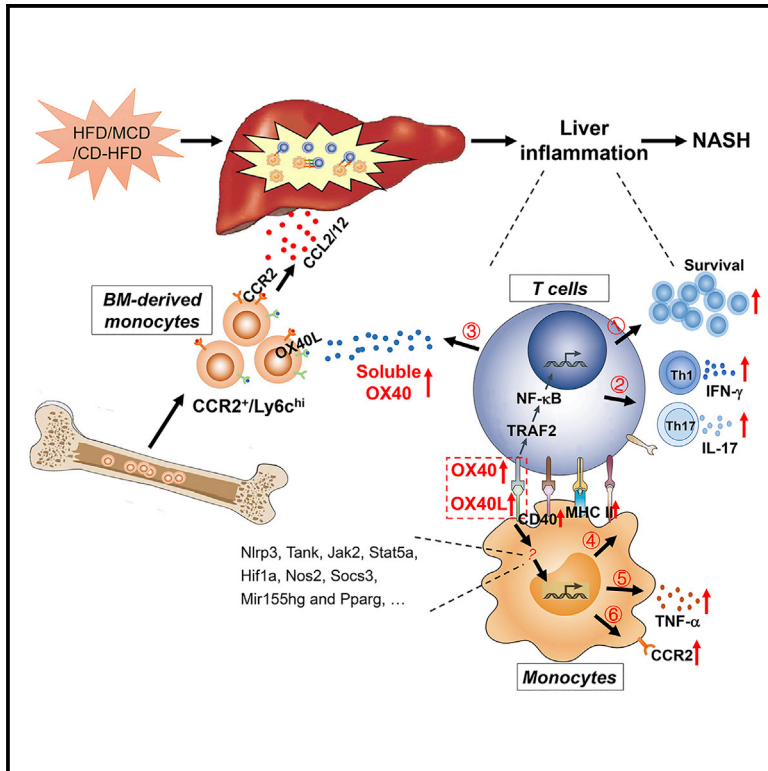


Cell Reports

OX40 Regulates Both Innate and Adaptive Immunity and Promotes Nonalcoholic Steatohepatitis

Graphical Abstract



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In Brief

Sun et al. show that OX40 is a key molecule in the regulation of both intrahepatic innate and adaptive immunity. OX40 promotes both proinflammatory monocyte and macrophage and T cell function, resulting in NASH development and progression. These findings suggest that OX40 could serve as a diagnostic index and therapeutic target in NASH.

Highlights

- TNF receptor family member OX40 is a key molecule in NASH development
- OX40 regulates both intrahepatic innate and adaptive immunity in NASH
- OX40 promotes hepatic monocyte but not Kupffer cell M1 polarization in NASH
- Plasma OX40 levels are positively associated with NASH in humans



OX40 Regulates Both Innate and Adaptive Immunity and Promotes Nonalcoholic Steatohepatitis

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SUMMARY

Both innate and adaptive immune cells are involved in the pathogenesis of nonalcoholic steatohepatitis (NASH), but the crosstalk between innate and adaptive immunity is largely unknown. Here we show that compared with WT mice, OX40^{-/-} mice exhibit decreased liver fat accumulation, lobular inflammation, and focal necrosis after feeding with diets that induce NASH. Mechanistically, OX40 deficiency suppresses Th1 and Th17 differentiation, and OX40 deficiency in T cells inhibits monocyte migration, antigen presentation, and M1 polarization. Soluble OX40 stimulation alone upregulates antigen presentation, chemokine receptor expression, and proinflammatory cytokine secretion by liver monocytes. Furthermore, plasma soluble OX40 levels are positively associated with NASH in humans, suggesting clinical relevance of the findings. In conclusion, we show a mechanism for T cell regulation of innate immune cells. OX40 is a key regulator of both intrahepatic innate and adaptive immunity, generates two-way signals, and promotes both proinflammatory monocyte and macrophage and T cell function, resulting in NASH development.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) represents a spectrum of progressive liver disease and has become one of the most common liver pathological conditions worldwide. NAFLD ranges from isolated intrahepatic triglyceride accumulation (simple steatosis) to intrahepatic triglyceride accumulation plus inflammation and hepatocyte injury (nonalcoholic steatohepatitis [NASH]) and ultimately progresses to fibrosis and cirrhosis and

potentially hepatocellular carcinoma (Angulo, 2002; Anstee et al., 2013; Arab et al., 2018; Borrelli et al., 2018; Lee et al., 2017; Suzuki and Diehl, 2017). Inflammation in the liver is crucial for the progression from hepatic steatosis to NASH, and infiltration of different subsets of inflammatory cells is the hallmark of steatohepatitis (Tilg and Moschen, 2010).

The liver is a central immunological organ with a high density of myeloid and lymphoid immune cells (Heymann and Tacke, 2016). Interactions among different immune cells are essential for the development and progression of NASH. In the early stage, steatotic hepatocytes release apoptotic bodies and modified lipoproteins, which activates proinflammatory cytokine and chemokine secretion by resident Kupffer cells, consequentially recruiting and activating inflammatory leukocytes (neutrophils and monocytes). In addition to metabolic factors, free fatty acids (FFAs), gut bacterial lipopolysaccharide (LPS), chemokines, cytokines, and adipokines are also contribute to the activation of innate immune cells (Ganz and Szabo, 2013; Tilg and Moschen, 2010). These cells directly promote additional fat accumulation in the liver and trigger adaptive immune cell recruitment and activation (Ganz and Szabo, 2013; Nati et al., 2016). CD4 and CD8 T lymphocytes, B lymphocytes, and natural killer T (NKT) cells are recruited within the liver in parallel with worsening parenchymal injury and lobular inflammation (Leroux et al., 2012; Sutti et al., 2014; Wolf et al., 2014b). The presentation of oxidative stress-derived antigens by liver antigen-presenting cells (APCs) to CD4 T helper lymphocytes leads to CD4 T cell activation that supports both cytotoxic CD8 T cell and B cell responses.

Costimulatory interactions likely mediate extensive crosstalk between innate and adaptive immunity during the pathogenesis of NASH. Crosstalk between B7-CD28 or CD40-CD40L plays a dual role in liver inflammation and steatosis by inducing protective regulatory T cell responses and eliciting effector T cell proinflammatory functions (Chatzigeorgiou et al., 2014; Guo et al., 2013; Wolf et al., 2014a). OX40 and OX40 ligand (OX40L) interaction is essential for regulating conventional T cell division, differentiation, and survival and plays an important role in inflammation development in models of multiple sclerosis (MS), colitis,



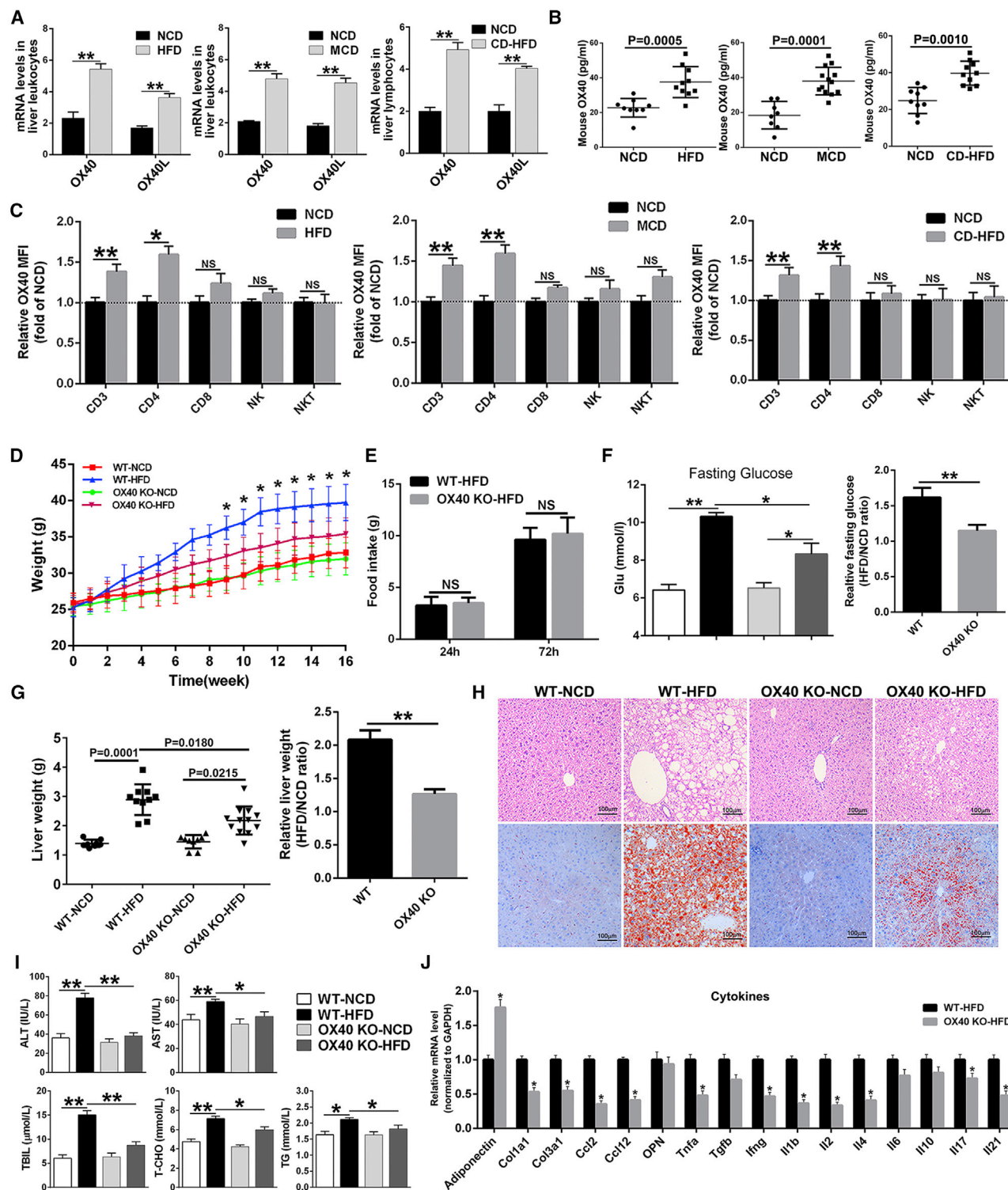


Figure 1. OX40 Expression Was Increased in Intrahepatic T Cells of HFD-, MCD-, and CD-HFD-Fed Mice, and OX40 Deficiency Alleviated Steatohepatitis in Mice

(A) OX40 and OX40L mRNA levels in the hepatic mononuclear cells of mice fed the NCD, high-fat diet (HFD) (left), MCD (middle), and CD-HFD (right). (B) Plasma levels of soluble OX40 were determined using ELISA in HFD-, MCD-, and CD-HFD-fed mice. (C) Statistical analysis of OX40 expression in intrahepatic lymphocytes in NCD-, HFD-, MCD-, and CD-HFD-fed mice, measured using flow cytometry. (D) Increases in body weight in NCD- and HFD-fed mice.

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rheumatoid arthritis (RA), graft-versus-host disease (GVHD), and leishmaniasis (Akiba et al., 2000; Croft, 2010; Higgins et al., 1999; Liu et al., 2017; Tsukada et al., 2000; Weinberg et al., 1999; Yoshioka et al., 2000; Zhao et al., 2016). In a previous study, we found that OX40 upregulation in CD4 T cells is crucial for adipose tissue inflammation and insulin resistance development (Liu et al., 2017). However, whether OX40-OX40L interaction can regulate innate immunity and promote hepatic inflammation and NASH development is largely unknown.

In this study, by inducing steatohepatitis in OX40-deficient mice by altering their diet, we provide evidence that OX40 plays a key role in regulating both liver innate and adaptive immunity in liver inflammation and NASH development.

RESULTS

OX40 Deficiency Ameliorated Steatohepatitis

To investigate the involvement of OX40 and OX40L in NASH development, we fed C57BL/6 mice a high-fat diet (HFD) for 16 weeks, a methionine and choline-deficient diet (MCD) for 4 weeks, or a choline-deficient HFD (CD-HFD) for 12 weeks. As shown in Figure 1A, OX40 and OX40L mRNA expression levels were strikingly increased in the liver mononuclear cells (MNCs) of mice receiving the HFD compared with those of lean animals, and similar results were found in MCD- and CD-HFD-fed mice (Figure 1A). Additionally, the plasma soluble OX40 levels in HFD, MCD, and CD-HFD mice were also remarkably higher than those in control mice (Figure 1B). The typical flow cytometry gating strategy to distinguish hepatic T cells is shown in Figure S1A. Compared with the normal control diet (NCD) controls, HFD-, MCD-, and CD-HFD-fed mice showed significantly increased OX40 levels in total CD3 T cells and CD4 T cells but not in CD8 T cells, natural killer (NK) cells, or NKT cells (Figures 1C and S1B).

The mechanism of OX40 upregulation is still largely unknown. OX40 is expressed predominantly on activated T cells. Interleukin (IL)-2, IL-4, and TNF have been reported to enhance or prolong OX40 expression (Croft, 2010). In this study, we found that IL-2, TNF- α , interferon (IFN)- γ , and leptin were highly expressed in liver tissues of NASH models, but only exogenous IL-2 stimulation upregulated OX40 expression in CD4 T cells (Figures S1C–S1F). Meanwhile, we also observed IL-2 inducing CD4 T cell upregulation of transcription factors Sp1/Sp3 and p65 (Figure S1G), which have been reported to bind to OX40 promoter and enhance OX40 expression (Tone et al., 2007). These results suggest that during NASH development, IL-2 secretion by activated CD4 T cells can further upregulate and maintain OX40 expression.

To determine the potential role of OX40 in the regulation of liver inflammation and NASH development, wild-type (WT) and OX40-

knockout (KO) mice were fed an HFD. OX40-KO mice gained remarkably less weight than age-matched WT mice (Figure 1D), although food intake between WT and OX40-KO mice was not significantly different (Figure 1E). After 16 weeks of HFD feeding, fasting glucose levels were significantly lower in OX40-KO mice than in HFD-fed WT mice (Figure 1F). Likewise, liver weight trended lower and liver size smaller in OX40-deficient mice compared with WT mice (Figure 1G). H&E and oil red O staining revealed decreased fat accumulation in the livers of OX40-KO mice compared with WT mice, accompanied by decreased hepatocellular ballooning and histological signs of inflammation (Figure 1H). OX40-KO mice fed the HFD exhibited significantly decreased plasma levels of alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin (TBIL), total cholesterol T-CHO, and triglyceride (TG) (Figure 1I). As shown in Figure 1J, OX40-deficient mice also exhibited decreased levels of *Ccl2*, *Ccl12*, *Tnfa*, *Tgfb*, *Ifng*, *Il1b*, *Il2*, *Il4*, *Il17*, and *Il21* in liver tissues. Fibrosis-related genes, such as *Col1a1* and *Col3a1*, were also downregulated in the liver of OX40-deficient mice. Similar results were also found in MCD- and CD-HFD-fed mice, and H&E and oil red O staining of liver samples demonstrated that OX40 deficiency restricted fat accumulation, lobular inflammation, and focal necrosis (Figures S2A and S2C). The mRNA levels of some proinflammatory cytokines, such as *Tnfa*, *Tgfb*, *Ifng*, *Il1b*, *Il6*, *Il10*, and *Il17*, were also downregulated in OX40-deficient livers compared with control livers (Figures S2B and S2D).

