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Supplemental information

Metformin inhibition of mitochondrial ATP and DNA synthesis abrogates NLRP3 inflammasome activation and pulmonary inflammation

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Figure S1

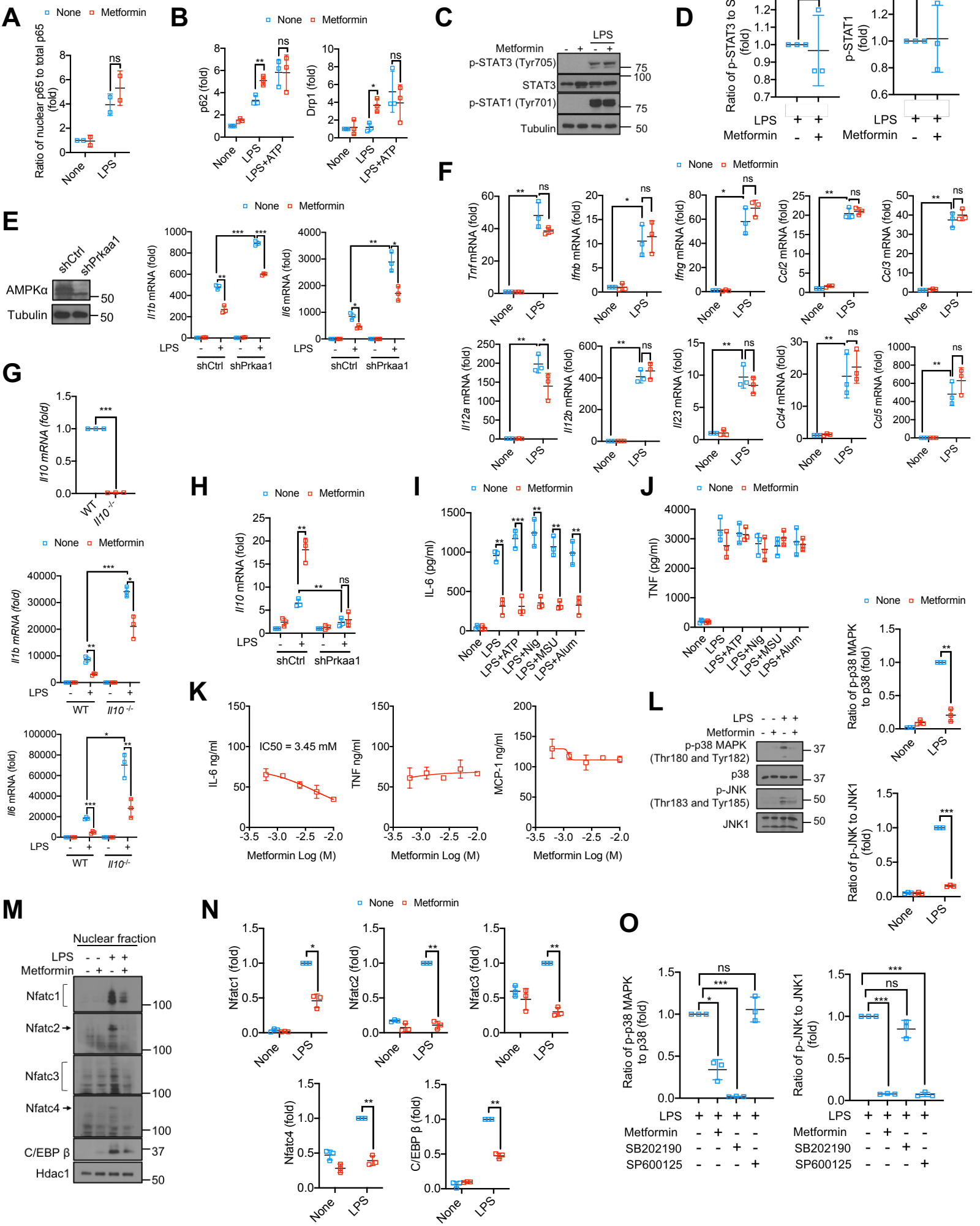

A
None
Metformin
Ratio of nuclear p65 to total p65 (fold)
ns
None
LPS
B
p62 (fold)
Drp1 (fold)
LPS+ATP
C
LPS
Metformin
p-STAT3 (Tyr705)
STAT3
p-STAT1 (Tyr701)
Tubulin
75
100
50
D
Ratio of p-STAT3 to STAT3 (fold)
p-STAT1 (fold)
E
shCtrl
shPrkaa1
AMPKα
Tubulin
Il1b mRNA (fold)
Il6 mRNA (fold)
F
Tnf mRNA (fold)
Ifnb mRNA (fold)
Ifng mRNA (fold)
Ccl2 mRNA (fold)
Ccl3 mRNA (fold)
Il12a mRNA (fold)
Il12b mRNA (fold)
Il23 mRNA (fold)
Ccl4 mRNA (fold)
Ccl5 mRNA (fold)
G
Il10 mRNA (fold)
WT
Il10 -/-
H
I
IL-6 (pg/ml)
LPS+Nig
LPS+MSU
LPS+Alum
J
TNF (pg/ml)
K
IC50 = 3.45 mM
IL-6 ng/ml
TNF ng/ml
MCP-1 ng/ml
Metformin Log (M)
L
p-p38 MAPK (Thr180 and Tyr182)
p38
p-JNK (Thr183 and Tyr185)
JNK1
Ratio of p-p38 MAPK to p38 (fold)
Ratio of p-JNK to JNK1 (fold)
M
Nuclear fraction
Nfatc1
Nfatc2
Nfatc3
Nfatc4
C/EBP β
Hdac1
N
O
SB202190
SP600125

Figure S1. Effect of metformin on different cytokines and chemokines, related to Figure 1

(A) Relative nuclear p65 to total p65 amounts by BMDM treated as in Figure 1A (n = 2).

(B) Relative p62 and Drp1 amounts in mitochondria isolated from BMDM treated as in Figure 1B (n = 3).

(C) IB analysis of phosphorylated STAT3 and STAT1 in lysates from BMDM pretreated -/+ metformin and stimulated with LPS. One representative IB out of 3 is shown.

(D) Relative phosphorylated STAT3 and STAT1 amounts in BMDM stimulated as in (C) (n = 3).

(E) Q-PCR quantitation of *Il1b* and *Il6* mRNAs (right) in shCtrl and shPrkaa1 (AMPKα1) primary BMDM pretreated or not with metformin (0.5 mM, 16 hrs) and stimulated with LPS (100 ng/ml) for 4 hrs. The left panel shows a blot that demonstrates knockdown efficiency (n = 3).

(F) Q-PCR quantitation of the indicated cytokine, chemokine and interferon mRNAs before or after LPS (100 ng/ml, 4 hrs) stimulation, -/+ metformin (0.5 mM, 16 hrs) pretreatment (n = 3).

(G) Q-PCR quantitation of *Il10*, *Il1b* and *Il6* mRNAs in WT and *Il10*$^{-/-}$ BMDMs treated as indicated (n = 3).

(H) Q-PCR quantitation of *Il10* mRNA in shCtrl and shPrkaa1 BMDM treated as in (E) (n = 3).

