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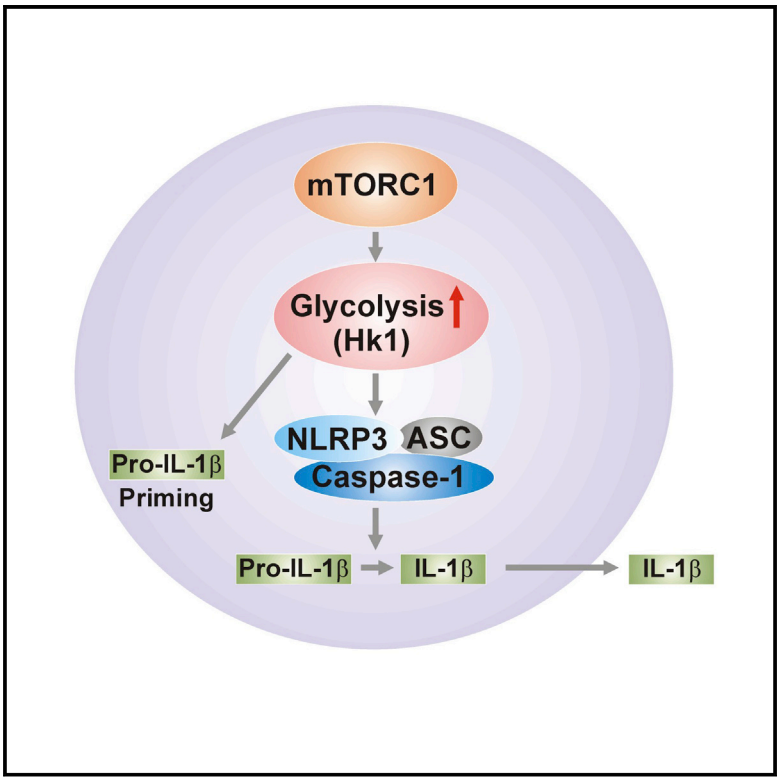
mTORC1-Induced HK1-Dependent Glycolysis Regulates NLRP3 Inflammasome Activation

Graphical Abstract



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In Brief

Moon et al. show that mTORC1-HK1-dependent glycolysis regulates NLRP3 inflammasome activation in macrophages. The inhibition of Raptor/mTORC affected the regulation of both pro-IL-1β maturation and caspase-1 activation during NLRP3 inflammasome activation. These findings suggest that a complex relationship exists between the NLRP3 inflammasome and cellular glycolysis in macrophages.

Highlights

- Inhibition of mTORC1 suppresses NLRP3 inflammasome activation
- Raptor/mTORC1 regulates HK1-dependent glycolysis
- HK1-dependent glycolysis is critical for NLRP3 inflammasome activation
- Activation of the glycolytic phenotype is linked to NLRP3 inflammasome activation

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mTORC1-Induced HK1-Dependent Glycolysis Regulates NLRP3 Inflammasome Activation

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SUMMARY

The mammalian target of rapamycin complex 1 (mTORC1) regulates activation of immune cells and cellular energy metabolism. Although glycolysis has been linked to immune functions, the mechanisms by which glycolysis regulates NLRP3 inflammasome activation remain unclear. Here, we demonstrate that mTORC1-induced glycolysis provides an essential mechanism for NLRP3 inflammasome activation. Moreover, we demonstrate that hexokinase 1 (HK1)-dependent glycolysis, under the regulation of mTORC1, represents a critical metabolic pathway for NLRP3 inflammasome activation. Downregulation of glycolysis by inhibition of Raptor/mTORC1 or HK1 suppressed both pro-IL-1β maturation and caspase-1 activation in macrophages in response to LPS and ATP. These results suggest that upregulation of HK1-dependent glycolysis by mTORC1 regulates NLRP3 inflammasome activation.

INTRODUCTION

Inflammasomes are multi-protein complexes that activate caspase-1 and downstream immune responses, including the maturation and secretion of pro-inflammatory cytokines (e.g., IL-1β and IL-18) (Franchi et al., 2009; Schroder and Tschopp, 2010; Sutterwala et al., 2006). The cytoplasmic nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) constitute critical components of the inflammasome. NLRs interact with the adaptor apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), which recruits pro-caspase-1 (Latz et al., 2013). Among the known NLR-containing inflammasomes, the NOD-, leucine-rich region-, and pyrin domain-containing 3 (NLRP3) inflammasome (also known as cryopirin or NALP3) responds to activation by a wide range of endogenous and exogenous agonists (Franchi et al., 2009; Schroder and Tschopp, 2010), and has been implicated in the pathogenesis of several diseases, including cancer, infectious diseases, and autoimmune diseases (Bruchard et al., 2013; Franchi et al., 2009; Schroder and Tschopp, 2010).

The mammalian target of rapamycin complex 1 (mTORC1) promotes activation of NK and T_{reg} cells (Marçais et al., 2014; Yang et al., 2013; Zeng et al., 2013), and acts as a crucial regulator of cellular energy metabolism (Cairns et al., 2011; Laplante and Sabatini, 2012). The mTORC1 is associated with activation of cellular glycolysis, which involves the increased translation of glycolytic enzymes or their transcriptional regulators (Düvel et al., 2010; Elstrom et al., 2004). Moreover, mTORC1 regulates translation and ribosome biogenesis through the phosphorylation of the translational regulators eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and S6 kinase 1 (S6K1) (Laplante and Sabatini, 2012; Ma and Blenis, 2009; Richter and Sonenberg, 2005). The phosphorylation of 4E-BP1 prevents its binding to the cap-binding protein eIF4E, enabling it to engage in the eIF4F complex that is required for the initiation of cap-dependent translation (Laplante and Sabatini, 2012; Ma and Blenis, 2009; Richter and Sonenberg, 2005). The activation of S6K1, through multiple effectors, leads to increased mRNA biogenesis as well as translational initiation and elongation (Laplante and Sabatini, 2012; Ma and Blenis, 2009; Richter and Sonenberg, 2005).

Glycolysis is a critical pathway in cellular glucose metabolism that provides intermediates for energy generation (DeBerardinis et al., 2008a, 2008b; Koppenol and Bounds, 2009; Vander Heiden et al., 2009). The phosphorylation of glucose by hexokinase represents the rate-limiting step in the regulation of glycolysis (Bustamante et al., 1981; Cairns et al., 2011). HKs play a vital role in the cellular uptake and utilization of glucose (Arora et al., 1990; Baijal and Wilson, 1995; Greiner et al., 1994). In mammals, four HK isozymes (HK1–4) have been identified, each with distinct subcellular localization, kinetics, substrate specificities, and physiological functions (Azoulay-Zohar and Aflalo, 1999, 2000; Baijal and Wilson, 1995; Crane and Sols, 1953; Mathupala et al., 2009; Parry and Pedersen, 1984; Wilson, 2003).

Recent studies suggested that glycolysis is involved in immune responses (Krawczyk et al., 2010; Masters et al., 2010; Tannahill et al., 2013; Zhou et al., 2010). The induction of glycolysis by Toll-like receptor (TLR) agonists facilitates the

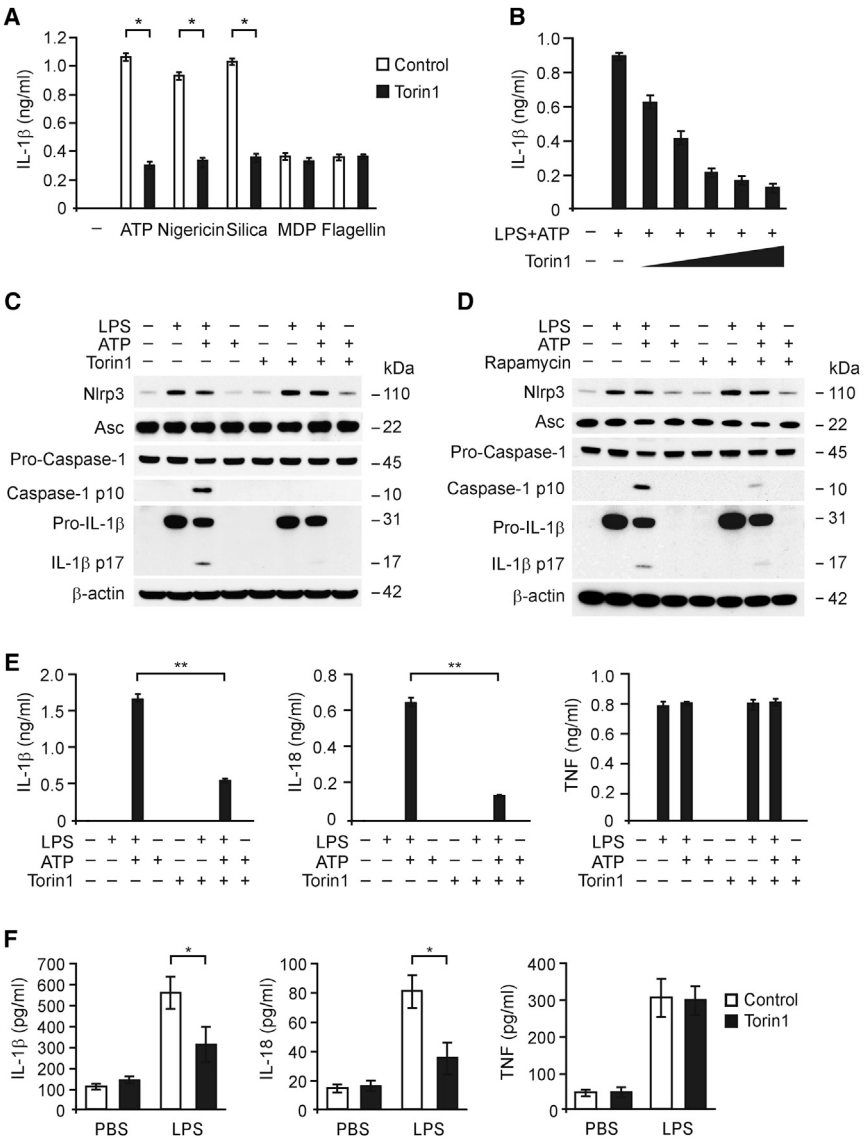

Figure 1. Inhibition of mTORC1 Suppresses NLRP3 Inflammasome Activation In Vitro and In Vivo

(A) ELISA assay shows IL-1β secretion in supernatants from wild-type peritoneal macrophages pre-treated with Torin1 (250 nM) for 1 hr before ATP (5 mM), nigericin (6.7 μM), silica (200 ng/ml), MDP (200 ng/ml), or flagellin (200 ng/ml) treatment, after LPS (500 ng/ml, 4 hr) stimulation. *p < 0.05 by ANOVA.

