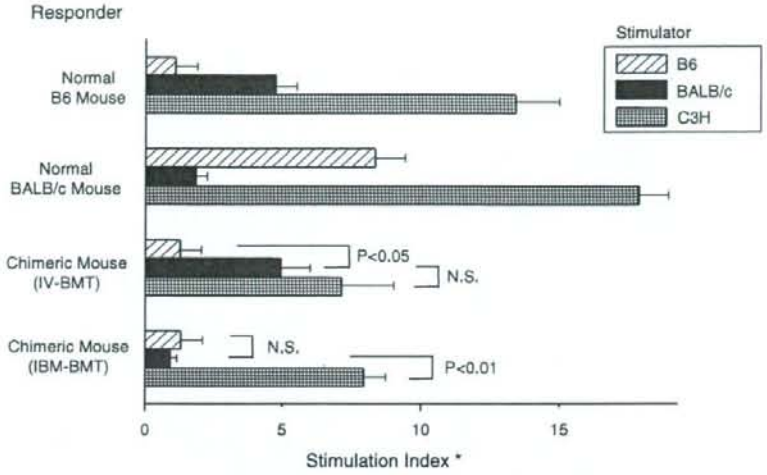

FIG. 5. MLR response of spleen cells obtained from chimeric mice that received 4.5 × 2 Gy of irradiation and IV- or IBM-BMT (8 weeks after transplantation). Spleen cells were collected from the chimeric mice 8 weeks after transplantation. Spleen cells were also obtained from untreated B6 or BALB/c mice. The cells were cultured in the absence or presence of stimulator cells. Three days later, [^{3}H]TdR uptake was measured. Tolerance to both donor and recipient types was observed in the chimeric mice in the IBM-BMT group. Mean ± SD of six wells. Representative data of three independent experiments. (*) Stimulation index = [^{3}H]TdR uptake on sample well (responder + stimulator)/[^{3}H]TdR uptake on control well (responder alone). N.S., Not significant.

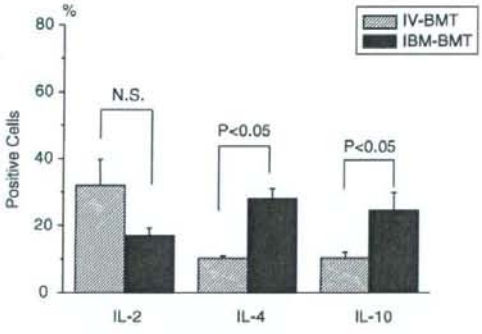

FIG. 6. Intracellular cytokine production in CD4$^+$ T cells obtained from chimeric mice that received 4.5 × 2 Gy of irradiation and intravenous (IV) or intra-bone marrow (IBM) bone marrow transplantation (BMT) (3 weeks after transplantation). Spleen cells collected from the chimeric mice were cultured in the presence of a leukocyte activation cocktail for 4 h. Cells treated in this manner were stained with anti-CD4 mAb-FITC. Intracellular cytokines (IL-2, IL-4, or IL-10) were detected by the permeabilization and the following staining with anti-cytokine mAb-PE. The percentages of double-positive cells (cytokine-producing cells in the CD4$^+$ T cell population) were measured using FACScan. Three chimeric mice per the IV- or IBM-BMT group were analyzed. Data represent the mean ± SD of three chimeric mice. Representative data of three independent experiments. N.S., Not significant.

skin and intestinal mucosa induced by the high-dose irradiation and therefore the general conditions of the recipient mice were greatly improved, (2) the second irradiation effectively removed the recipient HSCs/progenitor cells and the immunocompetent cells that have just re-entered the cell cycle from the dormant stage after the first irradiation. Therefore, if this irradiation protocol was applied to the conditioning before BMT and/or organ transplantation in humans, it might provide significant advantages for the engraftment of donor hematopoietic cells and organs. In the conditioning for patients with end-stage diseases, however, more mild myeloablative regimens [for example, an administration of fludarabine or donor lymphocyte infusion (DLI) combined with two lower doses of irradiation with a 4-h interval than in the present experimental protocol] should be considered.

In summary, the present study demonstrated that successful adult liver allografts could be achieved even in recipients that had been preconditioned with low-dose irradiation when donor BMCs were simultaneously engrafted via the IBM route.

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ORIGINAL ARTICLE

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

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

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Introduction

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

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

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

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

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

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Materials and methods

Mice

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

HSCs and thymus transplantation

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

IL-7 treatment in vivo

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

Reverse transcription-PCR

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

Western blotting

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

Analysis of surface markers and the numbers of lymphocytes by flow cytometry

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

Pathological findings

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

Statistical analysis

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

Results

Survival rates and chimerism in SLI mice receiving HSCs with or without thymus transplantation from variously aged donors

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

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

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

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

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

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

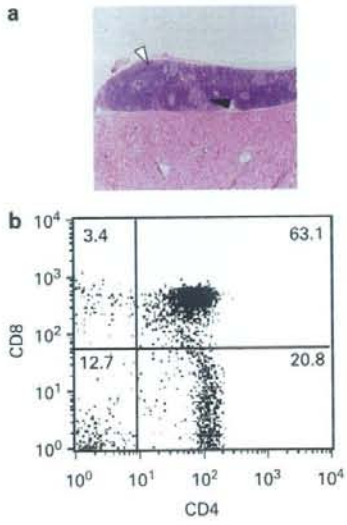

Figure 2 Histology and CD4/CD8 expression in transplanted thymus from SLI mice receiving NLCs plus NT transplantation. Histology (hematoxylin and eosin, ×200) (**a**) and percentages of $CD4^+$ and $CD8^+$ thymocytes (**b**) in engrafted NT from SLI BALB/c mice that had received 1×10^7 NLCs plus NT transplantation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

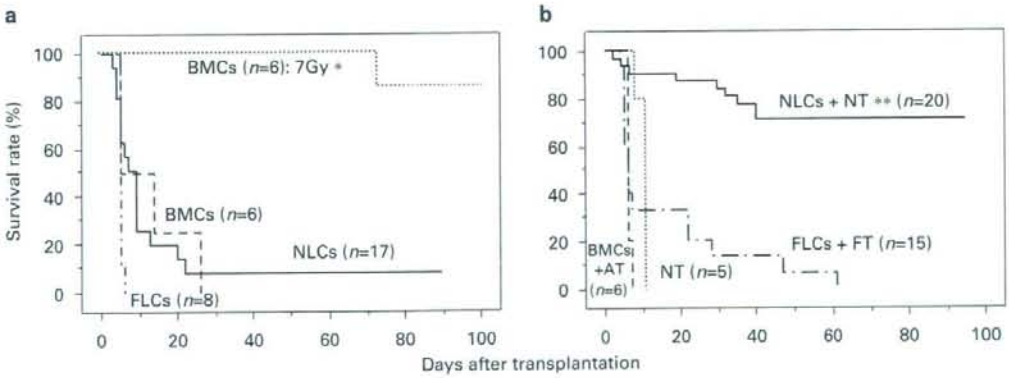

Figure 1 Survival rate in lethally irradiated or SLI mice receiving HSCs with or without thymus transplantation from variously aged donors. Survival rate for lethally irradiated mice (7 Gy) that had received 1×10^7 BMCs alone and SLI BALB/c mice (9.5 Gy) that had received 1×10^7 FLCs, NLCs or BMCs alone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

