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Salidroside Inhibits Lipopolysaccharide-ethanol-induced Activation of Proinflammatory Macrophages via Notch Signaling Pathway*

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Summary: Activation of macrophages is a key event for the pathogenesis of various inflammatory diseases. Notch signaling pathway recently has been found to be a critical pathway in the activation of proinflammatory macrophages. Salidroside (Sal), one of main bioactive components in *Rhodiola crenulata* (*Hook. F. et Thoms*) *H. ohba*, reportedly possesses anti-inflammatory activity and ameliorates inflammation in alcohol-induced hepatic injury. However, whether Sal regulates the activation of proinflammatory macrophages through Notch signaling pathway remains unknown. The present study investigated the effects of Sal on macrophage activation and its possible mechanisms by using both alcohol and lipopolysaccharide (LPS) to mimic the microenvironment of alcoholic liver. Detection of THP-1-derived macrophages exhibited that Sal could significantly decrease the expression of tumor necrosis factor-α (TNF-α), interleukin-1 beta (IL-1β) and IL-6 in the macrophages at both mRNA and protein levels. Furthermore, Sal significantly suppressed NF-κB activation via Notch-Hes signaling pathway in a dose-dependent manner. Moreover, in the microenvironment of alcoholic liver, the expression of Notch-dependent pyruvate dehydrogenase phosphatase 1 (PDP1) was elevated, and that of M1 gene expression [inducible NO synthase (NOS2)] was up-regulated. These changes could all be effectively ameliorated by Sal. The aforementioned findings demonstrated that Sal could inhibit LPS-ethanol-induced activation of proinflammatory macrophages via Notch signaling pathway.
Key words: THP-1 macrophages; Salidroside; Notch; tumor necrosis factor-α; monocyte chemoattractant protein-1

Inflammation represents a response to pathogenic invasion or tissue injuries and is an important contributor to the progression of various diseases, such as cardiovascular diseases, cancer, autoimmune diseases, alcoholic hepatitis and so forth[1–5]. During the process, the activated pro-inflammatory macrophages are known as key roles in the initiation of the inflammatory response and they over-produce pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, and inflammatory mediators, such as nitric oxide (NO) and prostaglandin (PG) E_2[6, 7].

Lipopolysaccharide (LPS) is one of the most potent activators of macrophages and it activates the macrophages by binding to its membrane receptor, Toll-like receptor 4 (TLR4)[8, 9]. In response to LPS, macrophages stage a wide range of responses, including secretion of proinflammatory mediators and cytokines, expression of adhesion molecules and coagulation factors, phagocytosis, and cytoskeletal rearrangement[8–12]. Upon binding onto circulating monocytes, LPS activates gene transcription and induces phosphorylation of signal transduction pathways, including phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinases (MAPKs), which, in turn, regulates the activation of nuclear factor-kappa B (NF-κB), and increases the expression of multiple genes implicated in the inflammatory response[13–15].

Moreover, the increased NF-κB activity is also regulated by Notch signaling pathway in the process of the LPS-induced macrophage activation[16]. Notch signaling pathway is a highly-conserved signaling pathway that regulates tissue development and homeostasis in a variety of ways[17]. In macrophages, Notch activation is triggered by LPS-induced TLR4

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activation plus the ligand interaction with Notch[18–20]. Following Notch activation, the expression of M1 gene, such as inducible NO synthase (NOS2), is up-regulated in macrophages, which favors the *in vitro* pro-inflammatory reactions.

As in many chronic inflammatory diseases, macrophages are recently found to be a critical player in the pathogenesis of alcoholic liver disease (ALD) and might serve as a therapeutic target for the treatment of the condition[21]. In the liver microenvironment, alcohol increases the sensitivity of macrophages to gut-derived endotoxin/LPS[22]. Upon the exposure to endotoxin in the portal circulation, such as LPS and alcohol, resident macrophages and infiltrated monocytes differentiate into a pro-inflammatory phenotype[23].

Salidroside (Sal) is a major component isolated from *Rhodiola crenulata* (*Hook. F. et Thoms*) *H. ohba*, and has long been used as a traditional Chinese medicinal agent for it possesses a wide range of important bioactivities, including anti-oxidative, anti-fatigue, anti-inflammatory, anti-cancer, anti-aging, hepatoprotective effects, among others[24]. Recent studies demonstrated that Sal could regulate inflammatory response in Raw264.7 macrophages via TLR4/TGF-activated kinase 1 (TAK1) and ameliorate inflammation in alcohol binge drinking-induced liver injury[25].

