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Original Paper

Anti-Inflammatory Activity of Dehydroandrographolide by TLR4/NF-κB Signaling Pathway Inhibition in Bile Duct-Ligated Mice

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Key Words
Dehydroandrographolide • Cholestatic liver injury • Inflammation • TLR4 • NF-κB activation

Abstract
Background/Aims: Clinically, biliary obstruction is often accompanied by progressive inflammation. Dehydroandrographolide (DA) possesses anti-inflammatory properties. However, the anti-inflammatory activities of DA in cholestatic liver injury remain unclear. ***Methods:*** Mice were administered with DA by intraperitoneal injection after bile duct ligation (BDL) on day 1. Then mice were subjected to an ileocecal vein injection of lipopolysaccharide (LPS). Liver function markers, histology, pro-inflammatory cytokine levels, NF-κB activation and fibrosis formation were evaluated in BDL mice with LPS. LPS binding to primary Kupffer cells was examined by high-content cytometers. ***Results:*** DA was shown to greatly lower initially higher than normal levels of alanine aminotransferase (ALT) and total bilirubin (TBIL) in the serum and liver of BDL mice with LPS. DA exerted hepatic protective effects that were also confirmed by prolonged survival of BDL mice with LPS. Liver histopathology showed reduced inflammatory cellular infiltration, bile duct proliferation, and biliary necrosis with DA treatment. Furthermore, DA reduced the expression levels of tumor necrosis factor (TNF)-α and interleukin (IL)-6 in liver tissue and plasma and showed decreased NF-κB activation in BDL mice with LPS. DA could prevent LPS binding to primary Kupffer cells in the normal liver and BDL mice liver. DA also suppressed LPS-stimulated inflammatory responses by blocking the interaction between LPS and TLR4 in primary Kupffer cells and human LX-2 cells, thereby inhibiting NF-κB activation. ***Conclusion:*** DA inhibition of inflammation against liver damage following BDL with LPS may be a promising agent for the treatment of cholestatic liver injury.

Z. Weng and Y. Chi contributed equally to this work.

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Introduction

Obstructive cholestasis is caused by occlusion of the common bile duct or its tributaries and is usually associated with life-threatening complications such as endotoxemia, systemic inflammatory responses and liver injury, leading to increased mortality [1]. Inflammation is known to contribute to the development of cholestatic liver injury from biliary obstruction in patients [2, 3]. Experimental studies have determined that a pro-inflammatory response was initiated by endotoxin or LPS treatment in animal models with biliary obstruction following an increase in cytokine production of TNF-α, IL-1β, and IL-6 [4-8]. Moreover, inhibition of the pro-inflammatory response by medicinal plants could effectively improve liver injury and survival via inhibiting Kupffer cell phagocytic activity and thereby decreasing the subsequent release of pro-inflammatory cytokines [9].

TLR4 is well-characterized as the receptor for Gram-negative bacterial endotoxins or LPS [10, 11] and is involved in inflammatory signaling responses. TLR4 is expressed in many types of cells including Kupffer cells of the liver. LPS activation of TLR4 on the cell surface leads to the activation of MAPK signaling proteins, translocation of NF-κB to the nucleus resultes in the transcription of genes encoding inflammation-associated molecules and cytokines, including TNF-α and IL-6 [12, 13]. Blockage of TLR4 reduces liver damage by biliary obstruction accompanied by bacterial infection [14]. In addition, pro-inflammatory cytokines, such as TNF-α and IL-6, reduces bile flow and further leads to cholestasis in animal models [15, 16], which may also worsen inflammation. Thus, TLR4 has become a potential therapeutic target for liver injury. An agent that inhibits TLR4-mediated cytokine expression could be a promising drug for the treatment of inflammation induced liver diseases.

Medicinal plants which have been traditionally used for treating liver diseases for centuries appear to produce lower side effects. Moreover, a previous study showed that the plant components for cytokine secretions or NF-κB transactivation activity in a murine macrophage cell line exerted anti-inflammatory effects in an LPS-induced inflammatory murine model [17, 18]. *Andrographis paniculata* (Burm. f.) Nees (Acanthaceae), known as Chuan-xin-lian in Chinese (AP), is widely used for treating infection, inflammation, common colds, fever, diarrhea, hypertension, and liver disorders [19-22]. DA [14-deoxy-11, 12-didehydroandrographolide, $C_{20}H_{28}O_4$], a diterpenoid compound that is purified from AP, has been shown to have anti-inflammatory, anti-platelet and anti-hypertensive pharmacological effects [23, 24]. DA was found to exert inhibitory activity on NF-κB-dependent transactivation in LPS-stimulated RAW 264.7 cells and to inhibit the release of pro-inflammatory cytokines [22]. Moreover, an AP extract has also been shown to inhibit LPS-induced acute inflammation in mice lung and brain [25].

The aim of this work was to elucidate the mechanism of DA for improving cholestatic liver injury in a mouse BDL model with subsequent LPS treatment to mimic the conditions of biliary obstruction with septic complications. Our findings demonstrated that DA significantly inhibited inflammation and subsequent liver damage following BDL in mice. To further investigate the mechanism, we confirmed that the effectiveness of DA treatment is due to its ability to prevent LPS binding to TLR4 in Kupffer cells, inhibit NF-κB activation and decrease pro-inflammatory cytokine TNF-α and IL-6 expression, which likely leads to reduced liver inflammation in cholestatic liver injury.

