

ORIGINAL ARTICLE

Dual roles of B lymphocytes in mouse models of diet-induced nonalcoholic fatty liver disease

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Funding Information

Huige Li and Ning Xia were supported by a research grant from the Boehringer Ingelheim Foundation for the CardioConsortium "Novel and neglected cardiovascular risk factors: molecular mechanisms and therapeutic implications" at the Johannes Gutenberg University Medical Center Mainz, Germany. Xiaoyu Wang was supported by an unrestricted PhD fellowship by Boehringer-Ingelheim. Detlef Schuppan received project-related support from the German Research

Abstract

Background and Aims: Growing evidence suggests an important role of B cells in the development of NAFLD. However, a detailed functional analysis of B cell subsets in NAFLD pathogenesis is lacking.

Approach and Results: In wild-type mice, 21 weeks of high fat diet (HFD) feeding resulted in NAFLD with massive macrovesicular steatosis, modest hepatic and adipose tissue inflammation, insulin resistance, and incipient fibrosis. Remarkably, B^{null} (*JHT*) mice were partially protected whereas B cell harboring but antibody-deficient *IgMi* mice were completely protected from the development of hepatic steatosis, inflammation, and fibrosis. The common feature of *JHT* and *IgMi* mice is that they do not secrete antibodies, whereas HFD feeding in wild-type mice led to increased levels of serum IgG2c. Whereas *JHT* mice have no B cells at all, regulatory B cells were found in the liver of both wild-type and *IgMi* mice. HFD reduced the number of regulatory B cells and IL-10 production in the liver of wild-type mice, whereas these increased in *IgMi* mice. Livers of patients with advanced liver fibrosis

Abbreviations: ACC, acetyl-CoA carboxylase 1; Adipoq, adiponectin; Adrp, adipose differentiation related protein; Alb, albumin; ALP, alkaline phosphatase; ALT, alanine transaminase; Arg1, arginase 1; AST, aspartate transaminase; Col1a1, collagen type 1 $\alpha 1$ chain; EAT, epididymal adipose tissue; FATP, fatty acid transporter protein; FFA, free fatty acids; GLUT4, glucose transporter 4; GTT, glucose tolerance test; HFD, high fat diet; HOMA-IR, homeostasis model assessment of insulin resistance; IHC, immunohistochemistry; IR, insulin resistance; ITT, insulin tolerance test; Leprb, leptin receptor b; MAIT, mucosal associated invariant T cell; MCP1, monocyte chemotactic protein 1; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NCD, normal control diet; Rbp4, retinol-binding protein 4; Scd1, stearoyl-CoA desaturase 1; T_h , T helper; Xbp1, X-box binding protein 1.

Martin Karl and Solveig Hasselwander contributed equally to this work.

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Foundation (DFG) Collaborative Research Center (CRC) grants SFB 1066 project B3 and CRC 1292 project B8, and by EU Horizon 2020 projects under grant agreements nr. 634413 (EPoS, European Project on Steatohepatitis) and nr. 777377 (LITMUS, Liver Investigation on Marker Utility in Steatohepatitis). Nadine Hövelmeyer received project-related support from the DFG CRC 1292 project 20N.

showed abundant deposition of IgG and stromal B cells and low numbers of IL-10 expressing cells, compatible with our experimental data.

Conclusions: B lymphocytes have both detrimental and protective effects in HFD-induced NAFLD. The lack of secreted pathogenic antibodies protects partially from NAFLD, whereas the presence of certain B cell subsets provides additional protection. IL-10-producing regulatory B cells may represent such a protective B cell subset.

INTRODUCTION

NAFLD can be considered the hepatic component of the metabolic syndrome. With worldwide increasing prevalence of obesity, NAFLD has become a global health problem affecting up to 40% of populations. Moreover, NAFLD is expected to become the leading cause of liver-related morbidity and mortality in upcoming years mainly due to advanced fibrosis, cirrhosis, and hepatocellular carcinoma.^[1] NAFLD is strongly associated with insulin resistance (IR), type 2 diabetes, and dyslipidemia.^[2] Importantly, NAFLD is not just a manifestation of a metabolic syndrome but has emerged as a driver of a systemic disease and an independent risk factor for cardiovascular mortality and morbidity.^[3]

NAFLD comprises a spectrum of liver abnormalities that range from simple steatosis (fatty liver) to NASH, characterized by hepatic inflammation, hepatocellular injury, and a varying degree of fibrosis.^[1] Although NAFLD in the stage of simple steatosis often remains clinically unremarkable, patients with NASH have a profound risk to progress to liver cirrhosis or hepatocellular carcinoma.^[1] Therefore, identifying the drivers that mediate the transition from steatosis to NASH and its severe complications is of particular importance.

Chronic hepatic inflammation has been shown to contribute to disease progression by promotion of advanced fibrosis. In fact, the onset of the inflammatory response is an early event that precedes the progression toward NASH.^[4] In mouse models of diet-induced obesity (DIO), infiltration of immune cells can be observed as early as four weeks on high fat diet (HFD).^[5] The initial inflammatory response is mediated by the activation of liver-resident cells, especially Kupffer cells, which expand rapidly in early stages and express proinflammatory cytokines, chemokines, and other mediators that attract and activate monocytes/macrophages and stimulate other liver-resident cells.^[6]

Growing evidence suggests an essential role for the adaptive immune system and, in particular, B cells in NAFLD development.^[5,7–11] Intrahepatic B cells make up a substantial proportion of the liver immune cell pool in patients with NASH.^[7] In murine models of NAFLD,

B cells are among the first to accumulate in response to HFD feeding.^[5,8] Here, the onset of the disease is characterized by B cell maturation to antibody-producing plasma cells correlating with elevated hepatic levels of IgG antibodies.^[8,9] In this line, circulating antibodies against oxidative stress-derived antigens appear to have a pathogenic role in the progression of NAFLD.^[9] HFD-experienced intrahepatic B cells also exhibited an enhanced capacity to activate proinflammatory T helper (T_h) 1 cells and M1-type macrophages *in vitro* indicating that B cells may have an important role in the orchestration of the immune response in NAFLD.^[5,8]

However, although interfering with the pathogenic activity of B cells in NAFLD may be a potential treatment for NASH, studies investigating whether certain B cell subsets may promote or possibly ameliorate the disease are lacking. Here, we made use of murine models with absent or restricted B cell function, *JHT* (B^{null}) and *IgMi* respectively, to study the role of B cell subsets in the pathogenesis of HFD-induced NAFLD. We found that B cell-deficient mice were partly protected from developing steatosis, NASH, and fibrosis. Strikingly, the two models exhibited remarkable differences in adipose tissue inflammation, adipokine signature, and lipid metabolism, indicating that B cells play a complex role in the pathology of NAFLD and adipose tissue dysfunction.

MATERIALS AND METHODS

Refer to the Supplement for detailed experimental procedures.

Mice and experimental diet

Male six-week-old wild-type or *JHT*^[12] and *IgMi*^[13,14] mice on a C57BL/6J genetic background were fed either a normal control diet (NCD) or a defined, lard-based HFD (Table S1) *ad libitum* for 21 weeks. The animal experiments were approved by the responsible regulatory authority (Landesuntersuchungsamt Rheinland-Pfalz; 23 177-07/G 17-1-020).

Immunohistochemistry on human livers

Human liver samples were obtained from 44 patients: those with advanced NASH ($n = 8$), alcoholic steatohepatitis ($n = 9$), viral hepatitis B ($n = 8$), and viral hepatitis C ($n = 9$) and unaffected resection margins of patients undergoing operations for hepatocellular carcinoma (normal controls, $n = 10$). Ethical approval was provided by and in accordance with the regulations of the Tissue Biobank of the University Medical Center Mainz after approval by the local ethics committee (Ethik-Kommission der Landesärztekammer Rheinland-Pfalz, 837.146.17 (10980), addendum 2018-13857_1 to DAR and BKS). Punch biopsies were used to generate a tissue array. Arrays were fixed, embedded, and deparaffinized, followed by antigen retrieval and staining with specific antibodies as described.^[15] Antibodies used are listed in Table S3. Cells positive for the target antigen were counted and results are shown in Table S4.

Statistics

Statistical testing between two groups was performed using Student *t* test (two-tailed, unpaired). One-way ANOVA was used for comparison between multiple groups. Significance levels are depicted as * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. Unless stated differently, data are presented as mean and SD in all graph types except box plot.

