Biochemical and Biophysical Research Communications 435 (2013) 306–312

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A protective role of IL-30 via STAT and ERK signaling pathways in macrophage-mediated inflammation

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ARTICLE INFO

Article history:
Received 10 March 2013
Available online 11 April 2013

Keywords:
IL-30
LPS
Liver injury
Macrophage
STAT/ERK

ABSTRACT

IL-30, the p28 subunit of IL-27, interacts with the Epstein–Barr virus-induced gene 3 (EBI3) to form IL-27, which modulates the inflammatory responses in autoimmune and infectious diseases. Several previous studies have provided evidence for the role of IL-30 in the anti-inflammatory process. However, the effect of IL-30 in macrophage-mediated immune responses is not well understood. With the recent observation in our experiment, we found that IL-30 exerted potent anti-inflammatory effects in the RAW 264.7 macrophages and in a lipopolysaccharide/D-galactosamine (LPS/D-GalN)-induced mouse model. IL-30 decreased the production of tumor necrosis factor (TNF)-α and IL-6 in LPS-stimulated RAW 264.7 macrophages in a dose-dependent manner. In the macrophage-mediated GalN and LPS model of acute liver injury, IL-30 prevented liver injury by suppressing serum enzyme activity and down-regulating the pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and interferon (IFN)-γ. IL-30 treatment decreased apoptosis in liver tissue and increased glutathione (GSH) levels. We postulated that IL-30 might function through gp130-mediated signaling pathways and then demonstrated that IL-30 affects LPS-induced inflammation through the STAT1, STAT3, and ERK signaling pathways. These data indicate that IL-30 can provide critical protection against macrophage-mediated liver inflammation through anti-apoptotic, anti-oxidant, and anti-inflammatory activities.

1. Introduction

Acute liver failure is a clinical manifestation of sudden and severe hepatic injury, with an extremely poor prognosis and high mortality due to a lack of effective preventive measures and therapies [1]. Therefore, effective therapeutic agents are needed for the prevention and treatment of liver injury.

Inflammation is an early host immune reaction mediated by cytokines secreted from immune cells. Macrophages are also involved in the pathogenesis of liver injury mediated by chemical substances, toxins, and pharmacological agents [2].

IL-30, the p28 subunit of IL-27, interacts with the Epstein–Barr virus induced gene 3 (EBI3) to form IL-27. In recent years, several studies have shown that IL-30 has functions independent of IL-27. IL-30 is a cytokine consisting of a 4-helix bundle that might be independently secreted. This is supported by the results of a study in which COS-7 cells transduced with the mouse *mp28* gene secreted a monomeric mp28 protein, whereas in the culture supernatants of cells transduced with the *mEBI3* gene, mEBI3 protein was not detected [3]. IL-30 acts as a natural antagonist of gp130 signaling by binding to gp130 and inhibiting the signaling of many cytokines such as IL-6, IL-11, and IL-27 [4]. Stumhofer and Laurence reported that IL-30 inhibited Th17 differentiation induced by IL-6 and transforming growth factor (TGF)-β *in vitro* through the STATs pathway [5]. Studies suggest that IL-30, independent of EBI3, modulates the immune response in autoimmune and infectious disease. Liu et al. found that lipopolysaccharide (LPS) and interferon (IFN)-γ treatment induced the expression of IL-30 in macrophages via binding of cRel to a distal nuclear factor-κB (NF-κB) site and binding of IFN regulatory factor-1 to a proximal IFN-stimulated response element site on the IL-30 promoter [6]. Gp130 is ubiquitously expressed in numerous cell types, including macrophages, and it serves as an essential co-receptor for many cytokines such as IL-6, IL-11, and IL-27 [7]. Activation of gp130 through its association with the α-chain/ligand complex

Abbreviations: ALT, alanine transaminase; AST, aspartate aminotransferase; DMEM, Dulbecco's modified eagle medium; EBI3, epstein barr virus induced gene 3; GalN, galactosamine; GSH, glutathione; IFN, interferon; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; NF-κB, nuclear factor-κB; TNF, tumor necrosis factor.

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0006-291X/$ - see front matter © 2013 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.bbrc.2013.03.136

activates 2 distinct signaling pathways, the STAT1/3 pathway and the mitogen activated protein kinase (MAPK) cascade [8,9]. However, the effect of IL-30 on macrophage-mediated inflammation and the underlying mechanism remain unknown. Therefore, we examined the effect of IL-30 on LPS-induced inflammation in macrophages *in vitro* and in a model of acute liver injury *in vivo*.

