Supporting Information

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SI Materials and Methods

Mice and Reagents. C57BL/6 and major histocompatibility complex class I-restricted OVA-specific T-cell–receptor (OT-I) transgenic mice were purchased from The Jackson Laboratory, and rederived stocks were maintained at the Washington University in St. Louis under protocols approved by the Institutional Animal Care and Use Committee. Polyclonal and OT-I naïve T (T_N) cell isolations from spleens and lymph nodes were done based on CD8 expression by using magnetic activated cell sorting (MACS) purification (Miltenyi Biotech). Polyclonal CD8 T_N cells ($CD44^{lo}$ $CD62L^{hi}$) and memory T (T_M) cells ($CD44^{hi}$ $CD62L^{hi}$) were isolated from naïve and *Listeria monocytogenes* expressing OVA (LmOVA)-infected mice (1×10^7 cfu per mouse i.p.) by using a FACSAriaII (BD Biosciences). The soluble peptides MIAAQLLAYYFTELK(beta-Ala)GYGR-KKRRQRRG-amide (HK-1), MIASHMIACLFTELN(beta-Ala) GYGRKKRRQRRG-amide (HK-2), and GYGRKKRRQRRG-amide (control) were produced by Selleckchem.

Flow Cytometry and Intracellular Cytokine Staining. All fluorochrome-conjugated monoclonal antibodies were purchased from BD Pharmingen, eBioscience, and Biolegend. All staining was performed as described (1). Mitotracker green and deep red (2) staining was performed according to the manufacturer's instructions (Invitrogen). To assess proliferation, cells were fluorescently labeled with CFSE or Cell Trace Violet (Invitrogen), activated with anti-CD3 (5.0 μg/mL) plus anti-CD28 (0.5 μg /mL), when indicated in the presence of 5 nM oligomycin, 200 μM etomoxir (Sigma), 25 μM clotrimazole (CLT; EMD Millipore), or 10 μM peptides. The 7AAD-negative cells were analyzed for dilution of the dye. For intracellular cytokine staining, cells were stimulated for 2 d with aCD3/CD28 and GolgiStop (BD Pharmingen) was added for the last 4 h.

In Vitro Cultures and Retroviral Transductions. OT-I splenocytes were activated with OVA-peptide and IL-2 (100 U/mL) for 3 d and subsequently cultured in the presence of either IL-2 or IL-15 (10 ng/mL) for 3–4 d to generate IL-2 T_E and IL-15 T_M cells, respectively (3). For (re)stimulation assays, cells were activated with anti-CD3 (5.0 μg/mL) plus anti-CD28 (0.5 μg /mL). For retroviral transduction experiments, OT-I splenocytes were activated, transduced with control (virus expressing shRNA against luciferase), virus expressing shRNA against CPT1a (hpCPT1a), or virus expressing shRNA against MPC1 (hpMPC1) 1 d later, and subsequently cultured as described. GFP is a marker of retroviral expression and was used to positively select transduced cells by using a FACSAriaII when required for the assay.

Metabolism Assays. Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured in XF media (nonbuffered RPMI medium 1640 containing 25 mM glucose, 2 mM L-glutamin, and 1 mM sodium pyruvate), under basal conditions and in response to 25 μM CLT, (5–20 μM) peptides, 200 μM etomoxir, (5 or 10 nM, or 1 μM) oligomycin, 1.5 μM FCCP, 100 nM rotenone plus 1 μM antimycin A, and PMA (5–50 ng/mL) plus ionomycin (500 ng/mL) (Sigma), or anti-CD3/CD28 coated beads (Gibco) using the XF-24 or XF-96 Extracellular Flux Analyzer (Seahorse Bioscience). ATP measurements were performed by using the ATP determination kit (Invitrogen).

