SUPPLEMENTAL METHODS

Pro-atherogenic flow increases endothelial stiffness via enhanced CD36-mediated oxLDL uptake

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Cellular Models:

HAECs (human aortic endothelial cells) and human microvascular ECs were grown using standard Endothelial Cell Growth Medium (EBM-2, Lonza) with supplements (EGM™-2 BulletKit™, Lonza), including 1% pen/strep and 2% FBS (Fetal bovine serum, Invitrogen). HAECs are used between passages 4-12. All cells are fed every 2-3 days and split every 4-7 days. Cell cultures were maintained in a humidified incubator at 37°C with 5% CO_2.

Mouse Models:

Wild Type (WT, C57BL/6) male mice were purchased from either Charles River or Jackson Lab. CD36 KO (B6.129S1-Cd36^{tm1Mfe}, Jackson Lab) were bred in-house. Mice are euthanized by CO_2, followed by cervical dislocation. This procedure is used for all experiments except for blood collection. In the latter experiments, mice are sedated using isoflurane, decapitated and drained of blood. The blood sat on ice for 20-30 minutes followed by centrifugation at 4°C for 10 minutes at 1500 rpm. The plasma top layer is collected for further analysis.

Diet Induced Hyperlipidemia: We used a well-established diet-induced mouse model of dyslipidemia described previously[1]. Eight to ten week old WT and CD36 KO male mice were fed a high-fat, high-cholesterol diet (Harlan, TD.88137) for one month. This atherogenic diet had 0.2% total cholesterol, 21% total fat of which >60% are saturated (42% kcal from fat). Mice were weighed at the start of the diet and at its conclusion.

Measuring LDL and oxLDL in Mouse Blood Plasma: Quantification of total cholesterol and LDL in the plasma was determined by a standard lipid profile analysis by IDEXX. OxLDL measurements were done using a sandwich ELISA from Elabscience Biotech following the manufacturer's protocol.

Mouse Vessel Extraction: After euthanasia, the descending aorta and aortic arch were extracted from male mice and cut longitudinally to expose the EC monolayer. After the harvest, the live tissue sample was glued with double sided tape to a glass coverslip with the endothelial surface on top and immersed in Hank's buffer solution (ThermoFisher). Immediately thereafter, endothelial surface was probed with the AFM. In the selected experiments, the samples were fixed and stained for PECAM-1. *All protocols adhered to the guidelines established by UIC's Institutional Animal Care and Use Committee.*

Isolation and oxidation of LDL:

Human blood plasma collected from normal volunteers was purchased from Life Source (Chicago). LDL was isolated by repeated centrifugations at 4°C using KBr (1.019-1.063 g/mL). Following isolation, the LDL is further purified by three rounds of dialysis in a 10mmol/L Tris/HCl buffer (4°C) to remove the KBr. To oxidize the LDL, copper sulfate (25 μmol/L) was added for 16 hours followed by the addition of 1mmol/L EDTA to stop the oxidation reaction. A TBARs (thiobarbituric acid-reactive substances) assay kit (ZeptoMetrix) was used to measure the degree of oxidation. 12-25 TBARs oxLDL was used in these experiments.

***AFM microindentation*:**

EC stiffness was assessed by measuring Young's elastic modulus using atomic force microscopy (AFM, Novascan Technologies; Asylum MFP-3D-Bio, Asylum Research, Santa Barbara, CA), as described previously[2, 3]. Briefly, a 10 μm diameter borosilicate glass bead affixed to the tip of a cantilever that served to indent endothelial cells or aortas endothelial monolayers. The force curves are generated from the laser's deflection on the cantilever as it approaches and indents the cell. The cantilever descended toward the cell at a velocity of 2 μm/s until a trigger force of 3 nN was reached, which corresponded to 0.5–1 μm indentation depth, or approximately 10–15% of the total cell height. The force-distance curves were collected and analyzed according to the Hertz model:

$$F = \frac{4}{3}\frac{E}{(1-\nu^2)}\,\delta^{3/2}\sqrt{R}$$

Where F is the loading force; δ is indentation depth; v is the cellular Poisson's ratio (assumed to be 0.5; R is the radius of the spherical indentor (5 μm), and E is the local Young's elastic modulus. The Asylum brand of AFM used a silicon nitride cantilever with a 35^{O} cone tip with the spring constant determined each experiment (range of 0.08-0.24 N/m). The bidomain polynomial model was fit to the experimental force curve using a standard least-squares minimization algorithm. *AFM measurements of cultured ECs* were taken in between the cell's edge and the nucleus to avoid the edge and perinuclear space. The upper panel of the flow chamber was removed after the cessation of the flow to allow the AFM tip access to the cell surface. Regions exposed to laminar vs. disturbed flow were identified by the proximity to the barrier: cells within 2 field views from the barrier (<2 mm) were exposed to DF and cells 10-20 mm away from the barrier were exposed to LF. Four force-distance curves were obtained for every cell and 15-30 cellular measurements were taken per condition per experiment. *AFM measurements of endothelial surface of intact arteries* were taken at 6-10 distinct tissue sites on each vessel with nine indentations per location providing a total of 50-90 curves per condition, or 20-40 distinct cellular measurements. The data are presented as histograms of the elastic moduli for each experimental condition.

Flow apparatus:

Parallel Plate Microfluidic Chamber: Cells are grown to confluency in disposable parallel–plate flow chambers with a step barrier, microfabricated as described below, and mounted into a flow apparatus consisting of a pressure pump and syringes containing cell medium (Ibidi, Integrated Biodiagnostic, Munchen, Germany). The device is designed to create a well-defined non-interrupted unidirectional laminar flow for prolong periods of time. For the duration of the flow experiment, the flow system is maintained in a humidified incubator at 37°C with 5% CO_2.
Microfabricated Flow Channel with Step Barrier: As previously described[4], a step barrier parallel plate chamber is used to mimic recirculating disturbed flow that occurs *in vivo*.

Computational fluid dynamics simulations: A modification of an earlier design was performed to maximize the region of low disturbed flow based on the step's height. Computational distribution of the flow and the shear stress were obtained by employing the microfluidic module of the commercial software Comsol Multiphysics 5.1 (Comsol Inc, Burlington, MA, USA) for an inlet pressure of 3400kPa and a normal grid mesh. The flowing medium is a water solution with viscosity and density negligibly higher than water. The Reynolds number (Re) on the cross section of the step is expected to be:

$$Re = \frac{uD_h}{v} = 216.22$$

Where u is the mean velocity of the flow, υ is the dynamic viscosity and D_h - a hydraulic diameter of the channel (which is proportional to the channel's cross-section divided by its

perimeter). A Reynolds number of 216 dictates laminar flow throughout the chamber, but unstable flow after the step barrier where recirculation occurs. The CAD, computer aided design, image of the chamber was created with AutoCAD software (Autodesk Inc., USA).
Microfabrication: The flow channel was fabricated at UIC's Microfabrication Foundry (PI: David Eddington, PhD) with PDMS (polydimethysiloxane, Ellsworth Adhesives) using photolithography, a well-established material and method for manufacturing patterned microfluidic devices for biological applications. The photomasks used during the fabrication of the molds for the photolithography process were made from Fineline Imaging (Colorado Springs, CO, USA). A subsequent flow channel design had a coverslip #1 bottom instead of PDMS to permit imaging with a confocal microscope. In this case, the PDMS step barrier and glass were plasma treated in order to adhere the step to the glass. In both designs, ECs are grown to confluency on the 0.2% gelatin (Sigma, 2 μg/mL) coated device [except those comparing ECs on a collagen (Corning, 40 μg/mL) versus fibronectin (10 μg/mL) coating, as previously described[5]], then exposed to physiologically relevant flow conditions for 48 hours: 10-20 dynes/cm^2 in laminar regions and low recirculating flow (0-5 dynes/cm^2) in the disturbed flow regions after the step barrier. If the cell loss was greater than 10-20% post-shear then that condition was excluded from the experiment.
Cone and Plate Flow Device: In a select set of experiments, we used a cone and plate apparatus system to generate physiologically-relevant shear stress patterns[6, 7] Athero-protective or athero-susceptible waveform from human carotid arteries was replicated using an *in vitro* dynamic flow system that is developed by Dai et al[6]. Briefly, flow device consisting of a computerized stepper motor UMD-17 (Arcus Technology) and a 1° tapered stainless steel cone. The flow devices were placed in 37°C incubator with 5% CO_2. HAEC at 100% confluence, maintained in EGM2 medium containing 4% dextran in 6-well plates, were subjected to 24 hour athero-protective flow that mimics the shear stress in human distal internal carotid or pro-atherogenic flow that models the flow waveform in carotid sinus.

