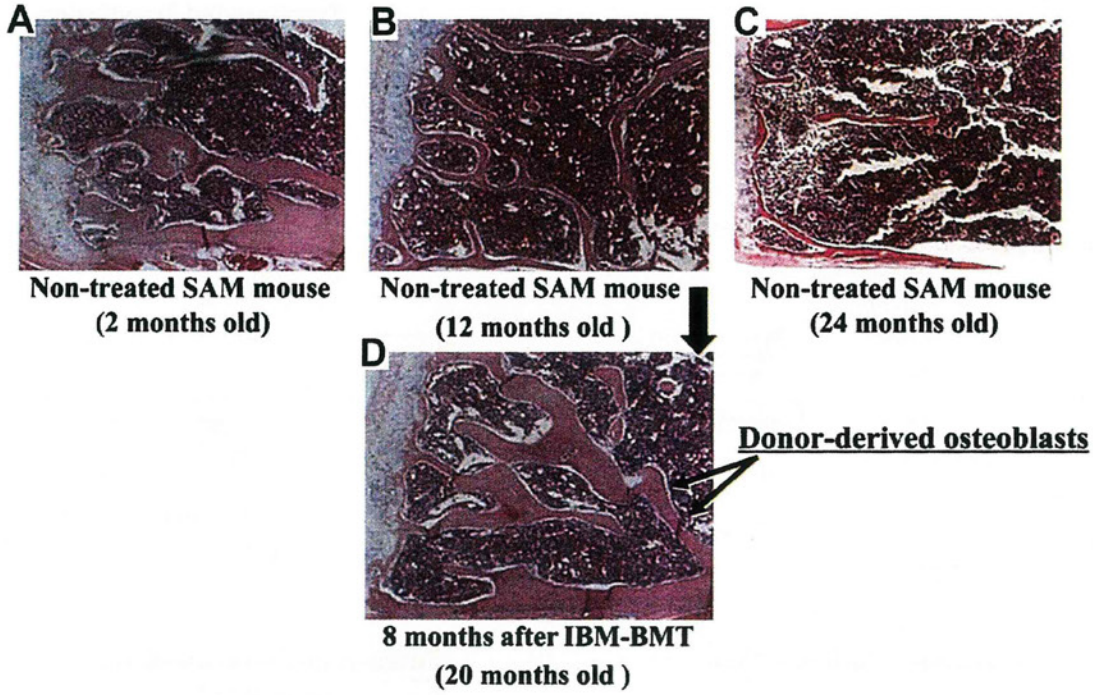

Fig. 3. Treatment of osteoporosis in SAMP6 mice by IBM–BMT from normal B6 mice.

BMT"-treated SAMP6 mice were replaced by donor stromal cells [18,19]. Thus, we succeeded in curing osteoporosis in SAMP6 mice by IBM–BMT, which can recruit both donor-derived HSCs and MSCs.

Since IBM–BMT appeared to be a powerful strategy in regeneration therapy, we next used tight-skin (Tsk) mice (an animal model for emphysema) to examine whether emphysema could be cured by IBM–BMT.

IBM-BMT was carried out from C3H mice into Tsk mice (8–10 weeks old) that had already shown emphysema. Eight months after the transplantation, the lungs of all the Tsk mice treated with IBM-BMT [C3H→Tsk] showed structures similar to those of normal mice, whereas the [Tsk→Tsk] mice showed emphysema, as seen in age-matched Tsk mice. Next, we attempted to transfer emphysema from Tsk mice to C3H mice by IBM–BMT. Six months after IBM-BMT, the [Tsk→C3H] mice showed emphysema [20]. These results strongly suggested that emphysema in Tsk mice originates from defects in the stem cells (probably MSCs and/or HSCs) in the bone marrow [20].

IBM–BMT + donor lymphocyte infusion (DLI) for treatment of malignant tumors

It is well known that the graft-versus-leukemia reaction (GvLR) can cure patients of a variety of hematological malignancies [21,22]. Recently, it has been reported that graft-versus-tumor (GvT) effects can induce partial (complete in some) remission of metastatic solid tumors such as breast cancer [23–25] and renal cell carcinoma [26–30]. Based on these findings, donor lymphocyte infusion (DLI) has recently been used for the treatment of malignant solid tumors even in humans. However, it is very difficult to completely eradicate the tumors, since extensive DLI induces graft-versus-host disease (GvHD). We therefore attempted to establish a new method for the treatment of malignant tumors, this method consisting of intra-bone marrow-IBM–BMT plus DLI, since we have recently found that IBM-BMT can allow a reduction in radiation doses as a conditioning regimen and prevent GvHD [31,32]. Using the Meth-A cell line (BALB/c-derived fibrosarcoma), we found that IBM-BMT plus the injection of $CD4^+$ T-cell-depleted (but not $CD8^+$ T-cell-depleted) spleen cells (as DLI) can prevent GvHD while suppressing tumor growth [33] (Fig. 4). In addition, we have found that IBM-BMT plus extensive DLI (3

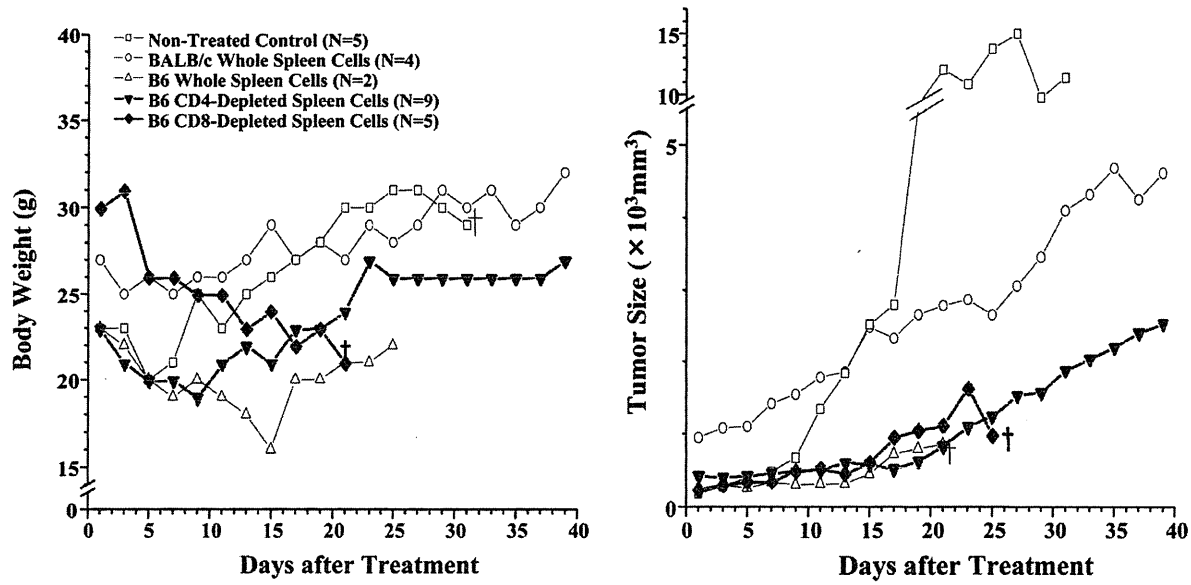

Fig. 4. Prevention of GvHD and suppression of tumor growth by IBM–BMT + DLI ($CD4^-$).

times every 2 weeks) leads to the complete rejection of the tumor, although the success rate (3/50) is not high so far [33].

In addition, we have examined whether this strategy (IBM-BMT plus DLI) is applicable to other tumors in other animals. We have obtained similar results in another system (colon cancer: ACL-15 in rats) [34]. We are now establishing more efficient strategies to eradicate malignant tumors.

IBM–BMT + thymus transplantation (TT) for modulation of age-associated diseases

We have recently proposed that age-associated diseases (AADs), such as osteoporosis and emphysema, are mesenchymal stem cell disorders.

Based on our findings, we attempted to prevent the progression of Alzheimer's disease using senescence-accelerated mice by IBM-BMT, and succeeded in preventing the development of Alzheimer's disease [35].

In addition, we succeeded in curing type 2 diabetes mellitus in db/db mice by IBM-BMT with TT [36].

These findings suggest that TT plays a crucial role in the prevention and treatment of AADs, since the recovery of T cell functions would be delayed after IBM–BMT alone (but not after IBM–BMT + TT).

Future directions

As described here, the new BMT method (PM + IBM–BMT) can be used to treat various otherwise intractable diseases, including i) autoimmune diseases, ii) AADs (osteoporosis, emphysema, etc.), iii) diseases curable by organ transplantation and iv) malignant tumors (including solid tumors) [33]. The PM can efficiently be used to collect whole BMCs (including HSCs and MSCs) without them being contaminated with T cells, and no GVHD therefore develops. IBM-BMT can efficiently transfer donor whole BMCs (both HSCs and MSCs) into recipients, and this method can therefore be used to quickly replace not only HSCs but also MSCs with donor-derived cells.

From the findings to date, it is conceivable that all the body's cells originate in the bone marrow, and that all diseases might therefore originate from defects in the bone marrow. One paper already suggests that gastric cancer originates from bone marrow-derived cells [37].

We believe that the development of our BMT method heralds a revolution in the field of transplantation (BMT and organ transplantation) and regeneration therapy.

Conflict of interest

No conflicts of interest to declare.

Acknowledgments

I thank Mr. Hilary Eastwick-Field and Ms. K. Ando for their help in the preparation of the manuscript. These studies were mainly supported by the 21st Century Center of Excellence (COE) program of the Ministry of Education, Culture, Sports, Science and Technology. Supported also by a grant from Haiteku Research Center of the Ministry of Education, Health and Labour Sciences Research Grants, a grant from the Science Frontier program of the Ministry of Education, Culture, Sports, Science and Technology, and a grant from the Department of Transplantation for Regeneration Therapy (sponsored by Otsuka Pharmaceutical Company, Ltd.), a grant from Molecular Medical Science Institute, Otsuka Pharmaceutical Co., Ltd., and a grant from Japan Immunoresearch Laboratories Co., Ltd. (JIMRO).

