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Supplemental Information

Mitochondrial Ca^{2+} Signaling Is an Electrometabolic Switch to Fuel Phagosome Killing

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Figure S1. Characterization of *Mcu-/-* macrophages at baseline (with Fig. 1)

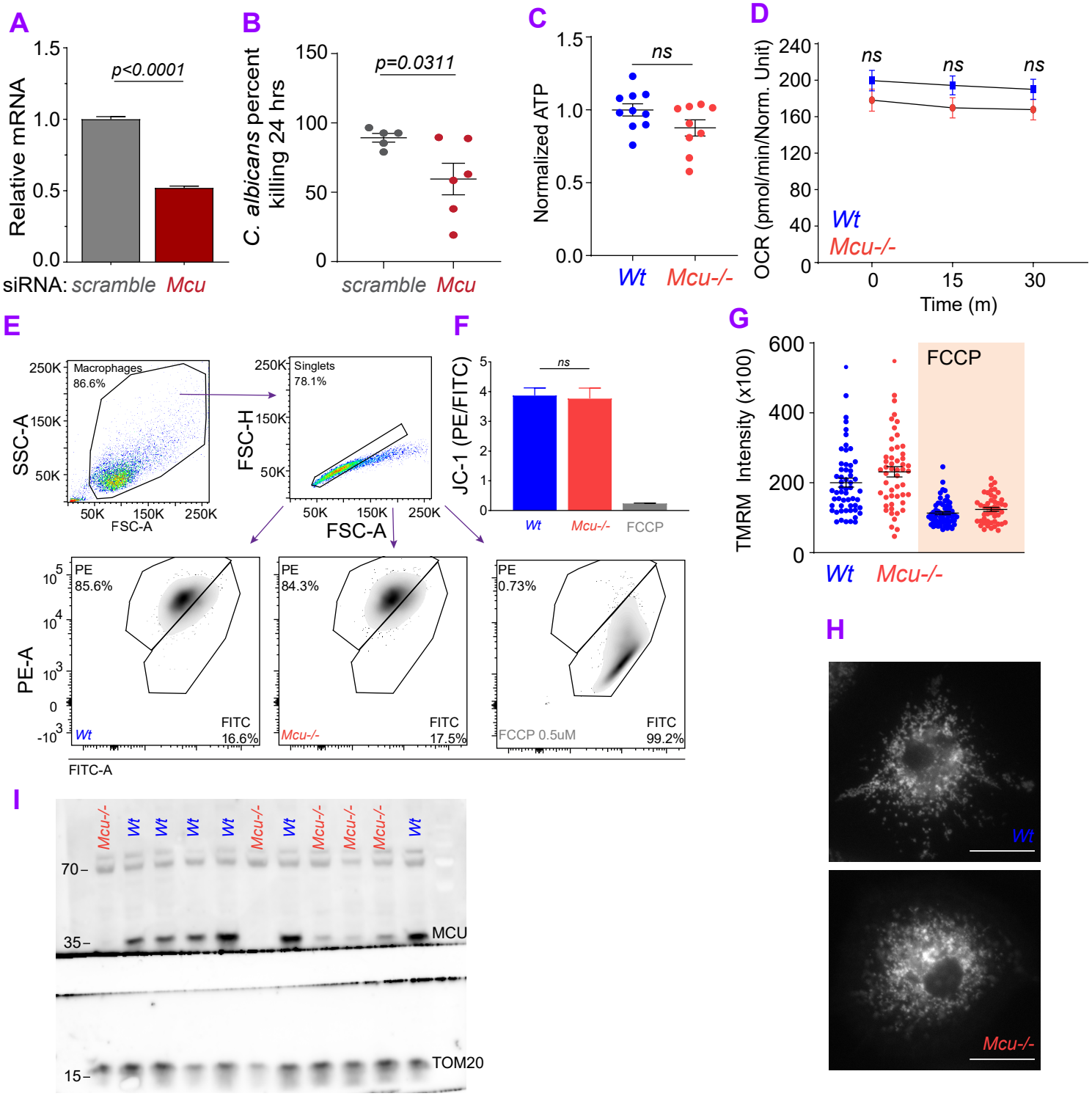


Figure S1. Characterization of *Mcu-/-* macrophages at baseline (with Fig. 1)

A. Gene expression analysis (qPCR) of *Mcu* mRNA in *scrambled* (n=3) and *Mcu* knockdown (n=3) RAW264.7 cells. Error bars represent SEM; *p<0.0001* according to Welch's t test, two-tailed

B. *C. albicans* killing by BMDMs transfected with *scrambled* (n=5) and *Mcu* siRNA (n=6). Error bars represent SEM; *p=0.0311* according to Welch's t test, two-tailed.

C. Normalized ATP levels in *wt* (n=10) and *Mcu-/-* (n=10) BMDMs. Error bars represent SEM of six independent experiments. No significant difference detected between *wt* and *Mcu-/-* according to Welch's t test, two-tailed.

D. Oxygen consumption rates (OCR) measured by Seahorse Analyzer across 30 min in *wt* (n=5) and *Mcu-/-* (n=5) BMDMs. Error bars represent SEM; no significant difference detected between *wt* and *Mcu-/-* according to Ordinary one-way ANOVA with multiple comparisons.

E. Gating strategy for flow cytometry of JC-1 dye. Viable Singlet BMDMs were gated prior to quantification of JC1 fluorescence in FITC and PE channels. Maximum mitochondrial depolarization was achieved by pretreating the BMDMs with FCCP (1μM) for 15 min prior to analysis.

