Gut-derived lipopolysaccharide promotes alcoholic hepatosteatosis and subsequent hepatocellular carcinoma by stimulating neutrophil extracellular traps through TLR4

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Abstract:

**Background:** Binge drinking leads to many disorders, including alcoholic hepatosteatosis, which is characterized by intrahepatic neutrophil infiltration and which increases the risk of hepatocellular carcinoma (HCC). Molecular mechanisms may involve the migration of bacterial metabolites from the gut to the liver and activation of neutrophil extracellular traps (NETs). **Methods:** Serum from both binge drinking and alcohol-avoiding patients was analyzed. Mouse models of chronic alcohol induced hepatosteatosis and HCC models were used. **Results:** A marker of NETs formation, lipopolysaccharide, was significantly higher in alcoholic hepatosteatosis and HCC patients and mice than in controls. Intrahepatic inflammation markers and HCC-related cytokines were decreased in mice with reduced NET formation due to neutrophil elastase (NE) deletion, and liver-related symptoms of alcohol were also alleviated in NE KO mice. Removal of intestinal bacteria with antibiotics led to decreases in markers of NETs formation and inflammatory cytokines upon chronic alcohol consumption, and genesis of alcoholic hepatosteatosis and HCC was also attenuated. These functions were restored upon supplementation with the bacterial product lipopolysaccharide (LPS). When mice lacking TLR4 received chronic alcohol feeding, intrahepatic markers of NETs formation decreased, and hepatosteatosis and HCC were alleviated. **Conclusion:** Formation of NETs following LPS stimulation of
TLR4 upon chronic alcohol usage leads to increased alcoholic steatosis and subsequent HCC.

**Keywords:** Neutrophil extracellular traps; alcoholic; hepatosteatosis; hepatocellular carcinoma; LPS; TLR4

**Background**

Alcohol consumption is becoming a leading risk factor impacting population health worldwide. According to an annual report of the World Health Organization, although the global percentage of drinkers has declined, the total alcohol consumption per capita has been increased steadily since 2000[1]. In 2018, worldwide annual average consumption was equivalent of 6.4 L of pure alcohol per person; when considering only people classified as “drinkers,” the average consumed increases to 15.1 L. Importantly, 39.5% of drinkers reported heavy episodic drinking[1].

These increasing numbers are noteworthy in part because chronic alcohol use can result in many diseases. The most common disease associated with alcohol is hepatosteatosis, which in the long term increases incidence of hepatocellular carcinoma (HCC)[2, 3]. Abuse of alcohol, defined as an average daily alcohol consumption of over 80 g, contributes to 15.7% of liver cancer cases in China[4]. In the United States, which is an area of lower HBV prevalence, it has been reported that 32% of HCC cases are related
to alcohol consumption[5]. As HBV vaccination becomes more prevalent and examination of blood used for transfusions becomes more effective, it can be anticipated that the proportion of HCC related to hepatic viruses will drop, and alcohol will eventually become the leading cause of HCC[6]. Therefore, understanding the etiology of HCC, especially in the context of alcohol consumption, is important for prevention and treatment.

Interestingly, binge drinking is associated with an accumulation of neutrophils in the liver, and this accumulation is a hallmark of alcohol consumption[7]. In addition, neutrophil extracellular traps (NETs) which could be formed after stimulation of lipopolysaccharide (LPS) both in vivo and in vitro could contribute to liver injury after binge alcohol use[8-10]. NETs are networks of extracellular fibers, composed of neutrophil DNA, myeloperoxidase (MPO), neutrophil elastase (NE) and citrullinated histone, that form in the presence of exogenous copper[11, 12]. While NETs are important in the removal of pathogens, NETs may sometimes also damage adjacent cells[13]. Studies have shown that inhibition of neutrophils can prevent alcoholic steatohepatitis and HCC that is induced by chemical carcinogens[14, 15]. For example, Kolaczkowska et al. showed that damage was decreased significantly when NETs were depleted in the context of bacterial-induced liver injury[16]. Thus, neutrophils and NETs
seem to play a critical role in several aspects of liver damage. Whether these factors also contribute to alcoholic hepatosteatosis and alcohol-related HCC remains unclear.

It is well known that alcohol contributes to hepatosteatosis and HCC through direct insult to the liver[3]; however, beyond this direct mechanism, alcohol may also damage the intestinal barrier and induce overgrowth of gut bacteria[17]. As a result, overgrowth of bacteria and overproduction of immunogenic components, including LPS, would translocate to the liver, potentially contributing to intrahepatic inflammation, which has been proved to be related to steatosis and carcinogenesis[17-19]. Although LPS has been proved to trigger NETs formation in many conditions, binge alcohol suppressed additional LPS-induced hepatic NET formation in mice[8]. Whether intestine-derived LPS is essential for NETs formation in alcoholic hepatosteatosis and subsequent HCC still unsure. According to the evidence presented above, we hypothesize that NETs and LPS play key roles in alcoholic hepatosteatosis and alcohol-induced HCC, and we performed the following studies to test this hypothesis.

