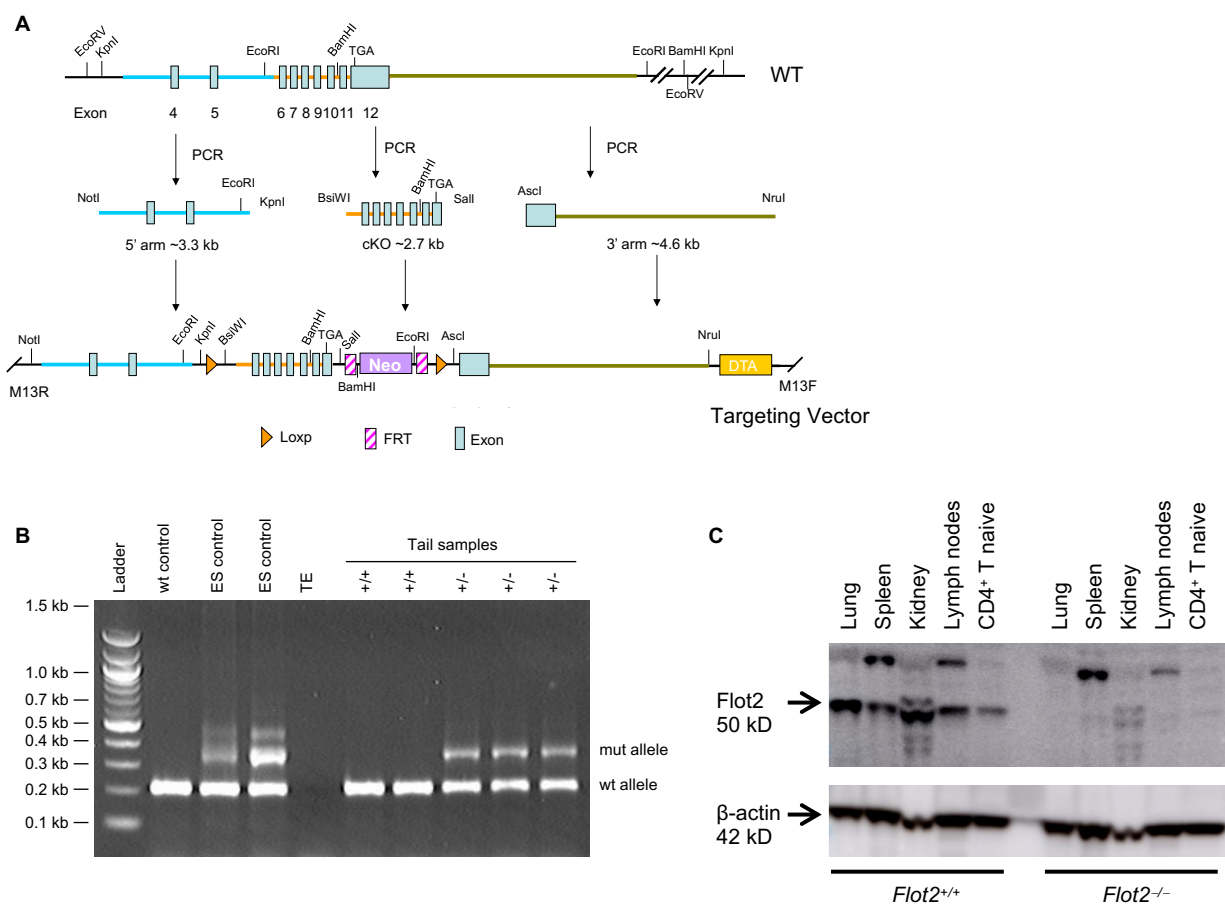
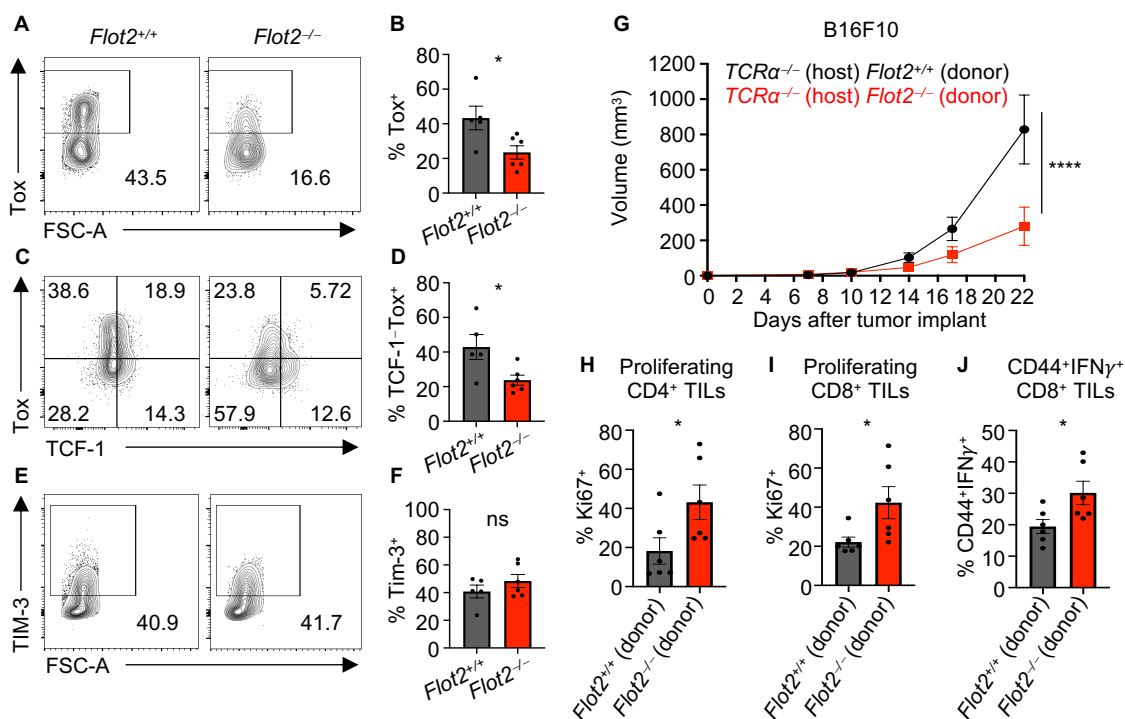


