Host NKT Cells Can Prevent Graft-versus-Host Disease and Permit Graft Antitumor Activity after Bone Marrow Transplantation[1]

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Allogeneic bone marrow transplantation is a curative treatment for leukemia and lymphoma, but graft-vs-host disease (GVHD) remains a major complication. Using a GVHD protective nonmyeloablative conditioning regimen of total lymphoid irradiation and antithymocyte serum (TLI/ATS) in mice that has been recently adapted to clinical studies, we show that regulatory host NKT cells prevent the expansion and tissue inflammation induced by donor T cells, but allow retention of the killing activity of donor T cells against the BCL_1 B cell lymphoma. Whereas wild-type hosts given transplants from wild-type donors were protected against progressive tumor growth and lethal GVHD, NKT cell-deficient $CD1d^{-/-}$ and $J\alpha\text{-}18^{-/-}$ host mice given wild-type transplants cleared the tumor cells but died of GVHD. In contrast, wild-type hosts given transplants from $CD8^{-/-}$ or $perforin^{-/-}$ donors had progressive tumor growth without GVHD. Injection of host-type NKT cells into $J\alpha\text{-}18^{-/-}$ host mice conditioned with TLI/ATS markedly reduced the early expansion and colon injury induced by donor T cells. In conclusion, after TLI/ATS host conditioning and allogeneic bone marrow transplantation, host NKT cells can separate the proinflammatory and tumor cytolytic functions of donor T cells. ***The Journal of Immunology,*** **2007, 178: 6242–6251.**

Graft-vs-host disease (GVHD)[3] remains a major complication of human hemopoietic cell transplantation in the treatment of leukemia and lymphoma (1). Although nonmyeloablative conditioning has markedly reduced the early toxicity after transplantation as compared with myeloablative conditioning, the incidence of the more severe manifestations of GVHD (grade II-IV) remains at ~20–50% of patients with HLA-matched related donors and considerably higher in patients with HLA-matched unrelated donors (1–4). T cell depletion of the transplants can markedly reduce the incidence of GVHD, but this is associated with immunodeficiency and loss of graft antitumor activity (5–7).

In a recent clinical study using a nonmyeloablative conditioning regimen of total lymphoid irradiation (TLI) and antithymocyte globulin (ATG) followed by HLA-matched, G-CSF-mobilized PBMC transplantation for the treatment of leukemia and lymphoma, the incidence of grade II-IV acute GVHD was <5% (8). Potent graft antitumor activity was retained as judged by conversion from partial to complete clinical remissions in patients with lymphoma (8). These results indicated that the TLI and antithymocyte Ab regimen protected recipients against GVHD while retaining antitumor activity. Preclinical studies of a TLI and antithymocyte serum (ATS) regimen in mice showed that the protection against GVHD was dependent upon regulatory host NKT cells (9, 10). Donor NKT cells in the bone marrow also protect against GVHD after transplantation (11).

Regulatory $CD4^+CD25^+$ T cells of donor origin can separate the graft antitumor from GVHD functions of donor T cells in mice given bone marrow transplants (12, 13). Whether regulatory NKT cells of host origin that prevent GVHD (9, 10, 14) will allow donor T cells to mediate antitumor activity remains unclear. We assessed whether posttransplant antitumor activity against a lethal BALB/c-derived B cell lymphoma (BCL_1) is retained after TLI and ATS conditioning of BALB/c hosts given C57BL/6 bone marrow transplants. We show that wild-type hosts were protected from both GVHD and progressive BCL_1 lymphoma growth using TLI/ATS as the preparative regimen. In NKT cell-deficient $J\alpha\text{-}18^{-/-}$ and $CD1d^{-/-}$ BALB/c hosts, we demonstrate that the donor T cells mediated both severe GVHD and graft antitumor activity. Using bone marrow transplants from wild-type, $CD8^{-/-}$, or $perforin^{-/-}$ C57BL/6 donors, we show that graft antitumor activity requires donor $CD8^+$ T cells as well as the perforin cytolytic molecule.

Materials and Methods

Mice

Wild-type male BALB/c ($H\text{-}2^d$) mice, 8–10 wk old, were obtained from The Jackson Laboratory and the Department of Comparative Medicine (Stanford University, Stanford, CA) and allowed to reach a minimum weight of 27 g/animal before initiation of host conditioning. $CD1d^{-/-}$ mice (15) were backcrossed >10 generations on the BALB/c background. $CD1d^{-/-}$ mice and $J\alpha18^{-/-}$ BALB/c mice (16) were bred in the Department of Comparative Medicine (Stanford University) as per existing protocols. Ten-week-old wild-type C57BL/6 ($H\text{-}2^b$), $CD8^{-/-}$ C57BL/6, and $perforin^{-/-}$ C57BL/6 male donor mice were also obtained from The Jackson Laboratory. The Stanford University Committee on Animal Welfare (Administration Panel of Laboratory Animal Care) approved all mouse protocols used in this study.

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[3] Abbreviations used in this paper: GVHD, graft-vs-host disease; TLI, total lymphoid irradiation; ATG, antithymocyte globulin; ATS, antithymocyte serum; BCL_1, B cell lymphoma; α-GalCer, α-galactosyl ceramide; DAPI, 4′,6′-diamidino-2-phenylindole; TBI, total body irradiation; MLN, mesenteric lymph node.

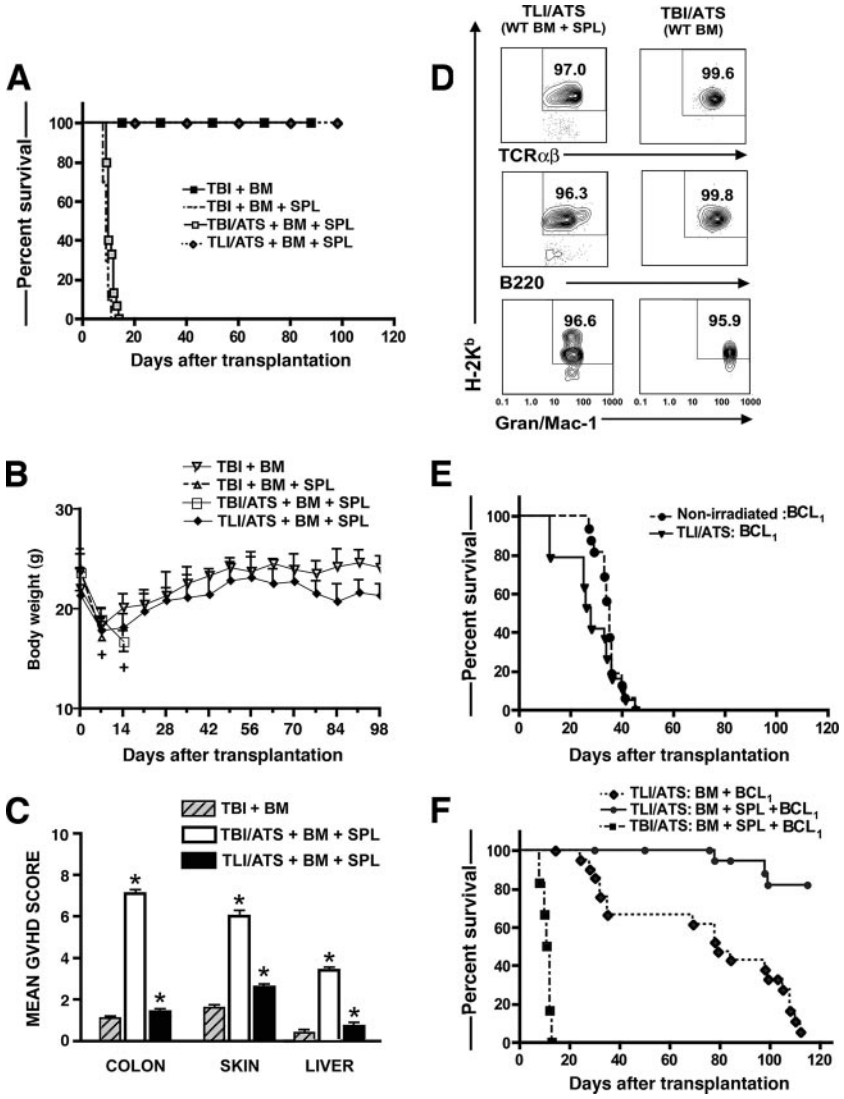

FIGURE 1. TLI/ATS-conditioned hosts are protected against GVHD and tumor progression. *A*, Wild-type BALB/c host survival after 800 cGy TBI ($n = 10$) or TBI + ATS ($n = 15$) vs TLI/ATS ($n = 15$) followed by transplantation of 50×10^6 whole bone marrow (BM) and 60×10^6 splenocytes (SPL) from WT C57BL/6 donors. Control hosts were given 800 cGy TBI followed by 50×10^6 whole BM alone ($n = 8$). *B*, Mean body weight (±SE) of groups shown in *A* at serial time points after transplantation. (+), Analysis was stopped when only two hosts remained in the group. *C*, Mean (±SD) histopathologic GVHD scores of colon, skin, and liver from three groups of hosts from *A*. (*), Statistically significant differences ($p < 0.05$) between two groups; $n = 5$ for all groups. *D*, Donor PBMC chimerism of WT TLI/ATS-conditioned hosts receiving donor BM and splenocytes is shown relative to control TBI/ATS-conditioned hosts receiving WT BM alone. Trilineage chimerism is shown from one representative animal in each group, assessed at day 28 following transplantation. Gated TCR$\alpha\beta^+$ T cells, B220$^+$ B cells, and Gran/Mac-1$^+$ granulocytes and macrophages were analyzed for the percentage of donor type (H-2K^{b+}) cells (enclosed in boxes) among the gated subsets of PBMC. *E*, Survival of untreated ($n = 9$) or TLI/ATS-conditioned ($n = 10$) WT BALB/c hosts given an i.v. infusion of 5×10^2 BCL_1 tumor cells without BM transplantation. *F*, Survival of WT BALB/c hosts receiving TBI/ATS ($n = 20$) vs TLI/ATS ($n = 20$) conditioning followed by injection of BCL_1 tumor cells with BM and splenocytes from WT C57BL/6 donors vs control hosts conditioned with TLI/ATS and injected with BCL_1 tumor cells and 50×10^6 WT C57BL/6 whole BM alone ($n = 15$).

