2-2021

Macrophage-Derived Thrombospondin 1 Promotes Obesity-Associated Non-Alcoholic Fatty Liver Disease

Taesik Gwag
University of Kentucky, taesik.gwag@uky.edu

Raja Gopal Reddy Mooli
University of Kentucky

Dong Li
University of Kentucky, Dong.Li@uky.edu

Sangderk Lee
University of Kentucky, sangderk.lee@uky.edu

Eun Young Lee
University of Kentucky, eylee@uky.edu

See next page for additional authors

Macrophage-Derived Thrombospondin 1 Promotes Obesity-Associated Non-Alcoholic Fatty Liver Disease

Digital Object Identifier (DOI)
https://doi.org/10.1016/j.jhepr.2020.100193

Notes/Citation Information

Published in *JHEP Reports*, v. 3, issue 1, 100193.

Authors

Taesik Gwag, Raja Gopal Reddy Mooli, Dong Li, Sangderk Lee, Eun Young Lee, and Shuxia Wang

Research article JHEP|Reports

Macrophage-derived thrombospondin 1 promotes obesity-associated non-alcoholic fatty liver disease

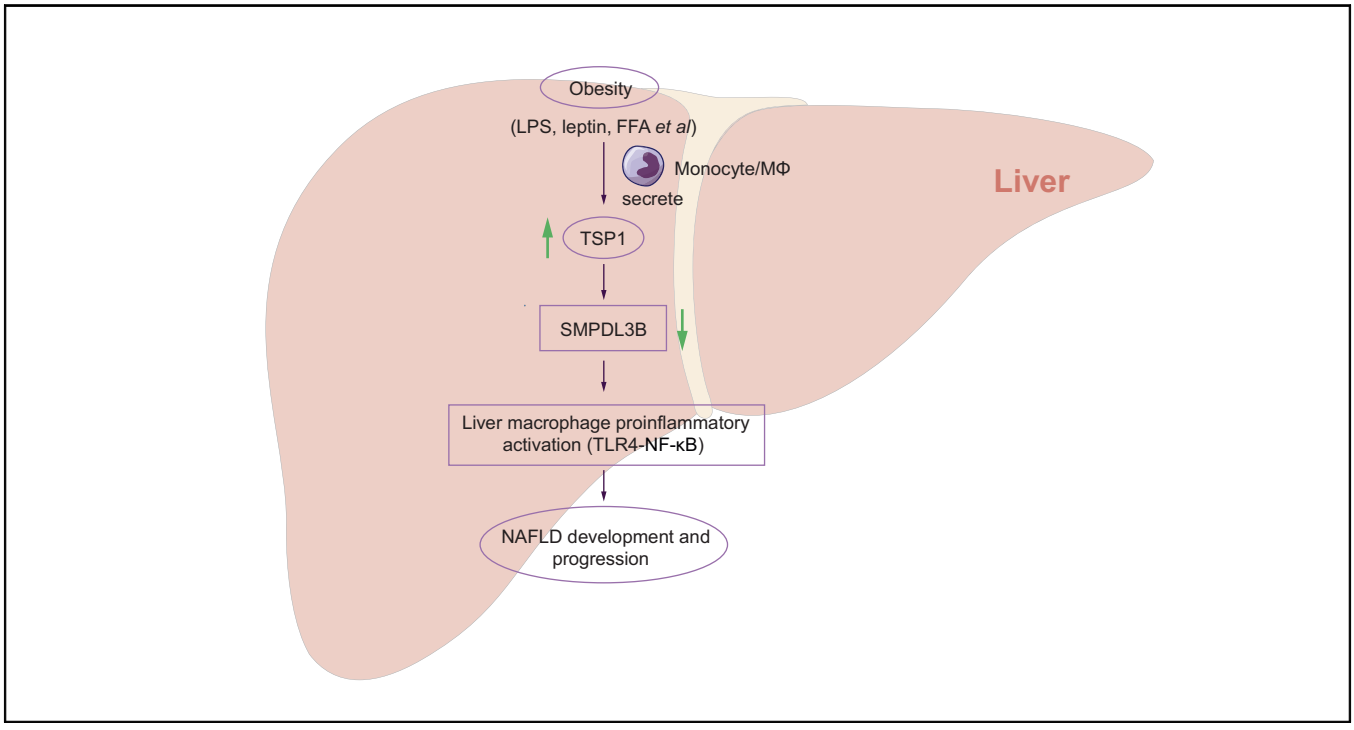
Authors

Taesik Gwag, Raja Gopal Reddy Mooli, Dong Li, Sangderk Lee, Eun Y. Lee, Shuxia Wang

Correspondence

swang7@uky.edu (S. Wang).

Graphical abstract



Highlights

- Obesity induces TSP1 expression in several tissues, such as adipose tissue and liver.
- Macrophages are an important cellular source of increased TSP1 in mouse NASH livers.
- Deletion of TSP1 in macrophages protects mice from diet-induced NASH.
- SMPDL3B negatively regulates TSP1-mediated macrophage activation.

Lay summary

Obesity-associated non-alcoholic fatty liver disease is a most common chronic liver disease in the Western world and can progress to liver cirrhosis and cancer. No treatment is currently available for this disease. The present study reveals an important factor (macrophage-derived TSP1) that drives macrophage activation and non-alcoholic fatty liver disease development and progression and that could serve as a therapeutic target for non-alcoholic fatty liver disease/steatohepatitis.

https://doi.org/10.1016/j.jhepr.2020.100193

Research article

JHEP|Reports

Macrophage-derived thrombospondin 1 promotes obesity-associated non-alcoholic fatty liver disease

Taesik Gwag,[1,†] Raja Gopal Reddy Mooli,[1,†] Dong Li,[1,†] Sangderk Lee,[1] Eun Y. Lee,[2] Shuxia Wang[1,*]

[1]*Department of Pharmacology and Nutritional Sciences, University of Kentucky, Lexington, KY 40536, USA;* [2]*Department of Pathology and Laboratory Medicine, University of Kentucky, Lexington, KY 40536, USA*

JHEP Reports **2021.** https://doi.org/10.1016/j.jhepr.2020.100193

Background & Aims: Thrombospondin 1 (TSP1) is a multifunctional matricellular protein. We previously showed that TSP1 has an important role in obesity-associated metabolic complications, including inflammation, insulin resistance, cardiovascular, and renal disease. However, its contribution to obesity-associated non-alcoholic fatty liver disease/non-alcoholic steatohepatitis (NAFLD or NASH) remains largely unknown; thus, we aimed to determine its role.
Methods: High-fat diet or AMLN (amylin liver NASH) diet-induced obese and insulin-resistant NAFLD/NASH mouse models were utilised, in addition to tissue-specific *Tsp1*-knockout mice, to determine the contribution of different cellular sources of obesity-induced TSP1 to NAFLD/NASH development.
Results: Liver TSP1 levels were increased in experimental obese and insulin-resistant NAFLD/NASH mouse models as well as in obese patients with NASH. Moreover, TSP1 deletion in adipocytes did not protect mice from diet-induced NAFLD/NASH. However, myeloid/macrophage-specific TSP1 deletion protected mice against obesity-associated liver injury, accompanied by reduced liver inflammation and fibrosis. Importantly, this protection was independent of the levels of obesity and hepatic steatosis. Mechanistically, through an autocrine effect, macrophage-derived TSP1 suppressed *Smpdl3b* expression in liver, which amplified liver proinflammatory signalling (Toll-like receptor 4 signal pathway) and promoted NAFLD progression.
Conclusions: Macrophage-derived TSP1 is a significant contributor to obesity-associated NAFLD/NASH development and progression and could serve as a therapeutic target for this disease.

Introduction

With the epidemic burden of obesity and metabolic diseases, non-alcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease in the Western world. NAFLD ranges from NAFL to non-alcoholic steatohepatitis (NASH), and can progress to cirrhosis and HCC. The rates for obesity-associated NAFLD/NASH continue to rise steadily.[1,2] Notably, there is no NAFLD/NASH therapy currently available. Lifestyle interventions, such as diet and exercise, are the only treatments available to patients with NAFLD. However, these interventions do not achieve satisfactory results. Therefore, an effective alternative strategy is urgently needed.

The pathogenesis of NAFLD/NASH is complex and controlled by coordinated actions of liver cells (*i.e.* hepatocytes, Kupffer cells [KCs]/macrophages, hepatic stellate cells, and endothelial cells). Accumulative experimental and clinical data demonstrate that macrophages have an important role in the initiation and progression of NAFLD.[3–9] KCs and infiltrating monocyte-derived macrophages (MDMs) are two hepatic macrophage populations that undergo expansion and functional changes during NASH development.[4,8,10–12] However, the driving factors that induce such changes in liver macrophages remain to be determined.