Materials and Methods

Reagents

DA was obtained from the National Institute for the Control of Pharmaceutical and Biological Products in China (Beijing, purity > 99% as determined by analytical HPLC). Ultrapure LPS (*Escherichia coli* 0111: B4) was purchased from List Biological Laboratories (Vandell Way, CA, USA). This LPS does not contain contaminating proteins that could stimulate TLR2 nonspecifically. Alexa Fluor 488-*E. coli* LPS was purchased from Molecular Probes (Eugene, OR, USA). Dulbecco's modified Eagle's medium (DMEM), fetal

bovine serum (FBS), dimethylsulfoxide (DMSO), type IV collagenase, Percoll, Kupffer cell isolation medium, ethylenediaminetetraacetic acid and Hoechst 33342 were purchased from Sigma (St. Louis, MO, USA). An alanine aminotransferase analysis kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). TNF-α and IL-6 enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D (Minneapolis, MN, USA). Total bilirubin ELISA kits were purchased from the Huamei Bioengineering Institute (Wuhan, China). Anti-mouse TLR4 and GAPDH antibodies were purchased from Abcam (Cambridge, UK). Anti-mouse TLR4-Cy3 and NF-κB-FITC and DAPI were purchased from Invitrogen (Carlsbad, CA, USA).

Experimental model

C57BL/6 male mice at 8-10 weeks of age were used for the present experiments. Mice were housed in a temperature- and humidity-controlled environment on a constant 12:12-h light-dark cycle. All animal studies were approved by the Animal Ethics Committee of Jinzhou Medical University. Mice were fasted for 12 h with water ad libitum before the operation.

Mice were anesthetized with 2% isoflurane, and common BDL was performed as previously described [26]. In brief, following a peritoneal cavity incision, the common bile duct was dissected and double-ligated with a 5-0 silk suture, and then the bile duct was cut between the ligatures. Controls underwent a sham operation in which the common bile duct was exposed but not ligated. A two-layer running suture was used for abdominal closure with 4-0 dexon and 2-0 nylon. Two concentrations of DA (25 or 50 mg/kg) were administered intraperitoneally daily at 9:00 AM beginning after the ligature on day 1. Four days after BDL, selected mice were subjected to an ileocecal vein injection of LPS under microscopy at a dose of 100 ng/gram of body weight (gbw), 1 μg/gbw or 5 μg/gbw diluted with 300 μl of PBS.

Blood biochemistry

Mice were anesthetized using an over-dose of phenobarbital sodium via intraperitoneal injection. Then, serum samples were collected from the sham, BDL and BDL with LPS mice at 0 h, 6 h, 12 h and 24 h. Serum ALT was measured as markers of hepatocellular injury using a commercial kit. TBIL levels in the plasma were determined using commercial ELISA kits [19].

Pro-inflammatory cytokine level analysis

Liver tissues, blood and cell culture supernatants were collected. Livers were weighed and washed in PBS and then homogenized immediately in 10 volumes of PBS at 37°C. After centrifugation, supernatants were collected and stored at -80°C. Levels of cytokines (TNF-α and IL-6) were measured using a commercial sandwich ELISA kit, according to the manufacturer's instructions. The absorbance was read on a microplate reader (Denley Dragon, Wellscan MK3, Thermo, Finland), and the concentrations were calculated based on a standardized curve.

Histological analysis

Mice were anesthetized using an over-dose of phenobarbital sodium via intraperitoneal injection. Liver tissue was fixed in buffered 10% formalin, paraffin embedded and prepared for histological slides. Slides were stained with hematoxylin & eosin (H&E) dye.

Primary Kupffer cell culture

Primary Kupffer cells were isolated from sham and BDL mice by type IV collagenase perfusion *in situ* and differential centrifugation using Percoll as described [27, 28] with slight modifications. Briefly, liver was perfused *in situ* through the portal vein using Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (37°C) containing 10 mM ethylenediaminetetraacetic acid at a speed of 1-3 ml/min for 5 minutes. Then perfusion was performed with HBSS solution (37°C) containing 0.1% collagenase IV and gradually increased the flow rate to 10 ml/mint for 5 minutes. The liver was removed and rapidly teared liver membrane using scissor to release all liver cells into the Kupffer cell isolation medium on ice. Liver cells were collected at 50g at 4°C for 2 minutes. Then supernatant was centrifuged at 1, 350g for 15 min to pellet non-parenchymal cells. The cell suspension was added the discontinuous isotonic gradient 25/50% Percoll and centrifuged at 900g for 20 minutes at 4°C to obtain the Kupffer cell. Cells were seeded on 0.01% gelatin-coated culture plates and cultured in DMEM: Ham's F-12 medium (1:1) with 15% fetal bovine serum and 10 mM hydroxyethylpiperazine-N-2 ethanesulfonic acid. After incubation for 30 minutes, nonadherent cells were

removed and 10 mM ethylenediaminetetraacetic acid was added in cell medium for 40 minutes on ice. After gently shaking the plate, the supernatant was collected and centrifuged at 300g for 5 minutes. The pellet was resuspended with 2×10^5 cells/ml in DMEM: Ham's F-12 medium (1:1) with 15% FBS, 15 mM HEPES, 1 mM insulin, 2 mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin. Cells were allowed attach after being cultured for 72 h (37°C, 5% CO_2) prior to treatment, the cell culture medium was changed to a serum-free medium.

Western blot analysis

Liver tissues were homogenized in RIPA lysis buffer containing protease inhibitors, followed by solubilization and centrifugation at 12, 000 rpm for 20 min at 4°C. Supernatants were stored at −80°C until assays were performed. Tissue protein concentrations were determined with a bicinchoninic acid (BCA) kit. Western blot analysis was performed as previously described [19]. Briefly, the supernatant was subjected to 10% SDS-polyacrylamide gel electrophoresis (20 μg of protein/lane) and transferred to a polyvinylidene difluoride membrane. Then the membranes were incubated with primary antibodies overnight at 4°C. After being washed with TBS, the membranes were incubated with horseradish peroxidase (HRP)-linked secondary antibodies at room temperature for 1 hour. Bands were developed using enhanced chemiluminescence. The relative quantities of protein expression were analyzed using ImageJ software.

Assay for LPS binding to Kupffer cells.