Monitoring of GVHD and survival

Survival and the signs of GVHD such as hair loss, hunched back, swollen face, and diarrhea were monitored daily and body weight was measured weekly. Mean body weights of surviving mice in each group were determined at day 100. Survival times include the day of euthanasia of any moribund tumor-bearing mice according to Stanford Animal Welfare protocol guidelines.

Irradiation

TLI was delivered to the lymph nodes, thymus, and spleen with shielding of the skull, lungs, limbs, pelvis, and tail as previously described (9). TLI began on day −24 before transplantation and 17 doses of 240 cGy each were administered. Total body irradiation was delivered as a single dose (800 cGy) to control BALB/c recipients 24 h before cell infusions. Irradiation was performed with a Philips x-ray unit (200 kV, 10 mA; Philips Electronic Instruments) at a rate of 84 cGy/minute with a 0.5-mm Cu filter. Mice were kept on antibiotic water (25 μg/ml neomycin/0.3 U/ml polymyxin B; Sigma-Aldrich) beginning 2 days before initiation of irradiation and continuing for the first 28 days after transplant.

Rabbit ATS

Rabbit ATS was purchased from Accurate Laboratories and adsorbed with BALB/c RBC before use. Host BALB/c mice were injected i.p. with 0.05 ml of ATS in 0.5 ml of sterile normal saline on days −12, −10, and −8 before bone marrow transplantation.

Cell preparation

Splenocytes were isolated in RPMI 1640 (Invitrogen Life Technologies) with 2% BSA. Femoral and tibial bones were taken from donor C57BL/6 mice and the residual muscle was carefully removed. Bone marrow suspensions were prepared by flushing the bones with RPMI 1640 with 2% BSA. Cell suspensions were filtered through nylon mesh, washed once, and resuspended in sterile PBS before infusion.

BCL_1 tumor cell passage and injection

BCL_1 is a BALB/c-derived B cell leukemia/lymphoma with an IgMλ surface Ig phenotype. This tumor was maintained by serial passage in untreated 8-wk-old male BALB/c mice as described previously (17–19). A total of 5×10^2 BCL_1 cells from spleen of tumor-bearing BALB/c mice were injected i.v. into BALB/c hosts 6 h before bone marrow transplantation.

Abs and FACS

Blood samples were hemolyzed with ammonium chloride potassium carbonate (ACK) buffer. Splenocytes were hemolyzed with ACK buffer in sterile RPMI 1640 with 2% BSA. Cells were washed twice with 0.05% sodium azide staining buffer and incubated on ice for 20 min with saturating concentrations of mAb mixtures as described previously (11, 13). All mouse cells were incubated with CD16/32 (2.4G2; BD Biosciences) before Ab staining to block FcR-γ II/IIIRs. The following conjugated mAbs were used: allophycocyanin-anti-TCR$\alpha\beta$ clone H57-597, PE anti-Ly6C (Gr-1) clone RB6-8C5, PE anti-CD11b (Mac-1α) clone M1/70, biotin-anti-CD8 clone 53-6.7, PE-anti-B220 clone RA3-6B2, and FITC anti-H-2K^b clone AF6-88.5 (BD Biosciences), and streptavidin-Texas Red (Molecular Probes). FITC-anti-BCL_1 Id was a gift from Dr. R. Levy (Stanford University). Propidium iodide was added before cytometric analysis to exclude dead cells. A modified dual-laser FACSVantage (BD Biosciences) in the Shared FACS Facility (Center for Molecular and Genetic Medicine,

Stanford University), using FlowJo software (Tree Star), was used for data analysis. Background staining for donor-type cells in normal control BALB/c mice was ≤0.5%.

Purification and adoptive transfer of NKT cells

Splenocyte suspensions were preincubated with CD1d dimer-mouse IgG fusion protein (BD Biosciences) loaded overnight at 37°C with the glycolipid α-galactosyl ceramide (α-GalCer), as well as with PE-conjugated CD1d tetramer reagent loaded with the glycolipid PBS-57 (obtained from the National Institutes of Health Tetramer Facility; www.niaid.nih.gov/reposit/tetramer/index.html) for 60 min on ice, and washed once with RPMI 1640/1% BSA. Samples were then enriched for CD1d dimer-positive cells with anti-mouse IgG1 magnetic beads using the MidiMACS system (Miltenyi Biotec). Column-enriched NKT cells were counterstained with allophycocyanin-conjugated anti-TCRαβ mAb and PE-conjugated CD1d tetramer reagent and FACS sorted on a modified dual-laser FACSVantage. A total of 5×10^5 sorted NKT cells were adoptively transferred into $J\alpha18^{-/-}$ BALB/c hosts treated with the TLI/ATS regimen. Sorted NKT cells (purity >95% by flow cytometry) were infused in sterile PBS via lateral tail vein on day 0 following TLI/ATS, 6 h before bone marrow and splenocyte transplantation.

Donor T cell accumulation

For day 6 donor T cell accumulation analyses, single-cell suspensions of lymphocytes from mesenteric lymph nodes and spleen of individual host mice were filtered through fine nitex membrane to remove aggregates. Mononuclear cells from the colon were isolated as described previously (20). Cells were stained with FITC-conjugated anti-H-$2K^b$, allophycocyanin-conjugated anti-TCRαβ, PE-conjugated anti-CD8, biotin-conjugated anti-CD4 mAb, and Texas Red-conjugated streptavidin.

Histopathology

Specimens from the spleen, skin, and colon of hosts were obtained at the time of death or at time of sacrifice of hosts after transplantation, fixed in 10% formalin, and embedded in paraffin blocks. Four- to 5-μm sections were stained with H&E using standard protocols. Microscopic images were obtained using an Eclipse E1000M microscope (Nikon) with a SPOT RT digital camera and acquisition software (Diagnostic Instruments) with final magnification (objective, ×20/0.45 numerical aperture) provided with each figure. Image processing was performed with Photoshop CS (Abode Systems), with standard adjustments of brightness, contrast, and color balance to the entire image.

Pathologic scoring for GVHD severity

At the time of histopathologic analysis of H&E-stained sections, the descending colon, skin, and liver were assigned a score assessing severity of GVHD as per previously published criteria (21). The mean GVHD score represents the mean ± SD among five animals per group.

Immunofluorescence staining of tissues

Tissues were embedded in OCT compound (Sakura Finetek), frozen in liquid nitrogen, and stored at −80°C. Tissues were cut into 4-μm-thick sections, fixed in acetone at −20°C for 10 min, and air-dried. Slides were incubated in 1% PBS for 5 min at room temperature, followed by staining with rat anti-mouse CD8 Ab (e-Bioscience) for 45 min at room temperature. Slides were rinsed three times with 1% PBS containing 0.1% Tween 20 (Aldrich) and incubated for 45 min with secondary Oregon green anti-rat Ig (Invitrogen Life Technologies) plus normal mouse serum (Jackson ImmunoResearch Laboratories) in the dark. Slides were rinsed three times with 1% PBS containing 0.1% Tween 20, mounted with 4′,6′-diamidino-2-phenylindole (DAPI), and examined with an Olympus BX51 fluorescent microscope, using the CytoVision 3.0 system (Applied Imaging). Three samples were analyzed in each group. Controls performed on each tissue specimen were stained with secondary Oregon green anti-rat Ig Ab plus normal mouse serum, and mounted with DAPI.

Serum cytokine analysis

At day 6 following transplantation, hosts were bled and serum frozen at −80°C. Samples were later thawed to room temperature and analyzed using a Th1/Th2 Mouse Cytometric Bead Array (BD Biosciences) according to the manufacturer's specifications. Cytokine bead staining was analyzed on a FACScan analyzer and data were compiled with BD Biosciences CBA software. A total of five samples were analyzed in each group, in two separate experiments. Statistical analysis for significance was performed using the two-tailed Student *t* test.

