Thrombospondin-1 (TSP1) Contributes to the Development of Vascular Inflammation by Regulating Monocytic Cell Motility in Mouse Models of Abdominal Aortic Aneurysm

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Running title: TSP1 and Abdominal Aortic Aneurysm

Subject codes:
[97] Other vascular biology
[98] Other research
[130] Animal models of human disease
[137] Cell biology/structural biology

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In April 2015, the average time from submission to first decision for all original research papers submitted to Circulation Research was 13.84 days.

DOI: 10.1161/CIRCRESAHA.117.305262
ABSTRACT

Rationale: Histological examination of abdominal aortic aneurysm (AAA) tissues demonstrates extracellular matrix (ECM) destruction and infiltration of inflammatory cells. Previous work with mouse models of AAA has shown that anti-inflammatory strategies can effectively attenuate aneurysm formation. Thrombospondin-1 (TSP1) is a matricellular protein involved in the maintenance of vascular structure and homeostasis through the regulation of biological functions such as cell proliferation, apoptosis, and adhesion. Expression levels of TSP1 correlate with vascular disease conditions.

Objective: To use TSP1 deficient (Thbs1-/-) mice to test the hypothesis that TSP1 contributes to pathogenesis of AAAs.

Methods and Results: Mouse experimental AAA was induced either through perivascular treatment with calcium phosphate, intraluminal perfusion with porcine elastase, or systemic administration of Angiotensin II. Induction of AAA increased TSP1 expression in aortas of C57BL/6 or apoE-/- mice. Compared to Thbs1+/+ mice, Thbs1-/- mice developed significantly smaller aortic expansion when subjected to AAA inductions, which was associated with diminished infiltration of macrophages. Thbs1-/- mononuclear cells had reduced adhesion and migratory capacity in vitro compared to wildtype counterparts. Adoptive transfer of Thbs1+/+ monocytic cells or bone marrow reconstitution rescued aneurysm development in Thbs1-/- mice.

Conclusions: TSP1 expression plays a significant role in regulation of migration and adhesion of mononuclear cells, contributing to vascular inflammation during AAA development.

Keywords: Abdominal aortic aneurysm, matrix protein, inflammation, monocyte, matricellular gene.
Nonstandard Abbreviations and Acronyms:
AAA      Abdominal Aortic Aneurysm
SMC      smooth muscle cells
ECM      extracellular matrix
MMP      matrix metalloproteinase
TSP1, TSP2  Thrombospondin-1, -2 (protein)
Thbs1, Thbs1−/−; Thbs1+/+  Thrombospondin-1 (gene); knockout (-/-); wildtype (+/+)
CD36, CD47, CD11b, CD68, CD3  cluster of differentiation ##
TGFβ      Transforming Growth Factor beta
CaPO4     calcium phosphate
IL6       interleukin-6
MCP-1     monocyte chemoattractant protein 1
CMFDA − 5  chloromethylfluorescein diacetate
BMM       bone marrow mononuclear
PDGF-BB   platelet derived growth factor, homodimer type BB
FAK       focal adhesion kinase
p-(p-FAK; pSmad3)  phosphorylated-
ApoE      Apolipoprotein E
CXCL10    C-X-C motif chemokine 10
PPARγ     peroxisome proliferator-activated receptor gamma
CBD       cell binding domain
CCR2/CCL2  CC chemokine receptor/ligand 2
E/IE      elastase/inactive elastase
APC       allophycocyanin
PI        propidium iodide
TUNEL     terminal deoxynucleotidyl transferase dUTP nick end labeling
DAPI      4′,6-diamidino-2-phenylindole
OD        optical density
MOMA2     monocyte/macrophage marker 2
NIMP-R14   anti-neutrophil antibody
LCCM      L-cell conditioned media
ANGII     Angiotensin II
IP         intraperitoneal
FACS      fluorescence-activated cell sorting

Thrombospondin molecule domain-related terms
NTD       N-terminal Domain
CTD       C-terminal domain
O         oligomerization sequence
C         pro-collagen molecule
NoC       N-terminal domain, oligomerization sequence, and procollagen molecule
DelNo     N-terminal domain and oligomerization sequence deleted
DelN      N-terminal domain deleted
P         properdin repeat (Type 1)
E         epidermal growth factor repeats (Type 2)
Ca        calcium repeats (Type 3)
INTRODUCTION

Abdominal aortic aneurysm (AAA), the progressive weakening and dilation of the aortic wall, is a common, age-related vascular disease. While the precise disease etiology remains elusive, women demonstrate protection from the disease prior to menopause, and both aging and smoking are to be major risk factors associated with AAA. Despite the many recent advances in AAA imaging and surgical interventions, the majority of patients who are diagnosed with small (5.5 cm for men and 5 cm for women), asymptomatic AAAs are left untreated due to the low benefit-to-risk ratio of surgical interventions in this patient population. Laboratory research that focuses on understanding the molecular and cellular mechanisms underlying aneurysm development and progression is vital to the design of effective pharmacological therapies with which to slow or reverse aneurysm progression in this population.

Over the past few decades, multiple studies have utilized both human tissues and animal models to explore the complex underlying pathophysiology of AAA. Animal models have been developed to replicate histological features of AAA such as disrupted elastin fibers, a diminished number of vascular smooth muscle cells (SMCs), and transmural infiltration of macrophages and lymphocytes. Evidence from these studies has indicated that the progressive disruption of extracellular matrix (ECM) proteins, particularly elastin and collagen, is a key event that leads to weakening and dilation of the aortic wall. Further, several classes of the matrix-degrading enzymes matrix metalloproteinases (MMPs) are implicated in aneurysm pathophysiology. Elevated MMP proteins found in human aneurysmal tissues were reported to correlate with aneurysm diameters. Additionally, manipulation of MMPs, either through pharmacological inhibition or through genetic deletion inhibits aneurysm development in mice. One such pharmacological inhibitor, doxycycline, is currently being tested in multi-centered clinical trials for the treatment of small aneurysm.

Infiltration of inflammatory cells is another pathological event that has received substantial attention in aneurysm research. Macrophages are believed to be the major source of MMPs and pro-inflammatory cytokines. Experimental strategies that reduce the macrophage population in the aneurysm wall, whether through macrophage depletion or cytokine inhibition, lead to lower MMP activity and attenuate aneurysm formation in mouse models of AAA.

Thrombospondin-1 (TSP1) is a member of the matricellular TSP protein family. Matricellular proteins are nonstructural extracellular proteins that integrate into the structural ECM. TSP1 exists naturally as a homotrimeric glycoprotein. Each monomer contains an N-terminal globular module (N), a central stalk region, and a globular C-terminal assemblage. Through these modules or domains, TSP1 binds to various matrix proteins, integrins, and cell surface receptors including CD36 and CD47. TSP1 modulates a wide range of biological functions including cell adhesion, inhibition of angiogenesis and endothelial cell proliferation, and activation of latent transforming growth factor-β (TGF-β). Additionally, TSP1 acts as a chemo-attractant that impacts various inflammatory cells, and enhanced levels of TSP1 are reported in conditions associated with tissue damage and inflammation. However, targeted gene deletion of Thbs1 produces mixed effects on the inflammatory responses depending upon the disease models. For instance, Moura et al demonstrated that Thbs1 deficiency accelerates atherosclerotic plaque maturation and is associated with higher macrophage-mediated inflammation in ApoE-/- mice. Using a diet-induced mouse model of obesity, Li et al reported that the lack of TSP1 reduces obesity-associated inflammation and improves insulin sensitivity specifically through the reduction of both adhesion, migration, and inflammatory signal in the Thbs1-/- macrophages. More recently, Csányi et al showed that TSP1, through its interaction with CD47, stimulates production of reactive oxygen species and attenuates vasodilatation. Conversely, in the retina, TSP1 mediates pro-inflammatory microglia such that a lack of TSP1 is associated with a spontaneous increase in...
inflammatory mediators. Further, TSP1 is involved in the maintenance of the immune privileged status of the ocular region.

TSP1 is present at low levels in the wall of healthy blood vessels, and its vascular expression is upregulated in animal models of atherosclerosis and ischemia-reperfusion injury. In general, high plasma TSP1 levels correlate positively with cardiovascular disease, however, serum levels of TSP1 were reported to be negatively associated with AAA. These findings are not necessarily contradictory nor definitive of any one disease state, as plasma and serum concentrations of TSP1 are sensitive to platelet number in the blood, platelet content of TSP1, and proportion of TSP1 released from platelets during preparation of plasma or serum. Thus, we aimed to evaluate the role of TSP1 in vascular inflammation and aneurysm pathogenesis through the use of two chemically-induced AAA models using Thbs1−/− mice. The results presented here show that TSP1 expression is required for macrophage adhesion to ECM proteins, migration toward chemokines, and recruitment to the vascular wall during AAA formation.

