The Journal of Immunology

Metformin Mediates Protection against *Legionella* Pneumonia through Activation of AMPK and Mitochondrial Reactive Oxygen Species

Chiaki Kajiwara, Yu Kusaka, Soichiro Kimura, Tetsuo Yamaguchi, Yuta Nanjo, Yoshikazu Ishii, Heiichiro Udono, Theodore J. Standiford and Kazuhiro Tateda

J Immunol published online 15 December 2017
http://www.jimmunol.org/content/early/2017/12/15/jimmunol.1700474

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Published December 15, 2017, doi:10.4049/jimmunol.1700474

Metformin Mediates Protection against *Legionella* Pneumonia through Activation of AMPK and Mitochondrial Reactive Oxygen Species

Chiaki Kajiwara,* Yu Kusaka,*,† Soichiro Kimura,* Tetsuo Yamaguchi,* Yuta Nanjo,*,‡ Yoshikazu Ishii,* Heiichiro Udono,§ Theodore J. Standiford,‡ and Kazuhiro Tateda*

In *Legionella pneumophila* infection, macrophages play a critical role in the host defense response. Metformin, an oral drug for type 2 diabetes, is attracting attention as a new supportive therapy against a variety of diseases, such as cancer and infectious diseases. The novel mechanisms for metformin actions include modulation of the effector functions of macrophages and other host immune cells. In this study, we have examined the effects of metformin on *L. pneumophila* infection in vitro and in vivo. Metformin treatment suppressed growth of *L. pneumophila* in a time- and concentration-dependent fashion in bone marrow–derived macrophages, RAW cells (mouse), and U937 cells (human). Metformin induced phosphorylation of AMP-activated protein kinase (AMPK) in *L. pneumophila*–infected bone marrow–derived macrophages, and the AMPK inhibitor Compound C negated metformin-mediated growth suppression. Also, metformin induced mitochondrial reactive oxygen species but not phagosomal NADPH oxidase–derived reactive oxygen species. Metformin-mediated growth suppression was mitigated in the presence of the reactive oxygen species scavenger glutathione. In a murine *L. pneumophila* pneumonia model, metformin treatment improved survival of mice, which was associated with a significant reduction in bacterial number in the lung. Similar to in vitro observations, induction of AMPK phosphorylation and mitochondrial ROS was demonstrated in the infected lungs of mice treated with metformin. Finally, glutathione treatment abolished metformin effects on lung bacterial clearance. Collectively, these data suggest that metformin promotes mitochondrial ROS production and AMPK signaling and enhances the bactericidal activity of macrophages, which may contribute to improved survival in *L. pneumophila* pneumonia. *The Journal of Immunology*, 2018, 200: 000–000.

Legionella is a Gram-negative pathogen that is widely distributed in nature, especially in water-associated environments. This organism primarily causes two types of infection: atypical pneumonia, referred to as Legionellosis, and an acute nonfatal respiratory disease known as "Pontiac fever." The prevention of *Legionella* pneumonia is important, because mortality is high, even in cases treated with appropriate antibiotics (1, 2). The majority of *Legionella* infections are caused by *L. pneumophila* (1, 2), and the most important pathogenic property of this organism is believed to be resistance to intracellular killing and proliferation within phagocytic cells, especially macrophages and monocytes (3–5). A/J mice, but not C57BL/6, BALB/c, and C3H/HeJ mice, are susceptible to *L. pneumophila*, and intrapulmonary challenge with *L. pneumophila* in A/J mice results in pneumonia mimicking human disease (6–9).

Metformin (dimethylbiguanide) is a drug used to treat type 2 diabetes that is derived from extracts of *Galega officinalis*, a leguminous plant (10). Although an antitumor effect of metformin was suggested dating back to the 1970s, Evans et al. (11) reported for the first time, in an epidemiological study, that the risk for cancer development was lower in diabetic patients treated with metformin. Since then, accumulating evidence has demonstrated antitumor effects of metformin in specific cancers of animals and humans (12–14). Mechanisms of antitumor activity of metformin have primarily been attributed to gluconeogenesis inhibition, reduction in insulin concentrations, or suppression of mTORC1 via activation of the AMP-activated protein kinase (AMPK) pathway (15–18). Recently, it was reported that metformin inhibits inflammatory responses in murine macrophages, in part through AMPK activation and RAGE/NFκB pathway suppression (19). These data suggested that metformin may act on cancer cells, as well as on several host inflammatory and immune cells. In addition, epidemiological research in *Mycobacterium tuberculosis* patients has found that the metformin-treated group had a better survival rate than the group not treated with metformin, and metformin's effect was correlated with an increase in specific T cell immune responses against *M. tuberculosis* (20). Studies using a lymphocytic choriomeningitis

*Department of Microbiology and Infectious Diseases, Faculty of Medicine, Toho University School of Medicine, Tokyo 143-8540, Japan; †Department of Respiratory Medicine, Tokyo Medical and Dental University, Tokyo 113-8519, Japan; ‡Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Michigan, Ann Arbor, MI 48109; and §Department of Immunology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan

ORCIDs: 0000-0001-6884-8110 (Y.K.); 0000-0002-5368-7091 (Y.N.); 0000-0002-9407-2296 (H.U.).

Received for publication April 4, 2017. Accepted for publication November 6, 2017.

This work was supported by a Grant-in-Aid for Private University Research Branding Project from the Ministry of Education, Culture, Sports, Science and Technology. Part of this project was supported by a Grant-in-Aid for Scientific Research (KAKENHI) for Young Scientists (B) from the Japan Society for the Promotion of Science.

Address correspondence and reprint requests to Prof. Kazuhiro Tateda, Toho University, 5-21-16 Omohinishi, Ota-ku, Tokyo 143-8540, Japan. E-mail address: kazu@med.toho-u.ac.jp

The online version of this article contains supplemental material.

Abbreviations used in this article: ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; BMDM, bone marrow–derived macrophage; CC, Compound C; cROS, cellular ROS; GSH, L-glutathione reduced; MOI, multiplicity of infection; mROS, mitochondrial ROS; qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species.

virus infection mouse model showed that pretreatment with metformin significantly enhanced CD1d-mediated NKT cell activation (21). These observations suggested an expanding potential therapeutic role for metformin, including not only diabetes, but also cancer, infectious diseases, and other inflammatory disease states.

Previous studies have demonstrated that reactive oxygen species (ROS) are essential components of innate immune responses against intracellular bacteria in macrophages. ROS are produced by catalyst of NADPH oxidase machinery (cellular ROS [cROS]) (22). Moreover, mitochondrial ROS (mROS) also contribute to mouse macrophage bacterial activity via TLRs (TLR1, TLR2, and TLR4) (23). Interestingly, it has been shown that *L. pneumophila* infection suppressed production of NADPH cROS in U937 cells during the course of infection (24).

In the current study, we examined the immunomodulatory roles of metformin in *L. pneumophila* infection in vitro and in vivo. Our data demonstrate that metformin reduces intracellular growth of bacteria, in part through activation of mROS in an AMPK-associated manner, which likely contributes to the metformin-induced survival benefit in *L. pneumophila* pneumonia.

Materials and Methods

Animals

Specific pathogen–free female A/J mice were purchased from Sankyo Labo Service (Tokyo, Japan). Mice were maintained under specific pathogen–free conditions within the animal care facility in the Laboratory Animal Research Center of Toho University School of Medicine until 9–12 wk of age. Animal and pathogen protocols were approved by the Institutional Care and Use Committee (approval numbers 15-54-220, 15-54-58).

L. pneumophila *inoculation and determination of bacterial number*

Clinical isolates of *L. pneumophila* Suzuki (serogroup 1) strain stocked at Toho University Hospital (25) were used for the pneumonia model, as previously reported (26). Animals were anesthetized i.m. with ketamine (50 mg/kg of body weight) and xylazine (10 mg/kg). Tracheas were exposed, and 30 μl of bacterial suspension was administered via a sterile 26-gauge needle. Skin incisions were closed with surgical staples. To quantitate bacterial number, at the indicated time points the lungs were removed and homogenized with a homogenizer (IKA Japan K.K., Osaka, Japan) in 1 ml of saline. Portions of homogenates (10 μl) were inoculated onto buffered charcoal–yeast extract agar supplemented with α-ketoglutaric acid (BCYEα) after serial 1:10 dilution in saline. Agar plates were incubated at 35˚C for 3–4 d, and the numbers of colonies were counted visually.

Bone marrow–derived macrophage and cell preparation

Bone marrow was harvested from A/J mice to isolate bone marrow–derived macrophages (BMDMs). The bone marrow cells were cultured in complete RPMI 1640 medium (10% FBS, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, penicillin-streptomycin, 2-ME) supplemented with L cell–conditioned medium and 2 ng/ml mouse rM-CSF (R&D Systems, Minneapolis, MN), as described previously (27, 28). BMDMs were used in experiments on day 5 of culture. The RAW 264.7 mouse macrophage cell line and U937 human monocytic cell line were originally obtained from the American Type Culture Collection. These cell lines were maintained in complete RPMI 1640 medium at 37˚C in 5% CO_2. To promote a macrophage-like phenotype, U937 cells were differentiated in 10 ng/ml PMA for 24 h.

In vitro L. pneumophila *infection*

On day 4 of BMDM preparation, the cells were lifted by Trypsin-EDTA (Life Technologies) and seeded at 1×10^5 cells per well in 96-well tissue culture plates (BD Falcon) overnight. BMDMs were infected at a multiplicity of infection (MOI) of 0.1 and incubated for 1 h after attachment by centrifugation. At the end of the infection period, nonphagocytosed and nonadherent bacteria were removed by washing three times with fresh medium. *L. pneumophila*–infected BMDMs were treated with various concentrations of metformin (Wako Pure Chemical) and subsequently incubated at 37˚C in 5% CO_2. At the indicated time points, culture supernatants were collected, and the infected macrophages were lysed for counting of viable bacterial number, as previously described (29). We have combined bacterial counts from macrophage lysates and supernatants to determine total intracellular bacteria, because *L. pneumophila* Suzuki do not proliferate outside of macrophages; thus, the supernatant bacteria could only come from dying macrophages. In some experiments, ROS scavengers were used: 10 mM L-glutathione reduced (GSH) or AMPK inhibitor, 25 μM Compound C (CC; both from Sigma-Aldrich). These scavengers were added to the cultures 30 min before metformin.

