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Growth differentiation factor 11 suppresses intrahepatic inflammation via restricting NLRP3 inflammasome activation in LPS-induced liver injury

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ABSTRACT

Growth differentiation factor 11 (GDF11) is reported as a member of TGF-β superfamily, which plays a key negative role in various tissue inflammation. However, the specific effect of GDF11 on infectious acute liver injury remains unknown. The current study is designed to certify the role of GDF11 both in LPS-induced RAW 264.7 cell line and rodent model of acute liver injury (ALI) and further investigate its molecular mechanism of inflammatory regulation. In vitro, LPS was used to stimulate the inflammatory activation of RAW 264.7 cells and then recombinant GDF11 (rGDF11) was used to treat the cells. In vivo, we injected LPS and rGDF11 in abdomen of mouse. The inflammatory indexes, GDF11 level, NLRP3 level, liver tissue injury, and liver function were examined using qRT-PCR, western blot, ELISA, IHC, IF and HE staining, respectively. Supplement of GDF11 protected the histology and function of liver tissue in LPS-induced ALI mice, in which the level of AST, ALT and TBiL associated with tissue damage were reduced after ALI. Moreover, increased GDF11 in RAW 264.7 cells and ALI mice reduced the expressions of COX-2, TNF-α, IL-1β, and IL-6 via inhibiting NLRP3 inflammasome activation, suggesting the anti-inflammatory role of GDF11 in ALI. Besides, owing to the protective role of GDF11, the apoptotic degree in liver after LPS insult was attenuated, such as the reduced c-caspase-3 and annexin-V expressions. The results indicate that overexpression of GDF11 plays an antagonistic role in LPS-induced inflammatory response after ALI. Therefore, GDF11 may become a promising target for preventing infectious acute liver injury.

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Introduction

Sepsis is a common but complicated disease following severe infection, trauma, burns, or surgery, leading to septic shock and even multiple organ dysfunction syndrome (MODS) (1). During the incubation of sepsis, the emergence of acute liver injury (ALI) due to pathogen infection brings great damage to digestive system (2), which holds an underlying problem that the outbreak of MODS. It is mentioned that the pathogenesis of ALI, violent inflammatory response and dysfunction of functional cells in liver impose a great burden on the body (3). Therefore, how to suitably mitigate intrahepatic inflammation and effectively protect survival of functional cells remains a formidable challenge in clinical therapeutics. Numerous studies (4-6) have sought to influential anti-inflammatory therapeutic targets in ALI in recent years, while the specific property of anti-inflammation in ALI is still elusive. Inflammasome, a complex of several proteins, was firstly reported to induce cellular stress and death under inflammatory conditions via Tschopp and his colleagues in 2002 (7). Mechanically, inflammasomes including NOD-like receptor protein (NLRP) 1, NLRP3, IPAF and AIM2 generally regulate the activation of caspase-1 and further promote the cleavage and maturation of pro-interleukin (IL)-1β and pro-IL-18 cytokines in the process of innate immune defense(8). It has been established that inflammasomes are involved in host defense responses against many pathogens (9), and play a vital role in inflammation of hepatic diseases (10,11). Importantly, inhibition of inflammasomes especially NLRP3 attenuates inflammation-related injury in liver diseases (12,13). GDF11 is identified as a transforming growth factor β (TGF-β) superfamily protein that modulates differentiation, maturation, and growth of cells (14). Recent reports have testified that GDF11 negatively regulates inflammatory response and promotes recovery in multiple diseases (14,15). More notably, GDF11 attenuates chondrogenic inflammation via lipopolysaccharides (LPS)-mediated injury (16). However, the function of GDF11 in LPS-induced ALI is never reported. Therefore, we designed to investigate the therapeutical effect and molecular mechanism of GDF11 in ALI following LPS insult. Herein, we showed that GDF11 reduced inflammation and apoptosis in the ALI model. In vitro results, increased GDF11 expression was proved to attenuate inflammatory response by inhibiting NLRP3 inflammasome activation in LPS-activated macrophage. The above exhibits that GDF11 may act as a promising target of clinical therapeutics in LPS-induced ALI.

