Supplementary Data for

Posttransplantation Cyclophosphamide Expands Functional Myeloid-Derived Suppressor Cells and Indirectly Influences Tregs

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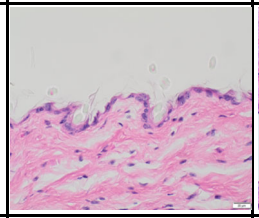
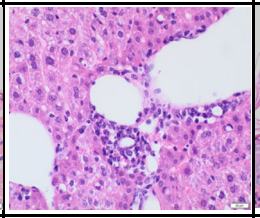
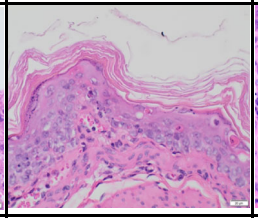
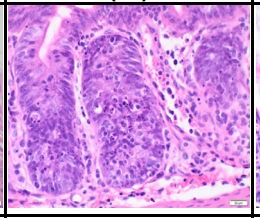
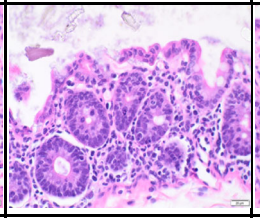
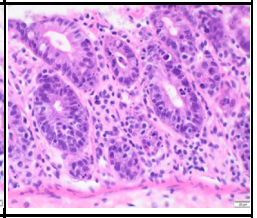
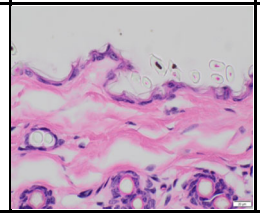
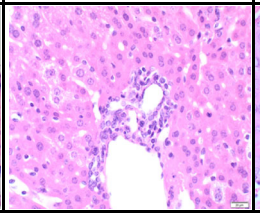
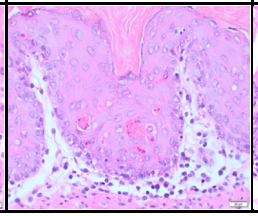
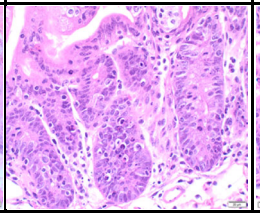
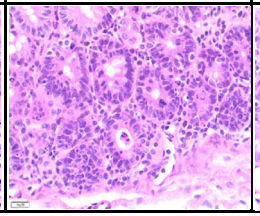
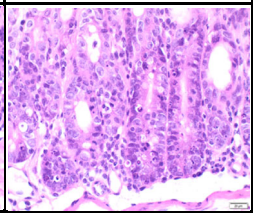
This PDF file includes:

Supplementary Methods

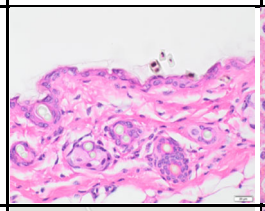
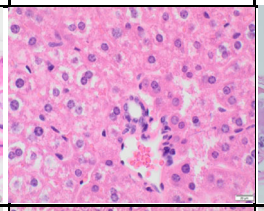
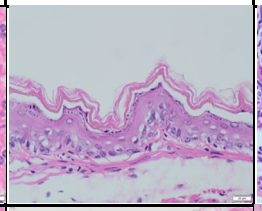
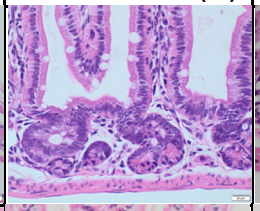
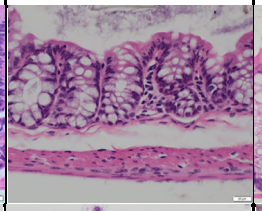
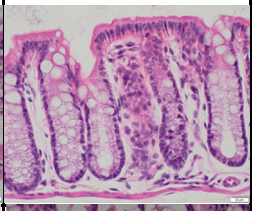
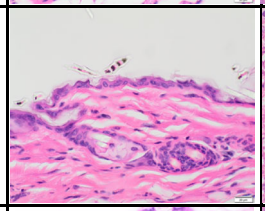
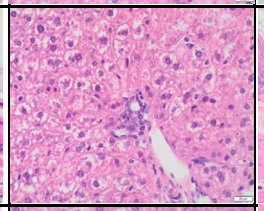
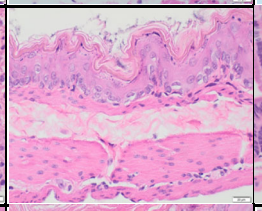
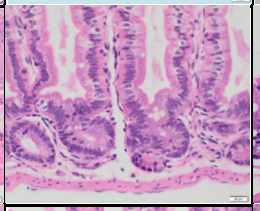
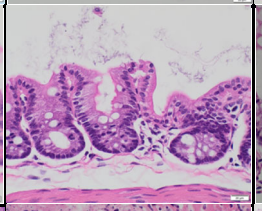
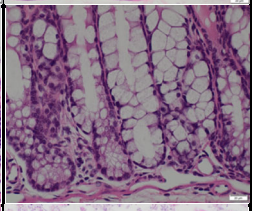
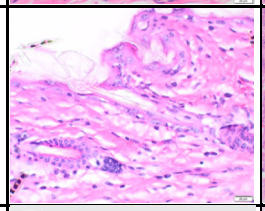
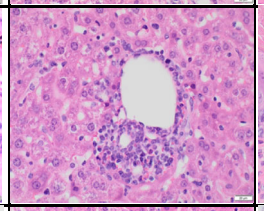
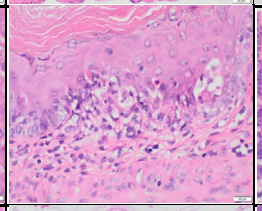
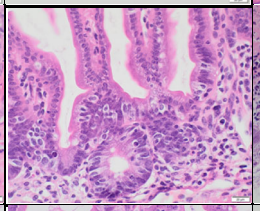
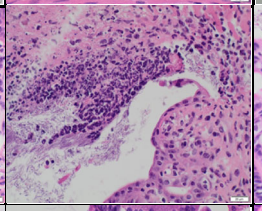
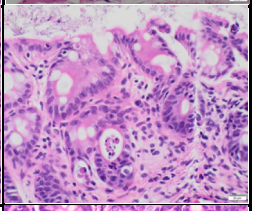
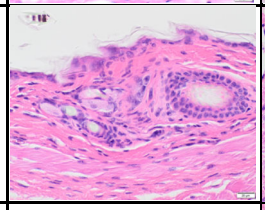
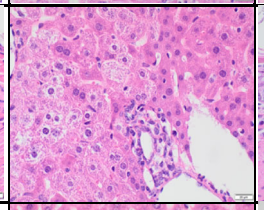
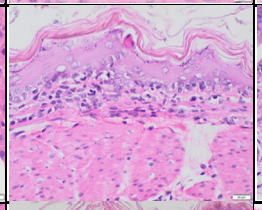
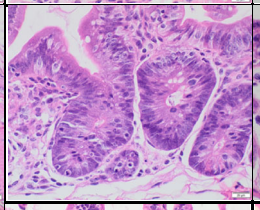
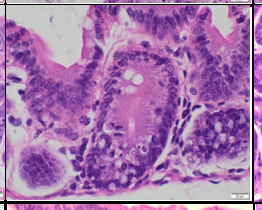
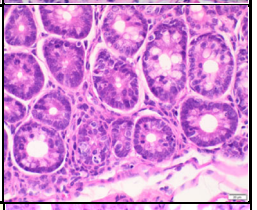
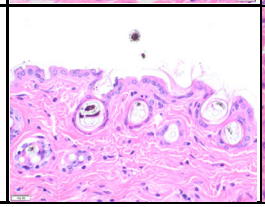
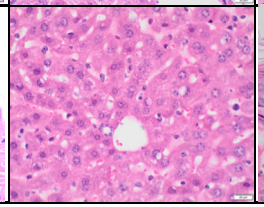
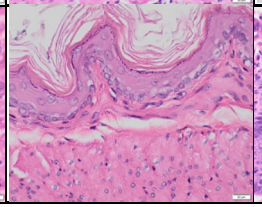
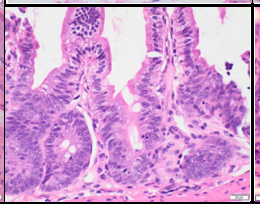
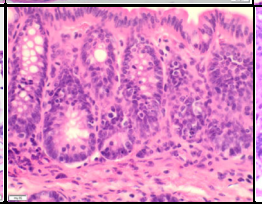
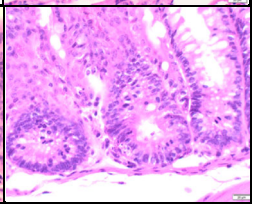
Antibodies for flow cytometry: Mouse fluorochrome-conjugated monoclonal antibodies used for flow cytometry included: Brilliant Ultraviolet (BUV) 395 anti-CD3 (clone 145-2C11), Brilliant Violet (BV) 786 anti-CD8 (clone 53-6.7), BUV661 anti-CD11b (clone M1/70), PE anti-CD11c (clone HL3), BV650 anti-CD19 (clone 1D3), PE-CF594 anti-CD25 (clone PC61), Alexa Fluor (AF) 700 anti-CD44 (clone IM7), PE anti-CD45.1 (clone A20), APC anti-CD45.2 (Clone 104), BUV737 anti-CD62L (clone MEL-14), BV786 anti-CD80 (clone 16-10A1), PE/Cy7 anti-CD90.2 (clone 53-2.1), BV421 anti-CD124 (Clone mIL4R-M1), BV711 anti-F4/80 (clone T45-2342), PE anti-$H2K^k$ (Clone 36-7-5), BV711 anti-$H2K^k$ (clone AF3-12.1), BV650 anti-MHC-II (clone M5/114.15.2), AF700 anti-Ly6G (Clone 1A8), and PE/Cy7 anti-PDL1 (clone 10F.9G2) from BD Biosciences; PE-Cy5 anti-CD8 (clone 53-6.7), APC/Fire 750 anti-CD40 (clone 3/23), APC anti-CD45.1 (clone A20), PE/Dazzle 594 anti-CD115 (CSF-1R) (clone AFS98), BV421 anti-CD11b (clone M1/70), AF647 anti-CD90.2 (clone 30-H12), PE anti-$H2k^d$ (clone SF1-1.1), PE-Cy7 anti-$H2K^d$ (clone SF1-1.1), BV605 anti-Ki-67 (clone 16A8), AF488 Ly6C (clone HK1.4), PE/Cy7 anti-Ly6G (clone 1A8), BV421 anti-NK1.1 (clone PK136), and BV711 anti-NK1.1 (clone PK136) from BioLegend; and APCefluor780 anti-CD4 (clone GK1.5), efluor450 anti-Foxp3 (clone FJK-16S), and PerCP-efluor710 anti-Vβ6 (clone RR4-7) from eBioscience. Human fluorochrome-conjugated monoclonal antibodies used for flow cytometry included BUV805 anti-CD14 (clone M5E2) and BUV395 anti-HLA-DR (clone G46-6) from BD Biosciences.

