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RESEARCH ARTICLE

MK2 mediates macrophage activation and acute lung injury by regulating *let-7e* miRNA

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Wu Y, He H, Ding Y, Liu S, Zhang D, Wang J, Jiang H, Zhang D, Sun L, Ye RD, Qian F. MK2 mediates macrophage activation and acute lung injury by regulating *let-7e* miRNA. *Am J Physiol Lung Cell Mol Physiol* 315: L371–L381, 2018. First published May 17, 2018; doi:10.1152/ajplung.00019.2018.—MAPK-activated protein kinase 2 (MK2) plays a critical role in the development of inflammation. However, the modulatory mechanisms in macrophage activation and acute lung injury (ALI) have not been completely defined. Here, we reported that MK2-deficient mice ($MK2^{-/-}$) protected against sepsis-induced ALI. In response to lipopolysaccharide (LPS) challenge, $MK2^{-/-}$ mice and myeloid cell-specific MK2 conditional knockout mice ($MK2^{Lyz2\text{-}KO}$) exhibited attenuated inflammatory response, especially producing fewer amounts of tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and macrophage inflammatory protein 2 (MIP-2). LPS treatment in vitro resulted in reduced cytokine expression in $MK2^{-/-}$ bone marrow-derived macrophages (BMDMs). Furthermore, we found that LPS-induced microRNA lethal-7e (*let-7e*) expression was significantly increased in $MK2^{-/-}$ macrophages. Transfection of *let-7e* antagomirs into $MK2^{-/-}$ BMDM rescued LPS-induced expression of TNF-α, IL-6, and MIP-2. In contrast, transfection of *let-7e* mimics into $MK2^{+/+}$BMDM decreased cytokine expression. Meanwhile, LPS-induced phosphorylation of cAMP response element-binding (CREB) protein, a substrate of MK2, was downregulated in $MK2^{-/-}$ BMDMs. Lin28, an inhibitory molecule of *let-7*, was significantly reduced in $MK2^{-/-}$ macrophages. Our results suggested that MK2 boosts LPS-induced macrophage activation and ALI via increasing activation of CREB and consequently, the expression of Lin28 and downregulation of *let-7e*.

acute lung injury, *let-7e*; lipopolysaccharides; macrophage; MAPK-activated protein kinase 2; miRNA; MK2

INTRODUCTION

Acute lung injury (ALI) and acute respiratory distress syndrome were first described in 1967 and are general symptoms in intensive care units with high mortality rate (46). In the early phase of ALI, inflammatory cell infiltration and leakage of edema fluid into the lung leads to lung inflammation, diffuse pulmonary interstitial and alveolar edema, and reduced lung compliance that leads to respiratory failure and death (36, 46, 50). Unfortunately, therapeutic approaches for these acute inflammatory disease and ALI are limited. Therefore, novel therapies are needed for clinical treatment of ALI.

Mitogen-activated protein kinase (MAPK)-activated protein kinase 2 (MK2) is a stress-activated serine/threonine-protein kinase that is involved in cytokine production, cell migration, cell cycle control, DNA damage response, and transcriptional regulation (20, 23, 35, 40). In response to stress, it is directly phosphorylated and activated by p38 MAPK, which drives MK2 signaling pathways in various cell types. For instance, stimulation of macrophages with the Toll-like receptor 4 (TLR4) agonist LPS, activates p38 MAPK and its substrate MK2. Subsequent MK2 activation is involved in inflammatory response by regulating production of cytokines such as TNF-α and IL-6 (16, 25). In addition, inhibition of the p38/MK2 downstream signaling pathway can inhibit inflammatory cell infiltration and the expression of inflammatory mediators, including cytokines, chemokines, and adhesion factors. It is well established that MK2 plays a critical role in the pathogenesis of acute and chronic diseases (9, 39), including arthritis (15), pancreatitis (42), skin inflammation (10), colitis (8), cardiac ischemia-reperfusion injury (38), and asthma (11) or ventilator-induced (4) lung injury. The crucial role of MK2 in regulating inflammatory factors' expression makes it an important therapeutic target for the treatment of inflammatory diseases (6).

MicroRNAs (miRNAs) are another class of gene expression regulators, which are short (18–25 nt), single-stranded noncoding RNA molecules. MiRNA binds to the 3′-untranslated region (UTR) elements of target mRNA, usually leading to mRNA instability and degradation, or recognizes 5′-promoter region to regulate gene expression (1, 2, 7, 29, 37, 45, 47, 49). Emerging evidence suggests a general role for miRNAs in regulating immunity and suppressing inflammatory reactions. For instance, an NF-κB signaling suppressor, miR (miRNA)-146, is identified as a negative regulator of the inflammatory response during microbial infection (41). Multiple members of the conserved lethal (*let*)-*7* miRNA family play important roles in regulating cell differentiation and inhibiting the development of certain diseases, including tumorigenesis and allergic lung disease (14, 19, 31). The RNA-binding protein Lin28 was found to selectively repress expression of *let-7* family miRNAs

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and rapidly reduce their levels (28, 44). *Let-7* could directly inhibit IL-6 expression, and reduced expression of *let-7* causes high levels of IL-6 to activate STAT3 and transforms epithelial cells (13). *Let-7a* could regulate the expression of IL-13, a cytokine essential for allergic lung disease (22). In addition, *let-7e* regulates TLR4 under the control of Akt1 (1). However, despite being the most abundant miRNAs in the lung (31), the potential regulatory role for *let-7* miRNAs in cytokine production and lung inflammatory processes has not been addressed.

Although the role of the MK2 pathway in regulating macrophage function has been studied in detail, we have recently been aware of a possible role for a member of *let-7* family miRNA in MK2-dependently controlled cytokine expression in LPS-challenged macrophages. In the present study, we examined the influence of MK2 gene deficiency in mice during cecal ligation and puncture (CLP)-induced sepsis as well as LPS-induced ALI. We further determined that LPS-induced inflammatory response is mediated by MK2 signaling in myeloid cells (mainly macrophages) using mice with myeloid MK2-conditional deficiency. Applying real-time reverse transcription PCR, we observed in vitro the aberrant expression of *let-7e* miRNA that could potentially contribute to MK2-mediated inflammation induced by LPS. Using gain- and loss-of-function experiments, we demonstrated the role of *let-7e* in suppressing inflammatory factor expression, especially cytokines IL-6 and TNF-α, and chemokine macrophage inflammatory protein (MIP)-2. Thus, our results provide insights into the role of MK2-regulated *let-7e* miRNA in macrophages and their contribution to the inflammation and mortality of ALI.

MATERIALS AND METHODS

Reagents and inhibitors. P38 inhibitor SB-203580 (Merck KGaA, Darmstadt, Germany), MK2 inhibitor CMPD1 (Merck KGaA), and cAMP response element-binding (CREB) protein inhibitor 666-15 (XingMo Biotechnology, Shanghai, China) were solubilized in DMSO as stock. Inhibitors were applied to cell culture 0.5 h before stimulation with PBS or LPS (100 ng/ml). Cells were collected after the indicated time and prepared for specific analysis.

The primers used in this study were synthesized by HuaGene Biotech (Shanghai, China). ELISA kits were purchased from R&D Systems (Minneapolis, MN). Other chemical reagents without special indication were obtained from Sigma.

Ethical approval of study protocol. Animal experiments were conducted in accordance with the care and use of laboratory animals from the National Institutes of Health (Bethesda, MD). The experimental and study protocols described in this study were approved by Animal Care and Use Committee of Shanghai Jiao Tong University (Shanghai, China).

Mice. The MK2-deficient mice (C57BL/6; B6.129P2-Mapkapk2$^{tm1Dgen/J}$, called MK2$^{-/-}$ here) and MK2$^{flox/flox}$ mice (C57BL/6; B6.Cg-Mapkapk2$^{tm1.1Yaff/J}$) were purchased from Jackson Laboratory (stock nos. 005850 and 024176, Sacramento, CA). All mice have been crossed back to C57BL/6 background at least 10 generations. Lyz2-cre knock-in mice were purchased from the model animal research center of Nanjing University (Nanjing, China). Myeloid cell-specific MK2-deficient mice (MK2$^{Lyz2\text{-}KO}$) were generated by mating MK2$^{flox/flox}$ mice with Lyz2-cre knock-in mice (C57BL/6) as described (3). MK2$^{+/+}$, MK2$^{-/-}$, and MK2$^{Lyz2\text{-}KO}$ mice were bred and maintained until 8–10 wk in the animal facility of Shanghai Jiao Tong University. Mice were housed five per cage in a climate-controlled room (25°C, 55% humidity, and 12-h:12-h light-dark cycle).