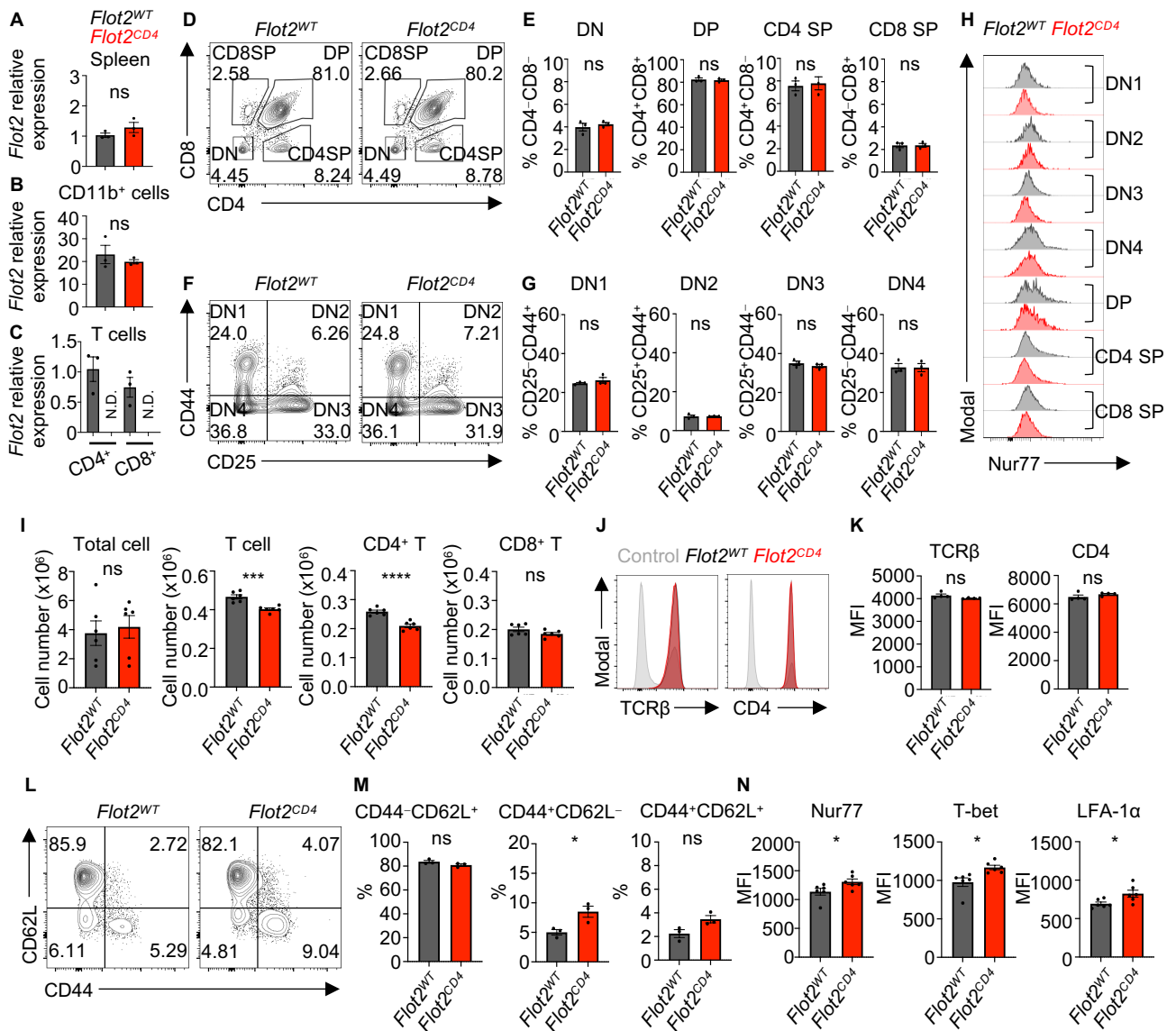


Supplemental Figure 1. Flotillin knockout mouse targeting vector strategy and confirmation.

(A) The vector targeting strategy for flanking the murine *Flot2* locus with loxP sites is shown, along with restriction sites. (B) Pups from chimera breeding were screened by PCR of tail samples using the following primers: 5'-ATCACTGTCTGTCTGTGAGGAGTGG-3' and 5'-AGGGCAAGAGCGTGTGGGTTGTGG-3' followed by gel electrophoresis as shown. (C) Loss of Flot2 expression at the protein level was confirmed in *Flot2*^{-/-} mice using Western blot analysis.

