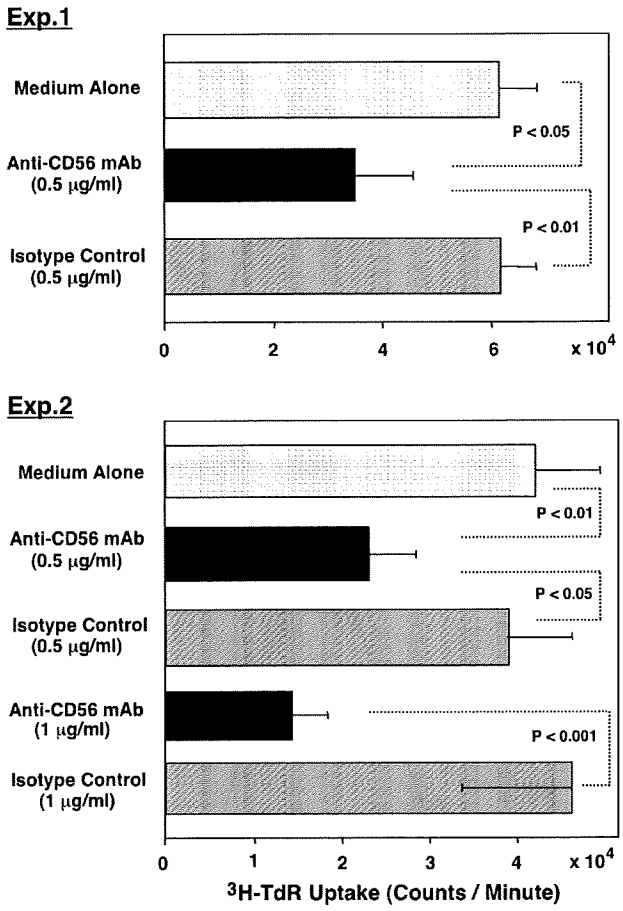

Fig. 4 Inhibitory effect of anti-CD56 mAb on monkey HSC proliferation. Partially purified HSCs were cultured on the BM adherent layer in the presence or absence of anti-CD56 mAb (five wells per sample). As a control, the same concentration of isotype-matched mouse normal IgG was added to the culture. Mean±SD of five wells. Representative data of three independent experiments

BM adherent cells. With the addition of anti-CD56 mAb, the number of cells adhering to the BM adherent cell layer decreased significantly (data not shown). As shown in Fig. 4, a marked decrease in the uptake of ^{3}H-TdR was seen in the wells with the anti-CD56 mAb, whereas no decrease was observed in the wells with isotype-matched control Ab. The proliferation of the HSCs was inhibited in a dose-dependent manner, as shown in experiment 2 in Fig. 4.

Detection of CD56-positive cells in monkey BM sections

We examined whether BMCs expressing CD56 molecules indeed exist in monkey BM tissues. The BM sections were immunostained with anti-CD56 mAb, and the cells showing stromal-cell-like features (characteristic nuclei with clear nucleolus and abundant cytoplasms) were stained positively with the anti-CD56 mAb. Some immature hematopoietic cells were also stained with the mAb. In addition, tight adhesions between the CD56-positive hemopoietic cells and the CD56-positive stromal cells were observed in some places (Fig. 5).

Differentiation ability of monkey BM adherent cells into osteoblasts and adipocytes

BM adherent cells were examined to see whether they have any characteristics of MSCs; we investigated their ability to differentiate into osteoblasts and adipocytes. BM adherent cells were cultured in the inductive medium into osteoblasts and adipocytes for 4 and 5 weeks and then stained with von Kossa and oil red O reagents, respectively. As shown in Fig. 6, calcium deposits were detected by the von Kossa staining, and intracellular lipid droplets were also detected

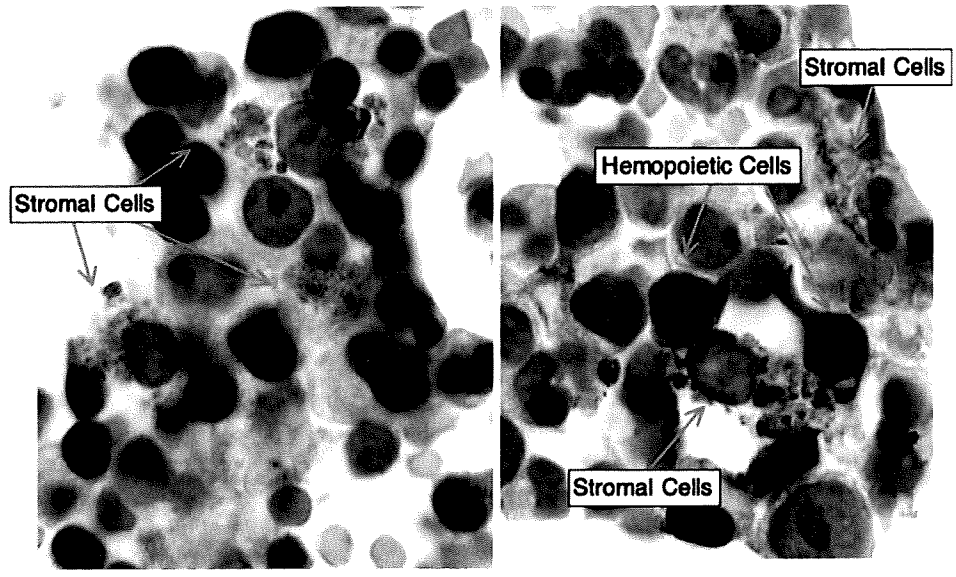

Fig. 5 Detection of CD56-positive stromal and hemopoietic cells in monkey BM tissues. BM sections were stained with anti-CD56 mAb and CD56-positive stromal cells were detected in many places. Immature hemopoietic cells, but not mature ones, were also positively stained with the mAb. The adhesion of the CD56-positive stromal cells and the CD56-positive immature hemopoietic cells was observed (×1,000). Representative data of six independent experiments

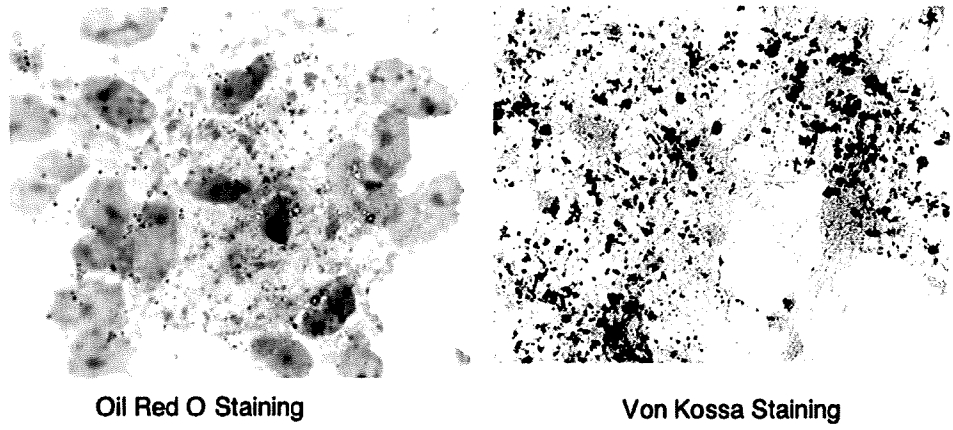

Fig. 6 Differentiation capacity of monkey BM adherent cells. The BM adherent cells were induced to differentiate into adipocytes and osteoblasts. Oil red O staining and von Kossa staining confirmed their differentiations (×1,000). Representative data of three independent experiments

by the oil red O staining, indicating that the cells do have the potential to differentiate into osteoblasts and adipocytes.

Discussion

Studies of the hemopoietic system of nonhuman primates have provided important information for understanding the mechanisms of human hemopoiesis [21, 22]. Recently, Lee et al. [23] demonstrated that BM adherent cells, prepared from the BMCs of fetal rhesus monkeys, had a high proliferative potential and had an ability to differentiate towards adipogenic, chondrogenic, and osteogenic lineages, indicating that the BM adherent cells have characteristics of MSCs. They also cultured adult BMCs collected by the conventional aspiration method from the iliac crest and obtained BM adherent cells, although the differentiation capacity of the adult BM adherent cells was not evaluated. In addition, Devine et al. have isolated BM adherent cells by culturing baboon BMCs obtained by the aspiration method, and the cells were shown to be MSCs; the cells were capable of differentiating along adipogenic and osteogenic lineages. When lethally irradiated baboons were administered the autologous (gene-marked) MSCs in conjunction with autologous HSCs by the intravenous route, the engraftment of the HSCs was facilitated, and the transgene was detected in the posttransplant BM biopsies [24]. These reports clearly demonstrate that cell populations that can be considered as MSCs exist in BMCs of nonhuman primates, as in humans and mice. We have previously shown that BMCs collected by the perfusion method contain a significantly higher number of hemopoietic colony-forming cells than those collected by the aspiration method [16, 17] since peripheral blood contamination was reduced to the minimum level in the perfusion method. There was a tendency for the number of colony-forming unit fibroblast (CFU-F) to be higher in the BMCs collected by the aspiration method than in those by the perfusion method, although the differences were not significant [17]. Thus, the perfusion method, which requires only two holes for the insertion of syringes in the bones and can be used to collect a sufficient number of BMCs with just one or two perfusions, provides a safe and convenient tool for harvesting BMCs that contain a high number of HSCs and CFU-F.

