INTRODUCTION

Hepatotoxicity is a serious human health problem worldwide. The pathogenesis of immune-mediated liver disease is complicated, which involves inflammatory cells and cytokines. Growing evidence suggests that major human liver diseases such as autoimmune and viral hepatitis are caused, in general, by activated T cell-mediated immune response (Chisari and Ferrari, 1995; Kita et al., 2001). Autoimmune hepatitis (AIH) is a severe form of liver disease characterized by progressive destruction of the hepatic parenchyma, cellular infiltration, and auto-antibodies. The prevalence of AIH is estimated to range between 50 and 200 cases per million population, and it accounts for 5.9% of all liver transplantations in the United States with all ages and ethnic groups (Czaja and Freese, 2002). Concanavalin A (Con A)-induced liver injury is an experimental model of immune-mediated liver injury (Tiegs et al., 1992). The T cell-dependent specific liver injury in mice induced by Con A is considered to be eligible for the study of pathophysiology of human liver diseases, such as viral hepatitis and AIH (Kimura et al., 1999; Sakamoto et al., 2000; Shirin et al., 1999; Cao et al., 1999; Fiorucci et al., 2000). Activated T lymphocyte infiltration in the portal area is regarded as the prominent character different from other models. Meanwhile, apoptosis and necrosis of hepatocyte, T cell activation and several cytokines secretion have also been proven to play a critical role in the process of Con A-induced liver injury (Okamoto et al., 1998b; Bozza et al., 1999; Sass

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undergoing elective liver resection without any potential perioperatively could attenuate hepatic injury in patients. A recent study had just demonstrated that DEX administered perioperatively could attenuate hepatic injury in patients undergoing elective liver resection without any potential risk. DEX also had been demonstrated to have protective effects in animal model of ischemia/reperfusion (I/R) liver injury. However, the precise mechanism of anti-inflammatory effect of DEX remains undefined. The dosage selection of Con A was based on a prior study and our preliminary experiments, wherein doses of DEX were determined by body surface area normalization method and confirmed to be suitable and effective in mice. All mice survived after 12 hr Con A administration. All mice were narcotized by sevoflurane and samples were harvested at 12 hr after Con A treatment. Blood was collected from heart puncture in polystyrene tubes with anticoagulant. Plasma was separated and stored at -20°C until analyzing serum levels of glutamic-pyruvic transaminase (ALT), glutamic oxalacetic transaminase (AST), and cytokines levels. The liver was quickly divided into portions. In our previous research, we have confirmed that ALT and AST levels peaked highest about 12 hr after Con A treatment. Therefore, in this study, mice were sacrificed at 12 hr after Con A injection. The liver was carefully excised and homogenized in ice-cold 0.15 M Tris-KCl buffer (pH 7.4) to yield a 10% (w/v) homogenate. The latter was next subjected to high-speed centrifugation for 10 min at 4°C. The resulting supernatant of liver was used for assaying of CD4+ T cell, CD8+ T cell, CD3+ CD25+ T cell, CD4+ CD25+ T cell and CD8+ CD25+ T cell. The homogenate of the liver was also used for western blot.

**Chemicals and reagents**

DEX was purchased from Jiangsu Hengrui Medicine Co., Ltd. (Nanjing, Jiangsu, China). Con A was purchased from Tianjin Bomeike Biotechnology Co., Ltd. (Tianjin, China). All other chemicals and reagents were of standard commercially available biochemical quality. Water was purified with a Milli-Q purification system (Millipore Corporation, Billerica, MA, USA) and was used to prepare all solutions.

**Animals**

Male mice of original C57BL/6, 10-12 weeks old, weighting 18-22 g, were used for the study. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the Chinese National Institutes of Health. The animals were obtained from the Animal Department of the Second Military Medical University (Shanghai, China) and were housed in a room maintained at 23 ± 2°C with relative air humidity of 45% to 55% on a 13-hr light/11-hr dark cycle. Mice were provided a standard laboratory chow and water ad libitum. The approval of this experiment was obtained from the Institutional Animal Ethics Committee of the Second Military Medical University (Shanghai, China).

