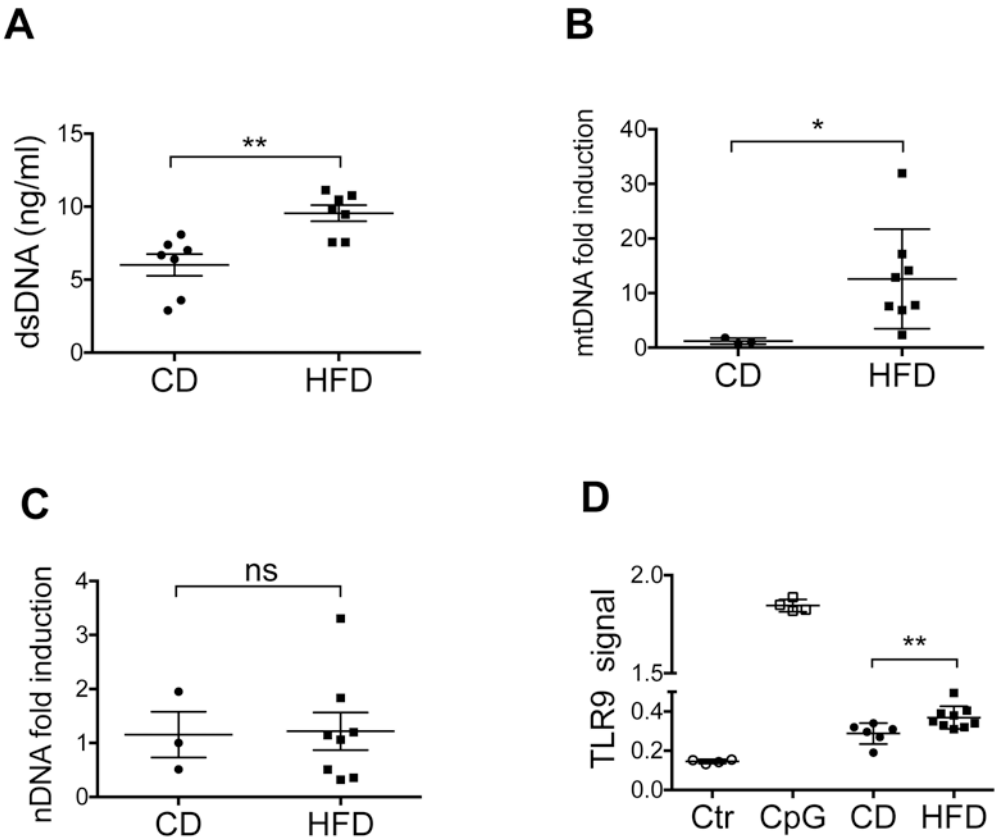
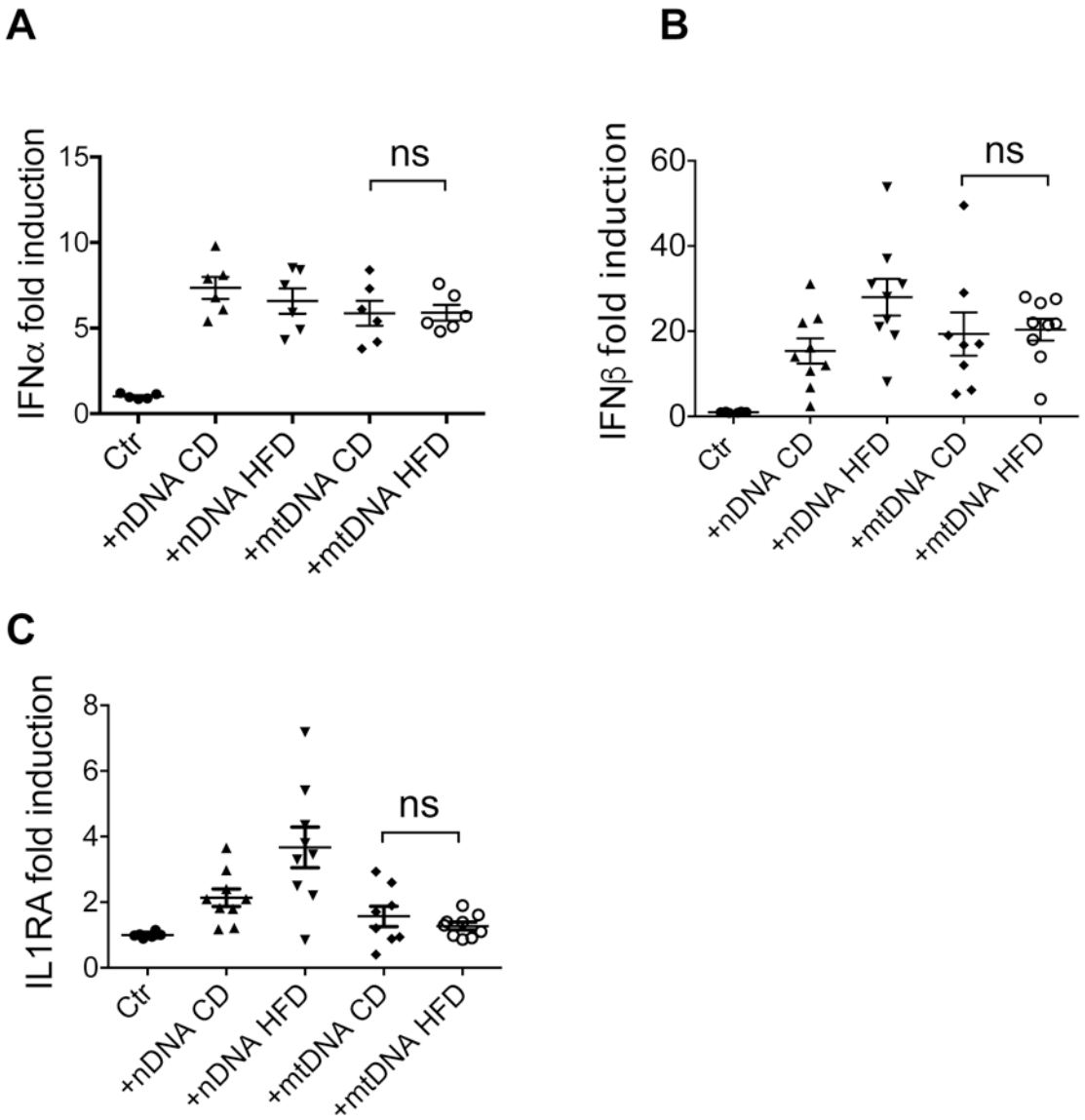


