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Supplemental information

Targeting SLP76:ITK interaction separates GVHD from GVL in allo-HSCT

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Supplemental Information

Transparent Methods

Mice: SLP76 Y145FKI mice were a kind gift of Dr. Martha S. Jordan (University of Pennsylvania) (Jordan et al., 2008). ROSA26-pCAGGs-LSL-Luciferase, Thy1.1 (B6.PL-Thy1a/CyJ), CD45.1 (B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ) and BALB/c mice were purchased from Charles River or Jackson Laboratory. Eomes$^{flox/flox}$, B6.129S1, and CD4cre mice were purchased from Jackson Laboratory. Mice expressing Cre driven by the CMV promoter **(**CMV-Cre) were purchased from the Jackson Laboratory and crossed to ROSA26-pCAGGs-LSL-Luciferase mice (B6-luc). B6-luc mice were bred with SLP76 Y145FKI mice to create SLP76 Y145FKI luc mice. Mice aged 8-12 weeks were used, and all experiments were performed with age and sex-matched mice. Animal maintenance and experimentation were performed in accordance with the rules and guidelines set by the institutional animal care and use committees at SUNY Upstate Medical University.

Reagents, cell lines, flow cytometry: Most monoclonal antibodies for flow cytometric analysis were purchased either from eBiosciences (San Diego, CA) or Biolegend (San Diego, CA). For TCR mediated activation, we used anti-CD3 and anti-CD28. For flow cytometry analysis, we used mouse antibodies anti-CD3-FITC, anti-CD8-FITC, anti-CD4-PE anti-BrdU-APC, anti-IFN-γ-APC, anti-TNF-α-PE, anti-CD122-APC, anti-CD25-BV421, and anti-FoxP3-APC. Mice migration studies we used anti CD45.1 APC, anti-CD45.2-PE, H2KB PerCP. Anti-CD44 Pacific Blue, anti-CD122 APC and anti-Eomes PE. Human antibodies: anti-CD3-APC, anti-CD4-PE, anti-CD8-Pacific Blue, anti-TNF-α-Pe/Cy7, anti-IFN-γ-APC/Cy7. For serum ELISAs, we used Biolegend LEGENDplex kits, some of which were custom ordered to detect both mouse and human cytokines. For bioluminescent imaging, luciferin was purchased from Gold Bio (St Louis, MO). To exclude dead cells from analyses, we used LIVE/DEAD Fixable Aqua Dead Cell staining. All flow cytometry was performed on a BD LSR Fortessa flow cytometer (BD Biosciences). Flow data were analyzed with FlowJo software (Tree Star,

Ashland, OR). T cells were purified with anti-CD8 or anti-CD4 magnetic beads using MACS columns (Miltenyi Biotec, Auburn, CA). Cells were sorted with a FACS Aria cell sorter (BD Biosciences). FACS-sorted cells showed > 95% purity. For signaling analysis, antibodies against for both human and mouse ITK, PLCγ1, ERK, GAPDH, AKT, PI3K and β-Actin (total and/or phosphoproteins) were purchased from Cell Signaling Technology (Danvers, MA). Cells culturing reagents were purchased from Invitrogen (Grand Island, NY) and Sigma-Aldrich (St. Louis, MO), unless otherwise specified. Primary mouse B-cell acute lymphoblastic leukemia (B-ALL) blasts and primary cells (Cheng et al., 2016) were transduced with luciferase, and cultured as described previously (Edinger et al., 2003). B-ALL was chosen for this model because (1) these cells are syngeneic with BALB/c mice and allogeneic to C57Bl/6 mice, and (2) B-ALL was selected to be more related to human disease (Cheng et al., 2016).

GVHD and GVL studies: Recipient BALB/c mice (MHC haplotype d) as recipients were lethally irradiated with 800 cGy total in two split doses of 400cGy. Bone marrow cells were harvested from mouse legs, and total bone marrow cells were incubated with CD90.2 beads, using 100ul of beads per mouse according to manufacturer protocols. We also depleted NK cells and by DX5 beads, and $CD122^+$ cells with anti PE beads. Recipient mice were injected intravenously with $10X10^6$ T cell-depleted bone marrow ($_{TCD}$BM) cells, with or without donor T cells, either $1X10^6$ or $2X10^6$ FACS-sorted $CD8^+$, $CD4^+$ T cells, or $CD8^+$ and $CD4^+$ cells mixed at a 1:1 ratio from WT or SLP76 Y145FKI mice. For GVL experiments, primary B cell acute lymphoblastic leukemia (B-ALL, syngeneic to BALB/c and allogeneic to C57Bl/6) blasts were transduced with luciferase as described previously (Cheng et al., 2016), and $2X10^5$ luciferase-expressing B-ALL-*luc* cells were used per recipient mouse unless otherwise specified. All recipient animals were examined for tumor burden twice a week from the time of challenge with B-ALL *luc* injection until 70 days post-transplant, using bioluminescence imaging with the IVIS 50 and IVIS 200 imaging systems (Xenogen) as previously described (Contag and Bachmann, 2002). Each mouse was injected with 10μg/g body weight of luciferin and imaged for 1

minute. The bioluminescence data were analyzed and quantified with Living Image Software (Xenogen) and Igor Pro (Wave Metrics, Lake Oswego, OR). Recipient animals were evaluated for clinical score 2-3 times per week by a scoring system that sums changes in 6 clinical parameters: (1) weight loss, (2) posture, (3) activity, (4) fur texture, (5) diarrhea and (6) skin integrity (Cooke et al., 1996). Animals which lost ≥ 30% of their initial body weight were euthanized.