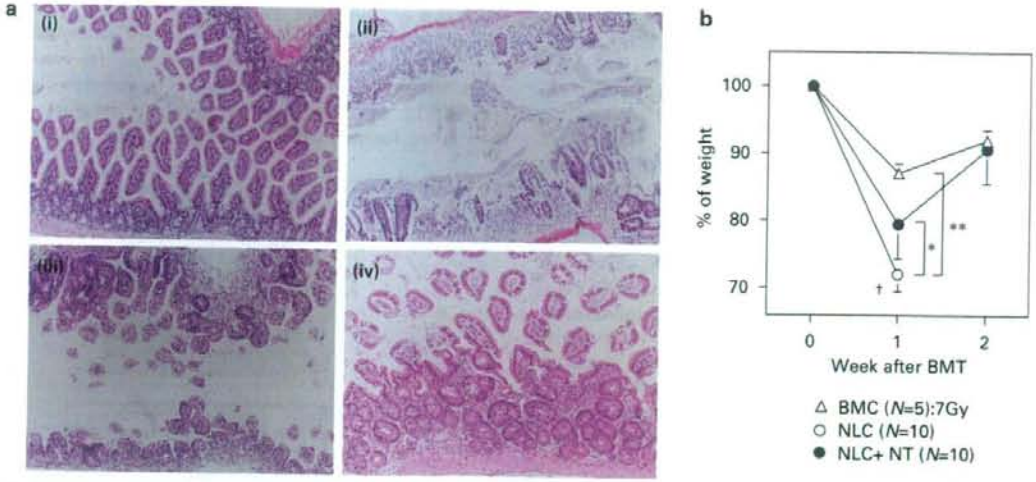

Figure 3 Small intestine histology and weight in SLI mice receiving NLCs with or without NT transplantation. Histology of the small intestine (hematoxylin and eosin, ×200) (**a**) and percentage of weight loss (**b**) in lethally irradiated (7 Gy) BALB/c mice that had received 1×10^7 BMCs or SLI BALB/c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

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

Analyses of IL-7 and KGF production in thymus grafts

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

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

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

Discussion

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

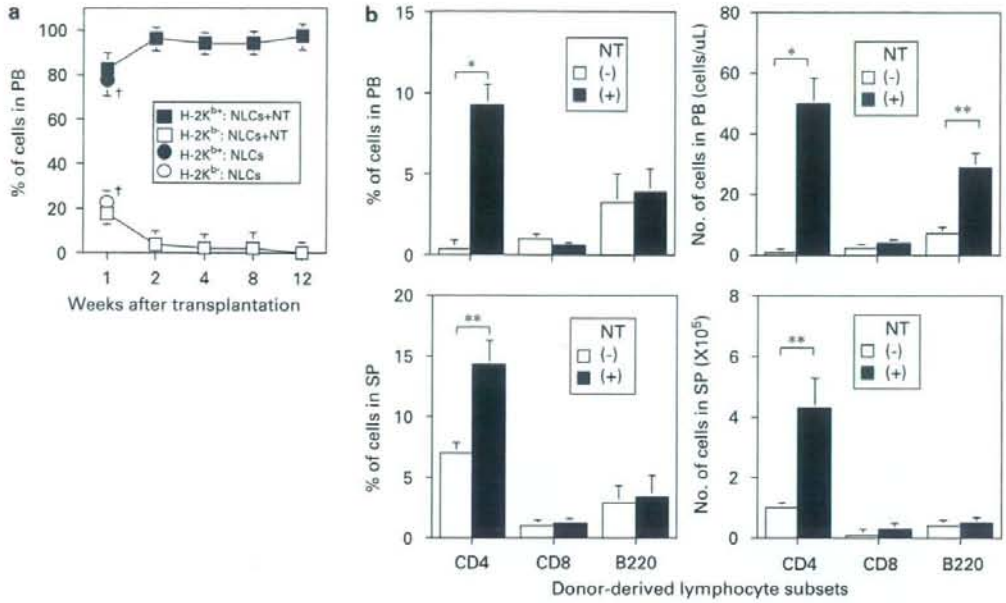

Figure 4 Analyses of chimerism and lymphocyte subsets in peripheral blood and spleen from SLI mice receiving NLCs plus NT transplantation at the early phase after transplantation. Chimerism of donor-derived cells (H-2K^{b+} cells) and host-derived cells (H-2K^{b-} cells) in the peripheral blood from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

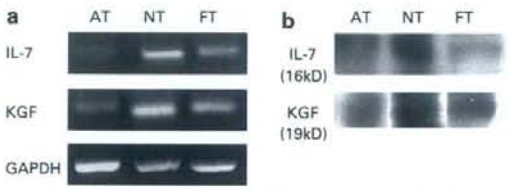

Figure 5 IL-7 and KGF levels of mRNA and protein in AT, NT and FT. The mRNA expression levels of IL-7, KGF and GAPDH according to RT-PCR (**a**) and protein levels of IL-7 and KGF by western blotting (**b**) were examined in freshly isolated AT, NT and FT from the aged donors. Representative data are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

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

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

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

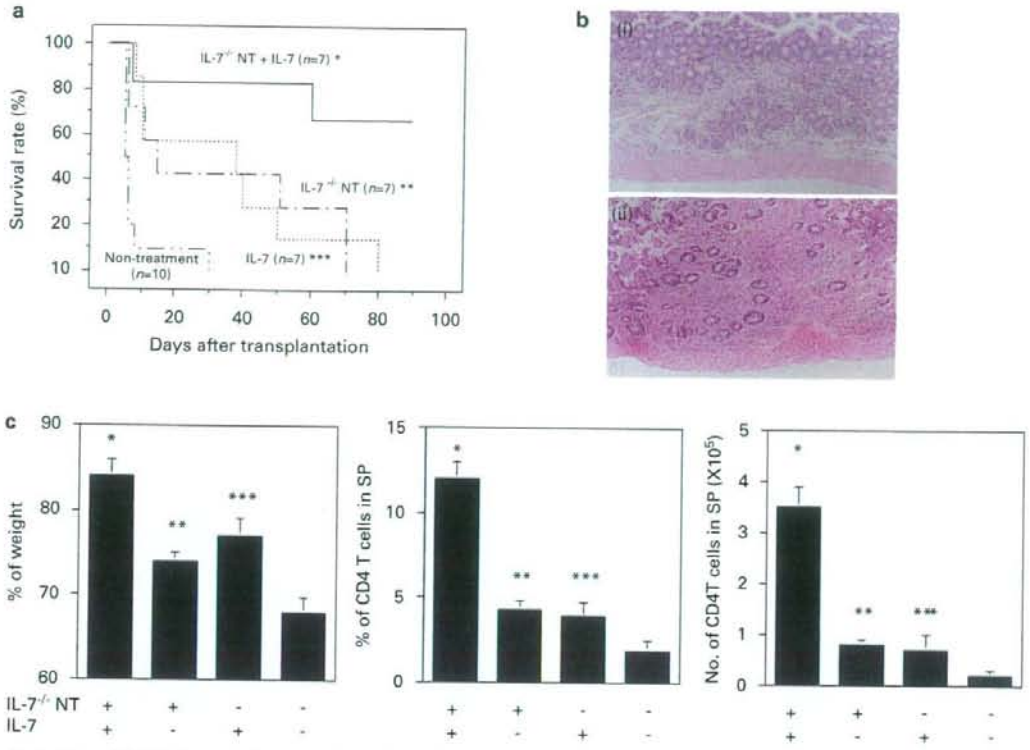

Figure 6 Analyses of survival rate, small intestinal histology, weight recovery and CD4 T-cell induction in spleen from SLI mice receiving NLCs with or without IL-7$^{-/-}$ NT transplantation in the presence or absence of IL-7 treatment. SLI BALB/c mice were transplanted with 1×10^7 NLCs with or without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

