

## Original Research Article

# Verteporfin mitigates sepsis-induced liver injury by blocking macrophage-derived inflammation

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### Abstract

**Purpose:** To study the role of yes-associated protein (YAP) inhibition by verteporfin (VTF) in LPS-induced acute liver injury (ALI) after sepsis.

**Methods:** *In vitro*, VTF was used to treat LPS-stimulated RAW 267.4 cells. *In vivo*, LPS was injected to induce sepsis in mice, followed by treatment with VTF. The inflammatory mediators were determined using quantitative real-time polymerase chain reaction (qRT-PCR), immunofluorescence spectroscopy (IF) and immunohistochemical staining (IHC), and the levels of YAP, P53 and ERK were measured by qRT-PCR, WB and IHC. Moreover, liver histology and liver function were examined using HE staining and ELISA respectively.

**Results:** The results showed that VTF reduced YAP expression and inhibited LPS-induced cell activation and inflammatory cytokine production such as IL-6 and IL-1 $\beta$ , by attenuating the expressions of p53 and ERK pathway in macrophages. The levels of AST, ALT and TBIL remarkably decreased in ALI mice after VTF treatment ( $p < 0.05$ ). Moreover, it was observed that inflammatory mediators, including inducible nitric oxide synthase (iNOS), IL-6 and IL-1 $\beta$ , decreased significantly in VTF treated mice ( $P < 0.05$ ).

**Conclusion:** VTF plays an antagonistic role in LPS-induced inflammatory response after ALI. Therefore, VTF is a potential medicinal agent for preventing infectious acute liver injury.

**Keywords:** Verteporfin, Sepsis, Liver injury, Macrophage, Inflammation

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## INTRODUCTION

Sepsis is one of the most aggressive syndromes caused by uncontrolled immune response to infection [1,2]. There are more than 18 million new cases of sepsis with high mortality rate worldwide each year [3]. The inflammatory cytokine storm causes multiple organ damage especially liver [4]. Sepsis-induced ALI is

considered as an early event and deteriorates into liver failure soon [5,6]. Therefore, how to suitably mitigate intrahepatic inflammation and effectively protect survival of functional cells remains a formidable challenge in sepsis therapeutics. Macrophages have routinely been considered as one of the most vital immune cells and plays a significant role in conferring innate and adaptive immunity to the host [7,8]. On the

basis of the surrounding conditions of the immune microenvironment in sepsis, macrophage activates by bacteria and traverses vessels into liver tissue, which sequentially secretes excess pro-inflammatory mediators [9]. Previous studies have shown that IL-1 $\beta$  and IL-6 secreted by activated macrophages recruit a large number of neutrophils into the liver, which release ROS and proteases, leading to necrosis of liver parenchymal cells [10].

Many scientists have been investigating the proper regulation of macrophage for the correction of inflammation-driven damage in liver. Yes-associated protein (YAP), a key transcription co-activator, plays a crucial role in the Hippo pathway, and is reported to be highly associated with cancers cells [11,12]. Activation of YAP leads to overgrowth and tumor phenotypes [13]. Hence, YAP has been considered independent predictor of prognosis [14]. Although Hippo-YAP signaling is important in oncogenesis, little is known about how YAP signals inflammation. Numerous researches showed that YAP aggravates inflammatory bowel disease, and inhibition of YAP activation suppress inflammatory response to LPS stimulation [15,16]. It is unknown whether Hippo-YAP signaling also regulates inflammatory response induced by sepsis.

Clinically, VTF has been utilized as a photosensitizing drug for the photodynamic treatment of macular degeneration. It can also inhibit interaction of the key components of the Hippo pathway [17]. Recent studies have shown that VTF inhibit lipopolysaccharide-induced inflammation by multiple functions in RAW 264.7 cells [18]. However, the function of VTF in macrophages and sepsis-induced ALI has remained elusive. In this study, we verified the role of YAP inhibition modulating inflammation in LPS-stimulated RAW 264.7 macrophage line and sepsis-induced ALI mice.

