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A new perspective of triptolide-associated hepatotoxicity: Liver hypersensitivity upon LPS stimulation

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Abstract

Objective: This study was designed to investigate whether the mice treated with triptolide (TP) could disrupt the liver immune homeostasis, resulting in the inability of the liver to eliminate the harmful response induced by lipopolysaccharide (LPS). In addition, we explored whether apoptosis and necroptosis played a critical role in the progression of the hepatotoxicity induced by TP-LPS co-treatment.

Methods: Female C57BL/6 mice were continuously administrated with two different doses of TP (250μg/kg and 500μg/kg) intragastrically for 7 days. Subsequently, a single dose of LPS (0.1mg/kg) was injected intraperitoneally to testify whether the liver possesses the normal immune function to detoxicate the exogenous pathogen’s stimulation. To prove the involvement of apoptosis and necroptosis in the liver damage induced by TP-LPS co-treatment, apoptosis inhibitor Z-VAD-FMK (FMK) and necroptosis inhibitor necrostatin (Nec-1) were applied before the stimulation of LPS to diminish the apoptosis and necroptosis respectively.

Results: TP or LPS alone did not induce significant liver damage. However, compared with TP or LPS treated mice, TP-LPS co-treatment mice showed obvious hepatotoxicity with a remarkable elevation of serum ALT and AST accompanied by abnormal bile acid metabolism, a depletion of liver glycogen storage, aberrant glucose metabolism, an up-regulation of inflammatory cell infiltration, and an increase of apoptosis and necroptosis. Intraperitoneal injection of FMK or Nec-1 could counteract the toxic reactions induced by TP-LPS co-treatment.

Conclusion: TP could disrupt the immune response, resulting in hypersensitivity of
the liver upon LPS stimulation, ultimately leading to abnormal liver function and cell death. Additionally, apoptosis and necroptosis played a vital role in the development of liver damage induced by TP-LPS co-treatment.

**Abbreviations**

TP, triptolide; LPS, lipopolysaccharide; RIPK1, receptor interacting serine/threonine kinase 1; MLKL, mixed lineage kinase domain like pseudokinase; FMK, Z-VAD-FMK; Nec-1, necrostatin-1; FXR, farnesoid X receptor; SHP, small heterodimer partner; BSEP, bile salt export pump; MRP2, multidrug resistance-associated protein 2; NTCP, Na\(^+\)-taurocholate cotransporting polypeptide; OATP1B, organic anion-transporting polypeptides 1B; PEPCK, phosphoenolpyruvate carboxykinase; cFlip, cellular FLICE-inhibitory protein; cIAP, cellular inhibitor of apoptosis protein; GLUT2, glucose transporter type 2; G6Pase, glucose-6-phosphatase; PARP, poly(ADP-ribose) polymerase.

**Keywords:** triptolide; LPS; hepatotoxicity; apoptosis; necroptosis

**1. Introduction**

The Chinese medicinal herb Tripterygium wilfordii Hook F. (TWHF) possesses numerous bioactivities and is widely used in China for centuries. Tripterygium glycosides tablets derived from TWHF has long been used for the treatment of autoimmune disease in clinical practice (Xi et al. 2017). Despite exhibiting a variety
of bioactivities, narrow therapeutic window and the high rate of adverse reactions of tripterysium glycosides limit its extensive application. According to the data from National Center for Adverse Drug Reaction Monitoring, China, treatment with tripterysium glycosides could increase the risk of multi-organ toxicity, such as liver, kidney, spleen, heart and gastrointestinal tract (Li et al. 2014b). Triptolide (TP), a major active and toxic component of tripterysium glycosides, has attracted numerous scholar’s attention because of its various therapeutic bioactivities (Li et al. 2015). Among other toxic effects, hepatotoxicity of TP needs serious attention.

With regard to TP-induced hepatotoxicity, several hypotheses have been proposed. Previous study indicated that oxidative stress, mitochondrial impairment, disruption of lipid and glucose metabolism, inflammation induced by Th17/Treg imbalance, and the inhibition of Sirt1/FXR pathways all are tightly associated with TP-induced hepatotoxicity (Jiang et al. 2016; Li et al. 2014a; Wang et al. 2013; Wang et al. 2014b; Yang et al. 2017a; Yao et al. 2008). Previously, the mechanisms involved in TP-induced hepatotoxicity are widely studied and these mechanisms are mainly focusing on the direct liver injury induced by TP. However, there is no existing research related to the indirect toxicity of TP.

Liver is one of the major organs in the body and is known for its vital role in metabolic homeostasis and immune regulation. As a metabolic organ, liver parenchymal cells (also known as hepatocytes) are responsible for glucose, lipid, and bile acid metabolism, taking the nutrients from portal vein to meet the basic energy needs of brain, muscle and other organs (Gong et al. 2017; Li et al. 2017; Petersen et
Meanwhile, the liver functions to metabolize drugs and toxins derived from plant, animals or fungi to avoid the cell damage induced by the exogenous substance (Blann 2014). Liver is also a central organ for immune system, containing a variety of immune cells, including Kupffer cells, Nature Killer cells, Nature Killer T cells, Dendritic cells and Hepatic Sinusoidal Endothelial Cells (Thomson and Knolle 2010). The blood derived from gut contains numerous bacterial products, environmental toxins, and food antigens, exposing the liver to sustained stimulation from pathogens and stimulates, such as lipopolysaccharide (LPS). At the same time, the liver relies on the appropriate phenotypic response generated by immune cells and hepatocytes, such as the recruitment of phagocytes through the production of inflammatory factors and the activation of transcriptional or proteolytic pathways, to destroy foreign pathogens and defend against the harmful stimulators without launching detrimental immune response (Gao et al. 2008). In contrast, the inability of the liver to clear the pathogens could result in systemic infections, autoimmune disease, tissue damage, and even cell death (Ebrahimkhani et al. 2014; Filliol et al. 2016; Medzhitov 2008).

