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Graft-versus-host disease can be separated from graft-versus-lymphoma effects by control of lymphocyte trafficking with FTY720

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Graft-versus-host disease (GvHD) mediated by donor T cells recognizing host alloantigens is associated with beneficial graft-versus-tumor effects in recipients of allogeneic hematopoietic cell transplants. Since leukemias and lymphomas reside largely within the lymphohematopoietic system, we have proposed that the desired graft-versus-leukemia or graft-versus-lymphoma effect can be separated from the complication of GvHD by confinement of the graft-versus-host alloresponse to the lymphohematopoietic tissues. Since the new sphingosine-1-phosphate receptor agonist immunosuppressive drug FTY720 leads to trapping of T cells in secondary lymphoid tissues, we evaluated the possibility that this drug could diminish GvHD, a disease involving epithelial target tissues, while permitting a beneficial alloresponse to take place within the lymphohematopoietic system, leading to graft-versus-lymphoma effects. We demonstrate here that FTY720 markedly reduces GvHD in a clinically relevant, haploidentical strain combination, while permitting antitumor effects against a T cell lymphoma of unshared host MHC haplotype to proceed unhindered. These results establish a potential new immunotherapeutic approach to separating graft-versus-leukemia effects from GvHD.

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Introduction

Allogeneic hematopoietic cell transplantation (HCT) is often the only potentially curative therapy for hematological malignancies and other diseases (1, 2). A major obstacle impeding the success and wider application of HCT is graft-versus-host disease (GvHD) (3). Significant acute GvHD (grades II–IV) occurs in 25–60% of HLA-identical related-donor HCT and in 45–70% of matched unrelated transplant recipients (4–6). The incidence is unacceptably high when major HLA barriers are traversed (7). Consequently, approximately 50% of patients who might benefit from HCT do not have this therapeutic option, due to the unavailability of an appropriately

matched related or unrelated donor. The prophylactic use of immunosuppressive drugs such as cyclosporine A, prednisone, and methotrexate (3, 8–13) has not successfully eliminated GvHD, even in HLA-identical allogeneic HCT (14, 15). One of the most effective approaches to GvHD prophylaxis, T cell depletion of the donor inoculum, is associated with increased risks of graft failure and relapse (6, 8, 16, 17). Experimental (18, 19) and clinical (20–22) data indicate that stronger graft-versus-leukemia/lymphoma (GvL) effects are obtained in the presence of the stronger graft-versus-host (GvH) alloreactivity associated with extensive MHC mismatches. Most individuals have haploidentical relatives who could potentially serve as HCT donors. Thus, efforts have been made to develop a safe approach to performing haploidentical HCT (23–25). However, the success of these approaches has been limited by GvHD and, when this is prevented by T cell depletion of the donor inoculum, by delayed immune reconstitution.

We have proposed that confining the GvH alloresponse to the lymphohematopoietic system could provide an approach to separating GvHD and GvL across MHC barriers. This suggestion was based on our observations of an association between delayed administration of donor lymphocyte infusions to established mixed chimeras and a GvH alloresponse that converts mixed chimeras to full chimeras (26, 27) and mediates GvL effects (28) without causing GvHD.

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Nonstandard abbreviations used: hematopoietic cell transplantation (HCT); graft versus host (GvH); graft-versus-host disease (GvHD); graft versus leukemia/lymphoma (GvL); donor leukocyte infusion (DLI); bone marrow transplant (BMT); bone marrow cell (BMC); white blood cell (WBC); biotin (Bio); spleen cell (SPC); median survival time (MST); cytotoxic T lymphocyte (CTL).

We have proposed that this confinement of the GvH alloresponse to the lymphohematopoietic system reflects the recovery from conditioning-induced inflammation by the time of donor lymphocyte infusion (DLI), and that such inflammation in the epithelial GvHD target tissues plays an important role in inducing GvH-reactive T cells to leave the lymphohematopoietic system and traffic to these target tissues (27). We have initiated clinical trials based on this concept and have indeed observed potent GvL effects in patients receiving DLI following induction of mixed chimerism. This has been achieved with nonmyeloablative conditioning that includes initial T cell depletion of the donor inoculum in vivo in order to minimize the GvH reaction at the time when conditioning-induced inflammation is present (29, 30). Thus, mixed chimerism is induced with a regimen that includes donor T cell depletion, and immunotherapy is given in the form of DLI several weeks or months later. A limitation of this approach is that, while the subsiding of conditioning-induced inflammation depends on the delay before administration of DLI as immunotherapy, many hematologic malignancies are rapidly progressive, making this delay undesirable. Thus, it would be desirable to develop an approach to confining the GvH alloresponse to the lymphohematopoietic system even in the presence of such inflammation, so that optimal GvL effects could be obtained immediately following HCT.

FTY720 (2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol hydrochloride) (31), hereafter referred to as FTY, is a novel immunosuppressive drug that is a metabolite of the ascomycete *Isaria sinclairii* and has shown promise alone or in combination therapy in various rodent, canine, and primate organ transplantation and autoimmune disease models (32–38). It has also been tested and has been well tolerated in human phase I and phase II trials (39) as chronic immunosuppressive therapy. FTY has also shown GvHD-inhibitory activity in a rat model in which spleen cells were transplanted from a parental strain into F₁ rats treated with 200 mg/kg cyclophosphamide (40) and in a rat small-bowel transplant model (41).

Recent findings suggest that FTY serves as a sphingosine-1-phosphate receptor agonist that acts on T cell G protein-coupled receptors (42, 43) to influence the migration of lymphocytes, resulting in trapping in the secondary lymphoid organs, diminished organ infiltration, and prevention of allograft rejection (33, 38, 44). We hypothesized that FTY could diminish GvHD-associated tissue infiltration while permitting antitumor effects to proceed unhindered within the lymphohematopoietic system, thus providing an approach to influencing lymphocyte trafficking so that GvH and GvL can be separated, even in the presence of conditioning-induced inflammation. We used a clinically relevant, haplotype-mismatched bone marrow transplant (BMT) model and a T cell lymphoma to demonstrate that this is in fact the case.

Methods

Animals. Female and male B6D2F1 (C57BL/6 × DBA/2, F₁; H2^{bxd}) and C3D2F1 (C3H × DBA/2, F₁; H2^{kxd}) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) and used at 8–12 weeks of age. All mice were housed in sterilized microisolator cages and received autoclaved feed and autoclaved drinking water. All manipulations were performed in a laminar-flow hood.

Preparation of bone marrow cells and spleen cells. Bone marrow was harvested and single-cell suspensions were prepared as described previously (45). Donor spleens were harvested and gently teased in ammonium chloride potassium-lysing buffer (BioWhittaker Inc., Walkersville, Maryland, USA). Single-cell suspensions were filtered through nylon mesh.

