

# Donor CD4 T Cells Are Critical in Allogeneic Stem Cell Transplantation against Murine Solid Tumor

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## Abstract

**Nonmyeloablative allogeneic stem cell transplantation (SCT) has been used for various malignancies, although detailed mechanisms of antitumor effects remain unclear. We showed that a nonmyeloablative allogeneic SCT regimen, which consists of mixed chimerism induced by an injection of donor spleen and bone marrow cells followed by cyclophosphamide treatment and a donor lymphocyte infusion (DLI), exerted antitumor effects on established murine bladder tumor, MBT-2. An expansion of donor CD4 T cells accompanied by transient but vigorous IFN- $\gamma$  production was detected shortly after DLI. *In vivo* neutralization of IFN- $\gamma$  or depletion of CD4 T cells from DLI abolished the antitumor effects, indicating an indispensable role of donor CD4 T cells producing IFN- $\gamma$ . Donor as well as host CD8 T cells accumulated in the tumor region with time. Importantly, depletion of CD8 T cells from DLI did not reverse the suppression of tumor growth, indicating that CD4 T cells play a more essential role in mediating early antitumor effects. Furthermore, tumor-specific response of host CD8 T cells was suggested. These results not only provide the first evidence of nonmyeloablative allogeneic SCT for the treatment of bladder tumor but also elucidate detailed mechanisms of antitumor effects provoked by DLI.** [Cancer Res 2009;69(12):5151–8]

## Introduction

Because it was revealed that nonmyeloablative allogeneic stem cell transplantation (SCT) efficiently provoked antitumor effects, nonmyeloablative allogeneic SCT has been applied as a treatment against hematopoietic malignancies as well as against solid tumors, including renal cell cancer, breast cancer, colon cancer, and ovarian cancer (1–11). Generally, antitumor effects of nonmyeloablative allogeneic SCT are attributed to graft-versus-tumor (GVT) activity, which is usually associated with inevitable graft-versus-host disease (GVHD; refs. 12–16). Therefore, various experimental animal models have been developed to elucidate the antitumor mechanisms of nonmyeloablative allogeneic SCT and to better control the balance between GVH and GVT effects (17–19).

We have recently established a unique model system of nonmyeloablative allogeneic SCT. The treatment regimen is based on our

previously reported cyclophosphamide-induced tolerance system, which is established by an i.v. injection of  $1 \times 10^8$  allogeneic spleen cells and  $2 \times 10^7$  bone marrow cells followed by an i.p. injection of 200 mg/kg cyclophosphamide 2 days later. Destruction of both donor-reactive T cells of host origin and host-reactive T cells of donor origin occurred in the induction phase, and then a stable mixed chimerism was induced with a tolerance to the allografts without GVHD (20, 21). We have applied the cyclophosphamide-induced tolerance system for the treatment of murine renal carcinoma, RENCA, by shifting the equal balance between GVH and host-versus-graft (HVG) reactions toward GVH reaction with an additional donor lymphocyte infusion (DLI; ref. 22). We found strong antitumor activity against RENCA induced by this method. Kinetics analysis revealed that the level of donor chimerism gradually decreased over time and was finally undetectable, whereas host T cells exerted tumor-specific immune responses against RENCA. Because low levels of mixed chimerism decrease the risk of GVHD, our novel model is thus considered ideal in terms of segregating GVT from GVHD. However, at present, detailed mechanisms of the induction of antitumor effects of the nonmyeloablative allogeneic SCT remain unclear.

To evaluate whether our nonmyeloablative allogeneic SCT system is applicable for other solid tumor, and to clarify the antitumor mechanisms of nonmyeloablative allogeneic SCT more in detail, we applied our nonmyeloablative allogeneic SCT model for the treatment of murine bladder tumor, MBT-2, using MHC-compatible but *mouse mammary tumor virus* (*Mtv*)-incompatible combination of C3H/HeN (H-2<sup>k</sup> *Mtv*1/6+) and AKR/J (H-2<sup>k</sup> *Mtv*1/6-) mice as host and donor, respectively (23). An advantage of using this combination of mouse strains is availability of monoclonal antibody (mAb) specific for V $\beta$  chain of T-cell receptor (TCR) that recognizes the *Mtv* antigens. Differential use of Thy1 antigens (Thy1.2 and Thy1.1 between C3H/HeN and AKR/J, respectively) enables us to follow either donor or host T cells. We here showed antitumor effects of our nonmyeloablative allogeneic SCT model against MBT-2 and an importance of host-reactive donor CD4 T cells producing IFN- $\gamma$  for the antitumor effects. It was also revealed that the nonmyeloablative allogeneic SCT treatment induced tumor antigen-specific effector CD8 T cells of host origin. These results thus revealed a cross-talk between alloreactive donor CD4 T cells and tumor-specific host CD8 T cells in antitumor activity of nonmyeloablative allogeneic SCT.

## Materials and Methods

**Animals.** Female C3H/HeN (H-2<sup>k</sup>) mice and female AKR/J (H-2<sup>k</sup>) mice were purchased from Japan Charles River at 7 wk of age. All mice were kept in specific pathogen-free conditions and were then used for experiments at 8 wk of age. All animal protocols were approved by the University Committee on the Use and Care of Animals at Kyushu University.

**Note:** This study was performed at the Division of Host Defense, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.

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**Tumor cells and the treatment protocol.** MBT-2 cell line, which had been derived from a carcinogen-induced bladder tumor in a C3H mouse, were maintained in DMEM containing 10% heat-inactivated FCS, 100 units/mL penicillin, 100  $\mu$ g/mL streptomycin, and 10 mmol/L HEPES. C3H/HeN mice were injected s.c. with  $2 \times 10^5$  MBT-2 cells. Considering the clinical application, we started the cancer treatment after establishing the injection tumors (usually 14 d after tumor inoculation). Initially, 1.0 mL of HBSS containing a set quantity of a mixture of  $1 \times 10^8$  spleen cells and  $2 \times 10^7$  bone marrow cells from donor AKR/J mice was injected i.v. into the tail vein of host C3H/HeN mice. Cyclophosphamide (Endoxan, Shionogi) dissolved in PBS (20 mg/mL) was injected i.p. at a dose of 200 mg/kg 2 d later. Donor AKR/J lymphocytes ( $1 \times 10^7$ ) from axillary, inguinal, and mesenteric lymph nodes were injected i.v. into the tail vein of the host C3H/HeN mice 1 d after cyclophosphamide injection. The sizes of primary tumors were determined every 2 or 3 d using a caliper. Body weights were also measured by a scale every 2 or 3 d. Tumor volume was calculated using the formula  $V = (A \times B^2)/2$ , where  $V$  is volume ( $\text{mm}^3$ ),  $A$  is long diameter (mm), and  $B$  is short diameter (mm).

