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The protective effect of cannabinoid type II receptor agonist AM1241 on ConA-induced liver injury in mice via mitogen-activated protein kinase signalling pathway

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Abstract

Introduction: The endocannabinoid system plays an important role in regulating the immune responses in inflammation. At present, there are no good clinical drugs for many immune liver diseases.

Methods: We explored the protective effect of the cannabinoid type II (CB2) receptor agonist AM1241 on the liver of mice with acute liver injury caused by concanavalin from the perspective of inflammation and immunity. Pathological evaluation in hepatic tissue was examined by haematoxylin and eosin (HE) staining and the levels of biochemical parameters in the serum were measured by automatic biochemical analysis. The content of inflammatory factors was measured by enzyme-linked immunosorbent assay and real-time quantitative reverse transcription polymerase chain reaction (real-time PCR). The liver apoptosis-related proteins were observed by immunohistochemistry. The expression of liver injury-related proteins was analysed by Western blot. Immune cells were isolated from the liver of mice and studied in vitro.

Results: Reduced levels of alanine transaminase and aspartate transaminase were observed in ConA-induced liver injury mice treated with AM1241, together with attenuated liver damage evidenced by H&E staining. Moreover, AM1241 inhibited the protein and gene expression levels of TNF-α, IL-6 and IFN-γ in the livers of mice. The phosphorylation levels of p38, JNK, ERK1/2, P65 and cAMP response element-binding protein (CREB) in the mouse were significantly reduced in AM1241 pretreatment, while the level of p-JNK increased. In addition, the P/T-P65 and P/T-CREB of the AM1241 pretreatment group were significantly reduced. The results of immunohistochemistry measurement are consistent with those of Western blotting. The CB2-mediated effect is through macrophage-like Kupffer cells.

Conclusion: Our study suggests that the ConA-induced liver injury model in mice is protected by CB2 agonist AM1241 by modulation of CB2 receptor-rich immune cells, for example, Kupffer cells. Reduced inflammatory responses regulate apoptosis/cell death in the liver particularly hepatocytes and other parenchymal cells.

Keywords

cannabinoid type II receptor, AM1241, mitogen-activated protein kinases, apoptosis

Introduction

Liver disease, which includes viral hepatitis, toxic liver disease, autoimmune hepatitis and alcoholic liver disease, represents a global health problem for humans.[1] However, only a small number of liver diseases can be managed with pharmacological interventions.[2] Therefore, the development of new drugs is required to provide additional therapeutic tools for the clinic. The pathophysiology of liver disease is complex. In autoimmune and viral hepatitis, immune-related inflammation is generally regarded as the basic pathogenic mechanism.[3,4] This inflammation is characterized by elevated enzymatic activity levels of aspartate transaminase (AST) and alanine transaminase (ALT) and accompanied by the activation of immune cells and the accumulation of inflammatory cytokines.[5]

ConA-induced acute liver inflammation is a widely used acute liver injury model in mice. The liver consists of resident macrophage or Kupffer cells and they are functionally indistinctive with macrophage when activated.[6] Apart from resident immune cells, the liver is also the source of a host of releasing factors from parenchymal cells (hepatocytes) including cytokines, chemokines and complement factors, which all contribute to immune response in acute liver injury.[7]

The endogenous cannabis system (ECS) plays an important role in regulating inflammation and oxidative stress and has anti-fibrosis effects.[8,9] Both the cannabinoid type I receptor and the cannabinoid type (CB2) II receptor play major roles in the ECS. Recent studies have shown that CB2 receptor agonists can protect the liver from ischaemia-reperfusion injury by alleviating oxidative stress and attenuating liver fibrosis by reducing the expression of platelet-derived growth factor.[10,11] CB2 receptors are upregulated and have protective effects in liver disease[12,13]; however, the protective effects of CB2 receptor agonists in acute immune liver injury and their mechanisms remain unclear. CB2 receptor was present in the Kupffer cell/macrophage and has critical role in alcoholic liver disease.[14]

Several studies have shown that the mitogen-activated protein kinase (MAPK) signalling pathway plays an important role in acute liver injury.[15,16] P38, JNK and ERK1/2 are members of the MAPK protein family and are activated in many diseases.[17,18] The liver contains a large number of immune cells. Previous studies have shown that immune cell proliferation and activation, inflammatory cytokine release and immune regulation disorders are characteristics of the acute liver injury model,[19] and liver macrophages play an important role.[20] We used ConA to generate a mouse model of acute immune liver injury to determine the effects and mechanisms of CB2 receptor activation on acute liver injury and hepatic macrophages.

Materials and methods

Animals

Wild-type (WT) and CB2$^{-/-}$ male mice on a C57BL/6 background were used at ages ranging from 8 to 10 weeks. The WT rodents were purchased from the animal centre of Guizhou Medical University (approval number SYXK (Guizhou) 2018–0001, Guiyang, China). The CB2$^{-/-}$ mice (B6.129P2-Cnr2tm1Dgen 005786) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed in temperature- and humidity-controlled rooms on a 12-h light–day cycle and provided with unrestricted access to food and water. All animal experiments were approved by the Guizhou Medical University Animal Care Welfare Committee.

Acute liver injury models

ConA was dissolved in a normal saline solution at a concentration of 2 mg/mL. Statistics require at least six available data for each group to be meaningful. Male WT mice were randomly divided into one of five groups: group I (saline only) included 10 mice that were injected with saline via the tail vein; group II (ConA-treated) included 10 mice that were injected with ConA (20 mg/kg) via the tail vein; group III (1 h after treatment with AM1241, 3 mg/kg) included 10 mice that were injected with AM1241 (dissolved in 1% DMSO, 1 mg/mL), which was administered via intraperitoneal injection 1 h before the injection with ConA via the tail vein; group IV (1 h after treatment with AM1241, 12 mg/kg) included 10 mice that were injected with AM1241 (dissolved in 1% DMSO, 1 mg/mL), which was administered via intraperitoneal injection 1 h before the injection with ConA via the tail vein; and group V (prior to AM1241 treatment) included 10 mice that were injected with AM630 (dissolved in 1% DMSO, 1 mg/mL) via intraperitoneal injection 1 h before they were injected with

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AM1241 (12 mg/kg) and ConA (20 mg/kg) via the tail vein. Male $CB2^{-/-}$ mice were randomly divided into two groups: group A (saline only) included eight mice that were injected with saline via the tail vein and group B (ConA treatment) included eight mice that were administered ConA (20 mg/kg) via the tail vein. All mice were killed 9 hours after the injection of ConA. Blood was collected for biochemical detection. Some liver samples were harvested for histology while others were frozen and stored at −80°C for further analysis.