**Preparation of mice and experimental design**

The mice were adapted to diet and environment for one week before the experiment began. After confirmation of the state, mice were randomly divided into three groups. In normal control (NS) group, mice were treated with normal saline (NS) (100 μL) and 1 hr later with NS (100 μL) again by caudal vein injection. In Con A treatment group, mice were treated with NS (100 μL) and 1 hr later with Con A (20 mg/kg, 100 μL) by caudal vein injection. In DEX treatment group, mice were treated with DEX (25 μg/kg, 100 μL) in 60 sec and 1 hr later with Con A (20 mg/kg, 100 μL) by caudal vein injection.

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**Serum enzyme and IL-6, IL-10, TNF-α, and IFN-γ assay**

Twelve hours after Con A treatment, blood was collected and separated to measure levels of ALT, AST, IL-6, IL-10, TNF-α, and IFN-γ. Plasma was collected following centrifugation of blood for 5 min at 2,000 rpm. Serum was stored at -20°C to measure serum biochemical parameters. The levels of ALT and AST were measured spectrophotometrically using an automatic biochemical analyzer (Hitachi Auto Analyzer 7170, Kobe, Japan). Levels of IL-6, IL-10, TNF-α, and IFN-γ in plasma were measured by ELISA using Mouse ELISA Kit (R&D system, Minneapolis, MN, USA) according to manufacture.
Dexmedetomidine attenuates liver injury

Assessment of CD4+, CD8+, CD3+CD25+, CD4+CD25+ and CD8+CD25+ T lymphocytes in liver

Single-cell suspensions of liver were obtained at 12 hr after Con A-induced hepatitis. Cells were then stained with fluorescence-labeled antibody (anti-CD3 Ab, anti-CD4 Ab, anti-CD25 Ab, anti-CD8 Ab). The count of infiltrating CD4+ and CD8+ T lymphocytes and the percent of CD3+CD25+, CD4+CD25+, and CD8+CD25+ T lymphocytes in the liver were analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA).

Histopathology

Mice were narcotized by sevoflurane 12 hr after Con A administration. Livers were fixed in 10% buffered formaldehyde, pH 7.4, and embedded in paraffin. Sections (5 μm) on slides were deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol, and stained with hematoxylin and eosin (H&E) and examined under an Olympus BH2 photomicroscope. Slides were examined by light microscopy for infiltrating leukocytes and tissue injury. All sections were scored blind by two pathologists under light microscopy using the following criteria (Zhu et al., 2012): 0, none; 1, individual cell necrosis; 2, ≤ 30% lobular necrosis; 3, ≤ 60% lobular necrosis; 4, > 60% lobular necrosis.

Western-blot assay of NF-κB

Mice were narcotized by sevoflurane 12 hr after Con A administration. Liver tissues from each mouse were then harvested and placed into lysis buffer (Thermo, Waltham, MA, USA). Liver tissues were homogenized, spun at 12,000 g for 10 min at 4°C. Cell lysates (45 g protein, as determined by a Bradford protein assay) were loaded equally onto 10% polyacrylamide gels (Life Technologies) and transferred to nitrocellulose membranes (Life Technologies). Membranes were washed three times in PBST, overnight at 4°C in 5% PBST. After primary antibody incubation, membranes were washed three times in PBST, and rabbit anti-mouse secondary antibody (Cell Signaling Technology, Danvers, MA, USA) was added at a concentration of 1:2,000 in 3% PBST. Membranes were incubated with fluorescence-labeled antibody (anti-CD3 Ab, anti-CD4 Ab, anti-CD25 Ab, anti-CD8 Ab). The count of infiltrating CD4+ and CD8+ T lymphocytes and the percent of CD3+CD25+, CD4+CD25+, and CD8+CD25+ T lymphocytes in the liver were analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA).