**Flow cytometric analysis.** The lymphocytes were stained with combinations of following mAbs and analyzed by using a FACSCalibur flow cytometer (Becton Dickinson). FITC-conjugated anti-Thy1.1 mAb (HIS51) and anti-Thy1.2 mAb (53-2.1), PE-conjugated anti-V $\beta$ 3 mAb (KJ25) and anti-CD8 mAb (53-6.7), and Cy-Chrome-conjugated anti-CD4 mAb (H129.19) were purchased from BD Biosciences. Live lymphocytes were carefully gated by forward and side scattering. The data were analyzed with CellQuest software (BD Biosciences).

**ELISA for measurement of IFN- $\gamma$ , tumor necrosis factor- $\alpha$ , and interleukin-12p40 production.** Commercial ELISA kits (DuoSet ELISA Development System, R&D Systems) were used to measure the levels of IFN- $\gamma$ , tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interleukin (IL)-12p40 in the serum or culture supernatants. To evaluate serum levels of three kinds of cytokines, blood from C3H/HeN mice was collected on days 4, 6, 24, and 30 after the transplantation of AKR/J spleen and bone marrow cells. To evaluate tumor-specific IFN- $\gamma$  production of spleen cells, C3H/HeN mice were sacrificed on 26 d after the transplantation of AKR/J spleen and bone marrow cells. Thy1.1 $^+$  spleen cells were depleted by negative selection using anti-Thy1.1 mAb (HIS51) and Dynabeads pan anti-mouse IgG (Invitrogen Life Technologies). The spleen cells ( $5 \times 10^5$  per well) were cultured with mitomycin C (MMC)-treated MBT-2 cells ( $1 \times 10^5$  per well) or MH134 cells, derived from a carcinogen-induced hepatoma in a C3H mouse ( $1 \times 10^5$  per well), in 96-well plates for 72 h in RPMI 1640 containing 10% FCS.

**Intracellular cytokine staining.** Lymph node cells were prepared on day 6 after the transplantation. The cells were incubated for 4 h at 37°C and 5% CO $_2$  with 10  $\mu$ g/mL brefeldin A (Sigma-Aldrich), 25 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich), and 1  $\mu$ g/mL ionomycin (Sigma-Aldrich) in 48-well flat-bottom plates at a concentration of  $2 \times 10^6$  per well (Falcon, BD Biosciences) in a volume of 1.0 mL of RPMI 1640 containing 10% FCS. After 4-h culture, the cells were harvested, surface stained, and then subjected to intracellular cytokine staining using a BD Fixation and Permeabilization system (BD Biosciences) according to the manufacturer's instructions in combination with FITC-conjugated IFN- $\gamma$  mAb (XMG1.2).

**In vivo blocking of IFN- $\gamma$  and depletion of CD4 or CD8 T cells from DLI.** To neutralize IFN- $\gamma$  *in vivo*, mice were injected i.p. with 100  $\mu$ g of anti-IFN- $\gamma$  mAb (XMG1.2) or control IgG on day 4 and 50  $\mu$ g on days 5, 6, and 7 after the transplantation. DLI CD4 T cells and DLI CD8 T cells from donor AKR/J mouse were depleted by negative selection using anti-CD4 mAb and anti-CD8 mAb with Dynabeads anti-rat IgG (Invitrogen). We confirmed efficient depletion of CD4 T cells or CD8 T cells by a flow cytometry (data not shown).

**Isolation of tumor-infiltrating lymphocytes.** Tumors were dissected from mice and minced to yield 1- to 2-mm pieces. To release tumor-

infiltrating lymphocyte (TIL), the tumor pieces were incubated in mixture of 1 mg/mL collagenase (Invitrogen) and 20  $\mu$ g/mL DNase (Sigma-Aldrich) in RPMI 1640 containing 10% FCS for 90 min at 37°C. Lymphocytes were separated by Percoll density gradient centrifugation (Amersham Biosciences) and analyzed by a flow cytometry.

**Statistical analysis.** The statistical significance of the data was determined by using the unpaired two-tailed Student's  $t$  test.  $P < 0.05$  was taken as the level of significance. Analysis was carried out using StatView 5.0 software (Abacus Concepts).

## Results

**Nonmyeloablative allogeneic SCT suppressed the growth of established murine bladder tumor.** We evaluated therapeutic potential of our nonmyeloablative allogeneic SCT model system against established murine bladder tumor, MBT-2. Syngeneic C3H/HeN mice were injected s.c. with  $2 \times 10^5$  MBT-2 cells. After 14 days, when the injected tumors were established, the mice were injected i.v. with a mixture of  $1 \times 10^8$  spleen cells and  $2 \times 10^7$  bone marrow cells from donor AKR/J mice followed by an i.p. injection of cyclophosphamide 2 days later. On the next day, a group of mice further received DLI of AKR/J mice. As shown in Fig. 1A, tumor growth was strongly suppressed in the C3H/HeN mice that had been treated with AKR/J spleen and bone marrow cells on day 0, cyclophosphamide on day 2, and AKR/J DLI on day 3. Suppression of tumor growth was also observed in C3H/HeN mice treated with AKR/J spleen and bone marrow cells on day 0 and cyclophosphamide on day 2 without DLI. However, the level of suppression is significantly less than those with DLI, and similar level of suppression was also seen in mice treated with cyclophosphamide alone, indicating that the antitumor effect was attributed to a direct cytotoxic effect of cyclophosphamide on tumor cells in these mice. These results thus revealed that DLI significantly suppressed the growth of MBT-2 cells. To our knowledge, this is the first demonstration of therapeutic effect of nonmyeloablative allogeneic SCT against bladder tumor. We also examined the degree of GVHD by assessing five clinical parameters: weight loss, posture (hunching), activity, fur texture, and skin integrity every day (24). All the mice with DLI exhibited mild skin ruffling with slight loss of body weight, which was not statistically significant compared with the mice without DLI (Fig. 1B). The symptoms of GVHD gradually disappeared over time. Any clinical symptoms of GVHD were not detected in other groups.

