SUPPLEMENTAL MATERIAL

DETAILED METHODS

***Thbs4*$^{-/-}$ mice.** The *Thbs4* gene was inactivated by replacing 14 nucleotides in the first intron of its genomic sequence with a LacZ-neomycin resistance cassette (Lac0-SA-IRES-lacZ-Neo555G/Kan). When *Thbs4* DNA was analyzed by Southern blots, the recombinant ES cell line showed an additional, single band of the predicted size. The mechanism for the insertional inactivation of *Thbs4* was essentially that of a promoter intron trap in which a splice acceptor sequence was fused 5' to a LacZ-Neo cassette that contained a poly A sequence at its 3' end. As a result, β-galactosidase was expressed under the control of the endogenous *Thbs4* promoter and the *Thbs4* gene was silenced[1]. Homologous recombination in the ES cell line used to generate the TSP-4-null mouse was verified by 3'PCR and 5' Southern blot analysis of genomic DNA (for additional information go to: http://jaxmice.jax.org/strain/005845.html). These procedures were conducted at Deltagen, Inc. (San Mateo, CA), and the resulting mice were genotyped and maintained at the Cleveland Clinic. *Thbs4*$^{-/-}$ mice were backcrossed onto a C57BL/6 background for 12 generations. The absence of TSP-4 mRNA and protein was confirmed by Northern and Western blots (Suppl. Fig.1) and by the absence of immunostaining of various tissues from *Thbs4*$^{-/-}$ mice with anti-TSP-4 antibody (the lack of staining in aortic lesions is shown in Suppl. Fig.5b). *ApoE*$^{-/-}$ C57BL/6 mice were purchased from Jackson Labs and mated with *Thbs4*$^{-/-}$ mice to produce groups of 12-15 mice of the same age, with the genotype *Thbs4*$^{-/-}$/*ApoE*$^{-/-}$. Mice were kept on a Western diet (42% calories from fat, Harlan Laboratories Inc., #TD.88137) or regular mouse chow diet (Harlan Laboratories Inc., # 8604 Teklad Rodent Diet) starting at 4 weeks of age, and they were sacrificed at 20 weeks of age. All animal procedures were performed according to NIH guidelines under protocols approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic.

Antibodies. The TSP-4 antibody used (R&D,Minneapolis, MN) was raised in a goat using a fragment of TSP-4 (Ala22-Asn961) that is highly homologous in the human and mouse proteins. This antibody recognized both human and mouse TSP-4 and did not react with tissues from *Thbs4*$^{-/-}$ mice in Western blots (Suppl. Fig. 1) or by immunohistochemistry (Suppl. Fig. 5a,b).

Rabbit anti-TSP-3 IgG was from Santa Cruz (Santa Cruz, CA). Rat anti-TSP-5 IgG was from Affinity BioReagents (Golden, CO). The specificity of these antibodies was confirmed by staining of atherosclerotic lesions with secondary antibody only (Suppl. Fig.5c,d) and by staining aortas from *Thbs3/Thbs4/Thbs5* triple KO mice (Suppl. Fig. 5e-h).

Mouse monoclonal antibodies to p38 MAPK and phospho-p38 MAPK were from Cell Signaling Technology (Danvers, MA). Anti-α-actin (#ab5694) rabbit polyclonal antibodies were from Abcam (Cambridge, MA), MOMA-2 (#MCA519) from Serotech (Raleigh, NC), TSP-1, TSP-2, (R&D Systems, Minneapolis, MN), FITC-CD106 (#553332), from BD Pharmigen (San Jose, CA), pan Macrophage Marker (#14-4801-85) and anti-MCP-1 (#ab7202) were from Abcam Inc. (Cambridge, MA), anti-MHC-1 from ATCC (Manassas, VA).

The antibodies used in adhesion and migration assays to block corresponding receptors were widely used, confirmed function blocking antibodies, and have been previously demonstrated to inhibit interactions of these receptors with their ligands, namely purified mouse monoclonal anti-mouse CD36 (Millipore, Billerica, MA)[2], purified rat anti-mouse integrin α_4 (clone PS/2) (Millipore)[3], ascites of rat anti-mouse integrin $\alpha_5\beta_1$, clone BMA5 (Millipore)[4], rabbit antiserum to human and mouse β_3-integrin (Millipore, cat# AB1932)[5], and purified rat anti-mouse α_M integrin, (clone M1/70, ATCC)[6].

An MCP-1 ELISA kit was purchased from R&D (Minneapolis,MN) and used according to the manufacturer's directions. Aortic tissue extracts were prepared from isolated aortic fragments,

including the aortic arch and the descending aorta down to iliac bifurcation. Homogenized tissue was incubated with RIPA buffer (ThermoScientific, Rockford, IL) for 20 min on ice, and a protease inhibitor cocktail (Roche, San Francisco, CA) was added as described previously[7].

Quantitative analysis of lesions in aortic root was performed as described by Baglione and Smith[8].

Quantitative Atherosclerosis Measurements in aortic arch and descending aorta.

At 20 weeks of age, the circulatory system of anesthetized mice was perfused with 0.9% NaCl by cardiac intraventricular canalization. The surface lesion area was determined by an *en face* method. The heart and ascending aorta, including the aortic arch were removed, and the heart containing the aortic root was fixed in phosphate-buffered formalin and processed for aortic root quantitative atherosclerosis measurements. The remaining aorta was removed, fixed in Histochoice (Electron Microscopy Science), opened by cutting longitudinally, stained with Oil Red O, and the area of lesions was quantified using ImagePro 6.3.

