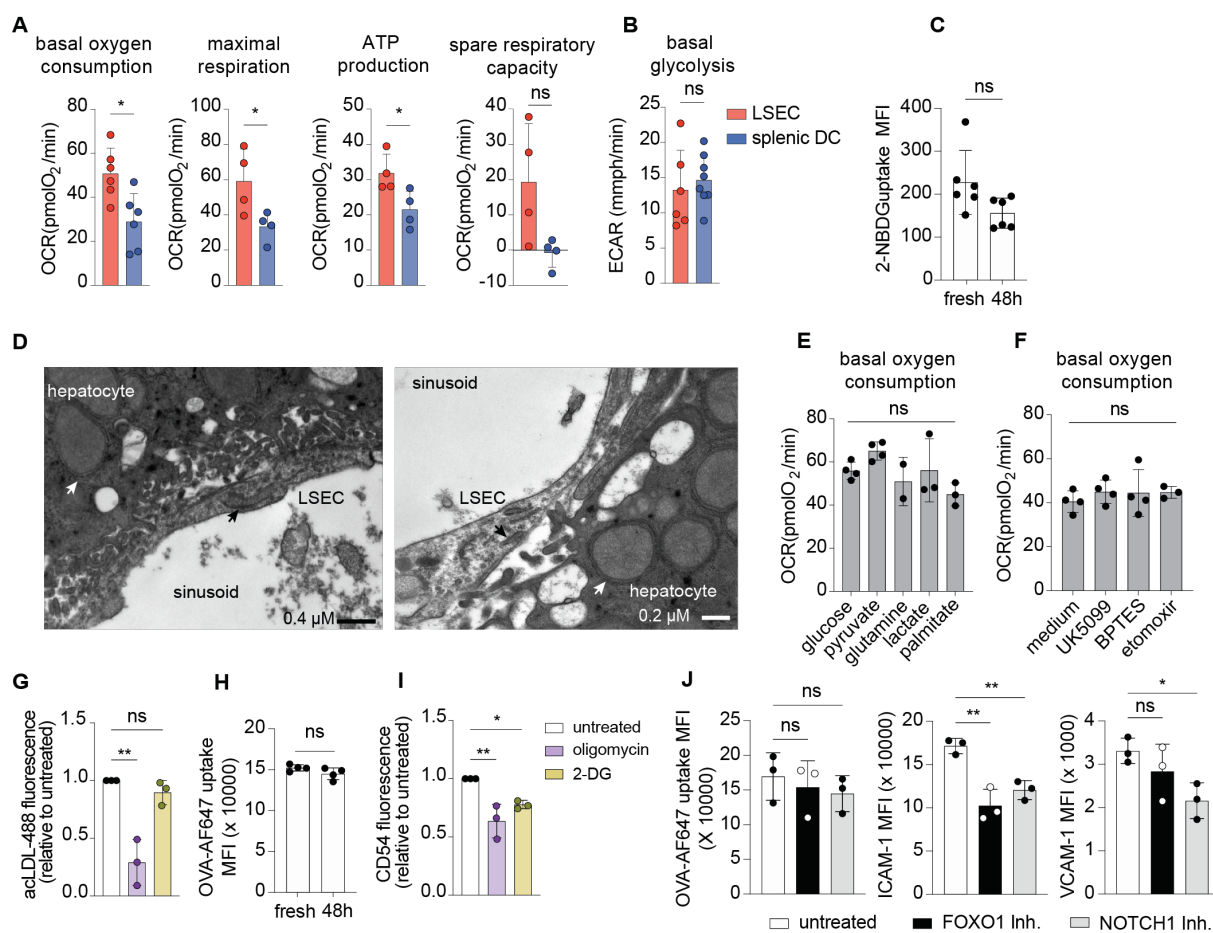


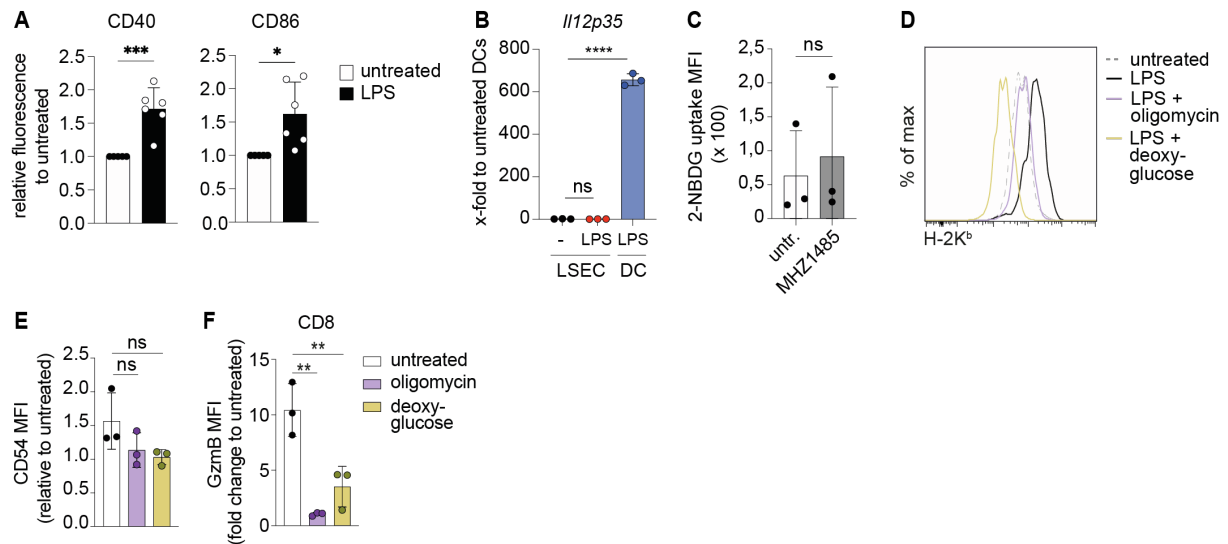
Supplemental information

**IL-6-induced FOXO1 activity determines
the dynamics of metabolism in CD8 T cells
cross-primed by liver sinusoidal endothelial cells**

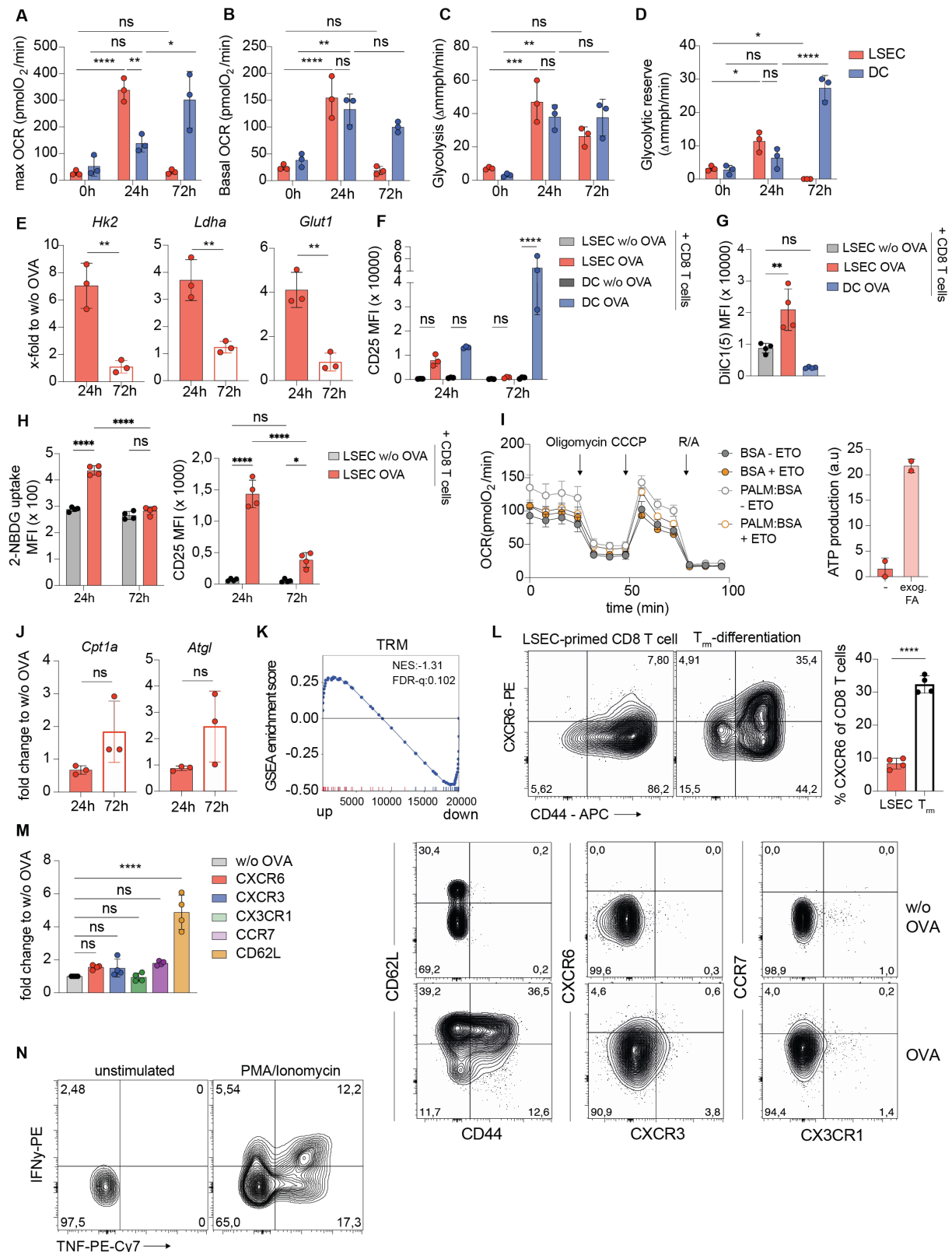
Michael Dudek, Kerstin Lohr, Sainitin Donakonda, Tobias Baumann, Max Lüdemann, Silke Hegenbarth, Lena Dübbel, Carola Eberhagen, Savvoula Michailidou, Abdallah Yassin, Marco Prinz, Bastian Popper, Stefan Rose-John, Hans Zischka, and Percy A. Knolle



Supplemental Figure 1: LSECs employ different energy substrates to fuel mitochondrial oxidative phosphorylation – related to Figure 1 (A,B) Values for basal oxygen consumption, maximal respiration, ATP production, spare respiratory capacity and basal glycolysis calculated from OCR and ECAR profiles ($n \geq 