Antitumor Activity Mediated by Double-Negative T Cells

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ABSTRACT

Allogeneic lymphocytes are potent mediators of leukemia and lymphoma remission. The goal of this study was to determine whether single MHC class I locus-mismatched lymphocytes could generate an antilymphoma activity in the absence of graft-versus-host-disease (GVHD) and to understand the underlying mechanisms. Immunoincompetent Scid or lethally irradiated mice were challenged i.v. with a lethal dose of A20 lymphoma cells together with an infusion of single MHC class I locus mismatched splenocytes. Mice that were challenged with A20 cells alone succumbed to lymphoma between 34 and 50 days after infusion. In contrast, >75% of mice that were coinfused with single class I MHC locus mismatched splenocytes survived indefinitely (n = 20) in the absence of GVHD. Interestingly, the number of CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> double-negative (DN) T cells increased 15-fold in mice that did not develop lymphoma. Both DN T cells isolated from the spleens of lymphoma-free mice and DN T cells cloned from naive mice were cytotoxic to A20 lymphoma cells in vitro. When DN T cell clones were infused into naive mice i.v. together with A20 lymphoma cells, 86% of recipient mice were protected from lymphoma onset and did not develop GVHD (n = 22). To assess whether the systemic injection of DN T cells can also suppress local tumor development, A20 cells were infused i.m., and at the same time DN T cell clones were infused either i.v. or i.m. Results indicated that DN T cells infused systemically (i.v.) could not prevent local tumor outgrowth, but DN T cells coinfused locally (i.m.) prevented local tumor development in 91% of animals (n = 11). Furthermore, we demonstrate that primary DN T cells were also able to prevent tumor growth in 75% of mice when infused together with A20 cells i.m. (n = 12). Together, these results demonstrate that an antilymphoma activity can be generated in mice without causing GVHD. Furthermore, DN T cells can suppress lymphoma cells in vivo and in vitro, suggesting that DN T cells could be used as a novel strategy for the treatment of lymphoma.

INTRODUCTION

The transplantation of stem cells after high-dose chemotherapy and irradiation is an effective treatment for many hematological malignancies (1). The success of this therapy was historically attributed to the conditioning regimen that was able to eliminate tumor cells. However, several studies recognized that antihost reactivity by transplanted grafts was associated with a decreased relapse of leukemia and increased survival (2–4). Moreover, this effect was not apparent in patients given T cell-depleted allografts (4–7) or patients who were given syngeneic bone marrow grafts (4). These early findings lead to the now widely accepted view that donor T cells can be induced to kill malignant cells, which is known as the GVL<sup>3</sup> effect.

Although donor lymphocytes are beneficial in preventing lymphoma relapse, their usefulness is hampered by their ability to respond to MHC and minor histocompatibility antigens on host cells and destroy host tissue. This effect, which is known as GVHD, represents a major factor responsible for the morbidity and mortality of allogeneic stem cell transplant recipients (8). To reduce the severity of GVHD, donor lymphocytes are often matched for MHC antigens with recipients, and recipients are given nonspecific immunosuppression. Although these approaches decrease the incidence and severity of GVHD, they are also associated with an increased leukemia/lymphoma relapse (9–11).

Several strategies have been developed to induce GVL in the absence of GVHD. Less toxic nonmyeloablative stem cell transplants (12, 13) and donor lymphocyte infusions (14) have been successfully used in patients with malignancies, and the adoptive transfer of lymphocytes mismatched with the host for minor histocompatibility antigens has been successful in eradicating tumors in mice (15). Additionally, accumulating evidence indicates that Treg lymphocytes play an important role in the down-regulation of immune responses to self or allogeneic antigens (16–25). Several groups have shown that Treg cells may be involved in suppressing GVHD (26–32), opening the door to a new strategy for treating or preventing GVHD. However, Treg cells have also been shown to inhibit antitumor cytotoxic T-lymphocyte responses (33–35).