Immunohistochemistry. Mice were sacrificed by CO_2 inhalation followed by cervical dislocation. Hearts and aortas were harvested and placed in Tissue-Tek O.C.T. Compound (Sakura Finetek U.S.A Inc., Torrance, CA), frozen in liquid nitrogen, and stored at -80^{o}C until processed. Frozen sections (10 -12 μm) were cut in a cryostat (Leica, Wetzlar, Germany) and placed on microscope slides (Superfrost; Fisher Scientific, Waltham, MA). Sections were immediately blocked by incubation in PBS containing 5% BSA (MB Biomedicals, OH) and donkey IgG (1:50) for 30 min at 4^{o}C. Following this treatment, the slides were incubated with primary antibodies for 2 h at 4^{o}C. After washing with PBS/BSA (3x10 min), primary antibodies were detected by incubating sections in Rhodamine Red-X (1:200) or FITC (1:100)-conjugated secondary antibodies for 45 min at 4^{o}C. Immunofluorescent images were acquired using an upright Leica DMR microscope (Leica Microsystems, GmbH, Wetzlar, Germany).

Confocal Microscopy. Z-stacks of images were collected using a Leica TCS-SP3-AOBS Laser Scanning Confocal Microscope (Leica Microsystems Inc., Bannockburn, IL) with an HCX Plan Apo 40x/1.25 NA oil immersion objective. All z-series were collected using the same number of slices, step size (0.4 μm), and collection parameters, including brightness, contrast and pinhole. Image stacks from the z-series were reconstructed and analyzed using Volocity 4.1.0 software (Improvision Inc., Lexington, MA).

Quantification of stained areas of lesions was performed using Adobe Photoshop CS2 and ImagePro6.3. The lesion area (including luminal endothelium) was copied using a magnetic lasso tool and pasted into a new image file, which was used further to measure the total number of pixels in the lesion and the number of pixels in the stained area. At least 3 animals per group were used, and 4 or more sections of aortic root / animal were examined.

Plasma Cholesterol Analysis. LDL/VLDL cholesterol was measured in mouse plasma using an HDL &LDL/VLDL Cholesterol Quantification Kit (BioVision Research Products).

Induction of peritonitis and isolation of peritoneal macrophages. Murine peritoneal macrophages were collected utilizing a thioglycolate inflammation model. Sterile 4% Brewer thioglycollate medium solution was injected intraperitoneally and after 72 hrs, when macrophage recruitment is maximal in this model[9], mice were sacrificed and macrophages were harvested by lavage of the peritoneal cavity with sterile PBS. FACS analysis with rat antibody to mouse macrophage antigen F4/80 (Abcam) confirmed that macrophages represented >95 % of all isolated peritoneal cells. The cells were washed once with serum-free RPMI1640 medium, counted, and used immediately in adhesion and migration assays. Cells from each mouse were counted using a hemacytometer, and an aliquot of the cells was stained with Gimsa/Wright stain

(FisherScientific, #123-869 (Rockford, IL) and examined microscopically to determine the % of macrophages.

Migration and adhesion assays.

Cell adhesion, migration, and MAPK phosphorylation were measured as described previously[7] using the RAW 264.7 macrophage cell line or thioglycollate-elicited peritoneal macrophages derived from wild-type C57BL/6 mice as described elsewhere[9]. Briefly, 96-well non-tissue culture-treated plates (Falcon, Becton Dickinson, San Diego, CA) were coated with recombinant TSP-4 (0-40 mg/ml)[10, 11] for 16 hrs at 4°C and then blocked with 0.5% polyvinylpyrrolidone (PVP) (Sigma Chemical, St Louis, MO) for 1 hr at room temperature. The RAW 264.7 cells or peritoneal macrophages were resuspended in serum-free RPMI1640 in the absence or presence of mouse recombinant MCP-1 (1 ng/ml) or Gro1$_\alpha$ (1-2ng/ml Invitrogen, Carlsbad, CA) and incubated for 20 min at 37°C. The cells were then seeded at 1.5×10^5 cells/well onto the coated plates and incubated at 37°C for 1hr. The plates were washed with PBS, and the number of adherent cells was quantified using a Cyquant Cell Proliferation Assay Kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions.

The migration of RAW 264.7 cells was assessed in serum-free RPMI 1640 medium using Costar 24-transwell plates with 8 μm-pore polycarbonate filters (Corning, Corning, NY) and with TSP-4 (0-40 mg/ml) immobilized on their lower surfaces. MCP-1 (1 ng/ml) was added to the lower chambers in a total volume of 600 μl medium, whereas the upper wells contained 2×10^5 cells in a final volume of 200 μl. The plates were incubated for 6 h in a humidified incubator at 37°C and 5% CO_2. The migrated cells were then quantified using a Cyquant cell proliferation kit. Murine RAW 264.7 macrophages were obtained from ATCC, maintained in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, and 1mM sodium pyruvate.. Undifferentiated, resting cells were used in adhesion and migration assays.

In inhibition experiments, cells were pretreated for 20 min at 37°C with the following blocking antibodies: rabbit anti-integrin β_3, rat anti-$\alpha_5\beta_1$, anti-α_4 (Chemicon) and anti-α_M (clone M1/70, ATCC), mouse anti-CD36 (Chemicon) and control anti-MHC-1 (W6/32, ATCC) prior to addition to the assays. After adhesion of macrophages from wild-type mice to TSP-4 for 45 min at 37°C, adherent cells were lysed, and 20 μg of lysate protein was subjected to SDS-PAGE followed by Western blotting using antibodies against p38 MAPK and phopsho-p38 MAPK, as previously described[10].

Foam Cell Formation was measured as described[12]. Briefly, peritoneal macrophages were isolated from mice and cultured in RPMI1640 medium with 10% FBS. After a 24 hr incubation with acetylated LDL, the cells were fixed with 4% FA [if this is formaldehyde, FA is not a recognized abbreviation] and stained with hematoxylin and Oil Red-O. Bright field images were acquired using an upright Leica DMR microscope (Leica Microsystems, GmbH, Wetzlar, Germany) equipped with a Retiga SRV cooled CCD camera, with aLiquid Crystal tunable [what is this?] RGB filter (QImaging, Surrey, BC Canada). Lipid droplet quantification was performed with ImagePro Plus software (Media Cybernetics, Bethesda, MD).

