SUPPLEMENTAL MATERIALS

Macrophage-specific IGF-1 overexpression reduces CXCL12 chemokine levels and suppresses atherosclerotic burden in Apoe-deficient mice

Patricia Snarski[1,2] BA, Sergiy Sukhanov[1,2] PhD., Tadashi Yoshida[1,2] PhD., Yusuke Higashi[1,2] PhD., Svitlana Danchuk[1,2] MS, Bysani Chandrasekar[3,4] PhD., DVM, Di Tian[6] MD, PhD, Vikara Rivera-Lopez[7], Patrick Delafontaine[1,2,5] MD.

[1]Section of Cardiology, John W. Deming Department of Medicine, Tulane University School of Medicine, New Orleans, LA
[2]Department of Physiology, Tulane University School of Medicine, New Orleans, LA
[3]Harry S. Truman Memorial Veterans' Hospital, Columbia, MO
[4]Department of Medical Pharmacology and Physiology, University of Missouri, Columbia, MO
[5]Department of Pharmacology, Tulane University School of Medicine, New Orleans, LA
[6]Department of Pathology, Tulane University School of Medicine, New Orleans, LA
[7]Loyola University, New Orleans, LA

SUPPLEMENTAL MATERIALS AND METHODS

Animals. The SRA-IGF-1-IRES-GFP vector was injected into zygotes of C57BL/6J mice (Jackson Laboratory #000664) by Caliper Life Sciences. Of the four lines of mice that had multiple tandem repeats of the IGF-1 gene, we decided to discard #31 because it had multiple single inserts as well, and #36 was lost because it did not breed well. The #12 and #17 transgenic animals were crossed with Apoe$^{-/-}$ mice (Jackson Laboratory #002052) to generate SRA-IGF1/ApoE$^{-/-}$ mice. Mice genotyping was carried out using the following primers: 5'-GCACTCTGCTTGCTCACCTTTA-3' and 5'-GGTGGAACGAGCTGACTTTGTA-3' targeting rat IGF1 and resulting in a transgenic band size of 280 BP (data not shown). Wild type littermates on an Apoe$^{-/-}$ background were used as controls. We found that the SRA-IGF1 #17 line (further referred to as MF-IGF1 mice) had significant increase in IGF-1 secretion by peritoneal macrophages so this line was selected for the current study. Eight-week old MF-IGF1 mice and control mice were fed with a high-fat diet (42% of total calories from fat, 0.15% cholesterol, Envigo TD.88137) for 12 weeks. The numbers of mice in each experiment are listed in the figure legends. Body weight, blood pressure, triglycerides, and cholesterol were measured every 2 weeks starting at the first week of mice feeding with high-fat diet. Animal number was calculated via power analysis using previously published data. We used the minimum number based on previous experiments. When analyzing all *in vivo* data, we did not see sex differences except as noted.

Cells. Human THP-1 mononuclear cells were obtained from ATCC (TIB-202) and grown in RPMI 1640 (ATCC 30-2001) supplemented with 10% HI FBS (Sigma F2442) and 50 nM 2-mercaptoethanol (BME, Thermo 21985023). Cells were plated at 6x10^6 cells/ml in 6 or 12 well plates and differentiated into macrophages by treatment with phorbol myristate acetate, (PMA, Sigma P1150) as we described previously[20]. After 24 hours incubation with PMA, cells were serum starved for another 24 hours, and then treated with human recombinant IGF-1 (Ipsen).

Laser capture microdissection (LCM). Hearts were embedded in optimal cutting temperature (OCT) compound (Fisher Scientific 23-730-571) and serial 10µm snap-frozen sections were obtained through the entire aortic valve. Sections were fixed with 75% ethanol and three to eight serial sections were stained with Histogene® LCM Frozen Section Staining Kit (Thermo Fisher, KIT0401) to distinguish between atherosclerotic plaque and medial layer. Plaque area was defined as the area bordered by internal elastic lamina to the edge of the intima[25]. LCM was performed with the Applied Biosystems® ArcturusXT™ LCM System by using a Nikon Eclipse Ti-E inverted research microscope (University of Missouri-Columbia) and a combination of IR and UV lasers to provide laser-capture and laser cutting into one modular platform. LCM dissected tissue was used for RNA isolation with the PicoPure Frozen RNA Isolation Kit (Thermo Fisher KIT0204) followed by cDNA synthesis with RiboAmp HS Plus cDNA kit (Thermo Fisher KIT0529) according to the manufacturer's instructions. RNA quality/concentration was evaluated with Tak3 plates on a Cytation5 imager (Bio-Tek). In addition, quality of RNA isolated from LCM dissected tissue was evaluated with RT² PCR Array Mouse RNA QC (Qiagen). Eight to forty nanograms of RNA was used for cDNA synthesis with Arcturus® RiboAmp® HS Plus cDNA kit according to the manufacturer's instructions. We confirmed proper dissection by measuring different markers in plaque and non-plaque isolates from LCM samples. α-Smooth Muscle Actin (αSMA) was present in both plaque and media samples, CD68 was elevated in plaque samples compared to media, and cardiac tissue showed high levels of troponin C type I while it was not detectable in plaque isolates (data not shown).

Oil Red O Assay. Foam cell formation was assayed using THP-1 cells as we described[26]. Briefly, cells were incubated in serum-free media (SFM) supplemented with PMA and BME and then treated with IGF-1. Oxidized LDL (oxLDL) or native LDL (ntLDL, control) (80µg/ml) was added to cells for 48 hours. Cells were fixed (4% paraformaldehyde, 1h), stained with Oil Red O (5mg/ml in 60% isopropanol, 30 min) and counterstained with hematoxylin (Abcam ab150678, 30 min). CellSens Dimension software (Olympus Life Science) was used for cell imaging (20x) and quantification of Oil Red O-positive area per cell is shown in the figures. In additional experiments, recombinant CXCL12 (80µg/ml, Abcam ab9798) was co-incubated with or without IGF-1 (100ng/ml) for twenty-four hours beforehand and the foam cell assay was performed as described above.

Isolation of peritoneal macrophages. Peritoneal macrophages were induced by injecting 4% thioglycolate broth followed by harvesting of peritoneal cells after 4 days. For cell harvesting, mice were euthanized with

CO^2 inhalation followed by cervical dislocation. Ice-cold PBS (10 ml) was injected into the peritoneum and peritoneal lavage was collected, washed, and treated with RBC lysis buffer (BioLegend 420300). Cells were plated at 1.2×10^6 cells/ml in RPMI-1640 media supplemented with 2% serum overnight. Adhered cells were confirmed to be macrophages based on their morphology and staining with Diff-Quik as we described[20]. For measuring IGF-1 levels in peritoneal macrophages, cells were incubated in SFM for 24 hours, supernatant was collected, and IGF-1 was measured by rat/mouse specific ELISA (RnD MG100). IGF-1 levels were normalized to cell protein (BCA protein assay, Thermo Pierce 23225).

PPP Inhibition. Peritoneal macrophages isolated from control and MF-IGF1 mice were plated at 600,000 cells/ml (2ml) in 6 well plates in RPMI with 10% FBS overnight. The cells were washed with SFM and then treated with nothing or picropodophyllin (PPP, 50nM, Selleck Chemicals S7688) for 48 hours. Cell lysate was collected and blotted for ABCA1 (Novus NB400-105).

Isolation of monocytes. Whole blood was treated with RBC lysis buffer (BioLegend 4203001) and the resulting sample was incubated with a cocktail of antibodies (all BioLegend) against T cells (Alexa Fluor 700 anti-mouse CD90.2 (Thy-1.2), B cells (Alexa Fluor 700 anti-mouse/human CD45R/B220 Antibody), natural killer cells (Alexa Fluor 700 anti-mouse NK-1.1 Antibody), granulocytes (Alexa Fluor 700 anti-mouse Ly6G Antibody), myeloid cells (APC anti-mouse/human CD11b Antibody), and leukocytes (PE/Cyanine7 anti-mouse CD45 Antibody). Circulating monocytes were identified as $CD45^+$, $CD11b^+$, and $(CD90/B220/CD49b/NK1.1)^-$ cells[17]. To isolate monocytes from spleen, whole spleen homogenates were obtained by using a Bullet Blender (Next Advance). For cell sort experiments, negative gating was not performed but additional positive selection was performed via staining for CD115-APC (BioLegend 135509), CD11b-FITC (BioLegend 101212), and CD45-PE/Cy7 (BioLegend 103114) and monocytes were identified as $CD115^+/CD11b^+/CD45^+$ positive cells. Monocyte identity was confirmed by assessment of cell markers (Table 1, Suppl.Fig.V.A,B).