CLP-induced sepsis. Polymicrobial sepsis was induced using the CLP method as described previously. Mice were anesthetized with intraperitoneal injection of pentobarbital sodium (5 mg/kg), followed by disinfection of the abdominal area after shaving. A 1.0-cm midline incision was made to expose the cecum under aseptic conditions. The ligation was performed with a 3-0 surgical silk at a distance of ~0.5–1.0 cm from the blind-ending cecum avoiding bowel obstruction. The distal cecum was then punctured with a needle (18 gauge). The cecum was gently squeezed to expel a small amount of feces, followed by cecum repositioning and skin closure.

LPS-induced sepsis. LPS from *Escherichia coli* (serotype 0111:B4, Sigma-Aldrich) were diluted in sterile PBS. For the induction of sepsis, MK2$^{+/+}$ and MK2$^{-/-}$ mice were grouped and intraperitoneally injected with 15 mg/kg LPS for survival assay. In addition, 5 mg/kg LPS was intraperitoneally administrated to establish sepsis-induced ALI mouse model. Each group contained at least five mice. Mice in the control group only received sterile PBS injection without LPS challenge. Mice were euthanized by CO_2 inhalation and samples were collected.

LPS-induced ALI. For the induction of ALI, wild-type (MK2$^{Lyz2\text{-}WT}$) and MK2$^{Lyz2\text{-}KO}$ mice were intratracheally injected with sterile PBS or 5 mg/kg LPS for 6 h. Each group contained at least five mice. Subsequently, mice were euthanized by CO_2 inhalation, and samples were collected.

Isolation of bone marrow-derived macrophages. Femoral and tibia bone marrow was isolated from MK2$^{+/+}$ and MK2$^{-/-}$ mice as previously described (33). Mouse bone marrow cells were flushed from femurs and tibias with PBS and then cultured in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, and 10% L929 cell-conditioned medium (or 10 ng/ml macrophage colony-stimulating factor) for 6 days. Then bone marrow-derived macrophages (BMDMs) were collected and prepared for subsequent stimulation and analysis.

Isolation of lung macrophages. The lungs were collected and perfused with sterile PBS. Macrophages were isolated from the whole lungs of mice via collagenase digestion [collagenase (1.0 mg/ml), DNase (25–50 U/ml)] as previously described (26).

Acquisition and analysis of bronchoalveolar lavage fluid. The lungs were lavaged three times with 1 ml PBS and bronchoalveolar lavage (BAL) fluid was centrifuged at 4°C. The cell-free supernatant was harvested for total protein analysis using the bicinchoninic acid protein assay kit (Beyotime, Shanghai, China).

Myeloperoxidase activity assay. The largest right lobes of the lungs were collected and subjected to three freeze-thaw cycles. Supernatants were collected at 4°C. Protein concentrations of the supernatants were determined as mentioned above. After adding substrate and catalyst into the supernatant, the change of absorbance at 655 nm was measured by the microplate reader (FlexStation 3, Molecular Devices). Myeloperoxidase (MPO) activity value was defined as the absorbance change per min per gram protein.

Histopathology. Lung tissues (left lobe) were fixed by 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm-thick sections in a microtome (RM-2235, Leica Biosystems, Wetzlar, Germany). Sections were stained with hematoxylin-eosin and images were captured by a microscope (RX-51, Olympus Optical, Tokyo, Japan).

Lung wet weight-to-dry weight ratio. Lung tissues were obtained after 18 h treatment of LPS and weighed immediately after removal (wet weight). Lungs were then heated at 80°C for 48 h to obtain the dry weight. The wet-to-dry (W/D) weight ratios were calculated to assess the lung edema.

ELISA. The concentrations of TNF-α, IL-6, and MIP-2 in serum, BAL fluid, or the supernatant of cell culture were quantified using ELISA kits (R&D Systems) according to the manufacturer's instructions. All the experiments were performed in triplicate.

Isolation of total RNA, reverse transcription, and quantitative PCR. Total cellular RNA was extracted from BMDMs using TRIzol Reagent (Invitrogen, Carlsbad, CA). Frozen lungs (one of right upper lobe) were homogenized and total RNA was isolated as described above. cDNA was prepared by ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) and amplified by real-time PCR on StepOne Plus

(Thermo Fisher Scientific, Waltham, MA) with primer sets for *let-7e* (forward, CATTCTCTCAGATGAGGTAGGAGG; reverse, TATGGTTGTTCTGCTCTCTGTGTC), U6 small nuclear 6 (snRNA RNU6B, referred to as U6, forward, ATTGGAACGATACAGAGAAGATT; reverse, GGAACGCTTCACGAATTTG).

Western blot analysis. Western blot analysis was conducted to detect protein expression as preciously described (32). BMDMs were plated on 6-well plates (1.0×10^6 cells per well) and incubated overnight (37°C, 5% CO_2). BMDMs were then stimulated with 100 ng/ml LPS for 0–8 h. Cells were collected and lysed in radioimmunoprecipitation assay buffer (WEIAO BioTech Co. Ltd, Shanghai, China) with 1 mM PMSF. The same amount of proteins was loaded on 10% SDS/PAGE gels, then transferred to PVDF membrane (Millipore, Bedford, MA), followed by incubation with indicated primary antibodies overnight at 4°C. The following antibodies were used as primary antibodies for Western blot analysis: The anti-Lin28 was ordered from Abcam (Burlingame, CA). The anti-p-CREB, anti-p-MK2, anti-MK2, anti-β-actin, and GAPDH were purchased from Cell Signaling Technology, Danvers, MA). Quantification of Western blots was performed with ImageJ software (National Institute of Mental Health, Bethesda, MD).

Flow cytometry assay. Cells collected from BAL fluid were incubated with Fc-blocking anti-mouse CD16/32 antibody (BD Biosciences, San Jose, CA) followed by incubation with FITC-conjugated anti-Ly-6G antibody (BD Biosciences). Cells were then analyzed on a flow cytometer (BD LSRFortessa, BD Biosciences) and data were analyzed with FlowJo software (FlowJo).

Transfection of Let-7e mimic or antagomir. BMDMs isolated from $MK2^{+/+}$ or $MK2^{-/-}$ mice were seeded in 24-well plates and were transfected with 50 nM *let-7e* mimic or 100 nM *let-7e* antagomir (GenePharma, Shanghai, China) or with a scrambled miRNA (GenePharma) by using HiperFect transfection reagent (Qiagen, Valencia, CA). Cells were challenged with LPS for 4 h after 24 h transfection. The supernatant and cell lysate were collected, respectively. ELISA assays and real-time PCR analysis were performed as described above.

Plasmid. The plasmids were constructed basing on PGL3 luciferase reporter vectors (Promega, Madison, WI). The 3′-UTR (778 bp) or 5′-promoter region (2,000 bp) of murine TNF-α were cloned into PGL3, respectively. The primer set for PGL3-mTNF-α-3′UTR: (forward: 5′-GATATGGTACCAGGGAATGGGTGTTCATCCA-3′, reverse 5′-GATATAAGCTTTCACATTTCTTTTCCAAGCGAT-3′); PGL3-mTNF-α-promoter (forward: 5′-GATATGGTACCTGGAGAAAGCTGCTCCCC-3′, reverse: 5′-GATATAAGCTTGGCTGGCTGTGCAGACG-3′).

Luciferase assay. Human embryonic kidney 293 T cells were seeded in 24-well plates and PGL3-mTNF-α-3′UTR or PGL3-mTNF-α-promoter were cotransfected with 50 nM *let-7e* mimic (GenePharma) or a scrambled miRNA (con mimic, GenePharma) by using Lipofectamine 2000 reagent (Invitrogen). Cell lysates were collected after 24-h transfection, and luciferase activity was measured using Luciferase Reporter Assay System Kit (Promega).

