

## Original Article

# Regulatory roles and mechanisms of *Hovenia dulcis* Thunb toward polarization of macrophage RAW264.7

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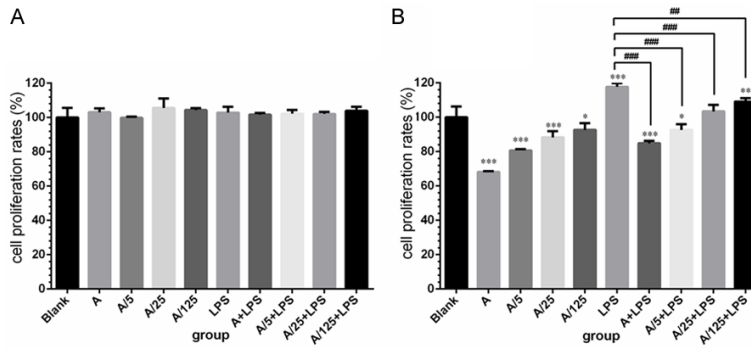
**Abstract:** This study aims to investigate the regulatory roles of *Hovenia dulcis* Thunb (HDT) on the polarization of lipopolysaccharide (LPS) induced macrophage RAW264.7 as well as its roles and related mechanisms on downstream inflammatory cytokines. The cell growth and polarization after drug treatment were determined by CCK-8 and flow cytometry. Enzyme linked immunosorbent assay (ELISA), Western blot and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) were performed to detect the expression levels of Toll-like receptor 4 (TLR4), p65, p-p65, as well as downstream inflammatory cytokines, including interleukin1 $\beta$  (IL-1 $\beta$ ), IL-6, IL-10, tumor necrosis factor $\alpha$  (TNF- $\alpha$ ), and transforming growth factor $\beta$  (TGF- $\beta$ ). HDT significantly inhibited the polarization of M1, promoted the polarization of M2, reduced the protein of TLR4 and phosphorylated p65, downregulated the mRNA expression of TLR4, IL-1 $\beta$ , IL-6, TNF- $\alpha$  and TGF- $\beta$ , and upregulated the expression of IL-10 mRNA in LPS-induced RAW264.7. However, it did not show much significance when cells were not disturbed by LPS. HDT may play its roles of reversing the polarization of LPS-induced RAW264.7 through reducing the expression of TLR4 and inhibiting the activation of nuclear factor $\kappa$ B (NF- $\kappa$ B), which thereby reduces the production of such downstream inflammation cytokines as IL-1 $\beta$  and IL-6.

**Keywords:** *Hovenia dulcis* Thunb, RAW264.7 cells, lipopolysaccharides, TLR4

## Introduction

Alcoholic liver disease (ALD) is a liver disease caused by long-term heavy drinking, which may proceed from common fatty liver (initial stage) to alcoholic hepatitis (AH), alcoholic liver fibrosis, and alcoholic liver sclerosis, and even induce extensive liver cell necrosis, liver failure, or liver cancer in severe alcoholics [1], and it has become one common disease that threatens public life and health all over the world currently. AH accounts for the highest rate in ALD, and the 30 d and 1-year mortalities of AH patients can be up to 15% and 39%, respectively [2], and the 6-month mortality of severe AH patients can be up to 40% [3]. Modern research has found [4-7] that alcohol can induce intestinal barrier damages, so a lot of intestinal lipopolysaccharide (LPS) can enter the liver, bind with liver cells-produced specific LPS binding proteins, and form LPS-LPS binding protein complex, which then binds with specific receptor

CD14 on KC surface and MD-2 regulatory protein-containing Toll-like receptor 4 (TLR4) in turn, followed by being transferred, with the assist of TLR4, into nuclei so as to form stimulation signals and activate the KC cells. Meanwhile, a series of inflammatory cascade reactions starting from the TLR4-MyD88 signaling pathway will activate nuclear factor $\kappa$ B (NF- $\kappa$ B)-regulated downstream inflammatory factors, thus resulting in the massive release of endogenous inflammatory cytokines (tumor necrosis factor $\alpha$  (TNF- $\alpha$ ), interleukin6 (IL-6), IL-1, and IL-12), as well as cell adhesion molecules and free radicals, and leading to the necrosis and apoptosis of a large number of liver cell. Therefore, it can be seen from the above that alcohol-induced immune disorders play an important role in the progression of ALD. *Hovenia dulcis* Thunb (HDT), also known as *Calligonum*, has a thousand-year history of being used as a drug in China for its sweet, acidic, and mild properties, and it has such



**Figure 1.** Effects of different concentrations of HDT extract on cell proliferation measured by CCK-8 at 0 h (A) and 24 h (B). The proliferation rate was shown as a fold of the blank control group. The bars represent the mean  $\pm$  SD of three independent experiments, performed each in triplicate samples. Note: "A" is the concentration of HDT extract, namely 4.0 mg/mL vs. blank control group, \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001; vs. LPS model group, ### $P$ <0.01, #### $P$ <0.001.

