International Immunopharmacology 72 (2019) 224–234

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

CXCR6 deficiency ameliorates ischemia-reperfusion injury by reducing the recruitment and cytokine production of hepatic NKT cells in a mouse model of non-alcoholic fatty liver disease

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ARTICLE INFO

Keywords:
Fatty liver
Ischemia-reperfusion injury
Hepatic NKT cells
CXCR6

ABSTRACT

Fatty liver is used for transplantation due to organ shortage, but prone to cause complications like ischemia-reperfusion injury (IRI). NKT cells as a bridge between innate and adaptive immunity were reported to infiltrate the liver at the early phase of IRI induced in normal liver. However, the localization mechanism of NKT cells is not precise, and the role of NKT cells in fatty liver IRI is poorly understood. In present murine IRI model of non-alcoholic fatty liver disease, we demonstrated that although the number reduced in fatty liver, NKT cells still activated and accumulated to fatty liver following IRI, and contributed to IRI by producing inflammatory cytokine IFN-γ. We revealed that NKT cells in fatty liver expressed more CXCR6, a vital chemokine receptor; meanwhile, the ligand CXCL16 mRNA expression level in fatty liver was up-regulated. The up-regulation of the CXCR6/CXCL16 axis in fatty liver happened in IRI, which maybe endow NKT cells more chemotaxis. We further found CXCR6 deficiency reduced the recruitment of NKT cells in a tissue-dependent manner, and impaired the IFN-γ producing capacity of hepatic NKT cells. Serum ALT level and hepatic histology were both improved in CXCR6 deficient mice. The results provide evidence of the pathogenic role of NKT cells in fatty liver IRI, and important localization mechanism involving up-regulated CXCR6/CXCL16. Deficiency of CXCR6 protects the fatty liver from IRI by reducing the recruitment and cytokine production of hepatic NKT cells.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) refers to an increasingly diagnosed condition involving fatty acid accumulation in hepatocytes resulting in a broad spectrum of liver injury [1]. NAFLD represents the most common chronic liver disease around the world, which affects approximately 30% of potential donors for liver transplantation [2]. The shortage of available liver organs led to the use of fatty liver for transplantation. Ischemia-reperfusion injury (IRI) is a significant complication of liver transplantation. Previous studies have shown that the degree of steatosis in NAFLD negatively impacts liver recovery from IRI [3,4]. Also, the use of fatty liver, especially with macrovesicular steatosis, has shown to be a risk factor for primary graft non-function [5], dysfunction [6], and decreased graft and patient survival [7,8]. Therefore, most transplant centers limit the donor organ percentage of steatosis below 60% [1,9,10], which reduces potential donor with steatosis significantly. Improve IRI in the fatty liver will help to increase available donor organs.

Hepatic IRI is mediated by a biphasic inflammatory response; the early phase involves Kupffer cell activation, $CD4^+$ cells accumulation and secretion of inflammatory cytokines and the later stage involves injury caused by neutrophil granulocytes [11]. $CD4^+$ cells accumulated in the initial period consist of mostly natural killer T (NKT) cells [12,13]. NKT cells are a unique but heterogeneous group of T cells that express both a TCR and NK markers, which recognize lipid antigens presented in the complex of MHC-I like molecule CD1d [14,15]. CD1d-restricted NKT cells are categorized into Type I and Type II NKT cells. Type I or invariant NKT cells express a semi-invariant T-cell receptor (TCR) encoded by the Vα14-Jα18 gene segments in mice and Vα24-Jα18 in human.

NKT cells are widely distributed in the body, whereas thymus and liver contain primary CD1d-dependent NKT cells, suggesting the

Abbreviations: NAFLD, non-alcoholic fatty liver disease; IRI, ischemia-reperfusion injury; TCR, T cells receptor; WT, wild-type; HF, high-fat; ND, normal diet; HMNCs, hepatic mononuclear cells; KO, knock-out; PMA, phorbol myfismte acetate; ALT, Alanine Aminotransferase

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https://doi.org/10.1016/j.intimp.2019.04.021
Received 31 December 2018; Received in revised form 26 March 2019; Accepted 8 April 2019
Available online 16 April 2019

character of tissue-specific of localization and activation of NKT cells [16]. Chemokine differential expressed in tissue and inflammatory microenvironments control homing and localization of leukocytes through various surface chemokine receptors [17]. Chemokine receptors expressed upon NKT cells are potentially crucial for trafficking and reveal their cytokine production capacity [18]. The previous study in mice indicated that chemokine receptor CXCR6 and its ligand could control the paroling of NKT cells during homeostasis [19]. CXCL16 can be released by endothelial cells to mediate chemokine-dependent activation of leukocytes, and CXCL16 functions as an adhesive molecule anchoring activated NKT cells [20]. CXCR6 and its ligand can localize NKT cells to cardiac allografts [21], kidney [22] and several inflammatory diseases [23–26].

The previous study has shown that in NAFLD, dietary factors alter the immune system, including NKT cells [27]. Several studies indicated that NKT cells contribute to IRI in normal livers [11,28]; however, to date, there is no evidence of the role of NKT cells in fatty liver IRI. Also, it is not clear whether and how chemokine receptor and its ligand contribute to the localization and recruitment of NKT cells in IRI. To address these issues, we established a murine model of hepatic IRI model in NAFLD. Our objectives were to determine the role and accumulation mechanism of NKT cells in fatty liver IRI and seek protective strategies.

2. Materials and methods

2.1. Animals

Adult (6–8 weeks) male wild-type (WT) C57BL6 mice and C57BL/6 background CXCR6 KO (CXCR6 knock-out, and CXCR6$^{gfp/gfp}$, in which a green fluorescence protein cDNA was used to replace the gene coding of CXCR6) mice were purchased from Jackson Laboratories (Bar Harbor, ME). CXCR6 KO mice were backcrossed to C57BL6 background. CD1d KO (CD1d knock-out) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were fed for 12 weeks with commercial high-fat diets (Bioserv, Frenchtown, NJ) with the percentage of calorie source from fat (59.1%) or normal diet (Bioserv, Frenchtown, NJ) with the percentage of calorie source from fat (18%). All mice were maintained in a temperature- and light-controlled facility, and permitted ad libitum consumption of water and pellet chow. All animal experiments fulfilled NIH and Institutional criteria for the humane treatment of laboratory animals.

2.2. Ischemia-reperfusion injury model

Mice were anaesthetized with isoflurane and oxygen inhalation (Baxter Healthcare, Deerfield, IL). After laparotomy, an atraumatic micro clip was applied to the hepatic triad of the three cephalad liver lobes. The caudal lobes retained intact blood circulation to prevent intestinal venous congestion. During the procedure, mice were avoided with a drop-in core temperature and dehydration. After 60 min of partial hepatic warm ischemia, the micro-clip was removed, initiating reperfusion and the abdominal wall was sutured, and the animals were returned to their cages. Mice were euthanized at 6 h or 24 h after reperfusion. Whole blood samples were collected before the removal of the cephalad liver lobes for histology. Sham controls underwent the same procedure but without vascular occlusion.

