Supplementary Data for

Post-Transplantation Cyclophosphamide Prevents Graft-Versus-Host Disease by Inducing Alloreactive T-cell Dysfunction and Suppression

Lucas P. Wachsmuth,[1] Michael T. Patterson,[1] Michael A. Eckhaus,[2] David J. Venzon,[3] Ronald E. Gress,[1] Christopher G. Kanakry[1]

[1]Experimental Transplantation and Immunology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA; [2]Division of Veterinary Resources, Office of Research Services, National Institutes of Health, Bethesda, Maryland, USA; [3]Biostatistics and Data Management Section, Office of the Clinical Director, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

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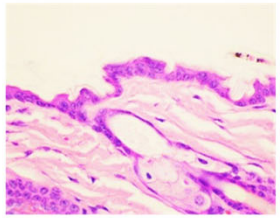
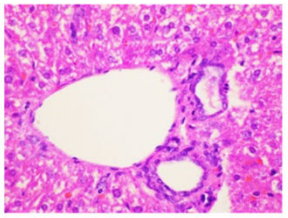
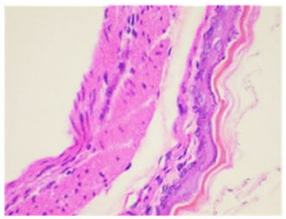
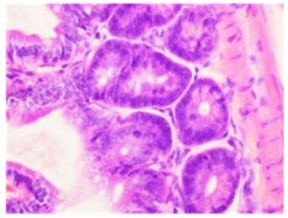
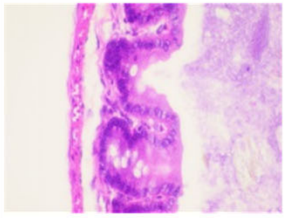
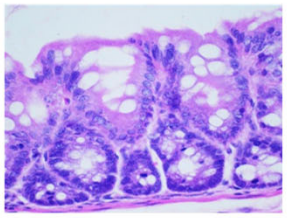
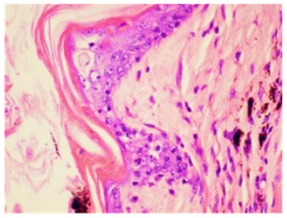
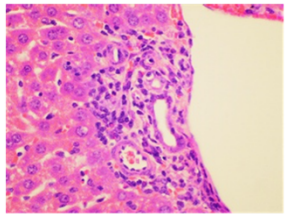
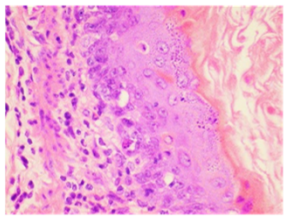
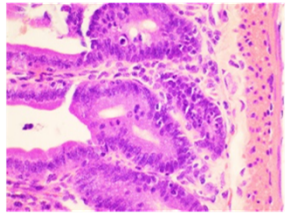
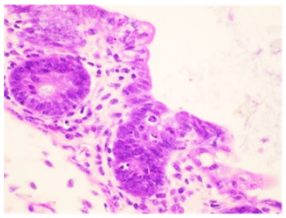
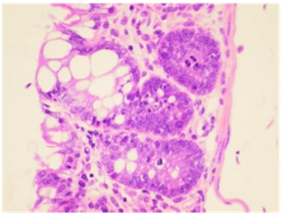
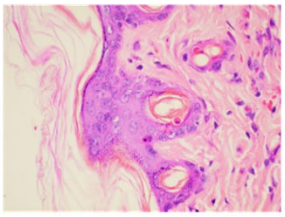
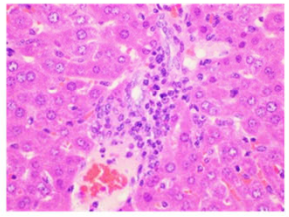
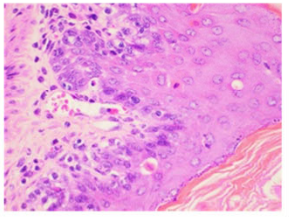
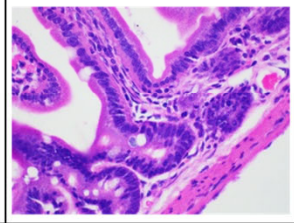
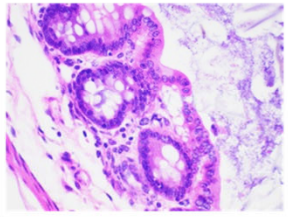
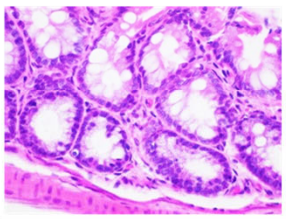
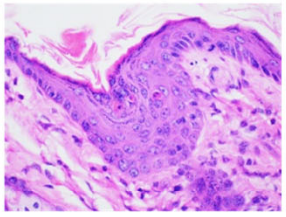
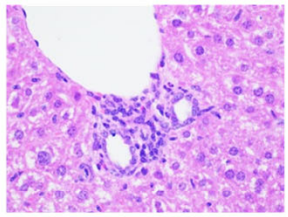
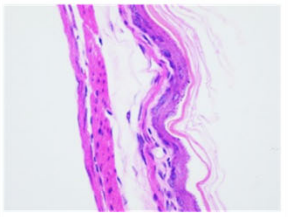
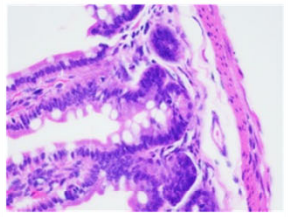
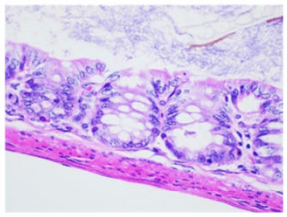
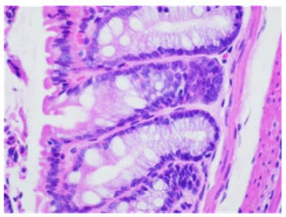
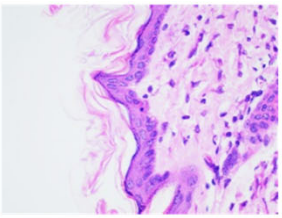
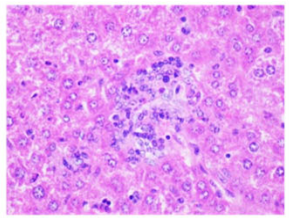
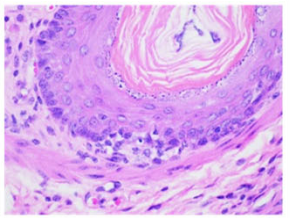
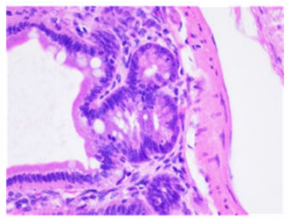
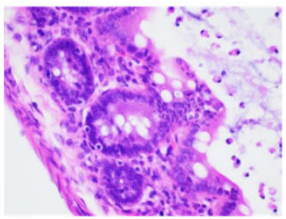
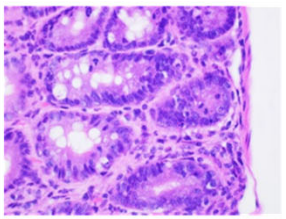
Table S1. Graft-versus-host disease (GVHD) histopathologic severity scoring rubric.

		Histologic Grade			
		1	**2**	**3**	**4**
Organ	**Skin**	· Mild hyperkeratosis and acanthosis · Rare dyskeratotic epithelial cells · Minimal numbers of lymphocytes adjacent to basal epithelium · Minimal intraepithelial lymphocytes multifocally · Some vacuolization of basal epithelium	· Mild hyperkeratosis and acanthosis · Small numbers of dyskeratotic epithelial cells · Small numbers of lymphocytes adjacent to basal epithelium · Rare to small numbers of intraepithelial lymphocytes · Some vacuolization of basal epithelium	· Mild to moderate hyperkeratosis and acanthosis · Small to moderate numbers of dyskeratotic epithelial cells · Increased numbers of lymphocytes adjacent to basal epithelium · Intraepithelial lymphocytes present · More prominent vacuolization of basal epithelium	· Moderate hyperkeratosis and acanthosis · Moderate numbers of dyskeratotic epithelial cells · Increased numbers of lymphocytes adjacent to basal epithelium · Intraepithelial lymphocytes present · More prominent vacuolization of basal epithelium · Microabscesses · Skin erosion and/or ulceration
	Liver	· Minimal periductal lymphoid cell infiltrates (5-10 cells)	· Mild periductal lymphoid cell infiltrates (10-20 cells)	· Moderate periductal lymphoid cell infiltrates (20-40 cells) with intraepithelial lymphoid cells · Single-cell hepatocellular necrosis	· Moderate to severe periductal lymphoid cell infiltrates (>40 cells) with intraepithelial lymphoid cells · Single-cell hepatocellular necrosis · Some bridging of portal zones with lymphoid infiltrates
	Stomach	*Squamous portion:* · Small numbers of lymphocytes adjacent to basal epithelium · Rare dyskeratotic epithelial cells	*Squamous portion:* · Small number of lymphocytes adjacent to basal epithelium · Small numbers of dyskeratotic epithelial cells · Some intraepithelial lymphocytes *Glandular portion:* · Intraepithelial lymphocytes with degeneration of Parietal cells	*Squamous portion:* · Small to moderate number of lymphocytes adjacent to basal epithelium · Moderate numbers of dyskeratotic epithelial cells · Small to moderate numbers of intraepithelial lymphocytes *Glandular portion:* · Intraepithelial lymphocytes with prominent degeneration of Parietal cells	*Squamous portion:* · Small to moderate number of lymphocytes adjacent to basal epithelium · Moderate numbers of dyskeratotic epithelial cells · Small to moderate numbers of intraepithelial lymphocytes · Mucosal erosion/ulceration *Glandular portion:* · Intraepithelial lymphocytes with prominent degeneration of Parietal cells · Mucosal erosion/ulceration
	Intestine	· Rare degenerative epithelial cells · Minimal to small numbers of infiltrating lymphocytes into crypt epithelium · Mild crypt hyperplasia	· Small numbers of degenerative epithelial cells in some crypts · Small numbers of infiltrating lymphocytes into crypt epithelium · Mild to moderate crypt hyperplasia	· Small to moderate numbers of degenerative epithelial cells in most crypts · Infiltrating lymphocytes into crypt epithelium · Moderate crypt hyperplasia · +/- Mucosal ulceration	· Most crypts have evidence of degeneration of crypt epithelial cells · Prominent numbers of infiltrating lymphocytes into crypt epithelium · Moderate crypt hyperplasia · Mucosal ulceration

Table S2. Representative examples of histopathologic assessment of different treatment groups.

	Skin	Liver	Stomach	Small Intestine (SI)	Cecum	Colon
T-cell-depleted Bone Marrow Only Total Score: 0 Skin: 0 SI: 0 Liver: 0 Cecum: 0 Stomach: 0 Colon: 0						
Vehicle Total Score: 14 Skin: 2 SI: 2 Liver: 1 Cecum: 3 Stomach: 3 Colon: 3						
PTCy 5 mg/kg Total Score: 10 Skin: 2 SI: 1 Liver: 2 Cecum: 1 Stomach: 3 Colon: 1						
PTCy 25 mg/kg Total Score: 4 Skin: 1 SI: 0 Liver: 1 Cecum: 1 Stomach: 0 Colon: 1						
PTCy 100 mg/kg Total Score: 6 Skin: 1 SI: 1 Liver: 1 Cecum: 1 Stomach: 1 Colon: 1						

Note: All groups received 10 x 10^6 T-cell-depleted B6C3F1 bone marrow on day 0. All groups except the T-cell-depleted Bone Marrow Only group also received 40 x 10^6 B6C3F1 splenocytes on day 0. PTCy or vehicle treatment was on days +3 and +4.

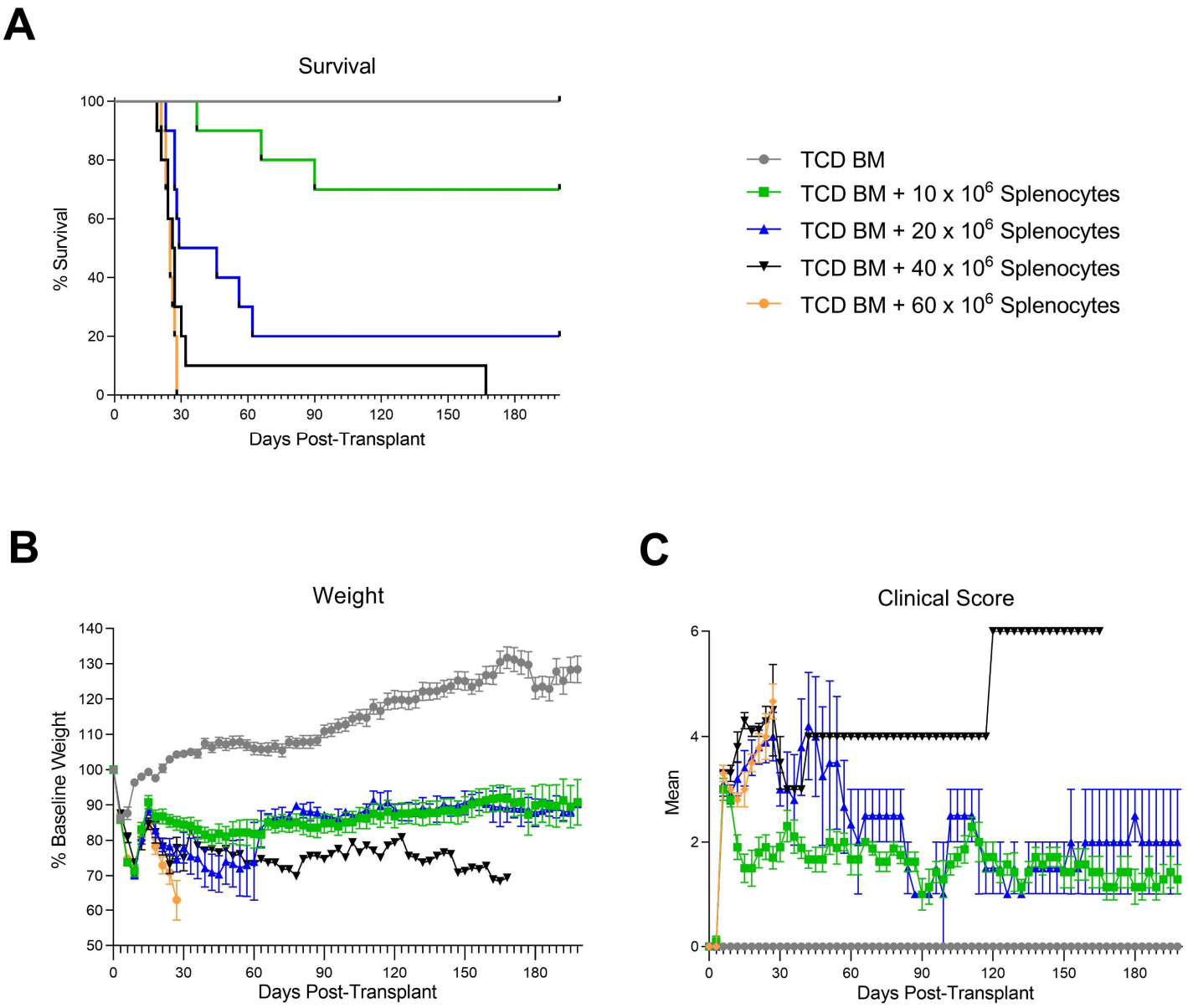


Figure S1. The severity and lethality of GVHD in the B6C3F1→B6D2F1 major histocompatibility complex (MHC)-haploidentical hematopoietic cell transplantation (HCT) model is splenocyte-dose-dependent. Recipient B6D2F1 mice were irradiated to 10.5 Gray and were injected intravenously with 10 x 10^6 B6C3F1 T-cell-depleted bone marrow (TCD BM) cells with or without B6C3F1 splenocytes. Increasing doses of splenocytes resulted in worse GVHD as measured by **(A)** survival, **(B)** weight, and **(C)** clinical score, with splenocyte doses ≥40 x 10^6 resulting in rapid onset of severe and ultimately fatal GVHD. Combined results are shown from two independent experiments of n=5 mice per group per experiment.