The primary Kupffer cells were incubated with DA (1, 2, and 4 μM) and anti-mouse TLR4 antibodies for 30 min at 37°C. The cells were washed, and the medium was changed to a serum-free medium in which the serum factors able to target LPS could not interfere with the results. Then, the cells were further incubated with 50 ng/ml LPS from *E. coli* serotype O55:B5 conjugated with Alexa Fluor 488 for 45 min at 37°C. After being washed twice, the cells were collected and analyzed with high-content cytometers. The assays were performed in triplicate for each preparation of the primary Kupffer cells obtained from five different donors. Specific LPS binding was estimated by subtracting the percentage of LPS-binding cells in the absence of LPS from that in the presence of LPS.

Statistical analysis

All data were analyzed using GraphPad Prism software (GraphPad, San Diego, CA). Data are expressed as the mean ± standard error. A Student's t-test was used to compare significant differences between the two groups. Significant differences among multiple groups were analyzed using a one-way ANOVA followed by Dunnett's test. A P value of < 0.05 was considered significant.

Results

Effect of DA on liver injury in BDL mice treated with LPS

DA has been shown to not only inhibit inflammation in various cell models, but to also act as a liver protectant. It is hypothesized that cholestatic liver injury would also be less severe with DA treatment in BDL following LPS. To investigate the effect of DA on liver injury in BDL-induced mice treated with LPS, we first assessed the cholestatic liver injury model by measuring serum levels of TBIL and ALT, which serve as the markers of cholestasis and hepatocyte injury in BDL mice with LPS. As shown in Fig. 1A, after 6 h, the BDL group had markedly elevated TBIL levels by more than 23-fold and 16-fold of ALT levels ($p < 0.01$) compared with the sham group, suggesting that significant cholestatic liver injury was induced successfully in this model. Levels of TBIL did not continue to increase in BDL mice after 6 hours post LPS administration (1 μg/gram of body weight), but the ALT levels increased further in a time-dependent manner (Fig. 1A). Fig. 1B shows the chemical structure of DA. Following the application of DA at 25 and 50 mg/kg treatment, with the concentrations obtained from an acute toxicity study, the levels of LPS-induced TBIL and ALT were significantly reduced in sham and BDL mice. Although TBIL levels were slightly decreased with an application of 25 mg/kg DA, they were not statistically significant ($p > 0.05$), whereas ALT levels were greatly reduced in BDL mice with LPS (Fig. 1C, $p < 0.01$).