4$). **(C)** Flow cytometry analysis of 2-NBDG uptake in LSECs directly after isolation (fresh) or after 48 hours of cell culture to measure glucose uptake ($n = 6$). **(D)** Ultrastructural analysis of LSECs *in situ*, arrows depicting mitochondria in LSECs (black) and hepatocytes (white), scale bar 200 nm. **(E)** Basal OCR of LSECs after incubation with minimal medium devoid of nutrients supplemented with glucose, pyruvate, glutamine, lactate or palmitate ($n \geq 2$). **(F)** Basal OCR in LSECs after pharmacological inhibition of substrate-specific mitochondrial uptake pathways (UK5099 for pyruvate, BPTES for glutamine and etomoxir for long chain fatty acids) ($n = 4$). **(G,I)** Influence of deoxyglucose or oligomycin on LSEC scavenger activity of acetylated-LDL-488 (AcLDL-488) ($n = 3$) and on surface expression of CD54 determined by flow cytometric measurement ($n = 3$). **(H)** Flow cytometry analysis of OVA-AF647 uptake in LSECs directly after isolation (fresh) or after 48 hours of *in vitro* culture ($n = 4$). **(J)** Flow cytometry analysis of OVA-AF647 uptake in LSECs and surface expression levels of ICAM-1 and VCAM-1 at 12 hours after isolation in the presence of inhibitors that block transcriptional activity of the FOXO1 (100ng/ml, AS1842856) or the Notch1 signaling pathway (10μg/ml, DAPT). Data are representative of at least two separate experiments. NS, not significant. MFI, mean fluorescence intensity. OCR, oxygen consumption rate. ECAR, extracellular acidification rate One-way analysis of variance (ANOVA) with Dunnett's (E-G,I,J) multiple comparison test and unpaired two-tailed *t*-test (A-C,H). In A-C,D-J data are mean \pm s.e.m., errors are shown as s.d.

