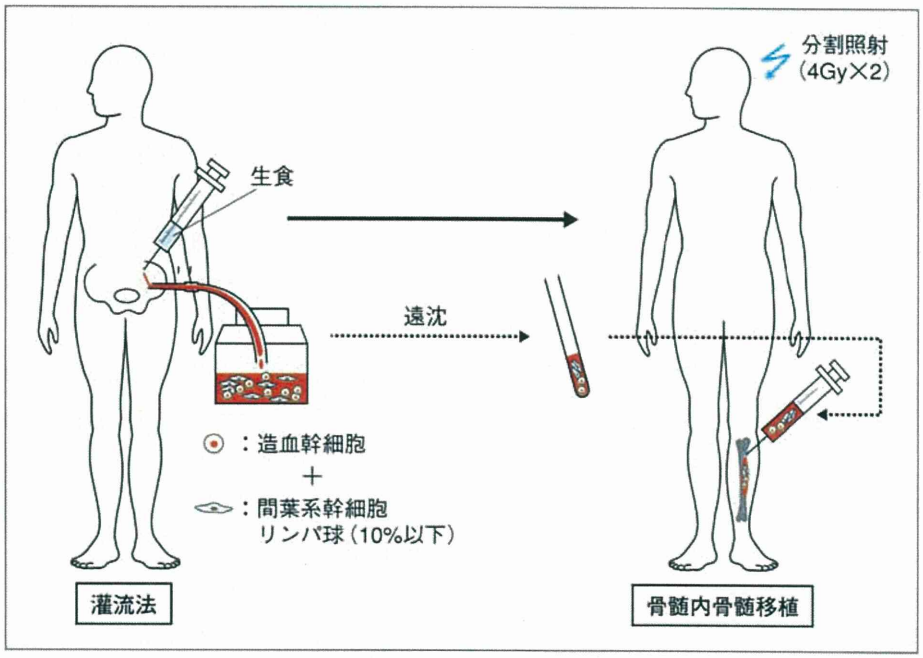

図 2　新しい骨髄移植の方法（灌流法＋骨髄内骨髄移植法）
腸骨（長管骨でも可）を用いて，2カ所に骨髄穿刺針を挿入し，片側から生理食塩水（生食）を注入し，もう一方から骨髄液を採取する（灌流法）．末梢血の混入が少ないため，T細胞の比率は10%以下で赤血球を除去する必要もない．遠沈後，できるだけ高濃度の細胞液を脛骨（両側）へ直接注入する．

換することが可能である[10]．

臓器移植への応用

マウスやラットにおいて，BMTと臓器移植を同時に行うと拒絶反応が起こらないことは古くから知られている．著者らも25年前にマウスで肝移植とBMTを同時に実施し，拒絶反応が起こらないことを証明している[14]．ヒトでも，BMTと臓器移植の同時移植はこれまで実施されてきたが，成功には至っていない．その理由は，アロのBMTの際，GvHDが発症しやすいことや，大量の放射線照射などの前処置が不可欠であるため，レシピエントに対する負担が大きいからである．そこで，mixed allogeneic chimerism（混合キメラ）の方法がIldstadとSachsらによって開発された[15]．しかし，長期間観察するとレシピエント側の造血系細胞が優位となり，著者らの膵島の移植実験ではドナーの膵島が拒絶されることも明らかになった[16]．

“IBM-BMT”は骨髄内にドナーの骨髄細胞を直接注入するため，ドナーのHSCのみならず，ドナーのストローマ細胞（MCSを含む）を効率よく移植することが可能である．そのため，従来の静脈内骨髄移植（IV-BMT）と比較して放射線量を減量しても移植臓器の拒絶を受けない．皮膚のアロ移植に関しても，IBM-BMTでは放射線の量を4 Gy×2（1回の照射であれば，6 Gyに相当）に減量しても100%の生着率が得られる[17]．この新しいBMTの方法を臓器移植と併用すると，免疫抑制剤を使用しなくても半永久的に拒絶反応が起こらない．

難病治療への応用

このようにHSCもMSCも正常のものと置換する，この新しいBMT法は，加齢とともに発症してくる骨粗鬆症，肺気腫，糖尿病，動脈硬化症，Alzheimer病，悪性腫瘍などの根本治療につながる可能性を秘めている．実際，著者らはモデル動物を用いて，骨粗鬆症[18]や肺気腫[19]が治療できるだけでなく，病気を正常マウスにtransferできることを明らかにし，間葉系幹細胞異常症という概念を提唱している[17,20]．

難病中の難病である悪性腫瘍に関しても，IBM-BMT法はドナーリンパ球輸注法（DLI）と併

用することによりDLIの副作用であるGvHDを抑制し，担癌動物に対して延命効果を発揮することを発見している[21-23]．

胸腺移植併用効果

著者らは，①骨髄細胞(HSC＋MSCを含む)，②胸腺，③環境，の3つが異なったトリプルキメラマウスの系においても，移植後長期間にわたって免疫学的寛容が誘導されることを証明した[24]．この事実は将来，脳死者から高齢者(胸腺の萎縮を認める)に対してIBM-BMTをする際，流産した第三者の胎児期胸腺(両親の許可を得て使用)の移植を併用することにより，高齢者の難病治療に役立てうる重要な発見と考える．

また著者らは，IBM-BMTによってドナー由来の間葉系の細胞が胸腺へ移住し，胸腺上皮に分化することを見出した[25]．この胸腺上皮はpositive selectionのみならず，negative selectionにも関与していることが判明している．

さらに著者らは，IBM-BMTに成体胸腺移植(持続的なドナーリンパ球輸注を目的として)を併用することによって，GvHDは抑制するが，強力な抗腫瘍効果を引き出せることを発見している[26]．

また，IBM-BMTと成体胸腺移植の併用は，mildな前処置(低放射線量＋低細胞数)でもアロの骨髄細胞を生着させ，長期の生存が可能となることを見出している[27]．

最近，著者らはIBM-BMT法に胸腺移植を併用すると，加齢に伴って発症してくるAlzheimer病や，2型糖尿病の進行を抑えることを発見した[28,29]．このようにBMTと胸腺移植，さらには臓器移植を組み合わせれば将来，かなりの難治性の疾患が治療可能と考えられる．

最近の国内外の動向

最近，臍帯血を腸骨内へ注入する腸骨内臍帯血移植がアメリカ[30]，イタリア[31]をはじめ，わが国の数カ所の施設でも開始されるようになった．しかし，この方法には2つの欠点がある．ひとつは臍帯血中にはストローマ細胞(MSCを含む)が骨髄細胞と比較してはるかに少量であること，もうひとつは血管の豊富な腸骨内への臍帯血移植は脛骨内BMTと異なり，移植細胞が骨髄内へtrapされずに循環系へと移行しやすいという点である．以上の理由で，著者らは灌流法で採取した純粋の骨髄細胞を脛骨内へ移植する脛骨内BMT法を推奨してきた．腸骨であれ脛骨であれ，ヒトでも骨髄内への造血系細胞を直接注入する技術の安全性が，国際的にも証明されつつあるものと考えられる．

今後，脳死や心臓死の症例だけでなく，ボランティアから骨髄細胞が灌流法で採取され，移植に利用されることを期待する．

おわりに

新移植技術(灌流法＋IBM-BMT)の特徴としては，灌流法での骨髄採取によって末梢血の混入が少ないこと，したがってGvHDが起こらないこと，一方，IBM-BMTを用いることによって効率よく，ドナーのHSCとMSCが移植可能であることがあげられる．それゆえ，造血幹細胞異常症のみならず，間葉系幹細胞異常症(加齢に伴って発症する疾患)の根治療法として，この新移植技術がクローズアップされてきている．

