

Supplementary data

Materials and Methods

Histological analysis

Liver tissue samples were fixed with 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin and eosin (H&E). Hepatic fibrosis was analyzed by Masson-trichrome staining. Images were captured using a BX60 camera (Olympus, Tokyo, Japan). The analysis was performed by investigators blinded to the experimental design. Slides were blindly evaluated and scored for steatosis, ballooning, and inflammation. Steatosis (0–4): 0 = <5%; 1 = 5–25%; 2 = 25–50%; 3 = 50–75%; 4 = 75–100%. Inflammation (0–4): 0 = absent; 1 = minimal (0–1 focus per 20×field); 2 = mild (two foci); 3 = moderate (three foci); 4 = severe (four or more foci). Ballooning (0–3): 0 = absent; 1 = mild (focal involving fewer than three hepatocytes); 2 = moderate (focal involving more than three hepatocytes or multifocal); 3 = severe (multifocal with more than two foci of three or more hepatocytes).

Biochemical analysis

Levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum were determined according to the enzymatic kinetic method by using an automatic biochemical analyzer (Olympus).

Levels of cholesterol in serum and liver were determined using a Cholesterol/Cholesteryl Ester Quantitation Kit (BioVision, Milpitas, CA, USA).

Levels of triacylglycerols in serum and livers were determined MaxDiscovery™ Triglycerides Enzymatic Assay Kit (Bioo, Austin, Texas USA).

Levels of IL-6 and TNF- α in livers were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA).

The final results of cholesterol, triacylglycerols, IL-6 and TNF- α in livers were normalized to total protein concentration.

Determination of hydroxyproline content

Samples from the livers were dried at 100°C for 24 hrs. The tissue was then hydrolyzed with 100 μ l of 4-mol/L NaOH at 120°C for 10 min. After neutralization with 100 μ l of 1.4-mmol/L citrate, oxidation of the hydrolyte (10 μ l) was initiated with the addition of chloramine-T reagent (1.0 ml) at 25°C. The reaction was stopped after 20 min by adding Ehrlich aldehyde reagent (1.0 ml). After incubation of samples at 65°C for 15 min, the absorbance of developed chromophore at 550 nm was measured. Levels of hydroxyproline were calculated from the standard curve and expressed as micrograms per milligram of sample dry weight.

Real-time PCR

In brief, the total RNA was isolated with Trizol according to manufacturer's protocol (Life Technologies Inc., Gaithersburg, USA) and reverse-transcribed using GenAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA). The sequences of forward and reverse primers are listed in Table S2. PCR cycles were programmed on

an ABI Prism 7000 Sequence Detection System (Applied Biosystems) and SYBR green PCR Master Mix was used. The relative quantities of target transcripts were calculated from duplicate samples after normalization to 18S.

Quantification nuclear DNA and mitochondrial DNA in plasma

DNA was extracted from 200ul of plasma with QIAmp DNA mini kit (QIAGEN, Duesseldorf, Germany) according to the manufacturer's instructions and a real-time qPCR was performed for the quantification of nDNA and mtDNA with a LightCycler 480 system (Roche, Basel, Switzerland). Quantitative real-time PCR (QPCR) was performed for nDNA and mtDNA using commercial primer probe sets and SsoAdvanced Universal Probes Supermix (BioRad, Hercules, CA, USA) in LightCycler 480 system (Roche). nDNA was quantified using mouse β -actin primers/Taqman 5'FAM-3'MGB probe (BioRad). mtDNA was quantified using mouse mt-ATP6 primers/Taqman 5'FAM-3'MGB probe (BioRad).

Mitochondrial isolation from hepatocytes of chow (Control diet, CD) and HFD fed mice and DNA extraction

Hepatocytes from CD and HFD mice were isolated by collagenase perfusion. Fresh hepatocytes were seeded on collagen-coated 6 well plates. Cells were washed after 3h to remove dead cells and fresh culture medium was added to incubate the cells overnight. Mitochondria were isolated using by Mitochondria Isolation Kit (Thermo Scientific). Mitochondrial DNA was purified using by QIAamp DNA mini kit (QIAGEN).