(B) ELISA assay shows IL-1β secretion in supernatants from wild-type peritoneal macrophages pre-treated with Torin1 (0, 60 125, 250, 500, or 1,000 nM) for 1 hr before ATP (5 mM, 30 min) after LPS (500 ng/ml, 4 hr) stimulation (representative of three independent experiments). *p < 0.05 by ANOVA.

(C) Immunoblot analysis for caspase-1, NLRP3, and ASC of cell lysates from wild-type BMDMs pre-treated with Torin1 (250 nM) for 1 hr before ATP (2 mM, 30 min) treatment, after LPS (500 ng/ml, 4 hr) stimulation. β-actin served as the standard.

(D) Immunoblot analysis for caspase-1, NLRP3, and ASC of cell lysates from wild-type BMDMs pre-treated with rapamycin (10 nM) for 1 hr before ATP treatment after LPS stimulation. β-actin served as the standard.

(E) ELISA assays show IL-1β, IL-18, and TNF secretions in supernatants from wild-type BMDMs pre-treated with Torin1 (250 nM) for 1 hr before ATP treatment, after LPS stimulation. **p < 0.01 by ANOVA.

(F) ELISA assays show IL-1β, IL-18, and TNF secretions in serum of wild-type mice after intraperitoneal injection of Torin (5 mg/kg) or DMSO for 14 hr, then LPS (10 mg/kg) or PBS for 8 hr (PBS, n = 6; LPS, n = 8). *p < 0.05 by ANOVA.

maturation and activation of dendritic cells (Everts et al., 2014; Krawczyk et al., 2010). High concentrations of glucose increase IL-1β secretion through an NLRP3-dependent mechanism (Zhou et al., 2010). The inhibition of glycolysis in macrophages suppressed IL-1β gene expression in response to lipopolysaccharide (LPS) treatment (Masters et al., 2010; Tannahill et al., 2013). The NLRP3 inflammasome has been implicated in the pathogenesis of metabolic disorders such as non-alcoholic fatty liver disease, obesity, and diabetes (Henao-Mejia et al., 2012; Jourdan et al., 2013; Vandanmagsar et al., 2011). However, the mechanisms by which glycolysis regulates NLRP3 inflammasome activation remain unclear.

In this paper, we demonstrate that genetic and pharmacologic inhibition of mTORC1 suppressed HK1-dependent glycolysis, caspase-1 activation, and the maturation and secretions of IL-1β and IL-18 in vitro and in vivo, under pro-inflammatory conditions. Furthermore, genetic and pharmacologic inhibition of HK1 suppressed caspase-1 activation and secretions of IL-1β and IL-18 in macrophages. Our results, taken together, suggest that mTORC1-induced HK1-dependent glycolysis provides an essential metabolic mechanism for NLRP3 inflammasome activation.

RESULTS

Inhibition of mTORC1 Suppressed NLRP3 Inflammasome Activation In Vitro and In Vivo

To investigate the function of mTORC1 in inflammasome activation, we inhibited mTORC activity in LPS-primed primary peritoneal macrophages using Torin1, a competitive mTORC1/2 inhibitor (Thoreen et al., 2009). We analyzed the secretion of IL-1β in LPS-primed macrophages that were treated with Torin1 for 1 hr before treatment with specific NLRP3 inflammasome activators, including ATP, nigericin, and silica. Torin1 suppressed IL-1β secretion in macrophages in response to treatment with NLRP3 inflammasome activators (Figure 1A). In contrast, Torin1 had no effect on the activation of IL-1β

secretion in response to treatment with muramyldipeptide (MDP), an NLRP1 inflammasome activator, or flagellin, an NLR family CARD domain-containing protein 4 (NLRC4) inflammasome activator (Figure 1A). Consistently, pre-treatment with Torin1 dose-dependently suppressed IL-1β secretion in response to ATP treatment in LPS-primed macrophages (Figure 1B). These results suggest that mTORC1/2 is involved in NLRP3 inflammasome activation in response to NLRP3 inflammasome activators.

Next, we analyzed caspase-1 activation in LPS-primed primary bone-marrow-derived macrophages (BMDMs) pre-treated with Torin1 for 1 hr before ATP treatment. Torin1 suppressed caspase-1 activation in wild-type BMDMs in response to LPS and ATP, compared to vehicle control, while NLRP3 and ASC expressions were unchanged (Figure 1C). Similarly, rapamycin, a selective mTORC1 inhibitor (Benjamin et al., 2011), suppressed caspase-1 activation in wild-type BMDMs in response to LPS and ATP, compared to vehicle control (Figure 1D). Moreover, the secretions of IL-1β and IL-18 were inhibited by Torin1 in wild-type BMDMs, while tumor necrosis factor (TNF) was unchanged (Figure 1E). Furthermore, Torin1 suppressed serum levels of IL-1β and IL-18 in response to LPS challenge in vivo, whereas TNF was unchanged (Figure 1F). These results suggest that the inhibition of mTORC1 suppressed NLRP3 inflammasome activation in vitro and in vivo.

mTORC1-Dependent Glycolysis Is a Critical Metabolic Pathway for NLRP3 Inflammasome Activation

Given that mTORC1 is associated with the activation of cellular glycolysis (Düvel et al., 2010; Laplante and Sabatini, 2012; Rowe et al., 2013), we investigated whether mTORC1-dependent glycolysis can regulate NLRP3 inflammasome activation. To analyze mTORC1-induced glycolysis during NLRP3 inflammasome activation, we measured the extracellular acidification rate (ECAR), as a measure of lactate production (a surrogate for the glycolytic rate), and the mitochondrial oxygen consumption rate (OCR) in wild-type peritoneal macrophages pre-treated with Torin1 for 1 hr before LPS and ATP stimulation. The ECAR was significantly increased by LPS and ATP stimulation compared to LPS alone or control treatments (Figure 2A). Importantly, Torin1 suppressed the induction of ECAR by LPS and ATP relative to vehicle control (Figure 2A). In contrast, the OCR measured after LPS and ATP treatment was not changed by Torin1 treatment compared to vehicle control (Figure 2B).

To confirm that the observed changes in ECAR correspond to glycolytic flux, we measured the levels of glycolytic metabolites in LPS-primed BMDMs pre-treated with Torin1 for 1 hr before ATP treatment. Glycolytic metabolites (i.e., phosphoenolpyruvate [PEP] and citrate) were induced ~2-fold by LPS relative to control (Figure 2C). Interestingly, glycolytic metabolites (i.e., PEP, citrate, and lactate) were also significantly increased in BMDMs by LPS and ATP, relative to LPS treatment alone or untreated control macrophages (Figure 2C). Importantly, Torin1 inhibited the activation of glycolysis, as indicated by the suppression of glycolytic metabolite production, in BMDMs in response to LPS and ATP, relative to vehicle control (Figure 2C). Similarly, rapamycin suppressed the activation of glycolytic metabolite (i.e., PEP, citrate, and lactate) production in response to LPS and ATP relative to vehicle control (Figure 2D). These results suggest that mTORC1-dependent glycolysis is a crucial metabolic pathway involved in NLRP3 inflammasome activation.

Inhibition of mTORC1 Suppressed the Increase in HK1 Expression during NLRP3 Inflammasome Activation

We investigated the underlying molecular mechanism by which mTORC1 regulates the high glycolytic phenotype observed during NLRP3 inflammasome activation. Given that mTORC1 regulates the translation of glycolytic enzymes and their transcriptional regulators (Düvel et al., 2010; Levine and Puzio-Kuter, 2010; Ma and Blenis, 2009), we first examined the regulation of glycolytic enzymes during NLRP3 inflammasome activation. We analyzed the protein expression of HK1, the first key enzyme in the glycolysis pathway, in mitochondrial and cytosolic protein lysates of BMDMs treated with LPS and ATP. HK1 was preferentially expressed in mitochondria of BMDMs (Figure S1A), consistent with previous reports that HK1 is primarily expressed in mitochondria (Azoulay-Zohar and Aflalo, 1999; Wilson, 2003). Importantly, the protein expression of HK1 was markedly increased in response to LPS and ATP, relative to LPS treatment alone or untreated control macrophages (Figure S1A). In contrast, we found that HK2, another major isoform of HK, was not expressed in BMDMs (Figures S1B and S1C).

Importantly, the inhibition of mTORC1 by Torin1 or rapamycin suppressed HK1 protein expression in BMDMs in response to LPS and ATP, compared to vehicle control (Figures 2E and 2F). In contrast, treatment with Torin1 or rapamycin had no effect on the activation of 5′-AMP-activated protein kinase (AMPK), which suggested that AMPK was not involved in mTORC1-dependent glycolysis (Figures 2E and 2F).

