

## Original Article

# Methane attenuates LPS-induced acute lung injuries by inhibiting inflammation and oxidation in mice

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**Abstract:** Background: Inflammation response and oxidative stress are involved in the pathogenesis of acute lung injuries (ALI). Methane has been reported to exert protective effects against I/R injuries and inflammation through anti-apoptotic, anti-oxidative, and anti-inflammatory actions. Methods: The current study used methane-rich saline (MS) on lipopolysaccharide (LPS)-induced acute lung injuries (ALI) in mice, investigating parameters of inflammation, apoptosis, oxidative stress, and possible mechanisms. Results: This study found that intraperitoneal injections with MS 20 mL/kg, 30 minutes after intratracheal LPS challenge, markedly ameliorated tissue injuries of the lungs, reducing wet-to-dry weight ratios, myeloperoxidase (MPO) activity, and pulmonary histopathological conditions. This study also observed reduced neutrophil infiltration, malondialdehyde (MDA), and inflammatory cytokines, as well as upregulation of super-oxide dismutase (SOD), in MS-treated LPS stimulated lungs. Results further show that MS markedly decreased the phosphorylation of ERK, p38, and p65 of lung tissues, as well as apoptosis of alveolar macrophages, an initial trigger of early stage inflammation of ALI. Conclusion: Taken together, present results indicate that MS exhibited protective effects on LPS-induced ALI through regulation of oxidative states and inflammation through ERK, p38, and p65. The current study suggests that methane be considered as a potentially effective candidate for treatment of ALI.

**Keywords:** Acute lung injury, methane, inflammatory cytokines, oxidation, alveolar macrophage

## Introduction

A life-threatening condition of acute lung injury (ALI), acute respiratory distress syndrome (ARDS) is characterized by bilateral pulmonary infiltrates, severe hypoxemia, and non-cardiogenic pulmonary edema [1]. Despite decades of research and numerous clinical trials, the mortality from ARDS still approaches 30%-50%, suggesting the need for new treatment.

LPS is a cellular wall component responsible for local and systemic toxicity of gram-negative bacteria [2]. LPS intratracheal administration causes neutrophil recruitment, pulmo-capillary permeability, and lung dysfunction, as well as increased cytokines and chemokines levels in bronchoalveolar lavage fluid (BALF) and lung parenchyma, with higher activities of oxidative stress [3, 4].

The simplest aliphatic hydrocarbon, methane is known as the main component of greenhouse gas. It has been widely used fuel. Mammalian methanogenesis has been exclusively the anaerobic fermentation in the intestines [5, 6]. Between 30-62% of healthy humans produce methane [7]. Human methane production rates [8] can be measured by clinical testing. Recent studies have revealed the possibility of non-microbial methane formation in eukaryote cells, plants, and animals [9]. Whole-body methane production of mice is significantly increased in endotoxemia in rats [9]. Furthermore, exogenous methane has been investigated in two animal models of ischemia-reperfusion (I/R). It has been shown to protect organs (liver and intestines) against I/R injury [2, 3].

Thus, it was hypothesized that methane exerts protective effects against lung injuries. In the

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mouse model of LPS intratracheal administration, it was found that the beneficial effects of methane treatment were linked to downregulation of inflammatory cytokines and oxidative stress, accompanied with reductions of ERK, p38, and NF- $\kappa$ B phosphorylation and anti-apoptotic effects of alveolar macrophages.

### Materials and methods

#### *Animal*

All animal experiments were conducted after approval of the Animal Care and Use Committee of Changhai Hospital. Male C57BL/6 mice, weighing 20-25 g, aged 8-10 weeks, were obtained from the Experimental Animal Center of the Second Military Medical University. Mice were bred in microisolator cages (24°C) under a 12:12-hour light-dark cycle, with free access to regular chow and water. Effort was made to minimize both animal suffering and the number of animals used.

#### *LPS-induced ALI model*

As previously described [4, 5], ALI was induced by intratracheal (i.t.) administration of LPS. Briefly, anesthesia was induced and maintained with sevoflurane (2%~3%) inhalation. They were given 10 mg/kg LPS (20  $\mu$ g~25  $\mu$ g/mouse; Escherichia coli O111:B4; Sigma, St. Louis, Mo) dissolved in 50  $\mu$ l sterile phosphate-buffered saline (PBS). The sham group was intratracheally given 50  $\mu$ l of PBS. Animals were randomly assigned to four groups: sham group, methane-rich saline (MS) group, LPS group, and LPS treated with methane-rich saline (LPS/MS) group. Animals in the LPS + CH<sub>4</sub> group received LPS. MS was administered with a syringe in one bolus injection intraperitoneally 30 minutes after LPS administration. The total volume of methane-rich saline was 500  $\mu$ l/mouse. The LPS group was treated with saline (250  $\mu$ l/mouse) the same time as the MS group. Both sham and LPS groups were given saline or methane-rich saline simultaneously (n = 10 per/group). Mice were euthanized 4 hours or 24 hours after surgery. The left lung was doused with bronchoalveolar lavage fluid (BALF). The isolated right lung was harvested and the right lower lobe was used for wet/dry (W/D) ratio measurement. The right middle lobe was fixed in 4% (vol/vol) neutral phosphate-buffered formalin and prepared for histo-

logical examinations. The other portion of the right lung was snap-frozen in liquid nitrogen for oxidative stress variables and Western blotting.