Thrombospondin 1 (TSP1), a 420–450-kDa homotrimer with individual subunits of ~145 kDa, is a multifunctional matricellular protein comprising several domains that can interact with different cell surface receptors. It also has a role in cardiovascular and renal diseases.[13,14] In addition, TSP1 is involved in tissue injury, inflammatory diseases, and NAFLD.[15–17] Recent studies demonstrated a novel role for TSP1 in both the recruitment and proinflammatory activation of macrophages.[18] Furthermore, TSP1-dependent modulation of macrophage function contributes to obesity-associated complications, such as chronic inflammation and insulin resistance.[19,20] However, whether TSP1 could serve as an important factor that drives macrophage activation and NAFLD development and progression is unknown and is investigated in the current study.

In this study, we show that TSP1 levels are increased in both animal and human NASH livers. By utilisation of tissue-specific TSP1-deficient mice in diet-induced obesity and insulin-resistant NAFLD/NASH models, we demonstrate that TSP1 deletion in adipocytes does not protect mice from obesity-associated NAFLD/NASH. However, myeloid/macrophage-specific TSP1 deficiency protects mice from obesity-associated liver inflammation and fibrosis. This study provides strong *in vivo* evidence that macrophage-derived TSP1 is an important contributor to obesity-associated NAFLD/NASH.

Keywords: TSP1; Obesity; NAFLD; NASH; SMPDL3B; Macrophage.
Received 17 June 2020; received in revised form 24 September 2020; accepted 27 September 2020; available online 9 October 2020
† These authors contributed equally.
* Corresponding author. Address: Department of Pharmacology and Nutritional Sciences, University of Kentucky, Wethington Building, Room 583, 900 South Limestone Street, Lexington, KY 40536, USA. Tel.: +1 859 218 1367; fax: +1 859 257 3646.
E-mail address: swang7@uky.edu (S. Wang).

Material and methods

Mice and diets

All the experiments involving mice conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Animals were housed individually in standard cages at 22°C in a 12:12-h light–dark cycle.

Mouse breeding

Female *Tsp1* floxed mice ($Tsp1^{fl/fl}$)[20] were bred with male Cre mice (Lyz-Cre for macrophage-specific deletion: $Tsp1^{\Delta M\Phi}$). Female offspring with a $Tsp1^{fl/+Cre-}$ genotype in the first generation were then bred with male $Tsp1^{fl/+Cre+}$ mice. Pups with a $Tsp1^{fl/fl/Cre+}$ genotype, $Tsp1^{fl/fl/Cre-}$ mice and littermate controls were used for the study.

AMLN diet-induced NAFLD/NASH

Male *C57BL/6 mice* (8-weeks old, Jackson Laboratories, Bar Harbor, ME, USA) were allowed *ad libitum* access to an amylin liver NASH (AMLN) diet (40% fat; 22% fructose and 2% cholesterol; D09100301, Research Diets, Inc, NJ, USA) and low-fat diet (LFD) (10% kcal, D1250B, Research Diets, Inc, NJ) (a control diet). The experiments were carried out with different time periods of 3 and 8 months to generate NAFL and NASH models, respectively. Each group contained 8–10 animals.

High-fat diet-induced NAFLD/NASH

The experiment was performed on 8-week-old male $Tsp1^{fl/fl}$, $Tsp1^{\Delta M\Phi}$ mice and their age and sex-matched littermates as controls. Mice were fed either a high-fat diet (HFD; 60% kcal, D12492) or LFD (10% kcal, D1250B) for 32 weeks with standard laboratory water. Each group contained 8 mice. Body weights were measured weekly. At the end of the study, mice were euthanised. Blood was collected and the livers were harvested for analysis.

Analysis of Tsp1 expression in available liver scRNA-Seq data sets of 3 controls and 3 mice with AMLN diet-induced NASH (GSE129516)

Seurat single-cell RNA sequencing (scRNA-seq) analysis R tools (https://satijalab.org/seurat/v3.2/immune_alignment.html) were utilised for clustering and cell type assignment of data sets from GSE129516.[11] A total of 22 clusters were identified and TSP1 expression in these clusters was further analysed.

Liver histology and NAFLD score

Liver histology and NAFLD activity score (NAS) were assessed using H&E stains in paraffin-embedded sections by using the standard method as previously described,[21] a composite parameter based on separate scores for steatosis (0–3), lobular inflammation (0–3), and hepatocellular ballooning (0–2). Liver fibrosis was assessed using Sirius Red and/or Mason's Trichrome stains in paraffin-embedded sections. The total positive staining (reported as the percentage of the total area) was assessed from 3–4 representative sections from each animal using Image J software. Details on the liver slides from human patients have been provided in the "Supplementary materials and methods".

Liver and plasma parameters

Total hepatic lipid was extracted from frozen livers using protocols as previously described.[22] Liver or plasma triacylglycerol (TG) and total cholesterol levels were measured enzymatically by using kits from the Wako Chemicals. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured by using the kits from Bioassay Systems according to the manufacture instructions.

Liver RNA-Seq transcriptome sequencing

Total RNA from liver samples of LFD or HFD-fed $Tsp1^{fl/fl}$ and $Tsp1^{\Delta m\phi}$ mice was extracted using the RNeasy Mini Kit (Qiagen, USA) and submitted to the BGI Americas (San Jose, CA, USA) for RNA-Seq transcriptome sequencing using the BGISEQ-500 platform. Data analysis was also performed by BGI Americas. DEseq2 algorithms were used to detect differentially expressed genes (DEGs) between 2 groups. Genes exhibiting false-adjusted p value <0.05 and the $\log_2$-fold change ≥1 or ≤−1 were considered significant. Data were deposited in the Gene Expression Omnibus with accession number GSE155973.

Real-time quantitative PCR

Total RNA from frozen liver tissues or cells was extracted using RNeasy Mini Kit (Qiagen). RNA was reverse transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Invitrogen, Carlsbad, CA, USA). Real-time quantitative PCR was performed on a MyiQ Real-time PCR Thermal Cycler (Bio-Rad) with SYBR Green PCR Master Kit (Qiagen). Relative mRNA expression was calculated using the MyiQ system software as previous reported[19] and normalised to *β-actin* mRNA levels. All primer sequences utilised in this study are detailed in Table 1.

Western blotting

Total protein was extracted from frozen liver tissue or cultured cells. From each sample, 30 μg protein was separated in a 10% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Protein expression was determined by immunoblotting with the following antibodies: anti-TSP1 (Novus Biologicals), anti-α-SMA (Sigma), anti-SMPDL3B (Proteintech, IL, USA), anti-Toll-like receptor 4 (anti-TLR4) (Sigma), and anti-β-actin (Sigma). Membranes were blocked and then incubated with primary antibodies, followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibodies. Labelled proteins were detected with an enhanced chemiluminescence system (Pierce).

Cell culture and treatment

RAW264.7 cell experiments

RAW264.7 cells (macrophage cell line from ATCC) were cultured and treated with the recombinant TSP1 (5 μg/ml, R&D system) for different time periods, and then harvested. SMPDL3B levels (mRNA and protein) in these cells were determined by quantitative (q)PCR and Western blotting, respectively. In addition, proinflammatory cytokine expression in these cells was determined by qPCR. In another set of experiments, RAW264.7 cells were transiently transfected with a SMPDL3B expression vector (pcDNA3.1-Smpdl3b from GenScript) or an empty control vector by using the FuGene 6 transfection reagent (Promega). After 48 h, cells were treated with TSP1 (5 μg/ml) for 6–24 h and the expression of SMPDL3B or proinflammatory cytokines was determined by qPCR. Interleukin (IL)-1β or tumour necrosis factor (TNF)-α levels in the cell condition media were determined by ELISA.

Primary mouse liver macrophage (KC) experiments

KCs were isolated from 6–8-week-old male C57BL6 mice using a 2-step Percoll gradient [25%/50% (vol/vol)] centrifugation

Table 1. Primer sequences used in the study.