## EXPERIMENTAL

### Cell culture and treatment

The RAW 267.4 line was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA) and cultured in 5  $\times$  5 cm 2 flasks (Corning, Corning, NY, USA). The RAW 264.7 cells were grown in Dulbecco's modified eagle medium (DMEM, Gibco, Rockville, MD, USA) supplemented with penicillin (100 IU/ml), streptomycin (100  $\mu$ g/mL) and 10 % fetal bovine serum (FBS, Gibco, Rockville, MD, USA). Cells were incubated at 37  $^{\circ}$ C with 5 % CO $_2$  until they reached more than 80 % confluence. The FBS-

free DMEM was used to replace the original medium overnight. Then, RAW 264.7 cells were stimulated with different concentrations of verteporfin (0.5, 1, or 2  $\mu$ M) for 2 h before treated with LPS (1  $\mu$ g/mL) for 12 h. All VTF treatments were done in the dark.

### Establishment of acute liver injury (ALI) model

Male C57BL/6J mice (6 – 8 weeks old, with mean weight of 20 g) were purchased from Kunming Medical University Animal Center. The mice were housed in Kunming Medical University Animal Center, where feed and tap water were available to them. The mice were randomly divided into vehicle, LPS and LPS + VTF groups (n = 6). Mice in the vehicle group were injected with PBS, with a volume equal to the LPS reagent; mice in the LPS group were administered with LPS at a dose of 10 mg/kg for 7 days; and mice in the LPS + VTF group were injected with VTF (100  $\mu$ g/kg) 4 h post-LPS injection via intraperitoneal injection for 3 days. This study was approved by the Animal Ethics Committee of Kunming Medical University Animal Center (no. 17-KMUAC-no. 03). All procedures were conducted in accordance with the 'Animal Research: Reporting in vivo Experiments guidelines 2.0' [19].

### Quantitative real-time polymerase chain reaction (qRT-PCR)

The cells and tissues were treated with 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). RT-PCR was performed in a condition including 40 cycles of denaturation at 95  $^{\circ}$ C for 5 s, annealing at 60  $^{\circ}$ C for 20 s, and extension at 72  $^{\circ}$ C for 30 s. The sequences of the primers were shown in Table 1.

### Western blot (WB) assay

Cells, after treatment, were collected and centrifuged. Then, radioimmunoprecipitation assay (RIPA) reagent (Solarbio, Beijing, China) was used to isolate the total protein. Bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA) was used to determine the concentration of protein. The proteins were separated using 10 % sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and then transferred to 0.45  $\mu$ m polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were incubated with primary antibodies at 4  $^{\circ}$ C overnight, and with corresponding secondary antibodies for 1 h at 37

°C. The primary and secondary antibodies are shown: p53 (1:1000, Abcam, Cambridge, MA, USA), p-ERK (1:1000, Abcam, Cambridge, MA, USA), ERK (1:1000, Abcam, Cambridge, MA, USA), GAPDH (1:2000, Abcam, Cambridge, MA, USA), HRP-Anti-Rabbit antibody (1:2000, Proteintech, Rosemont, IL, USA). Enhanced chemiluminescence method was used to measure the protein expression levels.

**Table 1:** Primer sequences for PCR

Gene		Primer sequence
YAP	Forward	5'- ACCCTCGTTTTGCCATGAAC- 3'
	Reverse	5'- TGTGCTGGGATTGATATTCC GTA-3'
p53	Forward	5'- CTCTCCCCGCAAAGAAAA A-3'
	Reverse	5'- CGGAACATCTCGAAGCGTTT A-3'
IL-6	Forward	5'- TAGTCCTTCTACCCCAATTT CC-3'
	Reverse	5'- TTGGTCCTTAGCCACTCCTT C-3'
IL-1 $\beta$	Forward	5'- GCAACTGTTCTGAACTCAA CT-3'
	Reverse	5'- ATCTTTTGGGGTCCGTC AAC T-3'
TNF- $\alpha$	Forward	5'- GCAACTGTTCTGAACTCAA CT-3'
	Reverse	5'- ATCTTTTGGGGTCCGTC AAC T-3'
$\beta$ -actin	Forward	5'- TGACGTGGACATCCGCAAAG -3'
	Reverse	5'- CTGGAAGGTGGACAGCGAG G-3'

