SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice and immunizations

C57BL/6, C57BL/6 CD45.1, C57BL/6 CD90.1, photo-activatable mitochondria (PhAM), and major histocompatibility complex (MHC) class I-restricted OVA specific TCR OT-I transgenic mice were purchased from The Jackson Laboratory. *Mfn1* and *Mfn2* conditional floxed mice were obtained from Dr. David C. Chan (California Institute of Technology, Pasadena, CA). *Opa1* conditional floxed mice were obtained from Dr. Hiromi Sesaki (Johns Hopkins University School of Medicine, Baltimore, MD). All conditional floxed mice were crossed to OT-I CD4 Cre transgenic mice to generate OT-I $Mfn1^{F/F}$ CD4 Cre, OT-I $Mfn2^{F/F}$ CD4 Cre, and OT-I $Opa1^{F/F}$ CD4 Cre mice. All mice were bred and maintained under specific pathogen free conditions under protocols approved by the AAALAC accredited Animal Studies Committee of Washington University School of Medicine, St. Louis, MO USA and the Animal Welfare Committee of the Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany. Age matched mice were injected intraperitoneally (i.p.) or intravenously (i.v.) as indicated with a sublethal dose of $1x10^6$ colony forming units (CFU) of recombinant *Listeria monocytogenes* expressing OVA deleted for *actA* (LmOVA) for primary immunizations and challenged with $5x10^7$ CFU for secondary immunizations. For tumor experiments, $1x10^6$ EL4 lymphoma cells expressing OVA (EL4-OVA) were injected subcutaneously (s.c.) into the right flank of mice.

Cell culture and drug treatments

OT-I splenocytes were activated with OVA-peptide (SIINFEKL, New England Peptide) and IL-2 (100 U/mL) for 3 days and subsequently cultured in the presence of either IL-2 or IL-15 (10 ng/mL) for an additional 3 days in TCM (RPMI 1640 media supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, and 55 μM β-mercaptoethanol). For drug treatment experiments, vehicle control (DMSO) or 10 μM Mdivi-1 + 20 μM M1 (Sigma) were added to cultures daily starting on day 3. For *in vitro* survival assays, cells were activated for 3 days as described, then cultured in either IL-2 at $5x10^4$ cells/mL or IL-15 at $1x10^5$ cells/mL in 96 well round bottom plates. Survival was analyzed by 7AAD exclusion using flow cytometry. Bone marrow cells were differentiated for 7 days into BM-Macs by culturing in complete medium (RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin/streptomycin, 2 mM L-glutamine) with 20 ng/mL mouse macrophage colony-stimulating factor (M-CSF; PeproTech) or into BM-DCs using 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; PeproTech). BM-Macs and BM-DCs were stimulated using 20 ng/mL LPS (Sigma), 50 ng/mL IFN-γ (R&D

Systems), or 20 ng/mL IL-4 (PeproTech). BM-DCs were cultured in 5 ng/mL GM-CSF during stimulation experiments.

Flow cytometry and spinning disk confocal microscopy

Fluorochrome-conjugated monoclonal antibodies were purchased from eBioscience, BD Pharmingen, or Biolegend and staining performed as previously described (Chang et al., 2015). OVA-specific $CD8^+$ T cells from spleen, lymph node, or blood were quantified by direct staining with H2-$K^b$$OVA_{257-264}$ (K^bOVA) MHC-peptide tetramers. MitoTracker, TMRE, CMxROS, MitoSOX, and Hoechst staining was performed according to the manufacturer's instructions (Life Technologies). Nos2 protein levels in BM-Macs were quantified after fixation and permeabilization using the transcription factor staining buffer set (eBioscience) and a directly conjugated antibody against Nos2 (clone CXNFT, eBioscience). Cells were collected on FACS Calibur, Canto II, LSR II, and Fortressa flow cytometers (BD Biosciences) and analyzed using FlowJo (TreeStar) software. Cells were sorted using a FACS Aria II. Cells were imaged live on glass bottom dishes coated with fibronectin or poly-D-lysine (Sigma) in TCM containing IL-2 or IL-15 (MatTek) using a LSM 510 META confocal scanning microscope (Zeiss), an Olympus Confocal Microscope FV1000, or a Zeiss spinning disk confocal with an Evolve (EMCCD) camera. Cells were kept in a humidified incubation chamber at 37°C with 5% CO_2 during image collection. Images were deconvolved and analyzed using ImageJ (NIH). Brightness and contrast were adjusted in Adobe Photoshop CS.

