RESEARCH PAPER

Shikonin inhibits myeloid differentiation protein 2 to prevent LPS-induced acute lung injury

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BACKGROUND AND PURPOSE

Acute lung injury (ALI) is a challenging clinical syndrome, which manifests as an acute inflammatory response. Myeloid differentiation protein 2 (MD2) has an important role in mediating LPS-induced inflammation. Currently, there are no effective molecular-based therapies for ALI or viable biomarkers for predicting the severity of disease. Recent preclinical studies have shown that shikonin, a natural naphthoquinone, prevents LPS-induced inflammation. However, little is known about the underlying mechanisms.

EXPERIMENTAL APPROACH

The binding affinity of shikonin to MD2 was analysed using computer docking, surface plasmon resonance analysis and ELISA. *In vitro*, the anti-inflammatory effect and mechanism of shikonin were investigated through ELISA, real-time quantitative reverse transcription PCR, Western blotting and immunoprecipitation assay. *In vivo*, lung injury was induced by intratracheal administration of LPS and assessed by changes in the histopathological and inflammatory markers. The underlying mechanisms were investigated by immunoprecipitation in lung tissue.

KEY RESULTS

Shikonin directly bound to MD2 and interfered with the activation of toll-like receptor 4 (TLR4) induced by LPS. In cultured macrophages, shikonin inhibited TLR4 signalling and pro-inflammatory cytokine production. These effects were produced through suppression of key signalling proteins including the NF- κ B and the MAPK pathway. We also showed that shikonin inhibits MD2–TLR4 complex formation and reduces LPS-induced inflammatory responses in a mouse model of ALI.

CONCLUSIONS AND IMPLICATIONS

Our studies have uncovered the mechanism underlying the biological activity of shikonin in ALI and suggest that the targeting of MD2 may prove to be beneficial as a treatment option for this condition.

Abbreviations

ALI, acute lung injury; BALF, bronchoalveolar lavage fluid; ICAM-1, intercellular cell adhesion molecule 1; MD2, myeloid differentiation protein 2; MPMs, mouse primary peritoneal macrophages; MPO, myeloperoxidase; MyD88, myeloid differentiation primary response gene 88; RFUs, relative fluorescence units; rhMD2, recombinant human myeloid differentiation protein 2; RT-qPCR, real-time quantitative reverse transcription PCR; SPR, surface plasmon resonance; TLR4, toll-like receptor 4; VCAM-1, vascular cell adhesion molecule 1

Introduction

Acute lung injury (ALI) is a major cause of morbidity and mortality in intensive care units (Maca *et al.*, 2017). ALI is characterized by lung oedema, haemorrhage, bronchiole epithelial desquamation, marked thickening of the alveolar wall and neutrophil infiltration resulting in respiratory failure (Ashbaugh *et al.*, 1967; Fulkerson *et al.*, 1996; Matthay *et al.*, 2012). Current treatments for ALI are mainly supportive and include lung-protective ventilation (Amato *et al.*, 1998; Villar *et al.*, 2006; Diaz *et al.*, 2010), fluid management (Staub, 1978; Matthay *et al.*, 2017) and glucocorticoid treatment (Bernard *et al.*, 1987; Steinberg *et al.*, 2006). Unfortunately, these current treatments do not significantly reduce lung injury and associated mortality in patients. The lack of success of other pharmacological therapies for ALI continues to present it as a clinical challenge.

There are a variety of causes of ALI. However, these generally include infectious pneumonia, sepsis and non-infectious lung insults. Endotoxin is thought to be an important pathogen that leads to the development of ALI (Welbourn and Young, 1992; Hudson *et al.*, 1995). **LPS**, the cell wall component of Gram-negative bacteria, is commonly used to model ALI in rodents (Matute-Bello *et al.*, 2008). LPS administration induces inflammatory cell infiltration, inflammatory factor release and alveolar epithelial cell death. LPS mediates these effects primarily by activating toll-like receptor 4 (**TLR4**) (Wright *et al.*, 1990; Yang *et al.*, 1998; Tapping *et al.*, 2000), a part of the innate immune system. The binding of LPS to TLR4 requires an accessory protein called myeloid differentiation 2 [**MD2 (lymphocyte antigen 96)**], which participates in the LPS–TLR4 complex by associating with the extracellular domain of TLR4 (Park *et al.*, 2009; Nijland *et al.*, 2014). Following the formation of this complex, the signal is transduced by two pathways comprising the myeloid differentiation primary response gene 88 (MyD88) pathway (Akira *et al.*, 2001; Takeda and Akira, 2004; Kenny and O'Neill, 2008; Xie *et al.*, 2016) and the TIR-domain-containing adapter-inducing IFN- β (TRIF) pathway (Yamamoto *et al.*, 2003; Akira and Takeda, 2004). Studies have shown that the ALI-like phenotype following LPS administration is dependent on the MyD88 pathway and not the TRIF pathway (Togbe *et al.*, 2007; Kumar *et al.*, 2011; Moghimpour Bijani *et al.*, 2012). MyD88 activation induces downstream mediators including NF- κ B and MAPKs, leading to pro-inflammatory cytokine production. One critical point of intervention in this axis is MD2, which is necessary for LPS recognition. Inhibition of TLR4 signalling by targeting MD2 may potentially be an effective way to treat sepsis and endotoxin-induced ALI (Duan *et al.*, 2014; Peri and Calabrese, 2014). We have recently shown that inhibiting MD2 by chalcone derivatives prevents the LPS-induced ALI phenotype in mice (Zhang *et al.*, 2016, 2017). Unfortunately, there are no MD2 inhibitors currently in the clinic.

Shikonin (Figure 1A) is an active component extracted from the Chinese herb *Radix Arnebiae*. Shikonin has been shown to possess biological activities in inflammatory and infectious diseases (Andujar *et al.*, 2013). Shikonin has also been recently shown to prevent LPS-induced ALI in mice by suppressing NF- κ B (Bai *et al.*, 2013; Liang *et al.*, 2013). Based on these findings, we tested the hypothesis that shikonin

prevents LPS-induced lung injury by inhibiting the TLR4 signalling pathway and that this mechanism involves MD2. Indeed, our results show that shikonin interferes with the LPS–MD2 interaction. Working as an MD2 inhibitor, shikonin markedly suppressed LPS-induced NF- κ B and MAPK activation in macrophages and reduced pro-inflammatory cytokine expression. In the mouse model of ALI, shikonin also inhibited MD2/TLR4-dependent inflammatory responses. Our study has shown that shikonin has a therapeutic effect in ALI through inhibiting MD2.

