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Preventive effects of interleukin-6 in lipopolysaccharide/D-galactosamine induced acute liver injury *via* regulating inflammatory response in hepatic macrophages

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ABSTRACT

Lipopolysaccharide/D-Galactosamine (LPS/D-Gal)-induced acute liver injury is characterized by significant inflammatory responses including TNF-α and interleukin-6 (IL-6) and is a widely applied experimental model for inflammation research. TNF-α is critical in the progression of LPS/D-Gal-induced liver injury. However, the role of IL-6 in this model is still unknown. In the present study, we aim to elucidate the involvement of IL-6 in the pathogenesis of acute liver injury induced by LPS/D-Gal in mice and its underlying mechanism. To induce acute liver injury, LPS (50 μg/kg body weight) and D-Gal (400 mg/kg body weight) were injected intraperitoneally in the C57BL/6 mice. The vehicle (saline) or a single dose of recombinant IL-6 (200 μg/kg body weight) was administered 2 h prior to LPS/D-Gal injection. Mice were sacrificed 2 h and 6 h after LPS/D-Gal injection. The results indicated that IL-6 treatment could protect mice from LPS/D-Gal-induced tissue damage, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) elevation, as well as hepatocyte apoptosis and inflammation. Furthermore, *in vitro* study showed that IL-6 treatment could significantly suppress LPS-triggered expression of proinflammatory cytokines and chemokines, TNF-α, RANTES and MCP-1 in macrophages while promoting the expression of M2 markers, such as Arg-1 and Mrc-1 in macrophages. Taken together, these findings revealed a novel and unexpected role of IL-6 in ameliorating LPS/D-Gal-induced acute liver injury *via* regulating inflammatory responses in hepatic macrophages.

1. Introduction

Acute liver injury is a highly destructive disorder without effective therapeutic strategies that is caused by sudden hepatocyte damage [1]. Drugs, hepatitis virus infections, and toxins are major etiologies associated with acute liver injury [2,3]. A combination injection of Lipopolysaccharide (LPS) and D-Galactosamine (D-Gal) causes acute liver injury in animals, which closely resembles the immuno-metabolic dysfunctions seen in the clinical syndrome [4]. Thus, LPS/D-Gal-induced acute liver injury in mice has been widely used as an animal model to investigate the pathogenesis of acute liver injury and to develop effective therapeutic approaches [4,5]. In this model, acute liver injury is triggered by LPS, which binds directly to Toll-like receptor 4 (TLR4) on intrahepatic macrophages and leads to their activation accompanied with secretion of numerous proinflammatory cytokines and chemokines. These proinflammatory molecules will further recruit substantial monocytes from the bloodstream to the liver to give rise to large numbers of monocyte-derived inflammatory macrophages, finally leading to hepatocyte injury [6]. Combining D-Gal with LPS injection is a general method to increase the sensitivity of hepatocytes to LPS stimulation [7].

Interleukin-6 (IL-6) is generally known as an inflammatory cytokine produced by a variety of cell types, especially macrophages at inflammatory sites [8]. Elevated plasma IL-6 levels have been widely accepted as a marker of inflammation in several diseases such as sepsis, liver disease and cardiovascular disease [9,10]. Previous studies also demonstrated that IL-6 was involved in the pathogenesis of acute and chronic liver diseases, such as ischemia/reperfusion (I/R) injury, nonalcoholic fatty liver and liver fibrosis [11]. Of note, exogenous administration of IL-6 ameliorated hepatic steatosis and I/R injury in fatty liver mice [12]. The IL-6-deficient mice presented increased hepatocyte apoptosis and necrosis, associated with increased liver inflammation

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and steatosis upon CCl4 injection or high fat diet treatment [13,14]. The IL-6R-deficient mice were also predisposed to liver steatosis and inflammation [15,16]. Taken together, these findings strongly suggested the potential use of IL-6 in treatment of liver pathologies [13,16]. Although elevated IL-6 in plasma was reported in LPS/D-Gal-induced acute liver injury [7], whether IL-6 promotes or ameliorates the development of LPS/D-Gal-induced acute liver injury remains unknown.

The present study aims at investigating the effect of exogenous IL-6 on acute liver injury induced by LPS/D-Gal. We examined liver function, histological changes, hepatic apoptosis and the production of inflammatory cytokines and chemokines *in vivo*. The possible mechanism involved in acute lever injury inflammatory activation was further elucidated *in vitro*.

