学位論文

Augmented Hepatic Toll-like receptors by fatty acids trigger the pro-inflammatory state of non-alcoholic fatty liver disease in mice

(脂肪酸が肝Toll-like receptorの発現を誘導し非アルコール性脂肪性肝疾患の前炎症状態を形成する)

旭川医科大学大学院医学系研究科博士課程医学専攻

澤田　康司

(　長谷部 拓夢、大竹 孝明、高後 裕　)

Augmented Hepatic Toll-like receptors by fatty acids trigger the pro-inflammatory state of non-alcoholic fatty liver disease in mice

Koji Sawada, Takumu Hasebe, Takaaki Ohtake, Yutaka Kohgo

Department of Medicine, Division of Gastroenterology and Hematology/Oncology, Asahikawa Medical University, Asahikawa, Japan

Abstract

Background: There is considerable evidence that intestinal microbiota are involved in the development of metabolic syndromes and consequently with that of nonalcoholic fatty liver disease (NAFLD). Toll-like receptors (TLRs) are essential for the recognition of microbiota. However, the induction mechanism of TLR signals through the gut-liver axis for triggering the development of nonalcoholic steatohepatitis (NASH) or NAFLD remains unclear. In this study, we investigated the role of fatty acids in triggering the development of a pro-inflammatory state of NAFLD.

Methods: NAFLD was induced in mice fed a high fat diet. The mice were sacrificed and the expressions of TLRs, tumor necrosis factor (TNF), interleukin 1β (IL-1β),

CXCL2, and phospho-interleukin-1 receptor-associated kinase 1 in the liver and small intestine were assessed. In addition, Huh7 and THP-1 cells, both of which are representatives of hepatocytes and Kupffer cells, respectively, were treated with palmitic acid, and the direct effects of fatty acids on TLR induction by these cells were evaluated.

Results: The expressions of inflammatory cytokines such as TNF, IL-1β, and TLRs-2, -4, -5, and -9 were increased in the liver, but decreased in the small intestine of high fat diet-fed mice *in vivo*. In addition, the expressions of TLRs in Huh7 and THP-1 cells were increased by treatment with fatty acids.

Conclusion: In the development of the pro-inflammatory state of NAFLD, fatty acids trigger the expressions of TLRs, which contribute to the induction of inflammatory cytokines through TLR signals by the intestinal microbiota.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a form of steatosis with or without inflammation of the liver, and is not related to excessive alcohol intake. NAFLD includes both simple steatosis and nonalcoholic steatohepatitis (NASH), the latter developing further into cirrhosis and hepatocellular carcinoma [(1)]. NAFLD is one of the

most common liver diseases worldwide and is considered to be related with obesity, insulin resistance, and metabolic syndrome [2].

A two-hit theory has been proposed to explain the pathogenesis of NASH [3]. First, simple steatosis is induced by obesity and insulin resistance. Second, NASH develops by several hits, including adipocytokines, iron, and bacterial endotoxins/lipopolysaccharide (LPS) derived from gram-negative bacteria [4, 5, 6].

Toll-like receptors (TLRs) recognize pathogen and endogenous damage-associated molecular patterns and activate nuclear factor-κB (NF-κB), which induces pro-inflammatory cytokines/chemokines, type 1 interferon through phosphorylation of interleukin-1 receptor-associated kinase 1 (IRAK1) and IRAK4 [7]; therefore, TLRs may play important roles in the activation of innate immunity. Among TLRs, TLR2, TLR4, TLR5, and TLR9 were identified as bacterial recognition receptors capable of recognizing lipopeptide, LPS, flagellin, and CpG-DNA, respectively [8]. Recently it was reported that TLR signal pathways, the ligands of which are bacterial components, play an important role in the pathogenesis of alcoholic liver disease and NASH [9]. In particular, the association between TLR4 signal pathways and the development of NASH was investigated [6, 10, 11]. More recently, Miura et al. reported that decreased levels of steatohepatitis and liver fibrosis in TLR9 knockout mice

compared with wild-type mice in a choline-deficient amino acid-defined (CDAA) diet-induced NASH model [12]. In contrast, in TLR2-deficient mice fed a methionine- and choline-deficient (MCD) diet, an increased level of liver injury was noted, suggesting a potential protective role of TLR2 in fatty liver [6].

An increasing proportion of the general population suffered from obesity, which with its related disorders such as metabolic syndrome, is an emerging global problem, and much recent evidence shows that microbiota are associated with these conditions [13-19]. In the intestine, TLRs are typically expressed in the epithelial cells and are involved in the production of immunoglobulin A (IgA), maintenance of tight junctions, proliferation of epithelial cells, and the expression of antimicrobial peptides [20]. TLR5, which specifically recognizes flagellin, is involved in promoting the pathophysiology of inflammatory bowel disease [21]. While the above reports suggest that intestinal TLRs play an important role in innate immunity of the gut, the association between its role in the small intestine and that in the development of NASH remains unclear.

In the present study, based on hypernutrition and obesity using a high-fat diet-induced NAFLD mouse model, the significance of TLRs and their signaling in the liver and small intestine was evaluated. In addition, a gut-sterilized mouse model treated

with antibiotics was used to confirm whether there is an association between intestinal microbiota and TLRs expression.

Materials and Methods

Animal studies

In a high-fat diet group, eight-week old male C57BL/6J mice (Charles River Japan, Inc., Tokyo, Japan) were fed a high-fat diet containing 82.0% of calories as fat (F2HFD2; Oriental Yeast Company Ltd, Tokyo, Japan). Control mice were fed a normal diet containing 12% of calories as fat (MF; Oriental Yeast Company Ltd). All mice were maintained under controlled conditions (22°C; humidity, 50%–60%, 12-h light/dark cycle) with food and water *ad libitum*. Mice from both groups were sacrificed at 4, 8, and 16 weeks for blood and tissue collection. These animals were fasted for 10-h before blood and tissue collection. After each mouse was anesthetized with diethyl ether and weighed, blood was collected by a cardiac puncture and subsequently assayed for biochemical parameters. Liver and small intestine were dissected, weighed, and frozen in liquid nitrogen. Samples of the resected liver were used later for histological and polymerase chain reaction (PCR) analysis. All experiments were performed in accordance with the rules and guidelines of the Animal Experiment Committee of

Asahikawa Medical University.

In vitro cultured human hepatocyte model

As a hepatocyte model, Huh7 human hepatoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin, streptomycin, and fetal bovine serum (FBS). Palmitic acid (PA) complexed with 1% bovine serum albumin (BSA) was added to the medium to attain a final concentration of 100 μM over 24 h [(22)].

In vitro cultued human Kupffer cell model

As a Kupffer cell model, THP-1 human acute monocytic leukemia cells were cultured in RPMI 1640 supplemented with penicillin, streptomycin, and 10% FBS. THP-1 cells were differentiated by 200 nM phorbol 12-myristate 13-acetate (PMA) (Calbiochem) for three days. After removal of PMA by washing, THP-1 cells were incubated in fresh RPMI 1640 for four days to enhance macrophage differentiation [(23)]. PA with 1% BSA was then added to the medium to attain a final concentration of 100 μM over 24 h.

Biochemical analyses

Serum alanine aminotransferase (ALT) and free fatty acids were measured using the Automatic Analyzer 7180 (Hitachi High-Technologies Corporation, Tokyo,

Japan).

Histopathological evaluation

Samples of remaining liver tissue were fixed in 10% formalin buffer, embedded in paraffin, cut, and stained with hematoxylin and eosin (H&E).

RNA isolation and first strand cDNA synthesis

Total RNA was isolated from the liver, small intestine, and Huh7 and THP-1 cell lines using QIAGEN RNeasy Mini Kit (QIAGEN, Hilden, Germany). RNA was reverse-transcribed by RETROscript using Random decamers (Ambion, Inc., Austin, TX, USA). Detailed methods were performed according to the manufacturers' instructions.