(I-J) IL-6 (I) and TNF (J) secretion by LPS (100 ng/ml, 4 hrs)-primed BMDM challenged with different NLRP3 activators as indicated, -/+ metformin (0.5 mM, 16 hrs) (n = 3).

(K) IL-6, TNF and MCP1 secretion by GM-CSF differentiated human macrophages pretreated with increasing concentrations of metformin for 2 hrs and stimulated with IFNγ (20 ng/ml) and LPS (100 ng/ml) for 24 hrs. Results are averages ± SD (n = 2 donors).

(L) IB analysis for p38 MAPK and JNK phosphorylation (left) and quantitation (right) in BMDM pretreated with metformin (0.5 mM, 16 hrs) stimulated with LPS (100 ng/ml) for 30 min. One representative IB out of 3 is shown.

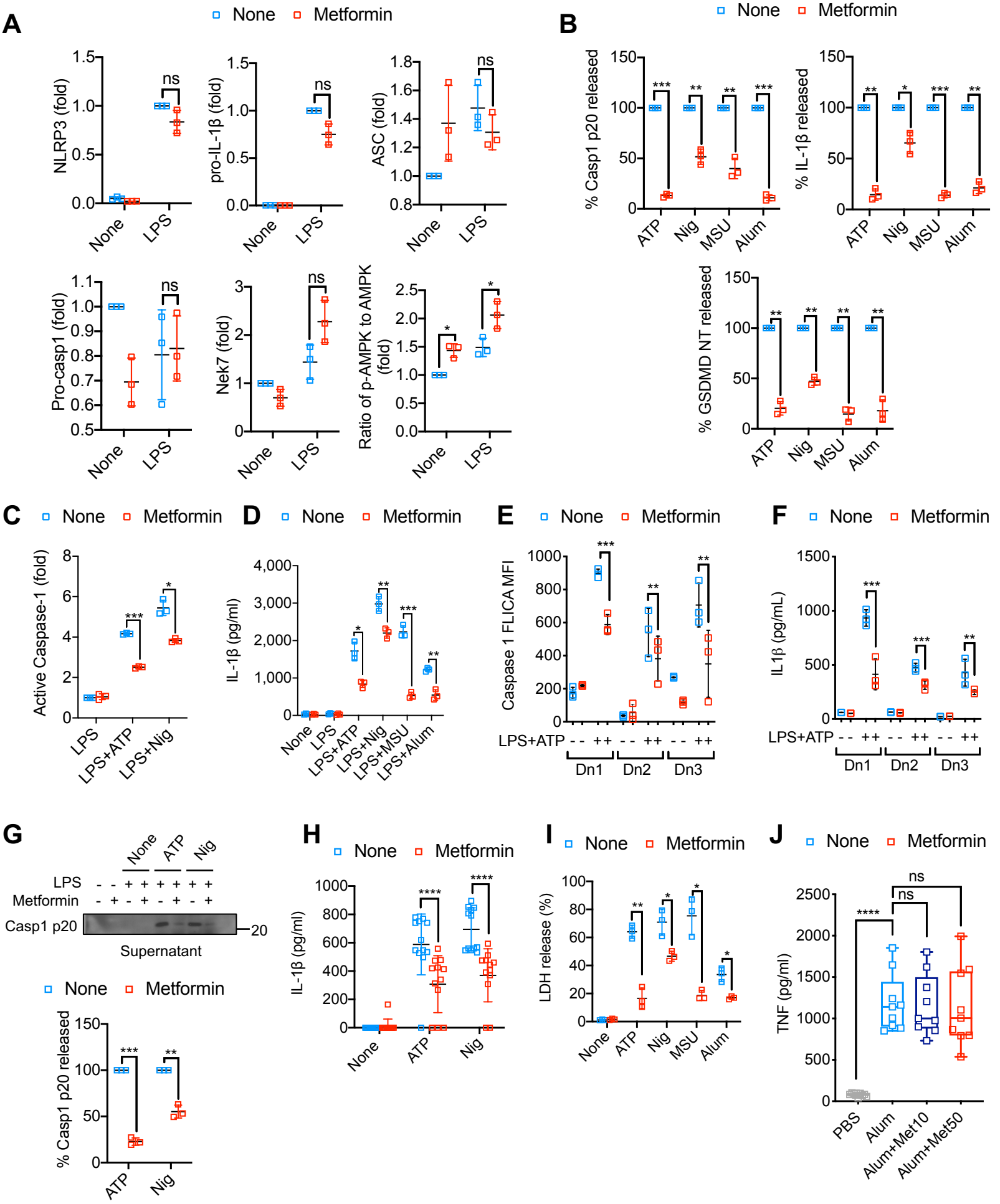
(M) IB analysis of indicated transcription factors in nuclear fractions from BMDM pretreated -/+ metformin (0.5 mM, 16 hrs) and stimulated with LPS (100 ng/ml, 4 hrs). HDAC1 was used as a loading control. One representative IB out of 3 is shown.

(N) Relative amounts of NFATc1, NFATc2, NFATc3, NFATc4 and c/EBPβ in BMDM treated as in (M) (n = 3).

(O) Relative amounts of phosphorylated p38 MAPK and JNK expression in BMDM treated as in Figure 1E (n = 3).

Results in (A, B, D-J, L, N and O) are averages ± SD. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant. Two-sided unpaired t-test.

Figure S2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None
Metformin
NLRP3 (fold)
pro-IL-1β (fold)
ASC (fold)
Pro-casp1 (fold)
Nek7 (fold)
Ratio of p-AMPK to AMPK (fold)
ns
None
LPS
B
% Casp1 p20 released
% IL-1β released
% GSDMD NT released
ATP
Nig
MSU
Alum
C
Active Caspase-1 (fold)
LPS
LPS+ATP
LPS+Nig
D
IL-1β (pg/ml)
LPS+MSU
LPS+Alum
E
Caspase 1 FLICA MFI
Dn1
Dn2
Dn3
F
IL1β (pg/mL)
G
Casp1 p20
Supernatant
H
I
LDH release (%)
J
TNF (pg/ml)
PBS
Alum
Alum+Met10
Alum+Met50

Figure S2. Metformin inhibits NLRP3 inflammasome activation, related to Figure 2

(A) Relative amounts of NLRP3 inflammasome components of BMDM as treated in Figure 2A (n = 3).

(B) Relative Casp1 p20, IL-1β and GSDMD NT secretion of BMDM treated as in Figure 2B (n = 3).

(C) Casp1 activity in LPS (100 ng/ml, 4 hrs)-primed BMDM stimulated with ATP (4 mM, 1hr) or nigericin (10 μM, 1 hr) -/+ metformin (0.5 mM, 16 hrs) pre-treatment was measured by FAM-FLICA® Caspase-1 (YVAD) Assay Kit (n = 3).

(D) IL-1β secretion by LPS (100 ng/ml, 4 hrs)-primed BMDM challenged with different NLRP3 activators as indicated, -/+ metformin (0.5 mM, 16 hrs) pre-treatment (n = 3).