2. Materials and methods

2.1. Reagents

Recombinant murine IL-6, interleukin-4 (IL-4) and interleukin-13 (IL-13) were purchased from Peprotech (Suzhou, China). Lipopolysaccharide (LPS), D-galactosamine (D-Gal) and all other chemicals were obtained from Sigma–Aldrich (Shanghai, China), unless otherwise noted. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Shanghai, China). *In Situ* Cell Death Detection Kit was purchased from Roche (Shanghai, China). TNF-α and RANTES enzyme-linked immunosorbent assay (ELISA) kits were purchased from R & D Systems (Shanghai, China). Alanine transaminase (ALT) and aspartate transaminase (AST) commercial assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. Animals and treatments

The C57BL/6 mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). All procedures were performed according to the guidelines for animal care and use and were approved by the Committee for Animal Research at Xiamen University. The mice were housed and maintained under controlled temperature (21–23 °C), humidity (55–60%) and lighting (12 h light/dark cycles) and given water *ad libitum*. To induce acute liver injury in 2 h and 6 h experiments, LPS and D-Gal were injected intraperitoneally in mice at the doses of 50 μg/kg and 400 mg/kg body weight respectively. The vehicle (saline) or a single dose of IL-6 (200 μg/kg dissolved in saline) was administered 2 h prior to LPS/D-Gal injection. Mice were sacrificed 2 h or 6 h after LPS/D-Gal injection.

2.3. Liver histological studies

Fresh liver biopsy specimens were fixed in 10% neutral-buffered formalin for 24 h, embedded with paraffin, sectioned using a Leica SM2010 R sliding microtome (Shanghai, China), and stained with hematoxylin-eosin (H & E) to assess liver histopathological damage. Stained areas were viewed and imaged under standard microscopy (Nikon, Shanghai, China).

2.4. TUNEL assay

Liver cell apoptosis was detected by a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay with an *In Situ* Cell Death Detection Kit according to the manufacturer's instructions. After TUNNEL labeling, the liver sections were counterstained with 4′-6-diamidino-2-phenylindole (DAPI) to label the nuclei. Images were observed under a fluorescence microscope (Olympus, Shanghai, China)

2.5. Plasma biochemistry and ELISA assays

For testing liver function, plasma aspartate transaminase (ALT) and alanine transaminase (AST) concentrations were determined by a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Shanghai, China) with commercial kits. The protein levels of plasma TNF-α and RANTES were analyzed using ELISA kits according to the protocol provided by the manufacturer.

2.6. Cell culture

RAW264.7 cells were obtained from the American Type Culture Collection (ATCC, Beijing, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). All cells were cultured in 6-well culture plates under 37 °C and 5% CO_2 in an incubator. The medium was replaced every two days, and the cells were harvested and diluted at a ratio of 1:3 every two days. In experiments, cells were pretreated with IL-6 (50 ng/mL) before stimulation with LPS (10, 100, 1000 ng/mL) or IL-4/13 (20 ng/mL).

2.7. RNA isolation, cDNA synthesis and real time PCR

Total RNA from liver cells was isolated using the TRIzol® reagent (Invitrogen, Shanghai, China) according to the manufacturer's instructions. cDNA was synthesized from total RNA using a FastQuant RT kit (Tiangen, Beijing, China) according to the manufacturer's instructions. Quantification of mRNA was carried out on a Roche LightCycler 480 Real time PCR Machine using SYBR® Premix Ex Taq™ II (Takara, Dalian, China). mRNA levels were normalized relative to the levels of β-actin.

2.8. Statistical analysis

Statistical calculations were performed with GraphPad Prism version 5.0 software for Windows. The results were expressed as the mean ± SEM of three independent experiments each performed in triplicate. Statistical analysis was performed *via* one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. IL-6 expression in LPS/D-Gal-induced acute liver injury mice

To evaluate the expression of IL-6 in acute liver injury *in vivo*, we first established a LPS/D-Gal-induced liver injury model using C57BL/6 mice and determined the hepatic and plasma IL-6 levels 2 h or 6 h post LPS/D-Gal stimulation. As shown in Figs. 1, 2 h or 6 h post LPS/D-Gal injection, IL-6 levels in the liver ($P < 0.05$, $P < 0.01$) and plasma ($P < 0.01$, $P < 0.01$) were dramatically increased compared to those of the control group receiving only the vehicle.

3.2. IL-6 ameliorated LPS/D-Gal-induced acute liver injury in mice

3.2.1. Effect of IL-6 on liver histological changes

Histological analysis was applied to evaluate the extent of liver injury induced by LPS/D-Gal injection (Fig. 2A). At 2 h after LPS/D-Gal injection, mild hepatocellular damage was observed in liver tissue. Furthermore, 6 h after LPS/D-Gal treatment, the liver tissue displayed more severe damages with disordered hepatic lobules. Of note, IL-6 pretreatment could efficiently ameliorate tissue damage and maintain more well-organized hepatic lobules.

3.2.2. Effect of IL-6 on plasma ALT and AST levels

Plasma aspartate transaminase (ALT) and alanine transaminase (AST) concentrations are essential biochemical markers of liver failure [17]. To further confirm the effect of IL-6 in protecting liver injury upon