Circulating Cell Quantification*.* Circulating leukocytes and endothelial progenitor cells populations were quantified via FACS. Myeloid and lymphocyte panels were modified from Liu et al.[30]. Briefly, whole blood was collected by cardiac puncture. Red blood cells were lysed with RBC buffer, and cells (100,000 per reaction) were incubated with a Fc block (Fc block CD16/CD32, BD 553141), then stained with 1µl each of the corresponding antibodies (all BioLegend unless noted), then fixed (4% paraformaldehyde, 30min) and stored in the dark overnight for analysis the next morning. Gating strategy was as follows: lymphoid derived leukocytes were gated as $CD45^+$ (CD45-Alexa Fluor 488 103122), $CD11b^-/CD172a^-$ (CD11b-Allophycocyanin/Cy7 101226, CD172a-PE-Cyanine7, eBioscience 25-172-182), then natural killer cells were gated as $NK1.1^+$ (NK-1.1-PE 108707), B cells as $CD19^+$ (CD19-APC 152409), and T cells were gated as $CD3e^+$ (CD3-Alexa Fluor 700 100215) (Suppl.Fig.V.D). Myeloid derived leukocytes were gated as $CD45^+/(CD11b/CD172a)^+$, then neutrophils were gated as $Ly6G^+$ (Ly-6G-Alexa Fluor 700 BD Biosciences 561236), Eosinophils were gated as Siglec-F^+ (Siglec-F-PE-Vio 615 Miltenyi Biotec 130-112-330) and monocytes were gated as $(Ly6G/\ Siglec\text{-}F)^-/CD115^+$ (CD115-PE/Cy7 135523) (Suppl.Fig.V.E). Based on previous reports[31, 103], endothelial progenitor cells were identified as $CD34^+$ (CD34-PerCP/Cyanine5.5 119327), $VEGFR2^+$ (VEGFR2/KDR/Flk-1-Alexa Fluor 700 R&D Systems FAB4432N) and $CD133^+$ (Mouse CD133-Alexa Fluor 750 R&D Systems FAB11332S) (Suppl.Fig.V.F). These cells were also stained with CD31 (CD31-PE 160203) to confirm endothelial identity (data not shown).

Monocyte recruitment into atherosclerotic plaque. To quantify monocyte recruitment into atherosclerotic plaque, mice were injected intravenously with fluorescent polychromatic red microspheres (1:25 dilution in PBS, Polyscience 19507-5) at 7 and 3 days before sacrifice[28]. The beads are excited at 488nm and emit at approximately 575 nm. Polychromatic red microspheres undergo specific uptake by only mononuclear cells in mouse models[28]. Serial snap-frozen aortic valve cross-sections were obtained and the number of red microsphere–positive cells were counted in plaque and normalized to the number of plaque cells. The efficiency of labeling of circulating monocytes with red microspheres was quantified by flow cytometry analysis and also by assessment of red microsphere–positive cells in the spleen[29]. Flow cytometry was done with a BD LRSFortessa X-20 and cell sorting was performed on a Beckman Coulter MoFlo XDP. Kaluza software (Beckman Coulter Life Sciences) was used to analyze flow cytometry data.

Atherosclerosis quantification. After 12 weeks of high fat diet, animals were placed in a surgical plane of anesthesia via a cocktail of Ketamine/Xylazine. Blood was collected via cardiac puncture, then animals were perfused with saline (5min) and then 4% buffered paraformaldehyde plus 5% sucrose (15min), and the heart and the aorta, connected to the heart down to the iliac branch were excised. Adventitial tissue was removed under a dissection microscope, and the heart was separated from the arch before the brachiocephalic branch. Both tissues were fixed overnight in 4% paraformaldehyde/5% sucrose and hearts were embedded into paraffin. Three aortic valve equally spaced sections were stained with H&E and lesions were manually outlined with CellSens Dimension software by two blinded investigators. The entire aorta was used to quantify aortic lipid accumulation via *en face* analysis as we have previously described[21]. Briefly, cleaned aortas were stained with Oil Red O overnight, then cut longitudinally (including the branches in the arch) and pinned onto a black polymer dish. Images were taken on a Leica MZ6 microscope and the total Oil Red O-positive area was quantified using CellSens Dimension software and normalized to total aortic area.

Atherosclerotic plaque composition. Plaque composition was assessed by immunostaining of aortic valve cross-sections for Mac3 (macrophage marker), calponin and αSMA (smooth muscle cell markers), and Masson's Trichrome staining was used to identify collagen. Cell apoptosis was quantified with TUNEL-Fluorescein kit (Roche 11684795910) with co-staining for Mac3 as described previously[20]. Macrophage cell apoptosis was defined as $TUNEL^+/Mac3^+$ cell number per 1000 $Mac3^+$ plaque cells. Other cell apoptosis was defined as $TUNEL^+/Mac3^-$ cell number per 1000 $Mac3^-$ plaque cells. Sections treated with DNAse I and sections stained with dUTP-omitted TUNEL mixture served as positive and negative controls for TUNEL assay, respectively. The necrotic core was identified as part of the atherosclerotic plaque which appeared to be acellular (hematoxylin-negative) and often contained cholesterol crystals. Necrotic core was measured using Image-Pro software and data presented as percent total plaque area. Antibodies for immunohistochemistry and immunoblotting are listed in the major resources table. Plaque macrophage content was quantified by immunohistochemistry with anti-Mac3 antibody. Briefly, aortic valve cross-sections were stained with anti-Mac3 antibody and DAPI. Mac3-immunopositive and total plaque area were manually outlined in CellSens Dimension software and macrophage plaque level was calculated as a ratio (%) of Mac3 plaque area/total plaque area. In addition, we quantified DAPI number in Mac3-positive area and in total plaque area to obtain $Mac3^+$ cell number per plaque cell number (%). To quantify CXCL12 macrophage levels, cross-sections were co-stained with Mac3 antibody followed by secondary antibody-AlexaFluor594 conjugate and with CXCL12 antibody followed by secondary antibody-AlexaFluor488. Mac3/CXCL12 positive plaque area appeared as "yellow" on RGB images. The "yellow" area was quantified by CellSens software and further normalized per cell number (DAPI). All immunohistochemistry IgG controls can be found in Supplementary Figure IX.

Immunoblotting analysis. In brief, cells were washed with PBS, collected in RIPA buffer (Thermo Fisher 89901) containing a protease and phosphatase inhibitor (Fisher PI78443). Lysates were run on a 10% gel SDS-PAGE and proteins were transferred to nitrocellulose membrane. Immunoblot detections were performed with Chemidoc XRS+ (Bio-Rad). Blots were probed with monoclonal anti-β-actin antibody (Sigma A3854) as a control for equal loading. Antibodies for immunohistochemistry and immunoblotting are listed in the major resources table. To analyze if rat or mouse IGF-1 has any difference in IGF1R signaling efficacy, mouse peritoneal macrophages isolated from control mice were treated with rat (Novus Biologicals NBP2-35219) or mouse (Abcam ab9861) recombinant IGF-1 at increasing dosages (0, 5, 10, 20 ng/mL) for 30 minutes, then cell lysate was collected and blotted for pAkt and Akt (Cell Signaling Technologies CST 9271, CST 4691).

Biochemical assays. Serum cytokine levels were quantified by Mouse Common Chemokine Multi-Analyte ELISArray (Qiagen MEM-009A) according to the manufacturer's instructions. CXCL12 levels were assessed with mouse CXCL12 ELISA (RnD MCX120). Human and mouse CXCL12 transcript and protein levels were measured via qRT-PCR or by immunoblotting, as were human and mouse ABCA1. IGF-1 levels were quantified with mouse/rat IGF-1 ELISA (RnD MG100). Triglycerides were measured using Triglyceride Colorimetric Assay Kit (Cayman Chemical Company 10010303) following manufacturer's protocol. Cholesterol was measured using the Total Cholesterol Assay Kit (Cell Biolabs STA-384) according to manufacturer's protocol. For triglyceride measurements, animals were fed high fat diet for 6 weeks starting at 8 weeks of age and serum was collected every two weeks.

