The Role of Intestinal C-type Regenerating Islet Derived-3 Lectins for Nonalcoholic Steatohepatitis

Sena Bluemel,[1] Lirui Wang,[1,2] Cameron Martino,[3] Suhan Lee,[1] Yanhan Wang,[1,2] Brandon Williams,[1] Angela Horvath,[4,5] Vanessa Stadlbauer,[4] Karsten Zengler,[3,6] and Bernd Schnabl[1,2]

C-type regenerating islet derived-3 (Reg3) lectins defend against pathogens and keep commensal bacteria at a distance. Deficiency of *Reg3g* and *Reg3b* facilitates alcohol-induced bacterial translocation and alcoholic liver disease. Intestinal *Reg3g* is down-regulated in animal models of diet-induced obesity, but the functional consequences for nonalcoholic steatohepatitis (NASH) are unknown. The aim of this study was to investigate the role of Reg3 lectins in NASH. NASH was induced by a Western-style fast-food diet in mice deficient for *Reg3g* or *Reg3b* and in transgenic mice overexpressing *Reg3g* in intestinal epithelial cells (*Reg3g*Tg). Glucose tolerance was assessed after 18 weeks and insulin resistance after 19 weeks of feeding. After 20 weeks, mice were assessed for features of the metabolic syndrome. Obesity was not different in genetically modified mice compared with their respective wild-type littermates. Glucose intolerance, liver injury, hepatic inflammation, steatosis, fibrosis, and bacterial translocation to mesenteric lymph nodes and to the liver were not different in *Reg3g*-deficient mice compared with wild-type littermates. Plasma endotoxin levels were higher in *Reg3g*-deficient mice. *Reg3b* deficiency protected against glucose intolerance, but liver disease, bacterial translocation, and plasma endotoxin levels were similar to wild-type littermates. Absence of either REG3G or REG3B protein in the ileum was not compensated for by up-regulation of the respective other REG3 protein. Transgenic *Reg3g* mice also developed liver injury, steatosis, and fibrosis similar to their wild-type littermates. *Conclusion:* In contrast to alcoholic liver disease, loss of intestinal Reg3 lectins is not sufficient to aggravate diet-induced obesity and NASH. This supports a multi-hit pathogenesis in NASH. Only glucose metabolism is affected by *Reg3b* deficiency. (*Hepatology Communications* 2018;2:393-406)

Introduction

Obesity and its complications are a globally increasing epidemic. Nonalcoholic fatty liver disease (NAFLD) has a prevalence of >70% in adults who are obese. Disease progression to nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and finally hepatocellular carcinoma worsens the prognosis, and therefore NASH is on the rise to become the leading indication for liver transplantation.[1] It is not well understood why some individuals with NAFLD develop progressive liver disease with inflammation and fibrosis.

Intestinal dysbiosis contributes to the development of NAFLD and NASH.[2-4] Transmission of the hepatic

Abbreviations: AST, aspartate aminotransferase; Ctrl, control chow; FFD, Western style fast food diet; GTT, glucose tolerance test; IL, interleukin; ITT, insulin tolerance test; LPS, lipopolysaccharide; MLN, mesenteric lymph nodes; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; Reg3b, regenerating islet-derived protein 3 beta; Reg3g, regenerating islet-derived protein 3 gamma; Tg, transgenic; TGFβ, transforming growth factor β; TLR-4, toll-like receptor 4; TNF-α, tumor necrosis factor α; WT, wild type.

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phenotype by microbiota transplantation to germ-free mice or cohousing of mice underlines the role of intestinal dysbiosis for liver disease.[5] Dysbiosis and/or poor nutrition can damage the intestinal epithelial barrier and lead to endotoxemia.[6] Lipopolysaccharide (LPS; or endotoxin) is a component in the outer membrane of gram-negative bacteria. Systemic LPS-binding protein is increased in patients with NAFLD compared to controls who are obese.[7] LPS binds and activates toll-like receptor (TLR)-4. TLR-4 expression in liver biopsies is elevated in patients with NASH compared with NAFLD.[8] Downstream signals of TLR activation, such as release of tumor necrosis factor α (TNFα) or transforming growth factor β (TGFβ), contribute to liver inflammation, fibrosis, and hepatocellular death.[9,10]

Maintaining the strict separation of resident bacteria in the intestinal lumen is mandatory for a healthy host. The C-type lectins regenerating islet-derived protein 3 beta (Reg3b) and 3 gamma (Reg3g) are antimicrobial molecules highly expressed especially in the intestine. REG3B and REG3G are up-regulated upon bacterial colonization of the gut and during intestinal infection and inflammation, thereby contributing to the spatial segregation of intestinal bacteria and the epithelium.[11-13] Bacteria are in closer contact to intestinal epithelial cells in the absence of REG3G.[14,15] Activation of TLR in both intestinal epithelial cells and Paneth cells stimulates expression of REG3G and REG3B in either cell type.[12,16] In colonic epithelial cells, REG3G and REG3B are also up-regulated by interleukin (IL)-22 that is mainly released from innate lymphoid cells upon stimulation of dendritic cells with IL-23.[17,18] We have recently demonstrated that ethanol-fed *Reg3b*$^{-/-}$ and *Reg3g*$^{-/-}$ mice have increased mucosa-associated bacteria and bacterial translocation. Absence of REG3B or REG3G renders mice more susceptible to alcoholic liver disease.[19] Intestine-specific overexpression of REG3G reduced mucosa adherent bacteria, decreased bacterial translocation, and protected mice from liver disease following chronic ethanol administration.[19] In animal models of diet-induced obesity, intestinal *Il-22* and *Reg3g* are down-regulated.[4,20,21] Here, we investigated the role of REG3B and REG3G on bacterial translocation and liver disease in a mouse model of diet-induced NASH.

Materials and Methods

MICE

Reg3g$^{-/-}$, *Reg3b*$^{-/-}$, and transgenic *Reg3g* (*Reg3g*Tg) mice with intestinal overexpression of *Reg3g* on a C57BL/6 background have been described.[14,19] Heterozygous *Reg3g*$^{+/-}$ and *Reg3b*$^{+/-}$ mice were bred to generate wild-type (WT) and knockout littermates. *Reg3g*Tg mice were bred to WT C57BL/6 mice to generate transgenic and WT littermates. Male mice were housed with littermates of the same genotype after weaning. At age 8 weeks, mice were started on a Western-style fast-food diet (FFD) (AIN-76A; TestDiet, St. Louis, MO) for 20 weeks. Drinking water was supplemented with 18.9 g/L glucose (G8270; Sigma Aldrich, St. Louis, MO) and 23.1 g/L fructose (F0127; Sigma Aldrich) to mimic soft drinks containing high-fructose corn syrup with 42% fructose.[22] Control mice (Ctrl) received standard chow (5053 PicoLab Rodent Diet; LabDiet, St. Louis, MO) and autoclaved tap water. Mice had free access to food and water and were kept with 7090 Teklad sani-chips bedding (Envigo, East Millstone, NJ). Animals were maintained on a 12-h:12-h light–dark cycle; experiments were performed during the light cycle.

