Thrombospondin-4 Regulates Vascular Inflammation and Atherogenesis

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Rationale: Thrombospondin (TSP)-4 is an extracellular protein that has been linked to several cardiovascular pathologies. However, a role for TSP-4 in vascular wall biology remains unknown.

Objective: We have examined the effects of TSP-4 gene (Thbs4) knockout on the development of atherosclerotic lesions in ApoE−/− mice.

Methods and Results: Deficiency in TSP-4 reduced atherosclerotic lesions: at 20 weeks of age, the size of the aortic root lesions in Thbs4−/−/ApoE−/− mice was decreased by 48% in females and by 39% in males on chow diets; in mice on Western diets, lesions in the descending aorta were reduced by 30% in females and 33% in males. In ApoE−/− mice, TSP-4 was abundant in vessel areas prone to lesion development and in the matrix of the lesions themselves. TSP-4 deficiency reduced the number of macrophages in lesions in all groups by ≥2-fold. In addition, TSP-4 deficiency reduced endothelial cell activation (expression of surface adhesion molecules) and other markers of inflammation in the vascular wall (decreased production of monocyte chemoattractant protein-1 and activation of p38). In vitro, both the adhesion and migration of wild-type macrophages increased in the presence of purified recombinant TSP-4 in a dose-dependent manner (up to 7- and 4.7-fold, respectively). These responses led to p38-MAP kinase activation and were dependent on β2 and β3 integrins, which recognize TSP-4 as a ligand.

Conclusions: TSP-4 is abundant in atherosclerotic lesions and in areas prone to development of lesions and may influence the recruitment of macrophages by activating endothelial cells and directly interacting with macrophages to increase their adhesion and migration. Our observations suggest an important role for this matricellular protein in the local regulation of inflammation associated with atherogenesis. (Circ Res. 2010;107:1313-1325.)

Key Words: atherosclerosis ■ thrombospondin-4 ■ monocyte/macrophage adhesion and migration ■ inflammation ■ endothelial cells

Thrombospondin (TSP)-4 is 1 of 5 members of the TSP family of secreted matrix proteins (TSP-1 through TSP-5). Within this family, 3 homologous TSPs (TSP-3, TSP-4, and TSP-5) form a subfamily (subgroup B) based on their shared secondary and tertiary structure. Whereas the functions of the antiangiogenic TSP-1 and TSP-2 (subgroup A) in the vascular wall have been studied extensively, the role of TSPs in subgroup B in vascular physiology and pathology remains unknown. In subgroup B, evidence suggesting a role of TSP-4 in vascular pathology is particularly strong. TSP-4 is expressed by endothelial cells (ECs) and smooth muscle cells (SMCs) of large blood vessels and is expressed abundantly in capillaries. Cell biology experiments in vitro suggest that TSP-4 promotes adhesion and migration of neutrophils, and proliferation of smooth muscle cells. In vivo, expression of TSP-4 increases dramatically in response to pressure overload, in failing hearts and heart hypertrophy, and in response to ischemia. Furthermore, multiple reports of various populations have documented a genetic association between TSP-4 and accelerated atherogenesis.

The effect of TSP-1 deficiency on the development of atherosclerotic lesions in a mouse model has been recently reported, namely promoting development of atherosclerotic lesions at the initial stages, but exerting a beneficial effect in...
the well-developed lesions by promoting the phagocytic activity of macrophages.

In view of the reported effects of TSPs in the vascular wall, the genetic information linking TSP-4 to atherogenesis, and yet the lack of information on the functions of TSP-4, we have investigated the effect of TSP-4 deficiency on the development of atherosclerotic lesions in ApoE/−/− mice. We report that TSP-4 is expressed in atherosclerotic lesions, and the size of the lesions and the inflammation in vascular wall is reduced in Thbs4/−/−/ApoE/−/− mice. Our data further suggest that the ability of TSP-4 to support macrophage adhesion, migration and proinflammatory signaling is an important contributor to the mechanism of its vascular effects. In addition to the direct interaction with monocytes/macrophages, TSP-4 is found to affect EC activation, as evidenced by their production of surface adhesion molecules and chemokines. These effects of TSP-4 on macrophage recruitment in atherosclerotic lesions suggest that TSP-4, which is abundant in the lesions in areas prone to lesion development, and in the subendothelial matrix of capillaries,7 is a local regulator of inflammation.

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

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 (LacZ-neo555G/Kan). The mouse was created at Delagen Inc (San Mateo, Calif), and the resulting mice were genotyped and maintained at the Cleveland Clinic. Thbs4/−/− mice were backcrossed into a C57BL/6 background for 12 generations. The absence of TSP-4 mRNA and protein was confirmed by Northern and Western blots (Online Figure I) and the absence of immunostaining of various tissues from Thbs4/−/− mice with anti-TSP-4 antibody (lack of staining in aortic lesions) is shown in Online Figure V (b).

ApoE/−/− and Thbs4/−/−/ApoE/−/− mice were kept on a Western diet (42% calories from fat) or regular mouse chow diet, starting at 4 weeks of age, and they were euthanized 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 are described in the Online Methods.

A monocyte chemoattractant protein (MCP)-1 ELISA kit was purchased from R&D (Minneapolis, Minn) and used according to the instructions of the manufacturer. Aortic tissue extracts were prepared from isolated aortic fragments, obtained from the aortic arch and descending aorta, down to the iliac bifurcation.

Quantitative analysis of lesions in aortic root was performed as described by Baglione and Smith.20

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 canulation. Surface lesion area was determined by an en face method. Oil red O-stained areas of lesions were quantified using ImagePro 6.3.