Table S1. Representative examples of histopathologic assessment at day +6.

	Skin	Liver	Stomach	Small Intestine (SI)	Cecum	Colon
Allo BM/Splen, PBS, Isotype Total Score: 9 Skin: 0 SI: 3 Liver: 1 Cecum: 1 Stomach: 2 Colon: 2						
Allo BM/Splen, PBS, Anti-Gr1 Total Score: 10 Skin: 0 SI: 3 Liver: 1 Cecum: 2 Stomach: 2 Colon: 2						

Notes: Allo, allogeneic; BM, bone marrow; Splen, splenocytes; PBS, phosphate-buffered saline.

Table S2. Representative examples of histopathologic assessment at day +10.

	Skin	Liver	Stomach	Small Intestine (SI)	Cecum	Colon
Syn BM/Splen, PTCy, Isotype Total Score: 0 Skin: 0 SI: 0 Liver: 0 Cecum: 0 Stomach: 0 Colon: 0						
Syn BM/Splen, PTCy, Anti-Gr1 Total Score: 0 Skin: 0 SI: 0 Liver: 0 Cecum: 0 Stomach: 0 Colon: 0						
Allo BM/Splen, PBS, Isotype Total Score: 10 Skin: 1 SI: 1 Liver: 2 Cecum: 2 Stomach: 2 Colon: 2						
Allo BM/Splen, PTCy, Isotype Total Score: 5 Skin: 0 SI: 1 Liver: 1 Cecum: 1 Stomach: 1 Colon: 1						
Allo BM/Splen, PTCy, Anti-Gr1 Total Score: 3 Skin: 0 SI: 1 Liver: 0 Cecum: 1 Stomach: 0 Colon: 1						

Notes: Syn, syngeneic; BM, bone marrow; Splen, splenocytes; Allo, allogeneic; PBS, phosphate-buffered saline.

