

# Toll-Like Receptor 4 Engagement Inhibits Adenosine 5'-Monophosphate-Activated Protein Kinase Activation through a High Mobility Group Box 1 Protein-Dependent Mechanism

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Despite the potent antiinflammatory effects of pharmacologically induced adenosine 5'-monophosphate kinase (AMPK) activation on Toll-like receptor 4 (TLR4)-induced cellular activation, there is little evidence that AMPK is activated during inflammatory conditions. In the present studies, we examined mechanisms by which TLR4 engagement may affect the ability of AMPK to become activated in neutrophils and macrophages under *in vitro* conditions and in the lungs during lipopolysaccharide (LPS)-induced acute lung injury. We found that incubation of neutrophils or macrophages with LPS diminished the ability of 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside (AICAR) or hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to activate AMPK. Although ratios of AMP to adenosine 5'-triphosphate (ATP) were increased in LPS-treated neutrophils and in the lungs of LPS exposed mice, a condition that should result in AMPK activation, no activation of AMPK was found. Immunocytochemistry and Western blot analysis revealed that nuclear to cytosolic translocation of the proinflammatory mediator high mobility group box 1 protein (HMGB1) correlated with inhibition of AMPK activation in LPS-stimulated macrophages. Moreover, while induced overexpression of HMGB1 resulted in inhibition of AMPK activation, Small interfering RNA (siRNA)-induced knockdown of HMGB1 was associated with enhanced activation of AMPK in macrophages incubated with AICAR. Increased interaction between liver kinase B1 (LKB1), an upstream activator of AMPK, and HMGB1 was found in LPS-stimulated macrophages and in the lungs of mice exposed to LPS. These results suggest that nuclear to cytoplasmic translocation of HMGB1 in TLR4-activated cells potentiates inflammatory responses by binding to LKB1, thereby inhibiting the antiinflammatory effects of AMPK activation.

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## INTRODUCTION

Adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) is a heterotrimeric serine/threonine kinase con-

sisting of a catalytic  $\alpha$  subunit and  $\beta$  and  $\gamma$  regulatory subunits. All three subunits of AMPK are necessary for the formation of a fully active complex (1,2). Classi-

cally, activation of AMPK has been described to occur under conditions of cellular stress that affect the balance between cellular adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP) and AMP and involves direct binding of AMP and ADP to the AMPK  $\gamma$  subunit, which then results in phosphorylation of Thr172 within the AMPK  $\alpha$  activating loop (3–8). Recent studies have shown that exposure of cells to reactive oxygen species or glycogen can induce AMPK activation independently of changes in cellular ATP-to-AMP ratios (9,10).

Although AMPK has primarily been characterized as a major regulator of

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metabolism, recent studies have shown that AMPK activation also has potent antiinflammatory effects in multiple cell populations, including neutrophils, macrophages and endothelial cells (11–15). For example, AMPK suppressed production of nuclear factor (NF)- $\kappa$ B-dependent cytokines in TLR4-stimulated cells (11,14). Treatment of mice with 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside (AICAR) or metformin, two inducers of AMPK activation, reduced the severity of lipopolysaccharide (LPS)-induced acute inflammatory lung injury (11,16). However, there is little evidence that AMPK is activated in inflammatory states, such as acute lung injury, despite the presence of conditions including increased release of reactive oxygen species and diminished generation of ATP, which would be expected to result in AMPK activation (9,17–19). Therefore, a potentially important, and presently unanswered question, relates to the mechanisms that may prevent activation of AMPK in such conditions.

In the present experiments, we explored potential mechanisms by which induction of cellular activation through TLR4 may modulate AMPK activation. We found that engagement of TLR4 inhibited activation of AMPK and also resulted in increased cytoplasmic interactions between high mobility group box 1 protein (HMGB1) and liver kinase B1 (LKB1), a kinase directly upstream to AMPK, in isolated cell populations and under *in vivo* conditions in the lungs of LPS-treated mice. Overexpression of HMGB1 suppressed AMPK activation, whereas the opposite effect was observed in cells in which HMGB1 was knocked down with small interfering RNA (siRNA). These findings provide new insights into mechanisms by which AMPK activation is regulated during inflammatory responses.

## MATERIALS AND METHODS

### Mice

Male C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD, USA). Male mice, 8–12 wks

old, were used for experiments. The mice were kept on a 12:12-hour light–dark cycle with free access to food and water. All experiments were conducted in accordance with institutional review board–approved protocols (University of Alabama at Birmingham Institutional Animal Care and Use Committee).

### Reagents

RPMI 1640 was purchased from BioWhittaker (Walkersville, MD, USA). Fetal bovine serum (FBS) and penicillin–streptomycin were obtained from Gemini Bioproducts (Calabasas, CA, USA). AICAR was purchased from Enzo Life Science (Plymouth Meeting, PA, USA). Antibodies against total and phosphorylated Thr172-AMPK and Ser79-acetyl-CoA carboxylase (ACC) and total LKB1 were purchased from Cell Signaling Technology (Beverly, MA, USA), whereas antibody to calcium/calmodulin-dependent protein kinase kinase (CaMKK) was purchased from Abcam (Cambridge, MA, USA). Custom antibody mixtures and negative selection columns for neutrophil isolation were purchased from Stem Cell Technologies (Vancouver, British Columbia, Canada). Culture medium, scrambled siRNA and siRNA to the HMGB1 were purchased from Thermo Scientific/Dharmacon (St. Louis, MO, USA). ANTI-FLAG<sup>®</sup> M2 monoclonal antibody and affinity agarose, tubulin and histone deacetylase (HDAC) antibodies and hydrogen peroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Alexa Fluor 594–conjugated phalloidin and Alexa Fluor 488–labeled secondary antibodies were purchased from Invitrogen (Carlsbad, CA, USA). Mouse monoclonal anti-HMGB1 antibody was obtained from R&D Systems (Minneapolis, MN, USA).

