Supplemental Materials and Methods

Induction and assessment of GVHD

BALB/c recipients were exposed to 850 cGy total body irradiation (TBI) with the use of a [137Cs] source 8 hours before HCT, and then given C57BL/6 donor spleen cells ($2.5x10^6$-5.0 x 10^6),Thy1.2^+ cells (1 x 10^6) and T cell-depleted BM (TCD-BM) cells (2.5 x 10^6) by tail vein injection. C57BL/6 recipients were exposed to 1300 cGy total body irradiation (TBI) with the use of a [137Cs] source 8 hours before HCT, and then given A/J donor spleen cells ($2.5x10^6$) and T cell-depleted BM (TCD-BM) cells (2.5 x 10^6) by tail vein injection. The bone marrow was depleted of T cells by using biotin-conjugated anti-CD4 and anti-CD8 mAbs, and streptavidin Microbeads (Miltenyi Biotec, Germany), followed by passage through an autoMACS Pro cell sorter (Miltenyi Biotec, Germany). For GVL experiments, Luc^+ B-cell leukemia/lymphoma 1 (BCL1) cells (5-10× 10^6) were injected intraperitoneally at the same time when donor bone marrow and spleen cells were injected intravenously. GFP+ blast crisis chronic myeloid leukemia (BC-CML) cells ($1x10^6$) were injected intravenously at the same time when donor bone marrow and spleen cells were injected. In vivo imaging of tumor growth was monitored by using Lago IVIS100 charge-coupled device imaging system. The assessment and scoring of clinical signs of acute GVHD has been described previously[1].

Cell lines

Luciference transfected BCL-1 cell line were provided by Dr. Christopher Contag at Stanford University (Stanford, CA). GFP+ blast crisis chronic myeloid leukemia (BC-CML) cell line were provided by Dr. Warren Shlomchik at Pittsburgh University (Pittsburgh, PA).

Antibodies and FACS analysis

Anti-IL-2 mAb (JES6-1A12, that is, JES6-1) , anti-IL-2 mAb (S4B6) and anti-NK1.1 (PK136) for in vivo treatment was purchased from Bio X Cell (West Lebanon, NH). ChromPure Rat IgG (012-000-003) was purchased from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA, USA). mAbs specific for MHCII (M5/114.15.2) and T-bet (4B10) were purchased from BD Bioscience. mAbs specific for TCRβ (H57-597), H-2Kb (AF6-88.5), CD4 (RM4-5), CD8a (53-6.7), CD11b (M1/70), CD11c (N418), Ly6G (RB6-8C5), Ly6C (HK1.4), pMTOR (MRRBY), CD39 (24DMS1) were purchased from ThermoFisher Bioscience. mAbs specific for CD24 (M1/69), Pro-IL-1β (NJTEN3), B7H1 (H1M5), pAKT473 (SDRNR), KLRG1 (2F1/KLRG1), CD107a (1D4B), GranzymeB (QA16A02), ly108 (330-AJ), TIM3 (RMT3-23), IFN-γ (XMG1.2), TNF-α (MP6-XT22), Eomes (Dan 11 mag) , GM-CSF (MP1-22E9), IL-10 (JES5-16E3), Blimp-1 (5E7) , perforin (eBioOMAK-D), CD127 (SB/199), IL-2 (JES5-5H4),Foxp3 (FJK-16s), NKp46 (29A1.4) and mouse Breg staining kit (anti-mouse CD19/CD5/CD1d) were purchased from Biolegend (San Diego, CA). CellROX™ Green Reagent (for detection of oxidative stress), anti-mouse CD3e (145-2C11), anti-mouse CD19 (eBio1D3) and anti-mouse NK1.1 (PK136) was purchased from ThermoFisher Scientific. Flow cytometry analyses were performed with an Attune NxT Cytometer (ThermoFisher Scientific) and BD LSRFortessa (Franklin Lakes, NJ), and the resulting data were analyzed with FlowJo software V10 (Tree Star, Ashland, OR).

Isolation of cells from spleen, mesenteric lymph node, liver and large Intestine

Spleen, lymph node and liver tissue were mashed through a 70 μm cell strainer, and MNC were isolated from the cell suspensions with percoll. Intestine was cut first longitudinally and then laterally into pieces of approximately 0.5 cm length. Tissue pieces were incubated with 20 mL of predigestion solution (1× HBSS) without containing 5 mM EDTA, 5% fetal bovine serum (FBS), 1 mM DTT) for 20 minutes at 37 °C under continuous shaking, then passed through 100 μm strainer and held for at least 10 minutes on ice, Intestine epithelia lymphoid cells in the

supernatant were collected. Then tissue pieces were digested with enzyme to isolate the lamina propria cells, following the protocol of Lamina Propria Dissociation Kit (Miltenyi Biotec).

Histopathology

Tissue specimens were fixed in formalin before embedding in paraffin blocks, sectioned and stained with H&E. Slides were examined at 100x (liver) or 200x (small intestine and colon) magnification and visualized with Zeiss Observer II. Tissue damage was blindly assessed according to a defined scoring system, as described previously[1]. Liver GVHD was scored by the severity of lymphocytic infiltrate, number of involved tracts and severity of liver cell necrosis; the maximum score is 9. Gut GVHD was scored by mononuclear cell infiltration and morphological aberrations (e.g. hyperplasia and crypt loss), with a maximum score of 8.

Bioluminescent imaging

Mice were given with luciferase$^+$ BCL1 cells (BCL1/ Luc$^+$) by i.p. injection. For in vivo imaging of tumor growth, 200µl firefly luciferin was injected i.p. (Caliper Life Sciences, Hopkinton, MA), and mice were anesthetized for analysis of tumor cell burden by using an IVIS100 (Xenogen) and AmiX (Spectral) imaging system. Data were analyzed by using Amiview software purchased from Spectral Instruments Imaging (New York, NY).

In vivo BrdU labeling

T cell proliferation was measured with a single i.p. injection of BrdU (2.5 mg/mouse, 100 mg/g) 3h before tissue harvesting. Analysis of donor CD8$^+$ T cells for BrdU incorporation was performed according to the manufacturer's instructions (BD Pharmingen).

mRNA sequencing library preparation and sequencing

RNA concentration was measured by NanoDrop 1000 (Thermo Fisher Scientific, Waltham Massachusetts, US), and RNA integrity was determined using Bioanalyzer (Agilent). Library construction of 280 ng total RNA for each sample was made by using KAPA Stranded mRNA-Seq Kit (Illumina Platforms) (Kapa Biosystems, Wilmington, USA) with 10 cycles of PCR amplification. Libraries were purified using AxyPrep Mag PCR Clean-up kit (Thermo Fisher Scientific). Each library was quantified using a Qubit fluorometer (Life Technologies), and the size distribution was assessed using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). Sequencing was performed on an Illumina® Hiseq 2500 (Illumina, San Diego, CA, USA) instrument using the TruSeq SR Cluster Kit V4-cBot-HS (Illumina®) to generate 51 bp single-end reads sequencing with v4 chemistry. Quality control of RNA-Seq reads was performed using FastQC. Bioconductor package “clusterProfiler” v3.10.1 was used for GSEA analysis to generate the NES and P value, while “enrichplot” v1.2.0 was used to generate the GSEA plot[2].

Single-cell sequencing library construction using the 10x Genomics Chromium Platform

Library preparation was done with the Chromium Single Cell 5' Reagent Kits from 10× Genomics according to manufacturer's protocol. Cellular suspensions were loaded on a Chromium Controller instrument (10x Genomics) to generate single-cell gel bead-in-emulsions (GEMs). GEM-reverse transcriptions (GEM-RTs) were performed in a Veriti 96-well thermal cycler (Thermo Fisher Scientific). After RT, GEMs were harvested, and the cDNAs were amplified and cleaned with the SPRIselect Reagent Kit (Beckman Coulter). Indexed sequencing libraries were constructed using the Chromium Single-Cell 5′ Library Kit (10x Genomics) for enzymatic fragmentation, end-repair, A-tailing, adaptor ligation, ligation cleanup, sample index PCR, and PCR cleanup. The purity and library size were validated by capillary electrophoresis using 2,100 Bioanalyzer (Agilent Technologies). The quantity was measured fluorometrically using Qubit dsDNA HS Assay Kit from Invitrogen.

Libraries were sequenced with a NovaSeq 6000 instrument (Illumina) to a depth of 35k-40k reads per cell. Raw sequencing data were processed using the 10x Genomics' Cell Ranger pipeline (version 3.1.0) to generate FASTQ files and aligned to mm10 genome to generate gene expression counts. The subsequent data analysis was performed using "Seurat v3.0" package and R scripts. Cells with mitochondrial read > 10% and < 200 detectable genes were considered as low-quality and filtered out. Normalized and scaled data were clustered using the top significant principal components of 2000 highly variable genes and resolution of 0.4 using "Seurat". The t-distributed stochastic neighbor embedding (t-SNE) algorithm was used to visualize the resulting clusters. Cluster specific markers were identified using "Seurat" to generate the heatmap of marker genes in these cell clusters. Genes were compared between different clusters using Bioconductor package "Limma" and log2 normalized data. Gene Set Enrichment analysis (GSEA) v3 was performed using genes ranked by the –log10 of "Limma" comparison P value to evaluate the significant activation or inhibition of the Hallmark gene sets in MSigDb (https://www.gsea-msigdb.org/gsea/msigdb/genesets.jsp?collection=H). All plots were generated using either "Seurat" or "ggplot2" package in R.

Statistical analysis

Data were displayed as mean ± SEM. Mortality rates in different groups were compared by log-rank test. Comparison of body weight in different groups was analyzed by nonlinear regression (curve fit). Comparison of means for more than two groups was analyzed by 1-way ANOVA or 2-way ANOVA multiple comparisons, while comparison of two means was analyzed by unpaired two-tailed Student t-test (Prism, version 8.0; GraphPad Software), *P* less than 0.05 was considered as statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

Reference

1. Chakraverty R, Cote D, Buchli J, et al. An inflammatory checkpoint regulates recruitment of graft-versus-host reactive T cells to peripheral tissues. *J Exp Med*. 2006;203(8):2021-2031.

2. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS*. 2012;16(5):284-287.