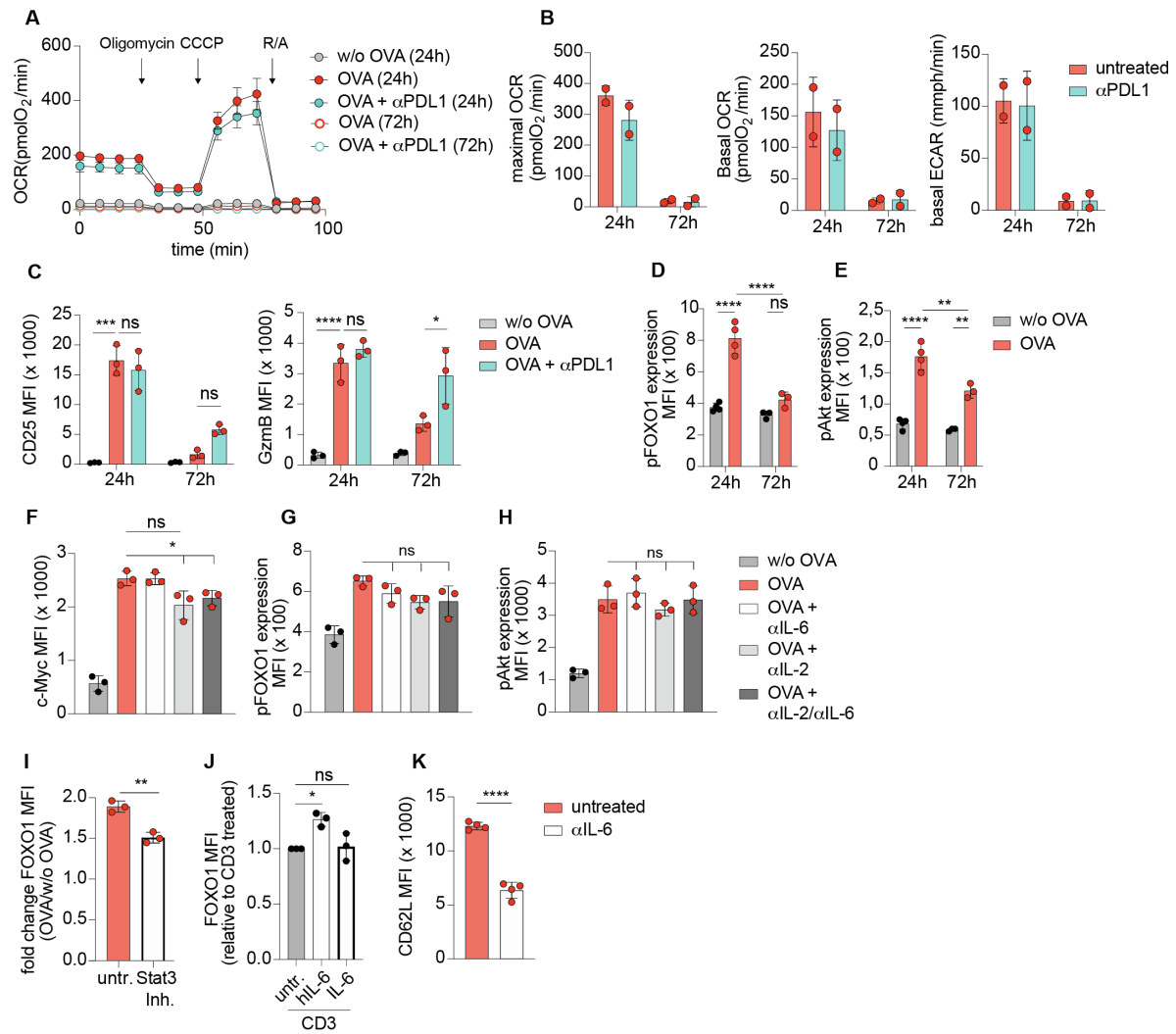


Supplemental Figure 2: Mitochondrial respiration and glycolysis are important for LSECs to efficiently prime CD8 T cells – related to Figure 2 (A) Flow cytometry analysis of surface expression levels of CD40 and CD86 on splenic CD11⁺ DC after LPS (100 ng/ml) stimulation for 24 hours ($n = 6$). (B) Gene expression level of *IL-12p35* in LSECs and splenic DCs treated with the TLR4-ligand LPS for 24 hours relative to untreated DCs and normalized to house-keeping gene *HPRT* ($n = 3$). (C) Glucose uptake by LSECs after mTOR agonist stimulation (MHY1485) ($n = 3$). (D,E) Histogram of H-2K^b expression (D) and quantification of CD54 expression (E) on LSECs after LPS challenge in absence or presence of deoxyglucose or oligomycin ($n = 3$). (F) GzmB expression in ovalbumin-specific H-2K^b-restricted CD8 T cells after activation by cross-presenting LSECs that were previously exposed to deoxyglucose or oligomycin ($n = 3$). Data are representative of at least two separate experiments. NS, not significant. MFI, mean fluorescence intensity. One-way analysis of variance (ANOVA) with Dunnett's (B,E,F) multiple comparison test and unpaired two-tailed *t*-test (A,C). In A-C,E,F data are mean \pm s.e.m., errors are shown as s.d.