Measurement of ROS production

RAW 264.7 cells were seeded in glass-bottom dishes, coated with poly-L-lysine (Matsunami), at 1×10^5 cells per dish and rested overnight. Cells were infected with *L. pneumophila*, as described above, and then treated with 2 mM metformin for 6 or 24 h. After washing, PBS with 5 μM MitoSOX Red (to measure mROS) or PBS with 5 μM CM-H_2DCFDA (to measure cROS) (Molecular Probes) was added to the cells. Cells were incubated at 37˚C in 5% CO_2 for 15 min and washed with PBS. Fluorescence intensities of ROS were analyzed using a Carl Zeiss confocal microscope (LSM 710 system). Three fields of view were examined for each sample, and the fluorescence intensity was adjusted by the number of cells.

Western blotting

Lungs were homogenized in 1 ml of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% Triton X-100) containing a complete protease inhibitor mixture tablet (Roche). The homogenates were incubated for 30 min on ice and then centrifuged at 12,000 × *g* for 20 min. Supernatants were collected and mixed with 4× sample buffer (4% SDS, 4% 2-ME, 20% glycerol, and 125 mM Tris-HCl). BMDMs were lysed with 4× sample buffer. The proteins were subjected to electrophoresis on a 15% SDS-polyacrylamide gel (Wako Pure Chemical), transferred to a polyvinylidene difluoride membrane, and blocked by 3% skim milk dissolved in TBS buffer (10 mM Tris-HCl [pH 7.5], 135 mM NaCl) with 0.05% Tween 20. The membrane was blotted with rabbit anti-AMPKα, phospho-AMPKα (Thr^{172}), and phospho–acetyl-CoA carboxylase (ACC; Thr^{79}; all from Cell Signaling Technology) Abs. Anti-GAPDH (Cell Signaling Technology) was used as an internal control. HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) was used as secondary Ab, and detection was conducted using a chemiluminescent reaction (Amersham Imager 600; GE Healthcare). The intensity of each band was normalized to the loading control GAPDH and analyzed with ImageJ software.

Isolation of lung cells and flow cytometric analysis

The excised lung tissue, separated from the associated lymph nodes, was minced and incubated at 37˚C in 5% CO_2 for 50 min in RPMI 1640 medium containing 2% FBS, 0.5 mg/ml collagenase D (Roche), and 150 μg/ml DNase (Roche). Single-cell suspensions were prepared by passing through a 70-μm cell strainer (BD Falcon). Detection of mROS in live cells was performed using a CellROX Green Flow Cytometry Assay Kit (Molecular Probes), according to the manufacturer's instructions. Cells were incubated with Abs for 10 min in PBS containing 0.5% BSA and 2 mM EDTA (FACS buffer). PE anti-mouse Ly6G (1A8), PE-Cy7 anti-mouse F4/80 (BM8), and allophycocyanin anti-mouse CD11b (M1/70; all from BioLegend) were used to identify phagocytic cells. Cells were washed, fixed with 4% paraformaldehyde, detected with a FACSCanto II flow cytometer (BD Biosciences), and analyzed with FlowJo software (TreeStar).

RNA isolation and gene expression analysis

Total RNA was isolated from mouse lungs or cells using TRIzol Reagent (Invitrogen), according to the manufacturer's instructions. For quantitative real-time PCR (qRT-PCR) analysis, 1 μg of total RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Data analysis, using the SYBR Green real-time RT-PCR technique, was performed on an ABI Prism 7000 sequence detector system (Applied Biosystems). We used the following PCR primers: IFN-γ, 5′-GAACTGGCAAAAGGATGGTGA-3′ (forward) and 5′-TGTGGGTTGTTGACCTCAAAC-3′ (reverse); IL-12p35, 5′-CACCCTTGCCCTCCTAAACC-3′ (forward) and 5′-CACCTGGCAGGTCCAGAGA-3′ (reverse); TNF-α, 5′-GCCTCCCTCTCATCAGTTCT-3′ (forward) and 5′-CACTTGGTGGTTTGCTACGA-3′ (reverse); and β-actin, 5′-AGAGGGAAATCGTGCGTGAC-3′ (forward) and 5′-CAATAGTGATGACCTGGCCGT-3′ (reverse). Relative fold changes in transcript levels were

calculated using the $2^{-\Delta\Delta CT}$ method (where *CT* is threshold cycle) (30), using the housekeeping gene β-actin as a reference standard for the amount loaded and the quality of the cDNA.

PCR array analysis

Total RNAs were isolated from *L. pneumophila*–infected cells ($n = 3$) in the presence or absence of metformin at 24 h, and PCR array analysis was performed (RT^2 Profiler PCR Array Mouse AMPK Signaling; catalog number PAMM-175Z; QIAGEN), according to the manufacturer's protocols.

ELISA

Cytokines in the culture supernatant were measured with IFN-γ, TNF-α, and IL-12p70 mouse ELISA kits (R&D Systems), according to the manufacturer's protocols.

Statistical analysis

All results are expressed as mean ± SD. Statistical analyses were performed using GraphPad Prism 6 software (GraphPad, La Jolla, CA). The Student *t* test was used for comparisons between two groups. ANOVA, followed by the Tukey multiple-comparison test, was performed for comparisons between more than two groups. Survival curves were constructed using the Kaplan–Meier method and were analyzed by the log-rank (Mantel–Cox) test. A p value < 0.05 was considered statistically significant.

Results

Metformin suppresses intracellular growth of L. pneumophila *in macrophages*

To assess the effects of metformin on host immune responses, we first examined whether metformin altered the ability of macrophages to inhibit the growth of *L. pneumophila* in vitro. BMDMs were prepared from A/J mice and infected with *L. pneumophila* (MOI 0.1). After 1 h of incubation, the cells were washed with culture medium to remove extracellular bacteria. The cells were cultured or not with metformin, and bacterial numbers were enumerated at the indicated time points. As shown in Fig. 1A, a time-dependent increase in bacterial number was observed in the control group during the 48-h incubation period. In contrast, a significant inhibition of intracellular bacterial number was observed in metformin-treated BMDMs from 12 to 48 h postinfection. Next, we examined the concentration-dependent effects of metformin on BMDMs and RAW cells (Fig. 1B, 1C). Significant suppression of *L. pneumophila* growth in BMDMs and RAW cells was observed at a metformin concentration of 2.5 mM. In U937 cells (a human macrophage cell line), the effect of metformin

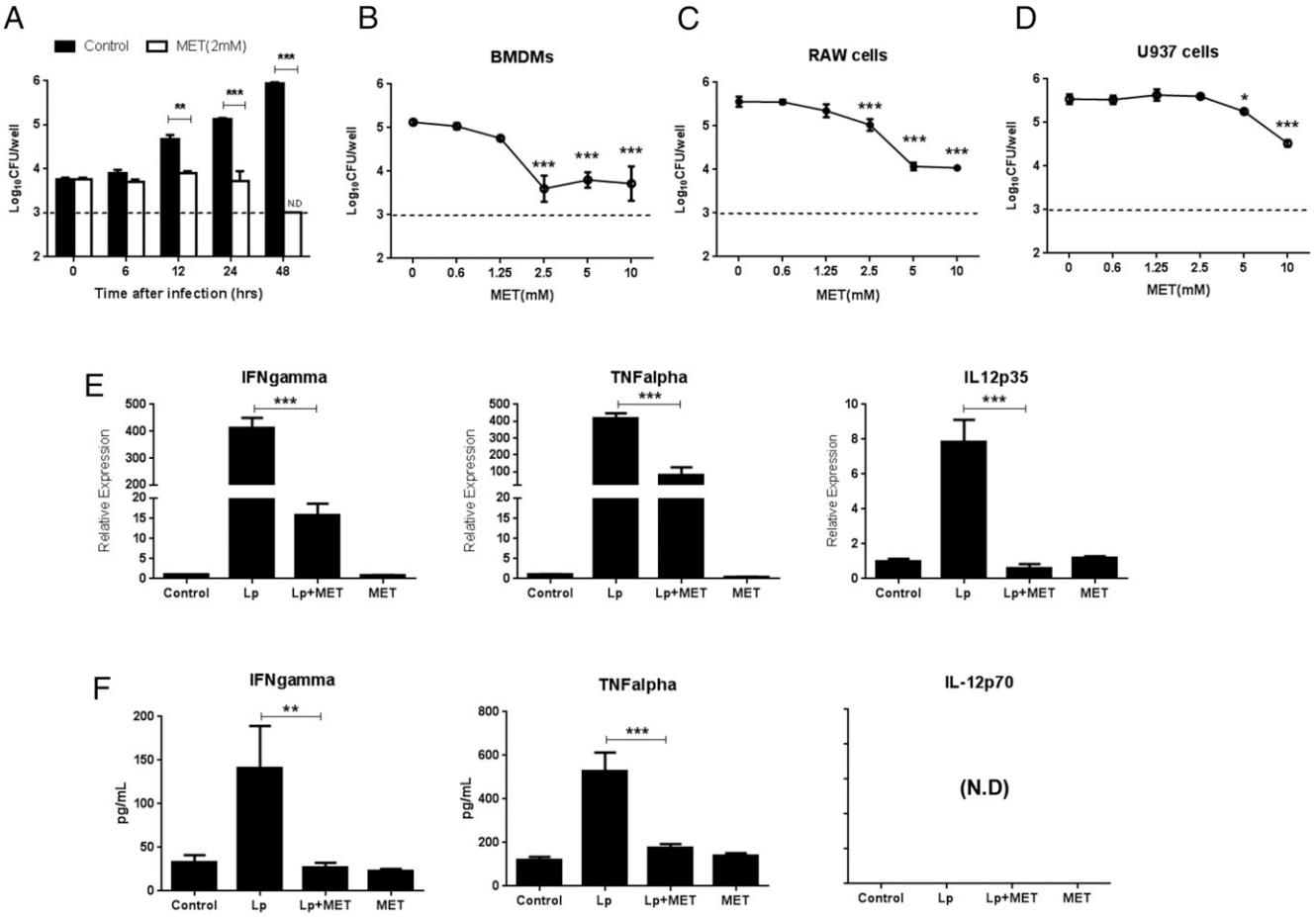


FIGURE 1. Effects of metformin on *L. pneumophila* infection in macrophages. (**A**) BMDMs infected with *L. pneumophila* for 1 h at an MOI of 0.1 were washed and then treated or not with metformin. After 6–48 h, BMDMs were collected, and CFU were enumerated at the indicated time points. (**B**) BMDMs infected with *L. pneumophila* were treated with different doses of metformin. After 24 h, BMDMs were collected, and CFU were enumerated. (**C**) RAW 264.7 cells were infected, and CFU were enumerated, as in (B). (**D**) U937 cells were infected, and CFU were enumerated as in (B). (**E**) qRT-PCR for IFN-γ, TNF-α, IL-12p35 associated with or without 2 mM metformin treatment in BMDMs at 24 h postinfection. (**F**) IFN-γ and TNF-α released by infected BMDMs was assessed by ELISA. Bars indicate mean ± SD, in triplicates. The dashed line indicates the detection limit. The results were confirmed by three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, two-tailed Student *t* test (A), one-way ANOVA followed by the Tukey multiple-comparison test (B–F). Control, no infection; Lp, *L. pneumophila* infection; Lp + MET, *L. pneumophila* infection + metformin treatment; MET, metformin treatment; N.D, not detected.

was somewhat less than that observed in mouse cell lines, but the growth of the bacteria was significantly inhibited at metformin concentrations ≥ 5 mM.

