Supplemental Data and Methods

Monocyte Tissue Factor-dependent Activation of Coagulation in Hypercholesterolemic Mice and Monkeys is inhibited by Simvastatin

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Condensed Title: Simvastatin attenuates hyperlipidemic induction of tissue factor expression

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METHODS

Human subjects

Human subjects with familial hypercholesterolemia (FH) were recruited at the Kansas University Medical Center (KUMC). The patient demographics are described in Supplemental Table 1. Apheresis was conducted utilizing either the heparin-induced extracorporeal LDL precipitation (HELP) system (n = 17, B. Braun, Melsungen AG, Germany) or a dextran-sulfate LDL adsorption (n = 8, Liposorber LA-15, Kaneka Pharma, Osaka, Japan), as described (1). Control plasma samples (n = 17) were purchased from Innovative Research (Novi, MI) and were matched for age, gender, and race (Supplemental Table 1). Pre-apheresis is defined as blood drawn from the body that has not yet passed over the lipid absorbing column. Post-apheresis blood was collected after lipid absorbing, but prior to re-entering the body. All blood was collected into 6 mL K2 EDTA 10.8 mg tubes (BD glass Vacutainers®, Becton Dickinson, Franklin Lakes, NJ). Blood was then centrifuged at 1,500 x g for 30 minutes using a fixed speed centrifuge, plasma collected, and stored at -80ºC. Blood from controls was collected in the same manner.

Human monocyte isolation

Human blood was collected into BD glass vacutainers® (3.2% sodium citrate) and placed into 50 mL sterile conical tubes. Blood was spun at 200 x g for 10 minutes. Plasma was removed and replaced with an equivalent volume of 10 mM EDTA in Hanks balanced salt solution (HBSS, Gibco). Blood was then layered onto sterile endotoxin

free ficoll-paque plus (GE Healthcare, Piscataway, NJ) and spun at 400 x g for 30 minutes with no brake. Peripheral blood mononuclear cells (PBMCs) were collected in the buffy coat layer and washed with autoMACS® running buffer (Mintenyi Biotech, Auburn, CA). Human monocytes were isolated utilizing indirect labeling of all non-monocytic cells (negative selection) with magnetic beads (monocyte isolation kit II, Miltenyi Biotech, Auburn, CA) using a LS column and a MidiMACS® separator. Monocytes were isolated, counted with a hemacytometer, and placed in ventilated 1.5 mL eppendorf tubes for treatment to avoid plating-induced monocyte to macrophage differentiation. Media and similar treatment reagents described in the THP-1 cell section were utililzed.

THP-1 cells

Human monocytic THP-1 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were passaged every three days in RPMI-1640 supplemented with 10% FBS, 1mM sodium pyruvate, 1mM HEPES, 1% glucose, and 1% penicillin/streptomycin. Cells were used between passage 1 and 10 and seeded at 10^6 per well in 12 well plates. After plating, cells were allowed a recovery period for 2 hours at 37ºC in 5% CO_2 until treatment. Cells were either untreated, treated with native LDL, or copper sulfate-oxidized LDL obtained from Biomedical Technologies, Inc. (Stoughton, MA) and were placed onto cells for a specified time-course. Each lot of oxLDL is tested for high ratio of ThioBarbituric Acid Reactive Substances (TBARS) and is membrane filtered, aseptically packaged, and endotoxin

tested (no detectable levels). On average, the lot of oxLDL obtained migrated 2.7 fold further than native LDL on agarose gel electrophoresis, and had increased TBARS from 0.2 nmoles of MDA/mg protein to 58.2 nmoles of MDA/mg protein after oxidation. The Toll-like receptor 4 (TLR4) inhibitor CLI-095 (1 µg/mL), also termed TAK-242, was obtained from Invivogen (San Diego, CA) and was incubated for 2 hours prior to treatment. The carboxylate form of simvastatin (20 µM) was purchased from Calbiochem (Gibbstown, NJ) and was incubated with cells overnight prior to treatment. Polymyxin B (10 µg/mL) was purchased from Sigma Aldrich/Fluka (St. Louis, MO) and was incubated with cells 30 minutes prior to treatment.

Cell viability assay

Viability was assessed using the Alamar Blue assay according to the manufacturers' specifications (Invitrogen, Carlsbad, CA). Briefly, cells were treated, as described above, and 100 µL of cell suspension was then collected and added to a black, clear bottom 96 well plate (BD Falcon Optilux plates™). 10 µL of Alamar Blue was then added and cells were incubated at 37°C in 5% CO_2 for 4 hours. Absorbance of the plate (544/590 nm) was then read using a SpectraMax M5 and analyzed using Softmax Pro v 5.2C software (Molecular Devices, Sunnyvale, CA).

Clotting assay

The procoagulant activity of cell lysates was measured using a 1-stage clotting assay with a STart 4 clotting machine (Diagnostica Stago). Briefly, cell pellets (1 x 10^6 cells/mL) were solubilized using a Misonix Microson XL2000 ultrasonic homogenizer for ~5 seconds. Cellular lysates (50 µl) were added to normal human plasma (50 µl, coagulation control level 1, Pacific Hemostasis) and allowed to incubate for 2 minutes at 37ºC. Calcium chloride solution (50 µl, 25 mM) was added and clotting times were recorded. Clotting times were converted to procoagulant TF activity by comparison with a standard curve generated with known concentrations of recombinant human relipidated TF (Innovin®, MedCorp, Brazil).

Microparticle (MP) tissue factor (TF) activity assay

MP TF activity was measured as described (2-3). Briefly, plasma MPs are pelleted at 20,000 x g for 20 minutes at 4ºC, washed twice and resuspended in Hanks Balanced Salt Solution with Bovine Serum Albumin (HBSA) buffer without calcium (137 mM NaCl, 5.38 mM KCl, 5.55 mM glucose, 10 mM HEPES, 0.1% BSA, pH 7.5). For measurement of TF-specific activity, samples are then incubated with either an inhibitory rat anti-mouse TF antibody (1H1, 100 µg/ml, Genentech, mouse plasma), a mouse anti-human TF antibody (HTF-1, 10 µg/mL, BD biosciences, monkey plasma), or the appropriate rat or mouse IgG controls (Sigma Aldrich) for 15 minutes at room temperature. Next, 10 nM mouse FVIIa (mouse plasma) or 10 nM human FVIIa (monkey plasma), 300 nM human FX, and 10 mM $CaCl_2$, was added to the sample and incubated for 2 hours at 37ºC in a 96 well plate. FXa generation was determined using

the chromogenic substrate, Pefachrome FXa 8595 (4 nM). Absorbance of the plate (405 nm) was then read using a SpectraMax M5 and analyzed using Softmax Pro v 5.2C software (Molecular Devices, Sunnyvale, CA). TF activity was extrapolated from a standard curve generated with recombinant human relipidated TF (0-14 pg/mL, Innovin®, MedCorp, Brazil). TF-specific activity was ascertained by subtracting the IgG control activity from the activity of the blocking antibody.

Mice and diet:

Male *Ldlr*$^{-/-}$ mice (*B6.129S7-Ldlr*^{tm1Her}, stock no. 002207, N12) were obtained from The Jackson Laboratory (Bar Harbor, MA). *Ldlr*$^{-/-}$/*Tlr4*$^{-/-}$ and *Ldlr*$^{-/-}$/*Tlr6*$^{-/-}$ were created by Linda Curtiss and Peter Tobias at The Scripps Research Institute (La Jolla, CA). *Ldlr*$^{-/-}$/*Tlr4*$^{+/+}$ and *Ldlr*$^{-/-}$/*Tlr4*$^{-/-}$ cousin littermate mice (N8) were generated by interbreeding *Ldlr*$^{-/-}$/*Tlr4*$^{+/-}$ mice, which were created by breeding *Ldlr*$^{-/-}$/*Tlr4*$^{-/-}$ onto the Jackson *Ldlr*$^{-/-}$ strain. A similar strategy was used to generate *Ldlr*$^{-/-}$/*Tlr6*$^{+/+}$ and *Ldlr*$^{-/-}$/*Tlr6*$^{-/-}$ littermates. *mTF*$^{-/-}$*, hTF*$^{+}$ (low TF) mice and littermate *TF*$^{+/-}$*, hTF*$^{+}$ controls were created by breeding *TF*$^{+/-}$ female mice with low TF male mice (4). All mice were fed a normal mouse laboratory diet and water *ad libitum*. To induce hypercholesterolemia, mice were fed a diet enriched with saturated milk fat (21% wt/wt) and cholesterol (0.15% wt/wt, diet TD.88137 from Harlan Teklad). For the simvastatin diet (50 mg/kg/day) (5), 350 mg simvastatin was added per kg diet based on a 5 g/day consumption rate in ~35 g mice (Harlan Teklad diet TD.88137 + simvastatin).

