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ORIGINAL PAPER

Methane alleviates carbon tetrachloride induced liver injury in mice: anti-inflammatory action demonstrated by increased PI3K/Akt/GSK-3β-mediated IL-10 expression

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Abstract The inflammatory response plays an important role in carbon tetrachloride (CCl_4)-induced acute liver injury and methane has been shown to exert beneficial effects on inflammation-associated diseases. Thus, we investigated the potential protective effects of methane-rich saline (MS) on CCl_4-induced acute liver injury and explored the underlying mechanism. A CCl_4-induced acute liver injury model was established by injection of CCl_4 (0.6 ml/kg, ip) in mice followed by treatment with MS (16 ml/kg, ip), 24 h later. All groups of mice were sacrificed and blood and liver tissues were collected. Serum aminotransferase, necrotic areas, and inflammatory cell infiltration in liver slices were enhanced after CCl_4 treatment but decreased with MS treatment. IL-6, TNF-α, IL-1β, IFN-γ, ICAM-1, CXCL1, MPO, NF-κB p65, ERK, JNK, and MAPK P38, expression in serum or liver homogenate were greater after CCl_4 treatment but comparatively less after MS treatment. Only IL-10 increased after MS treatment. Anti-IL10 blockade (1.5 mg/kg) restored MS-mediated attenuated phosphorylation of NF-κbB/MAPK and the protective effect of MS was abolished for all indices examined. The PI3K inhibitor, wortmannin had the same effects on MS as anti-IL-10 antibody. MS also induced phosphorylation of GSK-3β and AKT in CCl_4-treated mice. After pre-treatment with wortmannin (0.7 mg/kg), phosphorylation of GSK-3β and AKT proteins were reduced compared to its solvent control group-DMSO-treated animals. Thus, the data provide evidence that MS may activate the PI3K–AKT–GSK-3β pathway to induce IL-10 expression and produce anti-inflammatory effects via the NF-κB and MAPK pathways. The findings provide a new pharmacological strategy for management of inflammatory response after acute liver injury.

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Introduction

Carbon tetrachloride (CCl_4) is a hepatotoxicant that induces acute liver injury via generation of oxidative stress and recruitment of inflammatory cells (Mizuoka et al. 1999). CCl_4-induced hepatotoxicity is characterized by sinusoidal congestion, neutrophil invasion, and ballooning degeneration and features abnormal serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzyme activity. Several pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) contribute to hepatic fibrosis (Abdel-Moneim et al. 2015; Peng et al. 2009), indicating that inflammation is central to CCl_4-induced liver injury. CCl_4-induced liver injury differs from other drug-induced liver injury such as Con A-induced hepatotoxicity which resembles autoimmune liver disease.

Interleukin-10 (IL-10) is an anti-inflammatory pleiotropic cytokine which suppresses effector cells and mediates release of multiple cytokines (Si et al. 2010) to decrease T lymphocyte activity (Martin et al. 2010; Douglas et al. 2010) and strengthen immune tolerance. Previous work indicated that IL-10 can suppress ConA-induced liver injury (Erhardt et al. 2007; Shao et al. 2013) and ischemia–reperfusion injury (Ren et al. 2011). Dysregulated IL-10 production also contributes to inflammatory diseases (Ouyang et al. 2011; Yao et al. 2013).

The mechanism that controls IL-10 production in response to specific stimuli has been shown to include MAPK (Hovsepian et al. 2013) and several transcription factors. Nuclear factor κB (NF-κB) and MAPK regulate expression of many genes critical to the regulation of apoptosis, viral replication, tumorigenesis, inflammation, and various autoimmune diseases. Activation of NF-κB and MAPK may be a component of the stress response because they were activated by diverse stimuli including growth factors, cytokines, lymphokines, UV, pharmacological agents, and stress. Our work suggests that MS reduced phosphorylation of NF-κB, JNK, ERK and p38 in LPS-stimulated macrophages in an IL-10 dependent manner via enhanced activation of PI3K/AKT signal which involved activated GSK-3β (Zhang et al. 2016). Inhibition of GSK-3β was reported to reduce liver ischemia reperfusion injury via an IL-10-mediated immunoregulatory mechanism (Ren et al. 2011). Thus, we studied the relationship between PI3K–AKT–GSK-3β pathways and mechanisms of IL-10 production.