ヒトへの臨床応用としては現在，灌流法＋IBM-BMTの両技術のコンビネーションにおける安全性を最重点課題として，phase I studyを開始した．安全性が確認されればただちにphase II studyが実施できるように，臨床プロトコールを準備中である．新しいBMTの方法がヒトへ応用されるようになれば，骨髄ドナーの負担が軽減される．すなわち，骨髄穿刺針の穿刺部位が8カ所(従来の方法では100カ所以上)ですみ，麻酔からの覚醒後には痛みも少なく，歩行可能である[12]．それゆえ，骨髄バンクへの登録者が増加するし，たとえ，HLAが不一致でも新しい移植方法ではGvHDも起こらず，生着が促進されるため，前処置も軽減され，患者の負担も少なくなる．新技術により，これまで不治の病であった種々の難病が根治されれば，患者にとってこれ以上の福音はない．

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

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

Introduction

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

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

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

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

Materials and Methods

Mice

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

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

Cell lines

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

Inoculation of tumor cells

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

HSCT and TT

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

Histology

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

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

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

Mitogen responses

To analyze lymphocyte function, mitogen responses were examined in chimeric mice 2 months after the transplantation. For mitogen response, a total of 2×10^5 splenocytes collected from chimeric mice and nontreated B6 and BALB/c mice as responders were plated in 96-well flat-bottomed plates (Corning Glass Works, Corning, NY) containing 200 μL of RPMI1640 medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 2 μL of glutamine (Wako Pure Chemicals, Osaka, Japan), penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% heat-inactivated fetal calf serum. For mitogen responses, responder cells were incubated with 2.5 μg/mL of concanavalin A (ConA) (Calbiochem, San Diego, CA) or 25 μg/mL of lipopolysaccharide (LPS) (Difco Laboratories, Sparks, MI) for 48 or 72 h. During the last 18 h of the culture period, 20 mL of 0.5 μCi ^{3}H-thymidine (^{3}H-TdR; New England Nuclear) was introduced. Incorporation of ^{3}H-TdR was measured using Microbeta TriLux (PerkinElmer, Waltham, MA). The stimulation index was calculated as the average of ^{3}H-TdR incorporation in triplicate samples of responding cells with mitogen/^{3}H-TdR incorporation of responding cells in medium alone.

Statistical analyses

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

Results

Chimerism and tumor size

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

Survival period

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

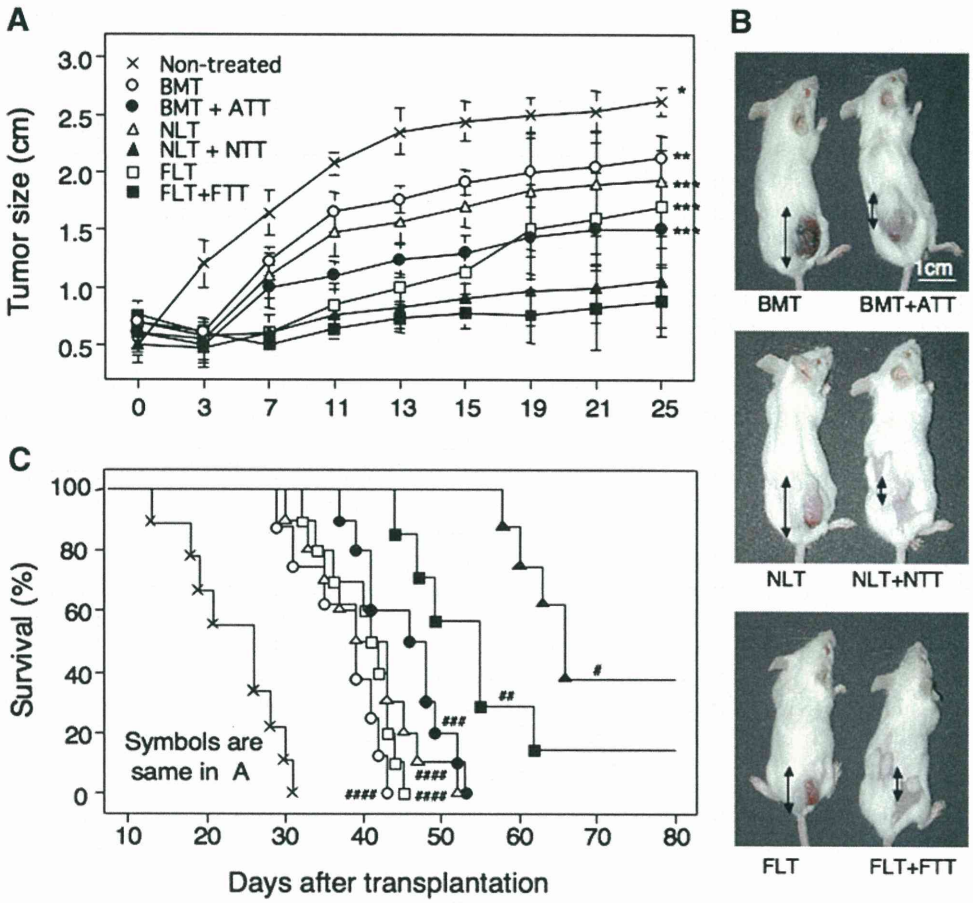

FIG. 1. Tumor size and survival rate in tumor-bearing mice treated with hematopoietic stem cell transplantation (HSCT) and thymus transplantation (TT) from various ages. Tumor size (**A, B**: representative data) and survival rate **(C)** are shown in tumor-bearing mice treated with bone marrow transplantation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

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

Analyses of TT

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

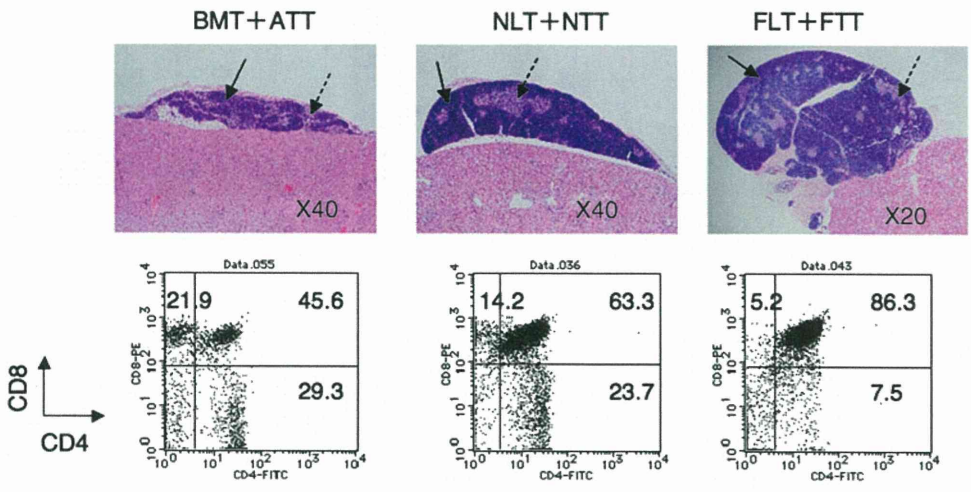

FIG. 2. Macroscopic and histological findings and FACS profiles of thymocytes in the transplanted thymus from tumor-bearing mice treated with HSCT and TT from various ages. Histological findings (*upper panels*, HE staining) and the FACS profiles of $CD4^+$ and $CD8^+$ cells in thymocytes (*lower panels*)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