Microvascular endothelial cell isolation and culture. Microvascular endothelial cells from murine lung were isolated as described[13]. Briefly, to isolate murine endothelial cells, lungs from 3 to up to 5 mice were removed and collected in a tissue culture plate, containing cold PBS and antibiotics. Tissues were trimmed of extraneous tissue, washed with PBS, finely minced, and incubated for 4 h at room temperature with collagenase/dispase (Roche, San Francisco, CA) and gentle agitation. Digested tissues were vigorously pipetted and filtered through a 50-μm mesh of a cell dissociated sieve- tissue grinder kit (Sigma-Aldrich, St. Louis, MO). The digested filtrate was centrifuged, and the pellet was washed and resuspended in DMEM/F12 medium. The cells were then incubated for 30 min at room temperature with 25 μl/ mouse of magnetic beads

conjugated with anti-mouse CD31 antibody (Invitrogen Dynal A.S, Oslo, Norway). The cells that were attached to the beads were collected using an MPC-S Magnetic Particle Concentrator (Dynal A.S, Oslo, Norway) and washed 6 times with PBS. The cells were then released from the beads by trypsinization and washed 6 times.. Washed cells were plated in endothelial medium into 25-cm^2 flasks precoated with fibronectin (Sigma-Aldrich, St. Louis, MO). After 24 hr, nonattached cells were removed, and fresh medium was added. Endothelial cells were plated on 6-well tissue culture plates and grown in DMEM/F12 medium with 10 mM HEPES, 20% FBS, 80 mg/500 ml ECGS, 40mg/500 ml heparin (Sigma-Aldrich, St. Louis, MO), and an antimycotic antibiotic. When a confluent layer was reached, the cells were washed with PBS and incubation was continued with Endothelial Cell Basal Medium (Lonza, Walkersville, MD) with 1% TSP4-free mouse serum and heparin for 2 days. To assess the expression of E-selectin (CD62E), ICAM (CD54), and VCAM (CD106), cells were stimulated with 100 ng/ml LPS (Sigma-Aldrich, St. Louis, MO) in the same medium for 4 hrs (for CD62E) and 17 hrs (for CD106 and CD54)**.**

Aortic endothelial cell isolation and culture. Endothelial cells from murine aorta were isolated as described[13]. Briefly, to isolate murine aortic endothelial cells, aortas of 3 to up to 5 mice were removed and collected in a tissue culture plate containing cold PBS and antibiotics. Aortas were trimmed of extraneous tissue, washed with PBS, and cut with surgical blade into 1-2 mm sections. Sections from one aorta were placed in one well of a matrigel (BD Bioscience, San Jose, CA) precoated 6-well plate. In 3-5 days, sections were removed. When the cells that migrated from sections of aortas into the matrigel became confluent, they were retrieved with dispase (BD Bioscience, San Jose, CA) treatment and cultured as regular endothelial cells.

FACS analysis. Endothelial cells were trypsinized, and the trypsin was inactivated by Trypsin Neutralizing Solution (Cambrex Bio Science, Walkersville, MD). The cells were harvested into DMEM/F12 medium, and washed. $0.6 – 0.8 \times 10^6$ cells were incubated with FITC-, PE-, biotin-conjugated antibodies for one hr at 4^oC. The cells were washed in PBS with 0.1% BSA and incubated (when needed) with Streptavidin-PE-Cy5 for 15 min at 4^oC, washed again and analyzed with a FACS Calibur (Becton Dickinson, San Jose, CA) using CellQuest Pro software (BD Biosciences, San Jose, CA). The antibodies used were FITC-CD106 (#553332), PE-CD54 (#553253), CD62E-biotin (#553750) from BD Pharmigen (San Jose, CA), and Streptavidin-PE-Cy5 (#15-4315-82) from eBioscience (San Diego, CA). Isotype-matched control antibodies were used as negative controls.

Statistical analysis. All data are presented as means ± SE or SD as indicated. Shapiro-Wilk test was used to evaluate normality of distribution in each data group. With the exception of data from females in Fig.1a and b and Fig. 2a, all data showed a normal distribution. Unpaired Student's t-test was used to compare the means between two independent groups with a single variable and normal distribution of data. The Mann-Whitney-Wilcoxon test was used to compare groups that were not normaly distibuted. Two-way ANOVA was used to compare groups with more than one variable. The significance level (p) was set at <0.05.

References

1. Raymond CS, Soriano P. Engineering mutations: deconstructing the mouse gene by gene. *Dev Dyn.* 2006;235:2424-2436.

2. Podrez EA, Febbraio M, Sheibani N, Schmitt D, Silverstein RL, Hajjar DP, Cohen PA, Frazier WA, Hoff HF, Hazen SL. Macrophage scavenger receptor CD36 is the major receptor for LDL modified by monocyte-generated reactive nitrogen species. *J Clin Invest.* 2000;105:1095-1108.

3. Garmy-Susini B, Jin H, Zhu Y, Sung RJ, Hwang R, Varner J. Integrin alpha4beta1-VCAM-1-mediated adhesion between endothelial and mural cells is required for blood vessel maturation. *J Clin Invest.* 2005;115:1542-1551.

4. Danen EH, Sonneveld P, Brakebusch C, Fassler R, Sonnenberg A. The fibronectin-binding integrins alpha5beta1 and alphavbeta3 differentially modulate RhoA-GTP loading, organization of cell matrix adhesions, and fibronectin fibrillogenesis. *J Cell Biol.* 2002;159:1071-1086.