Cytokine production assays: Animals were lethally irradiated and transplanted with donor T cells as described above. On Day 7 post-transplantation, serum was isolated from recipient mice to examine cytokines in circulation. Serum was examined for IL-33, IL-1α, IFN-γ, TNF-α and IL-17A by multiplex cytokine assays (Biolegend LEGENDplex). For restimulation, splenocytes were processed to obtain single cells, and T cells were stimulated with anti-CD3 and anti-CD28 for 6 hours in the presence of brefeldin A (10μM). After 6 hours, stimulated cells were stained for surface markers and stained intracellularly for cytokines (IFN-γ and TNF-α). As a control, T cells from the same spleen were stimulated with PMA and ionomycin in the presence of brefeldin A.

Proliferation Assays: For detection of BrdU, transplanted mice (as described above) were given BrdU with an initial bolus of BrdU (2 mg per 200 μl intraperitoneally) and drinking water containing BrdU (1 mg/ml) for 2 days. BrDU incorporation was performed using a BrDU kit (Invitrogen) according to the manufacturer's instructions.

Cytotoxicity assays: For cytotoxicity assays, luciferase-expressing A20 and B-ALL cells (both allogenic to BALB/c) were seeded in 96-well flat bottom plates at a concentration of $3X10^5$ cells/ml. D-firefly luciferin potassium salt (75 μg/ml; Caliper Hopkinton, MA) was added to each well and bioluminescence was measured with the IVIS 50 Imaging System. Subsequently, ex vivo effector cells

(MACS-sorted or FACS-sorted $CD8^+$ T cells from bone marrow-transplanted mice) were added at 40:1, 20:1, and 10:1 effector-to-target (E:T) ratios and incubated at 37°C for 4 hours. Bioluminescence in relative luciferase units (RLU) was then measured for 1 minute. Cells treated with 1% Nonidet P-40 were used as a measure of maximal killing. Target cells incubated without effector cells were used to measure spontaneous death. Triplicate wells were averaged and percent lysis was calculated from the data using the following equation: % specific lysis = 100 × (spontaneous death RLU–test RLU)/(spontaneous death RLU– maximal killing RLU)(Karimi et al., 2014).

Tissue Imaging: Allo-HSCT was performed with $10X10^6$ WT T cell-depleted BM cells and $1X10^6$ FACS-sorted $CD8^+$ or $1X10^6$ FACS-sorted $CD4^+$ T cells (from B6-luc or SLP76Y145FKI *luc* mice) and bioluminescence imaging of tissues was performed as previously described (Beilhack et al., 2005). Briefly, 5 minutes after injection with luciferin (10 μg/g body weight), selected tissues were prepared and imaged for 5 minutes. Imaging data were analyzed and quantified with Living Image Software (Xenogen) and Igor Pro (Wave Metrics, Lake Oswego, OR).

Migration assays: Lethally irradiated BALB/c mice were injected intravenously with $10X10^6$ WT $_{TCD}$BM cells from B6.PL-*Thy1*^{a}/CyJ mice, along with FACS-sorted $CD8^+$ or $CD4^+$ T cells from B6.SJL (Ly5 CD45.1) and SLP76Y145FKI (C57B16 background CD45.2) mice, mixed at a 1:1 (WT: SLP76Y145FKI) ratio. Seven days post-transplantation, the mice were sacrificed and lymphocytes from the liver, small intestine, spleen, and skin-draining lymph nodes were isolated. Livers were perfused with PBS, dissociated, and filtered with a 70μm filter. The small intestines were washed in media, shaken in strip buffer at 37°C for 30 minutes to remove the epithelial cells, and then washed, before digesting with collagenase D (100 mg/ml) and DNase (1mg/ml) for 30 minutes in 37°C, and followed by filtering with a 70 μm filter. Lymphocytes from the liver and intestines were further

enriched using a 40% Percoll gradient. The cells were analyzed for $CD8^+$ T cells and $CD4^+$ T cells and presence of $H2K^b$, $CD45.1^+$ and $CD45.2^+$ (to identify the transferred T cell populations) by flow cytometry, but we excluded any bone marrow-derived T cells ($Thy1.1^+$).

Western blotting: For protein analysis, T cells were either nonstimulated, or stimulated with anti-CD3 and anti-CD28 for 24 hours overnight, **and were** lysed with freshly prepared lysis buffer (RIPA Buffer(Fisher Scientific cat#PI89900) + cOmplete Protease Inhibitor Cocktail (Sigma-Aldrich; cat# 11697498001)) and centrifuged for 10 minutes at 14000rpm at 4°C. Protein aliquots of 70µg of protein were loaded on a 12-18% denaturing polyacrylamide gel and transferred to nitrocellulose membranes for immunoblot analysis using antibodies specific to proteins of interest.

qPCR assay: Post-transplanted donor $CD8^+$ and $CD4^+$ T cells from C57Bl/6 mice (MHC haplotype b) were FACS sorted from recipient mice on $H2K^b$ markers, and total RNA was isolated from T cells using the RNeasy kit from Qiagen (Germantown, MD). cDNA was made from total RNA using a cDNA synthesis kit (Invitrogen). qPCR assay was performed with a premade customized plate (CXCr3, CX3r1, CXCr1, CCR12, s1pR1, CrTAM, CXCR6, CCR9, CXCR5, CXCr4) (Fisher Scientific, Hampton, NH).

