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Tumor Necrosis Factor Receptor 1 Gain-of-Function Mutation Aggravates Nonalcoholic Fatty Liver Disease but Does Not Cause Insulin Resistance in a Murine Model

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Ectodomain shedding of tumor necrosis factor receptor 1 (TNFR1) provides negative feedback to the inflammatory loop induced by TNFα. As the significance of this mechanism in obesity-associated pathologies is unclear, we aimed to unravel how much TNFR1 ectodomain shedding controls the development of nonalcoholic fatty liver disease (NAFLD), as well as its role in the development of insulin resistance. We used knockin mice expressing a mutated TNFR1 ectodomain ($p55^{\Delta ns}$), incapable of shedding and dampen the inflammatory response. Our data show that persistent TNFα signaling through this inability of TNFR1 ectodomain shedding contributes to chronic low-grade inflammation, which is confined to the liver. In spite of this, hepatic lipid levels were not affected by the nonshedding mutation in mice fed a chow diet, nor were they worse off following 12 weeks of high-fat diet (HFD) than controls ($p55^{+/+}$) fed an HFD. We detected inflammatory infiltrates, hepatocellular necrosis, and apoptosis in livers of $p55^{\Delta ns/\Delta ns}$ mice fed an HFD, suggesting advanced progression of NAFLD toward nonalcoholic steatohepatitis (NASH). Indeed, fibrosis was present in $p55^{\Delta ns/\Delta ns}$ mice, but absent in wildtype mice, confirming that the $p55^{\Delta ns/\Delta ns}$ mice had a more severe NASH phenotype. Despite low-grade hepatic inflammation, insulin resistance was not observed in $p55^{\Delta ns/\Delta ns}$ mice fed a chow diet, and HFD-induced insulin resistance was no worse in $p55^{\Delta ns/\Delta ns}$ mice than $p55^{+/+}$ mice. *Conclusion*: TNFR1 ectodomain shedding is not an essential feedback mechanism in preventing the development of hepatic steatosis or insulin resistance. It is, however, pivotal in attenuating the progression from "simple steatosis" towards a more serious phenotype with many NASH features. Targeting TNFR1 could therefore be beneficial in attenuating NASH. (HEPATOLOGY 2013;57:566-576)

Driven by the obesity pandemic, nonalcoholic fatty liver disease (NAFLD) has become the main cause of chronic liver injury in Western societies. NAFLD is the hepatic component of the metabolic syndrome, a cluster of abnormalities predisposing to type 2 diabetes and cardiovascular disease.[1] NAFLD is characterized by the presence of lipid accumulation in the liver (simple steatosis). This benign

Abbreviations: ADAM17, ADAM metallopeptidase domain17; ALT, alanine aminotransferase, AST, aspartate transaminase; Bfl1, BCL2-related protein A1; Cd11b, integrin, alpha M (Mac 1); Cd68, cluster of differentiation 68; Ciap, cellular inhibitors of apoptosis; Col1a1, collagen type 1 alpha 1; HFD, high-fat diet; Il1β, interleukin-1β; Il6, interleukin-6; LDLR, low density lipoprotein receptor; MCD-diet, methionine choline-deficient diet; Mcp1, monocyte chemotactic protein-1; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; Mmp9, matrix metallopeptidase; NF-κB, nuclear factor kappa B; OGTT, oral glucose tolerance test; $p55^{\Delta NS/+}$, TNFR1 nonsheddable heterozygous mice; $p55^{\Delta NS/\Delta NS}$, TNFR1 nonsheddable homozygous mice; PBS, phosphate-buffered saline; Ppia, peptidylprolyl isomerase A (cyclophilin A); RT-PCR, real-time polymerase chain reaction; TACE, TNFα converting enzyme; TG, triglycerides; Timp1, tissue inhibitor of metalloproteinase 1; TNF, tumor necrosis factor; TNFR1, TNF receptor 1; TNFR1ns, TNFR1 nonsheddable; Traf1, TNFR-associated factor 1; wildtype mice, $p55^{+/+}$.

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and reversible state of NAFLD may, however, evolve into nonalcoholic steatohepatitis (NASH),[2] a condition of inflamed liver, which can further progress to liver fibrosis, cirrhosis, and ultimately hepatocellular carcinoma.[3]

Although the mechanisms underlying the pathogenesis of NAFLD are not fully understood, both human and animal studies support a central role for proinflammatory cytokines in the development of NASH (reviewed[4]). Tumor necrosis factor alpha (TNFα) is one of the most commonly described proinflammatory cytokines and plays a role in many types of liver injury. Increased gene expression of TNFα, its receptor TNF receptor 1 (TNFR1), and elevated levels of soluble TNFα have been reported in humans with NASH, suggesting a role for the TNFR1 pathway in its development.[5-7] However, whether the TNF system plays a causal role in the development and progression of NAFLD is still uncertain.[8-15]

There is also controversy on the importance of this inflammatory pathway in the setting of insulin resistance.[14,16-18] Several "loss-of-function" studies reported a partial protection against the development of insulin resistance in mice lacking TNFα or its receptors[16]; other studies suggested that the TNFR has no role,[17,19] or even a protective role[14] against the development of insulin resistance. Although blocking TNFα improved insulin resistance in rodents,[20] neutralizing antibodies against TNFα did not significantly improve insulin sensitivity in obese type 2 diabetic patients.[21]

Ectodomain shedding of TNFR1 provides negative feedback to the TNFα-induced inflammatory loop[22] and is therefore critical for damping the inflammatory response. The shedding is mediated by the cell surface metalloprotease TNFα-converting enzyme (TACE; also referred to as a disintegrin and metalloproteinase, ADAM17),[23] which also mediates the cleavage and release of membrane-bound pro-TNFα into the 17 kDa soluble form of TNFα.[24] Increased TACE activity in *Timp3−/−* (tissue inhibitor of metalloproteinase 3) mice contributes to hepatic steatosis and insulin resistance,[25] whereas treatment with the TACE-inhibitor Marimastat reverses hepatic steatosis and improves insulin resistance in ob/ob mice.[26] Moreover, mice heterozygous for TACE are protected from obesity-induced insulin resistance,[27] implying that TACE-mediated shedding plays a role in the pathogenesis of NAFLD and insulin resistance. However, TACE provides the broad sheddase activity required for ectodomain cleavage of a greater number of growth factors, cell adhesion molecules, cytokines, and cytokine receptors.[28] This makes it extremely difficult to delineate which shedding events are involved in obesity-associated pathologies.

Because the biological significance of TNFR1 shedding in NAFLD and insulin resistance was unclear, we aimed to unravel the extent to which it controls the initiation of NAFLD and the progression towards NASH, as well as its role in the development of insulin resistance. We used knockin mutant mice expressing nonsheddable TNFR1s ($p55^{\Delta ns}$ mice), which have been shown to exhibit persistent expression of the receptor at the cell surface. This dominant mutation leads to a spontaneous inflammatory response resulting in enhanced antibacterial host defenses, increased susceptibility to endotoxic shock, exacerbated TNF-dependent arthritis, and in a mild form of chronic hepatitis.[29]

Using this gain-of-function approach, we demonstrated that the inability of TNFR1 shedding did not result in obesity, insulin resistance, or hepatic steatosis in mice. However, $p55^{\Delta ns}$ mice showed a rapid progression towards NASH. Our data therefore suggest that activation of TNFR1 ectodomain shedding does not safeguard against the development of hepatic steatosis, obesity, or insulin resistance, although it is pivotal in attenuating the progression towards NASH.