Immunohistochemistry
Hearts and aortas were harvested and placed in Tissue-Tek OCT Compound (Sakura Finetek Inc, Torrance, Calif), frozen in liquid nitrogen, and stored at −80°C until processed. Frozen sections (10 to 12 μm) were incubated with primary antibodies for 2 hours at 4°C, followed by incubating sections in rhodamine Red-X—conjugated (1:200) or fluorescein isothiocyanate—conjugated (1:100) secondary antibodies for 45 minutes at 4°C.

Confocal Microscopy
Z-stacks of images were collected using a Leica TCS-SP3-AOBS Laser Scanning Confocal Microscope (Leica Microsystems Inc, Bannockburn, Ill) with an HCX Plan Apo ×40/1.25 NA oil immersion objective. Image stacks from the z-series were reconstructed and analyzed using Velocity 4.1.0 software (Improvision Inc, Lexington, Mass).

Quantification of stained area of lesions was performed using Adobe Photoshop CS2 and ImagePro6.3. At least 3 animals per group were used, and 4 or more sections of aortic root per animal were examined.

Plasma Cholesterol Analysis
Low-density lipoprotein/very-low-density lipoprotein (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 using a thioglycollate inflammation model. Sterile 4% Brewer thioglycollate medium solution was injected intraperitoneally and after 72 hours, when macrophage recruitment is maximal in this model,21 mice were euthanized and macrophages were harvested by lavage of the peritoneal cavity with sterile PBS.

Migration and Adhesion Assays
Cell adhesion and migration and MAPK phosphorylation were measured as described previously7 using the RAW 264.7 macrophage cell line or thioglycollate-elicited peritoneal macrophages derived from wild-type (WT) C57BL/6 mice, as described elsewhere.21 In inhibition experiments, cells were pretreated for 20 minutes at 37°C before addition to the following blocking antibodies: rabbit anti–integrin β1, rat anti-α3β1, anti-α5 (Chemicon), and anti-αM (clone M1/70, American Type Culture Collection), mouse anti-CD36 (Chemicon), and control anti–MHC-1 (W6/32, American Type Culture Collection).

Foam cell formation was measured as described.22

Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
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<td>EC</td>
<td>endothelial cell</td>
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<tr>
<td>FACS</td>
<td>fluorescence-activated cell-sorting</td>
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<td>ICAM</td>
<td>intercellular adhesion molecule</td>
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<td>KO</td>
<td>knockout</td>
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<td>LDL</td>
<td>low-density lipoprotein</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>MCP</td>
<td>monocyte chemoattractant protein</td>
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<tr>
<td>p38-MAPK</td>
<td>p38 mitogen-activated phosphokinesase</td>
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<td>SMC</td>
<td>smooth muscle cell</td>
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<td>TSP</td>
<td>thrombospinpin</td>
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<tr>
<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
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<td>VLDL</td>
<td>very-low-density lipoprotein</td>
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**Microvascular Endothelial Cell Isolation and Culture**
Microvascular ECs from murine lung were isolated as described. To assess the expression of E-selectin (CD62E), intercellular adhesion molecule (ICAM) (CD54), and vascular cell adhesion molecule (VCAM) (CD106), cells were stimulated with 100 ng/mL lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, Mo) in the same medium for 4 hours (for CD62E) and 17 hours (for CD106 and CD54).

**Fluorescence-Activated Cell-Sorting Analysis**
ECs were trypsinized. Cells were harvested into DMEM/F12 medium and washed. A total of 0.6 to 0.8×10^6 cells were incubated with fluorescein isothiocyanate–phycoerythrin–biotin-conjugated antibodies for 1 hour at 4°C, washed, and analyzed with a FACS Calibur (Becton Dickinson, San Jose, Calif) using CellQuest Pro software (BD Biosciences, San Jose, Calif). 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 Figure 1a and 1b and Figure 2a, all data showed a normal distribution. Unpaired Student test was used to compare the means between 2 independent groups with a single variable, with the exception of data from females in Figure 1a and 1b. With the exception of data from females in Figure 1a and 1b, we did not normally distributed. Two-way ANOVA was used to compare groups with more than 1 variable. The significance level (P) was set at <0.05.

**Results**

**Thbs4^{−/−} Mice**
Thbs4^{−/−} mice appeared normal at birth and displayed no obvious phenotype during their early and adult development. Their lifespan was similar to that of WT mice. Because β-galactosidase expression was detected in the ovary, seminal vesicles, prostate and uterus of Thbs4^{−/−} mice (http://www.informatics.jax.org/external/ko/deltagen/2006.html), the breeding status of these mice was evaluated. The duration and frequency of pregnancy were similar in WT and Thbs4^{−/−} mice and changed similarly with age. The fertility of males and females was also the same as assessed by mating Thbs4^{−/−} males with WT females and WT males with Thbs4^{−/−} females (data not shown). There was no difference in weight between Thbs4^{−/−}/ApoE^{−/−} mice and ApoE^{−/−} mice at the age of 20 weeks when all mice used in this study were killed. A slight decrease (10% to 15%) in body weight of the Thbs4^{−/−} mice was detected in mice of 87 to 90 weeks of age. The skeletal system of Thbs4^{−/−} and WT control mice was imaged using a custom-built microcomputed tomography system (Cleveland Clinic) at 30-μm voxel resolution, and visualized using the VolSuite 3D rendering software (Ohio SuperComputer Center, Columbus, Ohio). The resulting data suggested that the abnormality in bone density and structure observed in Thbs3^{−/−} mice24 was not present in the Thbs4^{−/−} mice. Furthermore, skeletal anatomic aberrations were absent by visual inspection. Thbs4^{−/−} mice were bred with ApoE^{−/−} mice to obtain Thbs4^{−/−}/ApoE^{−/−} mice. These mice also displayed no overt phenotype. With the absence of a spontaneous phenotype, we began our analyses of atherosclerotic lesions in Thbs4^{−/−}/ApoE^{−/−} mice.