Materials and Methods

Cell treatment

The RAW 264.7 line was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and seeded into 6-well plates (Corning, Corning,

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NY, USA). Culture dulbecco's modified eagle medium (DMEM, Gibco, Rockville, MD, USA) was supplemented with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin. When cells reached more than 80% confluence, the FBS-free DMEM was used to replace the original medium overnight. Then rGDF11 (50 ng/mL, PeproTech, Cranbury, NJ, USA) or vehicle (PBS) was added into complete medium for 48 h incubation followed by LPS (100 ng/mL, Sigma, St. Louis, MO, USA) treatment. After 12 h or 24 h incubation, cells were extracted to RNA or protein for qRT-PCR or western blot, respectively.

Acute liver injury (ALI)

The male C57/BL6J mice (20-22 g, 8 weeks) were selected for the ALI modeling and the procedure was approved by the Wenzhou Hospital of Integrated Traditional Chinese and Western Medicine. All the animals were purchased and housed in Wenzhou Medical University, in which the mice were allowed to obtain available food and tap water. All the animals were randomly divided into three groups: Vehicle group, in which the mice were injected with PBS whose volume is equal to the LPS reagent; LPS group, in which the mice were performed administration of LBS at the dose of 10mg/kg (i.p., q.d); LPS+rGDF11 group; in which rGDF11 (1 mg/kg) were conducted in the mice at 1h post-LPS injection (i.p., q.d).

qRT-PCR technique

The Cell and tissue were treated using 1 mL TRIzol reagent. Reverse transcription was conducted using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). RNA measurement was performed using a SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA) in final volume of 20 μL including 1 μg cDNA. The amplification procedure was performed using a MiniOpticon Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization using the comparative Ct method.

Immunoblot

Protein was isolated using a RIPA reagent (Solarbio, Beijing, China) according to the manufacturer's instructions. Protein concentration was detected using bicinchoninic acid (BCA) method. The protein was loaded in 10% SDS-polyacrylamide gel for electrophoresis and then was transferred to polyvinylidene difluoride membranes. The protein-loaded membranes were incubated with antibodies followed by examination using enhanced chemiluminescence in an exposure machine (Bio-Rad Laboratories, Hercules, CA, USA). Primary antibodies and Secondary antibodies were as follows: caspase 3 (1:1000, Abcam, Cambridge, MA, USA), caspase 8 (1:1000, Abcam, Cambridge, MA, USA), Bax (1:1000, Abcam, Cambridge, MA, USA), Bcl-2(1:1000, Abcam, Cambridge, MA, USA), GAPDH (1:2000, Abcam, Cambridge, MA, USA), HRP-Anti-Rabbit antibody (1:2000, Proteintech, Rosemont, IL, USA).

Immunohistochemical staining (IHC)

Mice were sacrificed after anesthesia using ketamine (80 mg/kg) and xylazine (4 mg/kg). Tissue was collected in 4% paraformaldehyde and made into 5 μm paraffin sections. Sections were incubated with primary antibodies [GDF11 (1:100, Abcam, Cambridge, MA, USA), NLRP3 (1:200, Abcam, Cambridge, MA, USA), IL-6, IL-1β, Annexin V (1:200, Abcam, Cambridge, MA, USA), c-caspase 3 (1:400, Cell signaling technique, Danvers, MA, USA)] at 4°C overnight. Washed by PBS, sections were incubated with corresponding secondary antibodies and then performed coloring treatment using a DAB staining Kit (Genentech, San Francisco, CA, USA). The nucleus was performed counterstaining with hematoxylin for 5 s. Then images were captured using a microscope (OLYMPUS, Tokyo, Japan).