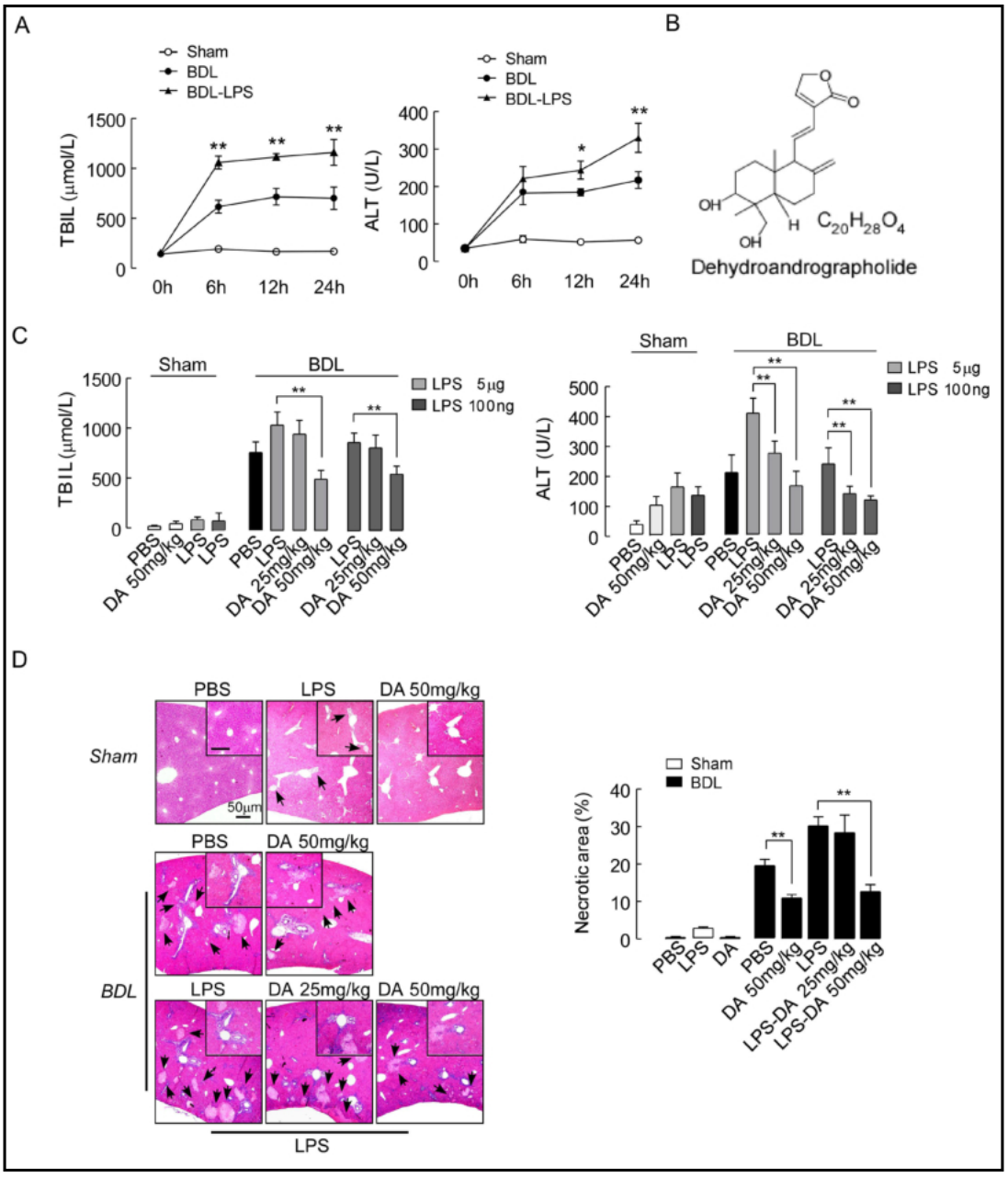

Fig. 1. DA reduced liver damage of BDL mice treated with LPS. (A) Serum levels of total bilirubin (TBIL; left) and alanine aminotransferase (ALT; right) were significantly elevated in BDL-LPS mice compared with BDL mice (*p<0.05, **p<0.01, respectively). (B) The chemical structure of DA. (C) Left: TBIL and ALT levels were reduced in BDL-LPS mice following DA (50 mg/kg) treatment compared to BDL-LPS mice, *p<0.05, **p<0.01. Right: A dose-response study of LPS (100 ng/gram of body weight and 5000 ng/ gram of body weight, respectively) to estimate DA inhibition of liver injury. (D) Representative H&E images of liver sections from sham and BDL mice treated with LPS. DA treatment significantly reduced liver biliary necrosis. Arrows show liver necrosis. The data are expressed as the mean ± SEM from three independent experiments. n=10, **p<0.01, between indicated groups.

Considering whether different doses of LPS would induce a different response of DA for liver injury, a dose-response study was performed using higher or lower doses of LPS (100 ng/ gram of body weight and 5000 ng/gram of body weight, respectively). Similar DA inhibition of liver injury patterns are shown in Fig. 1C.

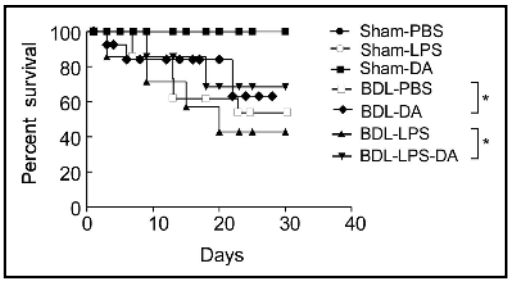

Fig. 2. DA prolonged survival in BDL mice treated with LPS. The ratio of survival between BDL-LPS mice following DA treatment and BDL-LPS mice at each time point. The data are expressed as the mean ± SEM from three independent experiments. n=30, *p<0.05, between indicated groups.

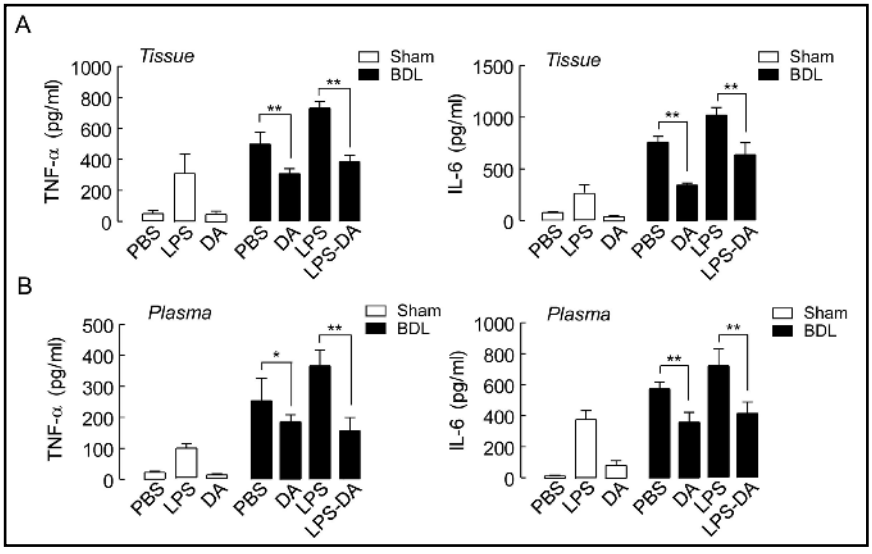

Fig. 3. DA inhibited pro-inflammatory cytokine production in BDL mice with LPS. An ELISA showed the inhibition of DA in the secretion of TNF-α and IL-6 both in liver tissue (A) and plasma (B) in BDL mice treated with LPS DA dose at 50 mg/kg and LPS (1 μg/gram of body weight). The data are expressed as the mean ± SEM from three independent experiments. n=10, *p<0.05, **p<0.01, between the indicated groups.

To confirm the effects of DA attenuated LPS-induced liver injury in BDL mice, liver histology was observed with H&E staining. Fig. 1D shows that DA dose at 25 or 50 mg/kg treatment significantly reduced the degree of periportal inflammation, cellular infiltration, bile duct proliferation, and biliary necrosis ($p < 0.01$) in BDL and BDL following LPS treatment mice compared with BDL or BDL mice following LPS only. Biliary necrosis was mainly observed around the periportal area in BDL mice. Taken together, these results suggested that DA pre-treatment can effectively reduce liver damage of BDL mice following LPS treatment. Thus, we chose a DA dose at 50 mg/kg and LPS (1 μg/gram of body weight) for our studies.

Effects of DA on Survival in BDL mice treated with LPS

To further test the protective effects of DA on the survival of mice with cholestatic liver injury, we treated the BDL mice using LPS at a dose of 1 μg/gram of body weight. Without DA pre-treatment, the BDL mice became ill and lethargic and lacked strength and coordination following LPS administration. However, DA treatment greatly improved the survival of BDL mice ($p < 0.05$). Moreover, the BDL mice with DA treatment had significantly improved survival with 73 % surviving following LPS administration vs 41 % of the PBS-treated BDL with LPS mice (Fig. 2). The results suggested that DA treatment not only protected the liver against cholestatic injury but also prolonged survival in BDL mice receiving LPS.