Macrophages play an important role in host defenses against noxious substances and infection and are involved in a variety of diseases, including autoimmune diseases and inflammatory disorders [10,11]. Inappropriate and prolonged macrophage activation is largely responsible for the pathology of acute inflammatory conditions [12,13]. LPS is a well-known inflammatory ligand that stimulates macrophages to release various inflammatory cytokines [14]. Treatment of mice with LPS in combination with D-galactosamine (GalN) generates a well-established model of macrophage-mediated liver injury [15,16]. GalN is a transcriptional inhibitor that sensitizes the liver to subtoxic amounts of LPS, leading to typical hepatic necrosis and apoptosis followed by fulminant hepatitis [17]. Here, we explored the role of IL-30 in LPS-induced inflammation in RAW 264.7 macrophages and in a GalN/LPS-induced mouse model of acute liver injury. In an attempt to determine the possible role of IL-30, STATs and ERK phosphorylation were analyzed by Western blotting. Our data show that IL-30 has a protective effect against macrophage-mediated liver inflammation and may offer a novel therapeutic strategy for inflammation.

2. Materials and methods

2.1. Animals

ICR mice (5–6-week-old) were purchased from Hua Fukang (Beijing, China). The mice were bred at 22 °C and 55.5% relative humidity with a 12-h light–dark cycle for at least 4–5 days before the experiment. Mice were treated in accordance with the guidelines of the local animal ethics committee.

2.2. Cell culture

RAW 264.7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum, 50 U/mL penicillin, and 50 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Production of recombinant mouse IL-30 protein

The mouse gene encoding IL-30 (GenBank Accession No. AY099297) was synthesized by Nanjing Genscript Biotechnology Co., Ltd. The mature 621-bp nucleic acid sequence encoding IL-30 was cloned into PET32α. Presence of a mature fragment of the IL-30 gene in the resulting plasmid was confirmed by nucleotide sequencing, and then the plasmid was transfected into competent *Escherichia coli* BL21 for overexpression. Inclusion bodies containing recombinant IL-30 were collected from the bacteria and processed through unfolding and refolding. After the proteins were folded, they were desalted and then loaded onto an ion-exchange column, followed by elution with a low-salt buffer. Proteins were concentrated and then filter-sterilized using a 0.2-μm filter. Recovery of recombinant proteins (>90% pure) was determined by SDS–PAGE and the protein was quantified using the Lowry assay.

2.4. In vivo GalN/LPS-induced liver injury

To prepare mice with GalN/LPS-induced fulminant hepatic failure, mice (except the normal controls) were injected intraperitoneally with GalN (700 mg/kg; Sigma, St. Louis, MO, USA) and LPS (10 μg/kg; *E. coli* O55:B5; Sigma, St. Louis, MO, USA) dissolved in phosphate-buffered saline. IL-30 was administered at a dose of 5 μg/kg by subcutaneous injection. Three treatment groups were examined: (1) vehicle-treated control, (2) vehicle-treated GalN/LPS, and (3) IL-30 (5 μg/kg)-treated GalN/LPS groups. After 8 h, the animals were sacrificed, and blood and liver samples were collected for further examination.

2.5. MTT cell viability assay

RAW 264.7 cells were seeded in 96-well plates at 4×10^5 cells/mL and incubated overnight at 37 °C and 5% CO_2. The cells were treated with different concentrations of IL-30 (0–5 μg/mL) for 2 h and then stimulated with LPS (1 μg/mL, *E. coli* O55:B5) for 22 h. MTT (20 μL of 5 mg/mL in FBS-free medium) was added to each well, and the cells were incubated for 4 h. The medium was carefully removed and 150 μL of DMSO was added. The optical density was measured at 570 nm using a microplate reader.

2.6. Cytokine assays

To investigate the effect of IL-30 on cytokine response from LPS-treated cells, RAW 264.7 cells (1×10^5 cells) were seeded in 96-well plates, incubated for 24 h, and then treated with 1.25, 2.5, or 5 μg/mL IL-30 for 2 h prior to treatment with 1 μg/mL LPS for 24 h. Cell-free supernatants were collected and stored at −20 °C until cytokine assay. The concentrations of TNF-α and IL-6 in the cell supernatants were determined according to the manufacturer's instructions by using ELISA kits (Neobioscience, Shenzhen, China).

2.7. Serum liver enzyme assay

Serum aspartate aminotransferase (AST) and alanine transaminase (ALT) levels were determined using a Hitachi 7020 automatic biochemical analyzer (Hitachi, Japan).