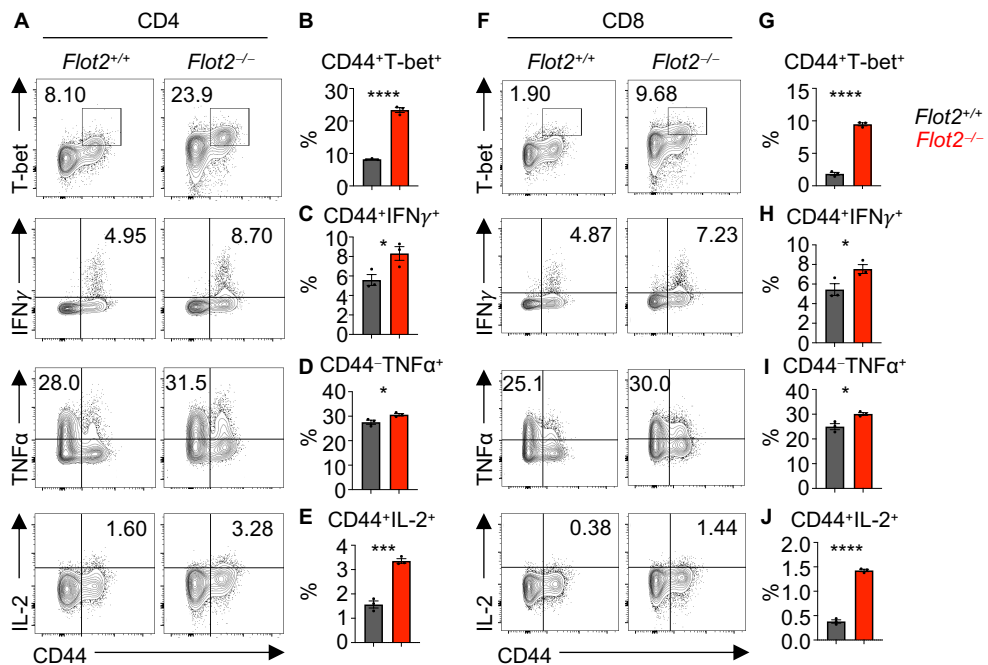


Supplemental Figure 2. Flot2 deficiency reduces T cell functional exhaustion and enhances anti-tumor T cell responses. (A-F) Flow cytometric analysis of TILs in MC38 tumor-bearing *Flot2*^{+/+} or *Flot2*^{-/-} mice: Representative plots (A, C, and E) are shown. Tox⁺ (B), TCF-1-Tox⁺ (D), and Tim-3⁺ (F) populations within 7AAD-CD45.2⁺TCRβ⁺CD8⁺ population are depicted. (G) B16F10 tumor volume in mixed bone marrow chimeras reconstituted with a 1:5 ratio mixture of bone marrow cells from either *Flot2*^{+/+} or *Flot2*^{-/-} mice and *TCRa*^{-/-} mice (n = 13 