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Contents

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Weekly Volume 26 Number 33 September 7, 2020

Contents

SYSTEMATIC REVIEWS

CASE REPORT

ABOUT COVER

Editorial Board of *World Journal of Gastroenterology*, Dr. Dario Sorrentino is a Professor of Medicine at Virginia Tech – Carilion School of Medicine and Research Institute (since 2013). His career research experience has ranged from the bench to the bedside focusing on IBDs, and carried out on three different continents. Fifteen years ago, he and his professional colleagues proposed a groundbreaking strategy to prevent post-surgical recurrence of Crohn's disease that has evolved into today's standard-of-care. More recently, he and his team developed a novel approach for diagnosing and treating pre-clinical Crohn's disease, representing a revolutionary approach to IBD management and research. Dr. Sorrentino has published > 150 high-quality publications and delivered speeches on his own research worldwide. His recent work in the United States has garnered awards of research funds exceeding 2 million dollars from major foundations and private sources. (L-Editor: Filipodia)

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ORIGINAL ARTICLE

Basic Study

Tumor necrosis factor alpha receptor 1 deficiency in hepatocytes does not protect from non-alcoholic steatohepatitis, but attenuates insulin resistance in mice

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Author contributions: Bluemel S performed experiments and wrote the manuscript; Wang Y and Lee S assisted with experiments; Schnabl B supervised the study and edited the manuscript; All authors approved the final version of the manuscript.

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Abstract

BACKGROUND

End-stage liver disease caused by non-alcoholic steatohepatitis (NASH) is the second leading indication for liver transplantation. To date, only moderately effective pharmacotherapies exist to treat NASH. Understanding the pathogenesis of NASH is therefore crucial for the development of new therapies. The inflammatory cytokine tumor necrosis factor alpha (TNF-α) is important for the progression of liver disease. TNF signaling *via* TNF receptor 1 (TNFR1) has been hypothesized to be important for the development of NASH and hepatocellular carcinoma in whole-body knockout animal models.

AIM

To investigate the role of TNFR1 signaling in hepatocytes for steatohepatitis development in a mouse model of diet-induced NASH.

METHODS

NASH was induced by a western-style fast-food diet in mice deficient for TNFR1 in hepatocytes ($TNFR1^{\Delta HEP}$) and their wild-type littermates ($TNFR1^{fl/fl}$). Glucose tolerance was assessed after 18 wk and insulin resistance after 19 wk of feeding. After 20 wk mice were assessed for features of NASH and the metabolic syndrome such as liver weight, liver steatosis, liver fibrosis and markers of liver inflammation.

Therabiomics, Intercept Pharmaceuticals and Patara Pharmaceuticals. Schnabl B's institution UC San Diego has received research support from Axial Biotherapeutics, BiomX, CymaBay Therapeutics, NGM Biopharmaceuticals, and Synlogic Operating Company.

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RESULTS

Obesity, liver injury, inflammation, steatosis and fibrosis was not different between TNFR1$^{\Delta HEP}$ and TNFR1$^{fl/fl}$ mice. However, *Tnfr1* deficiency in hepatocytes protected against glucose intolerance and insulin resistance.

CONCLUSION

Our results indicate that deficiency of TNFR1 signaling in hepatocytes does not protect from diet-induced NASH. However, improved insulin resistance in this model strengthens the role of the liver in glucose homeostasis.

Key words: Tumor necrosis factor alpha receptor 1; Non-alcoholic steatohepatitis; Non-alcoholic fatty liver disease; Type 2 diabetes; Insulin resistance; Glucose intolerance

Core tip: We investigated the role of hepatocellular tumor necrosis factor receptor 1 (TNFR1) signaling in diet-induced non-alcoholic steatohepatitis in mice with a deficiency for TNFR1 solely in hepatocytes. In contrast to most whole-body knock-out models, diet-induced non-alcoholic steatohepatitis is not aggravated by hepatocellular TNFR1 deficiency in our study. However, insulin resistance was markedly improved, which strengthens the role of the liver in glucose homeostasis.

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INTRODUCTION

Complications of the obesity epidemic are increasing globally. Over 70% of obese adults have non-alcoholic fatty liver disease (NAFLD)[1]. Disease progression to non-alcoholic steatohepatitis (NASH) and cirrhosis – often complicated by hepatocellular carcinoma (HCC) – worsens the prognosis. Therefore, NASH and its complications are becoming the second leading indication for liver transplantation[2,3]. Besides weight loss, only moderately effective therapies exist to reverse NASH[4]. Understanding the pathogenesis of NASH is therefore crucial for the development of novel treatment strategies.