Methane is the most common organic atmospheric gas and fuel source (Montano-Loza et al. 2017). Previous studies focused on biological characteristics of methane in the gastrointestinal tract (Ghoshal et al. 2016), specifically finding that exogenously applied methane has protective effects on the intestine (Varga et al. 2012; Boros et al. 2012), heart (Chen et al. 2015), abdominal skin flaps (Song et al. 2015), retina (Liu et al. 2016; Wu et al. 2015), and nervous system (Fan et al. 2016; Wang et al. 2016). Hepatoprotection is also reported to be conferred by methane (Strifler et al. 2016; Ye et al. 2015; He et al. 2016). Methane has been shown to inhibit the inflammatory response via modulating various pathways. Studies of methane include physiological saline for gas dissolution to reduce flammability and explosiveness of methane gas (Zhang et al. 2016). Methane gas is relatively stable for 1 month (Roccarina et al. 2010; Chen et al. 2015) and as such may have therapeutic use. Therefore, we studied the anti-inflammatory and protective effects of methane in a CCl_4-induced acute liver injury model by measuring cytokine IL-10 and associated intracellular signaling pathways.

Materials and methods

Animals and reagents

C57BL/6 mice (6–8 weeks-of-age; 18–22 g; male), were purchased from the Animal Experimentation Center of the Second Military Medical University. All animals were housed under specific pathogen-free conditions and were provided with Rodent Lab Chow and water ad libitum. CCl_4 was purchased from Sinopharm Chemical Reagent Co., Ltd (China) and was dissolved in olive oil. All other chemicals and reagents used were standard analytical grade.

Methane-rich saline production

Methane was stored in a gas canister and was dissolved in normal saline under high pressure (0.4 MPa) for 3 h to a saturated level. The saturated MS was stored under atmospheric pressure at 4 °C and was freshly prepared 1 day prior to the animal experiments to ensure stability. Gas chromatography (gas chromatography-9860, Qiyang Co., Shanghai, China) was used to measure methane in the saline solution according to published methods (Ren et al. 2011).

CCl_4-induced acute liver injury in mice

Mice were pretreated with DMSO, anti-IL10 antibody (1.5 mg/kg) or wortmannin (0.7 mg/kg) for 24 h. Acute liver injury was induced by injecting CCl_4 (0.6 ml/kg, ip, 12 µl:400 µl olive oil). MS (16 ml/kg) was administered 1 h after CCl_4 injection, and sham mice were treated with olive

oil (16 ml/kg). Serum and liver specimens were collected at the indicated time points.

Measurement of liver enzymes and cytokine production

Mice were anesthetized 24 h after CCl_4 treatment and blood was collected via heart puncture. Plasma was separated following centrifugation at 300×*g* for 10 min. Serum ALT and AST were measured as described by Magaye et al. (2016) with an automatic dry biochemical analyzer (Hitachi Auto Analyzer 7170, Japan). Serum IL-6, TNF-α, IL-1β, IFN-γ and IL-10 and myeloperoxidase (MPO), chemokine ligand 1 (CXCL1), Intercellular adhesion molecule-1 (ICAM-1) in liver homogenates were measured using ELISA (eBioscience, San Diego, CA) according to the manufacturer's instructions.

Histopathological assessment

Liver tissues were harvested in 24 h after CCl_4 administration. Liver samples were preserved in 4% paraformaldehyde for a minimum period of 72 h. Specimens were embedded in paraffin and cut into 4–5 μm sections for hematoxylin and eosin (H&E) staining. Inflammation and tissue damage were observed using light microscopy.

Real-time PCR

Total RNA was extracted from liver tissue using Trizol reagent (Takara, Japan). Concentration and purity of total RNA was measured using the absorbance ratios at 260/280 nm. Complementary DNA (cDNA) was reverse-transcribed using a Prime Script RT Reagent Kit (Takara). Quantification of IL-6, TNF-α, IL-1β, IFN-γ, CXCL1, ICAM-1 and IL-10 mRNA was conducted using Step One Plus Real Time PCR System (Applied Biosystems, CA). Murine primers were synthesized by Invitrogen and sequences were as follows:

IL-6:F5′-TAC CAC TCC CAA CAG ACC TG-3′(forward),5′-GGTACTCCAGAA ACCAGA GG-3′(reverse);TNF-α:5′-CACCATGAGCACAGA AAGCA-3′(forward), 5′-TAGACAGAAGAGCGT GGTGG-3′(reverse);IL-1β:5′-ACTCATTGTGGCTGT GGAGA-3′(forward),5′-TTGTTCATCTCGGAGCCT GT-3′(reverse);IFN-γ:5′-CCTCAA ACTTGG CAA TACTCA-3′(forward);5′-CTCAAGTGGCATAGA TGTGGA-3′(reverse);CXCL1:5′-GCTTGAAGGTGT TGCCCTCAG-3′(forward),5′-AGAAGCCAGCGT TCACCAGAC-3′(reverse);ICAM-1:5′-TTCACACTG AATGCCAGCCC-3′(forward);5′-GTCTGCTGAGAC CCCTCTTG-3′(reverse);IL-10:5′-TGCCACTCAGAA GACTGYGG-3′(forward),5′-GTCCTCAGTGTAGCC CAGGA-3′(reverse);GAPDH:5′-ATGGTGAAGGTC GGTGTGAA-3′(forward),5′-TGGAAGATGGTGATG GGCTT-3′(reverse).