Materials and Methods

Mice and Diet Intervention. Mice containing the TNFR1 nonsheddable mutation heterozygously and homozygously (referred to as $p55^{\Delta ns/+}$ and $p55^{\Delta ns/\Delta ns}$ mice, respectively) and their wildtype littermates ($p55^{+/+}$)[29] in a C57Bl/6 background were purchased from the European Mouse Mutant Archive (EMMA, Monterotondo Scalo, Italy) and crossed into a C57Bl/6 background for at least 10 generations. Mice were housed individually in a temperature- and light-controlled facility with *ad libitum* food and water. All

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experiments were performed according to Dutch law and approved by the Ethical Committee for Animal Experiments, University of Groningen, the Netherlands.

Age-matched mice (8-10 weeks) were fed either a high-fat diet (HFD) containing 36% fat from lard and 0.03% cholesterol (4031.45, Abdiets, Woerden, the Netherlands) or a regular chow diet (2181, RMH-B Arie Blok, Woerden, the Netherlands) for 12 weeks. All mouse experiments were carried out simultaneously, using diets from the same batch numbers. The mice were fasted for 4 hours before being given an intraperitoneal injection with saline or human recombinant insulin Actrapin (Novo Nordisk Canada, Ontario, Canada) (0.75 U/kg) 15 minutes before sacrifice. Tissues were isolated and snap-frozen in liquid nitrogen and stored either at −80°C, fixed in 4% paraformaldehyde, or stored in paraffin.

Nuclear Factor Kappa B (NF-κB) DNA Binding. Nuclear extracts were prepared from fresh liver tissue according to the manufacturer's protocol and binding of NF-κB to DNA was determined using the p65 TransAM enzyme-linked immunosorbent assay (ELISA) kit (Active Motif, La Hulpe, Belgium).

Lipid Analysis. Lipid extraction was performed as described.[30]

Oral Glucose Tolerance Test (OGTT). Mice were fasted 9 hours overnight and given 2 g/kg of a 20% glucose solution orally. Glucose levels were measured with a One Touch Ultra glucose meter before and 15, 30, 45, 90, and 120 minutes after the glucose administration. Insulin levels were determined with ALPCO immunoassays (ALPCO Diagnostics, Salem, NH) according to the manufacturer's instructions.

Liver Histology. Paraffin-embedded sections of the liver (4 μm) were stained with hematoxylin-eosin (H/E), Masson's Trichrome staining, or Oil Red O. Frozen-cut liver sections (5 μm) were fixed in 4% paraformaldehyde and stained with antibodies against Cd68 and Cd11b (Abcam, Cambridge, UK). We used a Leica DM 3000 microscope. Scoring of steatosis and ballooning of hepatocyte degeneration and inflammatory foci was done by a certified veterinary pathologist and based on a described method.[31]

Immunoblot Analysis. Tissues were homogenized and equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and the immune-complex was visualized using the molecular imager ChemiDoc xrs+ system (Bio-Rad).

Determination of Transaminases. The level of aspartate transaminase (AST) and alanine transaminase (ALT) plasma was measured according to the manufacturer's protocol (Spinreact, Santa Coloma, Spain).

Caspase 3 Activity Assay. Equal amounts of liver (300 μg) were incubated with Ac-DEVD-AMC Caspase-3 fluorogenic substrate (BD Pharmingen, San Jose, CA) at 37°C for 60 minutes. The amount of cleaved fluorescent AMC was quantified by a spectrofluometer at an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

Statistical Analysis. Data were statistically analyzed by performing a nonparametric Mann-Whitney test using GraphPad Prism to compare experimental groups (v. 5.00 for Windows, San Diego, CA). Data were expressed as mean ± standard error of the mean (SEM) and considered significant at $P < 0.05$.

Results

Gain-of-Function Mutation in TNFR1 Contributes to Liver but Not Adipose Tissue Inflammation in Mice. As mice harboring the knockin mutation in the TNFR1 are expected to display an inflammatory phenotype, we first evaluated inflammatory parameters in the liver, adipose tissue, and blood of $p55^{+/+}$, $p55^{\Delta ns/+}$, and $p55^{\Delta ns/\Delta ns}$ mice fed a regular chow diet. The livers of $p55^{\Delta ns/+}$ and $p55^{\Delta ns/\Delta ns}$ mice showed multifocal enhanced infiltration of macrophages, neutrophils, and lymphocytes within the hepatic lobules (Fig. 1A), as described.[29] Immunostaining for Cd68 and Cd11b confirmed the higher numbers of macrophages in livers of $p55^{\Delta ns/+}$ and $p55^{\Delta ns/\Delta ns}$ mice compared to $p55^{+/+}$ mice (Fig. 1A). In addition, the expression of genes encoding for proteins involved in macrophage infiltration (Ccl2, also known as Mcp1 and Cd68) and proinflammatory cytokines (interleukin (Il)6, Il1β, and Tnfα) was elevated in livers from $p55^{\Delta ns/\Delta ns}$ compared to $p55^{+/+}$ mice, with intermediate gene expression seen in $p55^{\Delta ns/+}$ mice (Fig. 1B). To determine the level of hepatic inflammation in $p55^{\Delta ns/\Delta ns}$ mice, C57Bl/6 mice were injected with TNFα and sacrificed after 1 or 2 hours. Hepatic expression of Tnfα, Mcp1, Il1β, and Il6 was drastically increased in mice subjected to TNFα treatment compared to phosphate-buffered saline (PBS) controls (Supporting Fig. 1A-D). In addition, hepatic inflammation induced by the incapability of TNFR1 ectodomain shedding was considerably lower than after the acute treatment of TNFα in C57Bl/6 mice (Supporting Fig. 1A-D), indicating that $p55^{\Delta ns}$ mice exhibit a chronic, low-grade inflammatory state in the liver.

Despite this chronic inflammation, we saw no differences in body weight (Fig. 1C), adipocyte size

