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Intestinal Helminths Regulate Lethal Acute Graft-versus-Host Disease and Preserve the Graft-versus-Tumor Effect in Mice

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Donor T lymphocyte transfer with hematopoietic stem cells suppresses residual tumor growth (graft-versus-tumor [GVT]) in cancer patients undergoing bone marrow transplantation (BMT). However, donor T cell reactivity to host organs causes severe and potentially lethal inflammation called graft-versus-host disease (GVHD). High-dose steroids or other immunosuppressive drugs are used to treat GVHD that have limited ability to control the inflammation while incurring long-term toxicity. Novel strategies are needed to modulate GVHD, preserve GVT, and improve the outcome of BMT. Regulatory T cells (Tregs) control alloantigen-sensitized inflammation of GVHD, sustain GVT, and prevent mortality in BMT. Helminths colonizing the alimentary tract dramatically increase the Treg activity, thereby modulating intestinal or systemic inflammatory responses. These observations led us to hypothesize that helminths can regulate GVHD and maintain GVT in mice. Acute GVHD was induced in helminth (*Heligmosomoides polygyrus*)–infected or uninfected BALB/c recipients of C57BL/6 donor grafts. Helminth infection suppressed donor T cell inflammatory cytokine generation and reduced GVHD-related mortality, but maintained GVT. *H. polygyrus* colonization promoted the survival of TGF-β–generating recipient Tregs after a conditioning regimen with total body irradiation and led to a TGF-β–dependent in vivo expansion/maturation of donor Tregs after BMT. Helminths did not control GVHD when T cells unresponsive to TGF-β–mediated immune regulation were used as donor T lymphocytes. These results suggest that helminths suppress acute GVHD using Tregs and TGF-β–dependent pathways in mice. Helminthic regulation of GVHD and GVT through intestinal immune conditioning may improve the outcome of BMT. *The Journal of Immunology*, 2015, 194: 1011–1020.

Graft-versus-host disease (GVHD) is a major and potentially severe complication of bone marrow transplantation (BMT). The disease is mediated by alloreactivity of donor T lymphocytes to recipient major or minor histocompatibility Ags (1, 2). Although the acute form of GVHD affects the skin, intestine, and liver, chronic GVHD exhibits multiorgan infiltration similar to various autoimmune diseases (3).

Intestinal inflammation in GVHD simulates inflammatory bowel diseases (IBDs), a group of immunological disorders that includes ulcerative colitis and Crohn's disease. Furthermore, allelic variants of the mammalian receptor protein for bacterial muramyldipeptide, CARD15/NOD2, influences the propensity to develop Crohn's disease, as well as GVHD (4, 5). Certain genetic variants of IL-23R protect individuals from these two disorders (6, 7). Inflammation in mouse models of IBD or GVHD is controlled by various immunomodulatory mechanisms that include regulatory T cells (Tregs) that suppress inflammation driven by effector T lymphocytes (1, 4, 8, 9). Tregs express the transcription factor Foxp3 and contribute to intestinal immune regulation by cell contact–dependent mechanisms or by the production of modulating cytokines, such as IL-10 and TGF-β (9–11). Helminthic regulation of intestinal immunity is associated with activation of Treg subsets and induction of regulatory cytokine production and depends on intact TGF-β circuitries (12–15).

The immune modulatory murine nematode *Heligmosomoides polygyrus* briefly resides in the submucosa of the mouse duodenum after oral administration and then remains in intestinal lumen without causing systemic infection, until the adult worm is expelled. We demonstrated previously that helminths like *H. polygyrus* trigger intestinal Treg activity and regulatory cytokine generation with consequent regulation of colitis in mice (13, 16). Other parasitic infestations may have immunosuppressive properties and were shown to reduce clinical activity in conditions such as multiple sclerosis, celiac disease, and IBD (17–21).

Although GVHD can be prevented by depleting donor T cells from the graft, recipients then are predisposed to severe infectious diseases. Engraftment, as well as the graft-versus-tumor (GVT) effect, may be diminished by donor T cell removal (1, 22, 23). In preclinical mouse models, several laboratories showed that GVHD can be prevented with preserved antitumor immunity (GVT) by coadministration of donor conventional T cells and Tregs given in equal numbers (22,

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The online version of this article contains supplemental material.

Abbreviations used in this article: BM, bone marrow; BMT, bone marrow transplantation; DN, dominant negative; GVHD, graft-versus-host disease; GVT, graft versus tumor; IBD, inflammatory bowel disease; LAP, latency-associated protein; MFI, mean fluorescent intensity; MLN, mesenteric lymph node; TBI, total body irradiation; TCD, T cell depleted; Treg, regulatory T cell; WT, wild-type.

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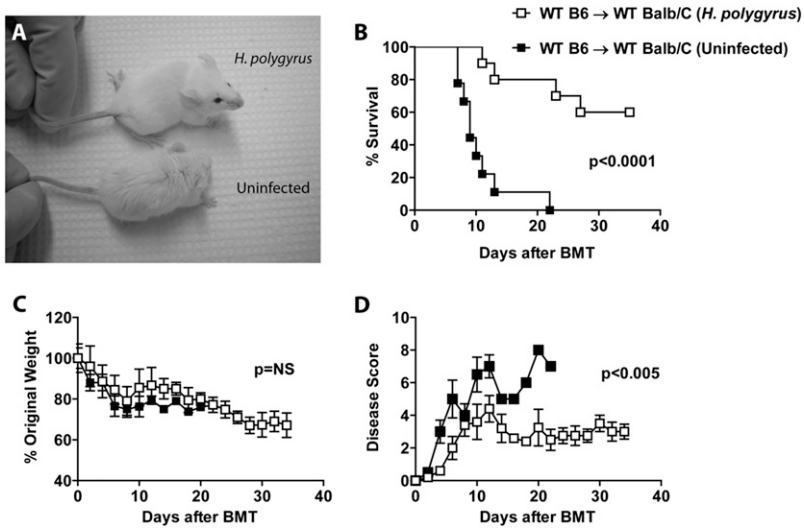

FIGURE 1. Helminths regulate acute GVHD in mice. (**A**) *H. polygyrus* infection protects mice from the severe inflammation during acute GVHD. Six days after BMT, *H. polygyrus*–infected animals displayed less skin discoloration or hunching body posture compared with uninfected mice, with representative examples shown. (**B**) Kaplan–Meier survival curves of *H. polygyrus*–infected or uninfected BALB/c recipients that received TCD BM and total splenic T cells from C57BL/6 mice. Cumulative data from three independent experiments (n = 9 uninfected, n = 10 infected). (**C**) Weight change in the mice in (B) during the follow-up period of the survival experiment. (**D**) Disease score for uninfected (n = 9) and *H polygyrus*–infected (n = 10) mice during the entire course of the survival experiment.

24–26). However, the production of high numbers of human Tregs suitable for infusion remains a technically challenging goal.

In this study, we show that *H. polygyrus* treatment of the recipient protects mice from fatal acute GVHD and sustains GVT. Regulation of GVHD is associated with induction of Tregs that may regulate Th1 inflammation by means of TGF-β expression and secretion. Helminths reduce GVHD-related Th1 inflammation. Furthermore, in a TGF-β–dependent manner, *H. polygyrus* administration decreases GVHD-related mortality. Because the intestine is a primary organ for GVHD generation (5, 27), our results open the possibility that intestinal immune conditioning of patients prior to BMT may be a useful strategy to reduce GVHD-related morbidity and mortality and preserve the donor graft's antitumor immunity.