As the major outer membrane component of gram-negative bacteria located in the digestive tract, LPS participates in the development of diverse diseases. Under physiological conditions, the low quantity of bacterial LPS reaches in the liver, binding to the innate immune receptors on the surface of the immune cells, promoting the release of the inflammatory mediators to attract the phagocytes and finally efficiently cleared by phagocytic hepatic cells without launching the sustained inflammatory reactions and harmful response (Medzhitov 2008). However, excessive
bacterial LPS translocation from the gastrointestinal tract into liver, the recruitment of massive immune cells, or the inability of liver to remove the LPS-induced excessive production of cytokines, could result in the progression of nonalcoholic steatohepatitis, cirrhosis, and hepatocellular carcinoma (Carnevale et al. 2017; Imajo et al. 2012; Roderburg and Luedde 2014). Recently, several groups revealed that the abnormality in the downstream of tumor necrosis factor-α (TNF-α) pathway, such as defective NF-κB mediated transcriptional activity or deficient receptor interacting serine/threonine kinase 1 (RIPK1) mediated scaffold function in hepatocytes, could result in cell death accompanied by severe liver damage upon LPS stimulation, expending our understanding of the immune system in protecting the cell from LPS induced cell death (Annibaldi and Meier 2018; Filliol et al. 2017; Geisler et al. 2007; Peltzer et al. 2016; Ting and Bertrand 2016). Nowadays, LPS was also widely used to testify the function of the certain protein related to the immune system (Filliol et al. 2017; Geisler et al. 2007). Considering the wide distribution of LPS and the function of immune system in protecting the mice from LPS stimulation without launching the harmful response, a low dose of LPS was selected as the stimulant to testify the effects of TP on liver immune homeostasis (Crispe 2009; Medzhitov 2008).

Apoptosis (especially external apoptosis) and necroptosis are the two types of cell death pathways activated by the stimulation of TNF-α, a cytokine produced by LPS. As a caspase-dependent cell death, a variety of liver diseases are accompanied by the occurrence of hepatocytes apoptosis (Luedde et al. 2014). Recently, a new type of cell death called “programmed necrosis” or necroptosis was proposed to involve in the
process of cholestasis, hepatic ischemia-reperfusion and ethanol-induced liver injury (Afonso et al. 2016; Hong et al. 2016; Roychowdhury et al. 2013). Although the binding of TNF-α to TNF-R1 could initiate the upstream signals of apoptosis and necroptosis, the pro-survival proteins, such as cFlip, cIAP1, and cIAP2, induced by TNF-α could counteract the pro-death signals, which ultimately leads to cell survival but not cell death in most cases (Chan et al. 2015).

In this study, we aimed to investigate whether the disruption of liver immune homeostasis was involved in TP-induced hepatotoxicity. LPS, a stimulant which was used to mimic the exogenous stimulation that liver was continuously exposed to, was chosen to examine the ability of the liver to neutralize the harmful response of TP-treated mice. We also explored several aspects, such as bile acid metabolism, glucose metabolism, and hepatic glycogen preservation to reflect normal liver function after TP-LPS co-treatment. Besides, pharmaceutical inhibitors were applied to confirm whether apoptosis and necroptosis played the crucial role in the pathogenesis of TP-LPS induced acute liver damage.

2. Material and methods

2.1 Material

Triptolide (>98%, HPLC) was purchased from Sanling Biotech (Guilin, China). LPS (L2755) was purchased from Sigma-Aldrich (MO, USA). Z-VAD-FMK (A1902) and Nec-1 (A4213) were purchased from Apexbio (HOU, USA). Trizol reagent, SYBR Green Master Mix and Reverse Transcription Kit for Q-PCR were purchased from Vazyme Biological Technology (Nanjing, China). Primers used for Q-PCR were
purchased from Invitrogen life Science (CA, USA).

Antibody against Cleaved Caspase-3 (9661), Cleaved Caspase-8 (8592), Cleaved PARP (9532), Cleaved Caspase-9 (9508), Bcl-2 (2870), Bax (2772) were purchased from Cell Signaling Technology (MA, USA). Antibody against FXR (bs-12867R) was purchased from Bioss (MA, USA). Antibody against GAPDH (sc-365062) was purchased from Santa Cruz Biotechnology (CA, USA). Antibody against RIPK1 (17519-1-AP), MLKL (66675-1-Ig), MPO (22225-1-AP) were purchased from Proteintech (Wuhan, China). Antibody against P-MLKL (Ser345) (ab196436) was purchased from Abcam (CA, USA).

2.2 Animals and pharmacological treatments

C57BL/6N mice (Female, 18-20g, 6-8 weeks of age), purchased from the Vital River Laboratory Animal Technology (Beijing, China), were selected in this study. Mice were given free access to food and water. All experiments and procedures involving mice were compliant with the guideline from Ethical Committee of China Pharmaceutical University and the Laboratory Animal Management Committee of Jiangsu Province (Approval No.2110748). The doses and route of administration of TP, LPS, FMK, and Nec-1 were chosen according to the published articles and our pre-experiment data (Gujral et al. 2004; Imajo et al. 2012; Ma et al. 2015; Wang et al. 2016b). After the administration of TP (intragastric administration), LPS (intraperitoneal injection), Nec-1 (intraperitoneal injection), FMK (intraperitoneal injection) or the same volume of solvent (0.5%Cmc-Na, PBS, or 2.5%DMSO) according to the body weight (10mL/kg) respectively, serum and the liver samples
were collected at the indicated time point for further experiments.

To investigate whether TP pre-treatment could increase the sensitivity of the liver to the stimulation of LPS, a total of 72 mice were randomly divided into 6 groups (n=12 per group) (Fig.1 to Fig.5). Each mouse was administrated with TP (250μg/kg or 500μg/kg) or the same volume of 0.5% Cmc-Na once a day for continuous 7 days followed by a single dose of LPS (0.1mg/kg, dissolved in PBS) or diluted PBS 2 hours after the last dose of TP on day 7. Mice were sacrificed 8 hours after LPS injection. The diagrammatic experimental procedure of this part was shown in Fig.1A.