Microscopy and Immunohistochemistry:

OxLDL uptake is quantified by adding 1 μg/mL of fluorescent rhodamine labeled oxLDL (DiI-oxLDL, Alfa Aesar) into the flow medium for the duration of the experiment (48 hours). In all experiments, full serum is substituted by 10% lipoprotein deficient serum from Sigma-Aldrich (instead of FBS). After the cessation of flow exposure, the cells are fixed and washed within the flow chamber: washed twice with PBS (with Ca/Mg), once with a 0.5 M NaCl 0.2M acidic acid wash and twice with PBS again. The intermediate step washing with the acid wash ensures the removal of any remaining attached DiI-oxLDL particles still bound to the EC surface. Cells were imaged using a Ziess Axiovert Fluorescent Microscope with a long-distance 40X lens. In a select set of experiments performed in microfluidic chambers with the glass bottom with a PDMS barrier, the imaging was performed with a Ziess Confocal Microscope. To avoid oversaturation of the images, each condition was initially imaged at 2-3 randomly selected locations per condition to determine the optimal settings for each experimental group. For confocal microscopy the optimal gain and offset was determined and for florescent microscopy the optimal exposure time for each condition (using the 'measure' function on the Ziess Axiovision 4.8 software). Average cellular fluorescence was assessed in ImageJ by outlining 2-7 cells per image, 6-12 images per condition for each experiment.

Immunohistochemistry: Briefly, cells are fixed in 3.8% Formaldehyde (Fisher), permeabilized, washed with PBS (Gibco), and blocked with 5% bovine serum albumin (Sigma) at room temperature (RT) for 2 hours. Cells are then washed and primary antibody (mouse monoclonal anti-CD36, Abcam, 185-1G2, 1:200) is applied overnight in 4°C on a slow rocker. Following several washes, the secondary antibody (AFF 488, donkey anti-mouse, 1:1000) is added at RT for 1 hour on a shaker, washed and mounted for imaging. Similarly to oxLDL uptake, imaging

was performed using Ziess Axiovert Fluorescent Microscope for cells grown in PDMS flow chambers and using Ziess Confocal Microscope for cells grown in a glass-bottom chambers. All experiments had an IgG control and a secondary only control. Average cellular fluorescence was quantified with ImageJ software.

Histology: Aortic arches and descending aortas were excised from WT and CD36 KO mice aged 5-6 months. The tissues were embedded in OCT (Optimal Cutting Temperature) Compound (Fisher), frozen on dry ice and sectioned at 8 μm. Tissues were then stained for 1 hour at RT with both rat anti-mouse PECAM-1 antibody (BD Biosciences, MEC 13.3, 1:50) and rabbit polyclonal anti-CD36 antibody (ProteinTech, #18836, 1:75) with a DAPI counterstain (Invitrogen ProLong™ Diamond Antifade Mountant with DAPI). After washing, secondary antibodies were applied for 1 hour at RT: AlexaFluor488 Goat-anti-rat (Jackson ImmunoResearch, 1:800) and Rhodamine-Red-X Goat-anti-rabbit (Jackson ImmunoResearch, 1:800). Consecutive histological sections were stained with Oil Red-O Stain (VWR) for 30 minutes at RT and counterstained with Hematoxylin Hydrate for three minutes (MP Biomedical). All stained tissues were imaged and analyzed using ImageJ and Axiovision software.

