2023 년 2 월

석사학위 논문

Inhibitory efficacy of *Castanopsis sieboldii* on hepatitis via kupffer cell regulation

조선대학교 대학원

약 학 과

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2023 년 2 월 24 일

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이 논문을 약학 석사학위신청 논문으로 제출함

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2022 년 12 월

조선대학교 대학원

CONTENTS

LIST OF FIGURES

ABBREVIATIONS

LPS	Lipopolysaccharide
ImKC	Immortalized murine kupffer cell
NO	Nitric Oxide
iNOS	Inducible nitric oxide synthase
NLRP3	NLR family pyrin domain containing 3
GSDMD	Gasdermin-D
CCl_4	Carbon tetrachloride
MAPK	Mitogen-activated protein kinase

국 문 초 록

Kupffer cell 조절을 통한 구실잣밤의 간염억제 효능 연구

김 재 민

지 도 교 수: 기 성 환

약학과

조선대학교 대학원

간은 인체에서 체내 대사 및 해독작용을 담당하는 가장 큰 기관으로 70%의 간 실질세포와 30%의 쿠퍼세포, 간성상세포를 포함하는 비 실질세포로 이루어져 있다. 과도한 알코올이나 고지방식이 섭취와 같은 요인들에 의해 장벽의 기능이 약화되면 장벽의 투과성이 증가하고 장내 내독소가 간으로 유입 될 수 있다. 이는 간에서 염증반응을 유도하여 간염증, 섬유증 및 간암과 같은 간질환을 유발할 수 있다. 쿠퍼세포는 간 내 상주하는 대식세포로써, 활성화되어 자극에 다라 전염증성 형태인 M1 표현형 또는 항염증성 형태인 M2 표현형으로 분극화 되며 간 내 면역 및 수복반응을 담당한다. 현재까지 보고 된 연구결과에 따르면 M1 포현형으로 분극화한 쿠퍼세포에서 분비된 사이토카인이 간성상세포의 활성화에 영향을 주어 간

섬유화 및 간염의 발병에 영향을 주는 것으로 알려져 있다. 따라서 쿠퍼세포의 분극화 제어 약물 발굴과 그 기전에 대한 이해가 간질환의 발병 및 진행에 중요하다.

최근 한반도 남부, 특히 제주 등지에 분포하는 난대수종 구실잣밤나무 열매 추출물의 항산화 및 세포사멸 억제 효능에 대한 연구결과가 보고 된 바 있다. 하지만 염증에 효과가 있다고 알려진 EGCG(epigallocatechin gallate)와 GA(gallic acid)가 높은 함량을 이루고 있지만 염증을 비롯한 면역반응 조절에 대한 구실잣밤나무 잎추출물의 효능연구는 거의 이루어지지 않고 있는 실정이다. 따라서 본 연구에서는 LPS(지질다당체, 내독소)를 이용한 염증성 손상 유도 시 간 내 대식세포인 쿠퍼세포의 분극화에 대한 구실잣밤나무 잎추출물의 효능 및 관련 보호기전 연구를 진행하고자 한다.

I. INTRODUCTION

The largest solid organ in the human body is the liver which is responsible for metabolism and detoxification. The liver mass is composed of 80% of parenchyma cells (hepatocytes) and 20% of non-parenchymal cells (lymphocytes, kupffer cells, hepatic stellate cells and endothelial cells) [1]. Kupffer cells, the liver-resident macrophage, play a central role in the pathogenesis of a diversity of liver diseases including alcoholic liver disease, steatohepatitis, intrahepatic cholestasis, viral hepatitis and liver fibrosis [2]. Activated Kupffer cells, in liver injury, are a major source of inflammatory mediators including nitric oxide, cytokines, chemokines, superoxide, eicosanoids [3], resulting in an excessive inflammatory response and hepatitis.

Hepatitis can be split into acute and chronic based on the duration of the inflammation of the liver. The natural recovery of acute hepatitis is sometimes possible [4], however, treatment is limited as antiviral agents or corticosteroids. The treatment of choice for patients with severe alcoholic hepatitis (AH), autoimmune hepatitis is use of corticosteroids. However, the use of corticosteroids acute hepatitis is still controversial until now. In contrast, chronic hepatitis can cause chronic liver diseases including liver fibrosis, cirrhosis, liver cancer leading to significant morbidity and mortality [5]. Current treatments for end stage of liver diseases such as liver failure or decompensated cirrhosis are still limited, and liver transplantation remnants the only available approach to improve survival. Thus,

research on identification of new drug targets and candidate drugs that can control acute hepatitis is very important.

Kupffer cells are responsible for immune and repair reactions in the liver and polarized into M1 phenotype (a pro-inflammatory form) or M2 phenotype (an anti-inflammatory form) [6, 7]. Polarization to the M1 phenotype, induced by LPS, TNF-α and IFN-γ and express pro-inflammatory cytokines, are involved in the pathogenesis of liver inflammation. When kupffer cells stimulated by LPS and/or IFN-γ, also formed the NLRP3 inflammasome and GSDMD is cleaved, resulting in pyroptosis. Pyroptosis, a kind of lytic programmed cell death, is stimulated by proinflammatory signals and connected with inflammation [8, 9]. Accumulation evidence show that pyroptosis in kupffer cells also contributes to sterile inflammatory liver diseases via release of pro-inflammatory cytokines like as IL18- or IL-1β [10]. However, there is no candidates for the treatment or prevention of acute hepatitis targeting pyroptosis in Kupffer cells.

Castanopsis sieboldii, a species of the warm-temperature tree species, is native to the southern part of the Korean Peninsula and Jeju Island. The warm-temperature tree species, distributed in subtropical regions, is gradually expanding on the Korean Peninsula due to global warming and climate change. The fruits of the *Castanopsis sieboldii* have been used for food since ancient times, and the its branches have recently been used as wood. In addition, it is known that the leaf extract of the *Castanopsis sieboldii* has high antioxidant and antibacterial activity

[11]. Recently, we identified that extract of *Castanopsis sieboldii* leaf (CSL) has cytoprotective and antioxidant efficacy in skin keratinocytes against UV-mediated phototoxicity [not published]. However, the role of the CSL extract in anti-inflammatory efficacy against pyroptosis-mediated hepatitis has not scrutinized.

Therefore, the aim of current study is examined whether CSL3 inhibited inflammatory response and pyroptosis by LPS or LPS/ATP-induced activated macrophages. Furthermore, we investigated *in vivo* effect CSL3 in acute hepatitis mice model.