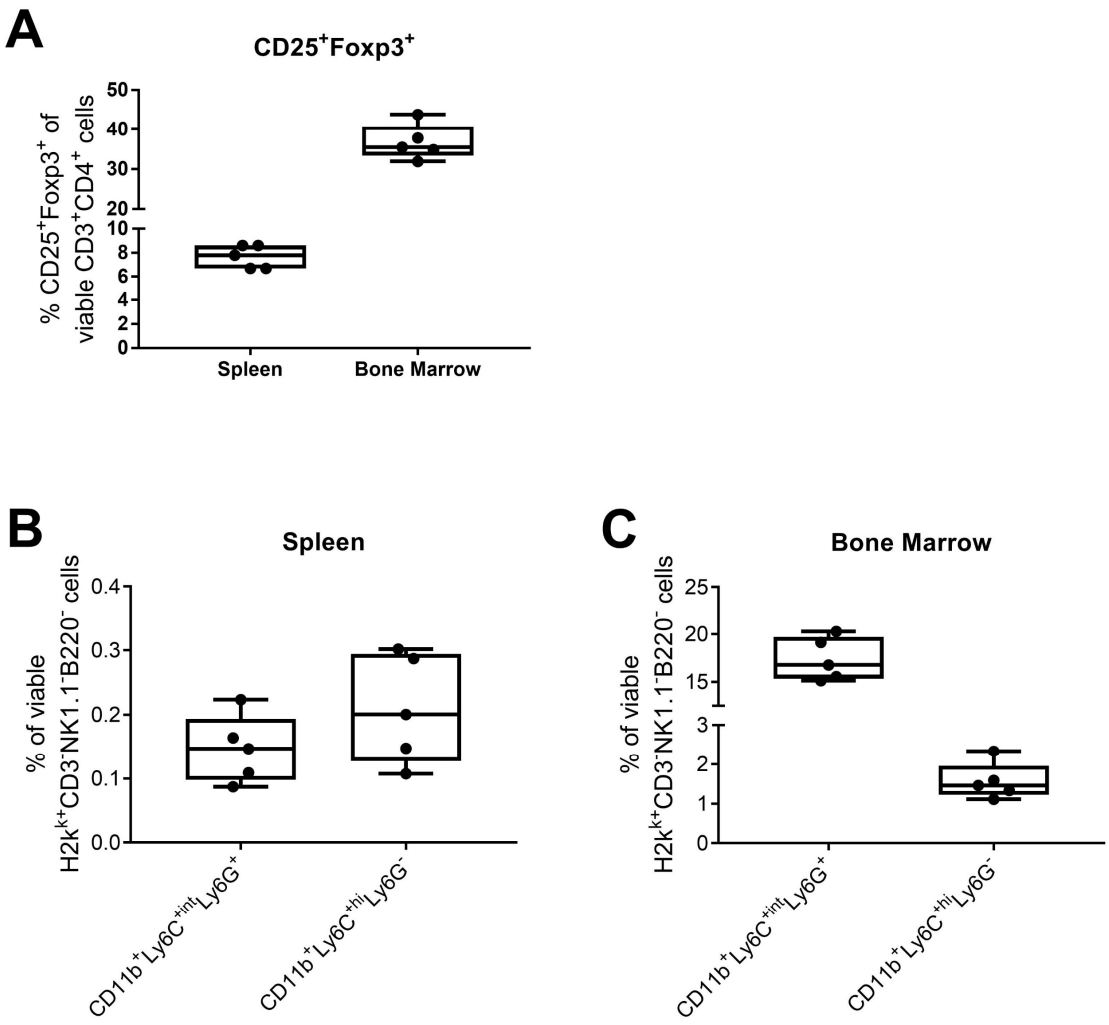


Figure S2. Frequency of phenotypic regulatory T cells and myeloid-derived suppressor cells in normal 12-week-old B6C3F1 female mice. (A) The percentages of $CD4^+$ T cells that were $CD25^+Foxp3^+$ are shown for the spleen and bone marrow. **(B-C)** The percentages of all viable cells that were phenotypically granulocytic ($CD11b^+Ly6C^{+int}Ly6G^+$) or monocytic ($CD11b^+Ly6C^{+hi}Ly6G^-$) myeloid-derived suppressor cells are shown for the **(B)** spleen and **(C)** bone marrow. N=5.

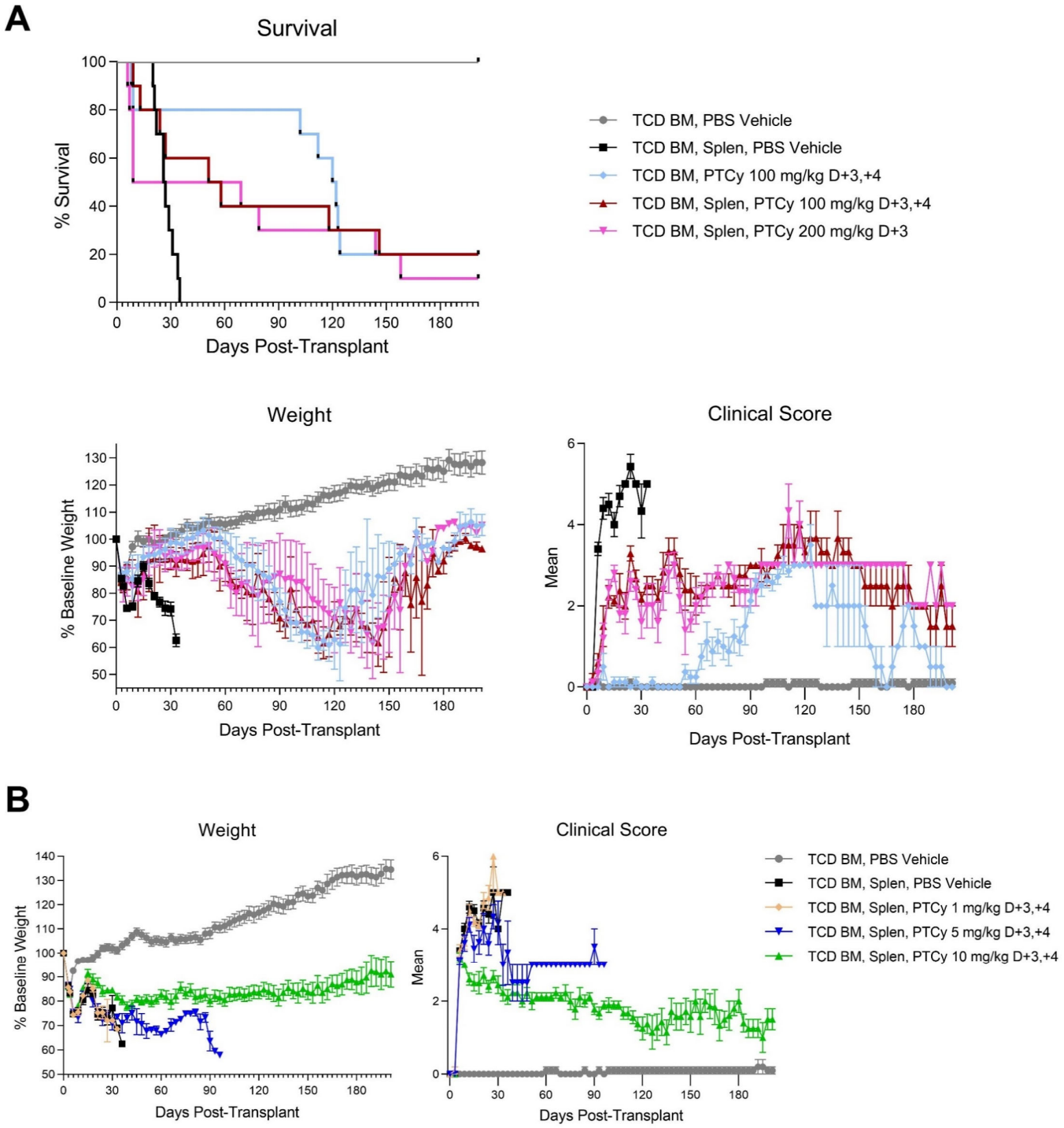
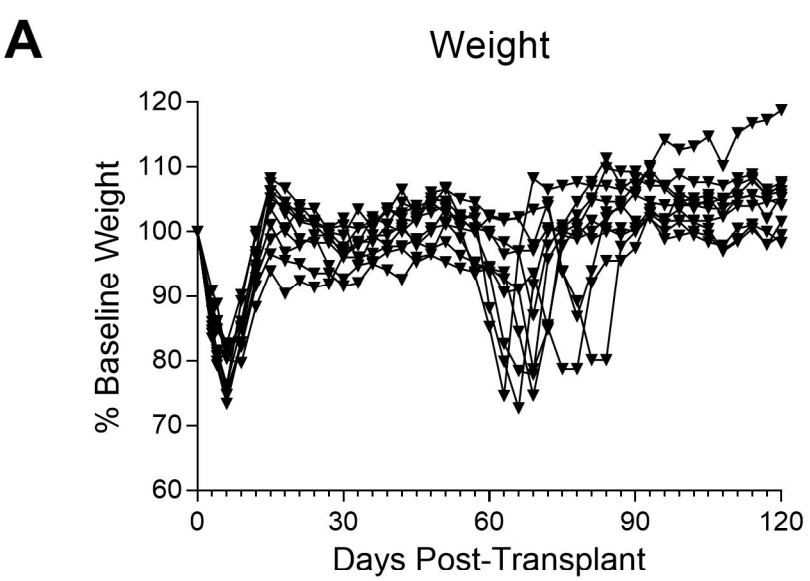


Figure S3. Post-transplantation cyclophosphamide (PTCy) doses ≤5 mg/kg/day or ≥100 mg/kg/day are ineffective in preventing lethality in the B6C3F1→B6D2F1 HCT model. The experiments in Figure 1A-B are shown here with additional data. Data from the PTCy 10, 25, and 50 mg/kg/day groups in the experiments in Figure 1A are not shown here as they are contained in their entirety in Figure 1A. **(A)** Doses of PTCy of 100 mg/kg/day on days +3 and +4 or 200 mg/kg on day +3 (the dose effective in the skin allografting models) resulted in mortality in the vast majority of mice, accompanied by significant deteriorations in weights and clinical scores. Mice receiving splenocytes and these PTCy doses had ~50% mortality prior to day +50, which was associated with increased histopathologic evidence of GVHD as shown in Figure 1F. Yet, mice receiving PTCy 100 mg/kg/day on days +3/+4 but no splenocytes (TCD BM cells only) had less early mortality than mice receiving splenocytes and PTCy 100 mg/kg/day as well as similar weight and clinical scores prior to day +50 compared with mice receiving TCD BM and PBS vehicle. Even so, mice receiving PTCy 100 mg/kg or 200 mg/kg, regardless of splenocyte administration, had marked deteriorations in their weights and clinical scores starting after day +50 that resulted in mortality in most of the remaining mice. In separate experiments [not shown], complete autopsies performed between days +50 and +80 on mice treated with splenocytes and PTCy 100 mg/kg/day on days +3/+4 showed only dehydration and anorexia but no GVHD. Similar autopsy findings were found at day +100 in mice treated with PTCy 100 mg/kg/day after TCD BM alone [n=6], which began to appear sick and die after day +60, suggesting that drug toxicity was a major contributing cause of late morbidity and mortality after the highest PTCy doses. **(B)** Doses of PTCy <10 mg/kg/day on days +3 and +4 were ineffective in preventing severe and fatal GVHD. For mice receiving splenocytes, PTCy 1 mg/kg/day resulted in outcomes superimposable with mice receiving vehicle, while PTCy 5 mg/kg/day led to a mild and transient benefit in a subset of mice. The survival data are shown in Figure 1B. For both **A** and **B**, combined results are shown for two independent experiments with 5 mice per group per experiment.



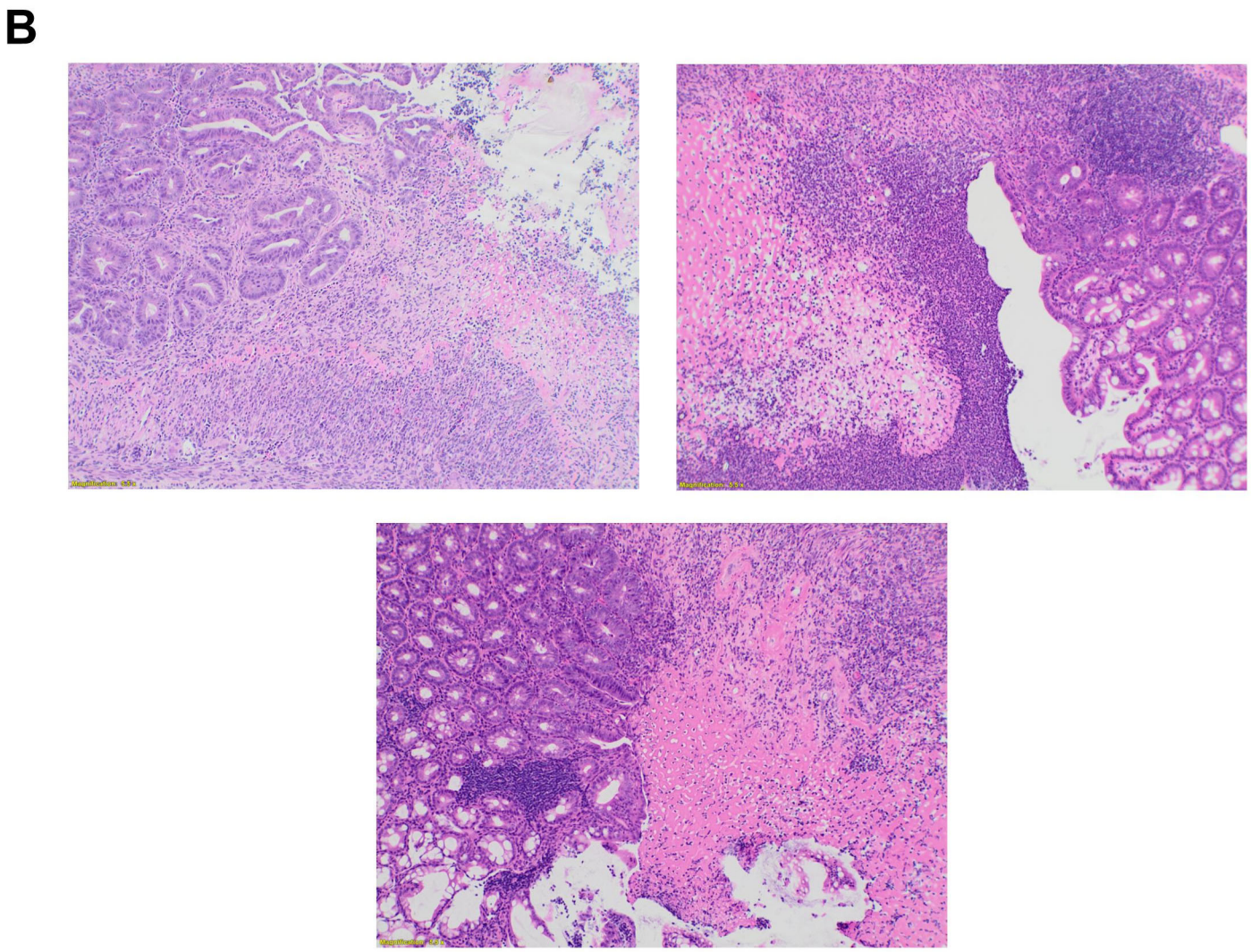


Figure S4. Transient clinical deterioration between days +50 and +90 in mice treated with PTCy 25 mg/kg/day appears related to focal inflammatory intestinal lesions. (A) As shown in Figure 1, mice treated with effective PTCy doses of 25 or 50 mg/kg/day on days +3 and +4 in the B6C3F1→B6D2F1 HCT model have reproducible declines in weight and worsening clinical scores between days +50 and +90. Although these changes appear to be uniform when viewed in aggregate, weights of individual mice rapidly declined and recovered within a matter of several days, while some mice did not have substantial weight changes during this period. Each line represents serial weight measurements for an individual mouse treated with 10 x 10^6 B6C3F1 TCD BM cells and 40 x 10^6 B6C3F1 splenocytes on day 0 followed by PTCy 25 mg/kg on days +3 and +4 (n=10). **(B)** Histopathologic assessment at day +50 of a representative mouse (total n=10) showed focal mucosal hyperplasia and focal mucosal ulceration in the **(Top left)** small intestine, **(Top right)** cecum, and **(Bottom)** colon. All three images are hematoxylin and eosin (H&E) stained slides photographed at 100X magnification.