Quantitative real-time PCR. Briefly, total cell RNA was isolated using TriPure Isolation Reagent (Sigma 11667165001) followed by purification with the RNeasy Mini Kit (Qiagen 74104). Complementary DNA was synthesized using the RT2 First Strand Kit (Qiagen 330404) or the Arcturus® RiboAmp® HS Plus cDNA kit and used for a 40-cycle 2-step PCR coupled with a melting curve protocol with sequence-specific primer pairs in the CFX Connect Detection System (Bio-Rad) and analyzed using Bio-Rad CFX Manager software. All RT-PCR primers were obtained from Qiagen (RT² qPCR Primer Assay) except the primers designed to detect both rat and mouse *Igf1*[18] and they are listed in the major resources table.

Long nested PCR. Primers (found in major resource table) were designed to target just upstream and downstream of the entire mouse CXCL12 locus (Ch6.117168535-117181367, 12,832 bp). The predicted targeted sequence size is 14,599 bp. The amplicon from this reaction was then used as a template and a region roughly half of that region (7409 bp) was amplified to confer specificity of the original primer set. 100ng of genomic DNA was isolated from mouse tail and DNA was isolated using the Monarch Genomic Purification kit (New England Biolabs T3010S). Q5 High-Fidelity PCR Kit (New England Biolab E0555S) was used for PCR amplification.

Cholesterol Efflux Assays. THP-1 cells were treated with human recombinant IGF-1 (100ng/ml, 24 hours) and then with IGF-1 and oxLDL (50µg/ml, 24 hours) in SFM. Efflux was measured according to manufacturer's instructions (Sigma-Aldrich MAK192). Briefly, after 4 hours with treatment with a cholesterol acceptor (ApoAI or HDL), cell media was transferred to a white fluorescent clear bottom plate and read at 482/515nm from the top using a Cytation 5 imager (Fm fluorescence). Cells were then lysed in lysis buffer for thirty minutes and the lysate fluorescence was read at 485/528nm from the bottom of the plate (Fc fluorescence). Cholesterol efflux was calculated as (Fm/(Fc+Fm))*100 and that value was normalized to the condition of no IGF-1 treatment. To ensure that IGF-1 did not change initial cholesterol loading of cells, both the Fm (media fluorescence) and the Fc (lysate fluorescence) was measured before adding a cholesterol acceptor and loading ratio was calculated using the equation: (Fc/(Fc+Fm))*100 (Suppl.Fig.VII B). All data is presented as fold change to control due to lot differences in oxLDL, ApoAI, and HDL, as these three components were all isolated from human patients. In THP-1 macrophages, control was designated as cells treated with 0ng/ml IGF-1, while in peritoneal macrophages, control was designated as cells isolated from control mice. All assays included a positive control (cells treated with cyclodextrin, a pan-cholesterol acceptor[27], and well as a negative control (no cholesterol acceptor) (Suppl.Fig.VII.C)." The cholesterol efflux measurement protocol was slightly modified when using peritoneal macrophages. All media was used was phenol free RPMI-1640 (Fisher Scientific 11-835-030). After overnight adherence, cells were washed and then switched to a media containing 10% lipoprotein deprived serum (Kalen BioMedical 880100). After six hours, cells were then switched to SFM containing oxLDL and cells were then labelled, equilibrated, and fluorescence was read as described above.

Smooth Muscle Cell Isolation and IGF-1 measurement. Aortic SMC were isolated as previously described[24]. To measure IGF-1 protein, 750µl of cultured supernatant in was collected from cells that were grown in DMEM/ 20% FBS for a week in a 48 well plate. There was no noted cross-reactivity of bovine IGF-1 in the assay.

MAJOR RESOURCES TABLE

In order to allow validation and replication of experiments, all essential research materials listed in the Methods should be included in the Major Resources Table below. Authors are encouraged to use public repositories for protocols, data, code, and other materials and provide persistent identifiers and/or links to repositories when available. Authors may add or delete rows as needed.

Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex	Persistent ID / URL
SRA 12/ApoE-null mice	In house	C57BL/6J	M:F, 50:50	N/A
SRA 17/ApoE-null mice	In house	C57BL/6J	M:F, 50:50	N/A
ApoE-null mice	Jackson Laboratory, #002052	C57BL/6J	M:F, 50:50	N/A

Genetically Modified Animals

	Species	Vendor or Source	Background Strain	Other Information	Persistent ID / URL
Parent – Male SRA 12/ApoE-null mice	SRA 12/ApoE-null mice	In house	C57BL/6J		N/A
Parent – Female SRA 12/ApoE-null mice	ApoE-null mice	In house	C57BL/6J		N/A
Parent – Male SRA 17/ApoE-null mice	SRA 17/ApoE-null mice	In house	C57BL/6J		N/A
Parent - Female SRA 17/ApoE-null mice	ApoE-null mice	In house	C57BL/6J		N/A
Parent – Male and Female ApoE-null mice	ApoE-null mice	Jackson Laboratory, #002052	C57BL/6J		N/A

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot # or Clone	Persistent ID / URL
Anti-α-smooth muscle actin	Abcam	ab21027	50 µg/ml	N/A	N/A
Anti-ABCA1	Novus Biologicals	NB400-105	1 µg/ml	N/A	N/A
Anti-CD107b (Mac3)	BioLegend	105502	20 µg/ml	N/A	N/A
Anti-Calponin	Abcam	ab46794	1 µg/ml	N/A	N/A
Anti-CXCL12	Cell Signaling	3740S	1:1000	N/A	N/A
Anti-beta-actin	Sigma-Aldrich	A3854	120 ng/ml	AC-15	N/A
MMP8 Antibody	Novus	AF3245	0.5 µg/ml	N/A	N/A
MMP14 Antibody	Novus	NBP267415	1 µg/ml	3-F7	N/A
Phospho Akt	Cell Signaling	CST 9271	1:1000	N/A	N/A
Akt (Pan)	Cell Signaling	CST 4691	1:1000	C67E7	N/A

Polychromatic red microspheres .05µm	Polysciences	19507-5	1:25	N/A	N/A
Anti-NK1.1- AlexaFluor700	BioLegend	108730	2.5 µg/ml	PK136	N/A
Anti-B220-AlexaFluor700	BioLegend	103231	2.5 µg/ml	RA3-6B2	N/A
Anti-CD90-AlexaFluor700	BioLegend	553005	20 µg/ml	53.21	N/A
Anti-CD115-APC	BioLegend	135509	20 µg/ml	AFS98	N/A
Anti-Ly-6C–FITC	BioLegend	553104	2.5 µg/ml	AL-21	N/A
Anti-CD45-PE/Cy7	BioLegend	103114	2 µg/ml	30-F11	N/A
Anti-CD11b-FITC	BDPharmingen	553310	2.5 µg/ml	M1/70	N/A
Anti-CD11b-APC	BioLegend	101212	2 µg/ml	M1/70	N/A
Anti-Ly-6G-AlexaFluor700	BioLegend	127622	2.5 µg/ml	1A8	N/A
Alexa Fluor 488 anti-mouse CD45	Biolegend	103122	20 µg/ml	30-F11	N/A
Anti-IgG2b, k Isotype Ctrl Rat Monoclonal Antibody (Alexa Fluor 488)	BioLegend	400625	20 µg/ml	RTK4530	N/A
APC anti-mouse CD19	BioLegend	152409	2.5 µg/ml	1D3/CD19	N/A
APC Rat IgG2a, κ Isotype Ctrl	BioLegend	400512	2.5 µg/ml	RTK2758	N/A
(Allophycocyanin)/Cy7) anti-mouse/human CD11b	BioLegend	101226	2 µg/ml	M1/70	N/A
Anti-IgG1, k Isotype Ctrl Rat Monoclonal Antibody (APC (Allophycocyanin)/Cy7))	BioLegend	400422	2 µg/ml	RTK2071	N/A
CD172a (SIRP alpha) Rat anti-Mouse, PE-Cyanine7	eBioscience Invitrogen	25-1721-82	2 µg/ml	P84	N/A
Anti-IgG2a, k Isotype Ctrl Rat Monoclonal Antibody (PE (Phycoerythrin)/Cy7))	BioLegend	400521	2 µg/ml	RTK2758	N/A
Anti-NK-1.1 Mouse Monoclonal Antibody (PE (Phycoerythrin))	BioLegend	108707	7.5 µg/ml	PK136	N/A
Anti-IgG2b, k Isotype Ctrl Rat Monoclonal Antibody (PE (Phycoerythrin))	BioLegend	400607	7.5 µg/ml	RTK4530	N/A
Anti-CD3 Rat Monoclonal Antibody (Alexa Fluor 700)	Biolegend	100215	7.5 µg/ml	17A2	N/A
Anti-IgG2b, k Isotype Ctrl Rat Monoclonal Antibody (Alexa Fluor 700)	Biolegend	400628	7.5 µg/ml	RTK4530	N/A
Mouse CD133 Alexa Fluor 750	R&D Systems	FAB11332S	7.5 µg/ml	217106	N/A
Rat IgG2B Alexa Fluor 750-Isotype Control	R&D Systems	IC013A	7.5 µg/ml	141945	N/A
Alexa Fluor® 700 Rat Anti-Mouse Ly-6G	BD Bioscience	561236	7.5 µg/ml	1A8	N/A
Rat IgG2B APC-conjugated Isotype Control	R&D Systems	17-4031-82	7.5 µg/ml	IC013A	N/A
Siglec-F Antibody, anti-mouse, PE-Vio 615, REAfinity	Miltenyi Biotec	130-112-330	0.5 µg/ml	REA798	N/A
REA Control Antibody, human IgG1, PE-Vio 615, REAfinity™	Miltenyi Biotec	130-113-451	0.5 µg/ml	REA293	N/A
Pe/cy7 anti-mouse CD115 (CSF-1R)	BioLegend	135523	7.5 µg/ml	AFS98	N/A
PerCP/Cyanine5.5 anti-mouse CD34	BioLegend	119327	7.5 µg/ml	MEC14.7	N/A