Analysis of Metabolites by GC-MS. OT-I cells were activated with OVA peptide and IL-2 for 3 d and subsequently cultured in IL-2 or IL-15 for 4 d to generate effector T (T_E) and T_M cells, respectively. Cells were pulsed for 4 h with [U-^{13}C] glucose (Cambridge Isotope Laboratories) before harvesting and fixing with 80% methanol (vol/vol) on dry ice. Lysates were sonicated and then centrifuged at 10,000 × *g* for 10 min at 4 °C, and the supernatants were stored at −80 °C until processing by GC-MS. For GC-MS analysis, dried samples were resuspended in 30 μL of anhydrous pyridine and added to GC-MS autoinjector vials containing 70 μL of *N*-(tert butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA) derivatization reagent. The samples were incubated at 70 °C for 1 h, following which aliquots of 1 μL were injected for analysis. GC-MS data were collected on an Agilent 5975C series GC/MSD system (Agilent Technologies) operating in election ionization mode (70 eV) and selected ion monitoring. The amount of each metabolite in the sample was calculated from the ratio of the integrated areas of the metabolite to the tricarballylic acid internal standard. This ratio was then compared with authentic sample calibration curves (all calibration curves were linear with R values better than 0.97). The amount of each reported metabolite was normalized to cell number.

Imaging. T_N and T_M cells were stained for Mitotracker green and DRAQ5 (Thermo Scientific).

RT-PCR and Western Blot Analysis. RNA isolations were done by using the RNeasy kit (Qiagen), and single-strand cDNA was synthesized by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Genomic DNA was extracted by using QIAamp DNA micro kit (Qiagen) to determine mtDNA/nDNA ratios (4). Primers were purchased from Applied Biosystems and real-time PCR was performed by the Taqman method using an Applied Biosystems 7000 sequence detection system. The expression levels of mRNA were normalized to the expression of a housekeeping gene (β-actin). Mitochondria were isolated by using the Mitosciences isolation kit. SDS/PAGE, electrophoretic transfer, immunoblotting, and development by using enhanced chemiluminescence were accomplished as described (1). The GAPDH, prohibitin I, and hexokinase I and II antibodies for Western blot analysis were purchased from Cell Signaling.

Statistical Analysis. Comparisons for two groups were calculated by using unpaired two-tailed Student's t tests.

1. Pearce EL, et al. (2009) Enhancing CD8 T-cell memory by modulating fatty acid metabolism. *Nature* 460(7251):103–107.
2. Zhou R, Yazdi AS, Menu P, Tschopp J (2011) A role for mitochondria in NLRP3 inflammasome activation. *Nature* 469(7329):221–225.
3. van der Windt GJ, et al. (2012) Mitochondrial respiratory capacity is a critical regulator of CD8+ T cell memory development. *Immunity* 36(1):68–78.
4. Guo W, Jiang L, Bhasin S, Khan SM, Swerdlow RH (2009) DNA extraction procedures meaningfully influence qPCR-based mtDNA copy number determination. *Mitochondrion* 9(4):261–265.

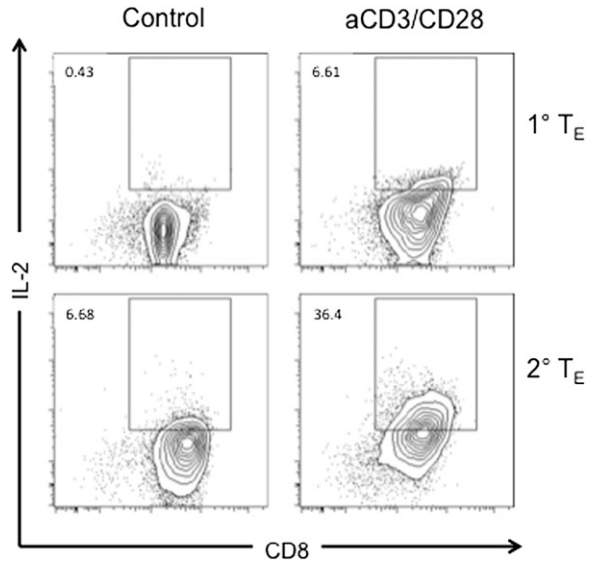

Fig. S1. Secondary T_E cells make more IL-2 than primary T_E cells. OT-I cells were activated with OVA peptide and IL-2 for 3 d and, subsequently, cultured in IL-15 for 3 d to generate IL-15 T_M cells. Purified naïve OT-I cells and IL-15 T_M cells were (re)stimulated with anti-CD3/28 for 2 d, and IL-2 production was determined. Data are representative of two independent experiments.