2.8. Assays for cytokine and glutathione (GSH) levels

The serum concentrations of TNF-α, IL-6, IL-1 β, and IFN-γ were measured using ELISA kits (Neobioscience, Shenzhen, China). GSH activity in the liver homogenates was quantified using a Glutathione Detection Kit (Nanjing Jiancheng Bioengineering Institute, China).

2.9. Histological examination of liver sections and TUNEL assay

Liver tissues were fixed with 10% buffered formalin/PBS, and embedded in paraffin. Liver sections (5 μm) were stained with hematoxylin and eosin by following a standard protocol. TUNEL assay was performed using the DeadEnd Fluorometric Tunel System (Promega, Madison, WI, USA) according to the manufacturer's instructions, and the cells were examined under a fluorescence microscope.

2.10. Semi-quantitative RT-PCR analysis

The expression of TNF-α, IL-1β, and IL-6 mRNA in liver tissue was measured by RT-PCR. Total RNA was isolated from cells or liver tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA (2 μg/reaction) was

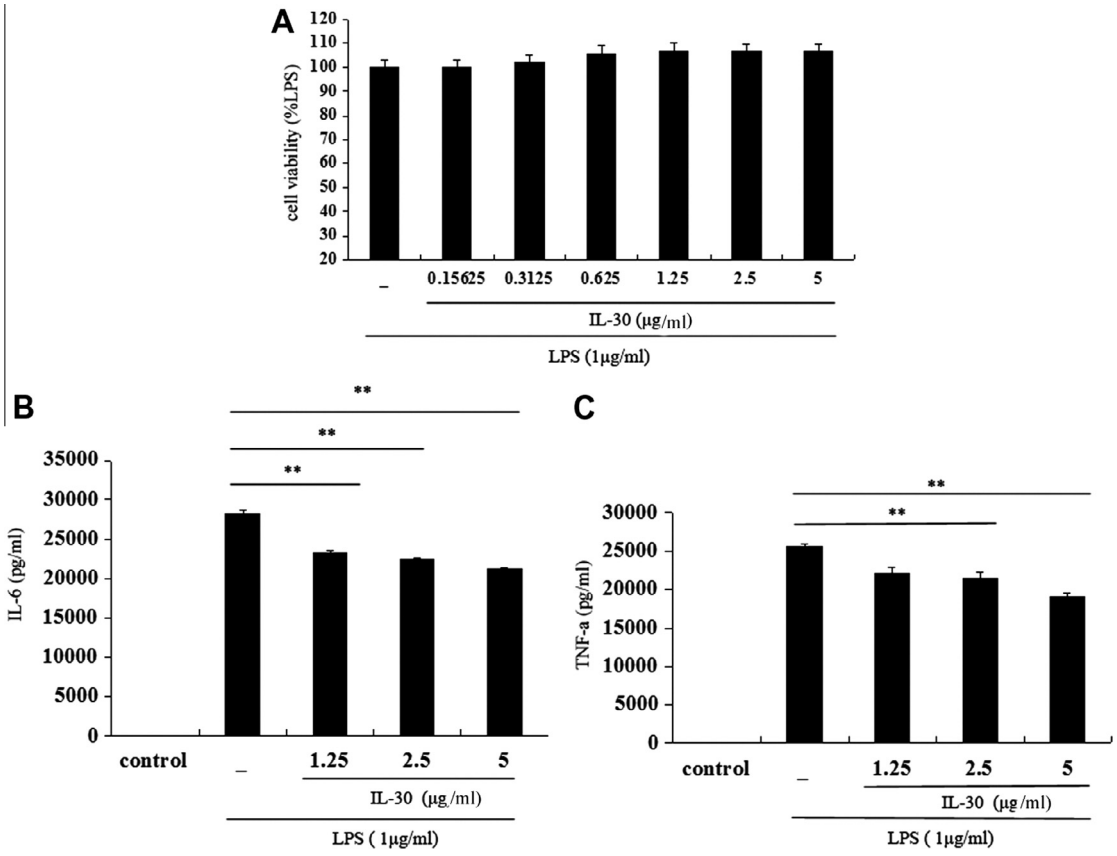

Fig. 1. IL-30 reduced the production of cytokines without cytotoxicity *in vitro* RAW 264.7 cells were treated with different concentrations of IL-30 (1.25, 2.5, and 5 μg/mL) in the absence or presence of lipopolysaccharide (LPS, 1 μg/mL) for 24 h. Cytokine levels in the culture medium were measured by ELISA (B and C). Cell viability was determined by the MTT assay (A). The data in the bar graph represents the mean ± SD of 3 independent experiments. The *P*-values were determined by ANOVA (**$P < 0.01$).

reverse transcribed to obtain cDNA by using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). The sequences of the primers used to amplify the cytokine genes were as follows:

TNF-α (307 bp), forward 5′-GGCAGGTCTACTTTGGAGTCATTGC-3′ and reverse 5′-ACATTCGAGGCTCCAGTGAATTCGG-3′; IL-1β (502 bp), forward 5′-TCATGGGATGATGATGATAACCTGCT-3′ and reverse 5′-CCCATACTTTAGGAAGACACGGATT-3′; IL-6 (497 bp), forward 5′-GGAAATCGTGGAAATGAG-3′ and reverse 5′-GCTTAGGC ATAACGCACT-3′; GAPDH (451 bp), forward 5′-ACCACAGTCCATGC-CATCAC-3′ and reverse 5′-CCACCACCCTGTTGCTGTAG-3′. PCR amplification was performed with 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 1 min.

2.11. Western blotting

RAW 264.7 cells were treated with 1.25, 2.5, or 5 μg/mL IL-30 for 2 h and then stimulated with 1 μg/mL LPS. After incubation for 2 h, the samples were homogenized in RIPA buffer and lysed for 30 min on ice for Western blotting. Protein was separated on sodium dodecyl sulfate–polyacrylamide gels (10–15%) and then electro-transferred to PVDF membranes. The membranes were incubated with a primary antibody (STAT1 and STAT3 from Cell Signaling Technologies; ERK from Santa Cruz Biotechnology) overnight at 4 °C, followed by incubation with a secondary antibody (anti-rabbit and anti-mouse horseradish peroxidase [HRP]-conjugated secondary antibodies [Santa Cruz Biotechnology]) and detected using an enhanced chemical luminescence kit. Membranes were also incubated with an anti-β-actin antibody to confirm equal loading of protein. Representative blots are shown.

2.12. Statistical analysis

Data are expressed as mean±SD. Statistical analysis was performed using ANOVA for multiple group comparisons. SPSS 16.0 (IBM, Chicago, IL, USA) was used for all statistical analyses. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. IL-30 showed no dose-dependent cytotoxicity

To determine whether IL-30 was toxic to RAW 264.7 cells, cell viability was evaluated at various concentrations of IL-30 by the MTT assay (Fig. 1A). The results showed that IL-30 alone (at concentrations of 1.25, 2.5, and 5 μg/mL) had no effect on the viability of RAW 264.7 cells.

3.2. IL-30 suppressed pro-inflammatory cytokine production in RAW 264.7 cells stimulated by LPS

Macrophage production of the pro-inflammatory cytokines TNF-α, IL-6, and IL-1β in response to inflammatory stimuli is well known. To explore the effects of IL-30 on inflammation, the levels of these inflammatory cytokines were determined in the different test groups. Our *in vitro* studies demonstrated that in the absence of IL-30, LPS treatment (1 μg/mL) dramatically increased IL-6 (Fig. 1B) and TNF-α levels (Fig. 1C). Treatment with IL-30 attenuated these increased TNF-α and IL-6 levels in a concentration-dependent manner (Fig. 1). These results suggest that IL-30