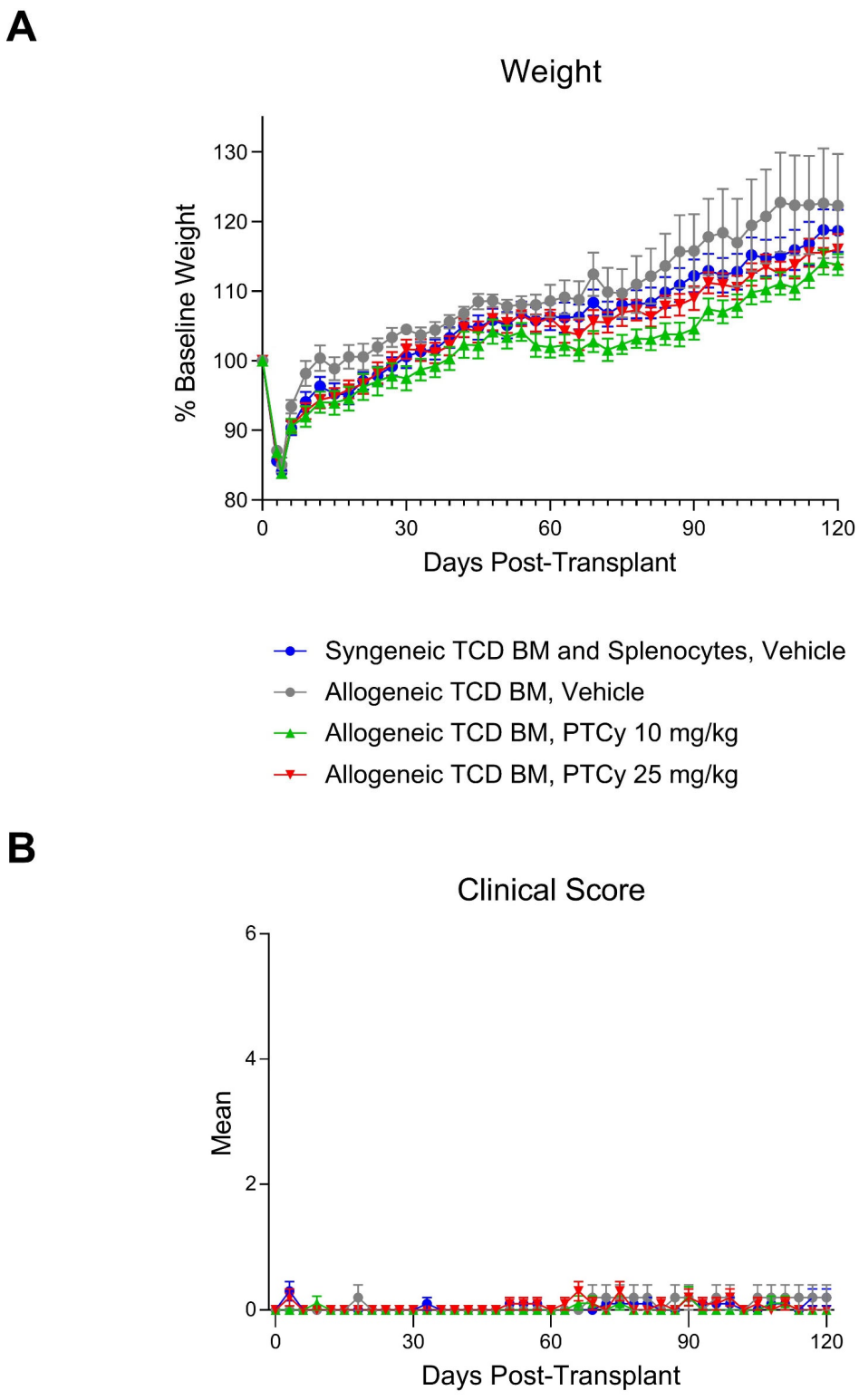


Figure S5. PTCy at 10-25 mg/kg/day has minimal associated toxicity. Recipient B6D2F1 mice receiving PTCy 10 or 25 mg/kg/day on days +3 and +4 after HCT with 10 x 10^6 allogeneic (B6C3F1) TCD BM (n=10 for each group) had **(A)** similarly steady improvements in weights and **(B)** consistently normal clinical scores compared with mice treated with allogeneic TCD BM HCT without PTCy (n=5) or with syngeneic (B6D2F1) T-cell-replete (10 x 10^6 TCD BM and 40 x 10^6 splenocytes) HCT (n=10). The survival data are not shown as they were uniformly 100% for all groups. Combined results are shown from two independent experiments.

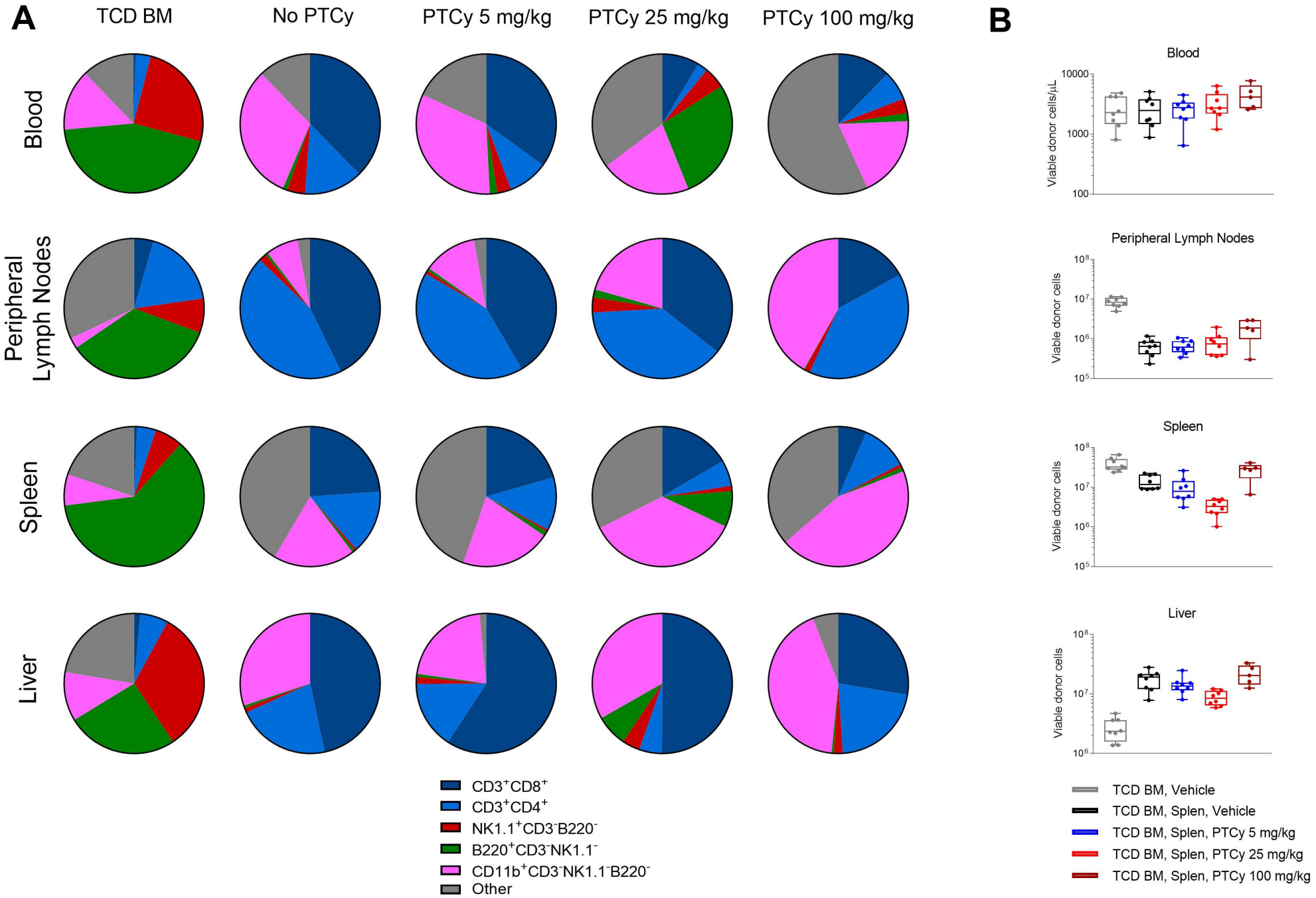


Figure S6. Leukocyte reconstitution of mice treated with PTCy after T-cell-replete HCT differs markedly from mice treated with T-cell-depleted bone marrow HCT. 10 to 12-week-old female B6D2F1 recipient mice were irradiated to 10.5 Gy and transplanted with 10 x 10^6 TCD BM alone on day 0 followed by vehicle on days +3 and +4 or with 10 x 10^6 TCD BM and 40 x 10^6 splenocytes followed by either vehicle (No PTCy) or PTCy on days +3 and +4. **(A)** Pie charts showing the relative composition of viable donor ($H2k^{k+}$) leukocytes in different tissue compartments at day +21. Median values for each subset were used for display purposes. **(B)** Total numbers of viable donor ($H2k^{k+}$) cells in each of these tissue compartments for these same day +21 experiments are shown. N=8 for all groups except n=5 for PTCy 100 mg/kg. Results are combined from two independent experiments.

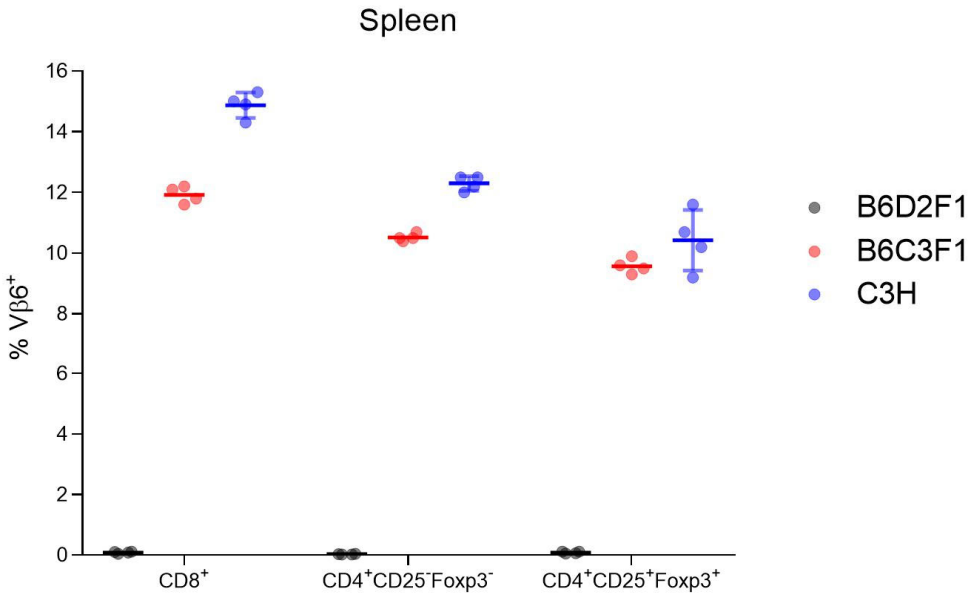


Figure S7. Percentages of Vβ6⁺ T-cell subsets in the spleens of normal mice of different strains. The percentage of $CD8^+$, $CD4^+CD25^-Foxp3^-$, and $CD4^+CD25^+Foxp3^+$ T cells that were $V\beta6^+$ are shown for splenocytes from female 10 to 12-week-old B6D2F1, B6C3F1, and C3H mice. N=4 for each strain.

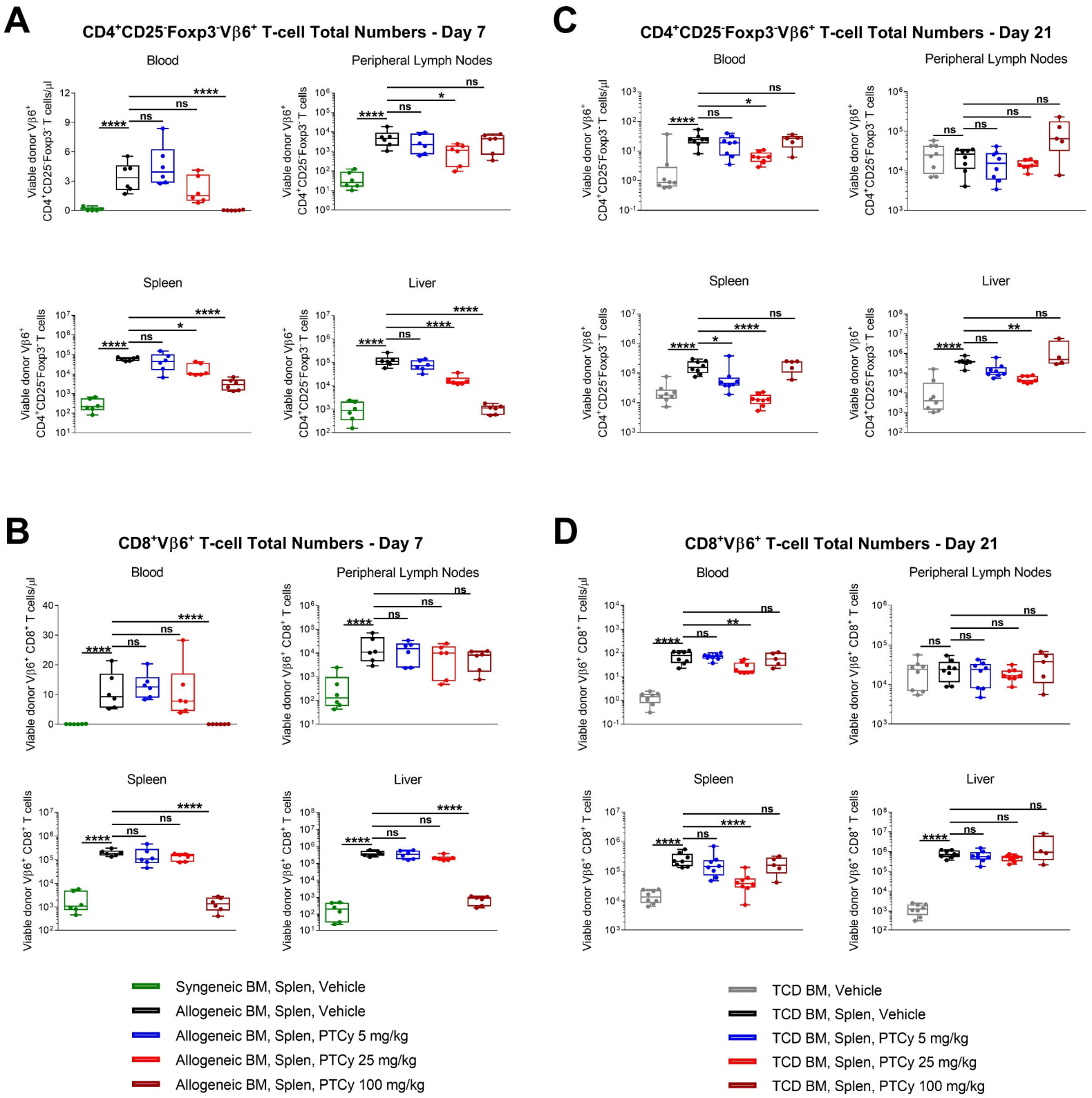


Figure S8. Total numbers of Vβ6⁺ conventional CD4⁺ and CD8⁺ T-cell subsets at days +7 and +21 post-transplant in the B6C3F1→B6D2F1 HCT model. The total numbers of viable donor **(A, C)** Vβ6⁺ CD4⁺CD25⁻Foxp3⁻ or **(B, D)** Vβ6⁺ CD8⁺ T-cell subsets are shown. These data correspond both to the data in Figure 2A-B showing that the total numbers of donor T cells were reduced by PTCy 25 or 100 mg/kg and to the data in Figure 2D-E showing similar percentages of Vβ6⁺ T cells between PTCy-treated and vehicle-treated mice. Consequently, total numbers of Vβ6⁺ CD4⁺, and in some tissues Vβ6⁺ CD8⁺, T cells also were reduced at day +7 after PTCy 25 or 100 mg/kg on days +3/+4. Expansion of Vβ6⁺ T cells from days +7 to +21 was constrained after PTCy 25 mg/kg. By contrast, in mice treated with PTCy 100 mg/kg, a dose associated with worse GVHD histopathologically, Vβ6⁺ T-cell numbers rebounded to vehicle-treated levels by day +21. Total numbers were calculated for each sample by taking the total number of gated events divided by the total number of flow cytometrically determined viable (LIVE/DEAD⁻) events and then multiplying by the total number of viable cells as determined by hematocytometric counting using trypan blue exclusion. All groups were allogeneic (B6C3F1→B6D2F1) unless specifically labeled as syngeneic (B6D2F1→B6D2F1). Combined results from **(A-B)** three (n=6/group) or **(C-D)** two (n=8/group except PTCy 100 mg/kg [n=5]) independent experiments are shown. *p≤0.05, **p≤0.01, ****p≤0.0001, and ns=not significantly different on one-way ANOVA followed by the Holm-Sidak post hoc test using the vehicle-treated splenocyte group as the control.