A glucose tolerance test (GTT) was performed after 18 weeks of feeding by injecting 1 μg glucose/g body weight intraperitoneally. After 19 weeks of feeding, an

ARTICLE INFORMATION:

From the [1]Department of Medicine, University of California San Diego, La Jolla, CA; [2]Department of Medicine, VA San Diego Healthcare System, San Diego, CA; [3]Department of Pediatrics, Division of Host-Microbe Systems and Therapeutics, San Diego, CA; [4]Department of Internal Medicine, Division of Gastroenterology and Hepatology, Medical University of Graz, Graz, Austria; [5]Center of Biomarker Research in Medicine, Graz, Austria; [6]Center for Microbiome Innovation, University of California San Diego, La Jolla, CA.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Bernd Schnabl, M.D.
Department of Medicine, University of California, San Diego
Biomedical Research Facility 2, Room 4A22
9500 Gilman Drive, MC0063
La Jolla, CA 92093
E-mail: beschnabl@ucsd.edu
Tel: +1-858-822-5311

insulin tolerance test (ITT) was performed by injecting insulin (Novolin N NPH; Novo Nordisk Inc., Princeton, NJ) at a concentration of 0.5 mU/g body weight intraperitoneally. Prior to GTT and ITT, mice were fasted for 6 hours. Tail vein blood for blood glucose levels was obtained before injection (t = 0 minutes) and at t = 15, 30, 60, 90, 120 minutes following the injection.

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

GENE EXPRESSION ANALYSIS

RNA of mouse liver and ileum was extracted using Ambion Trizol Reagent (15596; Thermo Fisher Scientific, Waltham, MA) following the manufacturer's protocol. Deoxyribonuclease treatment of RNA was performed using the RQ1 RNase-Free DNase (M6101; Promega, Madison, WI), and RNA was reverse transcribed using the Applied Biosystems High-Capacity Complementary DNA Reverse Transcription Kit (43688; Thermo Fisher Scientific). Real-time quantitative polymerase chain reaction was performed with iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) using an Applied Biosystems StepOnePlus thermocycler (4376600; Thermo Fisher Scientific). Primer sequences were obtained from the National Institutes of Health mouse qPrimerDepot. Gene expressions were normalized to the mouse 18S gene and expressed relative to Ctrl-fed or FFD-fed WT mice.

GENOMIC DNA ANALYSIS

Genomic DNA was isolated from sterile mouse liver to quantify bacterial 16S. The liver was homogenized in 1 mL sterile Hanks' balanced salt solution without calcium/magnesium/phenol red (14175; Thermo Fisher Scientific) using lysing matrix C tubes (MP116912; MP Biomedicals, Santa Ana, CA) and a Mini-BeadBeater-96 (GlenMills, Clifton, NJ). This was followed by digestion with ribonuclease A (19101; Qiagen, Valencia, CA), 10% sodium dodecyl sulfate (L3771; Sigma-Aldrich) and proteinase K (Am2546; Thermo Fisher Scientific) at 55°C for 1 hour. After adding phenol (15513; Thermo Fisher Scientific), suspensions were transferred to individual lysing matrix B tubes (MP116911; MP Biomedicals) and homogenized. The lysate was then extracted 3 times using phenol/chloroform/isoamyl alcohol (15593; Thermo Fisher Scientific) and once with chloroform with the addition of sodium acetate buffer solution (S7899; Sigma-Aldrich). DNA was finally precipitated and washed with ethanol and resuspended in sterile water (BP561; Thermo Fisher Scientific). The 16S ribosomal RNA gene was amplified using established primers (forward, 5′-GTG STG CAY GGY TGT CGT CA-3′; reverse, 5′-ACG TCR TCC MCA CCT TCC TC-3′),[23] and then gene expression was normalized to host 18S.

ANAEROBIC BACTERIAL CULTURES

At the time of harvesting, mesenteric lymph nodes (MLNs) were homogenized in sterile phosphate-buffered saline (BP661; Thermo Fisher Scientific) and plated on brucella agar with 5% sheep blood, hemin, and vitamin K_1 (297848; Becton, Dickinson and Company, Franklin Lakes, NJ). The plates were incubated for 72 hours at 37°C under strict anoxic conditions in an anaerobic chamber (Coy Lab Products, Grass Lake, MI) followed by the counting of colony-forming units.

PROTEIN ANALYSIS

Intestinal tissue was homogenized in Dignam C buffer.[24] We used 50 μg of protein homogenate for immunoblotting and the following primary antibodies: anti-Reg3b (MAB5110; R&D Systems, Inc., Minneapolis, MN), anti-Reg3g,[14] and anti-β-actin (A5441; Sigma-Aldrich). Immunoblots were developed using chemiluminescence (34580; Thermo Fisher Scientific) with a charge-coupled device camera (Gel Doc XR+ System; Bio-Rad), and bands were analyzed by densitometry using Image J version 1.49 (National Institutes of Health, Bethesda, MD).

BIOCHEMICAL ANALYSES

Biochemical analyses were performed according to the manufacturers' protocols. Aspartate aminotransferase (AST) was measured from plasma obtained from the inferior vena cava, using a kinetic assay (TR70121; Thermo Fisher Scientific). Plasma LPS levels were measured using a reporter cell line (hkb-htlr4; Invivogen, San Diego, CA).[25] LPS results were confirmed in FFD-fed animals (data not shown), using a commercially available enzyme-linked immunosorbent assay (CEB526Ge; Cloud-Clone Corp., Katy, TX).

Liver lipids were isolated from caudate lobes. The tissue was homogenized in phosphate-buffered saline, and lipids were precipitated using methanol and chloroform. Triglyceride levels were measured with a colorimetric endpoint assay (T7532; Pointe Scientific, Canton, MI). Liver hydroxyproline was extracted from 150-250 mg of mixed liver specimens from left and right liver lobes. The tissue was homogenized in 6 N HCl (3750-32; USABlueBook), using lysing matrix C tubes with the Mini-BeadBeater-96, and subsequently incubated at 110°C for 18 hours. The lysate was obtained using Whatman filter paper, grade 595 1/2 (WHA10311644; Sigma Aldrich). After incubation with chloramine T– (C9887; Sigma Aldrich) and Ehrlich's perchloric acid solution (AC168760250; Thermo Fisher Scientific), triplicates were measured at 558 nm (SpectraMax 190 Microplate Reader; Molecular Devices LLC, Sunnyvale, CA).[26,27]

TISSUE STAINING

At harvesting, the median liver lobe, including gall bladder, was fixed in 10% formalin (HT501128; Sigma-Aldrich) for 24 to 48 hours, then transferred to 70% ethanol and embedded in paraffin. We stained 3-μm paraffin sections with hematoxylin and eosin (38015 and 380161, SelecTech; Leica Biosystems Inc., Buffalo Grove, IL) or 0.1% picrosirius red (color index 35780, 365548; Sigma-Aldrich).

STATISTICAL ANALYSES

Numbers of biological replicates for each experiment are given in Supporting Table S1. Area under the curve and area over baseline of blood glucose levels were analyzed for ITT and GTT, respectively. Outliers were detected using the Tukey method. Normal distribution of values was assessed with the D'Agostino and Pearson omnibus normality test. Normally distributed groups were compared by the unpaired *t* test; non-normally distributed groups were compared by the Mann-Whitney test. All results are expressed as mean ± SEM. Analyses and data plots were done with GraphPad Prism 6.01 (GraphPad Software, Inc., La Jolla, CA). Differences are considered significant if $P < 0.05$.

Results

Reg3g$^{-/-}$, *Reg3b*$^{-/-}$, and *Reg3g*Tg mice were fed for 20 weeks with a Western-style FFD to test if Reg3 lectins are protective in diet-induced NASH.

Reg3g-DEFICIENT MICE DEVELOP SIMILAR METABOLIC FEATURES AS THEIR WT LITTERMATES

Reg3g deficiency did not affect body weight, epididymal fat weight, or brown fat weight (Fig. 1A), although food intake of FFD-fed *Reg3g*$^{-/-}$ mice was about 10% higher compared to their WT littermates (Fig. 1B). GTTs were performed after 18 weeks of feeding (Fig. 1C). FFD-fed cohorts had worse glucose tolerance than their Ctrl-fed littermates; differences between *Reg3g*$^{-/-}$ and WT mice did not exist. The effect of insulin on blood glucose levels was attenuated in the FFD-fed cohorts; this effect was also independent from the genotype (Fig. 1D). Taken together, metabolic features were mainly influenced by the type of diet and did not differ between *Reg3g*-deficient and WT littermate mice.