**Atherosclerotic Lesion Area Is Decreased in the Aortic Root and Descending Aorta of Thbs 4^{−/−}/ApoE^{−/−} Mice**
For the analysis of atherosclerotic lesions, 4 groups of mice (n=11) were analyzed; ApoE^{−/−} and Thbs4^{−/−}/ApoE^{−/−} mice were maintained either on a regular chow or a Western diet (42% calories from fat). All mice were analyzed for development of atherosclerosis at 20 weeks of age, 16 weeks after being placed on the 2 diets. In mice on the Western diet, lesions developed in the aortic root, aortic arch, and the descending aorta and were measured at these sites; in mice on the chow diet, in which atherosclerosis develops slower, and lesions at the age of 20 weeks are restricted to the aortic root, only lesions in the aortic root were measured (Figure 1a). These measurements revealed that a deficiency in TSP-4 has significant changes in the area of lesions.
The effect of the genotype on the size of the lesions was influenced by gender in the groups in Figure 1a and in the groups presented in Figure 1b as was demonstrated by a two-factor ANOVA.

**LDL/VLDL Levels in Thbs4<sup>-/-</sup>/ApoE<sup>-/-</sup> Mice**

The cholesterol in the LDL/VLDL lipoprotein fraction was measured as described in Methods (Figure 1c). There was no difference in LDL/VLDL levels between genotypes, suggesting that the effect of TSP-4 on development of atherosclerotic lesions is determined by its functions in the vascular matrix and in cell interactions with the matrix, rather than on global changes in lipoprotein metabolism. Total cholesterol and high-density lipoprotein cholesterol levels were similar in both genotypes (not shown).

**Cellular Content of Lesions in Thbs4<sup>-/-</sup>/ApoE<sup>-/-</sup> Mice**

The atherosclerotic lesions in aortic root of both genotypes were examined microscopically. Matrix deposition in the lesions was assessed by Masson trichrome staining, and no difference was observed between genotypes (Online Figure II, A, a through d). Collagens 1, 3, and 4 were visualized individually, and no differences were observed between the 2 genotypes (Online Figure II, B, a through f). However, microscopic evaluation suggested that the number and distribution of cells in the lesions were significantly different: Thbs4<sup>-/-</sup>/ApoE<sup>-/-</sup> mice had fewer cellular lesions with large acellular matrix depositions, and cells (apparently macrophages) concentrated closer to the luminal surface of the lesion. In contrast, lesions in ApoE<sup>-/-</sup> mice contained visibly higher numbers of cells, and these cells were more uniformly distributed throughout the lesion. The number of cells in the lesions was quantified as the area of lesions occupied by the nuclei (hematoxylin staining, fibrous cap and ECs included; Figure 2A). The quantification confirmed that the lesions of Thbs4<sup>-/-</sup>/ApoE<sup>-/-</sup> mice were less cellular in all groups: 14.5±3.1% of area versus 38.3±11.5% in males on chow (P=0.09); 12.6±4.1% versus 23.1±2.8% in females on chow (P=0.02); 9.3±1.3% versus 14.2±1.3 in males on Western diet (P=0.002) and 7.4±0.8% versus 15.3±1.2% in females on Western diet (P=0.0008).

To determine which cell type(s) account for these differences in cellularity, we examined ECs, smooth muscle cells.
and macrophages by immunohistochemistry. We did not detect significant numbers of CD31-positive cells (ECs) in the lesions. α-Actin–positive smooth muscle cells were detected in the lesions of male mice on the Western diet, both in ApoE<sup>-/-</sup> and in Thbs4<sup>-/-</sup>/ApoE<sup>-/-</sup> mice (Online Figure III), but their numbers were not different between the 2 genotypes: 11.7 ± 2.33% of lesion area in male ApoE<sup>-/-</sup> mice versus 15.7 ± 2.6% in male Thbs4<sup>-/-</sup>/ApoE<sup>-/-</sup> mice (P = 0.13) and 25.2 ± 3% in female ApoE<sup>-/-</sup> mice versus 25.9 ± 2.2% in male Thbs4<sup>-/-</sup>/ApoE<sup>-/-</sup> mice (P = 0.4). The difference in the total cellularity of the lesions was due primarily to the difference in the number of macrophages in the lesions (Figure 2B and 2C). The area of lesions occupied by macrophages was reduced ≥2-fold in all the groups (Figure 2B). Unlike for lipid accumulation (Figure 1), the effect of the genotype on cellularity and macrophage content was not influenced by the gender as determined by 2-factor ANOVA.

Furthermore, whereas the lesions of ApoE<sup>-/-</sup> mice (including smaller, less developed lesions) had a large number of macrophages evenly distributed throughout the lesion, most Thbs4<sup>-/-</sup>/ApoE<sup>-/-</sup> mice had lesions with fewer macrophages and these cells were restricted to the surface of the lesions with low cellular infiltration into the matrix-rich body of the plaque. This pattern was obvious in both smaller and larger lesions (Figure 2C, a through d). No correlation was observed between lesion size, as determined by lipid staining, and the accumulation of macrophages in the lesions in Western-diet-fed mice. Thus, although the quantification of lipid staining indicated an equal lesion size in the aortic root of mice of the 2 genotypes (Figure 1a), the area stained for macrophages in the lesions was twice smaller in Thbs4<sup>-/-</sup>/ApoE<sup>-/-</sup> mice than in the ApoE<sup>-/-</sup> mice, suggesting that macrophage recruitment was influenced by TSP-4 despite the equal lipid content of the lesions in mice of both genotypes.

