Supplemental Material

Sphingosine kinase 2 restricts T cell immunopathology but permits viral persistence

Caleb J. Studstill[1,#], Curtis J. Pritzl[1,#], Young-Jin Seo[2,#,*], Dae Young Kim[3], Chuan Xia[1], Jennifer J. Wolf[1], Ravi Nistala[4], Madhuvanthi Vijayan[1], Yong-Bin Cho[2], Kyung Won Kang[5], Sang-Myeong Lee[5,6], and Bumsuk Hahm[1,*]

[1]Departments of Surgery and Molecular Microbiology & Immunology, University of Missouri-Columbia, Medical Science Building M331, One Hospital Dr. Columbia, MO 65212, USA

[2]Department of Life Science, Chung-Ang University, Seoul 06974, Republic of Korea

[3]Veterinary Medical Diagnostic Laboratory, College of Veterinary Medicine, University of Missouri-Columbia

[4]Division of Nephrology, Department of Medicine, M754 Center for Precision Medicine, University of Missouri, One Hospital Dr. Columbia, MO 65211, USA

[5]Division of Biotechnology, College of Environmental and Bioresources, Jeonbuk National University, Iksan 54596, Republic of Korea

[6]College of Veterinary Medicine, Chungbuk National University, Cheongju, 28644, Republic of Korea

[#]These authors equally contributed to this work.

*Corresponding Authors

Supplemental Methods

Lymphocyte Isolation

Splenocyte suspensions were obtained as described in the methods section of the main text. Splenic dendritic cells (DCs) were obtained by treating spleens with collagenase D (1U/mL, Roche) for 10 minutes followed by EDTA treatment (1mM) for 5 minutes. For experiments investigating immune cell infiltration into kidneys, tissues were incubated at 37C in RPMI-1640 (Gibco) containing type 1 collagenase (2mg/mL, Alfa Aesar), DNase I (5U/mL, Thermo Scientific), FBS (10%, Sigma), HEPES (25mM, Gibco), and penicillin/streptomycin (100U/mL, 100µg/mL, Sigma) for 50 minutes followed by the addition of RPMI-1640 containing FBS (2%). Tissues were then forced through nylon mesh, and cells were used in downstream experiments.

Flow Cytometry Antibodies

Antibodies used were specific for murine CD11b (M1/70), CD11c (N418), MHC-I (H-2K^{b}, AF6.88.5), MHC-II (I-A^{b}, AF6-120.1), 4-1BBL (TKS-1), CD40 (HM40-3), CD8α (53-6.7), CD3 (145-2C11), CD4 (GK1.5, RM4-5, RM4-4), CD90.1 (Thy1.1, OX-7), CD45.1 (A20), Ly6C (HK1.4), Ly6G (1A8), B220 (RA3-6B2), IL-2 (JES6-5H4), Ki67 (SolA15), IFNγ (XMG1.2), Granzyme B (NGZB), TNFα (MP6-XT22), Tim-3 (RMT3-23), CD160 (CNX46-3), LAG-3 (C9B7W), and PD-1 (RMP1-30) (BD Pharmingen, eBioscience, Tonbo Biosciences, BioLegend, and Leinco Technologies).

Derivation of BM-DCs

The femurs and tibias of WT mice were harvested and washed with 70% EtOH. As much excess tissue was removed from the bone as possible. The ends of each bone were then clipped, and a 25-gauge needle and syringe were used to wash the inside of each bone with PBS. The collected cells were passed through a 40μm nylon mesh. Cells were then treated with NH_4Cl to remove RBCs and plated in cell dishes at a concentration of $1x10^7$ cells/plate. These cells were grown in complete RPMI-1640 (Gibco) supplemented with FBS (10%, Sigma), penicillin/streptomycin (100U/mL, 100μg/mL, Sigma), and 2-mercaptoethanol (50μM, Sigma), sodium pyruvate (1%, Gibco), HEPEs (1%, Gibco), and non-essential amino acid (1%, Gibco). Finally, the media was supplemented with 20ng/mL (200U/mL) rmGM-CSF (Peprotech). The cells were grown for 10 days, changing media on days 3, 6, and 8.

In vitro T cell proliferation assay

BM-DCs were treated with the TLR7 agonist loxoribine (0.5mM) in the presence of GP61 peptide (1ug/mL). The other process was performed as described in the Methods section of the main text.

Western Blot Analysis

$1x10^5$ GP61-specific $CD4^+$ or GP33-specific $CD8^+$ T cells were adoptively transferred to C57BL/6 mice. At 8 days post LCMV Arm or LCMV Cl 13 infection, $CD4^+$ or $CD8^+$ T cells

were obtained using EasySep $CD4^+$ or $CD8^+$ positive selection reagents (Stem Cell Technologies) according to the manufacturer's instructions. Splenocytes or positively selected $CD4^+$ and $CD8^+$ T cells were lysed utilizing a sample buffer containing glycerol (20%), Tris/HCl pH 6.8 (0.125M), SDS (0.016g/mL), bromophenol blue (0.2mg/mL), and 2-mercaptoethanol (0.358M). Lysates were resolved on 10% SDS-PAGE gels and transferred to nitrocellulose membrane. SphK2 (ab264042, Abcam), phospho-SphK2 (pSphK2, SP4631 ECM Biosciences or PA5-39812 Invitrogen), and GAPDH (5174, Cell Signaling) were identified using specific polyclonal antibodies and detected using a rabbit IgG-specific, HRP-linked secondary antibody (Thermo Fisher Scientific) and chemiluminescent substrate (Thermo Fisher Scientific) or a rabbit IgG-specific, IRDye 800CW antibody (LI-COR) and imaged with the Odyssey Fc Imaging System (LI-COR).