ABSENCE OF *Reg3g* MODESTLY ENHANCES FEATURES OF NASH

To determine the significance of REG3G for the development of NASH, parameters of liver injury, inflammation, steatosis, and fibrosis were investigated. Hepatic injury, expressed as plasma AST, was not significantly different between FFD-fed WT littermates and *Reg3g*$^{-/-}$ mice (Fig. 2B). The expression of inflammatory genes downstream of TLR activation (C-C motif chemokine ligand 2 [*Ccl2*] and *Tnf*) did not differ between FFD-fed *Reg3g*-deficient mice and their WT littermates (Fig. 2C,D). Similarly, hepatic steatosis, expressed as total liver triglycerides and relative liver weight, did not differ between FFD-fed *Reg3g*$^{-/-}$ mice and their WT littermates (Fig. 2E,F). Representative liver sections are depicted in Fig. 2B. Hepatic expression of genes related to fibrosis, including collagen type 1 α1 (*Col1a1*) and smooth muscle alpha-actin (*Acta2*), did not differ between FFD-fed WT littermates and *Reg3g*$^{-/-}$ mice (Fig. 2G,H). Fibrosis, expressed as total liver hydroxyproline, was highest in FFD-fed *Reg3g*$^{-/-}$ mice but not significantly different from their WT littermates (Fig. 2I,J). Taken together, *Reg3g* deficiency weakly affected liver injury and fibrosis following FFD feeding. The liver phenotype appeared to be more affected by the type of diet than by the deficiency of *Reg3g*.

EFFECT OF FFD-FEEDING ON BACTERIAL TRANSLOCATION IN *Reg3g*-DEFICIENT MICE

We next assessed if intestinal REG3G is restricting bacterial translocation with a Western-style diet. The

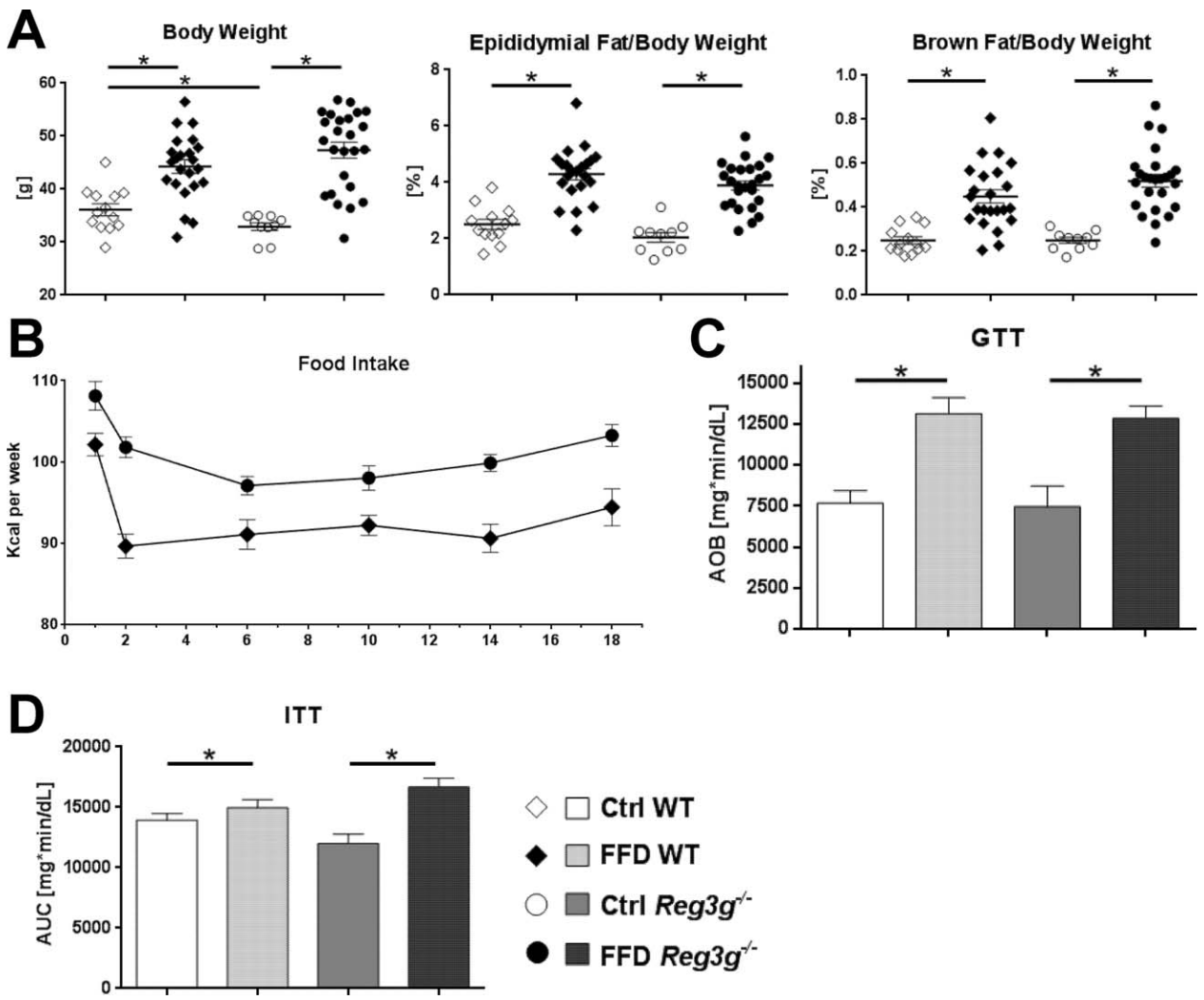

FIG. 1. Effect of *Reg3g* deficiency on body weight and metabolic function. (A) Body weight, and relative weights of epididymal and brown fat tissues after 20 weeks of feeding. (B) Food intake per group over the course of the experiment. (C) Glucose tolerance test performed after 18 weeks of feeding. (D) Insulin tolerance test performed after 19 weeks of feeding. Data represent mean ± SEM; $^*P < 0.05$. Refer to Supporting Table S1 for numbers of biological replicates. Abbreviation: AUC, area under the curve.

translocation of bacteria to MLNs and the liver was not increased in *Reg3g*$^{-/-}$ mice compared to their FFD-fed wild-type littermates (Fig. 3A). In contrast, translocation of bacterial endotoxins was elevated in FFD-fed *Reg3g*$^{-/-}$ mice but not in the Ctrl-fed littermate groups (Fig. 3B). As LPS is a ligand for the TLR-4, we examined *Tlr4* expression in the liver. However, while FFD increased hepatic *Tlr4*, *Reg3g* deficiency did not increase it further (Fig. 3C). To explain the unexpectedly weak effect of *Reg3g* deficiency, a possible intestinal compensation by REG3B was investigated. Ileal expression of REG3B did not differ between FFD-fed *Reg3g*$^{-/-}$ mice and their WT littermates (Fig. 3D). A representative western blot is depicted in Fig. 3E. There was also no difference in REG3B in the Ctrl-fed littermate groups (data not shown). Taken together, REG3G could not protect from translocation of bacteria and hepatic *Tlr4* expression but might be important for the reduction of endotoxemia in a model of diet-induced NASH. REG3G deficiency was not compensated by an increase in REG3B.