To testify whether the activation of apoptosis and necroptosis participated in the hepatotoxicity induced by TP-LPS co-treatment, another 72 mice were randomly divided into 6 groups (n=12 per group) (Fig.6 to Fig.7). Mice were administrated with 500μg/kg of TP or the same volume of 0.5% Cmc-Na once a day for 7 days; PBS or LPS (0.1mg/kg) was given 2 hours after the last dose of TP on day 7 while FMK (10mg/kg) and Nec-1 (1.65mg/kg) or diluted 2.5%DMSO were given 15 minutes before LPS injection respectively. Mice were sacrificed 8 hours after LPS administration. The diagrammatic experimental procedure of this part was shown in Fig.6A.

2.3 Blood chemical analysis

Serum Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and Alkaline Phosphatase (ALP) were detected using kits from Weiteman Biotech (Nanjing, China). Serum Total Bile Acid (TBA) was detected using the kit from Jiancheng Bioengineering Institute (Nanjing, China). Serum Glucose was detected
using the kit from Rongsheng Biotech (Shanghai, China). All kits were used according to the manufacturer’s instructions.

2.4 Histopathological evaluations

Liver tissues were fixed in 10% formaldehyde and embedded in paraffin for hematoxylin and eosin (H&E), immunohistochemistry (IHC) and Periodic Acid-Schiff (PAS) staining. H&E staining, immunolocalization of Cleaved Caspase-3, MLKL and MPO, PAS were carried out to investigate the liver morphological changes, apoptosis, necroptosis, the recruitment of neutrophils, and hepatic glycogen preservation respectively.

2.5 RNA extraction and real-time quantitative PCR

Total RNA was extracted from mice liver using TRIzol reagent. After the quantitation of RNA concentration with Nanodrop 2000 (Thermo, DE, USA), the same amount of RNA was reversed to cDNA. Q-PCR was performed using SYBR Green for target genes with specific primers on Stepone Plus (Thermo, DE, USA). The primers used in this article were listed on table 1 and GAPDH was used for normalizing the quantity of cDNA.

Table1. The primer sequences used for real-time quantitative PCR assay in mice

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>CTTTGGCATTTGTGGAAGGGCTC</td>
<td>GCAGGGATGATGTTCTGGGCAG</td>
</tr>
<tr>
<td>IL-1β</td>
<td>TGGACCTTCCAGGATGAGGACA</td>
<td>GTTCATCTCCGGAGCCTGTAGTG</td>
</tr>
<tr>
<td>IL-6</td>
<td>TACCACTTCACAAGTGGGGGGGC</td>
<td>CTGCAAGTGCATCACTCGTGTAGT</td>
</tr>
<tr>
<td>TNF-α</td>
<td>GGTGCCTATGTCTCAGCCTCTT</td>
<td>GCCATAGAACTGATGAGGAGGAG</td>
</tr>
<tr>
<td>Gene</td>
<td>5' Region</td>
<td>3' Region</td>
</tr>
<tr>
<td>-------</td>
<td>-------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>MPO</td>
<td>CGTGTCAAGTGCTGCTGCTAT</td>
<td>AACCAGCGTACAAAGGCACGCT</td>
</tr>
<tr>
<td>MCP-1</td>
<td>GCTACAAGAGGATCACACCAGCAG</td>
<td>GTCTGGACCCATTCTTCTTGG</td>
</tr>
<tr>
<td>FXR</td>
<td>GTTGTGATGTGCTACAAAGCTG</td>
<td>CGTGGTGATGGTTGAATGTCC</td>
</tr>
<tr>
<td>SHP</td>
<td>CCCCTATCTTCAGTACACATGGG</td>
<td>GACCATAAGGAGGACAAAGGTCT</td>
</tr>
<tr>
<td>BSEP</td>
<td>AGCAGGGCTCAGCTGATGAC</td>
<td>AATGGCCCAGCAATAGCAA</td>
</tr>
<tr>
<td>MRP2</td>
<td>AACTGCTCTCTCAGAATCTTA</td>
<td>GCCAGCCACGGAACAGCTGCT</td>
</tr>
<tr>
<td>OATP1B</td>
<td>GGGAAACATGCTCCTGGGATA</td>
<td>GGAGTTATGCGGACACTTCTC</td>
</tr>
<tr>
<td>NTCP</td>
<td>GCATGATGCCCCTCCTTTATAC</td>
<td>TACATAGTGGGCTTTTGAGCT</td>
</tr>
<tr>
<td>PEPCK</td>
<td>GGCAGTGACATTGGCTGGATGA</td>
<td>TGTCTTCAGTGAGGGAGCAGGA</td>
</tr>
<tr>
<td>G6Pase</td>
<td>AGGTTCGTGCTGAGGTGGTCTTC</td>
<td>GTAGCAGGTAATCAAGCGC</td>
</tr>
<tr>
<td>GLUT2</td>
<td>GTTGGAGAGAGGTAGTCAGGGA</td>
<td>ATCCAGGAGACCTTCTGCTAG</td>
</tr>
</tbody>
</table>

### 2.6 Western blot analysis

Total protein was extracted from mice liver using RIPA buffer from Beyotime Biotechnology (Shanghai, China). After quantification of the concentration of protein using the BCA protein assay kit, the protein was then mixed with 4× loading buffer from Bio-Rad laboratories (CA, USA). Proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. After blocking with 5% bovine serum albumin for one hour, the membranes were then incubated with primary antibody overnight at 4°C. The immunoblots were visualized with horseradish peroxidase-conjugated polyclonal secondary antibody using an ECL detection kit from Millipore (MA, USA).

### 2.7 Statistical analysis
The data in all experiments were expressed as means ± SEM and analyzed using GraphPad Prism 6. One-way analysis of variance (ANOVA) and two-way ANOVA followed by Tukey's Multiple Comparison Test were performed to analyze the differences between groups. P-values < 0.05 were considered to be statistically significant.