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

Analyses of lymphocyte subsets

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

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

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

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

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

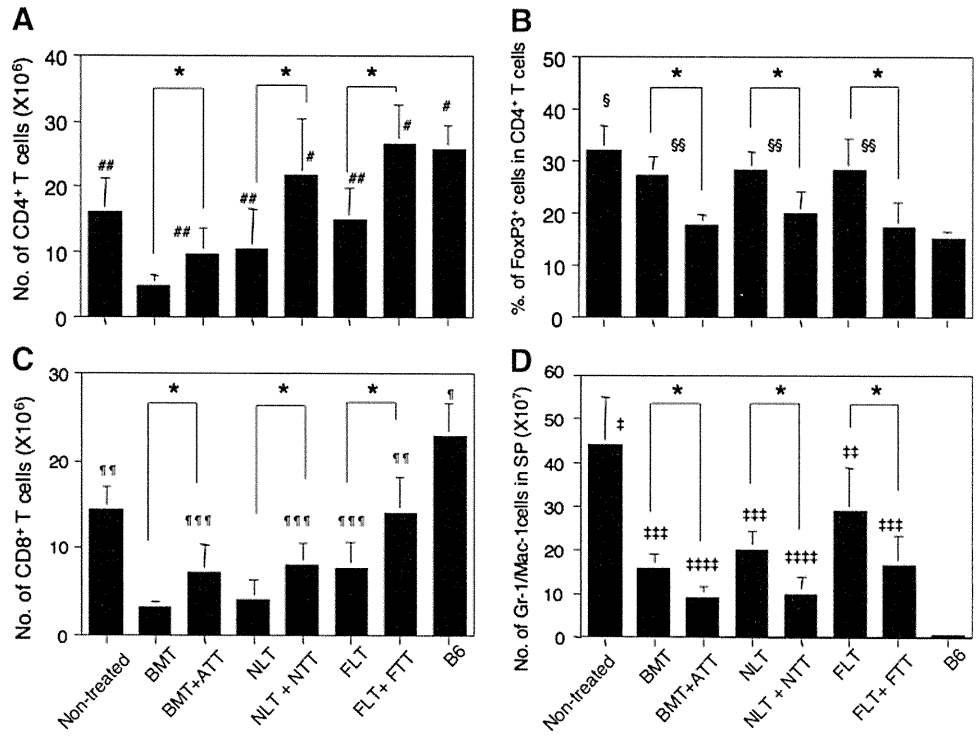

FIG. 3. Numbers of cells in the spleen from tumor-bearing mice treated with HSCT and TT from various ages. Numbers of $CD4^+$ T cells **(A)**, percentage of $FoxP3^+$ cells in $CD4^+$ T cells **(B)**, numbers of $CD8^+$ T cells **(C)**, and Gr-1/CD11b cells **(D)** in the spleen were evaluated in tumor-bearing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

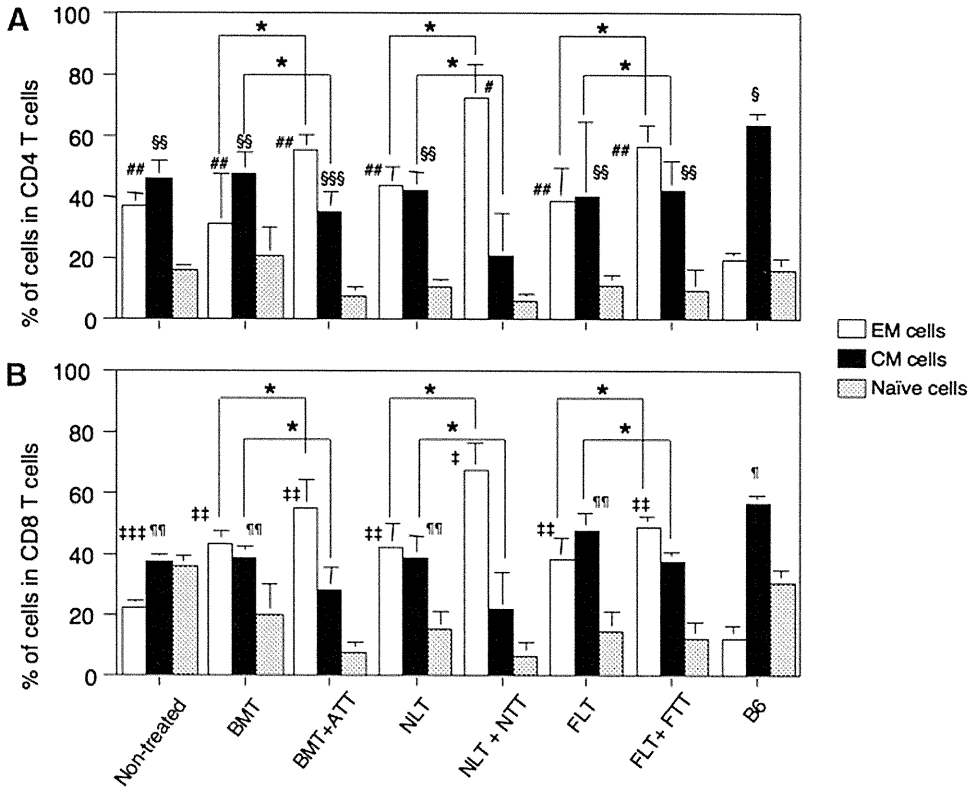

FIG. 4. Proportions of effector memory (EM), central memory (CM), and naïve T cells from tumor-bearing mice treated with HSCT and TT from various ages. Percentages of $CD62L^{-}CD44^{+}$ EM, $CD62L^{+}CD44^{+}$ CM, and $CD62L^{-}CD44^{-}$ naïve T cells in $CD4^{+}$ **(A)** and $CD8^{+}$ **(B)** subsets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

Analyses of lymphocyte function and cytokine production

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

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

Discussion

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

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

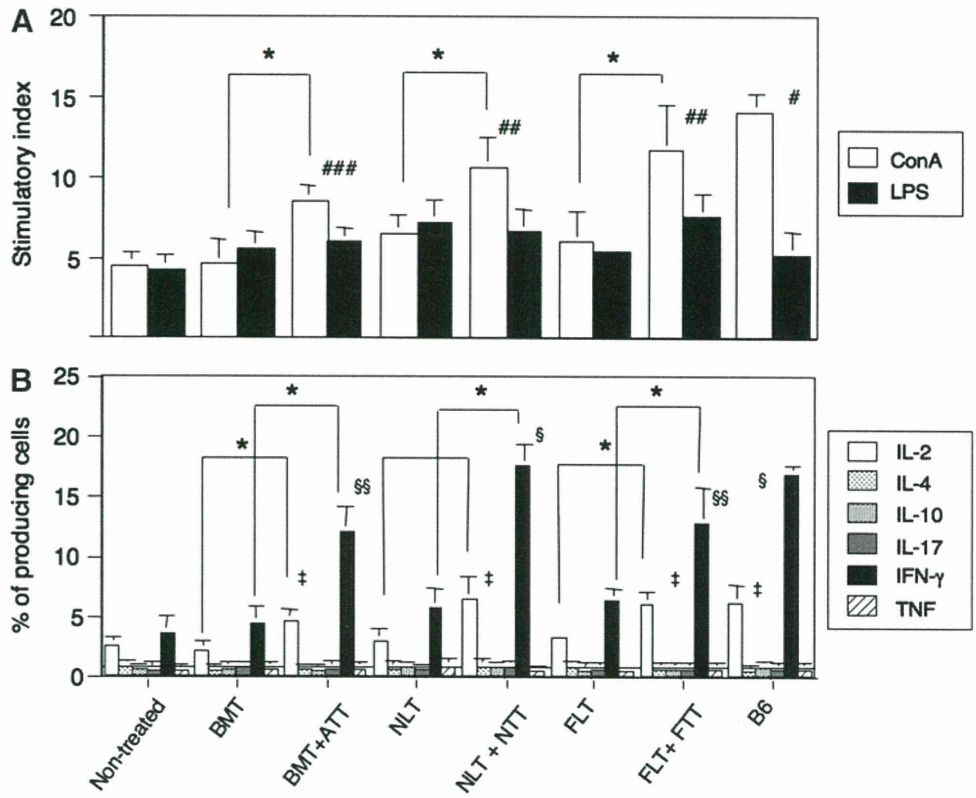

FIG. 5. Mitogen responses and percentages of cytokine-producing cells in the spleens from tumor-bearing mice treated with HSCT and TT from various ages. Mitogen responses: concanavalin A (ConA) and lipopolysaccharide (LPS) **(A)** and percentages of cytokine-producing cells **(B)** in the spleen were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

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

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

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

TABLE 1. SUMMARY OF DATA IN ALL GROUPS[a]

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

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

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

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

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

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

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

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

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

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Author Disclosure Statement

All authors state that no competing financial interests exist.