per group). (H-J) Flow cytometric analysis of TILs. Ki67⁺ populations among 7AAD-CD45⁺CD4⁺ population (H) or 7AAD-CD45⁺CD8⁺ population (I), and CD44⁺IFNγ⁺ populations within 7AAD-CD45⁺CD8⁺ population (J) are shown. Data are representative of two independent experiments (A-F and H-J) or pooled from two independent experiments (G). Data were analyzed by unpaired t-test (B, D, F, and H-J) or two-way ANOVA (G). Error bars denote SEM; *P < 0.05; ****P < 0.0001. ns = non-significant.



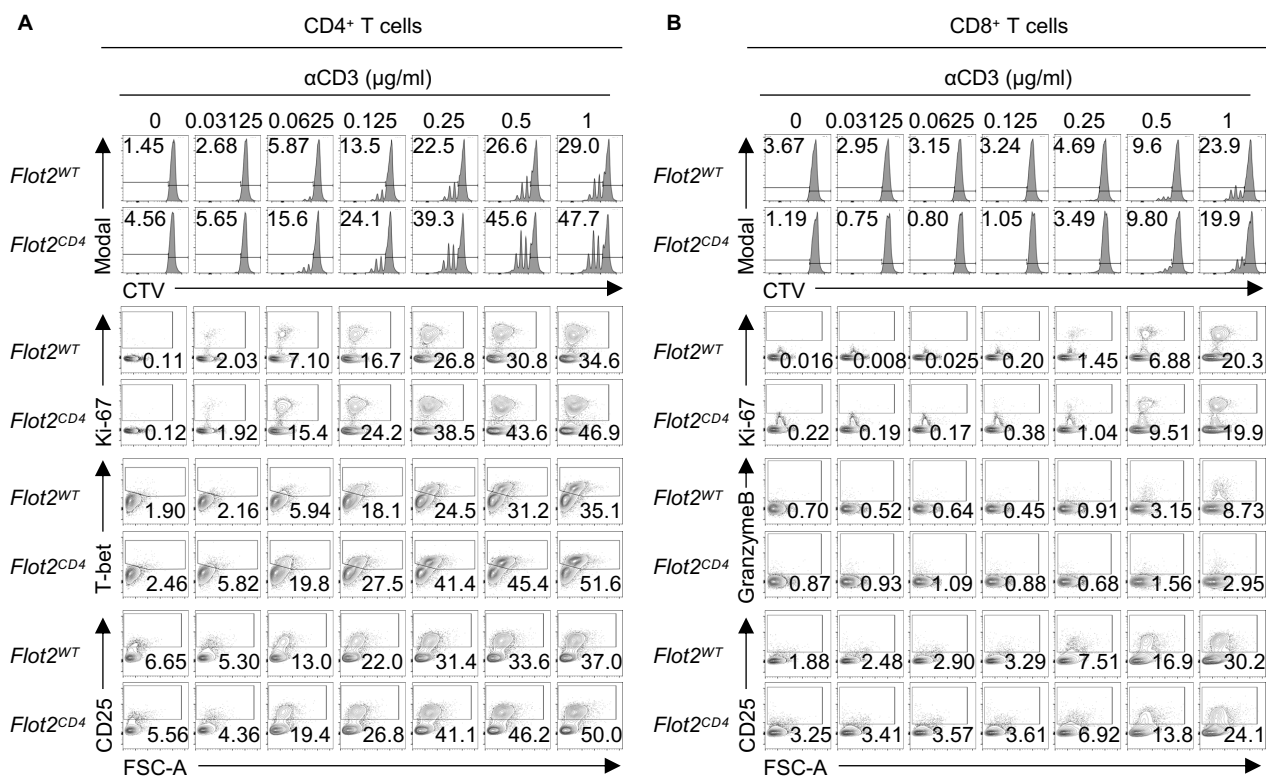
Supplemental Figure 3. Steady state analysis of *Flot2*^{WT} and *Flot2*^{CD4}.

