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Research Article **Immunology**

Programmed death ligand-1 (PD-L1) interacts with programmed death-1 (PD-1) and the immunostimulatory molecule CD80 and functions as a checkpoint to regulate immune responses. The interaction of PD-L1 with CD80 alone has been shown to exacerbate the severity of graft-versus-host disease (GVHD), whereas costimulation of CD80 and PD-1 ameliorates GVHD. Here we have demonstrated that temporary depletion of donor CD4+ T cells early after hematopoietic cell transplantation effectively prevents GVHD while preserving strong graft-versus-leukemia (GVL) effects in allogeneic and xenogeneic murine GVHD models. Depletion of donor CD4+ T cells increased serum IFN-γ but reduced IL-2 concentrations, leading to upregulation of PD-L1 expression by recipient tissues and donor CD8+ T cells. In GVHD target tissues, the interactions of PD-L1 with PD-1 on donor CD8+ T cells cause anergy, exhaustion, and apoptosis, thereby preventing GVHD. In lymphoid tissues, the interactions of PD-L1 with CD80 augment CD8+ T cell expansion without increasing anergy, exhaustion, or apoptosis, resulting in strong GVL effects. These results indicate that the outcome of PD-L1–mediated signaling in CD8+ T cells depends on the presence or absence of CD4+ T cells, the nature of the interacting receptor expressed by CD8+ T cells, and the tissue environment in which the signaling occurs.

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Programmed death ligand-1 (PD-L1) interacts with programmed death-1 (PD-1) and the immunostimulatory molecule CD80 and functions as a checkpoint to regulate immune responses. The interaction of PD-L1 with CD80 alone has been shown to exacerbate the severity of graft-versus-host disease (GVHD), whereas costimulation of CD80 and PD-1 ameliorates GVHD. Here we have demonstrated that temporary depletion of donor CD4+ T cells early after hematopoietic cell transplantation effectively prevents GVHD while preserving strong graft-versus-leukemia (GVL) effects in allogeneic and xenogeneic murine GVHD models. Depletion of donor CD4+ T cells increased serum IFN-γ but reduced IL-2 concentrations, leading to upregulation of PD-L1 expression by recipient tissues and donor CD8+ T cells. In GVHD target tissues, the interactions of PD-L1 with PD-1 on donor CD8+ T cells cause anergy, exhaustion, and apoptosis, thereby preventing GVHD. In lymphoid tissues, the interactions of PD-L1 with CD80 augment CD8+ T cell expansion without increasing anergy, exhaustion, or apoptosis, resulting in strong GVL effects. These results indicate that the outcome of PD-L1–mediated signaling in CD8+ T cells depends on the presence or absence of CD4+ T cells, the nature of the interacting receptor expressed by CD8+ T cells, and the tissue environment in which the signaling occurs.

Introduction

Allogeneic hematopoietic cell transplantation (HCT) is a curative therapy for hematological malignancies (i.e., leukemia and lymphoma), owing to graft-versus-leukemia/lymphoma (GVL) effects mediated by alloreactive T cells. These same T cells also mediate acute graft-versus-host disease (GVHD) and the subsequent development of chronic GVHD (1–5). Both alloreactive CD4+ and CD8+ T cells can mediate acute GVHD, and Th1 and Th17 cells play a critical role in initiating gut GVHD (6–10). While flow cytometry–sorted donor CD4+ T cells mediate severe GVHD through expression of FASL and production of proinflammatory cytokines (i.e., IFN-γ and TNF-α) (10, 11), sorted donor CD8+ T cells prevent graft rejection and mediate GVL effects through their expression of perforin/granzyme, without causing acute clinical GVHD in several mouse models (12, 13). However, the mechanisms whereby purified alloreactive CD8+ T cells mediate GVL effect without causing GVHD remain largely unknown.

Programmed death ligand-1 (PD-L1, also known as B7H1) functions as an immune checkpoint that interacts with pro-

grammed death-1 (PD-1) and CD80 (14, 15). PD-L1 is usually expressed by hematopoietic cells and by parenchymal cells under inflammatory cytokine (i.e., IFN-γ) induction (16). CD80 is constitutively expressed by T cells and is upregulated early after T cell activation (17), whereas PD-1 is expressed by T cells late after T cell activation (18). PD-L1 interaction with PD-1 induces anergy, exhaustion, and apoptosis of activated T cells (19, 20); on the other hand, PD-L1/CD80 interaction has been reported to inhibit CD28/CTLA4-deficient T cell proliferation in vitro (15).

Expression of PD-L1 in recipient tissues decreases the severity of GVHD in allogeneic recipients conditioned with conventional total body irradiation (21–23), while expression of PD-L1 by donor T cells increases the severity of GVHD by augmenting the expansion and survival of donor CD4+ and CD8+ T cells (24). We recently showed that the interaction of PD-L1 with CD80 in the absence of PD-1 worsened GVHD by augmenting alloreactive CD4+ T cell proliferation and expansion, although simultaneous interactions of PD-L1 with both CD80 and PD-1 ameliorated GVHD by augmenting apoptosis of activated alloreactive CD4+ T cells (25).

Regulation of anergy, exhaustion, and apoptosis through PD-L1 interactions with CD80 and PD-1 on CD8+ T cells in allogeneic HCT has not yet been well characterized. Our previous studies showed that the absence of host-tissue expression of PD-L1 contributed to expansion of infiltrating CD8+ T cells in GVHD target tissues in recipients with GVHD and lymphopenia (21). Other investigators have shown that host-tissue expression of PD-L1 caused exhaustion of alloreactive CD8+ T cells and reduced GVL

effects in GVHD recipients (26, 27). However, it was reported that in vivo expansion of alloreactive CD8+ T cells in lymphoid tissues (i.e., spleen) early after HCT, before the onset of GVHD, was not affected by host-tissue expression of PD-L1 (28).

In the current studies, we show that depletion of donor CD4+ T cells early after HCT led to an increase of IFN-γ and reduction of IL-2 in the serum, and increased expression of PD-L1 by GVHD target tissues and by donor CD8+ T cells. Interactions of PD-L1 with PD-1 on donor CD8+ T cells in GVHD target tissues induced tolerance through anergy, exhaustion, and apoptosis of effector T cells, thereby preventing GVHD. Interactions of PD-L1 with CD80 on donor CD8+ T cells in lymphoid tissues enhanced their expansion and activity against malignant cells in the recipient.

Results

Temporary depletion of donor CD4+ T cells immediately after HCT preserves strong GVL effects, while effectively preventing both acute and chronic GVHD in multiple models. In a previous study, we showed that sorted CD8+ T cells from C57BL/6 donors did not induce acute GVHD but they induced chronic GVHD in lethally irradiated BALB/c recipients, as indicated by histopathology in salivary glands, a prototypic target organ of chronic GVHD. Depletion of CD4+ T cells by treatment with anti-CD4 mAb on days 15 and 30 prevented the development of chronic GVHD, as indicated by prevention of tissue damage in all GVHD target tissues, especially in the salivary gland (7). In extending these results, we found that: (a) In vivo administration of anti-CD4 on the day of HCT was more effective in depleting donor CD4+ T cells as compared with ex vivo depletion of CD4+ T cells, as judged by percentage and yield of donor CD4+ T cells in the spleen of recipients at 7 days after HCT (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI91138DS1). (b) Although a low dose of CD4+ T cells (0.075 × 10⁶) or CD8+ T cells (1 × 10⁶) alone induced no sign of diarrhea, addition of the small numbers of CD4+ T cells to the CD8+ T cell graft induced severe diarrhea and death of all recipients (Supplemental Figure 1B). The addition of the small numbers of donor CD4+ T cells markedly reduced the apoptosis of colon tissue–infiltrating CD8+ T cells, resulting in marked expansion of donor CD8+ T cells in the colon tissue (Supplemental Figure 1C), and this effect was IL-2–dependent (Supplemental Figure 1D). (c) A single injection of anti-CD4 immediately following HCT effectively prevented acute GVHD but did not prevent chronic GVHD, which was associated with reconstitution of donor CD4+ T cells beginning by day 21 after HCT (Supplemental Figures 2 and 3). (d) Three injections of anti-CD4 on days 0, 14, and 28 effectively prevented both acute and chronic GVHD, and recovery of donor CD4+ T cells thereafter no longer caused chronic GVHD (Supplemental Figure 4, A–C). Prevention of chronic GVHD was indicated by the absence of tissue damage in GVHD target tissues at about 60 days after HCT (Supplemental Figure 4B). In addition, 3 injections but not 1 injection of anti-CD4 led to recovery of medullary thymic epithelial cells (mTECs) (Supplemental Figure 5, A–D). Therefore, GVHD, especially chronic GVHD, is more effectively prevented by temporary in vivo depletion of donor CD4+ T cells early after HCT than by ex vivo depletion of donor CD4+ T cells.

In the previous study, we did not evaluate the effect of in vivo CD4+ T depletion on GVL effects. To address this question, we first evaluated the impact of temporary in vivo CD4+ T cell depletion on GVL effects against BCL1 tumor cells (29). BALB/c recipients conditioned with total body irradiation were injected with luciferase-transfected BCL1 cells (BCL1/Luc, 5 × 10⁶ per mouse) together with T cell–depleted bone marrow (TCD-BM, 2.5 × 10⁶) alone or TCD-BM plus spleen cells (5 × 10⁶). Recipients given spleen cells were treated with anti-CD4 or control rat IgG on days 0, 14, and 28 after HCT. BCL1/Luc tumor cell–bearing recipients transplanted with TCD-BM alone all died with progressive tumor growth by 20 days after HCT (Figure 1, A–D). Recipients transplanted with BM and spleen cells and treated with control rat IgG eliminated BCL1/Luc tumor cells by 12 days after HCT, but all died with acute GVHD and severe diarrhea by 20 days after HCT. In contrast, recipients treated with anti-CD4 eliminated the tumor cells by 12 days after HCT, and all recipients survived for more than 100 days with mild and transient signs of GVHD (P < 0.001; Figure 1, A–D). These results indicate that in vivo depletion of donor CD4+ T cells early after HCT preserves GVL effects while preventing GVHD.

Leukemia and lymphoma cells also infiltrate liver tissues. In vivo bioluminescent imaging (BLI) indicated that tumor load started to decrease by day 7 and disappeared by day 12 after HCT (Figure 1, A and B). In addition, as compared with recipients given TCD-BM alone, anti-CD4–treated recipients showed mild and transient signs of acute GVHD, peaking at day 7 (Figure 1C). Thus, we tested whether CD8+ T cell–mediated GVL activity was associated with hepatocyte damage. We compared serum aspartate aminotransferase (AST) concentrations in anti-CD4–treated recipients with or without the presence of BCL1 cells. On day 7 after HCT, anti-CD4–treated recipients with BCL1 cells had significantly elevated serum AST concentrations as compared with recipients without BCL1 (P < 0.01; Figure 1E). By day 12, AST concentrations returned to normal, and no difference was observed among anti-CD4–treated recipients with or without BCL1 inoculation and recipients given TCD-BM alone. These results indicate that the GVL effect of CD8+ T cells is associated with mild hepatocyte damage, but this damage is self-limited and disappears after tumor cell eradication.

Second, we tested the GVL capacity of this regimen by using "GVL-resistant" blast-crisis chronic myelogenous leukemia (BC-CML) in C57BL/6 background. Murine BC-CML cells obtained from W. Shlomchik (University of Pittsburgh) were generated by retroviral transfer of bcr-abl and NUP98/HOXA9 fusion cDNAs. Like human BC-CML, murine BC-CML was relatively GVL resistant. At certain cell doses, allogeneic CD8+ T cells were not able to rescue recipients inoculated with BC-CML cells, although identical numbers of CD8+ T cells rescued almost all recipients inoculated with the same number of chronic-phase chronic myelogenous leukemia (CP-CML) cells (30).