PerCP/Cy5.5 Rat IgG2a, κ Isotype Ctrl	BioLegend	400531	7.5 µg/ml	RTK2758	N/A
Mouse VEGFR2 Alexa Fluor700	R&D Systems	FAB4432N	7.5 µg/ml	522302	N/A
PE anti-mouse CD31	BioLegend	160203	7.5 µg/ml	W18222B	N/A
Anti-IgG2a, k Isotype PE	BioLegend	400211	7.5 µg/ml	MOPC-173	N/A
Fc Block CD16/CD32	BD Bioscience	553141	10 µg/ml	AB_394656	N/A

Cultured Cells

Name	**Vendor or Source**	**Sex (F, M, or unknown)**	**Persistent ID / URL**
THP-1	ATCC, TIB-202	Male	N/A

Genotyping Primers

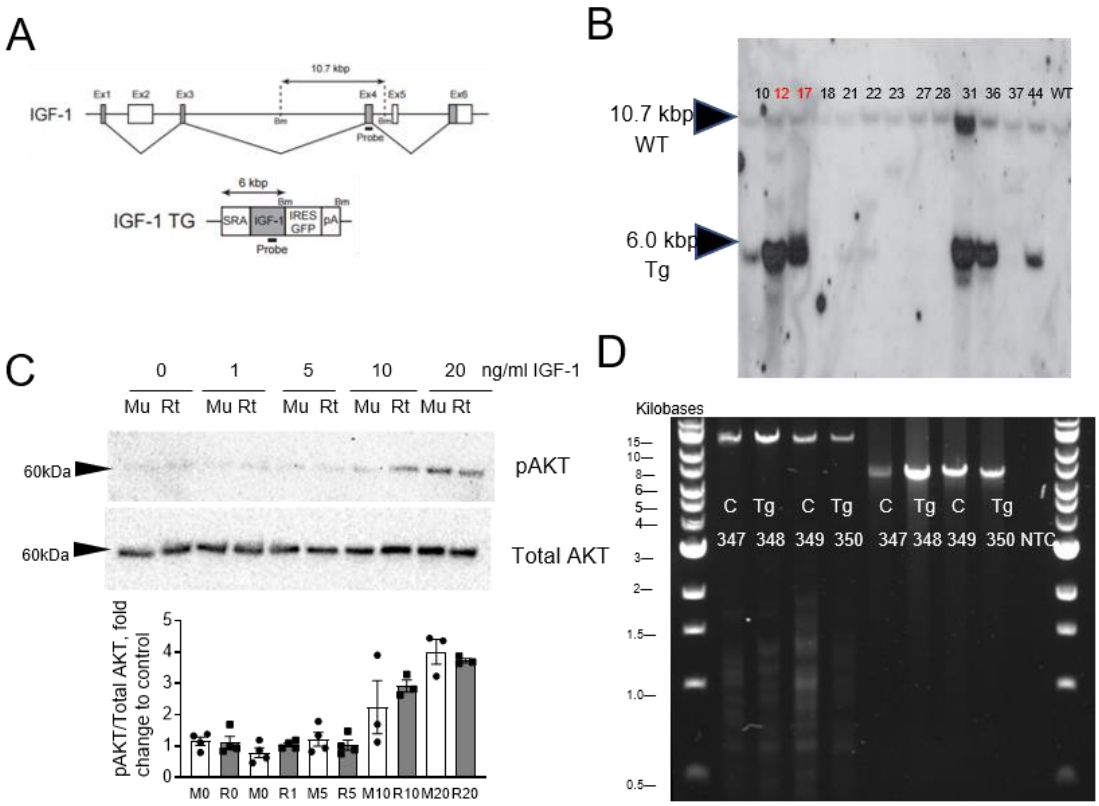
Description	**Primer Sequence 5'→3'**	**Persistent ID / URL**
Rat IGF-1 Transgene Forward	GCACTCTGCTTGCTCACCTTTA	N/A
Rat IGF-1 Transgene Reverse	GGTGGAACGAGCTGACTTTGTA	N/A
ApoE Common Forward	GCCTAGCCGAGGGAGAGCCG	N/A
ApoE Wildtype Reverse	TGTGACTTGGGAGCTCTGCAGC	N/A
ApoE Knockout Reverse	GCCCCGACTGCATCT	N/A
CXCL12 Seq Fwd 1 Out	AGCTGAGTCC CTAAAGTGTGTCTGACA	N/A
CXCL12 Seq Fwd 2 In	GAATGGATGC AAAGTGCTTA GCGGA	N/A
CXCL12 Middle FWD 1 Out	GTCAACACAAG ATCCGGCAGA GG	N/A
CXCL12 Internal REV 2 IN	CCTCTGCCGGAT CTTGTGTTGAC	N/A
CXCL12 Middle FWD 2 IN	CGGACCAATG CTGCAATGGA AGG	N/A
CXCL12 Internal REV 1 OUT	CCTTCCATTGCAG CATTGGTCCG	N/A
CXCL12 Seq REV 2 IN	CTTCCTCTGTCACCTTCCACCATGAC	N/A
CXCL12 Rev 1 OUT	AGCGTCCTCACCCAGGATAGG	N/A