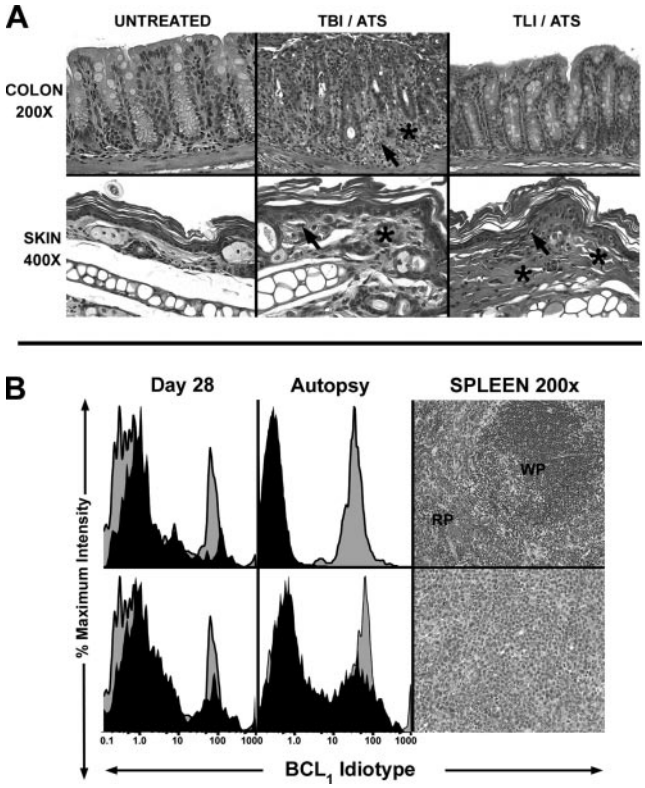

FIGURE 2. TLI/ATS-conditioned hosts eliminate BCL_1 Id-positive cells and are protected from GVHD. *A*, Microscopic examination of H&E-stained sections of colon and skin at autopsy in BALB/c hosts treated with 800 cGy TBI and ATS followed by BM, splenocyte, and BCL_1 tumor transfer. Evidence of crypt abscess (arrow) inflammatory infiltrate (*) and loss of normal crypt architecture in the colon is shown with separation of crypts by inflammation and lifting off of crypts from the basement membrane. Disruption of the dermal-epidermal junction with intense lymphocytic infiltrates (*) and increased apoptosis in the basal epidermis (arrow) are shown in skin sections. Hosts from groups given the same cell infusion after TLI/ATS conditioning revealed none of the changes in the colon upon autopsy at day 120. Skin specimens from the latter hosts showed scattered epidermal apoptosis (arrow), increased dermal collagen deposition (*), and minimal epidermal hyperplasia. Normal skin and colon sections from untreated mice are shown for comparison. Colon and skin specimens are shown at ×200 and ×400, respectively. *B*, Patterns of anti-BCL_1 Id immunofluorescent staining in peripheral blood (day 28) and spleen (autopsy). Hosts surviving 120 days were autopsied at that time. Gray histograms represent positive control animals receiving TLI/ATS and BCL_1 infusion without marrow transplantation. Controls showed 20–30% and 30–70% BCL_1 tumor Id-positive cells in the PBMC and spleen at autopsy, respectively. Black histograms represent experimental hosts. In 20 hosts, three patterns could be discerned: *upper panels,* 6 hosts (30%) displayed >1% BCL_1 Id positive cells in the peripheral blood at day 28, and <1% in the spleen at autopsy. Splenic red pulp (RP) and white pulp (WP) architecture was intact without histopathologic evidence of tumor clusters in the spleen at day 120 (*right panel*). In 10 of 20 hosts, <1% of Id-positive cells were present at day 28 and at autopsy (data not shown). *Lower panels*, In a third pattern, 20% ($n = 4$) of hosts demonstrated BCL_1 Id-positive cells in peripheral blood at day 28 and clinical progression of tumor. There was high intensity staining of BCL_1 Id-positive cells in the spleen at autopsy, and diffuse infiltration of the spleen by tumor with loss of red and white pulp architecture (*right lower panel*). Spleen sections were stained with H&E and examined at ×200 magnification. Control staining of normal BALB/c blood and spleen showed <1% BCL_1 Id-positive cells.

Statistical analysis

Kaplan-Meier survival curves were generated using Prism (GraphPad Software). Statistical differences in animal survival were analyzed by the log-rank

Table I. *Survival and BCL_1 tumor detection in BALB/c hosts conditioned with TLI/ATS and transplanted with C57BL/6 spleen and bone marrow cells*

Host	Donor Graft	Fraction with Detectable Tumor Day 28[a]	Fraction with Detectable Tumor at Autopsy[a]	Fraction Surviving Day 120
WT	WT SPL + BM	10/20	4/20	16/20
$CD1d^{-/-}$	WT SPL + BM	3/14	0/14	0/14
WT	$CD8^{-/-}$ SPL + BM	10/10	10/10	0/10
WT	$Prf^{-/-}$ SPL + BM	10/10	10/10	0/10
WT	WT BM only	14/15	15/15	0/15

[a] More than 1% BCL_1 idiotype-bright staining cells in peripheral blood (day 28) or spleen (autopsy). SPL, splenocytes; BM, bone marrow cells.

test. Differences in mean donor type T cell recovery in tissues of hosts and in serum cytokine levels between transplanted groups were analyzed using the two-tailed Student *t* test. For all tests, $p < 0.05$ was considered significant.

Results

MHC-mismatched mice conditioned with TLI/ATS are protected against lethal acute GVHD

Adult BALB/c male mice given a single dose of total body irradiation (TBI) (800 cGy) and an i.v. injection of 50×10^6 bone marrow cells from C57BL/6 donors survived at least 100 days without clinical signs of GVHD (Fig. 1*A*). Addition of 60×10^6 C57BL/6 splenocytes to the bone marrow cell infusion resulted in the death of all BALB/c hosts by 20 days, with typical features of GVHD, including diarrhea, weight loss, hunched back, and hair loss (Fig. 1*A*) ($p < 0.001$; log-rank test). Addition of three i.p. injections of rabbit ATS, a potent T cell-depletive reagent, on days −12, −10, and −8 before TBI (800cGy) failed to protect BALB/c hosts from GVHD induced by C57BL/6 bone marrow and splenocytes, and all died by day 20 (Fig. 1*A*). Control BALB/c hosts given TBI (800 cGy) without donor cell transplants all died by day 10 (data not shown).

Wild-type BALB/c hosts given TLI in 17 doses of 240 cGy each (total dose 4080 cGy) during a 24-day period accompanied by three i.p. doses of ATS (days −12, −10, and −8), followed by the infusion of 50×10^6 C57BL/6 bone marrow cells plus 60×10^6 splenocytes all survived for at least 100 days without clinical signs of GVHD (Fig. 1*A*). The survival of TLI/ATS-conditioned hosts given bone marrow and splenocytes was significantly increased as compared with the TBI/ATS-conditioned hosts as judged by the log-rank test ($p < 0.001$). Fig. 1*B* shows the mean serial body weights of the groups in Fig. 1*A*. The hosts given TBI and bone marrow cells alone or TLI/ATS, bone marrow cells, and splenocytes all recovered their mean body weights by 28 days after transplantation and remained stable thereafter. In contrast, the hosts given TBI or TBI/ATS, bone marrow cells, and splenocytes showed no recovery of their mean body weights before they all died by day 15. The mean histopathologic GVHD scores of the colon, skin, and liver of the groups given TBI/ATS and bone marrow cells alone, TBI/ATS with bone marrow cells and splenocytes, or TLI/ATS with bone marrow cells and splenocytes are shown in Fig. 1*C*. The mean GVHD scores of the group given TBI/ATS with bone marrow and splenocytes was significantly increased ($p < 0.01$) as compared with group given TLI/ATS with bone marrow and splenocytes in all three tissues examined. The TLI/ATS regimen was nonmyeloablative, as hosts given TLI/ATS without transplants survived at least 100 days (data not shown). In addition, hosts given TLI/ATS followed by infusion of 50×10^6 C57BL/6 bone marrow cells alone also survived 100 days without clinical or histopathologic evidence of GVHD (data not shown).

Trilineage chimerism in GVHD-protected hosts

All TBI- and TBI/ATS- treated hosts given bone marrow cells (50×10^6) from C57BL/6 (H-2^b) donors had at least 96% donor chimerism of peripheral blood T cells (TCR$\alpha\beta^+$), B cells ($B220^+$), granulocytes and macrophages (Gran/Mac-1^+) by day 28 following transplant (Fig. 1*D*) that remained stable through day 120. TBI- and TBI/ATS-treated hosts receiving C57BL/6 bone marrow plus splenocytes uniformly died of acute GVHD before day 28 and therefore donor chimerism could not be assessed in this group. Of TLI/ATS-treated BALB/c hosts given bone marrow and splenocytes from C57BL/6 donors, 15% ($n = 3$) developed stable mixed chimerism with <80% donor-type cells, and 85% ($n = 17$) developed high-level donor chimerism with >80% donor-type cells by day 28 as shown in the example in Fig. 1*D*. Chimerism in the latter mice remained stable through day 120.

TLI/ATS-conditioned hosts are protected against the BCL_1 lymphoma

TLI/ATS-treated hosts given an i.v. injection of 5×10^2 BCL_1 lymphoma cells without bone marrow transplantation died by day 50, and survival was similar to untreated hosts given 5×10^2 BCL_1 lymphoma cells ($p = 0.81$) (Fig. 1*E*). Transplantation of 50×10^6 C57BL/6 bone marrow cells together with 5×10^2 BCL_1 lymphoma cells into TLI/ATS-conditioned BALB/c hosts resulted in death of 50% of hosts by day 70, and 100% of hosts by day 120 (Fig. 1*F*), due to tumor progression (see below). Addition of 60×10^6 C57BL/6 splenocytes to 50×10^6 C57BL/6 bone marrow cells transplanted into hosts with the BCL_1 lymphoma resulted in death of 20% of hosts, and the remaining 80% of hosts survived without clinical evidence of tumor progression or GVHD (Fig. 1*F*). Survival of hosts given marrow plus splenocytes was significantly improved ($p < 0.01$; log rank test) as compared with hosts given bone marrow cells alone. In contrast, all hosts given TBI (800 cGy) with ATS followed by injection of C57BL/6 bone marrow, splenocytes, and BCL_1 lymphoma died uniformly by day 28 with clinical signs of acute GVHD (Fig. 1*F*). Survival of the latter group was significantly decreased ($p < 0.001$) as compared with the group given TLI/ATS conditioning.

The TBI/ATS group showed severe changes of GVHD on microscopic examination of colonic tissues obtained at autopsy, including loss of goblet cells, presence of inflammatory infiltrates surrounding crypts, and crypt atrophy associated with apoptosis of crypt cells as compared with the normal colon (Fig. 2*A*). Characteristic changes of GVHD were also observed in skin sections including thickening of the epidermis, dermal cell infiltrates, and subepidermal abscesses as compared with the normal skin (Fig. 2*A*). In contrast, colon specimens obtained from TLI/ATS-conditioned hosts after 120 days showed intact crypts with maintenance of goblet cells and little evidence of inflammatory infiltrate (Fig. 2*A*). Skin sections showed increased dermal collagen deposition consistent with chronic skin GVHD, mild epidermal hyperplasia, and rare scattered epidermal cell apoptosis (Fig. 2*A*).

