Host natural killer T cells induce an interleukin-4–dependent expansion of donor CD4$^+$CD25$^+$Foxp3$^+$ T regulatory cells that protects against graft-versus-host disease

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Although CD4$^+$CD25$^+$ T cells (T regulatory cells [Tregs]) and natural killer T cells (NKT cells) each protect against graft-versus-host disease (GVHD), interactions between these 2 regulatory cell populations after allogeneic bone marrow transplantation (BMT) have not been studied. We show that host NKT cells can induce an in vivo expansion of donor Tregs that prevents lethal GVHD in mice after conditioning with fractionated lymphoid irradiation (TLI) and anti–T-cell antibodies, a regimen that models human GVHD-protective nonmyeloablative protocols using TLI and antithymocyte globulin (ATG), followed by allogeneic hematopoietic cell transplantation (HCT). GVHD protection was lost in NKT-cell–deficient Jα18$^{-/-}$ hosts and interleukin-4 (IL-4)$^{-/-}$ hosts, or when the donor transplant was Treg depleted. Add-back of donor Tregs or wild-type host NKT cells restored GVHD protection. Donor Treg proliferation was lost in IL-4$^{-/-}$ hosts or when IL-4$^{-/-}$ mice were used as the source of NKT cells for adoptive transfer, indicating that host NKT cell augmentation of donor Treg proliferation after TLI/antithymocyte serum is IL-4 dependent. Our results demonstrate that host NKT cells and donor Tregs can act synergistically after BMT, and provide a mechanism by which strategies designed to preserve host regulatory cells can augment in vivo donor Treg expansion to regulate GVHD after allogeneic HCT. (Blood. 2009;113:4458-4467)

Introduction

Graft-versus-host disease (GVHD), a major complication of allogeneic bone marrow transplantation, causes injury to the host intestines, skin, and liver,[1-4] and is mediated by donor conventional CD4$^+$ and CD8$^+$ T cells.[2,5] Both natural killer T cells (NKT cells)[6-8] and CD4$^+$CD25$^+$ T regulatory cells (Tregs)[9-11] can regulate GVHD without prior exposure to the target antigens.[6-16] Host NKT cells express an invariant Vα14Jα18 T-cell receptor (TCR)α chain, and secrete large amounts of interleukin (IL)–4 and/or interferon (IFN)–γ within hours after activation via TCR recognition of the nonpolymorphic CD1d antigen-presenting molecule.[13,17-21] Host or donor NKT cell–mediated GVHD protection has been reported to be dependent on NKT cell secretion of cytokines, which induce T helper (Th) 2 polarization of conventional T cells,[8,22] and thereby attenuate their capacity to induce GVHD.[8,12,13,23-27] Naturally occurring CD4$^+$CD25$^+$ Tregs of either host or donor origin can protect against GVHD by suppressing the function of donor conventional T cells through direct cell contact[28-33] or secretion of cytokines, including IL-10.[9,32,34] In nontransplant settings, activation of NKT cells using α-galactosyl ceramide, a marine sponge-derived glycolipid, can activate Tregs via NKT-cell secretion of IL-2.[35,36]

We investigated whether host NKT cells can interact synergistically with donor Tregs to protect against GVHD after bone marrow transplantation. Hosts were conditioned with total lymphoid irradiation (TLI) and antithymocyte serum (ATS), a regimen that protects mice against lethal GVHD that has been successfully applied to hematopoietic cell transplantation in humans to achieve a low incidence of acute GVHD.[37,38] Our results demonstrate the novel finding that host NKT cells and donor Tregs interact to prevent conventional donor T cells from expanding and inducing lethal GVHD. Proliferation of donor Tregs in vivo occurred after transplantation and was dependent on host NKT cell secretion of IL-4.

Methods

Mice

Wild-type (BALB/cJ and BALB/cByJ) and IL-4$^{-/-}$ male BALB/c (H-2^d) male mice, 8 to 10 weeks old, were obtained from The Jackson Laboratory (Bar Harbor, ME) and the Department of Comparative Medicine, Stanford University (Stanford, CA). Jα18$^{-/-}$ BALB/c,[39] wild-type C57BL/6 CD45.2 (H-2^b), and C3H (H-2^k) mice were bred in the Department of Comparative Medicine, Stanford University. CD8$^{-/-}$, CD4$^{-/-}$, and wild-type CD45.2 and CD45.1 C57BL/6 donor mice were obtained from The Jackson Laboratory. The Stanford University Committee on Animal Welfare (Administration Panel of Laboratory Animal Care) approved all mouse protocols and guidelines for euthanasia.

Monitoring of GVHD and survival

Survival and the signs of GVHD, including hair loss, hunched back, swollen face, and diarrhea, were monitored daily, and body weight was measured weekly after transplantation, as described before.[7]

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Irradiation

Irradiation was performed with a Philips x-ray unit (200 kV, 10 mA; Philips Electronic Instruments, Rahway, NJ) at a rate of 84 cGy/minute with a 0.5-mm Cu filter. TLI was delivered to the lymph nodes, thymus, and spleen with shielding of the skull, lungs, limbs, pelvis, and tail, as previously described.[7] 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 BALB/c recipients 18 to 24 hours before cell infusions.

Rabbit ATS

ATS was purchased from ACCURATE Laboratories (New York, NY) and adsorbed with BALB/c red blood cells before use. Host BALB/c mice were injected intraperitoneally with 0.05 mL ATS in 0.5 mL sterile normal saline on days −12, −10, and −8 before transplantation.

Antibodies and flow cytometric analysis

All cells were incubated with anti-CD16/32 (2.4G2; BD Biosciences, San Diego, CA) before antibody staining to block FcR-γII/III receptors. The following reagents/mAbs were used: allophycocyanin anti-TCRαβ clone H57-597, phycoerythrin (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, FITC anti–H-2K^b clone AF6-88.5 (BD Biosciences), and streptavidin-Texas Red (Molecular Probes, Eugene, OR). Propidium iodide was used to exclude dead cells. A modified dual laser FACSVantage (BD Biosciences) or FACSAria in the Stanford Shared FACS Facility, and FlowJo software (TreeStar, Ashland, OR) were used for data analysis. Background staining for donor-type cells in normal control BALB/c mice was 0.5% or less.

Purification and adoptive transfer of NKT cells

Splenocyte suspensions were preincubated with CD1d dimer-mouse immunoglobulin (Ig)G fusion protein (BD Biosciences) and PE-conjugated CD1d tetramer reagent obtained from the National Institutes of Health (NIH) Tetramer Facility (http://www.niaid.nih.gov/reposit/tetramer/index.html) for 60 minutes on ice, washed once with RPMI 1640/1% BSA, enriched for CD1d dimer-positive cells with antimouse IgG1 magnetic beads using the MidiMACS system, stained with PE-CD1d tetramer and allophycocyanin anti-TCRαβ mAb, and sorted to more than 95% purity using a modified FACSVantage or FACSAria apparatus in the Stanford Shared FACS Facility.

Depletion of CD25$^+$ T cells from donor transplants

Wild-type C57BL/6 donor bone marrow and splenocytes were separately stained with PE-conjugated anti-CD25 mAb (clone PC61; BD Biosciences) and depleted using EasySep anti-PE selection and magnetic nanoparticles (StemCell Technologies, Vancouver, BC). CD25 depletion was performed separately on bone marrow and splenocyte suspensions. Undepleted cells were prepared using the same immunomagnetic bead separation, and subsequently adding back the CD25$^+$ cells to the CD25-depleted marrow or spleen cells, followed by counting cells and combining marrow and splenocytes to the final concentration of the donor transplant inoculum.