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

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

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

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

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

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

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IMMUNOLOGY ORIGINAL ARTICLE

Adult thymus transplantation with allogeneic intra-bone marrow–bone marrow transplantation from same donor induces high thymopoiesis, mild graft-versus-host reaction and strong graft-versus-tumour effects

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Summary

Although allogeneic bone marrow transplantation (BMT) plus donor lymphocyte infusion (DLI) is performed for solid tumours to enhance graft-versus-tumour (GVT) effects, a graft-versus-host reaction (GVHR) is also elicited. We carried out intra-bone marrow–bone marrow transplantation (IBM-BMT) plus adult thymus transplantation (ATT) from the same donor to supply alloreactive T cells continually. Normal mice treated with IBM-BMT + ATT survived for a long time with high donor-derived thymopoiesis and mild GVHR. The percentage of $CD4^+$ $FoxP3^+$ regulatory T cells in the spleen of the mice treated with IBM-BMT + ATT was lower than in normal B6 mice or mice treated with IBM-BMT alone, but higher than in mice treated with IBM-BMT + DLI; the mice treated with IBM-BMT + DLI showed severe GVHR. In tumour-bearing mice, tumour growth was more strongly inhibited by IBM-BMT + ATT than by IBM-BMT alone. Mice treated with IBM-BMT + a high dose of DLI also showed tumour regression comparable to that of mice treated with IBM-BMT + ATT but died early of GVHD. By contrast, mice treated with IBM-BMT + a low dose of DLI showed longer survival but less tumour regression than the mice treated with IBM-BMT + ATT. Histologically, significant numbers of $CD8^+$ T cells were found to have infiltrated the tumour in the mice treated with IBM-BMT + ATT. The number of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling (TUNEL)-positive apoptotic tumour cells also significantly increased in the mice treated with IBM-BMT + ATT. Allogeneic IBM-BMT + ATT thus can induce high thymopoiesis, preserving strong GVT effects without severe GVHR.

Keywords: graft-versus-host; graft-versus-tumour; intra-bone marrow–bone marrow transplantation; regulatory T cells; thymopoiesis; thymus transplantation

Introduction

Allogeneic bone marrow transplantation (BMT) has been used as a potentially curative therapy for patients with a wide variety of diseases, including haematological disorders, congenital immunodeficiencies, metabolic disorders, autoimmune diseases, and solid tumours.[1–7] However, BMT alone is not wholly effective against tumours, which tend to recur, particularly in the absence of T cells.[7] To enhance graft-versus-leukemia (GVL) or

Abbreviations: ATT, adult thymus transplantation; BM, bone marrow; BMC, bone marrow cell; BMT, bone marrow transplantation; DLI, donor lymphocyte infusion; FITC, fluorescein isothiocyanate; FoxP3, forkhead-box transcription factor p3; GVHD, graft-versus-host disease; GVT, graft-versus-tumour; HE, haematoxylin and eosin; HPF, high-power field; IBM-BMT, intra-bone marrow–bone marrow transplantation; IFN, interferon; IL, interleukin; IV-BMT, intravenous bone marrow transplantation; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; MSC, mesenchymal stem cell; PE, phycoerythrin; TREC, T-cell receptor rearrangement excision circle; Treg, regulatory T cell; TT, thymus transplantation; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling.

graft-versus-tumour (GVT) effects, donor lymphocyte infusion (DLI) is often performed following allogeneic BMT.[8–10] Although DLI can produce remission of leukemia[11] or the regression of solid tumours, GVL and GVT effects unfortunately seem to be closely associated with graft-versus-host disease (GVHD), which remains a major cause of post-transplantation morbidity and mortality.[12–14] New cellular-based methods are thus desired.

We have developed various new BMT methods. To supply recipients with major histocompatibility complex (MHC)-matched bone marrow (BM) stromal cells, we previously performed BMT plus bone grafts from the same donor.[5] For aging hosts with thymic involution, we performed thymus grafts with BMT.[15] To induce extramedullary haematopoiesis in the liver, we injected bone marrow cells (BMCs) from the portal vein.[16] Finally, we have recently developed intra-bone marrow (IBM)-BMT, in which BMCs are directly injected into the BM cavity.[17]

We have found that IBM-BMT not only allows us to use low-dose irradiation as a pre-conditioning regimen[17] but also helps to suppress GVHD,[18] as this IBM-BMT method can efficiently recruit donor-derived stromal cells [including mesenchymal stem cells (MSCs)], which can support donor-derived haemopoietic stem cells.[1,19–21] In addition, it has recently been shown, even in humans, that stromal cells or MSCs suppress GVHD.[22,23]

The thymus is an organ in which T cells can be induced to differentiate from precursor T cells. In addition, to maintain homeostasis during events such as autoimmune disease, infection, graft rejection and the growth of malignant tumours, the thymus itself regulates the production, proliferation and function of T cells not only by producing cytokines and hormones such as interleukin (IL)-4, IL-5 and IL-7, stem cell factor, thymopoietin and thymic stromal lymphopoietin,[24–26] but also by inducing functional subsets of T cells, including $CD4^+$ $CD25^+$ forkhead-box transcription factor p3 $(FoxP3)^+$ regulatory T cells (Treg), $CD4^+$ $CD25^-$ $FoxP3^-$ effector T cells and $CD8^+$ T cells.[27] Recently, Tregs have also been shown to preserve GVT effects while inhibiting GVH reactions (GVHRs).[28,29]

We have previously reported that fetal thymus transplantation in conjunction with allogeneic BMT from the same donor is successful for aged hosts who show low T-cell function.[15] In addition, we have also recently found that allogeneic BMT plus adult thymus transplantation (ATT) can be used to treat autoimmune diseases in chimeric-resistant MRL/Mp-Ipr/Ipr (MRL/Ipr) mice.[30] Interestingly, although T-cell functions were well restored or enhanced, concomitant GVHD was not observed. Thymus transplantation may thus represent an attractive method for improving T-cell functions.[31,32] However, thymus transplantation has only been clinically applied to patients with DiGeorge syndrome or human immunodeficiency virus infection who show hypoplasia of the thymus.[33,34] Its effectiveness in the treatment of other intractable diseases, including cancers, has not been examined in any detail.

In the present study, we attempt to carry out allogeneic IBM-BMT + ATT from the same donor for cancer therapy to recruit naïve allogeneic T cells continuously *in vivo*. We found that the high thymopoiesis induces strong GVT effects without inducing severe GVHR.

Materials and methods

Mice

Male C57BL/6 (B6:H-2^b) and female BALB/c (H-2^d) mice were obtained from Shimizu Laboratory Supplies (Kyoto, Japan). All mice were kept in our animal facilities under specific pathogen-free conditions. B6 mice were used as donors and BALB/c mice were used as recipients at the age of 6–8 weeks. All protocols for these animal experiments were approved under the Guideline for Animal Experimentation, Kansai Medical University.

Cell lines

Meth-A cells (H-2^d) are derived from methylcholanthrene-induced sarcoma in BALB/c mice. The cells were kindly provided by Dr Junko Yoshida of Kanazawa Medical School (Kanazawa, Japan). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum with antibiotics.