To assay hosts in the above groups for progression or eradication of the BCL_1 tumor, PBMC were analyzed for expression of

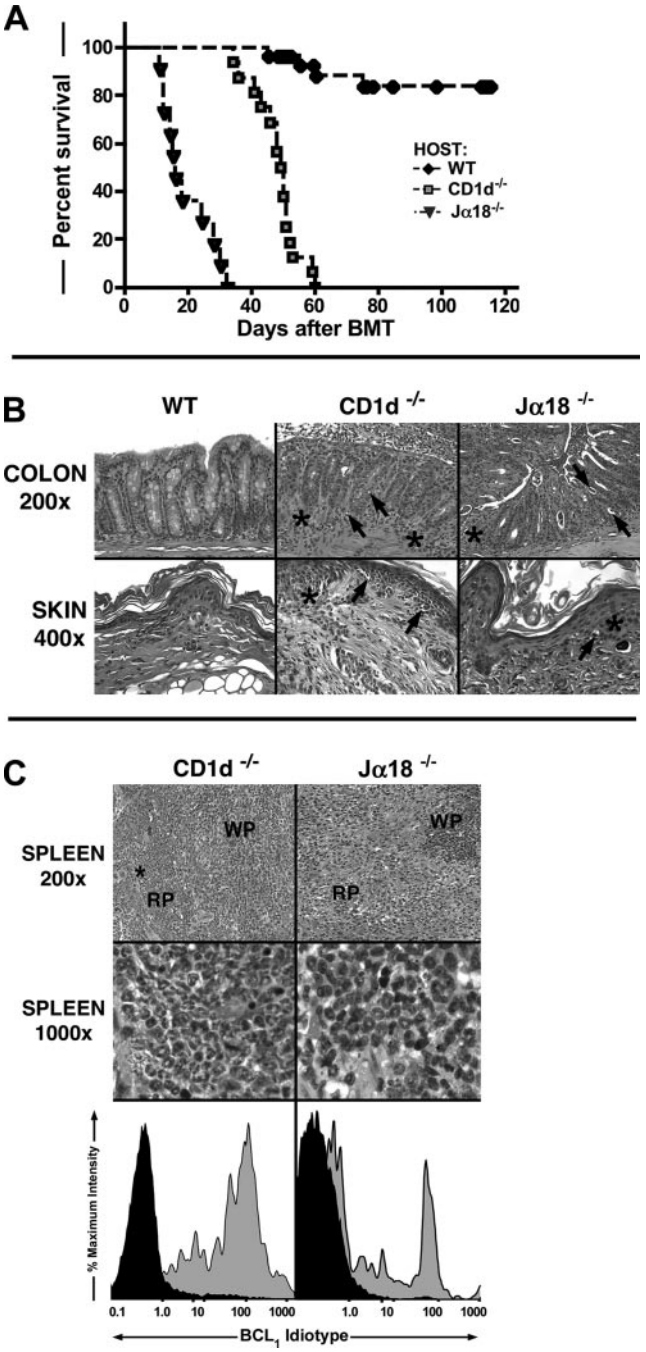

FIGURE 3. NKT cell-deficient $CD1d^{-/-}$ and $Ja18^{-/-}$ hosts are protected from tumor growth but develop lethal GVHD. *A*, Survival of WT ($n = 20$) vs NKT cell-deficient $CD1d^{-/-}$ ($n = 15$) and $J\alpha18^{-/-}$ ($n = 10$) hosts conditioned with TLI/ATS and injected with 5×10^2 BCL_1 tumor cells, 50×10^6 whole BM and 60×10^6 splenocytes from WT C57BL/6 donors. *B*, Representative histopathologic examination of colon and skin in $CD1d^{-/-}$ hosts reveals severe crypt apoptosis (arrows), inflammatory infiltrates (*), and crypt atrophy with loss of goblet cells in the colon. The skin showed disruption of the dermal-epidermal junction by an intense chronic inflammatory infiltrate (*) accompanied by increased apoptosis in the basal epidermis in the skin (arrow). Histopathologic examination of colon and skin in $J\alpha18^{-/-}$ hosts reveals similar severe crypt apoptosis (arrows), inflammatory infiltrate (*), and crypt atrophy with loss of goblet cells in the colon as well as disruption of the dermal-epidermal junction with intense inflammatory infiltrate (*) and increased apoptosis (arrow) in the basal epidermis of the skin, WT BALB/c hosts given the same transplants after TLI/ATS conditioning revealed minimal to no characteristic changes of GVHD in the colon or skin. Sections are stained with H&E and analyzed at ×200 magnification (colon) and ×400 magnification (skin). One representative animal is shown from each group. *C*, Spleen histopathologic section and splenic BCL_1 Id staining on day of autopsy of $CD1d^{-/-}$ hosts and $J\alpha18^{-/-}$ hosts. Fourteen $CD1d^{-/-}$ hosts and 10 $J\alpha18^{-/-}$ hosts showed no evidence of tumor Id (black filled histogram) relative to positive control WT BALB/c hosts given TLI/ATS and BCL_1 tumor alone (gray histograms). Similarly, none of the $CD1d^{-/-}$ hosts or $J\alpha18^{-/-}$ hosts showed microscopic evidence of BCL_1 tumor at autopsy. Instead, a neutrophilic infiltrate with many band forms was observed. Spleen sections are stained with H&E and analyzed at ×200 or ×1000 magnification.

the BCL_1 tumor Id at days 28 and 120 after transplantation. In addition, spleen cells were stained for surface BCL_1 Id expression after sacrifice of survivors at day 120. Positive control staining of TLI/ATS-treated BALB/c mice given BCL_1 tumor cells without transplants showed that ~20–30% of PBMC and 30–70% of spleen cells stained brightly for BCL_1 tumor Id at autopsy (Fig. 2*B*). At day 28 after transplantation, 10 of 20 TLI/ATS-conditioned hosts receiving marrow, splenocytes, and BCL_1 tumor cells showed <1% (limit of detection) of Id-positive cells in peripheral blood, and the rest showed up to 9% of Id-positive cells (Table I). At day 120 after transplantation, 16 of 20 hosts had <1% of bright-staining Id-positive cells in both peripheral blood and spleen (Fig. 2*B*, *upper panels*). None of the 16 hosts had microscopic evidence of splenic tumor cells or disruption of splenic architecture (Fig. 2*B*, *upper panels*). Thus, 6 of 20 hosts with detectable tumor cells at day 28 went on to clear the tumor by day 120 (Table I). There was no microscopic evidence of tumor clusters or disruption of architecture in the liver or colonic submucosal lymphoid tissue (data not shown). Four of the 20 TLI/ATS-treated hosts died and had histopathologic evidence of diffuse tumor infiltration of the spleen with disruption of splenic architecture (Fig. 2*B*, *lower panels*).

TLI/ATS-conditioned hosts without progression of the BCL_1 lymphoma showed stable high level (>80%) or complete (>95%) peripheral blood T cell donor chimerism (mean 91 ± 9%) at day 28 after transplantation, and three of the four hosts that succumbed to tumor progression demonstrated <70% peripheral blood donor T cell chimerism at day 28 (mean 42 ± 13%).

NKT cell-deficient hosts conditioned with TLI/ATS are protected against tumor growth but develop lethal GVHD

Our previous studies showed that protection against GVHD in TLI/ATS-conditioned hosts was dependent on the presence of host NKT cells, because NKT cell-deficient $CD1d^{-/-}$ hosts given TLI/ATS developed lethal GVHD and wild-type hosts did not (12). To further investigate the impact of NKT cell deficiency on GVHD and tumor protection in TLI/ATS-conditioned hosts, wild-type, and NKT cell-deficient $CD1d^{-/-}$ and $J\alpha\text{-}18^{-/-}$ BALB/c hosts were given TLI/ATS followed by BCL_1 tumor cells, and bone marrow and splenocytes from wild-type C57BL/6 donors. The NKT cell-deficient $J\alpha\text{-}18^{-/-}$ and $CD1d^{-/-}$ hosts developed uniform clinical signs of GVHD, and all died by days 30 and 60, respectively (Fig. 3*A*). Survival was significantly decreased ($p < 0.01$) as compared with wild-type hosts. Less than 1% of bright Id-staining cells were found in the spleen at autopsy in 14 of 14 $CD1d^{-/-}$ hosts analyzed (Fig. 3*C*, *lower panel*). Bright BCL_1 Id staining cells were >1% of mononuclear cells in the peripheral blood in 3 of 14 $CD1d^{-/-}$ hosts at day 28 (Table I).