OX40 Regulated Hepatic T Cell Activation and Survival and Decreased Th1 and Th17 Cell Differentiation during NASH Development

To elucidate the mechanisms underlying NASH amelioration observed in OX40-KO mice fed the HFD, we observed the liver T cell compositions of WT and OX40-KO mice. As shown in Figure 2A, deletion of the OX40 gene remarkably reduced the proportions of CD3 and CD4 T cells, but not CD8 T cells, in HFD-fed mice compared with WT mice. In addition, OX40 deficiency influenced hepatic T cell activation. As shown in Figure 2B, OX40 deficiency significantly lowered the proportion of CD69⁺ T cells in HFD-fed mice. Among CD3 T cells, the proportion of CD4⁺CD69⁺ T cells, but not CD8⁺CD69⁺ T cells, significantly decreased.

OX40-KO mice fed the HFD exhibited significantly lower expression levels of anti-apoptosis genes, such as *Bcl2*, *Bcl-xl*, and *Survivin* (Figure 2C). Annexin V staining of hepatic T cells further supported that the proportion of apoptotic T cells was increased in HFD-fed mice. Compared with WT mice, OX40-KO mice fed the HFD had a marked increase in their percentage of annexin V-positive CD3 and annexin V-CD4 T cells (Figure 2D). In addition, from each group, we also isolated and stimulated mice splenocytes with anti-CD3/CD28 antibodies. As shown in

(E) Food intake in WT and OX40-knockout (KO) HFD-fed mice for 24 and 72 hr.

(F) Plasma glucose levels and relative fasting glucose ratio were measured after the animals had fasted for 6 hr.

(G) Liver weights and relative liver weight ratio of mice receiving 16 weeks of NCD or HFD.

(H) Representative H&E (top) and oil red O (bottom) staining in liver paraffin sections.

(I) Plasma ALT, AST, TBIL, T-CHO, and triglyceride (TG) levels were measured.

(J) Relative proinflammatory cytokine mRNA levels in liver tissues were analyzed using quantitative real-time PCR.

Data are presented as mean $\pm$ SD; n = 6 in each group. *p < 0.05 and **p < 0.01; NS, not significant.

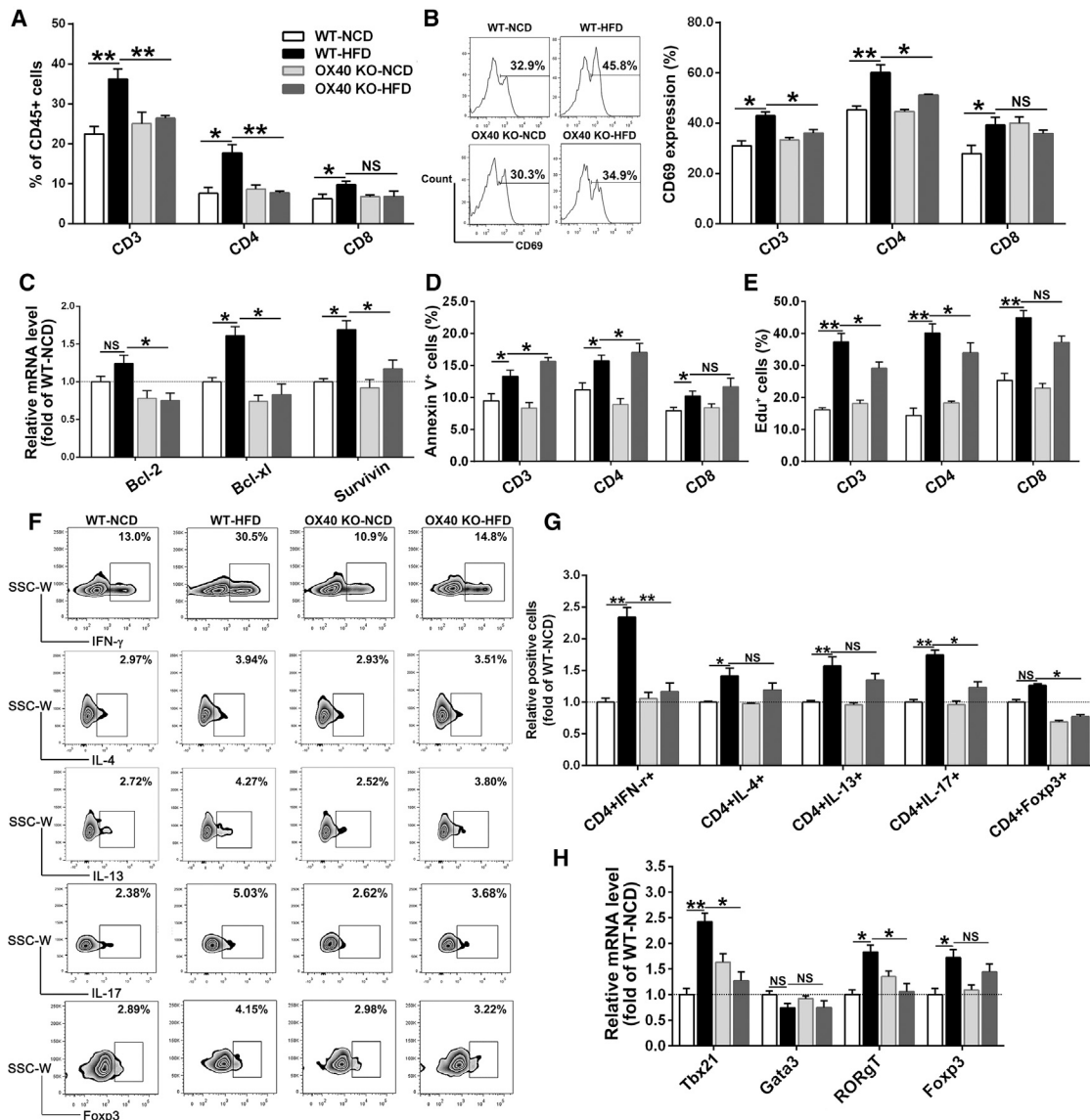


Figure 2. OX40 Deficiency Decreased Hepatic T Cell Activation, Survival, and Th1 and Th17 Cell Differentiation in Mice Fed the HFD

(A) T cell levels in mouse liver MNCs from each group.

(B) Representative flow cytometry image (left) and statistical analysis (right) of the percentages of CD69+ cells in CD3, CD4, and CD8 T cells.

(C) mRNA levels of apoptosis-related genes in hepatic MNCs.

(D) Annexin V+ cells relative to the total numbers of CD3 T cells, CD4 T cells, and CD8 T cells.

(E) EdU+ cells relative to the total numbers of CD3 T cells, CD4 T cells, and CD8 T cells.

(F) Representative flow cytometry images of IFN- γ , IL-4, IL-13, IL-17, and Foxp3+ cells of CD4 T cells.

(G) Changes in the percentages of IFN- γ + cells, IL-4+ cells, IL-13+ cells, IL-17+ cells, and Foxp3+ cells in CD4 T cells from the liver tissues of HFD WT and OX40-KO mice plotted as fold changes in the percentages of these cells in WT NCD-fed mice.

(H) Real-time PCR analysis of *Tbx21*, *Gata3*, *ROR γ T*, and *Foxp3* levels in the liver tissues of mice from each group.

Data are depicted as mean $\pm$ SD; n = 6 in each group. *p < 0.05 and **p < 0.01; NS, not significant.

Figure 2E, after 3 days of stimulation, HFD-fed WT mice had higher proliferation rates than NCD-fed WT mice. OX40 deletion suppressed CD3 and CD4 T cell division even for mice receiving the same HFD.

Among T cells, HFD-fed mice had significantly increased IFN- γ , IL-17-, IL-4-, and IL-13-producing CD4 T cells, while

OX40-KO mice fed the HFD only exhibited remarkably decreased percentages of IFN- γ - and IL-17-producing CD4 T cells. Additionally, OX40 deletion also lowered the proportion of Foxp3-positive cells in the total CD4 T cells of HFD-fed mice (Figures 2F and 2G). Furthermore, we quantified the expression of Th lineage-defining transcription factors in liver MNCs using

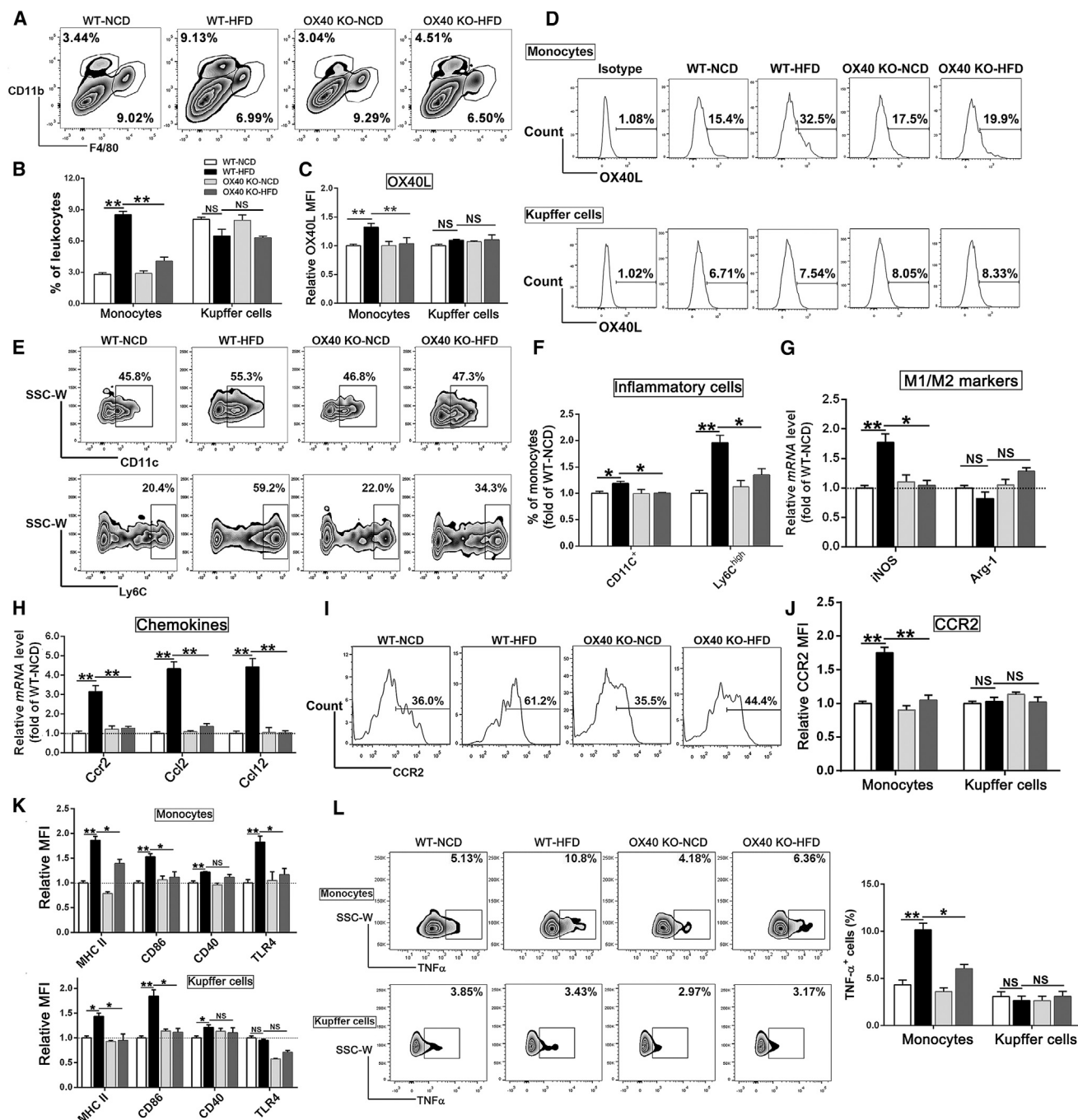


Figure 3. OX40 Deficiency Dampened Infiltrated Monocyte Hepatic Recruitment, Maturation, and Proinflammatory Cytokine Secretion In Vivo

(A) Flow cytometric images of liver monocytes and Kupffer cells.
(B) Quantification of hepatic Kupffer cells and monocytes as the percentage of total liver leukocytes.
(C) Relative changes in OX40L expression plotted as fold changes of OX40L expression induced by NCD administration in WT mice.
(D) Flow cytometric images of OX40L expression in monocytes and Kupffer cells.
(E) Flow cytometric images of CD11c⁺ and Ly6C^{high} cells.
(F) Relative changes of CD11c⁺ and Ly6C^{high} cell proportions plotted as fold changes of cell proportions induced by NCD administration in WT mice.
(G) Relative *iNOS* and *Arg-1* mRNA expression levels in liver monocytes.
(H) Relative *Ccl2*, *Ccl12*, and *Ccr2* mRNA expression levels in the liver.
(I) Flow cytometric images of CCR2⁺ cells from total liver monocytes.

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real-time PCR. As shown in Figure 2H, HFD-fed mice showed remarkably upregulated expression levels of transcription factors (*Tbx21*, *ROR γ T*, and *Foxp3*, but not *Gata3*), while OX40-KO mice fed the HFD expressed significantly lower levels of *Tbx21* and *ROR γ T*.

To further prove that CD4 T cells are required for NASH development, the mice in which CD4 T cells were depleted by antibody GK1.5 were fed the MCD for 4 weeks. As shown in Figure S3, CD4 T cell depletion significantly lowered hepatic steatosis and inflammation.

Taken together, these observations indicate that OX40 signaling contributes to hepatic inflammation and NASH development by promoting hepatic CD4 T cell activation, proliferation, survival, and Th1 and Th17 differentiation.

