Ursodeoxycholic Acid Exhibits Anti-inflammatory Activity against Concanavalin A-induced Immune Hepatitis in Mice

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Abstract

Objective

It was to evaluate the anti-inflammatory effect of ursodeoxycholic acid (UDCA) on concanavalin A (ConA)-induced immune hepatitis in mice and determine the molecular mechanism.

Methods

Female C57BL/6J mice were randomly classified into Control, ConA, and ConA+UDCA groups. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured. Quantitative real-time polymerase chain reaction (qRT-PCR) was applied to detect the expression of hepatic inflammatory factor tumor necrosis factor-α (TNF-α), and receptor-interacting protein (RIP)3 mRNA. The percentages of immune cells in liver and spleen were detected and analyzed by flow cytometry.

Results

UDCA decreased the serum ALT and AST levels, down-regulated the expression of cytokine TNF-α mRNA and necroptosis marker RIP3 mRNA in the liver tissue, up-regulated the percentages of immunomodulatory myeloid-derived suppressor cells (MDSCs) in liver and spleen tissues, and down-regulated the accumulation of liver macrophages of mice with immune hepatitis.

Conclusions

UDCA attenuates ConA-induced hepatic inflammation in mice by reducing the production of hepatic inflammatory factors, inhibiting the expression of necroptosis signal proteins in hepatocytes, down-regulating the accumulation of hepatic macrophages, and increasing the percentage of MDSCs with immunomodulatory properties.

Introduction

The etiology of autoimmune hepatitis (AIH) remains unclear, and its treatment mainly depends on immunosuppressants including glucocorticoids and azathioprine. Approximately 38–93% of patients achieve remission after treatment, but 90% relapse after drug withdrawal [Hoeroldt et al. 2011, Czaja et al. 2002]. Long-term use of immunosuppressants can lead to serious side effects such as osteoporosis and metabolic syndrome [Manns et al. 2010, Summerskill et al. 1975]. Therefore, actively exploring for new treatments is necessary. Ursodeoxycholic acid (UDCA) is a hydrophilic bile acid that is widely used for the treatment of various cholestatic diseases. This substance plays an immunomodulatory role in primary biliary cholangitis, but its liver protection and immunomodulatory effect in AIH remain unclear [Poupon 2012]. Necroptosis plays an important role in liver inflammation and injury because it is accompanied by the production of a good many of inflammatory factors and the release of injury-related molecules [Zhang et al. 2018]. Autoantibodies to hepatocytes and autoreactive T cells are produced in patients with

AIH, leading to liver inflammation. Owing to their immunosuppressive function, myeloid-derived suppressor cells (MDSCs) can inhibit T cell response and have protective effects on liver injury in both patients with AIH and mice with immune hepatitis [Diao et al. 2014, Zheng et al. 2018]. In this study, the mouse model of immune hepatitis induced by concanavalin A (ConA) was used to evaluate the effects of UDCA on serum transaminase, liver inflammatory factors, necroptosis-related molecules, and percentages of immune cells in liver and spleen. With this model, the anti-inflammatory effect of UDCA on immune hepatitis were determined, and the possible molecular mechanism was explored.

Materials And Methods

Experimental animals

Eighteen 6-week-old female C57BL/6J mice (Animal Certificate No.: 1103222011006028) weighing 20±2g were obtained from Beijing Huafukang Biotechnology Limited Company. The mice were housed in the Animal Experimental Center of Tianjin Medical University at a room temperature 24±2°C and a humidity 40–60% with a 12 h alternating light and dark cycle. Six mice were housed in a standard cage and allowed to drink and eat freely. All procedures were carried out in accordance with Guide for the Care and Use of Laboratory Animals, and the regulations of the People's Republic of China on the Administration of Experimental Animals and the requirements of ethics and morality.