Monkeys and diet

Adult male vervet monkeys (*Chlorocebus aethiops*) (n = 12, age 5-10 years) were obtained from St. Kitts Island. Monkeys were housed in an AAALAC accredited facility under the direct care of the Wake Forest University Health Sciences (WFUHS) animal resources program. Monkeys were singly housed in climate controlled conditions with 12 hour light and dark cycles. Monkeys were provided water *ad libitum* and were initially fed a chow diet (Monkey Diet 5038, Lab Diet), such that their daily caloric intake was 150 kcal/day/kg body weight. To induce hyperlipidemia, the monkeys were fed twice a day for 16 weeks a weighed amount of a high fat, high cholesterol semi-synthetic diet (Supplemental Table 3), such that their daily caloric intake was 70 kcal/day/kg body weight. Semi-synthetic diets were prepared at the Wake Forest Primate Center Diet Laboratory. The monkeys were then fed twice a day for 4 weeks a weighed amount of the high fat, high cholesterol diet containing simvastatin (Supplemental Table 3), which was provided by Merck and Co. (Rahway, NJ). For the simvastatin diet, the monkeys received 70 kcal/day/kg body weight and 10 mg simvastatin/day/kg body weight. During the simvastatin treatment phase, food intake was monitored daily.

Proprotein convertase subtilisin kexin type 9 (PCSK9) analysis

Plasma PCSK9 levels were determined using an enzyme-linked immunosorbent assay. 96-well Immulon plates (Thermo Scientific, Rochester, NY) were coated overnight with capture antibody (E07), and blocked for 1 hr at room temperature in tris-

buffered saline containing 0.05% Tween-20 (TBST) and 1% BSA. Standard curves (11-point 2-fold dilutions from 1 µg/mL) were generated using purified African green monkey PCSK9. Samples and standards (50 µL) were added to coated plates and incubated for 37°C for 1 hour. Following washing in TBST, plates were incubated with 1µg/mL biotinylated secondary antibody (B20) diluted in assay buffer (Perkin-Elmer Waltham, MA, product #1244-111) and incubated for 1 hour at room temperature. Following washing in TBST, Streptavidin/Europium indicator solution (Perkin-Elmer, Waltham, MA, product #1244-360) was added to plates and incubated for 20 minutes at room temperature. Enhancer solution was then added and plates were incubated for 1 hour at room temperature and data acquired using Europium reader (Envision, Perkin Elmer). E07 and B20 anti-PCSK9 antibodies were generated by Merck.

Bone marrow transplantation

Male recipient *Ldlr*$^{-/-}$ mice (8 weeks old) were irradiated with a total of 11 Gy (2 doses of 550 rads 4 hours apart) using a Cs^{137} irradiator (JL Shepherd, San Fernando, CA). Irradiated mice were re-populated with bone marrow harvested from *TF*$^{+/-}$ (n = 17) or low TF (n = 16) donor mice via retro-orbitally injected cells (1 x 10^7 cells per animal). Mice were allowed to recover for 4 weeks and then either fed a western diet (n = 10 *TF*$^{+/-}$; n = 9 low TF) or chow (n = 7 each group) for 12 weeks.

Inhibition of TF

Male *Ldlr*$^{-/-}$ mice (n = 14) were fed a HFD for 12 weeks. At 12 weeks, mice were split into two groups. One group received intraperitoneal injections of a neutralizing rat anti-mouse TF monoclonal antibody (1H1, 20 mg/kg, n = 7), while the other received the isotype control antibody (rat IgG2a; 20 mg/kg; n = 7; Sigma Aldrich). Injections were performed on day 0 and day 3 and mice were then euthanized and blood collected on day 6, as previously described (6-7).

Blood collection

Mice were sedated with 3% isofluorane and blood was collected from the inferior vena cava into a 25 gauge x 1' needle pre-coated with 3.8% sodium citrate. The mice were then humanely euthanized. Mouse blood was centifuged at 4,000 x g for 15 minutes to prepare platelet poor plasma (PPP) and stored at -80ºC. Monkeys were sedated with 10 to 15 mg/kg ketamine hydrochloride (depending on body weight) and blood was drawn from the median cubital vein into sodium citrate (BD glass vacutainers® 3.2% sodium citrate). Monkey blood was spun at 1,500 x g for 15 minutes to prepare PPP and stored at -80ºC. Human blood was collected from FH patients at the KUMC via the brachial vein or fistula into 6mL K2 EDTA 10.8mg tubes (BD glass vacutainers®). Blood was then spun at 1,500 x g for 30 minutes using a fixed speed centrifuge, plasma collected, and stored at -80ºC.

Monkey and mouse peripheral blood mononuclear cell (PBMC) and white blood cell (WBC) isolation

Monkey and mouse blood were collected as described in the previous section. For isolation of monkey PBMCs, blood was spun at 200 x g for 10 minutes. Plasma was removed and replaced with an equivalent volume of 10 mM EDTA in Hanks balanced salt solution (HBSS, Gibco). Blood was then layered onto sterile endotoxin free ficoll-paque plus (GE Healthcare, Piscataway, NJ) and spun at 400 x g for 30 minutes with no brake. Peripheral blood mononuclear cells (PBMCs) were collected in the buffy coat layer and washed with sterile HBSS. Cells were then spun down, and split equally into either 1 mL TRIzol® reagent or 1 mL sterile HBSS and frozen at -80ºC until processed. At most, 1 mL of blood is obtained from mice, which was deemed too small for PBMC isolation using Ficoll. Therefore, a WBC pellet was obtained from mouse blood spun at 4,000 x g for 15 minutes with the plasma removed. The erythrocytes were then lysed using a red blood cell lysis buffer (154 mM ammonium chloride, 0.127 mM EDTA, and 11.9 mM sodium bicarbonate in sterile ddH_2O). The white pellet remaining was deemed the WBC fraction.

Enzyme linked immunosorbent assay measurements

The following commercially kits were used: mouse IL-6 and human IL-8 (R&D Systems), human oxLDL (Caymen Chemicals), human hsCRP (ALPCO Immunoassays), human D-Dimer (Diagnostica Stago), and TAT Enzygnost micro kit (Dade Behring/Siemens).

MP PS equivalent assay

Plasma MPs were evaluated utilizing a phosphytidylserine (PS) capture and prothrombinase complex thrombin generation assay, per the manufacturer's instructions (Zymuphen MP Activity, Aniara, Mason, OH). The results are expressed in PS equivalents (nM), which are the amount of PS-positive MPs found in the plasma.

Enumeration of particles in plasma by Nanosight tracking analysis

Plasma was diluted in sterile filtered phosphate buffered saline to appropriate concentrations for the nanoparticle tracking analysis. The particle count in plasma was carried out using the Nanosight NS500 system (NanoSight, Amesbury, UK), which focused a laser beam through the plasma (8). Particles within the beam were visualized using a conventional optical microscope aligned normally to the beam axis, which collected light scattered from all particles in the field of view. Video (90 seconds) was recorded using an electron multiplying charge coupled device, and particle movement was analyzed by Nanoparticle Tracking Analysis software. Each particle was identified and its Brownian movement tracked and measured. The area of field of view obtained in the video is a known volume and thus the software calculates the number of particles per mL to which the dilution factor is applied to obtain number of particles per mL of plasma.