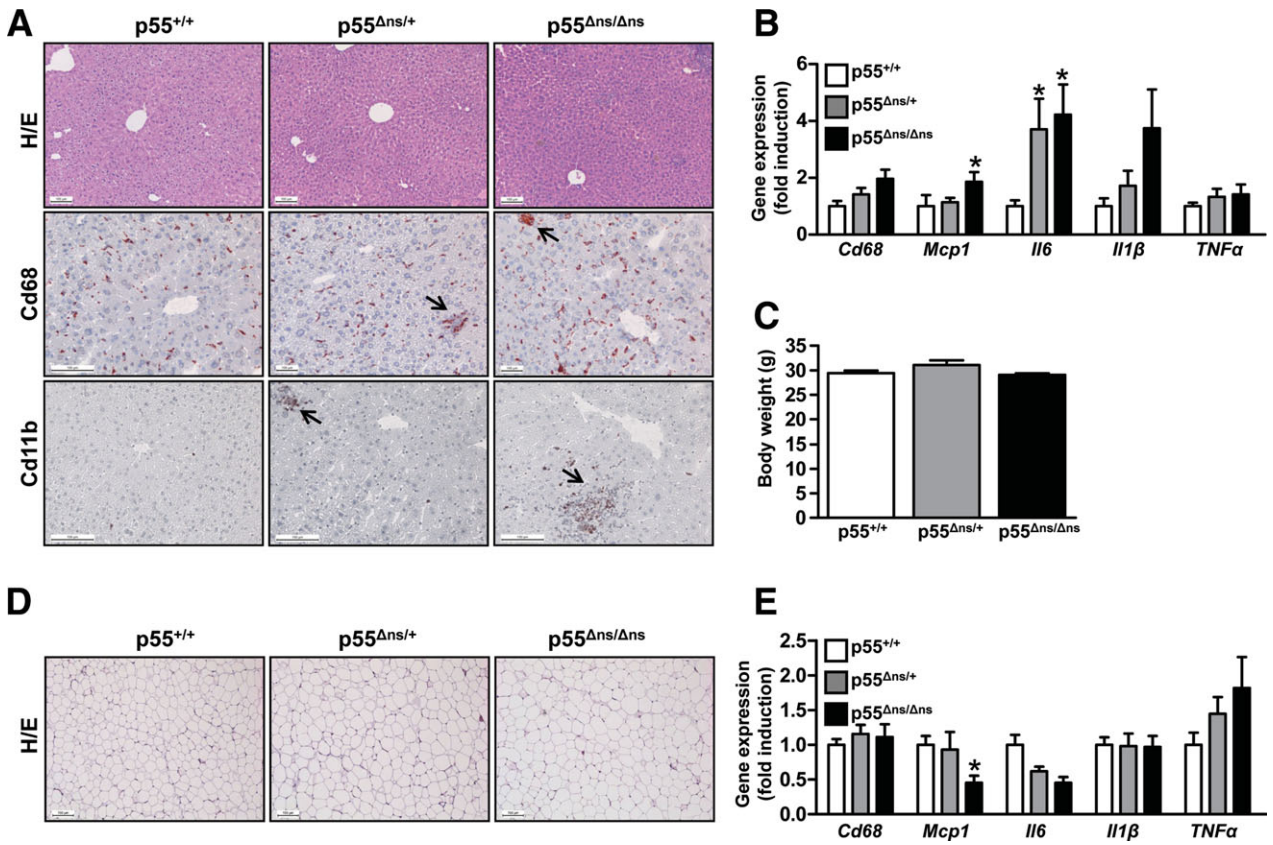

Fig. 1. Gain-of-function mutation in TNFR1 contributes to liver but not adipose tissue inflammation in mice. (A) Representative pictures of H/E, Cd68, and Cd11b-stained liver sections were taken from mice fed a normal chow diet (arrows indicate inflammatory infiltrates). (B) Gene expression in whole liver of Cd68, monocyte chemotactic protein-1 (Mcp1), interleukin-6 (Il6), interleukin-1β (Il1β), and tumor necrosis factor α (Tnfα) was determined by real-time polymerase chain reaction (RT-PCR) and expressed as fold induction versus $p55^{+/+}$. (C) Body weights after a normal chow diet. (D) Representative pictures of H/E-stained adipose tissue sections were taken from mice fed a chow diet. (E) Gene expression in white adipose tissue of Cd68, Mcp1, Il6, Il1β, and Tnfα was determined by RT-PCR and expressed as fold induction versus $p55^{+/+}$. (open square) = $p55^{+/+}$ mice, (grey square) = $p55^{\Delta ns/+}$ mice, and (black square) = $p55^{\Delta ns/\Delta ns}$ mice. N = 6. All error bars represent the mean ± SEM. *$P < 0.05$ versus $p55^{+/+}$.

(Fig. 1D), or adipocyte number (data not shown) in mice harboring the TNFR1 mutation compared to wildtype controls. Moreover, crown-like structures (dead adipocytes surrounded by macrophages) were not apparent in adipose tissue from $p55^{\Delta ns/+}$ and $p55^{\Delta ns/\Delta ns}$ mice (Fig. 1D), suggesting the absence of adipose tissue inflammation. No up-regulation in expression of proinflammatory genes and macrophage genes was seen in $p55^{\Delta ns/+}$ and $p55^{\Delta ns/\Delta ns}$ mice compared to $p55^{+/+}$ mice. Consistent with this, the protein levels of Il1β and Tnfα were not increased in $p55^{\Delta ns/\Delta ns}$ mice compared to $p55^{+/+}$ mice (Supporting Fig. 2A). In blood, cytokines were not increased (Supporting Fig. 2B), suggesting that the TNFR1 nonsheddable knockin mutation contributes to a more serious liver phenotype in mice.

Inability of TNFR1 Shedding Does Not Initiate Hepatic Steatosis and NAFLD. To assess whether defective TNFR1 shedding in hepatocytes results in increased TNFα signaling response, hepatocytes were isolated from wildtype and $p55^{\Delta ns/\Delta ns}$ mice and stimulated with TNFα (10 ng/mL) for 6 hours. As expected, Tnfα (Fig. 2A), Il1β (Fig. 2B), and Il6 (Fig. 2C) gene expression were dramatically increased in TNFα-stimulated $p55^{\Delta ns/\Delta ns}$ hepatocytes compared to the wildtype. Because TNFα has been shown to mediate lipid metabolism[32] and has been proposed to initiate hepatic steatosis,[8,33] we next assessed whether lipid accumulation was affected in male $p55^{\Delta ns/+}$ and $p55^{\Delta ns/\Delta ns}$ mice. Consistent with Oil Red O staining of liver sections (Fig. 2D), we saw no difference in hepatic triglyceride (TG) content between the mice (Fig. 2E). Moreover, 12 weeks of high-fat feeding did not exacerbate hepatic lipid levels in $p55^{\Delta ns/+}$ and $p55^{\Delta ns/\Delta ns}$ mice compared to littermate controls (Fig. 2D,E). The zonal distribution and severity of the microvesicular steatosis and hepatocyte ballooning did not differ between the genotypes (Fig. 2F). Although 12 weeks of high-fat feeding clearly increased body weight and raised plasma TG and cholesterol levels compared to a