Accordingly, A/J BM (10 × 10⁶) and spleen cells (10 × 10⁶) were transplanted into lethally irradiated (1,100 cGy) C57BL/6 recipients (31). The recipients were challenged with an i.v. injection of BC-CML (20 × 10³ cells per mouse) at the time of HCT (30). The tumor cells killed all (12 of 12) GVHD-free recipients given TCD-BM alone within 30 days, and moribund mice had high percentages of BC-CML cells in the spleen, liver, and BM (Figure 2, A and B, and Supplemental Figure 6A). In contrast, tumor cells were eliminated in IgG-treated GVHD recipients, although they
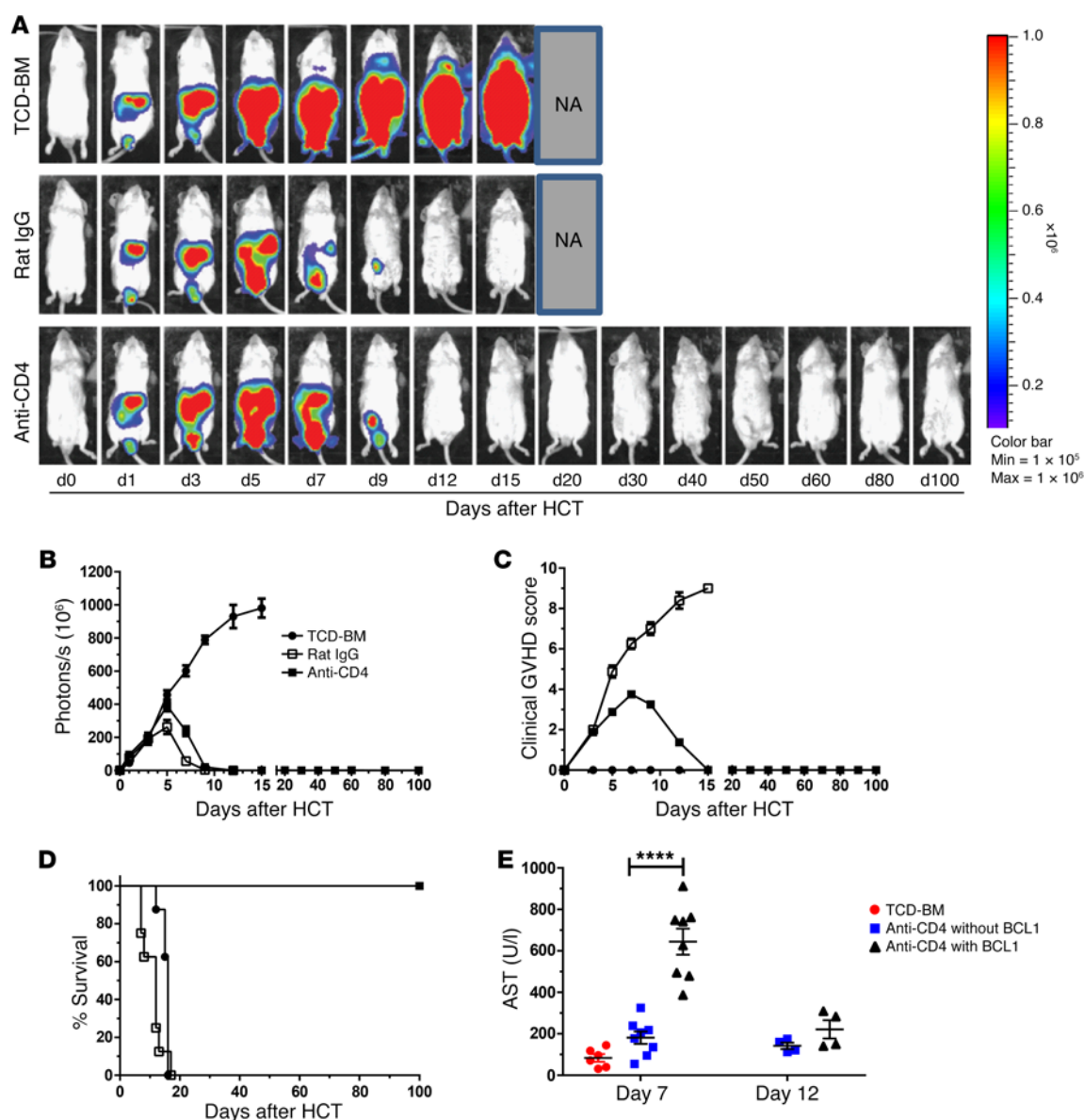
Figure 1. Three injections of anti-CD4 prevent both acute and chronic GVHD and preserve GVL effects after HCT with C57BL/6 donors and BALB/c recipients. Lethally irradiated BALB/c recipients transplanted with splenocytes (5×10^6) and TCD-BM (2.5×10^6) from C57BL/6 donors. Recipients were challenged with i.p. injection of BCL1/Luc cells (5×10^6 per mouse) and were given 3 i.v. injections of rat IgG or anti-CD4 mAb (500 μg/mouse) at days 0, 14, and 28 after HCT. Recipients given TCD-BM cells (2.5×10^6) alone were used as controls. Mice were monitored for tumor growth using in vivo biolumi-nescent imaging (BLI), clinical signs of GVHD, and survival. (**A**) One representative BLI image from each time point is shown for each group. (**B**) Summary of photons per second of recipients. (**C**) Clinical GVHD score. (**D**) Percentage of survival. (**E**) Serum AST concentrations on days 7 and 12 after HCT. n = 4–8 per group, combined from 2 replicate experiments. Data represent mean ± SEM. P values were calculated by multiple t test (**B** and **C**), log-rank test (**D**), or unpaired 2-tailed Student's t tests (**E**) (****$P < 0.0001$).

all (8 of 8) died due to GVHD within 15 days after HCT (Figure 2, A and B). The anti-CD4–treated, GVHD-free recipients given 10×10^6 donor spleen cells had significantly prolonged survival, as compared with TCD-BM recipients ($P < 0.01$; Figure 2A), but by day 100 after HCT, 70% (7 of 10) of the recipients died with progressive tumor growth (Figure 2, A and B, and Supplemental Figure 6A). The 3 recipients surviving more than 100 days after HCT had no detectable tumor cells. Thus, BC-CML cells appear to be partially resistant to GVL effects in anti-CD4–treated recipients given 10×10^6 spleen cells.

In further experiments, we increased donor spleen cells to 20×10^6 and 40×10^6 and extended the anti-CD4 treatment to day 60 after HCT. 37.5% (6 of 16) of recipients given 20×10^6 donor spleen cells died with progressive tumor growth; 62.5% (10 of 16) survived for more than 100 days without detectable tumor cells (Figure 2, A and B). All (12 of 12) recipients given 40×10^6 donor spleen cells survived for more than 100 days without detectable tumor cells in the spleen, liver, or BM (Figure 2, A and B, and Supplemental Figure 6A). The anti-CD4–treated recipients given 40×10^6 donor spleen cells showed recovery of CD4+ T cells to a level
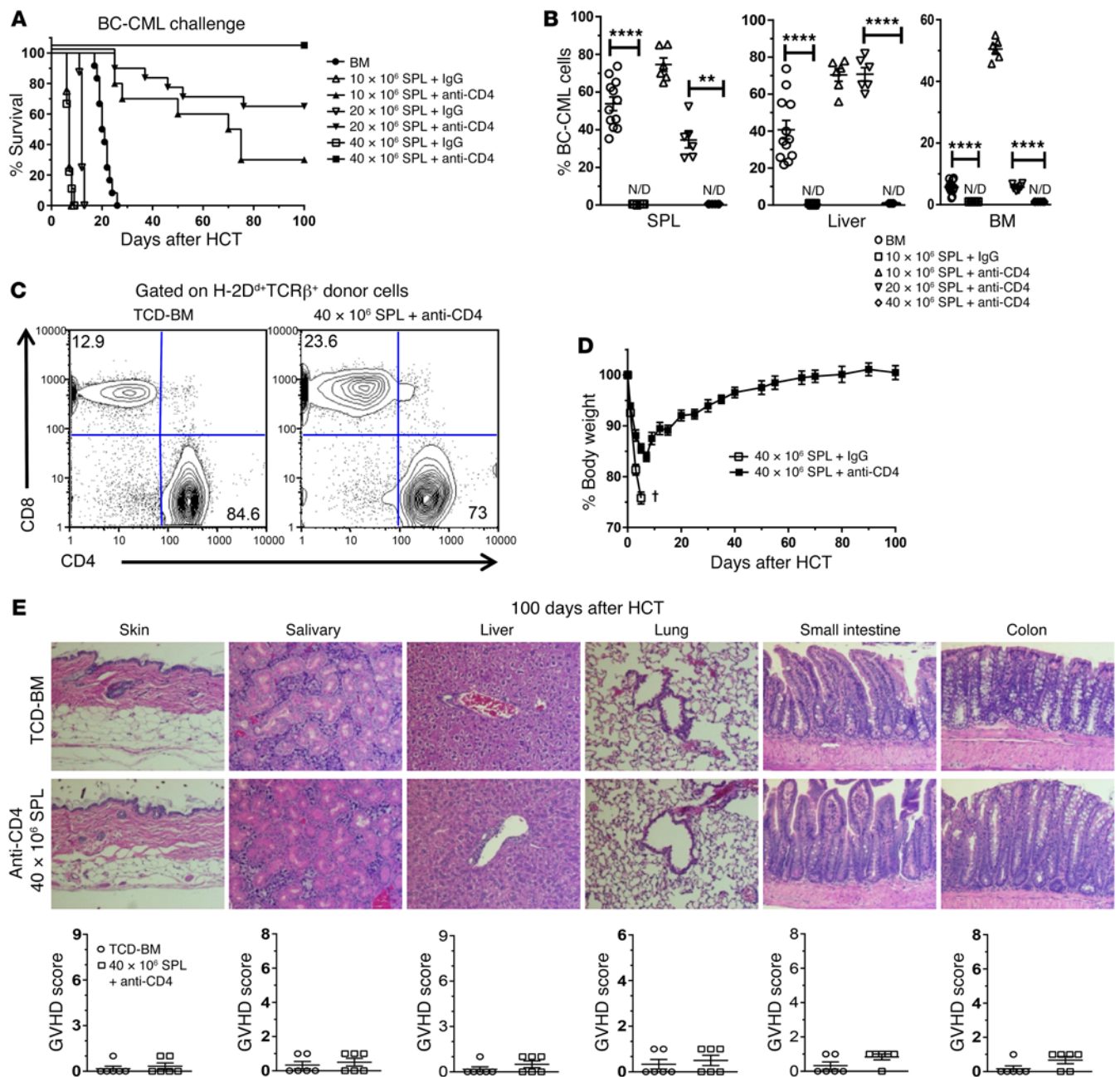
Figure 2. Depletion of donor CD4+ T cells preserves GVL effect while preventing GVHD after HCT with A/J donors and C57BL/6 recipients. Lethally irradiated C57BL/6 recipients transplanted with splenocytes (SPL; 10×10^6, 20×10^6, or 40×10^6) and BM cells (10×10^6) from A/J donors. eGFP+ blast-crisis chronic myelogenous leukemia cells (eGFP+ BC-CML, 20×10^3) were injected i.v. on day 0. Recipients were injected with either rat IgG or anti-CD4 mAb (500 μg/mouse) at days 0, 7, 14, 28, 45, and 60 after HCT. Recipients were monitored for signs of tumor burden and clinical GVHD. Data are combined from 2–4 replicate experiments. (**A**) Percentage of survival; n = 8–16 per group. (**B**) Moribund mice with or without GVHD during observation and mice at day 100 after HCT were checked for BC-CML tumor cells in the spleen, liver, and BM. Percentage of BC-CML cells in spleen, liver, and BM is shown; n = 6–12 per group. N/D, non-detectable. (**C**) One hundred days after HCT, splenocytes were stained with anti–H-2K^b, TCRβ, CD4, and CD8 mAbs and analyzed for CD4+ T cell recovery after anti-CD4 mAb treatment. One representative panel from 4 recipients in each group is displayed. (**D**) Percentage of body weight change in recipients transplanted with 40×10^6 splenocytes treated with either rat IgG or anti-CD4 antibody; n = 8–12 per group. (**E**) One hundred days after HCT, histopathology of skin, salivary gland, lung, liver (original magnification, ×200), small intestine, and colon (original magnification, ×400) was evaluated. A representative photomicrograph and mean ± SEM of histopathology scores are shown; n = 6 per group. Data represent mean ± SEM combined from 2–4 independent experiments. P values were calculated by log-rank test (**A**), unpaired 2-tailed Student's t tests (**B** and **E**), or multiple t test (**D**) (**P < 0.01, ****P < 0.0001). †, indicates all mice died.