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STEM CELLS AND DEVELOPMENT
Volume 21, Number 9, 2012

DOI: 10.1089/scd.2011.0358

Effects of Intrabone Marrow–Bone Marrow Transplantation Plus Adult Thymus Transplantation on Survival of Mice Bearing Leukemia

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We recently found that allogeneic intrabone marrow–bone marrow transplantation (IBM–BMT) plus adult thymus transplantation (ATT) from the same donor is effective in mice bearing solid tumors. In the current study, we examined the effects of this strategy on the survival of mice with leukemia. One week after intravenous injection of 1×10^6 leukemic cells (EL-4, H-2^b) into 8-week-old B6 (H-2^b) mice, the mice were 8 Gy irradiated and transplanted with 1×10^7 bone marrow cells (BMCs) from 8-week-old BALB/c mice (H-2^d) by IBM–BMT with or without donor lymphocyte infusion (DLI) or ATT. All the mice without treatment died within 70 days after injection of EL-4. About 40% of those treated with IBM–BMT alone died within 100 days due to tumor relapse. In contrast, those treated with IBM–BMT + DLI or ATT showed the longest survival rate without relapse of leukemia. In addition, the former showed less graft versus host disease (GVHD) than the latter. The mice treated with IBM–BMT + ATT also showed an intermediate percentage of effector memory (EM) and central memory (CM) cells between those treated with BMT alone and those treated with IBM–BMT + DLI. The numbers and functions of T cells increased in those treated with IBM–BMT + ATT with interleukin-2 and interferon-γ production. These results suggest that IBM–BMT + ATT is effective in the treatment of leukemia with strong graft versus leukemia without increased risk of GVHD.

Introduction

ALLOGENEIC BONE MARROW transplantation (allo-BMT) has been used for the radical treatment of leukemia. However, allo-BMT has some side effects. Graft versus host disease (GVHD) occurs if anti-host reaction in donor T cells is too strong, whereas relapse occurs if it is too weak [1]. In addition, a failure of bone marrow cell (BMC) engraftment in the early phase of transplantation may induce immunodeficiency, which, in turn, leads to severe infection [2]. Although donor lymphocyte infusion (DLI) is sometimes used to enhance engraftment and/or graft versus leukemia (GVL) activity [3], this is associated with an increased risk of GVHD [4]. Therefore, new cellular-based methods are required.

We recently developed a new BMT method, intrabone marrow–bone marrow transplantation (IBM–BMT), in which BMCs are directly injected into the bone marrow cavity [5]. IBM–BMT results in a reduced incidence of GVHD and greater engraftment of donor cells, including mesenchymal stem cells (MSCs) than the conventional intravenous method [6,7].

We have also developed a BMT method that is combined with thymus transplantation (TT), which includes the transplantation of adult thymus transplantation (ATT), newborn thymus, and fetal thymus. The combination of BMT + TT is effective in restoring donor-derived T cell function even in aged, chimeric-resistant, supralethally irradiated, and low-dose irradiated mice, mice with metabolic diseases, and also in mice injected with a small number of BMCs [8–12]. Further, we demonstrated that IBM–BMT + TT is effective for graft versus tumor (GVT) and long-term survival with a low risk of GVHD [13,14].

In the current study, we examined the BMT + ATT method in mice with leukemia. We also performed BMT alone and BMT + DLI in these mice and compared the survival rate, degree of GVHD, and T-cell functions.

Materials and Methods

Mice

Female 6- to 8-week-old 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. All

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protocols for these animal experiments were performed in accordance with the Guidelines for Animal Experimentation, Kansai Medical University, and received approval from the Committee on Animal Experiments. EL-4 cells (H-2^b) were derived from thymoma in B6 mice. Cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum with antibiotics. These cells were intravenously transferred to the recipients (B6 mice).

IBM–BMT and ATT

Recipient B6 mice with tumors were irradiated (8 Gy) by using a ^{137}Cs irradiator (Gammacell 40 Exactor; MDS Nordion International, Ottawa, ON, Canada) 7 days after transfer of the EL-4 cells. The next day, 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 [7]. Briefly, the knee was flexed to 90°, and the proximal side of the tibia was drawn to the anterior. A 26-gauge needle was inserted into the joint surface of the tibia through the patellar tendon and then inserted into the bone cavity. Simultaneously, one quarter of the AT was grafted under the renal capsule of the left kidney in some mice.

Histology

Histological studies were performed in the liver, intestine (for evaluation of GVHD), and engrafted tumors 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 by using hematoxylin and eosin. The degree of GVHD was evaluated by using a semiquantitative scoring system for abnormalities known to be associated with GVHD, as previously described [13].

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 by using a FACScan system (Becton Dickinson, Franklin Lakes, NJ). Fluorescein isothiocyanate (FITC)-conjugated anti-H-$2K^b$ (Pharmingen, San Diego, CA) mAbs and phycoerythrin (PE)-conjugated anti-H-$2K^d$ mAbs were used to determine chimerism. FITC, PE, or biotin-conjugated CD4, CD8, B220, CD44, or CD62L (Pharmingen) were used to analyze spleen cell subsets. Avidin-Cy5 (Dako, Kyoto, Japan) was used as the third color in the avidin/biotin system. Intracytoplasmic FoxP3 staining was performed by using an eBioscience FITC-anti mouse/rat FoxP3 staining kit in accordance with the manufacturer's instructions (eBioscience, San Diego, CA). Intracellular cytokines [interleukin (IL)-2, IL-4, IL-10, IL-17, interferon (IFN)-γ, and tumor necrosis factor] were detected by 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 transplantation. For mitogen response, a total of 2×10^5 splenocytes collected from chimeric mice and untreated 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, Tokyo, 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, Franklin Lakes, NJ) 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, Cambridge, MA) was introduced. Incorporation of ^{3}H-TdR was measured by using Microbeta TriLux (PerkinElmer, Wellesley, 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 and nonparametric analyses (Mann–Whitney *U*-test and log rank-test) were performed by using StatView software (Abacus Concepts, Berkley, CA). In all analyses, $P<0.05$ was taken to indicate statistical significance.

Results

Survival rate and body weight

First, we examined the effects of BMT alone, BMT + ATT, or BMT + DLI on the survival rate of mice transplanted with EL-4 (Fig. 1A). All the untreated mice transplanted with EL-4 died within 70 days due to tumor growth (Fig. 2B). Those treated with BMT alone showed a survival rate of about 60% 6 months after BMT. The remaining 40% of the mice died due to tumor growth. Interestingly, those treated with BMT + ATT or BMT + DLI showed the longest survival rate. Next, we investigated the weight of these mice. The mice not treated with EL-4 showed a gradual increase in weight (Fig. 1B), which was due to growth of the tumor (Fig. 2B). Those treated with IBM–BMT alone and IBM–BMT + ATT surviving for a long time showed a stable weight, and those treated with IBM–BMT + DLI showed a gradual weight loss.

Chimerism and histology

All mice treated with BMT showed donor-derived chimerism (H-$2K^d$), whereas untreated controls showed host-derived chimerism (H-$2K^b$) (Fig. 2A). The untreated mice showed massive infiltration of tumor cells throughout the whole body, including the liver, lung, mesenterium, muscle, and bone (Fig. 2B). All mice that died treated with BMT alone showed such tumor growth (as just mentioned). In contrast, most of those treated with BMT + ATT or BMT + DLI showed little tumor growth and long-term survival. The engrafted thymus showed a normal structure with cortical and medullar areas under the renal capsule (Fig. 2C). Normal T-cell differentiation was also observed in the thymus. In addition, those treated with BMT + ATT and BMT + DLI showed mild and moderate infiltrations of lymphocytes in the liver and small intestine, and the latter also showed some fibrosis with tissue destruction (Fig. 2D). Since the chimerism was of the donor type, this suggested the occurrence of

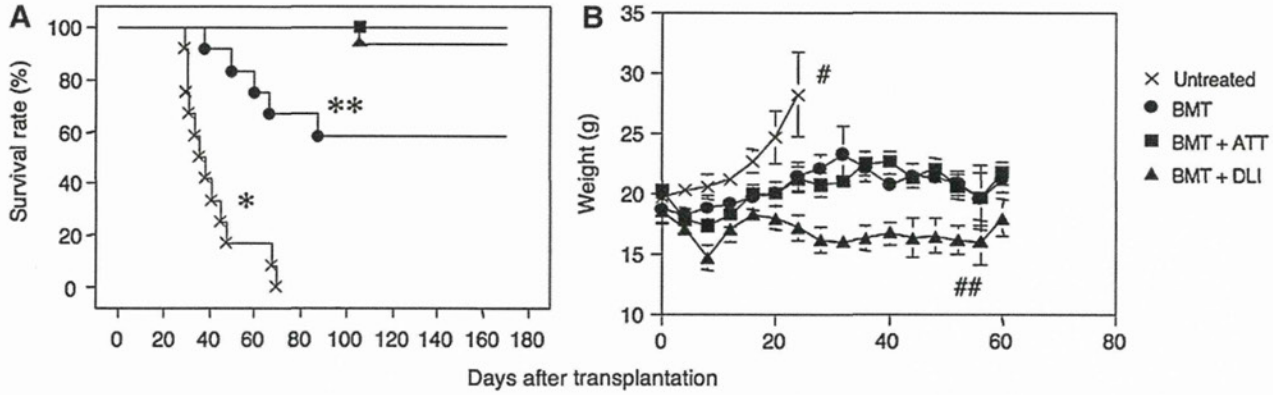

FIG. 1. Survival rate and body weight in mice with leukemia treated with BMT + TT. Survival rate **(A)** and weight **(B)** of mice with advanced tumors are shown. $^{*}P < 0.0001$ compared with those treated with BMT alone, BMT + ATT, or BMT + DLI. $^{**}P < 0.05$ compared with those treated with BMT + ATT or BMT + DLI. $^{\#}P < 0.05$ compared with those treated with BMT alone, BMT + ATT, or BMT + DLI. $^{\#\#}P < 0.05$ compared with those treated with BMT + ATT or BMT + DLI. Data are shown as means ± SE. Untreated controls ($n = 12$), those treated with BMT alone ($n = 12$), BMT + ATT ($n = 12$), or BMT + DLI ($n = 15$). ATT, adult thymus transplantation; BMT, bone marrow transplantation; DLI, donor lymphocyte infusion; TT, thymus transplantation.

GVHD. The GVHD scores are summarized in Fig. 2E. Those treated with BMT alone showed little GVHD, whereas those treated with BMT + ATT showed mild GVHD, and those treated with BMT + DLI showed moderate GVHD.