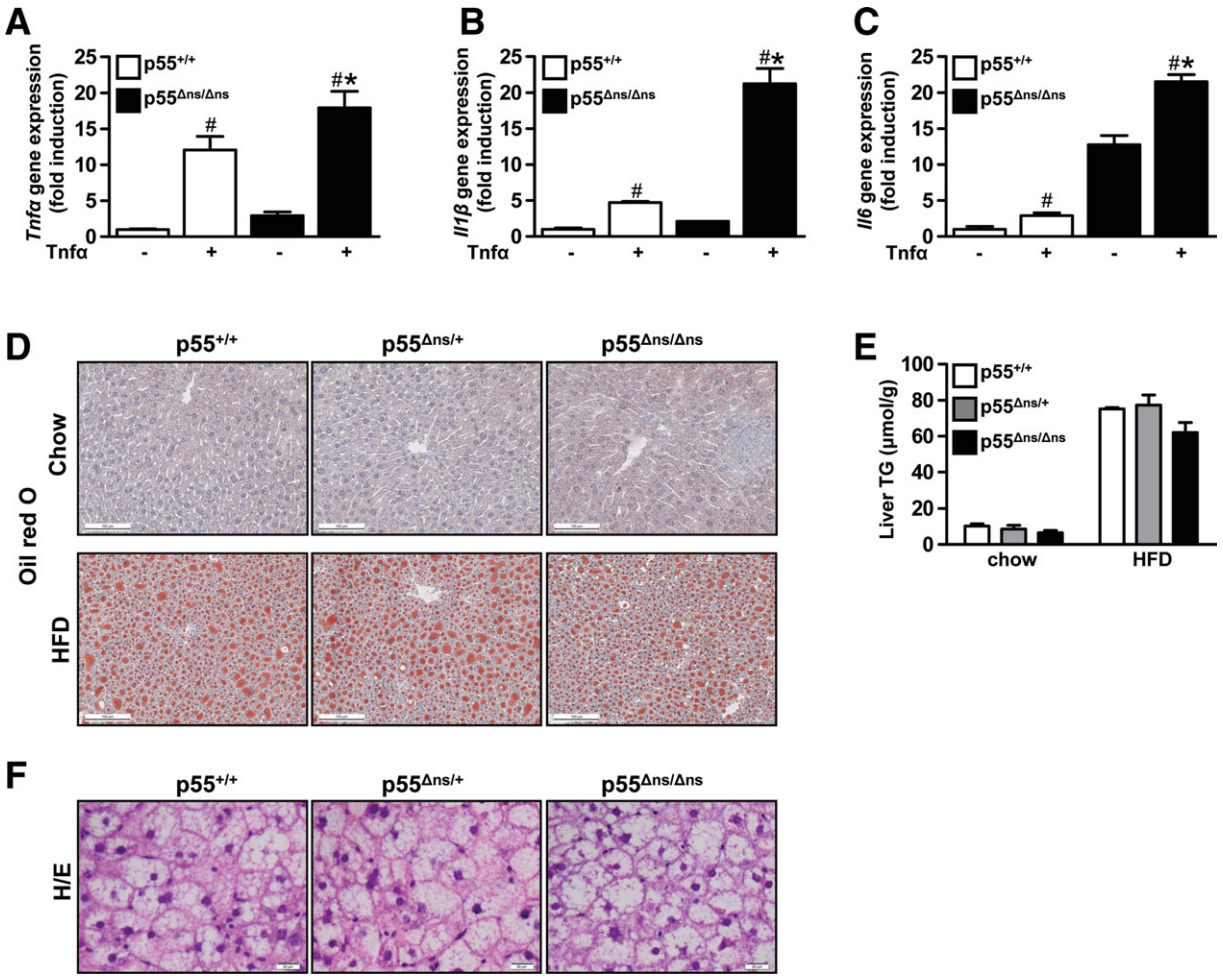

Fig. 2. Inability of TNFR1 shedding does not initiate hepatic steatosis and NAFLD. (A) Primary hepatocytes from $p55^{+/+}$ and $p55^{\Delta ns/\Delta ns}$ mice were stimulated for 6 hours with 10 ng/mL TNFα, after which inflammatory gene expression of (A) tumor necrosis factor-alpha (Tnfα), (B) interleukin-1β (Il1β), and (C) interleukin-6 (Il6) was measured. N = 3 per condition; data shown are representative of three independent experiments. (D) Representative pictures of Oil Red O staining after chow diet and 12 weeks of HFD. (E) Liver triglycerides (TG) after chow diet and 12 weeks of HFD. (F) Representative pictures of H/E stainings after 12 weeks HFD. (open square) = $p55^{+/+}$ mice, (grey square) = $p55^{\Delta ns/+}$ mice and (black square) = $p55^{\Delta ns/\Delta ns}$ mice. N = 6. All error bars represent the mean ± SEM. $^{*}P < 0.05$ versus $p55^{+/+}$; $^{\#}P < 0.05$ versus non-TNF-stimulated control.

normal chow diet, we saw no differences between the genotypes (Supporting Fig. 3A-C). Taken together, our data suggest that the inability to shed TNFR1, leading to liver inflammation, is not a critical factor in the induction of hepatic steatosis or NAFLD.

Inflammatory Infiltrations, Hepatocellular Necrosis, and Apoptosis in HFD-Fed $p55^{\Delta ns}$ Mice. Although the inability to shed TNFR1 may not be involved in the initial stage of NAFLD, it may still drive the progression from "simple steatosis" towards NASH (a more severe stage of NAFLD). We therefore investigated the steatotic livers of HFD-fed mice for inflammation, necrosis, and apoptosis. Following 12 weeks of high-fat feeding, the livers of $p55^{\Delta ns/+}$ and $p55^{\Delta ns/\Delta ns}$ mice clearly displayed more lobular inflammation than those of $p55^{+/+}$ mice (Fig. 3A), as well as larger inflammatory foci, covering an area of up to 20-30 hepatocytes in $p55^{\Delta ns/\Delta ns}$ mice (Fig. 3A, bottom panel). The foci are composed of macrophages, neutrophils, and lymphocytes. Moreover, enhanced clusters of inflammatory infiltrates were confirmed by macrophage Cd68 and Cd11b staining in $p55^{\Delta ns/+}$ and $p55^{\Delta ns/\Delta ns}$ mice compared to wildtype mice fed an HFD (Fig. 3B,C), contributing to an overt inflammatory phenotype in mice harboring the TNFR1 nonsheddable knockin mutation. This phenotype was associated with a gradual increase in DNA binding of the NF-κB subunit p65 (Fig. 3D) and elevated inflammatory gene expression (Fig. 3E) in livers from $p55^{\Delta ns/+}$ and $p55^{\Delta ns/\Delta ns}$ mice compared to wildtype controls.

$P55^{\Delta ns/\Delta ns}$ mice also displayed increased hepatocellular apoptosis and necrosis (Fig. 3A lower panel). Apoptosis in $p55^{\Delta ns/+}$ and $p55^{\Delta ns/\Delta ns}$ mice was confirmed by the detection of an increased protein