### Immunohistochemical staining (IHC)

Mice were sacrificed after anesthesia using ketamine (80 mg/kg) and xylazine (4 mg/kg), then liver tissue was extracted and placed into 4 % paraformaldehyde. After routine dehydration using a different alcohol gradient, the tissues were sliced into 5  $\mu$ m sections. Sections were incubated with primary antibodies [iNOS (1:200), YAP (1: 500), p53 (1:200), p-ERK (1:200), IL-6 (1:200), IL-1 $\beta$  (1:200)] at 4 °C. Washed with phosphate buffered saline (PBS), the sections were probed with secondary antibodies and

stained with a diaminobenzidine (DAB) staining Kit (Genentech, Shanghai, China). Then, the sections were counterstained with hematoxylin, and observed using a microscope.

### Immunofluorescence spectroscopy (IF)

Sections and cells were conducted with antigen blocking using 3 % bovine serum albumin (BSA) blocking buffer for 1h at room temperature. Then, the sections were incubated with primary antibodies [CD68 (1:200), iNOS (1:200), YAP (1:500), IL-6 (1:200), IL-1 $\beta$  (1:200)] overnight at 4 °C. Following incubation with secondary antibodies, sections were stained using the DAPI (Vectashield, Burlingame, CA, USA). A fluorescence microscope system (OLYMPUS, Tokyo, Japan) was used to capture images.

### Histological examination

In line with the manufacturers' instructions, the sections were stained with hematoxylineosin (H & E). The H & E Staining Kit (Servicebio, Wuhan, China) was used to measure histology. Images were collected using a microscope.

### Liver function test

Dirui CS-T300 Chemistry Analyzer and corresponding kits (Dirui Medical Technology, Changchun, China) were used to measure the levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBil) in accordance with the manufacturers' protocol.

### Statistical analysis

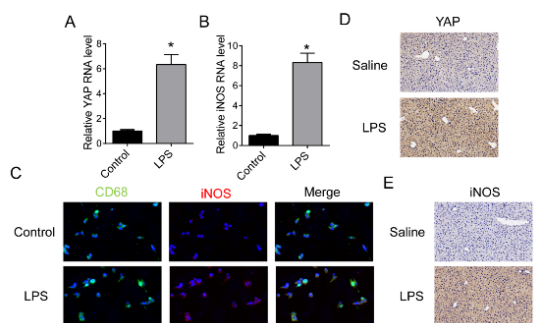
All experiments were performed in triplicate. SPSS statistical analysis software (version 26.0) was used for data analysis. Statistical data are expressed as mean and standard deviation (mean  $\pm$  SD) values. Statistical comparisons between groups were performed by the Student's t-test. The comparisons between the two groups were analyzed using one-way analysis of variance (ANOVA), followed by Post Hoc Test (Bonferroni).  $P < 0.05$  was considered statistically significant.

## RESULTS

### Hippo-YAP pathway regulated LPS-induced activation of macrophages

The RNA levels of YAP and iNOS were first determined in the macrophage and liver tissue after LPS treatment. Inducible nitric oxide synthase (iNOS) is a representative inflammatory

marker in activated macrophages, which increased sharply in correspondence with the RNA level of YAP after the induction of LPS in the cells (Figure 1 A and B). The results consistently showed that the expression of iNOS increased in LPS-stimulated macrophages when compared with those in control group (Figure 1 C). Moreover, IHC staining showed that the expressions of YAP and iNOS in mouse after LPS treatment were increased (Figure 1 D and E). The results indicate that both *in vitro* and *in vivo*, Hippo-YAP pathway may be essential for the regulation of LPS-induced activation of macrophage.