Nonetheless, to the best of our knowledge, whether Notch signaling pathway is involved in the anti-inflammatory effects of Sal on macrophages remains poorly understood. In this study, by simulating the microenvironment of alcoholic liver *in vitro* by co-stimulation with both LPS and alcohol, this study examined the effects of Sal on inflammatory responses in THP-1 macrophages elicited by alcohol and LPS and the possible roles of Notch signaling in this process, with an attempt to understand the anti-inflammatory mechanisms of Sal in the treatment of alcohol-induced hepatic injury.

1 MATERIALS AND METHODS

1.1 Materials

Sal of 98% purity was purchased from Chengdu Push Bio-technology Co., Ltd. (China). Phorbol 12-myristate 13-acetate (PMA) and LPS were bought from Sigma-Aldrich (USA). Anti-human TNF-α, IL-1β and IL-6 ELISA kits were procured from Dakewe Biotech Co., Ltd. (China). Real-time PCR Master Mix and ReverTra Ace qPCR RT Kit were from Toyobo Life Science Department (China). Trizol® Reagent was from Life Technologies Corporation (China). CCK8 kit and BCA protein assay kit came from Beyotime Institute of Biotchnology (China). Total protein extraction kits and nucleoprotein extraction kits were purchased from Key GEN Biotech (China). Anti-activated-notch1, hairy/enhancer of split 1 (HES1), phosphorylated-inhibiotr κBα (p-IκBα) and iNOS were bought from Abcam (USA). PDP1 was procured from WUHAN SANYING (China). Anti-IκBα was purchased from Cell Signaling Technology (USA). Anti-human LaminB antibody, anti-human β-actin antibody, horseradish peroxidase (HRP)-labeled anti-rabbit IgG (H+L) antibody and HRP-labeled anti-mouse IgG antibody were from Wuhan Boster Bio-engineering Co., Ltd. (China).

1.2 Cell Culture

Human monocyte-like cells (THP-1), were obtained from the Cell Culture Center of the Chinese Academy of Medical Sciences and cultured in DMEM medium supplemented with 15% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin.

1.3 CCK8 Assay for THP-1 Cells Viability

THP-1 cells (2×10^5 cells/mL) were seeded into 96-well plate and induced by PMA (100 nmol/L) for 72 h in an incubator at 37°C in 5% CO_2. Then, Sal at different concentrations was added to the plates. After 24 h, cytotoxicity was detected by CCK8 kit according to the manufacturers' instructions.

1.4 Macrophage Stimulation

THP-1 cells were pre-treated with PMA (100 nmol/L) for 72 h to induce macrophages. The macrophages obtained were stimulated with LPS (100 ng/mL, Sigma) and ethanol (25 mmol/L), with or without different concentrations of Sal for 12 h at 37°C in the presence of 5% CO_2[26]. The 25-mmol/L *in vitro* ethanol concentration is close to blood alcohol level of 0.1 g/dL, which, *in vivo*, results from a single moderate alcohol consumption and is marginally above the legal limit for blood alcohol concentration[27]. Cell viability was not affected by the treatment of LPS in combination with ethanol. Then, the cells were collected for real-time polymerase chain reaction (real-time PCR) and Western blotting, and culture supernatants were used for enzyme-linked immunosorbent assay (ELISA).

1.5 Quantitative Real-time PCR

Total RNA was extracted and dissolved in RNA-free water and quantified by using UV-clear microplates. Then, single-strand cDNA was synthesized from 2 μg total RNA by using Rever Tra Ace qPCR RT Kit. Real-Time PCR was performed using Real time PCR Master Mix. The primers used were as follows: GAPDH (sense: 5′-GGTCGGAGTCAACGGATTTG-3′; antisense: 5′-GGAAGATGGTGATGGGATT TC-3′); IL-1β (sense: 5′-AATGATGGCTTATTACAGTGGCA-3′; antisense: 5′-GCTGTAGTGGTGGTCGGAGATT-3′); TNF-α (sense: 5′-CTGCTGCACTTTGGAGTGATC-3′; antisense: 5′-GGTTCGAGAAGATGATCTGACTG-3′); IL-6 (sense: 5′-GTGAAAGCAGCAAAGAGGCA-3′; antisense: 5′-TTGGGTCAGGGGTGGTTATT-3′); MCP-1 (sense: 5′-TATTGTCCACTGACCCC-3′; antisense: 5′-CTT-CACCCTTGTCCTAAGGT-3′); NOS2 (antisense: 5′-

AGGTCCAAATCTTGCCTGGGA-3′; sense: 5′-ATCTGGAGGGGTAGGCTTGT-3′). Samples were measured in triplicate. Differences in gene expression were calculated by using the $2^{-\Delta\Delta CT}$ method.