Western blotting

Liver tissues from experimental animals were homogenized in protein lysis buffer (Thermo Fisher Scientific, location missing) with protease inhibitor (Gibco, location missing). After centrifugation (13,000×*g*, 4 °C, 10 min), protein was measured using a BCA protein assay kit (Thermo Fisher Scientific). Equal amounts of protein were loaded in each well and separated using 10% SDS-PAGE (Life Technologies, Carlsbad, CA). Gels were subsequently transferred to nitrocellulose membranes (Life Technologies) and membranes were blocked for 1 h in 5% non-fat dried milk and then incubated overnight at 4 °C with primary antibodies (Cell Signaling Technology). Membranes were washed with TBST and incubated with secondary antibody for 2 h at room temperature. Finally, protein bands were visualized using an ECL kit (Thermo Fisher Scientific, Waltham, MA). Band intensity was quantified using Image J software and protein expression was normalized according to expression of GAPDH protein.

Statistical analysis

Data are presented as means ± standard deviation (SD). Significant differences were confirmed with one-way ANOVA, followed by Turkey's test and two-way ANOVA. Prism 5 software package (GraphPad) was used to calculate p values and $p < 0.05$ was considered statistically significant.

Results

MS protects mice from CCl_4-induced liver injury and inhibits CCl_4-induced liver inflammation

Serum ALT and AST activity significantly increased after CCl_4 treatment and MS prevented this elevation (Fig. 1C, D). Histopathological studies supporting the biochemical data (Fig. 1A). Liver sections obtained from sham and treatment (sham + MS) groups had normal liver architecture (a, b, g, h) and no necrosis (Fig. 1B). CCl_4 caused large areas of extensive pericentral necrosis (Fig. 1B) with hepatic sinus congestion, neutrophil infiltration, ballooning degeneration, and loss of hepatic architecture (c, i). After MS administration to mice, the development of histopathological alterations induced by CCl_4 were inhibited (d, j), and necrosis was decreased (Fig. 1B). Data suggested that MS protected mice against CCl_4-induced liver injury.

