Supplement Material

Supplemental Methods

Animals and diets: *Tlr4*$^{-/-}$*Ldlr*$^{-/-}$ mice generated on a C57BL/6J genetic background were kindly provided by Dr. Linda Curtiss from the Scripps Research Institute (San Diego, CA) and bred and genotyped in our laboratory. 10-week old adult male *Tlr4*$^{-/-}$*Ldlr*$^{-/-}$ and littermate control *Ldlr*$^{-/-}$ mice were fed rodent chow providing 4% calories as fat and a "diabetogenic" high SFA-rich and carbohydrate-rich diet (DD, 60% calories from fat, Bioserv F1850) for a total of 24 weeks. Details of the diets have been published previously[1]. Mice were maintained in a temperature and light-controlled facility in cages with micro-isolator filter tops, and received the diets *ad libitum* prior to sacrifice. Body weights were measured weekly. Food intake, determined as the difference in the weight of unconsumed food between sequential days, was recorded for three sequential days after 16 weeks of diets. Metabolic variables were measured in blood samples obtained from the retro-orbital sinus at 12 weeks on the diets and on the day of sacrifice after a 4-hour fast. At sacrifice harvested tissues were snap-frozen in liquid nitrogen and stored at -70°C, or were fixed with 10% neutral-buffered formalin and embedded in paraffin wax. All experimental procedures were undertaken with approval from the Institution Animal Care and Use Committee of the University of Washington.

Analytical procedures: Total cholesterol and triglycerides in plasma were measured using colorimetric assay kits. Lipoprotein distribution was analyzed in pooled plasma by fast protein liquid chromatography (FPLC) as described previously[2]. Plasma insulin was measured using an enzyme-linked immunosorbent assay kit (Linco). Hepatic triglycerides content and adipose tissue lipid content were determined following lipid extraction using the Folch method[3]. Glucose and insulin tolerance tests were measured at 20-22 weeks, as previously described[4].

Body composition analysis: Body composition was performed on conscious immobilized mice using quantitative magnetic resonance (EchoMRI whole body composition analyzer, Echo Medical Systems, Houston, TX, USA).

Immunohistochemistry: Single-label immunohistochemistry was performed on adipose tissues as previously described[5]. Adipocyte cross-sectional area was measured by computer image analysis using a modification of techniques described previously[6]. Macrophage staining in adipose tissue sections was detected using a rat monoclonal antibody against Mac2 (1:2000 dilution, Cedarlane Laboratories, Burlington, NC). Area quantification for Mac2 was performed on digital images of immunostained adipose tissue sections using image analysis software (Image Pro Plus software, Media Cybernetics). All analyses were performed by a blinded observer.

Adipocyte sizing: Sections were stained with Movats Pentachrome. One randomized photomicrograph was analyzed at 20X objective (Canon EOS 5D Mark II, Olympus BX50) and quantified by manually outlining each individual adipocyte by hand (Image Pro Plus/Media Cybernectics software, Wacom Cintiq 21UX tablet).

Cell culture: 3T3-L1 murine pre-adipocytes, obtained from American Type Tissue Culture Collection, were propagated and differentiated according to standard protocol procedures[7], with the exception that media containing either 5 or 25 mmol/l glucose with or without 250μmol/l FFAs were replenished daily.

Real-time quantitative PCR analysis: Total RNA was extracted from 100mg of epididymal white adipose tissue and liver using RNeasy Lipid Tissue Kit (QIAGEN, Valencia, CA, USA) and Agilent Total RNA Isolation Kit (Agilent, Santa Clara, CA, USA), respectively, according to the manufacturer's protocols. After spectroscopic quantification, 2 μg of RNA was reverse-transcribed to cDNA and used for quantification of genes. Real-time quantitative PCR was performed with the TaqMan Master Kit (Applied Biosystems, Carlsbad, CA, USA) using an ABI 7900HT instrument. Primer and FAM probes for individual genes were purchased from Applied Biosystems (Assay-on-Demand). Relative quantities of mRNA were calculated using GAPDH as the reference gene. The amount of target gene was calculated using the $^{\Delta\Delta}$Ct formula.