Plasma lipid analysis

Mouse and human plasma lipids were analyzed with the following commercially kits: total plasma cholesterol (Free Cholesterol E), triglycerides (L-Type TG M), and HDL-C (L-Type HDL-C) from Wako Chemicals (Richmond, VA). LDL-C was calculated using the Friedewald equation where LDL-C = total cholesterol – HDL-C – (0.20 x triglycerides). When triglycerides were greater than 400 mg/dL, 0.20 was replaced with 0.16. VLDL-C was then calculated by subtracting HDL-C and LDL-C from total plasma cholesterol. Monkey total plasma cholesterol was measured using the Pointe Scientific Cholesterol Reagent Set (Canton, MI). Plasma lipoprotein cholesterol distribution was determined by on-line, high performance gel filtration chromatography as described previously (9).

Measurements of mouse plasma oxLDL

OxLDL was measured in the mouse plasma as described with modifications (10). Briefly, a microfluor2 white plate (Nunc) was coated overnight with 5 µg/mL (50 µl total) of anti human ApoB-100 IgG (Academy Bio-chemical Co, Houston, TX). The antibody was removed and the plate was blocked for 2 hours at room temperature with 1x PBS and 1% BSA (Sigma Aldrich, St. Louis, MO). Blocking buffer was removed and either plasma (30 µl of a 1:5 dilution) or controls malondialdehyde (MDA)-LDL (MDA modification of LDL), native LDL (nLDL, Kalen Biomedical, Montgomery Village, MD), or oxLDL (Kalen Biomedical, all controls diluted to 0.5 µg/mL, 50 µl volume) were added to the plate and incubated at room temperature for 2 hours. Plates were then washed and

either a mouse anti-MDA2-LDL antibody or a mouse OLF49 anti-oxLDL antibody were added to the wells (1:1000 dilution each antibody, 50 µl total) for 1 hour at room temperature. Wells were thoroughly washed and a goat anti-mouse IgG+IgM labeled with horse radish peroxidase (HRP) were added to the wells and incubated 30 minutes at room temperature (1 : 20,000 dilution, 50 µl total volume, Jackson Immunoresearch, West Grove, PA). Wells were washed, and signal was detected with Supersignal ELISA HRP substrate (Pierce, Rockford, IL) and a BMG Labtech POLARstar machine (Cary, NC). Data is expressed as relative light units (RLU) and subtracted from background.

Ferric chloride ($FeCl_3$) carotid arterial thrombosis model

The $FeCl_3$ carotid arterial thrombosis was performed as described (11). Briefly, *Ldlr*$^{-/-}$ mice fed either a chow or western diet for 12 weeks were utilized (n = 8 per group). Antibody inhibition studies were conducted as described above. In addition, *Ldlr*$^{-/-}$ mice were fed either a chow (n = 9) or Western diet (n = 9) for 12 weeks and then either remained on chow (n = 4) or Western diet (n = 4) or were switched to a chow (n = 5) or Western diet (n = 5) containing 50 mg/kg/day of simvastatin for a further 4 weeks. Mice were sedated with 1.5% isoflurane, the thoracic cavity was dissected, carotid artery isolated and cleaned, and baseline blood flow was analyzed using a microvascular ultrasonic flow probe (Transonic Systems, Ithica, NY). 5% $FeCl_3$ (Anhydrous, Sigma Aldrich, St. Louis, MO) was applied to a 1mm^2 filter paper (Whatman) for 2 minutes and incubated on the dorsal surface of the carotid artery for 3 minutes. The cavity was flushed with 37ºC sterile saline, and flow was monitored until

cessation of flow (0 mL/minute) for 30 seconds post $FeCl_3$ incubation. All mice were monitored for at least 30 minutes post injury.

Laser-injury thrombosis model

Intravital microscopy, laser-induced cremaster vessel-wall injury, and intravital imaging have all been described previously in detail (12). Briefly, *Ldlr*$^{-/-}$ mice on either a chow or western diet were subjected to injury. Platelets were labeled with an anti-CD42b antibody labeled with Dylight 488 (0.1 μg/g mouse, Emfret, Germany), and fibrin was labeled with an anti-fibrin antibody (0.5 μg/g mouse) purified in-house from the 59D8 hybridoma cell line (13) and labeled with Alexa 647 Invitrogen, Carlsbad, CA). Data are represented as plots of by median platelet and fibrin fluorescence arbitrary units versus time after injury. Images were captured using a Cooke SensiCam CCD camera (Auburn Hills, Michigan) under a 60x (NA 1.0) water immersion objective lens (LUMplan FL, Olympus). 10μm scale bars are included for size reference.

Real-time polymerase chain reaction (PCR) analysis of monkey PBMCs

Total ribonucleic acid (RNA) was isolated from cells using the TRIzol® method (Invitrogen, Carlsbad, CA) and reverse transcribed into complimentary deoxyribonucleic acid (cDNA) using random hexamers and a Moloney murine leukemia virus reverse transcriptase (M-MLV, Invitrogen, Carlsbad, CA). Levels of different messanger RNAs (mRNAs) were analyzed by real-time PCR using iTaq Supermix with ROX (Bio-Rad,

Hercules, CA) and realplex^2 Mastercycler (Eppendorf AG, Hamburg, Germany). We used TaqMan® probe sets (Applied Biosystems, Carlsbad, CA) to analyze mRNAs levels of human *CD36* (Hs00169627_m1), *TF* (Hs01076032_m1) and *TLR4* (Hs00370853_m1). For detection of human *TLR6*, we generated the following probe set (forward: 5'-TGG ACT CAT ATC AAG ATG CTC TG-3', reverse: 5'- AAT AGG TTC TTT GTC TTT GGT CAT G -3', probe: 5'-FAM-TGT TGC AGT GGC TAT CCT AAA GGG TT-TAMRA-3', Integrated DNA Technologies, Coralville, IA). Amplification of *18S* ribosomal RNA (rRNA, 4319413E, Applied Biosystems, Carlsbad, CA) was used to correct for variations in input RNA levels and efficiencies of the reactions.

Statistics and data representation

All bar and line graphs were created with Sigma Plot v.11 (SPSS, Chicago, IL). All statistical analysis was performed using SigmaStat, now incorporated into Sigma Plot v.11. Data are represented as mean ± SEM. For two group comparison of parametric data, a two-tailed Student's t-test was performed, while non-parametric data was analyzed with a Mann-Whitney Rank Sum. Statistical significance between multiple groups was assessed by One Way analysis of variance (ANOVA) on Ranks with a Dunn's post hoc, One Way ANOVA with Holm Sidak Post Hoc, or Two Way ANOVA with Holm Sidak Post Hoc, when appropriate. Values of $P < 0.05$ were considered statistically significant.

Study approval

All mouse studies were performed with the approval of the University of North Carolina at Chapel Hill (UNC-CH) Institutional Animal Care and Use Committee (IACUC). All studies with monkeys were approved by the WFUHS IACUC. All patients and controls provided written informed consent in accordance with KUMC (Kansas City, KS) and UNC (Chapel Hill, NC) Institutional Review Board (IRB) protocols for plasma and monocyte isolation, respectively.

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Supplemental Figure 1: LPS induction of TF expression and the release of TF^+ MPs from THP-1 cells and human monocytes in the presence and absence of a TLR4 inhibitor, simvastatin or polymyxin B. Time course of (A) cellular TF activity and (B) MP TF activity in the culture supernatant of THP-1 cells treated with LPS (1 µg/ml). LPS induction of (C) cellular TF activity and (D) MP TF activity in THP-1 cells pre-treated with simvastatin (10 µM, sim) for 12 hours, the TLR4 inhibitor TAK-242 (1 µg/mL) for 2 hours, or the LPS-neutralizing antibiotic peptide polymyxin B (10 µg/mL) for 30 minutes. Similar experiments were performed with human monocytes (panels E and F). Data are represented as mean ± SEM. All experiments were performed 5 times in triplicate. *$P < 0.001$ LPS treated versus 0 hour time-point. $^{\#}P < 0.001$ simvastatin, TAK-242, or polymyxin B pretreated cells versus LPS treated cells. A-B was analyzed with a One Way ANOVA with Holm-Sidak post hoc, while C-F was analyzed via Two Way ANOVA with Holm-Sidak post hoc.