(E-F) Active Casp1 measured by Caspase I FLICA dye (660-YVAD-FMK) staining and analyzed by flow cytometry (E) and IL-1β secretion measured by ELISA (F) in GM-CSF differentiated human macrophages LPS (20 ng/ml, 3 hrs)-primed BMDM stimulated with ATP (2 mM, 30 min) -/+ metformin (0.5 mM, 16 hrs) pre-treatment. Results are averages ± SD (n = 3 different donors, Dn1-3). **p<0.01; ***p < 0.005. Two-way ANOVA, Sydak's multiple comparison test.

(G-H) Casp1 p20 (G) and IL-1β (H) release by mouse microglia primed with LPS (100 ng/ml, 4 h) and challenged with different NLRP3 activators as indicated, -/+ metformin (0.5 mM, 16 hrs) pre-treatment, were determined by IB (G) or ELISA (H). (G) shows representative IB (top) and relative Casp1 p20 secretion (bottom) as averages ± SD (n = 3). **p < 0.01; ***p < 0.005. Two-sided unpaired t-test. (H) shows averages ± SEM (n = 12). ****p < 0.0001. Two way ANOVA and Sidak's multiple comparison post-hoc test.

(I) LDH release by LPS (100 ng/ml, 4 hrs)-primed BMDM stimulated with the indicated NLRP3 agonists, -/+ metformin (0.5 mM, 16 hrs) pre-treatment (n = 3).

(J) Peritoneal TNF in mice treated with 10 or 50 mg/kg metformin 30 min prior to i.p. alum (700 mg) injection (n = 9 mice per group). Peritoneal fluid was collected 4 hrs after alum injection. Results are averages ± SEM ****p < 0.0001; ns, not significant. Two-sided unpaired t-test.

Results in (A-D, G and I) are averages ± SD. *p<0.05; **p < 0.01; ***p < 0.001; ns, not significant. Two-sided unpaired t-test.

Figure S3

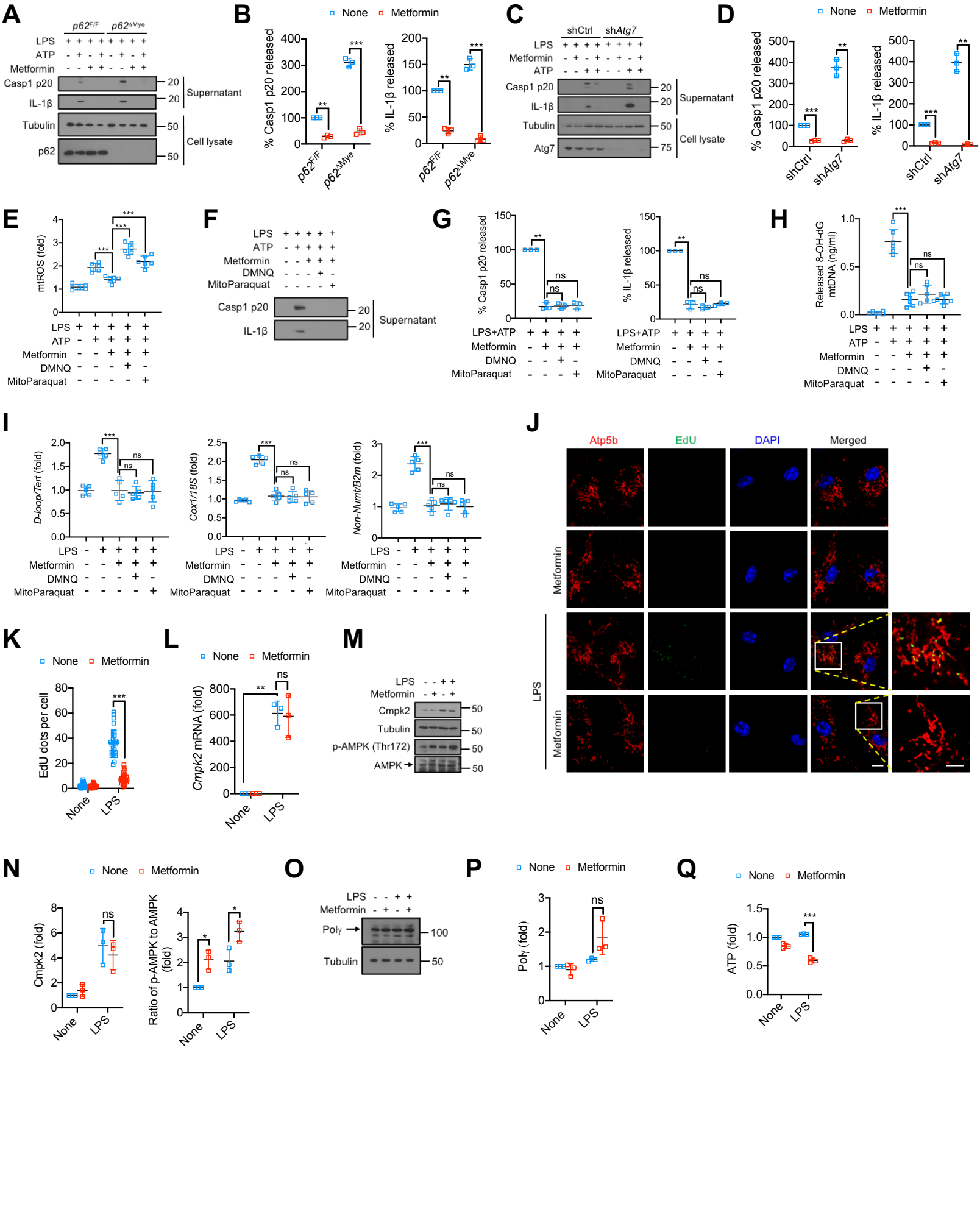

A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
None
Metformin
Supernatant
Cell lysate
Casp1 p20
IL-1β
Tubulin
p62
Atg7
LPS
ATP
Metformin
DMNQ
MitoParaquat
shCtrl
shAtg7
% Casp1 p20 released
% IL-1β released
mtROS (fold)
Released 8-OH-dG mtDNA (ng/ml)
D-loop/Tert (fold)
Cox1/18S (fold)
Non-Numt/B2m (fold)
Atp5b
EdU
DAPI
Merged
EdU dots per cell
Cmpk2 mRNA (fold)
Cmpk2
p-AMPK (Thr172)
AMPK
Cmpk2 (fold)
Ratio of p-AMPK to AMPK (fold)
Polγ
Polγ (fold)
ATP (fold)
ns

Figure S3. Metformin inhibits NLRP3 inflammasome activation independently of mitophagy, related to Figure 3

(A) IB analysis of Casp1 p20 and IL-1β in culture supernatants, and p62 and tubulin in cell lysates of LPS (100 ng/ml, 4 hrs)-primed *p62*$^{F/F}$ or *p62*$^{\Delta Mye}$ BMDM stimulated with ATP (4 mM, 1 hr), -/+ metformin (0.5 mM, 16 hrs) pre-treatment. One representative IB out of 3 is shown.

(B) Quantitation of above results, averages ± SD (n=3).