Donor T-cell accumulation

Single-cell suspensions from host mesenteric lymph nodes and spleen were prepared at day 6, as described previously.[3,7] Isolation of colonic lamina propria lymphocytes was achieved by harvesting entire individual colons (cecum to anal verge), washing in RPMI/10% fetal bovine serum (FBS)/1 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; RPMI-10/HEPES), and cutting colons into 5 mm × 5-mm sections, followed by 6 washes. Colon sections underwent 2 serial 1-hour digestions in collagenase-E (95 IU/mL; Sigma-Aldrich, St Louis, MO), and postdigestion tissue fragments were passed over nylon mesh to create a single-cell suspension. The mononuclear cells were enriched by layering single-cell suspensions in 44% Percoll onto 66% Percoll in RPMI-10/HEPES, and spinning at 800*g* for 20 minutes. Cells were stained with biotin- or FITC-conjugated anti–H-2K^b, Cy5PE- or allophycocyanin-conjugated anti-TCRαβ, PE- or Cy7allophycocyanin-conjugated anti-CD8, and biotin- or Pacific Blue–conjugated anti-CD4 mAbs. Texas Red- or Cy7PE-conjugated streptavidin was used with the biotin-conjugated mAbs.

Donor CD4$^+$CD25$^+$Foxp3$^+$ T-cell accumulation

Cells were analyzed using the eBioscience (San Diego, CA) mouse regulatory T-cell staining kit (No. 88-8111) with modifications. Briefly, 25 μg per sample of ethidium monoazide was added to exclude dead cells (Invitrogen, Carlsbad, CA; Live-Dead Aqua; was used in later experiments), and each sample was stained using anti-CD16/32, biotin-conjugated anti–mouse H-2K^b clone AF6-88.5 (BD Biosciences), FITC- or Pacific Blue–conjugated anti-CD4 mAb clone RM4-5, allophycocyanin-conjugated anti-CD25 mAb clone PC61.5 (eBioscience), and Texas Red– or Cy7PE-conjugated streptavidin for surface staining. PE-conjugated anti–mouse Foxp3 mAb clone FJK-16s or PE-conjugated rat IgG2a isotype control was used for intracellular staining. Staining with the rat isotype control resulted in less than 0.8% PE-positive cells among gated H-2K^{b+}CD4$^+$ cells in all cases.

Microscopic assessment of GVHD severity

Animals were killed at day 6 after transplantation, and GVHD was scored using guidelines described previously[7] if they survived without morbidity. Skin, spleen, liver, and small and large intestine were harvested from each host, fixed in 10% formalin, and embedded in paraffin blocks, and 4- to 5-μm sections were stained with hematoxylin and eosin using standard protocols.[7] The descending colon, skin, and liver were assigned a score assessing severity of GVHD, and microscopic images were obtained, as described previously.[7] The mean GVHD score represents the mean plus or minus SEM among 5 animals per group.

The 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester mixed leukocyte reaction suppression assay

Stimulator splenocytes from normal C57BL/6, BALB/c, and third-party C3H male mice were irradiated with 3000 cGy using a Cesium source. Responder cells were splenocytes from congenic (CD45.1) C57BL/6 mice that were labeled with 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) using the CellTrace CFSE assay system (Molecular Probes) per manufacturer's instructions. Responder cells (5×10^4 cells) were incubated with stimulators (10^5 cells), with or without sorted H-2K^{d-}CD4$^+$CD25$^+$ spleen cells (5×10^4 cells) from either normal C57BL/6 mice or chimeric BALB/c host mice, for 72 hours. Triplicate wells were harvested, pooled, and stained for CD45.1 and CD4 markers. Proliferation was measured using the proliferation calculation function of FlowJo software, with proliferating percentage defined by 2 or more divisions.

In vivo CFSE proliferation assays

Splenocytes from congenic (CD45.1) C57BL/6 mice were labeled with CFSE using the CellTrace system, per manufacturer's instructions, and injected with bone marrow from CD45.2 C57BL/6 mice, at a concentration of 50×10^6 bone marrow cells and 60×10^6 splenocytes per mouse. Spleens of transplanted hosts were harvested at day 6, pooled from 2 to 3 mice per analysis, and stained for CD45.1, CD4, CD8, Foxp3, and CD25 markers (eBioscience fixation/permeabilization system), and Live-Dead Aqua (Invitrogen) to exclude dead cells. Cells were analyzed by 8-color flow cytometry using a FACSAria (BD Instruments, Stanford Shared FACS Facility), setting CFSE voltage threshold using syngeneic transplanted host control day 6 splenocytes.

Cytokine profiling

H-2K^{b+}CD4$^+$CD25$^+$ splenocytes (10^5 cells/well) were sorted from either wild-type C57BL/6 mice or from the spleens of wild-type BALB/c hosts given TLI/ATS and transplanted with 50×10^6 bone marrow and 60×10^6 splenocytes from wild-type C57BL/6 donors (day 6). A total of 10^5 cells/well was incubated in duplicate wells for 40 hours in 5% CO_2 at 37°C in RPMI 1640/10% FBS/Pen-Strep/2-mercaptoethanol/glutamine

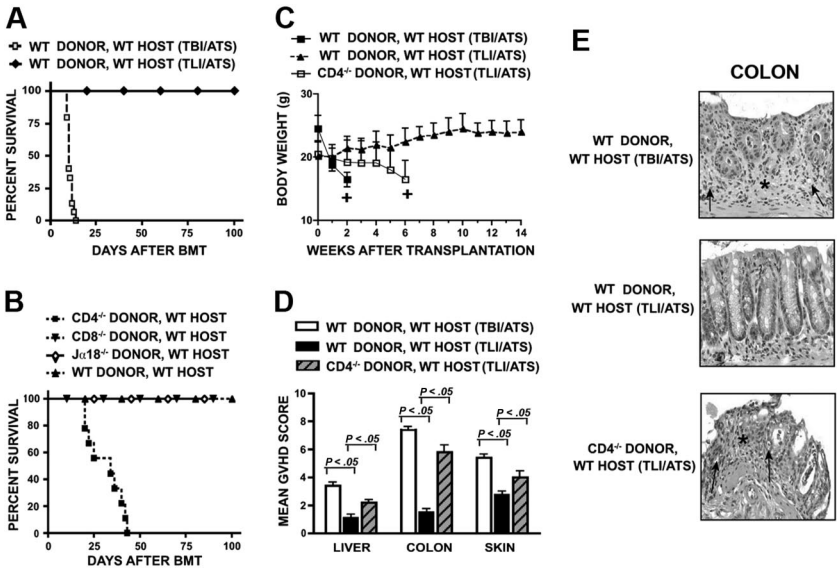

Figure 1. Protection against GVHD after TLI/ATS conditioning is dependent on CD4$^+$ T cells in the donor transplant. (A) Wild-type (WT) BALB/c host survival after 800 cGy TBI/ATS (n = 10) versus TLI/ATS (n = 8), followed by transplantation of 50 × 10^6 whole bone marrow cells and 60 × 10^6 splenocytes from wild-type C57BL/6 donors. Each group represents survival combined from 2 separate experiments. (B) Wild-type BALB/c host survival after TLI/ATS, followed by transplantation of 50 × 10^6 whole bone marrow and 60 × 10^6 splenocytes from CD4 T cell–deficient donors (CD4$^{-/-}$; n = 10), CD8 T cell–deficient donors (CD8$^{-/-}$; n = 10), invariant NKT cell–deficient donors (Jα18$^{-/-}$; n = 8), or WT donors (n = 8). Each group represents survival combined from 2 to 3 separate experiments. (C) Mean body weight (± SE) at serial time points after transplantation in wild-type BALB/c hosts receiving either 800 cGy TBI/ATS or TLI/ATS and 50 × 10^6 whole bone marrow and 60 × 10^6 splenocytes from wild-type donors, or wild-type TLI/ATS-conditioned hosts given CD4$^{-/-}$ donor bone marrow and splenocyte transplants. + indicates analysis was stopped when 2 hosts remained in the group. (D) Mean (± SE) histopathologic GVHD scores of colon, skin, and liver at day 6 from 3 groups of hosts from panel C. n = 5-8 for all groups. (E) Microscopic examination of hematoxylin/eosin-stained sections of colon at day 6 in 1 representative host from each group shown in panels C and D. WT BALB/c host given TBI/ATS with WT donor cells shows evidence of inflammatory infiltrate (*) and loss of normal crypt architecture in the colon, with separation of crypts by inflammation, lifting off of crypts from the basement membrane, and multiple areas of apoptotic crypt nuclei (→). WT BALB/c host given TLI/ATS conditioning with WT donor cells shows normal appearance. WT host given TLI/ATS and CD4$^{-/-}$ donor cells shows inflammatory infiltrate (*) and loss of normal crypt architecture in the host colon, with separation of crypts by inflammation and lifting off of crypts from the basement membrane. Specimens shown are at 300× final magnification.