#### *Methane-rich saline preparation*

Methane-rich saline (MS) was provided by the Department of Diving Medicine, Faculty of Navy Medicine, Second Military Medical University (Shanghai, China). Methane, stored in a gas canister, was dissolved in physiological saline for 6 hours under high pressure (0.4 MPa) to a supersaturated level. Methane-rich saline was freshly prepared one day before animal experiments to ensure a steady concentration of injections. The MS was stored under atmospheric pressure at 4°C for 24 hours. Concentrations of methane in the saline were measured using gas chromatography, as described by Oshawa et al. [6]. Concentration of the MS was 0.99 mmol/L, using methane-containing standard gas (Shanghai Jiliang Standard Gas Ltd., Shanghai, China) as a comparison.

#### *Lung damage and histologic examinations*

To quantify the magnitude of pulmonary edema, lung W/D weight ratios were evaluated. The harvested wet lung was weighed and placed in an oven for 24 hours at 80°C. It was then weighed when it was dried. The ratio of wet lung to dry lung was calculated. For histologic examinations, lung tissues were carefully removed from animals 24 hours after the LPS injection. Paraffin-embedded lung tissues were fixed in 4% paraformaldehyde, deparaffinized, and rehydrated. They were then sectioned into 5 mm slices. Samples were stained with hematoxylin and eosin, mounted, and analyzed blindly by two experienced pathologists using modified published criteria, as described previously [7, 8]. Briefly, lung injuries were assessed on a scale of 0-10 for each of the following criteria: 1) Neutrophils in the alveolar space; 2) Neutrophils in the interstitial space; 3) Number of hyaline membranes; 4) Amount of proteinaceous debris; and 5) Extent of alveolar septal thickening.

#### *BALF analysis*

Animals were subjected to bronchoalveolar lavage for collecting BALF, according to methods described previously [9]. BALF was obtained by

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cannulating the trachea with a 20-gauge catheter, 24 hours after LPS injury. The first volume of 0.5 mL of freezing PBS (pH 7.4) was instilled, gently aspirated, pooled, and re-aspirated. Lavage samples were centrifuged at 1,500 g for 10 minutes at 4°C. The supernatant was collected for determination of proinflammatory cytokines, including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL1- $\beta$ ), IL-6, and keratinocyte-derived chemokine (KC). The second volume of 0.5 mL of lavaged PBS was centrifuged to collect cell pellets. All collected cell pellets were resuspended in PBS. Subsequently, surface staining was performed for 15 minutes using standard methods. Antibodies included anti-Ly6g (BioLegend), anti-CD11b (eBioscience), anti-F4/80 (eBioscience), and Annexin V (eBioscience). Flow cytometric analysis (50,000 events/sample) was performed on a FACS Calibur Flow Cytometer (BD Biosciences). Further analysis was conducted with FlowJo software (Tree Star).

### *Oxygenation index analysis*

PaO<sub>2</sub>/FiO<sub>2</sub> was used to assess the oxygenation capability of the lungs. Mice were anesthetized and endotracheal intubated with a 20-gauge catheter, 24 hours after LPS administration. They were mechanically ventilated with pure oxygen at 7 mL/kg. The respiratory rate was set at 120 breaths/minutes. The animals were ventilated for 15 minutes before blood gas sampling. Arterial blood was obtained from carotid arteries and measured with GEM Premier 3000 gas analyzer (Instrumentation Laboratory, Milan, Italy).

### *Oxidative stress variable measurement*

Levels of MDA and SOD contained in lung tissues were measured using commercially available assay kits (Jiancheng Institute of Biotechnology, Nanjing, China), following manufacturer recommendations. MDA concentrations were calculated using the thiobarbituric acid method. Results are expressed as nanomoles per gram. SOD (units per mg protein) in lung tissue homogenates was estimated by evaluating the rate of inhibition of nucleotide oxidation. Lung leukocyte accumulation brought about by ALI was determined by measurement of the myeloperoxidase (MPO) activity and measurement of reduced glutathione (GSH). Concentrations of GSH in the lung homogenate

were measured according to methods described by Ellman (1959) [10].