IFN-γ, IL-12p35, and TNF-α expression was examined by qRT-PCR in BMDMs infected with *L. pneumophila*, with or without metformin, for 24 h. *L. pneumophila* infection strongly induced these cytokines, whereas significant suppression of these factors was observed in the presence of metformin, coincident with a decrease in the number of bacteria (Fig. 1E). In addition, when the protein expression level was measured by ELISA, IL-12 was below the detection limit, whereas the levels of IFN-γ and TNF-α were reduced at the protein level (Fig. 1F).

Because cell death is one mechanism by which host cells suppress intracellular proliferation of *L. pneumophila* (31–33), we calculated cell numbers at 6 and 24 h postinfection (Supplemental Fig. 1A, 1B). We did not observe any change in macrophage number among the *Legionella*, *Legionella* + metformin, metformin, and control groups. At 48 h, we observed damaged cells among *L. pneumophila*–infected cells, whereas metformin-treated infected cells showed no evidence of cytotoxicity (Supplemental Fig. 1C). We also examined effects on cell apoptosis (Supplemental Fig. 1D). Incubation with *L. pneumophila* induced apoptosis in macrophages. Metformin treatment resulted in a modest reduction in apoptosis, although the difference was not statistically significant. The effects observed were not due to the direct antibacterial effects of metformin, because 2 mM metformin did not alter the growth of *L. pneumophila* (Supplemental Fig. 2A, 2B).

Metformin enhances phosphorylation of AMPK in L. pneumophila*–infected macrophages*

Because previous reports demonstrated that metformin activates AMPK signaling (34), we next examined the effects of metformin on phosphorylation of AMPK in *L. pneumophila*–infected macrophages. BMDMs were infected with *L. pneumophila*, and total cell lysates were prepared at 6 and 24 h for Western blot analysis. In *L. pneumophila*–infected BMDMs, suppression of AMPK phosphorylation was observed at both time points compared with noninfected control cells. In contrast, addition of metformin induced phosphorylation of AMPK at both time points in *L. pneumophila*–infected BMDMs (Fig. 2A). The induction of AMPK phosphorylation was detected in metformin-treated groups, most prominently at the 24-h time point. Also, after 24 h, metformin increased the phosphorylation of AMPK and its downstream target ACC in a concentration-dependent manner (Fig. 2B). There was no change in the phosphorylation of the upstream target liver kinase B1 (data not shown). We next examined the effects of metformin on AMPK-related signaling molecules in a PCR array. Total RNA was isolated from *L. pneumophila*–infected cells ($n = 3$) in the presence or absence of metformin at 24 h, and a PCR array analysis (RT^2 Profiler PCR Array Mouse AMPK Signaling; catalog number PAMM-175Z; QIAGEN) was performed. Although the expression levels of AMPK subunits and ACC increased, the expression level of liver kinase B1 did not change, similar to the observed changes in protein expression (Supplemental Fig. 3).

We also examined whether metformin's effects on AMPK phosphorylation were associated with a reduction in *L. pneumophila* number in BMDMs. The effects of AMPK

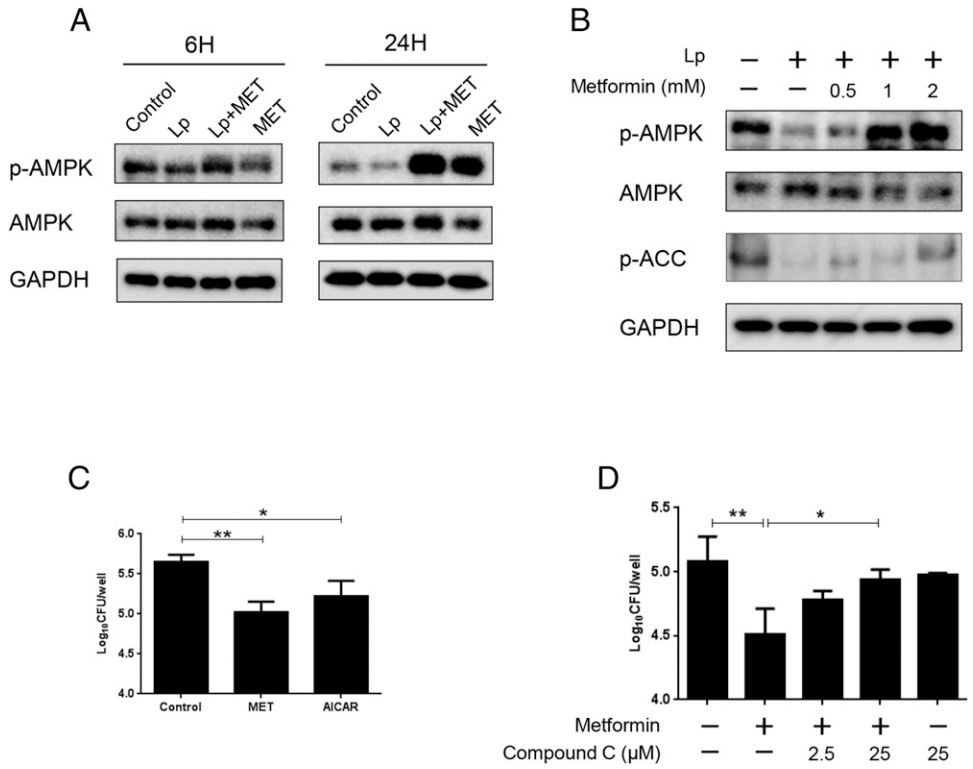


FIGURE 2. Effects of metformin on phosphorylation of AMPK and suppression of *Legionella* growth. (**A**) Western blot analysis was performed for p-AMPK in total cell lysates of BMDMs at 6 or 24 h postinfection. GAPDH was used as a loading control. (**B**) The Western blot analysis was performed for total cell lysates of BMDMs treated with different doses of metformin at 24 h postinfection. (**C**) Infected BMDMs were treated with metformin (2 mM) or AICAR (1 mM). After 24 h, BMDMs were collected, and CFU were enumerated. (**D**) BMDMs were treated with 2 mM metformin, with or without CC (2.5 or 25 μM), for 24 h. Then cells were washed and infected with *L. pneumophila*. CFU were enumerated after 24 h of incubation. Bars indicate mean ± SD, in triplicates. Different samples were used for triplicates. The results were confirmed by three independent experiments. $*p < 0.05$, $**p < 0.01$, one-way ANOVA followed by the Tukey multiple-comparisons test.

activator (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AICAR) and AMPK inhibitor (CC) were examined in *L. pneumophila*–infected BMDMs. As shown in Fig. 2C, a significant reduction in bacterial number was observed in AICAR-treated cells, which was similar to that of metformin. As expected, addition of AMPK inhibitor CC reversed the growth suppression of *L. pneumophila* by metformin in a concentration-dependent manner (Fig. 2D). These results demonstrated an association between AMPK phosphorylation and growth suppression of *L. pneumophila* with metformin treatment.

Metformin enhances the production of mROS

A variety of host defense systems are active against intracellular pathogens, including apoptosis, autophagy, and NO and ROS production in infected cells. In particular, ROS are essential components of the innate antibacterial responses. There are at least two forms of ROS: cROS and mROS. Metformin has been reported to inhibit mitochondrial respiratory chain complex I, resulting in activation of AMPK and increased production of mROS (35, 36). Other studies demonstrated that exposure to H_2O_2 was associated with cysteine oxidation in the AMPKα subunits and was able to directly activate AMPK (37). At the same time, AMPK activity was reported to suppress NADPH oxidase activation (38).