Statistical analysis. Data are presented as means ± SE obtained from at least three independent tests. Student's *t*-test (paired comparison) was performed using Prism 5 (GraphPad, San Diego, CA). $P < 0.05$ was considered statistically significant.

RESULTS

MK2-deficient mice are more resistant to sepsis induced by CLP and LPS. To understand the effects of MK2 in sepsis, we treated $MK2^{+/+}$ and $MK2^{-/-}$ mice with CLP or LPS to establish sepsis model. The expression of MK2 was measured by Western blotting in lung tissues of $MK2^{+/+}$ and $MK2^{-/-}$ mice, indicating MK2 is completely deficient in the lung tissues of $MK2^{-/-}$ mice (Fig. 1*A*). A survival assay was performed using CLP or intraperitoneally injected with LPS (15 mg/kg), respectively. Seventy-two hours after surgery, CLP induced 90% mortality in $MK2^{+/+}$ mice, whereas only 45% mortality in $MK2^{-/-}$ mice (Fig. 1*B*). For a long-term examination, the mortality of $MK2^{-/-}$ mice was increased but did not reach the mortality rate of $MK2^{+/+}$ mice (Fig. 1*B*). Furthermore, we carried out a survival assay in another sepsis model in which LPS (15 mg/kg) treatment caused 70% mortality in $MK2^{+/+}$ mice. In contrast, none of the $MK2^{-/-}$ mice died during 7 days after intraperitoneal injection of LPS (Fig. 1*C*). Collectively, these data indicated that MK2 deficiency protects against sepsis.

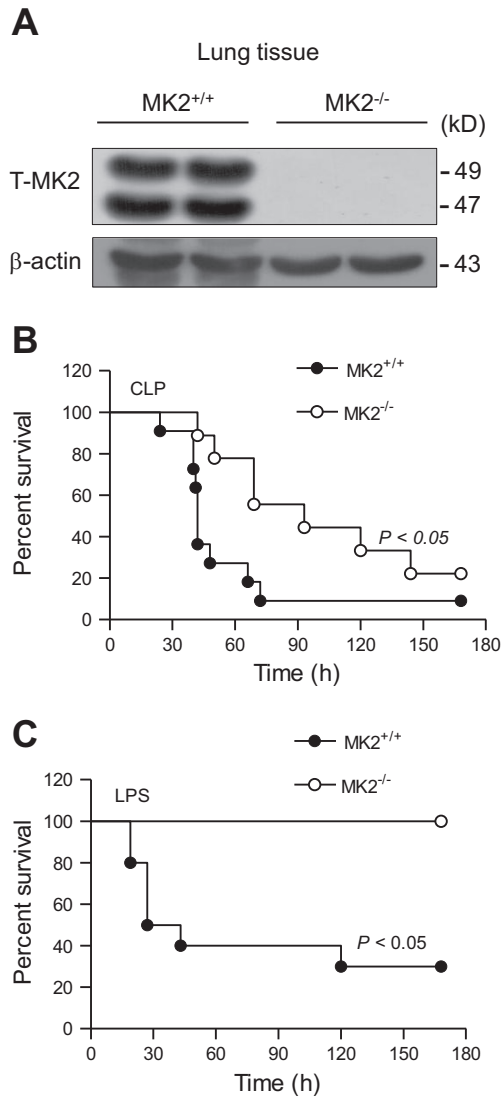

Fig. 1. MK2-deficient mice protect against CLP- and LPS-induced sepsis. *A*: lung lobes isolated from wild-type ($MK2^{+/+}$) and MK2-deficient ($MK2^{-/-}$) mice. The expression level of total MK2 protein was determined by Western blotting. β-actin was used as the loading control. *B*: survival curves of $MK2^{+/+}$ ($n = 11$) and $MK2^{-/-}$ ($n = 9$) mice that were subjected to CLP surgery. *C*: survival curves of $MK2^{+/+}$ ($n = 10$) and $MK2^{-/-}$ ($n = 10$) mice that were injected intraperitoneally with LPS (15 mg/kg of body weight). n = number of mice in each group. CLP, cecal ligation and puncture; MK2, MAPK-activated protein kinase 2; T-MK2, total MK2.

MK2 deficiency attenuates sepsis-induced ALI. To determine whether MK2 plays a critical role in sepsis-induced ALI, we treated $MK2^{-/-}$ and $MK2^{+/+}$ mice with intraperitoneal ad-

ministration of LPS (5 mg/kg). Mice were killed, and samples were collected at 6 and 18 h. The histologic assay displayed that LPS-induced ALI was significantly ameliorated in $MK2^{-/-}$ mice compared with that in $MK2^{+/+}$ mice, including decreased neutrophil infiltration and reduced thickening of interstitial alveolar regions (Fig. 2*A*). MPO activity of lung tissue is a specific marker representing the degree of neutrophil infiltration and the severity of pulmonary inflammatory injury. MPO activity was increased after LPS administration for 6 and 18 h in $MK2^{+/+}$ mice, which was greatly alleviated in the lungs of $MK2^{-/-}$ mice (Fig. 2*B*). The total protein concentrations in BAL fluid, a marker of microvascular permeability, raised ~four- to eightfold (Fig. 2*C*) in $MK2^{+/+}$ mice after 6 and 18 h of LPS challenge. Consistent with MPO activity assay, the $MK2^{-/-}$ mice showed reduced protein concentration compared with $MK2^{+/+}$ mice (Fig. 2*C*). In addition, the pulmonary edema was also assessed by measuring lung W/D ratio. After LPS challenge for 18 h, the lung W/D ratio was remarkably increased in $MK2^{+/+}$ mice, whereas the lungs of $MK2^{-/-}$ mice showed no significant change of W/D ratio (Fig. 2*D*). These results demonstrated that MK2 deficiency could protect against sepsis-induced lung inflammation.

MK2 promotes LPS-induced cytokine production in vivo. To determine the role of MK2 in the inflammatory cytokine production, we collected serum at 0, 3, 6, and 18 h after LPS treatment. ELISA assay was performed to determine the protein levels of TNF-α, IL-6, and chemokine MIP-2. At 3 and 6 h post-LPS administration, markedly elevated expressions of TNF-α (Fig. 3*A*), IL-6 (Fig. 3*B*), and MIP-2 (Fig. 3*C*) were detected in the serum from $MK2^{+/+}$ mice, which were substantially lower in that from $MK2^{-/-}$ mice (Fig. 3, *A*–*C*).

MK2 deficiency reduces cytokine expression in BMDMs in response to LPS challenge. To determine whether MK2 modulates macrophage activation, we isolated BMDMs from $MK2^{+/+}$ and $MK2^{-/-}$ mice and stimulate them with LPS (100 ng/ml) for 2 and 6 h. LPS challenge significantly induced phosphorylation of MK2, which was completely eliminated in $MK2^{-/-}$ BMDMs (Fig. 4*A*). LPS-induced production of TNF-α and MIP-2 was significantly decreased in $MK2^{-/-}$ BMDMs in comparison with that in $MK2^{+/+}$ BMDMs (Fig. 4, *B* and *C*). These results indicated that MK2 is involved in inflammatory cytokine and chemokine production both in vivo and in vitro.

