

## Innate immune reactivity of the liver in rats fed a choline-deficient L-amino-acid-defined diet

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Supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, No. 19590784

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Received: September 18, 2008 Revised: October 31, 2008

Accepted: November 7, 2008

Published online: November 21, 2008

TLR4 and CD14-mediated endotoxin liver damage may also occur in NASH.

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**Key words:** Nonalcoholic steatohepatitis; Kupffer cells; Toll-like receptor 4; CD14; Endotoxins; Tumor necrosis factor-alpha

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Kawaratani H, Tsujimoto T, Kitazawa T, Kitade M, Yoshiji H, Uemura M, Fukui H. Innate immune reactivity of the liver in rats fed a choline-deficient L-amino-acid-defined diet. *World J Gastroenterol* 2008; 14(43): 6655-6661 Available from: URL: <http://www.wjgnet.com/1007-9327/14/6655.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.6655>

### Abstract

**AIM:** To investigate the innate immune reactivity of tumor necrosis factor-alpha (TNF- $\alpha$ ), Toll-like receptor 4 (TLR4), and CD14 in the liver of non-alcoholic steatohepatitis (NASH) model rats.

**METHODS:** Male F344 rats were fed a choline-deficient L-amino-acid-defined (CDAA) diet. The rats were killed after 4 or 8 wk of the diet, and their livers were removed for immunohistochemical investigation and RNA extraction. The liver specimens were immunostained for TNF- $\alpha$ , TLR4, and CD14. The gene expressions of TNF- $\alpha$ , TLR4, and CD14 were determined by reverse-transcriptase polymerase chain reaction (RT-PCR). Kupffer cells were isolated from the liver by Percoll gradient centrifugation, and were then cultured to measure TNF- $\alpha$  production.

**RESULTS:** The serum and liver levels of TNF- $\alpha$  in the CDAA-fed rats increased significantly as compared with the control group, as did the immunohistochemical values and gene expressions of TNF- $\alpha$ , TLR4, and CD14 with the progression of steatohepatitis. TNF- $\alpha$  production from the isolated Kupffer cells of the CDAA-fed rats was elevated by lipopolysaccharide stimulation.

**CONCLUSION:** The expressions of TNF- $\alpha$ , TLR4, and CD14 increased in the NASH model, suggesting that

### INTRODUCTION

Some patients who are not regular drinkers of alcohol have hepatic histological features that mimic alcoholic hepatitis and can progress to liver cirrhosis<sup>[1]</sup>. This condition has been named non-alcoholic steatohepatitis (NASH) and is thought to begin with excessive fat accumulation in the liver (first hit), followed by aggravating factors such as oxidative stress, inflammatory cytokines, and endotoxins (second hit)<sup>[2]</sup>.

Kupffer cells, the resident macrophages in the liver, are localized in the non-parenchymal tissue and involved in the organ's innate immunity. Activation of Kupffer cells induces progression of NASH *via* tumor necrosis factor-alpha (TNF- $\alpha$ )<sup>[3]</sup>. The recent discovery of Toll-like receptor 4 (TLR4), a functional receptor on the surface of macrophages and various other types of cells that transmit Et signals, has led to recognition of molecules, including CD14, that bind to lipopolysaccharides (LPSs) as the mainstay of the mechanism that initiates signal transduction leading to LPS recognition and cellular activation<sup>[4]</sup>.

NASH is characterized by hepatic steatosis, inflammation, and fibrosis, with increased risk of developing cirrhosis and hepatocellular carcinoma (HCC)<sup>[5]</sup>. It is

known that the choline-deficient L-amino-acid-defined (CDAA) fed rat model shows fatty accumulation and fibrosis, and after 16 wk the liver shows cirrhotic changes and nodules appear like HCC<sup>[6]</sup>. This model is sometimes used as a hepatocarcinogenesis model. In this study, we used a CDAA diet to produce one of the experimental animal models of NASH<sup>[7]</sup>, to investigate the innate immune reactivity of the liver in NASH.

## MATERIALS AND METHODS

### Animal model

Male F344 rats (6-wk-old, weighing 180-200 g) were purchased from Japan SLC (Hamamatsu, Japan), and fed a CDAA diet for up to 8 wk to induce NASH. At 1, 4, and 8 wk, the control rats (fed a standard rat chow), and the CDAA-fed rats were killed and bled from the inferior vena cava (IVC). Liver tissues were excised after perfusion with PBS into the portal vein. All procedures were approved by our institutional animal care committee and conducted in accordance with Nara Medical University Guidelines for the Care and Use of Laboratory Animals.