Analysis of liver enzymes

Serum was separated by centrifugation at 3500 r/min at 4°C for 10 min. To assess the level of hepatocellular injury after ConA treatment, serum ALT and AST levels were measured using an automated chemistry analyser.

Histopathology

Mouse liver tissue was collected and stored in 4% paraformaldehyde for at least 24 h. Paraffin sections were stained with haematoxylin and eosin (H&E) to visualize the level of inflammation and tissue damage by light microscopy.

Real-time reverse transcriptase polymerase chain reaction analyses

Total RNA was extracted from frozen liver samples using the RNeasy Mini Kit (Axygen, USA). cDNA was synthesized from 1 μg of total RNA using the PrimeScript RT reagent kit with gDNA Eraser (Takara, Japan). Quantitative real-time PCR was carried out on a ViiA 7 system (Applied Biosystems, USA) in 20 μl reactions containing 2 μl of cDNA (1:10 dilution), 400 nM of primers (All primers were obtained from Sangon, China) and PowerUp SYBR Green Master Mix (Applied Biosystems, USA) according to the manufacturer's instructions. Data are expressed as the ratio of the target gene level to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the treated group relative to the sham group and were quantified using the comparative cycle threshold Ct method ($2^{-\triangle\triangle CT}$). The gene primer sequences were as follows:

TNF-α– 5'- GCGACGTGGAACTGGCAGAAG-3',
5'-GCCACAAGCAGGAATGAGAAGAGG-3'
IL-6– 5'- ACTTCCATCCAGTTGCCTTCTTGG-3',
5'- TTAAGCCTCCGACTTGTGAAGTGG-3'
IFN-γ– 5'- CAGGCCATCAGCAACAACATAAGC-3',
5'- AGCTGGTGGACCACTCGGATG-3'
GAPDH– 5'-AGGTCGGTGTGAACGGATTTG-3',
5'-TGTAGACCATGTAGTTGAGGTCA-3'.

Western blot analysis

Total protein was isolated from liver tissue and protein concentrations were quantified using the BCA protein estimation kit (Solarbio, China). For each sample, 20–40 μg of protein were loaded. The proteins were transferred onto PVDF membranes using the wet transfer method. Membranes were blocked in Western Blocking Buffer (Beyotime, China) for 1 h at room temperature and incubated with antibodies against P38 (1:1200, CST, USA), p-P38 (1:1200, CST, USA), JNK (1:1200, Abcam, USA), p-JNK(1:1200, Abcam, USA), ERk1/2 (1:1200, CST, USA), p-ERK1/2 (1:1200, CST, USA), NF-κB (1:1000, CST, USA), p-NF-κB (1:500, CST, USA), cleaved caspase-3 (1:1000, CST, USA), Bcl-2 (1:1000, Abcam, USA), Bax (1:1000, Abcam, USA), cAMP response element-binding protein (CREB) (1:1000, CST, USA) and p-CREB (1:1000, CST, USA). Blots were probed with anti-GAPDH (1: 10000, Abcam, USA) as the loading control. All antibodies were incubated with the membranes at 4°C overnight. The secondary antibody was an anti-rabbit HRP-conjugated antibody (1:20000, Lianke technology, China). Immunoblots were developed using the Immobilon ECL method (Millipore, Billerica, Massachusetts, USA).

Immunohistochemistry

Immunohistochemistry was carried out using rabbit monoclonal or polyclonal primary antibodies and horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies to detect cleaved caspase-3 (1:500, Wanlei, China), Bax (1:250, Abcam, USA), Bcl-2 (1:400, Abcam, USA) and F4/80 (1:100, Abcam, USA) expression. Expression is represented as a percentage of the total liver area using ImageJ after analysing five fields of stained liver sections per animal.

Cytokine quantification (ELISA)

The levels of cytokines in homogenized liver tissue, including IL-6, were detected by enzyme-linked immunosorbent assay (ELISA) using the Quantikine Elisa kit (R&D, USA), while TNF-α and IFN-γ were detected using the Valukine Elisa kit (Novus, USA) according to the manufacturer's protocol. The amount of protein in each sample was quantified via BCA, and the final result was expressed as the quantity contained in each milligram of protein.

Cell culture and Kupffer cell isolation

Hepatocytes (AML-12) were cultured in DMEM/F12 medium while macrophages (RAW264.7) were cultured in high glucose DMEM medium, both of which were

supplemented with 10% FBS and 1% cyan-streptomycin and cultured in a 37°C incubator with 5% CO_2. The protein of the cultured cells was extracted and the expression of the CB2 receptor was detected by Western blot. The Kupffer cells were isolated as described earlier.[21] Cells were processed and treated with TNF-α as described in figure.

Statistical analysis

Data are expressed as the mean ± SEM and were analysed using a one-way analysis of variance (ANOVA) followed by a Tukey's post hoc test using GraphPad Prism 5 software. Comparisons between the two groups were analysed using *t*-tests. In all comparisons, a $p < 0.05$ was considered to be statistically significant.

Results

ConA-induced liver injury is more severe in CB2-deficient mice

We first investigated whether the CB2 receptor had a protective role in the ConA-induced liver injury model. Treatment of mice with ConA for 9 h (20 mg/kg) induced a significant degree of liver injury in $CB2^{-/-}$ compared with the WT mice, which was characterized by inflammation, necrosis and inflammatory cell infiltration as shown by H&E (Figure 1(a)). Moreover, ALT and AST levels in the CB2-deficient mice were higher than the wild-type mice (Figure 1(b)). These data indicate that the CB2 receptor plays a protective role in ConA-induced liver injury.

AM1241 protects mice from ConA-induced liver injury

When administered after ConA injection, serum levels of ALT and AST increased in the ConA model group (ALT, 5757.5 ± 1036 U/L; AST, 4451 ± 1146 U/L) compared to the NC group (ALT, 28.5 ± 7.35 U/L; AST, 101.6 ± 26.35 U/L). The AM1241 pretreatment groups showed much lower levels of aminotransferase (ALT, 2697 ± 956.3 U/L; AST, 1911 ± 766 U/L and ALT, 500.7 ± 126.9 U/L; AST, 636 ± 99.3 U/L). However, pretreatment with AM630 prior to treatment with AM1241 inhibited the effect of AM1241 (ALT, 3395 ± 1811 U/L; AST, 2326 ± 1054 U/L) (Figure 1(c)). The changes in histopathology were consistent with the ALT and AST results (Figure 1(d)). We observed massive areas of necrosis in the group that was only treated with ConA. In contrast, the AM1241-treated groups showed only minor liver damage, which indicates that AM1241 treatment significantly reduced liver necrosis and the AM1241 (12 mg/kg) pretreatment group showed