Supplemental figures and figure legends

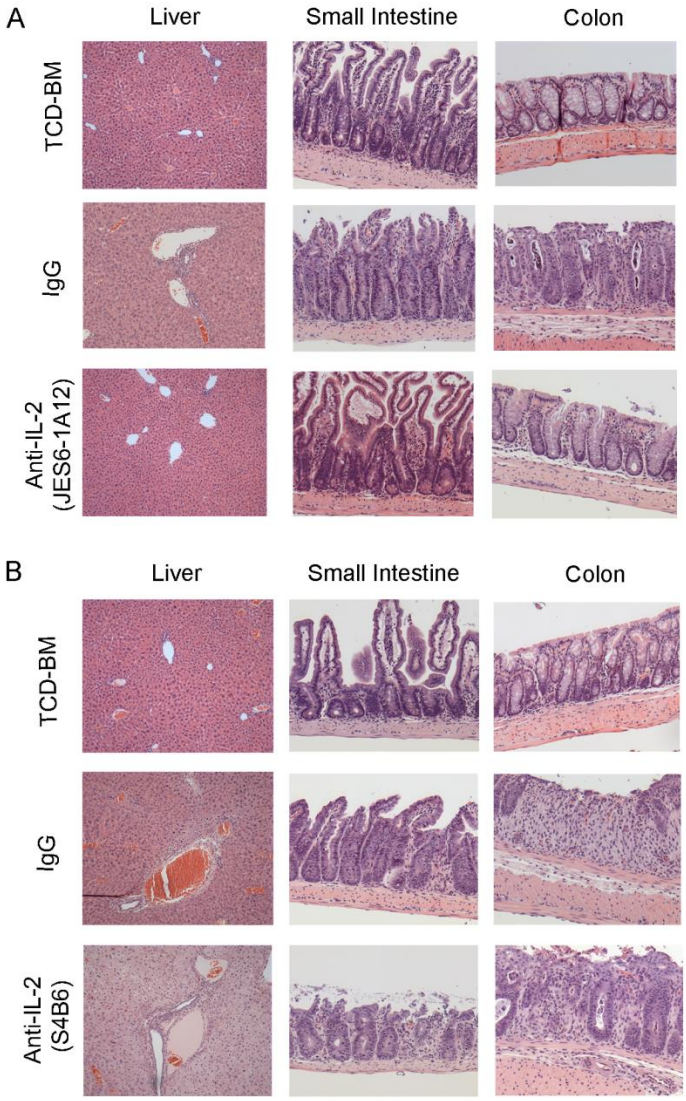

Figure S1: Prevention of aGVHD by tolerogenic anti-IL-2 mAb (JES6-1A12) but not by non-tolerogenic anti-IL-2 mAb (S4B6). Lethally irradiated WT BALB/c recipients were given splenocytes ($5x10^6$) and TCD-BM ($2.5x10^6$) from C57BL/6 donors. Recipients were given a total of 4 i.v. injections of control IgG, anti-IL-2 mAb (JES6-1A12) or anti-IL-2 mAb (S4B6) (500 µg/mouse) at days 0, 2, 4 and 6 after HCT. At 7 days after HCT, histopathology of liver, small intestine and colon was evaluated. A representative photomicrograph is shown, original magnification x100 (liver), x 200 (small intestine and colon).

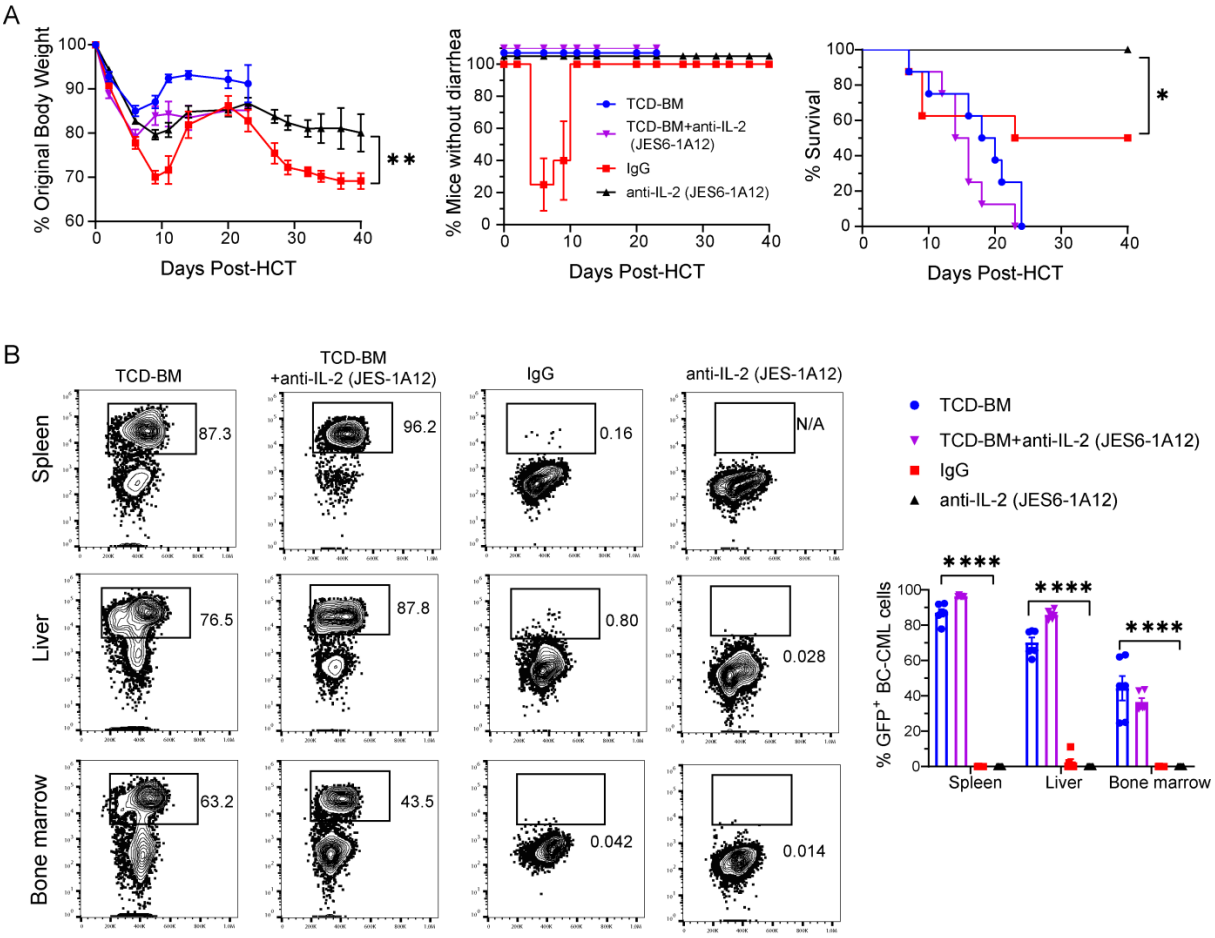

Figure S2: Tolerogenic anti-IL-2 mAb (JES6-1A12) preserves GVL activity against BC-CML tumor cell. Lethally irradiated C57BL/6 recipients were given TCD-BM ($2.5x10^6$) with or without splenocytes ($2.5x10^6$) from A/J donors. Recipients were challenged with i.v. injection of GFP+ BC-CML cells ($1x10^6$/mouse) at day 0 and were given at total of 4 i.v. injections anti-IL-2 mAb (JES6-1A12) (500 µg/mouse) or control IgG at days 0, 2, 4 and 6 after HCT. **(A)** Plots of % Original body weight, % mice without diarrhea and % survival. n=8 from two replicate experiments. **(B)** Moribund mice with or without GVHD during observation and mice at day 40 after HCT were checked for BC-CML tumor cells in the spleen, liver and bone marrow. Representative flow cytometry patterns and percentages of BC-CML cells in the spleen, liver and bone marrow from each group are shown. n=6 combined from duplicated experiments. Data represent mean ± SE. Nonlinear regression (curve fit) was used for body weight comparisons. Log-rank test was used for survival comparisons. 2-way ANOVA was used for BC-CML cell comparison (*p<0.05, ** p<0.01, **** p<0.0001).