TSP-4 in Atherosclerotic Lesions and Blood Vessels of ApoE<sup>-/-</sup> Mice

We examined the patterns of TSP-4 expression in the lesions and the adjoining area of blood vessels of ApoE<sup>-/-</sup> and in the aortas of WT mice. Remarkably, strong expression of TSP-4 was detected in areas of branching of intercostal arteries of ApoE<sup>-/-</sup> mice, sites especially prone to development of atherosclerotic lesions (Figure 3a). Such localization of TSP-4 was only detected in ApoE<sup>-/-</sup> mice but not in WT C57BL6 mice (Figure 3b). TSP-4 was found in the tunica adventitia of both genotypes (Figure 3, a through c).

TSP-4 was detected in all atherosclerotic lesions, regardless of their size (Figure 3d and 3e). It was abundant in the lesion matrix (Figure 3d and 3e) and was present within ECs, as shown by immunostaining (Figure 3d and 3e). Macrophages in the lesions of ApoE<sup>-/-</sup> mice were observed in close proximity to TSP-4 (Figure 3a, 3d, and 3e). However, no intracellular TSP-4 was detected in macrophages (unlike in ECs), suggesting that macrophages within lesions do not produce TSP-4. Blood monocytes or peritoneal macrophages of WT or ApoE<sup>-/-</sup> mice on chow or Western diet did not produce detectable TSP-4, as was documented in Western blotting and by ELISA (not shown).

TSP-4 is homologous to 2 other thrombospondins, TSP-3 and TSP-5, and we were concerned that the observed effects of TSP-4 deletion might be a consequence of compensatory expression and function of another TSP family member. To examine the relationships among the 3 TSPs in the lesions, we stained blood vessels of ApoE<sup>-/-</sup> mice for all 3 TSPs. Like TSP-4, both TSP-3 and TSP-5 were also present in the lesions. However, whereas TSP-3 was abundant in the tunica media and tunica adventitia, on the surface of luminal ECs and in the atherosclerotic lesions, TSP-5 was limited to the tunica media and was associated with cells of the media and with a few cells in the lesion (Online Figure IV, a through d). Furthermore, the expression patterns of the 3 TSPs were...
different; they appeared at different locations in the blood vessels and in atherosclerotic lesions, and, hence are likely to have distinct functions in the vascular wall. The specificity of observed immunostaining was confirmed by the lack of staining in lesions of Thbs4−/−/ApoE−/− mice (anti-TSP-4 antibody; Online Figure V, a and b), the lack of staining in lesions with the secondary antibody only (Online Figure V, c and d), and the lack of staining in aortas of Thbs3−/−/Thbs4−/−/Thbs5−/− mice (Online Figure V, e through h). TSP-1 was also abundantly present in the lesions of both ApoE−/− mice (Online Figure IV, e) and Thbs4−/−/ApoE−/− mice (Online Figure IV, f) in equal amounts, but only traces of TSP-2 were detected in the lesions of both genotypes (Online Figure IV, g and h).

As an independent approach to corroborate these findings, Western blotting was performed with extracts of aortas of WT and Thbs4−/− mice, and with extracts of aortas of ApoE−/− and Thbs4−/−/ApoE−/− mice on both diets. This analysis revealed that the levels of TSP-3 and TSP-5 were not increased in Thbs4−/− vessel tissue or in Thbs4−/−/ApoE−/− aortas from mice on chow diet (Online Figure VI). Western diet increased the levels of all 3 TSPs. Of the various comparisons, only the level of TSP-3 was slightly higher in Thbs4−/−/ApoE−/− aortas from mice on a Western diet as compared with aortas of ApoE−/− mice on a Western diet.

**TSP-4 and Macrophage Function**

Despite the absence of detectable TSP-4 in macrophages (Figure 3d and 3e), we considered that TSP-4 might influence macrophage function, and thus selected responses were evaluated.

**Effect of TSP4 on the Uptake of Acetylated LDL by Macrophages**

The uptake of acetylated LDL is a function that is critical to the formation of atherosclerotic lesions. Peritoneal macrophages from WT mice were cultured on 0 to 50 μmol/L recombinant TSP-4. TSP-4 had no effect on the formation of foam cells (data not shown).

**Migration and Adhesion of Thbs4−/− Peritoneal Macrophages**

Peritoneal macrophages were isolated from peritoneal fluid of ApoE−/− and Thbs4−/−/ApoE−/− mice on a chow or Western diet, 72 hours after injection of thioglycollate to induce peritonitis. At this time point, 95% of cells in peritoneal fluid were identified by microscopy or fluorescence-activated cell-sorting (FACS) analysis as macrophages. The cells were counted to assess the in vivo migratory ability of macrophages, but no difference in cell counts was found between the 2 genotypes (Online Figure VII, A).

The effect of TSP-4 deletion on adhesion and migration of peritoneal macrophages isolated from WT and Thbs4−/− mice was assessed. No difference was found between macrophages from the 2 genotypes to adhere or migrate in vitro in response to MCP-1 and Gro1α (Online Figure VII, B).

