Supplemental methods

16S rRNA gene sequencing analysis

DNA was extracted from mouse fecal samples using a phenol-chloroform bead beating protocol and genomic 16S ribosomal RNA gene V4-V5 variable region was amplified and sequenced on the Illumina MiSeq platform as previously described.[1,2] OTUs were clustered at 97% identity and classified to the species level against the Greengenes database.[3]

Immunofluorescence

T cells (0.5×10^6) were spun into each well of 4-well Millicell glass chamber slides (EMD Millipore, Burlington, MA) by low-speed centrifugation (300 rpm) and fixed with 4% paraformaldehyde (Sigma-Aldrich) for 20 minutes. After washing with PBS, cells were permeabilized in 0.1% Triton™ X-100 and incubated in 1% bovine serum albumin overnight with polyclonal anti-GPR109A antibody (1:250; Sigma-Aldrich). Cells were washed and stained with Alexa Fluor 488 goat anti-rabbit IgG antibody (1:500; Invitrogen, Waltham, MA), and nuclei were stained with 5 µg/mL 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI; Invitrogen, Waltham, MA). Images were captured on a Zeiss Axio Imager widefield fluorescence microscope.

In vivo virus infection

WT (CD45.2) and KO (CD45.2) BM was mixed at a 1:1 ratio and $4x10^6$ cell were transferred into irradiated (1150cGy) WT (CD45.1) mice. After 2 months mixed bone marrow chimera mice were infected by i.p. injection of $7.5x10^3$ PFU of MCMV. Mice were bled on day 7, 14 and 35 post-infection for flow cytometry analysis of specific T cell populations.

qRT-PCR

Total RNA from single-cell suspensions or snap-frozen colon pieces were isolated using a Direct-zol RNA kit (Zymo Research), followed by RT-PCR using a quantitect RT Kit (Qiagen). PowerUP SYBR green polymerase (Applied Biosystems) along with the following primers were used to detect the following transcripts: 5'-TGAGGCAGAGACAGATGGACAGAC-3' and 5'-AGAAGTTGGGGAAAGATGGGC-3' (*Gpr109a*); 5'-GCTCAGAAGCAAGGTGACTA-3' and 5'-CGGCATAACAGTGGAGACAa-3' (*Gpr43);* 5'-CCCTTGGAGACACAGGACTC-3' and 5'-GAGGCTGCAGTTGTCTAATTCC-3' (*Nlrp3*); 5'-ACTTTGGCCGACTTCACTGT-3' and 5'-GGGTTCACTGGCACTTTGAT-3' (*Il-18*); 5'- AGGATCAAGGAAGCTCTCAGTC-3' and 5'-ATTTCCATTCCGGATATCCCA-3' (*Retnlb*); 5'-TCCCAGGCTTATGGCTCCTA-3' and 5'-GCAGGCCAGTTCTGCATCA-3' (*Reg3b*); 5'-TTCCTGTCCTCCATGATCAAAA-3' and 5'-

CATCCACCTCTGTTGGGTTCA-3' (*Reg3g*); 5'-GCCTTCTCCATGGTGGTGAA-3' and 5'-GCACAGTCAAGGCCGAGAAT-3' (*Gapdh*). All reactions were performed according to manufacturer's instructions. Relative expression of target mRNAs was calculated by the ΔCt method and values were normalized to mRNA expression levels in controls.

Flow cytometry antibodies

The following antibodies were obtained from BD Biosciences: CD45 (30-F11), CD3e (145-2C11), CD25 (PC61), CD62L (MEL-14), Caspase-3 (C92-605), Annexin V and \Akt(pS473) (M89-61). The following antibodies were obtained from eBioscience: H-2k^{b} (AF6-88.5), Foxp3 (FJK-16s), and LPAM-1 (DATK32). The following antibodies were purchased from BioLegend: CD4 (RM4-5), CD8 (53-6.7), T-bet (4B10), and CD44 (IM7). The following antibodies were obtained from Cell Signaling Technology: Bim (2933) and ppERK1/2 (E10) and Rorgt (Q31-378) was obtained from BD Horizon.

Cytokine analyses

Blood was collected into microcentrifuge tubes, allowed to clot, centrifuged, and the serum was collected. Colon tissues were homogenized, and the supernatant was used to determine cytokine levels. ProcartaPlex Multiplex Immunoassay was conducted per the manufacturer's instructions (Affymetrix). Results were acquired with a Luminex 200 instrument and analyzed with xPONENT software (Luminex Corporation).

In vitro CD4+ T cell differentiation

Naïve CD4^{+} T cells were isolated using Miltenyi MACS naïve CD4^{+} T cell isolation kit for mouse and incubated with cytokine mixtures to polarize T helper subsets. For Th1 polarization, media contained IL-12 (10ng/ml), IFNg (2ng/ml), anti-IL-4 (1ug/ml) and IL-2 (30IU/ml). For Th2 polarization, media contained IL-4 (20ng/ml), anti-IFNg (1ug/ml) and IL-2 (30IU/ml). For Th17 polarization, media contained TGFb (5ng/ml), IL-6 (20ng/ml), IL-23 (5ng/ml), anti-IFNg (1ug/ml), and anti-IL-4 (1ug/ml). For Treg polarization, media contained TGFb (5ng/ml), anti-IFNg (1ug/ml), and anti-IL-4 (1ug/ml). Polarization of subsets was confirmed by flow cytometry. Th1 cells were confirmed by T-bet expression using T-bet (4B10), Th2 cells were confirmed by Gata-3 (TWAJ) from eBiosciences, Th17 cells confirmed by Rorgt (Q31-378) and Tregs confirmed by expression of CD25 (PC61) and Foxp3 (FJK-16s).

Treg suppression assay

Splenic T cells were isolated from a WT CD45.1 mouse using a Miltenyi MACS purification of Pan T cells (>90% purity) and Tregs were isolated from WT and KO mice using the Miltenyi MACS $CD4^+CD25^+$ Treg purification kit for mouse and purity was confirmed by flow cytometry, staining for FoxP3 (>90% purity). Responder T cells (CD45.1) were co-cultured with different ratios of Tregs (indicated in the figure) and anti-CD3/CD28 Dynabeads (Invitrogen, #11452D) for 96 hours. Total number of divided cells was calculated as well as proliferation using the FlowJo Proliferation Index.