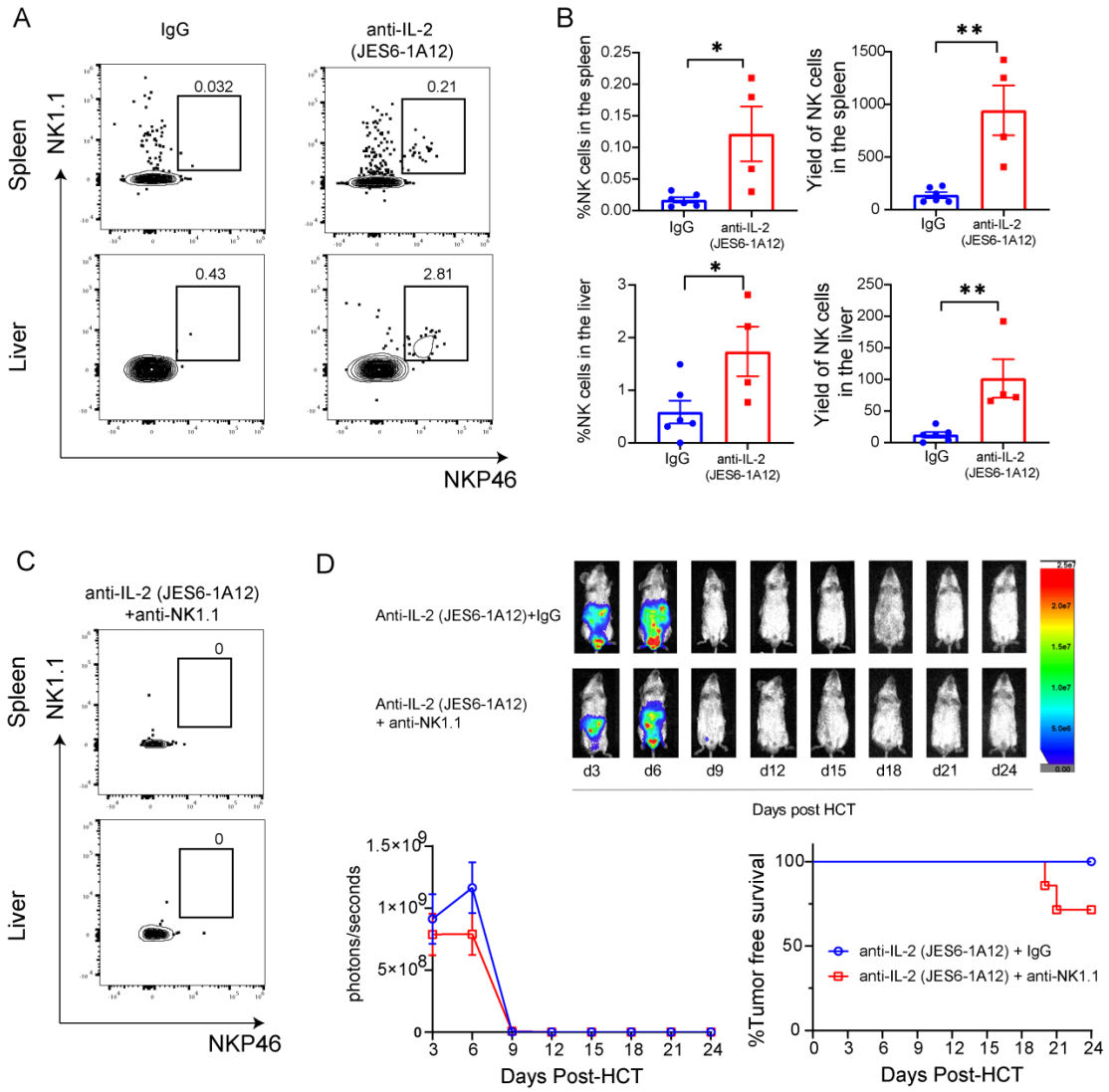

Figure S3: Tolerogenic anti-IL-2 mAb (JES6-1A12) augments NK cells expansion. (A-B) Lethally irradiated WT BALB/c recipients were given splenocytes ($5x10^6$) and TCD-BM ($2.5x10^6$) from WT C57BL/6 donors. Recipients were given a total of 4 i.v. injections of control IgG or anti-IL-2 mAb (JES6-1A12) (500 µg/mouse) at days 0, 2, 4 and 6 after HCT. At day 6 after HCT, NK cells were enumerated in the spleen and liver. Representative flow cytometry pattern **(A)**, percentage and yield of donor NK cells are shown **(B)**, n=4-6 from 2 replicates. **(C-D)** WT BALB/c recipients were engrafted with splenocytes and TCD-BM cells from WT C57BL/6 donors as described in Fig.1D. Recipients were given a total of 4 i.v. injections of anti-IL-2 mAb (JES6-1A12) (500 µg/mouse) at days 0, 2, 4 and 6 with anti-NK1.1 (200ug/mouse) or control IgG once a week after HCT. **(C)** Day 24 after HCT, NK cells were enumerated in the spleen and liver from recipients given anti-NK1.1 treatment. Representative flow cytometry pattern are shown. **(D)** Representative BLI image from each time point of one experiment is shown for each group. Summaries of photons/second and %survival of recipients are shown. n=7 combined from duplicated experiments. Data represent mean ± SE. *P* values were calculated by unpaired two-tailed Student t test (*p<0.05, ** p<0.01).

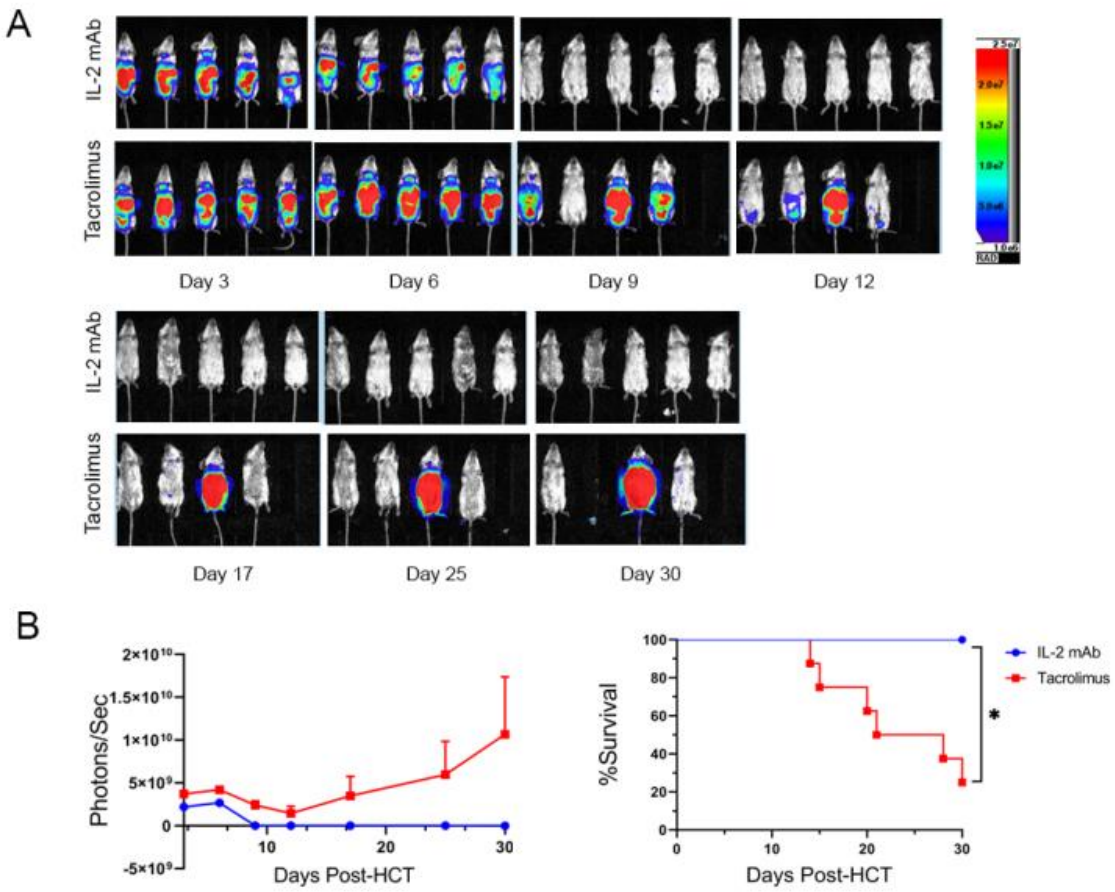

Figure S4: Tolerogenic anti-IL-2 mAb (JES6-1A12) prevents aGVHD and preserves GVL activity more effectively than tacrolimus. Lethally irradiated BALB/c recipients were given splenocytes ($1.25x10^6$) and TCD-BM ($2.5x10^6$) from C57BL/6 donors. Recipients were challenged with i.p. injection of BCL1/Luc cells ($5x10^6$/mouse) at day 0 and were given a total of 4 i.v. injections anti-IL-2 mAb (JES6-1A12) (500 µg/mouse) at days 0, 2, 4 and 6 after HCT or i.p. injections of tacrolimus (0.75 mg/Kg) daily until moribund with tumor growth. **(A)** Representative BLI image from each time point of one experiment is shown for IL-2 mAb and tacrolimus group. **(B)** Summary of photons/second and survival curve of recipients are shown. n=10 combined from duplicated experiments. Data represent mean ± SE. P values were calculated by log-rank test (**p*<0.05).

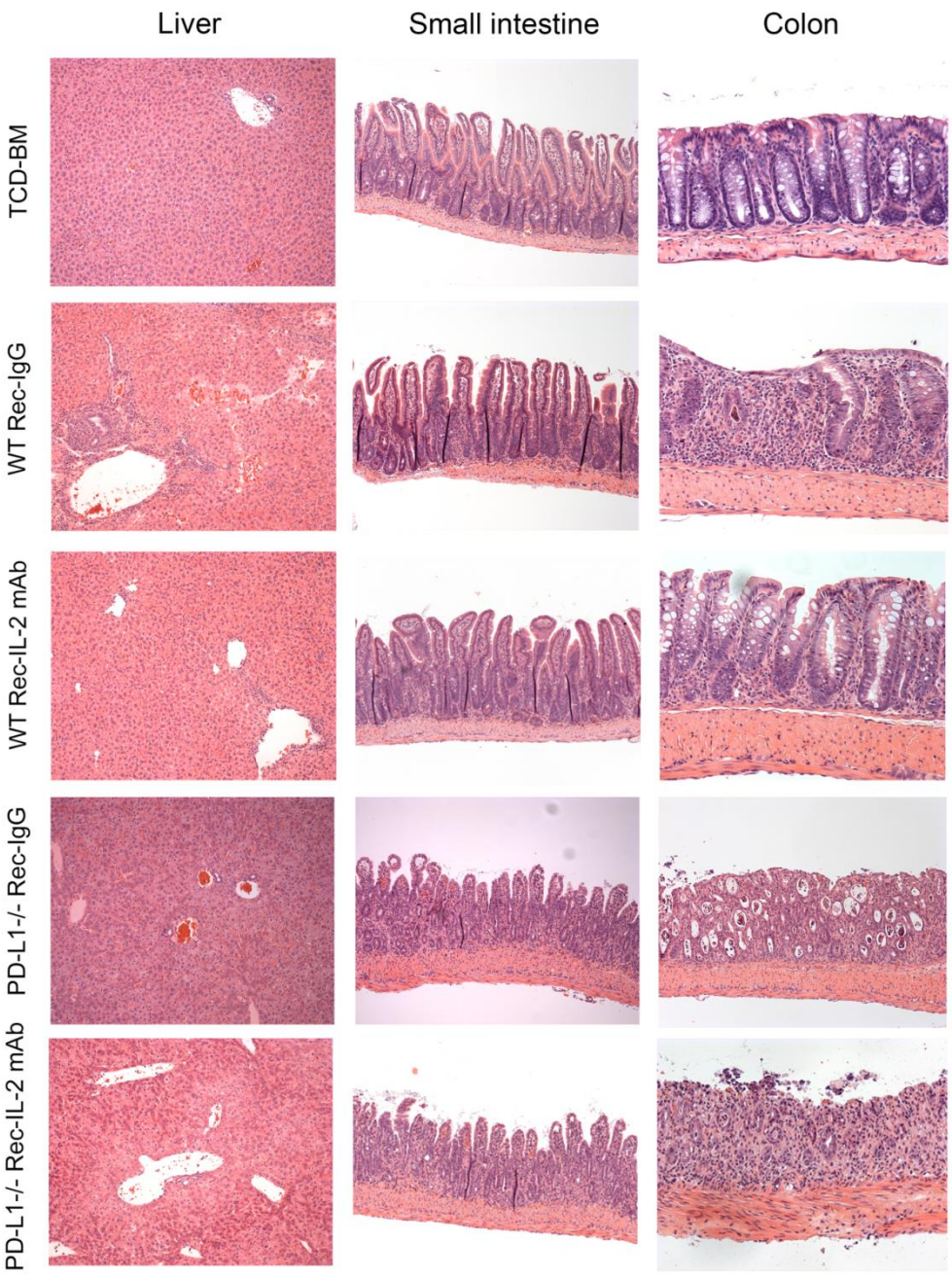

Figure S5: Prevention of aGVHD by tolerogenic anti-IL-2 mAb (JES6-1A12) requires PD-L1 expression by GVHD target tissues. Lethally irradiated WT or PD-L1$^{-/-}$ BALB/c recipients were given splenocytes ($5x10^6$) and TCD-BM ($2.5x10^6$) from C57BL/6 donors. Recipients were given a total of 4 i.v. injections of control IgG or anti-IL-2 mAb (JES6-1A12) (500 µg/mouse) at days 0, 2, 4 and 6 after HCT. At 7 days after HCT, histopathology of liver, small intestine and colon was evaluated. A representative photomicrograph is shown, original magnification x100 (liver), x 200 (small intestine and colon).