Supplemental Table I. Composition of the diets.

Supplemental Table I

Composition of the diets

Diet	Composition
Chow	Carbohydrate 68%, Protein 24%, Fat 4%, Fiber 4%
Diabetogenic diet (DD) **BioServ F1850**	Carbohydrate 36.3%, Fat 35.5% as lard*, Protein 20%, Fiber 0%.

All diets also contained: casein, methionine, vitamin and mineral mix.

* Lard adds small amounts of cholesterol (0.03%) – Source: USDA National Nutrient Database (http://www.nal.usda.gov/fnic/foodcomp/search/).

Supplemental Figure Legends:

Figure SI. Body weight and body composition is not changed by TLR4 deficiency. Body weight (A), percentage lean mass (B), percentage fat mass (C) in *Tlr4*$^{-/-}$*Ldlr*$^{-/-}$ mice fed DD (black bars and solid circle lines) and chow (grey bars and solid triangle lines), *Ldlr*$^{-/-}$ control mice fed DD (hatched bars and open circle dash lines) and chow (open bars and open triangle dash lines) (n=18-24, *P<0.05, **P<0.01 vs DD or chow groups).

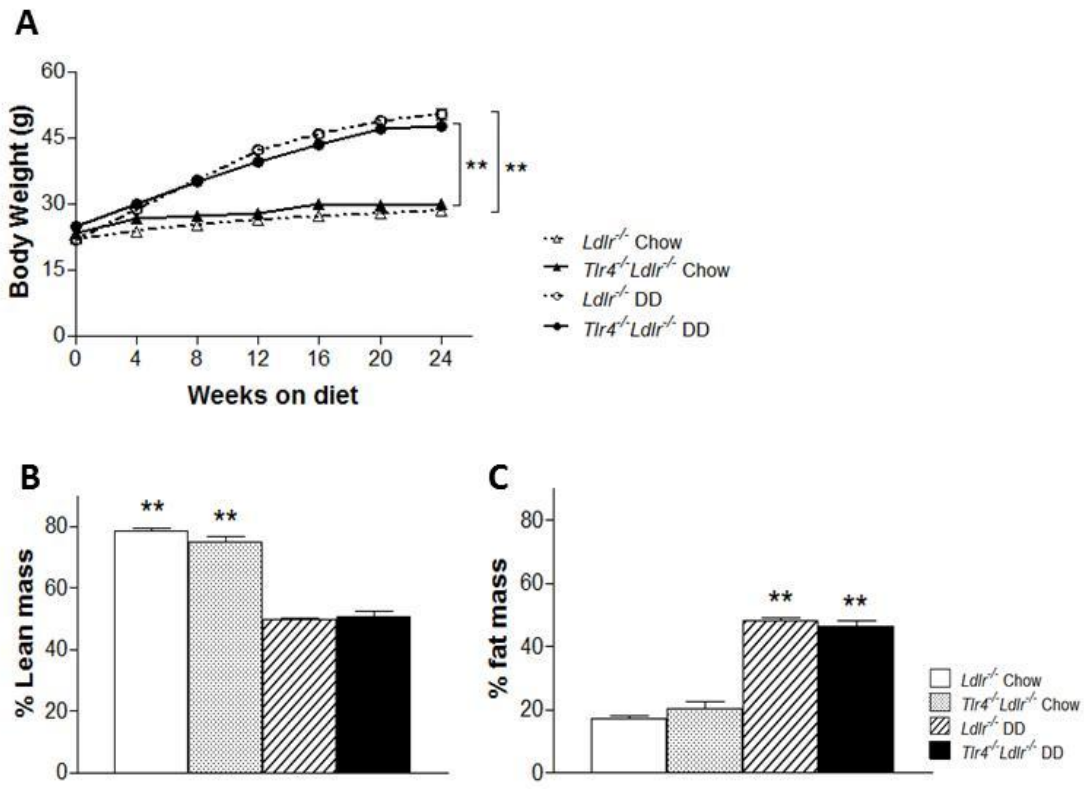
Figure SII. Insulin resistance is not attenuated in *Tlr4*$^{-/-}$*Ldlr*$^{-/-}$ mice. Plasma insulin levels at 30 minutes post injection in the glucose tolerance tests (n=9-11, *P<0.05, **P<0.01 vs DD or chow groups).