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Combination of Intra-Bone Marrow–Bone Marrow Transplantation and Subcutaneous Donor Splenocyte Injection Diminishes Risk of Graft-Versus-Host Disease and Enhances Survival Rate

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The combination of allogeneic bone marrow transplantation (allo-BMT) and donor lymphocyte infusion (DLI) is a useful method for establishing donor chimerism and preventing a relapse of leukemia/lymphoma. However, there is a risk of inducing uncontrollable fatal graft-versus-host disease (GVHD). In fact, allo-BMT plus intravenous (IV)-DLI using donor splenocytes induces fatal GVHD in recipient mice. In this study, we examined the effects of the combination of intra-bone marrow (IBM)-BMT and the subcutaneous injection of donor splenocytes (SC-DLI) on the allo-BMT system. Recipient BALB/c mice were conditioned by sublethal irradiation (5 Gy), followed by IBM-BMT plus IV-DLI or SC-DLI in C57BL/6 mice. The IV-DLI group showed better engraftment of donor hemopoietic cells than the control group (without DLI) but showed fatal GVHD. The SC-DLI group, however, showed good reconstitution and mild GVHD. These results suggest that the combination of SC-DLI and IBM-BMT promotes the reconstitution of hemopoiesis and helps reduce the risk of GVHD.

Introduction

BONE MARROW TRANSPLANTATION (BMT) was initially developed as a cure for certain diseases of the hematopoietic system such as aplastic anemia, leukemia, and immunodeficiencies [1–3]. Since then, BMT has been widely used for the treatment of autoimmune diseases, solid malignant tumors, multiple myelomas, myelodysplastic syndrome, and so on [4–9]. Allogenic-BMT (allo-BMT) is becoming more common owing to the discovery of more effective immunosuppressants, more powerful antibiotics, antithymocyte globulin, and fractionated irradiation [10–12]. Recently, we developed a new and powerful BMT method: intra-bone marrow (IBM)–BMT [13]. In this method, donor bone marrow cells (BMCs) are directly injected into the recipient's bone marrow (BM). A much greater number of donor hemopoietic stem cells and mesenchymal stem cells can therefore be inoculated into the recipient BM than by conventional intravenous BMT (IV-BMT). This results in rapid reconstitution of donor hemopoietic cells and permits a reduction in radiation doses as a pretreatment for BMT [14–16].

Donor lymphocyte infusion (DLI) is often used after allo-BMT to prevent disease relapse in the setting of T-cell–depleted BMT or nonmyeloablative conditioning regimens. It is also a combined method to convert mixed chimerism to full donor chimerism [17,18]. Donor T cells injected intravenously during DLI are activated in the host's lymphoid tissues, which then migrate to the target tissues of graft-versus-host disease (GVHD) and then mediate the GVHD. DLI, which is used as the combined conditioning therapy for BMT, helps to reduce relapse rates. However, DLI-induced GVHD is always associated with an increase in therapy-related morbidity because of its uncontrollable and fatal characteristics [19–26]. A key challenge for DLI is to balance the positive and negative effects of donor T cells in order to optimize the outcome.

In mice, allo-BMT plus IV-DLI using donor splenocytes can induce fatal GVHD due to the donor T-cell infiltration and proliferation in the GVHD target tissues such as the liver, spleen, intestine, and skin [27]. In this study, we examined the localizable effects of donor T cells (splenocytes) by subcutaneous injection (SC) of donor splenocytes in the

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allo-BMT system using a preconditioning regimen (sublethal irradiation). Compared with IV-DLI plus IBM-BMT, the SC-DLI plus IBM-BMT group showed good reconstitution and only mild GVHD. The survival rate in the SC-DLI group was much higher than in the IV-DLI group. These results suggest that the combination of SC-DLI and IBM-BMT promotes reconstitution of hemopoiesis and helps to reduce the risk of fatal GVHD.

Materials and Methods

Mice

C57BL/6 mice (B6) and BALB/c were purchased from Shimizu Laboratory Supplies (Shizuoka, Japan). All the mice were maintained in a pathogen-free room, and 8–10-week-old male mice were used in the present studies. The university's committee for animal research approved all experiments.

Reagents

The antibodies used in this study were as follows: fluorescein isothiocyanate (FITC)-labeled anti-mouse H-2^b Ab, phycoerythrin (PE)-labeled anti-mouse H-2^d Ab, peridinin chlorophyll protein (PerCP)-Cy5.5-labeled anti-mouse CD45 Ab, and anti-mouse CD3 Ab (BD Pharmingen, San Diego, CA). Lysing buffer (BD Pharmingen) was used for the lysis of erythrocytes. Collagenase type IV, used for hepatocyte isolation, was purchased from Sigma (Sigma-Aldrich, St. Louis, MO).

Whole-body irradiation of recipient mice

Gamma-irradiation was delivered by a Gammacell 40 Exactor (MDS Nordion, Kanata, ON, Canada) with two ^{137}Cs sources. Recipient mice were irradiated with 6, 5, or 4 Gy, the day before BMT.

IBM-BMT

BMCs were flushed from the medullary cavities of the femurs and tibias of donor mice with phosphate-buffered saline (PBS). After gentle dissociation, the BMC suspension was filtered through a 70-μm nylon mesh (Becton Dickinson Labware, Franklin Lakes, NJ). The BMC suspension was then centrifuged and the supernatant was aspirated. The BMCs were adjusted to 3×10^9 per mL. The thus-prepared BMCs (3×10^7) were injected directly into the tibial cavity of the recipient mice via the intra-bone marrow route (IBM-BMT) the day after irradiation, as previously described [13]. 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-G needle through the patellar tendon into the BM cavity. The BMCs (3×10^7/10 μL) were then injected into the BM cavity using a microsyringe (50 μL; Ito, Fuji, Shizuoka, Japan).

Donor lymphocyte infusion

Spleens were removed from donor B6 mice and then minced with scissors. Single cells were prepared by milling in steel mesh, followed by filtering through a 70-μm nylon mesh in PBS containing 2% fetal calf serum. After centrifugation, the pellets were suspended in 1×lysis buffer and kept for 15 min at room temperature for the lysis of erythrocytes. The erythrocyte-depleted splenocytes were adjusted to 5×10^7/0.2 mL (2.5×10^8 per mL) in PBS and were then injected intravenously into the tail vein in the IV-DLI group or subcutaneously in the back in the SC-DLI group. Figure 1 shows the experiment protocol, including the days for treatment.

GVHD analysis and scoring

The recipients were monitored daily for survival, and every 5 days for body weight changes and clinical signs of GVHD after BMT. The clinical scoring was based on 6 parameters: weight loss, posture, activity, fur texture, skin integrity, and diarrhea. A severity scale of 0 to 2 was used for each parameter, with a maximum score of 12 (Table 1). Clinical signs early after transplantation due to radiation toxicity were not considered as the appearance of GVHD [28,29].