effects as reducing fever, inducing urination, slaking thirst, cooling blood, invigorating spleen, reinforcing Qi, etc. In ancient times, HDT was mainly used as a diuretic so as to treat alcohol overdose, and is now widely used to treat a variety of liver diseases [8]. Studies in China [9-11] have also proved that HDT has higher superoxide scavenging activity, can improve liver functions in mice with acute alcoholic liver injuries, and can significantly reduce such indexes as alanine aminotransferase (ALT), glutamic-oxalacetic transaminase (AST), glutamyl transpeptidase (GGT), etc. Lots of studies have suggested that HDT may play a liver-protective role through a variety of ways, but the exact mechanism is not clear yet, especially the immunologic mechanism. Therefore, this study explored the impact of HDT on regulating LPS-induced polarization in macrophage RAW264.7, aiming to explore the underlying mechanisms and to provide theoretical basis and experimental evidence for the application of HDT in treating ALD.

**Materials and methods**

*Cell culture and dose groupings*

RAW264.7 (Shanghai Cell Bank, China) was cultured in the dulbecco minimum essential medium (DMEM) medium (Hyclone, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA) and 1% double-antibody (a mixture of penicillin and streptomycin) at 37°C and 5% CO<sub>2</sub> (Thermo, USA). The cells in the logarithmic growth phase

were then sampled and divided into four groups: the blank control group, the HDT-treated group, the model group induced by LPS (1 ug/ml, Sigma, USA), and the LPS model group pre-incubated with HDT. The HDT extract (Jiangyin Tianyin Pharmaceutical Co., China) was initially and successively diluted into four different concentrations according to corresponding protocol, to determine the effective concentration.

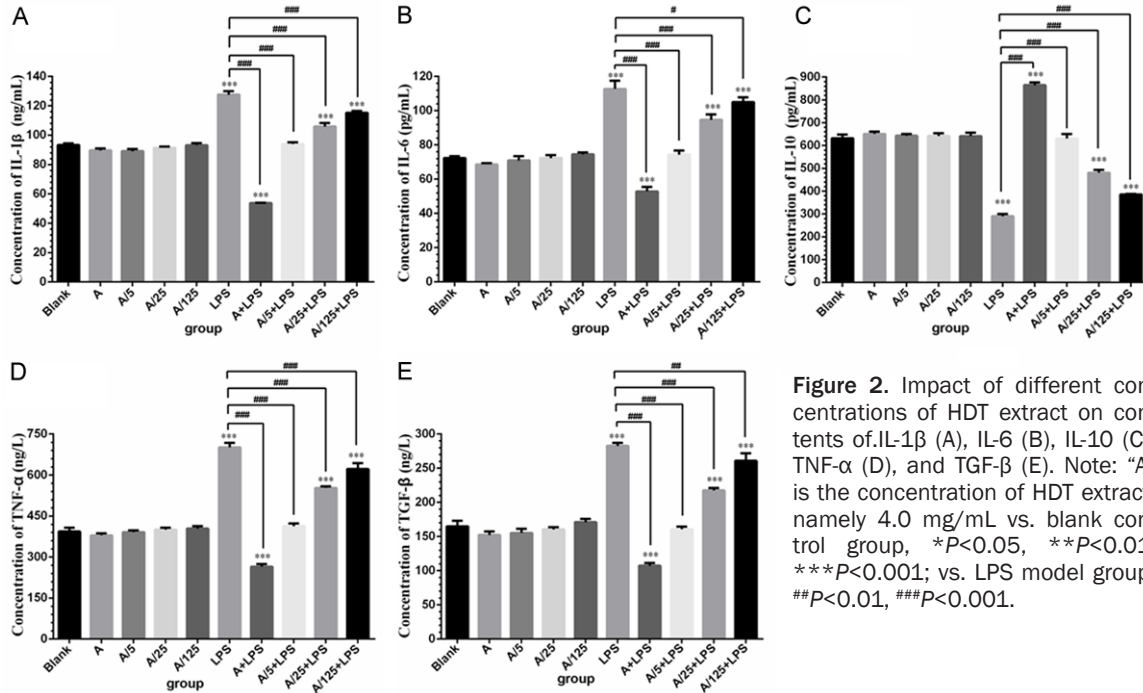
*Detection of cell proliferation*

The cells in the logarithmic growth phase were collected, digested using trypsin (Solarbio Co., China), counted under a microscope, and then prepared the cell suspension (1~5  $\times$  10<sup>4</sup> cells/ml). 100  $\mu$ L of such cell suspension was then added into 96-well culture plates, and each type of cell was repeated three times in each plate, 1~5  $\times$  10<sup>3</sup> cells/well, together with 100  $\mu$ L of culture medium as the blank control. After treated with corresponding drugs for 0 and 24 h, respectively, each well was mixed with CCK-8 (SAB, USA, 1:10 v/v) and serum-free essential basic medium; 100  $\mu$ L of the mixture was added into each sample well and incubated at 37°C and 5% CO<sub>2</sub> for 1 h; the OD values were then measured at 450 nm using a microplate reader (Multiscan MK3, Finland).

