

TITLE PAGE**Title:**

Hepatic vagus nerve regulates Kupffer cell activation via $\alpha 7$ nicotinic acetylcholine receptor in nonalcoholic steatohepatitis

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Abstract

Background: Nonalcoholic fatty liver disease ranges from simple steatosis to nonalcoholic steatohepatitis (NASH). Kupffer cells play a central role in promoting hepatic inflammation, which leads to the development of NASH. We investigated the anti-inflammatory effect of the hepatic vagus-mediated stimulation of $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR) on Kupffer cells in NASH pathogenesis.

Methods: Wild-type (WT) mice undergoing hepatic vagotomy (HV) were fed a methionine- and choline-deficient (MCD) diet for 1 week. $\alpha 7$ nAChR knockout ($\alpha 7$ KO) chimeric mice were generated by transplanting $\alpha 7$ KO bone marrow cells into irradiated and Kupffer cell-deleted WT recipients. Kupffer cells were isolated from WT mice and treated with $\alpha 7$ nAChR agonist under stimulation by lipopolysaccharide and/or palmitic acid.

Results: HV aggravated MCD diet-induced NASH in both steatosis and inflammation. The hepatic inflammatory response, including the upregulation of tumor necrosis factor alpha (TNF α), interleukin (IL)-12, and monocyte chemo-attractant protein-1 (MCP-1), was accelerated in HV mice, accompanied by the downregulation of PPAR α pathway genes. Kupffer cells were highly activated via the phosphorylation and nuclear translocation of nuclear factor-kappa B (NF- κ B) in MCD diet-fed HV mice. The $\alpha 7$ nAChR agonist suppressed the inflammatory response of primary Kupffer cells induced by lipopolysaccharide and palmitic acid by attenuating the NF- κ B cascade. $\alpha 7$ KO chimeric mice fed an MCD diet for 1 week developed advanced NASH with highly activated Kupffer cells. The hepatic expression of TNF α , IL-12, and MCP-1 was upregulated in $\alpha 7$ KO chimeric mice, accompanied by abnormal lipid metabolism.

Conclusions: Hepatic vagus activity regulates the inflammatory response of Kupffer cells via $\alpha 7$ nAChR in NASH development.

Keywords

NASH, NAFLD, fatty liver disease, $\alpha 7$ nAChR, vagotomy

Abbreviations

NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; TLR, Toll-like receptor; IL, interleukin; TNF α , tumor necrosis factor alpha; MCP-1, monocyte chemo-attractant protein-1; $\alpha 7$ nAChR, $\alpha 7$ nicotinic acetylcholine receptor; STAT3, signal transducer and activator of transcription 3; NF- κ B, nuclear factor-kappa B; MCD, methionine- and choline-deficient; WT, Wild type; $\alpha 7$ KO, $\alpha 7$ nAChR knockout; HV, hepatic vagotomy; CT, control; BM, bone marrow; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; LPS, lipopolysaccharide; PPAR α , peroxisome proliferator activated receptor alpha; AOX, acyl-CoA oxidase; L-FABP, liver-type fatty acid binding protein; SREBF-1c, sterol regulatory element binding factor-1c; FAS, fatty acid synthase; G6pc, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase 1; GFP, green fluorescent protein

Introduction

Nonalcoholic fatty liver disease (NAFLD), a hepatic consequence of metabolic syndrome, has become a common cause of chronic liver diseases worldwide as a result of an epidemic rise in obesity[1-3]. The disease spectrum of NAFLD ranges from simple steatosis to nonalcoholic steatohepatitis (NASH), which is characterized by steatosis with progressive inflammation and fibrosis, and eventually leads to liver cirrhosis and the development of hepatocellular carcinoma[4, 5]. The so-called “two-hit” model[6], which has been proposed as a potential mechanism of NASH pathogenesis for more than a decade, suggested that the first hit of steatosis and the second hit, which includes oxidative stress, innate immunity, adipocytokines, or gut-derived endotoxins, are required to progress from simple steatosis to NASH. On the other hand, recent studies have indicated that inflammation resulting from multiple parallel hits also precedes steatosis in NASH, as inflammatory events may lead to subsequent steatosis[7-9]. Elucidating the mechanism that regulates the initiation of the inflammatory response is essential for preventive and therapeutic approaches to treat patients with NASH.

The activation of the hepatic innate immune system is associated with the inflammatory response in NASH pathogenesis[7, 10]. Kupffer cells, which are liver-resident macrophages, play a central role in promoting hepatic inflammation by innate immune responses via Toll-like receptor (TLR) signaling[11-15]. Activated Kupffer cells induce liver injury by producing pro-inflammatory cytokines, e.g., interleukin (IL)-1 β , IL-12, tumor necrosis factor alpha (TNF α), and monocyte chemo-attractant protein-1 (MCP-1)[15-19]. Regulating Kupffer cell activity might be a key approach for suppressing NASH progression.

Currently, efferent vagus nerve-mediated cholinergic signaling is known to play an important role in regulating innate immune responses and inflammation, and this signaling has been defined as the cholinergic anti-inflammatory pathway, the efferent arm of inflammatory reflex[20-22]. The $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR), which is expressed in immune cells, such as macrophages and monocytes, is involved in mediating the inhibition of pro-inflammatory cytokine production via intracellular signaling pathways[23]. The pathways downstream of $\alpha 7$ nAChR upon cholinergic stimulation include the activation of Janus kinase 2 and the subsequent phosphorylation of signal transducer and activator of transcription 3 (STAT3), as well as the suppression of nuclear factor-kappa B (NF- κ B) nuclear translocation[24-27].

Several studies have reported that vagus nerve-mediated cholinergic activation has protective effects on obesity-related inflammation and other metabolic complications[28-30]. NASH, a hepatic result of chronic inflammation based on metabolic disorder, is likely to be affected by vagus nerve activity; however, the specific effect of hepatic vagus signaling on the modulation of inflammatory responses in NASH pathogenesis remains unclear.

In the present study, we sought to elucidate the role of the hepatic vagus nerve in the regulation of Kupffer cell-mediated inflammatory responses via $\alpha 7$ nAChR stimulation in the setting of NASH. We specifically focused on the early developmental phase by using a methionine- and choline-deficient (MCD) dietary experimental model.

Methods

Animal handling, surgical procedures, and diets.

Wild-type (WT) C57BL/6 mice, C57BL/6-Tg (CAG-EGFP) mice, and $\alpha 7$ nAChR^{-/-} ($\alpha 7$ KO) mice on the C57BL/6 background were obtained from Japan SLC (Shizuoka, Japan), CLEA Japan (Tokyo, Japan), and the Jackson Laboratory (Bar Harbor, ME). The mice were housed under a 12-hour light-dark cycle with free access to food and water. Animals received humane care, and all procedures were performed according to protocols approved by the animal research committee of Kyoto University.

Male WT mice (12 weeks old) were subjected to hepatic vagus denervation. A selective hepatic vagotomy (HV) was performed as previously described[31]. Briefly, a laparotomy was made at the midline, the fibrous attachment of the gastrohepatic ligament was dissected, and the stomach was gently pulled down. The ventral subdiaphragmatic vagus trunk was exposed along the descending esophagus. The common hepatic branch of the vagus nerve, which leaves the trunk and travels towards the hepatic portal region, was completely transected with ligation of each margin. Sham-operated mice underwent a laparotomy without the transection of the common hepatic vagus branch.

One week after surgery, the mice were fed a control (CT) or MCD diet (Research Diets #A07080109, 35 kcal% fat) for 1 week or 8 weeks. The mice were fasted for 12 hours before sacrifice, and samples were collected.

Generating chimeric mice.

To generate chimeric mice, WT recipients (male, 6 weeks old) were intraperitoneally injected with 200 μ L of liposomal clodronate (FormuMax Scientific, Sunnyvale, CA) to deplete Kupffer cells 24 hours before bone marrow (BM) transplantation. BM cells (2×10^7 cells) obtained from WT, α 7KO, or C57BL/6-Tg(CAG-EGFP) mice were transplanted by intravenous injection into lethally irradiated recipient mice (10 Gy). MCD diet feeding started 8 weeks after BM transplantation.

Histological examination.

Formalin-fixed specimens were embedded in paraffin, sectioned at a thickness of 4 μ m, and mounted on silanized glass slides. The paraffin-embedded sections were stained with hematoxylin and eosin, toluidine blue (Wako, Osaka, Japan), or Sirius Red (Wako). For Nile Red (Lonza, Basel, Switzerland) staining, the specimens were fixed in 4% paraformaldehyde, embedded in optimal cutting temperature compound (Sakura, Tokyo, Japan), and sectioned at a thickness of 4 μ m. For immunohistochemical staining, the specimens fixed in 4% paraformaldehyde and embedded in optimal cutting temperature compound were also sectioned at a thickness of 4 μ m. Antigen retrieval was performed by incubation in citric acid buffer at

90 °C for 20 minutes. After blocking, the sections were incubated with a primary antibody against F4/80 (Cat. No. 14-4801, eBioscience, San Diego, CA), phospho-NF- κ B p65 (Cat. No. ab106129, Abcam, Cambridge, MA), or α 7nAChR (Cat. No. ANC-007, Alomone Labs, Jerusalem, Israel) at 1:200 dilution overnight at 4 °C, followed by incubation with a corresponding secondary antibody at room temperature for 1 hour. Positively stained areas or cell counts were measured in 10 fields (at 100 $\times$ magnification) per specimen using the National Institutes of Health image software Image J[32]. The NAFLD activity score was determined by a pathologist (T.S.) according to the published criteria[33] in a blinded manner.