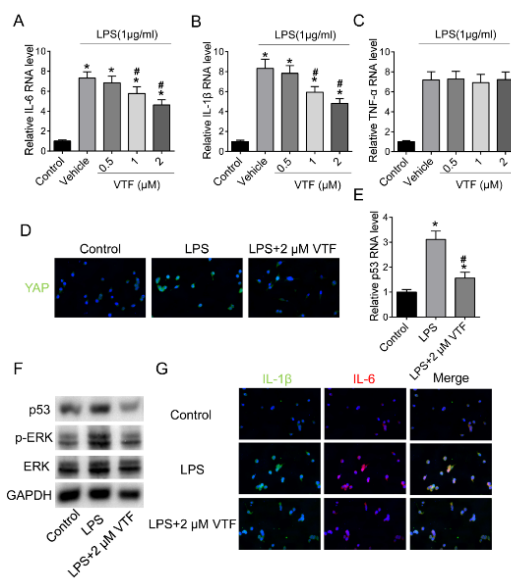


**Figure 1:** Hippo-YAP pathway regulates LPS-induced activation of macrophages. (A) The relative RNA expression of YAP. (B) Relative RNA expression of iNOS. (C) IF images of CD68 and iNOS post LPS treatment, (amplification: 200 $\times$ ). (D) Images of YAP IHC in liver post-LPS treatment, (amplification: 200 $\times$ ). (E) The images of iNOS IHC in liver post LPS treatment, (amplification: 200 $\times$ )

### YAP inhibition attenuated inflammation targeting p53 in macrophage

To ascertain the effect of YAP inhibition on macrophage-derived inflammation, various concentrations of VTF (0.5, 1.0 and 2.0  $\mu$ M) were added to the cells after LPS treatment. The RNA expressions of IL-6, IL-1 $\beta$  and TNF- $\alpha$  was measured using RT-PCR, showing that 1 and 2  $\mu$ M VTF administration significantly reduced IL-6 and IL-1 $\beta$  expression in a dose-dependent manner (Figure 2 A and B). However, no significant decrease was observed in the TNF- $\alpha$  level after VTF treatment in LPS-stimulated cells (Figure 2 C). Subsequently, 2.0  $\mu$ M VTF was selected for further investigation. To confirm the YAP inhibition mechanism in inflammatory response, the YAP expression was first examined using IF. The result displayed that the employment of VTF attenuated the level of YAP after LPS treatment (Figure 2 D). Gene p53 and ERK pathway have been reported to participate in the anti-inflammatory process [20], which was examined further by qRT-PCR and western blotting. The results showed that the expression

of p53 declined prominently following VTF treatment (Figure 2 E and F). Furthermore, the phosphorylation of ERK witnessed the same trend as p53 expression (Figure 2 F). The expressions of IL-6 and IL-1 $\beta$  in cells were determined using IF staining, showing that the employment of LPS increased the expressions of IL-6 and IL-1 $\beta$ . However, VTF reversed the expressions of IL-6 and IL-1 $\beta$  in cells (Figure 2 G). These results show that VTF inhibited LPS-induced inflammatory cytokine production such as IL-6 and IL-1 $\beta$ , by attenuating the expression of p53 and ERK pathway.