Induction of GvHD. Recipient B6D2F1 mice were lethally irradiated (9.75 Gy, ¹³⁷Cs source) and reconstituted within 4–8 hours by a single 1-ml i.v. inoculum containing 5 × 10⁶ allogeneic C3D2F1 bone marrow cells (BMCs) and 1 × 10⁶ to 3 × 10⁶ C3D2F1 spleen cells (GvHD group), or containing 5 × 10⁶ B6D2F1 BMCs with or without 1 × 10⁶ B6D2F1 spleen cells (syngeneic control group). To avoid bias from cage-related effects, animals were randomized before and after BMT as described previously (46).

Assessment of GvHD. Transplanted animals were monitored for 30, 100, or 114 days after BMT by an observer without knowledge of which treatment group the animals, which were randomized in different cages, belonged to. Body weights were measured before transplantation and daily from the day of transplantation until the end of drug administration, and then twice each week until the day of sacrifice. Animals were scored for clinical evidence of GvHD by assessment of changes in skin (alopecia and inflamed or scaly skin), generalized signs (ruffled fur texture, hunched posture, and inflammation of the eyes), and diarrhea. Each parameter was scored as follows: 0 = normal, 1 = mild, 2 = moderate, 3 = severe; for scoring of eye inflammation, 4 = eyes very severely inflamed, 5 = eyes very severely inflamed and closed; maximum total score = 11.

Histopathology. For histopathological analysis of GvHD target tissues, carcasses were saved after death. Samples were collected from small and large bowel, lung, liver, and skin (from the abdomen), and fixed in 10% formalin or Bouin's solution. The preserved tissue samples were embedded in paraffin, cut into 5-μm-thick sections, and stained with H&E for histological examination. Slides were coded without reference to mouse type or prior treatment status and were systematically examined by a pathologist (R. Bronson) without knowledge of the treatment of the animals.

Assessment of engraftment and chimerism. To determine the levels of donor-type hematopoietic reconstitution, we analyzed chimerism in white blood cells (WBCs) by two-color flow cytometry using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, California, USA). Peripheral blood was collected into heparinized Eppendorf tubes

(Brinkmann Instruments Inc., Westbury, New York, USA) and subjected to deionized-water lysis. For double staining, 1×10^6 WBCs were incubated in the presence of directly FITC-conjugated or biotin-conjugated (Bio-conjugated) or unlabeled mAb's for 30 minutes at 4°C. Development of Bio-labeled mAb's was performed by subsequent incubation with phycoerythrin-conjugated avidin for 10 minutes. To reduce nonspecific binding, 10 μ l of mAb 2.4G2 (anti-Fc γ -RII receptor, i.e., CDw32) (47) hybridoma supernatant was added to all tubes. The following antibodies were used for chimerism analyses in various cell lineages: anti-CD4-FITC, anti-CD8 β -FITC, anti-B220-FITC (all purchased from Pharmingen, San Diego, California, USA), and anti-Mac-1-FITC (Caltag Laboratories, Burlingame, California, USA). Nonreactive control mAb's HOPC-FITC and HOPC-Bio (mouse IgG2a prepared in our laboratory) were used as negative controls. Forward-angle and 90° light-scatter properties were used to distinguish lymphocytes, monocytes, and granulocytes in peripheral WBCs. We detected the recipients' cells (H2D^b) by KH95-Bio (Pharmingen) and the donors' cells (H2K^k) by 36-7-5-Bio (Pharmingen) and distinguished donor and host cells of various lineages. Using the recipient marker KH95-Bio, we calculated the relative percentage of recipient cells in a chimera using the formula: $100\% \times (\text{recipient phenotype percentage positive} - \text{isotype control}) / [(\text{recipient phenotype percentage positive} - \text{isotype control}) + (\text{recipient phenotype percentage negative} - \text{isotype control})]$. Subtraction of the relative percentage of recipient cells in a chimera from 100% yielded the relative percentage of donor cells in a chimera. Using the donor marker 36-7-5-Bio, we calculated the relative percentage of donor cells in a chimera using the formula: $100\% \times (\text{donor phenotype percentage positive} - \text{isotype control}) / [(\text{donor phenotype percentage positive} - \text{isotype control}) + (\text{donor phenotype percentage negative} - \text{isotype control})]$. Exclusion of dead cells was performed by propidium iodide staining and live gating on propidium iodide-negative cells. Ten thousand events were collected and analyzed. The different peripheral blood leukocyte populations were distinguished by their forward scatter (FSC) and side scatter (SSC) properties: lymphocytes had low FSC and low SSC, granulocytes had high SSC, and monocytes had high FSC and low SSC.

EL4 leukemia experiments. We used the EL4 leukemia/lymphoma model that we have previously described (48). EL4F cells (referred to here as EL4), a subline of B6 (H2^b) T cell leukemia/lymphoma EL4, were thawed from frozen vials and maintained in culture for 4 days before each experiment, and 5,000 cells were administered on day 0 along with BMCs and spleen cells in a single 1-ml i.v. injection. Carcasses were saved in formalin or Bouin's solution. The presence of tumor at death was determined by gross autopsy and/or histologic observation by an observer and/or pathologist who was unaware of which treatment group the carcasses belonged to, as previously described (44). EL4 cells were detected in lym-

phoid tissues by two-color flow cytometry using mAb's against V β 12 and Thy1.2 (Pharmingen).

Adoptive transfer of BMCs and spleen cells from surviving animals in GvL experiments. BMCs and spleen cells were harvested from individual surviving experimental or control animals, and single-cell suspensions were prepared as described above. Lethally irradiated secondary B6D2F1 recipients (9.75 Gy) were injected intraperitoneally with anti-CD4 mAb GK1.5, anti-Ly2.1 mAb 2.43, and anti-Ly2.2 mAb 116-13.1 on day -1 with respect to the cell transfer. All of the T cell-depleted BMCs and spleen cells from each experimental mouse were administered to a single lethally irradiated, in vivo T cell-depleted B6D2F1 secondary recipient. For in vitro T cell depletion, single-cell suspensions of BMCs and spleen cells from the individual surviving animals, or 5×10^6 BMCs and 1×10^6 spleen cells from normal C3D2F1 control mice, were filtered through nylon mesh and depleted of T cells with anti-CD4 mAb GK1.5 (49) and anti-CD8 mAb 2.43 (50) plus low-toxicity rabbit complement, as described previously (51). T cell depletion was analyzed by flow cytometry, and less than 1% of the cells of the depleted subset remained before DLI. Donor chimerism was followed by FACS analysis.

