Supporting Information

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SI Materials and Methods

Bone Marrow Chimeras. Mixed bone marrow chimeras were generated as described (1).

Flow Cytometry and Cell Sorting. Single-cell suspensions were prepared from organs of mice at 6–12 wk of age. T cells were purified from spleen and lymph nodes by positive immunomagnetic selection using CD4 and CD8 beads (Miltenyi or Stemcell Technologies) and sorted as naive ($CD44^{lo}CD62L^{hi}$) $CD4^+$ and $CD8^+$ cells. Cells were stained with fluorochrome-labeled Abs (BioLegend): CD4 (RM4-5), CD8α (53-6.7), TCRβ (H57-597), CD24 (M1/69), CD25 (3C7), CD44 (IM7), CD45.1 (A20), CD45.2 (104), CD62L (MEL-14), and CD69 (H1.2F3). Abs against CD5 (53-7.3) and CD98 (UM7F8) were from BD Biosciences. For intracellular flow cytometry, T cells were permeabilized with methanol as described (2) and stained with Abs (Cell Signaling Technology) against phosphorylated ribosomal protein $S6^{Ser235/236}$ (D57.2.2E), 4E-$BP1^{Thr37/46}$ (236B4), $ERK^{Thr202/Y204}$ (D13.14.4E), and Akt^{Thr308} (C31E5E). Ab against phosphorylated $STAT5^{Y694}$, Lck^{Y505}, $ZAP70^{Y319}$, $p38^{Thr180/Y182}$, and $JNK^{Thr183/Y185}$ were from BD Biosciences.

T-Cell Proliferation in Vivo. Purified naive CD4 (1.5–2 × 10^6) or CD8 (0.5–1 × 10^6) T cells from *Dnm2* HET ($CD45.1.2^+$) and KO ($CD45.2^+$) mice were mixed 1:1 and cotransferred into *Rag1* KO mice ($CD45.1^+$) by i.v. injection to measure lymphopenia-induced proliferation. To determine survival, a 1:1 mixture of naive CD4 (2.5–4 × 10^6) or CD8 (1.5–2 × 10^6) T cells from *Dnm2* HET ($CD45.1.2^+$) and KO ($CD45.2^+$) mice was adoptively transferred into C57BL/6 (B6) mice ($CD45.1^+$) with a full lymphocyte compartment. In some experiments, cells were labeled with CFSE (5 μM final concentration) before transfer. To analyze clonal expansion in response to foreign antigen, 1 × 10^5 naive CD8 T cells from OT-I *Dnm2*$^{flox/flox}$ and OT-I *GzmB*-cre*Dnm2*$^{flox/flox}$ mice were mixed 1:1 and cotransferred into B6 mice. One day after transfer, recipient mice were infected i.v. with 0.5–1 × 10^5 colony-forming units of LM-OVA.

T-Cell Stimulation in Vitro. Purified naive T cells were stimulated with plate-bound α-CD3 Ab (10 μg/mL unless indicated otherwise) and 5 μg/mL soluble α-CD28 Ab. Cells were cultured in 24-well plates at 1–3 × 10^6/mL in RPMI medium 1640 supplemented with 10% (vol/vol) FCS, 2 mM L-glutamine, 1% penicillin–streptomycin, and 55 μM β-mercaptoethanol at 37 °C/5% CO_2. To measure DNA synthesis, T cells (0.5–1 × 10^5 per well) were cultured in U-bottom 96-well plates in a final volume of 200 μL for 24–72 h before being pulsed with 1 μCi ^{3}H-thymidine per well (Amersham) for a further 18 h. ^{3}H-thymidine-uptake was determined by liquid scintillation counting. Cell survival was determined by 7-AAD and annexin V staining. IL-7 (Peprotech) was used at 10 ng/mL. In some experiments, T cells were treated with the indicated concentration of the lysosomal inhibitor bafilomycin A1 (BafA1).

