

ORIGINAL ARTICLE

iNKT cells prevent obesity-induced hepatic steatosis in mice in a C-C chemokine receptor 7-dependent manner

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Non-alcoholic fatty liver disease and non-alcoholic steatohepatitis are characterized by an increase in hepatic triglyceride content with infiltration of immune cells, which can cause steatohepatitis and hepatic insulin resistance. C-C chemokine receptor 7 (CCR7) is primarily expressed in immune cells, and CCR7 deficiency leads to the development of multi-organ autoimmunity, chronic renal disease and autoimmune diabetes. Here, we investigated the effect of CCR7 on hepatic steatosis in a mouse model and its underlying mechanism. Our results demonstrated that body and liver weights were higher in the CCR7^{-/-} mice than in the wild-type (WT) mice when they were fed a high-fat diet. Further, glucose tolerance and insulin sensitivity were markedly diminished in CCR7^{-/-} mice. The number of invariant natural killer T (iNKT) cells was reduced in the livers of the CCR7^{-/-} mice. Moreover, liver inflammation was detected in obese CCR7^{-/-} mice, which was ameliorated by the adoptive transfer of hepatic mononuclear cells from WT mice, but not through the transfer of hepatic mononuclear cells from CD1d^{-/-} or interleukin-10-deficient (IL-10^{-/-}) mice. Overall, these results suggest that CCR7⁺ mononuclear cells in the liver could regulate obesity-induced hepatic steatosis via induction of IL-10-expressing iNKT cells.

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INTRODUCTION

Obesity and diabetes are associated with mild but chronic inflammation in various tissues, thereby disrupting tissue homeostasis.^{1,2} The liver is an important organ for glucose homeostasis and metabolism, and excessive energy intake increases the accumulation of triglycerides in the liver. The accumulation of triglycerides results in the release of cytokines, causing steatosis to the liver with the consequent development of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH).^{3–5} NAFLD and NASH, which are closely associated with metabolic disease, often develop from type 2 diabetes or insulin resistance.^{5,6} Recent studies have demonstrated that the cause of this metabolic syndrome could be the accumulation of inflammatory immune cells that secrete pro-inflammatory cytokines into the peripheral tissues.^{2,7}

In association with the inflammatory infiltration in NAFLD and NASH, serum chemokines, including CXCL8, CCL2 and CCL19, are elevated in the livers of humans and rodents.^{8,9} Among them, the C-C chemokine receptor 7 (CCR7) ligand CCL19, which is constitutively expressed in the lymph nodes to recruit naïve T cells, is also known to be associated with the hepatic infiltration of lymphocytes such as CD8⁺ T cells¹⁰ and CD4⁺ T cells.^{11,12} However, several other reports have suggested that CCL19 is important for the emigration of CCR7⁺ effector cells out of inflamed tissue for the resolution of inflammation;¹³ thus, the role of this ligand remains elusive.

Several previous studies suggest that CCR7 deficiency may have an influence on regulatory immune cells as well as effector cells. The binding of CCR7 to CCL19 or CCL21 is required for the migration of Foxp3⁺CD4⁺ regulatory T (Treg) cells from the periphery to the draining lymph nodes, and thus, CCR7-deficient Treg cells cannot migrate to draining lymph nodes.¹⁴ In addition, CCR7 was found to regulate the development of invariant natural killer T (iNKT) cells in the thymus, and altered the balance of Foxp3⁺ Treg cells.^{15,16}

To follow-up on reports showing that CCR7^{-/-} mice exhibit enhanced inflammation and autoimmune disease phenotypes, especially diabetic nephropathy,¹⁷ in the present study, we evaluated the effects of CCR7 on obesity-associated metabolic syndrome, including NAFLD.

MATERIALS AND METHODS

Mice

C57BL/6 (Orient-Bio Ltd, Charles River Laboratories, Seoul, Korea), IL-10^{-/-}, CD1d^{-/-} (The Jackson Laboratory, Bar Harbor, ME, USA) mice were bred in the Kangwon National University (KNU). In addition, CCR7^{-/-} mice on a C57BL/6 background were generously provided by Dr Martin Lipp (Max Delbrück Center for Molecular Medicine, Berlin, Germany) and bred in the KNU. All male mice were purchased at 6 weeks of age and used in all of the experiments. The male mice were fed either a regular diet (RD) or a high-fat diet (HFD, with 60% of calories from fat, catalog No. D12492; Research Diets, New Brunswick, NJ, USA) for 10 weeks from 8 weeks of age. Data represent results of three independent experiments and are no blinding

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test. The animals were maintained in the KNU animal facility at 20–22 °C with 40–60% relative humidity and a 12 h/12 h light/dark cycle for at least 7 days before the experiment. The amount of HFD was adjusted according to changes in body weight for each mouse ($n=7$). Food and water intake, and body weight were measured weekly. All extracted tissues were immediately frozen in liquid nitrogen and stored at -80°C until analysis. All experiments were approved by the Institutional Animal Care and Use Committee of KNU (IACUC No. KW-150728-1). In addition, the ethical guidelines described in the KFDA Guide for the Care and Use of Laboratory Animals were followed throughout the experiments.

Hepatic mononuclear cells transplantation

Hepatic mononuclear cells were isolated from donor groups (C57BL/6, $\text{CCR7}^{-/-}$, $\text{IL-10}^{-/-}$ and $\text{CD1d}^{-/-}$). Six-week-old WT or $\text{CCR7}^{-/-}$ mice were i.v. injected once with 2×10^6 of the mixed hepatic mononuclear cells resuspended in 100 μl of phosphate-buffered saline (Gibco, Grand island, NY, USA). Recipient animals ($n=5$) adoptively transferred with hepatic mononuclear cells were fed either an RD or HFD for 10 weeks. We performed glucose tolerance tests to determine the glucose intolerance state of each group at the 16th week of the study. The mice were intraperitoneally injected with glucose (2 g kg^{-1} body weight) after an 8-h fasting period. The data represent the results of three independent experiments.

Flow cytometry

Immune cells isolated from the liver tissues were incubated with FcBlock (BD Biosciences, San Jose, CA, USA) in the dark at 4°C on a bidirectional shaker for 30 min, and double-stained with FITC-conjugated anti-CD11b and PE-conjugated anti-F4/80 (eBioscience, San Diego, CA, USA), or with FITC-conjugated anti-CD44 and -CD62L, and PE-conjugated anti-CD4 and -CD8 antibodies (eBioscience). For iNKT cell analysis, liver cell were labeled with anti-CD1d tetramer-APC, anti-TCR β -FITC, anti-CD3-FITC and anti-NK1.1-APC. The cells were then washed with fluorescence-activated cell sorting buffer and quantitated with a FACSVerse analyzer (BD Biosciences) with BD FACSuite (BD Biosciences) (see Supplementary Material).