Materials and Methods

Mice, H. polygyrus *administration, and egg counting*

We used wild-type (WT) C57BL/6 (H2b) and BALB/c (H2d) mice (Jackson Laboratory, Bar Harbor, ME), as well as a C57BL/6 mouse strain with

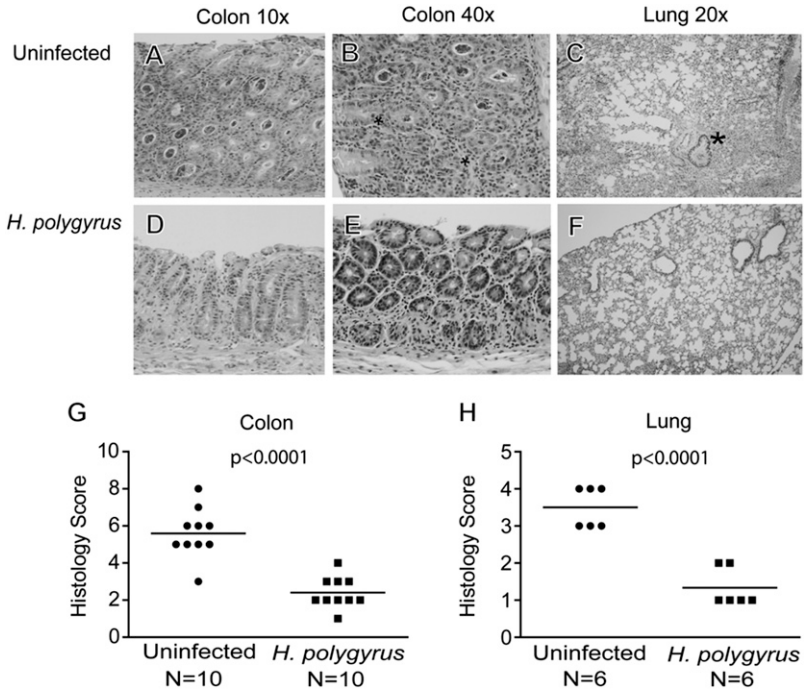

FIGURE 2. Helminth infection is associated with decreased GVHD-related inflammatory changes in the colon and the lungs. Six days after BMT, colons from uninfected (**A** and **B**) and helminth-infected (**D** and **E**) mice were isolated and fixed, and histopathological analysis was performed in 6-μm-thick sections under low (10×) (A and D) and high (40×) (B and E) magnification. The amount of inflammation and the number of apoptotic bodies in intestinal epithelium (*) were significantly decreased in *H. polygyrus*–infected mice (D and E) compared with tissues from uninfected animals (A and B). (**G**) Severity of colonic inflammation in colons from uninfected and *H. polygyrus*–infected mice was scored as described in *Materials and Methods*. Representative images (A, B, D, and E) and colitis scores (G) from 10 uninfected and 10 *H. polygyrus*–infected mice [each symbol in (G) represents the colonic GVHD score from an individual mouse] from three independent experiments. Horizontal lines represent the mean. Similarly, lungs from uninfected (**C**) and helminth-infected (**F**) mice were isolated, and histopathological analysis was performed in 6-μm-thick sections under low-power magnification (×20). Severity of inflammation in the lungs was scored as described in *Materials and Methods*. The dense inflammatory infiltrates (*) in samples from uninfected animals (C) were significantly decreased in lungs from helminth-infected mice (F). Representative images (C and F) and inflammatory scores (**H**) of GVHD-related inflammation in the lung from six uninfected and six *H. polygyrus*–infected mice [each symbol in (H) represents the lung GVHD score from an individual mouse] from two independent experiments.

a T cell–specific defect in TGF-β signaling (Cd4-TGFBR2, Jackson Laboratory #005551; also called TGF-βRII dominant negative [DN]) (H2b) (28). Five- to six-week-old BALB/c mice were inoculated with 150 *H. polygyrus* third-stage larvae by oral gavage. Infective *H. polygyrus* third-stage larvae [original specimens archived at the U.S. National Helminthological Collection no. 81930; also named *H. polygyrus (bakeri)* or *H. bakeri* in some publications (29, 30)] were obtained from mouse fecal cultures of eggs by the modified Baermann method and stored at 4˚C until used. The number of eggs in hydrated stool pellets was enumerated in duplicate at the indicated time points for each mouse and are shown as egg number/stool weight. Mice were housed and handled following national guidelines and as approved by the Animal Review Committee of the University of Iowa. Three weeks after initiation of helminth infection, mice underwent conditioning for BMT.

Cell purification for GVHD induction

Donor bone marrow (BM) cells were obtained from the femurs and tibias of uninfected, 5–8-wk-old C57BL/6 mice, and T cells were depleted using mouse pan-T cell beads (Dynal Biotech), according to the manufacturer's instructions. Donor T lymphocytes ($CD3^+$) were magnetically enriched from splenic single-cell suspensions of uninfected, 5–8-wk-old C57BL/6 and TGF-βRII DN mice using the Pan T Cell Isolation Kit (Miltenyi Biotec).

Cell purification for in vitro cultures

To determine TGF-β cytokine generation of Treg-enriched and Treg-depleted cultures, $CD4^+$ T cells were purified from splenic and mesenteric lymph node (MLN) single-cell suspensions of *H. polygyrus*–infected and uninfected BALB/c mice, using a CD4 T cell Isolation Kit (Miltenyi Biotec), and separated into $CD25^+$ and $CD25^-$ fractions using anti-CD25 PE labeling, followed by magnetic separation with anti-PE beads (Miltenyi Biotec). Enrichment or depletion efficiency was >98% with these techniques (data not shown). To determine helminthic regulation of donor T cell IFN-γ, TNF-α, and IL-4 cytokine output during GVHD, donor $CD3^+$ T cells were sorted from total anti-CD3 FITC–stained and anti-H2b PE–stained splenocytes from uninfected and *H. polygyrus*–infected BALB/c recipients 6 d after GVHD induction using a FACSVantage SE DiVa cell sorter (Becton Dickinson).

Total body irradiation and GVHD induction

Our studies used an MHC I/II mismatch, acute lethal GVHD model (26). Uninfected and helminth-infected BALB/c recipients (H2d) underwent lethal total body irradiation (TBI) from a [^{137}Cs] source (8.5 Gy in two divided doses given 4 h apart) and were administered 10×10^6 T cell–depleted (TCD) BM cells and 1.5×10^6 splenic T lymphocytes from uninfected C57BL/6 WT donors. To determine the effect of helminth infection on the conditioning regimen (TBI) without BMT, mice underwent irradiation with total doses ranging from sublethal 4 Gy to lethal 15 Gy, according to the same protocol. Similar split irradiation doses were used, except that the 4-Gy group received a single dose. To characterize the role of TGF-β signaling in helminth-induced regulation of donor T cell–mediated GVHD, 1.5×10^6 donor splenic T cells from TGF-βRII DN mice were administered along with 10×10^6 TCD BM cells from C57BL/6 WT mice into uninfected and *H. polygyrus*–infected BALB/c recipients. Mice were monitored daily for survival for up to 112 d in different experiments. Disease severity was scored daily based on animal weight, posture, activity, fur texture, and skin integrity (31–33). In parallel experiments, uninfected and helminth-infected mice were sacrificed 6 d after GVHD induction for cellular and histological analysis.