TCR Down-Modulation. Splenocytes were incubated with 10 μg/mL plate-bound α-CD3 Ab for the indicated time points at 37 °C/5% CO_2. After washing, cells were stained for flow cytometric analysis with antibodies against CD4, CD8, and TCRβ. Percent receptor down-modulation was calculated as follows: 100 × [MFI (cells on ice) − MFI (cells incubated with α-CD3 Ab)]/MFI (cells on ice).

Quantitative RT-PCR. Total RNA was extracted from purified cells with TRIzol reagent (Invitrogen) and used for cDNA synthesis with the SuperScript First-Strand Synthesis System (Invitrogen). Quantitative RT-PCR was performed on a 7500 fast real-time PCR system with primer-probe sets purchased from ABI.

Western Blot. Cell lysates were prepared in cell lysis buffer (Cell Signaling Technology) and proteins separated by SDS/PAGE using 4–12% Bis-Tris gels (Invitrogen). The following antibodies were used for immunoblotting (Cell Signaling Technology unless otherwise stated): c-Myc, phospho-S6K1/S6K1, phospho-Akt^{Ser473}/Akt, phospho-$AMPK\alpha^{Thr172}$/AMPKα, phospho-$Tsc2^{Ser939}$/phospho-$Tsc2^{Ser1387}$/Tsc2, phospho-$Raptor^{Ser792}$/Raptor, LC3B (MBL), Glut1 (Geoff Holman, University of Bath, Bath, United Kingdom), and actin (Santa Cruz Biotechnology).

1. Willinger T, Ferguson SM, Pereira JP, De Camilli P, Flavell RA (2014) Dynamin 2-dependent endocytosis is required for sustained S1PR1 signaling. *J Exp Med* 211(4):685–700.
2. Willinger T, Freeman T, Hasegawa H, McMichael AJ, Callan MF (2005) Molecular signatures distinguish human central memory from effector memory CD8 T cell subsets. *J Immunol* 175(9):5895–5903.

Fig. S1. Dynamin 2 is required for TCR down-modulation from the cell surface. Splenocytes from *Dnm2* HET or KO mice were stimulated with 10 μg/mL plate-bound α-CD3 antibody and flow cytometry was used to measure TCRβ surface expression. (*A*) Representative histograms showing TCRβ surface expression on CD4 and CD8 T cells. Green, blue, and red histograms represent freshly isolated cells, cells stimulated for 2 h, and cells stimulated for 8 h, respectively. (*B*) Antibody-induced TCR down-modulation is shown as percent surface down-modulation (calculated as described in *SI Materials and Methods*). All error bars represent SEM. Results are representative of three experiments.