METHODS

The detailed methods are shown in online supplements.

RESULTS

Levels of TSP1 are elevated in aneurysmal aorta.

Expression of TSP1 was evaluated in human aortic tissues using an anti-TSP1 antibody for immunohistochemical analysis. Compared to normal aortas, aneurysmal tissues displayed higher levels of TSP1 throughout the aortic wall (Fig. 1A). In order to evaluate the potential role for TSP1 in the development of aneurysm, we turned to murine models of the disease. First, we utilized the elastase-induced model of murine aneurysm, created by the brief perfusion of porcine pancreatic elastase (or equal concentration of heat-inactivated elastase) through the infrarenal region of the abdominal aorta. Following aneurysm induction, C57BL/6 male mice were harvested at various time points to evaluate TSP1 expression and aneurysmal features. In accordance with the established model, elastase perfusion produced gradual aortic dilation associated with elastin fragmentation and aortic inflammation (Supplemental Figure I). Immunohistochemical staining revealed a significantly higher expression of TSP1 in the elastase-treated aortas compared to inactivated elastase-treated controls (Fig. 1B). The upregulation of TSP1 was most noticeable in the adventitia, an area known to be subject to macrophage accumulation. Using an ELISA-based assay, we confirmed that elastase-treated aortic tissues contained a significantly higher level of TSP1 than inactive-elastase treated tissues, and that this difference occurred rapidly after surgery and declined over time (Fig. 1C). Furthermore, RT-PCR analysis revealed that elastase-treatment induced >4-fold increase in the level of Thbs1 mRNA in the aortic wall 3 days after surgery (Figure 1D). Co-immunostaining study in mouse aneurysm tissue revealed that CD68+ macrophages cluster in regions of the artery that are also positive for TSP1, and that TSP1 did not appear to co-localize with smooth muscle cells (myosin heavy chain 11, MHC) or neutrophils (myeloperoxidase, MPO) (Supplemental Figure II). Immunohistochemical analysis showed a similar upregulation of TSP1 in aneurysmal tissues harvested from ApoE−/− mice treated with Angiotensin II (Supplemental Figure IIIA) or in C57BL/6 mice treated with CaPO₄ (Supplemental Figure IIIB).
Mice deficient in Thbs1 are resistant to AAA induction.

To determine whether TSP1 contributes to aneurysm pathogenesis, we subjected Thrombospondin-1 deficient (Thbs1−/−) mice to aneurysm induction by either the elastase or CaPO4 model. Prior to aneurysm induction, the basic structure of the aortic wall of Thbs1−/− and wildtype mice appeared similar and contained comparable levels of Thrombospondin-2, the closest homolog within the TSP family to TSP1 (Supplemental Figure IV). Following aneurysm induction by elastase and CaPO4 aneurysm models, aortas harvested from Thbs1−/− mice displayed marked decreases in inflammatory responses and aortic dilation when compared to the aneurysmal responses seen in wildtype (Thbs1+/+) mice (Figure 2A, B and Supplemental Figure VA, B). In the elastase model, only 4 out of the 11 Thbs1−/− mice developed aneurysm, whereas all Thbs1+/+ mice developed aneurysm, defined as a 100% or larger increase in aortic diameter (Figure 2B). Furthermore, the 4 Thbs1−/− animals that developed aneurysm following elastase treatment displayed significantly smaller aortic expansion than the Thbs1+/+ mice (diameter increases 93.2±31% and 153.4±45.4% in Thbs1−/− and Thbs1+/+, respectively). In the CaPO4 model, none of the 7 Thbs1−/− mice developed aneurysmal dilation, as compared to 9 out of 10 Thbs1+/+ mice that developed aneurysm (diameter increases 67.9±17.9% and 119.4±32.3% in Thbs1−/− and Thbs1+/+, respectively) (Supplemental Figure VB).

Inflammation is diminished in aneurysms of Thbs1−/− animals.

Transmural infiltration of inflammatory cells, predominantly macrophages, is a major histological characteristic of aneurysm. Elastase- or CaPO4-induced aneurysmal tissues harvested from Thbs1+/+ mice displayed a significant infiltration of monocytes and macrophages, consistent with features of the models as reported in previous literature (Figure 2C, D and Supplemental Figure VC). Conversely, aortic samples harvested from Thbs1−/− mice treated either with elastase or CaPO4 contained significantly fewer macrophages measured by the aortic accumulation of CD68 positive cells (12.3±1.4% in Thbs1+/+ vs. 5.9±0.7% in Thbs1−/− in elastase treated tissues, Figure 2C, D; 9.7±1.2% in Thbs1+/+ vs. 4.2±0.6% in Thbs1−/− sections following CaPO4 treatment, Supplemental Figure VC). In alignment with diminished aortic dilation and macrophage infiltration, Thbs1−/− aortas displayed attenuated elastin degradation as compared to the Thbs1+/+ counterparts (Supplemental Figure VF).

The diminished inflammatory response caused by the lack of TSP1 was confirmed by decreased number of MOMA2 positive cells, which represent monocytes and macrophages (Supplemental Figure VI). Furthermore, following elastase treatment, the lack of TSP1 was associated with reduced infiltration of neutrophils (NIMP-R14 positive) by nearly 3-fold in elastase-treated tissues (18.7±5.9% of total cells in Thbs1+/+ animals compared to 6.1±2.5% of total cells in Thbs1−/−). However, T lymphocyte (CD3 positive) infiltration was not significantly changed between the two genotypes (Supplemental Figure VI). Immunohistochemical staining showed that IL-6 expression was reduced in Thbs1−/− arteries. MCP-1, a chemokine known to be critical for monocyte/macrophage recruitment during aneurysm development, appeared to be similarly upregulated in the tunica media of both genotypes (Supplemental Figure VII), although the adventitial accumulation of MCP-1 appeared to be reduced by TSP1 gene deficiency, likely due to the diminished presence of macrophages in the knockouts. Since TSP1 can activate TGFβ, we evaluated the TGFβ activity in the aortic wall following aneurysm induction by immunostaining for phosphorylated Smad3. Elastase treatment increased levels of Smad3 phosphorylation as compared to inactive elastase control, but differences between Thbs1−/− and Thbs1+/+ mice were insignificant (Supplemental Figure VIII A, B).
TSP1 is necessary for optimal mobility of monocytic cells.

The immunohistochemical evidence provided above suggests that the reduction in monocyte and macrophages found in aneurysmal Thbs1−/− aortas cannot be attributed simply to the cytokine milieu in these tissues. Thus, to determine which of the step(s) in the inflammatory response is affected by Thbs1 deficiency, we cultured monocytic cells from the bone marrow of Thbs1−/− and Thbs1+/+ mice. The bone marrow mononuclear cells (BMM) were labeled ex vivo with the fluorescent dye CMFDA and injected via the tail vein into Thbs1+/+ host mice. The host mice were then given an intraperitoneal (IP) injection of thioglycollate (Figure 3A). Twenty-four hours after thioglycollate injection, peritoneal cells were isolated and CMFDA-positive cells (donor monocytes) in CD11b (a monocyte/macrophage marker)-positive populations were identified by flow cytometry. As shown in Figure 3B, only 39.9±0.2% of the CD11b positive peritoneal-derived inflammatory cells were positive for CMFDA when Thbs1−/− BMM were used as donors as compared to 69.7±4.0% when Thbs1+/+ BMM were used, suggesting a compromised mobility of Thbs1−/− BMM.

Adoptive transfer of Thbs1+/+ bone marrow monocytic cells reverses the aneurysm-resistant phenotype of Thbs1−/− mice.