Primer	Forward	Reverse
TSP1	5′-CTA GGT GTC CTG TTC CTG TTG-3′	5′-AAG GAA GCC AGG AAG ATG AAG-3′
F4/80	5′-CTT TGG CTA TGG GCT TCC AGT C-3′	5′-GCA AGG AGG ACA GAG TTT ATC GTG-3′
IL-1β	5′-TGG AGA GTG TGG ATC CCA AGC AAT-3′	5′-TGT CCT GAC CAC TGT TGT TTC CCA-3′
TNF-α	5′-AGC CGA TGG GTT GTA CCT-3′	5′-TGA GTT GGT CCC CCT TCT-3′
TGF-β1	5′-ACA ATT CCT GGC GTT ACC-3′	5′-GGC TGA TCC CGT TGA TTT-3′
Collagen type I, α1	5′-TTCTCCTGGCAAAGACGGACTCAA-3′	5′-AGGAAGCTGAAGTCATAACCGCCA-3′
Collagen type III, α1	5′-TCCTAACCAAGGCTGCAAGATGGA-3′	5′-TCCTAACCAAGGCTGCAAGATGGA-3′
β-actin	5′-GGCTGTATTCCCCTCCATCG-3′	5′-CCAGTTGGTAACAATGCCATGT-3′
SMPDL3B	5′-GGGACTACCTCTGCGATTCTC-3′	5′-CCTCTCCTAGACTCTCATTGGG-3′

IL-, interleukin-; SMPDL3B, sphingomyelin phosphodiesterase acid-like 3; TGF, transforming growth factor; TNF, tumour necrosis factor; TSP1, thrombospondin 1.

method as described previously[23,24] and cultured in DMEM media containing 10% FBS and 1% penicillin-streptomycin. For characterisation, cells were fixed, stained with F4/80-Alexa 488 antibody (Bio-Rad, Hercules, CA, USA), mounted with a mounting medium containing DAPI, and visualised with a fluorescent microscope (Eclipse 80i, Nikon). In addition, KCs were cultured and transfected with a SMPDL3B expression vector and the effect of SMPDL3B overexpression on TSP1-induced proinflammatory cytokine production was determined as described earlier for the RAW264.7 cells.

Statistical analysis

Statistical analysis was performed using Prism version 8.0.2 (GraphPad Software, San Diego, CA, USA). Data are expressed as mean values ±SE. The statistical significance between 2 groups was determined using a 2-tailed Student's *t*-test. One-way ANOVA followed by Bonferroni's multiple comparisons test or 2-way ANOVA followed by Tukey's multiple comparisons test was applied for multigroup comparisons. A p value <0.05 was considered to be statistically significant.

Results

TSP1 is upregulated in animal and human NAFLD and NASH livers

In adult liver, TSP1 is expressed at very low levels under physiological conditions,[25] but increased under disease conditions.[26,27] We determined how the TSP1 level was altered in NAFLD/NASH using a AMLN diet-induced NASH mouse model (obese and insulin resistant).[28] Simple steatosis was induced by 3 months of an AMLN diet, whereas steatohepatitis and liver fibrosis were induced after 8 months. Liver TSP1 levels were increased in mice with simple steatosis and more pronounced in mice with NASH and fibrosis (Fig. 1A–D). Importantly, TSP1 expression was upregulated in human NAFLD liver as demonstrated by immunohistochemical staining of liver sections. Positive TSP1 staining was seen in livers with steatosis and was dramatically increased in NASH livers (Fig. S1). These data indicate that increased TSP1 expression occurs in both animal and human NAFLD and NASH livers.

To determine the cellular sources of increased TSP1 in NASH livers, we analysed a mouse liver scRNA-seq data set (GSE129516[11]), including 3 control and 3 AMLN diet-induced NASH liver samples, identifying 22 clusters (Fig. 1E–G). TSP1 was expressed in hepatic stellate cells (HSCs), cholangiocytes and macrophages, and was upregulated in NASH conditions. We also analysed a human liver scRNA-seq data set (GSE136103) that included healthy (n = 4–5) and cirrhotic liver samples (n = 4–5).[12] TSP1 was expressed in endothelial cells (ECs), epithelial cells, mesenchyme, and a subset of mononuclear phagocytes (MPs) (Fig. S2). Interestingly, cirrhosis increased TSP1 expression in the above-mentioned cell types except for MPs. This was in contrast to the mouse data and could be explained by the different stages of NAFLD analysed (NASH in mouse *vs.* cirrhosis in human). Nonetheless, both mouse and human data consistently showed that TSP1 is significantly expressed in liver nonparenchymal cells and has minimal expression in hepatocytes.

Macrophage-specific TSP1 deficiency protects mice from obesity-associated NASH

Tsp1 is expressed in liver macrophages and upregulated in NASH conditions (Fig. 1). Therefore, we further determined its contribution to obesity-associated NAFLD/NASH by utilisation of macrophage-specific *Tsp1* knockout mice ($Tsp1^{\Delta m\phi}$).[20] These mice were fed a HFD for 8 months. Despite similar obesity and hepatic steatosis, macrophage-specific TSP1 deletion was sufficient to reduce diet-induced hepatic injury, indicated by reduced plasma ALT and AST levels (Fig. 2A–C). HFD-induced liver *Tsp1* expression was significantly reduced in macrophage-specific *Tsp1*-knockout mice (Fig. 2D). These mice also had reduced markers for liver inflammation (*Tnf-α* and *Il-1β*) and fibrosis (Collagen I, Collagen III and α-smooth muscle actin [α-SMA]) (Figs. 2F–H and 3E). Furthermore, the reduction in liver fibrosis was confirmed with Sirius Red staining in HFD-fed $Tsp1^{\Delta m\phi}$ mice (Fig. 2E). Transforming growth factor (TGF)-β levels were comparable among all groups (Fig. 2F). Together, these data demonstrate that macrophage-derived TSP1 is a significant contributor to obesity-associated hepatic inflammation and fibrosis.

The contribution of other cellular sources of obesity-induced TSP1 (*e.g.* adipocytes[29,30]) to NAFLD/NSH development was determined by utilisation of adipocyte-specific TSP1-knockout mice ($Tsp1^{\Delta adipo}$).[20] $Tsp1^{\Delta adipo}$ mice fed a HFD for 8 months developed similar levels of obesity (Fig. S3). Liver weight, triglyceride levels, inflammatory markers, and positive Sirius red staining in liver sections were also similar between HFD-fed $Tsp1^{\Delta adipo}$ mice and control ($Tsp1^{fl/fl}$) mice (Fig. S3B–D). Other obesity-associated complications, such as glucose intolerance and insulin resistance, developed similarly in knockout mice compared with control ($Tsp1^{fl/fl}$) mice.[20] Collectively, these data suggest that adipocyte-derived TSP1 does not contribute significantly to obesity-associated NAFLD.

Additionally, the contribution of hepatocyte-derived TSP1 to NASH development was determined by utilisation of hepatocyte-specific TSP1-knockout mice ($Tsp1^{\Delta hep}$) (Fig. S4A). Control ($Tsp1^{fl/fl}$) and $Tsp1^{\Delta hep}$ mice developed a similar level of obesity over 20 weeks of receiving an AMLN diet (Fig. S4B). Compared with control mice, AMLN-fed $Tsp1^{\Delta hep}$ mice developed similar