(10% conditioned medium) with or without 10 ng/mL phorbol myristate acetate (PMA) plus 2 μM ionomycin. Supernatants were pooled from duplicate wells and analyzed by 21-plex Panomics Luminex assay, including IFN-γ, IL-4, and IL-10.

Statistical analysis

Kaplan-Meier survival curves were generated using Prism software (SAS Institute, Cary, NC), and statistical differences were analyzed using the log-rank (Mantel-Cox) test. Statistical significance in differences between mean weights, mean histopathologic GVHD scores, mean absolute numbers of isolated cell populations, mean percentage of proliferating cell populations, and cytokine concentrations was analyzed using the 2-tailed Student *t* test of means, assuming unequal variance. For all tests, a *P* value less than .05 was considered significant.

Results

CD4$^+$ T cells in the donor inoculum are required for protection against GVHD

BALB/c hosts conditioned with a single dose of total body irradiation (800 cGy TBI) and 3 doses of ATS conditioning died within 20 days after transplantation of C57BL/6 bone marrow cells (50 × 10^6) and spleen cells (60 × 10^6), whereas all TLI/ATS-conditioned hosts survived for at least 100 days (Figure 1A). TBI/ATS-treated hosts developed progressive weight loss (Figure 1C) and high histopathologic GVHD scores in the skin, liver, and colon at the time of autopsy (Figure 1D). The survival, kinetics of weight loss, and GVHD scores between the TBI/ATS and TLI/ATS groups were significantly different as judged by the log-rank test ($P < .001$, survival) or Student *t* test of independent means ($P = .04$, weight loss; $P = .04$, GVHD score). Both groups of mice became trilineage chimeras after transplantation (data not shown).

The role of donor CD4, CD8, and invariant NKT cells was investigated by comparing the development of GVHD using wild-type versus CD4$^{-/-}$, CD8$^{-/-}$, and invariant NKT cell–deficient Jα18$^{-/-}$ C57BL/6 donors. Surprisingly, the use of CD4$^{-/-}$ instead of wild-type donors resulted in the death of all hosts by day 45 (Figure 1B). In contrast, the use of CD8$^{-/-}$ and Jα18$^{-/-}$ donors gave a survival of at least 100 days. The hosts given CD4$^{-/-}$ donor transplants developed GVHD, as judged by significant weight loss in this group ($P < .01$; Figure 1C), and a significant increase in the mean histopathologic GVHD score at day 6 in the colon, skin, and liver ($P < .05$; Figure 1D). The most prominent early feature of GVHD in this model system, acute colitis at day 6, was not observed in TLI/ATS-conditioned hosts given wild-type C57BL/6 donor transplants (Figure 1E).

Both donor CD4$^+$CD25$^+$ Tregs and host NKT cells are required for protection against GVHD

We hypothesized that the donor CD4$^+$ cells likely to be required for GVHD protection in our model system were CD4$^+$CD25$^+$ Tregs. We depleted Tregs from the bone marrow and spleen cells of wild-type C57BL/6 donors by anti-CD25 mAb immunomagnetic bead separation, and reduced the Treg percentage among gated CD4$^+$ T cells from approximately 8% to less than 0.5% (Figure 2A). Undepleted transplants were prepared by anti-CD25 mAb separation of wild-type C57BL/6 bone marrow and splenocytes, followed by counting and recombining the CD25-depleted and CD25-enriched fractions before transplantation. When undepleted

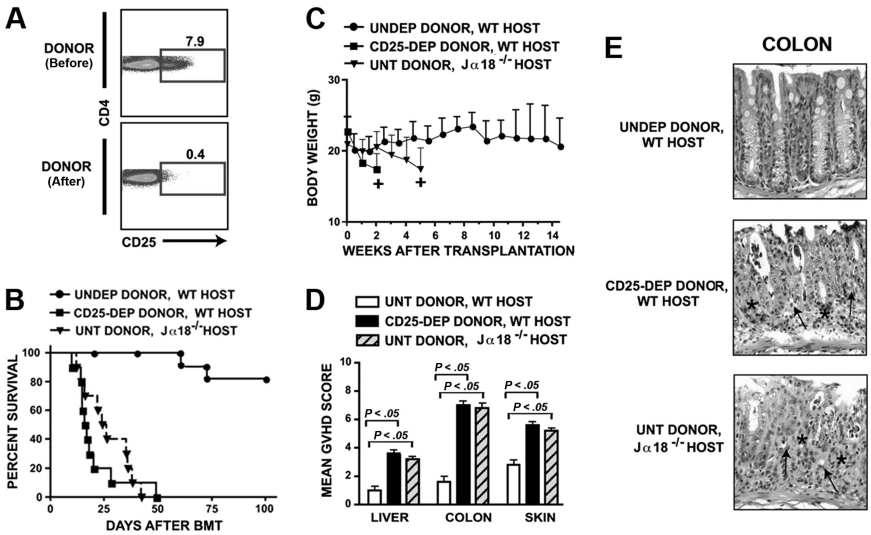

Figure 2. Protection against GVHD after TLI/ATS is dependent on both donor CD4$^+$CD25$^+$ Tregs and host invariant NKT cells. (A) Representative FACS patterns of the donor transplant inoculum before (PRE) and after (POST) CD25$^+$ T-cell depletion. Percentage of CD4$^+$CD25$^+$ T cells among gated CD4$^+$TCR$\alpha\beta^+$ T cells is shown enclosed in boxes. (B) Survival of wild-type BALB/c hosts after TLI/ATS conditioning, followed by transplantation of either undepleted (UNDEP DONOR, WT HOST; n = 8) or CD25$^+$ T cell–depleted (CD25-DEP DONOR, WT HOST; n = 9) donor grafts consisting of 50 × 10^6 bone marrow and 60 × 10^6 splenocytes from wild-type C57BL/6 donors. Also shown is survival of NKT cell–deficient Jα18$^{-/-}$ (n = 10) hosts conditioned with TLI/ATS and injected with 50 × 10^6 untreated whole bone marrow and 60 × 10^6 untreated splenocytes from wild-type C57BL/6 donors (UNT DONOR, Jα18$^{-/-}$ HOST). Each group represents survival combined from 2 to 3 separate experiments. (C) Mean body weight (± SE) of TLI/ATS-conditioned wild-type BALB/c hosts receiving either undepleted or CD25$^+$ T cell–depleted donor grafts or TLI/ATS-conditioned NKT cell–deficient Jα18$^{-/-}$ hosts receiving untreated donor grafts shown in panels A and B, at serial time points after transplantation. + indicates analysis was stopped when 2 hosts remained in the group. (D) Mean (± SE) histopathologic GVHD scores of colon, skin, and liver at day 6 from wild-type (WT) hosts given untreated or CD25-depleted transplants or Jα18$^{-/-}$ hosts given untreated transplants. n = 5 for all groups. (E) Microscopic examination of hematoxylin/eosin-stained sections of colon at day 6 in a representative host from each group shown in panel C. Comparison of WT BALB/c host given TLI/ATS and an undepleted transplant and WT BALB/c host given TLI/ATS and a CD25$^+$ T cell–depleted transplant demonstrates severe crypt apoptosis (→), inflammatory infiltrates (*), and crypt atrophy with loss of goblet cells in the latter compared with the former. NKT cell–deficient Jα18$^{-/-}$ host given TLI/ATS and untreated transplant reveals severe crypt apoptosis (→), inflammatory infiltrates (*), and crypt atrophy with loss of goblet cells. All micrographs are at 300× final magnification.