Lymphocyte subsets

We next analyzed the lymphocyte subsets in the spleen 4 weeks after transplantation. The number of $CD4^+$ T cells was significantly greater in the mice treated with BMT + ATT compared with those treated with BMT alone and those with BMT + DLI, in which the levels were comparable to those in normal BALB/c mice (Fig. 3). This was followed by those treated with BMT alone, followed by those treated with BMT + DLI, which showed the lowest levels. The percentage of $FoxP3^+$ regulatory T cells, which suppress immune responses, including GVH reactions [15,16], among $CD4^+$ T cells was the highest in the mice treated with BMT alone, the percentage being comparable to that in BALB/c mice. This was followed by those treated with BMT + ATT, whereas those treated with BMT + DLI showed the lowest percentage. The results for $CD8^+$ T cells were similar to those for $CD4^+$ T cells, although all values for mice treated with BMT were lower than those of BALB/c mice. The number of $B220^+$ T cells was lowest in those treated with BMT + DLI.

Effector memory, central memory, and naïve T cell subsets

T cells can be functionally divided into $CD62L^-CD44^-$ naïve, $CD62L^+CD44^+$ central memory (CM), and $CD62L^-CD44^+$ effector memory (EM) cells from prestimulation to terminal differentiation [17,18]. Therefore, we examined the proportions of these cells in both $CD4^+$ and $CD8^+$ subsets of T cells in the spleen. The percentage of EM among $CD4^+$ T cells was the highest in the mice treated with BMT + DLI followed by those treated with BMT + ATT (Fig. 4A). The lowest percentage of EM among $CD4^+$ T cells was seen in those treated with BMT alone, being comparable to that in BALB/c mice. Conversely, the percentage of CM was the highest in the mice treated with BMT alone and BALB/c mice, followed by those treated with BMT + ATT, and the lowest percentage of CM was seen in those treated with BMT + DLI. The percentage of naïve T cells was similar to that of CM cells, although the highest percentage was seen only in the BALB mice, and there were no significant differences between those treated with BMT + ATT and those treated with BMT + DLI. The results for $CD8^+$ T cells were similar to those for $CD4^+$ T cells (Fig. 4B).

Mitogen responses and cytokine production

Finally, we examined lymphocyte functions by monitoring mitogen responses (Con A for T cells and LPS for B cells) and cytokine production. The mice treated with BMT + ATT showed significantly increased Con A response compared with those treated with BMT alone, and the level was comparable to that in BALB/c mice (Fig. 5A). Those treated with BMT + DLI showed the lowest response. In contrast, the LPS response was almost the same in those treated with BMT alone, BMT + ATT, and BALB/c mice, and the lowest response was seen in BMT + DLI. With regard to cytokine production, those treated with BMT + ATT showed significant increases in IL-2 production compared with those treated with BMT alone and BMT + DLI, the level being comparable to that in BALB/c mice (Fig. 5B). In contrast, those treated with BMT + DLI showed significantly higher levels of IFN-γ production than those treated with BMT + ATT and BMT alone, although they did not reach the level of BALB/c mice. The production of IFN-γ was also elevated in those treated with BMT + TT, and was higher than in those treated with BMT alone.

Discussion

The current study was performed to examine the effects of BMT + ATT on leukemia in mice. The mice treated with BMT + ATT showed a longer survival than those treated with BMT alone, and milder GVHD than those treated with BMT + DLI. Leukemia showed little growth in BMT + ATT mice comparable to those treated with BMT + DLI. Those treated with BMT + ATT showed higher numbers of both

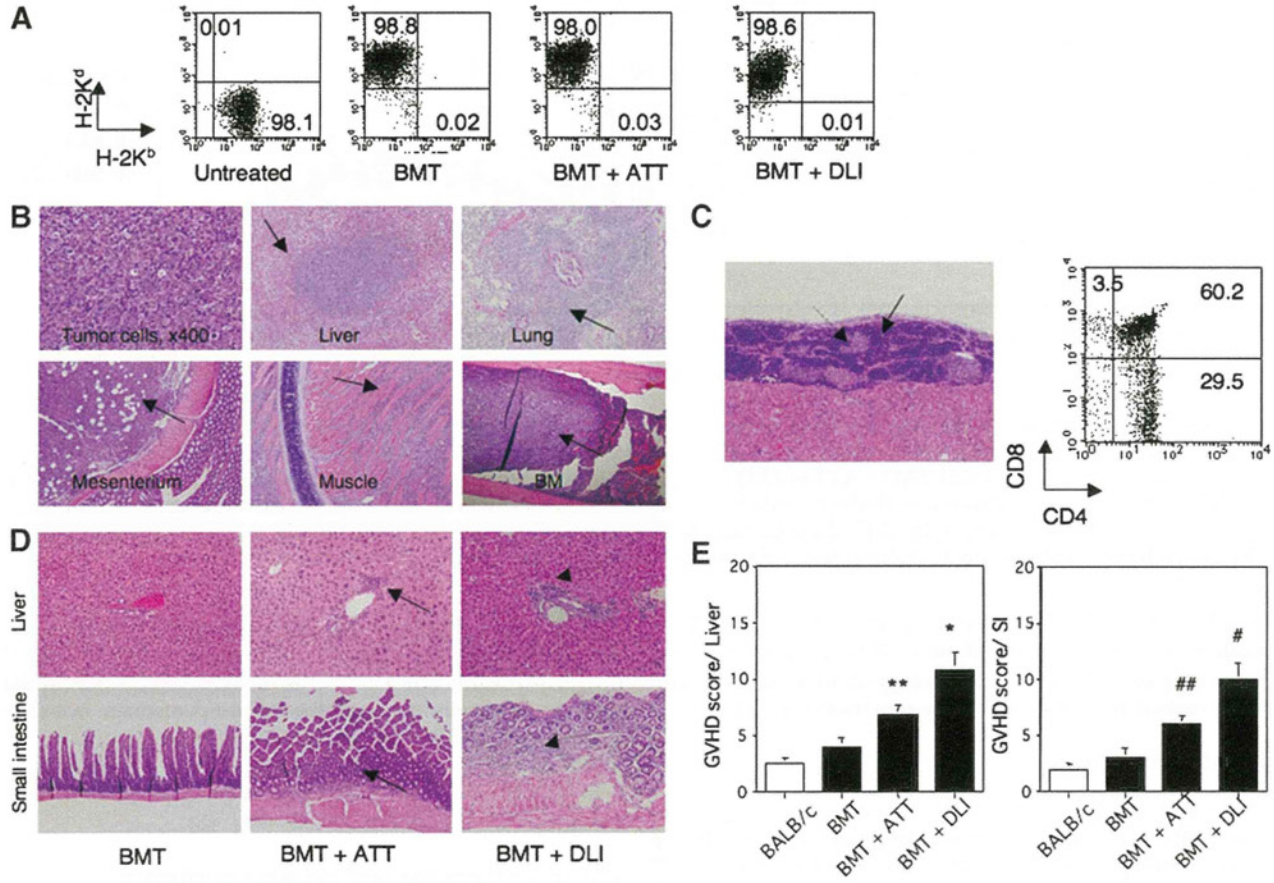

FIG. 2. Chimerism, histology, transplanted thymus, and GVHD in mice with leukemia treated with BMT + TT. Chimerism in experimental mice **(A)**, histology in the liver, lung, mesenterium, muscle, and BM from mice with leukemia **(B)**, histology in transplanted thymus and the FACS profile in **(C)**, histology for GVHD in chimeric mice **(D)** and the GVHD scores in the mice **(E)** are shown. Chimerism was analyzed in the spleens from mice transplanted with leukemia and those treated with BMT alone, BMT + ATT, or BMT + DLI **(A)**. The numbers in the profiles show the percentage. Tumor cells (*upper, left*) infiltrated the liver, lung, mesenterium, muscle, and BM in the mice transplanted with leukemia (*arrows*) **(B)**. Histological findings (*left*) and FACS profile (*right*) of thymocytes in the transplanted thymus from the mice treated with BMT and ATT **(C)**. *Plain arrow*, cortex; *dotted arrow*, medulla. The numbers in the profiles show the percentage. Representative data from 4 experiments are shown. The histology of GVHD is shown in the liver (*upper*) and small intestine (*lower*) **(D)**. Some lymphocytes infiltrated the liver and small intestine in BMT + ATT (*arrows*) and destroyed the tissue in BMT + DLI (*dotted arrow*). GVHD scores are shown in the liver (*left*) and small intestine (*right*) **(E)**. The GVHD score was calculated as described in Materials and Methods. $^{*}P < 0.05$ compared with normal BALB/c mice and mice treated with BMT alone and BMT + ATT. $^{**}P < 0.05$ compared with normal BALB/c mice and the mice treated with BMT alone. $^{\#}P < 0.05$ compared with normal BALB/c mice and mice treated with BMT alone and BMT + ATT. $^{\#\#}P < 0.05$ compared with normal BALB/c mice and mice treated with BMT alone. The mice transplanted with leukemia cells (EL-4) were analyzed 5 weeks after transplantation and those treated with BMT alone, BMT + ATT, or BMT + DLI were analyzed 8 weeks after treatment. Data are shown as means ± SE. Normal BALB/c ($n = 4$), BMT ($n = 4$), BMT + ATT ($n = 4$), BMT + DLI ($n = 4$). GVHD, graft versus host disease.

$CD4^+$ and $CD8^+$ T cell subsets than those treated with BMT alone or with BMT + DLI. Interestingly, the percentages of $FoxP3^+$ regulatory T cells, CM, and EM T cells in those treated with BMT + ATT were intermediate between those treated with BMT alone and those treated with BMT + DLI. T-cell functions with production levels of some cytokines were also elevated in those treated with BMT + ATT. These findings suggest that the BMT + ATT method is more effective in the treatment of leukemia than previous methods.

First, we examined the survival rates in association with GVH and GVL effects. All mice with the development of leukemia died early, whereas those without leukemia showed long-term survival, with or without GVHD, thus indicating that the presence of leukemia is the factor with the greatest influence on mortality. However, we did not examine the further long-term effects of GVHD, and chronic GVHD may also lead to death in the long term [19]. Therefore, these observations suggest that BMT + ATT is superior to BMT alone and BMT + DLI.