The carcasses of the recipients that had died or had been sacrificed at 3 months after BMT were kept in 10% formalin. Tissues from GVHD target organs (liver, intestine, and skin) were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Slides were observed using an Olympus BX41 light microscope (Olympus, Tokyo, Japan) with a UPlanFL N 20×/0.50 objective. An Olympus DP-25 color camera using DP2-BSW software was used to acquire the images.

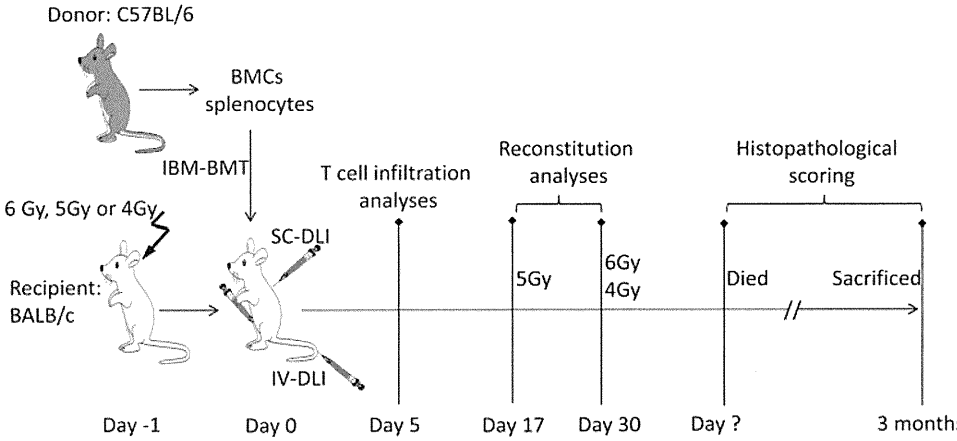

FIG. 1. Experiment protocol. The days of irradiation, BMT, and DLI are shown. The observed days of T-cell infiltration, reconstitution, and the analyses of histopathological scoring are also shown. BMT, bone marrow transplantation; DLI, donor lymphocyte infusion.

TABLE 1. CLINICAL SIGNS AND SCORING OF GVHD

Parameters	*Scale 0*	*Scale 1*	*Scale 2*
Weight loss	~10%	10%–25%	~25%
Posture	Normal	Hunching noted only at rest	Severe hunching impairs movement
Activity	Normal	Mildly to moderately decreased	Stationary unless stimulated
Fur texture	Normal	Mild to moderate ruffling	Severe ruffling/poor grooming
Skin integrity	Normal	Scaling of non–hair-bearing skin	Obvious areas of denuded skin
Diarrhea	Normal	Bits of residual perianal fecal material	A mass of residual perianal fecal material

The clinical signs of GVHD (based on 6 parameters: weight loss, posture, activity, fur texture, skin integrity, and diarrhea) were scored every 5 days after bone marrow transplantation. A severity scale of 0 to 2 was used for each parameter, with a maximum score of 12. GVHD, graft-versus-host disease.

Histopathological analysis was performed "single blind" by scoring the changes in the skin (dermal/epidermal lymphocyte infiltration, dyskeratotic epidermal keratinocytes, and epidermal thickening), the intestine (crypt regeneration, apoptosis in crypt epithelial cells, crypt loss, surface colonocyte attenuation, inflammatory cell infiltration in lamina propria, mucosal ulceration, and thickening of mucosa), and the liver (bile duct injury manifested by nuclear hyperchromasia, nuclear crowding, infiltrating lymphocytes, and cytoplasmic vacuolation and liver inflammation due to the infiltration of lymphocytes, neutrophils, and eosinophils). A severity scale from 0 to 4 was used, with a maximum score of 12 (Table 2) [29,30].

Analyses of donor cells in recipient peripheral blood, spleen, or liver by FACS

To detect the reconstitution of the recipients, the peripheral blood (PB) of the recipient mice was collected 17 days or 1 month after BMT. The PB was stained with FITC-labeled anti-H-2^b Ab, PE-labeled anti-mouse H-2^d Ab, and PerCP-Cy5.5-labeled anti-CD45 Ab. The erythrocytes were then lysed using lysing buffer. The stained cells were analyzed using FACScan (BD Biosciences, San Jose, CA). Leukocytes were first gated by $CD45^+$ cells, which were estimated as nuclear cells. The percentage of donor leukocytes was estimated as H-$2^{b+}/CD45^+$ cells.

To detect donor-derived T cells infiltrating the GVHD target tissues at 5 days after BMT, mononuclear cells (MNCs) from the recipient's spleen and liver were collected as follows: 0.5 mg/mL collagenase type IV solution was prepared by PBS dilution, and then after euthanasia, 2 mL collagenase solution was injected intraportally in the recipient mice. The spleen and liver were surgically excised and a single-cell suspension was prepared. The spleen and liver MNCs were then isolated by Lymphoprep (AXIS-SHIELD PoC AS, Oslo, Norway). The MNCs were stained with FITC-labeled anti-H-2^b Ab, PE-labeled anti-mouse H-2^d Ab, and PerCP-Cy5.5-labeled anti-CD3 Ab. The percentage of donor T cells was analyzed by FACScan estimated as H-$2^{b+}/CD3^+$ cells.

Statistical analysis

Survival data were analyzed using the Kaplan–Meier method in the Stat Mate software. Other results are represented as means ± standard deviation (SD). The Student's *t*-test was used to determine any statistical significance. A P value of <0.05 was considered to be a significant difference.

Results

No GVHD occurs in the BMT-only group

Previous studies have shown that, in contrast to humans and other primates, no or only mild GVHD is observed in the

TABLE 2. HISTOPATHOLOGICAL GVHD SCORING

Organ	*Parameters*	*Scale 0*	*Scale 0.5*	*Scale 1*	*Scale 2*	*Scale 3*	*Scale 4*
Skin	Dermal/epidermal lymphocyte infiltration, dyskeratotic epidermal keratinocytes, and epidermal thickening	Normal	Focal and rare	Focal and mild	Diffuse and mild	Diffuse and moderate	Diffuse and severe
Intestine	Crypt regeneration, apoptosis in crypt epithelial cells, crypt loss, surface colonocyte attenuation, inflammatory cell infiltration in lamina propria, mucosal ulceration, and thickening of mucosa						
Liver	Bile duct injury, infiltrating lymphocytes, and cytoplasmic vacuolation and liver inflammation						

Histopathological GVHD scoring was performed based on the changes in 3 GVHD target organs: the skin, intestine, and liver. A severity scale of 0 to 4 was used for each organ, with a maximum score of 12.

case of allo-BMT without DLI in the murine setting [31–33]. To confirm this, BMCs from B6 mice were transplanted into sublethally irradiated (6, 5, or 4 Gy) BALB/c recipients by IBM-BMT (control group). All the control groups (6, 5, or 4 Gy) survived until 90 days after BMT (Fig. 2). No severe complications, such as fatal GVHD, occurred in the BMT-only control group according to clinical observations (Fig. 4) and histopathological evaluation (Fig. 5).