TLR9 Knockdown

TLR9 shRNA (m) lentiviral particles (sc-40271-V, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and negative lentiviral particles were obtained from Santa Cruz Inc. KCs were transfected with TLR9 shRNA (m) lentiviral particles or negative lentiviral particles for 12 hrs.

Western blotting analysis

The protein concentration was determined with bovine serum albumin as a standard by a Bradford assay. Equal amount of protein preparations (15 μ g) were run on SDS-polyacrylamide gels, electrotransferred to polyvinylidene difluoride membranes, and blotted with primary antibodies overnight at 4°C. Then, they were blotted with HRP-conjugated secondary antibody (STING, ab92605, Abcam, Cambridge, MA, USA; TLR9, sc-47723, Santa Cruz Biotechnology). Immunoreactive bands were detected by a chemiluminescent reaction (ECL kit, GE Healthcare, Little Chalfont, Buckinghamshire, UK). The β -tubulin (Beyotime Institute of Biotechnology, Shanghai, China, AT809) was used as a loading control.

Luciferase assay

The NF- κ B-dependent Luciferase Reporter Vector (pGL3-NF- κ B) was ordered from Promega (Madison, WI, USA). Primary cultured KCs were seeded on 24-well plates

at a density of 10^6 /well on the day prior to transfection. The NF- κ B luciferase reporter and pRL-Thymidine Kinase (pRL-TK, Promega) were co-transfected into KCs using FUGENE®HD Transfection reagent (Roche). Luciferase activities were normalized to TK activities. After 6 hours, the cells were harvested, washed three times with PBS and lysed in 100 μ l of the passive lysis buffer (Promega). Cell debris was removed by centrifuging, and the supernatant was used for luciferase assays using Single-Mode Microplate Readers SpectraMax® (Molecular Devices, Sunnyvale, CA, USA).

Table S1: Composition of the control diet and MCD diet formulas

Ingredients (g)	MCD diet	Control diet
Protein	17.2	17.2
Carbohydrate	65.9	65.5
Fat	9.9	9.9
L-Alanine	3.5	3.5
L-Arginine	12.1	12.1
L-Asparagine-H ₂ O	6	6
L-Aspartate	3.5	3.5
L-Cystine	3.5	3.5
L-Glutamine	40	40
Glycine	23.3	23.3
L-Histidine-HCl-H ₂ O	4.5	4.5
L-Isoleucine	8.2	8.2
L-Leucine	11.1	11.1
L-Lysine-HCl	18	18
L-Phenylalanine	7.5	7.5
L-Proline	3.5	3.5
L-Serine	3.5	3.5
L-Threonine	8.2	8.2
L-Tryptophan	1.8	1.8
L-Tyrosine	5	5
L-Valine	8.2	8.2
Total L-Amino Acids	171.4	171.4
Sucrose	455.3	450.3
Corn starch	150	150
Maltodextrin	50	50
Cellulose	30	30
Corn oil	100	100
Mineral mix	35	35
Sodium bicarbonate	7.5	7.5
Vitamin mix V10001	10	10
DL-Methionine	0	3
Choline bitrate	0	2

Table S2. Sequences of oligonucleotides used as primers

Target gene		Sequence (5'-3')
F4/80	Sense	TGACTCACCTTGTGGTCCTAA
	Antisense	CTTCCCAGAATCCAGTCTTTCC
TNF- α	Sense	ACGTGGAAGTGGCAGAAGAG
	Antisense	GGTTGTCTTTGAGATCCATGC
Col1A1	Sense	ATGTTTCAGCTTTGTGGACCTC
	Antisense	CAGAAAGCACAGCACTCGC
α -SMA	Sense	GTCCCAGACATCAGGGAGTAA
	Antisense	TCGGATACTTCAGCGTCAGGA
IL-6	Sense	ATGAAGTTCCTCTCTGCAAGAGACT
	Antisense	CACTAGGTTTGTTTAATCTC
IL-1 β	Sense	GAAATGCCACCTTTTGACAGTG
	Antisense	TGGATGCTCTCATCAGGACAG
SREBP-1c	Sense	AACGTCACTTCCAGCTAGAC
	Antisense	CCACTAAGGTGCCTACAGAGC
FAS	Sense	AGCGGCCATTTCCATTGCC
	Antisense	CCATGCCCAGAGGGTGGTTG
PPAR α	Sense	AGAGCCCCATCTGTCCTCTC
	Antisense	ACTGGTAGTCTGCAAAACCAA
CD36	Sense	ATGGGCTGTGATCGGAACTG
	Antisense	TTTGCCACGTCATCTGGGTTT
IFN- α	Sense	GCTAGGCTCTGTGCTTTCCTGAT
	Antisense	AGGTCACATCCTAGAGAGCAGGTT
IFN- β	Sense	AGCTCCAAGAAAGGACGAACAT
	Antisense	GCCCTGTAGGTGAGGGTTGATCT
18S	Sense	ACGGAAGGGCACCACCAGGA
	Antisense	CACCACCACCCACGGAATCG