3. Results

3.1 Mice treated with TP sensitize the liver to LPS stimulation

To test whether TP could disrupt the liver immune homeostasis resulting in the inability of the liver to detoxicate the stimulation of LPS, mice were treated with two doses of TP (250μg/kg or 500μg/kg) continuously for 7 days followed by a single dose of LPS (0.1mg/kg) (Fig.1A). As shown in Fig.1B and 1C, TP only slightly increased serum ALT and AST at high dose (without the statistical difference), while low dose of TP and LPS had no impact on those two transaminases. However, TP and LPS co-treatment significantly increased the serum ALT and AST at TP high dose (Fig.1B and 1C). Histopathologic assessment revealed the administration of either LPS or TP alone had no or minor effect on normal liver histology, while TP-LPS co-treatment induced obvious apoptosis with massive fragmentation of cell nucleus and condensation of cytoplasm (shown by black arrows), necrosis, and inflammatory infiltration (Fig.1G). In addition, neither TP nor LPS affected the serum TBA, while TP-LPS co-treatment increased the serum TBA at TP high dose (Fig.1D). However, serum ALP remained unchanged (Fig.1E), indicating that the metabolic disorder of bile acid might not be related to the damage of bile duct. Moreover, sole treatment
with high dose of TP or LPS decreased the serum glucose; nonetheless, TP-LPS co-treatment further aggravated this reduction (Fig.1F). In this part, we proved that TP pre-treatment could decrease the ability of the liver to eliminate the stimulation of LPS, resulting in severe hepatotoxicity which could not be caused by TP or LPS alone.

3.2 Increased inflammatory infiltration of liver in mice treated with TP after the stimulation of LPS

To further prove the inflammatory infiltration induced by TP-LPS co-treatment, the expression of several key pro-inflammatory factors were determined using Q-PCR. As shown in Fig.2A to Fig.2E, TP treatment had little effect on the mRNA levels of IL-1β, IL-6, TNF-α, MCP-1, and MPO. Although LPS increased the expression of IL-1β, TNF-α, and IL-6, TP-LPS more significantly induce the transcription of those pro-inflammation factors, especially at high dose of TP (Fig.2A to Fig.2C). Compared with control group, LPS treatment could slightly up-regulate the expression of MPO (1.164 ± 0.263 to 2.875 ± 0.819) and dramatically increase the expression of MCP-1 (1.236 ± 0.417 to 51.633 ± 11.849), which could be additionally up-regulated by TP-LPS co-treatment (Fig.2D and Fig.2E). The inflammatory infiltration was also assessed by IHC staining of MPO, which could serve as an indicator of neutrophil infiltration. LPS-treated group slightly recruited the neutrophil, which was beneficial for the host to eliminate the LPS. However, compared with the LPS-treated group, much more neutrophil infiltration was observed on TP-LPS co-treatment group. An increase in the recruitment of neutrophils attracted more inflammatory cells and
worsened the liver injury (Fig. 2F, shown as black arrows). In this part, we confirmed that TP-untreated mice responded positively to the stimulation of a low dose of LPS, while TP-treated mice could lead to an intense inflammatory reaction after the stimulation of LPS.

3.3 Disruption of bile acid homeostasis in mice after TP-LPS co-treatment

The ability of the liver to synthesize and secrete bile acid represents its normal function (Boyer 2013). The impairment of bile acid secretion occurs in several liver diseases, including cholestasis and cholangitis (Jahn et al. 2018; Vaz and Ferdinandusse 2017). As the serum TBA level was significantly elevated following the administration of TP-LPS, we analyzed the expression of the key pathway and related transporters involved in bile acid metabolism. Among numerous transcriptional programs controlling the hepatic bile acid metabolic pathways, the nuclear receptors FXR and SHP axis plays an essential role (Fiorucci et al. 2007). As shown in Fig. 3A and Fig. 3B, LPS alone had no effect on the mRNA levels of FXR and SHP, while TP high dose could inhibit the expression of FXR and SHP. After the stimulation of LPS, the inhibition of FXR and SHP by TP was further exacerbated. The protein level of FXR was confirmed by western blot (Fig. 3G and Fig. 3H). The decreased expression of FXR and SHP could trigger overproduction of bile acid due to the loss of control of the bile acid synthesis pathway (Chiang et al. 2000). Bile acid transporters are tightly regulated via nuclear receptors to maintain proper bile acid homeostasis. NTCP and OATP1B function to uptake bile acid from portal blood to the hepatocytes. Meanwhile, at the canalicular membrane, bile acid was excreted by
hepatocytes to avoid the accumulation of toxic acid bile in the hepatocytes via bile acid efflux transporters, such as BSEP and MRP2. As shown in Fig.3C to Fig.3F, although LPS alone decreased the expression of BSEP, MRP2, OATP1B, and NTCP, it didn’t increase the serum TBA. Additionally TP treatment had little effect on the MRP2 and BSEP, while in addition inhibited the expression of OATP1B and NTCP (Fig.3C to Fig.3F), indicating the elevation of serum TBA might be accounted for the decreased intake of bile acid from blood to hepatocytes. Overall, TP-LPS co-treatment could affect bile acid metabolism, leading to an increase of serum TBA. The inhibition of FXR/SHP axis and decreased absorption of bile acid from sinusoidal blood to hepatocytes might be the reasons for the high levels of TBA in serum.

