Supplemental Figures

Figure S1. Related to Figure 1. T_E and T_M cells generated ± αCD28 display similar surface marker expression and viability and SRC early after activation is dependent on CD28 signals, independent of strength of signal by the TCR. **A)** Flow cytometric analysis of 2NBDG uptake during activation of cells activated with anti-CD3 ± anti-CD28. **B)** Seahorse assay showing oxygen consumption after consecutive injection of activator beads, Oligomycin, FCCP (F), and Rotenone and Antimycin (R+A) (n=3). **C)** ATP assay run on 10^5 cells activated ± αCD28 at 8 hours after activation (n=6). **D)** Histograms depicting CD44 and CD62L expression post activation. Top panels represent T_E cells, bottom panels T_M cells, blue histograms represent αCD3 priming, black histograms αCD3/28. Representative of >3 independent experiments. **E)** 7-AAD staining showed no difference in viability between in vitro T_M cells generated ± αCD28. **F)** T_M cells generated as in **Figure 1B** were restimulated with αCD3/28 beads and IL-2. ECAR was followed for 8 hr and maximal glycolytic capacity was assessed by sequential treatment with Oligomycin (Oligo), FCCP, Rotenone and Antimycin (R+A). **G)** Seahorse plots of TE cells under AGR conditions, where SRC is only present in cells that received CD28 mediated costimulation. **H)** Averaged bar graphs of 2 Independent experiments depicting SRC during AGR in TE cells initially stimulated ± αCD28. **I)** 7-AAD staining showed no difference in viability between in vitro T_E cells generated ± αCD28 in normal glucose or during AGR. Bar graphs depict mean ± SEM. **J)** Splenocytes from OTI mice were activated with high affinity peptide (SII**N**FEKL) or low affinity peptide (SII**Q**FEKL) and oxygen concentration and pH of extracellular media was measured during the first 48h after activation. Costimulation was blocked using CTLA4-Ig (n=2). Blocking costimulation via CD28 with CTLA4-Ig inhibits oxygen consumption in cells activated with high and low affinity peptides, although the effect is more marked in cells activated with the high affinity peptide. These data indicate that CD28 has the greatest effect on oxygen consumption of cells activated with stronger TCR stimuli, rather than boosting activation in cells activated with weaker stimuli. The data showing pH, suggest the same is true for aerobic glycolysis. **K)** Dilution series of anti-CD28 (μg/ml) during activation (n=3) * = $p < 0.05$ (unpaired t-test). **L)** T cell receptor beta (TCR-β) expression during activation (n=3).

Figure S2. Related to Figures 2 and 3. Imaris surface rendering as a quantifier for mitochondrial sphericity. Imaris software was used to quantify mitochondrial sphericity through surface rendering of the GFP signal localizing to the mitochondria.

A surface to volume algorithm was used to determine sphericity in images obtained from cells activated ± αCD28 at different time points after activation. **A)** Shown are representative images at 8 hours after activation of T_E cells activated ±CD28. Colour scheme depicts sphericity as binned in **Figures 2A-B** and **3D**. Elongated mitochondria are Blue / low sphericity (0), round mitochondria are Red / high sphericity (1). Scale bar 5 µm. **B)** dot plots representing individual measurements of mitochondria per group. High sphericity, Round (1) to low sphericity, elongated (0) depicted is Mean ± SEM, *** P < 0,001 by comparing two groups with two tailed students T test. **C)** High resolution EM micrographs of CD8 T cells stimulated with anti CD3 ± 28. Representative images of cells 8 hours after activation, T_E cells and T_M cells as in **Figure 2C-D)**

Figure S3. Related to Figures 3 and 4. CD28-mediated SRC and mitochondrial remodelling during metabolic stress is dependent on Cpt1a function. A) siRNA knockdown efficiency in T_N cells before activation (n=3) **B)** Activation of cells generated in panel **A**. for 8 hours, followed by injection of Oligomycin and FCCP. Graphs represent mean ± SEM of three biological replicates. **C)** Bar graphs depicting relative bodipy-labelled palmitate uptake at 8 and 24 hours after activation ± αCD28. No differences between activation conditions were observed (n=4). **D)** Seahorse plot of ex vivo T_M cells isolated from CD80/86$^{-/-}$ mice showing ETO injection (same experiment as shown in **Figure 1C**). **E)** T_N cells isolated from 3 mice were transduced with retrovirus expression Cpt1a or Luciferase shRNA, LUC-sh. 48 hours after transduction cells were activated and cultured as in figure 1b. Sorted GFP$^+$ T_M cells were reactivated with anti-CD3/28 and assayed for the production of IFN-γ. Histograms depict n=3 biological replicates. **F)** Cpt1a-sh retrovirus was used to transduce T_E cells to validate knockdown (n=2). * p<0.05 by two tailed student's T test. **G)** EM images of cells primed with αCD3/CD28 ±ETO. **H)** Maximal cristae width of WT and OPA^{Tg} naïve CD8 T cells activated for 8 hours using anti-CD3 ±CD28. Bar graph depicts mean ±SEM of maximum cristae width in arbitrary units, measured from 50 images from each group using Fiji/Image J software. Statistical comparisons for two groups were calculated by using unpaired two-tailed Student's ttest, ** P < 0.01; * P < 0.05.

Figure S4. Related to Figures 3 and 4. CD28 costimulation increased Cpt1a-dependent allocation of palmitate carbons to the TCA cycle. A) Activation ±CD28 does not alter allocation of carbons from Glucose **B)** or Glutamine. **C)** CD28 signals increase carbon allocation from palmitate to TCA intermediates at 24 hours

after activation. **D)** Schematic highlighting flux patterns of the metabolites shown in panels **A-C**.

Figure S5. Related to Figure 5. CD28 downstream signals suppress TXNIP in murine and human CD8$^+$ T cells, which leads to elevated levels of Cpt1a. A) Top 10 differentially expressed genes 24 hours after activation of CD4$^+$ T cells. Log fold change between αCD3 and αCD3/28 (n=4) ranked on p value as assessed by GEO2R (Analysis from data in ref 11). **B)** Western blot analysis at 48 hours after activation with αCD3 ± CD28. Signals shown from isolated lanes all developed on a single western blot. **C)** CD28 signals restrain TXNIP expression in human CD8$^+$ T cells 24 hours after activation. Histograms reflect densitometry of TXNIP bands normalised to GAPDH bands from the same sample. Western blots performed on samples from three healthy donors. **D)** Flow cytometric analysis of CPT1A expression in human T cells from 2 healthy donors 24 hours after activation (independent repeat experiment from data shown in **Figure 5D**). **E)** Ratio of CPT1A and TXNIP protein expression levels 24 hours after activation. N=3 healthy human controls. **F)** Flow cytometric analysis of CPT1A expression levels in CAR T cells after activation on target tumor cells. Adding soluble αCD28 enhances the expression of CPT1A in 1st generation CAR T cells.