Molecular Medicine

AMP-Activated Protein Kinase and Glycogen Synthase Kinase 3β Modulate the Severity of Sepsis-Induced Lung Injury

Zhongyu Liu,[1] Nathaniel Bone,[1*] Shaoning Jiang,[1] Dae Won Park,[1] Jean-Marc Tadie,[1] Jessy Deshane,[1] Cilina Ann Rodriguez,[2] Jean-Francois Pittet,[2] Edward Abraham,[3] and Jaroslaw W Zmijewski[1]*

[1]Department of Medicine, and [2]Anesthesiology, University of Alabama at Birmingham, Birmingham, Alabama, United States of America; [3]Office of the Dean, Wake Forest University School of Medicine, Winston-Salem, North Carolina, United States of America

Alterations in metabolic and bioenergetic homeostasis contribute to sepsis-mediated organ injury. However, how AMP-activated protein kinase (AMPK), a major sensor and regulator of energy expenditure and production, affects development of organ injury and loss of innate capacity during polymicrobial sepsis remains unclear. In the present experiments, we found that cross-talk between the AMPK and GSK3β signaling pathways controls chemotaxis and the ability of neutrophils and macrophages to kill bacteria *ex vivo*. In mice with polymicrobial abdominal sepsis or more severe sepsis induced by the combination of hemorrhage and intraabdominal infection, administration of the AMPK activator metformin or the GSK3β inhibitor SB216763 reduced the severity of acute lung injury (ALI). Improved survival in metformin-treated septic mice was correlated with preservation of mitochondrial complex V (ATP synthase) function and increased amounts of ETC complex III and IV. Although immunosuppression is a consequence of sepsis, metformin effectively increased innate immune capacity to eradicate *P. aeruginosa* in the lungs of septic mice. We also found that AMPK activation diminished accumulation of the immunosuppressive transcriptional factor HIF-1α as well as the development of endotoxin tolerance in LPS-treated macrophages. Furthermore, AMPK-dependent preservation of mitochondrial membrane potential also prevented LPS-mediated dysfunction of neutrophil chemotaxis. These results indicate that AMPK activation reduces the severity of polymicrobial sepsis-induced lung injury and prevents the development of sepsis-associated immunosuppression.

Online address: http://www.molmed.org
doi: 10.2119/molmed.2015.00198

INTRODUCTION

Severe infection accompanied initially by an overly exuberant inflammatory response and later by immunosuppression is frequently associated with dysfunction of vital organs and has a direct impact on morbidity and mortality in critically ill patients (1). Sepsis is the most frequent cause of death in hospitalized patients (2). Sterile inflammatory conditions linked to hemorrhage, trauma or burns worsen organ dysfunction in polymicrobial sepsis (3,4). Acute respiratory distress syndrome (ARDS) (5,6) frequently accompanies sepsis, and is associated with higher mortality rates in this setting (7). Effective pharmacologic interventions are not available for sepsis, a condition that affects more than a million patients each year in the United States (8). Similarly, there is no available pharmacologic intervention that improves the outcome from ARDS (9).

Excessive production of inflammatory mediators, including cytokines such as IL-1β and IL-17, disruption of endothelial and epithelial barriers with increased permeability, along with alterations in cellular bioenergetics and immunosuppression appear to contribute to organ dysfunction and mortality in sepsis (1,10–12). While innate immune cells play a central role in host response to infection, exaggerated macrophage and neutrophil proinflammatory activation is also implicated in increased severity of sepsis-induced organ injury (13–15). The late or adaptive phase of sepsis is associated with apoptosis of lymphocytes and with epithelial and endothelial cell dysfunction as well as with diminished activation of neutrophils, macrophages and other cell populations involved in innate immunity. Such late phase immunosuppression appears to contribute to enhanced susceptibility to secondary infections that result in increased mortality (16,17). Previous experiments have shown that loss of mitochondrial structure and function in immune cells is implicated in organ failure in sepsis (18,19).

**ZL and NB contributed equally to this work.*

Address correspondence to *Jaroslaw W Zmijewski, Division of Pulmonary, Allergy & Critical Care Medicine, University of Alabama at Birmingham, School of Medicine, 901 19th St. South, BMRII 304. Birmingham, AL 35294. Phone: 205-934-7793; Fax: 205-934-7437; E-mail: zmijewsk@uab.edu.*

Submitted September 3, 2015; Accepted for publication November 27, 2015; Published Online (www.molmed.org) November 30, 2015.

Of note, the extent of mitochondrial dysfunction in the lungs has been shown to correlate with mortality in sepsis (19,20). Approaches to prevent mitochondrial dysfunction or to restore mitochondrial bioenergetics may diminish the severity of sepsis-associated lung injury (21–23).

The ability of the AMP-activated protein kinase (AMPK) to detect metabolic alterations and to modulate cellular bioenergetic and redox states appears to contribute to mortality and organ dysfunction in sepsis as well as to recovery from this life-threatening condition (24–26). AMPK is a heterotrimer that consists of one catalytic α and two regulatory β and γ subunits (27). This serine/threonine kinase has a unique mechanism of activation that is coupled to increases in energy demand, typically either due to excessive energy expenditure and/or deficient energy production. Such situations are associated with increased AMP-to-ATP ratios followed by AMP-dependent binding to the AMPKγ subunit, allosteric domain rearrangement and phosphorylation of T172-AMPKα by upstream kinases (28). Both AMP binding and phosphorylation of T172 are required for optimal AMPK activation. Activated AMPK participates in limiting energy expenditure while promoting pathways of energy production, including fatty acid oxidation, glycolysis and enhanced oxidative phosphorylation (24). Although enhanced AMPK activation induced by pharmacologic agents, such as metformin, is an important therapeutic approach to type 2 diabetes, recent studies also show that administration of metformin can retard aging in experimental models, and has been suggested to be associated with an increased lifespan of diabetic patients (29,30). In addition to the effects of AMPK activation on glucose and lipid metabolism, studies, including those from our laboratory, indicate that activated AMPK has antiinflammatory effects in TLR4-activated neutrophils and macrophages, and also diminishes the severity of endotoxin-induced lung injury in preclinical models (31–33).

Although sepsis is accompanied by alterations in bioenergetics of immune and parenchymal cells, as well as an increase in generation reactive oxygen and nitrogen species (ROS/RNS), that should result in AMPK activation, activation of AMPK is often not found in such settings (34–37). More recently, we have shown that the Iκ B kinase beta (IKKβ)/glycogen synthase kinase beta (GSK3β) signaling axis contributes to preventing AMPK activation both after TLR4 engagement in neutrophils and macrophages, and in the lungs of mice subjected to sterile inflammatory conditions, including endotoxemia (38). However, it is not known whether this mechanism is operational during polymicrobial interabdominal infection, a clinically relevant issue in sepsis-induced ARDS. Because polymicrobial sepsis is linked to harmful inflammation and diminished capacity of the innate system for bacterial eradication, we also determined if AMPK activation contributes to subsequent development of immunosuppression. In particular, we examined whether AMPK activation affects clearance of *P. aeruginosa* lung infection following polymicrobial abdominal sepsis.

MATERIALS AND METHODS

Mice

Male C57BL/6 mice were purchased from the National Cancer Institute in Frederick, Maryland. Mice 10 to 12 wks of age were used for experiments. The mice were housed in the animal facility at the University of Alabama at Birmingham. All experiments were conducted in accordance with protocols approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Reagents and Antibodies

The GSK3β inhibitor BIO (6-bromoindirubin-3′-oxime) was purchased from R&D Systems whereas SB216763 was from Sigma-Aldrich. PS-1145 (IKK1/2 inhibitor), metformin (AMPK activator), LPS (TLR4 agonist) and brewer thioglycollate medium were obtained from Sigma-Aldrich. W-peptide (chemoattractant) was purchased from Phoenix Pharmaceuticals. Antibodies for phospho Thr172-AMPK, phospho Ser485-AMPK, total AMPK, phospho Ser79-ACC and total ACC were purchased from Cell Signaling Technology. HRP-conjugated β-actin antibody was from Santa Cruz Biotechnology. Anti-phospho-Thr479-AMPK antibody was generated as described previously (39) and was a gift from Ken Inoki of the University of Michigan. Anti-HMGB1 antibody was purchased from R&D Systems. Total OXPHOS Rodent WB Antibody Cocktail was obtained from Abcam. Hoechst dye and JC-1 probe were from Life Technologies. siRNA to the AMPKα1 subunit, scrambled siRNA and Accell medium were purchased from Thermo Scientific/Dharmacon.

Neutrophil and Peritoneal Macrophage Isolation

Bone marrow neutrophils were purified using a negative selection column (31,38). In brief, bone marrow cell suspensions were isolated from the femur and tibia of mice by flushing with RPMI 1640 medium with 5% fetal bovine serum (FBS). The cell suspension was passed through a glass wool column and collected by washing with phosphate buffer solution (PBS) containing 5% FBS. Negative selection to purify neutrophils was performed by incubation of the cell suspension with biotinylated primary Abs specific for the cell-surface markers F4/80, cluster of differentiation 4 (CD4), CD45R, CD5 and TER119 (Stem Cell Technologies) for 15 min at 4°C followed by subsequent incubation with antibiotin tetrameric Abs (100 μL; Stem Cell Technologies) for 15 min. The complex of antitetrameric Abs and cells was then incubated with colloidal magnetic dextran iron particles (60 μL; Stem Cell Technologies) for an additional 15 min at 4°C. The T cells, B cells, RBCs, monocytes and macrophages were captured in a column surrounded by a magnet, allowing the neutrophils to pass through. Neutrophil purity, as determined by Wright-Giemsa-stained cytospin preparations, was consistently >98%.

Peritoneal macrophages were isolated as described previously (38). Macrophages

were elicited in 10- to 12-wk-old mice by use of Brewer thioglycollate. Cells were collected 4 d after intraperitoneal (IP) injection of thioglycollate, cultured for 3 d *ex vivo* and then treated as described in the figure legends.