OX40 Deficiency Was Associated with the Downregulation of Infiltrated Monocytes Hepatic Recruitment, Maturation, and Proinflammatory Cytokine Secretion

To further explore the changes in liver immunity associated with NASH, the infiltrated monocytes and resident Kupffer cells of OX40-KO mice were analyzed. No significant differences in basal levels of bone marrow monocytes or liver monocytes were found between WT and OX40-KO mice (Figures S4A and S4B). However, as shown in Figures 3A and 3B, HFD-fed mice showed increased proportions of liver infiltrated monocytes (CD11b^{int}F4/80^{low}) but not resident Kupffer cells (CD11b^{low}F4/80^{hi}). OX40 deficiency decreased liver infiltrated monocytes in HFD-fed mice. The HFD-fed mice showed significantly upregulated OX40L expression in monocytes (1.52-fold, $p < 0.001$; Figures 3C and 3D). OX40-KO mice fed the HFD exhibited significantly lower expression levels of OX40L, while the OX40L expression levels in resident Kupffer cells were not significantly different.

The liver infiltrated monocytes of HFD-fed mice exhibited remarkably higher percentages of inflammatory macrophages (CD11b^{int}F4/80^{low}CD11c⁺, CD11b^{int}F4/80^{low}Ly6c^{high}) with *iNOS*, but not *Arg-1* mRNA expression. OX40 deletion suppressed *iNOS* mRNA expression and lowered the CD11c⁺ and Ly6c^{high} monocyte percentages (Figures 3E–3G), indicating that the OX40 and OX40L signal promotes liver monocyte and macrophage M1 polarization. CCR2 and its chemokine ligand CCL2/CCL12, which regulate bone marrow-derived monocyte recruitment to inflamed tissues during hepatic steatosis (Baeck et al., 2012; Obstfeld et al., 2010), were highly expressed in the liver tissues of HFD-fed mice (Figure 3H). The liver infiltrated monocytes of HFD-fed mice expressed higher levels of CCR2 than those of NCD-fed mice, while OX40 deficiency decreased CCR2 expression (Figures 3I and 3J). No significant difference in CCR2 expression was found in Kupffer cells from WT and OX40-KO mice fed the HFD (Figure 3J).

Furthermore, HFD-fed mice exhibited remarkably upregulated MHC II, CD86, CD40, and TLR4 expression in their infiltrated monocytes, while OX40-KO mice fed the HFD exhibited significantly lower levels of MHC II, CD86, and TLR4 (Figure 3K). The HFD also induced Kupffer cells to highly express MHC class II (MHC II), CD86, CD40, and TLR4, while OX40 deletion decreased MHC II and CD86 expression in HFD-fed mice.

We also observed higher TNF- α production in infiltrated monocytes after HFD feeding, and OX40 deficiency decreased TNF- α secretion in HFD-fed mice. Additionally, TNF- α production in Kupffer cells was not significantly different between the WT and OX40-KO groups fed the HFD (Figure 3L). The same analyses were performed in MCD- or CD-HFD-fed WT and OX40-KO mice, and similar results were obtained (Figures S4C–S4M and S5A–S5I).

These results suggest that OX40 deficiency is associated with the downregulation of infiltrated monocyte recruitment, maturation, and proinflammatory cytokine secretion, which may play important roles in the improvement of liver steatosis.

OX40 Deficiency in T Cells Ameliorated Steatohepatitis

To further prove that OX40 expression in T cells contributed to the phenotype observed in OX40-deficient mice, we selectively repopulated B6.Rag2 and Il2rg double-KO mice with purified CD3 T cells from WT or OX40-deficient mice. All mice consumed the HFD for 16 weeks (Figure 4A). The mice inoculated with OX40-deficient CD3 T cells gained remarkably less weight than age-matched mice inoculated with CD3 T cells (Figure 4B). Furthermore, mice inoculated with OX40-deficient CD3 T cells exhibited decreased plasma levels of soluble OX40 (Figure 4C), ALT, AST, and TG (Figure 4D) and reduced liver steatosis (Figure 4E), which demonstrated that OX40 deficiency in T cells restricts fat accumulation, lobular inflammation, and focal necrosis. Similar to OX40-KO mice, HFD-fed B6.Rag2 and Il2rg double-KO mice adoptively transferred with OX40-deficient CD3 T cells showed significantly increased proportions of apoptotic CD4 cells but not CD8 T cells (Figure 4F), decreased proportions of CD4⁺CD69⁺ T cells but not CD8⁺CD69⁺ T cells (Figure 4G), and reduced proportions of IFN- γ - and IL-17-producing CD4 T cells (Figure 4H).

In addition, OX40 deficiency in T cells also resulted in remarkably lower proportions of liver infiltrated monocytes but not resident Kupffer cells (Figure 4I). The liver infiltrated monocytes of HFD-fed B6.Rag2 and Il2rg double-KO mice adoptively transferred with OX40-deficient CD3 T cells showed remarkably lower OX40L, CCR2, MHC II, CD86, CD40 expression levels and TNF- α secretion; however, Kupffer cells in these mice exhibited lower expression levels of only CD86, as no changes in OX40L, CCR2, MHC II, CD40, and TLR4 expression or TNF- α secretion were observed (Figures 4J–4L).

These results indicate that OX40 in T cells contributes to liver inflammation by promoting hepatic CD4 T cell activation, Th1

(J) Relative changes in CCR2 levels plotted as fold changes of that induced by NCD administration in WT mice.

(K) Relative changes of antigen presentation-associated marker plotted as fold changes of the expression induced by NCD administration in WT mice.

(L) Flow cytometry image (left) and statistical analysis (right) of TNF- α ⁺ cells relative to the total numbers of monocytes and Kupffer cells.

Data are depicted as mean $\pm$ SD; $n = 6$ in each group. * $p < 0.05$ and ** $p < 0.01$; NS, not significant.

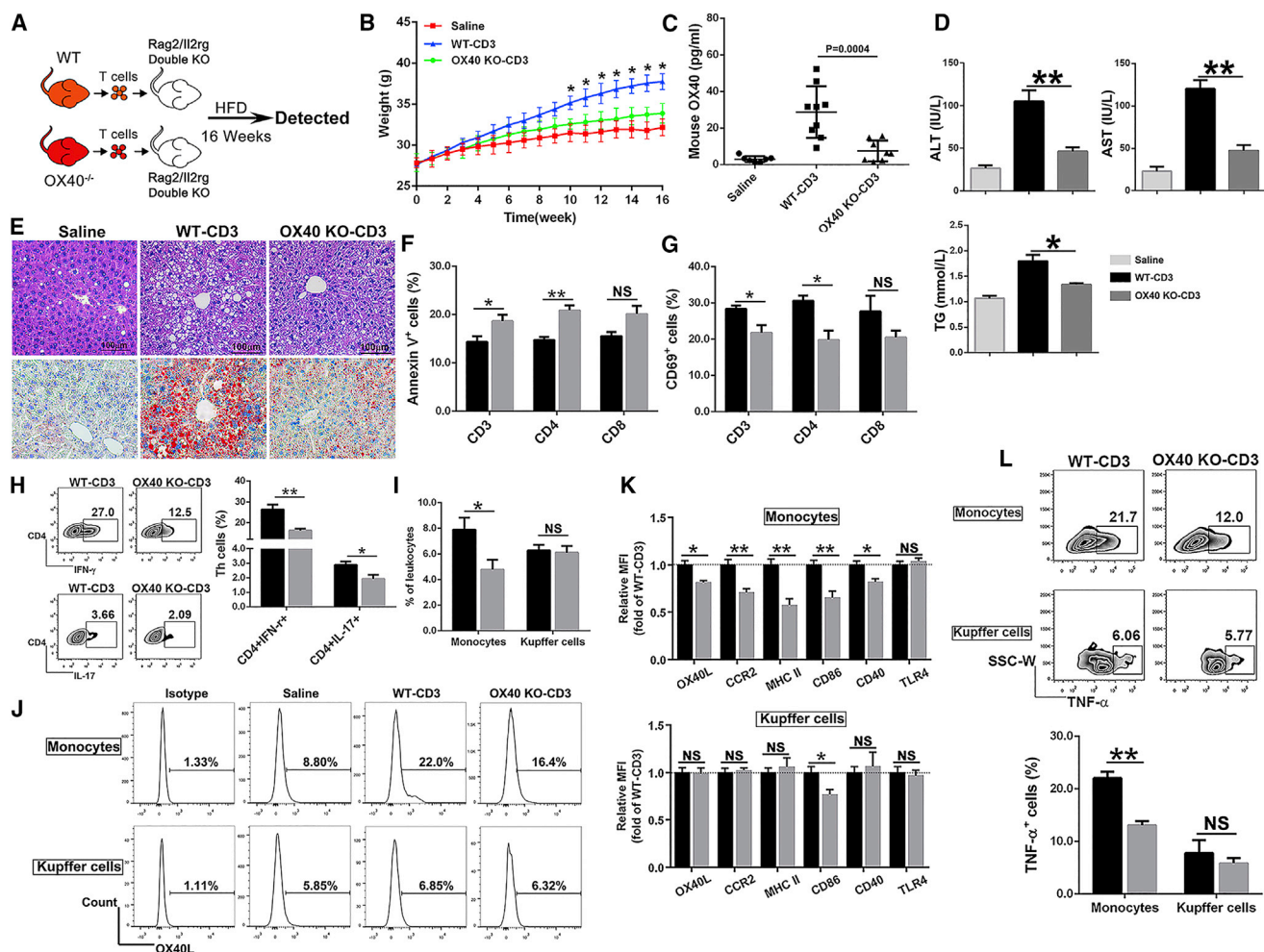


Figure 4. Compared with WT T Cells, Adoptively Transferred OX40^{-/-} T Cells Alleviated NASH Development

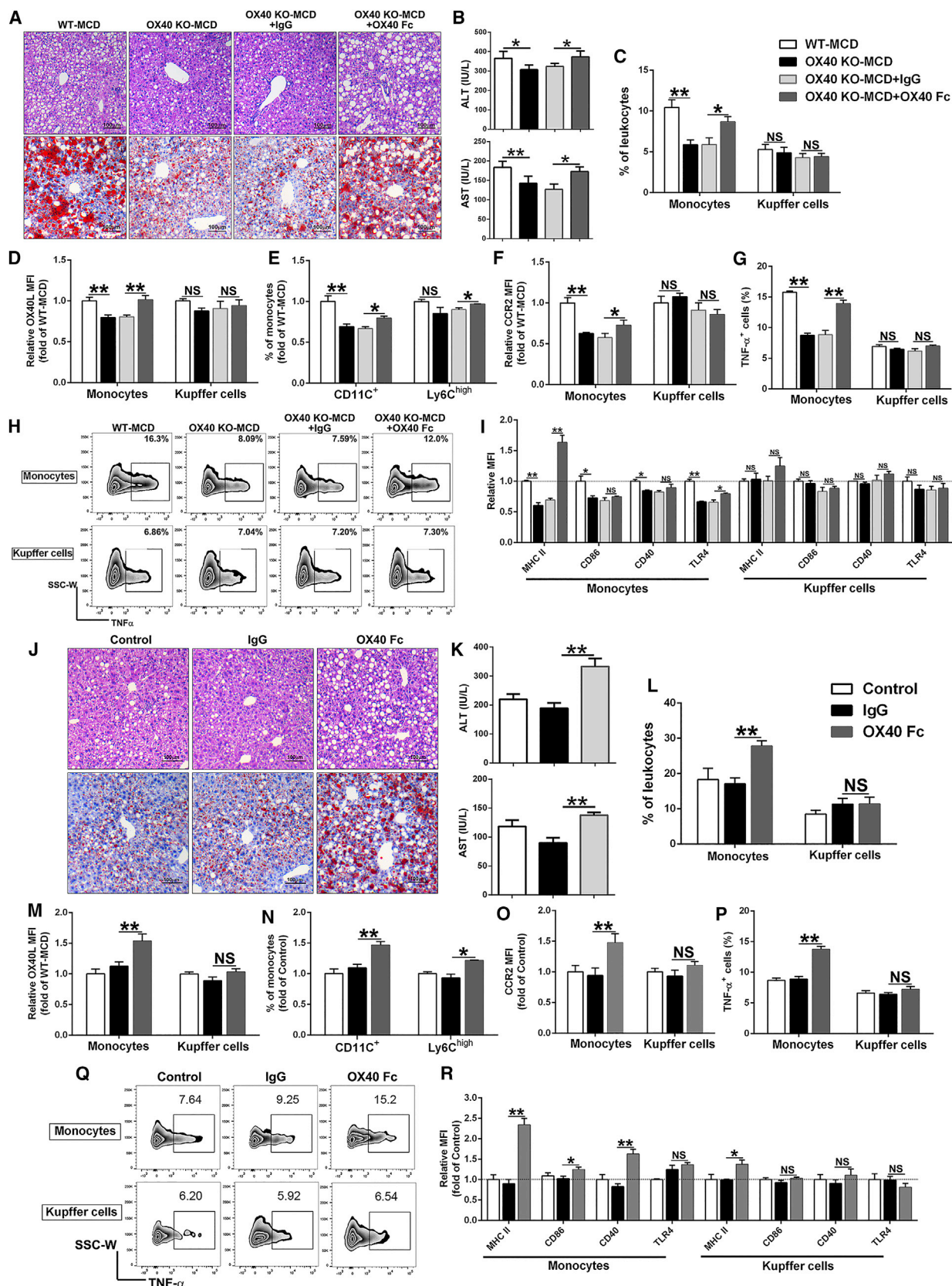
(A) Flowchart of adoptive transfer of WT or OX40-KO T cells into B6.Rag2 and Il2rg mice.
 (B) Body weights of B6.Rag2 and Il2rg double-knockout recipient mice.
 (C) Plasma soluble OX40 levels of each group.
 (D) Plasma ALT, AST, and TG levels of each group.
 (E) H&E (top) and oil red O (bottom) staining in liver paraffin sections from WT and OX40-KO CD3 T cell-inoculated recipients.
 (F) Annexin V⁺ cells relative to total T cells.
 (G) The percentages of CD69⁺ cells relative to the total numbers of T cells.
 (H) Flow cytometry image (left) and statistical analysis (right) of IFN-γ⁺ and IL-17⁺-producing CD4 T cells.
 (I) Statistical analysis of monocytes and Kupffer cells from liver leukocytes.
 (J) Flow cytometric images of OX40L expression in monocytes and Kupffer cells.
 (K) Cell surface markers expression levels in intrahepatic monocytes (top) and Kupffer cells (bottom), determined using flow cytometry. In addition, relative changes in the expression levels from each group were determined.
 (L) Flow cytometry image (top) and statistical analysis (bottom) of TNF-α⁺ cells relative to the total numbers of monocytes and Kupffer cells in the liver tissues. Data are depicted as mean ± SD; n = 6 in each group. *p < 0.05 and **p < 0.01; NS, not significant.

and Th17 differentiation, and monocyte M1 polarization during NASH development.