3.4 Disruption of glucose homeostasis in mice after TP-LPS co-treatment

Through the regulation of de novo glucose production and glycogen breakdown, the liver is able to maintain the blood glucose concentration within a relatively narrow range to meet the energy needs of other organs. LPS alone could result in hypoglycemia, which was relied on the inhibition of Phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), two key enzymes involved in the liver gluconeogenesis (Raetzsch et al. 2009; Tanaka et al. 2017) (Fig.1F, Fig.4A, and Fig.4B). The inhibition of PEPCK and G6Pase were further deteriorated by TP-LPS co-treatment (Fig.4A and Fig.4B). We supposed that the hypoglycemia induced by TP-LPS co-treatment could be, in some part, a result of the inhibition of gluconeogenesis. Meanwhile, during fasting state, the breakdown of liver glycogen contributes to hepatic glucose production which is crucial for the
maintenance of blood glucose levels (Ellingwood and Cheng 2018). Although LPS alone decreased the serum glucose level, it didn’t seem to affect the glycogen storage in hepatocytes (Fig.1F and Fig.4D). Also, the glycogen storage in the liver didn’t show visible change after TP-treatment. However, TP-LPS co-treatment, especially at TP high dose, almost depleted the whole glycogen storage in the liver (Fig.4D), which could reduce the glucose supply of the whole body under fasting state. The decrease of GLUT2 in TP-LPS co-treatment group also implied the reduction in the transfer rate of glucose between hepatocytes and blood (Fig.4C). This indicates that TP-LPS co-treatment could interrupt glucose metabolism, reflecting as the inhibition of gluconeogenesis and depleted the glycogen storage in hepatocytes.

3.5 Activation of intrinsic apoptosis and necroptosis in mice treated with TP after the stimulation of LPS

LPS, together with pro-inflammation cytokine TNF-α induced by LPS, coordinates tissue homeostasis by controlling the balance between cell survival and cell death. However, the loss of control of the checkpoints in TNF-α pathways can lead the cell to death on the stimulation of TNF-α (Annibaldi and Meier 2018). Apoptosis and necroptosis are two outcomes of the LPS/TNF-α induced cell death (Ting and Bertrand 2016). H&E staining in Fig.1G showed cell death is involved in the hepatotoxicity induced by TP-LPS co-treatment. To further prove this, we examined the apoptosis and necroptosis markers using western blot analysis. TP or LPS alone had a weak effect on the apoptosis and necroptosis markers. However, compared with TP or LPS treated group, TP-LPS co-treatment increased the protein
levels of Cleaved Caspase-3 and Cleaved PARP, two markers of apoptosis, which was further confirmed by Cleaved Caspase-3 IHC staining, revealing the activation of apoptosis (Fig.5A, Fig.5C, Fig.5E and Fig.5L, shown by black arrows). To our surprise, the levels of intrinsic apoptosis-related protein Bcl-2, Bax, and Cleaved Caspase-9 remained unchanged while extrinsic apoptosis-related protein Cleaved Caspase-8 remarkably increased, indicating the activation of extrinsic apoptotic pathway (Fig.5A, Fig.5D, and Fig.5F to Fig.5H). Apart from the caspase-dependent apoptosis, necroptosis is the caspase-independent but receptor interacting serine/threonine kinase 1 (RIPK1) and receptor interacting serine/threonine kinase 3 (RIPK3) dependent cell death. RIPK1 can associate with RIPK3 through receptor interacting protein homotypic interaction motifs to form a necrosome and further phosphorylate mixed lineage kinase domain like pseudokinase (MLKL), which subsequently translocates to the cell membrane, contributing to its rupture and the release of cellular contents (Hildebrand et al. 2014; Wang et al. 2014a). We also detected the level of necroptosis-related protein in the liver, finding that TP-LPS co-treatment significantly up-regulated the protein levels of MLKL, P-MLKL (Ser345) and RIPK1 (Fig.5B, Fig.5I to Fig.5K). The expression of MLKL was also confirmed by IHC staining (Fig.5L, shown by black arrows). Those results indicated that TP-LPS co-treatment activated the necroptosis, which had not been induced by TP or LPS alone.

Taken together, TP-LPS co-treatment resulted in severe liver damage, accompanied by internal apoptosis, necroptosis, inflammatory reactions, bile acid and
glucose metabolism disorders. However, we need to confirm that is there any connection between cell death, inflammatory reactions and metabolic disorders?

3.6 Pretreatment with FMK or Nec-1 protect mice from TP-LPS induced hepatotoxicity

To confirm whether the inhibition of apoptosis and necroptosis could relieve the liver injury induced by TP-LPS, we selected the pan-caspase inhibitor FMK and RIP1 kinase inhibitor Nec-1 to alleviate apoptosis and necroptosis respectively. The experimental scheme is shown in Fig.6A. As expected, FMK or Nec-1 pre-treatment diminished the protein levels of Cleaved Caspase-3, Cleaved PARP, RIPK1, P-MLKL (Ser345), MLKL induced by TP-LPS co-treatment, excluding the off-target effects of those two inhibitors (Fig.6F to Fig.6L). In addition, FMK and Nec-1 could hamper the increase of serum ALT, AST induced by TP-LPS (Fig.6B and Fig.6C). H&E staining revealed that FMK and Nec-1 pre-treatment also decreased the morphological changes induced by TP-LPS co-treatment, implying the protective effects of FMK and Nec-1 (Fig.7, shown as black arrows in H&E staining). Hence, we proved that the activation of apoptosis and necroptosis could aggravate the hepatotoxicity induced by TP-LPS co-treatment.

We also detected the relative index of bile acid, glucose metabolism, and inflammation after the treatment of FMK and Nec-1. Compared with TP-LPS co-treatment group, FMK and Nec-1 up-regulated the serum glucose content and restored the glycogen storage in the liver, indicating the disorder of glucose metabolism might be, in some part, the outcome of cell death (Fig.6C and Fig.7,
shown as PAS staining). Moreover, Nec-1 treatment decreased the elevation of serum TBA induced by TP-LPS co-treatment, while FMK had little influence on it (Fig.6D). Compared with apoptosis, the activation of necroptosis could promote the release of pro-inflammatory factors thus recruiting inflammatory cells (Newton and Manning 2016). The MPO staining showed in Fig.7 strongly proved this phenomenon as Nec-1 alleviated the neutrophil infiltration while FMK had little effect on this, revealing that the activation of necroptosis was responsible for the increased inflammatory infiltration (Shown as IHC staining of MPO in Fig.7).