RT-qPCR primers

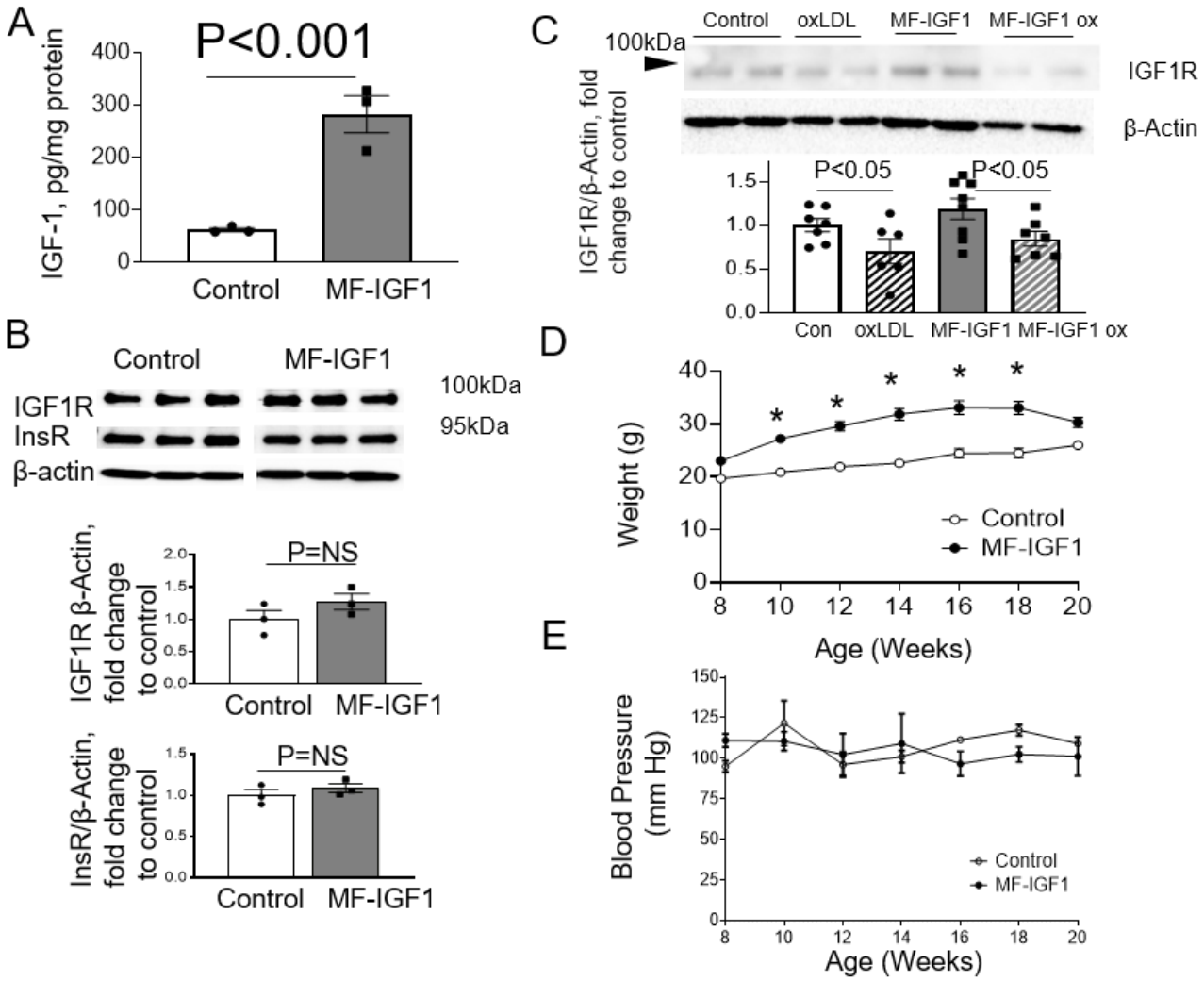
Primer Target	**Vendor**	**Product Number or sequence**
mIGF1R	Qiagen	PPM04714F
mCXCL12	Qiagen	PPM02965E
mB2M	Qiagen	PPM03562A
mABCA1	Qiagen	PPH02595A
hCXCL12	Qiagen	PPH00528B
hB2M	Qiagen	PPH01094E
Mouse/Rat Common IGF-1 F	In house	TGCGGGGCTGAGCTGGTG
Mouse/Rat Common IGF-1 R	In house	AGATCACAGCTCCGGAAGCA

Proteins and Biochemical Assays

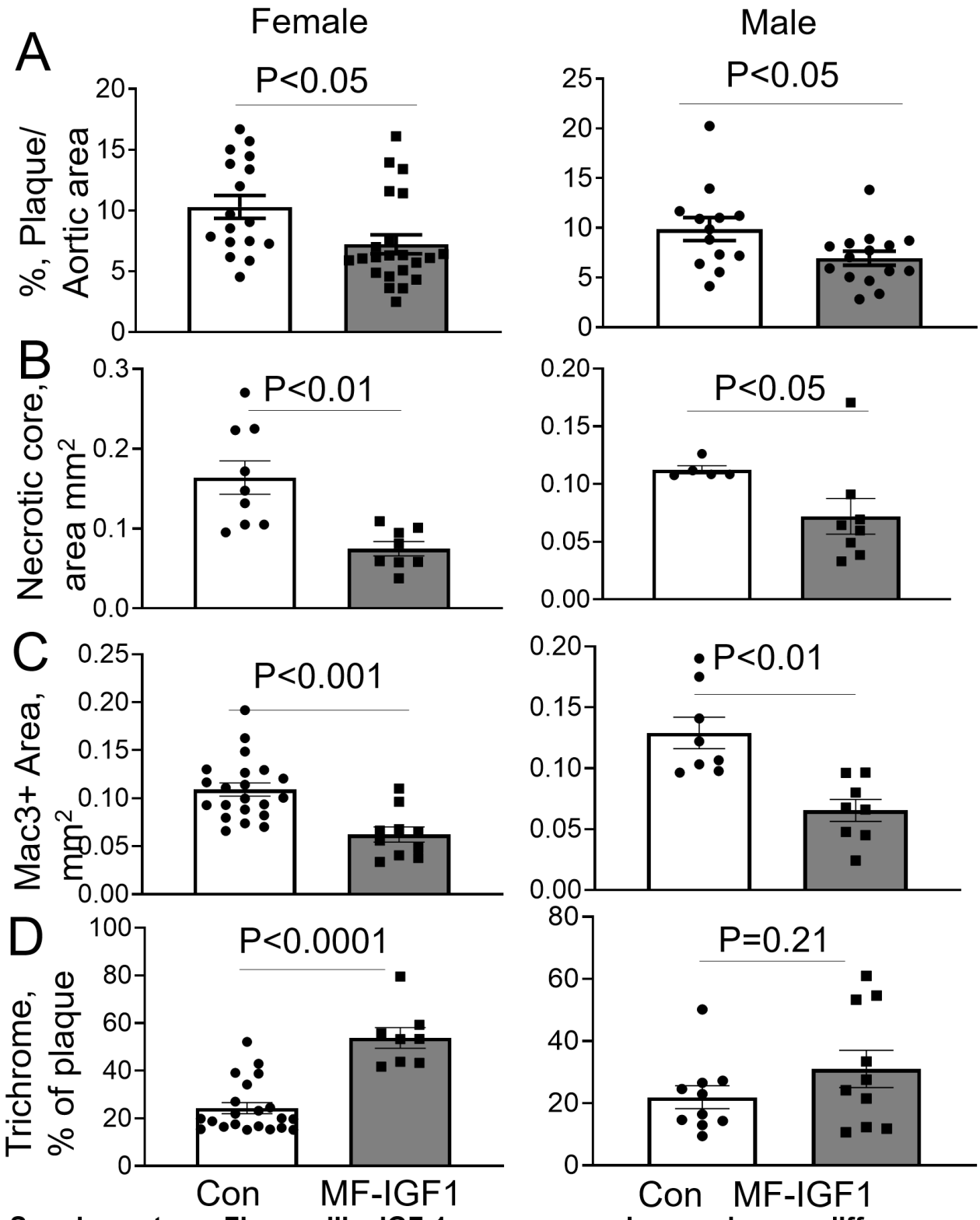
Protein or Kit	Vendor	Product Number
Human High-Density Lipoprotein	Kalen Biomedical	770300
Human Low-Density Lipoprotein	Kalen Biomedical	770252
Human High Oxidized Low-Density Lipoprotein	Kalen Biomedical	770200
Lipoprotein Deprived Serum	Kalen Biomedical	880100
Human recombinant IGF-1	Ipsen	Increlex™
Mouse recombinant IGF-1	Abcam	ab9861
Rat recombinant IGF-1	Novus Biologicals	NBP2-35219
m/rIGF-1 ELISA	RnD Systems	MG100
Cholesterol Efflux Kit	Sigma-Aldrich	MAK192
Recombinant CXCL12	Sigma-Aldrich	SRP4388
Recombinant Bioactive CXCL12	Abcam	ab9798
CXCL12 ELISA	RnD Systems	MCX120
Picropodophyllin	Selleck Chemicals	S7688
Monarch Genomic Purification Kit	New England Biolabs	T3010S
Oil Red O kit	Abcam	Ab150678
Cholesterol Efflux Assay Kit	Sigma-Aldrich	MAK192-1KT
Total Cholesterol Assay Kit (Colorimetric)	Cell Biolabs	STA-384
Triglyceride Colorimetric Assay Kit	Cayman Chemical Company	10010303
In situ TUNEL-Fluorescein kit	Roche	11966006001
Mouse Common Chemokine Multi-Analyte ELISArray	Qiagen	MEM-009A