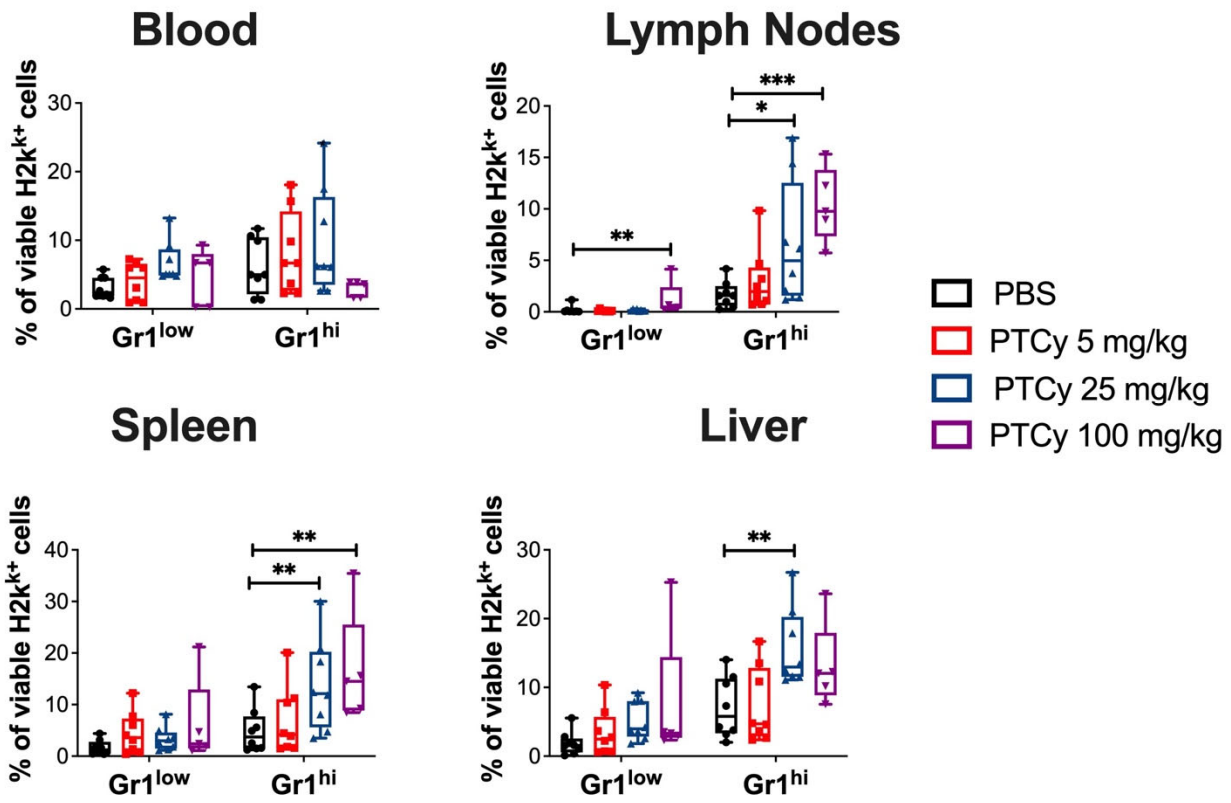


Figure S1. Intermediate and high doses of post-transplantation cyclophosphamide (PTCy) increase percentages of myeloid-derived suppressor cells (MDSCs) at day +21. On day 0, 10- to 12-week-old recipient female B6D2F1 mice were irradiated (10.5 Gy) and transplanted with 10×10^6 T-cell-depleted bone marrow cells from 10- to 12-week-old female wildtype B6C3F1 donors. Phosphate-buffered saline (PBS) vehicle or 25 mg/kg/day PTCy was administered on days +3 and +4. Mice were euthanized at day +21, and different tissues were processed and assessed by flow cytometry. CD11b and relative Gr1 expression were used to gate MDSC subsets off $B220^-NK1.1^-CD3^-$ donor cells. Class I ($H2k^k$ vs. $H2k^d$) expression was used to define donor, potentially leading to lower percentages of MDSCs than found when gating on $CD45.1^+$ cells in **Figure 3**. Combined results from two independent experiments are shown with n=4/group/experiment except for PTCy 100 mg/kg (n=5 total due to early deaths). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ on one-way ANOVA with the Holm-Sidak post hoc test using the PBS vehicle group as the control. Statistical comparisons with the PBS group that are not shown had $p > 0.05$.

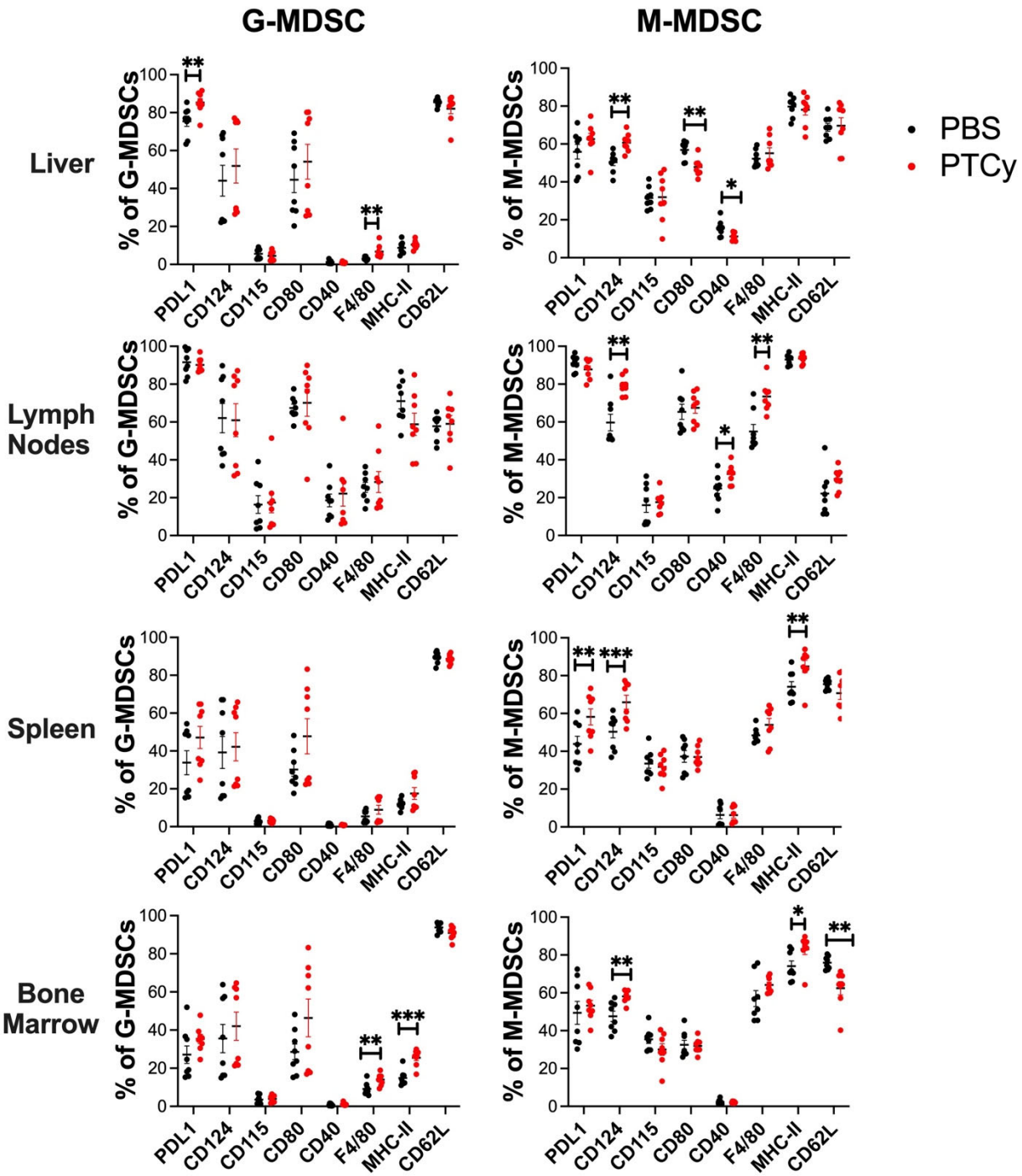


Figure S2. Effect of PTCy at day +7 on specific markers that can influence the suppressive capabilities of G-MDSCs and M-MDSCs. Mice transplanted in **Figure 3B** were assessed for a variety of cell surface markers associated with MDSCs. Combined results from two independent experiments are shown with n=4 mice/group/experiment. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ on unpaired t-test.