Methods

Reagents

Shikonin was purchased from Aladdin (Shanghai, China). LPS, **TLR2** agonist **Pam3CSK4** and **PMA** were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against **p38**, p-p38, **JNK**, p-JNK and GAPDH were from Cell Signaling Technology (Danvers, MA, USA). Antibodies against **ERK**, p-ERK, I κ B, MyD88, NF- κ B P65 subunit, TLR4 and macrophage marker CD68 were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). **TNF- α** antibody was obtained from Abcam (Cambridge, MA, USA). MD2 antibody was from eBioscience (San Diego, CA, USA). Recombinant human MD2 (rhMD2) protein was obtained from R&D Systems (Minneapolis, MN, USA). Mouse TNF- α and **IL-6** ELISA kits were obtained from eBioscience.

Cell culture

Human THP-1 monocytes were purchased from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China) and cultured in RPMI 1640 (Gibco, Eggenstein, Germany). Media for THP-1 cells were supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT, USA), 100 U·mL⁻¹ penicillin and 100 mg·mL⁻¹ streptomycin.

Mouse primary peritoneal macrophages (MPMs) were prepared from C57BL/6 mice as described previously (Pan *et al.*, 2012). Mice (total $n = 63$) were stimulated by i.p. injection of 6% thioglycollate solution (0.3 g beef extract, 1 g tryptone and 0.5 g sodium chloride dissolved in 100 mL ddH₂O and filtrated through a 0.22 μ m filter; 2 mL used per mouse) and kept in a pathogen-free condition for 3 days before cell isolation. Total MPMs were harvested by washing the peritoneal cavity with RPMI-1640 medium (8 mL per mouse). Cell suspension was centrifuged and suspended in RPMI-1640 medium with 10% FBS. Primary cultures were prepared at a density of 700 000 cells per 35 mm diameter well and used 24 h after plating. Non-adherent cells were removed by washing with medium 4 h after seeding.

Surface plasmon resonance analysis for shikonin and MD2 protein interaction

The binding affinity of shikonin with rhMD2 protein was determined using a Biacore T200 instrument (GE Healthcare Inc., Piscataway, NJ, USA) with a CM5 sensor chip (GE, #10248879). Briefly, protein was loaded to the sensors activated with N₁-(ethylimino)methylene)-N₃,N₃-dimethylpropane-1,3-diamine and *N*-hydroxysuccinimide. The shikonin samples (at 0, 1.56, 3.125, 6.25, 12.5, 25, 50

Figure 1

Shikonin is an inhibitor of MD2. (A) Chemical structure of shikonin (SHI). (B) The binding affinity of shikonin with rhMD2 was determined using a SPR assay. Increasing concentrations of shikonin were mixed with rhMD2, and binding was determined. Association and dissociation constants are shown. (C) Shikonin reduced LPS binding to MD2. MD2 antibody was coated onto 96-well plates to immobilize rhMD2, and biotin-labelled LPS was added. Displacement of LPS by shikonin at different concentrations was detected using streptavidin-conjugated HRP and TMB substrate. Absorbance values at 450 nm are shown (mean $\pm$ SEM of five separate experiments performed in duplicate; * $P < 0.05$ compared with buffer-alone group). (D) Bis-ANS displacement assay to detect binding of shikonin to MD2. Bis-ANS was incubated with rhMD2 to reach stable fluorescence under excitation at 380 nm. Different concentrations of shikonin were added, and emission at 430–570 nm was detected. Data shown as relative fluorescence units (RFU). (E) MPMs were pretreated with shikonin at 0.4, 1 and 2.5 μM for 30 min and then exposed to LPS for 5 min. The complexes of MD2–TLR4 and MyD88–TLR4 were detected by immunoprecipitation. (F) Molecular docking of shikonin with rhMD2 (PDB ID 2E56) was analysed with the Sybyl-2.0 molecular modelling software from Tripos. (G, H) Effect of shikonin on Pam3CSK4-induced cytokine production in MPMs. Cells were pretreated with vehicle or shikonin at 0.4, 1 or 2.5 μM for 30 min and then exposed to $0.1 \mu\text{g mL}^{-1}$ Pam3CSK4 for 12 h. Levels of TNF- α and IL-6 in culture media were measured by ELISA and normalized to the respective total protein content. Data are presented as % of Pam3CSK4 group (mean $\pm$ SEM; $n = 5$).

or 100 μM) were prepared with running buffer (PBS, 0.5% P20 and 5% DMSO). Sensor and sample plates were placed on the instrument, and then the shikonin samples flowed over the black and target sensors. Eight concentrations were injected successively at a flow rate of 30 $\mu\text{L}\cdot\text{min}^{-1}$ for a 180 s association phase, followed by a 270 s dissociation phase at 25°C. The final graphs were obtained by subtracting blank sensor grams and blank sample from the duplex. Data were analysed with Biacore™ T200 software Set V3.x. K_D was calculated by global fitting of the kinetic data from various concentrations of shikonin using a 1:1 Langmuir binding model.

LPS displacement assay

The ability of shikonin to interfere with LPS binding to MD2 was determined using a cell-free assay. Human MD2 antibody was coated onto 96-well plates using 10 mM Tris-HCl buffer (pH 7.5). The plates were incubated at 4°C overnight. Plates were then washed with PBST and blocked with 3% BSA for 1.5 h at room temperature. Following blocking, rhMD2 was added at 4 $\mu\text{g}\cdot\text{mL}^{-1}$ in 10 mM Tris-HCl buffer (pH 7.5), and the plates were further incubated for 1.5 h at room temperature. After washing with PBST, biotin-labelled LPS (Biotin-LPS, InvivoGen, San Diego, CA, USA) was added for 1 h at room temperature with or without shikonin (0.1, 1, 10, 100 or 1000 μM). Streptavidin-conjugated HRP (Beyotime, Shanghai, China) was added for 1 h at room temperature. HRP activity was then determined in SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA) at 450 nm after the addition of TMB substrate (eBioscience).