We have identified recently a novel CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> DN T-cell population from recipients who have permanently accepted donor skin allografts. This population of DN T cells differs from other T cells in their surface marker expression, cytokine profile, and mechanism of suppression (16), and can specifically suppress CD8<sup>+</sup> and CD4<sup>+</sup> T cells that are primed against the same alloantigen (16, 36, 37). Moreover, we have shown that DN T cells can attenuate GVHD caused by an infusion of single class I mismatched lymphocytes (32). The goal of this study was to determine whether GVL activity could also be established in mice that were resistant to GVHD. We show that the infusion of single class I locus mismatched lymphocytes into immunodeficient mice leads to an antilymphoma activity in the absence of GVHD. DN T cells increase in the periphery of recipient mice and are able to directly kill lymphoma cells in vitro. Furthermore, the infusion of DN T cells is able to prevent both systemic and local lymphoma outgrowth. Together, these data indicate the possibility of using DN T cells as a novel cellular therapy for tumors.

MATERIALS AND METHODS

Mice. C57BL/6 (B6), (B6xBALB/c)<sub>7</sub>, BALB/c H-2-dm2 (dm2), and B6MRL-Fas<sup>−/−</sup> lymphoproliferative (lpv) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Breeding stocks of 2C and H-Y transgenic mice were kindly provided by Drs. Dennis Y. Loh (Howard Hughes Medical Institute, St. Louis, MO; 38) and Hung-Sia Teh (University of British Columbia, Vancouver, British Columbia, Canada), respectively. 2C transgenic mice (on B6 background) carry functionally rearranged TCR α- and β-chain transgenes, which are specific for class I MHC L<sup>4</sup> (38) and are recognized by the mAb IB2 (39). 2C transgenic mice were bred with dm2 mice (a BALB/c L<sup>d</sup> loss mutant). The offspring, either (2Cxdm2)<sub>1</sub> mice (H-2<sup>dm2</sup>, L<sup>d</sup>, 1B2<sup>−</sup>) or (B6xdm2)<sub>1</sub> mice (H-2<sup>dm2</sup>, L<sup>L</sup>, 1B2<sup>−</sup>), were used as lymphocyte donors. C.B-17 Scid mice (effectively BALB/c congenic to B6 at the IgH locus; H-2<sup>d</sup>) were bred with B6 Scid mice. The resulting Scid/B6 mice (effectively BALB/c congenic to B6 at the IgH locus; H-2<sup>d</sup>) were sublethally irradiated (2.5 Gy) and used as recipients. Lethally

Received 5/27/03; revised 7/30/03; accepted 7/31/03.

Grant support: National Cancer Institutes of Canada 12160 (L.Z.) and Canadian Institutes of Health Research MT14431 (L.Z.).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: GVL, graft-versus-leukemia/lymphoma; GVHD, graft-versus-host-disease; Treg, regulatory T; DN, double-negative; TCR, T-cell receptor; mAb, monoclonal antibody; Scid, severe combined immunodeficient; IL, interleukin; NK, natural killer; Fasl, Fas ligand.
Irradiated (8.5 Gy; B6xBALB/c)F1 mice (H-2<sup>md</sup>, L<sup>d</sup>) were also used as recipients.

**Generation and Maintenance of 1B2<sup>+</sup>DN T-Cell Clones.** Spleen cells were collected from naïve (2Cxdm2)<sub>pr</sub> mice, and used to generate T-cell clones using standard cloning and subcloning procedures as described elsewhere (16). We have demonstrated previously that IL-2 and IL-4 are required for DN T-cell survival and function (16, 40). Therefore, to maintain the DN T-cell clones, 5 × 10<sup>5</sup> cells were cultured in a 24-well plate containing 5 × 10<sup>8</sup> irradiated L<sup>d</sup><sup>+</sup> cells in an α-MEM supplemented with 10% FCS and 30 IU/ml recombinant IL-2 and 30 IU/ml recombinant IL-4. The cells were incubated at 37°C with 5% CO<sub>2</sub>. The T-cell clones were stimulated in the above manner every 3–4 days.