To test whether the migratory defect of Thbs1−/− BMM is responsible for the aneurysm resistance of Thbs1−/− mice, CMFDA-labeled Thbs1+/+ BMM were adoptively transferred to Thbs1−/− mice every three days starting 1 day after the aneurysm induction (Supplemental Figure IX). As a control, CMFDA-labeled Thbs1−/− BMM was similarly administered to Thbs1−/− mice. Both groups of mice were sacrificed 14 days after elastase perfusion and analyzed for aortic dilation, inflammation, and elastin degradation. As shown in Fig. 4A and B, Thbs1−/− mice that received Thbs1+/+ BMM failed to develop aneurysm (57.2 ±23.1% diameter increase). In contrast, the mutant mice that received Thbs1+/+ BMM developed an aneurysmal dilation that was not statistically different from what was observed in Thbs1+/+ mice (138.1 29.3% vs. 162.6 19.6% diameter increase, respectively). Histologically, the Thbs1−/− mice receiving Thbs1+/+ BMM responded to aneurysm induction with elastin degradation, which was absent in those mice that were transferred with Thbs1−/− BMM (Fig. 4B). Furthermore, the transfer of Thbs1+/+ BMM to Thbs1−/− mice restored the infiltration of macrophages (CD68+) to numbers similar to those seen in Thbs1+/+ arteries (Fig. 4C). Fluorescent microscopy confirmed the vascular recruitment of the transferred Thbs1+/+ BMM (Fig. 4D). Taken together, these data suggest that inflammatory signals are intact in the aortic wall of Thbs1−/− mice, and that supplementing these mutant animals with exogenous Thbs1+/+ BMM can therefore restore the inflammatory response and other aneurysmal phenotypes.

Thbs1 gene deficiency in bone marrow cells dictates aneurysm resistance.

To further elucidate the importance of TSP1 in circulating inflammatory cells, we conducted bone marrow transplantations between Thbs1 knockout and wildtype. Since Thbs1+− mice were in C57B/6 background and express CD45.2, we used a substrain of C57B/6 expressing the CD45.1 allele (C57B/6-CD45.1) as the wildtype for easy monitoring (Supplemental Figure X). Six weeks after bone marrow transplant, recipient mice with confirmed bone marrow reconstitution determined by flow cytometry analysis of peripheral blood, were subjected to aneurysm induction using the elastase model. Aortic expansion as well as aneurysm-associated pathology was measured fourteen days after aneurysm induction. As shown in Figure 5A, transplantation of Thbs1−/− bone marrow cells to the wildtype mice (knockout → wildtype) inhibited aneurysm formation, producing an aortic expansion measuring 85.8±7.5%. In contrast, transplantation of Thbs1+− mice with the wildtype bone marrow (wildtype → knockout) developed aneurysm (114.9±13.1% increase in aortic diameter) (Figure 5A, B). The transplant procedure itself does not alter the aneurysm phenotype because the control groups, wildtype → wildtype or knockout → knockout, responded to aneurysm induction similar to the wildtype or knockout mice without bone marrow transplant (Supplemental Figure XI). Histological analyses revealed that the
aneurysmal characteristics, i.e. elastin degradation and monocyte/macrophage infiltration, were dictated by the \textit{Thbs}1 genotype of the bone marrow (Figure 5C-E).

\textit{TSP1 is required for optimal adhesion and migration of monocytic cells.}

Next, we examined whether \textit{Thbs}1 gene deficiency affects the ability of macrophages to adhere to matrix proteins in an \textit{ex vivo} adhesion assay. Aortic rings were prepared from \textit{Thbs}1\textsuperscript{+/-} mice 3 days after aneurysm induction with elastase and incubated with CMFDA-labeled peritoneal macrophages from \textit{Thbs}1\textsuperscript{+/-} or \textit{Thbs}1\textsuperscript{-/-} mice. The number of \textit{Thbs}1\textsuperscript{-/-} macrophages that adhered to the aortic tissues was 62\% less than that of the \textit{Thbs}1\textsuperscript{+/-} macrophages (Fig. 6A). Similarly, \textit{Thbs}1\textsuperscript{-/-} BMM showed reduced adhesion to fibronectin in an \textit{in vitro} culture system (Fig. 6B). To exclude a possible indirect effect of embryonic gene deletion, we transiently silenced \textit{Thbs}1 expression in RAW264.7 cells using an siRNA specific to TSP1. Compared to a scramble control, \textit{Thbs}1 siRNA attenuated RAW264.7 cell adhesion by 33\% (Fig. 6B). In a transwell migration assay, inhibition of TSP1 expression via gene deletion or siRNA completely abolished the ability of BMM or RAW264.7 cells to migrate toward chemokines (PDGF-BB or MCP-1) (Fig. 6C). Additionally, MCP-1-induced cellular adhesion, represented here by elevation of tyrosine phosphorylation of focal adhesion kinase (p-FAK 397 and 577), was diminished by \textit{Thbs}1 gene silencing in RAW264.7 (Fig. 6D).

\textit{The adhesive and migratory defects of \textit{Thbs}1\textsuperscript{-/-} monocytic cells are rescued by recombinant TSP1.}

The TSP1 molecule contains various domains that interact with matrix proteins, integrins, CD36, CD47, or other protein factors (Fig. 7A). We attempted to restore the ability of \textit{Thbs}1\textsuperscript{-/-} macrophages to adhere and/or migrate by supplementing them with recombinant TSP1 and to map the active region(s) of TSP1, with constructs comprising various domains within the TSP1 molecule (Figure 7B). Using fibronectin as an adhesion substrate, we demonstrated that recombinant TSP1 dose-dependently restored adhesion of BMM isolated from \textit{Thbs}1\textsuperscript{-/-} mice (Fig. 7C). In fact, in the presence of 50nM recombinant TSP1, \textit{Thbs}1\textsuperscript{-/-} BMM demonstrated no functional differences from \textit{Thbs}1\textsuperscript{+/-} BMM in this \textit{in vitro} adhesion assay. This same adhesion assay was performed using various TSP1 domain constructs containing or lacking the oligomerization domain that results in trimer formation. The N-terminal domain (NTD) trimer, (NoC), and the C terminal domain (CTD) monomer (DelNo) failed to rescue adhesion of \textit{Thbs}1\textsuperscript{-/-} BMM. However, the CTD trimer (DelN) restored adhesion of \textit{Thbs}1\textsuperscript{-/-} BMM, similar to the restoration obtained with whole TSP1. DelN contains 3 copies of the module that engages CD47. Next, we examined the specific effects of the CD47-binding domain through the administration of the 4N1K peptide (50nM), a CD47 agonist. 4N1K successfully restored adhesion of \textit{Thbs}1\textsuperscript{-/-} BMM to fibronectin. The 4NGG control peptide also produced a moderate, but significant, increase in adhesion. However, the 4N1K peptide fully restored \textit{Thbs}1\textsuperscript{-/-} BMM adhesion to the level of \textit{Thbs}1\textsuperscript{+/-} macrophages, and to a more significant degree than control 4NGG peptide (Figure 7C).

Recombinant TSP1 also dose-dependently rescued the chemotactic capability of \textit{Thbs}1\textsuperscript{-/-} BMM in an MCP-1-stimulated migration assay (Fig. 7D). In contrast to the results in the adhesion assay, administration of either monomeric DelNo or trimeric DelN successfully restored migration of \textit{Thbs}1\textsuperscript{-/-} BMM toward MCP-1. 4NIK and its control 4NGG peptides did not alter the inhibited migration of \textit{Thbs}1\textsuperscript{-/-} BMM. One of the mechanisms by which TSP1 is expected to influence inflammation is through the activation of TGF\textbeta\textsuperscript{30}. Using an MCP-1-driven transwell migration assay, we evaluated the migratory capacity of \textit{Thbs}1\textsuperscript{+/-} or \textit{Thbs}1\textsuperscript{-/-} peritoneal macrophages in the presence or absence of supplemental TGF\textbeta\textsuperscript{30} (5ng/mL). Similar to what we showed above with BMM cells, \textit{Thbs}1\textsuperscript{-/-} peritoneal macrophages migrated less efficiently as compared to \textit{Thbs}1\textsuperscript{+/-} peritoneal macrophages (Supplemental Figure VIIIC). Administration of TGF\textbeta\textsuperscript{30} did not restore the migratory capacity of \textit{Thbs}1\textsuperscript{-/-} peritoneal macrophages. In fact, TGF\textbeta\textsuperscript{30} significantly reduced migration of peritoneal macrophages of both genotypes, albeit more profoundly in the wildtype (Supplemental Figure VIIIC).
DISCUSSION

The present work explored, for the first time, the role of TSP1 in aneurysm pathophysiology using three established murine models of abdominal aortic aneurysm. TSP1 accumulation was notably increased in aneurysmal tissues, particularly adventitia, following either elastase perfusion or CaPO₄ application in C57BL/6 mice or following angiotensin II infusion in ApoE⁻/⁻ mice. Histological and morphological examinations of elastase- or CaPO₄-treated tissues harvested from Thbs1⁻/⁻ mice showed a profound reduction in the characteristic features of aneurysm including inflammation, disruption of elastin fibers, and aortic dilation. This aneurysm-resistant phenotype was “rescued” by adoptive transfer of the Thbs1⁻/⁺ mononuclear cells isolated from the bone marrow. These results are a striking example of uncovering the importance of a matricellular protein by stressing an organ and define a direct role for TSP1 in regulating mobility of monocytic cells, at least in the aneurysm setting.