1.6 Cytokine Assays

IL-1β, IL-6, MCP-1 and TNF-α in culture supernatants were detected by ELISA kits following instructions.

1.7 Western Blotting

The total protein and nucleoprotein of the cells were extracted by protein extraction kits according to the manufacturers' instructions. Protein concentration was then detected using BCA protein assay kit. Samples of cell lysates were separated by 12% SDS-PAGE and then transferred onto nitrocellulose membranes. After being placed into blocking buffer, the membranes were incubated with following primary antibodies: anti-activated notch1 (dilution at 1:300), anti-HES1 (dilution at 1:500), anti-PDP1 (dilution at 1:500), anti-IκB (dilution at 1:1000), anti-pIκB (dilution at 1:1000), anti-NOS2 (dilution at 1:1000) and anti-p65 (dilution at 1:700). Then, HRP-labeled secondary antibodies diluted at 1:40 000 were used. The protein bands were visualized by using the ECL kit. Then the intensities of the protein bands were analyzed by employing Gel-Pro 3.2 software package. β-actin protein was used as the internal control.

1.8 Statistical Analysis

All data were expressed as mean±standard deviation (SD). Database was set up with SPSS 11.0 software package (SPSS Inc., USA). Differences among groups were analyzed by one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls test, when applicable. A P value less than 0.05 was considered to be statistically significant.

2 RESULTS

2.1 Effects of Sal on THP-1 Cell Viability

To investigate the toxicity under the experimental condition, THP-1 cells were treated with different concentrations of Sal. As shown in fig. 1, CCK8 assay revealed that 25–200 μg/mL Sal did not exert any cytotoxic effects on THP-1 cells over a period of 24 h, which excluded non-specific cytotoxicity as a possibility for the decreased cytokines output.

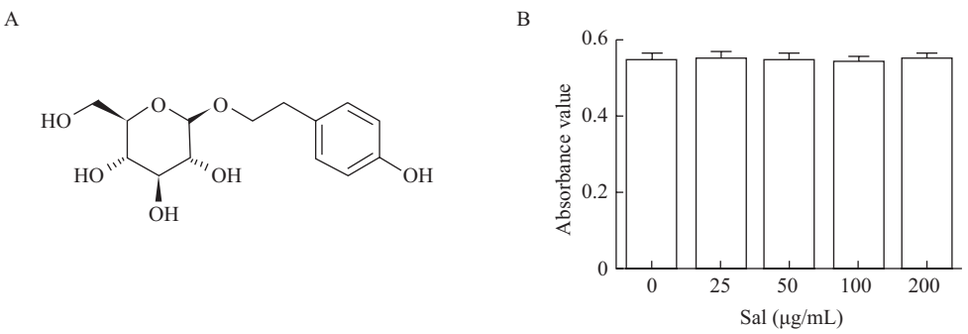

Fig. 1 The chemical structure of Sal and its effect on THP-1 cell viability
A: the chemical structure of Sal; B: the viability of THP-1 cells treated with a series concentration of Sal (0, 25, 50, 100 and 200 μg/mL)

2.2 Effects of Sal on Pro-inflammatory Cytokines Production in LPS- and Alcohol-stimulated THP-1 Cells

In the present study, the expression levels of MCP-1, TNF-α, IL-1β and IL-6 in the supernatant of LPS-stimulated THP-1 cells were increased significantly as compared with those in the control group (P<0.01). Different concentrations of Sal significantly decreased the levels of MCP-1, TNF-α, IL-1β and IL-6 as compared with the LPS+ethanol group (P<0.05 and P<0.01) (fig. 2A–2D). To further detect the changes of MCP-1, TNF-α, IL-1β and IL-6 in LPS- and alcohol-stimulated THP-1 cells, real-time PCR was used. Fig. 2E shows that the mRNA expression levels of MCP-1, TNF-α, IL-1β and IL-6 in THP-1 cells were significantly increased upon exposure to LPS and ethanol (all P<0.01). Treatment with Sal significantly inhibited LPS- and ethanol-induced mRNA expression of MCP-1, TNF-α, IL-1β and IL-6 (P<0.05 and P<0.01), and the inhibitory effect of Sal was dose-dependent.