α -SMA, alpha smooth muscle actin; PPAR α , peroxisome proliferator-activated receptor α ; SREBP-1c, sterol regulatory element binding protein 1c; FAS, fatty acid synthase; STING, stimulator of IFN genes; IFN, interferon

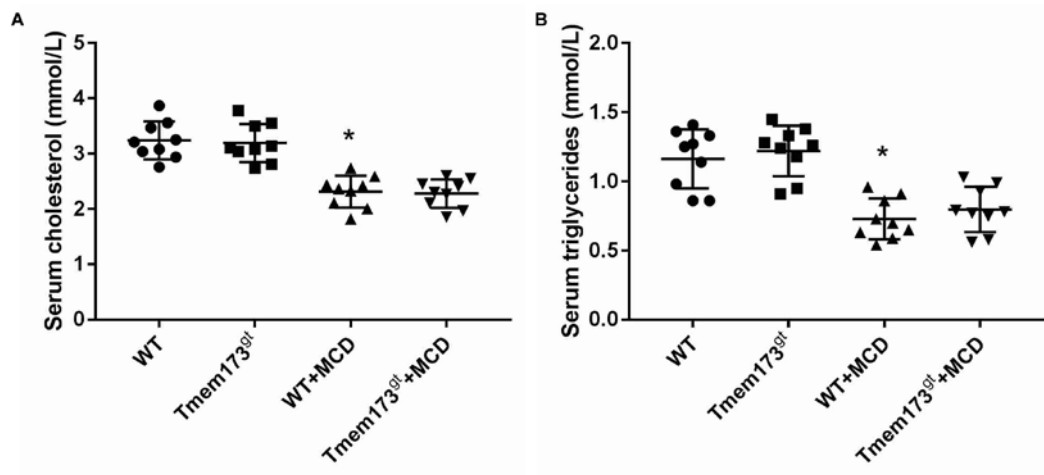


Figure S1. Levels of cholesterol (A) and triglycerides (B) in serum of mice fed with MCD for 8 weeks. n=9 in each group. Values are means \pm SD; * $P < 0.05$ versus WT mice fed with control diet. Statistical significance was determined using 1-way ANOVA followed by Tukey-Kramer multiple comparisons test.