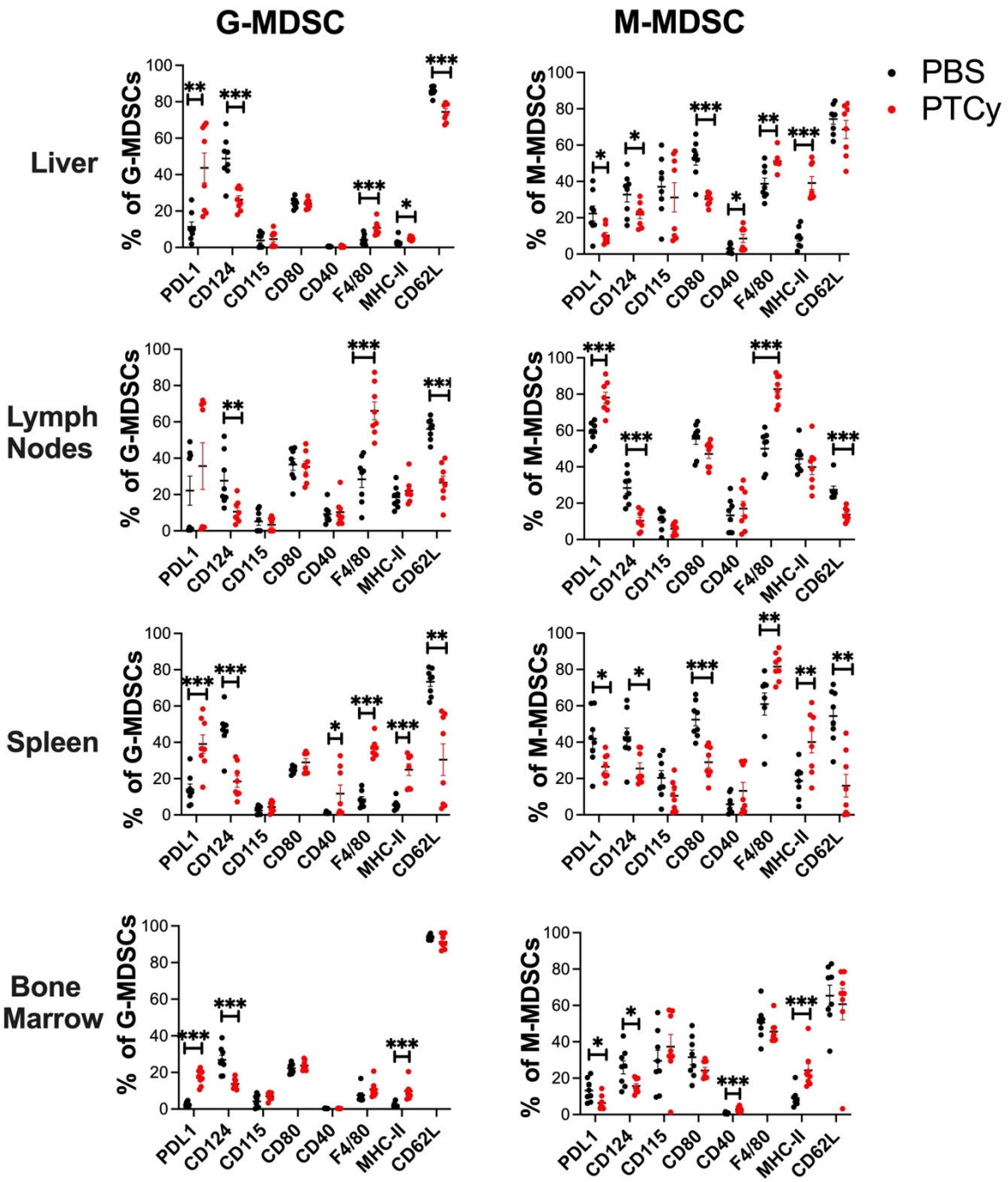


Figure S3. Effect of PTCy at day +21 on specific markers that can influence the suppressive capabilities of G-MDSCs and M-MDSCs. Mice transplanted in **Figure 3B** were assessed for a variety of cell surface markers associated with MDSCs. Combined results from two independent experiments are shown with n=4 mice/group/experiment. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ on unpaired t-test.