Preparation of FTY. FTY was kindly provided by Volker Brinkmann (Novartis Pharma AG, Basel, Switzerland) as dry powder and dissolved in sterile distilled water at a concentration of 1 mg/ml. The administration was performed by daily oral gavage at a dose of 6, 3, 1, or 0.1 mg/kg from day -2 or day 0 until day 29 or day 100 post-BMT as indicated.

Statistical analysis. Survival data were analyzed using the log rank test. Differences between group weights were tested using the nonparametric Mann-Whitney test or Student's *t* test of means. Chimerism was compared using Student's *t* test of means.

Results

Lack of toxicity of FTY in syngeneic BMT recipients. There was no clinical evidence of toxicity in syngeneic BMT recipients receiving FTY at doses of 6, 3, 1, or 0.1 mg/kg/d for 29 or 100 days. In mice receiving 3 mg/kg/d for 29 or 100 days, spleen, liver, lung, skin, and large and small bowel did not show any histological evidence of tissue damage (data not shown).

FTY decreases the mortality of GvHD and reduces clinical GvHD. To determine whether FTY could inhibit GvHD, we lethally irradiated male B6D2F1 (H2^{bxd}) mice (9.75 Gy) and reconstituted them with either syngeneic bone marrow (5×10^6 cells i.v.) for hematopoietic rescue, or an inoculum of haplotype-mismatched GvHD-inducing allogeneic marrow cells (5×10^6) and spleen cells (1×10^6) from male C3D2F1 (H2^{kxd}) donors. Three different doses of FTY (3 mg/kg, 1 mg/kg, or 0.1 mg/kg) or sterile H₂O as control were administered daily by oral gavage from day 0 until day 29 post-BMT. Mortality of the H₂O-treated GvHD control mice began on day 7 post-BMT, and five of ten mice succumbed by day 13 (Figure 1a). The remaining

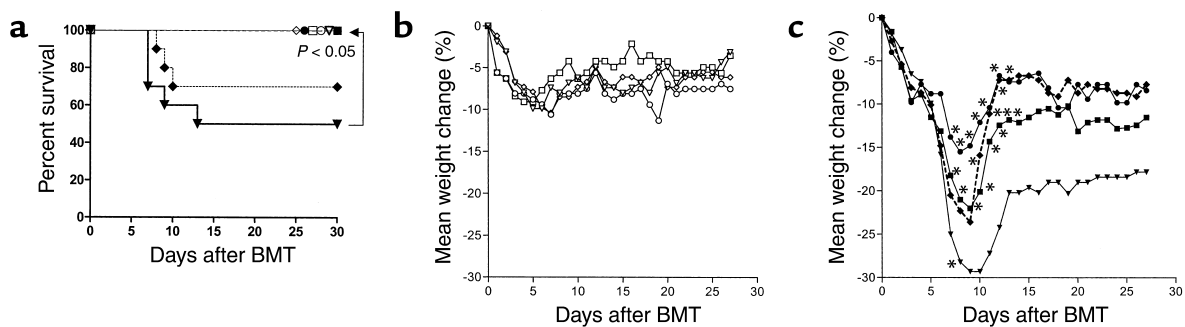


Figure 1

FTY decreases GvHD mortality and inhibits GvHD-associated weight loss. (a) GvHD mortality. (b and c) Weight curves of the syngeneic groups (b) and allogeneic groups (c). Lethally irradiated B6D2F1 mice received haplotype-mismatched GvHD-inducing C3D2F1 BMCs (5×10^6) and spleen cells (1×10^6), as well as daily treatment, by oral gavage on days 0–29 post-BMT, with either H₂O (filled inverted triangles; $n = 10$) or FTY at a dose of 3 mg/kg (filled circles; $n = 10$), 1 mg/kg (filled squares; $n = 10$), or 0.1 mg/kg (filled diamonds; $n = 10$). Syngeneic control groups received 5×10^6 B6D2F1 BMCs and either H₂O (open inverted triangles; $n = 5$) or different doses of FTY (3 mg/kg, open circles, $n = 5$; 1 mg/kg, open squares, $n = 5$; or 0.1 mg/kg, open diamonds, $n = 5$). *Statistically significant difference compared with H₂O-treated control group ($P < 0.05$).

mice in this group survived more than 29 days. In contrast, all recipients of FTY at a dose of 3 mg/kg ($n = 10$) or 1 mg/kg ($n = 10$) survived more than 29 days ($P < 0.05$ vs. allogeneic BMT with H₂O). Three of ten recipients of the lowest dose of FTY (0.1 mg/kg) died by day 10 post-BMT, and the remainder of the group survived more than 29 days. The syngeneic control groups receiving either H₂O or different doses of FTY (3 mg/kg/d, $n = 5$; 1 mg/kg/d, $n = 5$; 0.1 mg/kg/d, $n = 5$) all survived more than 29 days. Therefore, treatment with FTY at a daily dose of 3 or 1 mg/kg/d led to a decreased rate of mortality associated with haplotype-mismatched allogeneic marrow and spleen cell transplantation. The lowest dose of FTY (0.1 mg/kg/d) showed only weak protective activity.

We monitored all transplanted animals for clinical signs of GvHD. Figure 1, b and c, shows the average weight changes of the groups compared with those on day -1 pre-BMT. All transplanted animals, including syngeneic controls, showed initial weight loss. All syngeneic control groups later recovered their weights (Figure 1b). Control animals receiving allogeneic BMT without FTY rapidly lost more weight than syngeneic controls until day 9 post-BMT. The survivors began to regain weight on day 11 post-BMT ($P < 0.05$ on day 10 post-BMT vs. syngeneic BMT recipients treated with H₂O) (Figure 1c). The maximum weight loss of the allogeneic control animals receiving only H₂O was significantly greater than that in the group receiving allogeneic BMT and FTY at a daily dose of 3 mg/kg ($P < 0.05$ on days 7–13 post-BMT), 1 mg/kg ($P < 0.05$ on days 7–14 post-BMT), or 0.1 mg/kg ($P < 0.05$ on days 10–12 post-BMT), and the group receiving 3 mg/kg FTY showed less weight loss than did the groups treated with 1 or 0.1 mg/kg/d ($P < 0.05$) (Figure 1c). Thus, FTY at a dose of 3, 1, or 0.1 mg/kg/d alleviated GvHD-associated weight loss in a dose-related fashion.