The development of NAFLD and NASH is triggered by intestinal dysbiosis[5-13]. This is underlined by the transmission of the disease through co-housing experiments in mice and microbiota transplantation to germ-free mice[14,15]. Poor nutrition and dysbiosis can damage the intestinal epithelial barrier and facilitate endotoxemia[16]. Lipopolysaccharide (LPS), which is a component of the outer membrane of Gram-negative bacteria, acts as an endotoxin. LPS activates toll-like receptor 4 (TLR-4). TLR-4 expression is elevated in liver biopsies of patients with NASH compared with NAFLD[17]. Downstream signals of TLR activation, such as release of tumor necrosis factor alpha (TNF-α), contribute to liver inflammation and fibrosis[18-20].

TNF is a pro-inflammatory cytokine thought to be substantially involved in the progression of liver disease[18-20]. TNF signal transduction is mediated by TNF receptor 1 (TNFR1) and TNFR2. Whereas TNFR1 activation is thought to drive inflammation and metabolic alterations, TNFR2 regulates regeneration and immune response in a protective manner[21,22]. In obesity, adipocytes are a major systemic source of TNF, while in the liver TNF is mainly released from Kupffer cells[23,24]. Obese patients have higher serum levels of TNF, and patients with NASH have higher hepatic expression of *TNF* compared with NAFLD[25,26]. In a mouse model of NAFLD, TNF was suggested to be a driver of HCC development and tumor-associated inflammation[27,28]. Conversely, obese mice with whole-body knockout of *Tnfr1* have lower hepatic lipid accumulation, lower level of the pro-inflammatory cytokine IL-6, and lower hepatic accumulation of neutrophils and macrophages[27]. Moreover, inducers of hepatocellular endoplasmic reticulum (ER) stress, which promotes NASH progression, are lower in

Tnfr1-deficient mice[28,29]. This suggests that TNF signaling *via* TNFR1 is important for the development of steatohepatitis. However, results are conflicting, with other studies showing more hepatic steatosis as well as fibrosis and higher inflammatory markers in mouse livers of high-fat diet-induced NAFLD and dysfunctional TNFR1[30]. Therefore, the role of TNF and TNFR1 signaling in NAFLD and NASH remains unclear. The abovementioned studies on TNFR1 involvement in hepatocellular ER stress suggest that TNFR1 signaling in hepatocytes is crucial for NASH progression (rather than TNFR1 activation in other liver-homed cells, such as Kupffer cells or stellate cells).

To address this question, we investigated the role of TNFR1 signaling in hepatocytes on hepatic steatosis and steatohepatitis in a mouse model of diet-induced NASH.

MATERIALS AND METHODS

Mice

Mice with loxP sites inserted in the tumor necrosis factor receptor 1 gene (*Tnfr1*$^{flxneo/flxneo}$) were generated after receiving sperm from the European Mouse Mutant Archive[31]. These mice have exons 2 to 5 flanked with loxP sites and were crossed with albumin-Cre transgenic mice (The Jackson Laboratory, Sacramento, CA) to create mice with a deficiency of *Tnfr1* (*Tnfrsf1a*) specifically in hepatocytes (TNFR1$^{\Delta HEP}$). Albumin-Cre negative *Tnfr1*$^{flxneo/flxneo}$ littermates were used as controls (TNFR1$^{fl/fl}$). All mice were on a C57BL/6 background, and were maintained on a 12:12-h light-dark cycle. After weaning, male mice were housed with littermates of the same genotype for experiments. At age 8 wk, mice were started on a fast food diet (FFD) consisting of irradiated western-style diet (AIN-76A; TestDiet, St. Louis, MO, United States) for 20 wk[32]. Drinking water was supplemented with 23.1 g/L fructose (F0127; Sigma Aldrich, St. Louis, MO, United States) and 18.9 g/L glucose (G8270; Sigma Aldrich) to mimic high-fructose corn syrup containing soft drinks[3]. Control mice (Ctrl) received autoclaved tap water and irradiated standard chow (5053 PicoLab Rodent Diet; LabDiet, St. Louis, MO, United States). Mice had free access to food and water.

A glucose tolerance test (GTT) was performed after 18 wk of feeding by injecting 1 μg glucose/g body weight intraperitoneally[32]. After 19 wk of feeding, an insulin tolerance test (ITT) was performed by intraperitoneal injection of 0.5 mU/g body weight insulin (Novolin N NPH; Novo Nordisk Inc, Princeton, NJ, United States). Prior to both tests, mice were fasted for 6 h. Blood glucose levels were assessed from tail vein blood before injection (t = 0 min) and at t = 15, 30, 60, 90, and 120 min following injection.

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of the University of California, San Diego.