Quantification of tumor load and assessment of GVT by bioluminescent imaging

Luciferase-expressing A20 leukemia/lymphoma (A20-luc) cells syngeneic with recipients (H2d) were used for these experiments (34). Each recipient mouse received 3×10^5 A20-luc tumor cells i.v. within 24 h after BMT. Tumor load was assessed regularly in BMT recipient mice using an Ami 1000 Advanced Molecular Imager (Spectral Instruments, Tucson, AZ) live animal imaging system. Five minutes before bioluminescent imaging, mice were placed in an oxygenated isoflurane chamber and administered D-luciferin (Promega, Madison, WI) i.p. BMT recipient animals were imaged for 5 min, and tumor load was quantitated using Living Image software v2.50 (Caliper Life Sciences).

Flow cytometry

Six days after GVHD induction, uninfected and *H. polygyrus*–infected mice were sacrificed. Spleen and MLN cells were isolated for cellular analysis. For surface staining, cells were suspended at 2×10^7 cells/ml in PBS with 2% FCS, and FcRs were blocked with 2.4G2 mAb. Cells were stained with various combinations of anti-CD3 FITC, anti-CD3 PE-Cy7, anti-CD4 FITC, anti-CD4 PE-Cy7 (eBioscience), anti-latent TGF-β (latency associated peptide [LAP]) PE (BioLegend), anti-H2b PE, anti-H2d PE, and anti-H2b allophycocyanin (BD Biosciences). For the intracellular Foxp3 staining, cells were stained with anti-Foxp3 PE, Foxp3 PE-Cy7, or Foxp3 allophycocyanin using Foxp3 staining buffer (eBioscience), according to the manufacturer's instructions.

In vitro cell culture and cytokine ELISA

Eight- to nine-week-old uninfected or *H. polygyrus*–infected BALB/c mice were sacrificed 3 wk after initiation of helminth infection. Magnetically purified $CD4^+CD25^+$ (Treg-enriched) or $CD4^+CD25^-$ (Treg-depleted) splenic and MLN cells from uninfected or *H. polygyrus*–infected BALB/c mice were stimulated with plate-bound anti-CD3 and soluble anti-CD28 (each 1 μg/ml;

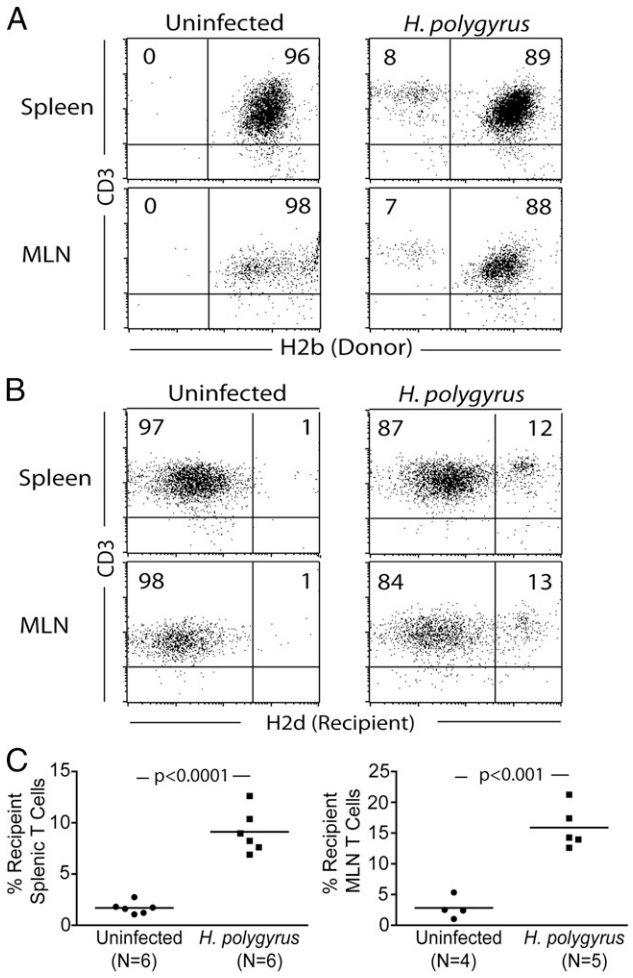

FIGURE 3. Helminths promote the survival of recipient T cells. (**A**) Splenic (*upper panels*) and MLN (*lower panels*) $CD3^+$ T cells isolated 6 d after BMT from *H. polygyrus*–infected mice (*right panels*) display a population of cells that does not stain for the donor marker H2b, whereas T cells from uninfected recipients with GVHD (*left panels*) uniformly stain for H2b. The percentage of cells in the corresponding quadrants is shown. Representative example from multiple experiments. (**B**) Splenic (*upper panels*) and MLN $CD3^+$ T cells (*lower panels*) isolated 6 d after BMT from *H. polygyrus*–infected mice (*right panels*) display a population of cells positive for the recipient marker H2d, whereas very few cells from uninfected recipients with GVHD (*left panels*) stain for the recipient marker. The percentage of cells in the corresponding quadrants is shown. Representative example from multiple experiments. (**C**) Statistical analysis of the percentage of splenic (*left panel*) and MLN (*right panel*) recipient T cells (N represents the number of mice used cumulatively in multiple experiments). Each symbol represents the percentage of host cells in a single mouse. Horizontal lines represent the mean percentages.