Examination for generalized signs of GvHD (fur texture, posture, and inflammation of the eyes) revealed

higher GvHD scores for the GvHD control group receiving allogeneic bone marrow and spleen cells (BMCs/SPCs) with H₂O compared with the FTY-treated allogeneic groups. By day 8, the maximum mean GvHD score of 6.58 ± 0.42 was reached in the allogeneic GvHD control group, and all animals had diarrhea (Table 1). The group receiving allogeneic BMCs/SPCs and the lowest dose of FTY (0.1 mg/kg/d) showed a maximum mean GvHD score of 5 ± 0 on day 7, with diarrhea in only two of ten animals. The recipients of allogeneic BMCs/SPCs and FTY at a dose of 3 mg/kg/d or 1 mg/kg/d showed further reductions in GvHD. The group receiving 3 mg/kg/d FTY had a maximum mean GvHD score of 3 ± 0 on days 9, 12, and 13, with no diarrhea in any of the animals ($n = 10$). The group receiving FTY at a dose of 1 mg/kg/d had a maximum mean GvHD score of 2 on days 8 and 9, and two of ten animals had diarrhea on day 9. All clinical signs of GvHD resolved by day 18 post-BMT in all groups. Thus, FTY at a dose of 3 mg/kg/d and 1 mg/kg/d led to a marked reduction in the severity of GvHD-associated clinical signs.

In order to explore the potential of FTY to inhibit a more rapidly lethal form of GvHD, we lethally irradiated B6D2F1 (H2^{bxd}) mice (9.75 Gy) and reconstituted them with either syngeneic bone marrow (5×10^6 cells i.v.) for hematopoietic rescue, or haplotype-mismatched allogeneic marrow (5×10^6 cells) with an increased spleen cell inoculum (3×10^6 cells) from C3D2F1 (H2^{kxd}) mice. To determine whether the presence of high levels of FTY at the time of transplant could better ameliorate GvHD, we included groups in which the administration of FTY was initiated on day -2 pre-BMT. To determine whether an augmented dose would further impede GvHD, we also evaluated a dose of 6 mg/kg/d of FTY. In groups receiving these doses starting on day -2 or day 0, FTY was continued until day 29 post-BMT. In order to determine whether continuation of treatment with FTY was required to maintain freedom from

GvHD in the group receiving FTY at a dose of 3 mg/kg/d beginning on the day of BMT, we discontinued FTY administration in about half of the mice ($n = 3$) on day 29 and continued it in the remaining animals until day 100 post-BMT ($n = 4$).

In these recipients of higher spleen cell doses, mortality in the control group receiving only sterile H₂O began on day 6 post-BMT (Figure 2a), and ten of ten mice succumbed by day 7. In contrast, recipients of 3 mg/kg/d FTY beginning on day 0, with discontinuation on day 29 or day 100, were almost completely protected from GvHD mortality ($P < 0.05$ vs. allogeneic BMT with H₂O). Recipients of 3 or 6 mg/kg/d FTY from day -2 until day 29 showed similar protection from GvHD mortality to that observed in recipients of 3 mg/kg/d FTY beginning on day 0.

The corresponding average weight changes compared with those on day 0 are shown in Figure 2, b and c. All experimental animals showed initial weight loss, but the control animals receiving only allogeneic BMCs/SPCs showed the greatest weight loss before they died. In contrast, all FTY-treated animals, regardless of the dose or time of initiation of administration, showed similar weight loss until day 7 post-BMT, then began to recover their weights on day 10. Beginning FTY on day -2, with or without an increase in dose to 6 mg/kg/d, did not further attenuate the weight loss compared with that observed in recipients of 3 mg/kg/d FTY beginning on day 0 (Figure 2c). Continuing the administration of FTY beyond day 29 did not result in any measurable difference in weight by day 100 compared with that of FTY-treated allogeneic recipients in which FTY was discontinued on day 29 (Figure 2c). The syngeneic controls treated with FTY (3 or 6 mg/kg/d) or water showed similar, milder weight

loss compared with the allogeneic BMT recipients and showed recovery and continuous weight increases regardless of FTY dose (Figure 2b).

In the group receiving allogeneic BMT with water alone, the mean GvHD score on day 6 was 2.5 ± 0.24 of a possible score of 11, and all animals died the following day. The GvHD scores of the recipients of allogeneic BMCs/SPCs and FTY at a daily dose of 3 mg/kg starting on day -2 (1 ± 0) or day 0 (2 ± 0) or 6 mg/kg beginning on day -2 (1 ± 0) were reduced on day 6, and clinical signs of GvHD resolved by day 15 post-BMT in all surviving groups (data not shown). Only one animal (receiving the highest dose of 6 mg/kg/d from day -2 until day 29) showed any subsequent evidence of GvHD. Neither starting administration prior to the day of BMT nor increasing the dose of FTY led to further protection above the potent inhibition of GvHD observed with FTY administered at a dose of 3 mg/kg/d from days 0-29.

FTY reduces GvHD-associated tissue infiltration. To evaluate our hypothesis that FTY could reduce GvHD-associated tissue infiltration, we lethally irradiated B6D2F1 (H2^{bxd}) mice (9.75 Gy) and reconstituted them with either syngeneic BMCs (5×10^6) and syngeneic spleen cells (1×10^6) ($n = 2$), or haplotype-mismatched GvHD-inducing allogeneic BMCs (5×10^6) and spleen cells (1×10^6) from C3D2F1 (H2^{kxd}) mice with ($n = 7$) or without ($n = 7$) FTY at a dose of 3 mg/kg/d beginning on day 0. Animals were sacrificed on day 11 or 13, and tissues (skin, lung, liver, and small and large bowel) were preserved for histological analysis. Tissue infiltration consisting of lymphocytes, macrophages, and neutrophils was assessed as mild, moderate, or severe. In the untreated allogeneic group, two of three mice sacrificed on day 11 showed severe large-intestinal infiltration consisting

Table 1
Clinically reduced GvHD after FTY at a dose of 3 or 1 mg/kg/d

Treatment	H ₂ O			FTY (3 mg/kg/d)			FTY (1 mg/kg/d)			FTY (0.1 mg/kg/d)		
	Days after BMT	Total GvHD score	Diarrhea	Survival	Total GvHD score	Diarrhea	Survival	Total GvHD score	Diarrhea	Survival	Total GvHD score	Diarrhea
7	5.1	4/7	7/10	2	0	10/10	1	0	10/10	5	02/10	10/10
8	6.6	7/7	7/10	1	0	10/10	2	01/10	10/10	4	01/09	9/10
9	6	3/6	6/10	3	0	10/10	2	02/10	10/10	4	0	8/10
10	5.4	2/6	6/10	2	0	10/10	1	0	10/10	2.3	0	7/10
11	5.8	1/6	6/10	2	0	10/10	1	0	10/10	1	0	7/10
12	4.7	1/6	6/10	3	0	10/10	1	0	10/10	1	0	7/10
13	3.4	0	5/10	3	0	10/10	1	0	10/10	2.1	0	7/10
14	1.8	0	5/10	2	0	10/10	1	0	10/10	1	0	7/10
15	1	0	5/10	0	0	10/10	0	0	10/10	1	0	7/10
16	0	0	5/10	0	0	10/10	0	0	10/10	1	0	7/10
17	1	0	5/10	1	0	10/10	0	0	10/10	1	0	7/10
18	0	0	5/10	0	0	10/10	0	0	10/10	0	0	7/10

Lethally irradiated B6D2F1 mice received haplotype-mismatched C3D2F1 BMCs (5×10^6) and spleen cells (1×10^6), as well as daily treatment, by oral gavage on days 0-29 post-BMT, with either H₂O ($n = 10$) or FTY at a dose of 3 mg/kg ($n = 10$), 1 mg/kg ($n = 10$), or 0.1 mg/kg ($n = 10$). Then the mice were scored for clinical evidence of GvHD by assessment of changes in skin (alopecia and inflamed or scaly skin), generalized signs (fur texture, posture, and inflammation of the eyes), and diarrhea. Shown are the average scores of the groups receiving allogeneic C3D2F1 bone marrow and spleen cells. The syngeneic control groups with or without FTY treatment remained healthy.

