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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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.

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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*$^{-/-}$ monocytic 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

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 [1, 2]. 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 5cm for women), asymptomatic AAAs are left untreated due to the low benefit-to-risk ratio of surgical interventions in this patient population [3, 4]. 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 [5–8]. 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 [9–11]. Additionally, manipulation of MMPs, either through pharmacological inhibition or through genetic deletion inhibits aneurysm development in mice [12–14]. One such pharmacological inhibitor, doxycycline, is currently being tested in multi-centered clinical trials for the treatment of small aneurysm [15–17].

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 [11, 13, 18, 19] and pro-inflammatory cytokines [20–22]. 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 [23–25].

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 [26]. TSP1 modulates a wide range of biological functions including cell adhesion [27], inhibition of angiogenesis and endothelial cell proliferation [28], and activation of latent transforming growth factor-β (TGF-β)[29, 30]. Additionally, TSP1 acts as a chemo-attractant that impacts various inflammatory cells [27], and enhanced levels of TSP1 are reported in conditions associated with tissue damage and inflammation [31, 32]. 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 [33]. 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 [34]. More recently, Csányi et al showed that TSP1, through its interaction with CD47, stimulates production of reactive oxygen species and attenuates vasodilatation [35]. Conversely, in the retina, TSP1 mediates pro-inflammatory microglia such that a lack of TSP1 is associated with a spontaneous increase in inflammatory mediators [36]. Further, TSP1 is involved in the maintenance of the immune privileged status of the ocular region [37].

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 [33, 38]. In general, high plasma TSP1 levels correlate positively with cardiovascular disease [39], however, serum levels of TSP1were reported to be negatively associated with AAA [40]. 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 [41]. 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 [42–44]. 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 *Apo*E$^{-/-}$ mice treated with Angiotensin II (Supplemental Figure IIIA) or in C57BL/6 mice treated with CaPO$_4$ (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 CaPO$_4$ 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 [26] (Supplemental Figure IV). Following aneurysm induction by elastase and CaPO$_4$ 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 CaPO$_4$ 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 [5, 17, 42, 45]. Elastase- or CaPO$_4$-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 [6, 46](Figure 2C, D and Supplemental Figure VC). Conversely, aortic samples harvested from *Thbs1*^{−/−} mice treated either with elastase or CaPO$_4$ 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 CaPO$_4$ 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 VD).

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 TSP1gene 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 VIIIA, 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 $39.9\pm0.2\%$ of the CD11b positive peritoneal-derived inflammatory cells were positive for CMFDA when $Thbs1^{-/-}$ BMM were used as donors as compared to $69.7\pm4.0\%$ 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 $85.8\pm7.5\%$. 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 *Thbs1* genotype of the bone marrow (Figure 5C–E).

TSP1 is required for optimal adhesion and migration of monocytic cells

Next, we examined whether *Thbs1* gene deficiency affects the ability of macrophages to adhere to matrix proteins in an *ex vivo* adhesion assay. Aortic rings were prepared from *Thbs1*$^{+/+}$ mice 3 days after aneurysm induction with elastase and incubated with CMFDA-labeled peritoneal macrophages from *Thbs1*$^{+/+}$ or *Thbs1*$^{-/-}$ mice. The number of *Thbs1*$^{-/-}$ macrophages that adhered to the aortic tissues was 62% less than that of the *Thbs1*$^{+/+}$ macrophages (Fig. 6A). Similarly, *Thbs1*$^{-/-}$ BMM showed reduced adhesion to fibronectin in an in vitro culture system (Fig. 6B). To exclude a possible indirect effect of embryonic gene deletion, we transiently silenced *Thbs1* expression in RAW264.7 cells using an siRNA specific to TSP1. Compared to a scramble control, *Thbs1* 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 *Thbs1* gene silencing in RAW264.7 (Fig. 6D).

The adhesive and migratory defects of Thbs1$^{-/-}$ 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 *Thbs1*$^{-/-}$ 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 *Thbs1*$^{-/-}$ mice (Fig. 7C). In fact, in the presence of 50nM recombinant TSP1, *Thbs1*$^{-/-}$ BMM demonstrated no functional differences from *Thbs1*$^{+/+}$ BMM in this 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 *Thbs1*$^{-/-}$ BMM. However, the CTD trimer (DelN) restored adhesion of *Thbs1*$^{-/-}$ 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 *Thbs1*$^{-/-}$ BMM to fibronectin. The 4NGG control peptide also produced a moderate, but significant, increase in adhesion. However, the 4N1K peptide fully restored *Thbs1*$^{-/-}$ BMM adhesion to the level of *Thbs1*$^{+/+}$ macrophages, and to a more significant degree than control 4NGG peptide (Figure 7C).