2.3 Effects of Sal on NOS2 Production in LPS- and Alcohol-stimulated THP-1 Cells

To evaluate the effects of Sal on NOS2 expression, THP-1 cells were induced to differentiate into macrophages by pre-treatment with PMA, and then they were challenged with LPS and ethanol with or without addition of Sal at different concentrations for 24 h. The protein levels of NOS2 were increased by the dual stimulation of both LPS and alcohol. However, Sal significantly decreased NOS2 production in a dose-dependent manner (fig. 3A and 3B). Moreover, to investigate the mechanism by which Sal inhibits NOS2 production, mRNA expression was detected by real-time PCR. The findings showed that the mRNA expression levels of NOS2 was significantly increased following LPS and ethanol stimulation. Sal at 25–100

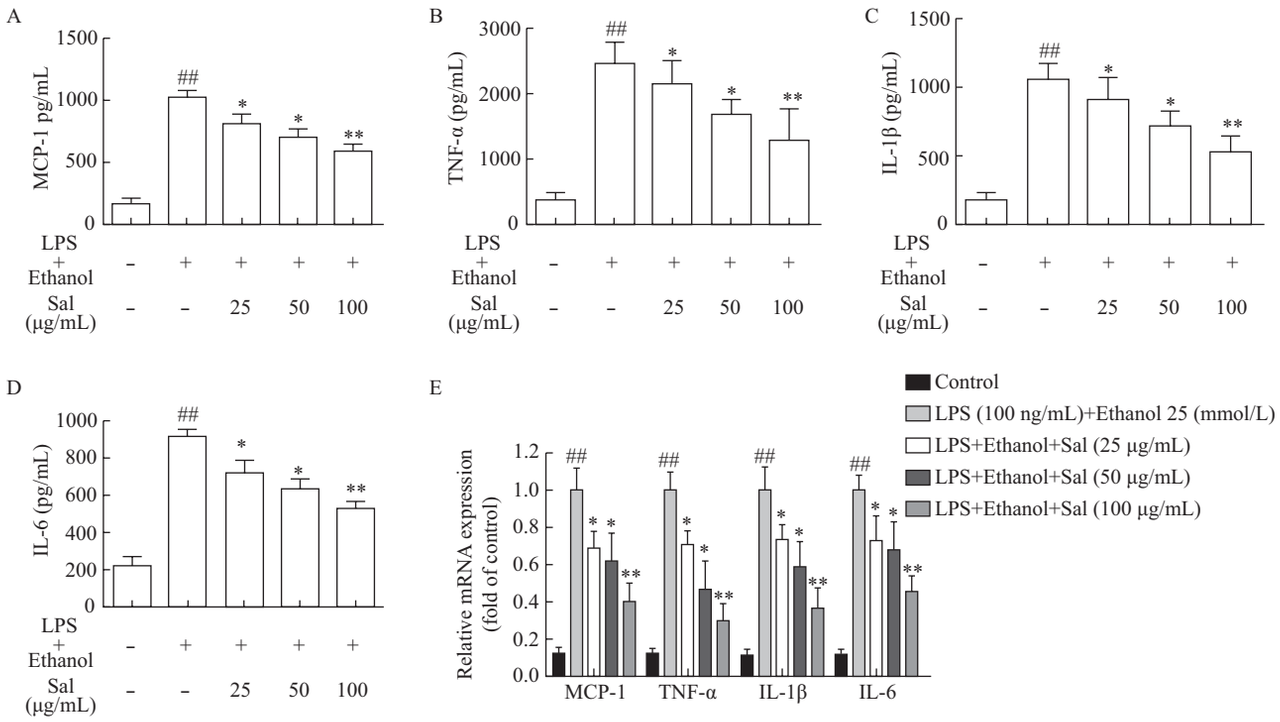

Fig. 2 Effects of Sal on LPS-alcohol-induced proinflammatory cytokines production in THP-1 cells
The levels of MCP-1 (A), TNF-α (B), IL-1β (C) and IL-6 (D) in the culture medium were measured by enzyme-linked immunosorbent assay (ELISA). The mRNA expression levels of MCP-1, TNF-α, IL-1β and IL-6 were assessed by real-time PCR (E). The values are expressed as the means±SD of three independent experiments. $^{\#\#}P<0.01$ *vs.* the control group; $^{*}P<0.05$, $^{**}P<0.01$ *vs.* the LPS+ethanol group