RESULTS

B cell deficiency normalizes IR in HFD-fed mice

To expand our understanding of the role of B cells in the development of NAFLD, we fed B cell-deficient mice an NCD (11 kJ% fat) or an HFD (45 kJ% fat; Table S1) for 21 weeks. In addition to the B^{null} model *JHT*,^[12] *IgMi* mice were investigated that exclusively express membrane-bound IgM and are unable to secrete antibodies of any type.^[13,14]

As expected, HFD feeding was accompanied by higher energy intake and resulted in increased body weight gain over the experimental time course in all mouse strains (Figures 1A, S1A,B). All mouse strains showed a comparable increase of epididymal adipose tissue (EAT) weight and adipocyte hypertrophy (Figures 1A, S1C,D, S2). However, when compared with wild-type *C57BL/6J* mice that exhibited a body weight increase 2.3× higher than seen in NCD-fed counterparts, weight gain of HFD-fed *JHT* and *IgMi* mice was significantly attenuated (1.6- and 1.4-fold versus NCD-fed mice, respectively; Figures 1A, S1A). Although the fat mass was comparable between *B6* and *IgMi* mice, *JHT* mice gained slightly less subcutaneous fat in response to an HFD (Figures 1A,

S3). Interestingly, *IgMi* mice showed a higher expression of uncoupling protein 1 (Ucp1) in epididymal and brown adipose tissue and increased peroxisome proliferator-activated receptor gamma coactivator 1- α (Pgc1) expression in brown adipose tissue. Ucp1 and Pgc1 were also up-regulated in *JHT* mice in inguinal adipose tissue and in EAT, respectively (Figure S4). Whether these genes contribute to the lower body weight gain in the *JHT* and *IgMi* mice warrants further studies.

Remarkably, insulin and glucose tolerance tests (ITT, GTT) indicated severe IR in HFD-fed wild-type mice (Figure 2A,B). Similarly, elevated levels of fasting glucose and insulin resulted in a highly significantly increased homeostasis model assessment of insulin resistance (HOMA-IR) score of 47 (Figure 2C,D). HFD-fed *JHT* and *IgMi* mice were protected from IR (HOMA-IR = 4.2 and 5.7, respectively), despite a fast recovery phase of blood glucose levels in the ITT in the *IgMi* mice (Figure 2A–D).

IgMi mice are protected from HFD-induced hepatic steatosis

HFD caused severe macrovesicular hepatic steatosis in wild-type mice accompanied by a mean liver weight increase of 83% (Figures 1A,B, S1C,E). Compared with wild-type mice, liver weight of HFD-fed *JHT* mice increased by only 21%, whereas *IgMi* mice were protected from an HFD-induced increase in liver weight and hepatic steatosis (Figures 1A,B, S1E, S2, S5A).

HFD has been shown to alter the expression profile of hepatocytes by inducing genes that are related to adipogenesis.^[16] Indeed, adipocyte protein 2 (encoded by *Fabp4*), a key regulator of the adipogenic transcriptional program, was up-regulated in livers of wild-type and B^{null} *JHT* mice but not *IgMi* mice (Figure 3A), which correlated with the extent of hepatic steatosis seen in histological analysis (Figure 1B).

Taken together, the studied B cell-deficient mice were protected from developing hepatic steatosis in a graded fashion. Remarkably, despite comparable characteristics in IR, body weight, and EAT phenotype, the complete lack of B cells in *JHT* mice attenuated hepatic lipid accumulation, whereas *IgMi* mice harboring B cells with restricted capabilities were almost completely protected from steatosis.

HFD-induced alterations in hepatic lipid metabolism are differentially attenuated in the B cell-deficient mice

To identify factors that contribute to the differences in the development of hepatic steatosis, we investigated serum lipid markers and key genes of hepatic lipid metabolism. Serum cholesterol levels increased in general

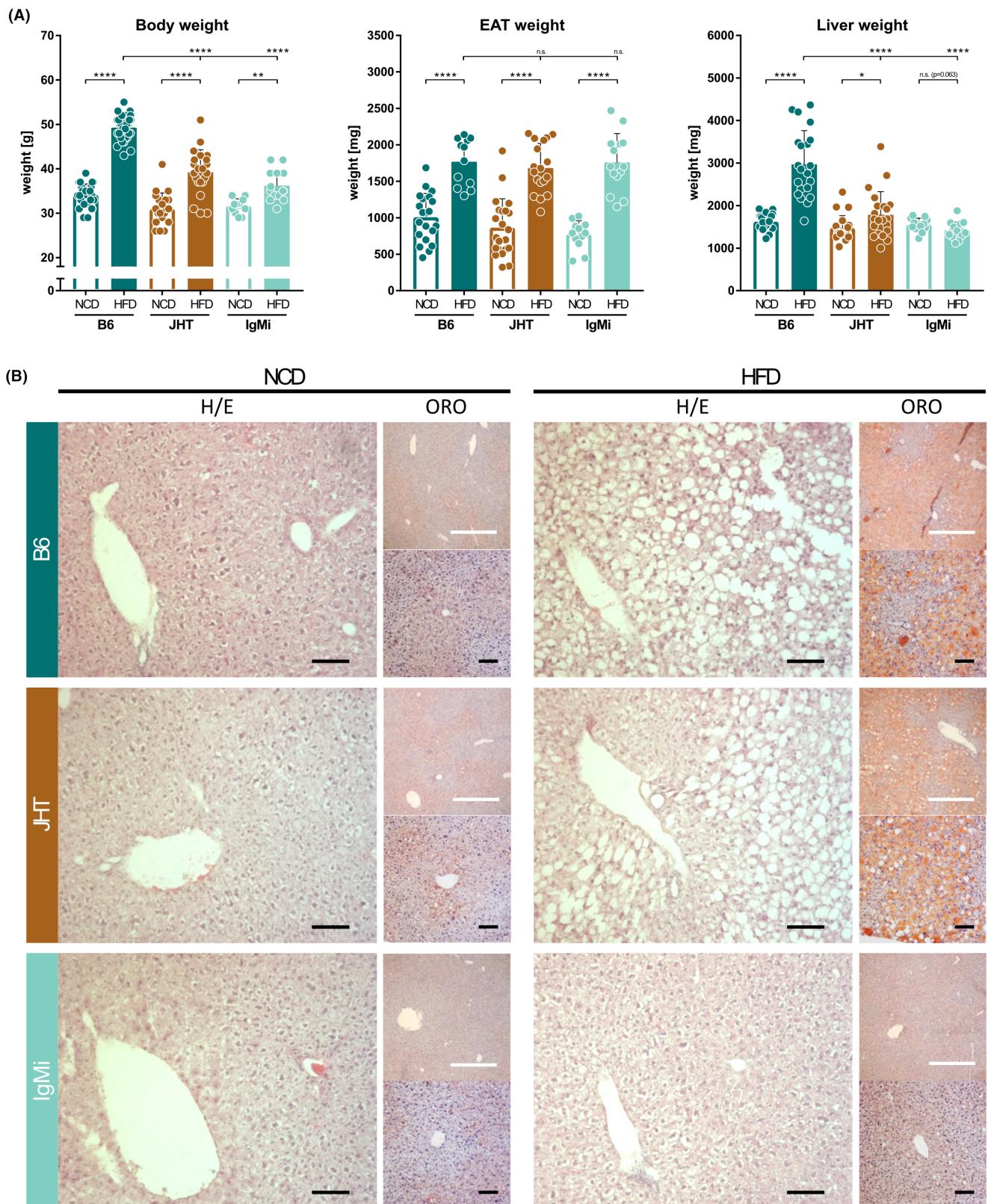


FIGURE 1 IgMi but not JHT mice are protected from HFD-induced hepatic steatosis. Wild-type mice (B6) and B cell models JHT and IgMi were fed an NCD or HFD for 21 weeks. (A) At the end of experimental feeding, total body weight, weight of EAT, and weight of liver were determined. Group numbers range from 11 to 22 individuals. (B) Cryosections of FA-fixed livers were examined using hematoxylin/eosin (H/E) or oil red O (ORO) stainings. Images were taken at 25 \times and 100 \times magnification (white and black scale bars illustrate 500 and 100 μ m, respectively). Presented images are representative of six individuals per group. Quantification results of ORO staining are shown as Figure S1E