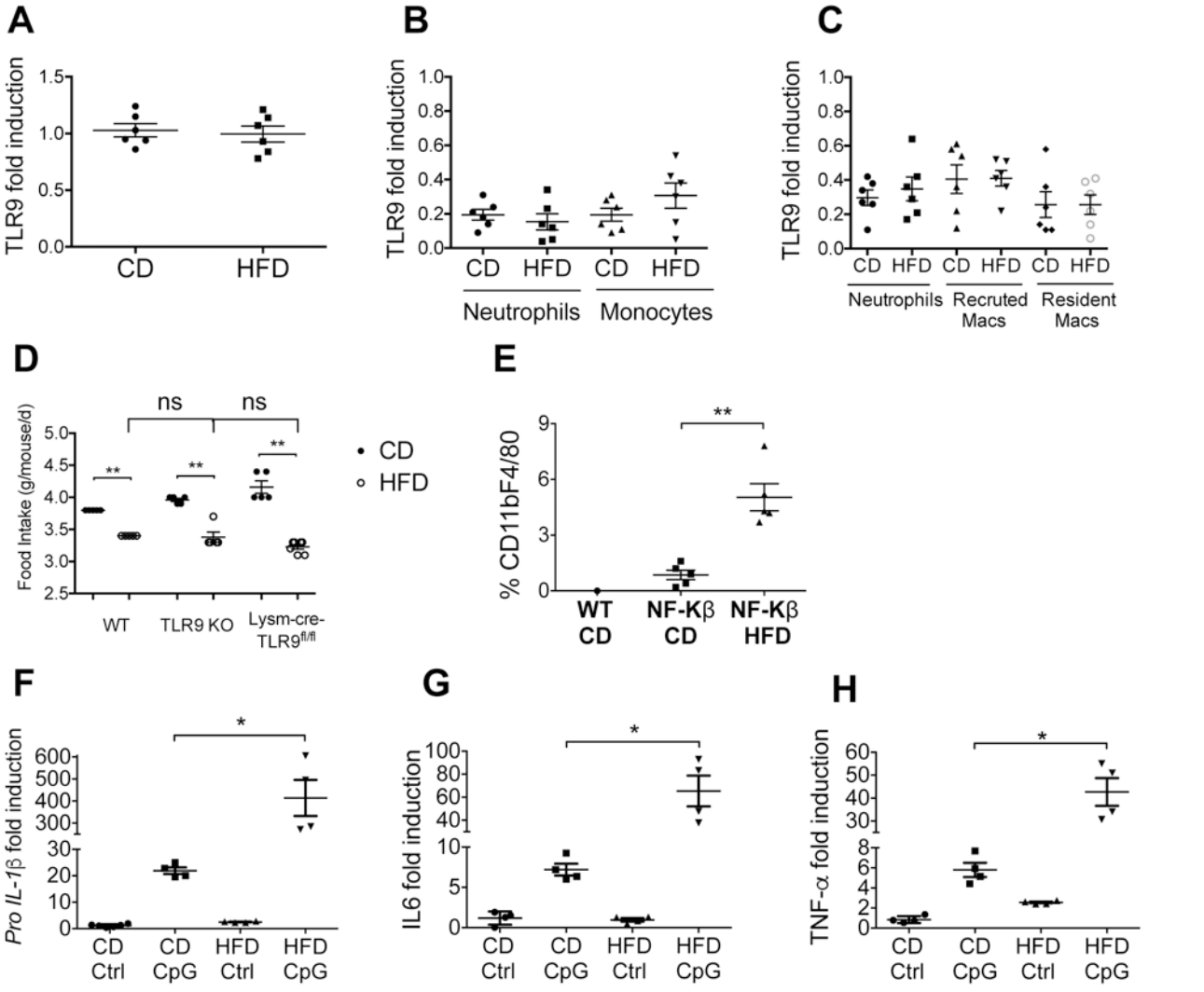
Supplementary figure 1. Plasma from mice on a high fat diet (HFD) has increased mtDNA, and greater ability to activate TLR9.