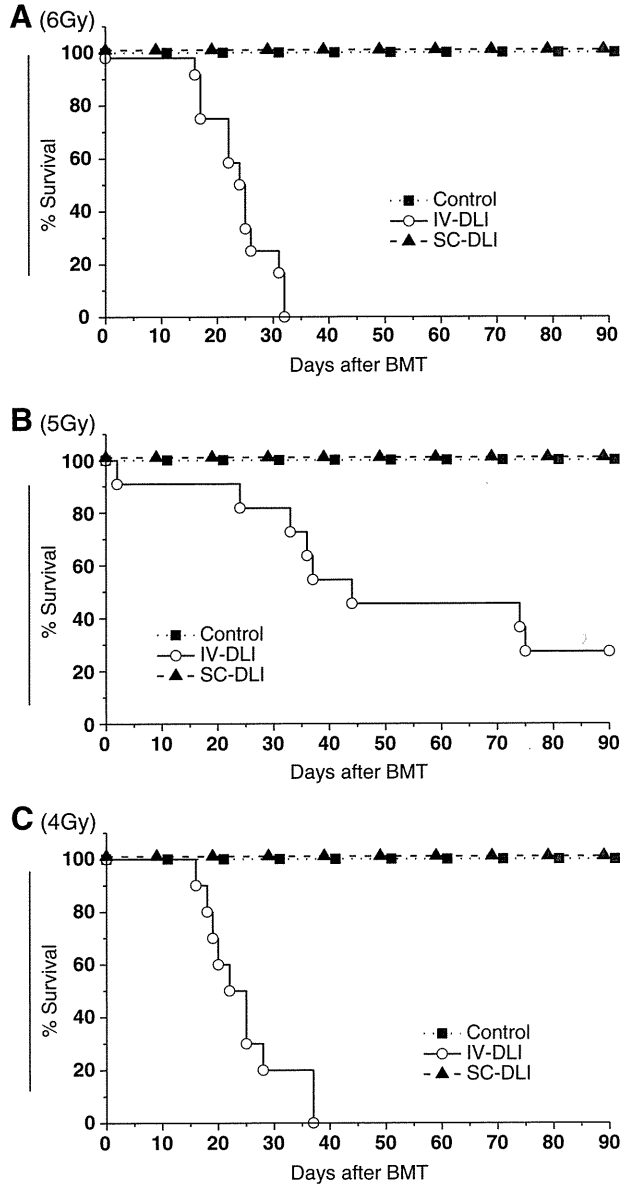

FIG. 2. IV-DLI shortens the survival but SC-DLI does not. Recipient (BALB/c) mice were irradiated at 6 Gy **(A)**, 5 Gy **(B)** or 4 Gy **(C)** on day −1. Bone marrow cells (3×10^7) from donors (B6) were injected by intra-bone marrow–BMT with or without 5×10^7 splenocytes DLI on day 0. The splenocytes were injected intravenously into the tail vein in the IV-DLI group or subcutaneously in the back side in the SC-DLI group. $n \geq 10$. IV, intravenous; SC, subcutaneous.

The SC-DLI group shows lower mortality than the IV-DLI group

The PB and spleen are commonly used as the source of lymphocytes for DLI. In the present study, we carried out DLI using the erythrocyte-depleted splenocytes (5×10^7 per mouse). After BMT, the recipients were monitored daily for survival. For the 6 Gy irradiated mice, all the mice in the IV-DLI group died within 32 days after BMT (Fig. 2), whereas all the mice in the SC-DLI group survived until 90 days after BMT. Similarly, high mortality rates were also observed in the IV-DLI group for 5 or 4 Gy irradiated recipients. To our surprise, the 5 Gy IV-DLI group showed significantly improved survival compared with the 4 Gy IV-DLI group. In contrast to the high mortality caused by IV-DLI, the SC-DLI groups (6, 5, or 4 Gy) showed a 100% survival rate, as seen in the control groups (Fig. 2).

Better reconstitution of donor hemopoietic cells is observed in both the IV-DLI and SC-DLI groups than the control group (without DLI)

Next, we examined the reconstitution degree of the recipients with donor-type cells in the PB of the recipient mice after BMT. In the experiment with preconditioning with 6 Gy irradiation, the PB was collected from the surviving recipient mice to analyze the chimerism at 1 month after BMT. All the recipients of IBM-BMT, either combined with DLI or not, showed nearly complete donor-type (H-2^d) hematopoietic cells (Fig. 3A). Moreover, there was no significant difference between the control group and the SC-DLI group: the mean and SDs of the percentages of donor hematopoietic cells were $92.4\% \pm 3.7\%$ and $95.3\% \pm 3.1\%$, respectively (Fig. 3D). We therefore reduced the radiation dose to 5 Gy. With 5 Gy, reconstitution was examined earlier (on day 17). Donor-type hematopoietic cells were detected in 4 of 10 control group recipients, 10 of 10 IV-DLI group recipients, and 9 of 11 SC-DLI group recipients (Fig. 3B). Statistically significantly higher percentages of reconstitution with donor cells were observed in the SC-DLI group than in the control group, and there was no significant difference between the IV-DLI and SC-DLI groups (Fig. 3E). Moreover, better reconstitution was confirmed by long-term (not transient) chimerism (data not shown). When the radiation dose was reduced to 4 Gy, neither the control group nor the SC-DLI group could reconstitute the recipients with donor BMCs. The two survivors in the IV-DLI group showed donor-type hematopoietic cells but these mice died soon (Fig. 3C, F).

Serious GVHD is observed in the IV-DLI group

After BMT, clinical signs of GVHD in the recipients (including weight loss, posture, activity, fur texture, skin integrity, and diarrhea) were assessed every 5 days and the score was calculated. In the 6 Gy IV-DLI group, all the recipient mice showed hunchback; several showed loss of weight, inaction, ruffled fur texture, and slight diarrhea on day 15. The signs of GVHD became progressively more serious: angular, severe hunching, stationary unless stimulated, severe ruffling, obvious areas of denuded skin, and severe diarrhea. The clinical score reached a peak on day 30: 7.33 ± 0.58. No obvious signs of GVHD appeared in the other