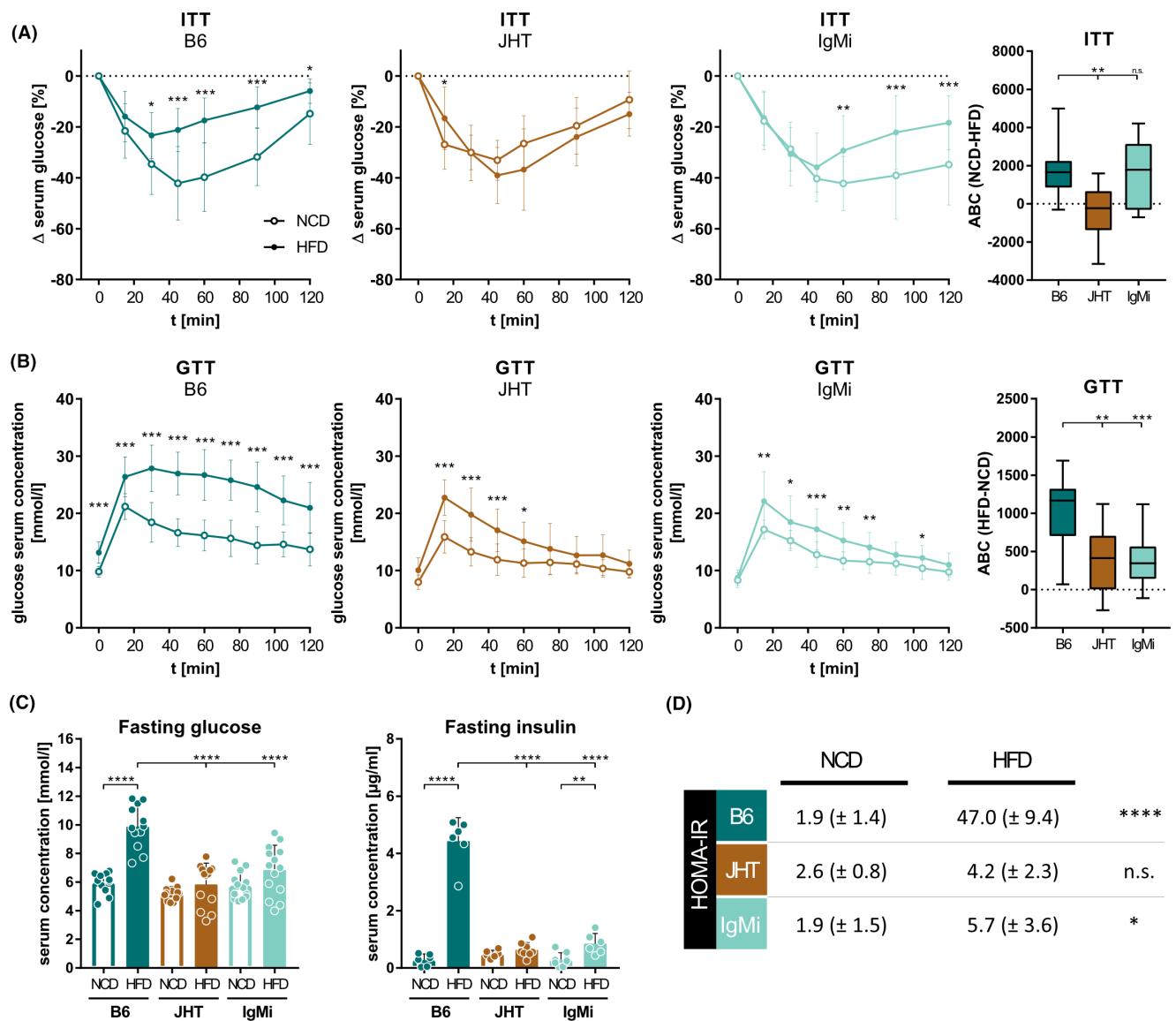


FIGURE 2 Attenuated development of IR in B cell deficiency models. During experimental feeding, parameters of blood glucose regulation were determined in wild-type (B6) mice and B cell deficiency models JHT and IgMi fed an NCD (empty symbols) or HFD (filled symbols). (A) An intraperitoneal ITT was conducted after 14 weeks of experimental feeding. The area between the curves of NCD and HFD-fed individuals is depicted as bar plot (right panel; n = 11–12 per group). (B) An intraperitoneal GTT was conducted after 16 weeks of experimental feeding (n = 12–14 per group). Statistical analysis in (A) and (B) was performed applying two-way ANOVA with Bonferroni correction. (C) Fasting glucose and insulin serum concentrations were determined after 18 weeks of experimental feeding (for fasting glucose, n = 12–14 per group; for fasting insulin, n = 6–8 per group). (D) The HOMA-IR was calculated on the basis of the mean shown in (C)

due to the HFD. However, compared with wild-type mice, mean cholesterol was significantly decreased in JHT and IgMi mice (increased by 88%, 39%, and 51%, respectively; Figure 3B). Remarkably, although higher total cholesterol levels in wild-type and JHT mice were mainly attributable to increased LDL, IgMi mice were characterized by augmentation of HDL (Figures 3B, S5C). An increase of HDL can be caused by a down-regulated hepatic scavenger receptor class B member 1 (SR-B1) or an up-regulated hepatic ATP-binding cassette transporter A1 (ABCA1).^[17,18] However, in livers of HFD-fed IgMi mice, SR-B1 expression was unchanged, and ABCA1 expression was even decreased

(Figure S6), implicating other effectors of the observed HDL increase.

Free fatty acids (FFA) and free cholesterol can cause hepatocyte endoplasmic reticulum stress.^[19,20] HFD feeding had no effect on hepatic free cholesterol but increased hepatic FFAs (Figure S7). However, the extent of HFD-induced increase was comparable between the three mouse strains (Figure S7).

Two sources primarily contribute to excessive lipid accumulation in NAFLD: increased uptake of FFA from serum and enhanced intrinsic *de novo* lipogenesis.^[21,22] Although expression of the very long-chain fatty acid uptake transporters fatty acid transporter protein 2

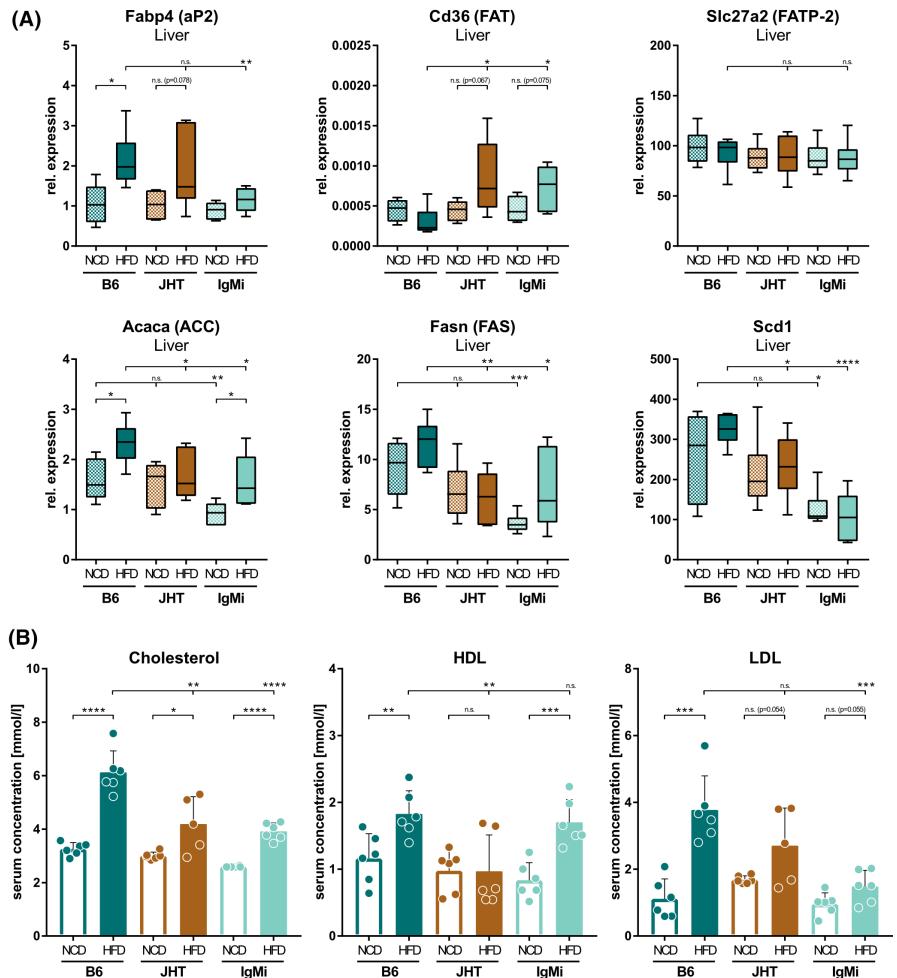


FIGURE 3 HFD-induced alterations in hepatic lipid metabolism are attenuated in B cell deficiency models. (A) After 21 weeks of experimental feeding, mRNA expression of genes related to lipid metabolism was determined in liver using qRT-PCR ($n = 6$ per group). (B) Blood serum concentrations of total cholesterol, HDL, and LDL were determined in random serum samples (per group $n = 6$, except $n = 5$ for JHT/NCD)