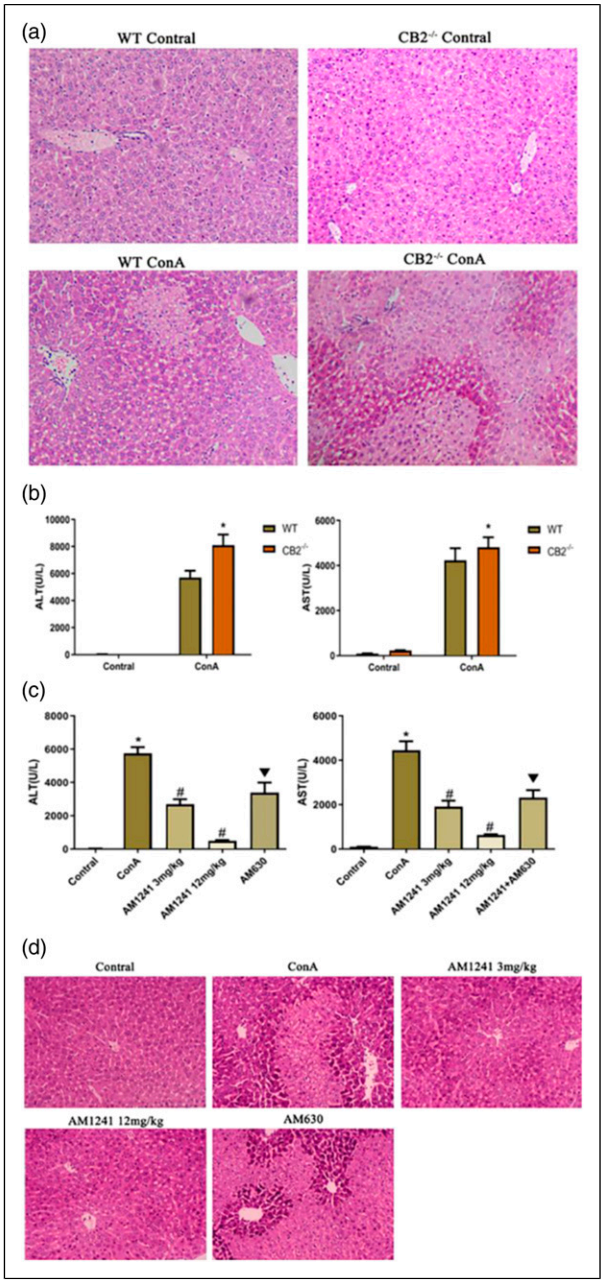

Figure 1. The effect of AM1241 and the cannabinoid type II receptor on ConA-induced autoimmune hepatitis. (a) Photomicrographs of representative livers collected 9 h after ConA injection in cannabinoid type II receptor$^{-/-}$ mice and wild-type mice and stained with H&E, ×200 magnification. (b) Protection of mouse liver by the cannabinoid type II receptor gene. Data are expressed as the mean ± SEM ($n = 6$; $^{*}p < 0.05$ for cannabinoid type II receptor $^{-/-}$ group versus wild-type group). (c) Effects of AM1241 on plasma alanine transaminase and aspartate transaminase levels in wild-type C57 mice. Data are expressed as the mean ± SEM ($n = 8$; $^{\#}p < 0.05$ for ConA versus control; $^{*}p < 0.05$ for AM1241 groups versus ConA; $^{\blacktriangledown}p < 0.05$ for AM630 versus AM1241 groups). (d) Photomicrographs of representative livers collected 9 h after ConA injection and stained with haematoxylin and eosin, ×200 magnification.

more effective protection than AM1241 (3 mg/kg) pretreatment group. These results showed that AM1241 treatment could ameliorate ConA-induced acute immune hepatitis in mice.

AM1241 inhibits cytokine release in ConA-induced hepatitis

Proinflammatory cytokines play a vital role in the progression of hepatitis, including TNF-α, IL-6, and IFN-γ. Therefore, we profiled the levels of these cytokines in liver homogenate by ELISA and their corresponding mRNA content in liver tissue by real-time PCR. Our results showed that the levels of these cytokines increased in the ConA-treated group following ConA induction. As expected, the production of TNF-α, IL-6 and IFN-γ was reduced in the AM1241-pretreatment group and AM630 was able to inhibit the action of AM1241 in the AM630 group. Additionally, the changes in mRNA levels were the same as observed for in cytokines, indicating that these changes were statistically significant (Figure 2).

The effect of AM1241 on the MAPK pathway in the ConA-induced hepatitis mouse model

Mitogen-activated protein kinases can change the expression levels of genes by phosphorylating transcription factors, which can mediate various physiological and pathological processes. Therefore, we examined the phosphorylation levels of P38, JNK and ERK1/2 proteins in each group. We found that p-p38 and p-ERK were significantly increased in the model group and decreased after pretreatment with AM1241, and pretreatment with the CB2 inhibitor AM630 was able to inhibit the effects of AM1241. However, we did not observe an increase in p-JNK in the model group despite increased expression after AM1241 was administered. The expression of p-38 and p-ERK1/2 were significantly increased in the AM1241-treated groups compared to the ConA-treated group and the AM630-pretreated group (Figure 3(a)). These data were analysed using ImageJ, which identified statistically significant differences among these changes (Figure 3(b)).

The effect of AM1241 on p-65 and CREB in the ConA-induced hepatitis mouse model

To investigate the possible association between the apoptosis results and the MAPK signalling pathway, we examined protein levels of NF-κB (P-65) and CREB in the liver tissue of each mouse group. As expected, p/T-P65 and p/T-CREB were significantly increased in the model group and pretreatment with AM1241 reduced their phosphorylation. Once again, AM630 reversed this effect. These changes were statistically significant (Figure 3).

The effects of AM1241 on apoptosis in the ConA-induced hepatitis mouse model

ConA-induced liver damage appeared to increase the amount of apoptosis in the liver. Interestingly, pro-apoptotic protein expression did not increase in the model group but increased levels of Bax and cleaved caspase-3 were observed in the AM1241 pretreatment group. Bcl-2 is known as an anti-apoptotic protein but was elevated in both the model group and in the AM630 pretreatment groups. These changes were statistically significant and contrary to our expectations. To further confirm these results, we measured the expression levels of Bax, Bcl-2 and cleaved caspase-3 in liver tissue using immunohistochemistry. As expected, the results were consistent with those of the Western blot. The statistical analyses were carried out using Image-Pro Plus 6.0 software. These data indicate that AM1241 can promote apoptosis in certain liver cells and that this phenomenon is inhibited by the CB2 inhibitor AM630 (Figure 4).