Recombinant TSP1 also dose-dependently rescued the chemotactic capability of *Thbs1*$^{-/-}$ 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 *Thbs1*$^{-/-}$ BMM toward MCP-1. 4NIK and its control 4NGG peptides did not alter the inhibited migration of *Thbs1*$^{-/-}$ BMM. One of the mechanisms by which TSP1 is expected to influence inflammation is through the activation of TGFβ [30]. Using an MCP-1-driven transwell migration assay, we evaluated the migratory capacity of *Thbs1*$^{+/+}$ or *Thbs1*$^{-/-}$ peritoneal macrophages in the presence or absence of supplemental TGFβ (5ng/mL). Similar to what we showed above with BMM cells, *Thbs1*$^{-/-}$ peritoneal macrophages migrated less efficiently as compared to *Thbs1*$^{+/+}$ peritoneal macrophages (Supplemental Figure VIIIC). Administration of TGFβ did not restore the migratory capacity of *Thbs1*$^{-/-}$ peritoneal macrophages. In fact, TGFβ 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 CaPO$_4$ application in C57BL/6 mice or following angiotensin II infusion in *Apo*E$^{-/-}$ mice. Histological and morphological examinations of elastase- or CaPO$_4$-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 [47, 48]. 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 [49, 50] and migration [28, 51] and induces apoptosis [52, 53]. In vascular SMCs, TSP1 has been shown to promote proliferation and migration [54]. 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 1 [9]. While oxidative stress has been well linked as an underlying pathogenic player in abdominal aortic aneurysm [5], 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 [44, 45, 55, 56].

Elevated levels of TSP1 in the vessel wall are observed in several other cardiovascular disorders including diabetes mellitus [57], atherosclerosis [58, 59], and ischemia-reperfusion injury [38, 60]. 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[61], whereas overexpression of TSP1 was found to negatively impact wound healing and vascularization in a wound healing model[62]. Early reports demonstrated that one characteristic of $Thbs1^{-/-}$ mice was a consistent and significant pulmonary inflammatory state[63]. 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 [33]. The authors attributed this pro-inflammatory phenotype to the diminished phagocytic ability of macrophages [33]. 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 [64] and the nuclear receptor peroxisome proliferator-activated receptor (PPAR)γ [65]. 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. [66, 67] 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 [68]. CD47 was initially identified for its association with $\alpha_v\beta_3$ 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 [69]. 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 [70]. Interestingly, our findings suggest that the CD47-binding capability of TSP1alone 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[71]. 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 [72]. 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 chemoattractant 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 [55, 73, 74]. However, knocking out MCP-1 (also called CCL2) has limited impact on aneurysm development, whereas knocking out its receptor, CCR2, significantly reduces aneurysm expansion [42, 45, 73], 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 [75]. In the case of atherosclerosis, the increased inflammation in the absence of TSP1 was found to be independent of TGFβ activation [33]. 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 [6]. 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[76]. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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)