**Transient expansion of host-reactive T cells in DLI followed by an establishment of enhanced mixed chimerism.** Kinetics of chimerism in the peripheral lymph nodes was analyzed by a flow cytometry using Thy1.1 as a marker of AKR/J donor cells. Engraftment of Thy1.1 $^+$  cells was clearly detected in the mice treated with donor spleen and bone marrow cells on day 0 and cyclophosphamide on day 2 (Fig. 2A). The percentage of Thy1.1 $^+$  cells in unmanipulated AKR/J mice was  $68.5 \pm 4.9\%$  (data not shown). The level of chimerism of donor lymphocytes in the mice without DLI was stable from day 4 to day 30 ( $5.1 \pm 0.3\%$  to  $6.8 \pm 2.2\%$ ) after injection of donor spleen and bone marrow cells. On the other hand, the level of chimerism of the mice treated with additional DLI was much higher than the mice without DLI. In the mice with DLI, the level of chimerism of donor origin was  $11.4 \pm 3.7\%$  on day 4, and the level of chimerism increased dramatically and reached to  $42.1 \pm 2.5\%$  on day 6 (3 days after DLI). Thereafter, the level of chimerism gradually decreased over time but was still  $18.6 \pm 1.9\%$  on day 30.

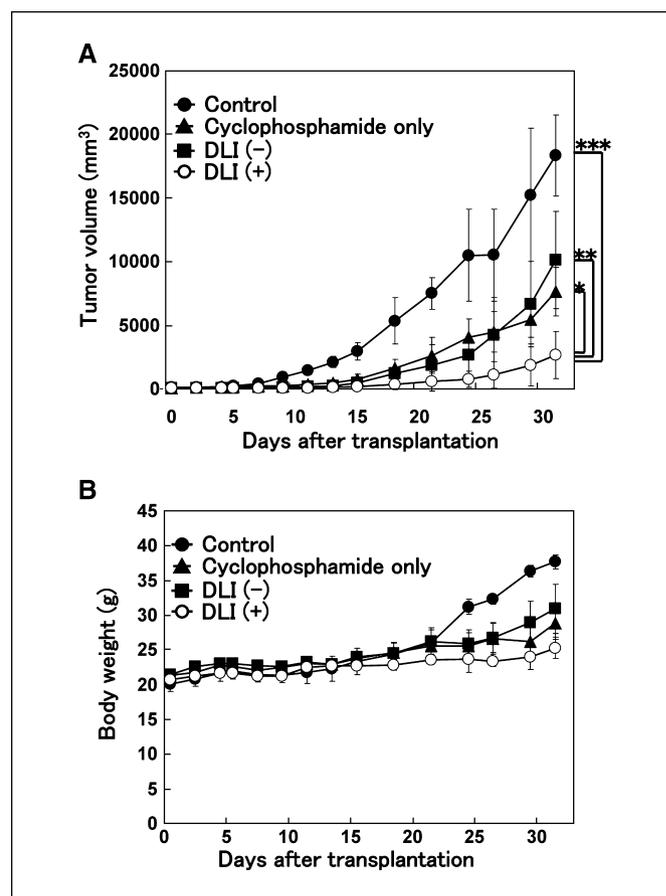
As we considered that the increased mixed chimerism in the mice with DLI was attributed to an expansion of host alloantigen-reactive T cells in DLI, we further examined expansion of AKR/J CD4 T cells bearing TCR V $\beta$ 3 that recognize superantigens encoded by *Mtv1* and *Mtv6* present in C3H/HeN mice (23). The percentage of V $\beta$ 3<sup>+</sup>CD4 T cells in unmanipulated AKR/J mice was about  $5.9 \pm 0.3\%$  (data not shown). However, in the mice that have been treated with AKR/J spleen and bone marrow cells on day 0 and on day 2 without additional DLI, the percentage of host-reactive V $\beta$ 3<sup>+</sup>CD4 T cells of donor AKR/J mice origin (Thy1.1<sup>+</sup>) was severely decreased (Fig. 2B). This was because of clonal destruction of host-reactive T cells by the cyclophosphamide treatment as we have shown previously (21). Similarly, donor-reactive host V $\beta$ 6<sup>+</sup>CD4 T cells, which recognize superantigens encoded by *Mtv7* in AKR/J mice, were also diminished as we have shown previously (data not shown; refs. 20, 25). On the other hand, in the mice with an additional DLI treatment, donor V $\beta$ 3<sup>+</sup>CD4 T cells vigorously expanded on day 6 ( $48.1 \pm 1.6\%$  on day 6,  $26.7 \pm 2.1\%$  on day 8; Fig. 2B and C). Slight increase of donor CD8 T cells was also detected after DLI, but it was not statistically different from mice without DLI (data not shown). The expanded V $\beta$ 3<sup>+</sup>CD4 T cells gradually decreased over time and finally decreased to the same levels as the mice without DLI on day 30, although there was still significant difference in the level of mixed chimerism between the mice with and without DLI (Fig. 2A). The movement of the absolute numbers of peripheral lymph nodes cells was similar to kinetics of percentage Thy1.1<sup>+</sup>V $\beta$ 3<sup>+</sup>CD4 T cells in mice (data not shown).