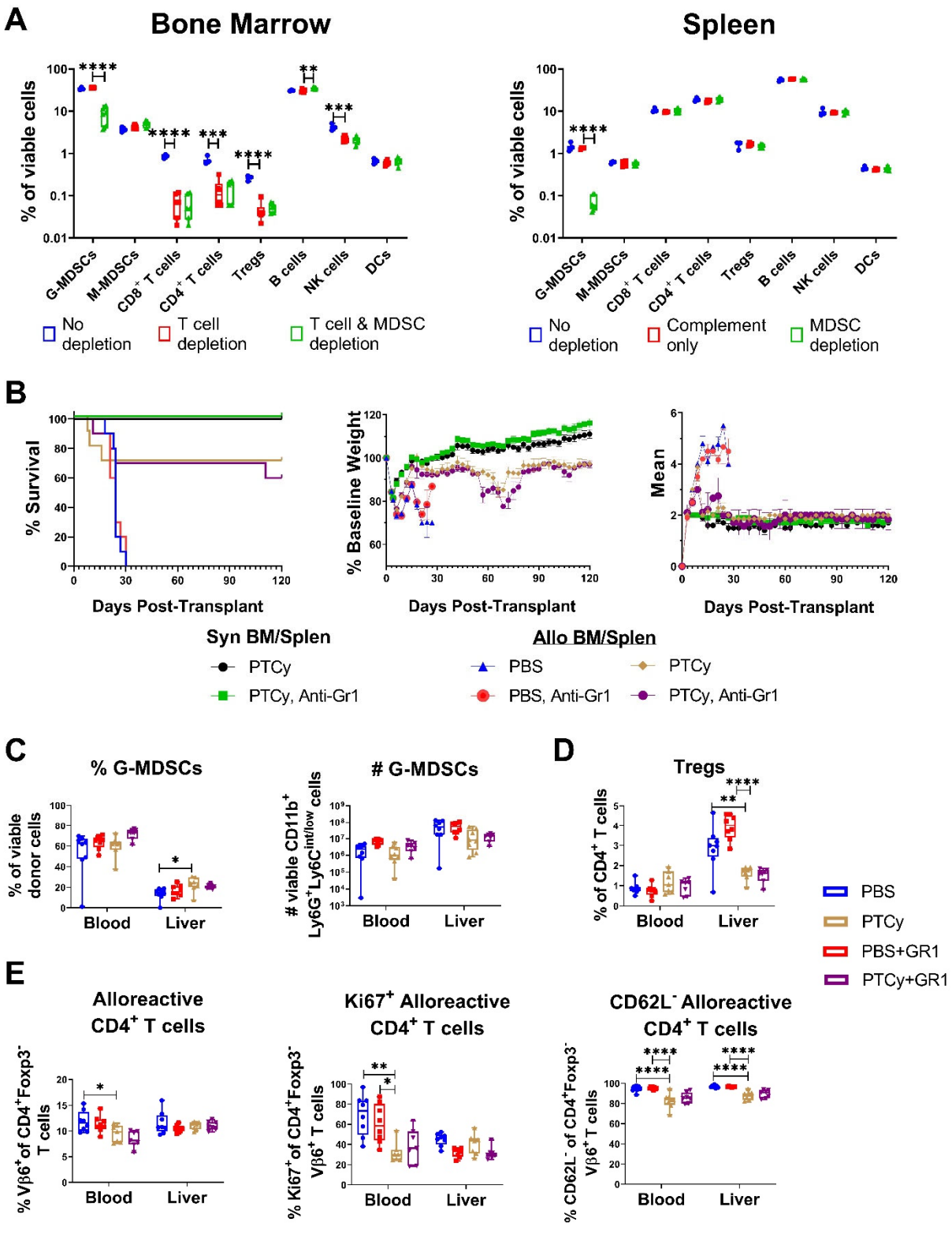


Figure S4. ***Ex vivo*** **anti-Gr1 depletion of the graft only transiently affects G-MDSCs and does not interfere with PTCy's impact on clinical GVHD, preferential MDSC recovery, or alloreactive T cells.** To attempt to deplete MDSCs within the allograft, bone marrow or spleen from donor B6C3F1 mice was

treated with anti-Gr1 antibody *in vitro*. These cells (or non-depleted cells) were used as the allografts for transplantation as per **Figure S1**. Syngeneic indicates B6D2F1→B6D2F1. **(A)** Anti-Gr1 *ex vivo* treatment substantially reduced percentages of G-MDSCs but did not affect M-MDSCs. This depletion was achieved with minimal impact on other immune subsets. Combined results of three independent experiments are shown. Comparisons were performed with one-way ANOVA followed by Holm-Sidak post tests using the T-cell depletion (bone marrow) or Complement only (spleen) groups as the comparator group. **(B)** *Ex vivo* anti-Gr1 depletion had no significant impact on survival or clinical GVHD in PTCy-treated mice. Combined results of two independent experiments of 5 mice/group/experiment are shown. **(C)** This lack of an effect likely was due to the very transient nature of the depletion. By day +7, G-MDSC levels in mice receiving G-MDSC-depleted grafts were similar to mice receiving non-depleted grafts. **(D-E)** Consequently, this G-MDSC transient depletion did not interfere with the impact of PTCy on **(D)** regulatory or **(E)** alloreactive ($V\beta6^+$) conventional $CD4^+$ T cells, including the percentages of alloreactive conventional $CD4^+$ T cells that were proliferating or differentiated. For **C-E**, combined results of two independent experiments are shown with n=4 mice/group/experiment. For **A, C-E**, comparisons were performed with one-way ANOVA followed by the Holm-Sidak post test. The comparator groups were the T-cell depletion (bone marrow) or Complement only (spleen) groups for **A** and the PTCy/no depletion group for **C-E**. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p<0.0001$. Only significant differences are shown.