### *Western blot analysis*

The right lobes were homogenized and analyzed for ERK, p38, and NF- $\kappa$ B (p65 subunit) phosphorylation. Protein concentrations were determined by BSA assay. Samples were electro-blotted onto PVDF membranes and probed with target primary antibodies, respectively. Membranes were then incubated with the secondary antibody (1:5000) and visualized with enhanced chemiluminescent reagent. This was followed by autoradiography. Mouse p-ERK, ERK, p-p65, p65, p-p38, p38, and GAPDH antibodies were purchased from Cell Signaling Technology (Danvers, MA).

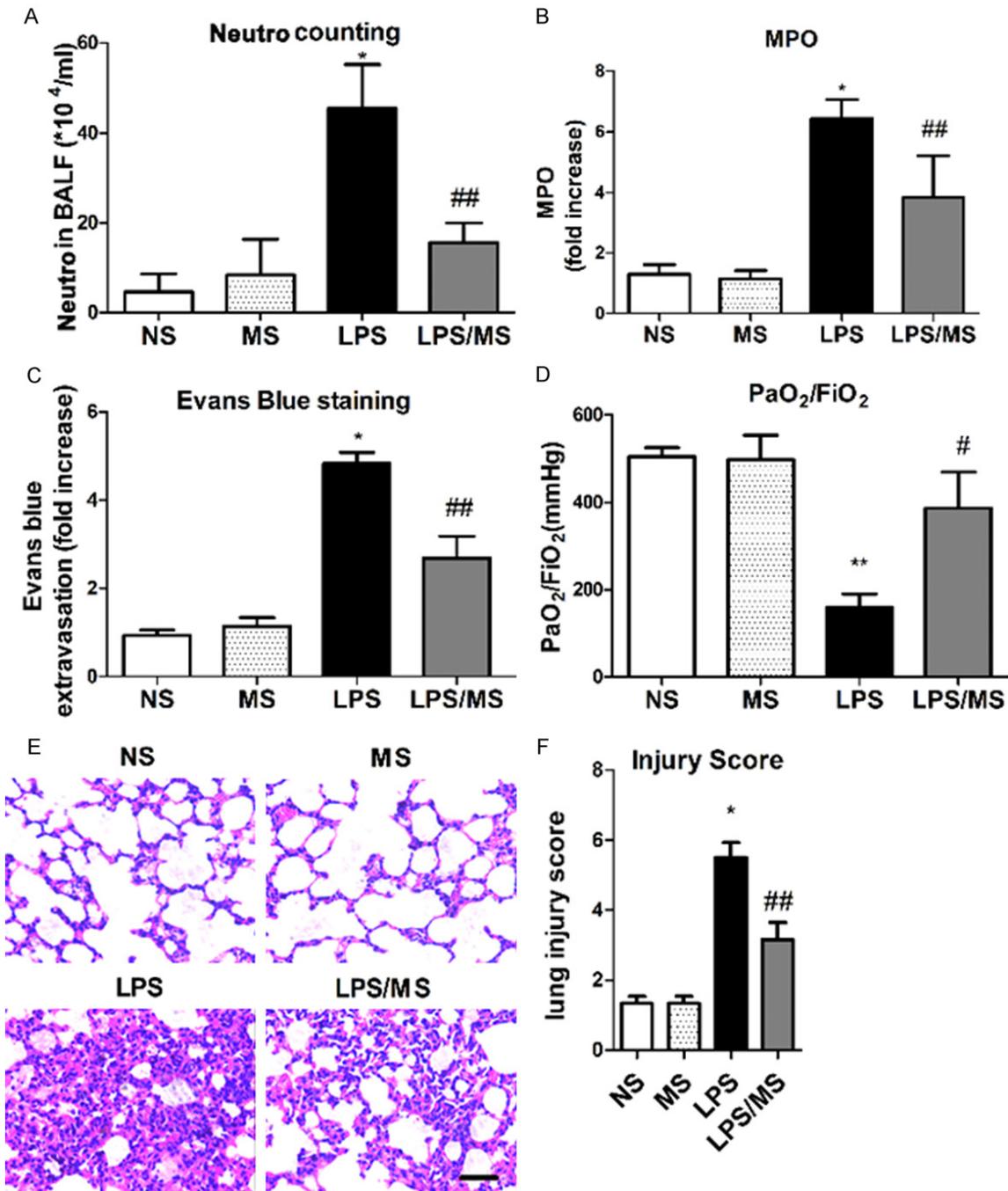
### *Statistical analysis*

Standard deviations were calculated either by Excel or GraphPad Prism. Further statistical analysis was performed using one-way ANOVA, followed by the Tukey's multiple comparison test. *P*-values are depicted only for relevant data pairs (\**P* < 0.05 vs. NS group; #*P* < 0.05 vs. LPS group).