RT-PCR assay for gene expression

Gene expression levels of hepatic C-X-C motif chemokine 10 (CXCL10) were assessed by Reverse Transcription-Polymerase Chain Reaction (PCR) as described. Total RNA was isolated from the homogenate of the liver after 12 hr Con A stimulations, with TRIzol reagent (Invitrogen, Waltham, MA, USA). Cellular RNA was treated with DNase1 and then primed with a DT oligonucleotide and reverse transcribed with Superscript II. For real-time assays, PCR reactions were prepared in SYBR Green PCR Master Mix. DNA targets were amplified and analyzed with a Chromo Real-Time PCR Detection System (Bio-Rad Life Sciences, Philadelphia, PA, USA). The murine primer sequences are shown as follows:

Mouse CXCL10 (F): 5’-TCCAAGTGAAGGAGCCCTTTagACC-3’ and (R): 5’-TGAAATCATCCCTGCGAGCTAT-3’ GAPDH (sense: 5’-AGAGTGAGGTGTGCTGTGTG-3’ antisense: 5’-GCTTCCGTGTTCCCTACC-3’). Total RNA was treated with DNase I to eliminate genomic DNA contamination, followed by synthesis of the first-strand using reverse transcription system. Reverse transcription was carried out as follows: 42°C for 60 min, 70°C for 10 min and first-strand cDNA was stored at -20°C. Real-time PCR was performed in a 20 μL of reaction solution containing SYBR Premix Ex Taq, primers, and cDNAs. The cycles for PCR were as follows: 95°C for 2 min, 40 cycles of 95°C for 15 sec, 58°C for 20 sec, and 72°C for 20 sec. Melting curves were determined by heated naturaling PCR products over a 35°C temperature gradient at 0.5°C/sec from 65 to 99.5°C. GAPDH was used as an internal control (Tu et al., 2011). The relative amount of mRNA was determined using the ΔΔCT technique (Livak and Schmittgen, 2001). The levels of mRNA were expressed as fold changes after normalization to GAPDH.

Statistical analysis

All results were presented as mean ± S.D. for ten mice in each group. To determine the effect of treatment, data were analyzed using one-way analysis of variance (ANOVA) repeated measures. For multi-group analysis, intergroup comparisons were performed by Dunn’s test. P-values of less than 0.05 were regarded as significant. Data were analyzed using the statistical package “SPSS 17.0 for Windows” and Prism 5.0 (Graph Pad Software, La Jolla, CA, USA).
RESULTS

**Dexmedetomidine decreased levels of ALT and AST after Con A challenge**

As Con A-induced hepatitis is an experimental model mirroring the pathology of human autoimmune hepatitis (Shao et al., 2013), we tested whether DEX could function as a remedy. Mice injected with Con A developed acute hepatitis as indicated by elevated serum ALT and AST levels in the Con A group compared with NS group. DEX ameliorated Con A-induced hepatitis as indicated by ALT and AST (Fig. 1A, B). This finding suggested that DEX antagonized hepatitis distinctly. We also injected DEX followed by Con A to mice to detect the treatment effect. However, there was no difference in levels of ALT and AST compared with NS group (demonstrated by Supplementary Fig. 1).

**Dexmedetomidine ameliorated Con A-induced liver injury by histological examination**

Liver histological examination was also used to determine the protective effect of DEX in Con A-induced hepatitis. Light microscopy showed normal tissue histology for NS-injected mice, but dramatic leukocyte infiltration and massive hepatocyte necrosis and disorder of hepatic sinusoids structure in Con A-injected mice. Con A-injected mice administrated with DEX showed a significant decrease in hepatic tissue injury and less leukocyte infiltration (Fig. 2A-C). The pathological scores showed that the severity of liver injury in the Con A group was much more serious than that in NS and was attenuated by DEX (Fig. 2D). These results indicated that there was a significant accumulation of liver damage of pathophysiology in the Con A-treated group, which could be largely eliminated by DEX.

**Dexmedetomidine inhibited infiltration of T cells in liver**

T cells play critical roles in Con A-induced hepatitis. Thus, we investigated whether DEX-induced suppression of hepatic injury results from inhibiting the infiltration of T cells in the liver. We isolated intrahepatic cells at 12 hr after Con A challenge and stained them with CD3, CD25, CD4 and CD8 markers. Con A administration resulted in a significant increase in the absolute number of CD4+ T cells in the liver compared with NS and DEX via flow cytometry (Fig. 3A). Among CD4+ T cells, the percentage of CD4+ CD25+ T cells increased significantly in the Con A group (Fig. 4B). Interestingly, we noted that CD8+ T cells didn’t increase in the Con A group compared with the NS group (Fig. 3B), while the percent of CD8+ CD25+ T cells among CD8+ T cells increased significantly compared with other groups (Fig. 4C). In addition, the percent of CD3+ CD25+ T cells in Con A group increased significantly compared with NS and DEX groups (Fig. 4A). These data suggested that DEX may afford protection to the hepatocytes from inhibiting specific T cells subpopulations.