HK1 exhibits a higher affinity for the voltage-dependent anion channel (VDAC)1 isoform than for other mammalian VDAC isoforms (Azoulay-Zohar and Aflalo, 1999, 2000). Given that VDAC is essential for NLRP3 inflammasome activation (Zhou et al., 2011), we investigated whether HK1 interacts with VDAC1 in the mitochondria, which may provide specific functional advantages for NLRP3 inflammasome activation. We observed that an interaction between HK1 and VDAC1, as assessed by co-immunoprecipitation (coIP), occurred in mitochondria of mouse J774A.1 macrophages and increased in response to LPS and ATP (Figure S2A). However, the inhibition of mTORC1 by Torin1 did not change the interaction between HK1 and VDAC1 (Figure S2B). These results, taken together, suggest that the induction of HK1 protein expression is responsible for the high glycolytic phenotype observed during NLRP3 inflammasome activation.

HK1 Is Regulated by NLRP3 Inflammasome Activation

To determine whether HK1-dependent glycolysis is regulated by activators of other inflammasomes, we examined HK1 expression in BMDMs in response to activators of the absent in melanoma 2 (AIM2) inflammasome relative to activators of the NLRP3 inflammasome pathway. In LPS-primed BMDMs, the protein expression of HK1 was increased by ATP treatment

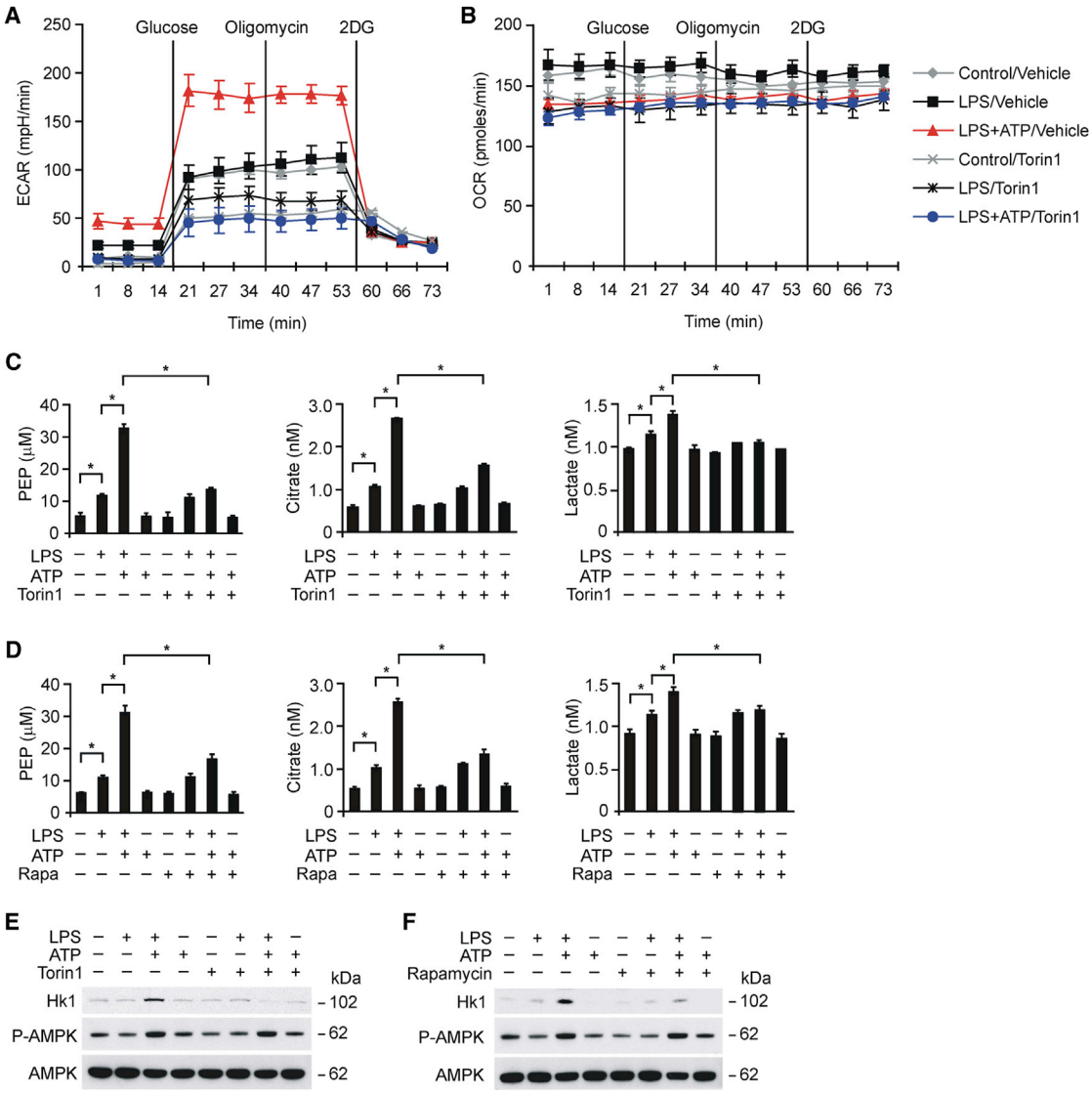

Figure 2. mTORC1-Dependent Glycolysis Is a Critical Metabolic Pathway in NLRP3 Inflammasome Activation

(A) ECAR was measured in wild-type peritoneal macrophages pre-treated with Torin1 (250 nM) for 1 hr before ATP (5 mM, 30 min) after LPS (500 ng/ml, 4 hr) stimulation. Data are mean ± SD.

(B) OCR was measured in wild-type peritoneal macrophages pre-treated with Torin1 (250 nM) for 1 hr before ATP (5 mM, 30 min) after LPS (500 ng/ml, 4 hr) stimulation. Data are mean ± SD.

(C) PEP, citrate, and lactate production assays from wild-type BMDMs pre-treated with Torin1 (250 nM) for 1 hr before ATP treatment after LPS stimulation are shown. *$p < 0.05$ by ANOVA.

(D) PEP, citrate, and lactate production assays from wild-type BMDMs pre-treated with rapamycin (10 nM) for 1 hr before ATP treatment after LPS stimulation are shown. *$p < 0.05$ by ANOVA.

(E) Immunoblot analysis for HK1 and phosphorylation of AMPK of cell lysates from wild-type BMDMs pre-treated with Torin1 (250 nM) for 1 hr before ATP after LPS stimulation. Total AMPK served as the standard.

(F) Immunoblot analysis for HK1 and phosphorylation of AMPK of cell lysates from wild-type BMDMs pre-treated with rapamycin (10 nM) for 1 hr before ATP after LPS stimulation. Total AMPK served as the standard.

See also Figures S1 and S2.

(Figure S3A). Consistently, caspase-1 activation and secretion of IL-1β were increased by ATP treatment, whereas TNF was unchanged (Figure S3B). In contrast, the protein expression of HK1 was not induced in LPS-primed BMDMs after treatment with poly(dA:dT), an AIM2 inflammasome activator, compared to NLRP3 inflammasome activation (Figure S3A). In contrast, caspase-1 activation and secretion of IL-1β were significantly increased by poly(dA:dT) in LPS-primed BMDMs (Figures S3A and S3B). These results suggest specificity of HK1 regulation during inflammasome activation.