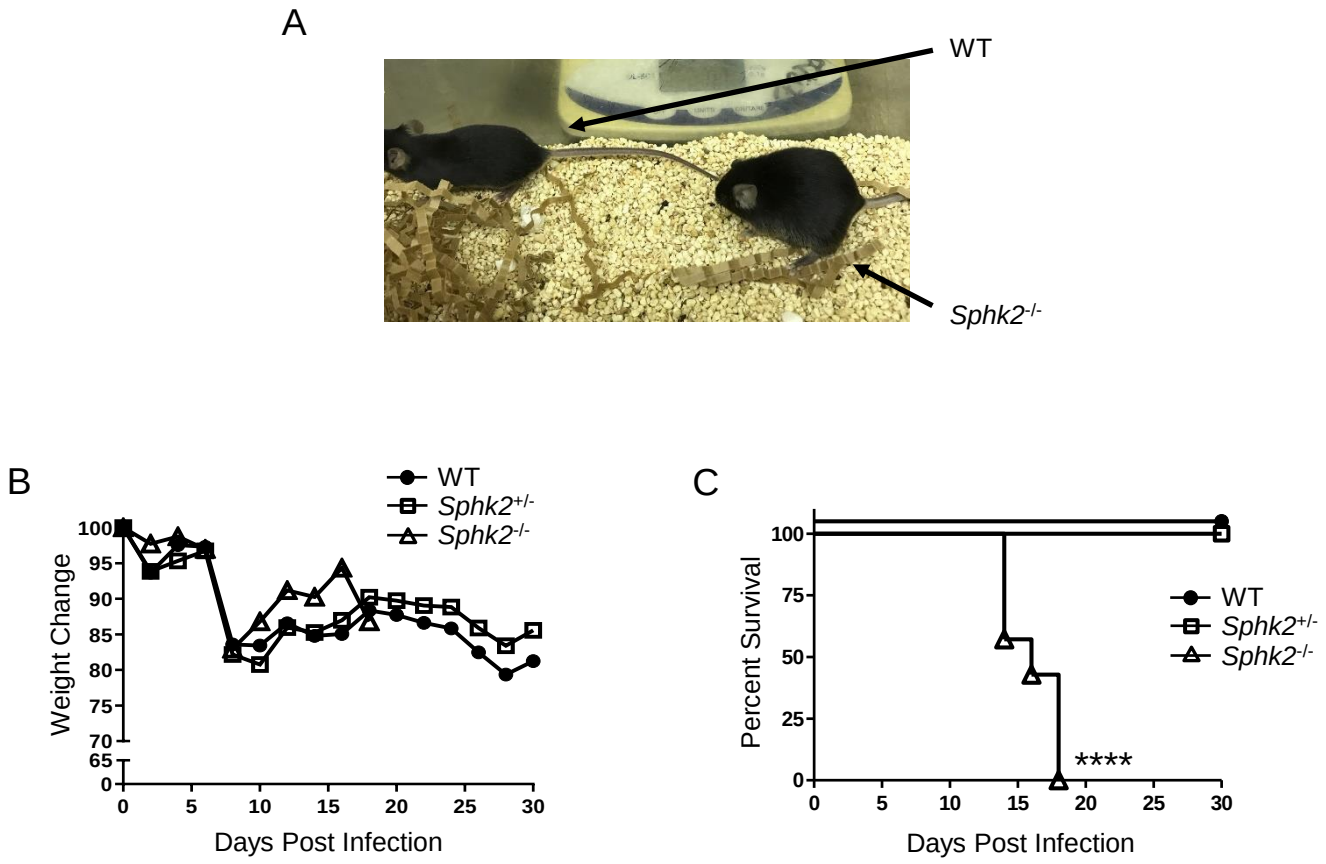
Antibody-Mediated Neutralization

Sphk2$^{-/-}$ mice were infected with $2x10^6$ PFU LCMV Cl 13 and then given 200µg Armenian hamster IgG (PIP, isotype control), anti-TNFα (TN3-19.12) (Leinco Technologies), or anti-FasL (MFL3) (Bio X Cell) i.p. on 4, 7, and 10 dpi. Survival was monitored subsequently.

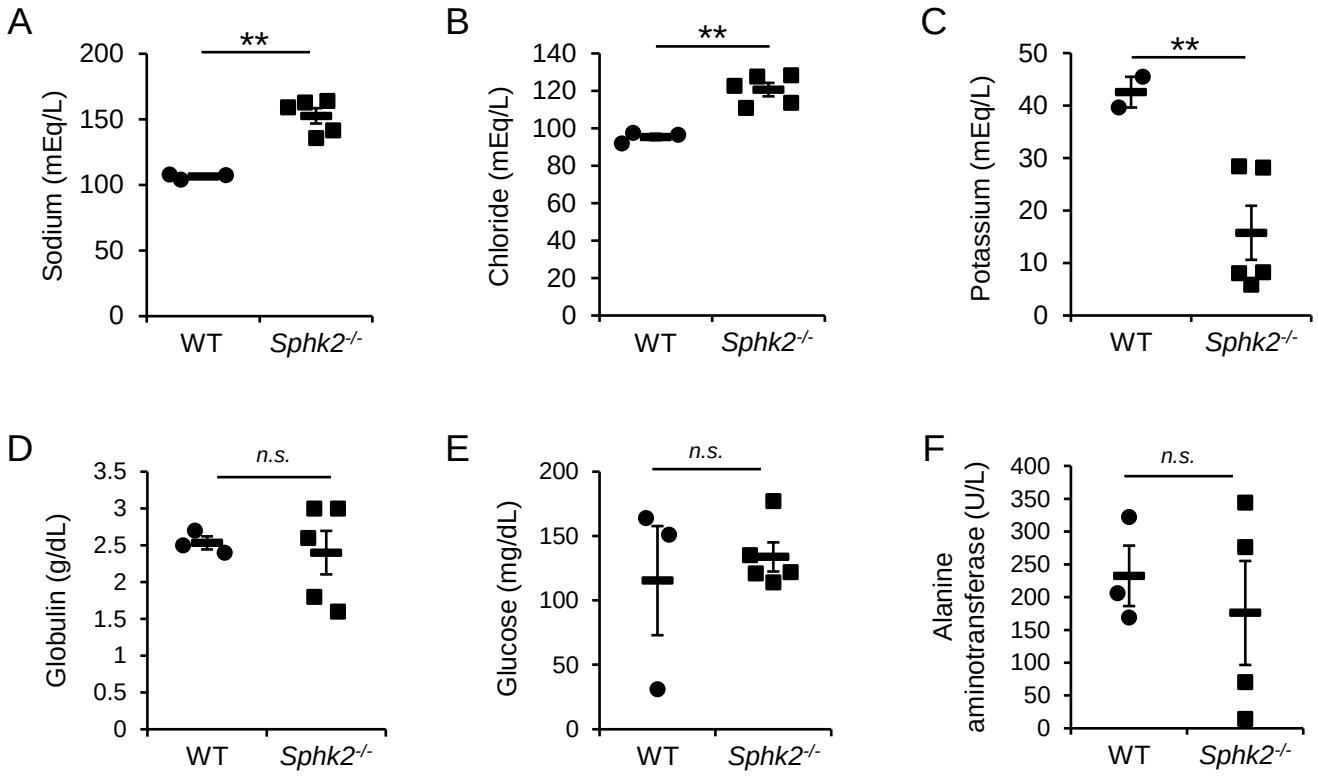
Focus-Forming Assay

Vero cells were infected with serum or homogenized kidney supernatants of mice, followed by overlaying with a 1% methylcellulose (Sigma-Aldrich) in DMDM. Forty-eight hours later, monolayer cells were fixed with 4% paraformaldehyde, permeabilized with 1% Triton X-100, and stained with rat anti-LCMV nucleoprotein antibodies (VL4, Bio X