RNAseq analysis

Spleens from *GPR109a*$^{+/+}$ and *GPR109a*$^{-/-}$ littermate mice were isolated and mashed through a 100um filter. Cells were stained for CD45, CD3, CD4 and CD8 and resuspended in DAPI in FACS wash buffer. Antibodies were obtained from BD Biosciences: PercP-Cy5.5 anti-CD45 (30-F11, #550994), APC-Cy7 anti-CD3e (145-2C11, #557596), FITC anti-CD4 (RM4-5, #553047) and PE-TR anti-CD8 (5H10, #MCD0817) was obtained from Caltag. $CD45^+CD3^+CD4^+CD8^-$ T cells were sorted using a FACS Aria. RNA was isolated using TRIzol (Invitrogen) and total RNA was amplified using SMART-seq V4 Low Input RNA Kit (Clontech). Next, amplified cDNA was used to prepare Illumina HiSeq libraries with the Kapa DNA library preparation chemistry (Kapa Biosystems). Samples were barcoded and run on Hiseq 2500 1T, in a 50bp/50bp paired-end run using the TruSeq SBS Kit v3 (Illumina). For analysis, paired-end reads were trimmed for adaptors and removal of low-quality reads using Trimmomatic (v.0.36). Trimmed reads were mapped to the *Mus musculus* genome (mm10 assembly) using Bowtied2 (v2.2.9). Differential gene expression was assessed with DESeq2 (v1.14.1) and genes were considered differentially expressed if they showed an FDR adjusted p value <0.3 (to allow for more gene candidates), adjusted for multiple hypothesis correction as calculated by the DESeq2 software.

Short-chain fatty acid analysis

Short-chain fatty acids were extracted from mouse cecal contents and measured by GC-MS as previously described.[27]

Mixed lymphocyte reaction

BMDCs were used as stimulators. First BM cells from WT-BALB/c mice were cultured with recombinant GM-CSF (20ng/ml; Peptrotech) and IL-4 (5ng/ml; Peptrotech) for 6 days. On day 6, BMDCs were matured with 50ng/ml of LPS (Sigma) for 24 hours and used in subsequent mixed lymphocyte reaction. Splenic T cells from WT and GPR109$^{-/-}$ mice were isolated using Miltenyi

MACS purification of Pan T cells (routinely >90% purity). 1 x 10^5 T cells were mixed with 1 x 10^4 stimulated BMDCs and co-cultured in a 96-well U-bottom plate for 96 hours, then T cells were analyzed by flow cytometry, using a FACS Symphony A5 (BD Biosciences)

In vitro CTL killing assay

Splenocytes isolated from BALB/c mice were activated with LPS (50ng/mL) for 24h and irradiated (2000cGy) prior to co-culture with sorted T cells derived from either WT or *Gpr109a*$^{-/-}$ mice using a Miltenyi MACS Pan T Cell Isolation Kit II for mouse. Cells were kept in culture for 5 days with the addition of hIL-2 (100U/mL). On day 3, $CD8^+$ T cells were then sorted with the Miltenyi MACS $CD8a^+$ T Cell Isolation Kit for mouse. Cell purity was analyzed by flow cytometry and H-$2kb^+$ $CD8^+$ T cell numbers were corrected accordingly for the cytotoxicity assay against target A20-luciferase cells. Cell killing was measured according to luciferase activity detected after 4h via bioluminescence using a Spark microplate reader (TECAN).

Metabolic flux analysis

T cells were activated *in vitro* and 4 x 10^5 cells/well were adhered to Seahorse 96-well plates pre-coated with Cell Tak (Corning) in serum-free XF Base Media (Agilent) containing 10mM glucose, 1mM pyruvate and 2mM glutamine. The oxygen consumption rate (OCR) was measured on an XF-96p extracellular flux analyzer (Agilent) under basal conditions, after which the following compounds were added sequentially: 1μM oligomycin, 1.5μM fluoro-carbonyl cyanide phenylhydrazone (FCCP), and 100nM rotenone plus 1μM antimycin A.

Supplemental figures:

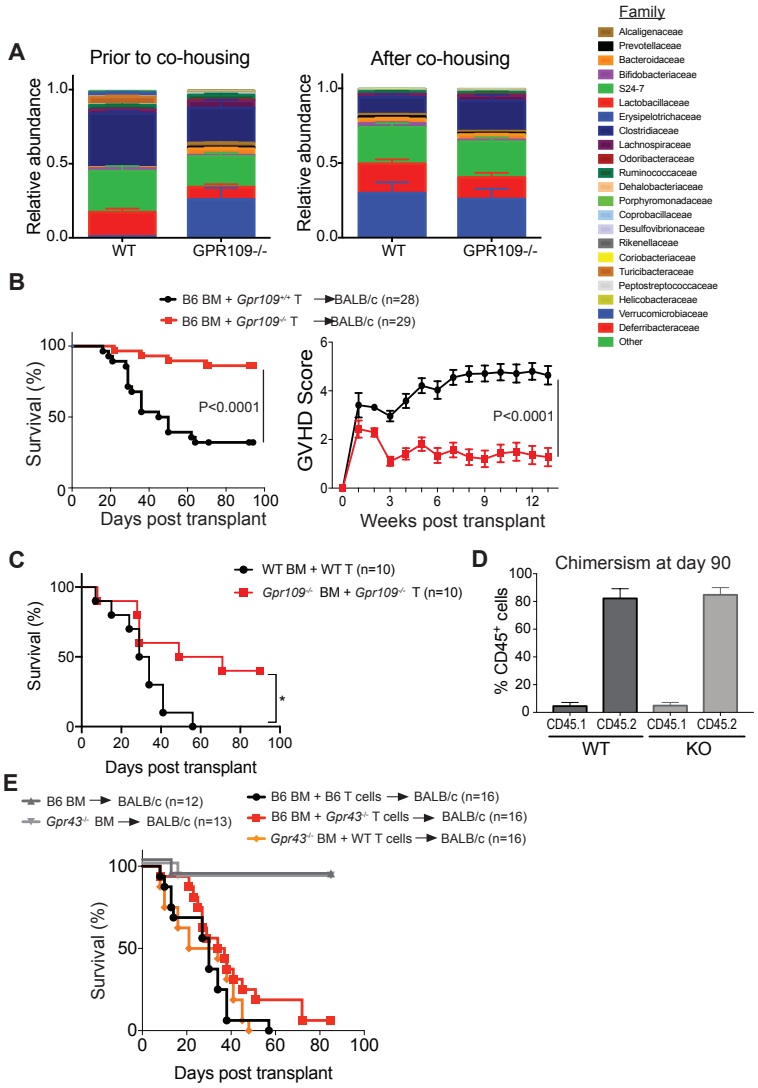

Figure S1: GPR109A$^{-/-}$ T cells cause less GVHD than WT T cells. (**A**) WT and *Gpr109a*$^{-/-}$ mice were co-housed for 14 days to equalize the flora and adjust for any minor differences between the genotypes. 16S rRNA sequencing revealed similar composition of microbiotas after co-housing. (**B**) Survival and clinical GVHD scores of lethally irradiated BALB/c mice transplanted with 5x10^6 T cell-depleted (TCD) BM cells and 1x10^6 WT or *Gpr109a*$^{-/-}$ T cells from littermate donors. (**C**) Survival of lethally irradiated BALB/c mice transplanted with 5x10^6 TCD WT or *Gpr109a*$^{-/-}$ BM cells and 1x10^6 WT or K *Gpr109a*$^{-/-}$ T cells from littermate donors. (**D**) Day 60 chimerism of lethally irradiated B6 CD45.1 mice transplanted with 5x10^6 WT CD45.2, or KO CD45.2 BM cells. (**E**) Survival of lethally irradiated BALB/c mice transplanted with 5x10^6 TCD BM cells and 1x10^6 WT T cells (B6 BM + B6 T cells), WT TCD BM with *Gpr43*$^{-/-}$ T cells (B6 BM + *Gpr43*$^{-/-}$ T cells), or *Gpr43*$^{-/-}$ TCD BM with WT T cells (*Gpr43*$^{-/-}$ BM + B6 T). Controls were transplanted with TCD BM only (B6 BM) or *Gpr43*$^{-/-}$ TCD BM only (*Gpr43*$^{-/-}$ BM) in a major (B6 into BALB/c) MHC-mismatched model of GVHD. Comparisons of groups for survival curves in (B), (C) and (E) were performed by Mantel-Cox log rank test for survival. For GVHD scores in (B), data represent the mean ± standard error, comparison of groups performed by two-way ANOVA. All results from two to three independent experiments.