(FATP-2; encoded by *Slc27a2*)^[22] and FATP-1 (encoded by *Slc27a1*) was comparable between strains and diets, transcripts for fatty acid translocase (FAT/CD36) were up-regulated by the HFD in both *JHT* and *IgMi* mice but not in wild-type controls (Figures 3A, S5D).

In contrast, key enzymes of *de novo* lipogenesis, acetyl-CoA carboxylase 1 (ACC, encoded by *Acaca*), fatty acid synthase (FAS, encoded by *Fasn*), and stearoyl-CoA desaturase 1 (*Scd1*) were significantly up-regulated in HFD-fed wild-type mice compared with both strains of B cell-deficient mice and most prominently up-regulated in the *IgMi* mice that have striking low levels on NCD (Figure 3A), thus indicating that the mild steatosis in HFD-fed *JHT* mice is driven primarily by FFA uptake rather than by *de novo* lipogenesis.

IgMi mice are protected from adipose tissue dysfunction

A major proportion of hepatic fat accumulating in the liver under the HFD arises from visceral adipose tissue,

represented by EAT, due to exhaustion of fat storage capability.^[23] In accordance with uniform EAT hypertrophy (Figures 1A, S1D), expression of regulators of adipocyte differentiation, peroxysome proliferator-activated receptor γ (*Pparg*) and adipose differentiation related protein (encoded by *Plin2*) were comparable between all mouse strains on the HFD (Figures S8A, S9A).

However, genes related to lipid metabolism were affected differentially by the HFD in EAT. Gene expression of transcription factors regulating lipogenesis or lipid storage, i.e., CCAAT/enhance binding protein α (*Cebpa*), sterol regulatory element-binding protein 1 (*Srebf1*), and X-box binding protein 1 (*Xbp1*), as well as of key enzymes of lipogenesis, *Acaca*, *Fasn*, *Scd1*, and *Slc27a1* (FATP-1), which are critically involved in insulin-stimulated FFA uptake by adipose tissue,^[24] was down-regulated by the HFD in both *B6* and *JHT* mice. In contrast, *IgMi* mice were widely protected from HFD-induced alterations of the lipogenic gene expression profile in EAT (Figure S8A).

Despite its direct role in lipid turnover and storage, adipose tissue, as an endocrine organ, regulates whole

body lipid metabolism through the secretion of adipokines. We analyzed gene expression of adipokines related to regulation of hepatic lipid metabolism, i.e., adiponectin (*Adipoq*), leptin (*Lep*), resistin (*Retn*), and retinol-binding protein 4 (*Rbp4*).^[25] As expected, the HFD fundamentally altered the adipokine expression profile in wild-type and JHT mice. Although transcripts for *Lep* tended to be up-regulated, those for *Adipoq*, *Retn*, and *Rbp4* were down-regulated in both strains. In contrast, *IgMi* mice were protected from these HFD-induced alterations of the adipokine expression profile (Figure S8B). Importantly, transcript levels of corresponding receptors adiponectin receptor 2, leptin receptor b (*Leprb*), and toll-like receptor 4 (*Tlr4*; a receptor also for resistin^[26]) in the liver were largely unaffected. However, expression of *Leprb* was up-regulated in livers of HFD-fed wild-type mice indicating enhanced hepatic leptin signaling specifically in wild-type mice (Figure S5D).

Defects in B cell biology alter the hepatic inflammatory response to HFD

Intrahepatic B cells have been shown to impact macrophage and T cell immunity in NAFLD.^[5,8] To investigate hepatic inflammation in our models, we performed flow cytometry and immunohistochemical (IHC) analysis of immune cell populations and assessed inflammation-related gene expression. Flow cytometry revealed that the absolute number of intrahepatic immune cells (CD45⁺) doubled in liver tissue of wild-type mice after 21 weeks of the HFD when compared with NCD mice, albeit lacking statistical significance ($p = 0.07$, Figure 4A). This increase was in part due to a highly significant accumulation of B cells, predominantly B2 cells, and elevated numbers of M1-type macrophages. In contrast, the number of Ym1⁺ or arginase 1 (Arg1)⁺ M2-type macrophages was decreased and that of total macrophages (M1 and M2) unchanged (Figures 4B, S10, S11). Although CD45⁺CD3⁺ T cell numbers remained unaltered, the ratio of CD8⁺ cells within the T cell pool increased in response to the HFD (Figures 4A, S12, S13A).

In HFD-fed B cell–deficient mice, the absolute numbers of intrahepatic immune cells were comparable to wild-type mice on HFD (Figure 4A). However, hepatic T cells were significantly increased, up to 2.5-fold, specifically in B cell–deficient mice when compared with HFD-fed wild-type controls (Figure 4A). IHC demonstrated that in the B cell deficiency models CD3⁺ cells accumulated in clusters with other nonparenchymal cells in response to the HFD (Figure S12).

Corresponding with high hepatic numbers of T cells in the B cell deficiency models, we noted a distinct expression pattern of cytokines that are prominently (but not exclusively) expressed by T cells, such as interferon

γ (*Ifng*) and $\text{II}17\alpha$ (Figures 4C, S14). Although the expression in wild-type mice tended to be down-regulated in response to the HFD, JHT and *IgMi* mice exhibited a higher and diet-independent expression of these cytokines. An altered expression of classical markers of T_h cell subtypes was not observed (Figure S14). However, markers for “innate-like” T cell populations exhibited model-specific expression patterns distinct from wild-type mice, i.e., master transcription factor promyelocytic leukemia zinc finger protein (*Zbtb16* gene) and the T cell receptor chain *Va14Ja18* associated with invariant natural killer T (iNKT) cells (Figure S14).

Remarkably, JHT mice exhibited high numbers of T cells and M1-type macrophages already on the NCD (Figure 4B). In *IgMi* mice, however, M1-type macrophages did not accumulate (Figure 4B). Moreover, in contrast to wild-type mice that showed a highly significant reduction of Ym1⁺ M2-type macrophages, numbers were unaltered in the B cell–deficient mice when fed the HFD (Figures 4B, S11). Strikingly, *IgMi* mice were also protected from an increase of intrahepatic B cells on the HFD (Figure 4A).

Genes related to immune cell recruitment/chemotaxis, i.e., monocyte chemotactic protein 1 (MCP1; *Ccl2*), C-C chemokine receptor type 2 (CD192; receptor to MCP1; *Ccr2*) and macrophage inhibitory protein Mip2a (homolog to human IL-8; *Cxcl2*) were significantly up-regulated due to the HFD in livers of wild-type mice, similar to JHT mice, whereas *IgMi* mice showed no up-regulation of these inflammatory mediators (Figure 4C). Accordingly, cell surface markers of cells of the innate immune system including $\beta 2$ integrin subunits αM (*CD11b*; *Itgam*) and αX (*CD11c*; *Itgax*) were also up-regulated by the HFD, especially in livers of wild-type mice correlating with the tendency of HFD-induced up-regulation of the proinflammatory cytokine TNF α (*tnf*) (Figure 4C). Although IHC staining showed no changes in CD68 (Figure 4B), a general macrophage marker, *Cd68* transcript levels were up-regulated in HFD-treated wild-type mice (Figure S14). In contrast, expression of another marker of the macrophage lineage, F4/80 (*Adgre1*), remained unaltered (Figure S14), which is likely attributable to its high expression by liver-resident Kupffer cells.^[27]

Visceral adipose tissue is discussed as a source of proinflammatory immune cells that migrate to the liver thereby driving NAFLD progression.^[28–31] Transcript levels for F4/80, CD68, CD11b, CD11c, MCP1, Mip1a (*Ccl3*), and TNF α were up-regulated in EAT of HFD-fed wild-type and JHT mice. Notably, this up-regulation was not observed in *IgMi* mice (Figure 5). Using protein arrays, CXCL1, CXCL10, MCP1, and TIMP-1 levels were found increased in the serum of HFD-treated B6 and JHT mice but not in *IgMi* mice (Figure S15).