CB2 receptor expression

To demonstrate that the target of AM1241 is not hepatocytes but instead some other non-parenchymal cells, including macrophages, we used cultured hepatocytes and macrophages. We found that both liver tissue and macrophages express the CB2 receptor and that the expression of CB2 is increased in the liver tissue of ConA-pretreated mice but hardly expressed in normal liver cells (Figure 5(a)).

The effect of AM1241 on macrophages in the ConA-induced hepatitis mouse model

After confirming the expression of the CB2 receptor in hepatic macrophages, we detected F4/80, which is a highly specific marker for macrophages, in the liver tissue of each group using immunohistochemistry. These data were analysed using Image-Pro Plus 6.0 software. The results showed a significant increase in F4/80 in the liver tissue of the ConA-treatment group but significant decreases after administration of AM1241, and AM630 once again inhibited the effect of AM1241 (Figures 5(b) and (c)).

The specific role of AM1241 on Kupffer cells/ macrophages in the ConA-induced hepatitis mouse model

To elucidate the specific role of resident macrophage, we examined the comparative expression of CB2 receptor in our RAW macrophage and isolated Kupffer cells from healthy pooled mice. The level of expression is quite similar between those two populations as evident from

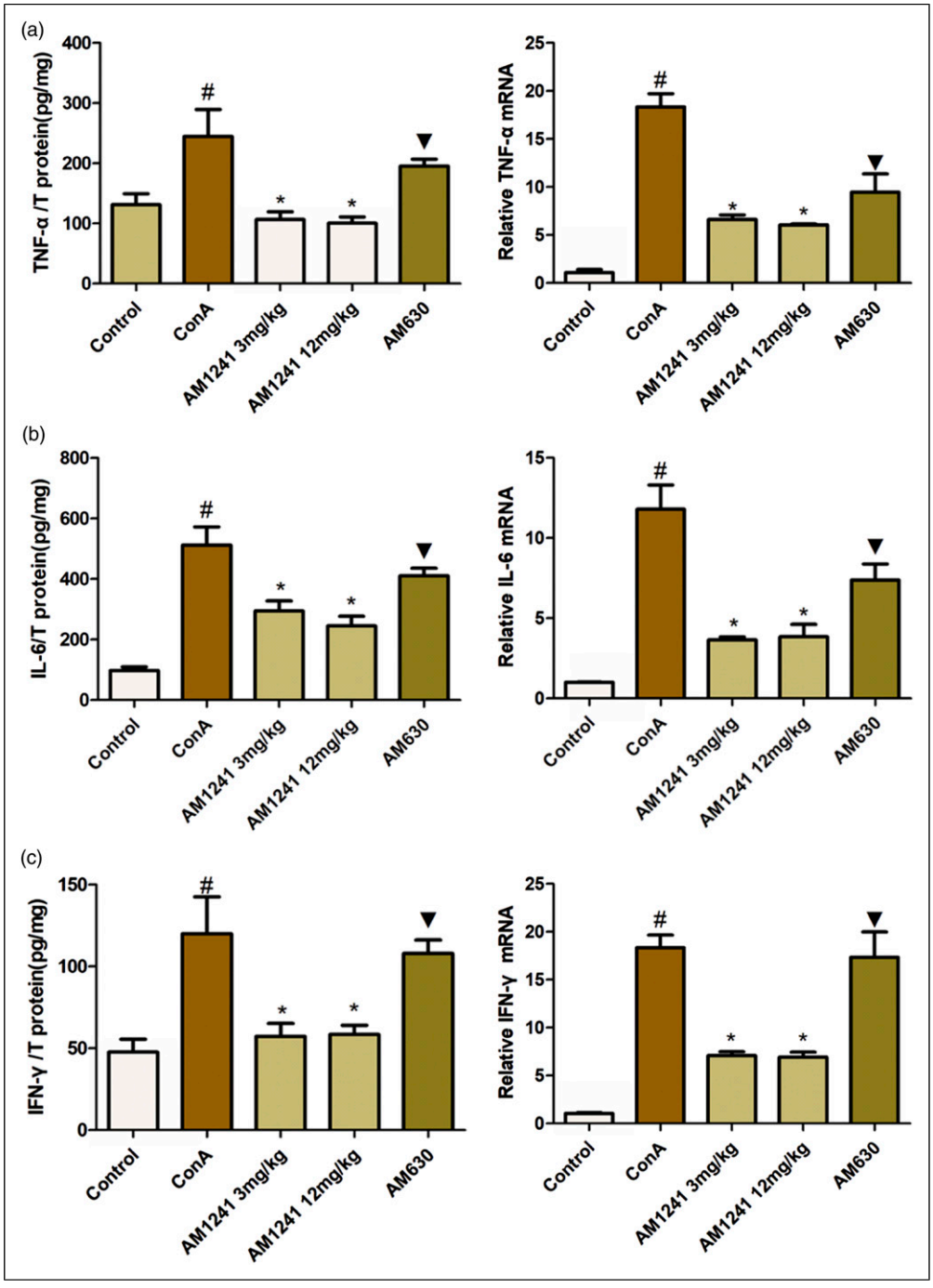

Figure 2. AM1241 inhibits cytokine release in ConA-induced hepatitis. (a) Serum levels of TNF-α were measured by enzyme-linked immunosorbent assay and mRNA levels were detected by real-time PCR. (b) Serum levels of IL-6 were measured by enzyme-linked immunosorbent assay and mRNA levels were detected by real-time PCR. (c) Serum levels of IFN-γ were measured by enzyme-linked immunosorbent assay and mRNA levels were detected by real-time PCR. Data are expressed as the mean ± SEM ($n = 6$; $^{\#}p < 0.05$ for ConA versus control; $^{*}p < 0.05$ for AM1241 groups versus ConA; $^{\blacktriangledown}p < 0.05$ for AM630 versus AM1241 groups).

real-time PCR-based Ct values (Figure 6(a)). We demonstrated a significant correlation of CB2 receptor expression and F4/80 expression in healthy and ConA liver samples (Figure 6(b)). We also induced activation in those isolated Kupffer cells by treatment with LPS in vitro with or without CB2 agonist AM1241 (Figure 6(c)). AM1241 treatment reduced inflammatory cytokine and thus associated hepatocytic cell death.

