SUPPLEMENTAL INFORMATION

mTORC1 induced HK1-dependent glycolysis regulates NLRP3 inflammasome activation

Jong-Seok Moon, Shu Hisata, Mi-Ae Park, Gina M. DeNicola, Stefan W. Ryter, Kiichi Nakahira & Augustine M. K. Choi

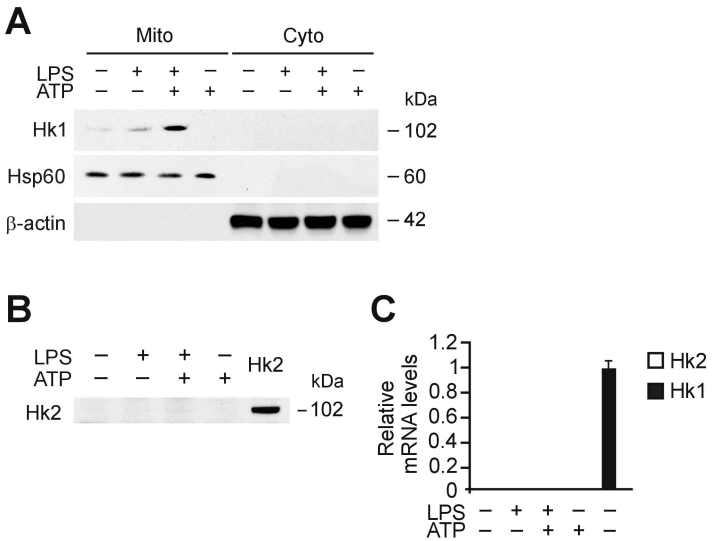

Figure S1. The mitochondrial HK1 expression is induced during NLRP3 inflammasome activation, Related to Figure 2.

(A) Immunoblot analysis for HK1 in mitochondrial (Mito) and cytosolic (Cyto) fractions of cell lysates from wild type BMDM treated with LPS (500 ng/ml) for 4 h, or LPS followed by incubation with ATP (2 mM) for 30 min. HSP60 served as the mitochondrial marker and loading control. β-actin served as the cytosolic marker and loading control. (B) Immunoblot analysis for HK2 in cell lysates from wild type BMDM treated with LPS (500 ng/ml) for 4 h, or LPS followed by incubation with ATP (2 mM) for 30 min. HK2 protein served as the positive control. (C) Quantitative PCR analysis for *Hk1* and *Hk2* gene expression from wild type BMDM treated with LPS (500 ng/ml) for 4 h, or LPS followed by incubation with ATP (2 mM) for 30 min.

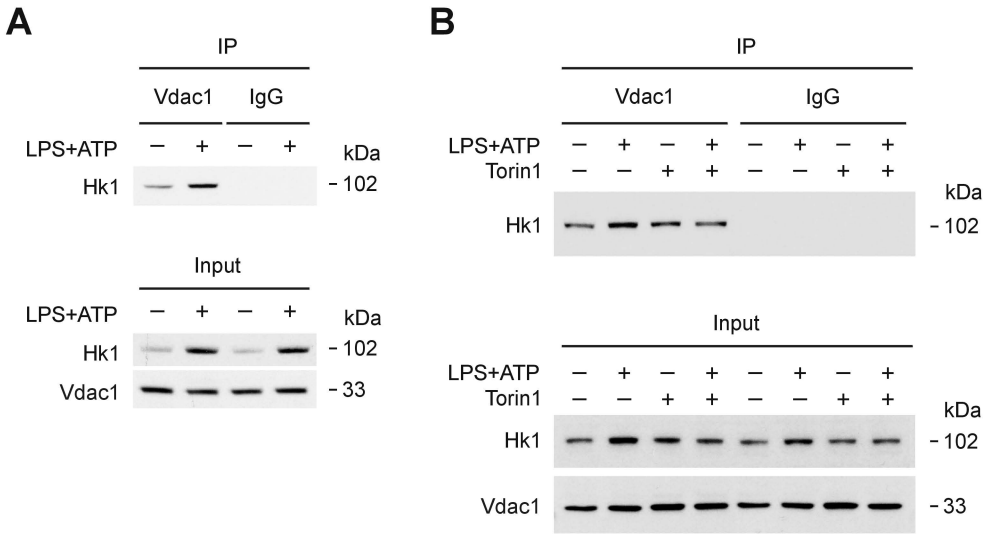

Figure S2. HK1 interacts with VDAC1 in mitochondria in macrophages, Related to Figure 2.