2.3. Preparation of liver and spleen mononuclear cells

Mouse livers were perfused with a sterile saline solution to remove blood cells, then carefully removed and minced. The liver and spleen were homogenized and passed through a 70-micron wire mesh to remove connective tissue. Hepatic mononuclear cells (HMNCs) were then isolated with a 37.5% Percoll gradient (Amersham Pharmacia Biotech) after centrifugation at 900 $\times g$ for 20 min. HMNCs and splenocytes were isolated after removal of red blood cells.

2.4. Flow cytometry

After blocking with anti-mouse FcR-γ (BD Pharingen, San Diego, CA), cells were incubated with a PBS57-loaded CD1d tetramer (NIH tetramer facility) or anti-mouse fluorescent antibodies against CD8, CD4, TCR-β, CD69 and NK1.1 (Biolegend, San Diego, CA). For intracellular staining, cells were incubated with phorbol myfismte acetate (PMA, 50 ng/ml), ionomycin (500 ng/ml), and Golgistop (0.7 μl/ml). Cells were labelled with surface antibody and then permeabilized with Cytoperm/Cytofix (BD Pharingen, San Diego, CA) according to the manufacturer's instruction. After permeabilization, cells were further labelled for intracellular cytokines IFN-γ (Biolegend, San Diego, CA). After incubation, cells were evaluated by flow cytometry.

2.5. Liver histology

Thin slices of liver were stained with hematoxylin and eosin. Liver tissue frozen sections were prepared from CXCR6 mice and analyzed by fluorescence microscope. An independent pathologist assessed histology and NAFLD activity score (NAS). Steatosis was graded (0–3), lobular inflammation was scored (0–3), and ballooning was rated (0–2).

2.6. Measurement of serum Alanine Aminotransferase (ALT) levels

ALT levels were measured with a multichannel autoanalyzer in the Clinical Chemistry Laboratory of the Johns Hopkins University Department of Comparative Medicine.

2.7. RNA preparation and qRT-PCR

The total RNA extracted from frozen liver tissue samples by using. cDNA was synthesized from total RNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). The primers used were 5′-TGCACCACCAACTGCTTAGC-3′ and 5′-GGCATGGACTGTGGTCATGAG-3′ for GAPDH, 5′-TGAACTAGGGACTGCTTTGAG-3′ and 5′-GCAAATGTTTTTGGTGGTGA-3′ for CXCL16, 5′-CCCTGTACTTTATGCCTTTG-3′ and 5′-CTTGGAACTGTCCTCAGAAG-3′ for CXCR6. Quantified PCR amplifications were performed using SYBR GREEN PCR Master Mix (Applied Biosystems) with Quantstudio 12k flex (Applied Biosystems). All estimated mRNA values were normalized to GAPDH mRNA levels. Each experiment was repeated at least twice, and representative data are shown.

2.8. Statistical analysis

All values are expressed as the means ± SEM. The group means were analyzed statistically by a Student *t*-test with Microsoft Excel (Microsoft, Redmond, WA) or GraphPad Prism5. p values of < 0.05 were considered statistically significant.

3. Result

3.1. NKT cells are reduced in high-fat diets induced macrovesicular hepatic steatosis

To establish a murine model of IRI in fatty liver, we fed mice with high-fat diets for 12 weeks. We found HF diet-fed mice gained more body weight ($p < 0.001$ vs ND, Fig. 1A) and liver weight (Fig. 1B). The histological examination of the livers showed that fatty droplets developed after the feeding of the HF diet, containing ballooning and predominant macrovesicular steatosis (Fig. 1D). NAS was assessed to quantify inflammation and steatosis. We found that high-fat diet fed mice had increased NAS (Fig. 1E). To explore the effect of high-fat diet and macrovesicular steatosis on NKT cells, we evaluated hepatic