Quantitative real-time polymerase chain reaction.

Total RNA was extracted from liver tissue or cell samples using TRIzol reagent (Life Technologies, Carlsbad, CA) and the RNeasy Mini Kit with on-column DNA digestion (Qiagen, Valencia, CA). The extracted RNA was reverse transcribed to complementary DNA using Omniscript RT Kit (Qiagen). The quantitative real-time polymerase chain reaction was performed with a KAPA SYBR Fast qPCR Kit (NIPPON Genetics, Tokyo, Japan) using a StepOnePlus (Applied Biosystems, Foster City, CA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and 18S rRNA were used as internal controls. Data normalized to 18S are shown. The primer sequences are summarized in **Table S1**.

Measurement of tissue triglyceride and acetylcholine content.

Hepatic triglycerides were extracted using the Folch method[34], and their levels were measured using Triglyceride E (Wako). Acetylcholine was isolated from the liver or spleen, and its concentration was measured using a Fluorimetric Acetylcholine Assay Kit (AAT Bioquest, Sunnyvale, CA). The resultant values were normalized to the tissue protein concentrations.

Western blot.

Western blot analysis was conducted as previously described[35]. Blots were incubated with primary antibody against $\alpha 7$ nAChR (Cat. No. ab23832, Abcam), phospho-p65 (Cat. No. ab106129, Abcam), p65 (Cat. No. ab7970, Abcam), phospho-STAT3 (Cat. No. ab30647, Abcam), STAT3 (Cat. No. ab68153, Abcam), or GAPDH (Cat. No. sc-25778, Santa Cruz Biotechnology, Dallas, TX) at 1:1000 dilutions. The band intensities were quantified using Image J.

Serum biochemistry.

The levels of aspartate aminotransferase, alanine aminotransferase, triglyceride, total cholesterol, and glucose were measured in serum samples obtained after 12 hours of fasting following CT or MCD diet feeding.

Cell culture and treatment.

Kupffer cells were isolated via the two-step collagenase-pronase perfusion of mouse livers followed by 15% Nycodenz (Nycomed Pharm, Oslo, Norway) two-layer discontinuous density gradient centrifugation as previously described[14]. The cell fraction was purified using magnetic antibody cell sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) with CD11b-conjugated microbeads (Miltenyi Biotec). Isolated cells were cultured for 16 hours in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics prior to stimulation by reagents. Lipopolysaccharide (LPS) (1 ng/ml) (Sigma-Aldrich, St. Louis, MO) and/or palmitic acid (400 μ M) (Sigma-Aldrich) were added to the culture medium, and the cells were stimulated for 6 hours. The cells were pre-treated with vehicle or α 7nAChR agonist (PNU 282987; Abcam) 30 minutes prior to the administration of LPS and/or palmitic acid.

Statistical analysis.

Data are presented as the mean $\pm$ SD. Differences between groups were compared using Student's *t* test or a one-way analysis of variance followed by the Tukey-Kramer test. *P* <0.05 was considered significant. The JMP pro 11 (SAS institute, Cary, NC) software was used for all statistical analyses.

Results

Hepatic vagus nerve denervation aggravates MCD diet-induced NASH.

WT mice were subjected to HV or a sham operation and were fed an MCD diet for 1 week. In the HV procedure, the common hepatic branch of the vagus nerve was transected, which was histologically confirmed in the resected tissue specimen (**Fig. S1a**). The hepatic acetylcholine content was significantly attenuated after HV, whereas the acetylcholine levels in the spleen were unaffected (**Fig. S1b**). Mice fed an MCD diet for 1 week developed early-stage steatohepatitis with mild inflammation (**Fig. 1a**). In this short time period, the attenuation of hepatic acetylcholine levels after HV was maintained in both MCD diet-fed mice and CT diet-fed mice (**Fig. S1c**). HV enhanced MCD diet-induced steatohepatitis, as demonstrated by aggravated histological findings of both steatosis and lobular inflammation (**Fig. 1a**), whereas it did not affect the normal livers of CT diet-fed mice. In the MCD diet-fed mice, hepatic lipid accumulation was significantly increased by HV, as indicated by the lipid staining of liver tissue and quantification of hepatic triglyceride content (**Fig. 1b, c**). The increased transaminase levels suggested that HV promoted NASH-induced liver damage (**Fig. 1d**). Weight loss and low total serum cholesterol levels were observed in MCD diet-fed mice, and these changes are features of the MCD dietary model due to the reduction of hepatic lipid secretion. HV did not affect body weight, adipose tissue weight, or the serum lipid levels (**Table 1**).

Hepatic vagus denervation promotes NASH-associated inflammatory responses and abnormal lipid metabolism.

Based on the aggravation of early-stage NASH by HV, we assessed the regulatory effect of the hepatic vagus nerve on inflammatory responses and lipid metabolism. The hepatic expression of pro-inflammatory cytokines associated with NASH pathogenesis, including TNF α and IL-12,

was significantly increased in MCD diet-fed mice that had undergone a HV. MCP-1, a key chemokine responsible for macrophage infiltration, was also upregulated. The levels of IL-1 β and IL-6 expression were not affected by HV (**Fig. 2a**).

Among lipid metabolism regulators, the expression of peroxisome proliferator activated receptor alpha (PPAR α)-pathway genes significantly decreased by HV in MCD diet-fed mice, including PPAR α , its downstream targets acyl-CoA oxidase (AOX) and liver-type fatty acid binding protein (L-FABP), which are key regulators of β -oxidation. The expression of genes involved in fatty acid synthesis, including sterol regulatory element binding factor-1c (SREBF-1c) and fatty acid synthase (FAS), were unaffected (**Fig. 2b**). Regarding glucose metabolism, the hepatic expression of gluconeogenic genes, including glucose-6-phosphatase (G6pc) and phosphoenolpyruvate carboxykinase 1 (PEPCK), was decreased in the MCD diet-fed mice, and it was significantly downregulated by HV, whereas the serum glucose level was not affected (**Fig. S2**). Conversely, the hepatic expression of G6pc was increased in the HV mice fed a control diet, indicating that attenuating the vagus-mediated suppressive effect on gluconeogenesis may upregulate hepatic gluconeogenetic gene expression in the normal liver [36].

Because liver fibrosis is a consequence of advanced NASH, we further investigated the influence of HV on NASH-induced liver fibrosis using mice fed an MCD diet for 8 weeks. These mice developed mild liver fibrosis but did not form septa or exhibit bridging (**Fig. S3a**). HV did not significantly affect the hepatic expression of genes involved in inflammation, fibrosis, or lipid

metabolism (**Fig. S3b, c, and d**). Notably, the chronic choline-deficient status of MCD dietary intake resulted in a slow decrease in the hepatic acetylcholine levels, which led to the attenuation of the HV effect in mice fed an MCD diet for 8 weeks, whereas this effect was maintained in mice fed a CT diet for the same period (**Fig. S4**). Under conditions of choline deficiency-based NASH pathogenesis, vagus cholinergic signaling thus appears to have a greater impact during the early phase before the attenuation of hepatic choline levels.

Hepatic vagus denervation promotes Kupffer cell activation in NASH.

To assess the vagus-mediated anti-inflammatory effect during NASH development, we focused on the behavior of Kupffer cells, which play a key role in hepatic inflammatory responses. MCD dietary intake induced hepatic F4/80⁺ cell activation, which was morphologically characterized by cytoplasm enlargement and process expansion, and these changes were generally accompanied by NF- κ B p65 phosphorylation and nuclear translocation (**Fig. 3a**). In the 1-week MCD diet model, HV promoted significant Kupffer cell activation, as represented by a larger area of F4/80⁺ cell infiltration as well as a higher proportion of phosphorylated p65 in these cells (**Fig. 3b**). Interestingly, the ratio of F4/80⁺ cell counts to total liver cells was unaffected by HV, and this ratio remained almost the same as that in the normal livers of CT diet-fed mice. This finding suggested that liver-resident macrophages, rather than those that secondarily migrate to the liver, were predominantly activated and modulated by hepatic vagus signaling during the early stages of NASH.

$\alpha 7$ nAChR agonist suppresses the inflammatory response of Kupffer cells.

We assessed the involvement of the vagus-mediated $\alpha 7$ nAChR pathway in the regulation of Kupffer cell responses using primary Kupffer cell cultures. To mimic NASH pathogenesis *in vitro*, Kupffer cells were stimulated with palmitic acid, a lipotoxic free fatty acid, and LPS, a TLR agonist. Kupffer cells were activated, as evidenced by increases in pro-inflammatory cytokine expression; specifically, palmitic acid and LPS stimulation synergistically upregulated TNF α and MCP-1 (**Fig. 4a**). The administration of an $\alpha 7$ nAChR agonist effectively downregulated cytokine expression in Kupffer cells activated by palmitic acid and/or LPS (**Fig. 4a**). The $\alpha 7$ nAChR agonist promoted STAT3 phosphorylation and suppressed p65 phosphorylation under TLR pathway stimulation by LPS (**Fig. 4b**). These modifications to STAT3 and NF- κ B were involved downstream of the $\alpha 7$ nAChR cascade and eventually led to the inhibition of pro-inflammatory cytokine transcription.