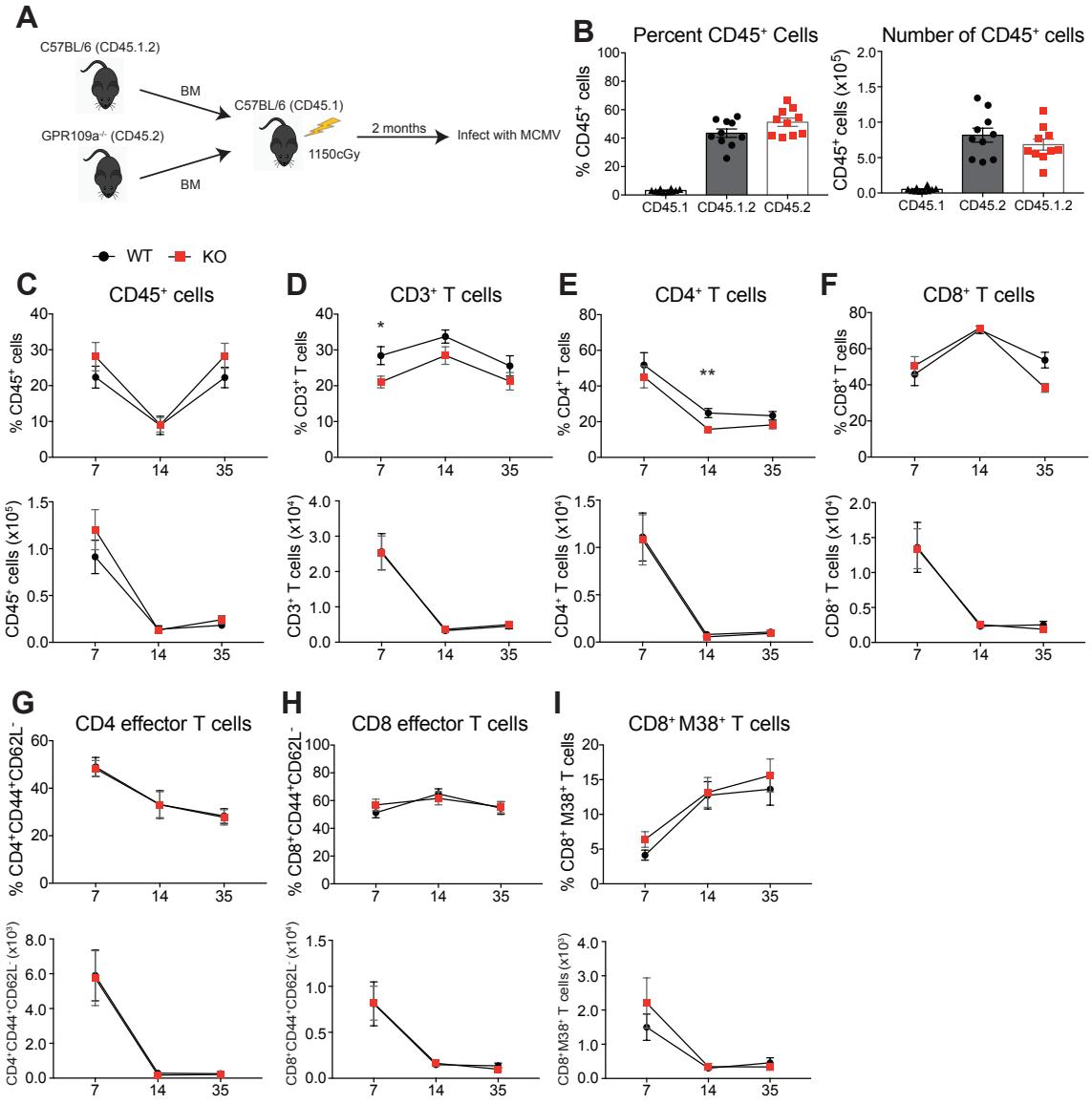

Figure S2: GPR109A$^{-/-}$ T cells expand to the same level as WT in a mouse viral infection. (**A**) schematic of mixed bone marrow chimera. CD45.1 mice were lethally irradiated at 1150cGy and transplanted with 2 x 10^6 T cell-depleted BM cells from WT (CD45.1.2) and *Gpr109a*$^{-/-}$ (CD45.2) mice and infected with MCMV two months later. (**B**) Percentage of host (CD45.1), WT (CD45.1.2) and KO (CD45.2) CD45$^+$ cells in peripheral blood and numbers of CD45$^+$ cells in 100ul of blood. (**C-F**) WT vs. KO percent (above) and number (below), in 100ul of blood, of CD45$^+$ cells (C), CD3$^+$ (D), CD4$^+$ (E), and CD8$^+$ T cells (F). (**G-H**) Percent (above) and number (below), in 100ul of blood, of effector memory CD4$^+$ (G) and CD8$^+$ T cells (H). (**I**) Percent of CD8$^+$ M38-tetramer MCMV specific T cells. All comparisons in (B) to (I) were performed by two-tailed unpaired Mann-Whitney Test. Values are means ± standard error. *P < 0.05, **P < 0.01, n = 10 mice per group. Results from two independent experiments.

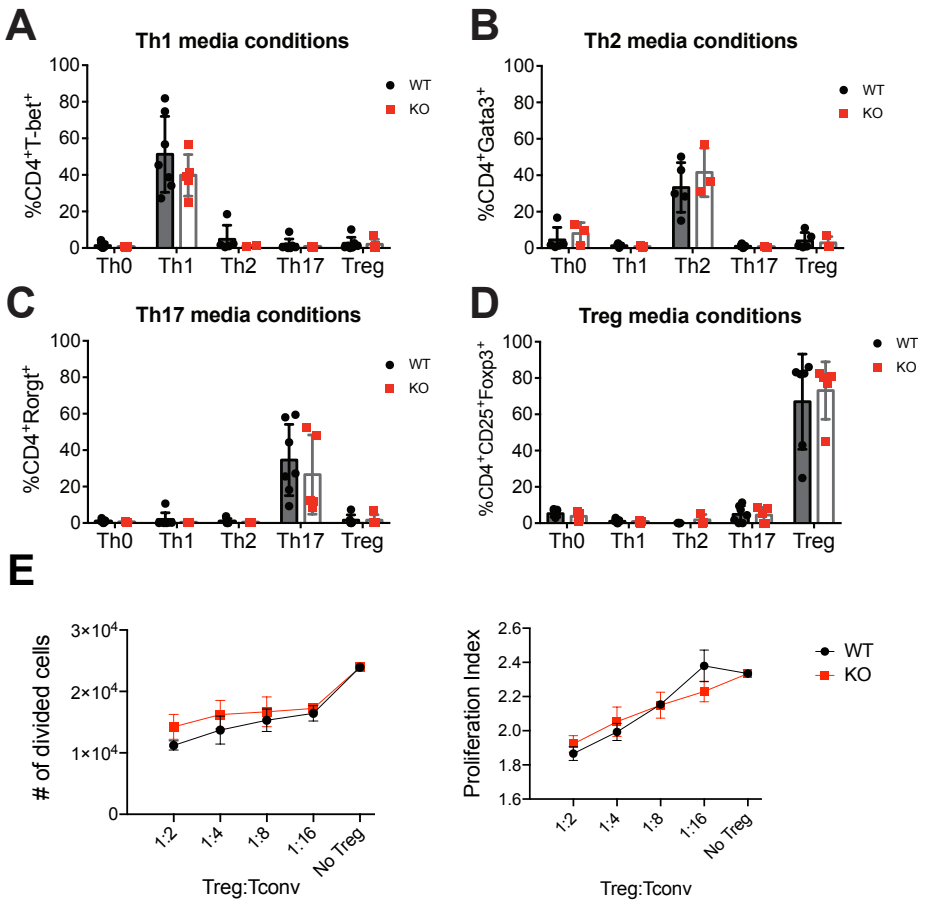

Figure S3: No difference in polarization of WT and GPR109A$^{-/-}$ T cells. (**A-D**) Naïve CD4$^+$ T cells were isolated from *Gpr109a*$^{+/+}$ and *Gpr109a*$^{-/-}$ littermate mice and incubated in different media conditions to polarize to T helper subsets, Th1, Th2, Th17, and Tregs. Polarization was determined by measuring frequency of CD4$^+$T-bet$^+$ for Th1 (A), CD4$^+$Gata3$^+$ for Th2 (B), CD4$^+$Rorgt$^+$ for Th17 (C) and CD4$^+$CD25$^+$Foxp3$^+$ for Tregs (D). (**E**) Total number of divided (left) and proliferated (right) responder T cells (CD45.1$^+$) after 96 hours of incubation with different ratios of Tregs. Ratio is indicated as Treg:Tconv (conventional T cells). All comparisons in (A) to (D) were performed by two-tailed unpaired Mann-Whitney Test. Values are means ± standard error. Results from two independent experiments.