Plasma from mice on a HFD (n=7) has an increase in total DNA and mtDNA, but not nDNA. Plasma from mice on a HFD (n=9) has a greater ability to activate TLR9 on a TLR9 reporter cell line **(A-D)**. Data are represented as mean+/- SEM. * $P<0.05$, and ** $P<0.01$, by Mann-Whitney Test.

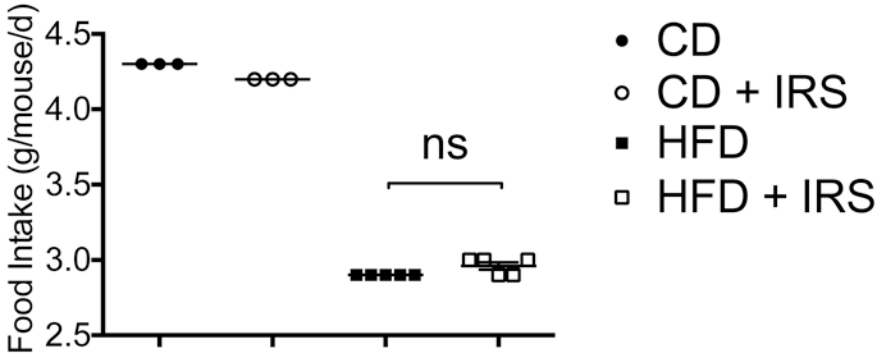


Supplementary figure 2. mtDNA from hepatocytes of HFD fed mice does not increase type 1 interferon and IL-1RA production from primary mouse Kupffer cells.

