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 specificed.

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 Hosptial, 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 *Thbs*1$^{-/-}$ 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 isoflourane and tissues were perfusion-fixed with 4% paraformaldehyde (PFA) in phospho-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)/Final measurement)*100. Aneurysm 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 *Thbs*1$^{-/-}$ and C57BL/6 mice (*Thbs*1$^{+/+}$), 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 100ºC 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 ($CaPO_4$) model: Male 12-week-old, C57BL/6 mice were subjected to $CaPO_4$ 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 $CaCl_2$ 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 0.5M PBS soaked gauze for 15 min.

Angiotensin II (AngII) model: The induction of AAA was carried outin male, 24-week-old, apolipoprotein E-deficient (*Apo*$E^{-/-}$; *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 Immunonohistochemistry

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 to that previously described [10] 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 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'-TCTGCAGCACCCCCTGAA-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= (E_{target} $^{\Delta CPtarget(control-sample)}$)/ ($E_{reference}$ $^{\Delta CPref(control-sample)}$), 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 [11]. 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 *Thbs*1$^{+/+}$ or *Thbs*1$^{-/-}$ macrophages were labeled *in vitro* with CMFDA. 0.2ml of T*hbs*1$^{+/+}$ or *Thbs*1$^{-/-}$ cell suspension (2.5×10^{6} 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 Flow Jo 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[14, 15]. 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 *Thbs*1$^{+/+}$ or *Thbs*1$^{-/-}$ mononuclear cells (2×10^5 cells in 100 μL of medium) for 4 hours at 37ºC. 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 4ºC, 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 4ºC 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 [16, 17]. 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 37ºC 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% Ethnol, 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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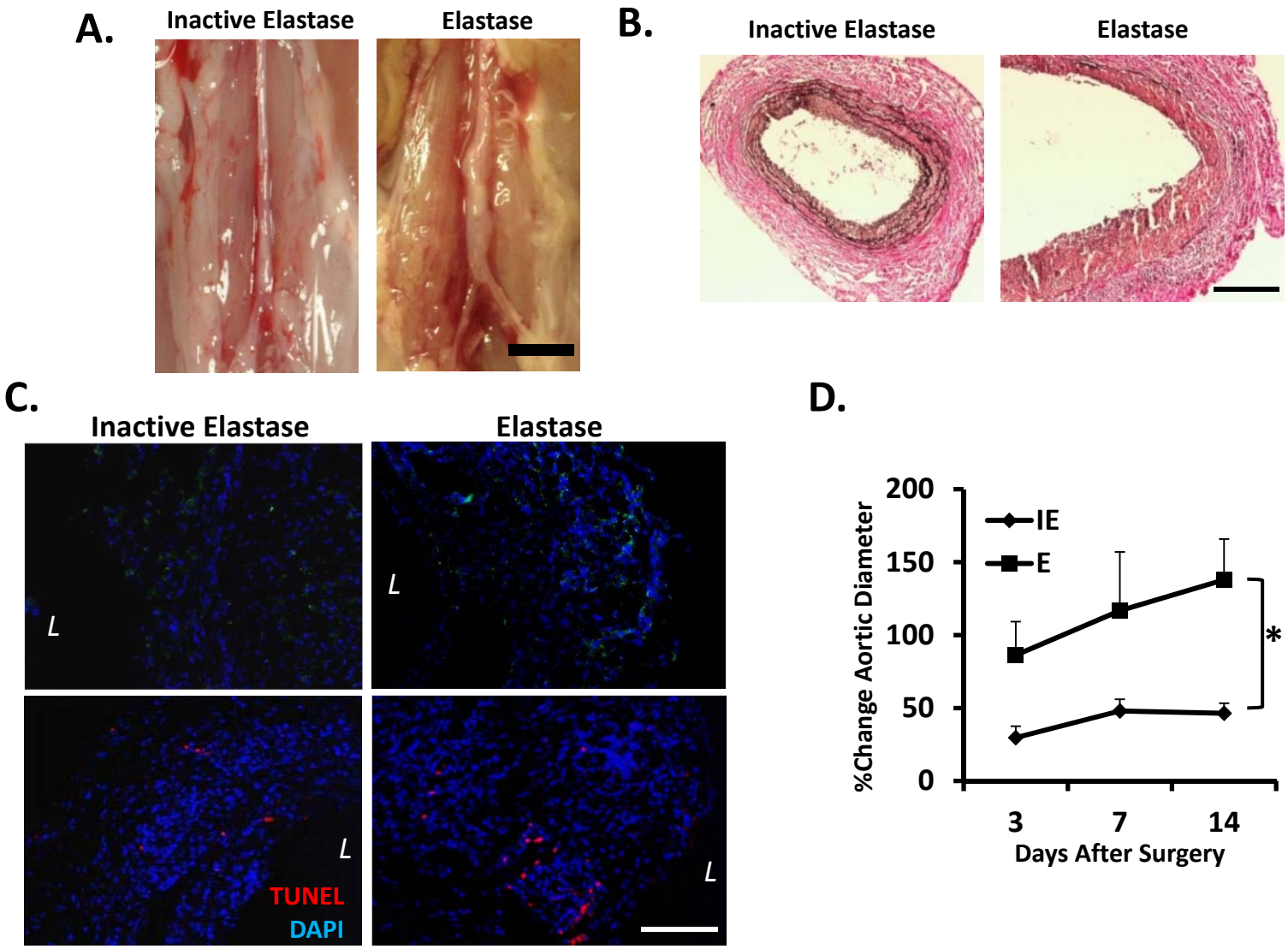
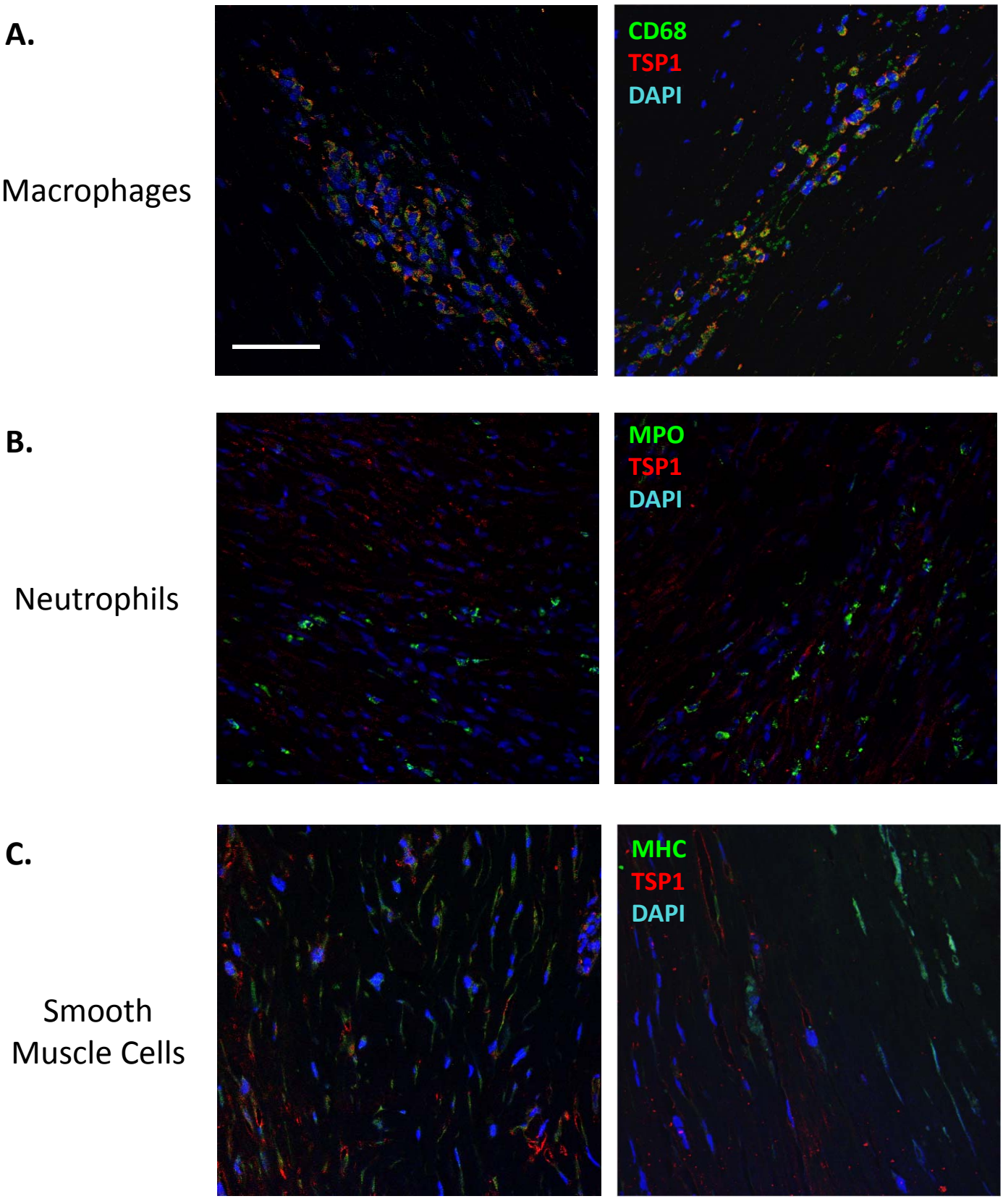