Let-7e miRNA is upregulated in MK2 deficient BMDMs challenged with LPS and participates in regulating inflammatory factor production. Given MK2 deficiency protected against LPS- and microbial-induced sepsis and inflammatory injury, we investigated whether miRNA is involved in MK2-dependant inflammatory response. As shown in Fig. 5*A*, in

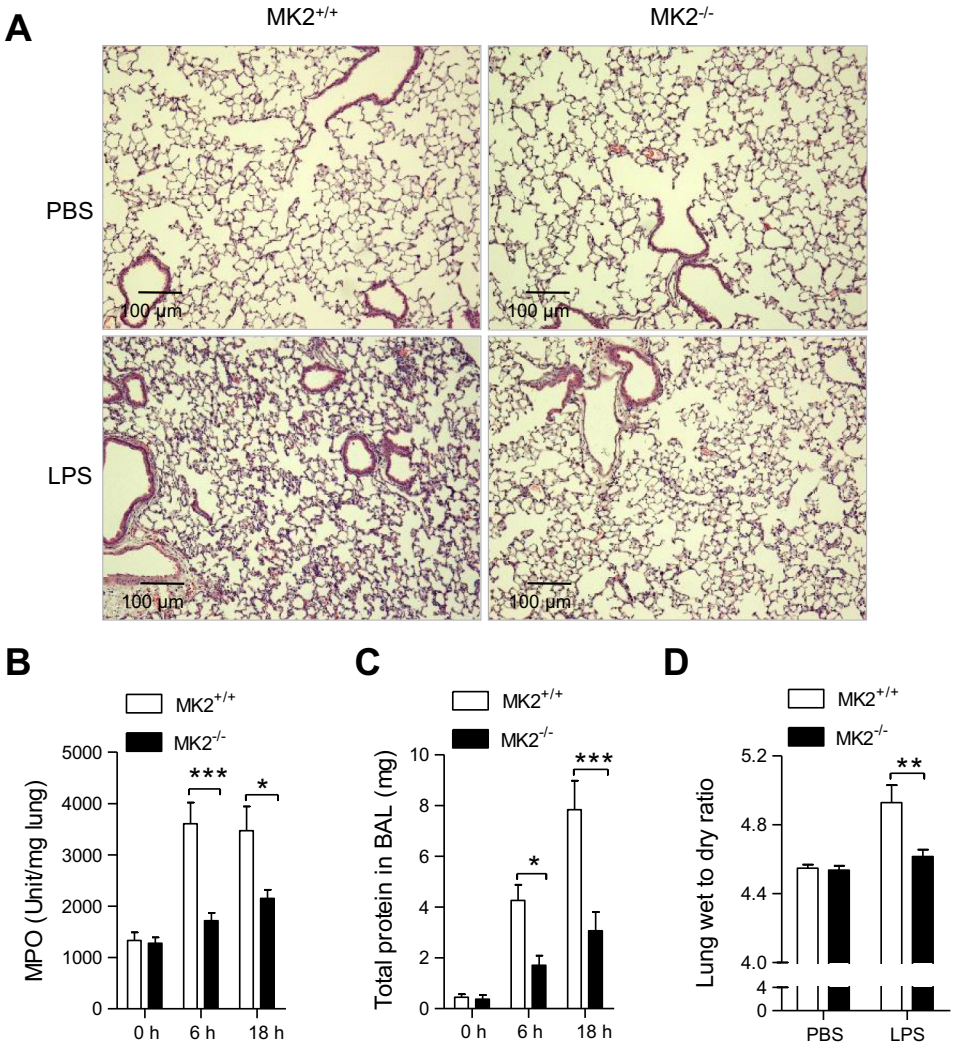

Fig. 2. $MK2^{-/-}$ mice show attenuated acute lung injury in response to LPS challenge. $MK2^{+/+}$ and $MK2^{-/-}$ mice were intraperitoneally injected with 5 mg/kg of LPS or equal volume of PBS. After 6 and 18 h, the lung lobes were collected and prepared for pathological analysis. *A*: hematoxylin-eosin staining was performed to illustrate the infiltration of inflammatory cells. The representative changes of the lungs of mice were shown with original magnification (×200). *B*: myeloperoxidase (MPO) activity in lung tissues from $MK2^{+/+}$ and $MK2^{-/-}$ mice was determined. *C*: total protein concentration in BAL fluid from $MK2^{+/+}$ and $MK2^{-/-}$ mice was measured. *D*: wet to dry ratio of lung lobes from $MK2^{+/+}$ and $MK2^{-/-}$ mice was determined after treatment with LPS for 18 h. $n = 5$ mice in each group. Data are shown as mean ± SE $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed Student *t*-test. BAL fluid, bronchoalveolar lavage fluid; MK2, MAPK-activated protein kinase 2.

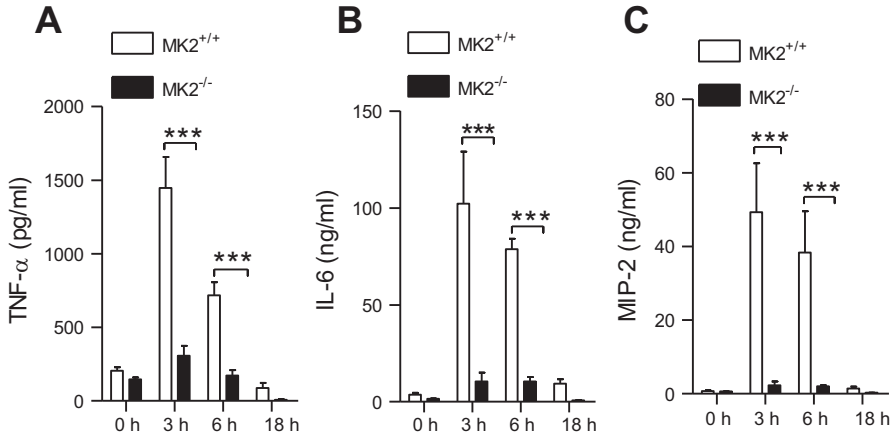

Fig. 3. MK2$^{-/-}$ mice display reduced production of TNF-α, IL-6, and MIP2 in response to LPS challenge. MK2$^{+/+}$ and MK2$^{-/-}$ mice were intraperitoneally injected with 5 mg/kg of LPS (n = 5). After 0, 3, 6, and 18 h treatment, the serum samples were collected and inflammatory cytokines TNF-α (*A*), IL-6 (*B*), and chemokine MIP-2 (*C*) were measured by ELISA kits. n = number of mice in each group. Data shown as means ± SE ***$P < 0.001$, two-tailed Student t-test. MIP-2, macrophage inflammatory protein 2; MK2, MAPK-activated protein kinase 2.

response to LPS stimulation, MK2 deficiency enhanced the expression of *let-7e* miRNA to ~3-fold at 8 h and over 10-fold at 24 h. In addition, we also challenged BMDMs isolated from MK2$^{Lyz2\text{-}WT}$ and MK2$^{Lyz2\text{-}KO}$ mice with LPS for 0, 8, and 24 h, and the expression of *let-7e* was increased in BMDMs from MK2$^{Lyz2\text{-}KO}$ mice (Fig. 5*B*).

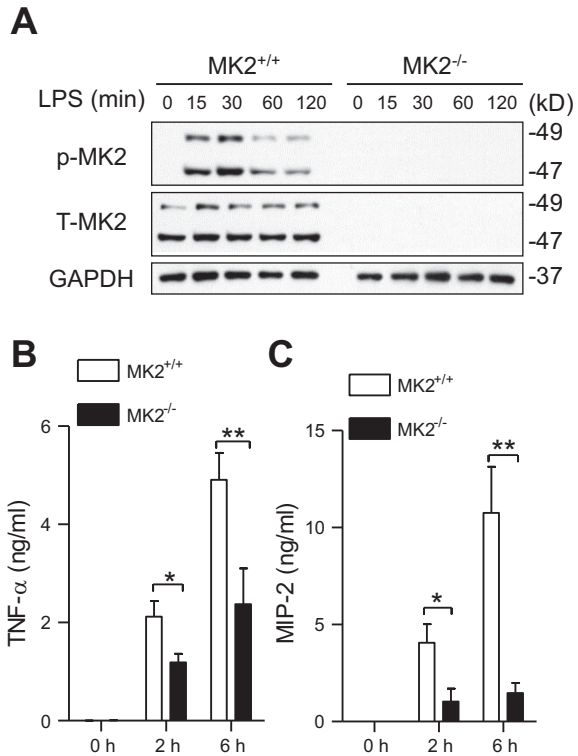

Fig. 4. MK2-deficient BMDMs produce less cytokines in response to LPS stimulation. BMDMs (1×10^6 cells/well) isolated from MK2$^{+/+}$ and MK2$^{-/-}$ mice were challenged with 100 ng/ml of LPS for 0, 15, 30, 60, and 120 min (*A*). Then Western blotting was performed to detect the expression of phosphorylated MK2 (p-MK2), total MK2 (T-MK2), and GAPDH. BMDMs were isolated and stimulated with LPS (100 ng/ml) for 0, 2, and 6 h, respectively (n = 3). The supernatants were collected for detection of TNF-α (*B*) and MIP-2 (*C*) production by ELISA kits. Data shown as means ± SE *$P < 0.05$, **$P < 0.01$, two-tailed Student t-test. n = number of mice in each group. BMDMs, bone marrow-derived macrophages; MIP-2, macrophage inflammatory protein 2; MK2, MAPK-activated protein kinase 2.