Biochemical analyses

Biochemical analyses were done according to the manufacturers' protocols. Alanine amino transferase (ALT) was measured from plasma obtained from inferior vena cava using a kinetic assay (TR71121; Thermo Fisher Scientific, Waltham, MA, United States). Triglyceride levels were measured with a colorimetric endpoint assay (T7532; Pointe Scientific, Canton, MI, United States) after tissue homogenization in phosphate-buffered saline, and precipitation of lipids using methanol and chloroform. Liver hydroxyproline was extracted from 150-250 mg of mixed liver specimens from the right and left liver lobes[32]. The tissue was homogenized in 6N HCl (3750-32; USABlueBook, Forest Park, GA, United States) using lysing matrix C tubes (MP116912; MP Biomedicals, Santa Ana, CA, United States) and a Mini-BeadBeater-96 (GlenMills, Clifton, NJ, United States)[32,33]. The homogenate was incubated at 110 °C for 18 h and subsequently filtered using Whatman® filter paper grade 595 1/2 (WHA10311644; Sigma Aldrich). The lysate was incubated with chloramine T- (C9887; Sigma Aldrich) as well as Ehrlich's perchloric acid solution (AC168760250; Thermo Fisher Scientific). Triplicates were measured at 558 nm (SpectraMax 190 Microplate Reader; Molecular Devices LLC, Sunnyvale, CA, United States)[33,34].

Tissue staining

At harvesting, the median liver lobe including the gall bladder was fixed in 10% formalin (HT501128; Sigma Aldrich) for 24 to 48 h, and then transferred to 70% ethanol and embedded in paraffin[32]. Five μm paraffin sections were stained with hematoxylin and eosin (H&E) (38015 and 380161 SelecTech; Leica Biosystems Inc., Buffalo Grove, IL, United States) or 0.1% picro Sirius red (color index 35780, 365548; Sigma-Aldrich),

respectively.

Gene expression analysis

Liver RNA was extracted using Ambion Trizol Reagent (15596; Thermo Fisher Scientific). RNA was treated with RQ1 RNase-Free DNase (M6101; Promega), and was reverse-transcribed using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (43688; Thermo Fisher Scientific). Real-time qPCR was performed on the Applied Biosystems StepOnePlus Thermocycler (4376600; Thermo Fisher Scientific) using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, United States). Primer sequences were obtained from the Harvard PrimerBank[35,36]. The primer used for proving the absence of *Tnfr1* was generated using NCBI primer blast; the sequences were: ACCGTGACAATCCCCTGTAA (Fwd) and CTCTTTGACA GGCACGGGAT (Rev). These primer amplified mRNA from exon 3 to exon 7. Gene expression was normalized to mouse TATA-Box binding protein gene and expressed relative to Ctrl-fed TNFR1$^{fl/fl}$ mice.

Statistical analyses

Numbers of biological replicates for each experiment are given in the respective figure legends. The area under the curve was used to compare blood glucose levels from ITT. The area over baseline (AOB) was calculated to compare blood glucose levels from GTT. Groups were compared by two-way analysis of variance with Tukey's post hoc test. All results are expressed as mean + standard error of the mean. Analyses and data plots were done with GraphPad Prism 6.01 (GraphPad Software, Inc., La Jolla, CA, United States). Significant differences are marked with (a) if $P < 0.05$.

RESULTS

TNFR1$^{\Delta HEP}$ mice were fed for 20 wk with a western-style diet (FFD) to evaluate, if the absence of TNFR1 in hepatocytes is protective against diet-induced NASH.