Many cell types within the aortic wall including endothelial cells, smooth muscle cells, and adventitial fibroblasts are capable of expressing TSP1. After aneurysm induction, TSP1 appeared to be more prominent in the adventitia and intima. As a potent anti-angiogenic factor, TSP1 inhibits endothelial cell proliferation and migration and induces apoptosis. In vascular SMCs, TSP1 has been shown to promote proliferation and migration. These diverse functions of TSP1 may differentially affect how the aortic wall responds to injuries associated with aneurysm. In the elastase-induced AAA model, reconstitution of wildtype mice with Thbs1⁻/⁻ bone marrow cells inhibited aneurysm formation. Thbs1⁻/⁻ inflammatory cells, whether delivered through bone marrow transplant or adoptive transfer, rescued aneurysm development in Thbs1⁻/⁻ mice. However, the aneurysm restoration was not 100% suggesting that the role for TSP1 is not entirely restricted to monocytic cells. Future studies are necessary to elucidate how TSP1 derived from mesenchymal cells (such as SMC) contributes to aneurysm pathogenesis.

It has been recently demonstrated that TSP1 stimulates reactive oxygen species production in vascular SMCs through a direct TSP1/CD47-mediated activation of NADPH oxidase. While oxidative stress has been well linked as an underlying pathogenic player in abdominal aortic aneurysm, our results from the adoptive transfer and ex vivo adhesion studies suggest that the reduction in inflammation protection is primarily caused by the reduced ability of Thbs1⁻/⁻ monocytes to migrate toward injured aorta. TSP1 was most prominently expressed in the adventitia of aneurysmal tissue, where infiltrating monocytes and macrophages accumulate. In contrast, the highest MCP-1 signal was detected in the aortic media; Thbs1 gene deficiency did not alter the medial MCP-1 accumulation nor apoptosis of vascular SMCs (data not shown), two well-established processes resulting from aneurysm induction.

Elevated levels of TSP1 in the vessel wall are observed in several other cardiovascular disorders including diabetes mellitus, atherosclerosis, and ischemia-reperfusion injury. TSP1 has been shown to have opposing roles within diseases and/or disease models, indicating that this highly complex molecule may interact with other factors and/or act dependent upon tissue types and/or diseases. For example, in a cardiac infarct model TSP1 expression appeared to confine the area of injury, whereas overexpression of TSP1 was found to negatively impact wound healing and vascularization in a wound healing model. Early reports demonstrated that one characteristic of Thbs1⁻/⁻ mice was a consistent and significant pulmonary inflammatory state. Adding to the contradictory evidence regarding TSP1 was a report that Thbs1 deficiency correlated with increased leukocyte and macrophage infiltration to mature plaques in Thbs1 and ApoE double knockout mice. The authors attributed this pro-inflammatory phenotype to the diminished phagocytic ability of macrophages. Our data demonstrate that TSP1 is one of the factors that differentiate aneurysm and atherosclerosis mechanistically, although many AAA patients are also diagnosed for atherosclerosis. Previous publications have revealed additional differentiating factors between these two diseases, such as the chemokine CXCL10 and the nuclear...
receptor peroxisome proliferator-activated receptor (PPAR)γ. Findings such as these further our understanding of the two diseases and may permit the identification of therapeutic targets. Further, various disease states are significantly impacted by the type and/or source of macrophages present in the tissue. While our study has not explored this concept thoroughly, we are tempted to speculate that the TSP1-dependent migration of bone marrow derived monocytes/macrophages is a significant contributor of inflammatory state associated with AAA.

Our in vitro rescue studies indicate that TSP1 promotes monocytic cell adhesion to ECM by multivalent binding to the C-terminal cell binding domain (CBD) where CD47 binds. CD47 was initially identified for its association with αβ3 integrin, and ligation of this transmembrane protein either through its natural ligand TSP or monoclonal antibodies recapitulate a series of cellular functions including adhesion, migration, proliferation, and apoptosis. Although previous reports have shown that the TSP1/CD47 interaction differentially modulates migration and/or adhesion, these varying results are suggested to depend upon the functional fragment of TSP1 that is most highly expressed in a biological situation. Interestingly, our findings suggest that the CD47-binding capability of TSP1 alone is not sufficient for the migration of monocytic cells in this setting, as the CD47 agonist peptide 4N1K did not significantly change the migration response of Thbs1−/− BMM toward MCP-1. However, the two TSP1 constructs containing the CD47-binding domain as well as Type 1-3 repeats successfully restored migration in Thbs1−/− BMM. In a related study, the integrins αMβ2 and β3 were found to be critical to the adhesive and migratory capability of macrophages. While the study by Frolova et al. focuses on TSP4, TSP1 and TSP4 share multiple features of their C-terminal regions reinforcing the importance of this segment. Further investigation will be required to better understand how the unique AAA environment influences these interactions.

Twenty years ago, TSP1 was reported to promote chemotaxis of human peripheral blood monocytes. This chemotactic function is believed to promote monocyte migration to the site of injury where TSP1 is upregulated. However, this chemotactic function seems to be less important in aneurysm pathophysiology. Interestingly, Thbs1−/− arteries were able to recruit normal (Thbs1+/+) monocytic cells, as evidenced by adoptive transfer and bone marrow transplant studies. A potential explanation is that there are redundant pro-inflammatory chemotactic signals produced by the injured aorta which is not dependent on TSP1. For example, MCP-1 production by injured SMCs has been shown to be important for aneurysm-associated inflammation. However, knocking out MCP-1, also called CCL2, has limited impact on aneurysm development, whereas knocking out its receptor, CCR2, significantly reduces aneurysm expansion, indicating a more complex role for CCL1/CCR2 signaling in aneurysm development. The co-localization between TSP1 accumulation and macrophages suggests that the elevated TSP1 is likely a result of inflammatory cell recruitment (i.e. is produced by infiltrating inflammatory cells). In the context of aneurysm, the primary function of TSP1 in inflammation is through regulation of adhesion and migration of monocytic cells. Again, this assertion is supported by the successful restoration of aneurysm by bone marrow transplant of Thbs1−/− to Thbs1+/+ recipients.

A potential mechanism by which TSP1 may affect inflammation is activation of TGFβ. However, TSP1 exerts its role in tissue repair via both TGFβ-dependent and independent mechanisms. In the case of atherosclerosis, the increased inflammation in the absence of TSP1 was found to be independent of TGFβ activation. Similarly, we found mice of both genotypes responded to aneurysm induction with comparable levels of Smad3 phosphorylation detected in the aortic tissues. We believe that TSP1 modulates aneurysm associated inflammation primarily through a TGFβ-independent mechanism. This notion is further supported by our in vitro study in which TGFβ failed to restore the migratory defect of Thbs1−/− BMM.
In summary, we conclude that TSP1 plays a key role in regulation of macrophage adhesion, migration, and recruitment in the inflammatory response during pathogenesis of AAA in mice. While murine models of AAA do not resemble the etiology of human aneurysm, these models reproduce the major pathological characteristics of the human disease including macrophage-mediated inflammation. However, the relationships between the models and the human disease are an important consideration. In our study, elastase perfusion induces an acute inflammatory response that culminates in aneurysm formation while the initiating and perpetuating events in the human condition are largely unknown. The relatively high level of TSP1 observed on day 1 following elastase perfusion may reflect this acute inflammatory response. Although the protective effect of Thbs1 gene deficiency in two distinct models of AAA is encouraging, significant work remains to be done regarding the role of TSP1 in human AAA. Despite this shortcoming, the current work underscores the importance of TSP1 in the recruitment of monocytic cells in the context of abdominal aortic aneurysm. Since many TSP1-derived activating/inhibitory peptides have been described and shown to be active in vivo, we will explore in the future whether manipulation of TSP1 functions would alter aneurysm formation in the Thbs1-deficient mice and more importantly in mice with existing aneurysm. Interestingly, a TSP1 antagonist peptide was shown to promote aneurysm expansion in established angiotensin II-induced aneurysms through the attenuation of TGFβ1 activation. Although the unaltered TGFβ signaling within the aortic wall shown in this paper suggests that the lack of TSP1 affects abdominal aortic aneurysm through non-TGFβ related functions of this matricellular protein, the study by Krishna et al. proved the feasibility of modifying aneurysm pathophysiology through tweaking TSP1 functions with peptides.