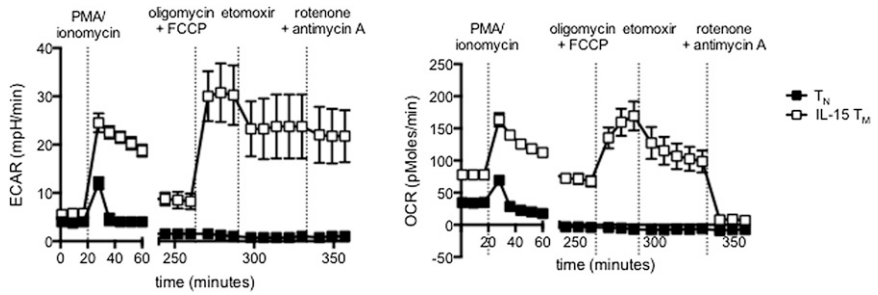

Fig. S2. IL-15 T_M cells maintain metabolic activity after PMA/iono stimulation. OT-I cells were activated with OVA peptide and IL-2 for 3 d and, subsequently, cultured in IL-15 for 3 d to generate IL-15 T_M cells. ECAR and OCR were measured in real-time in naïve OT-I and IL-15 T_M cells at basal levels, after stimulation with PMA (50 ng/mL) and ionomycin (500 ng/mL) (also shown in Fig. 4*C*), and after injection of oligo (1 μM) plus FCCP (1.5 μM), etomoxir (200 μM), and rotenone (100 nM) plus antimycin A (1 μM). Data are generated from the same experiment as shown in Fig. 4*C* and are representative of at least three independent experiments. Data are shown as mean ± SEM.

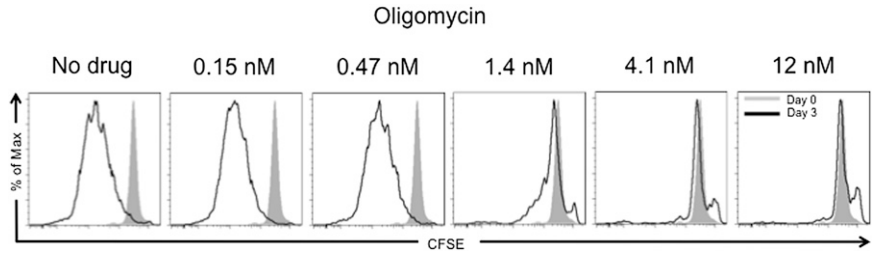

Fig. S3. Mitochondria-derived ATP is required for the initiation of proliferation in polyclonal CD8 T_N cells. Naïve polyclonal CD8 T cells were purified, CFSE labeled, and stimulated with anti-CD3/28 in the absence or presence of oligo (day 0). Stimulation induced proliferation is shown at day 0 and day 3. Data are representative of one experiment.

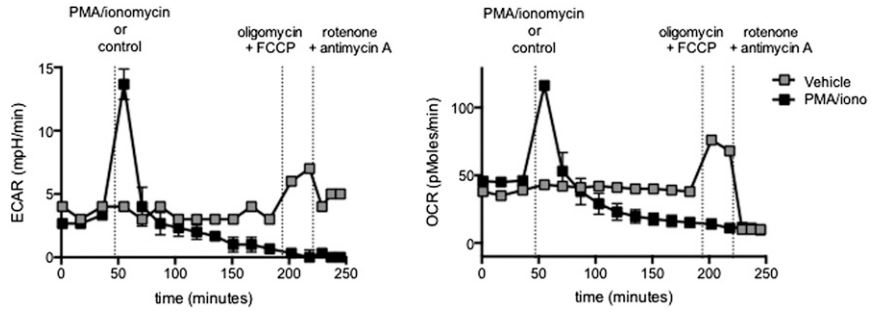

Fig. S4. T_N cells do not maintain metabolic activity after PMA/iono stimulation. $CD44^{lo}$ $CD62L^{hi}$ T_N cells were isolated from uninfected mice, and OCR and ECAR were measured in real-time, after stimulation with PMA (5 ng/mL) and ionomycin (500 ng/mL), and subsequently oligo (1 μM) plus FCCP (1.5 μM), and rotenone (100 nM) plus antimycin A (1 μM). Data are shown as mean ± SEM and representative of two independent experiments. T_N PMA group is the same as in Fig. 6C.

