Supplemental Materials

Detailed Methods

Cell culture, exogenous treatments, and transfection

Human aortic endothelial cells (HAECs), human coronary artery endothelial cells (HCAECs), and human umbilical vein endothelial cells (HUVECs) were purchased from Lonza or Cell Applications at passage 2-3. Endothelial cells were cultured in either EGM-2 (Lonza) supplemented with the Bullet Kit or in MCDB131 media supplemented with 0.16-0.18mg/ml bovine hypothalamus extract and 60 µg/ml heparin, and both with 10% fetal bovine serum (FBS, HyClone) and penicillin (100 U/ml)/streptomycin (100 µg/ml). HAECs were treated with 10 ng/ml TNFα, 5 ng/ml IL-1β (Fischer Scientific), 50-100 µg/ml oxidized LDL (Intracel or Kalen), or 2 µg/ml Fc-ephrinA1 (R&D Systems). LDL was oxidized in house by dialysis in PBS containing 13.6 µM $CuSO_4$ for 2-3 days, followed by 2 days in PBS containing 50µM EDTA to stop the reaction, and then stored under N_2 gas. Relative eletrophoretic mobility of oxLDL was consistently between 2 and 3[1, 2]. Fc-ephrinA1 was pre-clustered by incubation with 1 µg/ml goat anti-human Fc antibody for 30 minutes on ice prior to treatments. HAECs were transiently transfected with one of three different anti-EphA2 siRNA (Dharmacon, Sigma) to facilitate EphA2 knockdown, or with one siRNA (Sigma) for EphA4 knockdown. All transfections were performed using Lipofectamine2000 per manufacturer's instructions.

Immunoblotting

Cells were lysed in 2X Laemmli buffer, separated on 10% SDS-PAGE gels and transferred to PDVF membrane as described previously[3]. Membranes were blocked in 5% dry milk in TBST for 1 hour before immunoblotting. Antibodies for western blotting include rabbit anti-EphA2 (C-20), rabbit anti-EphA4 (S-20), rabbit anti-ephrinA1 (V-18), rabbit anti-VCAM-1 (C-19), and rabbit anti-ERK1/2 (K-23; Santa Cruz). For immunoblotting with the phospho-tyrosine specific Ab 4G10 (Upstate), PDVF membranes were blocked for 1 hour in 1% denatured BSA in PBS. Immunoblots were incubated HRP-conjugated secondary antibodies and with ECL Western Blotting Substrate (Pierce). Bands were visualized by exposing high performance chemiluminescence film (Amersham). Films were scanned and band intensity quantified using ImageJ software. Arbitrary absorbance units were normalized to the total protein or loading control and normalized to untreated controls.

Immunoprecipitation

Cells plated at confluency in 60mm culture plates were treated as indicated before lysis with 500µl of a cold lysis buffer (50mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 1% Triton X-100, 0.5% Igepal, 0.1 mM PMSF, protease inhibitor cocktail V (5µM AEBSF, 1.5µM Aprotinin, 10nM E-64, 10nM Leupeptin; RPI), and phosphatase inhibitor cocktail II (Imidazole 200µ, NaF 100µM, Sodium Molybdate 115µM, $NaVO_4$ 100µM, sodium tartrate dehydrate 400µM; RPI), pH 7.4). Lysates were pre-incubated 30 min with 40 µl ProteinA Sepharose beads (GE Healthcare), centrifuged. 450µl of cleared lysates were transferred to new tubes and incubated for 2 hours with 5 µg rabbit anti-EphA2 (C-20, Santa Cruz). Lysates were then incubated

with 40 µl of ProteinA sepharose beads for 2 hours, the beads were washed three times in lysis buffer (lacking protease/phosphatase inhibitors and detergents), and immunoprecipitated proteins were dissociated from the beads by the addition of 40 µl 2X Laemmli buffer followed by boiling at 95^{o}C for 10 minutes.

Quantitative Real Time-PCR (qRT-PCR)

mRNA was extracted using TRIzol (Invitrogen) per manufacturer's instructions and cDNA was synthesized using the iScript cDNA synthesis kit (Biorad). qRT-PCR was performed in a Biorad iCycler using Sybr Green Master mix (Biorad). qRT-PCR primers (Table SI) were designed using the Primer3 software (online) and validated using generic human cDNA pooled from 20 organs (Firstchoice® Total Human RNA, Ambion). PCR products were verified by the presence of a single peak in melt curve analysis and by DNA sequencing (SeqWright). Three different donors for each cell type (HAECs, HCAECs, and HUVECs) were used for mRNA analysis, and results were normalized to 18S or GAPDH mRNA. StellArray qRT-PCR was performed in 96-well plates containing primers to genes shown to regulate endothelial cell biology (Lonza). Expression of PCR products were normalized to 10 gene products exhibiting the lowest or no change in expression using Global Pattern Recognition Software 2.0 (GPR 2.0, Lonza). For analysis of gene expression in vivo, aortas of ApoE-null C5Bl/6J mice were harvested (see below) and cleaned of connective tissue. The aortic arch and a section of the thoracic aorta were excised, snap frozen in liquid nitrogen, and then sonicated briefly at low power in TRIzol reagent.

Animals, Tissue Harvesting, and Immunohistochemistry

Animal protocols were approved by the LSU Health Sciences Center-Shreveport IACUC committee, and all animals were cared for according to the National Institute of Health guidelines for the care and use of laboratory animals. All experiments using human tissue was deemed non human research by the local IRB due to the exclusive use of postmortem samples. Male ApoE null mice on the C57Bl/6J genetic background were purchased from Jackson Laboratories. Starting at 8 weeks of age mice were fed standard chow or a high fat, Western diet (TD 88137; Harlan-Teklad, Madison, WI) containing 21% fat by weight (0.15% cholesterol and 19.5% casein without sodium cholate) for 8 or 24 weeks. Mice were then euthanized, perfused with 4% formaldehyde, and atherosclerosis prone vessles (aortic arch, innominate artery, carotid sinus) were excised. Human tissue was excised postmortem during routine autopsy at the LSU Health Sciences Center. All tissue was fixed in 4% formaldehyde, embedded in paraffin, and cut into 5 µm sections. Immunohistochemistry was performed as previously described[4]. Antibodies used included rabbit anti-EphA2 (1:1000; Invitrogen), rabbit anti-VCAM-1 (1:200, Santa Cruz), goat anti-ephrinA1 (1:400; R&D), goat anti-PECAM-1 (1:50, Santa Cruz), rat anti-Mac2 (1:10,000, Accurate Chemical), rabbit anti-vWF (1:4000, Abcam), mouse anti-CD68 (1:200, Dako), and rabbit anti-ApolipoproteinB (1:10,000, Abcam) for staining, and staining was visualized with biotinylated secondary antibodies, the Vectastain ABC kit, and 3-3'-diaminobenzidine (DAB, Dako). For immunofluorescent staining, tissues were incubated with Alexa546- or Alexa488-conjugated donkey anti-rabbit IgG, Alexa350- or Alexa546-conjugated donkey anti-goat IgG, or Alexa488-conjugated donkey anti-rat IgG secondary antibodies (Invitrogen).