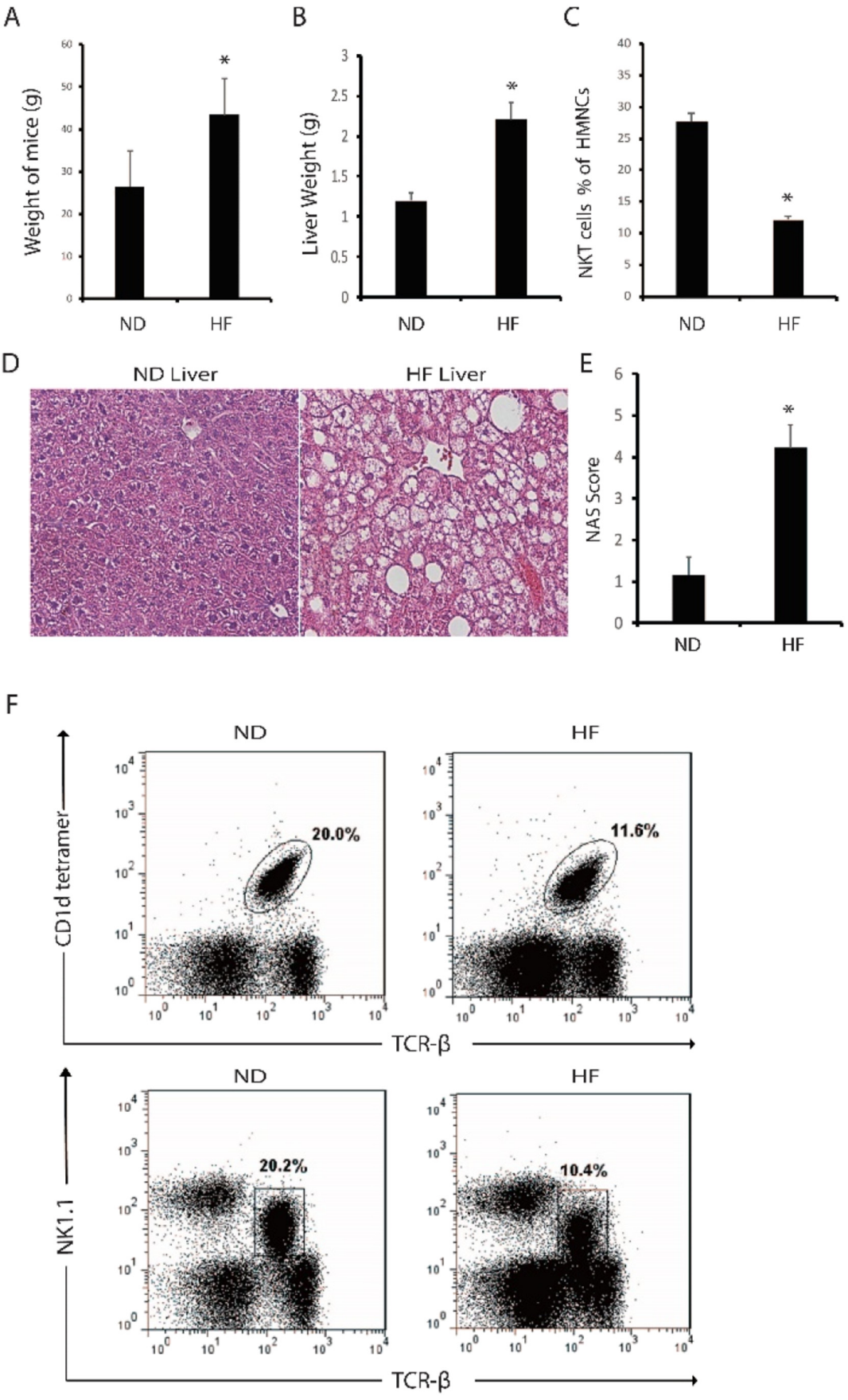

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Fig. 1. NKT cells are reduced in high-fat diets induced macrovesicular hepatic steatosis. Wild-type C57BL6 mice were fed high-fat (HF) or normal diet (ND) for 12 weeks. (A) Body weight of mice. (B) Liver weight of mice. (C) Histogram of percentage of NKT cells in HMNCs. (D) H&E staining images (magnification ×200) of liver sections from HF-fed mice and ND-fed mice (E) Graphical depiction of NAS between HF-fed mice and ND-fed mice. Data are presented as mean ± SEM with n = 10 mice/group with *$p < 0.05$ (HF vs ND). (F) HMNCs were stained with anti-TCR-β, -NK1.1 antibodies, and PBS57-loaded CD1d tetramer, and were analyzed by flow cytometry. Representative dot plots of percentages of NKT cells in livers from HF-fed mice and ND-fed mice. Results are representative of at least three independent experiments with similar results.

mononuclear cells using cell surface markers and flow cytometry. We found a significant reduction of hepatic NKT cells in mice fed a high-fat diet (Fig. 1C). We analyzed NKT cells by using two gating methods (Fig. 1F) and got the same result. Strictly, PBS57/CD1d tetramer$^+$TCR-β$^+$ stands for type I NKT cells, while NK1.1$^+$TCR-β$^+$ includes type I and type II NKT cells. However, the difference is not absolute in actual applications. Consequently, high-fat diets induce steatosis and cause hepatic NKT cells depletion. Interestingly, we observed that NKT cell counts remained constant among a small number of mice fed with high-fat diets but failed to develop fatty liver (data not shown).

3.2. Hepatic NKT cells activate and contribute to fatty liver IRI by producing inflammatory cytokine IFN-γ

We next aimed to determine the role of NKT cells in IRI. We examined the activation, number, and function of hepatic NKT cells. The activation of hepatic NKT cells during IRI was determined by the expression of the early surface activation marker CD69 [29]. The expression of CD69 on NKT cells is elevated in IRI (Fig. 2A). We found a significant increase in NKT cells in IRI groups (6 h and 24 h time points) compared with the sham group (Fig. 2B). The frequency of NKT cells in HMNCs in fatty liver increased firstly and then decreased up to 24 h ((5.35% → 26.3% → 9.68%, Fig. 2B). The increase of NKT cells from sham to IRI in fatty liver presented more dramatically than that in normal liver. We further examined inflammatory cytokines IFN-γ produced by NKT cells. IFN-γ$^+$ NKT cells increased significantly after reperfusion ($p < 0.05$ vs sham, Fig. 2D). Compared with normal liver, NKT cells in fatty liver produced significantly more inflammatory cytokine IFN-γ ($p < 0.05$ vs ND, Fig. 2D).

Also, serum ALT levels were measured at 6 h (the early stage) and 24 h (the late stage) after reperfusion. Compared with the sham group, IRI groups showed elevated ALT levels (Fig. 2E). Histologically, IRI groups showed increased infiltration of inflammatory cells, and more hepatic severe sinus congestion (Fig. 2F).

Percentage of NKT cells and IFN-γ producing NKT cells changed with the severity of the injury indicated by serum ALT level and liver histology, which suggests that the pathogenic role of NKT cells in inducing IRI not only in the normal liver but also in the fatty liver.

3.3. Improved IRI in the absence of NKT cells in the fatty liver

It is well documented that activation of NKT cells is CD1d restricted. To furtherly verify the pathogenic role of NKT cells in IRI, we compared CD1d KO mice and WT mice. The flowchart shows almost deficiency of hepatic NKT cells in CD1d KO mice (Fig. 3A). As a marker of hepatocellular damage, serum ALT enzyme was assessed in mice after 60 min of ischemia and 6 h of reperfusion. In CD1d KO mice, ALT levels are significantly decreased (Fig. 3B). Serum IFN-γ level in WT mice is significantly higher compared with that in CD1d KO mice (Fig. 3C). Liver tissue IFN-γ and TNF-α mRNA were quantified by real-time RT-PCR and normalized of GAPDH mRNA. Relative expressions of IFN-γ and TNF-α mRNA are significantly higher in WT mice compared with that in CD1d KO mice (Fig. 3D and E). Liver histology in CD1d KO mice showed alleviated IRI (Fig. 3F).

3.4. Fatty liver NKT cells express more CXCR6, and its ligand CXCL16 mRNA is up-regulated in the fatty liver during IRI

We firstly tested CXCL16 and CXCR6 mRNA expression during IRI in fatty liver tissue from WT mice and found the former up-regulated (Fig. 4B) while the latter down-regulated (Fig. 4A), compared with that in normal liver. The result of up-regulation of CXCL16 mRNA and protein in the steatotic liver is consistent with some previous studies. Our subsequent effort was directed at determining CXCR6 expression on hepatic NKT cells during IRI in WT mice. Data revealed that fatty liver NKT cells were expressing more CXCR6 (Fig. 4C).

To verify this result, we used CXCR6 deficient mice, in which the CXCR6 coding sequence was replaced by a reporter gene GFP. Although GFP (CXCR6) was expressed on CD4$^+$ T, NK, and CD8$^+$ T cells, most of GFP (CXCR6) expressing cells in liver were NKT cells (Fig. 4D), which could be found not only in the normal liver but also in the fatty liver. Although there were less GFP expressing cells among HMNCs in fatty liver (Fig. 4F), there were more GFP expressing cells among NKT cells in the fatty liver (Fig. 4E). In summary, fatty liver NKT cells in WT mice expressed more CXCR6, and fatty liver NKT cells in CXCR6 reporter mice expressed more GFP, these results were consistent and confirmed that fatty liver NKT cells express more CXCR6 during IRI. GFP expressing cells infiltrated in the liver can be detected directly by fluorescence microscopy (Fig. 4G).