Molecular docking of shikonin to MD2

A molecular docking simulation was performed using AutoDock version 4.2.6. The crystal structure of human MD2 lipid IVa complex (PDB code 2E59) was obtained from Protein Data Bank. The AutoDock Tools version 1.5.6 package was applied to generate the docking input files. A $60 \times 60 \times 60$ -point grid box with a spacing of 0.375 Å between the grid points was implemented. The affinity maps of MD2 were calculated by AutoGrid. One hundred Lamarckian genetic algorithm runs with default parameter settings were processed. We then analysed hydrogen bonds and bond lengths within the interactions.

Bis-ANS displacement assay

4,4'-Bis(phenylamino)-[1,1'-binaphthalene]-5,5'-disulfonic acid (bis-ANS, Carlsbad, CA, USA, 5 μM) and rhMD2 (5 nM) were mixed in PBS (pH 7.4) and allowed to reach stable fluorescence under excitation at 385 nm. Shikonin was then added for 5 min, and relative fluorescence units emitted at 430–570 nm were measured. Shikonin was tested at 2.5, 5, 10, 20 and 40 μM concentrations. Fluorescence measurements were performed with SpectraMax M5 at 25°C in 1 cm path-length quartz cuvettes.

Mouse model of ALI

All animal care and experimental procedures were approved by the Wenzhou Medical University Animal Policy and Welfare Committee (Approval Document No. wyd2016-0124), and all animals received humane care according to the National Institutes of Health (USA) guidelines. Animal studies

are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015).

Forty male C57BL/6 mice weighing 18–22 g were obtained from the Animal Centre of Wenzhou Medical University (Wenzhou, China). All animals were housed at a constant room temperature with a 12:12 h light–dark cycle and fed a standard rodent diet and given water *ad libitum*. Mice acclimatized to the laboratory for 7 days were randomly assigned to one of five groups ($n = 8$ per group). The groups consisted of vehicle control (CON), LPS-induced ALI group (LPS), shikonin treatment-only group (SHI; 25 $\text{mg}\cdot\text{kg}^{-1}$ shikonin), shikonin treatment of LPS-induced ALI (SHI + LPS 25; 25 $\text{mg}\cdot\text{kg}^{-1}$ shikonin) and a second shikonin treatment of LPS-induced ALI (SHI + LPS 12.5; 12.5 $\text{mg}\cdot\text{kg}^{-1}$ shikonin). Shikonin treatments were carried out for seven consecutive days prior to LPS challenge by gavage. Mice received shikonin p.o. (dissolved in 0.5% CMC-Na) at a volume of 200 $\mu\text{L} \times 20 \text{ g body weight}^{-1}$. CON and LPS groups received the same volume of 0.5% CMC-Na during this period. ALI was induced essentially as described previously (Yamada *et al.*, 2008; Zhang *et al.*, 2016; 2017). Briefly, after shikonin pretreatment period of 7 days, mice were anaesthetized with 2% sodium pentobarbital (80 $\text{mg}\cdot\text{kg}^{-1}$, Sigma-Aldrich, St. Louis, MO, USA) by i.p. injection. Heart rate, body temperature and toe pinch were consistently monitored to detect the depth of anaesthesia. Then, mice were challenged by intratracheal instillation of LPS (5 $\text{mg}\cdot\text{kg}^{-1}$, 0.9% saline) or equal volume 0.9% saline. Mice were killed 6 h after LPS challenge by an overdose of sodium pentobarbital. Serum, bronchoalveolar lavage fluid (BALF, see below) and lung tissue samples were collected and stored.

Serum was used for cytokine measurements using TNF- α and IL-6 ELISA kits. BALF samples were used for cytokine and cell measurements. From the lung tissues, the superior lobes of the right lungs were excised, and wet lung weight was estimated immediately after dissection. These sections were then dried at 60°C for 48 h and weighed again to obtain the dry lung weight. The ratio of the wet lung weight to the dry lung weight was calculated to assess lung oedema. Portions of lung sections were fixed in formalin and embedded in paraffin for histological analysis. The remaining lung tissues were used for RNA isolation and protein lysate preparation.

BALF preparation and analysis

A tracheal cannula was inserted into the primary bronchus, and BALF was performed through the cannula by using $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS. Approximately 0.8 mL BALF was acquired and centrifuged at $1000 \times g$ for 5 min at 4°C. The supernatant was immediately stored at -80°C . The sediment was resuspended in 50 μL 0.9% saline for determination of total number of cells and neutrophil count. Total cell number was acquired by use of a haemocytometer. Neutrophil count was acquired by counting 200 cells on a smear prepared by Wright–Giemsa stain (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Determination of TNF- α and IL-6 in culture media

MPMs were cultured at a density of 700 000 per well of six-well plates in normal growth media for 24 h. Cells were then

treated with shikonin at 0.4, 1 or 2.5 μM for 30 min. DMSO was used as vehicle control. Following pretreatment, cells were exposed to 0.5 $\mu\text{g}\cdot\text{mL}^{-1}$ LPS for 24 h. Culture media were collected and used to measure TNF- α and IL-6 levels using ELISA. Data were normalized to amount of total proteins from lysates of the same culture and expressed as a percentage of the LPS group in cell-based experiments (or were expressed as a percentage of one of mice in LPS group in animal-based experiments).

To test shikonin's effect on Pam3CSK4-induced TLR2 activation and inflammatory cytokine release, which is not dependent on MD2, MPMs were pretreated with shikonin for 30 min and exposed to the TLR2 agonist Pam3CSK4 at 0.1 $\mu\text{g}\cdot\text{mL}^{-1}$ for 12 h. TNF- α and IL-6 levels were measured as indicated above.

