

Synergistic Inhibitory Effect of the Gut Microbiome and Lithocholic Acid on Liver Fibrosis

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Research

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Abstract

Background: Lithocholic acid are essential signaling molecules that mediate the relationship between the gut microbiome and liver function by regulating inflammation. The purpose of this study is to investigate the role of lithocholic acid in liver fibrosis.

Methods: A liver fibrosis mouse model was induced by carbon tetrachloride followed by gavage of lithocholic acid, and the effects of lithocholic acid were evaluated by serum biochemical analysis and liver histology. Plasma cytokine levels and the number of immune cells were determined by cytometric bead array and flow cytometry, respectively.

Results: Lithocholic acid treatment increased the recruitment of NK cells and reduced the activation of NKT cells, and reduced M1 macrophages differentiation and increased M2 macrophages differentiation. Furthermore, the lithocholic acid prevented inflammatory liver disease by reducing TNF- α and IL-22 secretion. However, the effect of lithocholic acid disappeared when the host gut microbiome was treated with antibiotics.

Conclusions: It showed that the activation of lithocholic acid-mediated signaling was linked to the inhibition of inflammation and improvement of liver fibrosis. The role of lithocholic acid in liver fibrosis is mediated by the gut microbiome. The association between the gut microbiome, lithocholic acid, and liver function can serve as a therapeutic target for liver fibrosis.

Introduction

The enterohepatic circulation of bile acids (BAs) and blood circulation of the hepatic portal vein closely connect the gut microbiome with liver diseases, and form the gut microbiome-bile acids-liver triangle. However, the relationship between bile acids, the gut microbiome and liver diseases is incompletely understood. We speculate that the inflammatory response and innate immunity play an essential role between them.

Previous studies have suggested that bile acids are tissue-damaging agents that promote inflammation¹. In liver diseases, the accumulation of hydrophobic bile acids disrupts the mitochondrial membrane and the intestinal mucosal barrier and causes cell necrosis, allowing the gut microbiome and its metabolites to enter the liver^{2,3}. However, in recent years, studies have fully confirmed the role of bile acids in host metabolism and cancer progression, especially the effect of lithocholic acid (LCA) in innate immunity. As the strongest natural agonist of transmembrane G protein-coupled receptor (TGR5), LCA can reduce the phagocytic activity and the production of pro-inflammatory cytokine by activating TGR5 on monocytes and macrophages⁴. In addition, it can also inhibit the secretion of IL-6, IL-1A, and IL-1B induced by LPS and TNF secretion by Kupffer cells through a TGR5-cAMP-dependent pathway, ultimately inhibiting liver inflammation⁵. Because of the special anatomic relationship between the intestine and liver and the

bidirectional interaction between bile acids and the gut microbiome. This study investigates whether the gut microbiome can affect the liver inflammatory response and the progress of liver fibrosis through LCA.

Materials And Methods

Murine studies

C57BL/6 male mice aged 6-7 weeks were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. Animals were randomly assigned to the following groups: control, Carbon tetrachloride (CCl₄), CCl₄ + LCA, and CCl₄+ LCA +VM (vancomycin + ampicillin). The CCl₄ group received CCl₄ (Merck, Darmstadt, Germany) intraperitoneally twice a week (1mL/kg of body weight, 1:4 in olive oil). The CCl₄ + LCA group received CCl₄ intraperitoneally received LCA (1 mg/15 g of body weight, twice a week) by oral gavage. The CCl₄+ LCA + VM group received CCl₄ and LCA as described above and both ampicillin (MCE, 1g/L) and vancomycin (Sigma, 0.5 g/L) in the drinking water⁶. All mice were treated for 8 weeks and were sacrificed 48 hours after the last injection. The study conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the research ethics committee of the Zhejiang University School of Medicine.

Flow cytometry

Flow cytometry was performed on a BD LSRFortessa cell analyzer. The number of viable NKT cells and macrophages was determined using DAPI (BioLegend) and anti-7AAD-PerCP/Cy5.5 (BioLegend), respectively. The following fluorescent antibodies were used in flow cytometry: anti-CD45-PerCP/Cy5.5 (clone 30-F11, BD pharmigen), anti-CD3-FITC (clone 17A2, BD PharMingen), anti-NK1.1-Qdot 605 (clone PK136, BD PharMingen), anti-CXCR6-APC (clone SA051D1, BioLegend), anti-CD45-FITC (clone 30-F11, BioLegend), anti-CD11b-APC (clone M1/70, BioLegend), anti-F480-BV421 (clone BM8, BioLegend), anti-CD206-PE (clone C068C2, BioLegend), anti-MHCII-PECy7 (clone M5/114, BioLegend).