The present study clearly shows that CD56 is expressed on both BM adherent cells and HSCs (Figs. 1b and 2a) and that their interactions through the CD56 molecules are important for hemopoiesis because the proliferation of HSCs was suppressed markedly by the addition of anti-CD56 mAb (Fig. 4). As shown in Fig. 2c, the $CD34^{+}/56^{+}$ cells generated a comparable number of hemopoietic colonies to the $CD34^{+}/56^{-}$ cells in the methylcellulose assay and also showed the morphology of HSCs (Fig. 2b), indicating that the $CD34^{+}/56^{+}$ cells represent a population of HSCs but not artifacts produced by the HSC purification process. Our previous reports [7, 8] have shown that CD56 is expressed on mouse BM adherent cells as well as a stromal cell line (FMS/PA6-P) established from fetal mouse BMCs and contributes greatly to the supporting capacity of these stromal cells. However, we could not detect the expression of CD56 on highly purified mouse HSC populations, such as $Lin^{-}Sca\text{-}1^{+}$, $Lin^{-}CD34^{-/+}$, $Lin^{-}CD38^{-/+}$, or $Lin^{-}c\text{-}kit^{low/+}$ cells [7]. Therefore, it is conceivable that the CD56 molecules on mouse BM stromal cells might interact with HSCs through heterophilic bindings. Indeed, there are many reports showing that CD56 binds to heparan sulfate proteoglycan (extracellular matrix molecule) [25] and fibroblast growth factor receptors [26] in murine and chicken neural systems, although the biological significances of the heterophilic bindings have not yet been fully elucidated. The homophilic bindings of CD56 molecules are known to induce cell-to-cell tight adhesions because zipper-like CD56 complexes are generated by multiple cis- and trans-homophilic bindings of CD56. In this study, we provide evidence that CD56 molecules are also expressed on HSC-enriched populations in the monkey hemopoietic

system, indicating that HSCs can interact with BM stromal cells by homophilic bindings, in addition to heterophilic bindings. Such homophilic interactions would induce the tight adhesions between HSCs and stromal cells and, as a result, stable cellular adhesions and long-lasting cellular interactions would be achieved.

From the present results in monkeys, it can be speculated that CD56 is also expressed on human BM stromal cells (MSCs) and contributes to human hemopoiesis. Indeed, our preliminary study showed that 67% of commercially available human MSCs were positive for CD56. Thus, CD56 might be used as a new marker for MSCs in mice, monkeys, and humans. Moreover, BM stromal cells expressing CD56 molecules were detected in human BM sections by immunohistochemical staining. We also found that $CD34^{+}/56^{+}$ cells were contained in an HSC-enriched population of human cord blood cells at the percentage of 3–6% (manuscript in preparation). There is a possibility that human BMCs contain a higher amount of $CD34^{+}/56^{+}$ cells than human cord blood cells, although we have not assessed this yet.

Several adhesion molecules are known to regulate the interactions between hemopoietic stem–progenitor cells and BM stromal cells; for example, β-catenin–N-cadherin, integrin–VCAM, and $\beta_1$integrin–osteopontin interactions [27]. The present results indicate the important role of CD56 in the hemopoietic system, but they do not rule out the contribution of other adhesion molecules. We have very recently found that HSCs from MRL/lpr mice (autoimmune-prone mice) exhibit enhanced adhesion to the FMS/PA6-P stromal cell line, established in our laboratory [8], in vitro after the onset of autoimmune diseases: this is attributed to the increased expression of CD56 on the HSCs, but no increase was found in other adhesion molecules such as CD44, CD62L, VLA-4, and VCAM-1. The proliferation of HSCs on the FMS/PA6-P cells was significantly suppressed by the addition of anti-CD56 mAb, suggesting that the interaction between HSCs and stromal cells through CD56 molecules plays a major role in the abnormal proliferation (no MHC restriction) of HSCs in MRL/lpr mice [28].

More work is required before one can say that CD56 molecules contribute to hemopoiesis to a higher extent than other adhesion molecules. However, our previous [7, 8, 28] and present studies provide new understandings of the regulation mechanisms of hemopoiesis through CD56 molecules. To fully understand the physiological significance of CD56, we are now investigating the functional role of CD56 molecules in the monkey BM adherent cells and human MSCs.

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References

1. Dexter TM, Allen TD, Lajtha LG (1977) Conditions controlling the proliferation of hematopoietic stem cells in vitro. J Cell Physiol 91:335–344
2. Whitlock CA, Witte ON (1982) Long-term culture of B-lymphocytes and their precursors from murine bone marrow. Proc Nat Acad Sci U SA 79:3608–3612
3. Dorshkind K (1990) Regulation of hemopoiesis by bone marrow stromal cells and their products. Ann Rev Immunol 8:111–114
4. Verfaillie CM (1998) Adhesion receptors as regulators of the hematopoietic process. Blood 92:2609–2612
5. Charbord P (2001) The hematopoietic stem cell and the stromal microenvironment. Therapie 56:383–384
6. Izumi-Hisha H, Soe T, Ogata H et al (1991) Monoclonal antibodies against a preadipose cell line (MC3T3-G2/PA6) which can support hemopoiesis. Hybridoma 10:103–112
7. Wang X, Hisha H, Taketani S et al (2005) Neural cell adhesion molecule contributes to hemopoiesis-supporting capacity of stromal cell lines. Stem Cells 23:1389–1399
8. Wang X, Hisha H, Taketani S et al (2006) Characterization of mesenchymal stem cells isolated from mouse fetal bone marrow. Stem Cells 24:482–493
9. Thiery JP, Brackenbury R, Rutishauser U et al (1977) Adhesion among neural cells of the chick embryo. J Bio Chem 252:6841–6845
10. D'Eustachio P, Owens GC, Edelman GM et al (1985) Chromosomal location of the gene encoding the neural cell adhesion molecule (N-CAM) in the mouse. Proc Nat Acad Sci U S A 82:7631–7635
11. Van Camp B, Durie BGM, Spier C et al (1990) Plasma cells in multiple myeloma express a natural killer cell-associated antigen: CD56 (NKH-1; Leu-19). Blood 76:377–382
12. Cruse JM, Lewis RE, Pierce S et al (2005) Aberrant expression of CD7, CD56, and CD79a antigens in acute myeloid leukemias. Exp Mol Patho 79:39–41
13. Sillaber C, Walchshofer S, Mosberger I et al (1999) Immunophenotypic characterization of human bone marrow endosteal cells. Tissue Antigens 53:559–568
14. Nelissen JMDT, Torensma R, Pluyter M et al (2000) Molecular analysis of the hemopoiesis supporting osteoblastic cell line U2-OS. Exp Hematol 28:422–432
15. Kushida T, Inaba M, Ikebukuro K et al (2000) A new method for bone marrow cell harvesting. Stem Cells 18:453–456
16. Kushida T, Inaba M, Ikebukuro K et al (2002) Comparison of bone marrow cells harvested from various bones of cynomolgus monkeys at various ages by perfusion or aspiration methods: a preclinical study for human BMT. Stem Cells 20:155–162

17. Inaba M, Adachi Y, Hisha H et al (2007) Extensive studies on perfusion method plus intra-bone marrow-bone marrow transplantation using cynomolgus monkeys. Stem Cells 25:2098–2103
18. Hisha H, Nishino T, Kawamura M et al (1995) Successful bone marrow transplantation by bone grafts in chimeric-resistant combination. Exp Hematol 23:347–352
19. Hashimoto F, Sugiura K, Inoue K et al (1997) Major histocompatibility complex restriction between hematopoietic stem cells and stromal cells in vivo. Blood 89:49–54
20. Sugiura K, Hisha H, Ishikawa J et al (2001) Major histocompatibility complex restriction between hematopoietic stem cells and stromal cells in vitro. Stem Cells 19:46–58
21. Lee CC, Fletcher MD, Tarantal AF (2005) Effect of age on the frequency, cell cycle, and lineage maturation of rhesus monkey (*Macaca mulatta*) $CD34^+$ and hemopoietic progenitor cells. Pediatr Res 58:315–322
22. Shepherd BE, Kiem H-P, Lansdorp PM et al (2007) Hematopoietic stem cell behavior in non-human primates; HSC behavior in non-human primates. Blood 110:1806–1803 (prepublished online)
23. Lee CCI, Ye F, Tarantal AF (2006) Comparison of growth and differentiation of fetal and adult rhesus monkey mesenchymal stem cells. Stem Cells and Develop 15:209–220
24. Devine SM, Bartholomew AM, Mahmud N et al (2001) Mesenchymal stem cells are capable of homing to the bone marrow of non-human primates following systemic infusion. Exp Hematol 29:244–255
25. Storms SD, Rustishauser U (1998) A role for polysialic acid in neural cell adhesion molecule heterophilic binding to proteoglycans. J Biol Chemi 273:27124–27129
26. Kiryushko D, Korshunova I, Berezin V et al (2006) Neural cell adhesion molecule induces intracellular signaling via multiple mechanisms of Ca^+ homeostasis. Mol Biol Cell 17:2278–2286
27. Yin T, Li L (2006) The stem cell niches in bone. J Clin Invest 116:1196–1201
28. Wang X, Hisha H, Cui W et al (2007) The characteristics of hematopoietic stem cells from autoimmune-prone mice and the role of neural cell adhesion molecules in abnormal proliferation of these cells in MRL/lpr mice. Haematologica 92:300–307