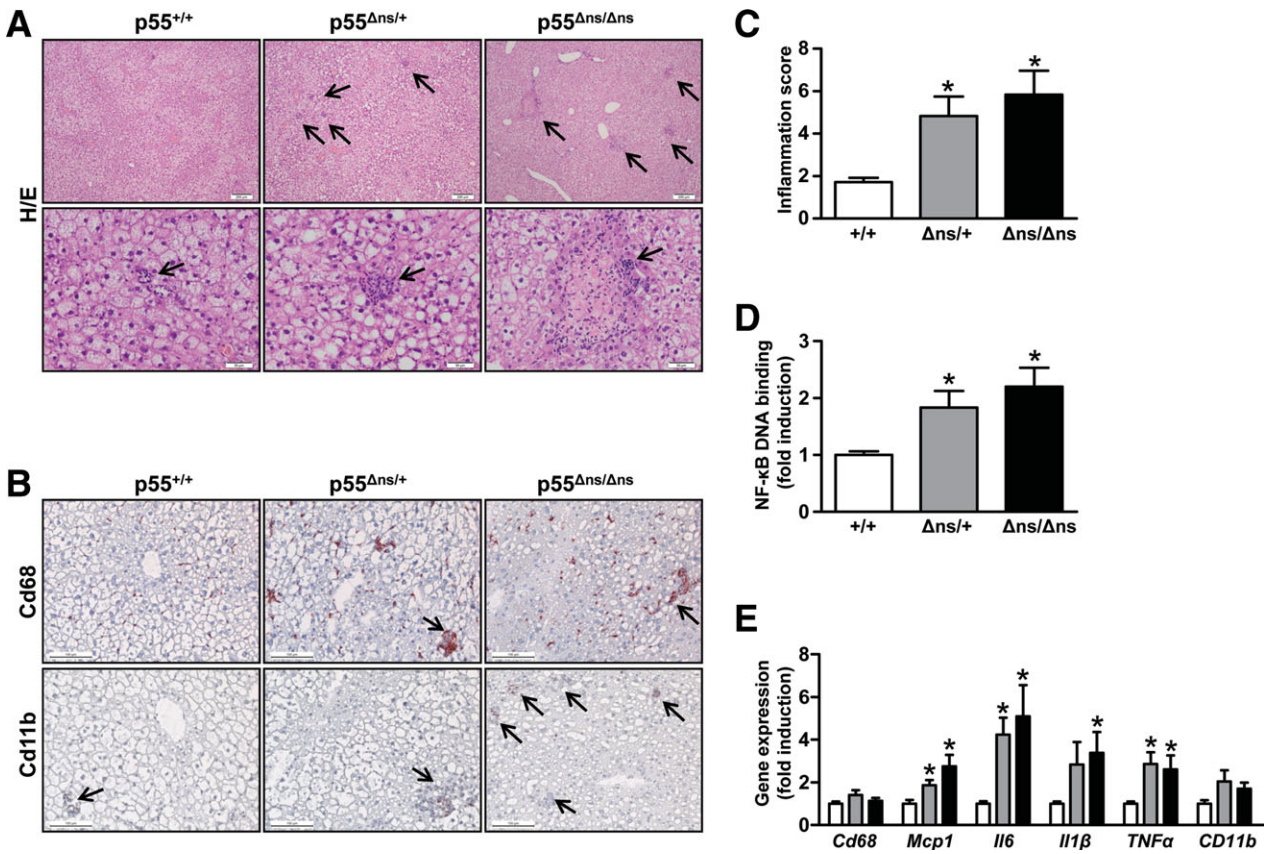

Fig. 3. Increased inflammatory infiltrations in HFD-fed $p55^{\Delta ns}$ mice. (A) Representative pictures of H/E stainings, with arrows indicating inflammatory foci. (B) Representative pictures of immunohistochemistry stainings with CD68 and CD11b, with arrows indicating inflammatory infiltrates. (C) Inflammation score was determined by counting the inflammatory foci per five random fields at 200×. (D) Nuclear extracts from liver were prepared and assessed by an ELISA kit for DNA-binding activity to a consensus NF-κB oligonucleotide and expressed as fold induction versus $p55^{+/+}$. (E) Hepatic gene expression of Cd68, monocyte chemotactic protein-1 (Mcp1), interleukin-6 (Il6), interleukin-1β (Il1β), tumor necrosis factor-alpha (Tnfα), and Cd11b was determined by RT-PCR and expressed as fold induction versus $p55^{+/+}$. (open square) = $p55^{+/+}$ mice, (grey square) = $p551^{\Delta ns/+}$ mice, and (black square) = $p55^{\Delta ns/\Delta ns}$ mice. +/+ = $p55^{+/+}$, Δns/+ = $p55^{\Delta ns/+}$, and Δns/Δns = $p55^{\Delta ns/\Delta ns}$. N = 6. All error bars represent the mean ± SEM. *$P < 0.05$ versus $p55^{+/+}$.

abundance of cleaved caspase 3 (Fig. 4A) and of higher caspase 3 activity compared to $p55^{+/+}$ mice (Fig. 4B). The presence of apoptosis was paralleled by an up-regulation of the antiapoptotic genes cellular inhibitors of apoptosis 1 and 2 (Ciap1 and Ciap2), BCL2-related protein A1 (Bfl1), and TNFR-associated factor 1 (Traf1) (Fig. 4C). The levels of transaminases ALT and AST, surrogate markers for liver damage, did not differ between the three genotypes (Fig. 4D).

Inability of TNFR1 Shedding Initiates a Liver Fibrosis Phenotype in HFD-Fed Mice. To investigate if the greater inflammation, apoptosis, and necrosis in $p55^{\Delta ns}$ mice were accompanied by increased hepatic fibrosis, a predominant feature of advanced NASH, we assessed the livers of mice fed an HFD for 12 weeks for collagen deposition. Whereas fibrosis was absent in wildtype mice, we saw mild fibrosis at inflammatory foci in $p55^{\Delta ns/\Delta ns}$ mice, as detected and quantified by Masson's Trichrome staining of liver sections (Fig. 4E). In line with this, the hepatic expression of some profibrotic genes (collagen type 1 alpha 1 (Col1a1), matrix metallopeptidase (Mmp9), and tissue inhibitor of metalloproteinase 1 (Timp1)) was elevated in $p55^{\Delta ns/\Delta ns}$ mice compared to wildtype controls fed an HFD (Fig. 4F).

Together, these data demonstrate an aggravation from "simple steatosis" towards a more severe NASH phenotype due to the defective TNFR1 shedding. It included not only inflammation, necrosis, and apoptosis, but also fibrosis.

Female Mice Are More Susceptible than Male Mice for TNFR1-Induced Fibrosis. Because it has been shown that male and female mice can exhibit differences in severity of NASH,[34,35] we also investigated female mice with the TNFR1 nonsheddable mutation. In all genotypes, the female mice showed less steatosis and ballooning than the males. In line with the males, $p55^{\Delta ns/\Delta ns}$ females showed increased inflammation, apoptosis, and necrosis versus $p55^{+/+}$ mice (Fig. 5A-D).