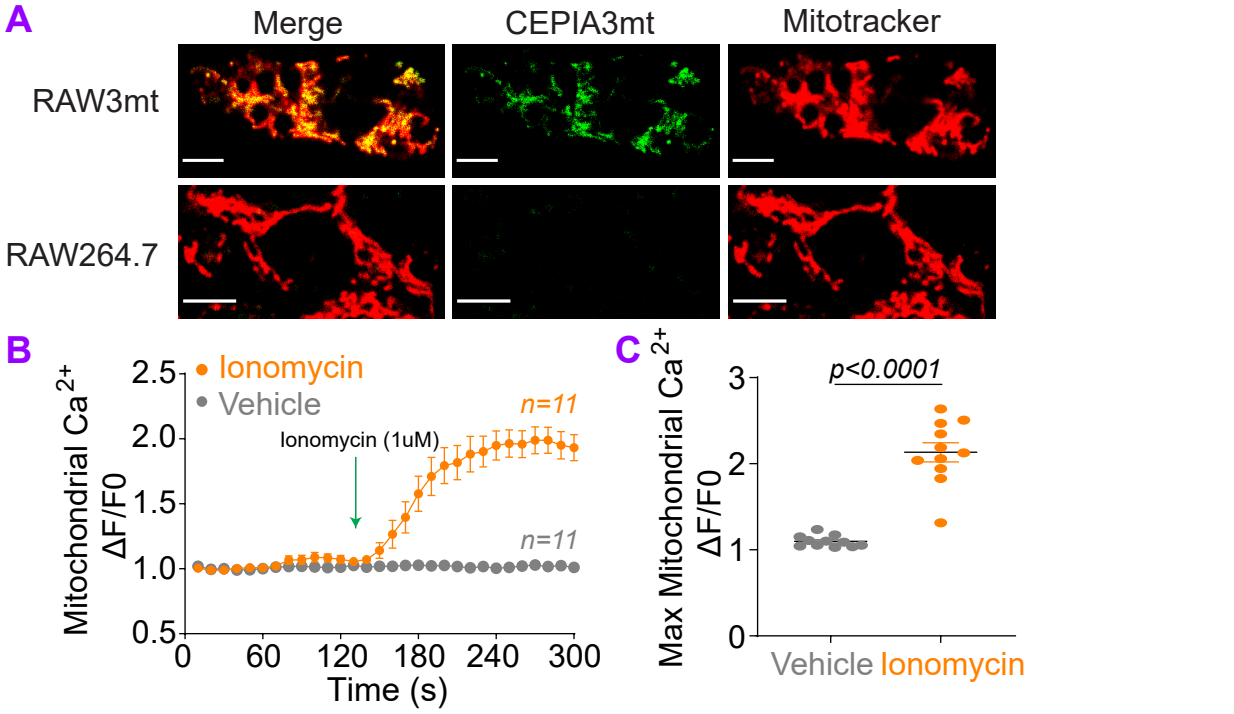
F. JC-1 PE:FITC ratio calculated for *wt* (n=4) and *Mcu-/-* (n=4) BMDMs. Error bars represent SEM; no significant difference was detected between *wt* and *Mcu-/-* according to Welch's t test, two-tailed.

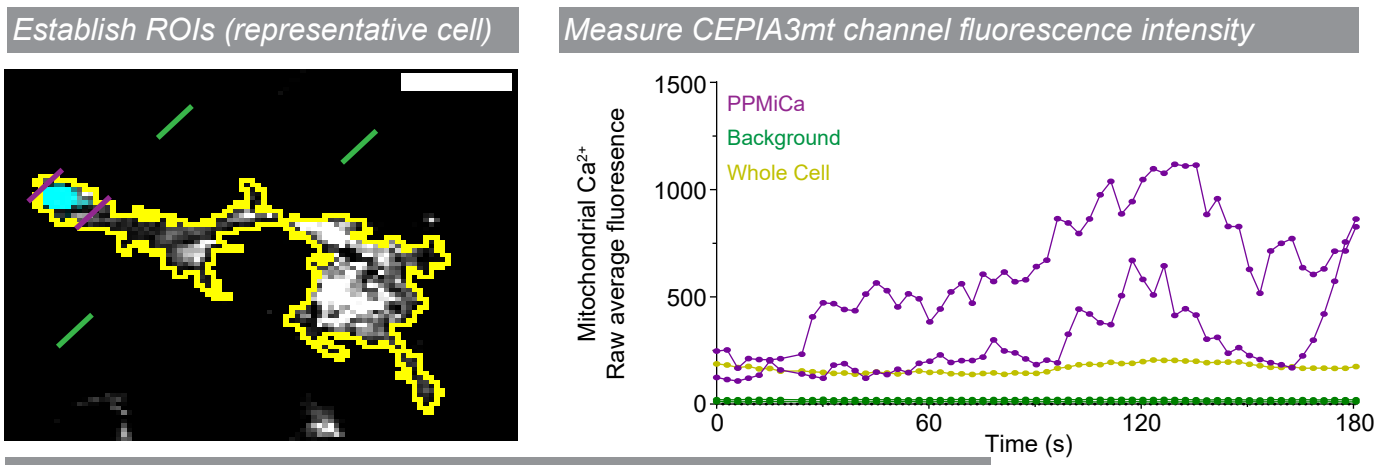
G. Quantification of TMRM (mitochondrial membrane potential dye) intensity in *wt* and *Mcu-/-* macrophages at baseline. FCCP (1 μM) was added to show maximum depolarization. No significant difference detected between *wt* and *Mcu-/-* according to Welch's t test, two-tailed.