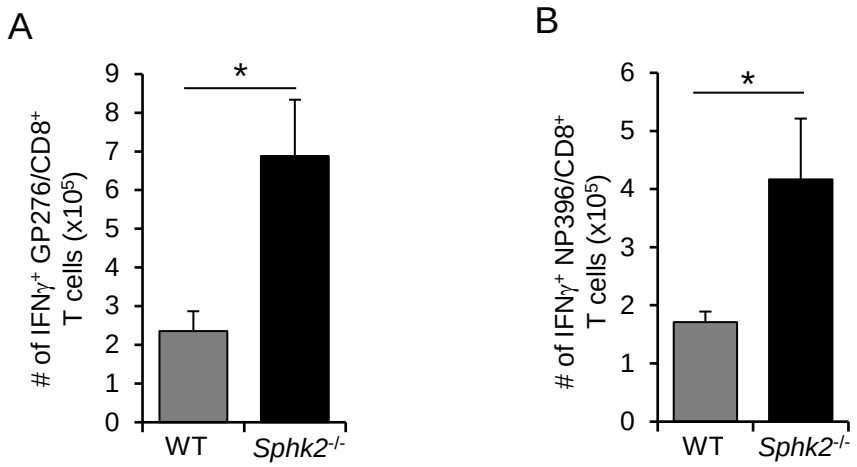
Cell). Following incubation with anti-rat IgG-HRP antibodies (Thermo Fisher Scientific), a colorimetric reaction with DAB (3,3´-diaminodbenzidine) solution (Thermo Fisher Scientific) was performed to visualize LCMV-infected Foci.



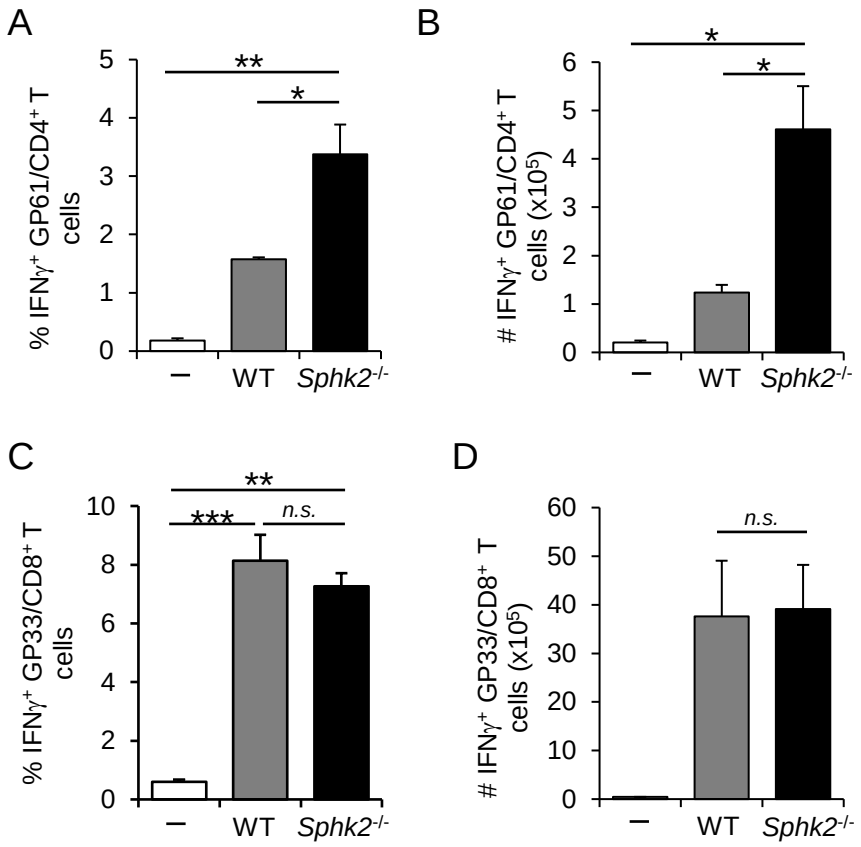
Supplemental Figure 1. *Sphk2*$^{-/-}$ mice experience severe morbidity following LCMV Cl 13 infection, and one genetic copy of *Sphk2* is enough to alleviate this morbidity. Groups of WT (●) (n = 5), *Sphk2*$^{+/-}$ (□) (n = 8), and *Sphk2*$^{-/-}$ (Δ) (n = 7) mice were infected with LCMV Cl 13 at 2 x 10^6 PFU. (A) Picture represents the morbidity seen in *Sphk2*$^{-/-}$ mice compared to WT mice 18dpi. Mice exhibit ascites, lethargy, and become hunched due to fluid retention. For 30 days following LCMV Cl 13 infection, the percent weight change (B) and percent survival (C) were compared between the three mice groups. ****p≤0.0001, Mantel-Cox Logrank test.



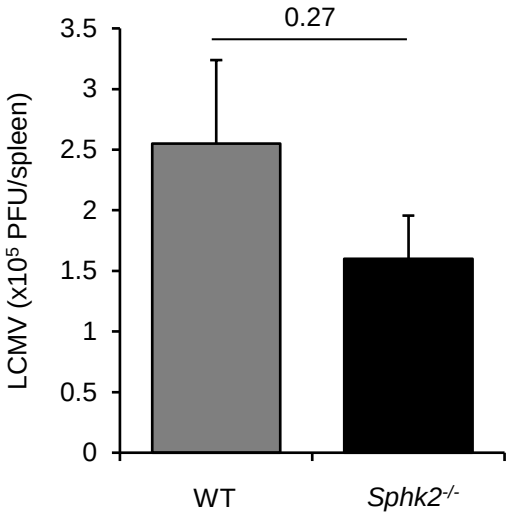
Supplemental Figure 2. Serum metabolite profile for *Sphk2*$^{-/-}$ mice indicates an electrolyte imbalance. WT (n = 3) and *Sphk2*$^{-/-}$ (n = 5) mice were infected with 2x10^6 PFU LCMV Cl 13 and monitored for morbidity. When *Sphk2*$^{-/-}$ mice showed signs of severe morbidity at 15-17dpi, they were sacrificed along with infected WT mice, and serum was assessed for a complete clinical chemistry profile analysis, including sodium (A), chloride (B), potassium (C), globulin (D), glucose (E), and alanine aminotransferase (ALT) (F) levels. **p≤0.01, *n.s.* not significant, bidirectional, unpaired Student's *t*-test.