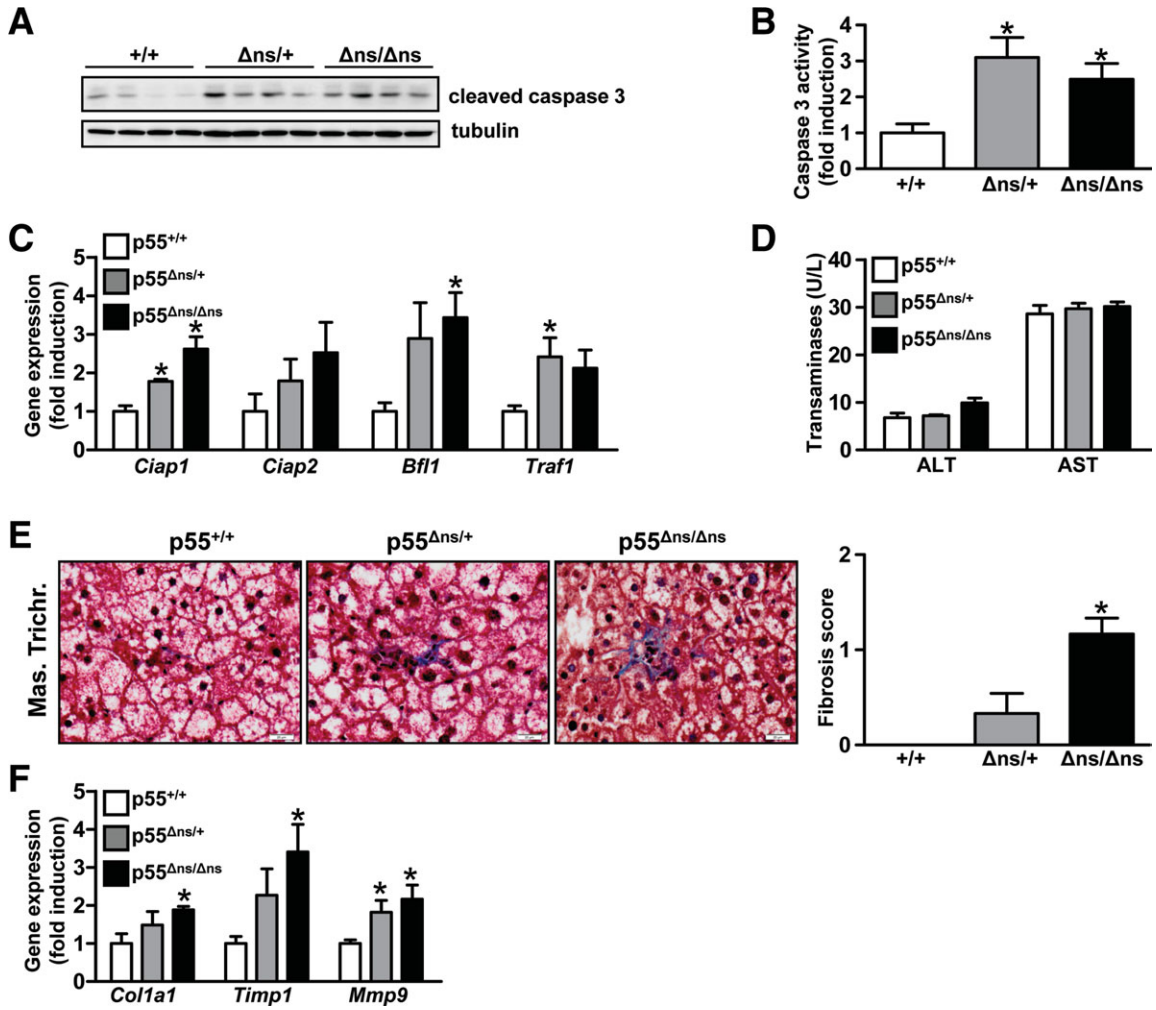

Fig. 4. Inability of TNFR1 shedding initiates hepatocellular necrosis, apoptosis and a liver fibrosis phenotype in HFD-fed mice. (A) Cleaved caspase 3 was determined by western blot analysis. (B) Caspase 3 cleavage activity was determined as described in Materials and Methods and expressed as fold induction versus $p55^{+/+}$. (C) Antiapoptotic gene expression (cellular inhibitors of apoptosis 1 and 2 (Ciap1 and Ciap2), BCL2-related protein A1 (Bfl1), and TNFR-associated factor 1 (Traf1)) was determined by RT-PCR and expressed as fold induction versus $p55^{+/+}$. (D) ALT levels in serum and AST levels in blood. (E) Representative pictures of Masson's Trichrome stainings are shown and fibrosis score. Stainings were categorized as 0 (no fibrosis), 1 (minimal fibrosis), or 2 (mild fibrosis). (F) Relative gene expression of the profibrotic genes Collagen 1a1 (Col1a1), tissue inhibitor of metalloproteinase 1 (Timp1) and metalloporoteinase-9 (Mmp9) were measured. (open square) = $p55^{+/+}$ mice, (grey square) = $p55^{\Delta ns/+}$ mice and (black square) = $p55^{\Delta ns/\Delta ns}$ mice. +/+ = $p55^{+/+}$, Δns/+ = $p55^{\Delta ns/+}$, and Δns/Δns = $p55^{\Delta ns/\Delta ns}$. N = 6. All error bars represent the mean ± SEM. *$P < 0.05$ versus $p55^{+/+}$.

The elevated fibrogenic phenotype detected and quantified in female $p55^{\Delta ns/\Delta ns}$ mice by Masson's Trichrome staining (Fig. 5A, lower panel, and Fig. 5E) was more severe than in male $p55^{\Delta ns/\Delta ns}$ mice (dashed line in Fig. 5E). Gene expression confirmed the NASH phenotype in female $p55^{\Delta ns/\Delta ns}$ mice fed an HFD (Fig. 5F).

These data demonstrate a greater susceptibility in female $p55^{\Delta ns/\Delta ns}$ mice than in the males for TNFR1-induced fibrosis.

Inability of TNFR1 Shedding Does Not Induce or Aggravate Insulin Resistance. Because NASH and insulin resistance are associated pathologies,[3] we tested whether the chronic, low-grade hepatitis seen in $p55^{\Delta ns/\Delta ns}$ mice contributed to the development of insulin resistance. In contrast to our expectation, $p55^{\Delta ns/\Delta ns}$ mice on a normal chow diet did not exhibit elevated fasting blood glucose levels nor was their glucose tolerance negatively affected (Fig. 6A). Furthermore, there was no difference between genotypes in the insulin levels obtained during the OGTT (Fig. 6B), and the mice remained insulin-sensitive compared to wildtype controls (data not shown), suggesting that the incapacity of shedding of TNFR1 does not result in insulin resistance. HFD-induced insulin resistance was not aggravated in $p55^{\Delta ns/\Delta ns}$ mice, as we saw no differences between genotypes for fasting blood glucose, glucose tolerance, or insulin levels during the