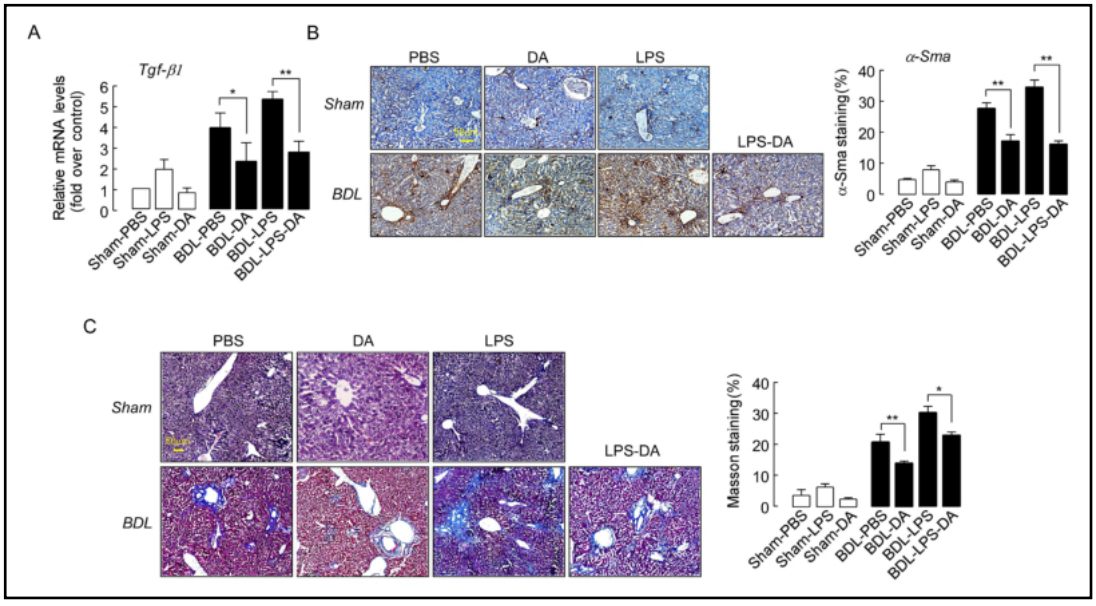

Fig. 4. DA treatment represses fibrosis formation in BDL with LPS mice. (A) Relative mRNA expression for Tgf-β1 in sham and BDL-LPS mice treated with either PBS or DA. Data are expressed in arbitrary units. n=3, *p<0.05, **p<0.01, between indicated groups. Data are expressed as the fold change relative to BDL-LPS mice. Data were normalized to α-actin. (B) Liver α-Sma protein expression was detected by immunohistochemistry staining in sham and BDL-LPS mice treated with either PBS or DA. n=3, **p<0.01, between indicated groups. (C) Representative images and densitometry data of masson's trichrome staining of liver sections from sham and BDL-LPS mice treated with either PBS or DA. Data represent means ± SEM of 3 independent experiments. n=5, *p<0.05, **p<0.01, between indicated groups.

Effects of DA on pro-inflammatory cytokine production and fibrosis formation in BDL mice treated with LPS

It is well known that inflammatory responses are major factors for liver damage induced by various causes. We next attempted to correlate the above findings with inflammatory cytokine production in response to LPS administration in sham and BDL mice. An ELISA test showed that the levels of TNF-α and IL-6 expression in liver tissues were greatly increased in BDL mice following LPS administration compared with the sham group (Fig. 3A, $p < 0.01$). Similarly, the plasma of BDL mice had significantly higher levels of TNF-α and IL-6 expression than the sham group, as shown in Fig. 3B. However, DA treatment greatly reduced the levels of pro-inflammatory cytokine production both in liver tissue and plasma in BDL and BDL mice following LPS administration (Fig. 3A, B, $p < 0.01$). The results for plasma and tissue contents provided strong evidence that DA had a protective role in the liver against injury via inhibition of pro-inflammation cytokines production in response to LPS.

Inflammation could induce Kupffer cells activation to produce TGF-β1 and are able to activate fibrogenic hepatic stellate cells. We evaluated whether DA treatment could affect hepatic expression of profibrogenic genes. qPCR demonstrated that the expression of Tgf-β1 gene in hepatic fibrogenesis was significantly decreased in DA-BDL mice with LPS compared to BDL mice with LPS (Fig. 4A, $p < 0.01$). An immunohistochemistry analysis also displayed a significant reduction in α-Sma protein expression in DA-BDL mice with LPS compared to BDL mice with LPS (Fig. 4B, $p < 0.01$). This result was also supported by analysis of Masson's Trichrome staining of the liver sections, which showed reduced thickening of the collagen fiber in the portal area and no marked fibrous septum in DA-BDL mice with LPS compared with BDL mice with LPS (Fig. 4C, $p < 0.05$). These data suggest that DA treatment represses the activation of Kupffer cells through inhibits inflammation, further reducing fibrosis formation in BDL mice with LPS.

Effect of DA on NF-κB activation in BDL mice with LPS and primary Kupffer cells

NF-κB refers to a family of transcription factors that play a crucial role in the inflammatory response [29]. Thus, we further examined NF-κB expression in Kupffer cells and tissues. Dramatically, with DA treatment, the BDL mice with LPS showed a markedly decreased NF-κB quantity compared with the BDL mice with LPS without DA treatment (Fig. 5A and B, $p < 0.01$). To further confirm this result, we isolated primary Kupffer cells from a wild type mouse and incubated with DA at 1, 2, 4 μM (LD_{50} 2 μM). High-content cell imaging of primary Kupffer cells displayed activation of NF-κB by LPS (1 μg/ml) that was significantly inhibited by DA at all given concentrations (Thermo, Cellomics ArrayScan VTIHCS Reader, USA) (Fig. 5C and D, $p < 0.01$). These results suggested that DA inhibited the activation of NF-κB to reduce the generation of pro-inflammatory cytokines.