### Neutrophil and Macrophage Isolation and Culture

Bone marrow neutrophils were purified using a negative selection column purification system, as previously described (16,20). Briefly, bone marrow cell suspensions were isolated from the femur and tibia of a mouse by flushing with

RPMI 1640 medium with 5% FBS. The cell suspension was passed through a glass wool column and collected by subsequent washing with phosphate-buffered saline (PBS) containing 5% FBS. Negative selection to purify neutrophils was performed by incubation of the cell suspension with biotinylated primary antibodies specific for the cell surface markers F4/80, CD4, CD45R, CD5 and TER119 (StemCell Technologies, Vancouver, BC, Canada; <http://www.stemcell.com>) for 15 min at 4°C followed by incubation with anti-biotin tetrameric antibodies (100  $\mu$ L; StemCell Technologies) for 15 min. The complex of anti-tetrameric antibodies and cells was then incubated with colloidal magnetic dextran iron particles (60  $\mu$ L; StemCell Technologies) for an additional 15 min at 4°C. The T cells, B cells, red blood cells, 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. Viability of purified bone marrow neutrophils was determined after trypan blue staining and was consistently >95%. In selected experiments, neutrophil viability was also determined using annexin/prodium iodide (PI) staining and flow cytometry. Peritoneal macrophages were elicited in 8- to 10-wk-old mice using Brewer thioglycollate (24). Cells were collected 5 d after intraperitoneal injection of thioglycollate. Macrophages were then cultured in 12-well plates ( $10^5$  cells/well) in RPMI 1640 medium containing 10% FBS at 37°C. Nonadherent cells were removed by washing cells after 1 h of culture.

### Expression of HMGB1-FLAG in MCF7 Cells

A human breast adenocarcinoma cell line (MCF7) cell line that inducibly expresses HMGB1-FLAG (MCF7-HMGB1-FLAG) was generated as described previously (21). The MCF7-HMGB1-FLAG cell line was maintained in RPMI 1640 media, supplemented with 10% FBS (Atlanta Biologicals), penicillin (100 U/mL) and strep-

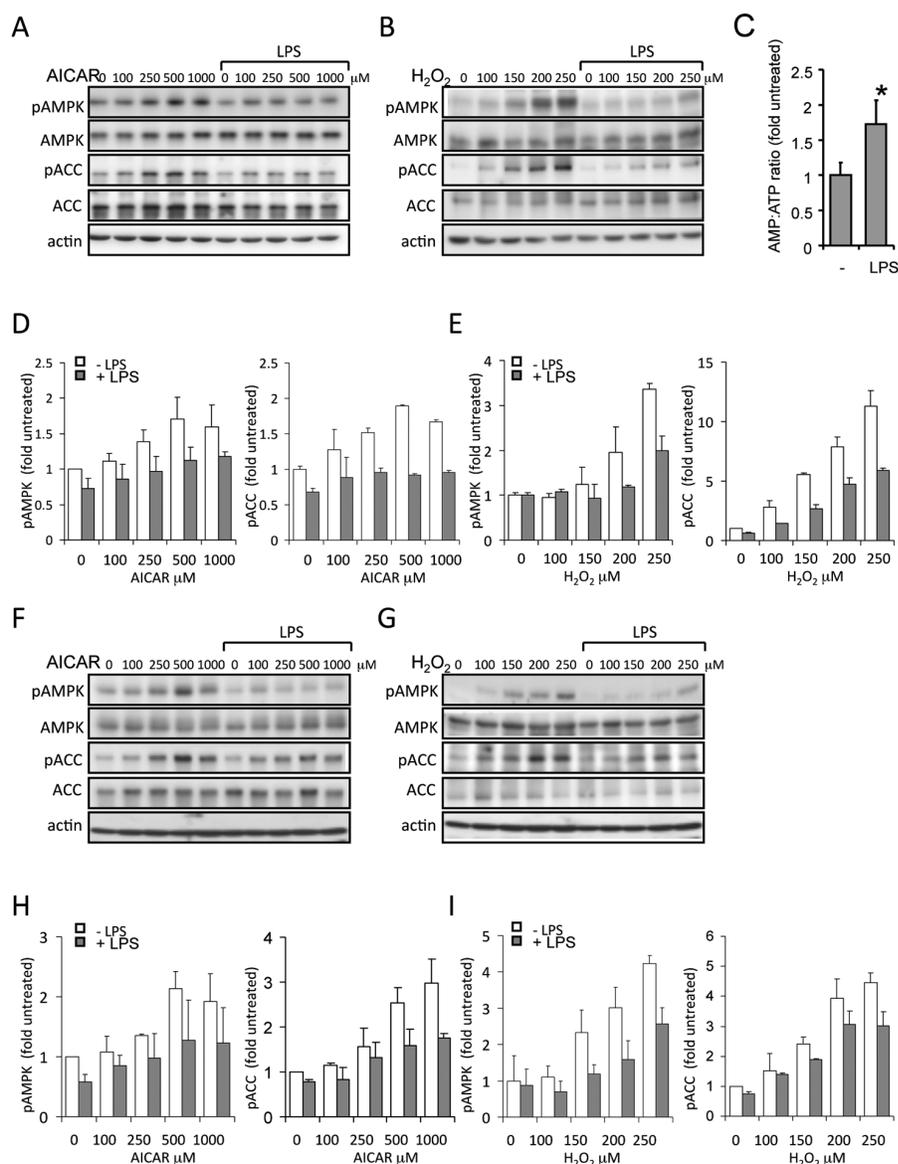
tomycin (100 ng/mL) at 37°C, 5% CO<sub>2</sub>. To induce expression of HMGB1-FLAG, ~80% confluent cells grown in 12-well plates were incubated with doxycycline (1 µg/mL) for 24 h. The amount of expressed HMGB1-FLAG was then determined using Western blot analysis.

