GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Fatty Acid Binding Protein 7 Regulates Phagocytosis and Cytokine Production in Kupffer Cells during Liver Injury

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Kupffer cells (KCs) are involved in the progression of liver diseases such as hepatitis and liver cancer. Several members of the fatty acid binding proteins (FABPs) are expressed by tissue macrophages, and FABP7 is localized only in KCs. To clarify the role of FABP7 in the regulation of KC function, we evaluated pathological changes of *Fabp7* knockout mice during carbon tetrachloride-induced liver injury. During liver injury in *Fabp7* knockout mice, serum liver enzymes were increased, cytokine expression (tumor necrosis factor- α , monocyte chemoattractant protein-1, and transforming growth factor- β) was decreased in the liver, and the number of KCs in the liver necrotic area was significantly decreased. Interestingly, in the FABP7-deficient KCs, phagocytosis of apoptotic cells was impaired, and expression of the scavenger receptor CD36 was markedly decreased. In chronic liver injury, *Fabp7* knockout mice showed less fibrogenic response to carbon tetrachloride compared with wild-type mice. Taken together, FABP7 is involved in the liver injury process through its regulation of KC phagocytic activity and cytokine production. Such modulation of KC function by FABP7 may provide a novel therapeutic approach to the treatment of liver diseases. (*Am J Pathol* 2014, 184: 2505–2515; <http://dx.doi.org/10.1016/j.ajpath.2014.05.015>)

Macrophages play a central role in inflammatory reactions by producing pro- and anti-inflammatory cytokines as well as by phagocytizing microorganisms. Recently, it has become evident that adipose tissue macrophages and liver macrophages are important not only in local inflammation and tissue damage but also in systemic diseases bound to metabolic abnormalities.^{1,2} Kupffer cells (KCs) are liver-resident macrophages that constitute approximately 70% to 80% of all macrophages in the body. KCs play an important role in normal physiology and homeostasis and participate in the acute and chronic response of the liver to toxic compounds. Activated KCs vigorously scavenge damaged hepatocytes while they release inflammatory and growth control mediators. This activation appears to modulate acute hepatocyte injury and chronic liver responses, including hepatic fibrosis. These specialized liver macrophages are primarily exposed to gut-derived antigens, and they orchestrate inflammatory processes in diseases such as alcoholic and nonalcoholic liver

disease.^{3,4} Although it seems that the role of KCs should be protective by removing gut-derived pathogens, studies have found that they contribute to the promotion of liver inflammation.^{5,6} Therefore, KCs can play both deteriorative and ameliorative roles in the pathogenesis of liver diseases according to their ambient conditions. Unraveling the controlling mechanism of the KC activation is essential for understanding the mechanism of the liver injury.

Fatty acid binding proteins (FABPs) can bind different FAs and their derivatives with distinct binding preferences and act as their intracellular chaperones.⁷ FABPs have been reported to govern the transcriptional activity of peroxisome proliferator-activated receptors by targeting the ligands to nucleus⁸ and to be involved in modulating macrophage function.^{9,10} FABPs

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are expressed in various macrophages with distinct patterns for the individual FABPs. For example, FABP4 is highly expressed in the activated macrophages of atherosclerotic lesions,^{11,12} whereas FABP5 is found in atretic ovarian follicles¹³ and alveolar macrophages express FABP1.¹⁴ Such differential expression of FABP subtypes might reflect the fact that macrophages adopt different regulatory systems to adjust to tissue-specific lipid environment. Macrophage-specific FABP4 deficiency protects against atherosclerosis in apolipoprotein E-deficient mice,¹¹ and pharmacological inhibition of FABP4 protects mice against atherosclerosis and compromises inflammatory responses in macrophages.¹⁵ Moreover, it has been reported that macrophage FABP5 deficiency suppresses atherosclerosis in low-density lipoprotein receptor-null mice.¹⁶ Among different macrophages, FABP7 is expressed only by KCs,¹⁷ suggesting its regulatory role by controlling lipid homeostasis in KCs. However, the contribution of FABP7 to the functional regulation of KCs remains unknown.

In this study, to explore the role of FABP7 in the process of acute liver injury and liver fibrosis, we examined the phenotype of *Fabp7*-knockout (KO) mice after carbon tetrachloride (CCl₄) or D-galactosamine (D-GalN)/lipopolysaccharide (LPS) treatment, both of which have been frequently used by many researchers because the important role of KCs in the clearance of dead cells and cytokine production has been well defined.^{6,18–20} We found that FABP7 regulates the phagocytosis of apoptotic cells and the production of cytokines, including fibrogenic factors in KCs.