Primer pairs of TLR-related molecules

Mouse 18srRNA was used as an endogenous amplification control. The use of this universally expressed housekeeping gene allows for correction of variation in the efficiency of RNA extraction and reverse transcription. Specific primer pairs were used on TLR2, TLR4, TLR5, TLR9, tumor necrosis factor (TNF), IL-1β, IL-8, CXCL2 (IL-8 mouse homolog), and 18srRNA.

Quantitative real-time PCR

The expressions of TLR2, TLR4, TLR5, TLR9, IL-1β, TNF, CXCL2, and IL-8

in mouse liver, small intestine, and THP-1 and Huh7 cells were evaluated by quantitative real-time PCR (7300 Real-time PCR system; Applied Biosystems). In this method, all reactions were run in 96-well plates with a total volume of 20 µl. The reaction mixture consisted of 10 µl TaqMan Universal PCR Master mix, 1 µl 18srRNA, 1 µl primer, 5 µl RNAase free water, and 3 µl cDNA. The PCR reaction involved the following steps: (1) 50°C for 2 min to prevent carryover of DNA; (2) 95°C for 10 min to activate polymerase; (3) 40 cycles each of 95°C for 15 s, 60°C for 15 s, and 72°C for 45 s.

Immunohistochemistry

Immunohistochemistry using F4/80 as a macrophage marker was performed on cryostatically sectioned liver, and staining was performed by immunofluorescence. The sections were fixed in 2 % paraformaldehyde for 10 min and washed three times with PBS for 5 min. Further, sections for F4/80 were blocked with 3% BSA/PBS for 1 h at room temperature, followed by incubation with monoclonal antibody against F4/80 (abcam) 1:100 diluted in 3% BSA/PBS for 1 h at room temperature. After washing, F4/80 slides were incubated with 1:200 diluted Alexa Fluor 488 goat anti- rat IgG (Invitrogen) for 1 h at room temperature and washed.

Western blotting analysis

Protein expression of phospho-interleukin-1 receptor-associated kinase1 (pIRAK1), the key mediator in the TLR signaling pathway, [24] was studied by western blot analysis in the liver (30 μg), small intestine (30 μg), and TPH-1 treated with PA (30 μg). Protein concentrations were measured by the Bradford method using the Pierce BCA Protein Assay kit (Thermo Scientific, Rockford, IL, USA) following the manufacturer's suggested procedure. Separation of 30 μg of protein was then performed by 12% Mini PROTEAN® TGX™ Precast Gels (BIO-RAD). After electrophoresis, proteins were transferred to nitrocellulose membranes (Amersham LIFE SCIENCE), blocked in 5% skim milk, 0.2% Tween20 in PBS (PBS-T) for 1 h at room temperature, reacted with rabbit polyclonal anti-pIRAK1 (abcam) or β-actin (BD Biosciences) as a control overnight at 4°C, washed with 0.2% PBS-T, reacted with secondary antibody horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG (RD, Minneapolis, MN, USA) for 1 h, and washed with PBS-T. After reaction with horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG immune complexes were visualized by super signal west Pico Chemoluminescent substrate (Thermo SCIENTIFIC) following the manufacturer's suggested procedure. pIRAK1 was analyzed by Image J under the area, which compensated for β-actin.

Statistical analysis

The results were expressed as mean ± SE. The two groups were assessed by Student's *t*-test. Data sets involving more than two groups were assessed by ANOVA. *P*-values of <0.05 were considered statistically significant.

Gut sterilization

Mice were treated with ampicillin (1 g/l; Sigma-Aldrich), neomycin (1 g/l; Sigma), metronidazole (1 g/l; Sigma), and vancomycin (500 mg/l; Sigma) in drinking water for 8 weeks [(25)]. This treatment was followed by feeding a high-fat diet for further 8 weeks.

Results

Fatty liver in mice fed a high-fat diet

At 16 weeks, body weight (BW) and serum ALT were significantly higher in mice fed a high-fat diet (F) than in those fed the control diet (C) (BW: C, 41.6 g; F, 51.0 g; serum ALT: C, 34 IU/L; F, 180 IU/L; Figure 1a, b). Histopathological findings in livers from group F demonstrated the absence of fat droplets until 4 weeks (Figure 1c, d), but at 8 weeks, the deposition of micronodular fat droplets in the centrilobular zone (Figure 1e) was observed, and at 16 weeks macronodular fat droplets and ballooning degeneration (Figure 1f) were observed, however, no obvious infiltration of

inflammatory cells was noted. F4/80 staining for macrophage markers did not demonstrate increased numbers of Kupffer cells (Figure 1g, h).

Upregulation of cytokines in fatty liver of mice fed a high-fat diet

Histopathological examination of group F livers demonstrated no obvious infiltration of inflammatory cells, while mRNA levels of the inflammatory cytokines/chemokines TNF, IL-1β, and CXCL2 were significantly higher at 16 weeks (Figure 2a, b, c).

Upregulation of Toll-like receptors in fatty liver of mice fed a high-fat diet

To confirm whether the expression of TLRs contribute to the induction of the above mentioned cytokines/chemokines, we analyzed the mRNA of TLR2, TLR4, TLR5, and TLR9 that recognize bacterial components in the liver. The expression of these TLRs in the liver was not different between the two groups at 4 and 8 weeks, but at 16 weeks, this was significantly higher in the F group than in the C group (Figure 2d, e, f, g). Western blot analysis also demonstrated that expression of pIRAK1 in the liver was significantly upregulated in the F group compared with the C group (Figure 2h). These findings suggest that upregulation of TLR contributes to the induction of cytokines/chemokines, and the TLR signal pathway is genetically enhanced in simple steatosis without inflammation.

Downregulation of TLRs and cytokines in the small intestine of mice fed a high-fat diet

The expression of TLRs that recognize bacterial components was significantly upregulated in the NAFLD liver. Because liver injury has a connection with exposure to bacterial components of intestinal origin, we then examined the small intestine of NAFLD model mice. The mRNA expression of small intestinal TLR2, TLR4, TLR5, and TLR9 was not significantly different between the two groups at 4 and 8 weeks. Histopathological examination of the small intestine revealed no difference between the groups at 16 weeks, however, mRNA expression of all four TLRs was significantly lower in the F group than in C group at 16 weeks (Figure 3a, b, c, d). Expressions of IL-1β, TNF, and CXCL2 were also downregulated at 16 weeks (Figure 3e, f, g). Moreover, pIRAK1 expression was also significantly decreased in the F group compared with the C group (Figure 3h). These findings indicate that the TLR signal pathway is genetically attenuated in the NAFLD small intestine.

Antibiotic treatment improved steatosis and TLRs expression in the liver

Small intestinal bacterial overgrowth (SIBO) was reported to coexist with NASH [14, 26], and some of the following factors can predispose to SIBO; morbid obesity [27], aging [28], concurrent use of proton pump inhibitors [29], and abnormal small

intestinal motility [30]. Therefore, we hypothesized that attenuation of TLR signal pathways might induce immunotolerance, altered levels of microbiota, and bacterial overgrowth in the NAFLD small intestine. To investigate whether intestinal microbiota contribute to TLR expression, we eliminated them by treatment with nonabsorbable, broad-spectrum antibiotics [25].

BW, serum ALT, and serum free fatty acids were significantly decreased in mice fed the high-fat diet and administrated antibiotics (FA) than in those fed the high-fat diet and water only (FC) (BW: control diet and water only (CC), 28.9 g; control diet and antibiotics (CA), 28.6 g; FA, 34.7 g; FC, 51.9 g; serum ALT: CC, 19.5 IU/L; CA, 23.4 IU/L; FA, 34.7 IU/L; FC, 147.6 IU/L; serum free fatty acids; CC, 677.1 μEq/l; CA, 818.7 μEq/l; FA, 635.6 μEq/l; FC, 962.7 μEq/l; Figure 4a, f, g). Histopathological findings from the livers of FC mice demonstrated the deposition of macronodular fat droplets in the centrilobular area and ballooning degeneration of hepatocytes. In contrast, FA mice showed a marked reduction in steatosis compared with FC (Figure 4b, c, d, e). The expression of TLRs (Figure 5a, b, c, d) and inflammatory cytokines/chemokines (Figure 5e, f, g) was also significantly lower in the liver of FA mice than in that of FC mice. These data indicate associations among intestinal microbiota, TLR expression, and fatty acid metabolism.