Because we were unable to detect differences in these macrophage responses and we could not detect TSP-4 in macrophages, we assessed the effect of exogenously added recombinant TSP-4 on the adhesion and migration of macrophages.

**Adhesion and Migration of Monocytoid Cells to Purified Recombinant TSP-4**

RAW 264.7 cells, a widely used murine macrophage-like cell line, were used initially to assess the effects of TSP-4 on adhesive and migratory responses. TSP-4 supported both the adhesion of RAW 264.7 cells in a dose-dependent manner, and a stimulus, MCP-1, did not affect the extent of adhesion (Figure 4A, a). RAW264.7 cells migrated onto TSP-4 in a dose-dependent manner (Figure 4A, b), and MCP-1 increased the migration almost two-fold. Maximum migration was reached at 5 μg/mL of rTSP-4 and decreased at higher concentrations, i.e., increased adhesion may impede migration as is often observed on matrix proteins. Macrophage adhesion and migration to extracellular matrix proteins such as the TSPs are integrin-mediated responses. We used a series of blocking antibodies to candidate integrins, as well as to nonintegrins as controls. As shown in Figure 4A, c and d, only 2 of the antibodies tested were effective inhibitors of macrophage adhesion and migration on TSP-4. These were antibodies to integrins α5β1 and to the β3 subunit. Antibodies to other integrins, αβ1, and the β3 subunit, and to nonintegrins, CD36 and MHC, had little effect.

Similar to the effect of TSP-4 on RAW264.7 cells, the adhesion and migration of peritoneal macrophages was supported by TSP-4 in a dose-dependent manner (Figure 4B, a and b, respectively). Antibodies to integrins α5β2 and to the β3 subunit again blocked both adhesion and migration, whereas anti-α5β1 and anti-MHC failed to affect either response, confirming that the interaction of macrophages with TSP-4 depends on β2- and β3-containing integrins (Figure 4B, c and d).

**Intracellular Signaling Initiated by TSP-4–Macrophage Interaction**

We have previously reported that interaction of neutrophils and ECs with TSP-4 initiates intracellular phosphorylation and activates intracellular kinases. In neutrophils adherent to TSP-4, phosphorylation of p38-MAPK was particularly robust. Increased phosphorylation of p38-MAPK was analyzed by Western blots with specific antibodies in peritoneal macrophages adherent to rTSP-4. A marked increase in phospho-p38-MAPK was noted in the cells adherent to TSP-4 (Figure 4C). The increase in phospho-p38-MAPK occurred in the absence of detectable changes in total cellular p38-MAPK. The increase in phosphorylation of p38-MAPK was prevented by preincubation with either anti-β3 or anti-α5β2 antibodies, suggesting that the activation of p38-MAPK is a consequence of engagement of these integrins by TSP-4.

To assess the activation of this important intracellular mediator of inflammatory signaling in lesions of ApoE−/− and Thbs4−/−/ApoE−/− mice on chow or Western diets, lesions in the aortic root of mice of all groups were stained with anti–phospho-p38-MAPK (Figure 5) and the area of staining was quantified (Figure 5A). The activation of p38-MAPK was decreased in Thbs4−/−/ApoE−/− mice in all groups, with an exception of males on a chow diet.
The role of $\alpha^M\beta_2$ and $\beta_3$ integrins in macrophage interactions with TSP-4 and initiation of proinflammatory signaling was confirmed using peritoneal macrophages from $\alpha^M\beta_2$ and $\beta_3$ subunit knockout (KO) mice (Figure 6). In both $\alpha^M\beta_2$ and $\beta_3$ subunit knockout macrophages, the ability to adhere (Figure 6A and 6B) and activate p38-MAPK (Figure 6C) was reduced, consistent with the inhibition of these functions by corresponding antibodies (Figure 4C). Macrophage migration was reduced in $\alpha^M\beta_2$-deficient macrophages, but not in $\beta_3$ subunit knockout cells (Figure 6B). The lack of effect of $\beta_3$ may be attributable to compensatory mechanisms that are known to occur in these mice.25

Reduced Vascular Inflammation and EC Activation in Thbs4$^{-/-}$/ApoE$^{-/-}$ Mice

As a secreted protein, TSP-4 in the matrix is available for interactions with many cell types and may influence proinflammatory processes in lesions in multiple ways. To assess the effect of a deficiency of TSP-4 on the levels of inflammatory mediators and chemoattractant MCP-1 in the aorta, we visualized MCP-1 in the lesions of mice of both genotypes on chow and Western diets (Figure 7A, a through d). In both genotypes, as expected, MCP-1 levels were increased by the Western diet in all the layers of aorta, but especially in the lesions themselves. However, there was also a remarkable decrease in MCP-1 levels in the lesions, in the tunica media and tunica adventitia of Thbs4$^{-/-}$/ApoE$^{-/-}$ mice as compared with a gender- and diet-matched control group of ApoE$^{-/-}$ mice.

MCP-1 levels were further evaluated in extracts of aortas by ELISA (Figure 7B, a). The difference observed by immunohistochemistry in aortas of mice on chow could not be confirmed by ELISA, because of either insufficient sensitivity of the assay or the smaller size of lesions, which may contribute a large part of the total MCP-1 in aortic tissue. However, in aortas of mice on a Western diet, in which both the levels of MCP-1 were higher and the lesions were larger, the 3-fold decrease in the MCP-1 level in Thbs4$^{-/-}$/ApoE$^{-/-}$ mice was confirmed.