nDNA and mtDNA (100 ng/ml) from hepatocytes of chow (n=9 and n=7, respectively) and HFD (n=8 and n=7, respectively) fed mice was added to KC for 8 hrs and the expression of the IFN-a, IFN-b and IL-1 receptor antagonist IL-1RA was quantified by qPCR. Macrophages without added DNA were used as controls **(A-C)**.

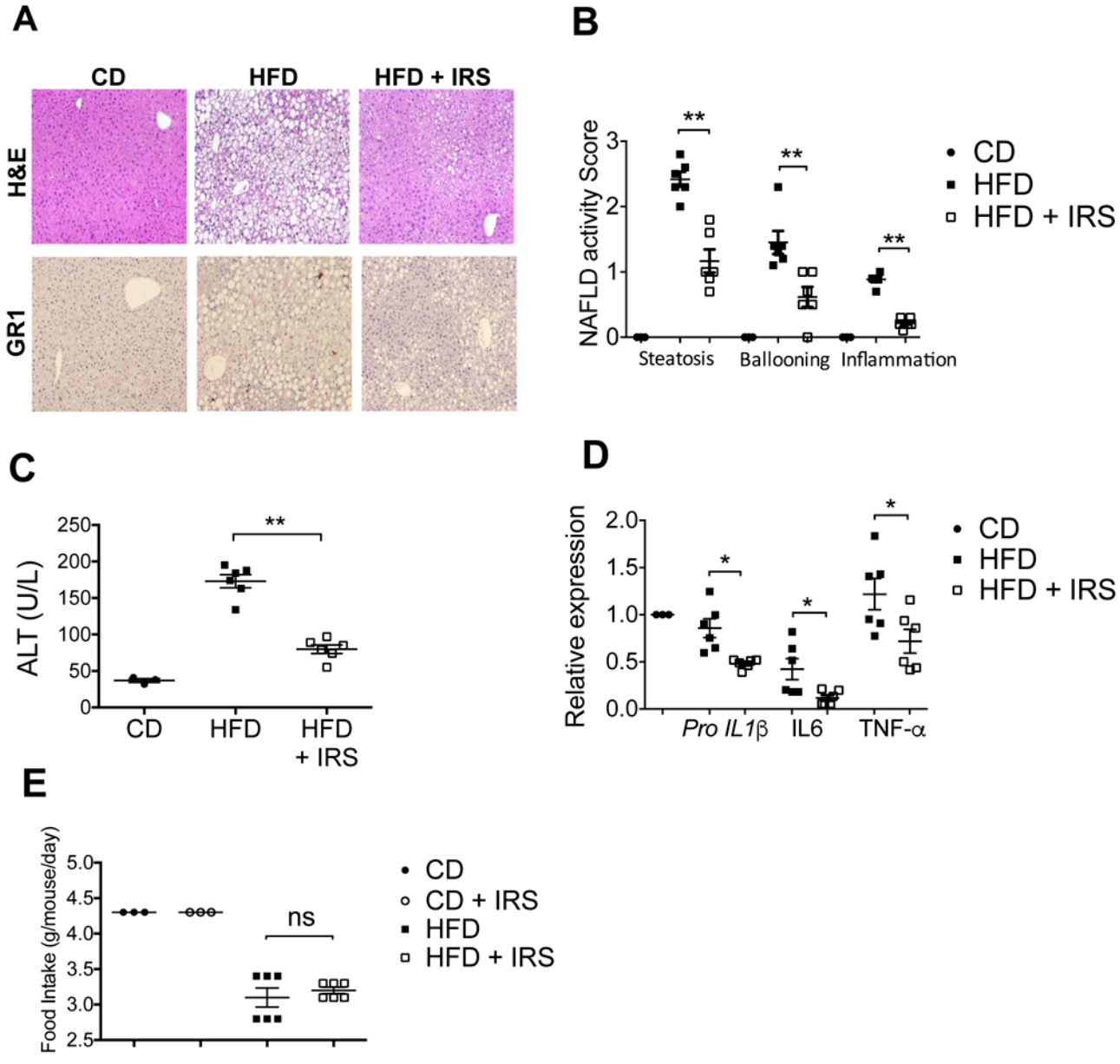


Supplementary figure 3. Increased responsiveness of Kupffer cells from high fat diet mice to TLR9 agonists. Relative expression of TLR9 in lysm-cre-TLR9$^{fl/fl}$ mice on chow (n=6) and HFD (n=6) from **(A)** splenic T cells, **(B)** peripheral blood neutrophils and monocytes, **(C)** liver neutrophils, recruited macrophages and resident macrophages. **(D)** have the same food intake as controls. **(E)** Up-regulation of NF-kB pathway in KC from NF-kB reporter mice on a HFD (CD n=5, HFD n=5). **(F-H)** KC were isolated from the livers of chow fed (n=4) and HFD (n=4) mice and stimulated with a TLR9 agonist (CPG ODN 1668 at concentration of 3uM for 8 hrs). There is greater up-regulation of inflammatory cytokine transcripts in KC from wild-type mice on a HFD, as compared to KC from chow fed diet. Data are represented as mean+/- SEM. * P<0.05, and ** P<0.01, by Mann-Whitney Test.



Supplementary figure 4. No change in food intake in wild-type mice given IRS954.

Wild-type mice were placed on a CD with (n=3) and without IRS (n=3), and also on a HFD with (n=8) and without IRS (n=8). The administration of IRS did not result in reduced food intake.



Supplementary figure 5. Weekly administration of a TLR9 antagonist after eight weeks of a high fat diet protects from NASH. (A-B) In a high fat model of NASH weekly subcutaneous administration of a TLR9 antagonist (IRS954) started after 8 weeks of a HFD, and analyzed at 12 weeks of a HD (n=6), results in reduced steatosis, ballooning and inflammation, **(C)** reduced serum ALT, and **(D)** reduced up-regulation of inflammatory cytokines, compared to HFD without IRS (n=6). **(E)** There was no difference in food intake between control and IRS receiving mice. Data are represented as mean+/- SEM.