METABOLIC PHENOTYPE IN *Reg3b*-DEFICIENT MICE

Reg3b deficiency did not affect body weight, epididymal fat weight, or brown fat weight (Fig. 4A). Food intake of

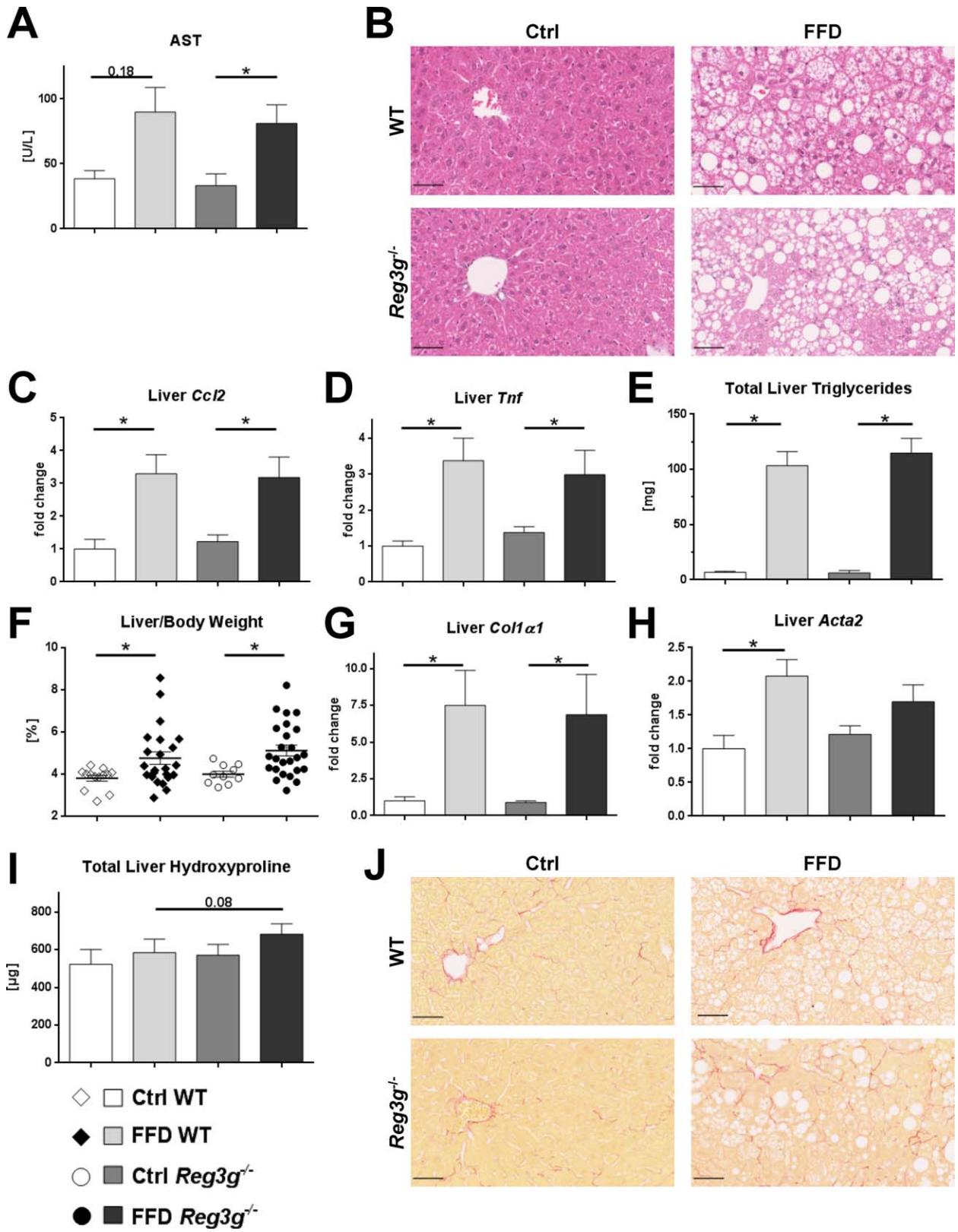

FIG. 2. Effect of *Reg3g* deficiency on liver phenotype. (A) AST plasma levels. (B) Representative hematoxylin and eosin-stained liver sections. (C,D) Expression of inflammatory genes in liver samples. (E) Total amount of triglycerides per liver. (F) Relative liver weight. (G,H) Expression of fibrosis-related genes in liver samples. (I) Total amount of hydroxyproline per liver. (J) Representative sirius red-stained liver sections. Scale bars, 50 μm. Data represent mean ± SEM; $^*P < 0.05$. Refer to Supporting Table S1 for numbers of biological replicates.

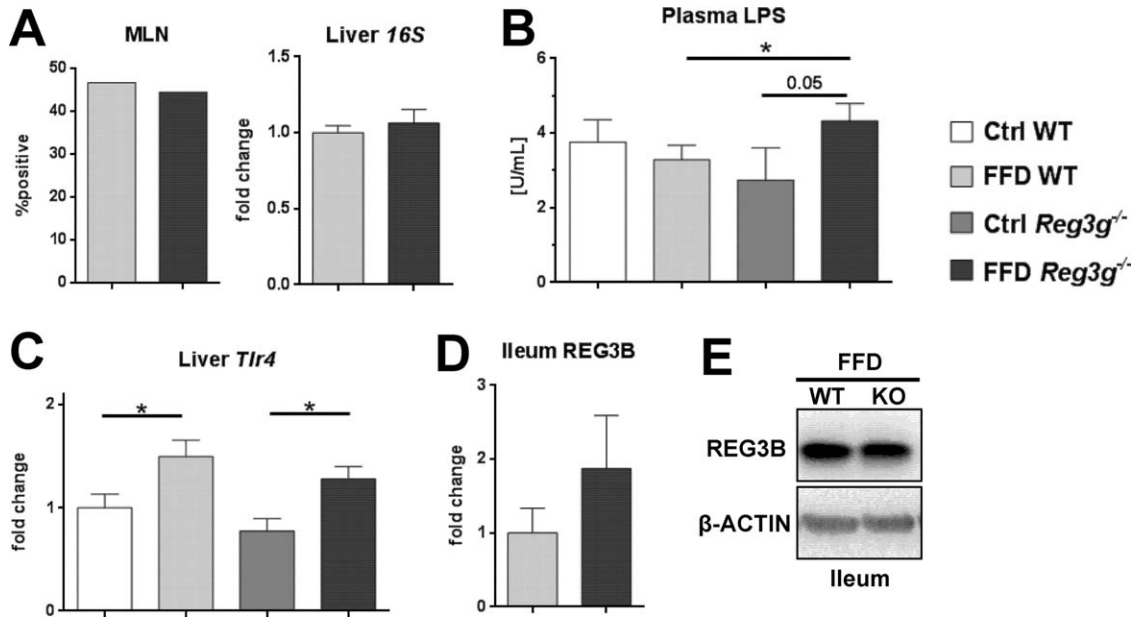

FIG. 3. Effect of *Reg3g* deficiency on bacterial translocation. (A) Results of anaerobic mesenteric lymph node cultures and total bacteria in liver specimens. (B) Plasma lipopolysaccharide levels. (C) Expression of *Tlr4* in liver samples. (D) REG3B protein expression in ileal samples. (E) Representative REG3B expression in ileal samples of FFD-fed WT and $Reg3g^{-/-}$ mice. Data represent mean ± SEM; $^*P < 0.05$. Refer to Supporting Table S1 for numbers of biological replicates. Abbreviation: KO, knockout.

FFD-fed $Reg3b^{-/-}$ mice was similar to their WT littermates (Fig. 4B). In contrast to the Reg3g experiment, the FFD-fed $Reg3b^{-/-}$ mice did not develop glucose intolerance compared to their Ctrl-fed littermates over the course of the feeding experiment (Fig. 4C). This resulted in better glucose tolerance of FFD-fed $Reg3b^{-/-}$ mice compared to their WT littermates (Fig. 4C). The result was confirmed with the ITT that showed a higher drop of blood glucose levels after insulin injections in FFD-fed $Reg3b^{-/-}$ mice compared to their WT littermates (Fig. 4D). Taken together, Western-style diet equally induced obesity in *Reg3b*-deficient and WT littermate mice. Interestingly, $Reg3b^{-/-}$ mice were resistant to the development of diet-induced glucose intolerance and insulin resistance.