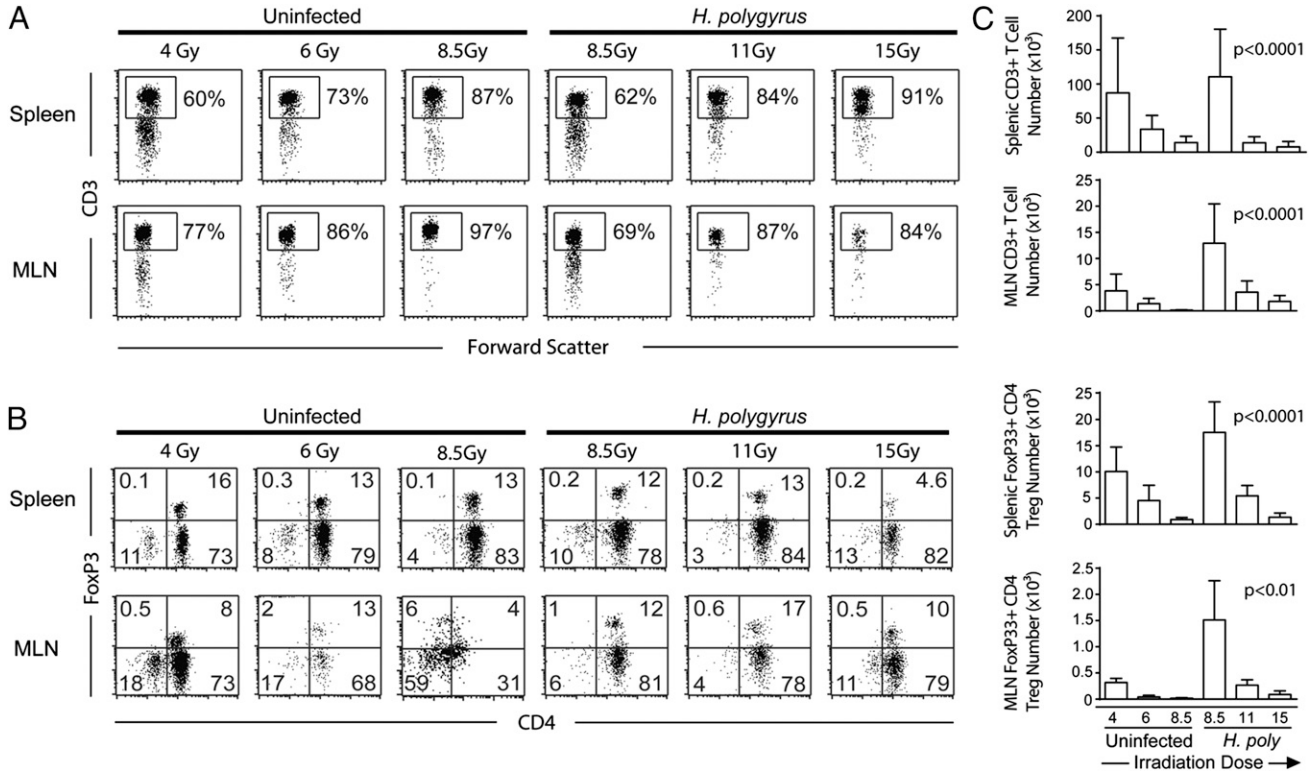

FIGURE 4. Helminths promote the survival of recipient T cells and Foxp3$^+$ CD4 Tregs after TBI. Spleen and MLN cells were isolated from uninfected or *H. polygyrus*–colonized mice 6 d after TBI. Cells were stained for CD3, CD4, and Foxp3. (**A**) Representative examples of splenocyte and MLN cell CD3 staining after different doses of TBI. (**B**) Representative examples of splenocyte and MLN cell CD4 and Foxp3 staining after different doses of TBI. Cells were gated on the CD3$^+$ lymphoid population. (**C**) The total number of splenic and MLN CD3$^+$ T lymphocytes (*upper panels*) or Foxp3$^+$ CD4 Tregs (*lower panels*) was calculated using the total number of cells isolated from uninfected and *H. polygyrus*–infected mice 6 d after TBI, the percentage of CD3$^+$ lymphocytes, and the percentage of Foxp3 staining CD4 T cells. Spleens were analyzed individually. The MLN cell number/animal was calculated by dividing the cell number from pooled MLNs by the number of mice in each sample. Each set of data is derived from at least three independent samples (experiments) for each group. Statistical analysis between groups was performed using ANOVA.

eBioscience) for 48 h in cell culture medium with 1% FCS and 1 mg/ml BSA (13, 35). TGF-β cytokine concentration in acidified and realkalinized supernatants was determined using Ab pairs (R&D Systems), according to the manufacturer's instructions. Results were calculated by subtracting the TGF-β concentration of culture supernatants from the TGF-β concentrations of the culture media. To determine helminthic regulation of donor T cell IFN-γ and TNF-α secretion, sorted donor splenic T cells (CD3$^+$ and H2b$^+$) from uninfected and *H. polygyrus*–infected BALB/c mice with GVHD were stimulated with plate-bound anti-CD3 and soluble anti-CD28 (each 1 μg/ml) for 48 h in lymphocyte growth medium containing 10% FCS (15). Supernatants were analyzed for IFN-γ, TNF-α, and IL-4 content using Ab pairs (R&D Systems). Similarly, sera isolated from uninfected and *H. polygyrus*–infected BALB/c mice 6 d after GVHD induction were analyzed for IFN-γ and TNF-α.

Histopathology

Six days after GVHD induction, colons, lungs, and livers from uninfected or *H. polygyrus*–infected mice were fixed in 4% neutral buffered formalin and processed, and 6-μm sections were stained with H&E. Tissues were analyzed for GVHD-related inflammation, and the severity of inflammation was scored in a blinded fashion by A.N.H. (31, 36–39). GVHD-related colitis was graded based on the degree of inflammation and the frequency of crypt apoptosis. Inflammation was graded as none (0), mild (1), moderate (2), severe without ulcer (3), or severe with ulcer (4). Crypt apoptosis was graded as rare (0), occasional per 10 crypts (1), few per 10 crypts (2), majority of crypts containing apoptotic bodies (3), or majority of crypts containing more than one apoptotic body (4). The minimal score in this grading system for colonic disease is 0, and the maximum score is 8. GVHD-related lung inflammation was graded based on the presence of perivascular cuffing, vasculitis, peribronchiolar cuffing, and alveolar hemorrhage. The minimal score in this grading system for lung inflammation is 0, and the maximum is 4.

Statistical analysis

Differences in survival between groups were determined by the Kaplan–Meier log-rank test. Differences in cell number and composition, serum cytokine content, differences in splenic donor T cell cytokine generation, differences in TGF-β cytokine output of in vitro–stimulated cell cultures, and histopathological GVHD scores between two groups were determined

Table I. Helminths increase the percentage and number of donor and recipient Foxp3$^+$ CD4 Tregs

Organ	CD3$^+$ T Cell Type	Foxp3$^+$ CD4 Tregs (%; Mean ± SD)[a]			No. of Foxp3$^+$ CD4 Tregs (Mean ± SD)[b]		
		Uninfected	*H. polygyrus*	*p* Value	Uninfected	*H. polygyrus*	*p* Value
Spleen (n = 7)[c]	Donor	0.62 ± 0.25	1.52 ± 0.83	<0.05	1.5 ± 1.1 × 10^4	3.3 ± 1.7 × 10^4	<0.05
	Recipient	2.75 ± 1.25	4.97 ± 2.36	<0.05	2.6 ± 2.2 × 10^3	18.0 ± 1.5 × 10^3	<0.05
MLN (n = 6)[c]	Donor	0.4 ± 0.1	2.2 ± 0.9	<0.001	0.3 ± 0.2 × 10^3	2.1 ± 1.0 × 10^3	<0.01
	Recipient	8.6 ± 3.8	14.1 ± 3.3	<0.05	0.4 ± 0.3 × 10^3	4.7 ± 2.6 × 10^3	<0.01

[a]The percentage of donor or recipient Foxp3$^+$ CD4 Tregs among all donor or recipient CD3$^+$ T cells.
[b]The number of Foxp3$^+$ MLN donor or recipient Tregs/mouse was calculated using the total number of mice used in each experiment, the total number of cells isolated from pooled MLN cells and the percentage of Foxp3$^+$ CD4$^+$ cells gated on CD3$^+$ lymphocytes.
[c]The number of experiments using a single spleen or cells from pooled MLNs from multiple mice.

Table II. Donor T cell numbers in spleens and MLN of uninfected or *H. polygyrus*–colonized BMT recipients

Organ	Uninfected (Mean ± SD)	*H. polygyrus* (Mean ± SD)	*p* Value
Spleen ($\times 10^6$/mouse)	3.7 ± 1.9 (n = 10)	4.8 ± 2.6 (n = 10)	NS
MLN ($\times 10^5$/mouse)	0.8 ± 0.4 (n = 4)	1.7 ± 0.9 (n = 5)	NS

using the Student t test. Differences in cell number and composition between multiple groups were analyzed by ANOVA.

Results

Helminth treatment of the recipient reduces GVHD

Acute GVHD was initiated in uninfected or *H. polygyrus*–infected irradiated BALB/c recipients by transfer of total splenic T cells and TCD BM cells from uninfected donor C57BL/6 mice. Mice started to display signs of GVHD 4–5 d later with this regimen, and GVHD was characterized by loss of activity, skin discoloration, hunched body posture, and bloody diarrhea (31). Beginning at this time, uninfected mice displayed severe GVHD compared with the relatively normal appearance of *H. polygyrus*–infected mice (Fig. 1A). *H. polygyrus* colonization of the recipient was associated with a significant increase in survival ($p < 0.001$) (Fig. 1B). Although weight loss associated with GVHD was not different between uninfected and *H. polygyrus*–exposed recipients (Fig. 1C), helminth-infected mice exhibited significantly decreased disease activity ($p < 0.005$) (Fig. 1D). Weight loss or significant disease activity were not seen in helminth-infected or uninfected BALB/c recipients that were administered TCD BM without splenic T cells from uninfected C57BL/6 donors (Supplemental Fig. 1A). To determine whether irradiation alters the parasite fecundity, 8–9-wk-old male WT BALB/c mice underwent lethal TBI (8.5 Gy) without BMT 3 wk after helminth infection. Stool egg counts were performed in no-irradiation control and lethally irradiated mice prior to and 6 d after TBI. Stool egg counts were similar between irradiated mice and control animals that did not receive irradiation (Supplemental Fig. 2).