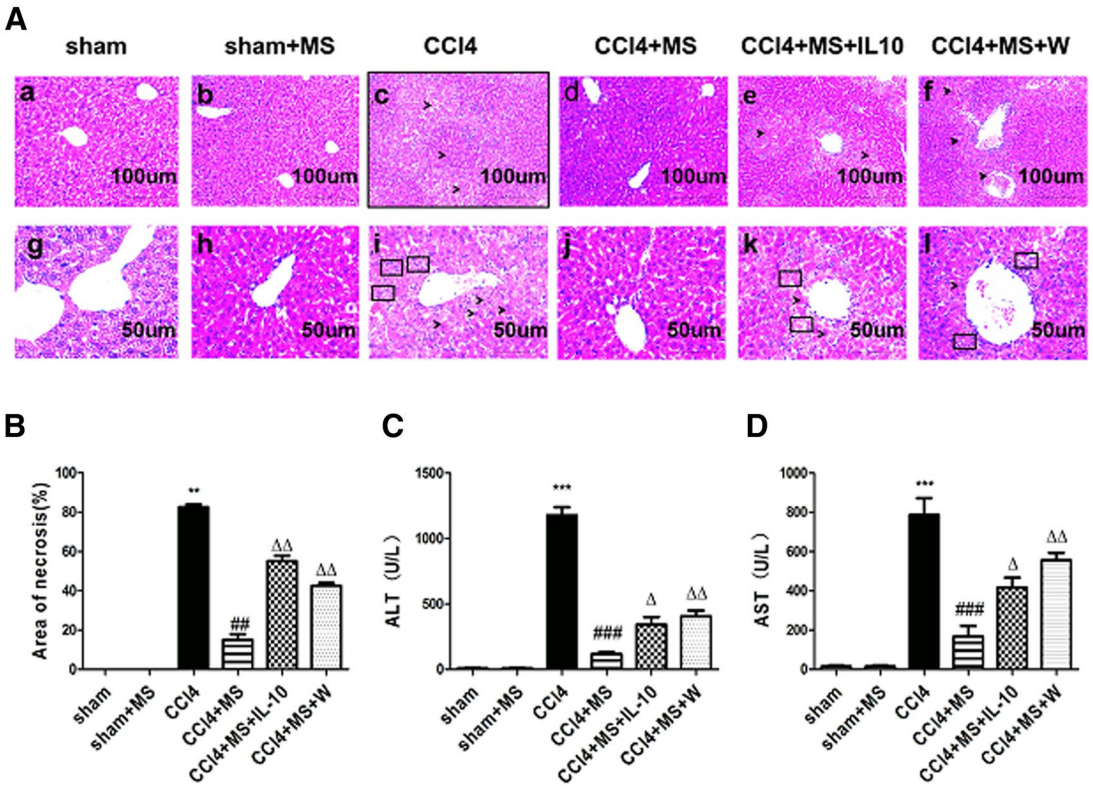

Fig. 1 MS protects mice from CCl_4-induced liver injury. Liver tissues were stained with H&E after collection from mice sacrificed 24 h after treatment with sham (*a, g*), sham + MS (*b, h*), CCl_4 (*c, i*), CCl_4 + MS (*d, j*), CCl_4 + MS + anti-IL10 (*e, k*), CCl_4 + MS + wortmannin (*f, l*) (*Scale bar* 100 μm, 50 μm), *Arrow* indicates preserved hepatic sinus congestion and ballooning degeneration; *squares* indicate areas of neutrophil infiltration and loss of hepatic architecture (**A**), necrotic areas were analyzed (**B**). Serum ALT and AST for each group (**C, D**). Data are as means ± SD (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. sham group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. CCl_4 group, $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$ vs. CCl_4 + MS group

Serum cytokines were measured after treatment with CCl_4 and IL-6, TNF-α, IL-1β and IFN-γ were elevated and MS treatment reduced these elevations (Fig. 2a–d). Relative IL-6, TNF-α, IL-1β and IFN-γ mRNA was significantly increased after CCl_4 treatment but decreased after MS treatment (Fig. 2e–h). Thus, MS protects liver function by inhibiting expression of inflammatory cytokines. We also measured neutrophils such as CXCL1, MPO and ICAM-1. Serum CXCL1, ICAM-1 and MPO activity and mRNA were increased in CCl_4-treated mice and decreased after treatment with MS (Fig. 2i–m). IL-10 expression and mRNA was not significantly changed after CCl_4 treatment but it was increased with CCl_4 and MS administration (Fig. 2n, o). Thus, anti-inflammatory effects of MS may be tied to IL-10.

Anti-IL-10 antibody and wortmannin reversed protective effects of MS in CCl4-induced acute liver injury

To study the contribution of IL-10 to the hepatoprotective effect of MS, we used an anti-IL-10 antibody and measured liver function. After CCl_4 and MS treatment with the antibody, serum ALT and AST were restored to normal (Fig. 1C, D). Similarly, histopathological studies indicated that the inhibitory effect of MS was partially reduced in mice in the presence of the anti-IL-10 antibody (Fig. 1A, B). Serum inflammatory cytokines (Fig. 2a–h) and neutrophil chemokines (Fig. 2i–m) and their mRNA were increased with concomitant administration of the anti-IL-10 antibody and MS. Thus, MS produced anti-inflammatory effects and restored liver function in CCl_4-treated mice via increased production of IL-10. GSK-3β is associated with IL-10 secretion via modulation of the PI3K–AKT pathway, so we inhibited GSK-3β with wortmannin and data show that the protective effect of MS was blocked.

MS reduces activation of NF-κB and MAPK in CCl_4-induced liver injury

The NF-κB and MAPK pathways are responsible for inflammatory cytokine production in mammals so whether MS influenced expression of NF-κB p65, ERK,