To determine the role of *let-7e* in MK2-mediated inflammation, we transfected *let-7e* mimic into MK2$^{+/+}$ BMDMs for 24 h, in which *let-7e* was successfully overexpressed in BMDMs (Fig. 5*C*). Then, *let-7e* mimic was transfected into MK2$^{+/+}$ BMDMs, and *let-7e* antagomir was transfected into MK2$^{-/-}$ BMDMs. Upon treatment with LPS for 4 h, *let-7e* mimic reduced the production of inflammatory cytokines TNF-α (Fig. 5*D*), IL-6 (Fig. 5*E*), and chemokine MIP-2 (Fig. 5*F*) in MK2$^{+/+}$ BMDMs; *let-7e* antagomir rescued the production of TNF-α (Fig. 5*G*), IL-6 (Fig. 5*H*), and MIP-2 (Fig. 5*I*) in MK2$^{-/-}$ BMDMs. Furthermore, to determine how *let-7e* regulate cytokine production, we cotransfected human embryonic kidney 293 T cells with PGL3-mTNF-α-3′UTR plasmid or PGL3-mTNF-α-promoter plasmid and 50 nM of *let-7e* mimic or a scrambled miRNA. After transfection for 24 h, cell lysates were collected, and luciferase activity was measured. *Let-7e* inhibited TNF-α expression by targeting the promoter of TNF-α (Fig. 5, *J* and *K*) but did not have effect on 3′-UTR of TNF-α (Fig. 5*L*). Taken together, these data suggested that MK2 mediates LPS-induced inflammatory factor production via regulating the expression of *let-7e* miRNA.

CREB and Lin28 are involved in MK2-dependent regulation of let-7e expression. Lin28, an RNA-binding protein, was reported to inhibit selectively the biogenesis of *let-7* family miRNA. To determine whether MK2 regulates *let-7e* expression through Lin28, we compared the Lin28 protein level in MK2$^{+/+}$ and MK2$^{-/-}$ BMDMs that were treated with LPS for 0, 0.25, 0.5, 1, 2, 4, and 8 h. Western blotting analysis showed that LPS induced the expression of Lin28 in MK2$^{+/+}$ BMDMs (Fig. 6*A*). Compared with MK2$^{+/+}$ BMDMs, in response to LPS administration, the expression of Lin28 was decreased in BMDMs from MK2$^{-/-}$ mice (Fig. 6, *A* and *C*), which indicates that MK2 deficiency caused a diminished expression of Lin28. The phosphorylation of CREB, a substrate of MK2, was reduced in MK2$^{-/-}$ BMDMs (Fig. 6, *A* and *B*). Pretreatment with 5 μM of 666-15 (CREB inhibitor), 10 μM of CMPD1 (MK2 inhibitor), and 10 μM of SB203580 (p38 inhibitor) for 0.5 h decreased the expression of Lin28 in MK2$^{+/+}$ BMDMs (Fig. 6, *D*, *F*, *G*, and *H*). Accordingly, LPS-induced *let-7e* miRNA expression in MK2$^{+/+}$ BMDMs was increased by treatment of these inhibitors (Fig. 6*E*), suggesting that MK2 regulates *let-7e* through inducing the expression of Lin28, which was probably dependent on phosphorylated CREB.