We next investigated the mechanism of these effects. The numbers of both T-cell subsets significantly increased in mice treated with BMT + ATT compared with those treated with BMT alone and BMT + DLI. The low numbers of T-cell subsets as well as B cells in those treated with BMT + DLI may have resulted in GVHD [20]. Interestingly, those treated with BMT + ATT showed a lower percentage of regulatory T cells

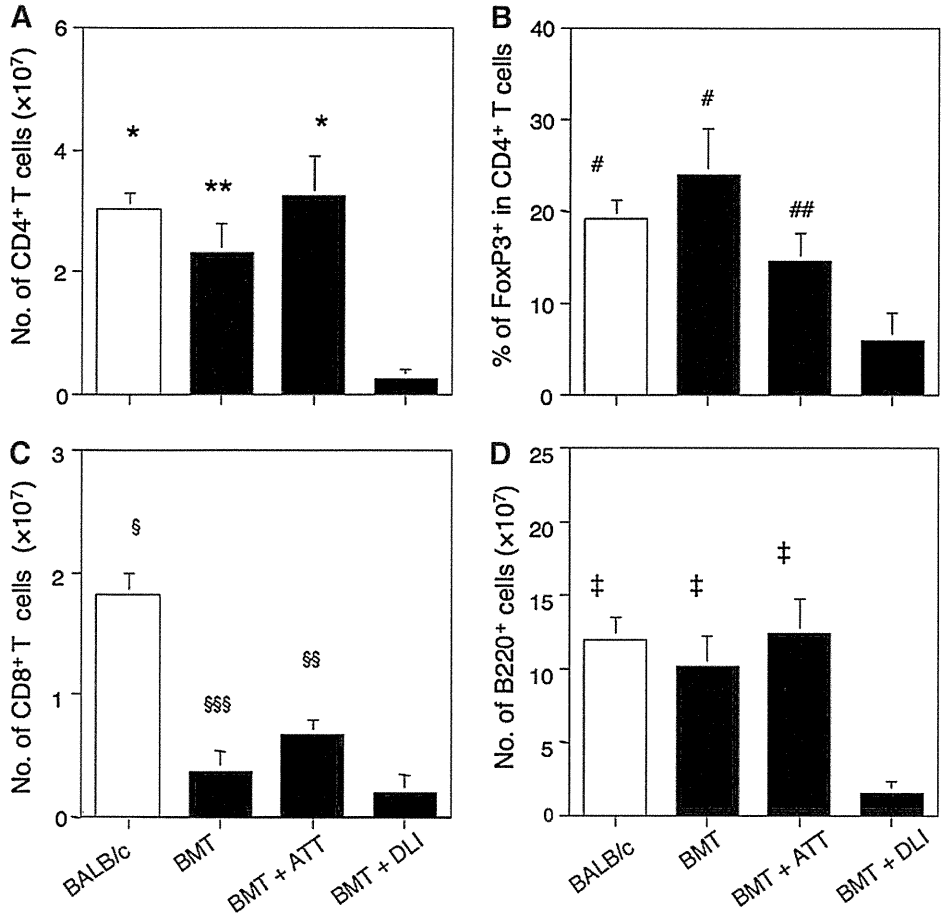

FIG. 3. Numbers of lymphocytes in the spleen from leukemia-bearing mice treated with BMT+ATT. Numbers of $CD4^+$ T cells **(A)**, percentage of $FoxP3^+$ cells in $CD4^+$ T cells **(B)**, numbers of $CD8^+$ T cells **(C)**, and $B220^+$ B cells **(D)** in the spleen were evaluated in normal BALB/c mice, leukemia-bearing mice treated with BMT alone, BMT+ATT, or BMT+DLI. The experiments were performed 5 weeks after BMT. $^*P<0.05$ compared with BMT alone and BMT+DLI. $^{**}P<0.01$ compared with BMT+DLI. $^{\#}P<0.05$ compared with BMT+ATT and BMT+DLI. $^{\#\#}P<0.05$ compared with BMT+DLI. $^{\S}P<0.01$ compared with BMT, BMT+ATT, and BMT+DLI. $^{\S\S}P<0.05$ compared with BMT and BMT+DLI. $^{\S\S\S}P<0.05$ compared with BMT+DLI. $^{\ddagger}P<0.01$ compared with BMT+DLI. Data are shown as means±SD. Normal BALB/c ($n=5$), BMT ($n=5$), BMT+ATT ($n=5$), and BMT+DLI ($n=5$).

than those treated with BMT alone, whereas they showed a higher percentage of regulatory T cells than BMT+DLI. Since regulatory T cells suppressed GVHD, preserving GVT and GVL [21,22] the intermediate percentage of these cells also played an important role in the mechanism underlying our results. The elevation of regulatory T-cell numbers compared with BMT alone suggested that some regulatory T cells in BMT+ATT may be produced from the TT [13].

The percentages of EM T cells were highest in the mice treated with BMT+DLI, followed by those treated with BMT+ATT, and lowest in those treated with BMT alone. In contrast, CM and naïve T cells were lowest in the BMT+DLI group, followed by the BMT+ATT group, and highest in those treated with BMT alone. Since freshly isolated CM and naïve T cells in DLI induce GVHD and GVL [23,24], the observation of the lowest numbers of these cells in BMT+DLI suggested that these cells in DLI had differentiated into EM T cells with activation and/or were consumed [25].

The mice treated with BMT+ATT showed sufficient mitogen responses to both T and B cells, whereas those treated with BMT alone showed low T-cell response, and those treated with BMT+DLI showed low responses to both T and B cells. Such immunodeficiency in BMT+DLI may be induced by GVHD [20]. These findings indicated that BMT+TT is the best method compared with BMT alone or BMT+DLI. Although the mechanism underlying the long-term survival in the BMT+DLI group is unclear, the SPF condition may have prevented severe infection. Alternatively, the IBM–BMT method itself may suppress lethal GVHD by DLI [6].

Analysis of cytokines indicated significantly elevated IL-2 production in the mice treated with BMT+ATT compared with those treated with BMT alone or with BMT+DLI, whereas IFN-γ production was significantly higher in those treated with BMT+DLI. Although several cytokines play a role in GVHD, IL-2 is effective in inducing strong GVL while avoiding GVHD [26]. In addition, the highest degree of elevation of IFN-γ production may contribute to GVHD with lymphoid hypoplasia (immunodeficiency) in BMT+DLI [27]. Therefore, the cytokine patterns may also be at least partly associated with the pathogenesis.

We thus found that donor-derived T cells play an important role in the treatment of leukemia. However, further gene analyses are needed for a more comprehensive understanding. For example, cytokine profiles at the transcriptional level should be analyzed by using DNA microarry in T cells [28]. In addition, differences in T-cell clones may become evident between GVL and GVH, or between host and donor-thymus derived by next-generation sequencing and/or other methods [29]. These findings would help detail the mechanism of BMT+TT.

We examined means of treating several intractable diseases and/or serious complications by BMT+TT [8–14]. In most studies, BMT+TT showed better results than BMT alone or BMT+DLI. The results of the current study were also compatible with these previous findings. The thymus is

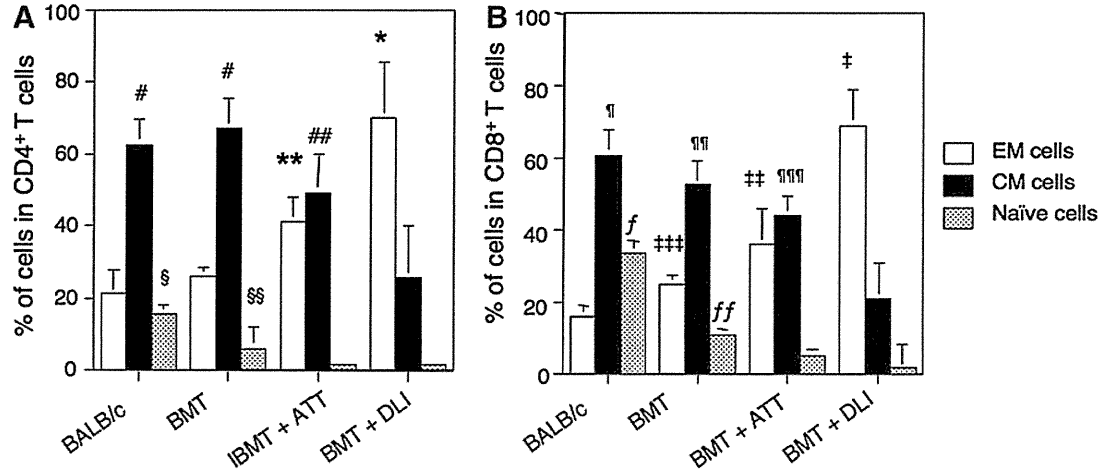

FIG. 4. Proportions of EM, CM, and naïve T cells from leukemia-bearing mice treated with BMT+ATT. Percentages of $CD62L^-CD44^+$ EM, $CD62L^+CD44^+$ CM, and $CD62L^-CD44^-$ naïve cells in $CD4^+$ **(A)** and $CD8^+$ **(B)** T cell subsets were analyzed in the spleens from normal BALB/c mice and leukemia-bearing mice treated with BMT alone, BMT+ATT, and BMT+DLI. Analyses were performed at the same time as for the experiment in Fig. 3. $^*P<0.01$ compared with BALB/c, BMT, and BMT+ATT. $^{**}P<0.01$ compared with BALB/c and BMT. $^{\#}P<0.05$ compared with BMT+ATT and BMT+DLI. $^{\#\#}P<0.01$ compared with BMT+DLI. $^{\S}P<0.01$ compared with BMT, BMT+ATT, and BMT+DLI. $^{\S\S}P<0.01$ compared with BMT+ATT and BMT+DLI. $^{\ddagger}P<0.01$ compared with BALB/c, BMT, and BMT+ATT. $^{\ddagger\ddagger}P<0.01$ compared with BALB/c and BMT. $^{\ddagger\ddagger\ddagger}P<0.01$ compared with BALB/c. $^{\P}P<0.05$ compared with BMT, BMT+ATT, and BMT+DLI. $^{\P\P}P<0.05$ compared with BMT+ATT and BMT+DLI. $^{\P\P\P}P<0.01$ compared with BMT+DLI. $^{f}P<0.01$ compared with BMT, BMT+ATT, and BMT+DLI. $^{ff}P<0.05$ compared with BMT+ATT and BMT+DLI. Data are shown as means±SD. Normal BALB/c ($n=5$), BMT ($n=5$), BMT+ATT ($n=5$), and BMT+DLI ($n=5$). CM, central memory; EM, effector memory.