Palmitic acid upregulated TLR2, TLR4, and TLR5 expressions in a human Kupffer cell model: THP-1 cells

Because both serum free fatty acids and TLR expression were coincidentally suppressed in the intestinal bacterial eradication model, we examined whether fatty acids would alter TLR expression. Firstly, we investigated TLR expression in THP-1 cells in a human Kupffer cell model. To differentiate THP-1 cells into macrophages, we added PMA to the cultured medium. Flow cytometry demonstrated that macrophage markers CD14, CD11b, and CD11c increased in PMA-treated THP-1 cells compared with control THP-1 cells, which suggests that the THP-1 cells so treated differentiated into Kupffer-like cells (Figure 6a, b).

To determine the effect of fatty acids on TLR expression in Kupffer cells, PA was added to differentiate THP-1 cells, where it induced the deposition of fat droplets (Figure 6c). The mRNA expression of TLR2, TLR4, and TLR5 was significantly higher in differentiated THP-1 cells treated with 100 μM PA than in control cells, but the expression of TLR9 was not different (Figure 6d). Western blot analysis demonstrated that the expression of pIRAK1 was upregulated in differentiated THP-1 cells treated with 100 μM PA compared with controls (Figure 6e). mRNA expression of TNF was also significantly higher in differentiated THP-1 cells exposed to 100 μM PA for 24 h

than in controls, but expression of IL-1b and IL-8 was not significantly different (Figure 6f, g, h). These findings indicate that PA may enhance the TLR signal pathway in Kupffer cells.

Palmitic acid upregulated TLR5 expression in a human hepatocyte model: Huh7 cells

Secondarily, we examined expression of TLR in Huh7 cells in a hepatocyte model, where PA induced the deposition of fat droplets in these cells (Figure 7a, b). The mRNA expression of TLR5 was significantly upregulated in Huh7 cells treated with 100 μM PA, but not that of TLR2, TLR4, and TLR9 (Figure 7c, d, e, f). The expression of IL-8 was significantly upregulated in Huh7 cells treated with 100 μM PA for 24 h but not that of TNF and IL-1β (Figure 7g, h, i). These findings suggest that fatty acids can, at least in part, enhance TLR signal pathways in hepatocytes.

Discussion

Mice fed the MCD diet demonstrated steatosis, macrophages accumulation, and clustering of neutrophils in the liver. Consequently, the expression of TLR4 and TNFα were increased but destruction of Kupffer cells prevented increase in TLR4 expression [(10)], indicating that increased levels of their expression had contributed to infiltration of inflammatory cells. It has been reported that fatty liver in NASH results in

increased liver injury and inflammation by intraperitoneal LPS injection in a MCD diet-induced NASH mouse model, suggesting that the MCD diet-induced NASH liver is sensitive to the TLR4 ligand LPS [6]. In our present model, mice fed the high-fat diet for 16 weeks developed steatosis with no histological evidence of inflammation and fibrosis, which is known as simple steatosis. However, the expressions of inflammatory cytokines/chemokines were upregulated, and our findings established that this is the mechanism by which TLR signal pathways are upregulated in the NAFLD liver prior to the development of steatohepatitis. Moreover, F4/80 staining revealed that this upregulation was not affected by altered numbers of Kupffer cells, but by changes in their activity in regard to inflammatory cytokine production levels. Our findings revealed that simple steatosis prior to NASH already demonstrated upregulation of TLR signal pathways and may be more sensitive to the ligands of intestinal microbial components. Bertola et al. reported that the expressions of TLRs, and certain cytokines/chemokines in genes were upregulated in biopsy specimens of morbidly obese patients with histologically normal liver or severe steatosis with or without NASH [31], and our findings from this animal study concur with this.

Though many studies have examined the association between TLR signal pathways and the pathogenesis of NASH, these reports focused mainly on hepatocytes,

Kupffer cells, and hepatic stellate cells [6, 10, 12, 32]. However, small intestinal microbiota are the cause of liver damage in NASH, and this is the first report to investigate the expression of intestinal TLR signal pathways in an NAFLD model and to reveal a discrepancy in TLRs expression in the gut-liver axis. The microbial TLR signal pathway and downstream cytokines/chemokines were downregulated in the small intestine of an NAFLD model. This downregulation may contribute the alternation of immune system and balance, and consequently, may alter microflora component ratio and induce SIBO with NAFLD.

We used a gut-sterilized mouse model with antibiotics to confirm whether there is an association between gut microbiota and TLR expression in NAFLD. In our model, oral caloric intake was not significantly different between FA and FC mice, however, BW, serum ALT, and serum free fatty acids were significantly suppressed in the FA mice and histopathological findings showed a marked reduction in steatosis in this group. Interestingly, the expressions of TLRs and downstream cytokines/chemokines were suppressed in FA mice as compared with FC mice. In contrast, in an EtOH-induced liver injury model, liver TLR expression was found to be independ of gut microbiota despite a reduction in fatty liver and liver injury by antibiotic administration [9]. Our findings suggest that, directly or indirectly, gut microbiota contribute to TLR

expression in the liver of a NAFLD model. Because antibiotics administration also reduced serum free fatty acids in FA mice, we focused on the association between fatty acid metabolism and gut microbiota. There are three reasons why serum fatty acids were suppressed in FA mice. The first reason is a reduction in adipocytes accompanied by suppression of increasing BW. This may be explained by the fact that obesity-associated microbiota, which increase the capacity to harvest energy from the diet, were eradicated by antibiotics [13]. We investigated 16s rRNA based analysis of fecal microbiota by the terminal restriction fragment length polymorphism (T-RFLP) method and confirmed the alternation in FA mice fecal microbiota compared with that of FC mice (data not shown). The second reason is a reduction in the release of free fatty acids from adipocytes. In obese individuals, it has been reported that gut microbiota might suppress the expression of fasting-induced adipose factor (Fiaf), which increases the activity of lipoprotein lipase, leading to the production of triglyceride storage in adipocyte and an increased supply of fatty acids [33]. The expression of intestinal Fiaf was significantly upregulated in FA mice compared with FC mice in our experiment (data not shown), indicating that gut-sterilization may contribute to a reduction in the release of serum free fatty acids from adipocytes through increased intestinal Fiaf expression. The third is a reduction in *de novo* liver lipogenesis. It is reported that microbiota might increase

hepatic lipogenesis through the expression of sterol response element binding protein 1 (SREBP1) and carbohydrate response element binding protein (ChREBP) [34]. The expressions of SREBP1 and ChREBP were significantly suppressed in FA mice compared with FC mice in our experiment (data not shown), indicating that the suppression of serum free fatty acids, at least in part, contributes to reduction in hepatic *de novo* lipogenesis through the suppression of SREBP1 and ChREBP. Our findings suggest both alternation of microflora and reduction of *de novo* lipogenesis in the liver of the gut-sterilized model, but it is unknown whether the release of fatty acids from adipocytes decreases.

Next, we hypothesized that fatty acids could alter the expression of TLRs in the liver. In one *in vitro* study, it was reported that the unsaturated fatty acids, eicosapentaenoic acid and docosahexaenoic acid, downregulated LPS-induced activation of NFκB via the PPAR-γ-dependent pathway [35], whereas saturated fatty acids conversely stimulated NFκB promoter activity through activation of TLR signal pathway [36, 37]. However, another recent *in vivo* mouse model study suggested that dietary saturated fat plays a protective role against MCD diet-induced NASH [32]. Therefore, the effects of saturated and unsaturated fatty acids on inflammation and the pathogenesis of NAFLD/NASH remain unclear, and it is unknown whether fatty acids

enhance the expression of TLRs. In regard to the above- mentioned *in vitro* experiment, the expression of TLR5 and downstream chemokine IL-8 was upregulated in Huh7 cells treated with PA. In contrast, the expressions of TLR2, TLR4, TLR5, and TNF were significantly higher in THP-1 cells treated with PA than in control cells. These findings suggest that hepatocytes and Kupffer cells are susceptible to bacterial components via upregulation of TLRs by free fatty acids in the pro-inflammatory state of NAFLD.