Soluble OX40 Enhanced Infiltrated Monocytes Hepatic Recruitment, Maturation, and Proinflammatory Cytokine Secretion and Promoted NASH Development

To demonstrate the important role of soluble OX40 in promoting NASH development, we injected soluble OX40/Fc into OX40-KO

mice fed the MCD. As shown in Figures 5A and 5B, OX40/Fc injection promoted OX40-KO mice hepatic steatosis and inflammation and upregulated plasma levels of ALT and AST. In addition, OX40/Fc injection also remarkably increased the proportion of liver infiltrated monocytes but not resident Kupffer cells (Figure 5C). The liver infiltrated monocytes of MCD-fed OX40-KO mice injected with OX40/Fc showed remarkably increased OX40L, CCR2, TLR4 expression, M1 polarization, and TNF-α



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secretion; however, Kupffer cells in these mice exhibited no changes in OX40L, CCR2, TLR4 expression, or TNF- α secretion (Figures 5D–5I). Meanwhile, we also observed similar results in B6.Rag2 and Il2rg double-KO mice injected with soluble OX40/Fc (Figures 5J–5R). These observations demonstrated that without CD4 T cell involvement, soluble OX40 *in vivo* stimulation alone can enhance infiltrated monocyte hepatic recruitment, maturation, and proinflammatory cytokine secretion and promote NASH development.

To further examine the mechanism of OX40 in regulating liver monocyte and macrophage inflammation during NASH development, highly purified (>98% purity) Kupffer cells and monocytes were isolated from the livers of WT mice and stimulated with recombinant soluble OX40/Fc (2 μ g/mL) with or without recombinant OX40L (0.5 μ g/mL) for 2 days. As shown in Figure 6A, the expression levels of antigen presentation-associated markers (MHC II, CD40, CD86, and OX40L) and chemokine receptors (CCR2, CCR5, and CCR9) in monocytes were markedly higher than those in the IgG-treated control groups. However, when soluble OX40L was presented in the culture, the responses of monocytes induced by OX40/Fc were neutralized. No such effects were observed in Kupffer cells, indicating that OX40 mainly regulates infiltrated monocyte antigen presentation and migration. Additionally, recombinant OX40/Fc stimulation could also elevate the mRNA levels of *Tnfa* and *Il1b* in isolated hepatic monocytes (Figure 6B). We also demonstrated these observations by stimulating bone marrow monocytes with OX40/Fc and OX40L (Figures 6C and 6D). Moreover, GFP⁺ monocyte migration was significantly increased upon treatment with OX40/Fc for 8 and 24 hr, while the GFP⁺ Kupffer cell numbers were not significantly different after OX40/Fc treatment (Figure 6E).

To better understand the role of OX40 in the regulation of monocyte activation and polarization, OX40/Fc- or control IgG-treated liver monocytes were compared in a transcriptome sequencing study (Figure 6F). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses (Figure 6G) revealed that upregulated genes from OX40-stimu-

lated monocytes are involved in the inflammatory response; cytokine production regulation; lipid, cytokine, and bacteria responses; the innate immune response; the enhanced cellular response to cytokines and cytokine-cytokine receptor interaction; and the phagosome and chemokine signaling pathway, among others. Compared with control monocytes, OX40/Fc-treated monocytes exhibited upregulated levels of cytokines (*Tnf*, *Il1a*, *Il1b*, *Il6*, and *Il12a*), cytokine receptors (*Il1r1*, *Il2r*, *Il18r*, and *Il23r*), chemokines (*Ccl3*, *Ccl4*, *Ccl22*, *Cxcl1*, *Cxcl2*, *Cxcl10*, and *Cx3cl1*), chemokine receptors (*Ccr2*, *Ccr12*, *Cxcr5*, and *Cxcr6*), and antigen processing and presentation molecules (*Cd40*, *Cd80*, *Cd86*, *Tlr1*, *Tlr2*, and *Ox40l*) (Figure 6H).

The coordinated action of various inflammatory modulators, signaling molecules, and transcription factors, including *Nlrp3*, *Tank*, *Jak2*, *Stat5a*, *Hif1a*, *Nos2*, *Socs3*, *Mir155hg*, and *Pparg*, was determined to be involved in the OX40 regulation of monocyte M1 polarization. *MyD88*, *Irf3*, *Irf5*, *Stat1*, *Nod1*, and *Nod2* may not be involved in OX40-triggered monocyte M1 polarization (Figure 6I).

OX40 Is Associated with NASH in Humans

To determine whether our findings are relevant for humans, we analyzed the plasma soluble OX40 (sOX40) levels of 54 NASH patients and 58 healthy controls in this observational study. Their demographic and clinical characteristics are shown in Table S1. Notably, we detected higher soluble OX40 plasma levels in NASH patients (11.29 ± 3.399 pg/mL) than in healthy controls (6.161 ± 1.213 pg/mL) ($p < 0.0001$; Figure 7A). In NASH patients, soluble OX40 levels were positively correlated with BMI ($\rho = 0.427$, $p = 0.001$), ALT ($\rho = 0.382$, $p = 0.004$), fasting glucose ($\rho = 0.399$, $p = 0.003$), liver steatosis ($\rho = 0.636$, $p = 0.001$), lobular inflammation ($\rho = 0.417$, $p = 0.002$), ballooning ($\rho = 0.304$, $p = 0.026$), and fibrosis ($\rho = 0.356$, $p = 0.008$). No correlations of soluble OX40 were found with T-CHO, TG, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) levels (Table S2). As shown in Figure 7B, notably, higher soluble OX40 plasma levels in NASH patients were associated with higher steatosis, activity, and fibrosis

Figure 5. The Soluble OX40 Stimulation Promoted Liver Steatosis and Liver Monocyte Proinflammation

Soluble OX40 was injected into OX40-KO mice fed an MCD for 4 weeks and mice injected with isotype-matched IgG as a control.

- Representative H&E (top) and oil red O (bottom) staining in liver paraffin sections.
 - Plasma ALT and AST levels were measured.
 - Quantification of hepatic Kupffer cells and monocytes as percentages of total liver leukocytes in each group.
 - Relative changes in OX40L expression plotted in each group.
 - Relative changes of CD11C⁺ and Ly6C^{high} cell proportions plotted in WT and OX40-KO MCD-fed mice injected with IgG or OX40/Fc.
 - Relative changes in CCR2 levels plotted in each group.
 - Statistical analysis (G) and flow cytometric images (H) of TNF- α ⁺ cells in monocytes and Kupffer cells.
 - Relative changes of antigen presentation-associated markers plotted in WT and OX40-KO mice injected with IgG or OX40/Fc. Soluble OX40/Fc was injected into B6.Rag2 and Il2rg mice fed an MCD for 4 weeks.
 - Representative H&E (top) and oil red O (bottom) staining in liver paraffin sections.
 - Plasma ALT and AST levels were measured.
 - Quantification of hepatic Kupffer cells and monocytes in liver leukocytes.
 - Relative OX40L mean fluorescence intensity (MFI) in monocytes and Kupffer cells plotted compared with that induced by MCD administration in B6.Rag2 and Il2rg mice (control group).
 - Relative changes of CD11C⁺ and Ly6C^{high} cell proportions plotted compared with those in control mice.
 - Relative CCR2 MFI in monocytes and Kupffer cells plotted in each group.
 - Flow cytometry images (Q) and statistical analysis (P) of TNF- α ⁺ cells in monocytes and Kupffer cells.
 - Relative MFI of antigen presentation-associated markers in monocytes and Kupffer cells plotted compared with those in control mice.
- Data are depicted as mean $\pm$ SD; n = 6 in each group. * $p < 0.05$ and ** $p < 0.01$; NS, not significant.

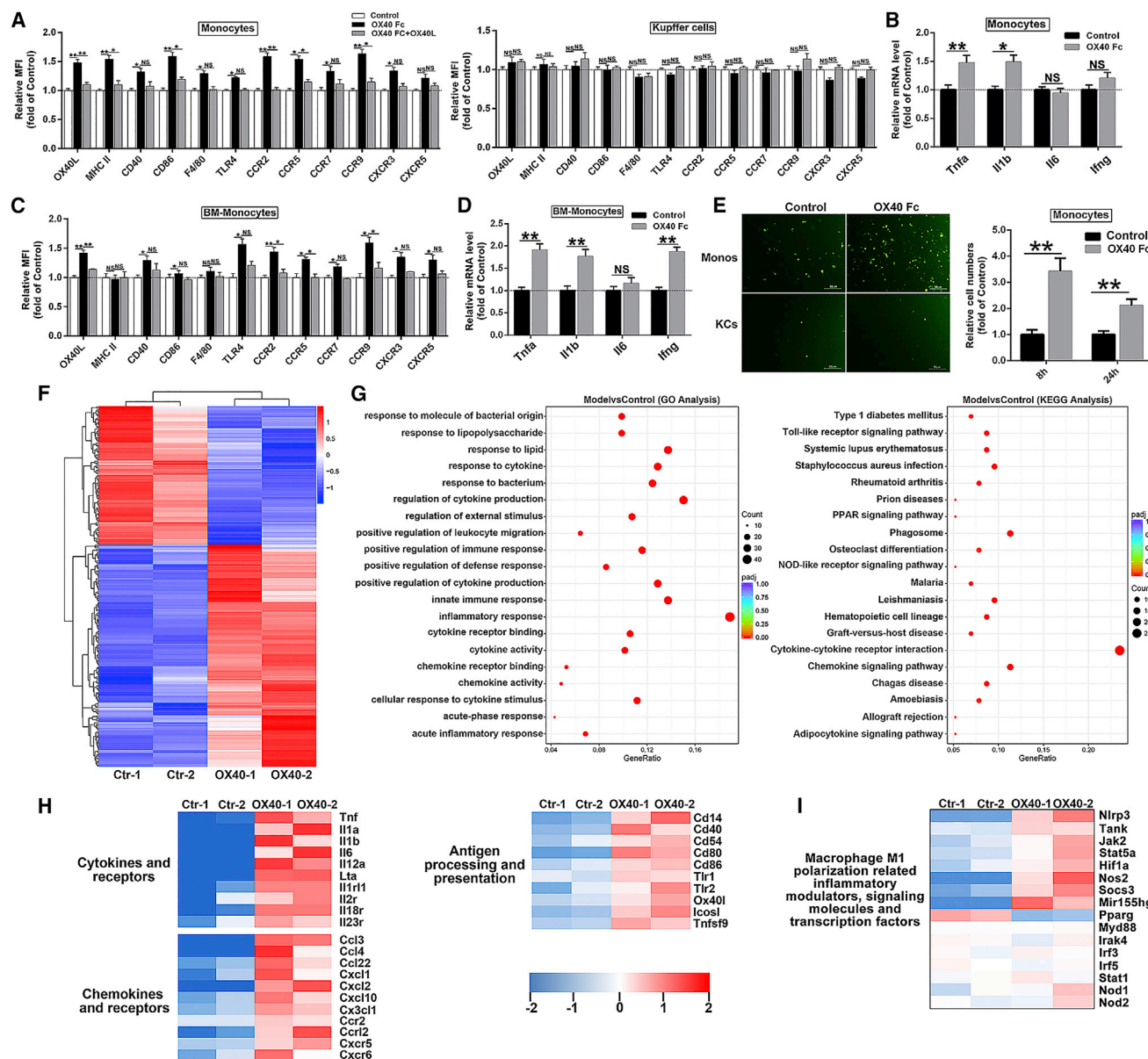


Figure 6. OX40 Promoted Monocyte Migration, Antigen Presentation, and Proinflammatory Cytokine Secretion

(A) Antigen presentation-associated markers and chemokine receptors in monocytes and Kupffer cells.
 (B) Real-time PCR analysis of proinflammatory cytokine mRNA levels in OX40/Fc-stimulated monocytes.
 (C) Antigen presentation-associated markers and chemokine receptors in bone marrow (BM) monocytes.
 (D) Real-time PCR analysis of proinflammatory cytokine mRNA levels in OX40/Fc-stimulated BM monocytes.
 (E) Representative images of monocyte and Kupffer cell migration with OX40/Fc treatment (left). Relative changes in monocyte numbers induced by OX40/Fc plotted as fold changes of cells induced by IgG treatment (right). Data are depicted as mean $\pm$ SD; $n = 6$ in each group. * $p < 0.05$ and ** $p < 0.01$; NS, not significant.
 (F) Transcriptome sequencing studies were performed on hepatic monocyte RNA after OX40 stimulation. The distributions of up- and downregulated genes (≥ 2 -fold difference, $p < 0.05$) compared with control IgG-treated monocytes are depicted in a heatmap.
 (G) Pathways comprising significantly up- or downregulated genes in OX40-treated monocytes compared with control cells are depicted.
 (H) Heatmap showing the up- and downregulated gene expression of cytokines, cytokine receptors, chemokines, chemokine receptors, and antigen processing and presentation molecules between control and OX40/Fc-treated monocytes.
 (I) Heatmap showing the up- and downregulated gene expression of inflammatory modulators, signaling molecules, and transcription factors between control and OX40/Fc-treated monocytes.