Quantitative real-time PCR

mRNA expression was assessed by quantitative real-time PCR. Primers are listed in supplementary material. Total RNA was extracted from mouse liver tissue. Reverse transcription was performed using the Takara-PrimeScript™ RT reagent Kit. PCR was carried out in 384-well plates using TaKaRa TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus), and a QuantStudio™Dx Real-Time PCR Instrument (Life technologies).

16S rRNA sequencing and analysis

The methods for analyzing the gut microbiome in mouse stool samples were described previously⁷.

Biochemical analysis

The levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using an automatic biochemical analyzer (DRI-CHEM 4000ie Fujifilm Corporation, Japan) and two test kits (FUJI DRI-CHEM Slide GOT/AST-PIII and GFP/ALT-PIII, respectively). Slide GOT/AST-PIII and GFP/ALT-PIII, respectively. Plasma cytokines were quantified using the Mouse Th17 Panel (8-Plex) with V-bottom Plate V02 (Cat. No. 740749) as per the manufacturer's instructions. The cytokines profiled were: interferon (IFN)- γ , tumor necrosis factor (TNF)- α , IL-6, IL-10, IL-22, IL-21, IL-17F and IL-17A.

Statistical analysis

Statistical analysis was performed using SPSS software version 21.0. Data were expressed as means and \pm standard deviations. The differences between groups were assessed by two-tailed unpaired Student's *t*-test or ANOVA. A *p*-value smaller than 0.05 was considered statistically significant.

Results

Lithocholic acid inhibits liver inflammation and improves liver fibrosis

The healthy liver is known for an active innate immune response and a weaker adaptive immune response⁸. Viruses, toxins, insulin resistance and hypoxia induce a chronic low-grade inflammation in the liver, and essentially creates a fibrotic environment⁹. Studies have shown that compensated cirrhosis is reversible after treating hepatitis B virus (HBV) and hepatitis C virus infections, which eliminates long-term stimulation of the liver by inflammation, demonstrating that controlling liver inflammation improves liver fibrosis and may lead to fibrinolysis^{10,11}.

The regulation of inflammation by LCA was investigated *in vivo*. For this purpose, LCA was fed to mice with CCl₄-induced liver fibrosis. The mRNA expression of liver fibrosis makers, including matrix metalloproteinase (MMP)-2, tissue inhibitor of matrix metalloproteinases 1 (Timp-1), α -smooth muscle actin (α -SMA), collagens I and III was measured. LCA treatment significantly reduced the mRNA expression of the markers in mice, except for the anti-fibrotic MMP-2 (Fig. 1A). Liver sections were stained with hematoxylin-eosin and Masson trichrome to evaluate the function of LCA on liver injury and fibrosis. The results showed that collagen deposition and inflammatory cell infiltration were lower in the CCl₄+LCA group than in the CCl₄ group. However, the anti-fibrotic effect of LCA was reduced in the CCl₄+LCA+VM group (Figs.1B and C). These data indicate that LCA can improve liver fibrosis progression by controlling liver inflammation, and that the anti-fibrotic effect of LCA is mediated by the gut microbiome.

Anti-fibrotic activity of hepatic immune cells

To investigate the mechanism underlying liver fibrosis suppression and inflammation, we analyzed the subsets of intrahepatic immune cells in mice. Macrophages are classified into two types: M1 with a pro-inflammatory effect; and M2 with anti-inflammatory and immunosuppressive effects¹². The results of multicolor flow cytometric analyses showed that the number of M1 macrophages decreased and the number of M2 macrophages increased in the CCl₄+LCA group compared with the CCl₄ group (Figs.2A

and B). However, after the LCA treated mice add antibiotics to destroy the homeostasis of the gut microbiome, the number of M1 and M2 macrophages not significantly different between the CCl4+LCA and CCl4+LCA+VM groups. The results suggest that LCA has anti-inflammatory activity in the liver and can modulate innate immunity by regulating macrophage differentiation. Nonetheless, the effect of LCA on macrophage differentiation was not mediated by the gut microbiome.