$\alpha 7$ nAChR deficiency in Kupffer cells aggravates MCD diet-induced NASH.

To assess the impact of cholinergic anti-inflammatory regulation via $\alpha 7$ nAChR on Kupffer cells *in vivo*, we generated chimeric mice by transferring $\alpha 7$ nAChR-deficient or WT BM cells into Kupffer cell-depleted WT recipients (**Fig. 5a**). Eight weeks after BM transplantation, mice were fed an MCD diet for 1 week. The reconstitution of Kupffer cells was assessed by immunohistochemical double staining for $\alpha 7$ nAChR and F4/80; the absence of $\alpha 7$ nAChR expression on Kupffer cells was verified in mice transplanted with $\alpha 7$ nAChR-deficient BM cells ($\alpha 7$ KO chimeric mice), whereas Kupffer cells in the mice transplanted with WT BM cells (WT

chimeric mice) maintained $\alpha 7$ nAChR expression (**Fig. 5b**). We further analyzed the efficacy of donor-derived Kupffer cell-reconstitution by depleting Kupffer cells and transplanting mice with green fluorescent protein-positive (GFP⁺) BM cells to generate chimeric mice (**Fig. S5a**). The F4/80⁺ cells highly corresponded with GFP⁺ cells (GFP⁺ per F4/80⁺ cell count, $98.4 \pm 0.5\%$; $n = 3$), indicating that Kupffer cells were precisely reconstituted with donor-derived cells. Conversely, the majority of intrahepatic GFP⁺ BM-derived cells acquired not only F4/80 expression (F4/80⁺ per GFP⁺ cell count, $97.8 \pm 0.2\%$; $n = 3$) but also the characteristic morphology and distribution of resident Kupffer cells (**Fig. S5b**).

The $\alpha 7$ KO chimeric mice, in which Kupffer cells were reconstituted with an $\alpha 7$ nAChR-deficient phenotype, developed more advanced steatohepatitis than the WT chimeric mice (**Fig. 5c**). Pathological steatosis, as well as lobular inflammation, was exacerbated in $\alpha 7$ KO chimeric mice, as also indicated by lipid staining and the hepatic triglyceride content (**Fig. 5d, e**). The $\alpha 7$ KO chimeric mice exhibited severe liver damage with significantly higher serum transaminase levels (**Fig. 5f**).

$\alpha 7$ nAChR-deficiency in Kupffer cells promotes NASH-associated inflammatory responses and abnormal lipid metabolism.

$\alpha 7$ nAChR deficiency in Kupffer cells worsened the outcome of steatohepatitis. Thus, we assessed the activity of $\alpha 7$ nAChR-deficient Kupffer cells in the liver tissue and its effect on the hepatic lipid metabolism. The Kupffer cells in $\alpha 7$ KO chimeric mice were highly activated, as evidenced by a larger F4/80⁺ area and a higher proportion of p65 phosphorylation. The ratio of

F4/80⁺ cell counts to total liver cells did not change (**Fig. 6a**). The hepatic expression levels of the pro-inflammatory cytokines TNF α , IL-12, and MCP-1 were significantly upregulated in $\alpha 7$ KO chimeric mice (**Fig. 6b**), consistent with the findings in the HV model. Regarding the modification of lipid metabolism, the expression of AOX, a regulator of the PPAR α pathway, was downregulated. The expression levels of SREBF-1c and FAS, which are involved in fatty acids synthesis, were significantly increased (**Fig. 6c**). With respect to glucose metabolism, the hepatic expression of G6pc was significantly downregulated, whereas the serum glucose level remained unchanged (**Fig. S6**), suggesting that $\alpha 7$ nAChR-deficiency in Kupffer cells may be associated with the suppression of the gluconeogenic gene expression.

Discussion

The vagus nerve is well known to play an essential role in maintaining metabolic homeostasis[31, 36-38]; it has also been reported to regulate innate immune responses and inflammation via the cholinergic stimulation of the $\alpha 7$ nAChR. Decreased vagus nerve activity was suggested to contribute to the obesity and metabolic syndrome-related complications resulting from chronic inflammation[30]. A recent study has shown that central insulin action suppresses hepatic vagal activity, and the $\alpha 7$ nAChR-mediated control of Kupffer cells is involved in the regulation of hepatic glucose production[39]. Given the vagus-mediated cross talk between metabolism and immunity, the liver-specific activity of the vagus nerve is likely to affect the pathogenesis of NASH, a hepatic result of metabolic disorders and chronic inflammation. Moreover, $\alpha 7$ nAChR stimulation has been suggested to exert a therapeutic effect on the experimental NASH[40]; however, the mechanism underlying the $\alpha 7$ nAChR-dependent control of hepatic inflammation

and the effect of this control on hepatic lipid metabolism remain to be elucidated. We focused on the specific interaction between the hepatic vagus cholinergic activity and the modulation of the Kupffer cell-mediated inflammatory response via $\alpha 7$ nAChR pathway during the developmental phase of NASH, and our findings might provide a perspective for preventive and therapeutic strategies that compensate for vagus activity in patients with obesity and metabolic syndrome.

In the present study, hepatic vagus denervation aggravated MCD diet-induced steatohepatitis. As a result, Kupffer cells were highly activated, and a high proportion of p65 was phosphorylated, which upregulated the expression of pro-inflammatory cytokines, including TNF α and IL-12, and a chemokine associated with macrophage activation, MCP-1. Likewise, $\alpha 7$ KO chimeric mice, in which Kupffer cells specifically lacked $\alpha 7$ nAChR expression, developed severe steatohepatitis and highly activated Kupffer cells. Moreover, the $\alpha 7$ nAChR agonist suppressed pro-inflammatory cytokine expression in primary Kupffer cells under LPS and palmitic acid stimulation. The STAT3 signaling pathway was involved downstream of the $\alpha 7$ nAChR, resulting in the inhibition of NF- κ B phosphorylation and the subsequent pro-inflammatory cytokine expression. The results of the *in vivo* and *in vitro* studies indicated that hepatic vagus cholinergic signaling has an anti-inflammatory effect on Kupffer cells via an $\alpha 7$ nAChR cascade during NASH pathogenesis.

In addition to the severity of inflammation, hepatic lipid accumulation was exacerbated in MCD diet-fed mice undergoing HV, and this change was accompanied by the downregulation of

PPAR α -pathway gene expression. The steatotic change was also accentuated in $\alpha 7$ KO chimeric mice, in which all liver parenchymal cells except for Kupffer cells were derived from the cells of the WT recipient. The modifications in lipid metabolism included the downregulation of AOX, which also occurred in the HV model, and the upregulation of SREBF-1c and its target FAS. The latter changes may reflect advanced metabolic disorder, partially due to the attenuated inhibition of SREBF-1c by the PPAR α pathway[41, 42]. The severe inflammatory response induced by the selective blockage of the $\alpha 7$ nAChR pathway in Kupffer cells secondarily affected lipid metabolism and steatotic change. In contrast, palmitic acid administration induced inflammatory cytokine expression in primary Kupffer cells. Specifically, free fatty acids activate TLRs by binding Fetuin-A contained in fetal bovine serum[43]. Stimulating Kupffer cells with palmitic acid and LPS synergistically upregulated TNF α and MCP-1, indicating that the lipotoxicity of free fatty acids as a consequence of hepatic lipid loading may also secondarily promote inflammation[44, 45]. Hepatic lipid metabolism and inflammatory responses closely interact with each other, which results in a vicious cycle of NASH progression that may be partially accelerated by decreased hepatic vagus activity and the subsequent attenuation of $\alpha 7$ nAChR signaling in Kupffer cells. These findings may provide insight into a potential role for the vagus nerve in mediating cross talk between metabolism and immunity.

The efferent vagus cholinergic signal was assumed to act directly on Kupffer cells and modulate their immune function. Hepatic vagus activity was suppressed after HV, as indicated by the significant decrease in hepatic acetylcholine content, resulting in severe hepatic inflammation and highly activated Kupffer cells. In addition to the direct mechanism of vagus-mediated anti-

inflammatory signaling, a subset of memory $CD4^+$ T cells was reported to provide an endogenous source of acetylcholine that stimulates the $\alpha 7nAChR$ cascade[30, 46]. The function of acetylcholine-synthesizing T cells is modulated by efferent vagus nerve signaling, which is translated into noradrenergic splenic nerve activity in the spleen[46]. The selective denervation of the hepatic vagus nerve might not directly attenuate the activity of such a T cell population, as expected from the preserved acetylcholine level in the spleen. In the present study, we instead focused on the direct effect of hepatic vagus-mediated cholinergic signaling, but the interaction between liver-resident Kupffer cells and acetylcholine-producing T cells in NASH pathogenesis should be the subject of further study. The residual amount of hepatic acetylcholine that remained after denervation might partially derive from this T cell production or from hepatic vagus innervation that escaped surgical resection, e.g., innervation originating from the dorsal vagal trunk or other intrahepatic cholinergic neuronal networks. The detailed anatomical distributions of vagus nerve fibers, postganglionic neurons, and target cells in the liver remain controversial, making the assessment of their specific functions difficult. The selective blockage of target receptors in Kupffer cells using chimeric mice should help to confirm a one-to-one correspondence between vagus efferent signals and hepatic innate immune modulation.