Western blotting

MPMs were treated with shikonin (0.4, 1, 2.5 μM) or vehicle control (DMSO) for 30 min and then incubated with LPS (0.5 $\mu\text{g}\cdot\text{mL}^{-1}$). LPS exposure was carried out for 25 min for MAPK activation (ERK, p38 and JNK) and 1 h for NF- κB activation (I κB). At the end of the time point, cells were lysed, and total protein was collected. The Bradford assay (Bio-Rad, Hercules, CA, USA) was used to detect the protein concentration. After being boiled in loading buffer (5 $\times$ loading buffer contain 0.5 M pH 6.8, Tris-HCl 2.5 mL, DTT 0.39 g, SDS 0.5 g, bromophenol blue 0.025 g and glycerol 2.5 mL) for 5 min, protein samples were separated by 10% SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad). Membranes were blocked with 5% milk in Tris-buffered saline containing 0.05% Tween 20 for 1.5 h at room temperature. Primary antibodies were applied, and membranes were incubated overnight at 4°C. The membranes were then washed in TBST and reacted with secondary HRP-conjugated antibodies (1:3000) for 1 h at room temperature. Blots were then visualized using an enhanced chemiluminescence reagent (Bio-Rad Laboratories, Hercules, CA, USA). The density of the immunoreactive bands was analysed using ImageJ software (NIH, Bethesda, MD, USA).

Assay of cellular NF- κB p65 translocation

MPMs were pretreated with shikonin (0.4, 1 and 2.5 μM) or vehicle control (DMSO) for 30 min and then incubated with LPS (0.5 $\mu\text{g}\cdot\text{mL}^{-1}$) for 1 h. Nuclear proteins were prepared using a cytoplasmic and nuclear protein extraction kit (KeyGEN, Nanjing, China). Levels of nuclear p65 subunit were then probed using Western blotting. In addition, NF- κB activation was assessed by staining cells for p65 translocation using a Cellular NF- κB p65 Translocation Kit (Beyotime Biotech, Nantong, China).

RNA extraction and real-time quantitative reverse transcription PCR assay

To obtain differentiated macrophages from THP-1 cells, THP-1 monocytes were incubated with PMA (250 $\text{ng}\cdot\text{mL}^{-1}$) for 24 h. MPMs and differentiated macrophages were treated with shikonin (0.4, 1 and 2.5 μM) or vehicle control (DMSO) for 30 min and then incubated with LPS (0.5 $\mu\text{g}\cdot\text{mL}^{-1}$) for 6 h. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA USA). Total RNA concentration was measured in duplicate

using SpectraMaxM5 Microplate Reader (Molecular Devices), and the purity of the samples was estimated by the OD ratio (A260/A280, ranging within 1.8–2.2). The expression of cytokines and adhesion molecules was also determined in lung tissues. Real-time quantitative reverse transcription PCR (RT-qPCR) was performed using the M-MLV Platinum RT-qPCR Kit (Invitrogen). Real-time quantitative PCR was carried out using the Eppendorf Realplex4 instrument (Eppendorf, Hamburg, Germany). Primers of genes encoding TNF- α , IL-6, **IL-1 β** , **COX-2**, intercellular cell adhesion molecule 1 (**ICAM-1**) and vascular cell adhesion molecule 1 (**VCAM-1**) were obtained from Invitrogen. The primer sequences are shown in Supporting Information Table S1. The relative amount of each gene was normalized to the amount of β -actin. The mRNA values for each gene were normalized to internal control β -actin mRNA and are expressed as a percentage of LPS group in cell-based experiments (or one of tissues in LPS group in animal-based experiments).

Immunoprecipitation assay to detecting MD2–TLR4 complexes

MPMs were treated with 0.5 $\mu\text{g}\cdot\text{mL}^{-1}$ LPS for 5 min in the presence or absence of shikonin. Cells were lysed with an extraction buffer containing mammalian protein extraction reagent, supplemented with protease and phosphatase inhibitor cocktails. Lysates were also prepared from lung tissues. Samples were centrifuged at 15 616 $\times$ g for 10 min at 4°C. A sufficient amount of TLR4 antibody was added to 400 μg protein, and samples were gently rotated at 4°C overnight. The immune complexes were collected with protein A + G agarose, and the precipitates were washed four times with ice-cold PBS. The proteins were then released by boiling in sample buffer, followed by Western blot analysis as described above.

Myeloperoxidase activity assay

To quantify neutrophil infiltration, myeloperoxidase (MPO) activity in the homogenized lung tissues was determined using an MPO Detection Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Lung tissue was homogenized in 1 mL of 50 mM potassium PBS (pH 6.0) containing 0.5% hexadecyltrimethylammonium hydroxide and centrifuged at 15 616 $\times$ g at 4°C for 20 min. Ten microlitres of the supernatant was transferred into PBS (pH 6.0) containing 0.17 $\text{mg}\cdot\text{mL}^{-1}$ 3,3'-dimethoxybenzidine and 0.0005% hydrogen peroxide (H_2O_2). MPO activity of the supernatant was determined by using absorbance values measured *via* spectrophotometry at 460 nm and presented as U $\cdot\text{g}^{-1}$ protein. Total protein content in the samples was analysed using total protein assay.

Lung histopathology

Formalin-fixed, paraffin-embedded lung tissues were sectioned at 5 μm thickness. Sections were stained with haematoxylin and eosin to estimate the degree of lung injury by light microscopy. Sections were also stained for TNF- α and macrophage marker CD68. Briefly, sections were deparaffinized in xylene and hydrated using an ethanol gradient. Heat-induced antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6.5). After blocking

endogenous peroxidase with 3% hydrogen peroxide, all sections were blocked in 5% BSA. Primary antibodies were applied, and slides were incubated overnight at 4°C. HRP-conjugated secondary antibody and 3,3-diaminobenzidine were used for detection.

Statistical analysis

All experiments are randomized and blinded. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). All data are reported as mean $\pm$ SEM. Statistical analysis was performed with GraphPad Prism 6.0 software (San Diego, CA, USA). In accordance with journal policy, statistical analysis was performed only when a minimum of $n = 5$ independent samples was acquired. We used one-way ANOVA followed by Dunnett's *post hoc* test when comparing more than two groups of data and one-way ANOVA, non-parametric Kruskal–Wallis test, followed by Dunnett's *post hoc* test when comparing multiple independent groups. P values of 0.05 were considered to be statistically significant. Post tests were run only if F achieved $P < 0.05$ and there was no significant variance in homogeneity.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a,b,c).