**Methods**

**Collection of human samples**

Serum samples were collected from patients hospitalized in the Liver Disease Center or General Surgery Department at the Second Affiliated Hospital of Xi'an Jiaotong
University. The diagnosis of alcoholic liver disease was based on the guideline which issued by Chinese Society of Gastroenterology [20], and all the HCC patients were diagnosed by histology. Control serum samples were collected from patients diagnosed with inguinal hernia, gallstones, and thyroid nodular goiter and without histories of alcohol consumption or any liver disease prior to their surgeries in the surgical clinic at the General Surgery Department, Second Affiliated Hospital of the Xi’an Jiaotong University. All human and animal experiments were reviewed and approved by the Ethics Committee of Second Affiliated Hospital of the Xi’an Jiaotong University (No. 2018-2115).

**Animals**

Wild type (WT) C57BL/6 mice were obtained from the Experimental Animal Center of Xi’an Jiaotong University School of Medicine. Neutrophil elastase (NE) (#006112) and Toll-like receptor (TLR) 4 knockout (KO) mice (#007227) (C57BL/6 background) were obtained from The Jackson Laboratory. Mice were maintained under 12 h light-dark cycles in specific-pathogen free (SPF) rooms of the Experimental Animal Center, Xi’an Jiaotong University School of Medicine.

**Animal models**

A mouse model of choronic plus binge feeding alcoholic hepatosteatosis was established by feeding male mice (8 to 10 weeks of age) a Lieber-DeCarli liquid alcohol
diet (Trophic Animal Feed High-Tech Co., China) for 4 weeks followed by three gavages of a single dose of ethanol (5 g/kg body weight per day). Control mice were fed with a Lieber-DeCarli regular control diet and saline. All mice were euthanized at 9 hours after last gavage for sample harvest.

An animal model of alcoholic HCC was also established Since HCC does not arise solely through the use of alcohol[21], mice were also treated with diethylnitrosamine (DEN) (Cool Chemistry, China. Cat.# 55-18-5) to induce HCC. Male mice (4 weeks of age) were injected intraperitoneally with DEN (75 mg/kg) once per week from weeks 4 through 6. From weeks 7 through 9, the dose was increased to 100 mg/kg once per week. Mice were fed a 4% Lieber-DeCarli liquid alcohol diet from weeks 10 to 17, and they were euthanized for sample harvest following week 17. Control mice were treated with same protocol except alcohol feeding. LPS (Sigma, US. Cat. #L5293) solution in PBS was daily administered by subcutaneous injection (300μg/kg/d).

A mouse model with depleted intestinal microbiota was established by treatment with a cocktail of antibiotics. The following antibiotics were dissolved in drinking water or in a liquid diet: neomycin (1 g/L) (Aladdin, China. Cat.# N109017), ampicillin (1 g/L) (Aladdin, China. Cat.# A102048), metronidazole (1 g/L) (Aladdin, China. Cat.# M109874), and vancomycin (0.5 g/L) (Aladdin, China. Cat.# V105495). Mice were treated with this
cocktail for 1 week in order to establish the model. Stool was collected and tested by plating on blood agar and counting colony forming units to quantify the depletion of bacteria.

**Immunofluorescence**

Fresh tissue was fixed in 4% paraformaldehyde overnight. Fixed tissues were dehydrated with a series of alcohol solutions, and the tissue was embedded into paraffin blocks. After slices were prepared, paraffin was removed with xylene, and the slice was hydrated with an ethanol gradient. Antigen was unmasked with sodium citrate and a methanol incubation. Non-specific binding sites were blocked with goat serum (Beijing Solarbio, China. Cat.# SL038), and the tissues were incubated with primary antibody (citrullinated histone H3 (Cit H3), Abcam, UK, Cat. # ab5103; NE, Bioss, China. Cat.# bs6982-r) overnight at 4 °C. After several washes, samples were incubated with a fluorescent dye-labeled secondary antibody for 1 h, and nuclei were counterstained with DAPI (Beyotime, China. Cat. # C1002). Slides were mounted with antifade mounting medium. The fluorescence intensity measurement was calculated from at least 3 adjacent sections using the ImageJ software (V1.8.0, National Institutes of Health, US).

**Quantitative RT-PCR**

Fresh tissues were homogenized in Trizol solution (Sangon Biotech, Shanghai, China.
Cat. # B511311), and then RNA was extracted according to the manufacturer’s instructions. RNA was diluted with RNA-free water, and DNA was removed with the TURBO DNA-free Kit (ThermoFisher, Waltham, MA, USA. Cat. # AM1907) following the manufacturer’s protocol. RNA concentrations were determined with a NanoDrop 2000. RNA (1 μg) was used to make cDNA with cDNA Synthesis SuperMix (Biolteke, China. Cat. # PR6502). A final qPCR 20 μL reaction mixture was made with 1 μL cDNA, 10 μL 2X SYBR GREEN MasterMix (Biolteke, China. Cat. # PR1702) and water, and qPCR was performed on an Exicycler™ 96 (Bioneer) machine. Primer sequences are shown in supplement Table 1. GAPDH was used as a control gene. Relative mRNA expression was calculated by the $2^{-\Delta\Delta CT}$ method with target gene expression in the sham treatment WT mice group set as 1.