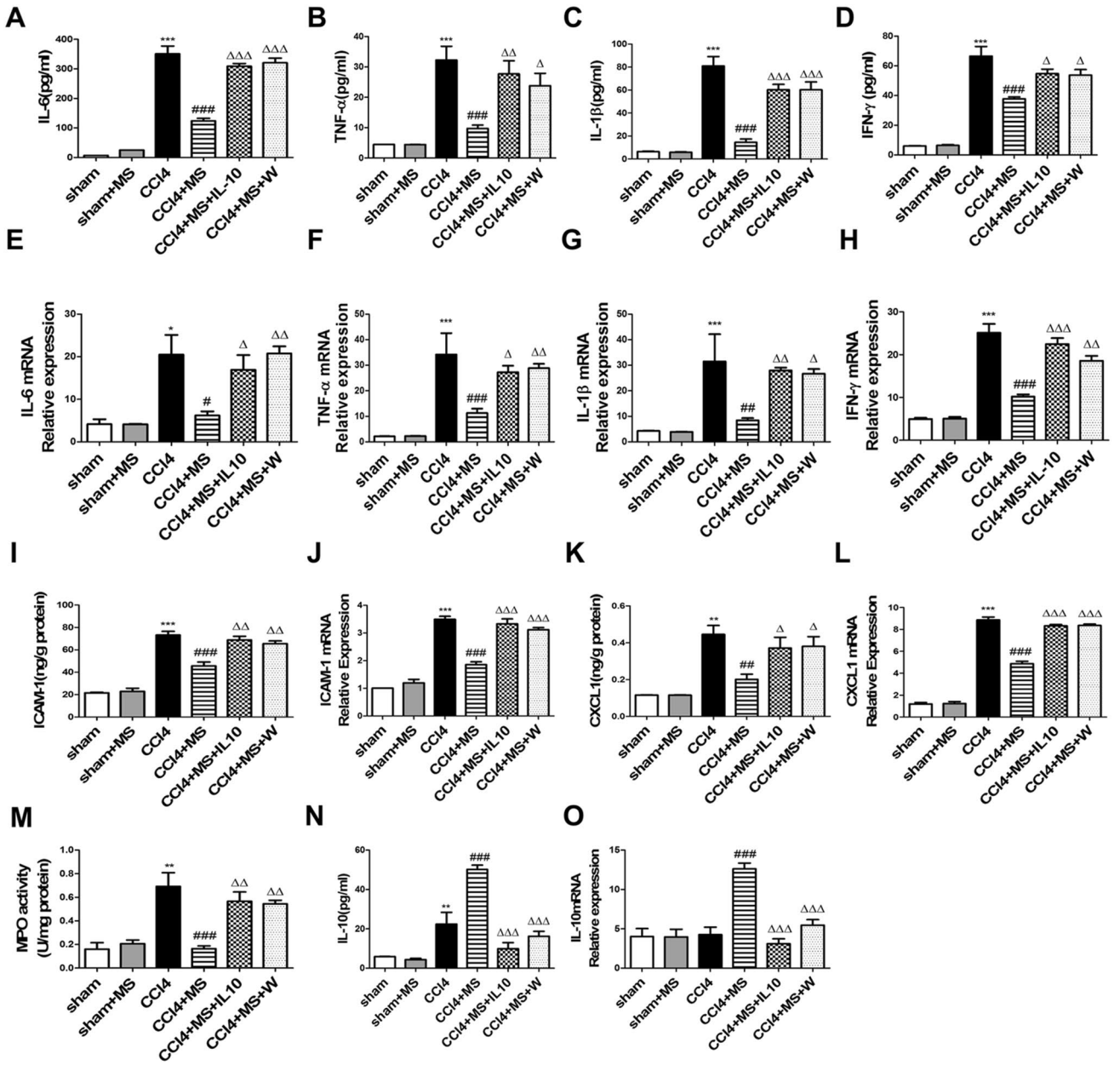

Fig. 2 MS inhibits inflammation in CCl_4-induced acute liver injury in mice. Serum IL-6, TNF-α, IL-1β, IFN-γ, IL-10 (**a–d, n**) and liver homogenate ICAM-1 (**i**), CXCL1 (**k**) were measured using ELISA. IL-6, TNF-α, IL-1β, IFN-γ (**e–h**), ICAM-1 (**j**), CXCL1 (**l**) and IL-10 (**o**) mRNA were measured using RT-PCR. MPO activity quantification in liver homogenates (**m**). Data are means ± SD (n = 6), $^{*}p < 0.05$, $^{**}p < 0.01$ vs. sham group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. CCl_4 group, $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$ vs. CCl_4 + MS group

JNK and MAPK P38 proteins was investigated and data show that NF-κB, ERK, JNK and p38 signaling pathways were activated by CCl_4. MS treatment inhibited phosphorylation of NF-κB P65, ERK, JNK and p38 proteins (Fig. 3a) and these results were statistically significant (Fig. 3c–f).

Anti-IL-10 and wortmannin reverses reduced NF-κB protein and MAPK phosphorylation in MS-treated mice

Studies suggest that GSK-3 acts on IL-10 and influences the NF-κB pathway. To explore anti-inflammatory