We sacrificed parallel groups of uninfected and *H. polygyrus*–administered mice at day six after GVHD induction and analyzed tissues by histopathology. Gut colonization with *H. polygyrus* was associated with reduced inflammatory infiltrates in the colon (mean inflammatory score, day 6 post-BMT, 2.4 ± 0.8 in *H. polygyrus*–infected mice versus 5.6 ± 1.3 in uninfected mice; n = 10/group for uninfected and helminth infected; $p < 0.001$) (Fig. 2). No inflammation was evident in the large intestine of helminth-infected or uninfected BALB/c mice without BMT (data not shown) or in mice that underwent TCD BM (Supplemental Fig. 1B). Numerous apoptotic bodies were evident in colonic samples from uninfected animals but not in samples from *H. polygyrus*–infected mice. Lung tissues from uninfected mice were characterized by dense mononuclear cell infiltrates, as well as alveolar hemorrhages, whereas samples from *H. polygyrus*–administered animals showed fewer infiltrates, with preservation of the air sacs (mean inflammatory score 1.3 ± 0.5 in *H. polygyrus*–infected mice versus 3.5 ± 0.5 in uninfected mice; n = 6/group for uninfected and helminth infected; $p < 0.001$) (Fig. 2). No inflammatory changes were evident in the lungs of helminth-infected or uninfected BALB/c mice without BMT (data not shown) or in mice that underwent TCD BM (Supplemental Fig. 1B). Liver tissues from uninfected or *H. polygyrus*–infected mice showed mild focal portal infiltrates, with no difference between groups (data not shown).

Helminth infection is associated with the persistence of recipient T cells

At day six after GVHD induction, the spleen and MLN cells were analyzed for donor and recipient markers. Most splenic or MLN cells in uninfected or *H. polygyrus*–infected BMT mice were $CD3^+$ T lymphocytes (Fig. 3). No B cells were seen by CD19 staining (Supplemental Fig. 3). Although >97% of splenic and 95% of MLN T cells were donor derived in uninfected mice, 9 ± 2% of splenic and 16 ± 2% of MLN cells were $H2d^+$ recipient cells in *H. polygyrus*–infected mice (Fig. 3). These data suggested that helminths stimulated the survival of recipient T lymphocytes.

Recipient T cell survival during GVHD may be due to helminth-induced protection from the TBI or due to suppression of donor T cell attack. To distinguish between these possibilities, we measured splenic and MLN T cell number and composition after TBI (conditioning regimen) without BMT. Eradication of T cells through TBI in uninfected mice was dose dependent, with <20,000 splenic and <1,000 MLN T cells surviving 8.5-Gy TBI, the dose used in BMT experiments (Fig. 4). Similarly, <2000 splenic and <100 MLN $Foxp3^+$ Tregs survived 8.5-Gy lethal TBI without BMT. In contrast, T cells from helminth-infected mice survived TBI doses of 8.5 Gy and higher (11 and 15 Gy), although the number of surviving T cells and Tregs gradually decreased with the increase in radiation dose (Fig. 4). Thus, helminths promoted the survival of recipient T cells and recipient $Foxp3^+$ CD4 Tregs to the conditioning regimen, making the recipient Tregs a dominant Treg pool in the early period after BMT (see also Table I).

Helminths regulate donor T cell cytokine generation but do not interfere with the engraftment or early in vivo expansion of donor $CD3^+$ T cells

Regulation of GVHD may involve suppression of early donor T cell proliferation (36). The number of splenic or MLN donor T cells was not different in helminth-infected recipients compared with uninfected mice (Table II). To determine the effect of helminth infection on donor T cell cytokine production, equal numbers of FACS-sorted splenic donor T cells from uninfected and *H. polygyrus*–infected recipients were isolated 6 d after GVHD induction and studied for in vitro cytokine output. Helminth infection stimulated donor T cell

Table III. Helminthic regulation of cytokine production during GVHD

Sample Source and Cytokine	Uninfected (Mean ± SEM)	*H. polygyrus* (Mean ± SEM)	*p* Value
Donor T cell IFN-γ (ng/ml)	153.4 ± 8.4 (n = 3)	54.7 ± 33.9 (n = 3)	<0.05
Donor T cell TNF-α (ng/ml)	8.0 ± 0.3 (n = 3)	5.1 ± 0.6 (n = 3)	<0.05
Donor T cell IL-4 (ng/ml)	0.5 ± 0.1 (n = 3)	2.8 ± 0.4 (n = 3)	<0.05
Serum IFN-γ (ng/ml)	2.0 ± 0.12 (n = 5)	0.37 ± 0.07 (n = 5)	<0.001
Serum TNF-α (ng/ml)	1.9 ± 0.3 (n = 5)	0.7 ± 0.1 (n = 5)	<0.01

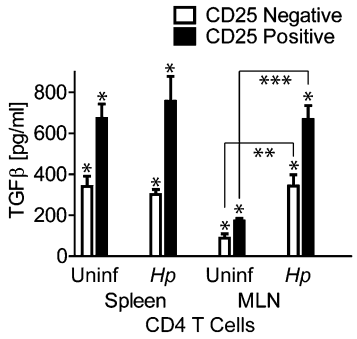

FIGURE 5. Helminths enhance TGF-β generation from Treg-enriched and Treg-depleted MLN CD4 T cells. TGF-β output of plate-bound anti-CD3–stimulated and soluble anti-CD28–stimulated splenic and MLN CD25-enriched or -depleted $CD4^+$ T cells from *H. polygyrus*–colonized (*Hp*) or uninfected (Uninf) BALB/c mice without BMT was measured by ELISA from the cell culture supernatants. Data are representative examples from at least three independent experiments. $*p < 0.05$, $CD25^-$ versus $CD25^+$, $**p < 0.01$ and $***p < 0.001$, corresponding MLN cell groups in uninfected versus *H. polygyrus*–infected mice.

IL-4 output and led to reduced anti-CD3/28–stimulated donor T cell inflammatory cytokine (IFN-γ, TNF-α) production (Table III). A parallel decrease in serum IFN-γ and TNF-α was observed in helminth-infected mice (Table III). Thus, *H. polygyrus* infection regulated donor T cells with suppression of inflammatory cytokine and stimulation of Th2 or regulatory cytokine production. Helminthic regulation of donor T cells did not suppress the engraftment and early expansion of donor T cells.

Helminths increase the percentage and number of donor and recipient Foxp3$^+$ Tregs

Helminths promote the survival of $Foxp3^+$ Tregs that were shown to regulate GVHD (22). To determine whether helminthic regulation of GVHD is associated with the induction of Tregs, we analyzed the percentage and total numbers of donor- or recipient-derived Tregs in the spleen and MLN cells of uninfected and *H. polygyrus*–infected mice 6 d after BMT. Helminth infection led to a significant increase in the percentage and number of donor, as well as recipient, $Foxp3^+$ CD4 Tregs in the spleen and MLNs (Table I). These data suggested that helminth-induced protection from GVHD was associated with increased donor and recipient Tregs in lymphoid compartments. Induction of Tregs may be one of the mechanisms of helminthic regulation of acute GVHD, because donor $CD25^+CD4^+$ T cells enriched for $Foxp3^+$ Tregs regulated acute GVHD when cotransferred with conventional T cells (Supplemental Fig. 4), confirming previous observations that studied regulation of GVHD by Tregs in adoptive-transfer models (22).