* P<0.05, and ** P<0.05, by Mann-Whitney Test.

Supplementary Information

Materials and Methods

Animals

7-8 week-old male C57BL/6N wild type (National Cancer Institute), TLR9 KO (Dr. R. Flavell, Yale University) and TLR9flox mice were used. Mice containing the floxed TLR9 allele were made in B6x129/Sv ES cells with the second exon, containing essentially all of the coding region, being flanked by LoxP sites. They were then backcrossed for more than 9 generations to B6. These mice will be described in detail elsewhere (Xu et al., submitted). The mice received care according to US National Institutes of Health (NIH) recommendations outlined in the "Guide for the Care and Use of Laboratory Animals".

Mice were divided into 2 groups: 1 with free access to a standard chow diet (CD, 6% fat, 20185 Harlan) and 1 with free access to a pelleted High Fat Diet (HFD, 45% fat, D12451 Research Diets). Animals were studied after 12 weeks of feeding. At the end of the protocols, blood was withdrawn and the animals were sacrificed and liver tissues were rapidly excised and frozen in liquid nitrogen or fixed in 10% formalin.

For TLR9 antagonism 8-week-old C57BL/6 mice were started on chow or HFD, and given a 5mg/kg subcutaneous dose of IRS954 (Dynavax Technologies) weekly starting at the time of starting the HFD, or 8 weeks after starting the HFD. Food intake was monitored twice per week, and mice were euthanized after 12 weeks of HFD. Eight hours after the last injection of IRS954, animals were sacrificed, and livers were removed and frozen or fixed in 10% formalin.