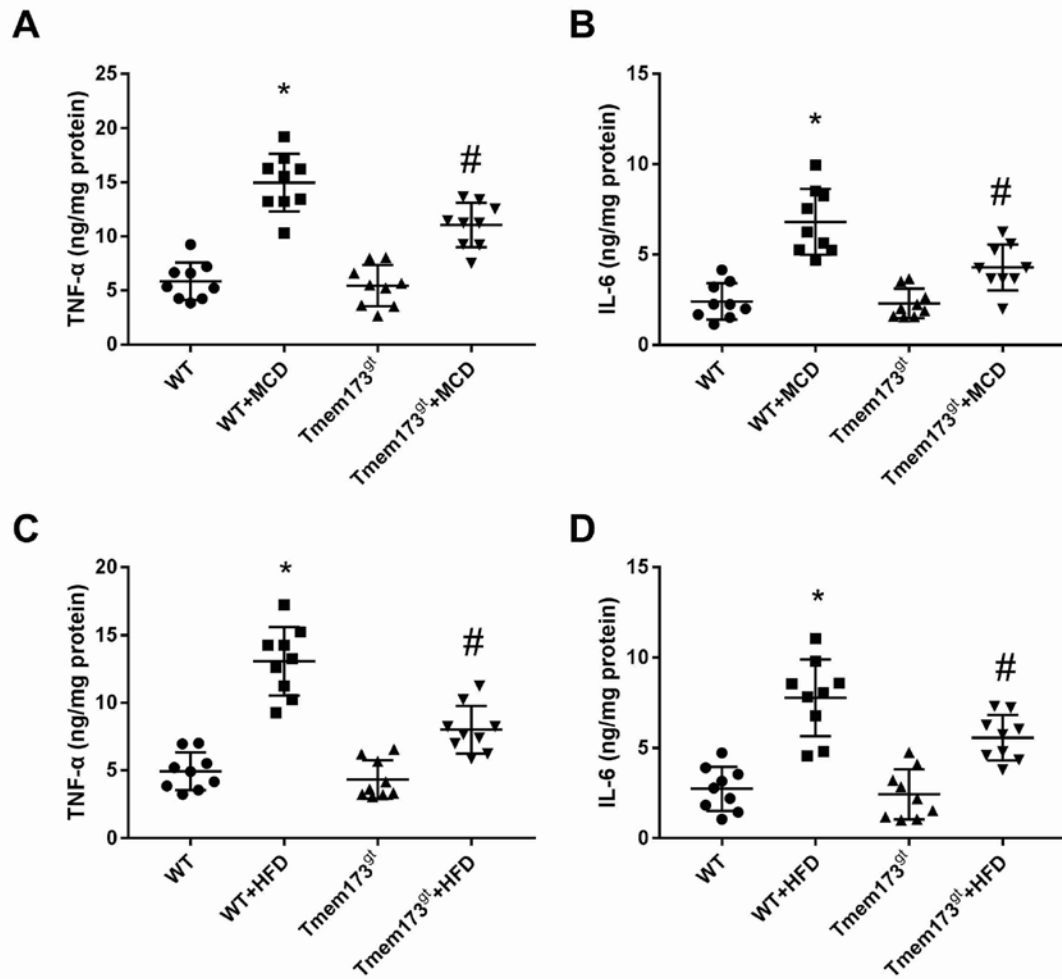


Figure S2. Deficiency of STING attenuated MCD or HFD-induced inflammation in livers of mice. WT or STING-deficient mice (Tmem173^{gt}) were exposed to MCD for 8 weeks or HFD for 26 weeks to induce NASH. Graphs showed the protein levels of TNF- α (A, C) and IL-6 (B, D) in livers of mice. n=9 in each group. Values are means \pm SD; * P < 0.05 versus WT mice fed with control diet; # P < 0.05 versus WT mice fed with MCD or HFD. Statistical significance was determined using 1-way ANOVA followed by Tukey-Kramer multiple comparisons test.