## Results

### *Methane-rich saline attenuated LPS-induced lung injury in mice*

MS attenuated neutrophil recruitment, pulmonary capillary permeability, and lung dysfunction of LPS-induced acute lung injuries. First, this study investigated concentrations of methane after methane-rich saline treatment in mice. It was found that concentrations of methane in plasma peaked at 30 minutes after methane-rich saline treatment in mice (see [Figure S1](#)). Thus, methane-rich saline was used 30 minutes after acute lung injury. MS significantly abrogated the changes in neutrophil numbers (**Figure 1A**) in BALF and MPO activities of lung tissue (**Figure 1B**) 24 hours after LPS injury. The change of the percentage of CD11b<sup>+</sup> Gr1<sup>+</sup> neutrophil cells in BALF showed the same with the neutrophil numbering ([Figure S2](#)). Vascular permeability in LPS-induced ALI was assessed by spectrophotometric analysis of Evans blue extravasation. LPS-challenged mice showed significant increases in lung Evans blue extravasation, which was reduced by MS treatment (*P* < 0.05, *n* = 10 per group, **Figure 1C**). This study

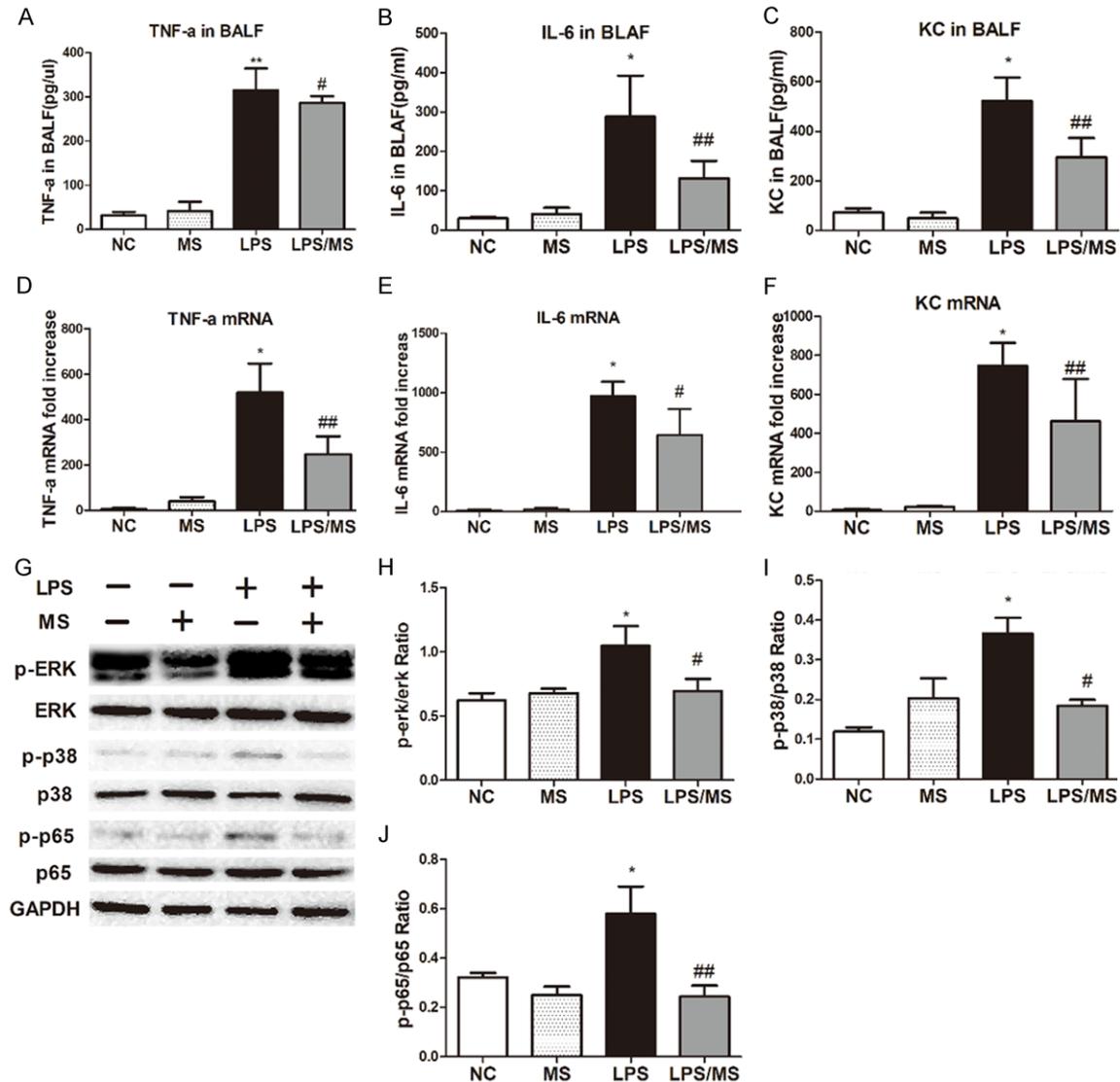


**Figure 1.** Methane-rich saline attenuated lung neutrophils recruitment, vascular integrity, and histopathology changes in LPS-challenged mice. The animals were i.t. challenged with PBS or LPS. MS was given intraperitoneal 30 minutes after LPS administration. Neutrophils in BALF (A), Lung MPO activity (B), Evans blue extravasation (C), and Oxygenation index ( $PaO_2/FiO_2$ ) (D) were measured 24 hours later. Pathologic analysis by hematoxylin and eosin staining (Original magnification  $\times 400$ , scale bars = 50  $\mu$ m) (E). Lung histologic scores based on neutrophils in the alveolar space, neutrophils in the interstitial space, number of hyaline membranes, amount of proteinaceous debris, and extent of alveolar septal thickening are shown as mean  $\pm$  SD (n = 10) (F). Statistical significance was determined by the nonparametric U-Mann-Whitney test. \*P < 0.05 vs. NS group, \*\*P < 0.01 vs. NS group; #P < 0.05 vs. LPS group, ##P < 0.01 vs. LPS group.

further investigated lung function changes, finding that exposure to LPS resulted in a sig-

nificant decrease in oxygenation index ( $PaO_2/FiO_2$ ) levels in lungs 24 hours later. These