H. Representative images from *wt* and *Mcu-/-* macrophages stained with TMRM (mitochondrial membrane potential dye). Cells were imaged every 10s. Baseline fluorescence was measured for 1 min followed by the addition of FCCP (1 μM). Scale bar is 6 μm.

I. Western blot of MCU and TOM20 in *wt* and *Mcu-/-* BMDMs.

Figure S2. RAW3mt validation, and analysis of PPMiCa Responses (with Fig. 4)





Calculate Phagosome:Whole Cell relative fluorescence intensity

Fluorescence Ratio =

All F values - $F_{Background}$ = "ΔF" → ΔF_{PPMiCa} / $\Delta F_{Whole\ Cell}$

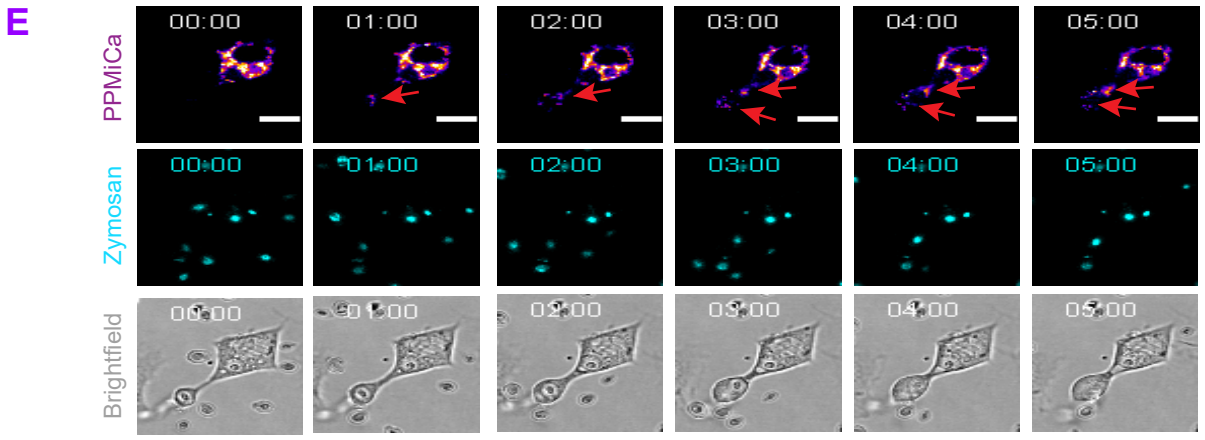


Figure S2. RAW3mt validation, and analysis of PPMiCa responses (with Fig. 4)

A. RAW3mt and RAW264.7 macrophages stained with MitoTracker Red (200 nM) and analyzed on confocal microscopy. Scale bar is 5 μm.

B. Whole cell mCa^{2+} elevations from ionomycin (1 μM) stimulated RAW3mt macrophages. Whole cell mCa^{2+} is taken from the average fluorescence intensity of ROIs drawn over entire mitochondrial network (See extended Figure 5). These values are calculated as a change in fluorescence/initial fluorescence (ΔF/F0). RAW3mt cells were imaged every 10 seconds; error bars represent SEM.

C. Quantification of peak whole cell mCa^{2+} elevations from FigureS2B Error bars represent SEM; $p<0.0001$ according to Welch's t test, two-tailed.

D. Representative analysis for PPMiCa and whole cell mCa^{2+} responses in RAW3mt. Note the entire mitochondrial network in the ROI. Scale bar is 10 μm.

E. Representative time course of PPMiCa responses in RAW3mt. *Red* arrows indicated PPMiCa responses near phagosome. CEPIA3mt (top images; Fire LUT), Zymosan (middle; cyan LUT), and brightfield (bottom image; grey LUT) were acquired using confocal microscopy (sampling rate: 1 frame/10s). Scale bar is 10 μm.

Figure S3. Gating strategy for flow cytometry (with Fig. 5)