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TRANSLATIONAL AND CLINICAL RESEARCH

Intra-Bone Marrow Injection of Donor Bone Marrow Cells Suspended in Collagen Gel Retains Injected Cells in Bone Marrow, Resulting in Rapid Hemopoietic Recovery in Mice

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Key Words. Intra-bone marrow bone marrow transplantation • Collagen gel • Colony-forming units of spleen • Reconstitution • Enhanced green fluorescent protein

ABSTRACT

We have recently developed an innovative bone marrow transplantation (BMT) method, intra-bone marrow (IBM)-BMT, in which donor bone marrow cells (BMCs) are injected directly into the recipient bone marrow (BM), resulting in the rapid recovery of donor hemopoiesis and permitting a reduction in radiation doses as a pretreatment for BMT. However, even with this IBM injection, some of the injected BMCs were found to enter into circulation. Therefore, we attempted to modify the method to allow the efficient retention of injected BMCs in the donor BM. The BMCs of enhanced green fluorescent protein transgenic mice (C57BL/6 background) were suspended in collagen gel (CG) or phosphate-buffered saline (PBS), and these cells were then injected into the BM of irradiated C57BL/6 mice. The numbers of retained donor cells in the injected BM, the day 12 colony-forming units of spleen (CFU-S) counts, and the reconstitution of donor cells after IBM-BMT were compared between the CG and PBS groups. The number of transplanted cells detected in the injected BM in the CG group was significantly higher than that in the PBS group. We next carried out CFU-S assays. The spleens of mice in the CG group showed heavier spleen weight and considerably higher CFU-S counts than in the PBS group. Excellent reconstitution of donor hemopoietic cells in the CG group was observed in the long term (>100 days). These results suggest that the IBM injection of BMCs suspended in CG is superior to the injection of BMCs suspended in PBS. STEM CELLS *2008;26:2211–2216*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Bone marrow transplantation (BMT) was originally developed to treat congenital immunodeficiencies and hematologic disorders [1, 2]. Recently, BMT-related methods have been improved, because of the discoveries of more effective immunosuppressants, powerful antibiotics, antithymocyte globulin, and fractionated irradiation, all of which add up to a better prognosis [3, 4]. BMT has, at this stage, been carried out for the treatment not only of immunodeficiencies and hemopoietic diseases but also of autoimmune diseases and solid malignant tumors [5–10]. However, BMT is still a difficult procedure because of the risk of lethal side effects, such as infection, graft-versus-host disease (GVHD), graft failure, and so on [11–13]. Recently, we developed a new and powerful BMT method: intra-bone marrow (IBM)-BMT [14]. In this method, donor bone marrow cells (BMCs) are injected directly into the recipient bone marrow (BM), and a much greater number of donor hemopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) can therefore be inoculated into the recipient BM than by conventional i.v. BMT, resulting in the rapid reconstitution of donor hemopoietic cells and permitting a reduction in radiation doses as a pretreatment for BMT [14, 15]. In addition, we have shown that the IBM-BMT can be used for organ transplantation, with the engrafted organs surviving long-term without the use of immunosuppressants [16, 17]. Collagen gel (CG) was originally used for the three-dimensional cell culture systems for isolated cells [18]. Cellmatrix (Nitta Gelatin, Inc., Yao, Japan, http://www.nitta-gelatin.com) is an acidic soluble type I collagen that is liquid on ice but forms a gel when warm. In this paper, we show that the CG helps retain more injected donor BMCs in the

Author contributions: M.S.: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript; Y.A.: conception and design, provision of study material or patients, collection and/or assembly of data, manuscript writing, final approval of manuscript; A.S., N.K.-K., W.F., and C.Y.: collection and/or assembly of data, final approval of manuscript; S.Y., Z.-X.L., and J.L.: data analysis and interpretation, final approval of manuscript; S.I.: financial support, administrative support, manuscript writing, final approval of manuscript.

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recipient BM upon IBM-BMT than phosphate-buffered saline (PBS), resulting in excellent reconstitution of donor cells and permitting a reduction in radiation doses as a pretreatment for BMT.

MATERIALS AND METHODS

Mice

C57BL/6 mice (B6) were purchased from SLC (Shizuoka, Japan, http://www.jslc.co.jp) for use as recipients, and enhanced green fluorescent protein (eGFP) transgenic (tg) mice, for use as donors, were kindly donated by Dr. Okabe (Osaka University, Osaka, Japan) [19]. These mice were used at 8–10 weeks of age. The university's committee for animal research approved all experiments.

Reagents

The antibodies (Abs) used in this study were as follows: phycoerythrin (PE)-labeled anti-mouse CD3 Ab, anti-mouse B220 Ab, anti-mouse Gr-1 Ab, peridinin chlorophyll protein (PerCP)-Cy5.5-labeled anti-mouse CD45 Ab, biotin-labeled anti-CD3 Ab, biotin-labeled anti-B220 Ab, biotin-labeled anti-CD11c Ab, biotin-labeled anti-Mac-1 Ab, biotin-labeled anti-NK1.1 Ab, biotin-labeled TER119 Ab, and biotin-labeled anti-Gr1 Ab (BD Pharmingen, San Diego, http://www.bdbiosciences.com/index_us.shtml). Cellmatrix, a purified collagen solution for cell culture, was purchased from Nitta Gelatin, Inc.

Whole-Body Irradiation of Recipient Mice

Gamma radiation was delivered by a Gammacell 40 Exactor (MDS Nordion, Kanata, ON, Canada, http://www.mds.nordion.com) with two ^{137}Cs sources. Recipient mice were irradiated with 7, 8, 9 or 10 Gy, the day before BMT.

Preparation of the Collagen Gel Matrix

The collagen gel matrix, Cellmatrix, was prepared following the manufacturer's instructions. That is, solutions A, B, and C were mixed at a ratio of 8:1:1 and were kept on ice to prevent gel formation until use. This is because the mixture is liquid on ice but becomes a gel when it is warmed. Hereafter, this mixture is referred to as collagen gel.

Treatment of Donor BMCs

BMCs were flushed from the medullary cavities of the femurs and tibias of donor mice with PBS. After gentle dissociation, the BMC suspension was filtered through a 70-μm nylon mesh (Becton Falcon, Franklin Lakes, NJ, http://www.bd.com). The BMCs were counted.

The BMCs were then divided into two groups: the CG group and the PBS group. The BMC suspension was centrifuged, and the supernatant was aspirated. The BMCs were suspended in 4°C PBS for the PBS group and ice-cold CG for the CG group and adjusted to 10^7, 10^8, or 10^9 cells per milliliter.

Bone Marrow Transplantation

One day after irradiation, the BMCs of eGFP tg mice were transplanted into recipient mice directly into the bone cavity via the intra-bone marrow route (IBM-BMT), as previously described [14]. Briefly, the mice were anesthetized, and the area from the inguinal region to the knee joint was shaved. The tibia was gently drilled with a 26-gauge needle through the patellar tendon into the BM cavity. BMCs suspended in PBS or CG were aspirated into a microsyringe (50 μl; Ito, Fuji, Shizuoka, Japan, http://www.ito-ex.co.jp) and then kept at room temperature. The BMCs (10^5, 10^6, or 10^7 in 10 μl) were then injected into the BM cavity using the microsyringe.

Analysis of Donor Cells in Recipient BM, Peripheral Blood, or Spleen

To detect injected donor BMCs in the recipient BM, the BMCs of eGFP tg mice (10^7 in 10 μl) or cultured MSCs (5×10^5 in 10 μl) were transplanted into the BM of B6 mice the day after 10-Gy irradiation. One hour after IBM-BMT, the B6 mice were sacrificed and BMC-injected bones were flushed to obtain the BMCs. The BMCs were then stained with biotin-labeled lineage Abs (anti-Gr1, anti-Mac-1, anti-TER119, anti-B220, anti-NK1.1, anti-CD3, and anti-CD11c Abs), PE-labeled anti-Sca-1 Ab, and PerCP-Cy5.5 labeled anti-CD45 Ab, followed by incubation with allophycocyanin (APC)-coupled streptavidin. The cells were analyzed using a FACSCalibur instrument (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com).

To detect donor-lineage$^-$ Sca-1$^+$ c-kit$^+$ cells (KSL cells) in the recipient mice after BMT, the BMCs of eGFP tg mice (1×10^6) were injected into 9-Gy-irradiated recipient B6 mice intravenously or into the left tibia (l-tibia). Spleen cells and BMCs from the BMC-injected l-tibia and noninjected right tibia (r-tibia) were obtained from the recipient mice, independently, at 5, 7, or 9 days after BMT. We calculated the numbers of nuclear cells using an SF-3000 autoanalyzer (Sysmex, Kobe, Japan, http://www.sysmex.co.jp/en) and stained the cells with APC-labeled anti-c-kit Ab (BD Pharmingen), PE-labeled anti-Sca-1 Ab (BD Pharmingen), and biotin-labeled lineage Abs followed by staining with PerCP-Cy5.5-coupled streptavidin (BD Pharmingen). The percentages of KSL cells in the spleen and the BM were analyzed using the FACSCalibur. The total numbers of donor KSL cells, which are eGFP$^+$, in the spleen and the BM were calculated by using the total cell numbers and percentage of donor KSL cells in the cells.