Figure SIII. TLR4 deficiency does not affect visceral fat pad weight and adipocyte size. Epididymal white adipose tissue weight (A), adipocyte size (B) and lipid content – triglyceride left, cholesterol right (C) (n=10-13, **P<0.01 vs DD or chow groups).

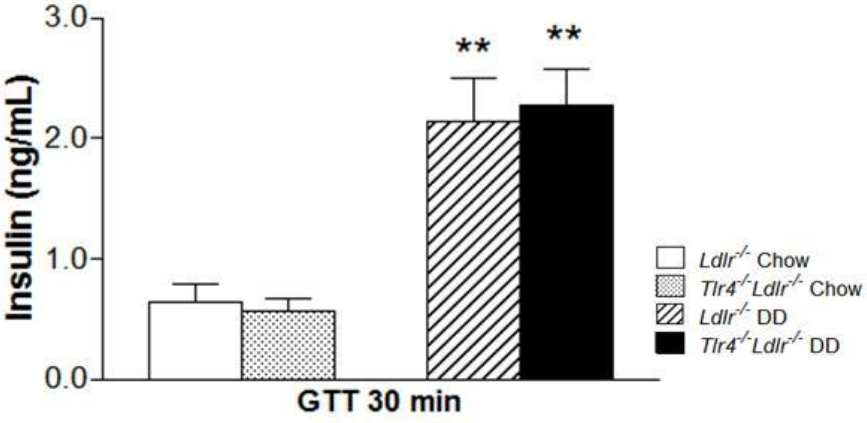
Figure SIV. TLR4 deficiency does not affect the expression of M1 and M2 markers in intra-abdominal adipose tissue of DD-fed *Tlr4*$^{-/-}$*Ldlr*$^{-/-}$ mice and *Ldlr*$^{-/-}$ mice. The mRNA levels of *Nos2* (A) and *Il-6* (B) as M1 markers, *Arg1* (C) and *Fizz1* (D) as markers in epididymal adipose tissue (n=10-13).

Figure SV. TLR4 deficiency does not affect the expression of cholesterol metabolism-related genes in liver of DD-fed *Tlr4*$^{-/-}$*Ldlr*$^{-/-}$ mice and *Ldlr*$^{-/-}$ mice. The mRNA levels of Apob (A), *Hmgcr* (B), *Acat1*(C), *Srebp1c* (D), *Dgat1* (E), *Mttp* (F) in liver (n=10-13).

Supplemental Figure SI.



Supplemental Figure SII.