*Enzyme linked immunosorbent assay (ELISA)*

The cell culture medium of each group was collected into sterile tubes, and after centrifuged (Shanghai Luxiangyi centrifuger Co. Ltd., China) at 2000-3000 r/min for 20 minutes, the supernatant was carefully collected; the standard reference was diluted into five gradients in accordance with the instructions, and the volume of each gradient added into each well was 50  $\mu$ L; meanwhile, blank wells and sample wells were also set simultaneously. 40  $\mu$ L and 10  $\mu$ L of diluted sample solution were added into each sample well successively, and after incubation at the room temperature and washing, 50  $\mu$ L of ELISA reagent was added into the sample wells, followed by incubation at the room temperature and washing five times

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**Figure 2.** Impact of different concentrations of HDT extract on contents of IL-1 $\beta$  (A), IL-6 (B), IL-10 (C), TNF- $\alpha$  (D), and TGF- $\beta$  (E). Note: "A" is the concentration of HDT extract, namely 4.0 mg/mL vs. blank control group, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; vs. LPS model group, ## $P < 0.01$ , ### $P < 0.001$ .

repetitively; each well was then added 50  $\mu$ L of Reagent A and B, respectively, and developed in darkness after 15 min; 50  $\mu$ L of stop buffer was then added, and the absorbance of each well (OD) was then measured at 450 nm.

### Flow cytometry

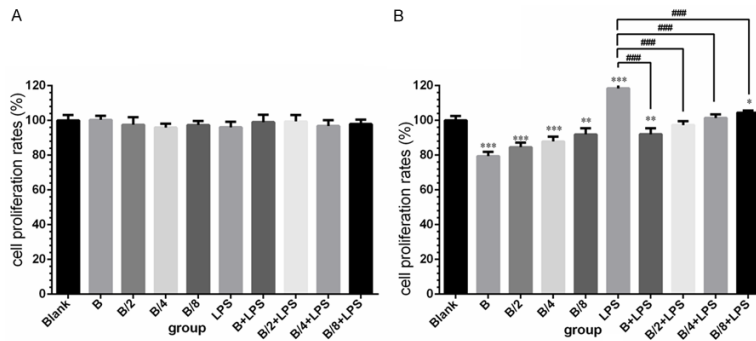
After digested using trypsin, the cells in the logarithmic growth phase were counted microscopically (Shanghai Caikon Optical Instrument Co., Ltd., China) and seeded into 6-well plates ( $5 \times 10^5$  cells/well); each type of cells was set three repetitive wells in each plate. Each plate was set four groups, and after treated for 24 h, the cells were digested using trypsin and then detected by flow cytometry. The mononuclear cell suspension was firstly counted, and then diluted with phosphate buffered saline (PBS) so as to adjust the concentration of the sample cells to be  $10^6$  cells/mL; 200  $\mu$ L of the cell suspension was then centrifuged at 1000 rpm for 5 min (4°C), followed by 1 ml pre-cooled PBS rinsing twice and resuspension into 100  $\mu$ L PBS. The test tubes were set as follows: negative control: only 100  $\mu$ L of cell suspension withno antibody; CD68 single standard tube: 100  $\mu$ L of cell suspension and 5  $\mu$ L of CD68 antibody (Biolegend, USA), gently mixed evenly; CD206 single standard tube: 100  $\mu$ L of cell suspension and 5  $\mu$ L of CD206 antibody (Biolegend,

USA), gently mixed evenly; CD68 + CD206 single standard tube: 100  $\mu$ L of cell suspension and 5  $\mu$ L of CD68 and CD206 antibodies, respectively, gently mixed evenly. All the tubes were incubated at 4°C in darkness for 1~2 h. After the incubation, all the test tubes were centrifuged at 3000 rpm for 10 min, discarded the supernatant, resuspended the precipitate using 200  $\mu$ L of PBS, and then detected using flow cytometer (BD, USA).

### Western blot

After digested using trypsin, the cells in the logarithmic growth phase were counted microscopically, and then seeded into 6-well plates ( $5 \times 10^5$  cells/well); each type of cells was set three repetitive wells in each plate. Each was set four groups, and 24 h after the treatment, the total protein was extracted and detected the concentration using the BCA protein quantification method (Thermo, USA), followed by PAGE-gel electrophoresis (BIO-RAD, USA), film-transferring, on-film protein detection, 1 h closure using 5% skim milk (BD, USA) at room temperature, overnight incubation with the primary antibody (Beyotime Biotechnology Co. Ltd., China) at 4°C, 1 h incubation with the second antibody (Beyotime Biotechnology Co. Ltd., China) at 37°C, and ECL coloration.

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**Figure 3.** Effects Impact of different concentrations of HDT extract on cell proliferation measured by CCK-8 at 0 h (A) and 24 h (B). The proliferation rate is shown as a fold of the blank control group. The bars represent the mean  $\pm$  SD of three independent experiments, performed each in triplicate samples. Note: "B" is the concentration of HDT extract, namely 0.8 mg/mL vs. Blank control group, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; vs. LPS model group, ## $P < 0.01$ , ### $P < 0.001$ .

### Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The total RNA was extracted using Trizol reagent (Invitrogen, USA), and the operation flow referred to the kit instructions (Thermo, USA). The PCR primers were designed according to gene sequences, so as to synthesize the first chain of cDNA. The primer sequences were as follows: TLR4, forward sequence GTCTCCCTCTTGCTTTC, reverse sequence GGACTGCTGTTGCTCTAC; IL-1 $\beta$ , forward sequence GAGCATCCAGCTTCAAATC, reverse sequence ACACCAGCAGTTATCATC; IL-6, forward sequence TGGAGCCCACCAAGAACGATAG, reverse sequence TGTCACCAGCATCAGTCCCAAG; IL-10, forward sequence AGGACTTTAAGGGTTACTTG, reverse sequence CAAATGCTCCTTGATTTCTG; TNF- $\alpha$ , forward sequence GGGTGTTCCATCCATTCTC, reverse sequence CCCAGCATCTTGTTTTC; TGF- $\beta$ , forward sequence CGAGAGGCAGAGATTTATCAG, reverse sequence ATGTGAAGATGGGCAAGAC; and GAPDH (internal reference), forward sequence ATCACTGCCACCCAGAAG, reverse sequence TCCACGACGGACACATTG. 2  $\mu$ L of cDNA was then used as the template for the RT-PCR amplification in one 25  $\mu$ L reaction system; the reaction conditions: denaturation at 95°C for 15 s, annealing at 60°C for 45 s, for a total of 40 cycles. ABI Prism 7300 SDS Software, which was provided by the instrument (ABI, USA), was used for the analysis.

### Statistical analysis

SPSS19.0 statistical software was used for the data analysis; all the data were expressed as mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ), and the pair

wise comparison between two groups used the LSD (Least Significant Difference) method in ANOVA, with  $P < 0.05$  considered as statistically significant.

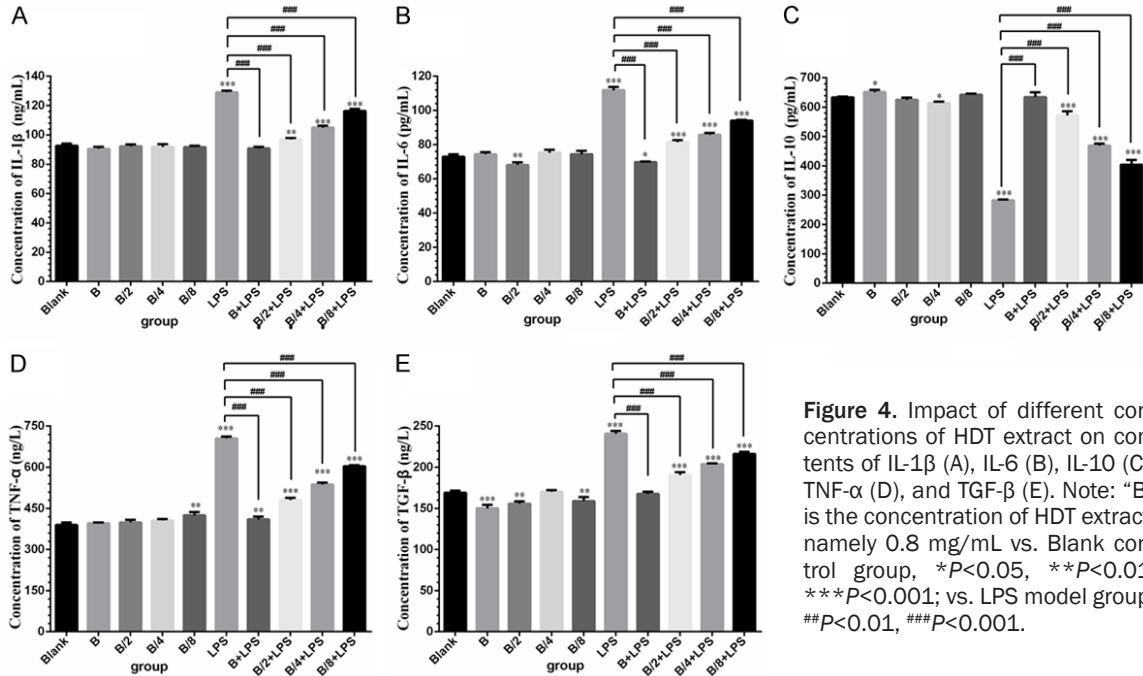
### Results

*The initial concentration of HDT extract screened according to the effects of HDT on cell proliferation and expression levels of inflammatory cytokines*

In order to choose the effective concentration, the HDT extract, with original dose of 4

mg/mL which was represented by "A", was successively diluted 5 times and sequentially finished at the doses of A/5, A/25 and A/125. We detected the role of HDT on cell proliferation by CCK-8. The **Figure 1A** showed that at 0 h, the conditions of cell proliferation in all the groups were the same and had no intergroup difference ( $P > 0.05$ ), indicating that the cells in all groups were the same before HDT treatments. After 24 h intervention (**Figure 1B**), compared with the blank control group, the cell proliferation rates in HDT-treated groups with different concentrations were significantly inhibited ( $P < 0.05$ ). Moreover, the cell proliferation rate in LPS model group was significantly increased compared to the blank control group ( $P < 0.001$ ), but was significantly decreased when incubated with varied doses of HDT. However, there was no difference between the model group and the one incubated with HDT at the concentration of A/25 ( $P > 0.05$ ).