### Western Blot Analysis

Western blot analysis was performed as previously described (9,22). Briefly, cell lysates of neutrophils ( $3.5 \times 10^6$ /well), macrophages ( $2 \times 10^5$ /well) or MCF7 cells ( $10^5$ /well) were prepared using lysis buffer containing Tris pH 7.4 (50 mmol/L), NaCl (150 mmol/L), Nonidet P40 (NP-40) (0.5%, vol/vol), ethylenediaminetetraacetic acid (EDTA) (1 mmol/L), ethyleneglycotetraacetic acid (EGTA) (1 mmol/L), okadaic acid (1 nmol/L) and protease inhibitors. Lysis buffer was also used to prepare lung homogenates, as described previously (11). Cell lysates or lung homogenates were sonicated and then centrifuged at 10,000g for 15 min at 4°C to remove insoluble material. The protein concentration in the supernatants was determined using the Bradford reagent (Bio-Rad) with bovine serum acid (BSA) as a standard. Samples were mixed with Laemmli sample buffer and boiled for 5 min. Equal amounts of proteins (50 µg/sample) were resolved by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Immobilon P; Millipore, Billerica, MA, USA). The membranes were probed with specific antibodies as described in the figure legends followed by detection with horseradish peroxidase–conjugated goat anti-rabbit IgG. Bands were visualized by enhanced chemiluminescence (Super Signal; Pierce Biotechnology, Rockford, IL, USA) and quantified by AlphaEaseFC software (Alpha Innotech, San Leandro, CA, USA). Each experiment was carried out two or more times using cell populations obtained from separate groups of mice.

### siRNA Knockdown of HMGB1

Murine macrophagelike RAW 264.7 cells were incubated with 2 µmol/L scrambled



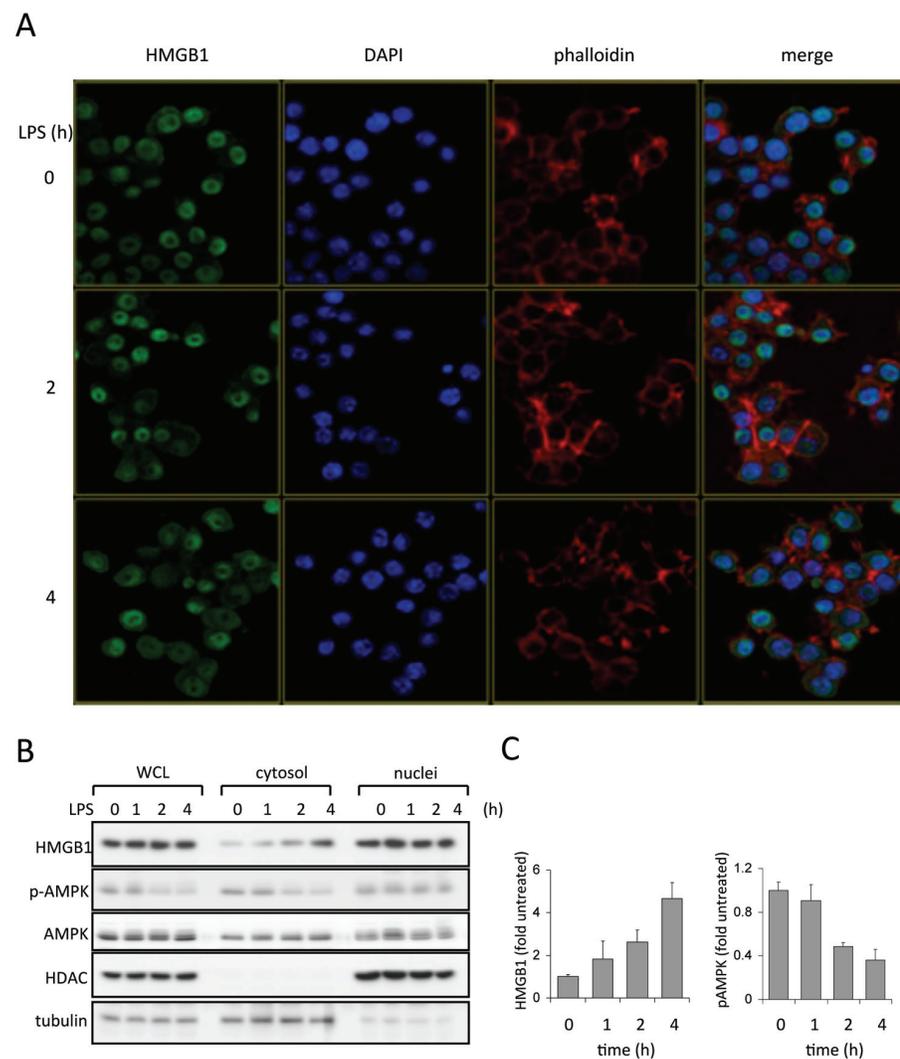
**Figure 1.** TLR4 engagement diminishes AMPK activation. Bone marrow neutrophils (A, B, D and E) or RAW 274.7 macrophages (F, G, H and I) were treated with LPS (0 or 100 ng/mL) for 4 h followed by culture with AICAR (0, 100, 250, 500 or 1,000 µmol/L) for an additional 90 min or H<sub>2</sub>O<sub>2</sub> (0, 100, 150, 200 or 250 µmol/L) for 45 min, as indicated. Representative Western blots and mean optical densitometry show amount of AMPK, phospho-Thr172AMPK $\alpha$ , ACC, phospho-Ser79ACC and actin. Data are means  $\pm$  standard deviation (SD). Two independent experiments were performed. (C) Average of AMP-to-ATP ratio in neutrophils cultured with or without LPS (100 ng/mL) for 4 h (means  $\pm$  SD,  $n = 4$  independent experiments,  $*P < 0.05$ ).

siRNA or siRNA specific to HMGB1 as described previously (23,24). Briefly, cells ( $3 \times 10^5$ /well) in 24-well plates were incubated in Accell media with scrambled siRNA (2 µmol/L) or siRNA (2 µmol/L) to HMGB1 for 72 h. Cells were treated as described in the figure legends and then sub-

jected to immunoprecipitation and Western blot analysis as indicated.