### Serum alanine aminotransferase (ALT)

Serum samples from the control and CDAA-fed rats were used to measure the serum ALT levels. The serum ALT levels were determined using a 7170 Clinical Analyzer (Hitachi High-Technologies, Tokyo, Japan).

### Serum TNF- $\alpha$

Serum samples (50  $\mu$ L) from the control and CDAA-fed rats were used to measure the TNF- $\alpha$  concentration by enzyme-linked immunosorbent assay (ELISA) kit (BioSource International, Camarillo, CA, USA).

### Liver TNF- $\alpha$

Liver tissues were frozen in liquid nitrogen, powdered, and 30 mg were homogenized in 1 mL of lysis buffer. The supernatant (50  $\mu$ L) was used to measure the TNF- $\alpha$  levels by ELISA kit (BioSource International).

### Histology

The liver tissues were fixed in 40 g/L formalin, embedded in paraffin, and 5- $\mu$ m thick sections were cut from the paraffin blocks for staining with hematoxylin-eosin (HE) and Azan stains. Histological grading and staging were performed using a modified scoring system based on the classification of either Matteoni *et al*<sup>[8]</sup> or Brunt *et al*<sup>[9]</sup> proposed NAFLD types 1-4 based on long-term outcome studies, while Brunt *et al*<sup>[9]</sup> proposed a system of grading and staging for NASH that follows methods of separate assessment for necroinflammatory lesions (grading) and fibrosis (staging) accepted in other forms of non-biliary chronic liver diseases.

### Immunohistochemistry

The TNF- $\alpha$  immunohistochemical staining was performed on the paraffin sections using an anti-

rat TNF- $\alpha$ /TNFSF1A antibody (R&D Systems, Minneapolis, MN, USA), and the TLR4 and CD14 immunohistochemical staining was performed on frozen sections using an anti-rat TLR4 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and an anti-rat CD14 antibody (Santa Cruz Biotechnology Inc.), respectively. After that, a Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA, USA), and DAB peroxidase substrate solution (Vector Laboratories Inc.), with counterstaining by Hematoxylin Mayer were used. The stained areas were analyzed by NIH image software (Version 1.61, U.S. National Institute of Health, Bethesda, MD, USA).

### Kupffer cell isolation and culture

Kupffer cells were isolated from the control and CDAA-fed rat livers according to a previously described procedure<sup>[10,11]</sup>. In brief, the liver was perfused through the portal vein with 100 mL of PBS containing 0.5 mmol/L EGTA (Research Organics, Cleveland, OH, USA) solution. The perfusion was carried out at 37°C at a flow rate of 10 mL/min. Subsequent perfusion with Hanks' balanced salt solution containing 0.5 g/L collagenase type IV (Wako, Osaka, Japan) (collagenase solution) was carried out at 37°C at a flow rate of 10 mL/min for 20 min. Then, the liver was excised and minced in the collagenase solution.

The liver slurry was filtered through gauze mesh, then washed with collagenase solution. Next, Percoll (Sigma-Aldrich Corp, St. Louis, MO, USA) was used for density gradient centrifugation of cells. Kupffer cells were collected from the layer between 1.04 and 1.06. They were then cultured in RPMI 1640 supplemented with 10% fetal bovine serum and SAG (50  $\mu$ g/mL streptomycin, 50  $\mu$ g/mL ampicillin, 0.3 g/L L-glutamine). The viability of the cultured Kupffer cells was determined by trypan blue exclusion. Kupffer cells were seeded at a density of  $5 \times 10^5$  cells/mL on a 12-well plate with RPMI 1640. For LPS-stimulated group, Kupffer cells were then exposed to 1  $\mu$ g/mL of LPS (Sigma-Aldrich Corp.) and incubated for 4.5 h at 37°C in a 50 mL/L CO<sub>2</sub>-humidified air atmosphere. The cells untreated with LPS were used as control. The supernatant was analyzed by ELISA kit (BioSource International) to measure the TNF- $\alpha$  concentration.