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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.
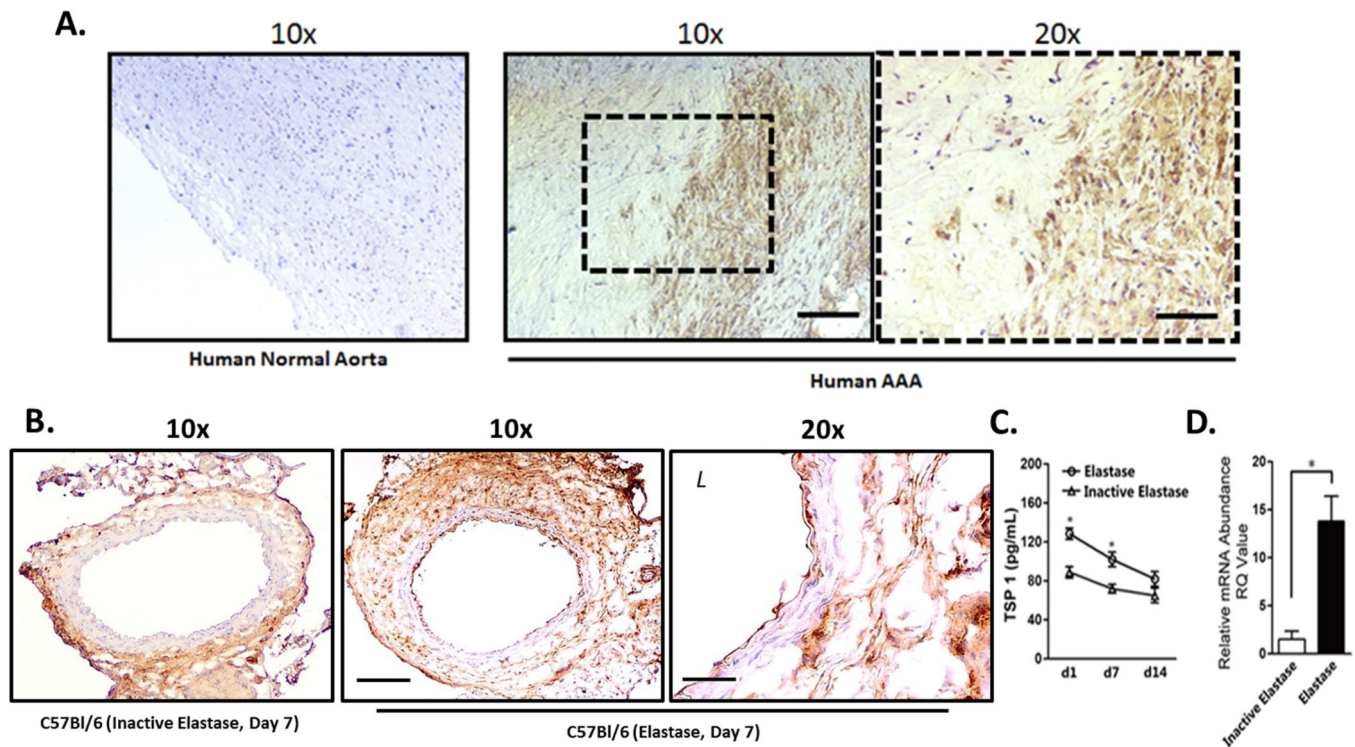
Figure 1. Thrombospondin 1 (TSP1) expression is increased in human and experimental aneurysm

(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. $*p<0.05$.
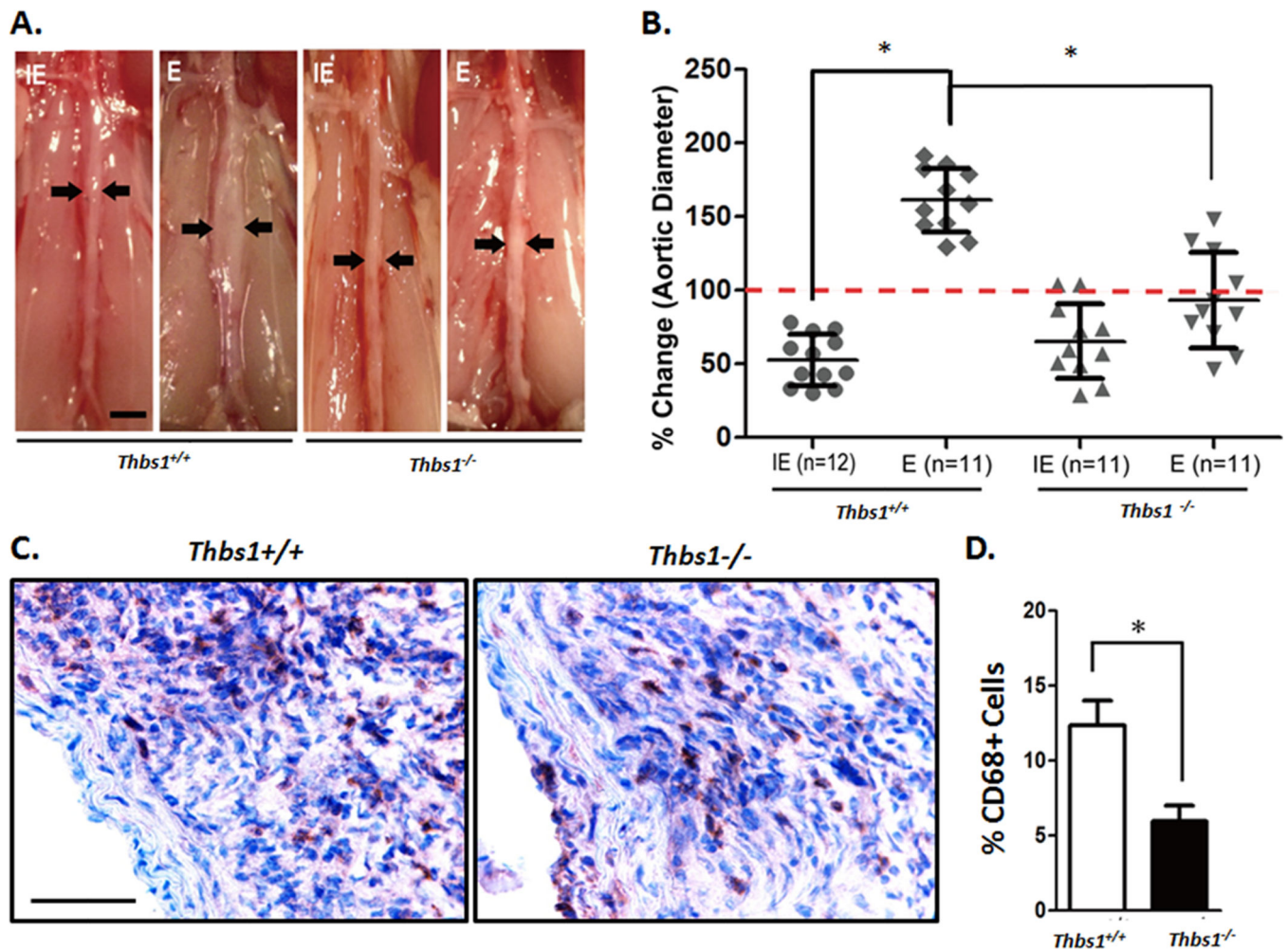
Figure 2. *Thbs1^{−/−}* mice are resistant to aneurysm induction