### Imaging HMGB1 in Macrophages

Cells were incubated with 4% paraformaldehyde in PBS for 20 min at room temperature and then washed with PBS



**Figure 2.** Translocation of HMGB1 from nucleus to cytosol is associated with diminished AMPK activation in LPS-stimulated macrophages. (A) Representative images show time-dependent accumulation of HMGB1 in the cytosol after culture of RAW 274.7 macrophages with LPS (100 ng/mL) for 0, 2 or 4 h. (B) Western blots show amounts of HMGB1, phospho- and total AMPK, and tubulin or HDAC in whole cell extracts as well as cytosolic or nuclear fractions obtained from RAW 264.7 macrophages incubated with LPS (100 ng/mL) for 0, 1, 2 or 4 h. (C) Mean band optical density of phospho-AMPK/AMPK and total HMGB1 in cytosolic fractions of macrophages treated as described in (B). Data are means  $\pm$  SD. Two independent experiments provided similar results.

and permeabilized with 0.1% TritonX-100/PBS for 4 min. The cells were then washed and incubated with 3% BSA in PBS for 45 min, followed by addition of anti-HMGB1 antibody overnight at 4°C. Next, cells were washed with PBS and then incubated with Alexa Fluor 488-labeled secondary antibody and Alexa Fluor 594-conjugated phalloidin for 90

and 20 min, respectively, at room temperature. After washing with PBS, the cells were mounted with emulsion oil solution containing 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei. Confocal microscopy was performed as previously described (24) using a confocal laser scanning microscope (model LSM 710 confocal microscope; Carl Zeiss MicroImaging,

Jena, Germany) provided by the High-Resolution Imaging Facility at the University of Alabama at Birmingham.

### Purification of Cytosolic and Nuclear Fractions

Separation of nuclear and cytosolic fractions was carried out as described previously (25). The cells were washed with PBS, and 500  $\mu$ L lysis buffer was added (10 mmol/L Tris, pH 7.5, 10 mmol/L NaCl, 3 mmol/L  $MgCl_2$ , 0.05% NP-40, 1 mmol/L EGTA, 1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride and protease inhibitors) was added. Lysed cells were centrifuged (2,700g) for 10 min at 4°C. The cytosol-containing supernatants were then centrifuged at 20,800g for an additional 15 min at 4°C to obtain the cytosolic fraction. The pellet-containing nuclei was washed three times (wash buffer: 10 mmol/L PIPES, pH 6.8, 300 mmol/L sucrose, 3 mmol/L  $MgCl_2$ , 1 mmol/L EGTA, 25 mmol/L NaCl, 1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride and protease inhibitors) and centrifuged at 2,700g for 5 min at 4°C. The nuclear pellet was suspended in 100  $\mu$ L wash buffer, layered over a cushion of 1 mL sucrose buffer (1 mol/L sucrose, 1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride and protease inhibitors) and centrifuged at 2,700g for 10 min. The pellet-containing nuclei were washed with 100  $\mu$ L wash buffer and centrifuged at 2,700g for 5 min at 4°C to remove residual sucrose buffer. Nuclear proteins were extracted by incubation of purified nuclei with lysis buffer for 30 min at 4°C. To remove insoluble material, the extract was centrifuged at 16,000g for 10 min at 4°C. Western blot analyses of subcellular fractions were performed using 60  $\mu$ g protein obtained from cytosolic and 5  $\mu$ g protein from nuclear fractions. Purity of obtained fractions was determined by reprobing the membranes with antibody to tubulin and HDAC, as indicated in the figure legends.