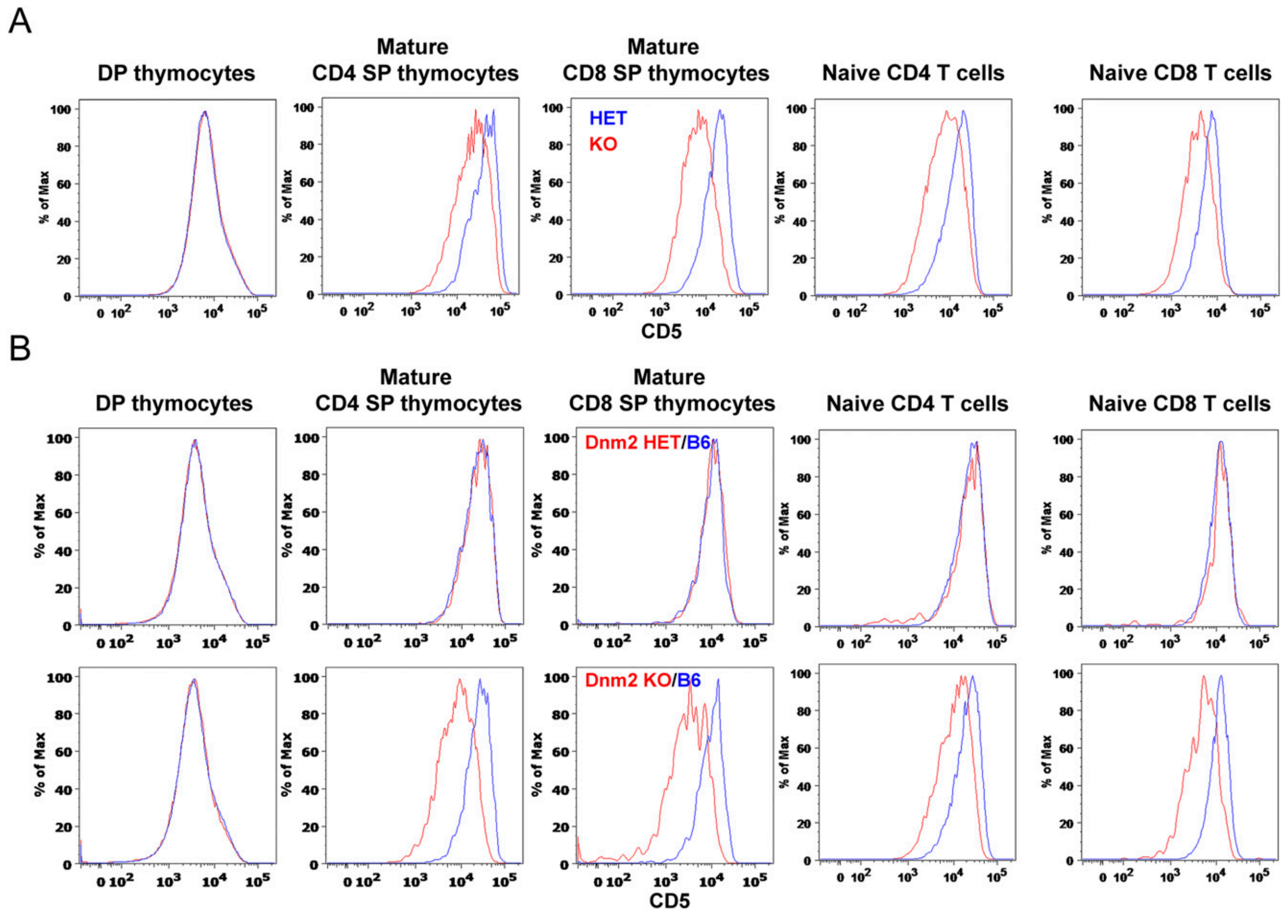


Fig. S2. Dynamin 2 deficiency causes reduced TCR signaling strength. (*A*) CD5 surface expression on thymocytes and peripheral CD4 and CD8 T cells from *Dnm2* HET and KO mice (n = 10) was measured by flow cytometry. Histograms show CD5 expression on double-positive (DP) thymocytes and mature (TCRβ^{hi}CD24^{lo}) single-positive (SP) thymocytes, as well as naive (CD44^{lo}CD62L^{hi}) T cells. (*B*) Bone marrow cells from *Dnm2* HET or KO mice (CD45.2^{+}) were mixed 1:1 with WT B6 cells (CD45.1^{+}) and injected into irradiated *Rag1* KO recipients (CD45.1^{+}) to generate bone marrow chimeras. CD5 surface expression on thymocytes and naive T cells from *Dnm2* HET/B6 and KO/B6 chimeras (n = 7) was measured by flow cytometry. Results are representative of two (*B*) or three (*A*) experiments.

Fig. S3. Blocking thymic egress does not cause reduced TCR signal strength. B6 mice were injected i.p. with PBS or FTY720 (1 mg/kg body weight) for 4 wk. (*A*) S1PR1 surface expression on mature ($TCR\beta^{hi}CD24^{lo}$) CD4 SP thymocytes was measured by flow cytometry. Red and blue histograms represent cells from PBS- and FTY720-treated mice, respectively. (*B* and *C*) Flow cytometric analysis of CD4 SP thymocytes (*B*) and blood T cells (*C*) from B6 mice treated with PBS or FTY720. (*D*) CD5 surface expression on mature CD4 SP thymocytes. Red and blue histograms represent cells from PBS- and FTY720-treated mice, respectively. Results are representative of two experiments ($n = 5$).