NKT cells are abundant in the liver, representing approximately 30% of hepatic lymphocytes¹³. Our data showed that NKT cells were depleted during liver fibrosis progression (Figs.3A and B). Consistent with other previous studies, the number of intrahepatic NKT cells was reduced in chronic liver injury, and this phenomenon is most significant in CCl4-induced toxic fibrosis¹⁴. This result is due to either activation-induced NKT cell death or loss of cell markers such as NK1.1^{15,16}. Our results suggest that gut microbiome can improve liver injury by suppressed NKT cells activation via LCA. However, the protective effect of LCA disappeared after treatment with antibiotics to reduce the gut commensal bacteria (Figs.1A, B, and C).

The human liver contains more NK cells than NKT cells, corresponding to 30-50% of total lymphocytes¹⁷. In our results, the number of NK cells was significantly higher in the CCl4 + LCA group than in the CCl4 group, and this number decreased after antibiotics treatment (Fig.2C). Consistent with our previous findings, the effect of LCA disappeared in the absence of gut commensal bacteria. The hepatoprotective effect of NK cells has been well documented in chronic liver diseases. NK cells can suppress inflammation by secreting anti-inflammatory factors. Moreover, activated NK cells have anti-fibrotic activity by killing pro-fibrotic hepatic stellate cells (HSCs), and this activity mediated by NKp46–NCR1 and NKG2D–MIC-A or MIC-B interactions, thereby reducing liver fibrosis in vivo¹⁸.

To connect these findings, several cytokines were measured in the peripheral blood of mice. Serum TNF- α levels of were significantly higher in the CCl4 group than in the CCl4+LCA group. However, TNF- α levels significantly increased after disrupting the gut microbiome with antibiotics (Fig.4A). M1 macrophage infiltration induces the production of inflammatory factors, including TNF- α , with strong pro-inflammatory or fibrotic effects on myofibroblasts and HSCs^{19,20}. Previous studies have shown that NKT cells release pro-inflammatory cytokines, including TNF- α , IL-4, and IFN-g, which promote fibrosis¹⁹. In other words, LCA can inhibits TNF- α secretion by reducing the activation of NKT cells and M1 macrophages, and this effect is mediated by the gut microbiome.

As expected, we also found that LCA can decreased the serum levels of IL-22, whereas changing the gut microbiome through antibiotics, which had little effect on IL-22 levels (Fig.4B). It is known that multiple intrahepatic immune cells, including macrophages, NK and NKT cells, and CD4 and CD8 T cells, secrete IL-22²¹. Interestingly, there are many articles confirming that IL-22 mediates liver protection functions in acute liver injury^{22,23}. However, increasing evidence has shown that IL-22 aggravates liver cell damage and inflammation in chronic liver disease²⁴. Many advances pointed out that the expression of IL-22 was

up-regulated in the liver of patients with chronic HBV infection, and promoted liver fibrosis by the inducing the migration of intrahepatic Th17 cells^{21,25}.

The gut microbiome mediates the anti-inflammatory effects of lithocholic acid

The liver is exposed to the gut microbiome and its metabolites through the intestinal circulation, which accounts for 70% of the blood supply to the liver²⁶. Changes in the gut microbiome can affect the function of hepatic immune cells, enterohepatic circulation, and LCA metabolism. Our results showed that antibiotics markedly reduced the abundance of gut commensal bacteria, increased the serum levels of ALT and AST (Fig.4C), and promoted the activation of NKT cells and M1 macrophages in mouse liver. Furthermore, antibiotic treatment aggravated liver inflammation and fibrosis, demonstrating that the anti-inflammatory and anti-fibrotic effects of LCA are reduced in the liver in the absence of a balanced gut microbiome.

Our study found that compared with the CCl₄ group, the Simpson's diversity index increased after LCA treatment (Fig.4D). Antibiotics treatment decreased microbial diversity and the relative abundance of Firmicutes. However, the Proteobacteria show excessive proliferation (especially Enterobacteriales) in CCl₄+LCA+VM group (Figs.4E and F). Studies commonly supported this concept that the excessive proliferation of Proteobacteria means that gut microbiome dysbiosis or an unstable gut microbial community structure²⁷. Generally, the human gut microbiome contains only a minor proportion of the phylum Proteobacteria under healthy conditions²⁸. However, this bacterial group causes colitis and inflammation under pathological conditions^{29,30}.

The relative abundance of Proteobacteria is positively correlated with intestinal inflammation, which may be because his bacterial taxon affects metabolism and immunity^{31,32}. In this respect, the oral administration of *Helicobacter typhlonius*, one of the Proteobacteria species also triggered colitis in mice lacking the recombinase-activating gene and the transcription factor T-bet³³. Antibiotics indreadsd serum TNF-a levels and abolished the anti-fibrotic effect of LCA (Fig. 4A and Figs.1A, B and C), which may be related to the increased relative abundance of *Escherichia coli* and Firmicutes, leading to local or systemic metabolic dysfunction^{34,35}. However, we suspect that LCA needs to be modified and/or transformed by the gut microbiome before it can play an anti-inflammatory and anti-fibrotic role in the liver.