The latter results indicate that 3 of 14 $CD1d^{-/-}$ hosts cleared BCL_1 tumor cells after day 28. The $CD1d^{-/-}$ and $J\alpha\text{-}18^{-/-}$ hosts had histopathologic studies of the spleen, colon, and skin at autopsy (Fig. 3, *B* and *C*). None had evidence of splenic tumor or loss of red and white pulp architecture, and all showed an intense neutrophilic infiltrate of the spleen (Fig. 3*C*). Whereas wild-type hosts

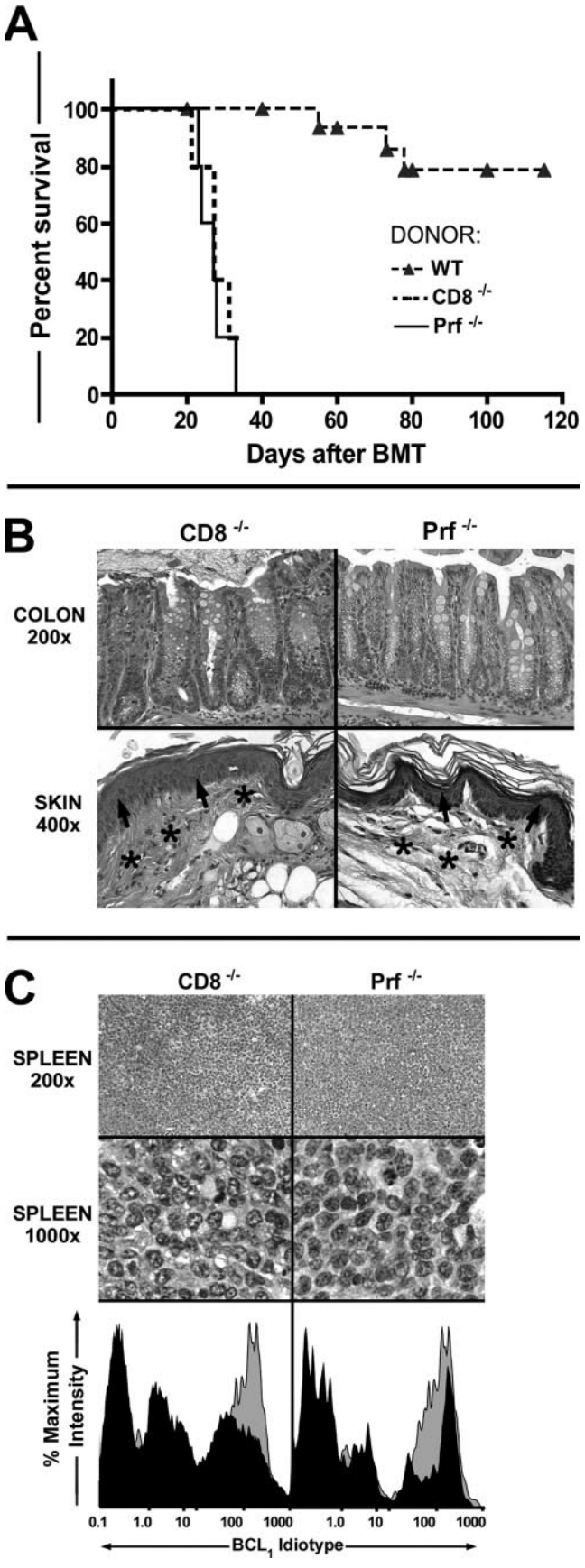

FIGURE 4. TLI/ATS-conditioned WT BALB/c hosts given transplants from CD8-deficient ($CD8^{-/-}$) or perforin-deficient ($Prf^{-/-}$) donors fail to clear tumor cells. *A*, Survival of WT BALB/c hosts conditioned with TLI/ATS and given injections of 5×10^2 BCL_1 tumor cells with BM and splenocytes from $CD8^{-/-}$ ($n = 10$), $Prf^{-/-}$ ($n = 10$), or WT ($n = 20$) donors. *B*, Microscopic examination of colon and skin from WT hosts given $CD8^{-/-}$ (*left panels*) and $Prf^{-/-}$ (*right panels*) transplants demonstrated no microscopic changes of acute GVHD in the colon. Skin sections showed dermal collagen deposition (*) and epidermal hyperplasia (arrows). One representative animal is shown per group. Sections shown were stained with H&E and viewed under ×200 magnification (colon) and ×400 magnification (skin), respectively. *C*, Splenic BCL_1 Id staining and histopathology in representative WT hosts receiving $CD8^{-/-}$ and $Prf^{-/-}$ donor transplants. All hosts had diffuse tumor infiltration of the spleen with disruption of splenic architecture (spleen, ×200, *upper panels*). Monomorphic infiltrates of atypical tumor cells were observed (spleen, ×1000, *middle panels*). Id staining of the spleens revealed considerable BCL_1 tumor Id-positive cells (black histograms, compared with positive control gray histograms from spleens of WT BALB/c hosts given TLI/ATS and BCL_1).

examined at 120 days had little or no evidence of GVHD in the colon and skin (Fig. 3*B*), $CD1d^{-/-}$ and $J\alpha\text{-}18^{-/-}$ hosts had severe submucosal inflammatory infiltrates, marked colonic crypt apoptosis, and crypt atrophy at autopsy. The skin of these $CD1d^{-/-}$ and $J\alpha\text{-}18^{-/-}$ hosts showed intense dermal infiltrates, epidermal apoptosis, and epidermal thickening (Fig. 3*B*).

Transplants from CD8-deficient and perforin-deficient donors fail to protect against progressive tumor growth

Transplantation of C57BL/6 bone marrow cells without donor peripheral T cells into BALB/c hosts conditioned with sublethal or lethal TBI has been reported to eliminate the BCL_1 tumor and antitumor activity was dependent on $CD8^+$ T cells in the donor marrow that expressed the cytolytic molecule, perforin (13, 19). In the current study, wild-type BALB/c hosts were given the TLI/ATS regimen followed by BCL_1 tumor cells, and bone marrow and splenocytes from $CD8^{-/-}$, $perforin^{-/-}$, or wild-type C57BL/6 donors. None of these hosts developed clinical signs of GVHD, but those given transplants from $CD8^{-/-}$ or $perforin^{-/-}$ donors died with progressive splenomegaly and pallor by day 35 (Fig. 4*A*). In contrast, 80% of hosts given transplants from wild-type donors survived at least 120 days. The difference in survival between hosts given $CD8^{-/-}$ transplants and those given wild-type transplants was statistically significant ($p < 0.01$), as was the difference in survival of hosts given $perforin^{-/-}$ transplants as compared with wild-type transplants ($p < 0.01$). Assessment of splenic BCL_1 tumor Id staining showed high levels of bright Id-positive cells at autopsy in hosts given transplants from $CD8^{-/-}$ and $perforin^{-/-}$ donors, (Fig. 4*C*, *lower panel*). Id-positive cells were easily detected in the blood at day 28 and their levels in the blood markedly increased at autopsy (data not shown). Hosts given $CD8^{-/-}$ or $perforin^{-/-}$ transplants demonstrated a diffuse tumor infiltrate in the spleen with loss of splenic architecture at autopsy (Fig. 4*C*, *upper panel*). Histopathologic analysis of the colonic mucosa in both groups of hosts showed a normal appearance with no crypt loss, inflammation, or increased crypt apoptosis (Fig. 4*B*). Skin sections showed mild epidermal hyperplasia and increased dermal collagen deposition consistent with mild chronic skin GVHD (Fig. 4*B*).

Host-type NKT cells markedly reduce the early rapid expansion of donor T cells in the lymphoid tissues and colon

We have previously shown that C57BL/6 donor T cells accumulate in the spleen, lymph nodes, and colon of BALB/c hosts conditioned with 800 cGy TBI by day 6 after bone marrow and spleen cell transplantation, in association with the onset of acute colitis and diarrhea (20). In the current study, we compared the accumulation of donor T cells in BALB/c hosts conditioned with either TBI/ATS or TLI/ATS at day 6 after transplantation of wild-type