4. Discussion

According to the data from the National Center for Adverse Drug Reaction Monitoring of China (http://samr.cfda.gov.cn/WS01/CL1989/70473.html), there are 633 adverse reaction cases (including 53 severe cases) that are caused by the application of Tripterysium Glycosides medications from 2004 to September 2011. Among the hepatotoxicity cases happened by the administration of Tripterysium Glycosides, some cases showed a dramatic increase in the level of serum transaminase (beyond 10 folds, even around 100 folds in severe condition). However, previously published research articles and also the results from our lab have shown that animals treated with Tripterysium Glycosides even at the high dose express only a slightly increase in serum ALT and AST (up to 2 to 3 folds), which is inconsistent with the clinical adverse reactions report (Wang et al. 2016a; Wang et al. 2015). As the main toxic component of Tripterysium Glycosides, TP has less toxic effect on the serum transaminase in mice, which is in line with Tripterysium Glycosides (Jiang et al.
These contradictory results between animal models and human beings have provoked us to figure out the exact cause of hepatotoxicity in TP treatment.

Previously, all the research projects have focused on the hepatotoxicity pathways regulated by only TP administration. However, we found that the modification of feeding environment from barrier system to conventional environment could significantly exacerbate the liver damage induced by TP, suggesting that the direct liver toxicity induced by TP may not be the only reason to be accounted for TP-induced hepatotoxicity (Supplementary material Fig.1A to Fig.1C). Compared with conventional environment, in barrier system we provided the mice with a sterile environment, which could sometimes reduce the incidence of infection. Additionally, clinical reports revealed that the long term usage of immunosuppressive agents could up-regulate the risk of infections in clinics because of the weakening of protective immune response. The deterioration of protective immune response might increase the risk of organ damage and even increase the mortality rate (Cajanding 2018; Orlicka et al. 2013). As an immunosuppressive agent, we don't know that whether there is a relationship between the immunomodulatory effects and hepatotoxicity induced by TP. We supposed that over-suppression of immune system by TP might result in the inability of liver to eliminate the toxic attack initiated by pathogens, ultimately leading to the hypersensitivity of liver to the stimulation of outside pathogens that can cause even cell death.

Our previous study revealed that female animals are more sensitive to TP-induced
hepatotoxicity (Jiang et al. 2016). Therefore, female C57BL/6N mice were selected in this study. For a more comprehensive understanding of TP-induced hepatotoxicity, we administered a low dose of LPS to female mice that got TP treatment before LPS administration to test whether TP could disrupt liver immune homeostasis, leading to the hypersensitivity of liver upon LPS stimulation. Under physiological conditions, although the normal liver routinely takes in the LPS from the portal vein, the outcome of the stimulation is immune tolerance but not overt immune response. This suggests that hepatic microenvironment possess a higher threshold of LPS stimulation. Consistent with this observation, our results showed that the low dose of LPS only initiated weak inflammatory reactions without causing liver damage (Fig.1G to Fig.2F). Although the low dose of TP plus LPS didn’t cause severe liver toxicity, it only showed the increased level of serum ALT, AST (Fig.1B and 1C). Importantly, a high dose of TP together with LPS induced intense elevation of serum transaminase, revealing TP-treated mice lost the ability to counteract the detrimental effects of LPS (Fig.1B and 1C). Excluding the changes in serum transaminases, we also found the abnormalities in bile acid and glucose metabolism through blood biochemical test.

Bile acids are a group of amphipathic steroid molecule synthesized in the liver, taking part in the transport, digestion, and absorption of nutrients, fat, and vitamins (Martinot et al. 2017). The abnormal bile acid metabolism leads to the development of multiple liver diseases. Thus, the homeostasis of bile acid metabolism reflects normal liver function. Previous reports showed that a high dose of LPS could cause cholestasis, with changes in FXR expression and bile acid transporters (Hao et al.
However, our results showed that low dose of LPS didn’t affect the serum TBA and ALP levels while LPS alone inhibited the bile acid transporters, which might be the inhibitory effects of pro-inflammatory factors produced by LPS (Geier et al. 2003). In contrast, TP-LPS co-treatment increased the serum content of TBA, revealing the abnormality of bile acid metabolism (Fig.1D). Our result showed that TP-LPS co-treatment decreased the mRNA and protein level of FXR (Fig.3A and 3G). The inactivation of FXR could up-regulate bile acid synthesis, resulting in the overload of bile acid in the hepatocytes and leading to liver damage (Martinot et al. 2017). Also, NTCP and OATP1B, two transporters involved in the transport of bile acid from serum into liver, were also decreased (Fig.3C and 3D). The reduction in NTCP and OATP1B might occur as a negative-feedback following the initial increase of bile acid in the liver to prevent the excessive accumulation of bile acids in hepatocytes. Hence, disorder in bile acid metabolism could be one reason responsible for the up-regulation of serum ALT and AST. Meanwhile, impairment of hepatocytes’ function, such as the tight junction, could also elevate the content of serum TBA (Rahner et al. 1996; Yang et al. 2017b). We proved that pre-treatment with Nec-1 could alleviate the elevation of TBA induced by TP-LPS, implying the necroptosis caused by TP-LPS might also be the one reason for the abnormality of bile acid metabolism (Fig.6D).

Liver glycogen is synthesized after the intake of food in response to the high concentration of blood glucose level and degraded to glucose when there is a need of energy supply to maintain the blood glucose homeostasis. Thus, liver glycogen acts as
an energy store to make sure the energy supply of the whole body. Previous study revealed the damage of liver could accelerate the depletion of liver glycogen and decrease the serum glucose level (Ferluga et al. 1979; Shen et al. 2014; Shibayama 1989). Meanwhile, glucose is the main source of energy for cell survival. Insufficient supply of ATP and biosynthesis resulting from the depletion of glucose could affect cell survival (Martinez-Outschoorn et al. 2017). A decrease in serum glucose level and loss of glycogen in the liver suggesting the abnormality in liver glucose metabolism, which was further confirmed by the mRNA levels of PEPCK, G6Pase and GLUT2 (Fig.4A to Fig.4C). After the inhibition of apoptosis and necroptosis by FMK and Nec-1 respectively, the restoration of glycogen level and increased serum glucose implied the glucose metabolism disorder as a secondary effects of apoptosis and necroptosis (Fig.6E and Fig.7). However, we can’t rule out the possibility that the hepatotoxicity of TP-LPS might be the result of insufficient glucose supply.