Discussion

Some studies demonstrated the anti-inflammatory effect of bile acids^{4,36,37}. In this respect, LCA can reduce the phagocytic activity and the production of pro-inflammatory cytokines in monocytes, macrophages, and other immune cells in the liver by activating TGR5⁴. With regard to the effect of LCA on liver inflammation and fibrosis, our results showed that (1) LCA regulated multiple intrahepatic immune cells, including NKT/NK cells and M1 and M2 macrophages; (2) LCA increased TNF-a and IL-22 and improved chronic hepatic inflammation and fibrosis by activating NKT cells and M1 macrophages;

and (3) The anti-inflammatory and anti-fibrotic effects of LCA required the participation of the gut microbiome.

Although, gut commensal bacteria are involved in immune regulation and inflammatory response mediated by LCA in the liver, how the gut microbiome affects the role of LCA in liver inflammation, the mechanism of action of LCA, and the immune pathways regulated by LCA in NKT/NK cells remain to be explored.

In summary, our study identified a mechanism by which gut commensal bacteria use LCA as messengers to regulate intrahepatic immune cells and improve fibrosis in the mouse liver. Therefore, maintaining the gut microbiome homeostasis while administering LCA is a potential therapeutic strategy to improve liver fibrosis even reverse it. Furthermore, a better understanding of the interactions of gut microbiome-bile acids-liver triangle in liver fibrosis may help develop effective microecological and immune interventions.

Abbreviations

bile acids	BAs
lithocholic acid	LCA
carbon tetrachloride	CCl4
natural killer	NK
natural killer T	NKT
G protein-coupled receptors	TGR5
NOD-, LRR- and pyrin domain-containing 3	NLRP3
T helper 17	TH17 cell
regulatory T	Treg
interleukin	IL
lipopolysaccharide	LPS
hepatic encephalopathy	HE
aspartate aminotransferase	AST
alanine aminotransferase	ALT
Immune interferon	IFN
tumor necrosis factor	TNF
standard deviations	SD
hepatitis B virus	HBV
matrix metalloproteinase	MMP-2
inhibitor of matrix metalloproteinases 1	Timp-1
α -smooth muscle actin	α -SMA
hematoxylin and eosin	H&E
hepatic stellate cells	HSC
transforming growth factor	TGF

Declarations

Ethics approval and consent to participate

The study conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the research ethics committee of the Zhejiang University School of Medicine.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to their confidentiality, but are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Authors' contributions

MD. Zhi Chen and Dr. Junwei Shao designed the experiments and wrote the manuscript. Dr. Tiantian Ge, Dr. Junwei Shao and Sengzhong Chen conducted the major parts of experiments.

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Tables

Table 1 Primers used in the RT-qPCR reactions		
Gene symbol	Murine forward primer (5'- 3')	Murine reverse primer (5'- 3')
Timp-1	GCATCTCTGGCATCTGGCATC	GGTATAAGGTGGTCTCGTTGA
Mmp-2	GCTGATACTGACACTGGTACTG	CAATCTTTTCTGGGAGCTC
α -SMA	CTGACAGAGGCACCACTGAA	GAAGGAATAGCCACGCTCAG
Collagen I	TCCTCCAGGGATCCAACGA	GGCAGGCGGGAGGTCTT
Collagen III	CTGGTCAGCCTGGAGATAAG	ACCAGGACTACCACGTTTAC
GAPDH	ACGGCAAATTCAACGGCACAG	AGACTCCACGACATACTCAGCAC

MMP2 metalloproteinase-2, Timp-1 tissue inhibitor of matrix metalloproteinases 1, α -SMA a-smooth muscle actin (α -SMA),GAPDH

glyceraldehyde-3-phosphate dehydrogenase.