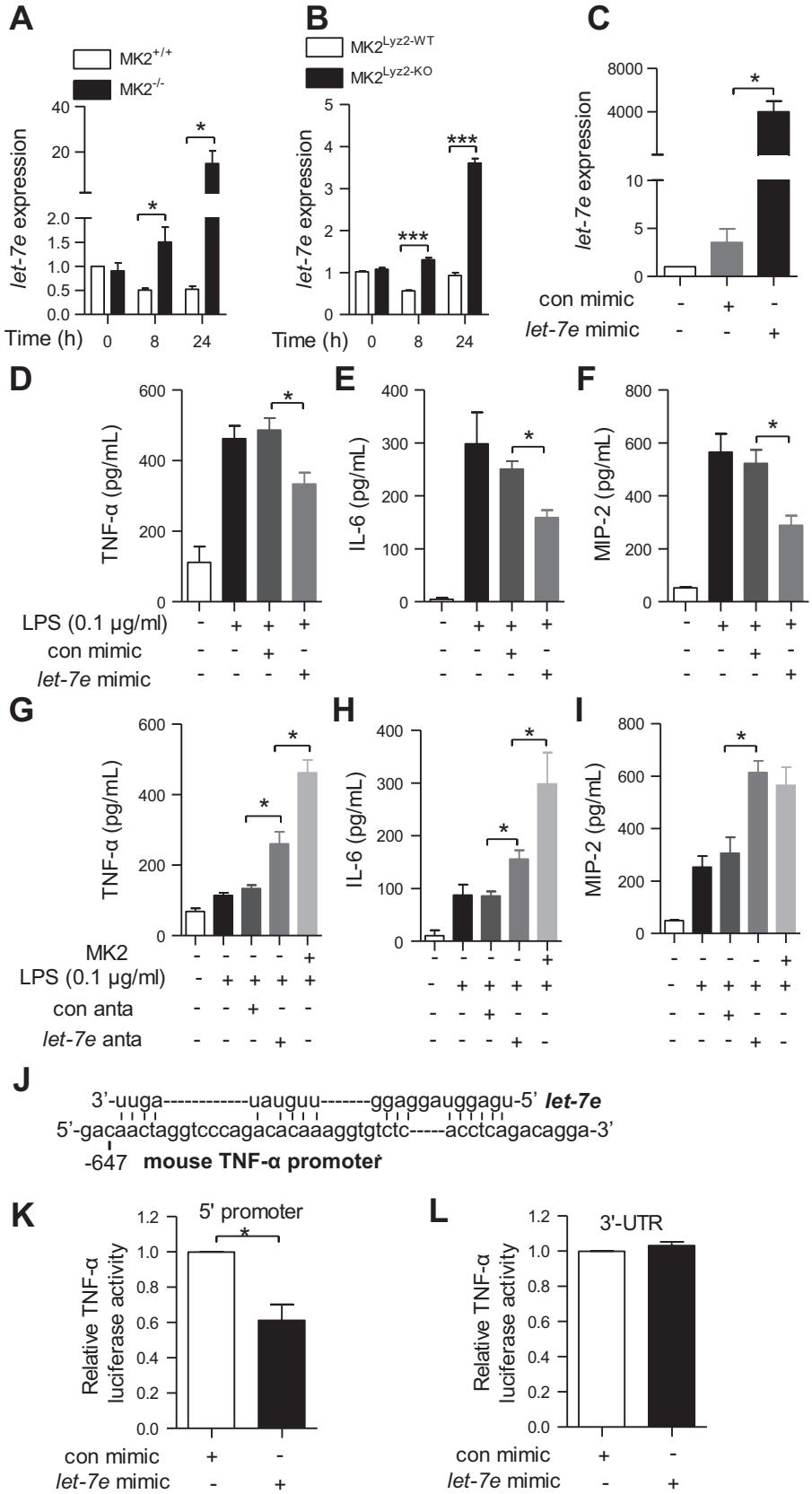

Fig. 5. The miRNA *let-7e* is involved in MK2-induced inflammatory cytokine production. BMDMs isolated from $MK2^{+/+}$ and $MK2^{-/-}$ mice (*A*) or $MK2^{Lyz2\text{-}WT}$ and $MK2^{Lyz2\text{-}KO}$ (*B*) mice were stimulated with LPS (100 ng/ml) for indicated time periods (0, 8, and 24 h). The RNA level of *let-7e* was analyzed by quantitative real-time PCR and normalized by the expression of U6. $MK2^{+/+}$ BMDMs were transfected with *let-7e* mimic or miRNA precursor negative control (con mimic) for 24 h using HiperFect transfect reagents (*C*). The level of *let-7e* was detected by quantitative real-time PCR. $MK2^{+/+}$ BMDMs were transfected with *let-7e* mimic or con mimic for 24 h, followed by treatment with LPS (100 ng/ml) for 4 h. The production of TNF-α (*D*), IL-6 (*E*), and MIP-2 (*F*) in supernatant was measured by ELISA. BMDMs from $MK2^{-/-}$ (−) mice were transfected with *let-7e* antagomir (*let-7e* anta) or miRNA precursor negative control (con anta) for 24 h and then challenged with LPS (100 ng/ml) for 4 h. The production of TNF-α (*G*), IL-6 (*H*), and MIP-2 (*I*) in supernatant was measured by ELISA. BMDMs from $MK2^{+/+}$ (+) mice stimulated with LPS for 4 h were used as positive control. The base pairs of mouse TNF-α promoter and *let-7e* (*J*). PGL3-mTNF-α-promoter plasmid (*K*) and PGL3-mTNF-α-3′UTR plasmid (*L*) were cotransfected with 50 nM *let-7e* mimic or a scrambled miRNA for 24 h, cell lysates were collected and luciferase activity was measured. Values represent means ± SE, $n \geq 3$, $^*P < 0.05$, $^{***}P < 0.001$, two-tailed Student *t*-test. BMDMs, bone marrow-derived macrophages; *let-7e*, lethal-7e; MIP-2, macrophage inflammatory protein 2; MK2, MAPK-activated protein kinase 2.

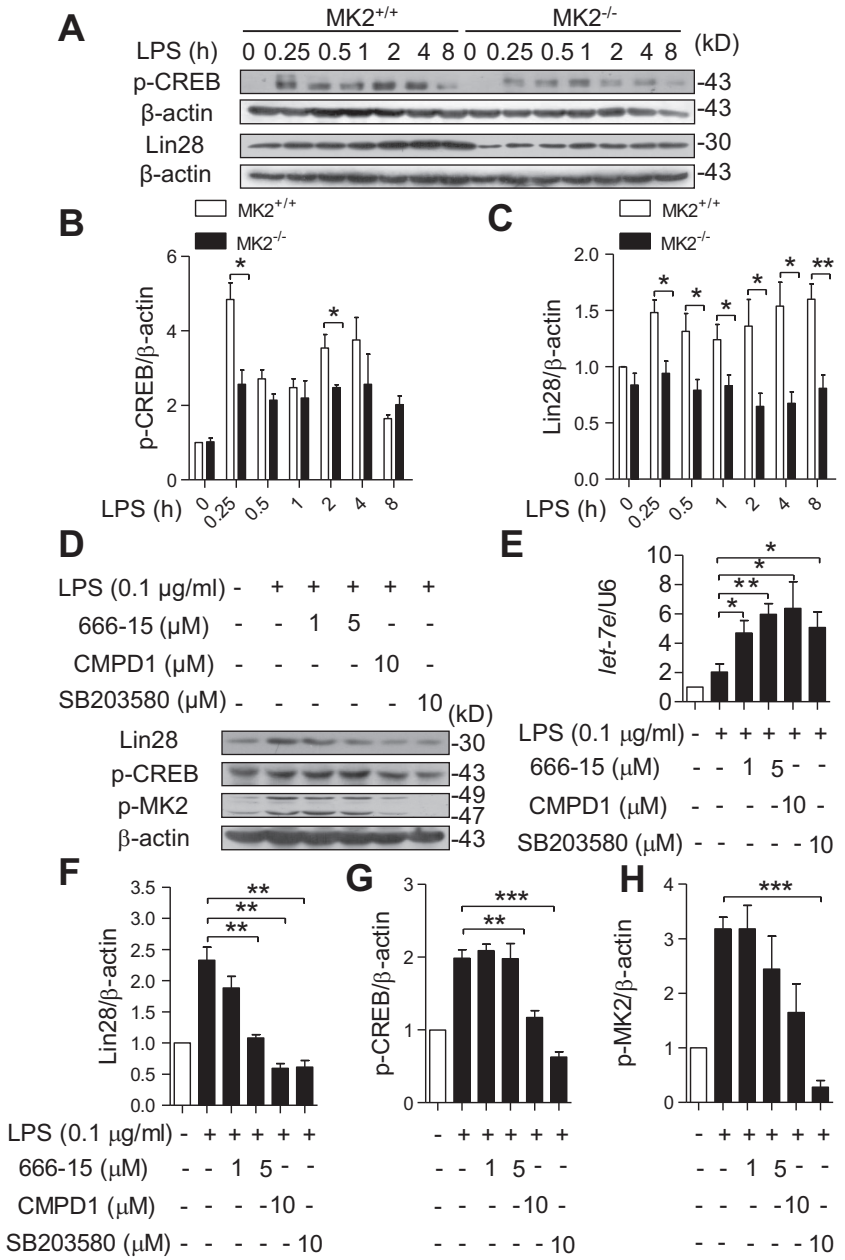

Fig. 6. CREB and Lin28 are implicated in the MK2-regulated *let-7e* expression. BMDMs isolated from $MK2^{+/+}$ and $MK2^{-/-}$ mice were stimulated with LPS (100 ng/ml) for indicated times (*A*). Total cell lysates were subjected to Western blotting analysis for detection of the phosphorylated (p-)CREB and the protein level of Lin28. β-actin was used as the loading control. The ratio of p-CREB/β-actin (*B*) and Lin28/β-actin (*C*) was analyzed in densitometry by ImageJ software. BMDMs isolated from $MK2^{+/+}$ mice were preincubated with inhibitors of CREB (666-15), MK2 (CMPD1), and p38 (SB203580) for 0.5 h and then challenged with LPS (100 ng/ml) for 2 h (*D*). The expression of Lin28, p-CREB, and phosphorylated MK2 (p-MK2) was examined by Western blotting. β-actin was used as the loading control. The ratios of Lin28/β-actin (*F*), p-CREB/β-actin (*G*), and p-MK2/β-actin (*H*) were analyzed in densitometry by ImageJ software. $MK2^{+/+}$ BMDMs were incubated with inhibitors of CREB (666-15), MK2 (CMPD1), and p38 (SB203580) for 0.5 h, followed by stimulation with LPS (100 ng/ml) for 24 h (*E*). Total RNAs were isolated and the mRNA level of *let-7e* was determined by quantitative real-time PCR. Values represent means ± SE, $n \geq 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed Student *t*-test. BMDMs, bone marrow-derived macrophages; CREB, cAMP response element-binding protein; MK2, MAPK-activated protein kinase 2.

MK2 conditional deficiency in cells of the myeloid lineage alleviates LPS-induced ALI in mice. To confirm whether MK2 modulated sepsis-induced ALI via activating macrophages, we generated the $MK2^{Lyz2\text{-}KO}$ mice by breeding $MK2^{flox/flox}$ mice with Lyz2-cre mice. Western blotting analysis confirmed that MK2 is deleted specifically in macrophages isolated from the lung tissues (Fig. 7, *A* and *B*). The ALI model was established by intratracheal injection of LPS (5 mg/kg) for 6 h in $MK2^{Lyz2\text{-}WT}$ and $MK2^{Lyz2\text{-}KO}$ mice. Histological analysis revealed that LPS-induced abundant inflammatory cell infiltration in $MK2^{Lyz2\text{-}WT}$ mice, whereas that was attenuated in $MK2^{Lyz2\text{-}KO}$ mice (Fig. 7*C*). MPO activity of the lung tissues (Fig. 7*D*) and protein leakage in BAL fluid (Fig. 7*E*) was attenuated in $MK2^{Lyz2\text{-}KO}$ mice compared with that in $MK2^{Lyz2\text{-}WT}$ mice. Furthermore, in response to LPS treatment, the percentage of pulmonary neutrophil infiltration was decreased from 86.7% of $MK2^{Lyz2\text{-}WT}$ to 30.8% of $MK2^{Lyz2\text{-}KO}$, based on FITC-anti-Ly-6G staining and flow cytometry analysis (Fig. 8*A*). In addition, LPS-induced total cell and neutrophil infiltration in BAL fluid were significantly reduced in $MK2^{Lyz2\text{-}KO}$ mice (Fig. 8, *A–D*). LPS-stimulated expression of inflammatory cytokines TNF-α (Fig. 8*E*) and IL-6 (Fig. 8*F*), as well as chemokine MIP-2 (Fig. 8*G*) was also decreased in $MK2^{Lyz2\text{-}KO}$ mice (Fig. 8, *E–G*). Altogether, these data demonstrated that MK2 modulates LPS-induced ALI via regulating macrophage activation.