II. MATERIALS AND METHODS

1. Materials

Antibodies against IκBα, iNOS, β-tubulin, p65 and NLRP3 were acquired by Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-p38, p38, phospho-ERK1/2, ERK1/2, phospho-JNK1/2, JNK1/2, phospho-IκBα, Lamin A/C, and cleaved-caspase-1 antibodies were acquired from Cell Signaling (Danvers, MA). Interleukin 1 beta (IL-1β) antibody was acquired from R & D system (Minneapolis, Minnesota). Gasdermin-D (GSDMDC1) antibody was acquired from NOVUS Biologicals (Littleton, CO). Horseradish peroxidase-conjugated goat anti-rabbit, anti-goat, anti-mouse antibodies were acquired from Invitrogen (Carlsbad, CA). ATP (Adenosine 5'-triphosphate), LPS (Escherichia coli 055:B5), β-actin antibody and sodium nitrite were acquired from Sigma Chemicals (St. Louis, MO).

2. Preparation of 70% ethanol extracts of *Castanopsis sieboldii* leaf (CSL3)

The CSL3 used in this study were collected around April 2020, supplied from Wando Arboretum (Wando-gun, Jeollanam-do, Korea), washed, dried with hot air, stored, and ground. 1.5 kg of dried and ground CSL3 were added to 70% EtOH (v/v) 15.0 L and immersed at room temperature for 2 weeks to extract them. The immersed sample was filtered using a vacuum filtration device and Whatman No. 1, and then the residue separated by this method was repeatedly carried out once under the same conditions. The filtrate obtained by filtration was concentrated in a rotary

vacuum evaporator at 37–40°C and then freeze-dried and used.

3. Cell Culture

RAW 264.7 cells, a murine macrophage cell line was purchased from ATCC (American Type Culture Collection, Rockville, MD, USA). Cells were preserved in DMEM (high glucose) added with 10% fetal bovine serum (FBS), 50 units/mL penicillin/streptomycin at 37 °C in a humidified 5% CO_2 atmosphere.

4. WST-1 cell viability assay

The cell viability in ImKCs and RAW264.7 cells were estimated using a EZ-Cytox kit (DoGenBio Co., LTD, Seoul, Korea). Cells were plated in 12 well plates incubated with CSL3 alone for 12 h. Cells were then changed medium with 5% WST-1 reagent for 0.5 h. Then, transferred 100 μl the cell medium to microplate. Absorbance at 450 nm (600 nm was used as reference wavelength) was analyzed on a microplate reader (Spectra MAX, Molecular Device).

5. Nitrite oxide (NO) production assay

NO production was evaluated by Griess reaction (Sigma, St. Louis, MO). Cells were pre-treated with CSL3 for 1 h, and then incubated with LPS for 15 h. Transferred 100 μl the cell medium to microplate then, added griess reaction 100 μl (0.04g/ml dissolved in DW) and reacted at room temperature for 0.5 h. Absorbance

at 540 nm was analyzed on a microplate reader (Spectra MAX, Molecular Device).

6. Immunoblot Analysis

Subcellular fractionation and protein extraction, SDS-polyacrylamide gel electrophoresis and immunoblot analysis were performed as previously published method. Protein extracts were separated via 7.5% and 12% gel by electrophoresis, and then electrophoretically transferred to nitrocellulose membranes. After the membranes were blocked with 5% skim milk for 0.5 h, they were incubated with primary antibody at 4℃ overnight with 20rpm shook and then incubated with a horseradish peroxidase-conjugated secondary antibody. Immunoreactive protein was visualized by ECL chemiluminescence detection kit. β-actin was used as control and β-tubulin or lamin A/C used as verified for integrity of subcellular fractionation.

7. RNA Isolation and RT-PCR Analysis

Total RNA was separated by TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. The cDNA acquired was escalated with a cDNA synthesis kit (Bioneer, Daejeon, Korea) using a thermal cycler (Bio-Rad, Hercules, CA, USA). To acquire cDNA, total RNA (2 μg) was reverse-transcribed by an oligo $(dT)_{18}$ primer. Primers were synthesized by Bioneer. The following primer sequences were used: mouse iNOS sense:5′-CCTCCTCCACCCTACCAAGT-3′,

antisense:5′-CACCCAAAGTGCTTCAGTCA-3′; mouse TNF-α sense:5′-AAGCCTGTAGCCCACGTCGTA-3′, antisense:5′-AGGTACAACCCATCGGCTGG-3′; mouse IL-6 sense:5′-TCCATCCAGTTGCCTTCTTG-3′, antisense:5′-TTCCACGATTTCCCAGAGAAC-3′; mouse IL-1β sense: , antisense:5′-AGAAGGTGCTCATGTCCTCA-3′; mouse GAPDH sense:5′-TGCCCCCATGTTTGTGATG-3′ and antisense:5′-TGTGGTCATGAGCCCTTCC-3′. GAPDH was used as a control for RT-PCR.

8. Reporter Gene Assay

To analyze the activities of the *iNOS* promoter constructs, luciferase reporter assays were established in cells stably transfected with the *iNOS* gene promoter, pGL-miNOS-1588, which contains the murine *iNOS* promoter from –1588 to +165 bp as previously described [12]. For the luciferase assay, cells were replated in 12-well plates overnight, and then treated with 0.1 μg/ml LPS in the presence or absence of CSL3 for 6h. After discarding medium, passive lysis buffer (Promega, Madison, WI) was immediately added to the cells. Luciferase assay reagent II (Promega) was added in lysates, and the luciferase activities were analyzed using a luminometer (Promega). The relative iNOS promoter driven-luciferase activity was calculated via normalizing luciferase activity to the protein concentration.

9. Enzyme-linked immunosorbent assay (ELISA)

IL-1β, IL-6, TNF-α were quantified using ELISA kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. IL-1β, IL-6, TNF-α contents in cell supernatant or serum were analyzed via ELISA, using anti-mouse IL-1β, TNF-α or IL-6 antibodies and biotinylated secondary antibodies according to manufacturer's recommendations.

10. Animals and Treatment

Male ICR mice (six weeks old) were purchased from Oriental Bio (Sung-nam, Korea) and acclimated for 1 wk. The mice (n = 8/group) were housed at 20 ± 2 ◦C under pathogenic-free air filtered at a 12-hour light/dark cycle and a relative humidity of 50 ± 5%, and food (Purina, Korea) and water were available. Mice (N=8/group) were treated with CSL3 (250 or 500 mg/kg CSL3) dissolved in 40% PEG for 5 day with oral administration. After that, to induce hepatitis, CCl_4 (1 mL/kg) dissolved in olive oil (10%) was intraperitoneally injected into the mice for 24 h. Mice were sacrificed after 24 h, and the blood and livers were harvested.

11. Blood chemistry

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed by commercial kits (Asan Pharmaceutical, Seoul, Korea).