CD4 T lymphocytes enriched for Foxp3$^+$ Tregs generate more TGF-β than do other peripheral CD4 T cells

Immune regulatory pathways involving TGF-β lead to peripheral induction and maintenance of Tregs (40) and may be essential in helminth-induced immune modulation (13, 14). We showed previously that *H. polygyrus* colonization stimulates T cell TGF-β generation that is essential for Treg functions, such as IL-10 production (13). We studied whether Tregs that are increased during GVHD in helminth-infected mice generated more TGF-β on a per-cell basis. Most $Foxp3^+$ CD4 T cells are found in the $CD25^+CD4^+$ T cell compartment. Plate-bound anti-CD3– and soluble anti-CD28–stimulated splenic and MLN $CD4^+CD25^+$ T cells from *H. polygyrus*–infected or uninfected BALB/c mice generated ∼2-fold more TGF-β compared with the $CD4^+CD25^-$ T cell fraction, as shown by ELISA from supernatants harvested 48 h after stimulation (Fig. 5). MLN T cell isolates from helminth-infected mice generated significantly more TGF-β compared with MLN T cell isolates from uninfected mice (Fig. 5). TGF-β cytokine content in anti-CD3/28–stimulated TCD parallel cultures was <20 pg/ml and did not increase with anti-CD3/28 stimulation (data not shown). These data suggested that, during GVHD, helminths induced the proliferation or generation or promoted the survival of TGF-β–producing $Foxp3^+$ CD4 Tregs.

Helminth infection is associated with an increase in MLN donor and recipient Treg latent TGF-β expression during GVHD

Pre–pro–TGF-β peptide is cleaved into N-terminal LAP and C-terminal TGF-β protein after transcription and translation (41). LAP is expressed on T cells that may regulate immune responses in a TGF-β–dependent manner (42). Therefore, we studied latent TGF-β

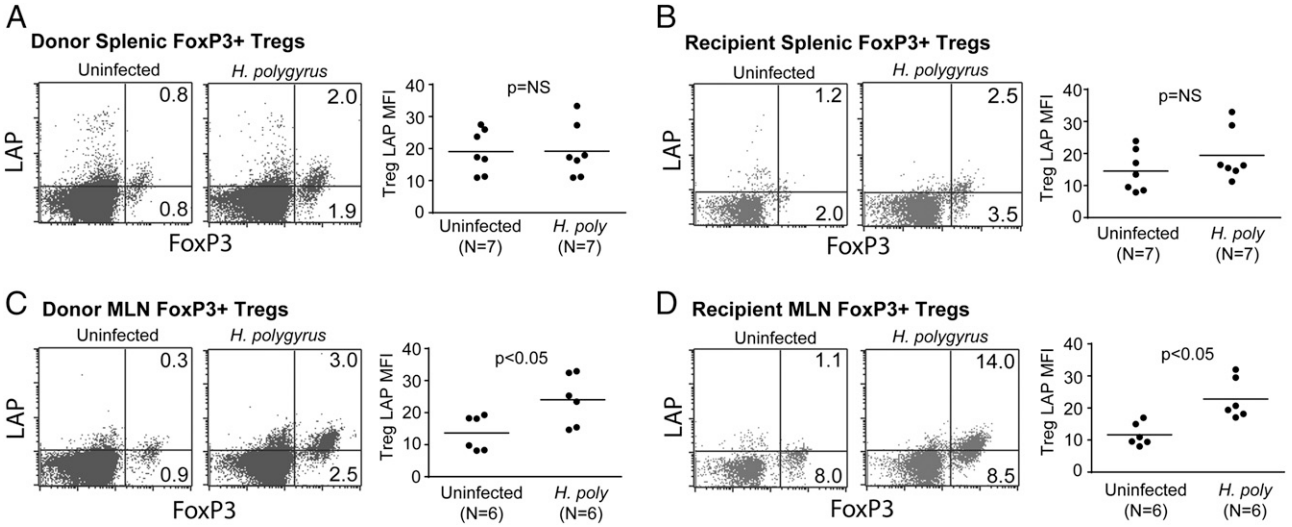

FIGURE 6. Helminths induce $Foxp3^+$ CD4 Treg LAP expression. Spleen and MLN cells from uninfected and helminth-infected (*H. polygyrus* or *H. poly*) mice were stained for CD3, CD4, CD8, H2b, H2d, Foxp3, and LAP. Representative dot plots from spleen (**A** and **B**) and MLN (**C** and **D**) cells isolated from uninfected and *H. polygyrus*–infected mice 6 d after BMT. Cells are gated on donor (A and C) or recipient (B and D) $CD3^+$ CD4 T cells; LAP and Foxp3 expression are displayed as dot plots with the numbers representing the percentage of events in the corresponding quadrants. The graphs show the MFI of LAP staining on $Foxp3^+$ CD4 Tregs; each symbol represents the data from one spleen or pooled MLN cells. *n* = number of independent samples.

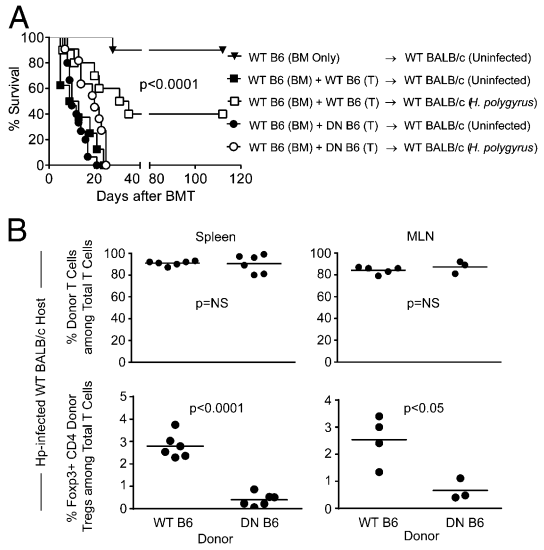

FIGURE 7. Helminths regulate donor T cell–mediated GVHD in a TGF-β–dependent manner. (**A**) Kaplan–Meier survival curves of uninfected BALB/c recipients of TCD BM cells only from uninfected C57BL/6 mice (WT B6) ($n = 10$) (▼), uninfected BALB/c recipients of TCD BM cells and total splenic T cells from uninfected C57BL/6 mice ($n = 10$) (■), uninfected BALB/c recipients of TCD-BM cells from uninfected C57BL/6 WT (B6 WT) mice and splenic T cells from uninfected TGF-βRII DN (DN B6) mice ($n = 15$) (●), *H. polygyrus*–infected BALB/c recipients of TCD BM and splenic T cells from uninfected C57BL/6 mice ($n = 10$) (□), and *H. polygyrus*–infected BALB/c recipients of TCD BM cells from uninfected C57BL/6 mice and splenic T cells from uninfected TGF-βRII DN mice ($n = 12$) (○). Cumulative data from three independent experiments with multiple mice from each group. (**B**) Spleen and pooled MLN cells were isolated from helminth-infected BALB/c (Hp-infected) recipients of C57BL/6 (B6) or TGF-βRII DN splenic T cell donors 6 d after BMT (all recipient mice also received C57BL/6 WT TCD BM cells). The percentages of total and donor $CD3^+$ T cells and $Foxp3^+$ CD4 T cells were determined by flow cytometry. Each symbol represents one experiment from a single spleen or pooled MLN. Percentage of donor $CD3^+$ T cells in all $CD3^+$ T cells (*upper panels*) or the percentage of donor $Foxp3^+$ CD4 Tregs in all $CD3^+$ T cells (*lower panels*) from spleen (*left panels*) or MLN (*right panels*) isolates. Each symbol represents the percentage from a single sample.