(A-C) *Flot2* mRNA expression was comparable in total spleen (A) or CD11b⁺ cells (B) but was fully deleted in both CD4⁺ and CD8⁺ T cells of *Flot2*^{CD4} mice (C). (D-H) At steady state, the thymus tissues from *Flot2*^{WT} and *Flot2*^{CD4} mice were removed, and thymocytes were isolated using mechanical dissociation, followed by FACS analysis. Representative plots (D and F) are shown. CD4-CD8⁻ (DN), CD4⁺CD8⁺ (DP), CD4⁺CD8⁻ (CD4 SP), and CD4⁺CD8⁺ (CD8 SP) populations among singlet thymocytes (E) and CD25⁻CD44⁺ (DN1), CD25⁺CD44⁺ (DN2), CD25⁺CD44⁻ (DN3), CD25⁻CD44⁻ (DN4) populations within the DN subset (G) in steady state *Flot2*^{WT} or *Flot2*^{CD4} mice are presented. Flow cytometric histogram plot of Nur77 expression at each stage of thymocytes are displayed (H). (I) Cell numbers of total cells, T cells, CD4⁺ T cells, CD8⁺ T cells in inguinal lymph nodes of steady state *Flot2*^{WT} or *Flot2*^{CD4} mice. (J and K) Flow cytometric analysis of TCRβ and CD4 expression on viable lymphocytes at steady state. Representative plots (J) and mean fluorescence intensity (MFI) quantification (K) are shown. (L-N) Flow cytometric analysis of naïve, effector, central memory populations and Nur77, T-bet, LFA-1α expression within steady state lymph node CD4⁺ T cells. Representative plots of each populations are shown (L). CD44-CD62L⁺ (naïve), CD44-CD62L⁻ (effector), CD44-CD62L⁺ (central memory) populations (M) and MFI quantification of Nur77, T-bet, LFA-1α (N) are provided. Data are representative of two independent experiments (A-N). Data were analyzed by unpaired t-test (A-C, E, G, I, K, M, and N). Error bars denote SEM; *P<0.05; ***P<0.001; ****P<0.0001. ns = non-significant. N.D. = not detected.



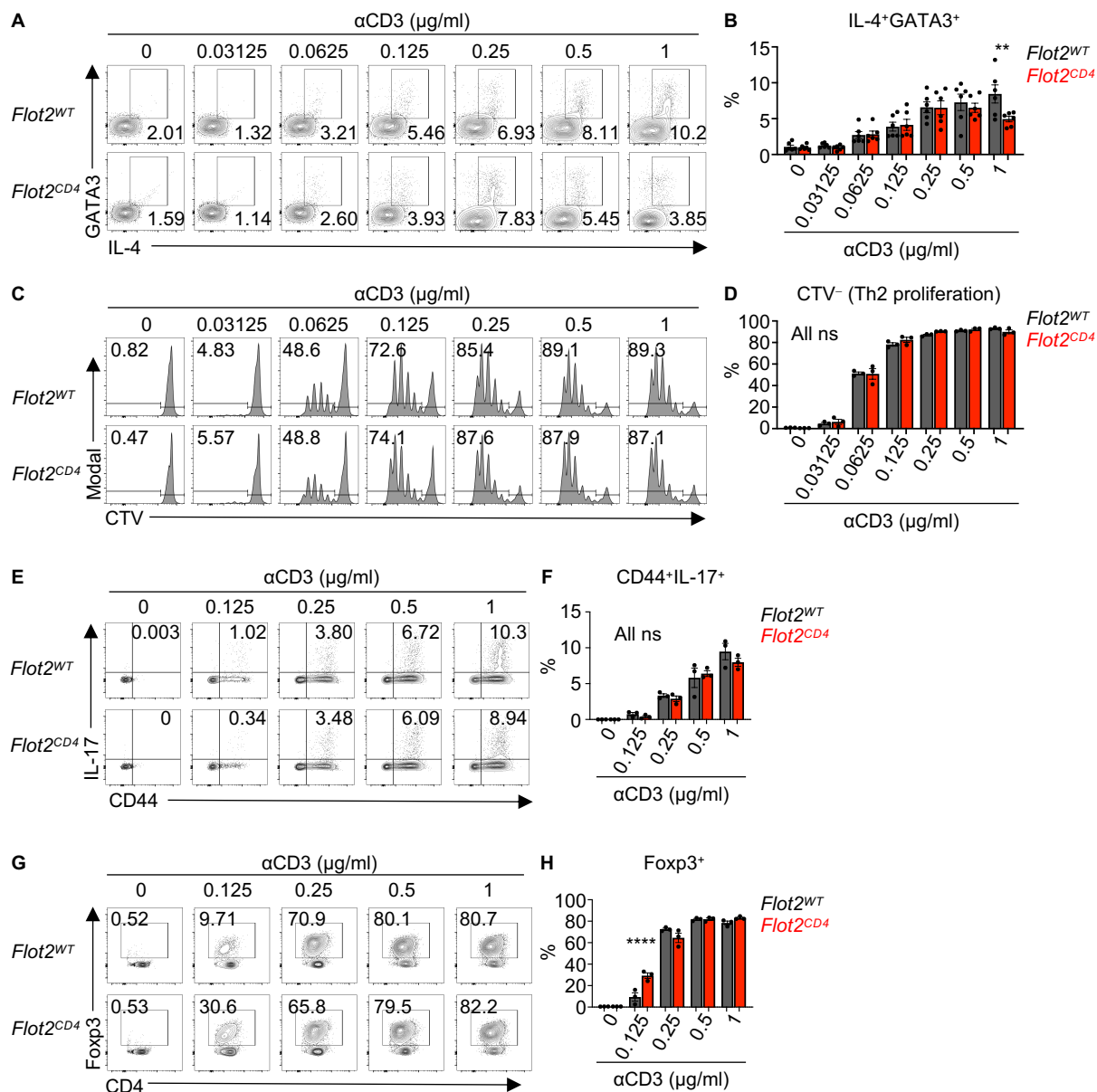
Supplemental Figure 4. *Flot2* deletion boosts effector T cell responses in *Listeria monocytogenes*-infected mice.