Staining was visualized using a Nikon Eclipse Ti inverted fluorescent microscope. Images were collected using the Photometrics CoolSNAP120 ES2 camera and the NIS Elements 3.00, SP5 imaging software.

Monocyte Adhesion Assay

HCAECs were plated at confluency in 24-well plates and serum starved overnight in 0.5% serum. HCAECs were treated with 2 µg/ml Fc-ephrinA1 (pre-clustered as described above) for 5 hours, and then rinsed twice in 2 volumes of HBSS containing Ca^{2+} and Mg^{2+}. THP-1 monocytes were labeled with Cell Tracker Green (Invitrogen) as per manufacturer's instructions, incubated 30 minutes, centrifuged and resuspended in HBSS containing Ca^{2+} and Mg^{2+}. $2.5x10^5$ THP-1 cells were incubated with HCAECs for 10 minutes. Non-adherent THP-1 cells were rinsed, collected, and centrifuged at 1000 rpm for 5 minutes. Adherent THP-1 cells and rinsed THP-1 cells were lysed in 100mM NaOH after removal of supernatants, and the percentage of adherent THP-1 cells was calculated by measuring fluorescence on a FLUOstar Optima fluorescent plate reader (BMG).

Statistical analysis

Student's t-test was utilized for timecourse and expression data performed with cytokines or oxLDL in Microsoft Excel software. Two-way Anova with Bonferroni post-test was performed with Graphpad Prism 5 software for data involving transient transfection with siRNA oligos before additional treatments.

Works Cited

1. Noble RP. Electrophoretic separation of plasma lipoproteins in agarose gel. *J Lipid Res.* 1968;9:693-700.
2. Yoshida H, Quehenberger O, Kondratenko N, Green S, Steinberg D. Minimally oxidized low-density lipoprotein increases expression of scavenger receptor A, CD36, and macrosialin in resident mouse peritoneal macrophages. *Arterioscler Thromb Vasc Biol.* 1998;18:794-802.
3. Orr AW, Pallero MA, Murphy-Ullrich JE. Thrombospondin stimulates focal adhesion disassembly through Gi- and phosphoinositide 3-kinase-dependent ERK activation. *J Biol Chem.* 2002;277:20453-20460.
4. Orr AW, Hahn C, Blackman BR, Schwartz MA. p21-activated kinase signaling regulates oxidant-dependent NF-kappa B activation by flow. *Circ Res.* 2008;103:671-679.

Supplemental Figure I

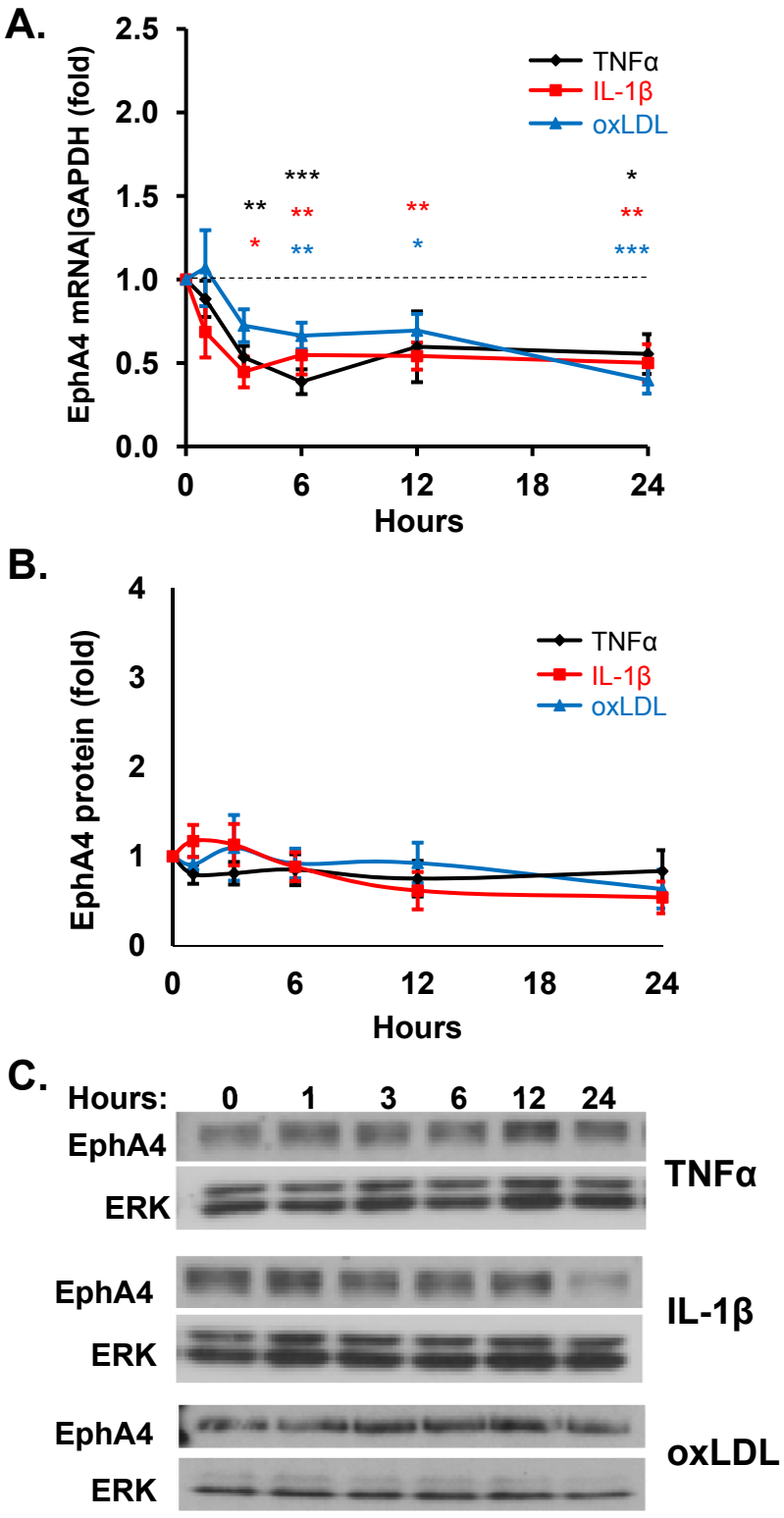
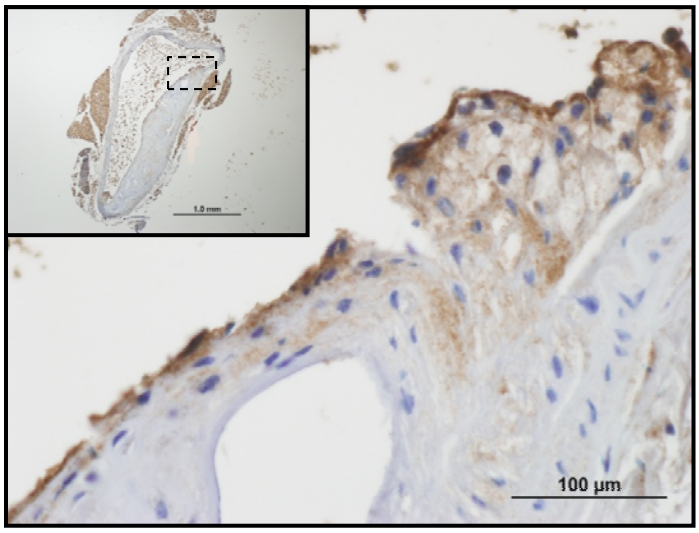