**Infusion of Allogeneic Lymphocytes and Tumor Cells.** Single viable cell suspensions were prepared from the spleen and lymph nodes of donor mice, and injected into sex-matched recipient mice via the tail vein (4 × 10<sup>7</sup> cells/mouse), DN T-cell clones were injected either i.v. (5 × 10<sup>5</sup> cells/mouse) or i.m. (1 × 10<sup>7</sup> cells/mouse). The A20 B-cell lymphoma line (BALB/c origin; Ref. 41) was obtained from American Type Culture Collectino (Manassas, VA), and 10<sup>5</sup> A20 cells were injected either i.v. or i.m. per mouse.

**Evaluation of GVHD and Tumor Burden.** Clinical signs of GVHD (42) such as weight loss, diarrhea, ruffled fur, hunched posture, and scaled ears were monitored several times weekly. In addition, tissue samples from major GVHD lesion sites (liver, skin, and intestine) were harvested, stained with H&E, and evaluated by light microscopy for GVHD according to standard methods (42). For evaluation of i.v.-injected tumor burden, major organs such as liver, spleen, lung, heart, kidney, intestine, and lymph nodes were examined for the presence of tumor cell infiltration. Morbidity was also monitored daily. For evaluation of i.m.-injected tumor, mice were monitored daily and graded from levels 1–4 based on tumor size, level 4 being the largest and causing impairment at which point the animal was sacrificed.

**Cell Surface Marker Staining.** Spleen cells were collected at various time points after reconstitution and triple stained with fluorescence-conjugated mAbs specifically recognizing the αβ-TCR (1B2), CD4, and CD8. In some mice staining was also performed using mAbs that recognized CD3, NK1.1, and Fas. All of the mAbs except 1B2 were purchased from BD PharMingen. Data were acquired and analyzed on an EPICS XL-MCL flow cytometer.

**Cytotoxicity Assays.** Purified DN T cells from lpr mice were stimulated with irradiated (B6xBALB/c)F1 splenocytes for 2–3 days in the presence of 30 units/ml IL-2 and 30 units/ml IL-4 before their use. Murine DN T cells were purified from the spleens of reconstituted mice by fluorescence activated cell sorting as described previously (16). Viable cells were harvested and seeded at various dilutions into 96-well plates and used as effector cells. Tumor target cells were labeled with 10 μCi/ml of [<sup>3</sup>H]thymidine at 37°C for 18 h and cultured for another 18 h in the presence of IL-2 and IL-4 either together or in the absence of DN T cells. Cytotoxicity was measured using a liquid scintillation counter as described previously (16). Specific cell killing was calculated using the equation: % specific killing = (S - E)/S × 100, where E (experimental) is cpm of retained DNA in the presence of effector cells and S (spontaneous) is cpm of retained DNA in the absence of effector cells.

**Statistical Analyses.** All of the statistical analyses were performed using Student’s t test and were calculated using Microsoft Excel XP. Error bars represent SD.

**RESULTS**

**Infusion of Transgenic Anti-L<sup>d</sup> Splenocytes Protects Immuno-deficient Mice from Developing Lymphoma Without Causing GVHD.** We have demonstrated previously that infusion of MHC class I L<sup>d</sup>-mismatched (2Cxdm2)<sub>pr</sub> splenocytes into Scid<sub>pr</sub> mice leads to an unresponsiveness of anti-L<sup>d</sup> CD8<sup>+</sup> T cells in the host (43, 44) and GVHD-free survival of recipient animals (32). To investigate whether the infused allogeneic lymphocytes could kill tumor cells, Scid<sub>pr</sub> mice were sublethally irradiated and infused i.v. with 4 × 10<sup>6</sup> viable spleen cells from L<sup>d</sup>-mismatched (2Cxdm2)<sub>pr</sub> mice, together with 10<sup>5</sup> A20 lymphoma cells, which were syngeneic to the host and mismatched at the L<sup>d</sup> locus with donor lymphocytes. As controls, Scid<sub>pr</sub> mice were infused with A20 lymphoma cells alone. All of the Scid<sub>pr</sub> mice that were infused with tumor cells alone developed lymphoma and were sacrificed between 28 and 42 days (median survival time 32 days; n = 11; Fig. 1A). Autopsies and histopathologic studies confirmed that lymphoma was the cause of death (Fig. 1B, middle). In striking contrast, 11 of 12 mice that were infused with (2Cxdm2)<sub>pr</sub> splenocytes and a lethal dose of A20 lymphoma cells remained healthy during the course of the study (>150 days; Fig. 1A). Neither tumor infiltration nor GVHD was found in any organ of these animals (Fig. 1B, bottom). These data demonstrate that infusion of
1.9% of the total splenocytes were NK1.1+/H11001.