To detect donor-derived peripheral blood nuclear cells, the peripheral blood (PB) of the recipient mice was examined at 2 weeks, 1 month, 2 months, and 100 days after BMT. The PB of each mouse was divided into three lots, and samples from each mouse were stained with PE-conjugated CD3 and PerCP-Cy5.5-conjugated CD45, PE-conjugated B220 and PerCP-Cy5.5-conjugated CD45, and PE-conjugated Gr1 and PerCP-Cy5.5-conjugated CD45. To examine the cells retained in the injected bone, the recipient mice were sacrificed 1 hour after IBM-BMT. The BMCs were flushed from the tibia that had been previously injected and were then suspended in ice-cold PBS. This step was carried out on ice. The number of BMCs was counted, and 10^6 BMCs were stained with PerCP-Cy5.5-conjugated CD45. The stained cells were analyzed using a FACSCalibur instrument equipped with CellQuest software.

Percentages of Donor Cells by FACS Analyses

Leukocytes were first gated by CD45$^+$ cells, which were estimated as nuclear cells. The percentage of donor T lymphocytes was estimated as eGFP$^+$/CD3$^+$cells. The percentages of donor B lymphocytes and granulocytes were estimated as eGFP$^+$/B220$^+$ cells and eGFP$^+$/Gr-1$^+$ cells, respectively.

Statistical Analysis

The results are represented as means ± SD. The Student t test was used to determine a statistical significance. A p value $<.05$ was considered a significant difference.

RESULTS

CG Retains Injected Donor BMCs in Recipient BM

First, we examined whether CG can retain more injected cells in the injected BM than PBS. We obtained BMCs from eGFP tg mice and suspended the BMCs in ice-cold CG (Cellmatrix) or PBS. These were then warmed to room temperature (>20°C) in a syringe, since Cellmatrix is liquid at 4°C but changes into the gel state when warm. The BMCs suspended in CG or PBS were injected directly into the BM, as previously described [14]. The mice were sacrificed 1 hour after

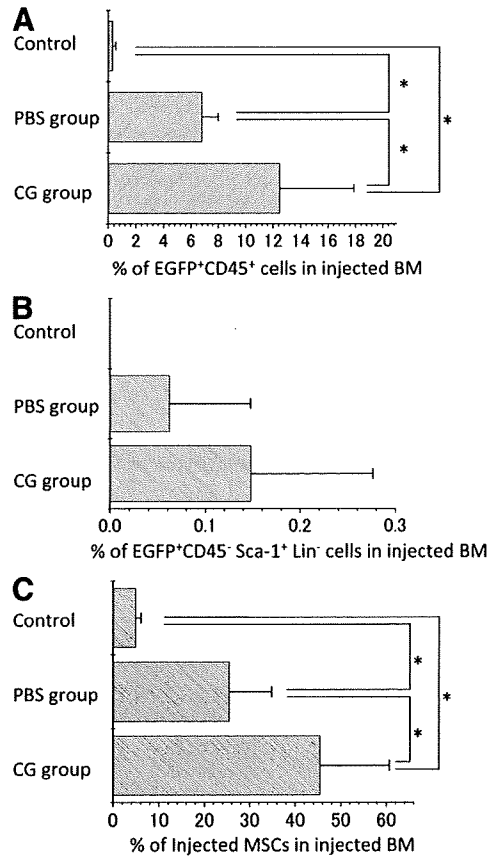

Figure 1. CG efficiently helps retain donor cells in recipient BM. Recipient (B6) mice were irradiated at 10 Gy 1 day before intra-bone marrow (IBM) bone marrow transplantation (BMT). BMCs were obtained from eGFP transgenic (tg) mice (donor mice). The BMCs ($1 \times 10^7/10$ μl) suspended in PBS (PBS group) or CG (CG group) were injected into the BM of the B6 mice. Control mice were injected with only PBS into the BM. One hour after IBM-BMT, the mice were sacrificed, and percentage of $CD45^+$ donor cells (**A**) and percentage of $CD45^-$lineage$^-$Sca-1^+ donor cells (**B**) in the BM injected with donor BMCs were analyzed by FACScan. $n = 3$ (control; mice injected with only PBS), 6 (PBS group), and 6 (CG group). The cells from control mice show autofluorescence. *, $p < .05$. (**C**): B6 mice were irradiated at 10 Gy 1 day before IBM-BMT. Cultured MSCs (5×10^5 cells per 10 μl) from eGFP tg mice suspended in PBS (PBS group) or CG (CG group) were injected into the BM of the B6 mice. Control mice were injected with only PBS into the BM. One hour after IBM-BMT, the mice were sacrificed, and percentages of donor cells were analyzed by FACScan. $n = 3$ (control; mice injected with only PBS), 5 (PBS group), and 6 (CG group). The cells from control mice show autofluorescence. *, $p < .05$. Abbreviations: BM, bone marrow; CG, collagen gel; EGFP, enhanced green fluorescent protein; MSC, mesenchymal stem cell; PBS, phosphate-buffered saline.

IBM-BMT, and the percentages of donor cells in the injected BM were examined. As shown in Figure 1A, many more donor BMCs remained in the injected BM cavity in the CG group than in the PBS group. Next, we examined whether IBM-BMT using CG helps more mesenchymal stem cells to be retained in the injected recipient BM. As shown in Figure 1B, CG helped more MSCs to be retained in the recipient BM, but there was no significant difference between the groups, possibly because of the large SDs. Therefore, we transplanted cultured MSCs of eGFP tg mice into the BM of B6 mice and analyzed the percentages of donor MSCs in the recipient BM. As shown in Figure 1C, a significantly larger number of donor MSCs existed in the recipient BM in the CG group than in the PBS group ($p < .05$). These results suggest that CG can help more injected cells (both HSCs and MSCs) be retained in the injected BM than PBS.

CG Retains KSL Cells in the Injected Site, Followed by Helping Proliferation and Migration of KSL Cells into the Spleen and Noninjected Sites

To examine the kinetics of the KSL cells in the recipients, we carried out IBM-BMT or i.v. BMT using BMCs suspended in PBS or CG from eGFP tg mice into B6 mice. As shown in Figure 2, on day 5, KSL cells were found only in the BM of the BMC-injected l-tibia in both PBS and CG groups; the number of KSL cells in the BMC-injected l-tibia was significantly higher in the CG group than in the PBS group. However, we could not detect KSL cells in the BM in the case of the i.v. group. In contrast, a greater number of KSL cells were detected in the spleen in the case of i.v. BMT. On day 7, we could still detect a high number of KSL cells in the BMC-injected l-tibia in the CG group. The KSL cells also appeared even in the noninjected r-tibia in the CG group, although no KSL cells were detected in the noninjected r-tibia in the PBS group in the case of IBM-BMT. On day 9, we still detected many KSL cells in the BMC-injected l-tibia in the CG group. There were no significant differences between the other groups due to the increases in the numbers of KSL cells. These findings suggest that immature hemopoietic progenitor cells (KSL cells) proliferate inside the BMC-injected bone cavity, followed by an acceleration of the migration of the KSL cells into the noninjected bone and the spleen in the CG group.

CG Group Shows Significantly Higher Day 12 Colony-Forming Units of Spleen Counts After IBM-BMT Than PBS Group

Next, we carried out day 12 colony-forming units of spleen (CFU-S) assays to examine the hemopoietic stem cell/immature hemopoietic progenitor activity after IBM-BMT using CG or PBS (Fig. 3). In the CG group, 16.1 $\pm$ 4.4 colonies per spleen (106.8 $\pm$ 35.9 mg of spleen weight) were observed on day 12, whereas 6.6 $\pm$ 3.4 colonies per spleen (68.2 $\pm$ 15.5 mg of spleen weight) were observed in the PBS group ($p < .05$). These results suggest that hemopoietic stem cell/progenitor cells can be more effectively retained in the CG group than in the PBS group.

Reduced Radiation Doses As Pretreatment for BMT

As described above, we have shown that CG can effectively help donor BMCs be retained in the recipient BM after IBM-BMT, resulting in the formation of significantly higher CFU-S counts. Next, we examined whether radiation doses could be reduced by the use of IBM-BMT with CG (Fig. 4). First, we irradiated recipient B6 mice at 8 Gy and reconstituted them with eGFP mouse BMCs by IBM-BMT. In this experiment, both the PBS and CG groups showed good reconstitution of donor hemopoietic cells 1 month after BMT. There was no significant difference between the two groups: the mean and SDs of the percentage of donor cells in the PBS group and the CG group were 89.3% $\pm$ 2.0% and 85.6% $\pm$ 5.0%, respectively, at 1 month after IBM-BMT. We therefore reduced the radiation dose (8 to 7 Gy). With 7 Gy of radiation, 1 month after BMT, significantly higher percentages of reconstitution with donor cells were observed in the CG group than in the PBS group. These results suggest that the IBM injection of BMCs suspended in CG is effective in reducing irradiation doses as a pretreatment.