Mice with a deficiency of TNFR1 in hepatocytes are not protected from obesity

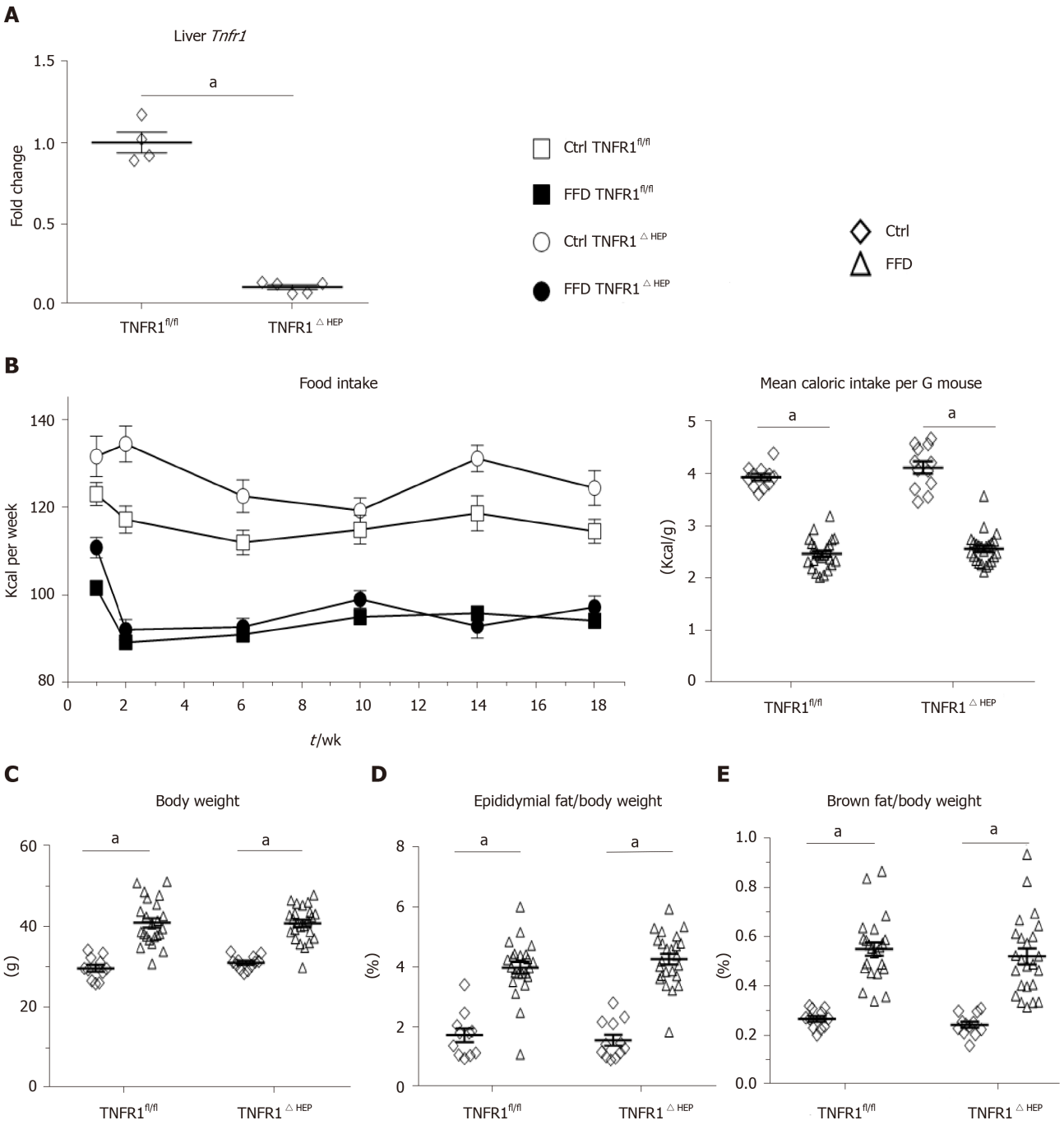
Lower *Tnfr1* expression in liver samples from mice with a deficiency of TNFR1 in hepatocytes is shown in Figure 1A. Food intake is given in Figure 1B. FFD led to an increase in body weight and adipose tissue (Figure 1C-F). However, FFD-fed TNFR1$^{\Delta HEP}$ mice did not differ from their TNFR1$^{fl/fl}$ littermates in terms of body weight, epididymal fat weight and brown fat weight (Figure 1C-E). Taken together, western-style diet-fed TNFR1$^{\Delta HEP}$ mice develop similar features of obesity compared with their TNFR1$^{fl/fl}$ littermates.

Mice with a deficiency of TNFR1 in hepatocytes are not protected from diet-induced steatohepatitis

To determine the significance of TNFR1 in hepatocytes for the development of NASH, parameters of liver injury, steatosis and fibrosis were investigated. Hepatic injury, expressed as plasma ALT, was not significantly different between FFD-fed TNFR1$^{\Delta HEP}$ mice and their TNFR1$^{fl/fl}$ littermates (Figure 2A and B). Similarly, hepatic steatosis, expressed as total liver triglycerides and relative liver weight, did not differ between FFD-fed TNFR1$^{\Delta HEP}$ mice and their TNFR1$^{fl/fl}$ littermates (Figure 2C and D). Representative liver sections are given in Figure 2B. Fibrosis, expressed as total liver hydroxyproline, was higher in FFD-fed mice, but not significantly different from control-fed mice in TNFR1$^{\Delta HEP}$ as well as TNFR1$^{fl/fl}$ mice (Figure 2E and F). Liver inflammation, assessed by gene expression for *Il1β*, *Tnf*, and *Ccl2*, did not differ between FFD-fed TNFR1$^{\Delta HEP}$ mice and TNFR1$^{fl/fl}$ littermates (Figure 2G). Taken together, western-style diet-fed TNFR1$^{\Delta HEP}$ mice develop similar signs of liver inflammation and fibrosis compared with TNFR1$^{fl/fl}$ littermates.