As became clear from the immunohistochemical staining, the source of MCP-1 (red staining in Figure 7A) was not limited to macrophages (green staining). We evaluated the effect of TSP-4 deficiency on production of MCP-1 by ECs isolated from WT, Thbs4$^{-/-}$, ApoE$^{-/-}$ and Thbs4$^{-/-}$/ApoE$^{-/-}$ mice (Figure 7B, b and c). As measured by ELISA in culture media, both basal and LPS-induced levels of MCP-1 released into the conditioned media of ECs derived from Thbs4$^{-/-}$/ApoE$^{-/-}$ mice were consistently and significantly lower than from ECs derived from ApoE$^{-/-}$ mice. ECs of the second passage were used in these experiments. The
levels of MCP-1 were normalized to the number of cells harvested from each plate at the end of this experiment.

Luminal aortic ECs produce TSP-4 (Figure 3d and 3e), and the MCP-1 levels in cultured cells of the 2 genotypes suggested that the activation of ECs may be influenced by TSP-4 and may contribute to the difference in macrophage recruitment between 2 genotypes. The expression of the surface adhesion molecules, E-selectin, VCAM-1 and ICAM-1, was assessed by FACS analysis in cultured ECs from WT, Thbs4−/−, ApoE−/−, and Thbs4−/−/ApoE−/− mice (Figure 8A). The expression of all 3 surface proteins was dramatically decreased in LPS-stimulated Thbs4−/− ECs as

Figure 5. Reduced activation of p38-MAPK in lesions of Thbs4−/−/ApoE−/− mice. Anti-p38 antibody was used for staining of aortic root (brown) as described in Methods and in the legend of Figure 2, and the stained area was quantified as percentage of lesion area. A, Percentage of stained lesion area (pixels) (means±SEM). *P<0.05, statistically significant changes. B, b, Sixteen weeks of Western diet, ApoE−/−, male; c, 16 weeks of Western diet, Thbs4−/−/ApoE−/−, male; d, 16 weeks of Western diet, ApoE−/−, female; e, 16 weeks of Western diet, Thbs4−/−/ApoE−/−, female. DKO indicates Thbs4−/−/ApoE−/− double KO mice.

Figure 6. Interaction of αM− and β3− integrin−deficient peritoneal macrophages with purified recombinant TSP-4. Cell adhesion (left graphs, A and B) and cell migration (right graphs, A and B) were measured as described in Methods and in the legend of Figure 4. Closed circles indicate WT macrophages; open circles, αM−integrin−deficient macrophages in a and β3−integrin−deficient macrophages in b. C, Reduced phosphorylation of p38-MAPK in αM−integrin−deficient macrophages (top) and β3−integrin−deficient macrophages adherent to rTSP-4 by Western blotting (bottom).
compared with those in WT ECs (Figure 8A, top). The differences became smaller in mice on an ApoE/H11002/H11002/H11002 background (Figure 8A, bottom). Of the 3 markers of endothelial activation tested in these in vitro experiments, E-selectin was the most consistently downregulated surface protein, whereas ICAM-1 and VCAM-1 showed smaller differences between the 2 genotypes on an ApoE background.

The EC environment in a cell culture model is different from the in vivo environment of luminal ECs of the aortic wall. We sought to confirm the reduced activation of Thbs4/H11002/H11002/H11002 animals in vivo (Figure 8B through 8D). The staining was visibly reduced in double knockout mice both on chow (Figure 8B through 8D, b) and on Western (Figure 8B through 8D, d) diets.

Discussion

TSPs are multifunctional matricellular proteins which interact with a wide variety of receptors and other binding partners to induce responses in numerous cell types, including many circulating blood cells and cells of the vessel wall.1,2,4 The influence of TSPs on cellular responses such as adhesion, migration and proliferation of vascular cells suggests that
they might exert a significant effect on atherogenesis, but very little information is available in this regard. In a recent study, deficient mice were used to show that TSP-1 influences early lesion formation and exerts a significant effect on the maturation of atherosclerotic lesions. TSP-4 has been linked to several cardiovascular pathologies, including myocardial infarction, through an unknown mechanism.

In the present study, we examined the effect of a lack of TSP-4 on the development of atherosclerotic lesions in ApoE^{-/-} mice. The absence of TSP-4 was protective in smaller, less developed lesions, eg, in the aortic roots of mice on chow diet and in the aortic arches and descending aortas of mice on a Western diet. The decrease in development of lesions in the aortic root (39% to 48%), was dramatic in mice on a regular chow diet and was observed in both genders. These differences were highly significant and the extent of this reduction is as dramatic (or greater) as that observed in mice deficient in other molecules with well established roles in atherogenesis, such as PAI-1 (40% decrease in lesion area), MCP-1 receptor CCR2 (42% decrease in lesion area) and iNOS (30% to 50% reduction).

However, the TSP-4 effect on the composition of the lesion and the extent of inflammation in the lesion was preserved in all groups, independent of the size and lipid content of lesions. VLDL/LDL levels were identical in both genotypes on the chow or Western diet, suggesting that the protective effect of TSP-4 deficiency is attributable to its effects in the vascular wall rather than on metabolism.

A striking difference in the lesions of Thbs4^{-/-}/ApoE^{-/-} mice compared with those of ApoE^{-/-} mice is the presence of substantially fewer cellular elements in Thbs4^{-/-} mice, regardless of lesion size. Detailed analysis of the cells within lesions revealed that the number of macrophages was reduced, and their location in the lesion was changed. Other cell types in the lesions were not affected.