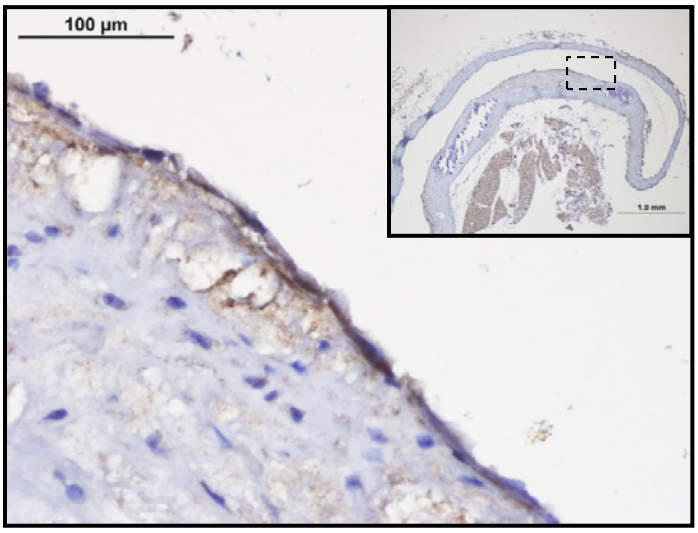
Figure SI. A-C, HAE cells were treated with 10 ng/ml TNF-α, 5 ng/ml IL-1β, or 50-100 ug/ml oxLDL for the indicated times. EphA4 mRNA expression was measured by qRT-PCR (A) and protein expression was measured by Western blotting (B,C) and normalized to ERK. Representative Western blots are shown. n=3-4.

Supplemental Figure II

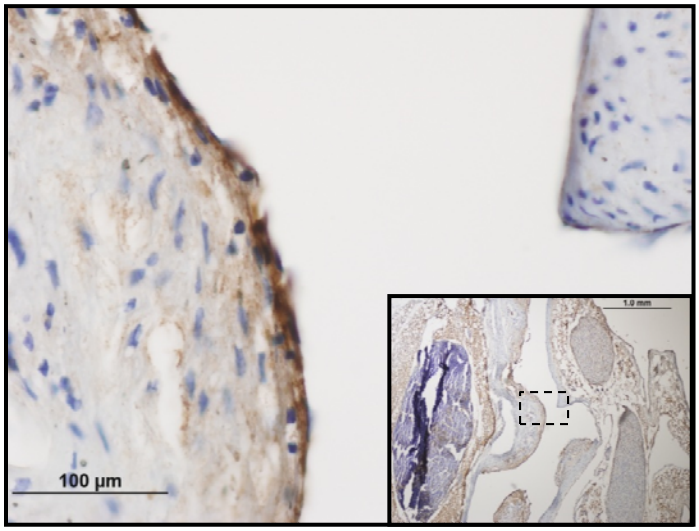
Innominate Artery



Aortic Arch



Abdominal Aorta near Renal Artery



Femoral Artery Bifurcation

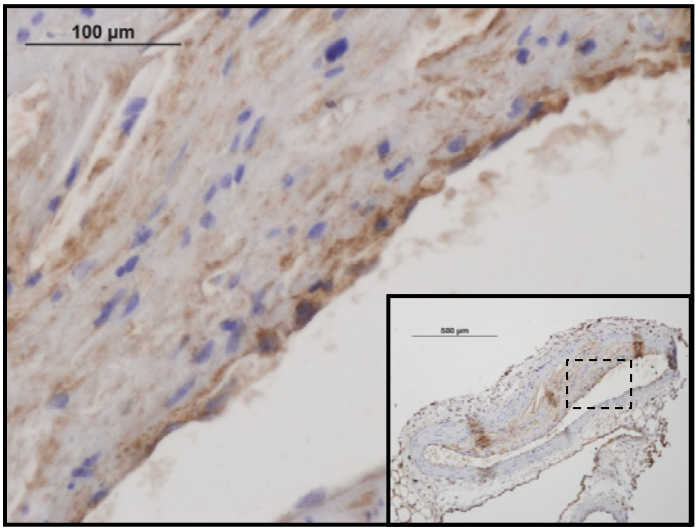


Figure SII. Atherosclerotic plaques from different vascular beds isolated from ApoE knockout mice were stained for EphA2 by immunohistochemistry. Images are 40X with either 4X or 10X inserts.