Inoculation of tumour cells

One day before inoculation of tumour cells, recipients (BALB/c mice) underwent total body irradiation (3 Gy) using a ^{137}Cs irradiator (Gammacell 40 Exactor; MDS Nordion International, Ottawa, Ontario, Canada). The next day, 2×10^6 Meth-A cells were subcutaneously inoculated into the right flank of the mice. We also examined the influence of 3-Gy irradiation in the mice before IBM-BMT. The lymphocytes recovered well after 2 weeks, which is the time required to grow the tumour sufficiently for IBM-BMT (described below). Therefore, the influence of irradiation was minimal.

BMT and thymus transplantation (TT)

Recipient 6- to 8-week-old BALB/c mice were irradiated (4·5 Gy × 2, at a 4-hr interval) using the ^{137}Cs irradiator 1 day before BMT. Bone marrow cells were flushed from the shafts of the femurs and tibias of donor 6- to 8-week-old B6 mice and single-cell suspensions were prepared. B6 BMCs (2×10^7) were directly injected into the BM cavity of the tibia, as previously described for the IBM-BMT method.[17] Simultaneously, a quarter of each of the

removed thymic lobes from the same donor B6 mice was grafted under the renal capsule of the left kidney, or transplanted splenocytes (1×10^7 or 3×10^6). from the same donor were injected intravenously into some mice as DLI. As thymic function is significantly age-dependent, we used young thymus grafts from the same donor 6- to 8-week-old B6 mice. We previously carried out TT in the muscle (intramuscle) of the thigh.[30] Although this is an effective method, grafting under the renal capsule is preferable because of the higher success rate. Therefore, we carried out TT under the renal capsule in the present study.

Histology

A histological study was performed on the liver, intestine and grafted tumour obtained from recipients 3 weeks after BMT. Tissues were fixed in 10% formaldehyde and embedded in paraffin. Serial tissue sections (4 μm thick) were prepared and stained using haematoxylin and eosin (HE). The degree of GVHD was evaluated using a semiquantitative scoring system for abnormalities known to be associated with GVHD, as previously described.[35,36] In the scoring system, for each parameter, 0 denotes normal, 0·5 focal and rare, 1 focal and mild, 2 diffuse and mild, 3 diffuse and moderate, and 4 diffuse and severe in GVHD. The maximum score for the liver was thus 40, and for the small intestine it was 28. We examined five to seven slides of tissue samples measuring > 10 × 5 mm from different sites of each organ in five or six mice from each group. The average scores were compared between the respective groups.

Analyses of tumour-cell apoptosis

Apoptosis of tumour cells was measured with the *in situ* terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling (TUNEL) method, using an *in situ* Apoptosis Detection Kit (Takara, Shiga, Japan), as previously described.[37] Tumour cells with TUNEL-positive nuclei were interpreted as displaying apoptotic changes. Positively stained cells were counted in 10 high-power fields (HPFs; ×400) in a blinded manner by two researchers, and the average was calculated as the number of apoptotic cells per HPF.[38] We examined five slides of tissue samples measuring > 5 × 5 mm from different sites of the tumours in five mice from each group. The average scores were compared between groups.

Immunohistochemistry

Tumour tissues were embedded in Tissue-Tek optimal cutting temperature (OCT) compound (Sakura Finetek, Tokyo, Japan) and stored at −40°. Cryosections (4 μm thick) were air-dried and fixed with acetone for 10 min. Specimens were treated using 0·5% bovine serum albumin in Tris-buffered saline (TBS) for 10 min, and then stained with biotin-conjugated H-2Kb or H-2Kd monoclonal antibodies (mAbs) and phycoerythrin (PE)-conjugated rat anti-mouse CD45, or with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD8 or CD4 mAbs (Pharmingen, San Diego, CA) for 1 hr. The reaction of avidin–FITC was followed by H-2 staining. Expressions were evaluated by confocal microscopy using an LSM 510 META microscope (Carl Zeiss, Minneapolis, MN). Numbers of positive cells per HPF were calculated using the same methods as those described above.

Analyses of surface marker antigens and intracellular FoxP3 and cytokines by flow cytometry and numbers of lymphocytes

Surface markers on lymphocytes from peripheral blood and the spleen were analysed with three-colour fluorescence staining using a FACScan system (Becton Dickinson, Franklin Lakes, NJ). FITC-conjugated anti-H-2K^b (Pharmingen) was used to determine chimerism. Phycoerythrin- or biotin-conjugated CD4 or CD8 (Pharmingen) was used to analyse lymphocyte subsets. Avidin-Cy5 (Dako, Kyoto, Japan) was used as the third colour in the avidin/biotin system. The percentage of T cells was evaluated by determining the per cent of CD4$^+$ plus CD8$^+$ T cells. Intracytoplasmic FoxP3 staining was performed using an FITC-anti mouse/rat FoxP3 staining set (eBioscience, San Diego, CA). The procedure was performed in accordance with the instructions of the manufacturer. Intracellular cytokines [IL-2, IL-4, IL-10, and interferon (IFN)-γ] were detected using an Intracellular Cytokine Staining Kit (Pharmingen). The procedure was also performed in accordance with the instructions of the manufacturer. The numbers of lymphocytes in the peripheral blood were calculated as the total numbers of white blood cells measured by SF-3000 with the SFVU-1 unit (Sysmex, Kobe, Japan). The numbers of T cells were calculated by the percentages of T cells.

Relative evaluation of T-cell receptor excision circle

The T-cell receptor rearrangement excision circle (TREC) was evaluated using real-time polymerase chain reaction (PCR), as previously described.[39] The method was modified in some parts for relative evaluation. A standard curve was obtained using thymocytes from donor B6 mice. A total of 1×10^7 thymocytes were stored at −80°. These cells were then lysed by incubation at 55° for 1 hr in 25 μl of 100 μg/ml proteinase K (TaKaRa, Tokyo, Japan) in 10 mM Tris. The sample was assayed at 5 μl per PCR reaction. In the samples, T cells enriched (purity > 98%) from 1×10^7 splenocytes using magnetic beads with anti-mouse CD45R, CD11b and Gr-1 Abs (BD Pharmingen) were used for assays. Cells were lysed with 25 μl of 100 μg/ml proteinase K (TaKaRa) in 10 mM Tris. For DNA purification, 5-μl

aliquots of the resulting samples were used. DNA was obtained from standard and experimental samples using a Puregene Cell and Tissue DNA purification kit (Gentra Systems, Minneapolis, MN). Real-time quantitative PCR was performed with standard thymocyte DNAs (diluted to 1/10, 1/100, 1/1000 and 1/10 000) and samples from chimeric mice containing 0·5 μM forward (CAT TGC CTT TGA ACC AAG CTG) and reverse (TTA TGC ACA GGG TGC AGG TG) primers of the T-cell receptor (TCR) α/δ locus gene, 0·3 μM fluorescent probe (FAM-CAG GGC AGG TTT TTG TAA AGG-QSY) and iQ Supermix (Bio-Rad, Hercules, CA). Amplifications were performed in duplicate on a DNA Engine OPTICON2 (MJ Research, Waltham, MA) and analysed using associated OPTICON MONITOR2 software (MJ Research). PCR conditions were 95° for 3 min followed by 50 cycles at 95° for 30 seconds and 63° for 30 seconds. A standard curve for TREC was obtained using serially diluted DNA samples from thymocytes of B6 mice, and the relative quantity of TREC in the spleen from chimeric mice was determined. Every assay was performed at least twice to confirm the results.