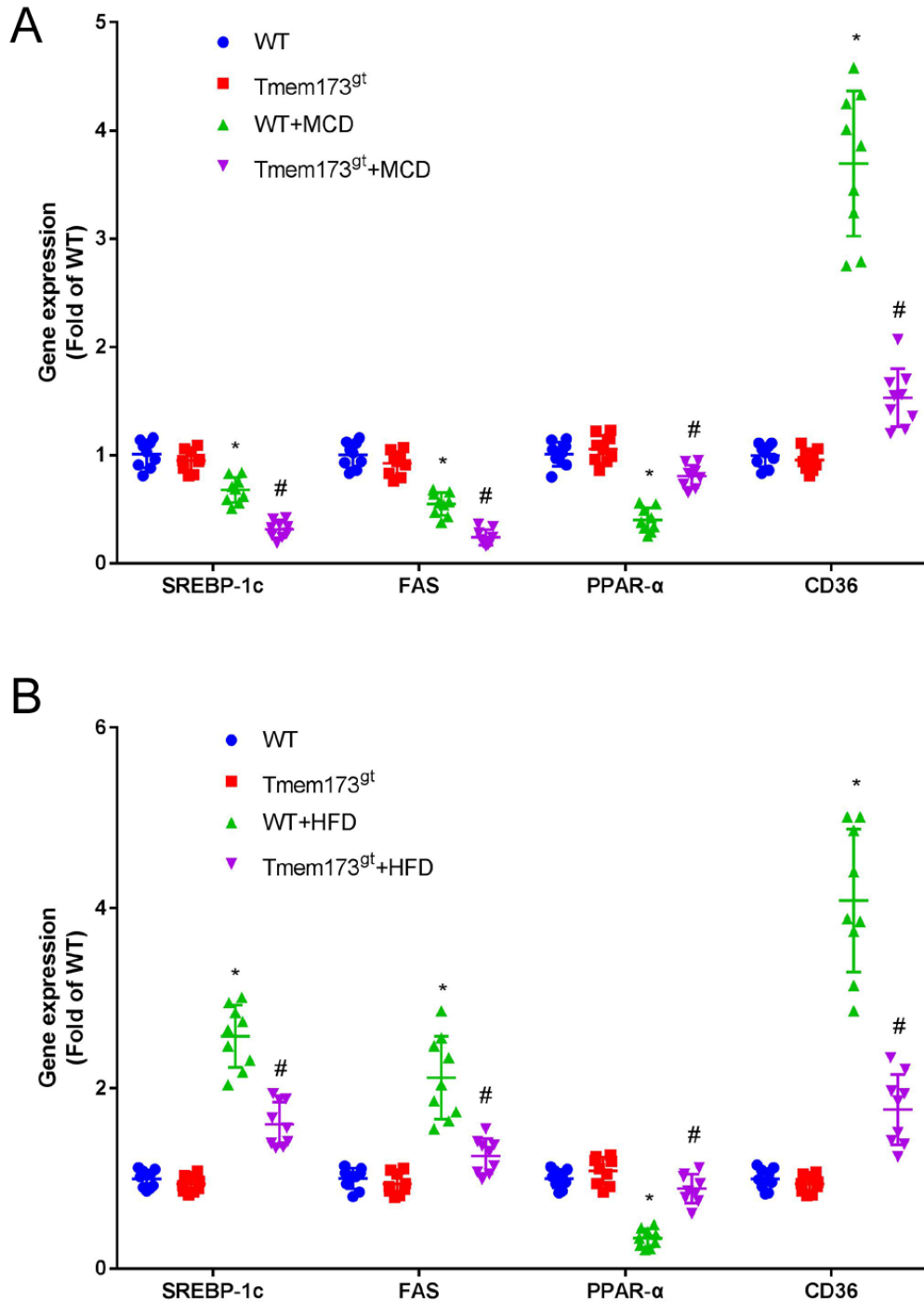


Figure S3. Deficiency of STING modulated mRNA expression of genes associated with fatty acid metabolism in livers of mice. WT or STING-deficient mice (Tmem173^{gt}) were exposed to MCD (A) for 8 weeks or HFD (B) for 26 weeks to induce NASH. Graphs showed the mRNA expression of SREBP-1c, FAS, CD36, and PPAR- α in livers. n=9 in each group. Values are means \pm SD; * P < 0.05 versus WT mice fed with control diet; # P < 0.05 versus WT mice fed with MCD or HFD. Statistical significance was determined using 1-way ANOVA followed by Tukey-Kramer multiple comparisons test.