Materials and Methods

Animals

We used 8- to 11-week-old male wild-type (WT) and *Fabp7*-KO²¹ C57BL/6 mice. Liver injury was induced by i.p. injection of CCl₄ (1 μ L/g body weight, dilution 1:3 in olive oil), once for the acute injury model and 16 i.p. injections (twice per week for 8 weeks) for the chronic fibrosis model. A suspension of liposome-encapsulated clodronate (Clo-lipo) prepared as described previously²² was administered by i.p. injection for the depletion of KCs. D-GalN (500 mg/kg body weight; Wako, Osaka, Japan) and LPS (5 μ g/kg body weight; Sigma-Aldrich, St. Louis, MO) were administered by i.p. injection. Mice were given i.p. injection of 2 mL of thioglycolate, and the peritoneal macrophages were harvested 4 days after injection. All experimental protocols were reviewed by the Ethics Committee for Animal Experimentation of Yamaguchi University School of Medicine and were performed according to the Guidelines for Animal Experimentation of the Yamaguchi University School of Medicine and under the law and notification requirements of the Japanese government.

Enrichment and Isolation of KCs from Mice

KCs were isolated according to the previously reported method^{23,24} with some modifications. Briefly, mice livers were

perfused initially by 0.03% collagenase (Wako). After perfusion, the liver was minced and incubated with 0.03% collagenase for 5 minutes at 37°C. The cell suspension was centrifuged at $50 \times g$ for 3 minutes, and the supernatant was centrifuged at $650 \times g$ for 5 minutes at 4°C. The pellet was used as KC-enriched fraction. The viability (propidium iodide) of KC-enriched fraction was 90%, and the purity of KCs (F4/80⁺) was approximately 50% by fluorescence-activated cell sorting analysis (data not shown). For further isolation of KCs, KC-enriched fraction was loaded on a 25% to 50% Percoll gradient and centrifuged at $1500 \times g$ for 15 minutes at 4°C. The layer of KCs was collected and used for experiments. The final purity of KCs was >80% and the viability was >90% (data not shown).

Cell Culture and *FABP7* Gene Transduction

Murine macrophage-like cell line J774 was obtained from Yamaguchi University Center for Gene Research. J774 cells were maintained by passage in RPMI 1640 medium (Life Technologies, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Waltham, MA), 100 U/mL penicillin, 100 μ g/mL streptomycin (Sigma-Aldrich), and 2 mmol/L L-glutamine (Life Technologies). J774 cells were transfected with either pcDNA3-FABP7 expression construct (FABP7/J774) or empty pcDNA3 vector (MOCK) by using Lipofectamine 2000 (Life Technologies). For transient expression, cells were analyzed 24 to 48 hours after transfection. For stable expression, the transfected clones were selected with 0.1 mg/mL G418 (Sigma-Aldrich) for 2 weeks.

Phagocytosis Assays

Apoptotic cells and necrotic cells were prepared according to the method previously reported²⁵ and were used as target cells for the phagocytosis assay. Briefly, thymocytes harvested from mice aged 4 to 7 weeks were incubated with 10 mmol/L dexamethasone for 5 hours in RPMI 1640 medium that contained 10% fetal bovine serum. Necrosis was induced by incubating the cells at 56°C for 10 minutes and was confirmed by trypan blue staining. Apoptotic thymocytes were labeled with 2 μ mol/L 5-chloromethylfluorescein diacetate (Cell-Tracker Green CMFDA; Life Technologies). CMFDA-labeled apoptotic thymocytes were intravenously injected into mice through the portal vein. Ten minutes after injection, the five largest lobes (five sections) of the liver were harvested, fixed with 4% paraformaldehyde, and stained for F4/80. CMFDA-positive thymocytes enclosed by F4/80⁺ KC cytoplasm were counted. The total number of ingested apoptotic thymocytes in 50 microscopic fields for five sections from different liver lobes was quantified as the phagocytic activity of each mouse. For the *in vitro* phagocytosis assay, 1×10^5 FABP7/J774 cells per well and 4×10^6 CMFDA-labeled apoptotic thymocytes cells per well were co-cultured for 120 minutes in a 24-well plate. After incubation, apoptotic thymocytes were removed by washing twice with phosphate-buffered saline (PBS) to break weak

cell-to-cell attachments, and the adhered cells were fixed with 4% paraformaldehyde. Phagocytosis and adhesion were evaluated by three different parameters: percent phagocytosis (the percentage of phagocytes ingesting at least one apoptotic thymocyte), percent adhesion (the percentage of phagocytes binding at least one apoptotic thymocyte), and adhesion index (percent adhesion $\times$ mean number of apoptotic thymocytes binding one phagocyte by using fluorescence microscopy).