**Determination of serum LPS concentration**

Serum LPS concentrations were measured with a chromogenic LPS detection kit (Xiamen Bioendo, China. Cat. #EC325455) according to the manufacturer’s instructions. Briefly, 50 μL of diluted serum samples (1:100) were incubated with 50 μL of a limulus amebocyte lysate for 12 min at 37 °C. Then, the mixture was incubated with 100 μL of the chromogenic substrate solution for 6 min at 37 °C. The absorbance ($\lambda = 410$ nm) was measured after the addition of 100 μL of acetic acid.
Statistical analysis

SPSS 20.0 software (IBM, US) was used for data analysis. Numerical data are shown as mean ± standard deviation. Student’s t-tests were used to compare differences between groups. The Pearson correlation method was used to perform correlation analysis between serum LPS and MPO-DNA. *P* values lower than 0.05 were considered significantly different.

Results

**NETs and inflammation increased in human ALD and alcoholic HCC**

Serum samples were collected from a total of 42 patients with alcoholic liver disease (ALD). The characteristics of patients are shown in Table 1 and some of parameters were different between control and ALD patients, HCC with and without ALD patients which imply the liver injury was present the patient with ALD. Based on our investigation, ALD patients had a higher serum concentration of MPO-DNA complexes than did normal control patients (*P* < 0.05) (Fig. 1A). Nonetheless, no significant difference was revealed after the subgroup analyzed the MPO-DNA level according to liver cirrhosis or tumor stage (Supplement Fig. 1A-B). Meanwhile serum concentrations of lipopolysaccharide (LPS) and IL-6 were also higher in ALD patients (Fig. 1B, C). Serum samples from 36 HCC patients without ALD and 31 patients with ALD and HCC patients were collected. The
characteristics of patients shows in Table 1. The serum concentration of MPO-DNA was significantly higher in ALD plus HCC patients than in HCC patients without ALD ($P < 0.05$) (Fig. 1A). Meanwhile, the serum LPS and IL-6 concentrations were significantly higher in ALD plus HCC patients than HCC without ALD patients ($P < 0.05$) (Fig. 1B-C). Interestingly, there was a significant correlation between serum LPS and MPO-DNA both in ALD and ALD plus HCC patients ($P < 0.05$) (Fig. 1D-E).

**Alcohol increases formation of intrahepatic NETs in mice**

Neutrophil infiltration is a typical pathologic feature of human alcoholic liver disease[22], and it could also be seen in a chronic-binge murine model of alcohol consumption[23]. Here, we found that chronic-binge alcohol consumption increased liver MPO-DNA in both alcoholic hepatosteatosis and alcoholic HCC models (Fig. 1F). We then investigated levels of other markers of NET formation, Cit H3 and NE, by immunofluorescence. This assay indicated more fluorescence in mice of alcoholic hepatosteatosis models as compared to paired mice fed control diets (Fig. 1 G, H). The strength of fluorescence was also significantly increased in alcoholic hepatosteatosis groups relative to pair feeding groups ($P < 0.05$) (Fig. 1 G, H). The fluorescence DEN + alcohol treatment group was significantly higher than the fluorescence in only DEN treatment mice ($P < 0.05$) (Fig. 1 G, H).
NETs promote alcohol-induced hepatosteatosis and alcohol-related HCC in mice

Since NE plays a critical role in lysing of neutrophil membranes in the formation of NETs, NETs do not form in NE KO mice. These mice, then, provided an important model to test the impact of NETs on development of alcohol-related disorders. In the study, we found that much less MPO-DNA and Cit H3 was observed in NE KO mice than WT mice in the model of alcohol-induced hepatosteatosis and alcohol-related HCC (Fig. 1F, G). After 5 weeks of alcohol consumption, positive regions stained by oil red, which stains intrahepatic fat, were significantly less in NE KO mice than in WT mice treated similarly (Fig. 2A). Liver triglyceride (TG) increased in binge alcohol-treated mice, but this increase in liver TG was attenuated in NE KO mice (Fig. 2B). Serum alanine aminotransferase (ALT) was elevated significantly in alcohol-fed mice as compared to paired controls, and this increase was attenuated in NE KO mice ($P < 0.05$) (Fig. 2C). Similarly, while liver mRNA expression of the inflammation markers tumor necrosis factor (TNF)-$\alpha$, IL-6, cyclin B1, C-X-C motif ligand 2 (CXCL2) and chemokine ligand 2 (CCL2) were increased upon binge alcohol administration for 5 weeks, these increases were significantly lower in NE KO mice ($P < 0.05$) (Fig. 2D).

In the murine model of alcoholic HCC, some tumors were formed in WT mice, but few tumors were seen in NE KO mice. Both average tumor number and maximum tumor
size were significantly higher in WT mice relative to NE KO mice ($P < 0.05$) (Fig. 2E). Meanwhile, serum ALT concentrations were decreased in NE KO mice as compared to WT mice (Fig. 2F). We further tested the liver mRNA levels of CCL2, cyclin B1, cyclin B2, IL-6, TNF-α and proliferating cell nuclear antigen (PCNA), and results showed that mRNA expression of all of these genes was lower in NE KO mice than WT mice ($P < 0.05$) (Fig. 2G).