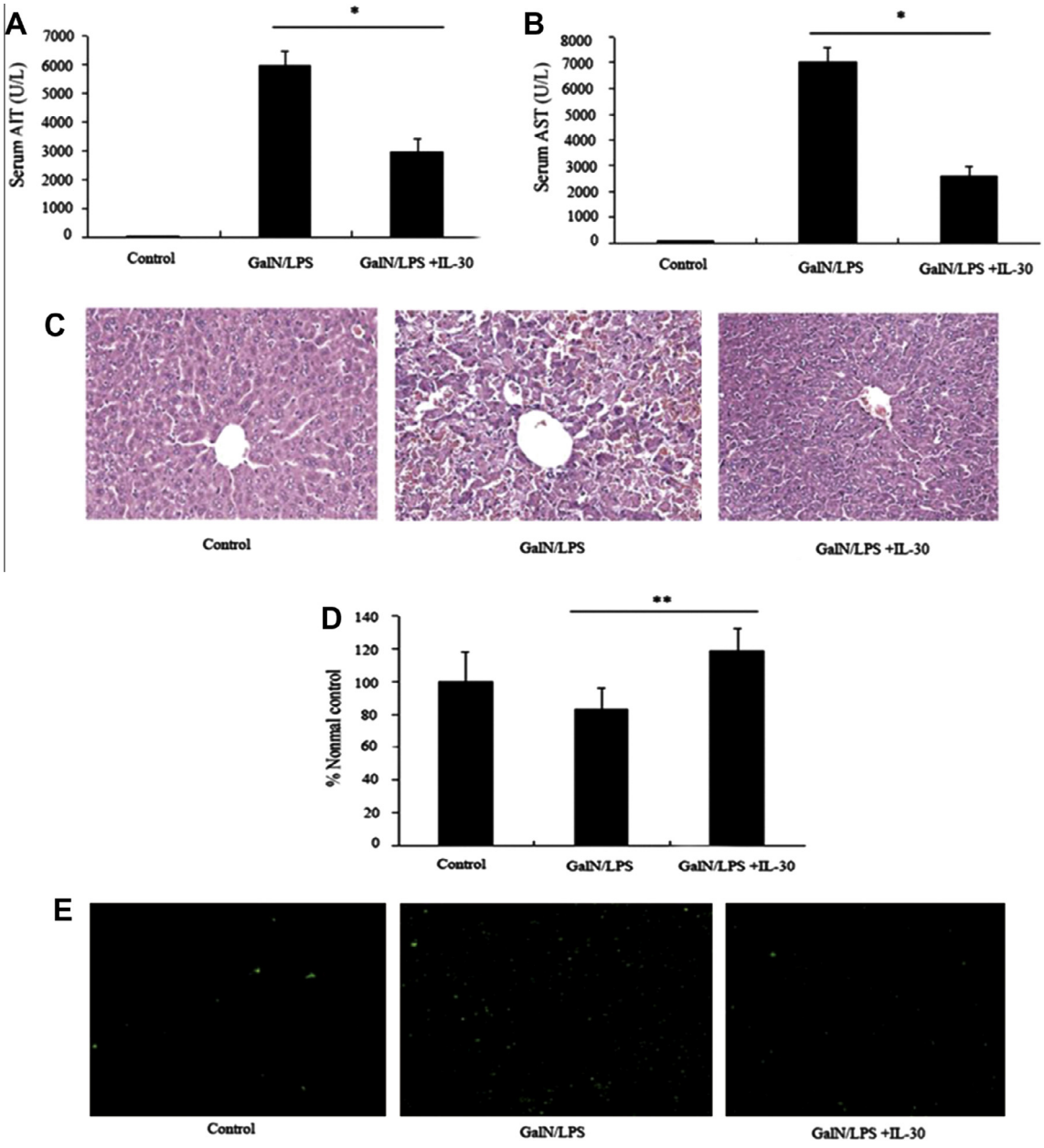

Fig. 2. Effects of IL-30 in a murine model of acute liver failure In A and B, mice were administered IL-30 (5 μg/kg) by subcutaneous injection and 8 h later, the serum levels of ALT and AST were determined. As shown in C, livers were dissected for histological analysis with H&E staining. Each group consisted of 6 mice. $^{*}P < 0.05$ vs. the GalN/LPS-treated group. Original magnification: 200×. In D, liver homogenates were collected to analyze glutathione (GSH) levels. The data shown are the means ± SEMs ($n = 6$ in each group). $^{**}P < 0.01$ vs. the GalN/LPS-treated group. In E, livers were analyzed by TUNEL staining as described in Section 2.

decreases inflammatory cytokine production in RAW 264.7 macrophages following LPS challenge.

3.3. IL-30 prevented GalN/LPS-induced acute liver injury

ALT and AST are considered reliable markers of hepatocyte damage. These markers were employed to examine the extent of acute liver injury and the effectiveness of IL-30 treatment. ALT and AST levels were significantly elevated 8 h after GalN/LPS challenge. Treatment with IL-30 prevented GalN/LPS-induced liver failure as evidenced by a reduction in the serum ALT and AST levels (Fig. 2A and B). In conjunction with these findings, the liver tissues of mice challenged with GalN/LPS showed substantial histological alterations consistent with LPS/D-GalN-induced hepatitis compared to liver tissues from vehicle-control mice (Fig. 2C). In the GalN/LPS group, liver sections showed massive necrosis associated with intralobular hemorrhage, destruction of hepatic architecture, and inflammatory cell infiltration. The control group did not show any abnormal changes in the liver architecture. The extent of tissue damage in mice treated with IL-30 was lesser than that in the GalN/LPS-only group (Fig. 2C).