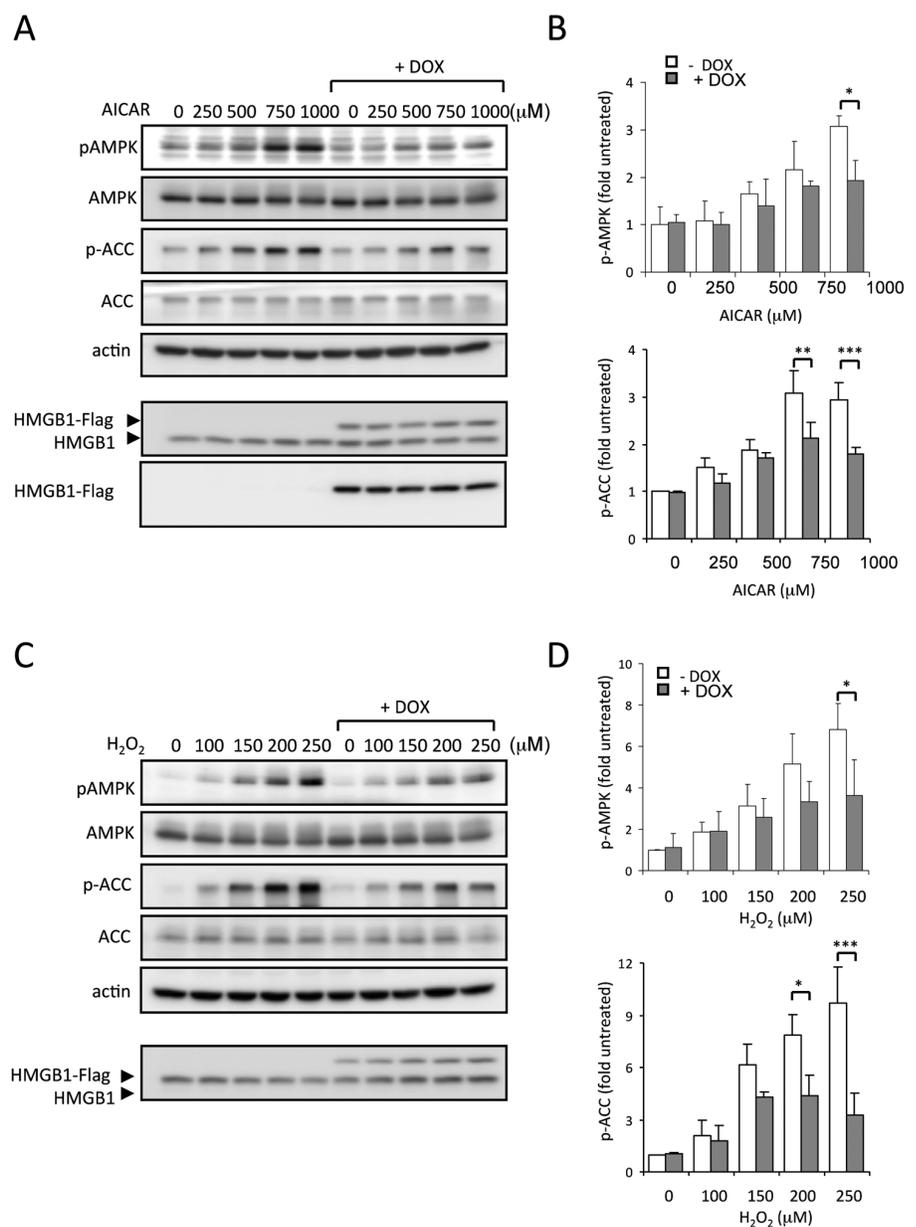
### Coimmunoprecipitation Assays

Cell extracts or lung homogenates (350  $\mu$ g/mL) prepared in immunopre-

cipitation buffer (26) were incubated with anti-AMPK, -LKB1 or -CaMKK $\beta$  antibodies (2  $\mu$ g/mL) overnight at 4°C. Samples were then incubated with protein-A agarose (25  $\mu$ L/sample) for an additional 60 min at 4°C. The protein-A agarose conjugates were then washed with immunoprecipitation buffer. The amount of HMGB1 associated with AMPK, LKB1 or CaMKK $\beta$  was determined by probing of the Western blot membrane with specific antibodies. In selected experiments, cell extracts obtained from the MCF7-HMGB1-FLAG cell line were incubated with ANTI-FLAG<sup>®</sup> M2 beads for 60 min at 4°C. The beads were then washed with immunoprecipitation buffer four times, and the amounts of HMGB1-FLAG associated with LKB1 were determined using Western blot analysis.

### Acute Lung Injury Model

Acute lung injury was induced by intratracheal administration of 1 mg/kg LPS in 50  $\mu$ L PBS as previously described (16,20,27,28). With this model, acute lung injury is characterized by neutrophil infiltration into the lung interstitium and airways, development of interstitial edema and increased pulmonary proinflammatory cytokine production, with the greatest degree of injury being present 24 h after LPS exposure (11). Briefly, mice were anesthetized with isoflurane, the tongue was gently extended and LPS in PBS or PBS alone (control) was deposited into the pharynx (53). Lungs were harvested 24 h after LPS administration, and homogenates were prepared using buffer containing Tris pH 7.4 (50 mmol/L), NaCl (150 mmol/L), NP-40 (0.5%, vol/vol), EDTA (1 mmol/L), EGTA (1 mmol/L), okadaic acid (1 nmol/L) and protease inhibitors. Of note, lungs were flushed with PBS to remove circulating cells before harvesting (lungs were not subjected to bronchoalveolar lavage [BAL] acquisition). Lung homogenates were then sonicated on ice for 90 s and centrifuged (16,000g for 10 min at 4°C), and protein concentration was determined using the Bradford assay.



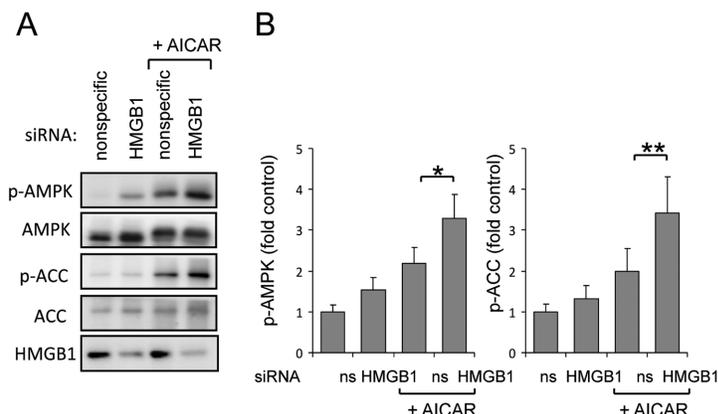
**Figure 3.** Effects of HMGB1 expression on AMPK activity. (A–D) The amounts of phosphorylated AMPK, ACC, total AMPK, ACC, HMGB1 and HMGB1-FLAG were determined in MCF7-HMGB1 cells incubated without (–DOX) or with doxycycline (+DOX) for 24 h followed by exposure to AICAR (A, B) or H<sub>2</sub>O<sub>2</sub> (C, D) for 1 h. Representative Western blots are shown. The amount of phospho-AMPK and phospho-ACC (B, D) were obtained from three independent experiments (means  $\pm$  SD, \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001).

### Statistical Analysis

Statistical significance was determined by the Wilcoxon rank-sum test (independent two-group Mann-Whitney  $U$  test) as well as the Student  $t$  test for comparisons between two groups. Multigroup comparisons were performed using one-

way ANOVA with the Turkey post hoc test. A value of  $P$  < 0.05 was considered significant. Analyses were performed on SPSS version 16.0 for Windows.

All supplementary materials are available online at [www.molmed.org](http://www.molmed.org).



**Figure 4.** Diminished expression of HMGB1 increases AMPK activity. (A, B) RAW 264.7 cells were treated with HMGB1-specific siRNA or control scrambled siRNA and then incubated with or without AICAR (1 mmol/L) for 1 h. Representative Western blots (A) and bend optical densitometry (B) show the levels of phosphorylated AMPK and ACC, as well as total AMPK, ACC and HMGB1 (means  $\pm$  SD,  $n = 3$ , \* $P < 0.05$ , \*\* $P < 0.01$ ). ns, Nonsignificant.