(A) Immunoprecipitation (IP) assay for the interaction of HK1 and VDAC1 in mitochondria of mouse J774A.1 macrophages treated with LPS and ATP. VDAC1 was immunoprecipitated with anti-VDAC1 antibody or rabbit IgG from mitochondrial fraction, followed by immunoblotting for HK1. (B) Immunoprecipitation (IP) assay for the interaction of HK1 and VDAC1 in mitochondria of mouse J774A.1 macrophages pre-treated with Torin1 (250 nM) for 1h before LPS and ATP stimulation. VDAC1 was immunoprecipitated with anti-VDAC1 antibody or rabbit IgG from mitochondrial fraction, followed by immunoblotting for HK1.

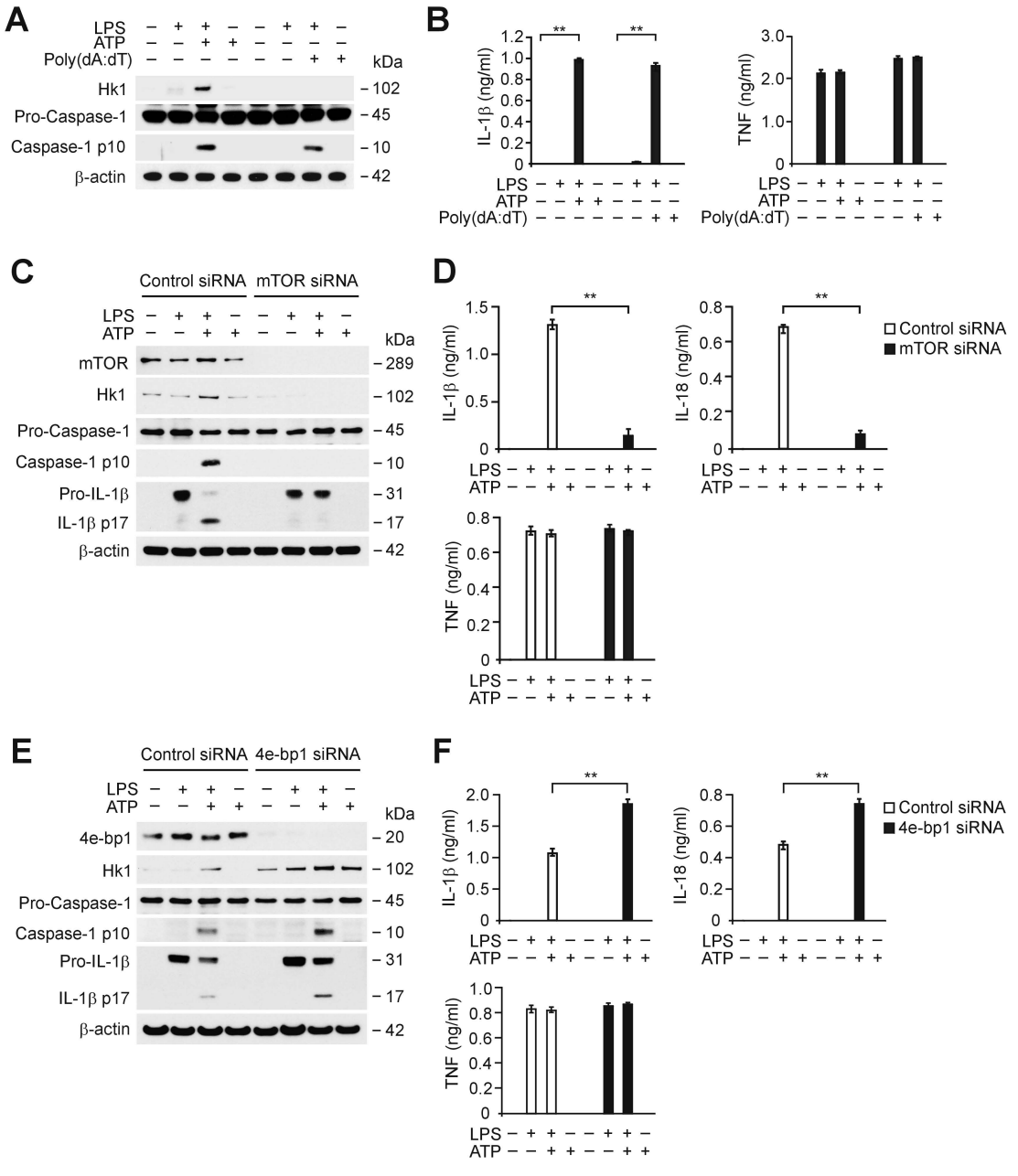

Figure S3. mTORC1 regulates HK1 expression during NLRP3 inflammasome activation, Related to Figure 5.

(A) Immunoblot analysis for HK1 and caspase-1 of cell lysates wild-type BMDM treated with LPS (500 ng/ml) and ATP (2 mM) for 30 min or poly (dA:dT) (1 μg/ml) for 6 h. β-actin served as the standard. (B) ELISA assay for IL-1β and TNF secretion in supernatants from A. ***P*<0.01 by ANOVA. (C) Immunoblot analysis for mTOR and caspase-1 of cell lysates from mouse J774A.1 macrophages transfected with control siRNA and siRNA for mTOR, treated with LPS (500 ng/ml) for 4 h, or LPS followed by incubation with ATP (2 mM) for 30 min. β-actin served as the standard. (D) ELISA assay for IL-1β, IL-18 and TNF secretion in supernatants from A. ***P*<0.01 by ANOVA. (E) Immunoblot analysis for 4E-BP1, HK1 and caspase-1 of cell lysates from mouse J774A.1 macrophages transfected with control siRNA or siRNA for 4E-BP1, treated with LPS (500 ng/ml) for 4 h, or LPS followed by incubation with ATP (2 mM) for 30 min. β-actin served as the standard. (F) ELISA assay for IL-1β, IL-18 and TNF secretion in supernatants from C. ***P*<0.01 by ANOVA.