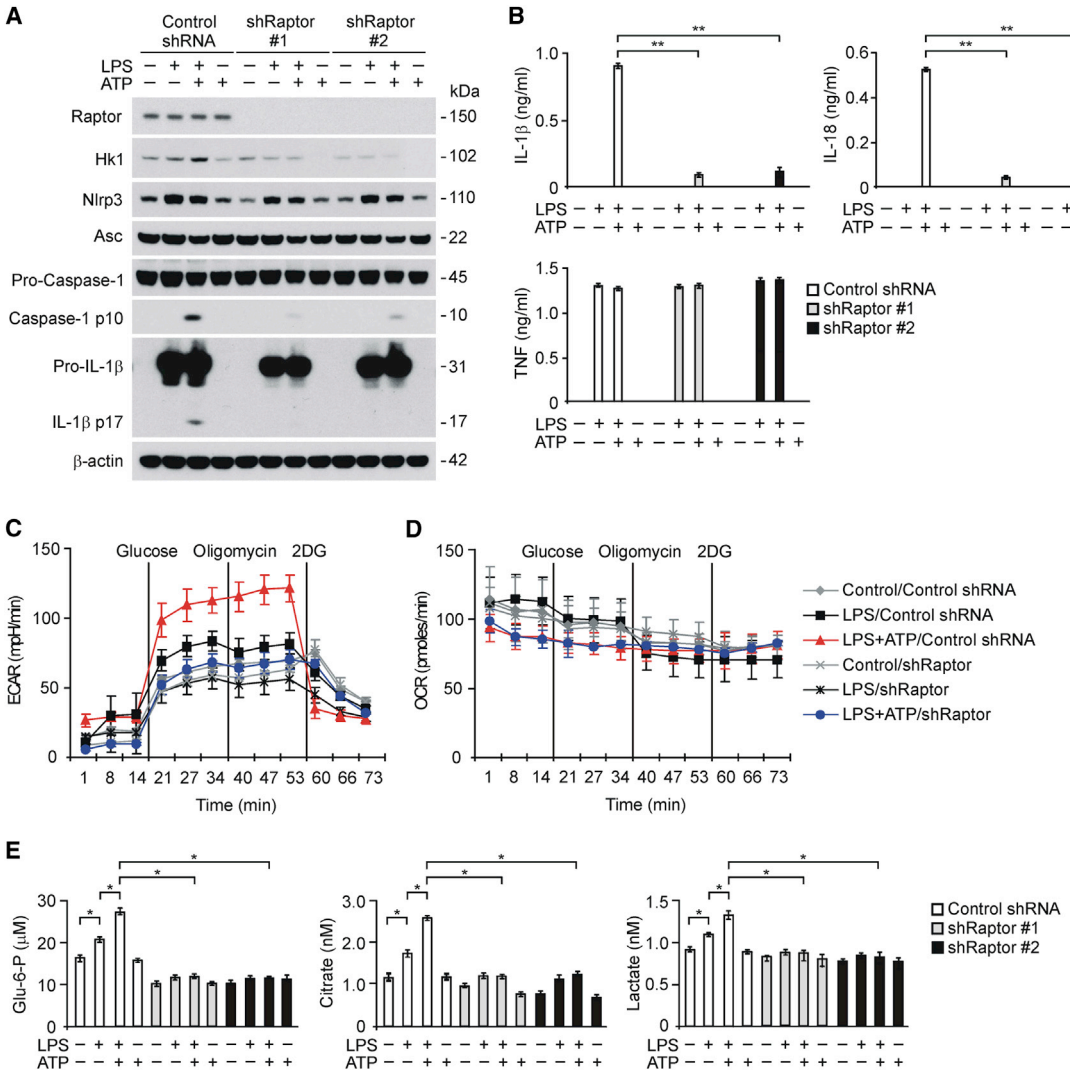

Figure 3. Deficiency of Raptor/mTORC1 Suppresses Caspase-1 Activation, HK1 Expression, and Glycolysis during NLRP3 Inflammasome Activation

(A) Immunoblot analysis for Raptor, HK1, caspase-1, and IL-1β in cell lysates from wild-type mouse peritoneal macrophages transduced with lentiviruses expressing non-target shRNA (control shRNA) or two independent shRNAs for Raptor (shRaptor 1 and 2) and stimulated with LPS and ATP. β-actin served as the standard.

(B) ELISA assays show IL-1β, IL-18, and TNF secretions from (A). **$p < 0.01$ by ANOVA.

(C) ECAR was measured in wild-type peritoneal macrophages transduced with lentiviruses expressing non-target shRNA (control shRNA) or shRNA for Raptor (shRaptor) and stimulated with LPS and ATP. Data are mean ± SD.

(D) OCR was measured in wild-type peritoneal macrophages transduced with lentiviruses expressing non-target shRNA (control shRNA) or shRNA for Raptor (shRaptor) and stimulated with LPS and ATP. Data are mean ± SD.

(E) Glucose-6-phosphate, citrate, and lactate production assays from wild-type peritoneal macrophages transduced with lentiviruses expressing non-target shRNA (control shRNA) or two independent shRNAs for Raptor (shRaptor 1 and 2) and stimulated with LPS and ATP are shown. *$p < 0.05$ by ANOVA.

See also Figure S3.

Raptor/mTORC1 Regulates Caspase-1 Activation and HK1 Expression during NLRP3 Inflammasome Activation

Next, we examined the specificity of mTORC1 in the regulation of caspase-1 activation and the protein expression of HK1 during NLRP3 inflammasome activation. We analyzed whether genetic deficiency of Raptor, a regulatory-associated protein of the mTORC1 complex (Kim et al., 2002), could suppress caspase-1 activation and the protein expression of HK1 in macrophages. We used two independent small hairpin RNA (shRNA) lentiviral constructs against mouse Raptor to deplete Raptor in wild-type peritoneal macrophages. The activation of caspase-1 and HK1 protein expression in response to LPS and ATP stimulation were suppressed by Raptor shRNA transduction relative to control shRNA transduction, while NLRP3 and ASC were unchanged (Figure 3A). Moreover, the secretions of IL-1β and IL-18 were inhibited by Raptor shRNA in wild-type peritoneal macrophages,

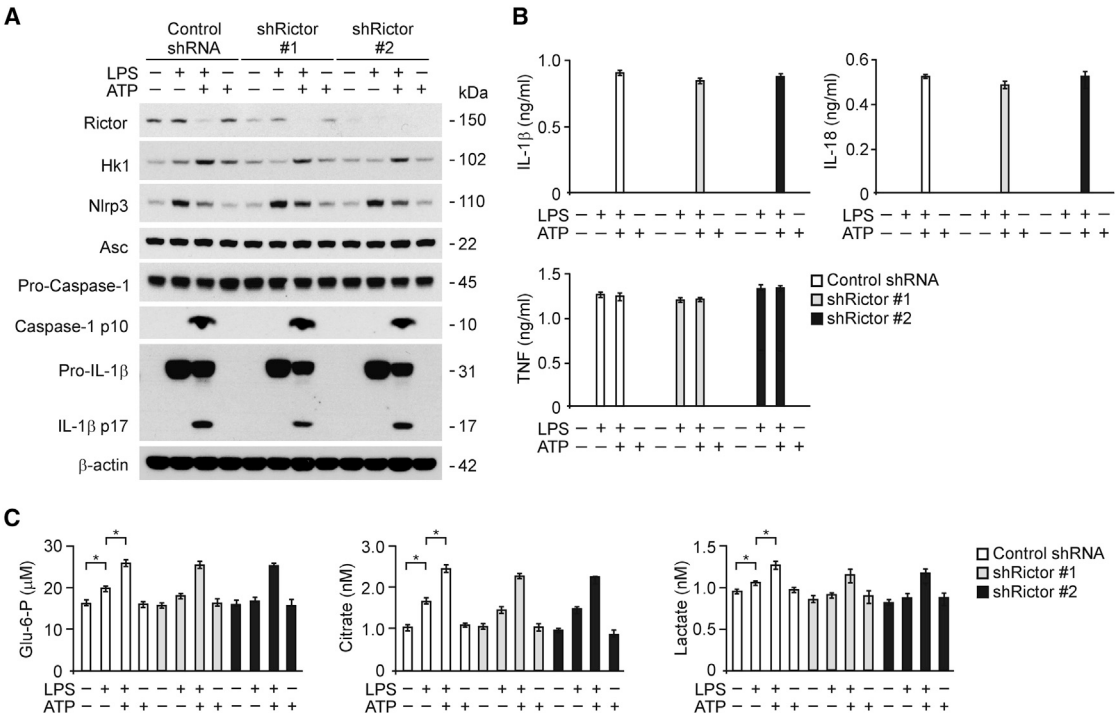

Figure 4. Deficiency of Rictor/mTORC2 Does Not Suppress Caspase-1 Activation and HK1 Expression during NLRP3 Inflammasome Activation

(A) Immunoblot analysis for Rictor, HK1, caspase-1, and IL-1β in cell lysates from wild-type mice peritoneal macrophages transduced with lentiviruses expressing non-target shRNA (control shRNA) or two independent shRNAs for Rictor (shRictor 1 and 2) and stimulated with LPS and ATP. β-actin served as the standard.

(B) ELISA assays show IL-1β, IL-18, and TNF secretions from (A).

(C) Glucose-6-phosphate, citrate, and lactate production assays from wild-type peritoneal macrophages transduced with lentiviruses expressing non-target shRNA (control shRNA) or two independent shRNAs for Rictor (shRictor 1 and 2) and stimulated with LPS and ATP are shown.

while TNF was unchanged (Figure 3B). Consistently, knockdown of mTOR by small interfering RNA (siRNA) inhibited the activation of caspase-1 and the secretions of IL-1β and IL-18 in mouse J774A.1 macrophages, relative to control siRNA-transfected cells (Figures S3C and S3D).

Consistent with the downregulation of HK1 protein expression, Raptor knockdown by transduction with Raptor shRNA suppressed the induction of ECAR in macrophages in response to LPS and ATP, relative to control shRNA transduction (Figure 3C). In contrast, the OCR measured after LPS and ATP treatment was not changed by Raptor knockdown in macrophages transduced with Raptor shRNA compared to control shRNA (Figure 3D). The production of glycolytic metabolites (i.e., glucose-6-phosphate, citrate, and lactate) in response to LPS and ATP was suppressed in wild-type peritoneal macrophages transduced with Raptor shRNA compared to control shRNA (Figure 3E).

To determine whether caspase-1 activation and the protein expression of HK1 are regulated by mTORC2 during NLRP3 inflammasome activation, we examined caspase-1 activation and HK1 expression in response to the depletion of Rictor, a regulatory-associated protein of the mTORC2 complex (Sarbassov et al., 2004), in macrophages. We used two independent shRNA lentiviral constructs against mouse Rictor to deplete Rictor in wild-type peritoneal macrophages. In contrast to Raptor knockdown, the activation of caspase-1 and HK1 protein expression in response to LPS and ATP stimulation were not suppressed by Rictor shRNA transduction relative to control shRNA transduction (Figure 4A). Similarly, the secretions of IL-1β, IL-18, and TNF were not inhibited by Rictor knockdown in wild-type peritoneal macrophages (Figure 4B). Also, the production of glycolytic metabolites was not changed by Rictor knockdown in wild-type peritoneal macrophages in response to LPS and ATP compared to control shRNA-transduced cells (Figure 4C). These results suggest that Raptor/mTORC1 regulates caspase-1 activation and HK1 expression during NLRP3 inflammasome activation.