Immunofluorescence technique (IF)

Paraffin sections were conducted antigen blocking using 3% BSA blocking buffer for 1h at room temperature. Then Sections were incubated with primary antibodies overnight at 4°C. Washed, sections were next performed using fluorescence secondary antibodies treatment for 1 h in the dark at room temperature. Fluorescence images were collected using a fluorescence microscope system (OLYMPUS, Tokyo, Japan).

Histology assessment

Haematoxylin-eosin (HE) staining was used to visualize the degree of damage and in liver. Staining was performed using HE Staining Kit (Servicebio, Beijing, China) according to the manufacturer's protocols, respectively. Photographs were gathered with a microscope.

Liver function

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBil) were examined using Dirui CS-T300 Chemistry Analyzer and corresponding kits (Dirui Medical Technology, Changchun, China).

Statistical analysis

Data were assessed using Statistic Package for Social Science (SPSS) 21.0 software (IBM, Armonk, NY, USA). Data were exhibited as the means ± standard deviations (SD). The difference between two groups was analyzed using Student's *t*-test in statistics, and the difference among more than two groups was evaluated using one-way or two-way ANOVA. When $P < 0.05$, the difference is verified as statistical significance.

Results

Supplement of GDF11 restrains deterioration of liver tissue and liver function after LPS insult

To clarify the alteration of GDF11 in liver tissue after LPS treatment, the expression of GDF11 was examined using IHC staining at 1 day and 3 days post-injury (dpi), respectively. The images showed that the positive focus of GDF11 were reduced following ALI, especially minimum of 3 days (Figure 1A). We thereby increased the expression of GDF11 via the injection in the abdomen and first evaluated the hepatological improvement. HE staining at 3 days exhibited severe tissue swell and excess hemorrhage, as well as leukocyte infiltration after LPS insult, while the employment of rGDF11 mitigated the above signs of inflammation in the liver treated with LPS (Figure 1B).

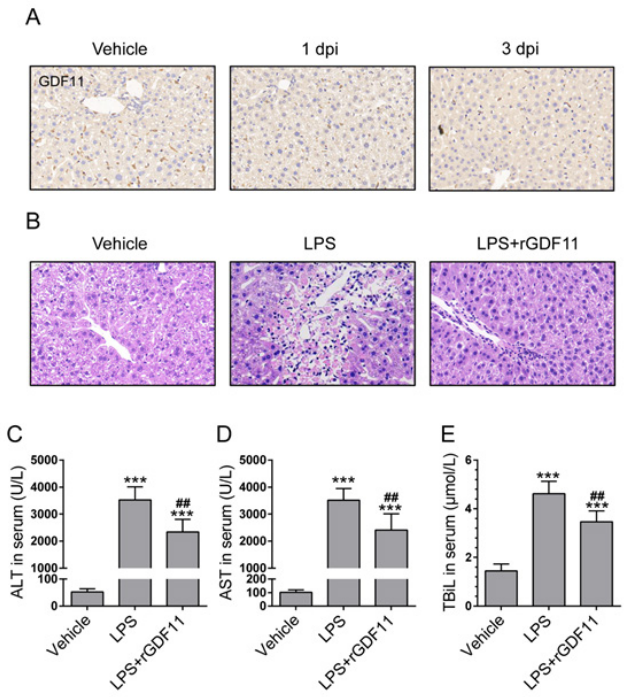

Figure 1. Supplement of GDF11 restrains deterioration of liver tissue and liver function after LPS insult. (A) Photos of GDF11 expression in Vehicle, 1dpi, 3dpi group after LPS treatment using IHC staining; amplification: 400×. (B) Images of liver tissue in Vehicle, LPS, and LPS+rGDF11 group using HE staining at 3 dpi; amplification: 400×. The levels of ALT (C), AST (D), and TBiL (E) in serum in Vehicle, LPS, and LPS+rGDF11 group at 3 dpi. "*" means vs. Vehicle group and "#" means vs. LPS group with statistical significance, "*" means P <0.05; "**" means P <0.01; "***" means P <0.001.