Supplemental Figure III

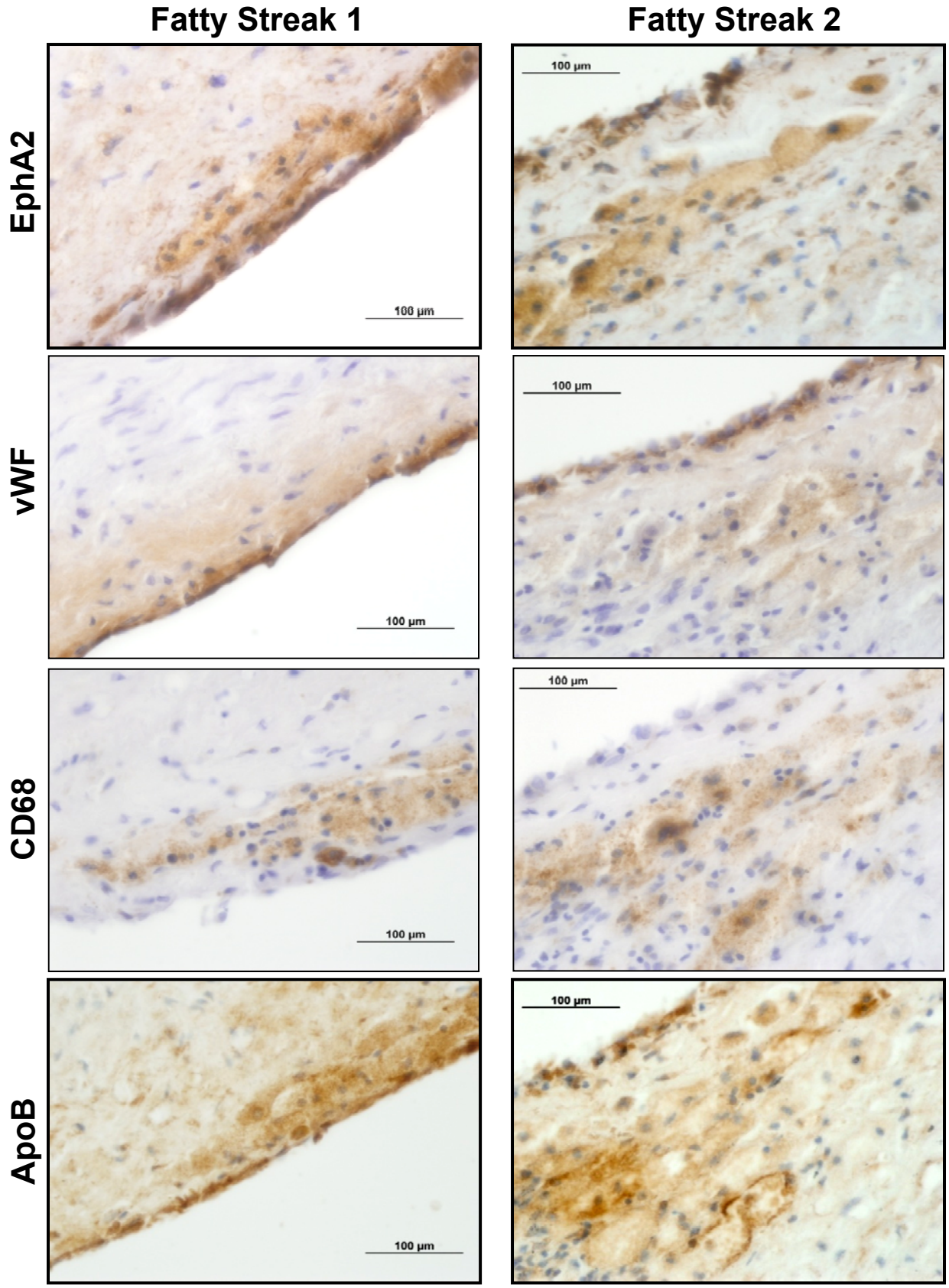


Figure SIII. Photomicrographs of fatty streaks isolated from human tissues depicting colocalization of EphA2 with intimal foam cells (CD68 and ApoB positive). EphA2 colocalization with lumenal endothelial cells (vWF) is also apparent.