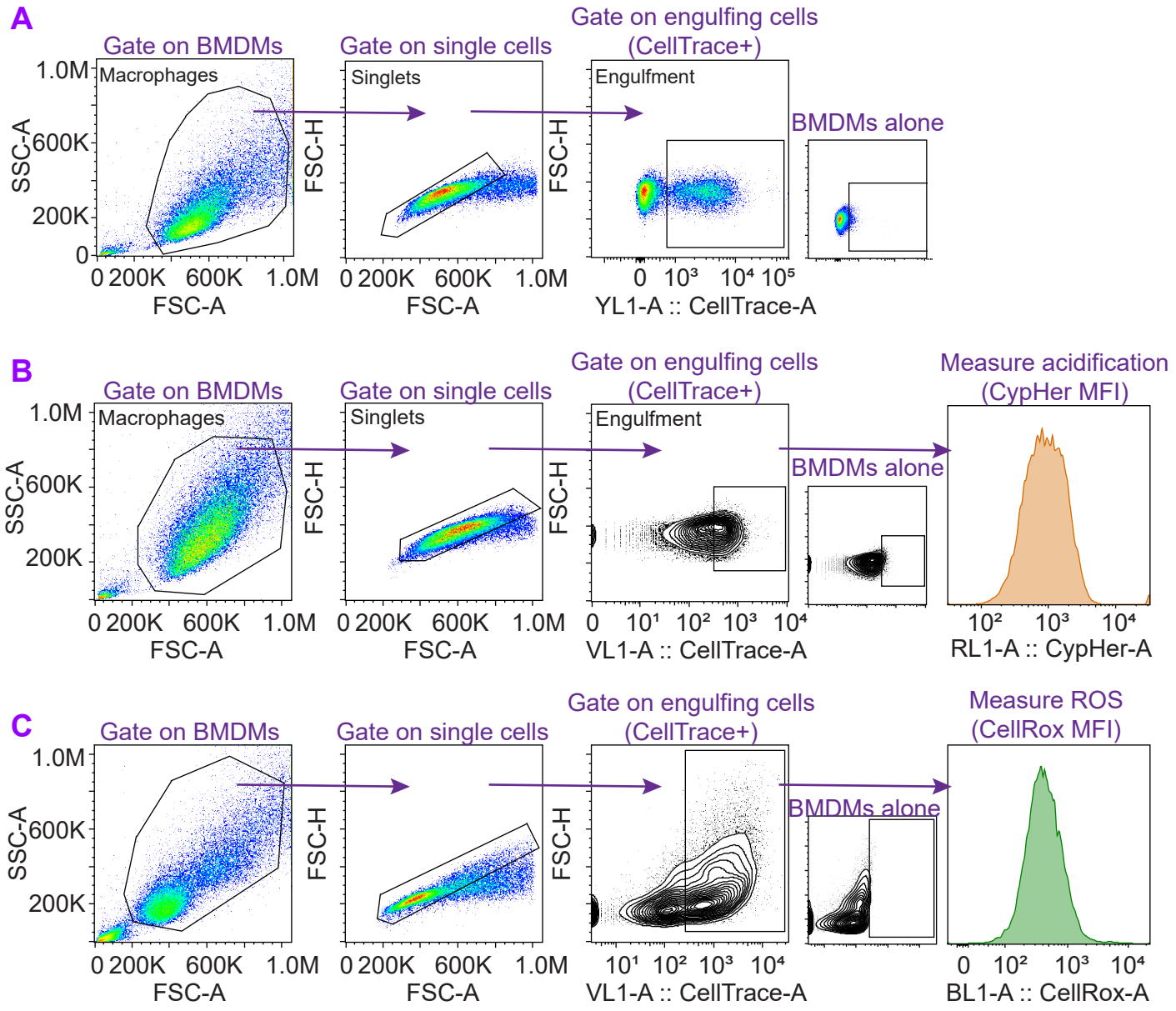


Figure S3. Gating strategy for flow cytometry (with Fig. 5)

A. Gating strategy for measuring engulfment of *C. albicans* in BMDMs.

B. Gating strategy for measuring phagosome acidification of *C. albicans* in BMDMs.

C. Gating strategy for measure phagosomal ROS in BMDMs.

Figure S4. Mitochondrial ROS, and validation of phagosomal ROS by flow cytometry (with Fig. 5)