(LAP) expression on donor and recipient Tregs during acute GVHD. LAP staining of $Foxp3^+$ CD4 Tregs gave a separate and bright signal on flow cytometry, with a significantly elevated mean fluorescent intensity (MFI) compared with Treg staining with an isotype Ab (data not shown). In splenic lymphocytes, LAP expression on donor and recipient $Foxp3^+$ CD4 Tregs did not change significantly after helminth infection (Fig. 6). However, in MLN cells, helminth infection increased LAP MFI on donor and recipient $Foxp3^+$ CD4 Tregs (Fig. 6). These results indicated that *H. polygyrus* infection promoted the induction and/or maintenance of $Foxp3^+$ Tregs, with enhanced TGF-β generation during acute GVHD.

Helminths regulate GVHD in a TGF-β–dependent manner

TGF-β is a strong regulator of Th1 immunity (40, 43). Because helminth infection suppressed Th1 inflammation during GVHD, thereby increasing T cells that secrete TGF-β and express LAP, we studied whether helminths regulated donor T cell–mediated GVHD in a TGF-β–dependent manner. In these experiments, we used donor T cells from uninfected TGF-βRII DN mice (H2b), in which the T cells are unresponsive to TGF-β–mediated immune regulation as a result of T cell–specific overexpression of a truncated TGF-βRII (28). Because the engineered CD4 promoter driving the truncated TGF-βRII expression lacked the CD8 silencer, both CD4 and CD8 T cells in these mice are unresponsive to TGF-β–mediated immune regulation. Uninfected and *H. polygyrus*–infected recipient male WT BALB/c mice were given TCD BM cells from uninfected 5–7-wk-old male C57BL/6 WT donors and splenic T cells from male 5–7-wk-old TGF-βRII DN mice (Fig. 7A). Other groups included uninfected BALB/c recipients given TCD BM cells from uninfected C57BL/6 mice and uninfected or *H. polygyrus*–infected recipient BALB/c recipients given TCD BM and splenic T cells from C57BL/6 WT donors. Transfer of TCD BM cells was associated with survival of 90% of mice, whereas GVHD was uniformly lethal in uninfected BALB/c recipients that also received C57BL/6 WT or TGF-βRII DN splenic T cells (Fig. 7A). Strikingly, 40% of *H. polygyrus*–infected recipients that received T cells from WT C57BL/6 mice survived the GVHD, whereas *H. polygyrus* infection did not protect against GVHD lethality in recipients that received TGF-βRII DN splenic T cells (Fig. 7A). Some of the helminth-infected surviving mice examined >200 d after GVHD induction maintained 60–80% of their original weight and displayed mild clinical disease activity (data not shown). Together, these data suggested that helminth infection regulated GVHD in recipient mice in a TGF-β–dependent manner.

Because helminths increased the percentage and number of GVHD-regulating donor Tregs, we investigated the role of TGF-β in donor Treg repopulation after GVHD. Splenic T cells from uninfected C57BL/6 WT or TGF-βRII DN mice contained comparable frequencies of $Foxp3^+$ Tregs (44), which we also found in our experiments ($6.7 \pm 0.7\%$ $Foxp3^+$ in TGF-βRII DN [$n = 3$] and $5.5 \pm 0.9\%$ in C57BL/6 WT [$n = 3$], p = not significant). Comparable donor T cell frequencies were observed in helminth-infected BALB/c BMT recipients that received splenic T cells from uninfected C57BL/6 or TGF-βRII DN mice (Fig. 7B). However, the percentage of donor $Foxp3^+$ CD4 Tregs was significantly lower in helminth-infected BMT recipients of TGF-βRII DN T cells compared with recipients of WT T cells (Fig. 7B). These results suggested that TGF-β induced during helminth infection plays an additional critical role in donor Treg expansion during GVHD.

Helminthic regulation of GVHD is associated with preserved antitumor immunity

To determine whether helminthic immune suppression preserves the donor T cell antitumor immunity (GVT), we administered A20-luc tumor cells within 24 h to BMT recipients. Live animal imaging demonstrated that uninfected or helminth-infected mice that received TCD BM without donor T cells developed tumors and died as a result of tumor burden within the first 24 d after BM and tumor transfer (Fig. 8). There was no difference in survival between these groups, suggesting that helminth infection did not have a beneficial or detrimental effect on tumor development. No tumor development was observed in uninfected or helminth-infected mice that received donor T cells other than TCD BM and A20 (Fig. 8). Although uninfected mice died of severe GVHD, five of nine helminth-infected mice survived without evidence of tumor until the end of the experiment (Fig. 8). These results suggested that helminths regulated GVHD and preserved GVT.

Discussion

Helminth infection of the gut was shown to suppress inflammation in various autoimmune, allergic, and immunological disorders (45). The major finding of the current study was that helminth infection also regulated inflammatory responses in an acute, lethal GVHD animal model, promoting the survival of *H. polygyrus*–administered recipients. *H. polygyrus* treatment resulted in regulation of donor T cell Th1 cytokine generation, regulation of colitis and