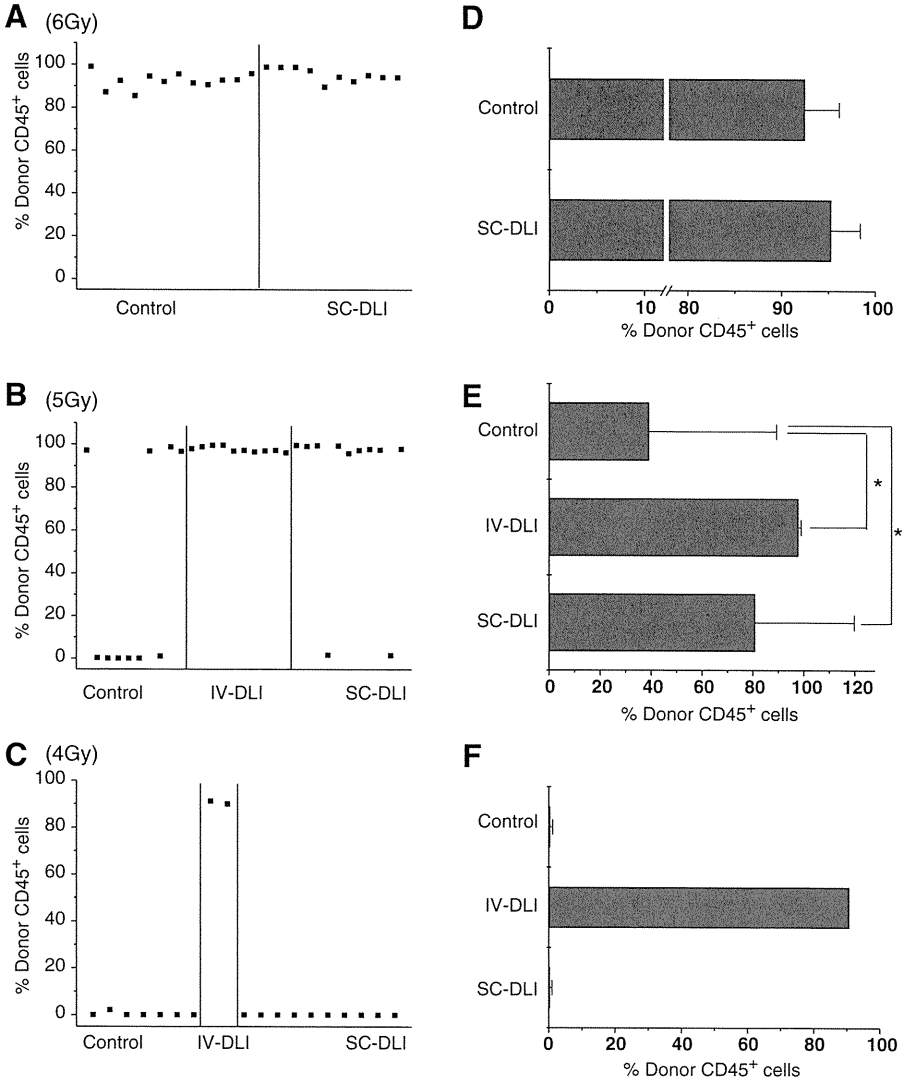

FIG. 3. Reconstitution in different irradiation doses. Recipient mice were irradiated with 4, 5, or 6 Gy on day −1. BMT and DLI were carried out on day 0. Peripheral blood of 6 Gy **(A** and **D)** and 4 Gy **(C** and **F)** irradiated mice was analyzed for reconstitution at 1 month after BMT. In the 5 Gy **(B** and **E)** experiment, peripheral blood was analyzed on day 17. Each small square (■) represents the percentage of reconstitution for each mouse in the left panels. The right panels show the percentage of donor $CD45^+$ by means ± standard deviation. $^*P <$ 0.05.

two groups: 0.25 ± 0.87 (control group) and 0.50 ± 0.71 (SC-DLI group) (Fig. 4A). In the 5 Gy experiment, the clinical scores in the control group, IV-DLI group, and SC-DLI group were 0.10 ± 0.31, 6.44 ± 2.60, and 0.81 ± 0.30, respectively, according to observations on day 30. The mice in the IV-DLI group retained the tendency to develop serious GVHD during the period from day 30 to day 70. The other two groups displayed mild GVHD (Fig. 4B).

In the 4 Gy experiment, the IV-DLI group displayed moderate but not severe GVHD, [although the recipient mice survived short-term (they died between days 16 and 37)]. Pathological diagnosis after autopsy indicated that infection due to graft failure (not GVHD) was the cause of death (data not shown).The clinical score was 4.00 (only two mice survived) on day 30 (Fig. 4C). It is not surprising that no GVHD was observed in either the control group or the SC-DLI group due to a failure of reconstitution.

GVHD is main cause of death in the IV-DLI group

We next examined the histopathological changes in the liver, skin, and intestine from randomly selected recipient mice that had died in the IV-DLI group or were sacrificed at 3 months after BMT in the control group and the SC-DLI group (four mice per group). The severity of histopathological GVHD nearly paralleled the clinical signs. In the IV-DLI group, either 6- or 5-Gy-irradiated recipients showed lymphocyte infiltration in the bile duct, epidermal thickening, and dermal lymphocyte infiltration, occasional crypt apoptosis, and mild inflammation in the intestine (Fig. 5A, C). The pathological scores in the IV-DLI group in the 6 and 5 Gy experiments were 5.75 ± 0.43 and 5.67 ± 0.47, respectively (Fig. 5B, D). In the control group and the SC-DLI group, no or only mild lesions were found in the GVHD target tissues, even in the skin at the site of the injection of splenocytes. The pathological scores in these two groups were also significantly lower than in the IV-DLI group in either radiation dose experiment. These data indicated that GVHD was the main cause of death in the IV-DLI group and that DLI via the SC route could diminish the risk of fatal GVHD.

SC-DLI reduces donor T-cell infiltration into GVHD target tissues

Previous studies reported that donor T cells reached a peak on day 5 after expansion and infiltration into target

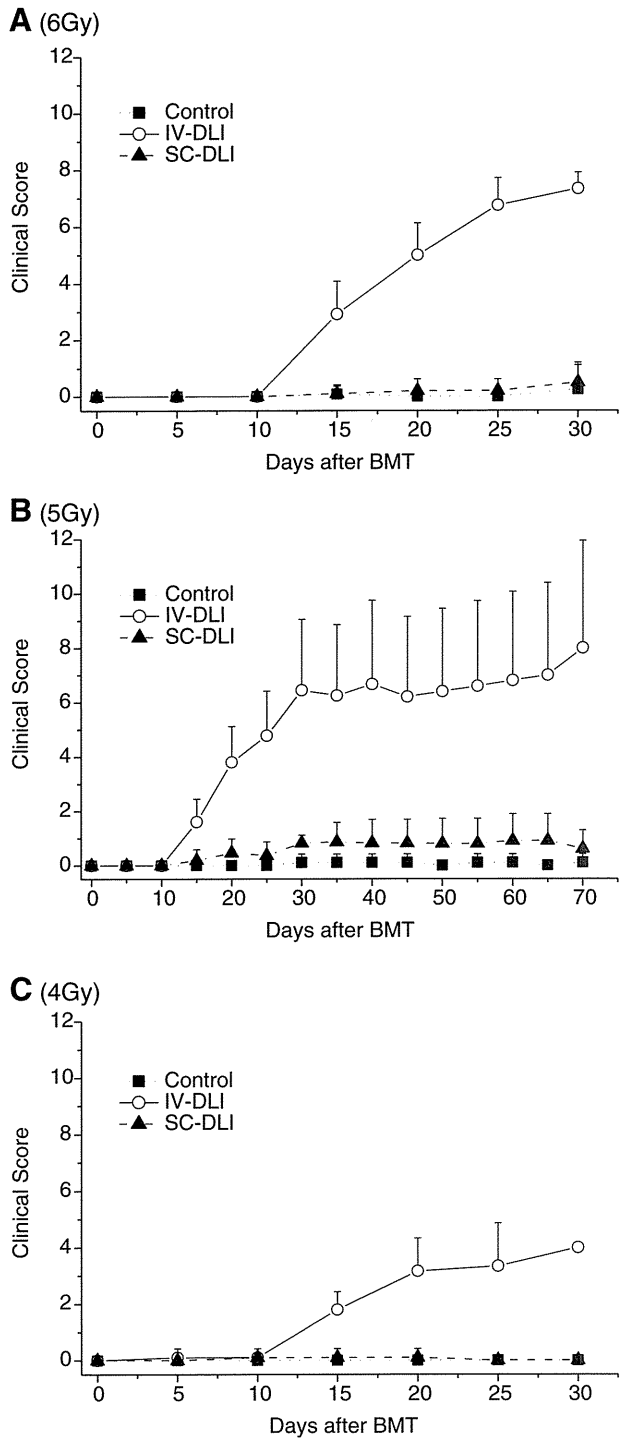

FIG. 4. SC-DLI significantly ameliorates GVHD. Recipient mice were irradiated at 6 Gy **(A)**, 5 Gy **(B)** or 4 Gy **(C)** on day −1. After BMT and DLI, the body weight of the recipients was recorded and clinical signs of GVHD were assessed every 5 days. The clinical scoring was based on 6 parameters: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