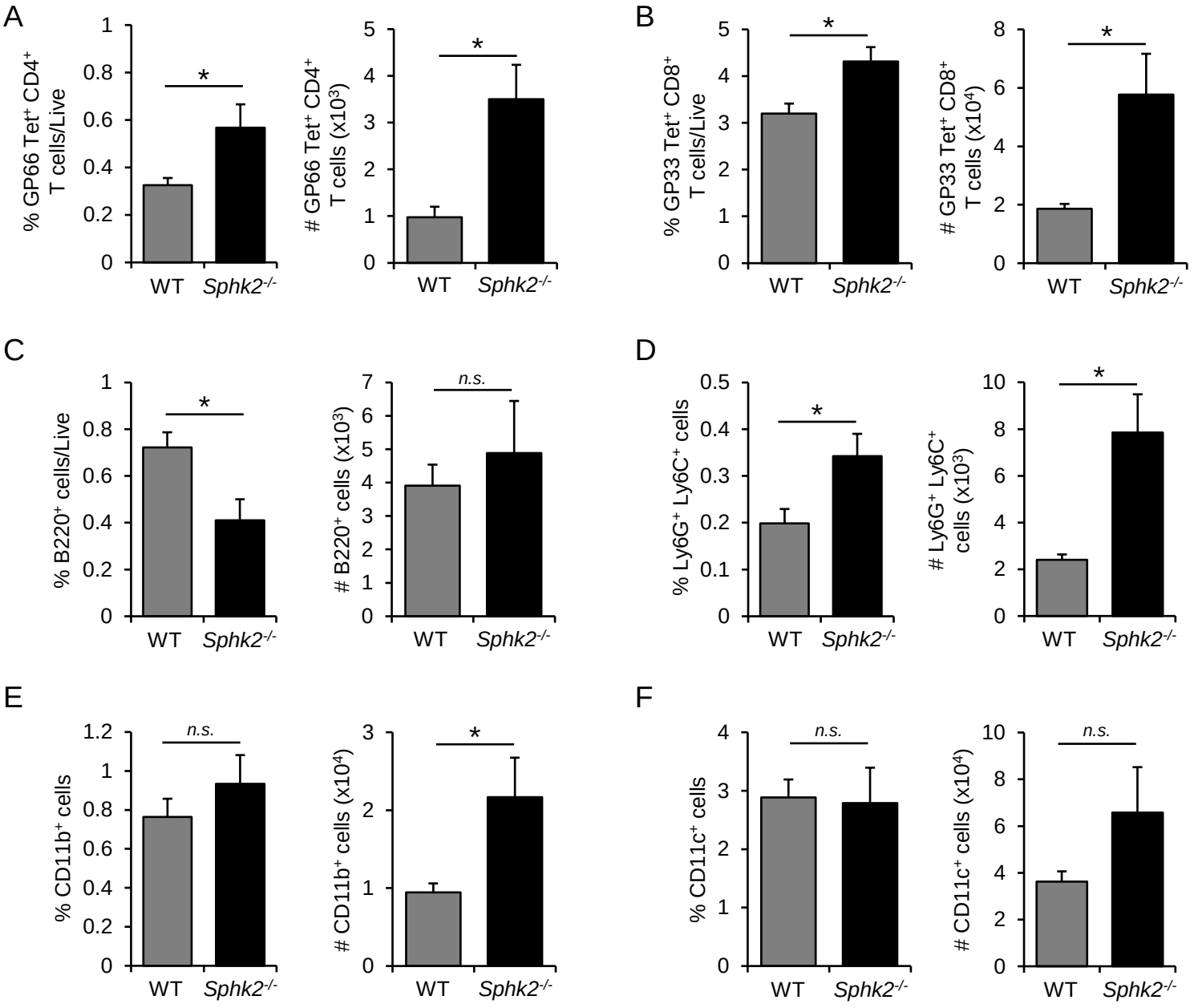
Supplemental Figure 3. Virus-specific CD8$^+$ T cell responses have collectively increased in *Sphk2*$^{-/-}$ mice during LCMV Cl 13 infection. Groups (n = 4-5 mice per group) of WT and *Sphk2*$^{-/-}$ were infected with LCMV Cl 13 at 2 x 10^6 PFU. At 7dpi, the numbers of IFNγ-positive LCMV GP276 (A) and NP396 (B)-specific CD8$^+$ T cells in the spleen were analyzed following re-stimulation with GP276- and NP396-peptides and intracellular staining. *p≤0.05, bidirectional, unpaired Student's *t*-test.



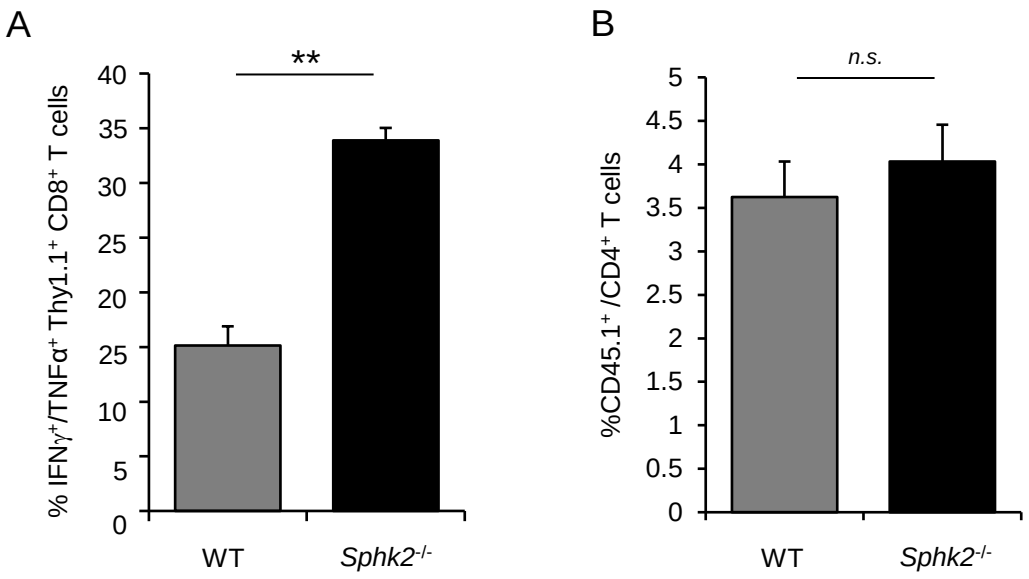
Supplemental Figure 4. SphK2 deficiency affects CD4$^+$, but not CD8$^+$ T cell responses to LCMV Arm infection. Groups of WT and *Sphk2*$^{-/-}$ mice (n = 5 mice per group) were infected with LCMV Arm. At 7dpi IFNγ$^+$ GP61/CD4$^+$ (Frequency, A; Number, B) and IFNγ$^+$ GP33/CD8$^+$ (Frequency, C; Number, D) T cells were determined in the spleens of infected mice by intracellular cytokine staining. ***p≤0.001, **p≤0.01, *p≤0.05, *n.s.* not significant, one-way ANOVA with Tukey's post-hoc test.