12. Histopathological examination

Liver tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin wax and cut into 3 μm thick sections, which were stained with hematoxylin and eosin (H&E) for routine examination. To estimate the histopathological changes, the stained tissue samples were identified under a light microscope. An arbitrary scope was given to each microscopic field viewed at magnifications of ×40–200. At least 5 fields were scored per liver section to acquire the mean value. The scoring system for CCl_4 chronic changes of hepatic inflammation, balloon degeneration and necrosis were evaluated according to a score proposed by previous described. The scores of hepatic inflammation are as follows: Absent: Score 0; Small amount of cells present at the junction of the necrotic zone: Score 1; Normal amount of cells present: Score 2; Predominantly neutrophils present: Score 3; Predominantly mononucleic cells present: Score 4 [13]. Necrosis extent was graded as: None: Score 0; Single cell necrosis: Score 1; –30%: Score 2; –60%: Score 3; >60%: Score 4 [14]. The scores of hepatocyte ballooning are as follows: None: Score 0; Few, in apparent: Score 1; Easily noted, many: Score 2 [15].

13. Statistical analysis

One-way analysis of variance (ANOVA) was used to evaluate statistical consequence of differences among treatment groups. For each statistically notable effect of treatment, the Newman-Keuls test was used for comparisons between

multiple group means. The data were expressed as means ± S.E.

III. RESULTS

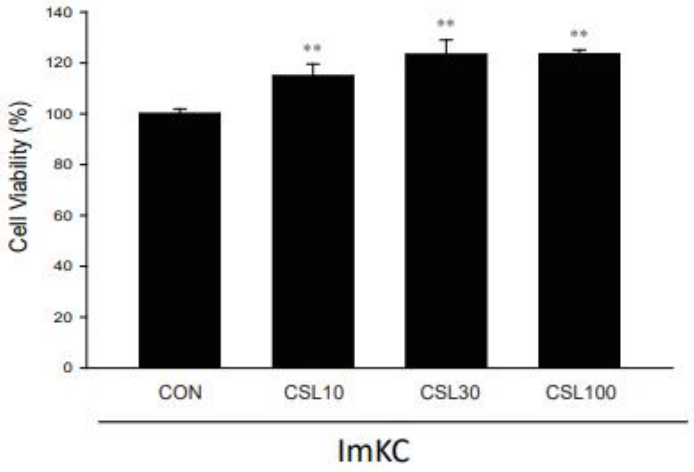
1. Suppression of LPS-induced iNOS expression and inflammatory cytokines by CSL3

For this study, we prepared were using CSL3. First of all, cytotoxicity of CSL3 in macrophage (ImKCs and Raw264.7 cells) was measured by MTT, and as a result, there was no cytotoxicity up to 100 μg/mL in both cells (Fig 1). The expression of the inducible nitric oxide synthase (iNOS) is one of the direct consequences of an inflammatory process [16]. Therefore, we investigate whether CSL3 can reduce inflammation effect, we determined level of inducible nitric oxide synthase (iNOS) expression in ImKCs and RAW264.7 cells. LPS-stimulated iNOS protein level was reduced by CSL3 treatment in a dose-dependent manner (Fig 2 and B). Then, we investigated whether inhibitory effect of CSL3 on iNOS transcriptional regulation in macrophages. Likewise, CSL3 inhibited LPS-induced mRNA level of iNOS in ImKCs and RAW264.7 cells (Fig 2C and D). Also, CSL3 treatment suppressed the increase of iNOS promoter activity by LPS in stably transfected RAW264.7 cells (Fig 2E). Nitric oxide (NO) produced by iNOS has been involved in both DNA damage induction and aberrant cell signalling in various tissue and cell backgrounds [17]. We thus evaluated whether CSL3 could inhibit NO production by LPS in ImKCs. The increased NO production due to LPS was reduced by CSL3 treatment in a concentration-dependent manner (Fig 2F).

Next, we established about effect of CSL3 on pro-inflammatory cytokines (TNF-α

and IL-6). We measured the mRNA expression of inflammatory cytokines (TNF-α and IL-6) by RT-PCR analysis and the release of cytokines into the media by ELISA. Treatment with LPS elevated the mRNA expression inflammatory cytokines and release of cytokines into the media, however pretreatment of CSL3 notably abolished them (Fig 3). In conclusion, our data showed that CSL3 alleviates LPS-induced NO production by reducing iNOS expression and release of pro-inflammatory cytokines.

A)



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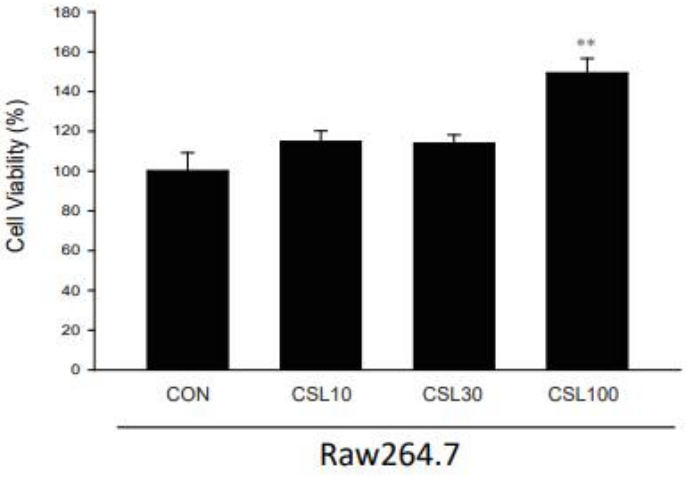


Figure 1. Cytotoxicity of CSL3 in macrophage (ImKCs and Raw264.7 cells) (A and B) Effect of CSL3 (10-100 μg/mL, 24 h) on cytotoxicity was estimated using MTT assays in ImKCs and Raw264.7 cells. Data represent the mean ± S.E. of three replicates; **P<0.01, significant versus vehicle-treated control