Figure 3. Depletion of donor CD4+ T cells preserves GVL effect while preventing GVHD in a xenogeneic GVHD model. NSG recipients transplanted with PBMCs (20 × 10^6 i.p.) from healthy human donors were injected with either IgG or anti–human CD4 mAb (200 μg/mouse, twice weekly for 4 weeks). 1 × 10^6 eGFP+ Raji cells were injected i.p. on day 0. Recipients were monitored for signs of tumor burden and clinical GVHD. (**A**) Percentage of body weight change, survival, and representative photograph of mice transplanted with 20 × 10^6 PBMCs at day 50 to 60 after HCT are shown; *n* = 12 per group. (**B**) Histopathology of skin, salivary gland, lung, and liver was evaluated 50–100 days after HCT. Tissues from the IgG-treated group were harvested approximately 50 days after HCT when the recipients had become moribund. Tissues from anti-CD4–treated recipients were harvested at 100 days after HCT when we ended the experiments. A representative photomicrograph (original magnification, ×200) and mean ± SEM of histopathology scores are shown; *n* = 6 per group. (**C**) Survival of recipients transplanted with 20 × 10^6 PBMCs and 1 × 10^6 Raji cells with IgG or anti–human CD4 mAb; *n* = 12 per group. Panels show eGFP staining to identify Raji cells in the spleen, liver, and BM with or without anti-CD4 treatment when mice became moribund or at day 100 after HCT when we ended the experiments. Percentages of Raji cells in spleen, liver, and BM are shown; *n* = 4 per group. Data represent mean ± SEM combined from 2 replicate experiments. *P* values were calculated by unpaired 2-tailed Student's *t* tests (**B** and **C**) or multiple *t* test and log-rank test (**A** and **C**) (*P < 0.05, **P < 0.01, ***P < 0.001). †, indicates all mice died.

similar to that of TCD-BM recipients by 100 days after HCT (Figure 2C). They showed no clinical evidence of GVHD. Body weight increased progressively, and histological evaluation showed no tissue damage at day 100, similar to results in TCD-BM control (Figure 2, D and E). The antitumor effect was donor CD8+ T cell–dependent, since injection of CD8+ T-depleted spleen cells (40 × 10^6) abolished GVL effects in anti-CD4–treated GVHD-free recipients, and all mice (8 of 8) died with progressive tumor growth by approximately 25 days after HCT (Supplemental Figure 6B). Taken together, these results show that temporary in vivo depletion of CD4+ T cells allows donor T cells to eliminate "GVL-resistant" BC-CML leukemia cells while effectively preventing GVHD.

Third, we tested whether administration of depleting anti–human CD4 mAb could prevent GVHD and preserve GVL effects in a xenogeneic model of GVHD (32). Immunodeficient NSG mice without or with human B cell lymphoma Raji cells (1 × 10^6 per mouse) were used for GVHD or GVL experiments. Healthy human peripheral blood mononuclear cells (PBMCs) (20 × 10^6) were injected i.p. into mice that were then treated with depleting anti–human CD4 (clone IT1208, 200 μg/mouse) or control IgG twice weekly for 4 weeks (33). We tested 4 human PBMC donors. For each donor, we used 16 NSG mice, 8 for GVHD experiments and 8 for GVL experiments. Within each experiment, 4 recipients were treated with control IgG and 4 were treated with anti-CD4.

Anti-CD4 treatment effectively prevented xenogeneic GVHD in experiments with 3 of the 4 donors, and the 12 GVHD-free anti-CD4–treated NSG recipients survived for more than 100 days after PBMC injection (Figure 3A). With cells from 1 donor, anti-CD4 treatment was only partially effective in preventing xenogeneic GVHD (Supplemental Figure 7). IgG-treated control NSG recipients all developed GVHD with weight loss, ruffled fur, and hair loss, and all died by about 60 days after PBMC injection (*P* < 0.01; Figure 3A). Anti-CD4 treatment prevented GVHD target tissue damage in the skin, salivary gland, liver, and lung (*P* < 0.01; Figure 3B).

In GVL experiments, control recipients given Raji cells alone all died with progressive tumor growth by 35 days. NSG mice given Raji cells and human PBMCs were treated with IgG or anti-CD4. All 12 GVHD-free anti-CD4–treated mice survived for more than 100 days after PBMC injection (*P* < 0.01; Figure 3C), but IgG-treated mice all died with GVHD by approximately 65 days after PBMC injection. Control NSG mice that died with progressive tumor growth had Raji cell infiltration in the spleen, liver, and BM, while the anti-CD4–treated GVHD-free NSG mice had no detectable tumor cells in these tissues (*P* < 0.01; Figure 3C). These results suggest that antibody-mediated in vivo depletion of donor CD4+ T cells immediately after HCT may be able to prevent GVHD while preserving GVL effects after allogeneic HCT in humans.

Depletion of donor CD4+ T cells immediately after HCT increases serum IFN-γ but decreases serum IL-2 concentrations. In experiments with C57BL/6 donors and BALB/c recipients, we explored how in vivo depletion of donor CD4+ T cells immediately after HCT prevented acute GVHD while preserving GVL effects. High serum levels of IFN-γ and TNF-α have been associated with acute GVHD (34). Contrary to expectation, depletion of donor CD4+ T cells increased serum IFN-γ concentrations approximately 3-fold at 7 days after HCT (*P* < 0.001). Serum IL-2 concentrations decreased by approximately 50% (*P* < 0.05), and serum TNF-α concentrations showed no significant differences from baseline (Figure 4A). The increased serum levels of IFN-γ are attributable to expansion of donor CD8+ T cells in lymphoid tissues, because the number of IFN-γ+CD8+ T cells in the spleen of anti-CD4–treated recipients was approximately 3-fold higher than in rat IgG–treated recipients (*P* < 0.001), although the percentage of IFN-γ+ cells among CD8+ T cells was similar in the 2 groups (Figure 4B). These results suggest that in vivo depletion of CD4+ T cells immediately after HCT may expand IFN-γ–producing CD8+ T cells in lymphoid tissues.

Depletion of donor CD4+ T cells immediately after HCT increases the numbers of donor CD8+ T cells in lymphoid tissues. Next, we kinetically evaluated the effects of in vivo CD4+ T cell depletion on donor CD8+ T cell expansion and tissue distribution. At 5 days after HCT, the numbers of H-2K^b+ donor-type CD8+ T cells in the spleen and mesenteric lymph nodes were lower in anti-CD4–treated recipients than in rat IgG–treated recipients (*P* < 0.01; Figure 4C). From 7 to 10 days after HCT, the numbers of donor CD8+ T cells in the spleen, popliteal lymph nodes, and mesenteric lymph nodes of anti-CD4–treated recipients were approximately 3-fold higher than those in the control IgG–treated recipients (*P* < 0.01), although the numbers subsided and differences diminished between the 2 groups by 14–21 days after HCT (Figure 4C). By day 28, donor CD8+ T cells expanded again in lymphoid tissues of anti-CD4–treated recipients, but not in IgG-treated recipients, and IgG-treated recipients showed severe lymphopenia (Figure 4C).

Furthermore, by using congenic markers (CD45.2 for the injected T cells and CD45.1 for T cells that were generated de novo from the donor marrow), we found that at 28 days after HCT, CD4+ and CD8+ T cells in the spleen of IgG-treated recipients were almost all derived from CD45.2+ mature T cells in the graft. In contrast, CD4+ T cells in the spleen of anti-CD4–treated recipients were almost all derived from the CD45.1+ donor marrow, while CD8+ T cells originated from both the injected CD45.2+ T cells and the CD45.1+ donor marrow (Supplemental Figure 8A). The yield of
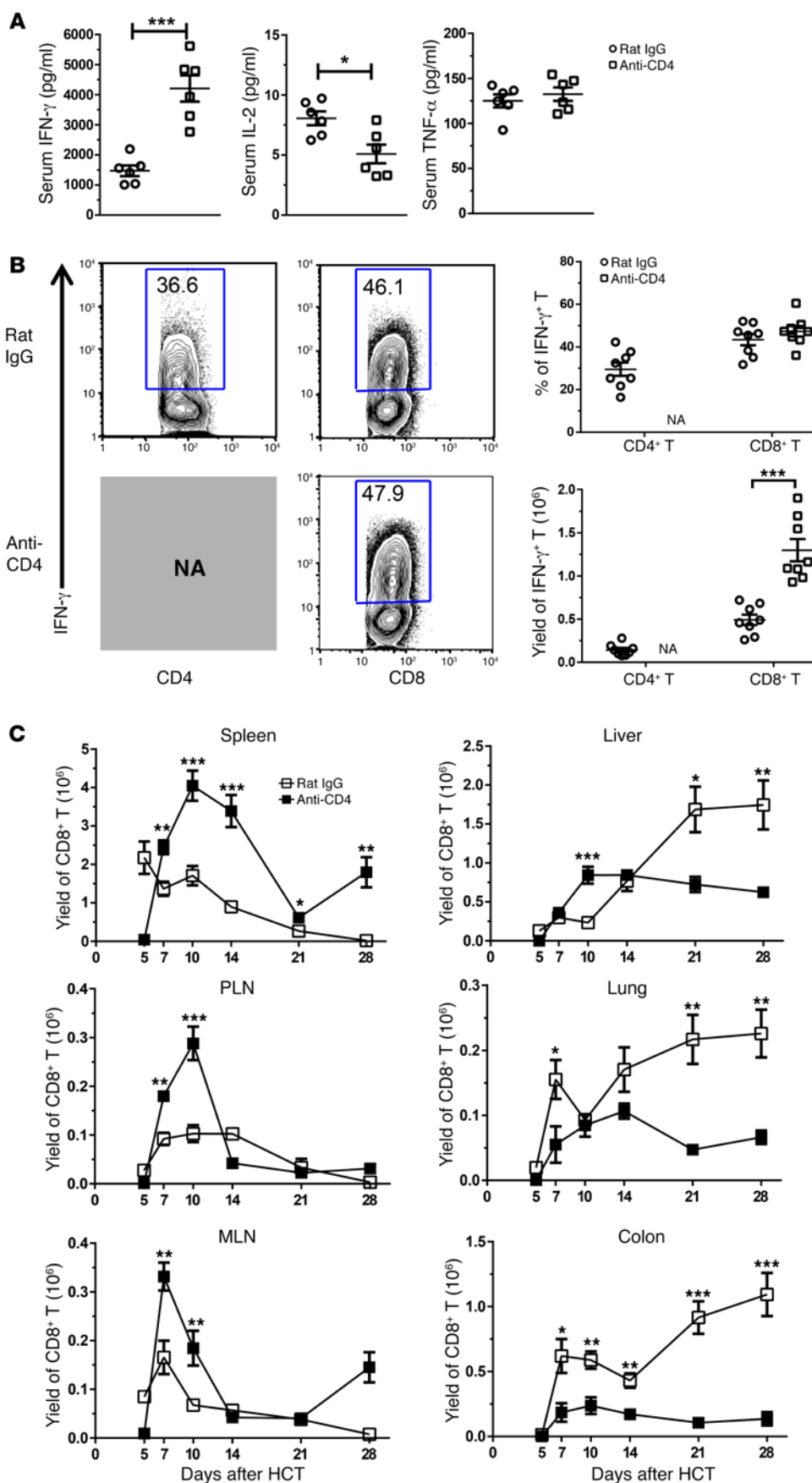
Figure 4. Depletion of donor CD4+ T cells increases serum IFN-γ concentrations but decreases IL-2 concentrations and augments CD8+ T cell expansion in lymphoid tissues but not in GVHD target tissues. BALB/c recipients transplanted with splenocytes (2.5 × 10⁶) and TCD-BM cells (2.5 × 10⁶) from C57BL/6 donors were injected with either rat IgG or anti-CD4 mAb (500 μg/mouse) at day 0 after HCT. (**A**) Concentrations of IFN-γ, IL-2, and TNF-α in serum from recipients 7 days after HCT; $n = 6$ per group. (**B**) Splenocytes from recipients at day 7 after HCT were gated on H-2K^b+TCRβ+ and displayed as IFN-γ versus CD4 or CD8. Representative patterns and mean ± SEM of the percentage and yield of IFN-γ+ donor T cells in the spleen are shown; $n = 8$ per group. (**C**) Kinetic changes in donor CD8+ T cell expansion and infiltration. At days 5, 7, 10, 14, 21, and 28 after HCT, spleen, popliteal lymph nodes (PLN), mesenteric lymph nodes (MLN), liver, lung, and colon of recipients were harvested for analysis of donor CD8+ T yield. Mean ± SEM of the yield of H-2K^b+TCRβ+ CD8+ T cells is shown; $n = 4$–6 per group. Data represent mean ± SEM combined from 2 replicate experiments. P values were calculated by unpaired 2-tailed Student's t tests (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