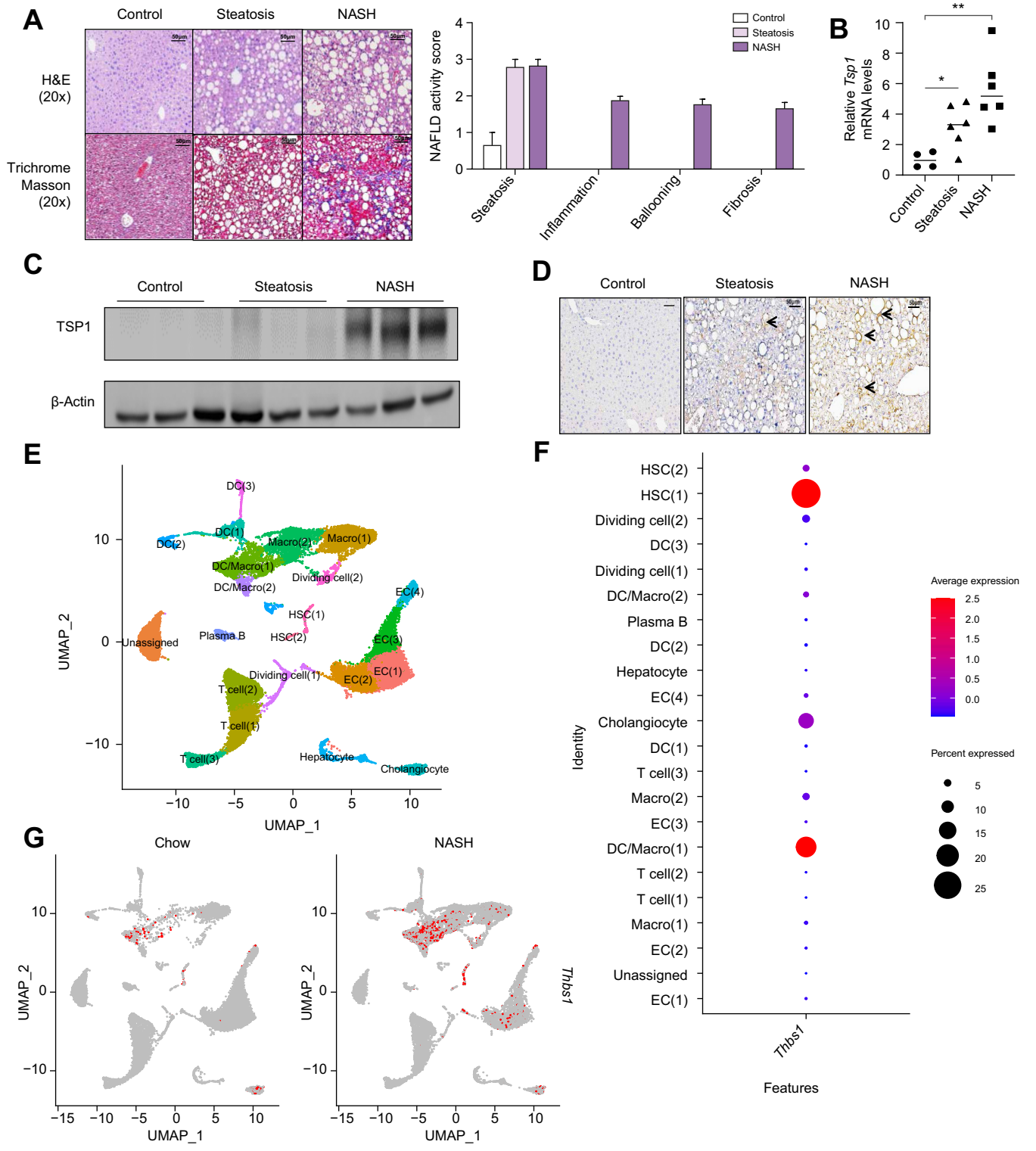


Fig. 1. TSP1 expression is increased in mouse NASH livers. C57BL6 male mice fed an AMLN-diet for 3 months or 8 months developed steatosis or NASH, respectively. (A) Representative images of H&E and Masson staining, and NAFLD activity scores. (B) Liver *Tsp1* mRNA levels. (C) Liver TSP1 protein levels. (D) Representative images of immunohistochemical staining for liver TSP1. Positive staining is in brown and indicated by the arrowhead; Data are mean ± SE (n = 4–6 mice/group). **p* <0.05, ***p* <0.01 (1-way ANOVA); (E–G) Analysis of *Tsp1* expression in a single-cell RNA sequence data set from mouse NASH livers (GSE129516) showing that *Tsp1* (*Thbs1*) was upregulated in macrophages, cholangiocytes, and HSCs from NASH livers. AMLN, amylin liver NASH; HSCs, hepatic stellate cells; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; TSP1 (THBS1), thrombospondin 1.

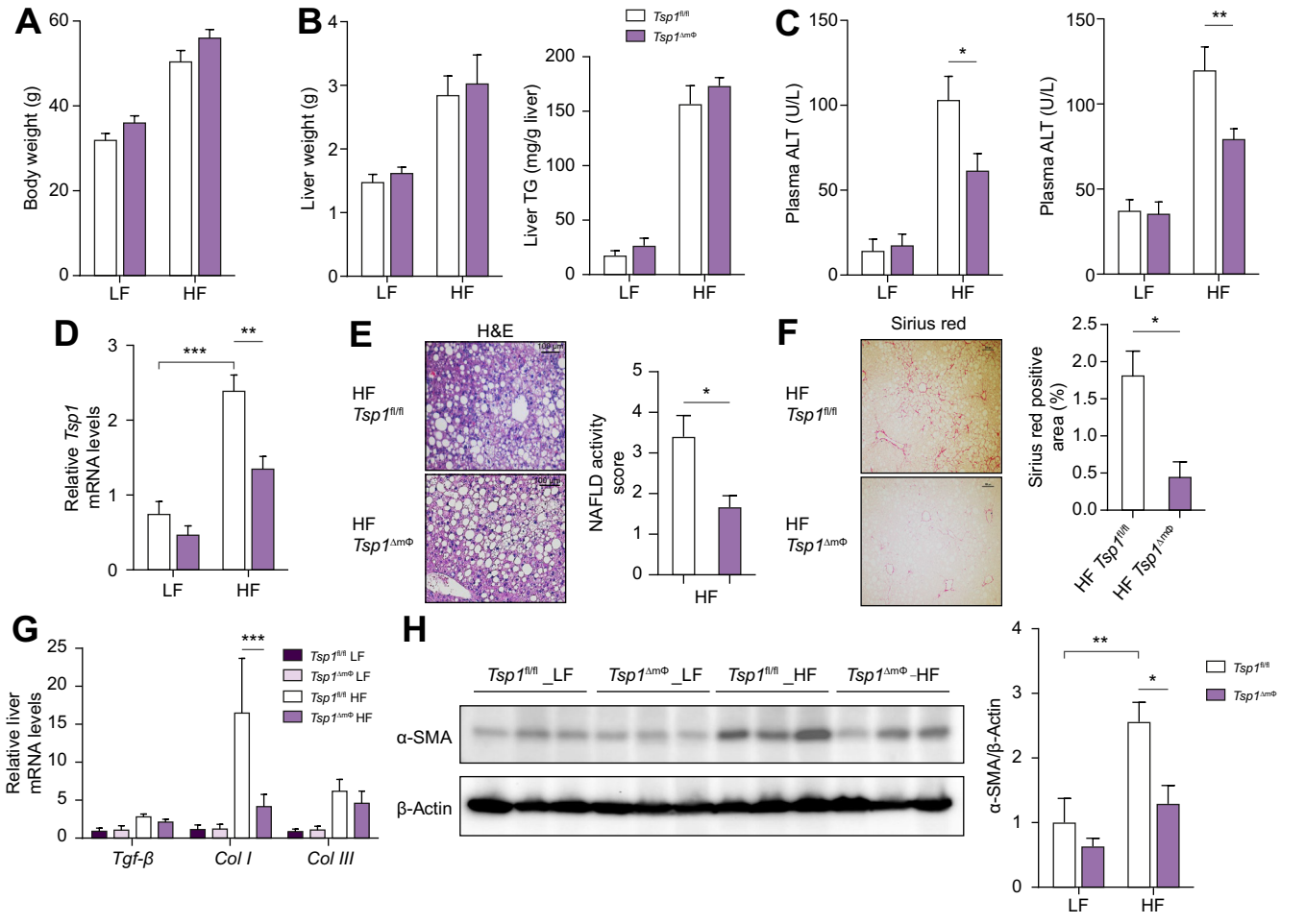


Fig. 2. Macrophage-specific TSP1 deficiency protects mice against diet-induced NAFLD/NASH. Male wild-type *Tsp1*$^{fl/fl}$ mice and *Tsp1*$^{\Delta m\phi}$ mice were fed either a LFD or HFD for 8 months. (A) Body weight changes in mice. (B) Liver weight and triglyceride levels from 4 groups of mice. (C) Plasma ALT and AST levels from mice. (D) Liver *Tsp1* expression based on qPCR. (E) Representative H&E-stained liver sections and NAFLD activity score from HFD-fed wild-type mice and *Tsp1*$^{\Delta m\phi}$ mice. (F) Sirius Red staining of liver sections and relative Sirius Red-positive area. (G) Fibrosis-related gene expression in liver based on qPCR. (H) Representative Western blotting of α-SMA levels in livers (normalised to β-actin). Data are mean ± SE (n = 6–7 mice/group). **p* <0.05, ***p* <0.01, ****p* <0.001 [2-way ANOVA (A–D,G,H) or Student's *t* test (E,F)]. α-SMA, α-smooth muscle actin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Col I/III; Collagen type I/III; HFD, high-fat diet; LFD, low-fat diet; qPCR, quantitative PCR; TSP1, thrombospondin 1; *Tsp1*$^{\Delta m\phi}$, macrophage specific *Tsp1*-knockout mice; *Tsp1*$^{fl/fl}$, *Tsp1* floxed mice.

levels of liver steatosis, inflammation and fibrosis (Fig. S4C–F). These data suggest that hepatocyte-derived TSP1 did not contribute significantly to diet-induced obesity and its associated NAFLD and NASH, which is consistent with the earlier described liver scRNA-seq data (Figs S1 and S2).