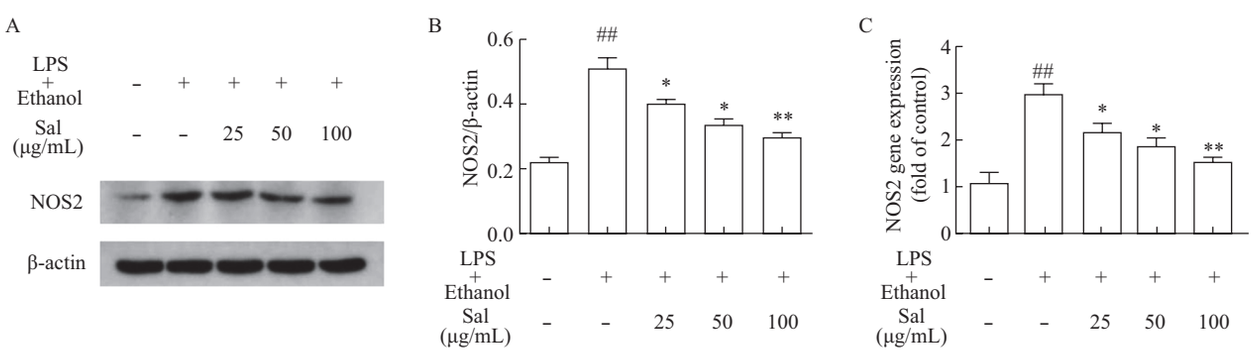

Fig. 3 Effects of Sal on NOS2 production in LPS- and alcohol-stimulated THP-1 cells
A: a representative Western blot result out of three experiments; B: means±SD of three independent experiments. C: The expression of NOS2 mRNA was assessed by Real-time PCR. $^{\#\#}P<0.01$ *vs.* the control group; $^{*}P<0.05$, $^{**}P<0.01$ *vs.* the LPS +ethanol group

μg/mL significantly reversed these abnormal changes compared to the LPS+ethanol group ($P<0.05$ and $P<0.01$), and a dose-effect relationship was found in the three Sal-treated groups (fig. 3C).

2.4 Effects of Sal on Notch1 and HES Activation in LPS- and Alcohol-stimulated THP-1 Cells

In this study, we found that upon the challenge with both LPS and alcohol, Notch1 was increased significantly. Sal at 25–100 μg/mL significantly suppressed the increase of Notch1 ($P<0.05$ and $P<0.01$) (fig. 4A) in a concentration-dependent manner (fig. 4B).

Since HES1 is a Notch-target gene that mediates part of its signaling activities, we further investigated the expression of HES by using Western blotting. As shown in fig. 4C, the HES level was remarkably increased in THP-1 cells stimulated by LPS and alcohol. Sal treatment decreased the HES1 expression in a concentration-dependent fashion (fig. 4C and 4D).

2.5 Effects of Sal on NF-κB Activation in LPS- and Alcohol-stimulated THP-1 Cells

Notch1 activation up-regulates LPS-induced

macrophage activation by increasing NF-κB activity[16]. In this study, in order to further understand the mechanism of Sal-mediated regulation in the activation of THP-1 cells, we investigated the expression of NF-κB in different groups. As shown in fig. 5A, the NF-κB p65 level was significantly up-regulated in the nucleus of THP-1 cells upon the stimulation with both LPS and ethanol. Following treatment with Sal, NF-κB level in the nucleus was remarkably down-regulated (fig. 5A). Moreover, we also observed that total IκBα was decreased with increase of p-IκBα in THP-1 cells subjected to the co-treatment of LPS and ethanol, and such change was inhibited by Sal (25–100 μg/mL) significantly (P<0.05 and P<0.01; fig. 5). These results indicated that Sal could modulate the activation of the NF-κB signaling cascade in macrophages simultaneously treated with LPS and alcohol.

2.6 Effects of Sal on PDP1 in LPS- and Alcohol-stimulated THP-1 Cells

To further investigate the mechanism of Sal inhibiting THP-1 activation induced by LPS in combination with alcohol, the effects of Sal on the expression of PDP1 was evaluated. This study showed that co-stimulation of LPS plus alcohol up-regulated PDP1 in THP-1 cells, while Sal at 25–100 μg/mL suppressed the increase of PDP1 (P<0.05 and P<0.01; fig. 6) in a concentration-dependent manner.

3 DISCUSSION

Previous studies have found that Sal could effectively inhibit inflammation, especially the LPS-induced inflammation, including mastitis, endotoxemia and acute lung injury in murine models[28–30]. In these inflammatory events, one or more MAPK signal pathways were gradually phorsphorylated and inhibited by Sal subsequently[28, 29]. However, the role of another important inflammatory regulator, Notch signaling pathway, in the process is still unknown. Moreover, various chronic inflammatory diseases are pathophysiologically mediated by a wide array of inflammatory and immune cells, such as macrophages and monocytes. With regard to the liver pro-inflammatory microenvironment, alcohol-sensitized macrophages are activated by LPS from bacteria translocated from the gut, which is a key event contributing to the pathogenesis of ALD[31]. Therefore, in this study, based on a cell model that mimics the hepatic inflammation microenvironment in ALD, we tried to prove that Sal might inhibit the production of inflammatory cytokines by THP-1 cells through Notch signaling pathway, which might be one of the mechanisms by which Sal inhibits inflammation.