Recent studies revealed that LPS-induced TNF-α plays a vital role in the activation of apoptosis and necroptosis. TNF-α is a pro-inflammatory cytokine that coordinates in tissue homeostasis by regulating cytokines production, cell survival, and cell death. Under normal conditions, the balance between pro-survival and pro-death signals regulated by TNF-α can protect cell from death. The disruption of this balance, such as inhibition of NF-κB mediated transcriptional activity or disruption in RIPK1 function could trigger the cell death in response to the stimulation of LPS/TNF-α (Annibaldi and Meier 2018; Peltzer et al. 2016). The increased density of cytoplasmic content, condensation of chromatin and the
appearance of apoptotic bodies in H&E staining gave us idea about the activation of apoptosis (Fig.1G). Protein level and IHC staining of Cleaved Caspase-3 confirmed our hypothesis (Fig.5A and 5L). Compared with the LPS group, TP-LPS co-treatment increased the level of pro-inflammatory factors and neutrophil infiltration, indicating the activation of apoptosis might not be the only reason resulting in the hepatotoxicity induced by TP-LPS (Fig.2). Considering the pro-inflammatory function of necroptosis, we detected the expression of RIPK1, P-MLKL, and MLKL by western blot (Fig.5B). As expected, TP-LPS co-treatment especially at TP high dose increase all of them (Fig.5I to Fig.5K), indicating the activation of necroptosis. Unlike apoptosis, the activation of necroptosis involved the loss of membrane integrity and could promote the production of cytokines through the cell-autonomous mechanism (Zhu et al. 2018). The release of pro-inflammatory mediators during necroptosis could also trigger more cellular death and further aggravate liver inflammation. A decrease in neutrophils recruitment after Nec-1 pre-treatment testified the inflammatory reaction was partly involved in the activation of necroptosis (Fig.7). As FMK and Nec-1 could both alleviate the hepatotoxicity induced by TP-LPS, this attractive phenomenon provoked us that the pro-survival and pro-death balance induced by LPS/TNF-α might be disrupted by TP (Fig.6B and Fig.6C). Among numerous factors determining cellular fate, transcription mediated up-regulation of pro-survival molecules can’t be ignored. Published data revealed that TP could inhibit RNA polymerase II-mediated transcription activity, implying transcriptional inhibition might be responsible for the activation of apoptosis and necroptosis on the stimulation of TP-LPS co-treatment
(Chen et al. 2015; Titov et al. 2011; Vispe et al. 2009). Further research is needed to explore the role of LPS-induced hepatotoxicity in TP-LPS co-treatment model to find out that how TP affected liver immune homeostasis can lead to hypersensitivity upon LPS stimulation.

In conclusion, we found that TP pre-treatment could disrupt liver immune homeostasis leading to the liver inability to neutralize the harmful response induced by LPS. Pre-treatment with TP sensitized the liver to the stimulation of LPS, resulting in impaired liver function and severe liver damage, reflected by the increased serum transaminase, apoptosis, necroptosis, inflammatory infiltration, glucose and bile acid metabolism disorder. Among them, necroptosis contributed to the inflammatory reaction, bile acid, and glucose metabolism disorder. While abnormal glucose metabolism was also the secondary effects of apoptosis, indicating that apoptosis and necroptosis were the most likely reasons for TP-LPS induced liver damage. Although this hypothesis has not been tested on human, our results indicated that when the patients are suffering from the diseases expressing liver damage due to excessive bacteria stimulation, such as gut microbial translocation or facing bacterial infections, drugs containing TP should not be administered. The complete scenario of hepatotoxicity mechanism of TP-LPS is fully illustrated in Fig.8.

**Conclusion**

This study provided a new perspective on TP-induced hepatotoxicity, which was different from the previous researches. We showed that the direct liver toxicity of TP in vivo was not the only reason accounting for the hepatotoxicity of TP. While TP
treatment could disrupt liver immune homeostasis, resulting in hypersensivity of the liver to the pathogen’s stimulation, such as LPS. We also found that apoptosis along with necroptosis played the predominant role in the liver damage induced by TP-LPS. Our research could contribute to a better understanding of the potential toxicity induced by TP.

Conflict of Interest

We declare that there is no conflict of interest.

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References

Hao, H., Cao, L., Jiang, C., Che, Y., Zhang, S., Takahashi, S., Wang, G. and Gonzalez, F.J. 2017. Farnesoid X Receptor Regulation of the NLRP3 Inflammasome Underlies Cholestasis-Associated
Petersen, M.C., Vatner, D.F. and Shulman, G.I. 2017. Regulation of hepatic glucose metabolism in
Wang, J., Miao, M., Qu, L., Cui, Y. and Zhang, Y. 2016a. Protective effects of geniposide against Tripterygium glycosides (TG)-induced liver injury and its mechanisms. The Journal of toxicological