Supplemental Figure 2: Thrombosis in the $FeCl_3$ model of arterial injury in chow fed mice or HFD mice in the treated with an anti-TF antibody or simvastatin. (A) Male *Ldlr*$^{-/-}$ mice were fed either chow (n = 7) or HFD (n = 7) for 12 weeks. An additional groups of mice were fed a HFD for 12 weeks and given two injections (three days apart) of either an IgG control antibody (n = 7) or an anti-TF antibody (n = 7). Time to occlusion was measured after application of 5% $FeCl_3$. (B) Thrombosis in male *Ldlr*$^{-/-}$ mice fed either chow (n = 4) or HFD (n = 4) for 12 weeks were compared with mice fed chow with simvastatin (statin) (n = 5) or HFD with simastatin (statin) (n = 5) for 4 weeks.

The time to occlusion is shown in minutes. *P < 0.001 HFD mice versus chow mice. #P < 0.001 treated groups versus chow group.

Supplemental Figure 3: Plasma lipoprotein cholesterol and PCSK9 levels in hypercholesterolemic monkeys treated with or without treatment simvastatin. African Green monkeys were fed a HFD for 16 weeks before being fed a modified HFD containing simvastatin (10 mg/kg/day) for an additional 4 weeks (total of 20 weeks total). Blood samples were collected at 0, 4, 8, 16 and 20 weeks. Levels of (A) TPC, (B) VLDL-C, (C) LDL-C, (D) HDL-C, (E) PCSK9, and (F) PBMCs TF expression are shown. Histobars represent means ± SEM of 12 monkeys. *P < 0.001 HFD versus chow, $^{\#}$P < 0.05 simvastatin treated versus 16 weeks HFD. Data was analyzed with a (A-D) One Way ANOVA with a Holm-Sidak post hoc, a One Way ANOVA on Ranks with Dunn's post hoc (F), or (E) a Mann Whitney Rank Sum.

Supplemental Figure 4: *TF*, *TLR4*, *TLR6*, and *CD36* mRNA expression in PBMCs from hypercholesterolemic monkeys with or without simvastatin. PBMCs were isolated before HFD (time 0), 16 weeks after HFD, and 4 weeks after simvastatin treatment (time 20) and RNA was isolated (n = 12 each time-point). Normalized ratios of (A) *TF*, (B) *TLR4*, (C) *TLR6*, and (D) *CD36* mRNA expression are shown compared to control *18S* rRNA. *P < 0.03 16 weeks versus 0, #P < 0.005 20 weeks versus 16 weeks, †P = 0.08 20 weeks versus 16 weeks. Data was analyzed with a One Way ANOVA on Ranks with a Dunn's post hoc analysis.

Supplemental Table 1: Human patient demographics and lipid profiles

Patients	Total Patients	Age (years)	Male	Female	TPC (mg/dL)	Total VLDL (mg/dL)	Total LDL (mg/dL)	Total HDL (mg/dL)	Triglycerides (mg/dL)
Healthy controls	17	61.2 ± 1.0	11	6	147.1 ± 10.3	13.8 ± 2.0	76.4 ± 11.0	56.2 ± 4.1‡	69.1 ± 10.2
FH pre-apheresis	24	59.1 ± 1.8	9	15	289 ± 10*	35.1 ± 3.2**	209.1 ± 9.2*	44.7 ± 2.4	211.6 ± 25.5**
FH post-apheresis	24	59.1 ± 1.8	9	15	142 ± 7	19.2 ± 1.4	82.1 ± 6.1	39.0 ± 2.0	120.2 ± 14.5†

Abbreviations: TPC-total plasma cholesterol; VLDL-very low density lipoproteins; LDL-low density lipoproteins; HDL-high density lipoproteins

*P<0.001 FH pre-apheresis versus healthy controls and post-apheresis. One Way ANOVA with Holm-Sidak post hoc analysis.

**P<0.001 FH pre-apheresis versus healthy controls and post-apheresis. One Way ANOVA on Ranks with Dunn's post hoc analysis.

†P<0.001 FH post-apheresis versus healthy controls. One Way ANOVA on Ranks with Dunn's post hoc analysis.

‡P<0.007 Healthy controls versus pre and post apheresis. One Way ANOVA with Holm-Sidak post hoc analysis.

Healthy controls: 6% African American, 12% Hispanic, and 82% Caucasian

FH patients: 4% Asian, 4% African American, 8% Hispanic, and 83% Caucasian

Supplemental Table 2: Mice weights and lipid profiles

Genotype	Gender	Mouse number	Diet	Treatment	HFD (weeks)	Weight (grams)	TPC (mg/dL)	Total VLDL (mg/dL)	Total LDL (mg/dL)	Total HDL (mg/dL)	Triglycerides (mg/dL)
Ldlr $^{-/-}$	Male	5	Chow	None	0	28.8 ± 0.8	117.8 ± 5.7	11.1 ± 0.4	41.4 ± 7.1	65.6 ± 6.2	69.2 ± 7.2
Ldlr $^{-/-}$	Male	5	HFD	None	2	29.2 ± 1.3	1336.8 ± 65.4*	88.3 ± 12.2*	1176.0 ± 19.4*	82.4 ± 7.1*	489.3 ± 45.8*
Ldlr $^{-/-}$	Male	5	HFD	None	4	30.1 ± 1.1	1356.8 ± 84.5*	88.0 ± 5.2*	1188.2 ± 76.3*	80.4 ± 6.9*	550.3 ± 74.3*
Ldlr $^{-/-}$	Male	5	HFD	None	8	34.1 ± 0.4*	1339.1 ± 137.3*	91.6 ± 5.2*	1174.3 ± 65.4*	73.2 ± 12.1	572.2 ± 100.3*
Ldlr $^{-/-}$	Male	5	HFD	None	12	37.2 ± 0.7*	1442.7 ± 94.3*	90.9 ± 7.4*	1276.5 ± 77.3*	75.2 ± 8.9	568.2 ± 85.2*
Ldlr $^{-/-}$	Male	10	HFD	Simvastatin	16	39.3 ± 0.5*	1394.5 ± 66.4*	94.0 ± 10.7*	1212.0 ± 64.9*	88.5 ± 4.7*	587.3 ± 67.0*
Ldlr $^{-/-}$	Male	7	HFD	Anti-TF Ab	13	35.3 ± 1.1	1480.9 ± 65.0	110.7 ± 11.5	1297.1 ± 52.4	73.1 ± 4.2	691.6 ± 81.4
Ldlr $^{-/-}$	Male	7	HFD	IgG Ab	13	36.8 ± 0.9	1462.2 ± 72.2	108.6 ± 11.1	1281.4 ± 50.7	72.5 ± 5.1	679.2 ± 75.2
Ldlr $^{-/-}$ with *TF* $^{+/-}$ BM	Male	7	Chow	Irradiation	0	26.6 ± 1.5	139.2 ± 23.5	11.1 ± 1.1	71.8 ± 17.0	48.0 ± 3.9	69.2 ± 7.7
Ldlr $^{-/-}$ with *TF* $^{+/-}$ BM	Male	10	HFD	Irradiation	12	38.8 ± 1.4*	1136.8 ± 23.5*	77.6 ± 11.3*	1014.2 ± 75.2*	83.2 ± 1.5*	495.0 ± 85.6*
Ldlr $^{-/-}$ with low TF BM	Male	6	Chow	Irradiation	0	24.8 ± 1.2	126.4 ± 19.5	12.3 ± 0.9	69.5 ± 20.7	44.6 ± 7.2	76.7 ± 6.6
Ldlr $^{-/-}$ with low TF BM	Male	9	HFD	Irradiation	12	29.9 ± 1.2*†	1175.0 ± 78.4*	75.4 ± 8.4*	981.1 ± 24.8*	80.2 ± 2.3*	471.5 ± 55.7*
Tlr4 $^{+/+}$ x *Ldlr* $^{-/-}$	Male	10	Chow	None	0	28.9 ± 1.3	212.3 ± 6.1	10.6 ± 1.0	124.7 ± 20.4	77.2 ± 7.2	65.4 ± 6.4
Tlr4 $^{+/+}$ x *Ldlr* $^{-/-}$	Male	15	HFD	None	12	37.1 ± 0.5*	1154.6 ± 107.8*	54.8 ± 12.1*	1018.6 ± 104.2*	81.9 ± 5.9	334.5 ± 49.5*
Tlr4 $^{-/-}$ x *Ldlr* $^{-/-}$	Male	10	Chow	None	0	29.9 ± 0.5	215.1 ± 15.1	11.0 ± 0.73	118.4 ± 11.8	85.2 ± 2.9	67.9 ± 3.4
Tlr4 $^{-/-}$ x *Ldlr* $^{-/-}$	Male	24	HFD	None	12	38.1 ± 0.9*	1185.9 ± 33.8*	62.4 ± 4.7*	1036.6 ± 36.8*	91.1 ± 5.2	383.7 ± 31.6*
Tlr6 $^{+/+}$ x *Ldlr* $^{-/-}$	Male	7	Chow	None	0	26.9 ± 1.7	219.4 ± 15.6	11.6 ± 0.8	112.7 ± 6.2	95.2 ± 7.9	72.2 ± 6.2
Tlr6 $^{+/+}$ x *Ldlr* $^{-/-}$	Male	10	HFD	None	12	37.2 ± 0.8*	1275.0 ± 71.5*	104.5 ± 9.3*	1086.9 ± 54.2*	83.9 ± 6.2	653.2 ± 67.2*
Tlr6 $^{-/-}$ x *Ldlr* $^{-/-}$	Male	7	Chow	None	0	28.9 ± 0.9	204.4 ± 21.3	11.6 ± 0.6	93.9 ± 11.3	99.8 ± 4.6	72.2 ± 6.2
Tlr6 $^{-/-}$ x *Ldlr* $^{-/-}$	Male	12	HFD	None	12	35.7 ± 1.1*	1272.4 ± 52.1*	110.8 ± 6.0*	1077.2 ± 46.0*	84.4 ± 4.5	692.2 ± 37.5*