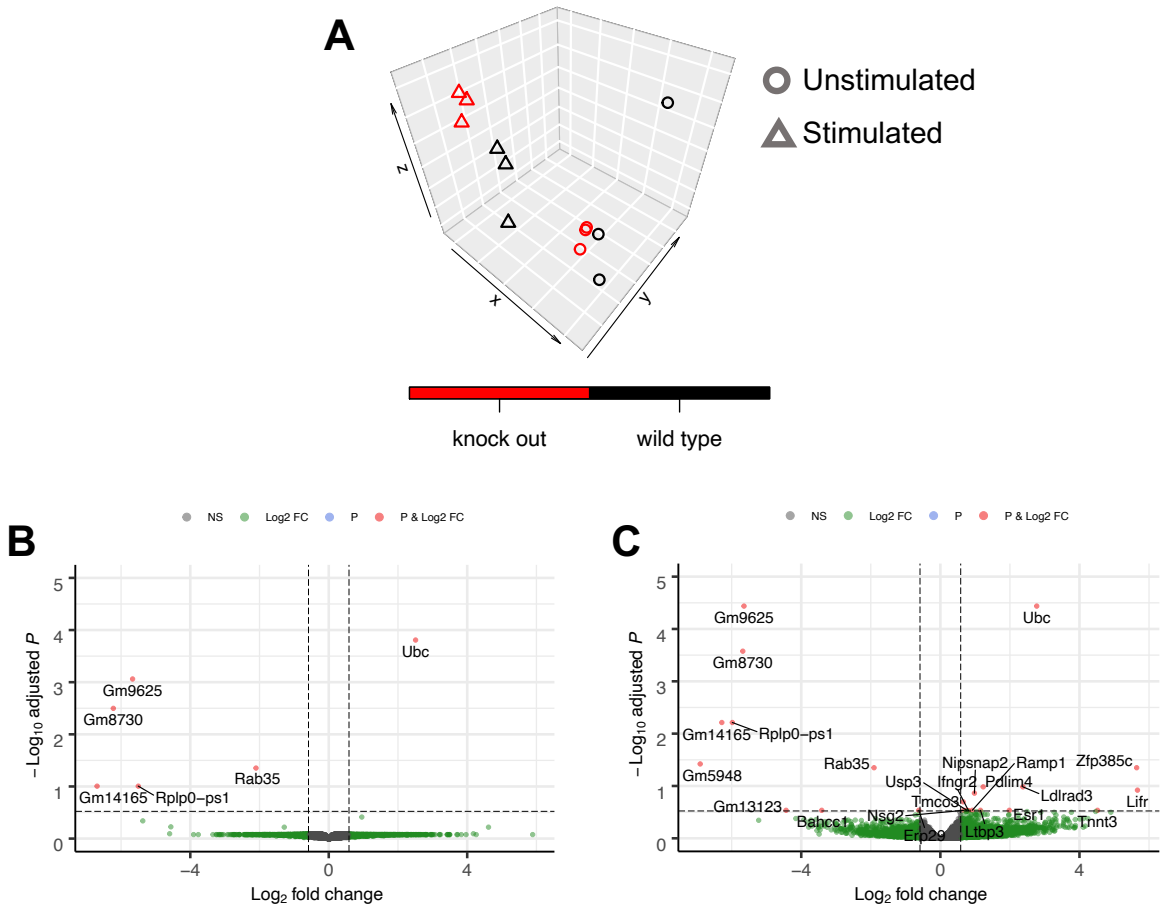

Figure S4: There are minimal transcriptional differences between WT and KO T cells. (**A**) PCA analysis of FACS sorted CD4+ T cells from WT and KO littermate mice. (**B-C**) Volcano plots of differentially expressed genes in KO compared to WT CD4+ unstimulated (B) and stimulated (C) T cells.