Mixed lymphocyte reaction

T cells that had been enriched (to a purity > 98%) using magnetic beads (Invitrogen, Carlsbad, CA) with anti-mouse CD45R, CD11b and Gr-1 (Pharmingen) were used for responders. The enriched T cells were incubated with 2×10^5 splenocytes irradiated at 15 Gy from various strains of mice including donor (B6) mice, recipient (BALB/c) mice, and third-party (C3H) mice as stimulators for 96 hr. Twenty millilitres of 0·5 μCi [^{3}H]thymidine (^{3}H-TdR; New England Nuclear, Cambridge, MA) was introduced during the last 18 hr of the culture period. The incorporation of ^{3}H-TdR was measured using Microbeta TriLux (Perkin-Elmer, Wellesley, MA). The stimulator index for the mixed lymphocyte reaction (MLR) was calculated as the average of ^{3}H-TdR incorporation (stimulator in medium)/^{3}H-TdR incorporation (medium) in triplicate wells.

Statistical analyses

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

Results

Effects of IBM-BMT + ATT on survival rate, body weight, chimerism and T-cell count in peripheral blood

First, we carried out conventional intravenous (IV)-BMT (intravenous injection of marrow cells) using low-dose irradiation (4·5Gy × 2) and radio-sensitive BALB/c mice as recipients. However, most of the (B6→BALB/c) chimeric mice (produced by IV-BMT) died of infection resulting from graft failure. Some chimeric mice survived but no donor-derived cells could be found. We therefore carried out IBM-BMT, as we know that IBM-BMT allows us to use low-dose irradiation, as previously described.[17,18,40,41]

We examined the effects of IBM-BMT + ATT on survival rate, weight, chimerism and T-cell count in the peripheral blood (Fig. 1a). BALB/c mice reconstituted with B6 BMCs by IBM-BMT with or without B6 ATT survived for a long time (> 100 days) and there was no significant difference in weight between mice with and without ATT. Regarding chimerism, all mice, regardless of ATT, showed approximately 100% donor-derived chimerism by 2 weeks after BMT. The number of lymphocytes increased to the same extent with and without ATT. Interestingly, both the percentages of T cells and the cell counts of T cells in the peripheral blood from the mice treated with IBM-BMT + ATT were significantly higher than in the mice treated with IBM-BMT alone. T-cell counts in the mice treated with IBM-BMT + ATT were about 1·5-fold higher than in the mice treated with IBM-BMT alone, and the counts remained elevated for up to 8 weeks after BMT.

Analyses of thymopoiesis induced by IBM-BMT + ATT

Next, we investigated thymopoiesis in the mice treated with IBM-BMT + ATT (Fig. 1b). Histologically, the transplanted thymus showed a normal appearance with both cortex and medullary constructions under the renal capsule 3 months after transplantation. Almost normal thymocyte differentiation was seen in $CD4^-$ $CD8^-$, $CD4^+$ $CD8^+$, $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ cells. In the spleen, although total cell counts did not differ between 3 weeks and 3 months after BMT, the numbers of both CD4 and CD8 T cells in the mice treated with IBM-BMT + ATT were significantly higher than in the mice treated with IBM-BMT alone 3 weeks after BMT. A significant number of T-cell subsets by IBM-BMT + ATT also increased 3 months after treatment (data not shown). Conversely, the number of $B220^+$ B cells was significantly lower in the mice treated with IBM-BMT + ATT than in the mice treated with IBM-BMT alone. The lymphocyte subset in the lymph nodes showed a similar tendency (data not shown). However, as it is unclear whether the high number of T cells was a result of peripheral proliferation or production by the transplanted thymus, we performed TREC analyses using real-time PCR on the spleens of chimeric mice. The relative quantity of TREC in the spleen cells of the mice treated with IBM-BMT + ATT

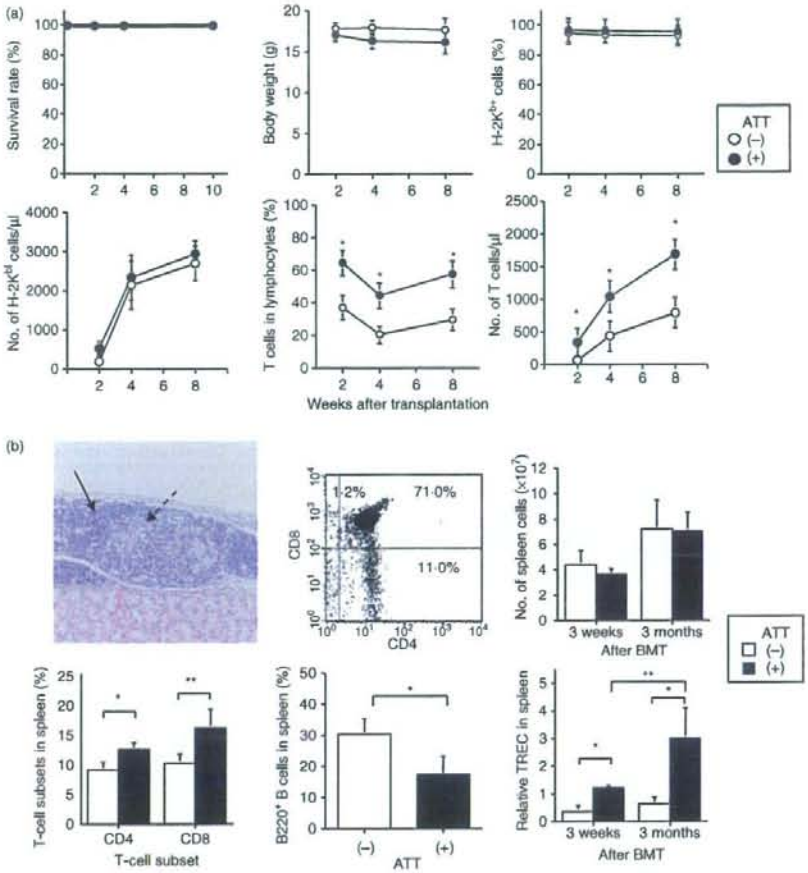

Figure 1. Effect of intra-bone marrow–bone marrow transplantation (IBM-BMT) + adult thymus transplantation (ATT) on survival rate, body weight, chimerism, percentage of T cells in peripheral blood and thymopoiesis. Lethally irradiated BALB/c mice underwent transplantation with 2×10^7 B6 bone marrow cells (BMCs) by IBM-BMT with or without ATT from the same donor. (a) The survival rate (upper left), body weight (upper middle), and percentages and numbers of donor-type H-2K^{b+} cells (upper left and lower left) and T cells (lower middle and right) in the peripheral blood are shown. The percentages and numbers of T cells in mice treated with IBM-BMT + ATT were significantly higher than in mice treated with IBM-BMT alone, whereas there were no differences in survival rate, body weight and chimerism. IBM-BMT alone, $n = 5$; IBM-BMT + ATT, $n = 6$. Data are shown as mean ± standard deviation (SD). *$P < 0{\cdot}05$ compared with IBM-BMT alone at the same time. (b) Histology of the thymus, fluorescence-activated cell sorter (FACS) profiles for CD4 and CD8 double-staining in thymocytes and the number of cells, T-cell receptor rearrangement excision circle (TREC) analyses and percentages of CD4 and CD8 T and B cells in the spleen. Thymus tissue was engrafted, and cortical (arrow) and medullary (dotted arrow) areas displayed fine construction [haematoxylin and eosin (HE) staining, ×400, upper left] with sufficient CD4 and CD8 subsets in thymocytes 3 months after transplantation (upper middle). Numbers of spleen cells (upper right) and relative TRECs in spleen cells (lower right) were determined 3 weeks and 3 months after transplantation. Percentages of CD4 and CD8 T cells and B220 B cells 3 weeks after transplantation in the spleen are shown (lower left and middle). Numbers of both CD4 and CD8 T cells and relative TRECs in mice treated with IBM-BMT + ATT were significantly higher than in mice treated with IBM-BMT alone, whereas the number of spleen cells was no different. IBM-BMT alone, $n = 5$; IBM-BMT + ATT, $n = 5$. Data are shown as mean ± SD. *$P < 0{\cdot}05$; **$P < 0{\cdot}01$.

was significantly greater than in the mice treated with IBM-BMT alone, both 3 weeks and 3 months after BMT. In addition, TREC at 3 months was higher than that at 3 weeks after IBM-BMT + ATT but not after IBM-BMT alone. These results indicate that the increase in T-cell numbers induced by IBM-BMT + ATT was attributable to continuous production by the transplanted thymus.