Mice with a deficiency of TNFR1 in hepatocytes are partially protected from glucose intolerance

To assess the metabolic phenotype, TNFR1$^{\Delta HEP}$ mice and their TNFR1$^{fl/fl}$ littermates were subjected to a glucose and an insulin tolerance test. The FFD-fed TNFR1$^{\Delta HEP}$ mice did not develop glucose intolerance compared to their Ctrl-fed littermates (Figure 3A). The result was confirmed with the insulin tolerance test that showed a higher drop of blood glucose levels after insulin injections in FFD-fed TNFR1$^{\Delta HEP}$ mice compared with TNFR1$^{fl/fl}$ littermates (Figure 3B). Taken together, although western-style diet equally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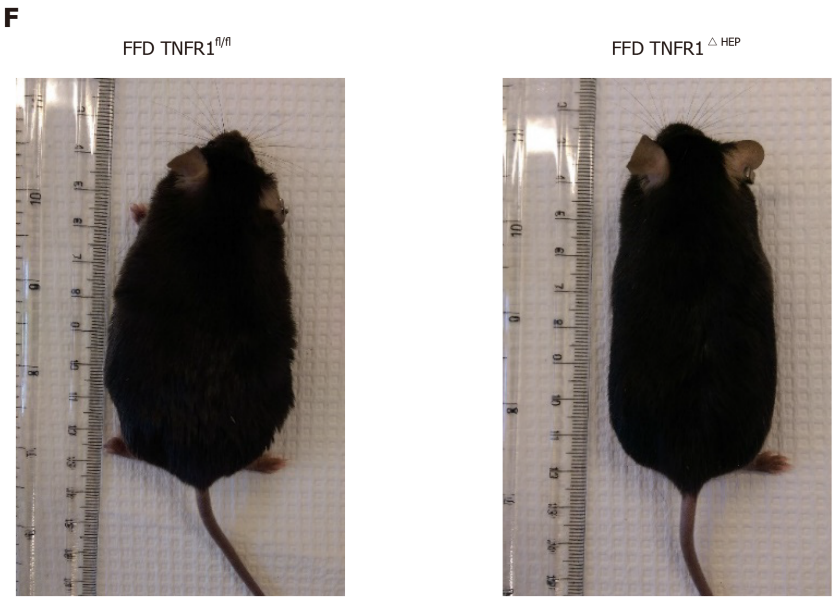


Figure 1 Effect of tumor necrosis factor alpha receptor 1 deficiency in hepatocytes on body weight. A: *Tumor necrosis factor alpha receptor 1 (Tnfr1)* expression in liver samples (n = 4-5); B: Food intake per group over the course of the experiment, given in kilocalories (Kcal) and normalized to the mouse weight; C: Body weight and relative weights of epididymal (D) and brown fat tissues (E) after 20 wk of feeding; F: Example appearance of mice with a knockout of TNFR1 in hepatocytes (TNFR1$^{\Delta HEP}$) and their TNFR1-expressing littermates (TNFR1$^{fl/fl}$) after 20 wk of fast food diet (FFD). Numbers of biological replicates: Ctrl/TNFR1$^{fl/fl}$ n = 11; FFD/TNFR1$^{fl/fl}$ n = 23; Ctrl/TNFR1$^{\Delta HEP}$ n = 12; FFD/TNFR1$^{\Delta HEP}$ n = 24. Line shows mean + standard error of the mean. Significant differences are marked with (a) if P < 0.05. Ctrl: Standard chow.