5. Chen N, Leu SJ, Todorovic V, Lam SC, Lau LF. Identification of a novel integrin alphavbeta3 binding site in CCN1 (CYR61) critical for pro-angiogenic activities in vascular endothelial cells. *J Biol Chem.* 2004;279:44166-44176.

6. Rogers C, Edelman ER, Simon DI. A mAb to the beta2-leukocyte integrin Mac-1 (CD11b/CD18) reduces intimal thickening after angioplasty or stent implantation in rabbits. *Proc Natl Acad Sci U S A.* 1998;95:10134-10139.

7. Stenina OI, Krukovets I, Wang K, Zhou Z, Forudi F, Penn MS, Topol EJ, Plow EF. Increased expression of thrombospondin-1 in vessel wall of diabetic Zucker rat. *Circulation.* 2003;107:3209-3215.

8. Baglione J, Smith JD. Quantitative assay for mouse atherosclerosis in the aortic root. *Methods Mol Med.* 2006;129:83-95.

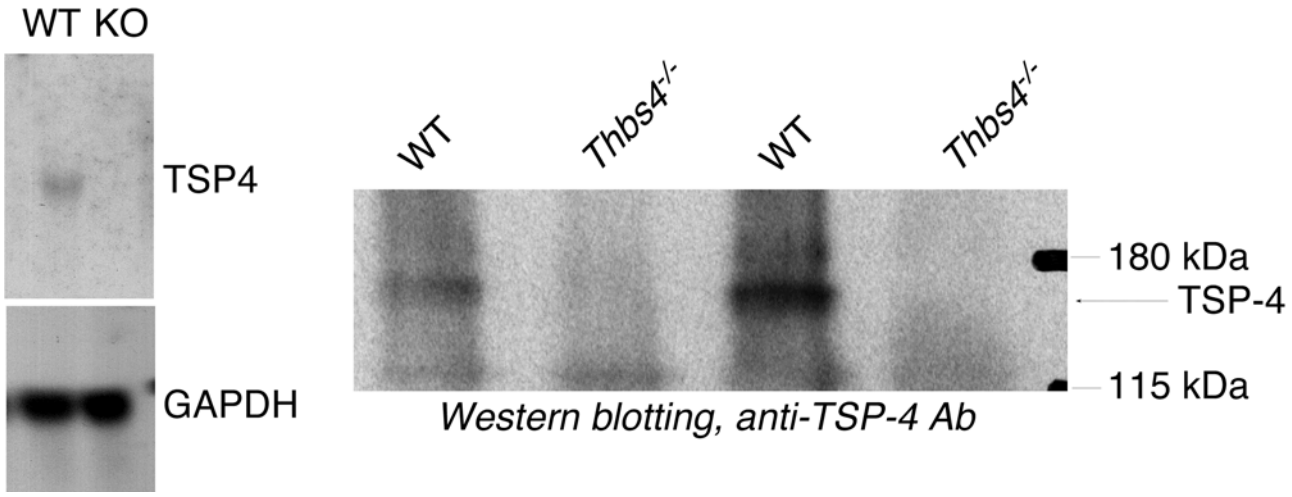
9. Ploplis VA, French EL, Carmeliet P, Collen D, Plow EF. Plasminogen deficiency differentially affects recruitment of inflammatory cell populations in mice. *Blood.* 1998;91:2005-2009.

10. Pluskota E, Stenina OI, Krukovets I, Szpak D, Topol EJ, Plow EF. Mechanism and effect of thrombospondin-4 polymorphisms on neutrophil function. *Blood.* 2005;106:3970-3978.

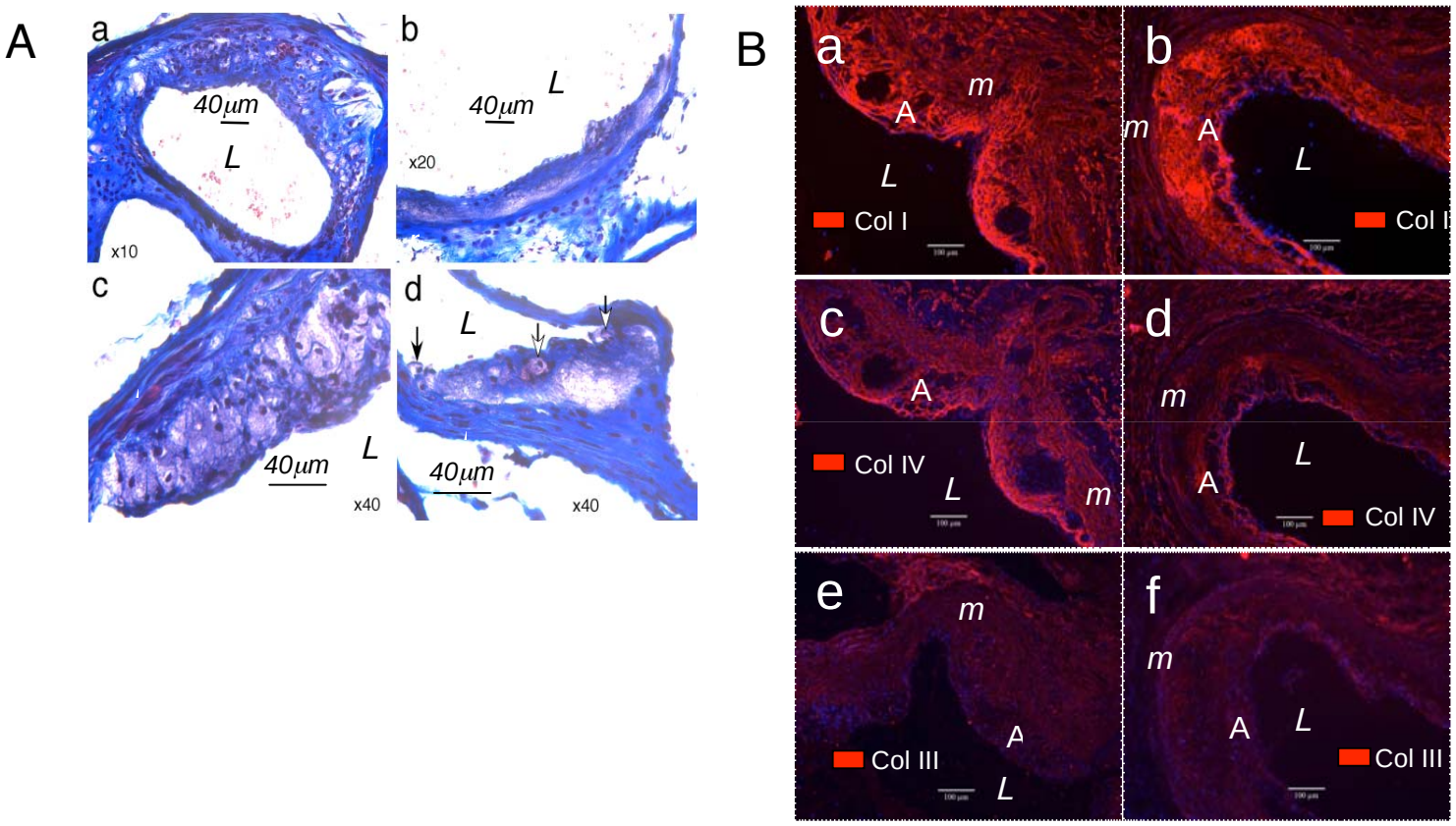
11. Stenina OI, Ustinov V, Krukovets I, Marinic T, Topol EJ, Plow EF. Polymorphisms A387P in thrombospondin-4 and N700S in thrombospondin-1 perturb calcium binding sites. *FASEB J.* 2005;19:1893-1895.