The effects of metformin on mROS and cROS were examined in *L. pneumophila*–infected macrophages. RAW cells were infected with *L. pneumophila* for 1 h at an MOI of 0.1 and then washed to remove extracellular bacteria. The infected cells were cultured for 6 or 24 h, with or without 2 mM metformin, and mROS and cROS production was evaluated by staining with MitoSOX Red and CM-H_2DCFDA, respectively. As shown in Fig. 3A and 3B, metformin treatment induced the production of mROS at 6 h in infected cells, with a further increase observed at 24 h of incubation. A marked induction of mROS was found in macrophages treated with metformin in the absence of *L. pneumophila* infection. In contrast, metformin did not alter the induction of cROS in *L. pneumophila*–infected or noninfected macrophages after 6 h of incubation (Fig. 3C, 3D). Interestingly, a clear suppression of cROS was exhibited in *L. pneumophila*–infected macrophages at 24 h of incubation, an effect that was not influenced by metformin. We next examined the effect of the ROS scavenger GSH on

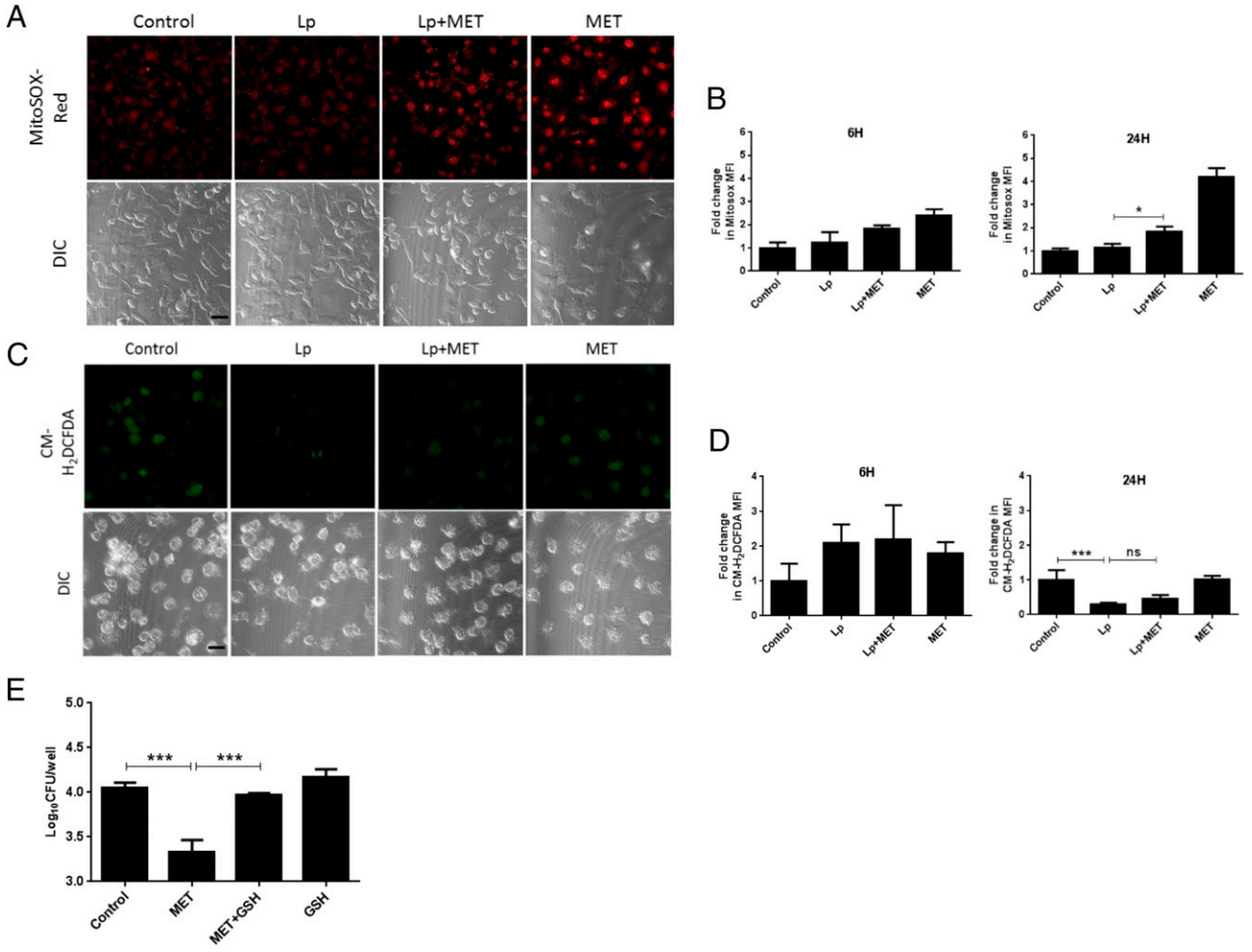


FIGURE 3. Effects of metformin on ROS and suppression of *Legionella* growth. (**A**) RAW 264.7 cells infected with *L. pneumophila* were incubated or not for 24 h with 2 mM metformin. The cells were stained with MitoSOX Red for detection of mROS and analyzed using a confocal laser microscope. Scale bar, 20 μm. (**B**) mROS in RAW 264.7 cells were examined at 6 or 24 h of infection. Mean fluorescence intensity (MFI) of MitoSOX Red–stained RAW 264.7 cells was calculated. (**C**) RAW 264.7 cells were infected with *L. pneumophila*, as in (A). The cells were stained with CM-H_2DCFDA for detection of cROS and analyzed using a confocal laser microscope. Scale bar, 20 μm. (**D**) cROS in RAW 264.7 cells were examined at 6 or 24 h of the infection. MFI of CM-H_2DCFDA–stained RAW 264.7 cells was calculated. (**E**) RAW 264.7 cells were treated with 2 mM metformin, with or without 10 mM GSH, for 24 h. Then cells were washed and infected with *L. pneumophila*. Bacterial numbers were enumerated after 24 h of incubation. Experiments in (A) and (C) were conducted three times. Data in (E) were confirmed by three independent experiments. Bars indicate mean ± SD, in triplicates. $*p < 0.05$, $***p < 0.001$, one-way ANOVA followed by the Tukey multiple-comparisons test. ns, not significant.

metformin-induced bacterial growth suppression in BMDMs (Fig. 3E). Incubation with GSH counteracted metformin-induced growth suppression of *L. pneumophila* in BMDMs. These data demonstrated that the induction of mROS, but not cROS, may be a mechanism for metformin-induced growth inhibition of *L. pneumophila* in macrophages.

Metformin effects in a murine model of L. pneumophila *pneumonia*

To assess the effect of metformin in vivo, we used a clinically relevant mouse model of *L. pneumophila* pneumonia. A/J mice received 5 mg/ml metformin in the drinking water starting from 7 d before to 16 d postinfection. On day 0, mice were intratracheally inoculated with 1.5×10^6 CFU *L. pneumophila*. Significant improvement in survival was observed in the metformin-treated group (Fig. 4A) compared with vehicle-treated control animals. When metformin was started at the time of bacterial administration, an improvement in mortality was not observed (data not shown). When the amount of bacteria administered was reduced by a log 10, the number of bacteria in the lungs peaked 2 d after the infection and gradually decreased thereafter. There was no difference in lung bacterial burden at 3 d after the infection; however, by day 4, a significant reduction in bacterial number was observed in metformin-treated mice (Fig. 4B). In addition, significant metformin effects on lung bacterial number were confirmed at days 3 and 4 when metformin treatment was completed on the day of *L. pneumophila* administration (Fig. 4C). Cytokine expression, as determined by qRT-PCR, was compared in the presence or absence of metformin (Fig. 4D). Consistent with the bacterial counts, cytokine mRNA expression in control mice increased at 2 d after the infection and decreased by day 3. There were no differences on day 2, whereas significant reductions in the expression of all cytokines, with the exception of IL-1β, were observed in the metformin group on day 3 (Fig. 4D).

Metformin effects on AMPK phosphorylation in the lungs of L. pneumophila*–infected mice*

We next investigated whether metformin induced the activation of AMPK in the lungs of mice infected with *L. pneumophila* in vivo. At 3 d of infection, lung tissue was removed and analyzed by Western blotting. As shown in Fig. 5, suppression of AMPK phosphorylation was observed in *L. pneumophila*–infected lungs