Effects of DA on LPS binding to the Kupffer cells

Many bacterial products and stimulation of a wide variety of cell-surface receptors lead to NF-κB activation and fair rapid changes in gene expression [30]. Toll-like receptors (TLRs) as specific pattern recognition molecules can lead to activation of NF-κB [31]. We investigated whether DA inhibited binding of LPS to TLR4 in primary Kupffer cells. LPS is a well-known TLR4 ligand that can bind to the TLR4 complex on host cell membranes such as monocytes and macrophages [32]. This leads to activation of inflammatory responses, and we therefore performed an experiment to evaluate the effect of DA on LPS binding to the cells using primary Kupffer cells. The cells were incubated with DA at 1, 2, and 4 μM, fluorescein-conjugated LPS and anti-TLR4 antibodies. Then, LPS binding was analyzed with high-content cell imaging. Anti-TLR4 antibodies were used as a positive control. As Fig. 6A shows, cells were incubated with anti-TLR4 antibodies, which led to almost complete blockage of the binding of LPS to primary Kupffer cells in which little fluorescence was observed around cells, whereas LPS-label fluorescence gradually increased in cells incubated with LPS only over an extended time. However, LPS binding could be blocked by DA in all tested concentrations and DA in 2 and 4 μM was statistically significant compared with LPS only in Kupffer cells.

To further confirm the inhibitory effect of DA on LPS-induced pro-inflammatory cytokine production, we examined LPS-induced cytokine levels using an ELISA in the media of Kupffer

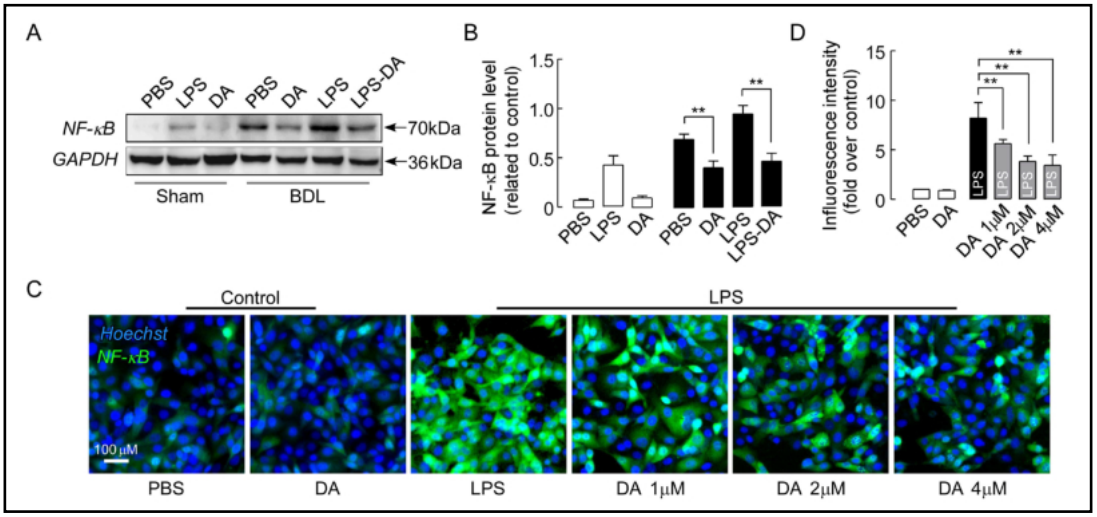

Fig. 5. DA treatment inhibited LPS-stimulated NF-κB activation in BDL mice and primary Kupffer cells. (A) Representative blots and densitometry data of liver NF-κB protein expression in BDL mice following LPS with and without DA treatment. (B) The data are expressed as the mean ± SEM from three independent experiments. n=5, **p<0.01, between indicated groups. Data were normalized to GAPDH. (C) High-content cell imaging showed DA inhibited activation of NF-κB by LPS-induced at all given concentration in Kupffer cells. (D) The data are expressed as the mean ± SEM from three independent experiments. n=3. **p<0.01, between indicated groups.