(C) IB analysis of Casp-1 p20 and IL-1β in culture supernatants and ATG7 and tubulin in cell lysates of LPS-primed control (shCtrl) or ATG7-deficient (shAtg7) iBMDM stimulated with ATP (4mM, 1 hr) -/+ metformin (0.5 mM, 16 hrs) pre-treatment. One representative IB out of 3 is shown.

(D) Quantitation of IB in (C), averages ± SD (n = 3).

(E) Relative mtROS amounts measured by MitoSOX staining of LPS (100 ng/ml, 4 hrs)-primed BMDM challenged with ATP (4 mM, 1hr) -/+ metformin (0.5 mM, 16 hrs), DMNQ (20 μM, 16 hrs), or MitoParaquat (5 μM, 1 hr) pretreatment (n = 6).

(F) IB analysis of Casp1 p20 and mature IL-1β in culture supernatants, of BMDM treated as above. One representative IB out of 3 is shown.

(G) Quantitation of IB in (F), averages ± SD (n = 3).

(H) 8-OH-dG amounts in cytosolic mtDNA of BMDM treated as above (n = 6).

(I) Relative total mtDNA amounts in LPS (200 ng/ml, 4 hrs)-primed BMDM treated -/+metformin (0.5 mM, 16 hrs), DMNQ (20 μM, 16 hrs), or MitoParaquat (5 μM, 1 hr) pretreatment. Shown are the ratios of *D-loop* mtDNA to *Tert* nuclear (n) DNA, *Cox1* mtDNA to *18S* nDNA, or mtDNA that is not inserted into nuclear DNA (*non-NUMT*) to *B2m* nDNA. Results are averages ± SD (n = 5).

(J-K) Representative fluorescent microscopy images (J) and quantification (K) of EdU-labelled BMDM that were co-stained for Atp5b and DAPI before and after stimulation with LPS (1 ug/ml, 4 hrs), -/+ metformin (0.5 mM, 16 hrs) pre-treatment. Scale bars, 10 μm and 2 μm. (K) shows results as average ± SD (n = 30 HMF per treatment in 3 independent experiments).

(L) Q-PCR quantitation of *Cmpk2* mRNA in BMDM treated with LPS (100 ng/ml, 4 hrs) -/+ metformin (0.5 mM, 16 hrs) (n = 3).

(M-N) IB analysis of CMPK2 expression and AMPK phosphorylation in LPS (100 ng/ml, 4 hrs)-primed BMDMs -/+ metformin (0.5 mM, 16 hrs) pre-treatment. (M) shows representative blot. (N) shows quantitation of above results, averages ± SD (n = 3).

(O-P) IB analysis of Polγ expression in BMDM treated as indicated. (O) shows one representative blot and (P) shows quantitation, averages ± SD (n = 3).

(Q) Relative cellular ATP amounts in LPS (100 ng/ml, 4 hrs)-primed BMDMs -/+ metformin (0.5 mM, 16 hrs) pre-treatment (n=3).

Results in (B, D, E, G-I, K, L , N, P and Q) are averages ± SD. *p<0.05; **p < 0.01; ***p < 0.001; ns, not significant. Two-sided unpaired t-test.