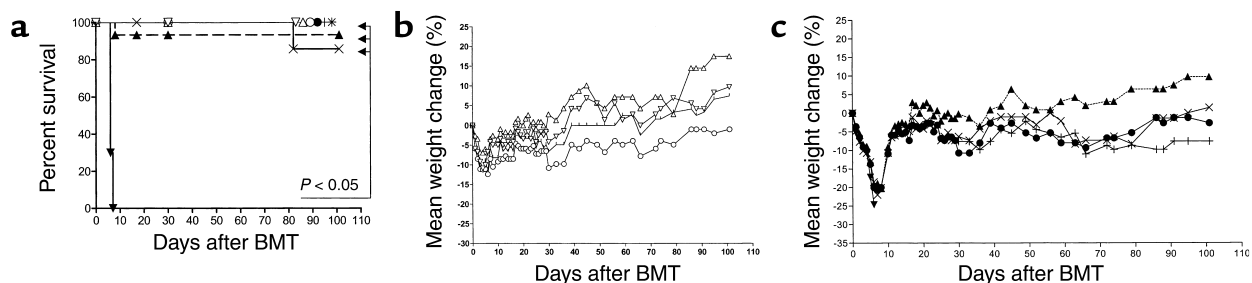


Figure 2

FTY inhibits GvHD induced by a rapidly lethal dose of allogeneic spleen cells. **(a)** GvHD mortality. **(b and c)** Weight curves of the syngeneic **(b)** and allogeneic **(c)** groups. Lethally irradiated B6D2F1 mice received haplotype-mismatched C3D2F1 BMCs (5×10^6) and an increased dose of spleen cells (3×10^6), as well as H₂O (filled inverted triangles; $n = 10$) or FTY at a dose of 3 mg/kg (filled triangles; $n = 10$) or 6 mg/kg (\times 's; $n = 7$), from day -2 pre-BMT until day 29 post-BMT. FTY was given at a dose of 3 mg/kg beginning on day 0 to an additional allogeneic group until day 29 post-BMT (filled circles; $n = 3$), or until day 100 post-BMT (+ 's; $n = 4$). Syngeneic control groups received 5×10^6 B6D2F1 BMCs, as well as H₂O (open inverted triangles; $n = 5$), different doses of FTY from day -2 until day 29 (3 mg/kg, open triangles, $n = 5$; 6 mg/kg, asterisks, $n = 5$), or 3 mg/kg FTY from day 0 until day 29 (open circles; $n = 5$).

of lymphocytes, macrophages, and neutrophils, thickening of the mucosa, and loss of crypts, and the third animal showed moderate infiltration. In contrast, in the allogeneic group receiving FTY, three of three mice showed only mild focal infiltration, with preserved crypts, less thickening of mucosa, and a few localized areas with loss of crypts and tissue infiltration, demonstrating a marked decrease in tissue pathology with FTY treatment. Among the untreated allogeneic control BMC/SPC recipients sacrificed on day 13, three of four animals showed severe, and the fourth animal showed moderate, inflammatory cell infiltration of the large bowel. In marked contrast, only one of four FTY-treated allogeneic recipients showed moderate infiltration, two of four showed mild focal infiltration, and one animal showed no pathological changes at all. Figure 3 depicts large-bowel tissues of representative animals from the syngeneic control group (Figure 3a), the untreated allogeneic group (Figure 3b), and the FTY-treated allogeneic group (Figure 3c), sacrificed on day 13. Syngeneic BMC/SPC control recipients showed no pathological changes on days 11 and 13, indicating recovery of the large intestine from the conditioning treatment. Liver, lung, and skin were normal in syn-

geneic and allogeneic groups on days 11 and 13, and the small intestine was largely unaffected in both allogeneic groups. Weight loss was more severe in the untreated than in the FTY-treated allogeneic group ($P < 0.05$ on day 10). These analyses demonstrate that FTY attenuates tissue infiltration associated with GvHD.

GvL effects proceed unhindered, while FTY inhibits GvHD. To determine whether inhibition of GvHD by FTY would be associated with impaired GvL effects, we used a previously established T cell leukemia/lymphoma, EL4 (52). This H2^b tumor is matched to the unshared host MHC haplotype. We lethally irradiated B6D2F1 (H2^{bxd}) mice (9.75 Gy) and administered either syngeneic marrow (5×10^6 cells i.v.) for hematopoietic rescue, or an inoculum of haplotype-mismatched GvHD-inducing allogeneic marrow cells (5×10^6) and spleen cells (3×10^6) from C3D2F1 (H2^{kxd}) mice; both groups also received 5,000 EL4 cells on the day of BMT. GvHD control groups received similar inocula without EL4.

Mortality induced by EL4 cells in the syngeneic control group receiving only H₂O began on day 19 post-BMT. Median survival time (MST) was 19 days, and five of five mice died by day 21 post-BMT (Figure 4b). Similarly, the syngeneic BMT group receiving EL4