Supplemental Figure IV

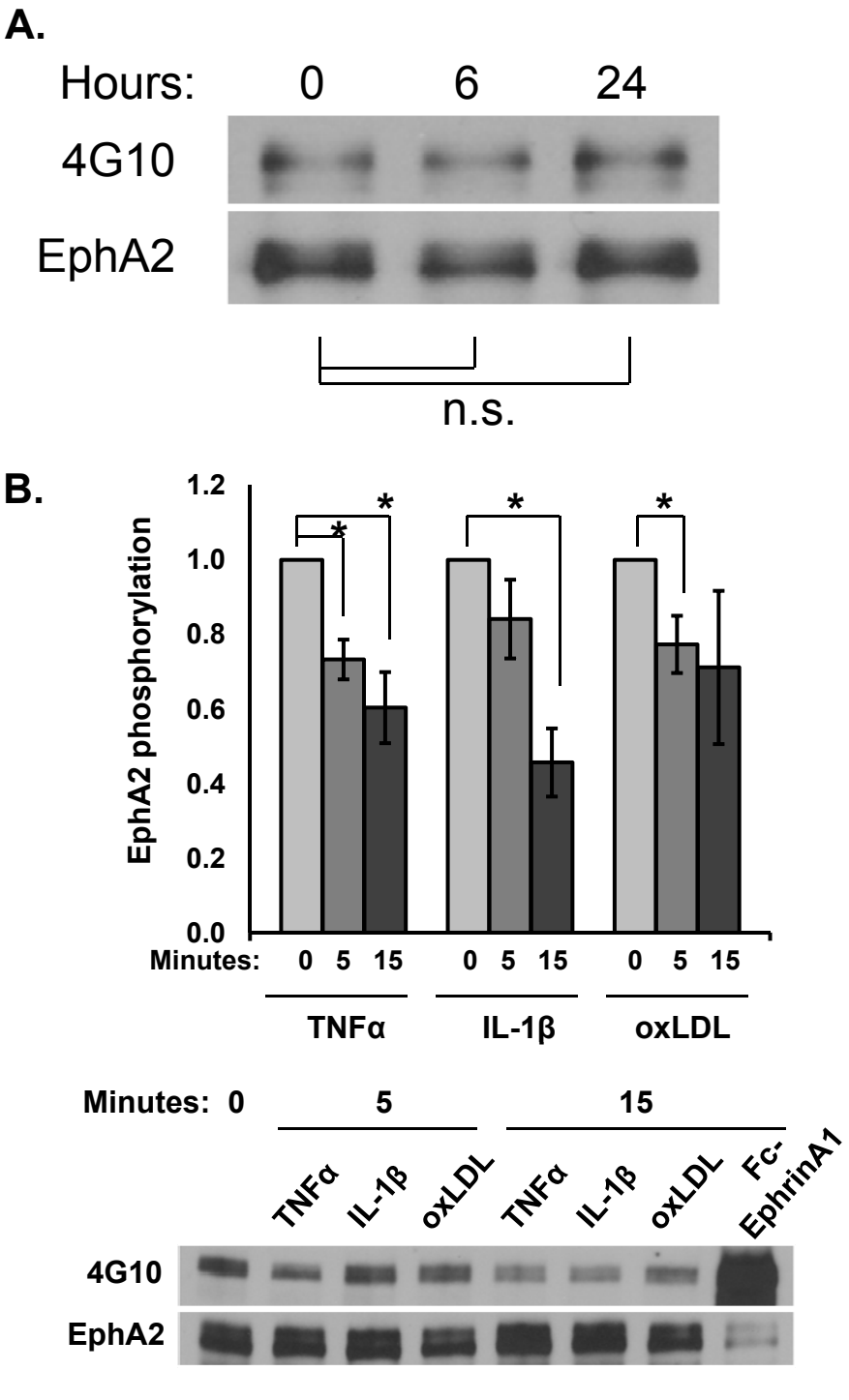


Figure SIV. A, HAE cells were treated with cell culture media for the indicated times. EphA2 activation was determined by EphA2 immunoprecipitation and immunoblotting for phospho-tyrosine (4G10), n = 3. B, HAE cells were treated with TNFα, IL-1β, or oxLDL for the indicated times and EphA2 activation was assayed as described in A. Representative Western blots are shown. n=3, * = $p < 0.05$, n.s. = not significant

Supplemental Figure V

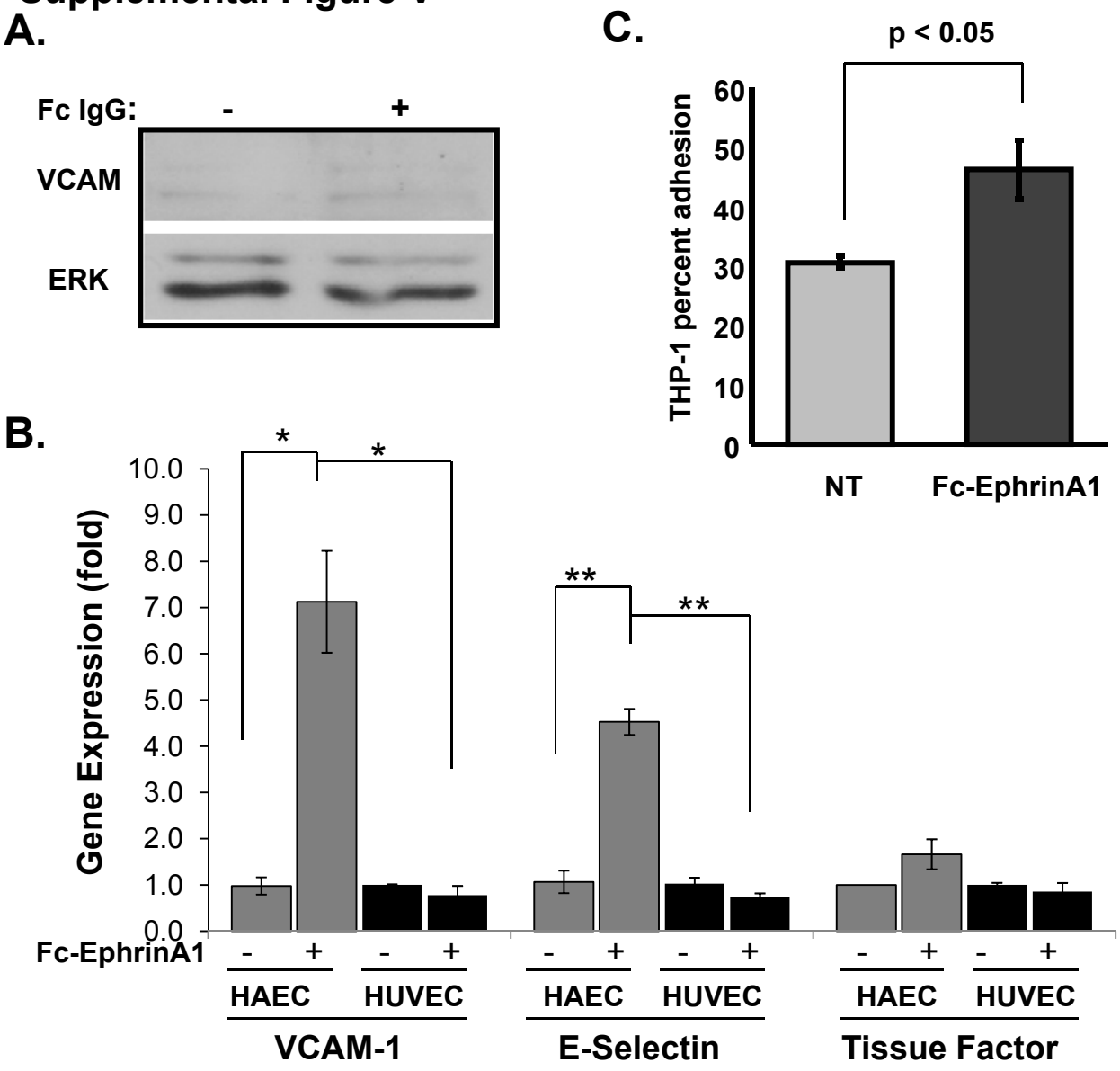


Figure SV. A, HAECs were treated with human Fc IgG fragment for 5 hours and VCAM-1 expression was determined by Western blotting. n = 3. B, HAECs and HUVECs were treated with Fc-ephrinA1 for 3 hrs and mRNA expression of VCAM-1, E-Selectin, and TF was determined by qRT-PCR and normalized to GAPDH. n = 3-5. C, HCAECs were treated with Fc-ephrinA1 for 5 hrs. were incubated for 10 minutes with ~2.5×10^5 THP-1 monocytes labeled with Cell Tracker Green. Non-adherent THP-1 monocytes were rinsed and collected. Adherent and non-adherent THP-1 monocytes were quantified using a fluorescence plate reader, and adherent cells were expressed as a percent of the total (n=5) * $p < 0.05$, ** $p < 0.01$.