We also assessed the effect of HDT on the expression levels of inflammatory cytokines with testing the cell supernatant by ELISA. It can be seen from **Figure 2** that compared with the blank control, different concentrations of HDT extract had no significant impact on the contents of IL-1 $\beta$ , IL-6, IL-10, TNF- $\alpha$ , and TGF- $\beta$  in non-LPS-treated RAW264.7 ( $P > 0.05$ ). Meanwhile, in LPS model group incubated with or without HDT at the concentration of A/25 and A/125, the pro-inflammatory cytokines including IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and TGF- $\beta$  were meaningfully increased, but the anti-inflammatory factor IL-10 was reduced ( $P < 0.001$ ), com-



**Figure 4.** Impact of different concentrations of HDT extract on contents of IL-1β (A), IL-6 (B), IL-10 (C), TNF-α (D), and TGF-β (E). Note: “B” is the concentration of HDT extract, namely 0.8 mg/mL vs. Blank control group, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; vs. LPS model group, ## $P < 0.01$ , ### $P < 0.001$ .

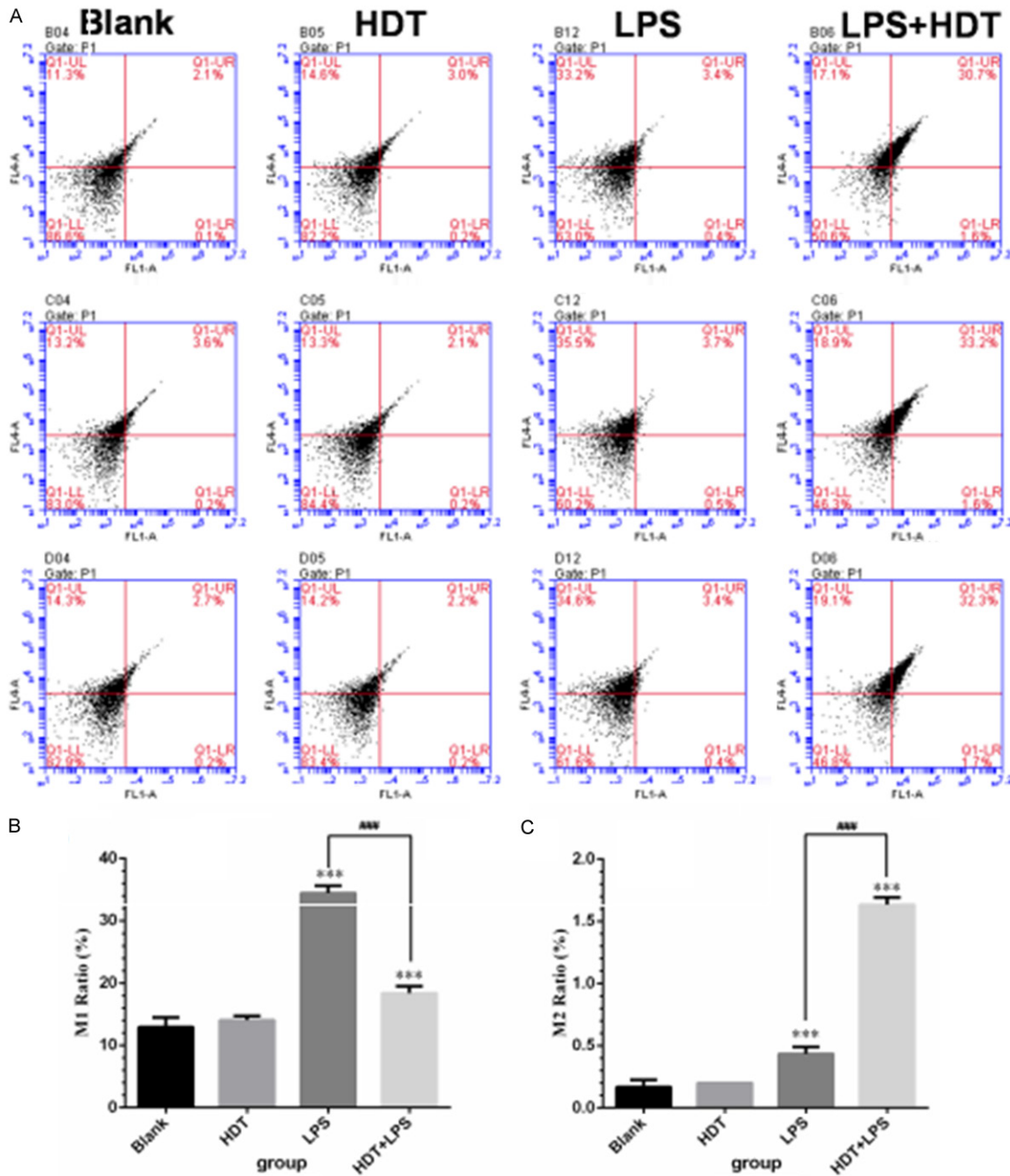
pared with the blank control group. However, the contents of IL-1β, IL-6, TNF-α, and TGF-β in the supernatant of LPS model group incubated with HDT at dose of A were decreased, but that of IL-10 was increased ( $P < 0.001$ ). Compared with model group, the contents of IL-1β, IL-6, TNF-α, and TGF-β in the supernatant of HDT-treated groups with different concentrations were reduced, but the content of IL-10 was increased ( $P < 0.05$ ). It also can be seen from the figure that after treated with LPS, the contents of IL-1β, IL-6, IL-10, TNF-α, and TGF-β in the supernatant of LPS model group incubated with HDT at the concentration of A/5 were the closest to those in the blank control group. Therefore, combining with the results of cell proliferation, the concentration of A/5, namely 0.8 mg/mL, was selected for subsequent experiments.