Figure S4

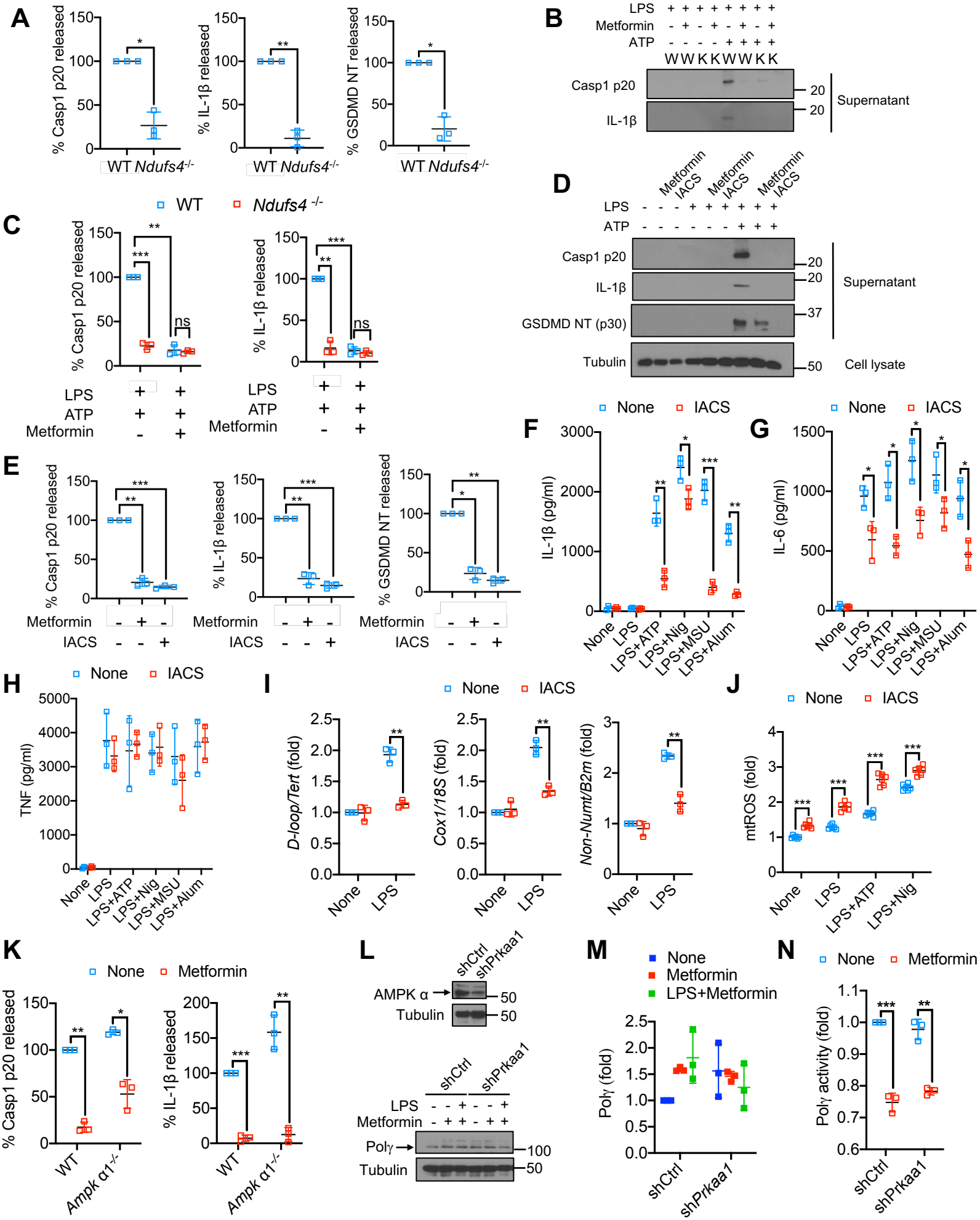

A
% Casp1 p20 released
% IL-1β released
% GSDMD NT released
WT
Ndufs4-/-
B
LPS
Metformin
ATP
W W K K W W K K
Casp1 p20
IL-1β
Supernatant
C
WT
Ndufs4 -/-
% Casp1 p20 released
% IL-1β released
ns
LPS
ATP
Metformin
D
Metformin IACS
Casp1 p20
IL-1β
GSDMD NT (p30)
Tubulin
Supernatant
Cell lysate
E
% Casp1 p20 released
% IL-1β released
% GSDMD NT released
Metformin
IACS
F
None
IACS
IL-1β (pg/ml)
None
LPS
LPS+ATP
LPS+Nig
LPS+MSU
LPS+Alum
G
IL-6 (pg/ml)
H
TNF (pg/ml)
I
D-loop/Tert (fold)
Cox1/18S (fold)
Non-Numt/B2m (fold)
J
mtROS (fold)
K
% Casp1 p20 released
% IL-1β released
WT
Ampk α1-/-
L
shCtrl
shPrkaa1
AMPK α
Tubulin
LPS
Metformin
Polγ
M
None
Metformin
LPS+Metformin
Polγ (fold)
shCtrl
shPrkaa1
N
Polγ activity (fold)

Figure S4. Metformin inhibits NLRP3 inflammasome activation independently of AMPK, related to Figure 4.

(A) Relative Casp1 p20, IL-1β and GSDMD NT release by LPS (100 ng/ml, 4 hrs)-primed wild type (WT) or *Ndufs4*$^{-/-}$ BMDM stimulated with ATP (4 mM, 1hr), shown as in Figure 4A (n = 3).

(B, C) IB analysis of Casp1 p20 and mature IL-1β in culture supernatants of LPS (100 ng/ml, 4 hrs)-primed wild type (W) or *Ndufs4*$^{-/-}$ (K) BMDM stimulated with ATP (4 mM, 1 hr), -/+ metformin (0.5 mM, 16 hrs) pretreatment. Representative IB of 3 independent experiments. (C) Quantitation of above results, averages ± SD (n = 3).

(D, E) IB analysis of Casp1 p20, mature IL-1β and cleaved gasdermin D (GSDMD NT (p30)) in culture supernatants, and tubulin in lysates of BMDM that were LPS (100 ng/ml, 4 hrs)-primed and ATP (4 mM, 1 hr) challenged as indicated, -/+ metformin (0.5 mM, 16 hrs) or IACS-010759 (20 μM, 16 hrs) pretreatment. (E) Quantitation of above results, averages ± SD (n = 3).

(F-H) IL-1β (F), IL-6 (G) and TNF (H) secretion by BMDM pretreated -/+ IACS-010759 (20 μM, 16 hrs) that were left unstimulated or LPS (100 ng/ml, 4 hrs)-primed and challenged with the indicated NLRP3 activators (n = 3).

(I) Relative total mtDNA amounts in LPS (200 ng/ml, 4 hrs)-primed BMDM -/+ IACS-010759 (20 μM, 16 hrs) pre-treatment. Shown are the ratios of *D-loop* mtDNA to *Tert* nuclear (n) DNA, *Cox1* mtDNA to *18S* nDNA, or mtDNA that is not inserted into nuclear DNA (*non-NUMT*) to *B2m* nDNA (n = 3).

(J) Relative mtROS amounts measured by MitoSOX staining of BMDM -/+ IACS-010759 (20 μM, 16 hrs) pretreatment that were left unstimulated or LPS (100 ng/ml, 4 hrs)-primed and challenged with the indicated NLRP3 activators (n = 6).

(K) Relative Casp1 p20 and IL-1β release by LPS (100 ng/ml, 4 hrs)-primed wild type (WT) or *Ampkα1*$^{-/-}$ BMDM stimulated -/+ ATP (4 mM, 1 hr), shown as in Figure 4C (n = 3).

(L, M) IB analysis of AMPKα1/2 (top), POLγ (bottom) and tubulin in lysates of shCtrl and shPrkaa1 treated BMDM that were stimulated with LPS (100 ng/ml, 4 hrs) -/+ metformin (0.5 mM, 16 hrs) pretreatment. The top panel demonstrates knockdown efficiency. (M) Quantitation of POLγ expression, averages ± SD (n = 3).

(N) Polγ activity in lysates of LPS (100 ng/ml, 4 hrs)-primed shCtrl and shPrkaa1 BMDM with or without metformin (0.5 mM, 16 hrs) pretreatment (n = 3).

Results in (A, C, E-K, and M-N) are averages ± SD. *p <0.05; **p < 0.01; ***p < 0.001; ns, not significant. Two-sided unpaired t-test.