ACKNOWLEDGEMENTS
The authors would like to thank Drs. K. Craig Kent and Jon Matsumura of the University of Wisconsin, Madison for intellectual inputs, and Dr. J Lawler at Harvard Medical School for the generous gift of Thbs1 knockout mice.

SOURCES OF FUNDING
This work was supported by the National Institute of Health R24EY022883 (NS), R01-HL054462 (DM), R01HL088447 (BL), R01CA152108 and R01HL113066 (JZ), American Heart Association 14PRE18560035 (QW), UW-Madison Ophthalmology Core grant P30EY016665 (NS), and an institutional training grant T32 HL110853 (SM).

DISCLOSURES
None.
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DOI: 10.1161/CIRCRESAHA.117.305262


FIGURE LEGENDS

Figure 1. Thrombospondin 1 (TSP1) expression is increased in human and experimental aneurysm. (A) Non-aneurysmal (Normal) and aneurysmal (AAA) human abdominal aorta stained for TSP1. Scale bar 200µm (10x) and 100µm (20x). (B) Experimental aneurysm tissues harvested from C57BL/6 mice 7 days after inactive elastase (control) or elastase treatment, stained for TSP1. Scale bar 200µm (10x) and 100µm (20x). (C) TSP1 expression as measured by ELISA at days 1 (d1), 7 (d7), and 14 (d14) after treatment with elastase (open circle) or inactive elastase (open triangle). (D) Real-time PCR (RT-PCR) results for TSP1 expression in the aortic wall 7 days after surgery. *p<0.05.

Figure 2. Thbs1<sup>−/−</sup> mice are resistant to aneurysm induction. (A) Representative images of wildtype (Thbs1<sup>+/+</sup>) and Thbs1<sup>−/−</sup> arteries 14 days after treatment with inactive elastase (IE) or elastase (E). Scale bar 2mm. (B) Graphic depiction of aortic dilation (%Change in aortic diameter) in IE- (circle) or E- (diamond) treated Thbs1<sup>−/−</sup> arteries and IE- (triangle) or E- (wedge) treated Thbs1<sup>−/−</sup> arteries. Red dotted line designates aneurysmal formation (100% change in aortic diameter); *p<0.05 as compared to E-treated Thbs1<sup>−/−</sup>. (C) Representative images of immunohistochemical stains for macrophages (CD68) in elastase treated arteries. Scale bar 100µm. (D) Quantification of macrophage infiltration, shown as % Positive Cells ((CD68+/nuclei)*100). *p<0.05.

Figure 3. Thbs1<sup>−/−</sup> bone marrow mononuclear cells demonstrate reduced tissue infiltration capability in vivo. (A) Schematic of experimental design. From left: fluorescence (CMFDA)-labeled bone marrow mononuclear cells (BMM) from Thbs1<sup>−/−</sup> or Thbs1<sup>+/+</sup> mice were injected via tail vein to Thbs1<sup>−/−</sup> mice, followed by intraperitoneal (IP) injection of thioglycollate. Peritoneal cells were analyzed by flow cytometry for live monocytes (CD11b APC+/PI-). Donor BMM was identified by CMFDA (Green CMFDA+). (B) Quantification of donor-derived infiltrating monocytes shown as %Donor Cells (number of Green CMFDA+ cells/number of CD11b APC+/PI- cells). *p<0.05.

Figure 4. Thbs1<sup>+/+</sup> bone marrow mononuclear cells restore aneurysm phenotype in Thbs1<sup>−/−</sup> mice. (A) Schematic of experimental design. Aneurysm was induced at day 0 (Elastase, red arrow). BMM cells were injected on days 1, 4, 7, and 10. Sacrifice and analysis was carried out on day 14 (red arrow). (B) Representative images of arteries at sacrifice, scale bar 2mm. Graphic representation of aortic expansion is shown at right. (C) Representative images of arterial cross sections stained with Van Giesson to demonstrate elastin fragmentation, scale bar 200µm. Semi-quantification of elastin fragmentation is shown on the right. (D) Representative images of immunohistochemical stains for macrophages (CD68+). Scale bar 100µm, inlaid image 10x. Number of infiltrating macrophages were counted and expressed as %Positive Cells ((CD68+/nuclei)*100. (E) Representative images of CMFDA-labeled donor cells (green) infiltrated to elastase-treated arteries, nuclei identified by DAPI (blue). Scale bar 200µm. Quantification of infiltrated donor-derived cells, expressed as %Positive Cells (fluorescent cells/nuclei), is shown on the right. *p<0.05.

Figure 5. Thbs1 gene deficiency in bone marrow cells dictates aneurysm formation. Results of a bone marrow chimera model with C57B/6-CD45.1 wildtype (CD45.1(WT)) and Thbs1 knockout (Thbs1-/-) mice. (A) Graphic representation of aneurysm expansion 14 days after elastase-induced aneurysm.; *p<0.05. (B) Representative images of arterial expansion at the time of expansion measurement; scale bar = 5mm. (C) Quantitative evaluation of macrophage (CD68+) infiltration; *p<0.05. (D) Representative images of macrophage (CD68+) infiltration, counterstain with DAPI (blue). L indicates lumen. (E) Representative elastin stains. Scale bar= 200um in 10x images, 100um in 20x images.

Figure 6. Monocytes/macrophages lacking TSP1 display reduced adhesion and migration capability. (A) Thbs1<sup>+/+</sup> aortic rings were incubated with CMFDA-labeled (green) peritoneal macrophages (Macs), nuclei labeled with DAPI (blue). Scale bar 200µm. Adhered CMFDA+ Macs from
Thbs1<sup>+/−</sup> (black) or Thbs1<sup>−/−</sup> (white) mice were counted and expressed as # cells/section. (B) in vitro adhesion assay on fibronectin-coated surface; graph at left showing RAW264.7 (RAW) cells treated with TSP1-specific siRNA (THBS1 siRNA, black bar) or scramble control (white bar), graph at right showing bone marrow mononuclear cells (BMM) harvested from Thbs1<sup>+/−</sup> (white bar) or Thbs1<sup>−/−</sup> (black bar) mice. (C) Chemotaxis toward PDGF (black) or MCP-1 (grey) or solvent (white) was assessed for RAW cells treated with scramble siRNA or TSP1-specific siRNA (THBS1 siRNA) (left graph) or BMM harvested from Thbs1<sup>+/−</sup> or Thbs1<sup>−/−</sup> mice (right graph). Results shown as number of migrated cells per high power field (HP). (D) Western blot measurement of phosphorylated (p-FAK) and total focal adhesion kinase (Total FAK) following administration of MCP1 in RAW264.7 cells after TSP1 knockdown (THBS1 siRNA) or scrambled siRNA control. β-actin used as loading control. *p<0.05.

**Figure 7. Rescuing adhesion and migration of Thbs1<sup>−/−</sup> BMM with exogenous TSP1 protein or fragments of TSP1 molecule.** (A) Cartoon representation of the TSP1 molecule, its domains and receptors. N-terminal domain (NTD), oligomerization sequence (O), procollagen molecule (C), properdin repeat (Type 1 (P)), EGF-like repeat (Type 2 (E)), calcium repeats (Type 3 (Ca)), C-terminal domain (CTD). Domain receptors indicated beneath the image. (B) Cartoon depiction of TSP1 fragments. (C) In vitro adhesion assay on fibronectin-coated plates using bone marrow mononuclear cells (BMM) from Thbs1<sup>+/−</sup> or Thbs1<sup>−/−</sup> mice quantified by optical density (OD) of adhered cells. (left) Rescue with recombinant TSP1 (0, 1, 10, 50nM). *p<0.05 compared to wildtype (Thbs1<sup>+/−</sup>). (center) Thbs1<sup>−/−</sup> BMM supplemented with solvent control (PBS), TSP1 fragments NoC, DelNo, DelN, or whole TSP1 (TSP1). *p<0.05 compared to wildtype (Thbs1<sup>+/−</sup>). (right) Rescue with CD47-binding domain peptide 4N1K or control peptide 4NGG compared to solvent (PBS). *p<0.05 compared to wildtype (Thbs1<sup>+/−</sup>). (D) In vitro chemotaxis toward MCP-1 using BMM from Thbs1<sup>+/−</sup> and Thbs1<sup>−/−</sup> mice, quantified as migrated cells counted per high power field (HP). (left) Rescue with recombinant TSP1 (0, 1, 10, 50nM). *p<0.05 compared to wildtype (Thbs1<sup>+/−</sup>). (center) Thbs1<sup>−/−</sup> BMM supplemented with PBS, TSP1 fragments NoC, DelNo, DelN, or TSP1. *p<0.05 compared to wildtype (Thbs1<sup>+/−</sup>). (right) Rescue with CD47-binding domain peptide 4N1K or control peptide 4NGG compared to solvent (PBS). *p<0.05 compared to wildtype (Thbs1<sup>+/−</sup>).
Novelty and Significance

What Is Known?