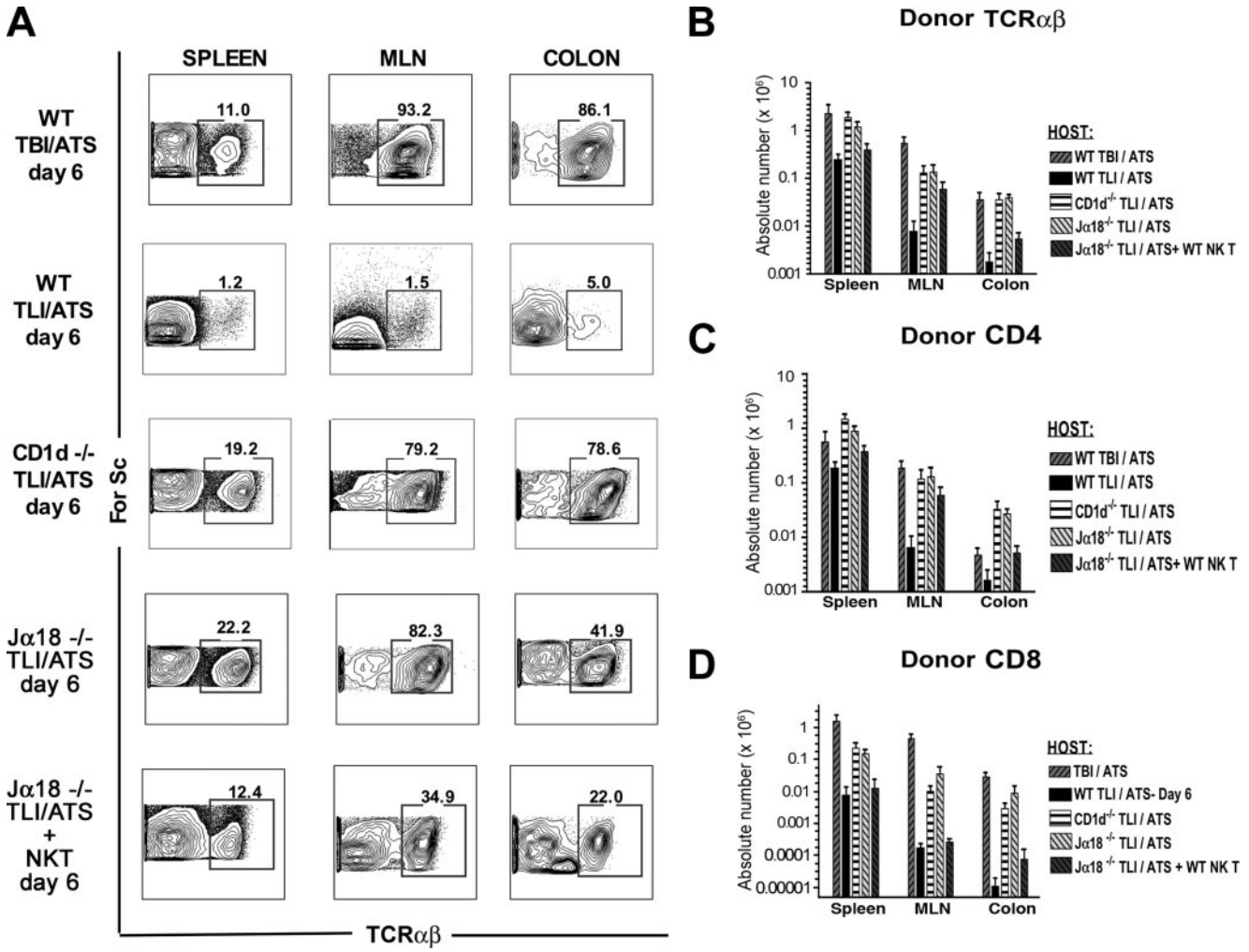

FIGURE 5. Host NKT cells inhibit accumulation of donor T cells in host lymphoid tissues and colon. *A*, Forward scatter vs TCR$\alpha\beta^+$ after gating on H-2K^{b+} in the spleen, mesenteric lymph nodes (MLN), and colon at day 6 following C57BL/6 BM and spleen cell transplantation in WT BALB/c hosts treated with TLI/ATS or TBI/ATS. Percentage of T cells is shown enclosed in boxes. Identical analyses are given for TLI/ATS-treated NKT cell-deficient CD1d$^{-/-}$ hosts and Jα18$^{-/-}$ hosts (*middle panels*) and TLI/ATS-treated Jα18$^{-/-}$ hosts that were given 0.5×10^6 purified WT BALB/c NKT cells (WT NKT) along with donor cells (*lower panel*). *B*, Comparison of mean absolute number of H-2K^{b+}TCR$\alpha\beta^+$ cells in spleen, MLN, and colon. *C*, Comparison of mean absolute number of H-2K^{b+}TCR$\alpha\beta^+$ CD4$^+$ cells in spleen, MLN, and colon. *D*, Comparison of mean absolute number of H-2K^{b+}TCR$\alpha\beta^+$CD8$^+$ cells in spleen, MLN, and colon. There were five to six hosts per group. Bars show means and brackets show SEs of two separate experiments.

C57BL/6 marrow cells (50×10^6) and spleen cells (60×10^6) without tumor cell injections. Fig. 5*A* shows the analysis of forward scatter vs TCR$\alpha\beta$ staining of the spleen, mesenteric lymph nodes (MLN), and colon of the hosts after gating on H-2K^{b+} cells. The most dramatic differences were observed in the MLN and colon, because T cells accounted for 86–93% of mononuclear cells in TBI/ATS-treated hosts but only 1–5% in TLI/ATS-treated hosts (Fig. 5*A*). To assess the affect of host NKT cells on the donor T cell accumulation, TLI/ATS-conditioned CD1d$^{-/-}$ and Jα-18$^{-/-}$ hosts were compared with the wild-type hosts. The NKT cell-deficient hosts showed a marked increase (at least 8-fold) in the percentage of donor T cells in the spleen, MLN, and colon as compared to wild-type hosts (Fig. 5*A*). Injection of 0.5×10^6 sorted NKT cells from untreated wild-type BALB/c mice into the Jα-18$^{-/-}$ hosts 6 h before the allogeneic transplant resulted in a marked reduction in the percentage of donor T cells in the lymphoid tissues and colon (Fig. 5*A*). The results indicate that conversion from mixed to full chimerism in the lymphoid tissues is slowed by the host NKT cells after TLI/ATS even though full chimerism is achieved by day 28 (Fig. 1*D*).

The mean ± SE absolute number of total (H-2K^{b+}) donor T cells, CD4$^+$ donor T cells, and CD8$^+$ donor T cells was also determined in the host tissues at day 6. Comparison of the total T cells showed a 10- to 50- fold decrease in TLI/ATS-conditioned wild-type hosts as compared with the TBI/ATS-conditioned hosts (Fig. 5*B*). Differences in the MLN and colon were highly significant ($p < 0.01$) between the two groups. In contrast, the differences in the absolute numbers of total donor T cells were not significant when CD1d$^{-/-}$ or Jα-18$^{-/-}$ TLI/ATS-treated hosts were compared with the TBI/ATS-treated hosts ($p > 0.05$). Differences in the absolute number of donor T cells between CD1d$^{-/-}$ or Jα-18$^{-/-}$ TLI/ATS-treated hosts and wild-type TLI/ATS-treated hosts were significant ($p < 0.05$) in both MLN and colon. Injection of sorted NKT cells into the Jα-18$^{-/-}$ TLI/ATS-treated hosts before transplantation significantly reduced the donor T cells in the colon ($p < 0.01$), but not in the spleen or MLN. When the absolute number of CD4$^+$ and CD8$^+$ donor T cells was analyzed separately in the different groups of hosts (Fig. 5, *C* and *D*), the addition of NKT cells was found to have a more profound impact ($p < 0.001$) in reducing the accumulation of CD8$^+$ T cells

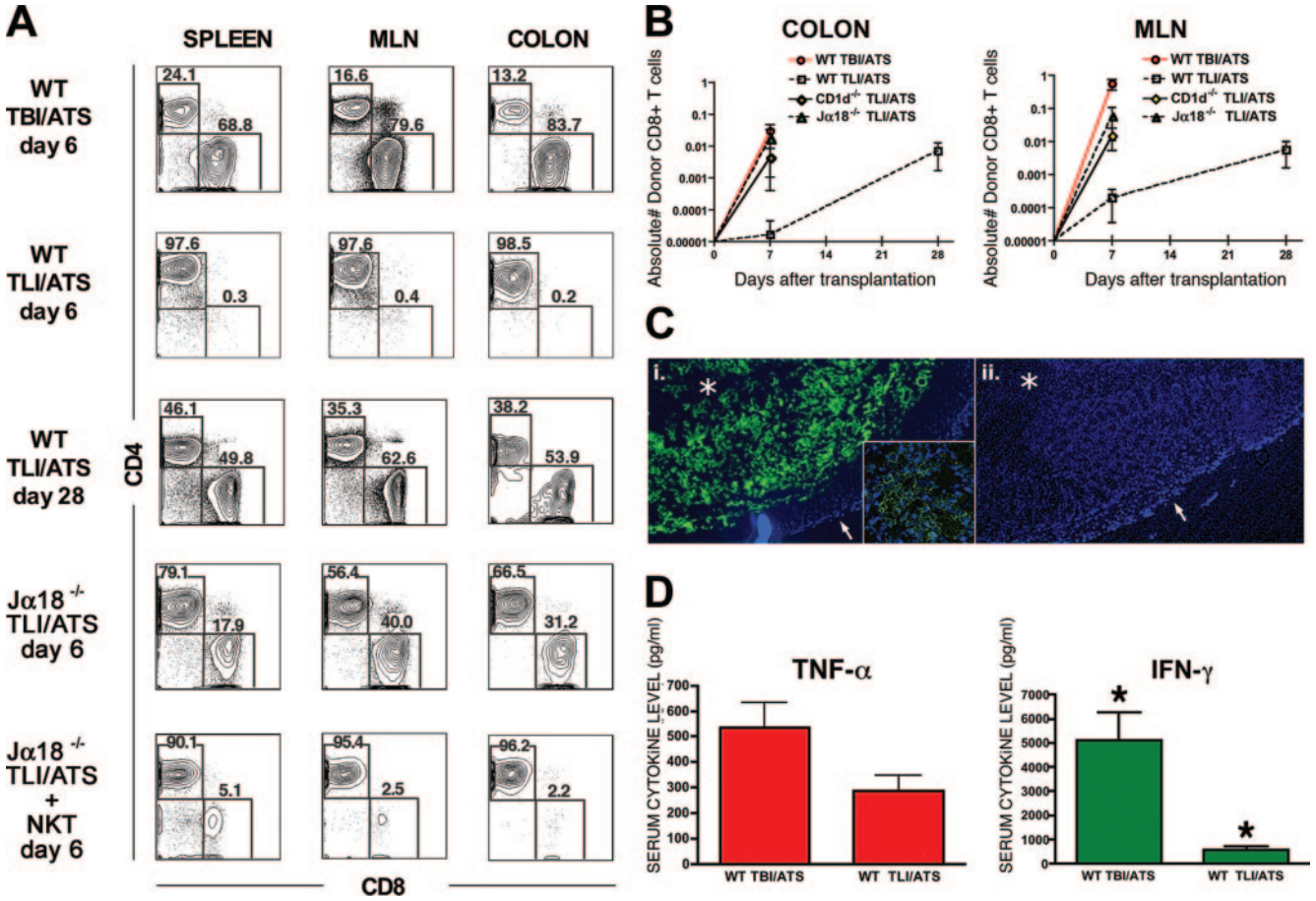

FIGURE 6. Host NKT cells inhibit early accumulation of donor $CD8^+$ T cells in host lymphoid tissues and colon, and TLI/ATS conditioned hosts show decreased serum levels of inflammatory cytokines after allogeneic transplantation. *A*, Representative analyses of gated donor H-$2K^{b+}TCR\alpha\beta^+$ cells, showing CD4 vs CD8 in spleen, MLN, and colon at day 6 following BM transplantation in WT hosts treated with TLI/ATS, WT hosts treated with TBI/ATS, $CD1d^{-/-}$ hosts treated with TLI/ATS, $J\alpha18^{-/-}$ hosts treated with TLI/ATS, and $J\alpha18^{-/-}$ hosts treated with TLI/ATS and injected with sorted WT NKT cells before transplantation. *B*, Comparison of mean absolute numbers of H-$2K^{b+}TCR\alpha\beta^+CD8^+$ T cells in colon and MLN of host groups represented in *A*. There were five to six hosts per group. Brackets represent SEs. *C*, Immunofluorescent staining of colon from TBI/ATS-treated vs TLI/ATS-treated hosts after transfer of 50×10^6 BM and 60×10^6 splenocytes from WT C57BL/6 donors. *i*, Colon (×40 final magnification) from a representative TBI/ATS-treated WT BALB/c host at day 6. DAPI stain shows nuclear detail of both host mucosal and donor cells (blue). Infiltrating $CD8^+$ lymphocytes (green) are seen (×40 final magnification), with a high power insert (×400) demonstrating clusters of $CD8^+$ lymphocytes (blue nuclear stain combined with green surface stain). The serosa is indicated by the bold white arrow (→), and lumen is labeled with an asterisk (∗). *ii*, Colon (×40) at day 6 in a TLI/ATS-treated host with a paucity of $CD8^+$ lymphocytes (green). Final magnifications include ×10 ocular and ×4 or ×40 objectives. *D*, Comparison of mean serum levels of TNF-α and IFN-γ at day 6 in WT BALB/c hosts given TBI/ATS vs TLI/ATS conditioning followed by transplantation of 50×10^6 BM and 60×10^6 splenocytes from WT C57BL/6 donors. Bars represent the means of five animals per group, and brackets show SEs.

in Jα-$18^{-/-}$ hosts as compared with the change in $CD4^+$ T cells ($p = 0.004$) in the colon.