CD36 Internalization: For one set of these experiments, a confluent monolayer of HAECs was treated to the live cells with a rhodamine-tagged plasma membrane marker (Cell Light^{TM}, BacMam 2.0) overnight, per the manufacturer's protocol. Following several washes with PBS, the cells were serum starved for 3 hours. To achieve staining of the cells for surface expression of CD36, the HAECs were put on ice and washed with 5% bovine serum albumin (BSA, Sigma) blocking solution for 10 minutes. Chilled primary mouse monoclonal anti-CD36 antibody (Abcam, 185-1G2, 1:75) was applied to the cells for 20 minutes in the cold, followed by washing and the application of FITC-labeled secondary antibody (Invitrogen) for 10 minutes. When cell surface staining of CD36 was desired, the cells were washed and fixed with 0.2% formaldehyde (FA) in the cold for 25 minutes. When CD36 endocytosis was desired, the cells were washed and placed in a 37^{o}C incubator without CO_2 for 30 minutes and then fixed at room temperature with 0.2% FA for 15 minutes. Cell surface staining and internalization of CD36 was visualized with 0.2 μm z-stack slices using a confocal microscope. The second set of these experiments was investigating DiI-oxLDL (1 μg/mL) uptake therefore the rhodamine-tagged plasma membrane marker was not used and the blocking step using BSA was omitted. All other steps remained the same.

Endothelial alignment was assessed by analyzing the brightfield images acquired from the Ziess Fluorescent Microscope. Using ImageJ, the long axis of the cell was determined and the cell angle measured (0 degrees being the direction of flow). The alignment was determined for 3-5 cells per image, 4-5 images per condition for each experiment. Three to four separate experiments were analyzed for each condition.

En face: Mouse vessels were opened up longitudinally, endothelial side up, and fixed in 3.8% paraformaldehyde for 1-2 hours. Fixed tissues were then washed with PBS, blocked with 5% bovine serum albumin, and rat anti-mouse PECAM-1 primary antibody (BD Pharimgen, MEC 13.3, 1:200) applied overnight on a rocker at 4°C. Samples are washed several times and secondary antibody is applied for 1 hour. Following several washes, samples are mounted for immediate imaging.

Total internal reflection fluorescence (TIRF) microscopy experiments were performed with a motorized Ziess Laser TIRF imaging system with a high-speed EMCCD camera (Quantem 512SC; Photometrics). Fluorescent images (FITC and rhodamine channels, excitation 488 and 561 nm, respectively) were acquired with a 63x/1.46 NA alpha Plan-Apochromat objective to observe the basal plasma membrane in 100 nm thick optical sections adjacent to the cover glass. The acquisition time, EMCCD gain and laser intensity was kept constant.

Real-time PCR, Western Blot and siRNA:

Real-time PCR

Total RNA was isolated using mirVana miRNA isolation kit (Life Technologies) or Direct-zol RNA MiniPrep (Zymo Research) and was reverse-transcribed into cDNA using SuperScript III (Life Technologies) or High Capacity cDNA Reverse Transcription kit (Life Technologies) following manual instruction. Absolute Quantitative PCR was performed on LightCycler 480 II (Roche) using SYBR Green I Master. Absolute quantification of CD36 or LOX-1 gene expression was normalized to the geometric mean of GAPDH, β-actin and ubiquitin.
PCR primers are listed as follows:
CD36 forward, 5'-GCAGCAACATTCAAGTTAAGCA-3';
CD36 reverse, GCTGCAGGAAAGAGACTGTGT-3';
LOX-1 forward, 5'-AAGTGGGGAGCCCAAGAAAG-3';
LOX-1 reverse, 5'-GGGCCACACATCCCATGATT-3';
GAPDH forward, 5'-TGCACCACCAACTGCTTAGC-3';
GAPDH reverse, 5'-GCATGGACTGTGGTCATGAG-3';
β-actin forward, 5'- TCCCTGGAGAAGAGCTACGA-3';
β-actin reverse, 5'- AGGAAGGAAGGCTGGAAGAG-3';
ubiquitin forward, 5'-ATTTAGGGGCGGTTGGCTTT-3';
ubiquitin reverse, 5'-TGCATTTTGACCTGTTAGCGG-3'.