Statistical analyses

All data are presented as mean \pm s.e.m. Statistical analysis was performed by one-way analysis of variance and Tukey's *post hoc* test for multiple comparison using SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA). The differences were considered statistically significant at $P < 0.05$.

RESULTS

High-fat diet increases obesity in $\text{CCR7}^{-/-}$ mice

To assess the role of CCR7 in the induction of obesity-associated hepatic steatosis and inflammation, we adopted a diet-induced steatohepatitis model. WT and $\text{CCR7}^{-/-}$ mice were fed an RD or HFD for 10 weeks. We found that HFD-fed $\text{CCR7}^{-/-}$ mice gained more weight than HFD-fed WT mice, whereas there was no significant difference in the body weight of WT and $\text{CCR7}^{-/-}$ mice fed an RD (Figure 1a). The final body weight was significantly higher in HFD- $\text{CCR7}^{-/-}$ mice compared with HFD-WT mice (Figures 1b and c). We confirmed that the difference in body weight was not due to appetite because food intake did not differ between the HFD-fed groups (Figure 1d).

CCR7 deficiency in mice diminishes the insulin response, resulting in hepatic steatosis

We next assessed whether the hepatic steatosis in HFD-fed $\text{CCR7}^{-/-}$ mice was associated with glucose tolerance. The intraperitoneal glucose tolerance test showed that the RD-fed $\text{CCR7}^{-/-}$ mice had significantly higher levels of plasma glucose compared with WT mice at 30 and 60 min, suggesting that impaired glucose tolerance was more severe in RD-fed $\text{CCR7}^{-/-}$ mice than in WT mice (Figures 2a and c). HFD-fed $\text{CCR7}^{-/-}$ mice had significantly higher levels of plasma glucose than HFD-fed WT mice at 15, 30 and 60 min (Figures 2a and c). The insulin tolerance

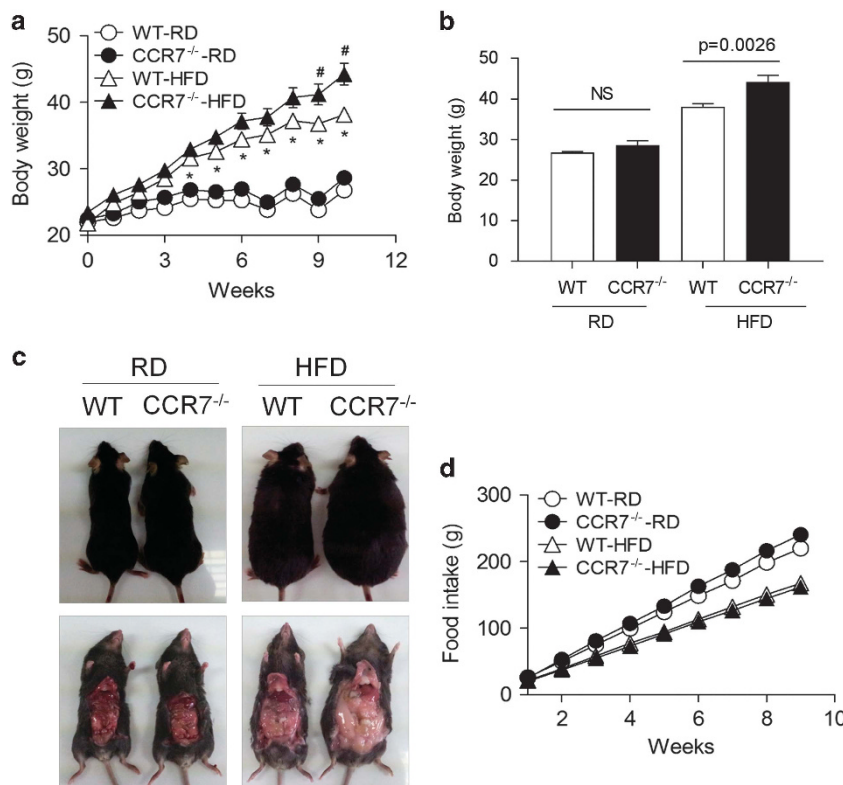


Figure 1. Body weight of $\text{CCR7}^{-/-}$ mice was increased compared with that of wild-type (WT) mice. WT and $\text{CCR7}^{-/-}$ mice were fed a high-fat diet (HFD) for 10 weeks. (a–c) Body weight changes of WT mice ($n=10$) and $\text{CCR7}^{-/-}$ mice ($n=10$). (d) Food intake changes of WT mice ($n=10$) and $\text{CCR7}^{-/-}$ mice ($n=10$). * $P < 0.05$ compared with RD-fed WT mice. # $P < 0.05$ compared with HFD-fed WT mice.