total CD4+ and CD8+ T cells in the spleen of IgG-treated recipients was significantly lower than that in anti-CD4–treated recipients ($P < 0.01$; Supplemental Figure 8A). Very few Foxp3+ Tregs derived from the injected CD4+ T cells were present in IgG-treated recipients, but Tregs represented approximately 10% of the CD4+ T cell population derived from the donor marrow in anti-CD4–treated recipients (Supplemental Figure 8B). These results indicate that IgG-treated recipients developed acute GVHD and lymphopenia. A single injection of anti-CD4 effectively depletes the injected CD4+ T cells and augments de novo regeneration of both CD4+ and CD8+ T cells, as well as Tregs.

Depletion of donor CD4+ T cells immediately after HCT decreases the numbers of donor CD8+ T cells in the intestine and lung but not in the liver. At 5 days after HCT, only a few donor CD8+ T cells infiltrated the colon, lung, and liver, with no difference between recipients treated with IgG or anti-CD4. From day 7 to day 28 after HCT, the numbers of donor CD8+ T cells in the colon were markedly lower in anti-CD4–treated recipients than in IgG-treated recipients ($P < 0.01$; Figure 4C). The pattern in the lung was similar to that in the colon. In the liver, the numbers of donor CD8+ T cells were higher in anti-CD4–treated recipients than in IgG-treated recipients at 10 days after HCT ($P < 0.01$), but by day 21, the numbers of CD8+ T cells in IgG-treated recipients surpassed the numbers in anti-CD4–treated recipients ($P < 0.01$; Figure 4C). The expansion of donor CD4+ and CD8+ T cells in GVHD target tissues of IgG-treated recipients was associated with recurrence of GVHD (Figure 4C and Supplemental Figure 4A).

We and others previously reported that donor T cell infiltration of gut tissues is regulated by their expression of gut tissue–specific homing and chemokine receptors ($\alpha_4\beta_7$, CCR9, CXCR3), and by tissue release of the corresponding chemokines (CCL25 and CXCL9–11) (35, 36). Although donor CD8+ T cell infiltration of intestinal tissues (i.e., colon) was markedly decreased in anti-CD4–treated recipients at 7 days after HCT (Figure 4C), donor CD8+ T cells did not show any significant reduction in the expression of $\alpha_4\beta_7$, CCR9, or CXCR3 (Supplemental Figure 9A). Expression of CCL25 in the small intestine and expression levels of CXCL9–11 in the colon were higher in anti-CD4–treated recipients than in IgG-treated recipients ($P < 0.05$; Supplemental Figure 9B). These results suggest that reduction of gut tissue infiltration by donor CD8+ T cells after depletion of donor CD4+ T cells is unlikely to be due to decreased CD8+ T cell migration into gut tissues.

Depletion of donor CD4+ T cells immediately after HCT augments donor CD8+ T cell apoptosis in the intestine and anergy/exhaustion in the liver, but not in the spleen. Next, we explored mechanisms whereby anti-CD4–treated GVHD-free recipients had reduced numbers of donor CD8+ T cells in the colon and similar or higher numbers in the liver, while having increased numbers of donor CD8+ T cells in the spleen, as shown in Figure 4. In the pathogenesis of GVHD, alloreactive donor T cells damage Paneth cells in the small intestine and disrupt epithelial junctions in the colon (37, 38). Consistently, anti-CD4–treated recipients without signs of diarrhea showed little damage to Paneth cells in the small intestine and little disruption of epithelial junctions in the colon (Figure 5, A and B).

Alloreactive T cell infiltration also plays a critical role in damage to the liver (39). Although the numbers of liver-infiltrating CD8+ cells were markedly higher in anti-CD4–treated recipients

than in control IgG–treated recipients on day 10 after HCT (Figure 4C), anti-CD4–treated recipients appeared to have little damage to liver or evidence of hepatocyte apoptosis, in contrast to IgG-treated control recipients ($P < 0.01$; Figure 5, C and D). Furthermore, liver-infiltrating CD8+ T cells from IgG-treated recipients at day 21 after HCT induced GVHD in secondary adoptive recipients, while CD8+ T cells from anti-CD4–treated recipients did not (Figure 5E). These results suggest that liver-infiltrating CD8+ T cells may be anergic or exhausted, such that they become nonpathogenic.

Therefore, we compared the proliferation and apoptosis of donor CD8+ T cells in the spleen, liver, and colon tissues at 7 and 10 days after HCT. At day 7, in vivo BrdU labeling showed that donor CD8+ T cells had significantly faster proliferation in the spleen, liver, and intestine tissues in anti-CD4–treated recipients as compared with IgG-treated recipients ($P < 0.01$; Figure 6A, middle column, and Supplemental Figure 10A). In contrast, apoptosis of donor CD8+ T cells was markedly reduced in the spleen ($P < 0.01$), not significantly changed in the liver, and markedly increased in the colon ($P < 0.01$) in anti-CD4–treated recipients as compared with IgG-treated recipients (Figure 6A, right column, and Supplemental Figure 10B). By day 10, donor CD8+ T cells in the spleen and liver of anti-CD4–treated recipients no longer proliferated better, although the apoptosis rate was still lower (Supplemental Figure 11A). Therefore, the increased proliferation and reduced apoptosis led to the increased numbers of donor CD8+ T cells in the spleen and liver of anti-CD4–treated recipients early after HCT.

To evaluate anergy and exhaustion of donor CD8+ T cells, we compared the CD8+ T cell expression levels (mean fluorescence index, MFI) of the anergy/exhaustion-related markers GRAIL, TIM-3, and IL-R7α. As compared with IgG-treated recipients, the CD8+ T cells from the spleen of anti-CD4–treated recipients did not have significant change in their expression of GRAIL, TIM-3, or IL-7Rα on day 7 (Figure 6B and Supplemental Figure 10C), but they had significantly downregulated expression of TIM-3 and upregulated expression of IL-7Rα on day 10 (Supplemental Figure 11B). In contrast, the CD8+ T cells from the liver of anti-CD4–treated recipients had significantly increased expression of GRAIL and downregulated expression of IL-7Rα on day 7, although the changes appeared to be small (Figure 6B and Supplemental Figure 10C), and on day 10 after HCT, they had upregulated expression of TIM-3 (Supplemental Figure 11B). In addition, comparing CD8+ T cells from the liver and spleen of anti-CD4–treated recipients, CD8+ T cells from the liver expressed significantly higher levels of GRAIL and TIM-3 and lower levels of IL-7Rα at 7 days after HCT ($P < 0.05$; Figure 6C); and higher levels of TIM-3 persisted at day 10 ($P < 0.01$; Supplemental Figure 11C). These results suggest that donor CD8+ T cells in the liver of anti-CD4–treated recipients become anergic and exhausted by 7–10 days after HCT, while those in the spleen do not.

EOMES regulates CD8+ T differentiation (40). EOMES+T-bet+ CD8+ T cells are effector cells with strong cytolytic function, while EOMES+PD-1+ CD8+ T cells are terminally differentiated exhausted cells (41, 42). Therefore, we evaluated the impact of depletion of CD4+ T cells on CD8+ T expression of EOMES, T-bet, and PD-1 in the spleen and liver at 7 and 10 days after HCT. CD8+ T cells from the spleen and liver of anti-CD4–treated recipients had significant increase in percentages of EOMES+T-bet+ and EOMES+PD-1+
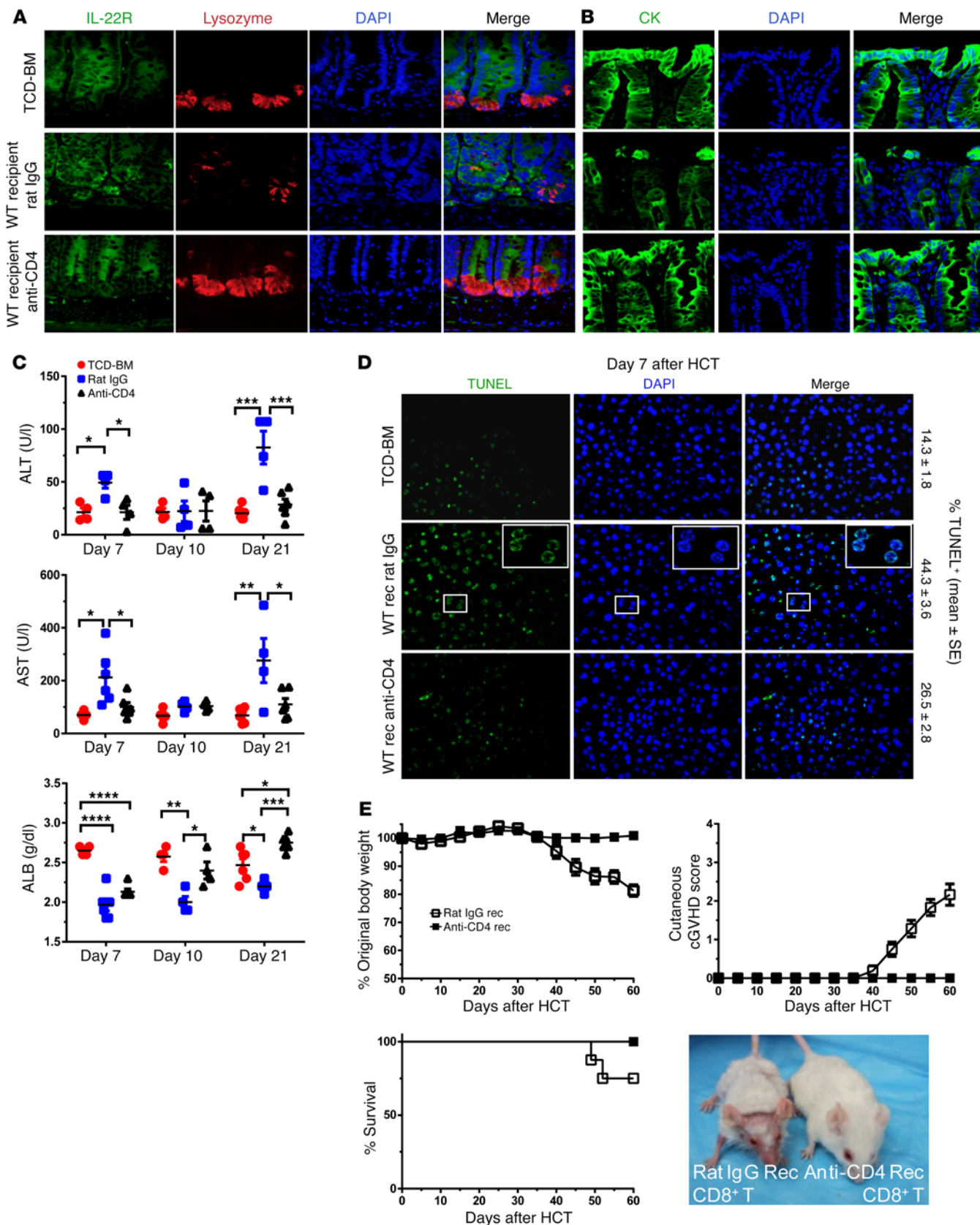
Figure 5. Depletion of donor CD4+ T cells protects Paneth cells, colonic epithelial cells, and hepatocytes. Lethally irradiated WT BALB/c recipients transplanted with TCD-BM alone or with TCD-BM cells and splenocytes (2.5×10^6) from C57BL/6 donors were injected with rat IgG or anti-CD4 mAb (500 μg/mouse) on day 0. Seven days after HCT, intestinal and liver tissue was analyzed. (**A**) Small intestine paraffin sections were stained with anti–IL-22R (green), anti-lysozyme (red), and DAPI (blue). (**B**) Colon paraffin sections were stained with anti-cytokeratin (CK) and DAPI (blue). (**A** and **B**) One representative photomicrograph (original magnification, ×400) is shown from 4 per group. (**C**) Liver enzymes in serum of recipients at days 7, 10, and 21 were measured. Mean ± SEM; $n = 4$–6 per group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$). ALT, alanine amino-transferase; AST, aspartate amino transferase; ALB, albumin. (**D**) TUNEL staining for hepatocyte apoptosis assay. A representative immunofluores-cent photomicrograph (original magnification, ×400) and mean ± SEM of percentage of TUNEL+ apoptotic hepatocytes are shown; $n = 4$ per group. (**E**) Recipients were sacrificed at day 21 after HCT, and sorted liver-infiltrat-ing donor CD8+ T cells (1×10^6) were transplanted together with TCD-BM (5×10^6) into secondary 200-cGy-irradiated $Rag2^{-/-}$ BALB/c mice. Mice were monitored for clinical GVHD. Percentage of body weight change, clinical cutaneous GVHD score, survival curve, and representative photo of mice at day 60 after HCT are shown; $n = 8$ per group combined from 2 replicate experiments. P values were calculated by 1-way ANOVA multiple-compari-sons (**C**) and multiple t test and log-rank test (**E**).