3.4. IL-30 treatment increased GSH levels in liver homogenates and reduced cell apoptosis

Oxidative stress is known to play an important role in the pathogenesis of acute liver failure. Therefore, we measured GSH levels in liver homogenates. GSH levels decreased 8 h after GalN/LPS injection, but were normal following treatment with 5 μg/kg IL-30 (Fig. 2D). To further evaluate the ability of IL-30 to protect

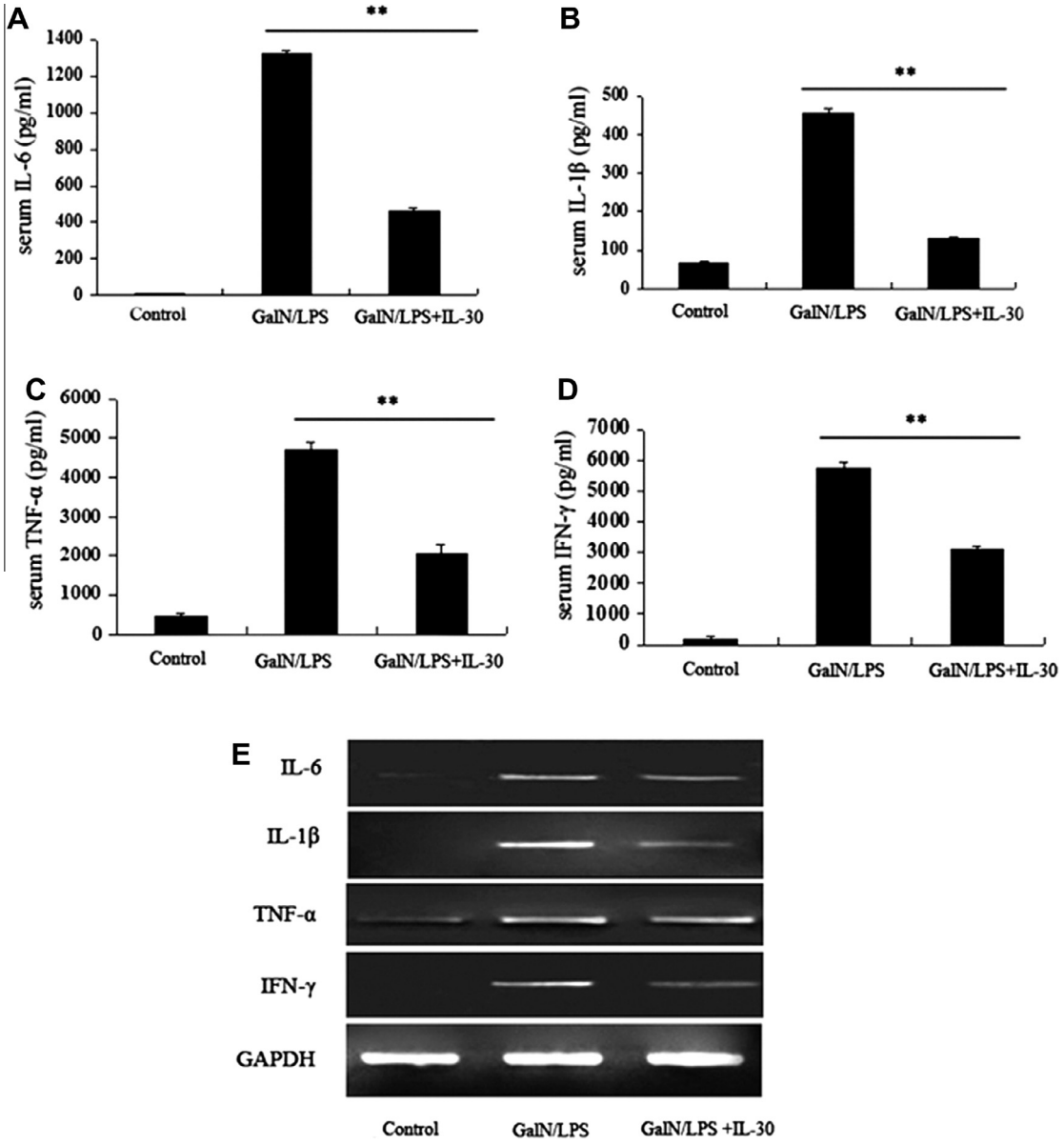

Fig. 3. Effects of IL-30 on pro-inflammatory cytokine expression in mice with acute liver failure In A, B, C, and D, the levels of IL-6, IL-1β, tumor necrosis factor-α (TNF-α), and interferon-γ (INF-γ) in the serum of mice administered vehicle (control), GalN/LPS, or GalN/LPS plus IL-30 were determined using ELISA. In E, semi-quantitative RT-PCR analysis of IL-6, IL-1β, TNF-α, and INF-γ was performed on total RNAs extracted from the livers of different treatment groups as described in Section 2. The data shown are the means ± SEMs ($n = 6$ in each group). $^{**}P < 0.01$ vs. the GalN/LPS-treated group.

against liver injury, we examined cell apoptosis in mouse livers by using the TUNEL assay. A large number of TUNEL-positive hepatocytes were observed in mouse liver tissues obtained 8 h after GalN/LPS treatment. However, only a few TUNEL-positive hepatocytes were observed after treatment with IL-30 (Fig. 2E).