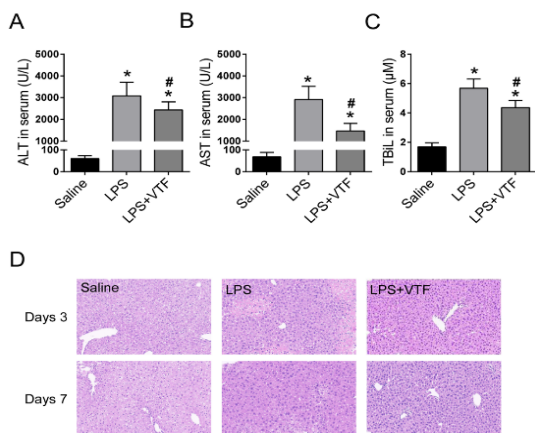


**Figure 2:** YAP inhibition attenuated inflammation targeting p53 in macrophages. Cells were incubated with various concentrations of VTF (0.5, 1.0 and 2.0  $\mu$ M) after LPS treatment. Relative RNA expressions of IL-6 (A), IL-1 $\beta$  (B), TNF- $\alpha$  (C). (D) Images of YAP expressions using IF staining in LPS and LPS+2.0  $\mu$ M VTF group at 12 h: ( $\times$ 200). (E) Relative RNA expressions of P53. (F) Protein levels of P53, p-ERK and ERK in LPS and LPS+2.0  $\mu$ M VTF group. (G) Images of IL-1 $\beta$  and IL-6 expressions using IF staining in LPS and LPS+2.0  $\mu$ M VTF group at 12 h (200 $\times$ )

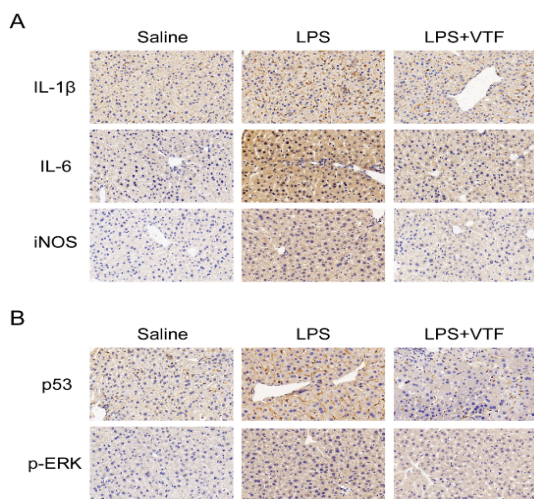
### VTF protects the liver from sepsis-induced injury in LPS-treated mice

At 7 days post-LPS injection, ALT, AST and TiBL were identified for liver function assessment, showing that ALT, AST and TiBL levels in serum were reduced after VTF treatment (Figure 3 A and C). Moreover, the liver histology in LPS-treated mice at 3 and 7 days was evaluated post-LPS treatment. HE staining showed that severe tissue destruction happened in the LPS group. However, VTF treatment mitigated the histologic disruption of liver tissue. Meanwhile, VTF administration significantly shrank the size of

hemorrhage and reduced infiltration of peripheral cells (Figure 3 D). These results suggest that VTF protects the liver from injury in sepsis mice.



**Figure 3:** Verteporfin protects the liver from sepsis-induced injury in LPS-treated mice. The levels of ALT (A), AST (B), and TBiL (C) in serum in Saline, LPS, and LPS+VTF group at 3 dpi. (D) The H & E images of liver in Saline, LPS, and LPS+VTF group at 3days and 7days post LPS and LPS+VTF treatment, (200×)



**Figure 4:** Inhibition of YAP decreases sepsis-induced inflammation in live. (A) The IHC images of IL-1β, IL-6 and iNOS in liver in Saline, LPS, and LPS +VTF group post LPS treatment (×200). (B) The IHC images of P53 and p-ERK in liver in Saline, LPS, and LPS +VTF group at 8W post LPS treatment (×200)

### Inhibition of YAP decreased sepsis-induced inflammation in liver

To further confirm whether inhibition of YAP is linked with inflammation *in vivo*, IHC staining was used to visualize the inflammatory level in liver at 3 days post LPS treatment, indicating that IL-6, IL-1β and iNOS positive area were reduced after VTF administration in the sepsis mice (Figure 4

A). Meanwhile, the protein levels of p53 and p-ERK were also measured in the liver using IHC, and a more positive expression of p53 and p-ERK was evident after LPS stimuli, despite a reduced expression of them in VTF treated mice (Figure 4 B). Taken together, it suggests that inhibition of YAP has an impeditive role in sepsis-induced inflammation in liver.