(A) Representative images of wildtype (*Thbs1^{+/+}*) and *Thbs1^{−/−}* 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^{+/+}* arteries and IE- (triangle) or E- (wedge) treated *Thbs1^{−/−}* arteries. Red dotted line designates aneurysmal formation (100% change in aortic diameter); *$p<0.05$ as compared to E-treated *Thbs1^{+/+}*. (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). *$p<0.05$.
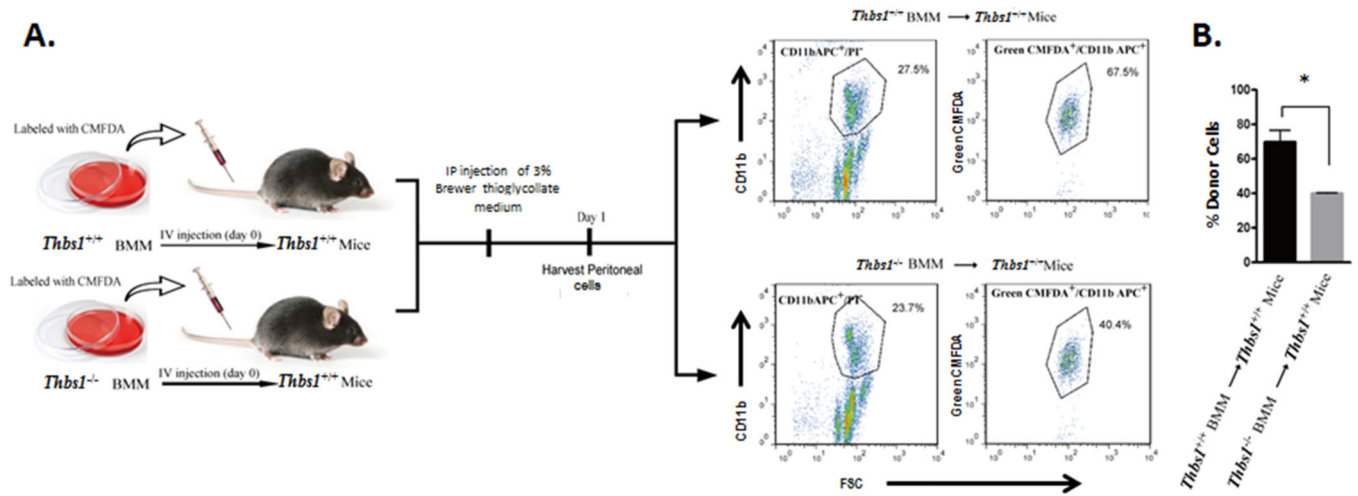
Figure 3. *Thbs1⁻/⁻* bone marrow mononuclear cells demonstrate reduced tissue infiltration capability in vivo