Immunohistochemistry, Immunofluorescence, TUNEL Staining, and Histochemistry

For immunohistochemistry, liver specimens were fixed with 10% formalin in 0.1 mol/L phosphate buffer, pH 7.4, and embedded in paraffin. Paraffin sections were stained with avidin-biotin complex immunohistochemistry (Vectastain kit; Vector Laboratories, Burlingame, CA). For immunofluorescence staining, liver specimens were fixed with 4% paraformaldehyde and embedded in OCT compound. Sections were stained with antibodies as follows. The first antibodies were FABP7 (rabbit polyclonal antibody),¹⁷ F4/80 (A3-1; Bio-Rad, Hercules, CA), desmin (Y-20; Santa Cruz Biochemistry, Dallas, TX), and α -smooth muscle actin (1A4; Dako, Glostrup, Denmark). The secondary antibodies were goat anti-rabbit IgG-Alexa Fluor 488 and goat anti-rat IgG-Alexa Fluor 594 (Life Technologies). Nuclei were stained by DAPI (Life Technologies).

Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). Briefly, liver sections were incubated with 100 mmol/L sodium cacodylate (pH 7.0), 1 mmol/L CoCl₂, 50 μ L/mL gelatin, 10 nmol/L biotin-16-dUTP (Roche, Basel, Swiss), and 100 U/mL TdT (TaKaRa, Shiga, Japan) for 60 minutes at 37°C. The sections were covered with Alexa Fluor 488-labeled streptavidin (Life Technologies) in Tris PBS for 60 minutes at room temperature. The sections were simultaneously stained with anti-F4/80 immunofluorescent antibody for the detection of KCs.

For the detection of collagenous fibers, paraffin sections from the chronic fibrosis models were stained by picrosirius red staining (Direct red 80; Sigma-Aldrich) and Azan staining.

Morphometric Analysis

The specimens were examined microscopically, and histological parameters were quantified with the imaging software NIS-Elements D 3.1 (Nikon, Tokyo, Japan). For assessment of KC distribution, two different quadrates (0.29 mm $\times$ 0.23 mm) sets (central vein area, including the central vein at the center; portal vein area, including the portal vein at the center) were used for counting in the liver specimens. The F4/80⁺ cells were counted in 10 randomly selected central vein and portal vein areas in the sections from normal livers. The hepatocytes enwrapped by F4/80⁺ cells were counted in six randomly selected areas in the liver sections 1 day after CCl₄ injection. Each area included both the central vein and the portal vein. For assessment of KC

infiltration into necrotic region, the number of F4/80⁺ cells inside the necrotic or the peripheral regions was counted separately. The F4/80⁺ cells were counted in 10 randomly selected necrotic and peripheral areas in the sections from injured livers. For measuring the size of necrotic region, 94 microscopic fields were randomly selected in each liver specimen from three mice, and the percentages of the necrotic regions, poorly stained with hematoxylin, in each microscopic field were evaluated. For evaluation of fibrosis, the percentage of positive area for collagen staining was measured.

Flow Cytometry

Isolated KCs and FABP7/J774 cells were suspended in 100 μ L of PBS that contained 2% fetal bovine serum. FcR γ was blocked with CD16/CD32 blocking antibody (Becton Dickinson, Franklin Lakes, NJ) for 5 minutes, and cell surface antigens were stained for 30 minutes. KCs were stained with the Alexa Fluor 488-labeled F4/80 (Cl:A3-1; Bio-Rad) and phosphatidylethanolamine-labeled CD36 (MF3; Bio-Rad) or with appropriate isotype controls (Serotec, Oxford, UK). Flow cytometry was performed with a Cytomics FC500 (Beckman Coulter, Fullerton, CA), running CXP Cytometer software version 2.0 for data collection and analysis.