Treatments of mice with UDCA and ConA

Eighteen 6-week-old female C57BL/6J mice were randomly classified into three groups each with six mice: normal control (Control group), model (ConA group) and UDCA pretreatment (ConA+UDCA group) groups. The mice in Control and ConA group were intragastrically administered with normal saline once a day for 1 week. The mice in ConA+UDCA group were intragastrically administered with UDCA (50 mg/kg, Aladdin Reagent, Shanghai) once a day for 1 week. ConA and ConA+UDCA groups were injected with ConA solution (15 mg/kg□Solarbio, China) via tail vein. After 12 hours post-ConA injection, the blood was collected to detect the levels of serum ALT and AST by using an automatic biochemical detector. The liver and spleen tissues were harvested and preserved in 4% tissue fixation solution, liquid nitrogen, and precooled phosphate buffer solution (PBS) for testing.

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

RNA was extracted from animal liver samples using the Trizol reagent (GIBCO, USA) in accordance with the experimental procedure. Total RNA was converted into cDNA using cDNA first strand synthesis kit (Beijing Tiangen Biotechnology Limited Company) following the experimental procedure. Real-time RT-PCR was performed in a Realtime PCR apparatus. The expression quantity of genes was calculated by $2^{-\Delta\Delta Ct}$. The primer sequences used were as follows: TNF-α gene, 5'-GTGCGTCCCTTGCAGCCACT-3' (forward) and 5'-GCAACAGCACCGCAGTA GCTGA-3' (reverse); GAPDH gene, 5'-CATCACTGCCACCCAGAAGACTG-3' (forward) and 5'-ATGCCAGTGAGCTTCCCGTTCAG-3' (reverse); RIP3

gene, 5'-AAGTGCAGATTGGGAACTACAACTC-3' (forward) and 5'-AGAATGTTGTGAG CTTCAGGAAGTG-3' (reverse).

Analysis of immune cells percentage in liver and spleen by flow cytometry

Flow-cytometric analysis of $CD45^+F4/80^+$ macrophage and $CD11b^+Gr\text{-}1^+$MDSCs was performed using fresh liver and spleen tissue samples following the protocol. Immediately after the mice were sacrificed, fresh liver and spleen tissue samples were collected in tubes filled with PBS solution precooled at 4°C. The tissues were cut into pieces, digested in trypsin (SIGMA, USA) at 37°C for 0.5–1 hour, ground into homogenate, filtered, and centrifuged. The supernatant was discarded. The cell precipitation was added with 30% Percoll solution (Solarbio, China) and 70% Percoll separation solution. The intermediate layer obtained after centrifugation was mononuclear cells. After being cleaned with PBS, the mononuclear cells were re-suspended, transferred into flow tube, and incubated with rabbit anti-mouse CD11b antibody, rabbit anti-mouse Gr-1 antibody, rabbit anti-mouse F4/80 antibody, and rabbit anti-mouse CD45 antibody (BD Pharmingen, USA) following the protocol. Flow Jo flow analysis software was used for data analysis.

Statistical analysis

SPSS 21.0 software was used for statistical analysis, and the measurement data were shown as mean ± standard deviation (±*s*). Independent sample T test was used for the comparison between two groups, and $P< 0.05$ was considered statistically significant.

Results

UDCA decreases the serum levels of ALT and AST in mice with immune hepatitis

After a single tail vein injection of ConA, liver inflammation and hepatocyte injury occurred in mice in a short time. Fig. 1 shows that compared with the Control group, ConA group had significantly increased serum ALT and AST levels. Compared with the ConA group, the ConA+UDCA group had significantly decreased serum ALT and AST levels. The results show that UDCA effectively protects against ConA-induced liver injury in mice.

UDCA improves the pathological manifestation of liver in mice with immune hepatitis

Figure 2 shows that 12 hours after a single injection of ConA via the tail vein, the liver and spleen of mice in ConA group became congested and swollen, and the liver granules were thickened. Under a microscope, hepatocyte edema, inflammatory cell infiltration, and patchy necrosis were observed in the liver of mice in ConA group. Only slight hepatocyte edema, inflammatory cell infiltration, and necrosis

were found in ConA+UDCA group. This result confirms that UDCA pretreatment can improve liver inflammation in mice with immune hepatitis.