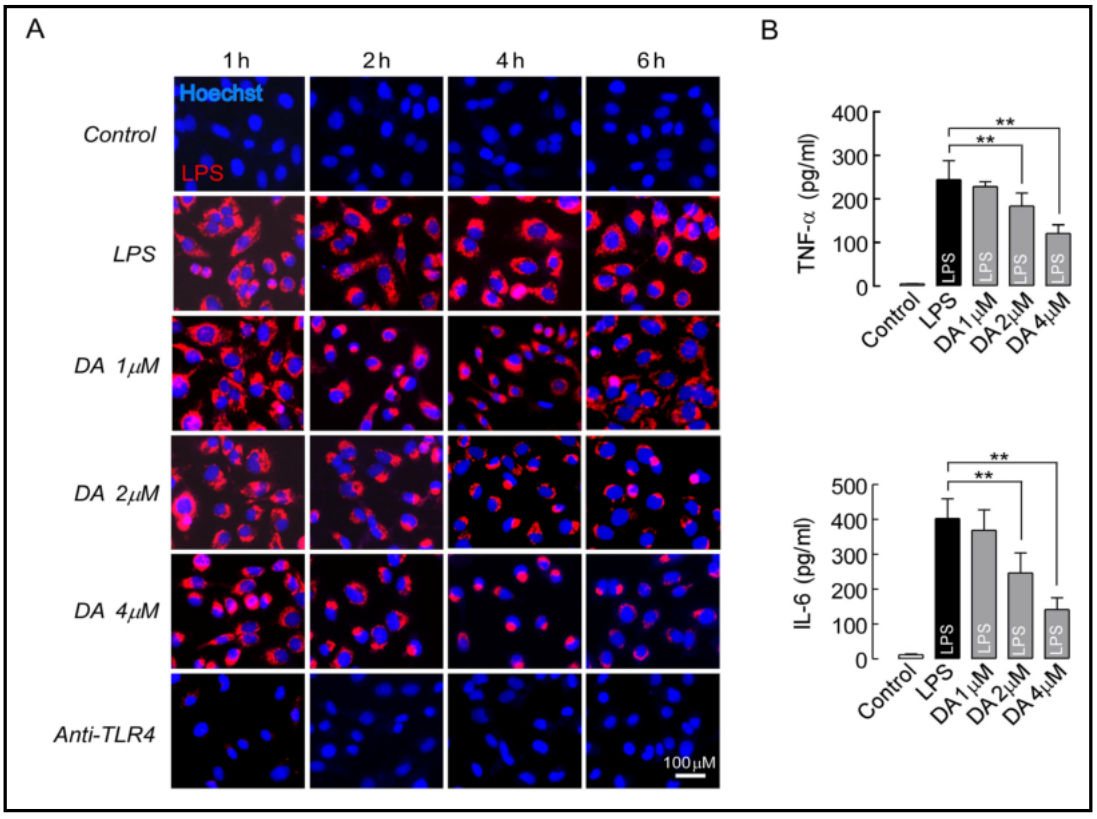

Fig. 6. DA treatment decreased LPS binding to Kupffer cells. (A) High-content cell imaging showed DA could effectively block red fluorescein-conjugated LPS binding to the primary Kupffer cells. Representative of 4 sets of experiments. (B) TNF-α and IL-6 production detected by an ELISA in the media of Kupffer cells stimulated with LPS (1 μg/ml) for 6 h alone or in combination with DA at 1, 2 or 4 μM. The data are expressed as the mean ± SEM from three independent experiments. n=4, **p<0.01, between indicated groups.

cells stimulated with LPS (1 μg/ml) for 6 h alone or in combination with DA at 1, 2, and 4 μM. As expected, DA at a 2 μM concentration could efficiency inhibit TNF-α and IL-6 production in a concentration-dependent manner, as shown in Fig. 6B ($p < 0.01$). In addition, we also observed the inhibitory effect of DA on LPS-induced pro-inflammatory cytokine production on primary Kupffer cells isolated from BDL mice and human LX-2 cells. Fig. 7A showed that DA at 2 and 4 μM concentration exerted inhibitory effect on TNF-α production ($p < 0.05$ or $p < 0.01$) on BDL-Kupffer cells and BDL-Kupffer cells with LPS (100ng/ml). Although DA at 2 and 4 μM concentration could efficiently inhibit IL-6 production ($p < 0.05$) on BDL-Kupffer cells, the effect of DA inhibition in BDL-Kupffer cells with LPS was at 4 μM concentration (Fig. 7B, $p < 0.05$). Moreover, DA at 2 or 4 μM also significantly inhibited LPS-stimulated production of TNF-α and IL-6 (Fig. 8, $p < 0.05$ or $p < 0.01$) in human LX-2 cells. These results of *ex vivo* suggest that DA alleviate LPS-induced inflammatory responses in primary Kupffer cells and hepatic stellate cells which could be through TLR4.

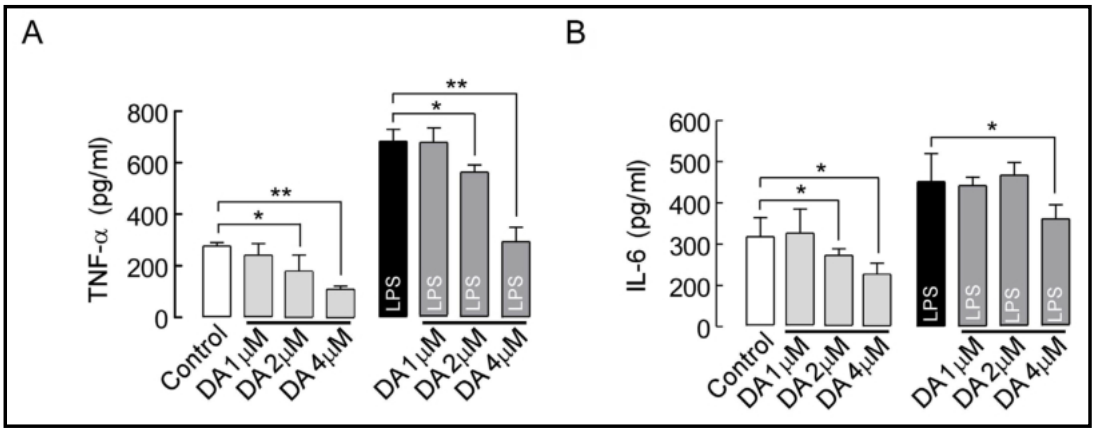

Fig. 7. DA treatment inhibited LPS-induced inflammatory cytokine production on primary Kupffer cells isolated from BDL mice. (A) TNF-α and (B) IL-6 production detected by ELISA in the media of Kupffer cells stimulated with LPS (100 ng/ml) for 6 h alone or in combination with DA at 1, 2 or 4 μM. The data are expressed as the mean ± SEM from three independent experiments. n=3, *p<0.05, **p<0.01, between indicated groups.

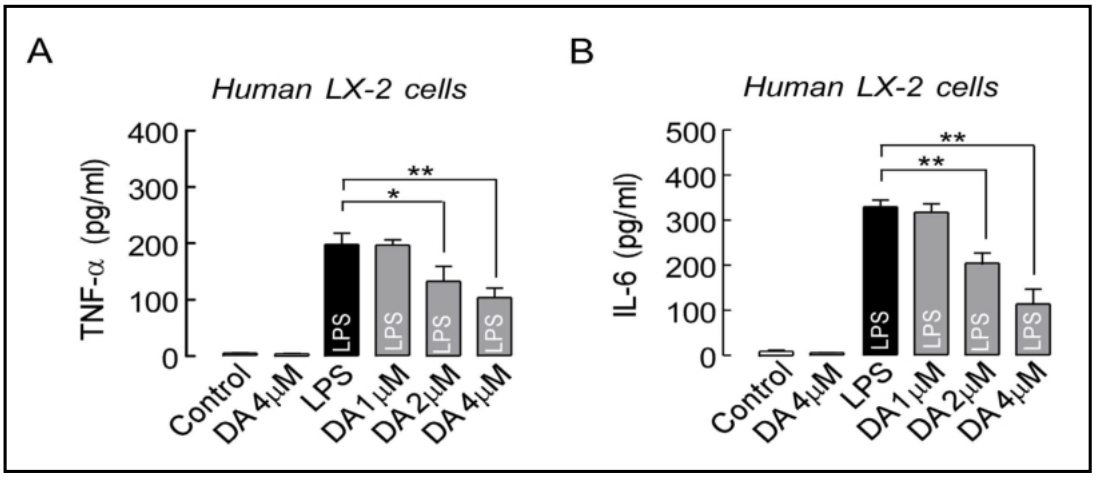

Fig. 8. DA treatment inhibited LPS-induced inflammatory cytokine production on human LX-2 cells. (A) TNF-α and (B) IL-6 production detected by ELISA in the media of human LX-2 cells stimulated with LPS (100 ng/ml) for 6 h alone or in combination with DA at 1, 2 or 4 μM. The data are expressed as the mean ± SEM from three independent experiments. n=3, *p<0.05, **p<0.01, between indicated groups.