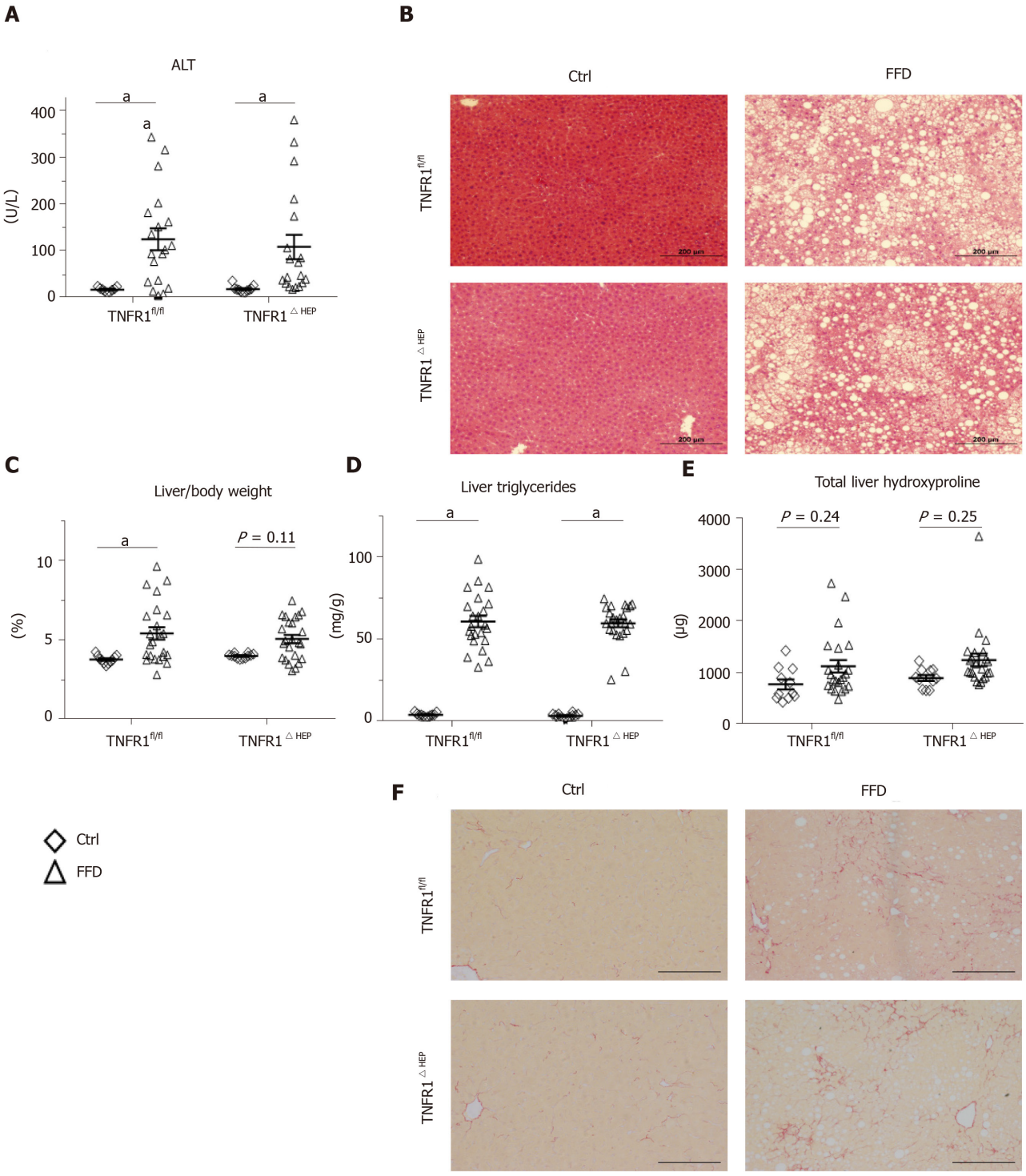
induced obesity in TNFR1$^{\Delta HEP}$ and TNFR1$^{fl/fl}$ littermate mice, interestingly, TNFR1$^{\Delta HEP}$ mice were resistant to the development of diet-induced glucose intolerance and insulin resistance.

DISCUSSION

The role of the TNF receptor 1 in hepatocytes for diet-induced NASH was investigated in mice with a hepatocyte-specific deficiency of TNFR1 (TNFR1$^{\Delta HEP}$). Obese mice with deficiency of TNFR1 in hepatocytes did not develop less liver disease than their TNFR1$^{fl/fl}$ littermates. However, TNFR1$^{\Delta HEP}$ mice were protected from glucose intolerance and insulin resistance.

Previous studies, using *Tnfr1* whole-body knockout animals, concluded that TNFR1 signaling is a driver for NASH. Constitutional activation of TNFR1 in mice promoted the progression of NASH, but protected from insulin resistance[37], in contrast to our findings and studies showing the importance of TNFR1 signaling in diabetes development[38-40] (discussed in more detail below). On the other hand, deficiency of *Tnfr1* was used to prove the contribution of TNFR1 signaling for downstream activation of STAT3 or the induction of ER stress both to promote NASH progression and HCC development[27,28]. In contrast to these studies, in our study *Tnfr1* was deleted in hepatocytes only. Kupffer cells are the primary source of hepatic TNF release[24]. Moreover, TNF acts on Kupffer cells in an autocrine manner[41]. Therefore, it can be speculated that the expected protective effect of hepatocyte-specific TNFR1 deficiency was reduced by increased TNFR1 signaling in Kupffer cells or recruited immune cells. These immune cells might have caused the release of different inflammation mediators, such as IL-1β or IL-6, which in turn resulted in activation of inflammatory pathways in hepatocytes that are independent from TNFR1 activation. This hypothesis is supported by a study that used mice with a whole-body knock out of *Tnfr1* and also found increased NASH features[30]. In this study by Lambertucci *et al*[30], *Tnfr1* deficiency resulted in increased numbers of both resident (*i.e.* Kupffer cells) and recruited macrophages into the liver, as well as up-regulation of IL-1β and IL-6 in the liver along with increased release into the plasma. IL-1β promotes alcoholic and non-alcoholic liver disease[42,43]. In the present study, we did not detect differences in IL-1β expression between obese mice with a hepatocyte-specific TNFR1 deficiency and their wild-type littermates.