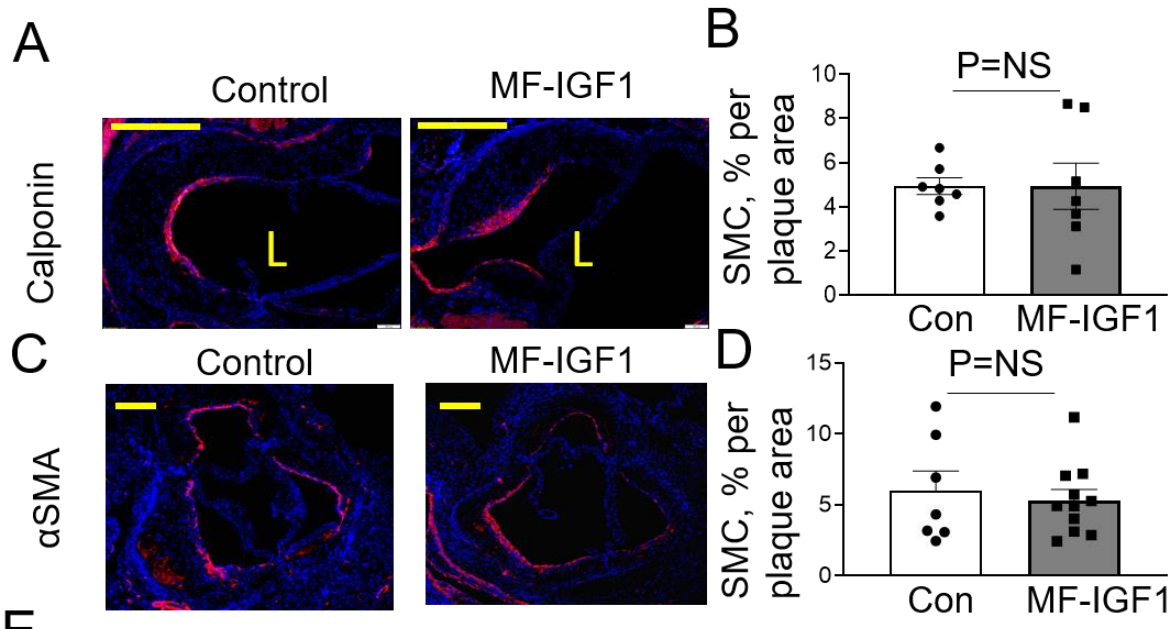
Supplemental Figure I: Construction of MF-IGF1 mice. A. Allele structures of IGF-1 genomic DNA (upper) and IGF-1 transgene (lower). In MF-IGF1 mice, Class 1-Ea isoform of IGF-1 (exon 1, 3, 4, and 6) was inserted downstream of SRA promoter/enhancer, and upstream of IRES-GFP-pA sequence. B. Using a probe against mouse IGF-1 exon 4, 13 lines of MF-IGF1 transgenic mice were analyzed by Southern blotting. Consistent with predicted band size, ~10.7 kbp WT IGF-1 bands were observed after BamHI (Bm) digestion in all of the animals (arrowhead). Digestion of transgenic mouse genomic DNA generated intense bands around 6 kbp in some of the animals, indicating that these animals have multiple copies of transgene inserted tandemly in the genome. C. Rat or mouse recombinant IGF-1 was incubated with control animal peritoneal macrophages and pAkt/Akt levels were measured by western blot (N=1-2 wells per group in two independent experiments). D. Nested PCR was performed on genomic DNA obtained from control (C) and transgenic animals (Tg) amplifying just outside of the entire CXCL12 gene and then two halves from the resulting amplicon (N=3-4 animals per group, representative image showing different amplicon sizes. Mu/M=Mouse, Rt/R=Rat. Any numeral following M or R are the concentration of IGF-1 in ng/ml.

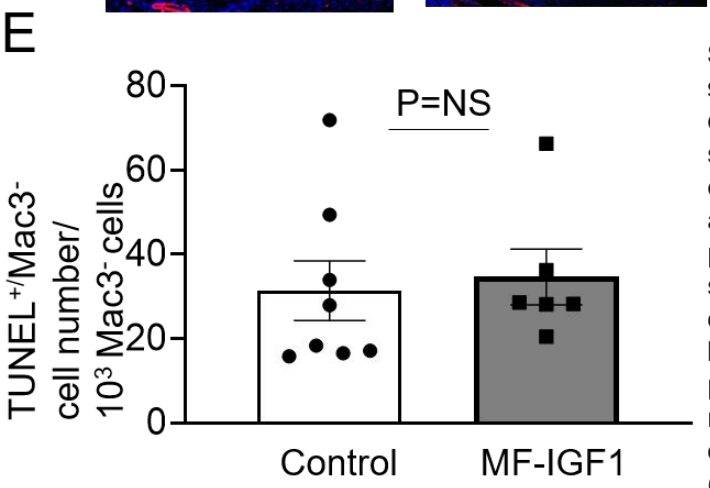


Supplemental Figure II: Phenotype of MF-IGF1 mice. A. IGF-1 was measured in peritoneal cell lysate by ELISA and normalized to total protein in the lysate. (N=3 mice per group). B. IGF1R and InsR protein expression was measured by immunoblotting in peritoneal macrophages isolated from MF-IGF-1 and control mice. (N=3 mice per group) C. Peritoneal macrophages isolated from mice were treated with oxLDL and then IGF1R protein levels were measured. (N=pooled cells from three mice per group, three wells per group). C,D. Mouse body weight (B, N=17-22 mice per group) and blood pressure (C, N=6 mice per group) were measured every two weeks over the course of feeding with high fat diet. All statistical tests are Student's T-test. *P<0.05 vs. control.

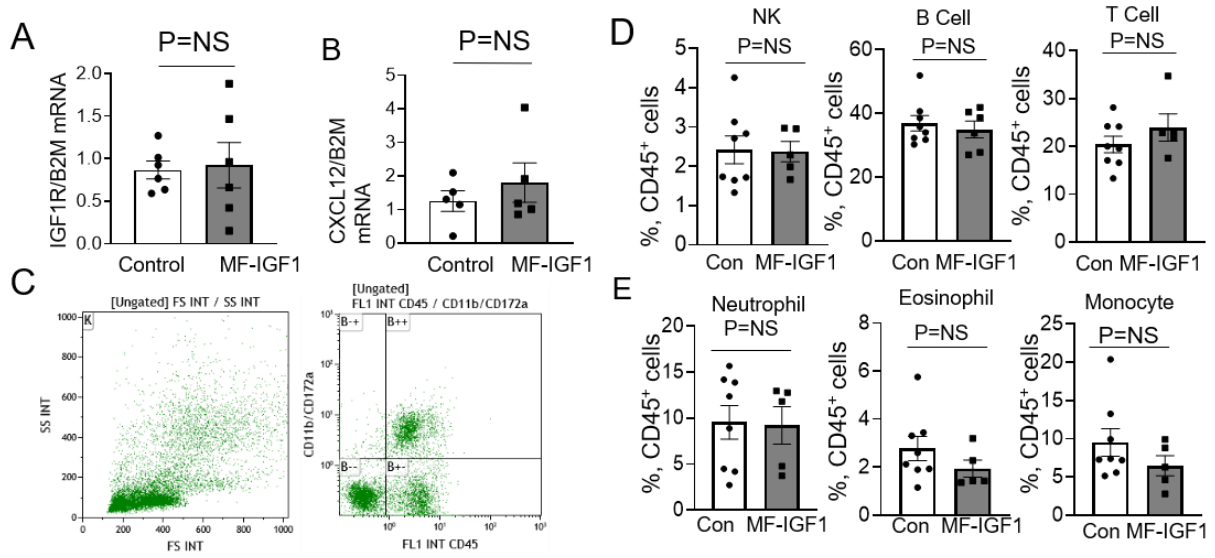


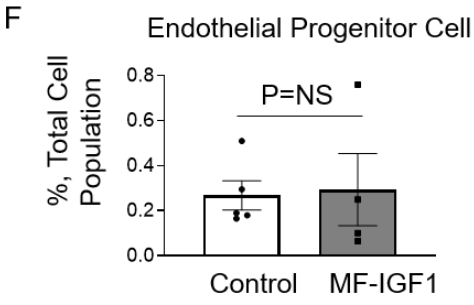
Supplementary Figure III: IGF-1 overexpression and sex differences in atherosclerotic measurements. All female measurement are on the left and all male measurements are on the right. A. *En face* plaque burden normalized to total aortic area (N=13-22 mice per group). B. Necrotic core area in atherosclerotic plaque (N=6-8 mice per group). C. Mac3 positive area inside the plaque (N=7-16 mice per group). D. Trichrome was used to stain collagen, then normalized to plaque area (N=8-22 mice per group). All statistical tests are student's t-test except for the males in B, which had a Welch's correction due to differences in SDs.