3.5. IL-30 reduced serum levels of pro-inflammatory cytokines

To explore the effects of IL-30 on pro-inflammatory cytokines *in vivo*, serum levels of TNF-α, IL-1β, INF-γ, and IL-6 were measured using ELISA kits. Mice injected with GalN/LPS had higher TNF-α, IL-1β, INF-γ, and IL-6 serum levels 8 h post-injection than the control mice. The concentrations of these 4 inflammatory cytokines were significantly lower in the IL-30-administrated mice than in the GalN/LPS-treated mice (Fig. 3A–D).

3.6. IL-30 reduced the mRNA levels of pro-inflammatory cytokines in GalN/LPS-treated liver tissue

We next examined whether IL-30 had an effect on the mRNA expression of pro-inflammatory cytokines following liver injury. Eight hours after GalN/LPS injection, TNF-α, IL-1β, INF-γ, and IL-6 mRNA expression levels were markedly elevated. Treatment with IL-30 significantly decreased the mRNA levels of TNF-α, IL-1β, INF-γ, and IL-6 (Fig. 3E).

3.7. IL-30 was regulated via the STAT and ERK-signaling pathways

Western blotting was performed to examine whether IL-30 could affect the STAT and ERK signaling pathways. We noticed that stimulation with LPS increased the phosphorylation of STAT1, STAT3, and ERK. IL-30 treatment reduced the levels of phosphorylated STATs

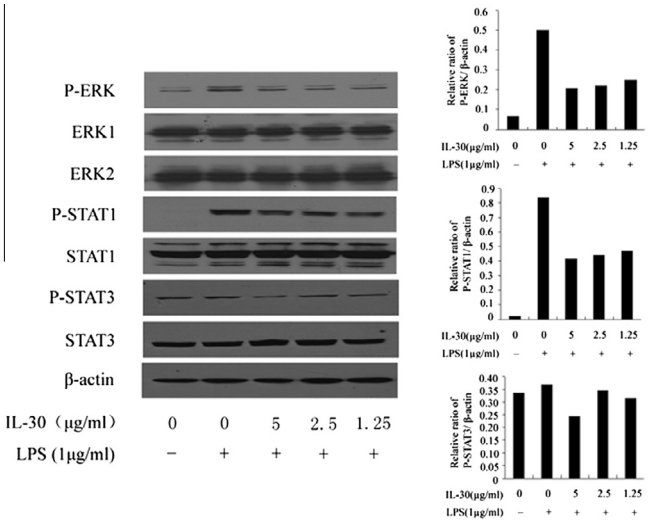

Fig. 4. IL-30 affected the LPS-induced cell-signaling pathways in RAW 264.7 cells RAW 264.7 cells were treated with 1.25, 2.5, or 5 μg/mL IL-30 for 2 h and then stimulated with LPS (1 μg/mL) for 24 h. Total cell lysates were used to detect phosphorylated and total STAT3, STAT1, and ERK by Western blotting.

and ERK. The amounts of non-phosphorylated STAT1, STAT3, and ERK 1/2 were unaffected by LPS or LPS plus IL-30 treatment (Fig. 4).

4. Discussion

Previous studies suggested that IL-30 had anti-inflammatory functions. Published studies have supported this suggestion because IL-30 inhibited pro-inflammatory cytokines such as IL-6 in gp130 signaling [4]. IL-30 expression in myoblasts has been shown to inhibit allogeneic T cell responses and prolonged graft survival, which supports the anti-inflammatory role of this cytokine [3,18]. Liu et al. showed that LPS and IFN-γ treatment could induce IL-30 expression in macrophages [6]. IL-30 also inhibits IL-12- and Con A-mediated liver toxicity through negative feedback regulation of IFN-γ expression [19]. However, to date, no study has focused on the mechanism underlying the role of IL-30 in macrophage-mediated liver inflammation. In the present study, we demonstrated that IL-30 possesses anti-inflammatory activity *in vitro* and that treatment with IL-30 attenuates GalN-sensitized LPS toxicity *in vivo*. Acute liver injury is a severe clinical syndrome characterized by hepatic cell injury resulting from a variety of hepatic disease processes, leading to multiorgan failure [20,21]. GalN/LPS-induced acute liver injury in mice has been widely used as an experimental animal model to investigate the mechanisms underlying clinical fulminant hepatic failure [21–23]. The levels of serum aminotransferases (AST and ALT) activity are used as biochemical markers of early acute hepatic damage [24]. Our data show that IL-30 treatment markedly decreases ALT and AST levels (Fig. 2A and B). Following stimulation with LPS, macrophages produce various pro-inflammatory cytokines including IL-6, IL-1β, and TNF-α. Our results show that IL-30 suppresses mRNA expression and production of these cytokines, thereby reducing liver injury (Fig. 3). LPS activates macrophages to produce TNF-α, which induces hepatocyte apoptosis in the early stage of GalN/LPS-induced hepatitis in mice [16]. Reduced GSH depletion has also been shown to sensitize primary mouse hepatocytes to TNF-α-mediated apoptosis [25].