SLP76pTYR Peptide: To generate a molecule that specifically inhibits the interaction between pY145 of SLP76 and the SH2 domain of ITK, we designed a peptide based on the amino acid sequence of SLP76 from N132 to A155, which contains a phosphorylated tyrosine residue at Y145 (**Fig. 5**). To ensure that our peptide easily enters cells and that its cellular localization can be monitored, we incorporated a C-terminal TAT peptide (GRKKRRQRRRPQ) and an N-terminal fluorescent FITC dye, respectively, and named it SLP76pTYR peptide (**Fig. 5**). Both SLP76pTYR peptide (FITC Dye - ^{132}NEEEEAPVEDDAD**pY**EPPPSNDEEA^{155}-TAT) and non-specific control peptide (FITC Dye-

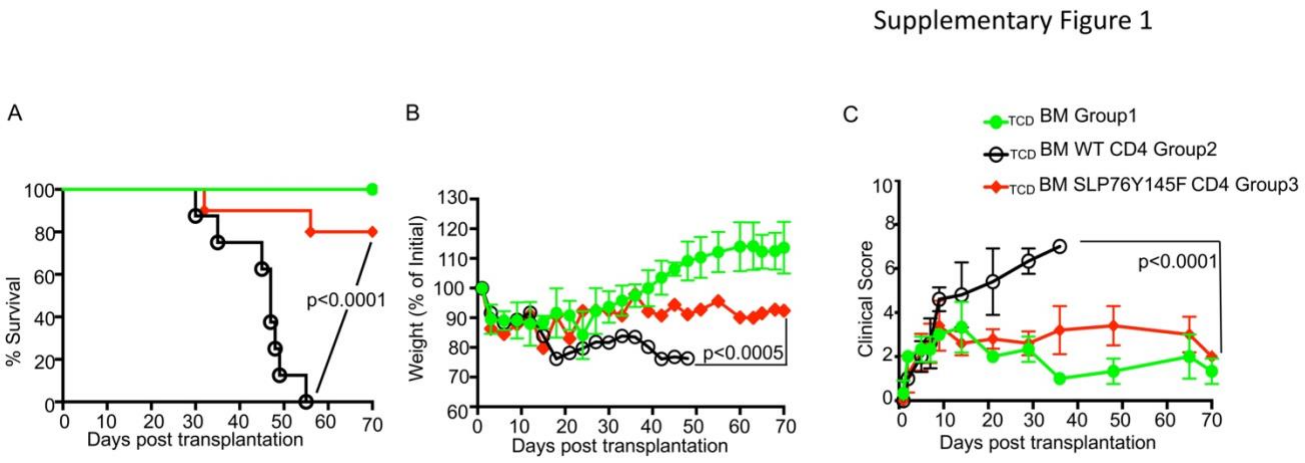
IIMTTTTNNKKSSRRVVVVAAAADD-TAT) were synthesized by Genscript Inc (Piscataway, NJ). These peptides were initially dissolved in 3% ammonia water to a final concentration of 10 μg/μL and then further diluted into PBS to a final concentration of 0.1 μg/μL. Fresh splenocytes were isolated from WT mice, and T cells were generated from splenocytes as previously described (Baker et al., 2001). Briefly, T cells were isolated from splenocytes using MACS beads (Miltenyi Biotec), then cultured in complete RPMI media ($3X10^6$ cells/mL) and plated on anti-CD3 (2.5 mg/ml; Biolegend; cat#100202) and anti-CD28 (2.5 mg/ml; Biolegend; cat#102116) antibody-coated tissue culture plates until otherwise specified. T cells were incubated with SLP76pTYR, control peptide or vehicle alone at different concentrations ranging from 100 ng/ml to 1 μg/ml in the presence of 4 μg/ml of protamine sulfate. Protamine sulfate significantly increased peptide delivery into primary cells. Within 60 minutes, we observed that peptides were inside the cells. Cells were cultured for 5 minutes prior to investigating signaling changes. Cells were examined for the presence of FITC by microscopy using a Leica DMi8 microscope equipped with an infinity total internal reflection fluorescence (TIRF) and DIC modules, a Lumencor SOLA SE II light box, a 150 mW 488 (GFP) laser and filter cube, a 100x/1.47 NA objective, and an Andor iXon Life 897 EMCCD camera. FITC expression was confirmed by flow cytometry as well. Cells were lysed and used in Western blots.

Human Samples: According to our IRB protocol (1140566-4), blood samples were obtained by vein puncture, and T cells were isolated from peripheral blood mononuclear cells (PBMC) of regular healthy donors as previously described(*56*). T cells were isolated from patient and healthy donor samples by Ficoll-Hypaque density centrifugation. The final product was resuspended at $3X10^6$ cells/ml in media and stimulated with OKT-3/anti-CD3 (2.5 mg/ml; Ortho Bio-Tech) and anti-human CD28 (2.5 mg/ml; Biolegend; cat#302902) presence of 4 μg/ml of protamine sulfate and 1 μg/ml SLP76pTYR or vehicle for five minutes**.** T cell lysates were used in western blot analysis.