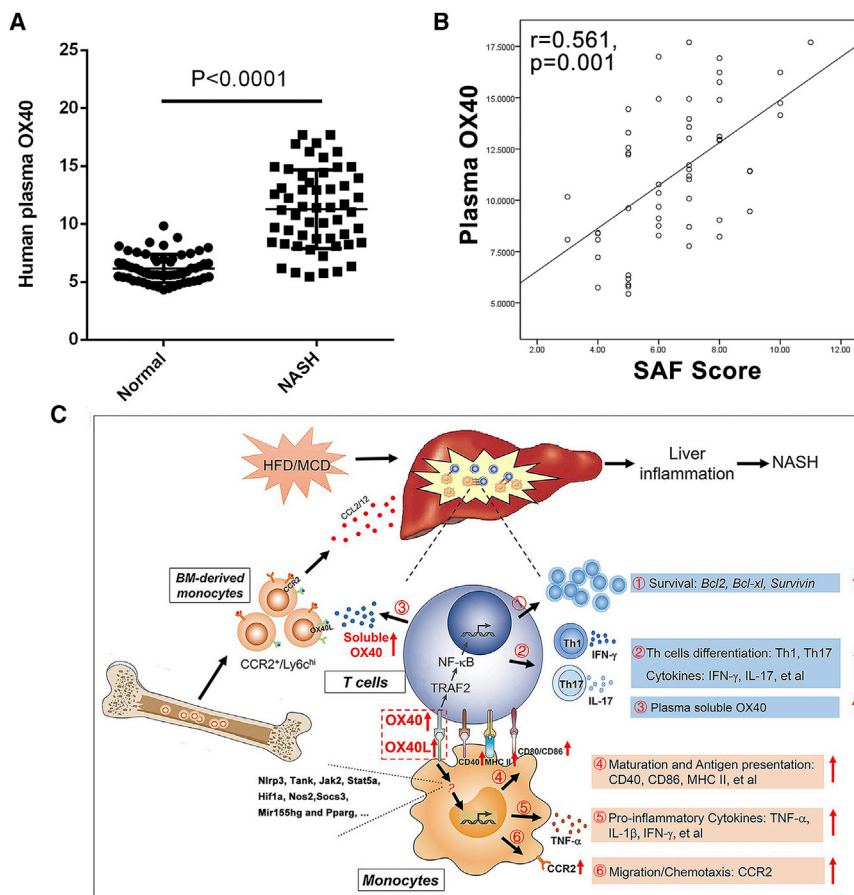


Figure 7. OX40 Was Associated with NASH in Humans

(A) Plasma samples collected from 54 NASH patients (NASH) and 58 healthy controls (Normal) were used to detect soluble OX40 levels. Data are depicted as mean $\pm$ SD, and p values are indicated in the graph.

(B) Plasma OX40 levels were plotted for the SAF scores of NASH patients.

(C) Intrinsic mechanisms of OX40 regulation on NASH development and progression.

interaction can prevent T cell responses and alleviate inflammation development in models of MS, colitis, RA, GVHD, and leishmaniasis (Akiba et al., 2000; Higgins et al., 1999; Tsukada et al., 2000; Weinberg et al., 1999; Yoshioka et al., 2000). In this study, OX40 engagement promoted upregulation of the *Tbx21* and *ROR γ T* transcription factors, which resulted in the differentiation of CD4 T cells into Th1 and Th17. Additionally, OX40 upregulation enhanced intrahepatic T cell activation and proliferation and decreased T cell apoptosis. This phenomenon was related to the upregulation of anti-apoptosis genes, such as *Bcl2*, *Bcl-xl*, and *Survivin*, in liver infiltrated T cells. These data revealed that OX40 positively regulates CD4 T cells toward proinflammatory Th1 and Th17 cell

(SAF) scores ($\rho = 0.561$, $p = 0.001$). These data suggest that OX40 is also involved in human NASH development and might act as an independent risk factor of human NASH.

DISCUSSION

T cells play a critical role in the development and progression of NASH. High-fructose diet-fed mice or MCD-fed mice lacking T cells fail to develop steatosis and hepatic inflammation (Bhat-tacharjee et al., 2014), and immune responses triggered by oxidative stress-derived antigens contribute to hepatic inflammation in experimental NASH by promoting Th1 activation of CD4 T cells (Sutti et al., 2014). These findings are validated in NASH patients, as they have increased frequencies of IFN- γ ⁺ memory T cells (Inzaugarat et al., 2011). Promoting the Th17 differentiation of activated CD4 T cells has also been linked to progressive NASH (Rau et al., 2016).

CD4 T cell activation requires costimulation in addition to MCH II-dependent antigen presentation. OX40 is predominantly expressed in activated CD4 and CD8 T cells, and OX40 signaling promotes T cell survival and division (Croft, 2010). In naive CD4 T cells, OX40 engagement can lead to either Th1 or Th2 cell generation in different disease models depending on the microenvironment (Croft et al., 2009; Kaur and Brightling, 2012; Ward-Kavanagh et al., 2016). Blocking the OX40-OX40L

differentiation, sustains liver infiltrated proinflammatory T cell survival, and promotes liver inflammation in diet-induced NASH.

The adoptive transfer of T cells from either WT or OX40-KO mice to immune-deficient B6.Rag2 and Il2rg double-KO mice fed an HFD or MCD provided evidence that OX40 in T cells contributes to monocyte migration, antigen presentation, and M1 polarization, thus promoting NASH development and progression. These phenomena could be explained by the fact that IFN- γ and IL-17 produced by Th1 and Th17 cells polarize monocytes to the M1 phenotype and promote liver inflammation. However, because we also observed markedly increased plasma soluble OX40 levels in HFD-, MCD-, and CD-HFD-fed mice, we speculated whether mechanisms other than controlling cytokine expression are involved in the T cell regulation of innate immune cells.

Studies on the OX40-OX40L interaction have focused mainly on the regulation of conventional T cells. One early study by Stuber and colleagues showed that the OX40-OX40L interaction was also critical for T cell-dependent B cell responses (Stüber et al., 1995; Stüber and Strober, 1996). OX40 cross-linking on CD40L-stimulated B cells, alpha IgD dextran-stimulated B cells, or both resulted in a significantly enhanced B cell proliferative response; however, OX40-Ig stimulation alone induced no B cell proliferation. Similarly, data from Uchiyama's group also showed that anti-OX40L monoclonal antibody (mAb) promoted TNF- α , IL-1 β , and IL-6 cytokine production and CD80, CD86,

and CD54 expression in CD40L-activated dendritic cells (DCs), while anti-OX40L mAb alone had no significant effect on DCs (Ohshima et al., 1997).

In the present study, similar to the results *in vivo*, OX40 engagement upregulated antigen presentation-associated molecules, chemokine receptors, and proinflammatory cytokines in monocytes. These phenomena could be blocked by recombinant OX40L. All these data indicate that OX40 in T cells or soluble OX40 alone can regulate monocyte maturation, migration and M1 polarization through the OX40-OX40L interaction. Although the coordinated action of various inflammatory modulators, signaling molecules, and transcription factors (including *Nlrp3*, *Tank*, *Jak2*, *Stat5a*, *Hif1a*, *Nos2*, *Socs3*, *Mir155hg*, and *Pparg*) was determined to be involved in the OX40 regulation of monocyte M1 polarization, the signaling pathways between OX40L engagement and inflammatory consequences in monocytes/macrophages still need to be further explored.

Soluble forms have been demonstrated for several members of the TNF receptor family, including TNF and NGF receptors, such as CD27, CD30, CD95, and CD137 (Cascino et al., 1995; Cheng et al., 1994; Kohno et al., 1990; Michel et al., 1998; Pizzolo et al., 1990). Similar to these receptors, soluble OX40 may be produced through cleavage from cell-surface OX40 or alternative splicing (Taylor and Schwarz, 2001). NASH is not only a liver-exclusive disease but also a hepatic manifestation of a systemic disease state characterized by obesity, insulin resistance, and chronic low-grade inflammation. The increased soluble OX40 in serum may also further promote systemic inflammation and onset of other inflammation-related diseases. To investigate the relevance of our findings to humans, we measured soluble OX40 in plasma from NASH patients and healthy controls. We observed that individuals with NASH exhibited significantly higher soluble OX40 plasma levels than healthy controls. These findings indicate that OX40 may also be an important regulator and potential contributor in human NASH development and progression.

In summary, during NASH development, the activation of T cells upregulated costimulatory OX40 expression, and OX40 further promoted intrahepatic T cell activation, proliferation, and differentiation into Th1 and Th17 cells. Additionally, OX40L was also upregulated in infiltrated monocytes during hepatic steatosis. The cross-linking of OX40 with its ligand OX40L enhanced the antigen presentation abilities of monocytes and macrophages, TNF- α secretion, migration, and recruitment in steatotic livers. Moreover, the soluble OX40 secreted by activated T cells could further promote bone marrow-derived monocyte hepatic recruitment and increase systemic inflammation (Figure 7C).

In conclusion, we have elucidated a novel pathway for T cell regulation of innate immune cells. OX40 is a key molecule that mediates crosstalk between intrahepatic innate and adaptive immunity, generates two-way signals, and promotes both proinflammatory monocyte and macrophage and T cell function, resulting in NASH development and progression. OX40 could serve as a diagnostic index and therapeutic target of NASH.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and three tables and can be found with this article online at <https://doi.org/10.1016/j.celrep.2018.12.006>.

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AUTHOR CONTRIBUTIONS

All listed authors participated meaningfully in the study and have seen and approved the submission of this manuscript. G.S., H.J., and C.Z. performed the research, analyzed the data, and initiated the original draft of the article. H.M. performed the clinical study. X.Z., D.W., X.O., Q.W., S.L., T.W., X.S., W.S., D.T., K.L., W.G., H.X., Y.T., X.L., and J.J. performed the research and collected the data. D.Z. established the hypotheses, supervised the studies, analyzed the data, and co-wrote the manuscript. Z.Z. supervised the clinical studies, analyzed the data, and co-wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
PE/Cy7 anti-CD11b (clone M1/70)	Thermo Fisher Scientific	Cat# 25-0112-82; RRID:AB_469588
FITC anti-CD11b (clone M1/70)	Thermo Fisher Scientific	Cat# 11-0112-82; RRID:AB_464935
PE anti-CD11b (clone M1/70)	Thermo Fisher Scientific	Cat# 12-0112-85; RRID:AB_465549
PerCP-Cy5.5 anti-CD3 (clone 145-2C11)	Thermo Fisher Scientific	Cat#35-0031-82; RRID:AB_11219266
APC anti-CD3 (clone 145-2C11)	Thermo Fisher Scientific	Cat#: 17-0031-83; RRID:AB_469316
PE/Cy7 anti-CD3 (clone 145-2C11)	Thermo Fisher Scientific	Cat#: 25-0031-82; RRID: AB_469572
PE/Cy7anti-CD4 (clone GK1.5)	Thermo Fisher Scientific	Cat#: 25-0041-82; RRID:AB_469576
PerCP-Cy5.5 anti-CD4 (clone GK1.5)	Thermo Fisher Scientific	Cat#: 45-0042-82; RRID:AB_1107001
FITC anti-CD4 (clone GK1.5)	Thermo Fisher Scientific	Cat#: 11-0041-85; RRID:AB_464893
APC anti-CD4 (clone GK1.5)	Thermo Fisher Scientific	Cat#: 17-0041-82; RRID:AB_469320
PerCP-Cy5.5 anti-CD8 (clone 53-6.7)	Thermo Fisher Scientific	Cat#: 45-0081-82; RRID:AB_1107004
FITC anti-CD8 (clone 53-6.7)	Thermo Fisher Scientific	Cat#: 53-0081-82; RRID:AB_469897
PE anti-CD69 (clone H1.2F3)	Thermo Fisher Scientific	Cat#: 12-0691-81; RRID:AB_465731
APC anti-CD69 (clone H1.2F3)	Thermo Fisher Scientific	Cat#: 17-0691-80; RRID:AB_1210796
PE/Cy7 anti-CD45 (clone 30-F11)	Thermo Fisher Scientific	Cat#: 25-0451-82; RRID:AB_469625
PE anti-CD45 (clone 30-F11)	Thermo Fisher Scientific	Cat#: 12-0451-82; RRID:AB_465668
APC anti-NK1.1 (clone PK136)	Thermo Fisher Scientific	Cat#: 17-5941-82; RRID:AB_469479
PE anti-NK1.1 (clone PK136)	Thermo Fisher Scientific	Cat#: 12-5941-83; RRID:AB_466051
FITC anti-NK1.1 (clone PK136)	Thermo Fisher Scientific	Cat#: 11-5941-82; RRID:AB_465318
FITC anti-TCR β (clone H57-597)	Thermo Fisher Scientific	Cat#: 11-5961-85; RRID:AB_465324
PerCP-Cy5.5 anti-TCR β (clone H57-597)	Thermo Fisher Scientific	Cat#: 45-5961-82; RRID:AB_925763
PE anti-Gr-1 (clone RB6-8C5)	Thermo Fisher Scientific	Cat#: 12-5931-83; RRID:AB_466046
APC anti-Gr-1 (clone RB6-8C5)	Thermo Fisher Scientific	Cat#: 17-5931-82; RRID: AB_469476
APC anti-Ly6G (clone 1A8-Ly6g)	Thermo Fisher Scientific	Cat#: 17-9668-82; RRID:AB_2573307
PE anti-OX40 (clone OX-86)	Thermo Fisher Scientific	Cat#: 12-1341-82; RRID:AB_465854
APC anti-OX40 (clone OX-86)	Thermo Fisher Scientific	Cat#: 17-1341-82; RRID:AB_10717260
APC anti-MHC II (clone M5/114.15.2)	Thermo Fisher Scientific	Cat#: 17-5321-82; RRID:AB_469455
APC anti-CD40 (clone HM40-3)	Thermo Fisher Scientific	Cat#: 17-0402-82; RRID:AB_10853008
PE anti-CD86 (clone P03.1)	Thermo Fisher Scientific	Cat#: 12-0861-83; RRID:AB_465766
PE/Cy7anti-CD86 (clone GL1)	Thermo Fisher Scientific	Cat#: 25-0862-82; RRID:AB_2573372
PE/Cy7 anti-TLR4 (clone MTS510)	Thermo Fisher Scientific	Cat#: 25-9924-82; RRID:AB_469689
APC anti-CCR2 (clone SA203G11)	Biolegend	Cat#: 150603; RRID:AB_2566139
FITC anti-CCR2 (clone SA203G11)	Biolegend	Cat#: 150607; RRID:AB_2616979
PE anti-CCR5 (clone HM-CCR5(7A4))	Thermo Fisher Scientific	Cat#: 12-1951-82; RRID:AB_657684
PE anti-CCR7 (clone 4B12)	Thermo Fisher Scientific	Cat#: 12-1971-82; RRID:AB_465905
PE anti-CCR9 (clone eBioCW-1.2)	Thermo Fisher Scientific	Cat#: 12-1991-82; RRID:AB_1257161
PE anti-CXCR3 (clone CXCR3-173)	Thermo Fisher Scientific	Cat#: 12-1831-82; RRID:AB_1210734
PE anti-CXCR5 (clone SPRCL5)	Thermo Fisher Scientific	Cat#: 12-7185-82; RRID:AB_11217882
PerCP-Cy5.5 anti-Foxp3 (clone FJK-165)	Thermo Fisher Scientific	Cat#: 45-5773-82; RRID:AB_914359
PE anti-IL-17 (clone eBio17B7)	Thermo Fisher Scientific	Cat#: 12-7177-81; RRID:AB_763582
PE anti-IFN- γ (clone XMG1.2)	Thermo Fisher Scientific	Cat#: 12-7311-82; RRID:AB_466193
PE anti-TNF α (clone MP6-XT22)	Thermo Fisher Scientific	Cat#: 12-7321-82; RRID:AB_466199