In the hepatic microenvironment, polarization of macrophages from M0 type to M1-proinflammatory

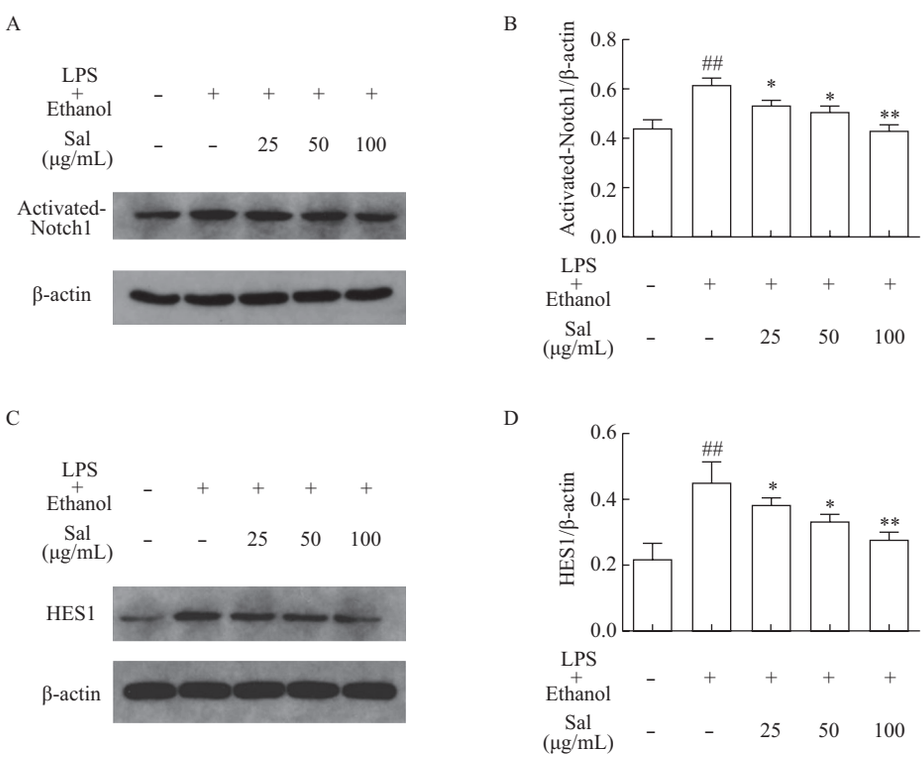

Fig. 4 Effects of Sal on Notch1 and HES1 activation in LPS combined with alcohol-stimulated THP-1 cells. THP-1 cells were co-stimulated with LPS (100 ng/mL) and alcohol in the presence or absence of Sal at different concentrations (25, 50 and 100 μg/mL) for 24 h. The expression of Notch1 (A, B), and HES1 (C, D) was then detected by Western blot. Representative Western blot result for three experiments and means±SD for three independent experiments. $^{\#\#}P$<0.01 *vs.* the control group; $^{*}P$<0.05, $^{**}P$<0.01 *vs.* LPS+ethanol group