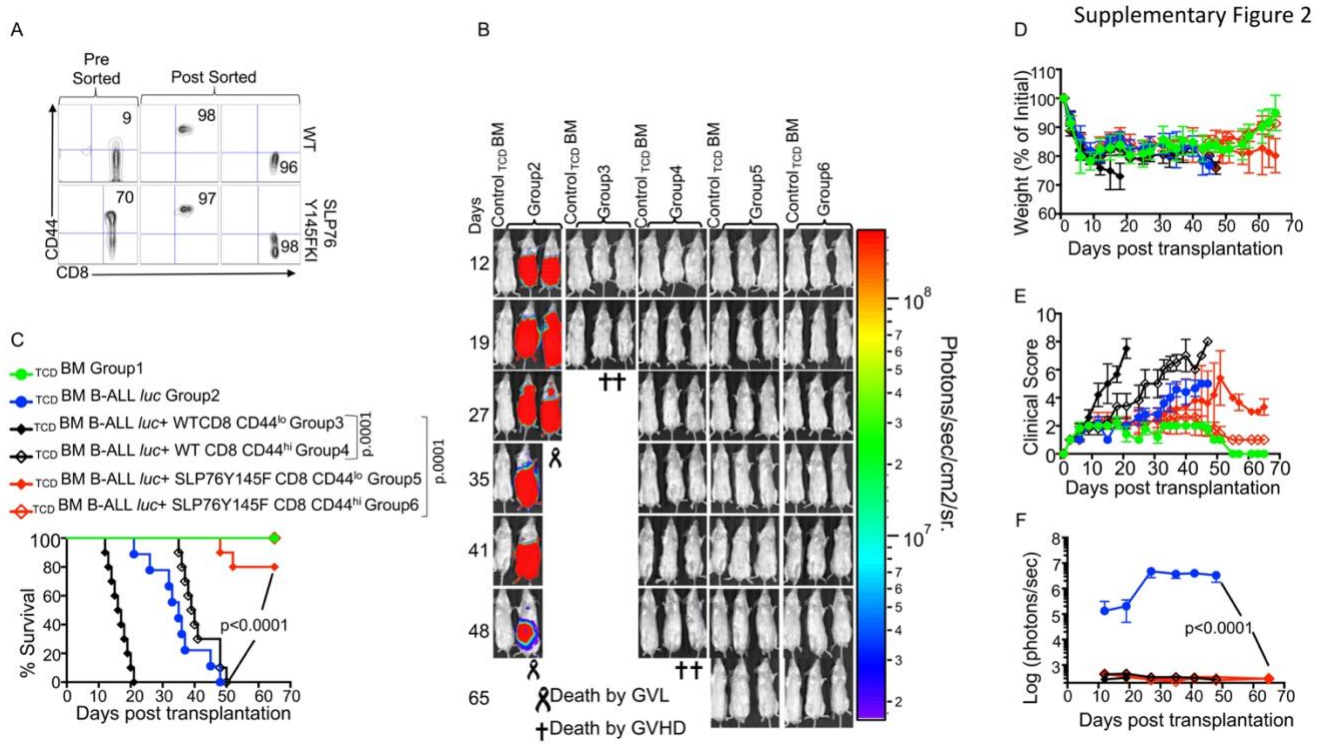
Transducing primary T cells with SLP76pTYR: We generated viruses that specifically express SLP76pTYR; the sequences encoding SLP76pTYR we cloned as a fusion protein with pCherry ordered through Integrated DNA Technology (IDT). The insert was cloned into a pQCX-I-X retroviral vector between MLU1 and Xho1 restriction sites, and the insert was confirmed by digestion and sequencing. To generate retroviral supernatants, Phoenix packaging cells were plated in 60 cm^2 dishes and transfected with 20 μg of the vector using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. The medium was changed after 8–12 hours, and viral supernatants were harvested after 24–36 hours. Concentrated viral supernatants were re-suspended in MDM media (Invitrogen) and used to transduce primary T cells in the presence of protamine sulfate (4μg/ml) to enhance transduction efficiency. T cells were transduced with viruses containing either SLP76pTYR-pCherry or empty plasmid for 24 hours and then injected into mice.

Statistics. All numerical data are reported as means with standard deviation. Data were analyzed for significance with GraphPad Prism. Differences were determined using one-way or two-way ANOVA and Tukey's multiple comparisons tests, or with a student's t-test when necessary. *P*-values less than or equal to 0.05 are considered significant. According to power analyses, all transplant experiments were done with N=5 mice per group and repeated at least twice. Mice were sex-matched and age-matched as closely as possible.

Supplementary Figure Legends

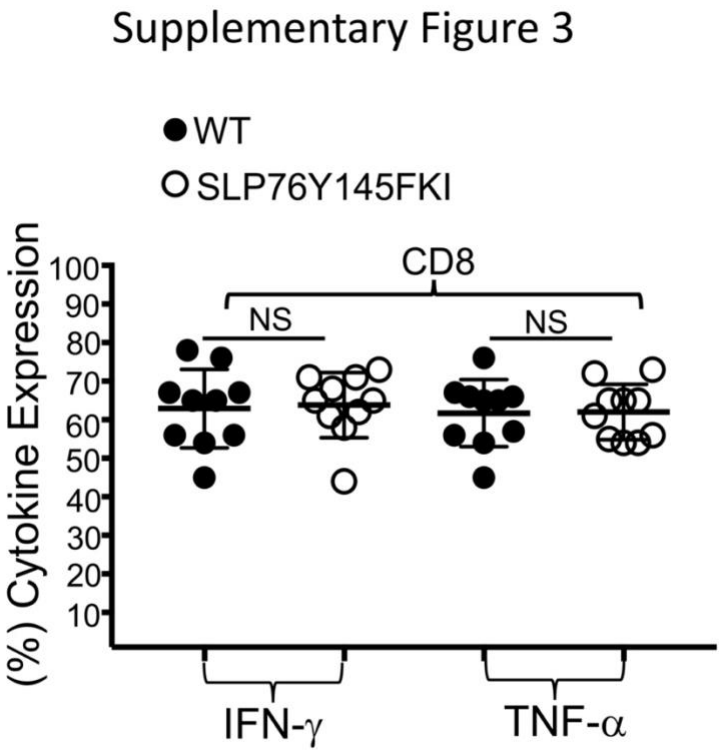


Supplementary Figure 1. SLP76 Y145FKI CD4$^+$ T cells exhibit attenuated induction of GVHD compared to WT T cells, Related to Figure 1. 10X10^6 T cell-depleted bone marrow cells and 1X10^6 purified WT or SLP76 Y154FKI CD4$^+$ T cells were transplanted into irradiated BALB/c mice. **(A)** The mice were monitored for survival, **(B)** changes in body weight, and **(C)** clinical score for 70 days post BMT. For weight changes and clinical score, one representative of 2 independent experiments is shown (n = 3 mice/group for BM alone; n = 5 experimental mice/group for all three groups). The *p* values are presented. Two-way ANOVA and Student's *t-test* were used for statistical analysis.