Further to better capture the underlying effect of GDF11 on liver function, the key biochemical indexes, such as alanine transaminase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBiL) were detected at 3 dpi, showing that LPS treatment elevated the levels of ALT, AST and TBiL, but utilization of rGDF11 decreased the LPS-induced high levels of them (Figure 1C-1E). It was indicated the protective role of GDF11 in liver function. Overall, the results suggest that an increase of GDF11 restrains the pathogenesis of LPS-induced ALI via the potential property of anti-inflammation.

Growing expression of GDF11 attenuates inflammatory response by inhibiting NLRP3 in RAW 264.7 cell

Next, we measured whether increased GDF11 could inhibit LPS-induced inflammation in the RAW 264.7 macrophage line and explained the specific molecular mechanism in vitro. To certain the anti-inflammation role in RNA level, the cells were extracted into RNA at 12 h post LPS treatment. The classical inflammatory enzyme, cyclooxygenase-2 (COX-2), and several cytokines including tumor necrosis factor (TNF)-α and interleukin (IL)-1β were detected by qRT-PCR. As Figure 2A-2C shows, the RNA levels of COX-2, TNF-α as well as IL-1β increased significantly following LPS treatment in macrophage yet treatment with rGDF11 markedly decreased the RNA levels of classical inflammatory factors. Moreover, the expressions both of COX-2 and GDF11 in cells were visualized using IF staining, showing LPS employment increased the expression of COX-2 but decreased GDF11 expression compared with those in Vehicle group. However, increased GDF11 reversed the expressions of COX-2 and GDF11 in cells at 24h post LPS stimulation (Figure 2D). Besides, to testify the mechanism of anti-inflammation, the NLRP3/ caspase 1 axis were examined using IF. The result displayed that LPS provoked overmuch caspase 1 and NLRP3 expressions in RAW 264.7 macrophage, whereas increase of GDF11 attenuated the levels of NLRP3/ caspase 1 axis post LPS treatment (Figure 2E). Taken together, the above results contribute to increased GDF11 mitigating inflammatory response via inhibition of the NLRP3/ caspase axis in vitro.

Increase in GDF11 reduces intrahepatic inflammation by inhibiting NLRP3 in ALI

To testify the anti-inflammatory role of GDF11 in vivo, we measured the intrahepatic inflammatory indexes at 3 dpi. The NLRP3 and GDF11 expressions in liver were firstly measured using IHC, exhibiting massive expression of NLRP3 and little expression of GDF11 in liver injured by LPS, but reduced expression of NLRP3 and enhanced levels of GDF11 in liver were witnessed in LPS+rGDF11 group (Figure 3A). Furthermore, the same indicators of inflammation in vitro were examined in vivo by qRT-PCR. The results indicated that the growing pro-inflammatory factors including COX-2, TNF-α and IL-1β declined prominently following GDF11 reinforcement in liver (Figure 3B-3D). The protein levels of IL-1β and IL-6 were also measured in liver using IHC at 3 dpi, displaying excessive positive expressions of IL-1β and IL-6 after LPS stimuli