**Fig.1** Mice treated with TP sensitized the liver to the stimulation of LPS. (A) Schematic representation of the experimental procedure for animal treatment in this part. (B-F) Changes of serum ALT, AST, ALP, TBA, Glucose in mice treated with TP, LPS, or both (n=12). (F) Pictures of liver tissue sections stained by H&E (200×). Results were expressed as means ± SEM. Statistical analysis was performed using two-way ANOVA following by Tukey’s Multiple Comparison Test. *, #, $P<0.05$, **, ##, $$P<0.01$, $$$, $$$P<0.001; {^*}, ^{##}, ^{###}, ^{####}P$, compared between TP-treated and TP-LPS co-treatment group; #P, ##P, ###P, compared between LPS-treated and TP-LPS co-treatment group; $^P$, $$P, $$$P$, compared between control and TP-treated group; ns, no statistical difference.
Fig. 2 Increased inflammatory infiltration in liver of mice treated with TP after the stimulation of LPS. (A-E) The relative mRNA levels of IL-1β, IL-6, TNF-α, MPO, and MCP-1 were determined by real-time PCR and normalized using GAPDH as the internal control (n=12). (F) Pictures of liver tissue sections analyzed by IHC for MPO. Results were expressed as means ± SEM. Statistical analysis was performed using two-way ANOVA following by Tukey’s Multiple Comparison Test. *, #, $P<0.05$, **, ##, $$$P<0.01$, ###, $$$P<0.001$; *, **, ***P, compared between TP-treated and TP-LPS co-treatment group; #P, ##P, ###P, compared between LPS-treated and TP-LPS co-treatment group; $P$, $$P$, $$$P$, compared between control and TP-treated group; ns, no statistical difference.
**Fig. 3** Disrupt of bile acid metabolic homeostasis in mice after TP-LPS co-treatment. (A-F) The relative mRNA levels of FXR-SHP signaling pathway and bile acid transporters were determined by real-time PCR (n=12). (G-H) Representative Western blot and relative intensity of protein band of FXR, with GAPDH as the loading control (n=4). Results were expressed as means ± SEM. Statistical analysis was performed using two-way ANOVA following by Tukey’s Multiple Comparison Test. *, #, ¥, $$$P<0.05, **, ##, $$$P<0.01, ###, $$$P<0.001; *P, **P, ***P, compared between TP-treated and TP-LPS co-treatment group; #P, ##P, ###P, compared between LPS-treated and TP-LPS co-treatment group; ¥P, $$$P, $$$P, compared between control and TP-treated group; ns, no statistical difference.
**Fig. 4** Disrupt of glucose metabolic homeostasis in mice after TP-LPS co-treatment. (A-C) The relative mRNA levels of the key enzyme involved in gluconeogenesis and transporter in liver glucose metabolism (n=12). (D) Pictures of liver tissue sections stained by PAS for the detection of glycogen (200x). Results were expressed as means ± SEM. Statistical analysis was performed using two-way ANOVA following by Tukey’s Multiple Comparison Test. *, #, $P<0.05$, **, ##, $$P<0.01$, ###, $$$P<0.001$; *, **P, ###P, compared between TP-treated and TP-LPS co-treatment group; #P, ###P, compared between LPS-treated and TP-LPS co-treatment group; $P$, $$P$, $$$P$, compared between control and TP-treated group; ns, no statistical difference.
**Fig. 5** Activation of intrinsic apoptosis and necroptosis in mice treated with TP after the stimulation of LPS. (A) The protein levels of Cleaved Caspase-3, Cleaved Caspase-8, Cleaved PARP, Cleaved Caspase-9, Bax, and Bcl-2 were determined by western blot analysis and normalized with GAPDH (n=3-6). (B) Analysis of the protein levels of RIPK1, MLKL, P-MLKL (Ser345) by western blot and GAPDH was used to normalize the loading control (n=3-6). (C-K) The relative intensity of protein bands of apoptosis and necroptosis related proteins. (L) Pictures of liver tissue sections analysis by IHC for Cleaved Caspase-3 and MLKL (200×). Results were expressed as means ± SEM. Statistical analysis was performed using two-way ANOVA following by Tukey’s Multiple Comparison Test. *, #, $P<0.05$, **, ##, $$P<0.01$, ###, $$$P<0.001; \ ^{*}, \ ^{*}, \ ^{**}, \ ^{***}, \ ^{#}, \ ^{##}, \ ^{$}, \ ^{$$}, \ ^{$$}$, compared between TP-treated and TP-LPS co-treatment group; \ ^{*}, \ ^{*}, \ ^{**}, \ ^{***}, \ ^{#}, \ ^{##}, \ ^{###}, \ ^{$$$}, \ ^{$$$}$, compared between LPS-treated and TP-LPS co-treatment group; \ ^{*}, \ ^{*}, \ ^{**}, \ ^{***}, \ ^{#}, \ ^{##}, \ ^{###}, \ ^{$$$}, \ ^{$$$}$, compared between control and TP-treated group; ns, no statistical difference.
Fig.6 Pretreatment with FMK or Nec-1 alleviated the activation of apoptosis, necroptosis, and hepatotoxicity induced by TP-LPS co-treatment. (A) Schematic presentation of the experimental procedure for animal treatment in this part. (B-E) The effect of FMK and Nec-1 on the serum ALT, AST, TBA, and glucose levels after TP-LPS co-treatment (n=12). (F-L) Representative Western blot and relative intensity of protein bands of Cleaved Caspase-3, Cleaved PARP, RIPK1, MLKL, P-MLKL (Ser345) after FMK or Nec-1 treatment, with GAPDH as the loading control (n=4). Results were expressed as means ± SEM. Statistical analysis was performed using One-way ANOVA following by Tukey’s Multiple Comparison Test. *, #P<0.05, **, ###P<0.01, ###P<0.001; *P, **P, ***P, compared between TP-treated group and TP-LPS co-treatment group; #P, ##P, ###P, compared between TP-LPS co-treatment and TP-LPS-FMK or TP-LPS-Nec-1 co-treatment group; ns, no statistical difference.
Fig. 7 Pretreatment of FMK or Nec-1 alleviated the morphological change, glycogen depletion and inflammatory infiltration induced by TP-LPS co-treatment. Representative photomicrographs of liver sections by H&E staining, PAS staining, and IHC for MPO after the treatment of FMK or Nec-1.
Fig. 8 Schematic presentation indicating the suggested mechanisms by which TP-LPS induced hepatotoxicity and the possible mechanisms of FMK and Nec-1 intervention for liver protection in TP-LPS induced liver injury.
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