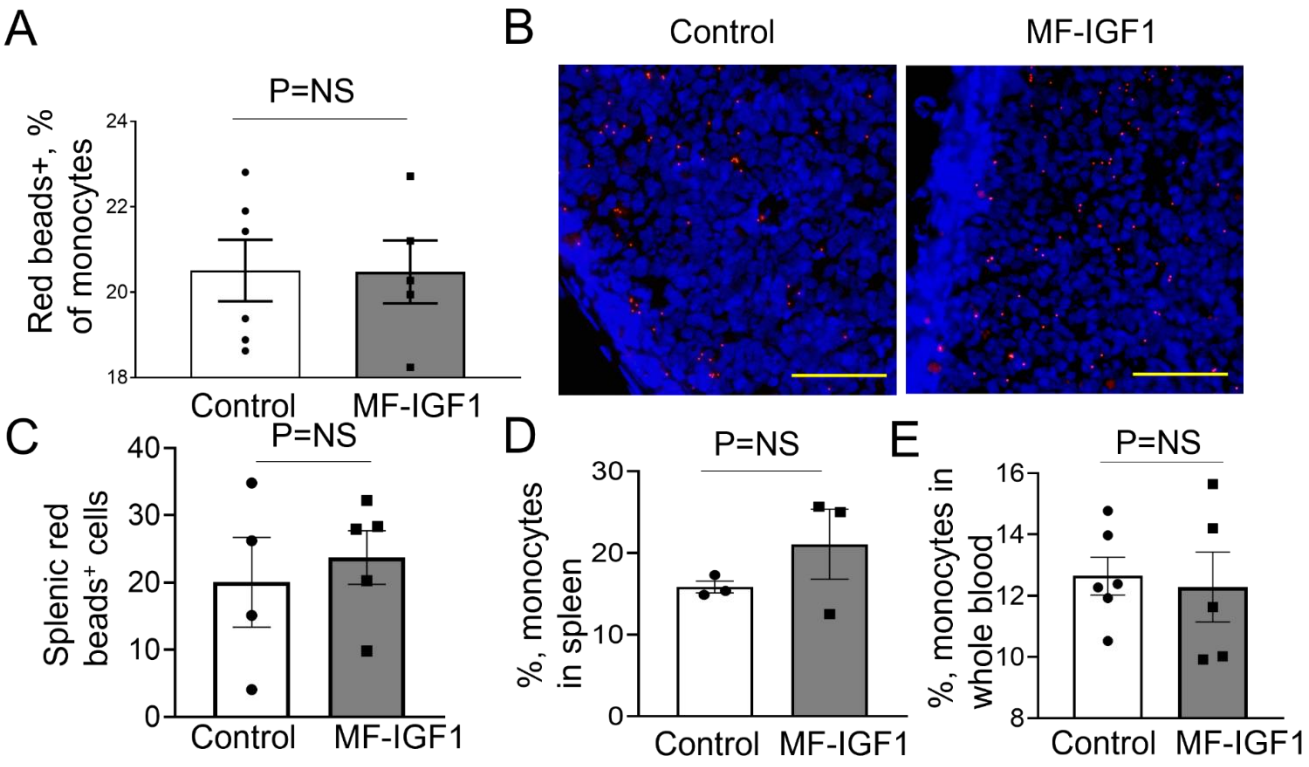


Supplemental Figure IV: Macrophage-specific IGF-1 overexpression did not change plaque SMC levels. A, B. Cross-sectional aortic root sections were stained with calponin antibody and calponin-immunopositive area was normalized to lesion area (N=7 mice per group). C, D. Cross sectional aortic root sections were stained with αSMA antibody and αSMA-immunopositive area was normalized to lesion area (N=7-11 mice per group). E. TUNEL positive/Mac3 negative cell number was normalized per 1000 Mac3 negative cells to quantify apoptosis in non macrophage cells (N=5-6 mice per group). All statistical tests are Student's Two Tailed T-test.

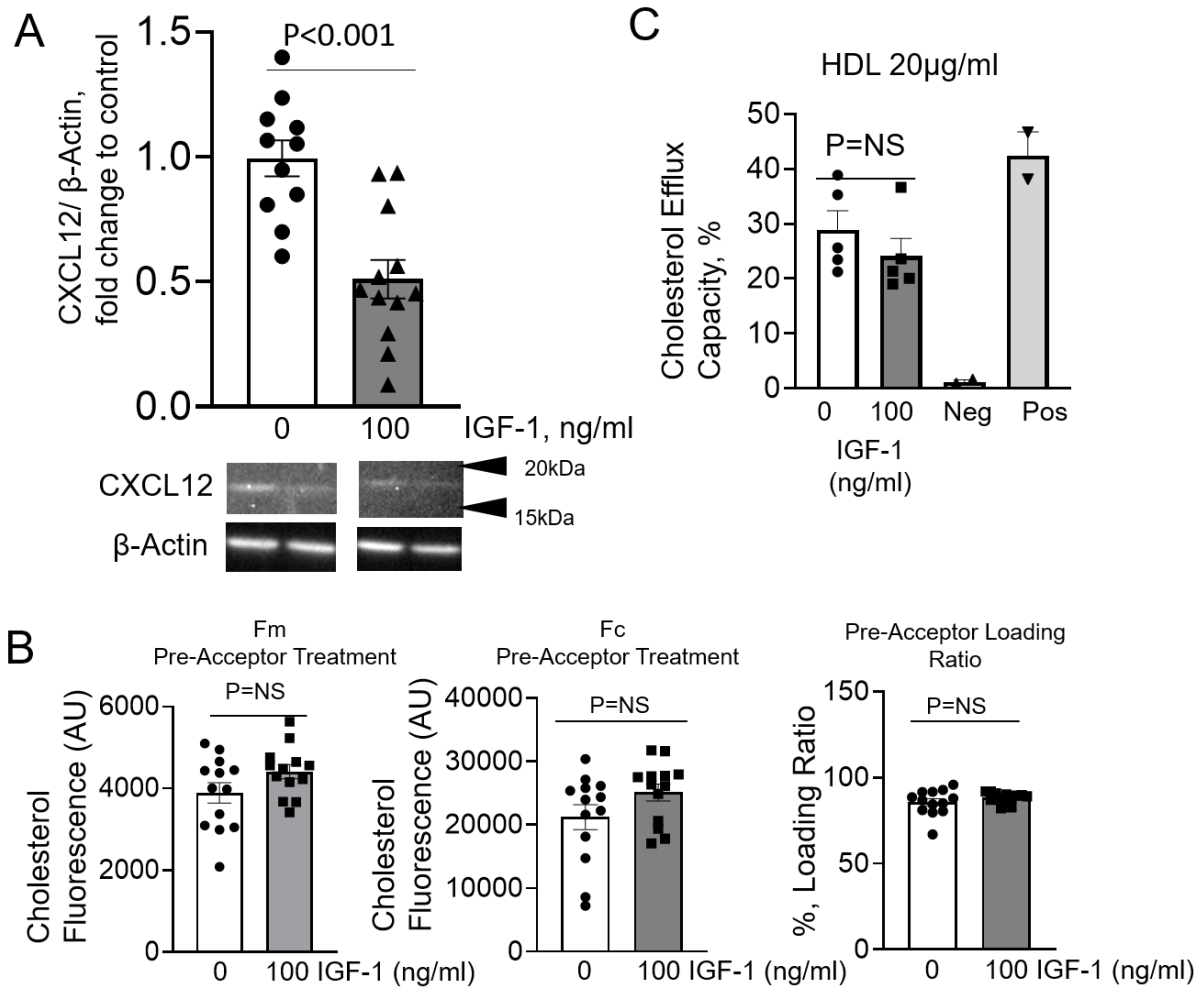




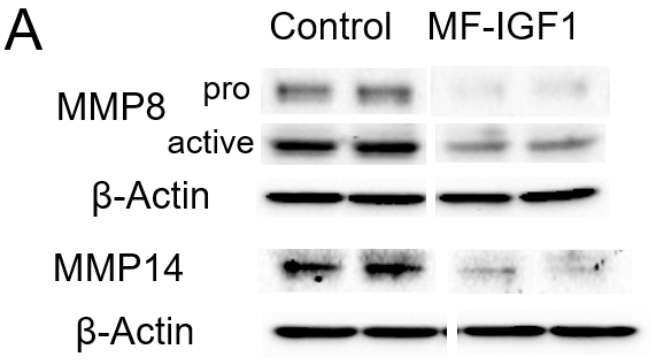
Supplementary Figure V: Monocyte quantification and isolation by flow cytometry. A. IGF1R mRNA transcripts were measured in $(CD11b/F4/80/CD115)^+$ circulating monocytes (N=6 mice per group). B. CXCL12 mRNA levels were measured in circulating monocytes (N=5 mice per group). C. Representative images of the lymphocyte gating strategy. D. Lymphoid lineage leukocytes in the circulation (n=5-6 mice per group). E. Myeloid lineage leukocytes in the circulation (n=5-6 mice per group). F. Endothelial progenitor cell percentage of total cell population in the circulation (n=4-5 mice per group). All statistical tests are Student's Two-Tailed t-Test.