### Reverse transcriptase-PCR (RT-PCR) analysis

The total RNA was extracted from the powdered frozen liver samples using an RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Briefly, 2  $\mu$ g of the RNA sample were used for cDNA synthesis using Moloney Murine Leukemia Virus (M-MLV), Reverse Transcriptase RNaseH Minus (Toyobo, Osaka, Japan), and RNase Inhibitor (Toyobo) to investigate the expressions of TNF- $\alpha$  mRNA, TLR4 mRNA, and CD14 mRNA. PCR procedure was performed as follows: the samples were heated at 94°C, subjected to 30 cycles of 45 s at 94°C for denaturing, 45 s at 60°C for annealing,

Table 1 Primers for the target genes used in RT-PCR

Target gene	Primer
<i>TNF-<math>\alpha</math></i>	5'-TACTGAACTTCGGGGTGATTGGTCC-3' (F) 5'-CAGCCTTGCCCTTGAAGAGAACC-3' (R)
<i>TLR4</i>	5'-CGCTTTCAGCTTGGCTTCATTAC-3' (F) 5'-TGCTACTTCTTGTGCCCTGTGAG-3' (R)
<i>CD14</i>	5'-GTGCTCCTGCCAGTGAAAGAT-3' (F) 5'-GATCTGTCTGACAACCCTGAGT-3' (R)
<i><math>\beta</math>-actin</i>	5'-ACCACAGCTGAGAGGGAAATCG-3' (F) 5'-AGAGGTCTTACGGATGTCAAAG-3' (R)

F: Sense; R: Antisense.

and 2 min at 72°C for extension. Following RT-PCR, 10- $\mu$ L samples of the amplified products were resolved by electrophoresis on 15 g/L agarose gel, and stained with ethidium bromide. Each PCR product was analyzed by NIH image software. The specific primers for *TNF- $\alpha$* , *TLR4*, *CD14*, and  *$\beta$ -actin* used are described in Table 1.

### Statistical analysis

All results are expressed as mean  $\pm$  SD. The analysis was carried out using Statview software (version 5.0, SAS Institute, Cary, NC, USA). Statistical differences between groups were evaluated using the paired *t*-test.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Serum ALT

The mean serum ALT level in the control rats was  $41.7 \pm 7.4$  IU/L, as compared with  $524.6 \pm 101.7$  IU/L,  $267.9 \pm 47.5$  IU/L, and  $251.4 \pm 81.6$  IU/L in the 1-, 4-, and 8-wk CDAA-fed rats, respectively. All CDAA-fed groups showed a statistically significant elevation in the serum ALT level as compared with the control group ( $P < 0.01$ ).

### Serum *TNF- $\alpha$*

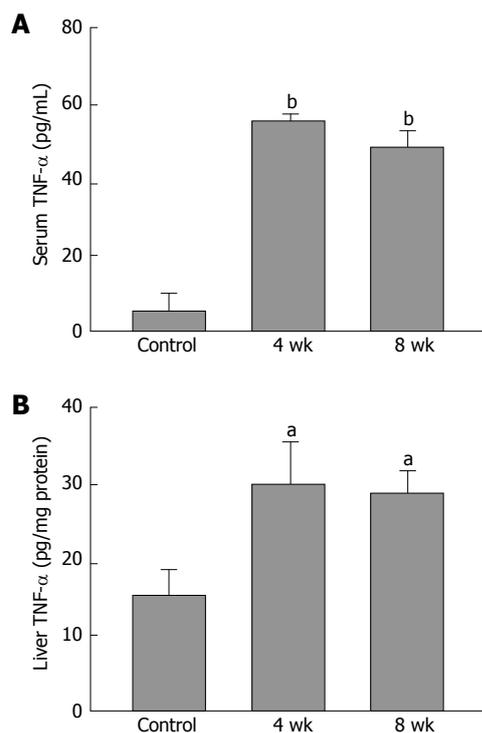
As compared with the control rats ( $5.2 \pm 4.4$  pg/mL), the serum *TNF- $\alpha$*  level in the 4-wk CDAA-fed rats was elevated ( $55.9 \pm 1.7$  pg/mL,  $P < 0.01$ ), and that in the 8-wk CDAA-fed rats ( $48.2 \pm 5.3$  pg/mL,  $P < 0.01$ ) was elevated (Figure 1A).