UDCA down-regulates the expression of cytokine TNF-α mRNA and necroptosis marker RIP3 mRNA in the liver tissue of mice

To explore the mechanisms of UDCA protection against liver injury, we detected the expression of hepatic inflammatory factor TNF-α and RIP3 mRNA by qRT-PCR. Compared with the Control group, ConA group had significantly increased expression levels of TNF-α and RIP3 mRNA. Compared with the ConA group, ConA+UDCA group had significantly decreased expression levels of TNF-α and RIP3 mRNA. (Fig. 3). These results indicate that UDCA pretreatment can reduce the release of TNF-α and inhibit the expression of signal protein in hepatocyte necroptosis pathway.

UDCA regulates immune cell infiltration in the liver and spleen of mice with immune hepatitis

To determine whether the protective effect of UDCA pretreatment on liver is related to MDSCs with immunosuppressive effect, the cell percentages of $CD45^+F4/80^+$ macrophages and $CD11b^+Gr\text{-}1^+$MDSCs in liver and $CD11b^+Gr\text{-}1^+$MDSCs in the spleen tissues of mice were detected by flow cytometry (Fig. 4 and Fig. 5). Compared with the Control group, ConA group had increased percentage of liver macrophages and MDSCs in liver and spleen. Compared with ConA group, ConA+UDCA group had significantly decreased percentage of liver macrophages and significantly increased percentage of MDSCs in liver and spleen. These results show that UDCA can up-regulate the percentages of immunomodulatory MDSCs in liver and spleen tissues and down-regulate the accumulation of liver macrophages.

Discussion

AIH is an inflammatory lesion of the liver caused by autoantibodies to hepatocytes and autoreactive T cells. Its etiology is still unclear and is considered to be related to genetic susceptibility and molecular simulation. There are significant differences in the response to the existing immunosuppressant therapy and prognosis of different AIH patients. Poor response can often lead to liver cirrhosis [Gleeson et al. 2011], liver failure, and even death, hence, suitable treatment is needed. UDCA is the most hydrophilic and least toxic bile acid formed by the isomerization of 7α-hydroxy-chenodeoxycholic acid by bacteria in the intestine. This substance reaches the liver through enterohepatic circulation in the body and has the functions of cholagogic, antioxidant, anti-inflammatory, immunomodulatory, and cellular protection. UDCA is widely used in many liver diseases, especially cholestatic diseases [Roma et al. 2011]. This bile acid can improve the biochemical and immunological indexes of patients with AIH [Li et al. 2015], but the specific mechanism is unknown.

ConA-induced liver injury in mice is currently recognized as an immune-mediated liver injury model; ConA induces circulating T lymphocytes to converge into the hepatic sinusoid and locally proliferate, thus

release various of cytokines and stimulating macrophages to produce TNF-α, the most important cytokine leading to ConA-indued liver injury [Trautwein et al. 1998, Watanabe et al. 1996, Tiegs et al. 1992]. In this experiment, the serum ALT and AST levels in mice were significantly increased 12 hours after ConA was injected via the tail vein. Congestion and edema of liver and spleen were visible even to naked eyes, and hepatocyte edema, inflammatory cell infiltration, and patchy necrosis were observed in mouse liver under a microscope. These findings are consistent with previous reports. In UDCA-pretreated group, the serum ALT and AST levels decreased, the congestion of liver and spleen improved, and only slight hepatocyte edema, inflammatory cell infiltration, and necrosis were observed. These results confirm that UDCA can improve liver inflammation in mice with immune hepatitis.

Necroptosis is regulated by signal molecules and driven by the activation of RIP1, RIP3, and MLKL. The best initiating factor is cytokine TNF-α produced by macrophages [Galluzzi et al. 2008]. RIP3 is a key molecule in necroptosis pathway, and its knockout blocks cell necrosis induced by LPS and TNF-α [Kearney et al. 2014]. RIP3-mediated inflammatory signaling pathway may be involved in the occurrence and development of AIH and thus could be a potential therapeutic target [Zhang et al. 2018]. In this study, the expression of TNF-α and RIP3 mRNA in liver tissue of mice with immune hepatitis induced by ConA increased, suggesting the destruction of hepatocytes and the activation of necroptosis pathway, and this finding was consistent with previous reports [Trautwein et al. 1998, Deutsch et al. 2015]. UDCA pretreatment decreased the expression of TNF-α and RIP3 mRNA in liver tissue, indicating that it can reduce the release of TNF-α and inhibit the expression of signal protein in hepatocyte necroptosis pathway.