NASH FEATURES IN *Reg3b*-DEFICIENT MICE

To determine the significance of REG3B for the development of NASH, parameters of liver injury, inflammation, steatosis, and fibrosis were investigated. Plasma AST and the expression of inflammatory genes encoding for *Ccl2*, *Tnf*, and *Tgfb1* were elevated in the FFD-fed cohorts but did not differ between WT littermate and $Reg3b^{-/-}$ mice (Fig. 5A-E). Hepatic steatosis, expressed as total liver triglycerides (Fig. 5F) and relative liver weight (Fig. 5G), also did not differ between FFD-fed $Reg3b^{-/-}$ mice and their WT littermates. Representative liver sections are depicted in Fig. 5B. Measures of liver fibrosis were induced in FFD-fed animals. Total liver hydroxyproline was highest in FFD-fed $Reg3b^{-/-}$ mice but not statistically different to WT littermates (Fig. 5H). Representative liver sections are depicted in Fig. 5I. The investigated fibrosis-related genes were induced in FFD-fed animals (Fig. 5J,K). The expression of *Col1a1* did not differ between $Reg3b^{-/-}$ and WT littermate mice (Fig. 5J). Tissue inhibitor of metalloproteinase 1 *(Timp1)* expression was highest in FFD-fed $Reg3b^{-/-}$ mice but also did not differ from WT littermates (Fig. 5K). Taken together, *Reg3b* deficiency does not aggravate diet-induced NASH.

EFFECT OF INTESTINAL REG3B ON BACTERIAL TRANSLOCATION

We next investigated if intestinal REG3B is restricting bacterial translocation with a Western-style diet. Bacterial translocation to MLNs rarely occurred and was not different between groups (data not shown). Bacterial translocation to the liver was also not affected by *Reg3b* deficiency in FFD-fed mice

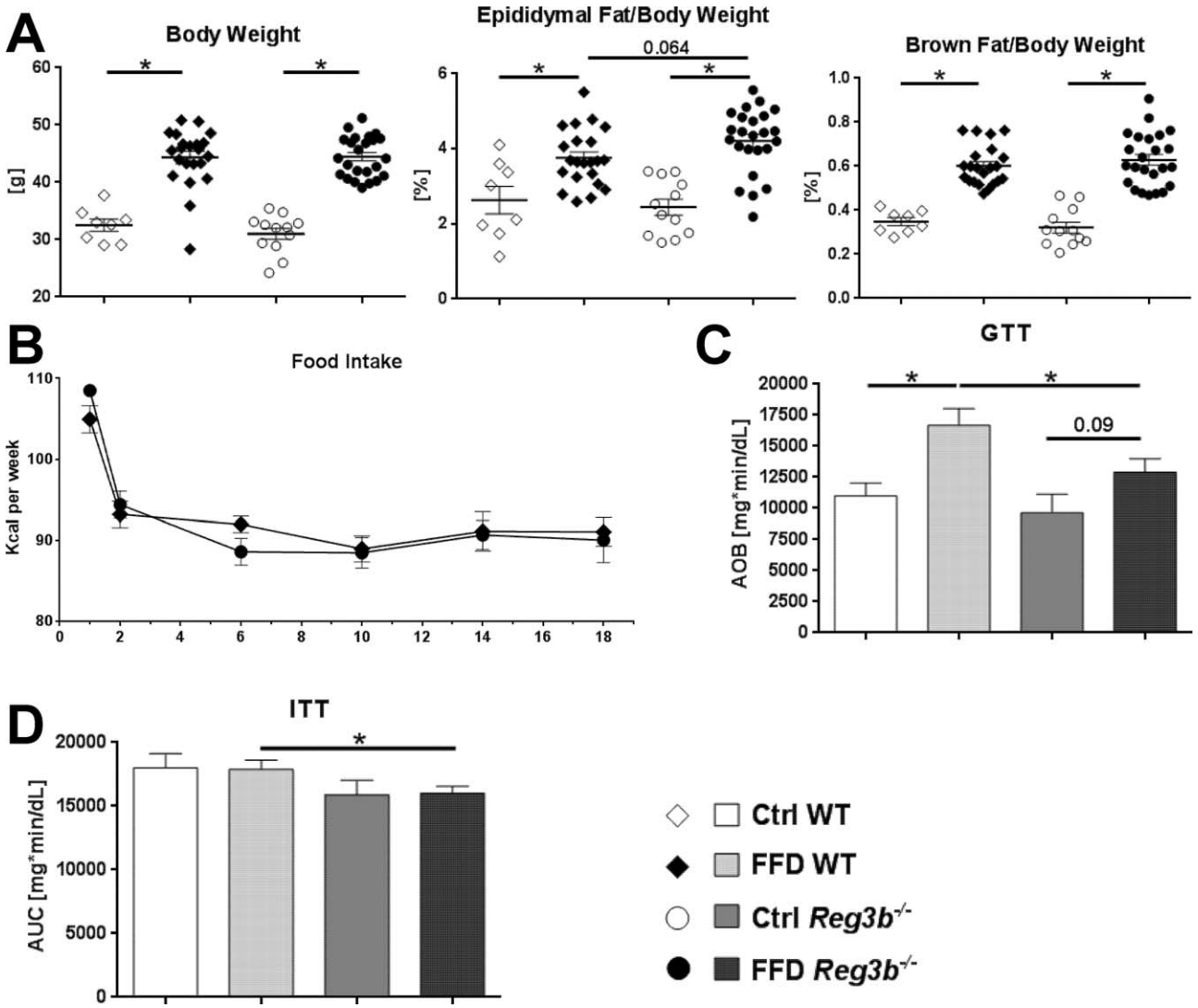

FIG. 4. Effect of *Reg3b* deficiency on body weight and metabolic function. (A) Body weight and relative weights of epididymal and brown fat tissues after 20 weeks of feeding. (B) Food intake per group over the course of the experiment (C) Glucose tolerance test performed after 18 weeks of feeding. (D) Insulin tolerance test performed after 19 weeks of feeding. Data represent mean ± SEM; $^*P < 0.05$. Refer to Supporting Table S1 for numbers of biological replicates. Abbreviations: AOB, area over baseline; AUC, area under the curve.

(Fig. 6A). In contrast to the *Reg3g*-deficient mice, absence of *Reg3b* did not lead to an increase of plasma LPS levels (Fig. 6B). Hepatic expression of *Tlr4* was increased with the FFD but not with *Reg3b* deficiency (Fig. 6C). Comparable to the Reg3g cohort, there was no compensation by REG3G in the ileum of FFD-fed *Reg3b*-deficient mice (Fig. 6D). A representative western blot is depicted in Fig. 6E. There was also no difference in REG3G in the Ctrl-fed littermate groups (data not shown). Taken together, this study could not find evidence that REG3B protects from translocation of bacteria and endotoxins in a model of diet-induced NASH. The deficiency of REG3B was not compensated by an increase of REG3G in the ileum.