(A) Schematic of experimental design. From left: fluorescence (CMFDA)-labeled bone marrow mononuclear cells (BMM) from *Thbs1⁻/⁻* or *Thbs1⁺/⁺* mice were injected via tail vein to *Thbs1⁺/⁺* 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). *$p<0.05$.
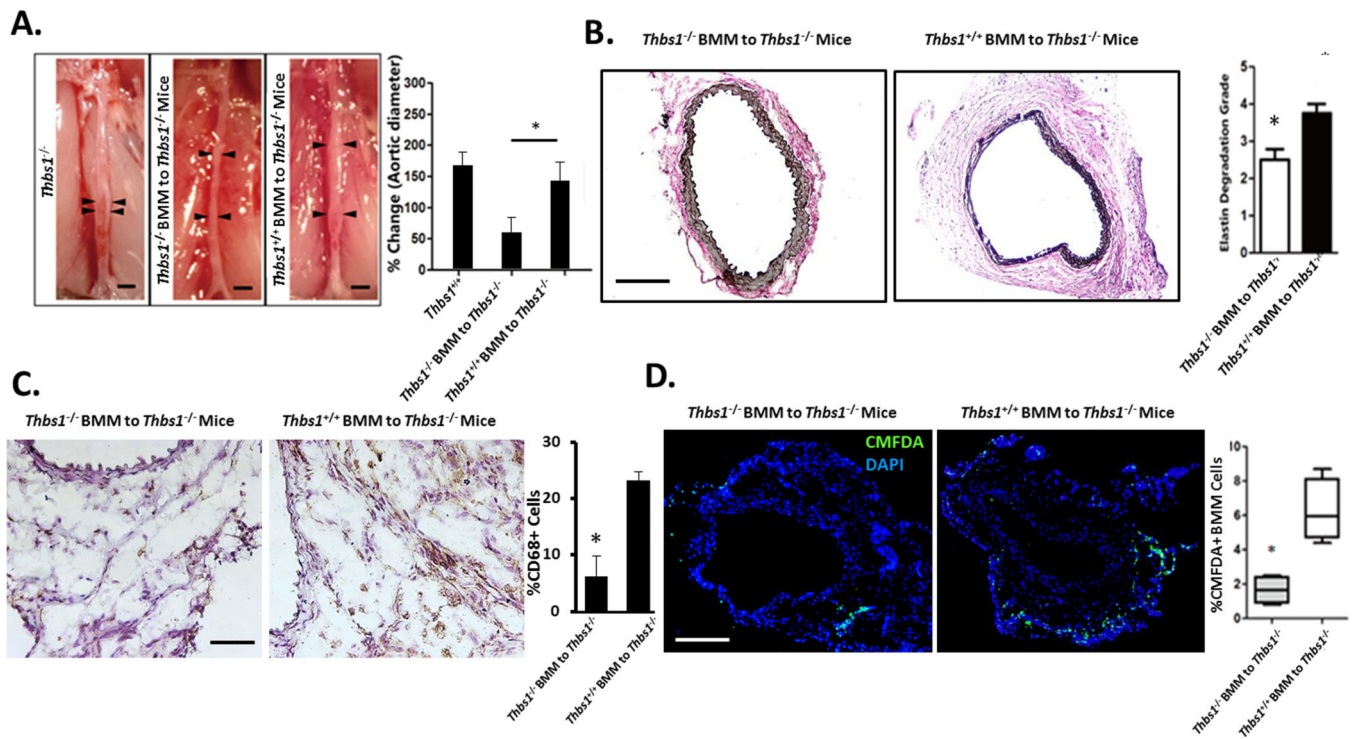
Figure 4. *Thbs1*$^{+/+}$ bone marrow mononuclear cells restore aneurysm phenotype in *Thbs1*$^{-/-}$ mice