Figure S5

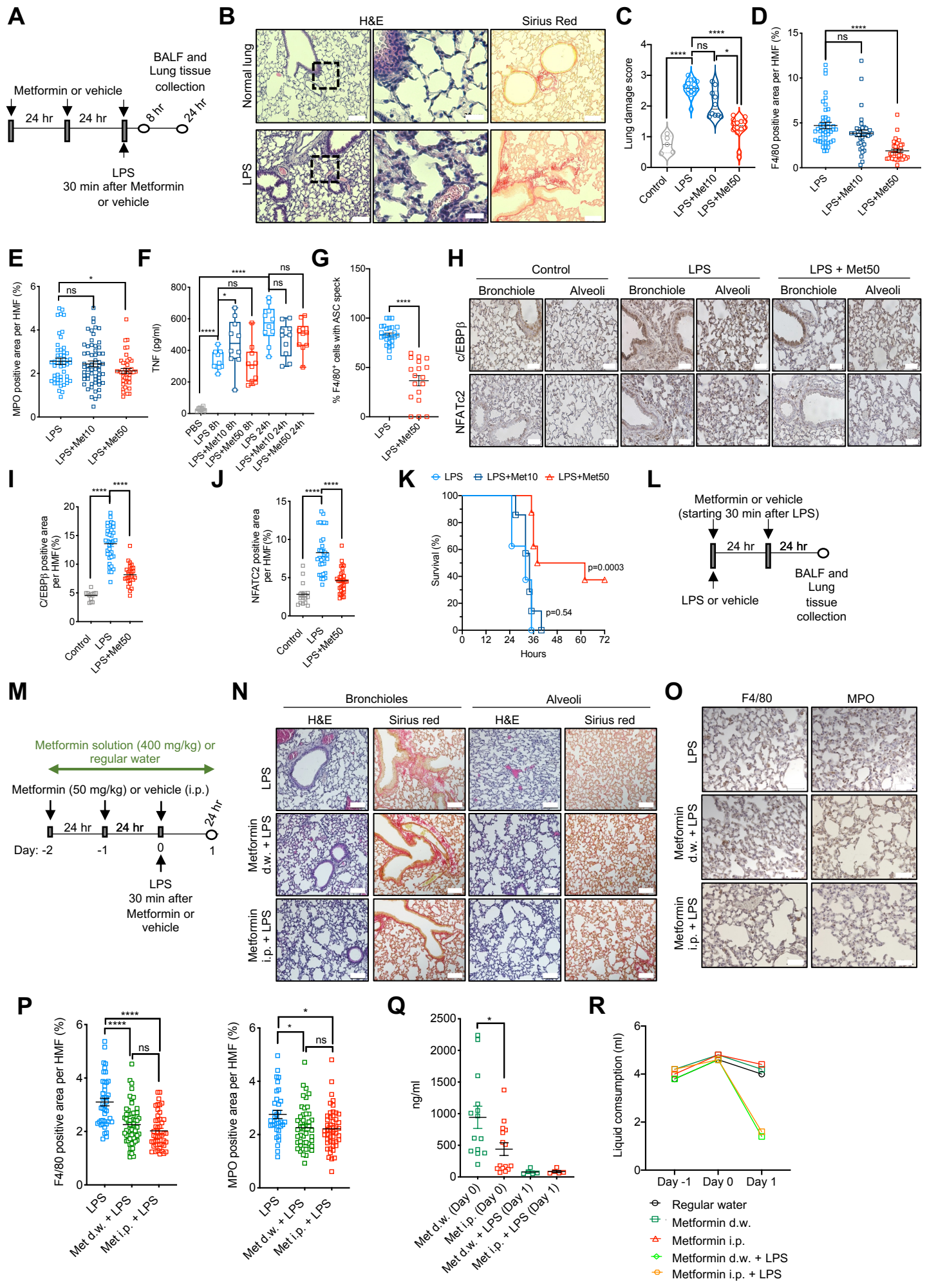

A
Metformin or vehicle
24 hr
24 hr
8 hr
24 hr
BALF and Lung tissue collection
LPS
30 min after Metformin or vehicle
B
H&E
Sirius Red
Normal lung
LPS
C
Lung damage score
Control
LPS
LPS+Met10
LPS+Met50
D
F4/80 positive area per HMF (%)
E
MPO positive area per HMF (%)
F
TNF (pg/ml)
PBS
LPS 8h
LPS+Met10 8h
LPS+Met50 8h
LPS 24h
LPS+Met10 24h
LPS+Met50 24h
G
% F4/80+ cells with ASC speck
H
Control
LPS
LPS + Met50
Bronchiole
Alveoli
c/EBPβ
NFATc2
I
C/EBPβ positive area per HMF (%)
J
NFATC2 positive area per HMF (%)
K
LPS
LPS+Met10
LPS+Met50
Survival (%)
Hours
p=0.0003
p=0.54
L
Metformin or vehicle (starting 30 min after LPS)
24 hr
24 hr
LPS or vehicle
BALF and Lung tissue collection
M
Metformin solution (400 mg/kg) or regular water
Metformin (50 mg/kg) or vehicle (i.p.)
24 hr
24 hr
24 hr
Day: -2
-1
0
1
LPS
30 min after Metformin or vehicle
N
Bronchioles
Alveoli
H&E
Sirius red
LPS
Metformin d.w. + LPS
Metformin i.p. + LPS
O
F4/80
MPO
P
F4/80 positive area per HMF (%)
MPO positive area per HMF (%)
Met d.w. + LPS
Met i.p. + LPS
Q
ng/ml
Met d.w. (Day 0)
Met i.p. (Day 0)
Met d.w. + LPS (Day 1)
Met i.p. + LPS (Day 1)
R
Liquid comsumption (ml)
Day -1
Day 0
Day 1
Regular water
Metformin d.w.
Metformin i.p.
Metformin d.w. + LPS
Metformin i.p. + LPS

Figure S5. Metformin inhibits LPS-induced ARDS, related to Figure 5.

(A) Treatment and analysis scheme. Mice were i.p. injected with metformin (10 or 50 mg/kg), or vehicle daily for three days. LPS was i.p. injected 30 minutes after the last metformin injection. Lung tissue and BALF were collected 8 and 24 hrs after LPS administration.

(B) H&E and Sirius red staining of lung tissue isolated at the 24 hrs time point from control mice or mice i.p. injected with LPS (5 mg/kg). Scale bar 100 μm. Scale bar in highlighted area 20 μm. n = 10 mice per group. Ten to twelve images per mouse were evaluated. The figure shows a representative image for each group.

(C) The degree of lung damage and inflammation was blindly scored by two mouse lung pathology experts. The degree of lung damage and inflammation was assigned an arbitrary score of 0 (baseline, no inflammation, no airway thickening, no edema), 1 (minimal cellular infiltration, and minimal edema), 2 (mild-moderate cellular infiltration, plus mild airway thickening and mild edema), 3 (severe cellular infiltration, plus diffuse airway thickening and severe edema). The scores were averaged and are shown as average ± SEM. n = 10-16 mice. *p<0.05; ****p<0.001; ns, not significant. All groups were compared to each other using ANOVA Kruskal-Wallis test.

(D-E) Area (in %) occupied by F4/80 stained macrophages (D), and MPO stained neutrophils (E), in lung sections from Figure 5B are shown as averages ± SEM. n = 4 mice per group. Ten to twelve HMF per group were quantified using ImageJ. *p<0.05; ****p < 0.001; ns, not significant. All groups were compared to each other using unpaired T-test and Mann-Whitney test.

(F) TNF amounts in BALF from mice in Figure 5A measured by ELISA. n = 10 mice per group. *p < 0.05; ****p < 0.001; ns, not significant. Unpaired T-test and Mann-Whitney test.

(G) Percentage of F4/80^{+} cells with ASC specks in lung sections from mice treated as in Figure 5E. Cells were quantified in 18-26 high magnification fields from 4-5 mice per group. Data are shown as average ± SEM. ****p < 0.001. Unpaired T-test and Mann-Whitney test.

(H) C/EBPβ and NFATc2 staining of lung tissue isolated at the 24 hrs time point from control mice or mice i.p. injected with LPS (5 mg/kg). Scale bar 50 μm. n = 5 mice per

group. Ten to twelve images per mouse were evaluated. The figure shows a representative image for each group.

(I-J) Area (in %) occupied by C/EBPβ (I), and NFATc2 staining (J), in lung sections from (H) are shown as averages ± SEM. n = 3 mice per group. Ten to twelve HMF per group were quantified using ImageJ. ****p<0.001. All groups were compared to each other using unpaired T-test and Mann-Whitney test.

(K) Kaplan Meyer survival analysis of mice pretreated with vehicle or metformin (10 and 50 mg/kg) and i.p. injected with LPS (30 mg/kg). n = 8 mice per group. Log-rank (Mantel-Cox) test, p = 0.0003 LPS+Met50 vs LPS; p = 0.54 LPS+Met10 vs LPS.

(L) Treatment and analysis scheme of therapeutic administration of metformin. Mice were i.p. injected with 50 mg/kg metformin or vehicle daily for two days starting 30 minutes after i.p. injection of 5 mg/kg LPS. Lung tissue and BALF were collected 48 hrs after LPS administration.

(M) Treatment and analysis scheme for comparison the prophylactic i.p. versus oral administration of metformin. Metformin was provided to the mice in the drinking water (ad libitum at 400 mg/kg) or i.p. (daily at 50 mg/kg), starting two days before LPS challenge with 5 mg/kg. Lung tissue and plasma were collected 24 hrs after LPS administration.