cells, as compared with control IgG–treated recipients at days 7 and 10 after HCT ($P < 0.01$; Figure 6D, Supplemental Figure 10D, and Supplemental Figure 11D). The increase of EOMES+T-bet+ cells was dominant among splenic CD8+ T cells on days 7 and 10, while the increase of EOMES+PD-1+ cells was dominant among CD8+ T cells in the liver at day 7, with no difference on day 10 (Figure 6E and Supplemental Figure 11E). These results indicate that anti-CD4 depletion of donor CD4+ T cells early after HCT leads to preferential cytotoxic differentiation of CD8+ T cells in the spleen and preferential terminal differentiation and exhaustion of CD8+ T cells in the liver.

Depletion of donor CD4+ T cells immediately after HCT allows host-tissue expression of PD-L1 to tolerize infiltrating donor CD8+ T cells in GVHD target tissues but not in lymphoid tissues. PD-L1/PD-1 interaction leads to T cell anergy and exhaustion (18), and simultaneous PD-L1/PD-1 and PD-L1/CD80 interactions aug-ment apoptosis of activated alloreactive CD4+ T cells early after HCT (25). Depletion of donor CD4+ T cells increased serum lev-els of IFN-γ (Figure 4A), and IFN-γ induces tissue expression of PD-L1 in GVHD target tissues (21, 23). Although IL-27 upregulates PD-L1 expression (43), we did not see differences in serum IL-27 concentrations in recipients with or without anti-CD4 treatment (Supplemental Figure 12). Thus, we first tested whether donor cell IFN-γ production and tissue expression of PD-L1 contributed to prevention of GVHD in anti-CD4–treated recipients. Spleen cells (5×10^6) from IFN-γ–deficient ($Ifng^{-/-}$) and WT C57BL/6 donors were transplanted into lethally irradiated BALB/c recipients. Indeed, anti-CD4 treatment did not prevent acute GVHD mediat-ed by transplants from $Ifng^{-/-}$ donors. All recipients showed severe diarrhea and weight loss, and approximately 80% (8 of 10) of the recipients died by 30 days after HCT (Supplemental Figure 13A). The CD8+ T cells, CD11c+ DCs, and Mac-1/Gr-1+ myeloid cells in the spleen and liver of recipients given $Ifng^{-/-}$ transplants all had significantly downregulated expression of PD-L1 early after HCT (Supplemental Figure 13B). These results suggested that IFN-γ
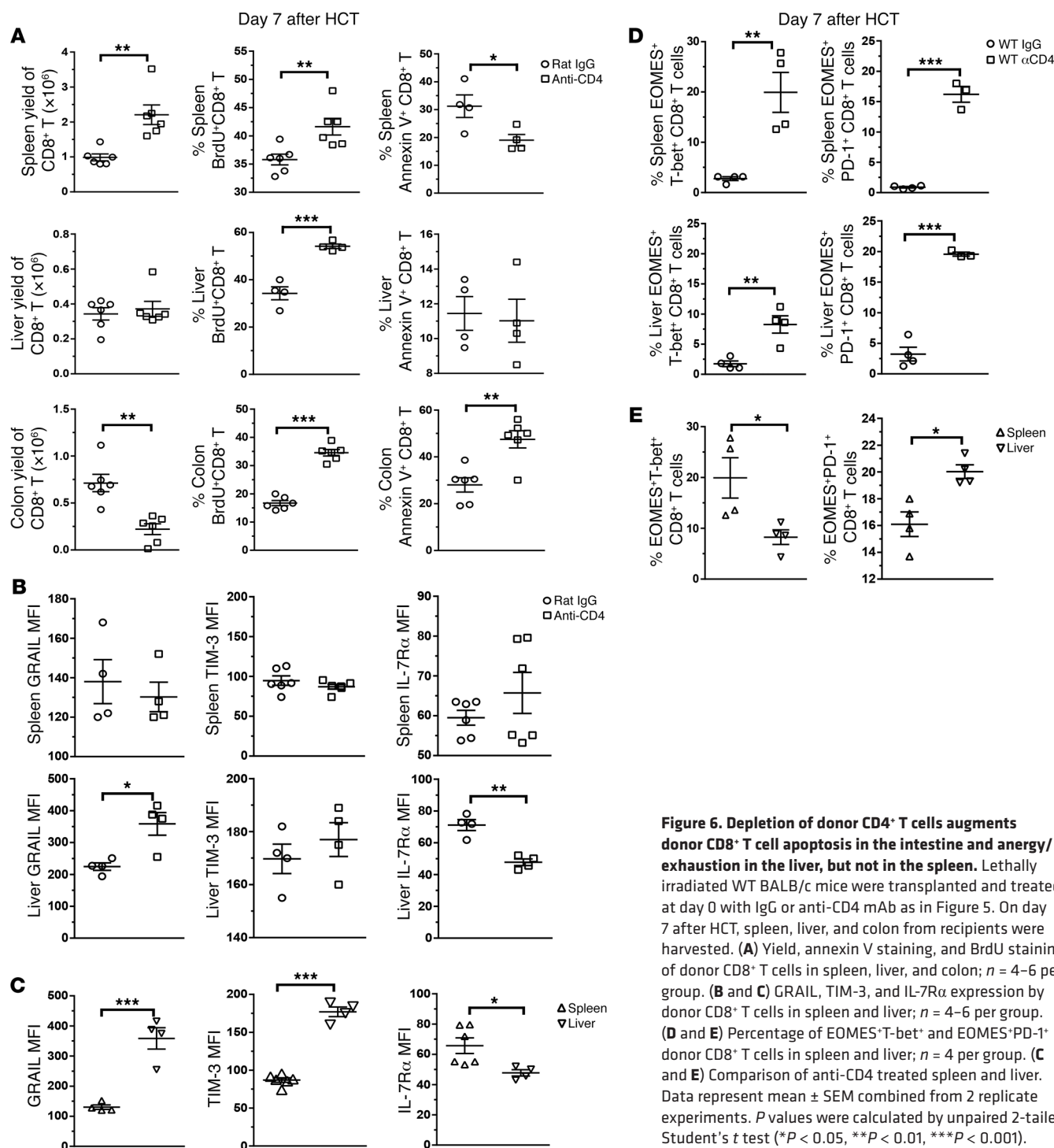
production and tissue PD-L1 expression contribute to GVHD pre-vention by administration of anti-CD4 early after HCT.

Furthermore, we found that elevation of IFN-γ in anti-CD4–treated BALB/c recipients given WT C57BL/6 transplants was associated with upregulation of host intestinal epithelial cell expression of PD-L1 (Supplemental Figure 14A), and in contrast to acute GVHD-free anti-CD4–treated WT recipients, anti-CD4–treated $Pdl1^{-/-}$ recipients showed severe acute GVHD, as judged by body weight loss, severe diarrhea, and death within 10 days after HCT (Supplemental Figure 14B). The acute GVHD was associated with liver dysfunction, hepatocyte apoptosis, and loss of Paneth cells and colon epithelial integrity ($P < 0.01$; Supplemental Fig-ure 14, C and D). The severity of acute GVHD in $Pdl1^{-/-}$ recipients appeared to be similar to that in IgG-treated control WT recipients (Supplemental Figure 14, B–D).

In addition, we directly tested the role of host-tissue PD-L1 on acute GVHD severity induced by sorted CD4+ or CD8+ T cells. While 2.5×10^6 or 5×10^6 sorted CD8+ T cells induced very little evidence of acute GVHD, the same numbers of donor CD4+ T cells induced severe lethal acute GVHD, and all the recipients died with-in 10 days ($P < 0.01$; Supplemental Figure 15A). Conversely, 2.5×10^6 or 5×10^6 sorted CD8+ T cells induced severe lethal acute GVHD in $Pdl1^{-/-}$ recipients, and the severity was similar to that induced by the same number of donor CD4+ T cells (Supplemental Figure 15B). Taken together, these results indicate that host-tissue expression of PD-L1 plays a critical role in preventing acute GVHD mediated by donor CD8+ T cells in the absence of donor CD4+ T cells.

Furthermore, we evaluated the effect of host-tissue expression of PD-L1 on the proliferation, apoptosis, and anergy/exhaustion of CD8+ T cells in the spleen, liver, and colon tissues of anti-CD4–treated recipients on day 7 after HCT. As compared with anti-CD4–treated WT recipients, anti-CD4–treated $Pdl1^{-/-}$ recipients had no changes in proliferation or apoptosis of donor CD8+ T cells in the spleen (Figure 7A and Supplemental Figure 16, A and B), with no difference in the numbers of CD8+ T cells between $Pdl1^{-/-}$ recipi-ents and controls. Anti-CD4–treated $Pdl1^{-/-}$ recipients had a signifi-cant decrease in CD8+ T proliferation and apoptosis in the liver and colon, and the reduction of apoptosis outweighed the reduction of proliferation ($P < 0.01$; Figure 7A and Supplemental Figure 16, A and B), such that higher numbers of donor CD8+ T cells infiltrat-ed the liver and colon of $Pdl1^{-/-}$ recipients as compared with con-trols. These results indicate that host-tissue expression of PD-L1 augments the apoptosis of infiltrating CD8+ T cells in the liver and intestine but not in the spleen of anti-CD4–treated recipients.