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**Fig. 1.** DEX pretreatment attenuated liver damage followed by Con A administration. A and B, DEX reduced levels of ALT and AST in mice 12 hr after Con A administration. **P < 0.01 versus NS group, #P < 0.05, ## P < 0.01 versus Con A group (n = 10 mice per group).
Dexmedetomidine protected against Con A-induced hepatitis by suppressing cytokines secretion

Con A-induced hepatitis is associated with the production of various cytokines. To determine whether DEX exerts its effect by modulating levels of cytokine, and to identify the mechanism by which DEX antagonists the effect of Con A on liver maintenance, we next measured a series of cytokines including TNF-\(\alpha\), IFN-\(\gamma\), IL-10, and IL-6 by ELISA. As shown in Fig. 5A-D, Con A challenge led to elevation of most of cytokines tested compared with NS and/or DEX injected group, including TNF-\(\alpha\), IFN-\(\gamma\), IL-6, and IL-10. Administration of DEX before Con A injection resulted in a significant suppression of inflammatory cytokines. These results suggested that DEX may retard immune response by restraining inflammatory cytokine secretion.

Dexmedetomidine suppressed phosphorylation of NF-\(\kappa\)B I\(\kappa\)B\(\alpha\) and p65

We found that Con A stimulated TNF-\(\alpha\), IFN-\(\gamma\), IL-10 and IL-6 secretion and DEX can block the activity. The production of inflammatory cytokines was partly regulated by the activation of transcription factors such as NF-\(\kappa\)B. To substantiate the hypothesis, we performed western-blot to determine the effect of DEX on NF-\(\kappa\)B activity. Our results indicated that DEX attenuated I\(\kappa\)B\(\alpha\) and p65 phosphorylation (Fig. 6).
Dexmedetomidine inhibited CXCL10 mRNA production

Previous studies indicated that CXCL10 expression is increased in experimental models of Con A-induced liver disease (Tu et al., 2011) and associated with the migration of T lymphocytes. In the present study, as shown in Fig. 7, the expression of CXCL10 mRNA in liver was enhanced significantly in the Con A group compared with the NS group, while it was down-regulated significantly by DEX pretreatment. Given to the tendency of CXCL10 and its function, we speculated that the shift of T lymphocytes to the liver may be modulated by CXCL10 partly.

DISCUSSION

Hepatotoxicity has been linked to hepatitis virus infection which is a major human health problem. Con A-induced hepatitis is a stable experimental model mirror-
The pathology of human autoimmune hepatitis (Shao et al., 2013). T cell-mediated hepatitis can be induced in mice by intravenous injection of Con A, which leads to polyclonal activation of T cells, resulting in clinical and histological patterns of acute hepatitis (Tiegs et al., 1992). After injection of Con A, the blood level of IFN-γ increased dramatically (Zhang et al., 2010), indicating that the CD4+ T helper (Th) cell was involved in liver injury. It is reported that CD4+ Th cells recognize the Con A modified major histocompatibility complex (MHC) structures of macrophages and become activated (Varthaman et al., 2010). In vivo administration of pretreatment DEX alleviated Con A-induced hepatitis as indicated by decreased transaminase levels and markedly attenuated inflammatory lesions in liver.

Among several pro-inflammatory cytokines involved, TNF-α and IFN-γ play a critical role in protecting mice from Con A-induced liver injury (Gantner et al., 1995; Kusters et al., 1996). Earlier reports demonstrated that cytokines TNF-α (Schumann et al., 2000) and IFN-γ (Sarra et al., 2013) were essential to trigger liver injury in Con A model, and we found that Con A stimulation triggers higher TNF-α and IFN-γ expression in serum. These variations of cytokine expression were in accordance with the observed changes in aminopherase activity and histopathology. In the current model, Con A maybe act directly on enhancing T cell function in the presence of Con A to affect cytokine secretion.