## RESULTS

### TLR4 Engagement Inhibits Activation of AMPK

Activation of AMPK inhibits TLR4-induced activation of neutrophils or macrophages, including NF- $\kappa$ B activation and cytokine production (11,13,29). However, little is known about how inflammatory stimuli, such as TLR4 engagement, may affect AMPK activation. To address this question, neutrophils were incubated with or without LPS (100 ng/mL) for 4 h followed by exposure to the AMPK activator AICAR for an additional 90 min. As shown in Figures 1A and C, AICAR dose-dependently increased phosphorylation of AMPK and phosphorylation of ACC, a downstream target of AMPK. In contrast, pretreatment with LPS resulted in decreased activation of AMPK by AICAR. Such diminished AICAR-dependent activation of AMPK may result from the enzymatic conversion of AICAR to ZMP (5'-phosphorylated form of AICAR) in LPS-treated cells, thereby reducing the ability of AICAR to affect AMPK (30). However, similar inhibitory effects of LPS treatment on AMPK activation were found in neutrophils cultured with H<sub>2</sub>O<sub>2</sub>, a direct activator of AMPK (Figures 1B, D) (9). There was no significant

difference between the viability of control (untreated) neutrophils and neutrophils treated with the combination of LPS and AICAR or LPS and H<sub>2</sub>O<sub>2</sub>.

We determined if alterations in the ATP-to-AMP ratio in neutrophils cultured with LPS were responsible for the lack of activation of AMPK under such conditions. In particular, if the ATP-to-AMP ratio increased after LPS exposure, this could explain the diminished ability of AICAR or H<sub>2</sub>O<sub>2</sub> to activate AMPK. However, as shown in Figure 1E, the ATP-to-AMP ratio decreased after culture of neutrophils with LPS. Although such change in the ATP-to-AMP ratio should promote AMPK activation, Western blot analysis revealed that neither LPS alone nor subsequent exposure of LPS-stimulated cells to AICAR or H<sub>2</sub>O<sub>2</sub> potentiated phosphorylation of AMPK or ACC. Of note, such inhibitory effects of TLR4 engagement on AMPK activation were not limited to neutrophils, since pretreatment of RAW 264.7 macrophages with LPS also diminished activation of AMPK by AICAR or H<sub>2</sub>O<sub>2</sub>. Similarly to our findings obtained with neutrophils or cell lines, exposure to LPS diminished the ability of peritoneal macrophages to activate AMPK after culture with AICAR or H<sub>2</sub>O<sub>2</sub> (Supplementary Figure S1).

### Time-Dependent Changes in the Nuclear to Cytosol Translocation of HMGB1 Correlate with the Diminished Activation of AMPK in LPS-Treated Macrophages

HMGB1 is almost exclusively located in the nucleus of unstimulated cells (Figure 2A) but demonstrates time-dependent translocation from the nucleus to cytosol after culture of macrophages with LPS. The cytosolic accumulation of HMGB1 in LPS-stimulated RAW 264.7 macrophages was confirmed using cell fractionation and Western blot analysis (Figure 2B). These results indicated that modest accumulation of HMGB1 was found in the cytosol after LPS exposure, while most of the HMGB1 was retained in the nucleus. However, a time-dependent increase in cytosolic HMGB1 in LPS-stimulated macrophages was associated with decreased phosphorylation of AMPK. Although AMPK can be readily detected in the nucleus, exposure of cells to LPS did not affect AMPK phosphorylation in this compartment, indicating that the diminished phosphorylation of AMPK in TLR4-activated macrophages was primarily a cytoplasmic event.

### Ectopic Expression of HMGB1 Diminishes AMPK Activation

Although the above experiments showed an inverse correlation in TLR4-stimulated macrophages between HMGB1 translocation to the cytoplasm and decrease in AMPK phosphorylation, they did not establish whether HMGB1 was directly involved in the inhibition of AMPK phosphorylation that occurred in these cells. This issue was examined using MCF7 cells that inducibly express FLAG-tagged HMGB1 (21). Under control conditions, the amounts of HMGB1-FLAG were undetectable, whereas doxycycline treatment resulted in upregulation of HMGB1-FLAG (Figure 3A). As shown in Figure 3, exposure to AICAR or H<sub>2</sub>O<sub>2</sub> resulted in robust activation of AMPK in control MCF7 cells (-DOX), whereas only a modest increase in the phosphorylation of AMPK and ACC was found in

doxycycline-treated cells that expressed HMGB1-FLAG (+DOX).

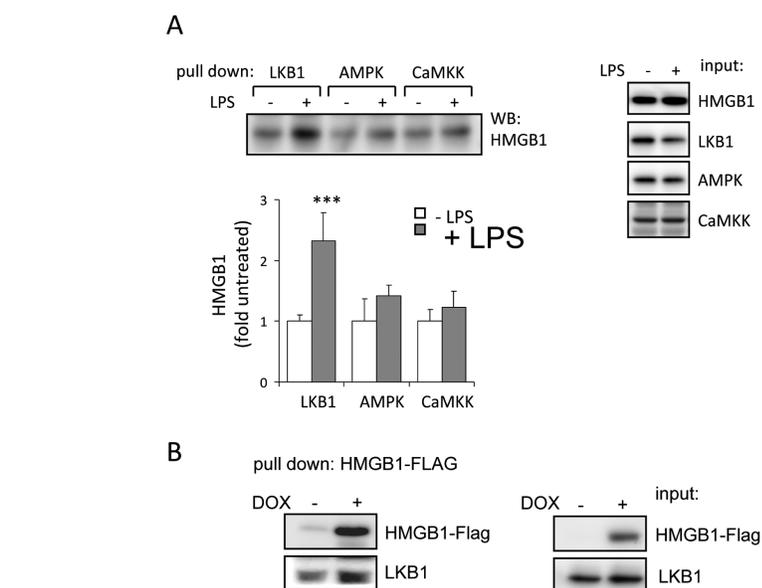
We next determined whether knock-down of HMGB1 affects the activation of AMPK. Treatment of RAW 264.7 macrophages with HMGB1-specific siRNA decreased HMGB1 levels compared with cells treated with nonspecific siRNA (Figure 4). Knockdown of HMGB1 resulted in increased AMPK phosphorylation as well as enhanced activation of AMPK after culture of the macrophages with AICAR. These results show that HMGB1 is directly involved in regulating AMPK activity, as enhanced expression of HMGB1 diminished, whereas knockdown of HMGB1 increased AMPK activation.