MDSCs are derived from bone marrow progenitor cells and immature myeloid cells. These cells can differentiate into mature granulocytes, dendritic cells, and macrophages and enter corresponding organs and tissues to exert normal immune function. Under the conditions of tumor, infection, and inflammation, these progenitor cells fail to mature and instead become immunosuppressive MDSCs that inhibit the activation of macrophages, T cells, B cells, and natural killer cells; activate Treg helper cells; and exert immune regulation [Souliotis et al. 2015]. MDSCs have a protective effect on liver injury in patients with AIH and immune hepatitis mouse models [Diao et al. 2014, Zheng et al. 2018]. Flow cytometry was used to detect and analyze the percentages of $CD45^+F4/80^+$macrophages and $CD11b^+Gr\text{-}1^+$MDSCs in liver tissues and $CD11b^+Gr\text{-}1^+$MDSCs in the spleen tissues of mice. The results showed that the percentages of these immune cells increased, confirming the infiltration of macrophages in liver tissues. The percentage of MDSCs in liver tissues and spleen tissues also increased in mice with immune hepatitis induced by ConA, and this finding was consistent with previous reports [Diao et al. 2014, Zheng et al. 2018]. The percentage of $CD45^+F4/80^+$ macrophages in liver tissues of mice pretreated with UDCA was significantly lower than that in ConA group, whereas the percentage of $CD11b^+Gr\text{-}1^+$MDSCs in liver tissues and spleen tissues increased. These results shows that UDCA can up-regulate the percentages of immunomodulatory MDSCs in liver and spleen tissues and down-regulate the accumulation of liver macrophages.

The successfully established mouse model of immune hepatitis induced by ConA confirmed the increase in transaminase and the release of inflammatory cytokine TNF-α, expression of necroptosis pathway signal protein RIP3, macrophage accumulation, and up-regulated percentages of MDSCs with immunomodulatory properties in mouse liver and spleen. UDCA pretreatment could reduce ConA-induced liver inflammation in mice possibly by reducing the release of hepatic inflammatory factors, inhibiting the expression of necroptosis signal proteins in hepatocytes, down-regulating the accumulation of liver macrophages, and increasing the percentages of MDSCs with the immunomodulatory properties of liver and spleen. These results provide a theoretical basis for the clinical application of UDCA in AIH.

Abbreviations

UDCA
Ursodeoxycholic acid
ConA
Concanavalin A
AIH
Autoimmune hepatitis
ALT
Alanine aminotransferase
AST
Aspartate aminotransferase
TNF-α
Tumor necrosis factor-α
RIP
Receptor-interacting protein
MDSCs
Myeloid-derived suppressor cells.

Declarations

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None.

Authors' Contributions

Guixian Ji, Man Liu, and Yi Zhou contributed equally to this work. All authors read and approved the final manuscript.

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Availability of data and materials

Data will be available on request.

Ethics approval

All procedures were carried out in accordance with Guide for the Care and Use of Laboratory Animals, and the regulations of the People's Republic of China on the Administration of Experimental Animals and the requirements of ethics and morality.

Consent to participate

Not applicable.

Consent for publication

All authors read and approved the manuscript for publication.

Competing interests

The authors declare that there is no conflict of interest.

