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Interleukin-17 mediates triptolide-induced liver injury in mice

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ABSTRACT

Triptolide (TP)-induced liver injury can be attributed to the Th17/Treg imbalance with the enhancement of the expansion of Th17 cells and suppression of the production of Tregs, especially the significant increase of interleukin (IL)-17 secreted by helper T (Th) 17 cells. To further investigate the involvement of IL-17-mediated immune response in the TP-induced hepatotoxicity, we examined the plasma transaminase, histopathological changes, hepatic frequencies of Th17 cells, hepatic expression of transcriptional factors and cytokines genes and plasma IL-17 levels after administration of TP (600 μg/kg) by oral gavage to female C57BL/6 mice. Mice treated with TP displayed acute liver injury with significantly increased hepatic frequencies of Th17 cells, mRNA expression of retinoid-related orphan receptor (ROR)-γt and plasma IL-17 level as well as the plasma ALT and AST. Neutralization study using anti-IL-17 antibody ameliorated TP-induced liver injury. In contrast, when challenged by coadministration of recombinant IL-17, hepatotoxicity was exacerbated in the triptolide-administered mice. In summary, this report was demonstrated for the first time that IL-17-mediated immune response is involved in the pathogenesis of TP-induced liver injury in mice, which may shed light on the mechanisms of TP-induced liver injury.

1. Introduction

Triptolide (diterpenoid triepoxide, TP) is a principal active component isolated from the traditional Chinese medicine *Tripterygium wilfordii* Hook F (TWHF), which exhibits notable immune-regulative effects (Chen, 2001). Although the ethyl acetate and methanol/chloroform extracts of TWHF have been used in the treatment of systemic lupus erythematosus, rheumatoid arthritis and nephritis (Liu et al., 2005; Zhang et al., 2010), TWHF and TP have been noted to induce severe adverse reactions, especially hepatotoxicity (Wang et al., 2013; Zhang et al., 2012). However, the mechanisms of TP-induced liver injury have not been fully clarified.

Helper T (Th) cell-mediated immune responses contribute to the pathogenesis of drug-induced liver injury (DILI) (Heneghan and McFarlane, 2002). The effect of Th cells in the liver is exerted through the production of cytokines, which target on the hepatocytes and immune cells by stimulating multiple signaling cascades (Oo and Adams, 2010). Th cells can be subdivided into Th1, Th2, regulatory T cells (Treg) and Th17 subsets by their unique transcription factors and characteristic secretion of cytokines (Table 1) (Kidd, 2003; Steinman, 2007). Thus, it is pivotal to understand the critical immune cells and cytokines that mediate DILI.

IL-17 and Th17 cells influence various liver injuries, such as DILI (Kobayashi et al., 2009), non-alcoholic fatty liver disease (Tang et al., 2011) and hepatocellular carcinoma (Kuang et al., 2010). It is well known that IL-17 plays important roles in activating chemotaxis and granulopoiesis through the stimulation of endothelial and epithelial cells to secrete macrophage inflammatory protein-2, granulocyte-colony stimulating factor and keratinocyte cytokine (Kolls and Linden, 2004). In addition, IL-17 acts mainly as a pro-inflammatory mediator activating and recruiting neutrophils into the liver (Ouyang et al., 2008). Activated neutrophils in the liver

Abbreviations: DILI, drug-induced liver injury; IL-17, interleukin-17; Th17 cells, T helper 17 cells; TP, triptolide; TWHF, *Tripterygium wilfordii* Hook F; Treg, regulatory T cells; T-bet, T-box expressed in T cells; GATA-3, GATA-binding domain-3; FoxP3, Forkhead box P3; ROR-γt, retinoid-related orphan nuclear receptor γt; ELISA, enzyme linked immunosorbent assay; ALT, alanine transaminase; AST, aspartate transaminase; H&E, hematoxylin and eosin; MPO, myeloperoxidase; MIP-2, macrophage inflammatory ptrotein-2; TNFα, tumor necrosis factor α; CXCL-1, C–X–C chemokine ligand-1.

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Table 1
Scheme for Th cells.

Th cells	Transcription factors	Cytokines	Function
Th1	T-bet	IFN-γ	Mediating cellular immunity to kill intracellular pathogens
Th2	GATA-3	IL-4	Driving humoral immunity against extracellular parasites
Treg	FoxP3	IL-10, TGF-β1	Immunosuppression and maintenance of immune tolerance
Th17	RORγt	IL-17	Protection against microbial challenges and the induction of autoimmune diseases

T-bet, T-box expressed in T cells; IFN-γ, interferon-γ; GATA-3, GATA-binding domain-3; IL-4, interleukin-4; FoxP3, Forkhead box P3; TGF-β1, transforming growth factor-β1; RORγt, retinoid orphan nuclear receptor γt.

have been reported to release protease that can directly cause liver injury and to act as effector cells through cytotoxicity leading to hepatocyte necrosis (Jaeschke et al., 1999).

Our previous investigations have reported that TP can break the hepatic Th17/Treg equilibrium in TP-induced liver injury and Th17/Treg ratios positively correlated with serum levels of ALT and AST (Wang et al., 2014). Moreover, the hepatic frequencies of Th17 cells, the hepatic expression of IL-17 as well as the hepatic IL-17 level increased considerably, which was an interesting phenomenon to be further investigated. Clarifying the mechanisms of TP-induced liver injury helps attenuation of toxicity and provides theoretical guidance for its structural transformation. Based on these considerations, we investigated involvement of IL-17-mediated immune response in TP-induced liver injury in C57BL/6 mice following exposure to TP (600 μg/kg). Firstly, the hepatic expressions of transcription factors specific for Th1, Th2, Treg and Th17 cells were analyzed after TP administration to investigate the involvement of Th cells in the TP-induced liver injury. Secondly, neutralization and administration of recombinant IL-17 were performed, and the plasma IL-17 levels were measured to investigate the IL-17 involvement. The results indicated the liver injury induced by TP was partly mediated by activation of Th17 cells and secretion of IL-17, which has not been reported before and may represent a novel pathogenesis of TP-induced liver injury. In addition, neutralization of IL-17 suppressed hepatotoxicity whereas exogenous administration of recombinant IL-17 exacerbated hepatotoxicity suggesting that effective regulating strategy can be developed to control the progression of TP-induced liver injury.

2. Materials and methods

2.1. Chemicals

TP (purity, >98%) was a gift from the Dermatological Disease Research Institute of the Chinese Academy of Medical Sciences (Nanjing, China). Human/mouse myeloperoxidase (MPO) antibody, monoclonal anti-mouse IL-17A antibody (monoclonal rat IgG2a Clone#50104), rat IgG2a isotype and recombinant mouse IL-17A were from R&D Systems (Abingdon, UK).

2.2. Animals groups and TP administrations

Female C57BL/6 mice, 6–8 weeks of age, were purchased from the Vital River Experimental Animal Technology, Co., Ltd. (Beijing, China). All of the mice were housed under pathogen-free conditions and were provided with mouse chow and water ad libitum. The animals were raised at a controlled temperature (22 °C ± 2 °C) and photoperiod (12 h of light and 12 h of dark). The animals were acclimated to the laboratory for 1 week before the experiments. The animal experiments were conducted in compliance with standard ethical guidelines and with the approval of the faculty ethical committee. TP was reconstituted in propylene glycol and stored at −20 °C. TP was freshly diluted to the appropriate concentrations with a 0.2% carboxymethylcellulose solution before use in the experiments. Female C57BL/6 mice were dosed by oral gavage with TP at a dose of 600 μg/kg per mouse, and the mice were sacrificed 6 h, 12 h, 24 h and 48 h after receiving the dose. The degree of liver injury was assessed by hematoxylin–eosin (H&E) staining, and the plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT) was analyzed using an automatic clinical analyzer (7080, HITACHI Ltd., Tokyo, Japan). The neutrophil infiltration was assessed by immunostaining for MPO.

2.3. Isolation of mononuclear cell and intracellular cytokine and transcription factor labeling

Mononuclear cells were isolated and labeled as previously described (Guebre-Xabier et al., 2000). For intracellular cytokine labeling, mononuclear cells were incubated with phorbol 1, 2-myristate 1, 3-acetate (PMA, 50 ng/mL; Sigma–Aldrich, St Louis, MO, USA), ionomycin (500 ng/mL; Sigma–Aldrich) and BFA (1 mg/mL; Sigma–Aldrich). Next, the cells were labeled with anti-mouse CD4 antibodies (Becton Dickinson, San Diego, CA, USA) before permeabilization with Cytoperm/Cytofix (Becton Dickinson) according to the manufacturer's instructions. After permeabilization, the cells were incubated with labeled antibodies which were specific to mouse IL-17A (Becton Dickinson). Next, the cells were centrifuged, and the pellets were washed to remove unbound antibodies. After surface and intracellular labeling, mononuclear cells were evaluated by flow cytometry (Calibrate; Becton Dickinson, Palo Alto, CA, USA) and the data were analyzed using CellQuest software (Becton Dickinson).

2.4. RNA extraction and real-time PCR

RNA was isolated from the liver sections with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster city, CA, USA) according to the manufacturer's instructions. Real-time PCR was performed in a 20 μL that contained 10 μL of 1 × SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA), 2 μL of cDNA, 6 μL of RNase/DNase-free water and 500 nM of each primer. The thermal cycler conditions included holds for 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C and 10 s at 60 °C. A melting curve analysis was performed for each reaction with a 65–95 °C ramp. The threshold cycle at which the fluorescent signal reached an arbitrarily set threshold near the middle of the log-linear phase of the amplification for each reaction was calculated, and the relative quantity of mRNA were determined. The mRNA levels were normalized against the mRNA levels of the housekeeping gene, GAPDH. The primer sequences for real-time PCR are shown in Table 2.

2.5. Administration of anti-mouse IL-17 antibody or recombinant mouse IL-17

Mice were administered anti-mouse IL-17A antibody by intraperitoneal injection (100 μg antibody in 0.5 mL of sterile PBS) at 9 h after TP administration. Rat IgG2a was intraperitoneally administered (100 μg of rat IgG2a in 0.5 mL of sterile PBS) as a control. Recombinant mouse IL-17A was administered by intraperitoneal injection (1 μg of recombinant IL-17A in 0.2 mL of sterile PBS containing 0.5% BSA) immediately after TP administration.

2.6. Detection of plasma IL-17 level by enzyme linked immunosorbent assay (ELISA)

The plasma IL-17 level was measured using enzyme linked immunosorbent assay (ELISA) (eBioscience, San Diego, CA, USA), according to the manufacturers' protocol. All of the assays were performed in triplicate.

2.7. Statistical analysis

The data were expressed as the mean ± standard deviation (SD). The groups were evaluated using student's *t*-test between two groups and a one-way analysis of variance (ANOVA) and Dunnett's *t*-test among groups that is more than two. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Time-dependent liver injury induced by TP in mice

The time-dependent hepatotoxicity of TP was investigated at a dose of 600 μg/kg (Fig. 1A and B). Plasma AST and ALT showed tendencies to increase at 6 and 12 h, peaked at 24 h after TP challenge,