(A-J) Flow cytometric analysis of splenic T cells from *Listeria*-infected *Flot2*^{+/+} or *Flot2*^{-/-} mice: Representative plots (A and F) are shown. CD44⁺T-bet⁺ (B), CD44⁺IFN γ ⁺ (C), CD44⁺TNF α ⁺ (D), and CD44⁺IL-2⁺ (E) populations within viable CD45⁺TCR β ⁺CD4⁺ population, and CD44⁺T-bet⁺ (G), CD44⁺IFN γ ⁺ (H), CD44⁺TNF α ⁺ (I), and CD44⁺IL-2⁺ (J) populations within viable CD45⁺TCR β ⁺CD8⁺ population are shown. Data are representative of two independent experiments (A-J). Data were analyzed by unpaired t-test (B-E and G-J). Error bars denote SEM; *P<0.05; ***P<0.001; ****P<0.0001.



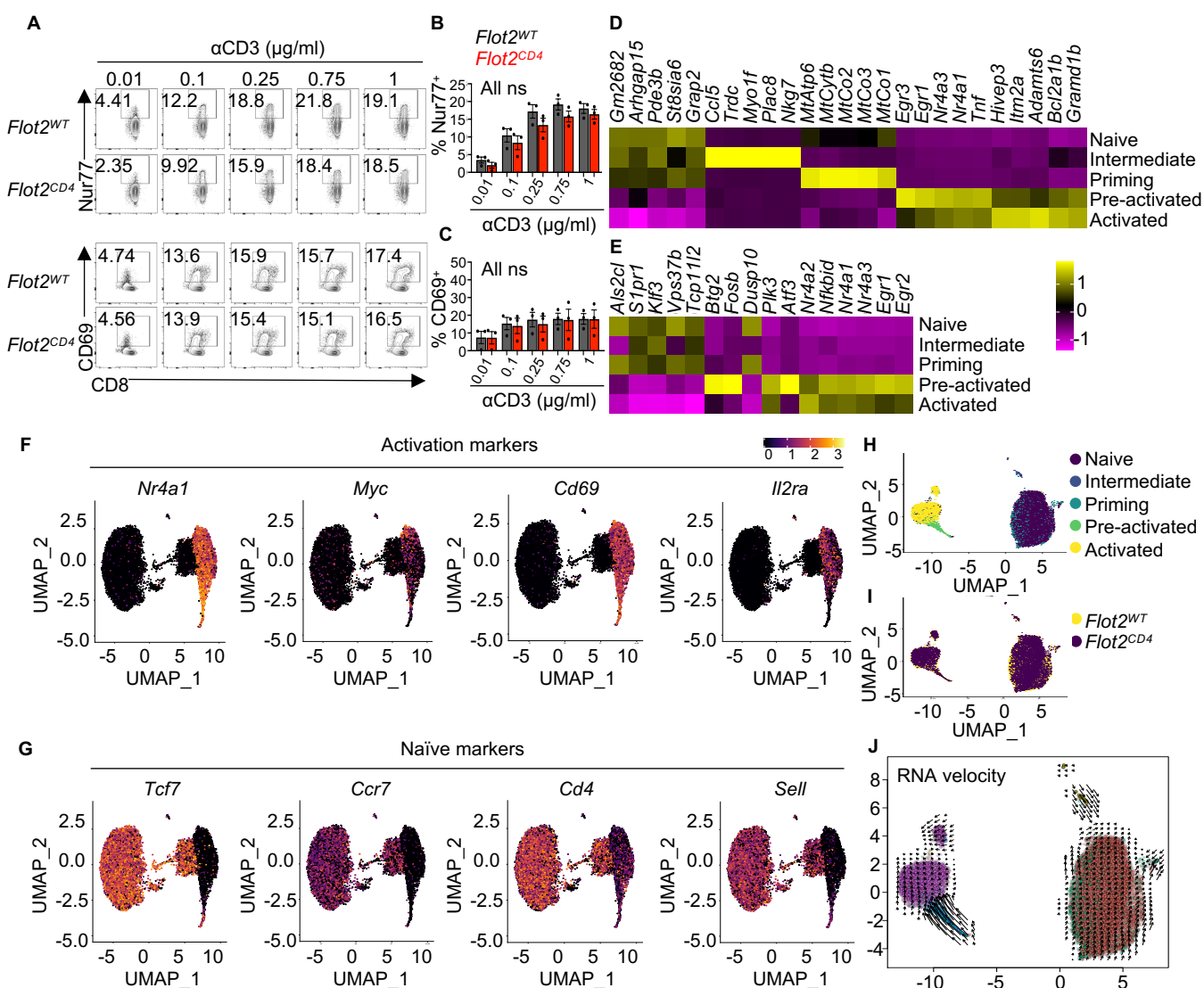
Supplemental Figure 5. *Flot2*^{CD4} CD4⁺ but not CD8⁺ T cells displayed enhanced responses to in vitro stimulation.

Representative plots of flow cytometric analysis of naïve CD4⁺ (A) or CD8⁺ T cells (B) that were stimulated in vitro for 72 hours with varying doses of plate-bound α CD3, alongside a fixed dose of soluble α CD28 (1 μ g/ml). CTV⁻, Ki67⁺, T-bet⁺, and