(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.
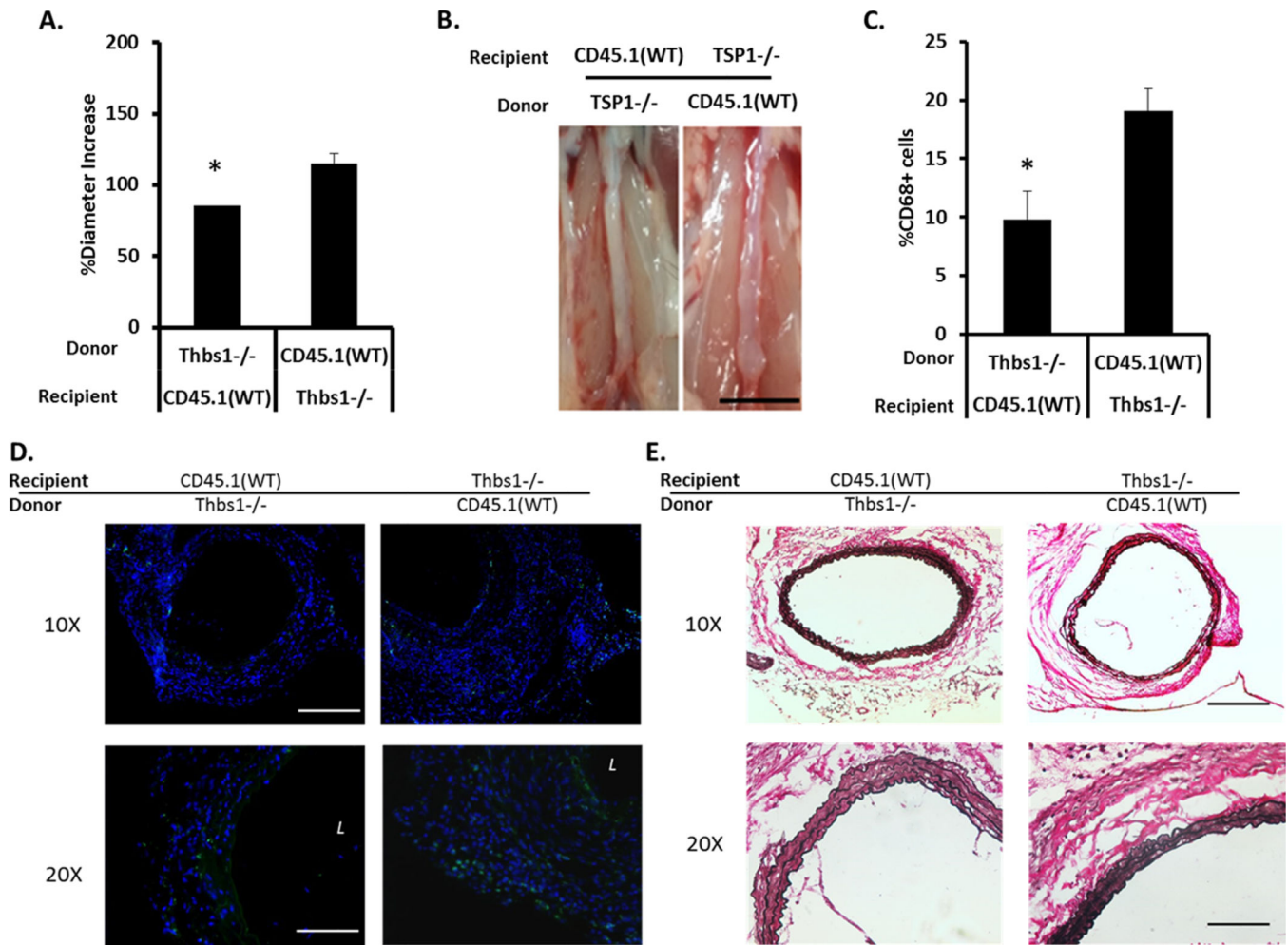
Figure 5. Thbs1 gene deficiency in bone marrow cells dictates aneurysm formation
Results of a bone marrow chimera model with C57B/6-CD45.1 wildtype (CD45.1(WT)) and