Transwell Migration Assay

Bone marrow neutrophils (10^6 cells/well) were incubated with PS1145 (0 or 10 μmol/L), BIO (0 or 5 μmol/L), SB216763 (0 or 30 μmol/L) or metformin (0 or 500 μmol/L) for 2 h followed by exposure to LPS (300 ng/mL) for an additional 60 min. Transwell migration assay was performed using 24-well cell plate BD Falcon cell culture inserts (pore size 3 μm) (Translucent PET Membrane, BD Biosciences). Briefly, bone marrow neutrophils (10^6/mL) were placed into the upper reservoir, and chemotaxis initiated by inclusion of W-peptide (50 nmol/L) in the lower reservoir of transmigration chamber. Chemotaxis was determined after neutrophils were allowed to migrate for 60 min in RPMI media supplemented with FBS (5%). Cells in capillary structures of transmigrating chamber were subjected to Wright-Giemsa-staining followed by image acquisition using light microscopy. Each condition was tested three or more times using independent cell populations.

In Vitro Killing Assay

Neutrophils (5×10^6 cells/mL) cultured in RPMI 1640 (0.5% FBS) were pretreated with PS1145 (0 or 10 μmol/L) or SB216763 (0 or 30 μmol/L) for 60 min or metformin (0 or 500 μmol/L) for 2 h, followed by inclusion of *Pseudomonas aeruginosa* (PAK; 5×10^7/mL; 1:10 ratio neutrophil/PAK) for an additional 90 min. Similar to neutrophils, PS1145-, SB216763- or metformin-treated peritoneal macrophages (5×10^6 cells/mL) were incubated with PAK (macrophage/PAK; 1:10 ratio) for 90 min. The cell/bacterial solutions were centrifuged at 375*g* for 5 min, and then the cell pellets were lysed by adding 100 μL of Triton-X 100 (0.1%). The lysates were then plated on agar plates with ampicillin and incubated overnight at 37°C. Colony-forming units (CFUs) were calculated by counting bacterial colonies grown on agar plates using colony counter software (Bio-Rad) and expressed as a percentage of colonies obtained from untreated neutrophils or macrophages.

siRNA Knockdown of AMPKα1

Peritoneal macrophages were incubated with scramble (1 μmol/L) or AMPKα1-specific siRNA (1 μmol/L), as described previously (40). Briefly, cells (5×10^5/well) in 12-well plates were incubated in Accell medium (serum free) containing siRNA (1 μmol/L) for AMPKα1 for 72 h. Cells were then subjected to AMPK Western Blot analysis or exposure to GSK3β inhibitor followed by TNF-α enzyme-linked immunosorbent assay (ELISA).

Cecal Ligation and Puncture (CLP)-Induced Sepsis

CLP was performed in 10- to 12-wk-old male C57BL/6 mice as described before (41). Briefly, the cecum was ligated 1.0 cm from the tip of cecum, which was an approximately 50% cecum ligation. A through-and-through puncture was performed with a 21-gauge needle and then a drop of feces was extruded to the peritoneal cavity. Saline (0.9%; 500 μL) was then applied into the peritoneal cavity and the abdominal wall incision was closed in two layers. The control group of mice (sham) underwent surgery without CLP.

A Mouse Model of Hemorrhage and Resuscitation

Hemorrhage was performed using the previously described method (42). C57BL/6 male mice were anesthetized by inhalation of isoflurane (5%), and then both femoral arteries were cannulated with catheters (Braintree Scientific). The systemic arterial pressure line was continuously measured, independently from the hemorrhage/resuscitation catheter line. Blood withdrawal was performed for 60 minutes with a 25 ± 5 mmHg mean arterial pressure (MAP), typically a resultant of nearly 60% (~800 μL) blood loss. Next, mice were fully resuscitated with Hanks' Balanced Salt solution (HBSS; Sigma-Aldrich) for 30 min. CLP procedure was conducted within 24 h, as described above.

Application of Metformin or GSK3β Inhibitor SB216763 in Mice Subjected to Sepsis-Induced Lung Injury

Mice were treated with metformin (100 mg/kg) or control (saline) IP applications in three doses; 48 h, 24 h and 30 min prior to CLP. In selected experiments, the second dose of metformin was given before hemorrhage. Mice were given the GSK3β inhibitor SB216763 (20 mg/kg) dissolved in 500 μL of DMSO/saline (1:40) or control vehicle (DMSO/saline 1:40) IP three times, that is, 48 h, 24 h and 30 min prior to CLP. Mice were euthanized 24 h after CLP, followed by preparation of lung homogenates for Western blot analysis, lung sections for H&E staining and collection of BAL fluids for cytokine ELISA. In particular, BAL fluids were collected by lavaging the lungs three times with 1 mL of PBS followed by measurement of inflammatory cytokines and protein content. Independent groups of mice were used to measure wet-to-dry ratios to determine the extent of pulmonary edema. In particular, after measuring the weight of freshly harvested (wet) lungs, the lungs were kept in an incubator for 7 d at 80°C. Next, the weight of dry lungs was measured followed by calculation of wet-to-dry ratio. Independent groups of mice were used to prepare lung homogenates in RIPA buffer (Sigma-Aldrich) followed by Western blot analysis of phosphorylated and total amounts of AMPK.

Cytokine ELISA

ELISA was used to measure cytokines in bronchoalveolar lavage (BAL) fluids. The amounts of tumor necrosis factor alpha (TNF-α), MIP-2, IL-6 and KC were determined by using commercially available ELISA kits (R&D Systems) according to the manufacturer's instructions and as previously described (38,43).

Macrophage Endotoxin Tolerance Assay

Peritoneal macrophages (3×10^5/well) were first treated with LPS (0 or 10 ng/mL) for 24 h then media washed three times followed by incubation for an additional 60 min. Next, cells were exposed to a second stimulation with LPS (10 ng/mL) for 4 h. In selected experiments, macrophages were also treated with metformin (1 mmol/L), AICAR (500 μmol/L) or SB216763 (30 μmol/L) for 60 min followed by incubation with LPS (first stimulation) for an additional 24 h.

Measurement of Mitochondrial Membrane Potential (mΔΨ)

Bone marrow neutrophils were seeded 80% (confluent) in a 4-well chambered coverslip coated with fibronectin (40 μg/mL). The cells were left unaltered or treated with AICAR (250 μmol/L), metformin (500 μmol/L) or BIO (20 μmol/L) for 60 min followed by inclusion of LPS (300 ng/mL) for an additional 60 min. The JC-1 probe (100 ng/mL) and Hoechst (1 μg/mL) were applied 30 min before image acquisition. Microscopy was performed using a confocal laser scanning microscope (model LSM 710 confocal microscope; Carl Zeiss MicroImaging). Quantitative fluorescent intensity (red/green pixel intensity) of the images was processed using IPLab Spectrum. In an additional experiment, mitochondrial membrane potential was also measured after mitochondrial depolarization with FCCP (100 nmol/L).

In Vivo Bacterial Killing Assay

Briefly, mice were anesthetized with isoflurane and then suspended by their upper incisors on a 60° incline board. The tongue was gently extended and wild-type PAK strain of *Pseudomonas aeruginosa* (2.5×10^7/mouse) suspension in PBS (50 μL) or PBS alone (control; 50 μL) was deposited into the pharynx followed by bacterial aspiration into the lungs, similar to the method that was described previously (32). Lung homogenates were prepared 4 h after *P. aeruginosa* instillation and serial dilutions used to determine CFUs/mL. Number of bacterial colonies grown on agar plates (CFUs) were measured using colony counter software (Bio-Rad).

Protein Concentration and Cell Counts in BAL Fluid

Briefly, protein concentration in BAL fluid was determined by Bradford method with Bio-Rad protein assay dye reagent concentrate (Bio-Rad). The numbers of neutrophils in BAL fluid were determined after cytospin and Wright-Giemsa staining followed by image acquisition using light microscopy.

Western Blot Analysis

Western blot analysis was performed as described previously (34,38). Each experiment was carried out three or more times with peritoneal macrophages or lung homogenates obtained from separate groups of mice. In selected experiments, BAL fluids (30 μL) were mixed with Laemmli sample buffer and boiled for 5 min followed by Western blot analysis of HMGB1.

Statistical Analysis

Multigroup comparisons were performed using one-way analysis of variance (ANOVA) with Tukey *post hoc* test. Statistical significance was determined by the Student *t* test for comparisons between two groups. A value of $P < 0.05$ was considered significant. Analyses were performed on SPSS version 16.0 (IBM) for Windows (Microsoft).

All supplementary materials are available online at www.molmed.org.

RESULTS

Participation of AMPK and GSK3β Signaling Pathways in Neutrophil- and Macrophage-Dependent Bacterial Killing

We have recently found that in LPS/TLR4 stimulated macrophages had diminished Thr172-AMPK phosphorylation and increased GSK3β-dependent Thr479-AMPK inhibitory phosphorylation (Supplementary Figure 1) (38). However, it is not known whether similar mechanisms are operational during infection, including development of lung injury due to polymicrobial interabdominal infection. Therefore, we investigated if the clearance of bacteria *ex vivo* may be affected by AMPK activation, and specifically examined the role of IKKβ/GSK3β signaling pathways in this process. Neutrophils or peritoneal macrophages were incubated with or without the AMPK activator metformin (500 μmol/L), the IKK1/2 inhibitor PS1145 (10 μmol/L) or the GSK3β inhibitor SB216763 (30 μmol/L), and then were cultured with *P. aeruginosa* (PAK; 5×10^7) for 90 min followed by measuring bacterial survival using CFU assays. As shown in Figures 1A, B and C, inclusion of metformin, IKK1/2 or GSK3β inhibitors had no adverse effects on neutrophil- or macrophage-dependent killing of bacteria. Indeed, the numbers of CFUs (an indicator of bacteria survival) were reduced after incubation of the macrophages or neutrophils with metformin or PS1145. Exposure of neutrophils to SB216763 reduced the amounts of bacteria, though such treatment was less effective in macrophages (Figures 1B, C). Both metformin and the AMPK activator AICAR, as well as the GSK3β inhibitor SB216763, resulted in degradation of NLRP3, a major regulator of cell death, inflammatory organ injury and production of IL-1β in stimulated macrophages (44). These results suggest that in spite of the antiinflammatory effects associated with AMPKα1 activation during sterile inflammatory processes, metformin-enhanced AMPK activity does not diminish neutrophil- and macrophage-dependent bacterial killing. Of note, while AMPKα1 and AMPKα2 subunits are typically found in stromal cells, neutrophils and macrophages exclusively express the AMPKα1 subunit (40).