Transmission electron microscopy

Cells were fixed in 2% paraformaldehyde, 2.5% glutaraldehyde in 100 mM sodium cocodylate containing 0.05% malachite green. Following fixation, samples were washed in cocodylate buffer and post fixed in 1% osmium tetroxide. After extensive washing in H_2O, samples were stained with 1% aqueous uranyl acetate for 1 hour and washed again. Samples were dehydrated in ethanol and embedded in Eponate 12 resin (Ted Pella). Cut sections were stained with uranyl acetate and lead citrate and then imaged using a JOEL 1200 EX transmission electron microscope equipped with an 8 MP ATMP digital camera (Advanced Microscopy Techniques).

Metabolism assays

Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured in XF media (non-buffered RPMI 1640 containing 25 mM glucose, 2mM L-glutamine, and 1 mM

sodium pyruvate) under basal conditions and in response to 200 μM etomoxir (Tocris), 1 μM oligomycin, 1.5 μM fluoro-carbonyl cyanide phenylhydrazone (FCCP) and 100 nM rotenone + 1 μM antimycin A, or 50 ng/mL phorbol 12-myristate 13-acetate (PMA) + 500 ng/mL ionomycin (all Sigma) using a 96 well XF or XFe Extracellular Flux Analyzer (EFA) (Seahorse Bioscience). For mitochondrial fission inhibition experiments, cells were plated in XF media containing 10 μM Mdivi-1 or vehicle control (DMSO), followed by injection into port A with XF media, αCD3/CD28-conjugated beads (1 bead/cell; Dynabeads), or 20 ng/mL LPS ± 50 ng/mL IFN-γ.

Glucose tracing

Cells were activated with OVA peptide and cultured in glucose free TCM (prepared with dialyzed FBS) supplemented with 11 mM glucose. On day 3 of culture, cells were washed and cultured overnight in TCM replaced with 11 mM D-[1,2^{13}C] labeled glucose. For harvest, cells were rinsed with cold 150 mM ammonium acetate (NH4AcO), and metabolites extracted using 1.2 mL of 80% MeOH kept on dry ice. 10 nM norvaline (internal standard) was added. Following mixing and centrifugation, the supernatant was collected, transferred into glass vials and dried via centrifugal evaporation. Metabolites were resuspended in 50 μL 70% ACN and 5 μL of this solution used for mass spectrometer-based analysis performed on a Q Exactive (Thermo Scientific) coupled to an UltiMate 3000RSLC (Thermo Scientific) UHPLC system. Mobile phase A was 5 mM NH4AcO, pH 9.9, B was ACN, and the separation achieved on a Luna 3u NH2 100A (150 × 2.0 mm) (Phenomenex) column. The flow was kept at 200 μL/min, and the gradient was from 15% A to 95% A in 18 min, followed by an isocratic step for 9 min and re-equilibration for 7 min. Metabolites we detected and quantified as area under the curve (AUC) based on retention time and accurate mass (≤ 3 p.p.m.) using TraceFinder 3.3 (Thermo Scientific) software.

Adoptive transfers

For *in vivo* memory T cell experiments, ≤1x10^4 OT-I$^+$ CD8$^+$ cells/mouse from donor splenocytes were transferred intravenously (i.v.) into congenic recipient mice. Blood samples or spleens were collected at indicated time points and analyzed by flow cytometry. For *in vivo* survival experiments, 1-2x10^6 day 6 IL-2 T_E treated cells/mouse were injected i.v. into naïve C57BL/6 mice. Cells were recovered two days later from the spleen or lymph nodes and analyzed by flow cytometry or isolated from spleens >3 weeks 6 days after LmOVA infection. For adoptive cellular immunotherapy experiments, 1-5x10^6 day 6 IL-2 T_E treated cells/mouse were injected i.v. into previously EL4-OVA tumor inoculated mice and measured for tumor volume growth.