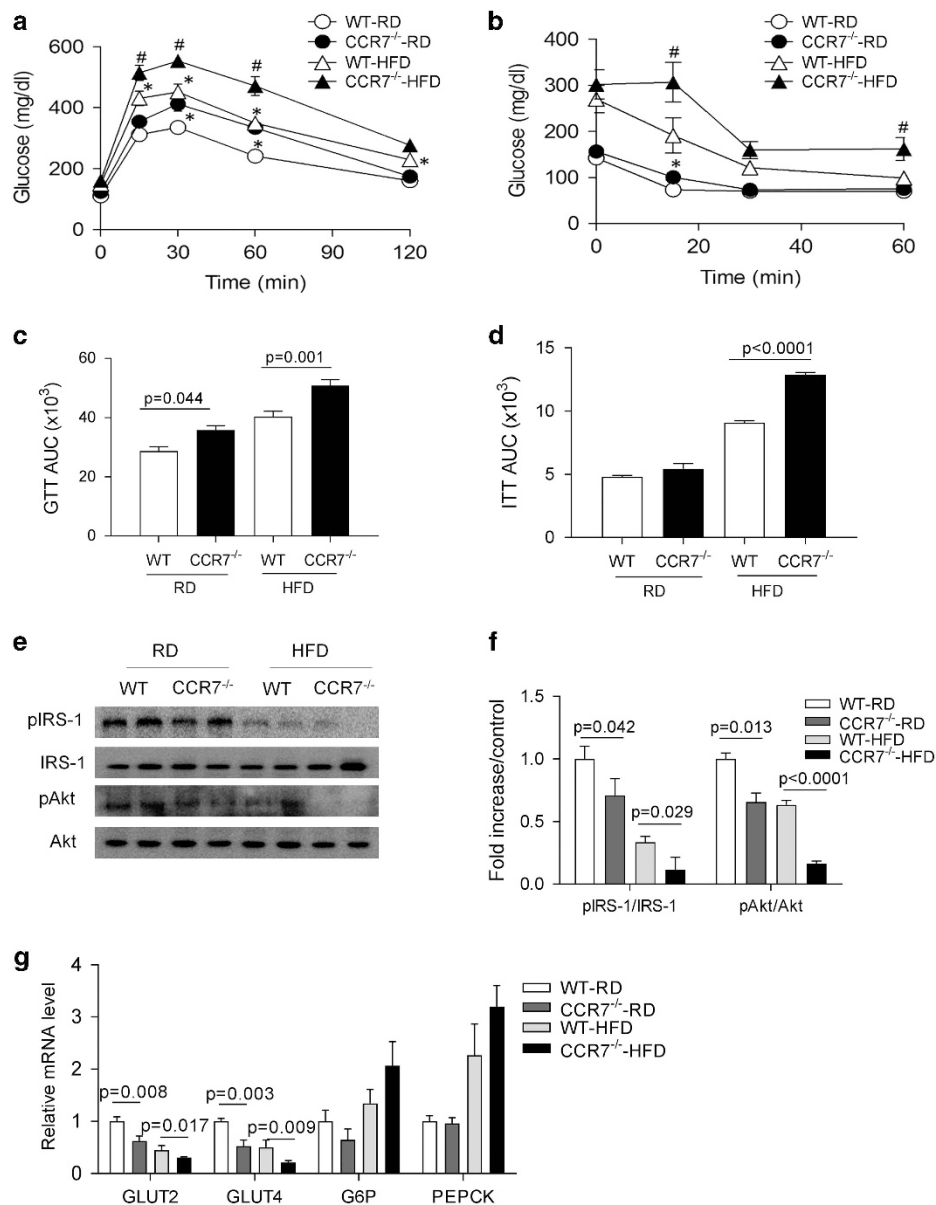


Figure 2. CCR7^{-/-} mice display increased insulin resistance. (a–d) Mice underwent glucose (2 g kg⁻¹) and insulin (0.75 U kg⁻¹) tolerance tests at 8 weeks (n = 10/each group). *P < 0.05 compared with the RD-fed WT mice. #P < 0.05 compared with HFD-fed WT mice. (e, f) Western blots of insulin signaling molecules, pIRS-1 and pAkt, in the liver. Cropped membrane was used in western blot. The band intensities were measured using Image J. Results shown are means ± s.e.m. *P < 0.05 compared with RD-fed WT mice. (g) mRNA levels of glucose regulator genes (*Glut2*, *Glut4*, *G6p* and *Pepck*) in the livers of mice.

test showed that HFD-fed CCR7^{-/-} mice had higher insulin resistance than HFD-fed WT mice at 15 and 60 min (Figures 2b and d). Insulin inhibits the production of glucose in the liver by inducing the phosphorylation of insulin receptor substrate (IRS), and accelerates glucose uptake by inducing the translocation of the glucose transporter GLUT4.¹⁸ We found that levels of phosphorylated IRS-1 and Akt were significantly lower in the livers of CCR7^{-/-} mice (Figures 2e and f). Likewise, the transcription of *Glut2* and *Glut4* was also decreased in HFD-fed CCR7^{-/-} mice (Figure 2g). These findings suggest that the CCR7^{-/-} mice had defects in insulin signaling in the liver, which aggravated glucose tolerance and insulin resistance. The fasting serum insulin (1.98 ± 0.23 vs 2.91 ± 0.37 ng ml⁻¹), total cholesterol (80.00 ± 1.73 vs 95.91 ± 7.77 mg dl⁻¹), triglyceride (21.89 ± 1.44 vs 47.25 ± 7.81 mg dl⁻¹) and aspartate aminotransferase (36.39 ± 5.07 vs 58.58 ± 13.14 IU l⁻¹) levels in RD-fed CCR7^{-/-} mice were

significantly higher than in RD-fed WT mice (Supplementary Table S1). In addition, serum insulin (3.23 ± 0.65 vs 4.28 ± 1.11 ng ml⁻¹) and total cholesterol (106.35 ± 3.38 vs 123.05 ± 6.20 mg dl⁻¹) levels were significantly higher in HFD-fed CCR7^{-/-} mice than in HFD-fed WT mice, respectively (Supplementary Table S1). No significant differences in serum glucose and alanine aminotransferase levels were found between CCR7^{-/-} and WT mice fed an RD (Supplementary Table S1). Collectively, these results suggested that insulin resistance in diet-induced obese CCR7-deficient mice was increased compared with that in WT mice.

CCR7-deficient mice show increased hepatic steatosis

We investigated the liver tissue to examine the effect of enhanced insulin resistance in diet-induced obese CCR7^{-/-} mice. HFD-fed CCR7^{-/-} mice had larger lipid droplets in the liver compared with

HFD-WT mice, suggesting that hepatic steatosis was also significantly higher in HFD-fed CCR7^{-/-} mice than in WT mice (Figures 3a and b). The levels of liver glycerolipids in HFD-fed CCR7^{-/-} mice were significantly higher than those in HFD-fed WT mice (9.44 ± 0.88 vs 15.84 ± 3.40 mg g⁻¹ liver weight, respectively) (Figure 3c). Using transmission electron microscopy, we also confirmed that mitochondrial degradation in the hepatocytes was markedly increased in CCR7^{-/-} mice (Figure 3d). Next, we assessed whether CCR7 deficiency induced hepatic steatosis and apoptosis of liver hepatocytes. The levels of sterol regulatory element-binding protein 2, which regulates lipid and cholesterol metabolism,¹⁹ were significantly increased in RD- or HFD-fed CCR7^{-/-} mice when compared with those in the RD- or HFD-fed WT mice, respectively (Figures 3e and f). No differences in sterol regulatory element-binding protein 1 expression were found

within the CCR7^{-/-} or WT groups of mice. In addition, the expression of PGC-1 α , which mediates mitochondrial biogenesis and oxidative phosphorylation,²⁰ was significantly lower in RD- or HFD-fed CCR7^{-/-} mice than in RD- or HFD-fed WT mice (Figures 3e and f). Immunohistochemical analysis of liver tissues showed that the numbers of caspase-3-expressing cells and TUNEL-positive cells in the liver were highly increased in RD- or HFD-fed CCR7^{-/-} mice compared with those in RD- or HFD-fed mice (Supplementary Figure S1a). We also confirmed that apoptotic (red circles) and necrotic (HFD-CCR7^{-/-}) dead cells were markedly increased in CCR7^{-/-} mice using transmission electron microscopy (Supplementary Figure S1b). The caspase-3 levels and phosphorylation of the apoptosis-related p38 protein were also significantly higher in HFD-fed CCR7^{-/-}-deficient mice than in HFD-fed WT mice (Supplementary Figures S1c and d).