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
APC anti-OX40L (clone OX86)	Thermo Fisher Scientific	Cat#: 17-1341-82; RRID:AB_10717260
Annexin V-PE kit	BD PharMingen	Cat#: 559763
Biological Samples		
NASH patients and healthy controls plasma	Clinical Data and Biobank Resource of Beijing Friendship Hospital	OD16-metabolic diseases (Biobank at Beijing friendship Hospital)
Chemicals, Peptides, and Recombinant Proteins		
Recombinant mouse OX40/Fc Chimera Protein	R&D systems	Cat#: 1256-OX-050
Recombinant mouse OX40L Protein	R&D systems	Cat#: 1236-OX-025/CF
Cell Stimulation Cocktail	Thermo Fisher Scientific	Cat#: 00-4975-03
Collagenase type IV	Sigma	Cat#: 559763
DNase I	Roche	Cat#: 11284932001
Percoll	GE Health	Cat#: 17-0891-09
Critical Commercial Assays		
anti-CD4 monoclonal antibody (clone GK1.5)	BioXCell	Cat#: 5730-1-3/0515
Purified anti-mouse CD3e (clone 145-2C11)	BD Biosciences	Cat#: 550275
Purified anti-mouse CD28 (clone 37.51)	BD Biosciences	Cat#: 553294
Permeabilization Buffer 10x	Thermo Fisher Scientific	Cat#: 00-8333-56
Fixation/Permeabilization Concentrate	Thermo Fisher Scientific	Cat#: 00-5123-43
Fixation/Permeabilization Diluent	Thermo Fisher Scientific	Cat#: 00-5223-56
Fixation Buffer	BioLegend	Cat#: 420801
Cell-Light EdU Apollo®643 <i>in Vitro</i> Flow Cytometry Kit	RiboBio Corporation	Cat#:C10338-3
Mouse OX40 ELISA Kit	Boster Biological Technology	Cat#: EK0998
Soluble OX40 Human ELISA Kit	Thermo Fisher Scientific	Cat#: BMS296
RNeasy Plus Mini Kit	QIAGEN	Cat#: 74136
SuperScript III RT kit	Invitrogen	Stock#: 18080051
Alanine Transaminase (ALT) Detection Kits	NanJing JianCheng Biochemical Institute	Cat#: C009-2
Aspartate Transaminase (AST) Detection Kits	NanJing JianCheng Biochemical Institute	Cat#: C010-2
TG Detection Kits	NanJing JianCheng Biochemical Institute	Cat#: A110-1
Total Bilirubin (TBIL) Detection Kits	NanJing JianCheng Biochemical Institute	Cat#: C019-1
Total Cholesterol (T-CHO) Detection Kits	NanJing JianCheng Biochemical Institute	Cat#: A111-1
Deposited Data		
Raw and analyzed data	This paper	GEO: GSE105187
Experimental Models: Organisms/Strains		
Mouse: C57BL/6	The Jackson Laboratory	Stock#: 000664
Mouse: C57BL/6 OX40 KO	The Jackson Laboratory	Stock#: 012838
Mouse: C57BL/6-GFP	The Jackson Laboratory	Stock#: 004353
Mouse: B6.Rag2 and Il2rg double KO	Taconic Bioscience	Model#: 4111-F
Oligonucleotides		
See Table S3 for gene expression analysis primers	This paper	N/A
Software and Algorithms		
FlowJo software version 10.5.2	Treestar	N/A
GraphPad Prism 6	GraphPad software	N/A
SPSS software	SPSS Inc	N/A
Other		
Diet: Normal Control Diet (NCD)	Beijing HFK Bioscience	Cat#: 1035
Diet: High Fat Diet (HFD)	Beijing HFK Bioscience	Cat#: 1042
Diet: Methionine/choline-deficient Diet (MCD)	Beijing HFK Bioscience	Cat#: 10401

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Diet: Choline-deficient High Fat Diet (CD-HFD)	Research Diets	Cat#: D05010402
Gentle-MACS dissociator	Miltenyi Biotec	Cat#: 130-093-235
ABI 7500 sequence detection system	Applied Biosystems	N/A
BD FACS Aria II	BD Biosciences	N/A

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed and will be fulfilled by the Lead Contact, Dong Zhang (zhangd2010@hotmail.com).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

Eight weeks old weight-matched male wild-type (WT) C57BL/6, C57BL/6 OX40-KO and C57BL/6-GFP mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). B6.Rag2 and Il2rg double KO mice were purchased from Taconic Bioscience (Germantown, NY, USA). These mice were fed either a normal control diet (NCD), a HFD (45 kcal% fat, Beijing HFK Bioscience, Beijing, China), a methionine/choline-deficient diet (MCD, Beijing HFK Bioscience), a choline-deficient high fat diet (CD-HFD, Research Diets, D05010402, New Brunswick, NJ, Canada). The mice were maintained in a pathogen-free, temperature-controlled environment under a 12-hour light/dark cycle at Beijing Friendship Hospital, and all animal protocols were approved by the Institutional Animal Care and Ethics Committee.

METHOD DETAILS

Isolation of liver immune cells by enzymatic digestion

After mice were anesthetized, each mouse liver was perfused with 30 mL of normal saline (NS) through left cardiac perfusion until its liver changed to a pale color, at which point the liver was carefully removed and digested with 0.01% type IV collagenase for 30 min at 37°C. The mixture was then dissociated using a gentle-MACS dissociator (Miltenyi Biotec, Bergisch-Gladbach, Germany). The cell suspension was filtered through a 70-μm nylon cell strainer and centrifuged at 50 x g for 5 min to obtain the supernatant, which was then centrifuged at 500 x g for 5 min. The cell pellet was used to detect hepatic infiltrated monocytes and Kupffer cells. The above-mentioned cell pellet was resuspended in 30% Percoll in Hanks' Balanced Salt Solution (HBSS, 1 ×), gently overlaid onto 70% Percoll and centrifuged at 800 x g for 25 min. The cells were collected from the interface to detect intrahepatic T cells.

Real-time PCR

Total RNA was isolated from liver tissue, splenocytes, liver immune cells using an RNeasy Plus Mini kit (QIAGEN, Valencia, CA, USA) and reverse transcribed into cDNA using a SuperScript III RT kit (Invitrogen, Carlsbad, CA, USA). Specific message levels were quantified by real-time PCR using the ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). Amplicon expression in each sample was normalized to that of *GAPDH*, and after normalization, gene expression was quantified using the $2^{-\Delta\Delta C_t}$ method. The genes and primer sequences are shown in [Table S3](#).

Flow cytometry analysis

Liver immune cells were harvested and analyzed to determine the expression levels of various cell surface and intra-cellular markers. All samples were acquired on a FACS Aria II flow cytometer (BD Biosciences, CA, USA), and the data were analyzed using FlowJo software (Treestar, Ashland, OR, USA).

T cell isolation and T cell proliferation assay

Splenocytes from WT and OX40-KO mice were cultured in triplicate in wells pre-coated with the anti-CD3 mAb (3 μg/mL, BD Biosciences) and the soluble anti-CD28 mAb (1 μg/mL, BD Biosciences) at 37°C with 5% CO₂. After 72 hours of incubation and 12 hours before harvest, 5-ethynyl-2'-deoxyuridine (EdU) was added to the plates (final concentration of 50 μM). Cell proliferation was measured via EdU incorporation according to the manufacturer's instructions (EdU staining kit, RiboBio Corporation, Guangzhou, China).

CD4⁺ T cell depletion *in vivo*

Mice were intraperitoneally injected with 400 μg of rat anti-CD4 monoclonal antibody (GK1.5) every other day for the first week and twice a week for the following 3 weeks before detection for NASH development, during these 4 weeks, mice were fed with MCD.

Adoptive transfer of T cells

CD3 T cells (5×10^6 , purity > 97%) were sorted from splenocytes of naive WT C57BL/6 or OX40-KO mice, and transferred into B6.Rag2 and Il2rg double KO mice by tail vein injection. The control mice were injected with saline. Recipient mice subsequently consumed HFD for 16 weeks and were detected for NASH development.

Soluble OX40 stimulation *in vivo*

The OX40-KO mice or B6.Rag2 and Il2rg double KO mice were intraperitoneally injected with 1 μ g of OX40/Fc (Cat no. 1256-OX-50, R&D Systems, Minneapolis, MN, USA) or control IgG 3 times a week for total 4 weeks. During these 4 weeks, mice were fed with MCD. After 4 weeks OX40/Fc injection, recipient mice were detected for NASH development.

Transwell assays

Monocytes and Kupffer cells were isolated from the livers of GFP⁺-C57BL/6 mice, and isolated GFP⁺ cells (0.5×10^6) were pretreated with OX40/Fc (2 μ g/ml) or the IgG control with serum-starved media overnight. The starved monocytes or Kupffer cells were then placed in the top chamber, and Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 2 μ g/ml CCL2 was placed in the lower chamber. Transwell plates were incubated at 37°C with 5% CO₂ for 6 hours, after which the upper chambers were removed. Then, GFP⁺ cells that had migrated to the lower wells were counted, and migration was quantified using inverted fluorescence microscopy by analyzing at least 10 random fields from each of three replicate filters for each experimental condition.

Clinical study

The observation study involved 54 patients with NASH who underwent liver biopsy at Beijing Friendship Hospital. The NASH diagnosis was confirmed by the liver biopsy specimen showing steatosis with lobular inflammation and hepatocyte ballooning with or without fibrosis in the setting of increased BMI or metabolic risk factors (type 2 diabetes, hypertension, hyperlipidemia). For each biopsy, a SAF score summarizing the main histological lesions was defined based on the grade of steatosis, the grade of activity (hepatocytes ballooning and lobular inflammation) and the stage of fibrosis ([Bedossa and FLIP Pathology Consortium, 2014](#)). NASH patients were consuming less than 10 g/alcohol per day for women and 20 g/alcohol per day for men, and other causes of steatosis or chronic liver disease were excluded. Fifty-eight patients visiting the Physical Examination Center Beijing Friendship Hospital for routine physical examinations served as healthy controls. Healthy subjects were required to be nondiabetic and free of major organ disease, chronic inflammatory conditions, cancer, active psychiatric diseases and surgical history. Plasma and sera were collected in vacutainer tubes, and fasting serum glucose, ALT, AST, cholesterol and TG levels were measured using standard laboratory techniques. All subjects provided written informed consent to participate in the study, and the study protocol was approved by the Human Institutional Review Board of Beijing Friendship Hospital.

QUANTIFICATION AND STATISTICAL ANALYSIS

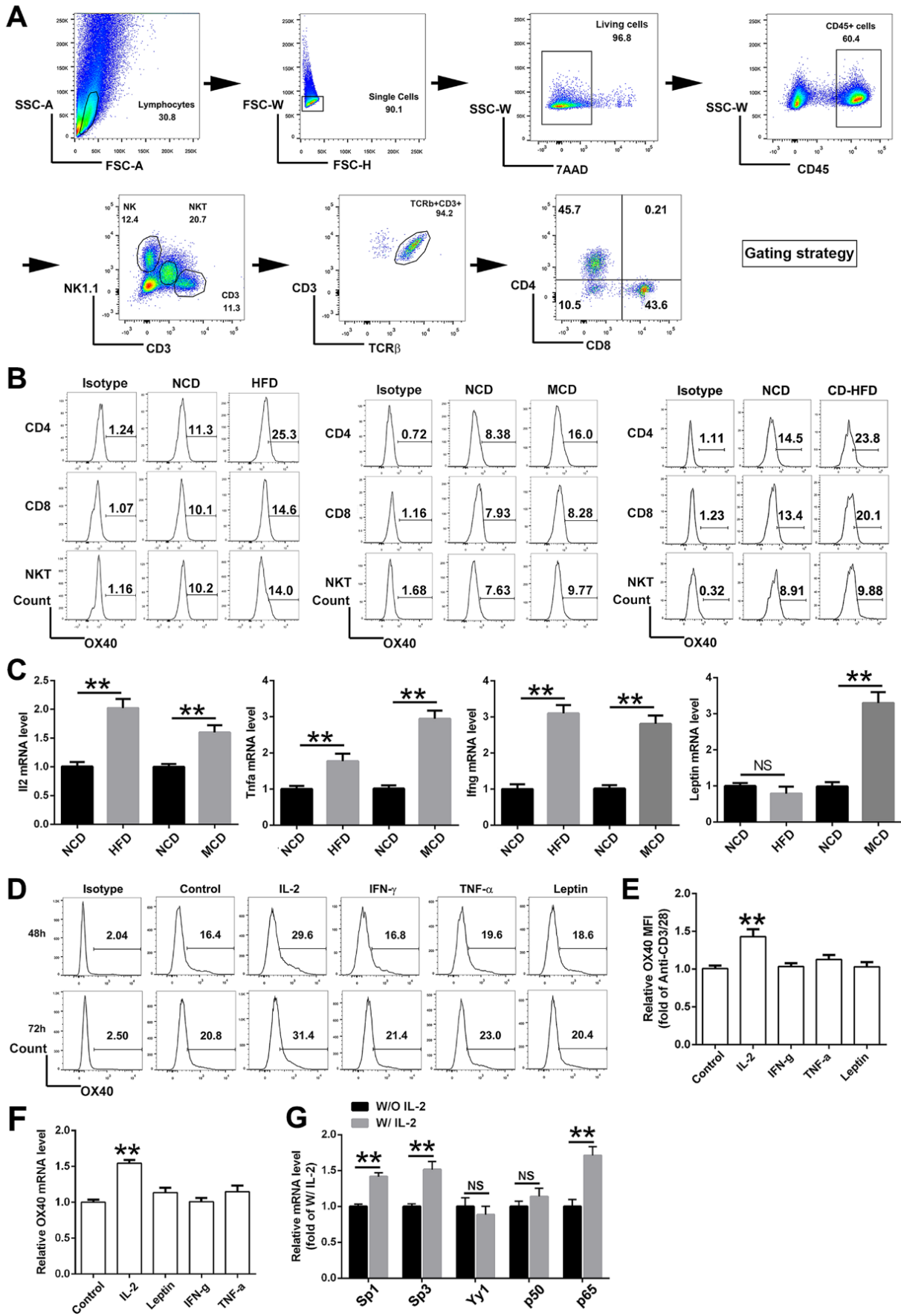
Statistical analysis was performed with SPSS software (SPSS Inc), and values are expressed as the mean $\pm$ standard deviation (SD). Significant differences were analyzed using Student's t tests and one-way analysis of variance (ANOVA). In the clinical study, normal variable distributions were tested with the Shapiro-Wilk test. Differences between groups were compared by t tests for normal variables and the Mann-Whitney U-test for non-normal variables. Spearman's correlation coefficient was used to estimate the association of serum OX40 levels and several factors of interest. *p* values < 0.05 were considered significant.