Supplemental Figure VI

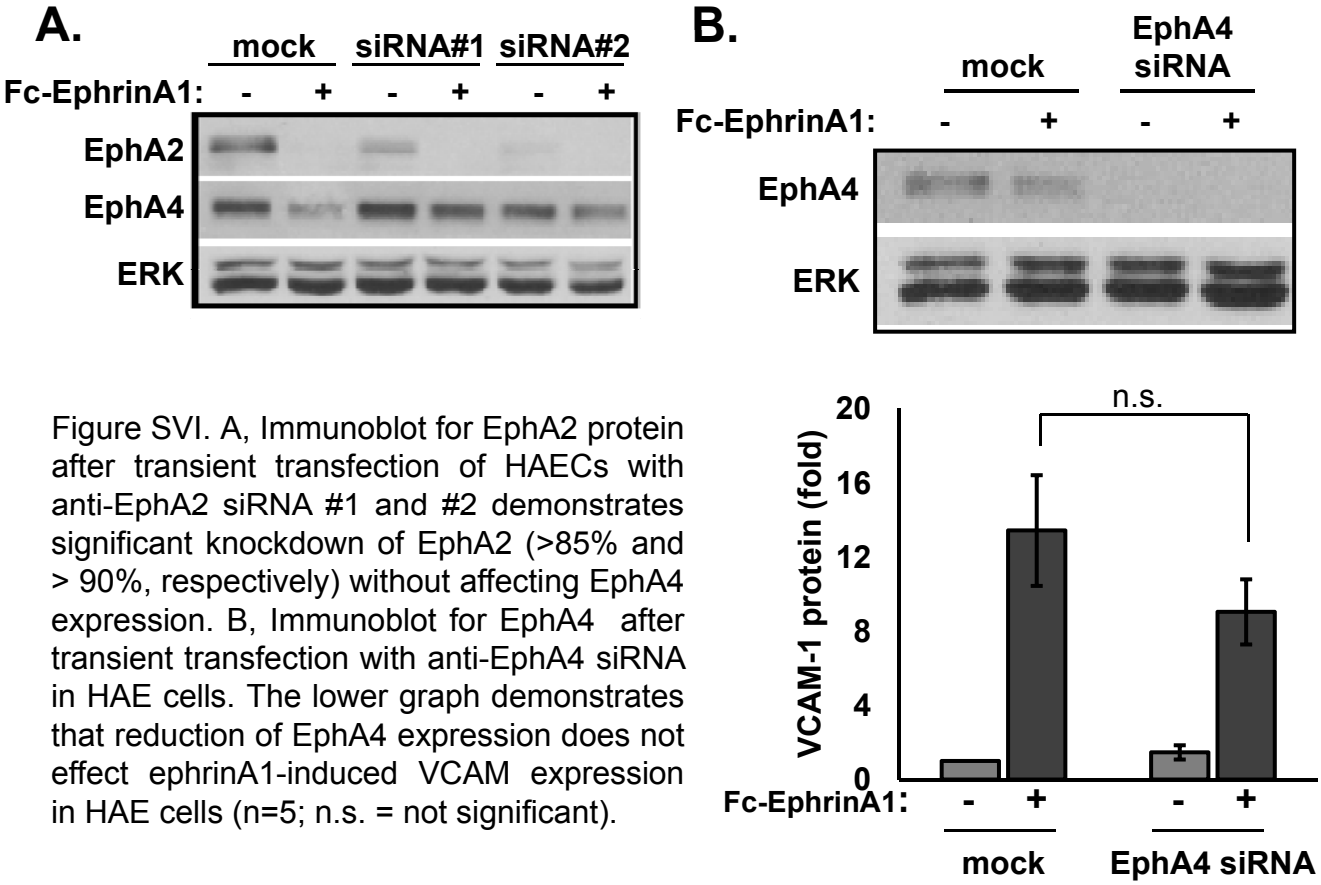


Figure SVI. A, Immunoblot for EphA2 protein after transient transfection of HAECs with anti-EphA2 siRNA #1 and #2 demonstrates significant knockdown of EphA2 (>85% and > 90%, respectively) without affecting EphA4 expression. B, Immunoblot for EphA4 after transient transfection with anti-EphA4 siRNA in HAE cells. The lower graph demonstrates that reduction of EphA4 expression does not effect ephrinA1-induced VCAM expression in HAE cells (n=5; n.s. = not significant).