SMPDL3B expression is increased in livers from *Tsp1*$^{\Delta M\phi}$ mice, accompanied by reduced liver TLR4 signalling

Liver RNA-seq analysis was performed to further determine the mechanisms underlying the protective effect of macrophage-specific TSP1 deficiency on HFD-induced liver phenotype (Fig. 2). There were 367 significantly upregulated genes and 162 downregulated genes in *Tsp1*$^{fl/fl}$ mice fed a HFD *vs.* a LFD, respectively. Kyoto Encyclopedia of Genes and Genomes (KEGG) enriched pathways revealed several upregulated pathways, such as the focal adhesion, TNF-α, extracellular matrix (ECM)–receptor interaction and phagosome pathways. A comparison of HF-fed *Tsp1*$^{\Delta M\phi}$ with HF-fed *Tsp1*$^{fl/fl}$ mice identified 31 upregulated and 16 downregulated genes. As expected, the TNF-α and nuclear factor (NF)-κB pathways were significantly reduced in livers from HFD-fed *Tsp1*$^{\Delta M\phi}$ mice (Fig. 3A). This was consistent with previous studies showing that TSP1-null macrophages had a reduced proinflammatory phenotype.[19] Sphingomyelin phosphodiesterase acid-like 3B (*Smpdl3b*) was one of the differentially regulated genes (Fig. 3B). It is a glycosylphosphatidylinositol (GPI)-anchored membrane protein on macrophages and negatively regulates TLR signalling.[31] Increased *Smpdl3b* in the liver from HFD-fed *Tsp1*$^{\Delta M\phi}$ mice was confirmed by qPCR (Fig. 3C). This alteration was associated with reduced TLR4 and proinflammatory cytokine (*e.g. Tnf-α*, *Il-1β*) expression (Fig. 3D,E). In addition, *Smpdl3b* levels in adipose tissue were comparable among all groups (data not shown), suggesting a tissue-specific effect of TSP1 on the regulation of *Smpdl3b* levels.

SMPDL3B negatively regulates TSP1-induced macrophage proinflammatory status

TSP1 is an important regulator of macrophage function. TSP1-null bone marrow-derived macrophages (BMDMs) have a

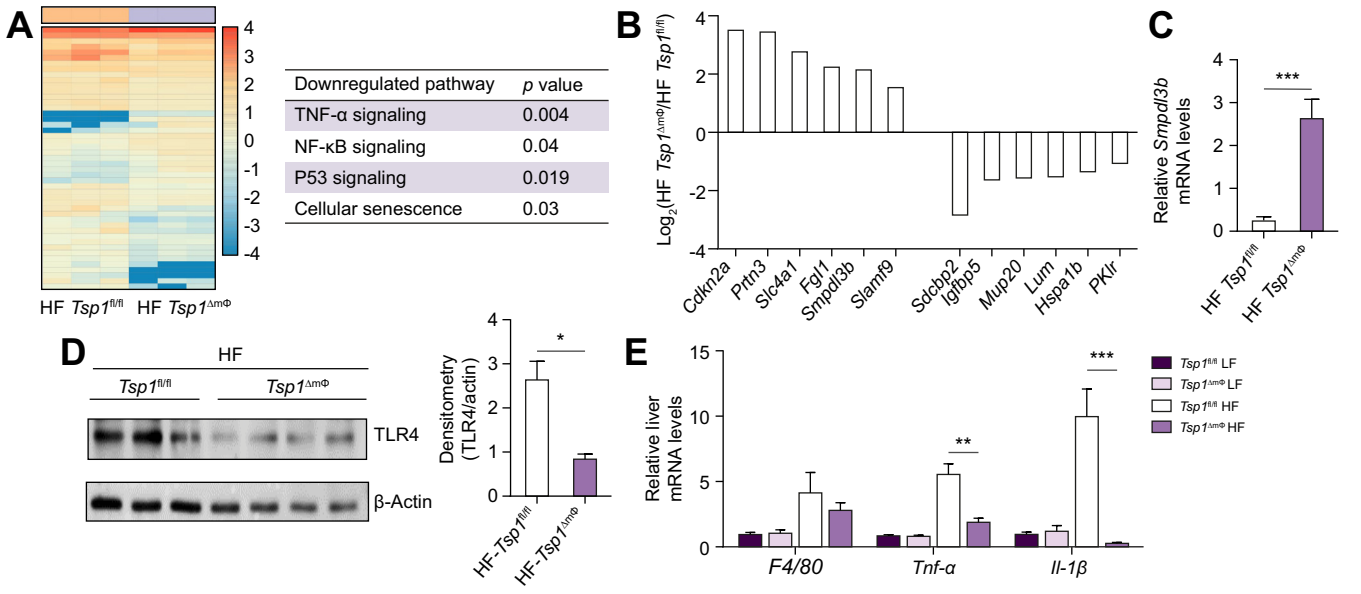


Fig. 3. Liver SMPDL3B is increased in macrophage-specific *Tsp1*-null mice (*Tsp1*$^{\Delta m\phi}$), accompanied by reduced expression of TLR4 and proinflammatory cytokines. Transcriptome analysis of liver from HFD-fed *Tsp1*$^{fl/fl}$ and HFD-fed *Tsp1*$^{\Delta m\phi}$ mice revealed significantly downregulated (A) signalling pathways and (B) genes. (C) qPCR of *Smpdl3b* in livers from HFD-fed *Tsp1*$^{\Delta m\phi}$ and *Tsp1*$^{fl/fl}$ mice. (D) Western blots of liver TLR4 levels from HFD-fed *Tsp1*$^{\Delta mΦ}$ and *Tsp1*$^{fl/fl}$ mice. (E) Inflammation-related gene expression in liver based on qPCR. Data are mean ± SE (n = 3–5 mice/group). **p* <0.05, ***p* <0.01 and ****p* <0.001 (2-way ANOVA [E] or Student's *t* test for [C,D]). HFD, high-fat diet; qPCR, quantitative PCR; TLR4, Toll-like receptor 4; TSP1, thrombospondin 1; *Tsp1*$^{\Delta m\phi}$, macrophage-specific TSP1-knockout mice; *Tsp1*$^{fl/fl}$, TSP1 floxed mice.

reduced proinflammatory phenotype,[19] whereas TSP1 treatment stimulates macrophages to produce proinflammatory cytokines, associated with increased TLR4 expression and NF-κB activity.[18] Despite research showing that SMPDL3B negatively regulates TLR signalling in macrophages,[31] whether SMPDL3B has a role in TSP1-induced macrophage activation was unclear and, thus, was investigated here.

BMDMs isolated from TSP1-null mice had increased SMPDL3B levels, associated with reduced proinflammatory cytokine production (Fig. S5). TSP1 treatment downregulated *Smpdl3b* expression in RAW264.7 cells (Fig. 4A,B), which was associated with increased expression of proinflammatory cytokines (Fig. 4C), indicating that TSP1 suppresses macrophage *Smpdl3b* expression. Moreover, overexpression of *Smpdl3b* abolished TSP1-induced IL-1β and TNF-α expression as well as secretion in RAW264.7 cells (Fig. 4D–E). To confirm the above findings in primary liver macrophages, KCs from 8-week-old male C57BL6 mice were isolated and utilised in the experiment. The purity of these cells was demonstrated by F4/80 immunofluorescence staining (Fig. 5A). Similarly, overexpression of *Smpdl3b* in KCs abolished TSP1-induced proinflammatory cytokine production (Fig. 5B–D). Collectively, these data suggest that SMPDL3B negatively regulates TSP1-induced macrophage activation.

Discussion

TSP1 is a multifunctional secreted matricellular protein that is involved in the pathogenesis of various diseases, such as cardiovascular disease, renal complications, obesity, and insulin resistance. However, the role of TSP1 in NAFLD/NASH is largely unknown.[16,17] By using tissue-specific TSP1-knockout mice, we demonstrated that macrophage-specific TSP1 deletion but not adipocyte-specific TSP1 deletion protected mice against obesity-associated liver injury, accompanied by reduced liver inflammation and fibrosis. Importantly, this protection was independent of the levels of obesity and hepatic steatosis. Mechanistically, macrophage-derived TSP1 suppresses *Smpdl3b* expression in liver through an autocrine effect, amplifying liver proinflammatory signalling and promoting NAFLD progression.