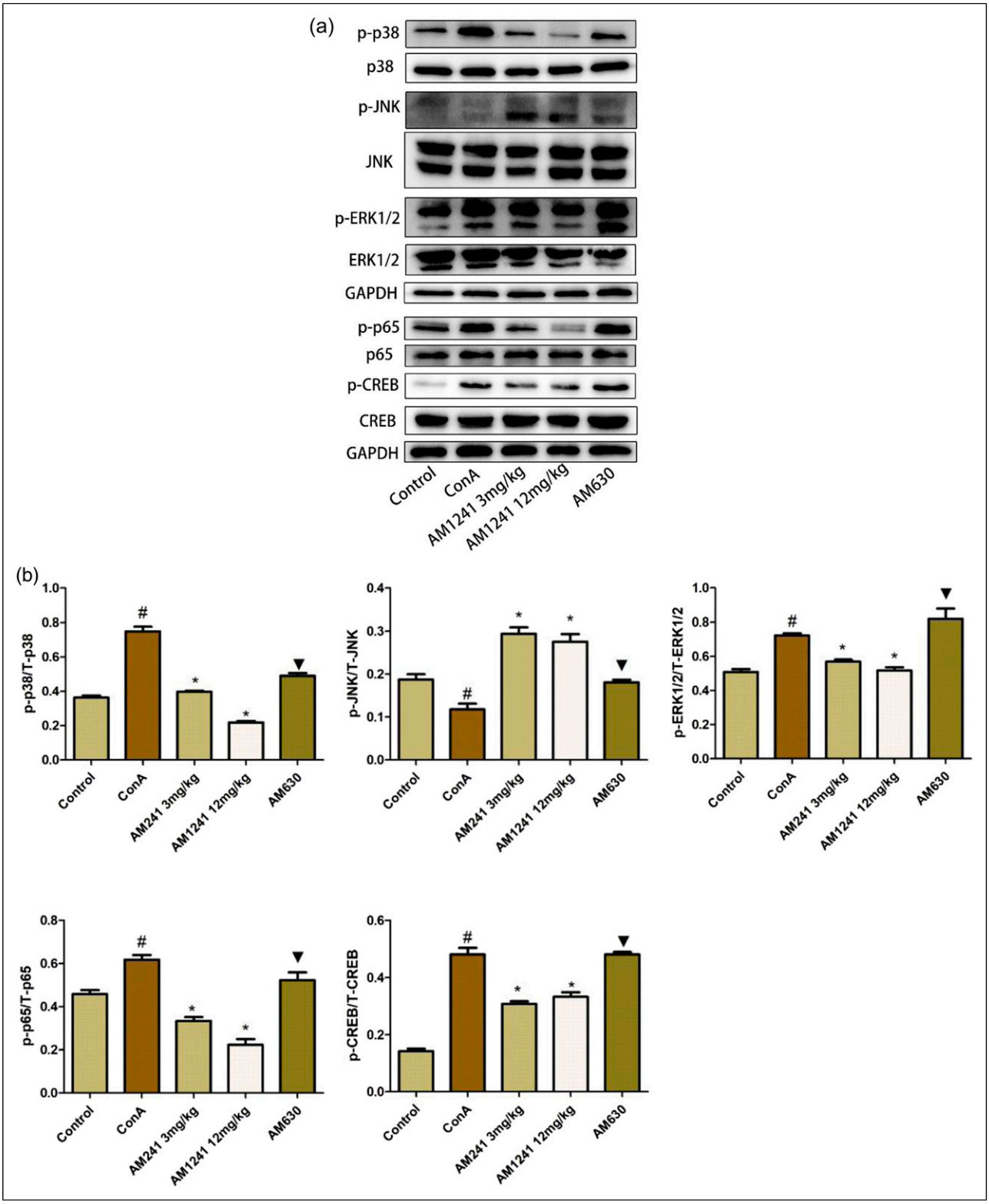

Figure 3. The effects of AM1241 on the mitogen-activated protein kinase pathway in the ConA-induced hepatitis mouse model. (a) The expression of p38, p-p38, JNK, P-JNK, ERK1/2, p-ERK1/2, p65, p-p65, cAMP response element-binding protein and p-cAMP response element-binding protein levels were detected using Western blot. (b) The results of the Western blots were analysed with ImageJ. Data are expressed as the mean ± SEM ($n = 6$; $^{\#}p < 0.05$ for ConA versus control; $^{*}p < 0.05$ for AM1241 groups versus ConA; $^{\blacktriangledown}p < 0.05$ for AM630 versus AM1241 groups).