TSP-1 and TSP-4 are highly homologous in the region that in TSP-1 stimulates SMC proliferation through integrin. We have observed that the proliferation of cultured SMCs is increased in the presence of recombinant C-terminal fragments of TSP-4, and αβ3 has been identified as one of the receptors for TSP-4 in this report and previously. However, there was no difference in SMC numbers between the lesions of Thbs4^{-/-}/ApoE^{-/-} and ApoE^{-/-} mice.

We investigated further the interaction of macrophages with recombinant TSP-4, focusing on the formation of foam cells, adhesion and migration. No effect of TSP-4 on uptake of acetylated LDL and formation of foam cell was detected. However, TSP-4 supported adhesion and migration of macrophages in a dose-dependent manner. These functional
activities of TSP-4 were observed with a macrophage cell line as well as with peritoneal macrophages and may account for the decrease in the accumulation of macrophages in atherosclerotic lesions in Thbs4−/−/ApoE−/− mice, as compared with that in ApoE−/− mice.

In investigating the mechanism by which TSP-4 might influence macrophage adhesion and migration, we found that antibodies to 2 integrins blocked these responses in a murine macrophage cell line. Antibodies to integrin α3β2 and the integrin β3 subunit (integrin α3β3, is the only β3 expressed by these cells) were potent inhibitors of macrophage adhesion and migration on TSP-4. Of note, in a previous study of neutrophils, we implicated these 2 integrins in the adhesion and migration of these cells by using both antibody inhibition and expression approaches. As in neutrophils, we noted that adhesion of macrophages to TSP-4 led to phosphorylation of p38-MAPK, a reflection of intracellular signaling. This activation was inhibited by antibodies to α3β2, but not to α3β3 integrin. Deficiencies of both of these integrins and inhibition of p38-MAPK have been shown to affect vascular wall remodeling, inflammation in the vascular wall, and atherogenesis in mice. α3β3 integrin was atheroprotective and antiinflammatory in the published reports. The discrepancy between the 2 models in their effects on atherogenesis may be attributable to compensatory mechanisms in the α3β3 integrin KO mouse or to a predominant effect of other ligands for the integrin or other receptors for TSP-4. Indeed, our data provide direct evidence for involvement of a second integrin, α3β2, in TSP-4-induced responses. Of note, the lack of the effect of α3 integrin knockout on migration (Figure 6B, right) was inconsistent with the results of experiments using the inhibitory antibody (Figure 4A, d; Figure 4B, d), also suggesting a compensation in the β3 integrin knockout mouse. Such differences between antibody inhibition and β3 integrin knockout mice were previously observed in the angiogenic response and were ascribed to a compensatory mechanism (upregulation of VEGFR2 [vascular endothelial growth factor receptor 2]) in the KO mice.

Although antibodies specific for α3β2 were very effective in blocking macrophage responses to TSP-4, we do not exclude that other β2 integrins might recognize TSP-4 and thereby contribute to atherogenesis. Wu et al reported that CD11c (α3β2) is upregulated by hypercholesterolemia in apoE mice on a high fat diet and CD11c KO mice are resistant to diet-induced atherosclerosis. Of note, α3β2 and α3β2 share many ligands in common with α3β2.

The interaction of macrophages with TSP-4 induced activation of p38-MAPK, a major intracellular mediator of the production of cytokines and inflammatory mediators, as well as endothelial-leukocyte interactions and motility in macrophages. p38-MAPK has been implicated in signaling, leading to the production of MCP-1, MIP-1β and -1α (macrophage inflammatory protein), interleukin (IL)-1β, IL-6, IL-8, IL-10, GM-CSF (granulocyte–macrophage colony-stimulating factor), and tumor necrosis factor by macrophages. Immunosnoaining of lesions revealed that both p38-MAPK and MCP-1 levels in the lesions are reduced in Thbs4−/−/ApoE−/− mice, which in turn would reduce inflammation and leukocyte recruitment into the lesion, in addition to the direct effect of the lack of TSP-4.

As a result of TSP-4 deficiency, vascular inflammation was reduced through multiple mechanisms, including reduced EC activation, monocyte/macrophage recruitment, macrophage migration inside the lesion and proinflammatory signaling in macrophages. Consequently, the cellularity and inflammation within lesions became substantially reduced, even when the lipid content within the lesions of ApoE−/− mice was similar.

Thus, we propose that TSP-4 may provide an important substrate for the recruitment of monocytes/macrophages into lesion-prone areas. The absence of TSP-4 in the Thbs4−/− mice may impede initiation and progression of atherogenesis. Although some of the effects of TSP-4 and TSP-1 deficiencies on atherogenesis appear to be similar, these proteins seem to influence the advanced lesions differently. Thus, whereas TSP-1 deficiency results in accelerated maturation of the lesion by inhibiting phagocytosis in macrophages, TSP-4 deficiency reduces macrophage recruitment into the lesion, their migration in the lesion and proinflammatory signaling in macrophages and ECs. In view of differences in structure and binding partners for TSP-1 and TSP-4, it is not surprising that they have different functions in the vascular wall and differentially affect the macrophages in the lesions.

Monocytes/macrophages or any other blood cell types do not produce detectable amounts of TSP-4, and Thbs4−/− macrophages do not differ from WT macrophages in their ability to adhere and migrate to chemoattractants. TSP-4 is always expressed in atherosclerotic lesions, independent of their size and location, and is clearly not solely responsible for macrophage recruitment. However, it is noteworthy that TSP-4 is abundant in areas of blood vessels that are prone to the development of lesions, eg, sites of branching of intercostal arteries. These findings suggest that in these areas, the adhesion and migration of monocytes/macrophages is enhanced, compared with these processes in adjacent areas. In other parts of aorta and in other large arteries and veins, TSP-4 was found in the tunica adventitia, but its presence there was discontinuous.