- Abdominal aortic aneurysm (AAA) is characterized by an inflammatory response dominated by macrophages.

- Thrombospondin 1 (TSP1) is a matricellular protein that regulates multiple biological functions including cell proliferation and adhesion and angiogenesis.

What New Information Does This Article Contribute?

- Levels of TSP1 are elevated in human and mouse aneurysmal tissues.

  TSP1, primarily in monocytes and macrophages, is critical for vascular inflammation and aortic expansion in murine models of AAA.

- TSP1 promotes monocyte/macrophage adhesion through its C-terminal domain.

This study addresses a knowledge gap regarding the contribution of extracellular proteins in the pathogenesis of aneurysm. We demonstrate a critical role of TSP1 expression, particularly in monocytic cells, in AAA development. Although macrophage infiltration is known to contribute to aneurysm development, the role for TSP1 in this process has not yet been explored. We found that monocytic cells require TSP1 for extracellular matrix adhesion as well as migration, and that aneurysm development is dependent upon this molecule. These findings shed new light on the pathogenesis of AAA and provide new insights into the development of new therapeutic approaches to AAA, for which no effective pharmaceutical treatment currently exists.
Figure 1

A. 10x

Human Normal Aorta

Human AAA

B. 10x 10x 20x

CS7Bl/6 (Inactive Elastase, Day 7)

CS7Bl/6 (Elastase, Day 7)

C.

D.

Relative mRNA Abundance

Elastase

Inactive Elastase

TSP 1 (pg/mL)

d1 d7 d14

40 80 120 160 200

+ + +

Inactive Elastase

Elastase

+ + +
Figure 4
Thrombospondin-1 (TSP1) Contributes to the Development of Vascular Inflammation by Regulating Monocytic Cell Motility in Mouse Models of Abdominal Aortic Aneurysm

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_Circ Res._ published online May 4, 2015;
_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2015/05/04/CIRCRESAHA.117.305262

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2015/05/04/CIRCRESAHA.117.305262.DC1

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Supplemental Material

Materials and Methods

General Materials

Fetal Bovine Serum (FBS), Dulbecco’s Modified Eagles Medium (DMEM) and cell culture reagents were from Gibco BRL LifeTechnologies (Carlsbad, CA). All other chemicals were purchased from Sigma Chemical Co (St. Louis, MO) unless otherwise specified.

Human AAA Samples

Human abdominal aortic aneurysm (AAA) tissues were obtained from patients undergoing open AAA repair, and control tissues were obtained from organ transplantation donors. The use of human tissue was approved by the Health Sciences Research Board of Sir Run Run Shaw Hospital, Zhejiang University. Samples were processed for paraffin embedding and cut 8µm thick using a Reichert-Jung 2050 SuperCut Microtome.

Mouse Models of AAA

Mice: Thrombospondin-1 deficient mice (Thbs1-/-; n=47) on the C57BL/6 background were generated as previously described 1,2 and maintained by mating Thbs1-/- males with Thbs1+/- females. C57BL/6 mice, purchased from the Jackson laboratory (Bar Harbor, Me; #000664) were used as the Thbs1+/- controls (n=122). All mice had free access to a normal diet and water. Mice were anesthetized using continuous flow of 1-2% Isofluorane. Surgical procedures were carried out under an operative microscope (Carl Zeiss, Thornwood, NY). Following completion of surgical
procedures, Buprenorphine was administered subcutaneously at a dose of 0.05mg/kg. Subsequently, a 2.5% Xylocaine topical ointment was applied to the suture site. Additional doses of Buprenorphine were given via intraperitoneal injection every 8-12 hours after surgery for the first 48 hours. At selective time points, mice were sacrificed by an overdose of isoflurane and tissues were perfusion-fixed with 4% paraformaldehyde (PFA) in phosho-buffered saline (PBS). For each mouse model of aneurysm, the maximum external diameter of the infrarenal aorta was measured using a digital caliper (VWR Scientific, Radnor, PA) prior to treatment (initial measurement) and at the time of tissue harvest (final measurement). Aortic expansion (% Aortic Dilation) was determined by aortic expansion relative to pre-treatment diameter ((Final measurement – initial measurement)/Initial measurement)*100. Anurysm was defined as a 100% increase in aortic diameter.

All experiments were conducted in accordance with experimental protocols that were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison (Protocol M02284).

Elastase model: Male Thbs1^-/- and C57BL/6 mice (Thbs1^+/+) 12 weeks old were subjected to aneurysm induction with luminal infusion of porcine pancreatic elastase as described previously 3,4. The aorta was isolated from the renal vein to the iliac bifurcation and occluded with silk suture proximally and distally of the isolation points. Then, an aortotomy was made with a 30-gauge needle and a catheter was inserted and secured with silk ligature. Heat-tapered polyethylene tubing (IN-10, ROBOZ, MD) was introduced through the aortotomy and secured with a tie. This catheter was used to perfuse the artery with 0.45 U/mL type I porcine pancreatic elastase saline solution for 5 min (E-1250, Sigma, St. Louis, MO). Control mice were similarly infused with the elastase solution.
that had been heated at 100°C for 15 min (heat-inactivated elastase). All animals were treated with elastase from the same lot. The aortotomy was closed with 11-0 suture after removal of the catheter.

Calcium phosphate (CaPO₄) model: Male 12-week-old, C57BL/6 mice were subjected to CaPO₄ treatment as described ⁵. Briefly, the infrarenal region of the abdominal aorta was isolated following a midline incision. A small piece of gauze soaked in 0.5M CaCl₂ was applied perivascularly for 10 min. The gauze was replaced with another piece of PBS-soaked gauze for 5 minutes. The Control mice received one treatment of PBS soaked gauze for 15 min.

Angiotensin II (AngII) model: The induction of AAA was carried out in male, 24-week-old, apolipoprotein E-deficient (ApoE⁻/⁻; n=8) mice with a C57BL/6 background from Jackson Laboratories (Bar Harbor, Me) as previously described ⁶. AngII (1000ng/kg per minute) or saline was administered subcutaneously by Alzet osmotic minipump (model 2004; Alzet, Cupertino, CA) for 28 days ⁷.

Morphometric Analysis and Immunohistochemistry

Tissues meant for immunohistochemical analyses were imbedded in optimal cutting temperature (OCT) Compound (Sakura Tissue Tek, Netherlands), and tissues meant for morphological analyses were processed for paraffin embedding. All frozen sections were cut to 5μm thick using a Leica CM3050S cryostat and paraffin sections were cut to 8μm thick using a Reichert-Jung 2050 SuperCut Microtome. Van Geison stains were carried out using Chromaview Van Gieson kit (Richard Allan Scientific, Kalamazoo, MI) according to provided protocol. Elastin integrity was evaluated using a semi-quantitative methodology described previously ⁴: (1, no elastin degradation or mild elastin degradation; 2, moderate; 3, moderate to severe; and 4, severe elastin
degradation)⁸,⁹. Each section was numbered and photographed at 10x and 20x magnification, maintaining their respective numbers. Then, an objective participant graded the photographs according to the aforementioned scale and recorded the grade with the section number.

Human aortic sample sections were stained with thrombospondin 1 (TSP1) antibody (1:300 dilution, Abcam) for immunohistochemistry.

OCT embedded murine arterial samples were permeabilized with 0.1% TritonX for 10 minutes at room temperature. Non-specific sites were blocked using 5% bovine serum albumin (BSA), 3% normal donkey serum in Tris-buffered Saline and Tween 20 (TBS-T) for 1 hour at room temperature. Primary antibodies included: anti-TSP1 (Invitrogen, CA, 1:300); anti-TSP2 (Biorbyt, CA, 1:300); anti-IL6 (1:100), anti-MCP-1 (1:100), anti-Neutrophil (NIMP-R14, 1:100), and anti-CD3 (1:100) from Santa Cruz Biotech (Santa Cruz, CA); anti-CD68 (AbD Serotec, Kidlington, UK, 1:200); anti-MOMA2 (Abcam, 1:300); and phosphorylated Smad3 (Cell Signaling Technology, 1:100). 4’6-diamidino-2-phenyl-indole, dihydrochloride (DAPI, Invitrogen, CA) was used to detect nuclei. Staining was visualized with a Nikon Eclipse Ti inverted microscope system and digital images were acquired using a Nikon DS-Ri1 digital camera. Microscope exposure settings were held contstant for all images taken amongst experimental groups sets. Quantification of stains was performed in a manner consistent with previously described methods¹⁰ using Image J Software as provided by the National Institutes of Health. Data quantification was performed using at least 3 sections per artery.