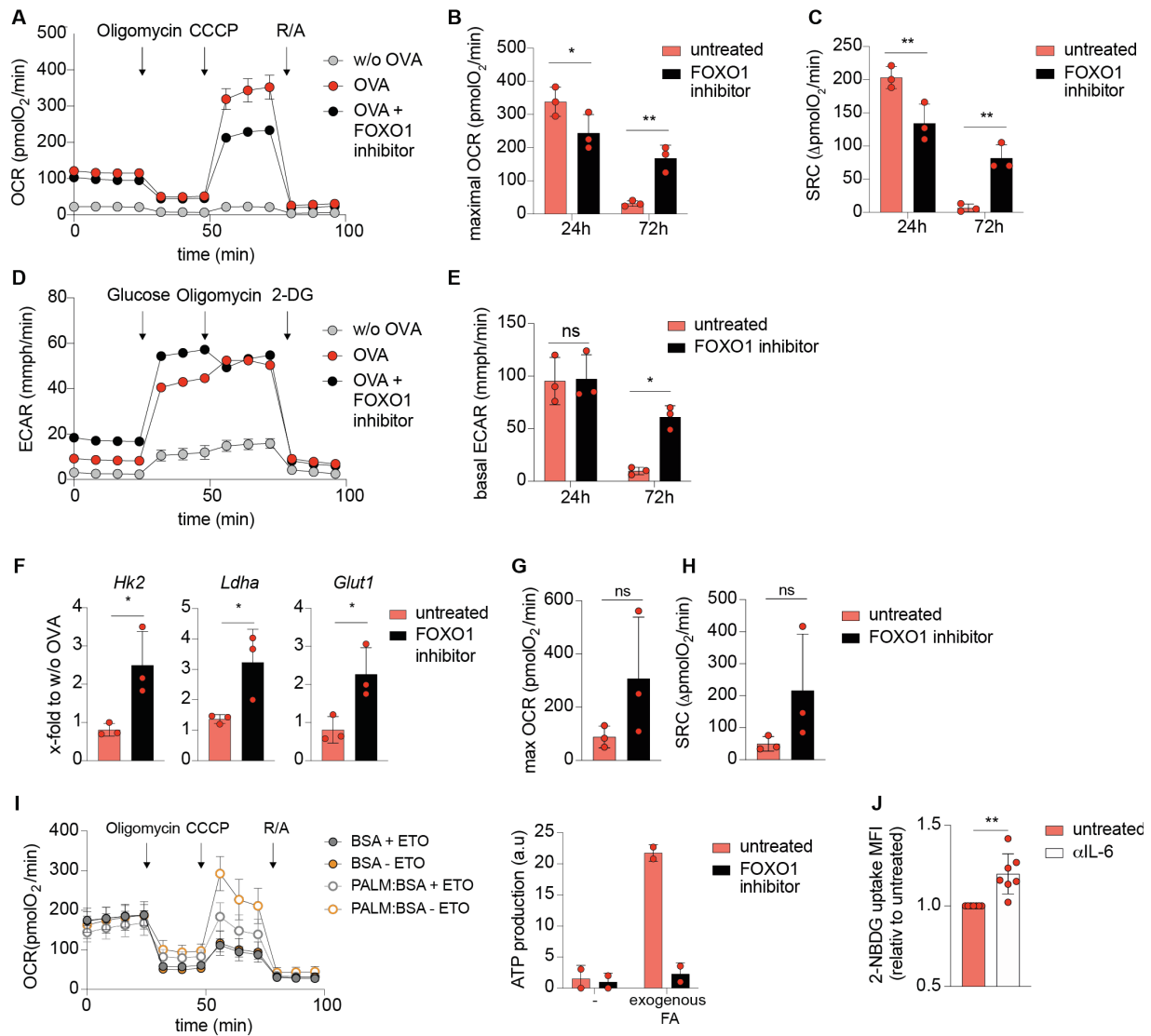


Supplemental Figure 3: Phenotype of LSEC and DC-primed CD8 T cells parallels metabolic dynamics in antigen-presenting cells – related to Figure 3. (A-D) Calculation of maximal respiration (A), spare respiratory capacity (B), glycolysis (C) and glycolytic reserve (D) from oxygen consumption rate (OCR) during mitochondrial stress test and extracellular acidification rate (ECAR) during glucose stress test measured by extracellular flux analysis in SIINFEKL-specific OT1 CD8 T cells after 24 and 72 hours of priming by LSECs and DCs ($n = 3$). (E) Gene expression levels of *Hk2*, *Ldha* and *Glut1* in LSEC-primed CD8 T cells after 24 and 72 hours of priming

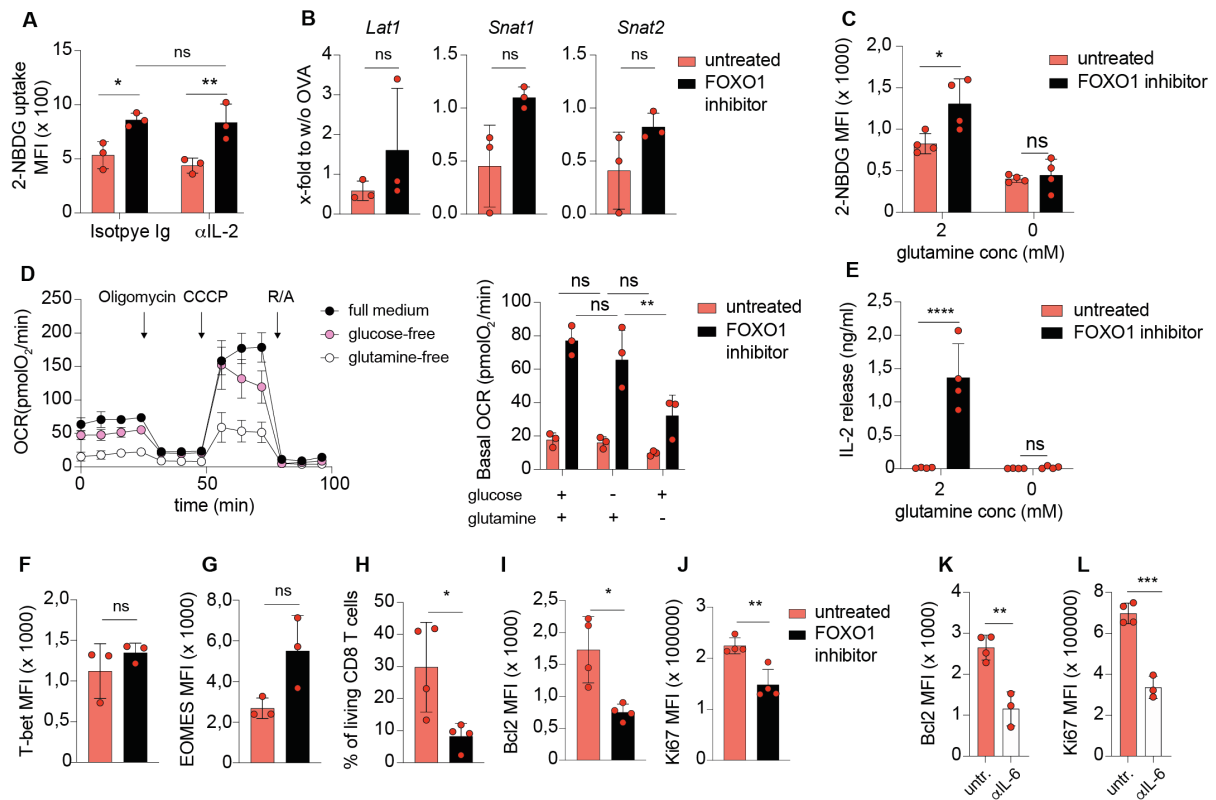
relative to SIINFEKL-specific OT1 CD8 T cells without stimulation (w/o OVA) normalized to house-keeping gene *I8s* ($n = 3$). **(F)** Flow cytometry analysis of CD25 and CD44 expression of LSEC- and DC-primed CD8 T cells ($n = 3$). **(G,H)** One hour after mice received intravenous injection of ovalbumin (400 μ g/mouse) or PBS (w/o OVA), LSECs and splenic DCs were isolated and 16 hours later they were co-cultured with naïve OT-1 CD8 T cells for 24 and 72 hours. Flow cytometry analysis of DilC1(5), 2-NBDG uptake and CD25 expression in CD8 T cells ($n = 4$). **(I)** Representative metabolic flux profile of OCR in CD8 T cells at 72 hours after LSEC-priming. Fatty acid oxidation was determined by injections of palmitate (PALM), a free fatty acid conjugated to bovine serum albumin (BSA), and etomoxir, a CPT-1 inhibitor. ATP production of LSEC-primed CD8 T cells during FAO assay measured by metabolic flux analyzer ($n = 2$). **(J)** Relative gene expression levels of *Cpt1a* and *Atgl* in LSEC-primed CD8 T cells from **(E)** normalized to house-keeping gene *I8s* ($n = 3$). **(K)** GSEA of differential expressed genes (DEG) of liver-primed CD8 T cells (Böttcher *et al.