These *in vivo* data provide, for the first time, compelling evidence for the significant contribution of macrophage-derived TSP1 to obesity-associated NAFLD/NASH. The role of macrophages in the development and progression of NAFLD is supported by previously published experimental and clinical data.[4,5,8,11,32,33] In liver, KCs and infiltrating MDMs are two hepatic macrophage populations that change dramatically during NASH development.[4,7,8,10–12] Recent studies demonstrated that KCs and MDMs have distinct morphology and transcriptional profiles in experimental NASH models.[4,8] Moreover, infiltrating MDMs produce more TSP1 in NASH livers than do KCs.[4] Given that KCs have a role in the initiation and/or onset of NAFLD,[34,35] infiltrating MDM-derived TSP1 might stimulate NAFLD progression. Consistently, we found that macrophage-specific TSP1 deletion protected mice from steatohepatitis and fibrosis. Interestingly, diet-induced steatosis was not improved in macrophage-specific TSP1-null mice, although these mice had reduced hepatic TLR4 or TNFα levels. Research has shown that TNF-α stimulated sterol regulatory element binding protein-1 c (SREBP1c) expression and induced *de novo* lipogenesis and hepatic steatosis.[36] However, in the current study, the expression of genes relating to the hepatic lipid metabolism (*e.g. Cd36*, *Srebp1*, *Fas*, *Scd1*, and *Dgat1* and *2*) (data not shown) was comparable in livers between wild-type and macrophage-specific TSP1-null mice. Therefore, other mechanisms warrant further investigation in future studies.

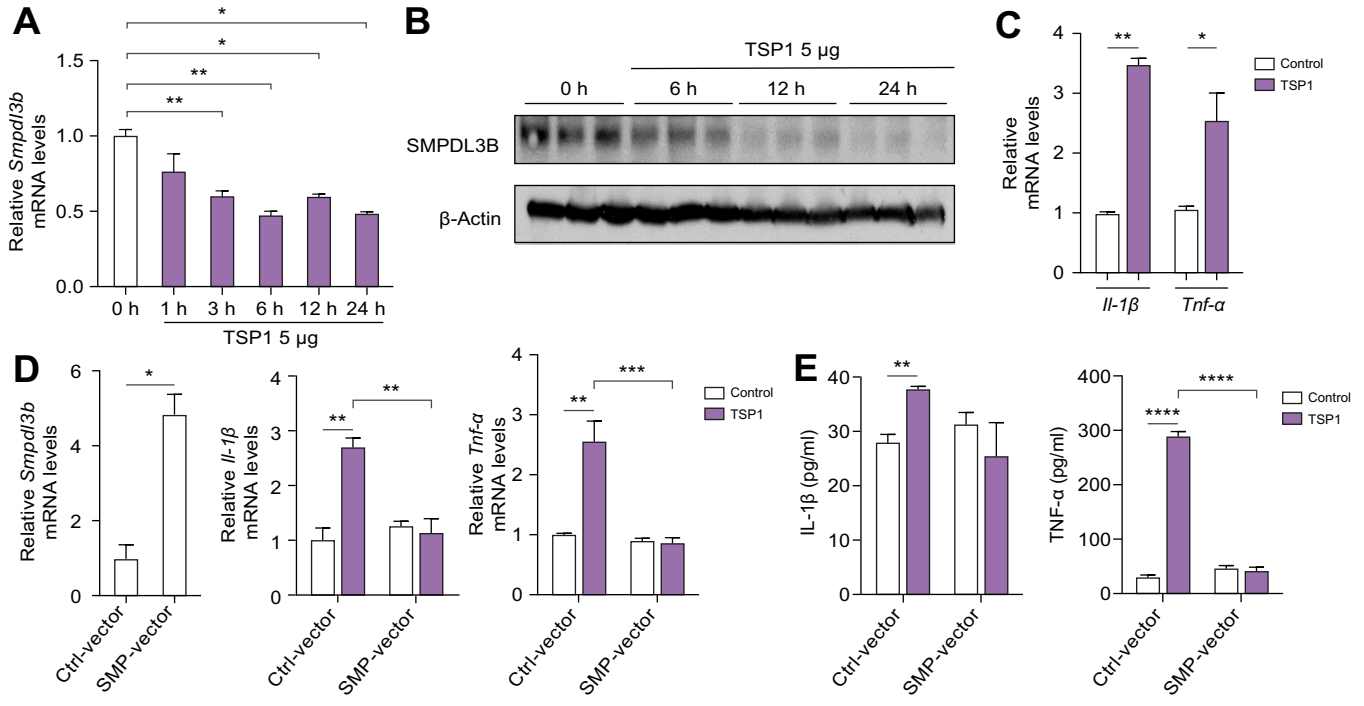


Fig. 4. SMPDL3B negatively regulates TSP1-induced macrophage proinflammatory status in RAW264.7 cells. TSP1 treatment (5 μg/ml) downregulated *Smpdl3b* mRNA (A) and protein (B) levels in RAW264.7 macrophages, associated with increased proinflammatory cytokine expression (C). Overexpression of *Smpdl3b* in RAW264.7 macrophages by transfection with a pcDNA 3.1-SMPDL3B vector abolished TSP1-induced *Il-1β* and *Tnf-α* expression based on PCR (D) and secretion in condition media by ELISA (E). Data are mean ± SE (n = 3 experiments). *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001 (1-way ANOVA [A], 2-way ANOVA [C–E], or Student's t test [D]). SMPDL3B, sphingomyelin phosphodiesterase acid-like 3; TNF, tumour necrosis factor; TSP1, thrombospondin 1.

Our results suggest monocyte and/or macrophage-derived TSP1 as a therapeutic target for obesity-associated NASH. However, how monocytes and/or macrophages produce more TSP1 under obese conditions is unknown. Under obese conditions, factors including fatty acids (FFA) and bacterial endotoxins [*i.e.* lipopolysaccharides (LPS) resulting from small intestinal bacterial overgrowth and increased intestinal permeability] have been reported as putative factors for macrophage activation and NASH development.[7,10,37–39] Both FFA and LPS stimulate TSP1 expression in monocytes and/or macrophages (including human THP-1 monocytes and/or macrophages and BMDM). Interestingly, neither T cells (T cells, T helper type [Th] 1 or Th17 cells) nor dendritic cells (DCs) respond to LPS to produce TSP1.[40–42] Collectively, these studies suggest that factors (*e.g.* LPS or FFA) involved in gut–liver and adipose–liver crosstalk drive TSP1 expression in liver macrophages and contribute to NAFLD progression.

Research previously demonstrated a novel role for TSP1 in both the recruitment and proinflammatory activation of macrophages.[18] In the current study, SMPDL3B was identified as a new mediator of TSP1-induced macrophage proinflammatory activation in liver. SMPDL3B is a GPI-anchored membrane-associated protein with homology to acid sphingomyelinase (ASMase) and is involved in sphingomyelin catabolism.[43] SMPDL3B also has a role in podocyte injury from diabetic nephropathy or focal segmental glomerulosclerosis[44,45] as well as in inflammatory processes. A negative regulatory role in innate immune signalling has been identified in macrophages, where SMPDL3B is found on the cell surface. Its knockdown altered cellular lipid composition and membrane fluidity changes, leading to enhanced responsiveness to TLR stimulation.[31] However, whether SMPDL3B is involved in NAFLD/NASH development is unknown, although accumulating evidence suggests the contribution of altered sphingolipid metabolism to NAFLD.[46] In the current study, SMPDL3B was increased in livers from macrophage-specific TSP1-deficient mice, associated with reduced liver TLR4 and proinflammatory cytokine expression. Interestingly, SMPDL3B levels in adipose tissue were comparable between wild-type and knockout mice, suggesting that SMPDL3B specifically negatively regulates TSP1-induced liver macrophage/KC activation. This tissue-specific effect highlights the potential involvement of SMPDL3B in the development and progression of NAFLD/NASH, which warrants further investigation.

In addition to regulating liver macrophage proinflammatory status, macrophage-derived TSP1 might directly modulate the function of other liver cells, such as hepatocytes, stellate cells or sinusoidal ECs, and impact the development and progression of NAFLD/NASH through paracrine effects.[17,47,48] TSP1 inhibition or knockdown in cultured stellate cells attenuated TGF-β-induced stellate cell activation (*e.g.* production of α-SMA and collagen).[17] In addition, platelet-derived growth factor-induced TSP1 expression in hepatic SCs is a prerequisite for the effect of TGF-β on SC activation.[48] These studies suggest a role for TSP1 in modulating SC function and its contribution to liver fibrosis. TSP1 also causes a dose-dependent loss of fenestrae in liver sinusoidal ECs and affects their function.[49] Together with our results of the effect of TSP1 on macrophage function, these additional effects of TSP1 together contribute to obesity-associated NAFLD/NASH development and progression.