ELISA
Enzyme-linked immunosorbent assay was used to detect TSP1 secreted by mouse abdominal aortic aneurysm using mouse TSP1 ELISA kit (BD Biosciences, San Diego, CA) according to the manufacturer’s protocol.

**Real-Time PCR Analysis**

Total RNA was isolated from abdominal aortic aneurysm tissue by using Trizol reagent (Invitrogen, CA) according to the manufacturer’s protocol. cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA) on a Veriti 96-well Thermal Cycler (Applied Biosystems, CA). The primer sequences for THBS1 were forward, 5’-TGGCCAGCGTTGCCA-3’, and reverse, 5’-TCTGCAGCACCACCTGGA-3’. Amplification was detected using SYBR Green PCR Master Mix (Applied Biosystems, CA). Real-time PCR was carried out using a 7500 Fast Real-time PCR System Machine (Applied Biosystems, CA). RQ value, where \( RQ = \frac{(E_{\text{target}} \Delta C_{P\text{target}}(\text{control-sample}))}{(E_{\text{reference}} \Delta C_{P\text{ref}}(\text{control-sample}))} \), the reference gene was GAPDH, and CP is defined as a ‘crossing point’, was used to compare expression of target cytokines.

**Cell Isolation and Culture**

The murine macrophage cell line RAW264.7 was obtained from American Type Culture Collection (ATCC, Manassas, VA). Peritoneal macrophages were isolated 24 hours after the thioglycollate injection using a previously described method. Briefly, animals were anesthetized and the skin and fur was removed from the peritoneum. 3mL of phospho-buffered saline (PBS) was then injected to the cavity, followed by gentle agitation. The PBS was then collected from the cavity using a syringe, and cells were washed twice with additional PBS before use.

**Adoptive Transfer of Bone Marrow derived Macrophages**
Bone marrow mononuclear (BMM) cells were cultured using a method modified from a previously published by Zhang et al. \(^{12}\) Bone marrow was flushed from long bones and washed with PBS. Bone marrow was then suspended in 10% DMEM supplemented with 10% L-cell conditioned media (LCCM). LCCM media was collected from L929 cells cultured in T-75 cm\(^2\) filter cap flasks in DMEM for 7 days and filtered through 0.2µm \(^{13}\). 3 days after harvest, all non-adherent cells were removed and remaining cells were washed. BMM were then trypsinized from culture, labeled with Cell Tracker Green 5-chloromethylfluorescein diacetate (CMFDA; Life technologies, C7025), washed with PBS, and resuspended to 1.25×10\(^7\) cells/mL. 200µL of cell suspension was injected intravenously (IV) into the tail vein every three days beginning one day after surgery.

### Murine Bone Marrow Transplant

Bone marrow transplant was executed using a protocol adapted from Zhang et al (14). C57B/6 mice (8 weeks of age) were lethally irradiated with a cesium source for a single dose of 8 Gy. To establish the method, we used bone marrow cells harvested from long bones of GFP+ mice in C57B/6 background. 24 hours after irradiation, about 1 million donor bone marrow cells suspended in 150µl were delivered to the irradiated recipients through a retro-orbital injection. Peripheral blood was collected 6 weeks after cell injection and analyzed for the presence of GFP+ cells by flow cytometry analysis. Supplemental Figure 10A shows a dominant presence of GFP+ donor cells in peripheral blood of the chimeric.

To study the functional importance of TSP1 in circulating or arterial resident cells, we conducted \(Thbs1^{-/-}\) to wildtype (C57B/6-CD45.1, NCI) or vice versa bone marrow transplantations. Our \(Thbs1^{-/-}\) mice are also in the C57B/6 background, but carry the CD45.2
allele, which allows us to distinguish donor from recipient cells by using antibodies specifically to CD45.1 or CD45.2 (Supplemental Figure 10B) (14). Briefly, blood cells, following elimination of erythrocytes with ACK buffer (Life Technologies), were stained with anti-mouse CD45.1-FITC and anti-mouse CD45.2-PE (Tonbo Biosciences) antibodies. Total CD45 was determined as the sum of CD45.1 and CD45.2 positive cells, and percent of donor CD45 allele was determined as the number of either CD45.1 or CD45.2 divided by total CD45. Flow cytometric data were collected on a BD FACS Calibur Flow Cytometer equipped with a Cytek 633 laser (Freemont, CA) and analysis was performed using Flow Jo software (TreeStar, Inc.). Successful bone marrow reconstitution, defined here as 95% donor CD45 allele, was achieved by 6 weeks after transplantation (Supplemental Figure 10C). After confirming transplant success, recipient mice underwent aneurysm induction as described above.

**Peritoneal Inflammatory Model**

To determine the origin and evaluate the mobility of inflammatory cells, allogeneic peritoneal Thbs1+/+ or Thbs1−/− macrophages were labeled in vitro with CMFDA. 0.2ml of Thbs1+/+ or Thbs1−/− cell suspension (2.5×10⁶ cells) was injected to C57BL/6 mice by tail vein at the same time as an injection of 4% thioglycollate (BD Biosciences) intraperitoneal (IP) injection. Twenty four hours after, peritoneal macrophages were isolated and subjected to flow cytometry analysis.

**Flow Cytometry**

Isolated cells were washed with cold PBS and then suspended in ice cold fluorescence-activated cell sorting (FACS) buffer (0.5% BSA in TBS-T) and labeled with both propidium iodide (PI; Biolegend,)

and allophycocyanin (APC)-conjugated CD11b (Tonbo, CA) and incubated in the dark at room temperature for 30 minutes prior to analysis. Cells were washed with additional FACS buffer. Flow cytometric data was collected on a BD FACS Calibur Flow Cytometer equipped with a Cytek 633 laser (Freemont, CA) and analysis was performed using FlowJo software (TreeStar, Inc.). To determine the percentage of infiltrating, donor-derived monocytes, the monocyte population was identified as CD11b+ and PI-. The percent of monocytes that were also CMFDA+ was identified as the donor-derived monocytes. Experiments were performed in duplicate and the mean of 3 mice per genotype calculated.

**Ex vivo Adhesion Experiment**

Bone marrow derived mononuclear cells were labeled with CMFDA and applied to aortic vessel explants as previously described. Briefly, C57BL/6 male mice underwent the elastase model procedure as described above. The treated portion of each aorta was harvested 3 days after elastase-treatment, segmented, and incubated in vitro with CMFDA-labeled Thbs1+/+ or Thbs1−/− mononuclear cells (2×10^5 cells in 100 μL of medium) for 4 hours at 37°C. Aortic sections were washed and embedded in optimal cutting temperature (OCT) media and sectioned to 8 μm sections. Fluorescent cells present in each section were counted and recorded.

**Western Blot Analyses**

Cells were lysed in radioimmunoprecipitation (RIPA) buffer (50 mMTris, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, and 10 μg/ml aprotinin). 20μg of protein from each sample were separated on 10 % SDS-PAGE gels and then transferred to nitrocellulose membranes. Protein expression was confirmed by immunoblotting with the following antibodies: total FAK, p-FAK397, p-FAK577 and β-actin (Cell
Signaling, Boston, MA). Primary antibody incubation was carried out overnight with gentle agitation at 4°C, followed by 1 hour room temperature (RT) incubation with appropriate, horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA). Labeled proteins were visualized with an enhanced chemiluminescence system (Perkin-Elmer, Boston, MA).