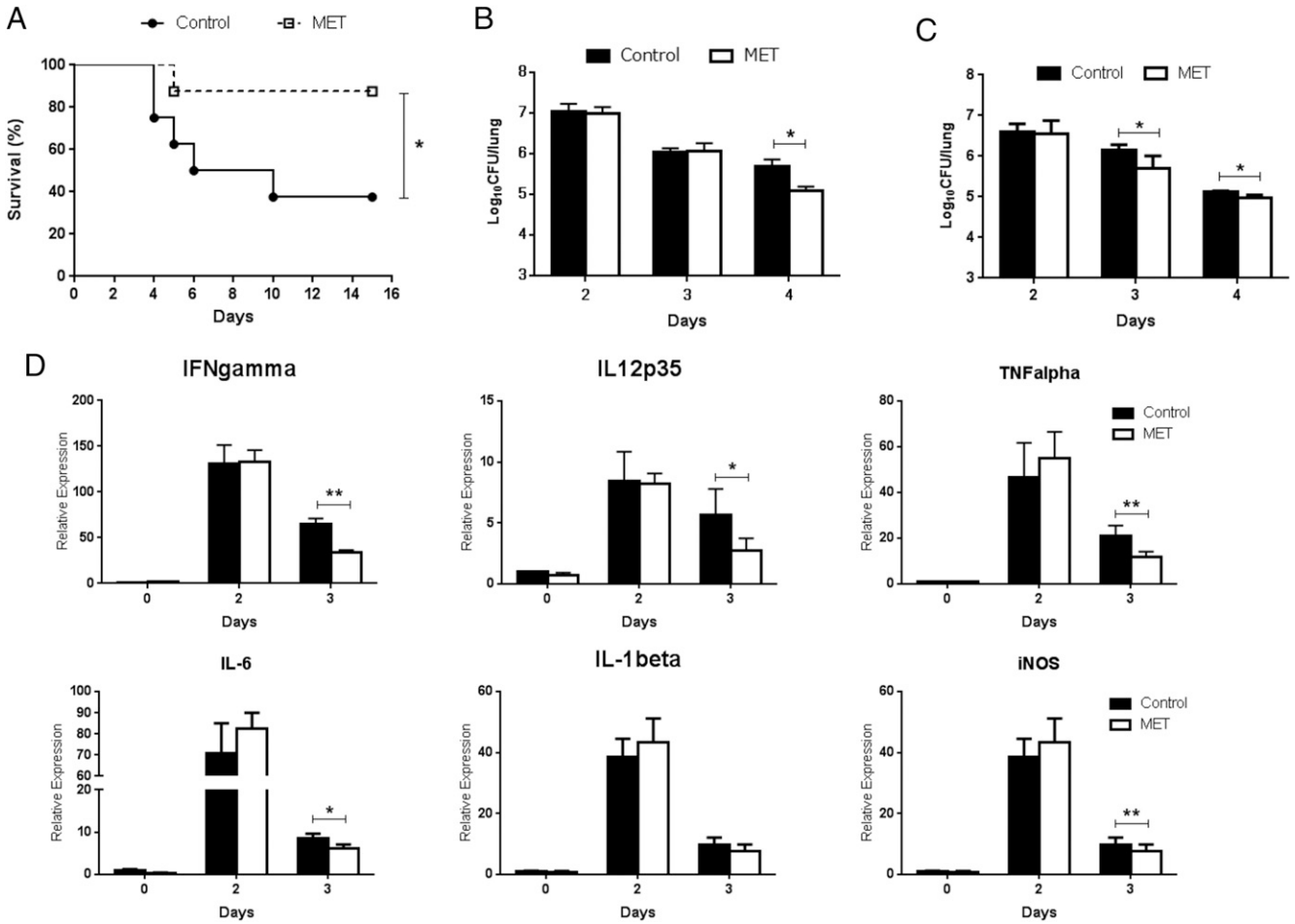


FIGURE 4. Effects of metformin on mice model of *L. pneumophila* pneumonia. (**A**) Mice were infected intratracheally with ~1.5×10^6 CFU *L. pneumophila*. The metformin treatment group received 5 mg/ml metformin in drinking water from 7 d before the infection until the end of experiment ($n = 8$ per group). (**B**) Mice were infected intratracheally with 10^5 CFU *L. pneumophila*. Numbers of bacteria in the lungs were calculated 2, 3, and 4 d postinfection. Bars indicate mean ± SD ($n = 5$ per group). (**C**) Mice were infected intratracheally with 10^5 CFU *L. pneumophila*. Mice received metformin from 7 d before until the day of infection. Numbers of bacteria in the lungs were calculated 2, 3, and 4 d postinfection. Bars indicate mean ± SD ($n = 5$ per group). (**D**) mRNA levels of inflammatory markers in the lungs were examined 2 and 3 d postinfection. The metformin group received 5 mg/ml metformin in drinking water from 7 d before until the day of infection. Data are expressed as fold increase. Bars indicate mean ± SD ($n = 5$ per group). All data in Fig. 4 were confirmed by two independent experiments. Survival curves were constructed using the Kaplan–Meier method and were analyzed using the log-rank test (A), and qRT-PCR were analyzed using the two-tailed Student *t* test (B–D). $^*p < 0.05$, $^{**}p < 0.01$.

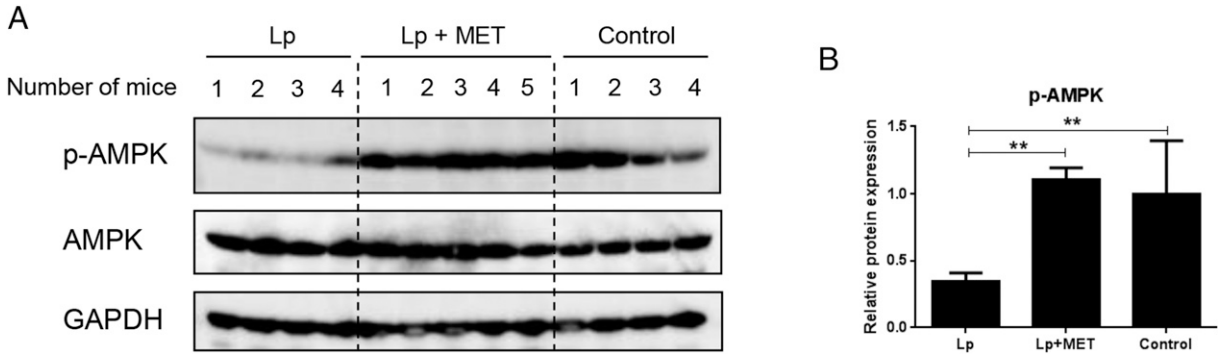


FIGURE 5. Metformin effects on phosphorylation of AMPK in lungs with *L. pneumophila* pneumonia. (**A**) Mice were inoculated intratracheally with ~1.5 × 10^5 CFU *L. pneumophila.* The mice received 5 mg/ml metformin in drinking water from 7 d before the infection. On day 3 postinfection, the lungs were removed and analyzed for p-AMPK by Western blotting (n = 4 or 5 per group). GAPDH was used as a loading control. (**B**) Bar graph of p-AMPK intensity after correction with GAPDH, as shown in (A). The results were confirmed by two independent experiments. $^{**}p < 0.01$, one-way ANOVA followed by the Tukey multiple-comparisons test.

compared with noninfected lungs. Metformin treatment significantly restored phosphorylation of AMPK in the lungs of mice with *L. pneumophila* pneumonia (Fig. 5).

Metformin enhances lung mROS in mice infected with L. pneumophila

To investigate the mechanisms of the anti–*L. pneumophila* effects of metformin in vivo, we examined the production of mROS in infected lungs. Total pulmonary cells were harvested from the lungs at day 3 of infection, and production of mROS in lung macrophages was evaluated by CellROX Green staining. As shown in Fig. 6A, *L. pneumophila* infection strongly reduced mROS, whereas restoration of mROS production by metformin was reflected in the frequency of $mROS^+$ cells and the intensity of mROS in macrophages (Fig. 6B). As expected, GSH treatment reduced mROS in macrophages during metformin treatment (Fig. 6A, 6B). These metformin effects were not observed in neutrophils (Supplemental Fig. 4). Furthermore, we observed mitigation of the metformin-mediated reduction in *L. pneumophila* burden in the lungs when the mice were treated with GSH (5 mg/ml) at 5 d postinfection (Fig. 6C). Our data demonstrated that metformin-mediated mROS induction may be one mechanism for the reduction in lung bacterial burden in mice with *L. pneumophila* pneumonia.

Discussion

The present study demonstrated a protective role for metformin in *L. pneumophila* infection in vitro and in vivo. We examined

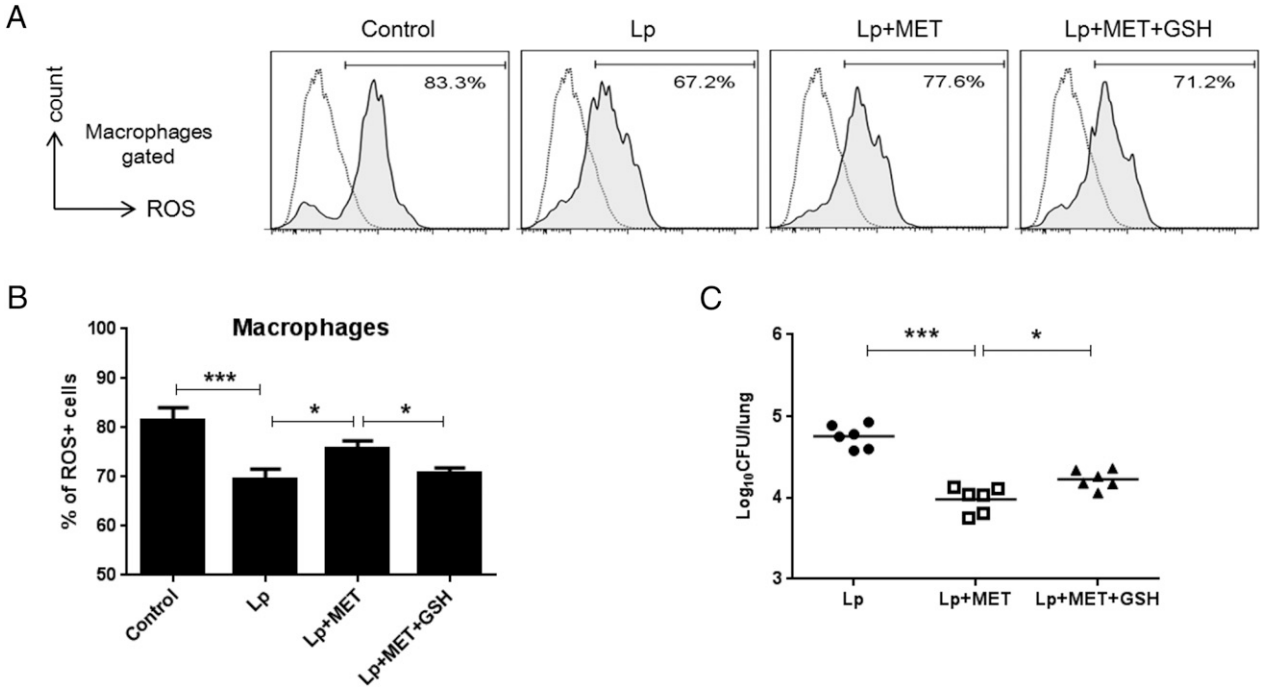


FIGURE 6. Effects of metformin on mROS production and bacterial number in the lungs. (**A**) Mice infected with ~1.5 × 10^5 CFU *L. pneumophila* received 5 mg/ml metformin and 5 mg/ml GSH in drinking water from 7 d before until the day of infection. Total lung cells were isolated from the mice on day 3 and stained for mROS by CellROX Green. The cells were stained by Abs to detect macrophages ($CD11b^+$, $F4/80^+$), washed, and fixed in 4% paraformaldehyde. The open graph in each plot indicates unstained control (n = 3 per group). The results were confirmed by two independent experiments. (**B**) Proportions of $mROS^+$ cells were calculated from data in (A). (**C**) Numbers of bacteria in lungs infected with *L. pneumophila* were enumerated on day 5 (n = 6 per group). The horizontal lines indicate mean values. The results were confirmed by two independent experiments. $^*p < 0.05$, $^{***}p < 0.001$, one-way ANOVA followed by the Tukey multiple-comparisons test.

the metformin-specific effects on macrophages and proved that activation of AMPK and mROS may be a mechanism of the anti-*Legionella* activity of metformin. These data represent another example of the host defense–modifying activity of metformin in acute *L. pneumophila* pneumonia, likely through the activation of AMPK and mROS. Because metformin effects were observed when administered prophylactically, it may be relevant to diabetes patients who were treated chronically with metformin. Also, the present data identified the possible contribution of mROS in macrophages, but not neutrophils, to protective responses in *Legionella* infection.

Although metformin has been used for many years as an oral treatment for type 2 diabetes, recent reports have described additional unanticipated effects of this drug (39). Insulin-based diabetes treatment is associated with an increased cancer risk, whereas metformin use has been shown to decrease the frequency of specific cancers (40–42). The anticancer mechanism of metformin may be attributable to effects on metabolism, cell cycle, angiogenesis, inflammation, and immunity (43). Within the tumor microenvironment, metformin can stimulate immune cells, particularly T cells. Indeed, metformin increases the number of $CD8^+$ tumor-infiltrating lymphocytes and promotes the generation of memory CD8 T cell (44). In our experiments, we have examined the effects of metformin on CD8 T cells but observed only minor changes in the frequency and activation of lymphocytes in *L. pneumophila*–infected mice (data not shown).