Fig. S4. Dynamin 2 is essential for T-cell homeostasis. (*A*) Flow cytometric analysis of CD4 and CD8 T cells from spleen and lymph node of *Dnm2* HET and KO mice. (*B*) Numbers of naive- ($CD44^{lo}$) and memory-phenotype ($CD44^{hi}$) T cells in spleen and lymph node of *Dnm2* HET and KO mice ($n = 10$). All error bars represent SEM. $P > 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by unpaired Student's *t* test (*B*). Results are representative of more than five (*A*) or combined from two (*B*) experiments.

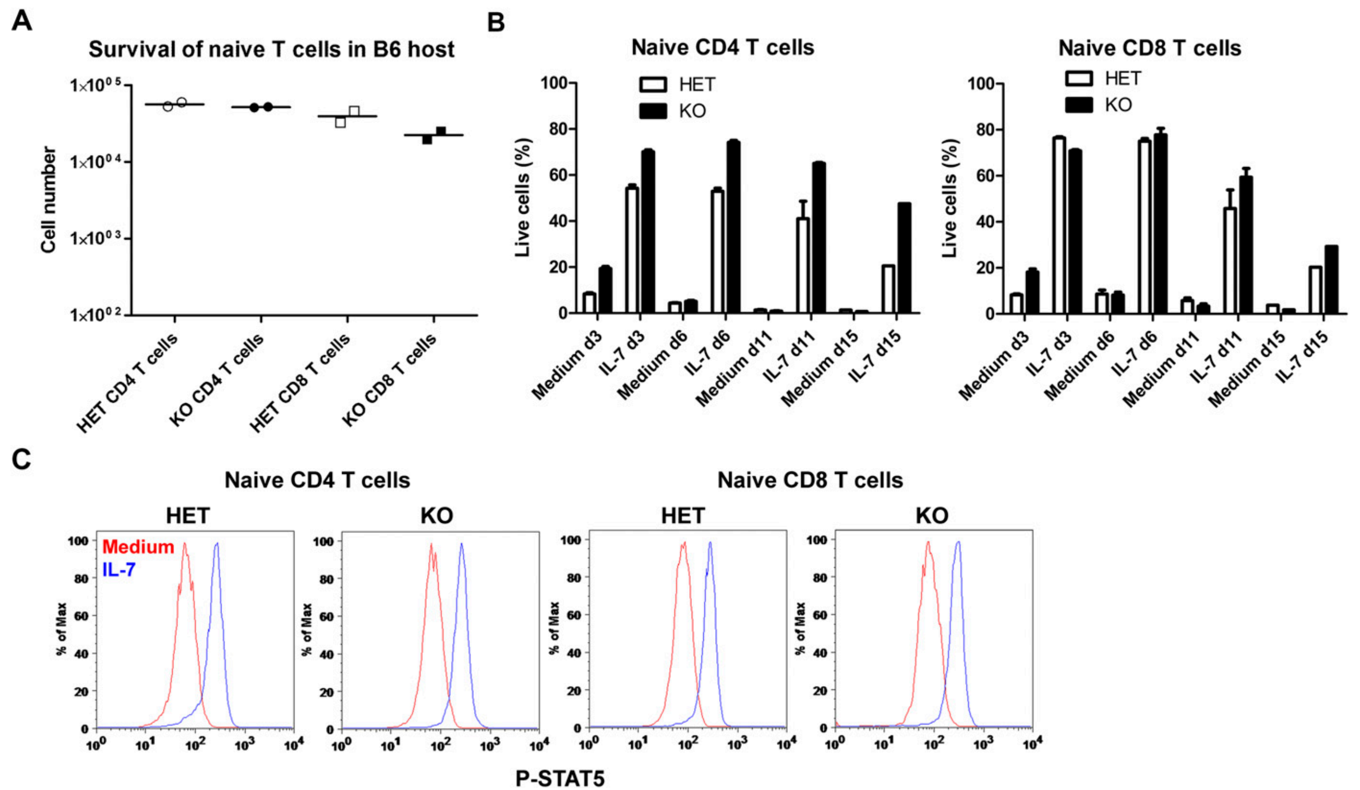


Fig. S5. Dynamin 2-deficient T cells have normal homeostatic survival and respond to IL-7 signals. (*A*) Naive CD4 or CD8 T cells from *Dnm2* HET (CD45.1.2^{+}) and KO (CD45.2^{+}) mice were mixed 1:1 and injected into B6 recipients (CD45.1^{+}). Graph shows the number of transferred T cells that were recovered from spleen and lymph nodes 8 d after transfer. (*B*) Naive CD4 or CD8 T cells from *Dnm2* HET and KO mice were cultured in medium alone or in medium with 10 ng/mL IL-7. The frequency of live T cells (7-AAD^{-}annexinV^{-}) was determined by flow cytometry. (*C*) Phosphorylated STAT5 (P-STAT5) protein was measured in naive CD4 and CD8 T cells cultured in medium alone or stimulated with IL-7. Blue and red histograms represent HET and KO cells, respectively. Results are representative of or combined from two (*A* and *C*) or three (*B*) experiments.