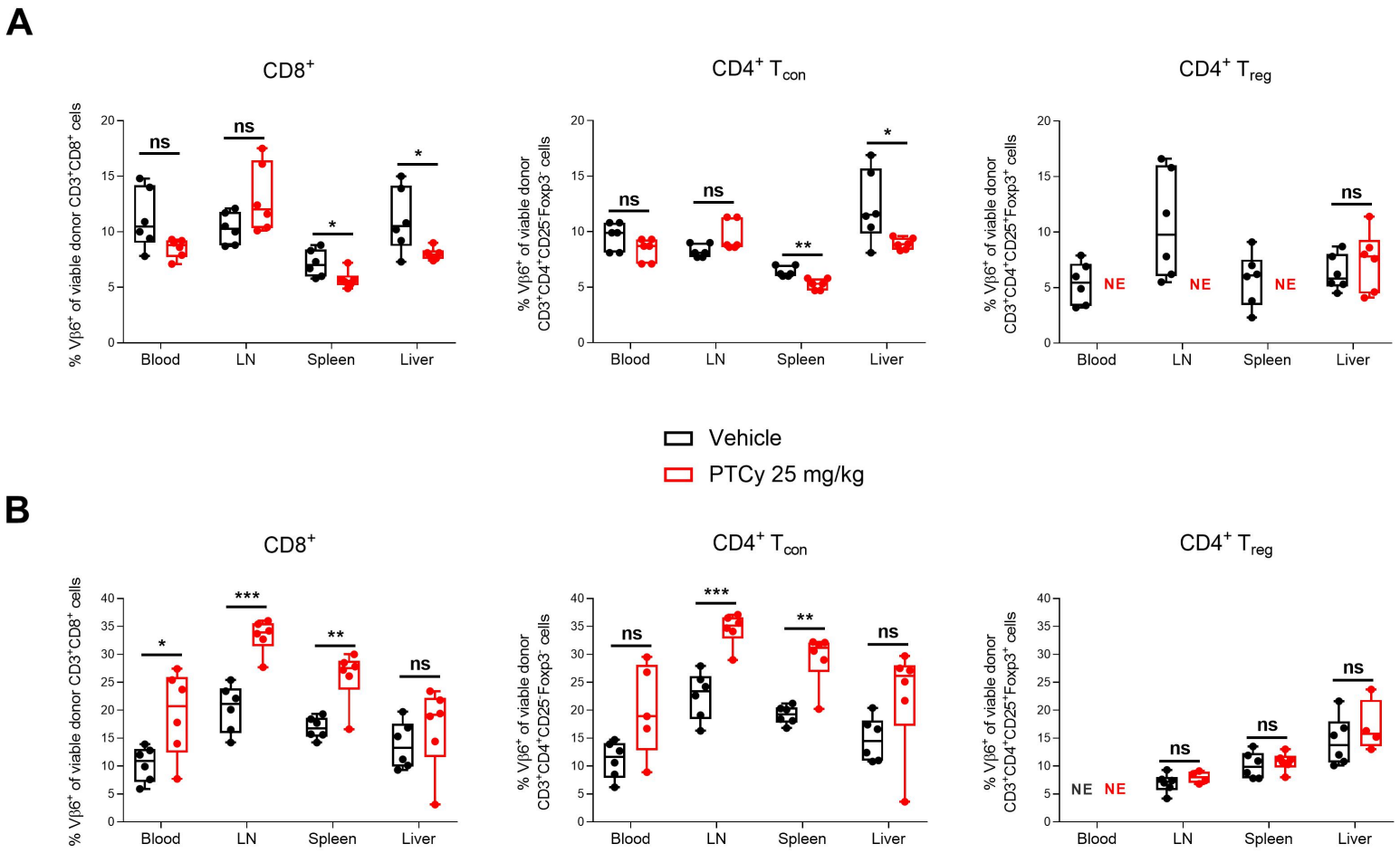


Figure S9. Alloreactive Vβ6⁺ T cells are not eliminated in the MHC-haploidentical B6→B6D2F1 HCT model, regardless of conditioning. Ten to 12-week-old female B6D2F1 recipient mice were either **(A)** irradiated to 10.5 Gy or **(B)** not irradiated and then transplanted with 10 x 10^6 B6 TCD BM cells and 40 x 10^6 B6 splenocytes followed by PTCy 25 mg/kg/day or vehicle on days +3 and +4. Regardless of tissue compartment or T-cell subset ($CD8^+$, $CD4^+CD25^-Foxp3^-$ conventional [$CD4^+$ T_{con}], or $CD4^+CD25^+Foxp3^+$ [$CD4^+$ T_{reg}]), Vβ6⁺ T cells were not eliminated by PTCy and continued to persist near or even above the percentages found in donors or vehicle-treated mice. Combined results from two independent experiments of 3 mice per group per experiment are shown. NE indicates specific subsets in which all samples were not evaluable due to the parent populations all being <100 cells. * indicates p≤0.05, ** indicates p≤0.01, *** indicates p≤0.001, and ns indicates not significantly different on unpaired t-test with Welch's correction.

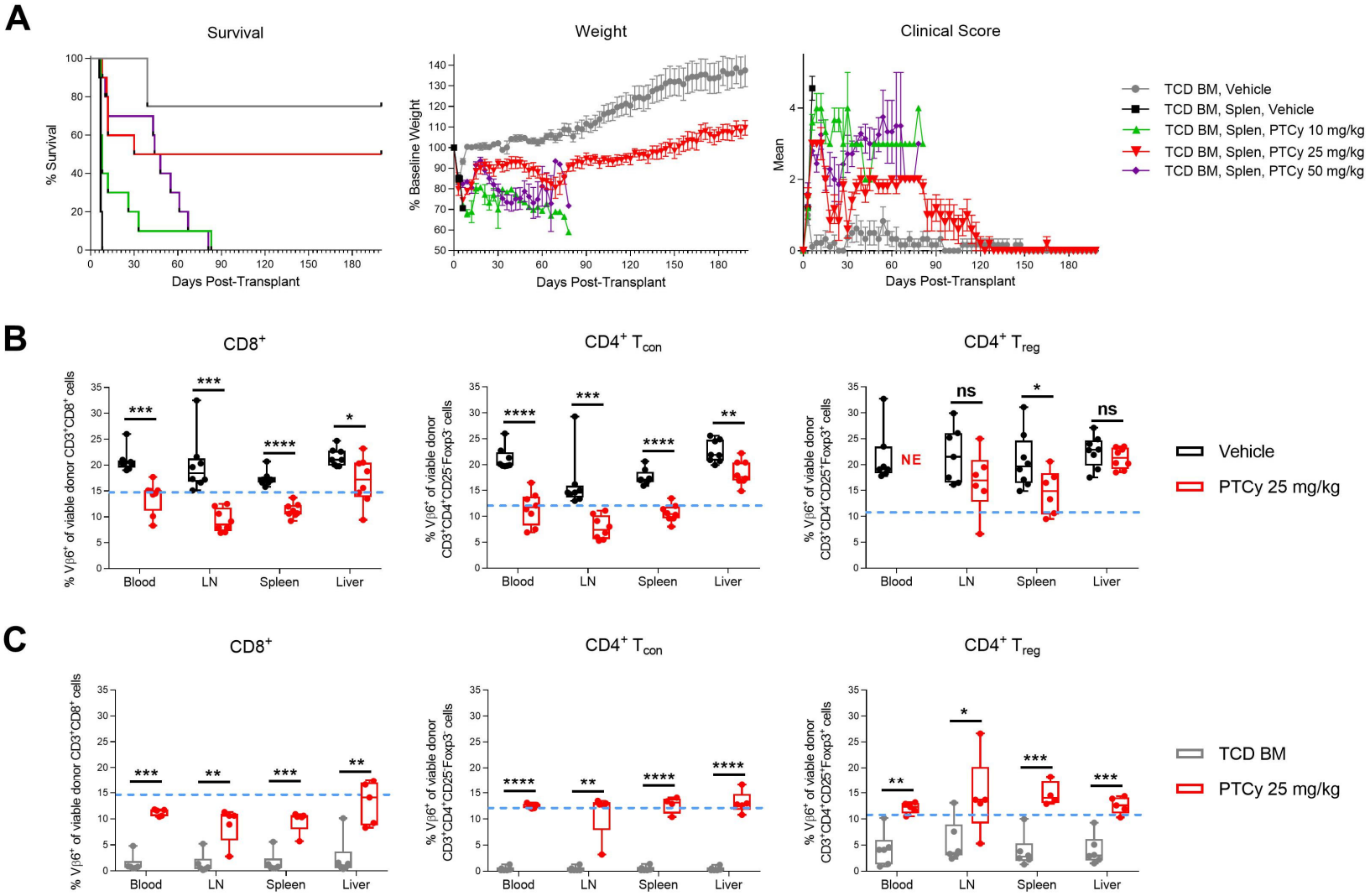


Figure S10. PTCy 25 mg/kg/day on days +3 and +4 is effective GVHD prophylaxis in an MHC-disparate C3H→B6D2F1 HCT model, but does not eliminate alloreactive Vβ6⁺ T cells at early (day +6) or late (day +200) timepoints. On day 0, 10 to 12-week-old female B6D2F1 recipient mice were irradiated (10.5 Gy) and received allografts intravenously from 10 to 12-week-old female C3H donors. On days +3 and +4, mice received PTCy or vehicle intraperitoneally. **(A)** 5 x 10^6 TCD BM cells with or without 50 x 10^6 splenocytes were transplanted. The splenocyte dose was chosen to normalize the number of infused T cells between models. The PTCy doses tested were those that were effective in preventing fatal GVHD in the B6C3F1→B6D2F1 model. In this C3H→B6D2F1 model, the GVHD induced was severe and rapid, resulting in death in all vehicle-treated mice within 8 days. All PTCy doses prolonged survival in a subset of mice, but only PTCy 25 mg/kg (the dose also optimal in the B6C3F1→B6D2F1 model) fully abated GVHD and produced long-term survivors. Combined results from two independent experiments are shown. All groups had 5 mice/group/experiment except the TCD BM group which had 4. **(B)** For all experiments testing the impact on Vβ6⁺ T cells, the cell dose infused was standardized to be identical across models. Therefore, mice were transplanted with 10 x 10^6 TCD BM and 40 x 10^6 splenocytes and assessed by flow cytometry at day +6. Although the relative expansion of alloreactive Vβ6⁺ T cells was restrained by PTCy, these cells persisted near or even above donor percentages (medians 14.9% for $CD8^+$, 12.3% for $CD4^+CD25^-Foxp3^-$, and 10.4% for $CD4^+CD25^+Foxp3^+$ T cells from Figure S7 are indicated by the dotted blue lines in this figure). Combined results from two independent experiments are shown with n=8/group. **(C)** Mice from **A** surviving to day +200 (TCD BM, n=6; PTCy 25 mg/kg, n=5) were assessed for the frequency of Vβ6⁺ T cells. Mice treated with splenocytes and PTCy 25 mg/kg had persistence of $CD4^+$Vβ6⁺ T cells at approximately donor percentages and $CD8^+$Vβ6⁺ T cells at slightly below donor percentages. Both were distinct from mice treated with TCD BM without splenocytes or PTCy. NE indicates specific subsets in which all samples were not evaluable due to the parent populations all being <100 cells. *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001, and ns indicates not significantly different on unpaired t-test with Welch's correction.