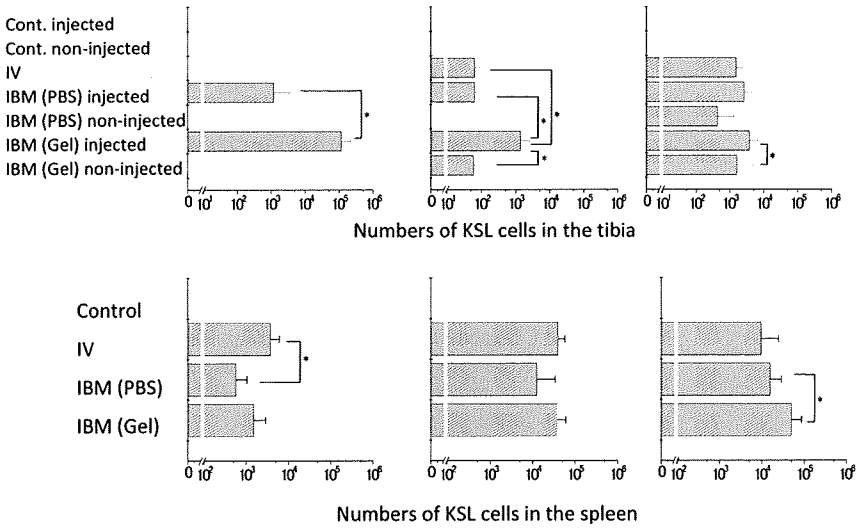

Figure 2. Collagen gel (CG) augments increment of KSL cells in injected bone marrow (BM) followed by increment of KSL cells in noninjected BM and spleen. B6 mice were irradiated at 9 Gy 1 day before bone marrow transplantation (BMT). BMCs from enhanced green fluorescent protein (eGFP) transgenic (tg) mice (1×10^6 cells per 10 μl) suspended in CG were injected into the left tibia. In the IV group, 1×10^6 BMCs from eGFP tg mice suspended in PBS were injected intravenously. As a control, the mice were injected with only PBS into the left tibia. The mice were sacrificed 5, 7, or 9 days after BMT. Total cell numbers of the spleen, the left tibia (injected tibia), and right tibia (noninjected tibia) were calculated with Sysmex, and percentages of donor-derived KSL cells were examined with FACSCalibur. Next, we calculated the total number of eGFP-positive KSL cells in the spleen, the BMC-injected tibia, and the noninjected tibia. n = 5–11. *, $p < .05$. Abbreviations: IBM, intra-bone marrow; IV, intravenous; KSL, donor-lineage$^-$ Sca-1$^+$ c-kit$^+$; PBS, phosphate-buffered saline.

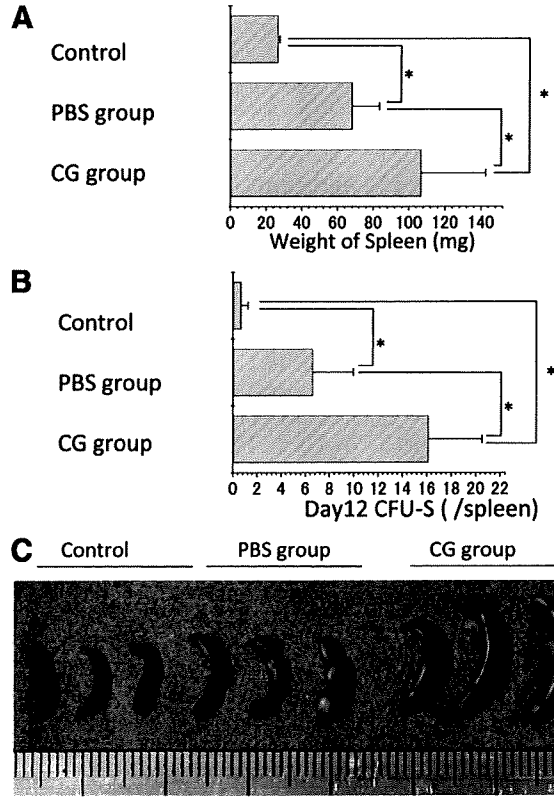

Figure 3. Heavier spleen weight and higher day 12 CFU-S counts in the CG group than in the PBS group. Recipient (B6) mice were irradiated at 9 Gy, 1 day before intra-bone marrow (IBM) bone marrow transplantation (BMT). BMCs (1×10^5 cells per 10 μl) from enhanced green fluorescent protein transgenic mice were injected into the bone marrow of the B6 mice. Twelve days after IBM-BMT, the spleens of the B6 mice were weighed, and numbers of CFU-S were counted. **(A):** Means and SDs of the weight of spleens. **(B):** Means and SDs of CFU-S. **(C):** Representative photograph of spleens. There were three mice in the control group (mice injected with only PBS), five in the PBS group, and eight in the CG group. *, $p < .05$. Abbreviations: CFU-S, colony-forming units of spleen; CG, collagen gel; PBS, phosphate-buffered saline.

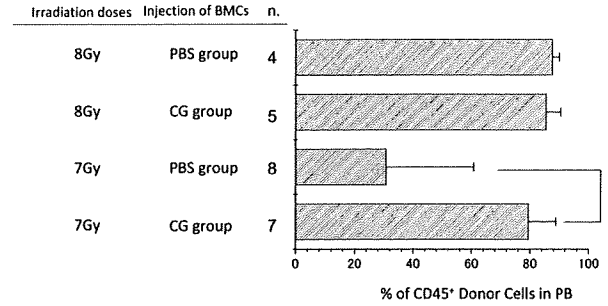

Figure 4. Better reconstitution of donor hemopoietic cells in the CG group than the PBS group. Recipient (B6) mice were irradiated at 7 or 8 Gy, 1 day before intra-bone marrow (IBM) bone marrow transplantation (BMT). BMCs (1×10^6 cells per 10 μl) from enhanced green fluorescent protein mice were injected into the bone marrow of the B6 mice. One month after IBM-BMT, nuclear cells in the PB were obtained from the mice, and percentages of donor cells were analyzed by FACScan. *, $p < .05$. Abbreviations: BMC, bone marrow cell; CG, collagen gel; PB, peripheral blood; PBS, phosphate-buffered saline.

Next, we examined whether cells of various lineages can differentiate in the CG group. As shown in Table 1, donor BMCs had differentiated into multilineage hemopoietic cells in both the CG and PBS groups. The percentages of donor cells (all lineage cells) in the CG group were higher than those in the PBS group even on day 100 after IBM-BMT (Table). We also calculated the percentage of donor cells in various lineages at 6 months after IBM-BMT and obtained results similar to those at 100 days after IBM-BMT (data not shown). These results suggest that CG is effective in reconstituting recipients with donor cells by IBM-BMT not only in short-term observation but also in long-term observation after IBM-BMT.

DISCUSSION

In the present study, we have shown that IBM-BMT using CG helps transplanted BMCs to be retained in the recipient BM upon IBM-BMT and permits a reduction in radiation doses as a pretreatment for BMT. In 2001, we reported that IBM-BMT promotes the rapid recovery of donor hemopoiesis in BMT [14] and that IBM-BMT can be used to treat intractable autoimmune diseases in MRL/Mp-lpr/lpr mice, which are resistant to conventional BMT

Table 1. Short-term (1 month) and long-term (100 days) reconstitution of donor-derived hemopoietic cells between the PBS group and the CG group

		% Of donor cells			
	n	CD45	CD3	B220	Gr-1
1 Month after IBM-BMT					
PBS group	8	30.8 ± 30	22.6 ± 16.2	51.0 ± 35.1	34.1 ± 35.0
CG group	7	79.5 ± 9.3[a]	52.5 ± 17.5[a]	91.4 ± 4.2[a]	85.8 ± 7.0[a]
100 Days after IBM-BMT					
PBS group	8	31.3 ± 40.3	16.8 ± 23.3	35.9 ± 43.3	26.6 ± 38.0
CG group	7	87.3 ± 8.7[a]	57.1 ± 17.4[a]	94.7 ± 3.3[a]	80.1 ± 20.8[a]

[a] $p < .05$ versus PBS group.
Abbreviations: CG, collagen gel; IBM-BMT, intra-bone marrow bone marrow transplantation; PBS, phosphate-buffered saline.

therapy [14]. Since then, we have shown the advantages of IBM-BMT not only in the treatment of hemopoietic diseases but also in organ transplantations and solid malignant tumors [20–24]. In these papers, we have shown that IBM-BMT induces rapid recovery of donor hemopoietic cells, easily induces tolerance in the recipients, reduces the severity of GVHD, and permits a reduction in radiation doses as a pretreatment for BMT. The mechanisms underlying the effects of IBM-BMT are (a) the efficacy of injection of donor BMCs (the direct injection of HSCs into the recipient BM) and (b) the injection of donor MSCs into the recipient BM. However, as there is an abundance of blood vessels in the BM, we have found that some of the injected BMCs get into the blood vessels, and thereby into peripheral circulation, even with IBM-BMT. Therefore, there was a need to modify the method of IBM-BMT to promote a greater retention of the injected BMCs at the site of injection in the BM. In this paper, we used CG (Cellmatrix) to help retain the donor BMCs in the recipient BM. Cellmatrix is liquid on ice but becomes a gel when warmer [18]. Therefore, we expected that the warmed CG containing BMCs would prevent the BMCs entering into circulation. As shown in Figure 1, we found a significantly higher number of donor BMCs in the injected bone of the recipients when donor BMCs were suspended in CG. As we expected, higher CFU-S counts were found in the CG group than in the PBS group, and the peripheral blood cells showed better reconstitution with donor hemopoietic cells. These results suggest that the more BMCs are injected and are retained in the recipient BM upon IBM-BMT, the better the reconstitution of the recipient mice. It has been reported that the interaction of stromal cells (particularly MSCs) and HSCs is crucial for the differentiation of hemopoietic cells and the maintenance of HSCs [16, 25, 26]. Therefore, it is necessary that the injected HSCs migrate to the BM and interact with stromal cells, including MSCs, for the production of mature hematopoietic cells and the maintenance of the HSCs. Recently, we have found that unique HSCs exist in the human cord blood [27]. These HSCs cannot migrate to the BM when they are injected into the vein. However, they can differentiate into mature hematopoietic cells and produce HSCs, resulting in long-term hematopoiesis. Therefore, we assume that some HSCs cannot participate in hematopoiesis if they are injected into the vein, possibly because of a lack of some important receptor(s) for migration to the bone marrow niche. These results and concepts suggest that BMT via the vein results in the loss of some multipotent HSCs and that IBM-BMT is effective in retaining the injected HSCs in the BM. However, in the present study, some of the HSCs were found to have entered into peripheral circulation even after IBM-BMT. IBM-BMT using CG could help retain more HSCs in the BM than conventional IBM-BMT. As shown in Figure 2, it is likely that KSL cells (immature hemopoietic progenitor cells) of the CG group proliferate more rapidly in the injected bone than those of the PBS and IV groups even 5 days after BMT, and that they migrate to the other bones and the spleen more effectively, resulting in the expansion of hemopoiesis followed by high CFU-S counts. Thus, CG is a candidate for improving conventional IBM-BMT, since more donor BMCs can be retained in the recipient BM upon IBM-BMT and since CG itself does not induce severe inflammation or foreign body granuloma when it is injected into mice (data not shown).