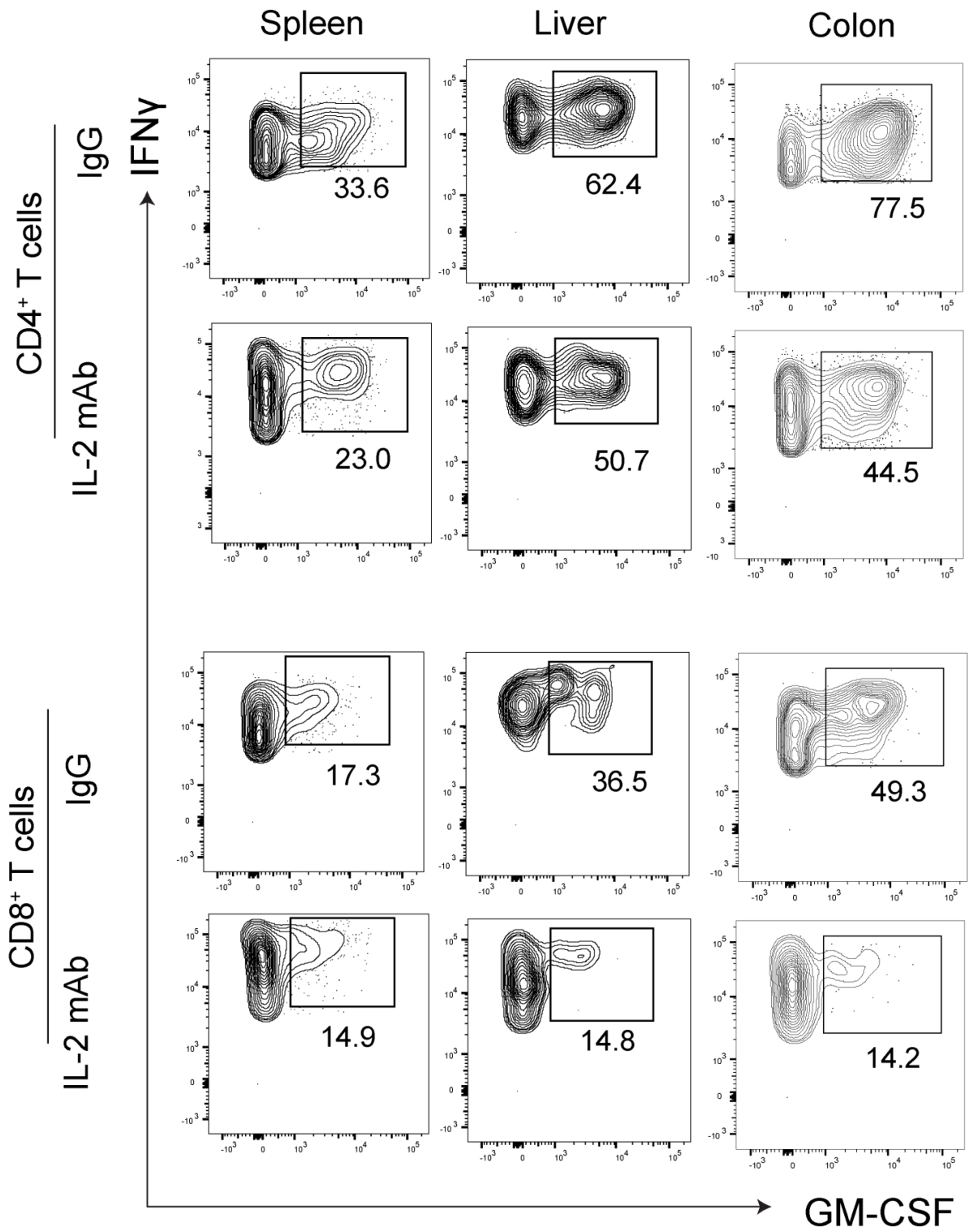

Figure S6: Representative flow cytometry patterns of Fig.3A.

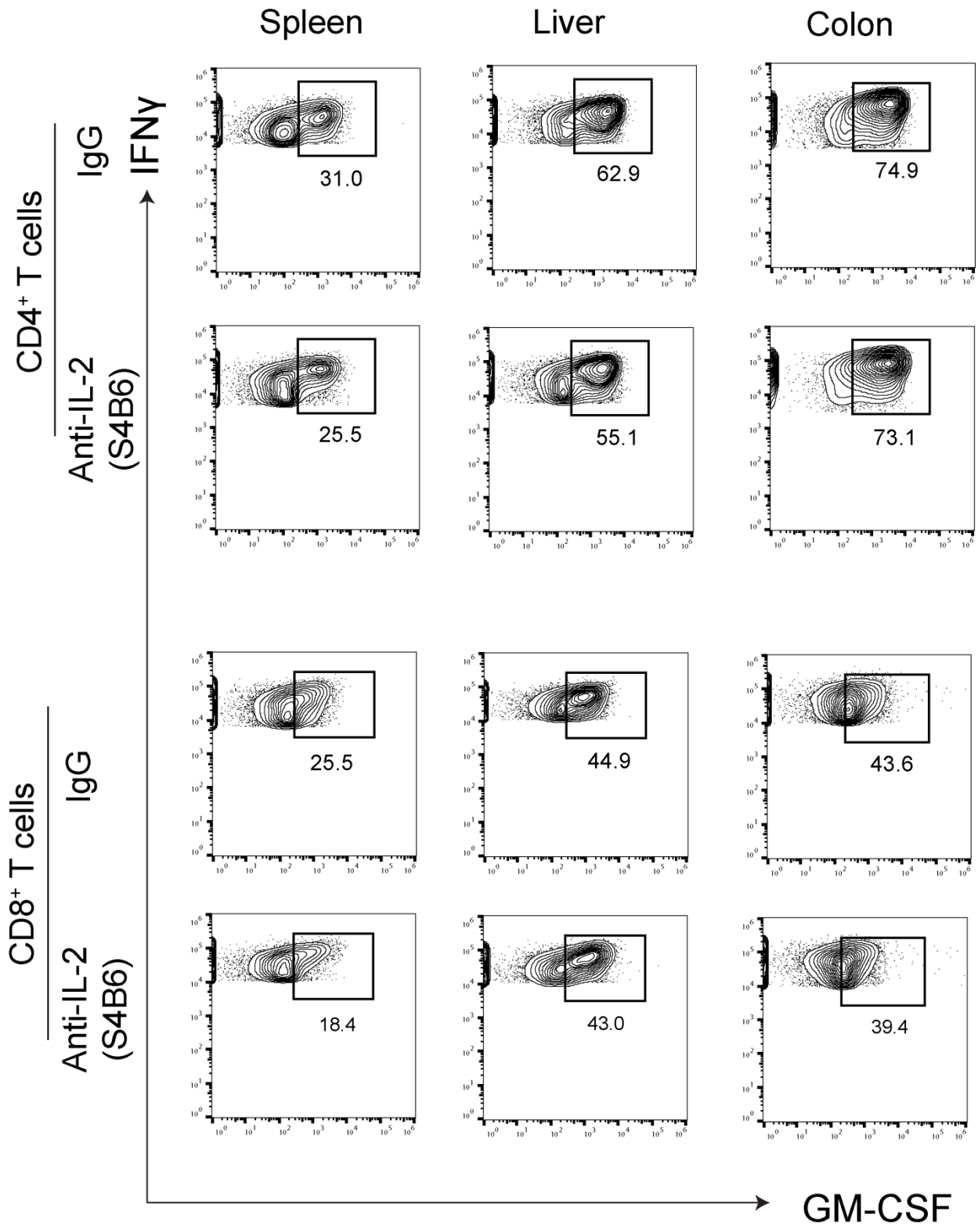

Figure S7: Representative flow cytometry patterns of Fig.3B.

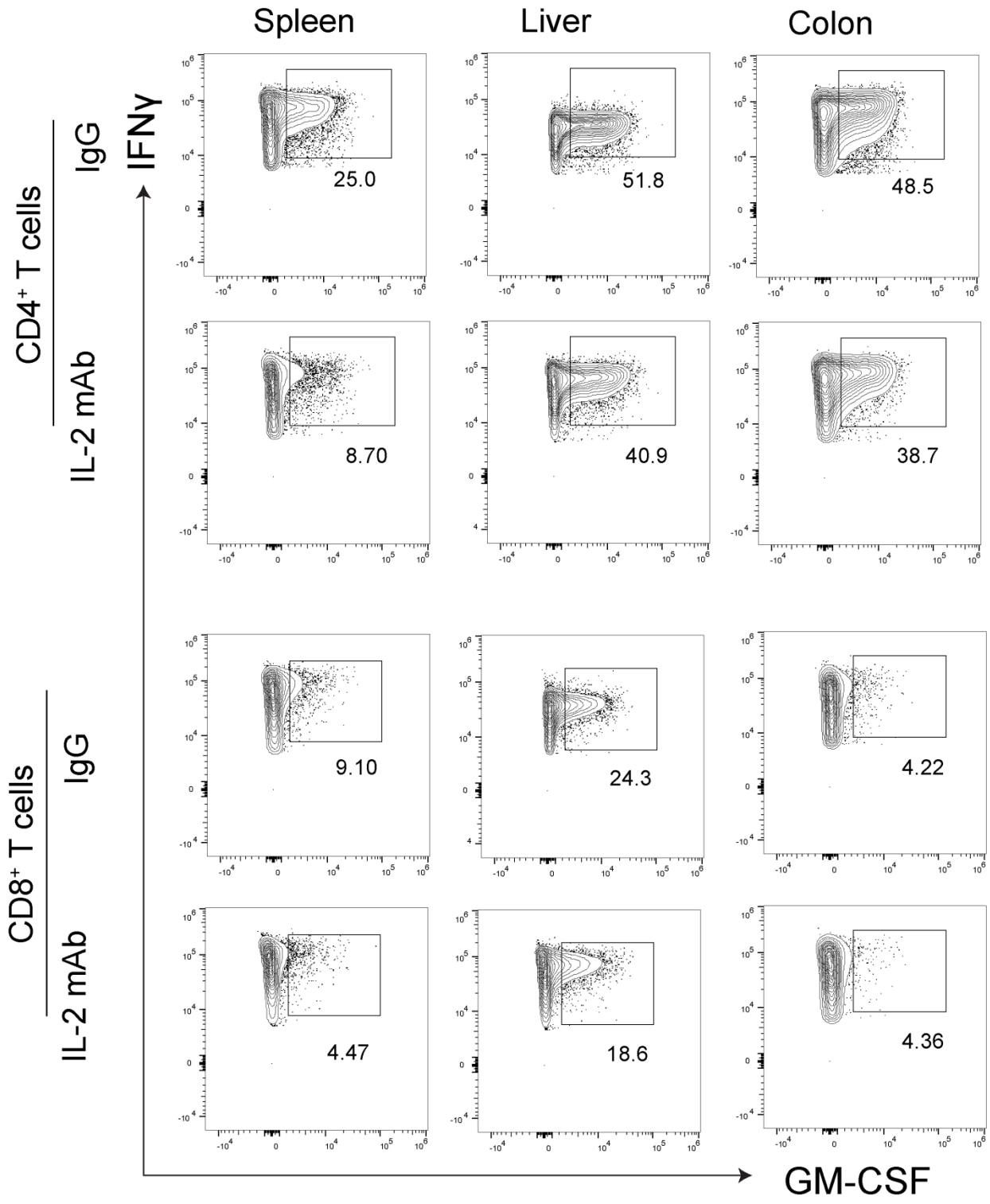

Figure S8: Representative flow cytometry patterns of Fig.3C.