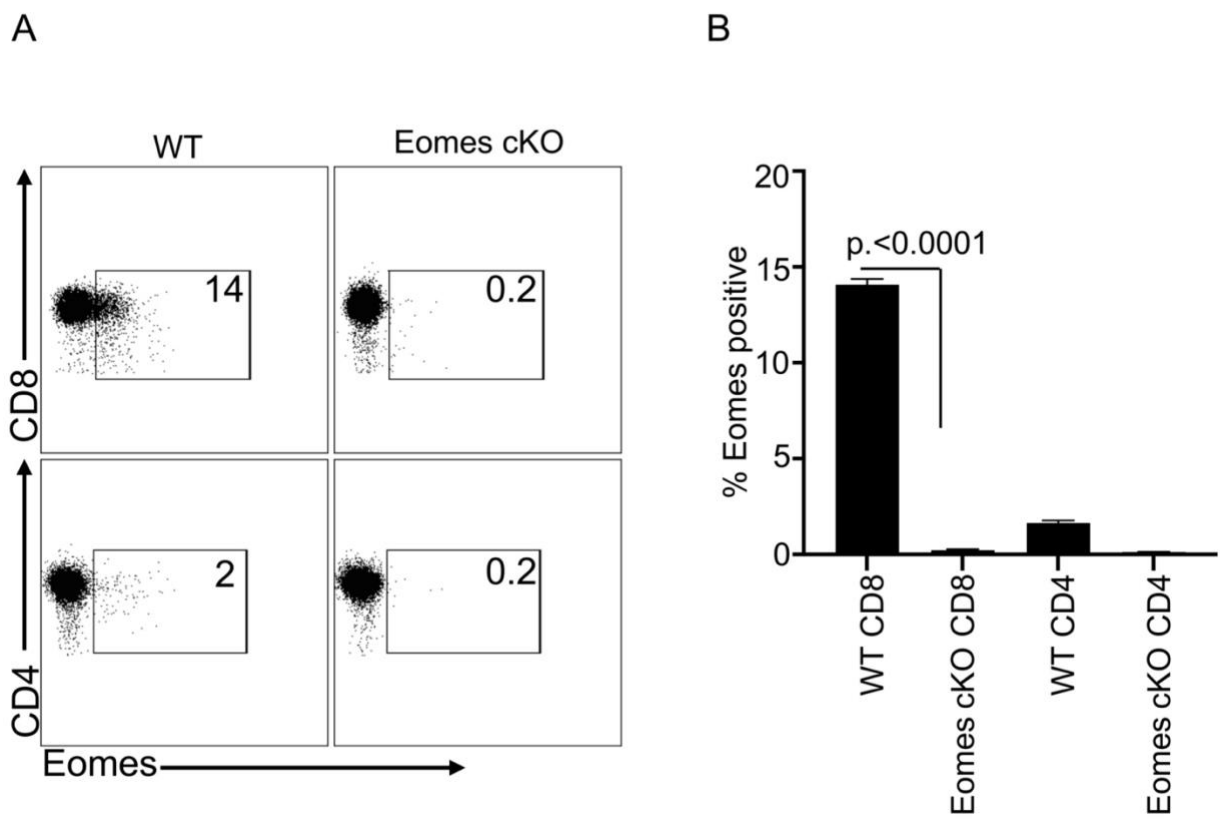
Supplementary Figure 2. The innate memory phenotype of CD8$^+$ T cells does not separate GVHD and GVL effects, Related to Figure 1. (A) Purified T cells from WT and SLP76Y145FKI mice were examined for expression of CD44 pre- and post-sort. (**B**) All recipient BALB/c mice were lethally irradiated and divided into six different groups. Group one was transplanted with 10X10^6 $_{TCD}$BM. Group 2 was transplanted with 10X10^6 $_{TCD}$BM and 1X10^5 B-ALL-*luc*. Group 3 was transplanted with 10X10^6 $_{TCD}$BM along with 1X10^6 purified WT CD8$^+$ CD44^{lo} T cells and 1X10^5 B-ALL-*luc*. Group 4 was transplanted with 10X10^6 $_{TCD}$BM along with 1X10^6 purified WT CD8$^+$ CD44^{hi} T cells, and 1X10^5 B-ALL-*luc*. Group 5 was transplanted 10X10^6 $_{TCD}$BM along with 1X10^6 purified SLP76Y145FKI CD8$^+$ CD44^{lo} T cells and 1X10^5 B-ALL-*luc*. Group 6 was transplanted with 10X10^6 $_{TCD}$BM and 1X10^6 purified SLP76Y145FKI CD8$^+$ CD44^{hi} T cells and 1X10^5 B-ALL-*luc*. These mice were monitored for tumor growth using the IVIS 50 system. **(C)** The mice were monitored for survival, **(D)** changes in body weight, and **(E)** animal clinical score for 65 days post BMT. For body weight changes and clinical score, one representative of 2 independent experiments is shown (n = 3

mice/group for BM alone; n = 5 experimental mice/group for all other groups. (**F**) Quantitated luciferase bioluminescence of luciferase-expressing B-ALL cells. Statistical analysis for survival and the clinical score was performed using the log-rank test and two-way ANOVA, respectively. *P* values are presented with each figure. *Note: Controls are naïve for tumor, but transplanted* with *10X10^6 T cell depleted bone marrow alone ($_{TCD}$BM) and used as a negative control for BLI.*