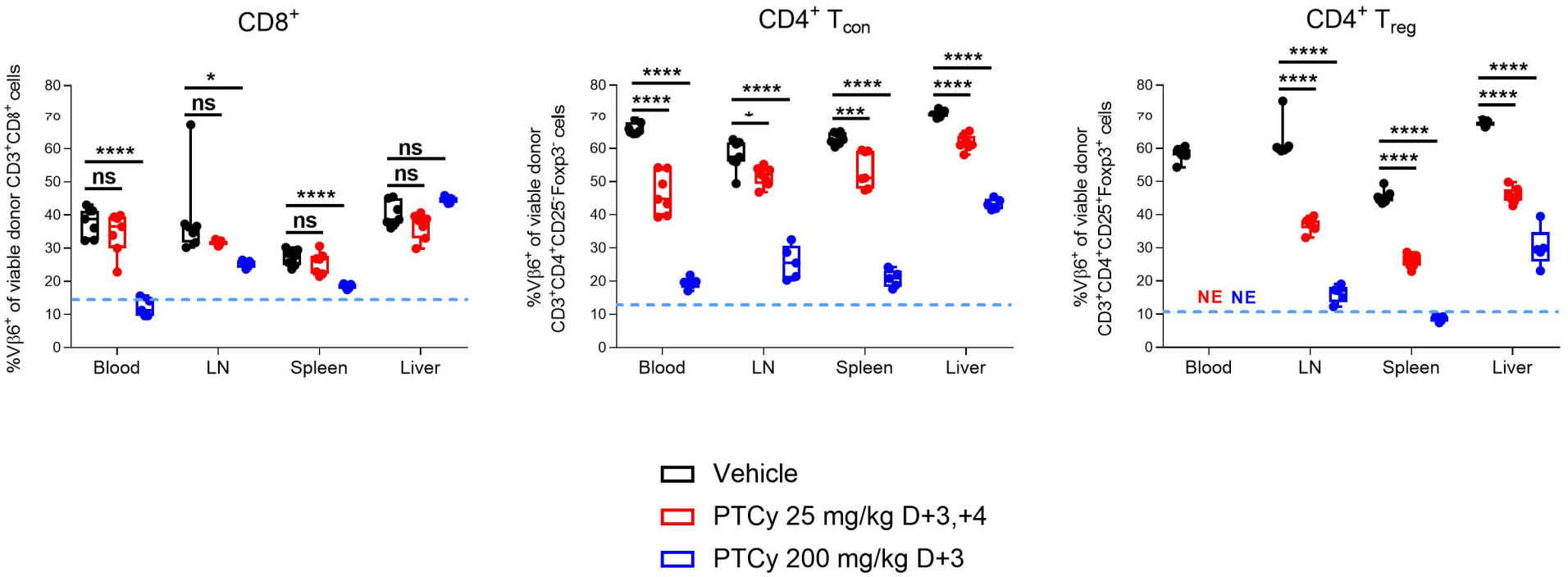


Figure S11. Alloreactive Vβ6⁺ T cells preferentially survive despite PTCy in an MHC-matched C3H→AKR model, regardless of PTCy dose. AKR→C3H was one of two primary models employed in the MHC-matched murine skin allografting models used to assess the impact of PTCy on host-versus-graft immunity. Therefore, we utilized the C3H→AKR model to study parallel effects in graft-versus-host immunity in HCT. On day 0, AKR mice were irradiated to 10.5 Gy and received 10 x 10^6 C3H TCD BM and 40 x 10^6 C3H splenocytes. Mice received vehicle on days +3 and +4, PTCy 25 mg/kg/day on days +3 and +4, or PTCy 200 mg/kg on day +3 and vehicle on day +4; this last group was included to parallel the dose and timing used in the skin allografting models. Mice were euthanized at day +7 and assessed by flow cytometry. In vehicle-treated mice, Vβ6⁺ T cells expanded markedly across all T-cell subsets and tissue compartments assessed. This expansion was partially restrained in mice treated with PTCy, but the percentage of Vβ6⁺ T cells generally remained near or frequently above donor levels (medians 14.9% for $CD8^+$, 12.3% for $CD4^+CD25^-Foxp3^-$, and 10.4% for $CD4^+CD25^+Foxp3^+$ T cells from Figure S7 are indicated by the dotted blue lines in this figure). Combined results from two independent experiments are shown. N=7 for each of the vehicle and PTCy 25 mg/kg groups, and n=5 for the PTCy 200 mg/kg group. NE indicates specific subsets in which all samples were not evaluable due to the parent populations all being <100 cells. * indicates p≤0.05, *** indicates p≤0.001, **** indicates p≤0.0001, and ns indicates not significantly different on one-way ANOVA followed by the Holm-Sidak post hoc test.

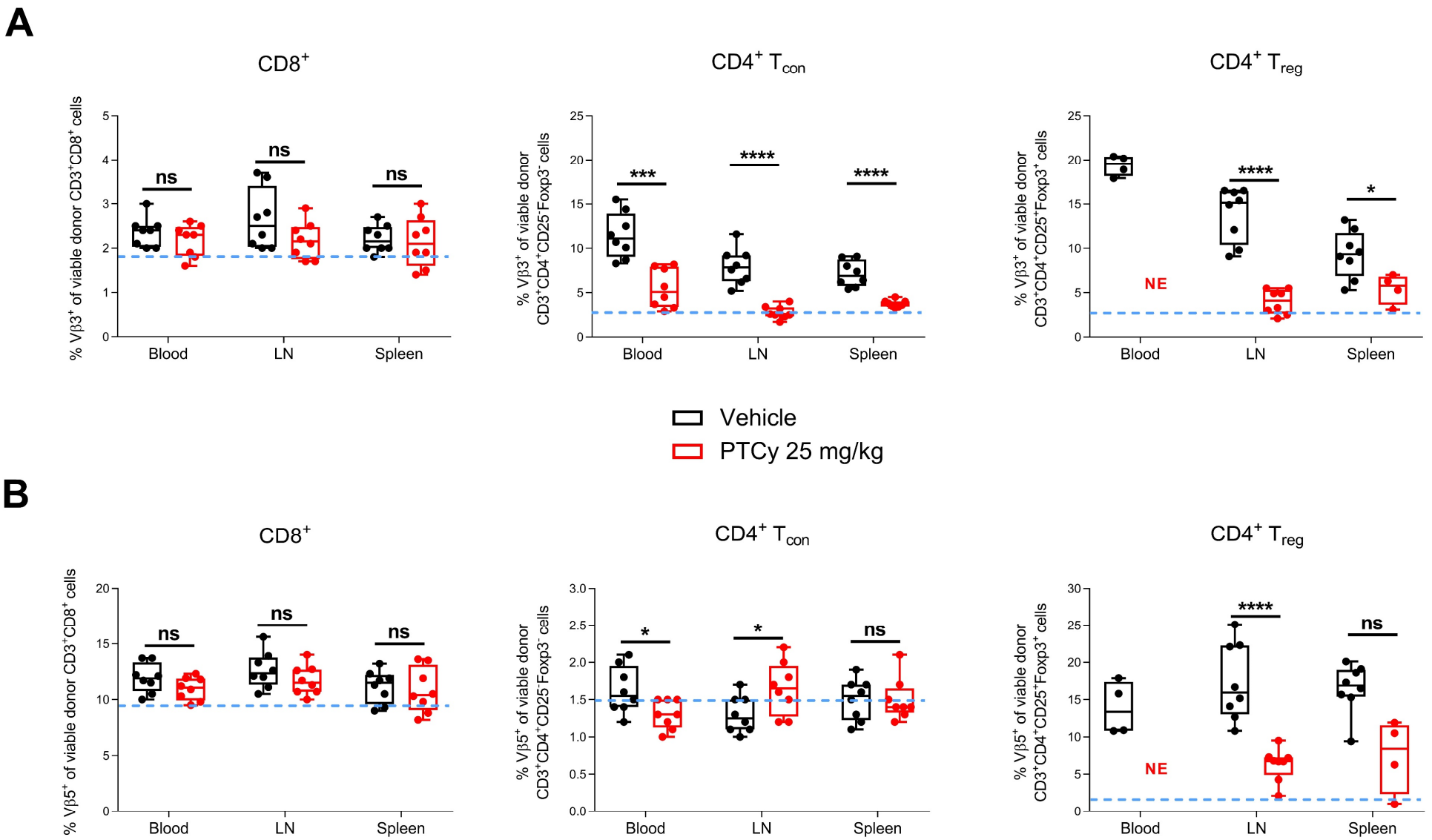


Figure S12. Alloreactive Vβ3⁺ and Vβ5⁺ T cells persist near or above donor percentages after PTCy 25 mg/kg/day on days +3 and +4 in an MHC-haploidentical B6→B6C3F1 HCT model. Day +7 flow cytometry data shown for the experiments in Figure 3D-F also were assessed for the presence and frequency of alloreactive **(A)** Vβ3⁺ or **(B)** Vβ5⁺ T cells. Alloreactive CD4⁺ T-cell expansion was restrained in this model, but persisted near or above donor percentages (normal B6 donor levels: Vβ3⁺, CD8⁺ 1.9%, CD4⁺ 2.9%; Vβ5⁺, CD8⁺ 9.5%, CD4⁺ 1.5%, are indicated by the dotted blue lines). N=8 per group. Combined results from two independent experiments are shown. NE indicates specific subsets in which all samples were not evaluable due to the parent populations all being <100 cells. * indicates p≤0.05, *** indicates p≤0.001, **** indicates p≤0.0001, and ns indicates not significantly different on unpaired t-test with Welch's correction.

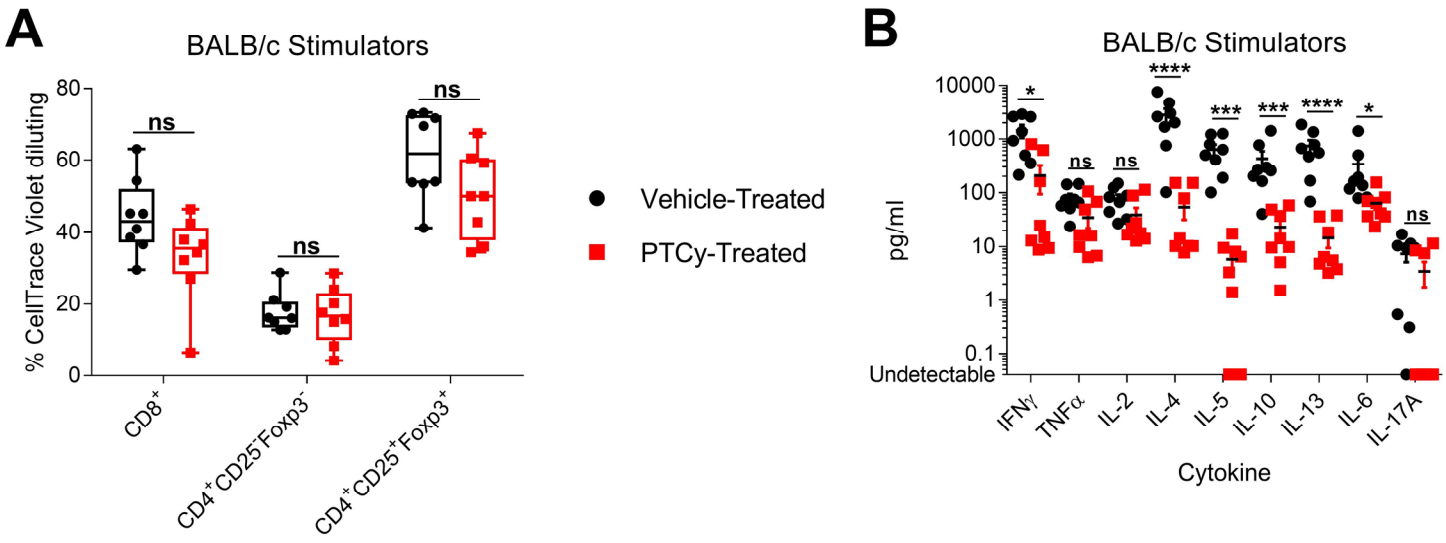


Figure S13. PTCy-treated T cells also have impaired functionality in response to a third-party MHC-mismatched strain that expresses the H2^{d} haplotype. B6D2F1 mice were transplanted with 10 x 10^{6} B6C3F1 TCD BM and 40 x 10^{6} B6C3F1 splenocytes and treated with vehicle or PTCy 25 mg/kg/day on days +3 and +4. At day +21, liver-infiltrating cells were isolated and re-stimulated *in vitro* with third-party (BALB/c) irradiated (30 Gy) splenocytes that shared the mismatched MHC haplotype of the host (H2^{d}). 2 x 10^{5} of each cell type were plated per well in 96-well round bottom plates. Proliferation was measured by dilution of CellTrace Violet on day 5 of *in vitro* culture, and cytokine production was measured in cell culture supernatant obtained at 24 hours after culture initiation. T cells isolated from PTCy-treated mice had lower **(A)** CD8^{+} proliferation and **(B)** inflammatory cytokine production compared with T cells isolated from vehicle-treated mice. These results are overall very similar to the responses to DBA/2 stimulators shown in Figure 6C-D; the p-value for the CD8^{+} proliferation difference, while not significant as it was for the DBA/2 stimulators, was p=0.08. Combined results of two independent experiments are shown with n=8. * indicates p≤0.05, *** indicates p≤0.001, **** indicates p≤0.0001, and ns indicates not significantly different on unpaired t-test with Welch's correction. Statistical testing of cytokines was adjusted for multiple comparisons by the Holm-Sidak method.

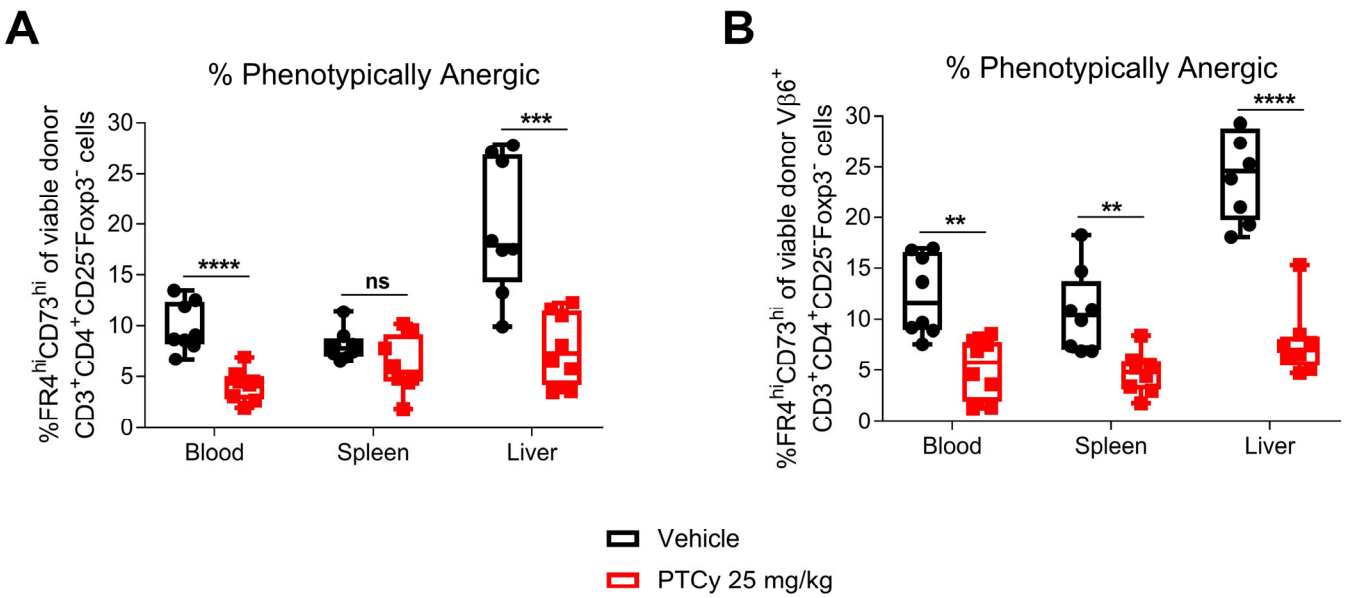


Figure S14. PTCy does not induce an anergic phenotype in Vβ6⁺ alloreactive CD4⁺CD25⁻Foxp3⁻ T cells. Mice were transplanted as in Figure 1, treated with PBS vehicle or PTCy 25 mg/kg/day on days +3 or +4, and then evaluated by flow cytometry on day +21. The percentages of **(A)** all or **(B)** Vβ6⁺ donor CD4⁺CD25⁻Foxp3⁻ T cells in the blood, spleen, or liver at day +21 that were folate receptor-4 (FR4)^{hi} and CD73^{hi} are shown. Combined results of two independent experiments of n=4 per group per experiment are shown. **p≤0.01, ***p≤0.001, ****p≤0.0001, and ns=not significantly different on unpaired t-test with Welch's correction.