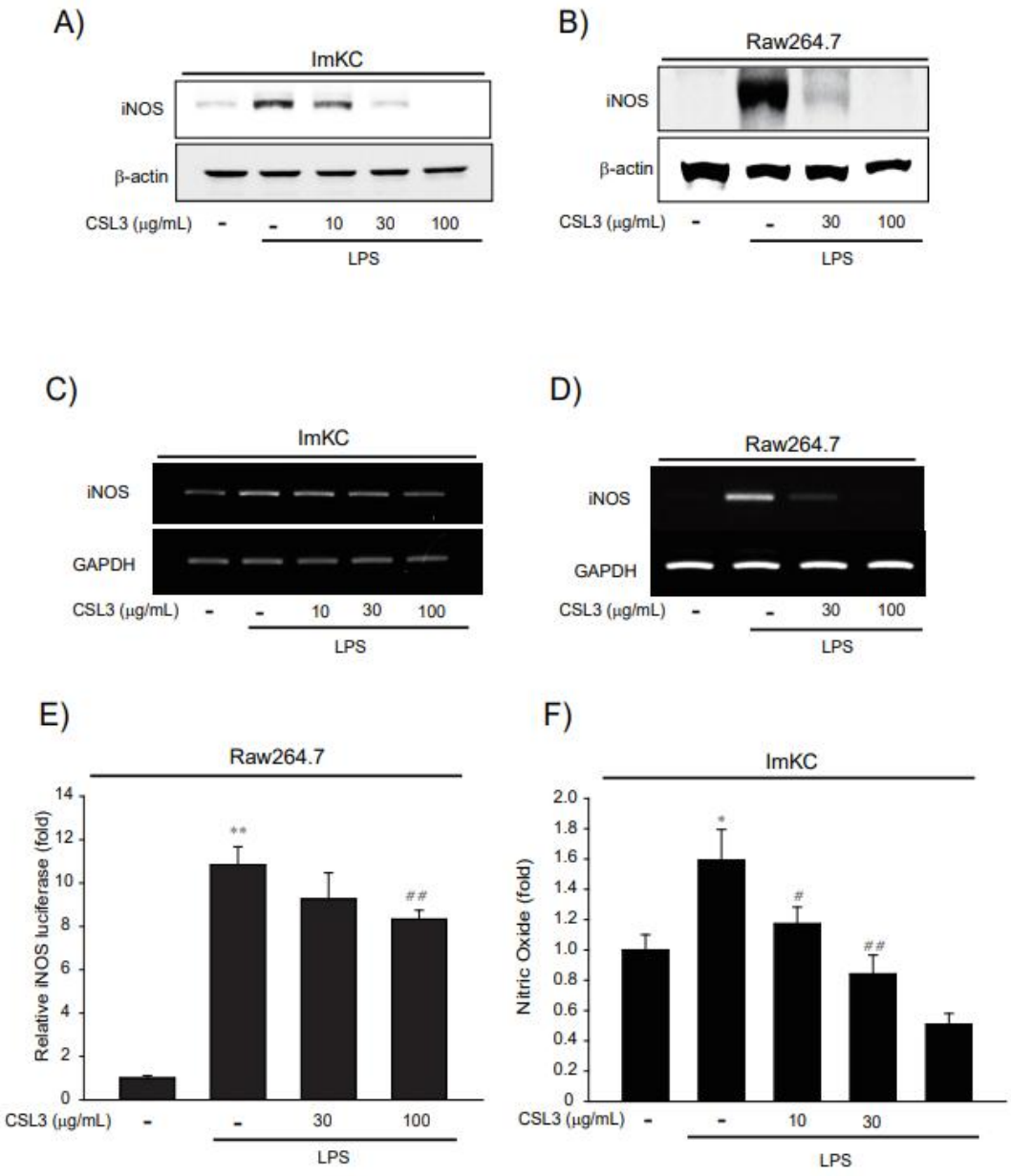


Figure 2. Suppression of iNOS expression and NO production by CSL3 in LPS-activated macrophage

(A and B) The expression level of iNOS protein in macrophages. ImKC (A) or Raw264.7 cell (B) were pre-treated with CSL for 0.5 h before LPS for 12 h. L iNOS protein expression was visualized by immunoblotting and results were verified by repeated experiments. (C, D) The inhibition effect of CSL3 on iNOS transcriptional regulation by LPS in macrophages. ImKC (C) or Raw264.7 cell (D) were pre-treated with CSL3 1 h and treated with LPS. After 3 h, expression level of iNOS was evaluated by PCR analysis and results were confirmed by repeated experiments. (E) iNOS luciferase assays were performed in cells stably transfected with pGL-miNOS-1588, which contains murine *iNOS* promoter from −1588 to +165 bp and exposed to LPS and/or CSL3 in RAW264.7 cells for 12 h. (F) NO production was measured by Griess reaction. ImKCs were pretreated with CSL3 for 1 h, and then incubated with LPS for 15 h. (n = 3, significant when compared with controls, *$p<0.05$, compared with the LPS treatment group, ## $p<0.01$, # $p<0.05$). (n = 3, significant when compared with controls, **$p<0.01$, compared with the LPS treatment group, ## $p<0.01$).

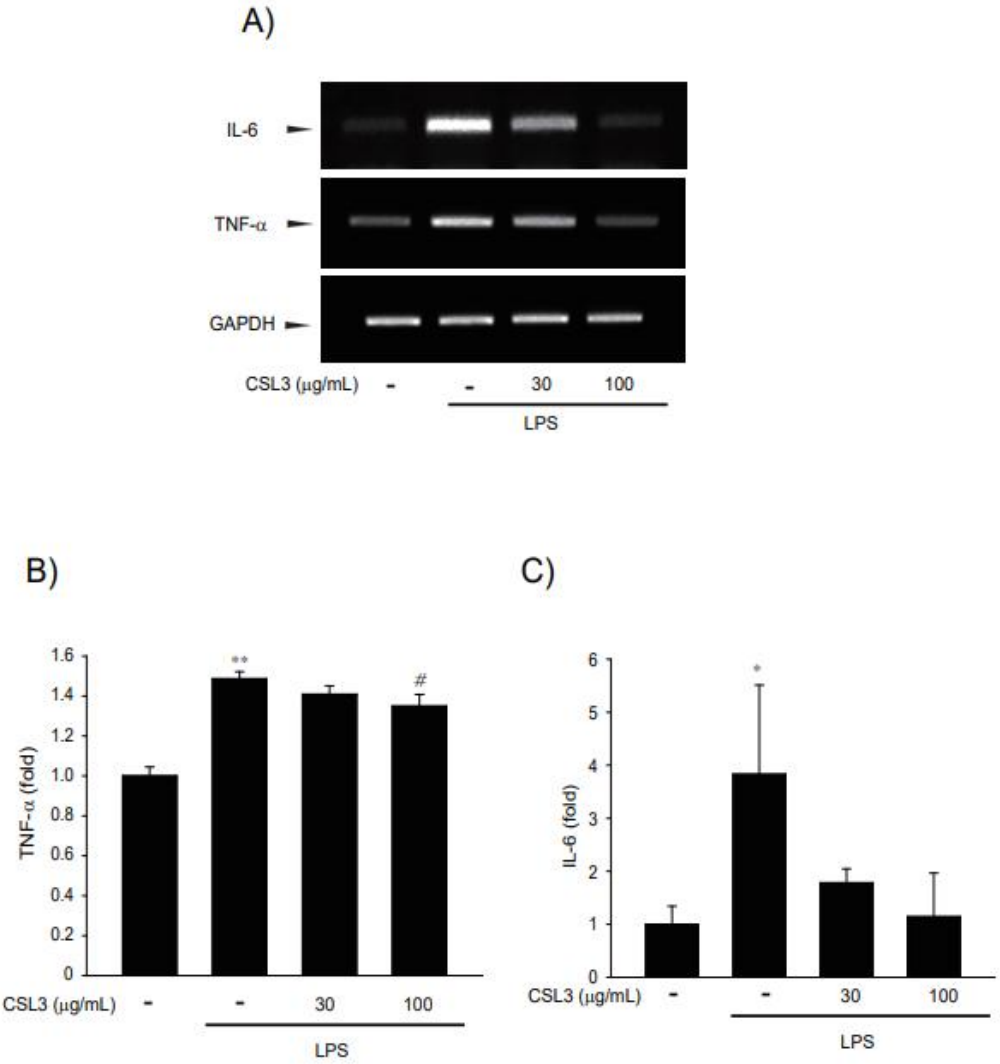


Figure 3. Suppression of inflammatory gene expression through CSL3 in LPS-activated ImKC