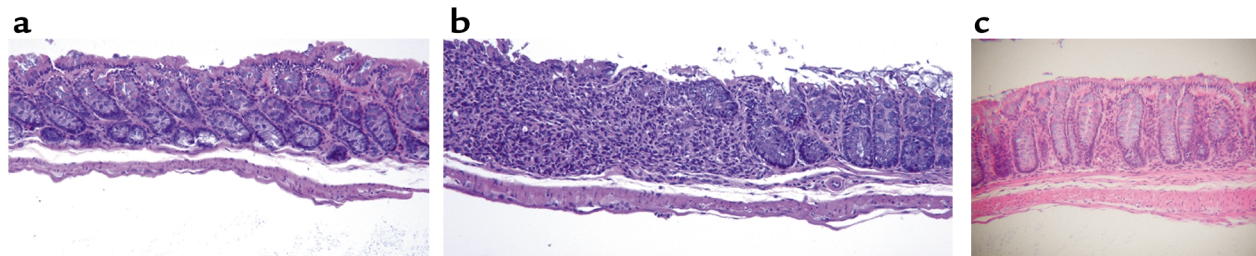


Figure 3

FTY reduces GvHD-associated tissue infiltration. Large-bowel tissues are shown ($\times 50$) from one representative animal in each group. Lethally irradiated B6D2F1 mice (9.75 Gy) were reconstituted with either syngeneic BMCs (5×10^6) and spleen cells (1×10^6) for hematopoietic rescue ($n = 2$), or an inoculum of haplotype-mismatched GvHD-inducing allogeneic BMCs (5×10^6) and spleen cells (1×10^6) from C3D2F1 mice with ($n = 7$) or without ($n = 7$) 3 mg/kg/d FTY (days 0-11 or 0-13). Tissues are from representative animals of the syngeneic control group **(a)**, the untreated allogeneic group **(b)**, and the FTY-treated allogeneic group **(c)** that were sacrificed on day 13 post-BMT.

tumor and FTY treatment (3 mg/kg/d) showed mortality beginning on day 18 post-BMT, and six of six mice succumbed by day 21. Therefore, FTY had no measurable antitumor effect against EL4 in mice receiving syngeneic BMT. Macroscopic examination of carcasses in these groups showed clearly enlarged lymph nodes and spleen, and nodular infiltration of spleen, liver, and kidney (data not shown), consistent with EL4-induced death (48).

The GvHD control group receiving allogeneic BMCs and 3×10^6 donor spleen cells began showing mortality on day 7 post-BMT, and six of seven mice died by 8 days post-BMT (Figure 4a). In contrast, all FTY-treated mice (3 mg/kg from day 0 to day 29 post-BMT) that received similar inocula survived until day 100 post-BMT ($n = 9$; $P < 0.05$ vs. allogeneic group without FTY treatment) (Figure 4a). The allogeneic group receiving tumor (without FTY) began showing mortality on day 6 post-BMT, apparently due to GvHD, and eight of ten mice died by day 9 post-BMT (Figure 4b). Thus, a GvL effect could not be measured in this group, presumably because of rapid GvHD mortality. While several mice in the allogeneic tumor group receiving 3 mg/kg/d FTY treatment (days 0–29 post-BMT) died between day 5 and day 7 post-BMT, MST for the group was 32 days, and three of ten mice survived more than 100 days ($P < 0.05$ vs. allogeneic tumor group without FTY treatment, and $P < 0.05$ vs. syngeneic groups receiving EL4 with or without FTY) (Figure 4b). Thus, simultaneous protection from lymphoma and from GvHD mortality was observed in FTY-treated recipients of allogeneic BMCs/SPCs.

All animals showed initial weight loss (data not shown). The greatest weight loss occurred, as seen previously, in the allogeneic BMT groups that did not receive FTY treatment ($P < 0.05$ vs. allogeneic group with FTY). Clinical signs of GvHD were also most severe in the group receiving allogeneic BMT with water alone: on day 7 the mean GvHD score was 6.57 ± 0 , and all animals died by day 10. In contrast, the average GvHD score of the recipients of allogeneic BMCs/SPCs and FTY at a dose of 3 mg/kg/d (days 0–29) was lower on day 7 (1.25 ± 0), and all surviving animals were free of signs of GvHD by day 11.

Thus, FTY treatment markedly inhibits GvHD mortality while allowing a significant antitumor effect to occur. Since allogeneic control mice that received tumor but not FTY died early from GvHD, an antitumor effect could only be seen in the FTY-treated mice that were protected from GvHD.

To compare the magnitude of GvL effects in allogeneic BMC/SPC recipients treated or not treated with FTY, we repeated the experiment with the allogeneic spleen cell dose (1×10^6) that did not previously cause lethal GvHD in control mice that did not receive FTY. We lethally irradiated B6D2F1 ($H2^{bxd}$) mice (9.75 Gy) and administered either syngeneic marrow (5×10^6 cells i.v.) for hematopoietic rescue, or an inoculum of haplotype-mismatched allogeneic

marrow cells (5×10^6) and spleen cells (1×10^6) from C3D2F1 ($H2^{kxd}$) mice; both groups also received 5,000 EL4 cells on the day of BMT. Control groups received similar inocula without EL4. As in other experiments, allogeneic recipients of 3 mg/kg/d FTY with and without EL4 showed significantly less weight loss than the corresponding groups receiving similar inocula without FTY ($P < 0.05$ on days 7 and 8 post-BMT without EL4, $P < 0.05$ on days 7, 9, and 10 post-BMT with EL4). Almost all nonleukemic allogeneic control animals, with or without FTY treatment, survived until the end of the experiment, permitting comparison of GvL effects in untreated and FTY-treated allogeneic recipients. EL4-induced mortality in the syngeneic control group began on day 19 post-BMT, and seven of seven animals succumbed by day 21 post-BMT (Figure 5a). In contrast, in the allogeneic group receiving EL4 cells and H_2O , leukemic mortality began 21 days post-BMT, MST was 50 days, and six of ten animals died by day 50 post-BMT ($P < 0.05$ vs. syngeneic BMT with EL4). Thus, allogeneic BMC/SPC administration significantly delayed and reduced the mortality associated with EL4, as we have previously reported (48). In this experiment, mortality in the FTY-treated allogeneic recipients was similar to that in the allogeneic group receiving EL4 cells and sterile H_2O only. Leukemic mortality in the FTY-treated group began on day 25 post-BMT, MST was 32 days, and seven of ten animals died by day 42 post-BMT ($P < 0.05$ vs. syngeneic BMT with EL4; no significant difference vs. allogeneic BMT with EL4 and H_2O) (Figure 5a). Thus, results of this experiment indicated that the magnitude of the GvL effect in mice receiving FTY was similar to that in the group receiving

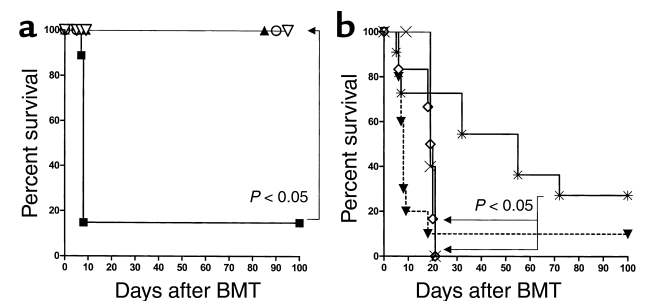


Figure 4

FTY inhibits GvHD mortality while permitting GvL effects to be observed. Survival curves of nonleukemic control groups (a) and leukemic groups (b) are shown. (a) Nonleukemic groups received syngeneic B6D2F1 BMCs (5×10^6) with either H_2O (open inverted triangles; $n = 7$) or FTY (3 mg/kg/d; open circles; $n = 4$); or they received allogeneic haplotype-mismatched C3D2F1 BMCs (5×10^6) and spleen cells (3×10^6) with either H_2O (filled squares; $n = 10$) or 3 mg/kg/d FTY from day 0 until day 29 post-BMT (filled triangles; $n = 10$). (b) Leukemic groups received 5,000 EL4 cells on day 0. Mice received 5×10^6 syngeneic B6D2F1 BMCs alone (x's; $n = 6$) or with FTY (3 mg/kg/d; open diamonds; $n = 6$). Allogeneic groups received haplotype-mismatched C3D2F1 BMCs (5×10^6) and spleen cells (1×10^6) with either H_2O (filled inverted triangles; $n = 10$) or FTY treatment (*; $n = 10$).