Induction of mild GVHR by IBM-BMT + ATT

If the increase in the number of T cells after allogeneic IBM-BMT + ATT is the result of ATT, GVHR should occur. In the analysis of donor-derived lymphocytes, we found a small number of donor-derived H-2K^{b+} CD45^{+} cells, but no host-derived H-2K^{d+} CD45^{+} cells in the small intestine and liver from mice treated with IBM-BMT + ATT (Fig. 2a). Histologically, a small number of lymphocytes infiltrated the portal area of the liver (Fig. 2a) and the mucosa of the small intestine (Fig. 2b) with some fibrosis in the mice treated with IBM-BMT + ATT, indicating the development of mild GVHD. However, the degree of GVHD was much less than that in the group treated with IBM-BMT plus 1×10^7 B6 spleen cell injection (DLI), which induced severe GVHD with mortality by 3 weeks after the transplantation.[41] The mice treated with IBM-BMT alone showed no pathological findings. We also investigated the specific responses for MHC determinants in MLR

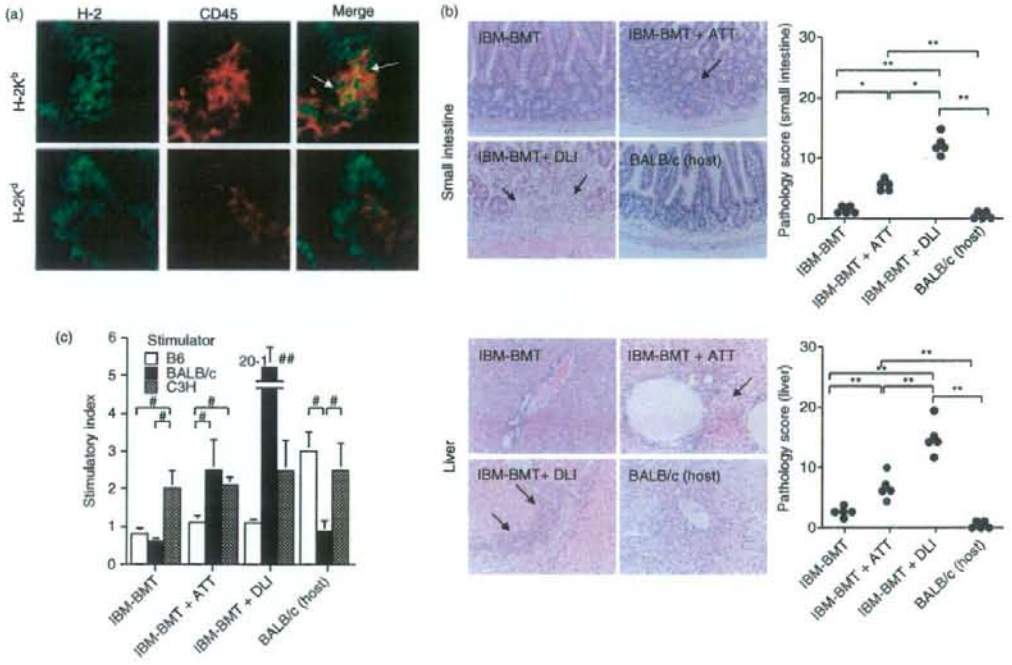

Figure 2. Analyses of donor-derived lymphocytes, histology and mixed lymphocyte reaction (MLR) for graft-versus-host disease (GVHD) induced by intra-bone marrow–bone marrow transplantation (IBM-BMT) + adult thymus transplantation (ATT). Lethally irradiated BALB/c mice underwent transplantation with 2×10^7 B6 bone marrow cells (BMCs) by IBM-BMT with or without ATT, or injection of 1×10^7 spleen cells from the same donor. At 3 weeks after transplantation, donor-derived lymphocytes (H-2K^{b+} CD45^{+}) and host-derived lymphocytes (H-2K^{d+} CD45^{+}) in the mice treated with IBM-BMT + ATT (a) and histology (b) in the small intestine (upper) and the liver (lower) were analysed. Donor-derived lymphocytes (H-2K^{b+} CD45^{+} cells), but not host-derived lymphocytes (H-2K^{d+} CD45^{+} cells), were observed in the small intestine from the mice treated with IBM-BMT + ATT (arrows) (×1000) (a). H-2K^{d+} CD45^{-} cells may be epithelial cells in the intestine. (b) Representative histology is shown [left; haematoxylin and eosin (HE) staining; ×200]: lymphocytes infiltrate the mucosa of the small intestine with fibrosis and the portal area of the liver as GVHD (arrows) in mice with IBM-BMT + ATT or 1×10^7 spleen cell injection [donor lymphocyte infusion (DLI)] from the same donor. However, the degree of GVHD in mice treated with IBM-BMT + ATT was significantly lower than in mice treated with IBM-BMT + DLI (right; pathology scores). Few or no specific pathological findings were observed in mice treated with IBM-BMT alone or untreated host BALB/c mice, and the degree of GVHD in these mice was significantly lower than in the case of IBM-BMT + ATT or DLI. IBM-BMT alone, $n = 5$; IBM-BMT + ATT, $n = 5$; IBM-BMT + DLI, $n = 5$; untreated donor B6 spleen cells, $n = 5$. Data are shown as mean ± standard deviation (SD). $^{*}P < 0{\cdot}01$, $^{**}P < 0{\cdot}0005$. (c) MLRs in splenocytes are shown for mice treated with IBM-BMT, IBM-BMT + ATT or IBM-BMT + DLI or BALB/c mice. The stimulatory index was calculated as the average [^{3}H]thymidine (^{3}H-TdR) incorporation of triplicate samples of responding cells with either mitogen or stimulating cells/^{3}H-TdR incorporation of responding cells in medium alone. $^{\#}P < 0{\cdot}05$; $^{\#\#}P < 0{\cdot}01$ compared with B6 and C3H stimulators and the BALB/c stimulator in mice treated with IBM-BMT + ATT.

assays. The MLR showed a slight response to host (BALB/c mice) in the mice treated with IBM-BMT + ATT, but not in the mice treated with IBM-BMT alone (Fig. 2c). However, the level was significantly lower than that in the mice treated with IBM-BMT + DLI. All the mice showed comparable responses to the third party (C3H).