Taken together, deficiencies in B cell function altered the intrahepatic immune cell composition, both under the NCD and further in response to the HFD. In

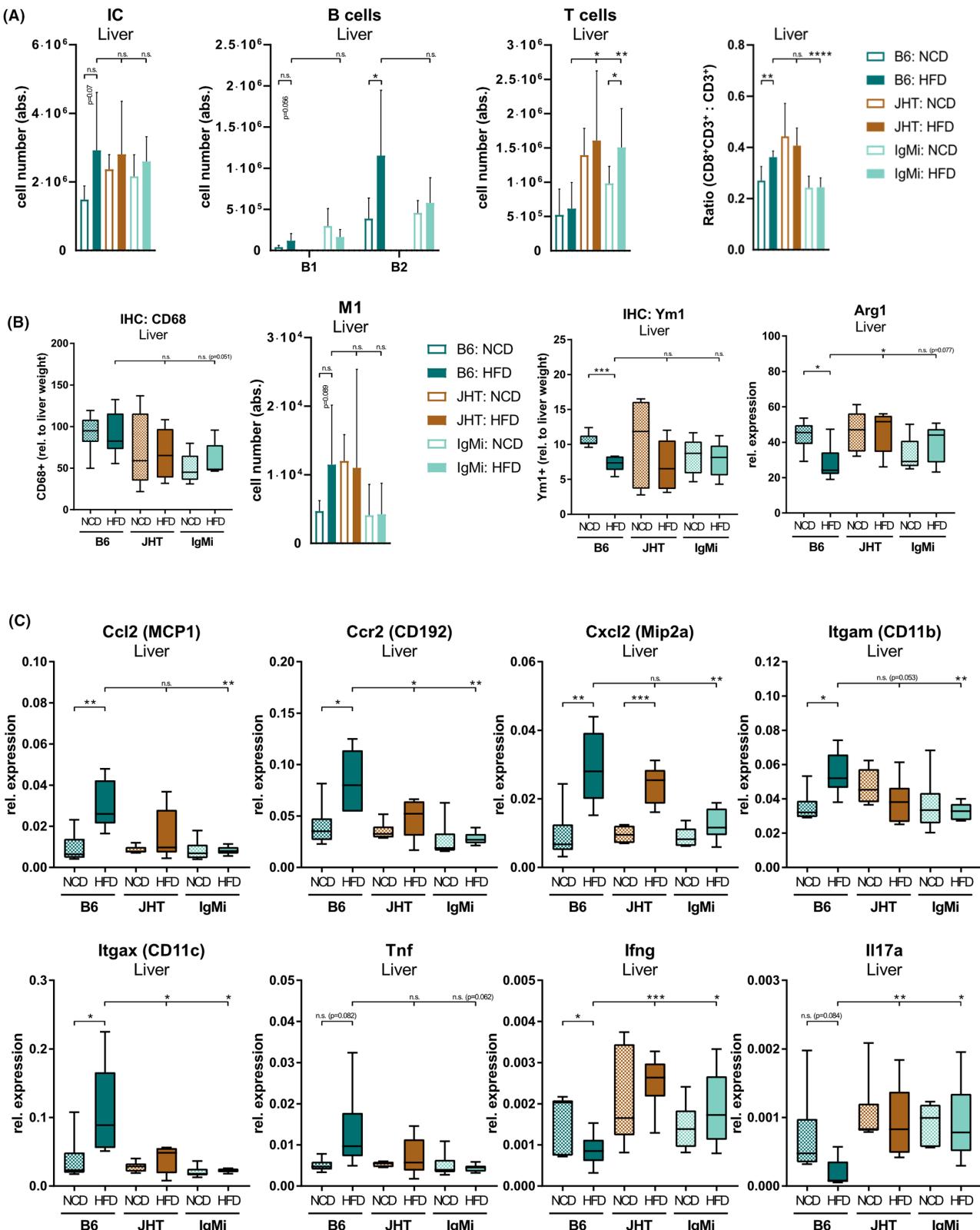


FIGURE 4 The intrahepatic immune response to HFD is altered in B cell deficiency models. (A) Intrahepatic immune cells (IC, CD45⁺), B cell subtypes (B1, CD19⁺ CD23⁻; B2, CD19⁺ CD23⁺), and subpopulations of T cells (CD3⁺) were determined by flow cytometry. Absolute cell numbers refer to the analyzed liver tissue and are presented for 6 (B6/both diets, IgMi/HFD) or 9 (JHT/both diets, IgMi/NCD) mice per group. (B) Intrahepatic M1-type macrophages (CD11b⁺ F4/80⁺ CD11c⁺ CD206⁻) were determined by flow cytometry. Liver tissue was analyzed immunohistochemically for CD68 (Figure S10) or Ym1 (Figure S11). Presented are average numbers of positive cells normalized to liver weight (n = 6 for each group). mRNA expression of Arg1 was determined in liver tissue using qRT-PCR (n = 6 per group). (C) mRNA expression of genes related to inflammation was determined in liver tissue using qRT-PCR (n = 6 per group).

contrast to wild-type mice, HFD-induced inflammation by the innate immune system was attenuated in the B cell models, albeit to varying extent. Remarkably, *IgMi* mice showed no signs of an HFD-mediated innate immune response.

B cell-deficient mice are protected from the development of NASH and fibrosis

Steatosis, hepatic inflammation, and hepatocyte injury, as observed in HFD-fed wild-type mice, are main criteria for the diagnosis of NASH in patients.^[32,33] In our study, serum markers of liver damage, i.e., alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) were significantly increased in HFD-fed wild-type mice, whereas these markers were in the normal or even subnormal range in the B cell models (Figure 6A).

Hepatocellular ballooning, which is used as a histological marker to diagnose hepatocyte lipoapoptosis in patients with NASH, is hard to detect in mice.^[33] However, lipotoxicity has been shown to increase the expression of TNF-related apoptosis-inducing ligand receptor 2 (TRAIL-R2, or DR5, encoded by *Tnfrsf10b*), thereby sensitizing hepatocytes for TRAIL-mediated

apoptosis.^[34] *Tnfrsf10b* expression was up-regulated in steatotic livers of HFD-fed wild-type and to a lesser extent in JHT but not *IgMi* mice (Figure 6B).

Advanced chronic liver disease inevitably results in an impairment of liver function. Investigating the expression of central liver functional markers, we found decreased expression of albumin, *Arg1*, and the genes encoding several coagulation factors (FII, FVII, FVIII, FXI, subunits of FI) especially in HFD-fed wild-type mice in line with elevated parameters of NASH in these mice. In contrast, expression of these genes was largely normal in the two HFD-fed B cell deficiency models (Figures 6B, S16B,C).