Figures

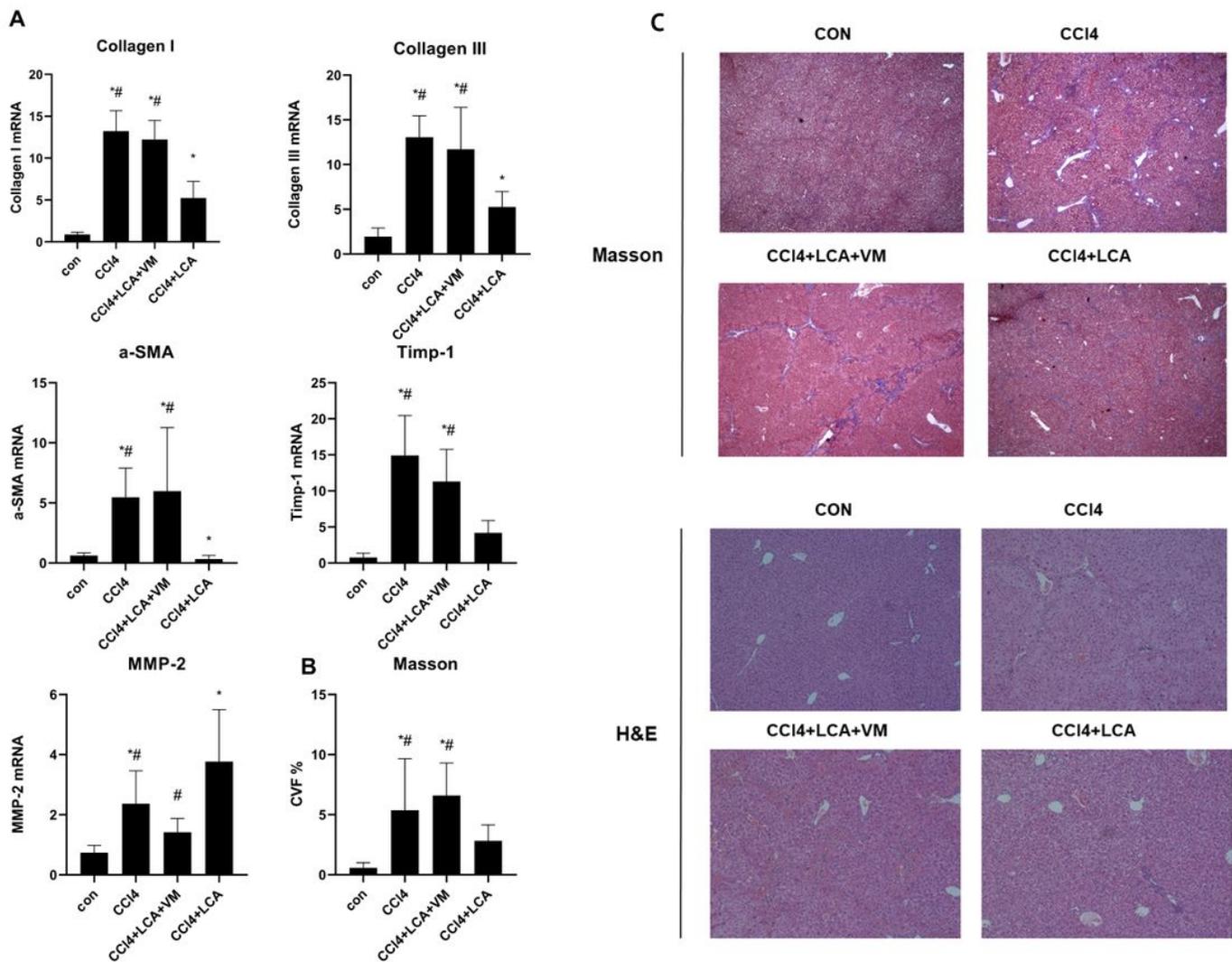


Figure 1

Lithocholic acid inhibits liver inflammation and alleviates liver fibrosis. (A) The mRNA expression levels of collagen (types I and III), α -SMA, TIMP-1, and MMP-2 in liver tissues of mice from each group. (B) The level of fibrosis in the liver was evaluated by the collagen volume fraction. (C) Representative images of liver tissue sections stained with hematoxylin and eosin (H&E) (200X) and Masson's trichrome staining. Collagen fibers (blue) and nuclei (brown) were shown (200X). *Represents the $P < 0.05$ compared with the normal control group; # represents $P < 0.05$ compared with the CCI4+LCA group. CCI4 carbon tetrachloride, VM vancomycin + ampicillin, LCA lithocholic acid, con control group, MMP-2 matrix metalloproteinase, Timp-1 inhibitor of matrix metalloproteinases 1, α -SMA α -smooth muscle actin, CVF collagen volume fraction, H&E hematoxylin and eosin.