Supplemental Information

OX40 Regulates Both Innate and Adaptive Immunity and Promotes Nonalcoholic Steatohepatitis

Guangyong Sun, Hua Jin, Chunpan Zhang, Hua Meng, Xinyan Zhao, Dan Wei, Xiaojuan Ou, Qianyi Wang, Shuxiang Li, Tianqi Wang, Xiaojing Sun, Wen Shi, Dan Tian, Kai Liu, Hufeng Xu, Yue Tian, Xinmin Li, Wei Guo, Jidong Jia, Zhongtao Zhang, and Dong Zhang

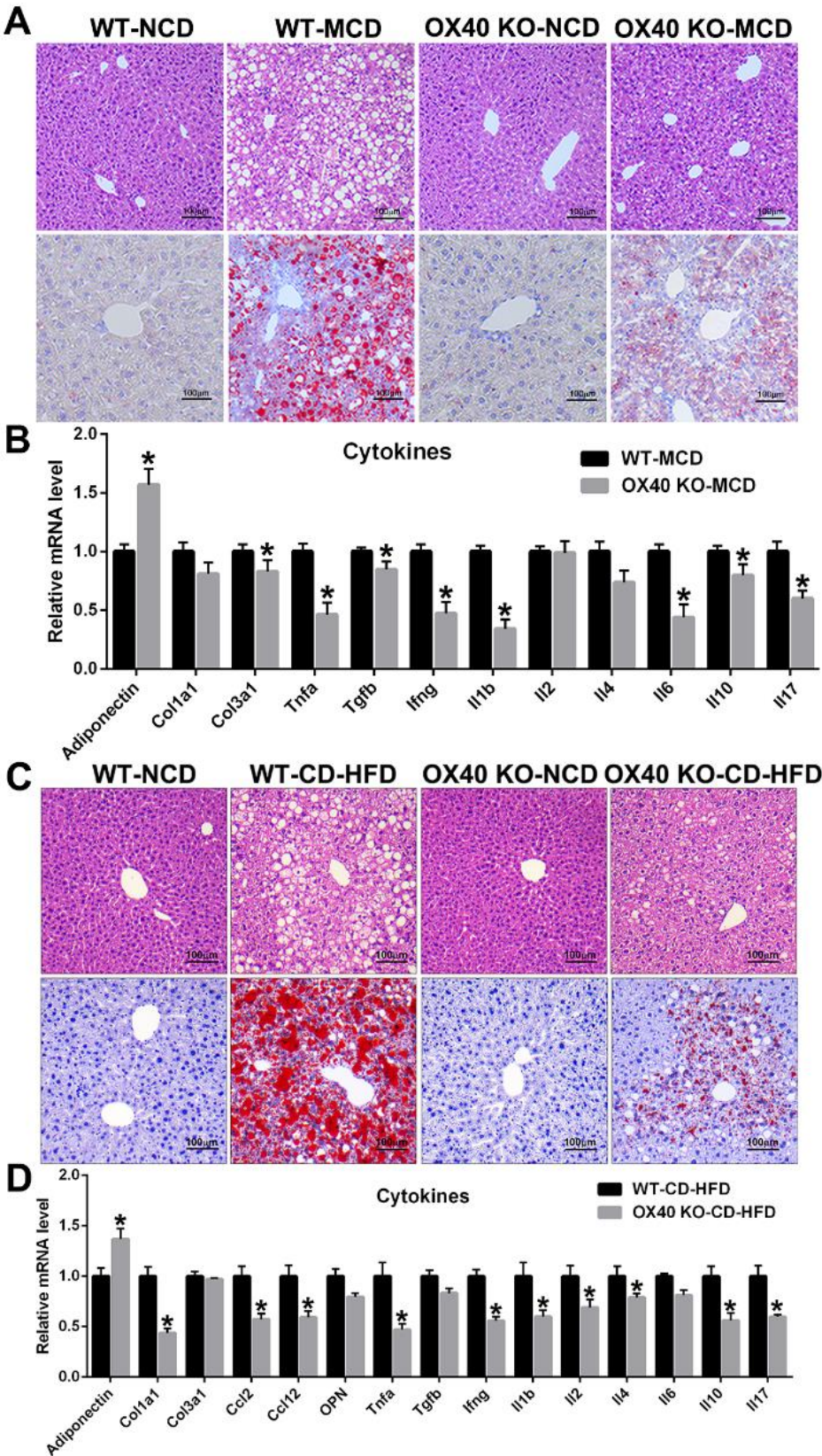
Figure S1



Supplementary Figure 1. IL-2 promoted OX40 expression in CD4 T-cells. Related to Figure 1.

A: Representative flow cytometry images of the gating strategy used for flow cytometry analysis. **B:** Flow cytometry images of OX40 expression in different liver lymphocytes. **C:** Relative *Il2*, *Tnfa*, *Ifng* and *Leptin* mRNA expression levels in normal and NASH liver tissues. **D and E:** Flow cytometry image and the statistical analysis of OX40 expression in CD4 T-cells after stimulation with IL-2, IFN- γ , TNF- α or Leptin. **F:** Relative *OX40* mRNA levels after stimulation with different cytokines. **G:** Relative mRNA levels of the transcription factors *Sp1*, *Sp3*, *Yy1*, *p50* and *p65* in the presence or absence of IL-2 in CD4 T-cells. The data are depicted as the mean $\pm$ SD, and n=6 in each group. * $p<0.05$, ** $p<0.01$, NS: no significance.

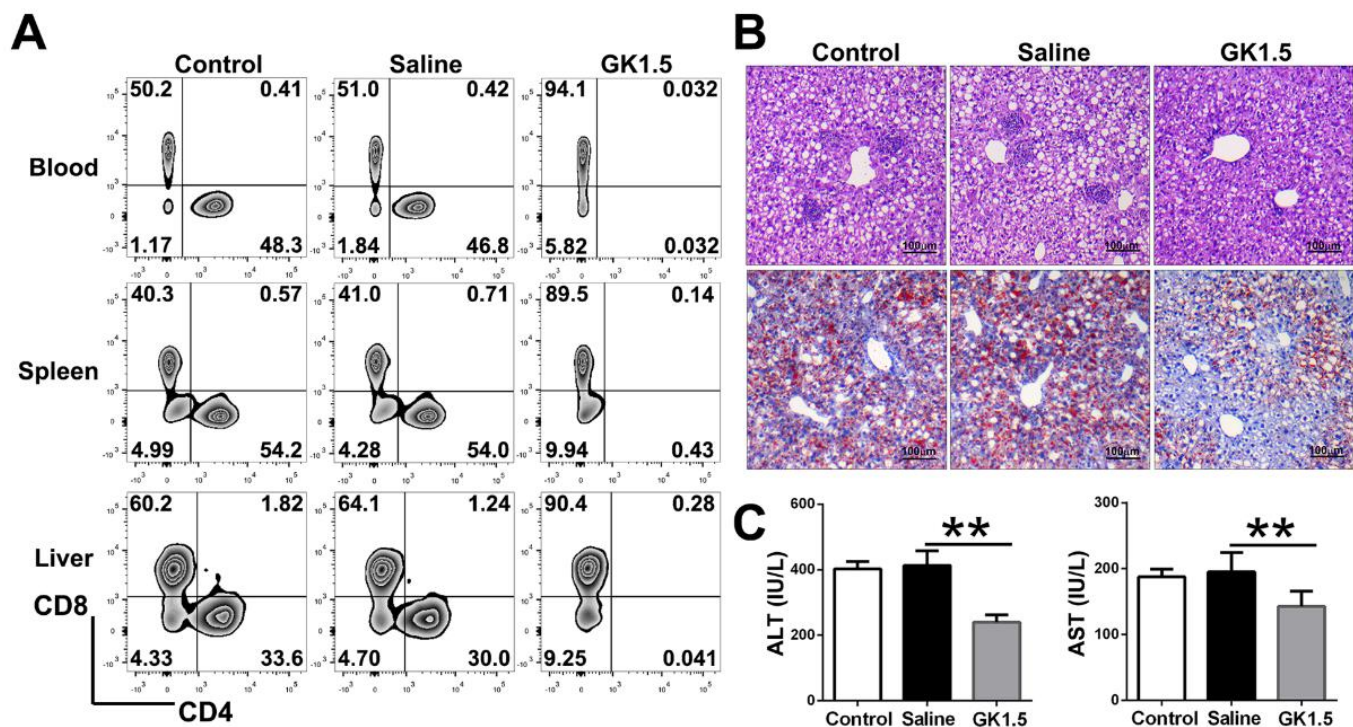
Figure S2



Supplementary Figure 2. OX40 deficiency alleviated steatohepatitis in mice received MCD or CD-HFD. Related to Figure 1.

A: Representative hematoxylin and eosin staining (upper panel) and Oil Red O staining (lower panel) in liver paraffin sections from WT and OX40-KO mice fed the NCD or MCD. **B:** Relative proinflammatory cytokine mRNA levels in liver tissues were analyzed by quantitative real-time PCR in WT and OX40-KO mice fed the NCD or MCD for 4 weeks. **C:** Representative hematoxylin and eosin staining (upper panel) and Oil Red O staining (lower panel) in liver paraffin sections from WT and OX40-KO mice fed the NCD or CD-HFD. **D:** Relative proinflammatory cytokine mRNA levels in liver tissues were analyzed by quantitative real-time PCR in WT and OX40-KO mice fed the NCD or CD-HFD for 12 weeks. The data are depicted as the mean $\pm$ SD, and n=6 in each group. * $p<0.05$, ** $p<0.01$, NS: no significance.

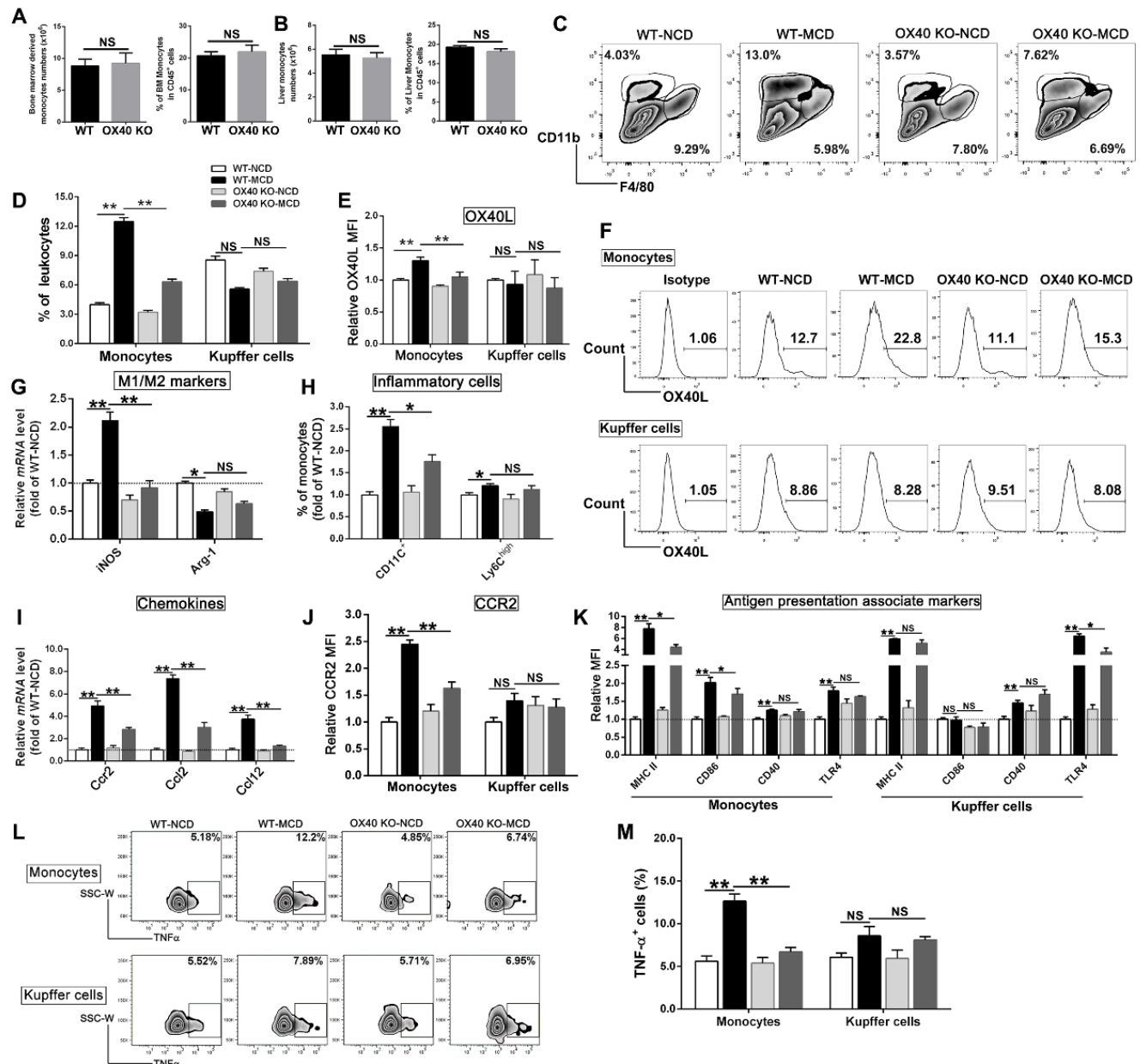
Figure S3



Supplementary Figure 3. CD4 T-cells depletion ameliorated diet induced hepatic steatosis. Related to Figure 2.

A: The proportions of T-cells in blood, spleen and liver after injection with saline or GK1.5 antibody. **B:** Representative hematoxylin and eosin staining (upper panel) and Oil Red O staining (lower panel) in liver paraffin sections from the mice in each group. **C:** Plasma ALT and AST levels were measured. The data are depicted as the mean±SD, and n=6 in each group. * $p<0.05$, ** $p<0.01$, NS: no significance.