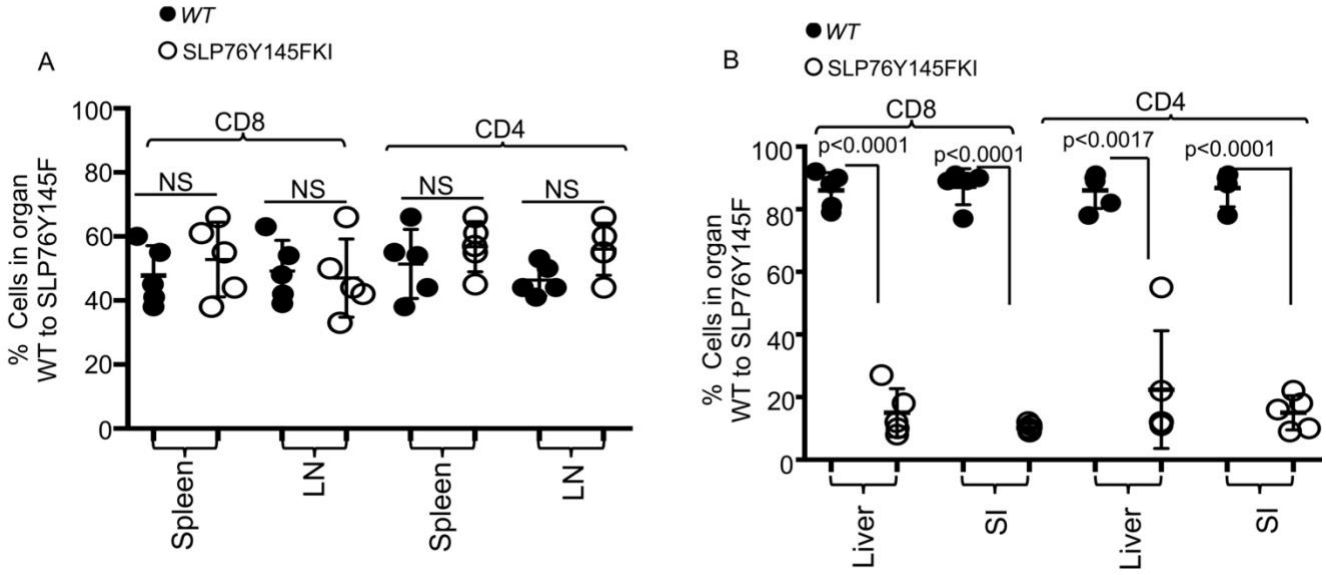
Supplementary Figure 3. SLP76Y145FKI T cells are capable of cytokine production, Related to Figure 2. Purified T cells from WT and SLP76Y145FKI C57Bl/6 mice were transplanted into irradiated BALB/c mice (MHC haplotype d) as recipients. On day 7, donor T cells were gated for expression of H-2K^b, CD45.2, and CD45.1, and analyzed for intracellular expression of IFN-γ and TNF-α following *ex vivo* stimulation with PMA/ionomycin. Data from several experiments were combined, and statistical analysis performed using two-way ANOVA and Student's *t-test*, with *p* values presented.

Supplementary Figure 4



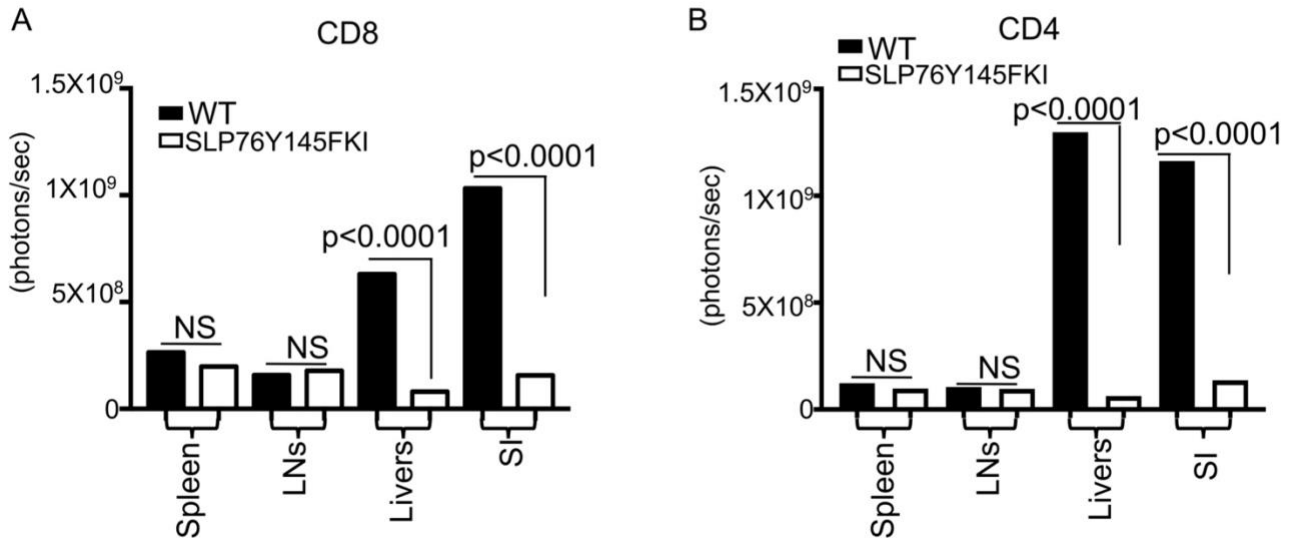
Supplementary Figure 4. Eomes deletion on CD8$^+$ and CD4$^+$ T cells, Related to Figure 3. (A) Purified donor CD8$^+$ and CD4$^+$ T cells from either WT or WT Eomes-deficient (Eomes cKO) mice on a C57Bl/6 background were examined for Eomes expression. **(B)** Quantitative analysis from flow cytometry data of several experiments. For statistical analysis we used two-way ANOVA and student's *t* test, *p* values are presented.