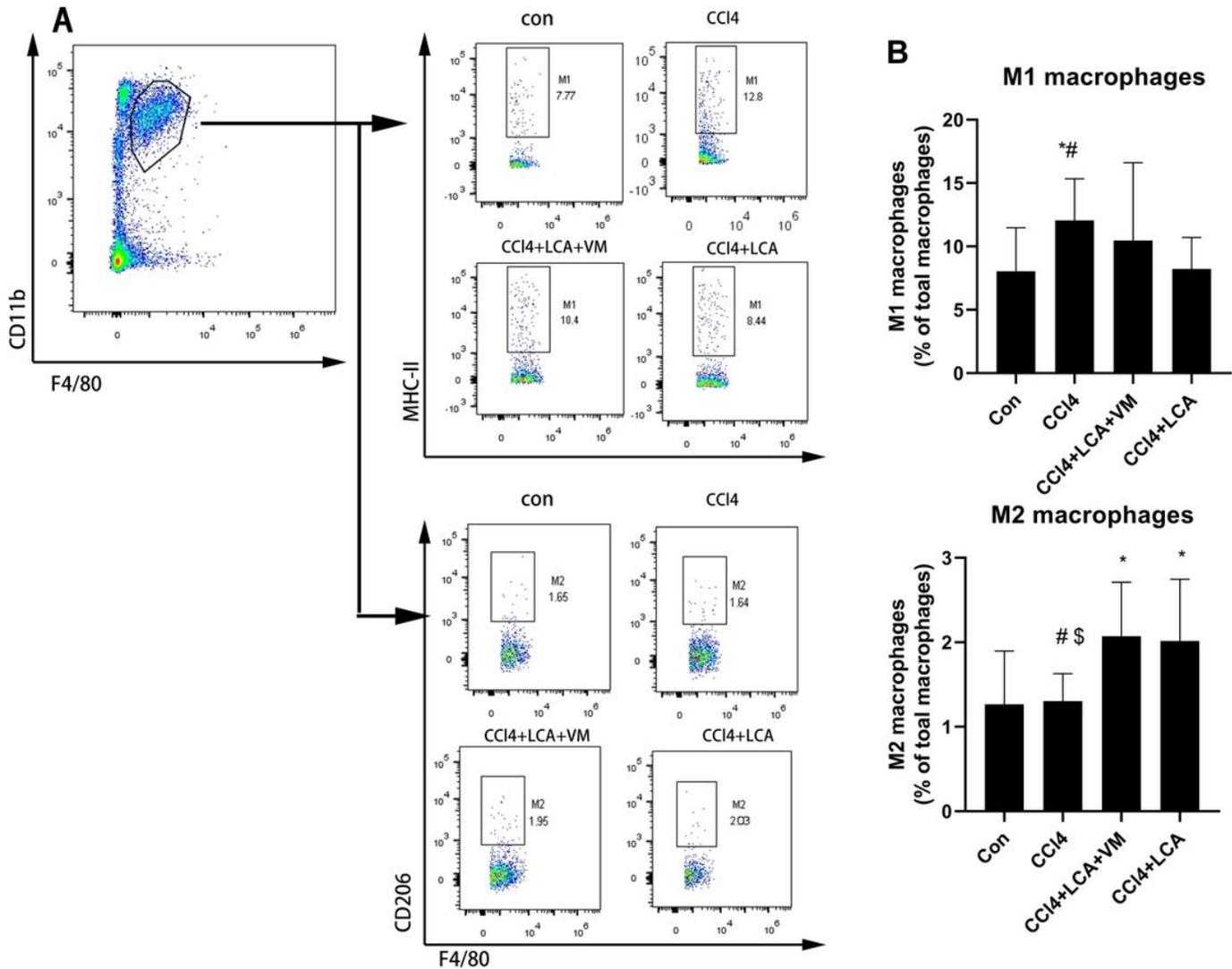


Figure 2

Lithocholic acid regulates the differentiation of macrophages in the liver. (A) Flow cytometry analysis of M1 macrophage and M2 macrophage in different group. (B) The percentage of M1 macrophages and M2 macrophages in the liver tissue of mice with different treatments. *Represents the $P < 0.05$ compared with the normal control group; # represents $P < 0.05$ compared with the CCI4+LCA group; \$ represents $P < 0.05$ compared with the CCI4+LCA+VM group. CCI4 carbon tetrachloride, VM vancomycin + ampicillin, LCA lithocholic acid, con control group.