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**Figure 2.** Effects of MS on TNF- $\alpha$ , IL-6, and KC levels in the BALF and in the lung tissues of mice after LPS stimulation. MS was given intraperitoneal 30 minutes after LPS administration. TNF- $\alpha$  (A, D), IL-6 (B, E), and KC (C, F) protein levels of the BLAF and RNA levels of lung homogenates were measured 24 hours after LPS challenge. Lungs were harvested 24 hours after LPS challenge. Q-PCR results shown are averages ( $n = 8$ ) with standard deviations. Statistical significance was determined with Student's t-test. Western blots of lung homogenates were employed to monitor ERK (H), p38 (I), p65 (J) phosphorylation of lung tissue homogenates. Representative blots of each group for each protein are shown (G). Results are shown as mean  $\pm$  SD ( $n = 8-10$ ). \* $P < 0.05$  vs. NS group, \*\* $P < 0.01$  vs. NS group; # $P < 0.05$  vs. LPS group, ## $P < 0.01$  vs. LPS group.

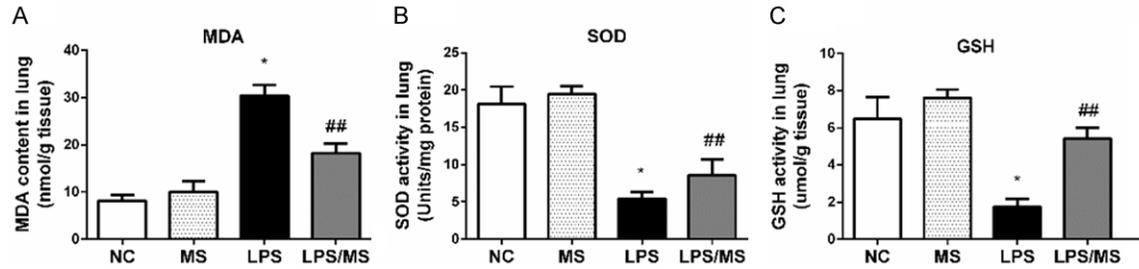
changes, induced by LPS, were all blocked significantly by MS ( $P < 0.05$ ,  $n = 10$  per group, **Figure 1D**). Pathological improvement was observed in the MS treated group (**Figure 1E**). Moreover, a scoring system to grade the degree of lung injury was used. Control samples taken from the sham-operated group had a median grade of injury of 1.3 (0.51), respectively. LPS-challenged mice showed a significant increase in lung histologic scores of a median grade of 5.5 (1.05), whereas MS treatment reduced

lung injury and its median grade decreased to 3.2 (1.17) ( $P < 0.05$ ,  $n = 10$  per group, **Figure 1F**).

### *MS treatment downregulated inflammatory response in the lungs of LPS-challenged mice*

Pro-inflammatory cytokine and chemokine changes are the secondary mediators of the necessary signals on pulmonary dysfunction evolved in LPS-induced acute lung injury. Since

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**Figure 3.** Effects of methane-rich saline on MDA content, SOD, and GSH activities in lungs of mice after LPS stimulation. Lungs were harvested 24 hours after LPS challenge. Lung homogenates were prepared for measuring MDA (A), SOD (B), and GSH (C). Results are shown as mean  $\pm$  SD ( $n = 8$ ). \* $P < 0.05$  vs. NS group; ## $P < 0.05$  vs. LPS group (Student's *t*-test).