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Figures

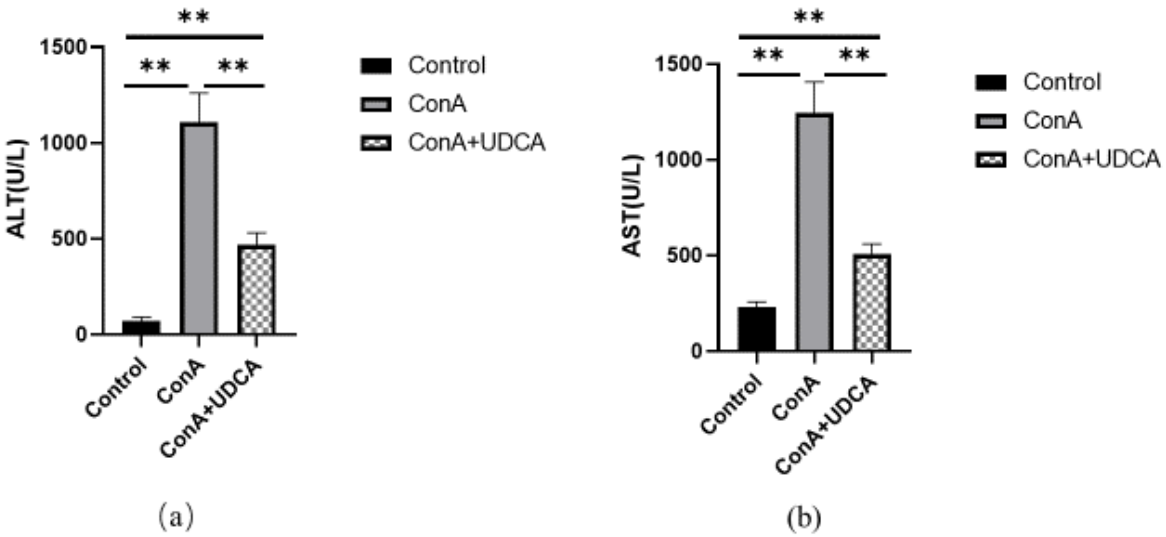

Figure 1

UDCA decreases the serum levels of ALT and AST in mice with immune hepatitis. Notes: mice were induced by intragastric administration with normal saline or UDCA (50 mg/kg) at 7 days before injection of ConA (15 mg/kg). ALT (a) and AST (b) levels were determined 12 h after ConA injection. The data were expressed as mean ± SD (n = 6, *P<0.05, ** P<0.01).

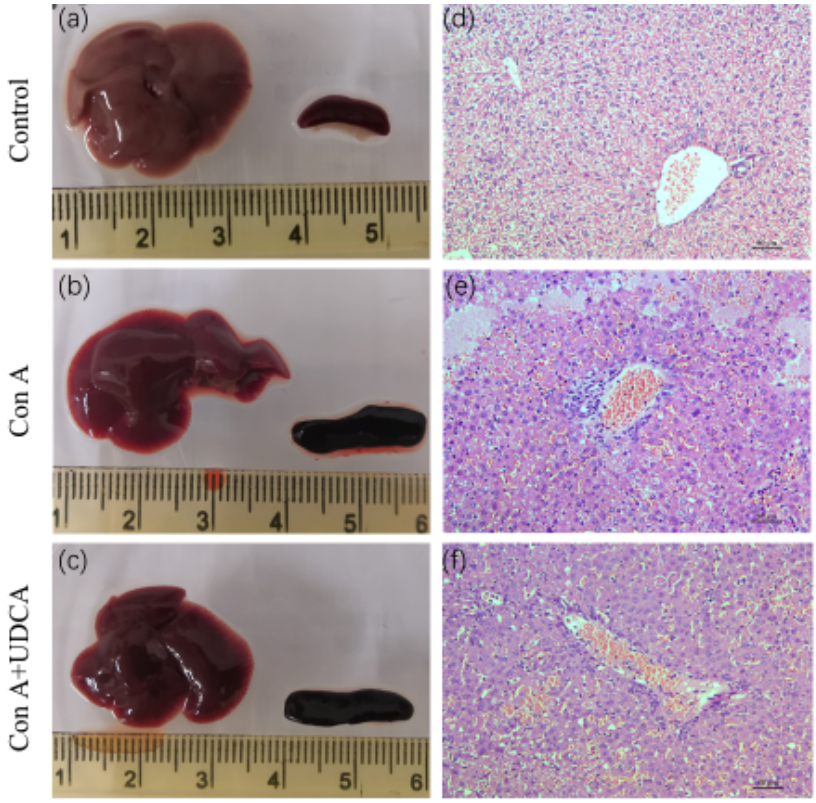

Figure 2

UDCA improves the pathological manifestation of liver in mice with immune hepatitis. Notes: mice were induced by intragastric administration with normal saline or UDCA (50 mg/kg) at 7 days before injection of ConA (15 mg/kg). Mice were sacrificed at 12 h after ConA injection. The livers and spleens were harvested from three groups. Liver tissues from Control (a, d), ConA (b, e), and ConA+UDCA (c, f) groups were fixed and stained with H&E. H&E: hematoxylin and eosin.