Both T cells and NK cells have been shown to have an antitumor activity, we first analyzed the proportions of T cells and NK cells in recipient mice. Splenocytes were collected from recipient mice 60 days after reconstitution and stained for either NK1.1 or CD3. As shown in Fig. 3, the proportions of T cells and NK cells in recipient mice. Splenocytes into Naïve Mice.

To investigate whether DN T cells have an antitumor activity, we first studied whether mice that did not develop lymphoma after an initial coinjection of Ld-mismatched donor lymphocytes and A20 lymphoma cells splenocytes mismatched for a single class I locus antigen does not result in GVHD but can protect immunodeficient recipients from dying of lymphoma.

An Antilymphoma Activity Is Established in the Absence of GVHD After the Infusion of Nontransgenic Ld Mismatched Splenocytes into Naïve Mice. The above observations are based on a transgenic-Scid model and, thus, may not be representative of true pathophysiological responses. To validate the findings in a nontransgenic model, (B6xBALB/c)F1 mice were lethally irradiated, and infused with a lethal dose of A20 lymphoma cells and 4 × 10⁷ viable spleen cells from (B6xdm2)F1 mice. As a control, a group of mice were infused with A20 lymphoma alone. All of the control mice succumbed to lymphoma within 50 days of injection of A20 cells. In contrast, 6 of the 8 (B6xBALB/c)F1 mice that were infused with both (B6xdm2)F1 spleen cells and A20 tumor cells remained free from both GVHD and tumor invasion for >150 days (Fig. 2). The remaining 2 mice were sacrificed at the indicated periods of time, and histopathological analyses revealed that the mice died of lymphoma and had no signs of GVHD (data not shown). These results confirm the findings in the transgenic-ScidF1 model, and demonstrate that infusion of MHC class I Ld-mismatched lymphocytes allow immune-incompetent animals to reject tumors without causing GVHD.

DN T Cells Increase in Immunodeficient Mice After Reconstitution. Both T cells and NK cells have been shown to have an antilymphoma activity. To identify the type of cells that might be involved in the observed antilymphoma activity, we first analyzed the proportions of T cells and NK cells in recipient mice. Splenocytes were collected from recipient mice 60 days after reconstitution and stained for either NK1.1 or CD3. As shown in Fig. 3, A and B, only 1.9% of the total splenocytes were NK1.1⁺, whereas 22.7% were CD3⁺. Although not completely eliminating the possibility that NK cells are involved in preventing tumor onset, these results led us to additionally study the potential role of T cells in the prevention of lymphoma. The fate of anti-Ld 1B2⁺ T lymphocytes was monitored after the infusion of sublethally irradiated ScidF1 mice with (2Cxdm2)F1 lymphocytes and A20 lymphoma cells (Fig. 3C). Consistent with previous reports (43, 45), a vigorous expansion followed by a massive depletion of antihost 1B2⁺CD8⁺ T cells was observed in the spleen of recipients. 1B2⁺CD4⁺ T cells also increased in the first 35 days after infusion of donor lymphocytes but remained relatively unchanged thereafter. Interestingly, we observed a steady increase in the number of donor-derived 1B2⁺DN T cells in these recipient mice, which became the dominant population of T cells over time (Fig. 3C). A similar change was also observed in sublethally irradiated ScidF1 mice that had been given an infusion of Ld-mismatched lymphocytes alone (32), although the increase observed here was greater. These results support the possibility that DN T cells might be involved in the prevention of lymphoma onset.