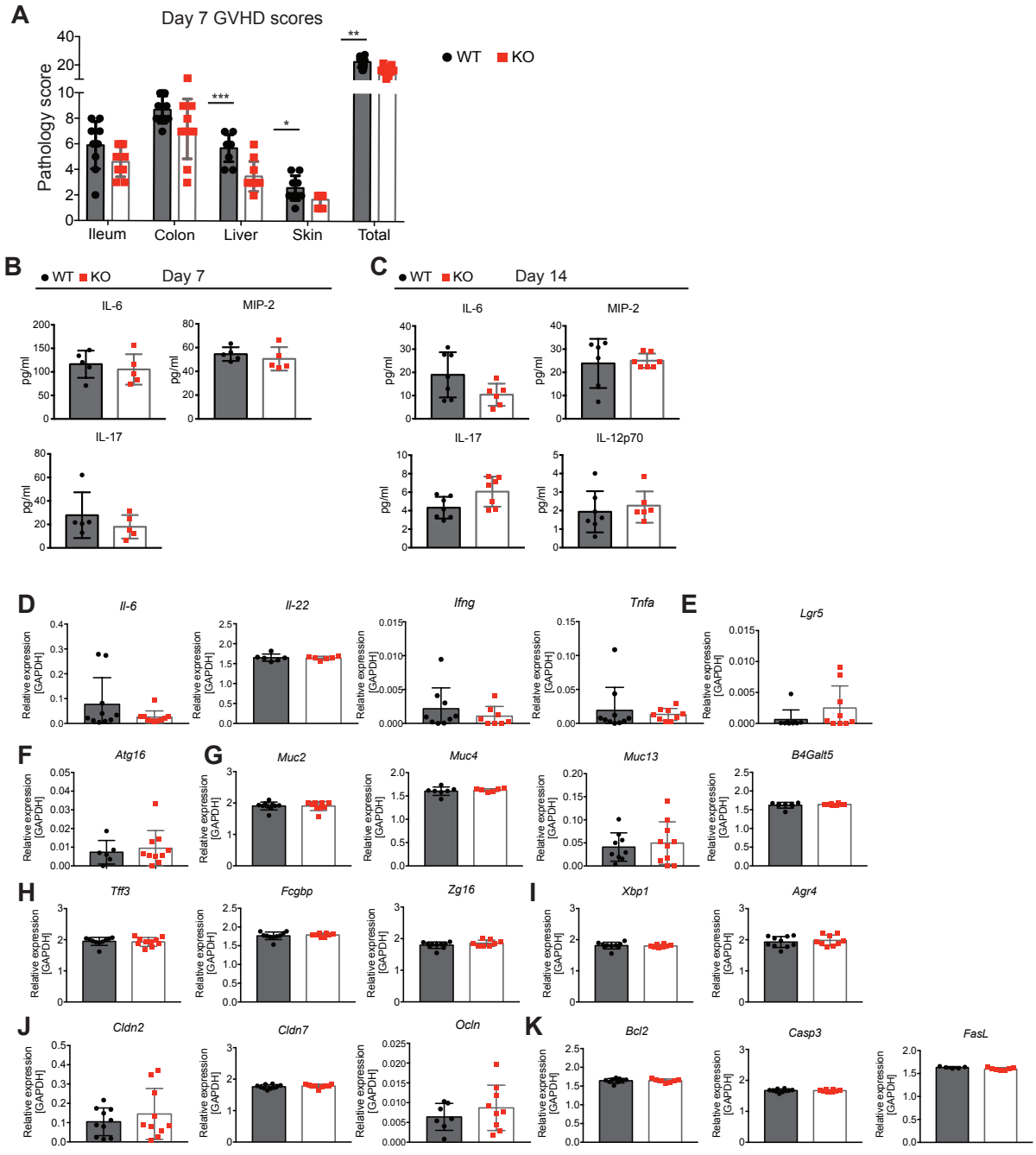

Figure S5: GPR109A$^{-/-}$ T cell recipients have reduced pathology and inflammation. Lethally irradiated BALB/c recipients received B6 WT TCD BM and 0.5x10^6 WT or KO T cells. (**A**) Organs were scored for histopathalogic damage at day 7. (**B-C**) Serum cytokines were measured at 7 days post allo-HCT (**B**) and 14 days post allo-HCT (**C**). (**E-J**) mRNA was extracted from colonic tissue at day 7 post allo-HCT to measure expression of specific genes, such as cytokine genes (D), stem cell gene (E), autophagy gene (F), mucin and mucin production genes (G), mucin-associated genes (H), tight junction genes (J) and apoptotic genes (K). All comparisons in (A) to (K) were performed by two-tailed unpaired Mann-Whitney Test. Values are means ± s.d. *P < 0.05, **P < 0.01, ***P <0.001, n = 8-15 mice per group. Results from two to three independent experiments.

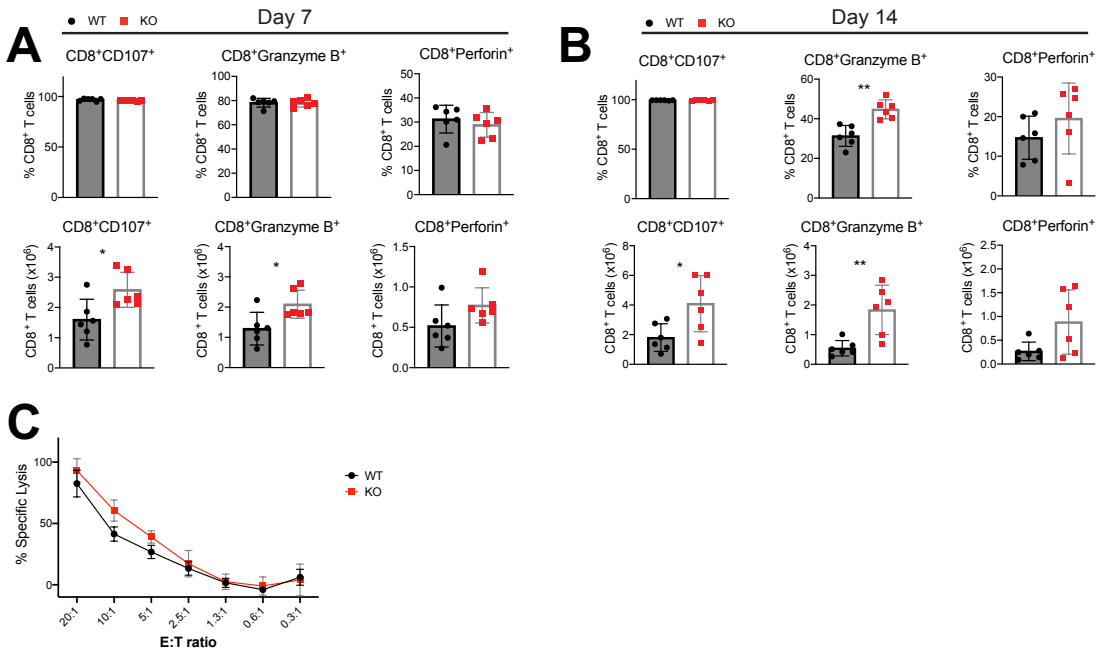

Figure S6: WT an KO CD8$^+$ T cells have similar lysing/killing abilities (**A-B**) Lethally irradiated BALB/c recipients received B6 WT TCD BM and 0.5x10^6 WT or KO T cells. Percentage and number of splenic donor CD8$^+$CD107$^+$, CD8$^+$Granzyme B$^+$, and CD8$^+$Perforin$^+$ T cells were analyzed on day 7 (E) and day 14 (F) post allo-HCT. (**C**) Previously activated WT or KO CD8+ T cells incubated with A20-luciferase cells at effector to T cell (E:T) ratios serially diluted from 20:1 to 0.3:1. All comparisons in (A) and (B) were performed by two-tailed unpaired Mann-Whitney Test. Values are means ± standard error. *P < 0.05, **P < 0.01, n = 6 mice per group. Results from two independent experiments.