The anti–*M. tuberculosis* effects of metformin are believed to be largely dependent on the activation of AMPK and ROS in macrophages, which may have disparate effects on the immune response and the inflammatory response (20). The protective antimicrobial effect is mediated by increased host cell production of mROS and increased acidification of mycobacterial phagosomes (20). Notably, mROS produced upon mitochondrial recruitment to phagosomes is instrumental in the killing of intracellular bacteria by macrophages (23). Other investigators have reported the potential of AMPK activators, including metformin, to enhance chemotaxis and phagocytic ability in macrophages and neutrophils (45–47). Our data are consistent with these previous observations and further support the contributions of metformin-induced activation of AMPK and mROS to protection against *L. pneumophila* pneumonia. The AMPK activator AICAR promotes an anti-*Legionella* effect similar to metformin, whereas the AMPK blocker CC abolished metformin effects at the cellular level. In addition, treatment with GSH, a ROS scavenger, negated the metformin-induced induction of mROS and the reduction in bacterial number in vitro and in vivo. These data strongly implicate activation of AMPK and mROS as a mechanism for the anti-*Legionella* activity of metformin. Because *L. pneumophila* is an intracellular pathogen, it would be of interest to examine the effects of metformin on macrophages in other types of intracellular pathogens, including nontuberculosis mycobacteria.

It is generally accepted that metformin effects are due to AMPK, a crucial regulator of energy and homeostasis (48), which also inhibits NF-κB signaling as a negative regulator of inflammation during several infectious diseases (34). Metformin and AICAR have been reported to exert immunomodulatory activities in several pathological conditions, such as LPS-induced sepsis (49–51) and lipoteichoic acid–induced lung inflammation (52), in which activation of AMPK was suggested to be involved. Furthermore, metformin inhibited TNF-α–induced IL-6 production in endothelial cells (53) and HMGB1 release in LPS-treated cells (54). These data suggested that activated AMPK may attenuate proinflammatory responses induced by several stimuli. In the current study, the metformin-induced decreases in inflammatory cytokines, such as IFN-γ, IL-12, and TNF-α, were apparent on day 3, but not day 2, in the infected lungs. It is difficult to conclude that this is due to a direct effect of metformin on cytokine expression, because a reduction in bacterial numbers was also observed at this time point.

A previous study demonstrated that virulent *L. pneumophila*, but not less virulent strains, suppressed production of cROS in a macrophage cell line (24). In the current study, *L. pneumophila* infection inhibited AMPK activation and cROS/mROS in cultured cells, as well as dramatically suppressed AMPK and mROS in the infected lungs. It is plausible that *L. pneumophila* suppresses host AMPK and ROS as a potential virulence mechanism, and restoration of these factors by metformin may be beneficial for the host. Interestingly, our data demonstrated that metformin's effect on ROS was limited to the mitochondrial form and not NADPH-associated ROS. Further studies are required to elucidate a mechanism accounting for the differential actions of metformin on ROS-related host defense systems to identify more specific and efficient therapeutic applications for this compound.

The current study has several limitations. Metformin administration was initiated 1 wk prior to challenge with *L. pneumophila*. As such, this represents a preventative, rather than a therapeutic, strategy. Although diabetes is one of the risk factors for *L. pneumophila* pneumonia, there are no epidemiological reports comparing acquisition and frequency of *L. pneumophila* pneumonia between metformin users and nonusers in patients with type 2 diabetes (55, 56). Although serum metformin concentration is reported to be ~10 μM in humans (43), the concentration of the drug in mice administered metformin in free drinking water is not known. Also, although we focused on the effects of metformin on macrophage effector function, several other types of immune cells, such as dendritic cells and NKT cells, may also be regulated by metformin and, thus, be candidates for future investigation.

Disclosures

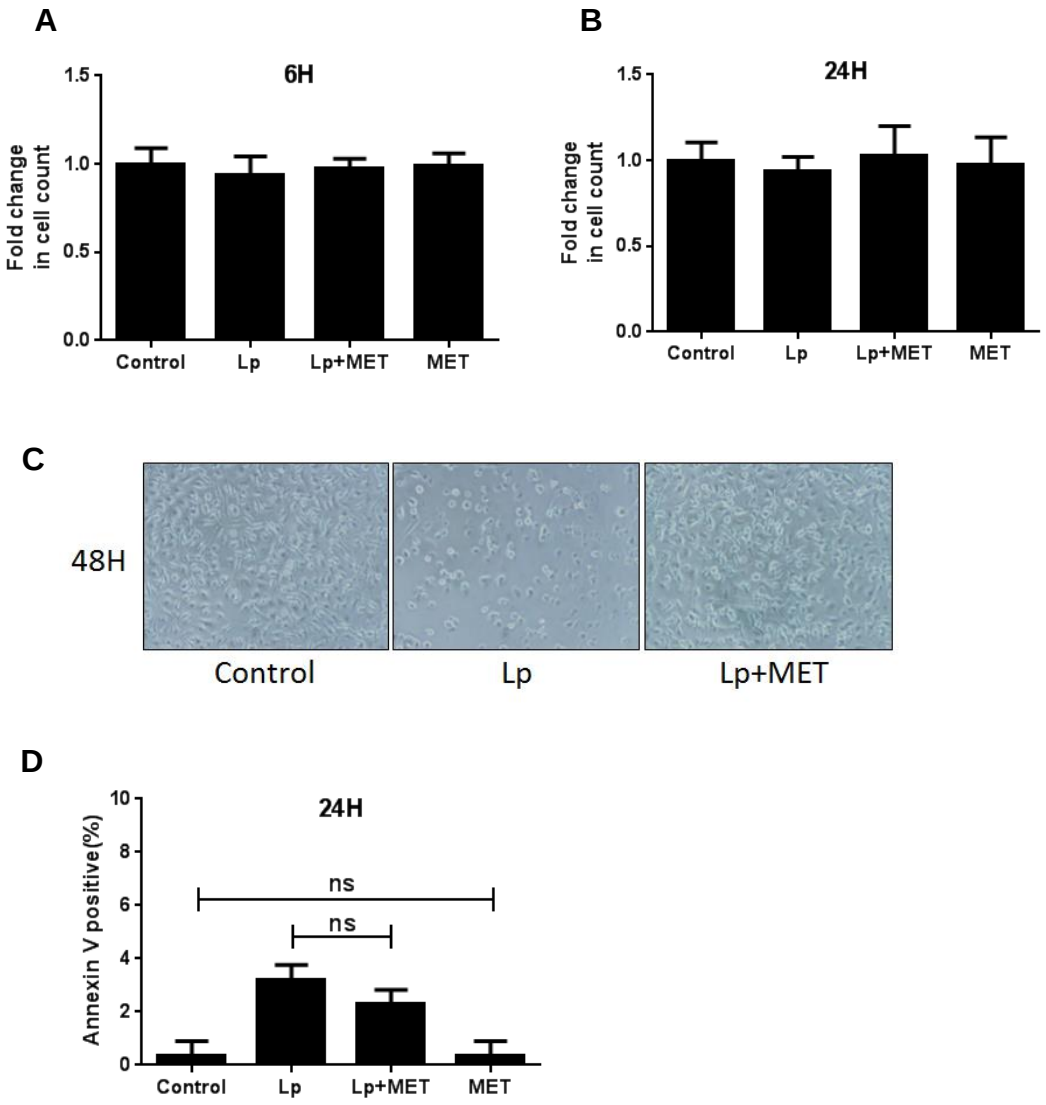
The authors have no financial conflicts of interest.

References

1. Reingold, A. L. 1988. Role of Legionellae in acute infections of the lower respiratory tract. *Rev. Infect. Dis.* 10: 1018–1028.
2. Marston, B. J., H. B. Lipman, and R. F. Breiman. 1994. Surveillance for Legionnaires' disease. Risk factors for morbidity and mortality. *Arch. Intern. Med.* 154: 2417–2422.
3. Friedman, H., Y. Yamamoto, and T. W. Klein. 2002. *Legionella pneumophila* pathogenesis and immunity. *Semin. Pediatr. Infect. Dis.* 13: 273–279.
4. Ninio, S., and C. R. Roy. 2007. Effector proteins translocated by *Legionella pneumophila*: strength in numbers. *Trends Microbiol.* 15: 372–380.
5. Shin, S., and C. R. Roy. 2008. Host cell processes that influence the intracellular survival of *Legionella pneumophila. Cell. Microbiol.* 10: 1209–1220.
6. Losick, V. P., K. Stephan, I. I. Smirnova, R. R. Isberg, and A. Poltorak. 2009. A hemidominant Naip5 allele in mouse strain MOLF/Ei-derived macrophages restricts *Legionella pneumophila* intracellular growth. *Infect. Immun.* 77: 196–204.
7. Wright, E. K., S. A. Goodart, J. D. Growney, V. Hadinoto, M. G. Endrizzi, E. M. Long, K. Sadigh, A. L. Abney, I. Bernstein-Hanley, and W. F. Dietrich. 2003. Naip5 affects host susceptibility to the intracellular pathogen *Legionella pneumophila. Curr. Biol.* 13: 27–36.
8. Yamamoto, Y., T. W. Klein, C. A. Newton, R. Widen, and H. Friedman. 1988. Growth of *Legionella pneumophila* in thioglycolate-elicited peritoneal macrophages from A/J mice. *Infect. Immun.* 56: 370–375.
9. Brieland, J., P. Freeman, R. Kunkel, C. Chrisp, M. Hurley, J. Fantone, and C. Engleberg. 1994. Replicative *Legionella pneumophila* lung infection in intratracheally inoculated A/J mice. A murine model of human Legionnaires' disease. *Am. J. Pathol.* 145: 1537–1546.
10. Bailey, C. J., and R. C. Turner. 1996. Metformin. *N. Engl. J. Med.* 334: 574–579.
11. Evans, J. M., L. A. Donnelly, A. M. Emslie-Smith, D. R. Alessi, and A. D. Morris. 2005. Metformin and reduced risk of cancer in diabetic patients. *BMJ* 330: 1304–1305.
12. Bodmer, M., C. Meier, S. Krähenbühl, S. S. Jick, and C. R. Meier. 2010. Long-term metformin use is associated with decreased risk of breast cancer. *Diabetes Care* 33: 1304–1308.
13. Bowker, S. L., S. R. Majumdar, P. Veugelers, and J. A. Johnson. 2006. Increased cancer-related mortality for patients with type 2 diabetes who use sulfonylureas or insulin. *Diabetes Care* 29: 254–258.