### Liver *TNF- $\alpha$*

The liver *TNF- $\alpha$*  was  $15.8 \pm 3.6$  pg/ $\mu$ g protein in the control rats,  $30.3 \pm 5.4$  pg/ $\mu$ g protein in the 4-wk CDAA-fed rats, and  $29.6 \pm 4.4$  pg/ $\mu$ g protein in the 8-wk CDAA-fed rats. The CDAA-fed groups showed a statistically significant elevation in the liver *TNF- $\alpha$*  level as compared with the control group ( $P < 0.05$ ) (Figure 1B).

### Histological findings

Histologically, the livers of the 1-wk CDAA-fed rats showed inflammation and fat deposits, but no fibrosis, and were classified as Matteoni's type 2. The livers of the 4-wk CDAA-fed rats showed more inflammation, fat deposits, and fibrosis, equivalent to Matteoni's type 3



**Figure 1** Serum *TNF- $\alpha$*  levels and liver *TNF- $\alpha$*  (ELISA method) compared with the control group ( $n = 5$ ). A: Serum *TNF- $\alpha$*  levels were significantly elevated in the livers of the 4- and 8-wk CDAA-fed rats; B: Liver *TNF- $\alpha$*  was significantly elevated in the livers of the 4- and 8-wk CDAA-fed rats. <sup>a</sup> $P < 0.05$  vs the control; <sup>b</sup> $P < 0.01$  vs the control.

and grade 2/stage 2 of Brunt's NASH classification. The histological findings in the livers of the 8-wk CDAA-fed rats were equivalent to Matteoni's type 4 and Brunt's grade 2/stage 3 (Figure 2).

### Immunohistochemical findings

The *TNF- $\alpha$*  immunoreactivities in the tissue samples from the livers of the 4- and 8-wk CDAA-fed rats were increased as compared with those in the control rats. The *TLR4* and *CD14* immunohistochemical findings in the 4- and 8-wk CDAA-fed rats were also increased as compared with those in the control rats. Moreover, the immunopositive areas in those animals were localized either in the periphery of fatty spots or non-parenchymal cells. The semiquantitative analysis of *TNF- $\alpha$* , *TLR4*, and *CD14* staining revealed significantly increased immunoreactivity in the livers of the 4-wk ( $14600 \pm 5700$ ,  $26400 \pm 2300$ ,  $17400 \pm 6000$ , respectively) and 8-wk ( $10600 \pm 1800$ ,  $21600 \pm 4700$ ,  $21300 \pm 3900$ , respectively) CDAA-fed rats as compared with the control rats ( $1560 \pm 1080$ ,  $3160 \pm 1000$ ,  $4290 \pm 1110$ , respectively) ( $P < 0.01$ ) (Figure 3).

### Kupffer cell isolation and culture

The viability of the cultured Kupffer cells was over 98% as determined by trypan blue exclusion. The basic *TNF- $\alpha$*  production of the Kupffer cells isolated from 4- ( $26.4 \pm 2.3$  pg/mL) and 8-wk CDAA-fed rats ( $26.6 \pm 1.4$  pg/mL) was equal to that of the control rats ( $23.4 \pm 1.8$  pg/mL). After LPS stimulation, the *TNF- $\alpha$*