RT-PCR and qPCR

Total RNA was isolated from liver tissue and cultured cells and reverse transcribed with the use of the First Strand cDNA Synthesis Kit (Roche). The following primers were used in this study: FABP7, 5'-GGGTAAGACCCGAGTTCCTC-3' (forward) and 5'-GAGCTTGTCTCCATCCAACC-3' (reverse); and β -actin, 5'-CAGGAGATGGCCACTGCCGCA-3' (forward) and 5'-CTCCTTCTGCATCCTGCAGCA-3' (reverse). The PCR program was as follows: initial denaturation at 94°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for 3 minutes. Each cDNA sample was analyzed for gene expression by quantitative real-time PCR (qPCR) with the TaqMan Probe reagent on an Applied Biosystems Step One Plus thermal cycler according to the manufacturer's protocol. Gene expression levels were calculated after normalization to the standard housekeeping gene *GAPDH* by using the $\Delta\Delta$ CT method. The following TaqMan probes were used: Gapdh, Mm 03302249_g1; Fabp7, Mm 0044225_m1; Emr1 (F4/80), Mm 00802529_m1; Tnfa, Mm 00443258_m1; Ccl2 (MCP1), Mm 00441242_m1; Tgfb1, Mm 00441724_m1; Cd36, Mm 01135198_m1; Msr1, Mm 00446214; Tyro3, Mm 00444547_m1; Axl, Mm 00437221_m1; and MERTK, Mm 00434920_m1.

Western Blot Analysis

Total protein was extracted from tissues and cells by homogenization in lysis buffer [100 mmol/L Tris-HCl (pH

6.8), 2% sodium dodecyl sulfate, 20% glycerol]. Western blot analysis was used as described previously.¹⁷

ELISA

Serum after D-GalN/LPS administration and cell culture supernatants after 15 hours of LPS stimulation were collected. The concentration of tumor necrosis factor (TNF)- α and monocyte chemoattractant protein (MCP)-1 in serum and cell culture supernatant was measured by ELISA kits (R&D Systems, Minneapolis, MN).

To assess the oxidative stress in CCl₄-induced liver injury, liver was homogenized in lysis buffer [0.5% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100 in 0.1 mol/L PBS], and then the soluble material was separated by centrifugation at 20,000 $\times$ *g* for 20 minutes at 4°C. The concentration of nitrotyrosine in liver homogenate was detected by ELISA kit (OxisResearch; Percipio Bio Sciences, Burlingame, CA).

Measurement of Serum Liver Enzymes

Serum was harvested from mice by cardiac puncture at 1, 2, and 4 days after CCl₄ administration, and serum liver enzymes were analyzed by SRL Inc. (Tokyo, Japan).

Statistical Analysis

All data are expressed as means $\pm$ SEM. Statistical comparisons of means were made by Student's *t*-test. *P* < 0.05 was considered statistically significant.

Results

Localization of FABP7 in KCs

We compared the FABP7 expression in the macrophage-abundant tissues by Western blot analysis. FABP7 was highly expressed in liver; faintly expressed in peritoneal macrophages; and undetectable in lung, spleen, and bone marrow

Figure 1 Exclusive expression of FABP7 in KCs. **A:** Western blot analysis for FABP7 expression. **B:** Immunostaining of normal liver for FABP7 and F4/80. **C:** Measurement of the percentage of F4/80⁺ cells within FABP7⁺ cells and the percentage of FABP7⁺ cells within F4/80⁺ cells in WT liver 2 days after CCl₄ administration. Immunostaining of CCl₄-injured liver for FABP7, desmin, or α -smooth muscle actin (**D**) and FABP7 (**E**). **E:** Arrows indicate lymphocytes and the arrowhead indicates neutrophil. **F:** Quantification of F4/80⁺ cells in the WT (black bars) and *Fabp7*-KO (white bars) liver before and after Clo-lipo administration. **G:** Quantification of the percentage of FABP7⁺ cells within F4/80⁺ cells in WT liver before and after Clo-lipo administration. **H:** Quantification of the number of F4/80⁺ cells in PV and CV areas and *Emr1* expression in normal liver from WT and *Fabp7*-KO mice. Data are expressed as means $\pm$ SEM. *n* = 4 (**C**); *n* = 4 to 6 (**F** and **G**, each time course); *n* = 6 (**H**). Scale bars: 50 μ m (**B** and **D**); 10 μ m (**E**). Original magnifications: $\times 200$ (**B** and **D**); $\times 400$ (**E**). BM, bone marrow; CV, central vein; OB, olfactory bulb (positive control); ND, not detected; PEM, thioglycolate-induced peritoneal macrophage; PV, portal vein; α -SMA, α -smooth muscle actin.