Development of NASH is usually linked to liver fibrosis, which may lead to end stage liver disease.^[1,35] HFD-fed wild-type mice did not develop a relative increase in collagen proportionate area, as quantified by sirius red morphometry, in line with prior reports of DIO models.^[36] However, total liver collagen content nearly doubled when the almost 2-fold expansion of the liver volume by the HFD was factored in. Accordingly, they displayed a significant increase in activated myofibroblasts/hepatocytic stellate cells, as quantified by α -smooth muscle actin (α SMA) deposition (Figures 7A,B, S17–S19). Similarly, a broad panel of key fibrosis related transcripts were up-regulated in HFD-fed wild-type mice (collagen type 1 α chains

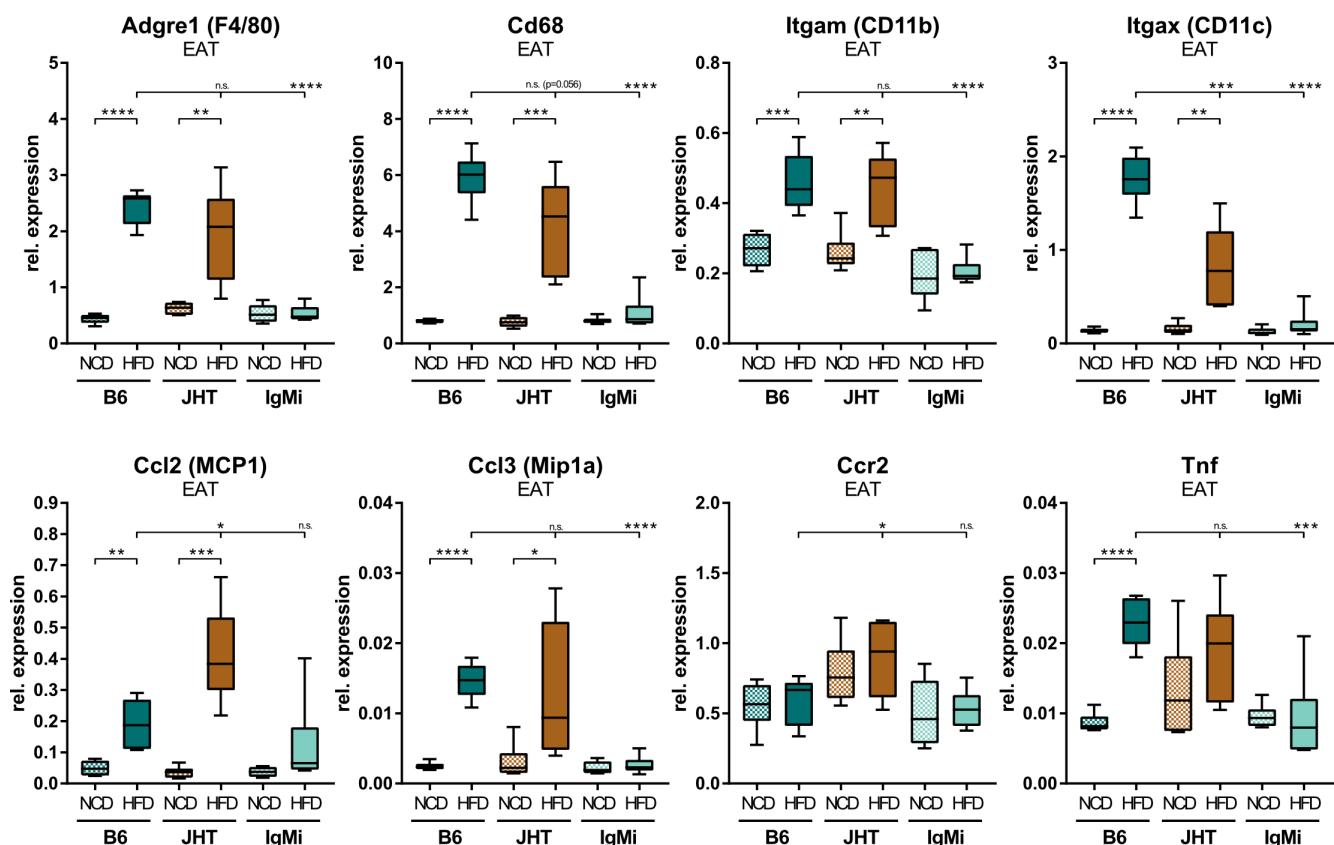


FIGURE 5 *IgMi* mice lack signs of inflammation in EAT. mRNA expression of genes related to inflammation was determined in EAT using qRT-PCR (for each group n = 6)

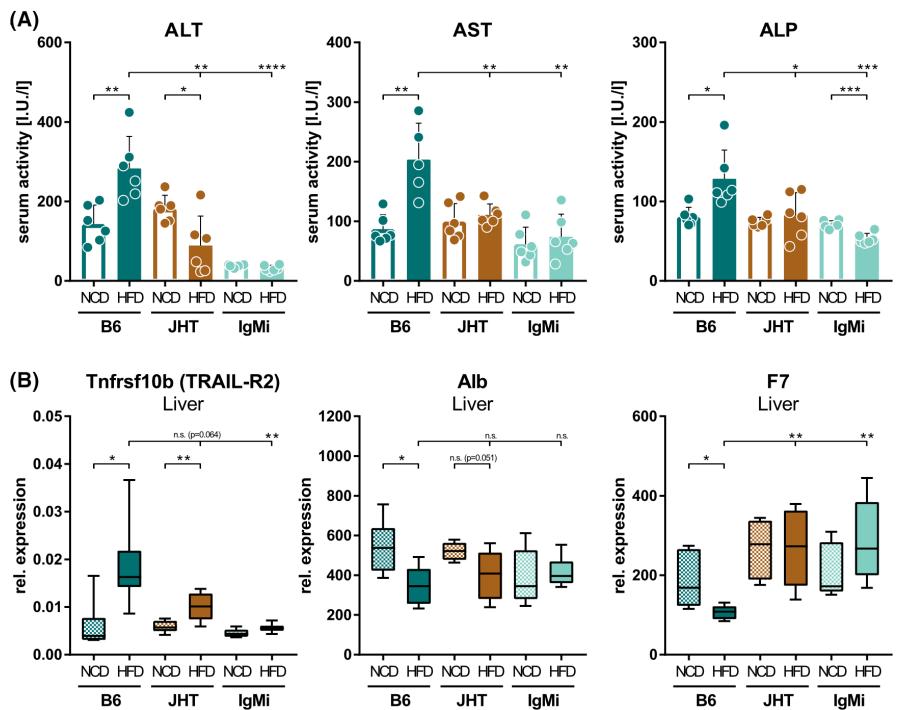


FIGURE 6 B cell deficiency models are protected from HFD-induced liver tissue injury and liver function impairment. (A) Serum levels of liver enzymes ALT, AST, and ALP were determined for six individuals per group (except n = 5 for AST/B6/HFD). (B) mRNA expression of genes related to apoptosis and liver function was determined in liver using qRT-PCR (n = 6 per group)

Col1a1, *Col1a2*, *Col3a1*, and *Col4a1*; matrix metalloproteinase *Mmp2*, *Mmp8*, and *Mmp13*; *Timp1*; and *Pai1*), all in all indicating a highly up-regulated extracellular matrix (ECM) metabolism in diet-induced NASH, with a balanced increase in fibrogenesis and fibrolysis, resulting in no net increase in fibrosis. Notably, the up-regulation of these genes was (significantly) attenuated in the HFD-fed *JHT* and especially *IgMi* mice (Figures 7C, S16A).

Commonalities and differences between JHT and IgMi mice

The common feature of *JHT* and *IgMi* mice is that they do not secrete antibodies, neither under basal conditions nor in response to HFD feeding. In contrast, wild-type mice showed increased serum levels of IgG2c antibodies in response to HFD feeding (Figure 8A). Moreover, *JHT* mice completely lack B cells, whereas *IgMi* mice have a normal number of B cells. Because regulatory B cells (Breg) can down-regulate immune response by producing IL-10,^[37] we explored whether this may contribute to the liver phenotypes observed in *IgMi* mice. Although serum levels of IL-10 were not changed in any mouse strains in response to HFD (Figure S15), an increased mRNA expression of IL-10 was observed in the livers of HFD-treated *IgMi* mice (Figure 8B), indicating that the increase of IL-10 may originate in the liver. FACS analyses showed that HFD feeding reduced

the number of hepatic Breg of wild-type mice, whereas it increased the number of hepatic total and IL-10 expressing Breg in *IgMi* mice (Figure 8C,D).

To translate our findings to the human system, we studied a collective of 44 liver sections from patients with advanced chronic liver disease due to NASH, alcoholic steatohepatitis, and chronic viral hepatitis B and C infection for B and T cell infiltration and expression of IgG and IL-10 and compared the findings to normal control livers. In line with our mouse experimental data, B cell numbers were increased prominently in stromal areas along with a marked deposition of IgG next to portal parenchymal interfaces, hepatocytes, and sinusoids, whereas IL-10 expressing cells were scarce, especially in NASH and HBV (Figure S20, Table S4).