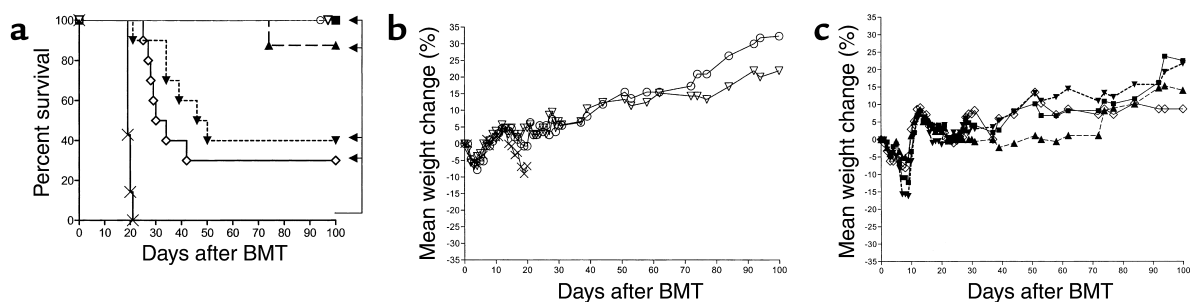


Figure 5

FTY reduces clinical GvHD while preserving GvL. (a) Mortality. (b and c) Weight curves of the syngeneic (b) and allogeneic (c) groups. Lethally irradiated B6D2F1 mice received haplotype-mismatched allogeneic C3D2F1 BMCs (5×10^6) and spleen cells (1×10^6), with either H₂O (filled squares; $n = 10$) or 3 mg/kg FTY treatment from day 0 until day 29 post-BMT (filled triangles; $n = 10$). Five thousand EL4 cells were administered to allogeneic groups on the day of BMT with H₂O (filled inverted triangles; $n = 10$) or with FTY treatment (open diamonds; $n = 10$). Syngeneic control groups received 5×10^6 B6D2F1 BMCs with H₂O (open inverted triangles; $n = 5$), with FTY (3 mg/kg; open circles; $n = 5$), or with 5,000 EL4 cells (x's; $n = 5$).

allogeneic BMT with no GvHD prophylaxis. This observation was confirmed in another experiment involving administration of 2,000 or 5,000 EL4 cells (data not shown).

To determine whether the tumor had been completely eradicated in the surviving allogeneic BMC/SPC recipients of EL4, we performed an adoptive transfer of spleen cells from individual surviving animals that had received allogeneic BMCs, spleen cells, and EL4 with ($n = 3$) or without ($n = 4$) 3 mg/kg FTY, into lethally irradiated host-type B6D2F1 secondary recipients. To eliminate any donor T cells that might have mediated an ongoing GvL effect at the time of sacrifice, these T cells were depleted *ex vivo* from both spleen cell and BMC inocula before transfer to secondary recipients. To ensure that residual donor T cells did not survive in the secondary recipients, these mice were treated with T cell-depleting mAb's (anti-CD4 mAb GK1.5, anti-Ly2.2 mAb 2.43, and anti Ly2.1 mAb 116-13.1) on day -1 with respect to the cell transfer. EL4 does not express CD4 or CD8 (data not shown). All of the T cell-depleted BMCs and spleen cells from each experimental mouse were administered to a single B6D2F1 secondary recipient. As a control, one recipient was given T cell-depleted BMCs (5×10^6) and spleen cells (1×10^6) from a normal C3D2F1 donor. All recipients survived and remained healthy until day 100 post-BMT, when the experiment was terminated. These results suggest that EL4 had been completely eradicated in the original full-haplotype MHC-disparate BMT recipients treated or not treated with FTY (data not shown).

A therapeutic dose of FTY does not directly reduce EL4 proliferation or migration in vivo. We addressed the possibility that the antitumor effects observed in FTY-treated mice reflected, in part, a direct effect of FTY on EL4 tumor proliferation or migration into tissues. Thirty thousand EL4 cells were administered to normal B6 mice, which were sacrificed 5, 10, 17, or 24 days later ($n = 3$ per group per time point). Treatment with 3 mg/kg/d FTY for the duration of the experiment did

not diminish the number of $V\beta 12^+Thy1^+$ EL4 cells in the lymphoid tissues and did not delay or decrease the level of tumor infiltration in other tissues (kidney, liver, and lungs) compared with that in controls receiving EL4 with H₂O treatment alone (data not shown). Thus, FTY had no direct antitumor effect on EL4 and did not decrease its migration into nonlymphoid organs.

FTY does not directly sensitize EL4 to alloreactive cytotoxic T lymphocytes. FTY has been reported to have a direct toxic effect on human and mouse tumor cell lines *in vitro* (53–56). Although the above studies in normal B6 mice and syngeneic BMT recipients rule out a direct antitumor effect of FTY *in vivo*, we performed an *in vitro* experiment to address the possibility that the drug might enhance the sensitivity of EL4 cells to killing by alloreactive cytotoxic T lymphocytes (CTLs), which we have previously shown to be the major effectors of GvL in the EL4 model (51). BALB/c anti-B6 CTLs were generated using standard techniques (57), and cytolytic activity was assessed using EL4 targets that were cultured in medium alone, or with 0.5, 1, or 2 μ M FTY (concentrations similar to those used in the studies cited above; ref. 56) for the last 4 hours before ⁵¹Cr labeling. In two similar experiments, culture with FTY did not affect the lysis of EL4 by alloreactive CTLs compared with that of EL4 cultured in medium alone (data not shown).

A GvHD-inhibitory dose of FTY does not prevent donor-marrow engraftment. To determine whether full donor chimerism developed in animals protected from GvHD by FTY, chimerism was evaluated by FACS analysis of the peripheral WBCs on day 63 post-BMT in the FTY-treated animals presented in Figure 2. The results confirmed that the animals that received allogeneic BMCs/SPCs and that were protected from GvHD by FTY, reconstituted fully with donor-type WBCs in all lineages (data not shown). Thus, FTY inhibits GvHD induced by allogeneic BMCs/SPCs while permitting full donor-type hematopoietic reconstitution.