NF-κB reporter mice were kindly provided by Dr. Christian Jobin (University of Florida). Mice were fed with CD or HFD and after 12 weeks liver tissue was excised and GFP signal from macrophages were analyzed by flow cytometry.

Patients

The 47 subjects were recruited from the well-studied Yale NAFLD/NASH cohort that is carefully phenotyped with respect to quantification of hepatic fat content and abdominal fat distribution using MRI, systemic biomarkers of apoptosis as non-invasive indicators of NASH, fasting lipid and lipoprotein profile, and glucose homeostasis(9).

DNA Quantification

Quantification of plasma total DNA by direct fluorescent PicoGreen staining DNA quantification was performed using PicoGreen dsDNA kit (Life Technologies), according to the manufacturer's instructions. Each sample DNA was analyzed in two duplicated dilution series. Black microtiter plates were read in a plate reader (BioTek) at an emission wavelength of 520 nm and excitation of 480 nm.

DNA was extracted from 200ul of plasma with QIAmp DNA mini kit (Qiagen) 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). Quantitative real-time PCR (QPCR) was performed for nDNA and mtDNA using commercial primer probe sets and SsoAdvanced Universal Probes Supermix (BioRad) in LightCycler 480 system (Roche). nDNA was quantified using mouse or human 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) and human mt-ATP6 primers/Taqman 5'FAM-3'MGB probe (Applied Biosystems).

TLR9 ligand activity was monitored with HEK-blue TLR9 Reporter Cells (InvivoGen). Cells were cultured and stimulated with ODN1688 (InvivoGen) or mice/human plasma or with nDNA, mtDNA from hepatocytes and incubated overnight. The supernatant was incubated with Quanti-Blue detection medium (InvivoGen) and the activity was read in a plate reader (Biotek) at 630 nm.

Histopathology, immunohistochemistry and plasma assays

Plasma alanine aminotransferase (ALT) activity was measured by a standard clinical chemistry assay.

Liver tissue was fixed in 10% formalin. Specimens were routinely processed, embedded in paraffin, and sections were stained with hematoxylin and eosin (H&E). Hepatic steatosis, inflammation and ballooning Scoring analysis was based on Kleiner NAFLD activity score in a blinded manner.

Immunohistochemistry for detection of neutrophil infiltration was performed using an anti-mouse Ly-6B.2 antibody (AbD serotec, catalogue number MCA771GA).

Cell Isolation and Treatment

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. Nuclear and Mitochondrial fractions were extracted from hepatocytes with Nuclei EZ Prep Nuclei Isolation Kit (Sigma-Aldrich) and Mitochondria Isolation Kit for cultured cells (Thermo Scientific), respectively.

Mouse KCs were isolated by the density gradient separation of Percoll (GE Healthcare Life Sciences), and then plates were gently washed and media was replenished after seeding cells for 2 h to raise KC purity. Cells were stimulated with ODN1688 (InvivoGen) at 3μM or nDNA and mtDNA from hepatocytes at 100ng/ml. Peritoneal exudate cells from WT and lysm-cre-TLR9$^{fl/fl}$. mice were isolated by peritoneal lavage 3 days after intraperitoneal injection of 4% thioglycollate solution (B2551, Fluka). Cells were plated at the density of 3x10^{6} cells in 12-well dishes and non-adherent cells were removed after 3 h. RNA was extracted with TRIzol and TLR9 expression measured by qPCR.

Quantitative Real-Time Polymerase Chain Reaction Analysis

Total RNA from livers and cells was extracted with the TRIzol Reagent (Invitrogen) and were subjected to reverse transcription and subsequent quantitative real time polymerase chain reaction (PCR) using a LightCycler 480 system (Roche). q-PCR was performed for TNFα, IL1β, IL6, IFNα, IFNβ, IL1RA, TLR9, using LightCycler 480 SYBR Green I master mix (Roche). Genes were normalized with β-actin as an internal control.