12. Kar NS, Ashraf MZ, Valiyaveettil M, Podrez EA. Mapping and characterization of the binding site for specific oxidized phospholipids and oxidized low density lipoprotein of scavenger receptor CD36. *J Biol Chem.* 28 2008;283:8765-8771.

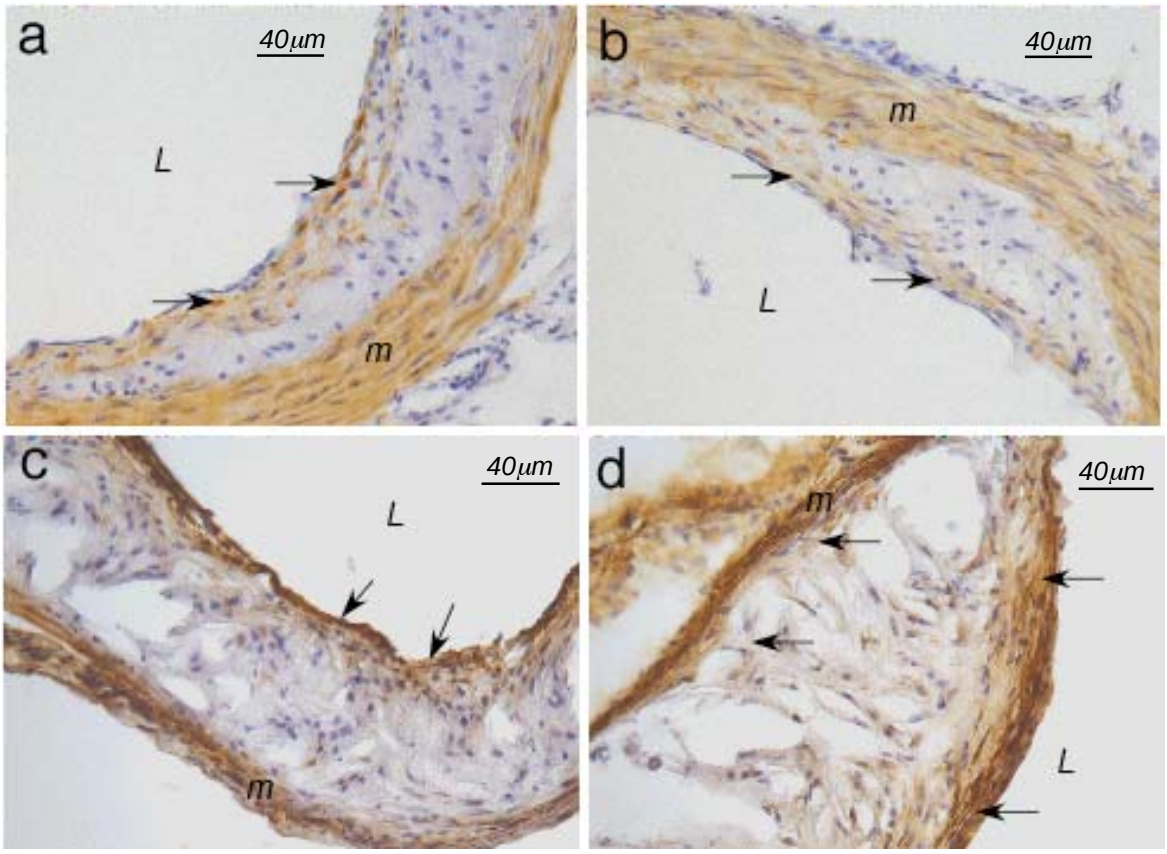
13. Mahabeleshwar GH, Somanath PR, Byzova TV. Methods for isolation of endothelial and smooth muscle cells and in vitro proliferation assays. *Methods Mol Med.* 2006;129:197-208.