HK1 Expression Is Related to mTORC1-Dependent Translational Regulation during NLRP3 Inflammasome Activation

Next, we investigated the molecular mechanism by which mTORC1 regulates the protein expression of HK1 during NLRP3 inflammasome activation. Importantly, the inhibition of mTORC1 by Torin1 suppressed HK1 protein expression in response to LPS and ATP, compared to vehicle control (Figure 2D), while HK1 mRNA levels were unchanged (Figure 5A). Consistent with the downregulation of HK1 protein expression, Torin1 and rapamycin suppressed translation by inhibiting the phosphorylation of eIF4E and ribosomal protein S6, which are integrated into two key translational processes (Ma and Blenis,

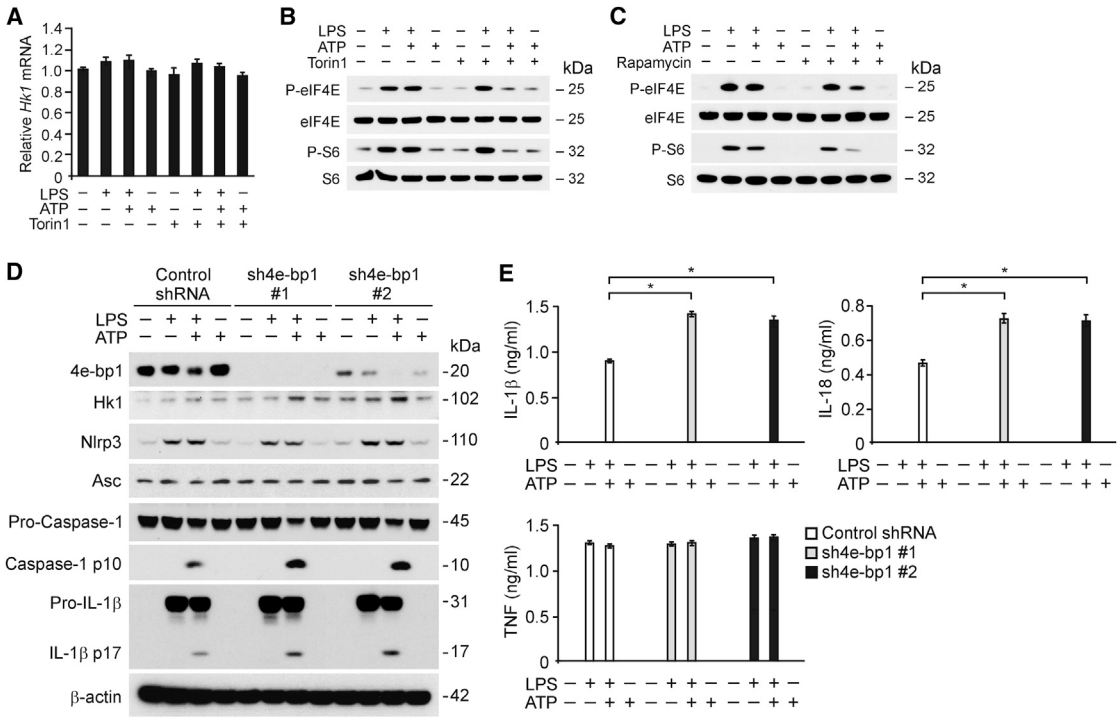

Figure 5. mTORC1 Regulates HK1 Expression during NLRP3 Inflammasome Activation

(A) The qPCR analysis for *Hk1* gene expression from wild-type BMDMs pre-treated with Torin1 (250 nM) for 1 hr before ATP treatment after LPS stimulation is shown.

(B) Immunoblot analysis for phosphorylation of eIF4E and S6 of cell lysates from wild-type BMDMs pre-treated with Torin1 (250 nM) for 1 hr before ATP treatment after LPS stimulation is shown.

(C) Immunoblot analysis for phosphorylation of eIF4E and S6 of cell lysates from wild-type BMDMs pre-treated with rapamycin (10 nM) for 1 hr before ATP treatment after LPS stimulation is shown.

(D) Immunoblot analysis for 4E-BP1, HK1, caspase-1, and IL-1β in cell lysates from wild-type mice peritoneal macrophages transduced with lentiviruses expressing non-target shRNA (control shRNA) or two independent shRNAs for 4E-BP1 (sh4E-BP1 1 and 2) and stimulated with LPS and ATP. β-actin served as the standard.

(E) ELISA assays show IL-1β, IL-18, and TNF secretions from (D). *$p < 0.05$ by ANOVA.

See also Figure S3.

2009; Richter and Sonenberg, 2005): (1) the cap-dependent translation by eIF4E, and (2) translation by ribosomal protein S6 kinase (p70S6K) (Figures 5B and 5C). The translational inhibitory protein 4E-BP1 plays a major role in translation by inhibiting cap-dependent translation initiation through the regulation of eIF4E phosphorylation by reversible interaction (Richter and Sonenberg, 2005).

We therefore examined the effect of translation activation by 4E-BP1 knockdown using two independent shRNA lentiviral constructs against mouse 4E-BP1 on HK1 protein expression and caspase-1 activation in wild-type peritoneal macrophages. Importantly, the expression of 4E-BP1 was diminished in response to LPS and ATP, with a corresponding increase in HK1 expression, in control shRNA-transduced macrophages (Figure 5D). Translation activation by 4E-BP1 knockdown using 4E-BP1 shRNA increased the expression of HK1 and caspase-1 activation in response to LPS and ATP in wild-type peritoneal macrophages, relative to control shRNA transduction (Figure 5D). Moreover, 4E-BP1 knockdown by 4E-BP1 shRNA increased the secretions of IL-1β and IL-18 in response to LPS and ATP in wild-type peritoneal macrophages compared to control shRNA, whereas TNF was unchanged (Figure 5E). Similarly, 4E-BP1 knockdown by 4E-BP1 siRNA increased the expression of HK1 and caspase-1 activation in response to LPS and ATP in mouse J774A.1 macrophages, compared to control siRNA transfection (Figure S3E). Moreover, 4E-BP1 knockdown increased the secretions of IL-1β and IL-18 in response to LPS and ATP in mouse J774A.1 macrophages in comparison to control, while TNF was unchanged (Figure S3F). These results suggest that the elevation of HK1 protein expression during NLRP3 inflammasome activation is related to mTORC1-dependent translational regulation.

mTORC1-Dependent Glycolysis Is a Critical Metabolic Pathway for NLRP3 Inflammasome Activation

To examine the role of glycolysis activation in NLRP3 inflammasome activation, we inhibited glycolysis in macrophages using 2-deoxyglucose (2DG), a potent glycolysis inhibitor (Pelicano et al., 2006). To determine the inhibitory effect of 2DG on glycolysis in macrophages, we measured ECAR in wild-type peritoneal

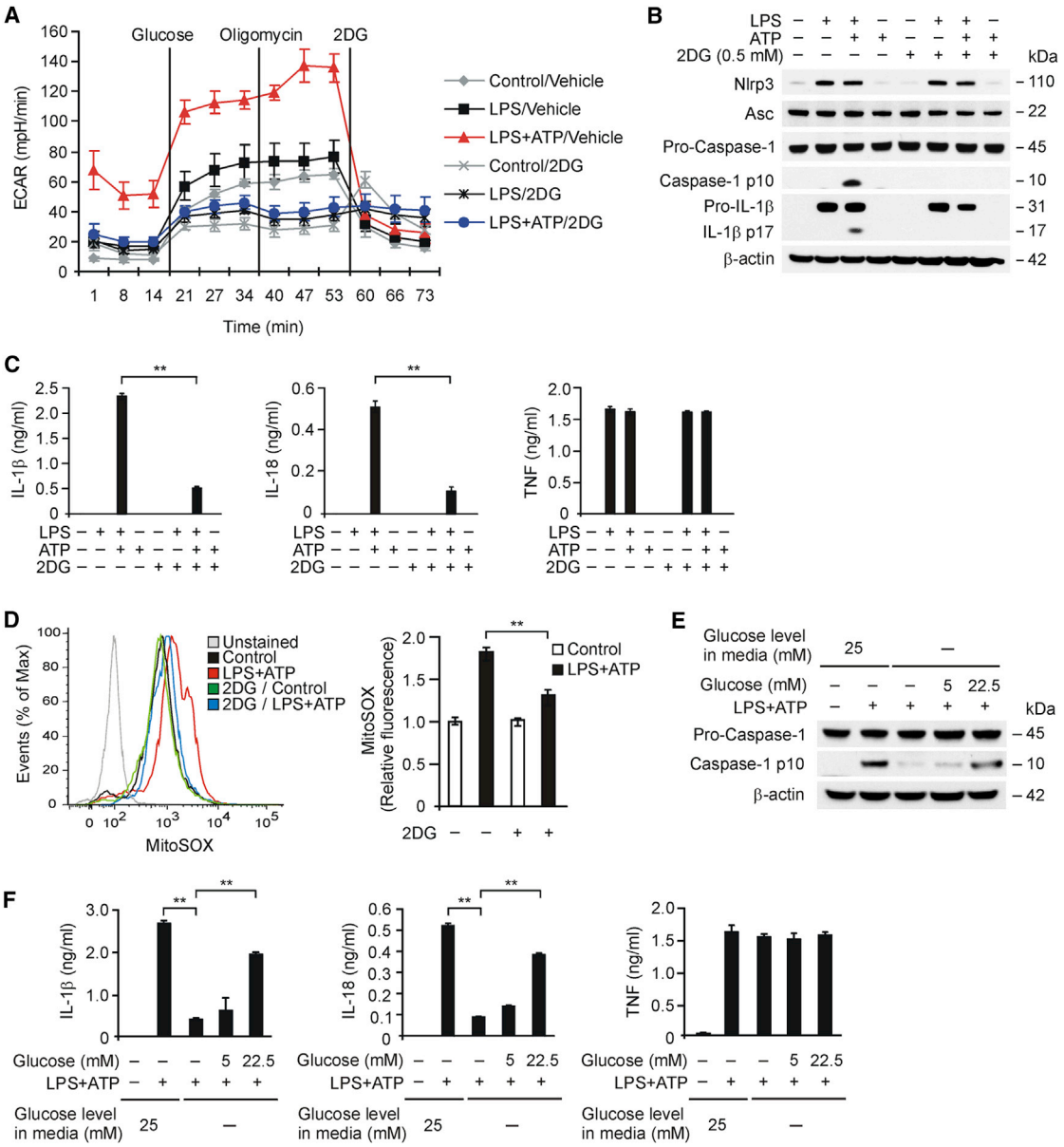

Figure 6. HK1-Dependent Glycolysis Is Required for NLRP3 Inflammasome Activation

(A) ECAR was measured in wild-type peritoneal macrophages pre-treated with 2DG (0.5 mM) for 2 hr before ATP (5 mM, 30 min) after LPS (500 ng/ml, 4h) stimulation. Data are mean ± SD.