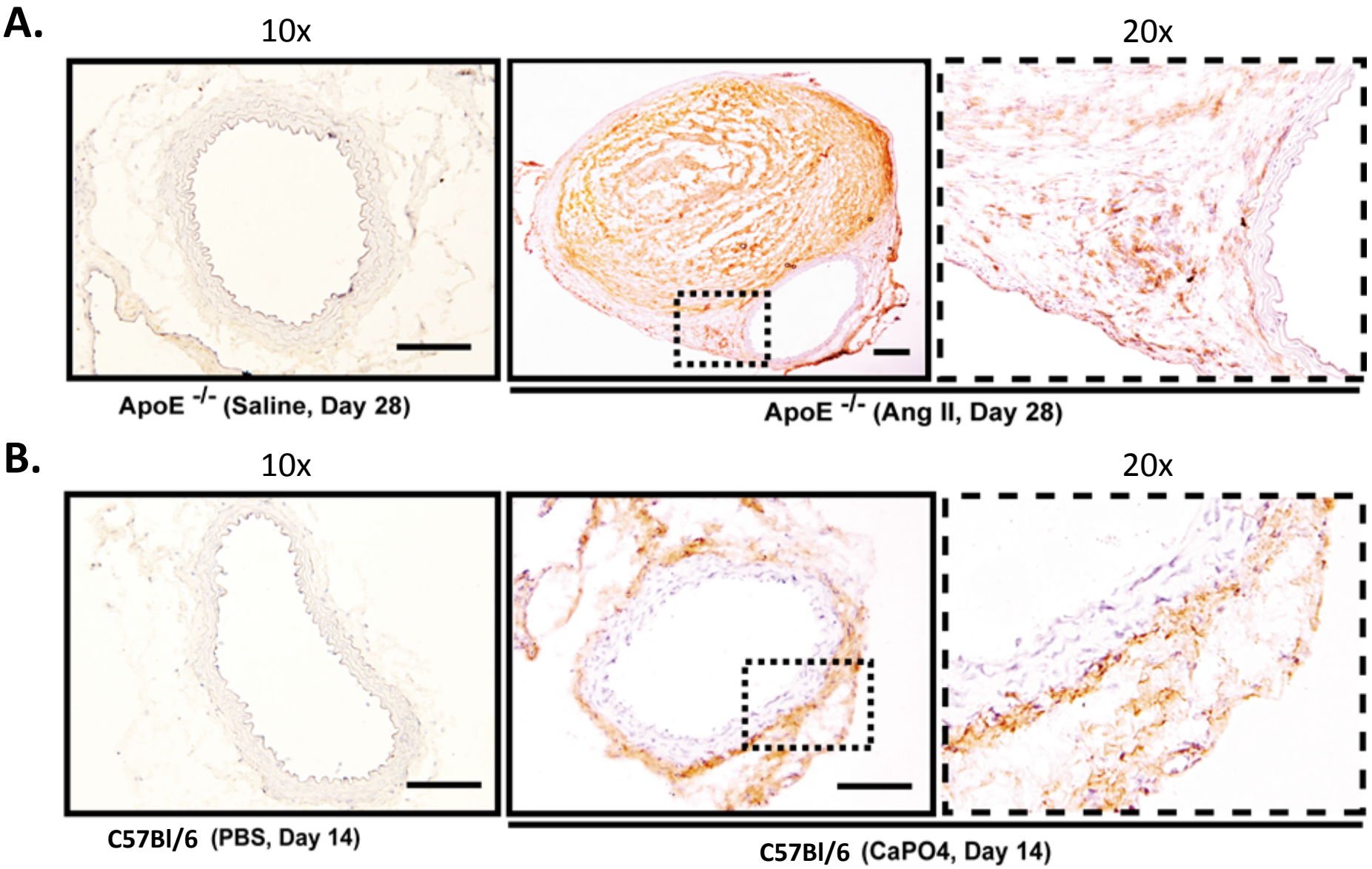


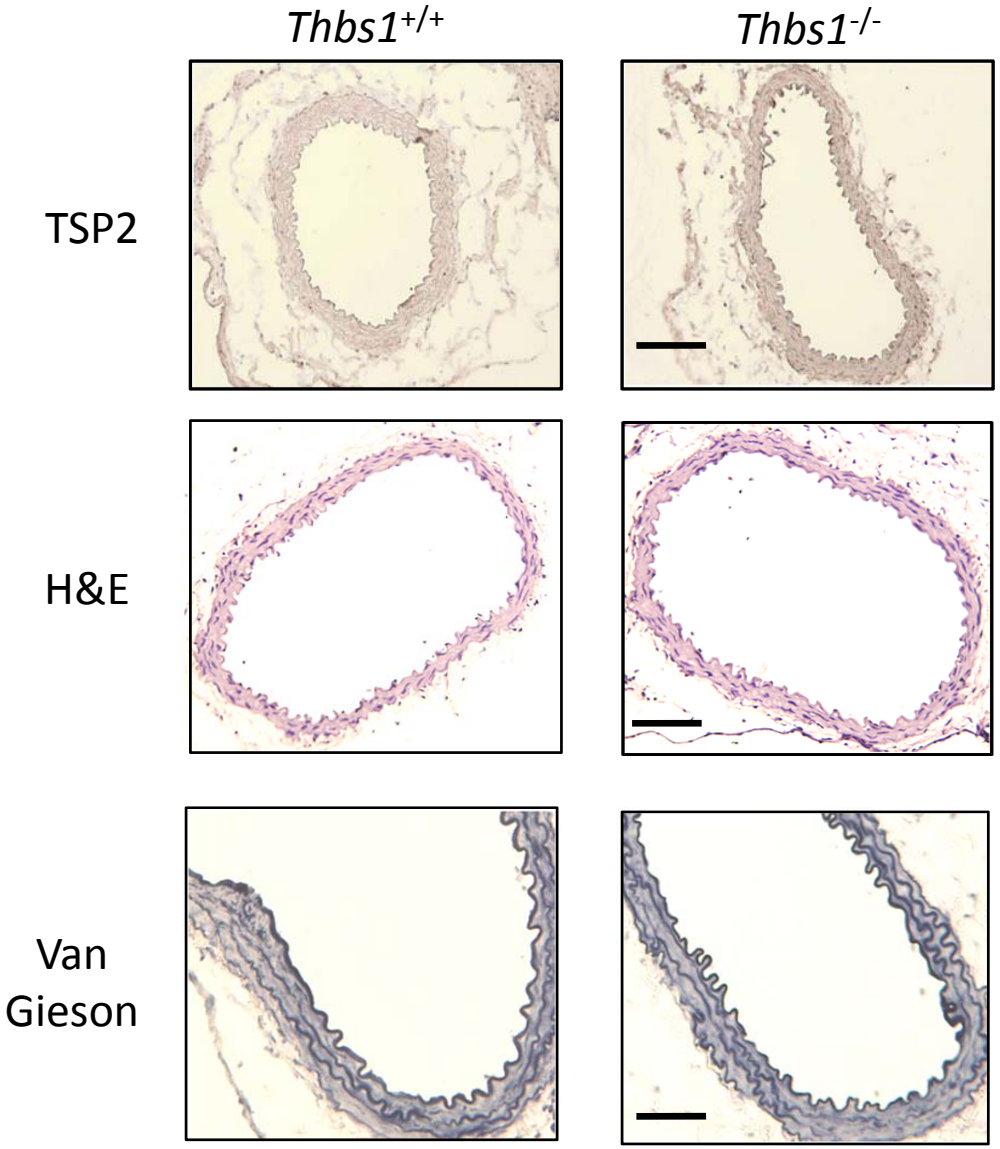
Supplemental Figure I. Characterization of aneurysm induced by elastase infusion. A) Representative photos of treated aortas 14 days after surgery; scale bar=5mm. B) Representative Van Gieson stains for elastin integrity in aortic tissues harvested 14 days after surgery, scale bar=100µm. C) Representative 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.



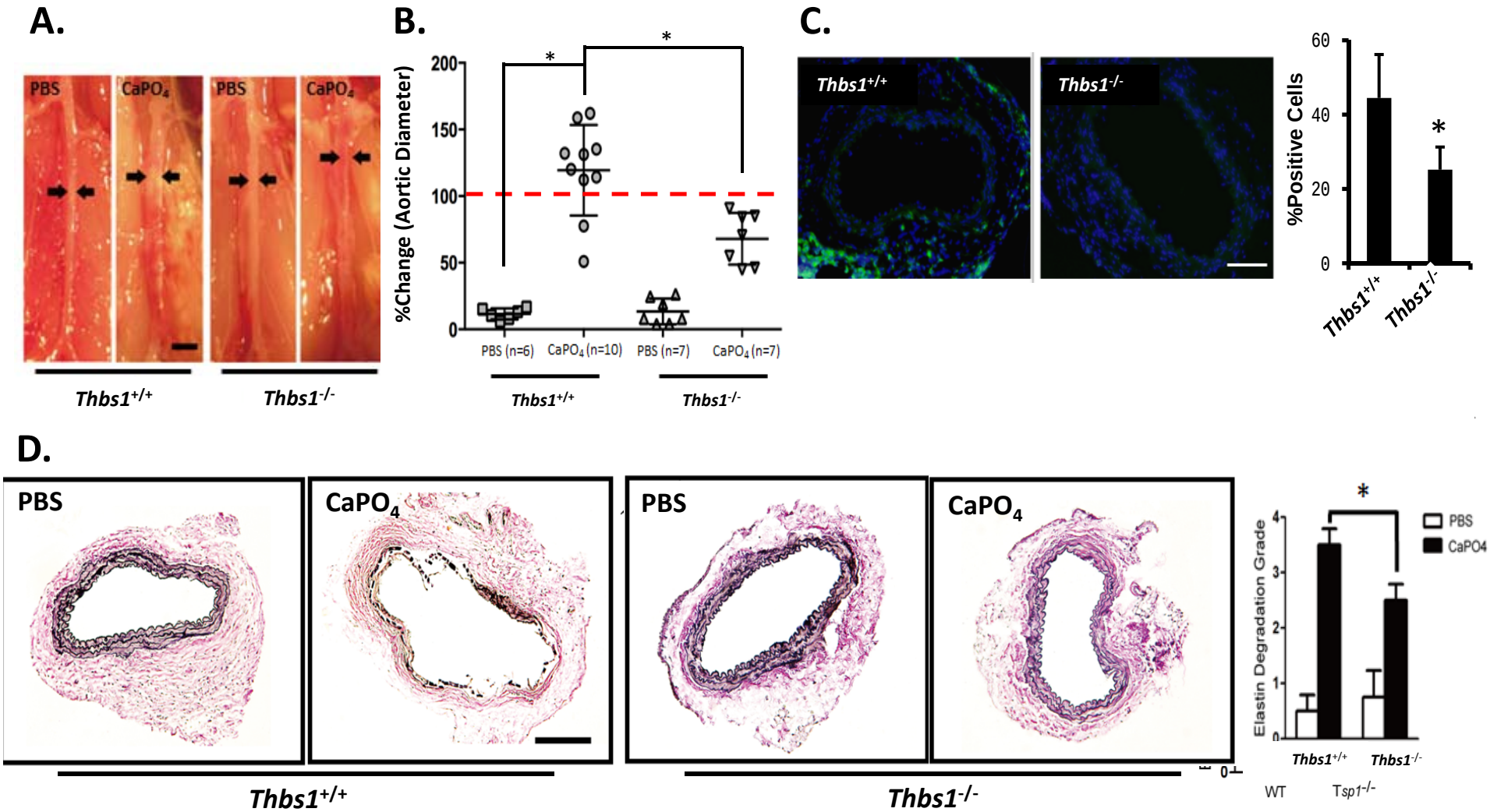
Supplemental Figure II. TSP1 co-localizes with a macrophages marker, to a lesser extent with smooth muscle cells or neutrophils. Two similar representative confocal images of human AAA tissues are provided. (A) Co-stain with macrophage marker CD68 (green), thrombospondin-1 (TSP1, red), and nuclei (DAPI, 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.