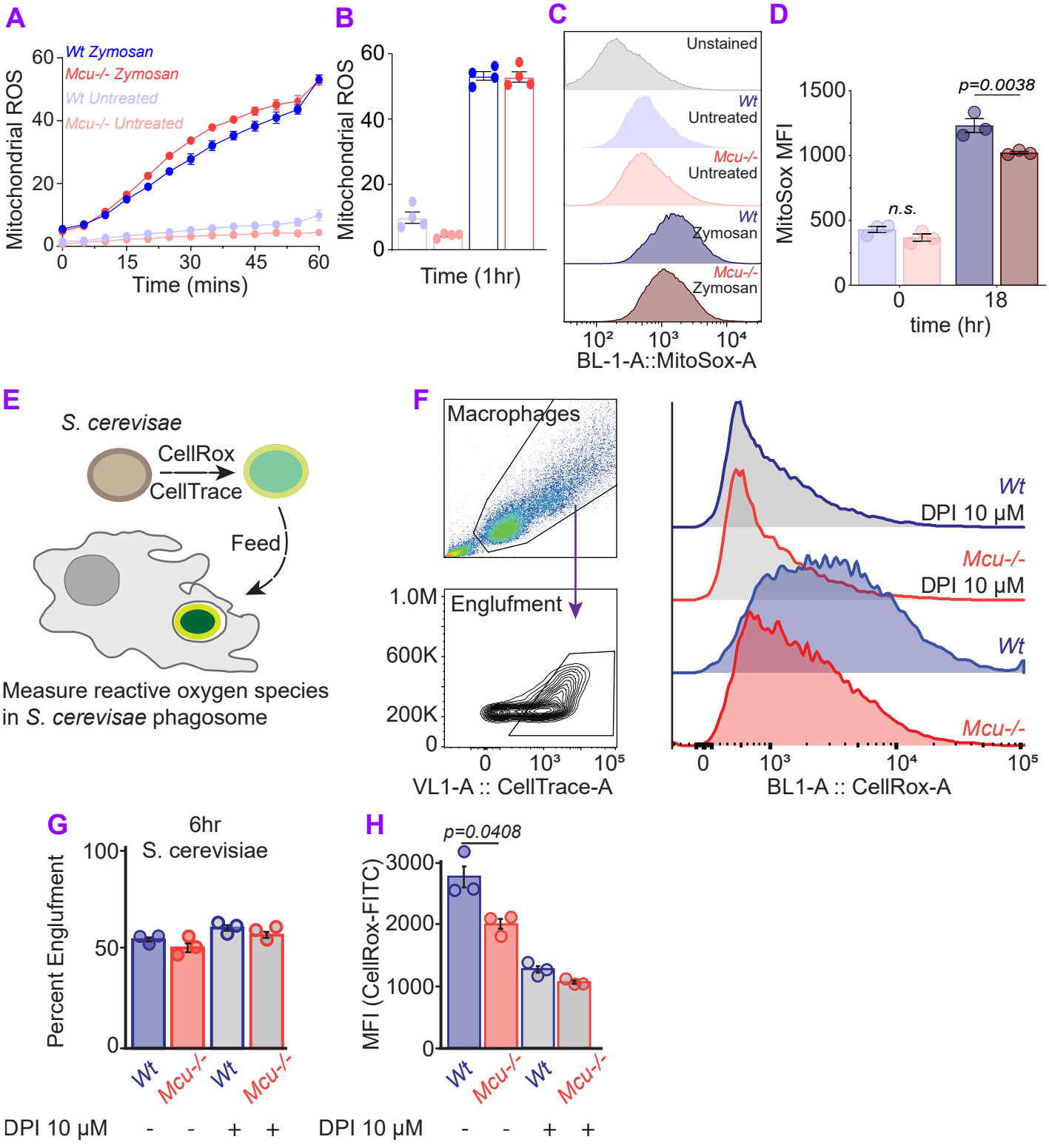


Figure S4. Mitochondrial ROS, and validation of phagosomal ROS by flow cytometry (with Fig. 5)

A. Change in MitoSox fluorescence in wt (n=4) and Mcu-/- (n=4) macrophages stimulated with zymosan. Fluorescence was recorded every 5 min (Ex: 510, Em: 580). Zymosan was added at 2 particles per cell prior to recording. Error bars represent SEM.

B. Quantification of MitoSox fluorescence at 60 min post zymosan stimulation. No significant difference was found between wt (n=4) and *Mcu-/-* (n=4) BMDMs. Error bars represent SEM; no significance was detected according to Welch's t test, two-tailed.

C. Representative histograms from *wt* and *Mcu-/-* BMDMs.

D. Quantification of MitoSox flow cytometry from *wt* (n=3) and *Mcu-/-* (n=3) bone-marrow derived macrophages. Cells were treated for 18 h with Zymosan 2:1, particles per cell. Error bars represent SEM; *p=0.0038* according to 2-way ANOVA, two-tailed.

E. Schematized experimental design to measure phagosomal ROS in BMDMs. *S. cerevisiae* were labeled with CellRox (reports oxidative stress) and CTV (to confirm engulfment) prior to phagocytosis.

F. Flow cytometry gating strategy for measuring phagosomal ROS in BMDMs engulfing *S. cerevisiae*.

G. Quantification of CTV+ cells measures engulfment) at 6 h post phagocytosis for *wt* (n=3), *Mcu-/-* (n=3) BMDMs. Macrophages were treated with DPI (10 μM, inhibitor of NADPH oxidase) or vehicle control (DMSO) for the duration of the experiment. Error bars represent SEM; no significant difference was detected between *wt* and *Mcu(M)-/-* in either condition according to Welch's, two-tailed test.

H. Quantification of phagosomal ROS (CellRox fluorescence) in *wt* (n=3) and *Mcu-/-* (n=3) BMDMs, after 6h of phagocytosis. Macrophages were treated with DPI (10μM) or vehicle control (DMSO) for the duration of the experiment. Error bars represent SEM; *p=0.0408* according to Welch's, two-tailed test.

Figure S5. NADPH/NADP+ biochemical analysis, and Seahorse quantification (with Fig. 6)