Induction of Tregs by IBM-BMT + ATT

To explore the mechanism underlying GVHD, we next analysed $CD4^+$ $FoxP3^+$ Tregs and $CD4^+$ $FoxP3^-$ T effector cells. We first analysed the number of CD4 T cells in the spleen (Fig. 3a). Three weeks after the transplantation, there was a significantly greater number of these cells in the mice treated with IBM-BMT + ATT than in the mice treated with IBM-BMT alone. In contrast, the number of these cells in the mice treated with IBM-BMT + DLI was, as a result of GVHD, significantly lower than in the mice treated with IBM-BMT alone. Interestingly, although the numbers of both $CD4^+$ $FoxP3^+$ Tregs and $CD4^+$ $FoxP3^-$ effector T cells were low in the mice treated with IBM-BMT alone (Fig. 3a,b), the percentages of Tregs in total $CD4^+$ T cells of the spleen were comparable to those in the untreated donor B6 spleen (Fig. 3c). In contrast, although numbers of both $CD4^+$ $FoxP3^+$ Tregs and $CD4^+$ $FoxP3^-$ effector T cells increased in the spleen of mice treated with IBM-BMT + ATT, the number of effector T cells increased to a greater extent. However, the

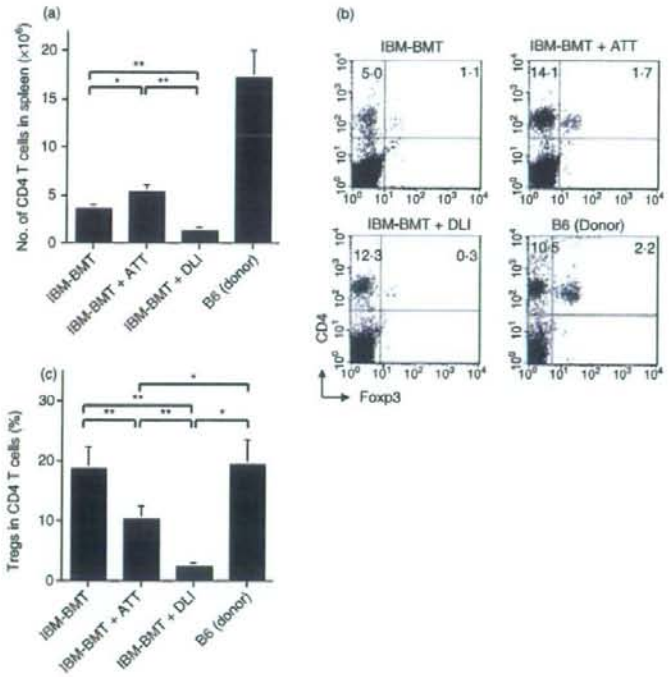

Figure 3. Number of CD4 T cells and percentages of $CD4^+$ $FoxP3^+$ regulatory T cells (Tregs) and $CD4^+$ $FoxP3^-$ effector cells induced by intra-bone marrow–bone marrow transplantation (IBM-BMT) + adult thymus transplantation (ATT). Lethally irradiated BALB/c mice underwent transplantation with 2×10^7 B6 bone marrow cells (BMCs) by IBM-BMT with or without ATT, or injection of 1×10^7 spleen cells [donor lymphocyte infusion (DLI)] from the same donor. At 3 weeks after transplantation, the numbers of CD4 T cells (a), $CD4^+$ $FoxP3^+$ Tregs and $CD4^+$ $FoxP3^-$ effector cells (b) were analysed in spleen cells. Representative fluorescence-activated cell sorter (FACS) profiles for $CD4^+$ $FoxP3^+$ Tregs and $CD4^+$ $FoxP3^-$ effector cells in the spleen (b) and the analysis for the percentage of Treg cells in $CD4^+$ cells (c) are shown: the number of CD4 T cells in the mice treated with IBM-BMT + ATT was significantly higher than in those treated with IBM-BMT alone 3 weeks after transplantation. In addition, the cell number in the mice treated with IBM-BMT + DLI was significantly reduced compared with that in the mice treated with IBM-BMT alone or plus ATT (a). The percentage of $CD4^+$ $FoxP3^+$ Tregs in the mice treated with IBM-BMT alone and B6 mice (donor) was significantly higher than in the mice treated with IBM-BMT + ATT or DLI. In the latter, the percentage of cells in those treated with IBM-BMT + ATT was significantly higher than in those treated with IBM-BMT + DLI (b). IBM-BMT alone, $n = 6$; IBM-BMT + ATT, $n = 6$; IBM-BMT + DLI, $n = 5$; untreated donor B6 spleen cells, $n = 5$. Data are shown as mean ± standard deviation (SD). $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}005$.

number of Tregs markedly decreased in the spleen of mice treated with IBM-BMT + DLI. As a result, the percentages of Tregs in total $CD4^+$ T cells in the IBM-BMT + DLI groups were significantly lower than in the IBM-BMT group and in untreated donor B6 mice (Fig. 3c). In contrast, the percentage of Tregs in total $CD4^+$ T cells in the mice treated with IBM-BMT + ATT was still significantly higher than in the mice treated with IBM-BMT + DLI. There was thus a negative correlation between the Tregs in total $CD4^+$ T cells and the degree of GVHD.

GVT effects of IBM-BMT + ATT

Next, we examined GVT effects in the mice treated with IBM-BMT + ATT. Meth-A sarcoma cells were subcutaneously inoculated into BALB/c mice, and IBM-BMT was performed when tumours had reached 5 mm in diameter. Interestingly, IBM-BMT + ATT significantly inhibited tumour growth, compared with non-treatment and IBM-BMT alone, after 14 days (Fig. 4a,b). Moreover, all mice that had a high dose of DLI (1×10^7 of spleen cells) died within 21 days from severe GVHD even with a strong GVT effect comparable to that of IBM-BMT + ATT. In contrast, the mice with a low dose of DLI (3×10^6 of spleen cells) survived for a long time, but showed weaker GVT effects than those treated with IBM-BMT + ATT.

Mechanisms underlying tumour regression induced by IBM-BMT + ATT

We analysed the mechanisms of tumour regression. Although the tumour-bearing mice treated with IBM-BMT with or without ATT clearly displayed donor-derived chimerism in both CD4 and CD8 T cells in the spleen (Fig. 5a), the percentages of both subsets in the mice treated with IBM-BMT + ATT were higher than those in the mice treated with IBM-BMT alone. Histologically, in contrast to the infiltration of a few lymphocytes in the tumours of the mice treated with IBM-BMT alone, numerous lymphocytes had infiltrated the tumours in the mice treated with IBM-BMT + ATT (Fig. 5b; HE staining). The cells were $H\text{-}2K^{b+}$ $CD45^+$ (not $H\text{-}2K^{d+}$ $CD45^+$), indicating that they were donor-derived lymphocytes (Fig. 5c). The analyses of T-cell subsets in