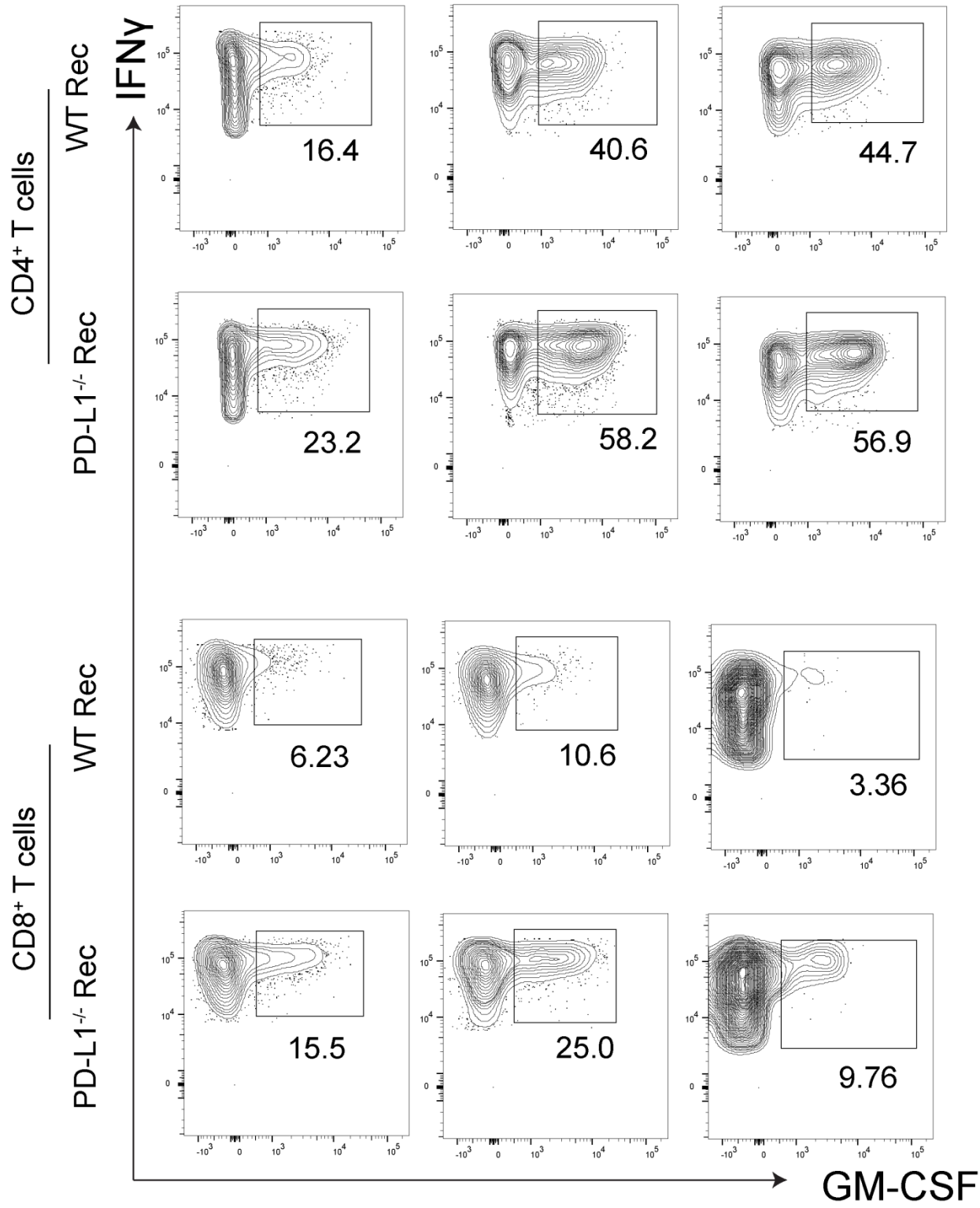

Figure S9: Representative flow cytometry patterns of Fig.3D.

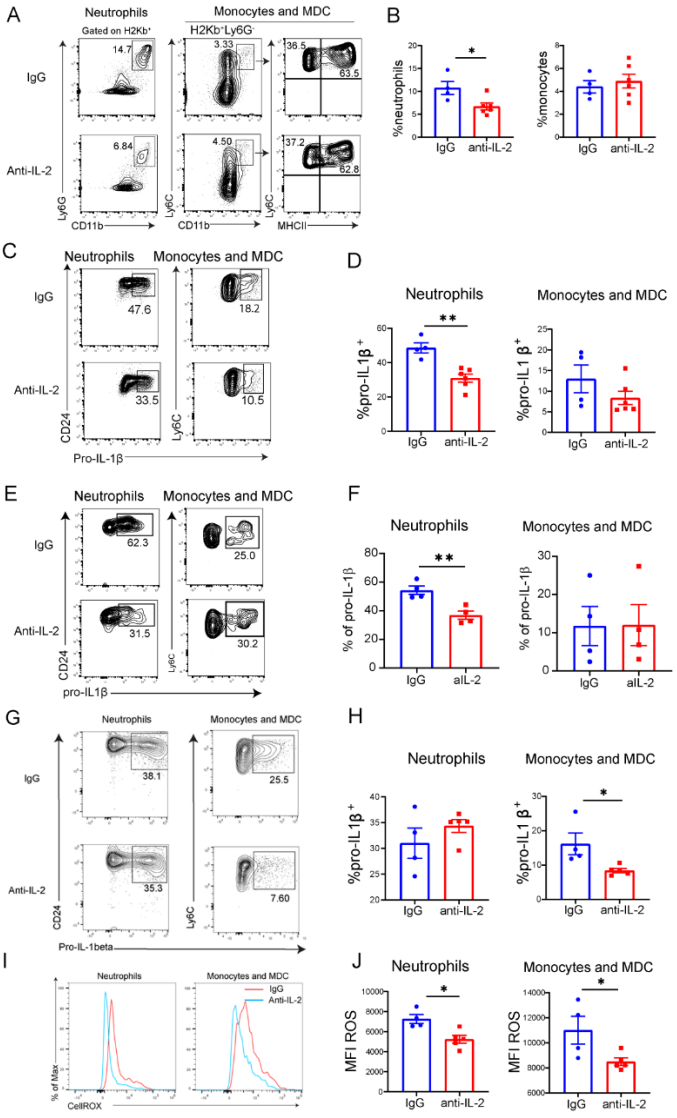

Figure S10: Tolerogenic anti-IL-2 mAb (JES6-1A12) results in the reduction of donor myeloid cells. Lethally irradiated WT BALB/c recipients were given splenocytes ($2.5x10^6$) and TCD-BM ($2.5x10^6$) from C57BL/6 donors. Recipients were given a total of 4 i.v. injections of control IgG or anti-IL-2 mAb (JES6-1A12) (500 µg/mouse) at days 0, 2, 4 and 6. At day 12 after HCT, cells from the spleen (SPL), liver and colon were analyzed by flow cytometry. **(A-B)** Example gating **(A)** and quantification of myeloid cell populations **(B)** in the liver. **(C-D)** Example gating **(C)** and frequency of pro–IL-1β–producing neutrophils and monocytes within the $H2Kb^+$ $CD45^+$ population from liver **(D). (E-F)** Example gating **(E)** and frequency of pro–IL-1β–producing neutrophils and monocytes within the $H2Kb^+$ $CD45^+$ population from colon **(F**). **(G-H)** Example gating **(G)** and frequency of pro–IL-1β–producing neutrophils and monocytes within the $H2Kb^+$ $CD45^+$ population from spleen **(H)**. **(I-J)** Flow cytometric analysis of ROS (CellROX reagent) in the spleen showing representative histograms **(I)** and quantification of median fluorescence intensity (MFI) for neutrophils, monocytes, and MDCs **(J).** Data represent two experiment with n = 4 to 6 per group. Data represent mean ± SE. *P* values were calculated by unpaired two-tailed Student t tests (*$p < 0.05$, **$p < 0.01$).