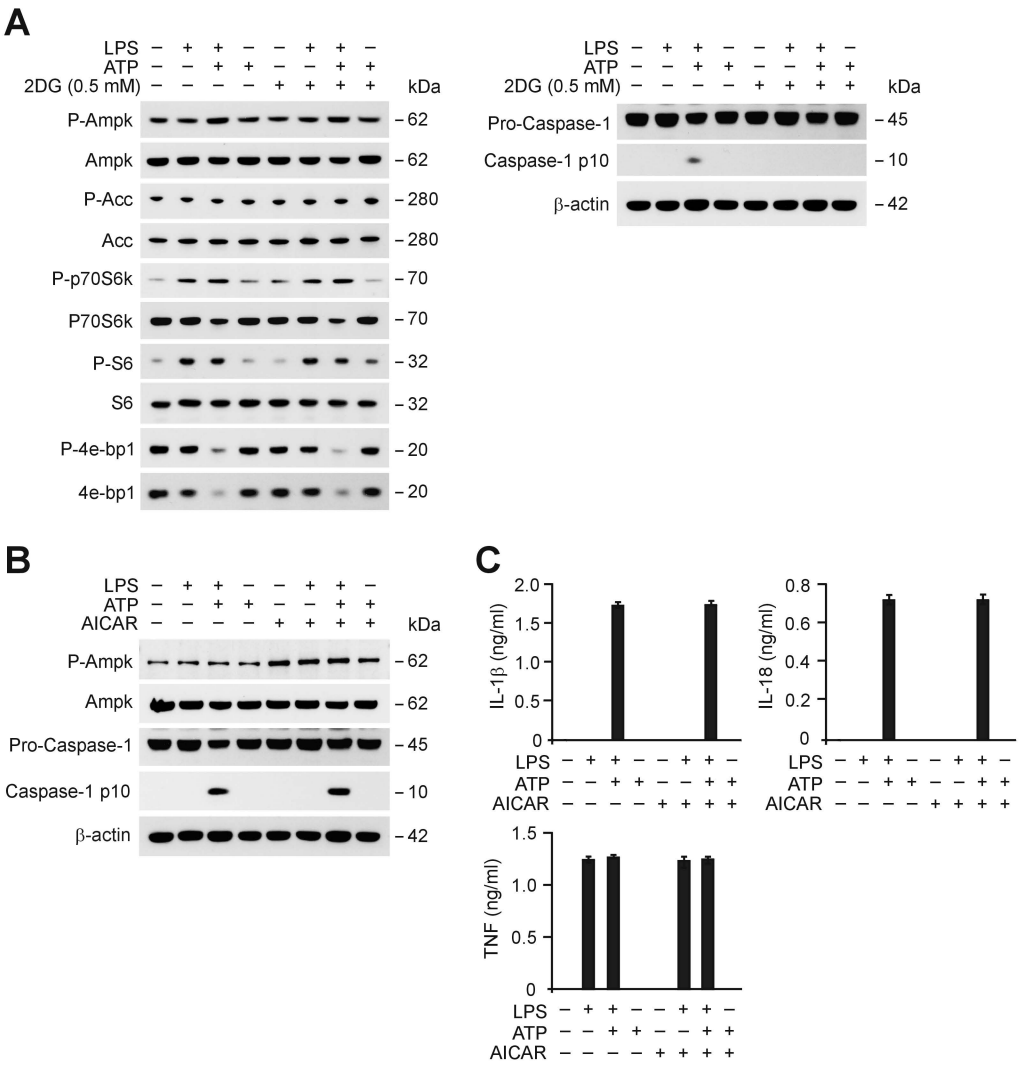

Figure S4. Inhibition of glycolysis by 2DG did not affect the activation AMPK on NLRP3 inflammasome activation, Related to Figure 6.

(A) Immunoblot analysis for phosphorylation of Ampk, Acc, p70S6k, S6, 4e-bp1 and caspase-1 of cell lysates wild-type BMDM treated with 2DG (0.5 mM) for 2h before ATP (2mM, 30 min) after LPS (500 ng/ml, 4h) stimulation. β-actin served as the standard. (B) Immunoblot analysis for phosphorylation of Ampk and caspase-1 of cell lysates wild-type BMDM treated with AICAR (100 μM) for 1h before ATP (2mM, 30 min) after LPS (500 ng/ml, 4h) stimulation. β-actin served as the standard. (C) ELISA assay for IL-1β, IL-18 and TNF secretion in supernatants from wild type BMDMs pre-treated with AICAR (100 μM) for 1h before ATP (2mM, 30 min) after LPS (500 ng/ml, 4h) stimulation.