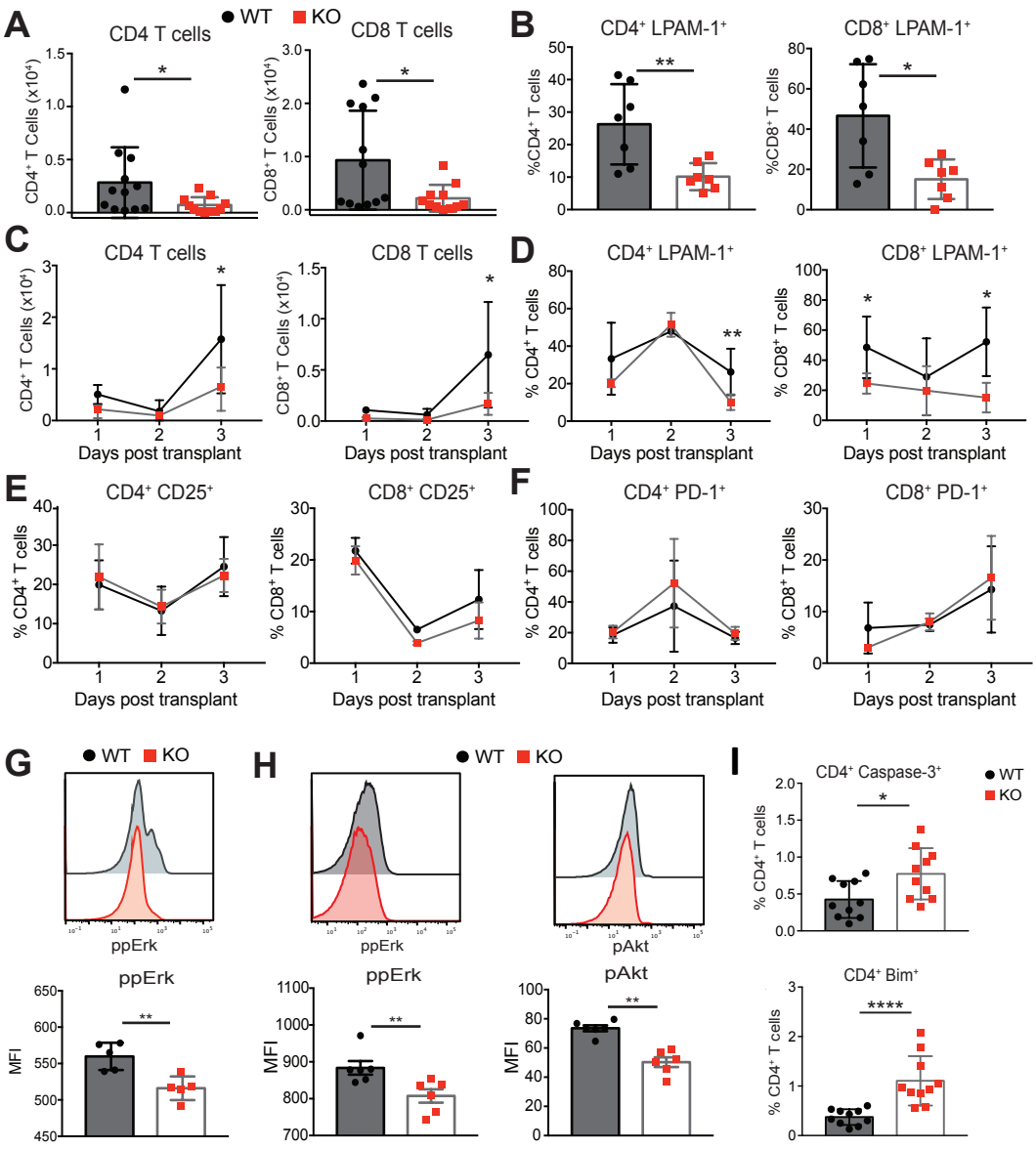

Figure S7: GPR109A$^{-/-}$ T cells become less activated. Lethally irradiated BALB/c recipients received B6 WT TCD BM and 5x10^6 WT or KO T cells. (**A**) Donor CD4$^+$ and CD8$^+$ T cell numbers were analyzed from mLN on day 3 post allo-HCT. (**B**) Frequency of LPAM-1 on mLN donor CD4$^+$ and CD8$^+$ T cells analyzed on day 3 post allo-HCT. (**C-F**) Timecourse early after transplant (day 1, 2, and 3) showing frequency of CD4$^+$ and CD8$^+$ T cells (C), LPAM-1$^+$ CD4$^+$ and CD8$^+$ T cells (D) and cells expressing activation markers CD25 (E) and PD-1 (F) on day 3 post allo-HCT from mLNs. (**G**) Mean fluorescence intensity (MFI) of phosphorylated Erk1/2 on splenic donor CD3$^+$ T cells measured on day 3 post allo-HCT with representative histogram above. (**H**) MFI of phosphorylated proteins Erk1/2 and Akt on splenic donor CD3$^+$ T cells on day 7 post allo-HCT with representative histograms above. (**I**) Frequency of CD4$^+$ T cells from mLN expressing apoptotic markers cleaved Caspase-3 and Bim on day 3 post allo-HCT. All comparisons in (A) to (I) were performed by two-tailed unpaired Mann-Whitney Test. Values are means ± s.d. *P < 0.05, **P < 0.01, ****<0.0001, n = 8-15 mice per group. Results from two to three independent experiments.

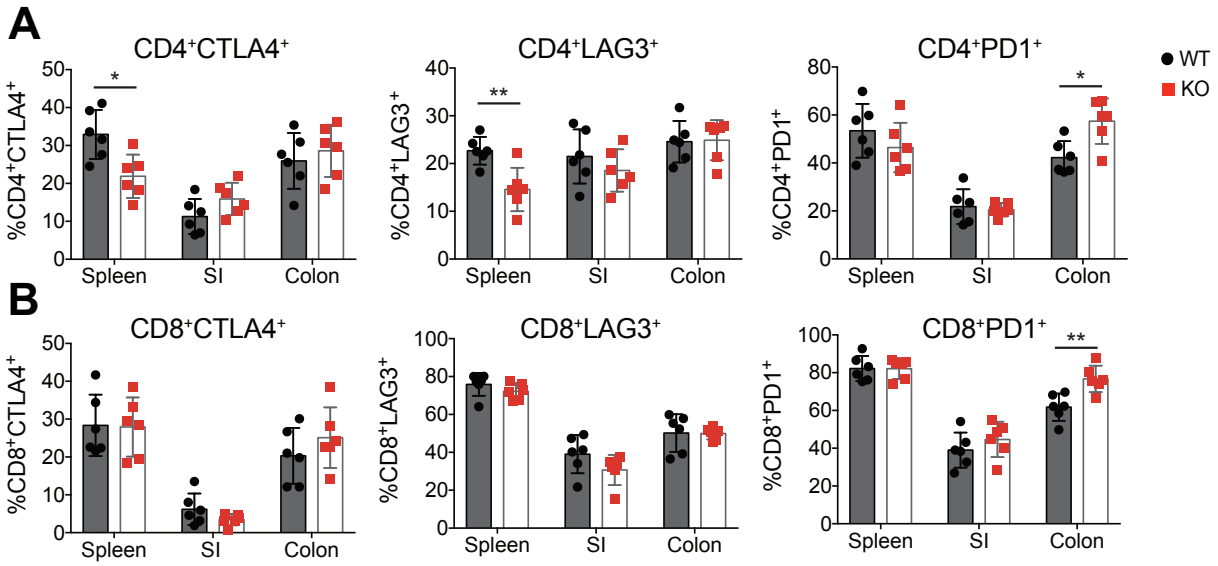

Figure S8: GPR109A$^{-/-}$ T cells do not exhibit increased exhaustion. Lethally irradiated BALB/c recipients received B6 WT TCD BM and 5x10^6 WT or KO T cells. (**A**) Percentages of live, CD45$^+$, CD3$^+$, H-2kb$^+$ donor CD4$^+$ T cells expressing exhaustion/activation markers CTLA4, LAG3 and PD1 on day 7 post allo-HCT. (**B**) Percentages of live, CD45$^+$, CD3$^+$, H-2kb$^+$ donor CD8$^+$ T cells expressing exhaustion/activation markers CTLA4, LAG3 and PD1 on day 7 post allo-HCT. All comparisons were performed by two-tailed unpaired Mann-Whitney Test. Values are means ± s.d. *P < 0.05, **P < 0.01, n = 8-15 mice per group. Results from two to three independent experiments.