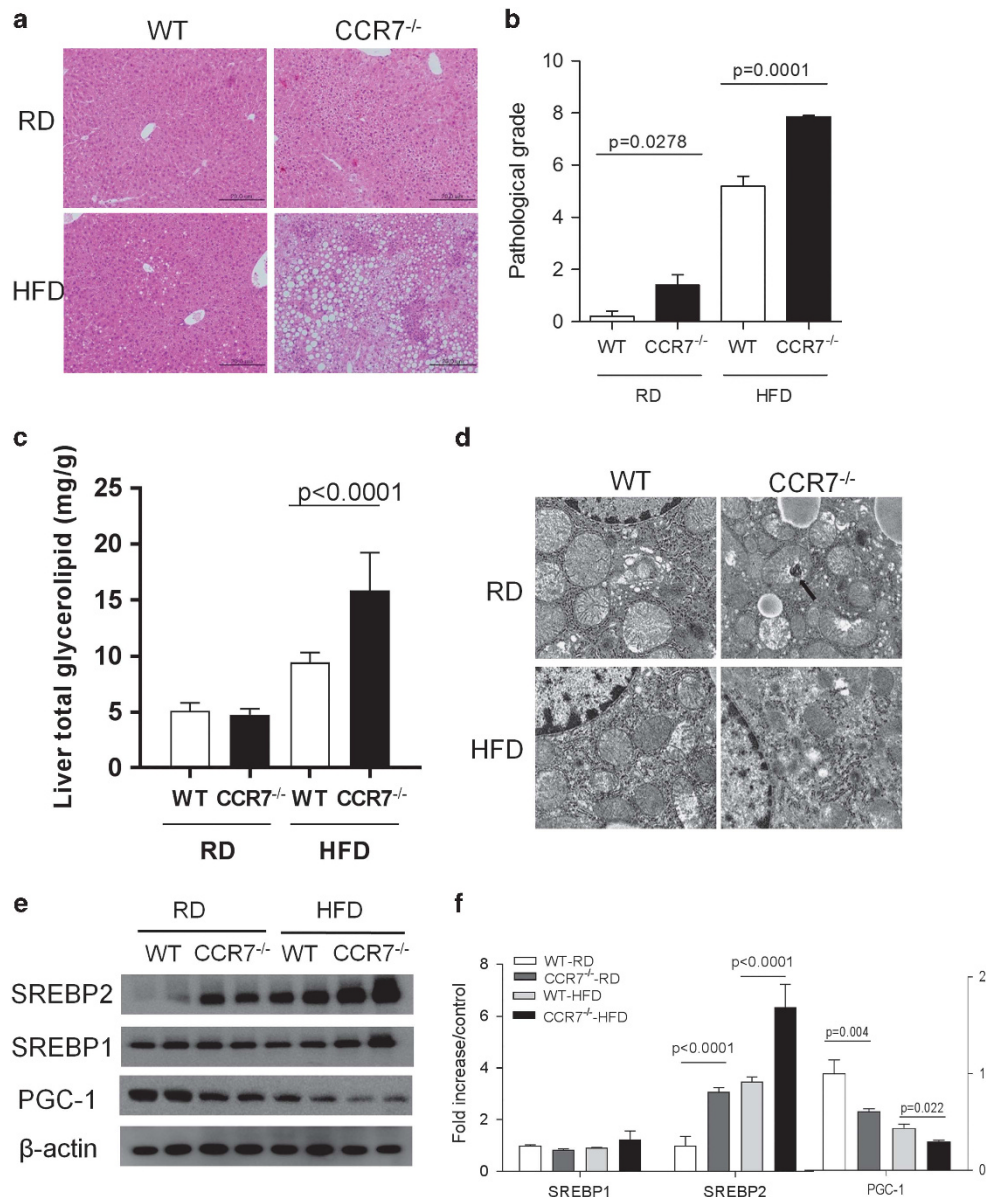


Figure 3. Glycerolipids content and liver steatosis were enhanced in high-fat diet CCR7^{-/-} mice. **(a, b)** Pathological grade from histological examination of the livers stained with hematoxylin and eosin. **(c)** Glycerolipids contents of the livers from RD- or HFD-fed WT and CCR7^{-/-} mice ($n = 10$ /each group). **(d)** Electron microscopy analysis of mitochondria of hepatocytes from WT and CCR7^{-/-} mice (arrow = mitochondria damage). **(e, f)** Western blots of SREBP1, SREBP2 and PGC-1 in the liver. Cropped membrane was used in western blot. The band intensities were measured by Image J. Results shown are means \pm s.e.m. Abbreviation: SREBP, sterol regulatory element-binding protein.

However, there was no significant difference in the extent of liver fibrosis between WT and CCR7-deficient obese mice (Supplementary Figure S2). We also observed a significant increase in the weights of epididymal fat, brown fat and muscle in CCR7-deficient obese mice compared with those in WT obese mice (Supplementary Figures S3a and b). Morphological characteristics of the adipose tissue, brown adipose tissue and muscle were not significantly different between RD- or HFD-fed CCR7-deficient and WT mice (Supplementary Figure S3c). We next obtained liver tissue samples and assessed the expression of several pro-inflammatory and anti-inflammatory cytokines. RD-fed CCR7^{-/-} mice had higher levels of pro-inflammatory cytokines, including tumor necrosis factor- α and interleukin-6 (IL-6), in the liver than in WT mice (Supplementary Figures S4a and b). Notably, the expression of hepatic anti-inflammatory cytokines, including IL-10 and IL-13, was significantly lower in RD-fed CCR7^{-/-} mice than in WT mice (Supplementary Figures S4c and d). Similar results were obtained in HFD-fed CCR7^{-/-} mice. Collectively, these

observations suggest that CCR7 deficiency increases hepatic steatosis under both normal and obese conditions.

Adoptive transfer of CCR7-sufficient hepatic mononuclear cells to CCR7-deficient mice ameliorates obesity-induced hepatic inflammation and glucose tolerance

To assess whether CCR7 deficiency in hepatic immune cells is directly associated with the enhanced hepatic steatosis induced in HFD-fed CCR7^{-/-} mice, we conducted adoptive transfer experiments using hepatic mononuclear cells obtained from RD-fed WT or CCR7^{-/-} mice. Adoptive transfer of WT hepatic mononuclear cells into CCR7^{-/-} mice resulted in decreased weight gain compared with when CCR7^{-/-} cells were transferred to CCR7-deficient mice after HFD feeding (Figure 4a). More importantly, the intraperitoneal glucose tolerance test demonstrated that the transfer of WT cells into CCR7^{-/-} mice improved glucose tolerance (Figure 4b). Adoptive transfer of WT hepatic mononuclear cells to CCR7^{-/-} mice was significantly reduced in hepatic steatosis as