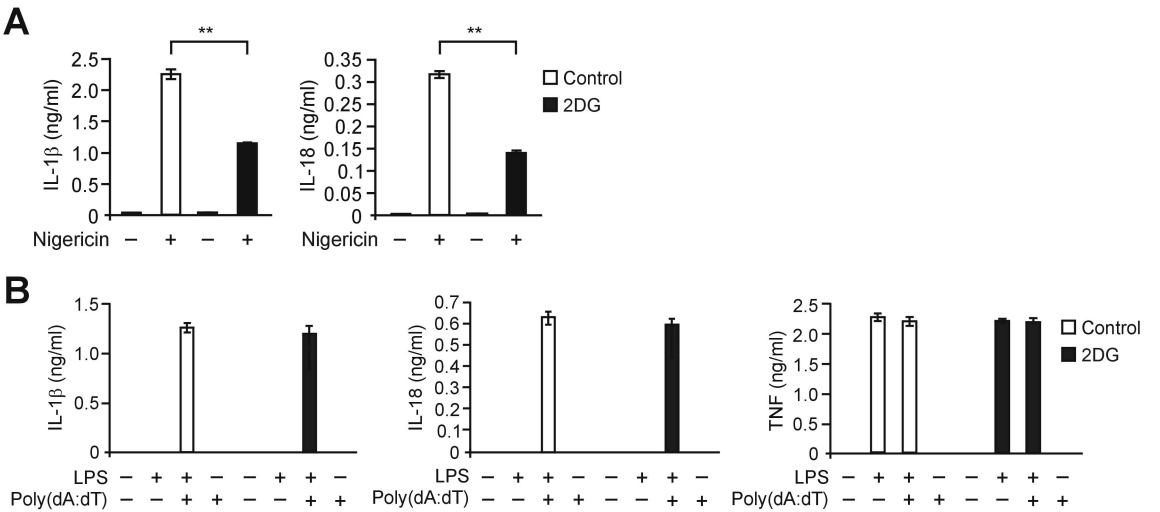

Figure S5. Inhibition of glycolysis by 2DG suppresses NLRP3 inflammasome activation, Related to Figure 6.

(A) Luminex assay for IL-1β and IL-18 secretion in supernatants from human THP-1 monocyte-derived macrophages pre-treated with 2DG (0.5 mM) for 2 h before stimulation with nigericin (6.7 μM) for 6 h. $^{**}P < 0.01$ by ANOVA. (B) ELISA assay for IL-1β, IL-18 and TNF secretion in supernatants from wild type BMDMs pre-treated with 2DG (0.5 mM) for 2 h before stimulation with LPS (500 ng/ml) for 4 h, followed by transfection with poly(dA:dT) (1 μg/ml) for 6 h.