ImKC were pretreated with CSL3 for 1 h and treated with LPS for 3 h. (A) mRNA levels of inflammatory cytokines (IL-6 and TNF-α) were evaluated by RT-PCR using GAPDH as control and results were verified by repeated experiments. (B) IL-6 and TNF-α release into cell supernatant was identified by ELISA analysis (n = 3, significant when compared with controls, **$p<0.01$, *$p<0.05$, compared with the LPS treatment group, ## $p<0.01$, # $p<0.05$).

2. Suppression of LPS-induced NF-κB, AP-1 and MAPK activation by CSL3 on ImKCs

NF-κB and AP-1 can be activated in cells treated with inflammatory stimuli and regulate the transcriptional activation of inflammatory gene expression [18, 19], and nuclear translocation of NF-κB is preceded by phosphorylation and subsequent degradation of IκBα [20]. LPS increases phosphorylation and degradation of IκBα, and this step was completely blocked by pretreatment with CSL3 (Fig 4A and B). Furthermore, treatment of CSL3 inhibited the increase p65 protein levels by LPS in the nuclear (Fig 4C). The inducible transcriptional complex AP-1 (composed of c-Fos and c-Jun proteins) is crucial for cell adaptation to many environmental changes [21]. To determine the inhibitory effect of CSL3 on LPS-induced AP-1 activation, we performed immunoblot analysis. LPS increased the phosphorylation of c-Jun and c-Fos in the lysate cells but not in those of CSL3 treated cells (Fig 4D).

MAPKs including ERK, p38 and JNK initiates the downstream induction of transcription factors, like NF-κB and AP-1 of the pro-inflammatory genes [22]. Therefore, we estimated the effect of CSL3 on the LPS-induced activation of MAPKs. Phosphorylation of MAPKs (ERK, p38 and JNK) were increased by LPS alone, whereas were decreased in cells treated with CSL3 (Fig 4E). These results indicate that anti-proinflammatory effect of RSL3 might be involved suppression of NF-κB, AP-1 and MAPK activation.

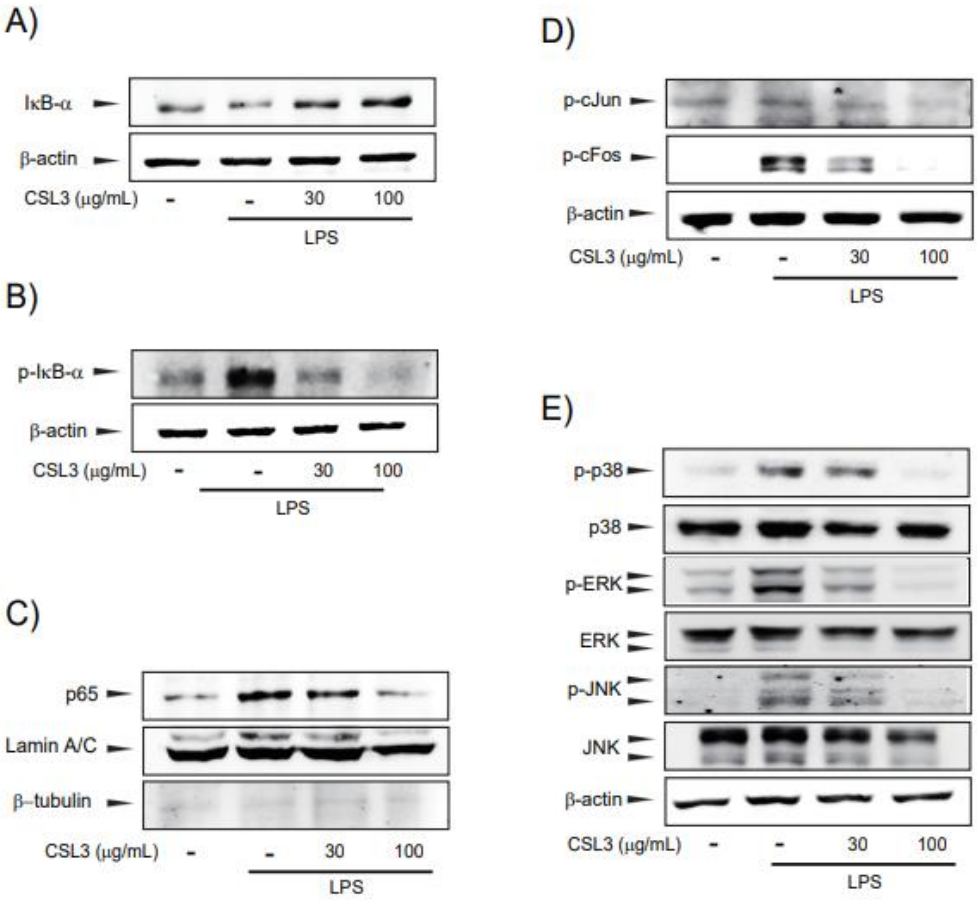
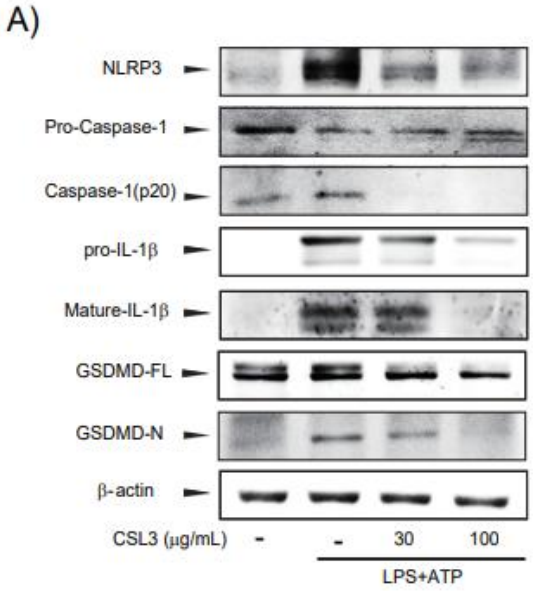