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

Discussion

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

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

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

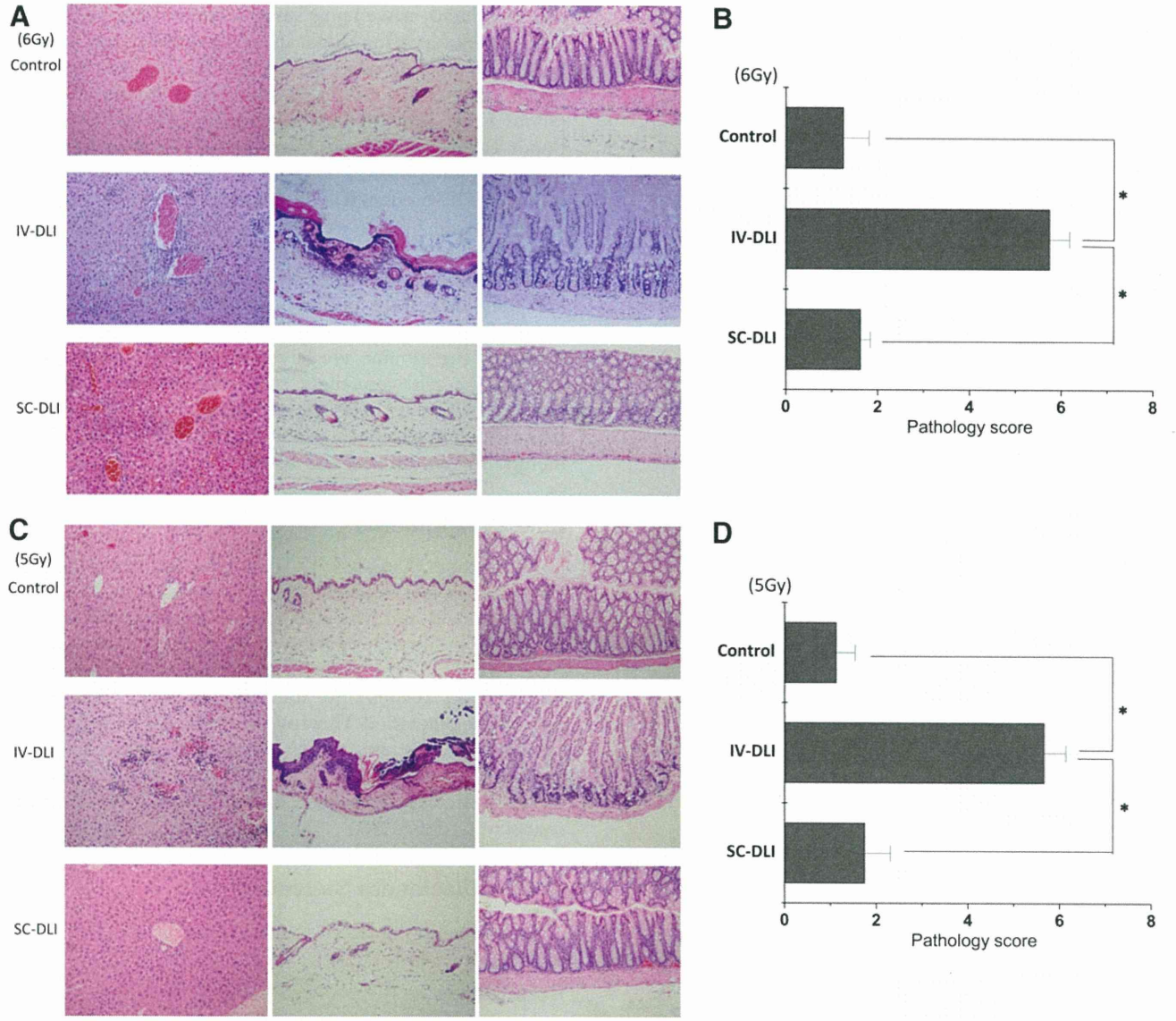

FIG. 5. Histopathological score and representative photographs of GVHD target tissues: liver, skin, and intestine. Autopsies were performed on mice that had died (IV-DLI group) or had been sacrificed at 3 months after BMT (control group and SC-DLI group). Tissues from GVHD target organs (liver, skin,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

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

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

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

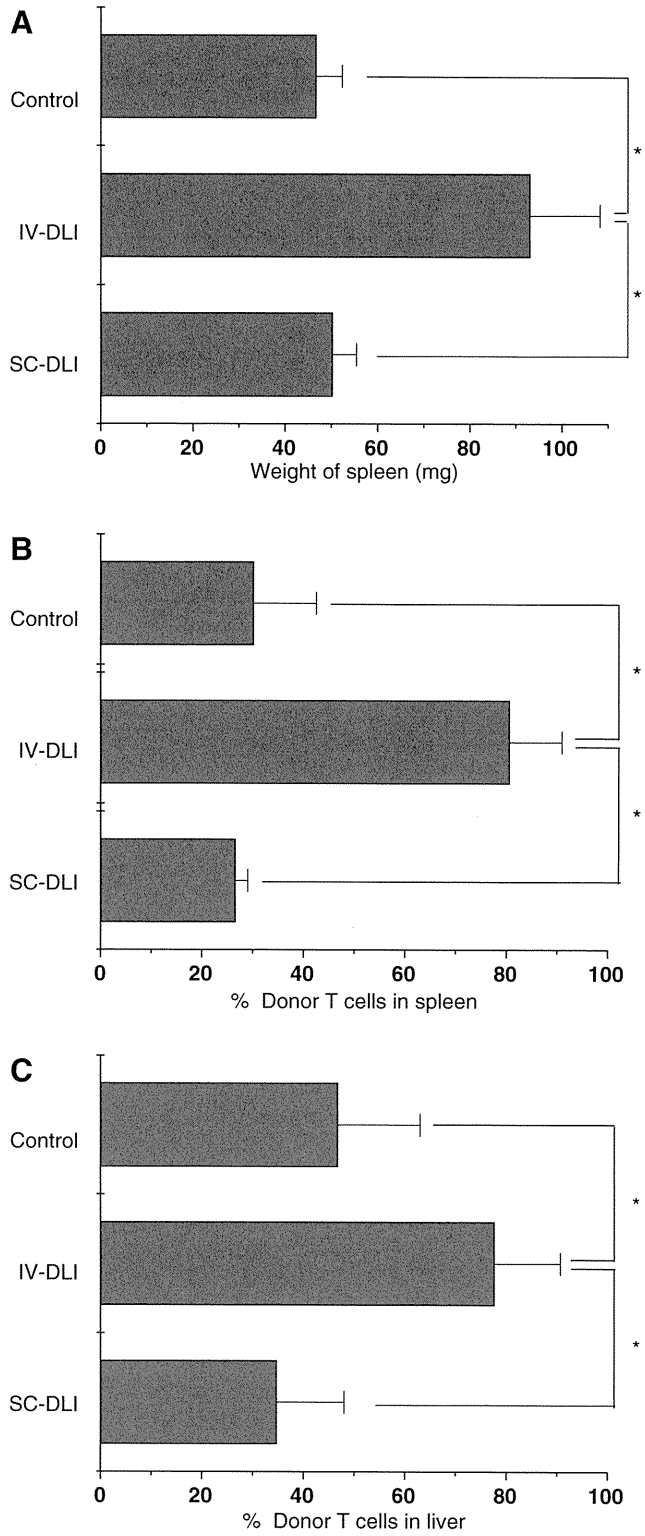

FIG. 6. SC-DLI inhibited donor T-cell infiltration of the GVHD target tissues. Five days after BMT and DLI, the weight of the spleen **(A)** and the percentage of donor T cells in the spleen **(B)** and liver **(C)** were compared between groups. Control group: $n=3$; IV-DLI group and SC-DLI group: $n=4$;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

the other hand, as we know, subcutaneous injection is widely used as a long-acting depot injection method, and the skin, which is a physical obstacle, can localize the large number of donor splenocytes ($5\times10^7/0.2$ mL) in a not-so-small area (about 7 cm^2, measured after injection). The speed at which donor T cells migrate into the recipient's lymphoid tissues is limited by the physical obstacle. Therefore, the downstream of the GVHD process will be slowed down. However, donor T cells appear to migrate in a long-lasting way and the development of GVHD is thus at a low-enough level to be controllable. Moreover, the subpopulation of donor T cells and the mechanisms underlying the reduction of GVHD in the SC-DLI group should be further investigated.

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

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Author Disclosure Statement

No competing financial interests exist.

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