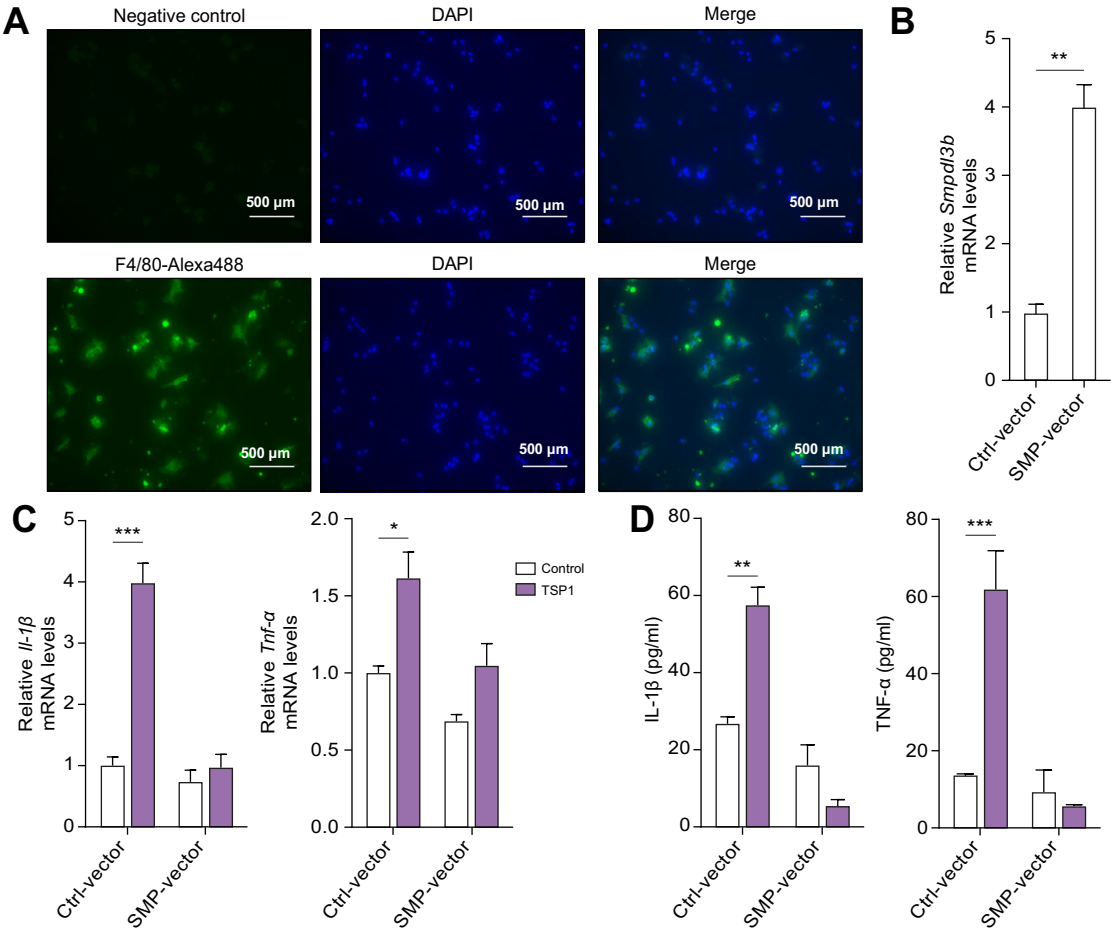


Fig. 5. Overexpression of SMPDL3B abolishes TSP1-induced proinflammatory cytokine expression in liver Kupffer cells. (A) Representative immunofluorescence images of primary liver Kupffer cells isolated from 8-week-old male C57BL6/j mice, cultured and stained with F4/80-Alexa488. Overexpression of *Smpdl3b* in Kupffer cells by transfection with a pcDNA 3.1-SMPDL3B vector (B) abolished TSP1 (5 μg/ml)-induced *Il-1β* and *Tnf-α* gene expression, as shown by PCR (C) and secretion in condition media by ELISA (D). Data are mean ± SE (n = 3 experiments). *p <0.05, **p <0.01, ***p <0.001 [2-way ANOVA (C,D) or Student's t test (B)]. IL, interleukin; SMPDL3B, sphingomyelin phosphodiesterase acid-like 3; TNF, tumour necrosis factor; TSP1, thrombospondin 1.

In summary, by using tissue-specific TSP1-knockout mice, our research reveals a crucial role of macrophage-derived TSP1 in obesity-associated NAFLD/NASH development and progression. It also suggests macrophage-derived TSP1 as a therapeutic target for obesity-associated comorbidities.

Abbreviations

α-SMA, smooth muscle actin; ALT, alanine aminotransferase; AMLN, amylin liver NASH; ASMase, acid sphingomyelinase; AST, aspartate aminotransferase; BMDM, bone marrow-derived macrophage; DEG, differentially expressed gene; ECM, extracellular matrix; EC, endothelial cell; GPI, glycosylphosphatidylinositol; HFD, high-fat diet; HSC, hepatic stellate cell; IL-, interleukin-; KC, Kupffer cell; KEGG, Kyoto Encyclopedia of Genes and Genomes; LFD, low-fat diet; LPS, lipopolysaccharide; MDM, monocyte-derived macrophage; MP, mononuclear phagocyte; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; NF-κB, nuclear factor-κB; qPCR, quantitative PCR; scRNA-seq, single-cell RNA sequencing; SMPDL3B, sphingomyelin phosphodiesterase acid-like 3B; SREBP1c, sterol regulatory element-binding protein-1 c; TGF, transforming growth factor; Th, T helper type; TLR, Toll-like receptor; TNF, tumour necrosis factor; TSP1, thrombospondin 1; $Tsp1^{\Delta adipo}$, adipocyte-specific TSP1-knockout mice; $Tsp1^{\Delta m\phi}$, macrophage-specific TSP1-knockout mice; $Tsp1^{fl/fl}$, TSP1 floxed mice.

Financial support

This work was supported by the Department of Veterans Affairs Merit Review Award (to S.W.), a National Institutes of Health (NIH) Grant (DK098176 to S.W.) and an Institutional Development Award (IDEA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number P30 GM127211.

Conflict of interest

The authors declare no conflicts of interest that pertain to this study.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Conceived the study design: S.W.
Performed the experiments and analysed the data: T.G., R.M. and D.L.
Performed the liver pathology analysis: E.L.
Analysed the scRNA-seq data set: S.L.
Wrote the manuscript: R.M. and S.W.

Data availability statement

RNA-seq dataset has been deposited to GEO (Gene Expression Omnibus) with accession number GSE155973.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2020.100193.

References

Author names in bold designate shared co-first authorship

[1] Ratziu V, Bellentani S, Cortez-Pinto H, Day C, Marchesini G. A position statement on NAFLD/NASH based on the EASL 2009 special conference. J Hepatol 2010;53:372–384.

[2] Farrell GC, Wong VW, Chitturi S. NAFLD in Asia–as common and important as in the West. Nat Rev Gastroenterol Hepatol 2013;10:307–318.

[3] Vonghia L, Van Herck MA, Weyler J, Francque S. Targeting myeloid-derived cells: new frontiers in the treatment of non-alcoholic and alcoholic liver disease. Front Immunol 2019;10:563.

[4] McGettigan B, McMahan R, Orlicky D, Burchill M, Danhorn T, Francis P, et al. Dietary lipids differentially shape nonalcoholic steatohepatitis progression and the transcriptome of Kupffer cells and infiltrating macrophages. Hepatology 2019;70:67–83.

[5] Kazankov K, Jorgensen SMD, Thomsen KL, Moller HJ, Vilstrup H, George J, et al. The role of macrophages in nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. Nat Rev Gastroenterol Hepatol 2019;16:145–159.

[6] Li C, Menoret A, Farragher C, Ouyang Z, Bonin C, Holvoet P, et al. Single cell transcriptomics based-MacSpectrum reveals novel macrophage activation signatures in diseases. JCI Insight 2019;5:e126453.

[7] Liao CY, Song MJ, Gao Y, Mauer AS, Revzin A, Malhi H. Hepatocyte-derived lipotoxic extracellular vesicle sphingosine 1-phosphate induces macrophage chemotaxis. Front Immunol 2018;9:2980.

[8] Krenkel O, Puengel T, Govaere O, Abdallah AT, Mossanen JC, Kohlhepp M, et al. Therapeutic inhibition of inflammatory monocyte recruitment reduces steatohepatitis and liver fibrosis. Hepatology 2018;67:1270–1283.

[9] Oates JR, McKell MC, Moreno-Fernandez ME, Damen M, Deepe GS Jr, Qualls JE, et al. Macrophage function in the pathogenesis of non-alcoholic fatty liver disease: the Mac attack. Front Immunol 2019;10:2893.

[10] Krenkel O, Tacke F. Liver macrophages in tissue homeostasis and disease. Nat Rev Immunol 2017;17:306–321.

[11] **Xiong X**, **Kuang H**, **Ansari S**, **Liu T**, Gong J, Wang S, et al. Landscape of intercellular crosstalk in healthy and NASH liver revealed by single-cell secretome gene analysis. Mol Cell 2019;75:644–660.