Abbreviations: HFD: high fat diet; TPC-total plasma cholesterol; VLDL-very low density lipoproteins; LDL-low density lipoproteins; HDL-high density lipoproteins; BM: bone marrow; Tlr: toll-like receptor

Studies are separated by thicker lines.

*$P<0.009$ HFD versus chow controls. One Way ANOVA with Holm-Sidak post hoc analysis.

†$P<0.005$ Low TF BM HFD versus *TF* $^{+/-}$ BM HFD. One Way ANOVA with Holm-Sidak post hoc analysis.

Supplemental table 3. Composition of semisynthetic diets fed to African Green monkeys

Ingredient	High fat, high cholesterol diet	Simvastatin diet
	g/100g dry weight	
ACHumko Oleic Blend	16.4000	16.4000
Fish Oil (Omega Protein)	0.2000	0.2000
Casein, USP	9.0000	9.0000
Lactalbumin	5.0000	5.0000
Dextrin	9.6000	9.6000
Sucrose	10.0000	10.0000
Wheat Flour, self-rising	35.0000	35.0000
Alphacel	7.1490	7.0911
Crystalline Cholesterol	0.1200	0.1200
Vitamin Mixture, Teklad	2.5000	2.5000
Hegsted Salt Mixture	5.0000	5.0000
B-sitosterol (ICN)	0.0068	0.0068
Tenox 20A	0.0080	0.0080
MTS-50	0.0122	0.0122
Vit E 5-67	0.0040	0.0040
Simvastatin	0.0000	0.0579
TOTAL	100.0000	100.0000

	% of Calories
Fat	37
Carbohydrate	46
Protein	17

mg cholesterol/Kcal	0.03

Supplemental Table 4: African Green monkey weights and lipid profiles

Diet (weeks)	Number of monkeys	Weight (Kg)	TPC (mg/dL)	Total VLDL (mg/dL)	Total LDL (mg/dL)	Total HDL (mg/dL)	Triglycerides (mg/dL)
0	12	6.5 ± 0.2	133.4 ± 12.4	1.1 ± 0.1	49.2 ± 4.7	83.1 ± 8.2	32.3 ± 2.2†
4	12	6.8 ± 0.3	346.0 ± 30.7*	14.7 ± 2.1*	251.6 ± 30.7*	79.2 ± 6.7	21.2 ± 2.1
8	12	6.8 ± 0.3	328.5 ± 22.7*	10.5 ± 1.1**	256.9 ± 26.6*	61.1 ± 6.6	26.0 ± 2.2
16	12	7.1 ± 0.3	339.7 ± 20.4*	17.1 ± 2.6*	258.9 ± 23.2*	63.7 ± 5.1	30.7 ± 2.6†
20	12	7.0 ± 0.3	352.7 ± 34.5*	17.0 ± 3.8*	264.4 ± 35.8*	71.4 ± 6.3	34.0 ± 2.2†

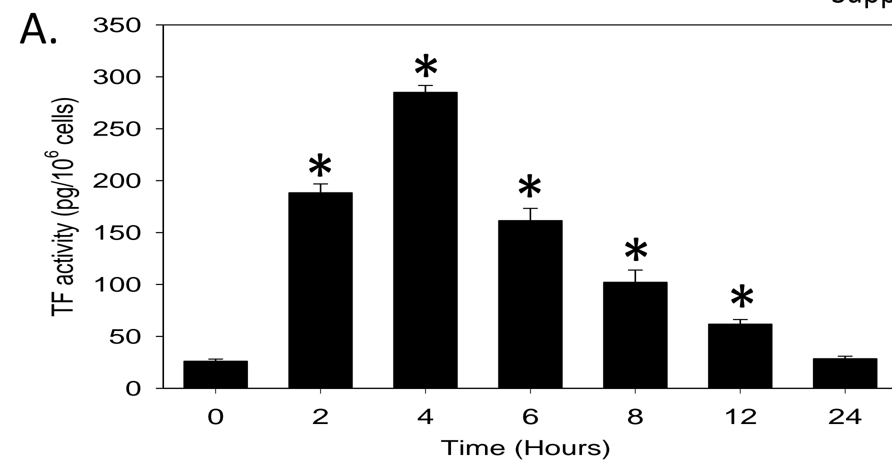
Abbreviations: TPC-total plasma cholesterol; VLDL-very low density lipoproteins; LDL-low density lipoproteins;
HDL-high density lipoproteins

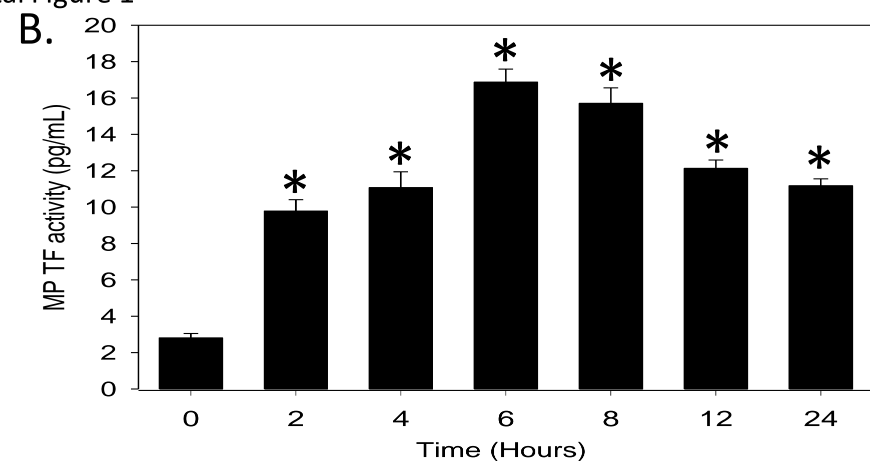
*P<0.001 all timepoints versus 0 weeks. One Way ANOVA with Holm-Sidak post hoc analysis.