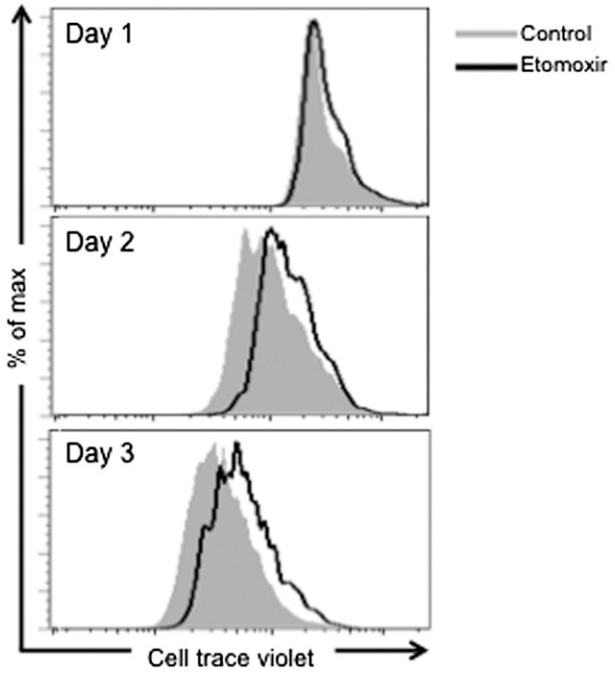

Fig. S5. Inhibition of fatty acid oxidation impairs proliferation of IL-15 T_M cells. OT-I cells were activated with OVA peptide and IL-2 for 3 d and, subsequently, cultured in IL-15 for 3 d to generate IL-15 T_M cells. These cells were restimulated with anti-CD3/28 and induced proliferation (as shown by Cell Trace Violet dilution) in the absence or presence of etomoxir is shown. Data are a different experiment as shown in Fig. 7*B* and is representative of four independent experiments.

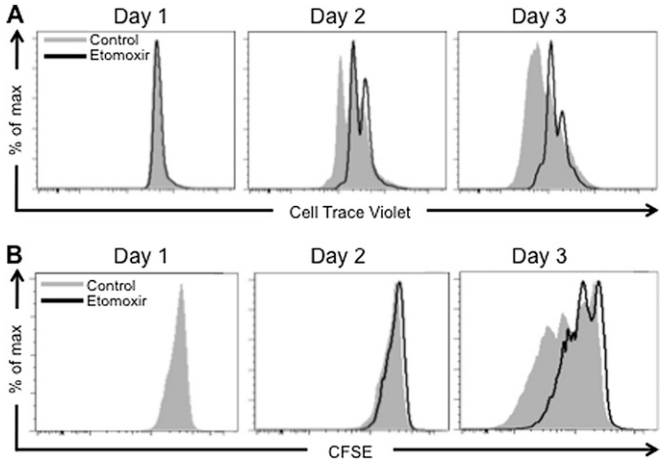

Fig. S6. Inhibition of fatty acid oxidation impairs proliferation of T_N cells. (*A*) Purified naïve OT-I cells were stimulated with anti-CD3/28, labeled with Cell Trace Violet, and proliferation was observed in the absence or presence of etomoxir is shown. Data are representative of three independent experiments. (*B*) Polyclonal CD8 T_N cells were isolated from LmOVA-infected mice based on $CD44^{lo}$ and $CD62L^{hi}$ expression, stimulated with anti-CD3/28 in the absence or presence of etomoxir (day 0), and (re)stimulation-induced proliferation (as shown by CFSE dilution) is shown at day 1, 2, and 3. Data are representative of one independent experiment. Control group is the same as in Fig. 6*G* and Fig. S12*B*.