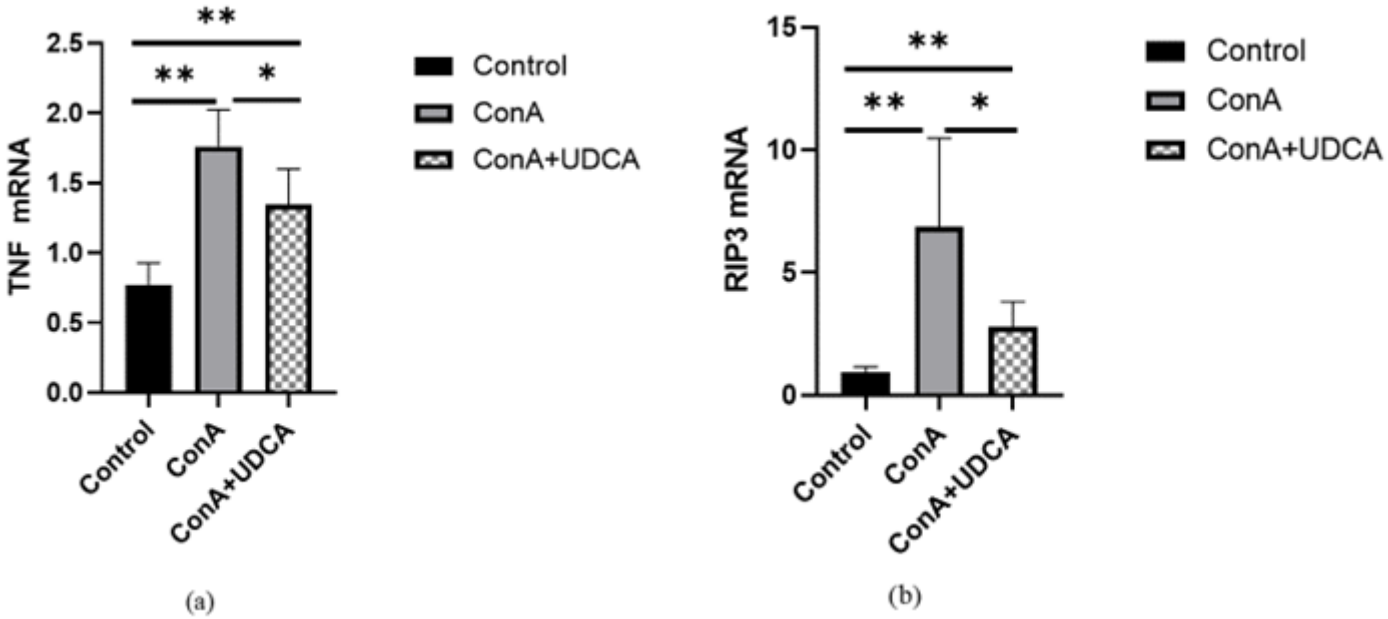

Figure 3

Levels of TNF-α and RIP3 mRNA in the liver tissues of mice. Notes: female C57BL/6J mice were induced by intragastric administration with normal saline or UDCA (50 mg/kg) at 7 days before injection of ConA (15 mg/kg) via the tail vein. qRT-PCR was used to detect the expression of TNF-α (a) and RIP3 (b) mRNA 12 hours after ConA injection. The data were expressed as mean ± SD (n = 6, *P<0.05, ** P<0.01).