DN Treg Cells Possess an Antilymphoma Activity. To investigate whether DN T cells have an antitumor activity, we first studied whether mice that did not develop lymphoma after an initial coinjection of Ld-mismatched donor lymphocytes and A20 lymphoma cells...
weeks after coinjection with (2Cxdm2)F1 and A20 cells, and assessed for their ability to kill A20 lymphoma cells in vitro. As shown in Fig. 4B, purified 1B2⁺ DN T cells were able to lyse allogeneic A20 tumor cells in a dose-dependent manner but could not kill syngeneic EL4 tumor targets. To assess whether nontransgenic DN T cells were also able to kill A20 cells, DN T cells were purified from (B6xBALB/c)F1 mice 8 weeks after infusion of (B6xdm2)F1 splenocytes and A20 lymphoma cells. As shown in Fig. 4C, purified nontransgenic DN T cells were also able to kill A20 lymphoma cells in a dose-dependent manner. Together, these data indicate that in addition to suppressing syngeneic antihost T cells (32), both transgenic and nontransgenic DN T cells from reconstituted mice are capable of killing allogeneic A20 tumor cells in vitro.

**DN T Cell-Mediated Cytotoxicity to Lymphoma Cells Requires Fas/FasL Interactions.** We have demonstrated previously that DN T-cell clones are able to kill activated syngeneic CD8⁺ T cells through a Fas/FasL-mediated pathway (16). To gain an insight into the mechanism involved in DN T cell-mediated killing of allogeneic lymphoma cells, we evaluated the role of Fas/FasL in the cytototoxicity to tumor cells. DN T-cell clones generated from (2Cxdm2)F1 mice (16) were used as effector cells. Tumor cell lines that are Ld⁺ (Fig. 5A, left) and either Fas high (A20; Fig. 5A, right) or Fas low (B16; Fig. 5A, right) were used as targets in a killing assay. Fas high tumor cell lines were killed by DN T cell clones in a dose-dependent manner, whereas killing of Fas low tumor targets was much less efficient (Fig. 5B), suggesting that Fas/L interactions are involved for DN T cell-mediated cytototoxicity of tumor cells. To additionally test the role of Fas/L interactions in DN T cell-mediated killing of tumor cells, FasL was blocked on DN T cells using Fas-Fc Fusion protein. As shown in Fig. 5C, Fas-Fc partially inhibited the killing of A20 lymphoma cells mediated by DN Treg clones. These findings indicate that Fas/L interactions are important for DN T-cell-mediated cytototoxicity to A20 lymphoma cells.

**DN T-Cell Clones Can Prevent Systemic Lymphoma Outgrowth.** To determine the antilymphoma activity of DN T cells in vivo and to study the therapeutic potential of using in vitro generated DN T cells for the treatment of lymphoma, nonirradiated (B6xBALB/c)F1 mice were coinjected with various doses of 1B2⁺ DN T-cell clones and a lethal dose of A20 tumor cells. All of the mice that received A20 cells alone died of lymphoma between 30 and 40 days after injection (Fig. 6A; Fig. 6B, top). Interestingly, infusion of 5 x 10⁶ DN T-cell clones with A20 lymphoma cells prevented ≥80% of the mice from developing lymphoma. These mice lived for >150 days (Fig. 6A) with no detectable clinical or histopathological signs of lymphoma or GVHD (Fig. 6B, bottom). The infusion of 10 x 10⁶ DN T cells similarly protected mice from lymphoma and did not cause GVHD (data not shown). These data demonstrate that in vitro generated DN T cells can prevent the onset of lymphoma and suggest that DN Treg cells may be used as a novel cellular therapy for lymphoma.