C57BL/6 donor cell inocula were compared with the CD25-depleted cells for the ability to induce GVHD in TLI/ATS-conditioned wild-type BALB/c hosts, CD25-depleted cells induced uniform death of hosts by day 50, whereas the undepleted cells induced only 20% mortality during the 100-day observation ($P = .001$; Figure 2B). CD25 depletion caused a significant worsening of weight loss ($P < .01$; Figure 2C), increase in GVHD ($P = .03$; Figure 2D), and characteristic histopathologic changes of GVHD, including crypt drop-out, marked mononuclear cell infiltrates surrounding crypts, and loss of goblet cells (Figure 2E).

As we have reported previously,[7] TLI/ATS-conditioned NKT cell–deficient Jα18$^{-/-}$ hosts given untreated donor cells died by approximately day 40 (Figure 2B), and developed acute GVHD, as judged by more profound weight loss ($P < .01$), higher GVHD scores than wild-type hosts ($P < .05$; Figure 2C,D, vs Figure 1C,D), and severe colitis (Figure 2E).

Accumulation of donor CD4$^+$CD25$^+$Foxp3$^+$ Tregs in the host spleen is augmented by host NKT cells

We compared the accumulation of donor Tregs in the host spleen 6 days after transplantation in TBI/ATS versus various TLI/ATS-conditioned hosts. We used intracellular Foxp3 as a specific Treg marker,[40] because CD25 can be expressed both on donor Tregs and on donor nonregulatory (conventional) T cells activated by host alloantigens.[2,5,28-30] Figure 3A shows a representative analysis of gated H-2K^{b+}CD4$^+$ T cells in the pretransplant donor splenocytes, in which 13.7% of the gated H-2K^{b+}CD4$^+$ T cells were Foxp3$^+$. More than 95% of these gated H-2K^{b+}CD4$^+$Foxp3$^+$ cells were CD25$^+$ (data not shown). In TBI/ATS-conditioned wild-type BALB/c hosts, approximately 0.6% of gated H-2K^{b+}CD4$^+$ T cells in the host spleen at day 6 were Foxp3$^+$ cells (Figure 3A). In TLI/ATS-conditioned hosts, approximately 5% were Foxp3$^+$ (Figure 3A), of which 90% or more were CD25$^+$ (data not shown). In congenic marker transplants of CD45.1$^+$ splenocytes and CD45.2$^+$ marrow cells from wild-type C57BL/6 donors, the H-2K^{b+}CD4$^+$CD25$^+$Foxp3$^+$ Tregs in the day 6 host spleen were predominantly CD45.1$^+$ (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article), that is, they derived from the splenocyte fraction of the donor graft. Sorted day 6 donor CD4$^+$CD25$^+$ T cells obtained from these hosts were suppressors of the mixed leukocyte reaction of normal responder C57BL/6 CD4$^+$ T cells to both BALB/c and C3H stimulator cells, with potency similar to that of sorted Tregs from untreated wild-type mice (Figure S2A). These in vivo expanded donor Tregs secrete significantly increased concentrations of IL-10 after activation compared with Tregs from untreated C57BL/6 donors (Figure S2B).

When TLI/ATS-conditioned Jα18$^{-/-}$ BALB/c hosts were given wild-type transplants, the percentage of CD4$^+$Foxp3$^+$ T cells was reduced to 0.3% of donor CD4$^+$ T cells (Figure 3A). Injection of 0.5 × 10^6 sorted NKT cells from wild-type BALB/c mice into TLI/ATS-conditioned Jα18$^{-/-}$ BALB/c hosts given transplants resulted in an increase in the Treg percentage to 3.5% (Figure 3A). The mean absolute numbers of H-2K^{b+}CD4$^+$CD25$^+$Foxp3$^+$ cells in the spleen of hosts are shown in Figure 3B. The mean number of donor Tregs in the spleen of wild-type BALB/c hosts conditioned with TLI/ATS (0.49 ± 0.04 × 10^5 cells) was at least 10-fold higher than the number recovered in NKT cell–deficient Jα18$^{-/-}$ BALB/c hosts (0.05 ± 0.03 × 10^5 cells; $P < .01$). The low number of donor Tregs observed in the Jα18$^{-/-}$ BALB/c hosts was not significantly different ($P = .94$) from that observed in wild-type BALB/c hosts conditioned with TBI/ATS (Figure 3B). Injection of wild-type host