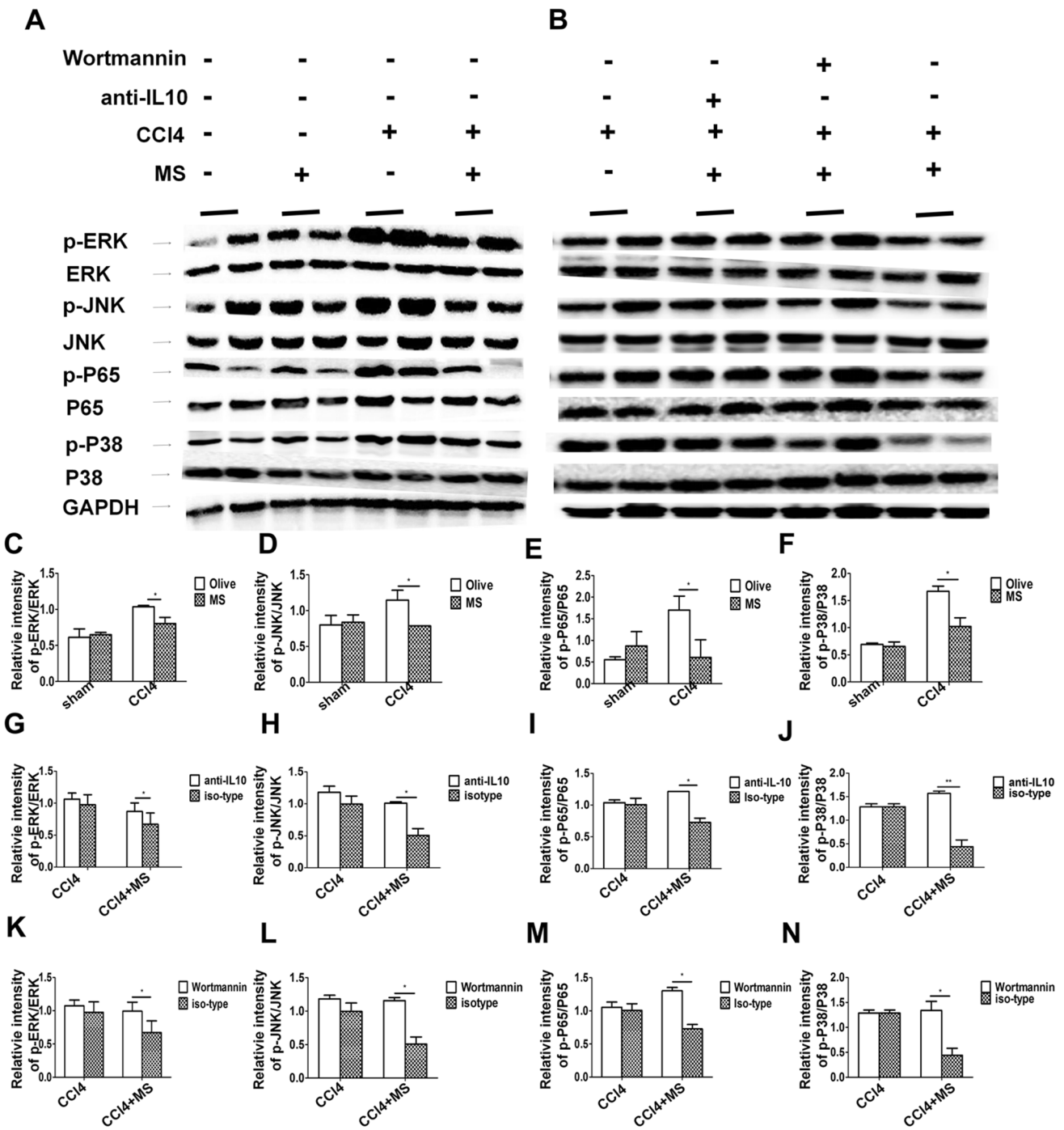

Fig. 3 Anti-IL-10 antibody and wortmannin reversed NF-κB and MAPK protein phosphorylation in MS-treated mice according to Western blot of liver homogenates (**a, b**). Band intensities were quantified as ratios of phosphorylated signaling molecules to total molecules (**c**–**n**). $*p < 0.05$, $**p < 0.01$

mechanism of MS, mice were pretreated with an anti-IL-10 antibody and wortmannin as previously described. NF-κB and MAPK protein expression was measured before and after inhibition of PI3K enzyme and IL-10 (Fig. 3b). Pretreatment anti-IL-10 antibody and wortmannin increased the ratio of phosphorylated to total the NF-κB and MAPK proteins (Fig. 3g–n) in MS-treated mice, suggesting that these proteins regulate anti-inflammatory processes in CCl_4-treated mice. So, anti-IL-10 antibody and wortmannin abolished the inhibitory effect of MS via increased phosphorylation of MAPK proteins and NF-κB.

IL-10 expression was mediated by the PI3K–AKT–GSK-3β pathway in MS-treated mice

MS diminished expression of pro-inflammatory factors and protected livers against injury induced by CCl_4. MS augmented expression of IL-10 and inhibited the phosphorylation of NF-κB, ERK, JNK, and p38 (Figs. 1, 2, 3). Anti-IL-10 antibody and wortmannin treatment reversed hepatoprotection offered by MS. Thus, an association between IL-10 and PI3K was investigated (Fig. 4) and treatment with MS increased phosphorylation of GSK-3β and AKT after CCl_4-induced liver injury and wortmannin treatment inhibited activation of GSK-3β and AKT (Fig. 4). Wortmannin treatment decreased IL-10 (Fig. 2n, o), suggesting that GSK-3β was essential for IL-10 expression. This, data suggest that MS activates the PI3K–AKT pathway and promotes IL-10 expression via activation of GSK-3β which then produces an anti-inflammatory effect via the NF-κB and MAPK pathways.

Discussion

Methane is one of the simplest organic compounds (Zhang et al. 2016) and it is increasingly being studied for medical applications. We reported that MS protected against LPS-induced inflammation and ConA-induced liver injury in several animal models (Zhang et al. 2016; He et al. 2016) and here we report that MS was protective against CCl_4-induced acute liver injury. CCl_4 caused extensive necrotic areas and inflammatory infiltration in mouse liver sections. Serum ALT and AST were used to establish the severity of liver injury (Maes et al. 2016; Szabo and Petrasek 2015). Treatment with MS significantly decreased necrotic areas and inflammatory infiltration and reduced serum ALT and AST. Thus, MS reduced liver damage induced by CCl_4.