methane-rich saline ameliorated major pathological changes of LPS-induced acute lung injuries, this study further detected pro-inflammatory cytokine and chemokine changes in the air space and lung parenchyma between the MS group and LPS group, 24 hours after LPS injury. TNF- $\alpha$ , IL-6, and KC protein levels in the BALF were significantly increased in LPS-treated mice ( $P < 0.05$ ,  $n = 10$  per group, **Figure 2A-C**). MS inhibited these cytokines concentrations in the BALF. TNF- $\alpha$ , IL-6, and KC mRNA level changes in lung parenchyma showed the same results ( $P < 0.05$ ,  $n = 10$  per group, **Figure 2D-F**). Mitogen activated protein kinases (MAPKs) are critical mediators for activation of transcription factors, such as ERK, p38, and NF- $\kappa$ B, which lead to cytokine processing following inflammatory stimuli. MS treatment resulted in an overall decrease in phosphorylation of ERK (**Figure 2G**) and p38 (**Figure 2H**). Additionally, lung tissue analysis revealed a decrease in NF- $\kappa$ B activity with MS treatment, compared to mice treated with saline, as measured by the phosphorylation of NF- $\kappa$ B p65 (**Figure 2I**).

### *MS treatment released oxidative stress in mice lungs with LPS-induced ALI*

To explore possible mechanisms of MS protection on LPS induce ALI, this study measured MDA, SOD, and GSH levels of lung homogenates 24 hours after LPS injury. MDA concentrations were determined as an indicator of lipid peroxidation in the lung tissues. Superoxide dismutase (SOD) levels were used to indicate antioxidant levels. LPS challenges resulted in a significant increase in MDA levels ( $P < 0.05$ ,  $n = 10$  per group, **Figure 3A**), while GSH and SOD activities were markedly decreased ( $P < 0.05$ ,  $n = 10$  per group, **Figure 3B, 3C**). These results

highly suggest that methane interferes with ALI process through the antioxidant pathway. These changes induced by LPS challenges were all blocked significantly by MS treatment.

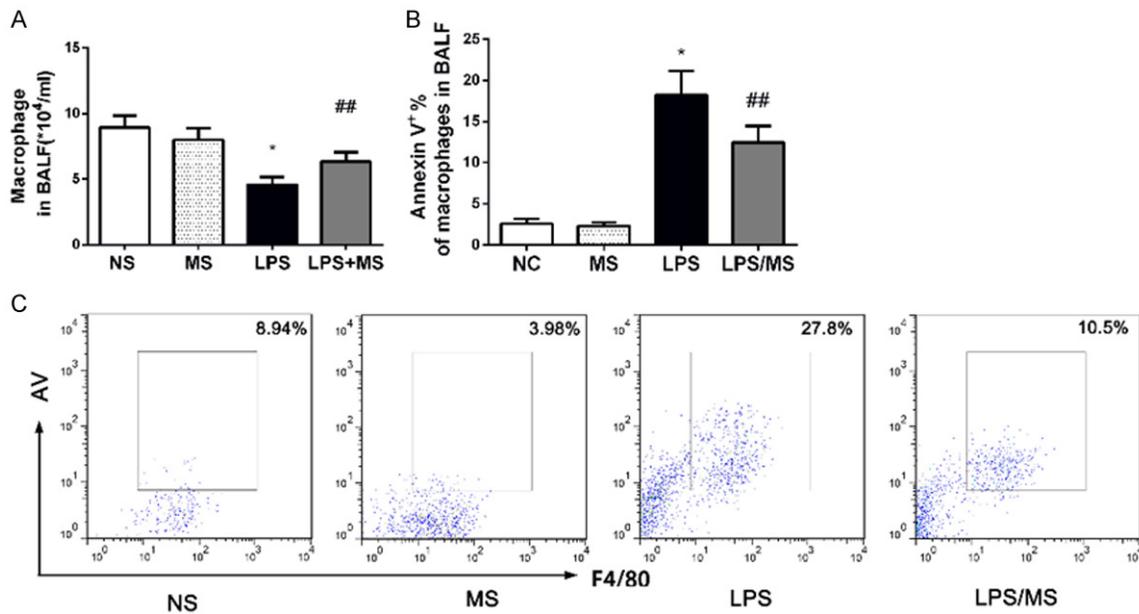
### *MS treatment prevented the alveolar macrophages apoptosis of LPS-challenged mice*

Since AMs have been shown to be critical for maintaining immune homeostasis in the lungs, this study examined whether AMs were involved in MS treatment mechanisms protecting against LPS induced ALI. BAL cells were isolated 4 hours post LPS. They were analyzed by flow cytometry for F4/80 (alveolar macrophages) and Annexin V, detecting apoptotic and necrotic cells. LPS-challenged mice showed a decrease in AMs counting (**Figure 4A**) and percentage (**Figure S3**), and also a significant increase in AMs apoptosis, which was reduced by MS treatment (**Figure 4B**). Representative images are shown (**Figure 4C**). ( $P < 0.05$ ,  $n = 10$  per group).