CD25⁺ populations within viable TCR β ⁺CD4⁺ population (A) or CTV⁻, Ki67⁺, Granzyme B⁺, and CD25⁺ populations within viable TCR β ⁺CD8⁺ population (B) are shown. Data are representative of two independent experiments (A and B).



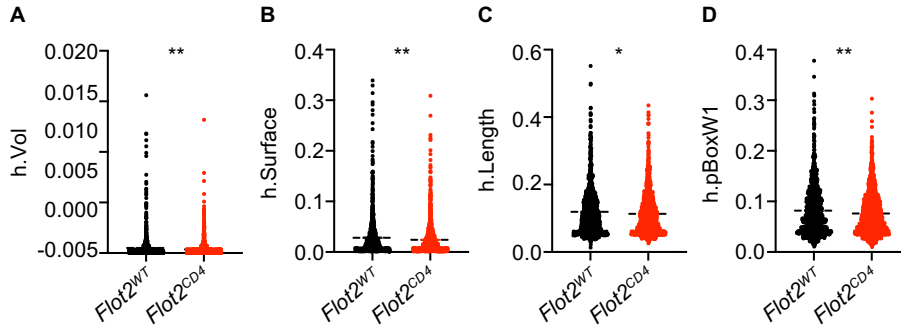
Supplemental Figure 6. Flot2 ablation does not affect Th2 and Th17 differentiation, while enhancing Treg differentiation at weak TCR stimulation.

(A-H) Naïve CD4⁺ T cells were purified and differentiated towards the Th2 (A-D), Th17 (E and F) and Treg (G and H) subtypes in vitro, followed by flow cytometric analysis. Representative plots (A, C, E, and G) are shown. IL-4⁺GATA3⁺ (B), CTV⁻ (D), CD44⁺IL-17⁺ (F), and Foxp3⁺ (H) populations within viable TCRβ⁺CD4⁺ population are shown. Data are representative of two independent experiments (A-H). Data were analyzed by one-way ANOVA followed with Sidak's multiple comparison tests (B, D, F, and H). Error bars denote SEM; **P<0.01; ****P<0.0001. ns = non-significant.