Fig. 6*A* shows that although the $CD8^+$ donor T cells represented the large majority of the gated donor T cells in all three tissues in TBI/ATS-treated hosts at day 6, the $CD8^+$ donor T cells were at background levels in TLI/ATS-treated hosts. Interestingly, there was a slow accumulation of $CD8^+$ donor T cells in all three tissues such that they became the major subset in wild-type TLI/ATS-treated hosts at day 28 (Fig. 6*A*). The deficiency of NKT cells in Jα-$18^{-/-}$ hosts treated with TLI/ATS resulted in a marked increase in the percentage of $CD8^+$ donor T cells at day 6, and addition of wild-type NKT cells resulted in a marked decrease (Fig. 6*A*).

The rapid early rise in the absolute number of $CD8^+$ donor T cells in the colon and MLN of TBI/ATS-conditioned wild-type hosts, as well as in $CD1d^{-/-}$ and Jα-$18^{-/-}$ TLI/ATS-conditioned hosts is shown in Fig. 6*B*. All of these hosts died within 28 days following bone marrow and splenocyte transplantation (Fig. 1*A*). In contrast, the slow accumulation of $CD8^+$ donor T cells in wild-type TLI/ATS-conditioned hosts by day 28 (Fig. 6*B*) was associated with the absence of clinical signs of GVHD and survival for at least 100 days (Fig. 1*A*).

$CD8^+$ T cell accumulation in the colon and the production of key inflammatory cytokines are reduced In TLI/ATS Vs TBI/ATS-conditioned hosts

Immunofluorescent staining and analysis of the distribution of $CD8^+$ T cells in the colon in TBI/ATS-conditioned hosts at day 6 showed that $CD8^+$ T cells were observed infiltrating the areas between and below crypts with disruption of crypt architecture (Fig. 6*Ci*). In wild-type TLI/ATS-conditioned hosts at day 6, the $CD8^+$ T cells were rare in the mucosa, with preservation of the colonic architecture (Fig. 6*Cii*). At day 28, $CD8^+$ T cells remained rare in the mucosa, despite the increase in the absolute numbers of $CD8^+$ T cells in the colons of TLI/ATS-treated hosts (data not shown). The mean concentrations of the serum cytokines TNF-α and IFN-γ were reduced in TLI/ATS-conditioned and -transplanted wild-type hosts at day 6 relative to TBI/ATS-conditioned

hosts (Fig. 6*D*). The difference in TNF-α was not statistically significant ($p = 0.06$), whereas that of IFN-γ was statistically significant ($p = 0.02$).

Discussion

Our data demonstrate that BALB/c mice given nonmyeloablative conditioning with TLI and ATS followed by MHC-mismatched bone marrow and splenocyte transplants from C57BL/6 donors are protected not only against GVHD, but also against the progressive growth of the BCL_1 tumor. Some modifications of the donor cell transplant were made from our previous studies (9, 10); high doses of splenocytes (60×10^6) and bone marrow cells (50×10^6) were infused to maximize donor chimerism and graft antitumor activity. Using this regimen, almost all wild-type BALB/c hosts conditioned with TLI and ATS became >80% donor-type chimeras in all cell lineages tested (T cells, B cells, monocytes, and granulocytes) in the peripheral blood when assayed at day 28 after transplantation. The dose of infused donor T cells per kilogram of host body weight ($\sim 600 \times 10^6$/kg) is ~2-fold higher than that infused with G-CSF-mobilized PBMC transplants in humans conditioned with TLI/ATG for treatment of leukemia and lymphoma (8). These patients had a very low incidence of acute GVHD (<5%). Retained graft antitumor activity was documented in those who entered the study with lymphomas in partial remission and subsequently achieved complete remission (8).

TLI/ATS conditioning protected BALB/c hosts from acute lethal GVHD as compared with conditioning with a single dose of TBI or TBI and ATS. Protection was dependent upon residual host invariant NKT cells, because all Jα18$^{-/-}$ BALB/c hosts that lack the invariant Vα14Jα18 TCRα-chain that recognizes CD1d succumbed to acute GVHD after TLI/ATS conditioning. The latter subset of NKT cells (type I) is distinguished from the type II subset that recognizes CD1d but does not express the invariant TCR (22). The type II NKT cells have been shown to impair tumor surveillance by inhibiting $CD8^+$ T cell tumor killing function (23, 24). Our previous studies showed that almost all residual NKT cells in the spleen after TLI or TLI/ATS conditioning are type I, and ~80% of these are $CD4^+$ NKT cells that show a marked Th2 cytokine bias as judged by their vigorous secretion of IL-4 (9). The remaining 20% of the residual host splenic NKT cells after TLI/ATS conditioning are $CD4^-CD8^-$, and this subset of cells has recently been shown to facilitate in vivo tumor killing in murine sarcoma and melanoma clearance models (25). The latter host NKT cell subset may indeed contribute to the observed in vivo antitumor activity after TLI/ATS conditioning and transplant.

Although the presence of host regulatory NKT cells is required to prevent GVHD in the TLI/ATS-conditioned hosts, other regulatory T cells such as $CD4^+CD25^+Foxp3^+$ cells of host or donor origin may be required as well. In fact, our recent studies show that acute GVHD protection afforded by TLI/ATS conditioning is lost when donor cells are depleted of $CD4^+CD25^+$ regulatory T cells before transplantation (A. Pillai, et al., manuscript in preparation). In addition, changes in the function of host APCs may play a critical role in preventing GVHD and facilitating the elimination of tumor cells (26). In our ongoing studies of GVHD protection in the TLI/ATS system, we are investigating the role of different purified populations of regulatory T cells cotransferred alone or in combination with GVHD inducing T cells into myeloablated hosts with and without tumor cells.

Graft antitumor activity was clearly demonstrated in our study by the survival of hosts given TLI/ATS followed by allogeneic bone marrow and splenocyte transplantation, compared with the uniform tumor progression in wild-type hosts given the TLI/ATS-conditioning regimen and BCL_1 lymphoma without transplants. Progressive tumor growth was also seen after the transplantation of donor bone marrow cells without splenocytes, or after the transplantation of bone marrow and spleen cells from $CD8^{-/-}$ or perforin$^{-/-}$ donors. The results indicate that donor peripheral $CD8^+$ T cells expressing the perforin cytolytic molecule were required for killing and clearance of the BCL_1 tumor cells. It is possible that other pathways of $CD8^+$ T cell cytolysis of tumor cells including Fas ligand and granzyme molecules may contribute to BCL_1 tumor clearance as well. Donor $CD8^+$ T cells have been shown to eliminate BCL_1 tumor cells in TBI-conditioned hosts (13, 19) and in hosts conditioned with TLI and cyclophosphamide (3). In the latter report, tumor cells were injected before transplant conditioning of the host. Neither radiation nor chemotherapy contributed to tumor cell killing in the present report, because tumor cells were infused after host conditioning.

We demonstrate that regulatory NKT cells inhibit GVHD and allow donor $CD8^+$ T cell-mediated protection against progressive growth of BCL_1 tumor cells. The NKT cells may have attenuated graft antitumor activity in some hosts, because 20% (4 of 20) of wild-type hosts conditioned with TLI/ATS and given donor marrow and spleen cells had progressive growth of the tumor at autopsy. By contrast, none of the NKT cell-deficient CD1d$^{-/-}$ hosts (0 of 14) given the same regimen demonstrated progressive tumor growth at autopsy, but all died of GVHD. The kinetics of graft antitumor activity may be slowed by the NKT cells due to the slowed expansion of donor $CD8^+$ T cells during the first month after transplantation. However, it is notable that 80% of wild-type hosts had no detectable tumor cells in microscopic sections of the spleen at day 120 after transplantation.

Donor regulatory $CD4^+CD25^+$ T cells can also inhibit GVHD while retaining graft antitumor activity (12). However, host regulatory $CD4^+CD25^+$ T cells are not effective inhibitors of GVHD in MHC-mismatched transplant systems such as those used in the current study (13, 27). In this regard, $CD4^+CD25^+$ T cells differ in suppressive activity from the NKT cells, because both donor and host NKT cells have been shown to inhibit GVHD in MHC-mismatched systems (9, 11). The suppressive activity of host NKT cells has been reported to be enhanced by activation with the NKT cell-specific ligand, α-GalCer (14, 28). Enhancement of the GVHD suppressive activity and facilitation of the tumor killing activity of CD8 T cells by donor NKT cells has been reported after treatment of the donors with the fusion protein progenipoietin-1, a potent chimeric cytokine that stimulates both G-CSF and Flt-3L receptors (29).