Discussion

Despite attempts to prevent GvHD by the prophylactic use of immunosuppressive drugs (3), GvHD remains a major cause of morbidity and mortality in patients undergoing allogeneic bone marrow transplantation (14, 15). Since GvH alloreactivity induces both detrimental GvHD and desired GvL effects, the goal of separating GvHD and GvL is of major importance. The strength of the alloresponse across MHC barriers, combined with the greater availability of haploidentical than of closely HLA-matched donors, makes the separation of GvHD and GvL effects across full-haplotype MHC barriers particularly desirable. We have previously shown that this can be achieved by administering DLI after the recipient has recovered from conditioning-induced inflammation and is less predisposed to develop GvHD (26–28). Conditioning such as total body irradiation induces upregulation of tissue cytokines (58), chemokines (M. Mapara and M. Sykes, unpublished observations), and adhesion molecules. These changes subside over time in the absence of GvH alloreactivity (M. Mapara and M. Sykes, unpublished observations), possibly leading to reduced migration of GvH-reactive T cells to the target tissues, and hence to reduced propensity to develop GvHD, with increasing time after conditioning (26–28, 59–61). However, this approach to separating GvHD and GvL entails an undesirable delay before immunotherapy, in the form of DLI, is given. The studies presented here suggest that FTY can achieve a similar result, i.e., can reduce the predisposition of the GvH alloresponse to cause GvHD in the epithelial target tissues, while completely preserving the salutary GvL effect of lymphohematopoietic GvH reactions. Our results show that this can be achieved despite the administration of donor T cells in the presence of proinflammatory conditions associated with lethal total body irradiation (58–61). The observed reduction in clinically and histologically detectable GvHD in FTY-treated mice receiving allogeneic BMCs/SPCs immediately following lethal total body irradiation suggests that FTY's powerful lymph node-trapping effect inhibits tissue infiltration under even these most proinflammatory conditions.

Our data show that FTY markedly decreases GvHD in a dose-related fashion. However, the peak effect appears to be achieved at a dose of 3 mg/kg/d, with no further protection seen at a higher dose of 6 mg/kg/d. While clinical use of FTY to prevent graft rejection usually has been in combination with other immunosuppressive agents and has involved lower FTY doses than those used in our mice (62, 63), doses of 3 mg/kg/d have been used in nonhuman primate studies as a monotherapy with no apparent toxicity (64). A higher dose of 5 mg/kg/d (days –5 and –4 pre-BMT) in a canine non-meloablative BMT model induced hemorrhagic enteritis in one of five animals (65). We have not evaluated FTY in combination with other immunosuppressants such as cyclosporine, but other studies (37) show synergy between such agents (35), suggesting that a lower

dose of FTY might be effective in the BMT setting as well, if used in combination with cyclosporine. Continuation of the administration beyond day 29 post-BMT did not show a beneficial effect over a limited 29-day course of FTY. This may reflect the reduced predisposition of GvH-reactive T cells to cause injury in GvHD target tissues after conditioning-induced inflammation has subsided. However, further studies are needed to address this hypothesis.

Since no difference in the magnitude of GvL effects was found between the allogeneic groups receiving tumor with or without FTY, our results demonstrate that GvL can proceed unhindered while GvHD is significantly alleviated in FTY-treated mice. Although it has been reported that FTY may have a direct effect on human and mouse tumor cell lines (53–56), a direct toxic effect of FTY on the EL4 tumor is ruled out by our *in vivo* studies showing that the control group receiving syngeneic BM, EL4, and FTY succumbed to the tumor in a time frame similar to that in animals receiving treatment without FTY. Furthermore, the addition of host-type spleen cells in association with FTY administration did not lead to a measurable antitumor effect. We also demonstrated that FTY did not directly sensitize EL4 cells to killing by alloreactive (anti-H-2^b) CTLs *in vitro* and did not directly diminish EL4 proliferation or tissue migration in normal B6 mice. Thus, we can conclude that the GvL effect against EL4 in this model, as in other previously reported studies using this model (48), is dependent on a preserved GvH alloresponse.

The mechanism by which FTY inhibits GvHD is most likely altered lymphocyte trafficking. Increased homing into secondary lymphoid tissues, with decreased infiltration into grafted organs, has been well described in association with FTY administration, which leads to a reversible decrease in circulating lymphocytes and increased counts in secondary lymphoid tissues (38). FTY seems to induce lymph node sequestration of lymphocytes by affecting chemokine-driven cell trafficking involving CCR7 and other G-protein-coupled receptors. CCR7 is expressed on naive and central memory T cells and regulates T cell recirculation to secondary lymphoid tissues (44, 66–68). Consistent with published results (36, 38), we found a reduced percentage of circulating CD4 and CD8 cells and B cells in animals receiving FTY until day 100 post-BMT compared with controls (data not shown).

Since leukemias and lymphomas reside largely within the lymphohematopoietic system, we hypothesized that FTY-induced inhibition of migration to the parenchymal tissues would not impede antitumor effects against these types of malignancies. Our results are consistent with this possibility. The demonstration that this separation of GvHD and GvL can be achieved with FTY in a haploidentical strain combination is very encouraging with respect to the potential for clinical transplantation from haploidentical related donors. The possibility of using such donors without unacceptable GvHD could greatly expand the donor pool,

making hematopoietic stem cell transplantation available to almost all patients who could benefit from it, while allowing the enhanced GvL effects associated with MHC disparity (19) to be enjoyed.

Most other strategies for preventing and treating GvHD are globally immunosuppressive, and infectious complications have been a major barrier to their use, particularly in extensively HLA-mismatched transplants such as those involving haploidentical donors (21, 69). The use of FTY, on the other hand, may not cause the global immunosuppression associated with current methods of GvHD prophylaxis and treatment. Animals receiving therapeutic doses of FTY show normal primary and memory antibody responses to lymphocytic choriomeningitis virus and vesicular stomatitis virus infection and show increased numbers of virus-specific cytotoxic CD8 T lymphocytes in the lymph nodes, thereby allowing complete clearance of the virus. Bacterial defense seems to be intact as well (39, 70). Furthermore, it has been shown that FTY does not inhibit T cell activation and proliferation (36), cytokine production (33, 36), or antibody production in other settings (70). These data are consistent with the interpretation that FTY does not markedly impair T or B cell activation or differentiation, and that the preserved GvL effects while GvHD is inhibited reflect altered lymphocyte trafficking induced by this novel agent. Thus, FTY provides a possibly clinically applicable new strategy for the separation of GvHD and GvL.

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