We also compared the expression levels (MFI) of GRAIL, TIM-3, and IL-7Rα and the percentage of EOMES+T-bet+ CD8+ and EOMES+PD-1+ CD8+ T cells in the spleen and liver of $Pdl1^{-/-}$ recip-ients and controls at 7 days after HCT. The absence of host-tissue expression of PD-L1 did not significantly change donor CD8+ T expression of GRAIL or TIM-3 in the spleen, although expression of IL-7Rα was higher in $Pdl1^{-/-}$ recipients than in WT recipients (Figure 7B and Supplemental Figure 16C). On the other hand, the absence of host-tissue expression of PD-L1 reduced expression of GRAIL and increased expression of IL-7Rα by CD8+ T cells in the liver, with no significant changes in TIM-3 expression (Fig-ure 7B and Supplemental Figure 16C). The absence of host-tissue PD-L1 did not significantly change the percentages of EOMES+

Figure 6. Depletion of donor CD4+ T cells augments donor CD8+ T cell apoptosis in the intestine and anergy/exhaustion in the liver, but not in the spleen. Lethally irradiated WT BALB/c mice were transplanted and treated at day 0 with IgG or anti-CD4 mAb as in Figure 5. On day 7 after HCT, spleen, liver, and colon from recipients were harvested. (**A**) Yield, annexin V staining, and BrdU staining of donor CD8+ T cells in spleen, liver, and colon; n = 4–6 per group. (**B** and **C**) GRAIL, TIM-3, and IL-7Rα expression by donor CD8+ T cells in spleen and liver; n = 4–6 per group. (**D** and **E**) Percentage of EOMES+T-bet+ and EOMES+PD-1+ donor CD8+ T cells in spleen and liver; n = 4 per group. (**C** and **E**) Comparison of anti-CD4 treated spleen and liver. Data represent mean ± SEM combined from 2 replicate experiments. P values were calculated by unpaired 2-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

T-bet+ or EOMES+PD-1+ CD8+ T cells in the spleen. The absence of host-tissue PD-L1 did not significantly change the percentage of EOMES+T-bet+ CD8+ T cells in the liver, but the percentage of EOMES+PD-1+ CD8+ T cells in the liver was lower in *Pdl1*-/- recipients compared with WT recipients (Figure 7C and Supplemental Figure 16D). In addition, the presence of BCL1 tumor cells did not have a significant impact on the induction of tolerance in donor CD8+ T cells infiltrating the liver (Supplemental Figure 17), suggesting that donor CD8+ T cells in the liver tissues are able to eliminate the infiltrating tumor cells before becoming completely

anergic or exhausted. Taken together, these results show that in anti-CD4–treated allogeneic mouse recipients, host-tissue expression of PD-L1 plays an important role in the induction of anergy, exhaustion, and apoptosis of donor CD8+ T cells infiltrating the liver but not in the spleen.

We also found that human T cells could interact with mouse PD-L1 (Supplemental Figure 18A), and blockade of PD-L1 interaction with its receptors by administration of anti–mouse PD-L1 led to development of lethal xenogeneic GVHD in anti-human CD4–treated NSG recipients given human PBMCs, whereas the
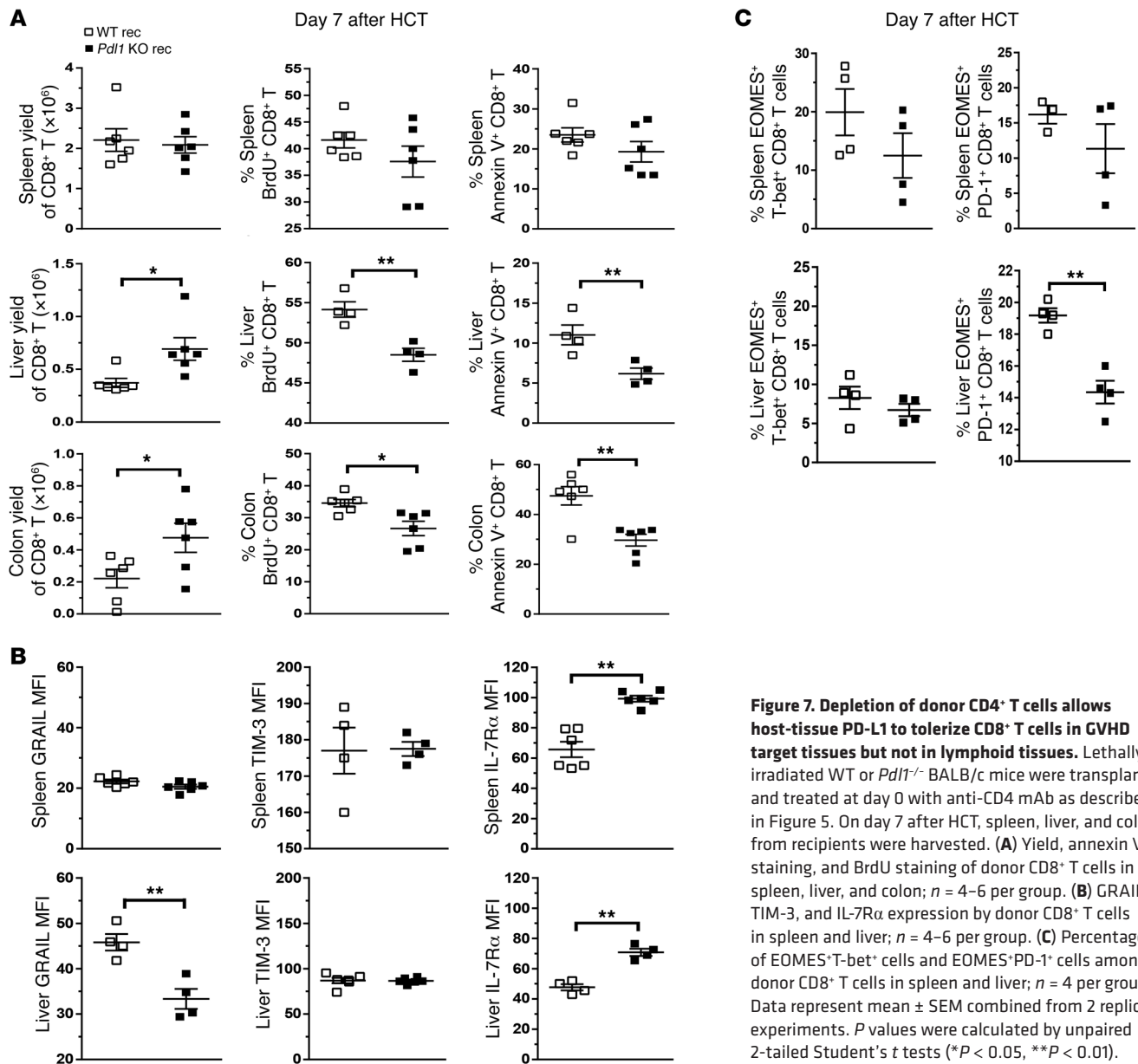
Figure 7. Depletion of donor CD4+ T cells allows host-tissue PD-L1 to tolerize CD8+ T cells in GVHD target tissues but not in lymphoid tissues. Lethally irradiated WT or *Pdl1−/−* BALB/c mice were transplanted and treated at day 0 with anti-CD4 mAb as described in Figure 5. On day 7 after HCT, spleen, liver, and colon from recipients were harvested. (**A**) Yield, annexin V staining, and BrdU staining of donor CD8+ T cells in spleen, liver, and colon; *n* = 4–6 per group. (**B**) GRAIL, TIM-3, and IL-7Rα expression by donor CD8+ T cells in spleen and liver; *n* = 4–6 per group. (**C**) Percentage of EOMES+T-bet+ cells and EOMES+PD-1+ cells among donor CD8+ T cells in spleen and liver; *n* = 4 per group. Data represent mean ± SEM combined from 2 replicate experiments. *P* values were calculated by unpaired 2-tailed Student's *t* tests (**P* < 0.05, ***P* < 0.01).

control recipients without anti–PD-L1 blockade showed no signs of xenogeneic GVHD (Supplemental Figure 18B). Blockade with anti–PD-L1 led to significant downregulation of PD-1 expression by CD8+ T cells and significant augmentation of CD8+ T cell expansion in the liver and lung (Supplemental Figure 18, C and D). These results suggest that tissue expression of PD-L1 contributes to tolerization of human donor CD8+ T cells in xenogeneic recipients in the absence of human CD4+ T cells.

Depletion of donor CD4+ T cells immediately after HCT leads to donor CD8+ T cell upregulated expression of PD-L1 and CD80 in lymphoid tissues, which preserves GVL effects. Since donor T cell expression of PD-L1 augments acute GVHD lethality in recipients transplanted with both CD4+ and CD8+ T cells (24), we evaluated the effect of donor CD8+ T expression of PD-L1 in the expansion and GVL activity of CD8+ T cells in GVHD-free anti-CD4–treated recipients. Anti-CD4 treatment significantly upregulated CD8+ T cell expression of PD-L1 in the spleen and liver but not in the colon (Figure 8A and

Supplemental Figure 19A). Anti-CD4 treatment also significantly upregulated CD8+ T cell expression of PD-1 and CD80 early after HCT (Figure 8A and Supplemental Figure 19A). CD8+ T cells in the spleen had the highest expression of PD-L1 and CD80, with the lowest expression of PD-1. In contrast, CD8+ T cells in the colon had the lowest expression of PD-L1 and CD80, with the highest expression of PD-1. The pattern for CD8+ T cells in the liver fell in between, as indicated by the ratio of PD-1 to CD80 (Figure 8A). Consistent with a previous report (44), non-T cells such as CD11c+ DCs and CD11b/Gr-1+ myeloid cells in the spleen expressed much higher levels of PD-L1 as compared with those in the liver and colon. Anti-CD4 treatment did not significantly change the high expression of PD-L1 by non-T cells in the spleen (Supplemental Figure 20). Therefore, we tested the impact of donor CD8+ T cell PD-L1 and CD80 interactions on donor CD8+ T expansion and GVL effects.