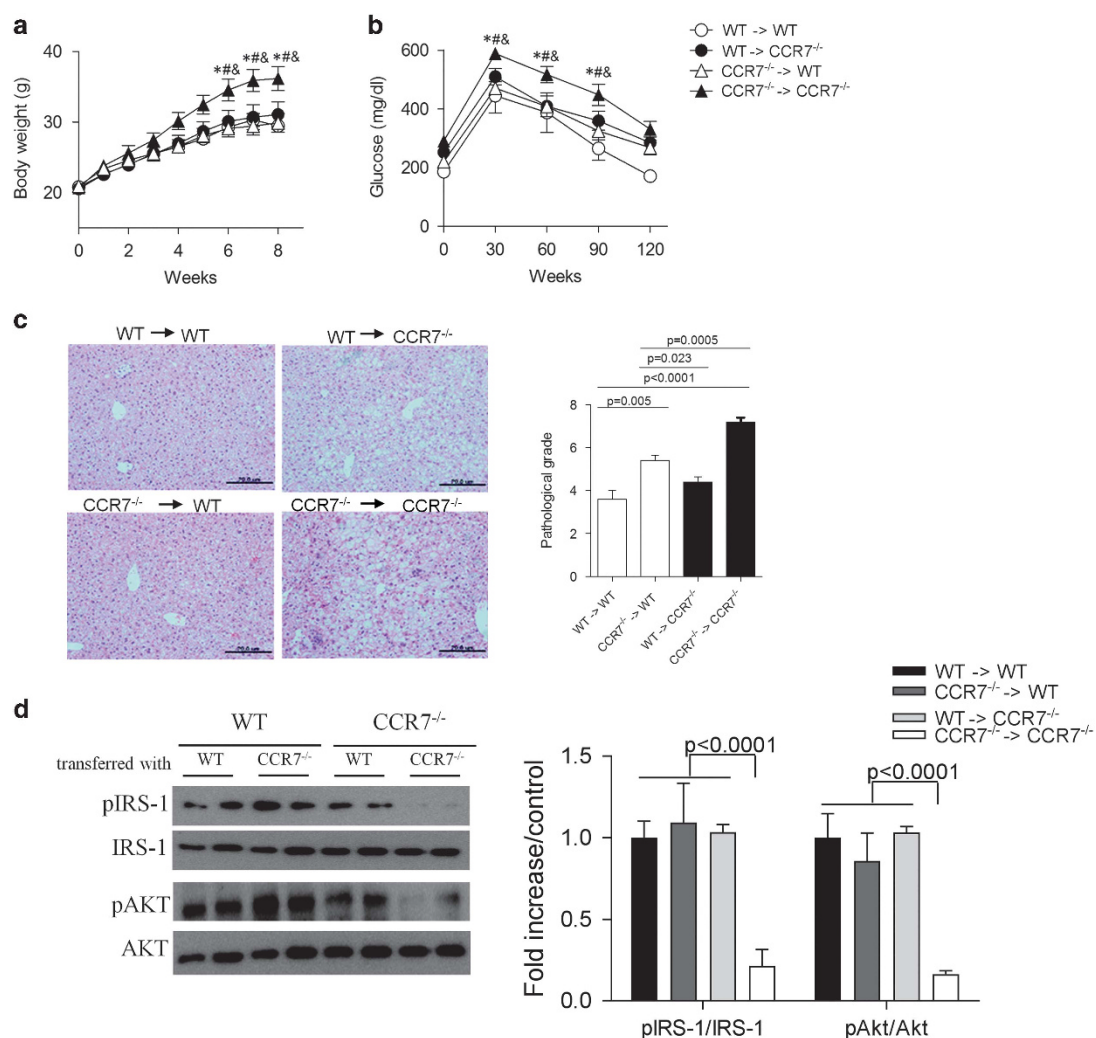


Figure 4. Adoptive transfer of CCR7-sufficient hepatic mononuclear cells to CCR7^{-/-} mice restored obesity-induced hepatic inflammation and glucose tolerance. WT ($n = 10$) and CCR7^{-/-} mice ($n = 10$) were transplanted with WT or CCR7^{-/-} hepatic mononuclear immune cells and fed an HFD for 8 weeks. **(a)** The body weight of mice was monitored for 8 weeks following transplantation. **(b)** Mice were fasted for 16 h and then intraperitoneally injected with glucose (1 g kg^{-1} body weight) to test glucose tolerance. * $P < 0.05$ compared with the WT mice transplanted with WT cells, # $P < 0.05$ compared with CCR7^{-/-} mice transplanted with WT cells, and & $P < 0.05$ compared with WT mice transplanted with CCR7^{-/-} cells. **(c)** Representative hematoxylin and eosin-stained images and pathological grades based on histological examination of the liver. *** $P < 0.001$, NS; not significant. **(d)** Western blots of insulin signaling molecules, pIRS-1 and pAkt, in the liver. Cropped membrane was used in western blot.

compared with that in CCR7^{-/-} mice transferred with CCR7^{-/-} hepatic mononuclear cells (Figure 4c). We found that the levels of phosphorylated IRS-1 and phosphorylated AKT were significantly higher in the livers of CCR7^{-/-} mice transferred with WT hepatic mononuclear cells compared with those in CCR7^{-/-} mice transferred with CCR7^{-/-} cells (Figure 4d). Thus, these observations suggest that the hepatic mononuclear cells present in the WT liver might play a role to improve glucose tolerance and ameliorate the hepatic steatosis in an obese condition via CCR7.

iNKT cells were diminished in the livers of CCR7^{-/-} mice

Based on the observation of severe hepatic inflammation with increased cellular infiltrates in the absence of CCR7 from liver histology, we next assessed the changes in inflammatory and immuno-regulatory cells in the liver. The percentage of iNKT cells (NK1.1^{mid}/CD3^{mid} or TCRβ⁺/CD1d tetramer⁺) in the liver was markedly reduced in CCR7^{-/-} mice compared with that in WT mice regardless of diet type (Figures 5a–c and Supplementary Figure S5a). Instead, the hepatic infiltration of CD8⁺ T cells and regulatory CD4⁺ T cells was significantly increased in HFD-CCR7^{-/-} mice compared with that in HFD-fed WT mice (Supplementary Figures S5b and c), whereas there was no significant difference in the infiltration of CD4⁺ T cells between RD- or HFD-fed CCR7^{-/-} mice and WT mice (Supplementary Figure S5d).

We also analyzed the infiltration of inflammatory mononuclear cells into the liver; however, there were no significant differences in the percentages of infiltrated neutrophils and macrophages, although CCR7 deficiency tended to induce liver leukocyte recruitment (Supplementary Figures S6a–d). In addition, in the lymph nodes, there were no significant alterations in the proportions of immune cells, including CD4⁺ and CD8⁺ T cells, and dendritic cells between HFD-fed CCR7^{-/-} and WT mice (Supplementary Figure S7). Therefore, we hypothesized that obesity-induced hepatic inflammation, resulting in insulin resistance and glucose tolerance could be associated with defective iNKT cells in CCR7^{-/-} mice.