Results

Shikonin prevents MD2–LPS interaction

Of the numerous TLRs expressed in the lung tissue, data indicate that TLR2 and TLR4 promote inflammation in response to LPS as well as non-microbial stimuli (Jiang *et al.*, 2006; Qureshi *et al.*, 2006). Since MD2 acts as an important accessory protein in TLR4 (Shimazu *et al.*, 1999; Akashi *et al.*, 2000) and TLR2 (Dziarski *et al.*, 2001) pathways, we reasoned that the beneficial effects of shikonin in LPS-induced ALI involves inhibition or alteration of MD2. To test this idea, we first determined whether shikonin interacts with MD2. We achieved this by using surface plasmon resonance (SPR) to study the direct interaction between shikonin and rhMD2. The SPR assay showed that shikonin directly binds to rhMD2 protein in a dose-dependent manner (Figure 1B) with a K_D value of $5.67 \text{E} - 05 \text{ M}$. Meanwhile, shikonin showed no interaction with rhTLR4 protein (Supporting Information Figure S1). Next, we examined the effect of shikonin on the LPS–MD2 interaction through two displacement assays. We immobilized rhMD2 and added biotin-labelled LPS (biotin-LPS). Displacement of biotin-LPS was then probed following addition of increasing concentrations of shikonin. Our results showed that shikonin significantly decreases the binding of LPS to rhMD2 (Figure 1C). This competitive displacement also suggests that the binding site of shikonin on MD2 may overlap with the binding site of

LPS. To build on these findings, we used an MD2-bis-ANS displacement assay. Bis-ANS is a fluorescent probe, which is used to map the hydrophobic binding sites in proteins (Pastukhov and Ropson, 2003). We mixed bis-ANS with rhMD2 protein and observed increased fluorescence indicating binding (Figure 1D). The addition of shikonin to this mixture decreased the fluorescence intensity of bis-ANS in a dose-dependent manner, showing that shikonin binds to MD2 and displaces bis-ANS.

We next wanted to confirm the binding of shikonin to MD2 in functional assays. To do this, we exposed MPMs to LPS, which caused the formation of MD2–TLR4–MyD88 complex (Figure 1E). The addition of shikonin suppressed this complex formation in a dose-dependent manner (Figure 1E), confirming an inhibitory effect of shikonin. To investigate the underlying structural mechanism of this inhibition, we performed molecular simulation of shikonin–MD2 interaction. As shown in Figure 1F, shikonin fits into the binding pocket of MD2, displaying close contact with hydrophobic residues in the most energetically favourable simulation. The whole shikonin molecule was buried inside the lipid-binding pocket, indicating overlap and competition between shikonin and LPS sites. The computer-assistant prediction showed that several binding conformations of shikonin may exist in the MD2 pocket.

We next determined whether MD2 inhibition by shikonin plays a role in TLR2-mediated signalling or whether it is specific to TLR4. We exposed MPMs to Pam3CSK4, a synthetic triacylated lipopeptide, which specifically activates TLR2 (Ozinsky *et al.*, 2000). MPMs showed increased production of TNF- α and IL-6 in response to Pam3CSK4 (Figure 1G, H). However, treatment of MPMs with shikonin, at concentrations that inhibited MD2–TLR4–MyD88 complex formation, failed to dampen TLR2-mediated cytokine production. Collectively, these results show that shikonin binds and inhibits MD2 and prevents MD2–TLR4–MyD88 complex formation without altering TLR2 signalling.

Shikonin attenuates LPS-induced expression of pro-inflammatory factors in macrophages by inhibiting MAPK and NF- κ B

Our results showing that shikonin prevents the formation of the MD2–TLR4–MyD88 complex suggested that shikonin may attenuate LPS-induced activation of macrophages and subsequent cytokine production. To test this, we pretreated MPMs with different concentrations of shikonin for 30 min and then exposed the cells to LPS for 24 h. LPS increased the production of both TNF- α (Figure 2A) and IL-6 (Figure 2B) and shikonin pretreatment of cells significantly decreased this LPS-induced cytokine production. As expected, shikonin alone had no effect on inflammatory cytokine release. To profile other cytokines and inflammatory factors, which may be inhibited by shikonin, we utilized qPCR. Using the experimental platform shown in Figure 2, we exposed MPMs and differentiated macrophages from human THP-1 monocytes to LPS and assessed the effect of shikonin. Our results show that LPS induced marked increases in inflammatory cytokines including TNF- α , IL-6, IL-1 β and COX-2 (Figure 3A–D, G–J). In addition, ICAM-1 and VCAM-1 (Figure 3E, F, K, L) were increased by LPS in both MPMs

Figure 2

Shikonin (SHI) inhibited LPS-induced inflammatory cytokine production in MPMs. MPMs were pretreated with shikonin at 0.4, 1 or 2.5 μM for 30 min and then treated with LPS at 0.5 μg·mL⁻¹ for 24 h. DMSO was used as vehicle control for shikonin. (A) TNF-α and (B) IL-6 cytokine levels in the culture medium were measured by ELISA. Data were normalized to total protein concentration from the same plate and presented as % of LPS group (mean ± SEM of five independent experiments; *P < 0.05 compared with LPS group).

and differentiated macrophages. Shikonin pretreatment inhibited the LPS-induced increase in expression levels of all of these inflammation-associated compounds. These results provide further evidence that shikonin exhibits anti-inflammatory properties *in vitro*.

As LPS engages TLR4 to induce pro-inflammatory cytokines through the activation of MAPK and NF-κB, we assessed the effect of shikonin on these mediators. LPS significantly increased the phosphorylation of p38, ERK and JNK in MPMs (Figure 4A and Supporting Information Figure S2A–C). In addition, the NF-κB pathway was activated by LPS as illustrated by decreased levels of IκB levels (Figure 4B and Supporting Information Figure S2D) and increased nuclear levels of p65 subunit of NF-κB (Figure 4C and Supporting Information Figure S2E, F). A similar result was observed with p65 staining assay in MPMs (Figure 4D). Pretreatment of cells with shikonin markedly suppressed LPS-induced p65 nuclear translocation from the cytoplasm to the nucleus. We noted a decreased phosphorylation of MAPK pathway proteins, restoration of IκB levels and prevention of nuclear p65 in cells following shikonin treatment (Figure 4). These results are consistent with the inhibition of MD2 by shikonin and prevention of TLR4 signalling pathway.