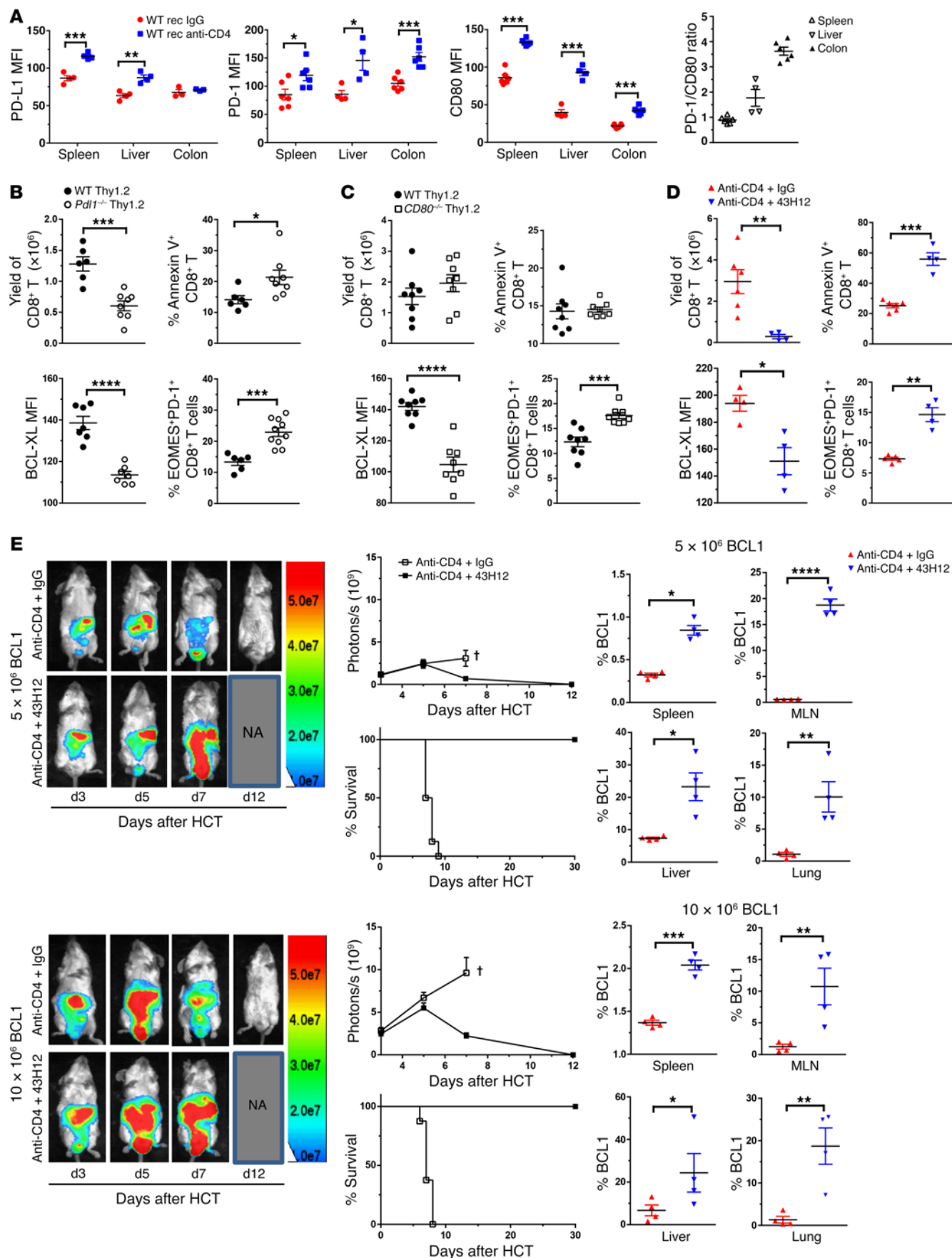Transplantation of sorted Thy1.2+ T cells from *Pdl1−/−* C57BL/6 donors and TCD-BM cells from WT C57BL/6 donors led to

Figure 8. Donor CD8+ T-T PD-L1/CD80 interactions augment CD8+ T expansion and GVL effects in lymphoid tissues. (**A**) Lethally irradiated WT BALB/c recipients received HCT as described in Figure 5. PD-L1, PD-1, and CD80 expression on donor CD8+ T cells in spleen, liver, and colon on day 7 after HCT; PD-1/CD80 ratio is for comparison of spleen, liver, and colon in anti-CD4 treated recipients; n = 4–6 per group. (**B**) WT BALB/c recipients were transplanted with 1×10^6 Thy1.2+ splenocytes from WT or $Pdl1^{-/-}$ C57BL/6 donors and TCD-BM cells from WT C57BL/6 and given anti-CD4 mAb (500 μg/mouse) on day 0. Yield, annexin V staining, BCL-XL staining, and percentage of EOMES+PD-1+ cells among donor CD8+ T cells in spleen are shown; n = 6–10 per group, combined from 2 replicate experiments. (**C**) 1×10^6 Thy1.2+ splenocytes from $CD80^{-/-}$ donors were used to repeat experiments described in **B**; n = 8. (**D**) Anti-CD4–treated WT BALB/c recipients were injected with IgG or PD-L1–specific mAb 43H12 (500 μg/mouse) on days 0 and 2 after HCT. Yield, annexin V staining, BCL-XL staining, and percentage EOMES+PD-1+ cells among donor CD8+ T cells in spleen are shown; n = 4–6 per group. (**E**) Anti-CD4–treated BALB/c recipients of splenocytes, TCD-BM, and BCL1/Luc+ cells were treated with 43H12 mAb or control IgG on days 0 and 2. Recipients were monitored for survival and tumor burden as described in Figure 1. A representative BLI for each group, photons per second of BLI, survival, and percentage of BCL1 cells in the spleen, mesenteric lymph nodes, liver, and lung are shown; n = 4–8 per group. Data represent mean ± SEM combined from 2 replicate experiments. P values were calculated by unpaired 2-tailed Student's t tests (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001). †, indicates all mice died.

marked reduction of donor CD8+ T expansion in the spleen of anti-CD4–treated recipients early after HCT, as compared with Thy1.2+ T cells from WT donors (P < 0.001; Figure 8B). This finding was associated with increased apoptosis, downregulated expression of BCL-XL, and an increased percentage of CD8+ T cells that express PD-1 and EOMES (P < 0.01; Figure 8B and Supplemental Figure 19B). Similarly, transplantation of Thy1.2+ T cells from $CD80^{-/-}$ donors and TCD-BM cells from WT donors also led to significant downregulation of BCL-XL and an increased percentage of CD8+ T cells that express PD-1 and EOMES (P < 0.01) as compared with T cells from WT donors, although expression of annexin V and expansion of the CD8+ T cells were similar in the 2 groups (Figure 8C and Supplemental Figure 19C). These results indicate that expression of PD-L1 and CD80 by donor CD8+ T cells are both required in order to augment their survival and expansion in the spleen of anti-CD4–treated recipients early after HCT.

To further evaluate the role of PD-L1/CD80 interaction in CD8+ T cell survival and expansion, we used an anti–PD-L1 mAb (43H12) that specifically blocks PD-L1/CD80 interaction without interfering with PD-L1/PD-1 interaction (20). The 43H12 mAb was injected i.p. into anti-CD4–treated WT recipients on days 0 and 2 after HCT. As compared with control IgG treatment, blockade of PD-L1/CD80 interaction also markedly decreased donor CD8+ T cell expansion in the spleen. This finding was associated with augmented apoptosis, reduced expression of BCL-XL, and an increased percentage of EOMES+PD-1+ cells (Figure 8D and Supplemental Figure 19D). Taken together, these results indicate that donor CD8+ T-T PD-L1/CD80 interactions play a critical role in augmenting donor CD8+ T survival and expansion in the spleen of anti-CD4–treated recipients early after HCT.

Finally, we evaluated the impact of PD-L1/CD80 interaction on GVL activity in anti-CD4–treated recipients. Since BCL1/Luc+ tumor cells in anti-CD4–treated recipients were eliminated within 12 days after HCT without relapse by 100 days after HCT (Figure

1C), we focused on comparing the tumor load early after HCT in recipients with or without blockade of PD-L1/CD80 interactions via 43H12 mAb treatment on days 0 and 2 after HCT. Anti-CD4–treated recipients were challenged with 5×10^6 and 10×10^6 BCL1/Luc cells. Treatment with anti–PD-L1 (43H12) significantly augmented tumor growth as visualized with in vivo BLI (P < 0.01; Figure 8E). Although all (8 of 8) anti-CD4–treated recipients eliminated tumor cells before day 12 after HCT, blockade of PD-L1/CD80 augmented tumor growth, as indicated by in vivo BLI, resulting in death of all (8 of 8) recipients given 5×10^6 or 10×10^6 BCL1/Luc cells by approximately day 10 after HCT (Figure 8E). 43H12 mAb treatment also markedly increased the tumor load in the spleen, mesenteric lymph nodes, liver, and lungs at 7 days after HCT. Taken together, these results show that donor CD8+ T cell expression of PD-L1 and its interaction with CD80 augments donor CD8+ T survival and expansion in the spleen, resulting in strong GVL activity without causing GVHD early after HCT in anti-CD4–treated recipients.

Discussion

We have shown that temporary in vivo depletion of adoptively transferred mature donor CD4+ T and de novo–generated CD4+ T cells early after HCT prevents both acute and chronic GVHD, while augmenting donor early CD8+ T expansion in lymphoid tissues and preserving strong GVL effects. This outcome does not simply reflect depletion of donor CD4+ T cells that recognize recipient alloantigens, but results from several newly observed mechanisms.

Expression of PD-L1 in recipient tissues can prevent both acute and chronic GVHD after effective depletion of donor CD4+ T cells early after HCT, and temporary depletion for only 30–60 days after HCT is sufficient. We observed that a single injection of anti-CD4 effectively prevented acute GVHD, but the recipients still develop chronic GVHD with damage in GVHD target tissues. We also observed that at least 3 injections were required to effectively prevent chronic GVHD. Three injections of anti-CD4 allowed mTEC recovery and restoration of thymic negative selection, but a single injection was not sufficient. Our recent publication (7) indicated that de novo–generated CD4+ T cells early after HCT could perpetuate CD8+ T-mediated damage in the thymus, leading to autoimmune-like chronic GVHD. Consistently, although a single injection of anti-CD4 prevented acute GVHD and augmented de novo generation of donor-type CD4+ T cells, it did not prevent thymus damage mediated by de novo–generated donor CD4+ T cells early after HCT. On the other hand, in the absence of donor CD4+ T cells, donor CD8+ T cells infiltrating thymic tissues were tolerized by host-tissue PD-L1, and thymus damage mediated by the donor CD8+ T cells was self-limited. Therefore, anti-CD4 treatment has the important effect of temporarily depleting both the injected mature CD4+ T cells and also the CD4+ T cells generated de novo from the marrow progenitors early after HCT, thereby allowing sufficient time for mTECs to recover and restore effective thymic negative selection. This time period is approximately 30–60 days after HCT. CD4+ T cells generated from the donor marrow progenitors after this time point no longer cause chronic GVHD.

We observed that increased IFN-γ concentrations were associated with enhanced expression of PD-L1 by colon epithelial cells,
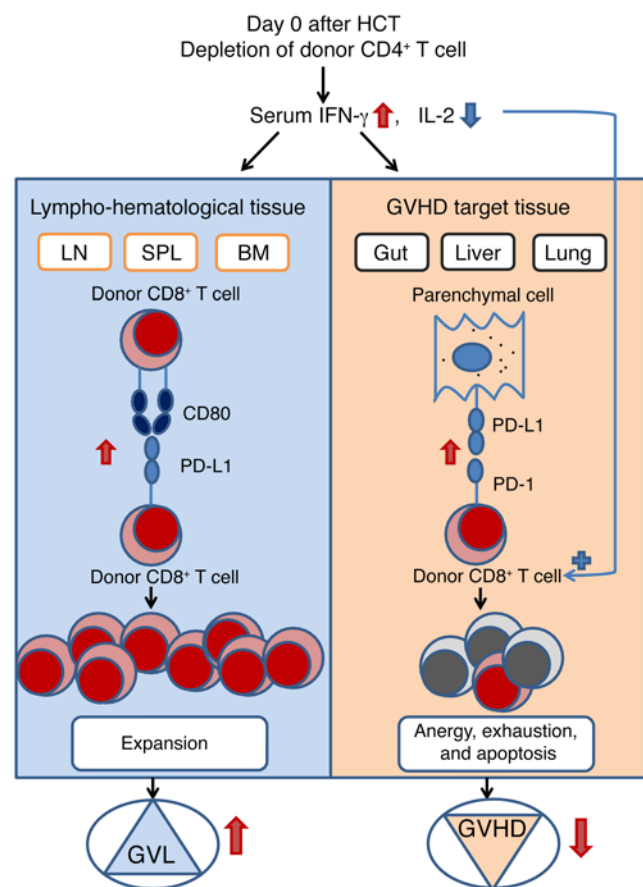
Figure 9. Summary diagram of donor and host-tissue cell expression of PD-L1 in regulation of donor CD8⁺ T expansion and tolerance in the lymphoid and GVHD target tissues. LN, lymph node; SPL, spleen.

and IFN-γ deficiency was associated with downregulation of PD-L1 expression. These observations are consistent with results of a previous study showing that in recipients with acute GVHD, upregulation of PD-L1 expression in host tissues requires IFN-γ (23). Although host-tissue PD-L1 had little impact on donor CD8⁺ T cell proliferation or survival in the spleen early after HCT, depletion of donor CD4⁺ T cells led to induction of apoptosis of infiltrating donor CD8⁺ T cells by PD-L1 in colon tissue and induction of anergy and exhaustion by PD-L1 in liver tissue. The differential effect of PD-L1–mediated signaling on donor CD8⁺ T cells in the colon and liver was associated with differential expression of PD-1 and CD80 by donor CD8⁺ T cells in these tissues. The ratio of PD-1 to CD80 MFI on donor CD8⁺ T cells was significantly higher in the colon as compared with the liver. Further studies are needed to address how donor CD8⁺ T cells express different levels of PD-1 and CD80 in the different tissues, and how different ratios of PD-1/CD80 regulate PD-L1–mediated tolerance mechanisms in CD8⁺ T cells. Other mechanisms may also contribute to GVHD prevention in anti-CD4–treated recipients with elevation of IFN-γ. For example, IFN-γ augments tissue release of indoleamine 2,3-dioxygenase (IDO), which could ameliorate acute GVHD (45). Depletion of donor CD4⁺ T cells also prevents marrow GVHD (46).