*The final concentration further determined by the results of cell proliferation and contents of IL-1β, IL-6, IL-10, TNF-α, and TGF-β*

In order to determine the final concentration, the HDT extract, with dose of 0.8 mg/mL which was marked by “B”, was repeatedly diluted and finished at the doses of B/2, B/4 and B/8. The assessment was performed again as above. **Figure 3** show that at 0 h, the conditions of cell proliferation in all the groups were the same and had no difference ( $P > 0.05$ ), indicating that the cells in all the groups were the same at 0 h;

24 h after the treatment, compared with the blank control group, the cell proliferation rates in HDT-treated groups with different concentrations were significantly inhibited ( $P < 0.01$ ); and after the LPS treatment, the cell proliferation rate in LPS model Group incubated with HDT at dose of B/2 and B/4 showed no significant increase or decrease ( $P > 0.05$ ), but that in model group was significantly increased ( $P < 0.001$ ); the cell proliferation rates in model group were significantly decreased when incubated with HDT at different concentrations ( $P > 0.05$ ).

It can be seen from **Figure 4** that compared with the blank control group, the HDT-treated groups with different concentrations had certain impacts on the contents of IL-6, IL-10, TNF-α, and TGF-β in a non-dose-dependent manner; after the LPS treatment, the contents of IL-1β, IL-6, TNF-α and TGF-β, in the supernatant of LPS model groups incubated with or without HDT at the concentration of B/2, B/4 and B/8, were increased, but that of IL-10 was reduced ( $P < 0.01$ ). The contents of IL-6 and TNF-α in the supernatant of LPS model group incubated with HDT at the concentration of B were increased slightly ( $P < 0.05$ ). Compared with model group, the contents of IL-1β, IL-6, TNF-α, and TGF-β in the supernatant of LPS model group incubated with HDT at different concentrations were reduced, but the content of IL-10



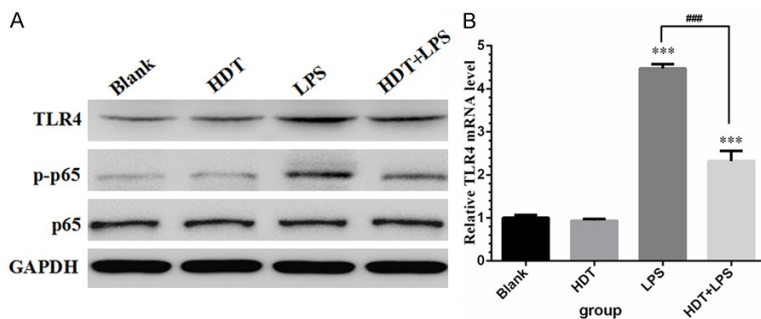
**Figure 5.** Effects of HDT extract on polarization of RAW264.7 determined by flow-cytometric analysis. A. Results of flow cytometry. UL: CD68+; LR: CD206+; UR: CD68+CD206+; B. Analysis graph for M1 polarization; C. Analysis graph for M2 polarization. Vs. blank control group, \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ ; vs LPS model group, ## $P<0.01$ , ### $P<0.001$ . Note: The concentration of HDT is 0.8 mg/mL.

was increased ( $P<0.001$ ). Based on the above, the final concentration for subsequent experiments was determined to be 0.8 mg/mL.

*Effects of HDT on polarization of RAW264.7*

Compared with the blank control group, HDT treatment had no meaningful impact on the

polarization of M1 and M2 in macrophage cell RAW264.7 ( $P>0.05$ , **Figure 5**). The polarization of M1 or M2 was aggravated in the cells when induced by LPS ( $P>0.001$ ), whether there was HDT treatment or not. Moreover, compared with the model group, the HDT extract can differentially inhibit the polarization of M1 and promote the polarization of M2 ( $P<0.001$ ).



**Figure 6.** Result of TLR4 expression and p65 phosphorylation by Western blot and qRT-PCR. Left: Representative Western blotting; Right: The mRNA expression of TLR4. Note: The concentration of HDT is 0.8 mg/mL vs. Blank control group, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; vs. LPS model group, ## $P < 0.01$ , ### $P < 0.001$ .