Shikonin inhibited LPS-induced acute lung injury in mice

LPS is widely used to produce a murine model of ALI (Yamada *et al.*, 2008; Zhang *et al.*, 2016, 2017). As shikonin exhibited MD2 inhibitory activity *in vitro*, we examined the effects of shikonin on LPS-induced cellular and molecular deficits in the lungs of this animal model of ALI. Firstly, we detected the levels of MD2 and TLR4 in lung tissues of mice exposed to LPS; intratracheal instillation of LPS induced marked increases in both MD2 and TLR4 (Figure 5A, B and Supporting Information Figure S3). Histological analyses revealed that LPS triggered inflammatory cell infiltration, a thickening of alveolar walls and disrupted the normal lung tissue architecture (Figure 5C). Further evidence of LPS-induced structural deficits in lungs is illustrated by increased lung injury scores

(Figure 5D), increased wet-to-dry lung weight ratio (Figure 5E) and protein concentration in BALF (Figure 5F). These indices replicate those seen with ALI in humans. Pretreatment of mice with 12.5 or 25 mg·kg⁻¹ shikonin for 7 days prior to LPS challenge markedly inhibited this ALI-like pathology. Shikonin prevented the structural changes in the lung tissue, the infiltration of inflammatory cells and improved injury scores and oedema. It is interesting to note that both doses of shikonin tested in our study had an inhibitory effect on LPS-induced lung injury.

Shikonin inhibited inflammatory responses in lung tissues of mice by preventing MD2–TLR4 complex formation

The BALF of patients with ALI shows a neutrophilic inflammatory response with increased levels of pro-inflammatory cytokines (Pittet *et al.*, 1997). We examined whether shikonin pretreatment would attenuate LPS-induced inflammatory cell counts in BALF obtained from mice. LPS increased the total number of cells (density) as well as neutrophil counts in BALF obtained from mice (Figure 6A, B). However, a significant reduction in total cell and neutrophil counts was noted in BALF from mice pretreated with shikonin prior to LPS challenge. Although both doses were effective, a greater reduction was seen with the higher dose of 25 mg·kg⁻¹ shikonin. Moreover, LPS-challenged animals showed a significant increase in lung tissue MPO activity, a marker of neutrophils, while pretreatment with shikonin dose-dependently reduced the LPS-increase in MPO activity (Figure 6C). In addition to neutrophils, shikonin also reduced LPS-induced cytokine levels in BALF (Figure 6D, E). Interestingly, a reduction in IL-6 was only seen with the 25 mg·kg⁻¹ but not the 12.5 mg·kg⁻¹ dose. Overall, it also appeared that shikonin had a more pronounced effect on TNF-α. Similar results were obtained when TNF-α and IL-6 levels were probed in serum samples from these mice (Figure 6F, G). Shikonin reduced serum TNF-α, but had a smaller effect on IL-6 levels. This difference may result from the different role of TNF-α and IL-6 in the pathogenesis of ALI.

Figure 3

Shikonin (SHI) inhibits expression of pro-inflammatory cytokines by LPS. (A–F) MPMs were pretreated with shikonin at 0.4, 1 or 2.5 μM for 30 min, followed by exposure to LPS at 0.5 μg mL⁻¹ for 6 h. DMSO was used as vehicle control for shikonin. (G–L) Differentiated macrophages from human THP-1 monocytic line were pretreated with shikonin for 30 min, followed by exposure to LPS for 6 h. The mRNA levels of inflammatory cytokines: (A, G) TNF-α, (B, H) IL-6, (C, I) IL-1β, (D, J) COX-2 and adhesion molecules (E, K) ICAM-1 and (F, L) VCAM-1 were measured by RT-qPCR. Data were normalized to β-actin and are expressed as % of LPS group (mean ± SEM of five independent experiments; *P < 0.05 compared with LPS group).

Immunohistochemical staining of lung tissues harvested from mice following LPS administration showed increased reactivity to the macrophage marker CD68 (Figure 6H, I) and TNF-α (Figure 6J, K). Consistent with our other results, shikonin pretreatment was associated with reduced immunoreactivity of CD68 and TNF-α. This anti-inflammatory readout was also obtained when we assessed the expression of other cytokines and adhesion molecules through qPCR. The mRNA levels of cytokines (TNF-α, IL-6 and IL-1β), COX-2

and adhesion molecules (ICAM-1 and VCAM-1) showed that shikonin pretreatment prevented the LPS-mediated increases in these molecules (Figure 7A–F).

Our last objective was to examine the effects of shikonin on LPS-induced MD2–TLR4 complex formation in lung tissues. Since shikonin inhibited the formation of the MD2–TLR4 complex in cultured macrophages, the same was expected in lysates prepared from lung tissues. In LPS-challenged mice the formation of the MD2–TLR4 complex

Figure 4

Shikonin (SHI) inhibits LPS-induced MAPK and NF- κ B activation. (A, B) MPMs were pretreated with shikonin for 30 min followed by exposure to LPS for 25 min. Activation of the MAPK pathway (A) was assessed by measuring p-ERK, p-p38 and p-JNK. Total ERK, p38 and JNK were used as control. (B) NF- κ B activation was determined by measuring I κ B levels. GAPDH was used as loading control. (C) MPMs were pretreated with shikonin for 30 min followed by LPS exposure for 1 h. Cytosolic (upper blot) and nuclear (lower blot) P65 levels were determined by Western blot. GAPDH and lamin B were used as loading controls. (D) MPMs were treated as in panel C; p65 staining was carried out, and positivity was detected by Cy3-conjugated secondary antibody (red). Cells were counterstained with DAPI (blue) (scale bar = 100 px). Representative blots from five independent experiments are shown in panels A–C, and their densitometric quantifications are shown in Supporting Information-Figure S2.

was increased, while pretreatment with shikonin prevented this increase (Figure 7G, H). Taken together, these results reveal that shikonin exhibits its protective effects on LPS-induced ALI through targeting MD2 and disrupting TLR4 signalling.