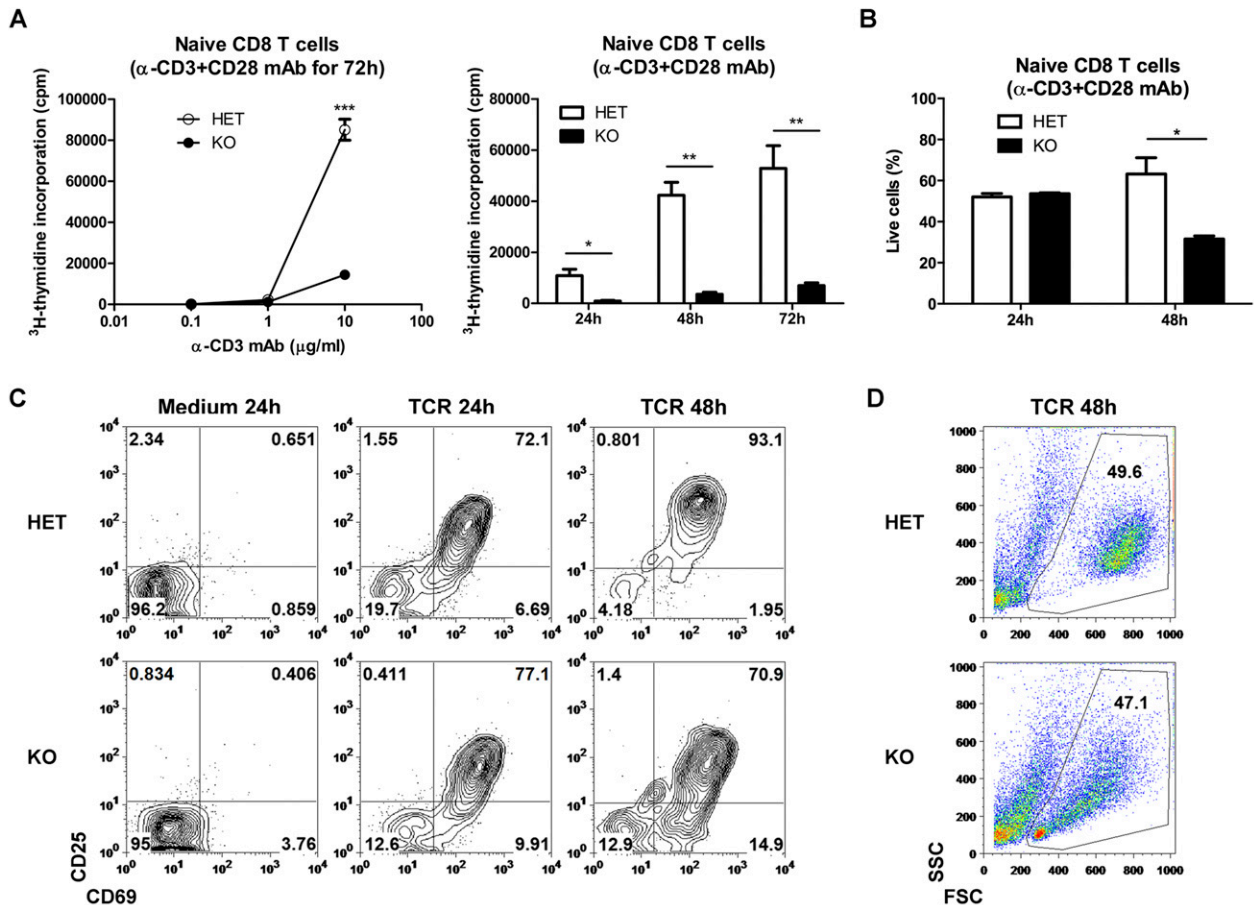
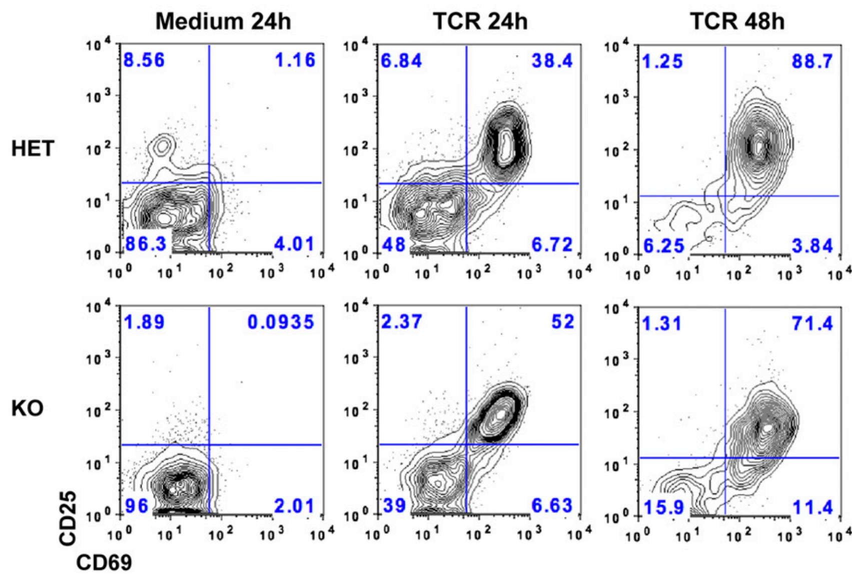


Fig. S6. Dynamin 2 supports the growth of naive CD8 T cells. Naive CD8 T cells from *Dnm2* HET and KO mice were stimulated with plate-bound α-CD3 Ab and soluble α-CD28 Ab in vitro. (*A*) DNA synthesis was measured in *Dnm2* HET and KO cells by ^{3}H-thymidine incorporation as cpm. Graph on the *Left* shows DNA synthesis in naive CD8 T cells stimulated for 72 h with various concentrations of α-CD3 Ab and with 5 μg/mL α-CD28 Ab. Graph on the *Right* shows DNA synthesis in naive CD8 T cells stimulated for the indicated times with 10 μg/mL α-CD3 Ab and 5 μg/mL α-CD28 Ab. (*B*) Frequency of live *Dnm2* HET and KO CD8 T cells 24 and 48 h after stimulation with 10 μg/mL α-CD3 Ab and 5 μg/mL α-CD28 Ab. Live cells were gated as 7-AAD$^-$annexinV$^-$ cells using flow cytometry. (*C*) Flow cytometric analysis of CD25 and CD69 surface expression on *Dnm2* HET and KO CD8 T cells that were either unstimulated (medium) or stimulated with α-CD3 and α-CD28 Abs (TCR) for 24 and 48 h. Numbers next to outlined areas indicate the percentages of cells. (*D*) Representative dot plots showing forward scatter (FSC) and side scatter (SSC) profile of naive CD8 T cells from *Dnm2* HET and KO mice stimulated for 48 h with α-CD3 and α-CD28 Abs. All error bars represent SEM. $P > 0.05$; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by unpaired Student's *t* test. Results are representative of or combined from three (*B* and *C*), four (*A*), or more than five (*D*) experiments.