In conclusion, the expression of TLRs was downregulated in the NAFLD small intestine, and this may contribute to an increase in fatty acids through alteration of gut microbiota. In contrast, the hepatic TLR signal pathway was upregulated and susceptible to microbial components by increased fatty acids in the pro-inflammatory state of NAFLD. Our findings suggest that discrepancy in TLR signals in the gut-liver axis may be associated with the pathogenesis of progression to NASH through an increase in free fatty acids. Because there is no specific treatment for human NASH, early intervention in the pro-inflammatory state may be important in the prevention of its development from simple steatosis. As fatty acids play an important role in development to pro-inflammatory state of NAFLD, we thus considere that both fatty acid metabolism and gut microbiota in the pro-inflammatory state might be useful targets with preventive treatment for development to NASH.

Acknowledgements

This work was supported in part by Grants-in-Aid for Young Scientists (B) provided by Ministry of Education, Culture, Sports, Science, and, Technology, Japan.

References

(1) Ludwig J, Viggiano TR, McGill DB, Brewer NS. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hithero unnamed disease. *Mayo Clin Proc* 1980; **55:** 482-8.

(2) Lazo M, Clark JM. The epidemiology of nonalcoholic fatty liver disease: a global perspective. *Semin Liver Dis* 2008; **28:** 339-50.

(3) Day CP, James OF. Steatohepatitis: a role of two “hit”? *Gastroenterology* 1998; **114:** 842-5.

(4) Marra F, Bertolani C. Adipokines in Liver Disease. *Hepatology* 2009; **50:** 957-62.

(5) George DK, Goldwurm S, MacDonald GA, Cowley LL, Walker NI, Ward PJ, et al. Increased hepatic iron concentration in nonalcoholic steatohepatitis is associated with increased fibrosis. *Gastroenterology* 1988; **114:** 311-8.

(6) Szabo G, Velayudham A, Romics L Jr, Mandrekar P. Modulation of non-alcoholic

steatohepatitis by pattern recognition receptors in mice: the role of toll-like receptor 2 and 4. *Alcohol Clin Exp Res* 2005; **29(11 Suppl):** 140S-5S.

(7) Seki E, Brenner DA. Toll-like receptors and adaptor molecules in liver disease: updata. *Gastroenterology* 2008; **48:** 322-35.

(8) Takeda K, Akira S. TLR signaling pathway. *Semin in Immunol* 2004; **16:** 3-9.

(9) Gustot T, Lemmers A, Moreno C, Nagy N, Quertinmont E, Nicaise C, et al. Differential liver sensitization to Toll-like receptor pathways in mice with alcoholic fatty liver. *Hepatology* 2006; **43:** 989-1000.

(10) Rivera CA, Adegvoyage P, van RooijenN, Tagalicud A, Allman M, WallaceM. Toll-like receptor-4 signaling and Kupffer cells play pivotal roles in the pathogenesis of non-alcoholic steatohepatitis. *J Hepatol* 2007; **47:** 571-9.

(11) Imajo K, Fujita K, Yoneda M, Nozaki Y, Ogawa Y, Shinohara Y, et al. Hyperresponsivity to low-dose endotoxin during progression to nonalcoholic steatohepatitis is regulated by leptin mediated signaling. *Cell Metab* 2012; **16:** 44-54.

(12) Miura K, Kodama Y, Inokuchi S, Schnabl B, Aoyama T, Ohnishi H, et al. Toll-like receptor 9 promotes steatohepatitis by induction of interleukin-1β in mice. *Gastroenterology* 2010; **139:** 323-34.

(13) Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An

obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 2006; **444:** 1027-31.

(14) Shanab AA, Scully P, Crosbie O, Buckley M, O'Mahony L, Shanahan F, et al. Small intestinal bacterial overgrowth in nonalcoholic steatohepatitis: association with toll-like receptor 4 expression and plasma levels of interleukin 8. *Dig Dis Sic* 2011; **56:** 1524-34.

(15) Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Human gut microbes associated with obesity. *Nature* 2006; **444:** 1022-3.

(16) Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in gut microbiota control metabolic endotoxemia-induce inflammation in high fat diet induced obesity and diabetes in mice. *Diabetes* 2008; **57:** 1470-81.

(17) Bajzer M, Seeley RJ. Obesity and gut flora. *Nature* 2006; **444:** 1009-10.

(18) de La Serre CB, Ellis CL, Lee J, Hartman AL, Rutledge JC, Raybould EE. Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota gut inflammation. *Am J Physiol Gastrointest Liver Physiol* 2010; **299:** G440-8.

(19) Vijay-Kumar M, Aitken JD, Carvalho A, Cullender TC, Mwangi S, Srinivasan S, et al. Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5.

Sicence 2010; **328:** 228-31.

(20) Santaolalla R, Fukata M, Abreu M. Innate immunity in the small intestine. *Curr Opin Gastroenterol* 2011; **27:** 125-31.

(21) Lodes MJ, Cong Y, Elson CO, Mohamath R, Landers CJ, Targan SR, et al. Bacterial flagellin is a dominant antigen in Crohn disease. *J Clin Invest* 2004; **113:** 1296-306.

(22) Gómez-Lechón, Donato MT, Martinez-Romero A, Jimenéz N, Castell JV, O'connor JE. A human hepatocellular in vitro model to investigate steatosis. *Chem Biol Interact* 2007; **165:** 106-16.

(23) Daigneault M, Preston JA, Marriotto HM, Whyte MK, Dockrell DH. The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. *PLoS One* 2010; **13:** e8668.

(24) Gottipati S, Rao NL, Fung-Leung WP. IRAK1: A critical signaling mediator of innate immunitiy. *Cell signal* 2008; **20:** 269-76.

(25) Seki E, Minicis SD, Osterreicher CH, Kluwe J, Osawa Y, Brenner DA, et al. TLR4 enhances TGF-β signaling and hepatic fibrosis. *Nat Med* 2007; **13:** 1324-32.

(26) Wigg AJ, Roberts-Thomson IC, Dymock RB, McCarthy PJ, Grose RH, Cummins AG. The role of small intestinal bacterial overgrowth, intestinal permeability,

endotoxaemia, and tumor necrosis factor alpha in the pathogenesis of non-alcoholic steatohepatitis. *Gut* 2001; **48:** 206-11.

(27) Sabate JM, Jouet P, Harnois F, Mechler C, Msika S, Grossin M, et al. High prevalence of small intestinal bacterial overgrowth with morbid obesity: a contributor to severe hepatic steatosis. *Obes Surg* 2008; **18:** 371-7.

(28) Pillotto A, Franceschi M, Del Favero G, Fabrello R, Di Mario F, Valerio G. The effect of aging on oro-cecal transit time in normal subjects and patients with gallstone disease. *Aging (Milano)* 1995; **7:** 234-7.

(29) Elphick DA, Chew TS, Higham SE, Bird N, Ahmad A, Sanders DS. Small bacterial obergrowth in symptomatic older people: can it be diagnosed earlier? *Gerontology* 2005; **51:** 396-401.

(30) Soza A, Riquelme A, GonzalezR, Alvarez M, Perez-Ayuso RM, Glasinovic JC, et al. Increased orocecal transit time in patients with nonalcoholic fatty liver disease. *Dig Dis Sci* 2005; **50:** 1136-40.

(31) Bertola A, Bonnafous S, Anty R, Patouraux S, Saint-Paul MC, Iannelli A, et al. Hepatic expression patterns of inflammatory and immune response genes associated with obesity and NASH in morbidly obese patients. *PLoS ONE* 2010; **5:** e13577.

(32) Rivera CA, Gaskin LT, Allman M, Pang J, Brady K, Adegboyega P, et al. Toll-like

receptor 2 deficiency enhances non-alcoholic steatohepatitis. *BMC Gastroenterol* 2010; **10:** 52.

(33) Backhed F, Ding H, Wang T, Hooper LV, Hoh GY, Nagy A, et al. The gut microbiota as an environmental factor that regulates fat storage. *Proc Nalt Acad Sci USA* 2004; **101:** 15718-23.