DISCUSSION

We investigated the impact of restricted B cell function (*IgMi*) or a complete lack of B cells (*JHT*) on the development of HFD-induced NAFLD, NASH, and liver fibrosis. HFD feeding of wild-type mice for 21 weeks resulted in manifest NAFLD that was characterized by (i) macrovesicular steatosis associated with massively increased liver, body, and EAT weight and up-regulation of genes related to *de novo* lipogenesis, (ii) a proinflammatory intrahepatic immune response, with elevated numbers of myeloid inflammatory cells and transcripts

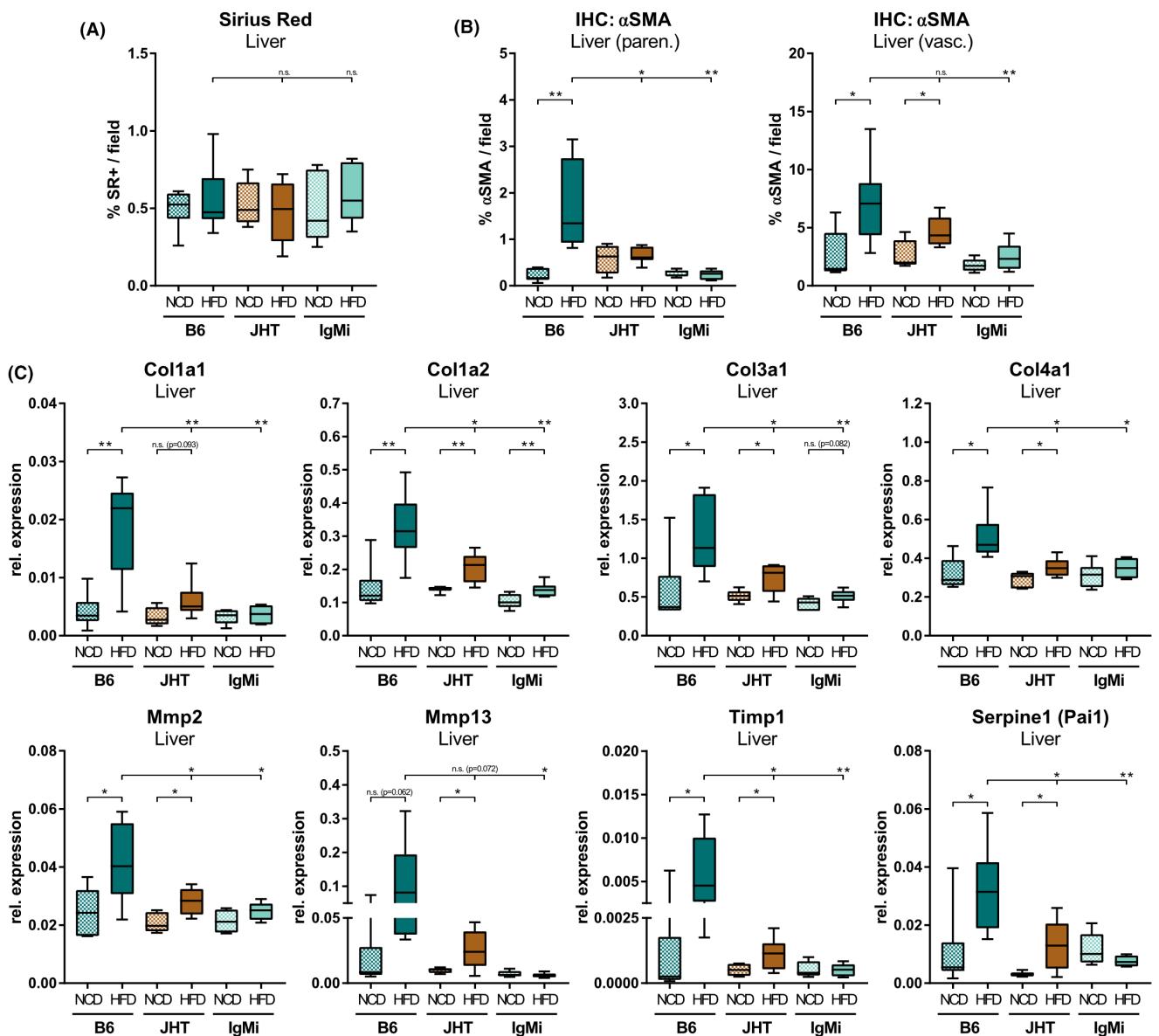


FIGURE 7 B cell deficiency models are protected from HFD-induced liver tissue remodeling. Cryosections of FA-fixed liver tissue were examined histologically using sirius red (A) or immunohistochemically for α SMA (B) in parenchyma (paren.; left panel) and in areas of large vessels (vasc.; right panel). Presented are mean percentages of positive area per field ($n = 6$ for each group). (C) mRNA expression of genes related to fibrosis were determined in livers using qRT-PCR ($n = 6$ per group)

both in the liver and visceral EAT, (iii) marked IR, and (iv) signs of a highly enhanced ECM turnover. This is in line with central features of human NASH, despite a rather modest increase in fibrosis.^[32,33] B cell deficiency, IgMi more than JHT, was associated with a significantly attenuated NAFLD phenotype. In fact, these models were largely protected from the establishment of NASH and the associated proinflammatory and profibrotic immune response in the liver and EAT and the resultant metabolic alterations and liver functional abnormalities.

In a murine B2 cell deficiency model (B cell-activating factor^{-/-}), HFD was recently shown to result in reduced steatosis and liver weight increase.^[10]

Attenuated steatosis in these mice as well as in our B cell deficiency models correlated with protection from enhanced hepatic *de novo* lipogenesis as can be concluded from the decreased serum triglycerides and a lack of up-regulation of genes of lipogenic enzymes ACC, FAS, and SCD1. In addition, damped up-regulation of *Tnfrsf10b* (TRAIL-R2), regarded as an indicator of lipid-induced hepatocyte stress, indicates damped lipotoxicity in HFD-fed B cell-deficient mice when compared with wild-type mice. Therefore, attenuation of hepatic steatosis in JHT mice appears to be a result of protection from HFD-induced hepatic *de novo* lipogenesis and is likely attributable to the lack of B2 cells.