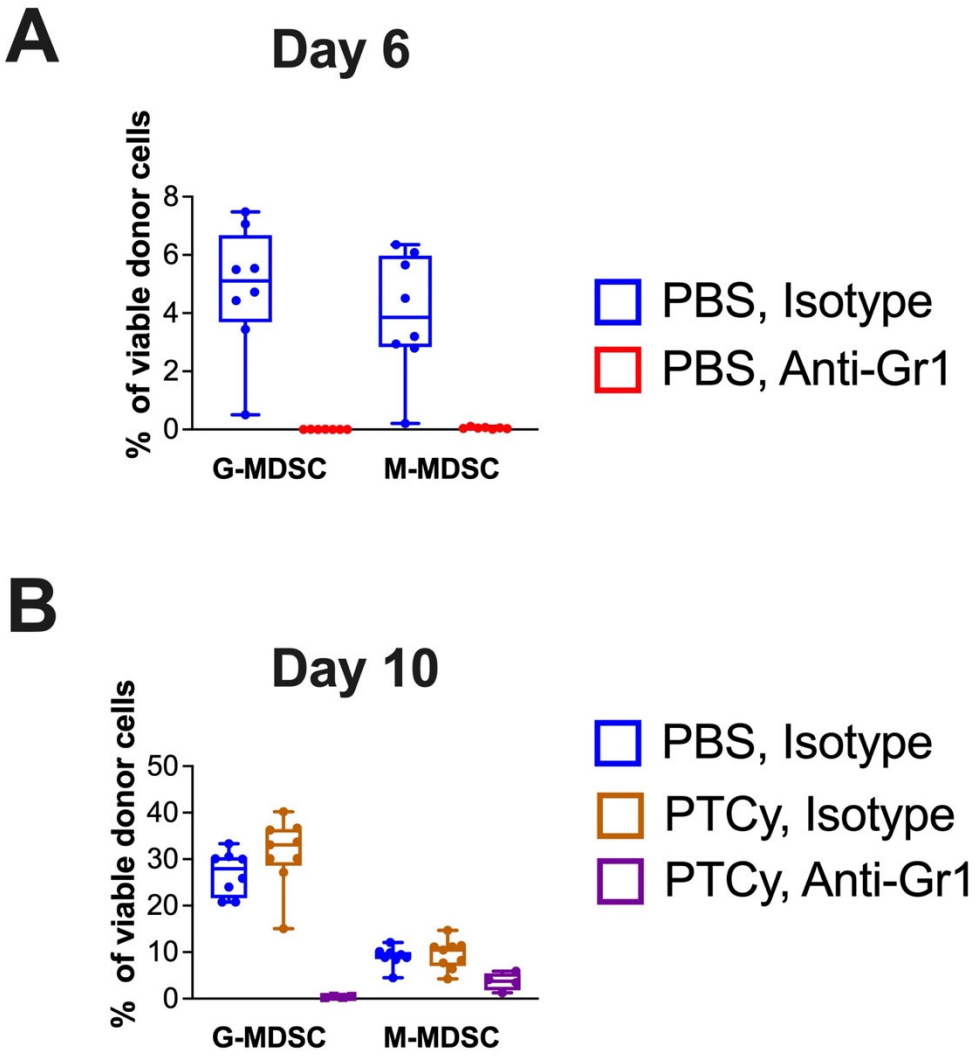


Figure S5. Efficacy of anti-Gr1 depletion in transplanted mice treated with or without PTCy. For mice transplanted in **Figure 7**, percentage of viable donor cells that were G-MDSCs ($CD11b^{+}Ly6G^{+}Ly6C^{int/low}$) or M-MDSCs ($CD11b^{+}Ly6G^{-}Ly6C^{high}$) were assessed in the liver. Combined results from two independent experiments are shown with total n=4/group/experiment except for the Allo PTCy Anti-Gr1 group (n=4 total due to excess deaths prior to day +10).