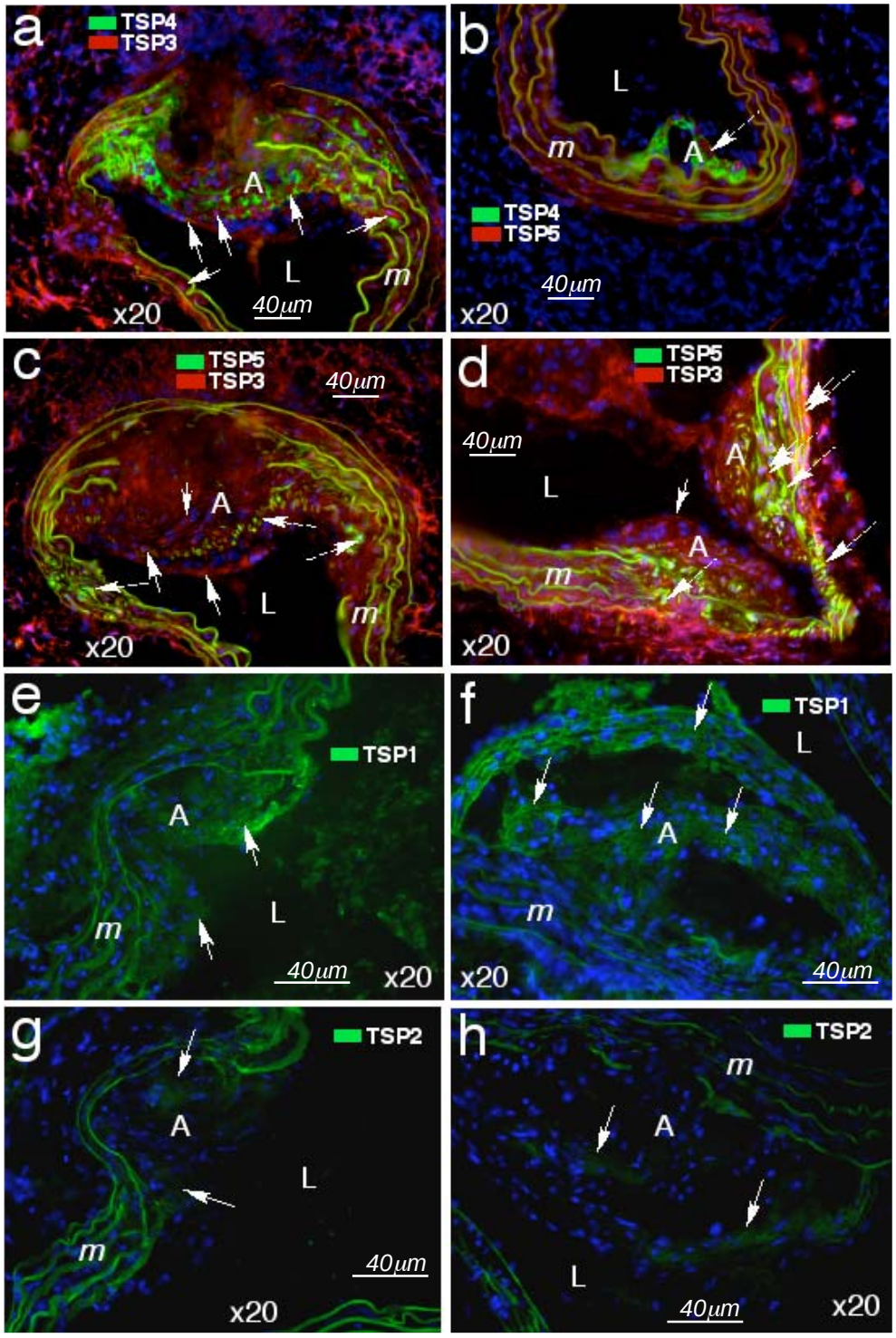
Online Figure I. Left panel: 40 μg of total RNA from brain of wild type and *Thbs4*$^{-/-}$ mice was analyzed by Northern blotting. TSP-4 cDNA was used as a probe. A GAPDH probe was used to assure equal loading. Right panel: 15 μg of protein of the extract of aortic tissue was used in Western blotting to detect TSP-4.