Thbs1 knockout (Thbs1−/−) mice. (A) Graphic representation of aneurysm expansion 14
days after elastase-induced aneurysm.; *p<0.05. (B) 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)
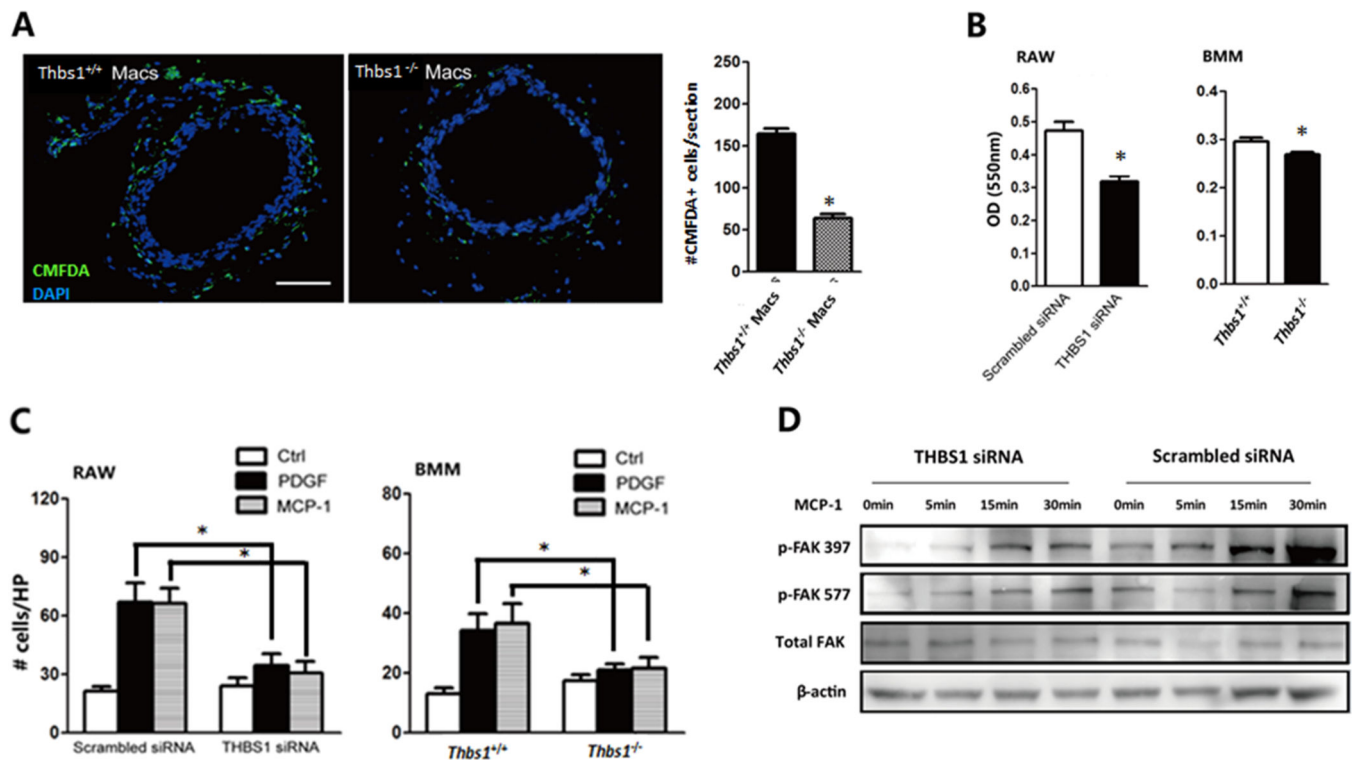Representative elastin stains. Scale bar= 200um in 10x images, 100um in 20x images.