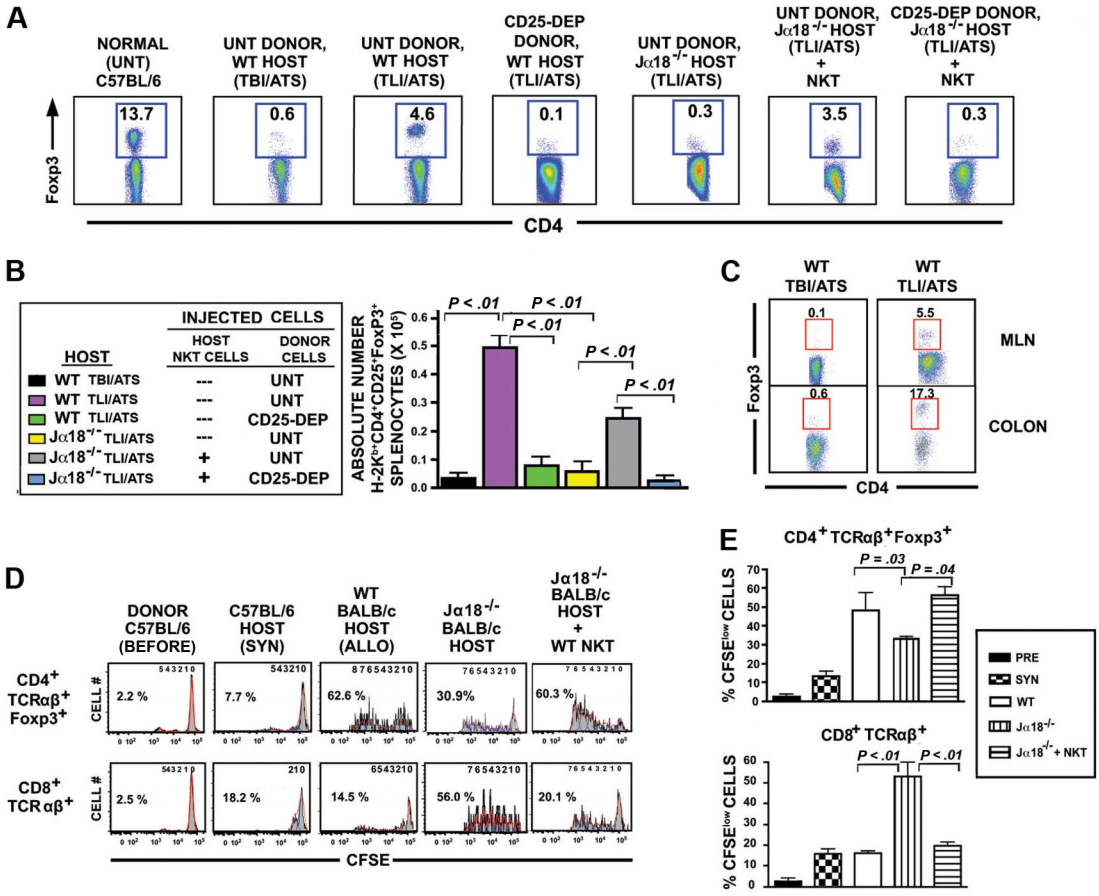

Figure 3. Host NKT cells facilitate the early proliferation of chimeric donor CD4$^+$Foxp3$^+$ Tregs. (A) Representative FACS analyses of CD4 versus intracellular Foxp3 staining at day 6 of gated H-2K^{b+}CD4$^+$ splenocytes from normal untreated (UNT) C57BL/6 control mice and from the following hosts: 800 cGy TBI/ATS wild-type BALB/c hosts or TLI/ATS wild-type BALB/c hosts given untreated transplants, TLI/ATS wild-type BALB/c hosts given CD25 T-cell–depleted transplants, TLI/ATS Jα18$^{-/-}$ BALB/c hosts given untreated transplants, TLI/ATS Jα18$^{-/-}$ BALB/c hosts given 0.5×10^6 sorted wild-type BALB/c NKT cells with untreated donor cell transplants, and TLI/ATS Jα18$^{-/-}$ BALB/c hosts given 0.5×10^6 sorted BALB/c NKT cells with CD25$^+$ T-cell–depleted donor cell transplants. Percentage of CD4$^+$Foxp3$^+$ cells among gated CD4$^+$ donor cells is shown enclosed in boxes. (B) Mean (± SE; $\times 10^5$) absolute number of H-2K^{b+}CD4$^+$CD25$^+$Foxp3$^+$ cells isolated from spleen at day 6 in groups of transplanted BALB/c hosts from panel A. n = 5-10 per group. (C) Representative FACS plots showing CD4 versus intracellular Foxp3 staining in gated H-2K^{b+}CD4$^+$ cells from MLN and colon of wild-type BALB/c hosts conditioned with either TBI/ATS or TLI/ATS at day 6 after injection of untreated donor cell transplants. Percentage of Foxp3$^+$ cells is shown. (D) Representative histograms showing CFSE-staining intensity versus cell number for gated CD45.1$^+$CD4$^+$TCRαβ$^+$Foxp3$^+$ cells and CD45.1$^+$CD8$^+$TCRαβ$^+$ cells from the pretransplant donor cell inoculum at day 0 (DONOR C57BL/6 [PRE]) and cells isolated from spleen of transplanted TLI/ATS-conditioned hosts at day 6. SYN, Wild-type CD45.2 C57BL/6 TLI/ATS-conditioned hosts given C57BL/6 CD45.1$^+$ splenocytes and CD45.2$^+$ marrow cells; ALLO, wild-type CD45.2 BALB/c TLI/ATS-conditioned hosts given C57BL/6 CD45.1$^+$ splenocytes and CD45.2$^+$ marrow cells. n = 2-3 pooled animals per group. Percentage of cells with 2 or more divisions is shown. (E) Mean (± SE; %) gated CD45.1$^+$CD4$^+$TCRαβ$^+$Foxp3$^+$ cells and CD45.1$^+$CD8$^+$TCRαβ$^+$ CFSE^{low} cells in the groups shown in panel D. Mean of 2 to 3 experiments. n = 2-3 pooled animals per group. Determination of CFSE^{low} cells is detailed in "Methods."

NKT cells into the Jα18$^{-/-}$ hosts significantly increased the mean absolute number of donor Treg cells, to $0.25 \times 10^5 \pm 0.03 \times 10^5$ cells ($P < .01$; Figure 3B).

CD25 depletion of the donor cell transplants resulted in a 50-fold decrease in the percentage of Foxp3$^+$ cells (0.1%) among gated donor CD4$^+$ T cells in the day 6 host spleen (Figure 3A), and a significant decrease in the mean absolute number of donor Tregs ($P < .01$; Figure 3B) compared with that in hosts given untreated donor cell transplants. Notably, the injection of host-type NKT-cells into Jα18$^{-/-}$ hosts conditioned with TLI/ATS did not result in a significant increase ($P = .92$) in either the percentage or absolute number of gated CD4$^+$Foxp3$^+$ donor cells in the spleen at day 6 when CD25-depleted transplants were used (Figure 3A,B). This indicates that the day 6 donor Tregs in the TLI/ATS-conditioned host spleen were derived from the original CD4$^+$CD25$^+$ cells in the donor cell inoculum, rather than from the CD4$^+$CD25$^-$ cells, as described elsewhere.[41-45]

We examined donor CD4$^+$Foxp3$^+$ T cells in other key GVHD target organs, including mesenteric lymph nodes (MLN) and colon at day 6 in wild-type BALB/c hosts conditioned with either TBI/ATS or TLI/ATS. The percentage (Figure 3C) and absolute number (data not shown) of CD4$^+$Foxp3$^+$ T cells were significantly higher in both MLN and colon in wild-type TLI/ATS-conditioned hosts than in those given TBI/ATS ($P < .01$).

Host NKT cells facilitate the early proliferation of donor CD4$^+$CD25$^+$Foxp3$^+$ Tregs

We performed transplants of 60×10^6 CFSE-labeled CD45.1 donor splenocytes injected with 50×10^6 CD45.2 donor C57BL/6 bone marrow cells into TLI/ATS-conditioned C57BL/6 (syngeneic) CD45.2 wild-type and BALB/c (allogeneic) CD45.2 wild-type or NKT-cell–deficient Jα18$^{-/-}$ hosts. Gated donor CD45.1$^+$TCRαβ$^+$CD4$^+$Foxp3$^+$ cells and CD45.1$^+$TCRαβ$^+$CD8$^+$ cells isolated from the spleen at day 6 after transplantation were evaluated by FACS analysis for CFSE-staining intensity, and percentage of proliferation was calculated using FlowJo proliferation software (Figure 3D,E). There was significantly increased

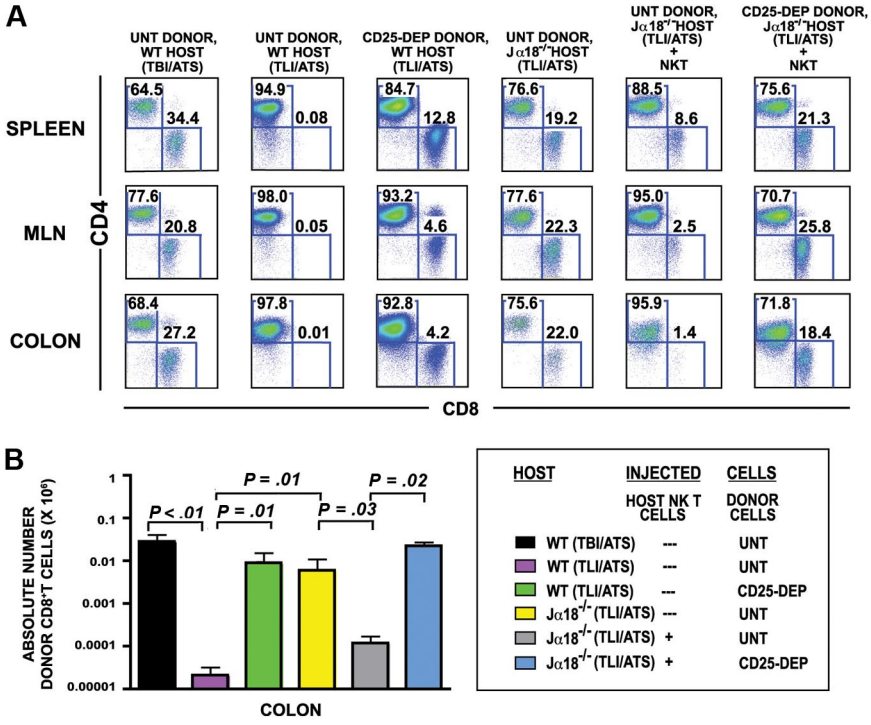

Figure 4. Host NKT cells and donor CD4$^+$CD25$^+$ T cells inhibit accumulation of donor CD8$^+$ T cells in TLI/ATS-conditioned host tissues. (A) Representative analyses of gated donor H-2K^{b+}TCR$\alpha\beta^+$ cells, showing CD4 versus CD8 in spleen, MLN, and colon at day 6 after bone marrow and splenocyte transplantation in TBI/ATS-conditioned BALB/c hosts given wild-type C57BL/6 transplants and in TLI/ATS-conditioned hosts in the following groups: wild-type BALB/c hosts given untreated C57BL/6 transplants, wild-type BALB/c hosts given CD25$^+$ T cell–depleted C57BL/6 transplants, invariant NKT cell–deficient Jα18$^{-/-}$ BALB/c hosts given untreated C57BL/6 transplants, and Jα18$^{-/-}$ BALB/c hosts receiving 0.5×10^6 purified wild-type BALB/c NKT cells 4 hours before either untreated or CD25 T cell–depleted C57BL/6 transplants. Percentages of CD4 or CD8 T cells are shown enclosed in boxes. (B) Comparison of mean absolute number (± SE; $\times 10^6$) of H-2K^{b+}CD8$^+$TCR$\alpha\beta^+$ cells isolated at day 6 from the colon of groups shown in panel A. n = 4-8 per group.

proliferation of CD45.1$^+$TCR$\alpha\beta^+$CD4$^+$Foxp3$^+$ cells ($P = .03$) and significantly decreased proliferation of CD45.1$^+$TCR$\alpha\beta^+$CD8$^+$ cells ($P < .01$) in the wild-type compared with the Jα18$^{-/-}$ allogeneic hosts (Figure 3E). Adoptive transfer of 0.5×10^6 wild-type BALB/c NKT cells into BALB/c Jα18$^{-/-}$ hosts significantly increased donor Treg proliferation ($P = .04$) and decreased CD8$^+$ T-cell proliferation ($P < .01$; Figure 3E). CFSE-staining intensity of Treg and CD8$^+$ T cells in the donor graft before transplantation and in syngeneic C57BL/6 host controls at day 6 after transplantation is shown for comparison (Figure 3D,E).