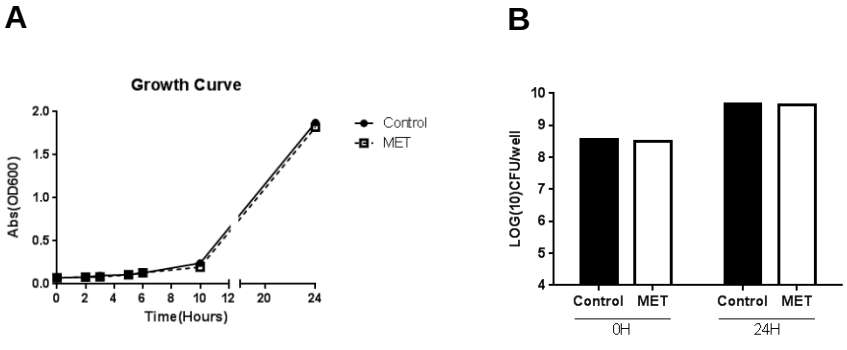
14. Libby, G., L. A. Donnelly, P. T. Donnan, D. R. Alessi, A. D. Morris, and J. M. Evans. 2009. New users of metformin are at low risk of incident cancer: a cohort study among people with type 2 diabetes. *Diabetes Care* 32: 1620–1625.
15. Sato, A., J. Sunayama, M. Okada, E. Watanabe, S. Seino, K. Shibuya, K. Suzuki, Y. Narita, S. Shibui, T. Kayama, and C. Kitanaka. 2012. Glioma-initiating cell elimination by metformin activation of FOXO3 via AMPK. *Stem Cells Transl. Med.* 1: 811–824.
16. Song, C. W., H. Lee, R. P. Dings, B. Williams, J. Powers, T. D. Santos, B. H. Choi, and H. J. Park. 2012. Metformin kills and radiosensitizes cancer cells and preferentially kills cancer stem cells. *Sci. Rep.* 2: 362.
17. Mohammed, A., N. B. Janakiram, M. Brewer, R. L. Ritchie, A. Marya, S. Lightfoot, V. E. Steele, and C. V. Rao. 2013. Antidiabetic drug metformin prevents progression of pancreatic cancer by targeting in part cancer stem cells and mTOR signaling. *Transl. Oncol.* 6: 649–659.
18. Gong, J., L. A. Robbins, A. Lugea, R. T. Waldron, C. Y. Jeon, and S. J. Pandol. 2014. Diabetes, pancreatic cancer, and metformin therapy. *Front. Physiol.* 5: 426.
19. Zhou, Z., Y. Tang, X. Jin, C. Chen, Y. Lu, L. Liu, and C. Shen. 2016. Metformin inhibits advanced glycation end products–induced inflammatory response in murine macrophages partly through AMPK activation and RAGE/NFκB pathway suppression. *J. Diabetes Res.* 2016: 4847812.
20. Singhal, A., L. Jie, P. Kumar, G. S. Hong, M. K. Leow, B. Paleja, L. Tsenova, N. Kurepina, J. Chen, F. Zolezzi, et al. 2014. Metformin as adjunct antituberculosis therapy. *Sci. Transl. Med.* 6: 263ra159.
21. Webb, T. J., G. B. Carey, J. E. East, W. Sun, D. R. Bollino, A. S. Kimball, and R. R. Brutkiewicz. 2016. Alterations in cellular metabolism modulate CD1d-mediated NKT-cell responses. *Pathog. Dis.* 74. DOI: 10.1093/femspd/ftw055.
22. Lambeth, J. D. 2004. NOX enzymes and the biology of reactive oxygen. *Nat. Rev. Immunol.* 4: 181–189.
23. West, A. P., I. E. Brodsky, C. Rahner, D. K. Woo, H. Erdjument-Bromage, P. Tempst, M. C. Walsh, Y. Choi, G. S. Shadel, and S. Ghosh. 2011. TLR signalling augments macrophage bactericidal activity through mitochondrial ROS. *Nature* 472: 476–480.
24. Harada, T., M. Miyake, and Y. Imai. 2007. Evasion of *Legionella pneumophila* from the bactericidal system by reactive oxygen species (ROS) in macrophages. *Microbiol. Immunol.* 51: 1161–1170.
25. Tateda, K., T. Matsumoto, Y. Ishii, N. Furuya, A. Ohno, S. Miyazaki, and K. Yamaguchi. 1998. Serum cytokines in patients with *Legionella* pneumonia: relative predominance of Th1-type cytokines. *Clin. Diagn. Lab. Immunol.* 5: 401–403.
26. Tateda, K., J. C. Deng, T. A. Moore, M. W. Newstead, R. Paine, III, N. Kobayashi, K. Yamaguchi, and T. J. Standiford. 2003. Hyperoxia mediates acute lung injury and increased lethality in murine *Legionella* pneumonia: the role of apoptosis. *J. Immunol.* 170: 4209–4216.
27. Byrne, B., and M. S. Swanson. 1998. Expression of *Legionella pneumophila* virulence traits in response to growth conditions. *Infect. Immun.* 66: 3029–3034.
28. Kimizuka, Y., S. Kimura, T. Saga, M. Ishii, N. Hasegawa, T. Betsuyaku, Y. Iwakura, K. Tateda, and K. Yamaguchi. 2012. Roles of interleukin-17 in an experimental *Legionella pneumophila* pneumonia model. *Infect. Immun.* 80: 1121–1127.
29. Yoshizawa, S., K. Tateda, T. Matsumoto, F. Gondaira, S. Miyazaki, T. J. Standiford, and K. Yamaguchi. 2005. *Legionella pneumophila* evades gamma interferon-mediated growth suppression through interleukin-10 induction in bone marrow-derived macrophages. *Infect. Immun.* 73: 2709–2717.
30. Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. *Methods* 25: 402–408.
31. Derré, I., and R. R. Isberg. 2004. Macrophages from mice with the restrictive Lgn1 allele exhibit multifactorial resistance to *Legionella pneumophila. Infect. Immun.* 72: 6221–6229.
32. Fortier, A., C. de Chastellier, S. Balor, and P. Gros. 2007. Birc1e/Naip5 rapidly antagonizes modulation of phagosome maturation by *Legionella pneumophila. Cell. Microbiol.* 9: 910–923.
33. Santic, M., R. Asare, M. Doric, and Y. Abu Kwaik. 2007. Host-dependent trigger of caspases and apoptosis by *Legionella pneumophila. Infect. Immun.* 75: 2903–2913.
34. Salminen, A., J. M. Hyttinen, and K. Kaarniranta. 2011. AMP-activated protein kinase inhibits NF-κB signaling and inflammation: impact on healthspan and lifespan. *J. Mol. Med. (Berl.)* 89: 667–676.
35. Luengo, A., L. B. Sullivan, and M. G. Heiden. 2014. Understanding the complex-I-ty of metformin action: limiting mitochondrial respiration to improve cancer therapy. *BMC Biol.* 12: 82.
36. Dugan, L. L., Y. H. You, S. S. Ali, M. Diamond-Stanic, S. Miyamoto, A. E. DeCleves, A. Andreyev, T. Quach, S. Ly, G. Shekhtman, et al. 2013. AMPK dysregulation promotes diabetes-related reduction of superoxide and mitochondrial function. *J. Clin. Invest.* 123: 4888–4899.
37. Zmijewski, J. W., S. Banerjee, H. Bae, A. Friggeri, E. R. Lazarowski, and E. Abraham. 2010. Exposure to hydrogen peroxide induces oxidation and activation of AMP-activated protein kinase. *J. Biol. Chem.* 285: 33154–33164.
38. Wang, S., M. Zhang, B. Liang, J. Xu, Z. Xie, C. Liu, B. Viollet, D. Yan, and M. H. Zou. 2010. AMPKalpha2 deletion causes aberrant expression and activation of NAD(P)H oxidase and consequent endothelial dysfunction in vivo: role of 26S proteasomes. *Circ. Res.* 106: 1117–1128.
39. Nikolai, S., K. Pallauf, P. Huebbe, and G. Rimbach. 2015. Energy restriction and potential energy restriction mimetics. *Nutr. Res. Rev.* 28: 100–120.
40. Kumar, S., A. Meuter, P. Thapa, C. Langstraat, S. Giri, J. Chien, R. Rattan, W. Cliby, and V. Shridhar. 2013. Metformin intake is associated with better survival in ovarian cancer: a case-control study. *Cancer* 119: 555–562.
41. Nangia-Makker, P., Y. Yu, A. Vasudevan, L. Farhana, S. G. Rajendra, E. Levi, and A. P. Majumdar. 2014. Metformin: a potential therapeutic agent for recurrent colon cancer. *PLoS One* 9: e84369.
42. Higurashi, T., K. Hosono, H. Takahashi, Y. Komiya, S. Umezawa, E. Sakai, T. Uchiyama, L. Taniguchi, Y. Hata, S. Uchiyama, et al. 2016. Metformin for chemoprevention of metachronous colorectal adenoma or polyps in post-polypectomy patients without diabetes: a multicentre double-blind, placebo-controlled, randomised phase 3 trial. *Lancet Oncol.* 17: 475–483.
43. Daugan, M., A. Dufaÿ Wojcicki, B. d'Hayer, and V. Boudy. 2016. Metformin: an anti-diabetic drug to fight cancer. *Pharmacol. Res.* 113: 675–685.
44. Eikawa, S., M. Nishida, S. Mizukami, C. Yamazaki, E. Nakayama, and H. Udono. 2015. Immune-mediated antitumor effect by type 2 diabetes drug, metformin. *Proc. Natl. Acad. Sci. USA* 112: 1809–1814.
45. Park, D. W., S. Jiang, J. M. Tadie, W. S. Stigler, Y. Gao, J. Deshane, E. Abraham, and J. W. Zmijewski. 2013. Activation of AMPK enhances neutrophil chemotaxis and bacterial killing. *Mol. Med.* 19: 387–398.
46. Bae, H. B., J. W. Zmijewski, J. S. Deshane, J. M. Tadie, D. D. Chaplin, S. Takashima, and E. Abraham. 2011. AMP-activated protein kinase enhances the phagocytic ability of macrophages and neutrophils. *FASEB J.* 25: 4358–4368.
47. Quan, H., J. M. Kim, H. J. Lee, S. H. Lee, J. I. Choi, and H. B. Bae. 2015. AICAR enhances the phagocytic ability of macrophages towards apoptotic cells through P38 mitogen activated protein kinase activation independent of AMP-activated protein kinase. *PLoS One* 10: e0127885.
48. O'Neill, L. A., and D. G. Hardie. 2013. Metabolism of inflammation limited by AMPK and pseudo-starvation. *Nature* 493: 346–355.
49. Kim, J., H. J. Kwak, J. Y. Cha, Y. S. Jeong, S. D. Rhee, K. R. Kim, and H. G. Cheon. 2014. Metformin suppresses lipopolysaccharide (LPS)-induced inflammatory response in murine macrophages via activating transcription factor-3 (ATF-3) induction. *J. Biol. Chem.* 289: 23246–23255.
50. Vaez, H., M. Najafi, M. Rameshrad, N. S. Toutounchi, M. Garjani, J. Barar, and A. Garjani. 2016. AMPK activation by metformin inhibits local innate immune responses in the isolated rat heart by suppression of TLR 4-related pathway. *Int. Immunopharmacol.* 40: 501–507.
51. Escobar, D. A., A. M. Botero-Quintero, B. C. Kautza, J. Luciano, P. Loughran, S. Darwiche, M. R. Rosengart, B. S. Zuckerbraun, and H. Gomez. 2015. Adenosine monophosphate-activated protein kinase activation protects against sepsis-induced organ injury and inflammation. *J. Surg. Res.* 194: 262–272.
52. Hoogendijk, A. J., S. S. Pinhanços, T. van der Poll, and C. W. Wieland. 2013. AMP-activated protein kinase activation by 5-aminoimidazole-4-carbox-amide-1-β-D-ribofuranoside (AICAR) reduces lipoteichoic acid-induced lung inflammation. *J. Biol. Chem.* 288: 7047–7052.
53. Huang, N. L., S. H. Chiang, C. H. Hsueh, Y. J. Liang, Y. J. Chen, and L. P. Lai. 2009. Metformin inhibits TNF-alpha-induced IkappaB kinase phosphorylation, IkappaB-alpha degradation and IL-6 production in endothelial cells through PI3K-dependent AMPK phosphorylation. *Int. J. Cardiol.* 134: 169–175.
54. Tsoyi, K., H. J. Jang, I. T. Nizamutdinova, Y. M. Kim, Y. S. Lee, H. J. Kim, H. G. Seo, J. H. Lee, and K. C. Chang. 2011. Metformin inhibits HMGB1 release in LPS-treated RAW 264.7 cells and increases survival rate of endotoxaemic mice. *Br. J. Pharmacol.* 162: 1498–1508.
55. Geerlings, S. E., and A. I. Hoepelman. 1999. Immune dysfunction in patients with diabetes mellitus (DM). *FEMS Immunol. Med. Microbiol.* 26: 259–265.
56. Den Boer, J. W., J. Nijhof, and I. Friesema. 2006. Risk factors for sporadic community-acquired Legionnaires' disease. A 3-year national case-control study. *Public Health* 120: 566–571.