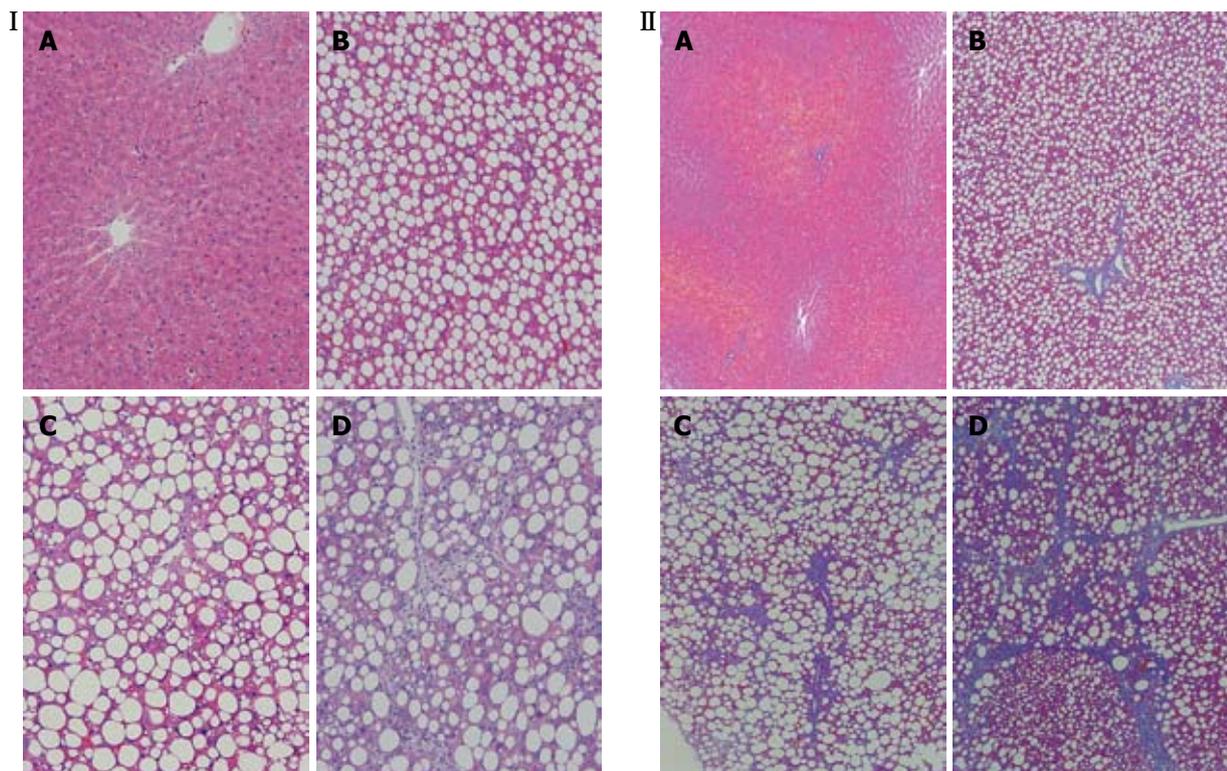


Figure 2 Inflammation and fibrosis in the rat liver. I: HE, x 200; II: Azan, x 100. A: Control rat; B: 