Supplementary Figure 5

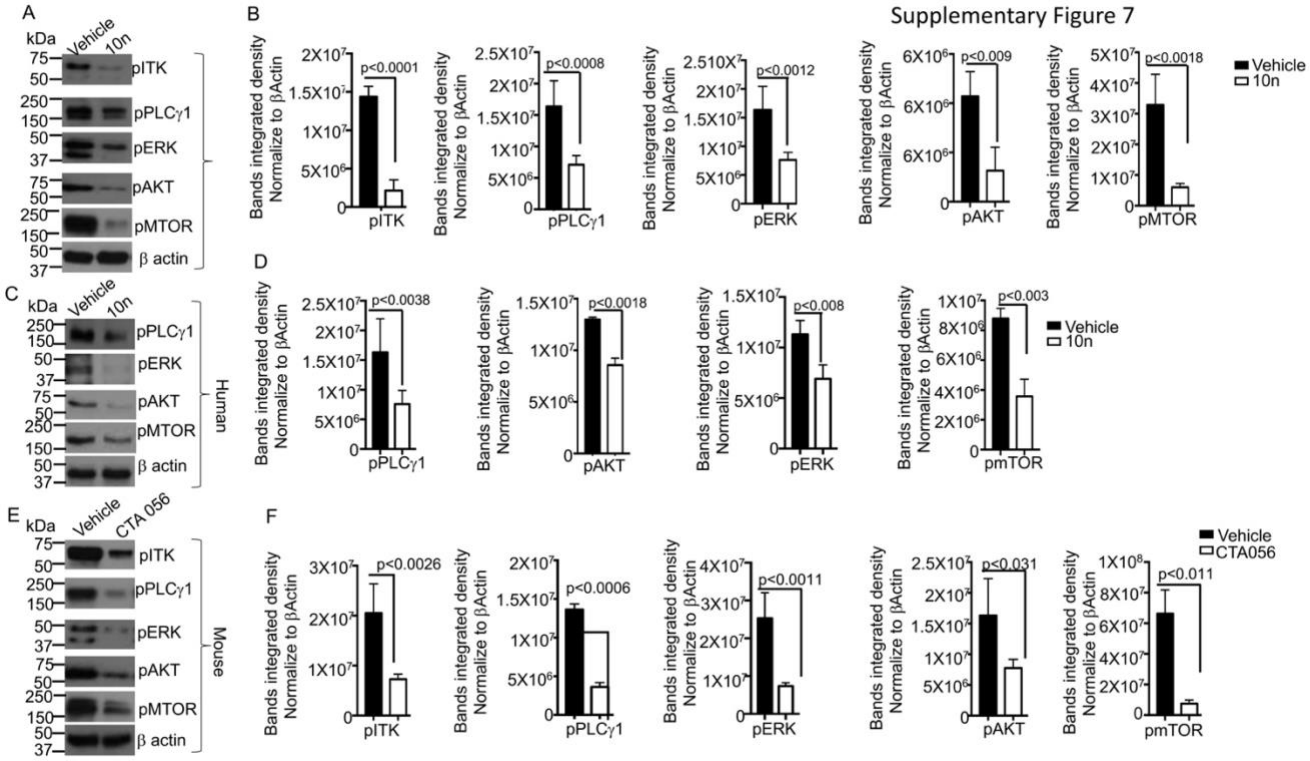


Supplementary Figure 5. Quantitative analysis of donor T cells in secondary lymphoid organs and GVHD target organs, Related to Figure 4. (A) Quantitative analysis from flow cytometry data. $CD8^+$ and $CD4^+$ T cells from WT and SLP76Y145FKI C57Bl/6 mice were transplanted into BALB/c mice (MHC haplotype d) as recipients. In several experiments, donor $CD4^+$ and $CD8^+$ T cells were analyzed for migration in the secondary lymphoid organs spleens and lymph nodes. **(B)** Quantitative analysis from flow cytometry data. In several experiments donor $CD4^+$ and $CD8^+$ T cells were analyzed for the presence of donor T cells in GVHD target organs, liver and small intestine. For statistical analysis we used two-way ANOVA and student's *t* test, *p* values are presented.