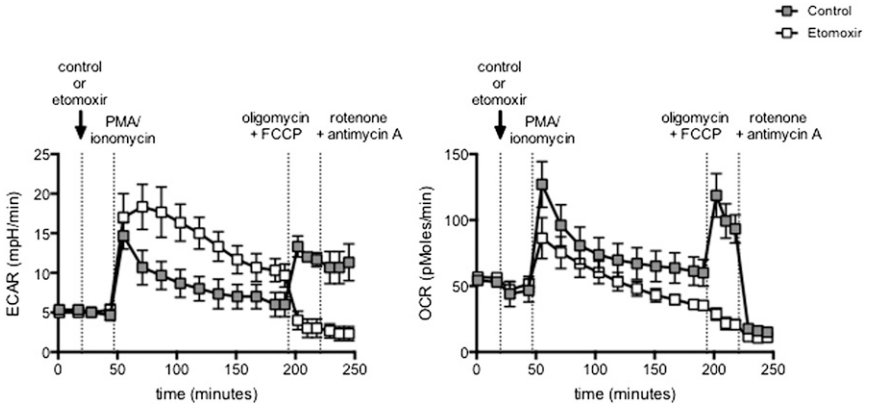

Fig. S7. Inhibition of fatty acid oxidation impairs OXPHOS in T_M cells after PMA/iono stimulation. T_M cells were isolated from LmOVA-infected mice and restimulated with PMA (5 ng/mL) and iono (500 ng/mL) in the presence or absence of etomoxir, subsequently exposed to oligo (1 μM) plus FCCP (1.5 μM), and rotenone (100 nM) plus antimycin A (1 μM), and OCR and ECAR were measured in real-time. Data are shown as mean ± SEM and representative of two independent experiments. T_M control group is the same as in Figs. 6*C* and 8*F*.

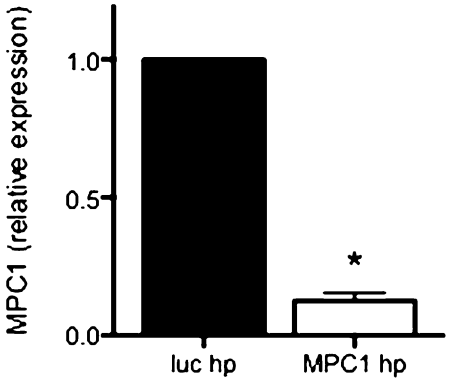

Fig. S8. MPC1 expression. OT-I cells were activated with OVA peptide and IL-2 for 3 d and subsequently cultured in IL-15 for 3 d to generate IL-15 T_M cells. Relative MPC1 RNA expression is shown in IL-15 T_M cells transduced with either control (virus expressing shRNA against luciferase) or virus expressing shRNA against MPC1 (MPC1 hp). Data are shown as mean ± SEM; figure is generated from two independent experiments; $*P < 0.0001$.