3.5. CXCR6-expressing CD8, CD4 T cells and NK cells do not significantly change in CXCR6 deficient mice

Besides to NKT cells, CXCR6 expressing cells also include CD4, CD8 T cells, and NK cells (Fig. 4D). It is questionable whether liver infiltrated CD4, CD8 T cells and NK cells are involved in mediating IRI. A previous study has proved that conventional T cells were not crucial in hepatic IRI, in which the injury was reduced by depletion of NKT cells but not by depletion of CD4, CD8 or NK cells [30]. To understand the relationship of CXCR6 and these liver infiltrated cells. We analyzed the percentage of CD4, CD8 T cells and NK cells by flow cytometry between WT mice and CXCR6 KO mice. We found no significant difference in the portion of CD8 T cells between WT mice and CXCR6 KO mice (Fig. 5A and B). CD4 T cells (Fig. 5C and D) and NK cells (Fig. 5E and F) are not significantly changed. The data suggest that liver infiltrated CD4, CD8 T cells and NK cells express CXCR6, but not CXCR6 dependent. There might be other chemokines involved in the infiltrating of these cells.

3.6. CXCR6 deficiency protects the fatty liver from IRI by reducing hepatic recruitment of NKT cells and IFN-γ production

Given the different level of CXCR6 expression on NKT cells, we speculated that CXCR6 regulate NKT localization and function. We firstly compared NKT between CXCR6 KO and WT mice in normal liver. Flow cytometry analysis revealed that hepatic NKT cells decreased significantly in CXCR6 KO mice, while there was no significant alternation in splenic NKT cells (Fig. 6A and B), which might be concluded that CXCR6 KO reduced the recruitment of NKT cells in a tissue-dependent manner. It is possible that hepatic NKT cells are unique subsets and more dependent on CXCR6, or splenic NKT cells have other mechanisms for localization. We next compared the frequency of NKT cells in the fatty liver during IRI and found fewer NKT cells accumulated in the injured liver in CXCR6 deficiency mice (Fig. 6C). These results

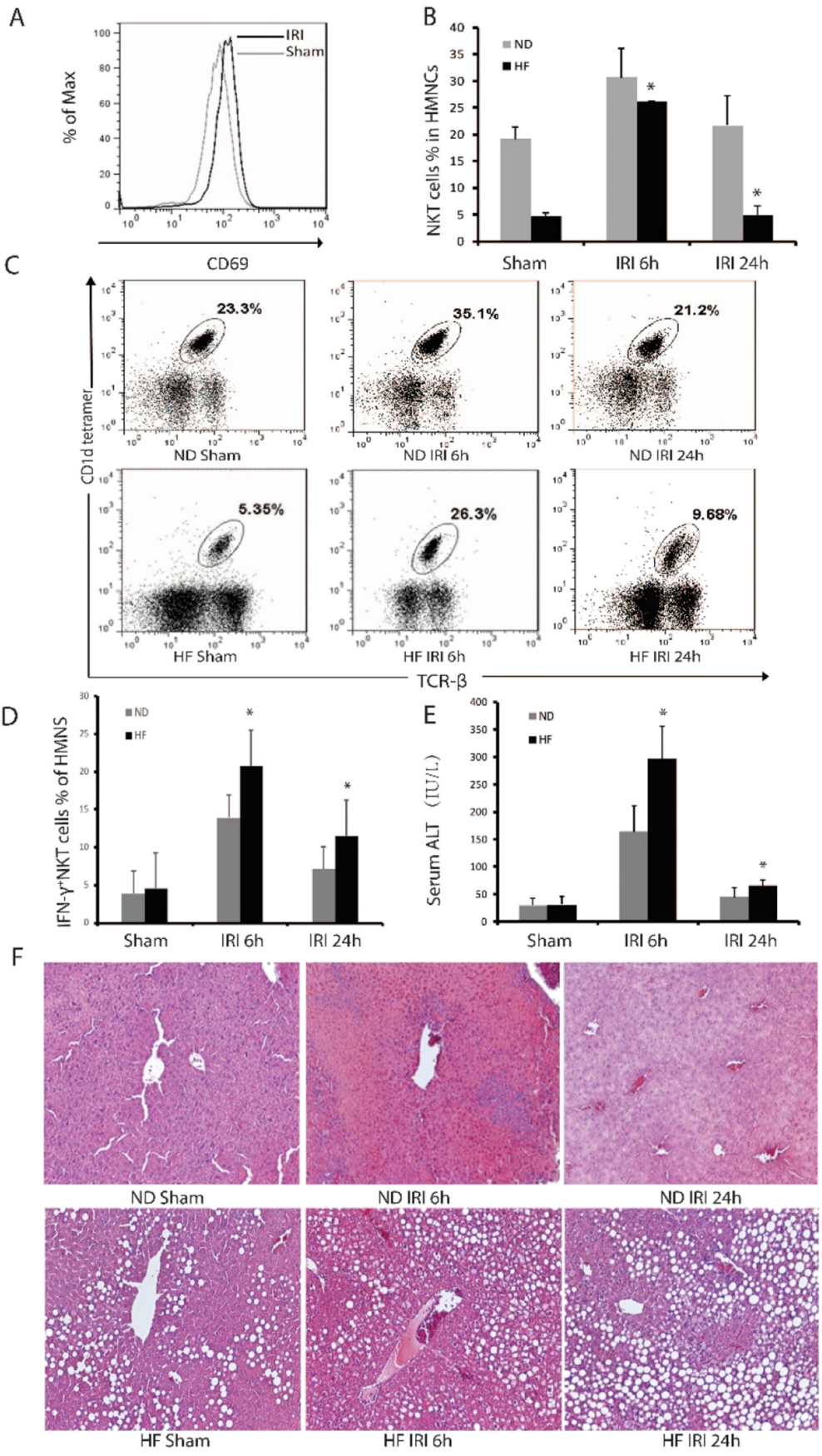

Fig. 2. Hepatic NKT cells activate and contribute to fatty liver IRI by producing inflammatory cytokine. ND and HF diet-fed wild-type C57BL6 mice were sacrificed at 6 h and 24 h after reperfusion. HMNCs were stained with anti-TCR-β, -CD69, -IFN-γ antibodies and PBS57-loaded CD1d tetramer, and were analyzed by flow cytometry. (A) Surface activation marker CD69 expression on NKT cells. (B) Histogram of percentages of hepatic NKT cells during IRI. (C) Representative dot plots of percentages of NKT cells in HMNCs during IRI. (D) Percentage of IFN-γ producing NKT cells in HMNCs. (E) Serum ALT level during IRI. Data are presented as mean ± SEM with n = 10–15 mice/group with *$p < 0.05$ (IRI vs Sham). (F) Histological injury by H&E staining images (magnification ×100) of liver sections from HF-fed mice at different time points after reperfusion.