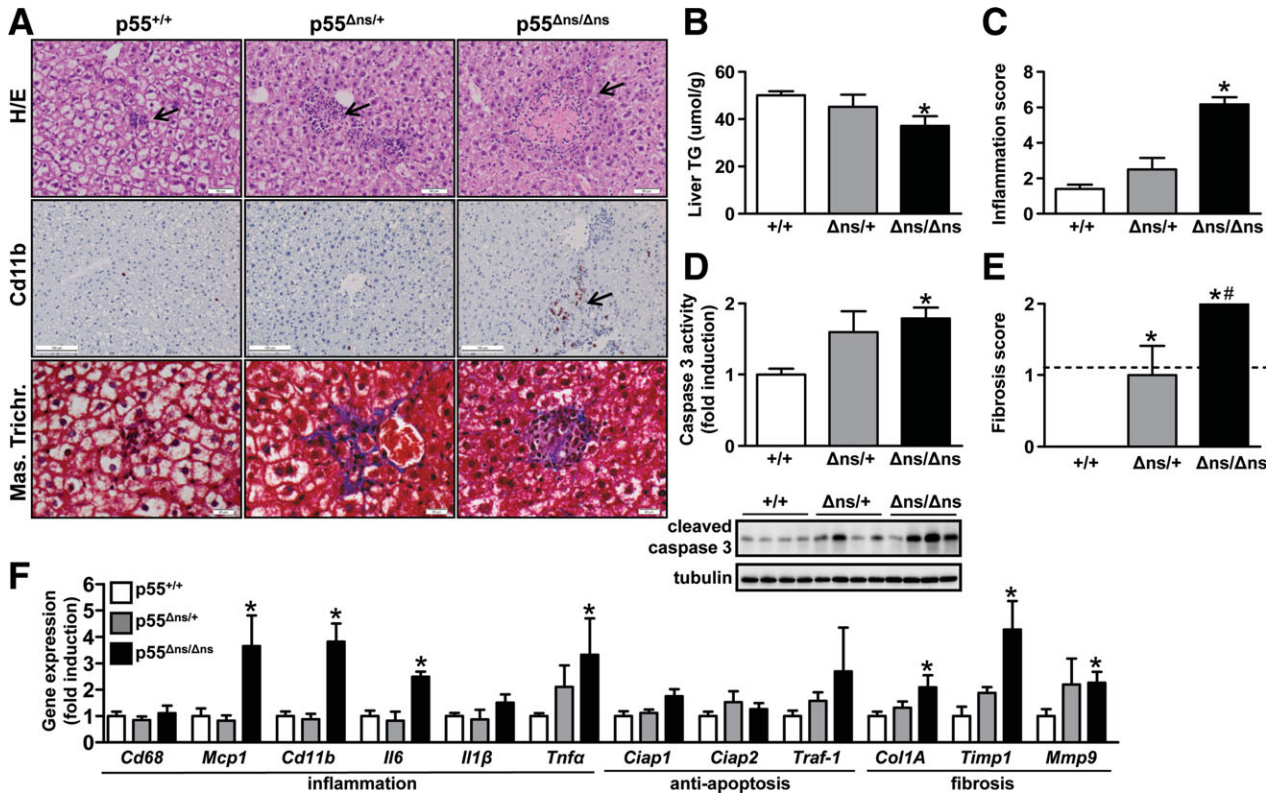

Fig. 5. Female mice are more susceptible than male mice to TNFR1-induced fibrosis. (A) Representative pictures of H/E, Cd11b, and Masson's Trichrome stainings, with arrows indicating inflammatory foci. (B) Liver triglycerides (TG) were quantified biochemically. (C) Inflammation score was determined by counting the inflammatory foci per five random fields at 200×. (D) Cleaved caspase 3 was determined by western blot analysis and caspase 3 cleavage activity was determined as described (Materials and Methods) and expressed as fold induction versus $p55^{+/+}$. (E) Fibrosis score, stainings were categorized as 0 (no fibrosis), 1 (minimal fibrosis), or 2 (mild fibrosis). The dashed line represents the level of fibrosis in $p55^{\Delta ns/\Delta ns}$ male mice. (F) Hepatic expression of proinflammatory, antiapoptotic, and profibrotic genes was determined by RT-PCR and expressed as fold induction versus $p55^{+/+}$. (open square) = $p55^{+/+}$ mice, (grey square) = $p55^{\Delta ns/+}$ mice and (black square) = $p55^{\Delta ns/\Delta ns}$ mice. +/+ = $p55^{+/+}$, Δns/+ = $p55^{\Delta ns/+}$, and Δns/Δns = $p55^{\Delta ns/\Delta ns}$. N = 6. All error bars represent the mean ± SEM. *$P < 0.05$ versus $p55^{+/+}$. #$P < 0.05$ versus male $p55^{\Delta ns/\Delta ns}$ mice.

OGTT (Fig. 6A,B). Hepatic insulin sensitivity was not affected in $p55^{\Delta ns/\Delta ns}$ mice on chow (Fig. 6C) or HFD (Fig. 6D) compared to wildtype mice, measured by the phosphorylation status of Protein Kinase B (Akt) in livers of saline- and insulin-injected $p55^{+/+}$ and $p55^{\Delta ns/\Delta ns}$ mice. The messenger RNA (mRNA) levels of phosphoenolpyruvate carboxykinase and glucose-6-phosphate, the rate-limiting enzymes in gluconeogenesis, were also not increased in $p55^{\Delta ns/\Delta ns}$ mice compared to wildtype controls, suggesting there was no hepatic insulin resistance in $p55^{\Delta ns/\Delta ns}$ mice (Fig. 6E,F).

Discussion

There is growing evidence connecting obesity and its associated pathologies, like NAFLD and insulin resistance, to the presence of a chronic low-grade inflammatory state.[36,37] We aimed to gain more insight into the biological significance of shedding of the TNFR1 ectodomain in these pathologies by studying the extent to which ectodomain shedding of the TNFR1 controls the initiation and progression of NAFLD towards NASH and the development of insulin resistance. Using knockin mice expressing a mutated nonsheddable TNFR1,[29] we demonstrated for the first time that ectodomain shedding of the TNFR1 is not an essential feedback mechanism in preventing the development of hepatic steatosis and insulin resistance. However, this mechanism of the TNFα-inflammatory loop is pivotal for protecting against the transition from "simple steatosis" towards NASH.

We have shown that $p55^{\Delta ns/\Delta ns}$ mice on a normal chow diet do not develop hepatic steatosis, despite increased hepatic inflammation (Fig. 2E). Moreover, 12 weeks of HF feeding did not exacerbate hepatic lipid levels, nor alter the zonal distribution or severity of microvesicular steatosis in $p55^{\Delta ns/\Delta ns}$ mice compared to controls (Fig. 2D-F), suggesting that shedding of TNFR1 does not prevent the development of hepatic steatosis. It was known that the shedding of