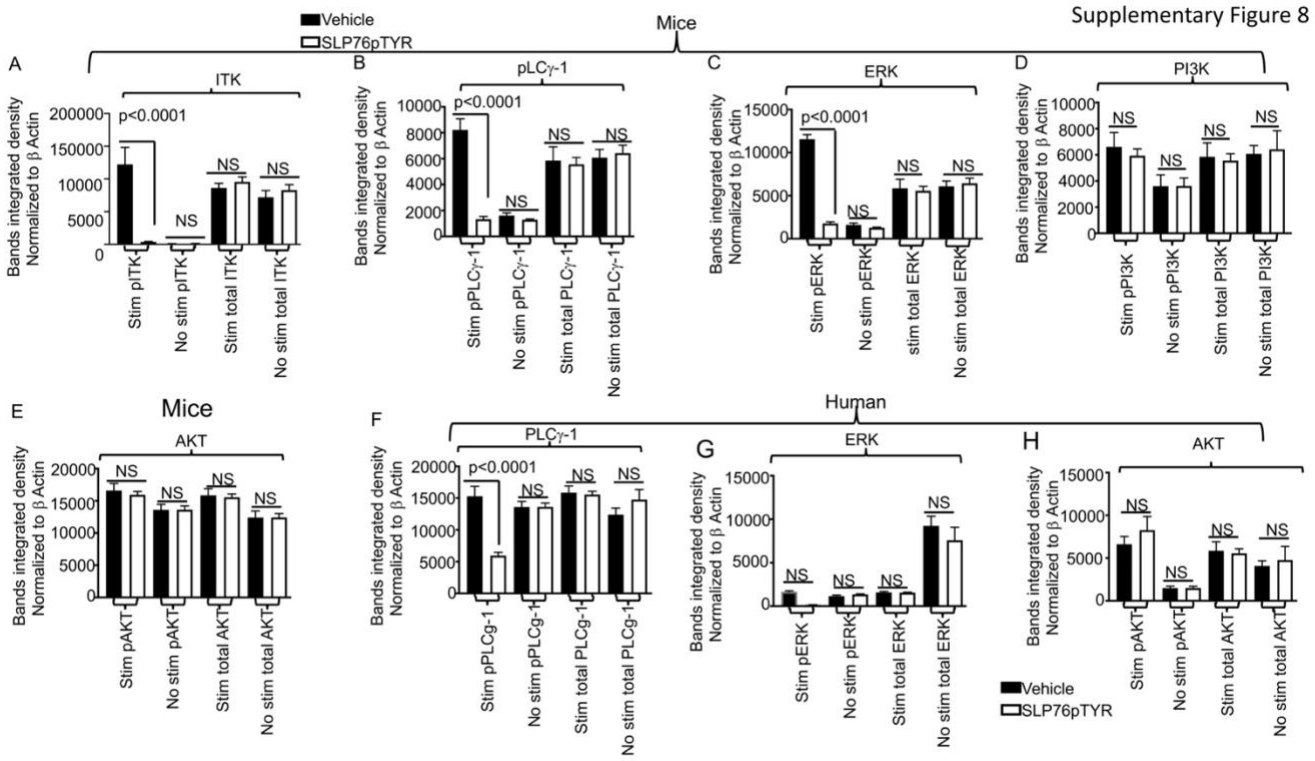
Supplementary Figure 6



Supplementary Figure 6. Quantitative analysis of tissue bioluminescence imaging (BLI), Related to Figure 4. For tissue imaging experiments, allo-HSCT was performed with $10X10^6$ WT $_{TCD}$BM cells and $1X10^6$ FACS-sorted $CD8^+$ T cells (A) or $CD4^+$ T cells (B) (from B6-luc or SLP76Y145FKI *luc* mice) and bioluminescence imaging of tissues was performed as previously described (Mammadli et al., 2020). Briefly, 5 minutes after injection with D-luciferin (10 μg/g body weight), selected tissues were prepared and imaged for 1 minute. Imaging data were analyzed and quantified with Living Image Software (Xenogen) and Igor Pro (Wave Metrics, Lake Oswego, OR)



Supplementary Figure 7. ITK inhibitors 10n and CTA056 are not specific for ITK, Related to Figure 5. (A) WT mouse T cells were cultured with either 10n or vehicle, then lysed post-incubation, and lysates were western blotted for pITK (size 50-75kDa) , pPLCγ1 (size ~155kDa), pERK (size ~42kDa), pAKT (size ~60kDa), and pmTOR(size 240kDa). (**B**) Western blots from three experiments were quantitated and normalized to actin. **(C)** T cells from primary human PBMCs were isolated and cultured with commercially available 10n or vehicle and western blotted for pPLCg1, pERK, pAKT, and pmTOR. **(D)** Western blots from three experiments were quantitated and normalized to actin. **(E)** Mouse T cells were cultured with either CTA056 or vehicle, the cells were lysed post-incubation, and lysates were western blotted for pITK, pPLCγ1, pAKT, pmTOR, and pERK. **(F)** Western blots from three experiments were quantitated and normalized to β-Actin. Two-way ANOVA and Student's *t-test* were used for statistical analysis.



Supplementary Figure 8. Quantitative analysis of SLP76:ITK signaling protein expression in cells treated with peptide SLP76pTYR, Related to Figure 6. (A-E) Quantitative analysis of cell lysates were obtained from mouse T cells stimulated with anti-CD3 and anti-CD28 in the presence of SLP76pTYR, or vehicle alone. Lysate from stimulated cells or non-stimulated cells were examined for phosphorylated ITK, total ITK, phosphorylated PLCγ1, total PLCγ1, phosphorylated ERK, total ERK, phosphorylated PI3K, total PI3K, phosphorylated AKT, and total AKT. n=3 and one representative experiment is shown. **(A-E)** Quantitative analysis of cell lysates from human T cells, non-stimulated or stimulated with OKT3 for 5min in the presence of SLP76pTYR or vehicle alone, were examined for phosphorylated pPLCγ1 and total PLCγ1 on stimulated and non-stimulated T cells. Cell lysate from stimulated and non-stimulated cells were examine for pERK and total ERK . Lysates from stimulated and non-stimulated were also examined for phosphorylation and total AKT. n=3 and one representative experiment is shown.

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