RT-PCR and western blotting

RNA isolations were done by using the RNeasy kit (Qiagen) and single-strand cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Genomic DNA was extracted using the QIAamp DNA micro kit (Qiagen) to determine mtDNA/nDNA ratios. All RT-PCR was performed with Taqman primers using an Applied Biosystems 7000 sequence detection system. The expression levels of mRNA were normalized to the expression of a housekeeping gene (β-actin). For western blot analyses, cells were washed with ice cold PBS and lysed in 1x lysis buffer (Cell Signaling Technologies) supplemented with 1 mM PMSF. Samples were freeze-thawed 3 times and centrifuged at 20,000xg for 10 min at 4°C. Cleared protein lysate was denatured with LDS loading buffer for 10 min at 70°C. For native lysis, cells were resuspended in native lysis buffer (Life Technologies), supplemented with increasing percentages of digitonin, MgCl, and micrococcal nuclease. After nuclease incubation at RT for 1h, lysates were cleared by centrifugation at 20,000xg for 30 min at 4°C. For mitochondrial membrane solubilization analyses, both the cleared supernatant and pellet were denatured with LDS loading buffer for 10 min at 70°C. Samples were run on precast 4-12% bis-tris protein gels (Life Technologies). Proteins were transferred onto nitrocellulose membranes using the iBLOT 2 system (Life Technologies). Membranes were blocked with 5% w/v milk and 0.1% Tween-20 in TBS and incubated with the appropriate antibodies in 5% w/v BSA in TBS with 0.1% Tween-20 overnight at 4°C. The following antibodies were used: Opa1 (BD), rodent OXPHOS complex proteins cocktail (Abcam), Calnexin (Santa Cruz), and β-Actin, Mfn2, Drp1, Drp1^{pS616} (Cell Signaling Technologies). All primary antibody incubations were followed by incubation with secondary HRP-conjugated antibody (Pierce) in 5% milk and 0.1% Tween-20 in TBS and visualized using SuperSignal West Pico or femto Chemiluminescent Substrate (Pierce) on Biomax MR film (Kodak).

Retroviral transduction

Activated OT-I splenocytes were transduced with control (empty vector) or Mfn1, Mfn2, Opa1 expressing retrovirus by centrifugation for 90 minutes in media containing hexadimethrine bromide (8 μg/mL; Sigma) and IL-2 (100 U/mL). GFP or human CD8 were markers for retroviral expression.

Cytotoxicity assay

EL4-OVA tumor cells were pre-treated with 100 U/mL murine IFN-γ for 24 hours before use. To generate target cells, $1x10^6$ tumor cells were labeled with 0.5 μM Cell Proliferation Dye e670 (eBioscience) in PBS for 8 minutes at room temperature, washed twice with PBS and 10,000 cells were seeded per well in 96-well round bottom plates. IL-2 T_E cells treated with DMSO or M1+Mdivi-1 were co-cultured with target cells at the indicated effector/target cell ratios and incubated for 12 hours at 37°C in 5% CO2. To generate reference cells, $1x10^6$ tumor cells were labeled with 5 μM Cell Proliferation Dye e670 in PBS and incubated on ice. 10,000 reference cells were added before cells were stained with Po-Pro™-1 dead cell staining dye (Life Technologies). IL-2 T_E cell killing efficiency was analyzed by flow cytometry and data defined as percentage of live cells normalized to reference cells.

Statistical analysis

Comparisons for two groups were calculated using unpaired two-tailed student's *t*-tests, comparisons for more than two groups were calculated using one-way ANOVA followed by Bonferroni's multiple comparison tests. Comparisons over time were calculated using two-way ANOVA followed by Bonferroni's multiple comparison tests.

SUPPLEMENTAL REFERENCES

Chang, C. H., Qiu, J., O'Sullivan, D., Buck, M. D., Noguchi, T., Curtis, J. D., Chen, Q., Gindin, M., Gubin, M. M., Van Der Windt, G. J., Tonc, E., Schreiber, R. D., Pearce, E. J. & Pearce, E. L. (2015). Metabolic Competition in the Tumor Microenvironment Is a Driver of Cancer Progression. *Cell*, 162, 1229-41.