### TLR4 Engagement Increases Complex Formation between HMGB1 and LKB1

To explore whether the inhibitory effects of HMGB1 on AMPK activation are due to interactions between HMGB1 and AMPK subunits and/or with kinases upstream of AMPK, including LKB1 or CaMKK, RAW 264.7 macrophages were incubated with or without LPS, and then interactions between AMPK $\alpha$ 1, LKB1, CaMKK $\beta$  and HMGB1 were examined. As shown in Figure 5A, TLR4 engagement resulted in marked increase of HMGB1-LKB1 complex formation, compared with only slight enhancement of interactions between HMGB1 and AMPK $\alpha$ 1 or CaMKK $\beta$ . The ability of HMGB1 to bind LKB1 was further confirmed using MCF7 cells that inducibly express HMGB1-FLAG (Figure 5B). These results suggest that a potential mechanism by which HMGB1 inhibits AMPK activation may be through enhanced interactions with LKB1, thereby preventing LKB1-induced activation of AMPK.

### AMPK Activity Is Decreased in Lungs of Mice with LPS-Induced Acute Lung Injury

To determine if LPS exposure also inhibits AMPK activation *in vivo*, phosphorylation of AMPK and ACC was determined in lung homogenates obtained



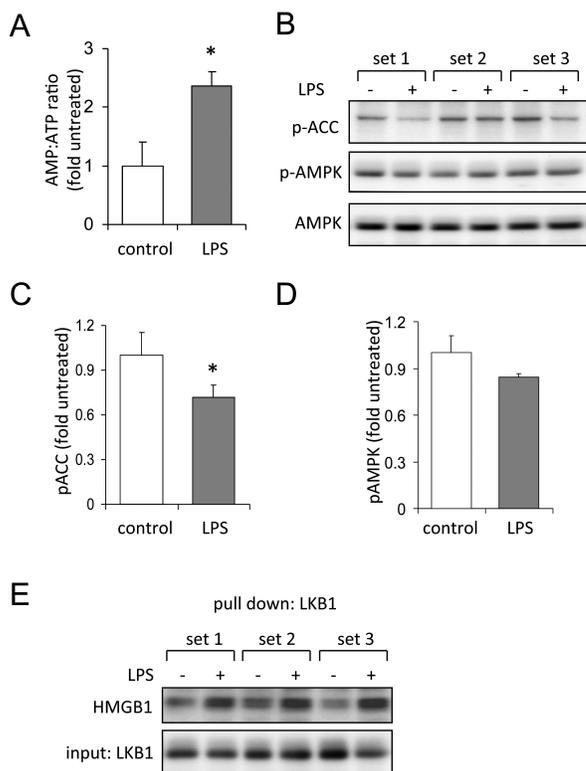
**Figure 5.** Stimulation with LPS or overexpression of HMGB1 enhances interactions between HMGB1 and LKB1. (A) RAW 264.7 macrophages were treated with LPS (0 or 100 ng/mL) for 4 h, and then cell extracts were subjected to immunoprecipitation using antibodies to LKB1, AMPK or CAMKK. Representative Western blots and quantitative analysis show amounts of HMGB1 associated with LKB1, AMPK or CAMKK after immunoprecipitation (left panel) as well as total HMGB1, CAMKK, AMPK and LKB1 in cell extracts before (right panel) immunoprecipitation (means  $\pm$  SD,  $n = 3$ ,  $***P < 0.001$ ). (B) Complex formation between HMGB1 and LKB1 was determined in control MCF7-HMGB1 cells (-DOX) or after doxycycline-induced expression of HMGB1-FLAG (+DOX). Cell extracts were subjected to immunoprecipitation with ANTI-FLAG<sup>®</sup> M2 monoclonal antibody followed by Western blot analysis of LKB1. Representative blots show amounts of LKB1 after HMGB1-FLAG pull-down (left panel) as well as before immunoprecipitation (right panel). A second independent experiment provided similar results.

24 h after LPS administration. As shown in Figure 6A, whereas a significant increase in AMP-to-ATP ratios was found in the lungs of LPS-treated mice, there was a modest decrease in the phosphorylation of AMPK and a significant decrease in the levels of phospho-ACC (Figures 6B–D). These results are consistent with our *in vitro* data using TLR4-stimulated cells (Figure 1E). Similar to results obtained *in vitro*, immunoprecipitation of LKB1 from the lungs of LPS-treated mice showed increased interactions between HMGB1 and LKB1.

### DISCUSSION

The present results show that TLR4-induced stimulation of neutrophils or macrophages is associated with inhibition of AMPK activation. In particular,

we found that culture of macrophages with LPS decreased the ability of two potent activators of AMPK (AICAR and H<sub>2</sub>O<sub>2</sub>) to efficiently promote phosphorylation of AMPK and ACC, a downstream target of AMPK. Furthermore, whereas a significant increase in intracellular AMP-to-ATP ratios was found after culture of neutrophils with LPS, a situation that should result in increased AMPK activity, AMPK activation was not altered (11,16). Although TLR4 engagement can potentially affect AMPK activation by inhibiting enzymatic conversion of AICAR to ZMP, a similar inhibition of AMPK activation was found in LPS-stimulated cells that were then treated with H<sub>2</sub>O<sub>2</sub>, compared with cells treated with H<sub>2</sub>O<sub>2</sub> alone. Of note, previous studies have shown that H<sub>2</sub>O<sub>2</sub> can activate AMPK both as a



**Figure 6.** Lack of AMPK activation, despite increase in AMP-to-ATP ratio, in the lungs of LPS-treated mice. The amounts of AMP, ATP (A) and phosphorylation of AMPK and ACC (B–D) were determined in lung homogenates obtained from mice given saline or LPS (1 mg/kg) intratracheally 24 h previously. Representative Western blots (B) and quantitative data (C and D) are shown (mean  $\pm$  SD,  $n = 6$ ,  $*P < 0.05$  compared with control mice). (E) Representative Western blots show the amounts of LKB1 and of HMGB1 in whole lung homogenates after immunoprecipitation of LKB1 from lung homogenates of control and LPS-treated mice (three mice per group).