Another possible explanation for our finding might be an enhanced signal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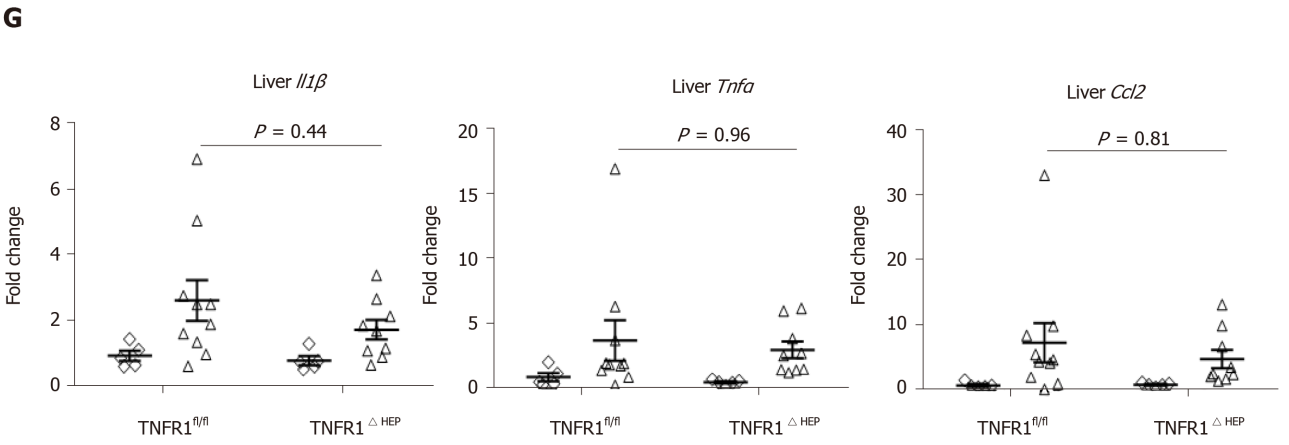

Figure 2 Effect of tumor necrosis factor alpha receptor 1 deficiency in hepatocytes on liver phenotype. A: Plasma levels of alanine amino transferase (ALT); B: Representative hematoxylin and eosin-stained liver sections; C: Relative liver weight; D: Relative amount of triglycerides normalized to liver weight. E: Total amount of hydroxyproline per liver; F: Representative Sirius Red-stained liver sections; G: Expression of the inflammatory genes interleukin 1-β (*Il1-β*), tumor necrosis factor alpha (*Tnfa*), and C-C motif chemokine ligand 2 (*Ccl2*). Scale bars: 200 μm. Number of biological replicates: Ctrl/tumor necrosis factor alpha receptor 1-expressing littermates (TNFR1$^{fl/fl}$) *n* = 9-11; fast food diet (FFD)/TNFR1$^{fl/fl}$ *n* = 19-23; Ctrl/TNFR1$^{\Delta HEP}$ *n* = 11-12; FFD/TNFR1$^{\Delta HEP}$ *n* = 19-24. Line shows mean + standard error of the mean. Significant differences are marked with (a) if $P < 0.05$. Ctrl: Standard chow.

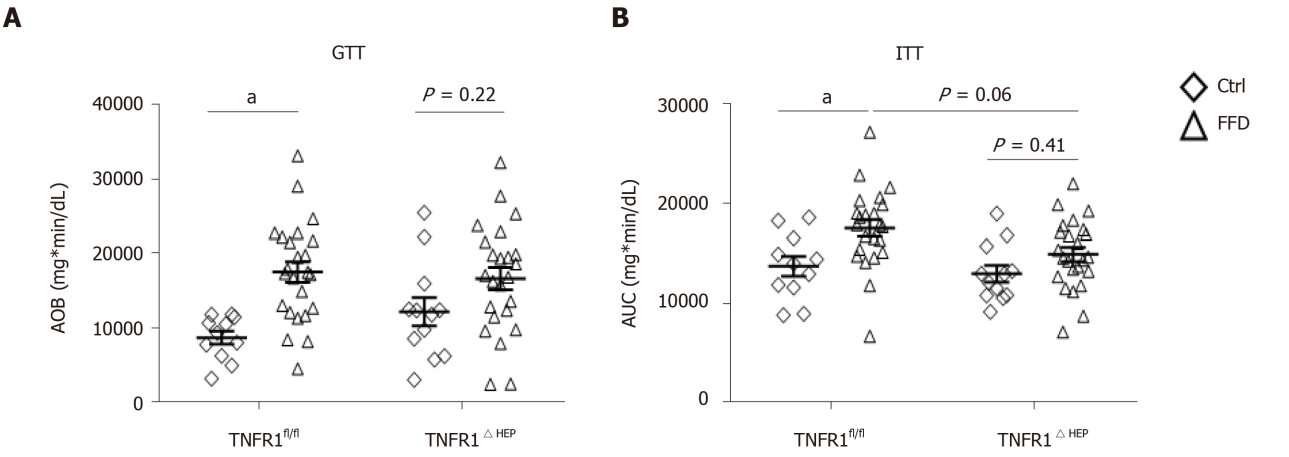

Figure 3 Effect of tumor necrosis factor alpha receptor 1 deficiency in hepatocytes on metabolic function. A: Glucose tolerance test performed after 18 wk of feeding; B: Insulin tolerance test performed after 19 wk of feeding. Numbers of biological replicates: Ctrl/tumor necrosis factor alpha receptor 1-expressing littermates (TNFR1$^{fl/fl}$) *n* = 11; fast food diet (FFD)/TNFR1$^{fl/fl}$ *n* = 23-24; Ctrl/TNFR1$^{\Delta HEP}$ *n* = 12; FFD/TNFR1$^{\Delta HEP}$ *n* = 24. Line shows mean + standard error of the mean. Significant differences are marked with (a) if $P < 0.05$. Ctrl: Standard chow; GTT: Glucose tolerance test.