(34) Prakash S, Rodes L, Coussa-Charley M, Duchesneau CT. Gut mictobiota: next frontier in understanding human health and development of biotherapeutics. *Biologics* 2011; **5:** 71-86.

(35) Li H, Ruan XZ, Powis SH, Fernando R, Mon WY, Wheeler DC, et al. EPA and DHA reduce LPS-induced inflammation responses in HK-2 cells: evidence for a PPAR-gamma-dependent mechanism. *Kidney Int* 2005; **67:** 867-74.

(36) Lee JY, Plakidas A, Lee WH, Heikkinen A, Chanmugam P, Bray G, et al. Differential modulation of Toll-like receptors by fatty acids: preferential inhibition by n-3polyunsaturated fatty acids. *J Lipid Res* 2003; **44:** 909-16.

(37) Lee JY, Gao Z, Youn HS, Lee WH, Zhao L, Sizemore L, et al. Reciprocal modulation of Toll-like recptor-4 signaling pathways involving MyD88 and phosphatidylinositol 3-kinase/AKT by saturated and polyunsaturated fatty acids. *J Biol Chem* 2003; **278:** 37041-51.

Figure legends

Figure 1. Body weight, serum ALT, and histopathological findings in high-fat diet fed and control mice.

Body weight (a) and serum ALT (b) were significantly higher in mice that were fed a high-fat diet (F) than in controls at 16 weeks. Histopathological liver findings in F mice at 4, 8, and 16 weeks with H&E staining (×400) [control (c); 4 weeks (d); 8 weeks (e); 16 weeks (f)]. Micronodular fat droplets deposited in hepatocytes in the centrilobular zone at 8 weeks (e). Macronodular fat droplets and ballooning degeneration were marked at 16 weeks, but no obvious infiltration of inflammatory cells was observed (f). F4/80 staining for macrophage markers did not demonstrate any increase in Kupffer cells [control (g); 16 weeks (h); white arrows indicate Kupffer cells] (* $p < 0.05$).

Figure 2. mRNA expressions of inflammatory cytokines and Toll-like receptors in liver of high-fat diet fed and control mice.

mRNA expression of TNF (a), IL-1β (b), and CXCL2 (c) was significantly higher in the liver of mice that were fed a high-fat diet (F) than in that of controls (C) at 16 weeks. The expression of TLR2 (d), TLR4 (e), TLR5 (f), and TLR9 mRNA (g) was

significantly higher in F than C livers at 16 weeks. Western blot analysis demonstrated a higher expression of pIRAK1 in F than C livers (h) (*$p < 0.05$, **$p < 0.01$)

Figure 3. mRNA expressions of inflammatory cytokines and Toll-like receptors in small intestine of high-fat-diet fed and control mice.

mRNA expression of TLR2 (a), TLR4 (b), TLR5 (c), and TLR9 (d) in the small intestine was significantly lower in mice that were fed a high-fat diet (F) than control diet (C) at 16 weeks. mRNA expression of TNF (e), IL-1β (f), and CXCL2 (g) in the small intestine was also lower in F than C at 16 weeks. Western blot analysis demonstrated a lower expression of pIRAK in F than C (h) (*$p < 0.05$, **$p < 0.01$)

Figure 4. Body weight, serum ALT, serum free fatty acids, and histopathological findings in high-fat diet fed and control mice with or without antibiotics.

CC, control diet and water only; CA, control diet and antibiotics; FC, high-fat diet and water only; FA, high-fat diet and antibiotics. Body weight was significantly higher in FC mice than in FA mice (a). Histopathological liver findings for CC (b), CA (c), FC (d), and FA (e) with H&E staining (×100) demonstrated macronodular fat droplets and ballooning degeneration in FC mice liver, but steatosis was obviously suppressed in FA.

Serum ALT (f) and serum free fatty acids (g) were also suppressed in FA in comparison with FC (*$p < 0.05$, **$p < 0.01$)

Figure 5. mRNA expressions of Toll-like receptors and inflammatory cytokines in liver of high-fat diet fed and control mice with or without antibiotics.

Expression of TLR 2 (a), TLR4 (b), TLR5 (c), and TL9 (d) was suppressed more in the livers of FA mice as compared with FC mice. Expression of TNF (e), IL-1β (f), and CXCL2 (g) was also suppressed. (*$p < 0.05$, **$p < 0.01$)

Figure 6. The expressions of Toll-like receptors and inflammatory cytokines in THP-1 cells treated with palmitic acid.

Flow cytometry of THP-1 cells treated with phorbol 12-myristate 13-acetate (PMA, a) and controls (b). Palmitic acid (PA)-induced deposition of fat droplets in THP-1 cells (c). The expression of TLR2, TLR4, and TLR5 was significantly higher in THP-1 cells treated with 100 μM PA for 24 h (d). Western blot analysis demonstrated that the expression of pIRAK was upregulated in THP-1 cells treated with 100 μM PA as compared with control cells (e). TNF expression was significantly higher in THP-1 cells treated with PA [TNF (f); IL-1β (g); IL-8 (h)] (*$p < 0.05$, **$p < 0.01$)

Figure 7. **The expressions of Toll-like receptors and inflammatory cytokines in Huh7 cells treated with palmitic acid.**

Palmitic acid (PA, 100 μM)-induced deposition of fat droplets in Huh7 cells (a) and controls (b). The expression of TLR5 mRNA was significantly upregulated in Huh7 cells treated with 100 μM PA (TLR2 (c); TLR4 (d); TLR5 (e); TLR9 (f)). Expression of IL-8 was significantly higher in Huh7 cells treated with PA [TNF (g); IL-1β (h); IL-8 (i)] (*$p < 0.05$, **$p < 0.01$)

Figure 1

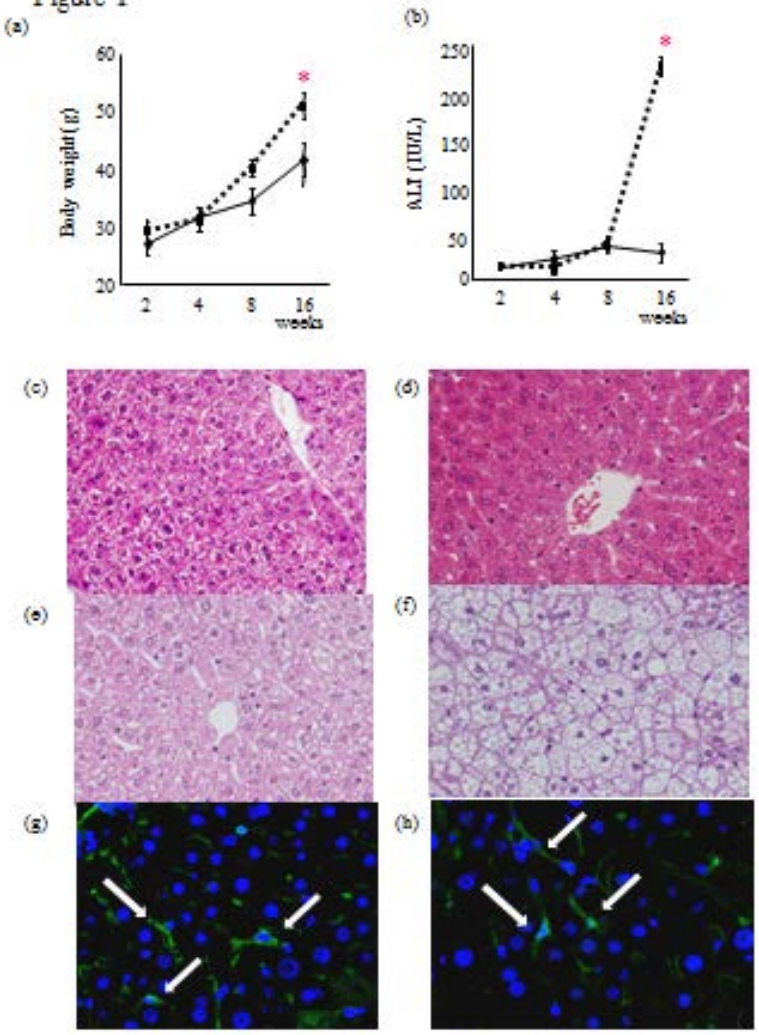

(a)
Body weight (g)
60
50
40
30
20
2
4
8
16
weeks
*
(b)
ALT (IU/L)
250
200
150
100
50
0
2
4
8
16
weeks
*
(c)
(d)
(e)
(f)
(g)
(h)