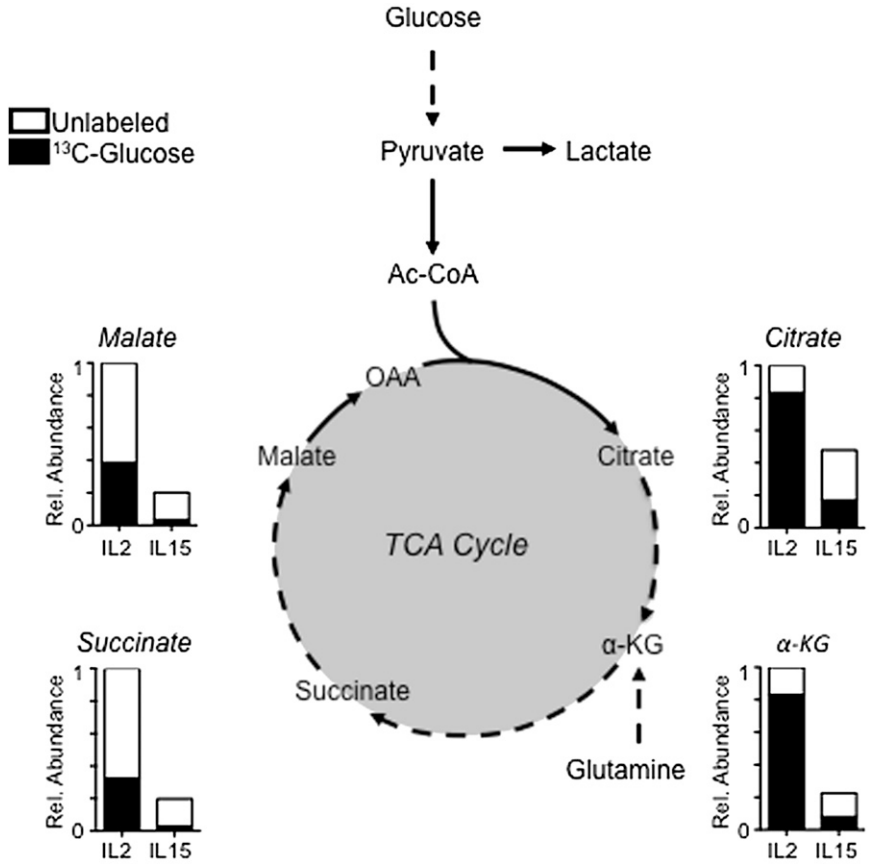

Fig. S9. Metabolite analysis of IL-2 T_E and IL-15 T_M cells. OT-I cells were activated with OVA peptide and IL-2 for 3 d and, subsequently, cultured in IL-2 or IL-15 for 4 d to generate IL-2 T_E and IL-15 T_M cells, respectively. Cells were pulsed for 4 h with U-^{13}C-glucose and relative abundance of tricarboxylic acid (TCA) cycle intermediates is shown. Data are representative of one experiment.

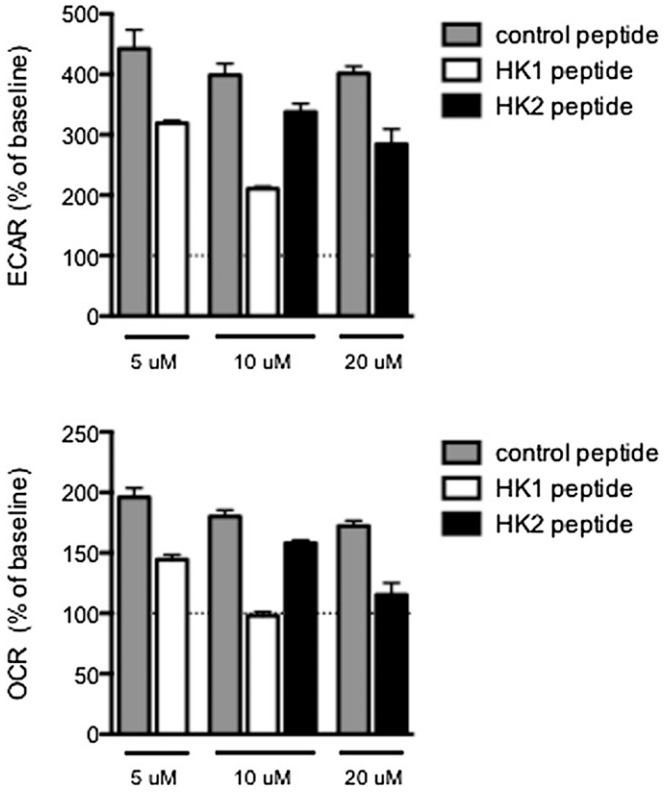

Fig. S10. OCR and ECAR after varying doses of HK-dissociating peptides. IL-15 T_M cells were incubated with control, HK-1 or HK-2 peptides and ECAR and OCR were measured after restimulation with PMA (50 ng/mL) and ionomycin (500 ng/mL) (first measurement after activation is shown relative to baseline). Data are representative of one independent experiment.