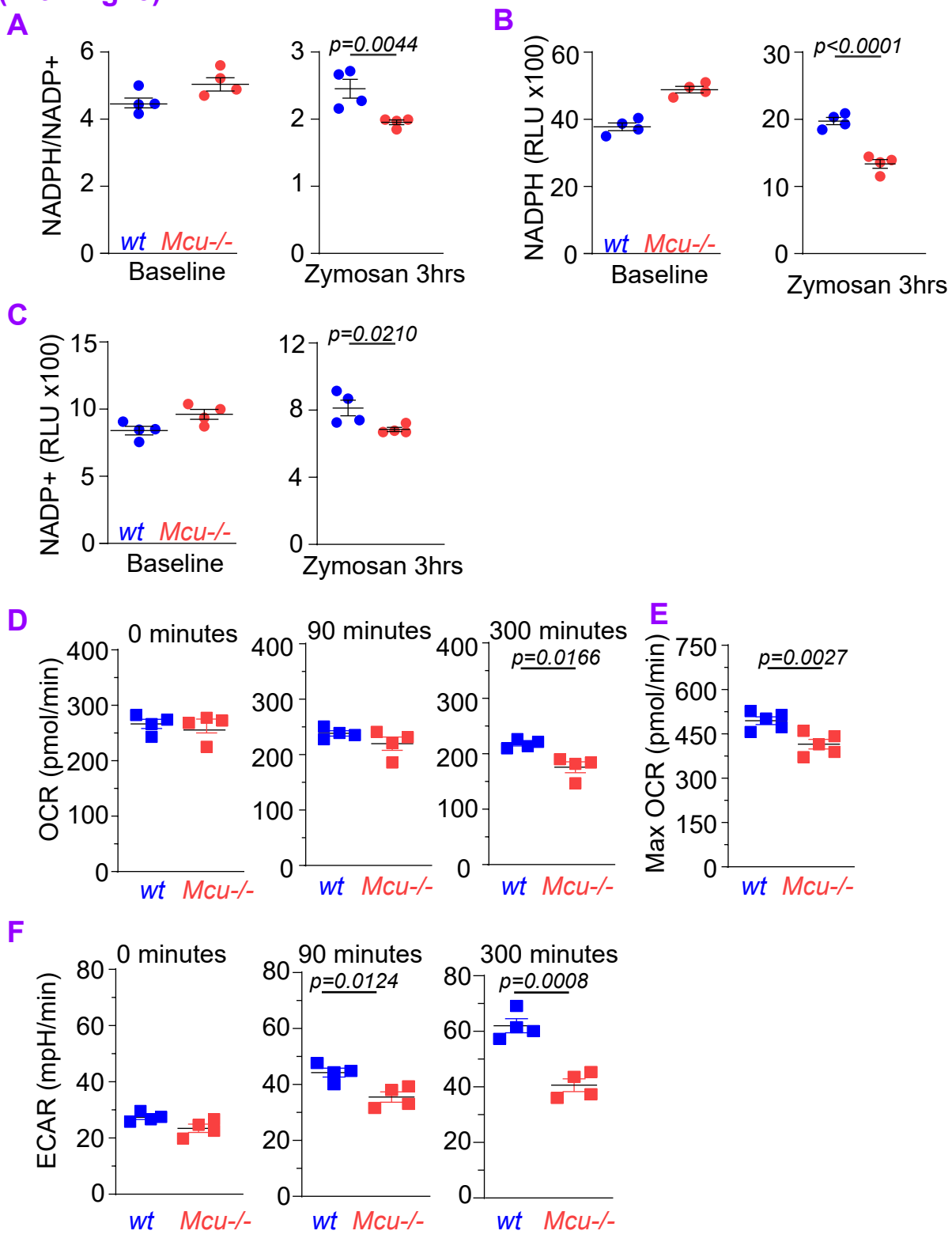


Figure S5. NADPH/NADP+ biochemical analysis, and Seahorse quantification (with Fig. 6)

A. NADPH/NADP+ ratios in *wt* and *Mcu-/-* BMDMs at baseline (left panel) and after zymosan stimulation (3h, right panel). Error bars represent SEM; *p=0.0044* between zymosan treated *wt* (n=4) and *Mcu-/-* (n=4) according to Welch's, two-tailed test.

B. NADPH luminescence in *wt* and *Mcu-/-* macrophages (same experiment as panel A). Error bars represent SEM; *p<0.0001* between zymosan treated *wt* (n=4) and *Mcu-/-* (n=4) according to Welch's, two-tailed test.

C. NADP+ luminescence in *wt* and *Mcu-/-* macrophages. Error bars represent SEM; *p=0.0210* between zymosan treated *wt* (n=4) and *Mcu-/-* (n=4) according to Welch's, two-tailed test.

D. Quantification of OCR at baseline, 90 min and 300 min from Figure 6C. Error bars represent SEM; No significant differences were detected between zymosan treated *wt* (n=4) and *Mcu-/-* (n=4) according to Welch's, two-tailed test.

E. Quantification of maximal OCR following the addition of BAM15 (mitochondrial uncoupler) from Figure 6C. Error bars represent SEM; *p=0.0059* between zymosan treated *wt* (n=4) and *Mcu-/-* (n=4) according to Welch's, two-tailed test.

F. Quantification of ECAR at baseline, 90 min and 300 min from Figure 6D. Error bars represent SEM; *p=0.0351* at 90 min and *p=0.0003* at 300 min between zymosan treated *wt* (n=4) and *Mcu-/-* (n=4) according to Welch's, two-tailed test.

Figure S6. Metabolomic analysis of glycolysis and pentose phosphate pathway (with Fig. 7)