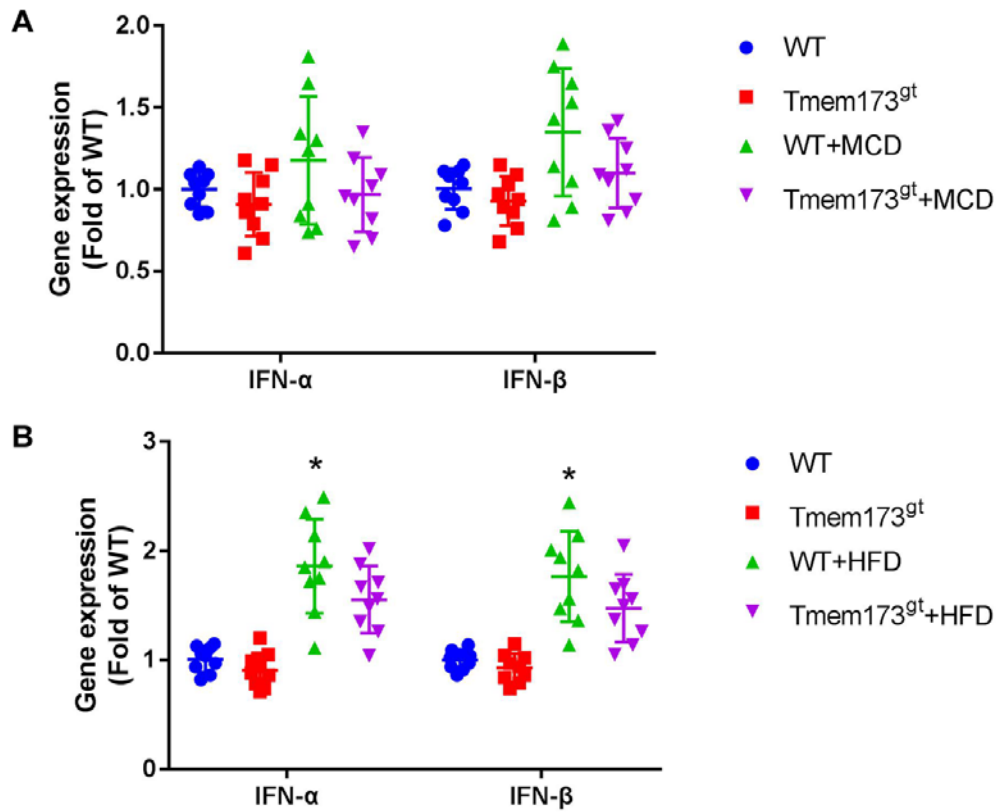


Figure S4. Deficiency of STING did not affect mRNA expression of type I IFN (including IFN- α and β) in livers of mice fed with control chow or MCD/HFD. WT or STING-deficient mice (Tmem173^{gt}) were exposed to MCD (A) for 8 weeks or HFD (B) for 26 weeks to induce NASH. Graphs showed the mRNA expression of IFN- α and IFN- β in liver of mice fed with MCD (A) or HFD (B). n=9 in each group. Values are means \pm SD; * P < 0.05 versus WT mice fed with control diet. Statistical significance was determined using 1-way ANOVA followed by Tukey-Kramer multiple comparisons test.

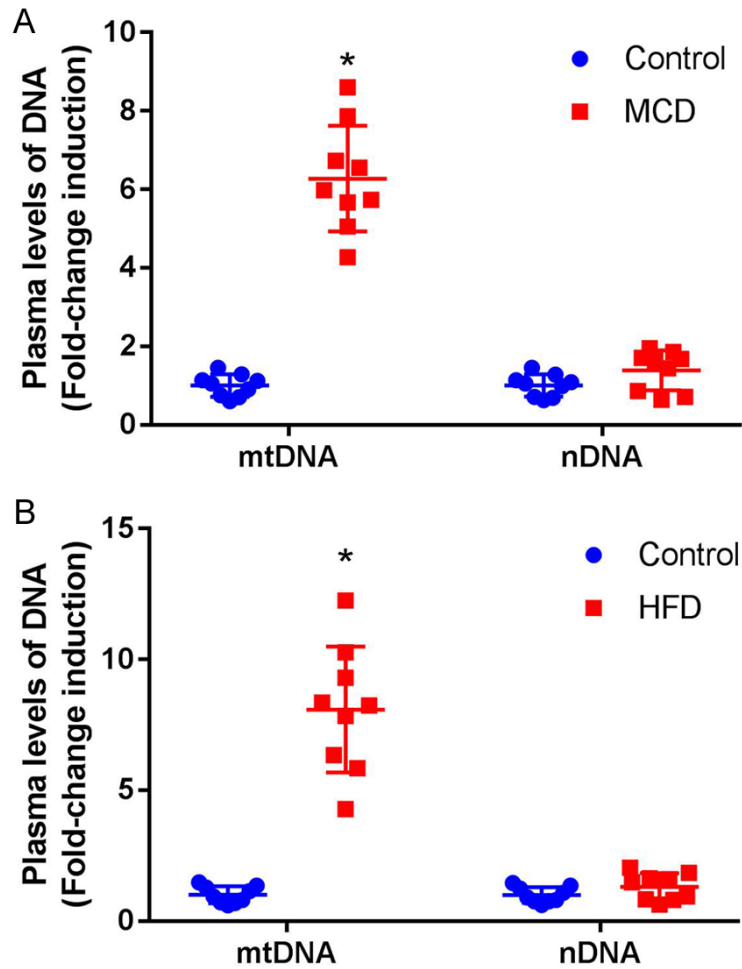


Figure S5. Plasma from NASH mice had increased mtDNA levels. WT mice were exposed to MCD for 8 weeks or HFD for 26 weeks to induce NASH. Graphs showed the levels of mtDNA and nDNA in plasma of mice fed with MCD (A) or HFD (B). n=9 in each group. Values are means \pm SD; * $P < 0.05$ versus WT mice fed with control diet. Statistical significance was determined using 1-way ANOVA followed by Tukey-Kramer multiple comparisons test.