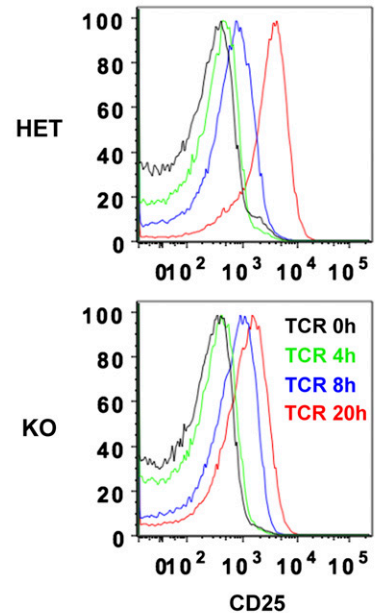


Fig. S7. Expression of activation markers in dynamin 2-deficient CD4 T cells. Naive CD4 T cells from *Dnm2* HET and KO mice were stimulated with plate-bound α-CD3 Ab and soluble α-CD28 Ab in vitro. (*A*) Flow cytometric analysis of CD25 and CD69 surface expression on *Dnm2* HET and KO CD4 T cells that were either unstimulated (medium) or stimulated with α-CD3 and α-CD28 Abs (TCR) for 24 and 48 h. Numbers next to outlined areas indicate the percentages of cells. (*B*) CD25 surface expression was determined in naive *Dnm2* HET and KO CD4 T cells activated with α-CD3 and α-CD28 mAbs for the indicated time periods. Results are representative of two experiments.

Fig. S8. MAP kinase and tyrosine kinase signaling in T cells lacking dynamin 2. Naive CD4 T cells from *Dnm2* HET and KO mice were activated with α-CD3 and α-CD28 mAbs for the indicated time periods and phosphorylated ERK (*A* and *B*), JNK (*C*), p38 (*D*), Lck (*E*), and ZAP70 (*F*) measured by intracellular flow cytometry. MFI, mean fluorescence intensity. Results are representative of or combined from two experiments.