Accumulation of donor CD8$^+$ T cells in the host lymphoid tissues and colon is regulated by a combination of host NKT cells and donor CD4$^+$CD25$^+$Foxp3$^+$ Tregs

TLI/ATS conditioning had a marked impact on the accumulation of donor CD8$^+$ T cells in host tissues, because these cells account for less than 0.1% of donor cells in the spleen, MLN, and colon of TLI/ATS-conditioned hosts compared with greater than 20% in TBI/ATS-conditioned hosts (Figure 4A). Therefore, we focused subsequent studies on the donor CD8$^+$ T-cell subset.

When untreated transplants were given to TLI/ATS-conditioned Jα18$^{-/-}$ instead of wild-type BALB/c hosts, the percentage of donor CD8$^+$ T cells increased to approximately 20% of all donor T cells (Figure 4A), and the mean absolute number of donor CD8$^+$ T cells in the colon increased 100-fold ($P = .01$; Figure 4B). Injection of 0.5×10^6 sorted NKT cells from wild-type BALB/c mice into the Jα18$^{-/-}$ BALB/c hosts reduced the percentage (Figure 4A) and the mean absolute number (Figure 4B) of donor CD8$^+$ T cells in the colon approximately 20-fold ($P = .03$), to a level similar to that observed in TLI/ATS-conditioned wild-type hosts ($P > .05$, data not shown).

When CD25$^+$ T cells were depleted from the donor cell inoculum, there were marked increases in the percentage of donor CD8$^+$ T cells in the lymphoid tissues and colon (Figure 4A), and significant increases in the mean absolute number of donor CD8$^+$ T cells in the colon ($P = .01$; Figure 4B) of wild-type TLI/ATS-conditioned hosts. The absolute number of donor CD4$^+$ T cells also increased significantly after CD25$^+$ T-cell depletion (data not shown). Notably, the add-back of host NKT cells to TLI/ATS-conditioned Jα18$^{-/-}$ hosts failed to significantly reduce donor CD8$^+$ T-cell accumulation ($P = .63$) when CD25$^+$ T cell–depleted donor cells were used (Figure 4B). CD25 depletion of the donor cell inoculum resulted in significant increases in donor CD8$^+$ T-cell accumulation in the colon of TLI/ATS-conditioned Jα18$^{-/-}$ hosts given wild-type host NKT cells, compared with those given host NKT cells and untreated wild-type donor cell transplants ($P = .02$; Figure 4A,B).

IL-4 from hosts protects against GVHD and augments proliferation of donor CD4$^+$CD25$^+$Foxp3$^+$ Tregs

IL-4$^{-/-}$ hosts conditioned with TLI/ATS had significantly reduced survival ($P < .01$; Figure 5A), increased weight loss (Figure 5B), and increased colon inflammation (Figure 5C) compared with wild-type hosts given untreated donor transplants. The percentage and absolute number of donor Tregs in the host spleen were significantly lower ($P < .01$) in IL-4$^{-/-}$ versus wild-type hosts (Figure 5D,E), and the percentage of donor CD8$^+$ T cells in the colon was significantly higher ($P < .01$; Figure 5D vs Figure 4A). Injection of 0.5×10^6 sorted wild-type BALB/c NKT cells into the IL-4$^{-/-}$ hosts resulted in significant increases in the percentage and absolute number of splenic donor Tregs ($P = .02$; Figure 5D,E) and significant decreases in the absolute number (data not shown) and percentage of colonic donor CD8$^+$ T cells ($P < .01$; Figure 5D). In contrast, no significant change in the absolute number of day 6 donor Tregs was observed when IL-4$^{-/-}$ BALB/c NKT cells were injected into TLI/ATS-conditioned IL-4$^{-/-}$ hosts ($P = .15$; Figure 5E). We analyzed donor T-cell proliferation using CFSE-labeled wild-type C57BL/6 donor cells infused into IL-4$^{-/-}$ BALB/c hosts, with or without injection of wild-type NKT cells. The injection of wild-type NKT cells resulted in a significantly