Supplemental Figure VII

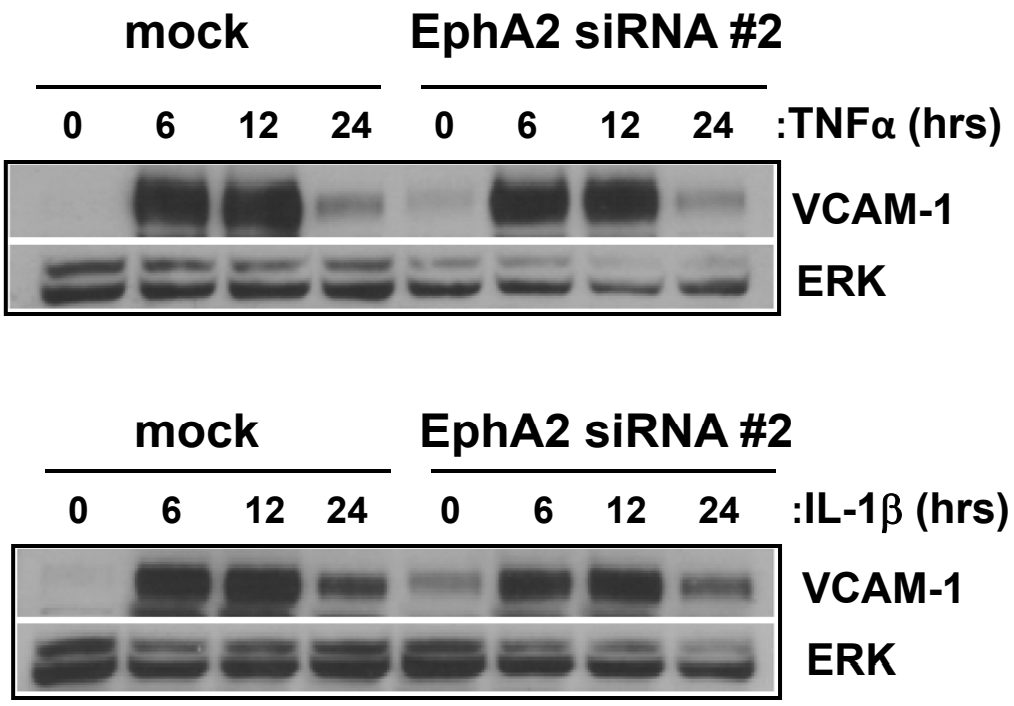


Figure SVII. HAECs were transiently transfected with anti-EphA2 siRNA before treatment with the cytokines TNFα (10 ng/nl) or IL-1β (5 ng/ml) at the indicated times. Immunoblots are representative of three independent experiments.

Supplemental Figure VIII

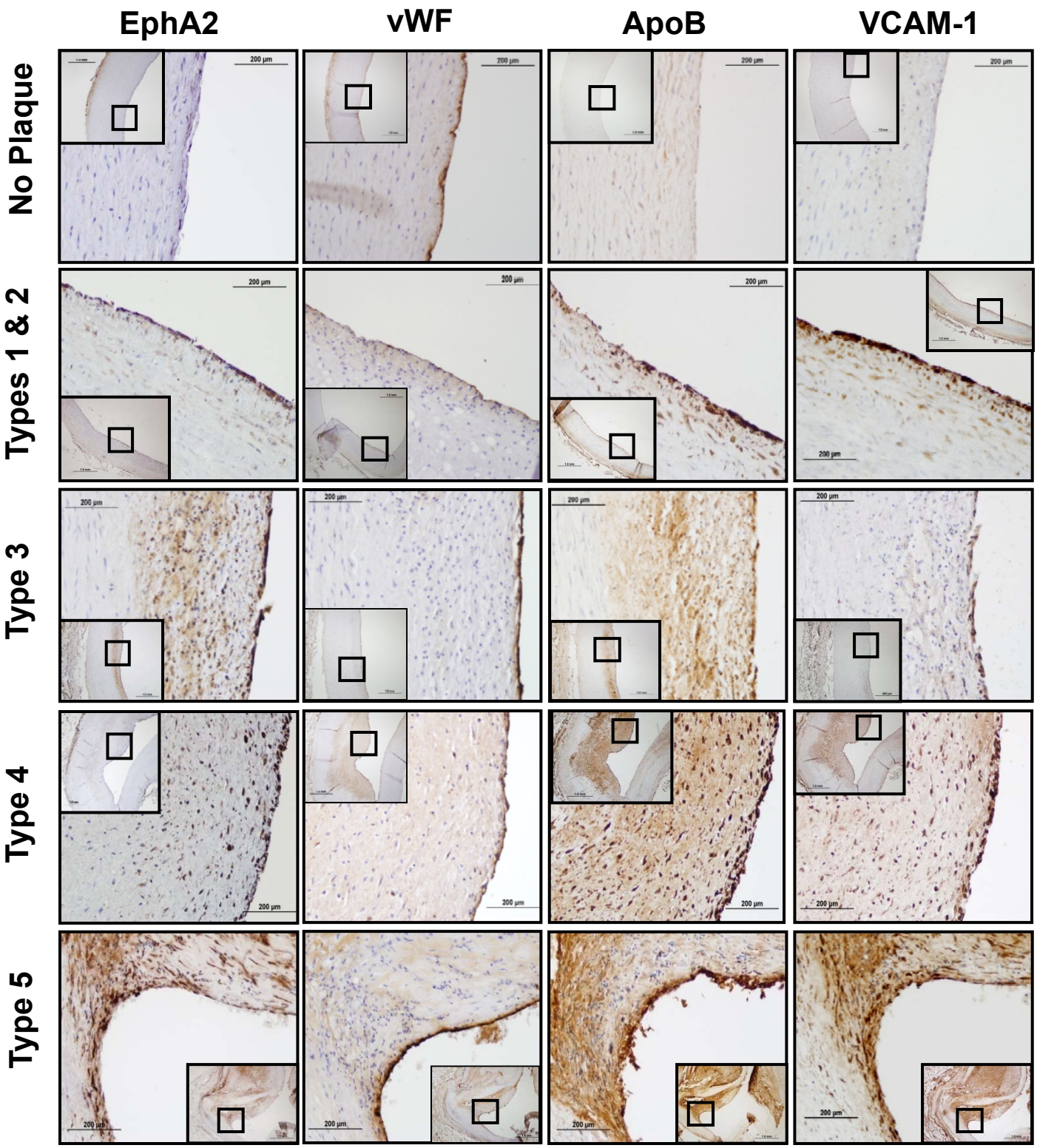


Figure SVIII. Photomicrographs of atherosclerotic plaques isolated from human tissues depicting colocalization of EphA2 with low density lipoproteins (ApoB) and VCAM-1 in the endothelial cell layer (vWF).