Conclusion

Collectively, our results show that CG helps retain more injected donor BMCs in the recipient BM upon IBM-BMT than PBS, resulting in excellent reconstitution of donor cells and permitting a reduction dose as a pretreatment for BMT in mice.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

References

1 Gatti RA, Meuwissen HJ, Allen HD et al. Immunological reconstitution of sex-linked lymphopenic immunological deficiency. Lancet 1968;2: 1366–1369.

2 Bach FH, Albertini RJ, Joo P et al. Bone marrow transplantation in a patient with the Wiskott-Aldrich syndrome. Lancet 1968;2:1364–1366.

3 Gocheva L. Total body irradiation prior to bone marrow transplantation; some aspects of fifty year experience. J BUON 2004;9:147–160.

4 Carella AM. Treatment of hematological malignancies with allogeneic nonmyeloablative stem cell transplantation: Conditioning regimens with fludarabine. Hematol J 2004;5(suppl 1):S68–S75.

5 Marmont AM. Stem cell transplantation for autoimmune disorders. Coincidental autoimmune disease in patients transplanted for conventional indications. Best Pract Res Clin Haematol 2004;17:223–232.
6 Burt RK, Traynor AE. SLE—Hematopoietic stem cell transplantation for systemic lupus erythematosus. Arthritis Res Ther 2003;5:207–209.
7 Burt RK, Traynor AE. Hematopoietic stem cell transplantation: A new therapy for autoimmune disease. STEM CELLS 1999;17:366–372.
8 Baron F, Storb R. Allogeneic hematopoietic cell transplantation following nonmyeloablative conditioning as treatment for hematologic malignancies and inherited blood disorders. Mol Ther 2006;13:26–41.
9 Bregni M, Dodero A, Peccatori J et al. Nonmyeloablative conditioning followed by hematopoietic cell allografting and donor lymphocyte infusions for patients with metastatic renal and breast cancer. Blood 2002; 99:4234–4236.
10 Eibl B, Schwaighofer H, Nachbaur D et al. Evidence for a graft-versus-tumor effect in a patient treated with marrow ablative chemotherapy and allogeneic bone marrow transplantation for breast cancer. Blood 1996; 88:1501–1508.
11 Cutler C, Giri S, Jeyapalan S et al. Acute and chronic graft-versus-host disease after allogeneic peripheral-blood stem-cell and bone marrow transplantation: A meta-analysis. J Clin Oncol 2001;19:3685–3691.
12 Bacigalupo A, Lamparelli T, Barbanti M et al. Improved results in marrow transplantation from unrelated donors. Genoa BMT Group. Haematologica 1999;84(suppl EHA-4):50–22.
13 Parody R, Martino R, Rovira M et al. Severe infections after unrelated donor allogeneic hematopoietic stem cell transplantation in adults: comparison of cord blood transplantation with peripheral blood and bone marrow transplantation. Biol Blood Marrow Transplant 2006;12:734–748.
14 Kushida T, Inaba M, Hisha H et al. Intra-bone marrow injection of allogeneic bone marrow cells: A powerful new strategy for treatment of intractable autoimmune diseases in MRL/lpr mice. Blood 2001;97:3292–3299.
15 Ikehara S. New strategies for BMT, organ transplantation, and regeneration therapy. Hematology 2003;8:77–81.
16 Esumi T, Inaba M, Ichioka N et al. Successful allogeneic leg transplantation in rats in conjunction with intra-bone marrow injection of donor bone marrow cells. Transplantation 2003;76:1543–1548.
17 Kaneda H, Adachi Y, Saito Y et al. Long-term observation after simultaneous lung and intra-bone marrow-bone marrow transplantation. J Heart Lung Transplant 2005;24:1415–1423.
18 Elsdale T, Bard J. Collagen substrata for studies on cell behavior. J Cell Biol 1972;54:626–637.
19 Okabe M, Ikawa M, Kominami K et al. 'Green mice' as a source of ubiquitous green cells. FEBS Lett 1997;407:313–319.
20 Wang YB, Ogawa Y, Doi H et al. Long-term immunologic induction of donor-specific tolerance to skin allografts by bone marrow transplant in rabbits. Plast Reconstr Surg 2003;111:291–297.
21 Suzuki Y, Adachi Y, Minamino K et al. A new strategy for treatment of malignant tumor: Intra-bone marrow-bone marrow transplantation plus CD4- donor lymphocyte infusion. STEM CELLS 2005;23:365–370.
22 Taira M, Inaba M, Takada K et al. Treatment of streptozotocin-induced diabetes mellitus in rats by transplantation of islet cells from two major histocompatibility complex disparate rats in combination with intra bone marrow injection of allogeneic bone marrow cells. Transplantation 2005; 79:680–687.
23 Baba S, Inaba M, Iwai H et al. Intra-bone marrow-bone marrow transplantation facilitates hemopoietic recovery including dendritic cells. Immunobiology 2005;210:33–42.
24 Takada K, Inaba M, Ichioka N et al. Treatment of senile osteoporosis in SAMP6 mice by intra-bone marrow injection of allogeneic bone marrow cells. STEM CELLS 2006;24:399–405.
25 Sugiura K, Inaba M, Hisha H et al. Requirement of major histocompatibility complex-compatible microenvironment for spleen colony formation (CFU-S on day 12 but not on day 8). STEM CELLS 1997;15:461–468.
26 Hashimoto F, Sugiura K, Inoue K et al. Major histocompatibility complex restriction between hematopoietic stem cells and stromal cells in vivo. Blood 1997;89:49–54.
27 Wang J, Kimura T, Asada R et al. SCID-repopulating cell activity of human cord blood-derived CD34- cells assured by intra-bone marrow injection. Blood 2003;101:2924–2931.

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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