## Discussion

Methane has been suggested to exert protective effects against ischemia/reperfusion (I/R) injuries. Hence, in the current study, methane was used to treat LPS induce acute lung injuries, examining promising protective effects. LPS-induced ALI is an animal model that covers several key pathologic processes of ALI, including neutrophil recruitment, pulmo-capillary permeability, and lung dysfunction, as well as increased cytokine and chemokine levels in BALF and lung parenchyma, with higher activities of oxidative stress. Results demonstrated that methane-rich saline treatment, 30 minutes after LPS administration, inhibited neutrophilic infiltration and ameliorated vascular in-

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**Figure 4.** Effects of methane-rich saline on alveolar macrophages (AMs) of mice after LPS stimulation. Mice BALF was collected 4 hours after LPS challenge. AMs were stained with annexin V-FITC and analyzed by flow cytometry. Apoptosis levels in the AMs (annexin V-FITC positive and PI negative) are shown by histogram (A). Live AMs numbers (annexin V and PI negative) were assessed in BALF by flow cytometry (B). Representative images of each group were shown (C). Results are shown as mean  $\pm$  SD (n = 8). The nonparametric U-Mann-Whitney test was performed to evaluate flow cytometry data. \*P < 0.05 vs. NS group; #P < 0.05 vs. LPS group.

tegrity and lung histopathological changes. The current study investigated the mechanisms involved in the protective effects of methane-rich saline treatment in attenuating LPS induced acute lung injuries. Protective effects of methane-rich saline were linked to downregulation of the inflammatory cytokines and oxidative stress. Moreover, methane-rich saline treatment prevented alveolar macrophages apoptosis of LPS-challenged mice and reduced ERK, p-38, and NF- $\kappa$ B phosphorylation of LPS-injured lungs.

Many gaseous molecules, like Hydrogen ( $\text{H}_2$ ),  $\text{H}_2\text{S}$ , generated by an-aerobic bacteria in the gastrointestinal of human body, are biologically active [7]. Exogenous  $\text{H}_2$  (inhalation drinking or intraperitoneal) exerts protecting effects in many animal disease models, including acute lung injury animal models, suggesting its antioxidant action. Since exogenous methane has manifested anti-inflammatory and antioxidant properties in two animal models of I/R, it was hypothesized that methane may play the same protective role in LPS- induced lung injuries. To the best of our knowledge, this is the first study demonstrating that methane-rich saline can

significantly attenuate LPS induced lung injuries. Present results suggest that methane-rich saline treatment could significantly ameliorate LPS induced ALI by reducing BAL fluid PMN, albumin, proinflammatory cytokines levels, and AMs apoptosis, which are associated with decreased oxidative stress.

ALI animal models are closely linked to the magnitude of inflammatory response. TNF- $\alpha$  and IL-6 are potent proinflammatory cytokines that play a role in the initiation and amplification of inflammatory responses during ALI [11]. Inhibiting the overproduction of pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-6, promotes the lessening of pulmonary injury in a CLP-induced ALI model [12]. It was found that TNF- $\alpha$  and IL-6 protein levels in the BALF were significantly increased at 4 hours in LPS-treated mice. MS treatment showed inhibitory effects on TNF- $\alpha$  and IL-6 protein levels in the BALF. In addition to pro-inflammatory cytokines, the current study proved that phosphorylation and consequent activation of ERK, p38 MAPK, and NF- $\kappa$ B in the lung homogenates were blocked by methane-rich saline.

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To further explore underlying mechanisms of the protective effects of methane, its influence on production of free radicals was examined. ALI is considered as a state of increased oxidative stress and reduced anti-oxidative ability. Malondialdehyde (MDA) levels were measured as a marker of lipid oxidation. Superoxide dismutase (SOD) levels were used to indicate anti-oxidant levels. The present study proved that levels of MDA significantly increased, while GSH and SOD activity markedly decreased in lungs 24 hours after LPS challenge. Similar to Ye's research [3], these parameters became obviously improved by intraperitoneal injections of methane-rich saline. CH<sub>4</sub> supplementation significantly diminished many of the damaging consequences of disturbed redox balance of ALI.

Alveolar macrophage (AM) is the main driver of early inflammation in LPS-induced ALI [8]. AMs depletion in the very early stages of inflammation significantly increases neutrophil recruitment in sepsis or lipopolysaccharide (LPS)-induced animal models of ALI [13]. After LPS instillation, PMN numbers increased over time in the BALF, whereas AM numbers decreased 4 hours later. They increased as monocyte recruitment started. Present data shows that methane-rich saline treatment can attenuates pathological changes of LPS induced ALI. Thus, this study next explored whether AMs were involved in protective mechanisms of MS. AMs apoptosis, which increased in LPS challenges, was reduced by methane-rich saline treatment. The counting of annexin V-FITC negative AMs was also increased in the LPS/MS group, compared to the LPS group.