*, 2013) using gene set for liver residency (Zhao *et al.*, 2020). **(L)** Percentage of CXCR6⁺ T cells from CD8 T cells at 72 hours after LSEC-priming and from splenic CD8 T cells differentiated into T_{rm}-like CD8 T cells. **(M)** Flow cytometry analysis and dot plots of surface expression of CXCR6, CXCR3, CX3CR1, CCR7 and CD62L on LSEC-primed CD8 T cells after 72 hours of priming ($n = 4$). Fluorescence intensity is shown as fold change compared to naïve CD8 T cells that were cultured with LSECs which were not loaded with ovalbumin. **(N)** Expression of intracellular cytokines IFN γ and TNF in CD8 T cells after 72 hours of LSEC-priming restimulated for 4 hours with CD3/CD28 and IL-12 in the presence of the vesical transport inhibitor Brefeldin-A (BFA) and monensin. Data are representative of at least three separate experiments. NS, not significant. MFI, mean fluorescence intensity. NES, normalized enrichment score. FDR, false discovery rate. OCR, oxygen consumption rate. Two-way analysis of variance (ANOVA) with Tukey's **(A-D,F,H)** and One-way ANOVA with Dunnett's **(G,M)** multiple comparison test and unpaired two-tailed *t*-test **(I,K)**. In **A-D,F-J,L,M** data are mean \pm s.e.m., errors are shown as s.d.