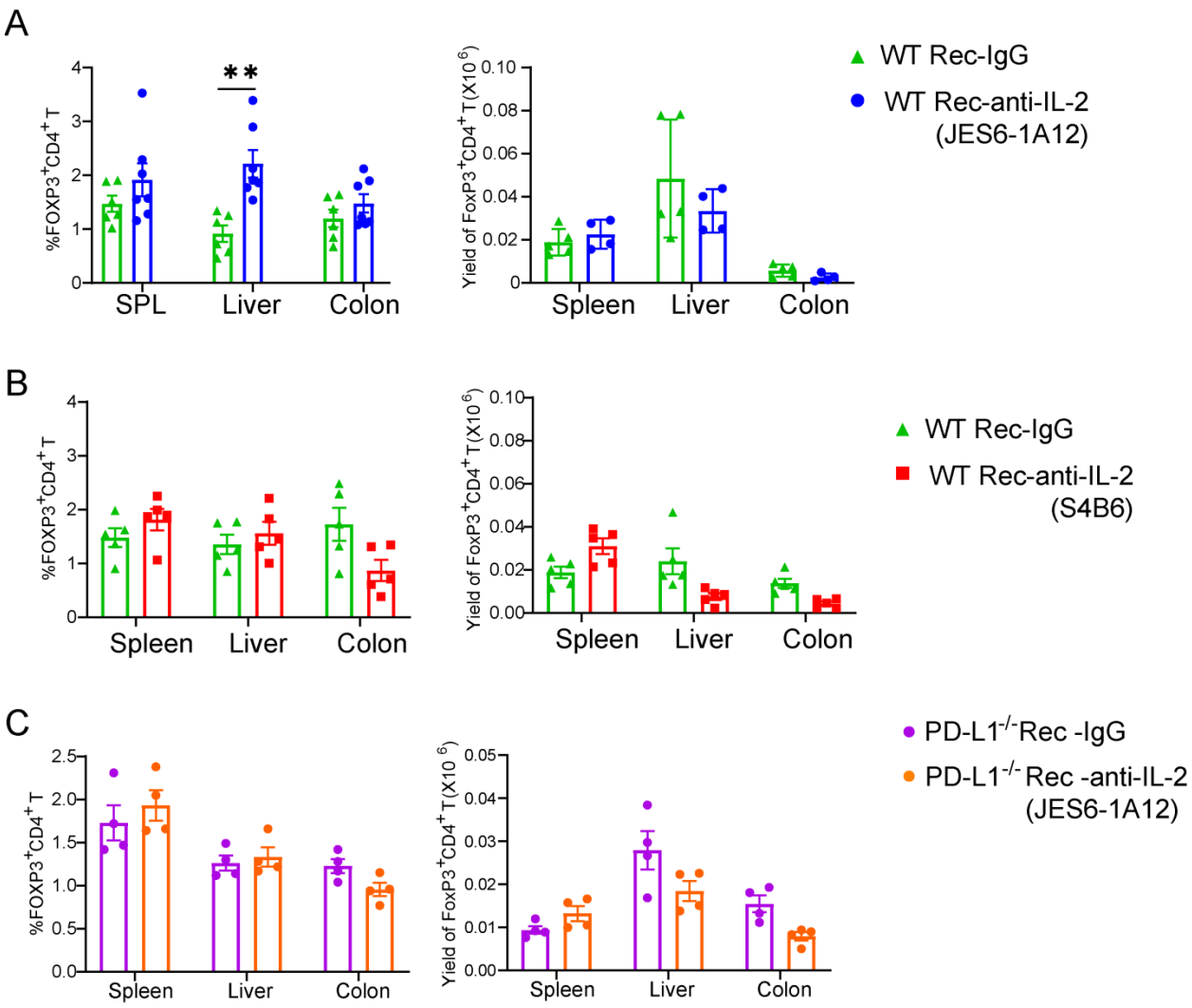

Figure S11: Tolerogenic (JES6-1A12) and non-tolerogenic (S4B6) anti-IL-2 mAb showed different impact on Foxp3$^+$ Treg cells. Lethally irradiated WT **(A-B)** or PD-L1$^{-/-}$ BALB/c **(C)** recipients were given splenocytes ($2.5x10^6$) and TCD-BM ($2.5x10^6$) from C57BL/6 donors. Recipients were given a total of 3 i.v. injections of rat-IgG or 500 µg/mouse anti-IL-2 mAb (JES6-1A12) **(A)** or anti-IL-2 mAb (S4B6) **(B)** at days 0, 2, and 4 after HCT. At day 6 after HCT, FoxP3$^+$ Treg cells were measured in the spleen, Liver and Colon. n=4-5 per group. Combined from two experiments. Data represent mean ± SE. *P* values were calculated by 2-way ANOVA (* $p < 0.05$, ** $p < 0.01$).

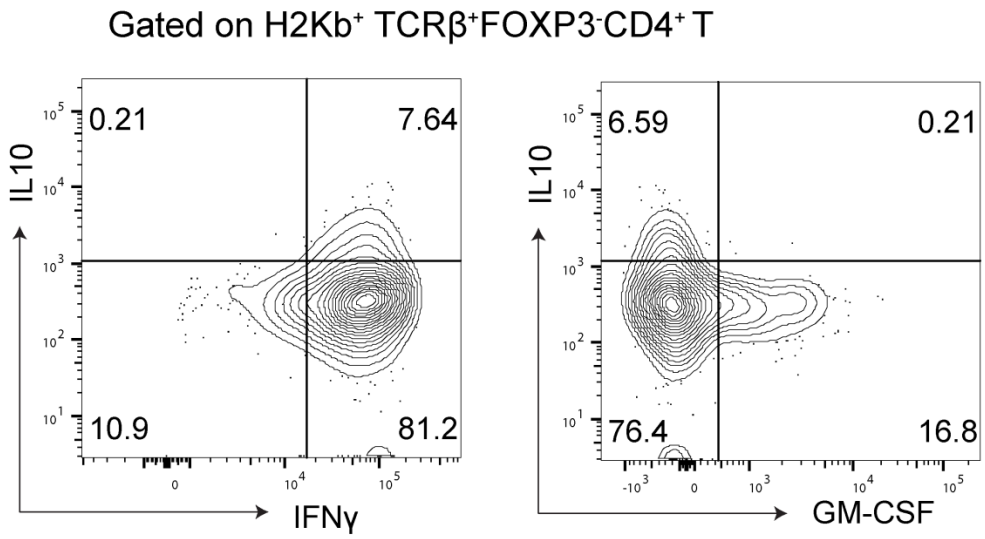

Figure S12: H2Kb^{+}TCRβ^{+} IL-10^{+}FoxP3^{-} CD4^{+} Tr1 cells are IFN-γ^{+} but GM-CSF^{-}. One representation is shown from four replicate experiments.

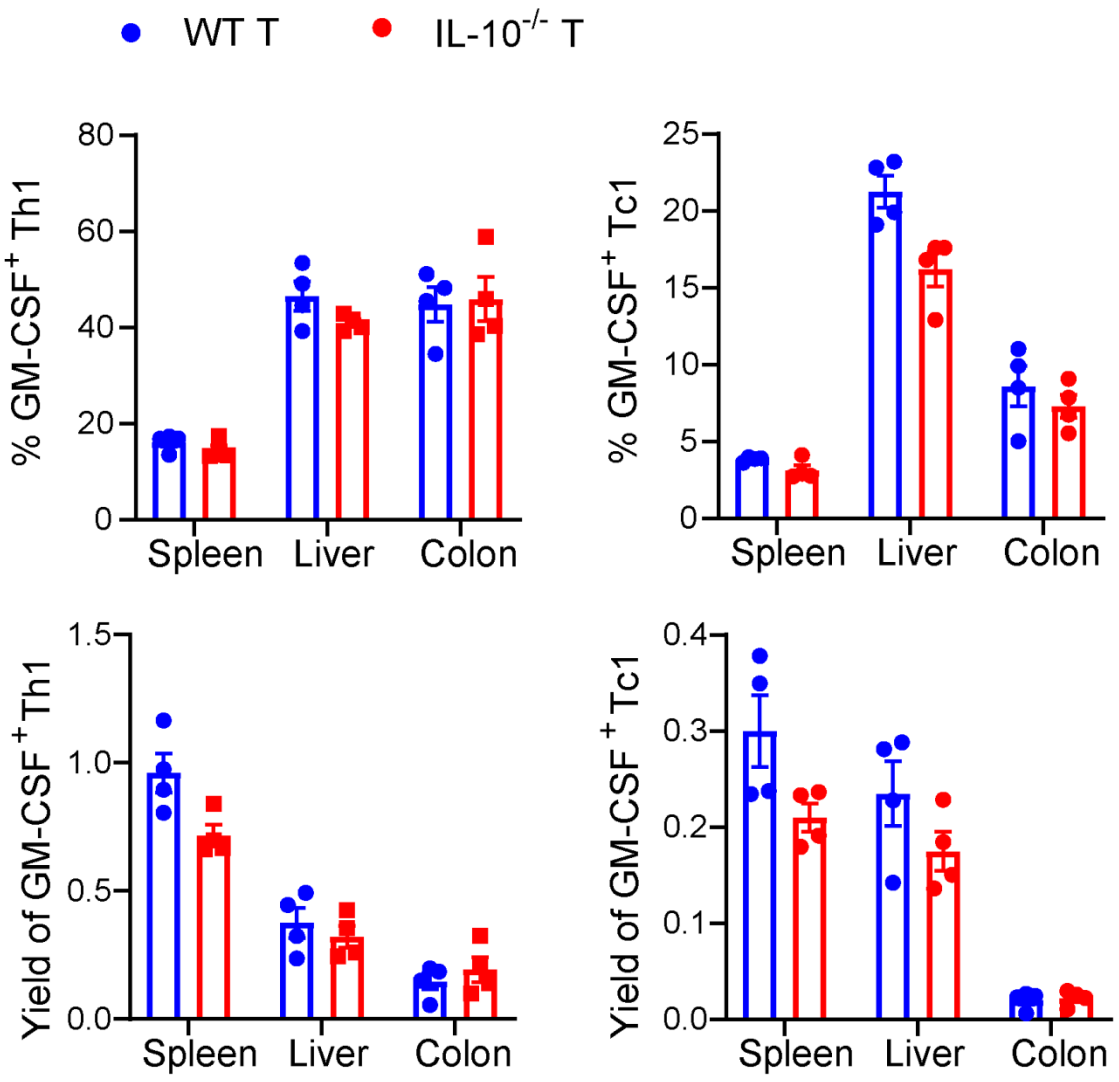

Figure S13: IL-10 deficiency in donor T cells did not affect the percentages or yields of GM-CSF^{+} Th/Tc1 cells. Lethally irradiated WT BALB/c recipients were given T cells (1.0 x10^{6}) from WT or IL-10$^{-/-}$ C57BL/6 donors with TCD-BM (2.5x10^{6}) from WT C57BL/6 donors. On day 6 after HCT, GM-CSF+ Th/Tc1 cells were enumerated in the spleen, liver and colon by flow cytometry. % and yield of GM-CSF+ Th/Tc1 are shown. n=4 per group. Data represent mean ± SE combined from two replicate experiments.