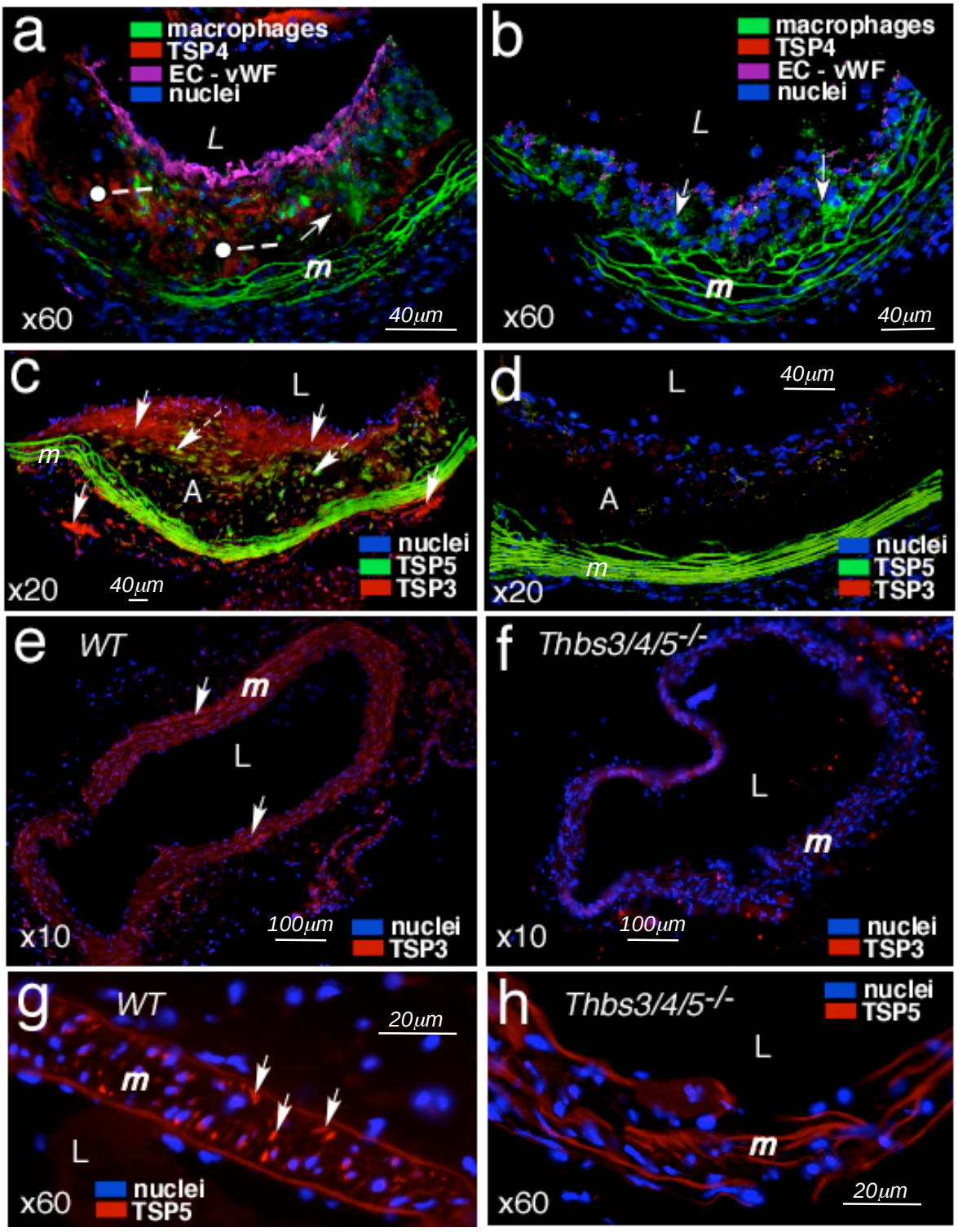
Online Figure II. Extracellular matrix of the atherosclerotic lesion of *ApoE*$^{-/-}$ and *Thbs4*$^{-/-}$ /*ApoE*$^{-/-}$ mice. A: Extracellular matrix was visualized by Masson trichrome staining, light microscopy: a, c – *ApoE*$^{-/-}$ mouse, chow diet; b, c – *Thbs4*$^{-/-}$/*ApoE*$^{-/-}$ mouse, chow diet. Arrows mark foam cells. B: Collagens I (a,b), IV (c,d) and III (e,f) in atherosclerotic lesion of *ApoE*$^{-/-}$ (a,c,e) and *Thbs4*$^{-/-}$/*ApoE*$^{-/-}$ (b,d,f) mice. Immuostaining (red). L = lumen; A = atherosclerotic lesion; m = tunica media; scale bars = 100 μm.



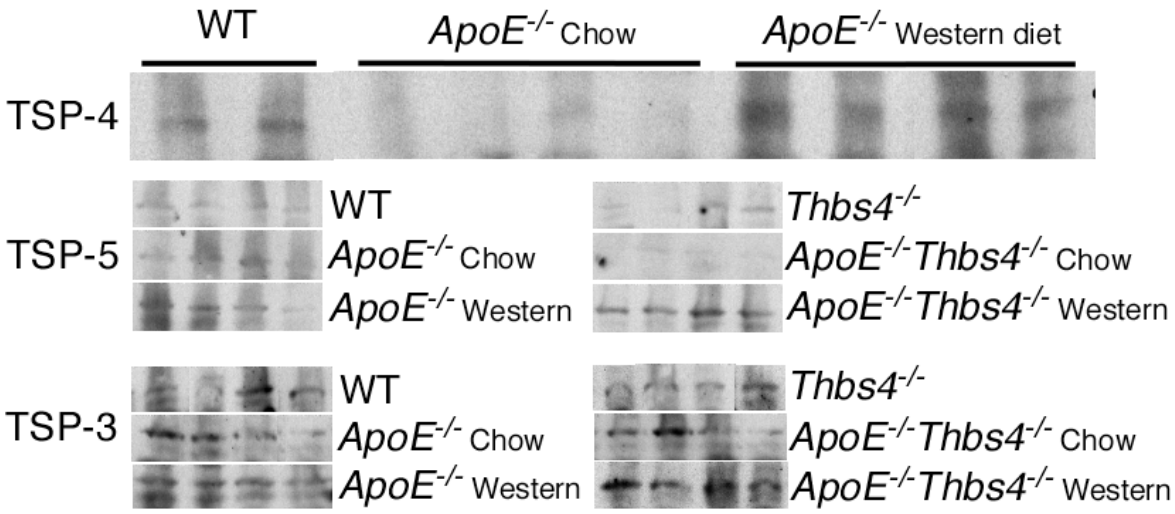
Online. Figure III. Smooth muscle cells in atherosclerotic lesions of *ApoE*$^{-/-}$ and *Thbs4*$^{-/-}$ /*ApoE*$^{-/-}$ mice. a – d: immunostaining with anti-α-actin (brown color, arrows). a,b – males, Western diet; c,d – females, Western diet; m = tunica media, L = lumen.