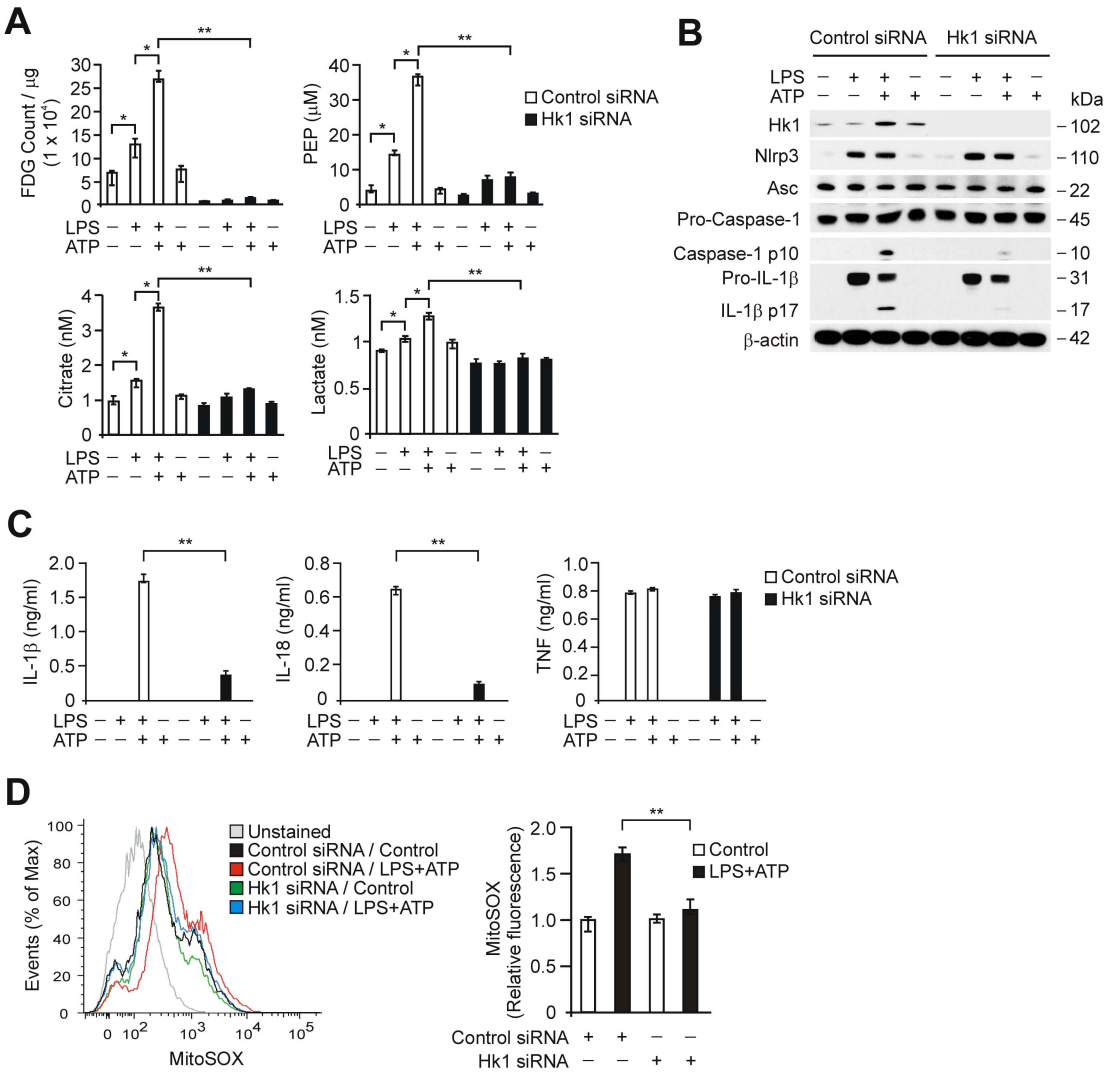

Figure S6. HK1 dependent glycolysis is critical metabolic pathway in NLRP3 inflammasome activation, Related to Figure 7.

(A) ^{18}F-FDG uptake assay, PEP, citrate and lactate production assay from mouse J774A.1 macrophages transfected with control siRNA and siRNA for HK1, treated with LPS (500 ng/ml) for 4 h, or LPS followed by incubation with ATP (2 mM) for 30 min. ***P*<0.01, **P*<0.05 by ANOVA. (B) Immunoblot analysis for HK1, caspase-1, NLRP3 and ASC of cell lysates from mouse J774A.1 macrophages transfected with control siRNA and siRNA for HK1, treated with LPS (500 ng/ml) for 4 h, or LPS followed by incubation with ATP (2 mM) for 30 min. (C) Luminex assay and ELISA assay for IL-1β, IL-18 and TNF secretion in supernatants from mouse J774A.1 macrophages transfected with control siRNA and siRNA for HK1, treated with LPS (500 ng/ml) for 4 h, or LPS followed by incubation with ATP (2 mM) for 30 min. ***P*<0.01 by ANOVA. (D) Flow cytometry analysis of mouse J774A.1 macrophages transfected with control siRNA and siRNA for HK1, treated with LPS (500 ng/ml) for 4 h, or LPS followed by incubation with ATP (2 mM) for 30 min. Cells were stained with the mitochondrial superoxide-specific stain MitoSOX and then left unstimulated. ***P*<0.01 by ANOVA.