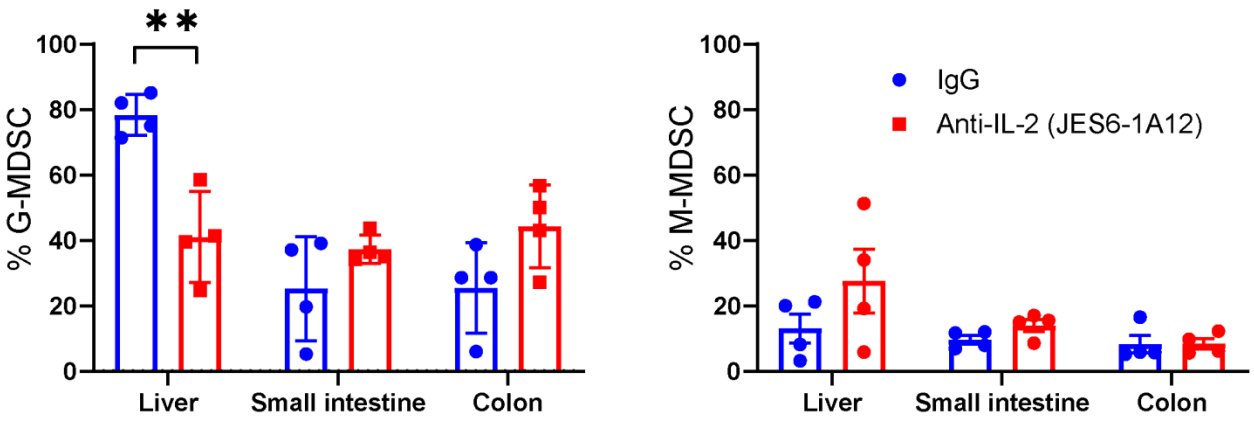

Figure S14: Tolerogenic anti-IL-2 mAb (JES6-1A12) decreases the percentage of donor G-MDSC in the liver. Lethally irradiated WT BALB/c recipients were given splenocytes ($2.5x10^6$) and TCD-BM ($2.5x10^6$) from C57BL/6 donors. Recipients were given a total of 4 i.v. injections of control IgG or anti-IL-2 mAb (JES6-1A12) (500 µg/mouse) at days 0, 2, 4 and 6. At day 12 after HCT, cells from the spleen (SPL), liver and colon were analyzed by flow cytometry. Percentages of G-MDSC and M-MDSC are shown. n=4, data represent mean ± SE combined from two replicate experiments. *P* values were calculated by 2-way ANOVA (** $p<0.01$).

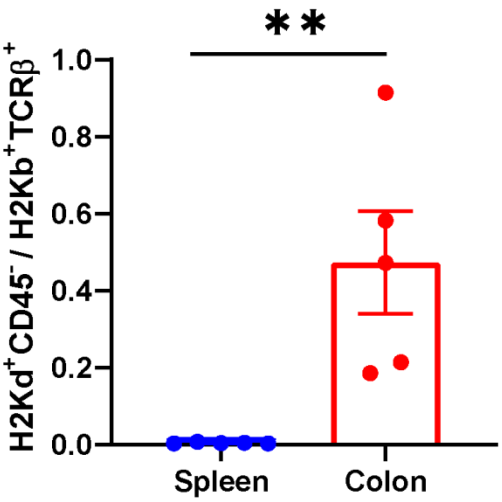

Figure S15: Comparison the percentage of host parenchymal cells versus donor T cells. Lethally irradiated WT BALB/c recipients were given splenocytes ($2.5x10^6$) and TCD-BM ($2.5x10^6$) from C57BL/6 donors. Recipients were given a total of 4 i.v. injections of anti-IL-2 mAb (JES6-1A12) (500 µg/mouse) at days 0, 2, 4 and 6 after HCT. Day 7 after HCT, spleen and colon were harvested for analysis. The percentage of $H2Kd^+CD45^-$ cells versus $H2Kb^+TCR\beta^+$ cells is shown. n=5 per group. Data represent mean ± SE combined from two replicate experiments. P values were calculated by unpaired two-tailed Student t tests (** $p<0.01$).

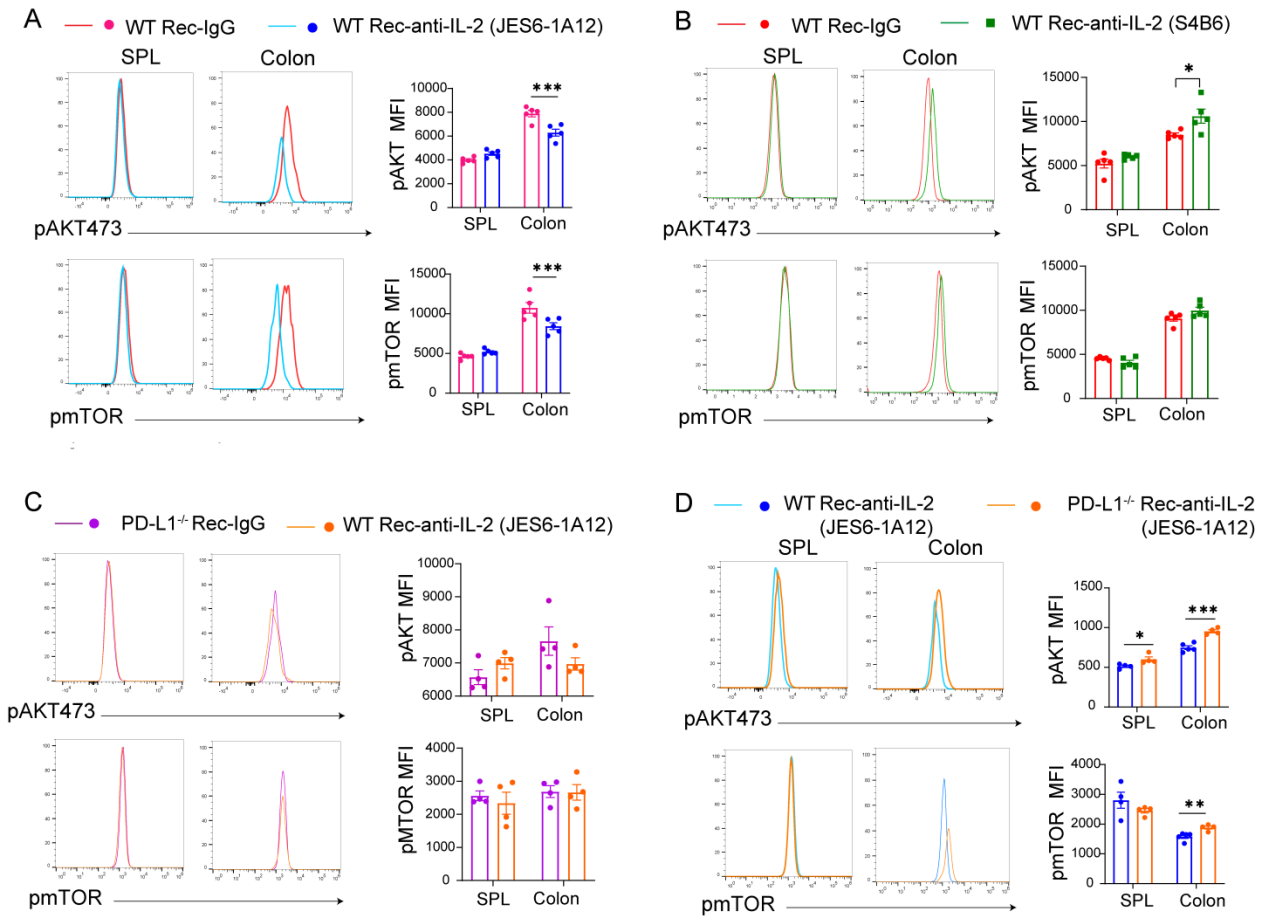

Figure S16: Tolerogenic anti-IL-2 (JES6-1A12) but not non-tolerogenic anti-IL-2 (S4B6) treatment inhibits activation of AKT-mTOR pathway in CD8$^+$ T cells in a host-tissue PD-L1-dependent manner. Lethally irradiated WT or PD-L1$^{-/-}$ BALB/c recipients were given splenocytes (2.5x10^6) and TCD-BM (2.5x10^6) from C57BL/6 donors. Recipients were given a total of 3 i.v. injections of rat-IgG or anti-IL-2 mAb (500 µg/mouse) at days 0, 2, and 4 after HCT. At day 6 after HCT, spleen and colon were harvested for analysis. **(A)** pAKT and pMTOR expression on of donor CD8$^+$ T cells in spleen and colon of WT recipients treated with anti-IL-2 (JES6-1A12) or control IgG; n=5 per group. **(B)** pAKT and pMTOR expression on of donor CD8$^+$ T cells in spleen and colon of WT recipients treated with anti-IL-2 (S4B6) or control IgG; n=5 per group. **(C)** pAKT and pMTOR expression on of donor CD8$^+$ T cells in spleen and colon of PD-L1$^{-/-}$ recipients treated with anti-IL-2 or control IgG; n=4 per group. **(D)** pAKT and pMTOR expression on of donor CD8$^+$ T cells in spleen and colon of WT or PD-L1$^{-/-}$ recipients treated with anti-IL-2; n=4-5 per group. Data represent mean ± SE combined from two replicate experiments. P values were calculated by 2-way ANOVA (*p<0.05, ** p<0.01, ***p<0.001).