Discussion

The aim of this study was to investigate the mechanism of the effects of shikonin on LPS-induced pro-inflammatory responses and lung tissue damage indicative of ALI. The key findings of our study include the discovery that shikonin binds and interferes with the interaction of LPS with MD2. This inhibitory effect on MD2 reduced MD2–TLR4 complex formation and downstream activation of NF- κ B and MAPK pathways. The overall effect of MD2 inhibition by shikonin was attenuation of inflammatory factors in cultured cells and in the mouse model of LPS-induced ALI. Furthermore, we showed that shikonin prevented the structural alterations

in lung tissues of mice challenged with LPS. Thus, our studies show that shikonin may serve as a candidate for ALI therapy as it inhibited the initiation of the deleterious TLR4 signalling cascade through disrupting MD2.

Although shikonin has been tested in various disease models, the underlying mechanisms of these effects have remained elusive. Most studies, however, have shown some alteration of MAPK/NF- κ B pathways. For example, shikonin administration to ovalbumin-challenged mice prevented pathological airway remodelling, the infiltration of inflammatory cells and collagen deposition through suppression of ERK and NF- κ B (Wang *et al.*, 2017). In fibroblast-like synoviocytes, which are commonly used to model rheumatoid arthritis, shikonin decreased LPS-induced IL-10 and TNF- α expression by inactivating NF- κ B (Sun *et al.*, 2017). Similar results were obtained when microglial cells were treated with shikonin prior to LPS challenge (Prasad *et al.*, 2015). These studies and others (Lee *et al.*, 2010; Liang *et al.*, 2013) clearly show that shikonin inhibits NF- κ B activity. NF- κ B plays an important role in regulating immune

Figure 5

Shikonin (SHI) prevents LPS-induced acute lung injury in mice. (A, B) Protein levels of (A) TLR4 and (B) MD2 in mouse lung tissues challenged with intratracheal LPS. GAPDH used as loading control. (C) Representative histological images of lung tissues harvested from mice following LPS challenge showing the effect of shikonin. Tissues were stained with haematoxylin and eosin (scale bar = 100 μ m). (D) Lung injury score as assessed by histological analysis of lung tissues. (E) Lung wet/dry ratio was determined at 6 h after LPS challenge. (F) BALF was collected 6 h after LPS challenge, and the amount of protein was measured. Data are reported as mean $\pm$ SEM; $n = 8$ mice per group; * $P < 0.05$ compared with LPS group.

responses. However, aberrant activation of NF- κ B is linked to an up-regulation of pro-inflammatory mediators in ALI (Fan *et al.*, 2001). NF- κ B binding sequences have also been identified in pro-inflammatory genes such as inducible NO synthase, COX-2 and TNF- α (Kempe *et al.*, 2005). In our study, we showed that shikonin inhibited LPS-induced NF- κ B activation in cultured macrophages. This inhibition was associated with a reduced expression and release of TNF- α and IL-6. Furthermore, COX-2 levels were suppressed by shikonin.

Based on our results, we do not expect shikonin to directly target NF- κ B activation or mediate its plethora of effects through regulating NF- κ B only. Our studies showed that shikonin also decreases the MAPK signalling pathway. This effect was seen at a very short time period following LPS exposure. Furthermore, shikonin has been shown to regulate the activities of ERK and JNK in cancer cells (Xuan and Hu, 2009; Wu *et al.*, 2013; Zhang *et al.*, 2013). Also, in a gastric cancer cell line, shikonin inhibited NF- κ B through a mechanism involving TLR2 (Liu *et al.*, 2015). These studies, in different systems, allude to a mechanism with a wide-spectrum of responses. As TLR2 and TLR4 mediate the responses to LPS in the lung (Jiang *et al.*, 2006; Qureshi *et al.*, 2006) and MD2 acts as the co-receptor or accessory protein for both TLR4 and TLR2 (Shimazu *et al.*, 1999; Akashi *et al.*, 2000; Dziarski *et al.*, 2001), we suspected MD2 as the target of shikonin. Indeed, using SPR and displacement assays, we showed that shikonin prevents the binding of LPS to MD2. In cell systems, we showed that this inhibitory effect of shikonin on MD2

prevents the subsequent LPS-mediated activation of TLR4. We also studied the potential mechanism of shikonin binding to MD2 using a molecular docking assay. Figure 1F shows that Arg⁹⁰ may be the key residue for the shikonin-MD2 interaction. In our previous studies, we found that three chalcone compounds, L6H21, L6H20 and L2H21, bind MD2 through the residues Arg⁹⁰ and Tyr¹⁰² (Wang *et al.*, 2015; Zhang *et al.*, 2016; 2017). Compounds L6H21/20/L2H21 have the structure of chalcone, but shikonin has a structure of flavone. Although their MD2 binding affinities are similar, the binding mode of shikonin is different from that of chalcones. Thus, the binding mode of shikonin is different from that of chalcone compounds. Our data suggest that Arg⁹⁰ may be a more important residue for discovering the small molecular inhibitors of MD2. Interestingly, these effects are specific to TLR4 as shikonin was unable to prevent TLR2-mediated increased cytokine expression. We challenged macrophages with Pam3CSK4 that mimics the acylated amino terminus of bacterial lipoproteins and specifically activates TLR2 (Ozinsky *et al.*, 2000). Pam3CSK4 is also a potent activator of NF- κ B (Aliprantis *et al.*, 1999). However, pretreatment of cells with shikonin only slightly inhibited the Pam3CSK4-induced increased cytokine expression. These findings support the notion that shikonin specifically targets MD2/TLR4 signalling.

Bronchial instillation of LPS in humans (O'Grady *et al.*, 2001) at small doses ranging from 1 to 4 ng-kg⁻¹ is shown to induce the early phase of ALI, as characterized by increases in neutrophils as well as pro-inflammatory cytokines in BALF.