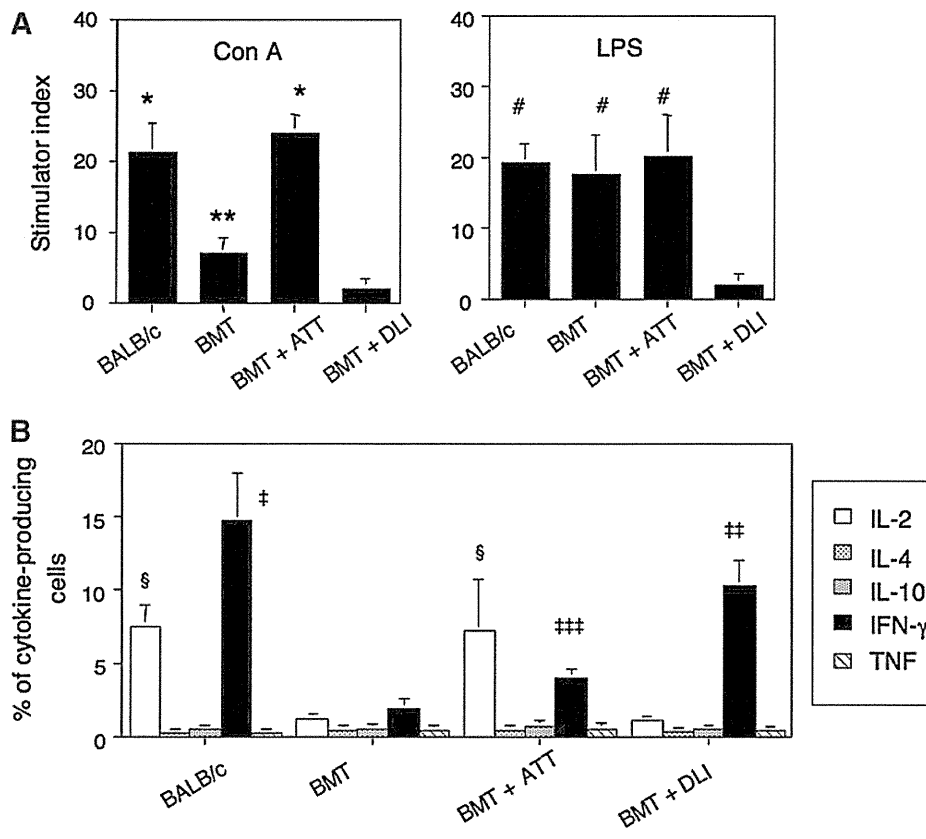

FIG. 5. Mitogen responses and percentages of cytokine-producing cells in the spleens from leukemia-bearing mice treated with BMT and ATT Mitogen responses: Con A and LPS **(A)** and percentages of cytokine-producing cells **(B)** in the spleen were evaluated in the spleens from normal BALB/c mice and leukemia-bearing mice treated with BMT alone, BMT+ATT, and BMT+DLI. Analyses were performed at the same time as for the experiment in Fig. 3. $^*P<0.05$ compared with BMT and BMT+DLI. $^{**}P<0.05$ compared with BMT+DLI. $^{\#}P<0.05$ compared with BMT+DLI. $^{\S}P<0.05$ compared with BMT and BMT+DLI. $^{\ddagger}P<0.05$ compared with BMT, BMT+ATT, and BMT+DLI. $^{\ddagger\ddagger}P<0.05$ compared with BMT and BMT+ATT. $^{\ddagger\ddagger\ddagger}P<0.05$ compared with BMT. Data are shown as means±SD. Normal BALB/c ($n=4$), BMT ($n=4$), BMT+ATT ($n=4$), and BMT+DLI ($n=4$). LPS, lipopolysaccharide.

an organ involved in the maintenance of homeostasis itself and regulates the production of not only T cells but also several cytokines and hormones in a feedback mechanism [30]. Thus, TT is different from DLI, which supplies mature lymphocytes in one direction. Hence, TT is a type of functional organ transplantation, and may represent an approach that significantly regulates the immune function of T cells in vivo for the benefit of the host.

Overall, we found that allogeneic IBM–BMT + ATT induces strong GVL effects with mild GVHD. Although it may be both ethically and technically difficult to obtain adequate thymus tissue in clinical cases (including the question of donor age), grafts could be obtained from patients with congenital heart diseases or from aborted fetuses, as previously utilized [31,32]. We have recently found that even if the thymus donor is different from the BMC donor, then the effect is comparable to that seen with transplantation from the same donor using triple chimeric mice [33]. In addition, a method of regenerating the thymus has also been developed [34–37]. Therefore, IBM–BMT + ATT could become a viable strategy for the treatment of malignant hematological tumors in humans.

Acknowledgments

This work was supported by a grant from Haiteku Research Center of the Ministry of Education; a grant from the Millennium Program of the Ministry of Education, Culture, Sports, Science, and Technology; a grant from the Science Frontier Program of the Ministry of Education, Culture, Sports, Science, and Technology; a grant from The 21st Century Center of Excellence (COE) Program of the Ministry of Education, Culture, Sports, Science, and Technology; a Research Grant B from Kansai Medical University, Health and Labor Sciences (Research on Human Genome, Tissue Engineering Food Biotechnology); a grant from the Department of Transplantation for Regeneration Therapy (sponsored by Otsuka Pharmaceutical Co., Ltd.); a grant from the Molecular Medical Science Institute (Otsuka Pharmaceutical Co., Ltd.); and a grant from Japan Immunoresearch Laboratories Co., Ltd. (JIMRO). The project was supported by Guangdong Natural Science Foundation. The authors wish to thank Ms. Y. Tokuyama and Ms. A. Kitajima for their technical assistance, and Mr. Hilary Eastwick-Field and Ms. K. Ando for their help in the preparation of this article.

Author Disclosure Statement

The authors disclose no commercial association that might create a conflict in connection with the submitted article. No competing financial interests exist.

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Received for publication July 7, 2011
Accepted after revision August 19, 2011
Prepublished on Liebert Instant Online August 23, 2011

IVYSPRING INTERNATIONAL PUBLISHER

International Journal of Biological Sciences

2012; 8(10):1335-1344. doi: 10.7150/ijbs.4654

Research Paper

Stem Cell Transplantation Increases Antioxidant Effects in Diabetic Mice

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Received: 2012.05.26; Accepted: 2012.09.13; Published: 2012.10.27

Abstract

Intra bone marrow-bone marrow transplantation (IBM- BMT) + thymus transplantation (TT) has been shown to reduce the incidence of graft versus host disease (GVHD) and restore donor-derived T cell function. In addition, an increase in insulin sensitivity occurred in db/db mice after IBM-BMT+TT treatment. Heme oxygenase (HO)-1 is a stress inducible enzyme which exert antioxidant, antiapoptotic, and immune-modulating properties. We examined whether IBM-BMT+TT could modulate the expression of HO-1 in the kidneys of db/db mice. Six-week-old db/db mice with blood glucose levels higher than 250 mg/dl were treated with IBM-BMT+TT. Six weeks later, the db/db mice showed decreased body weight, blood glucose levels and insulin, and increased plasma adiponectin levels. The upregulation of HO-1 was associated with significantly ($p<0.05$) increased levels of peNOS and pAKT, but decreased levels of iNOS in the kidneys of db/db mice. Plasma creatinine levels also decreased ($p<0.05$), and the expression of type IV collagen was improved. Thus IBM-BMT+TT unregulated the expression of HO-1, peNOS and pAKT, while decreasing iNOS levels in the kidney of db/db mice. This was associated with an improvement in renal function.

Key words: IBM-BMT+TT, antioxidant, HO-1, diabetic nephropathy.

Introduction

Heme oxygenase (HO)-1 is a stress inducible enzyme that catalyzes the degradation of heme proteins into free iron, CO and biliverdin, which is then rapidly converted into bilirubin. These catabolic end products exert antioxidant, antiapoptotic, and immune-modulating properties, rendering the overall function of HO-1 to be cytoprotective [1, 2]. Graft versus host disease (GVHD) is a major side effect of allogenic bone marrow transplantation (BMT) [3]. Intra bone marrow- BMT (IBM-BMT) appears to offer the best approach for allogenic BMT, as manifested by a reduced incidence of GVHD and the restoration of donor-derived T cell function [4-6]. We recently reported that, in db/db mice treated with IBM-BMT+ thymus (TT) transplantation, CD4/CD8 ratios were normalized with a consequent increase in plasma adiponectin levels and insulin sensitivity. In addition, the expression of pancreatic phosphorylated AK transforming factor (pAKT), phosphorylated liver kinase B1 (pLKB1), phosphorylated adenosine monophosphate-activated protein kinase (pAMPK) and HO-1 increased in thus-treated db/db mice [7].

The db/db mouse presents with renal abnormalities including proteinuria, glomerular hypertrophy, and glomerulosclerosis [8, 9], thereby making it an ideal animal model in which to study renal and

vascular dysfunction in diabetic nephropathy (DN). DN is the major cause of end-stage renal failure, mainly due to the increased incidence of type 2 diabetes mellitus [10]. Oxidative stress (OS) is considered a significant pathogenic factor in the development of diabetic vascular complications, including nephropathy [11, 12]. The beneficial effect of increased HO-1 expression and HO activity further suggests that the abnormality in endothelial progenitor cells is due to a mesenchymal stem cell (MSC) disorder exacerbated by OS and decreased levels of adiponectin [13].

In the present study, we examined whether allogenic IBM-BMT+TT modulated the expression of HO-1 in the kidneys of db/db mice and show that this intervention does indeed upregulate the expression of HO-1, phosphorylated endothelial nitric oxide synthase (peNOS) and pAKT, decrease inducible NOS (iNOS), and improve renal function, therefore providing a mechanistic basis for the therapeutic use of IBM-BMT+TT.