Figure 2

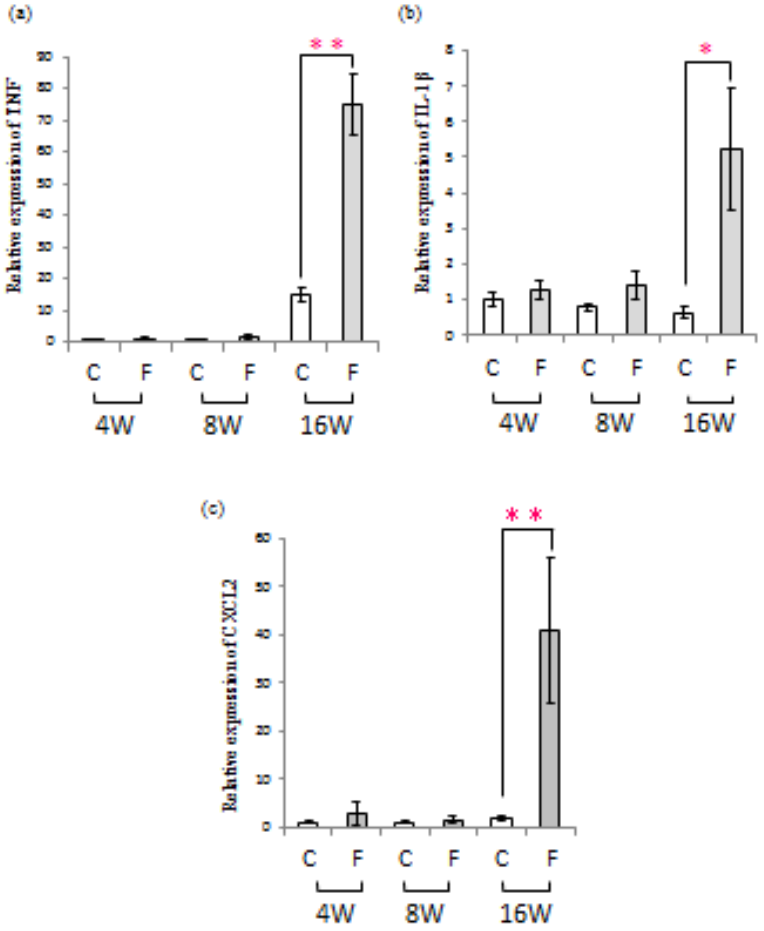


Figure 2

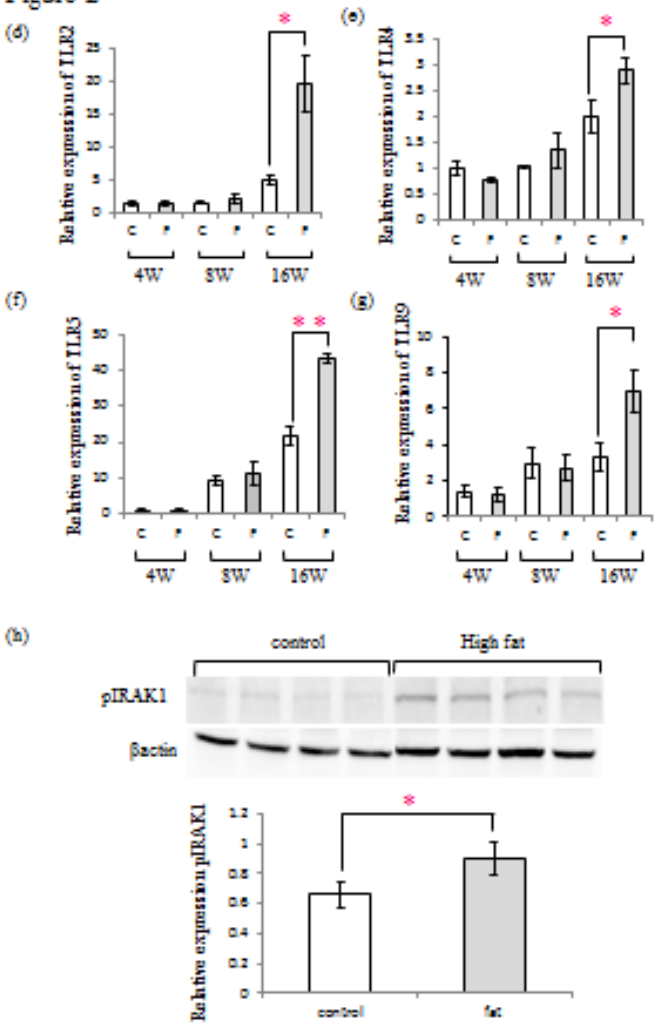


Figure 3

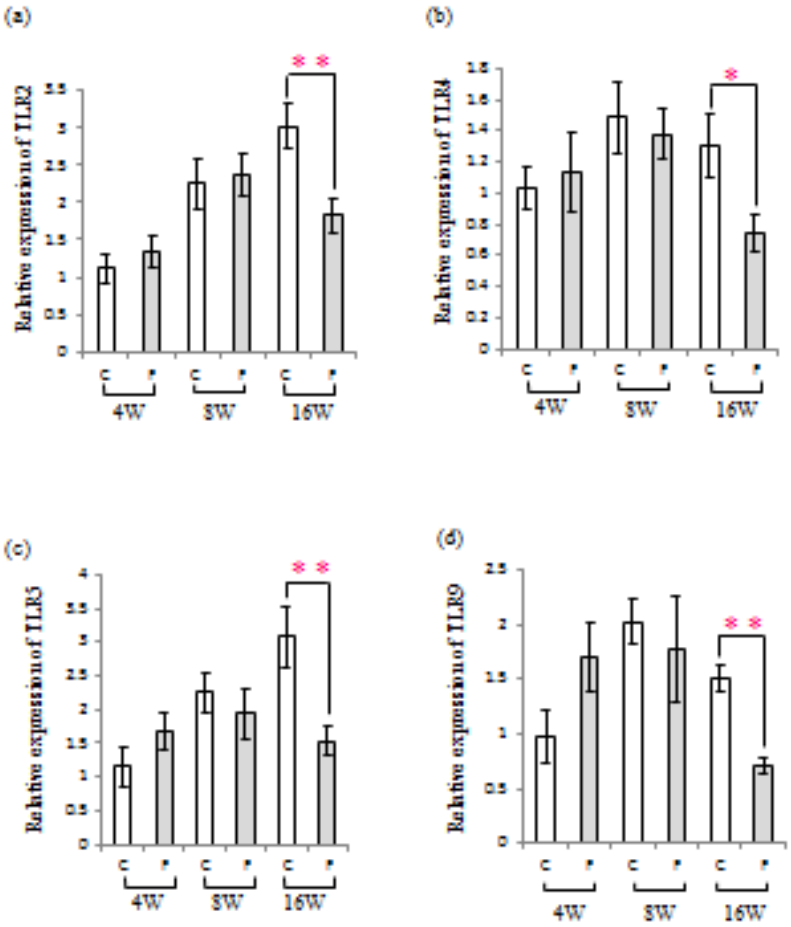


Figure 3

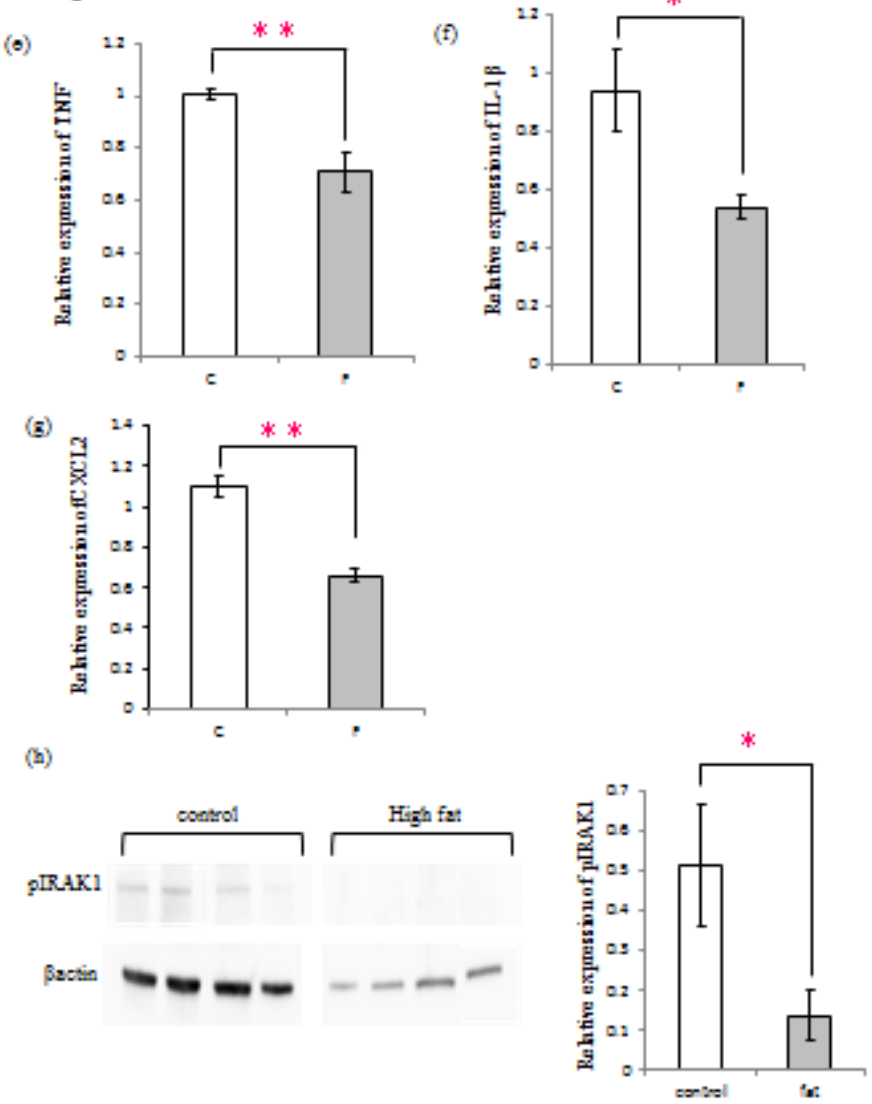


Figure 4

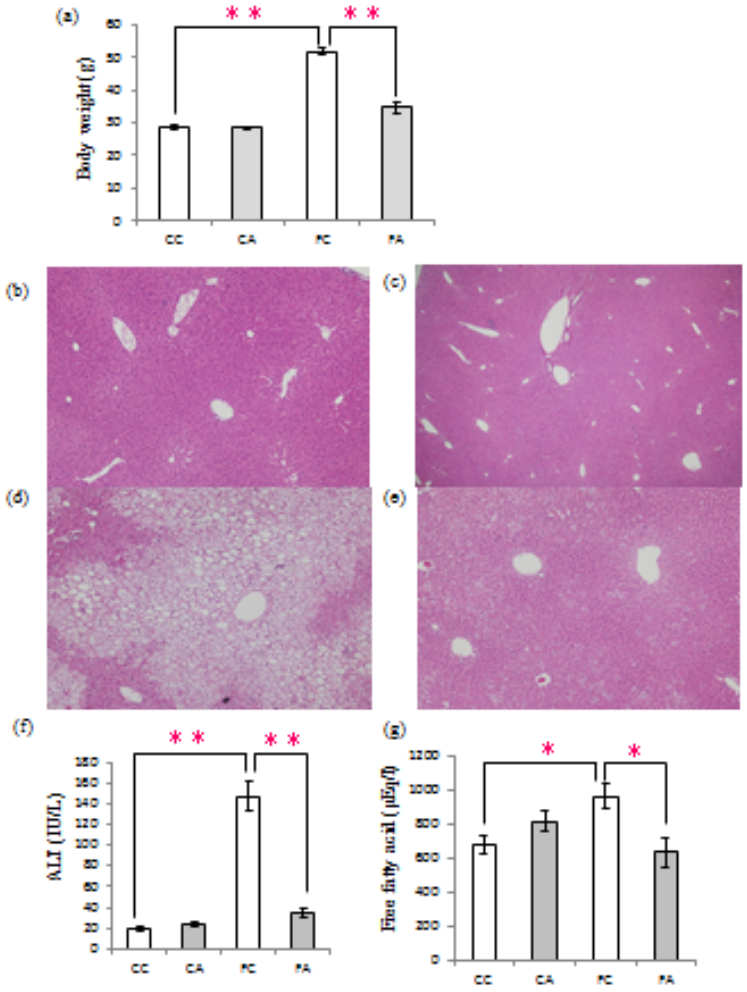