AMPK Activation by Metformin or through GSK3β Inhibition Decreased the Severity of Lung Injury following Polymicrobial Sepsis

Given the ability of AMPK to modulate proinflammatory activation of macrophages and neutrophils, but without adverse effects on bacterial killing, we next

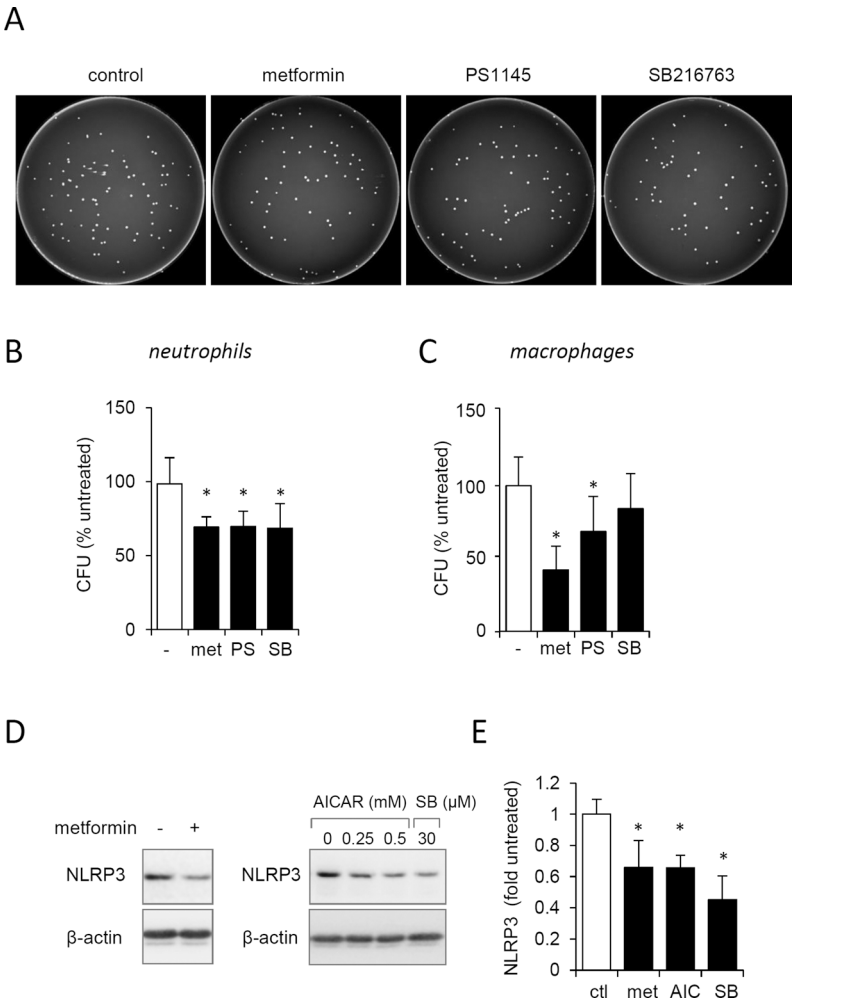

Figure 1. Metformin or IKK1/2 or GSK3β inhibitors affect bacterial killing by neutrophils or macrophages. (A,B,C) Bone marrow neutrophils or peritoneal macrophages were treated with metformin (0 or 1 mmol/L), PS1145 (0 or 10 μmol/L) or SB216763 (0 or 30 μmol/L) and then bacterial killing determined after exposure to *P. aeruginosa*. (A) Images show agar plates with bacterial colonies, an indicator of amounts surviving bacteria after coincubation of neutrophils with *P. aeruginosa*. (B and C) CFUs were calculated after treatment with (B) neutrophils or (C) macrophages with or without metformin, PS1145 or SB216763 followed by exposure to *P. aeruginosa*. Means ± SD (n = 3), *p < 0.05. (D,E,F) Western blot analysis shows the extent of NLRP3 degradation in peritoneal macrophages treated with (D) metformin (0 or 500 μmol/L), (E) AICAR (0, 0.25 or 0.5 mmol/L) or (F) SB216763 (0 or 30 μmol/L) for 8 h. Means ± SD (n = 3), *p < 0.05.

examined if AMPK activation diminishes the severity of lung injury in mice with intraabdominal sepsis. Mice were treated with either metformin (100 mg/kg; IP) or SB216763 (20 mg/kg; IP) once a day for 2 d, followed by cecal ligation and puncture (CLP). All mice subjected to CLP (50% cecum ligation and double puncture) or combined CLP and metformin survived 24 h. Neutrophil accumulation, increased lung permeability and significantly increased amounts of TNF-α, MIP-2, IL-6 and KC were found in bronchioalveolar lavages of CLP-treated mice (Figure 2). However, pretreatment with the AMPK activators metformin or SB216763 prevented such adverse effects of CLP (Figures 2A–E).

AMPK Activation Diminishes the Severity of Lung Injury following Hemorrhage and Intraabdominal Sepsis

Because the model of CLP used in our experiments resulted in modestly severe ALI and a lack of mortality at 24 h, we examined the effects of metformin-induced AMPK activation in a more severe model of sepsis produced by the combination of hemorrhage and CLP (45). In particular, mice were first subjected to hemorrhage (~60% total blood volume) followed 60 min later by resuscitation with buffered saline. CLP was performed 24 h after hemorrhage. As compared with the lack of mortality 24 h after CLP alone, hemorrhage and subsequent CLP resulted in a 30% mortality rate (Figure 3B). Pretreatment with metformin prevented mortality in hemorrhage/CLP group, and also diminished the severity of pulmonary edema and increased lung permeability, as determined by lung wet-to-dry ratios and BAL protein content (Figures 3B, C). Consistent with the antiinflammatory effects of AMPK activation, reduced amounts of TNF-α, MIP-2, IL-6 and KC were found in the BALs of mice subjected to hemorrhage/CLP and pretreated with metformin, as compared with control hemorrhage/CLP mice (Figure 3D). Western blot analysis showed accumulation in BAL fluid of high mobility group box 1 (HMGB1), an important marker and mediator of organ injury, in mice subjected to hemorrhage and CLP, and diminished levels of pulmonary HMGB1 accumulation in metformin-pretreated mice (Figure 3E).

AMPK Activation Prevents Dissipation of Mitochondrial ATP Synthase (Complex V) and Increases the Amounts of ETC Complexes III and IV in the Lungs of Septic Mice

Severe sepsis is associated with mitochondrial dysfunction in critically ill patients (18). Consistent with this, Western blot analysis of mitochondrial ETCs demonstrated a substantial decrease in

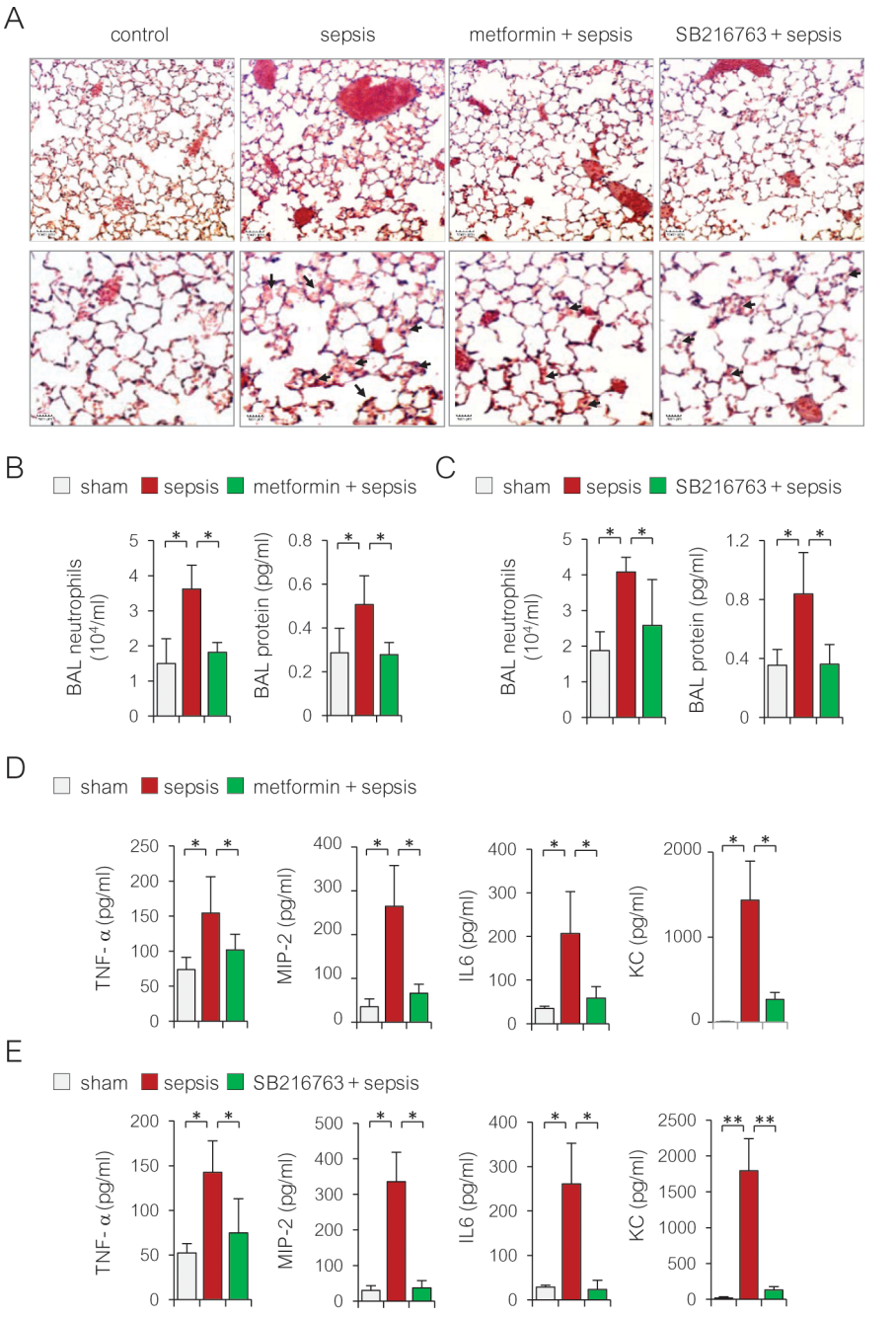

Figure 2. (A–E) The effects of AMPK activator metformin or GSK3β inhibitor SB216763 on the severity of lung inflammation in mice subjected to CLP-induced sepsis. (A) Images show lung sections obtained of control or mice treated with metformin or GSK3β inhibitor SB216763 following CLP. Bars: 1,000 μm (upper panel) or 100 μm (lower panel). Arrows indicate spaces filled with a mixed mononuclear/neutrophilic infiltrate, cellular debris and proteinaceous material. Alveolar walls are also thickened, and the septa are edematous. (B) and (C) show the ability of metformin or SB216763 to diminish the amount of BAL neutrophils and to reduce lung permeability (BAL proteins). (D and E) Metformin lowers BAL proinflammatory cytokines content, including TNF-α, MIP-2, KC and IL-6 in mice subjected to CLP and metformin or CLP and SB216763, compared with control (sham) or mice with CLP alone (means ± SD, $n = 6$ mice/group, $^{*}p < 0.05$, $^{**}p < 0.01$).