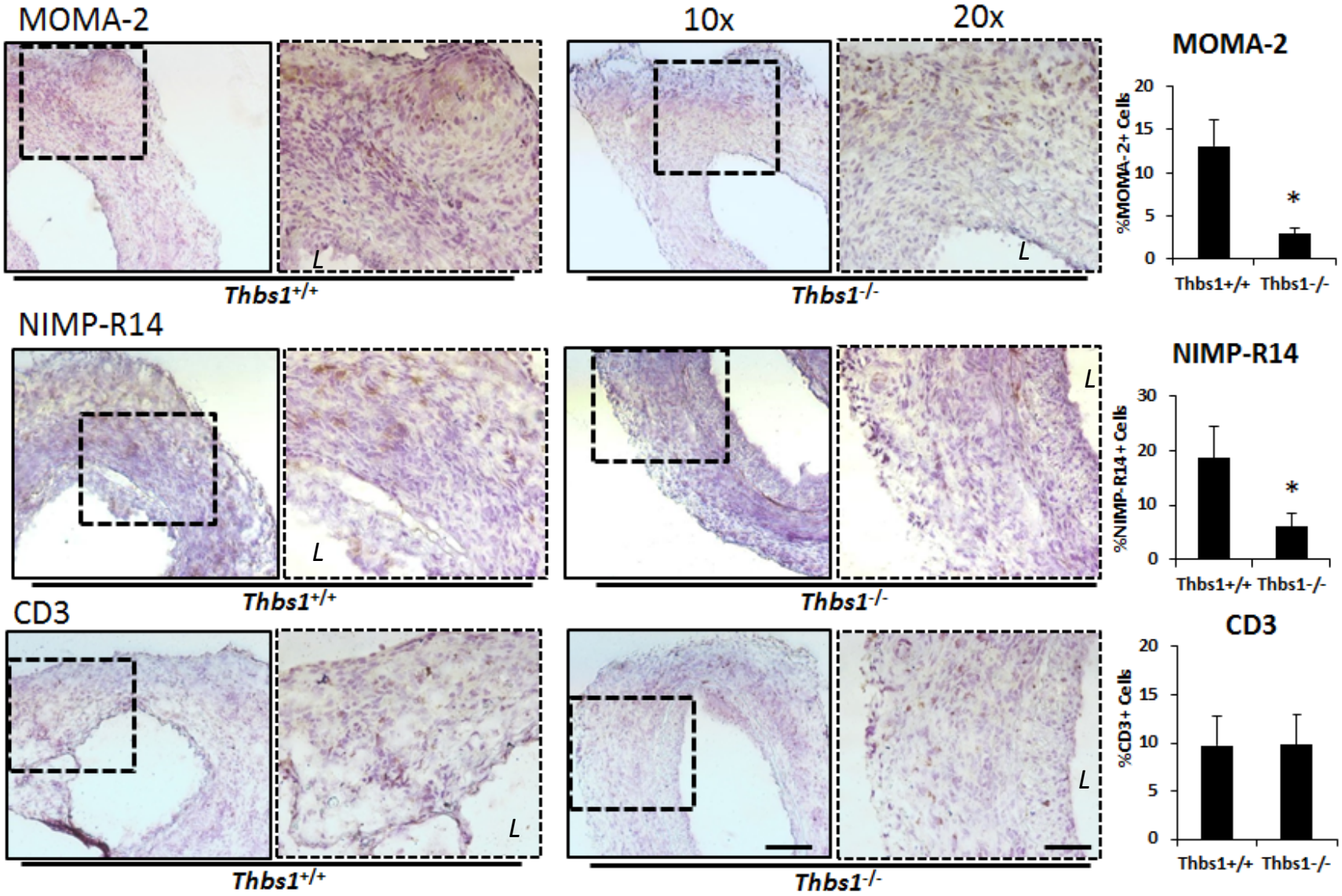
Supplemental Figure III. Levels of TSP1 are elevated in two additional models of murine aneurysm. Representative images of immunohistochemical stains for TSP1 in (A) Angiotensin II (AngII)-treated ApoE$^{-/-}$ mice, control treatment (saline, far left), and (B) $CaPO_4$-treated C57Bl/6 mice arteries, control treatment Phospho-buffered saline (PBS, far left). Scale bar 200µm (10x) and 50µm (20x).