**Intestinal bacteria and LPS promotes NETs formation in the mice liver upon chronic alcohol consumption**

After we confirmed that alcohol stimulated the formation of intrahepatic NETs and further relate this increase to alcohol-induced hepatosteatosis and HCC, we endeavored to figure out an upstream inducer of NET formation. Since the gut-liver axis has been shown to play a critical role in many liver diseases, we considered that some product of intestinal bacteria could be the key factor. When we depleted gut bacteria with a cocktail of antibiotics in mice of the alcohol-induced hepatosteatosis model, we found that intrahepatic MPO-DNA was significantly decreased ($P < 0.05$) (Fig. 3A). In addition, upon antibiotic treatment, fewer cells staining positive for intrahepatic Cit H3 and NE were observed (Fig. 3B, C) indicating lower NET formation.

When mice were treated with both antibiotics and LPS, although the bacterial
population of stool was significantly reduced, both stool and liver LPS levels were increased (Supplement Figure 2A, C). After a 5 weeks binge alcohol treatment, liver MPO-DNA was recovered with the combined treatment, and intrahepatic NE and Cit H3-positive cells also increased relative to mice treated only with antibiotics (Fig. 3A-C).

Similar results were also seen in the alcoholic HCC model. Depletion of intestinal bacteria by a cocktail of antibiotics attenuated intrahepatic LPS (Supplement Figure 2), and liver MPO-DNA and intrahepatic cells positive for NE and Cit H3 also significantly decreased (Fig. 3D-F). On the other hand, in this model, antibiotics failed to attenuate liver MPO-DNA level or the fluorescence of NE and Cit H3-positive cells when mice were also treated with LPS (Fig. 3D-F).

**LPS plays a role in mice alcoholic hepatosteatosis and alcohol-related HCC**

A We have shown that LPS plays a role in the alcohol-induced formation of intrahepatic NETs, and NETs are known to correlate with alcoholic hepatosteatosis and alcohol-related HCC. Therefore, we predicted that LPS contributes to these diseases. Accordingly, in the model of alcohol-induced hepatosteatosis, antibiotics decreased the ballooning degeneration of hepatocytes and inflammation of intrahepatic cells as demonstrated by hematoxylin and eosin (HE) staining, while these phenomena increased when antibiotic treatment was combined with LPS administration. Similarly, upon staining
with oil red, less red staining was observed in mice treated with antibiotics, but more staining was observed when antibiotics were combined with LPS (Fig. 4A). Next, we determined concentrations of TG in the liver and found that it was decreased in the antibiotics group but was recovered in the group treated with antibiotics and LPS (Fig. 4B). Serum ALT levels were much lower in the antibiotics group and were increased with LPS (Fig. 4C). Meanwhile, liver expression of the mRNAs of TNF-α, cyclin B1, CCL2 and CXCL2 were significantly decreased after treatment with antibiotics, while this effect was attenuated upon treatment with LPS (P < 0.05) (Fig. 4D).

In the alcohol-related HCC model, the use of antibiotics attenuated HCC carcinogenesis. The number of tumors and the maximum tumor size were significantly reduced in mice administered antibiotics, while the number and size increased upon combining of antibiotics with LPS treatment (Fig. 4E). At the time of sacrifice, serum ALT concentrations were significantly lower in antibiotic-treated mice than in mice not treated with antibiotics, and these concentrations were recovered upon co-administration of LPS (Fig. 4F). Similarly, liver expression of the mRNA of CCL2, cyclin B1, cyclin B2, TNF-α, IL-6, and PCNA was significantly decreased upon treatment of mice with antibiotics, and expression of these genes was increased again upon co-treatment of mice with antibiotics and LPS (Fig. 4G).
Lack of TLR4 decrease NETs formation in mice chronical alcohol consumption

After confirming that intestinal derived LPS promotes the formation of NETs during chronic alcohol consumption, we considered that TLR4, which is LPS receptor, may also play a role in the formation of NETs. A strain of WT and TLR4 KO mice was used in the study. There was no difference in the concentration of intrahepatic LPS between the mice with and without TLR4 in the model of alcohol-induced hepatosteatosis (Supplement Figure 2C). However, when we examined expression of Cit H3 and NE in the liver by immunofluorescence, we observed fewer positive cells in TLR4 KO mice than in WT mice (Fig. 5A, B). The liver MPO-DNA level decreased significantly in the absence of TLR4, as well (Fig. 5C).

The same pattern was observed in the alcohol-related HCC model. The intrahepatic LPS was constant upon depletion of TLR4, but fewer cells were stained positive for Cit H3 and NE in TLR4 KO mice than in WT mice (Fig. 5D, E). Similarly, the liver MPO-DNA concentration was reduced significantly in TLR4 KO mice relative to WT mice (Fig. 5F).

Lack of TLR4 decreases formation of NETs and HCC in the mouse model of alcohol-induced HCC

Finally, we investigated whether TLR4 also plays a role in alcohol-induced hepatosteatosis and alcohol-induced HCC by comparing WT and TLR4 KO mice. In the
murine model of alcohol-induced hepatosteatosis, fewer ballooning degeneration hepatocytes and intrahepatic inflammation cells could be seen in the liver by H&E staining. Intrahepatic fat deposition, as revealed by oil red O staining, was less in TLR4 KO mice than in WT mice (Fig. 6A). Serum ALT and liver TG concentrations were much decreased in TLR4 KO mice (Fig. 6B-C). Liver expression of CXCL2, CCL2, IL-6, TNF-α and cyclin B1 mRNA was higher in WT mice than in TLR4 KO mice (Fig. 6D).