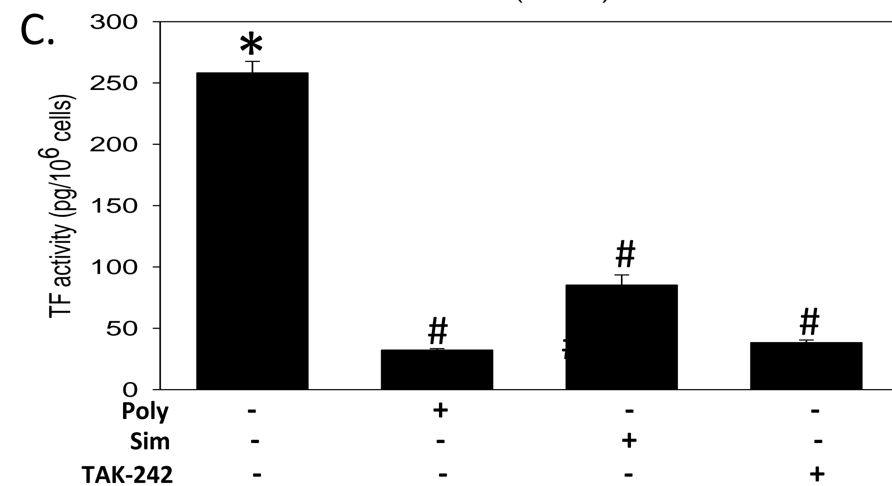
**P<0.042 8 weeks VLDL versus 0 weeks. One Way ANOVA with Holm-Sidak post hoc analysis.

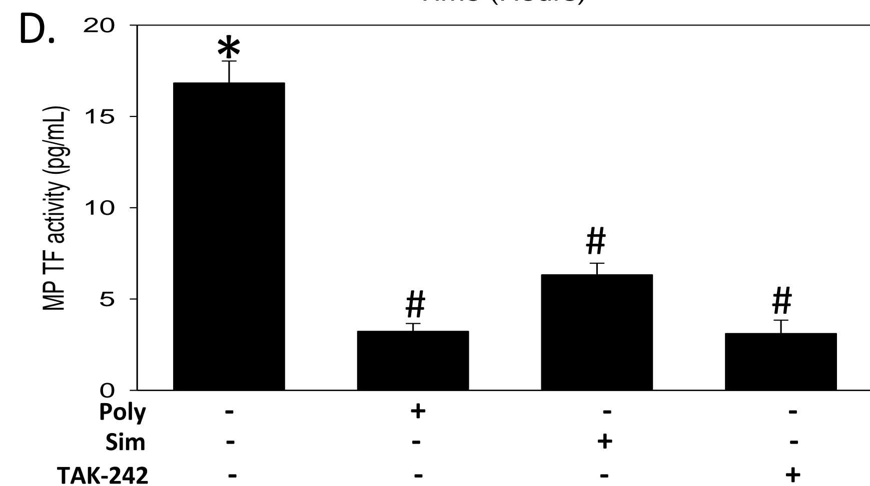
†P<0.033 20, 16, and 0 weeks versus 4 weeks triglycerides. One Way ANOVA with Holm-Sidak post hoc analysis.

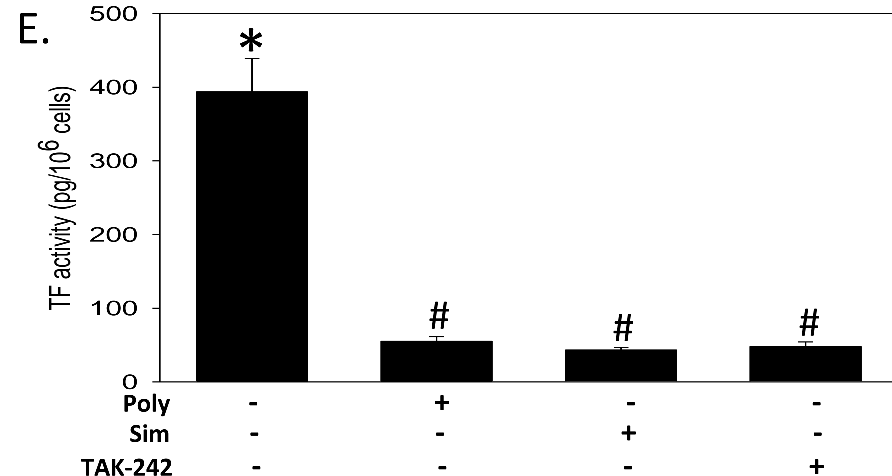
Supplemental Figure 1

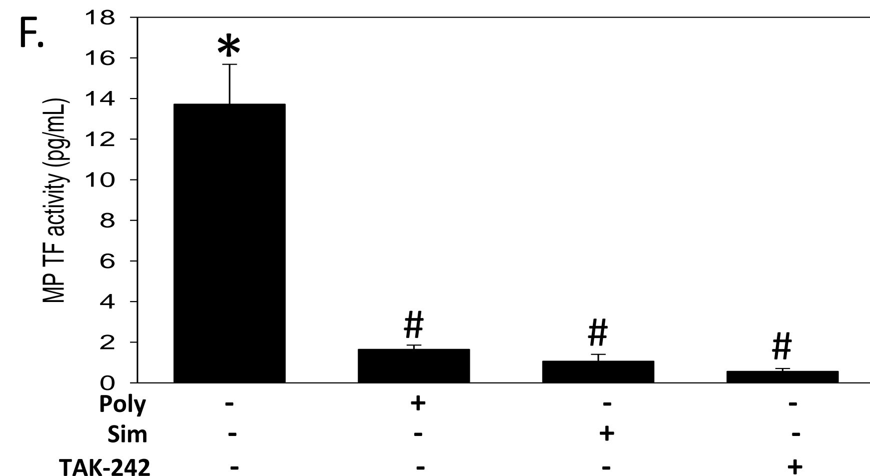












Supplemental Figure 2

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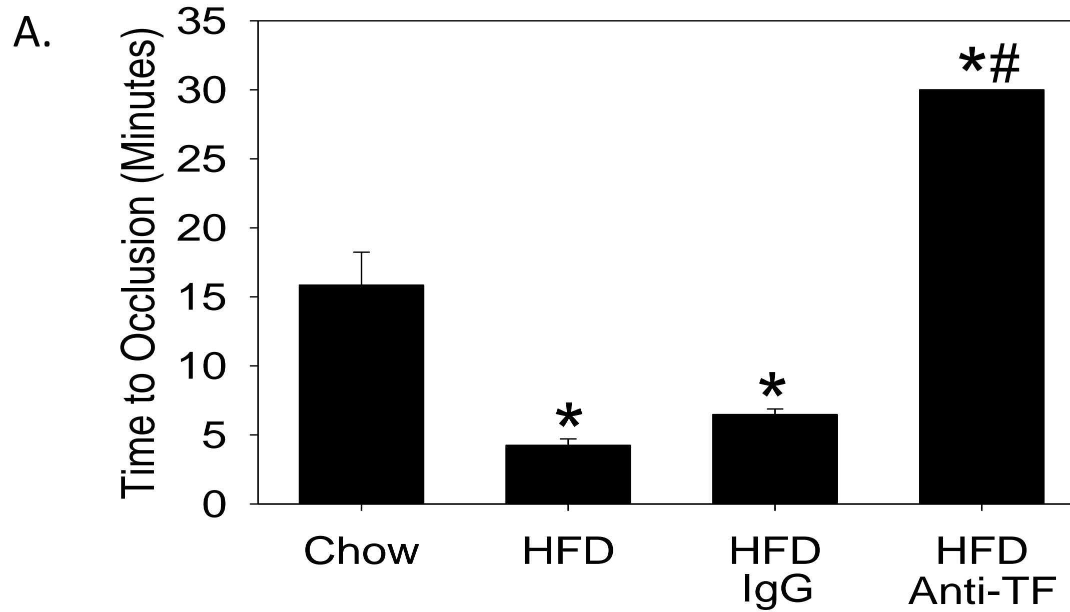