The limitations of the present study were mainly associated with the MCD dietary model. In this experimental NASH model, hepatic steatosis and inflammation are certainly accentuated, while peripheral insulin resistance is preserved, and the mice do not develop obesity or metabolic syndrome but instead lost weight[47, 48]. The MCD dietary model does not strictly reproduce human NASH; specifically, it lacks extrahepatic effects, e.g., insulin resistance and gut- or

adipose tissue-derived factors associated with obesity. Nevertheless, because we focused on liver-specific modulation by vagus efferent signals, this model might be well suited to assess the interaction between neural signals and NASH pathogenesis by minimizing such extrahepatic effects. Although the serum glucose levels were not affected in the MCD diet-fed HV mice or $\alpha 7$ KO chimeric mice, the dysregulation of Kupffer cell activity by attenuated vagus-mediated cholinergic signal significantly downregulated hepatic gluconeogenic genes. These findings were consistent with the findings of a previous report on gluconeogenic suppression by $\alpha 7$ nAChR-dependent Kupffer cell activation[39] and consequently may support the reproducibility and validity of the HV and the $\alpha 7$ KO chimeric mice models used in the present study. Additionally, the relatively short-term induction of steatohepatitis by the MCD diet compared with other dietary models, e.g., a high-fat diet and a Choline- and L-amino acid-deficient diet, might be advantageous to assess the maximum influence of vagus activity before the effect of denervation declines. However, this model failed to assess the influence of the hepatic vagus nerve on the long-term consequences of NASH fibrogenesis because of the faint fibrogenic effect of the MCD diet and the reduced potency of denervation due to chronic choline deficiency, which might affect the baseline activity of the cholinergic signal. Based on the clinical features of choline deficiency in patients with NASH[49, 50], the impact of vagus-mediated cholinergic signaling might be strongest at the onset of NASH development, as we successfully demonstrated its effect on the inflammatory response during early-stage NASH.

In conclusion, the present study demonstrated that the hepatic vagus nerve plays a key role in regulating the inflammatory response in Kupffer cells via the $\alpha 7$ nAChR pathway and eventually

contributed to the suppression of NASH progression in its early phase. Our study may help elucidate the mechanisms by which the failure to modulate inflammatory events results in advanced disease in the NAFLD population.

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Conflict of interest

The authors declare no conflicts of interest.

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Table 1. Body, liver, and adipose tissue weight and serum lipid levels after MCD diet feeding.

		CT diet						MCD diet					
		Sham (N=6)			HV (N=6)			Sham (N=12)			HV (N=12)		
BW change	(%)	2.9	±	3.3	1.1	±	2.3	-12.0 *†	±	2.3	-13.2 *†	±	2.6
Liver weight	(% BW)	3.9	±	0.6	4.1	±	0.5	4.3	±	0.3	4.5	±	0.4
SAT weight	(% BW)	0.84	±	0.27	0.92	±	0.12	0.91	±	0.30	0.90	±	0.15
VAT weight	(% BW)	0.86	±	0.36	1.00	±	0.23	0.92	±	0.28	0.94	±	0.25
Serum TG	(mg/dL)	28.8	±	7.1	26.3	±	11.3	21.8	±	4.2	27.1	±	12.3
Serum TC	(mg/dL)	85.1	±	10.9	82.5	±	13.4	45.2 *†	±	8.8	38.8 *†	±	6.8

Values are presented as the mean ± SD.

* $p < 0.001$ vs. CT diet + Sham

† $p < 0.001$ vs. CT diet + HV

MCD, methionine- and choline- deficient; CT, control; HV, hepatic vagotomy; BW, body weight; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; TG, triglyceride; TC, total cholesterol

Figure legends

Fig. 1. Hepatic vagus denervation aggravates MCD diet-induced NASH. WT mice underwent a sham operation or HV and were fed a CT or MCD diet for 1 week. (a) *Left*: Hematoxylin and eosin staining of liver sections. *Right*: NAFLD activity score. (b) *Left*: Nile Red staining of liver sections. *Right*: Quantification of Nile Red-stained area. Original magnification $\times 100$; scale bars, 100 μm . (c) Hepatic triglyceride content. (d) Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. $n=6$ per CT-diet group; $n=12$ per MCD-diet group. Data represent the mean $\pm$ SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. ND, not detected.

Fig. 2. Hepatic vagus denervation promotes NASH-associated inflammatory responses and abnormal lipid metabolism. (a, b) Hepatic mRNA expression of pro-inflammatory cytokine genes (a) and lipid metabolism-associated genes (b). Data were normalized to 18S. $n=6$ per CT-diet group; $n=12$ per MCD-diet group. Data represent the mean $\pm$ SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Fig. 3. Hepatic vagus denervation promotes Kupffer cell activation in NASH. (a, b) Immunohistochemical staining of liver sections for F4/80 and phospho-p65 (p-p65). (a) Liver sections from mice fed an MCD diet for 1 week. Original magnification $\times 200$; scale bars, 100 μm . (b) *Top*: Liver sections in sham-operated or HV mice fed an MCD diet for 1 week. *Bottom*: Percentage of F4/80-stained area, F4/80^+ per total liver cell count, and both F4/80^+ and p-p65^+

per F4/80⁺ cell count were measured. Original magnification $\times 100$; scale bars, 100 μm . $n=4$ per CT-diet group; $n=6$ per MCD-diet group. Data represent the mean $\pm$ SD. *** $p < 0.001$.

Fig. 4. $\alpha 7\text{nAChR}$ agonist suppresses the inflammatory response in Kupffer cells. Primary Kupffer cells were treated with $\alpha 7\text{nAChR}$ agonist under stimulation by LPS (1 ng/mL) and/or palmitic acid (400 μM). (a) mRNA expression of pro-inflammatory cytokine genes. Data were normalized to 18S. (b) Western blotting for $\alpha 7\text{nAChR}$, phospho-p65 (p-p65), p65, phospho-STAT3 (p-STAT3), or STAT3. Density ratios of bands of p-STAT3 to STAT3 and p-p65 to p65 were quantified. $n=3$ to 5, each condition. Data represent the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 5. $\alpha 7\text{nAChR}$ deficiency in Kupffer cells aggravates MCD diet-induced NASH. Chimeric mice in which WT or $\alpha 7\text{KO}$ BM cells were transplanted into WT recipients were fed an MCD diet for 1 week. (a) Procedure for generating chimeric mice. (b) Immunohistochemical staining of liver sections for F4/80 and $\alpha 7\text{nAChR}$. (c) *Left*: Macroscopic findings and hematoxylin-and-eosin-stained liver sections. *Right*: NAFLD activity score. (d) *Left*: Nile Red staining of liver sections. *Right*: Quantification of Nile Red-stained area. Original magnification $\times 100$; scale bars, 100 μm . (e) Hepatic triglyceride content. (f) Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. $n=6$ per group. Data represent the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 6. $\alpha 7$ nAChR deficiency in Kupffer cells promotes NASH-associated inflammatory responses and abnormal lipid metabolism. (a) *Top*: Immunohistochemical staining of liver sections for F4/80 and phospho-p65 (p-p65). *Bottom*: Percentage of F4/80 stained area, F4/80⁺ per total liver cell count, and both F4/80⁺ and p-p65⁺ per F4/80⁺ cell count were measured. Original magnification $\times 100$; scale bars, 100 μ m. (b, c) Hepatic mRNA expression of pro-inflammatory cytokine genes (b) and lipid metabolism-associated genes (c). Data were normalized to 18S. n=6 per group. Data represent the mean $\pm$ SD. * p <0.05, ** p <0.01, *** p <0.001. NS, not significant.