Tissue inflammation contributes to liver pathology and pro-inflammatory cytokines such as IL-6, TNF-α, IL-1β, IFN-γ are associated with the pathogenesis of drug-induced liver injury (Mackenzie et al. 2013; Juhaszova et al. 2004). We hypothesized that MS exerted effects by reducing inflammation so we measured IL-6, TNF-α, IL-1β and IFN-γ mRNA and protein and found that they

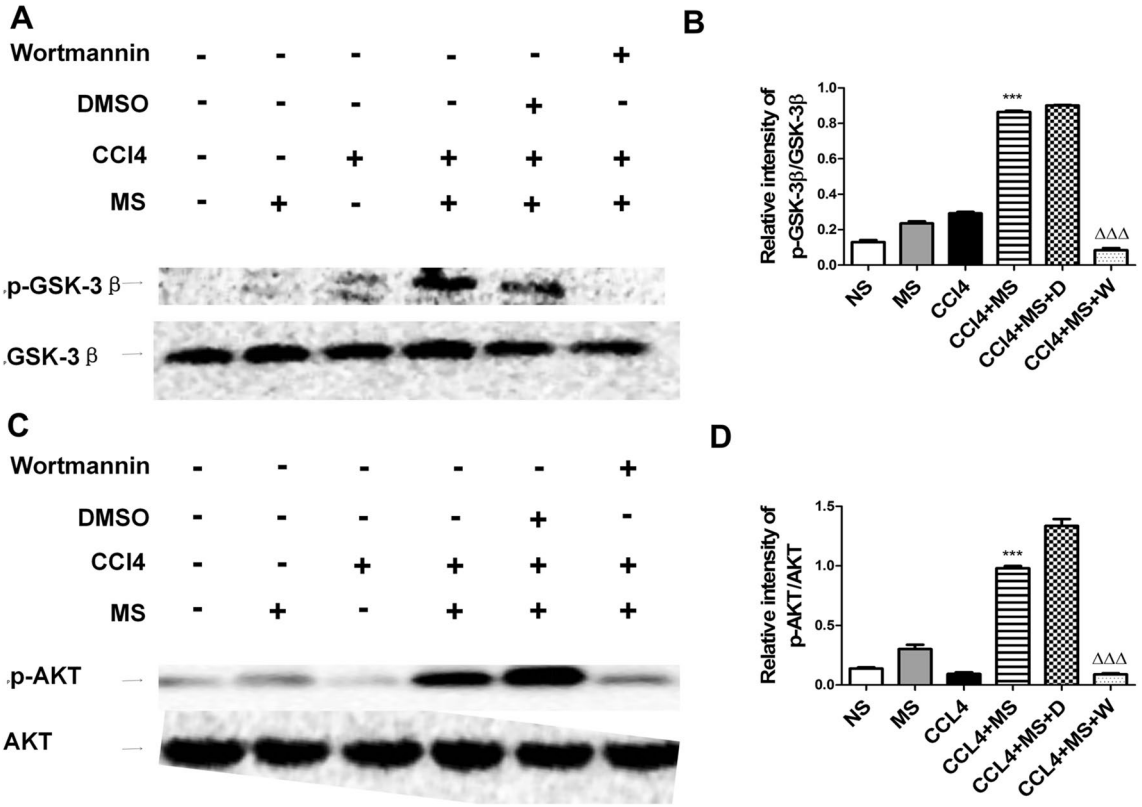

Fig. 4 GSK-3β-mediated IL-10 expression in MS-treated mice is promoted via the activation of PI3K–AKT. Mice were pre-treated with DMSO or wortmannin (0.7 mg/kg) for 24 h and Western blot was used to quantify protein (**a, c**). Band intensities were quantified as ratios of phosphorylated signaling proteins to total proteins (**b, d**). $^{**}p < 0.01$ vs. sham group, $^{\Delta\Delta}p < 0.01$ vs. CCl_4 + MS group

were increased in CCl_4-treated mice. Treatment with MS after CCl_4 administration reduced elevations IL-6, TNF-α, IL-1β and IFN-γ. CXCL1, MPO and ICAM-1 expression are key to the migration of neutrophils (Özdemir-Kumral et al. 2016; Zhao et al. 2016) and MS treatment decreased chemokines protein and mRNA, confirming anti-inflammatory effects of MS in our mouse model.

IL-10 blocks the inflammatory response (Bekker et al. 2016; Yao et al. 2013a, b) and may protect against hepatic injury (Peng et al. 2009; Rood et al. 2016). MS treatment increased IL-10 protein and mRNA expression and inhibition of IL-10 expression with an anti-IL-10 antibody reduced elevated ALT and AST, pro-inflammatory cytokines and neutrophilic chemotactic factor. Thus, IL-10 may protect the liver from injury.