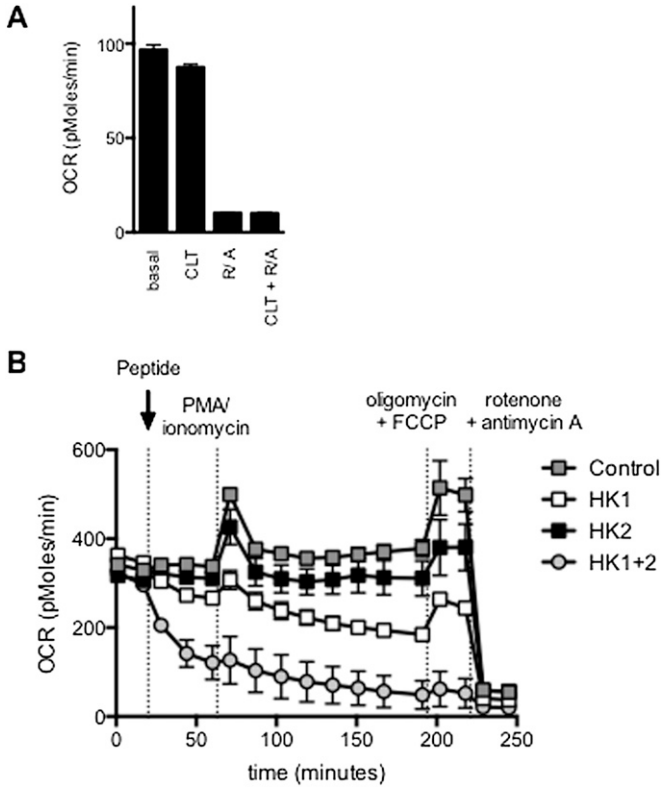

Fig. S11. IL-15 T_M cells maintain mitochondrial function after activation in the presence of HK competing peptides. (*A*) OCR of IL-15 T_M cells was measured in basal state, after injection of CLT or rotenone plus antimycin A, and after injection of CLT and subsequent rotenone plus antimycin A. (*B*) OCR of IL-15 T_M cells was measured in real-time in basal state, after injection of control, HK1, HK2 or HK1+2 competing peptides (10 μM), and subsequent oligo plus FCCP and rotenone plus antimycin A. Data are shown as mean ± SEM and representative of two independent experiments.

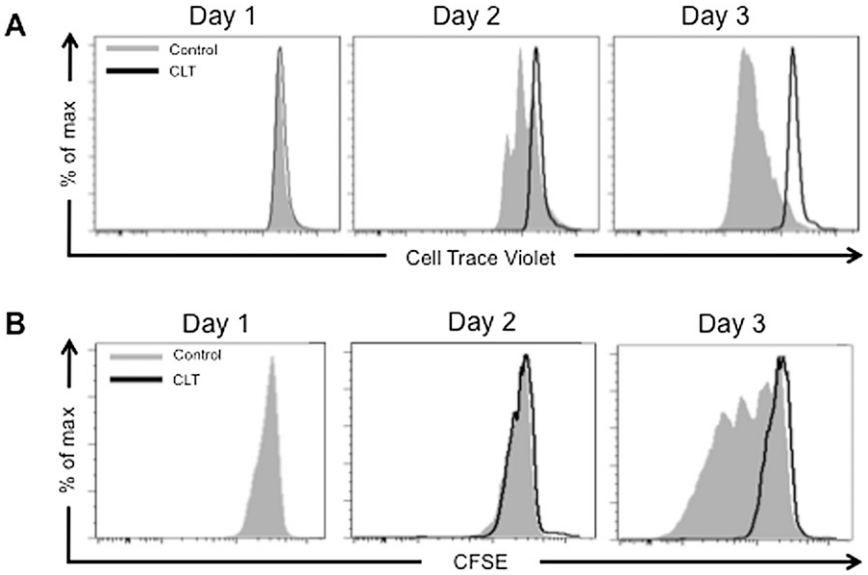

Fig. S12. Dissociation of hexokinase from mitochondria impairs the proliferation of T_N cells. (*A*) Purified naïve OT-I cells were stimulated with anti-CD3/28 and induced proliferation (as shown by Cell Trace Violet dilution) in the absence or presence of CLT is shown. Data are representative of three independent experiments. (*B*) Polyclonal CD8 T_N cells were isolated from LmOVA-infected mice based on $CD44^{lo}$ and $CD62L^{hi}$ expression and stimulated with anti-CD3/28 in the absence or presence of CLT (day 0), and (re)stimulation-induced proliferation (as shown by CFSE dilution) is shown. Data are representative of one independent experiment. Control group is the same as in Fig. 6*G* and Fig. S6*B*.