Fig. 1

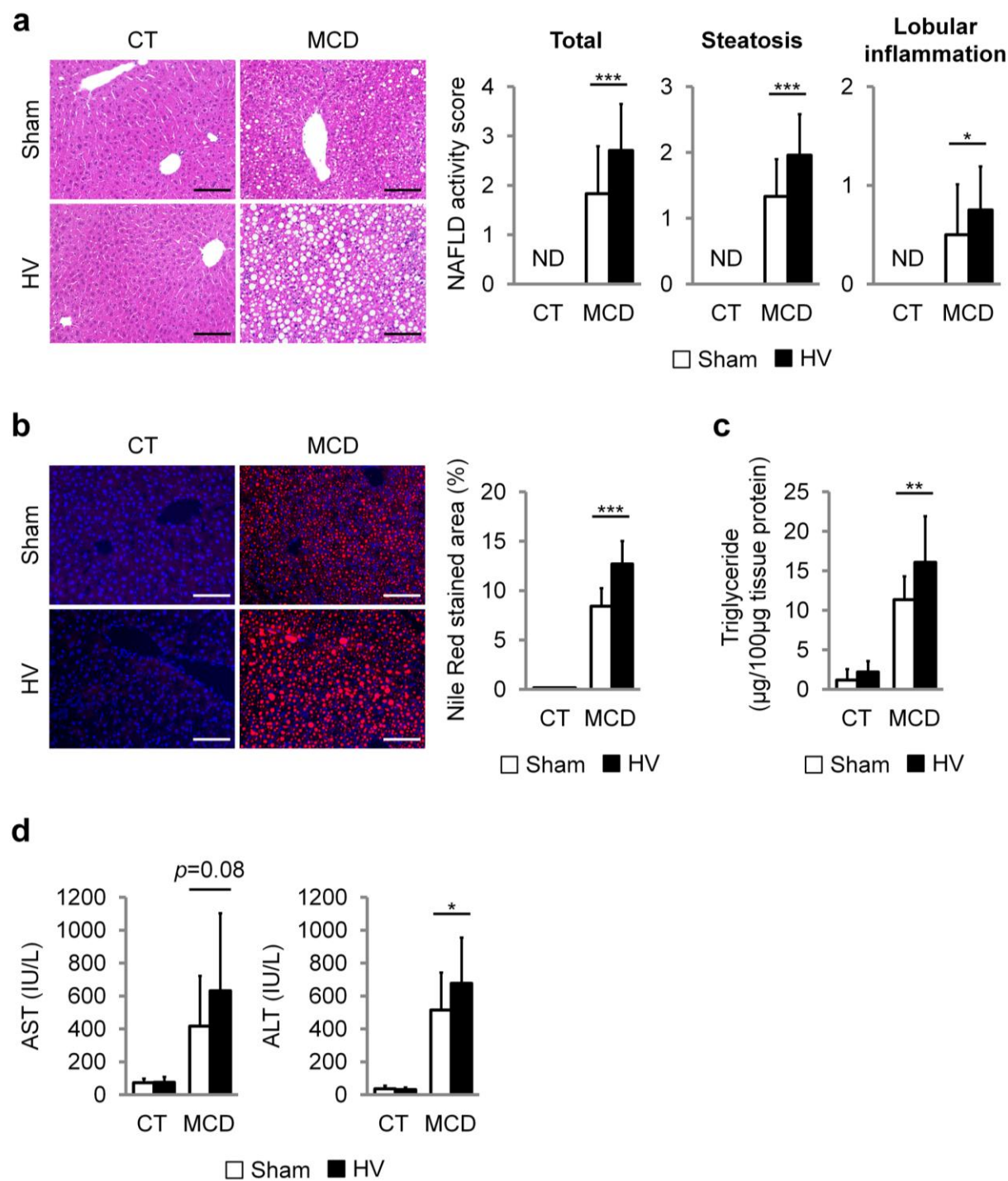


Fig. 2

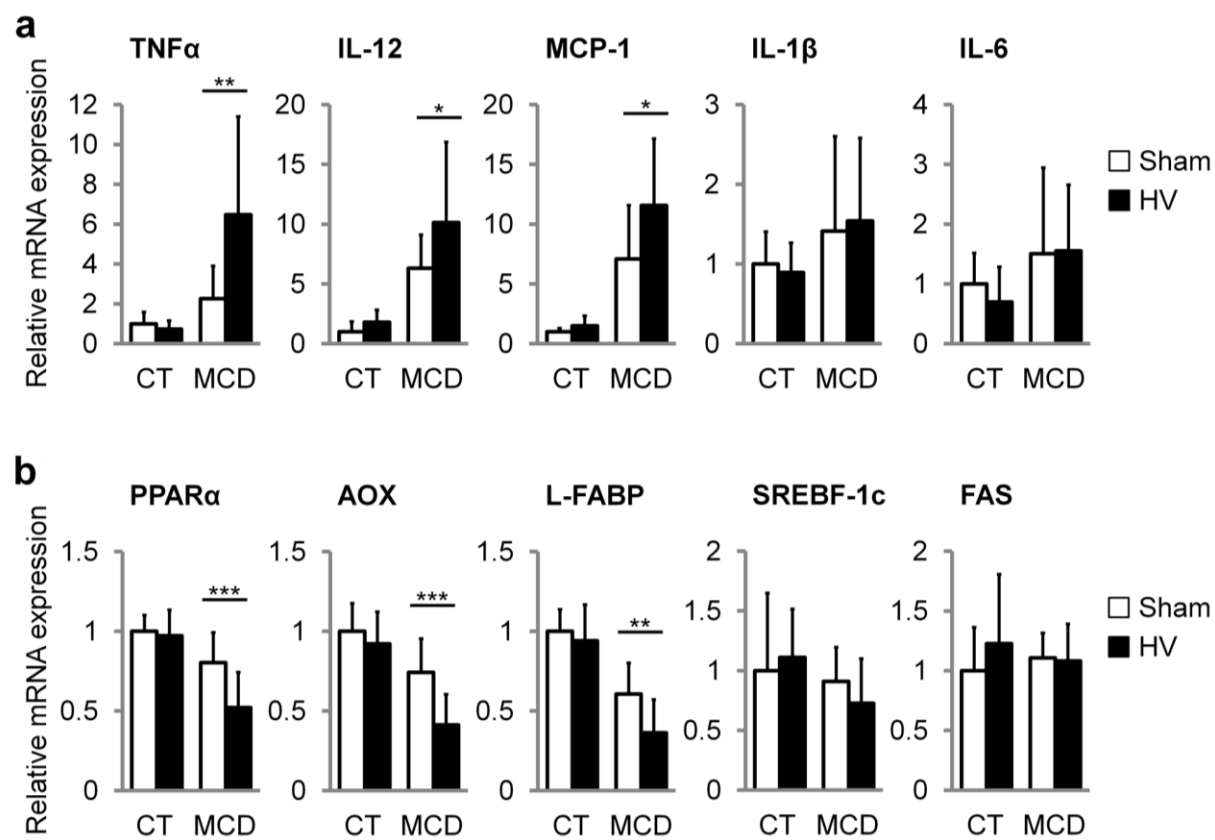


Fig. 3

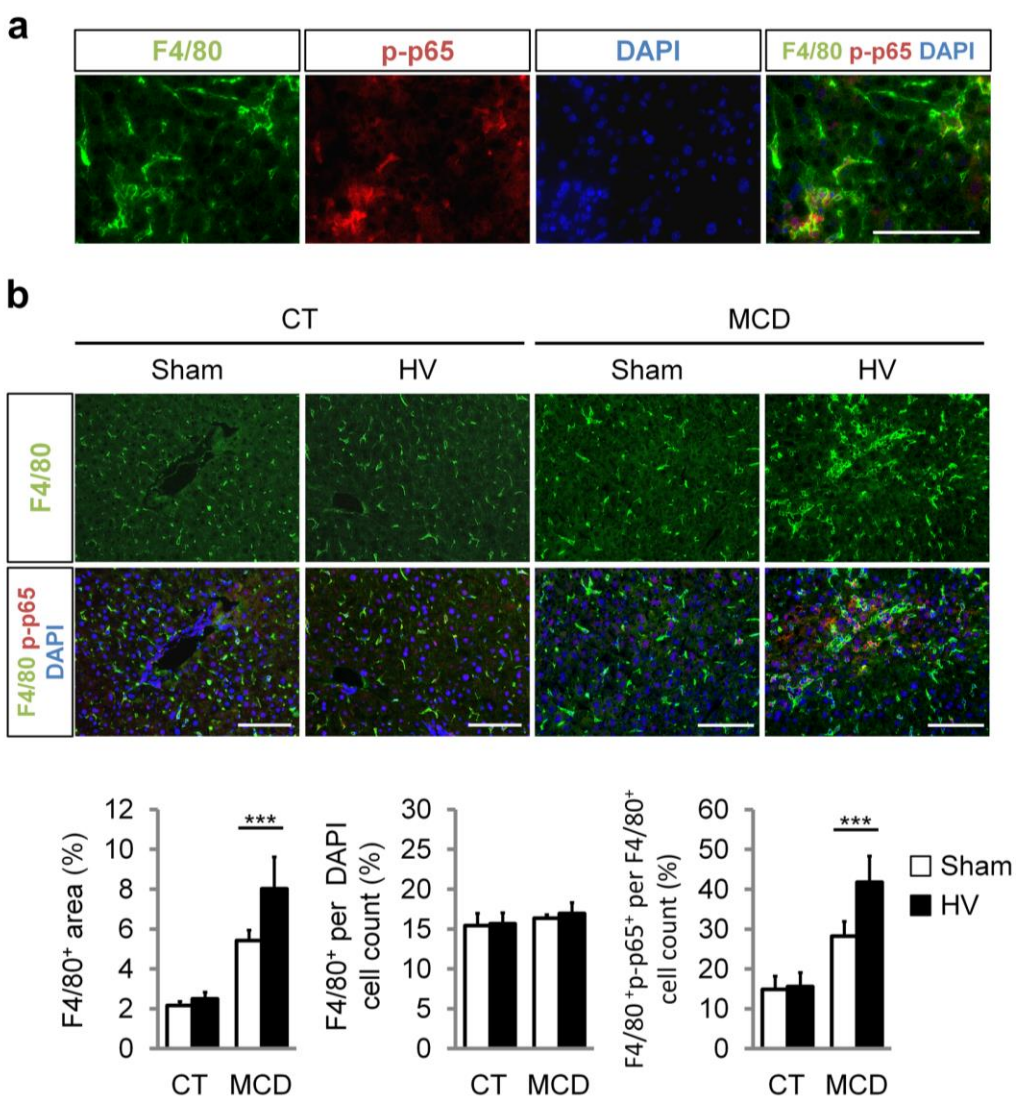


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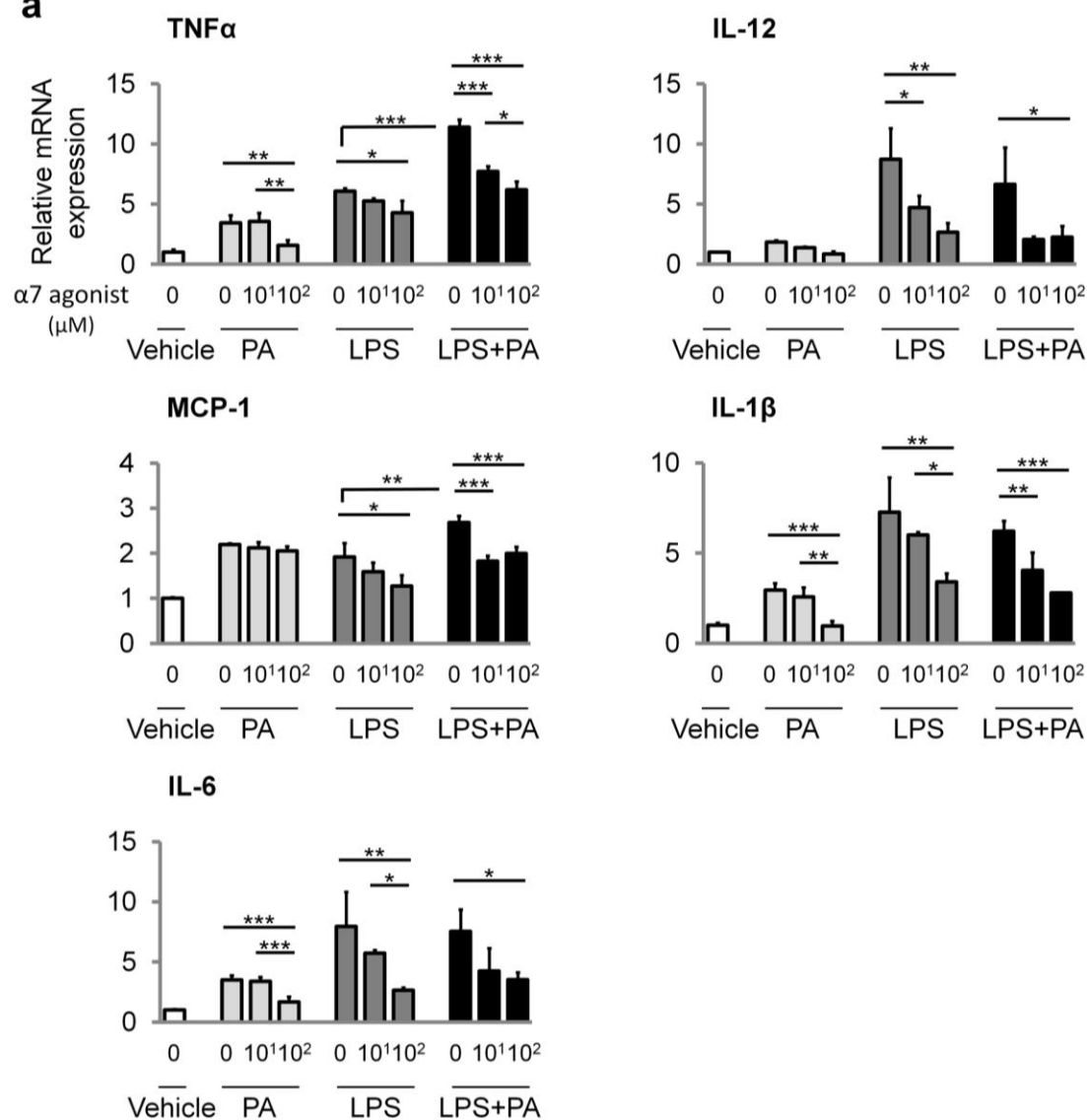