1-wk CDAA-fed rat; C: 4-wk CDAA-fed rat; D: 8-wk CDAA-fed rat.

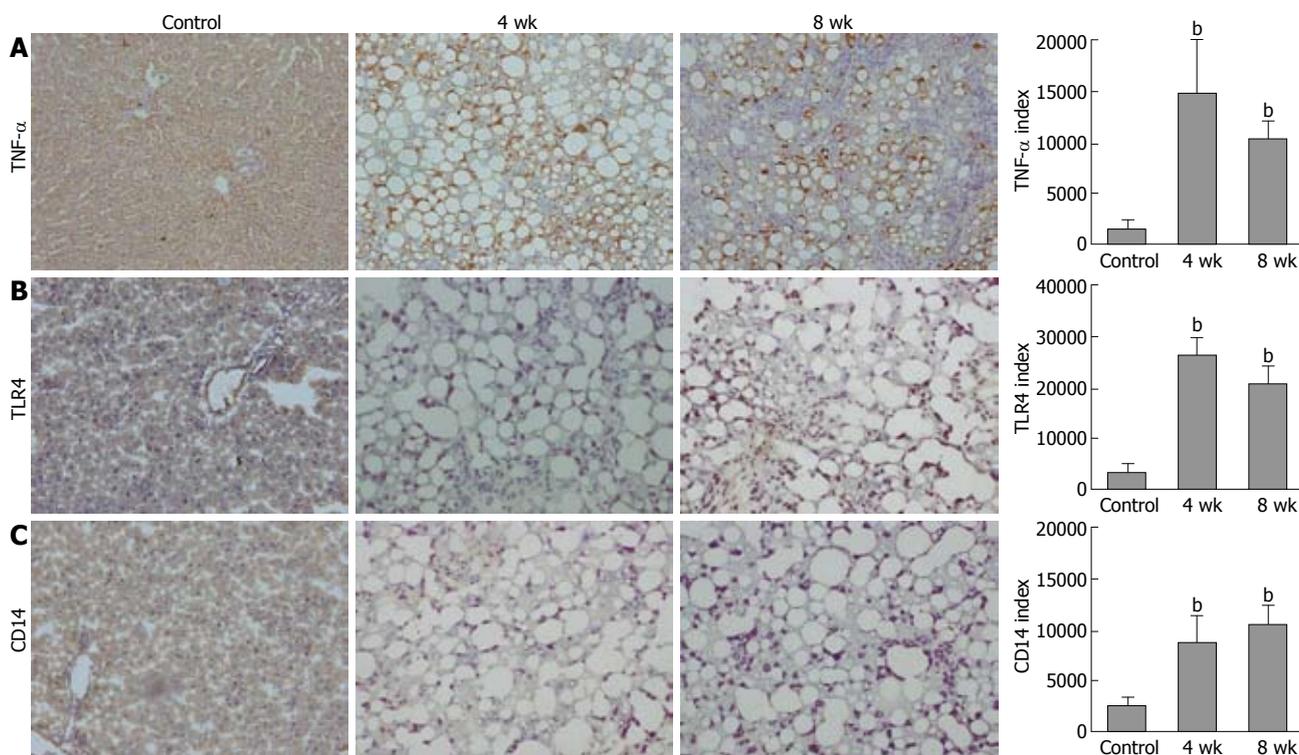
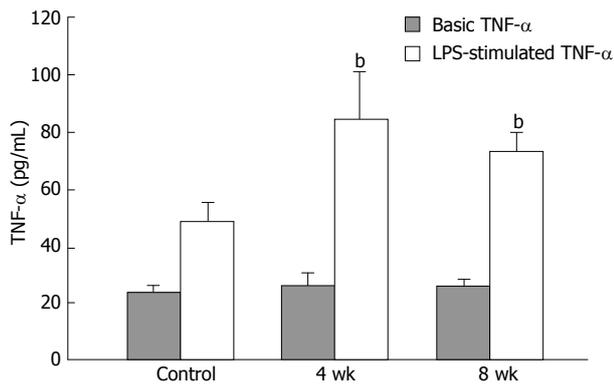