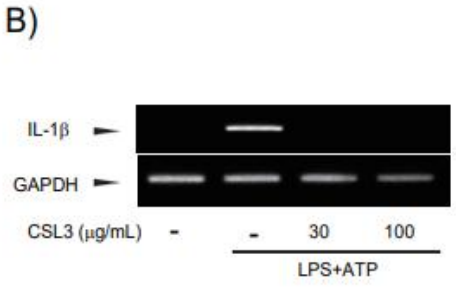
Figure 4. Suppression of NF-κB and MAPK activation by CSL3 in LPS-activated ImKC

(A and B) Immunoblotting for total (A) or phosphorylated IκBα (B). The cells were pretreated with CSL3 for 1 h before LPS stimulation for 15 min. Total or phosphorylated IκBα was immunoblotted in the cell lysate. Results were verified by repeated experiments. (C) The expression level of p65 protein in ImKCs nuclear with nuclear fraction. Using Lamin A/C as control for nuclear fraction and β-tubulin as control for cytosolic fraction. ImKCs were pretreated CSL3 for 1 h and then incubated with LPS for 30 min. Results were verified by repeated experiments. (D) The expression of phosphorylated c-Fos and c-Jun protein in ImKCs. The cells were pretreated with CSL3 for 1 h before LPS stimulation for 2h. c-Fos and c-Jun phosphorylation were immunoblotted in the cell lysate. Results were validated by repeated experiments. (E) The expression level of phosphorylation MAPK phosphorylation in ImKCs. Cells were treated LPS and/or CSL3 in RAW264.7 cells for 1 h. The cell lysates were immunoblotted and results were validated by repeated experiments.

3. Suppression of LPS/ATP-stimulated pyroptosis by CSL3 on ImKCs

Pyroptosis, a programmed cell death caused by inflammation, has some similarities to apoptosis, like as DNA damage and chromatin condensation [23]. Whereas, the unique characteristics of pyroptosis are different from apoptosis [24]. Pyroptosis is mediated by inflammasome assembly, which is accompanied via NLRP3 activation, cleavage of caspase-1 and GSDMD, and IL-1β release [25]. Therefore, we investigated whether CSL3 inhibits NLRP3 inflammasome activation and pyroptosis by LPS/ATP in ImKCs. As result, LPS/ATP treatment increases IL-1β maturation, cleavage of caspase-1 and GSDMD, and NLRP3 expression, whereas CRSL3 treatment decreased them (Fig 5A). Also, CSL3 suppressed level of IL-1β mRNA expression, and decreased IL-1β release in ImKC culture supernatant (Fig 5B and C).





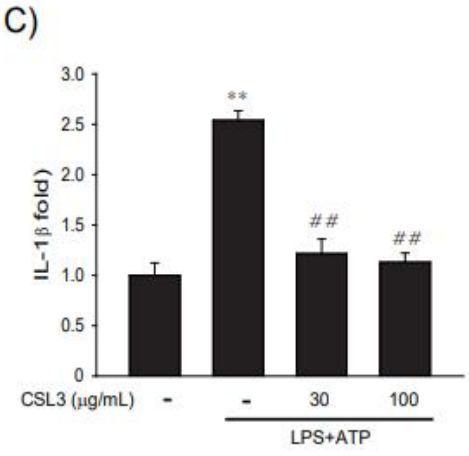


Figure 5. Inhibition of pyroptosis by CSL3 on LPS/ATP-stimulated ImKC

(A) The protein expression level of inflammasome and pyroptosis markers were evaluated by immunoblotting. ImKC. pretreated with CSL3 incubate with LPS for 6 h and then treatment of ATP for 0.5 h. Results were validated by repeated experiments. (B) mRNA levels of inflammatory cytokine IL-1β was evaluated by RT-PCR using GAPDH as control. Cells pretreated with CSL3 incubate with LPS for 3 h and then treatment of ATP for 0.5 h. Results were validated by repeated experiments. (C) Enzyme-linked immunosorbent assay (ELISA). ELISA measured by ImKCs supernatant treated LPS/ATP and/or CSL3 in RAW264.7 for 3h. (n = 3, significant when compared with controls, **$p<0.01$, compared with the LPS treatment group, ## $p<0.01$).

4. Improvement effect of CSL3 on CCl_4-induced acute hepatitis

The carbon tetrachloride (CCl_4)-induced acute hepatitis is the most popular in vivo model to study intrinsic drug-induced liver injury [26, 27]. Therefore, we used CCl_4-induced acute liver injury model for investigating liver protection effect of CSL3. CSL3 was treated 5 times in mice before sacrificed (Fig 6A, upper). The CCl_4 alone treatment group had injury characteristics in liver, however CCl_4+CSL3 group showed a hepato-protective effect of CSL3 (Fig 6A, down). Then, the activity of serum ALT and AST was reduced in the CCl_4+CSL3 group compared to the CCl_4 alone group (Fig 6B). Similarly, H&E staining of liver tissue showed that liver injury caused by CCl_4 chiefly caused by lesions around the central vein, but the CCl_4+CSL3 group showed liver protection effects about liver injury (Fig 6C).

Next, the expression of inflammatory cytokines (IL-6 and TNF-α) in mouse serum was investigated using an ELISA kit. As a result, it was validated that IL-6 and TNF-α reduced in the CSL3-treated group (Fig. 7A and B). And CSL3 treatment significantly decreased protein level of iNOS (Fig 7C).