**DN T Cell Clones Can Prevent the Outgrowth of Solid Tumor.** To determine whether systemically injected DN T cells are also able to prevent solid tumor outgrowth, (B6xBALB/c)F1 mice were injected i.v. with DN T-cell clones and at the same time given an i.m. injection of A20 lymphoma cells into their right hind leg. As controls, mice were given an i.m. injection of A20 lymphoma cells with no other treatment. Eight of 9 mice that received A20 lymphoma alone developed large tumors in their hind legs within 30 days and were sacrificed when their tumors reached a level 4 (Fig. 7A). Similarly, 75% of the animals that were infused with A20 i.m. together with DN T-cell clones injected i.v. developed lesions within 30 days (Fig. 7A). Together, these results indicate that systemically infused DN T cells are unable to prevent local solid tumor outgrowth.

The inability of DN T cells to kill local tumor cells could be
explained by DN T cells being unable to migrate to this tissue or exert their effect in the muscle. To test these possibilities, DN T cells were coinfected with lymphoma cells i.m., and recipient mice were monitored for tumor growth. As seen previously, 89% of control mice developed tumor lesions in their hind legs within 30 days of tumor infusion. Interestingly, when DN T-cell clones were coinfused with A20 tumor cells, tumor outgrowth was prevented in 91% of recipient animals (Fig. 7B). These results indicate that DN T-cell clones are able to prevent the outgrowth of tumor locally when infused directly into the muscle and demonstrate that DN T cells are able to exert their effect locally.

Primary DN T Cells Have an Antilymphoma Activity. DN T cells are difficult to isolate from naïve nontransgenic mice because of their low numbers and the lack of specific markers on DN T cells. We have demonstrated previously that DN T cells from lpr mice are able to suppress syngeneic CD8⁺ T cells as well as enhance skin graft survival (37). Therefore, these mice provide a source of primary DN T cells, which can be used to verify our findings using DN T clones. To ensure that DN T cells from lpr mice are not NK T cells, splenocytes from lpr mice were stained using CD3, CD4, CD8, and NK1.1. Fig. 8A shows that the vast majority of DN T cells are NK1.1⁺ (66.2% versus 0.49%). DN T cells were subsequently isolated as described in “Materials and Methods,” and were tested for their ability to kill A20 lymphoma cells. Fig. 8B shows that lpr DN T cells were able to dose-dependently kill A20 tumor cells in vitro. Next, (B6xBALB/c)F₁ mice were infused i.m. with A20 tumor cells together or in the absence of 1 × 10⁶ lpr DN T cells. All of the mice that received A20 lymphoma cells alone developed lesions within 30 days and were sacrificed. In contrast, 75% of mice that were coinfused with

Fig. 5. Fas/FasL interactions are required for DN T cell mediated cytotoxicity. A, A20 (gray) and EL4 (white) tumor cell lines were stained with biotinylated anti-Ld mAb followed by streptavidin-FITC (left), or anti-Fas phycoerythrin-conjugated mAb (right). Negative controls are shown in ——. B, varying numbers of 1B2 DN T cell clones were used as effector cells in cytotoxicity assays as described previously (68). A20 (●), A20-Ld/Fasnerg, and P815 (●, Ld/Faslow) tumor cells were used as targets. The data show the mean percentage of specific killing of 3 replicates and are representative of three independent experiments; bars, ±SD. C, 1B2 DN T-cell clones were preincubated with various concentrations of Fas-Fc fusion protein before coincubation with A20 tumor cells or left untreated as controls. The percentage of inhibition of killing of tumor cells by Fas-Fc-treated DN T cells compared with controls is shown. The experiment was repeated two times each with three replicates, and similar results were obtained. Bars, ±SD.

Fig. 6. DN T-cell clones prevent systemic lymphoma outgrowth. (A; B6xBALB/c) mice were infused with either a lethal dose of A20 cells alone (▼, n = 10), or together with either L12.2 (●, n = 8) or CN04 (▲, n = 14) DN T-cell clones. Survival and clinical signs of GVHD were monitored for >150 days. B, mice injected with A20 alone showed dense tumor infiltrates in the liver at 30 days (B, top). Mice that were coinfected with DN T-cell clones had normal liver structure with no infiltrating tumor cells or signs of GVHD at 100 days (B, bottom).
lpr DN T cells showed no signs of tumor growth and survived tumor free indefinitely (Fig. 8C). Together, these results verify our finding with transgenic DN T cell clones and additionally demonstrate that DN T cells have an antilymphoma activity in vivo.