ATP synthase (complex V) in the lungs of mice with sepsis, as compared with controls (Figures 4A, B). We also observed similar decreases of ATP synthase in the lungs of mice subjected to intratracheal (i.t.) instillation of LPS (2 mg/kg, i.t.) 24 h previously (data not shown). Treatment of mice with metformin partially prevented sepsis-induced depletion of complex V and also increased the total amounts of ETC complex III and IV (Figures 4A, B).

GSK3β Regulates AMPK Activity in the Lungs of Septic Mice

Western blot analysis showed that phosphorylation of Thr172-AMPK and S79-ACC (a downstream target of AMPK) was diminished in the lungs of mice with sepsis, as compared with control animals (Figures 5A, B). In contrast, increased T172-AMPK and S79-ACC phosphorylation was found when metformin was administered before CLP. Of note, metformin increased T172-AMPK phosphorylation in mice with sepsis to levels similar to those found in control groups. In addition to the modest increases in phospho-T172-AMPK detected in the metformin/sepsis group, increased phosphorylation of S485-AMPK and T479-AMPK, events associated with inhibition of AMPK activation, were present in the lungs of septic mice pretreated with metformin (Figures 5A, 6C). These results suggest that metformin might interact with a limited pool of AMPK, and particularly AMPK that was not affected by IKKβ and GSK3β inhibitory phosphorylation of S485 and T479 in AMPK. To examine this issue, AMPK phosphorylation was determined in lung homogenates obtained from mice treated with the GSK3β inhibitor SB216763 before the induction of sepsis. Western blot analysis demonstrated that SB216763 increased phosphorylation of T172-AMPK and its downstream target S79-ACC (Figures 5D, E). Of note, exposure to SB216763 resulted in higher levels of phospho T172-AMPK in the lungs of septic mice as compared with that present in controls (Figures 5D, E). As anticipated, SB216763 diminished GSK3β-mediated

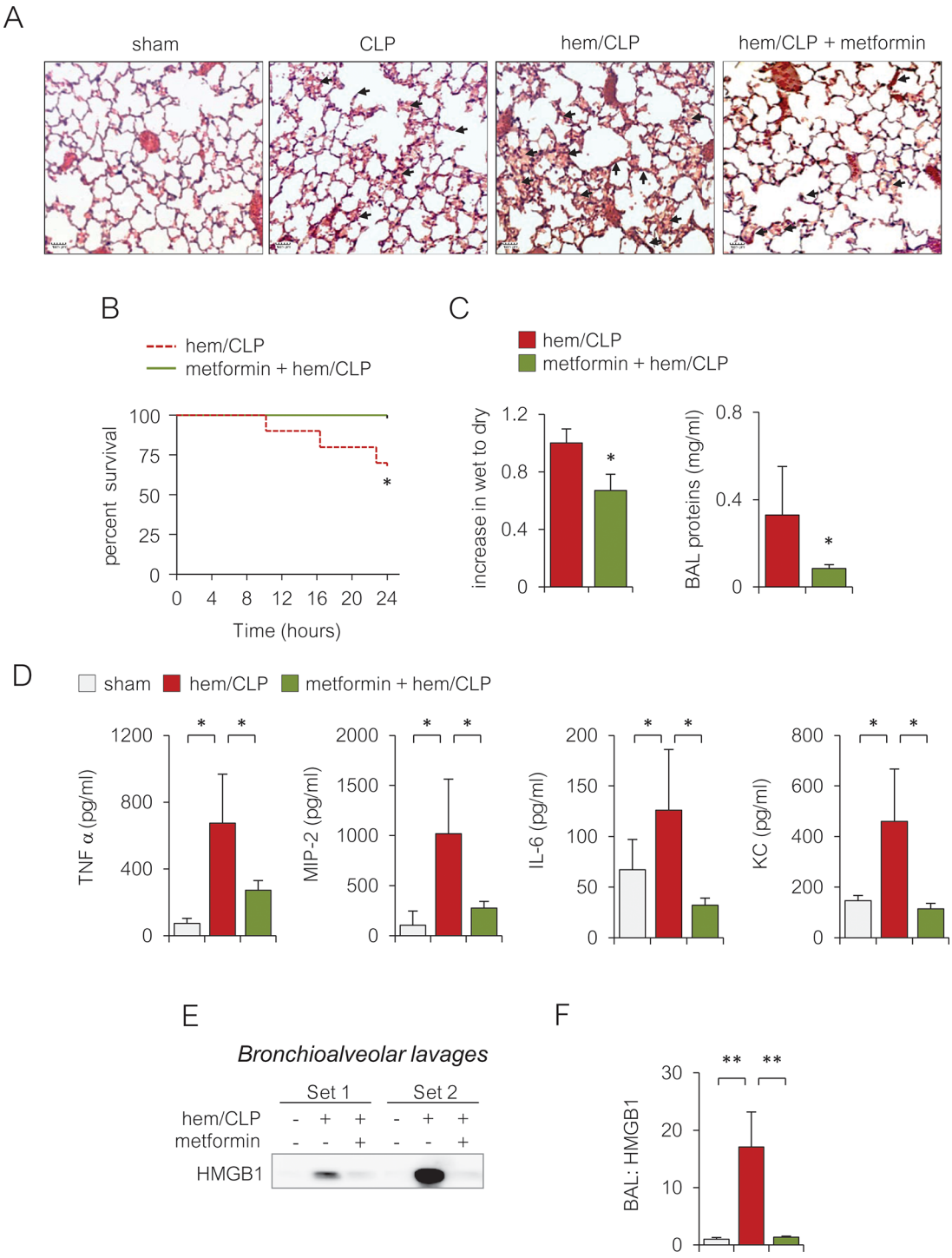

Figure 3. Metformin diminishes the severity of ALI in mice subjected to hemorrhage and CLP-induced sepsis. (A) Images show lung sections of control (sham), CLP- or hemorrhage/CLP- or metformin and hemorrhage/CLP-treated mice. Bars: 100 μm. Arrows indicate spaces filled with a mixed mononuclear/neutrophilic infiltrate, cellular debris and proteinaceous material. Alveolar walls are also thickened, and the septa are edematous. (B) Metformin increased survival rates following hemorrhage and CLP. (C) Decrease of pulmonary edema and lung permeability was evidenced by measurement of lung wet-to-dry ratios and BAL proteins in mice treated with metformin and hemorrhage/CLP, as compared with hemorrhage/CLP group. Panels (D), (E) and (F) show amounts of BAL TNF-α, MIP-2, KC, IL-6 or HMGB1 in sham, hemorrhage/CLP- or metformin and hemorrhage/CLP-treated mice (means ± SD (n = 5 mice/group), *$p < 0.05$, **$p < 0.01$).

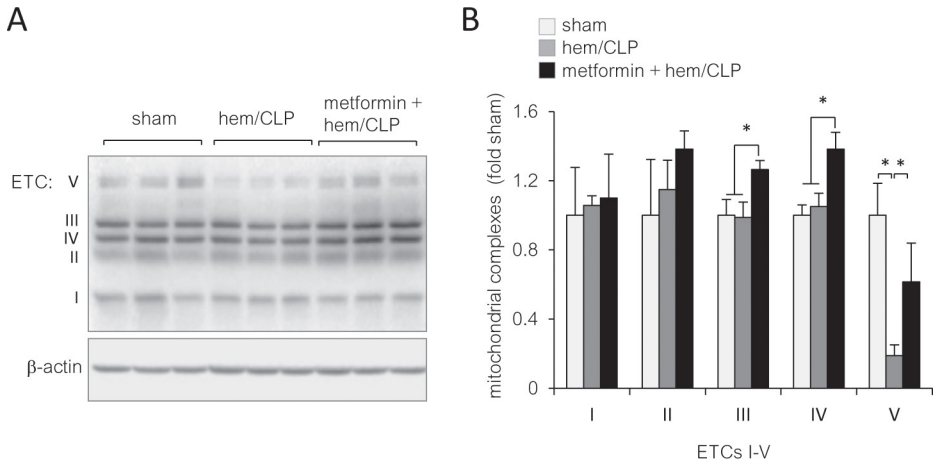

Figure 4. (A,B) Metformin prevents the loss of mitochondrial ETC complex V and increased ETC complexes III and IV in lungs of mice subjected to sepsis-induced ALI. (A) Representative Western blots and (B) quantitative data show the amount of ETC complexes I–V in control (sham), hemorrhage/CLP- or metformin and hemorrhage/CLP-treated mice (means ± SD, $n = 3$, $^*p < 0.05$). Comp. I: NDUFB8; Comp. II: 30 kDa FeS complex; Comp. III: Core protein 2; Comp. IV: subunit I; Comp. V: α subunit.