In this study, we found that IL-30 had anti-apoptotic and anti-oxidative effects in macrophage-mediated liver injury. These data indicate that IL-30 might be a promising therapeutic tool for the treatment of macrophage-mediated liver injury.

Although exogenous introduction of IL-30 plasmids via gene therapy has been shown to significantly reduce IL-12/ConA-induced hepatotoxicity by preventing IFN-γ expression in the liver and lowering circulating serum IFN-γ levels [19], no previous studies have reported on the effects of IL-30 in macrophage-mediated liver injury. To investigate the mechanisms underlying the hepato-protective effects of IL-30, we analyzed the gp130-mediated signaling that IL-30 might target.

IL-30 is a 4-helix bundle protein that binds EBI3 to form IL-27 [26]. IL-27 signaling occurs through a heterodimeric receptor (IL-27R) consisting of WSX-1 and gp130 subunits, which is a common receptor used by several cytokines, including IL-6 and IL-27 [27]. However, in the absence of WSX-1, IL-30 seems to act as a natural antagonist of gp130 signaling, which suppresses IL-17 production in TGF-β- and IL-6-stimulated $CD4^+$ T cells *in vitro* [5]. IL-30 can interact with gp130 in the absence of EBI3 and might antagonize the ability of IL-6 and IL-27 to signal through the immunoglobulin-like domain of gp130 in $CD4^+$ T cells [4]. Gp130 is ubiquitously expressed in cells, including macrophages. Several cytokines, including IL-6 and IL-27, bind to the gp130 subunit of cytokine receptors that inhibit macrophage activation, blocking its pro-inflammatory effects [28,29].

Macrophages play an important role in inflammatory disease by releasing inflammatory factors in response to pathogen- and host-derived molecules such as LPS and IFN-γ [13,30]. Inflammatory factors such as IL-6, IL-27, and TNF-α play pivotal roles in the pathogenesis of the endotoxin-induced experimental liver injury model [31]. Here we attempted to elucidate the anti-inflammatory effects of IL-30 in macrophage-mediated inflammation. Our *in vitro* RAW 264.7 cell experiments suggested that IL-30 reduced the production of IL-6, IL-1β, and TNF-α (Fig. 1) in macrophage-mediated inflammation. We infer that IL-30 may antagonize cytokine gp130-mediated signaling through IL-6 or IL-27, thereby suppressing the production of IL-1β and TNF-α. LPS-induced signaling via Toll-like receptor 4 (TLR)-4 enhances the expression of the STAT and ERK protein families, leading to translocation of NF-κB/Rel family members into the nucleus [32]. IL-30 seems to antagonize the interaction of IL-6 and IL-27 with gp130 in $CD4^+$ T cells, leading to markedly reduced STAT1 and STAT3 phosphorylation [4]. STAT3 is a downstream effector of multiple signaling pathways, such as those involving IL-6, IL-21, IL-23, and IL-27, and its inhibition probably has broad effects. ERK plays a critical role in the LPS-induced immune response signaling pathway in mammalian cells [33]. We sought to identify the signaling pathways necessary for the anti-inflammatory effects of IL-30 in macrophage-mediated inflammation. Our results suggest that IL-30 inhibits the phosphorylation of STATs and ERK (Fig. 4). The protective effect of IL-30 could be due to attenuation of anti-inflammation, and the mechanism may be related to the STAT and ERK signaling pathways.

In summary, our results clearly demonstrated that IL-30 has a hepatoprotective effect during macrophage-mediated liver injury. The protective effect of IL-30 could be due to attenuation of anti-inflammation and oxidative stress and inhibition of hepatocyte apoptosis, and the underlying mechanism may be related to the induction of STAT1, STAT3, and ERK. Therefore, IL-30 may be a useful therapeutic tool for the treatment of acute hepatic damage.

Acknowledgments

This work was supported by the National Science and Technology Major Project of China (No. 2012ZX10002006-003-001).

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