In the murine model of alcoholic HCC, some tumors had occurred by the time of harvest, but few tumors were observed in TLR4 KO mice, and TLR4 KO mice had significantly decreases in both the average number of tumors and the size of largest tumor (Fig. 6E). Upon euthanizing, serum levels of ALT were found to be significantly decreased in TLR4 KO relative to WT mice (Fig. 6F). Meanwhile, mRNA expression of PCNA, CCL2, TNF-α, IL-6, cyclin B1 and cyclin B2 were decreased in TLR4 KO mice as compared to WT mice(Fig. 6G).

Discussion

Binge drinking is a global problem, and it is well known that a potential consequence of binge drinking is hepatic steatosis, which can lead to HCC. In fact, many clinical studies have shown that chronic alcohol consumption is an independent risk factor for HCC[5, 24], and the WHO lists alcohol as a risk factor for HCC occurrence[1]. In
animal studies, although HCC does not occur only with long-term alcohol administration, alcohol consumption significantly accelerates the genesis of HCC and increases tumor load when combined with a chemical inducer[25].

Intrahepatic neutrophil infiltration is a hallmark of binge alcohol consumption, and neutrophils have been proved to play an important role in the occurrence of HCC[14]. Neutrophils are also believed to be the major source of many cytokines, including matrix metalloproteinase (MMP)-9, IL-10 and CCL2, which are thought to play roles in enhanced tumorigenesis, invasion and growth. A strong infiltration of neutrophils into the liver is also believed to be independently associated with poorer survival in human HCC[26]. It has been reported that neutrophils contribute to ALD[27] and that antibody-mediated depletion of hepatic neutrophils will attenuate HCC[14]. Intestinal bacterial has also been considered to contribute ALD[28]. However, the mechanisms behind the involvement of intestinal bacteria and neutrophils in alcoholic HCC has not been fully revealed. In particular, NETs are a neutrophil-formed structure that have been proved to form in nonalcoholic steatohepatitis and HCC and to be involved with metastasis of HCC and with many other liver diseases[29, 30]. However, as in the case of neutrophils, the precise role of NETs in alcohol-related HCC has not been revealed.

According to our initial data involving human subjects, we found that ALD and
alcohol-related HCC correlated with increased serum levels of MPO-DNA complexes, which are indicators of the formation of NETs. However, without liver biopsy samples, we could not confirm whether NETs were formed in the liver or in extrahepatic organs, so a murine study was necessary. With mice chronic alcohol feeding models, we found an abundance of NETs formed in the liver under conditions of chronic alcohol consumption.

The process of NETosis, which is a form of programmed cell death, and the elimination of antigens by NETs, an abundance of reactive oxygen species (ROS), cytokines and enzymes are released[31]. This release would not only remove the pathogen but would also impair the surrounding healthy cells[31]. Kolaczkowska et al. found that intrahepatic neutrophil infiltration is critical for bacterial-induced liver injury, and liver injury decreased 80% when NET formation was inhibited in the liver[16]. Similarly, NETs have been found to contribute to liver injury associated with sepsis that is induced by binge alcohol consumption[8], along with many other liver disorders not associated with alcohol, such as non-alcoholic steatohepatitis, ischemia reperfusion injury and portal hypertension[31, 32].

However, whether NET formation is a risk factor for alcohol-induced HCC remains unproven. In this study, a NE KO mice, in which neutrophils fail lyse and form NETs, were used. Using this model, we found that alcoholic hepatosteatosis together with many
inflammation markers were significantly attenuate upon loss of NET formation. It is well known that chronic inflammation relate to carcinogenesis, and hepatosteatosis is also known as a risk factor for HCC. So there is no surprise that the absence of NETs delays HCC occurrence.

While we confirmed that NETs are related to alcoholic hepatosteatosis and HCC, the reason for intrahepatic accumulation of NETs upon alcohol consumption had still not been fully revealed. Notably, alcohol binge consumption not only insults the liver but also affect the intestinal tract. Alcohol and its metabolites can injure the intestinal mucosa directly and contribute to intestinal inflammation, which result in an increasing of gut permeability[33]. In addition, consuming alcohol can also slow intestinal movement and result in overgrowth of intestinal bacteria[34, 35]. Together, these factors lead to the occurrence of leaky gut and a movement of bacteria and bacterial products into the liver via the portal vein[34, 35]. The role of the gut-liver axis has been implicated in many liver diseases, including some alcohol-related liver diseases, and LPS has been shown to be a mediator of the communication between the liver and the gut[36, 37]. However, with leaky gut in alcohol consumption, there remains some additional bacterial products that might be involved in intrahepatic translocation, including LPS.