Supplemental Table I

Quantitative Real Time PCR primers

Gene	Forward	Reverse
18S	5' - CGGCTACCACATCCAAGGAA - 3'	5' - AGCTGGAATTACCGCGGC - 3'
EphA1	5' - GCCTGACACCACATACATCG - 3'	5' - GAAAACGAGAATCCCAAGCA - 3'
EphA2	5' - ATGACCAACGACGACATCAA - 3'	5' - GCAGGGGGAGGAAAGAACTA - 3'
EphA3	5' - CCAGTTCCCGTGTAAAGCAC ' 3'	5' - CCCACCCATCCAATAAATGA - 3'
EphA4	5' - TTTCGCCCTATTTTCGTGTC - 3'	5' - GTTATTCTGGCTGGGTTCCA - 3'
EphA5	5' - CTACACCACAAGGGGAGGAA - 3'	5' - AGAGCAGCAGGACAATCCAT - 3'
EphA6	5' - ATGCCCCTTCACGTGTCGTA - 3'	5' - AACCAGCCAATCTCCATCAG - 3'
EphA7	5' - CAGATTCGGGCTTTTACTGC - 3'	5' - AGTGCCTTCTCCCAATGATG - 3'
EphA8	5' - CAGCACACAAGAAAGCCAGT - 3'	5' - AGATGCGGAGAGAGAGGATG - 3'
EphA10	5'- TTGAAAAGGACCAAGAGACCA - 3'	5' - AACCACTGCCAACAAAATGA - 3'
EphrinA1	5' - AGGCTGAAGAGAGGGACAGG - 3'	5' - AGGAATGGTGGGAAAGGACT - 3'
EphrinA2	5' - ACGGTGGAGGTGAGCATC - 3'	5' - AGGGAGAAGGGCGTGAAG - 3'
EphrinA3	5' - CTCTGGGCTACGAGTTCCAC - 3'	5' - CCGCTGATGCTCTTCTCAAG - 3'
EphrinA4	5' - CTCCAGGTGTCTGTCTGCTG - 3'	5' - CTTGGGATGAGAGGGAAGGT - 3'
EphrinA5	5' - CCAGAAGATAAGACTGAGCGC - 3'	5' - CCATTATCTGGGATTGCAGAGG - 3'
GAPDH	5' -GAAGGTGAAGGTCGGAGTC - 3'	5' - GAAGATGGTGATGGGATTTC - 3'
VCAM-1	5' - ATGAGGGGACCACATCTACG - 3'	5' - CACCTGGATTCCTTTTTCCA - 3'
E-Selectin	5' - GGTACAGGCGAGTTGGAG - 3'	5' - GATGGCGTCACAGGTCAC - 3'
TF	5' -AGGATGGATGCCAAGATGTA - 3'	5' - TCTTGCCTTTCAGATTGTCC - 3'
PGK1	5' - GCAGATTGTTTGGAATGGTC - 3'	5' - TGCTCACATGGCTGACTTTA - 3'
PP1A	5' - AGCTCTGAGCACTGGAGAGA - 3'	5' - GCCAGGACCTGTATGCTTTA - 3'

Supplemental Table II

Summary of pathological scoring of human patient tissues.

Plaque Type	# Samples	Age Range	Median Age	# Patients	M:F
1	6	34-47	39	5	3:2
2	14	21-49	24	11	9:2
3	7	22-47	24	6	5:1
4	5	26-47	45	5	5:0
5	13	43-71	55	11	6:5

Supplemental Table III

Etiology of human patient tissues

Patient #	Site	Lesion Type	Luminal Narrowing	Sex	Age	CAUSE OF DEATH
1	LAD	5	20	F	63	Hypertensive/Atherosclerotic CVD
2	LAD	5	20	F	49	No definitive cause, but had Brain Edema
2	Rco	2	10	F	49	No definitive cause, but had Brain Edema
3	CC	4	10	M	47	Gunshot wound
3	LAD	1	10	M	47	Gunshot wound
3	Rco	2	10	M	47	Gunshot wound
4	LCS	5	10	M	57	Suicide by hanging
5	LAD	3	10	M	22	Idiopathic cardiomegaly
5	Rco	2	10	M	22	Idiopathic cardiomegaly
6	LAD	2	5	M	23	Drug Overdose
7	CC	3	5	F	44	Blunt Force, Motor vehicle accident
7	LAD	5	30	F	44	Blunt Force, Motor vehicle accident
7	LCS	5	10	F	44	Blunt Force, Motor vehicle accident
8	LAD	5	15	M	71	Hypertensive/Atherosclerotic CVD
9	Rco	4	50	M	26	No def. cause
10	LAD	4	50	M	45	Hypertensive CVD/Cardiomegaly
11	Rco	5	30	M	71	Hypertensive/Atherosclerotic CVD
12	CC	2	0	M	34	Blunt Force, Motor vehicle accident
12	LAD	1	5	M	34	Blunt Force, Motor vehicle accident
12	LCS	3	10	M	34	Blunt Force, Motor vehicle accident
13	Rco	5	70	F	60	Hypertensive, intracranial hemorrhage
14	LAD	3	10	M	22	Obese, post operative ileus
14	Rco	3	5	M	22	Obese, post operative ileus
15	CC	2	0	M	21	Blunt Force, Motor vehicle accident
15	LCS	2	5	M	21	Blunt Force, Motor vehicle accident
16	Rco	1	5	F	40	Bronchopneumonia
17	LAD	5	90	M	53	Mod. atherosclerosis & hypertension & cocaine abuse
17	LCS	5	40	M	53	Mod. atherosclerosis & hypertension & cocaine abuse
18	LCS	2	5	M	21	Blunt Force, Motor vehicle accident
18	Rco	2	10	M	21	Blunt Force, Motor vehicle accident
19	LAD	4	20	M	47	Bronchopneumonia
19	LCS	3	10	M	47	Bronchopneumonia
19	Rco	2	10	M	47	Bronchopneumonia
20	LAD	1	10	F	38	Blunt Force, Motor Vehicle Accident
20	LCX	2	10	F	38	Blunt Force, Motor Vehicle Accident
20	Rco	1	5	F	38	Blunt Force, Motor Vehicle Accident
21	CC	4	40	M	40	Blunt Force, Motor Vehicle Accident
21	LAD	2	10	M	40	Blunt Force, Motor Vehicle Accident
21	LCX	1	10	M	40	Blunt Force, Motor Vehicle Accident
22	Rco	5	20	M	55	Bronchopneumonia/cirrhosis
23	LAD	2	10	M	24	GSW to head
23	LCS	3	10	M	24	GSW to head
23	Rco	2	5	M	24	GSW to head
24	Rco	5	60	M	61	Drowning (stented LAD)
25	Rco	5	40	F	43	Myocarditis