In conclusion, the TLI and ATS-conditioning regimen prevented GVHD and retained protection against progressive tumor growth in 80% of recipients given MHC-mismatched bone marrow transplants. Prevention of lethal GVHD was dependent upon the presence of host NKT cells, and graft antitumor activity was dependent upon donor $CD8^+$ T cells and their production of perforin. The results help elucidate the mechanisms of GVHD protection and retention of graft antitumor activity in clinical trials using TLI/ATG conditioning (8).

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Disclosures

The authors have no financial conflict of interest.

References

1. Ferrara, J. L. M., and J. H. Antin. 1999. The pathophysiology of graft-versus-host disease. In *Hematopoietic Stem Cell Transplantation*, 2nd Ed. E. D. Thomas, K. G. Blume, and S. J. Forman, eds. Blackwell Science, Malden, pp. 310–315.
2. Giralt, S., E. Estey, M. Albitar, K. van Biesen, G. Rondon, P. Anderlini, S. O'Brien, I. F. Khouri, J. Gajewski, R. Mehra, et al. 1997. Engraftment of allogeneic hematopoietic progenitor cells with purine analog-containing chemotherapy: harnessing graft-versus-leukemia without myeloablative therapy. *Blood* 89: 4531–4536.
3. Khouri, I. F., M. Keating, M. Korbling, D. Przepiorka, P. Anderlini, S. O'Brien, S. Giralt, C. Ippoliti, B. von Wolff, J. Gajewski, et al. 1998. Transplant-lite: induction of graft-versus-malignancy using fludarabine-based nonablative chemotherapy and allogeneic blood progenitor-cell transplantation as treatment for lymphoid malignancies. *J. Clin. Oncol.* 16: 2817–2824.
4. Slavin, S., A. Nagler, E. Naparstek, Y. Kapelushnik, M. Aker, G. Cividalli, G. Varadi, M. Kirschbaum, A. Ackerstein, S. Samuel, et al. 1998. Nonmyeloablative stem cell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases. *Blood* 91: 756–763.
5. Curtis, R. E., L. B. Travis, P. A. Rowlings, G. Socie, D. W. Kingma, P. M. Banks, E. S. Jaffe, G. E. Sale, M. M. Horowitz, R. P. Witherspoon, et al. 1999. Risk of lymphoproliferative disorders after bone marrow transplantation: a multi-institutional study. *Blood* 94: 2208–2216.
6. Martin, P. J., J. A. Hansen, C. D. Buckner, J. E. Sanders, H. J. Deeg, F. R. Appelbaum, R. Clift, A. Fefer, and R. P. Witherspoon. 1985. Effects of in vitro depletion of T cells in HLA-identical allogeneic marrow grafts. *Blood* 66: 664–672.
7. Burnett, A. K., I. M. Hann, A. G. Robertson, M. Alcorn, B. Gibson, I. McVicar, L. Niven, S. Mackinnon, H. Hambley, and A. Morrison. 1988. Prevention of graft-versus-host disease by ex vivo T cell depletion: reduction in graft failure with augmented total body irradiation. *Leukemia* 2: 300–303.
8. Lowsky, R., T. Takahashi, Y. P. Liu, S. Dejbakhsh-Jones, F. C. Grumet, J. A. Shizuru, G. G. Laport, K. Stockerl-Goldstein, L. Johnston, R. Hoppe, et al. 2005. Protective conditioning for acute graft-versus-host disease. *N. Engl. J. Med.* 353: 1321–1331.
9. Lan, F., D. Zeng, M. Higuchi, P. Huie, J. P. Higgins, and S. Strober. 2001. Predominance of NK1.1$^+$TCR $\alpha\beta^+$ or DX5$^+$TCR $\alpha\beta^+$ T cells in mice conditioned with fractionated lymphoid irradiation protects against graft-versus-host disease: "natural suppressor" cells. *J. Immunol.* 167: 2087–2096.
10. Lan, F., D. Zeng, M. Higuchi, J. P. Higgins, and S. Strober. 2003. Host conditioning with total lymphoid irradiation and antithymocyte globulin prevents graft-versus-host disease: the role of CD1-reactive natural killer T cells. *Biol. Blood Marrow Transplant.* 9: 355–363.
11. Zeng, D., D. Lewis, S. Dejbakhsh-Jones, F. Lan, M. Garcia-Ojeda, R. Sibley, and S. Strober. 1999. Bone marrow NK1.1$^-$ and NK1.1$^+$ T cells reciprocally regulate acute graft versus host disease. *J. Exp. Med.* 189: 1073–1081.
12. Edinger, M., P. Hoffmann, J. Ermann, K. Drago, C. G. Fathman, S. Strober, and R. Negrin. 2003. CD4$^+$CD25$^+$ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat. Med.* 9: 1144–1150.
13. Hoffmann, P., J. Ermann, M. Edinger, C. G. Fathman, and S. Strober. 2002. Donor-type CD4$^+$CD25$^+$ regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J. Exp. Med.* 196: 389–399.
14. Hashimoto, D., S. Asakura, S. Miyake, T. Yamamura, L. Van Kaer, C. Liu, M. Tanimoto, and T. Teshima. 2005. Stimulation of host NKT cells by synthetic glycolipid regulates acute graft-versus-host disease by inducing Th2 polarization of donor T cells. *J. Immunol.* 174: 551–556.
15. Sonoda, K. H., M. Exley, S. Snapper, S. P. Balk, and J. Stein-Streilein. 1999. CD1-reactive natural killer T cells are required for development of systemic tolerance through an immune-privileged site. *J. Exp. Med.* 190: 1215–1226.
16. Cui, J., T. Shin, T. Kawano, H. Sato, E. Kondo, I. Toura, Y. Kaneko, H. Koseki, M. Kanno, and M. Taniguchi. 1997. Requirement for Vα14 NKT cells in IL-12-mediated rejection of tumors. *Science* 278: 1623–1626.
17. Warnke, R. A., S. Slavin, R. L. Coffman, E. C. Butcher, M. R. Knapp, S. Strober, and I. L. Weissman. 1979. The pathology and homing of a transplantable murine B cell leukemia (BCL_1). *J. Immunol.* 123: 1181–1188.
18. Palathumpat, V., S. Dejbakhsh-Jones, and S. Strober. 1995. The role of purified CD8$^+$ T cells in graft-versus-leukemia activity and engraftment after allogeneic bone marrow transplantation. *Transplantation* 60: 355–361.
19. Lan, F., D. Zeng, P. Huie, J. P. Higgins, and S. Strober. 2001. Allogeneic bone marrow cells that facilitate complete chimerism and eliminate tumor cells express both CD8 and T-cell antigen receptor-$\alpha\beta$. *Blood* 97: 3458–3465.
20. Dutt, S., J. Ermann, D. Tseng, Y. P. Liu, T. I. George, C. G. Fathman, and S. Strober. 2005. L-selectin and β_7 integrin on donor CD4 T cells are required for the early migration to host mesenteric lymph nodes and acute colitis of graft-versus-host disease. *Blood* 106: 4009–4015.
21. Kaplan, D. H., B. E. Anderson, J. M. McNiff, D. Jain, M. J. Shlomchik, and W. D. Shlomchik. 2004. Target antigens determine graft-versus-host disease phenotype. *J. Immunol.* 173: 5467–5475.
22. Seino, K. I., and M. Taniguchi. 2005. Functionally distinct NK T cell subsets and subtypes. *J. Exp. Med.* 202: 1623–1628.
23. Terabe, M. S., J. Swann, E. Ambrosino, P. Sinha, S. Takaku, Y. Hayakawa, D. I. Godfrey, S. Ostrand-Rosenberg, M. J. Smyth, and J. A. Berzofsky. 2005. A nonclassical non-Vα14Jα18 CD1d-restricted (type II) NKT cell is sufficient for downregulation of tumor immunosurveillance. *J. Exp. Med.* 202: 1627–1633.
24. Terabe, M. S., J. M. Matsui, M. Park, M. Mamura, N. Noben-Trauth, D. D. Donaldson, W. Chen, S. M. Wahl, S. Ledbetter, and B. Pratt. 2003. Transforming growth factor-β production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence. *J. Exp. Med.* 198: 1741–1752.
25. Crowe, N. Y., J. M. Coquet, S. P. Berzins, K. Kyparissoudies, R. Keating, D. G. Pellici, Y. Hayakawa, D. I. Godfrey, and M. J. Smyth. 2005. Differential anti-tumor immunity mediated by NKT cell subsets in vivo. *J. Exp. Med.* 202: 1279–1288.
26. Reddy, P., Y. Maeda, C. Liu, O. I. Krijanovski, R. Korngold, and J. L. M. Ferrara. 2005. A crucial role for antigen-presenting cells and alloantigen expression in graft-versus-leukemia responses. *Nat. Med.* 11: 1244–1249.
27. Anderson, B. E., J. M. McNiff, C. Matte, I. Athanasiadis, W. D. Shlomchik, and M. J. Shlomchik. 2004. Recipient CD4$^+$ T cells that survive irradiation regulate chronic graft-versus-host disease. *Blood* 104: 1565–1573.
28. Morecki, S., S. Panigrahi, G. Pizov, E. Yacovlev, Y. Gelfand, O. Eizik, and S. Slavin. 2004. Effect of KRN7000 on induced graft-vs-host disease. *Exp. Hematol.* 32: 630–637.
29. Morris, E. S., K. P. MacDonald, V. Rowe, T. Banovic, R. Kuns, A. L. Don, H. M. Bofinger, A. C. Burman, S. D. Olver, N. Kienzle, et al. 2005. NK T cell-dependent leukemia eradication following stem cell mobilization with potent G-CSF analogs. *J. Clin. Invest.* 115: 3093–3103.