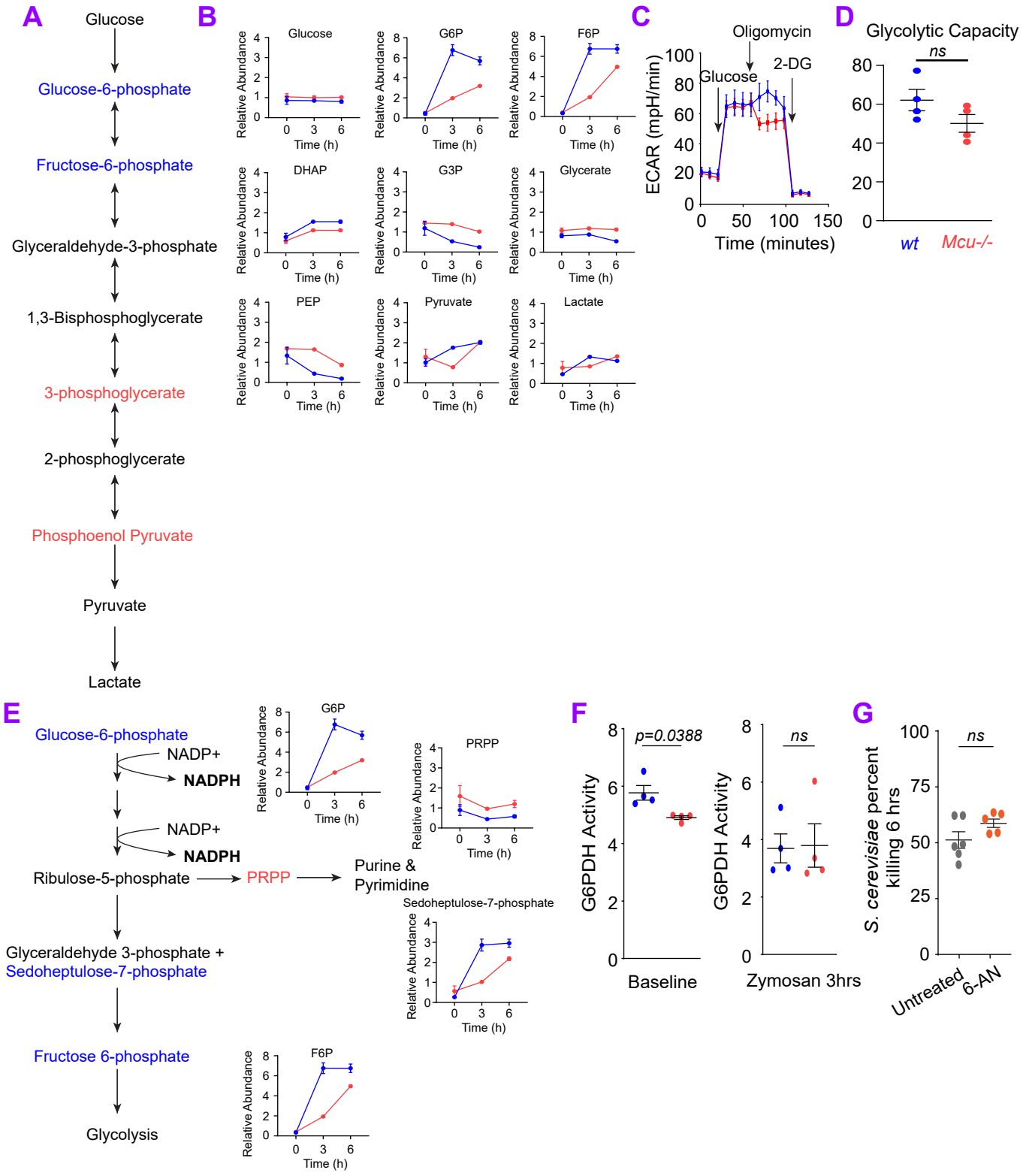


Figure S6. Metabolomic analysis of glycolysis and pentose phosphate pathway (with Fig. 7)

A. Schematic of glycolysis. Metabolites reduced in *Mcu-/-* BMDMs responding to zymosan (6h) are blue. Metabolites that increased in *Mcu-/-* BMDMs are red.

B. Glycolytic metabolites measured at 0, 3 and 6 h after zymosan stimulation in *Mcu-/-* (n=3) and *Wt* (n=3) macrophages. Error bars represent SEM.

C. Glycolytic Stress Test (GST) on *wt* (n=4) and *Mcu-/-* (n=4) macrophages following 6 h of zymosan stimulation. Injections are indicated with arrows.

D. Glycolytic capacity of *wt* and *Mcu-/-* macrophages following 6 h of zymosan stimulation Error bars are representing SEM, and significance was determined by Welch's, two-tailed t test.

E. Schematic of pentose phosphate pathway. Metabolites reduced in *Mcu-/-* BMDMs responding to zymosan (6h) are blue. Metabolites that increased in *Mcu-/-* BMDMs are red. Pentose phosphate pathway metabolites measured at 0, 3 and 6 h after zymosan stimulation in *Mcu-/-* (n=3) and *Wt* (n=3) macrophages. Error bars represent SEM.

F. G6PDH activity was assessed using a colorimetric assay. *Mcu-/-* macrophages showed a modest reduction in G6PDH activity at baseline but not after 3 h of zymosan stimulation. Error bars represent SEM (n=4); *p=0.0196* according to 2-way ANOVA, two-tailed

G. Killing of *S. Cerevisiae* by BMDMs treated with vehicle (n=6) or 100 μM 6-Aminonicotinamide (n=5). Error bars represent SEM from five independent experiments. No significant difference was detected by Welch's t test, two tailed

Figure S7. Metabolomic analysis of TCA cycle and quantification of pPDH IF (with Fig. 7)