Discussion

Several lines of evidence have shown that biliary obstruction causes endotoxemia, systemic inflammatory responses and liver injury, leading to increased mortality, and these complications can be life threatening [1, 33]. In addition, biliary obstruction results in function impaired of intestinal barrier and the translocation of enteric bacteria to the systemic circulation, patients are susceptible to septic complications not only with cholangiovenous reflux but also with increased bacterial translocation from the gut to the systemic circulation. However, there is still no good method for treating biliary obstruction clinically. In the present study, we showed that LPS administration in cholestatic mice greatly induced hepatocellular damage and the expression levels of proinflammatory cytokines; it also induced a significant increase in NF-κB activation and a significant increase in the level of TLR4 expression and resulting in a significant increase in lethality. DA treatment could reverse the adverse effects of LPS in cholestatic mice and thus reduce mortality in BDL mice.

Andrographis paniculata is widely used as a Chinese herbal remedy to reduce inflammation and remove toxins. It has traditionally been used to treat liver diseases. Moreover, accumulating evidence has demonstrated that andrographolide has anti-inflammatory effects [34]. DA is a natural compound that is purified from *Andrographis paniculata* and possesses anti-inflammatory, anti-platelet and anti-hypertensive pharmacological effects. In our previous study, we showed that DA treatment protected against liver damage and fibrosis through development of a hepatic adaptive response and inhibition of the activation of HSCs following BDL in mice [19]. Thus, we hypothesize that DA can also inhibit inflammation and increase the effect of protection against liver damage in biliary obstruction mice. To accurately reproduce the clinical conditions of biliary obstruction and inflammation, we developed a mouse model of BDL with LPS injection via the ileocecal vein because LPS can induce a higher grade of liver necrosis and mortality in jaundiced rats [35]. Even a very small dose of injected LPS can also result in severe liver damage. After DA treatment, H&E staining showed a greatly improved phenotype of BDL following LPS-induction, which reduced inflammatory cell infiltration, bile duct proliferation and biliary necrosis in mice (Fig. 1D). The levels of TBIL and ALT were significantly decreased in BDL-LPS mice treated with DA (Fig. 1A). Moreover, DA also significantly prolonged survival of BDL mice with LPS (Fig. 2). According to these results, DA exerts a hepatic protective effect in liver injury caused by biliary obstruction in mice.

Many molecules participate in every stage of the early immune response and inflammatory reactions and are modulated by NF-κB, including TNF-α, and IL-6 [36]. In particular, NF-κB is an essential transcription factor that is necessary for TNF-α and IL-6 transcription [37]. In addition, pro-inflammatory cytokines such as TNF-α and IL-6 can decrease bile flow, further leading to cholestasis [4]. In contrast, the development of cholestasis can also result in inflammation and further deterioration. Previous studies have demonstrated that DA can reduce inflammatory responses [38]. To clarify the mechanisms underlying the effect of DA on inflammatory response in the BDL mice with LPS, we assessed the levels of TNF-α and IL-6 expression and NF-κB activation by ELISA and Western blot. As expected, DA treatment reduced NF-κB quantities (Fig. 5), accompanied by decreased TNF-α and IL-6 expression (Fig. 3) in BDL mice with LPS.

In addition, Kupffer cells express TLR4 and is capable of respond to extremely low concentrations of LPS in liver [14], and liver Kupffer cells also play a critical role in inflammation [39-42]. LPS binds to TLR4 and transduces subsequent signals that result in upregulation of the inflammatory response [35, 43]. It was also clearly demonstrated that blocking of TLR4 signaling or NF-κB activation effectively reduced liver damage in biliary obstruction. Our data displayed that LPS-induced activation of NF-κB could be inhibited by DA at all given concentrations in Kupffer cells (Fig. 5). Accordingly, we hypothesized that DA could block LPS binding to TLR4 in primary Kupffer cells and regulates subsequent signaling pathways that lead to decreased inflammatory responses. In this study, we used high-content cell imaging to demonstrate that DA inhibited the combination of LPS and TLR4 in primary Kupffer cells (Fig. 6A). Furthermore, we also found that the expressions of TNF-α and IL-6 by LPS-induction were decreased in Kupffer cells with DA treatment (Fig. 6B). According to these data, DA inhibition of pro-inflammatory cytokine production depended on TLR4 in primary Kupffer cells. It also implied that there is a regulatory relationship between DA, TLR4, NF-κB, TNF-α and IL-6 in the inflammatory processes induced by LPS. Moreover, the exact mechanism for how TLR4 adjusts the subsequent signaling pathway will be elucidated in the future.

Conclusion

Our studies demonstrated that DA could decrease TNF-α and IL-6 production in BDL mice or primary Kupffer cells treated with LPS. This effect was mediated through the prevention of LPS binding to TLR4 and subsequent inhibition of the NF-κB signaling pathway.

DA may become a novel therapeutic drug for patients with biliary obstruction and a severe inflammatory reaction.

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Conceived and designed the experiments: LHL, ZYW. Performed the experiments: ZYW, YC, JX, XFL, JHH, FY, LHL. Analyzed the data: LHL, ZYW, YC. Contributed reagents/materials/analysis tools: LHL, ZYW. Wrote the paper: LHL.

Disclosure Statement

The authors declare no competing financial interests.

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