Figure 6

Shikonin (SHI) reduces LPS-induced infiltration of inflammatory cells and cytokine levels. (A, B) Shikonin reduced the number of (A) total cells and (B) neutrophils in BALF following LPS challenge (mean $\pm$ SEM; $n = 8$ per group; $*P < 0.05$ compared with LPS). (C) Shikonin inhibited the LPS-induced MPO activity in lung tissue. (D–G) Shikonin inhibited LPS-induced inflammatory cytokines, (D, F) IL-6 and (E, G) TNF- α in BALF and serum. BALF levels are shown in (D) and (E), and serum levels are shown in (F) and (G). (H) Immunohistochemical staining for macrophage marker CD68 in lung tissues of mice challenged with LPS. Immunoreactivity is shown in brown (scale bar = 100 μm). (I) Quantification of CD68 positivity. Data shown as ratio of positive staining area to total area. (J) Immunohistochemical staining for TNF- α in lung tissues (scale bar = 100 μm). (K) Quantification of TNF- α positivity. Data shown as ratio of positive staining area to total area. Data are reported as mean $\pm$ SEM; $n = 8$ mice per group; $*P < 0.05$ compared with LPS group. All images are representative of eight mice per group. IOD, integral optical density.

LPS, not surprisingly, is widely utilized in a number of animal models of asthma, lung injury and ischaemic stroke (Lee *et al.*, 2010; Liang *et al.*, 2013; Wang *et al.*, 2014). In these models, shikonin prevents the pathological changes induced by LPS. The notion that shikonin may serve as a therapeutic candidate for ALI is intriguing as the only intervention proven to be effective in phase 3 trials in ALI is a procedure involving lung-protective ventilation (Acute Respiratory Distress Syndrome *et al.*, 2000). Even targeting specific pro-

inflammatory cytokines including TNF- α has not improved the outcome of patients. Furthermore, inhibiting one of the most prominent indicators of ALI, an infiltration of neutrophils, with neutrophil elastase inhibitors has been shown not to improve the outcome (Iwata *et al.*, 2010). Therefore, the utilization of shikonin and targeting of MD2 in ALI warrants further investigation. In recent years, inhibition of MD2 has been shown to attenuate inflammatory diseases, such as sepsis (Duan *et al.*, 2014), lung inflammation (Hadina

Figure 7

Expression of inflammatory cytokines in lung tissues. (A–F) mRNA levels of inflammatory cytokines (A) TNF- α , (B) IL-6, (C) IL-1 β , (D) COX-2 and adhesion molecules (E) ICAM-1 and (F) VCAM-1 in lung tissues of mice were determined by RT-qPCR. Data normalized to β -actin (mean $\pm$ SEM; $n = 8$ mice per group; * $P < 0.05$ compared with LPS group). (G) Shikonin (SHI) inhibited the formation of MD2/TLR4 complex in lung tissues. (H) Densitometric quantification of MD2/TLR4 complex levels (mean $\pm$ SEM; $n = 8$ mice per group; * $P < 0.05$ compared with LPS group).

et al., 2008) and asthma (Hosoki *et al.*, 2016). Furthermore, nasal aspiration of LPS does not produce pulmonary inflammation in MD2 knockout mice (Hadina *et al.*, 2008). In our study, we showed that shikonin targeted MD2 and markedly reduced LPS-induced pulmonary oedema, inflammatory cell infiltration and production of pro-inflammatory cytokines. These changes were mediated by a reduced formation of the MD2–TLR4 complex in lung tissue lysates. Collectively, these studies show that MD2 inhibition by shikonin has potential as a therapeutic approach for ALI.

In summary, our studies show that shikonin effectively inhibits LPS-induced pro-inflammatory responses in macrophages and in the mouse model of ALI. We have discovered that MD2 is a direct target of shikonin. Shikonin prevented LPS binding to MD2 and its subsequent presentation to TLR4. Following this disruption of the MD2–TLR4 complex by shikonin, the downstream signalling cascades are dampened. These downstream mediators include MAPK and NF- κ B signalling proteins. Reduced MAPK/NF- κ B activation translates to reduced cytokine expression, as seen in the cultured cells and mice *in vivo*. We also showed that MD2 is increased in lung tissues of mice following LPS challenge,

implicating its role in the pathogenesis of ALI. Therefore, MD2 may be an excellent target to pursue for the development of therapies for ALI. Our findings also suggest that shikonin may have therapeutic utility as it specifically inhibited MD2.

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Author contributions

Y.Z., T.X., Z.P. and C.S. contributed to the conception and design, collection, analysis and interpretation of data and manuscript writing; X.G., C.L., H.C. and Z.X. to the collection,

interpretation and analysis of data; and B.Z., Y.D. and G.L. to the conception and design, interpretation of data and manuscript revision.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.

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Table S1 The primer sequences of genes in real-time qPCR assay.

Figure S1 shikonin has no direct binding with rhTLR4. RhTLR4 protein was loaded to the sensors activated with EDC and NHS. The SHI samples (at 0, 1.56, 3.125, 6.25, 12.5, 25, 50, or 100 μ M) were prepared with running buffer (PBS, 0.5% P20, 5% DMSO). Sensor and sample plates were placed on the instrument, then the SHI samples were flowed the black and target sensors. Eight concentrations were injected successively at a flow rate of 30 μ L/min for a 180 s association phase, followed by a 270 s dissociation phase at 25 °C. The final graphs were obtained by subtracting blank sensor grams and blank sample from the duplex. Data were analyzed with Biacore™ T200 software Set V3.x. KD was calculated by global fitting of the kinetic data from various concentrations of SHI using a 1:1 Langmuir binding model.

Figure S2 Densitometric quantifications for western blots in Figure A-C. The band intensity on the western blots was analyzed by Image J software. All data are from 5 independent experiments. [Data are reported as mean $\pm$ SEM. * P < 0.05 compared to LPS group].

Figure S3 The densitometric quantification for MD2 or TLR4 expression in lung tissue presented in Figure A-B. The band intensity on the western blots was analyzed by Image J software. Data are from 8 mice per group. [Data are reported as mean $\pm$ SEM. # P < 0.05 compared to CON group].