Figure 5

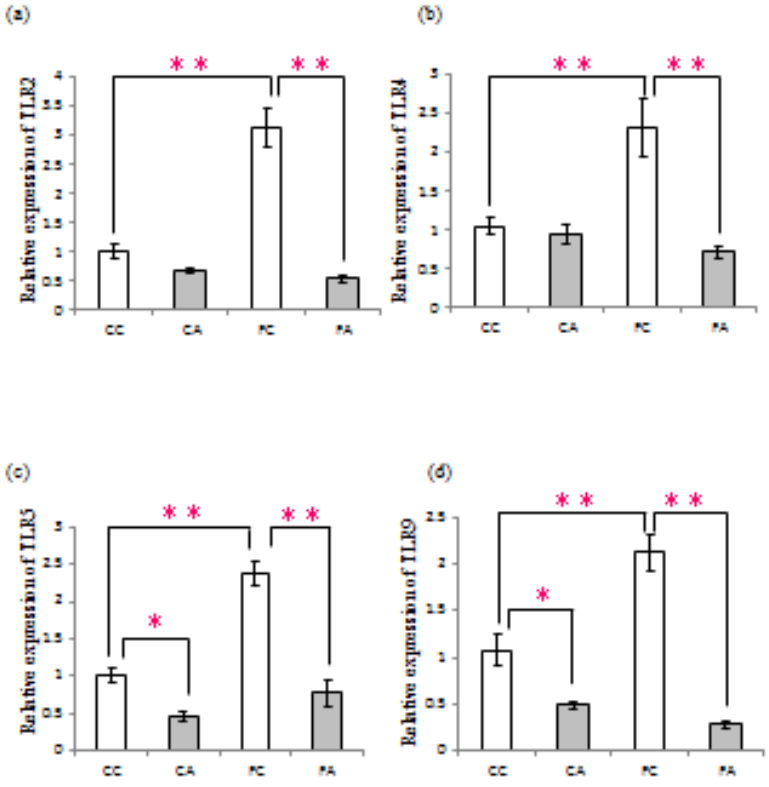


Figure 5

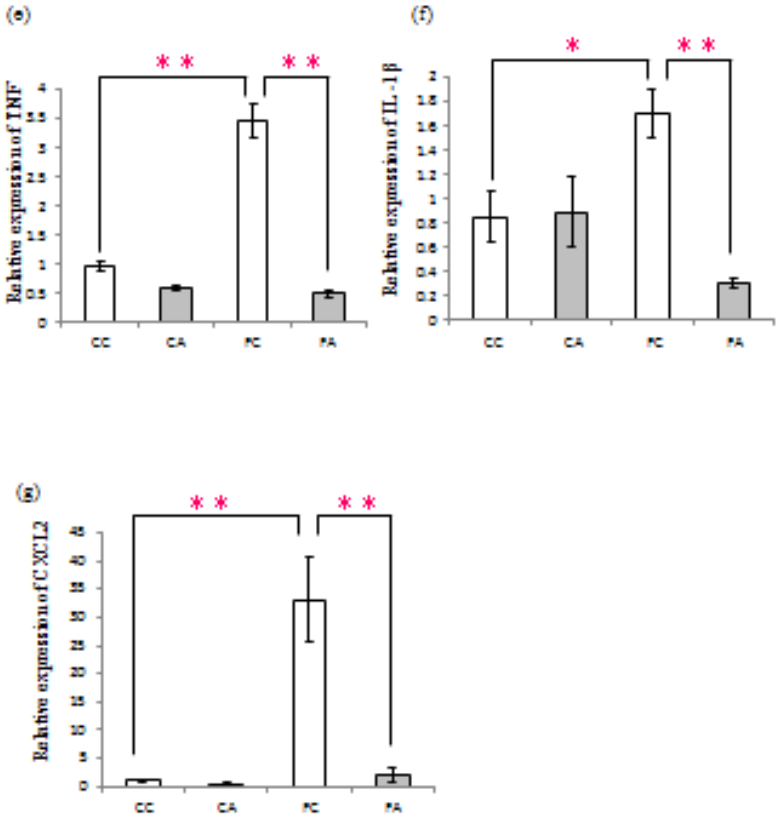


Figure 6

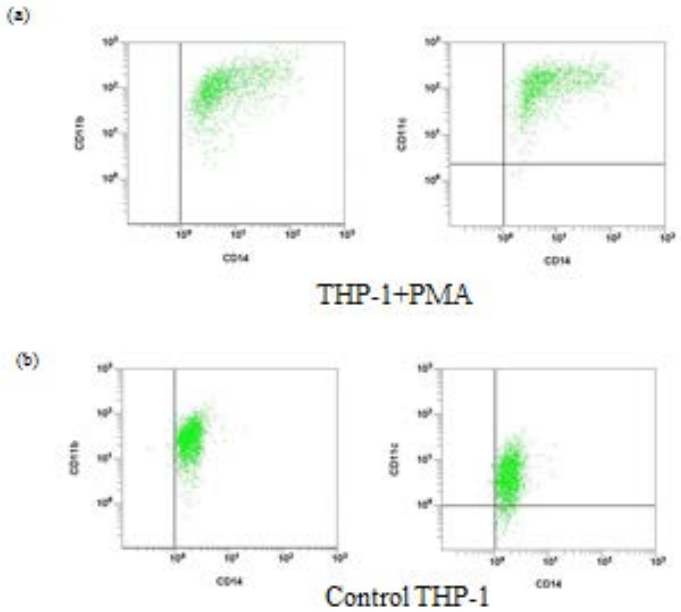


Figure 6

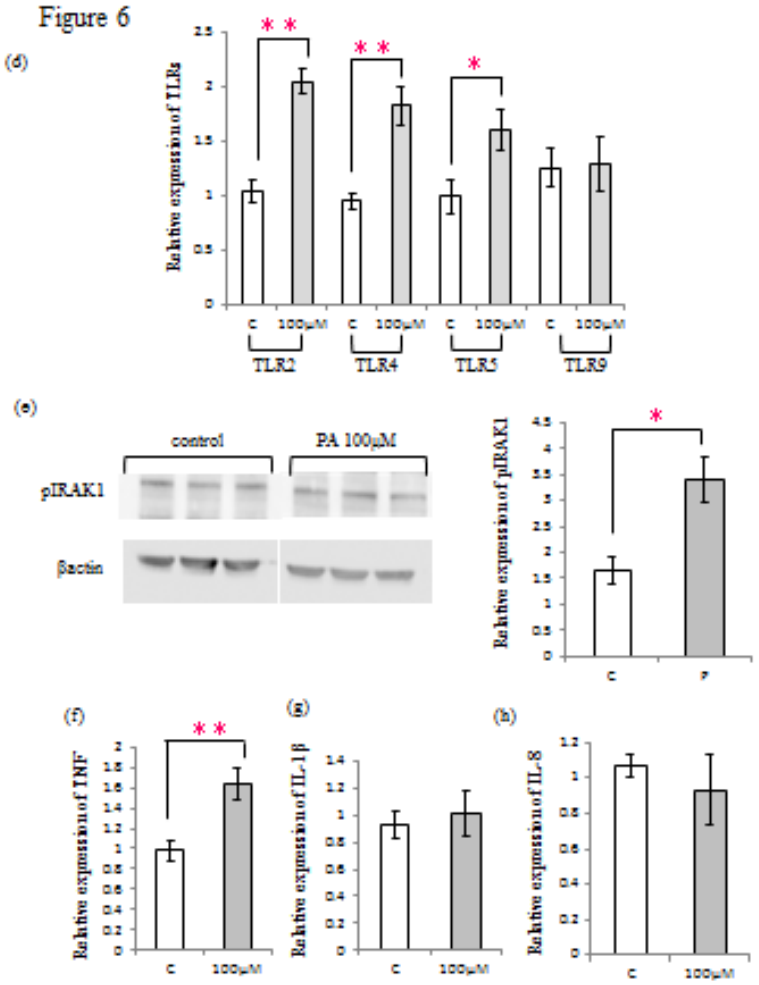
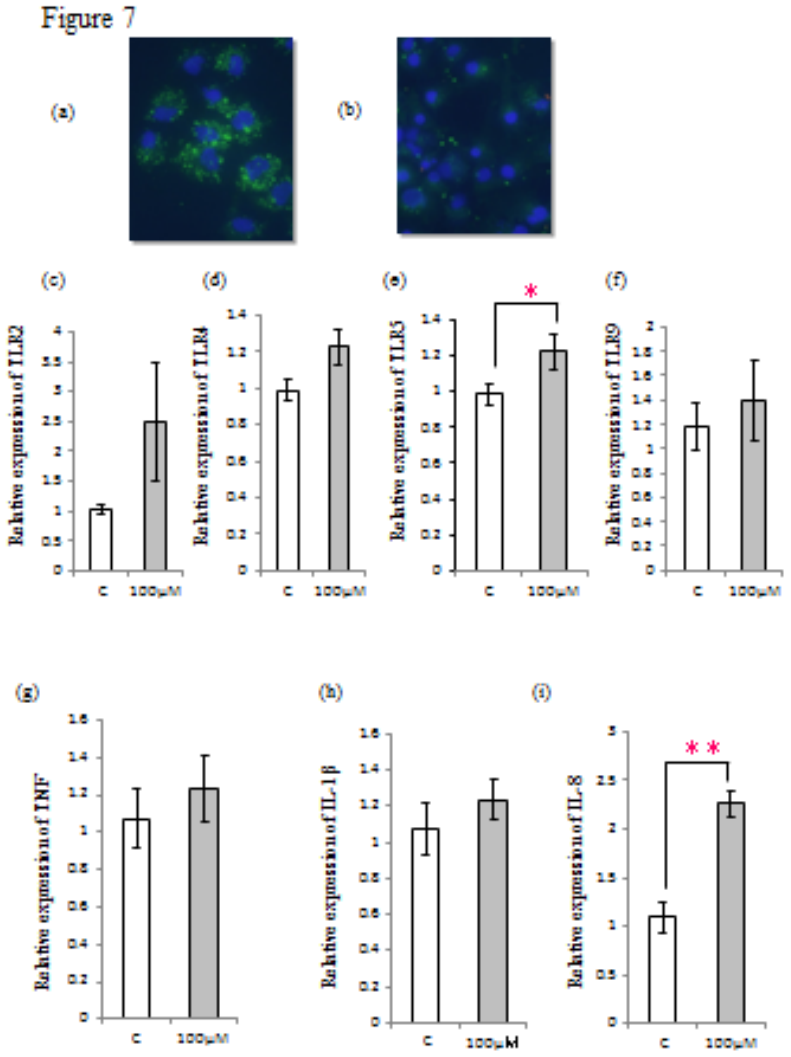


Figure 7


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
Relative expression of TLR2
Relative expression of TLR4
Relative expression of TLR5
Relative expression of TLR9
Relative expression of TNF
Relative expression of IL-1β
Relative expression of IL-8
C
100μM
*
**