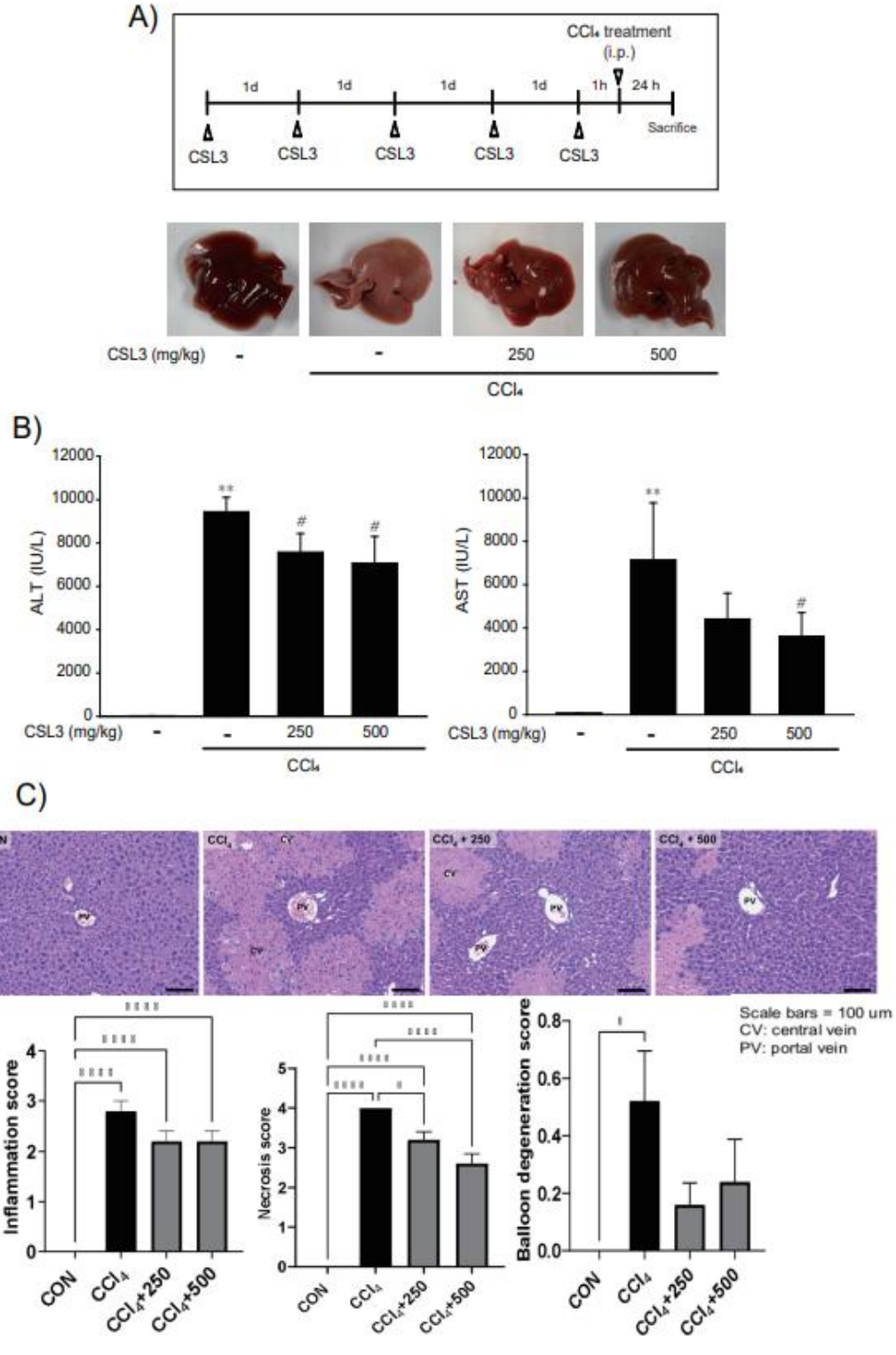
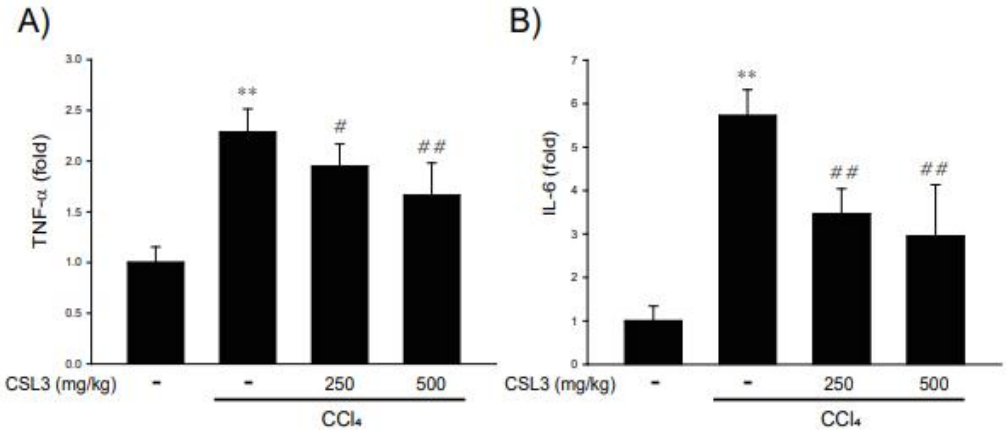


Figure 6. Suppression of CCl_4-induced acute liver injury through CSL3

(A) Male ICR mice were caused liver injury by intraperitoneal injection of CCl_4. CSL3 was orally administered to mice at a dose of 250 or 500 mg/kg/day for 5 consecutive days, followed by subcutaneous injection of CCl_4 (1ml/kg, dissolved in olive oil [10%]) for 24 h to induce acute liver injury. (B) The activities of serum ALT and AST were analyzed by using an automated blood chemistry analyzer All values were expressed as mean ± SE of 5 mice serum (notable as compared with vehicle control, **p<0.01; notable as compared with CCl_4 alone, ##p < 0.01, #p < 0.05). (C) Mice liver H & E staining by intraperitoneal injection of CCl_4. Scale bars indicate 120 μm.



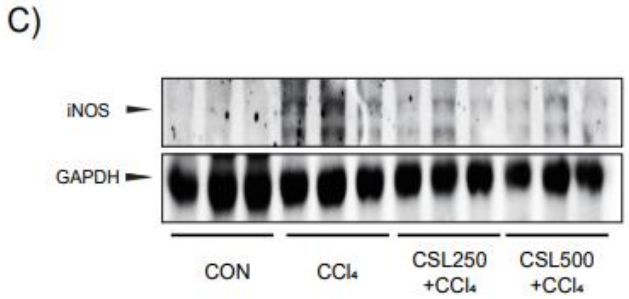


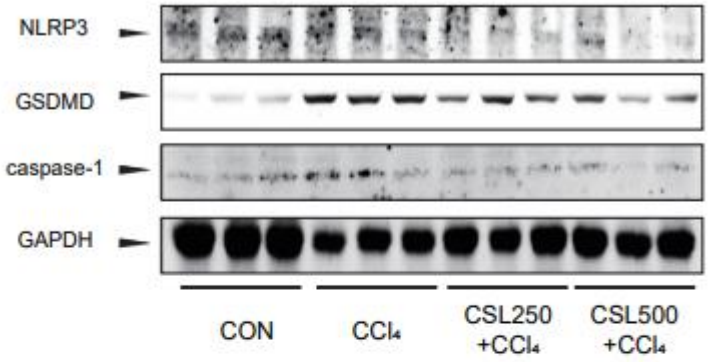
Figure 7. Inhibition of inflammatory gene expression through CSL3 in CCl_4-injected mice

(A, B) ELISA. IL-6 and TNF-α release into serum was established by using ELISA. (C) The effect of CSL3 on CCl_4-induced iNOS protein expression All values were expressed as mean ± SE of 3 mice (notable as compared with vehicle control, **p<0.01; notable as compared with CCl_4 alone, ##p < 0.01, #p < 0.05).