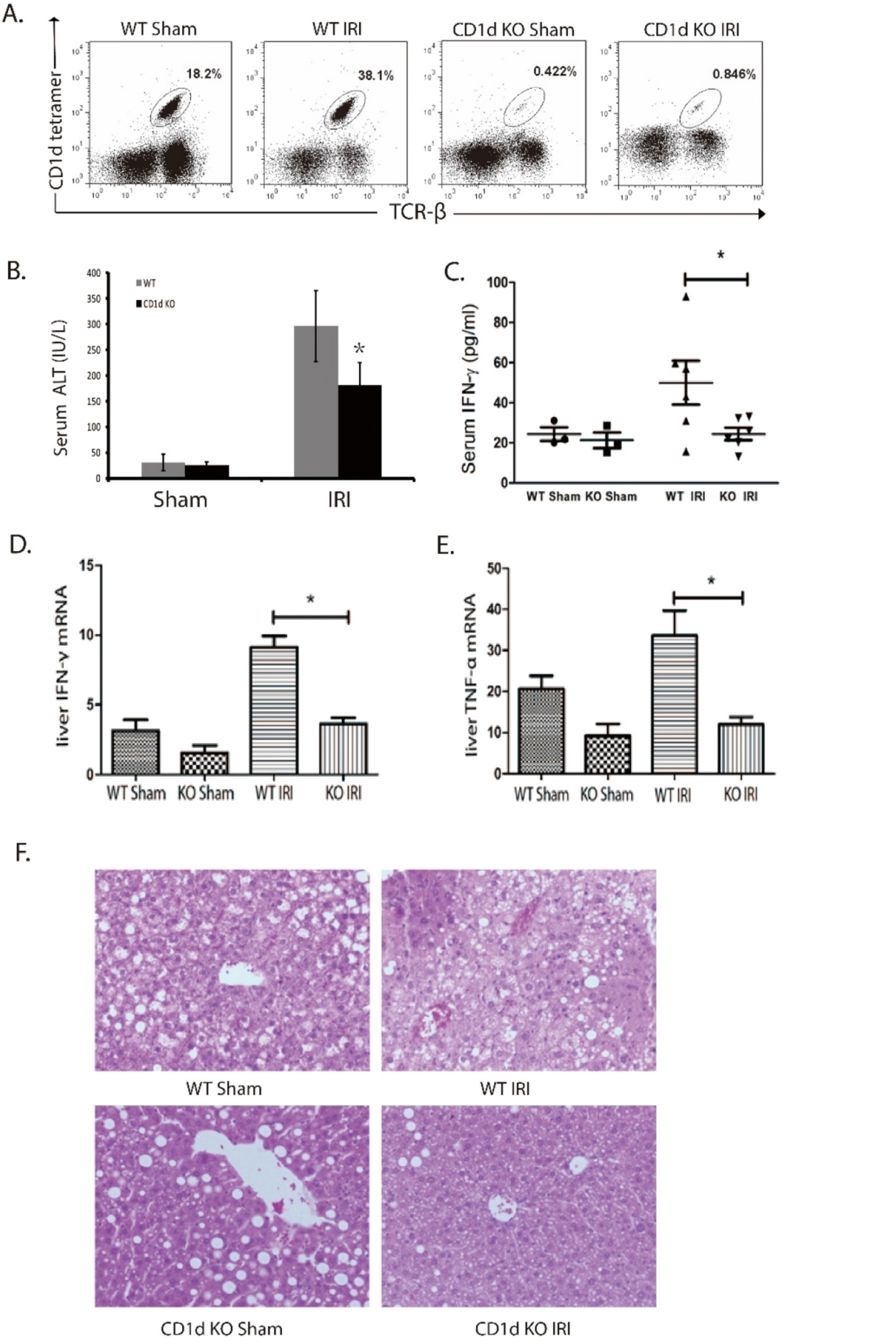

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Fig. 3. Improved IRI injury in the absence of NKT cells in the fatty liver (A–B) HF diet-fed WT C57BL6 mice and CD1d KO mice were sacrificed at 6 h after reperfusion or Sham surgery. (A) HMNCs were stained with anti-TCR-β and PBS57-loaded CD1d tetramer and were analyzed by flow cytometry, representative dot plots of percentages of NKT cells in HMNCs in IRI and sham surgery. (B) Bar graphs depict serum ALT levels of IRI and Sham group. (C) ELISA of serum IFN-γ levels between IRI and Sham groups. (D–E) Total RNA in liver tissue was extracted, and IFN-γ and TNF-α mRNA were quantified by real-time RT-PCR and normalized of GAPDH mRNA. (D) IFN-γ mRNA relative expression in liver tissue. (E) TNF-α mRNA relative expression in liver tissue. (F) Histological injury by H&E staining images (magnification ×200) of liver sections from WT and CD1d KO mice at 6 h after reperfusion.

indicate that CXCR6 deficiency reduces recruitment of hepatic NKT cells not only in the normal liver but also in fatty liver, especially during IRI.

The previous study has shown that cytokines-producing capacity of NKT cells responding to the stimulation with α-Galcer was impaired in CXCR6 KO mice [31]. In the present study, we investigated intracellular IFN-γ production from fatty liver NKT cells during IRI. Percentage of CD1d-tetramer$^+$ and IFN-γ$^+$ cells was significantly reduced in CXCR6 KO mice (Fig. 6D). Our study indicated that CXCR6 deficiency decreased the recruitment of hepatic NKT cells during IRI, and inhibited its capacity to produce inflammatory cytokine. To explore the relevance of this mechanism on the outcome of fatty liver injury, we measured serum ALT level. Serum ALT level of CXCR6 KO mice was significantly decreased compared with wild-type mice (Fig. 6E). Histologically, liver sections from CXCR6 KO mice showed reduced infiltration of inflammatory cells and improved hepatic sinus congestion (Fig. 6F).

4. Discussion

Although fatty liver is currently used for transplantation according to extended criteria due to donor shortage, clinical data have shown that liver allograft with macrovesicular steatosis is more susceptible to injury, leading to elevated serum ALT level and diminished liver function [32]. Fatty liver can be classified as macrovesicular or microvesicular steatosis, depending on the size of the lipid vacuoles. Here we found high-fat diet feeding for 12 weeks in mice can develop macrovesicular hepatic steatosis successfully.

We found NKT cells significantly decreased in the liver with steatosis and this is consistent with several previous studies. In these studies, nonalcoholic fatty liver mice models were induced by high-fat diet, choline-deficient diet or high-fat and high carbohydrate diet feeding for 3–12 weeks [27,33–37]. High-fat diet increased apoptosis of hepatic NKT cells, and the reduction of NKT cells in the steatotic liver may be attributed to Kupffer cells derived IL-12 [27,33]. However, a study found enrichment of liver NKT cells in NASH fibrosis in mice fed by MCD-fed for 8 weeks. Mice with excessive hedgehog signaling harbored increased NKT cells and developed increased fibrosis in diet-induced nonalcoholic steatohepatitis [38]. This result was supported by a finding that intrahepatic CD3$^+$CD56$^+$ NKT cells are grown in NAFLD patients as NAS increased [39]. Recently, hepatic infiltration of NKT cells was observed in another nonalcoholic steatohepatitis model, in which mice were fed by a high-fat diet and a drinking water with high fructose and sucrose for 16 weeks [40]. The dietary composition and feeding method in this study was quite different from the diet in our current study. Also, 16-week feeding period is more extended than ours. These differences may lead to different severity of NAFLD or existing progressive fibrosis, which might influence the presentation of glycolipid antigens by CD1d to NKT cells and impact NKT cells accumulation or depletion. Better comparative studies will be required in future.