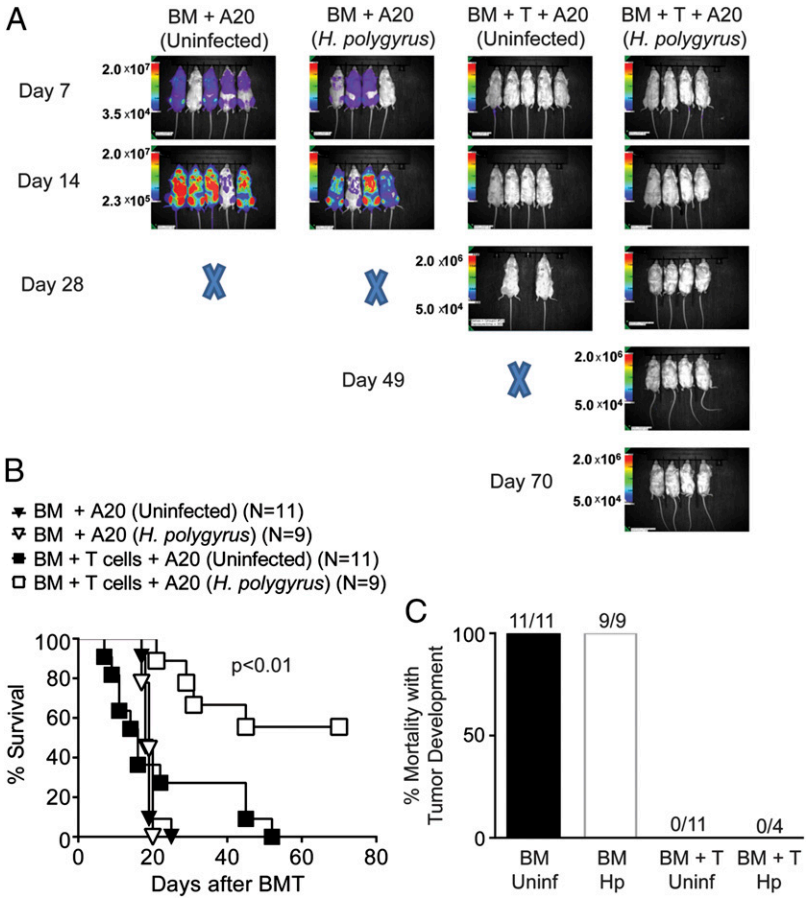

FIGURE 8. Helminths regulate GVHD and preserve GVT. (**A**) Live animal imaging of all surviving mouse populations from a single experiment at the indicated time points; the bioluminescence scale was kept the same at each time point for all groups. (**B**) Kaplan–Meier survival curves of uninfected BALB/c recipients of TCD BM cells from uninfected C57BL/6 mice (WT B6) and A20-luc tumor cells (▼), uninfected BALB/c recipients of TCD BM cells, total splenic T cells from uninfected C57BL/6 mice, and A20-luc tumor cells (■), helminth (*H. polygyrus*)–infected BALB/c recipients of TCD BM cells from uninfected C57BL/6 mice (WT B6) and A20-luc tumor cells (▽), and helminth (*H. polygyrus*)–infected BALB/c recipients of TCD-BM cells (BM), total splenic T cells (T) from uninfected C57BL/6 mice and A20-luc tumor cells (□). N = cumulative number of mice. $p < 0.001$, uninfected versus helminth-infected mice that received TCD BM, splenic T cells, and A20-luc. (**C**) Percentage mortality associated with tumor development. Proportions represent the number of mice with tumor/total number of mice that died during the experiment.

lung inflammation, and enrichment for donor Tregs that generate high quantities of TGF-β (42) and that regulate GVHD (22). Another novel finding of the study was helminth-induced survival of recipient T cell subsets after TBI that led to persistence of recipient T cells and Tregs during GVHD. Helminths stimulated MLN recipient Treg TGF-β secretion, where MLN recipient Tregs continued with enhanced TGF-β expression during GVHD. Helminths suppressed GVHD and increased survival in a TGF-β–dependent manner, while preserving GVT.

TGF-β suppresses Th1 cytokine–driven inflammation models in mice, such as IBD, experimental allergic encephalomyelitis, or donor T cell–mediated acute GVHD (1, 41, 43). Induction of host TGF-β pathways is critical in helminthic regulation of intestinal immunity, as shown by our group (13) and other investigators (14). Helminths may trigger these regulatory pathways by increasing TGF-β production from host cells. Nematodes also produce factors with TGF-β–like activity that stimulate mammalian TGF-β signaling and induce T cell Foxp3 gene expression (14). In this study, we broadened these observations on helminthic immune regulation to another disease model, GVHD, further indicating the importance of the gut immune system and luminal microenvironment in the initiation and regulation of mucosal, as well as systemic, alloreactivity (27, 46–48). When we used donor T lymphocytes that were unresponsive to TGF-β–mediated immune regulation, helminthic protection from lethal GVHD was abrogated. Therefore, intestinal *H. polygyrus* larvae may regulate donor T lymphocyte alloreactivity through promoting host, as well as donor, T cell TGF-β synthesis or by generating TGF-β–like factors. These observations attest to a mechanistic link between helminthic stimulation of TGF-β pathways and suppression of acute GVHD.

One source of TGF-β in helminth-infected BMT recipients is the recipient Treg population. Recipient Tregs that survive the conditioning regimen may contribute to immune regulation after experimental hematopoietic stem cell transplantation (49). However, they are too few in number after lethal TBI and major mismatch BMT to help suppress acute GVHD. In this study, we show that helminths stimulate recipient Treg survival after TBI and BMT and augment recipient MLN Treg TGF-β expression before and after BM transfer. TGF-β–generating recipient Tregs may be a critical regulatory cell population in helminth-infected BMT recipients, because B cells are absent during this acute GVHD and because helminths do not promote TGF-β generation by innate immune cells (50, 51). In addition to regulating donor T cell Th1 alloreactivity, TGF-β–generating recipient T cells and Tregs from helminth-infected mice may facilitate donor T cell engraftment (52).

Helminth-induced TGF-β may regulate donor T cell Th1 inflammatory cytokine generation by directly inhibiting donor T cell Th1 differentiation (41) or by stimulating T cell or Treg IL-10 production. Helminths induce T cell IL-10 generation in a TGF-β–dependent manner (13, 25). Helminths also may regulate GVHD-related Th1 responses through induced Th2 cytokine generation, as we demonstrate in this study.

In addition to regulating Th1 inflammation, TGF-β is important for the induction and maintenance of Tregs, implying that helminthic induction of TGF-β may regulate GVHD through the induction of various immune-regulatory pathways, such as stimulation of donor as well as recipient Tregs and through direct regulation of Th1 responses. Previous studies showed contradictory data for the role of TGF-β in Treg expansion (44, 53–56). No role for TGF-β was suggested if T cell subpopulations were analyzed 6–8 wk after experimentation, whereas TGF-β appeared critical in studies that examined Treg expansion within a few days after T cell transfer or very early in life. Consistent with these

early-response experiments, we demonstrated that TGF-β is critical for rapid and robust donor Treg expansion and is crucial in regulating acute lethal GVHD.

TGF-β is produced as a pre–pro-peptide where the N-terminal cleaved portion of the protein, LAP, is secreted from the cell and noncovalently attached to the cleaved C-terminal part of the original pre–pro-peptide (41). The C-terminal protein is the TGF-β cytokine. Separation of the noncovalently attached N-terminal LAP leads to activation of TGF-β, permitting the binding of TGF-β to the TGF-βR complex and triggering signal transduction. In addition to the secreted TGF-β cytokine, noncovalently attached LAP and TGF-β proteins are present in membrane-bound forms on regulatory cell subsets that dampen immune responses in a cell contact– and TGF-β–dependent manner (42). We found that *H. polygyrus* infection was associated with an increase in MLN donor and recipient Foxp3$^+$ Tregs expressing LAP and that protection from acute GVHD requires donor T cell TGF-β signaling. This confirms the importance of TGF-β in regulating intestinal immunity (57) and the importance of the gut immune system in regulating GVHD.

GVHD has remained a challenge of clinical practice, with increasing cases of hematopoietic stem cell transplantation to treat various hematological or nonhematological diseases (23). Various studies showed the importance of Tregs in regulating effector donor T cells and suppressing acute or chronic GVHD (22, 26, 58–61), with ex vivo Tregs being a new area of clinical investigation in BMT. Purification and in vitro expansion of Tregs to a sufficient dose to manage GVHD is a challenge in clinical practice. Therefore, new clinical strategies to expand Tregs in vivo are being investigated (62). Our results suggest that in vivo induction of Tregs by self-limited colonization of the gut with helminths and use of the TGF-β pathway may suppress donor T cell Th1 immune reactivity and enable regulated donor T cell engraftment.

Helminths have been used in patients to successfully treat inflammation (17). Helminths may regulate immunity directly or through enriching the intestinal microbiome for beneficial or probiotic strains (63), because GVHD is associated with major shifts in the composition of intestinal flora in animal models or patients (46). So helminths may also regulate GVHD through modulating the gut flora. With recent evidence showing that helminth products may regulate inflammatory responses similar to helminth infections (64), exposure to helminths or helminth products may become a novel and safe therapy for GVHD with preserved antitumor immunity (GVT), allowing the broader use of BMT.

Disclosures

The authors have no financial conflicts of interest.

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