*Effects of HDT on the expression of TLR4 and NF- $\kappa$ B signaling pathway in RAW264.7*

We further explored the possible related mechanisms, that is TLR4 and NF- $\kappa$ B signaling pathway. From **Figure 6**, it was suggested that the HDT extract played no significant role in protein expression or mRNA expression of TLR4 and p65 phosphorylation ( $P > 0.05$ ), while LPS disturbance significantly upregulated the expression level of TLR4 and phosphorylated p65 ( $P < 0.05$ ) compared with the blank control group. However, the promoting effect of LPS as above, could be distinctively eased if combined with HDT ( $P < 0.05$ ).

*Effects of HDT on the mRNA expression of downstream inflammatory cytokines including IL-1 $\beta$ , IL-6, IL-10, TNF- $\alpha$  and TGF- $\beta$  in RAW264.7*

It can be seen from **Figure 7** that compared with the blank control, the expressions of IL-1 $\beta$ , IL-6, IL-10, TNF- $\alpha$ , and TGF- $\beta$  mRNA had no significant changes when no LPS treatment was applied ( $P > 0.05$ ); after the LPS treatment, the expressions of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and TGF- $\beta$  mRNA in model group were upregulated, and that of IL-10 mRNA was downregulated ( $P < 0.001$ ); compared with model group, the expressions of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and TGF- $\beta$  mRNA in HDT-treated model group were significantly downregulated, and that of IL-10 mRNA was upregulated ( $P < 0.001$ ).

**Discussion**

Alcohol is mainly converted into acetaldehyde under the catalysis of ethanol dehydrogenase

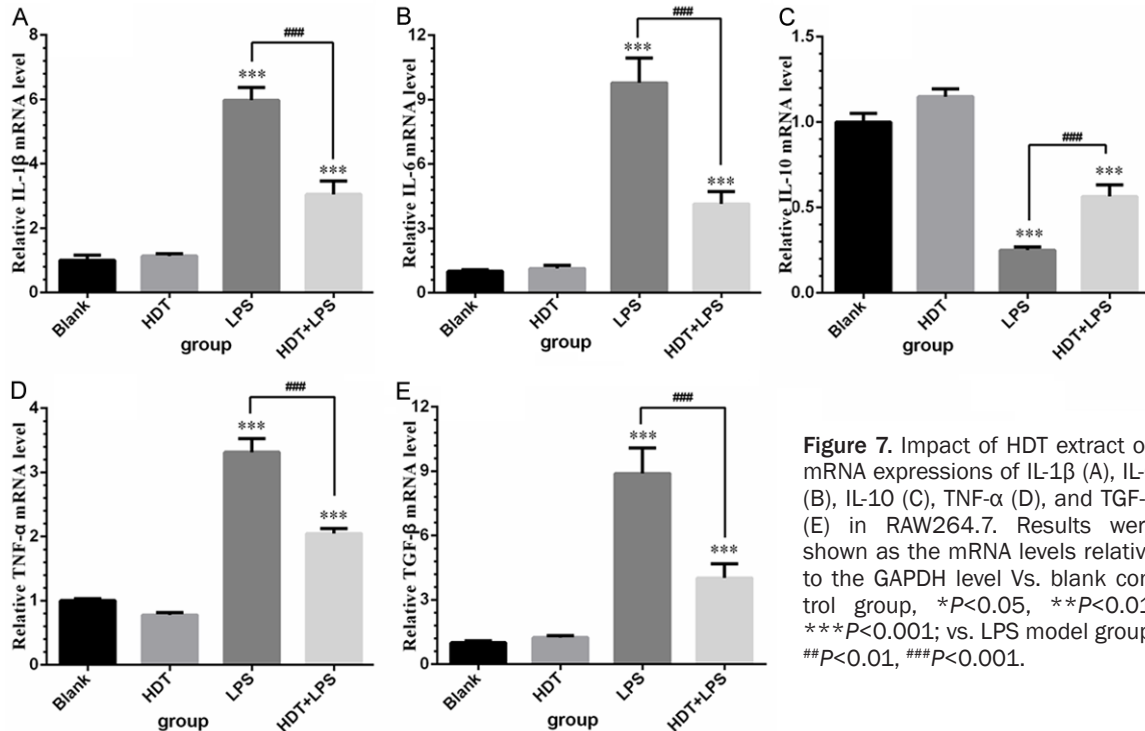
which has strong liver toxic effects and can combine with albumin, lipoproteins, etc., in the circulation, thus generating new antigens and stimulating the body's immune responses, followed by liver inflammation, necrosis, or fibroplasia [4, 12, 13]. Endotoxin is also known as LPS, the component of the cell wall of Grand negative bacteria, and continuous alcohol intake may induce intestinal endotoxemia, in which the intestinal permeability is increased

so that excessive LPS can enter the hepatic portal circulation, thus promoting the process of ALD [14]. Macrophages are important inflammatory response cells in human beings, and the Kupffer cells are the macrophages inside the hepatic blood sinusoid cavity. The KC cells are involved in the lipid peroxidation process of oxidative stress and can generate a variety of inflammatory cytokines and reactive oxygen species, thus promoting the development of ALD [15].