**DISCUSSION**

It has been well established that allogeneic lymphocytes play a role in the eradication of leukemia and lymphoma. Here we demonstrate that the reconstitution of immunoincompetent mice with single class I mismatched lymphocytes leads to tumor- and GVHD-free survival of recipients. We have shown that DN T cells expand in the periphery of reconstituted mice and form the majority of anti-Ld lymphocytes. These cells are able to eliminate allogeneic tumor cells in vitro through a mechanism that involves Fas/FasL. In addition, DN T cells that were cloned from (2C|xdm2)F1 mice or purified from lpr mice have a similar ability to eliminate lymphoma cells in vitro and are also able to prevent the outgrowth of both systemic and solid tumor in vivo.

These data indicate that the infusion of DN T cells are sufficient to prevent lymphoma onset. Whereas these data do not preclude a role for other lymphocytes, such as NK or CD4+ T cells, in mediating GVL in reconstituted animals, they indicate the importance of DN T cells in mediating GVL and their potential use as a novel cellular antitumor therapy.

Although allogeneic lymphocytes are an effective treatment for eliminating cancer cells, this therapy is limited by the risk of developing GVHD (46). For example, a recent study demonstrated that more than half of patients with chronic myeloid leukemia, who were given a nonmyeloablative stem cell transplant, developed acute GVHD despite being given immunosuppressive drugs (13). Also, in a study conducted by Childs et al. (12), nonmyeloablative stem cell transplants successfully induced tumor regression only in those patients that developed GVHD. Furthermore, patients who did not develop GVHD also did not develop an antitumor effect (12). These data demonstrate that despite modern immunotherapeutic regimens, GVHD remains one of the largest barriers to successful treatment of cancer using allogeneic lymphocytes.

Several recent studies have been designed to assess the possibility

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**Fig. 7.** Local tumor outgrowth can be prevented by locally injected DN T cells. **A,** (B6xBALB/c)x1 mice were i.m. injected with 10^5 A20 tumor cells alone (■, n = 9) or along with i.v. injection of 5 × 10^4 CN04 DN T-cell clone (n = 4). **B,** (B6xBALB/c)x1 mice were injected i.m. with 10^5 A20 tumor cells either alone (■, n = 10) or together with 1 × 10^5 CN04 DN T-cell clone (▲, n = 10). Mice were monitored and sacrificed when the tumor appearance was a level 4, as described in "Materials and Methods.”

**Fig. 8.** Primary DN T cells are NK1.1 and can prevent tumor outgrowth. **A,** LN and spleen cells were isolated from lpr mice and stained with anti-CD4/8 FITC, anti-NK1.1 PhycoErythrin, and anti-CD3 CyChrome. The histogram is gated on the CD3+ T-cell population, and the percentages of the CD4+CD8+ and DN T cells are explicitly indicated. **B,** DN T cells were purified from the lymph nodes and spleen of lpr mice as described in "Materials and Methods.” and stimulated with irradiated (B6xBALB/c)x1 splenocytes for 4 days. The proportion of DN T cells was then determined by flow cytometry and used as effector cells in various dilutions as indicated. A20 tumor cells were labeled overnight with 10 μCi of [3 H]thymidine, and used as target cells. The data shown is the percentage of specific killing for three replicates, and the experiment is representative of two independent experiments. **C,** (B6xBALB/c)x1 mice were injected i.m. with A20 tumor cell either alone (■, n = 10) or together with 1 × 10^5 purified lpr DN T-cell clones (▲, n = 12). Tumor growth and recipient survival were monitored.
of infusing lymphocytes with an antitumor activity into patients without causing GVHD, especially as part of a nonmyeloablative regimen (12, 25, 47). One such approach involved the insertion of a thymidine kinase suicide gene into allogeneic lymphocytes so that infused lymphocytes could be eliminated at the onset of GVHD (48). Whereas thymidine kinase was successful in controlling GVHD, less than half of the patients had a complete remission. Other groups have attempted to induce T-cell responses against tumor-associated antigens (49) such as MART-1, gp100, and tyrosinase on melanoma cells (50). However, many of these tumor-associated antigens are also expressed on normal melanocytes, resulting in autoimmunity and degeneration (51). Autoimmunity can be avoided by using low avidity T cells (52, 53); however, successful tumor rejection will only be achieved if the level of self-antigen expression on the tumor is higher than that expressed on the healthy tissues. Hence a dichotomy exists between successfully eradicating tumor cells, and avoiding GVHD and autoimmune diseases. We demonstrate here that although DN T cells are allogeneic to the host, they do not cause GVHD or autoimmune disease in recipient mice. Most of the recipient mice that have been injected with either DN T cells or clones survived indefinitely, and none showed signs of GVHD. Moreover, almost all CD4+ and CD8+ T cells, which are susceptible to activation-induced cell death (54), DN Treg cells are resistant to TCR cross-linking-induced apoptosis in vitro (40) and activation-induced cell death in vivo (Fig. 3C). Whether DN T cells can also mediate a GVL activity in humans without causing GVHD is currently under study, and the results derived from these studies will determine the efficacy of using DN T cells as an antitumor therapy.