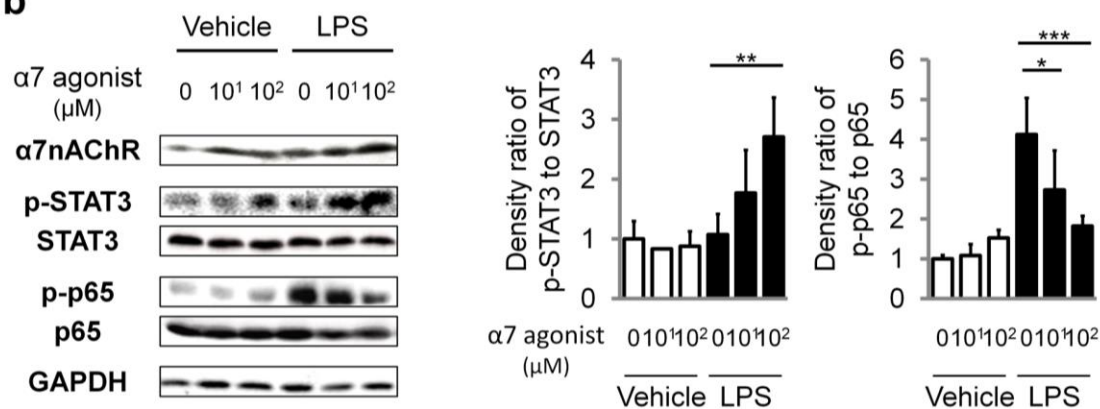
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Fig. 5

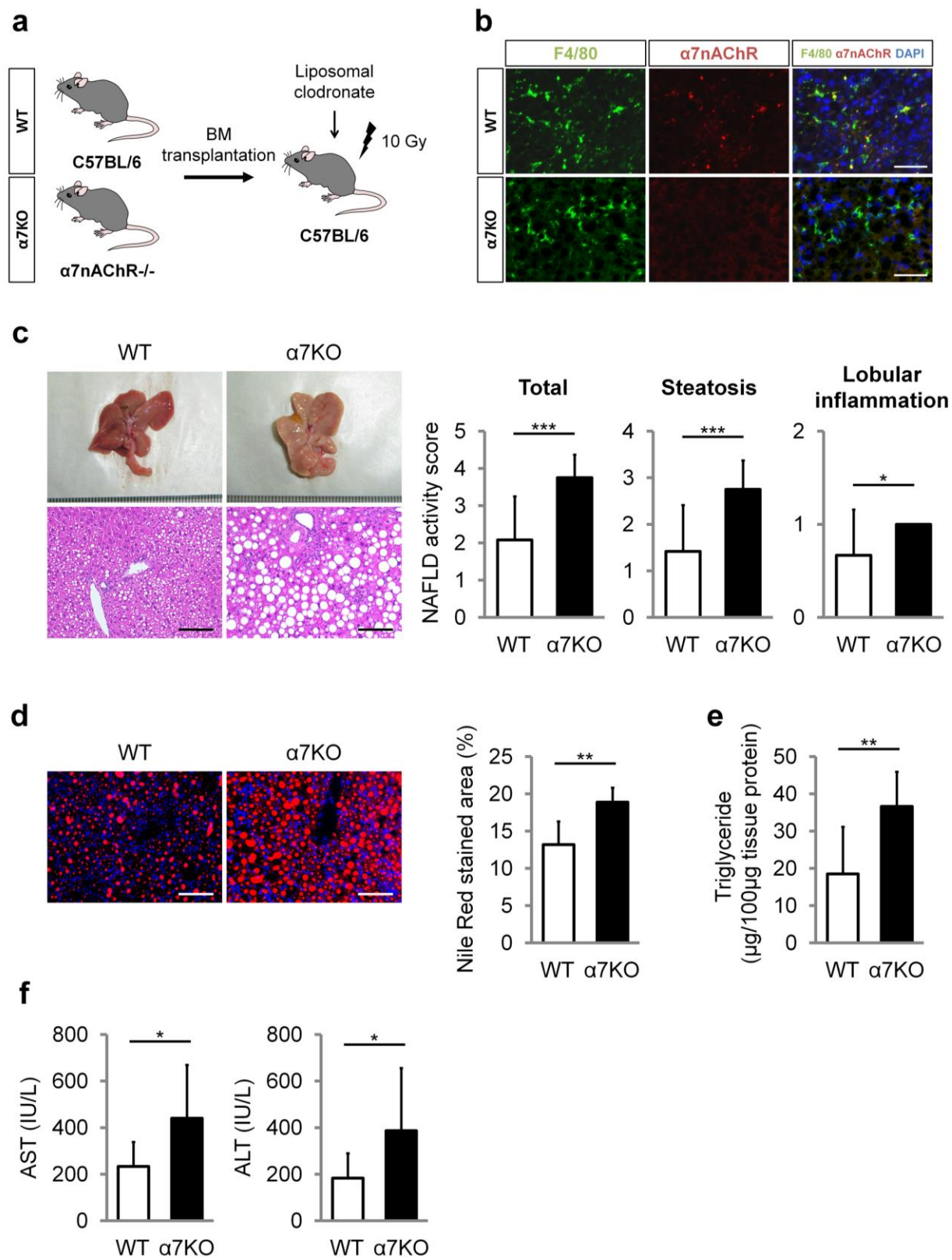
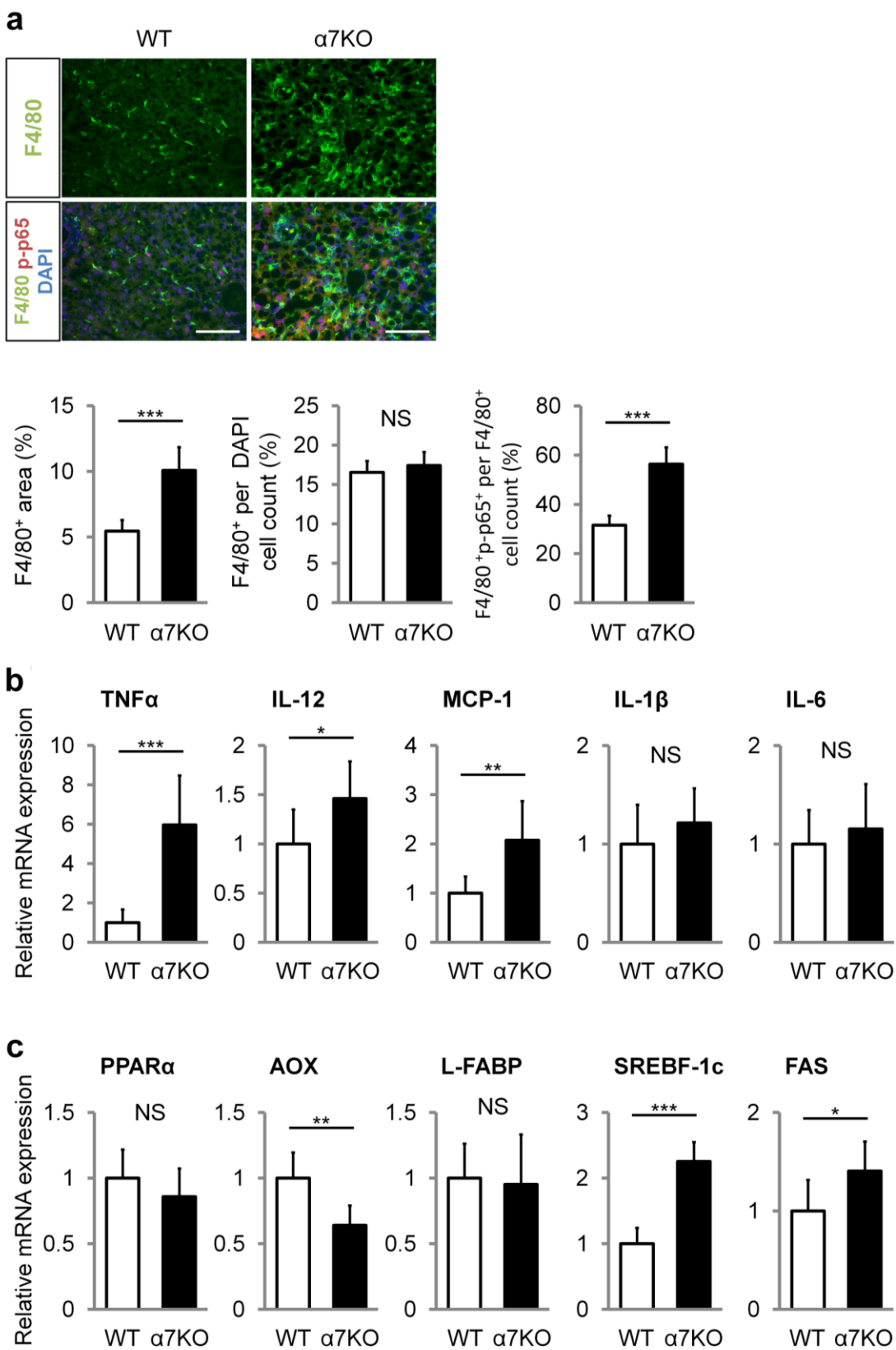