Figure 3 Immunohistochemical staining and semiquantification in the rat liver (x 200). A: For TNF- $\alpha$ ; B: For TLR4; C: For CD14. <sup>b</sup> $P < 0.01$  vs the control.

production in the control rats ( $48.9 \pm 7.5$  pg/mL) was significantly higher than the basic control levels, and that in the 4-wk ( $85.8 \pm 16.8$  pg/mL) and 8-wk ( $73.8 \pm 8.4$  pg/mL) CDAA-fed rats was significantly higher than in the control rats ( $P < 0.01$ ) (Figure 4).

**RT-PCR analysis**

The expression of both *TNF- $\alpha$*  mRNA and *TLR4* mRNA was significantly increased in the livers of the 4- and 8-wk CDAA-fed rats as compared with those in the control group ( $P < 0.01$ ). The expression of *CD14*

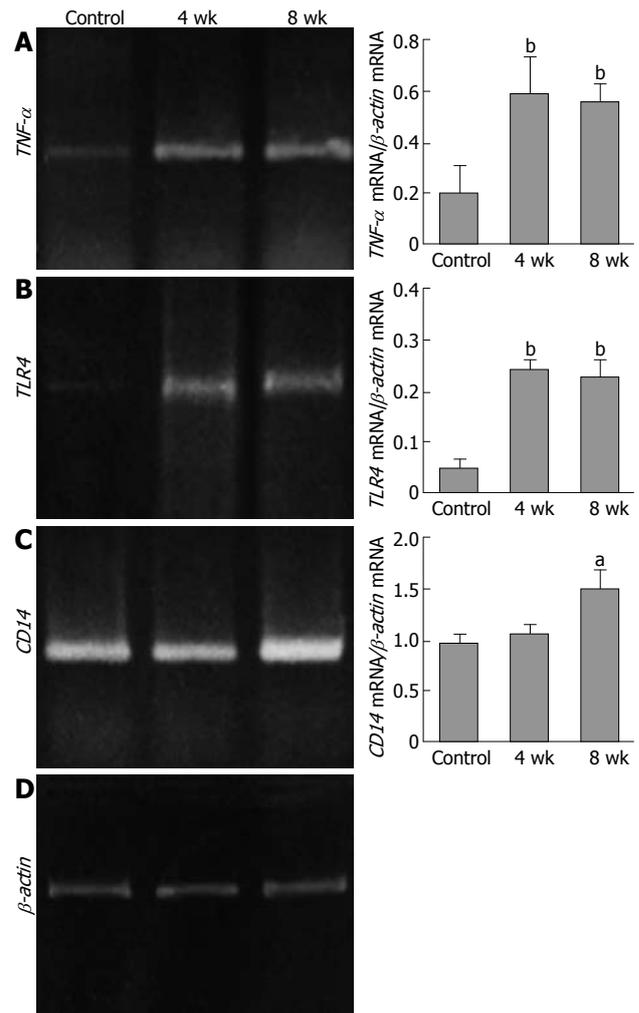


**Figure 4** TNF- $\alpha$  production of Kupffer cells *in vitro*. The basic TNF- $\alpha$  production of Kupffer cells isolated from 4- and 8-wk CDAA-fed rats was equal to that of the control rats. After LPS stimulation, the TNF- $\alpha$  production of the control rats was significantly higher than the basic control levels, and that of the 4- and 8-wk CDAA-fed rats was significantly higher than that of the control rats. <sup>b</sup> $P < 0.01$  vs the control.

mRNA was increased in the livers of the 8-wk CDAA-fed rats as compared with that in the control group ( $P < 0.05$ ) (Figure 5).

## DISCUSSION

Kupffer cells secrete inflammatory cytokines<sup>[12]</sup> and reactive oxide<sup>[13]</sup>, which affect the hepatocytes, stellate cells, endothelial cells, *etc.*<sup>[14]</sup>. In alcoholic hepatitis, inflammatory cytokines, such as TNF- $\alpha$ , induce the liver injury<sup>[15]</sup>. After chronic ethanol feeding, Kupffer cells exhibit enhanced sensitivity to LPS-stimulated TNF- $\alpha$  production<sup>[16]</sup>. Increased serum levels of TNF- $\alpha$  have been noticed in patients with NASH<sup>[17]</sup>. The mechanism of the latter may be secretion from adipocytes, as a result of obesity, or secretion from Kupffer cells activated by endogenous endotoxin originating from enteric bacteria. The activated Kupffer cells induce increases in the levels of cytokines, such as TNF- $\alpha$  or reactive oxide, which may be involved in the progression of NASH. Rats fed a high-fat diet become obese and show insulin resistance. In addition, the activities of both nuclear factor kappa enhancer binding protein (NF- $\kappa$ B) and the inhibitor of NF- $\kappa$ B kinase beta (IKK $\beta$ ) significantly increase in the liver, and the expression of inflammatory cytokines such as TNF- $\alpha$  becomes excessive<sup>[18]</sup>. Our results showed that the livers of CDAA-fed rats developed inflammation in the early stage, and this was sustained by chronic feeding of the diet. After 4 wk or more, the livers of the CDAA-fed rats developed progressive steatohepatitis. In the present study, we confirmed by ELISA, immunohistochemistry, and RT-PCR that both the serum level of TNF- $\alpha$  and the liver TNF- $\alpha$  expression were increased, suggesting involvement of TNF- $\alpha$  in the development and progression of NASH similar to that in alcoholic hepatitis. Moreover, the TNF- $\alpha$  production of the isolated Kupffer cells was equal in the NASH model. Kupffer cells from the control rats were sensitive to LPS stimulation. Furthermore, those from NASH were more sensitive to LPS stimulation, suggesting that the continuous inflammation leads to up-regulation of the LPS-



**Figure 5** RT-PCR and semiquantification ( $n = 5$ ). A: TNF- $\alpha$  mRNA; B: TLR4 mRNA; C: CD14 mRNA; D:  $\beta$ -actin mRNA. <sup>a</sup> $P < 0.05$  vs the control, <sup>b</sup> $P < 0.01$  vs the control.

stimulated TNF- $\alpha$  production by the activated Kupffer cells.