(N) H&E and Sirius red staining of lung tissue collected from mice in (B). Scale bar 100 μm. n = 8 mice per group. Ten to twelve images per mouse were evaluated. The figure shows a representative image for each group.

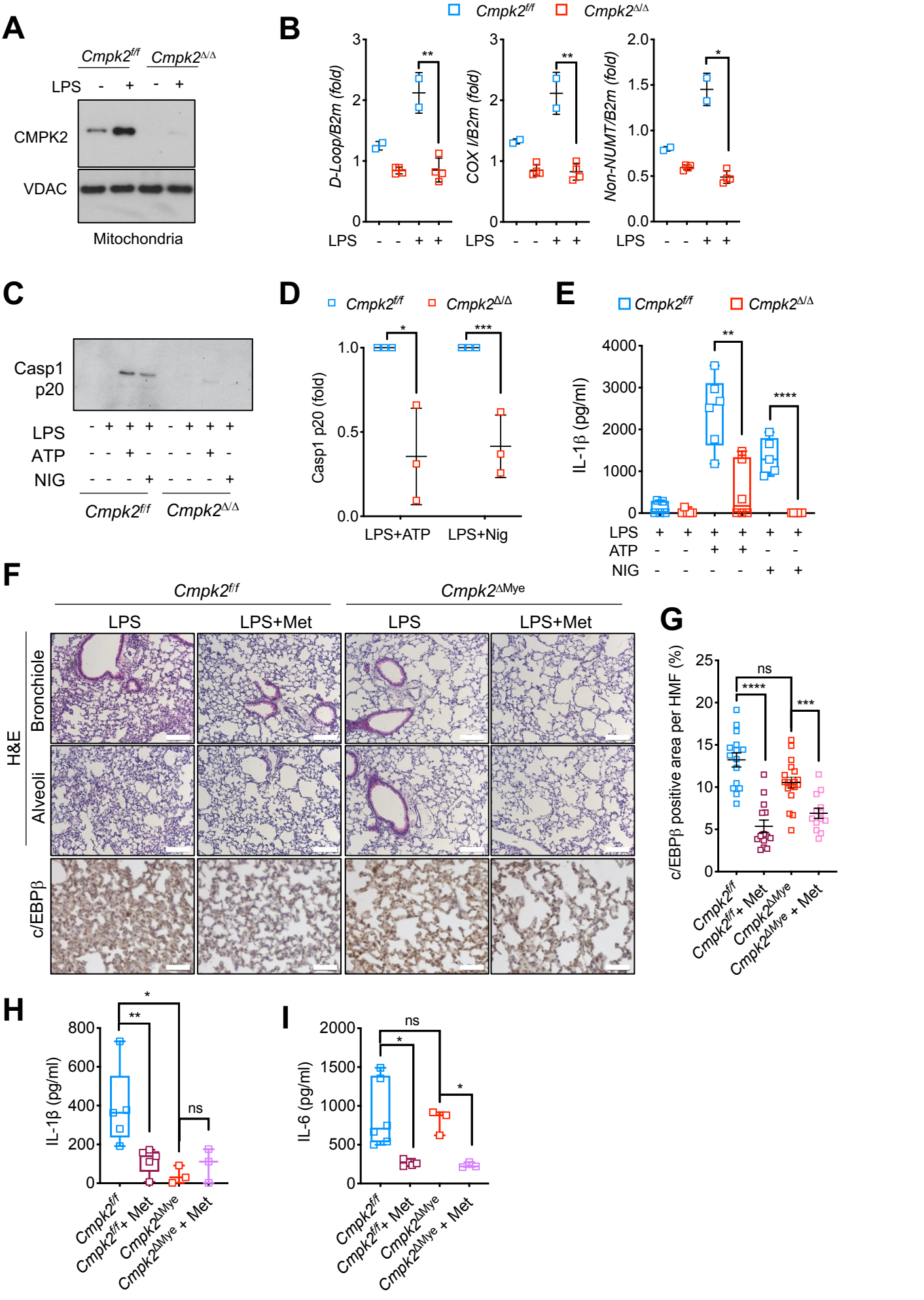
(O) Lung sections from mice in (N) were stained with F4/80 and MPO antibodies. Scale bar 50 μm. n = 4 mice per group. Ten to twelve images per mouse were evaluated. The figure shows a representative image for each group.

(P) Area (in %) occupied by F4/80 stained macrophages (left), and MPO stained neutrophils (right), in lung sections from (O) are shown as averages ± SEM. n = 4 mice per group. Ten to twelve HMF per group were quantified using ImageJ. *p<0.05; ****p<0.001; ns, not significant. All groups were compared to each other using unpaired T-test and Mann-Whitney test.

(Q) Metformin concentration in plasma from mice in (B) collected 1 hr after i.p. metformin on day 0 and 24 hrs after LPS challenge (day 1). n = 5-14 mice per group. * p < 0.05. Unpaired T-test and Mann-Whitney test.

(R) Liquid consumption by mice in (N). Data are averages of 5 mice per group.

Figure S6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Cmpk2f/f
Cmpk2Δ/Δ
LPS - + - +
CMPK2
VDAC
Mitochondria
B
Cmpk2f/f
Cmpk2Δ/Δ
D-Loop/B2m (fold)
COX I/B2m (fold)
Non-NUMT/B2m (fold)
LPS - - + +
C
Casp1 p20
LPS - + + + - + + +
ATP - - + - - - + -
NIG - - - + - - - +
Cmpk2f/f
Cmpk2Δ/Δ
D
Cmpk2f/f
Cmpk2Δ/Δ
Casp1 p20 (fold)
LPS+ATP
LPS+Nig
E
Cmpk2f/f
Cmpk2Δ/Δ
IL-1β (pg/ml)
LPS + + + + + +
ATP - - + + - -
NIG - - - - + +
F
Cmpk2f/f
Cmpk2ΔMye
LPS
LPS+Met
LPS
LPS+Met
H&E
Bronchiole
Alveoli
c/EBPβ
G
c/EBPβ positive area per HMF (%)
ns
****
***
Cmpk2f/f
Cmpk2f/f + Met
Cmpk2ΔMye
Cmpk2ΔMye + Met
H
IL-1β (pg/ml)
ns
Cmpk2f/f
Cmpk2f/f + Met
Cmpk2ΔMye
Cmpk2ΔMye + Met
I
IL-6 (pg/ml)
ns
Cmpk2f/f
Cmpk2f/f + Met
Cmpk2ΔMye
Cmpk2ΔMye + Met

Figure S6. Myeloid specific CMPK2 ablation decreases IL-1β but not IL-6 amounts in BALF, related to Figure 6.

(A) IB analysis of CMPK2 in mitochondria from BMDM isolated from *Cmpk2*^{ff} and *Cmpk2*$^{\Delta Mye}$ mice (*Cmpk2*^{ff} and *Cmpk2*$^{\Delta/\Delta}$, respectively), that were stimulated with LPS (200 ng/ml) for 4 hrs.