Figure 6. Monocytes/macrophages lacking TSP1 display reduced adhesion and migration capability

(A) *Thbs1*$^{+/+}$ aortic rings were incubated with CMFDA-labeled (green) peritoneal macrophages (Macs), nuclei labeled with DAPI (blue). Scale bar 200μm. Adhered CMFDA + Macs from *Thbs1*$^{+/+}$ (black) or *Thbs1*$^{-/-}$ (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*$^{+/+}$ (white bar) or *Thbs1*$^{-/-}$ (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*$^{+/+}$ or *Thbs1*$^{-/-}$ 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. *$p<0.05$.
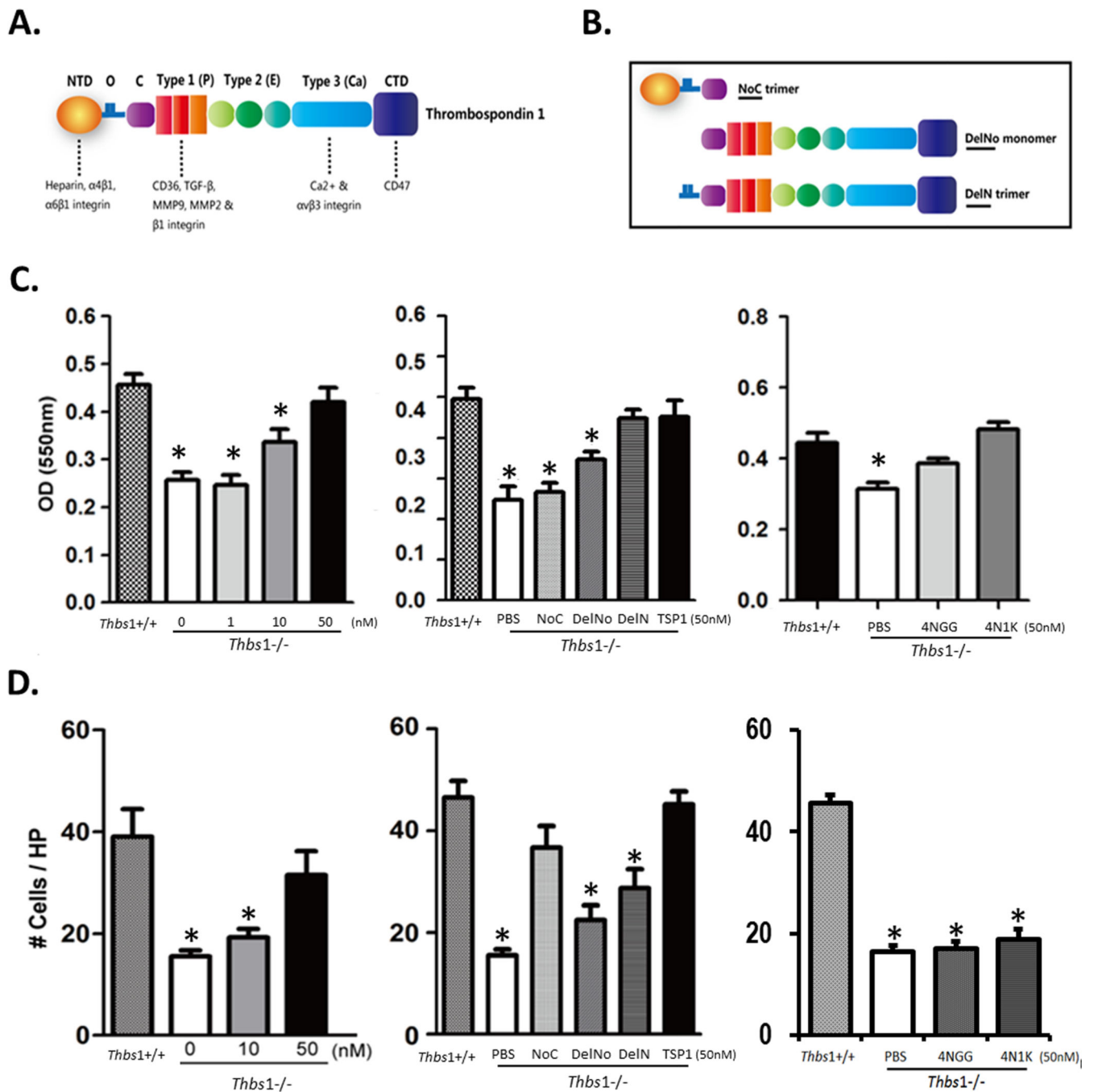
Figure 7. Rescuing adhesion and migration of *Thbs1*−/− BMM with exogenous TSP1 protein or fragments of TSP1 molecule

(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*+/+ or *Thbs1*−/− 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*$^{+/+}$). (center) *Thbs1*$^{-/-}$ BMM supplemented with solvent control (PBS), TSP1 fragments NoC, DelNo, DelN, or whole TSP1 (TSP1). *$p<0.05$ compared to wildtype (*Thbs1*$^{+/+}$). (right) Rescue with CD47-binding domain peptide 4N1K or control peptide 4NGG compared to solvent (PBS). *$p<0.05$ compared to wildtype (*Thbs1*$^{+/+}$). (D) In vitro chemotaxis toward MCP-1 using BMM from *Thbs1*+/+ and *Thbs1*−/ − 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*$^{+/+}$). (center) *Thbs1*$^{-/-}$ BMM supplemented with PBS, TSP1 fragments NoC, DelNo, DelN, or TSP1. *$p<0.05$ compared to wildtype (*Thbs1*$^{+/+}$). (right) Rescue with CD47-binding domain peptide 4N1K or control peptide 4NGG compared to solvent (PBS). *$p<0.05$ compared to wildtype (*Thbs1*$^{+/+}$).