To elucidate the cellular source for the high level of circulating IFN-γ and TNF-α, previous studies demonstrated that CD4+ T cells were the main source of increased IFN-γ (Zhang et al., 2013; Kato et al., 2013) and TNF-α (Kato et al., 2013) after Con A challenge. Our study demonstrated that DEX pretreatment inhibited IκBα and p65

Fig. 5. Cytokine secretion measured by ELISA. A-D. DEX reduced serum cytokines secretion induced by Con A challenge. *P < 0.05, **P < 0.01 versus NS group, ###P < 0.01 versus Con A group. (n = 10 mice per group).
phosphorylation in Con A-induced hepatitis. We speculated that the attenuation of the severity of Con A-induced liver injury by DEX pretreatment is partly mediated by inhibiting phosphorylation of NF-κB.

Consistently, active T cells have been documented to be essential for Con A hepatitis. In the present study, the percent of activated T cells (CD3+CD25+) increased dramatically in the Con A group. CD4+ T cells are the predominant lymphocytes recruited to liver after intravenous administration of Con A. Accumulating evidence suggests that CD4+ T cells play an important role in Con A-induced hepatitis, and anti-CD4 monoclonal antibodies could prevent liver damage following Con A injection (Tiegs et al., 1992). T cells may contribute to liver damage by producing IFN-γ and TNF-α (Diao et al., 2004). Lymphocytes in liver can be classified into two groups, exogenous and endogenous (van der Meer et al., 2007). The endogenous lymphocytes are mainly T cells and lymphocyte infiltration in liver is mainly in the portal area (Kage, 2007). Our study demonstrated that CD4+ T cells increased in liver after Con A challenge, while DEX pretreatment was associated with a dramatic reduction in infiltrating CD4+ T cells. Furthermore, the percent of CD4+CD25+ T cells in the Con A group increased significantly. Cytotoxic CD8+ T lymphocyte (CTL) may also contribute to liver injury, but not as the major factor and the main mechanism of the Con A model (Wang et al., 2012). Although the counts of CD8+ T cells in infiltrating in liver didn’t increase after Con A administration, DEX down-regulated the percent of active CD8+ T cells in the present study. These findings suggested that pretreatment of DEX prevented the severity from Con A-induced liver damage by recruiting CD4+ T lymphocytes and activating the percent of T cells in the liver. This may result from induction of Th cells, as well as possible increased migration of T cells into the liver. Further studies were needed to investigate the tendency and effect of CTL in the model.

It should be noted that there were some limitations in our present study. Firstly, DEX was used in one dose in the model, which may ignore its dose-dependent effect in the model. Secondly, the mRNA expression of CXCL10 was measured in liver, while its protein level was not detected. Thirdly, cytokine secretion from Th1 and Th2 cells was detected in the study, but the effect of Th1 and Th2 cells, especially T regulatory cells, was not further explored in the study. Finally, the NF-κB pathway was a general molecule signaling in the immune inflammatory

Fig. 6. Effect of DEX pretreatment on NF-κB protein expression. DEX pretreatment combine with Con A administration attenuated NF-κB IκBα and p65 phosphorylation. **P < 0.01 versus NS group, #P < 0.05 versus Con A group. (n = 10 mice per group).

Fig. 7. CXCL10 mRNA expression measured by RT-PCR. DEX alleviated intrahepatic mRNA expression of CXCL10 induced by Con A challenge. *P < 0.05, **P < 0.01 versus NS group, ##P < 0.01 versus Con A group. (n = 10 mice per group).
response, which may not explain the effect exactly and directly. In other words, further studies were needed to verify these effects.

In conclusions, taken together, our data suggested that DEX inhibited pro-inflammatory cytokine secretion, and CD4+ T cell infiltration in liver, thereby preventing Con A-induced liver injury. The phosphorylation of NF-κB IkBα and p65 was inhibited by DEX pretreatment, which strongly suggests that NF-κB singling pathway was partly involved in the inflammatory response. In addition, the expression of CXCL10 mRNA, down-regulated by DEX, which suggests that CXCL10 might inhibit T cell infiltration in Con A-induced acute hepatitis. These findings raise the promising potential of developing novel pharmacological treatments for T-cell mediated liver diseases.

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Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES


