Th17/Treg imbalance in triptolide-induced liver injury

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ABSTRACT

The study was designed to investigate the immune-modulatory effects of triptolide (TP) on CD4+ T cell responses during liver injury. Previous studies have suggested that TP plays a critical role in modulating both innate and adaptive immune reactions, but its effects on the Th17/Treg balance during TP-induced liver injury remain unknown. In this study, female C57BL/6 mice were administered by oral gavage with TP at a dose of 250 or 500 μg/kg per mouse. We examined the plasma biochemical parameters, histopathological changes, hepatic frequencies of Th17 cells and Treg cells, hepatic expression of transcriptional factors and cytokine genes and hepatic interleukin (IL)-17 and IL-10 levels in TP-administered mice. Mice treated with TP displayed liver injury with markedly increased plasma transaminase as well as hepatic mRNA expression of retinoid related orphan receptor (ROR)-γt and hepatic IL-17 level at 24 h. However, hepatic frequencies of Tregs and hepatic expression of forkhead/winged-helix family transcriptional repressor p3 (FoxP3) decreased at 24 h after TP administration. Furthermore, we found that elevated serum biochemical parameters positively correlated with the Th17/Treg ratios. Taken together, these results revealed a novel and interesting phenomenon of TP in the enhancement of the expansion of Th17 cells and suppression of the production of Tregs during liver injury, which may represent a new pathogenesis of TP-induced liver injury.

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1. Introduction

Triptolide (diterpenoid triepoxide, TP) is isolated from the extracts of the traditional Chinese medicine Tripterygium wilfordii Hook F (TWHF), which exhibits notable immune-regulative effects [1, 2]. TWHF has demonstrated a promising effect in the treatment of rheumatoid arthritis and systemic lupus erythematosus [3, 4]. Evidence also suggests that TP pretreatment effectively attenuated the hepatotoxicity induced by LPS/D-GalN or ischemia/reperfusion in mice by reducing oxidative stress, lipid peroxidation and NF-κB activation in the liver [5–7]. However, the salvage effect of TP was not obvious with post treatment [5]. Despite these benefits it provides, the side effects of TP cannot be ignored, such as gastrointestinal injury, antifertility and liver injury [8–10]. The accumulated proofs indicate that hepatotoxicity is one of the main adverse reactions of TP [8, 10–12]. Pharmacological or toxicological effects exhibited by TP mostly depend on the dosage. Researches have proved that the dose of TP for treatment is less than 200 μg/kg with no discernable adverse effects [5, 6, 13, 14]. The administration of high doses (300–1000 μg/kg) of TP has been shown to cause liver injury by chemical stress responses, lipid peroxidation...
and hepatocyte necrosis [8,12]. However, the mechanism of hepatic injury induced by TP is still unclear.

Drug-induced liver injury can be attributed to the innate immune response generated by lymphocytes [15]. The adaptive immune system can also be influenced by the innate immune response, which leads to liver damage. Helper T (Th) cells are an important regulator of acquired immunity. The action of Th cells in the liver is mediated through the release of a variety of cytokines, which target hepatocytes and immune cells by activating multiple signaling cascades [16]. Recently, studies have identified that CD4+ T cells can differentiate into four subgroups at different circumstances, including Th1, Th2, Treg and Th17 cells, which maintain the balance of immunological effect [17]. It is important to investigate which cells of the immune system are activated and recruited into the liver during drug-induced hepatotoxicity [18].

IL-17 produced by Th17 cells can induce abundant inflammatory cytokines and chemokines, and thus has a pivotal role in neutrophil infiltration and activation [19]. In addition, IL-17 has cytotoxicity and directly causes tissue injury [20,21]. IL-10 and TGF-β1 are mainly secreted by Tregs to exert immunosuppressive effects [22]. Th17 cells are closely related to Treg cells, as both arise from naïve CD4+ T cell precursors and share a requirement for TGF-β1 in their differentiation [23]. Accumulating evidence suggests that the expansion of Th17 cells influences various liver injuries, such as non-alcoholic fatty liver disease [24], hepatocellular carcinoma [25] and drug-induced liver injury [26,27]. Moreover, the deficit of hepatic Treg cells is directly correlated with high-fat diet-induced steatosis, which results in increased hepatic inflammation [28]. The Th17/Treg balance is so important for immune homeostasis that the disturbance of this equilibrium may lead to the liver injury [29]. The identification of Th17 cells and Treg cells has opened up a vast field of researches in the potential roles of these cells in liver diseases.

Studies have reported that TP can break the Th17/Treg equilibrium in animal models and a hepatic Th17/Treg imbalance could induce liver injury [29,30]. TP is a natural reactive electrophile containing three epoxide groups, which are usually linked to hepatotoxicity via their ability to covalently bind to a 90-kDa protein (XPB) [31,32]. Thus, the effect of TP on the activity of transcription factors, such as NF-κB and p53, at the transactivation step can be explained by the inhibition of XPB activity. NF-κB and p53 play important roles in the development and functional divergence of the Th17/Treg subsets [33,34], by which TP may exert effect on the Th17/Treg imbalance. Therefore, we hypothesize that the hepatotoxicity of TP is correlated with its effect on the breakdown of the Th17/Treg balance. We investigate the hepatic frequencies of Th17 cells and Treg cells as well as the hepatic expressions of retinoid orphan nuclear receptor γ (RORγt) and forkhead/winged-helix family transcriptional repressor p3 (FoxP3), which are transcriptional factors for Th17 and Treg cells respectively. In addition, the Th17-related cytokines IL-17 and Treg-related cytokines IL-10 were measured and hepatotoxicity of TP was evaluated by biochemical and histopathologic data. Furthermore, we also analyzed correlation between the levels of serum biochemical parameters and Th17/Treg ratios in TP-administered group to further investigate the Th17/Treg imbalance in TP-induced liver injury. These data would provide valuable information for further understanding of TP-induced hepatotoxicity.

2. Materials and methods

2.1. Chemicals and animals

TP (purity, >98%) was a gift from the Dermatological Disease Research Institute of the Chinese Academy of Medical Sciences (Nanjing, China). 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 maintained 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.

2.2. Animals groups and TP administrations

TP was reconstituted in propylene glycol and stored at −20 °C. TP was freshly diluted to the appropriate concentrations with a 0.5% carboxymethylcellulose solution before use in the experiments. Female C57BL/6 mice were administered by oral gavage with TP at a dose of 250 or 500 μg/kg per mouse.

2.3. Blood chemistry analysis

The blood was collected in tubes without an anticoagulant and was analyzed for alanine transaminase (ALT), aspartate transaminase (AST), γ-glutamyl transpeptidase (GGT), total bilirubin (TBIL) and cholesterol (CHO) levels using an automatic clinical analyzer (7080, HITACHI Ltd., Tokyo, Japan).

2.4. Histopathological evaluations

Sections from the livers were removed and fixed in 10% neutral-buffered formalin. For the histopathological examination, all of the fixed organs were processed for embedding in paraffin, sectioned, and stained with hematoxylin and eosin (H&E).

2.5. Mononuclear cell isolation and labeling

Mononuclear cells were isolated and labeled as previously described [35], following incubation 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 that were specific for either mouse IL-17 or FoxP3 (Becton Dickinson). Then, 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).
2.6. 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 hold 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 was determined. The mRNA levels were normalized against the mRNA levels of the housekeeping gene, β-actin. The primer sequences for real-time PCR are shown in Table 1.

2.7. Detection of cytokines by enzyme linked immunosorbent assay (ELISA)

Hepatic homogenates were used to determine the concentrations of IL-17 and IL-10 by ELISA (eBioscience, San Diego, CA, USA), according to the manufacturer’s protocol. All of the assays were performed in triplicate.

2.8. Statistical analysis

The data were expressed as the mean ± standard deviation (SD). The groups were evaluated using a one-way analysis of variance (ANOVA) and Dunnett’s t-test. Correlation between serum biochemical parameters and Th17/Treg ratios was evaluated by Pearson’s correlation analysis. P-values < 0.05 were considered to be statistically significant.

3. Results

3.1. Dose- and time-dependent hepatotoxic effects of TP in mice

TP was administered by oral gavage at a dose of 250 or 500 μg/kg to female C57BL/6 mice. A dose-dependent increase of ALT and AST was demonstrated at a dose of 500 μg/kg compared with vehicle-administered control mice (Fig. 1A and B), thus we adopted a dose of 500 μg/kg in the subsequent experiments. The time-dependent hepatotoxic effect of TP was investigated at a dose of 500 μg/kg (Table 2). Plasma biochemical parameters showed tendencies to increase at 6 and 12 h after the administration of TP, yet significant increases in the ALT, AST, GGT, TBIL and CHO levels were observed at 24 h after the administration of TP. Histopathological changes in livers at 24 h after TP administration (500 μg/kg) indicated focal necrosis with inflammatory cell infiltration in hepatocytes (Fig. 1C).

3.2. Time-dependent changes of frequencies of Th17 cells, Tregs and Th17/Treg ratios in the liver

In this study, we detected the changes of the percentages of Th17 cells and Tregs in the liver according to their surface marker (CD4), intracellular cytokine (IL-17) and transcription factor (FoxP3) using flow cytometry. Th17 cells were defined as CD4+IL-17+ T cells, and Tregs were defined as CD4+FoxP3+ T cells. Mice treated with TP had significantly higher frequencies of hepatic CD4+Th17 than the control group at 24 h, yet the percentage of hepatic Th17 cells didn’t change significantly at 6 h and 12 h (Fig. 2A and B). In addition, there was a decreased prevalence of hepatic Tregs at 24 h in the mice administered with TP compared with the control group, whereas the percentage of hepatic Tregs at 6 h and 12 h wasn’t affected by TP compared with the control (Fig. 2C and D). The significance of increased Th17 cells and decreased Treg cells was further explored by calculation of Th17/Treg percentage ratios (Fig. 2E). A sharp elevation of Th17/Treg ratios at 24 h compared with that of controls (P < 0.05). Moreover, there was no obvious bias of Th17/Treg ratios at 6 h and 12 h groups. Such results indicated that there was a Th17/Treg imbalance in TP-induced liver injury.

3.3. Time-dependent alterations of transcription factors and cytokine genes expression in TP-administered mouse liver

To investigate the involvement of immune-related factors in the TP-induced liver injury, the hepatic mRNA expression levels of transcriptional factors and cytokine genes were measured by real-time RT-PCR (Fig. 3). Previous studies have confirmed that the expression levels of mRNA and protein were similar in ILS [26,27]. The hepatic mRNA expression level of IL-17 increased in a time-dependent manner and was nearly 5-fold higher at 24 h after TP administration compared with that of control mice (Fig. 3A). The hepatic expression of RORγt, which is the master regulator of Th17 cells, increased significantly at 12 and 24 h after TP administration compared with the control mice (Fig. 3B). Hepatic mRNA expression levels of FoxP3, which is required for the development of Treg cells, showed tendencies to decrease at 6 h and decreased significantly at 12 h and 24 h in TP-administered mice (Fig. 3C).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>5'-GCTGTGCGTCCCTGTATGCTCT-3'</td>
<td>5'-CCCTCCGCTGTGGTGAAGG-3'</td>
</tr>
<tr>
<td>IL-17(A)</td>
<td>5'-TCAGGGTCTGGAGGACTGAG-3'</td>
<td>5'-GGCCGGAGCTTGAAACTT-3'</td>
</tr>
<tr>
<td>RORγt</td>
<td>5'-TCCACCCGCTGTTCTACCTG-3'</td>
<td>5'-AGTACCCCATGATCTGCT-3'</td>
</tr>
<tr>
<td>FoxP3</td>
<td>5'-CACCTATCCACCTTATGCG-3'</td>
<td>5'-CATGCGCTAAACCAATGTT-3'</td>
</tr>
</tbody>
</table>
3.4. Time-dependent changes in hepatic IL-17 and IL-10 levels

To find whether the changes in liver injury observed in TP-administered mice resulted from the increase of pro-inflammatory cytokines and decrease of anti-inflammatory cytokines, hepatic IL-17 and IL-10 levels were measured using ELISA. IL-17 produced by Th17 cells contributes to neutrophil infiltration and activation [21]. IL-10, which is mainly secreted by Treg, promotes the degradation of mRNA for the pro-inflammatory cytokines [22]. The hepatic IL-17 levels in TP-administered mice significantly increased at 24 h compared with the control mice without obvious changes at 6 h and 12 h (Fig. 4). In addition, the IL-10 levels decreased significantly at 24 h with minor changes at 6 h and 12 h.

Table 2
Serum parameter changes in the female C57BL/6 mice that were administered triptolide.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Time after triptolide (500 μg/kg) administration</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>49.80 ± 6.00</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>201.10 ± 12.86</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>3.53 ± 1.20</td>
</tr>
<tr>
<td>TBIL (μmol/L)</td>
<td>3.98 ± 1.13</td>
</tr>
<tr>
<td>CHO (mmol/L)</td>
<td>2.45 ± 0.16</td>
</tr>
</tbody>
</table>

Alanine transaminase (ALT), aspartate transaminase (AST), γ-glutamyl transpeptidase (GGT), total bilirubin (TBIL) and cholesterol (CHO) were measured in the serum using an automatic clinical analyzer (HITACHI). All of the values are the mean ± SD.

** P < 0.01 vs. control.
*** P < 0.005 vs. control.

Fig. 1. Dose-dependent changes of plasma ALT and AST and histopathological examination in the liver of triptolide-administered mice. Triptolide (TP; 250 or 500 μg/kg; o.p.) was administered to mice and plasma samples were collected at 24 h after the administration for the assessment of ALT (A) and AST (B). (C) Histopathological examination of the livers from TP-administered mice (500 μg/kg, at 24 h after the administration). The liver tissue sections were stained with hematoxylin and eosin (H&E, 20×). All of the values are the mean ± SD. * P < 0.05, ** P < 0.01, *** P < 0.005 vs. control.
3.5. Correlation of elevated serum chemistry parameters with increased Th17/Treg ratios in TP-administered mice

Further analysis revealed a positive correlation between elevated levels of serum biochemistry and increased Th17/Treg ratios at 24 h in TP-administered group by Pearson’s correlation analysis. As shown in Fig. 5, Th17/Treg ratios positively correlated with serum concentrations of ALT ($r = 0.8713, P < 0.01$) and AST ($r = 0.9089, P < 0.01$) in TP-administered 24 h group. The changes in Th17/Treg ratios correlated well with the serum biochemical parameters.

4. Discussion

Our previous researches have shown that mitochondrial respiratory chain inhibition and hepatocyte apoptosis are involved in TP-induced liver injury [8,10,12]. Emerging evidence suggests that the direct effects of drugs on liver cells may be an initiating event for immune response, which determines the extent of liver damage. Furthermore, it has been reported that focal necrosis with inflammatory cell infiltration in hepatocytes is involved in TP-induced liver injury [8,10,12] and glucocorticoid receptor agonist dexamethasone alleviated TP-induced liver injury in rats (unpublished data), suggesting that TP-induced hepatotoxicity may involve immune-mediated reactions. These lines of background prompted us to investigate whether immune-mediated factors play a role in TP-induced liver injury.

To investigate the involvement of immune-related factors in the TP-induced hepatotoxicity, we established the dosing condition for TP induced acute liver injury in C57BL/6 mice at first. An acute toxicity analysis demonstrated that the oral LD50 value for TP in mice was 1200 μg/kg [6]. In this study, we administered TP at a dose of 250 or 500 μg/kg, which was less than a half of the oral LD50 value. After investigating different conditions, we found that administration of TP 500 μg/kg caused acute liver injury at 24 h. The degree of liver injury was assessed by plasma biochemical parameters and H&E staining. Plasma ALT, AST, GGT, TBIL and CHO levels significantly increased with inflammatory cell infiltration at
24 h after TP administration (Fig. 1). However, a slight increase of plasma biochemical parameters was observed at 6 h and 12 h after the administration of TP.

In this study, we demonstrated a relationship between TP-induced liver injury and immune-related factors. At 24 h after the administration of TP (500 μg/kg) the hepatic frequencies of Th17 cells, the hepatic expression of ROR-γt and IL-17 as well as the hepatic IL-17 level significantly increased, whereas the hepatic frequencies of Treg cells, the hepatic expression of FoxP3 as well as the hepatic IL-10 level significantly decreased (Figs. 2, 3 and 4). ROR-γt is a critical transcription factor for Th17 cells. IL-17 is mainly secreted by Th17 cells [19]. FoxP3 is indispensable for Treg differentiation. IL-10 is produced by Treg principally to exert anti-inflammatory effects [22]. The balance between Th17 and Treg subsets has been implicated in the regulation of many immune responses and is known to be impaired in liver diseases [36]. Moreover, Th17/Treg ratios positively correlated with serum level of ALT and AST, which further indicated the implication of Th17/Treg imbalance in TP-induced liver injury. As a result, a novel hypothesis has been proposed that the Th17/Treg imbalance may be involved in the pathogenic mechanism of TP-induced liver injury and may be considered as a characteristic feature.

As for the low frequencies of Treg cells in TP-induced liver injury, one of the possible explanations is that hepatic cytokine environment of acute liver injury state is not in favor of the differentiation of Treg cells. Cytokine in liver tissue may provide suitable environment for the differentiation of Th17 cells but not suitable for Treg cells. Studies have reported that the administration of TP at a low dose (100–250 μg/kg) protected mice from ischemia/reperfusion injury by inhibition of NF-κB activation [6, 7]; however, the administration of high doses (300–1000 μg/kg) of TP has been shown to cause liver injury by chemical stress responses and unknown mechanisms [8, 12]. Our results indicated for the first time that there would be Th17/Treg imbalance in TP-induced liver injury, which is an interesting phenomenon to be further investigated.

In summary, Th17/Treg imbalance can be associated with the immune tolerance breakthrough and exacerbation of liver inflammation in TP-induced liver injury. The role of Th17/Treg imbalance in TP-induced liver injury suggests that effective regulating strategy can be developed to control the progression.

Fig. 4. Triptolide administration induces changes of IL-17 and IL-10 secretion in the liver. The mice were sacrificed at 6 h, 12 h and 24 h after the administration of triptolide (TP, 500 μg/kg). The concentrations of hepatic cytokines were determined using ELISA. (A) IL-17 levels. (B) IL-10 levels. All of the values are the mean ± SD. ∗P < 0.05, ∗∗P < 0.01, ∗∗∗P < 0.005 vs. control.

Fig. 5. Correlation of elevated serum chemistry with increased Th17/Treg ratios in triptolide-administered mice. Further analysis revealed a positive correlation between elevated levels of serum chemistry parameter and increased Th17/Treg ratios at 24 h in triptolide (TP)-administered group by Pearson’s correlation analysis. (A) Correlation between serum ALT and hepatic Th17/Treg ratios (r = 0.8713, P < 0.01), (B) Correlation between serum AST and hepatic Th17/Treg ratios (r = 0.9089, P < 0.01). ∗P < 0.05, ∗∗P < 0.01, ∗∗∗P < 0.005 vs. control.
of TP-induced liver injury, which makes Th17/Treg imbalance a potential target for future prediction or prevention of hepatotoxicity. Though the detailed investigations on mechanisms need to be carried out in future study, these findings may shed light on the mechanisms of TP-induced liver injury.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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