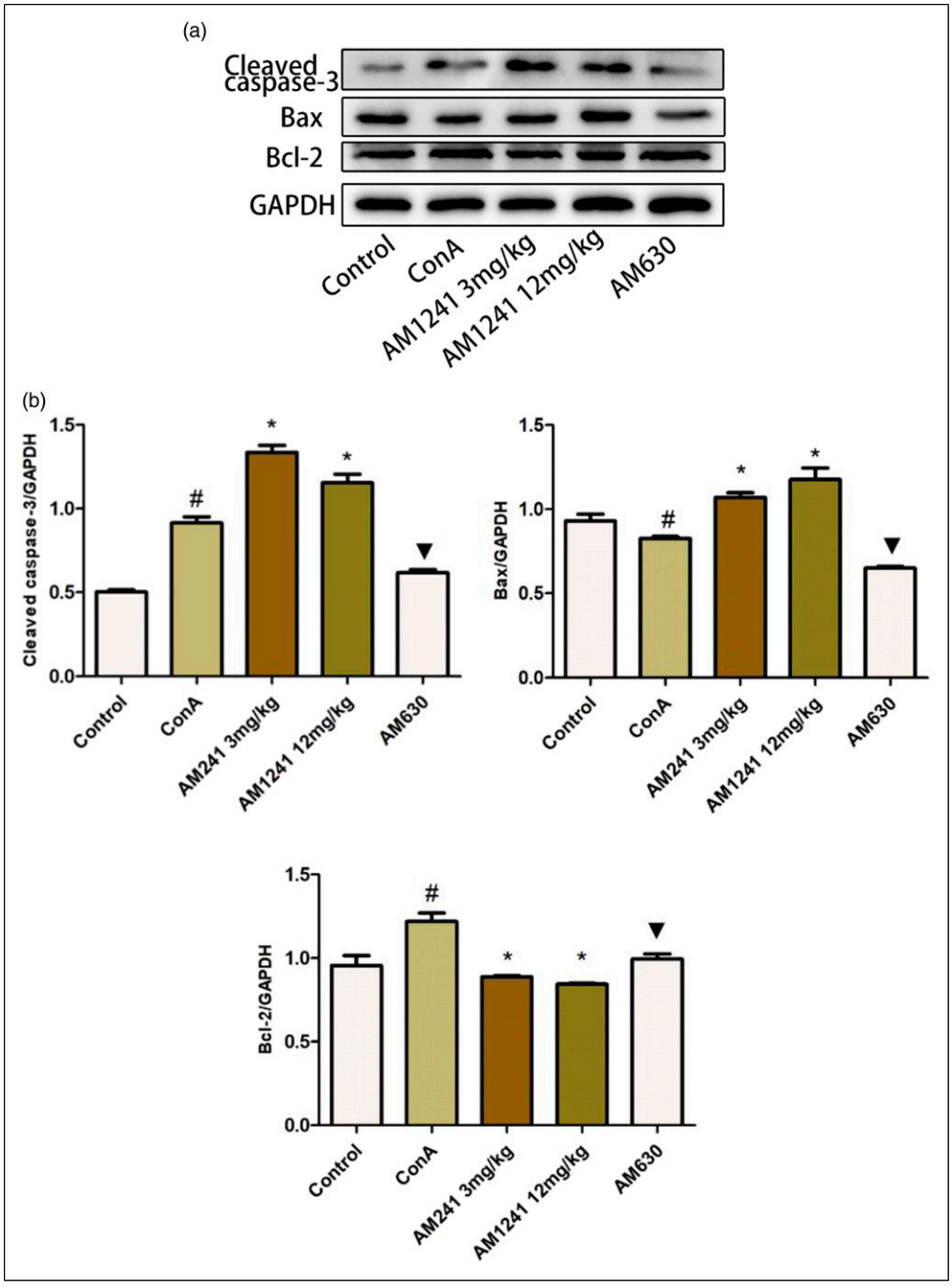

Figure 4. Continued.

Discussion

Billions of people around the world suffer from hepatitis, including viral hepatitis, autoimmune hepatitis, alcoholic and liver damage.[22] Although the liver is capable of repairing itself, long-term damage repair can destabilize its internal environment.[23-25] There are no specific drugs that treat autoimmune and viral hepatitis, and the current interventions can only control and delay the progression of the disease.[2,26] Hepatitis is closely related to cirrhosis and liver cancer, and it is vital that we develop new drugs and identify new drug targets. The role of immune cell activation and the release of various cytokines in the pathogenesis of viral hepatitis and autoimmune hepatitis has been widely accepted. In this study, the results of ALT, AST and H&E staining demonstrated that the CB2 receptor agonist-AM1241 protected mice from ConA-induced liver injury and massive levels of liver cell death. We confirmed

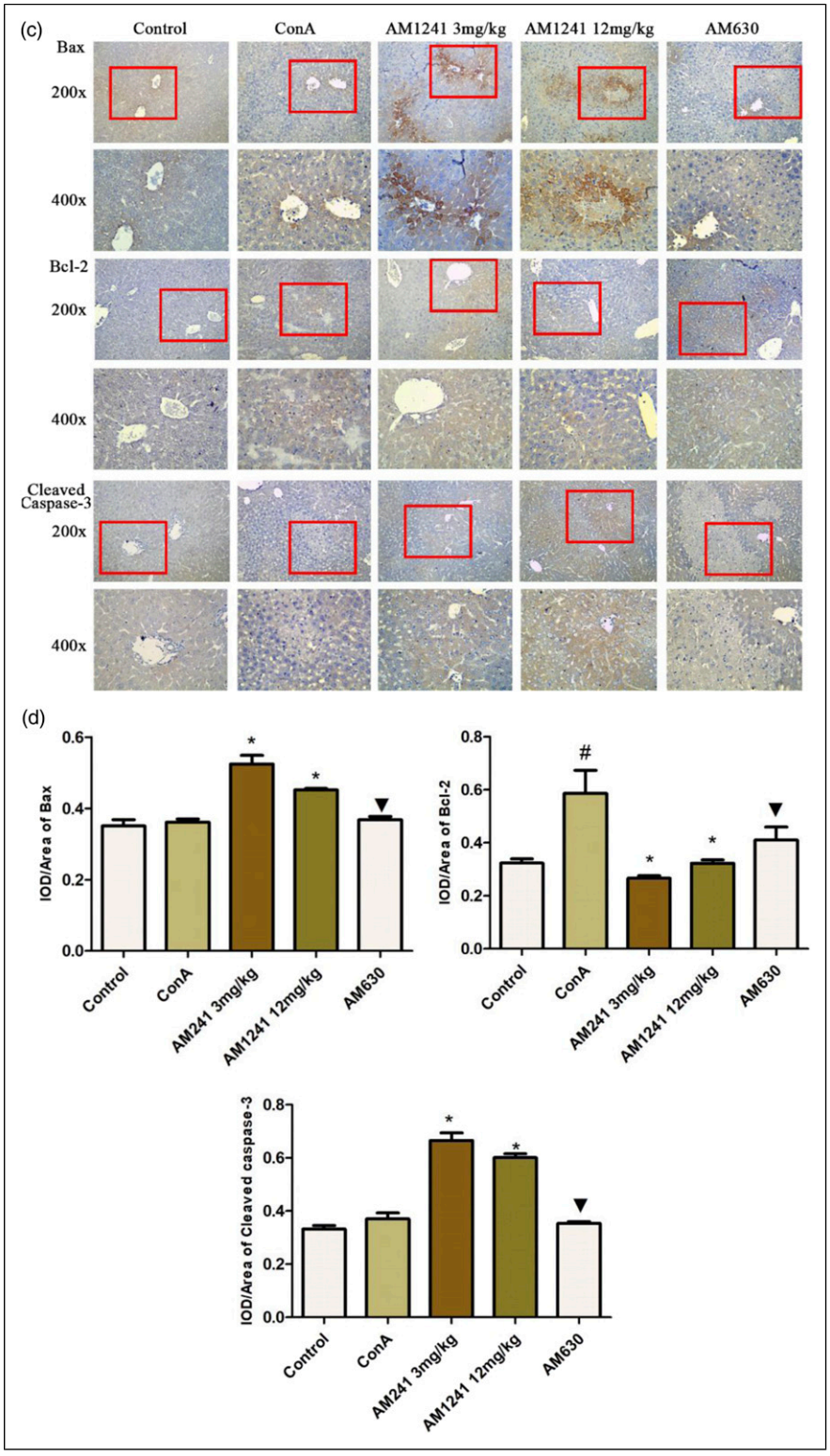

Figure 4. The effects of AM1241 on apoptosis in ConA-induced hepatitis mouse model. (a) The expressions of cleaved caspase-3, Bax and Bcl-2 protein levels were detected using Western blot. (b) The results of the Western blots were analysed with ImageJ. (c) The expressions of cleaved caspase-3, Bax and Bcl-2 protein levels were detected by immunohistochemistry staining in hepatic tissues at 9 h (×200 and ×400 magnification). (d) The results were analysed using Image-Pro Plus 6.0. Data are expressed as the mean ± SEM ($n = 6$; $^{\#}p < 0.05$ for ConA versus control; $^{*}p < 0.05$ for AM1241 groups versus ConA; $^{\blacktriangledown}p < 0.05$ for AM630 versus AM1241 groups).