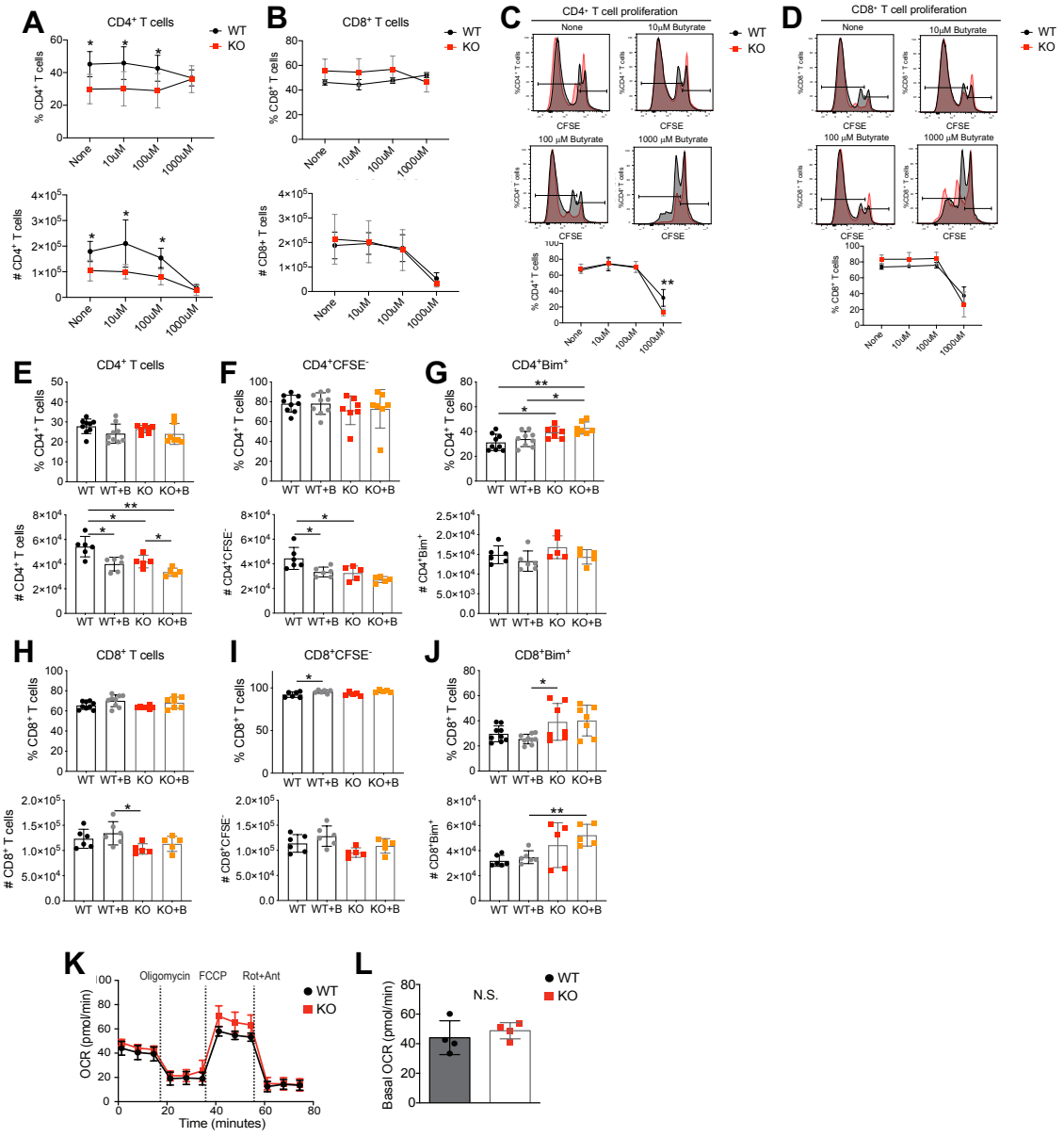

Figure S9: The phenotype of GPR109A$^{-/-}$ T cells is likely butyrate independent. (A-D) T cells from *Gpr109a*$^{+/+}$(WT) and *Gpr109a*$^{-/-}$ (KO) littermate mice were isolated, stained with CFSE and incubated with increasing concentrations of butyrate (10μM, 100μM, 1000μM) and stimulated with anti-CD3/CD28 beads for 72 hours (A-D). (**A-B**) Percentage (top) and number (bottom) of total CD4^{+}(A) and CD8^{+}(B) T cells. (**C-D**) Representative concatenated CFSE line graphs for each concentration of butyrate shown in the upper panel and quantification showing percentage of CFSE$^{medium/low}$ WT and KO CD4^{+} (C) and CD8^{+} (D) T cells in the bottom panel. (**E-J**) Mixed lymphocyte reaction (MLR) of bone marrow derived dendritic cells from a BALB/c mouse and either WT or KO T cells with or without 100μM butyrate. Percentage and number of CD4^{+}(E), CD4^{+}CFSE^{-}(F), and CD4^{+}Bim^{+}(G), as well as CD8^{+}(H), CD8^{+}CFSE^{-}(I), and CD8^{+}Bim^{+}(J) were analyzed. (**K-L**) Oxygen consumption rate (OCR) of unstimulated WT (black) or KO (red) T cells, as measured under basal conditions (L) and in response to oligomycin, FCCP, and rotenone and antimycin A (Rot + Ant) (K). All comparisons in (A) to (L) were performed by two-tailed unpaired Mann-Whitney Test. Values are means ± standard s.d. *P < 0.05, **P < 0.01. Results are from two to three independent experiments.

1. Shono Y, Docampo MD, Peled JU, et al. Increased GVHD-related mortality with broad-spectrum antibiotic use after allogeneic hematopoietic stem cell transplantation in human patients and mice. *Sci Transl Med*. 2016;8(339):339ra371.
2. Stein-Thoeringer CK, Nichols KB, Lazrak A, et al. Lactose drives Enterococcus expansion to promote graft-versus-host disease. *Science*. 2019;366(6469):1143-1149.
3. DeSantis TZ, Hugenholtz P, Larsen N, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol*. 2006;72(7):5069-5072.