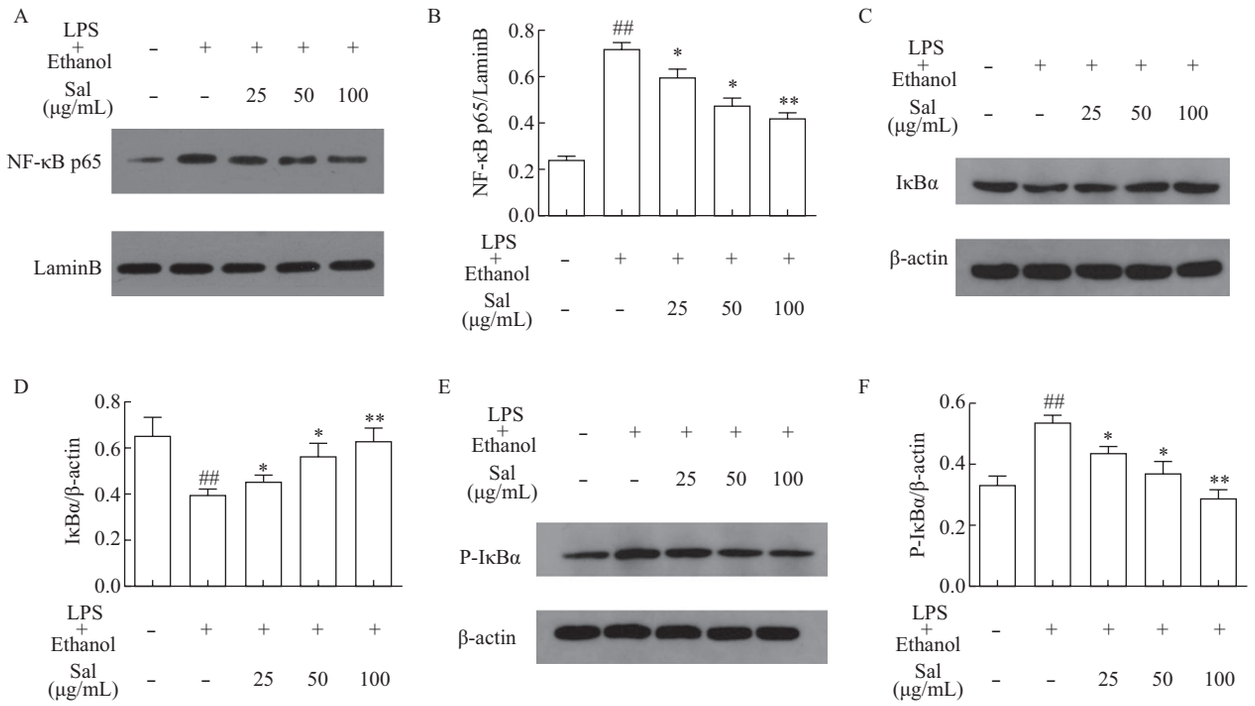

Fig. 5 Effects of Sal on NF-κB activation in LPS- and alcohol-stimulated THP-1 cells
THP-1 cells were co-stimulated with LPS (100 ng/mL) and alcohol in the presence or absence of Sal at different concentrations (0, 25, 50 and 100 μg/mL) for 24 h. The nucleus NF-κB p65 (A, B), IκBα (C, D) and total p-IκBα (E, F) were then detected by Western blot. Representative Western blot result for three experiments and means±SD for three independent experiments. $^{\#\#}P$<0.01 *vs.* the control group; $^{*}P$<0.05, $^{**}P$<0.01 *vs.* the LPS+ethanol group

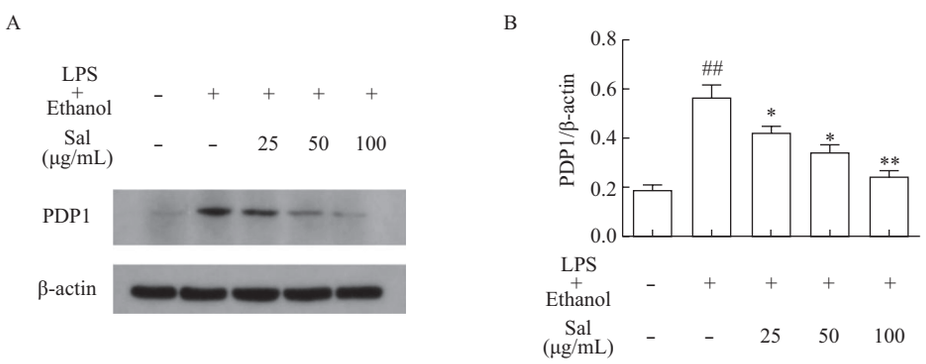

Fig. 6 Effects of Sal on PDP1 in LPS- and alcohol-stimulated THP-1 cells
A: Western blotting for PDP1 (a representative result out of three experiments); B: relative ratio of PDP1/β-actin. Data are shown as the mean±SD (n=3). $^{\#}P$<0.05, $^{\#\#}P$<0.01 *vs.* the control group; $^{*}P$<0.05, $^{**}P$<0.01 *vs.* the LPS+ethanol group

type plays an essential role in the pathogenesis of liver diseases such as ALD[26]. In response to chronic alcohol consumption, macrophages, stimulated by ethanol from hepatic microcirculation and LPS from bacteria translocated from intestines, release inflammatory cytokines, such as TNF-α, IL-6, IL-1β and MCP-1, thereby leading to injury of hepatocytes and recruitment of extrahepatic immune cells. These inflammatory mediators and cells result in inflammatory injury in liver microenvironment and subsequently contribute to liver injury, which progresses to advanced stages, including alcoholic hepatitis, alcoholic fibrosis and cirrhosis[32]. Moreover, TNF-α and MCP-1 have also been proved to be the upstream regulators promoting excessive fatty deposition in liver cells by increasing lipid synthesis and decreasing lipid degradation[26]. Mechanistically, TNF-α and MCP-1 inhibit peroxisome proliferator-activated receptor alpha (PPARα) nuclear translocation and its downstream gene expression for lipid oxidation, and enhance the Sterol-regulatory element binding protein 1 (SREBP1) nuclear translocation and its downstream gene expression[33–35]. Therefore, regulation on the activation of M1-proinflammatory macrophages in liver microenvironment is an important