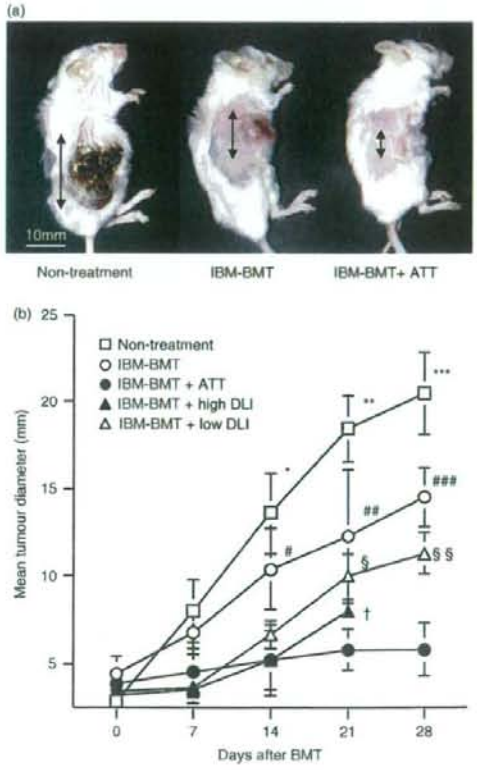

Figure 4. Graft-versus-tumour (GVT) effect induced by intra-bone marrow–bone marrow transplantation (IBM-BMT) with or without adult thymus transplantation (ATT). Lethally irradiated BALB/c mice with Meth-A sarcoma underwent transplantation with 2×10^7 B6 bone marrow cells (BMCs) by IBM-BMT with or without ATT or spleen cell injection [donor lymphocyte infusion (DLI)] from the same donor. (a) Representative findings for tumours (arrows) in non-treated mice, or in mice treated with IBM-BMT with or without ATT 28 days after BMT. (b) The time-course of tumour growth after transplantation in mice following IBM-BMT with or without ATT or DLIs (high dose, 1×10^7; low dose, 3×10^6 B6 spleen cells) or non-treatment. The mice treated with IBM-BMT + ATT showed significant tumour regression, in contrast to the non-treated mice and the mice treated with IBM-BMT or IBM-BMT + a low DLI. The mice treated with IBM-BMT + a high DLI showed similar results, but they died early as a result of GVHD. IBM-BMT alone, $n = 8$; IBM-BMT + ATT, $n = 12$; IBM-BMT + a high DLI, $n = 8$; IBM-BMT + a low DLI, $n = 10$; non-treatment, $n = 7$. Data are shown as mean ± standard deviation (SD). $^*P < 0.05$ compared with IBM-BMT + a low dose of DLI, IBM-BMT + a high dose of DLI and IBM-BMT + ATT; $^{**}P < 0.05$ compared with IBM-BMT, IBM-BMT + a low dose of DLI, IBM-BMT + a high dose of DLI and IBM-BMT + ATT; $^{***}P < 0.05$ compared with IBM-BMT, IBM-BMT + a low dose of DLI and IBM-BMT + ATT. $^{\#}P < 0.05$ compared with IBM-BMT + a low dose of DLI, IBM-BMT + a high dose of DLI and IBM-BMT + ATT; $^{\#\#}P < 0.05$ compared with IBM-BMT + ATT; $^{\#\#\#}P < 0.05$ compared with IBM-BMT + a low dose of DLI and IBM-BMT + ATT. $^{\S}P < 0.05$ compared with IBM-BMT + ATT; $^{\S\S}P < 0.05$ compared with IBM-BMT + ATT. $^{\dagger}P < 0.05$ for short survival time in IBM-BMT + a high dose of DLI compared with other groups.

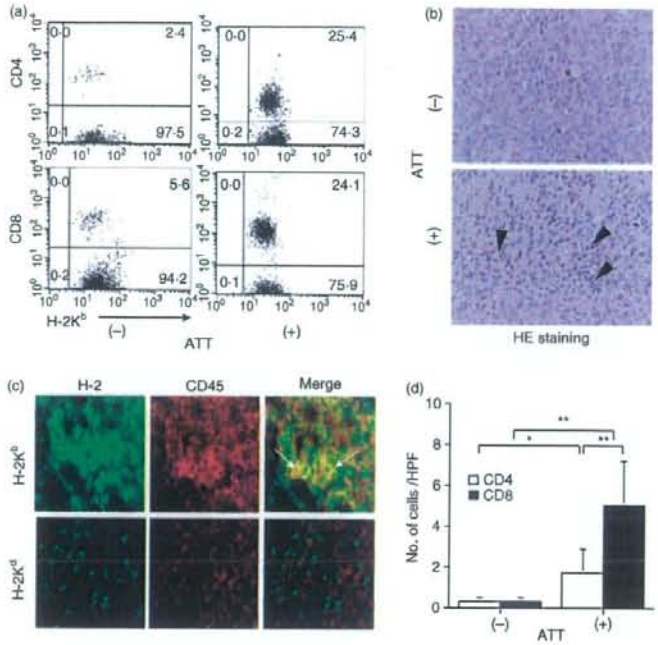

Figure 5. Analysis of donor-derived T-cell subsets in the spleen and infiltrated T-cell subsets in tumours following intra-bone marrow–bone marrow transplantation (IBM-BMT) with or without adult thymus transplantation (ATT). Lethally irradiated BALB/c mice with Meth-A sarcoma underwent transplantation with 2×10^7 B6 bone marrow cells (BMCs) by IBM-BMT with or without ATT from the same donor. Tumours were removed 4 weeks after transplantation. (a) Fluorescence-activated cell sorter (FACS) profile for donor-derived H-2K^{b+} and CD8 or CD4 T cells in the spleen. Elevated numbers of both CD4 and CD8 T cells were found in the mice treated with IBM-BMT + ATT compared with those treated with IBM-BMT alone. (b) Representative findings for tumour cells with haematoxylin and eosin (HE) staining (upper) and the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling (TUNEL) method (lower) following IBM-BMT with or without ATT (×800). Numerous lymphocytes had infiltrated the tumours in the mice treated with IBM-BMT + ATT compared with those treated with IBM-BMT alone (arrowhead). Donor-derived lymphocytes (H-2K^{b+} CD45$^+$) and host-derived lymphocytes (H-2K^{d+} CD45$^+$) in tumours from the mice treated with IBM-BMT + ATT were examined (×1000) (c). Donor-derived lymphocytes (H-2K^{b+} CD45$^+$ cells), but not host-derived lymphocytes (H-2K^{d+} CD45$^+$ cells), were observed in the tumour from the mice treated with IBM-BMT + ATT (arrows). H-2K^{b+} CD45$^-$ cells may be tumour cells. (d) Comparison of the CD4 and CD8 T-cell subsets in tumours by IBM-BMT with or without ATT. Both subsets in the mice treated with IBM-BMT + ATT were significantly higher than those in the mice treated with IBM-BMT, and predominantly CD8 T cells infiltrated the tumour compared with CD8 T cells in those treated with IBM-BMT + ATT. IBM-BMT + ATT, $n = 5$; IBM-BMT alone, $n = 5$. Data are shown as mean ± standard deviation (SD). $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$.

the tumour revealed that numbers of both CD4 and CD8 T cells were significantly higher in the mice treated with IBM-BMT + ATT than in the mice treated with IBM-BMT alone (Fig. 5d). Interestingly, CD8 T cells predominantly infiltrated the tumours in the mice treated with IBM-BMT + ATT.

Cytokine production and apoptosis in tumours from mice treated with IBM-BMT + ATT

We next investigated cytokine production in spleen cells from tumour-bearing non-treated mice and tumour-bearing mice treated with IBM-BMT with or without ATT (Fig. 6a). The production of IFN-γ was higher in the mice treated with IBM-BMT + ATT than in the mice treated with IBM-BMT alone. The non-treated mice showed the lowest level of IFN-γ. Although IL-2 was slightly elevated in these mice, no significant difference was observed. IL-4 and IL-10 production was very low compared with IFN-γ and IL-2. Finally, we performed TUNEL staining for the detection of apoptotic cells in the tumour. The positive cell counts were much higher in the mice treated with IBM-BMT + ATT than in the mice treated with IBM-BMT alone (Fig. 6b,c). The non-treated mice showed few apoptotic cells in the tumour (data not shown).