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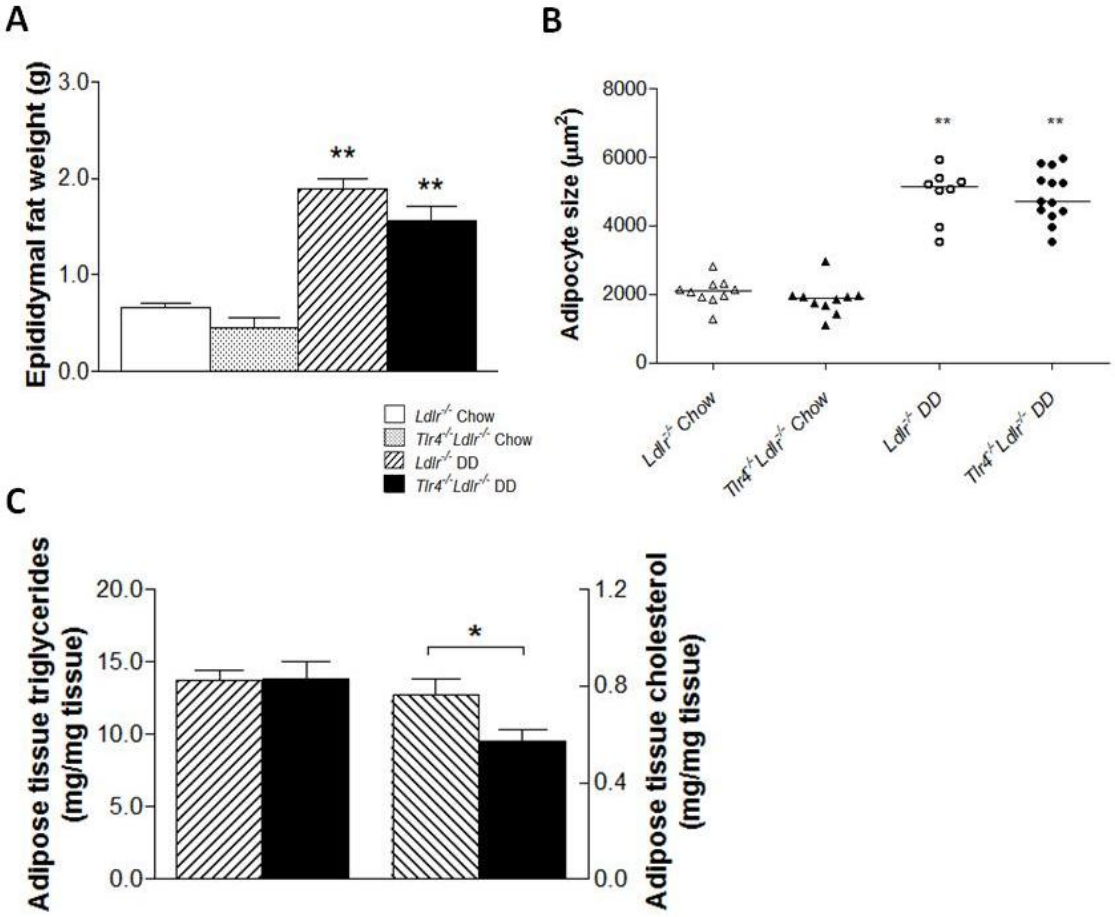

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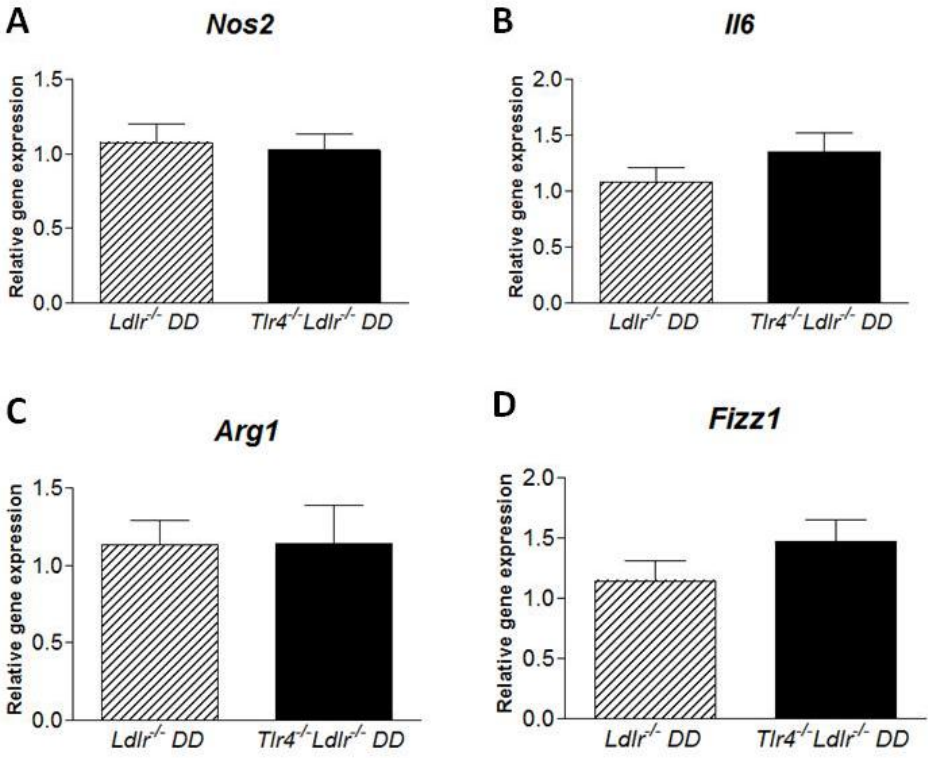