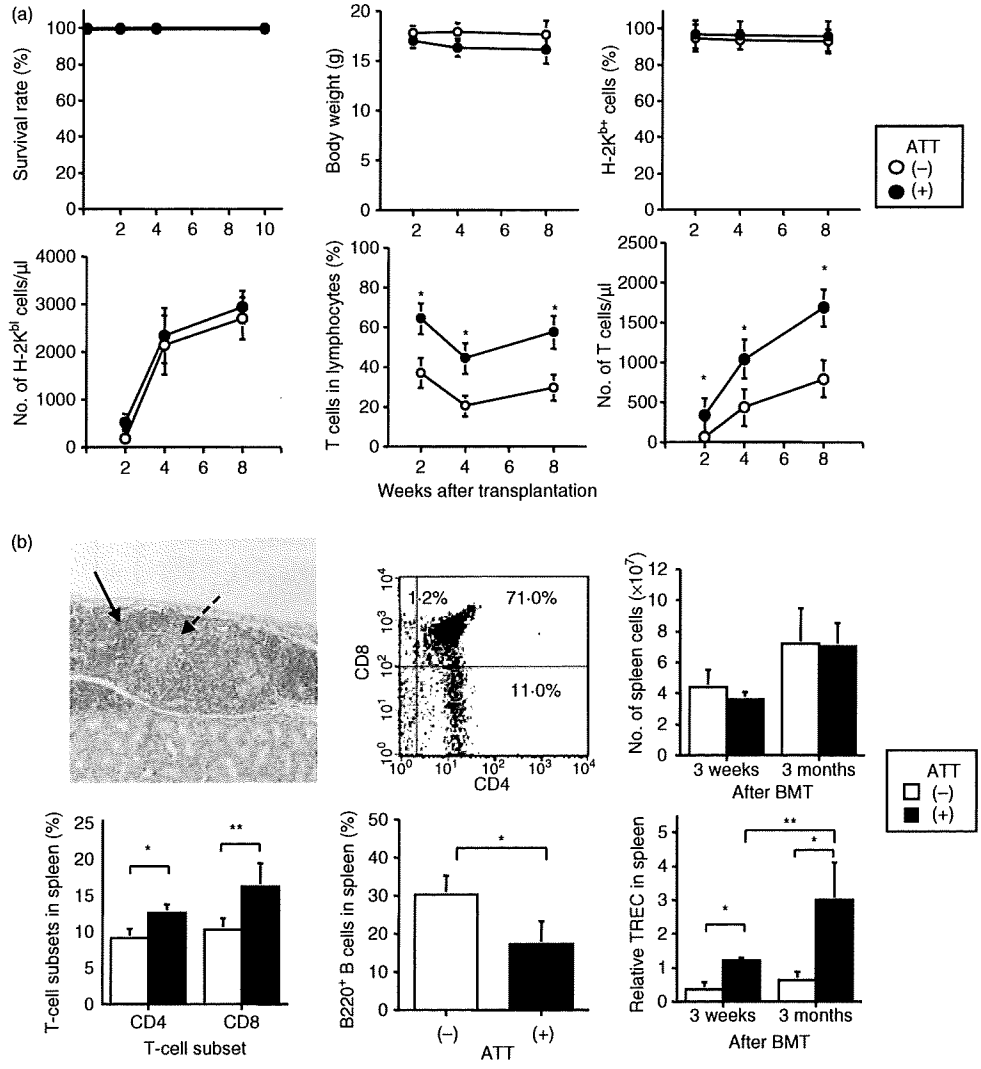

Figure 1. Effect of intra-bone marrow–bone marrow transplantation (IBM-BMT) + adult thymus transplantation (ATT) on survival rate, body weight, chimerism, percentage of T cells in peripheral blood and thymopoiesis. Lethally irradiated BALB/c mice underwent transplantation with 2×10^7 B6 bone marrow cells (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

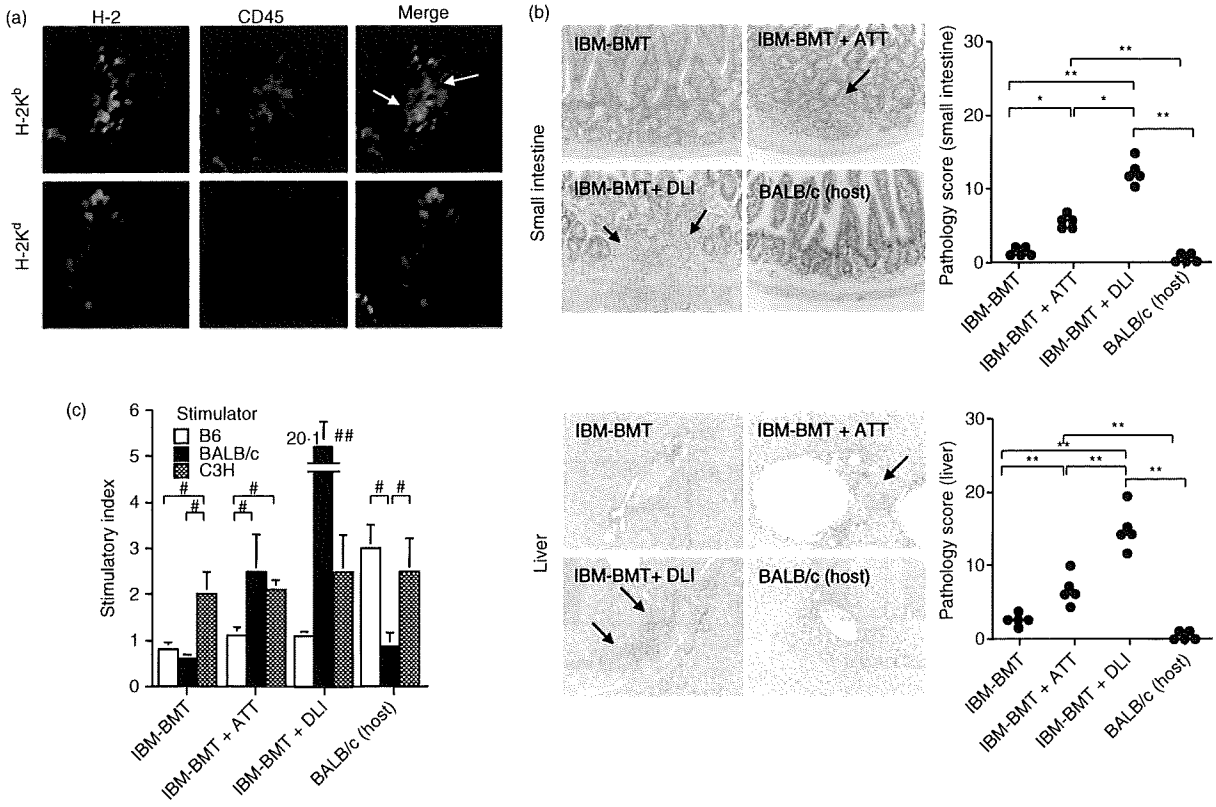

Figure 2. Analyses of donor-derived lymphocytes, histology and mixed lymphocyte reaction (MLR) for graft-versus-host disease (GVHD) induced by intra-bone marrow–bone marrow transplantation (IBM-BMT) + adult thymus transplantation (ATT). Lethally irradiated BALB/c mice underwent transplantation with 2×10^7 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

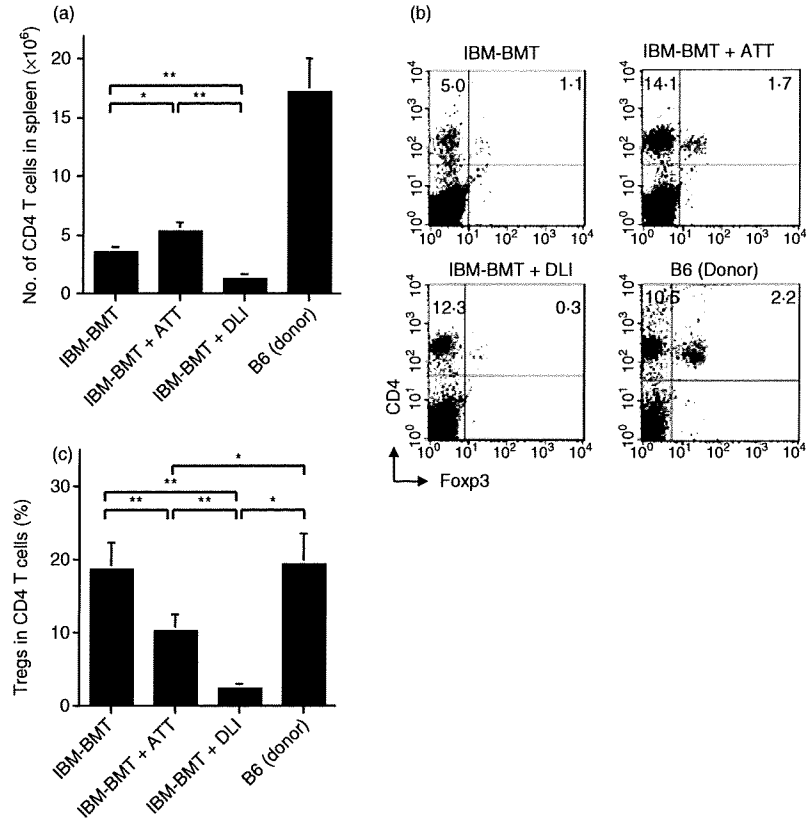

Figure 3. Number of CD4 T cells and percentages of $CD4^+$ $FoxP3^+$ regulatory T cells (Tregs) and $CD4^+$ $FoxP3^-$ effector cells induced by intrabone marrow–bone marrow transplantation (IBM-BMT) + adult thymus transplantation (ATT). Lethally irradiated BALB/c mice underwent transplantation with 2×10^7 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