Supplemental Figure 4. PD-1/PD-L1 signaling does not dampen the initial metabolic burst of LSEC-primed CD8 T cells – related to Figure 4. (A,B) Representative profile of OCR during mitochondrial stress test (A) measured by extracellular flux analysis in SIINFEKL-specific OT1 CD8 T cells after 24 and 72 hours of priming by LSECs treated with anti-PDL1 antibodies (10 µg/ml). Basal and maximal OCR and basal ECAR (B) were quantified after consecutive injections of several compounds ($n = 2$). (C) Flow cytometry analysis of CD25 and GzmB expression in LSEC-primed CD8 T cells from (A) ($n = 3$). (D-E) Phosphorylation levels of FOXO1 and Akt and c-Myc expression in SIINFEKL-specific OT1 CD8 T cells at 24 and 72 hours after cross-priming by LSECs ($n = 3-4$). (F-H) Phosphorylation levels of FOXO1 and Akt and c-Myc expression in LSEC-primed CD8 T cells treated with blocking antibodies against IL-6 (10 µg/ml) and IL-2 (10 µg/ml) at 24 hours after cross-priming ($n = 3$). (I) FOXO1 expression in LSEC-primed CD8 T cells at 24 hours after priming and treatment with Stat3 inhibitor S3I-201 (100 µM) ($n = 3$). (J) FOXO1 expression in splenic CD8 T cells stimulated with antibodies against CD3 (5 µg/ml) and treated with hyper IL-6 (10 ng/ml) and IL-6 (10 ng/ml) for 24 hours ($n = 3$). (K) CD62L expression on SIINFEKL-specific OT1 CD8 T cells after 72 hours of priming by LSECs and IL-6 inhibition ($n = 4$). Data are representative of at least two separate experiments. NS, not significant. MFI, mean fluorescence intensity. OCR, oxygen consumption rate. ECAR, extracellular acidification rate. Two-way analysis of variance (ANOVA) with Tukey's (C-E) multiple comparison test, one-way analysis of variance (ANOVA) with Tukey's (F-H) and Dunnett's (I) multiple comparison test and unpaired two-tailed t -test (I,K). In B-D data are mean \pm s.e.m., errors are shown as s.d.