(B) Immunoblot analysis for caspase-1, NLRP3, ASC, and IL-1β of cell lysates from wild-type BMDMs pre-treated with 2DG (0.5 mM) for 2 hr before ATP (2 mM) for 30 min after LPS stimulation. β-actin served as the standard.

(C) ELISA assays show IL-1β, IL-18, and TNF secretions from (B). **$p < 0.01$ by ANOVA.

(D) Flow cytometry analysis shows wild-type BMDMs stained with the mitochondrial superoxide-specific stain MitoSOX and then left unstimulated, or treated with 2DG for 2 hr before incubation with ATP (2 mM) for 30 min after LPS stimulation. **$p < 0.01$ by ANOVA.

(E) Immunoblot analysis for caspase-1 of cell lysates from wild-type BMDMs pre-treated with glucose-free media for 2 hr, followed by the addition of increasing concentrations of glucose (0, 5.0, and 22.5 mM) before stimulation with LPS and ATP. β-actin served as the standard.

(F) Luminex assay and ELISA assay for IL-1β, IL-18, and TNF secretions in supernatants from (E) are shown. **$p < 0.01$ by ANOVA.

See also Figures S4 and S5.

macrophages pre-treated with 2DG (0.5 mM) for 2 hr before LPS and ATP stimulation. 2DG significantly suppressed the increase of ECAR in response to LPS and ATP compared to vehicle control (Figure 6A).

Next, to investigate the effect of glycolysis inhibition by 2DG on NLRP3 inflammasome activation, we analyzed caspase-1 activation in LPS-primed BMDMs pre-treated with 2DG (0.5 mM) for 2 hr before ATP treatment. Consistent with the

results obtained with Torin1, 2DG inhibited caspase-1 activation, while NLRP3 and ASC expressions were unchanged (Figure 6B). Moreover, the secretions of IL-1β and IL-18 were decreased by 2DG treatment, while TNF was unchanged (Figure 6C). Since we have reported previously that mitochondrial reactive oxygen species (mtROS) are required for NLRP3-dependent caspase-1 activation (Nakahira et al., 2011), we analyzed the effect of 2DG on mtROS generation. 2DG treatment significantly suppressed mtROS generation in response to LPS and ATP compared to control (Figure 6D).

We investigated whether the effects of 2DG related to glycolysis inhibition could involve regulation of the energy-sensing kinase AMPK. Treatment with 2DG had no effect on the activation of AMPK at the doses of 2DG tested, whereas 2DG inhibited caspase-1 activation (Figures S4A and S4B). Consistently, 2DG did not significantly affect translational activation through the phosphorylations of p70S6K, S6, and 4E-BP1 (Figure S4A). Moreover, AICAR, an activator of AMPK, did not affect caspase-1 activation and secretions of IL-1β and IL-18 in wild-type BMDMs in response to LPS and ATP compared to control, as previously reported (Wen et al., 2011; Figure S4C). These results suggest that AMPK was not involved in the suppression of caspase-1 activation by inhibition of glycolysis.

We also examined the requirement for glycolysis in NLRP3 inflammasome activation by using deprivation of glucose to inhibit glycolysis (Greiner et al., 1994). Similar to the effects of 2DG, glucose deprivation inhibited caspase-1 activation and secretions of IL-1β and IL-18 in wild-type BMDMs in response to LPS and ATP, while TNF secretion was unchanged (Figures 6E and 6F). Importantly, the inhibitory effects of glucose deprivation on caspase-1 activation and secretions of IL-1β and IL-18 were dose-dependently reversed by increasing the concentration of glucose in the media, while TNF was unchanged (Figures 6E and 6F). Consistent with the results observed in BMDMs, nigericin-induced secretions of IL-1β and IL-18 in human THP-1 macrophages were inhibited by 2DG (Figure S5A). In contrast, 2DG did not significantly affect the secretions of IL-1β and IL-18 in wild-type BMDMs subjected to LPS and poly(dA:dT) treatment, which activates the AIM2 inflammasome pathway (Figure S5B). These results suggest that mTORC1-dependent glycolysis is a critical metabolic pathway involved in NLRP3 inflammasome activation.

HK1-Dependent Glycolysis Is Required for NLRP3 Inflammasome Activation

To analyze whether HK1 can play a critical role in NLRP3 inflammasome activation, we investigated the effect of HK1 deficiency on NLRP3 inflammasome activation. Genetic deletion of HK1 results in early embryonic lethality in mice, precluding the use of *Hk1*$^{-/-}$ mice in the current study (Fueger et al., 2003). Hence, we used two independent shRNAs to downregulate HK1 expression in wild-type peritoneal macrophages. The activation of caspase-1 in response to LPS and ATP stimulation was suppressed by HK1 shRNA transduction relative to control shRNA transduction, while NLRP3 and ASC expressions were unchanged (Figure 7A). Moreover, the secretions of IL-1β and IL-18 were inhibited by HK1 shRNA in peritoneal macrophages, while TNF was unchanged (Figure 7B). Consistent with the results observed with 2DG treatment, HK1 knockdown by HK1 shRNA suppressed the production of glycolytic metabolites (i.e., glucose-6-phosphate, citrate, and lactate) in peritoneal macrophages in response to LPS and ATP, compared to control shRNA-transduced cells (Figure 7C).

Similarly, increased glucose uptake and the PEP, citrate, and lactate productions induced by LPS and ATP were significantly suppressed by HK1 knockdown, using HK1 siRNA in mouse J774A.1 macrophages prior to stimulation with LPS and ATP (Figure S6A). Knockdown of HK1 using HK1-specific siRNA inhibited the activation of caspase-1 in mouse J774A.1 macrophages in response to LPS and ATP, compared to control siRNA-transfected cells (Figure S6B). Moreover, knockdown of HK1 inhibited the secretions of IL-1β and IL-18 in response to LPS and ATP relative to control siRNA-transfected cells, while TNF was unchanged (Figure S6C). Consistent with the results observed with 2DG treatment, knockdown of HK1 by transduction with HK1 siRNA inhibited mtROS generation in response to LPS and ATP, compared to control siRNA-transfected cells (Figure S6D).

Next, we sought to investigate whether overexpression of HK1 could rescue the effect of mTORC1 inhibition on NLRP3 inflammasome activation. We overexpressed HK1 in mouse J774A.1 macrophages and validated the expression levels by western immunoblot analysis (Figure 7D). The mTORC1 inhibition by Torin1 suppressed caspase-1 activation and secretions of IL-1β and IL-18 in response to LPS and ATP stimulation, relative to control (Figures 7D and 7E). Importantly, the inhibitory effects of Torin1 treatment on caspase-1 activation and secretions of IL-1β and IL-18 were reversed by overexpression of HK1, while TNF was unchanged (Figures 7D and 7E).

To evaluate whether glycolysis activation can serve as an upstream mechanism for NLRP3-mediated caspase-1 activation, we analyzed caspase-1 activation and glycolysis activation represented by the production of glycolytic metabolites (i.e., glucose-6-phosphate, citrate, and lactate) in wild-type BMDMs pre-treated with Z-VAD, a selective caspase-1 inhibitor, for 1 hr before LPS and ATP stimulation. Z-VAD suppressed caspase-1 activation and secretions of IL-1β and IL-18 in response to LPS and ATP stimulation relative to vehicle control, while TNF was unchanged (Figures S7A and S7B). In contrast, Z-VAD did not change the production of glycolytic metabolites (i.e., glucose-6-phosphate, citrate, and lactate) by LPS and ATP stimulation relative to vehicle control (Figure S7C). These results suggest that HK1-dependent glycolysis is required for NLRP3 inflammasome activation.

DISCUSSION

In this paper, we show that mTORC1-induced glycolysis provides a critical mechanism for NLRP3 inflammasome activation in macrophages. We also demonstrate that HK1-dependent glycolysis, under the regulation of mTORC1, represents a crucial metabolic pathway that is required for NLRP3 inflammasome activation. Furthermore, we show that activation of the glycolytic phenotype is linked to the immune response.