An important task of the innate immune system is to rapidly eliminate bacteria and
their metabolites from the circulation. In a mouse model of sepsis, most bacteria are trapped in the liver immediately upon entry into the blood. Subsequently, neutrophil granulocytes were also immediately recruited to the liver [38]. In the present study, by completely removing the intestinal bacteria in a mouse model, it was found that intestinal bacteria are the cause of the formation of NETs induced by alcoholic steatohepatitis and subsequent HCC. However, it should be noted that existing intestinal sterilization methods that eliminate various intestinal bacteria also eliminate relevant chemical components, including LPS and LTA. Therefore, it is possible that one or both of these chemicals influences NET formation. A relationship between LTA production and NET formation has been demonstrated [39]; other studies have even demonstrated that LTA protects against NET-induced liver damage [9, 40]. Similarly, multiple studies have proved that LPS, a pathogen-associated molecular pattern molecule produced by Gram-negative bacteria, can promote adhesion of neutrophil granulocytes in the hepatic sinusoid and promote NET formation through activation of the TLR4 receptor [9, 10]. The LPS-TLR4 pathway has been found to participate in the progress of both non-alcoholic steatohepatitis and HCC, and NET formation has also been proved to be correlated with such chemical-induced HCC [29, 41, 42].

Therefore, both LTA and LPS were candidates for a chemical mediator of the link
between intestinal bacteria and NET formation. Upon removal of intestinal bacteria, we found the formation of NETs was recovered by supplementing with LPS. In addition, the formation of NETs was inhibited in mice lacking TLR4. Together, these results indicate that intestinal LPS is the key factor leading to NET formation during alcohol intake, and it thereby contributes to the development of alcoholic hepatosteatosis and subsequent HCC.

Activation of TLR4 can also stimulate the formation of NETs through non-bacterial factors. For instance, the platelets of patients with ANCA-associated vasculitis can promote the formation of NETs in vitro through TLR4 without the assistance of bacteria [43]. High mobility group box 1 also causes adjacent damage by promoting formation of NETs through TLR4 in liver ischemia-reperfusion injury [13]. Activation of the LPS-TLR4 pathway has been found to be involved in cell damage and an increase in intracellular ROS upon inhalation of alcohol, thus leading to the release of several damage-associated molecular pattern molecules [44]. Although our study clearly demonstrated a role for TLR4 in the formation of NETs upon alcohol consumption via the study of TLR4 KO mice, the impact of TLR4 on cellular NET formation may be direct or it may be indirect, as LPS activation of TLR4 on nearby cells could stimulate the release of cytokines that would stimulate NET formation. The actual mechanism could also involve both direct and
indirect pathways, and elucidating the mechanism will be a subject of further research.

Our findings are consistent with the results of several studies that have proved that antibiotics can prevent the occurrence of ALD and non-alcoholic HCC [45-47]; in this study, we identified a role for intestinal bacteria in HCC, and antibiotics seemed to prevent alcoholic HCC. However, chronic antibiotic treatment is associated with multiple negative side effects, so it is not suitable for HCC prophylaxis. Instead, our results suggest that targeting the gut-liver axis or the LPS-TLR4 pathway, maintaining the gut barrier, and detoxifying LPS are likely to represent superior prophylactic strategies [48, 49].

Conclusions

In conclusion, our data show that intestinal derived LPS stimulates NET formation via the TLR4 pathway and increases intrahepatic inflammation in chronic alcohol consumption, which in turn increases the genesis of alcoholic hepatosteatosis and subsequent HCC.

Declarations
Ethics approval and consent to participate: All human sample collections and animal experiment were reviewed and approved by the Ethics Committee of Second Affiliated Hospital of the Xi’an Jiaotong University (No. 2018-2115).

Consent for publication: Not applicable

Availability of data and material: The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare no competing interests.

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Authors’ contributions: Y.L.: Designed research, conducted experiments, acquired data, analyzed data, wrote the manuscript. S.C., J.W.: Conducted experiments, acquired data, analyzed data, and wrote the manuscript. X.Z.: Designed research, conducted experiments, analyzed data. S.Y., Y.L, M.X., W.Q, H.A., H.L., T.S., J.W.: Acquired data, conducted experiments. G.C.: Designed research, analyzed data, revised the manuscript. All authors
revised and approved the manuscript for publication.

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**List of Abbreviations:** alanine aminotransferase (ALT); body mass index (BMI); chemokine ligand 2 (CCL2); citrullinated histone H3 (Cit H3); C-X-C motif ligand 2 (CXCL2); diethylnitrosamine (DEN); hepatocellular carcinoma (HCC); hematoxylin and eosin (HE); knockout (KO); lipopolysaccharide (LPS); myeloperoxidase (MPO); nonalcoholic steatohepatitis (NASH); neutrophil elastase (NE); neutrophil extracellular traps (NETs); reactive oxygen species (ROS); proliferating cell nuclear antigen (PCNA); specific-pathogen-free (SPF); triglyceride (TG); Toll-like receptor (TLR); tumor necrosis factor-α (TNF-α); wild type (WT).