In the adoptive transfer experiment, HFD-fed CCR7^{-/-} mice receiving WT hepatic mononuclear cells had significantly higher numbers of iNKT cells than HFD-fed CCR7^{-/-} mice receiving CCR7^{-/-} cells (Figures 5d and e), although there were no significant alterations in the proportions of immune cells, including CD4⁺ and CD8⁺ T cells, and macrophages between HFD-fed CCR7^{-/-} mice receiving WT cells or CCR7^{-/-} cells (Supplementary Figure S8). These results suggest that iNKT cells might represent a critical cell subset that has regulatory roles in suppressing obesity-associated hepatic inflammation, implying that defects in the regulatory function of iNKT cells might be responsible for the liver inflammation observed in obese CCR7^{-/-} mice.

IL-10-producing iNKT cells restored the hepatic steatosis in HFD-fed CCR7^{-/-} mice

IL-10 is a well-known immunomodulatory cytokine, and the presence of IL-10-producing iNKT cells (iNKT10 cells) with regulatory potential was recently reported as a novel subset of iNKT cells *in vivo* under steady-state conditions.²¹ This prompted us to assess the role of IL-10-producing iNKT cells in hepatic inflammation, insulin resistance and glucose tolerance in the HFD-fed CCR7^{-/-} condition. In this regard, we adoptively transferred hepatic mononuclear cells obtained from CD1d^{-/-} or IL-10^{-/-} mice into HFD-fed CCR7^{-/-} mice and assessed the levels of obesity-induced hepatic inflammation. Notably, liver inflammation was exacerbated in HFD-fed CCR7^{-/-} mice receiving hepatic mononuclear cells from CD1d^{-/-} or IL-10^{-/-} mice compared with that in mice receiving hepatic mononuclear cells from WT mice (Figures 6a and b). We next assessed whether the iNKT cells in the liver expressed IL-10. Interestingly, however, we found that the

number of IL-10-producing non-iNKT cells did not significantly differ between the WT and CCR7^{-/-} mice (Supplementary Figure S9). More importantly, the percentage of iNKT10 cells (TCRβ⁺/CD1d tetramer⁺/IL-10⁺) in the liver was markedly reduced in HFD-fed CCR7^{-/-} mice compared with that in HFD-fed WT mice. However, HFD-fed CCR7^{-/-} mice transplanted with WT hepatic mononuclear cells had a higher proportion of iNKT10 cells than those obtained from HFD-fed CCR7^{-/-} mice (Figures 6c and d). Further, HFD-fed CCR7^{-/-} mice also had some IL-10-producing non-iNKT cells, as there were non-iNKT cells producing IL-10 in HFD-fed WT mice, which was not significantly increased even after hepatic mononuclear cell transplantation (Supplementary Figure S9). This suggests that iNKT10 cells might have regulatory roles in suppressing obesity-associated liver inflammation.

DISCUSSION

Obesity is closely associated with the development of NAFLD, but the underlying mechanism has not yet been clarified. A recent study showed that infiltration of CCR7-expressing cells in the adipose tissue is associated with insulin resistance in obesity.²² However, the link between hepatic inflammation and obesity in CCR7-deficient animals had not been reported, and the role of CCR7-expressing immune cells in relation to the development of obesity-associated inflammation has remained unclear until now. CCR7 is a critical molecule for the migration of immune cells to the lymph nodes, and defects in CCR7 have been confirmed to be associated with autoimmune-like inflammation in several organs, including the lacrimal and submandibular glands and the kidney. Furthermore, a previous report demonstrated the onset of diabetes in CCR7-deficient mice.¹⁷

In the current study, we investigated the effect of CCR7 on NAFLD and NASH in an HFD-induced obese mouse model. NAFLD and NASH are commonly characterized by inflammation in the liver, and are the most prevalent chronic liver diseases.⁷ Several studies showed that fat accumulation in the liver inhibited insulin receptor signaling and induced insulin resistance.⁵ In addition to the accumulation of hepatic glycerolipids, there might be involvement of several other lipid classes for the induction of steatohepatitis and hepatic insulin resistance. In this regard, other recent studies suggested that several lipids second mediators such as diacylglycerols and sphingolipids were shown to play major roles in lipid-induced hepatic insulin resistance. Accumulation of diacylglycerols induced PKCε activation, and which in turn increased hepatic insulin resistance via inhibition of IRS-2 in the liver.^{7,23} In addition, it was shown that sphingolipids enhanced mitochondrial dysfunction, reactive oxygen species production and inflammation in liver.^{24,25} In the present study, body and liver weights were significantly different between WT obese and CCR7^{-/-} obese mice. In addition, hepatic glycerolipids levels were increased in CCR7^{-/-} obese mice, resulting in the release of chemokines, and hence the recruitment of inflammatory immune cells to the liver. These results suggest that CCR7 regulates hepatic lipid homeostasis and body fat accumulation.

In contrast to a recent report demonstrating that CCR7 deficiency improved glucose tolerance and insulin sensitivity,²² CCR7-deficient mice were shown to develop autoimmunity in various organs,¹⁷ and it was proposed that CCR7^{-/-} mice might carry an increased predisposition for the development of diabetes. In addition, it was shown that the lack of CCR7 induced the dysregulation of mucosal tissue homeostasis and development of autoimmune gastritis and exocrinopathy.^{26,27} We found that CCR7 deficiency upregulated fasting glucose and increased plasma insulin levels. There was also a significant decrease in the level of Akt phosphorylation and IRS-1 phosphorylation in the livers of CCR7^{-/-} mice.

Recent studies have investigated the role of infiltration and activation of inflammatory immune cells in hepatic insulin