and promising bridge linking hepatic immunity and lipid metabolism in ALD. In this study, after co-stimulation with alcohol plus LPS, inflammatory cytokines, such as TNF-α, IL-6, IL-1β and MCP-1, were increased in THP-1 macrophages with elevated expression of M1 gene NOS2. On the contrary, Sal suppressed this M1-proinflammatory activation significantly, as demonstrated by decreased production of TNF-α, IL-6, IL-1β and MCP-1 and down-regulated expression of NOS2. Our results were consistent with findings reported by previous studies, which also showed Sal had a similar inhibitory effect on mouse RAW 264.7 macrophages and could attenuate inflammatory responses in LPS-induced murine endoxemia, mastitis and acute lung injury[29].

It is well documented that the LPS-induced transcriptional regulation of most pro-inflammatory genes is largely dependent on NF-κB activation[36]. In general, NF-κB is located in the cytoplasm as an inactive complex bound to IκBα. When stimulated, NF-κB is dissociated from IκBα and translocated into the nucleus, thus leading to transcription of inflammatory mediators. Moreover, NF-κB activation is the principal pathway of Notch signaling pathways that regulate macrophage-involved inflammatory responses. In macrophages, different Notch receptors and ligands are expressed on cellular surface. Under certain circumstances, such as LPS challenge or activation of other TLR ligands, Notch1 expression is up-regulated and Notch signaling is activated subsequently. In these processes, Notch1 up-regulates LPS-induced macrophage activation by increasing NF-κB activity, favoring the expression of genes implicated in the inflammatory response, such as TNF-α, IL-6, IL-1β and MCP-1[37]. Upon dual stimulation of both alcohol and LPS, Notch signaling and NF-κB activity were increased markedly, as demonstrated by enhanced Notch1/Hes1 expression and increased p-IκBα and decreased total IκBα.

These changes were blocked by Sal in a dose-dependent fashion. These data suggested that Sal could down-regulate Notch1-HES1 signaling to suppress the activation of M1-macrophages, which might be achieved by inhibiting NF-κB activity. The exact molecular mechanism warrants further study.

Recently, mounting evidence revealed that metabolic reprogramming drives activated macrophages to switch into M1 pro-inflammatory phenotype in response to LPS stimulation. Under this circumstance, macrophages' metabolism withdraws from oxidative phosphorylation (OXPHOS) and switches towards glycolysis in order to quickly generate ATP that favors the pro-inflammatory state, in which mitochondrial reactive oxygen species (ROS) generation is critical to the determination of the inflammatory phenotype of macrophages[38, 39]. Similarly in ALD, LPS-mediated activation of Notch signaling increases the uptake and lysis of glucose to stimulate the generation of ATP and mtROS, resulting in M1 macrophage activation as indicated by up-regulated TNF-α, IL-1β and iNOS[40]. In these processes, Notch1 increases the expression of PDP1, which subsequently enhances glycolysis and concurrent glucose flux to the TCA cycle via the PDP1-mediated activation of pyruvate dehydrogenase (PDH). In this study, the expression of Notch1 and PDP1 was increased significantly in response to the combined stimulation of both alcohol and LPS in THP-1 macrophages. Interestingly, the Notch-dependent induction of PDP1 was decreased by Sal. As the concentration of Sal increased, these effects became stronger. These results suggested that Sal might also affect the Notch-mediated metabolic reprogramming in alcohol-LPS-stimulated THP-1 macrophages. Further studies are needed to elucidate the precise mechanism of Sal regulating the metabolic switch of OXPHOS to glycolysis in macrophage polarization, and such research is now underway.

In conclusion, in this study, by using alcohol in combination with LPS to activate THP-1 macrophages *in vitro* and to mimic liver microenvironment in ALD, we demonstrated that Sal achieved its effect by regulating M1 proinflammatory activation via dual Notch signaling pathways. Regulation of NF-κB activation by Notch1 and PDP1-mediated mitochondrial metabolism may be implicated in such regulation. Our study suggests that Sal, due to its role in Notch signaling pathway, might be therapeutically valuable for the treatment of ALD.

Conflict of Interest Statement

We declare that there are no conflicts of interests.

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