Isolation and characterization of microparticles from human plasma

Microparticles were isolated from human plasma samples. Platelet-poor plasma (PPP) was centrifuged at 2,000 g for 12 minutes in order to get the platelet-free plasma (PFP). PFP was additionally centrifuged at 15,000 g for 30 minutes at 4°C to pellet microparticles.

MP membranes were labeled with the PKH67 green dye (Sigma-Aldrich) and mitochondria with MitoTracker Deep Red (Life Technologies), following the manufacturer's instructions.

After fixation and permeabilization (eBioscience) labeled MPs were co-incubated with mouse anti-8-OH dG IgG2a monoclonal antibody (Rockland, catalogue number 200-301-A99) over night at 4°C and incubated the next day with goat anti-mouse IgG2a-CF405 (Biotium). Microparticles acquisition was performed by BD LSRII Flow Cytometer System (BD Biosciences) and the data were analyzed using FACSDiva Software (BD Biosciences). In order to determine a proper gating of the microparticles, green beads of 0.2, 0.5, 0.8 μm (Spherotech) size were used. Microparticles were gated on a forward scatter/side scatter plot in the 0.2-1.0 μm region.

The presence of proteins of hepatic origin were identified on plasma MP by Western Blot. Microparticles were lysed in RIPA buffer (10mM phosphate buffer pH 7.4, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with complete protease inhibitor cocktail (Roche). Lysates were resolved in 4–12% Tris-glycine gradient gels (Invitrogen) and transferred to nitrocellulose (Invitrogen) by electroblotting. The following antibodies were used: polyclonal sheep anti-Arginase1 (R&D Systems, catalogue number IC5868P), mouse anti-CD41 (eBioscience, catalogue number 12-0419-41), rabbit anti-sheep HRP (Santa Cruz technologies, catalogue number sc-2770).

Statistical Analysis

Differences between the 2 groups were compared with Mann-Whitney *U* test. (GraphPad Prism 6; GraphPad Software Inc,). A *P* value of <0.05 was considered significant.

Study Approval

All animal experiments were approved by the Yale University Animal Care and Use Committee. Approval from the Yale Human Investigation committee was used for study of plasma from study subjects.

Author contribution:

IG, NS, YC, RH, XO: Experimental work, data analysis and manuscript review.

RLC, AC, MC: Experimental design, data analysis and manuscript review.

SC: Clinical management and phenotypic characterization of subjects.

GM and WZM: Experimental design, data analysis, manuscript preparation.

Table 1

	1: Lean (n=19) Median (range)	**2: Obese normal ALT (n=19) Median (range)**	**3: Obese High ALT (n=9) Median (range)**
Age (years)	15.4 (9-16.5)	13.7 (9.5-18.8)	15.1 (9.8-17.4)
Sex (M/F)	11/8	9/10	6/4
Race (C/AA/H)	9/3/7	6/5/8	3/2/4
GT (NGT/IGT/T2D)	17/2/0	11/3/1	7/2/0
Z-score BMI	0.41 (-1.70-1.04)	2.16 (1.75-2.67)	2.61 (1.94-2.82)
Fasting Glucose (mg/dl)	86 (77-89)	87.5 (71-96)	91.5 (81-101)
2-h glucose (mg/dl)	110 (74-138)	117 (92-219)	125 (102-166)
Fasting Insulin (mcU/ml)	14 (7-46)	16 (4-109)	40 (20-85)
Insulin sensitivity index	3.64 (2.52-5.53)	2.91 (0.48-8.1)	1.02 (0.51-1.70)
ALT (UI/L)	13 (8-27)	18 (9-21)	102 (81-177)

Table 1. Characteristics of patients groups.

Demographic, biometric and metabolic characteristics of the three patient groups. Normal ALT are less than 35 U/L.