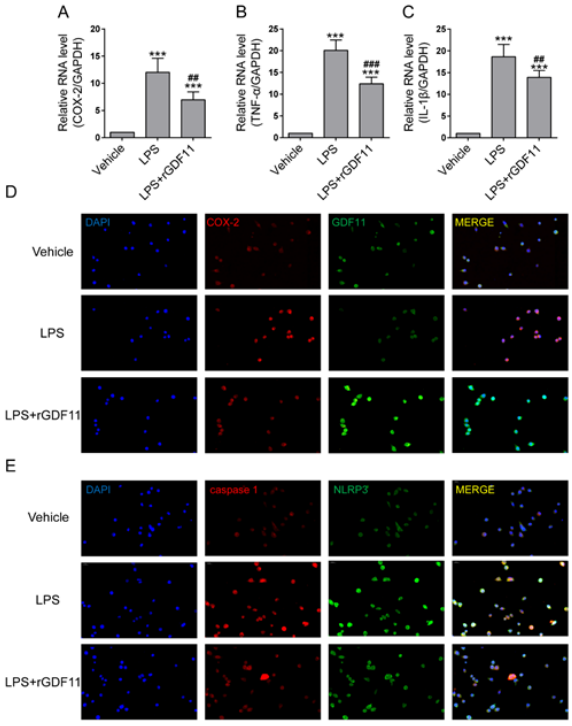

Figure 2. Growing expression of GDF11 attenuates inflammatory response by inhibiting NLRP3 in RAW 264.7 cells. The levels of COX-2 (A), TNF-α (B), and IL-1β (C) in cells in Vehicle, LPS, and LPS+rGDF11 group at 12 h. (D) Images of COX-2 (red) and GDF11 (green) expressions using IF staining after LPS treatment for 24 h; amplification:400×. (E) Images of caspase 1 (red) and NLRP3 (green) expressions using IF staining after LPS treatment for 24 h; amplification:400×. "*" means vs. Vehicle group and "#" means vs. LPS group with statistical significance, "*" means P <0.05; "**" means P <0.01; "***" means P <0.001.

yet reduced expressions via increased GDF11 treatment (Figure 3E). Hence, the overall results imply that increased GDF11 expression decreases intrahepatic inflammation provoked by LPS via negative regulation of NLRP3 inflammasome post-ALI.

The potential protective role of GDF11 in apoptosis after LPS-induced ALI

Persistent impact of inflammation can aggravate liver tissue damage and promote apoptosis. To certain whether the anti-inflammatory role of GDF11 influences the degree of apoptosis in the liver after LPS treatment, the apoptosis-related factors like Bax, Bcl-2, caspase 8 and caspase 3 were monitored using western blot at 3 dpi. The images showed that LPS stimuli increased the protein levels of Bax, caspase 8, and caspase 3 but decreased Bcl-2 expression, whereas the supplement of GDF11 reversed the expressions of each protein, which significantly reduced the ratio of Bax/Bcl-2 and the expressions of caspase 3/8 (Figure 4A-4D). Moreover, the biomarkers of apoptosis including Annexin V and cleaved-caspase 3 (c-caspase 3) were visualized using IHC at 3 dpi, exhibiting that LPS induced increased positive cells of Annexin V and c-caspase 3, while increased GDF11 expression reduced the count of Annexin V^+ and c-caspase 3^+ cells in liver post-LPS insult