(Figure 1A). Double immunostaining with F4/80 and FABP7 showed that the FABP7⁺ cells overlapped entirely with the F4/80⁺ cells in normal liver (Figure 1B). In the liver sections from three normal WT mice, FABP7 was expressed in 100% of the F4/80⁺ cells, indicating that FABP7 was expressed exclusively by KCs under normal conditions (data not shown). We further examined the FABP7 expression during CCl₄-induced inflammation. In the liver 2 days after CCl₄ injection, FABP7 was expressed in 98.2% ± 1.2% of the F4/80⁺ cells and F4/80 was expressed in 97.5% ± 0.3% of FABP7⁺ cells (Figure 1C). In the double immunostaining with F4/80, immunopositive cells for desmin and for α -smooth muscle actin, the markers of activated hepatic stellate cells and myofibroblasts, respectively, were both distinct from FABP7⁺ cells (Figure 1D). In addition, FABP7 localization was not detected in the neutrophils and lymphocytes that infiltrated into the damaged area (Figure 1E).

To examine the relationship between FABP7 expression and macrophage infiltration into liver, we examined the recovery of F4/80⁺ cells and FABP7 expression in those cells in the liver sections after KC elimination by Clo-lipo. F4/80⁺ cells disappeared from the liver 2 days after Clo-lipo injection. No significant difference was found in the recovery of KCs in *Fabp7*-KO liver compared with WT liver (Figure 1F). In the population of recovering WT KCs, within F4/80⁺ cells, the FABP7 expression was recovered to 82% at day 7 (Figure 1G) and fully recovered by day 14 (data not shown).

In *Fabp7*-KO mice, no significant difference was detected compared with WT mice in the KC distribution in both portal and central vein areas by immunohistochemistry and in the *Emr1* expression by qPCR (Figure 1H).

Increase in Serum Liver Enzymes in *Fabp7*-KO Mice

Because KCs play a key role in liver inflammation, we evaluated the effect of FABP7 deficiency in KCs by using the CCl₄-induced acute liver injury model, in which the central role of KCs has been well defined.⁶ We estimated hepatocyte damage by measuring serum liver enzymes and nitrotyrosine formed by oxidative stress.²⁶ In the sera on 1, 2, and 4 days after CCl₄ injection, levels of alanine aminotransferase, aspartate transaminase, and lactate dehydrogenase in *Fabp7*-KO mice were significantly higher than levels in WT mice on day 1 after CCl₄ injection, and they returned to normal by day 4 (Figure 2A). The amount of nitrotyrosine in the supernatant of the liver homogenate was not significantly different between WT and *Fabp7*-KO mice 1 day after CCl₄ injection (Figure 2B). In addition, serum liver enzymes were measured after D-GalN/LPS administration, which is known to induce acute hepatitis.²⁷ Serum liver enzymes in *Fabp7*-KO mice were significantly higher than enzymes in WT mice 1 and 4 hours after D-GalN/LPS administration (Figure 2C).

Altered Cytokine Production in *Fabp7*-KO Mice

KCs play important roles in liver injury by producing cytokines and chemokines. We measured the mRNA expression of *Tnfa*, *Ccl2*, and *Tgfb1*, all of which are mainly derived from activated KCs in the acute phase of CCl₄-induced liver injury. The decreased expression of these molecules was observed in *Fabp7*-KO liver 2 days after

Figure 2 Increase of serum liver enzymes in *Fabp7*-KO mice. Quantification of ALT, AST, and LDH in the serum (A) and nitrotyrosine in the liver (B) of WT (black bars) and *Fabp7*-KO (white bars) mice. C: Quantification of ALT, AST, and LDH in the serum of WT and *Fabp7*-KO mice before and after D-GalN/LPS administration. Data are expressed as means ± SEM. *n* = 6 (A–C, each time course). **P* < 0.05, ***P* < 0.01. ALT, alanine aminotransferase; AST, aspartate transaminase; LDH, lactate dehydrogenase.

CCl₄ injection compared with WT liver (Figure 3A). Significant decrease of TNF- α and MCP-1 was also detected in the sera of *Fabp7*-KO mice 1 hour after D-GalN/LPS administration (Figure 3B). Furthermore, to analyze the cytokine expression levels of KCs, expression of *Tnfa* and *Ccl2* in isolated KCs after LPS stimulation was measured by qPCR. *Tnfa* expression level was significantly decreased in *Fabp7*-KO KCs compared with WT KCs (Figure 3C). A clonal macrophage cell line stably expressing FABP7 (FABP7/J774) (Figure 3D) consistently showed significant increase of TNF- α production after LPS stimulation compared with control (Figure 3E).