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Fig.1. Alcohol consumption increased NETs both in human and mice. A: A-C: Serum MPO-DNA, LPS and IL-6 level from ALD, HCC, ALD+HCC and control patients were
detected by ELISA. D-E: Correlation between serum LPS and MPO-DNA in the patients with ALD and ALD + HCC. F: Liver MPO-DNA levels in the mouse models of alcoholic hepatosteatosis and HCC were detected by ELISA. G-H: Mouse livers were tested for NETs by detecting the expression of Cit H3 and NE by immunofluorescence in both alcoholic hepatosteatosis and alcoholic HCC models (Scale bar, 50 μm). *: p < 0.05 compared between groups (5–8 mice per experimental group).
Fig. 2. Lack of NETs leads to alleviation of alcoholic hepatosteatosis and subsequent
**HCC in mice.** A: Mouse liver tissues were visualized by H&E staining and oil red O staining after 5 weeks of alcohol treatment (Scale bar, 100μm). B-C: Liver TG and serum ALT levels from alcoholic hepatosteatosis model mice were determined. D: WT and NE KO mice underwent alcohol treatment for 5 weeks, and liver levels of TNF-α, IL-6, Cyclin B1, CXCL-2 and CCL2 genes were determined by qPCR. E: Liver tumors from alcoholic HCC model mice were analyzed by H&E staining (Scale bar, 200μm), photography and MRI scanning. The arrows indicate the location of tumors in MRI scanning. F: Serum ALT levels were determined in models of alcoholic HCC. G: WT and NE KO mice underwent the procedure to establish the alcoholic HCC model for 13 weeks, whereupon liver levels of TNF-α, IL-6, Cyclin B1, Cyclin B2, CCL2 and PCNA genes were determined by qPCR. *: p < 0.05 compared between groups (5–8 mice per experimental group).
Fig. 3. Gut-derived LPS promotes intrahepatic NET formation during mice alcoholic hepatosteatosis and subsequent HCC. A: Liver MPO-DNA was determined by ELISA in the mouse model of alcoholic hepatosteatosis. B-C: Alcoholic hepatosteatosis livers were tested for NETs by the expression of Cit H3 and NE, which were determined by immunofluorescence (Scale bar, 50μm). D: Alcoholic HCC liver MPO-DNA was determined by ELISA in the mouse model of alcoholic HCC. E-F: Alcoholic HCC livers were tested for NETs by expression of Cit H3 and NE, which were determined by immunofluorescence (Scale bar, 50μm). *: p < 0.05 compared between groups (5–8 mice per experimental group).
Fig. 4. Intestinal LPS contributes to alcohol induced hepatosteatosis and subsequent HCC in mice. A: Hepatosteatosis was determined by H&E and oil red O staining after
treatment with alcohol for 5 weeks (Scale bar, 100μm). B-C: Liver TG and serum ALT concentrations were determined in the mouse model of alcoholic hepatosteatosis. D: Liver levels of TNF-α, IL-6, Cyclin B1, CXCL-2 and CCL2 genes were determined by qPCR in the alcoholic hepatosteatosis model. E: Mouse liver tumors were presented by photography, H&E staining (Scale bar, 200μm) and MRI scan after development of the alcoholic HCC model. The arrows indicate the location of tumors in MRI scanning. F: The serum ALT concentration was determined in alcoholic HCC mice. G: Liver levels of TNF-α, IL-6, Cyclin B1, Cyclin B2, CCL2 and PCNA genes were determined by qPCR in the alcoholic HCC model. *: p < 0.05 compared between groups (5–8 mice per experimental group).
Fig. 5. Lack of TLR4 results in less intrahepatic NET formation during the process of alcoholic hepatosteatosis and subsequent HCC in mice. A-B: Intrahepatic NETs were determined by immunofluorescence staining of Cit H3 and NE in both WT and TLR4 KO mice after treatment with alcohol for 5 weeks (Scale bar, 50 μm). C: Liver MPO-DNA was tested by ELISA in both WT and TLR4 KO mice in a model of alcoholic hepatosteatosis.
D-E: Livers from WT and TLR4 KO mice were tested for NETs by expression of Cit H3 and NE, which were determined by immunofluorescence in the model of alcoholic HCC (Scale bar, 50μm). F: Liver MPO-DNA was determined by ELISA in the alcoholic HCC model in both WT and TLR4 KO mice. *: \( p < 0.05 \) compared between groups (5–8 mice per experimental group).
Fig. 6. Mice alcoholic hepatosteatosis and subsequent HCC are attenuated in the absence of TLR4. A: Hepatosteatosis was determined by H&E and oil red O staining.
after treatment with alcohol for 5 weeks in WT and TLR4 KO mice (Scale bar, 100μm). B-C: Liver TG and serum ALT concentrations were determined in WT and TLR4 KO mouse models of alcoholic hepatosteatosis. D: WT and TLR4 KO mouse liver levels of TNF-α, IL-6, Cyclin B1, CXCL-2 and CCL2 genes were determined by qPCR in the alcoholic hepatosteatosis model. E: WT and TLR4 KO mouse liver tumors are presented by photography, HE staining (Scale bar, 200μm) and MRI scanning after development of the alcoholic HCC model. The arrows indicate the location of tumors in MRI scanning. F: Serum ALT concentrations were determined in the alcoholic HCC model in WT and TLR4 KO mice. G: Liver levels of TNF-α, IL-6, Cyclin B1, Cyclin B2, CCL-2 and PCNA genes were determined by qPCR in alcoholic HCC model in WT and TLR4 KO backgrounds. *: p < 0.05 compared between groups (5–8 mice per experimental group).
<table>
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<tr>
<th>Character</th>
<th>ALD (n=42)</th>
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<th>ALD plus HCC (n=31)</th>
<th>HCC (n=36)</th>
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<tr>
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<td>11</td>
<td>31*</td>
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<tr>
<td>Female</td>
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<td>8</td>
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<td>HCC (x10^9/L)</td>
<td>ALD Plus HCC (x10^9/L)</td>
<td>HCC (x10^9/L)</td>
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<td>Platelets</td>
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<td>94 (54-179)</td>
<td>103 (67-178)</td>
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<td>ALT (IU/L)</td>
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<td>22 (9-36)</td>
<td>73 (27-101) *</td>
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<tr>
<td>AST (IU/L)</td>
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<td>TBIL (umol/L)</td>
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<td>38.1 (26.7-)</td>
<td>44.8 (34.9-)</td>
<td>35.8 (30.2-43.7)</td>
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<td>ALB (g/L)</td>
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<td>Scr (μmol/L)</td>
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<td>91 (57-121)</td>
<td>94 (70-118)</td>
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</table>