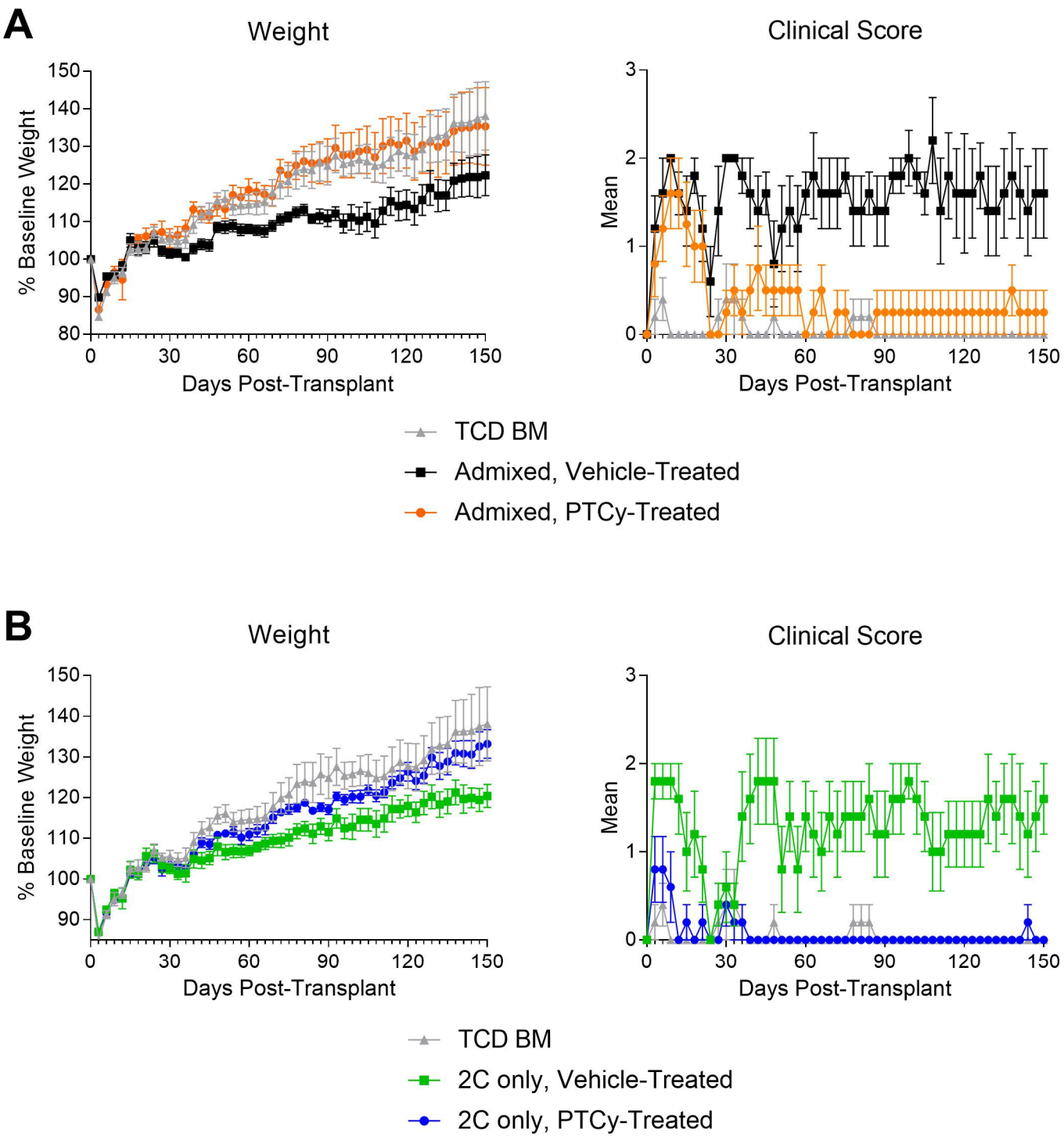


Figure S15. 2C TCR$^+$ alloreactive T cells have reduced potential to cause GVHD after PTCy regardless of the composition of the graft at the time of PTCy. Irradiated B6D2F1 recipient mice were transplanted with 40 x 10^6 2C TCR$^+$ admixed B6C3F1 splenocytes and 10 x 10^6 wild-type TCD BM cells as described in Figure 3 (8% of CD8$^+$ T cells were 2C TCR$^+$) or with 40 x 10^6 splenocytes and 10 x 10^6 TCD BM from 2C TCR$^+$ B6C3F1 donors. Mice received either vehicle or PTCy 25 mg/kg/day on days +3 and +4. On day +5, splenocytes from these mice were flow cytometrically sorted to isolate viable 2C TCR$^+$ T cells (LIVE/DEAD$^-$ CD8$^+$Vβ8.1/8.2$^+$1B2$^+$). 0.5 x 10^6 2C TCR$^+$ T cells from mice that had received admixed grafts or 1 x 10^6 2C TCR$^+$ T cells from mice that had received only 2C splenocytes were transplanted along with 5 x 10^6 2C BM cells (from new 2C donors) flow cytometrically depleted of Thy1.2$^+$ cells into new, irradiated (10.5 Gy), thymectomized, B6D2F1 recipients. These mice did not receive any post-transplant treatment (i.e. no PTCy or vehicle on days +3 and +4). The experiments in Figure 7A are shown here with the **(A)** admixed and **(B)** 2C only cells experiments plotted separately, showing overall similar results. N=5 per group per experiment except the admixed/PTCy-treated group, n=4. The TCD BM group (n=5) is shown in both parts for comparison purposes.

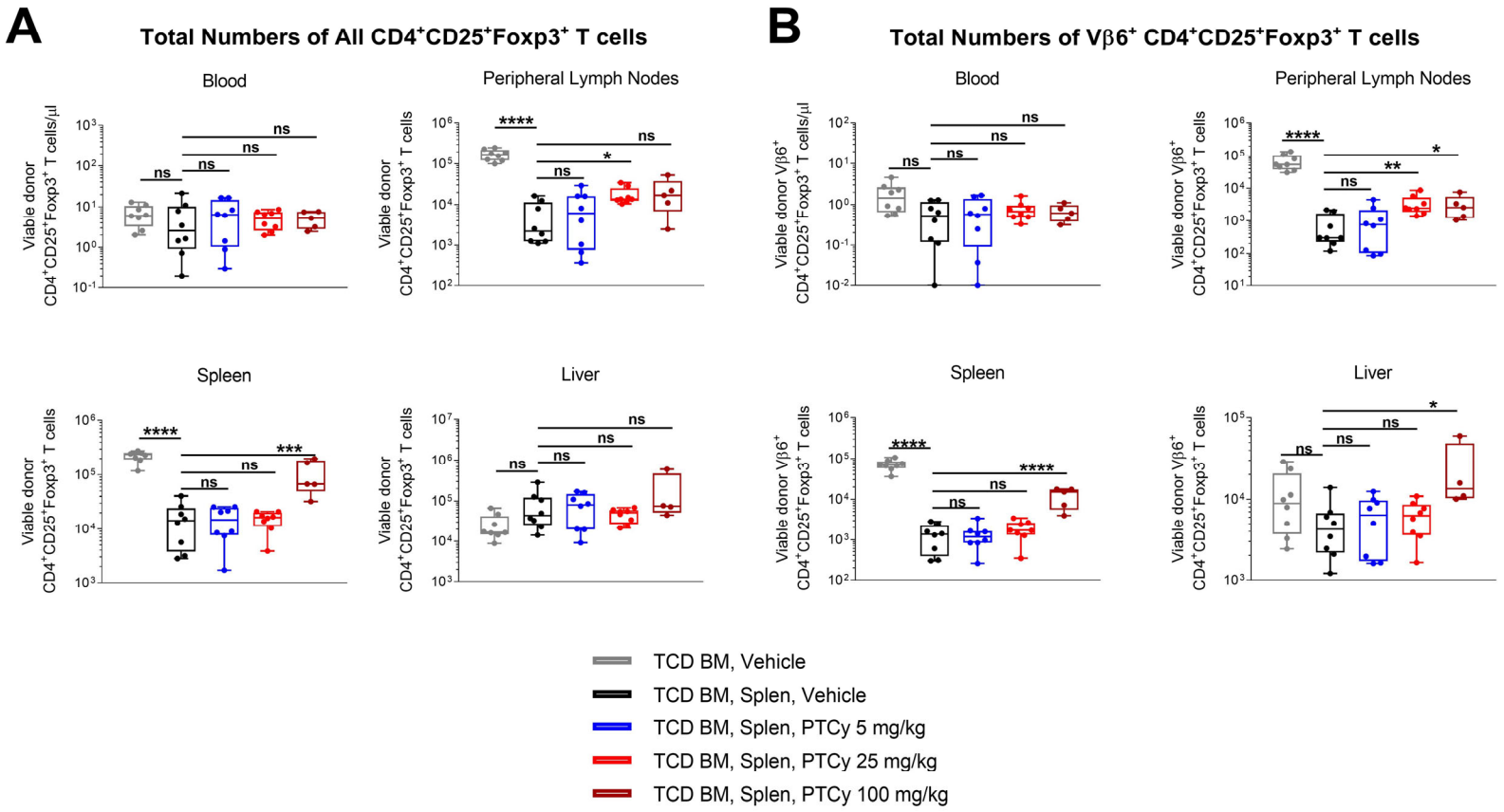


Figure S16. Rapid numerical recovery of CD4⁺CD25⁺Foxp3⁺ T cells at day +21 post-transplant in the B6C3F1→B6D2F1 HCT model. Mice were transplanted as in Figure 2. Total numbers of **(A)** all or **(B)** Vβ6⁺ donor CD4⁺CD25⁺Foxp3⁺ T cells are shown. These data correspond to the percentage data shown in Figure 8B and 8E. CD4⁺CD25⁺Foxp3⁺ T-cell numbers were similar or even significantly higher in mice treated with PTCy 25 or 100 mg/kg compared with vehicle-treated mice; . *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001, and ns=not significantly different on one-way ANOVA followed by the Holm-Sidak post hoc test using the vehicle-treated splenocyte group as the control.

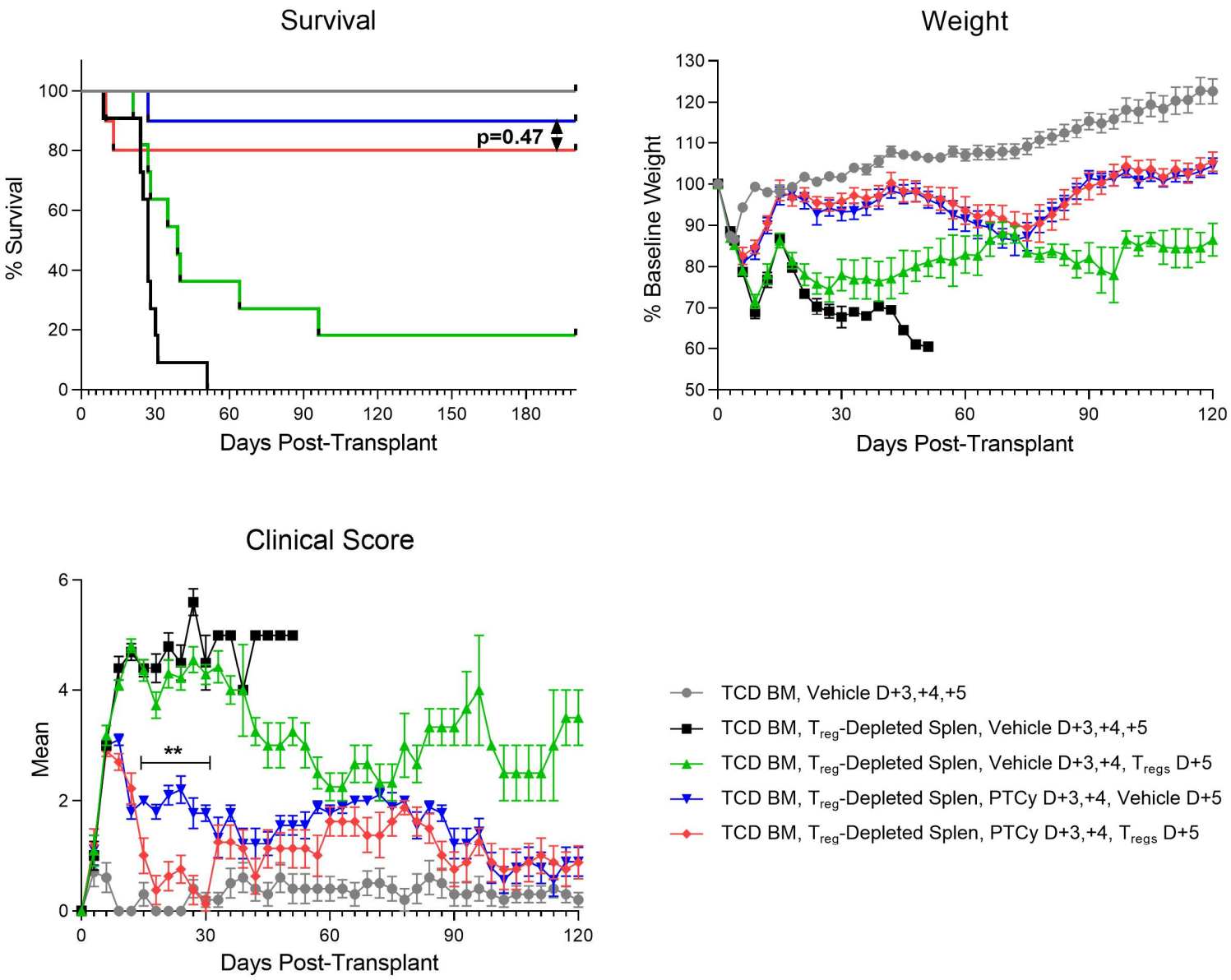


Figure S17. Eighty percent reduction in CD4$^+$CD25$^+$ T cells via magnetic column depletion has minimal impact on outcomes in the B6C3F1→B6D2F1 HCT model. Using an autoMACS Pro Separator, B6C3F1 CD4$^+$CD25$^+$ cells were successfully depleted by approximately 80% with the CD25$^+$ content of spleen CD4$^+$ T cells being decreased from 10-12% to 1.8-2.5%. 40 x 10^6 B6C3F1 splenocytes that were depleted of CD4$^+$CD25$^+$ T cells were given with 10 x 10^6 B6C3F1 TCD BM cells on day 0 to B6D2F1 recipient mice that had been irradiated (10.5 Gy) 8 hours earlier. PTCy 25 mg/kg/day or PBS vehicle was administered on days +3 and +4. 8 x 10^5 CD4$^+$CD25$^+$ T cells (the approximate number contained in the 40 x 10^6 splenocytes normally infused on day 0 in this model had the splenocytes not been CD4$^+$CD25$^+$ depleted; purity >97%) or RPMI vehicle were administered intravenously on day +5. Survival and weights were not significantly different between PTCy-treated mice receiving or not receiving the add-back of CD4$^+$CD25$^+$ T cells (T_{regs}). However, there were significant differences ($p<0.0085$ for each timepoint) in the clinical scores of these groups between days +15 and +30. Areas under the curve (AUCs) of the clinical scores between days 0 and 30 also differed between treatment groups ($p=0.021$). Combined results from two independent experiments are shown with n=5-6 mice per group per experiment.