Fig. 6



Supplementary material

Table S1. Primer sequences for quantitative real-time polymerase chain reaction.

Gene	Sequence (5'→3')	
	Forward primer	Reverse primer
AOX	TCGAAGCCAGCGTTACGAG	GGTCTGCGATGCCAAATTCC
α SMA	GTCCCAGACATCAGGGAGTAA	TCGGATACTTCAGCGTCAGGA
Coll α 1	TGTGTGCGATGACGTGCAAT	GGGTCCCTCGACTCCTACA
FAS	AGAGATCCCGAGACGCTTCT	GCTTGGTCCTTTGAAGTCGAAGA
G6pc	CGACTCGCTATCTCCAAGTGA	GTTGAACCAGTCTCCGACCA
GAPDH	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA
IL-1 β	GAAATGCCACCTTTTGACAGTG	CTGGATGCTCTCATCAGGACA
IL-6	CCAGTTGCCTTCTTGGGACTG	CAGGTCTGTTGGGAGTGGTATCC
IL-12	TGGTTTGCCATCGTTTTGCTG	ACAGGTGAGGTTCACTGTTTCT
L-FABP	ATGAACTTCTCCGGCAAGTACC	GGTCCTCGGGCAGACCTAT
MCP-1	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
PPAR α	AACATCGAGTGTCGAATATGTGG	CCGAATAGTTCGCCGAAAGAA
PEPCK	AGCATTCAACGCCAGGTTC	CGAGTCTGTCAGTTCAATACCAA
SREBF-1c	TGACCCGGCTATTCCGTGA	CTGGGCTGAGCAATACAGTTC
TNF α	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG

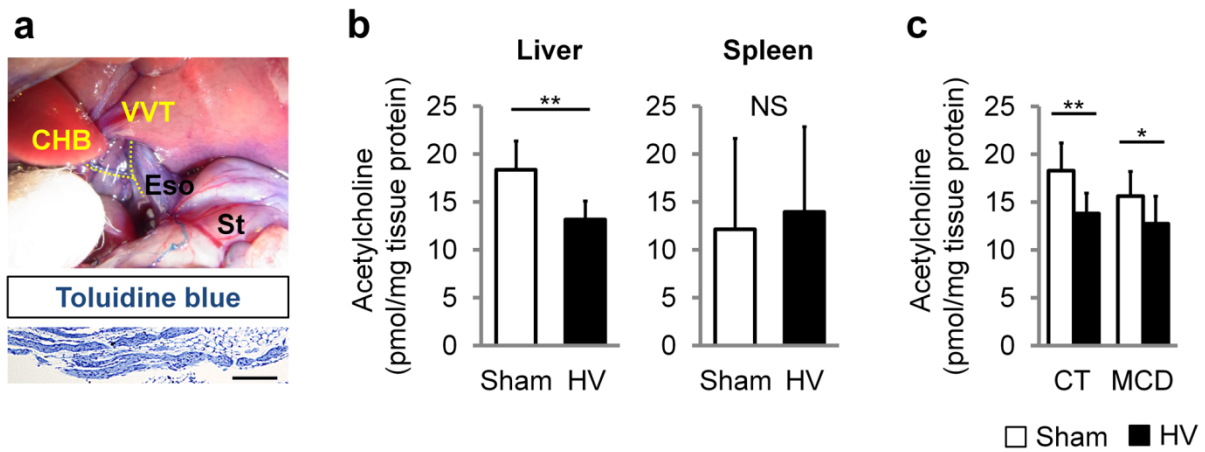


Fig. S1. HV attenuates hepatic acetylcholine content. (a) *Top*: Surgical anatomy of the hepatic vagus nerve. The ventral subdiaphragmatic vagus trunk (VVT) was exposed along the descending esophagus (Eso) with the stomach (St) pulled down. The common hepatic branch (CHB) was transected. *Bottom*: Toluidine blue staining of a section of resected CHB. (b) Acetylcholine content in the liver or spleen. WT mice underwent sham operation or HV and were sacrificed 2 weeks after surgery. n=10 per group. (c) Hepatic acetylcholine content. WT mice undergoing sham operation or HV were fed with CT diet or MCD diet for 1 week. n=8 to 10 per group. Data represent the mean $\pm$ SD. * p < 0.05, ** p < 0.01. NS, not significant.

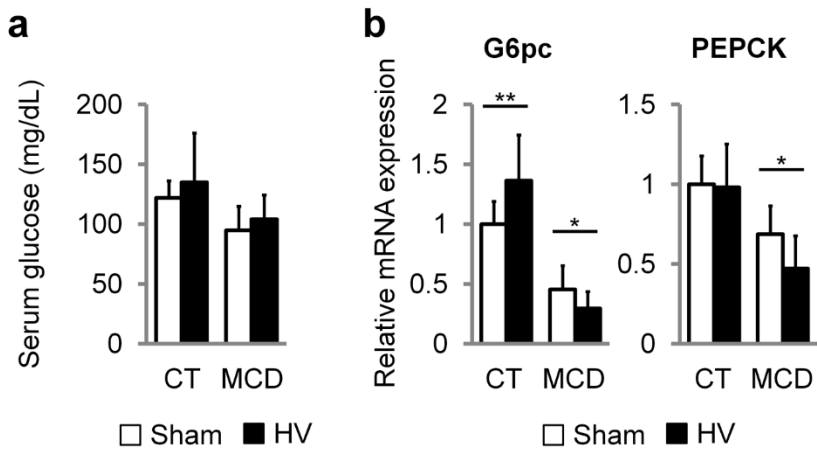


Fig. S2. HV suppresses hepatic gluconeogenic gene expression in MCD diet-induced NASH. (a) Serum glucose levels. (b) Hepatic mRNA expression of gluconeogenic genes. Data were normalized to 18S. n=6 per CT-diet group; n=12 per MCD-diet group. Data represent the mean $\pm$ SD. * p < 0.05, ** p < 0.01.