INTESTINAL *Reg3g* OVEREXPRESSION DOES NOT PROTECT FROM DIET-INDUCED NASH

To cross-validate the findings, an identical feeding experiment was performed in *Reg3g*Tg mice, overexpressing Reg3g in intestinal epithelial cells under the control of the villin promoter.[19]

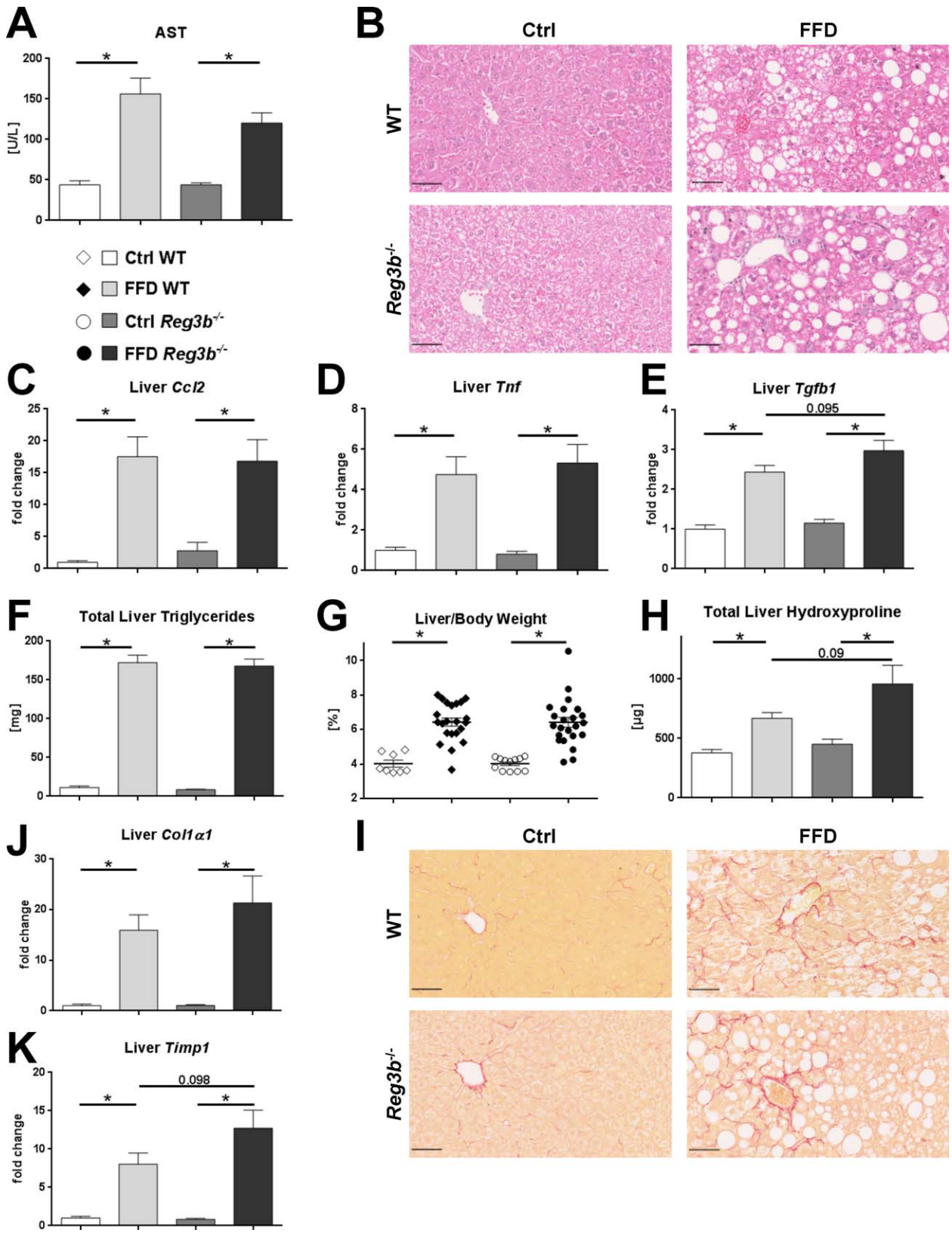

FIG. 5. Effect of *Reg3b* deficiency on liver phenotype. (A) AST plasma levels. (B) Representative hematoxylin and eosin-stained liver sections. (C-E) Expression of inflammatory genes in liver samples. (F) Total amount of triglycerides per liver. (G) Relative liver weight. (H) Total amount of hydroxyproline per liver. (I) Representative sirius red-stained liver sections. (J,K) Expression of fibrosis-related genes in liver samples. Scale bars, 50 μm. Data represent mean ± SEM; $^*P < 0.05$. Refer to Supporting Table S1 for numbers of biological replicates.

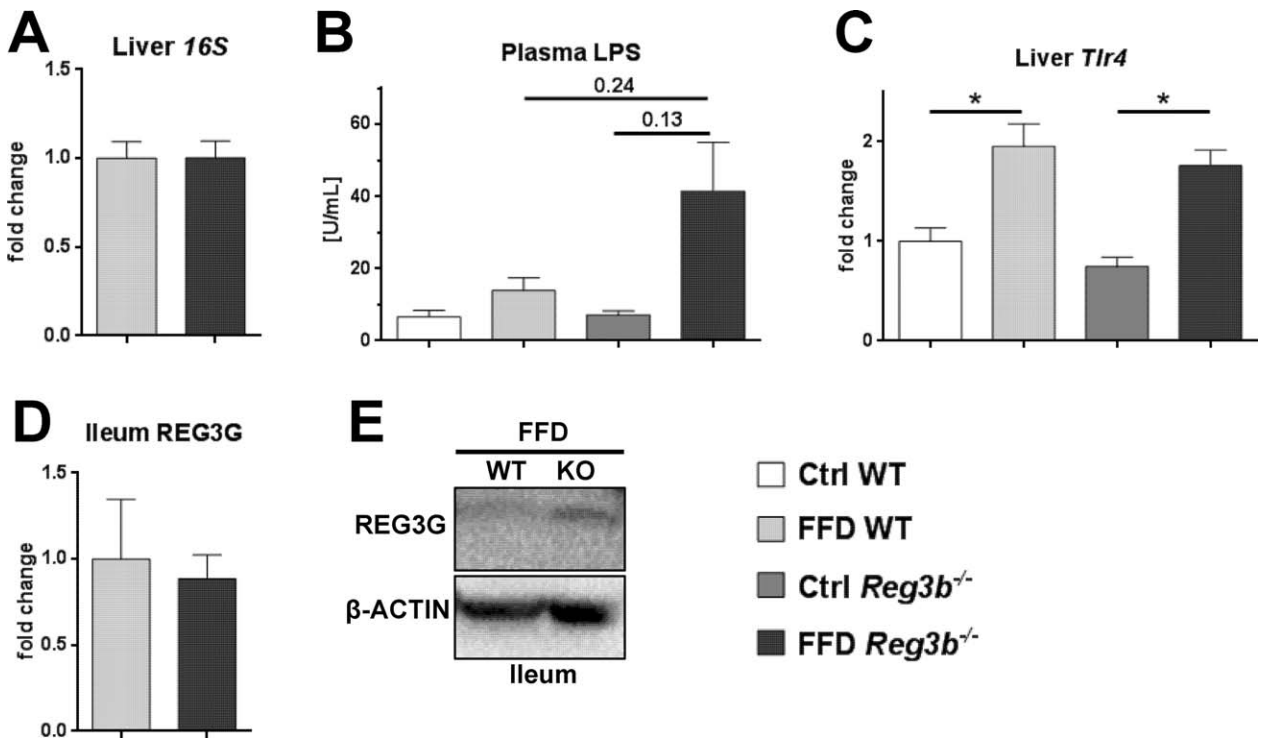

FIG. 6. Effect of *Reg3b* deficiency on bacterial translocation. (A) Total bacteria in liver specimens. (B) Plasma LPS levels. (C) Expression of *Tlr4* in liver samples. (D) REG3G protein expression in ileal samples. (E) Representative REG3G expression in ileal samples of FFD-fed WT and *Reg3g*$^{-/-}$ mice. Data represent mean ± SEM; *$P < 0.05$. Refer to Supporting Table S1 for numbers of biological replicates. Abbreviation: KO, knockout.