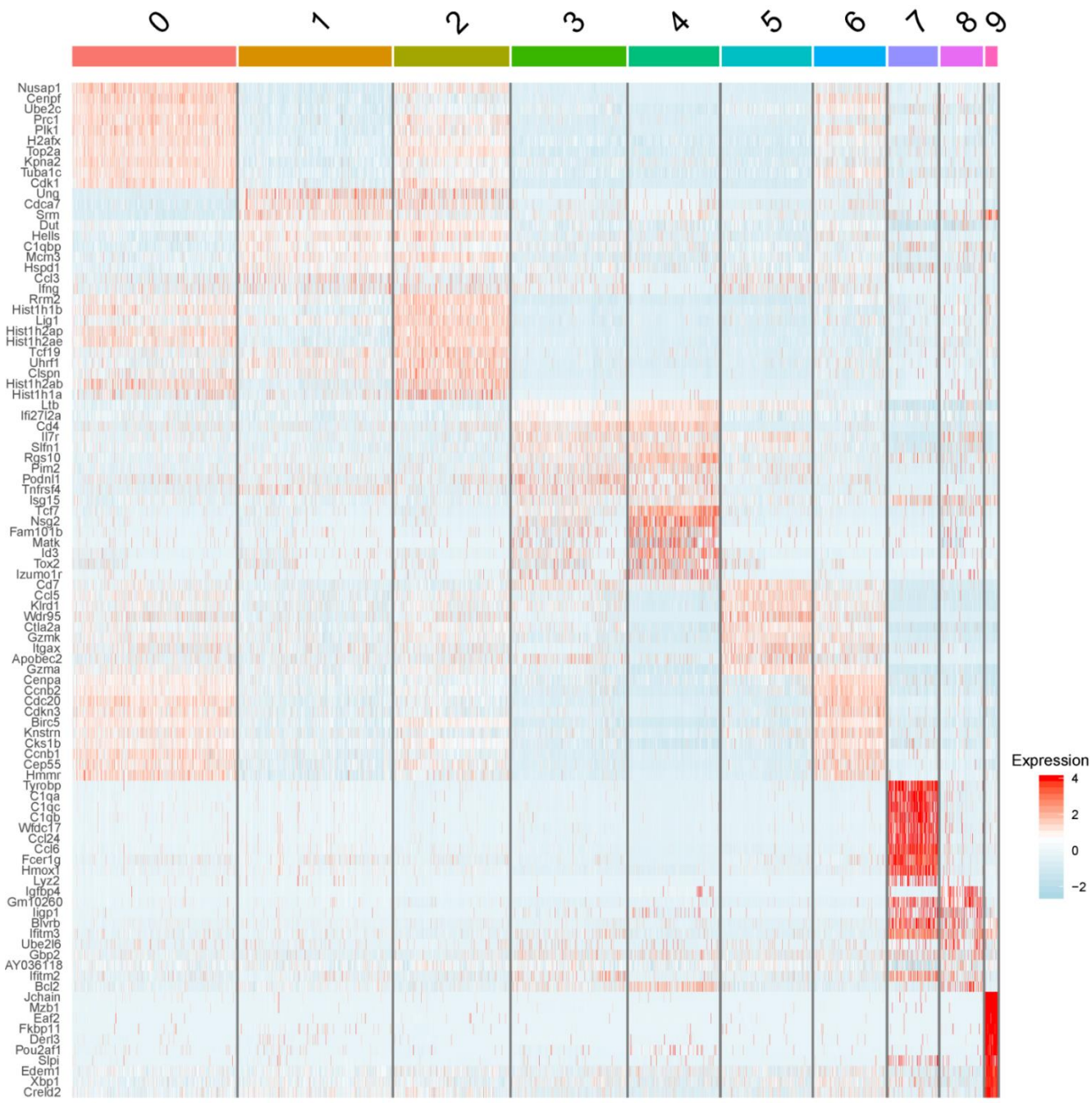

Figure S17: scRNA-Seq reveals distinct subpopulations and transcriptional signatures of CD8 T cells after treatment with tolerogenic anti-IL-2 (JES6-1A12) mAb or tacrolimus. Heatmap showing differentially expressed coding genes for the 9 clusters in Figure 6A.

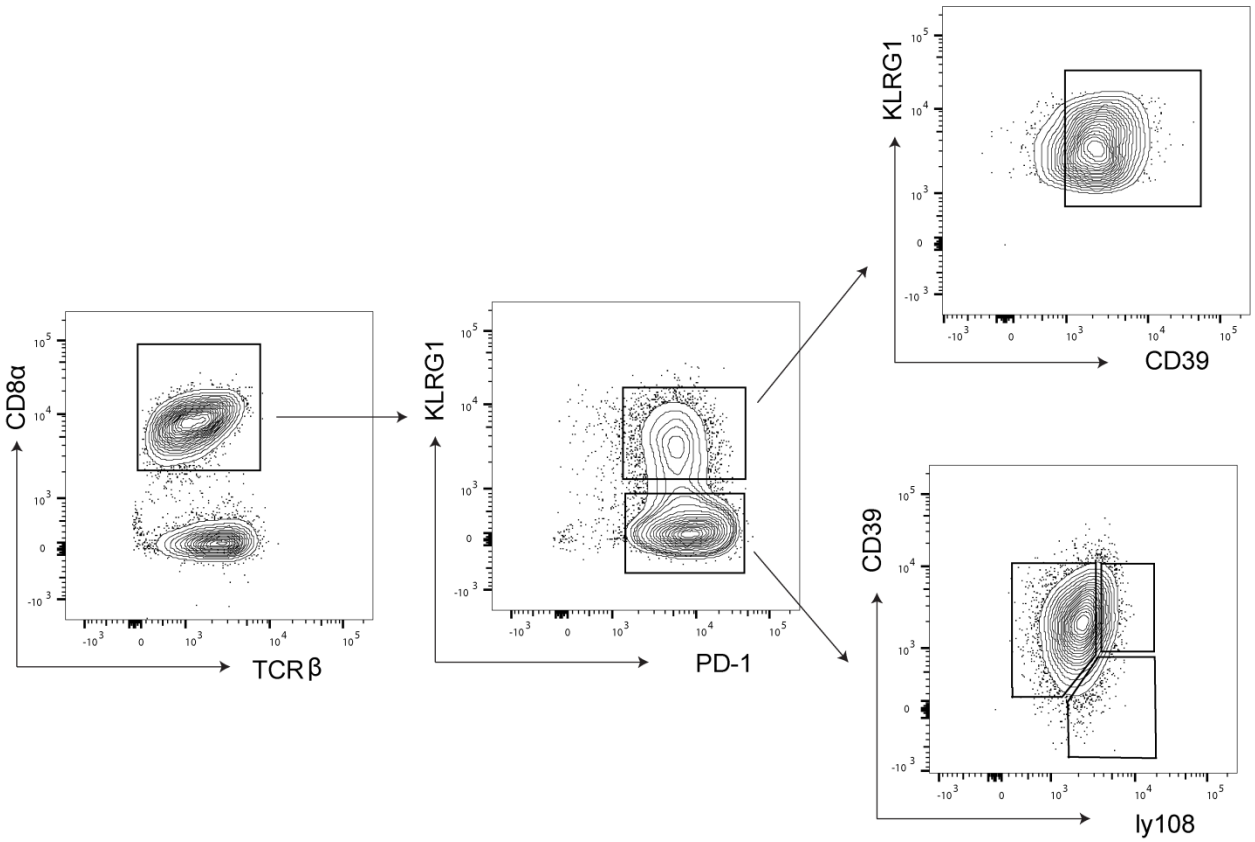

Figure S18: Gating strategy showing the Teff, Tex, Tex and Ter-Teff CD8^{+} T cell subsets in the spleen of the WT recipients treated with tacrolimus.

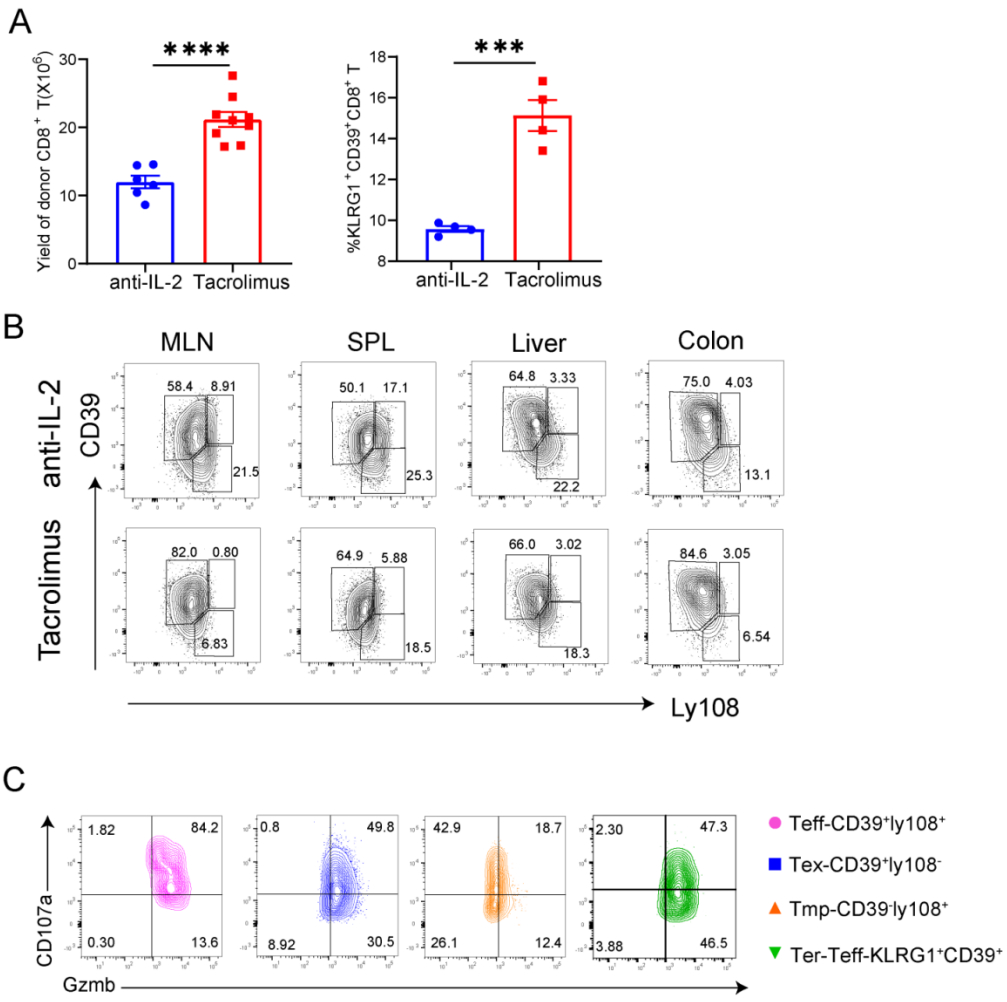

Figure S19: Tolerogenic anti-IL-2 (JES6-1A12) treatment is more effective than TAC treatment for preserving CD8$^+$ T memory progenitors and functional effectors that mediate GVL effect in lymphoid tissues. Lethally irradiated WT BALB/c recipients were given splenocytes (2.5x10^6) and TCD-BM (2.5x10^6) from C57BL/6 donors. Recipients were given a total of 4 i.v. injections of anti-IL-2 mAb (JES6-1A12) (500 µg/mouse) at days 0, 2, 4 and 6 after HCT or i.v. injections of tacrolimus (0.75 mg/Kg) at days 0-6 after HCT. On day 7 after HCT, mesenteric lymph node (MLN), spleen (SPL), liver and colon were harvested for analysis. **(A)** Yield of total donor CD8$^+$ T cells and % of KLRG1$^+$CD39$^+$ CD8$^+$ T cells in the spleen from anti-IL-2 or tacrolimus-treated recipients are showed, n=4-9, combined from 2 experiments. **(B)** Representative flow cytometry patterns showing the expression of Ly108 and CD39 expression in mesenteric lymph node, spleen, liver and colon from anti-IL-2 or tacrolimus-treated recipients. **(C)** Representative flow cytometry pattern showing CD107a, Granzyme B expression among Teff, Tex, Tmp and Ter-Teff cells in the spleen from anti-IL-2 or tacrolimus-treated recipients.