(B) Relative total mtDNA amounts *Cmpk2*^{ff} and *Cmpk2*$^{\Delta/\Delta}$ BMDM stimulated with LPS as above. Shown are the ratios of *D-loop* mtDNA, *Cox1* mtDNA, or mtDNA that is not inserted into nuclear DNA (*non-NUMT*) to *B2m* nDNA (n = 4). Results are averages ± SD. *p <0.05; **p < 0.01; Two-sided unpaired t-test.

(C-D) IB analysis of Casp1 p20 (C) and quantification (D) in culture supernatants of *Cmpk2*^{ff} and *Cmpk2*$^{\Delta/\Delta}$ BMDM that were LPS-primed and stimulated with ATP (4 mM) or nigericin (10 μM) (n = 3). Results are average ± SD.

(E) IL-1β concentrations in culture supernatants of *Cmpk2*^{ff} and *Cmpk2*$^{\Delta/\Delta}$ BMDM treated as indicated in (C). Results are averages ± SEM. **p < 0.01; ****p <0.001. Two-sided unpaired t-test.

(F) H&E and c/EBPβ staining of lung tissue collected from *Cmpk2*^{ff} and *Cmpk2*$^{\Delta Mye}$ mice pretreated with 50 mg/kg metformin daily starting 2 days before LPS challenge. Scale bar 100 μm for H&E and 50 μm for C/EBPβ. n = 4 - 8 mice per group. Ten to twelve images per group were evaluated. The figure shows a representative image for each group.

(G) Area (in %) occupied by C/EBPβ positive cells is shown as averages ± SEM. n = 4 mice per group. Fifteen to twenty HMF images per group were quantified using ImageJ. ***p<0.005; ****p<0.001; ns, not significant. Unpaired T-test Mann-Whitney test.

(H-I) IL-1β (H) and IL-6 (I) concentrations in bronchioalveolar lavage fluid from *Cmpk2*^{ff} and *Cmpk2*$^{\Delta Mye}$ mice pretreated with 50 mg/kg metformin or vehicle starting 2 days before i.p. LPS administration. n = 3 - 5 mice per group. *p<0.05; **p<0.01; ns, not significant. Unpaired T-test Mann-Whitney test.

Figure S7

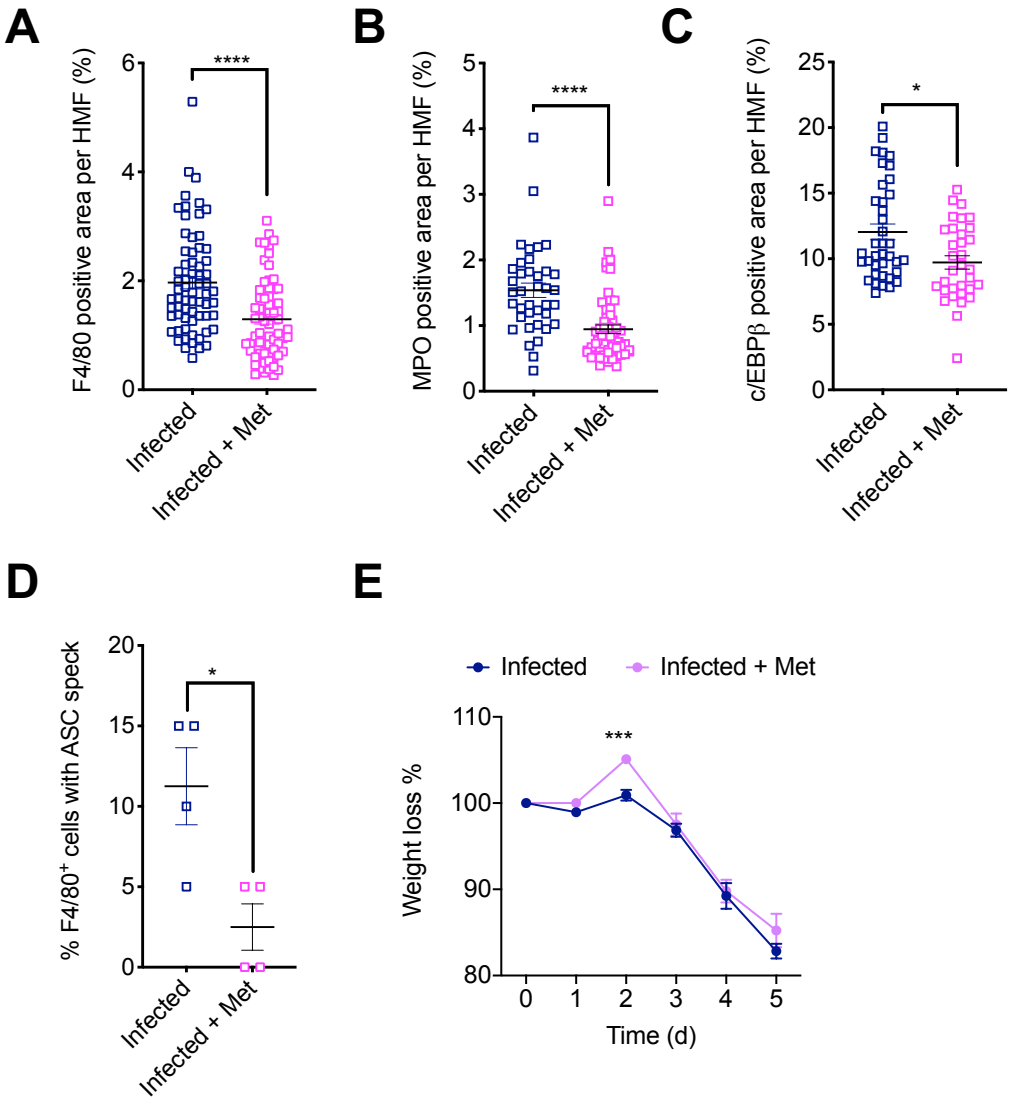
A
F4/80 positive area per HMF (%)
****
Infected
Infected + Met
B
MPO positive area per HMF (%)
****
Infected
Infected + Met
C
c/EBPβ positive area per HMF (%)
*
Infected
Infected + Met
D
% F4/80+ cells with ASC speck
*
Infected
Infected + Met
E
Infected
Infected + Met
Weight loss %
***
Time (d)

Figure S7. Metformin ameliorates ARDS induced by SARS-CoV-2 infection of hACE2 mice, related to Figure 7.

(A-C) Area (in %) occupied by F4/80 stained macrophages (A), MPO stained neutrophils (B), and c/EBPβ positive cells (C), in lung sections from hACE2 Tg mice infected with SARS-CoV-2 (Figure 7F). Data are averages ± SEM. n = 4 mice per group. Ten HMF per group were quantified using ImageJ. *p<0.05; ****p<0.001. Unpaired T-test and Mann-Whitney test.

(D) Percentages of $F4/80^{+}$ cells with ASC specks in lung sections from mice in Figure 7G. Cells were quantified in 8 high magnification fields from 4 mice per group. Data are shown as average ± SEM. *p<0.05. Unpaired T-test and Mann-Whitney test.

(E) Percentage of weight loss during the course of SARS-CoV-2 infection (day 0 to day 5). n = 8 - 9 mice per group. ***p<0.005. Two-way ANOVA Sydak's multiple comparison test.