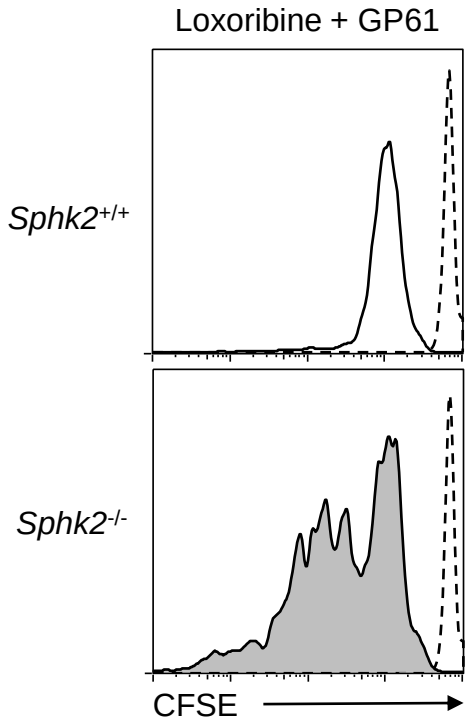
Supplemental Figure 5. Analysis of virus titers in *Sphk2*$^{-/-}$ mice upon LCMV Cl 13 infection. WT and *Sphk2*$^{-/-}$ mice (n = 4 mice per group) were infected with LCMV Cl 13. At 7dpi, virus titers in the spleen were measured by plaque assay. Bidirectional, unpaired Student's *t*-test.



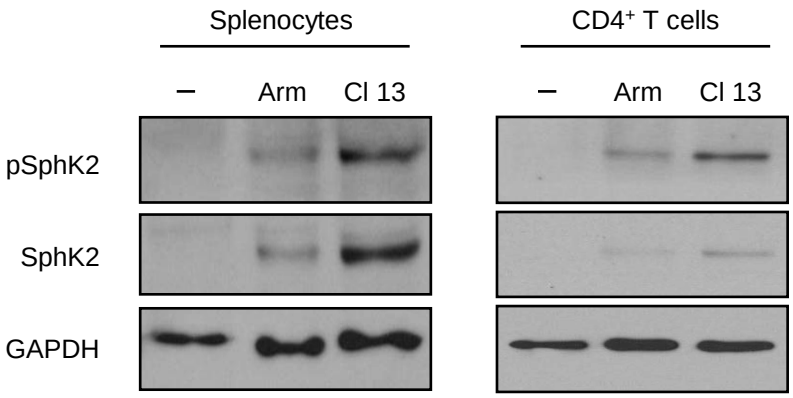
Supplemental Figure 6. Virus-specific T cells and neutrophils infiltrate the kidneys of *Sphk2*$^{-/-}$ mice following LCMV infection. (A-F) WT and *Sphk2*$^{-/-}$ mice (n = 4-5 mice per group) were infected with 2x10^6 PFU LCMV Cl 13. At 8dpi, mice were sacrificed, and kidneys were analyzed for immune cell infiltration via flow cytometry. The percent and number of LCMV GP66 (GP_{66-77}) tetramer$^+$ CD4$^+$ T cells (A), LCMV GP33 (GP_{33-41}) tetramer$^+$ CD8$^+$ T cells (B), B220$^+$ cells (C), neutrophils [Ly6G(1A8)$^+$ Ly6C$^+$ cells] (D), CD11b$^+$ cells (E), and CD11c$^+$ cells (F) are shown. *p≤0.05, *n.s.* not significant, bidirectional, unpaired Student's *t*-test.



Supplemental Figure 7. *Sphk2*$^{+/+}$ CD8^{+} T cells function better in a *Sphk2*$^{-/-}$ environment, whereas *Sphk2*$^{+/+}$ CD4^{+} T cells do not expand differentially based on the environment. (A) 1x10^{4} *Sphk2*$^{+/+}$ Thy1.1^{+} GP33/CD8^{+} T cells were transferred into WT or *Sphk2*$^{-/-}$ mice (n = 4-5 mice per group). Mice were infected with 2x10^{6} PFU LCMV Cl 13 one day later. 7dpi, the transferred cells were re-stimulated and analyzed for the percentage of IFNγ/TNFα-double positive CD8^{+} T cells via flow cytometric analysis. (B) 1x10^{4} *Sphk2*$^{+/+}$ CD45.1^{+} GP61/CD4^{+} T cells were transferred into WT or *Sphk2*$^{-/-}$ mice (n = 4 mice per group). Mice were infected and 7dpi, transferred cells were analyzed for the percentage of CD45.1^{+} cells out of total CD4^{+} T cells by flow cytometric analysis. **p≤0.01, *n.s.* not significant, bidirectional, unpaired Student's *t*-test.