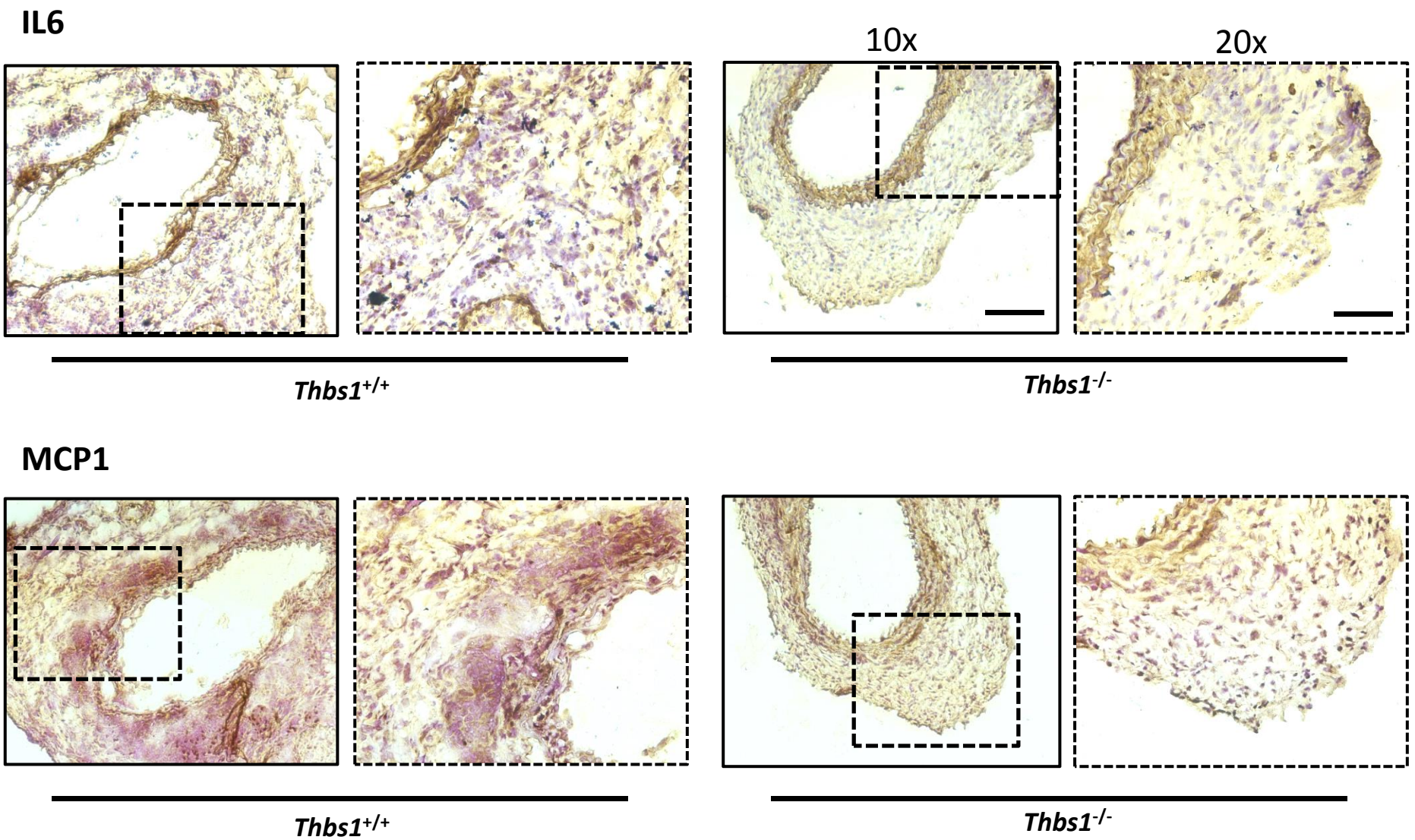
Supplemental Figure IV. Untreated *Thbs1*$^{+/+}$ and *Thbs1*$^{-/-}$ arteries appear similar histologically. TSP2 expression (top row), hematolxilin and eosin (H&E) stains (center row), and elastin integrity (Van Gieson, bottom row). Scale bar 200μm (TSP2 and H&E) and 100μm (Van Gieson).