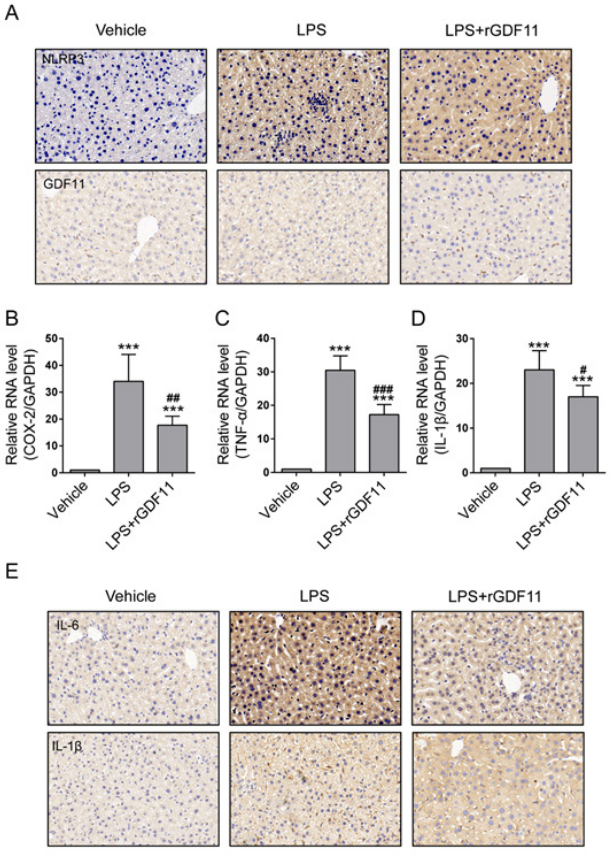

Figure 3. Increase of GDF11 reduces intrahepatic inflammation by inhibiting NLRP3 in ALI. (A) Images of NLRP3 and GDF11 expressions using IHC staining at 3dpi; amplification: 400×. The levels of COX-2 (B), TNF-α (C), and IL-1β (D) in liver tissue in Vehicle, LPS, and LPS+rGDF11 group at 3dpi. (E) Images of IL-6 and IL-1β expressions using IHC staining at 3dpi; amplification: 400×. "*" means vs. Vehicle group and "#" means vs. LPS group with statistical significance, "*" means P <0.05; "**" means P <0.01; "***" means P <0.001.

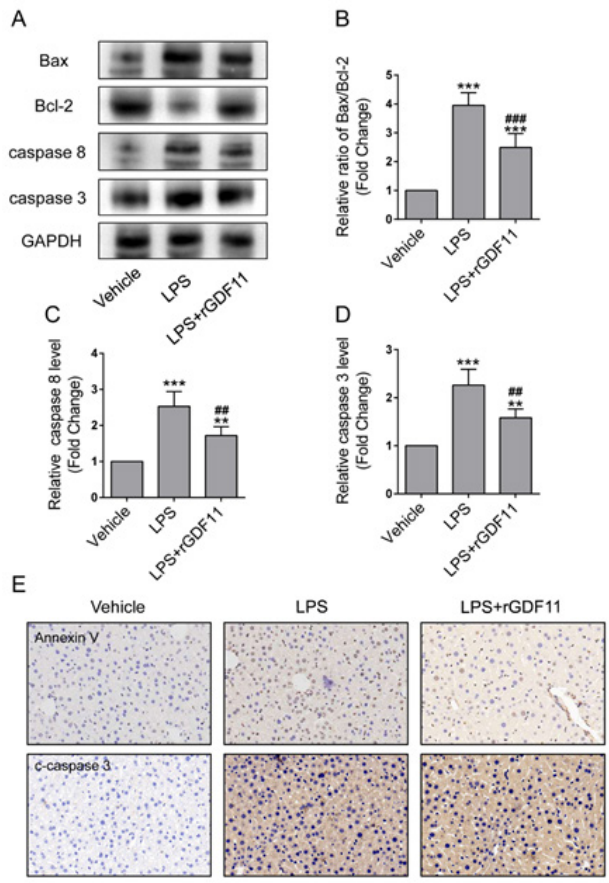

Figure 4. The potential protective role of GDF11 in apoptosis after LPS-induced ALI. (A) Protein bands including Bax, Bcl-2, caspase 8, and caspase 3 in liver tissue in Vehicle, LPS, and LPS+rGDF11 group at 3 dpi. The quantification includes the ratio of Bax/Bcl-2 (B), the expressions of caspase 8 (C) and caspase 3 (D) in Vehicle, LPS, and LPS+rGDF11 groups. (E) Images of Annexin V and c-caspase 3 expressions using IHC staining at 3dpi; amplification: 400×. "*" means vs. Vehicle group and "#" means vs. LPS group with statistical significance, "*" means P <0.05; "**" means P <0.01; "***" means P <0.001.

(Figure 4E). Taken together, it is suggested that GDF11 has a potential mitigative role in apoptosis post-LPS-induced ALI.