Materials and methods

Animals

Six-, 12- and 30-wk-old BKS.Cg-m+Lepr^{db}/+Lepr^{db}/J (H-2K^{d}) (db/db) mice and age-matched lean mice were purchased from Charles River Laboratories (Yokohama, 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. Blood glucose levels higher than 250 mg/dl on two consecutive measurements were considered to indicate the onset of diabetes. Each experiment was repeated three times.

IBM-BMT+TT

Six-wk-old db/db mice with blood glucose levels greater than 250 mg/dl and proteinuria greater than ++ on two consecutive measurements received fractionated irradiation twice a day (5.0 Gy×2, 4-hour interval). One day after irradiation, whole BMCs from B6 mice were injected into the recipient mice (1×10^7/mouse) by IBM-BMT using our previously-described method [7]. Simultaneously, the thymus from newborn B6 mice was grafted under the renal capsule of the left kidney of the recipient mice. All treated mice were sacrificed 6 wks after IBM-BMT+TT treatment.

Immunochemistry and measurement of nitric oxide (NO) levels

The pancreata, livers, kidneys and bones of lean and db mice were removed at each time point. After the tissues were fixed in 10% formalin for 24 hours at room temperature, they were embedded in paraffin. The sections (3-μm thick) were stained with hematoxylin and eosin. The pancreata were stained with polyclonal guinea pig anti-swine insulin antibody (N1542, Dako Cytomation, CA). The kidneys were stained with collagen type IV (Sigma-Aldrich, MO), pAKT, iNOS and eNOS (Abcam PLC, Cambridge, UK) antibodies. The stained sections were examined under a microscope.

NO levels in the kidneys were measured according to the manufacturer's protocol using a Nitric Oxide Assay Kit (BioAssay Systems, Hayward, CA).

Electron microscopy

The fixed renal cortices were embedded in epoxy resin using conventional methods [14]. Kidney tissue was fixed in 2.5% glutaraldehyde in 0.1M PBS, pH 7.4. Samples were washed and post fixed with 2% aqueous OsO_4. The samples were then dehydrated with serially-increasing concentrations of ethanol (60 to 100%), and were infiltrated with epon mixed with methyl nadic anhydride (TAAB Laboratories, UK), Dodecenyl succinic anhydride (TAAB Laboratories), and Quetol-812 and DMP-30 (Nishin Company, Japan). Ultrathin sections were prepared, stained using uranyl acetate and lead citrate, and then examined with a JEM-1400A electron microscope (JEOL, Tokyo, Japan). The measurements were taken from electron micrographs with magnifications of x2,500 and x12,000.

Adiponectin, insulin, creatinine and pancreatic insulin content measurements

Adiponectin was 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).

Plasma creatinine was measured according to the manufacturer's protocols using a plasma creatinine detection kit (Arbor Assays, Michigan).

For insulin content measurement, the pancreas was isolated and weighed, and then placed in 3ml cold acid ethanol (0.18M HCl in 70% ethanol) and homogenated. This was kept overnight at 4°C and centrifuged at 2400rpm for 30min at 4°C. The supernatant was transferred and stored at -20°C for measurement using ELISA.

Western blot analysis of kidney HO-1, peNOS, AKT, pAKT, and iNOS

At sacrifice, kidneys were dissected, then pooled for each mouse in order to measure signaling molecules. Specimens were stored at -140°C until assayed. Frozen kidney 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, and the supernatant was then isolated and protein levels were obtained by immunoblotting with the relevant antibodies. Antibodies against HO-1, AKT, phosphorylated AKT (pAKT), eNOS, peNOS and iNOS were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Antibodies were prepared by dilution as described previously [15, 16].

Flow cytometric analyses

Peripheral blood mononuclear cells were obtained from the tail vein of mice 30 days after transplantation. These cells were stained with antibodies against PE-H-2K^d, PE-CD4, PE-CD8a and FITC-H-2K^b (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). Isotype-matched immunoglobulins were used as controls.

Statistical analysis

Statistical significance between experimental groups was determined by the Fisher method of analysis of multiple comparisons. 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. Statistical significance was regarded as significant at $p<0.05$.

Results

Body weight, thymus weight, and morphology of pancreas and bone, and insulin content of pancreas islet

We first confirmed the parameters (body weight, thymus weight and morphology of pancreas and bone marrow) of the db/db mice and lean mice (as control) maintained in our animal facilities (Fig.1), since food and environment may affect some parameters of db/db mice. As seen in Fig. 1A, the body weight of 12-wk-old lean mice was significantly higher than that of 6-wk-old lean mice (30.4 ±0.5 vs 22.6±0.1 g, $p< 0.001$). Similar results occurred in the db/db mice (53.2 ±1.7 vs 33.5±0.03 g, $p< 0.001$). However, although the body weight of 30-wk-old db/db mice was significantly higher than that of 12-wk-old db/db mice (61.2±0.6 vs 53.2± 1.7 g, $p< 0.01$), there was no significant difference in body weight between 30-wk-old and 12-wk-old lean mice (32.4±0.5 vs 30.4± 0.5 g). As shown in Fig. 1B, thymus weight was significantly lower in the 6-wk-, 12-wk and 30-wk-old db/db mice than in the age-matched lean mice (51.7 ±4.7 vs 75.7±3.3, 21.3 ±0.9 vs 49±4.0, 20.5 ±1.2 vs 43±4.1mg, $p < 0.05$ at each time point).

On HE staining, 12-wk- and 30-wk-old db/db mice (Fig. 1E and F) exhibited larger islets than 6 wk-old db/db mice and lean mice (Fig. 1C and D). More adipocytes were present in the bone marrow of 12-wk- and 30-wk-old db/db mice (Fig. 1H-J) than in lean mice (Fig. 1G).

The db/db mouse shows insulin resistance and insulin-positive cell failure. The insulin content (brown color) was much lower in the larger islets of 6-wk-, 12-wk- and 30-wk-old db/db mice (Fig. 1L-N) than in those of lean mice (Fig. 1K), suggesting that more insulin was secreted into the peripheral blood in the db/db mice. Pancreatic insulin contents of lean mice (2336±84.5 pg/ml per mg pancreas), and 6-wk- (1149±37.5 pg/ml per pancreas), 12-wk- (521±21.7 pg/ml per mg pancreas), and 30-wk- (180±21.3 pg/ml per mg pancreas) old db/db mice were measured by ELISA.

Plasma creatinine levels and histology of the kidneys of db/db mice

As shown in Fig. 1O, plasma creatinine levels were significantly higher in 12-wk-old when compared to 6-wk-old db/db mice (3.41±0.20 vs 1.47±0.46 mg/dl, $p<0.05$), and in 30-wk-old compared to 12-wk-old db/db mice (4.65±0.03 vs 3.41±0.20 mg/dl, $p<0.05$). Electron microscopic images of the kidneys are shown in Fig. 1P-S. 12-wk and 30-wk db/db mice exhibited severe podocyte foot fusion. Electron dense deposits and increased numbers of mesangium cells were observed in the 30-wk-old db/db mice (Fig. 1R and S).

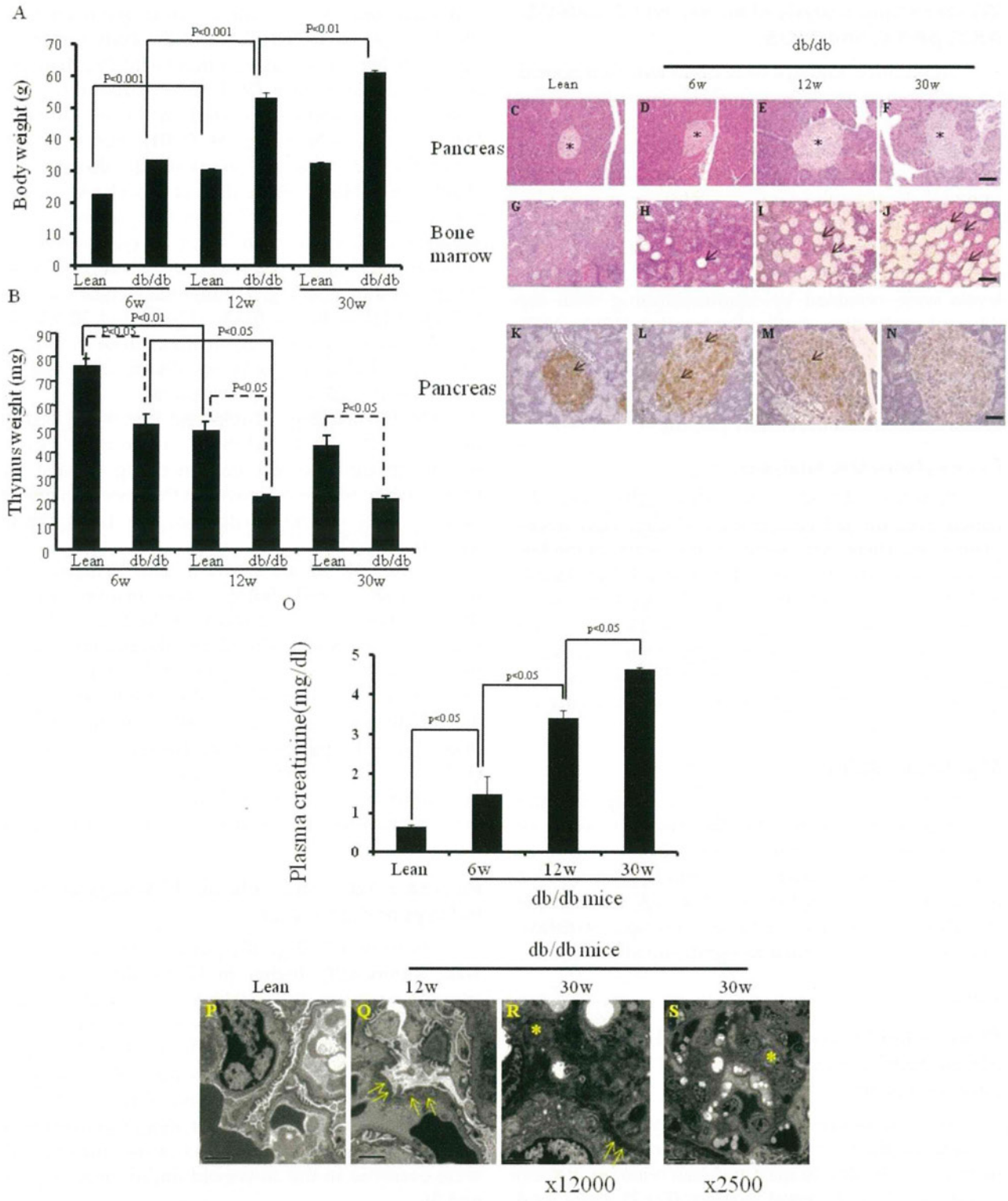

Figure 1. Body weight, thymus weight and the morphology of pancreas and bone marrow, and expression of insulin on the pancreata, and plasma creatinine levels and images of electron microcopy. (A) Body weights are shown (*p< 0.01). (B) Thymus weights are shown (*p<0.05). (C-J) HE staining of pancreas and bone marrow of the lean and db/db mice at each age. The islets are shown (* in Fig.C-F). The adipocytes are shown in db/db mice (arrows in Fig. H-J). (K-N) Immunochemistry staining for insulin (arrows) was performed. Scale bar =50μm in Fig. C-J, Scale bar =25μm in Fig. K-N. (O) Plasma creatinine levels of lean, (p< 0.05). (P-S) EM of kidneys. Podocyte foot fusion is shown in Fig. 3Q and R (arrows). Electron dense deposits and increased mesangium cells (*) are shown in Fig. 1R and S. Scale bar =2μm in Fig. P-R, Scale bar =10μm in Fig. S. The results are mean±SE, n=6 in each group.