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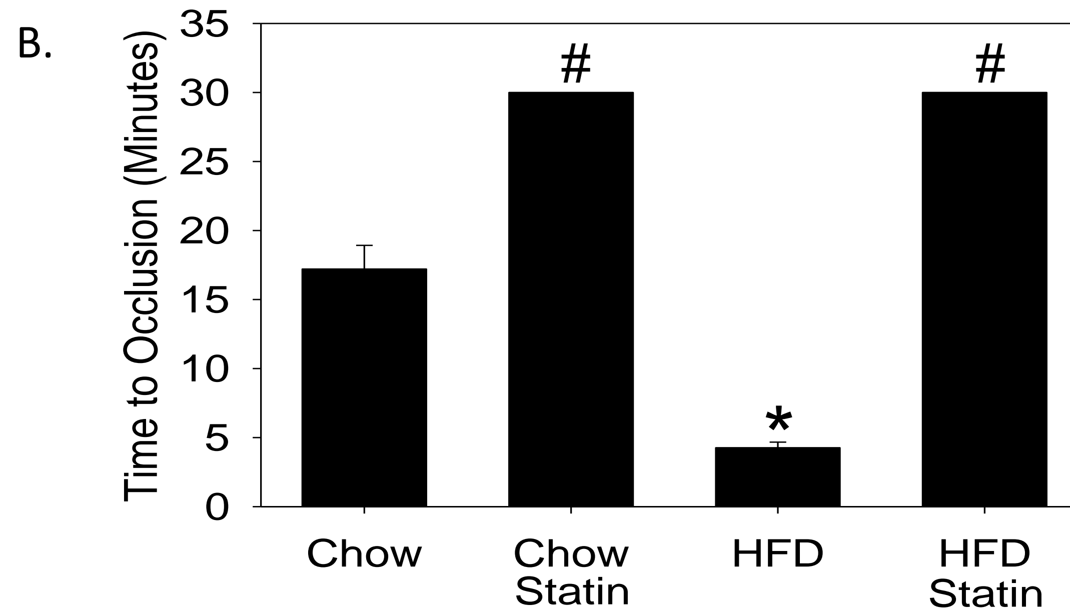
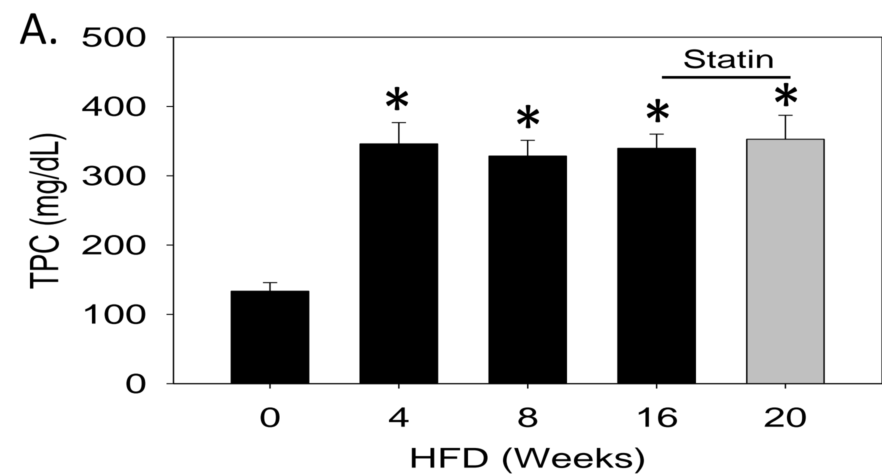
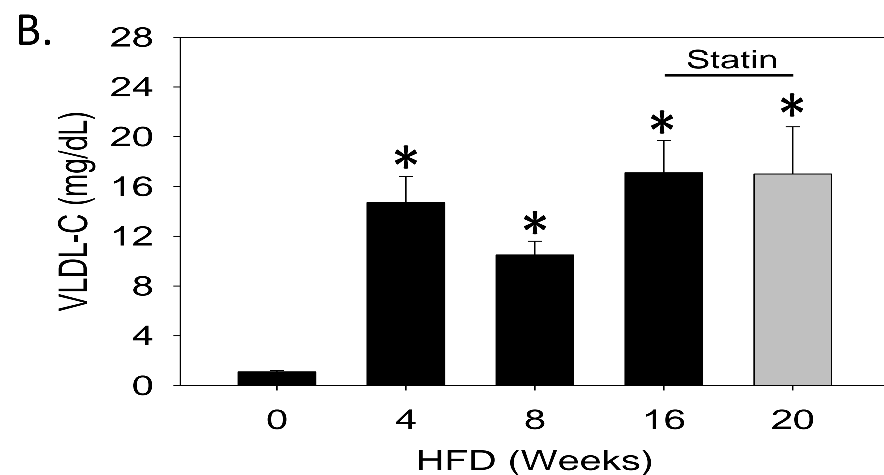
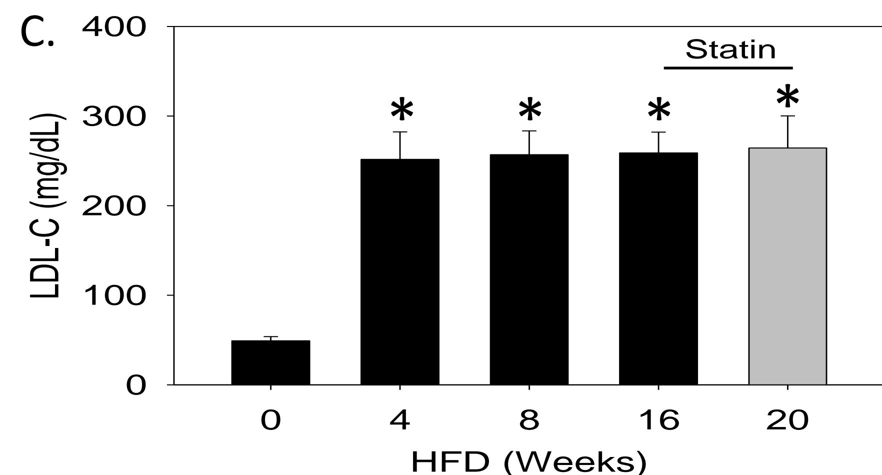
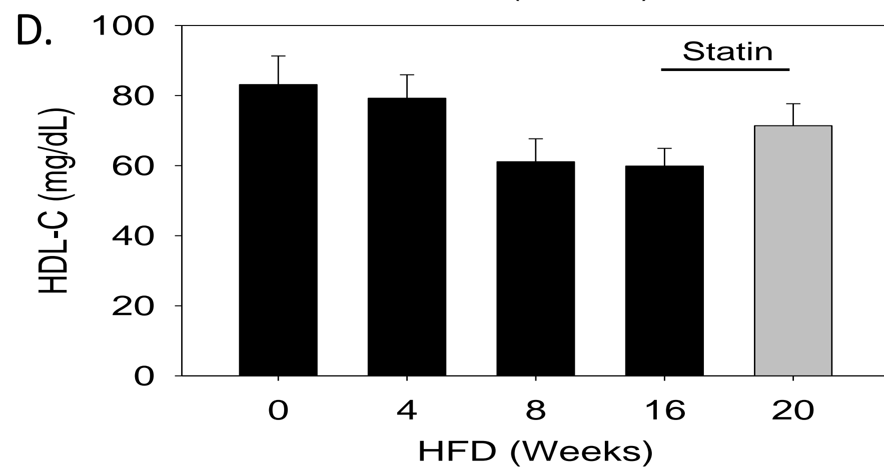
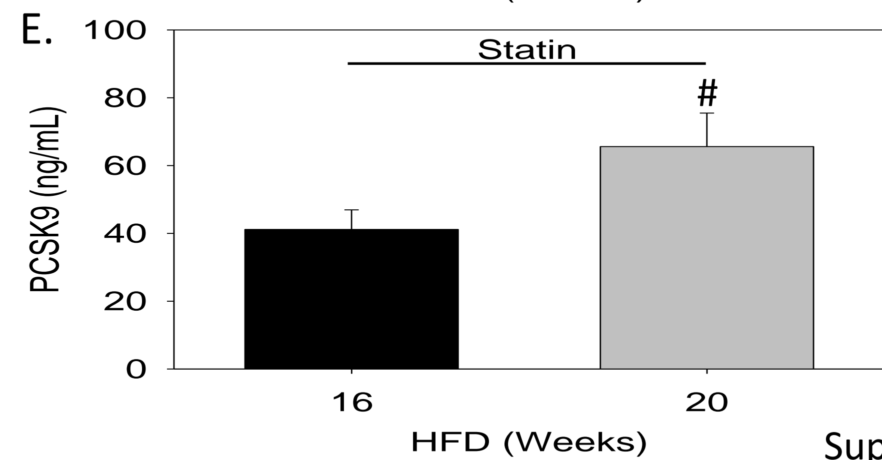