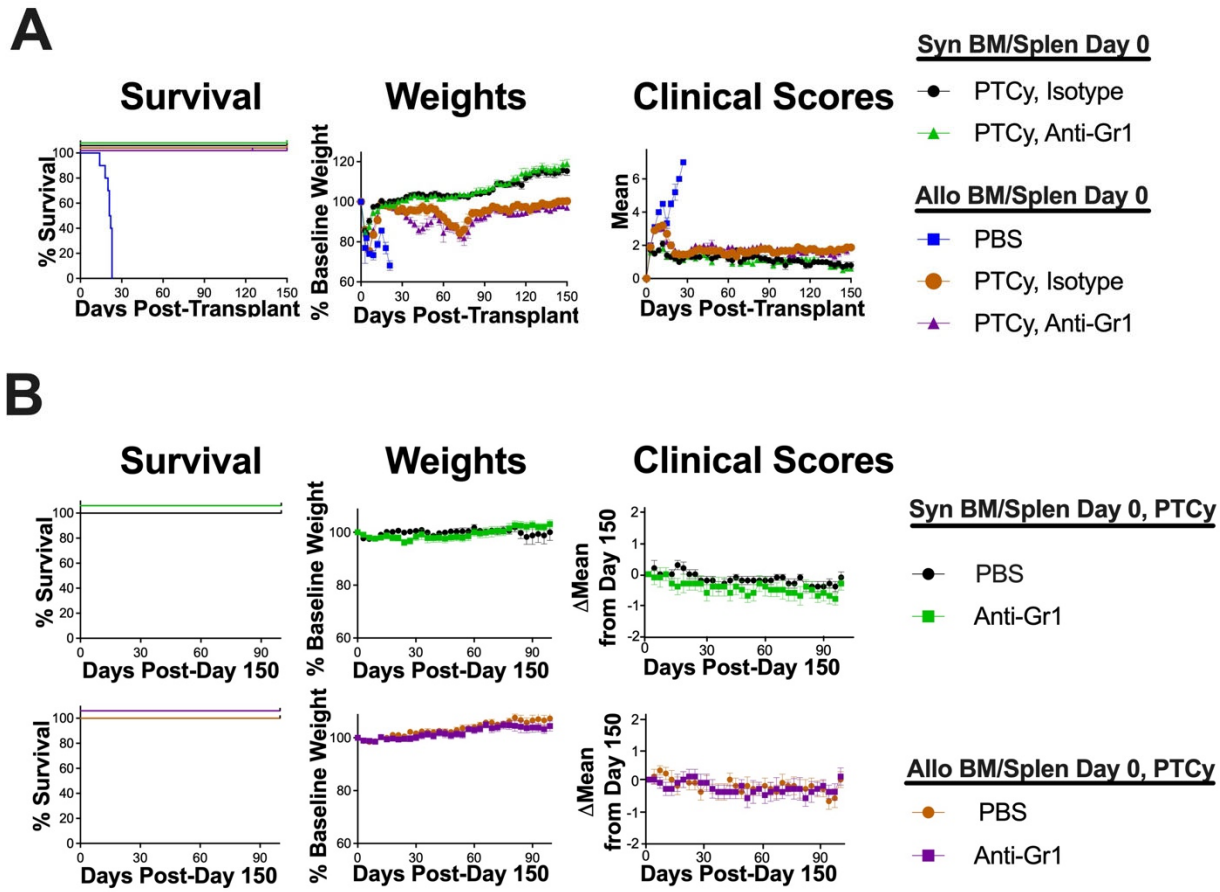


Figure S6. Gr1$^+$ cell depletion at intermediate (day +28) and late (day +150) posttransplant time points has minimal impact on PTCy-treated mice. Mice were treated as in **Figure 6** except that anti-Gr1 or isotype control antibody was given on **(A)** days +28, +32, +36, and +40 or **(B)** days +150, +154, +158, and +162. Combined results from **(A)** two or **(B)** four independent experiments with n=5 and n=2-3 mice/group/experiment, respectively. Baseline weight for **B** was the day +150 weight at the start of treatment for each mouse.

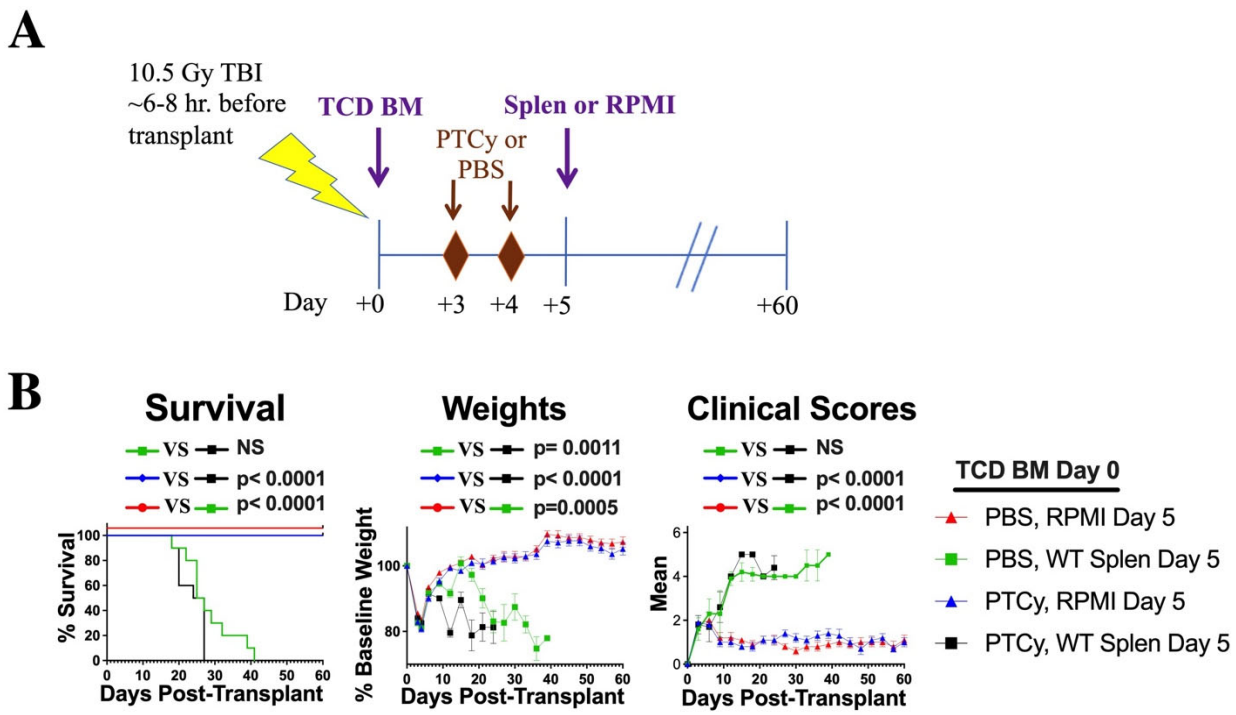


Figure S7. PTCy does not protect against fatal GVHD induced by additional splenocyte infusion when PTCy is given to mice transplanted with only T-cell-depleted bone marrow. On day 0, 10- to 12-week-old recipient female B6D2F1 mice were irradiated (10.5 Gy) and transplanted with 10×10^6 T-cell-depleted (TCD) bone marrow (BM) cells from 10- to 12-week-old female wildtype B6C3F1 donors. Phosphate-buffered saline (PBS) vehicle or 25 mg/kg/day PTCy was administered on days +3 and +4. On day +5, 40 x 10^6 red-blood-cell-depleted wildtype splenocytes or RPMI were infused. Combined results are shown from two independent experiments of n=5 mice/group/experiment. Survival outcomes were compared using the exact log-rank test, and area-under-the-curve comparisons of weights and clinical scores were performed using Wilcoxon's rank sum test.