result of increasing intracellular AMP/ATP levels as well as through additional mechanisms, including direct oxidative modification of the AMPK $\alpha$ 1 subunit (9,31,32). Our results indicate that, whereas exposure of cells to H<sub>2</sub>O<sub>2</sub> and/or an increase in the AMP-to-ATP ratio normally robustly activates AMPK, such activation is inhibited in TLR4-stimulated cells. Previous studies (11,16) have shown that AMPK can be effectively activated before TLR4 engagement and also that AMPK activation diminished proinflammatory response of neutrophils, macrophages and endothelial cells, including release of HMGB1 after exposure of macrophages to LPS. However, our current studies demonstrated that the ability of cells to activate AMPK

is diminished after exposure to LPS. In particular, a significant decrease, but not complete inhibition, of AMPK activation was found when cells were first cultured with LPS before treatment with AICAR or H<sub>2</sub>O<sub>2</sub>. These results suggest that counterregulatory mechanisms affect the activation of AMPK in LPS-treated cells.

HMGB1 is a 215-amino acid nuclear protein with highly conserved sequence among species. Although the initial function described for HMGB1 was as a nuclear protein involved in regulating gene expression (33,34), HMGB1 has been shown to be released from injured and dying cells and actively secreted by stimulated macrophages (35–38). Extracellular HMGB1 has potent proinflammatory actions and contributes to inflammation

through several mechanisms, including by enhancing cellular activation induced through Toll-like receptors, including TLR2, TLR4 and TLR9, or via the receptor for advanced glycation end products (RAGE) (39–41). HMGB1 has also been shown to enhance neutrophil chemotaxis and to diminish the uptake and clearance of apoptotic cells by macrophages and other phagocytes (42, 43). Increased circulating concentrations of HMGB1 are present in septic patients, as well as in animal models of endotoxemia, hemorrhage/ischemia and sepsis (44–46). Neutralization of HMGB1 with specific antibodies diminished mortality in murine endotoxemia and sepsis and also decreased pulmonary injury associated with sepsis, hemorrhage or experimental arthritis (46–49).

Although nuclear to cytoplasmic translocation of HMGB1 is a well-known event in TLR4-stimulated cells, association between this event and AMPK activation has not previously been described. In the present studies, we found an inverse relationship between accumulation of HMGB1 in the cytoplasm of LPS-stimulated macrophages and AMPK phosphorylation, suggesting that HMGB1 might be involved in the suppression of the ability of AMPK to be activated in TLR4-activated cells. In our experiments, overexpression of HMGB1 diminished, whereas knockdown of HMGB1 increased, AMPK activity. Recent studies have shown that stimulation of macrophages with LPS is associated with inhibition of LKB1 activation, a kinase upstream to AMPK (50). In the present studies, we found enhanced LKB1-HMGB1 complex formation in MCF7 cells that overexpressed HMGB1, in macrophages incubated with LPS and in the lungs of LPS-treated mice. Such findings provide important insights into the mechanism by which HMGB1 may affect AMPK activation; in particular, because LKB1 plays an important role in AMPK activation by AICAR and hydrogen peroxide (51–54), increased interactions between LKB1 and HMGB1 after TLR4-induced cellular activation, with con-

comitant inhibition of interactions between LKB1 and AMPK, may be responsible for suppression of AMPK activation under such conditions.

As observed in macrophages and neutrophils after culture with LPS, there was a decrease in the phosphorylation of AMPK and also of ACC, an important downstream target of AMPK, in the lungs of LPS-treated mice, despite increased AMP-to-ATP ratios—an event that would be expected to activate AMPK in a potent manner. Enhanced generation of ROS has been shown to occur in the lungs during LPS-induced acute lung injury (55–58) and also should result in AMPK activation. However, despite such potent inducing factors for AMPK activation, we did not find any evidence that AMPK was activated in the lungs of LPS-treated mice. Such results are consistent with the finding that AMPK is not activated in the lungs of patients with acute lung injury, despite reduced ATP levels and increased production of ROS being present under such conditions (57–59). Consistent with the results obtained from cultured cells, enhanced interaction between HMGB1 and LKB1 was found in the lungs of mice with LPS-induced acute lung injury.

HMGB1 has been shown to have potent proinflammatory activity and to contribute to organ injury during inflammatory processes initiated by infectious and noninfectious etiologies (39). Unlike the proinflammatory properties of HMGB1, activation of AMPK has antiinflammatory effects and has been shown to decrease proinflammatory cytokine production by LPS-stimulated macrophages and neutrophils, as well as diminish organ injury after LPS-induced lung inflammation, sepsis, hemorrhage and ischemic cardiovascular events (11,16,60,61).

## CONCLUSION

Our present findings suggest a novel mechanism by which HMGB1 may contribute to enhancing inflammatory processes. In particular, by inhibiting activation of AMPK through complex formation with LKB1, an upstream activator

of AMPK, HMGB1 may suppress antiinflammatory regulatory events that normally would diminish tissue injury induced by TLR4 engagement and probably by other mediators capable of producing inflammation. Our findings demonstrating the ability of HMGB1 to prevent AMPK activation suggest that therapeutic approaches that inhibit translocation of HMGB1 from the nucleus to the cytoplasm and/or prevent interactions between LKB1 and HMGB1 may be beneficial in acute and chronic inflammatory conditions, such as sepsis and rheumatoid arthritis, pathophysiologic states in which HMGB1 plays a contributory role (62–64). Of note, nonselective blockade of the intracellular actions of HMGB1 are unlikely to be efficacious given the important transcriptional functions of HMGB1 (33,65). Because binding between HMGB1 and inflammatory mediators, including LPS, potentiates their ability to activate cells through TLR-dependent mechanisms (66), therapeutic approaches to neutralize circulating HMGB1 may also restore the antiinflammatory actions of AMPK through preventing TLR4-induced inhibition of AMPK activation.

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## 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.

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