Altered Distribution of *Fabp7*-KO KCs in Acute Injured Liver

Next, to determine the role of FABP7 in KCs during the course of liver injury, we examined histological features of KCs in the CCl₄-induced acute liver injury model. In the

normal liver, no significant difference was found in the number of KCs between WT and *Fabp7*-KO mice (Figures 1G and 4A). In the liver 24 hours after CCl₄ injection, WT KCs were frequently observed to enwrap damaged hepatocytes (Figure 4, A and B), consistent with findings from previous studies that hepatocytes undergo apoptosis by 24 hours after CCl₄ injection and numerous activated KCs incorporate the dying hepatocyte.^{20,28} However, in *Fabp7*-KO liver, KCs were rarely observed to enwrap damaged and/or TUNEL⁺ dying hepatocytes (Figure 4, B–D). Furthermore, in the *Fabp7*-KO liver 2 days after CCl₄ injection, it was surprisingly found that the number of KCs inside the necrotic region, poorly stained with hematoxylin, and that of the peripheral area was significantly decreased compared with WT mice (Figure 4, A and E), whereas the extent of necrotic area did not show any difference between WT and *Fabp7*-KO mice (Figure 4F). As for the survival of *Fabp7*-KO KCs, we examined by using TUNEL and F4/80 double staining whether apoptotic KCs were increased in *Fabp7*-KO mice

Figure 3 Altered cytokine production in *Fabp7*-KO mice. **A:** The expression of *Tnfa*, *Ccl2*, and *Tgfb1* in the liver of WT (black bars) and *Fabp7*-KO (white bars) mice was analyzed by qPCR. **B:** Quantification of TNF- α and MCP-1 levels in the serum from WT and *Fabp7*-KO mice after D-GalN/LPS administration. **C:** The expression of *Tnfa* and *Ccl2* in isolated KCs from WT and *Fabp7*-KO mice was analyzed by qPCR. **D:** RT-PCR and Western blot analysis confirm the increase in gene and protein expression for FABP7 in FABP7/J774. **E:** Quantification of TNF- α and MCP-1 levels produced by FABP7/J774 with (dark gray bars) or without (light gray bars) LPS challenges. Data are expressed as means $\pm$ SEM. $n = 3$ independent experiments; $n = 6$ mice (**A** and **C**, each time course); $n = 5$ mice (**B**, each time course). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Conc., concentration.

during the process of acute liver injury, and no significant difference was detected between WT KCs and *Fabp7*-KO KCs (data not shown). In addition, the degree of liver injury was evaluated histopathologically from 1 to 4 days after CCl₄ administration (Supplemental Figure S1), and the extent of

necrotic area was quantified. As a result, no significant difference was found in the extent of damaged area between WT and *Fabp7*-KO mice at each time point examined (Figure 4F). Furthermore, we examined the liver histopathology at 1 and 4 hours after D-GalN/LPS treatment (Supplemental Figure S2);

Figure 4 Altered distribution of *Fabp7*-KO KCs in acute injured liver. **A:** Immunostaining of liver sections for F4/80 before and after CCl₄ administration. **B:** Immunostaining of liver sections for F4/80 1 day after CCl₄ administration. **C:** TUNEL and F4/80 staining of liver sections 1 day after CCl₄ administration. **Arrows** indicate TUNEL⁺ cells. **D:** Quantification of hepatocytes wrapped by F4/80⁺ cells 1 day after CCl₄ administration. **E:** Quantification of F4/80⁺ cells in necrotic and peripheral regions 2 days after CCl₄ administration. **F:** Quantification of percentage of necrotic area within total area in WT (black bars) and *Fabp7*-KO (white bars) liver. Data are expressed as means ± SEM. *n* = 5 (**D** and **E**, each genotype); *n* = 6 (**F**, each time course). Scale bar: 100 μm (**A**); 50 μm (**B–D**). Original magnifications: ×40 (**A**); ×200 (**B–D**). ***P* < 0.01, ****P* < 0.001. C, central vein; P, portal vein.

no significant difference was found in the extent of necrotic area between WT and *Fabp7*-KO mice.