DISCUSSION

MK2 is involved in regulating various cellular processes, including cell migration, cytoskeleton dynamics, apoptosis, and inflammation. As one of the downstream effectors of p38

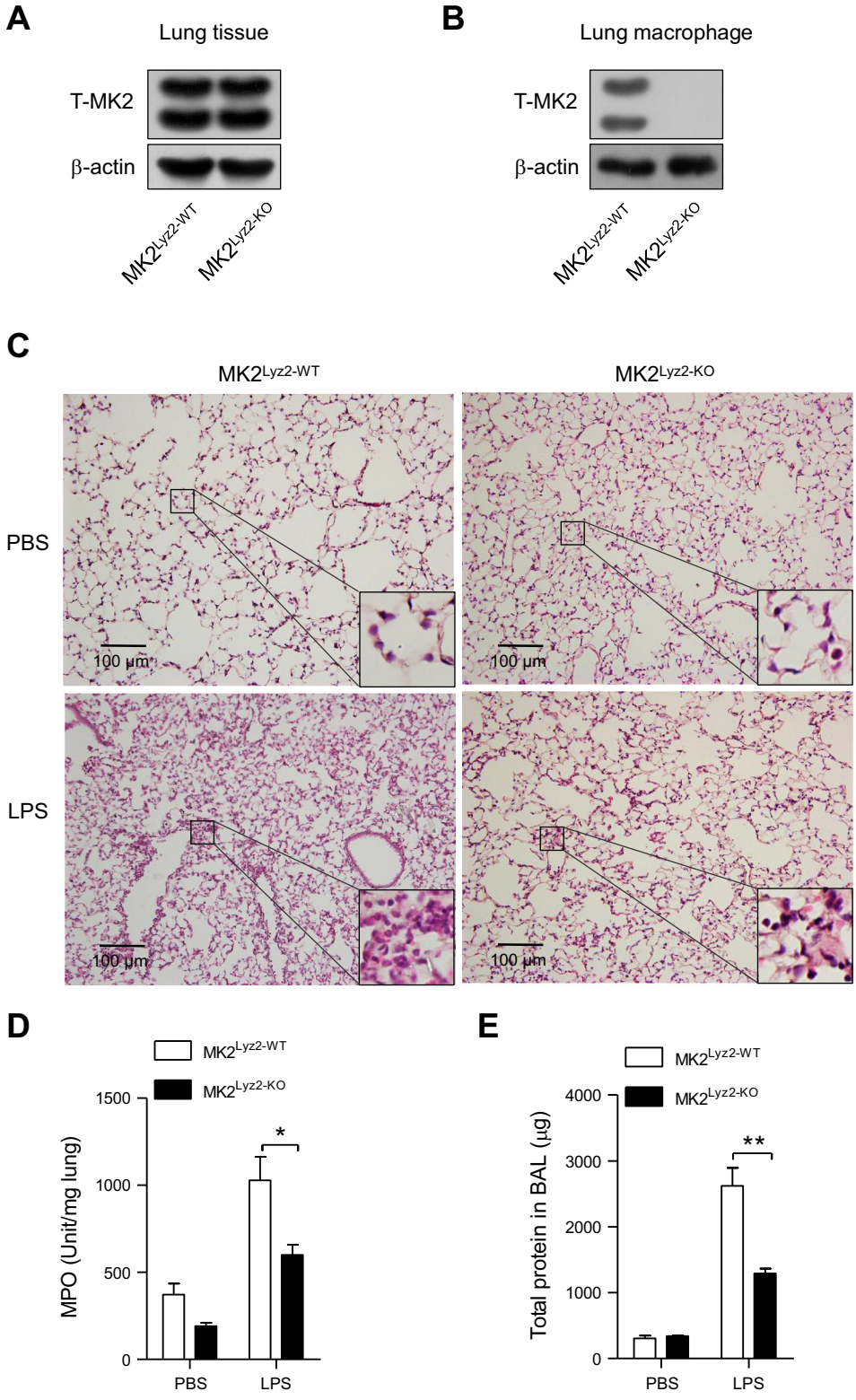

Fig. 7. LPS-induced acute lung injury is decreased in MK2$^{Lyz2\text{-}KO}$ mice. *A* and *B*: alveolar macrophages were isolated from lung lobes of MK2$^{Lyz2\text{-}WT}$ and MK2$^{Lyz2\text{-}KO}$ mice, respectively. Western blotting analysis was performed to detect MK2 total protein expression in lung tissues and alveolar macrophages. β-actin was used as the loading control. *C*: MK2$^{Lyz2\text{-}WT}$ and MK2$^{Lyz2\text{-}KO}$ mice were intratracheally injected with LPS (5 mg/kg) or equal volume of PBS for 6 h. The lung sections were stained with hematoxylin-eosin (original magnification, ×200). *D*: lung tissues were homogenized and the MPO activity was determined as described above. *E*: total protein concentration in BAL fluid was measured. Data shown as means ± SE. $*P < 0.05$, $**P < 0.01$, two-tailed Student *t*-test. BAL fluid, bronchoalveolar lavage fluid; MK2, MAPK-activated protein kinase 2; MPO, myeloperoxidase; T-MK2, total MK2.

MAPK, a major transducer of cell stress responses, it is well known that MK2 plays a crucial role in the cell response to inflammatory stress. In a previous report (21), MK2-deficient mice are shown to be resistant to LPS/D-Galactosamine-induced hepatitis. In another report (43), MK2-deficient mice were shown to be resistant to endotoxic shock but highly sensitized to TNF-induced mortality and CLP-induced sepsis. The hyperacute phenotype was attributed to the instability of the MK2-deficient endothelial barrier under conditions of oxidative stress. Concerns were then raised that systemic inhibition of this potential drug target probably has dangerous side effects in treating various inflammatory diseases. Our study