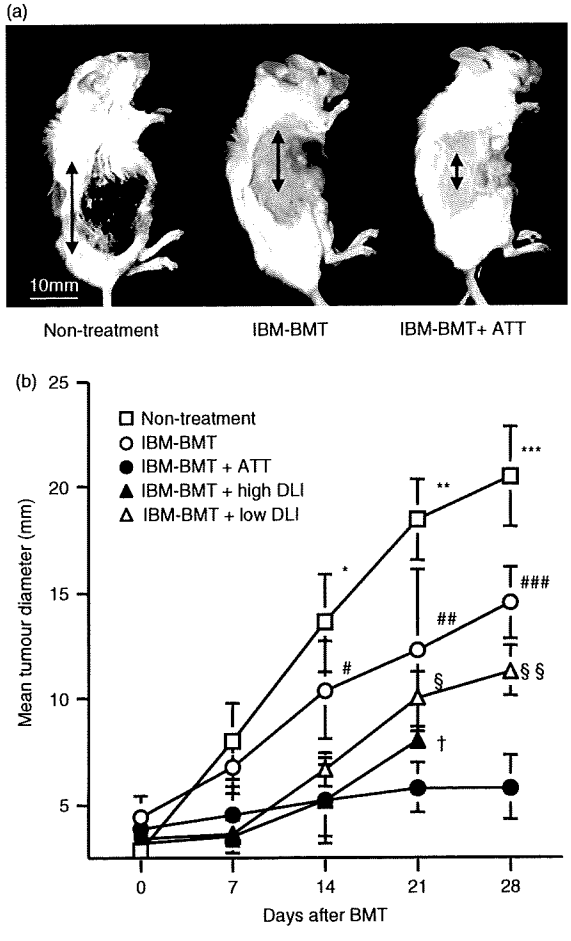

Figure 4. Graft-versus-tumour (GVT) effect induced by intra-bone marrow–bone marrow transplantation (IBM-BMT) with or without adult thymus transplantation (ATT). Lethally irradiated BALB/c mice with Meth-A sarcoma underwent transplantation with 2×10^7 B6 bone marrow cells (BMCs) by IBM-BMT with or without 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). $^*P < 0{\cdot}05$ compared with IBM-BMT + a low dose of DLI, IBM-BMT + a high dose of DLI and IBM-BMT + ATT; $^{**}P < 0{\cdot}05$ compared with IBM-BMT, IBM-BMT + a low dose of DLI, IBM-BMT + a high dose of DLI and IBM-BMT + ATT; $^{***}P < 0{\cdot}05$ compared with IBM-BMT, IBM-BMT + a low dose of DLI and IBM-BMT + ATT. $^{\#}P < 0{\cdot}05$ compared with IBM-BMT + a low dose of DLI, IBM-BMT + a high dose of DLI and IBM-BMT + ATT; $^{\#\#}P < 0{\cdot}05$ compared with IBM-BMT + ATT; $^{\#\#\#}P < 0{\cdot}05$ compared with IBM-BMT + a low dose of DLI and IBM-BMT + ATT. $^{\S}P < 0{\cdot}05$ compared with IBM-BMT + ATT; $^{\S\S}P < 0{\cdot}05$ compared with IBM-BMT + ATT. $^{\dagger}P < 0{\cdot}05$ for short survival time in IBM-BMT + a high dose of DLI compared with other groups.

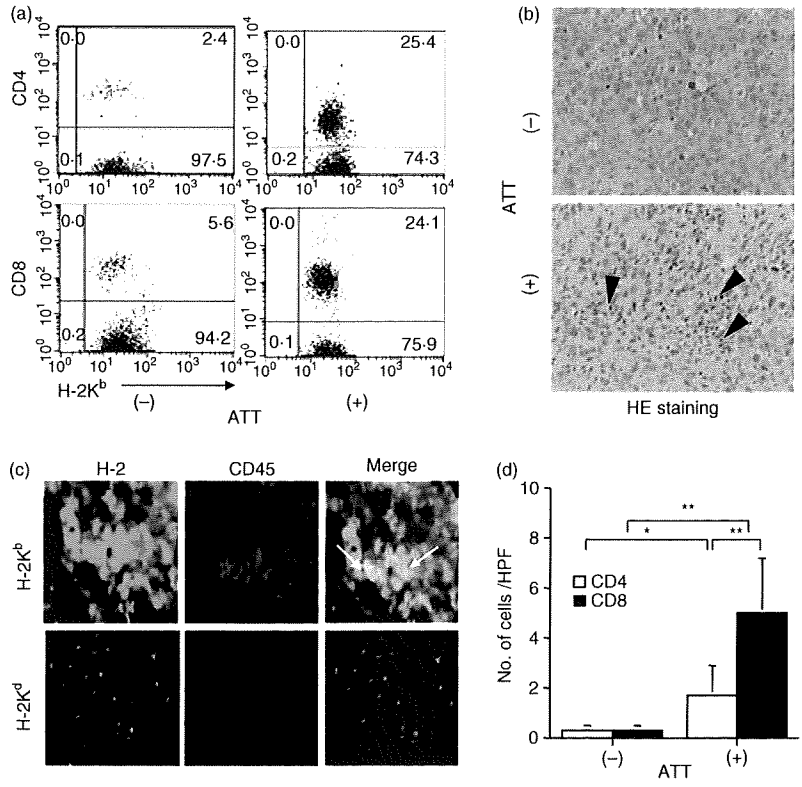

Figure 5. Analysis of donor-derived T-cell subsets in the spleen and infiltrated T-cell subsets in tumours following intra-bone marrow–bone marrow transplantation (IBM-BMT) with or without adult thymus transplantation (ATT). Lethally irradiated BALB/c mice with Meth-A sarcoma underwent transplantation 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).

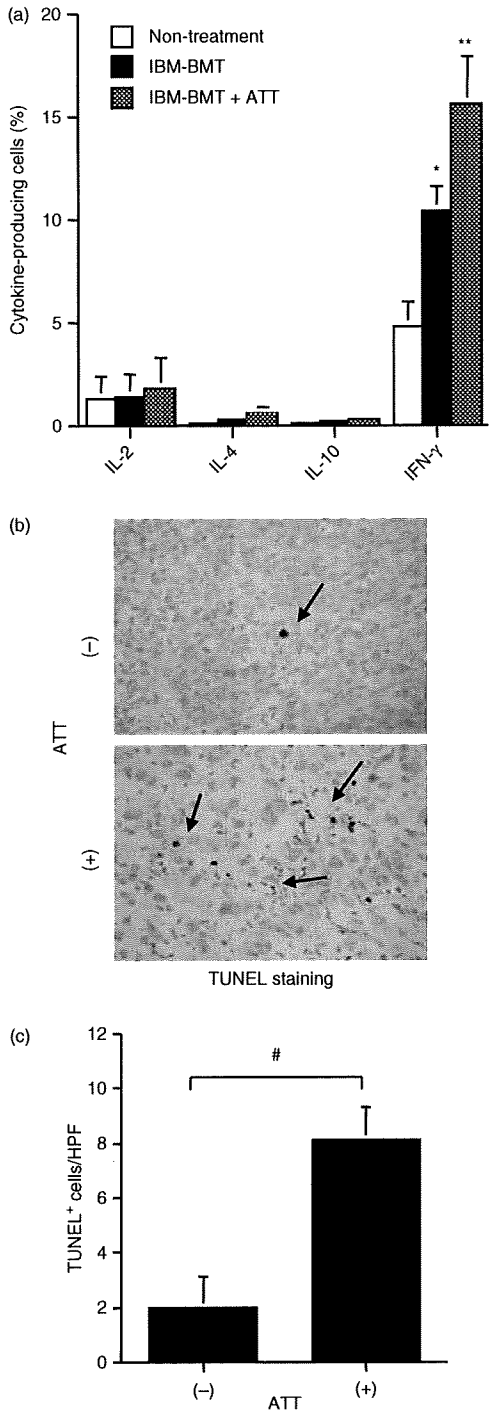

Figure 6. Analysis of cytokine production and apoptotic cells in tumours following intra-bone marrow–bone marrow transplantation (IBM-BMT) with or without adult thymus transplantation (ATT). (a) Spleen cells were intracytoplasmically stained with phycoerythrin-anti-interleukin (IL)-2, IL-4, IL-10 or interferon (IFN)-γ monoclonal antibodies (mAbs) to determine the per cent of IL-2-, IL-4- or IL-10-producing cells. Non-treatment, $n = 4$; IBM-BMT alone, $n = 4$; IBM-BMT + ATT, $n = 4$. $^{*}P < 0{\cdot}05$ compared with non-treatment; $^{**}P < 0{\cdot}05$ compared with non-treatment and IBM-BMT alone. (b) Representative findings for tumour cells with terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling (TUNEL) staining (×400). TUNEL-positive tumour cells were observed (arrows). Numbers of TUNEL^{+} cells after treatment with IBM-BMT with and without ATT were compared. Numbers of TUNEL-positive tumour cells were significantly elevated in the mice treated with IBM-BMT + ATT in comparison with those treated with IBM-BMT. IBM-BMT + ATT, $n = 5$; IBM-BMT alone, $n = 5$. Data are shown as mean ± standard deviation (SD). $^{\#}P < 0{\cdot}01$.

Discussion

In the present study, we have demonstrated that allogeneic IBM-BMT + ATT successfully induces high thymopoiesis. Although this treatment induced mild GVHR, survival rate, weight and donor-derived chimerism did not differ from those in mice that received allogeneic IBM-BMT alone. Interestingly, when IBM-BMT + ATT was performed in tumour-bearing mice, tumour growth was significantly inhibited, compared with non-treatment or IBM-BMT alone. IBM-BMT + DLI did not produce a longer survival time than IBM-BMT + ATT. The donor-derived CD8 T cells markedly infiltrated the tumour after IBM-BMT + ATT, and the tumour cells underwent apoptosis as a result of lymphocyte infiltration with elevation of IFN-γ. These findings strongly indicate that IBM-BMT + ATT induces high thymopoiesis, thereby eliciting strong GVT effects with mild GVHR.

We first examined the effects of IBM-BMT + ATT on normal mice. The transplanted thymus showed a normal structure under the renal capsule and normal thymocyte differentiation. Although the number of spleen cells did not differ between the mice treated with IBM-BMT alone and the mice treated with IBM-BMT + ATT, the numbers of both CD4 and CD8 T-cell subsets were significantly higher with ATT than without ATT. In addition, the number of TRECs in the mice treated with IBM-BMT + ATT was significantly higher than in the mice treated with IBM-BMT alone, with the number increasing over time. These results indicate the successful induction of high and continuous thymopoiesis by IBM-BMT + ATT, and that most T cells are derived from TT.

As some T cells were produced by the transplanted allogeneic thymus in the mice treated with IBM-BMT + ATT, the cells should display anti-host activity, as seen in GVHR. However, in contrast to IBM-BMT + DLI, which induces severe GVHD with rapid mortality,[41] IBM-BMT + ATT elicits only mild GVHD.

We next attempted to explain the present data by the frequency of Tregs, detected as FoxP3^{+} cells in CD4^{+} T cells; this frequency was slightly lower in the recipients of IBM-BMT + ATT than in the recipients of IBM-BMT