Improved body weight, blood glucose and plasma adiponectin, and insulin levels 6 weeks after treatment with IBM-BMT +TT

As seen in Fig.2A, body weight was significantly ($p<0.01$) lower in the IBM-BMT+TT treated db/db mice than in the age-matched non-treated db/db mice. In addition, fasting blood glucose levels (Fig. 2B) were significantly ($p<0.01$) lower in the treated db/db mice than in the non-treated db/db mice. Indeed IBM-BMT+TT reduced blood glucose to the levels found in age-matched lean mice. As shown in Fig. 2C, the non-treated db/db mice exhibited a significantly ($p<0.05$) lower level of plasma adiponectin than age-matched lean mice. However, the plasma adiponectin levels were significantly ($p<0.05$) increased in the treated db/db mice when compared to the non-treated db/db mice (Fig. 2C). Plasma insulin levels were higher ($p<0.05$) in the non-treated db/db mice than in the age matched lean mice. IBM-BMT+TT treatment resulted in a decrease ($p<0.05$) in plasma insulin levels to the levels found in age-matched lean animals (Fig. 2D).

Lymphocyte subpopulations in peripheral blood and cell number of thymus 6 weeks after treatment with IBM-BMT +TT

The relative percentage of the total cell number in the thymus was significantly ($p<0.05$) lower in the non-treated db/db mice than in the lean mice (65.13% of that of lean mice). The total cell number of the thymus was significantly higher ($p<0.05$) increased in the treated mice (97.9% of lean mice) (Fig. 3A). Figure 3B shows the percentages of donor-derived cells ($H\text{-}2K^b$) in the peripheral blood of the treated db/db mice. The percentages of donor-derived $CD4^+$ and $CD8^+$ were 10.16% and 7.81% respectively, Figure 3C and D.

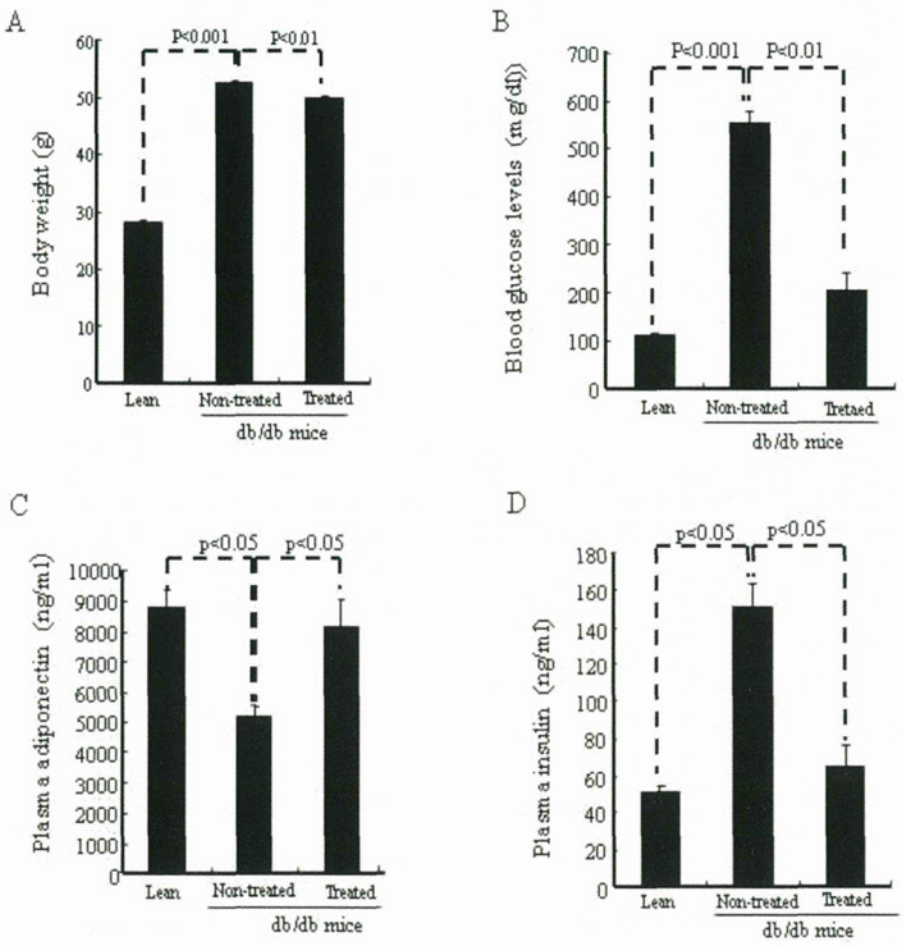

Figure 2. Body weight, blood glucose levels, plasma adiponectin, insulin. (A) Body weights are shown. (B) Blood glucose levels are shown. (C) Plasma adiponectin. (D) Plasma insulin.

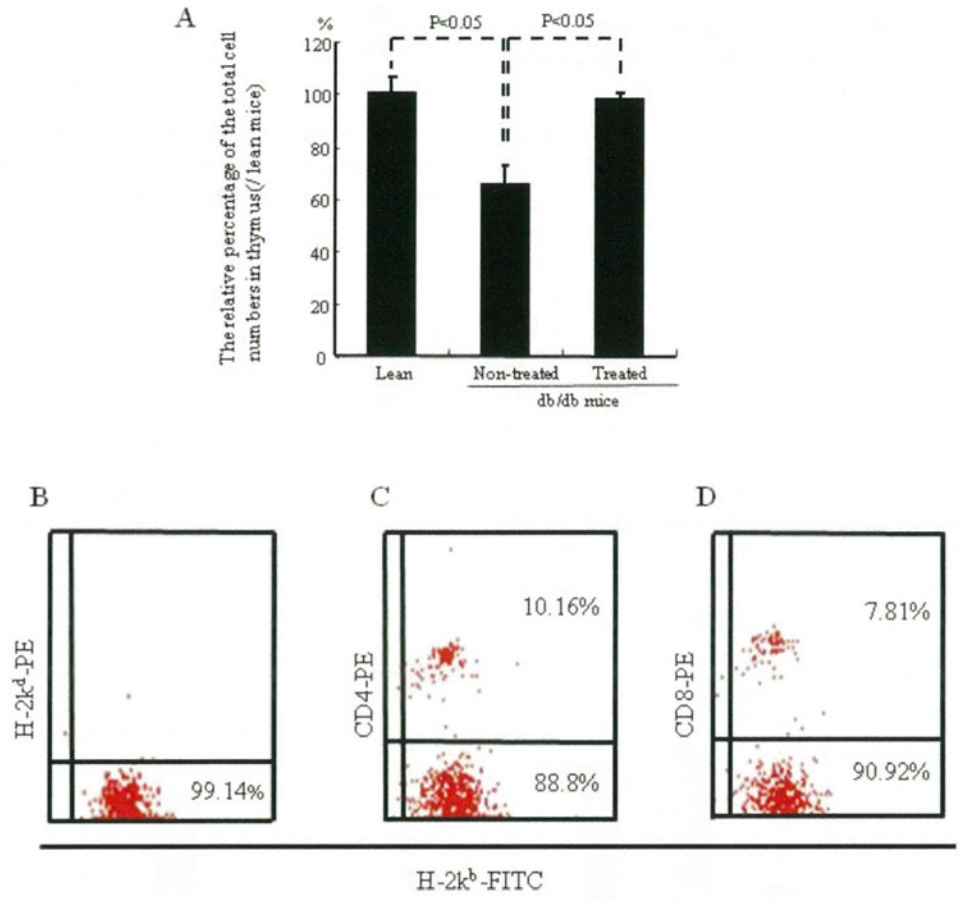

Figure 3. Cell number of thymus and lymphocyte subpopulations in peripheral blood. (A) The relative percentage of total cell numbers in the thymus, which was significantly increased in the db/db mice treated with IBM-BMT+TT. (B-D) Donor-derived cells in peripheral blood, $CD4^+$ cells and $CD8^+$ cells are shown. The results are mean±SE, n=6 in each group.

Lymphocyte function

Approximately 98% of hematolymphoid cells were of donor-origin in the peripheral blood of the recipients one month after IBM-BMT+TT treatment. Spleen cells of the recipient animals demonstrated mitogen responses to lipopolysaccharide, in contrast to those of non-treated db/db mice: 26296±2780.93 vs 19292±1310.42, respectively, $p<0.05$. In lean mice the mitogen response was 31406±2780.93. These findings suggest that IBM-BMT+TT treatment restores lymphocyte function in the db/db mice.

Expression of HO-1, iNOS, peNOS and pAKT in the kidney of db/db mice treated with IBM-BMT+TT

The expression of renal HO-1 was significantly lower ($p<0.05$) in the non-treated db/db mice when compared to lean mice, but significantly increased ($p<0.05$) in db/db mice treated with IBM-BMT+TT, when compared with non-treated db/db mice (Fig. 4A and B). The expression of iNOS was the receptacle of HO-1 expression and was significantly ($p<0.05$) higher in the non-treated db/db mice than in the lean mice. Densitometry analyses revealed a significant decrease in iNOS ($p<0.05$) in the kidney of treated db/db mice when compared with non-treated db/db mice (Fig. 4A and C).