[12] Ramachandran P, Dobie R, Wilson-Kanamori JR, Dora EF, Henderson BEP, Luu NT, et al. Resolving the fibrotic niche of human liver cirrhosis at single-cell level. Nature 2019;575:512–518.

[13] Wang S. Thrombospondin1 deficiency attenuates obesity-associated microvascular complications in ApoE-/- mice. Sci Rep 2015;10:e0121403.

[14] Wang S, Lincoln TM, Murphy-Ullrich JE. Glucose downregulation of PKG-I protein mediates increased thrombospondin1-dependent TGF-{beta} activity in vascular smooth muscle cells. Am J Physiol Cell Physiol 2010;298:C1188–C1197.

[15] Lopez-Dee Z, Pidcock K, Gutierrez LS. Thrombospondin-1: multiple paths to inflammation. Mediators Inflamm 2011;2011:296069.

[16] Li Y, Turpin CP, Wang S. Role of thrombospondin 1 in liver diseases. Hepatol Res 2016;47:186–193.

[17] Min-DeBartolo J, Schlerman F, Akare S, Wang J, McMahon J, Zhan Y, et al. Thrombospondin-I is a critical modulator in non-alcoholic steatohepatitis (NASH). PLoS One 2019;14:e0226854.

[18] Li Y, Qi X, Tong X, Wang S. Thrombospondin 1 activates the macrophage Toll-like receptor 4 pathway. Cell Mol Immunol 2013;19:32.

[19] Li Y, Tong X, Rumala C, Clemons K, Wang S. Thrombospondin1 deficiency reduces obesity-associated inflammation and improves insulin sensitivity in a diet-induced obese mouse model. PLoS One 2011;6:e26656.

[20] Memetimin H, Li D, Tan K, Zhou C, Liang Y, Wu Y, et al. Myeloid-specific deletion of thrombospondin 1 protects against inflammation and insulin resistance in long-term diet-induced obese male mice. Am J Physiol Endocrinol Metab 2018;315:E1194–E1203.

[21] Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005;41:1313–1321.

[22] Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957;226:497–509.

[23] Teratani T, Tomita K, Furuhashi H, Sugihara N, Higashiyama M, Nishikawa M, et al. Lipoprotein lipase up-regulation in hepatic stellate cells exacerbates liver fibrosis in nonalcoholic steatohepatitis in mice. Hepatol Commun 2019;3:1098–1112.

[24] Byun JS, Suh YG, Yi HS, Lee YS, Jeong WI. Activation of toll-like receptor 3 attenuates alcoholic liver injury by stimulating Kupffer cells and stellate cells to produce interleukin-10 in mice. J Hepatol 2013;58:342–349.

[25] Lawler J, Sunday M, Thibert V, Duquette M, George EL, Rayburn H, et al. Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. J Clin Invest 1998;101:982–992.

[26] El-Youssef M, Mu Y, Huang L, Stellmach V, Crawford SE. Increased expression of transforming growth factor-beta1 and thrombospondin-1 in congenital hepatic fibrosis: possible role of the hepatic stellate cell. J Pediatr Gastroenterol Nutr 1999;28:386–392.

[27] Smalling RL, Delker DA, Zhang Y, Nieto N, McGuiness MS, Liu S, et al. Genome-wide transcriptome analysis identifies novel gene signatures implicated in human chronic liver disease. Am J Physiol Gastrointest Liver Physiol 2013;305:G364–G374.

[28] Clapper JR, Hendricks MD, Gu G, Wittmer C, Dolman CS, Herich J, et al. Diet-induced mouse model of fatty liver disease and nonalcoholic steatohepatitis reflecting clinical disease progression and methods of assessment. Am J Physiol Gastrointest Liver Physiol 2013;305:G483–G495.

[29] Garcia-Diaz DF, Arellano AV, Milagro FI, Moreno-Aliaga MJ, Portillo MP, Martinez JA, et al. Glucose and insulin modify thrombospondin 1 expression and secretion in primary adipocytes from diet-induced obese rats. J Physiol Biochem 2011;67:453–461.

[30] Varma V, Yao-Borengasser A, Bodles AM, Rasouli N, Phanavanh B, Nolen GT, et al. Thrombospondin-1 is an adipokine associated with obesity, adipose inflammation, and insulin resistance. Diabetes 2008;57:432–439.

[31] Heinz LX, Baumann CL, Koberlin MS, Snijder B, Gawish R, Shui G, et al. The lipid-modifying enzyme SMPDL3B negatively regulates innate immunity. Cell Rep 2015;11:1919–1928.

[32] Malehmir M, Pfister D, Gallage S, Szydlowska M, Inverso D, Kotsiliti E, et al. Platelet GPIbalpha is a mediator and potential interventional target for NASH and subsequent liver cancer. Nat Med 2019;25:641–655.

[33] Xu L, Kitade H, Ni Y, Ota T. Roles of chemokines and chemokine receptors in obesity-associated insulin resistance and nonalcoholic fatty liver disease. Biomolecules 2015;5:1563–1579.

[34] Reid DT, Reyes JL, McDonald BA, Vo T, Reimer RA, Eksteen B. Kupffer cells undergo fundamental changes during the development of experimental NASH and are critical in initiating liver damage and inflammation. PLoS One 2016;11:e0159524.

[35] Huang W, Metlakunta A, Dedousis N, Zhang P, Sipula I, Dube JJ, et al. Depletion of liver Kupffer cells prevents the development of diet-induced hepatic steatosis and insulin resistance. Diabetes 2010;59:347–357.

[36] Endo M, Masaki T, Seike M, Yoshimatsu H. TNF-alpha induces hepatic steatosis in mice by enhancing gene expression of sterol regulatory element binding protein-1c (SREBP-1c). Exp Biol Med 2007;232:614–621.

[37] Ohtani N, Kawada N. Role of the gut-liver axis in liver inflammation, fibrosis, and cancer: a special focus on the gut microbiota relationship. Hepatol Commun 2019;3:456–470.

[38] Cheung O, Sanyal AJ. Abnormalities of lipid metabolism in nonalcoholic fatty liver disease. Semin Liver Dis 2008;28:351–359.

[39] Farrell GC. PNPLeAse get the fats right: does lipogenesis or lipolysis cause NASH? Hepatology 2010;52:818–821.

[40] Gokyu M, Kobayashi H, Nanbara H, Sudo T, Ikeda Y, Suda T, et al. Thrombospondin-1 production is enhanced by *Porphyromonas gingivalis* lipopolysaccharide in THP-1 cells. PLoS One 2014;9:e115107.

[41] Fordham JB, Hua J, Morwood SR, Schewitz-Bowers LP, Copland DA, Dick AD, et al. Environmental conditioning in the control of macrophage thrombospondin-1 production. Sci Rep 2012;2:512.

[42] Finlin BS, Zhu B, Starnes CP, McGehee RE Jr, Peterson CA, Kern PA. Regulation of thrombospondin-1 expression in alternatively activated macrophages and adipocytes: role of cellular cross talk and omega-3 fatty acids. J Nutr Biochem 2013;24:1571–1579.

[43] Gorelik A, Heinz LX, Illes K, Superti-Furga G, Nagar B. Crystal structure of the acid sphingomyelinase-like phosphodiesterase SMPDL3B provides insights into determinants of substrate specificity. J Biol Chem 2016;291:24054–24064.

[44] Mitrofanova A, Mallela SK, Ducasa GM, Yoo TH, Rosenfeld-Gur E, Zelnik ID, et al. SMPDL3b modulates insulin receptor signaling in diabetic kidney disease. Nat Commun 2019;10:2692.

[45] Yoo TH, Pedigo CE, Guzman J, Correa-Medina M, Wei C, Villarreal R, et al. Sphingomyelinase-like phosphodiesterase 3b expression levels determine podocyte injury phenotypes in glomerular disease. J Am Soc Nephrol 2015;26:133–147.
[46] Regnier M, Polizzi A, Guillou H, Loiseau N. Sphingolipid metabolism in non-alcoholic fatty liver diseases. Biochimie 2019;159:9–22.
[47] Chavez-Tapia NC, Rosso N, Tiribelli C. Effect of intracellular lipid accumulation in a new model of non-alcoholic fatty liver disease. BMC Gastroenterol 2012;12:20.
[48] Breitkopf K, Sawitza I, Westhoff JH, Wickert L, Dooley S, Gressner AM. Thrombospondin 1 acts as a strong promoter of transforming growth factor beta effects via two distinct mechanisms in hepatic stellate cells. Gut 2005;54:673–681.
[49] Venkatraman L, Tucker-Kellogg L. The CD47-binding peptide of thrombospondin-1 induces defenestration of liver sinusoidal endothelial cells. Liver Int 2013;33:1386–1397.