Among the family of NLRs, the NLRP3 inflammasome is unique in its ability to recognize molecular patterns associated

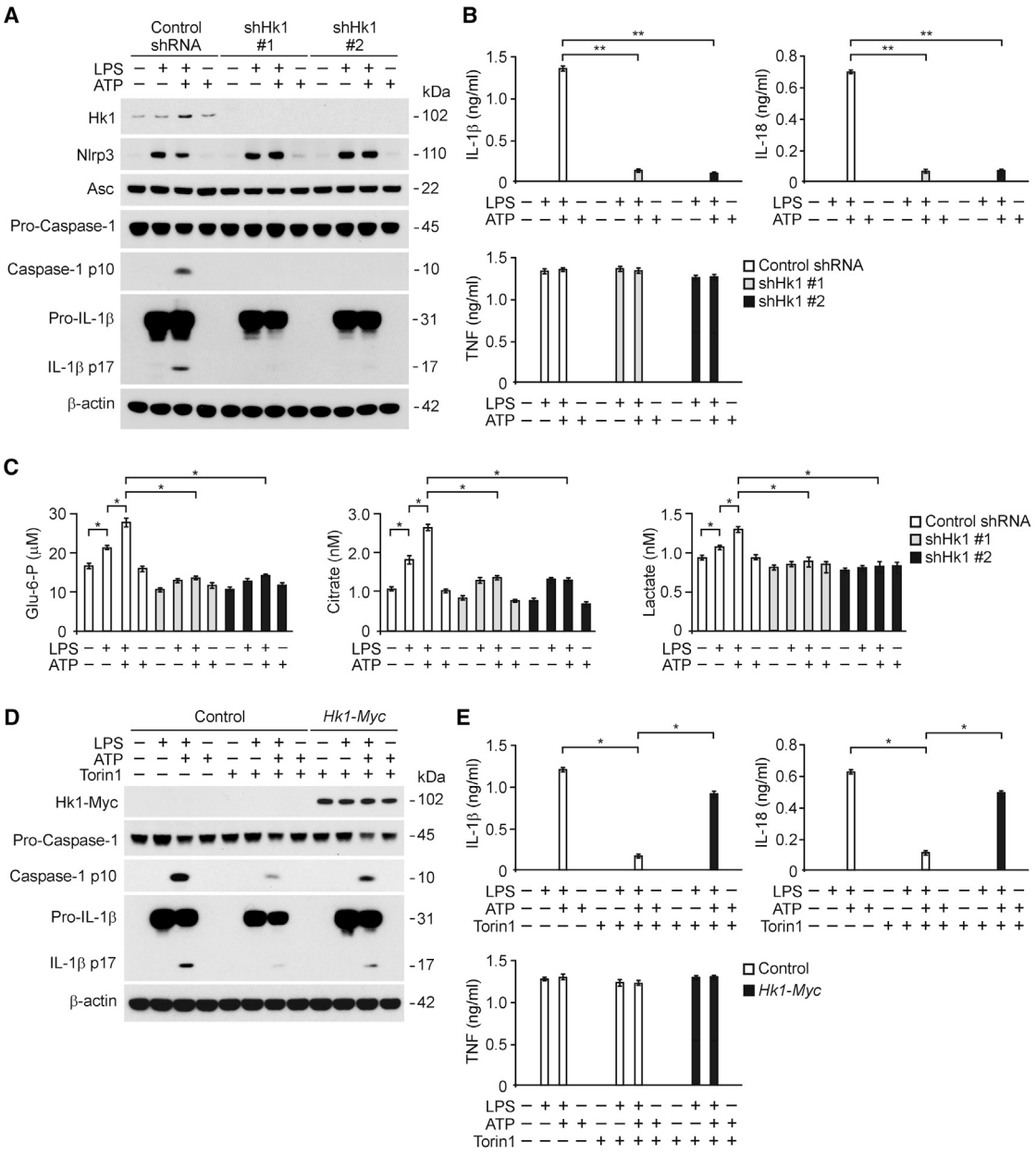

Figure 7. Deficiency of HK1-Dependent Glycolysis Suppresses NLRP3 Inflammasome Activation

(A) Immunoblot analysis for HK1, caspase-1, and IL-1β in cell lysates from wild-type mice peritoneal macrophages transduced with lentiviruses expressing non-target shRNA (control shRNA) or two independent shRNAs for HK1 (shHK1 1 and 2) and stimulated with LPS and ATP. β-actin served as the standard.

(B) ELISA assays show IL-1β, IL-18, and TNF secretions from (A). **$p < 0.01$ by ANOVA.

(C) Glucose-6-phosphate, citrate, and lactate production assays from wild-type peritoneal macrophages transduced with lentiviruses expressing non-target shRNA (control shRNA) or two independent shRNAs for HK1 (shHK1 1 and 2) and stimulated with LPS and ATP are shown. *$p < 0.05$ by ANOVA.

(D) Immunoblot analysis for Myc-tagged HK1, caspase-1, and IL-1β of cell lysates from mouse J774A.1 macrophages transfected with control vector or Myc-tagged Hk1 expression vector, treated with LPS (500 ng/ml) for 4 hr, and followed by incubation with ATP (5 mM) for 30 min. β-actin served as the standard.

(E) ELISA assays show IL-1β, IL-18, and TNF secretions from (D). *$p < 0.05$ by ANOVA.

See also Figures S6 and S7.

with host-derived metabolites, such as glucose or saturated fatty acids (Jourdan et al., 2013; Tannahill et al., 2013; Zhou et al., 2010). Although recent reports indicate that the NLRP3 inflammasome is regulated by metabolic changes in association with high-fat diet-induced obesity (Stienstra et al., 2011; Vandanmagsar et al., 2011), it has remained unclear whether cellular glycolysis can regulate NLRP3 inflammasome activation. Here we have demonstrated that mTORC1-HK1-dependent glycolysis

was required for NLRP3 inflammasome activation in response to pro-inflammatory stimuli. Genetic and pharmacologic downregulation of Raptor/mTORC1 or HK1 inhibited the activation of glycolysis and NLRP3 inflammasome-mediated caspase-1 activation in macrophages. The inhibition of the mTOR pathway by downregulation of Raptor/mTORC1 affected the regulation of both pro-IL-1β maturation and caspase-1 activation during NLRP3 inflammasome activation. Consistently, we also have shown that glucose deprivation markedly suppressed NLRP3 inflammasome-mediated caspase-1 activation in response to LPS and ATP. In contrast, we have shown that the inhibition of glycolysis by 2DG did not affect AIM2 inflammasome activation. Similarly, we have demonstrated that activation of HK1-dependent glycolysis was not involved in AIM2 inflammasome activation. Collectively, our results suggest that the activation of glycolysis is required for NLRP3 inflammasome activation.

Glycolysis is a critical pathway in cellular glucose metabolism that provides intermediates for energy generation. The increased expression and activity of HK is critical for persistent consumption of glucose-6-phosphate by the glycolytic pathway in order to attain a high glycolytic phenotype (Azoulay-Zohar and Aflalo, 1999, 2000; Baijal and Wilson, 1995; Crane and Sols, 1953; Mathupala et al., 2009; Parry and Pedersen, 1984; Wilson, 2003). Anaerobic glycolysis is an effective means of energy production during short intense exercise, providing energy for short periods ranging from 10 s to 2 min. The speed at which ATP is produced is about 100 times that of oxidative phosphorylation (OXPHOS) (Koppenol and Bounds, 2009; Vander Heiden et al., 2009). Recently, HK2 also has been shown to be responsible for TLR-driven high glycolytic rates in dendritic cells (Everts et al., 2014). In contrast, we found that HK1 is the major isoform of HK expressed in BMDMs, whereas HK2 is not expressed in these cells. In LPS-primed macrophages, glucose utilization and glycolytic metabolites were rapidly increased via increased HK1 protein expression as early as 30 min after ATP treatment. The rapid increase in HK1 protein levels may explain, in part, how glucose uptake and glycolytic metabolites were markedly increased with short kinetics. We suggest that the high glycolytic phenotype observed during NLRP3 inflammasome activation is regulated by the rapid increase of HK1 protein in macrophages.

Among four HK isozymes (HK1–4), HK1 is ubiquitously expressed in all mammalian tissues (Azoulay-Zohar and Aflalo, 1999, 2000; Baijal and Wilson, 1995; Crane and Sols, 1953; Mathupala et al., 2009; Parry and Pedersen, 1984; Wilson, 2003). However, the mechanism by which HK1 expression is regulated remains poorly understood. In our study, we found that the protein expression of HK1 was regulated by mTORC1-dependent translation in response to LPS and ATP in macrophages. The inhibition of translation by Torin1 or Raptor/mTORC1 knockdown suppressed HK1 protein levels. Consistently, the activation of translation by knockdown of the inhibitory protein 4E-BP1 increased HK1 protein levels. The initiation of translation by mTORC1 is regulated through the phosphorylation state of eIFs and other factors (Richter and Sonenberg, 2005). The eIF4E activity is tightly regulated by reversible interaction with 4E-BP (Richter and Sonenberg, 2005). Among three known 4E-BPs (4E-BP1, 4E-BP2, and 4E-BP3), 4E-BP1 specifically plays a major role in translation by inhibiting cap-dependent translation initiation through the regulation of eIF4E phosphorylation (Richter and Sonenberg, 2005). Previous studies have demonstrated a critical role of 4E-BP1 in adipogenesis and metabolism in mammals, such that *4ebp1*$^{-/-}$ mice displayed increased metabolic rate. Furthermore, deficiency of 4E-BP1 increased the protein levels of the mitochondrial biogenesis factor peroxisome proliferator-activated receptor-γ co-activator 1 (PGC1) via stimulating translation, whereas the mRNA levels of this factor were unaffected (Tsukiyama-Kohara et al., 2001). According to our data, we suggest that HK1 protein expression was regulated by mTORC1-dependent translation via 4E-BP1 in macrophages.

Several proteins that have a metabolic function, such as thioredoxin-interacting protein (TXNIP), VDACs, and mitochondrial antiviral signaling proteins (MAVs), have been associated with NLRP3 inflammasome activation (Subramanian et al., 2013; Zhou et al., 2010, 2011). While the protein expression of HK1 was not induced in response to AIM2 inflammasome activation, the expression of HK1 was selectively increased during NLRP3 inflammasome activation. A considerable variation in the proportion of HK activity bound to the mitochondria occurs in different cell types and tissues, with the majority found in the bound form in tissues, such as brain and kidney, where HK1 is highly expressed (Azoulay-Zohar and Aflalo, 1999, 2000; Baijal and Wilson, 1995; Crane and Sols, 1953; Mathupala et al., 2009; Parry and Pedersen, 1984; Wilson, 2003). Consistent with previous results, we have shown that HK1 expression was found mainly in the mitochondrial fraction of macrophages. HK1 exhibited a higher affinity for the VDAC1 isoform than for other mammalian VDAC isoforms (Azoulay-Zohar and Aflalo, 1999, 2000). Given that VDAC is essential for NLRP3 inflammasome activation (Zhou et al., 2011), it is likely that the interaction of HK1 with VDAC1 in mitochondria can confer specific functional advantages for NLRP3 inflammasome activation. In our study, we demonstrated the interaction of HK1 with VDAC1 in the mitochondria of macrophages. We suggest that mitochondrial interaction of HK1 and VDAC1 may represent another factor essential for NLRP3 inflammasome activation in macrophages.