5. Suppression of inflammasome and pyroptosis marker expression by CSL3 in in CCl_4-treated mice

Carbon tetrachloride (CCl_4)-induced acute liver injury probably via suppressing the anti-oxidant pathway and inducing NLRP3 inflammasome activation [28, 29]. Therefore, we verified that CCl_4-induced inflammasome and pyroptosis protein expression (like as NLRP3, cleaved caspase-1 and GSDMD) was suppressed by CSL3 treatment (Fig 8A). It was confirmed that CCl_4-induced release of IL-1β in mouse serum was decreased by CSL3 treatment (Fig 8B).

A)



B)

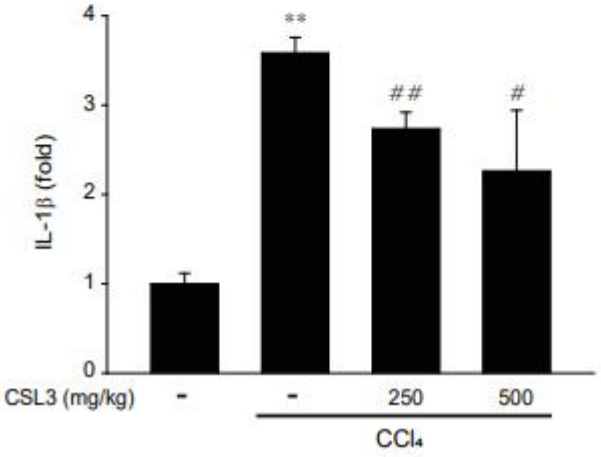


Figure 8. Suppression of CCl_4-induced inflammasome and pyroptosis by CSL3

(A) The protein expression level of inflammasome and pyroptosis markers were evaluated by immunoblotting using GAPDH as control in liver tissue (n=3). (B) ELISA. IL-1β release into serum was established by using ELISA kit. All values were expressed as mean ± SE of 3 mice (notable as compared with vehicle control, **p<0.01; notable as compared with CCl_4 alone, ##p < 0.01, #p < 0.05).

DISCUSSION

There is a need for study about resource conversion and food materialization of the warm-temperature tree species because of climate change. The fruits of the *Castanopsis sieboldii*, a kind of warm-temperature tree species, have been used for food for a long time, and recently its branches have been recognized for their value as wood. In addition, it is known that the leaf extract of the *Castanopsis sieboldii* (extracted with ethanol and then solvent fractionated with ethyl acetate) has high antioxidant and antibacterial activity [11]. Albeit, the pharmacological efficacy of leaves of *Castanopsis sieboldii* is still unveiled. Therefore, in this study, we verified that CSL3 has protective effect by suppressing inflammatory response and inflammasome in *in vitro* and *in vivo* model of acute hepatitis.

Activation of TLR4 (Toll-like receptor 4, CD284) by LPS cause phosphorylation and degradation of IκB-α [30]. Degradation of IκB-α leads to activate NF-κB causing translocation into the nucleus of p65 (a subunit of NF-κB transcription complex) to produce NO and pro-inflammatory cytokine including TNF-α, interleukin (IL)-1α/β, IL-6, IL-12, IL-18 [31].

We first established whether CSL3 has anti-inflammatory efficacy in LPS-activated macrophage (ImKC: immortalized murine kupffer cell and Raw264.7 cell: monocyte/macrophage-like cells, originating from Abelson leukemia virus-transformed cell line derived from mice). CSL3 significantly inhibited iNOS

expression and NO production in both ImKC and Raw264.7 cells. CLS3 treatment antagonized increased mRNA expression of inflammatory cytokines (IL-6 and TNF-α) by LPS alone (Fig 2 and 3).

In addition, we verified which transcription factors including NF-κB and AP-1, crucial transcription factors for inflammatory gene expression) were affected the inhibition of inflammatory gene expression by CSL3. As a result, CSL3 suppressed the activation of NF-κB and AP-1 which were mediated via inhibition of phosphorylation of MAPKs (ERK, JNK, and p38) (Fig 4).

Then, we examined whether CSL3 inhibited LPS+ATP-induced inflammasome and pyroptosis in ImKC. LPS activation of macrophages can cause an increase in oxygen absorption, leading to a range of reactive oxygen species (ROS), which are the key factors that drive oxidative stress-stimulated inflammation in immune cells [32]. In the extracellular environment, ATP is markedly released in response to tissue damage and cellular stress [33]. ATP, generally secreted from dying and stressed cells, is used as a DAMP to activate NLRP3 inflammation [34, 35]. Canonical pyroptotic cell death is mediated by NLRP3 inflammasome assembly, which is accompanied by GSDMD cleavage and IL-1β and IL-18 release [36]. The NLRP3 inflammasome triggers caspase-1 activation, it induces cleavage of GSDMD, and N-terminal domain can oligomerize to form pores in the cell membrane, causing cell membrane rupture [37]. Based on these facts [38], we

treated LPS+ATP for inducing inflammasome and pyroptosis in Kupffer cells. As a result of the experiment, we confirmed that CSL3 pretreatment inhibited expression of NLRP3, inflammasome sensor, and blocked cleavage of caspase-1 and GSDMD. CSL3 also decreased production of pro-inflammatory cytokine like as IL-1β related to inflammasome and pyroptosis (Fig 5).

Finally, we investigate that CSL3 has the liver protection efficacy against CCl_4-induced hepatitis *in vivo*. CCl_4 is widely used as animal model that induces acute hepatitis and even liver fibrosis [39, 40]. Recently, it was reported that inflammasomes are induced in the CCl_4-induced mice model [26, 29]. CSL3 was orally administered for 5 days, followed by CCl_4 via intraperitoneal, and blood and liver tissues were obtained by sacrifice after 24 hours. The intensified levels of ALT and AST by CCl_4 were decreased by CSL3, and H&E staining showed that liver damage was protected by CSL3(Fig 6). Moreover, CSL3 treatment reduced production of inflammatory cytokines (IL-6 and TNF-α) in serum and protein expression of iNOS in the liver tissue (Fig 7). Then, we confirmed that CSL3 inhibited expression of proteins related to inflammasome and pyroptosis such as NLRP3, GSDMD, and caspase-1 and significantly reduced production of IL-1β in serum (Fig 8).

In conclusion, our data provide that CSL3 has hepatoprotective effect against CCl_4 -induced acute liver injury via suppression of inflammation and pyroptosis. Based

on these results, we propose the potential of CSL3 as a therapeutic drug candidate for acute hepatitis.

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