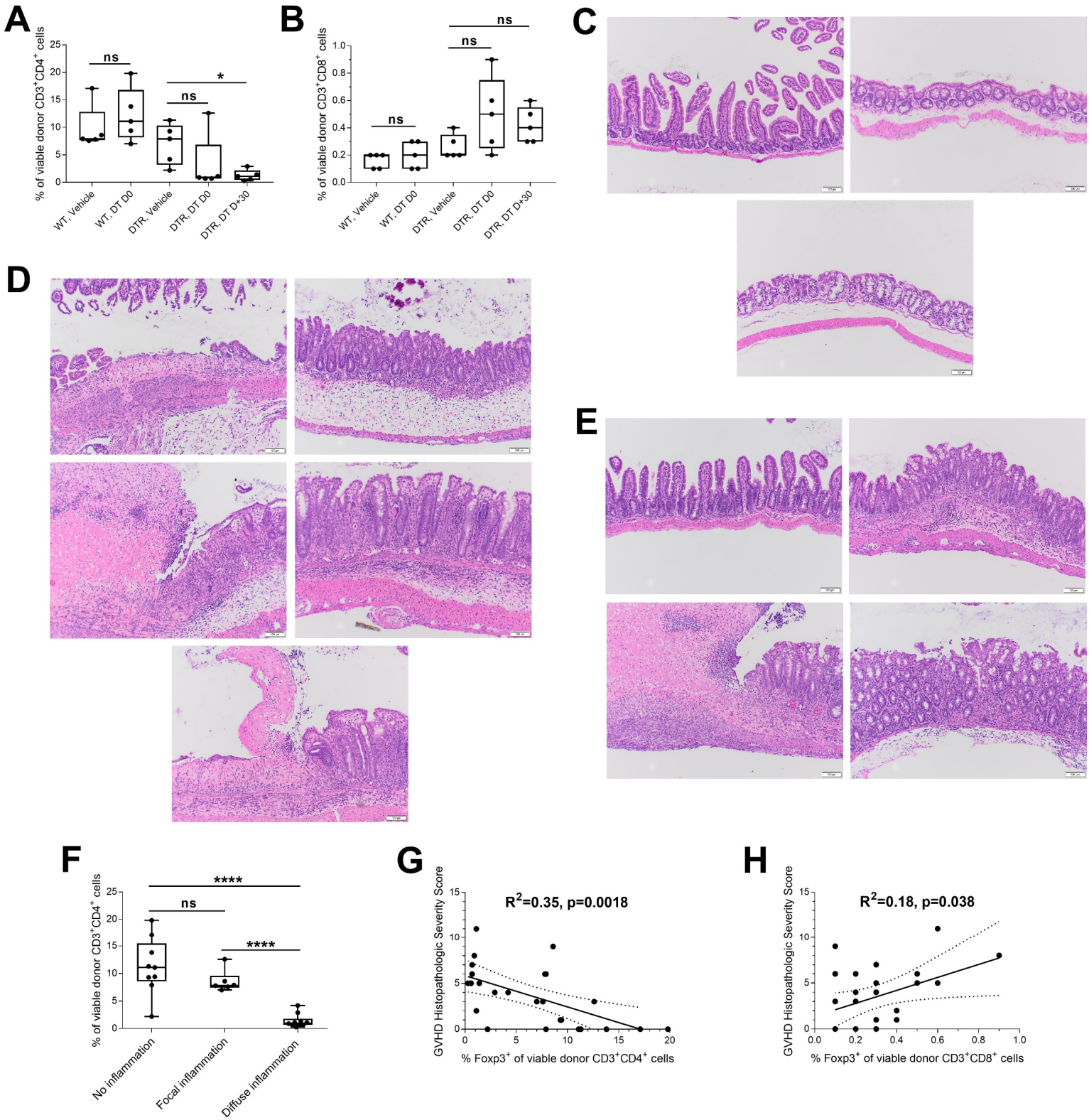


Figure S18. Foxp3⁺ cell depletion early post-transplant induces histopathologic evidence of GVHD, the severity of which inversely correlates with the level of liver-infiltrating CD4⁺Foxp3⁺ T cells. Thymectomized female B6D2F1 recipient mice were irradiated (10.5 Gy) and transplanted with 40 x 10^6 splenocytes and 10 x 10^6 TCD BM cells from either (B6.Foxp3-diphtheria toxin receptor [DTR]^{GFP} x C3H)F1 or wild-type (WT) male B6C3F1 donors. PTCy was given to all recipients at 25 mg/kg/day on days +3 and +4. DT was given intraperitoneally at 25 μg/kg/day on days 0, +1, +6, and +7 (DT D0) or on days +30, +31, +36, and +37 (DT D+30) to overcome the T_{reg} rebound observed ~7 days after depletion using this system.*(42)* Mice not receiving DT were given PBS vehicle over the same schedule. Recipients were euthanized at day +50 for histopathologic and flow cytometric assessments. **(A)** DT treatment at day 0 or +30 of mice that had received Foxp3-DTR grafts resulted in decreased percentages of CD4⁺Foxp3⁺ T cells in the liver at day +50. **(B)** However, the low Foxp3⁺ content within CD8⁺ T cells was not lower in the liver at this timepoint. **(C-E)** DT treatment resulted in GVHD histopathologically only in susceptible mice. 100X H&E photographs from a representative mouse from each of the **(C)** WT cells, DT D0; **(D)** DTR cells, DT D0; and **(E)** DTR cells, DT D+30 groups. **(C)** Normal **(Top left)** small intestine, **(Top right)** cecum, and **(Bottom)** colon. **(D) Top left:** Small focal mucosal ulceration in the small intestine. **Top right:** Cecum showing diffuse mucosal hyperplasia and **(Center left)** multifocal ulceration adjacent to atrophic Peyer's patches. **Center right:** Colon showing diffuse mucosal hyperplasia with **(Bottom)** multifocal mucosal ulceration adjacent to atrophic Peyer's patches. **(E) Top left:** Normal small intestine. **Top right:** Cecum showing moderate diffuse mucosal hyperplasia. **Bottom:** Colon showing moderate diffuse mucosal hyperplasia **(Bottom left)** with focal mucosal ulceration. **(F)** The diffuseness of the GVHD inflammatory lesions in the gastrointestinal tract inversely correlated more closely with the liver CD4⁺Foxp3⁺ content than it did with the original treatment group (shown in **A**). **(G-H)** The GVHD histopathologic severity also inversely correlated with the liver CD4⁺Foxp3⁺ T-cell content, but did not have an inverse correlation with the liver CD8⁺Foxp3⁺ T-cell content, which was a very minor population of CD8⁺ T cells. *p≤0.05, ****p≤0.0001, and ns=not significantly different.

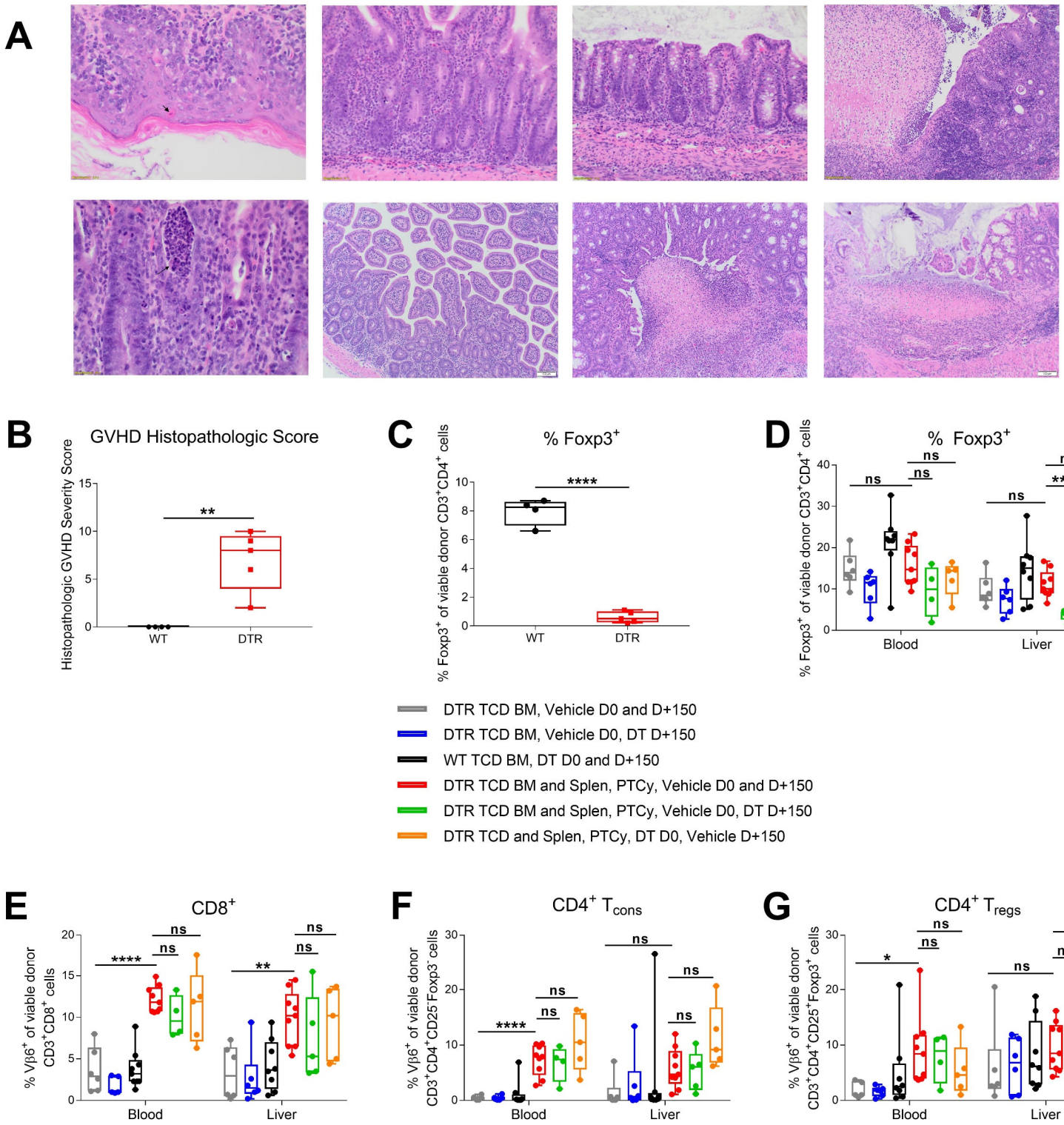


Figure S19. Foxp3⁺ cell depletion starting at day +150 induces severe histopathologic GVHD accompanied by prolonged CD4⁺Foxp3⁺ T-cell lymphopenia in the liver. Mice were transplanted as per Figure S18 except that the TCD BM only groups did not receive PTCy. DT 25 μg/kg or vehicle was started at day 0 (days 0, +1, +6, +7) and/or day +150 (days +150, +151, +156, +157). **(A)** DT treatment starting at day +150 of mice transplanted with Foxp3-DTR donor cells resulted in severe histopathologic evidence of GVHD at early (day +164) and, in survivors, late (day +250) timepoints. **Top left:** Day +164: Stomach showing dyskeratotic epithelial cells (arrow), intraepithelial lymphocytes, and submucosal lymphocytic infiltration. 400X. **Top center left:** Day +164: Small intestine showing diffuse mucosal hyperplasia with degenerative epithelial cells. 200X. **Top center right:** Day +164: Cecum showing moderate mucosal hyperplasia. 200X. **Top right:** Day +164: Cecum showing mucosal hyperplasia and ulceration. 100X. **Bottom left:** Day +164: Colon showing crypt abscess with degenerative epithelial cells (arrow). 400X. **Bottom center left:** Day +250: Proliferative lymphocytic duodenitis with mucosal hyperplasia and increased numbers of lymphocytes in the lamina propria. 100X. **Bottom center right:** Day +250: Colonic mucosal ulceration with mucosal hyperplasia. 100X. **Bottom right:** Day +250: Cecum showing ulcerative typhlitis with prominent mucosal ulceration and large numbers of neutrophils and fibrin on mucosal surface. 100X. **(B)** GVHD histopathologic severity scores at day +164 for mice transplanted with WT or Foxp3-DTR splenocytes and BM and treated with DT starting at day +150. **(C)** This worse GVHD correlated with successful CD4⁺Foxp3⁺ T-cell depletion. The data here show liver-infiltrating T cells. **(D-G)** Flow cytometric data at day +250 comparing several treatment groups from three experiments. **(D)** Despite persistent GVHD histopathologically and clinically, CD4⁺Foxp3⁺ percentages in the blood had recovered, while CD4⁺Foxp3⁺ levels in the livers of mice depleted at day +150 remained low. **(E-G)** Despite all recipient mice being thymectomized, mice treated with TCD BM (but no PTCy) had low levels of alloreactive Vβ6⁺ CD4⁺ and CD8⁺ T cells, distinct from mice treated with T-cell-replete grafts and PTCy. These results suggest that clonal deletion after TCD BM transplants also occurs through peripheral (thymic-independent) mechanisms and that these deleting mechanisms are not active in mice treated with T-cell-replete grafts and PTCy

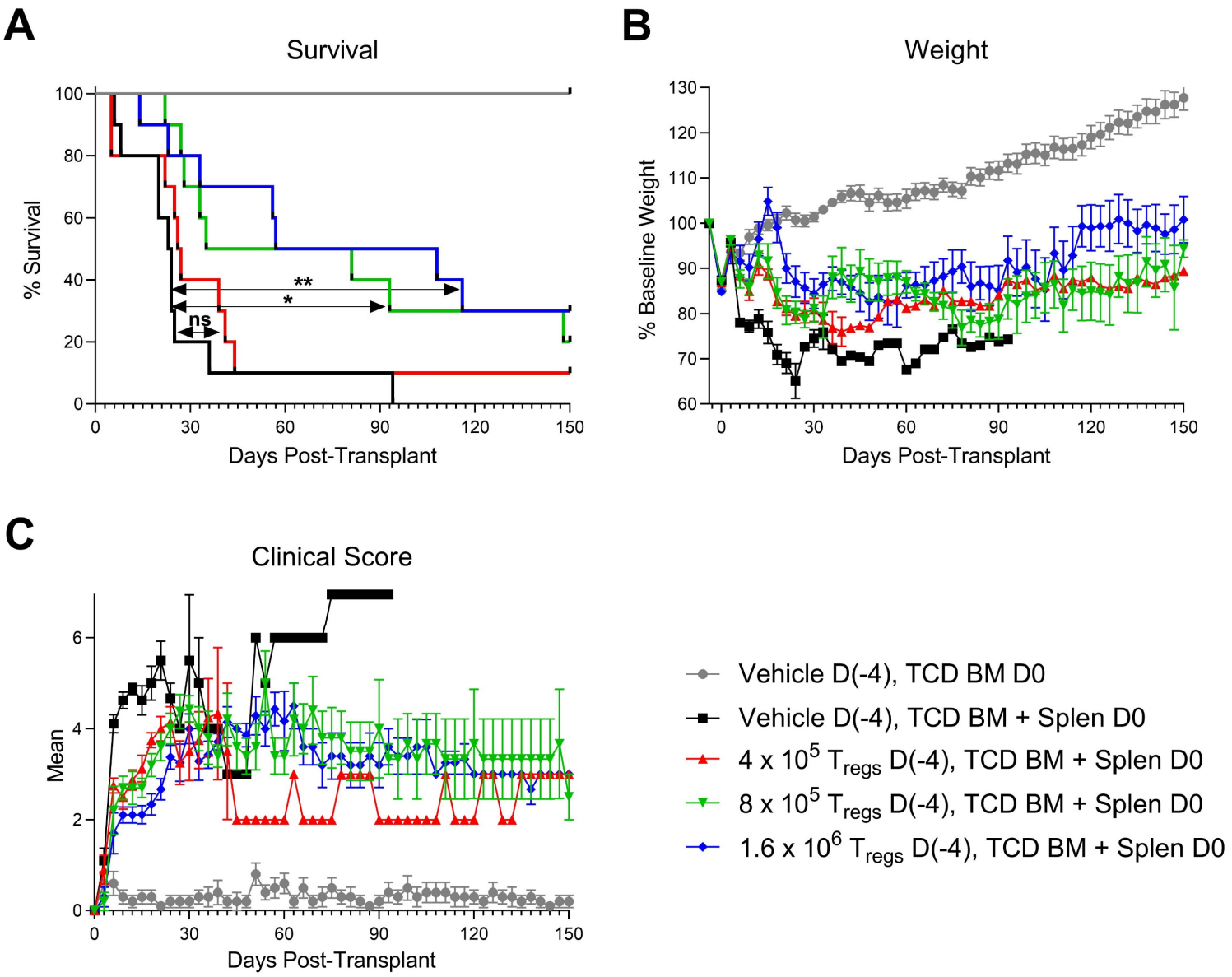


Figure S20. The pre-transplant infusion of CD4⁺CD25⁺ T cells delays GVHD severity and prolongs survival, but ultimately is insufficient to prevent severe and fatal GVHD in the B6C3F1→B6D2F1 MHC-haploidentical model. B6C3F1 CD4⁺CD25⁺ T cells (T_{regs}) were isolated via an autoMACS Pro Separator using magnetic column separation (purity >97%) and were intravenously administered on day -4 to B6D2F1 recipient mice that had been irradiated to 10.5 Gy 6-8 hours earlier. The doses of CD4⁺CD25⁺ T cells (4 x 10^5, 8 x 10^5, or 1.6 x 10^6 cells) were 0.5, 1, or 2 times, respectively, the approximate amount of CD4⁺CD25⁺ T cells that would be contained in 40 x 10^6 B6C3F1 splenocytes (the standard dose given in our B6C3F1→B6D2F1 HCT model). On day 0, the B6D2F1 mice received 10 x 10^6 T-cell-depleted bone marrow (TCD BM) and 40 x 10^6 unfractionated splenocytes. **(A)** Survival was significantly prolonged with the pre-transplant administration of 8 x 10^5 or 1.6 x 10^6 T_{regs} (p=0.017 and p=0.0071, respectively). However, survival in all groups was inferior to mice receiving TCD BM alone (comparisons with RPMI vehicle, 4 x 10^5, 8 x 10^5, and 1.6 x 10^6 cells: p<0.0001, p=0.0001, p=0.0007, and p=0.0031, respectively). **(B-C)** As the majority of vehicle-treated mice transplanted with splenocytes died by day +20, comparisons of weights and clinical scores were only performed through day +18. **(B)** Areas under the curve (AUCs) of weights over days 0 to +18 of the vehicle-treated splenocyte group were statistically smaller than all T_{reg}-treated groups (p-values p=0.001, p=0.0021, and p=0.003 for comparisons with the 4 x 10^5, 8 x 10^5, and 1.6 x 10^6 T_{reg} groups, respectively). These differences also were statistically significant (p≤0.01) on point-wise comparison between days +6 and +18 for each of the T_{reg} groups compared with the vehicle-treated splenocyte group. **(C)** AUCs of the clinical scores were p=0.0001, p<0.0001, and p<0.0001 on comparisons of the vehicle-treated splenocyte group with the 4 x 10^5, 8 x 10^5, and 1.6 x 10^6 T_{reg} groups, respectively. These differences also were statistically significant (p≤0.006) on point-wise comparisons between days +6 and +18 for each of the T_{reg} groups compared with the vehicle-treated splenocyte group. Combined results from two independent experiments are shown with n=5 mice/group/experiment.