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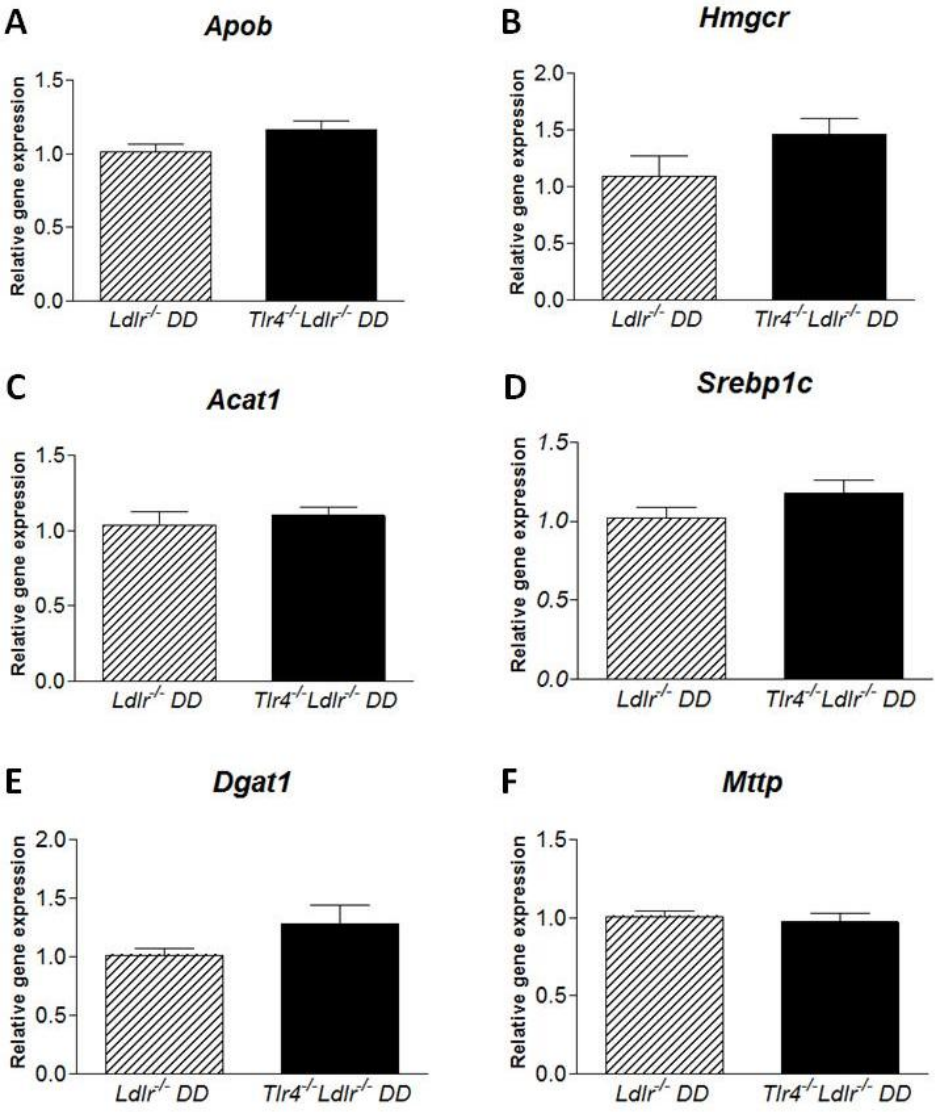

References

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