NKT cells, myeloid-derived suppressor cells, and regulatory T cells can suppress GVHD (3, 45), and some of these cells express

CD4 and could be depleted by anti-CD4 treatment. However, depletion of donor CD4⁺ T cells together with those CD4⁺ regulatory cells was able to effectively prevent GVHD, suggesting that in the absence of donor CD4⁺ T cells, tissue-protective mechanisms are sufficient to prevent GVHD mediated by CD8⁺ T cells, and CD4⁺ regulatory T cells are dispensable.

We also found that small numbers of donor CD4⁺ T cells in the graft could augment acute GVHD by markedly reducing the apoptosis of CD8⁺ T cells infiltrating the colon, and this effect was IL-2–dependent. In addition, although sorted CD8⁺ T cells induced little and sorted CD4⁺ T cells induced severe acute GVHD in PD-L1–sufficient WT recipients, sorted CD8⁺ and CD4⁺ T cells both induced lethal acute GVHD with similar severity in PD-L1–deficient recipients. These results suggest that CD8⁺ T cells are more sensitive than CD4⁺ T cells to host-tissue PD-L1–mediated apoptosis, and CD4⁺ T cell help early after HCT can make donor CD8⁺ T cells resistant to host-tissue PD-L1–mediated apoptosis. This observation is consistent with a previous report that IL-2 from CD4⁺ T cells may prevent apoptosis induced by PD-1 signaling in CD8⁺ T cells that are deficient in IL-2 production (47). Our observations also explain why host-tissue expression of PD-L1 could ameliorate GVHD only to a certain degree when whole spleen cells with both CD4⁺ and CD8⁺ T cells were transplanted, as indicated by comparison of WT and *Pdl1⁻/⁻* recipients (21, 22).

Depletion of donor CD4⁺ T cells early after HCT not only prevented GVHD, but also enabled donor CD8⁺ T cell expression of PD-L1 to mediate their own expansion in lymphoid tissues and mediate strong GVL activity that could overcome "GVL-resistant" BC-CML tumor cells. The high concentrations of IFN-γ associated with CD4⁺ T cell depletion could contribute to the preservation of GVL activity, since Yang et al. (48) showed that in the absence of donor CD4⁺ T cells, IFN-γ–deficient donor CD8⁺ T cells had lower GVL activity than WT donor CD8⁺ T cells.

We observed that augmentation of donor CD8⁺ T cell expansion in the lymphoid tissues after anti-CD4 treatment is dependent on donor CD8⁺ T expression of both PD-L1 and CD80, and host-tissue expression of PD-L1 has little impact. The lack of impact from host PD-L1 is likely due to relative paucity of host parenchymal cells that express PD-L1 in the lymphoid tissues. The expansion of donor CD8⁺ T cells in lymphoid tissues most likely results from T-T interaction via PD-L1/CD80, although we cannot entirely exclude the possibility that CD8⁺ T interaction with non-T cells via PD-L1/CD80 also plays a role. First, PD-L1 deficiency on donor CD8⁺ T cells, but not PD-L1 deficiency on donor non-T cells (data not shown), markedly reduced donor CD8⁺ T cell survival and expansion. Second, CD80 deficiency on donor CD8⁺ T cells also reduced donor CD8⁺ T expression of survival gene BCL-XL and increased CD8⁺ T cell exhaustion. Third, anti-CD4 treatment early after HCT upregulated PD-L1 and CD80 expression by donor CD8⁺ T cells but not by non-T cells (i.e., DCs and myeloid cells). Finally, specific blockade of PD-L1/CD80 interaction by anti–PD-L1 (43H12) markedly reduced donor CD8⁺ T survival and expansion in the spleen and abolished GVL effects. This observation is consistent with previous findings that PD-L1 on CD8⁺ T cells was required for survival of activated CD8⁺ T cells (49). In contrast, PD-L1/CD80 interactions augment apoptosis of activated CD4⁺ T cells (25). Mechanisms that account for the differential

effects of PD-L1/CD80 interactions on donor CD8^{+} T cells and CD4^{+} T cells are under investigation.

Saha et al. showed that PD-L1 deficiency in donor T cells reduced proliferation and survival of donor T cells and delayed GVHD lethality in recipients given both CD4^{+} and CD8^{+} donor T cells (24). This observation suggests that even when donor T cells have reduced survival capacity due to lack of expression of PD-L1, host-tissue expression of PD-L1 is still unable to tolerize tissue-infiltrating T cells to prevent GVHD when both donor CD4^{+} and CD8^{+} T cells were present. Consistent with this report and adding one step further, we showed that depletion of donor CD4^{+} T cells early after HCT allowed host PD-L1 to effectively tolerize infiltrating CD8^{+} T cells in GVHD target tissues and completely prevent acute GVHD. At the same time, PD-L1/CD80 interactions among donor CD8^{+} T cells in the lymphoid tissues augment CD8^{+} T cell survival and expansion as well as their GVL activity.

On the other hand, using a nonlethal murine model of GVHD mediated by H-Y antigen–specific transgenic CD8^{+} T cells, Michonneau et al. reported that the transgenic CD8^{+} T cells were not able to eliminate host-type tumor cells in lymphoid tissues because of enhanced PD-L1/PD-1 interactions between PD-1^{+} transgenic CD8^{+} T cells and PD-L1^{+} CD11c^{+} DCs and F4/80^{+} macrophages (44). Similarly, Mueller et al. showed that PD-L1 expressed by hematopoietic cells suppressed viral-specific CD8^{+} T cell activation and expansion (50). Although we observed that CD11^{+} DCs and Mac-1/Gr-1^{+} myeloid cells in the spleen expressed much higher levels of PD-L1 as compared with those in the liver, the high levels of PD-L1 on DCs and myeloid cells did not appear to interfere with the GVL activity of donor CD8^{+} T cells in the lymphoid tissues of anti-CD4–treated recipients, since different types and doses of tumor cells were all eliminated in anti-CD4–treated recipients.

Several explanations might account for the different impact of PD-L1 expressed by hematopoietic cells on GVL effects in the lymphoid tissues of recipients given H-Y–specific CD8^{+} T as compared with WT alloreactive CD8^{+} T cells. First, PD-L1 expressed by hematopoietic cells mainly controls activation and expansion of naive T cells (50). In anti-CD4–treated recipients, alloreactive CD8^{+} T cells are activated by recipient APCs that are rapidly eliminated. Therefore, PD-L1 expression by donor hematopoietic-derived APCs does not play an important role in donor T cell activation and expansion. Second, H-Y–specific transgenic CD8^{+} T cells in male recipients appeared to have very weak alloreactivity as indicated by lack of GVHD mortality even after blockade of PD-1. Their alloreactivity was easily controlled by PD-L1/PD-1 interactions between CD8^{+} T cells and DCs and macrophages in the lymphoid tissues. In contrast, the alloreactivity of WT alloreactive CD8^{+} T cells is much stronger, as indicated by their ability to cause rapidly lethal GVHD in Pdl1^{−/−} recipients. Their alloreactivity cannot be controlled by PD-L1/PD-1 interactions between CD8^{+} T cells and DCs and macrophages. Third, H-Y–specific transgenic CD8^{+} T cells might not express PD-L1, or PD-L1 might not play a role in their survival and expansion, unlike WT alloreactive T cells (24).

In summary, as depicted in the diagram (Figure 9), depletion of donor CD4^{+} T cells immediately after HCT increases serum IFN-γ but decreases IL-2 concentrations. Increase of IFN-γ augments expression of PD-L1 by donor CD8^{+} T cells and host tissues, while increasing expression of PD-1 and CD80 by donor CD8^{+} T

cells. Donor CD8^{+} T cells express higher levels of PD-L1 and CD80 but a lower level of PD-1 in the spleen, promoting PD-L1/CD80 interactions among donor CD8^{+} T cells. In contrast, donor CD8^{+} T cells express a higher level of PD-1 and lower levels of PD-L1 and CD80 in GVHD target tissues, promoting host-tissue PD-L1 interaction with PD-1 on donor CD8^{+} T cells. CD8^{+} T cells are defective in IL-2 production, and in the absence of IL-2 help from CD4^{+} T cells, donor CD8^{+} T cells may become more sensitive to the tolerizing effects of PD-L1/PD-1 signaling. Donor CD8^{+} T-T and PD-L1/CD80 interactions augment donor CD8^{+} T survival and expansion in lymphoid tissues, resulting in strong GVL effects. Dominant host PD-L1 interaction with PD-1 on CD8^{+} T cells mediates donor CD8^{+} T cell anergy, exhaustion, and apoptosis in GVHD target tissues, thereby preventing GVHD.

Our results provide an answer to the unanswered historical question of why sorted donor CD8^{+} T cells facilitate engraftment and mediate GVL effect without causing GVHD (4, 13). Our results might explain why ex vivo depletion of donor CD4^{+} T cells did not effectively prevent GVHD in a previous human trial (51), because very small numbers of donor CD4^{+} T cells in the graft could have expanded after HCT, and they could have worked together with donor CD4^{+} T cells generated from the marrow progenitors early after HCT to help donor CD8^{+} T cells resist host-tissue PD-L1–mediated apoptosis or other tolerance mechanisms. Our studies also indicate that temporary in vivo depletion of donor CD4^{+} T cells immediately after HCT may represent a novel approach to prevent GVHD while preserving strong GVL effects. Temporary in vivo depletion of donor CD4^{+} T cells for a period of approximately 30−60 days after HCT may not only allow GVHD target tissues to tolerize infiltrating donor CD8^{+} T cells while preserving GVL effects in the lymphoid tissues, but may also allow regeneration of mTECs and restoration of thymic negative selection for durable prevention of chronic GVHD.

Methods

Mice. WT and gene-manipulated C57BL/6, BALB/c, and other mice were purchased from the National Cancer Institute animal production program (Frederick, Maryland, USA) or Jackson Laboratory or were bred at City of Hope Animal Research Center. Detailed strain information and sources are described in Supplemental Methods. All mice were maintained in a pathogen-free room in the City of Hope Animal Research Center.

Experimental procedures and materials. Induction and scoring of acute and chronic GVHD, in vivo BLI, in vivo BrdU labeling of proliferating T cells, TUNEL staining, tissue cell isolation, intracellular staining of cytokines, antibodies, flow cytometry analysis and sorting, histopathology, histoimmunofluorescent staining, and real-time PCR are described in our previous publications (7, 23, 25) and in Supplemental Methods.

Statistics. Data are displayed as mean ± SEM. Body weight, diarrhea, cutaneous damage scoring, GVHD, and survival in different groups were compared by the multiple t test or log-rank test. Comparison of 2 means was with an unpaired 2-tailed Student's t test; comparison of multiple means was with 1-way ANOVA (Prism, version 6.0; GraphPad Software). P less than 0.05 is considered as significant.

Study approval. All animal procedures were approved by the IACUC of the Beckman Research Institute of City of Hope.

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