T479-AMPK phosphorylation, but had no effect on IKKβ-dependent phosphorylation of S485-AMPK (Figures 5D, F). These results suggest that metformin activates a specific AMPK pool not affected by GSK3β-dependent inhibition in the lungs of septic mice (Figure 5G).

AMPK Activation Prevents Macrophage Reprogramming into an Endotoxin Tolerogenic Phenotype

Recent studies indicate that proinflammatory stimulation, including LPS/TLR4 engagement, is associated with reprogramming of monocytes that results in diminished responses to second challenge with LPS, a condition known as endotoxin tolerance (46,47). AMPK activation has been shown to reduce proinflammatory cytokine production effectively in LPS-stimulated macrophages (38,48,49). However, it is not clear whether AMPK activation participates in the development of endotoxin tolerance. To examine this question, macrophages were first pretreated with LPS (0 or 10 ng/mL), LPS and metformin (1 mmol/L) or LPS and SB216763 (30 μmol/L per mL) for 24 h (Figure 6A). Next, the cells were washed and incubated for an additional 60 min prior to exposure to second dose of LPS (0 or 10 ng/mL). As shown in Figure 6B, robust proinflammatory activation was found in LPS-treated macrophages, whereas preexposure to LPS-induced endotoxin tolerance. In contrast, we observed more than a 50% increase of LPS-stimulated TNF-α production in macrophages that were pretreated with the combination of LPS and metformin or LPS and SB216763. These results indicate that AMPK activation inhibits the development of LPS-induced endotoxin tolerance.

AMPK Activation Diminishes HIF-1α Production in Macrophages

Recent studies have shown that enhanced expression of hypoxia-inducible factor 1α (HIF-1α) participates in sepsis-induced immunosuppression (50). Thus, we investigated whether AMPK activation affects HIF-1α accumulation in LPS-treated macrophages. As shown in Figures 6C and D, incubation of macrophages with metformin, AICAR or the GSK3β inhibitor SB216763 resulted in diminished LPS-induced accumulation of HIF-1α. These results suggest that cross-talk between AMPK and GSK3β may be implicated in regulating development of macrophage immunosuppressive phenotypes.

AMPK and GSK3β Signaling Pathways Participate in Modulating Neutrophil Chemotaxis

Innate immune activation is the host's first-line defense against microbial infection. However, sepsis-induced accumulation of inflammatory mediators and microbial products in the circulation has adverse effects on chemotaxis of neutrophils (51,52). Given the importance of mitochondria in regulating neutrophil chemotaxis (53) we used a JC-1 probe to measure if LPS affected mitochondrial membrane potential. As shown in Figures 7A and B, culture neutrophils with LPS resulted in significant mitochondrial membrane depolarization. Furthermore, inclusion of metformin or GSK3β inhibitor (BIO) prevented the effects of LPS. Of note, BIO was used because SB216763 produced substantial fluorescence and interfered with JC-1 fluorescence. Next, we examined if the AMPK and/or GSK3β signaling axis also participate(s) in modulating neutrophil migration after LPS/TLR4 engagement. Neutrophils were pretreated with the AMPK activator metformin (0 or 500 μmol/L), GSK3β inhibitors SB216763 (0 or 30 μmol/L) or BIO (0 or 5 μmol/L) for 60 min. To establish the effects LPS/nuclear factor kappa B (NF-κB) signaling, cells were also pretreated with IKK1/2 inhibitor PS1145 (0 or 10 μmol/L). Next, cells were incubated with LPS (0 or 300 ng/mL) for 60 min, washed and then added into the upper reservoir of transmigration chambers. Neutrophil migration through pore structures was initiated by inclusion of the chemoattractant W-peptide (50 nmol/L, an equivalent of human IL-8) into the lower reservoir. As shown in Figure 7C, exposure to LPS nearly completely immobilized neutrophils, whereas pretreatment with metformin, PS1145, SB216763 or BIO diminished such inhibitory effects of LPS. These results indicate

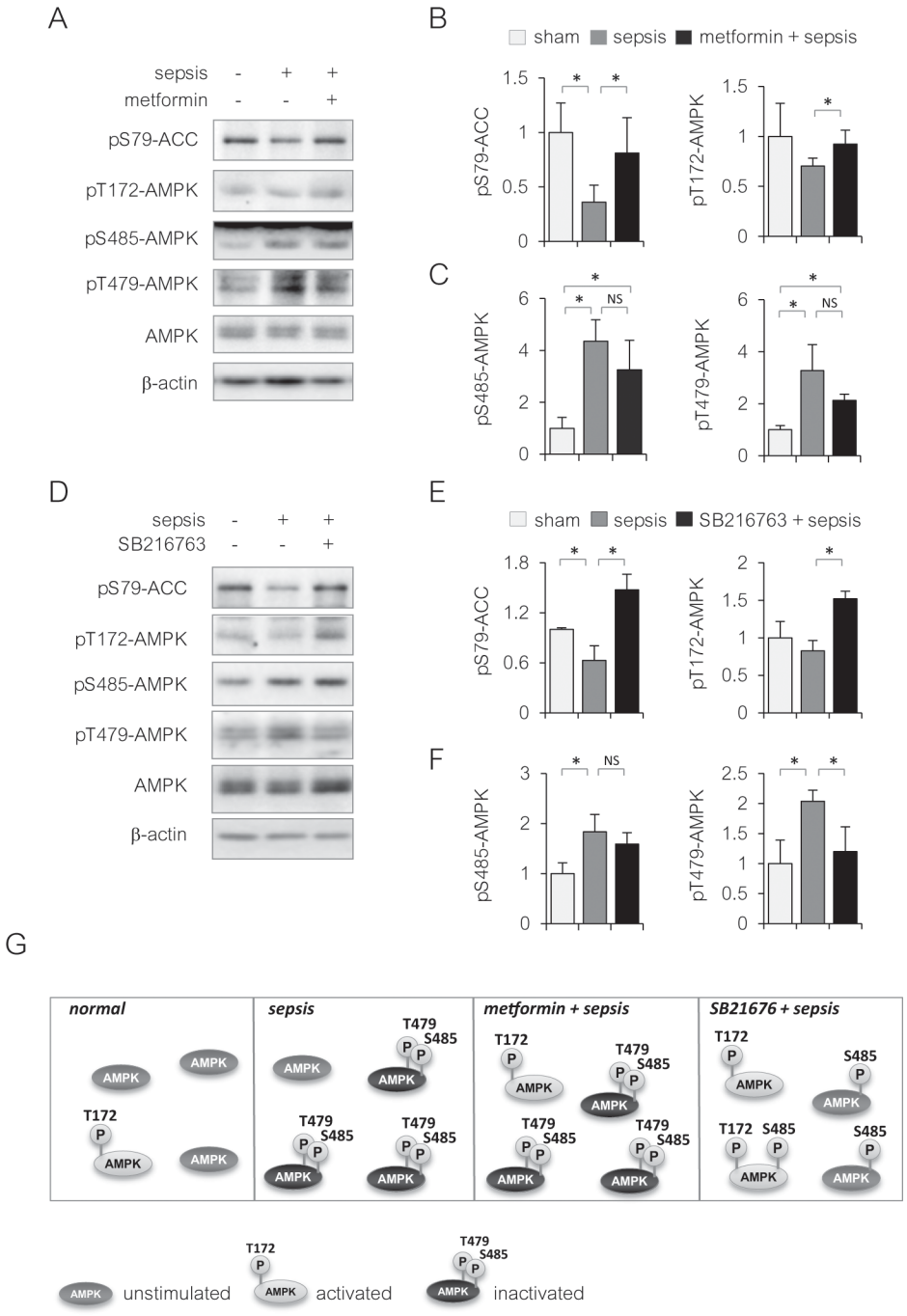

Figure 5. Metformin and GSK3β inhibitor SB216763 affect T172-AMPK phosphorylation in lungs of mice subjected to sepsis. Representative Western blots and quantitative data show the extent of pT172-AMPK, pS485-AMPK, pT479-AMPK and AMPK downstream target pS79-ACC in lung homogenates obtained from mice treated with (A,B,C) metformin or (C,D,E) GSK3β inhibitor SB216763 and then subjected to CLP-induced sepsis (means ± SD, n = 3–5, *p < 0.05, NS = not significant). (G) Cross-talk between metformin-stimulated pT172-AMPK and inhibitory IKKβ/GSK3β-induced S485/T479-AMPK phosphorylation modulates AMPK activity in lung of mice subjected to abdominal sepsis.

that both AMPK and GSK3β are involved in regulating LPS-dependent mitochondrial membrane depolarization and inhibition of neutrophil chemotaxis.

AMPK Activation Diminished the Onset of Immunosuppression in Mice with Sepsis

P. aeruginosa is frequently associated with sepsis and ventilator-associated secondary infection, including *P. aeruginosa* (16,54). Given the ability of AMPK activators to preserve neutrophil and macrophage function, we examined if metformin improves immune homeostasis and efficient killing of bacterial *in vivo*. Mice were treated with two doses of metformin (100 mg/kg; IP), given 24 h and 30 min prior to i.t. instillation of *P. aeruginosa* (PAK; 2.5 × 10^7). As shown in Figures 8A and B, there was significant decrease in CFUs in lung homogenates of PAK infected mice treated with metformin, compared with mice treated with PAK alone. Next, we determined if metformin affects bacterial clearance in the lungs of mice subjected to sepsis. As compared with control mice, CLP-induced sepsis resulted in a robust increase in CFUs (~10 fold) in lung homogenates. Metformin partially prevented such adverse effects of sepsis (Figures 8C, D). In additional experiments, we found that metformin also diminished the amounts of bacteria in samples obtained from peritoneal lavages of CLP mice (Figure 8E).