In conclusion, our results establish the effects of a deficiency in TSP-4 on atherogenesis and suggest a role for TSP-4 in the vascular wall and in inflammation. Inflammation drives the development of lesions from their initiation to the formation of the vulnerable plaque. The inflammatory status of the lesion correlates with thrombosis and clinical manifestations. As suggested by our data, increased TSP-4 expression may affect local inflammation through several mechanisms: increased adhesion and migration of monocytes/macrophages through the blood vessel resulting from the direct interaction of monocytes/macrophages with TSP-4, abundant presence in lesions, the activation of ECs and increased expression of surface adhesion proteins on their surface, and the increased inflammatory and atherogenic stimuli produced by both ECs and macrophages. These observations provide a framework within which the role of TSP-4 in the development of cardiovascular disease in humans can be considered.
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Disclosures
None.

References

**Novelty and Significance**

**What Is Known?**
- TSP-4 is an extracellular matrix protein that regulates cell–matrix interaction and intracellular signaling in several types of vascular cells.
- TSP-4 is associated with several cardiovascular pathologies. TSP-4 variant is associated with atherothrombosis and the increased expression of TSP-4 was detected in failing and ischemic hearts.

**What New Information Does This Article Contribute?**
- TSP-4 modulates development of atherosclerotic lesions.
- TSP-4 regulates local inflammation during formation of atherosclerosis in the vessel wall.
- TSP-4 induces proinflammatory signaling in endothelial cells and monocytes/macrophages and promotes the accumulation of macrophages in atherosclerotic lesions.

Atherothrombosis is the leading cause of death in the developed world. Our progress in understanding the mechanisms underlying the pathogenesis of atherothrombosis has been heavily focused on invoking specific cell type in the disease. Yet, we now know that it is not only specific cell type that is important but also the nature of the interaction with extracellular matrix that controls the initiation and the progression of atherothrombosis. The goal of this work was to provide novel insights into the role of TSP-4, an extracellular matrix protein, in atherogenesis. Evidence emerging over the past decade suggests a close correlation between TSP-4 and atherothrombosis. However, TSP-4 functions in the cardiovascular system remains unknown. Our study of atherosclerotic lesions in Thbs4−/−/ApoE−/− mice revealed that TSP-4 controls lesion development and local inflammation within the vascular wall. Mechanistically, TSP-4 regulates the number of macrophages accumulating in lesions through several complementary mechanisms. These include activation of endothelial cells and increased expression of surface adhesion proteins, increased proinflammatory signaling and production of inflammatory regulators, and direct binding of monocytes/macrophages that controls their adhesion/migration. This study provides new insights into mechanisms of regulation of vascular function and local inflammation by extracellular matrix and specifically by TSP-4, a protein with poorly understood but important functions in the cardiovascular system.
Thrombospondin-4 Regulates Vascular Inflammation and Atherogenesis
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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. 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](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), purified rat anti-mouse integrin α4 (clone PS/2) (Millipore), ascites of rat anti-mouse integrin αβ1, clone BMA5 (Millipore), rabbit antiserum to human and mouse β3-integrin (Millipore, cat# AB1932), and purified rat anti-mouse αM integrin, (clone M1/70, ATCC).

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

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 CO₂ 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°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°C. Following this treatment, the slides were incubated with primary antibodies for 2 h at 4°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°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 thioglycollate inflammation model. Sterile 4% Brewer thioglycollate medium solution was injected intraperitoneally and after 72 hrs, when macrophage recruitment is maximal in this model, 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.
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\(^8\). 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 Gro\(_1\) (1-2ng/ml Invitrogen, Carlsbad, CA) and incubated for 20 min at 37°C. The cells were then seeded at 1.5 \(\times\) 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 \(\mu\)m-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 \(\mu\)l medium, whereas the upper wells contained 2 \(\times\) 10\(^5\) cells in a final volume of 200 \(\mu\)l. 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 \(\beta_3\), rat anti-\(\alpha_5\)\(\beta_1\), anti-\(\alpha_4\) (Chemicon) and anti-\(\alpha_M\) (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 \(\mu\)g of lysate protein was subjected to SDS-PAGE followed by Western blotting using antibodies against p38 MAPK and phospho-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 a Liquid 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-\(\mu\)m 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 \(\mu\)l/ 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-cm² flasks precoated with fibronectin (Sigma-Aldrich, St. Louis, MO). After 24 hr, non-attached 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. 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 x10⁶ cells were incubated with FITC-, PE-, biotin-conjugated antibodies for one hr at 4°C. The cells were washed in PBS with 0.1% BSA and incubated (when needed) with Streptavidin-PE-Cy5 for 15 min at 4°C, 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 normally distributed. Two-way ANOVA was used to compare groups with more than one variable. The significance level (p) was set at <0.05.
References

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<sup>−/−</sup> and Thbs4<sup>−/−</sup>/ApoE<sup>−/−</sup> mice. A: Extracellular matrix was visualized by Masson trichrome staining, light microscopy: a, c – ApoE<sup>−/−</sup> mouse, chow diet; b, c – Thbs4<sup>−/−</sup>/ApoE<sup>−/−</sup> mouse, chow diet. Arrows mark foam cells. B: Collagens I (a,b), IV (c,d) and III (e,f) in atherosclerotic lesion of ApoE<sup>−/−</sup> (a,c,e) and Thbs4<sup>−/−</sup>/ApoE<sup>−/−</sup> (b,d,f) mice. Immuno staining (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 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.