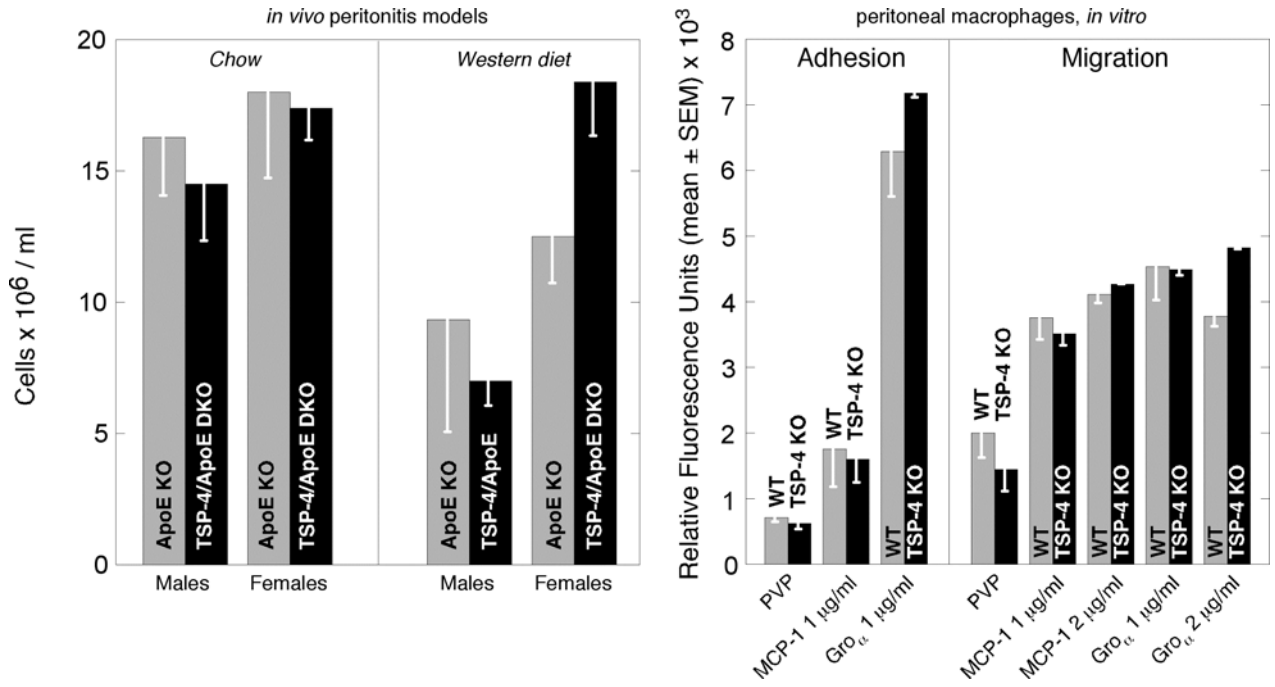
Online Figure IV. Comparative localization of TSP-4 and other thrombospondins in atherosclerotic lesions. a – d: co-staining with anti-TSP-4 and anti-TSP-3 and TSP-5 antibodies. a, c, d - TSP-3 (red staining) is abundant in *tunica media* (m, elastic lamina have green autofluorescence) and *tunica adventitia*, and on the surface of the luminal endothelial monolayer of aorta, as well as in atherosclerotic lesions (marked with solid arrows). TSP-5 staining (red in b, green in c and d) is associated with the cells of the *tunica media* and is deposited in the media, including some areas overlapping with TSP-3 staining (d, double dashed arrows). Nuclei are stained with DAPI (blue color). e ,f – staining of TSP-1 (green); g,h – TSP-2 (green); e,g - *ApoE*$^{-/-}$, f,h - *Thbs4*$^{-/-}$/*ApoE*$^{-/-}$ mice. A = atherosclerotic lesion, L = lumen, m = tunica media.



Online Figure V. Specificity of anti-TSP antibodies. The specificity of anti-TSP-4, anti-TSP-3 and anti-TSP-5 was verified using vascular tissues from corresponding knockout mice - *Thbs4*$^{-/-}$ /*ApoE*$^{-/-}$ mice for anti-TSP-4 antibody (b) and *Thbs3*$^{-/-}$/ *Thbs4*$^{-/-}$/ *Thbs5*$^{-/-}$ mice for anti-TSP-3 and anti-TSP-5 antibodies (f, h), as well as staining of the lesion with the secondary antibody only (d). a – TSP-4 staining (red) in the atherosclerotic lesion of *ApoE*$^{-/-}$ mouse (green – macrophages, blue – nuclei, magenta – EC); b – same as a, *Thbs4*$^{-/-}$/*ApoE*$^{-/-}$ mouse; c – TSP-3 (red) and TSP-5 (green) staining in the atherosclerotic lesion of *ApoE*$^{-/-}$ mouse; d – same tissue specimen and processing, no primary antibody; e – TSP-3 staining (red), wild type mouse; f – same, *Thbs3*$^{-/-}$/ *Thbs4*$^{-/-}$/ *Thbs5*$^{-/-}$ mouse; g – TSP-5 staining (red), wild type mouse; h – same, *Thbs3*$^{-/-}$/ *Thbs4*$^{-/-}$/ *Thbs5*$^{-/-}$ mouse. A = atherosclerotic lesion, L = lumen, m = tunica media.



Online Figure VI. Levels of TSP-4, TSP-3 and TSP-5 in aortas of *ApoE*$^{-/-}$ and *Thbs4*$^{-/-}$/*ApoE*$^{-/-}$ mice. Extracts of aortas from mice of all groups were prepared as described in Methods; 15 mg of protein was resolved in SDS-PAGE and blotted with anti-TSP-4, anti-TSP-3, and anti-TSP-5 antibodies.



Online Figure VII. Migration and adhesion of *Thbs4*$^{-/-}$ macrophages. Left panel: The *in vivo* migration of macrophages was assessed in the thioglycollate peritonitis model in all animal groups used in atherosclerosis experiments. Right panel: purified peritoneal macrophages were used in adhesion and migration assays as described in Methods and in Fig.5 legend.