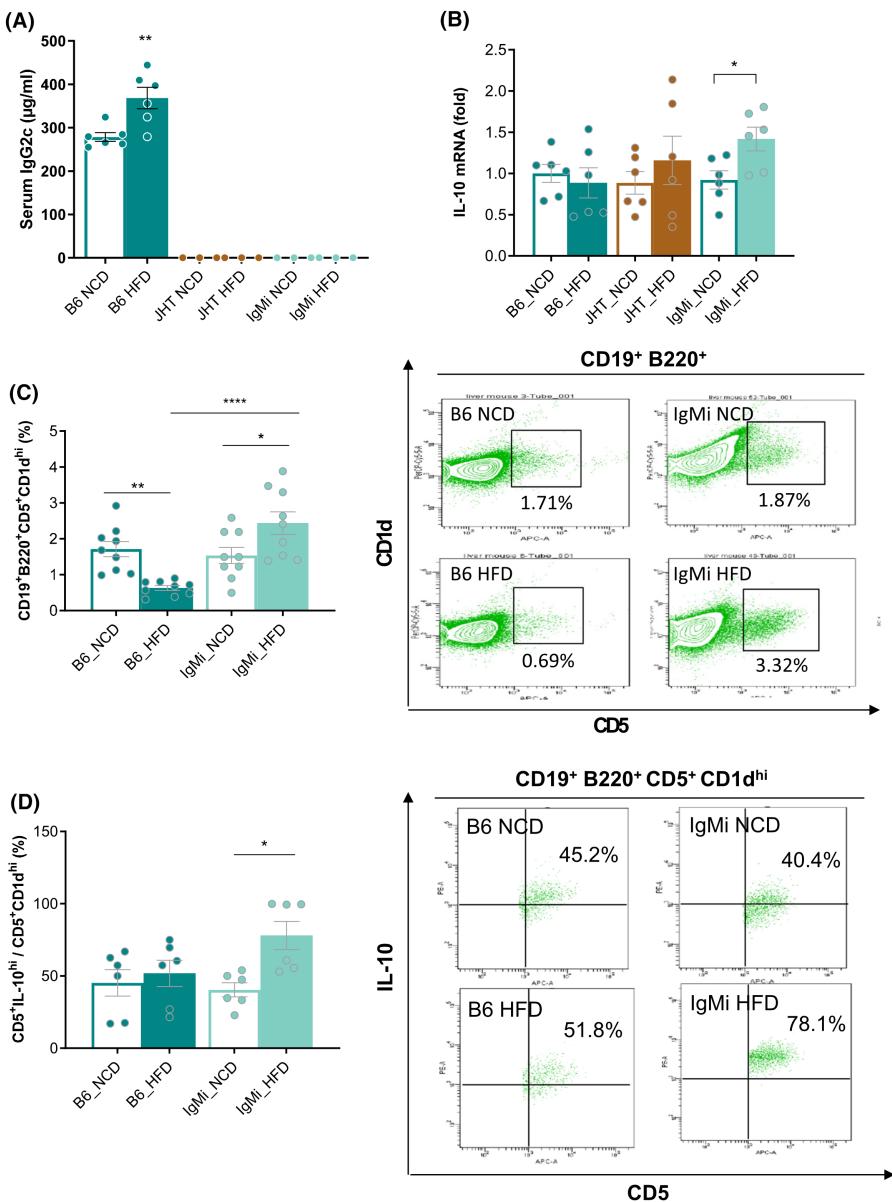


FIGURE 8 Mechanisms of B cell effects in HFD-induced NAFLD. Wild-type (B6) mice and B cell–deficient *JHT* and *IgMi* mice were fed an NCD or HFD for 15 weeks. Mice were sacrificed without fasting. IgG2c serum levels were determined by ELISA (A). (B) mRNA expression of IL-10 in the liver was determined using qRT-PCR ($n = 6$ per group). (C) Regulatory B cells (Breg; CD45⁺ CD19⁺ B220⁺ CD5⁺ CD1d^{high}) were determined by flow cytometry. Shown are Breg numbers as percent of total hepatic CD45⁺ immune cells. (D) IL-10-positive cells (CD5⁺ IL-10^{high}) were determined among Breg by flow cytometry. Data are presented as mean \pm SEM.

Although *IgMi* mice have B2 cells, these B2 cells do not secret antibodies. *IgMi* mice were almost completely protected from an HFD-induced enhancement of hepatic *de novo* lipogenesis and inflammation. In stark contrast to B^{null} *JHT* mice, the presence of B cells in *IgMi* mice largely prevented adipose tissue dysfunction that included normalized expression of adipokines (*Adipoq*, *Retn*, *Rbp4*) as well as a (partial) rescue of the expression of lipogenic enzymes. In particular, gene expression of *Scd1*, which is a strong indicator of metabolic deterioration,^[38] was unaffected in *IgMi* mice. Similarly, *IgMi* mice were devoid of signs of an activated (innate) immune response in EAT. Hence,

metabolic dysfunction and inflammation of the EAT strongly correlated with—if not preceded—the presence or absence of liver steatosis and inflammation in the investigated mouse models, supporting the notion that adipose tissue dysfunction, rather than hepatic *de novo* lipogenesis, is causing hepatic steatosis in HFD-fed *JHT* mice.

B cells display several capacities that might mediate their effects in NAFLD as well as IR. *JHT* and *IgMi* mice are deficient in antibody secretion and are both protected from NAFLD development. Therefore, HFD-induced antibody production may play a significant role in the enhancement of NAFLD. Indeed, previous

studies have provided evidence that antibodies are involved in the pathology of metabolic disease. In a cohort of patients with NAFLD, the serum level of IgA was elevated and emerged as an independent predictor of advanced fibrosis.^[39] In mouse models, NAFLD is characterized by maturation of B2 lymphocytes to plasma cells and by an elevated circulating IgG.^[8,9] The authors incriminated intrahepatic B in NAFLD development by secreting IgG2a subclass antibodies (equivalent to IgG2c in B6 mice^[40]).^[8] Our data in HFD-fed wild-type mice that display elevated IgG2c serum levels are consistent with these findings and a previous study with an HFD-induced obesity model.^[41]

B2 cell-derived pathogenic antibodies have been shown to induce an accumulation of M1 macrophages and T_h1 cells.^[41,42] In our HFD-fed wild-type mice, the number of total liver macrophages was unchanged compared with NCD-fed controls. However, the ratio of intrahepatic M1 versus M2-type macrophages was increased in HFD-fed wild-type mice. This increase was prevented in *JHT* and *IgMi* mice. Therefore, we hypothesize that the protection of NAFLD development in *JHT* and *IgMi* mice may be partly attributable to the lack of pathogenic antibodies that would increase M1 and reduce (protective) M2-type macrophages.

The most striking finding of the present studies is that *IgMi* mice are more protected than the *JHT* mice. The lack of pathogenic antibodies should protect *JHT* and *IgMi* mice to a similar extent. Therefore, the additional protection observed in the *IgMi* mice may result from the presence of certain B cell subsets. Breg are plausible candidates of such protection, because they can suppress the differentiation of proinflammatory lymphocytes, including cytotoxic CD8⁺ T cells.^[43,44] Moreover, IL-10 and Breg can promote M2-type macrophage polarization.^[45,46] This is in line with our observation of an increased M1 over M2-type macrophage ratio and increased CD8⁺ T cells in livers of HFD-fed wild-type mice, which were prevented in *IgMi* mice; accordingly, *IgMi* mice showed increased hepatic Breg numbers and IL-10 levels.

Our study had limitations. First, *JHT* and *IgMi* mice gained less body weight on HFD than the wild-type mice. However, we do not think that this is the reason for the observed liver phenotype, because EAT weight gain was similar among the three mouse strains. In addition, *JHT* and *IgMi* mice showed less adipose tissue dysfunction and inflammation. Hence, the difference in liver phenotype is more likely a specific effect due to the deficiency of B cells. Second, we observed an increased serum level of IgG2c in HFD-fed wild-type mice. This is consistent with previous findings showing the role of B2 cells and pathogenic antibodies in NAFLD. Nevertheless, demonstrating a causal role of IgG2c in NAFLD needs further studies. Third, the

comparison of *JHT* and *IgMi* mice indicates a protective role of certain B cell populations in NAFLD. Our results support the concept that Breg are likely to be this B cell subset. Further studies are needed to confirm their protective role in NAFLD and possibly other chronic liver diseases.

In summary, comparative analysis of genetically modified B cell models unraveled differential roles of distinct B cell subsets in NAFLD. Importantly, all B cell deficiency models exhibited an attenuated development of NAFLD under metabolic stress, in line with recently published reports that suggested a pathogenic role for B cells in NAFLD.^[5,8–11] The pathogenic B cell population is likely represented by B2 cells that produce pathogenic antibodies such as IgG2c, as is also supported by our correlative studies in a collective of 44 human specimens, including NASH, ASH, HBV, HCV, and normal control livers as comparators. On the other hand, *IgMi* mice that are deficient in antibody production but do harbor otherwise functional B cells were almost completely protected from HFD-induced NAFLD, which together with our characterization of B cell subsets supports a role of IL-10 expressing Breg as prime candidates mediating protective effects in NAFLD. Our data merit further studies that may lead to therapies to enhance protective B cell subsets in NAFLD.

ACKNOWLEDGMENTS

The *JHT* and *IgMi* mouse strains were kindly provided by Ari Waisman (Institute for Molecular Medicine, Johannes Gutenberg University Medical Center, Mainz, Germany).

CONFLICT OF INTEREST

Dr. Schuppan consults for, advises, and received grants from NorthSea. He has other interests in Boehringer Ingelheim.

AUTHOR CONTRIBUTIONS

Martin Karl designed and performed the experiments, analyzed data, and wrote the manuscript; Solveig Hasselwander designed and performed the experiments and analyzed data; Yawen Zhou, Gisela Reifenberg, Yong Ook Kim, Kyoung-Sook Park, Dirk A. Ridder, Xiaoyu Wang, Eric Seidel, Beate K. Straub, and Ning Xia conducted the experiments, analyzed data, and provided technical advice on experiments; Nadine Hövelmeyer and Beate K. Straub provided advice on experimental design and critically revised the manuscript; Huige Li, Detlef Schuppan, and Ning Xia designed and supervised the experiments and revised the manuscript.

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How to cite this article: Karl M, Hasselwander S, Zhou Y, Reifenberg G, Kim YO, Park K-S, et al. Dual roles of B lymphocytes in mouse models of diet-induced nonalcoholic fatty liver disease. *Hepatology.* 2022;76:1135–1149. <https://doi.org/10.1002/hep.32428>