To date, 13 TLRs have been described in mice and 11 in humans. Recently, TLR4 has been highlighted as an important membrane protein<sup>[19,20]</sup> and is now established as the receptor for LPS. CD14 binds LPS and subsequently presents it to TLR4 and MD-2, which activate the intracellular signaling pathway *via* myeloid differentiation factor 88 (MyD 88), resulting in NF- $\kappa$ B activation<sup>[21]</sup>. Experiments using TLR4 knockout (KO) mice or CD14 KO mice have revealed a reduced reaction to LPS and a reduced expression of TNF- $\alpha$ <sup>[22,23]</sup>, indicating that LPS signaling induces TNF- $\alpha$  production *via* TLR4 or CD14. The intake of alcohol facilitates the permeability of endotoxin from the gut to the portal vein, and the increased concentration of endotoxin in the portal vein leads to Kupffer cell activation *via* TLR4, with consequent increases in the liver expression of TNF- $\alpha$ , progression of inflammation, and fibrosis of the liver<sup>[24]</sup>. In the experimental chronic liver injury model, the expression of TLR4 in isolated Kupffer cells increases with progression of liver injury<sup>[25]</sup>, suggesting a correlation between inflammation and expression of

TLR4. The present study showed increased expression of mRNA levels and immunohistochemical values of TNF- $\alpha$ , TLR4, and CD14 in the livers of rats with NASH induced by CDAA diet, suggesting that TLR4-mediated endotoxin liver damage may also occur in NASH. Rivera *et al*<sup>[26]</sup> also demonstrated enhanced TLR4 expression together with steatohepatitis in the liver of mice fed a methionine choline-deficient (MCD) diet. They further reported that the liver injury was attenuated in the TLR4 mutant mice and in Kupffer cell-depleted mice, and pointed out the importance of TLR4 signaling and Kupffer cells in the pathogenesis of steatohepatitis. Although these models lack obesity and insulin resistance, two major characteristics of NASH in humans, the possible relation of Kupffer cells with inflammation, steatosis, and fibrosis may resemble the human NASH. We also demonstrated decreased Kupffer cell phagocytic activity in this model<sup>[27]</sup>. The possible Kupffer cell dysfunction characterized by decreased phagocytic activity and increased cytokine production should be investigated in future studies.

Although the pathogenic mechanism of NASH is still unclear, its histopathology is similar to that of alcoholic hepatitis. Enhanced activation of the TLR4 dependent signaling pathways was also observed after chronic ethanol feeding<sup>[28]</sup>. There is a possibility that TLR4-dependent innate immunity induced by intestinal bacterial endotoxin may work as a common pathway in these two types of steatohepatitis. Further studies are needed to confirm this hypothesis.

In conclusion, our study focused on the innate immune reactivity of livers in the rat model of NASH, and we found overexpressions of TNF- $\alpha$ , TLR4, and CD14 in association with liver fibrosis and inflammation. These results suggest that TLR4 and CD14-mediated endotoxin liver damage may also occur in NASH.

## COMMENTS

### Background

Recently, non-alcoholic steatohepatitis (NASH) has become a common disease in Japan. The two-hit theory has been widely accepted to explain the progression from simple steatosis to NASH. Moreover, NASH is known as a disease progressing to liver cirrhosis or hepatocellular carcinoma (HCC). The mechanism of NASH progression has not been clarified yet. Authors investigated the engagement of Toll-like receptor 4 (TLR4) or CD14 in the rat NASH model.

### Research frontiers

This is the first report showing the innate immune response in the rat NASH model. Authors found overexpression of tumor necrosis factor-alpha (TNF- $\alpha$ ), TLR4, and CD14 in association with liver fibrosis and inflammation. These results suggest that TLR4 and CD14-mediated endotoxin liver damage may occur in NASH.

### Innovations and breakthroughs

The present study demonstrated the overexpression of TNF- $\alpha$ , TLR4, and CD14 in the rat NASH model. This result suggests that the involvement of endotoxin-induced TLR4 activation led to the NASH progression.

### Applications

The present study is an experimental research using the rat NASH model, which was made by feeding a choline-deficient L-amino-acid-defined diet to rats, to examine their innate immunity. For understanding NASH progression, further evaluation of the cytokine production from the Kupffer cells and the expressions of TLR4 and CD14 from the isolated Kupffer cells should be

employed in the subsequent experiments.

### Terminology

TLR4 is a transmembrane receptor for lipopolysaccharides (LPS) on the surface of macrophages. LPS stimulation activates TLR4 by binding with CD14, resulting in pro-inflammatory cytokine production, like TNF- $\alpha$ .

### Peer review

Authors described the importance of innate immune responses to the pathogenesis of rat model of NASH. The findings are important.

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S- Editor Li DL L- Editor Logan S E- Editor Lin YP