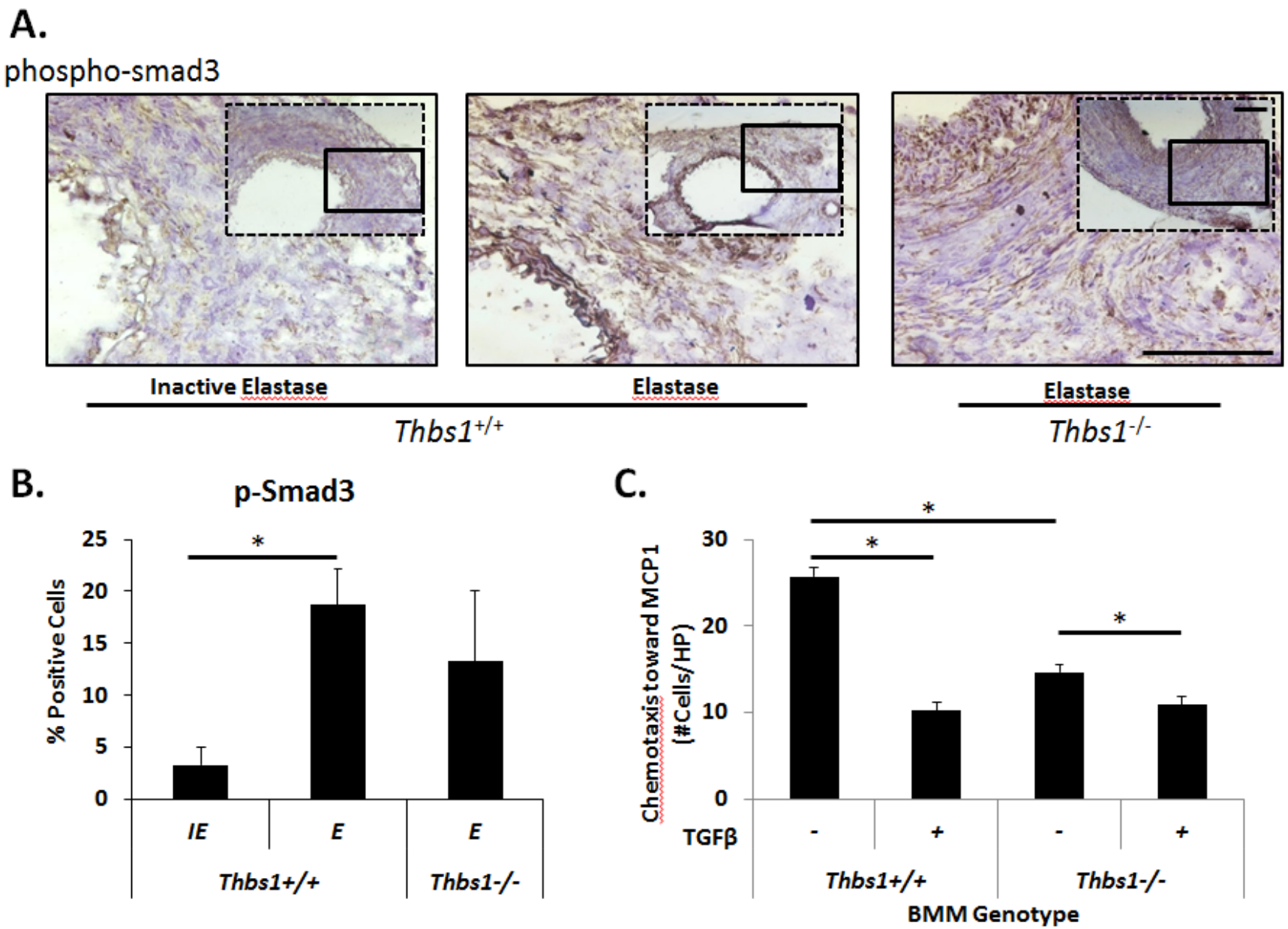
Supplemental Figure V. *Thbs1*$^{-/-}$ mice are resistant to aneurysm induction by $CaPO_4$ model. (A) Representative images of wildtype (*Thbs1*$^{+/+}$) and *Thbs1*$^{-/-}$ arteries treated with PBS (control) or Calcium phosphate ($CaPO_4$) 14 days after surgery. Scale bar 2mm. (B) Graphic depiction of aortic dilation (%Change in aortic diameter) in PBS- (grey square) or $CaPO_4$- (grey circle) treated *Thbs1*$^{+/+}$ arteries and PBS- (grey triangle) or $CaPO_4$- (grey wedge) treated *Thbs1*$^{-/-}$ arteries. Red dotted line designates aneurysmal formation (100% change in aortic diameter); *$p<0.05$ as compared to $CaPO_4$-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, *$p<0.05$.



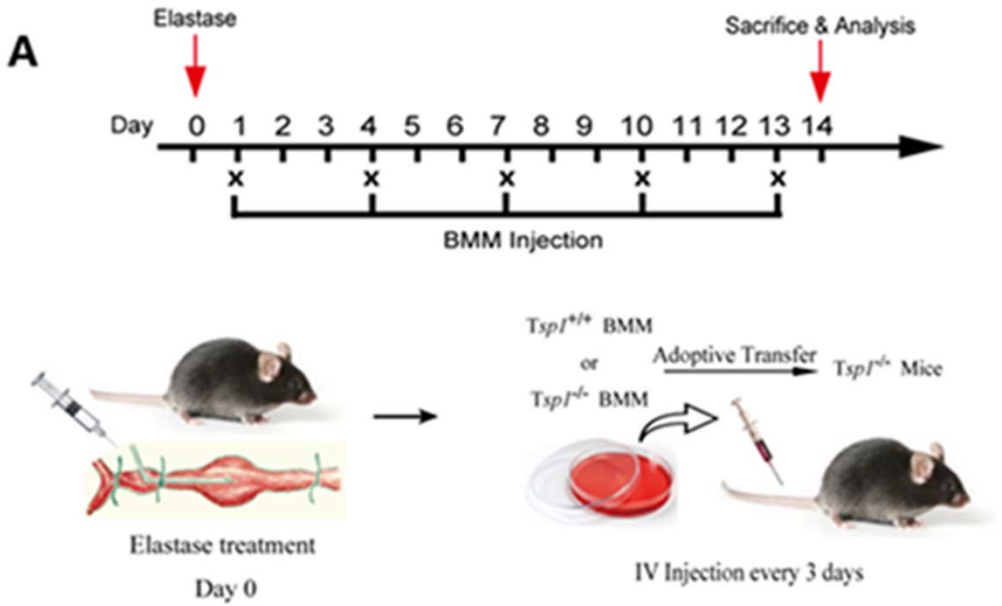
Supplemental Figure VI. Infiltration of inflammatory cells to *Thbs1*$^{+/+}$ and *Thbs1*$^{-/-}$ arteries 7 days after treatment with elastase. Monocytes and Macrophages (MOMA2), Neutrophils (NIMP-R14), (C) T lymphocytes (CD3). Scale bar 100μm (10x) and 50μm (20x). Quantification for each inflammatory 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.