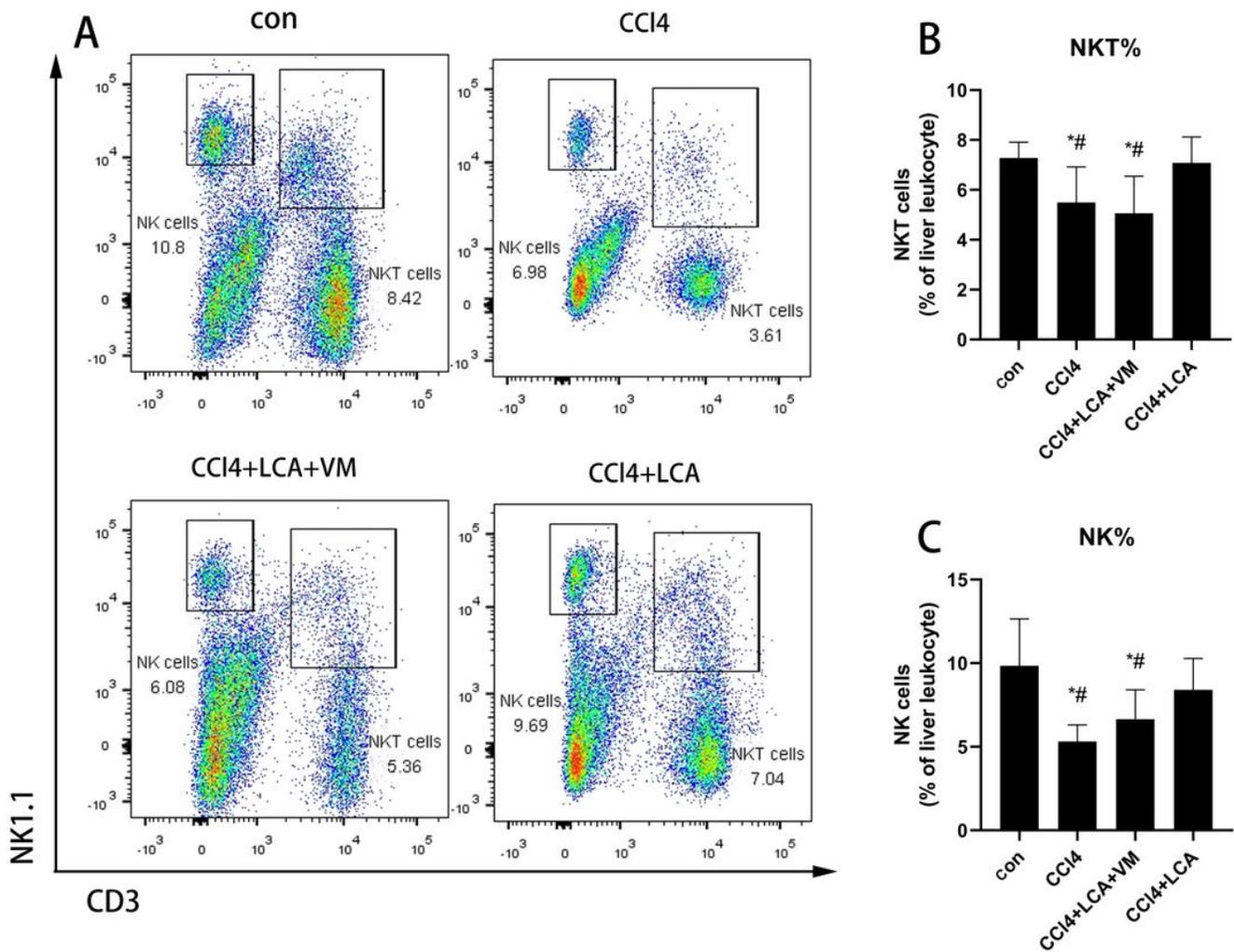


Figure 3

Hepatic NKT cells and NK cells increase after giving LCA gavage. (A) Flow cytometry analysis of hepatic NKT cells and NK cells in the liver tissue of C57BL/6 mice with different treatments. (B and C) The percentage of NKT/ NK cells in the liver tissue of mice with different treatments. *Represents the $P < 0.05$ compared with the normal control group; # represents $P < 0.05$ compared with the CCl4+LCA group. CCl4 carbon tetrachloride, VM vancomycin + ampicillin, LCA lithocholic acid, con control group.