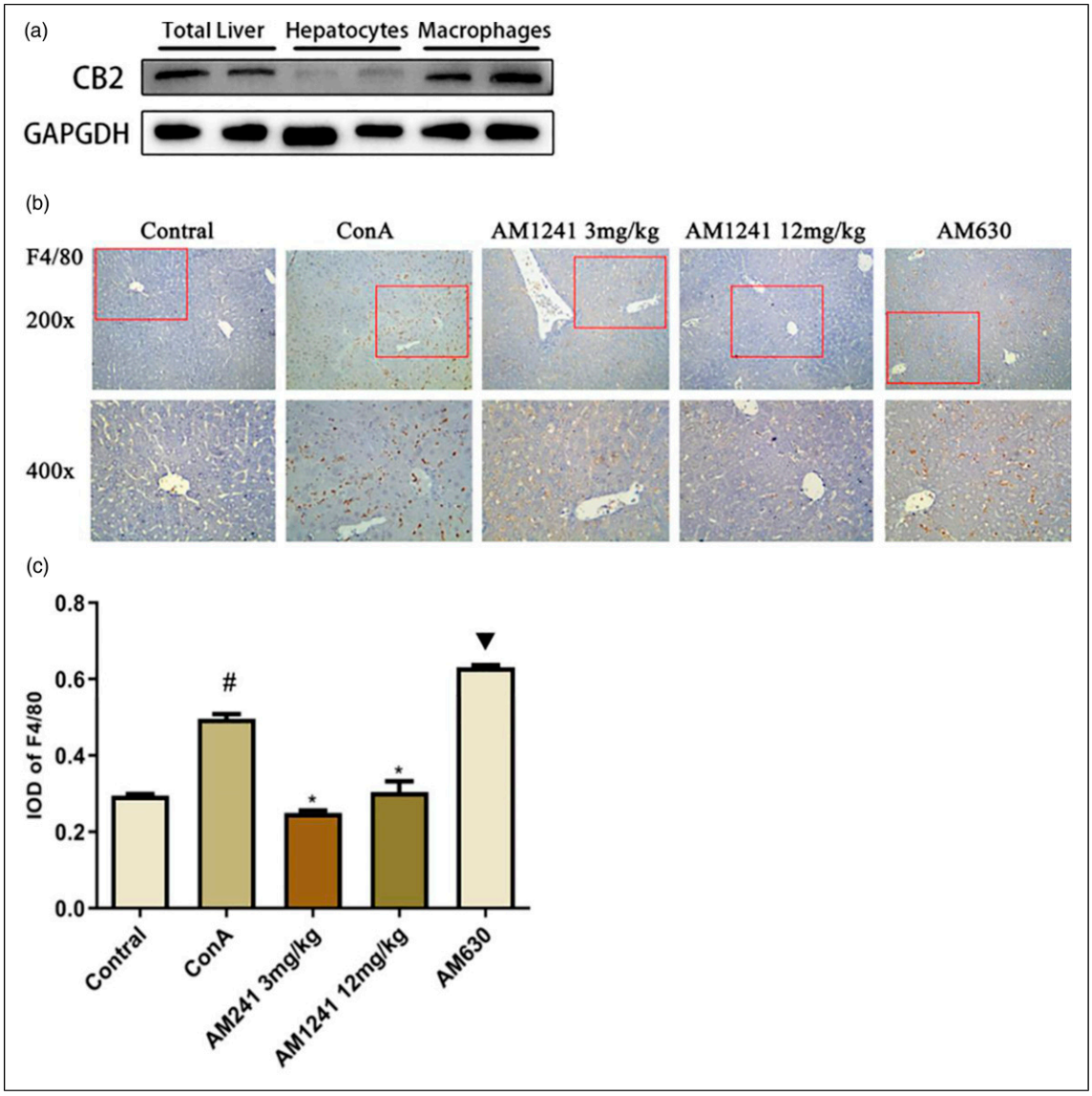

Figure 5. The effects of AM1241 on macrophages and cannabinoid type II receptor expression in ConA-induced hepatitis mouse model. (a) The expressions of cannabinoid type II receptor protein levels in total liver, hepatocytes and macrophages were detected using Western blot. (b) The expressions of F4/80 protein levels were detected using immunohistochemistry staining. (c) The results were analysed using Image-Pro Plus 6.0. Data are expressed as the mean ± SEM ($n = 6$; $^{\#}p < 0.05$ for ConA versus control; $^{*}p < 0.05$ for AM1241 groups versus ConA; $^{\blacktriangledown}p < 0.05$ for AM630 versus AM1241 groups).

the protective effects of the CB2 receptor in mouse liver by comparing ALT, AST and H&E staining results between ConA-pretreated CB2-knockout mice and WT mice.

The MAPK signalling pathways are important in vivo and are involved in the regulation of various physiological and pathological processes such as cell proliferation, growth, differentiation, apoptosis and the inflammatory response.[27] A primary goal of this study was to investigate whether AM1241 was involved in the induction of apoptosis in hepatocyte by regulating the MAPK signalling pathway. Previous studies have shown that the activation of JNK plays a central role in inducing apoptosis.[28] In contrast, the activation of ERK plays an important role in cell proliferation and differentiation and is thought to regulate cell survival.[29] The role of NF-κB in immunity and inflammation has been thoroughly explored, but it also has anti-apoptotic effects,[30] that may be closely related to p38, and some studies have shown that several NF-κB target genes may be dependent on p38-driven chromatin modifications.[31] The activation of NF-κB also stimulates the rapid accumulation of A20. Increased levels of A20 induce the synthesis of TNF-α but also reduces apoptosis through