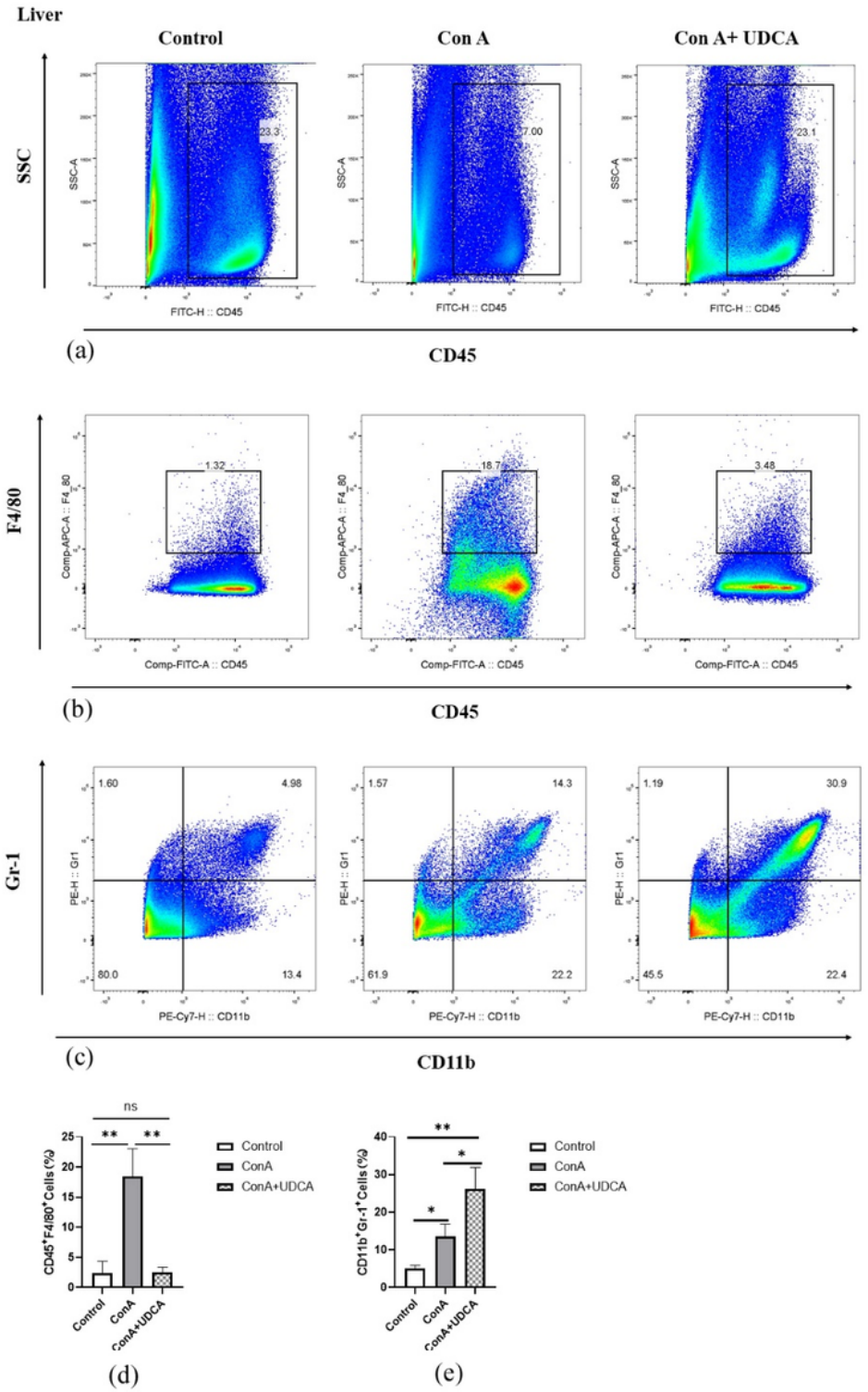

Figure 4

Effect of UDCA on immune cell infiltration of liver in mice with immune hepatitis. Notes: mice were induced by intragastric administration with normal saline or UDCA (50 mg/kg) at 7 days before injection of ConA (15 mg/kg) via the tail vein. Percentages of CD45+F4/80+ macrophages (a, b, d) and CD11b+Gr-1+MDSCs (c, e) in liver were detected by flow cytometric analysis 12 h after ConA injection.