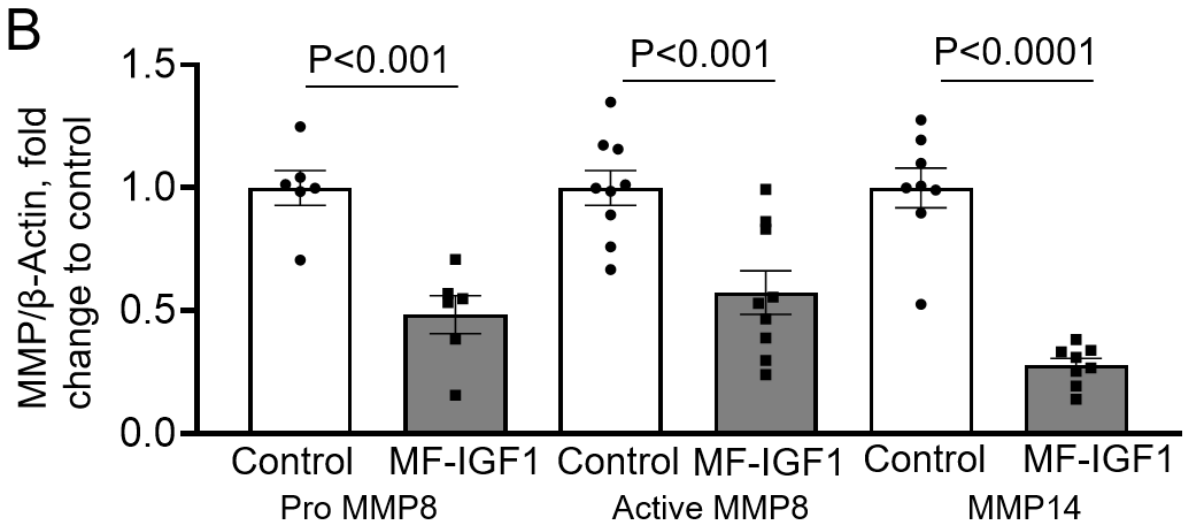
Supplementary Figure VI: Monocyte labelling with red latex beads. A. Red bead signal was quantified in $(CD11b/CD45)^{+}$, $(CD90/B220/CD49b/NK1.1/Ly\text{-}6G)^{-}$ gated monocytes. (N=6 mice per group) B. Representative images of splenic sections stained with DAPI (blue) and imaged for red fluorescent signal. C. Quantification data of red beads counted per image (N=4-5 mice per group). D. Whole spleens were homogenized and labelled with the same gating strategy as in C. Monocytes were normalized to total leukocytes ($CD45^{+}$ cells) present in the spleen. (N=3 mice per group) E. Monocytes identified by the above strategy were normalized to total leukocyte number in the circulation. (N=5-6 mice per group). Scale bar, 100 μm. All statistical tests are Student's two-tailed t-test.

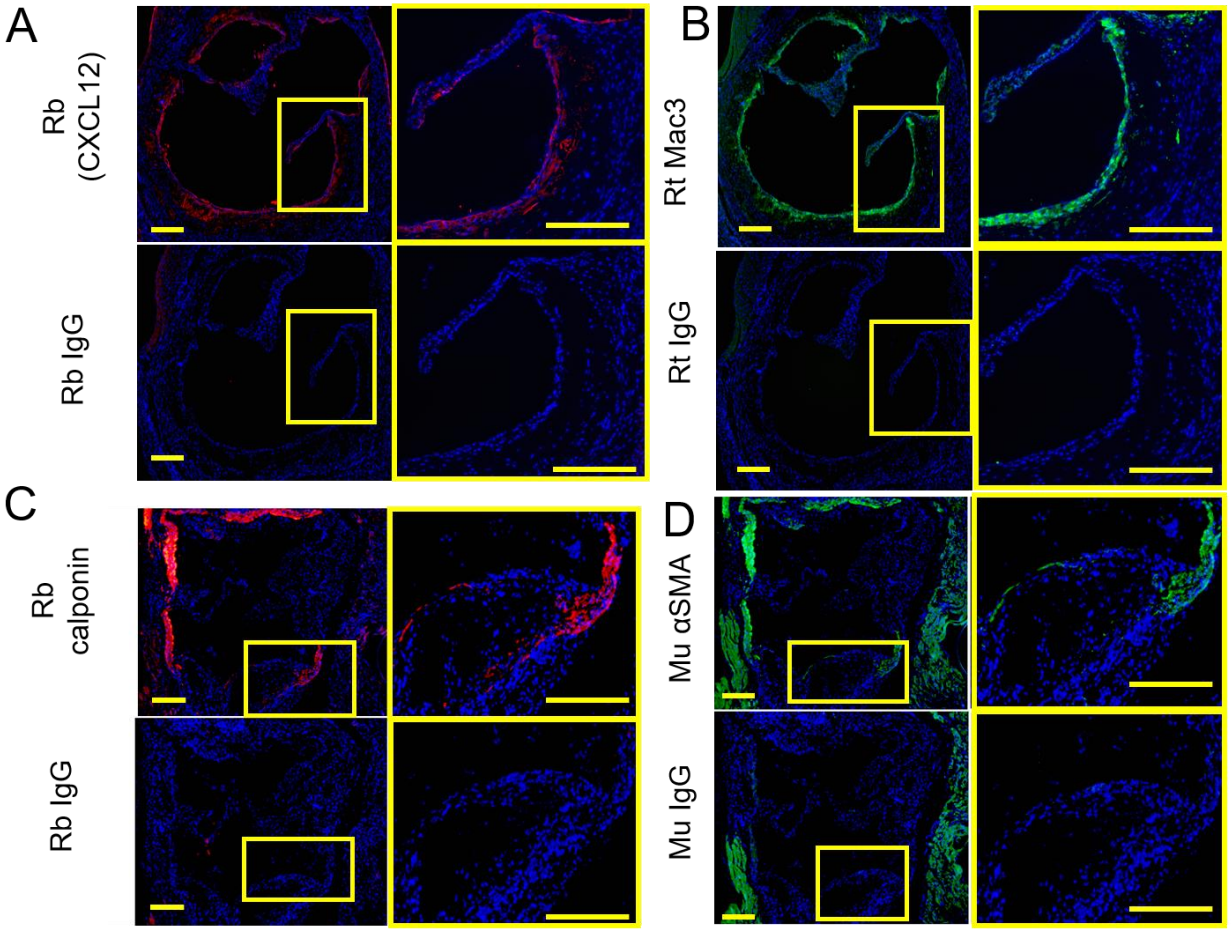


Supplementary Figure VII: A. CXCL12 protein expression in THP-1 macrophages exposed to oxLDL (N=3 wells per group per experiment, 4 independent experiments). B. IGF-1 treatment does not change initial cholesterol loading. Cholesterol fluorescence was measured before adding a cholesterol acceptor to oxLDL-treated THP-1 cells in the presence or absence of IGF-1. Fluorescence was measured in the media (Fm) and cell lysate (Fc), then the loading ratio ((Fc/(Fc+Fm))*100) was calculated. (N=4 wells per group in three independent experiments). C. IGF-1 does not affect HDL mediated cholesterol efflux in THP-1 macrophages. HDL was used at a concentration of 20µg/mL. (N=5 wells in one experiment). Also included is representative values for negative control (no cholesterol acceptor) and positive control (cyclodextrin as non-specific cholesterol acceptor). Fm=Fluorescence in culture supernatant. Fc=Fluorescence in cell lysate. AU=Arbitrary units All statistical tests are Student's two-tailed t-test.



Supplementary Figure VIII: Matrix metalloproteinase expression in peritoneal macrophages: A. Representative images of Western blots for peritoneal macrophages from control and MF-IGF1 mice. B. Quantitative data (N=9 animals per group). All statistical tests are Student's two-tailed t-test.





Supplemental Fig. IX: Representative IHC IgG controls. A. IgG for mouse CXCL12 antibody. B. IgG control for Mac-3 antibody. C. IgG control for calponin. D. IgG control for α-SMA. Rb=Rabbit, Rt=Rat, Mu=Mouse. Scale bar, 100 μm.