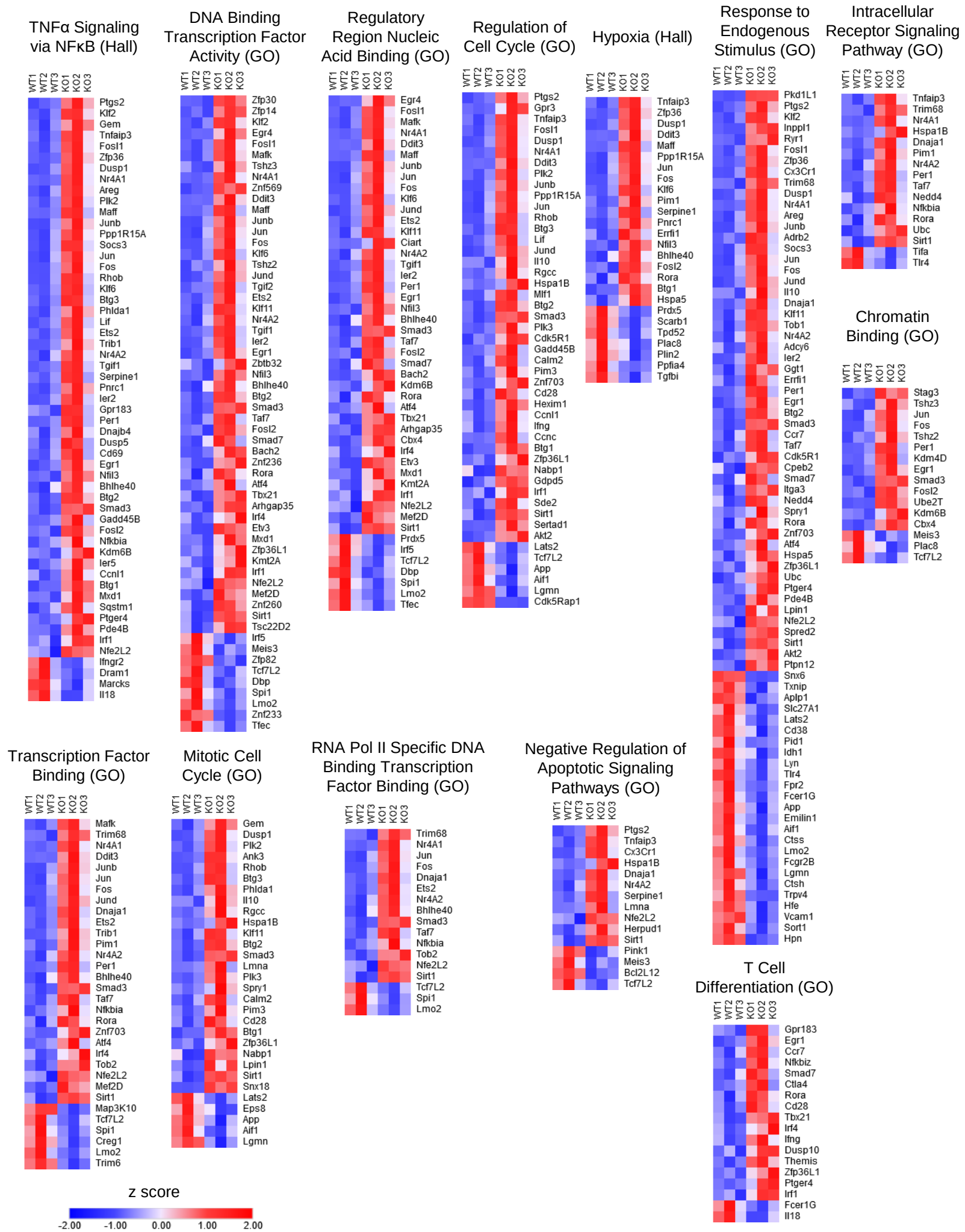


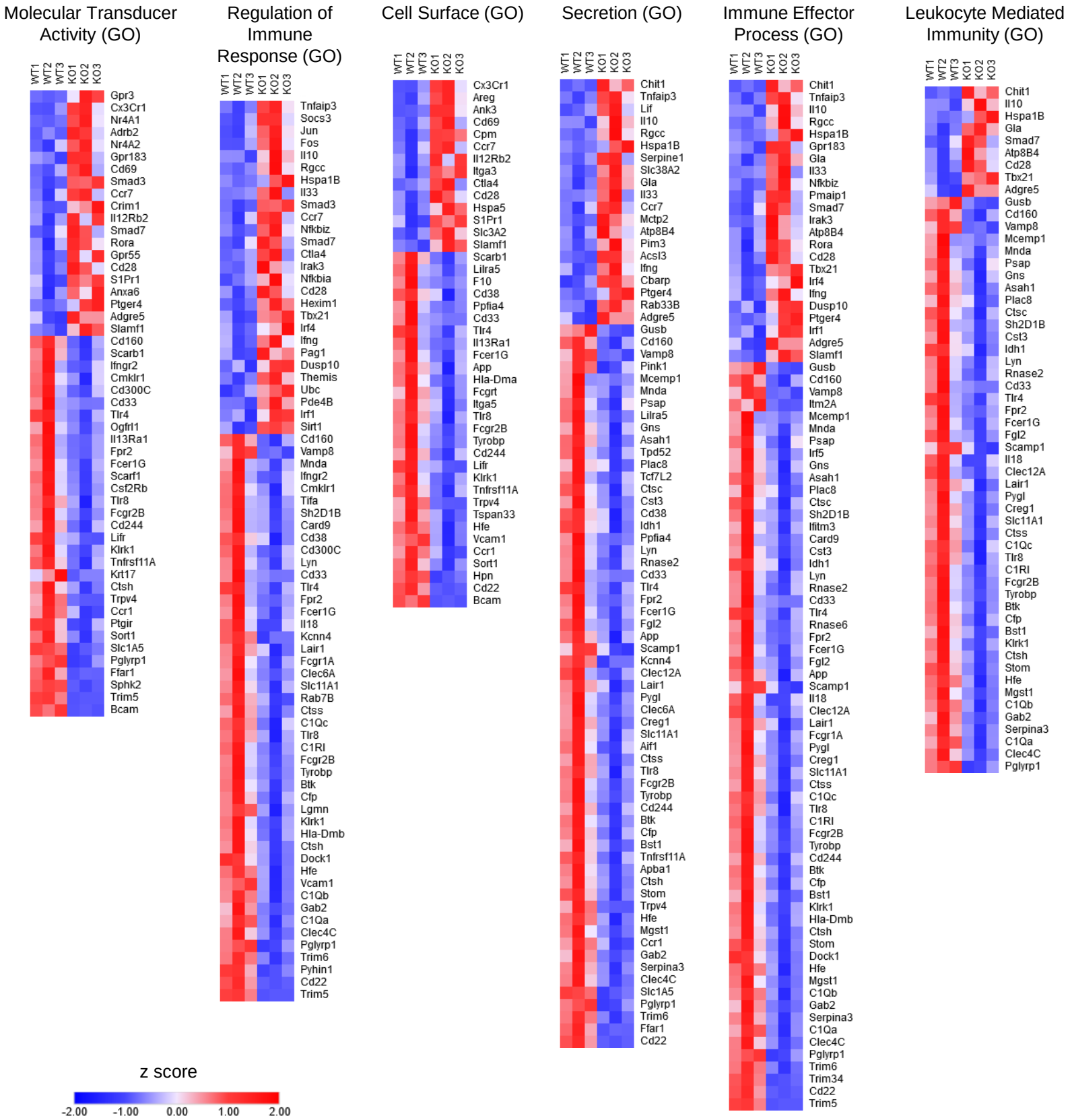
Supplemental Figure 8. *Sphk2*$^{-/-}$ CD4^{+} T cells proliferate at a higher rate than *Sphk2*$^{+/+}$ CD4^{+} T cells when stimulated by DCs that were activated with a TLR7 ligand. Naïve CD4^{+} T cells were isolated from *Sphk2*$^{+/+}$ (upper panel; open histogram) or *Sphk2*$^{-/-}$ (lower panel; filled histogram) CD45.1^{+} GP66/CD4^{+} mice. Bone marrow-derived dendritic cells (BM-DCs) were incubated with 0.5mM loxoribine and 1µg/mL GP61 peptide for 1 hour. CFSE-stained *Sphk2*$^{+/+}$ or *Sphk2*$^{-/-}$ CD4^{+} T cells were then co-cultured with the treated BM-DCs and harvested 5 days later. CFSE dilution was checked by flow cytometry analysis and compared to the cells measured at day 0 (dotted histogram).