According to the activation status and different functions, the KC cells can be divided into type M1 (classic activated macrophages) and type M2 (alternatively activated macrophages) [16, 17]; the former can activate the Th1 cells and be mainly involved in forward immunity and antigen presenting, as well as promoting the secretion of pro-inflammatory cytokines IL-1b, TNF- $\alpha$ , IL-6, IL-8, etc., thus playing the roles of killing intracellular pathogens and inhibiting tumor growth, etc.; the latter can activate the Th2 cells and be mainly involved in downregulating the immune responses of inhibitory inflammatory cytokines IL-10 and TGF- $\beta$ , so it has the roles of anti-inflammation and promoting the immune escape of tumors. Foreign studies have shown [18, 19] that reducing the polarization of M1-type KC can reduce the progression of ALD. Meanwhile, certain in vivo and in vitro study also has proved [20] that M2-type KC can promote the senescence of liver cells through secreting IL-6 and limit alcohol-related liver fatty degeneration and apoptosis, thus playing significant roles in early ALD.

NF- $\kappa$ B normally binds with inhibitory proteins and forms inactive complex, and when it's sub-

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**Figure 7.** Impact of HDT extract on mRNA expressions of IL-1 $\beta$  (A), IL-6 (B), IL-10 (C), TNF- $\alpha$  (D), and TGF- $\beta$  (E) in RAW264.7. Results were shown as the mRNA levels relative to the GAPDH level Vs. blank control group, \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001; vs. LPS model group, ### $P$ <0.01, #### $P$ <0.001.

jected to a variety of stimuli (oxidative stress, bacteria, viruses, etc.), it can play its roles by phosphorylation, namely the expressions of NF- $\kappa$ Bp65 and p-p65 can be significantly increased, thus promoting the further development of inflammation. One study has confirmed [5] that LPS can be identified by CD14 and then activate the TLR4 receptor, which further activates the I $\kappa$ B kinase (IKKS) for its phosphorylation through a series of cascade so as to expose the binding site on NF- $\kappa$ B subunit p65, promote the transferring of NF- $\kappa$ B into nuclei, increase the intranuclear NF- $\kappa$ Bp65 level, and combine with the promoters of downstream inflammatory factors, thus inducing the generation of huge amounts of inflammatory factors. Another studies have suggested that [6, 21] chronic alcohol intake can make increase the transcription activities of TLR4 mRNA in liver cells, KC, and hepatic stellate cells (HSCs), and significantly enhance the expressions of liver proinflammatory cytokines IL-1, IL-6, TNF- $\alpha$ , and TGF- $\beta$ 1.

This study found that the polarization of M1 and M2 in LPS-induced RAW264.7 was increased, the protein expressions of TLR4 and p-p65 were increased, and the mRNA expressions of such proinflammatory cytokines as IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and TGF- $\beta$  were increased;

however, the content of anti-inflammatory factor IL-10 was reduced, consistent with the results of other modern research; HDT (0.8 mg/mL) can inhibit the polarization of M1 while promote the polarization of M2, reduce the contents of TLR4 and p-p65, significantly downregulate the mRNA expressions of TLR4, IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and TGF- $\beta$ , but upregulate the mRNA expression of IL-10, consistent with our expected results. One research in the same period also [22] revealed that the 70%-ethanol extract of HDT can act on LPS-treated RAW264.7, effectively inhibit the expressions of CO, COX-2, IL-1 $\beta$ , and TNF- $\alpha$ , as well as inhibiting the phosphorylation of NF- $\kappa$ Bp65, consistent with our experimental results. We consider the possible mechanisms may be related to the facts that HDT can inhibit the polarization of M1 and promote the polarization of M2 in LPS-induced macrophages, which thus reduces the production of downstream inflammatory cytokines, eases the progression of liver inflammation. So, it can be speculated that HDT can adjust intrahepatic immunity and exhibit exact significant effects in improving hepatic inflammation.

Meanwhile, this study also has some shortcomings: first, the concentrations of HDT extract



and alcohol are single, if we cannot figure out whether this immunomodulatory effects are associated with the concentration; second, in addition to this cell signaling pathway, we cannot confirm whether other signaling pathways are also involved in; third, HDT is rich of various ingredients, whether the results of this study are caused by one main component or various ingredients is still not clear; fourth, this study is one in vitro experiment, the impact (pathological changes) and specific mechanisms of HDT on alcohol-induced hepatocyte injuries still remain to be further studied. Through this study, we can conclude that HDT can improve liver inflammation, which not only is related to its roles of antioxidation, liver cell-protection, and transaminase reduction but also can be achieved via the immune system. Thereafter, we will further improve HDT-related cell and animal studies so as to clear the specific mechanisms of HDT, thus providing basis for the clinical applications of HDT in treating ALD.

### Conclusion

HDT may play its roles of reversing the polarization of LPS-induced RAW264.7 through reducing the expression of TLR4 and inhibiting the activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B), which thereby reduces the production of such downstream inflammation cytokines as IL-1 $\beta$  and IL-6. It also clearly suggests HDT can obviously inhibit the continued inflammation progress due to the use of alcohol, so as to achieve the purpose of treating ALD, making HDT has more reasonable substantial theoretical basis in the clinical application. In view of HDT has good treatment effect on ALD, together with its approach of achieving conveniently and lower price in our country, it must have a very good application prospect.

### Disclosure of conflict of interest

None.

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