Impaired Phagocytosis of Apoptotic Cells in *Fabp7*-KO KCs

To investigate the role of FABP7 in the phagocytosis against damaged cells, CMFDA-labeled apoptotic thymocytes were administered via the portal vein, and the number of phagocytosed apoptotic thymocytes surrounded by KC cytoplasm was counted in the liver sections (Figure 5A). WT KCs ingested a significantly larger number of apoptotic thymocytes than did *Fabp7*-KO KCs (Figure 5A). In contrast, no significant difference was detected in phagocytosis of necrotic thymocytes between WT and *Fabp7*-KO KCs (Figure 5A). Therefore, we compared the mRNA expression of phagocytic receptors *Msr1*, *Cd36*, and TAM receptors (*Tyro-3*, *Axl*, and *Mertk*), which may be associated with apoptotic cell clearance by macrophages,^{29,30} between WT and *Fabp7*-KO KCs. Expression of *Cd36* mRNA was found to decrease in the *Fabp7*-KO KC-enriched cell fraction compared with WT by qPCR analysis (Figure 5B), whereas *Emr1* expression did not show any difference. Flow cytometric analysis also showed that the proportion of CD36 highly positive cells within the

F4/80⁺ cell population was decreased in *Fabp7*-KO liver compared with WT liver (Figure 5C). Consistently, FABP7/J774 cells showed significant increase in *Cd36* mRNA expression (Figure 5D) and in phagocytosis (percent phagocytosis) and cell-to-cell adhesion (percent adhesion and adhesion index) after incubation with CMFDA-labeled apoptotic thymocytes (Figure 5E).

Altered Fibrogenic Response in *Fabp7*-KO Liver

On the basis of the results that FABP7 was involved in the cytokine production and phagocytosis in KCs, we finally examined the effect of FABP7 deficiency on liver fibrosis in which such KC functions have been indicated as important factors for its pathology.⁶ After continuous administration of CCl₄, the area of collagenous tissue by Sirius Red staining showed a significant decrease of collagenous fibers in the *Fabp7*-KO mice compared with WT mice (Figure 6A), which was also confirmed by Azan staining (Figure 6B).

Discussion

In the present study, FABP7 was exclusively expressed by liver KCs in normal and inflammatory conditions. With the

Figure 5 Impaired phagocytosis of apoptotic cells in *Fabp7*-KO KCs. **A:** Quantification of the number of CMFDA-labeled ATCs or NTCs surrounded by F4/80⁺ cells. **Arrows** indicate CMFDA-labeled cells. **B:** Quantification of the mRNA level of phagocytic receptors analyzed in KC-enriched fraction from WT (black bars) and *Fabp7*-KO (white bars). **C:** Flow cytometric analysis of CD36 expression on F4/80⁺ cells. **D:** Quantification of mRNA level of phagocytic receptors in FABP7/J774 cells. **E:** Quantification of phagocytosis against ATCs (percentage of phagocytosis) and adhesion to ATCs (percentage of adhesion and adhesion index) of FABP7/J774. Data are expressed as means ± SEM. *n* = 6 ATCs (**A**, of each genotype); *n* = 4 NTCs (**A**, each genotype); *n* = 6 mice (**B**, both WT and *Fabp7*-KO); *n* = 5 independent experiments (**C**); *n* = 3 (**D**, each group); *n* = 6 (**E**, each group). **P* < 0.05, ***P* < 0.01. Scale bar = 50 μm (**A**). Original magnification: ×400 (**A**). ATC, apoptotic thymocyte; FITC, fluorescein isothiocyanate; NTC, necrotic thymocyte.

use of *Fabp7*-KO mice, FABP7 was found to be involved in cytokine and chemokine expressions in KCs during acute liver injury. Moreover, FABP7-deficient KCs were impaired in the clearance of apoptotic cells, likely because of decreased expression of phagocytic receptor CD36, and in the migration into the injured liver. We further suggested the association of FABP7 in KCs with liver fibrogenic response. These results strongly suggested that FABP7 is deeply involved in the acute and chronic liver injury process through its control of gene expression in KCs.

FABPs, as transporters of hydrophobic FAs in cytoplasm, may participate in the regulation of cellular activities through modulation of membrane constituents or of FA-dependent signal transduction. In the immune tissues or cells, FABPs have so far been suggested to play roles in the regulation of cytokine production. FABP4 promoted LPS-induced TNF- α production in macrophages,⁹ and the inhibition of FABP4 attenuated the atherosclerosis lesions through the suppression of inflammatory cytokine production.^{10,11} The FABP5 deficiency suppressed the expression of macrophage inflammatory genes.¹⁶ FABP7 expressed by fibroblastic reticular cells in lymph nodes is associated with lymphocyte maturation.³¹ In this study, the production of inflammatory cytokines such as TNF- α was decreased in *Fabp7*-KO mice as observed in two liver injury models (CCl₄ or D-GalN/LPS administration). Expression of transforming growth factor- β , one of the important fibrogenic factors produced by liver stromal cells,³² was also down-regulated in the *Fabp7*-KO mice after acute liver injury. The decrease of transforming growth factor- β , which promotes the differentiation the hepatic stellate cells into myofibroblasts, may largely contribute to the decrease of fibrosis in *Fabp7*-KO mice in experiments of chronic liver injury. The mechanism by which FABP7 regulates the cytokine gene transcription in KCs should be examined in the future study.