Supplemental Figure 5. Increase in metabolism in LSEC-primed CD8 T cells after inhibition of FOXO1 activity at late time points – related to Figure 5. (A-E) Representative profiles of OCR (A) and ECAR during glucose stress test (D) measured by extracellular flux analysis in OT1 CD8 T cells at 24 hours after cross-priming by LSECs and treatment with FOXO1 Inhibitor AS1842856 (100 nM). Calculation of maximal respiration (B), spare respiratory capacity (C) and basal ECAR (E) ($n = 3$). (F) Gene expression levels of *Hk2*, *Ldha* and *Glut1* in LSEC-primed CD8 T cells at 72 hours after cross-priming and treatment with FOXO1 Inhibitor AS1842856 relative to untreated OT1 CD8 T cells without stimulation (w/o OVA) normalized to *18s* ($n = 3$). (G,H) Calculation of maximal respiration (G) and spare respiratory capacity (H) from *in vitro*-differentiated memory CD8 T cells at day 6 treated with FOXO1 Inhibitor AS1842856 (100 nM) for the last two days of culture ($n = 3$). (I) Representative metabolic flux profile and ATP production of LSEC-primed CD8 T cells treated with FOXO1 Inhibitor AS1842856 during FAO assay measured by metabolic flux analyzer ($n = 2$). (J) 2-NBDG uptake of SIINFEKL-specific CD8 T cells after 72 hours of priming by LSECs shown as fold change between CD8 T cells with IL-6 inhibition or not ($n = 7$). Data are representative of at least two separate experiments. NS, not significant. MFI, mean fluorescence intensity. OCR, oxygen consumption rate. ECAR, extracellular acidification rate. Two-way analysis of variance (ANOVA) with Tukey's (B,C,E) multiple comparison test and unpaired two-tailed *t*-test (F,H,J). In B,C,E,F,H,J data are mean \pm s.e.m., errors are shown as s.d.



Supplemental Figure 6: Deregulated metabolism in FOXO1-inhibited LSEC-primed CD8 T cells is glutamine-dependent – related to Figure 6. (A) Glucose uptake of LSEC-primed CD8 T cells treated with FOXO1 inhibitor at 72 hours after priming in the presence of neutralizing antibody against IL-2 or control immunoglobulin ($n = 3$). (B) Gene expression levels of glutamine transporters *Lat1*, *Snat1* and *Snat2* in LSEC-primed CD8 T cells at 72 hours after priming treated with FOXO1 Inhibitor AS1842856 relative to untreated SIINFEKL-specific OT1 CD8 T cells without stimulation (w/o OVA) and normalized to house-keeping gene *18s* ($n = 3$). (C) Flow cytometry analysis of glucose uptake with 2-NBDG in FOXO1-inhibited SIINFEKL-specific OT1 CD8 T cells after 72 hours of priming by LSECs cultured in glutamine-free medium ($n = 4$). (D) Representative metabolic flux profile of OCR during mitochondrial stress of FOXO1-inhibited LSEC-primed CD8 T cells at 72 hours after LSEC priming cultured in glutamine-or glucose-free medium and quantification of basal OCR ($n = 3$). (E) Determination of IL-2 release by ELISA from (C) ($n = 4$). (F,G) Fluorescence intensity of intracellular T-bet and EOMES detected by flow cytometry from LSEC-primed CD8 T cells at 72 hours after priming treated with FOXO1 Inhibitor AS1842856 (100 nM) ($n = 3$). (H) Flow cytometry analysis of the percentage of living FOXO1-inhibited LSEC-primed CD8 T cells 24 hours after restimulation with CD3/CD28 + IL-12 ($n = 4$). (I,J) Intracellular expression of Bcl2 and Ki67 in LSEC-primed CD8 T cells treated with FOXO1 Inhibitor AS1842856 (H,I) or with neutralizing antibodies against IL-6 (K,L) after restimulation with CD3/CD28 + IL-12 for 24 hours ($n = 4$). Data are representative of at least three separate experiments. NS, not significant. MFI, mean fluorescence intensity. OCR, oxygen consumption rate. Two-way analysis of variance (ANOVA) with Tukey's (A-E) multiple comparison test and unpaired two-tailed t -test (F-L). In A-L data are mean \pm s.e.m., errors are shown as s.d.