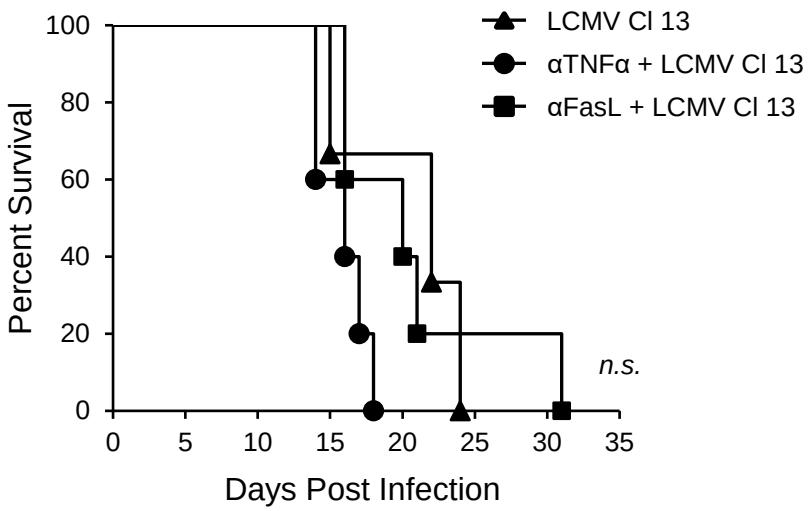
Supplemental Figure 9. LCMV infections increase the levels of SphK2 and pSphK2 in splenocytes and CD4⁺ T cells. WT mice were uninfected or infected with LCMV Arm or LCMV Cl 13. Total splenocytes and CD4⁺ T cells were harvested from mice at 8dpi. The levels of SphK2 and pSphK2 were detected by western blot analysis. The pictured blot is a representative of at least 3 mice per group.