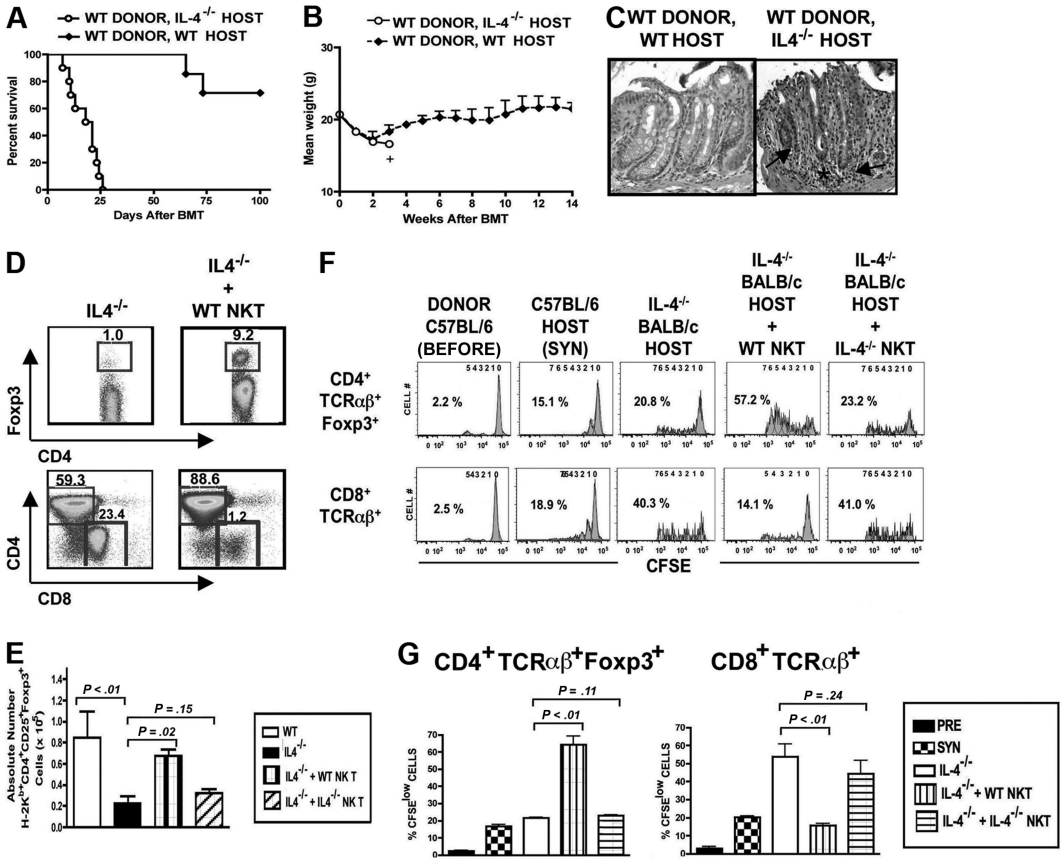

Figure 5. Host IL-4 is required for protection against GVHD, NKT-cell regulatory activity, and proliferation of donor Tregs after transplantation. (A) Wild-type (WT) versus IL-4$^{-/-}$ BALB/c host survival after TLI/ATS, followed by transplantation of 50 × 10^6 bone marrow and 60 × 10^6 splenocytes from wild-type C57BL/6 donors (IL-4$^{-/-}$, n = 10; WT, n = 7). Each group represents survival combined from 2 to 3 separate experiments. (B) Mean body weight (± SE) at serial time points after transplantation in hosts shown in panel A. + indicates analysis was stopped when 2 hosts remained in the group. (C) Microscopic examination of hematoxylin/eosin-stained sections of colon at day 6 in one representative host from each group shown in panels A,B. IL-4$^{-/-}$ BALB/c host given TLI/ATS with WT donor cells shows evidence of inflammatory infiltrate (*) in colonic crypts and multiple areas of apoptotic crypt nuclei (→). WT BALB/c host given TLI/ATS conditioning with WT donor cells shows normal appearance. Specimens shown are at ×300 final magnification. (D) Representative FACS analyses of CD4 versus intracellular Foxp3 staining of gated H-2K^{b+}CD4$^+$ splenocytes (top panels) and CD4 versus CD8 staining of gated H-2K^{b+}TCRαβ$^+$ splenocytes (bottom panels) 6 days after transplantation of untreated donor cells into TLI/ATS-treated IL-4$^{-/-}$ BALB/c hosts with and without injections of 0.5 × 10^6 sorted wild-type BALB/c NKT cells. NKT cells were injected 4 hours before donor cell transplantation. (E) Mean (± SE; ×10^5) absolute number of H-2K^{b+}CD4$^+$CD25$^+$Foxp3$^+$ cells isolated from spleen at day 6 from groups of transplanted BALB/c hosts from Figure 3D. n = 4-6 per group. Values for additional control wild-type BALB/c hosts given untreated donor cell transplants are shown for comparison (n = 5). (F) Representative histograms from 1 of 2 to 3 similar experiments showing CFSE-staining intensity versus cell number for gated CD45.1$^+$CD4$^+$TCRαβ$^+$Foxp3$^+$ cells and CD45.1$^+$CD8$^+$TCRαβ$^+$ cells from the pretransplant donor cell inoculum (DONOR C57BL/6 (PRE)) and cells isolated from spleen of transplanted TLI/ATS-conditioned hosts at day 6. *SYN*, Wild-type CD45.2 C57BL/6 TLI/ATS-conditioned hosts given C57BL/6 CD45.1$^+$ splenocytes and CD45.2$^+$ marrow cells; IL-4$^{-/-}$ CD45.2 BALB/c TLI/ATS-conditioned hosts were given no NKT cells (IL-4$^{-/-}$ BALB/c HOST), 0.5 × 10^6 sorted wild-type BALB/c NKT cells (IL-4$^{-/-}$ BALB/c HOST + WT NKT), or 0.5 × 10^6 sorted IL-4$^{-/-}$ BALB/c NKT cells (IL-4$^{-/-}$ BALB/c HOST + IL-4$^{-/-}$ NKT) 4 hours before untreated donor cell transplantation. n = 2-3 pooled animals per group. (G) Mean ± SD (%) gated CD45.1$^+$CD4$^+$TCRαβ$^+$Foxp3$^+$ cells and CD45.1$^+$CD8$^+$TCRαβ$^+$ CFSE^{low} cells in the groups shown in panel F. Mean of 2 to 3 experiments. n = 2-3 pooled animals per group.

higher proliferation of donor Tregs ($P = .001$) and significantly lower proliferation of donor CD8$^+$ T cells ($P = .004$). However, changes were not significant for either Tregs ($P = .11$) or donor CD8$^+$ T cells ($P = .24$) when IL-4$^{-/-}$ BALB/c NKT cells were adoptively transferred into IL-4$^{-/-}$ BALB/c hosts (Figure 5F,G).

We also compared the day 6 accumulation of donor CD8$^+$ T cells and donor Tregs in the spleen, MLN, and colon of control IFN-γ$^{-/-}$ and IL-10$^{-/-}$ BALB/c hosts conditioned with TLI/ATS to that of wild-type TLI/ATS-conditioned hosts. We found no significant differences in the accumulation of donor Tregs or donor CD8$^+$ T cells in IFN-γ$^{-/-}$ or IL-10$^{-/-}$ hosts (Figure S3) versus wild-type BALB/c hosts (Figures 3B, 4B).

Accumulation of donor CD8$^+$ T cells in the host lymphoid tissues and colon after TLI/ATS is affected by Tregs directly, without the requirement for conventional CD4$^+$CD25$^-$ donor T cells

It has been shown that TLI and anti-thymocyte antibody conditioning can induce a potent Th2 polarization of donor CD4$^+$ T cells,[23-24] and that Th2 polarization of conventional donor T cells can contribute to GVHD protection in conventional TBI transplant models.[25-26] To examine this possibility, we compared the day 6 accumulation of donor CD8$^+$ T cell GVHD effectors in wild-type BALB/c hosts given CD4$^{-/-}$ donor spleen and marrow cells with and without purified wild-type donor splenic Tregs in our TLI/ATS model. In these experiments, we found a significant ($P < .001$) reduction in donor CD8$^+$ T-cell accumulation at day 6 in both MLN and colon when wild-type Tregs were added at day 0 to CD4$^{-/-}$ donor cell transplants, without added conventional CD4$^+$CD25$^-$ T cells (Figure 6). Histopathologic changes of colon injury at day 6 were also markedly reduced when donor Tregs were added to CD4$^{-/-}$ donor cells (data not shown).

Discussion

We investigated the potential for cooperative interaction between host NKT cells and the naturally occurring donor CD4$^+$CD25$^+$

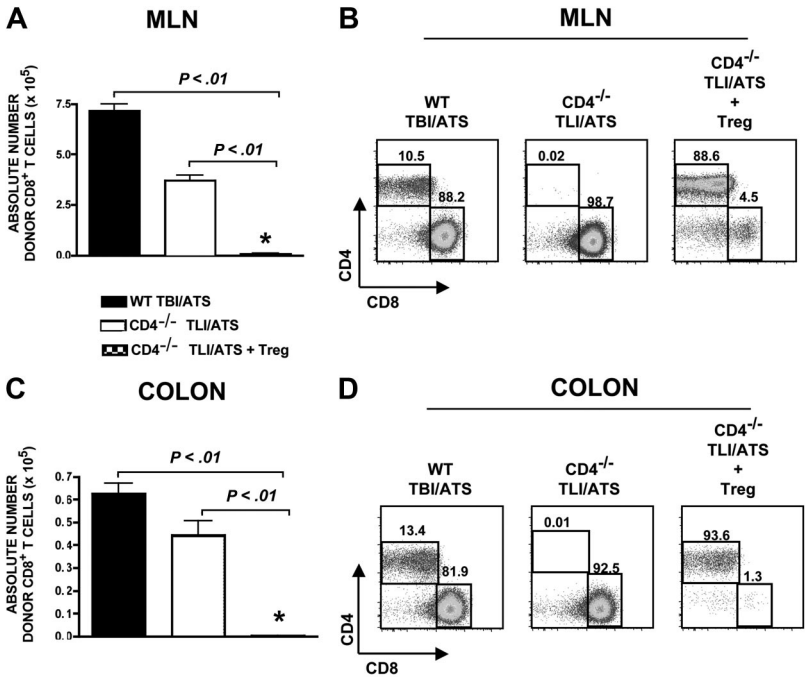

Figure 6. Donor Tregs added to CD4$^{-/-}$ donor transplants protect against donor CD8$^+$ T-cell infiltration in the colon. (A) Mean absolute number (± SD) H-2K^{b+}TCR$\alpha\beta^+$CD8$^+$ cells recovered from MLN of wild-type BALB/c hosts given 800 cGy TBI/ATS and wld-type C57BL/6 donor bone marrow and splenocytes, or TLI/ATS and transplantation of 50 × 10^6 bone marrow and 60 × 10^6 splenocytes from CD4$^{-/-}$ C57BL/6 donors with and without addition of 1.5 × 10^6 purified wild-type C57BL/6 CD4$^+$CD25$^+$ Tregs (n = 3, TBI/ATS; n = 5, CD4$^{-/-}$ TLI/ATS; n = 4, CD4$^{-/-}$ TLI/ATS + Treg). Each group represents data combined from 2 separate experiments. (B) FACS analyses of gated donor H-2K^{b+}TCR$\alpha\beta^+$ cells, showing CD4 versus CD8 in MLN at day 6 after bone marrow and splenocyte transplantation in 1 representative host from each group shown in panel A. (C) Mean absolute number (± SD) H-2K^{b+}TCR$\alpha\beta^+$CD8$^+$ cells recovered from colons of wild-type BALB/c hosts shown in panel A. (D) FACS analyses of gated donor H-2K^{b+}TCR$\alpha\beta^+$ cells, showing CD4 versus CD8 in colon at day 6 after bone marrow and splenocyte transplantation in 1 representative host from each group shown in panel C.