*: P<0.05 compared between ALD and healthy control patients.

#: P<0.05 compared between ALD plus HCC and HCC patients.
Supplement methods:

Oil red O staining

Liver tissues were frozen in Tissue-Tek O.C.T. compound and cut into 10 μm sections. Slices were fixed with ice cold 10% formalin. The slices were washed with 60% isopropanol and then stained with oil red O solution for 5 min (Sigma, US. Cat. # O0625). Following another 60% isopropanol wash, nuclei were stained with hematoxylin, and slices were mounted. Oil red O positive area was calculated from at least 3 adjacent sections using ImageJ software (V1.8.0, National Institutes of Health, US).

Liver triglyceride (TG) detection

Fresh liver samples were weighed and homogenized in PBS on ice. Heat the samples to 80 °C in a water bath for 5 minutes and cold down to room temperature. The soluble fraction was collected following centrifugation (10000rpm, 5min), and the protein was detected with a bicinchoninic acid (BCA) assay using bovine serum albumin as a reference. Samples were diluted with PBS to achieve equal protein concentrations, and TG was quantified with the Triglycerides Assay Kit (Nanjing Jiancheng Bio. Co. Cat.# F001-1-1). Briefly, 2.5 μL of the sample was incubated with 200 μL of the working solution for 10 min at 37 °C, and the absorbance at 510 nm was determined. TG concentration was determined via comparison to a standard curve.
ELISA

Human serum MPO-DNA (Boster, US. Cat. # EK0850) and serum IL-6 (Elabscience, China. Cat. # E-EL-M0044c), mouse liver MPO-DNA was (Boster, US. Cat. # EK0943) levels were detected by ELISA kit following the manufacturer’s instructions. Briefly, liver samples were weighed and homogenized in cell extraction buffer with protease inhibitors on ice. After samples were vortexed for 30 min, the supernatant was collected with centrifugation at 5000 rpm, and the protein content was quantified with a BCA Assay using bovine serum albumin as a reference. Samples were diluted with PBS to achieve equal protein concentrations. First, 96 well plates were coated with coating solution, and plates were then washed with washing buffer. Next, wells were blocked with blocking buffer, and standards and samples (100 µL) were added into wells. After an incubation, wells were washed, and the detection antibody solution was added, followed by addition of streptavidin-horseradish peroxidase solution. Next, TMB substrate solution was added, and added stop solution was added after a 30 min incubation. Absorbances were read at 450 nm. Concentrations were determined based on a standard curve.

Determination of serum alanine aminotransferase (ALT) activity

Human and mouse sera, diluted 1:20, were assayed for ALT using an ALT Activity Assay Kit (Elabscience, China. Cat. # E-BC-K235-M) according to the manufacturer’s
instructions. Briefly, samples of the diluted sera (20 μL) were incubated with 100 μL working solution for 5 min. ALT was quantified by measuring the resulting fluorescence ($\lambda_{ex} = 535$ nm; $\lambda_{em} = 587$ nm).
**Supplement Table 1.** The sequences of primers that were used in the study.

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<th>Sequence</th>
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<td>TNF-α F</td>
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<td>IL-6 F</td>
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<td>IL-6 R</td>
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<td>Cyclin B1 F</td>
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Supplement Figure 1. Serum MPO-DNA level in ALD, HCC, and ALD plus HCC patients. Liver MPO-DNA levels in the mouse models of alcoholic hepatosteatosis and HCC were detected by ELISA. A: The subgroup analyzed the MPO-DNA level according to whether the patients were with or without liver cirrhosis. B: The subgroup analyzed the MPO-DNA level according to tumor stage.
Supplement Figure 2. Stool bacteria and serum LPS levels in both alcoholic hepatosteatosis and alcoholic HCC mice. A, B: Stool CFU was tested every week in the mouth model of alcoholic hepatosteatosis and alcoholic HCC. C, D: Serum LPS was tested after euthanised in a mouse model of alcoholic hepatosteatosis and alcoholic HCC model. *: $p < 0.05$ compared between groups (5–8 mice per experimental group).