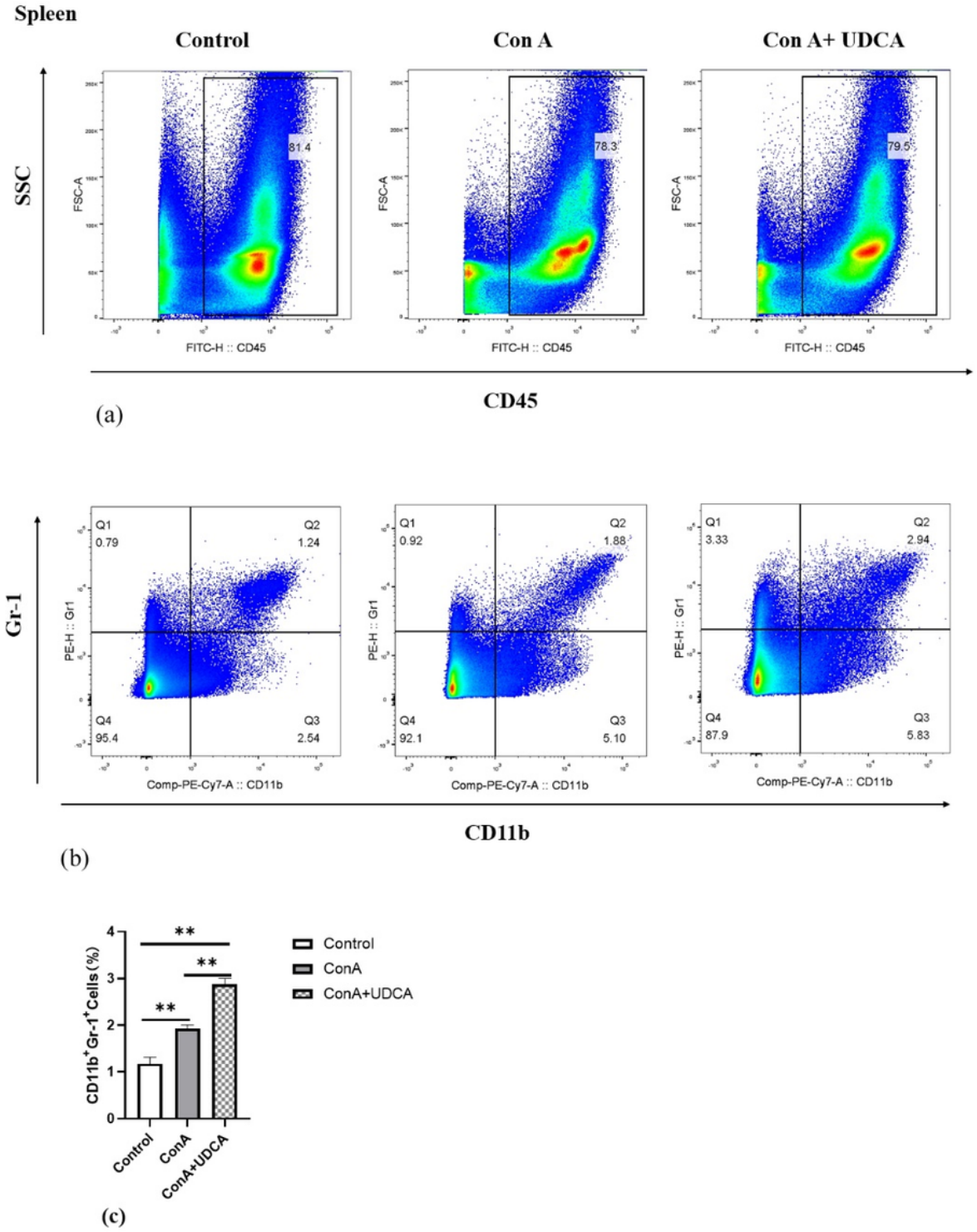

Figure 5

UDCA enhances splenic MDSCs infiltration in mice with immune hepatitis. Notes: mice were induced by intragastric administration with normal saline or UDCA (50 mg/kg) at 7 days before injection of ConA (15 mg/kg) via the tail vein. Percentages of CD11b+Gr-1+MDSCs in spleen (a, b, c) were detected by flow cytometric analysis 12 h after ConA injection.