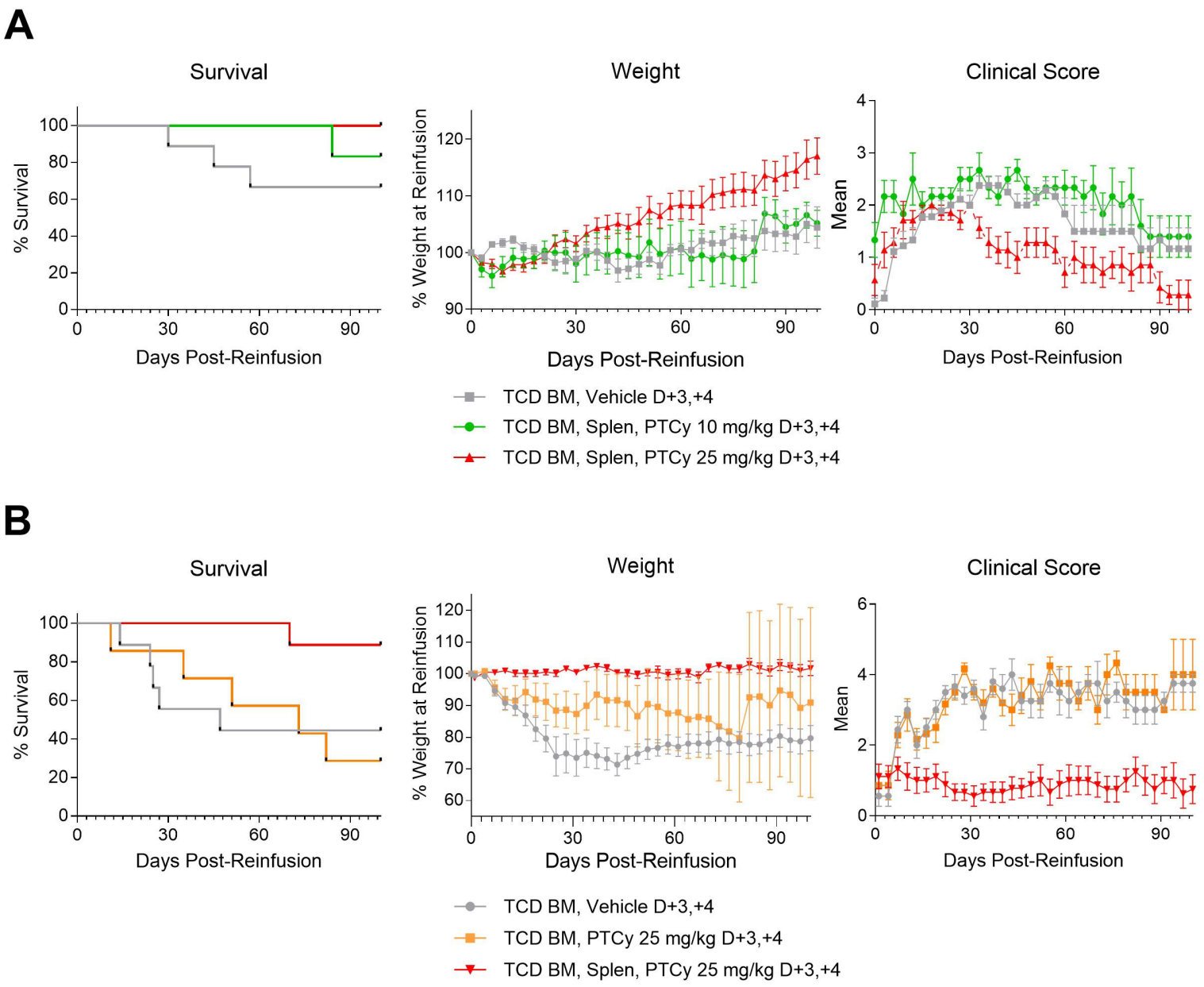


Figure S21. Infusion of new donor splenocytes does not cause severe GVHD in non-thymectomized or thymectomized mice treated with T-cell-replete grafts and PTCy. (A) Non-thymectomized or **(B)** thymectomized B6D2F1 recipient mice were transplanted with 10 x 10^6 B6C3F1 TCD BM cells alone or with 10 x 10^6 B6C3F1 TCD BM cells and 40 x 10^6 B6C3F1 splenocytes followed by vehicle or PTCy on days +3 and +4. At day **(A)** +150 or **(B)** +200, the mice were reinfused with 40 x 10^6 splenocytes from new B6C3F1 donors. **(A)** Mice treated with PTCy 25 mg/kg/day had 100% survival after reinfusion and significantly higher weights and lower clinical scores compared with the TCD BM group (AUC: weights p=0.0079, clinical scores p=0.016). **(B)** Mice treated with splenocytes and PTCy 25 mg/kg/day had significantly better survival after splenocyte reinfusion compared with mice treated with TCD BM and either vehicle or PTCy 25 mg/kg (p=0.029 and p=0.023, respectively). The splenocyte/PTCy group also had superior weights and clinical scores compared with the TCD BM/vehicle (AUCs: weights, p<0.0001; clinical scores, p<0.0001) and TCD BM/PTCy (AUCs: weights, p=0.091; clinical scores, p=0.0002) groups. Combined results from two independent experiments are shown for both parts. N=9, 6, and 7, respectively, for **A**, and n=9, 7, and 9, respectively, for **B**. The numbers of mice reflect all surviving mice to that timepoint for all groups from other experiments.

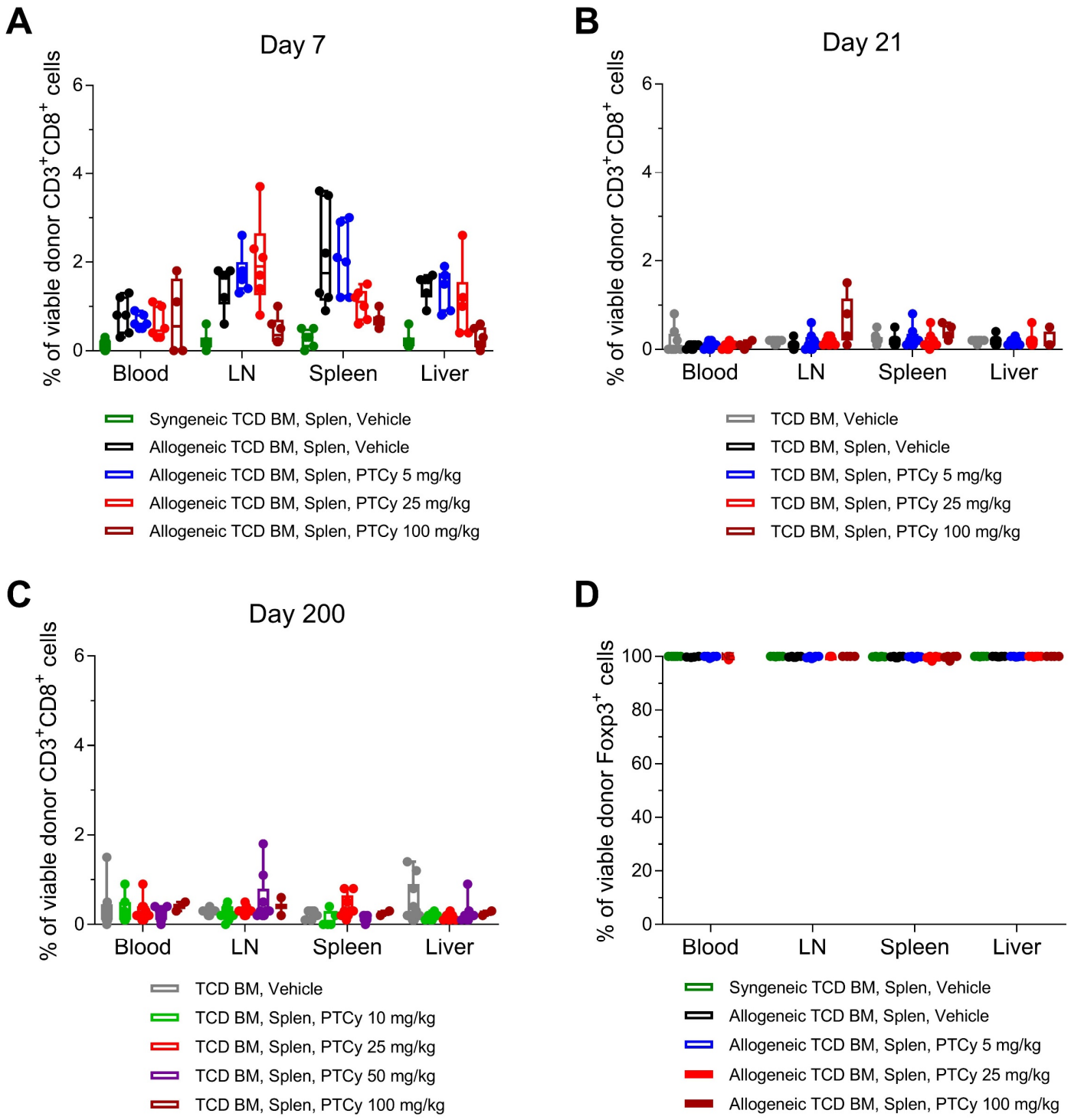


Figure S22. Foxp3⁺ cells are all CD3⁺ and are only a minor population within CD8⁺ T cells. In the B6C3F1→B6D2F1 HCT model, only a very small percent of viable donor CD8⁺ T cells were Foxp3⁺ at day **(A)** +7, **(B)** +21, or **(C)** +200, and the percentages did not appear to consistently track with the treatment group. **(D)** Nearly all viable donor Foxp3⁺ cells at day +7 were CD3⁺. Donor was defined as H2k^{k+} for the allogeneic groups in **A** and **D** and for all groups in **B** and **C** and as H2k^{d+} for the syngeneic group in **A** and **D**. Combined results from **(B, C)** two or **(A, D)** three independent experiments. N=6 for all groups in **A** and **D**. N=8 for all groups in **B** except for the PTCy 100 mg/kg group (n=5). N=2-10 for **C** reflecting all surviving mice to that timepoint from an initial 10 mice/group that were transplanted.

A

Criteria	Grade 0	Grade 1	Grade 2
Posture	No hunching	Mild hunching at rest or with activity	Moderate to severe hunching with movement
Activity	Normal to mildly reduced	Moderately to severely reduced	Stationary unless stimulated
Fur texture	No ruffling or fur loss not attributable to grooming	Mild to moderate ruffling	Severe ruffling or bare patches not attributable to grooming
Skin Integrity	No scaling or denuded skin	Scaling of paws/tail	Areas of denuded skin
Eyes	Both eyes fully open	One or both eyes persistently maintained as partially closed or one eye persistently maintained as fully closed	Both eyes persistently maintained as fully closed except with extreme stimulation

B

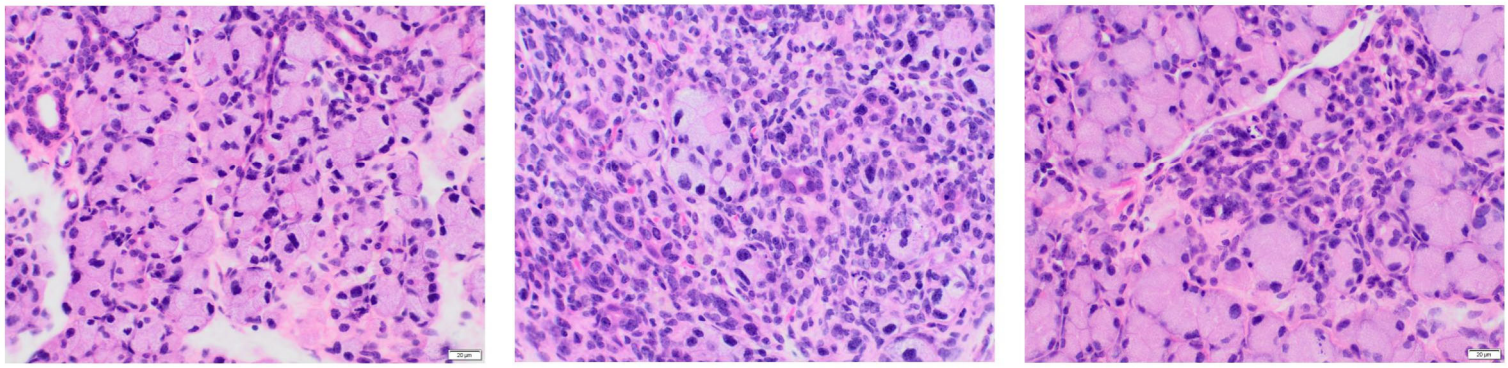


Figure S23. Scoring rubric for clinical evaluation of mice. (A) The scoring rubric used for clinical evaluations of mice is shown. Each criterion is graded from 0 to 2 to generate a combined score of 0 to 10. This rubric was adapted from a previous scoring rubric,*(59)* but was modified to best evaluate mice treated with the B6C3F1→B6D2F1 model. There were only rare skin integrity findings seen in this model. However, there were prominent eye findings observed in mice suffering from severe GVHD, and the severity of those eye findings correlated with the other clinical parameters and the intensity of GVHD histopathologically. Thus, this criterion was added to better distinguish gradations in the severity of GVHD in this HCT model. Compared with the previous scoring rubric, mildly reduced activity also was downgraded to a grade 0 (from grade 1) in an attempt to minimize the subjectivity of this new scoring rubric. **(B)** The eye findings seen were confirmed to be GVHD-related by histopathologic examination. After 10.5 Gy radiation, recipient B6D2F1 mice were transplanted with 10 x 10^6 B6C3F1 TCD BM cells and 40 x 10^6 B6C3F1 splenocytes followed by vehicle or PTCy 25 mg/kg/day on days +3 and +4. Mice were euthanized on day +21, and the exorbital lacrimal glands were assessed by histopathology. **Left:** Normal lacrimal gland from a mouse treated with PTCy. 400X H&E. **Center:** Lacrimal gland from a mouse treated with vehicle, showing moderate acinar atrophy, mild lymphocytic adenitis, and minimal acinar cell necrosis. 400X. **Right:** Lacrimal gland from a second mouse treated with vehicle, showing mild multifocal lymphocytic adenitis, acinar atrophy, and minimal acinar cell necrosis. 400X