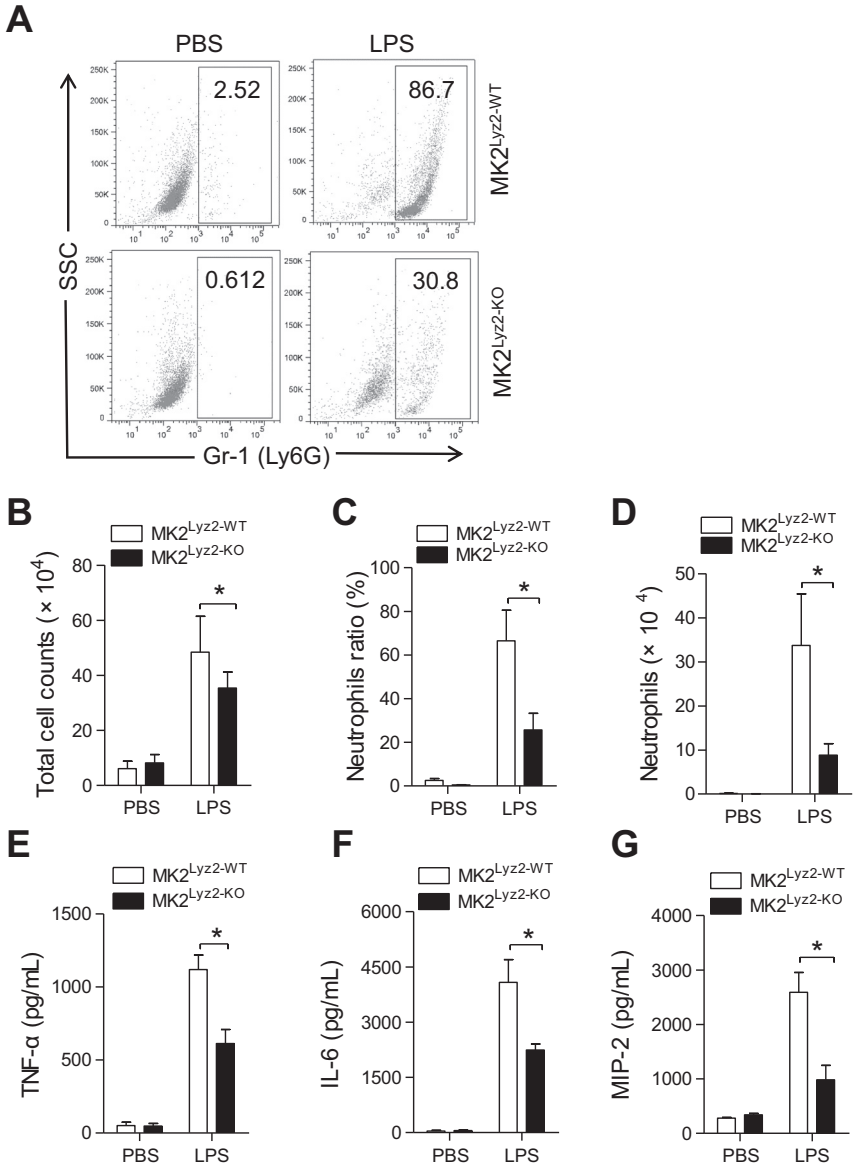

Fig. 8. MK2 in myeloid lineage cells is required for LPS-induced lung inflammation. $MK2^{Lyz2\text{-}WT}$ and $MK2^{Lyz2\text{-}KO}$ mice were treated with LPS (5 mg/kg) or equal volume of PBS through intratracheal injection for 6 h. Infiltrated neutrophils in BAL fluid were stained with a FITC-conjugated anti-Gr-1 (Ly6G) antibody and analyzed by flow cytometry (*A*). The counts of total infiltrated cells (*B*), neutrophils (*C*), and the ratio of neutrophils (*D*) in BAL fluid were calculated. Production of TNF-α (*E*), IL-6 (*F*), and MIP-2 (*G*) induced by LPS in BAL fluid were measured by ELISA. Values represent means ± SE, $n = 5$ mice in each group. *$P < 0.05$, two-tailed Student *t*-test. BAL fluid, bronchoalveolar lavage fluid; MIP-2, macrophage inflammatory protein 2; MK2, MAPK-activated protein kinase 2.

aimed to explore the role of MK2 in regulating inflammatory responses induced by bacterial stressors; therefore, we checked the survival rate of MK2-deficient mice that were subjected to CLP surgery and LPS injection. The results confirmed that MK2 deficiency renders mice highly resistance to LPS-induced mortality. Curiously, in contrast to the previous report mentioned above, the CLP-induced mortality was also significantly reduced in $MK2^{-/-}$ mice. This divergence might be caused by the different sources of MK2 knockout mice used in the CLP models. To ensure the reliability of the results obtained in our study, the $MK2^{-/-}$ mice were originally purchased from Jackson Laboratory. In addition, a small needle size (22-gauge) was used in their puncture surgery, leading to little amounts of bacterial leak into abdomen. Thus, the multibacterial-induced inflammation might not be a major contribution to the observed acute mortality, which is caused more likely by high reactive oxygen species levels and the resulting tissue damage. As we are interested in the inflammation-driving role of MK2 under inflammatory conditions, a bigger size of needle (18-gauge) was chosen in this study. In comparison with the previous report, our wild-type mice showed more acute and higher mortality, whereas our $MK2^{-/-}$ mice were apparently protected against this inflammation-induced mortality.

MK2 is also implicated in the regulation of cytokine mRNA metabolism. This was first shown in a model of acute hepatitis, in which TNF biosynthesis was changed in response to a combined LPS/D-Galactosamine challenge (21). Multiple studies have highlighted a role for MK2 in the posttranscriptional regulation of proinflammatory cytokine production, including TNF-α (21), IL-6 (25), and IFN-γ (27). One of the best characterized mechanisms is that activated MK2 can stabilize TNF mRNA through phosphorylation of tristetraprolin, an mRNA-binding protein that destabilizes and degrades mRNAs by binding to adenylate-uridylate-rich elements in a nonphosphorylated form (5). Recently, it was revealed that microRNAs (miRNAs) plays a role in regulation of cytokine expression and orchestrates inflammatory signaling (18, 30, 48). For instance, Akt1 was found to regulate the response of macrophages to

LPS by controlling miRNA expression, including *let-7e* and miR-155 (1). It was also reported that differential activation and expression of MK2, miR-125b, and miR-155 are involved in regulating TNF-α biosynthesis in human macrophage stimulated by *Mycobacterium tuberculosis* and *Myocobacterium smegmatis* (34). However, the potential connection of MK2 and miRNAs in regulating complex inflammatory processes is lacking. Our results reveal for the first time, to our knowledge, that the expression of miRNA *let-7e* is significantly enhanced in MK2-deficient macrophages. The *let-7* family miRNAs are well identified as regulators of development, morphogenesis and tumorigenesis. As inflammation is linked to cell transformation and tumorigenesis, a regulatory role for *let-7* miRNAs in inflammation is possible. It was reported that *let-7* directly inhibits IL-6 expression and is involved in a regulatory circuit in cancer cell lines and tissues (19). There is also evidence that IL-10 is one of the functional targets of *let-7e* (12). In this study, we found that the *let-7e* miRNA level is negatively associated with the LPS-induced expression of proinflammatory cytokines TNF-α and IL-6, as well as chemokine MIP-2. The *let-7e* partially matched the sequence of TNF-α promoter and decreased the production of TNF-α. Given that MK2 can trigger TNF-α expression by induction of tristetraprolin phosphorylation (16, 24), we predicted that MK2 modulates TNF-α expression by two different pathways. In addition, TLR4 is also the target of *let-7e* (1); therefore, it is possible that *let-7e* modulates various cytokine expression through downregulating TLR4. These results suggested that *let-7e* mediates another alternative signal pathway in regulation of inflammatory cytokine expression.

Lin28, an RNA-binding protein, is a specific regulator of *let-7* family miRNAs processing and biogenesis in tumor cells (28, 44). MK2 deficiency decreased the protein level of Lin28 in macrophages, especially under treatment with LPS. Inhibition of p38 MAPK/MK2/CREB signaling pathways impaired the Lin28 expression in response to LPS. In TLR-4-mediated signal pathways, the axis of p38 MAPK/MK2 delivers an activation signal to induce Lin28 expression that reduced *let-7e* miRNA. Therefore, our findings revealed a new regulatory signal pathway for controlling cytokine production.

In summary, our in vivo and in vitro data confirmed that MK2 deficiency protected against CLP- or LPS-induced sepsis. MK2 deficiency resulted in the increased expression of *let-7e* in macrophages in response to LPS, leading to reduced production of inflammatory factors, including TNF-α, IL-6, and MIP-2. Our results also demonstrated that RNA-binding protein Lin28 might be involved in the regulation of *let-7e* expression in macrophages. The expression of *let-7e* and Lin28 was found to be associated with the activation of CREB, a transcriptional factor and one of the substrates of MK2. Our studies suggested that MK2 plays a critical role in macrophage activation and ALI via regulating *let-7e* miRNA expression.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

H.H. and F.Q. conceived and designed research; Y.W., Y.D., and S.L. performed experiments; Y.W., Y.D., S.L., and J.W. analyzed data; Y.W. prepared figures; H.H., Depeng Zhang, and H.J. interpreted results of experiments; Y.W., H.H., and F.Q. drafted manuscript; Y.W., J.W., Dan Zhang, L.S., and R.D.Y. edited and revised manuscript; H.H., Depeng Zhang, H.J., Dan Zhang, L.S., R.D.Y., and F.Q. approved final version of manuscript.

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