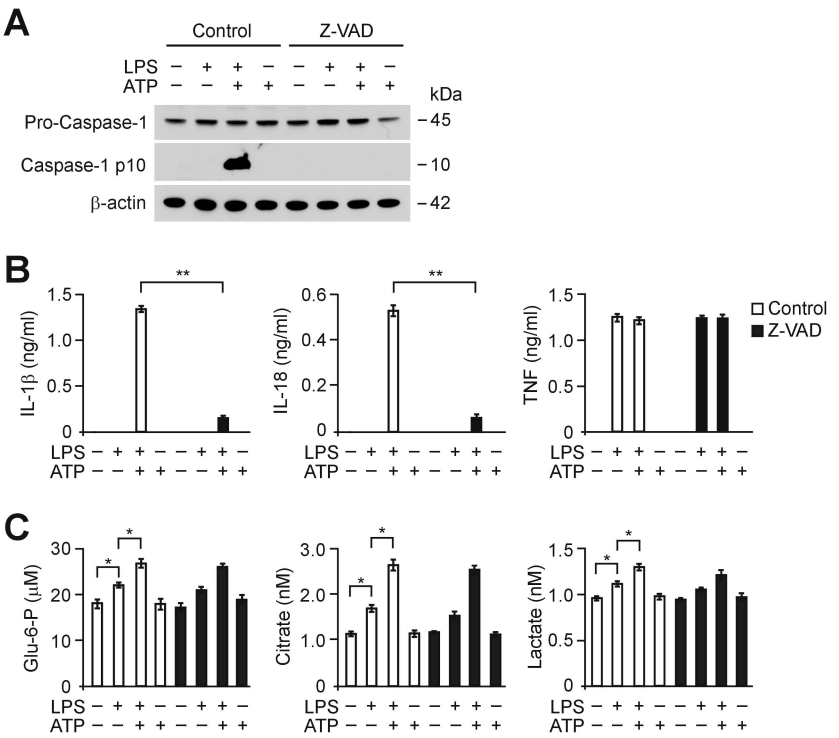

Figure S7. Inhibition of caspase-1 activation did not suppress glycolysis in NLRP3 inflammasome activation, Related to Figure 7.

(A) Immunoblot analysis for caspase-1 of cell lysates from wild type BMDM pre-treated with Z-VAD (10 μM) for 1 h before incubation with ATP (2 mM) for 30 min after LPS stimulation. β-actin served as the standard. (B) ELISA assay for IL-1β, IL-18 and TNF secretion in supernatants from A. $^{**}P<0.01$ by ANOVA. (C) Glucose-6-phosphate assay, citrate and lactate production assay from wild type BMDM pre-treated with Z-VAD (10 μM) for 1 h before incubation with ATP (2 mM) for 30 min after LPS stimulation. $^{*}P<0.05$ by ANOVA.

Supplemental Experimental Procedures

Cell culture

Bone marrow collected from mouse femurs and tibias was plated on sterile petri dishes and incubated for 7 days in DMEM media (Invitrogen) containing 10% (*vol/vol*) heat-inactivated FBS, 100 units/ml penicillin, 100 mg/ml streptomycin, and 25% (*vol/vol*) conditioned medium from mouse L929 fibroblasts. Cells were incubated for 4 h with LPS (500 ng/ml) and then were treated with ATP (2 mM) for 30 min as described. Mouse J774A.1 macrophages were cultured in DMEM medium containing 10% (*vol/vol*) FBS, penicillin and streptomycin. Human THP-1 monocyte-derived macrophages were grown in RPMI-1640 media (Invitrogen) containing 10% (*vol/vol*) FBS, penicillin and streptomycin. THP-1 cells were differentiated for 16 h with 50 nM phorbol 12-myristate-13-acetate. Cells were incubated for 6 h with nigericin (6.7 μM).

Reagents

LPS (Escherichia coli) (tlrl-pelps) was from InvivoGen. ATP (A2383), nigericin (N7143), 2DG (D3875), AICAR (A9978) and poly(dA:dT) (P0883) were from Sigma-Aldrich. Torin1 (4247) and Z-VAD-FMK (2163) was from Tocris Bioscience.