Given NKT cells were reduced in fatty liver, it is questionable whether the role of NKT cells can be ignored in the injury because growing evidence indicated that NKT cells mediate injury by expanding themselves and secreting pro-inflammatory cytokines. However, it was also reported that hepatic innate immune system in NAFLD was changed, manifested by more production of IFN-γ from HMNCs and T cells, especially NKT cells [27]. In the present study, we found that hepatic NKT cells during IRI expressed more activation marker and accumulated more of their population to the injury site. The kinetic alternations of NKT cells, not only in the population but also in the production of inflammatory cytokine, were keeping pace with the degree of the injury. Also, in the absence of NKT cells, IRI was improved, which further suggested the pathogenic role of NKT cells in inducing the injury.

The previous study indicated CXCR6 expressing hepatic lymphocytes increased in cases of hepatitis C-infected patients [41], and CXCR6 expression increased slightly in patients with NAFLD [42]. Moreover, CXCR6 and its ligand CXCL16 were up-regulated in chronic liver disease, such as fibrosis and steatosis [42]. Analogous to human disease, CXCL16 serum level was significantly elevated in murine models of MCD diet-induced chronic and CCl4 induced acute liver injury [43]. In this study, we found down-regulation of CXCR6 mRNA expression but up-regulation of CXCL16 mRNA expression in the fatty liver during IRI. The discrepancy with prior reports may be related to the model we used that was acute injury not chronic.

To some extent, our finding proved that CXCR6/CXCL16 axis was up-regulated in fatty liver and this happened only when the liver suffered IRI because there was no significant expression difference of CXCR6 mRNA and CXCL16 mRNA in the sham group. The result that fatty liver NKT expressed more CXCR6 can also be supported by the observation from CXCR6 gene reporter mice, as GFP (CXCR6) expressing cells were mainly NKT cells. Interestedly, by using these mice, the localization and distribution of hepatic NKT cells during IRI can be visualized. Behavior and activity of NKT cells can even be recorded by intravital fluorescence microscopic system [19].

As described in the Introduction section, CXCR6 and CXCL16 could mediate the trafficking and localization of lymphocytes in inflammation and injury. We speculate up-regulated CXCR6/CXCL16 axis might endow NKT cells more chemotaxis drive to the injured and inflamed liver. More importantly, up-regulation of CXCR6 on NKT cells may be indispensable to the function character of NKT cells in the fatty liver (more Th1-type cytokine production), because CXCR6 was reported as a marker of IFN-γ producing effector cells [44]. Indeed, we found that CXCR6 deficiency negatively impacted the IFN-γ producing capacity of hepatic NKT during IRI. It is that this inhibition of inflammatory cytokine production caused by CXCR6 lack, making strategies based on CXCR6 or CXCL16 possible to improve the injury. More importantly, pathogenic hepatic NKT cells were significantly reduced in CXCR6 deficiency mice during IRI, suggesting that CXCR6 deficiency negatively affect NKT cells in the function as well as in the population. CXCR6 deficiency decreased the hepatic accumulation of NKT cell displayed a tissue specificity that can also be observed at the aspect of IL-4 secretion of NKT cells, in that study, intracellular staining of IL-4 was reduced in liver but not in the spleen [41]. CXCR6 deficiency decreased the significantly hepatic accumulation of NKT cell but not other CXCR6-expressing cells, which suggests that recruitment of hepatic NKT cells is CXCR6-dependent.

Several studies have indicated that hepatic NKT cells decreased in chronic liver disease [27,33], and this can be supported by clinical data from patients with hepatic steatosis and fibrosis [33]. To some extent, it may be complicated to seek strategies involving NKT cells for protecting acute hepatic inflammatory injury induced in chronic NAFLD. Effective methods reported to preserve the normal liver from IRI focused on how to reduce CD1d dependent activation of NKT cells [45], inhibit type I and activate type II NKT cells [11]. From another view, we targeted on inhibiting localization and accumulation of NKT cells and improved the injury.