In conclusion, we found that mTORC1-HK1-dependent glycolysis regulates NLRP3 inflammasome activation in macrophages. We also determined that the activation of the glycolytic phenotype is linked to the immune response. Given the complex relationship between the NLRP3 inflammasome and cellular glycolysis, our findings may have broad implications for therapeutic targeting in human diseases, not only limited to metabolic diseases but also diseases in which inflammation plays a key role.

EXPERIMENTAL PROCEDURES

Mice

Mice were genotyped with standard PCR of tail DNA. All animal experimental protocols were approved by the Harvard Standing Committee for Animal Welfare (protocol 04435, Harvard Medical School) and the Institutional Animal Care and Use Committee (protocol 2013-0108, Weill Cornell Medical College).

Reagents and Antibodies

LPS (*Escherichia coli*) (tlrl-pelps), MDP (tlrl-mdp), and flagellin (Salmonella typhimurium) (tlrl-stfla) were 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) were from Tocris Bioscience. Rapamycin (9904) was from Cell Signaling Technology. The following antibodies were used: polyclonal rabbit anti-caspase-1 for mouse caspase-1 (SC-514, Santa Cruz Biotechnology); polyclonal goat anti-IL-1β for mouse IL-1β (AF-401-NA, R&D Systems); monoclonal mouse anti-NLRP3 for mouse NLRP3 (ALX-804-881-C100, Enzo Life Sciences); polyclonal rabbit anti-ASC for mouse ASC (AP07815PU-N, Acris Antibodies); glycolysis antibody sampler kit for anti-HK1 and anti-glycolytic enzymes (8337, Cell Signaling Technology); translational control antibody sampler kit for phospho-eIF4E, phospho-p70 S6 kinase, phospho-S6, and phospho-4E-BP1 (9918, Cell Signaling Technology); AMPK and ACC antibody sampler kit for phospho-AMPKα, AMPKα, phospho-ACC, and ACC (9957, Cell Signaling Technology); monoclonal rabbit anti-Raptor (2280, Cell Signaling Technology); monoclonal rabbit anti-Rictor (9476, Cell Signaling Technology); monoclonal rabbit anti-mTOR (2983, Cell Signaling Technology); monoclonal rabbit anti-4E-BP1 (9644, Cell Signaling Technology); monoclonal rabbit anti-Myc-Tag (2278, Cell Signaling Technology); and monoclonal mouse anti-β-actin (A5316, Sigma-Aldrich).

Cell Culture

BMDMs were prepared as described previously (Sutterwala et al., 2006). Bone marrow collected from mouse femurs and tibias was plated on sterile petri dishes and incubated for 7 days in DMEM (Invitrogen) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 mg/ml streptomycin, and 25% (v/v) conditioned medium from mouse L929 fibroblasts (CCL-1, ATCC). Cells were incubated for 4 hr with LPS (500 ng/ml) and then were treated with ATP (2 mM) for 30 min as described. Mice were injected with thioglycollate broth medium (1.0 ml, i.p.). After 4 days, peritoneal cells were collected from mice with cold PBS. Cells were incubated for 2 hr with DMEM containing 10% (v/v) heat-inactivated FBS, penicillin, and streptomycin in six-well tissue culture plates. Non-adherent cells were removed by gently washing with PBS. After cells were cultured overnight, cells were incubated for 4 hr with LPS (500 ng/ml) and then were treated with ATP (5 mM) for 30 min as described. Mouse J774A.1 macrophages (TIB-67, ATCC) were cultured in DMEM containing 10% (v/v) FBS, penicillin, and streptomycin. Human THP-1 monocyte-derived macrophages (TIB-202, ATCC) were grown in RPMI-1640 media (Invitrogen) containing 10% (v/v) FBS, penicillin, and streptomycin. THP-1 cells were differentiated for 16 hr with 50 nM phorbol 12-myristate-13-acetate. Cells were incubated for 6 hr with nigericin (6.7 μM).

Immunoblot Analysis

Cells or tissues were harvested, lysed in 2× SDS loading buffer or NP40 Cell Lysis Buffer (FNN0021, Invitrogen), and then briefly sonicated. Lysates were centrifuged at 15,300 × *g* 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 (10600001, GE Healthcare).

Cytokine Analysis

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

qRT-PCR

Total RNA was isolated from cultured cells and tissues using the TRIzol reagent (15596-018, Invitrogen), according to the manufacturer's instructions. For qRT-PCR, cDNA was synthesized from 4 μg total RNA using random hexamers and SuperScript Reverse Transcriptase II (18064-014, 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 2× SYBR Green PCR Master Mix (4309155, 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′- CTTGACG GAGGCCGTTGGGTT-3′; mouse *Gapdh* forward, 5′-GGTGAAGGTCGGTGT GAACGGA-3′ and reverse, 5′-CCAAAGTTGTCATGGATGACCTTGG-3′.

Transduction of shRNA and Transfection of siRNA

For stable knockdown of mouse *Raptor*, two independent shRNAs (TRCN0000077469 and TRCN0000077471, Sigma-Aldrich) were used. For stable knockdown of mouse *Rictor*, two independent shRNAs (TRCN0000123394 and TRCN0000123395, Sigma-Aldrich) were used. For stable knockdown of mouse *Hk1*, two independent shRNAs (TRCN0000297076 and TRCN0000278141, Sigma-Aldrich) were used. For stable knockdown of mouse *4e-bp1*, two independent shRNAs (TRCN0000335449 and TRCN0000335450, Sigma-Aldrich) were used.

Mouse peritoneal macrophages (5×10^5 cells/well) were seeded in six-well plates and were transduced with shRNA lentiviral constructs against mouse Raptor, Rictor, Hk1, or non-target shRNA (SHC016, Sigma-Aldrich) for control. For transient knockdown of mouse *mTOR*, *Hk1*, and *4e-bp1*, siRNAs of *mTOR* (EMU047451), *Hk1* (EMU086531), and *4e-bp1* (EMU029571) were obtained from Sigma-Aldrich. Mouse J774A.1 macrophages (2×10^5 cells/well) were seeded in six-well plates and were transfected with siRNAs 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 overexpression of HK1, mouse Hk1 cDNA ORF clone (Myc-DDK tagged) (MR204388, OriGene) was used. Mouse J774A.1 macrophages (2×10^5 cells/well) were seeded in six-well plates and were transfected with Hk1 cDNA ORF clone or control vector using Lipofectamine LTX Reagent with Plus reagent (15338-100, Invitrogen), according to the manufacturer's instructions. For AIM2 inflammasome activation, LPS-primed wild-type BMDMs were transfected with poly(dA:dT) (1 μg/ml) (Sigma-Aldrich) using Lipofectamine with Plus reagent (15338-100, Invitrogen), according to the manufacturer's instructions.

mtROS Production Assay

mtROS were measured by MitoSOX (M36008, 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% (v/v) heat-inactivated FBS. Data were acquired with a FACSCanto II (BD Biosciences) and were analyzed with FlowJo analytical software (Tree Star).

Glycolytic Function Assay

For the glycolytic function assay, peritoneal cells (5×10^4 cells/well) were plated on XF96 cell culture microplates (101085-004, Seahorse Bioscience). ECAR or OCR, as parameters of glycolytic flux, was measured on a Seahorse XF96 bioanalyzer, using the XF Glycolysis Stress Test kit according to the manufacturer's instructions (102194-100, Seahorse Bioscience).

Glucose Uptake, Glucose-6-Phosphate, PEP, Citrate, and Lactate Production Assays

For the glucose uptake assay, cells (5×10^5 cells/well) were plated on six-well plates. Cells were then incubated in a glucose-free DMEM (Invitrogen) for 1 hr. After the addition of ^{18}F-FDG (0.3 MBq), the cells were incubated at 37°C for 30 min. The cells were washed twice with PBS. Cells were harvested in PBS for radioactivity measurement using a 1480 Wizard 3 γ-counter (PerkinElmer). Data shown are the mean ± SD of triplicate samples from a representative experiment. The PEP production was measured using a PEP fluorometric assay kit, according to the manufacturer's instructions (700780, Cayman Chemical). The glucose-6-phosphate (657-100, BioVision Technologies), citrate (K655-100, BioVision Technologies), and lactate (K607-100, BioVision Technologies) productions were measured using colorimetric assay methods, according to the manufacturer's instructions.

Statistical Analysis

All data are mean ± SD, combined from three independent experiments. All statistical tests were analyzed by Student's two-tailed t test for comparison of two groups and ANOVA (with post hoc comparisons using Dunnett's test) with a statistical software package (GraphPad Prism version 4.0) for comparison of multiple groups. p values of less than 0.05 were considered statistically significant. Survival was analyzed with the log-rank test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.05.046.

AUTHOR CONTRIBUTIONS

J.-S.M., K.N., and A.M.K.C. conceived the study with assistance from S.W.R. J.-S.M. and G.M.D. performed the in vitro experiments. J.-S.M., M.-A.P., and S.H. performed the in vivo experiments. J.-S.M., K.N., S.W.R., and A.M.K.C. wrote the paper. A.M.K.C. supervised the entire project.

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