Tregs (nTregs) in the bone marrow graft and its effect on GVHD. We used the TLI/ATS-conditioning regimen because it has been shown to protect against acute GVHD in both murine and human studies compared with TBI or TBI/ATS,[7,12,13,37,38] and to alter host residual T cells to markedly increase the percentage of NKT cells among total host T cells.[12,13,37]

Host invariant NKT cells, host IL-4, and donor CD4$^+$CD25$^+$ nTregs are clearly required for protection against GVHD after TLI/ATS conditioning, because all Jα18$^{-/-}$ and IL-4$^{-/-}$ hosts given wild-type donor grafts and all wild-type hosts given nTreg-depleted grafts developed lethal GVHD after transplantation. In addition, there was no significant early requirement for conventional CD4$^+$CD25$^-$ donor T cells for protection, because add-back of purified nTregs to grafts from CD4$^{-/-}$ donors provided protection from donor CD8$^+$ T-cell accumulation in the colon at day 6. The results of our CD25-depletion studies further indicate that the protective donor Tregs were not induced from CD4$^+$CD25$^-$ donor T cells and are therefore unlikely to represent iTreg (induced Treg) cells. Whereas IL-4 has been shown to inhibit (via activation of GATA-3 and STAT-6) the TGF-β–dependent induction of Foxp3$^+$ iTregs from their CD4$^+$CD25$^-$ precursors,[42-45] IL-4 has been shown by other groups to either promote the proliferation or prevent apoptosis of nTregs, with variable effects on nTreg suppressor capacity.[46-48] We demonstrated an important influence of host NKT cells and host IL-4 on the accumulation of donor Tregs in the TLI/ATS-conditioned host spleen 6 days after transplantation. Donor Treg cell division and accumulation were markedly reduced in IL-4$^{-/-}$ or NKT cell–deficient Jα18$^{-/-}$ hosts compared with wild-type hosts. Injection of wild-type BALB/c NKT cells markedly increased donor Treg cell division and accumulation in both Jα18$^{-/-}$ and IL-4$^{-/-}$ hosts. The ability of NKT cells to restore Treg cell division was dependent on their production of IL-4, because IL-4$^{-/-}$ NKT cells failed to significantly increase Treg expansion. It remains to be determined whether IL-4 induces nTreg expansion directly, via IL-4 receptors on Tregs, or indirectly via other cells such as antigen-presenting cells. Activation of Tregs by NKT cell IL-2 secretion has been previously demonstrated using unirradiated murine or human cells.[35,36] NKT cells from irradiated mice develop a Th2 bias via unknown direct or indirect mechanisms, with increased IL-4 secretion after activation.[12,13,23] Reports suggest that IL-4-biased NKT cells may be resistant to apoptosis,[49] which could confer a survival advantage to these host cells after sublethal host-conditioning strategies. These findings may account for the importance of IL-4 in our system, where NKT cells predominate among host T cells after TLI.[12,13] In control experiments, we have additionally shown that 2 other key cytokines secreted by host NKT cells, IFN-γ and IL-10,[17,20-21] are not required for either donor Treg expansion or protection against early GVHD colitis after TLI/ATS and allogeneic bone marrow transplantation. It is of interest that expanded donor Tregs in GVHD-protected transplanted wild-type hosts secreted significantly higher levels of IL-4 and IL-10 than Tregs from untreated donor mice. This cytokine bias in donor Treg after TLI/ATS may reflect the influence of host NKT cell–derived IL-4, an area currently under study using adoptive transfers of IL-4–deficient NKT cells and/or sorted donor cell populations that are nonresponsive to IL-4.

Donor CD8$^+$ T-cell division, as well as accumulation in the colon, increased in NKT cell– or IL-4–deficient versus wild-type TLI/ATS-conditioned hosts and/or when CD4$^+$CD25$^+$ T-cell–deficient donor transplants were given. Injection of wild-type NKT cells into IL-4$^{-/-}$ hosts was effective in blocking donor CD8$^+$ T-cell division and accumulation, but the ability to block these was lost when IL-4$^{-/-}$ NKT cells were used. Notably, injection of BALB/c NKT cells into Jα18$^{-/-}$ hosts reduced the donor CD8$^+$ T-cell accumulation in the colon only if CD4$^+$CD25$^+$ T cells were present in the donor cell inoculum. In addition, donor Tregs were able to significantly reduce the accumulation of donor CD8$^+$ T cells in the host colon at day 6 even when conventional donor CD4$^+$CD25$^-$ T cells were absent from the donor graft. When conventional donor CD4$^+$CD25$^-$ T cells were present, Tregs reduced accumulation of both CD4$^+$ and CD8$^+$ T cells in the colon, although the reduction was more dramatic with donor CD8$^+$ T cells. Reduction of CD4$^+$ and CD8$^+$ T-cell accumulation was associated with reduced colon injury. This effect could potentially be explained by the potent IL-10–secreting capacity of the in vivo expanded Tregs. We are exploring the mechanisms by which these

expanded Tregs reduce conventional T-cell accumulation, including the effect of altered donor Treg cytokine secretion profiles, in more detail. In addition to NKT cells and Tregs, we are also currently investigating the potential for a larger cellular network of immunoregulation, including the role of host and donor APC populations expressing the critical NKT-stimulatory molecule CD1d.[17-19,21,33]

In conclusion, we have elucidated a key in vivo posttransplant interaction between 2 naturally occurring regulatory cell populations, host NKT cells and donor Tregs, after a nonmyeloblative bone marrow transplantation regimen that protects against GVHD. Unlike data in the existing literature, GVHD protection required neither activation of NKT cells with glycolipids nor pretransplant exposure of Tregs to alloantigens. Previous murine studies in which donor Tregs ameliorated GVHD in myeloablative TBI-conditioned hosts used donors tolerized to host alloantigens after donor-specific transfusions and/or costimulatory blockade,[50] or transplants in which the ratio of donor Tregs to conventional donor T cells has been manipulated ex vivo to as high as 10:1 in the pretransplantation donor cell inoculum.[9,10,14,15] Instead, we achieved GVHD protection through host conditioning that alters the balance of residual T-cell subsets to enrich host innate regulatory cells, and thereby increases the expansion of donor Tregs in vivo. Such immune regulatory cell interactions provide new insight into mechanisms of GVHD protection after TLI and anti-thymocyte antibody conditioning in humans given allogeneic hematopoietic cell transplants for treatment of leukemia and lymphoma.[37] These results open important new avenues in the design of host conditioning and transplant regimens that harness these protective immunoregulatory interactions, and point to potential host-conditioning strategies that could facilitate in vivo donor Treg expansion from the unmanipulated donor cell graft.

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Authorship

Contribution: A.B.P. designed the research, performed experiments, collected and analyzed data, prepared figures, and wrote the paper; T.I.G. analyzed data and prepared figures; S.D. performed experiments and collected data; and S.S. analyzed data and wrote the paper.

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