IL-10 is reported to modulate the inflammatory response by inhibiting NF-κB, ERK/MAPK pathways (Peng et al. 2009; Mittal and Roche 2015; Gabrysova et al. 2014), and transcription factors such as CREB, NF-κB p50 homodimers, and C/EBPb (Ananieva et al. 2008; Cao et al. 2006; Nandan et al. 2012; MacKenzie et al. 2013; Sanin et al. 2015). However, we did not observe upregulation of activation of p38MAPK in MS-treated mice. To investigate the down-stream mechanism of IL-10, we neutralized IL-10 expression with an anti-IL-10 antibody and measured phosphorylation of proteins activated by NF-κB transcription factor and MAPK. Data show that neutralization of IL-10 partially reversed MS-induced decreased NF-κB p65, ERK, JNK and P38 protein phosphorylation. Thus, IL-10 may inhibit pro-inflammatory cytokine expression via the NF-κB and MAPK pathway in MS-treated mice.

The PI3K–AKT–GSK-3β pathway is thought to be a significant producer of IL-10 (Beurel et al. 2010, 2015) and the PI3K inhibitor wortmannin inhibited protective and anti-inflammatory effects of MS after liver injury induced by CCl_4. MS treatment increased IL-10 and wortmannin decreased them. MS increased and prolonged expression of p-GSK-3β and p-AKT, and PI3K inhibition reduced these effects indicating involvement of the PI3K–AKT–GSK-3β pathway in MS-induced IL-10 production.

Akt is activated by phosphorylation of various enzymes, kinases, transcription factors and other downstream factors and this downregulates IL-10. The mammalian target of rapamycin (mTOR), activation of GSK-3β, and phosphorylation of Foxo138-40, so mTOR and Foxo1 may also act upstream to mediate p-GSK-3β-induced IL-10 production in MS-treated mice.

IκB kinase (IKK) is activated by Akt and this leads to the degradation of NF-κβ inhibitor, Iκβ, and enhances expression of NF-κB and promotes cell survival. As NF-κB was not upregulated in MS-treated mice, how MS exerts effects on the PI3K–AKT pathway is unclear, but it may act on membrane channels of G-proteins, membrane receptor-mediated signaling, or acetylcholine-activated ion channel kinetics (Kai et al. 1998; Sokoll et al. 1989; Puig et al. 1988). Alternatively, MS may accumulate at interfaces of cell membranes to modulate the function of membrane-bound enzymes (Ghyczy et al. 2008). MS easily penetrates membranes and diffuses into organelles (Pimentel et al. 2006; Venardos et al. 2007), so it may penetrate and activate PI3K–AKT, a hypothesis that supports our observation MS peaked in the circulation of mice in 10 min.

The molecular mechanism underlying GSK-3β regulation of anti-inflammatory cytokine expression in our study is poorly characterized. Active GSK-3β may reduce NF-κB activation by enhancing interactions of CREB with the CBP, which leads to reduced CBP binding with NF-κB (53). Alternatively, GSK-3β may facilitate NF-κB activity by activation of NF-κB p65 (Viatour et al. 2004) and limitation of NF-κB activation in unidentified pathways (Schwabe et al. 2002). It is more likely that reduced activation of NF-κB and p38 MAPK contributes to GSK-3β-mediated IL-10 production in our study, because neutralization of IL-10 partially reversed decreased activation of NF-κB and MAPK. Because the intracellular signaling pathways controlling IL-10 production are complex, how p-GSK-3β regulates IL-10 expression in our study was not resolved.

In conclusion, MS protected against CCl_4-induced acute liver injury as evidenced by liver function enzymes and reduced liver injury. MS treatment significantly inhibited inflammatory responses likely via activation of the PI3K–AKT–GSK-3β pathway and increased production of IL-10 and suppressed NF-κB and MAPK signaling in CCl_4-treated mice. Furthermore, inhibition of IL-10 and GSK-3β reduced the protective effects of MS in CCl_4-treated mice, suggesting a requirement of IL-10 for this signaling pathway and the contribution of IL-10 production to the protective effect of MS.

Our study has some limitations. CCl_4-induced liver injury has been confirmed in neutrophils and Kupffer cells so in future studies, we will focus on IL-10 activity in different cell types. Also, different time points and a MS concentration gradient are needed to understand dose and time-responses of MS for hepatic protection against acute injury. We must clarify whether hepatoprotective effect is restricted to CCl_4 hepatotoxicity as well. Despite the aforementioned limitations, MS offers a new therapeutic strategy for clinical application and our data suggest a rationale for developing new pharmacological strategies to treat acute liver injury.

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Compliance with ethical standards

Conflicts of interest All authors have no potential financial or ethical conflicts of interest regarding this paper.

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