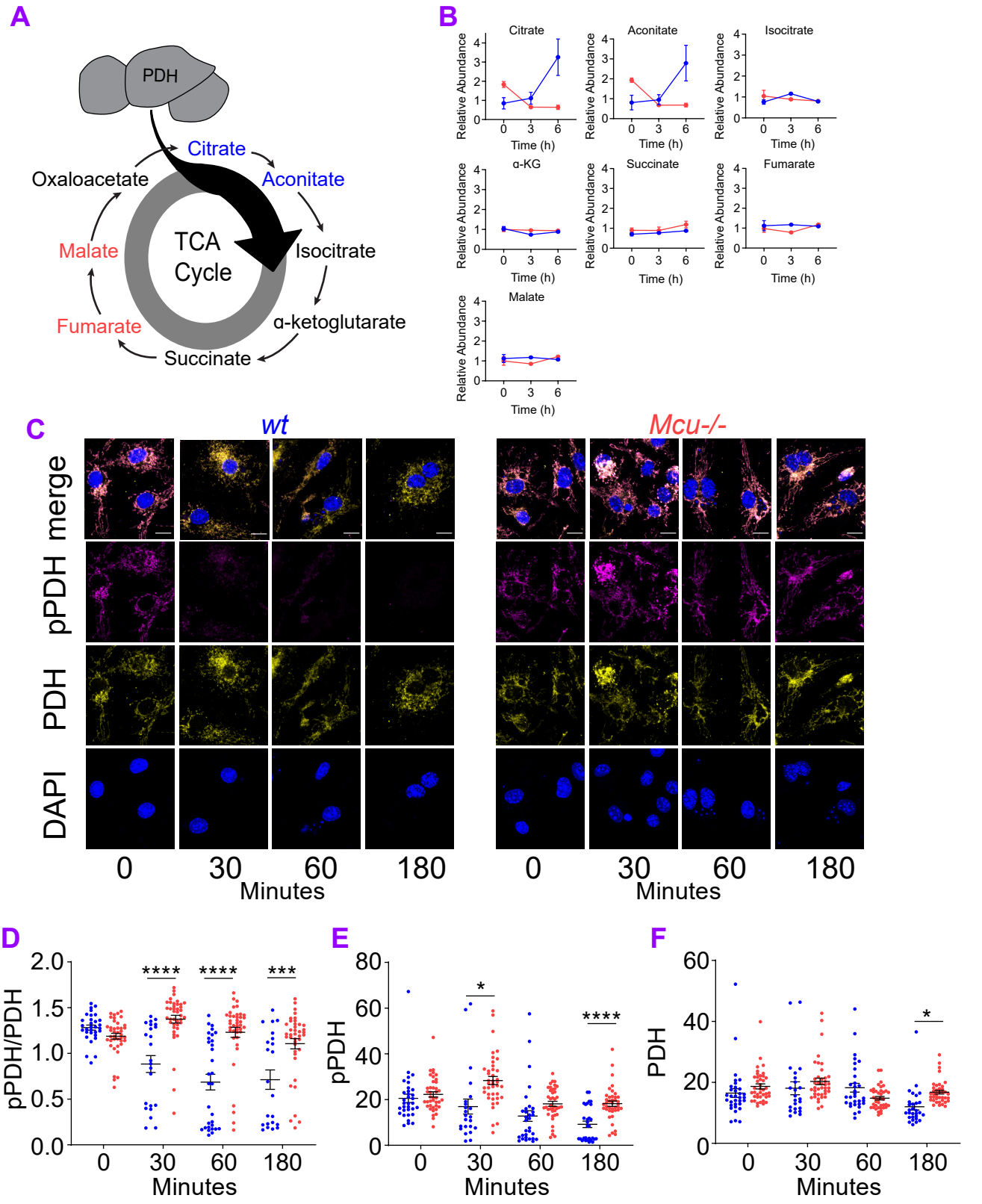


Figure S7. Metabolomic analysis of TCA cycle and quantification of pPDH IF (with Fig. 7)

A. Schematic of TCA cycle. Significantly decreased metabolites are blue and significantly increased metabolites are red. Significance was determined by Welch's, two-tailed t test.

B. TCA cycle metabolites measured at 0, 3 and 6 hours after zymosan stimulation in *Mcu-/-* (n=3) and *Wt* (n=3) macrophages. Error bars represent SEM.

C. Representative IF images from *wt* and *Mcu-/-* BMDMs responding to *C. albicans*. pPDH (magenta LUT), PDH (yellow LUT), and nucleus (DAPI; blue LUT) were acquired using LSM880 confocal microscope. Scale bar is 10 μm.

D. Quantification of pPDH/PDH from *wt* and *Mcu-/-* macrophages in response to *C. albicans*. Cells quantified for each time point are 0 h (n=31), 30 min (n=23), 60 min (n=31), and 180 min (n=22) for *wt*. For *Mcu-/-* all time points n=40 cells were quantified. Error bars represent SEM; *p=values (***p=0.0001,****p<0.0001)* was detected according to 2-way ANOVA, two-tailed.

E. Quantification of pPDH from *wt* and *Mcu-/-* macrophages in response to *C. albicans*. Cells quantified for each time point are 0 h (n=31), 30 min (n=23), 60 min (n=31), and 180 min (n=22) for *wt*. For *Mcu-/-* all time points n=40 cells were quantified. Error bars represent SEM; *p=values (***p<0.001, ****p<0.0001)* according to 2-way ANOVA, two-tailed.

F. Quantification of PDH from *wt* and *Mcu-/-* macrophages in response to *C. albicans*. Cells were combined from three independent experiments. Error bars represent SEM; **p=0.0109* was detected according to 2-way ANOVA, two-tailed.

Supplementary Methods

Clinical scoring parameters for *in vivo* candidiasis:

Scoring Parameter:	**0**	**1**	**2**
Conjunctivitis	Normal	Single eye open with visible discharge	Eyes closed with discharge and swelling
Lethargy	Normal locomotion and reaction, >3steps	Inactive, <3 steps after moderate stimulation, slight hunching	Only lifting of head after moderate stimulation <1 step, severe hunching
Hair Coat	Well-groomed with smooth coat	Rough coat, minor ruffling	Unkempt fur, dull coat
Grimace Pain	Normal	Moderate orbital tightening or nose bulge	Sever orbital tightening, nose bulge, and collapsed ear position
Stool	Normal	Visible diarrhea	Bloody diarrhea, sunken in abdomen