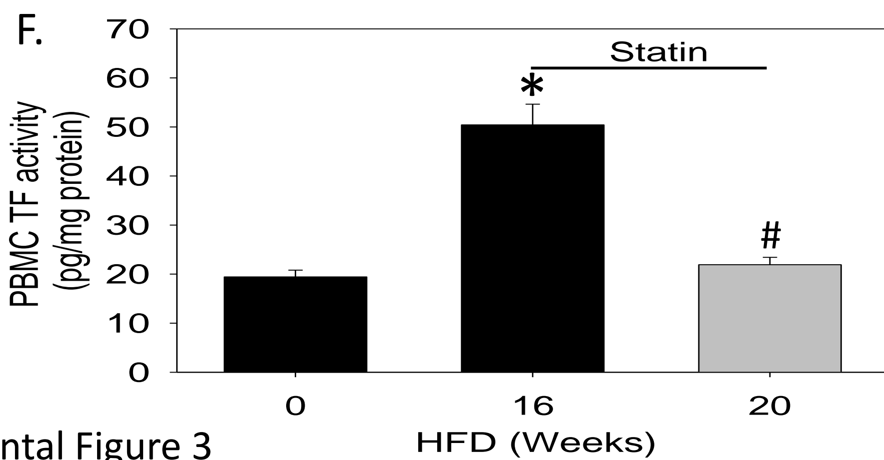








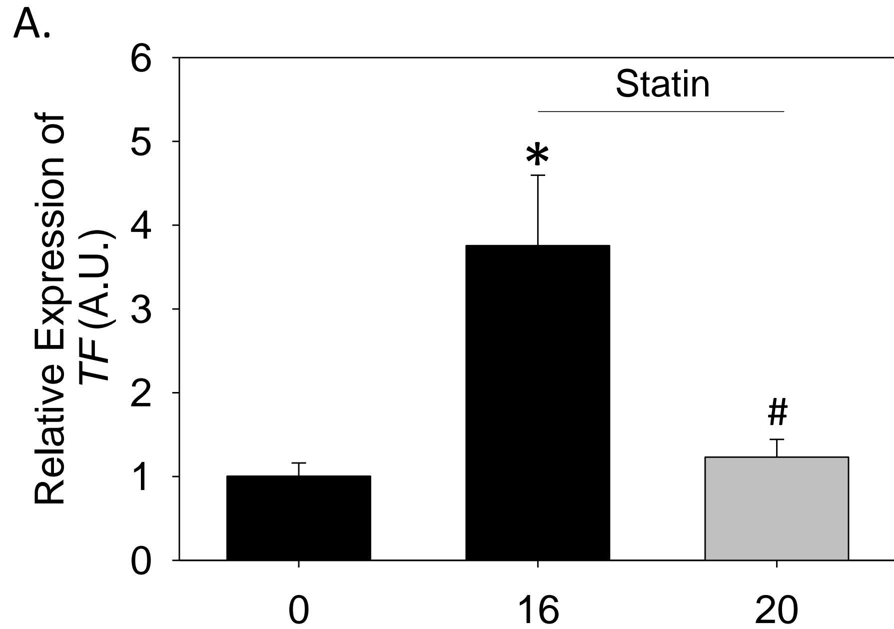




Supplemental Figure 3

Supplemental Figure 4

A.



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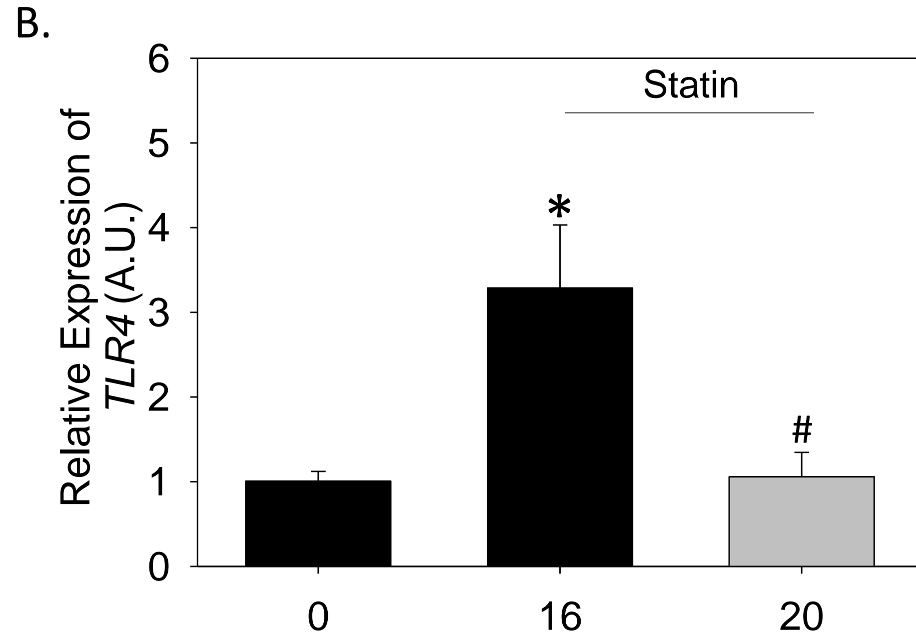

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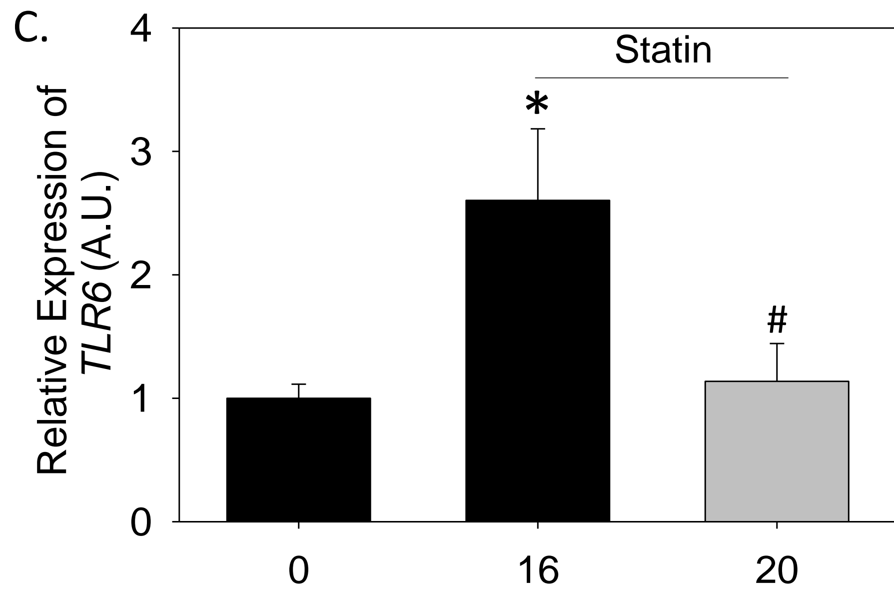

D.