DISCUSSION

In this study, we found that cross-talk between AMPK and GSK3β was involved in regulating lung inflammation and development of lung injury in experimental models of polymicrobial abdominal sepsis or by the more severe combination of hemorrhage and abdominal sepsis. The AMPK activator metformin and the GSK3β inhibitor SB216763 prevented the decrease in neutrophil chemotaxis induced by LPS, and also enhanced the ability of neutrophils and macrophages to kill bacteria. *In vivo* treatment with metformin improved

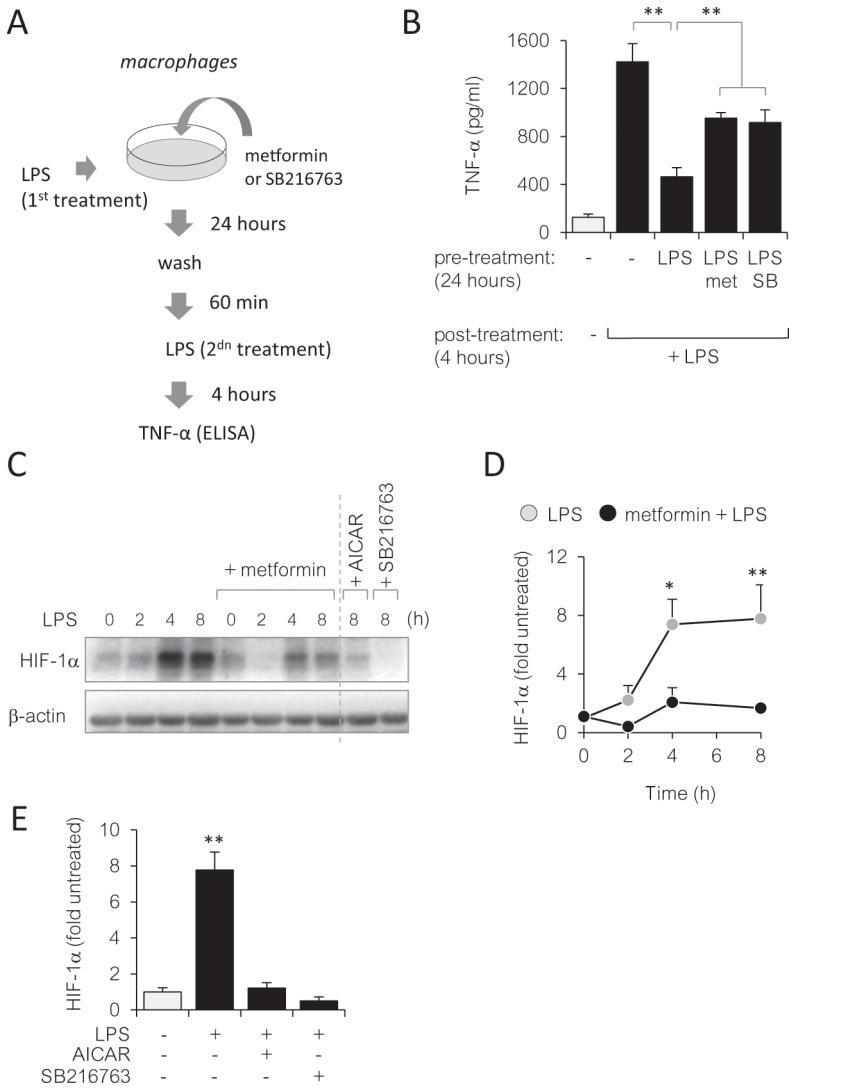

Figure 6. AMPK activation or inhibition of GSK3β diminishes development of endotoxin tolerance in LPS-treated macrophage. (A,B) Macrophages were first incubated with LPS (0 or 10 ng/mL) for 24 h. Next, cells were washed and treated with a second dose of LPS (0 or 10 ng/mL) for 4 h followed by measurement of TNF-α in culture media (ELISA). As indicated, cells were also treated with AMPK activators metformin (0 or 500 μmol/L) or GSK3β inhibitor SB216763 (0 or 30 μmol/L) for 60 min followed by inclusion of LPS (first dose). (C,D,E) AMPK activators or GSK3β inhibition prevented HIF-1α accumulation in LPS-treated macrophages. Peritoneal macrophages were treated with metformin (0 or 1 mmol/L) and LPS (30 ng/mL) for 0, 2, 4, 8 h followed by Western blot analysis of HIF-1α and β-actin. Cells were also treated with AICAR (0 or 300 μmol/L) or SB216763 (0 or 30 μmol/L) and then cultured with LPS for an additional 8 h (means ± SD, $n = 3$, $^*p < 0.05$, $^{**}p < 0.01$).

survival of mice with polymicrobial abdominal sepsis, stabilized mitochondrial complex V and increased the amounts of mitochondrial complexes III and IV. Although activated AMPK diminished production of proinflammatory mediators in LPS-treated macrophages, this event was not associated with diminished bacterial killing. Indeed, metformin or SB216763 effectively prevented development of LPS-induced macrophage immunosuppressive phenotypes. Similarly, metformin increased bacterial clearance in the lungs of mice with sepsis.

In spite of increases in AMP-to-ATP ratios and ROS formation, which normally result in AMPK activation, there was no increase in AMPK activity in the experimental models of ARDS or in critically ill patients (35–37). Recent studies demonstrated that interactions between PI3K/AKT and GSK3β as well as between IKKβ and GSK3β promoted direct phosphorylation and inactivation of AMPK, thereby suggesting potential mechanisms for the lack of AMPK activation in preclinical models of sepsis and in patients with critical illness (38,39). Such findings are consistent with the ability of GSK3β inhibitors to diminish monocyte proinflammatory activation and to reduce mortality in experimental sepsis, ischemic organ injury and endotoxin-induced lung injury (38,55,56).

Previous studies, including results obtained in our laboratory, have shown that enhanced AMPK activation diminished the severity of lung injury in experimental models of sterile inflammation, such as after exposure of the lungs to LPS or peptidoglycan (PGN) (31,32,36). Our new data indicate that AMPK activation also decreases pulmonary injury in the setting of polymicrobial sepsis. Of note, despite the inhibitory activity of AMPK activation on proinflammatory cytokine release in the lungs, bacterial clearance was increased in the lungs of septic mice treated with metformin and in mice with *Pseudomonas aeruginosa* pneumonia. It is important to note that while moderate inflammation is necessary to initiate the accumulation of immune cells to sites of infection, diminishing exaggerated and deleterious proinflammatory activation is not necessarily associated with loss of innate immune function (1,8,17). For example, a recent study has shown that mice treated with the specific NF-κB inhibitor BMS-345541 had reduced severity of lung injury following CLP-induced sepsis (57). Similarly, we found that treatment with an IKK1/2 inhibitor or activation of AMPK by metformin or GSK-3β inhibitors had

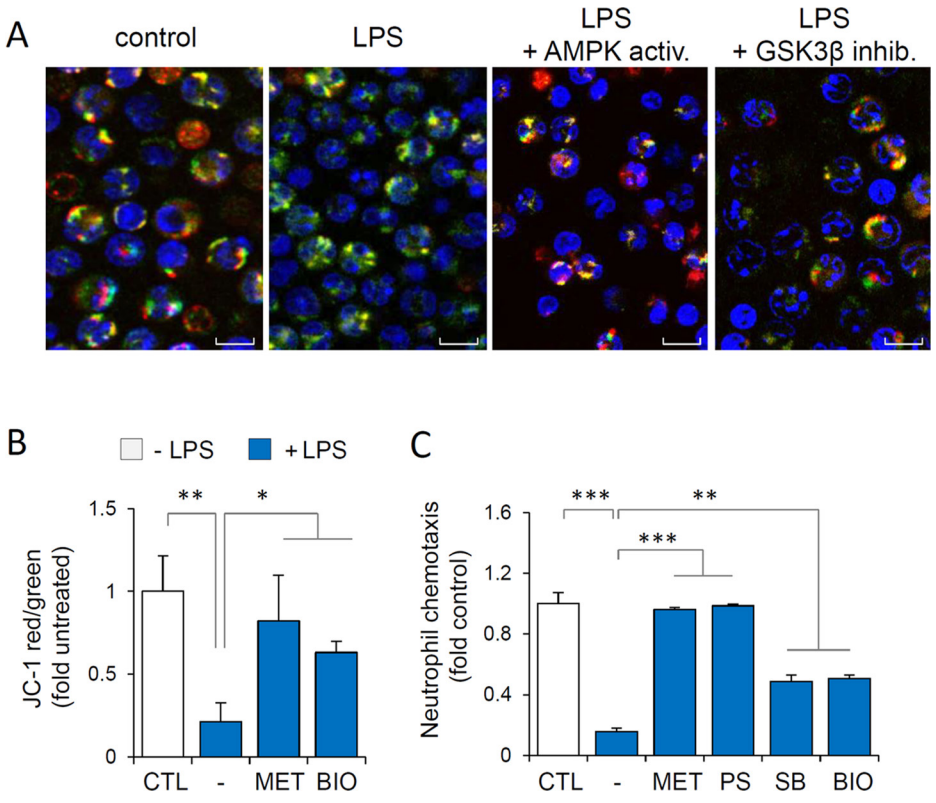

Figure 7. GSK3β inhibition and AMPK activation prevented mitochondrial membrane depolarization and chemotaxis dysfunction in LPS-treated neutrophils. Bone marrow neutrophils were pretreated with metformin (0 or 500 μmol/L) or GSK3β inhibitor (BIO; 0 or 20 μmol/L) for 60 min prior to inclusion of LPS (300 mg/mL) for an additional 60 min. (A) Representative images show JC-1 and nuclei fluorescence in control and treated neutrophils. Red-high Δψ; green-low Δψ; blue-nuclei (Bars; 2 μm). (B) Means ± SD red/green pixel intensity, $n = 5$, $^{*}p < 0.05$, $^{**}p < 0.01$. (C) Neutrophils were pretreated with AMPK activator metformin (0 or 500 μmol/L), IKK1/2 inhibitor PS1145 (0 or 10 μmol/L), or GSK3β inhibitors SB216763 (0 or 30 μmol/L) for 120 min followed by incubation with LPS (0 or 300 ng/mL) for an additional 60 min. Quantitative data show the amount of neutrophils that migrated from upper to lower reservoir of transmigrating chamber (B) Means ± SD ($n = 3$), $^{**}p < 0.01$, $^{***}p < 0.001$.

no adverse effects on bacterial killing by neutrophils or macrophages *ex vivo*. Of note, activation of AMPK has been shown to increase phagocytosis in neutrophils and macrophages (58–61).