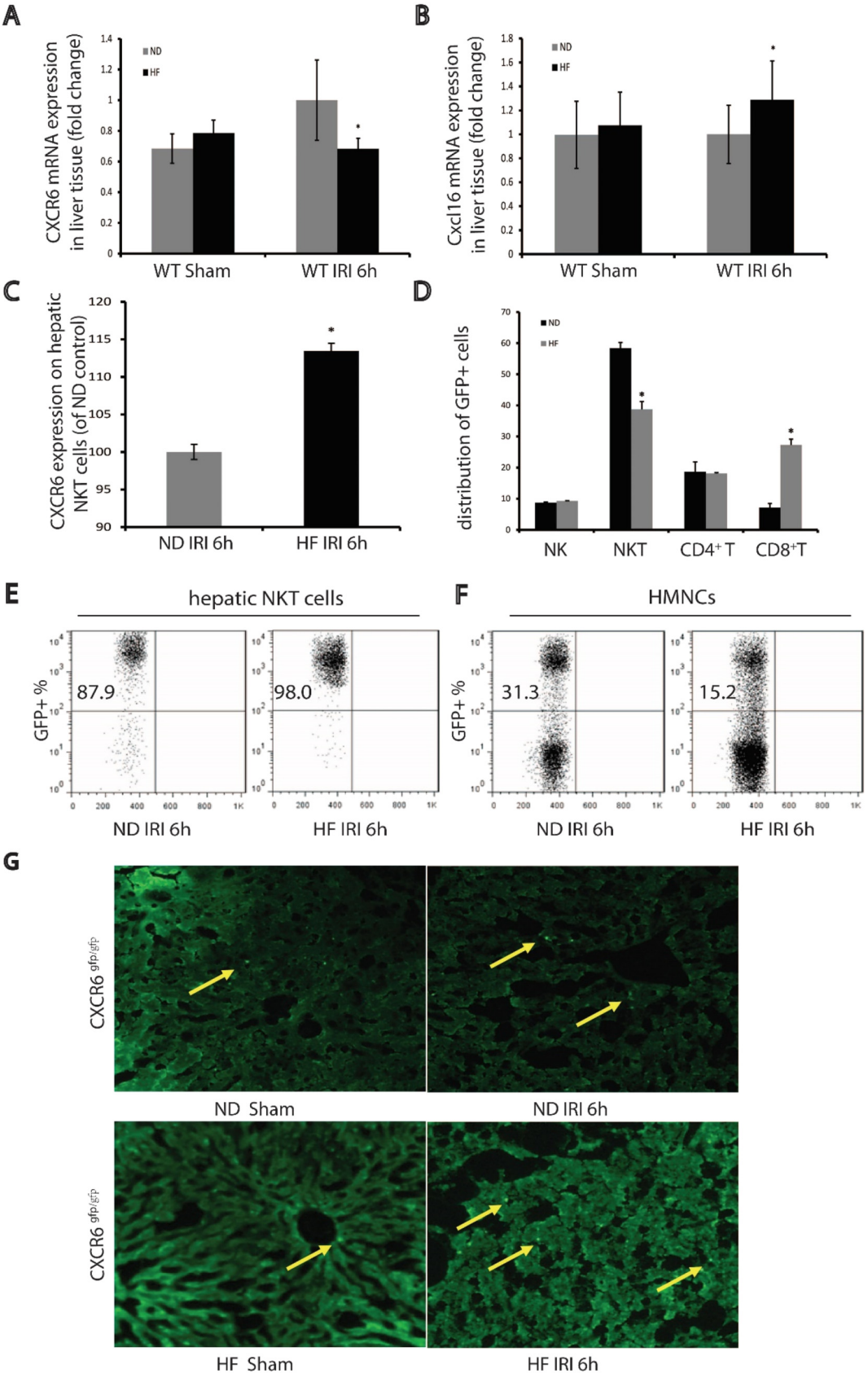

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Fig. 4. Fatty liver NKT cells express more CXCR6, and its ligand CXCL16 mRNA is up-regulated in the fatty liver during IRI. (A–C) HF diet or ND-fed WT C57BL6 mice were sacrificed at 6 h after reperfusion. Total RNA in liver tissue was extracted, and CXCR6 and CXCL16 mRNA were quantified by real-time RT-PCR and normalized of GAPDH mRNA. (A) CXCR6 mRNA relative expression in liver tissue. (B) CXCL16 mRNA relative expression in liver tissue. HMNCs were stained with anti-TCR-β, -CXCR6 and PBS57-loaded CD1d tetramer, and were then analyzed by flow cytometry (C) CXCR6 expression on NKT cells. HMNCs were stained with anti-TCR-β, -CXCR6 and PBS57-loaded CD1d tetramer, and were then analyzed by flow cytometry. (D-G) HF diet or normal diet-fed CXCR6 KO mice ($CXCR6^{gfp/gfp}$) were sacrificed at 6 h after reperfusion, and HMNCs were analyzed by flow cytometry. (D) Distribution of hepatic infiltrated GFP^+ cells, NK cells ($NK1.1^+TCR\text{-}\beta^-$), NKT cells ($CD1d\ tetramer^+\ TCR\text{-}\beta^+$ cells), $CD4^+T$ ($CD4^+TCR\text{-}\beta^-$), $CD8^+T$ ($CD8^+TCR\beta^-$). (E) Representative dot plot of percentages of GFP^+ cells in hepatic NKT cells. (F) Representative dot plot of percentages of GFP^+ cells in HMNCs. (G) Liver frozen sections from ND mice and HF mice were prepared and analyzed by fluorescence microscope. Arrows were pointed to hepatic infiltrated GFP^+ cells.