The expression of pAKT and peNOS in the kidney was significantly lower ($p<0.05$) in the non-treated db/db mice when compared to the lean mice, but there was a significant increase in the expression of both pAKT and peNOS in the IBM-BMT+TT treated db/db mice, when compared with non-treated db/db mice (Fig. 4D-G, $p<0.05$). The expression of type IV collagen (brown color) was greater in the kidney of the non-treated db/db mice (Fig. 4I) than in the lean mice (Fig. 4H). However, it was significantly lower in the kidney (arrows in Fig. 4J) in the treated db/db mice than in the non-treated db/db mice.

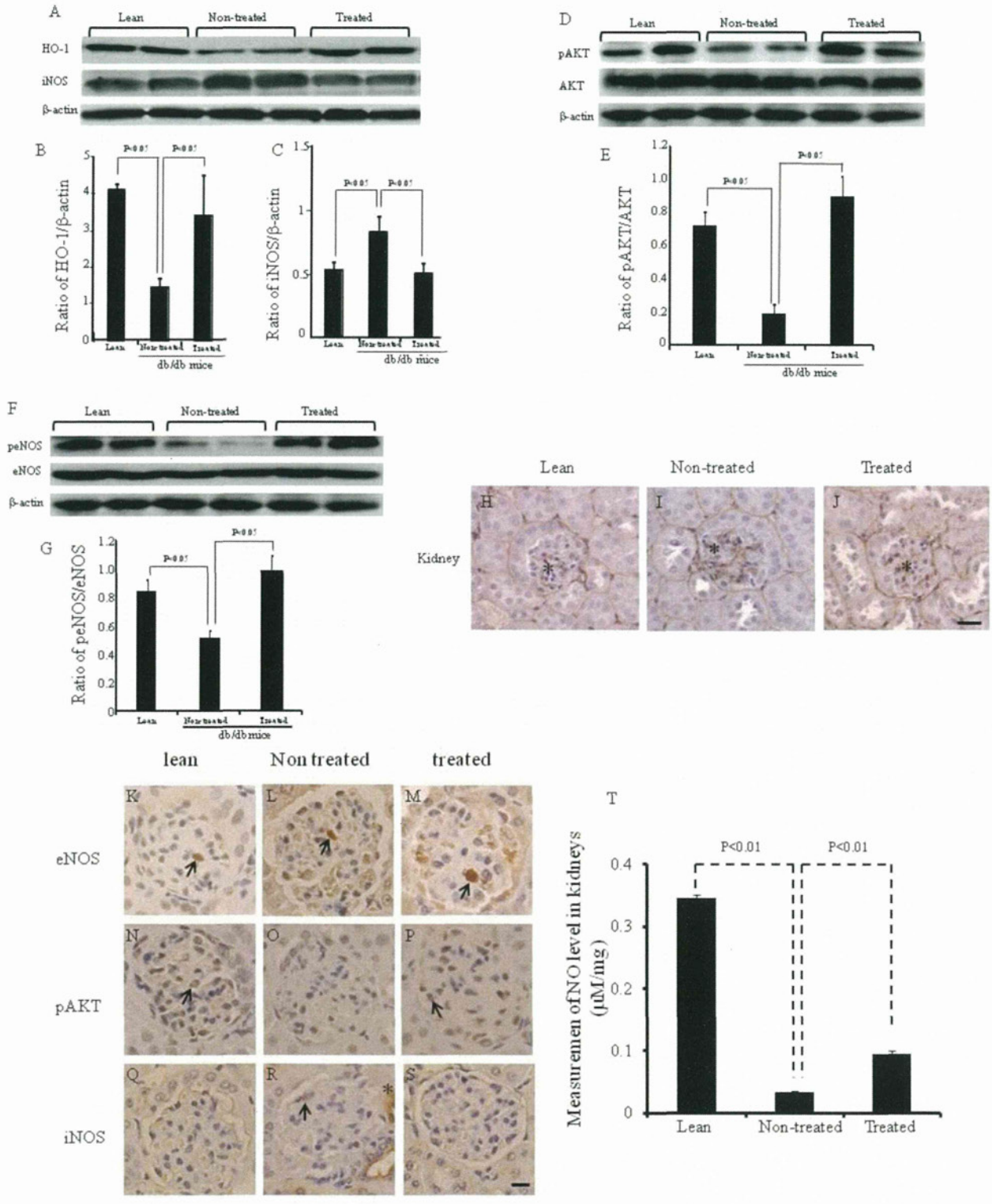

Figure 4. Expression of HO-1, iNOS AKT, pAKT, eNOS and peNOS in the kidney, and expression of type IV collagen in the kidneys. (A-C) Western blot and densitometry analysis of HO-1, iNOS and actin proteins in kidneys of lean, non-treated db/db mice and treated db/db mice. (D and E) Western blot and densitometry analyses of AKT and pAKT and actin proteins in kidney of lean, non-treated db/db mice and treated db/db mice are shown. (F and G) Western blot and densitometry analyses of eNOS and peNOS and actin proteins in kidney of lean, non-treated db/db mice and treated db/db mice are shown. Representative immunoblots are shown. There were significant differences between non-treated and treated groups ($p<0.05$). (H-J) Immunochemistry staining for type IV collagen (*). (K-S) Immunochemistry staining for eNOS, pAKT and iNOS. Scale bar =25μm. (T) NO levels in the kidney.

The expression of peNOS, pAKT and iNOS was confirmed by immunochemistry staining (Fig. 4K-T). eNOS positive cells were identified in the kidneys of lean, non-treated and treated db/db mice (arrow in Fig. 4K-M). pAKT positive cells were identified in the kidney of lean and treated db/db mice (arrow in Fig. 4N and P). However, none were found in the non-treated db/db mice. iNOS positive cells were identified in the kidney of non-treated db/db mice (arrow and *in Fig. 4R).

As shown in Fig. 4T, NO levels in the kidney were significantly lower in non-treated db/db mice when compared to lean mice (0.35±0.004 vs 0.03±0.002 μM/mg, $p<0.01$). However, the NO levels were significantly higher in the kidney (0.09±0.005 vs 0.03±0.002 μM/mg, $p<0.01$) in the treated db/db mice than in the non-treated db/db mice.

Discussion

Leptin receptor-deficient db/db mice exhibit severe hereditary obesity [17] and display hormonal imbalances and hematolymphoid defects [18, 19]. db/db mice exhibit a marked reduction in the size and cellularity of the thymus [20, 21]. Thus we firstly confirmed the body weight and thymus weight of db/db and lean mice at each age that were maintained in our animal facilities. Meantime, the morphology of the pancreas, kidney and bone marrow was also confirmed (Figure 1- 3). There were significant differences in body weight and thymus weight between 6-wk-and 12-wk-old mice. The pancreas, kidney and bone marrow showed differences in morphology at each time point. Previous studies have shown that IBM-BMT+TT improved insulin sensitivity in db/db mice 7 wks after treatment [7]. However, there are no reports indicating whether IBM-BMT+TT increased HO-1 expression, or benefited the kidney in db/db mice, although a protective effect of BMT on renal pathology has been reported [22].

HO-1 is the inducible form of HO (HO-2 is the constitute form) and is increased in response to OS. Increased levels of HO-1 slow the rate of weight gain and decrease levels of TNF-α and IL-6, but increase serum levels of adiponectin in obese diabetic mice [15]. db/db mice treated with IBM-BMT+TT exhibited a larger decrease in body weight and plasma insulin levels than age-matched non-treated db/db mice. Insulin administration to treated db/db mice produced a rapid decrease in blood glucose levels, suggesting improved insulin sensitivity after IBM-BMT+TT treatment. In the present study, HO-1 expression increased in the kidney after IBM-BMT+TT and was accompanied by an enhanced expression of peNOS and pAKT. A reduction in the production of nitric oxide [23] and a decrease in the expression of eNOS are reportedly associated with advanced DN [24]. An increase in AKT signaling is considered an important metabolic response to OS through the attenuation of ROS-mediated endothelial dysfunction [25]. The up regulation of renal HO-1 expression with a resultant increase in signaling molecules, including adiponectin via the pAKT-pAMPK-peNOS pathway, prevents the development of metabolic syndrome and improves both vascular and renal function [26].

HO has been shown to be important for attenuating the overall production of reactive oxygen species (ROS) through its ability to degrade heme and to produce carbon monoxide (CO), biliverdin/bilirubin, and the release of free iron, which possess potent antioxidant properties and antiapoptotic effects [26]. Recently, the induction of HO-1 has been found to reduce diabetic induced-glomerular injury and apoptosis, and these effects are associated with decreased NF-κB-induced inflammation and oxidative stress [27]. MSCs have the capacity to repair renal injury, accelerate tubular proliferation and improve renal function, and upregulate HO-1 expression and increase HO activity, all are essential for MSC growth and differentiation to the osteoblast lineage, which is consistent with the role of HO-1 in hematopoietic stem cell differentiation [28]. Induction of HO-1 by cobalt-protoporphyrin IX in recipient mice before conditioning and bone marrow transplantation (BMT) results in a reduction of GVHD and improved survival [29]. One report has indicated that HO-1 in dendritic cells may function as an inhibitor of the alloimmune response mediated by CD4+ T cells. HO-1 could thus play a key role in the design of therapies to prolong allograft function, based on the potent alloimmunity modulating capacity of this enzyme [30].

Plasma adiponectin levels are also significantly higher in treated db/db mice than in non-treated db/db mice. Adiponectin is a key regulator of albuminuria, suppressing not only i) OS and albuminuria but also ii) high levels of adiponectin phosphorylase and iii) active AMPK, presumably via adipoR1, which prevents OS and the fusion of the podocyte foot processes [31]. There is a report supporting the hypothesis that adiponectin is critical for both endothelial cell survival and function via the activation of eNOS and crosstalk between pAKT and pAMPK [32].

Bone marrow cells have the capacity to differentiate into mesangial cells [33, 34] and to transdifferentiate into podocytes. This is accompanied by the re-expression of the defective collagen chains and improved renal histology and function [35]. MSCs have the capacity to repair renal injury, accelerate tubular proliferation and improve renal function [36,