Discussion

In the present study, we prove that GDF11 exerts a protective role in LPS-induced ALI targeting inflammation. Following LPS injection, we find the decreasing GDF11 expression in liver tissue, which suggests that GDF11 is involved in ALI pathogenesis. The enhanced expression of GDF11 in liver is verified to correct the hepatic histology associated with decreased ALT, AST as well and TBiL. Hence, we are certain that GDF11 is a crucial factor of liver protection in the ALI process. Several studies have shown that GDF11 plays an antagonistic role in inflammatory damage. In LPS-mediated injured chondrogenic ATDC5 cells, GDF11 was shown as a promising anti-inflammatory target repressing the activation of NF-κB and MAPK signaling [16]. More notably, GDF11 was found a potential neuroprotective effect via amending edema, inflammation, apoptosis, oxidative stress and mitochondria damage in an experimental intracerebral hemorrhage

model (17). Consistent with the above findings, we here discover that GDF11 is an antagonism of inflammation in the ALI model. In vitro LPS-medicated RAW 264.7 cells, increased GDF11 expression reduced COX-2 expression in both transcription and translation levels, a key enzyme that promotes prostaglandin synthesis, leading to the restraining of pro-inflammatory procedure. Moreover, the classical cytokines including TNF-α and IL-1β decreased their levels after the GDF11 supplement. In TNF-α-induced inflammatory arthritis, Li and his workmates demonstrated GDF11 likewise reduced COX-2 TNF-α, and IL-1β expressions (18). Numerous evidences have testified that GDF11 antagonizes inflammation via suppression of the NF-κB pathway (16,18,19). Differently, we certify that GDF11 increase inhibits the expression of NLRP3/caspase 1 axis in RAW 264.7 cells. The findings suggest that GDF11 negatively regulates inflammatory response via restricting the NLRP3/caspase 1 pathway in LPS-activated macrophages. NLRP3 is a well-known inflammatory promoter in various infectious diseases including sepsis-induced ALI(20-22). Importantly, growing studies have presented that Inhibition of NLRP3 inflammasome can alleviate hepatitis damage during ALI (12,23,24). Consistently, the current study found that a supplement of GDF11 in ALI mice reduced pro-inflammatory markers expression via inhibition of NLRP3 inflammasome level, suggesting that GDF11 exerts an anti-inflammation effect via reducing NLRP3 expression. These findings may complement the molecular mechanism of GDF11 in NF-κB/NLRP3 signaling pathway, but the specific interactions between GDF11 and NF-κB/NLRP3 axis need to be further investigated. Besides, inflammation-related apoptosis is a critical aspect of ALI. Evidences have shown that apoptosis is related to liver damage after ALI and inhibition of inflammation can attenuate apoptosis development (25-27). Herein, we examined the degree of apoptosis after NLRP3 inhibition by increased GDF11, finding that decreased expressions of caspase 3/8 were accompanied by a reduced ratio of Bax/Bcl-2. In Parkinson's disease, therapy against NLRP3 inflammasome prevented subsequent apoptosis in neurons (25). We also prove that the reinforcement of GDF11 against NLRP3 mitigates apoptosis characterized by as reduced count of Annexin V and c-caspase 3 positive cells in live tissue. GDF11 replenishment has been reported to alleviate apoptosis in cardiomyocytes by regulating autophagy (28). Although we witnessed the alleviative effect of GDF11 on apoptosis, whether the underlying mechanism is to inhibit inflammation or promote autophagy is unknown. Hence, more attention would be paid to the potential anti-apoptotic mechanism of GDF11 in subsequent studies of ALI. Overall, the present study certifies that the loss of GDF11 occurs in liver tissue after LPS stimulation and replenishment of GDF11 protects liver tissue and function. Increased expression of GDF11 attenuates intrahepatic inflammation via inhibiting NLRP3/caspase 1 pathway after ALI and mitigates cell apoptosis in liver tissue. Therefore, GDF11 may be a promising regulated target of future inflammatory studies and be a selected intervention of therapeutic schedule in ALI.

Conclusions

We prove that GDF11 suppresses LPS-induced macrophage and intrahepatic inflammatory response via restricting NLRP3/caspase 1 inflammasome signaling. Increased GDF11 protected liver histology and function from damage in ALI. Hence, GDF11 is suggested as a promising anti-inflammatory target in ALI process.

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