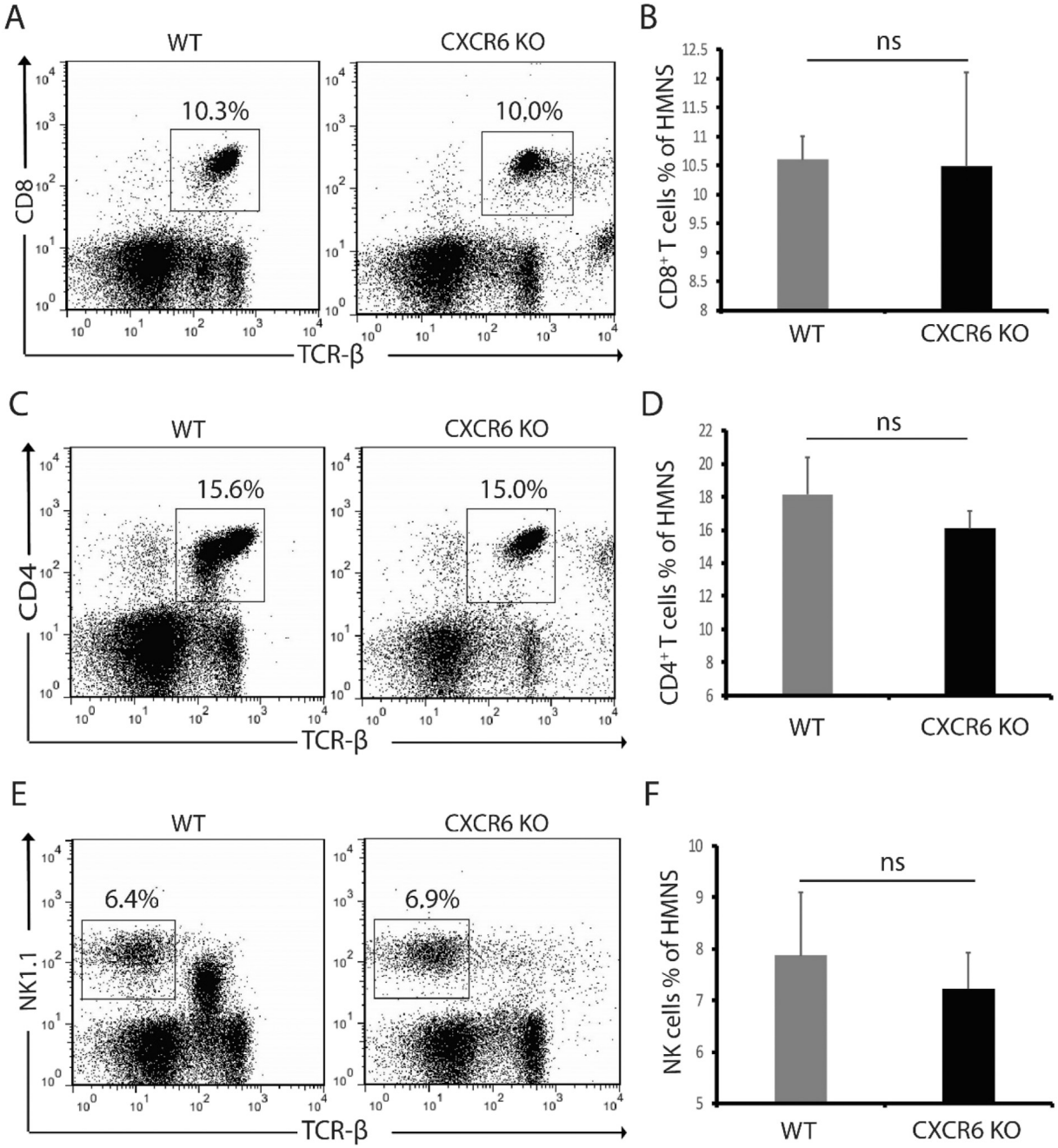

Fig. 5. CXCR6-expressing CD8, CD4 T cells and NK cells do not significantly change in CXCR6 deficient mice. (A–F) Wild-type C57BL6 mice and CXCR6 KO mice were sacrificed. HMNCs were stained with anti-CD8, -CD4, -NK1.1 and -TCR-β antibody and analyzed by flow cytometry. (A) Representative dot plots of hepatic $CD8^+$ T cells. (B) Histogram of percentages of hepatic $CD8^+$ T cells. (C) Representative dot plots of hepatic $CD4^+$ T cells. (D) Histogram of percentages of hepatic $CD4^+$ T cells. (E) Representative dot plots of hepatic NK cells. (F) Histogram of percentages of hepatic NK cells. Data are presented as mean ± SEM with n = 5 mice/group with $^*p < 0.05$ (WT vs CXCR6 KO).

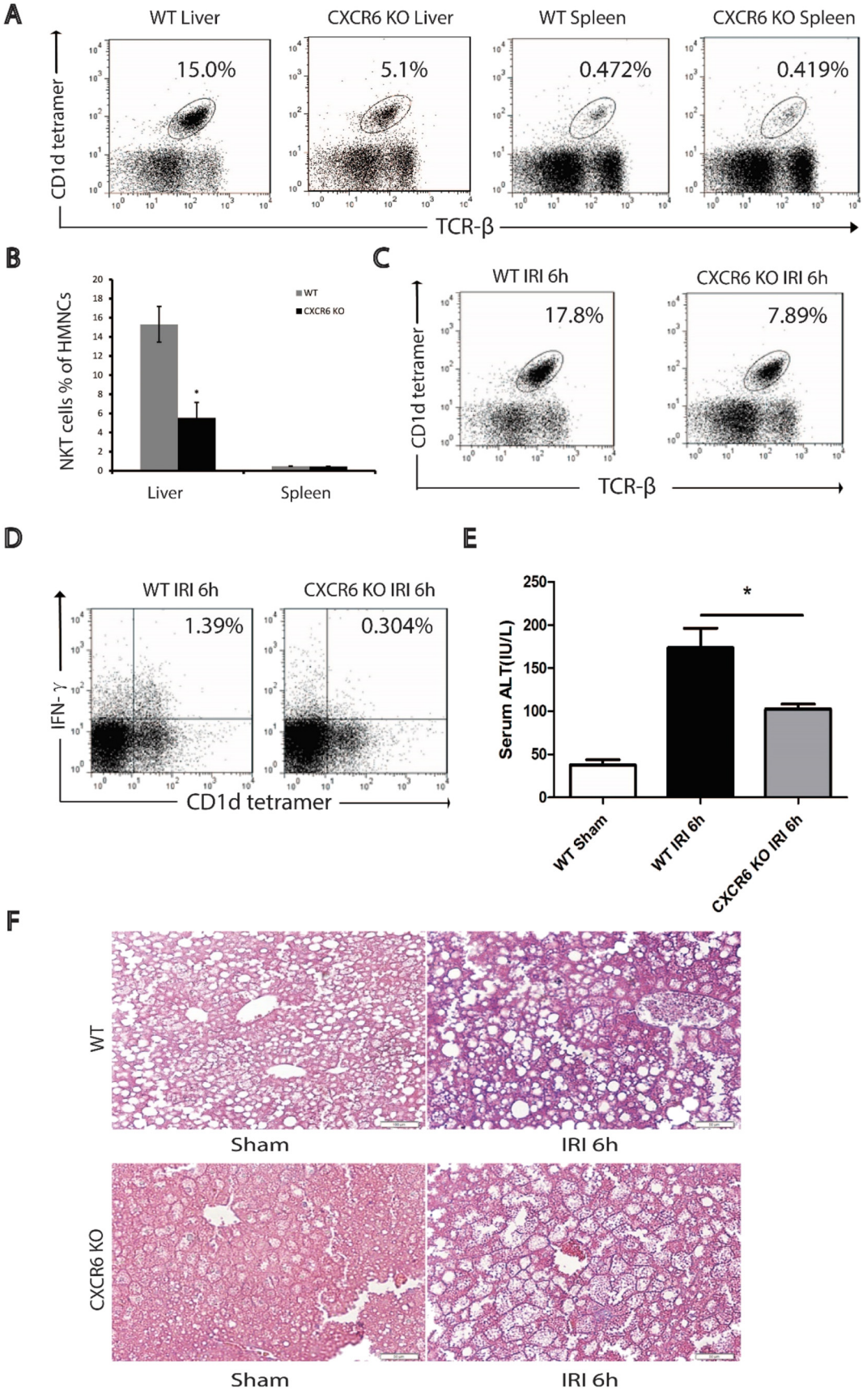

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Fig. 6. CXCR6 deficiency protects the fatty liver from IRI by reducing hepatic recruitment of NKT cells and IFN-γ production. (A–B) HF diet-fed wild-type C57BL6 mice and CXCR6 KO mice were sacrificed. HMNCs and splenic mononuclear cells were stained with anti-TCR-β antibody and PBS57-loaded CD1d tetramer and analyzed by flow cytometry. (A) Representative dot plots of hepatic and splenic NKT cells. (B) Histogram of percentages of hepatic and splenic NKT cells. (C–F) Wild-type C57BL6 mice and CXCR6 KO mice were fed with HF diet for 12 weeks, and were sacrificed at 6 h after reperfusion (C) Representative dot plots of percentage of NKT cells in HMNCs. (D) Representative dot plots of percentage of IFN-γ^+ CD1d Tetramer$^+$ cells in HMNCs. Flow cytometry results are representative of three independent experiments with similar results. (E) Serum ALT level. Data are presented as mean ± SEM with n = 5–7 mice/group with *p < 0.05 (CXCR6 KO vs WT). (F) Histological injury by H&E staining images (magnification ×200) of liver sections from WT and CXCR6 KO mice at 6 h after reperfusion.

5. Conclusions

Taken together, we demonstrated that even though NKT cells decreased in the liver with macrovesicular steatosis, hepatic NKT cells still play a pathogenic role in inducing IRI in NAFLD. NKT cells in the fatty liver during IRI were endowed more chemotaxis character by up-regulated CXCR6/CXCL16 axis. Deficiency of CXCR6 reduced the accumulation and cytokine secretion of hepatic NKT cells and further protected fatty liver form IRI. Strategies based on this mechanism might be used to treat other inflammatory and injury diseases.

Acknowledgments

We thank NIH tetramer facility for providing mouse CD1d tetramer. This work was supported by NIH/NIDDK R01DK075990, National Natural Science Foundation of China, 81172036.

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