Transfection

For transient knockdown of mouse *mTOR*, *Hk1* and *4e-bp1*, small interfering RNA of *mTOR* (EMU047451), *Hk1* (EMU086531) and *4e-bp1* (EMU029571) was from Sigma-Aldrich. Mouse J774A.1 macrophages (2 x 10^5 cells/well) were seeded in 6-well plates and were transfected with small interfering RNA for mouse *mTOR*, *Hk1*, *4e-bp1* or siRNA Universal Negative Control (SIC001, Sigma-Aldrich) (200 ng/well) using Lipofectamin® RNAiMAX reagent (13778-075, Invitrogen) according to the manufacturer's instructions. For AIM2 inflammasome activation, LPS primed wild type BMDM were transfected with poly(dA:dT) (1 μg/ml) (Sigma) using Lipofectamine® with Plus reagent (Invitrogen), according to the manufacturer's instructions.

Immunoblot analysis

Cells or tissues were harvested and lysed in 2 x SDS loading buffer or lysis buffer (Invitrogen) and then briefly sonicated. Lysates were centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatants were obtained. The protein concentrations of the supernatants were determined using the Bradford assay (Bio-Rad). Proteins were

electrophoresed on NuPAGE 4-12% Bis-Tris gels (Invitrogen) and transferred to Protran nitrocellulose membranes (Whatman).

Cytokine analysis

Cell culture supernatants or serum were measured for mouse and human IL-1β and IL-18 using ELISA (R&D Systems) and luminex multiplex cytokine assays (Luminex) according to the manufacturer's instructions. Cell culture supernatants or serum were measured for mouse tumor necrosis factor using ELISA (R&D Systems).

Quantitative real-time PCR

Total RNA was isolated from cultured cells and tissues using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For quantitative RT-PCR, cDNA was synthesized from 4 µg of total RNA using random hexamers and SuperScript Reverse Transcriptase II® (Invitrogen) according to the manufacturer's instructions. A 10 µl mixture containing the diluted cDNA and a set of gene-specific primers was mixed with 10 µl of 2 x SYBR Green PCR Master Mix (Applied Biosystems) and then subjected to RT-PCR quantification using the ABI PRISM 7500 real-time PCR system (Applied Biosystems). The following primers were used: mouse *Hk1* forward, 5'-TGCCATGCGGCTCTCTGATG-3' and reverse, 5'- CTTGACGGAGGCCGTTGGGTT-3'; mouse *Hk2* forward, 5'- GGGCATGAAGGGCGTGTCCC-3' and reverse, 5'-TCTTCACCCTCGCAGCCGGA-3'; mouse *Gapdh* forward, 5'-GGTGAAGGTCGGTGTGAACGGA-3' and reverse, 5'-CCAAAGTTGTCATGGATGACCTTGG-3'.

Isolation of mitochondrial fraction

Mitochondrial fractions were isolated from cells using a mitochondria/cytosol fraction kit (#K265-25, Biovision).

Immunoprecipitation

Mouse J774A.1 macrophages were incubated for 4 h with LPS (500 ng/ml) and then were treated with ATP for 30 min (5 mM). Mitochondrial fractions were isolated from cells using a mitochondria/cytosol fraction kit (#K265-25, Biovision). Mitochondrial fraction extracts were incubated overnight with anti-VDAC1 antibody or rabbit IgG.

These extracts were immunoprecipitated with protein A/G plus agarose beads (SC-2003, Santa Cruz Biotechnology) and then were assessed by immunoblotting using anti-HK1 antibody.

Mitochondrial ROS production assay

Mitochondrial ROS were measured by MitoSOX (Invitrogen) staining. Cells were incubated with MitoSOX (5 μM) for 15 min at 37 °C. Cells were washed with PBS, treated with trypsin and resuspended in PBS containing 1% (*vol/vol*) heat-inactivated FBS. Data were acquired with a FACSCanto II (BD Biosciences) and were analyzed with FlowJo analytical software (TreeStar).