transduction *via* TNFR2. TNFR2 is a mediator of systemic inflammation and enhances cytotoxicity of monocytes[44-46]. Reduced expression of adhesion molecules VCAM-1 and ICAM-1, which both facilitate leukocyte infiltration, was described in a double-knockout model of *Tnfr1* and *Tnfr2*, along with a reduction of hepatic steatosis and fibrosis[47]. However, a more prominent role of TNFR2 would have occurred in the whole-body knockout models as well. Higher serum level of soluble TNFR2 were detected in patients with HCC and hepatitis C[48]. The role of TNFR2 for NASH development is not conclusive, but it is thought to be protective[49].

The increase in hepatic triglyceride content in NAFLD is associated with hepatic insulin resistance[50]. This is mediated by the insulin receptor substrate IRS-2 and protein kinase-Cε. We did not detect a difference in hepatic triglycerides between obese *Tnfr1* deficient mice and their *Tnfr1*$^{fl/fl}$ littermates. However, compared to lean controls, only *Tnfr1*$^{fl/fl}$ mice had high blood glucose level after glucose and insulin challenge. Our observation that mice with a deficiency of *Tnfr1* in hepatocytes are protected from insulin resistance, strengthens previous findings of *Tnfr1* deficiency and improved insulin resistance[38,49]. TNF-mediated insulin resistance occurs *via* phosphorylation of the insulin receptor substrate IRS-1, which inhibits insulin-action in hepatocytes, and therefore promotes insulin resistance[39,40]. Phosphorylation of IRS-1 was reduced in mice with a whole-body deficiency of *Tnfr1*, thus enhancing insulin-

uptake into hepatocytes[38]. A similar mechanism occurs in brown adipose tissue and myocytes – alternative major sites of insulin action[51]. The effect seen in our study emphasizes the important role of hepatocytes for glucose homeostasis during obesity development.

Our findings were limited by the selective deficiency of TNFR1 in hepatocytes. As mentioned above, selective deficiency of TNFR1 in Kupffer cells might have clarified the cell type specific role for the pathogenesis of NASH. Another limitation was that we could only speculate about the compensating role of TNFR2. As TNFR2 signaling is expected to be protective, a selective manipulation of TNFR2 would be an interesting target for future studies. Furthermore, we could not investigate the mechanisms leading to insulin resistance in more details, because samples were not taken from starved animals, as usually done in metabolic studies.

In conclusion, our results do not indicate that loss of TNFR1 in hepatocytes can protect from diet-induced NASH. However, improved insulin resistance in this model confirms the important role of the liver for glucose homeostasis during obesity.

ARTICLE HIGHLIGHTS

Research background

Understanding the pathogenesis of non-alcoholic steatohepatitis (NASH) is crucial for the development of new therapies. The inflammatory cytokine tumor necrosis factor alpha (TNF-α) is important for the progression of liver disease. It binds to two receptors, TNF receptor 1 (TNFR1) and TNFR2.

Research motivation

TNF signaling *via* TNFR1 has been hypothesized to be important for the development of NASH and hepatocellular carcinoma in whole-body knockout animal models.

Research objectives

The aim of our study was to investigate the hepatocyte specific role of TNFR1 signaling for diet-induced steatohepatitis.

Research methods

NASH was induced by a 20-wk western-style fast-food diet in mice deficient of TNFR1 in hepatocytes ($TNFR1^{\Delta HEP}$) and their wild-type littermates ($TNFR1^{fl/fl}$). Features of NASH as well as glucose tolerance and insulin resistance were assessed.

Research results

Obesity, liver injury, inflammation, steatosis, and fibrosis was not different between $TNFR1^{\Delta HEP}$ and $TNFR1^{fl/fl}$ mice. However, *Tnfr1* deficiency in hepatocytes protected mice against glucose intolerance and insulin resistance.

Research conclusions

Our results do not indicate that inhibition of TNFR1 signaling in hepatocytes can protect from diet-induced NASH. However, improved insulin resistance in this model confirms the important role of the liver for glucose homeostasis during obesity.

Research perspectives

Compensatory mechanisms, possibly occurring *via* TNFR2 signaling, need to be investigated in future studies.

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