Our results show that AMPK activation provided substantial protection against sepsis-induced lung injury. However, the exact role of AMPK that plays during recovery of immune and peripheral tissue homeostasis needs to be further examined. A possible mechanism by which AMPK activation may modulate acute inflammatory responses, such as sepsis-induced lung injury, is linked to cellular bioenergetics. Previous studies have shown that sepsis-mediated organ injury was associated with alterations in mitochondrial structure and function (19). Mitochondrial impairment in peripheral tissues and in immune cells has been correlated with morbidity and mortality associated with sepsis (18,62). For example, significant loss of ATP synthase (complex V) has been found in circulating monocytes in patients with sepsis, and is likely to participate in disrupted immune bioenergetic homeostasis (63). Our results showed that AMPK activation effectively prevented loss of ATP synthase (ETC complex V) in the lungs of septic mice. Of note, besides direct immunoregulatory action, AMPK activation is also involved in preservation of epithelial and endothelial bioenergetics and in recovery of lung tissue homeostasis, including restoration of intercellular connections (33,36). A recent study indicates that AMPK activation in the brain also diminished LPS-mediated development of sepsis-ALI, evidence for more diverse mechanism of AMPK action (64). Of note, while AMPK is an established metabolic sensor and regulator, MKK3 signaling axis has been also shown to affect mitochondrial function in sepsis/ALI (65).

Diminished macrophage and neutrophil proinflammatory activation, as well as T-cell exhaustion, is characteristic of the immunosuppression described in late sepsis (16,66). While engagement of TLR4 in macrophages and neutrophils diminishes AMPK activity (36,38), HIF-1α has been shown to promote an immunosuppressive status in monocytes during human sepsis (50). Our results indicate that AMPK activation prevented both accumulation of HIF-1α and development of endotoxin tolerance in LPS-treated macrophages. These new findings are similar to the ability of AMPK activation to inhibit HIF-1α expression in cancer cells and insulin- and IGF-1-induced expression of HIF-1α in endothelial cells (67,68).

Our data suggest that therapeutic interventions that induce AMPK activation may be beneficial in diminishing organ dysfunction, enhancing bacterial clearance and improving survival in severe polymicrobial sepsis. Although this hypothesis is primarily supported through the use of metformin as an AMPK activator, similar results were found using other AMPK activators, including GSK-3β inhibitors, as performed in the present experiments. Previous studies have suggested that metformin can be used safely in patients with critical illness, COPD or asthma (48–50). More than 50 million type 2 diabetics are taking

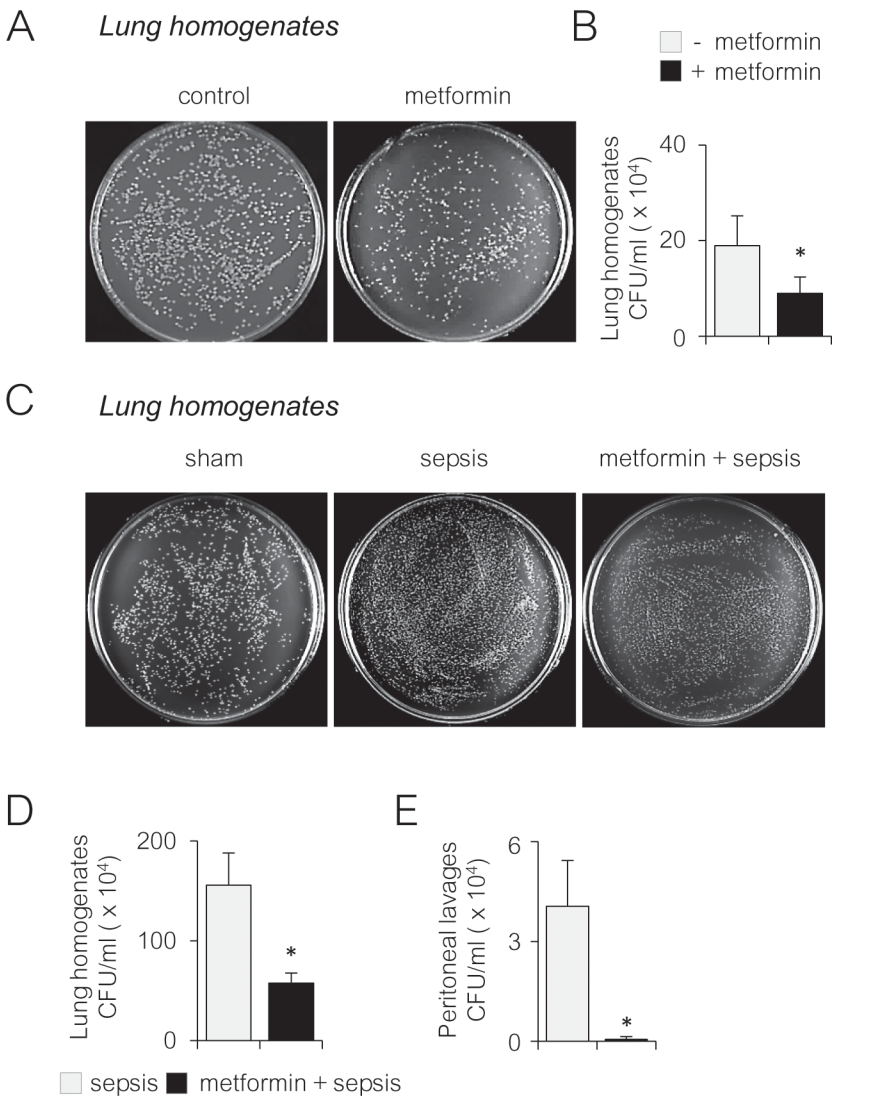

Figure 8. Metformin diminishes sepsis-mediated immunosuppression. Mice were treated with saline, metformin, CLP or metformin and CLP for 24 h followed by i.t. instillation of *P. aeruginosa* (PAK, 2.5 × 10^7/mouse) for an additional 4 h. Next, amounts of surviving bacteria were determined in lung homogenates using CFU assay. (A,C) Representative images show the amounts of bacterial colonies grown on agar plates, whereas (B,D) show CFU data obtained from lung homogenates of control, metformin, CLP, or metformin and CLP mice. Panel (E) shows the amounts of bacteria in peritoneal lavages of mice treated with CLP or a combination of metformin and CLP for 7 d (means ± SD $n = 5$, $^*p < 0.05$).

metformin daily worldwide, and recently metformin was selected for a clinical trial to evaluate its effect on human longevity (ClinicalTrials.gov NCT02432287) (69). Given the safety of metformin in many humans with diverse pathophysiologic conditions, and the suggestion that metformin may have beneficial effects in diminishing inflammation-associated organ dysfunction, it may be appropriate to consider its use in clinical trials enrolling severely ill septic patients with organ dysfunction.

ACKNOWLEDGMENTS

We thank Ken Inoki from the University of Michigan for the anti-phospho-Thr479-AMPK antibody. Funding was provided by National Institutes of Health Grant HL107585 to JW Zmijewski.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES

1. Deutschman CS, Tracey KJ. (2014) Sepsis: current dogma and new perspectives. *Immunity*. 40:463–75.
2. Angus DC, van der Poll T. (2013) Severe sepsis and septic shock. *N. Engl. J. Med*. 369:2063.
3. Hotchkiss RS, Monneret G, Payen D. (2013) Immunosuppression in sepsis: a novel understanding of the disorder and a new therapeutic approach. *Lancet Infect. Dis*. 13:260–8.
4. Lord JM, *et al*. (2014) The systemic immune response to trauma: an overview of pathophysiology and treatment. *Lancet*. 384:1455–65.
5. Wheeler AP, Bernard GR. (2007) Acute lung injury and the acute respiratory distress syndrome: a clinical review. *Lancet*. 369:1553–64.
6. Force ADT, *et al*. (2012) Acute respiratory distress syndrome: the Berlin Definition. *JAMA*. 307:2526–33.
7. Sheu CC, *et al*. (2010) Clinical characteristics and outcomes of sepsis-related vs non-sepsis-related ARDS. *Chest*. 138:559–67.
8. Fink MP, Warren HS. (2014) Strategies to improve drug development for sepsis. *Nat. Rev. Drug. Discov*. 13:741–58.
9. Standiford TJ, Ward PA. (2016) Therapeutic targeting of acute lung injury and acute respiratory distress syndrome. *Transl. Res*. 167:183–91.
10. Flierl MA, *et al*. (2008) Adverse functions of IL-17A in experimental sepsis. *FASEB J*. 22:2198–205.
11. Bosmann M, Ward PA. (2012) Therapeutic potential of targeting IL-17 and IL-23 in sepsis. *Clin. Transl. Med*. 1:4.
12. Mariathasan S, *et al*. (2004) Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature*. 430:213–8.
13. Brown KA, *et al*. (2006) Neutrophils in development of multiple organ failure in sepsis. *Lancet*. 368:157–69.
14. Kolaczkowska E, Kubes P. (2013) Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol*. 13:159–75.
15. Abraham E. (2003) Neutrophils and acute lung injury. *Crit. Care Med*. 31:S195–9.
16. Hotchkiss RS, Monneret G, Payen D. (2013) Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. *Nat. Rev. Immunol*. 13:862–74.
17. Gentile LF, *et al*. (2012) Persistent inflammation and immunosuppression: a common syndrome and new horizon for surgical intensive care. *J. Trauma Acute Care Surg*. 72:1491–501.