**IFN- $\gamma$  production by donor CD4 T cells was indispensable for the antitumor effects of DLI.** To elucidate the mechanisms of the antitumor effects of DLI, we measured the concentration of cytokines in the serum. Serum levels of IFN- $\gamma$  rapidly elevated from day 4 to day 6 after the transplantation and then were decreased and returned to the basal levels by day 24 in the mice treated with AKR/J spleen and bone marrow cells on day 0 and cyclophosphamide on day 2 followed by AKR/J DLI on day 3 (Fig. 3A). The kinetics of serum IFN- $\gamma$  was clearly correlated with the expansion of host-reactive V $\beta$ 3<sup>+</sup>CD4 T cells of DLI (Fig. 2B). In the mice of other groups, levels of serum IFN- $\gamma$  were not elevated through the observation period (Fig. 3A). Serum levels of TNF- $\alpha$  and IL-12p40 were not significantly elevated in all groups (Fig. 3B). To define cellular source of IFN- $\gamma$ , lymph node cells were analyzed by intracellular staining on day 6 after the transplantation. It was revealed that the donor Thy1.1<sup>+</sup>CD4 T cells produced IFN- $\gamma$  ( $20.4 \pm 3.2\%$ ; Fig. 4A). Furthermore, most of the IFN- $\gamma$ <sup>+</sup> cells in the donor lymphocytes were the host-reactive V $\beta$ 3<sup>+</sup>CD4 T cells ( $18.9 \pm 3.0\%$ ; Fig. 4B). On the other hand, both of donor and host CD8 T cells produced very little amount of IFN- $\gamma$ . To directly investigate the role of the transient IFN- $\gamma$  production in antitumor activity in our model system of nonmyeloablative allogeneic SCT, we neutralize IFN- $\gamma$  by administrating anti-IFN- $\gamma$  mAb daily from day 4 to day 7. As shown in Fig. 4C, suppression of tumor growth in the mice with DLI was reversed by the anti-IFN- $\gamma$  mAb treatment. Because we have found that IFN- $\gamma$  was mainly produced by donor CD4 T cells after DLI (Fig. 4A), we examined the effect of depleting CD4 T cells from DLI. As shown in Fig. 4D, antitumor activity of DLI was diminished when CD4 T cells were depleted from DLI, again indicating the importance of donor CD4 T cells as the source of IFN- $\gamma$ .

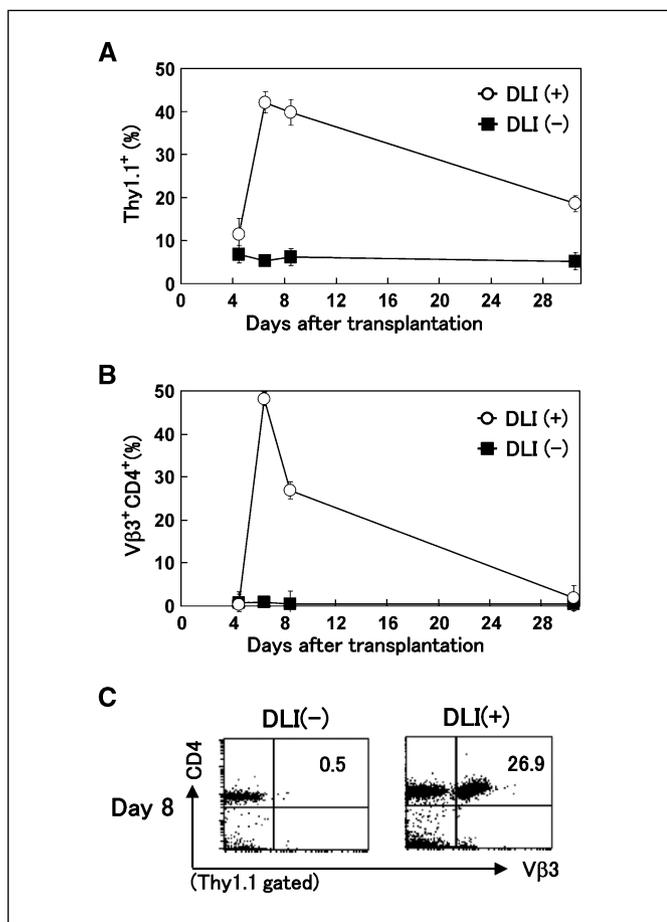
**Initial infiltration of donor CD4 T cells followed by an increase of both donor and host CD8 T cells in the tumor region of mice with DLI.** Next, we analyzed the population of TIL by

a flow cytometry to further investigate the effector antitumor mechanisms of the nonmyeloablative allogeneic SCT. As early as on day 6 after the transplantation, donor Thy1.1<sup>+</sup> T cells were detected in the TIL of mice with DLI, which was nearly absent in those without DLI (Fig. 5A). The percentage of donor T cells remained high even on day 26 ( $66.0 \pm 2.3\%$  of the TIL) when the level of mixed chimerism was decreased in the periphery (Fig. 2). Majority of the donor T cells in TIL were V $\beta$ 3<sup>+</sup>CD4 T cells on day 6, but these cells were decreased to only  $5.9 \pm 2.8\%$  of TIL on day 26 (Fig. 5B). Instead, CD8 T cells increased to  $>70\%$  among donor T cells on day 26 ( $71.3 \pm 2.4\%$ ). Interestingly, similar kinetics in the appearance of CD4 T cells and CD8 T cells after DLI was detected in host T-cell populations. CD4 T cells accounted  $58.5 \pm 8.1\%$  of TIL on day 6, whereas CD8 T cells accounted  $78.6 \pm 1.9\%$  on day 26. An increase of CD8 T cells was also observed in TIL of the mice without DLI, but the percentage of CD8 T cells reached at most 45% on day 26 (data not shown).

**Donor CD8 T cells were not essential for the antitumor effects of DLI in the early stage.** The predominant infiltration of CD8 T cells suggests a role of CD8 T cells as effectors of antitumor



**Figure 1.** Growth of MBT-2 cells was significantly suppressed by DLI. *A*, the C3H/HeN mice were injected s.c. with MBT-2 cells ( $2 \times 10^5$ ). After establishing the injected tumors (usually 14 d after tumor inoculation), the C3H/HeN mice ( $n = 5$ ) were treated with cyclophosphamide injection alone on day 2 (▲), transplantation of AKR/J spleen and bone marrow cells on day 0 and cyclophosphamide injection on day 2 (■), and transplantation of AKR/J spleen and bone marrow cells on day 0 and cyclophosphamide injection on day 2 and AKR/J DLI on day 3 (○) or untreated (●). Tumor volume of mice was inspected every 2 or 3 d. Data were obtained from three separate experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , compared with control group on day 31 after the transplantation. *B*, body weight of mice in each group was also measured by a scale every 2 or 3 d.