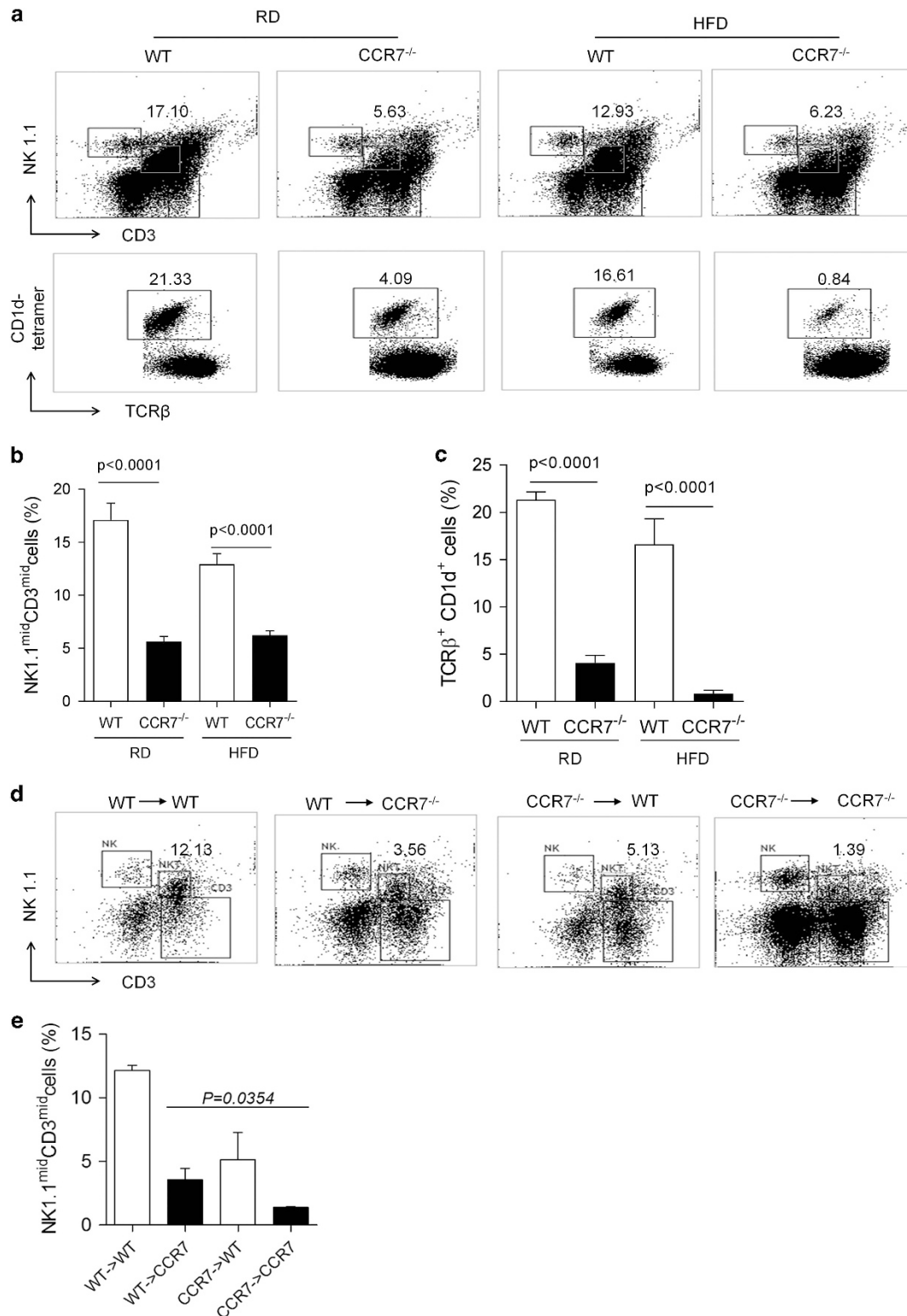


Figure 5. Invariant natural killer T (iNKT) cells decreased in the liver of CCR7^{-/-} mice. (a–c) iNKT cells represented by the NK1.1⁺ CD3⁺ or TCRβ⁺ CD1d tetramer⁺ population were analyzed from the livers of mice. (d and e) NK1.1⁺ CD3⁺ iNKT cells were analyzed from the livers of WT and CCR7^{-/-} mice transplanted with WT or CCR7^{-/-} hepatic mononuclear immune cells.

resistance.^{7,28,29} Immune cells, including CD4⁺ and CD8⁺ T cells, iNKT cells, B cells, neutrophils and macrophages, release inflammatory cytokines and chemokines. Inflammatory immune cells can induce insulin resistance in the liver.³⁰ Wolf *et al.*³¹ showed that CD8⁺ T cells and iNKT cells produce NASH; however,

they also showed that the liver CD3⁺/NK1.1⁺/αGC⁺ iNKT cell population was decreased in NASH. Our study suggests that increased hepatic inflammatory immune cell (CD4⁺, CD8⁺ T cells, granulocytes, and macrophages) infiltration in CCR7^{-/-} mice might activate an inflammatory response in the liver. Interestingly,