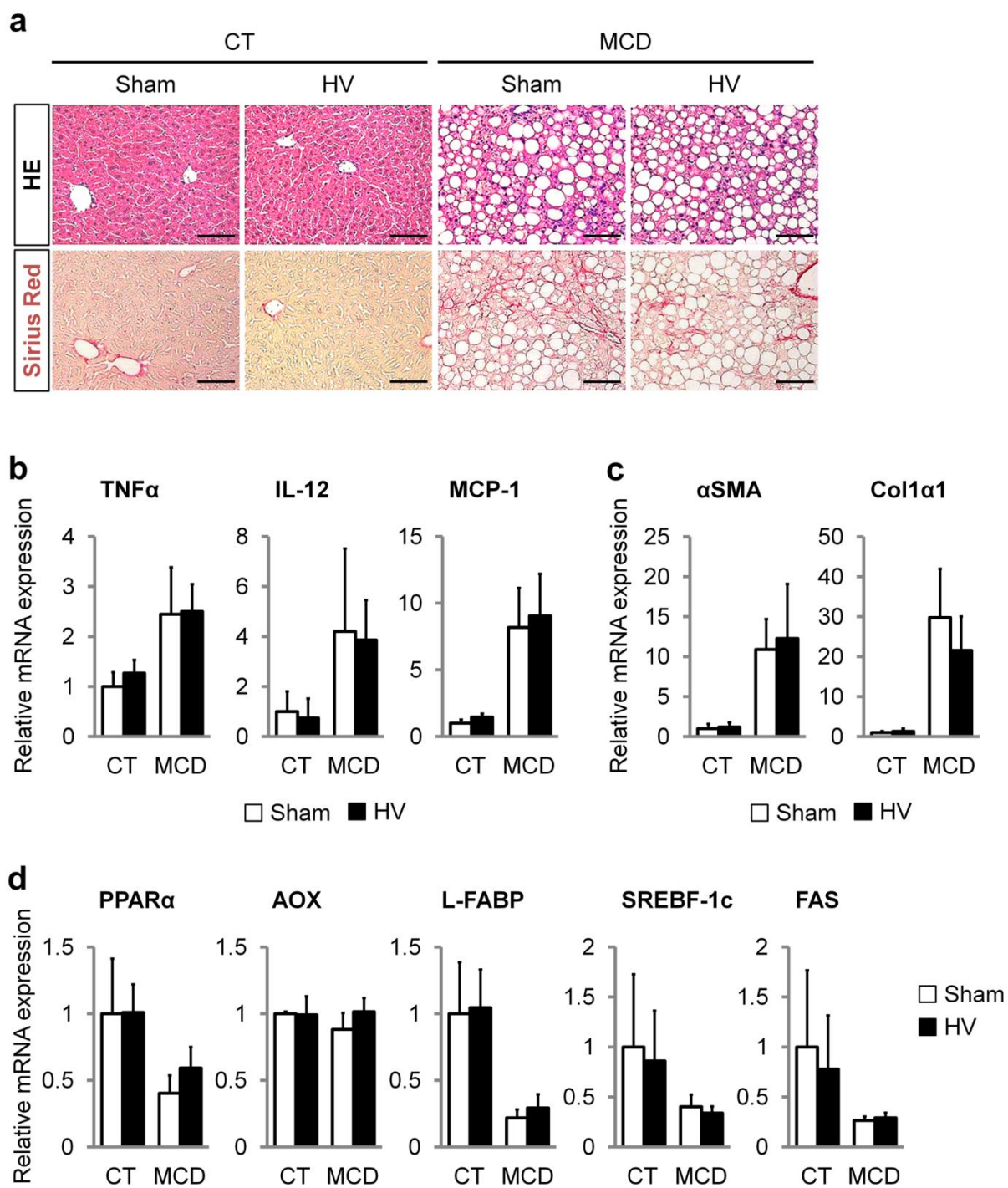


Fig. S3. HV does not significantly affect fibrogenesis in mice fed an MCD diet for 8 weeks. (a) Hematoxylin and eosin (HE) and Sirius Red staining of liver sections. Original magnification $\times 100$; scale bars, 100 μm . (b, c, d) Hepatic mRNA expression of proinflammatory cytokine genes (b), fibrogenic genes (c), and lipid metabolism-associated genes (d). Data were normalized to 18S. n=4 per CT-diet group; n=9 per MCD-diet group. Data represent the mean $\pm$ SD. αSMA , alpha smooth muscle actin; $\text{Coll}\alpha 1$, type 1 collagen alpha 1.

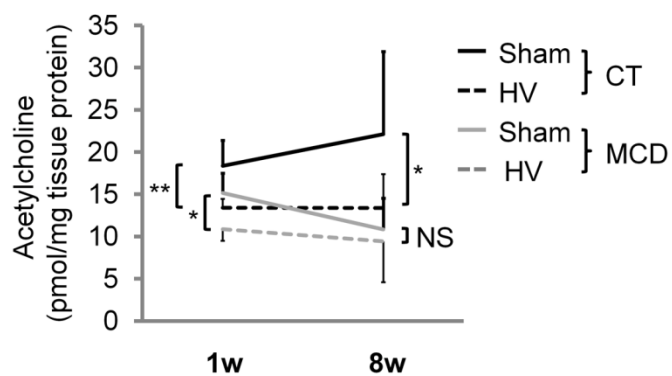


Fig. S4. Effect of HV is attenuated in mice fed an MCD diet for 8 weeks. Hepatic acetylcholine content in mice undergoing sham operation or HV fed a CT or MCD diet for 1 week (1 w) or 8 weeks (8 w). $n=4$ to 7 per group. Data represent the mean $\pm$ SD. $*p < 0.05$, $**p < 0.01$. NS, not significant.

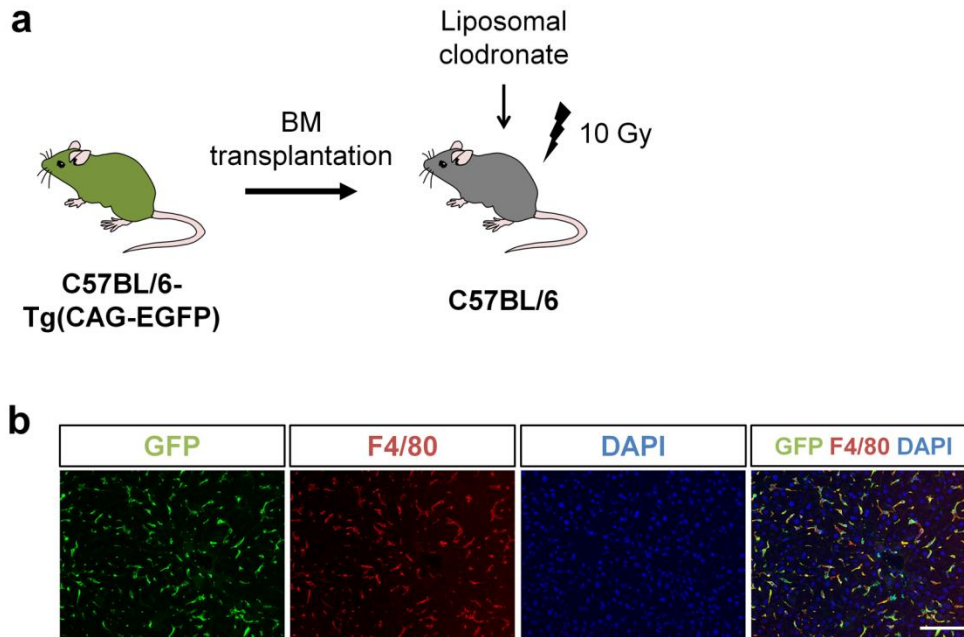


Fig. S5. Efficacy of the reconstitution of Kupffer cells in chimeric mice. (a) Procedure for generating chimeric mice in which GFP⁺ cells were transferred into irradiated and Kupffer cell-depleted WT recipients. Mice were sacrificed 8 weeks after BM transplantation. (b) Immunohistochemical staining of liver sections for F4/80. Original magnification $\times 100$; scale bar, 100 μm .

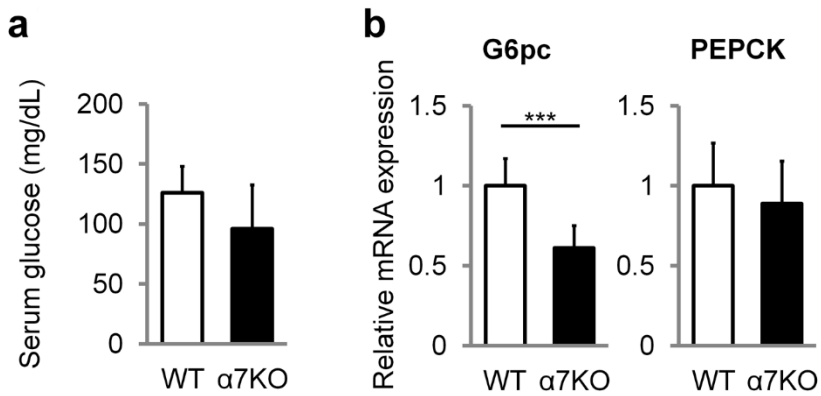


Fig. S6. $\alpha 7$ nAChR deficiency in Kupffer cells suppresses hepatic gluconeogenic gene expression.

(a) Serum glucose levels. (b) Hepatic mRNA expression of gluconeogenic genes. Data were normalized to 18S. n=6 per group. Data represent the mean $\pm$ SD. *** $p < 0.001$.

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