Western Blot

HAECs were washed with cold PBS to remove residual media and then lysed in lysis buffer with protease inhibitor cocktail. Cells were sonicated briefly, and spun for 10 minutes at 14,000 x g at 4°C. The protein concentration of the supernatant was determined by a Bio-Rad protein assay reagent. 30 µg total protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF (polyvinylidene difluoride) membranes. The membranes were then blocked in Tris-buffered saline with 0.1% Tween 20 (TBST) containing 5% BSA, and probed with rabbit polyclonal anti-CD36 (Proteintech, #18836, 1:1000) and rabbit polyclonal anti-Lox1 (Abcam, #60178, 1:1000) overnight at 4°C. After applying with secondary antibody, the membranes were washed with TBST and developed with enhanced chemiluminescence (Thermo Scientific). Quantitative analysis was performed with ImageJ software.

Knockdown using siRNA: HAECs were seeded and grown to 95-100% confluence. The cells were transfected with FlexiTube Gene Solution for OLR1 (Lox1), CD36, with All Star Negative siRNA from Quiagen. SiRNAs were prepared per manufactures instruction. In brief, a mixture of Lipofectamine RNA mix, Invitrogen RNA max, OptiMEM and the siRNA were added together and incubated for 30 minutes at RT, after which it was added to the cells for a final siRNA concentration of 50 nmol/L. Cells were treated with the siRNA for 48 hours prior to using them for the experiments. Initially, four siRNAs for Lox1 and four siRNAs for CD36 were tested for reduction in mRNA expression. The two siRNAs for each gene that had the greatest decrease in mRNA expression (at least 80% decrease) versus scrambled control was chosen for further analysis for protein expression knockdown using western blot.

Over-expression of CD36 in CHO (Chinese Hamster Ovary) cells was achieved by using WT human CD36, cloned in pcDNA3.1/myc-His A vector (Invitrogen, #V800-20). CHO cells, which were cultured in Opti MEM Reduced Serum, were appropriately split to reach ~90% confluency at the time of the transfection. Transfection was performed using the Lipofectamine 2000 reagent, according to manufacturer's instructions. The CD36 plasmid and lipofectamine were diluted separately in an antibiotic and fetal bovine serum free in Opti MEM at RT for 5 minutes. Then, the two were combined and allowed to sit for 30 minutes at room temperature. CHO cells were exposed to this lipofectamine/CD36 mixture and after 24 hours cells were washed and

lysed in RIPA buffer. Western blotting technique was used to confirm successful CD36 transfection.

Liquid Chromatography-Electrospray Ionization Tandem-Mass Spectrometric (LC-ESI-MS/MS) Analysis was performed as previously described[8]. In brief, sterols and oxysterols were quantified using an EST-LC-MS/MS approach using AB Sciex 6500 QTRAP mass spectrometer. Internal deuterated cholesterol and oxysterol standards were used. Cholesterol and oxysterols compositions were quantified using an isotope dilution approach.

Bone Marrow Transfer:

Two to three month old host WT and CD36 KO male mice were exposed to 10 Gy radiation followed by bone marrow transfer of donor WT macrophages 4-6 hours later. The donor WT mouse (Ly5.1) carries the differential Ptprca leukocyte marker commonly known as CD45.1 while WT C57BL/6 inbred mice express the Ptprcb (CD45.2) allele. Mice were monitored either every day or twice per week following the standard post-bone marrow transfer (BMT) protocol. One month post-BMT, blood from each mouse was collected and used to determine the degree of donor macrophage incorporation into each host mouse, using flow cytometry. Stiffness measurements were performed on the arch and descending aortic tissues from the recipient WT and CD36 KO mice two months post-BMT, using AFM.

Statistical Analysis:

One way ANOVA, analysis of variance, (α=0.05) was performed to detect differences between the mouse weights, total cholesterol, LDL and oxLDL in WT LFD vs HFD fed mice. All other statistical analysis was done with a two-way ANOVA with replication (α=0.05) was performed using Excel to determine statistical significance. Further statistical analysis was performed using a standard t-test assuming two-tailed distributions with unequal variances. A p-value less than 0.05 was considered significant.

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