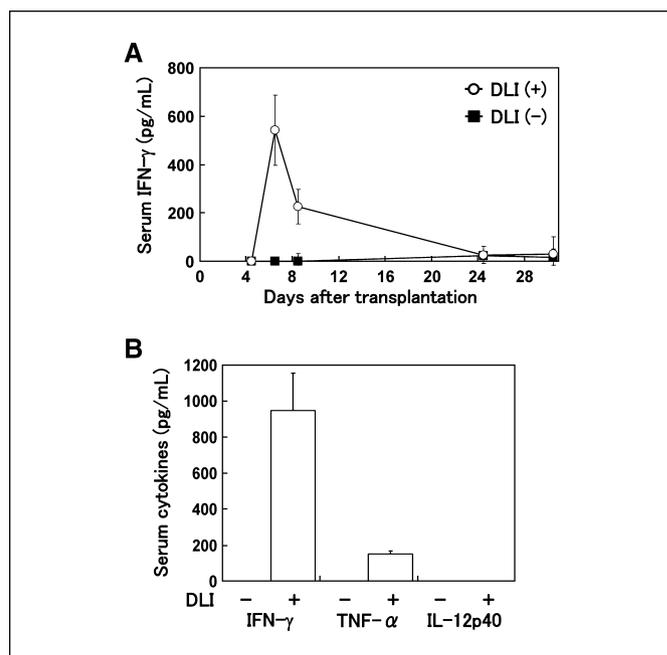
**Figure 2.** Vigorous expansion of host-reactive donor T cells and high levels of mixed chimerism after DLI treatment. C3H/HeN mice ( $n = 3$ ) were treated with transplantation of AKR/J spleen and bone marrow cells on day 0 and cyclophosphamide injection on day 2 (■) and transplantation of AKR/J spleen and bone marrow cells on day 0 and cyclophosphamide injection on day 2 and AKR/J DLI on day 3 (○). *A*, kinetics of donor chimerism (Thy1.1<sup>+</sup>) in the host lymph nodes. *B*, kinetics of the frequency of host-reactive donor T cells (Vβ3<sup>+</sup>CD4<sup>+</sup> in Thy1.1<sup>+</sup> cells) in the lymph nodes. *C*, representative dot plots of flow cytometric analysis on day 8 after the transplantation. Experiments were repeated three times in total with three mice for each experiment. Representative data are shown.

effects of DLI, although relative importance of donor or host CD8 T cells is unclear. It is also an important question to be addressed whether the effector CD8 T cells recognize tumor-specific antigens or alloantigens, both of which might be expressed on the tumor cells. To evaluate the role of CD8 T cells in DLI in our system, we examined antitumor effects of CD8 T-cell-depleted DLI. As shown in Fig. 6A, there was no difference in the suppression of tumor growth between the mice injected with whole donor lymphocytes and those with CD8 T-cell-depleted lymphocytes, suggesting that CD8 T cells in DLI were not essential for the antitumor effects in the early stage. We also examined tumor-specific T-cell responses in the spleen on day 26. Spleen cells produced high level of IFN-γ in response to MBT-2 but not to control MH134 cells, although both tumors were derived from C3H/HeN strain of mice (Fig. 6B). Importantly, spleen cells depleted of donor-derived Thy1.1<sup>+</sup> cells still produced high levels of tumor-specific IFN-γ and these levels were significantly higher than those produced by spleen cells of mice without DLI. Although we cannot completely deny the role of tumor-specific CD4 T cells and donor-derived CD8

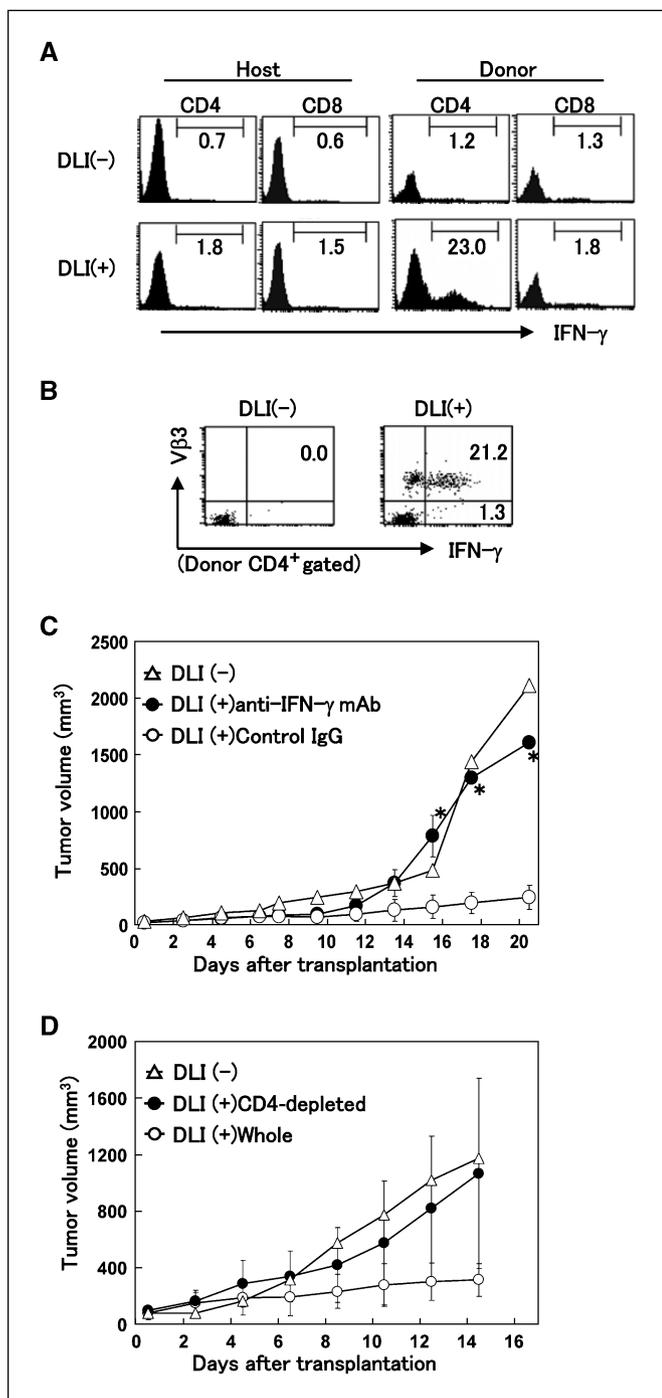
T cells in this experiment, these results indicate that host CD8 T cells, which recognize tumor-specific antigens, clearly contribute to antitumor activity in the late stage of our model of nonmyeloablative allogeneic SCT.