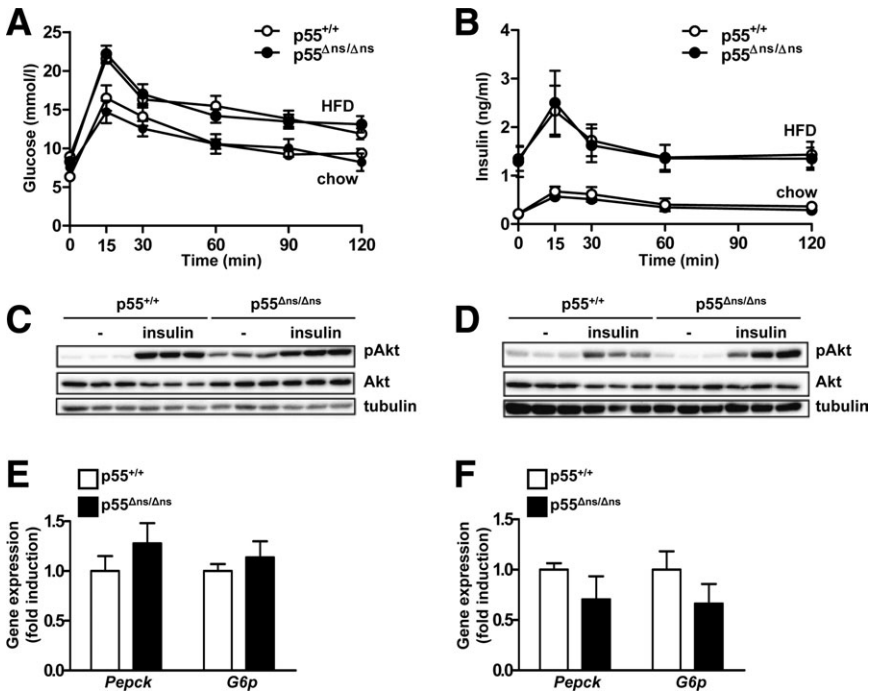

Fig. 6. Inability of TNFR1 shedding does not induce or aggravate insulin resistance. At several timepoints during the performance of an OGTT, plasma glucose (A) and plasma insulin concentrations (B) were measured in chow-fed and HFD-fed $p55^{+/+}$ and $p55^{\Delta ns/\Delta ns}$ mice. Chow-fed (C) and HFD-fed (D) $p55^{+/+}$ and $p55^{\Delta ns/\Delta ns}$ mice were injected with saline or insulin (0.75 U/g) 15 minutes prior to sacrifice, after which phosphorylation status of Akt was determined by western blot. Representative blots are shown. Gene expression of phosphoenolpyruvate carboxykinase (Pepck) and glucose 6 phosphate (G6p) was determined by RT-PCR from livers of chow-fed (E) and HFD-fed (F) $p55^{+/+}$ and $p55^{\Delta ns/\Delta ns}$ mice and expressed as fold induction versus $p55^{+/+}$. (open circle/square) = $p55^{+/+}$ mice and (filled circle/square) = $p55^{\Delta ns/\Delta ns}$ mice. N = 6. All error bars represent the mean ± SEM. *$P < 0.05$ versus $p55^{+/+}$.

TNFR1 ectodomains attenuates the inflammatory response induced by TNFα,[23] but our data now show that persistent TNFR1 signaling is not involved in the initiation of NAFLD. Consistent with this, mice with a genetic deletion of TNFα or TNFR1 are not protected against developing obesity-induced hepatic steatosis.[10,13,14] However, TNFα has been shown to be a potent lipid metabolism regulator[38] and many studies in rodents have described a role for TNFα in the development of hepatic steatosis.[8,33] Most of these have studied the effects of TNFα within 24 hours of a high dose of human recombinant TNFα. Although administration of TNFα induces acute hepatitis, it does not mimic the chronic low-grade inflammation associated with obesity. The inflammatory gene expression seen in livers from $p55^{\Delta ns/\Delta ns}$ mice was approximately 10- to 100-fold lower than that seen after a single injection with TNFα (Supporting Fig. 1); it thus led to a more physiologically relevant situation of chronic low-grade hepatic inflammation in our study.

Although our data do not support a role for shedding of TNFR1 in the initiation of steatosis, we did see an advanced NASH-like phenotype in the livers of $p55^{\Delta ns/\Delta ns}$ mice fed an HFD compared to wildtype mice. This included the presence of inflammatory infiltrates, apoptotic hepatocytes, and large areas of hepatocellular necrosis surrounded by neutrophils and lymphocytes (Fig. 3A,B). Because our data indicated an important role for TNFR1 in the progression of NAFLD towards NASH, we investigated the effect of ectodomain shedding of TNFR1 on hepatic fibrosis, an advanced hallmark of NASH. $P55^{\Delta ns/\Delta ns}$ mice demonstrated increased levels of collagen staining, as detected by Masson's Trichrome staining (Fig. 4E). The presence of hepatic fibrosis was confirmed by an up-regulation of fibrotic gene expression in $p55^{\Delta ns/\Delta ns}$ mice compared to wildtype mice (Fig. 4F). Moreover, a recent study with cultured hepatic stellate cells deficient for TNFR1 or TNFR2, or both TNFR1 and TNFR2,[12] confirmed a critical role for TNFR1 in the development of liver fibrosis. Notably, the level of fibrosis observed in $p55^{\Delta ns/+}$ mice was significantly lower compared to $p55^{\Delta ns/\Delta ns}$ mice, being an exception of the dominant nature of the TNFR1 mutation. The reason for this is still unclear.

Elevated levels of the liver enzymes ALT and AST are often used as surrogate markers for advanced liver injury. However, ALT levels are persistently normal in more than half of the patients with NAFLD and biopsy-proven NASH,[39] suggesting that the presence of NASH does not necessarily correlate with higher levels of these liver transaminases. In line with this, we demonstrated that AST and ALT levels were not increased in HFD-fed $p55^{\Delta ns/\Delta ns}$ mice despite the existence of NASH. A NASH-like phenotype without overt changes in liver enzymes has also been observed in the low-density lipoprotein receptor (LDLR) knockout mice fed a high fat cholesterol diet.[40] As elevated levels of circulating liver enzymes are a prerequisite for patients to undergo a liver biopsy,[3,39] we urgently need better, non-invasive methods to assess hepatic inflammation and fibrosis and properly diagnose disease severity.

As chronic low-grade inflammation has a broad role in driving the pathogenesis of systemic insulin resistance,[37] we assessed whether hepatic inflammation in $p55^{\Delta ns/\Delta ns}$ mice was associated with the development of hepatic or systemic insulin resistance. We found no signs of glucose intolerance or hepatic insulin resistance in $p55^{\Delta ns/\Delta ns}$ mice fed a chow diet compared to littermate controls (Fig. 6A,C,E). Insulin resistance developed readily in mice fed an HFD for 12 weeks, but was no worse in $p55^{\Delta ns/\Delta ns}$ mice with the nonshedding mutation (Fig. 6B,D,F). Furthermore, older $p55^{\Delta ns/\Delta ns}$ mice fed a chow diet for 1 year were not prone to developing insulin resistance (data not shown), nor did 12 weeks of HFD starting at the age of 1 year accelerate the development of insulin resistance in $p55^{\Delta ns/\Delta ns}$ mice compared to control mice (data not shown). Our data therefore indicate that TNFR1 signaling is not essential for the development of insulin resistance in mice. As several reports have raised doubts on the importance of the TNFR1, TNFR2, and TNFα signaling in contributing to obesity-induced insulin resistance,[14,17,19] our data provide yet another piece of evidence against the prevailing concept that the TNFα pathway mediates HFD-induced insulin resistance in the obesity research field. Moreover, ob/ob mice lacking TNFα/TNFR-function are only partly protected from obesity-induced insulin resistance,[16] which also suggests that other pathways play an important role in its development. Obesity, insulin resistance, hepatic steatosis, and adipose tissue remodeling induced by an HFD was markedly improved by systemic deletion of TACE,[41] the enzyme responsible for ectodomain cleavage of TNFR1. However, TACE is required for the shedding of many cytokines and cytokine receptors, growth factors, and cell adhesion molecules.[28] As shedding of TNFR1 ectodomains does not contribute to the etiology of insulin resistance, the question remains as to which TACE-mediated shedding event is truly pivotal for the induction of insulin resistance.

Although our data indicate that hepatic inflammation does not contribute to insulin resistance, the inability of TNFR1 ectodomains shedding did not affect adipose tissue remodeling or contribute to adipose tissue inflammation (Fig. 1D). Given the direct link between adipose tissue inflammation and systemic insulin resistance,[37] this may explain the dissociation of hepatic inflammation and insulin resistance we observed.

We have shown that shedding of TNFR1 ectodomains does not play a pivotal role in the development of hepatic steatosis and insulin resistance in mice, although it does appear to protect them from low-grade hepatic inflammation and NASH. We therefore propose that the TNFR1-signaling pathway plays an important role in aggravating a state of "simple steatosis" towards a phenotype with many features of NASH. Our results suggest that targeting the TNFR1 pathway may help in attenuating NASH.

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