**Cell Adhesion Assay**

96-well-plate was coated with fibronectin (FN, 10 μg/ml, Invitrogen ) at 4°C over night. Cell suspension was adjusted to 4×10^5 cells/ml. The construction and expression of TSP1 constructs including NoC (N-terminal domain (N), oligomerization sequence (O), and procollagen molecule (C)), DelNo (N-terminal domain and oligomerization sequence deleted), and DelN (N-terminal domain deleted) were as previously reported. The CD47 agonist peptide 4N1K (KRFYVVMWKK) and its control 4NGG (KRFYGGMWKK) were synthesized at University of Wisconsin Biotechnology Center. Constructs and peptides were used at 50nM, recombinant TSP1 (R&D Systems, MN) was used at concentrations between 1 and 50nM. 100μl cell suspension added to each well and incubated at 37°C for 45 minutes. Washed 3 times with PBS and fixed 3 minutes with 4% paraformaldehyde (PFA). Cells were then stained with Crystal Violet (5mg/ml in 2% Ethanol, Sigma) for 10min. Wells were washed with PBS 3 times and then turned upside down to dry completely. Each well was then incubated in 2% SDS 30 minutes at RT, plate was read on a FlexStation 3 (Molecular Devices, CA) at 550nm.

**Transwell Migration Assay**

RAW267.4 or bone marrow monocytic cells were starved 24 hours prior to assay set up. Cells were lifted and counted to adjust to 1×10^4 cells/200μl. TSP1 domain constructs or peptides (described
above), recombinant TSP1 (R&D Systems, MN), or TGFβ (5ng/mL; R&D Systems) were added to the cell suspension and total mixture was placed in a 5µm pore transwell insert (Corning Inc, Wilkes Barre, PA). 0.5% FBS medium containing chemotactic agents PDGF (5ng/mL; R&D Systems), MCP-1 (100 ng/mL; R&D Systems), and TGFβ (2.5 ng/mL; R&D Systems) was placed in the bottom of standard 24 well plate and the transwell inserts were added to appropriate wells. Following 6 hour incubation at 37°C, inserts were removed and washed with PBS, fixed with ice cold 70% Ethanol and stained with hematoxylin for nuclei visualization. The mean value of migrated cells counted in eight high-power fields per membrane was used as a measurement of migration.

**Statistical Analysis**

Values were expressed as mean ± standard deviation (SD). Experiments were repeated at least three times unless stated otherwise. Differences between 2 groups were analyzed by Student’s t test after the demonstration of homogeneity of variance with an F test. One-way ANOVA analysis was followed by Bonferroni’s test to adjust for multiple comparisons. Values of P<0.05 were considered significant. Statistical analysis was done with GraphPad Prism 5 (GraphPad Software Inc.).


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Supplemental Figure I. Characterization of aneurysm induced by elastase infusion. A) Representative photos of treated aortas 14 days after surgery; scale bar=5mm. B) Representative Van Gieson stains for elastin integrity in aortic tissues harvested 14 days after surgery, scale bar=100µm. C) Representative immunofluorescence stains for macrophages (CD68, green, top panels; Scale bar=100µm) and apoptosis (TUNEL, red, lower panels; Scale bar=200µm). Nuclei identified by DAPI (blue). D) Temporal graphical aortic dilation following surgery. Treatment effect *p<0.0001.
Supplemental Figure II. TSP1 co-localizes with a macrophages marker, to a lesser extent with smooth muscle cells or neutrophils. Two similar representative confocal images of human AAA tissues are provided. (A) Co-stain with macrophage marker CD68 (green), thrombospondin-1 (TSP1, red), and nuclei (DAPI, blue). (B) Co-stain with neutrophil marker myeloperoxidase (MPO, green), TSP1 (red), and DAPI (blue). (C) Co-stain with smooth muscle cell marker myosin heavy chain 11 (MHC), TSP1 (red), and DAPI (blue). Scale bar = 100µm.
Supplemental Figure III. Levels of TSP1 are elevated in two additional models of murine aneurysm. Representative images of immunohistochemical stains for TSP1 in (A) Angiotensin II (AngII)-treated ApoE<sup>-/-</sup> mice, control treatment (saline, far left), and (B) CaPO<sub>4</sub>-treated C57Bl/6 mice arteries, control treatment Phospho-buffered saline (PBS, far left). Scale bar 200µm (10x) and 50µm (20x).
Supplemental Figure IV. Untreated Thbs1<sup>+/+</sup> and Thbs1<sup>-/-</sup> arteries appear similar histologically. TSP2 expression (top row), hematoxylin and eosin (H&E) stains (center row), and elastin integrity (Van Gieson, bottom row). Scale bar 200µm (TSP2 and H&E) and 100µm (Van Gieson).
Supplemental Figure V. *Thbs1*^+/−^ mice are resistant to aneurysm induction by CaPO4 model. (A) Representative images of wildtype (*Thbs1*^+/+) and *Thbs1*^−/−^ arteries treated with PBS (control) or Calcium phosphate (CaPO4) 14 days after surgery. Scale bar 2mm. (B) Graphic depiction of aortic dilation (%Change in aortic diameter) in PBS- (grey square) or CaPO4- (grey circle) treated *Thbs1*^+/+^ arteries and PBS- (grey triangle) or CaPO4- (grey wedge) treated *Thbs1*^−/−^ arteries. Red dotted line designates aneurysmal formation (100% change in aortic diameter); *p*<0.05 as compared to CaPO4-treated *Thbs1*^+/+. (C) Representative images of immunohistochemical stains for macrophages (CD68, green) in elastase treated arteries, nuclei stained with DAPI (blue). Scale bar 100µm. Quantification at right, shown as % Positive Cells. *p*<0.05. (D) Representative stains for elastin degradation (Van Gieson), scale bar 100µm. Quantification at right, *p*<0.05.
Supplemental Figure VI. Infiltration of inflammatory cells to Thbs1^{+/+} and Thbs1^{-/-} arteries 7 days after treatment with elastase. Monocytes and Macrophages (MOMA2), Neutrophils (NIMP-R14), (C) T lymphocytes (CD3). Scale bar 100µm (10x) and 50µm (20x). Quantification for each inflammatory cell shown at right of images as number of infiltrating inflammatory cells counted and expressed as %Positive Cells ((number of positive cells / number of nuclei)*100). *p<0.05.
Supplemental Figure VII. Inflammatory cytokine expression in *Thbs1<sup>+/+</sup>* and *Thbs1<sup>−/−</sup>* arteries 7 days after treatment elastase. Representative immunohistochemical stains for interleukin 6 (IL6) or monocyte chemoattractant protein 1 (MCP1) in *Thbs1<sup>+/+</sup>* and *Thbs1<sup>−/−</sup>* arteries 7 days after treatment with elastase. Scale bar 200µm for 10x and 100µm for 20x.
Supplemental Figure VIII. TGFβ activation is not changed in Thbs1−/− arteries. (A) Representative immunohistochemical stains for phosphorylated Smad3 in Thbs1+/+ and Thbs1−/− mice 7 days after treatment with inactive elastase (control) or elastase. Scale bar 100µm; inlay 10x scale bar 100µm. (B) Quantification of phosphorylated Smad 3 (pSmad3), shown as % Positive cells ((number of pSmad3+ cells/nuclei)*100). *p<0.05. (C) Chemotaxis toward MCP1 was assessed for Thbs1−/− and Thbs1+/+ BMM supplemented with 5ng/mL TGFβ (+) or solvent (-). Results shown as number of migrated cells per high power field (HP). *p<0.05.
Supplemental Figure IX. Schematic of adoptive transfer experimental design.
Aneurysm was induced at day 0 (Elastase, red arrow). BMM cells were injected on days 1, 4, 7, and 10. Sacrifice and analysis was carried out on day 14 (red arrow).
Supplemental Figure X. Flow cytometry results representing the bone marrow transplant model. (A) Circulating blood from C57B/6 (Recipient) mice receiving GFP+ (Donor) bone marrow expresses GFP 6 weeks after transplant (C57B/6+GFP). (B) Flow cytometry confirms that the wildtype C57B/6-CD45.1 (CD45.1(WT)) mice express CD45.1 allele and Thbs1-/- mice express the CD45.2 allele. (C) Representative flow cytometry results from circulating blood confirming success of the bone marrow transplant model.
Supplemental Figure XI. Bone marrow transplant procedure does not alter aneurysm expansion. Results of a bone marrow chimera model with C57B/6-CD45.1 wildtype (CD45.1(WT)) and Thbs1 knockout (Thbs1-/-) mice. (A) Graphic representation of aneurysm expansion 14 days after elastase-induced aneurysm.; n=3 WT, n=2 Thbs1-/- (B) Representative images of arterial expansion at the time of expansion measurement; scale bar = 5mm. (C) Quantitative evaluation of macrophage (CD68+) infiltration; *p<0.05. (D) Representative images of macrophage (CD68+) infiltration, counterstain with DAPI (blue). L indicates lumen. (E) Representative elastin stains. Scale bar= 200um in 10x images, 100um in 20x images.