## Discussion

Nonmyeloablative allogeneic SCT has widely been applied for the treatment of hematologic malignancies as well as solid tumors (1–11). Recently, we have developed a murine model of nonmyeloablative allogeneic SCT system, which was designed based on our previously established cyclophosphamide-induced tolerance model and was proved to be effective against murine renal cell carcinoma, RENCA (22). In this model, DLI was carried out 1 day after the cyclophosphamide treatment of that tolerance-inducing system. In the present study, we found that our nonmyeloablative allogeneic SCT regimen was also effective for the treatment of established murine bladder tumor, MBT-2. The necessity of DLI was further supported in the present study because no antitumor effects were observed against murine bladder tumor without DLI (Fig. 1A). Similarly, in the mice without the transplantation of spleen and bone marrow cells on day 0, antitumor effects were not observed (data not shown). In such mice without spleen and bone marrow cells, the level of donor chimerism was <10% on day 6 and rapidly decreased thereafter. If the transplantation of spleen and bone marrow cells was not performed before cyclophosphamide treatment, the tolerance to donor cells was not induced at the time of DLI



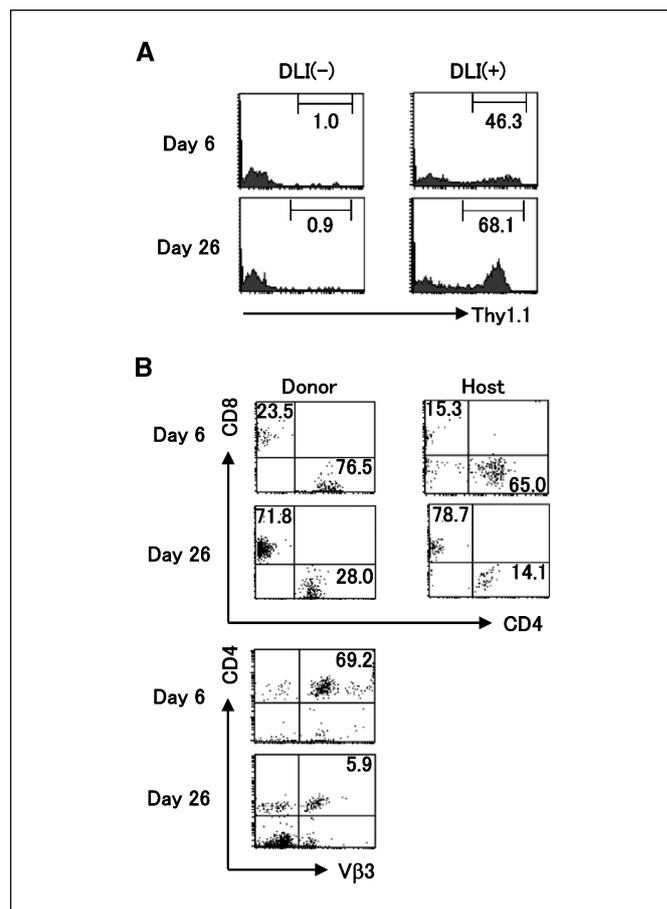
**Figure 3.** A transient and vigorous IFN-γ production after DLI. *A*, the concentration of serum IFN-γ was measured by ELISA. The C3H/HeN mice ( $n = 3$ ) were treated with transplantation of AKR/J spleen and bone marrow cells on day 0 and cyclophosphamide injection on day 2 (■) and transplantation of AKR/J spleen and bone marrow cells on day 0 and cyclophosphamide injection on day 2 and AKR/J DLI on day 3 (○). Sera of the C3H/HeN mice were collected on days 4, 6, 8, and 30 after the transplantation. Data were obtained from three separate experiments. *B*, concentration of IFN-γ, TNF-α, and IL-12p40 in the serum on day 6. C3H/HeN mice ( $n = 3$ ) were treated with transplantation of AKR/J spleen and bone marrow cells on day 0 and cyclophosphamide injection on day 2 (■) and transplantation of AKR/J spleen and bone marrow cells on day 0 and cyclophosphamide injection on day 2 and AKR/J DLI on day 3 (○).



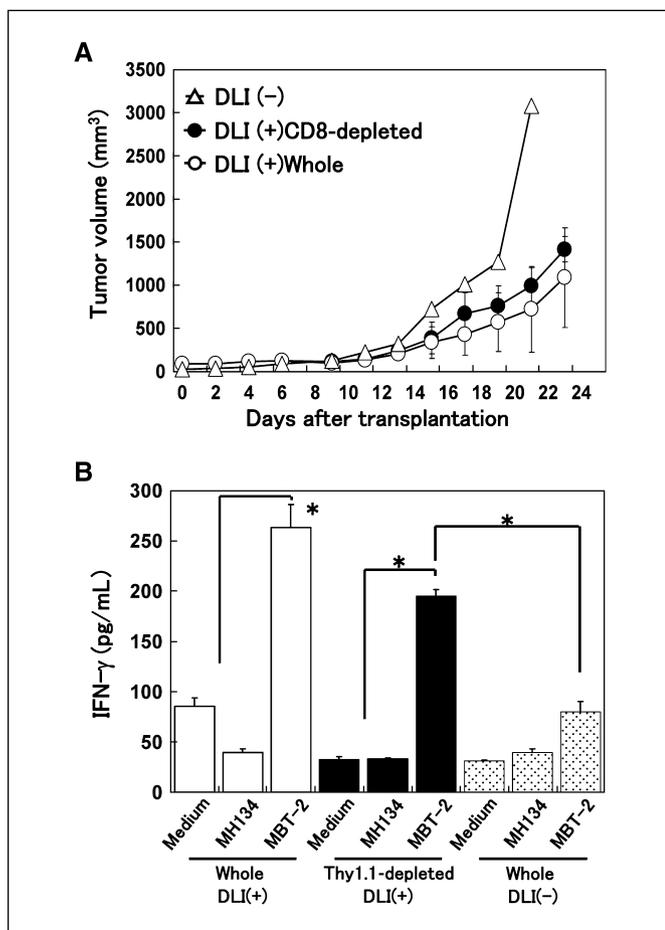
**Figure 4.** IFN- $\gamma$  produced by host-reactive donor CD4 T cells is essential for antitumor activity. **A** and **B**, lymph node cells were analyzed by intracellular staining for IFN- $\gamma$  on day 6 after transplantation. Analysis gate was set either on Thy1.2<sup>+</sup> (Host) or Thy1.2<sup>-</sup> (Donor) lymphocytes. Furthermore, these cells were gated by CD4<sup>+</sup> or CD8<sup>+</sup>, respectively. Experiments were repeated three times in total with three mice for each experiment. Representative data are shown. **C** and **D**, the C3H/HeN mice were injected s.c. with MBT-2 cells ( $2 \times 10^5$ ). After establishing the injected tumors, the C3H/HeN mice ( $n = 4$ ) were treated with the transplantation of AKR/J spleen and bone marrow cells on day 0, cyclophosphamide injection on day 2, and AKR/J DLI on day 3. **C**, DLI-treated C3H/HeN mice were injected i.p. with anti-IFN- $\gamma$  mAb (●) or control IgG (○), 100  $\mu$ g on day 4 and 50  $\mu$ g on days 5, 6, and 7 after the transplantation. \*,  $P < 0.01$ , compared with control IgG group on days 15, 17, and 20 after the transplantation. Control mice;  $\Delta$ , without AKR/J DLI. Data were obtained from three separate experiments. **D**, DLI-treated C3H/HeN mice received CD4 T-cell-depleted DLI (●) or whole DLI (○). Control mice;  $\Delta$ , without AKR/J DLI. Data were obtained from three separate experiments.