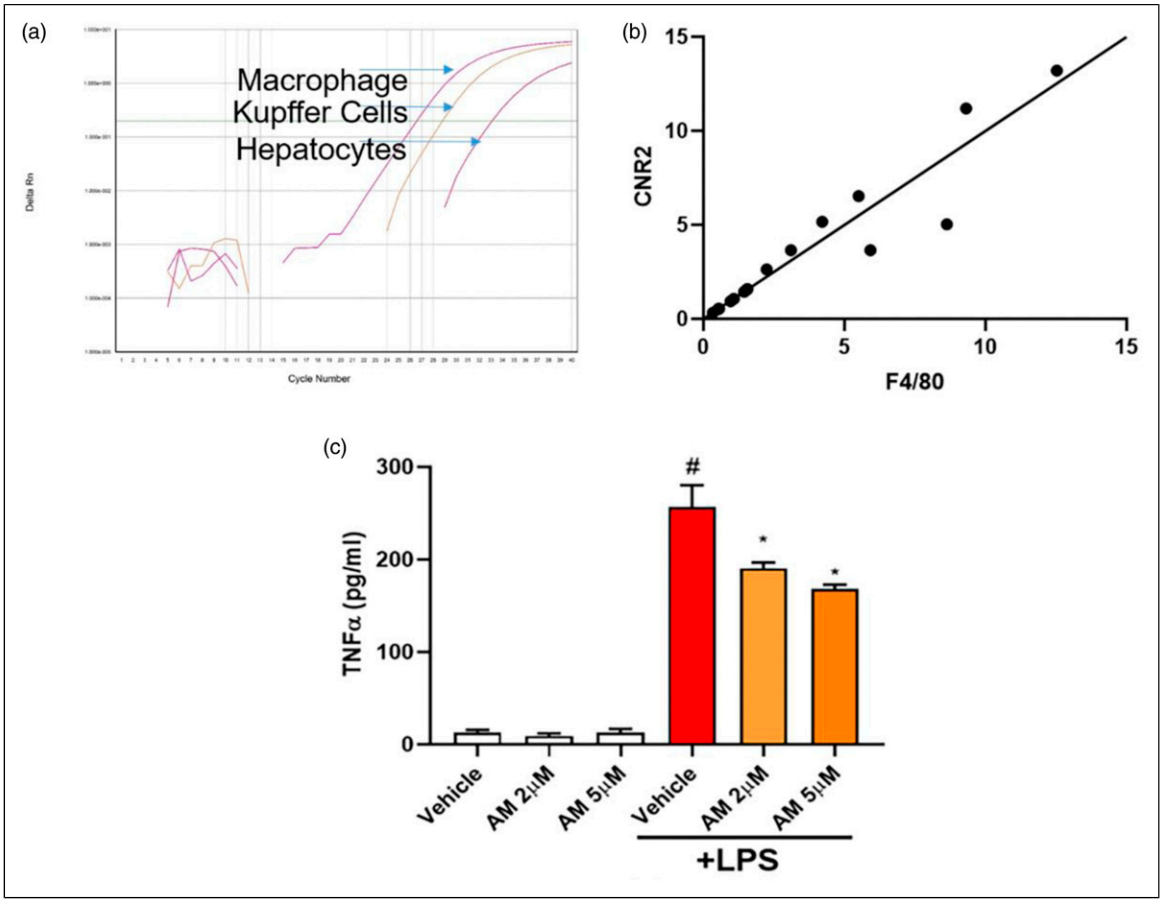

Figure 6. Cannabinoid type II receptor expression and the effects of AM1241 on Kupffer cell activation from mice. (a) Representative amplification plot from real-time PCR of cannabinoid type II receptor expression in macrophage, Kupffer cell and hepatocytes from mice. (b) Correlation analyses of F4/80 and cannabinoid type II receptor gene expression in liver from control and ConA-treated mice. (c) TNF-α level measured by Quantikine kit (R&D, USA) from cell culture supernatant from isolated Kupffer cell from control mice with or without LPS ($^{\#}p < 0.05$ for LPS group versus Vehicle; $^{*}p < 0.05$ for AM1241 groups versus Vehicle+LPS).

the inhibition of JNK phosphorylation by targeting the apoptosis signal-regulating kinase 1 (ASK1).[32] The CREB regulates gene transcription and participates in synaptic plasticity, memory and survival and also has roles in the pathophysiology of tissue injury.[33-35] Studies have shown that the activation of ERK1/2 significantly upregulates the expression of p-CREB, which promotes the expression of the anti-apoptotic protein Bcl-2 and promotes cell survival.[36,37]

The expression of p-ERK1/2, p-p38, p-p56, p-CREB and Bcl-2 was significantly increased in the ConA-pretreated mouse liver tissue but decreased in the AM1241 pretreatment group. In the AM1241 pretreatment group, p-JNK, Bax and activated caspase-3 were significantly increased. However, AM630 can almost completely reverse the changes induced by AM1241. This suggests that some of the cells that promote inflammation and hepatocyte necrosis undergo apoptosis in response to AM1241 pretreatment. While we observed the expression of the CB2 receptor in liver tissue, hepatocytes and macrophages, the subsequent Western blot analysis revealed that hepatocytes were nearly absent of the CB2 receptor while the RAW264.7 macrophages strongly expressed it. Taken together with the results of the inflammatory cytokine profiling of TNF-α, IL-6 and IFN-γ, we propose a model of acute immune liver injury caused by ConA in which hepatic macrophages proliferate and activate in large quantities and AM1241 influences the regulation of MAPKs, which promotes cell death in these hepatic parenchymal cells such as hepatocytes. Previous studies have shown that there are many non-parenchymal cells in the liver including B cells, NK cells, T cells and others that express the CB2 receptor.[38] Understanding their roles is beyond the scope of this study, However, that the liver resident macrophages play an important role in this disease process by their activation. To test our hypothesis, we examined the expression of the F4/80 protein in the liver tissue of each group by immunohistochemistry. As

expected, F4/80 was strongly expressed in the liver tissue of ConA-pretreated mice. After pretreatment with AM1241, the expression of F4/80 was significantly inhibited. The inflammatory response leads to hepatocytic cell death and causing a new cycle of inflammatory response. The protective effect of CB2 receptor agonists may be due to multiple mechanisms on different cell types. We cannot exclude other possible mechanisms such as its effect on smooth muscle cells, endothelial cells, T cells, and other immune cell types. In this study, we explored the specific role of Kupffer cells/macrophage in CB2 receptor-mediated protection in ConA-induced acute liver injury.

Overall, we showed that the CB2 receptor agonist AM1241 protected mice from hepatic macrophage–mediated liver injury that was induced by ConA. This liver injury results from imbalances in inflammatory cytokine-dependent immunity and liver cell death driven primarily by TNF-α, IL-6 and IFN-γ. AM1241 promotes apoptosis/cell death in liver particularly hepatocytes and other parenchymal cells, reduces the production of inflammatory cytokines through regulation of the MAPK signalling pathway and promotes the expression of cleaved caspase-3. The present study focused on the preventive effects of AM1241 on ConA-induced liver injury. Our results did not clarify the signal pathways or molecular mechanisms involved in the protective effect of AM1241 on the liver. Thus, the deeper molecular mechanism could be confirmed in the later research that would be useful in the development of its drug.

Conclusion

In our findings, it was clearly stated that after mice were injected with ConA, the liver had obvious damage. This damage was more serious in CB2 gene knockout mice, which proved that the CB2 gene has a certain hepatoprotective effect. Along with the present data and its ability to prevent ConA-induced liver injury, these data suggest that endocannabinoid receptor agonists AM1241 have a better protective effect on the liver.

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Declaration of conflicting interests

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Data availability statement

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

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