Body weight and epididymal fat weight did not differ between the FFD-fed cohorts. Only the relative brown fat weight was lower in FFD-fed *Reg3g*Tg mice compared to their WT littermates (Fig. 7A). Food intake of the FFD-fed cohorts was identical (Fig. 7B).

Interestingly, Ctrl- and FFD-fed cohorts showed similar glucose tolerance after 18 weeks of feeding (Fig. 7C). The overexpression of *Reg3g* was not beneficial. However, the FFD-fed *Reg3g*Tg mice showed more sensitivity to insulin than their WT littermates (Fig. 7D).

Liver injury was equally present in the WT and *Reg3g*Tg littermate mice (Fig. 8A). Similarly, hepatic steatosis did not differ between the FFD-fed cohorts (Fig. 8B,C). FFD equally induced fibrosis in the transgenic and the WT animals. According to the liver hydroxyproline levels, FFD-fed *Reg3g*Tg mice were not protected from liver fibrosis compared to their WT littermates (Fig. 8E). Bacterial translocation to MLNs rarely occurred and was not lower in the FFD-fed transgenic *Reg3g* mice (data not shown).

Taken together, FFD-fed *Reg3g*Tg mice were only protected from insulin resistance but not from all other parameters of diet-induced metabolic syndrome; a protection from bacterial translocation or NASH was also not present in FFD-fed *Reg3g*Tg mice.

Discussion

The role of Reg3 lectins in diet-induced NASH was investigated in *Reg3g*$^{-/-}$, *Reg3b*$^{-/-}$, and *Reg3g*Tg mice. Despite elevated endotoxemia, *Reg3g*-deficient mice did not develop more liver disease than their WT littermates. Both *Reg3b*-deficient and *Reg3g*Tg mice had similar liver phenotypes as their WT littermates. *Reg3b*-deficient mice were protected from glucose intolerance and insulin resistance. Overall, our results do not indicate that loss of *Reg3g* or *Reg3b* is sufficient to aggravate NAFLD or that intestinal overexpression of *Reg3g* can protect against NASH.

We have recently demonstrated a protective role of Reg3 lectins in alcoholic liver disease.[19] After ethanol feeding, *Reg3g*$^{-/-}$ and *Reg3b*$^{-/-}$ mice had increased bacterial translocation to mesenteric lymph nodes and liver and mice were more susceptible to ethanol-

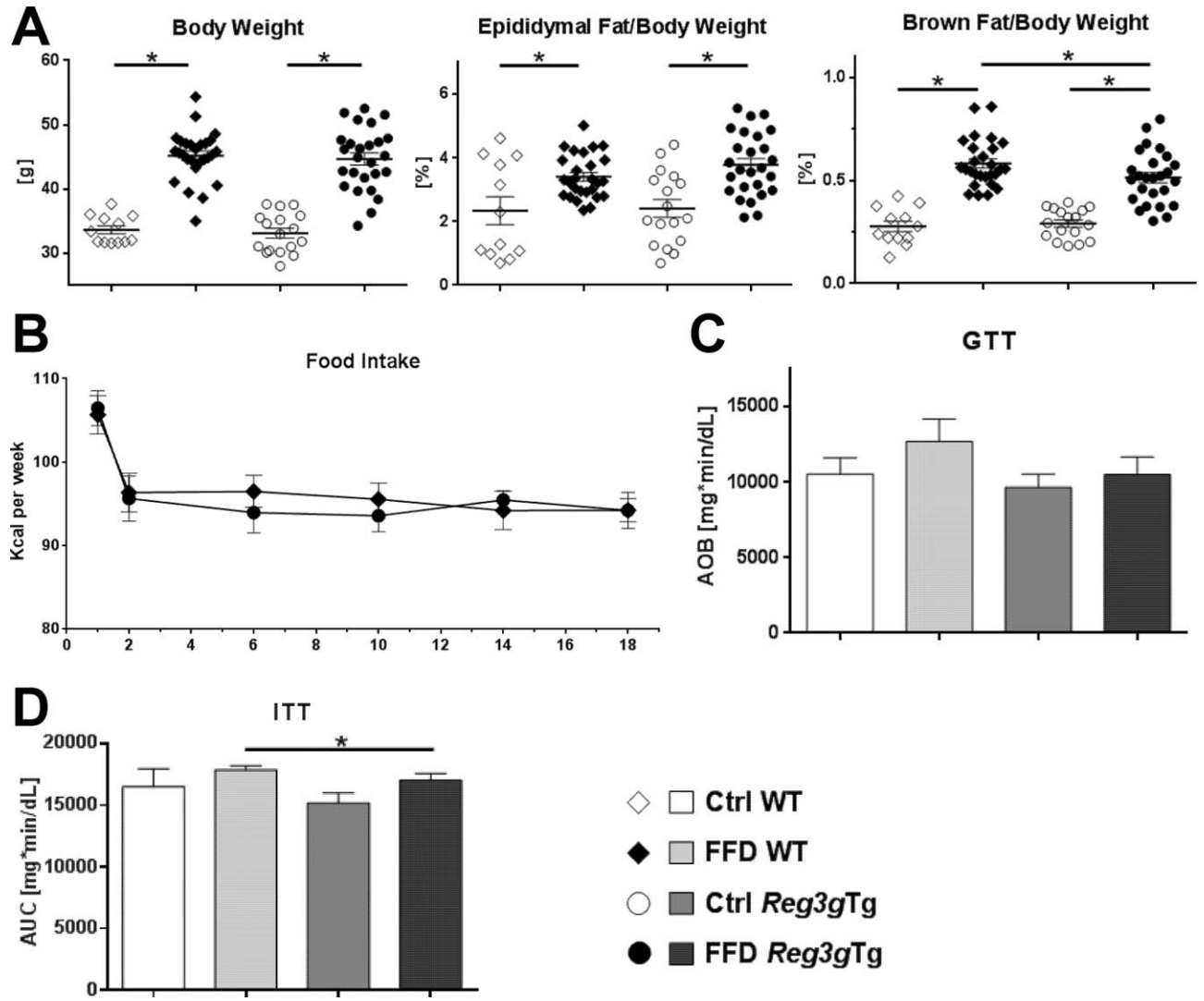

FIG. 7. Effect of *Reg3g* overexpression on body weight and metabolic function. (A) Body weight and relative weights of epididymal and brown fat tissues after 20 weeks of feeding. (B) Food intake per group over the course of the experiment. (C) Glucose tolerance test performed after 18 weeks of feeding. (D) Insulin tolerance test performed after 19 weeks of feeding. Data represent mean ± SEM; $^*P < 0.05$. Refer to Supporting Table S1 for numbers of biological replicates. Abbreviations: AOB, area over baseline; AUC, area under the curve.

induced liver disease.[19] Intestinal overexpression of REG3G reduced bacterial translocation and protected mice from alcoholic liver disease. A possible explanation for the relevance of Reg3 lectins in alcoholic liver disease but not in NASH might originate from the toxic effects of ethanol or its metabolites on the intestinal epithelium. Increased gastrointestinal permeability has long been recognized in acute and chronic alcohol consumption.[28] Alcohol-associated gut dysbiosis induces intestinal inflammation and increases intestinal permeability in mice.[29] Acetaldehyde, which is a product of ethanol metabolism, and the generation of reactive oxygen species through cytochrome P450 2E1 induction might contribute to tight junction disruption leading to increased permeability.[6] Increased bacterial translocation adds to chronic ethanol-induced hepatic inflammation long before chronic liver disease occurs. Absence of Reg3 lectins enhances this mechanism.[19] In NAFLD and NASH, mechanistic data of dietary effects on the intestinal barrier are less stringent. While several mouse models with gene knockouts of intestinal defense mechanisms, such as junctional adhesion molecule A and myeloid differentiation protein 88, provide hints for the interrelation of intestine and liver in