## DISCUSSION

Previous studies have reported that iNOS is a biomarker of pro-inflammatory macrophages [21]. It has been shown that the RNA level of iNOS increased in LPS-stimulated macrophages, indicating that macrophages were activated upon LPS treatment. Furthermore, the RNA level of YAP was found to have increased after LPS treatment both *in vivo* and *in vitro*. Thus, the Hippo-YAP pathway is essential for regulating LPS-induced activation of macrophage. Moreover, IL-6 and IL-1β have been demonstrated to be markers for a variety of inflammatory diseases such as arthritis and glomerulonephritis.

This study has demonstrated that YAP promotes pro-inflammatory response by increasing IL-6 expression. Conversely, the inhibition of YAP leads to an impaired inflammatory process as well as an enhanced reparative response [16]. Given its ability to inhibit Hippo-YAP pathway, VTF has the potential to fight against inflammation following sepsis. Results show that concentration-dependent VTF significantly decreases IL-6 and IL-1β *in vitro*, especially in the group treated with (2 μM) high dose. TNF-α also plays a major role in inflammatory system diseases [22]. However, there is little direct evidence that any dose of VTF has the potential to inhibit the inflammatory process by reducing TNF-α, which is a potent promoter of NF-κB, which activation also increases TNF-α, and forms a positive feedback loop.

Previous research clearly demonstrated that TNF-α is able to inhibit sepsis-induced inflammation via NF-κB [22]. Hence, the association between VTF and TNF-α is still controversial. Mounting evidence has proven the important roles of the p53 and ERK signaling pathway in pro-inflammation activation of macrophages [23]. It is worth mentioning that many studies have verified that the p53 and ERK pathway increased expression in LPS-activated macrophages [24]. The results in this study demonstrate that phosphorylated ERK protein and LPS stimulation increased, as confirmed by previous reports. Administration of VTF in macrophages protects the cell, possibly by

inhibiting the activation of the p53 and ERK pathway. In addition, findings in the study has shown that VTF prevented LPS-induced activation of the macrophage by reducing the level of ERK phosphorylation.

These results underscore the suppression role of p53 and ERK pathway in inflammatory system. It should be noted that IL-1 $\beta$  and IL-6 are the inflammatory magnifiers, increased expression of the Hippo-YAP pathway was presented to be highly involved with an inflammatory response. On the one hand, over-accumulated inflammatory magnifiers exert pro-inflammatory effects via provoking macrophage activation and polarization, thereby enlarging the inflammatory area in the injured liver. On the other hand, AIL induces rapid increases in pro-inflammatory cytokines and chemokines which enhance macrophage migration towards the injured area and lead to a more severe liver injury. Results demonstrated that the addition of VTF attenuated tissue edema and extensive hemorrhage around the lesion 7 days, which potentially affects the inflammatory level.

Moreover, *in vivo* the treatment of AIL models with VTF effectively decreased the levels of AST, AIL and TBiL, suggesting that macrophage inflammation may be mediated negatively by VTF via inhibition of the Hippo-YAP pathway. Collectively, this study has demonstrated that the treatment of VTF after LPS stimulation protects liver tissue and liver function. VTF attenuates intrahepatic inflammation via inhibiting hippo-YAP pathway after ALI. Based on the present evidence, VTF may be repositioned as a selected intervention of therapeutic schedule in ALI and a promising adjuvant drug.

## CONCLUSION

The primary finding of the present study shows that VTF, an inhibitor of YAP, reduces macrophage-induced inflammatory response and mitigates sepsis-induced liver injury. Hence, VTF might be a suitable therapeutic agent management of inflammatory diseases.

## DECLARATIONS

### Conflict of Interest

No conflict of interest associated with this work.

### Contribution of Authors

We declare that this work was done by the authors named in this article, and all liabilities

pertaining to claims relating to the content of this article will be borne by the authors. Yaopeng Yang and Anxiu Wang contributed equally to this work.

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