Supplemental Figure 1.



Supplemental Figure 1: Effects of metformin on growth and cell death of BMDMs. **A** and **B**. BMDMs were seeded at 2 x 10^6 cells per wells in 6-well tissue culture plates (BD Falcon) for overnight. BMDMs infected with *L. pneumophila* were treated with 2 mM of metformin. After 6 h or 24 h, the cells were removed with trypsin-EDTA, mix with trypan blue solution and count the numer of live cells. The results were confirmed by three independent experiments. **C**. After 48 h of infection, cell morphology was compared in *Legionella*, *Legionella*+metformin and control (no infection). **D**. BMDMs were seeded in glass bottom dishes coated at 1 x 10^5 cells per dish and rested overnight. The cells were infected with *L. pneumophila* and then treated with 2 mM metformin for 24 h, and stained with PI and annexin V for detection of dead cells and early apoptosis cells. Samples were analyzed by fluorescence microscope. Three fields of view were taken for each sample, in each sample 100 to 200 cells per field were counted. The results were confirmed by two independent experiments, and were statistially analyzed using one-way ANOVA followed by a Tukey's multiple comparisons test.

Supplemental Figure 2.



Supplemental Figure 2: Effects of metformin on the growth of *L. pneumophila*. **A** and **B**. Growth of *L. pneumophila* (starting OD of 0.07) were examined with or without 2 mM of metformin for 24 h. At the indicated time points, growth of bacteria was measured by OD600 nm and CFU. The results were confirmed by two independent experiments.

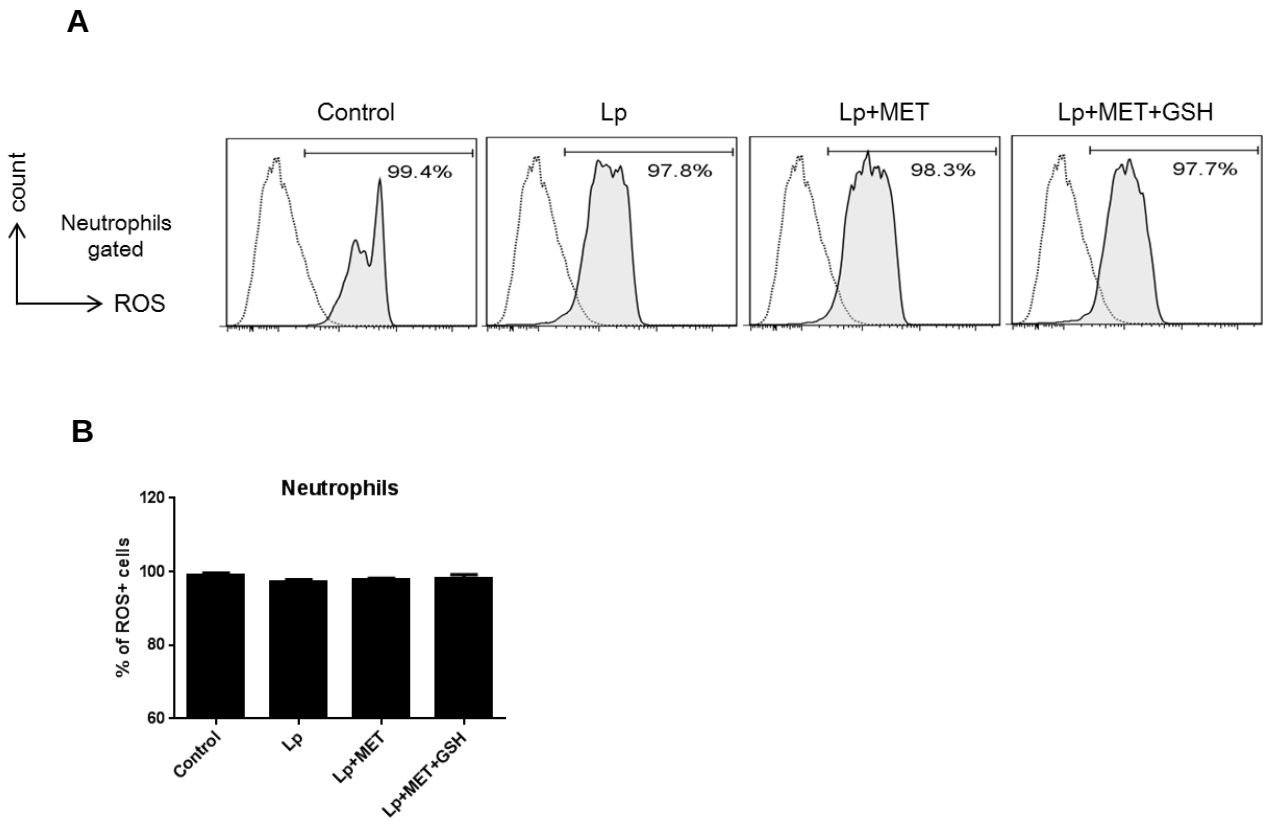
Supplemental Figure 3.

	I / U	M / I
AMPK Catalytic Subunits PrKaa2	-2.87	2.90
AMPK Regulatory Subunits Prkag2	-8.44	2.44
Acetyl-CoA carboxylase Acacb	-18.82	4.37
Liver kinase B1 Stk11	-2.01	1.23

I : Infected cells
U : Un-infected cells
M : MET-treated infected cells

Supplemental Figure 3: Effects of metformin on AMPK-related signaling molecules in PCR-array. Relative values of mRNA expression level were shown. PrKaa2, Prkag2, Acacb and Stk11 were gene names, respectively.

Supplemental Figure 4.



Supplemental Figure 4: Effects of metformin on mROS production of intrapulmonary neutrophils. **A**. Mice infected with approximately 1.5 x 10^5 CFU of *L. pneumophila* received 5 mg/mL metformin and 5 mg/ml of GSH in drinking water from 7 days before until the day of infection. Total lung cells were isolated from the mice on day 3, and stained for mROS by CellROX-Green. The cells were stained by Abs to detect neutophils ($CD11b^+$, $Ly6G^+$), then cells were washed, fixed 4% PFA. The dotted line of each plot indicates unstained control. *n* = 3 in each group. The results are confirmed by two independent experiments. **B**. Proportions of mROS positive cells were calculated from data of A. C. The total number of cells and proportions of macropheges, neutrophils in the lungs infected with *L. pneumophila* were enumerated, *n* = 3 in each group. Actual different samples were used for triplicates. The results are confirmed by two independent experiments.