18. Singer M. (2014) The role of mitochondrial dysfunction in sepsis-induced multi-organ failure. *Virulence*. 5:66–72.
19. Brealey D, *et al*. (2002) Association between mitochondrial dysfunction and severity and outcome of septic shock. *Lancet*. 360:219–23.
20. Singer M. (2007) Mitochondrial function in sepsis: acute phase versus multiple organ failure. *Crit*. *Care Med*. 35:S441–8.
21. Srivastava A, *et al*. (2015) MKK3 deletion improves mitochondrial quality. *Free Radic*. *Biol*. *Med*. 87:373–84.
22. Matthay MA. (2015) Therapeutic potential of mesenchymal stromal cells for acute respiratory distress syndrome. *Ann*. *Am*. *Thorac*. *Soc*. 12 Suppl 1:S54–7.
23. Rocha M, Herance R, Rovira S, Hernandez-Mijares A, Victor VM. (2012) Mitochondrial dysfunction and antioxidant therapy in sepsis. *Infect*. *Disord*. *Drug Targets*. 12:161–78.
24. Hardie DG, Ross FA, Hawley SA. (2012) AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat*. *Rev*. *Mol*. *Cell*. *Biol*. 13:251–62.
25. Eid AA, Lee DY, Roman LJ, Khazim K, Gorin Y. (2013) Sestrin 2 and AMPK connect hyperglycemia to Nox4-dependent endothelial nitric oxide synthase uncoupling and matrix protein expression. *Mol*. *Cell*. *Biol*. 33:3439–60.
26. Colombo SL, Moncada S. (2009) AMPKalpha1 regulates the antioxidant status of vascular endothelial cells. *Biochem*. *J*. 421:163–9.
27. Chen L, *et al*. (2009) Structural insight into the autoinhibition mechanism of AMP-activated protein kinase. *Nature*. 459:1146–9.
28. Woods A, *et al*. (2003) Identification of phosphorylation sites in AMP-activated protein kinase (AMPK) for upstream AMPK kinases and study of their roles by site-directed mutagenesis. *J*. *Biol*. *Chem*. 278:28434–42.
29. Martin-Montalvo A, *et al*. (2013) Metformin improves healthspan and lifespan in mice. *Nat*. *Commun*. 4:2192.
30. Bannister CA, *et al*. (2014) Can people with type 2 diabetes live longer than those without? A comparison of mortality in people initiated with metformin or sulphonylurea monotherapy and matched, non-diabetic controls. *Diabetes Obes*. *Metab*. 16:1165–73.
31. Zhao X, *et al*. (2008) Activation of AMPK attenuates neutrophil proinflammatory activity and decreases the severity of acute lung injury. *Am*. *J*. *Physiol*. *Lung Cell*. *Mol*. *Physiol*. 295:L497–504.
32. Zmijewski JW, *et al*. (2008) Mitochondrial respiratory complex I regulates neutrophil activation and severity of lung injury. *Am*. *J*. *Respir*. *Crit*. *Care Med*. 178:168–79.
33. Jian MY, Alexeyev MF, Wolkowicz PE, Zmijewski JW, Creighton JR. (2013) Metformin-stimulated AMPK-α1 promotes microvascular repair in acute lung injury. *Am*. *J*. *Physiol*. *Lung Cell*. *Mol*. *Physiol*. 305:L844–55.
34. Jiang S, *et al*. (2014) Human resistin promotes neutrophil proinflammatory activation and neutrophil extracellular trap formation and increases severity of acute lung injury. *J*. *Immunol*. 192:4795–803.
35. Tadie JM, *et al*. (2012) Toll-like receptor 4 engagement inhibits adenosine 5′-monophosphate-activated protein kinase activation through a high mobility group box 1 protein-dependent mechanism. *Mol*. *Med*. 18:659–68.
36. Xing J, *et al*. (2013) Inhibition of AMP-activated protein kinase accentuates lipopolysaccharide-induced lung endothelial barrier dysfunction and lung injury in vivo. *Am*. *J*. *Pathol*. 182:1021–30.
37. Meares GP, Qin H, Liu Y, Holdbrooks AT, Benveniste EN. (2013) AMP-activated protein kinase restricts IFN-gamma signaling. *J*. *Immunol*. 190:372–80.
38. Park DW, *et al*. (2014) GSK3β-dependent inhibition of AMPK potentiates activation of neutrophils and macrophages and enhances severity of acute lung injury. *Am*. *J*. *Physiol*. *Lung Cell*. *Mol*. *Physiol*. 307:L735–45.
39. Suzuki T, *et al*. (2013) Inhibition of AMPK catabolic action by GSK3. *Mol*. *Cell* 50:407–19.
40. Jiang S, *et al*. (2013) Mitochondria and AMP-activated protein kinase-dependent mechanism of efferocytosis. *J*. *Biol*. *Chem*. 288:26013–26.
41. Rittirsch D, Huber-Lang MS, Flierl MA, Ward PA. (2009) Immunodesign of experimental sepsis by cecal ligation and puncture. *Nat*. *Protoc*. 4:31–6.
42. Rodriguez C, *et al*. (2009) Sodium nitrite therapy attenuates the hypertensive effects of HBOC-201 via nitrite reduction. *Biochem*. *J*. 422:423–32.
43. Zmijewski JW, *et al*. (2009) Antiinflammatory effects of hydrogen peroxide in neutrophil activation and acute lung injury. *Am*. *J*. *Respir*. *Crit*. *Care Med*. 179:694–704.
44. Latz E, Xiao TS, Stutz A. (2013) Activation and regulation of the inflammasomes. *Nat*. *Rev*. *Immunol*. 13:397–411.
45. Ayala A, *et al*. (2002) Shock-induced neutrophil mediated priming for acute lung injury in mice: divergent effects of TLR-4 and TLR-4/FasL deficiency. *Am*. *J*. *Pathol*. 161:2283–94.
46. LaRue KE, McCall CE. (1994) A labile transcriptional repressor modulates endotoxin tolerance. *J*. *Exp*. *Med*. 180:2269–75.
47. McClure C, Brudecki L, Yao ZQ, McCall CE, El Gazzar M. (2015) Processing body formation limits proinflammatory cytokine synthesis in endotoxin-tolerant monocytes and murine septic macrophages. *J*. *Innate Immun*. 7:572–83.
48. Sag D, Carling D, Stout RD, Suttles J. (2008) Adenosine 5′-monophosphate-activated protein kinase promotes macrophage polarization to an anti-inflammatory functional phenotype. *J*. *Immunol*. 181:8633–41.
49. Jeong HW, *et al*. (2009) Berberine suppresses proinflammatory responses through AMPK activation in macrophages. *Am*. *J*. *Physiol*. *Endocrinol*. *Metab*. 296:E955–64.
50. Shalova IN, *et al*. (2015) Human monocytes undergo functional re-programming during sepsis mediated by hypoxia-inducible factor-1α. *Immunity*. 42:484–98.
51. van der Poll T, Opal SM. (2008) Host-pathogen interactions in sepsis. *Lancet Infect*. *Dis*. 8:32–43.
52. Tavares-Murta BM, *et al*. (2002) Failure of neutrophil chemotactic function in septic patients. *Crit*. *Care Med*. 30:1056–61.
53. Fossati G, *et al*. (2003) The mitochondrial network of human neutrophils: role in chemotaxis, phagocytosis, respiratory burst activation, and commitment to apoptosis. *J*. *Immunol*. 170: 1964–72.
54. Barbier F, Andremont A, Wolff M, Bouadma L. (2013) Hospital-acquired pneumonia and ventilator-associated pneumonia: recent advances in epidemiology and management. *Curr*. *Opin*. *Pulm*. *Med*. 19:216–28.
55. Martin M, Rehani K, Jope RS, Michalek SM. (2005) Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. *Nat*. *Immunol*. 6:777–84.
56. Rocha J, *et al*. (2015) Inhibition of glycogen synthase kinase-3β attenuates organ injury and dysfunction associated with liver ischemia-reperfusion and thermal injury in the rat. *Shock*. 43:369–78.
57. Li H, *et al*. (2013) NF-κB inhibition after cecal ligation and puncture reduces sepsis-associated lung injury without altering bacterial host defense. *Mediators Inflamm*. 2013:503213.
58. Park DW, *et al*. (2013) Activation of AMPK enhances neutrophil chemotaxis and bacterial killing. *Mol*. *Med*. 19:387–98.
59. Bae HB, *et al*. (2011) AMP-activated protein kinase enhances the phagocytic ability of macrophages and neutrophils. *FASEB J*. 25: 4358–68.
60. Mounier R, *et al*. (2013) AMPKα1 regulates macrophage skewing at the time of resolution of inflammation during skeletal muscle regeneration. *Cell Metab*. 18:251–64.
61. Labuzek K, Liber S, Gabryel B, Adamczyk J, Okopien B. Metformin increases phagocytosis and acidifies lysosomal/endosomal compartments in AMPK-dependent manner in rat primary microglia. *Naunyn Schmiedebergs Arch*. *Pharmacol*. 381:171–86.
62. Zolfaghari PS, *et al*. (2015) Skeletal muscle dysfunction is associated with derangements in mitochondrial bioenergetics (but not UCP3) in a rodent model of sepsis. *Am*. *J*. *Physiol*. *Endocrinol*. *Metab*. 308:E713–25.
63. Japiassu AM, *et al*. (2011) Bioenergetic failure of human peripheral blood monocytes in patients with septic shock is mediated by reduced F1Fo adenosine-5′-triphosphate synthase activity. *Crit*. *Care Med*. 39:1056–63.
64. Mulchandani N, *et al*. (2015) Stimulation of brain AMP-activated protein kinase attenuates inflammation and acute lung injury in sepsis. *Mol*. *Med*. 21:637–44.

65. Mannam P, *et al*. (2014) MKK3 regulates mitochondrial biogenesis and mitophagy in sepsis-induced lung injury. *Am. J. Physiol. Lung Cell. Mol. Physiol*. 306:L604–19.
66. Ward PA. (2011) Immunosuppression in sepsis. *JAMA*. 306:2618–9.
67. Faubert B, *et al*. (2013) AMPK is a negative regulator of the Warburg effect and suppresses tumor growth in vivo. *Cell Metab*. 17:113–24.
68. Treins C, Murdaca J, Van Obberghen E, Giorgetti-Peraldi S. (2006) AMPK activation inhibits the expression of HIF-1alpha induced by insulin and IGF-1. *Biochem. Biophys. Res. Commun*. 342:1197–202.
69. Metformin in Longevity Study (MILES) [Internet]. (2016) [updated 2015 Dec 8; cited 2016 Jan 29]. Available from: https://clinicaltrials.gov/ct2/show/record/NCT02432287 ClinicalTrials.gov identifier: NCT02432287.

Cite this article as: Liu Z, *et al*. (2015) AMP-activated protein kinase and glycogen synthase kinase 3β modulate the severity of sepsis-induced lung injury. *Mol. Med*. 21:937–50.