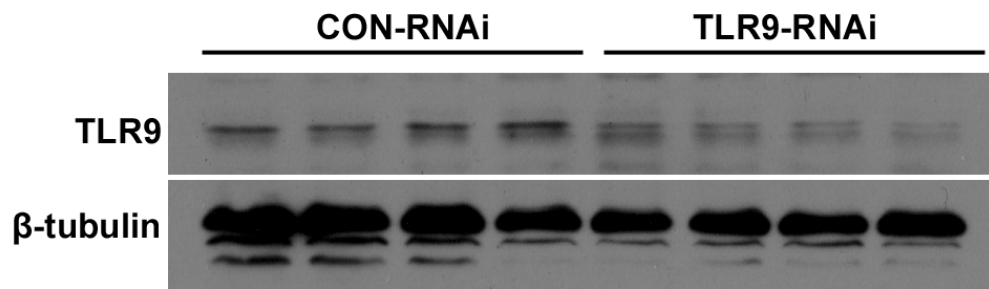


Figure S6. KCs isolated from WT mice were transfected with TLR9 shRNA lentiviral particles or negative lentiviral particles according to manufacturer's instructions. 24 hrs later, TLR9 protein expression was determined by Western blotting analysis.

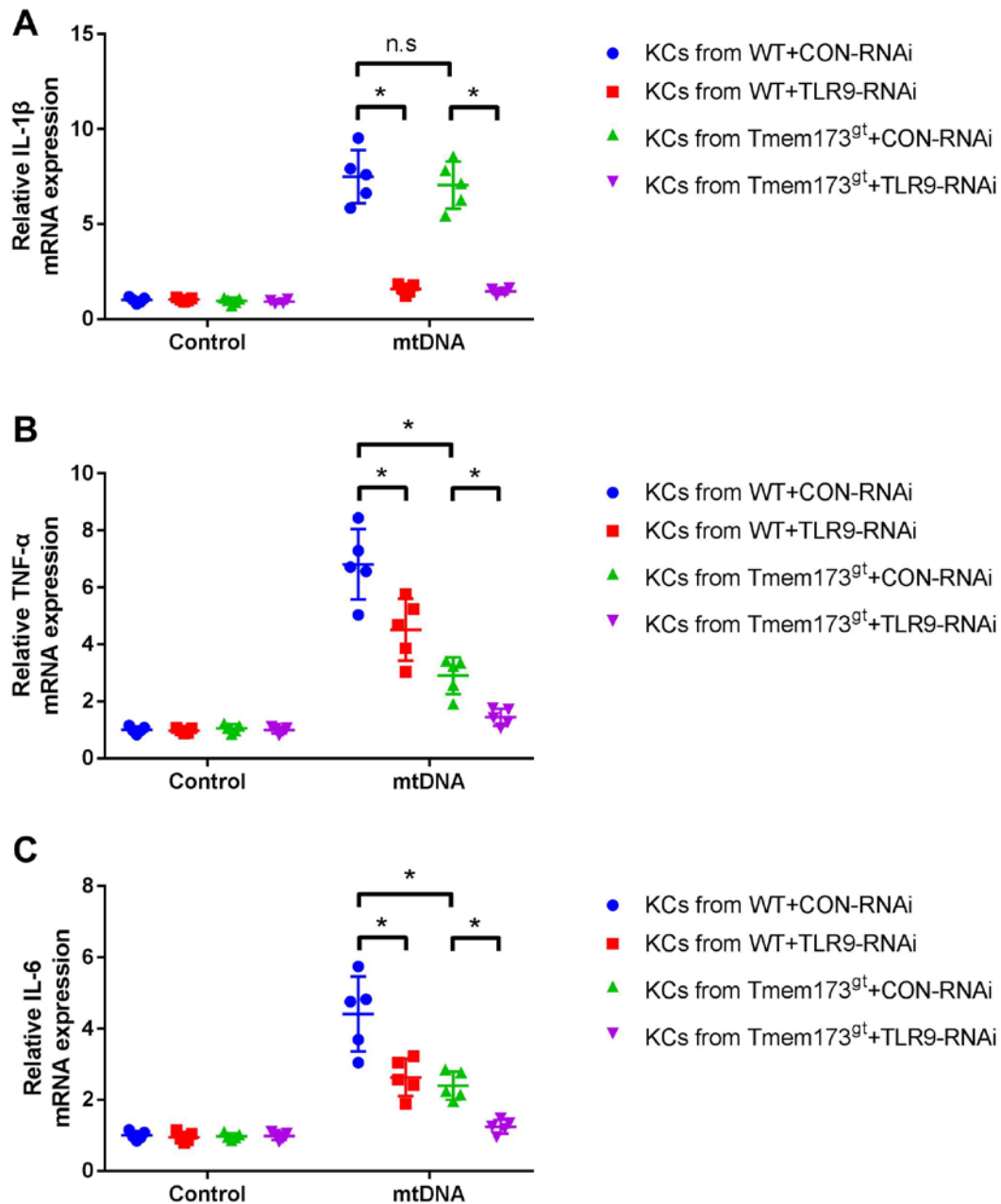


Figure S7. KCs isolated from WT mice and STING-deficient mice fed with control chow were transfected with TLR9 shRNA lentiviral particles or negative lentiviral particles according to manufacturer's instructions. 24 hrs later, the cells were exposed to the mtDNA (100ng/ml) from hepatocytes of HFD (mtDNA HFD) fed mice or not for 12 hrs. The mRNA expression of IL-1 β (A), TNF- α (B), and IL-6 (C) was determined by RT-PCR. The in vitro