Both DN T cells (32) and CD4+CD25+ Treg cells (27, 28) have been shown to suppress GVHD. For example, DN T cells have been shown to attenuate GVHD caused by the infusion of allogeneic CD8+ T cells (32), and CD4+CD25+ Treg cells suppress acute GVHD after the infusion of allogeneic CD4+CD25- T cells (27, 28). However, there is evidence that CD4+CD25- Treg cells additionally inhibit antitumor T-cell responses and could ultimately contribute to the progression of cancer (33–35, 55–58). For example, it has been shown recently that autologous antitumor T cells are inhibited by CD4+CD25+ Treg cells in both mouse and human models (33–35, 55). Large numbers of CD4+CD25+ Treg cells have also been shown to infiltrate the tumor microenvironment (34, 55, 59) and have been found in patients immunized with known melanoma antigens (56). Furthermore, tumors were efficiently rejected by the host immune system in mice that were depleted of CD4+CD25+ Treg cells using antiCD25 mAb (35, 57, 58). In contrast to CD4+CD25+ Treg cells, which inhibit antitumor immune responses, we show here that DN T cells augment the antitumor immune responses.

The dual role of DN T cells to inhibit GVHD and promote GVL can be explained as follows. DN T cells are activated after their infusion into Ld+ recipient mice (60). After activation, DN T cells expand and become the major subset of T cells (Fig. 3C). These cells are then able to acquire allo-MHC molecules from antigen-presenting cells, and present these molecules on their surface. Antithost T cells with the same TCR specificity as DN T cells can recognize the acquired MHC molecules on the DN T-cell surface and are subsequently killed by the DN T cells through Fas/FasL interaction (32). Hence, GVHD is prevented. On the other hand, DN T cells can also kill Ld+ A20 tumor cells through the Fas/FasL pathway (Fig. 4, B and C: Fig. 5C), therefore mediating an antilymphoma response. The finding that Fas-Fc fusion protein does not completely block DN T cell-mediated killing indicates that there may be other pathways involved in the elimination of tumors by DN T cells. However, it is not clear why host tissues, which express both Ld and Fas, are not damaged by DN T cells. Several studies have shown that although endothelial cells express Fas they are normally resistant to Fas-induced apoptosis (61, 62). Additionally, evidence suggests that IFN-γ can cause T-cell immunodeficiency (63) and protect mice from GVHD (64–67) by indirectly inhibiting donor T-cell activation and expansion (64). We found that both in vitro and in vivo activated DN Treg cells produce large amounts of IFN-γ (16; data not shown), and may therefore protect hosts from tissue damage. In conclusion, we demonstrate here that DN T cells have a novel antitumor function both in vitro and in vivo. This potent antitumor activity makes DN T cells a prospective candidate for cellular immune therapy.

REFERENCES


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