treatment (20), and donor T cells were considered to be rejected by HVG reaction. Taken together, we conclude that tolerance-inducing system with the transplantation of spleen and bone marrow cells followed by cyclophosphamide is also indispensable for antitumor activity against murine bladder tumor. To our knowledge, this is the first demonstration of a therapeutic effect of nonmyeloablative allogeneic SCT against bladder tumor, indicating a possible application for the treatment of human bladder tumor as well.

Because MBT-2 cells are originated from C3H mice, we took advantage of the use of a combination of C3H and AKR/J strains, which enabled us to identify alloantigen-specific T cells by means of V $\beta$  chain-specific mAbs (23). It was revealed that the host (C3H)-reactive donor (AKR/J) V $\beta$ 3<sup>+</sup> alloreactive CD4 T cells producing IFN- $\gamma$  expanded transiently in the lymph nodes as well as in the tumor region at the early stage after DLI. An importance of the initial IFN- $\gamma$  production for the antitumor effects of DLI and an importance of these CD4 T cells as the source of IFN- $\gamma$  were clearly shown by *in vivo* neutralization of IFN- $\gamma$  (Fig. 4C) and depleting CD4 T cells from DLI (Fig. 4D), respectively. Similar to our results, an importance of IFN- $\gamma$  for the induction of antitumor effect in a different system of nonmyeloablative allogeneic SCT



**Figure 5.** Infiltration of CD4 and CD8 T cells in the tumor region. **A** and **B**, the C3H/HeN mice were injected s.c. with MBT-2 cells ( $2 \times 10^5$ ). After establishing the injected tumors, the C3H/HeN mice ( $n = 3$ ) were treated with the transplantation of AKR/J spleen and bone marrow cells on day 0 and cyclophosphamide injection on day 2, with AKR/J DLI on day 3 or without DLI. TILs were analyzed on days 6 and 26 after the transplantation. Analysis gate was set on lymphocytes (**A**) or Thy1.1<sup>+</sup> (Donor) or Thy1.2<sup>+</sup> (Host) lymphocytes (**B**). Experiments were repeated three times in total with three mice for each experiment. Representative data are shown.



**Figure 6.** Antitumor activity of host CD8 T cells. *A*, the C3H/HeN mice were injected s.c. with MBT-2 cells ( $2 \times 10^5$ ). After establishing the injected tumors, the C3H/HeN mice ( $n = 4$ ) were treated with the transplantation of AKR/J spleen and bone marrow cells on day 0, cyclophosphamide injection on day 2, and AKR/J DLI on day 3. DLI-treated C3H/HeN mice received CD8 T-cell–depleted DLI (●) or whole DLI (○). Control mice; Δ, without AKR/J DLI. Data were obtained from three separate experiments. *B*, spleen cells from the C3H/HeN mice treated with DLI were harvested on day 26 after the transplantation and cultured with medium alone, MMC-treated MH134 (H-2<sup>b</sup>), or MBT-2 for 3 d. IFN- $\gamma$  in the culture supernatant was measured by ELISA. ■, spleen cells depleted of donor (Thy1.1<sup>+</sup>) cells; □, whole spleen cells. Control mice; ▨, whole spleen cells of C3H/HeN mice without AKR/J DLI. \*,  $P < 0.001$ .

was also reported (26, 27). As T helper 1 (Th1) cells promote induction of CTL, especially by conditioning dendritic cell (28, 29), it is reasonably assumed that these alloreactive Th1 cells exerted antitumor activity by enhancing CTL activity. In addition to these indirect effects, CD4 T cells may directly exert antitumor activity. Because MBT-2 cells do not express MHC class II molecules, CD4 T cells might recognize tumor antigen peptides presented by MHC class II molecules on dendritic cell or macrophages infiltrated in the tumor or in the draining lymph nodes and activate them to produce cytotoxic mediators such as nitrate oxides (30–32).

Although our results clearly showed an importance of CD4 T cells in the antitumor effects of nonmyeloablative allogeneic SCT, there may be some criticism on generalization of our data. In our experimental model, AKR/J mice have unusually high frequency of the alloreactive V $\beta$ 3<sup>+</sup>CD4 T cells, which comprise ~5% of CD4 T cells even in naive mice, suggesting that it is a special case. In fact, most of the expanded CD4 T cells were V $\beta$ 3<sup>+</sup> cells after DLI, which were also found in the tumor region. However,

Chakraverty and colleagues (33) also reported a requirement of CD4 T cells primed by host antigen-presenting cells (APC) for CD8 T-cell–mediated graft-versus-leukemia responses in a model using a combination of MHC disparate B10.A (H-2<sup>a</sup>) and C57BL/6 (H-2<sup>b</sup>) strains. An important feature shared by the two studies is induction of mixed chimerism in the host before DLI. It has become clear that mixed chimerism augments antitumor effects following DLI treatment more efficiently than complete chimerism (34). Because MHC class II molecules are expressed on bone marrow–derived APCs but usually not on tumor cells or other cells of host origin, it is reasonably assumed that mixed chimerism is required for activation of allo-MHC–reactive helper CD4 T cells, which efficiently induce antitumor CD8 T-cell responses.