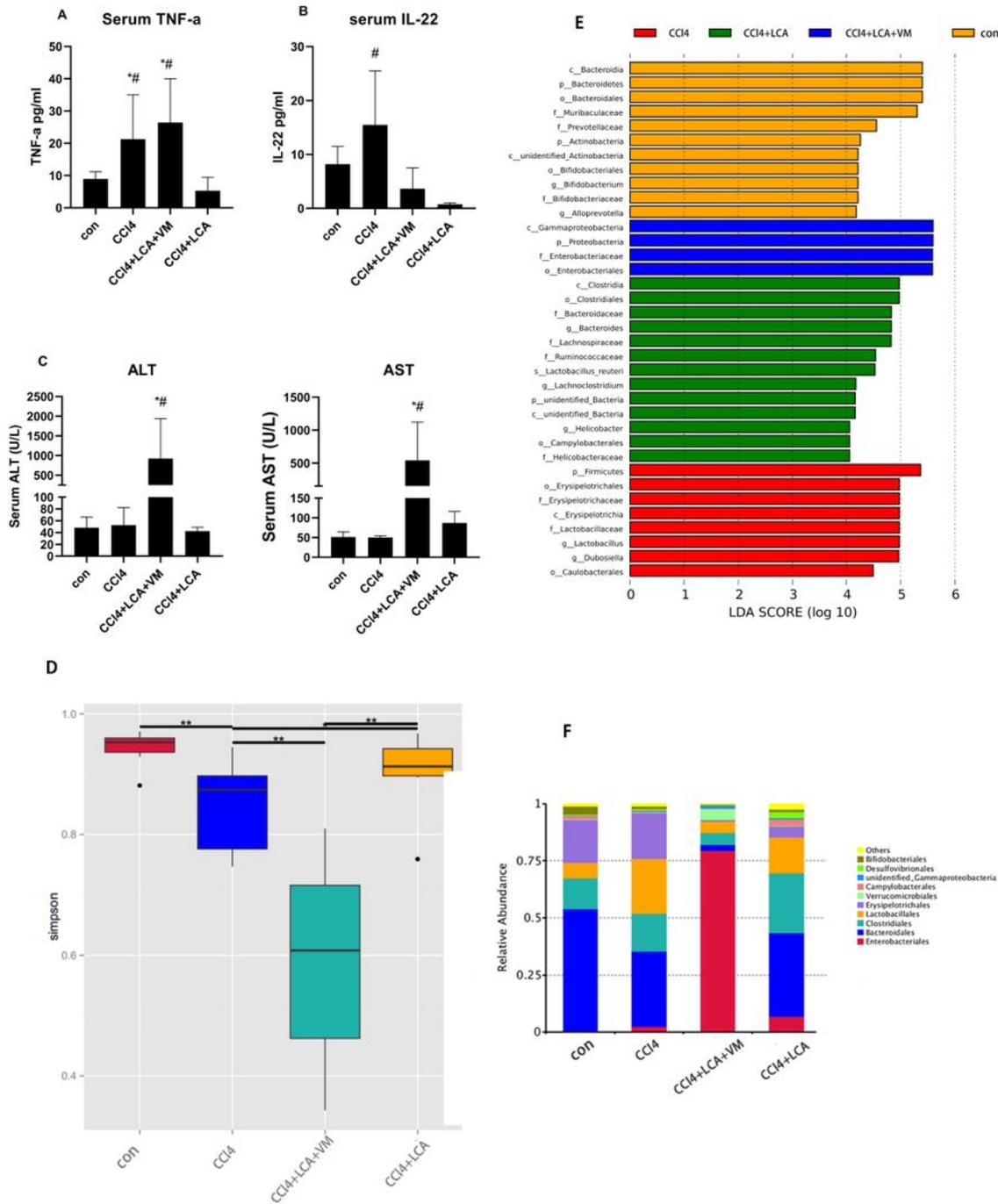


Figure 4

Gut microbiome affect the liver inflammatory response and the progress of liver fibrosis through LCA. (A and B) Serum TNF- α and IL-22 levels of mice in different groups. (C) Serum ALT and AST levels of mice in different groups. (D) Simpson indices of 16S rRNA genes from the gut microbiome in different treatment groups. (E) Functional microbial genes shown were enriched in the respective groups and had an LDA score higher than 4. (F) The relative abundance of the 10 most-represented OTUs in the mice fecal.

*Represents the $P < 0.05$ compared with the normal control group, ** $p < 0.001$; # represents $P < 0.05$ compared with the CCl₄+LCA group. CCl₄ carbon tetrachloride, VM vancomycin +ampicillin, LCA lithocholic acid, con control group, AST aspartate aminotransferase, ALT alanine aminotransferase, IL interleukin, TNF tumor necrosis factor, LDA linear discriminant analysis.