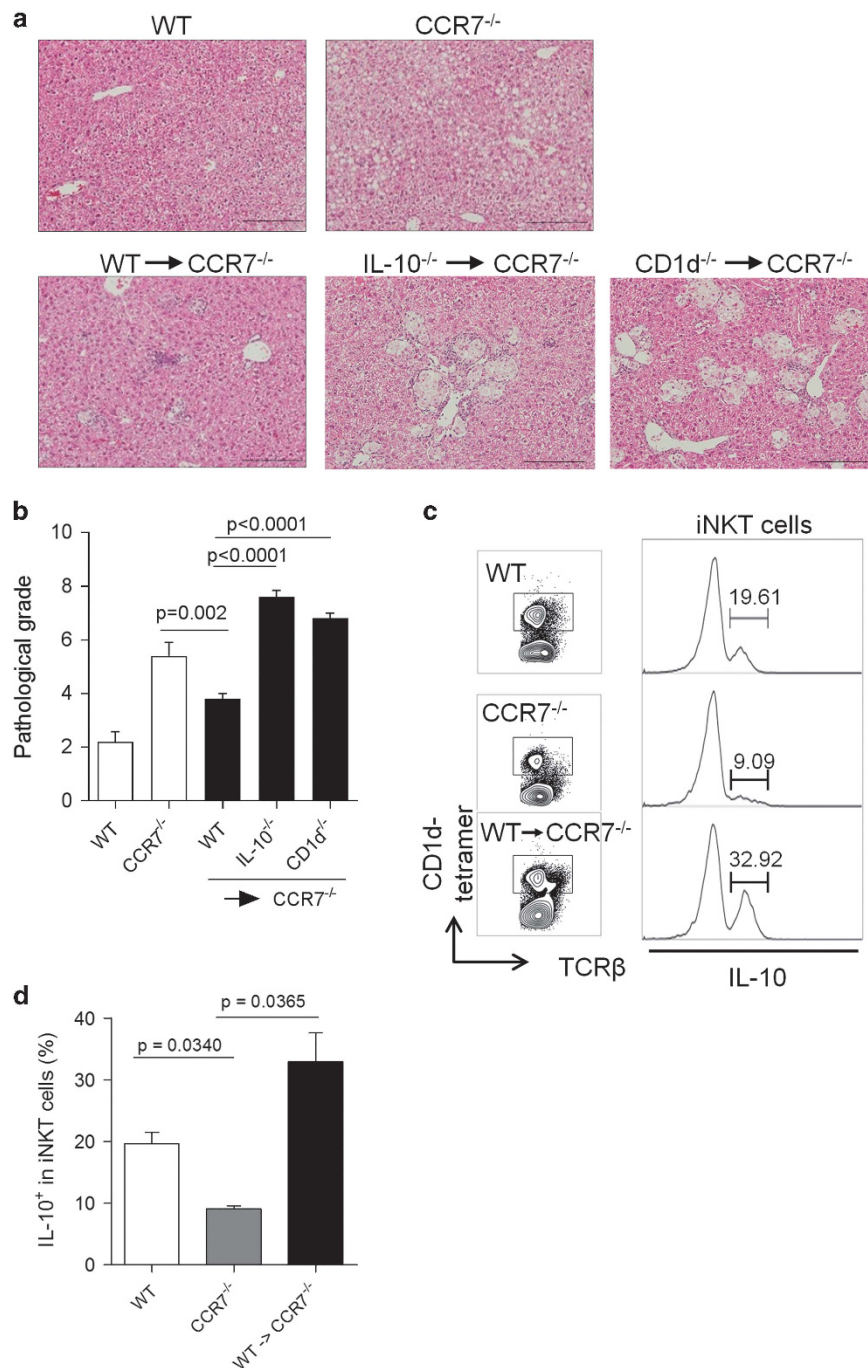


Figure 6. IL-10-expressing invariant natural killer T (iNKT) cells restored the hepatic steatosis in obese CCR7^{-/-} mice. CCR7^{-/-} mice ($n = 10$) were transplanted with WT, IL-10^{-/-} or CD1d^{-/-} hepatic mononuclear immune cells and then fed an HFD for 8 weeks. **(a)** Representative hematoxylin and eosin-stained images and **(b)** pathological grades based on histological examination of the liver. **(c)** TCRβ⁺/CD1d tetramer⁺/IL-10⁺ iNKT population were analyzed from the liver of WT mice, CCR7^{-/-} mice and CCR7^{-/-} mice which were transplanted with WT hepatic mononuclear immune cells. For the determination of iNKT cell expressing IL-10, liver cells were stained with anti-CD1d tetramer-APC and anti-TCRβ-FITC. The level of IL-10 expression in CD1d tetramer⁺ TCRβ⁺ iNKT cells was determined after intracellular cytokine staining. For intracellular staining for IL-10, the cells were stimulated with PMA (50 ng ml⁻¹)/ionomycin (750 ng ml⁻¹) and then fixing and permeabilization buffer were used as per the manufacturer's instructions. **(d)** Percentage of IL-10 expressing iNKT cells.

the proportion of iNKT cells was significantly decreased in CCR7^{-/-} mice. Cowan *et al.*¹⁶ showed that CCR7 regulated the development of iNKT cells in the thymus, and influenced the balance of Foxp3⁺ Treg cells. This result is in accordance with that from a previous report, suggesting that CCR7 is required for the intrathymic development of iNKT cells, and that CCR7 deficiency significantly reduced the percentage and absolute numbers of

total thymic iNKT cells.¹⁶ Thus, we think that the reduction of iNKT cell development in the thymus may result in the decreased number of hepatic iNKT cells in CCR7^{-/-} mice. However, we could not rule out the possibility that iNKT cells could migrate into the liver in a CCR7-dependent manner or that the decreased migration of CCR7-deficient cells results in the accumulation of other hepatic mononuclear cells other than iNKT cells. In the other

hand, liver-associated Treg cells were increased in CCR7^{-/-} obese mice compared with WT obese mice, suggesting that Treg cells might not be functional in the liver, as previously suggested using a colitis model.¹⁵ Type II iNKT cells have shown a protective effect against autoimmune hepatitis.³² Interactions between iNKT cells and hepatic dendritic cells induced iNKT cell activity, and activated iNKT cells increased the prevalence of inflammatory diseases, including autoimmunity.³³ Thus, the present results suggest that CCR7 also regulates the iNKT cells in the liver.

iNKT cells have a lipid antigen-binding major histocompatibility complex class I-like molecule (CD1d). Recent studies showed that the number of iNKT cells changed significantly in a variety of metabolic-related inflammatory liver diseases; in particular, iNKT cells were significantly decreased in the liver of HFD-induced obese and *ob/ob* mice.^{34,35} In addition, CD1d-deficient mice had impaired glucose tolerance, adipose tissue inflammation and hepatic steatosis. Adoptive transfer of iNKT cells was shown to ameliorate glucose intolerance and hepatic steatosis in *ob/ob* mice.³⁴ Similarly, upon injection of an activator of iNKT cells (α -galactosylceramide), proliferation of iNKT cells and improved glucose tolerance in *ob/ob* mice were observed.³⁶ Ji *et al.*³⁶ suggested that iNKT cells regulate hepatic M2 macrophage polarization in the adipose tissue. Our data showed that the adoptive transfer of hepatic mononuclear cells obtained from CD1d-deficient mice into CCR7-deficient mice resulted in increased hepatic inflammation compared with that observed after the adoptive transfer of hepatic mononuclear cells from WT mice. This result suggests that iNKT cells might be critical for the prevention of obesity-related hepatic steatosis and inflammation.

We found that iNKT10 cells were significantly increased in HFD-fed CCR7^{-/-} mice after adoptive transfer of WT hepatic mononuclear cells. Thus, we conclude that the lower number of iNKT10 cells in HFD-fed CCR7^{-/-} mice might be a major cause of the hepatic steatosis. However, it remains unclear which mechanism ultimately accounts for the phenotypic differences, which requires further studies.

iNKT cells regulate innate and adaptive immunity via cytokine production (such as IL-4, IL-10, and IL-13). IL-10 is secreted from various cells in many organs, including the liver, and the production of IL-10 has been reported in endothelial cells, Kupffer cells and lymphocytes of the liver.³⁷ This cytokine inhibits the production of inflammatory cytokines.^{38,39} Many studies have demonstrated that low levels of IL-10 are associated with insulin resistance in obese patients.^{40,41} In addition, IL-10 regulates NAFLD via M2 macrophages, through the promotion of M1 macrophage apoptosis.⁴² In the current study, obesity-induced hepatic inflammation was increased in CCR7-deficient mice receiving hepatic mononuclear cells from IL-10-deficient mice compared with that in mice receiving hepatic mononuclear cells from WT mice.

In conclusion, we provide the first demonstration of the link between CCR7 and hepatic lipid homeostasis via hepatic immune cells. The absence of CCR7 decreased IL-10-producing iNKT cells in the fatty liver and exacerbated NAFLD/NASH. These results suggested that IL-10-producing iNKT cells could regulate obesity-induced hepatic inflammation and prevent hepatic steatosis in a CCR7-dependent manner.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

HMK and BRL conducted the experiments, data analysis and wrote the manuscript. ESL, MHK, JHH, B-EK and E-KP conducted the experiments and data analysis. S-YC, M-NK and P-HK contributed to the development of analytical tools and discussion. S-YC and M-NK contributed to the experimental design and discussion. H-JK and CHC contributed to the experimental design and wrote the manuscript.

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