Due to safety concerns, methane administration via inhalation was not chosen for the present study. Methane saline was intraperitoneally administered with a syringe in one bolus injection right after the challenge of LPS. Methane-rich saline could be administered more than once after intratracheal LPS. Protective effects of the methane were strengthened. However, considering the possibility of over fluid load due to excessive intraperitoneal saline, one bolus injection was given. The current research did not measure methane concentrations in lung parenchyma, since airway of the lung opens to the atmosphere, unlike livers or intestine, which are organs folded in the peritoneal cavity.

### Conclusion

In conclusion, present results suggest that methane plays an important role in protecting lungs from the inflammatory effects of LPS-induced ALI. The effects of methane-rich saline are mainly mediated by modulating the inflammatory-anti-inflammatory and oxidative-anti-oxidative balance in the early phases of LPS-induced ALI.

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### Disclosure of conflict of interest

None.

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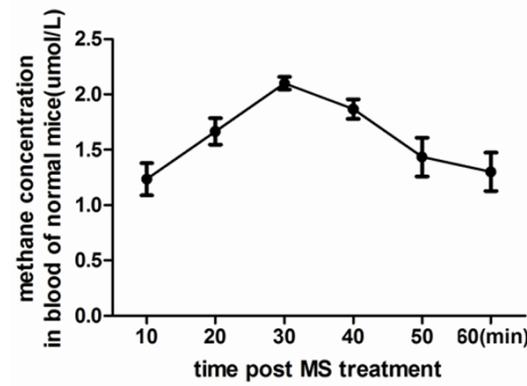
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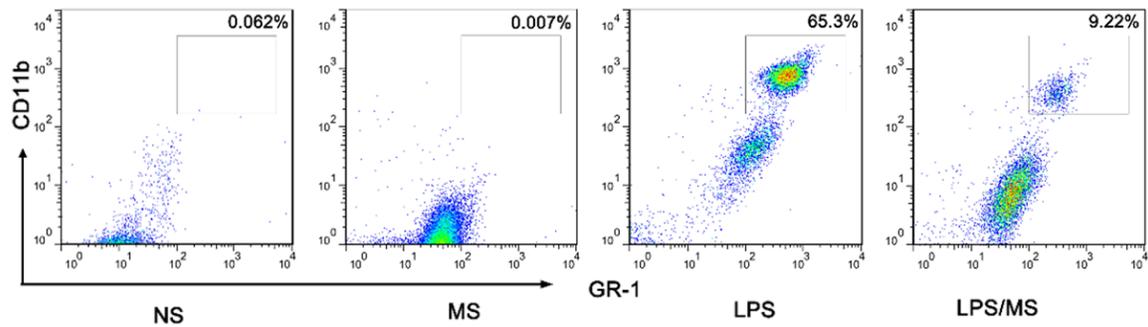
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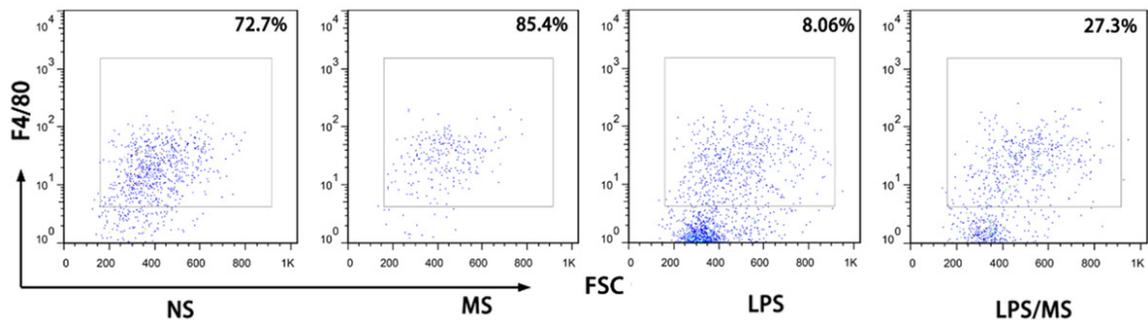
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**Figure S1.** Methane levels in plasma of mice after MS treatment. Mice blood was collected at indicated times after MS intraperitoneal injections. They were sent for gas chromatography measurement. Results are shown as mean  $\pm$  SD (n = 6).



**Figure S2.** Frequency of neutrophils in BALF. The animals were i.t. challenged with PBS or LPS. MS was given intraperitoneal 30 minutes after LPS administration. 24 hours after LPS injury, BALF were analyzed by the flow cytometry. The representative dot plots (from n = 10) of the frequency of CD11b<sup>+</sup> Gr1<sup>+</sup> Neutrophils in BALF.



**Figure S3.** The frequency of macrophages in BALF. The animals were i.t. challenged with PBS or LPS. MS was given intraperitoneal 30 minutes after LPS administration. Next, 4 hours after LPS injury, BALF were analyzed by flow cytometry. The representative dot plots (from n = 8) of the frequency of F4/80<sup>+</sup> AV<sup>+</sup> macrophages in BALF.