We found that CD8 T cells of both donor and host origin were infiltrated in the tumor after DLI, especially at the late stage. Such predominance of CD8 T cells was not observed in the tumor region of the mice without DLI. To prove that these CD8 T cells in TIL were tumor specific, we tried to examine CTL assay. However, because of lack of sufficient cell number for CTL assay, we could not prove that these cells were tumor specific. Instead of TIL, we showed tumor-specific response by using spleen cells in the late stage. And it is widely accepted that TIL is basically tumor-specific lymphocytes. Taken together, we believe that the TIL in our system also has the tumor-specific activity. Importantly, we found that CD8 T cells in DLI are not essential for the antitumor effects in the early stage, which was revealed by depleting CD8 T cells in DLI, although MBT-2 cells likely express alloantigens on their MHC class I molecules. Furthermore, we detected tumor-specific T-cell response, which was still evident after depleting donor Thy1.1<sup>+</sup> cells. Even after depleting donor Thy1.1<sup>+</sup> cells, tumor-specific T-cell responses were significantly higher than those without DLI (Fig. 6B). Taken together, these results clearly showed induction of host CD8 T-cell responses against the tumor in this model of nonmyeloablative allogeneic SCT. Notably, by using the same regimen but different combination of mouse strains (BALB/c and DBA/2) and tumor (RENCA), we also found that host rather than donor T cells were essential for rejecting the tumor (22). Similar to our results, Steljes and colleagues (35) also showed induction of antitumor response that was mediated by tumor-associated antigen-specific CD8 T cells in parent into F1 models of GVHD (BALB/c or C57BL/6 to BALB/c  $\times$  C57BL/6 F1). However, these results are in contrast to other models of nonmyeloablative allogeneic SCT, in which alloantigen-specific donor CD8 T cells seem to be the major effectors of tumor eradication, which is called GVT effect (36, 37). Although we do not know the exact reason for this discrepancy, it is of note that remission is observed in some patients who lost donor chimerism after allogeneic hematopoietic cell transplantation, suggesting the induction of antitumor effector cells of recipient origin (38). These observations also support clinical relevance of our findings. However, in most human nonmyeloablative SCT model, mixed chimerism is usually transient and is followed by increasing and usually full chimerism. On the other hand, mixed chimerism gradually decreased in our model. About this point, our model is not typical and we may overemphasize the role of host-derived T cells in mediating antitumor effects. However, there is no disadvantage of induction of tumor-specific response in nonmyeloablative allogeneic SCT. One of the major obstacles of clinical application of nonmyeloablative allogeneic SCT was an association between GVH and GVT effect (39). Induction of efficient tumor-specific immune response without alloreactivity to host is the ultimate goal of nonmyeloablative

allogeneic SCT-based tumor immunotherapy. In this regard, we believe that our model has a great advantage because it induces efficient antitumor immune responses with mild GVHD (22, 40, 41). Indeed, Takahashi and colleagues recently reported that GVT effects against human kidney cancer can be associated with tumor-specific immune responses to tumor antigens but not associated with GVHD. They also emphasized the importance of tumor-specific reactions in allogeneic SCT (42).

By comparing depletion of CD4 cells with CD8 cells in DLI, donor CD4 T cells were more important than donor CD8 T cells (Fig. 4D versus Fig. 6A). Induction of cytokine storm, especially IFN- $\gamma$ , was indispensable to activate immune system in tumor-bearing host (Fig. 4C). However, in the early stage, donor CD8 T cells did not produce IFN- $\gamma$  (Fig. 4A), which might explain the reason why donor CD8 T cells were not essential for antitumor effects. However, we cannot deny the role of donor-derived tumor-specific CD8 T cells. In the late stage, the level of tumor-specific IFN- $\gamma$  was significantly higher in the spleen cells of the mice with DLI than without DLI. And spleen cells of the mice with DLI still produced high IFN- $\gamma$  even after depletion of Thy1.1<sup>+</sup> spleen cells. This result indicated that host CD8 T cells were activated by DLI treatment, and these activated cells contributed to tumor-specific antitumor effects. In other words, tumor-specific host CD8 T cells, which originally existed in host before DLI treatment, were considered to be activated by cytokine storm induced by donor CD4 T cells. This fact revealed a cross-talk between alloreactive donor CD4 T cells and tumor-specific host CD8 T cells in antitumor activity of nonmyeloablative allogeneic SCT. Of course, although data of Fig. 6B cannot completely deny the role of tumor-specific CD4 T cells in the late stage, predominant CD8 T cells in TIL (Fig. 5B) support the important role of tumor-specific host CD8 T cells at this timing. We tried to deplete host-derived CD8 T cells in the late stage. How-

ever, as far as we were able to search, there were no CD8 knockout (KO) mouse of C3H/HeN origin and it was impossible to distinguish host CD8 T cells from donor CD8 T cells by using anti-CD8 depletion antibody. In the future, to prove directly that host CD8 T cells are essential for antitumor effects in our model, it is necessary to use different combination of murine strains that have CD8 KO mice and syngeneic tumor model.

In summary, this study showed involvements of host-reactive donor CD4 T cells and tumor-reactive host CD8 T cells in the antitumor effects of nonmyeloablative allogeneic SCT in a murine bladder tumor model. Furthermore, although we could not deny the role of donor-derived tumor-specific CD8 T cells, donor CD8 T cells were not essential for the antitumor effects in the early stage. It is important to further clarify the antitumor mechanisms as well as therapeutic potentials to various malignancies, which will develop more effective and safer strategy of nonmyeloablative allogeneic SCT for clinical application.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Donor CD4 T Cells Are Critical in Allogeneic Stem Cell Transplantation against Murine Solid Tumor

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