It was shown that KCs infiltrate and phagocytose apoptotic hepatocytes during 6 to 48 hours after CCl₄ administration²⁰ and that the clearance of liver enzymes by KCs is closely associated with their serum levels after liver injury.^{33,34} We demonstrated that there was a decrease in the hepatocyte-enwrapping KCs and in the infiltration of KCs into the necrotic region of *Fabp7*-KO liver. However, the serum liver enzymes were higher in *Fabp7*-KO mice during acute liver injury than in WT mice, despite that no significant difference in the extent of necrotic area and the amount of the oxidative stress were detected in *Fabp7*-KO liver. It is likely that FABP7-deficient KCs were impaired in their clearance of liver enzymes leaked from dying hepatocytes. Consistently, we confirmed a significant decrease of phagocytosis against apoptotic cells and infiltration into the necrotic area in *Fabp7*-KO KCs. As for the impairment of KC infiltration, decreased expression of MCP-1, which is one of the macrophage chemoattractants in the liver of *Fabp7*-KO mice, should be noted as a possible mechanism.

The scavenger receptor family expressed on the surface of macrophages was shown to recognize the apoptotic cells and to play a pivotal role in their phagocytosis.²⁸ It is well documented with the use of several animal models that CD36, which is one of the type B scavenger receptors, is important in the clearance of apoptotic cells by macrophages.^{35–37} In the present study, expression of CD36 was decreased in *Fabp7*-KO KCs. FABP7 overexpression in the macrophage cell line (FABP7/J774) drastically increased Cd36 mRNA expression, and the phagocytic activity against apoptotic cells was also increased. Therefore, the impairment of apoptotic cell clearance found in FABP7-deficient KCs is partly explained by the decreased CD36 expression, although the involvement of other phagocytic receptors in the present phenotype cannot be fully excluded. The mechanism underlying the CD36 expression control by FABP7 in KCs remains to be elucidated.

Figure 6 Altered fibrogenic response in *Fabp7*-KO liver. Liver sections were stained by Sirius red (**A**) and Azan (**B**) staining after continuous CCl₄ administration in WT and *Fabp7*-KO mice. The percentage of the positive area within total area was measured. Data are expressed as means $\pm$ SEM. $n = 5$ WT mice (**A** and **B**); $n = 4$ *Fabp7*-KO mice (**A** and **B**). * $P < 0.05$, ** $P < 0.01$. Scale bar = 100 μ m (**A** and **B**). Original magnifications: $\times 100$ (**A** and **B**).

A close relationship has been found between macrophage function and its cellular FA homeostasis. The changes in lipid uptake can modulate their phagocytic activity³⁸ and cytokine production.³⁹ FABP family members have been suggested to promote or inhibit the targeting of their ligands to the functional domains within the cells.⁴⁰ In this study, *Fabp7*-KO mice showed decreased fibrogenic responses. Considering the evidence that FABP7 binds to n-3 polyunsaturated FAs (PUFAs)⁴¹ and that n-3 PUFAs have beneficial effects on liver damage, including fibrosis,⁴² FABP7 likely inhibited the effects of n-3 PUFA action by restricting the availability of n-3 PUFAs in KCs. In this context, the finding of Makowski et al¹² should be noted that FABP4 blocks the action of bioactive lipid mediators, including Fas, in atherosclerotic macrophages.

KCs are constantly exposed to blood similar to atherosclerotic macrophages, thereby rendering them distinct from other tissue macrophages in response to various cellular environments, including gut-derived endotoxin or serum components in blood such as lipids. Possible involvement of dietary n-3 PUFAs and KCs has so far been suggested in the pathology of hepatitis⁴³ and nonalcoholic fatty liver disease.⁴⁴ In this context, analysis of FABP7 function in KCs by using other liver injury models such as the steatosis model should be done. Further investigation of FABP7-dependent regulation of KCs may provide deeper understanding of PUFA effects on liver diseases such as nonalcoholic fatty liver disease and a new approach for its treatment and may contribute to the treatment outcomes of liver diseases.

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Supplemental Data

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