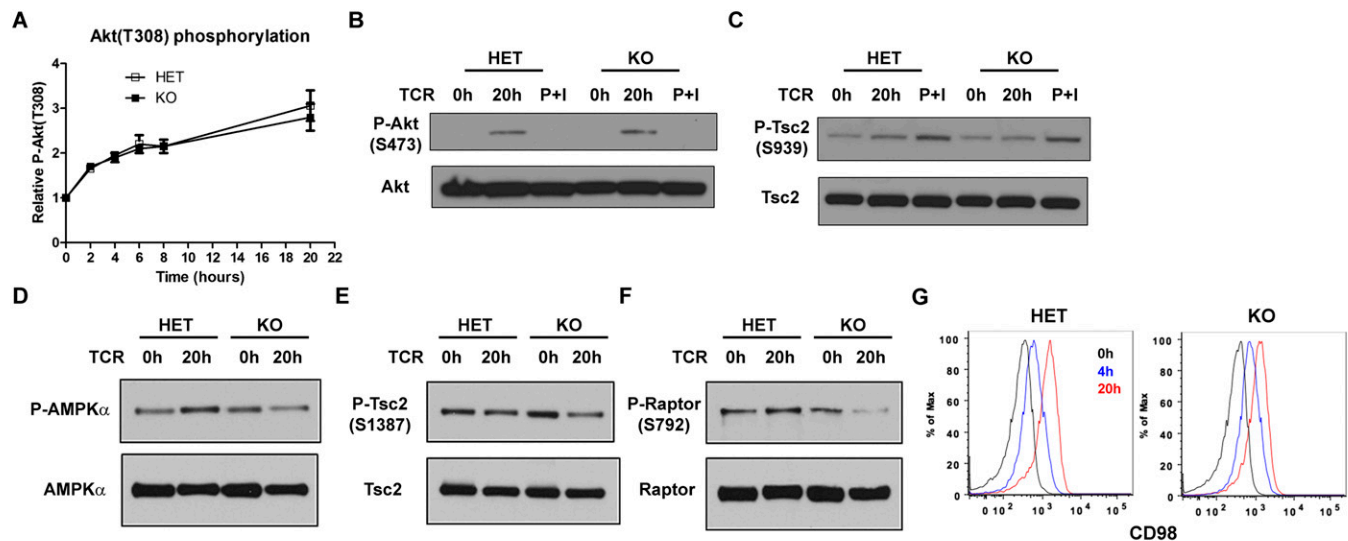


Fig. S9. Upstream mTORC1 regulators in dynamin 2-deficient T cells. (*A*) Phosphorylation of Akt on threonine 308 was measured in *Dnm2* HET and KO CD4 T cells by intracellular flow cytometry. Graph shows the mean fluorescence intensity of phosphorylated Akt in activated T cells relative to unstimulated cells (0 h). (*B–F*) Western blot analysis of phosphorylated Akt^{Ser473} (*B*), $Tsc2^{S939}$ (*C*), AMPKα (*D*), $Tsc2^{S1387}$ (*E*), and Raptor (*F*) in activated CD4 T cells from *Dnm2* HET or KO mice. P + I, PMA + ionomycin. (*G*) Flow cytometric analysis of CD98 surface expression on naive CD4 T cells from *Dnm2* HET and KO mice. Black, blue, and red histograms represent freshly isolated cells, cells stimulated for 4 h, and cells stimulated for 20 h, respectively. All error bars represent SEM. Results are representative of or combined from two experiments.

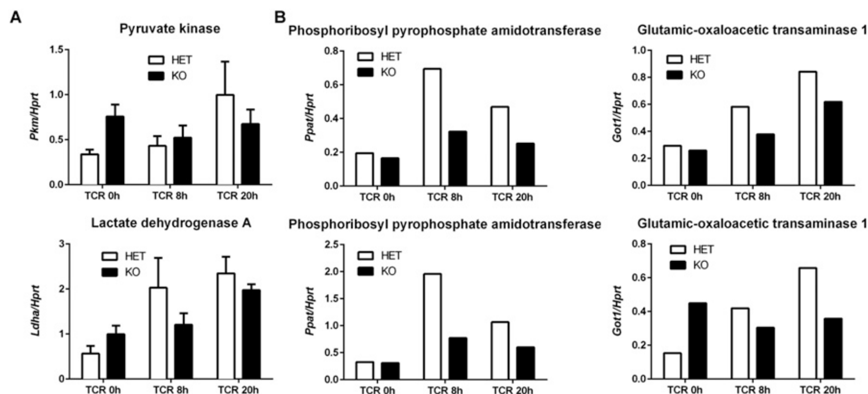


Fig. S10. Dynamin 2 promotes the induction of glutaminolytic enzymes in T cells. (*A*) Pyruvate kinase (Pkm) and lactate dehydrogenase A (Ldha) mRNA expression in CD4 T cells from *Dnm2* HET and KO mice was measured by quantitative RT-PCR ($n = 5$). All error bars represent SEM. (*B*) mRNA expression of phosphoribosyl pyrophosphate amidotransferase (Ppat) and glutamic-oxaloacetic transaminase 1 (Got1) in *Dnm2* HET and KO CD4 T cells. Two individual experiments are shown.