experiments were performed five times and each experiment was performed with replicates. Statistical significance was determined using 1-way ANOVA followed by Tukey-Kramer multiple comparisons test. * P <0.05.

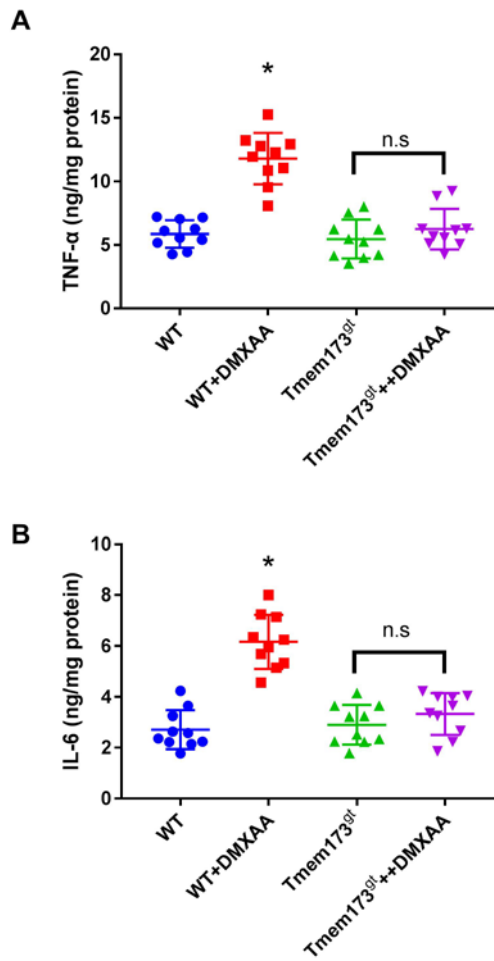


Figure S8. DMXAA induced inflammation in livers of mice. WT or STING-deficient mice (Tmem173^{et}) were exposed to DMXAA (a known activator of mouse STING, 25mg/kg/2 days, i.p.) for 8 weeks. Graphs showed the protein levels of TNF- α (A) and IL-6 (B) in livers of mice. n=10 in each group. Values are means \pm SD; * P < 0.05 versus WT mice fed with control diet; # P < 0.05 versus WT mice fed with MCD or HFD. Statistical significance was determined using 1-way ANOVA followed by Tukey-Kramer multiple comparisons test.