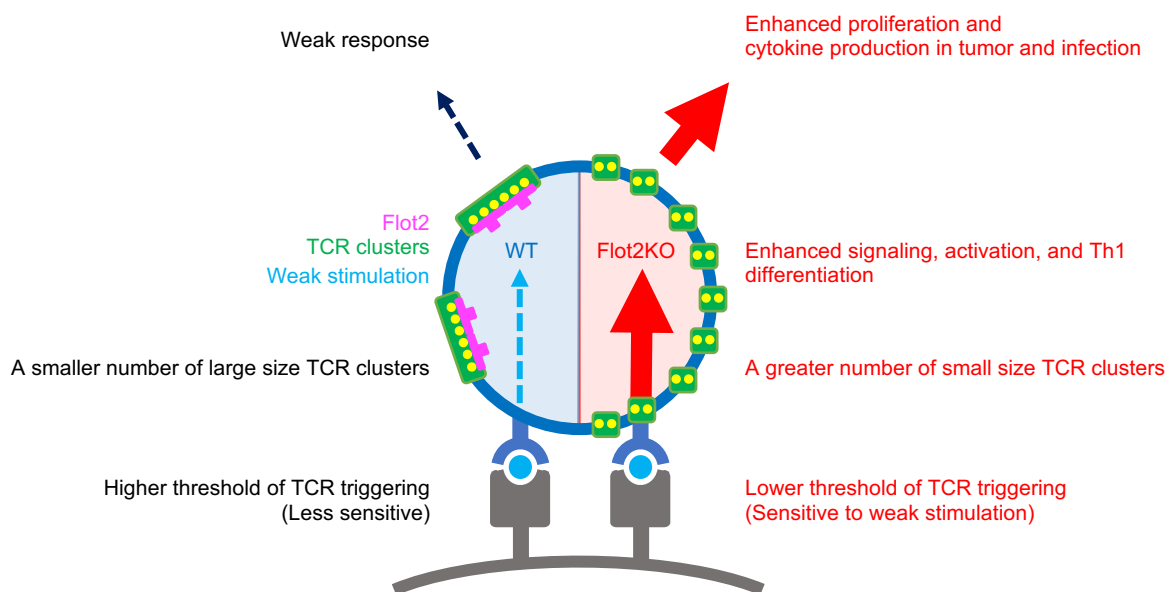
Supplemental Figure 7. Flot2 ablation does not impact the early activation of CD8⁺ T cells upon in vitro stimulation, nor does it affect the transcriptional activation trajectory of CD4⁺ T cells.

(A-C) Naive CD8⁺ T cells were purified and stimulated in vitro for 3 hours (B) or 24 hours (C) with varying doses of plate-bound α CD3, alongside a fixed dose of soluble α CD28 (1 μ g/ml), followed by flow cytometric analysis to assess TCR signaling (Nur77) and early T cell activation (CD69). Representative plots (A) are shown. Nur77⁺ (B) and CD69⁺ (C) populations within viable TCR β ⁺CD8⁺ population are indicated. (D and E) Gene expression heatmap from scRNA-seq analysis. Top markers for clustering (D) and gene set related to early T cell activation (E) are shown. (F and G) Expression of T cell activation (F) or naive state (G) marker genes over the UMAP dot plots. (H-J) T cell activation trajectory fitted by RNA velocity analysis. T cell functional clustering (H), genotype (I), and RNA velocity (J) results are shown. Data are pooled from three independent experiments and were analyzed by one-way ANOVA followed with Sidak's multiple comparison tests (A-C). Error bars denote SEM. ns = non-significant.



Supplemental Figure 8. Convex hull geometry analysis of TCR nanoclusters.

(A-D) Convex hull geometry analysis of TCR β ⁺ nanoclusters in *Flot2^{WT}* and *Flot2^{CD4}* naïve CD4⁺ T cells: Volume enclosed by the convex hull of the cluster in cubic micrometers (h.Vol; **A**), surface of the convex hull of the cluster in squared micrometers (h.Surface; **B**), the largest length of the convex hull of the cluster in micrometers (h.Length; **C**), and the largest width of the convex hull principal box perpendicular to the convex hull length in micrometers (h.pBoxW1; **D**) are quantified and displayed. Data are representative of two independent experiments (A-D). Data were analyzed by unpaired t-test (A-D). Error bars denote SEM; *P<0.05; **P<0.01.



Supplemental Figure 9. Flotillin-2 dampens T cell antigen-sensitivity and functionality.