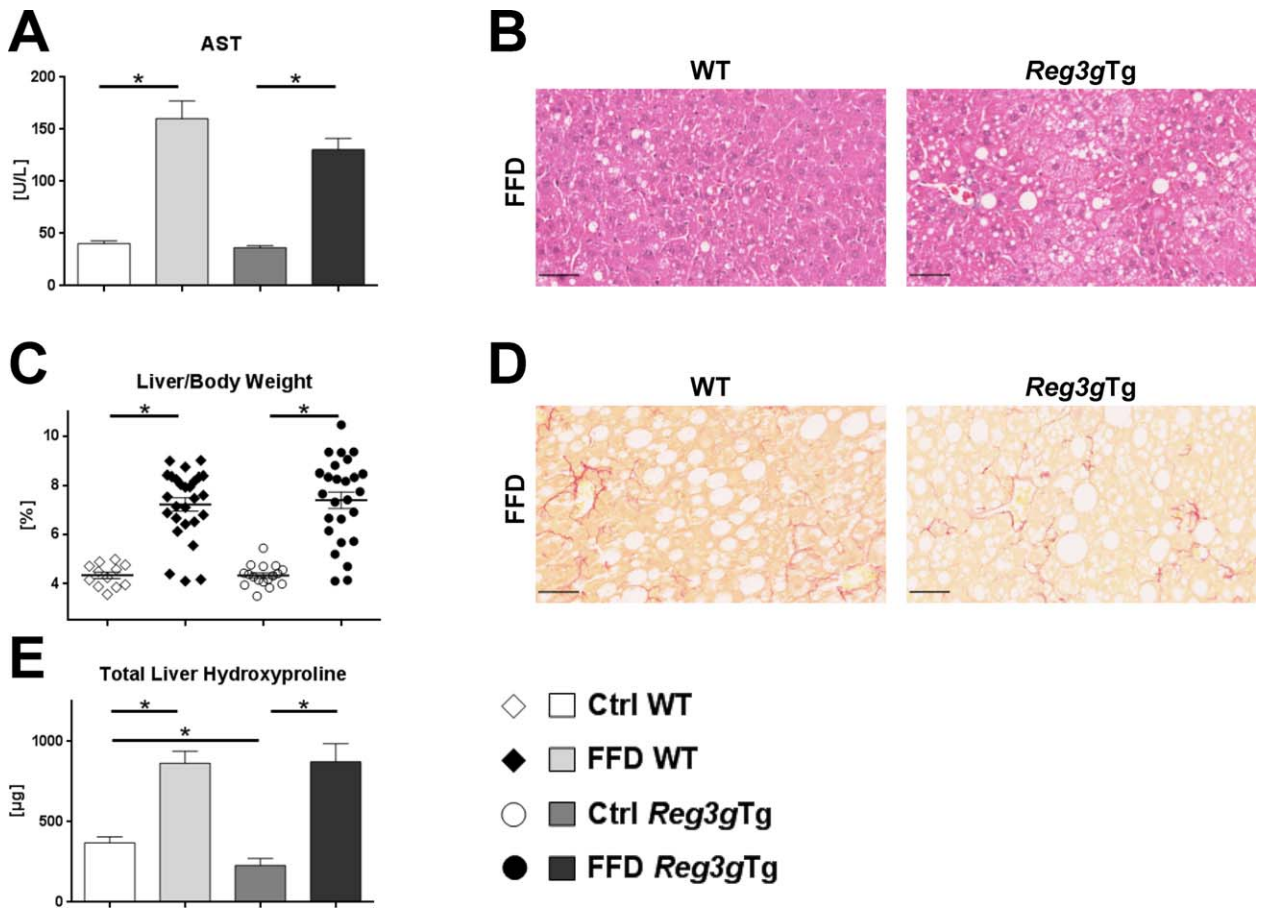

FIG. 8. Effect of *Reg3g* overexpression on liver phenotype and bacterial translocation. (A) AST plasma levels. (B) Representative hematoxylin and eosin-stained liver sections. (C) Relative liver weight. (D) Representative sirius red-stained liver sections. (E) Total amount of hydroxyproline per liver. Scale bars, 50 μm. Data represent mean ± SEM; $^*P < 0.05$. Refer to Supporting Table S1 for numbers of biological replicates.

NASH, the causality of diet-induced damage of the intestinal barrier and subsequent liver disease is not as clear as in alcohol consumption.[30,31] Investigation of the gut–liver axis in fatty liver disease might, in part, be hampered by numerous dietary regimes that are used to experimentally induce NASH in rodents.[22] In human studies, the investigation of endotoxemia using LPS or LPS-binding protein showed inconsistent results for NAFLD and NASH.[7,8,32-34] Data on bacterial translocation in human NAFLD or NASH are scarce. Recently, increased 16S gene copies and reduced bacterial diversity were reported in the blood-obtained buffy coat of patients with biopsy-confirmed liver fibrosis compared to fatty liver alone.[35] To the best of our knowledge, no paper investigating bacterial translocation to livers of patients with NAFLD or NASH has been published. We used a Western diet model for 20 weeks to induce NASH in mice.[36] The Western diet did not induce translocation of viable bacteria to mesenteric lymph nodes or increase 16S gene copies in the liver in WT mice compared with Ctrl-fed mice (data not shown). Although systemic endotoxin levels were elevated in *Reg3g*-deficient mice, this did not result in enhanced features of NASH. Alcohol-associated liver disease is dependent on increased translocation of microbial products [29,37] or viable bacteria,[19,38] while our current data indicate that increased intestinal permeability for endotoxins is not a major contributor to NAFLD disease progression. Both alcoholic liver disease and NASH are dysbiosis-driven diseases and can be transmitted by microbiota transfer.[2,39,40] It is likely that functional microbiota changes associated with dysbiosis, such as ethanol production, choline metabolism, and trimethylamine synthesis, and changes in bile acids are more important for the pathogenesis of NAFLD and

NASH progression.[41-43] With regard to our findings, changes in intestinal *Reg3g* expression in previous mouse studies applying either harmful high-fat diet or beneficial fibers [20,21,44] might be of minor meaning for the progression of NAFLD.

An interesting finding was the reduced glucose intolerance and insulin resistance in obese, FFD-fed, *Reg3b*-deficient mice. REG3B might influence the development of diabetes as it is expressed not only in the intestine but also in the pancreas, acting in pancreatic β-cell proliferation and regeneration.[45,46] Goto-Kakizaki rats that spontaneously develop diabetes showed overexpression of islet *Reg3b*.[47] This was explained as a response to the inflammation leading to islet injury.[48] In addition, human beta-cell tissues of type 2 diabetics had increased expression of human Reg family members, presumably as a response to inflammation.[49] This further supports a possible role of REG3B in islet injury. Using a high-fat diet to induce type 2 diabetes, obese mice with pancreatic β-cell-specific overexpression of REG3B had higher blood glucose levels than their obese WT controls.[50] In summary, *Reg3b* deficiency might be protective in diet-induced type 2 diabetes, as it ameliorates the inflammation leading to islet injury and subsequent decrease of insulin secretion over time.

In conclusion, deficiency of Reg3 lectins alone is not sufficient to influence the gut–liver axis in the development of NASH by facilitated bacterial translocation. *Reg3*-deficient mice do not develop more severe liver disease, and intestinal overexpression of *Reg3g* does not protect from NASH. This is in contrast to alcoholic liver disease and supports a multi-hit pathogenesis in NASH. *Reg3b* might be important for the development of type 2 diabetes, but this requires further investigation.

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Author names in bold designate shared co-first authorship.

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