Supplemental Figure 10

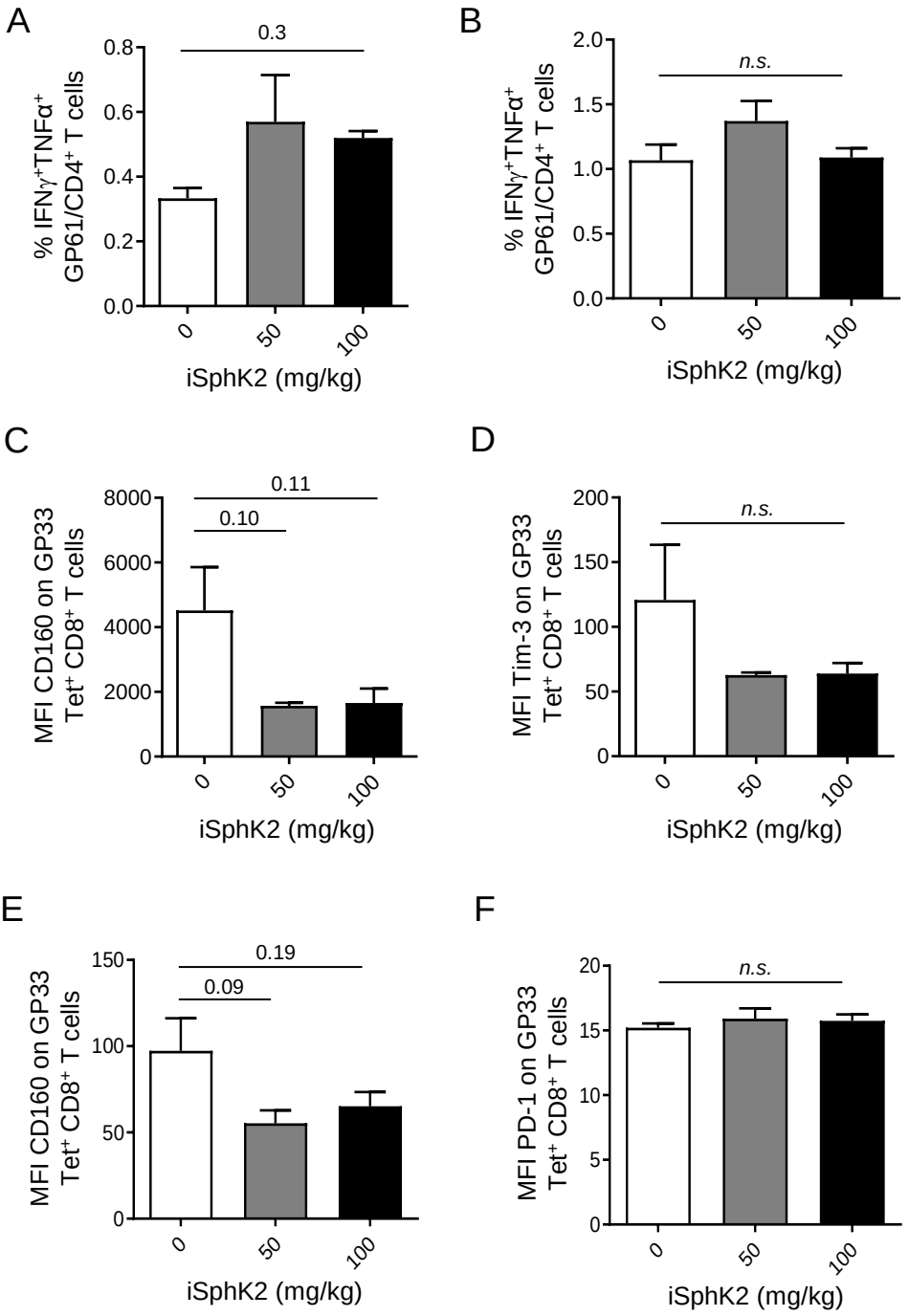




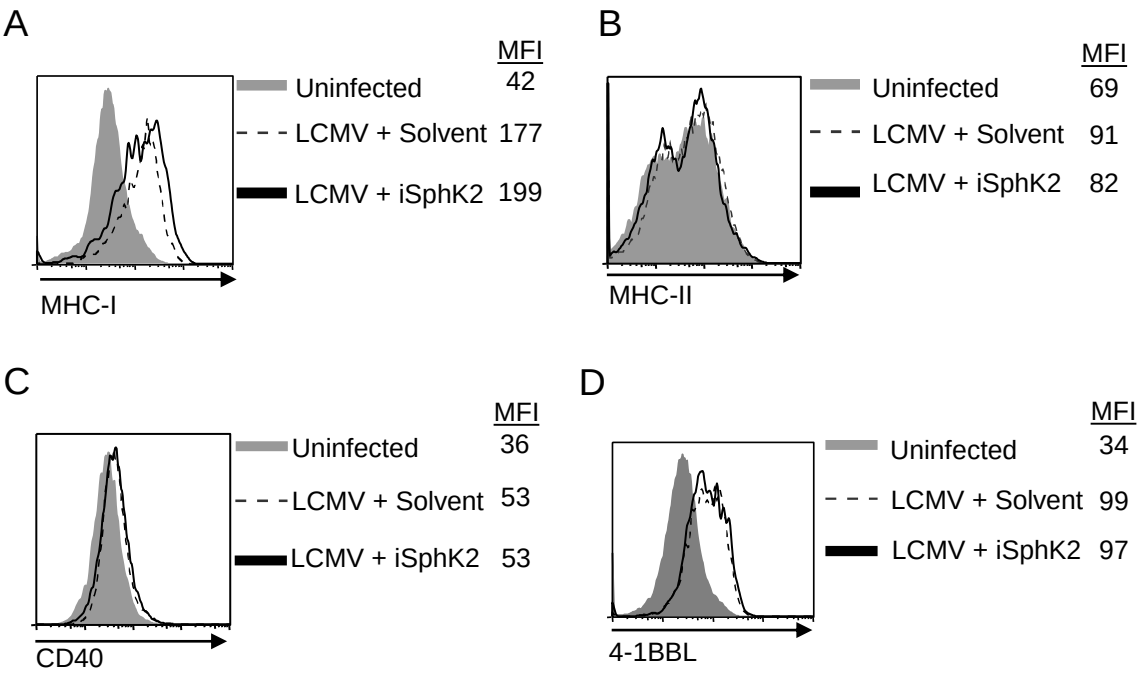
Supplemental Figure 10. Heatmaps for top relevant genes sets from GSEA. Following differential expression analysis and GSEA (see Fig. 6), heatmaps were generated for significant, representative gene sets from gene ontology (GO) and hallmark (Hall) molecular signature databases. The heatmaps depict colors relating to the z scores for increased (positive values, red) or decreased (negative values, blue) normalized expression values of genes from *Sphk2*$^{+/+}$ (WT) or *Sphk2*$^{-/-}$ (KO) CD4^{+} T cells (n = 3 samples per group).



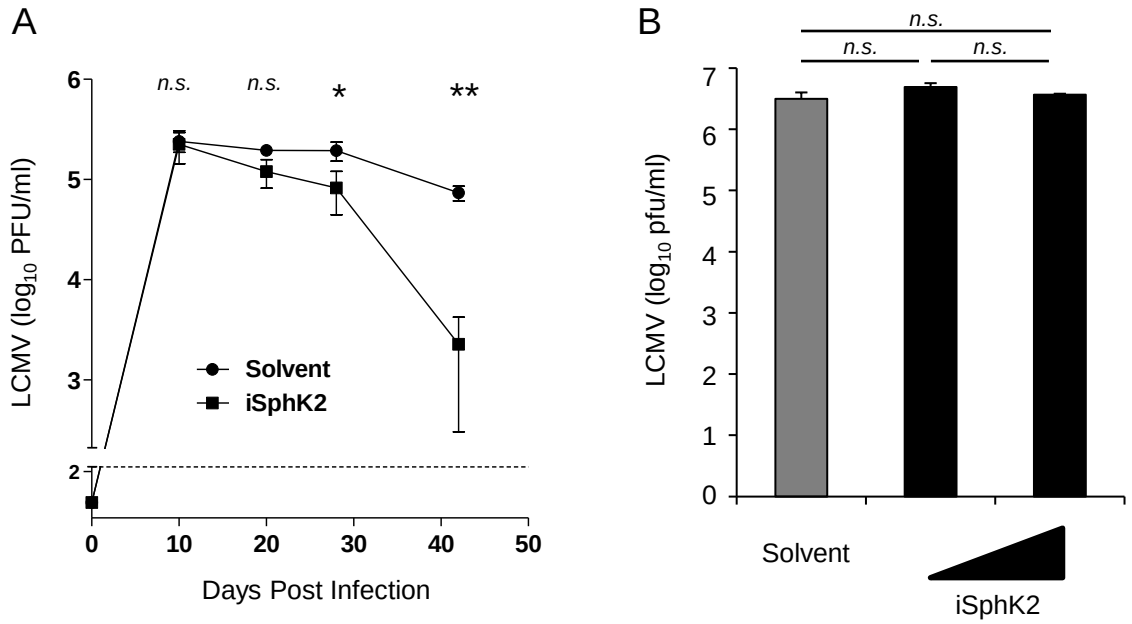
Supplemental Figure 11. Neutralization of TNFα or FasL does not prevent the death of *Sphk2*$^{-/-}$ mice following LCMV Cl 13 infection. *Sphk2*$^{-/-}$ mice were infected with 2x10^{6} PFU LCMV Cl 13. On 4, 7, and 10dpi mice were given i.p. 200ug of hamster IgG control Ab (n = 3), anti-TNFα Ab (n = 5), or anti-FasL Ab (n = 5). Survival was monitored for 32 days. *n.s.* not significant, Mantel-Cox Logrank test.



Supplemental Figure 12. Inhibition of SphK2 regulates virus-specific T cells. (A-F) LCMV Cl 13-infected mice (n = 3-5 mice per group) were treated with solvent (0mg/kg), 50mg/kg, or 100mg/kg iSphK2. At 40dpi, spleen (A, C-D) and liver (B, E-F) tissues were analyzed by flow cytometry for IFNγ+TNFα+ GP61/CD4+ T cells (A, B) and the mean fluorescent intensity (MFI) of CD160 (C, E), Tim-3 (D), and PD-1 (F) on GP33 Tet+ CD8+ T cells. *n.s*. not significant, one-way ANOVA with Tukey's post-hoc test (A-F).



Supplemental Figure 13. SphK2 deficiency does not regulate DC activation. (A-D) C57BL/6 mice were infected with LCMV Cl 13 (n = 3 mice per group) and given iSphK2 (100mg/kg) or its solvent starting the day of infection for two days. The expression levels of MHC-I (A), MHC-II (B), CD40 (C), and 4-1BBL (D) were measured on splenic $CD11c^+$ cells of mice at 2dpi. $CD11c^+$ DCs from uninfected mice were used as a control. Mean fluorescence intensities (MFIs) are depicted.



Supplemental Figure 14. Inhibition of SphK2 promotes clearance of persistent LCMV infection but does not impair LCMV replication in vitro. (A) C57BL/6 mice (n = 5 mice per group) were infected with LCMV Cl 13 and treated daily for 7 days with 100mg/kg iSphK2 or solvent by oral gavage. At 10, 20, 28, and 42dpi, LCMV titers were determined in the serum of infected mice by plaque assay. (B) BHK cells were infected with LCMV Cl 13 (MOI = 0.1) and treated with 25 or 50μM iSphK2 or solvent control for 48 hours. LCMV Cl 13 titers were determined in the supernatants by plaque assay. **p≤0.01, *p≤0.05, *n.s*. not significant, bidirectional, unpaired Student's *t*-test (A), one-way ANOVA with Tukey's post-hoc test (B).