Figure S4

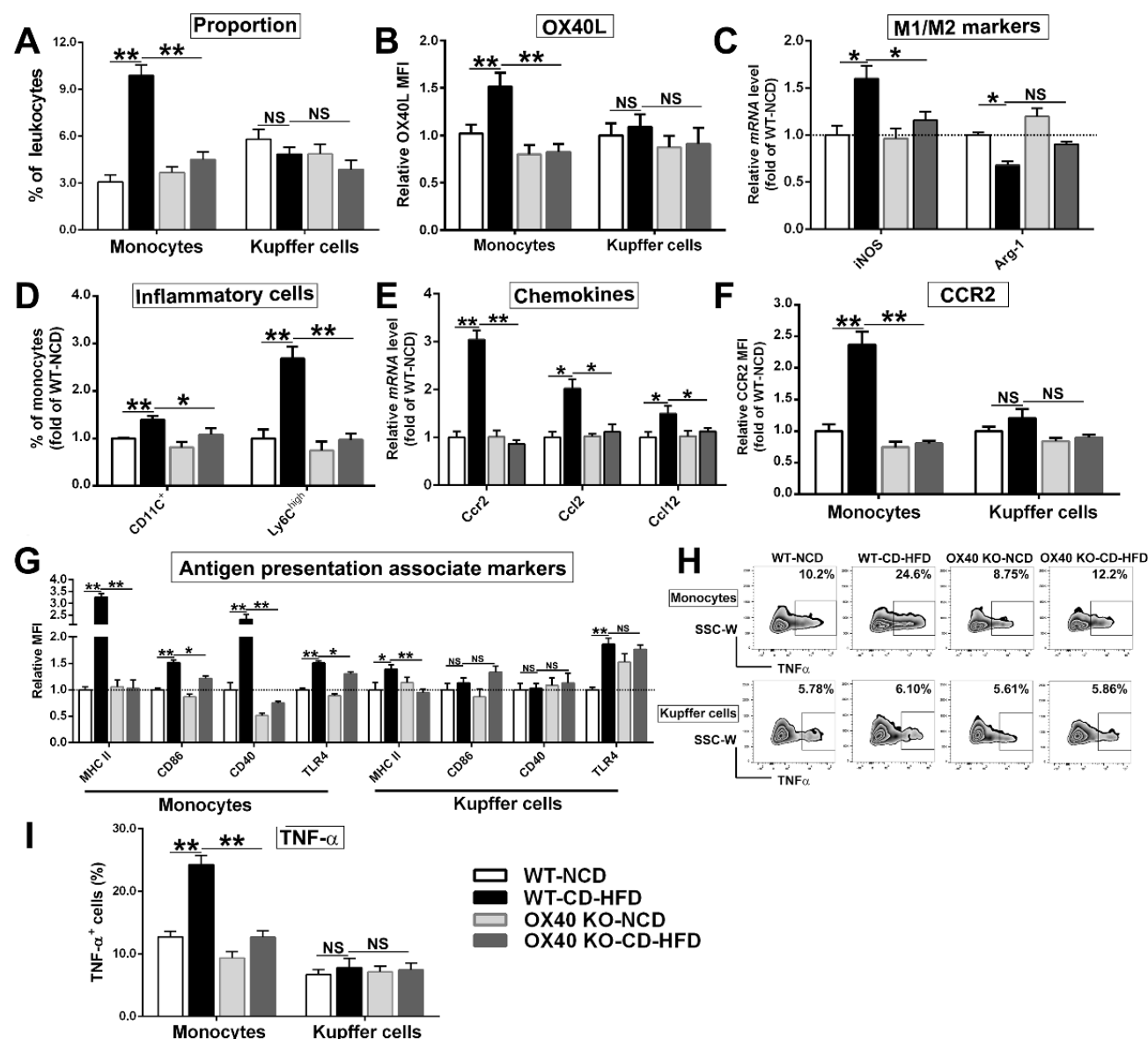


Supplementary Figure 4. OX40 deficiency dampened infiltrated monocyte hepatic recruitment, maturation, and proinflammatory cytokine secretion in MCD-fed mice. Related to Figure 3.

A: Statistical analysis the basal numbers and proportions (% CD45⁺ leukocytes) of bone marrow derived monocytes in naïve WT and OX40-KO mice. **B:** Statistical analysis of liver monocytes numbers and proportions (% CD45⁺ leukocytes) in naïve WT and OX40-KO mice were determined by flow cytometry. **C:** Representative flow cytometric plots from liver leukocytes showing monocyte and Kupffer cell populations in each group. **D:** Quantification of hepatic Kupffer cells and monocytes as the percentage of total liver leukocytes of WT and OX40-KO mice fed the MCD or NCD. **E:** Statistical analysis of

OX40L expression in the liver monocytes and Kupffer cells of WT and OX40-KO mice, as measured via flow cytometry. In addition, relative changes in OX40L expression were plotted as fold changes of OX40L expression induced by NCD administration in WT mice. **F:** Flow cytometric images of OX40L expression in monocytes and Kupffer cells. **G:** Relative *iNOS* and *Arg-1* mRNA expression levels in each group. **H:** Statistical analysis of CD11C⁺ and Ly6C^{high} cells (% CD11b^{int}F4/80^{low} monocytes) in the livers of mice from each group, as determined by flow cytometry. In addition, relative changes of the cell proportions were plotted as fold changes of the cell proportions induced by NCD administration in WT mice. **I:** Relative chemokine (*Ccl2*, *Ccl12*) and chemokine receptor (*Ccr2*) mRNA expression levels in each group. **J:** Statistical analysis of CCR2 levels in the liver monocytes and Kupffer cells of mice from each group, as determined by flow cytometry. In addition, relative changes in CCR2 levels were plotted as fold changes of that induced by NCD administration in WT mice. **K:** Statistical analysis of antigen presentation-associated marker (MHC II, CD86, CD40 and TLR4) expression in intrahepatic monocytes and Kupffer cells, as determined by flow cytometry. In addition, relative changes in the expression were plotted as fold changes of expression induced by NCD administration in WT mice. **L:** Flow cytometry analysis of TNF- α ⁺ cells relative to the total numbers of monocytes and Kupffer cells in the liver tissues of mice from each group. **M:** The statistical analysis of TNF- α ⁺ cells relative to the total numbers of monocytes and Kupffer cells in the liver tissues of mice from each group, as determined by flow cytometry. The data are depicted as the mean $\pm$ SD, and n=6 in each group. * $p<0.05$, ** $p<0.01$, NS: no significance.

Figure S5



Supplementary Figure 5. OX40 deficiency decreased infiltrated monocyte hepatic recruitment, maturation, and proinflammatory cytokine secretion in mice fed the CD-HFD. Related to Figure 3.

A: The proportion of monocytes and Kupffer cells in total liver leukocytes of WT and OX40-KO mice fed with NCD or CD-HFD. **B:** Relative OX40L expression in each group was determined by flow cytometry. **C:** Relative *iNOS* and *Arg-1* mRNA expression levels in each group. **D:** Statistical analysis of CD11c⁺ and Ly6C^{high} cells (% CD11b^{int}F4/80^{low} monocytes) in the livers of mice from each group, as determined by flow cytometry. **E:** Relative chemokine (*Ccl2*, *Ccl12*) and chemokine receptor (*Ccr2*) mRNA expression levels in each group. **F:** Relative CCR2 expression in each group was determined by

flow cytometry. **G:** Statistical analysis of antigen presentation-associated marker (MHC II, CD86, CD40 and TLR4) expression in intrahepatic monocytes and Kupffer cells, as determined by flow cytometry. **H** and **I:** Flow cytometry image and the statistical analysis of TNF- α^+ cells relative to the total numbers of monocytes and Kupffer cells in each group. The data are depicted as the mean $\pm$ SD, and n=6 in each group. * $p<0.05$, ** $p<0.01$, NS: no significance.

Table S1. Demographic and clinical characteristics of the human study population. Related to Figure 7.

	Control	NASH	P-value
ALL	58	54	
Male (Female)	21 (37)	13 (41)	0.2175
Age (yr)	30.74 $\pm$ 4.96	32.53 $\pm$ 9.12	0.1939
Body weight (kg)	63.02 $\pm$ 10.62	105.73 $\pm$ 21.91	0.0001
BMI	22.41 $\pm$ 2.85	38.34 $\pm$ 6.73	0.0001
ALT (IU/L)	14.51 $\pm$ 6.53	51.88 $\pm$ 42.11	0.0001
AST (IU/L)	16.43 $\pm$ 3.73	31.17 $\pm$ 21.38	0.0001
Fasting Glucose (mmol/L)	4.98 $\pm$ 0.33	6.54 $\pm$ 2.75	0.0001
T-CHO (mmol/L)	4.23 $\pm$ 0.68	4.86 $\pm$ 0.81	0.0001
TG (mmol/L)	0.95 $\pm$ 0.44	1.98 $\pm$ 1.03	0.0001
HDL-C (mmol/L)	1.45 $\pm$ 0.32	1.08 $\pm$ 0.18	0.0001
LDL-C (mmol/L)	2.06 $\pm$ 0.49	2.83 $\pm$ 0.58	0.0001
Steatosis grade 1/2/3		24/21/9	
Lobular inflammation 1/2		20/34	
Ballooning 0/1/2		1/15/38	
Fibrosis 0/1/2/3/4		4/28/14/7/1	

BMI, body-mass index; ALT, Alanine transaminase; AST, Aspartate transaminase; T-CHO, total cholesterol; TG, triglycerides; HDL-C, high-density cholesterol; LDL-C, low-density cholesterol. Data are presented as mean $\pm$ standard deviation (SD).

Table S2. Correlations with OX40 in NASH patients. Related to Figure 7.

	OX40	
	rho	<i>p value</i>
Age	0.102	0.463
BMI	0.427	0.001
ALT	0.382	0.004
AST	0.263	0.055
Fasting Glucose	0.399	0.003
T-CHO	0.175	0.262
TG	0.14	0.314
HDL-C	0.118	0.395
LDL-C	0.178	0.197
Steatosis	0.636	0.001
Lobular inflammation	0.417	0.002
Ballooning	0.304	0.026
Fibrosis	0.356	0.008

BMI, body-mass index; ALT, Alanine transaminase; AST, Aspartate transaminase; T-CHO, total cholesterol; TG, triglycerides; HDL-C, high-density cholesterol; LDL-C, low-density cholesterol.

Table S3. Primer sequences used for real-time PCR
(Related to KEY RESOURCES TABLE in STAR METHODS)

Gene	Strand	Primer sequence (5'-3')
<i>OX40</i>	Sense	GGGCAGGGAACACAGTCAAC
	Antisense	CAGAATTGCACACCTACTCAG
<i>OX40L</i>	Sense	TTGACTTTGCCCTTATTG
	Antisense	GCCTTAGCGTCTTCTTC
<i>Tbx21</i>	Sense	GTTCCCATTCCTGTCCTTCA
	Antisense	ACCCACTTGCCGCTCTG
<i>Gata3</i>	Sense	GGGTTCGGATGTAAGTCGAG
	Antisense	CCACAGTGGGGTAGAGGTTG
<i>RORγT</i>	Sense	TACCTTGGCCAAAACAGAGG
	Antisense	ATGCCTGGTTTCCTCAAAA
<i>Foxp3</i>	Sense	CACCCAGGAAAGACAGCAACC
	Antisense	GCAAGAGCTCTTGTCCATTGA
<i>Inos</i>	Sense	ATCTTTGCCACCAAGATGGCCTGG
	Antisense	TTCCTGTGCTGTGCTACAGTTCGG
<i>Arg-1</i>	Sense	TGACTGAAGTAGACAAGCTGGGGAT
	Antisense	CGACATCAAAGCTCAGGTGAATCGG
<i>TGFb</i>	Sense	GAGGTCACCCGCGTGCTA
	Antisense	TGTGTGAGATGTCTTTGGTTTTCTC
<i>Col1a1</i>	Sense	CTGCTGGCAAAGATGGAGA
	Antisense	ACCAGGAAGACCCTGGAATC
<i>Col3a1</i>	Sense	CAAATGGCATCCCAGGAG
	Antisense	CATCTCGGCCAGGTTCTC
<i>Adiponectin</i>	Sense	GGAGATGCAGGTCTTCTTGG
	Antisense	CGAATGGGTACATTGGGAAC
<i>OPN</i>	Sense	GATGATGATGACGATGGAGACC
	Antisense	CGACTGTAGGGACGATTGGAG
<i>IL-1β</i>	Sense	CCCTGCAGCTGGAGAGTGTGGA
	Antisense	TGTGCTCTGCTTGTGAGGTGCTG
<i>IL-2</i>	Sense	TTCAATTGGAAGATGCTGAGA
	Antisense	ATCATCGAATTGGCACTCAA
<i>IL-4</i>	Sense	AGC AGT TCC ACA GGC ACA AG
	Antisense	AGC AGT TCC ACA GGC ACA AG
<i>IL-6</i>	Sense	CTTCCATCCAGTTGCCTTCTTG
	Antisense	AATTAAGCCTCCGACTTGTGAAG
<i>IL-10</i>	Sense	AGAAAAGAGAGCTCCATCATGC
	Antisense	TTATTGTCTTCCCGGCTGTACT
<i>IL-17</i>	Sense	GCTCCAGAAGGCCCTCAGACT
	Antisense	CCAGCTTTCCCTCCGCATTGA
<i>IL-21</i>	Sense	CAC AGA CTA ACA TGC CCT TCA T
	Antisense	GAA TCT TCA CTT CCG TGT GTT C

<i>Tnfa</i>	Sense	TCCCAGGTTCTCTTCAAGGGA
	Antisense	GGTGAGGAGCACGTAGTCGG
<i>Ifng</i>	Sense	GGCCATCAGCAACAACATAAGCGT
	Antisense	TGGGTTGTTGACCTCAAACCTGGC
<i>Ccl2</i>	Sense	CAGCAAGATGATCCCAATGA
	Antisense	GTAGGTTCTGATCTCATTTG
<i>Ccl12</i>	Sense	ACTTCTATGCCTCCTGCTC
	Antisense	CACTGGCTGCTTGTGATTC
<i>Ccr2</i>	Sense	TTTGTTTTTGCAGATGATTCAA
	Antisense	TGCCATCATAAAGGAGCCAT
<i>Survivin</i>	Sense	GCGGAGGTTGTGGTGAC
	Antisense	AGGGCAGTGGATGAAGC
<i>Bcl-2</i>	Sense	GGAAGGTAGTGTGTGTGG
	Antisense	ACTCCACTCTCTGGGTTCTTGG
<i>Bcl-xl</i>	Sense	AACATCCCAGCTTCACATAACCCC
	Antisense	GCGACCCCAGTTTACTCCATCC
<i>GAPDH</i>	Sense	AAGGTCATCCCAGAGCTGAA
	Antisense	CTGCTTCACCACCTTCTTGA