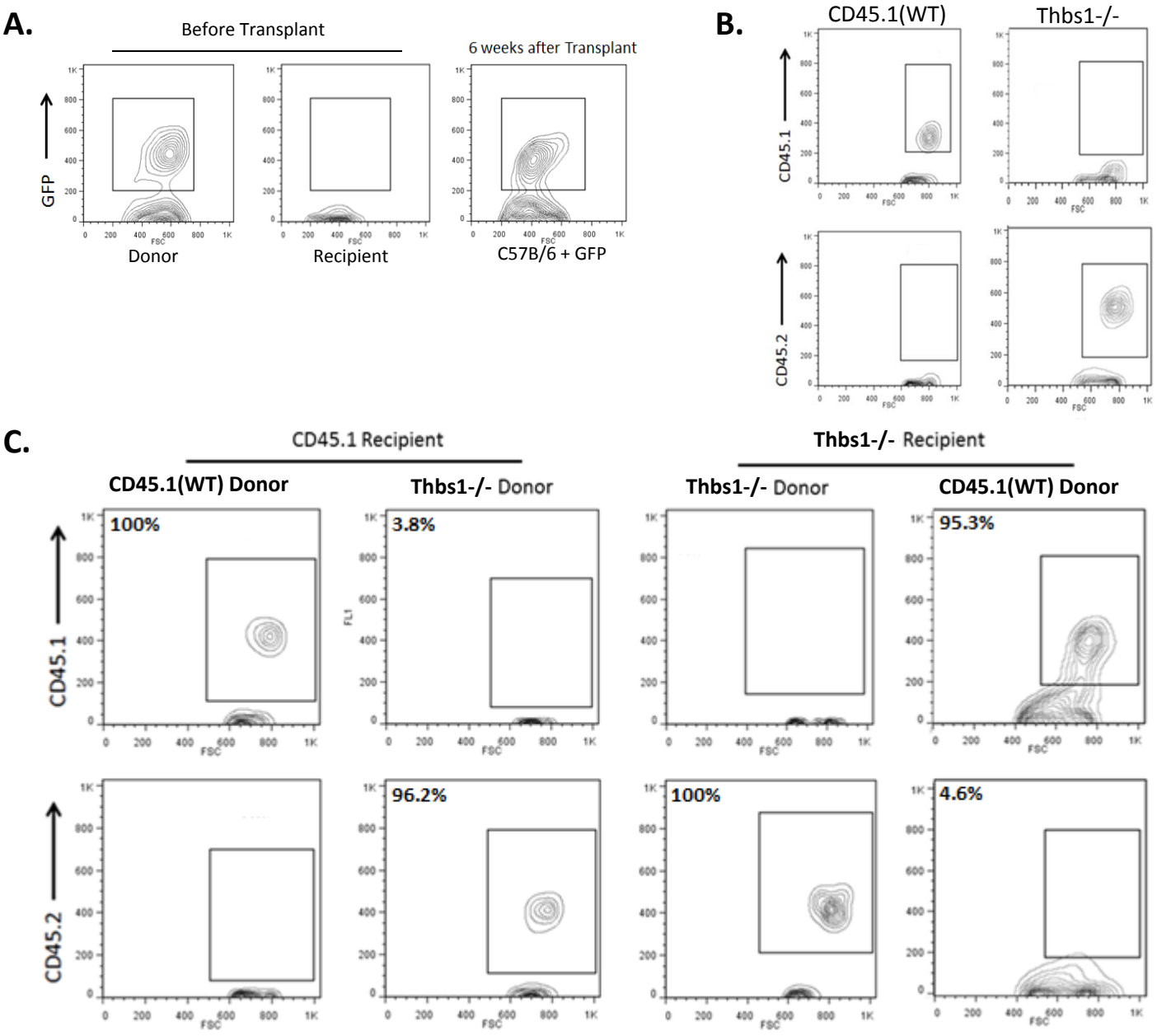
Supplemental Figure VII. Inflammatory cytokine expression in *Thbs1*$^{+/+}$ and *Thbs1*$^{-/-}$ arteries 7 days after treatment elastase. Representative immunohistochemical stains for interleukin 6 (IL6) or monocyte chemoattractant protein 1 (MCP1) in *Thbs1*$^{+/+}$ and *Thbs1*$^{-/-}$ arteries 7 days after treatment with elastase. Scale bar 200μm for 10x and 100μm for 20x.



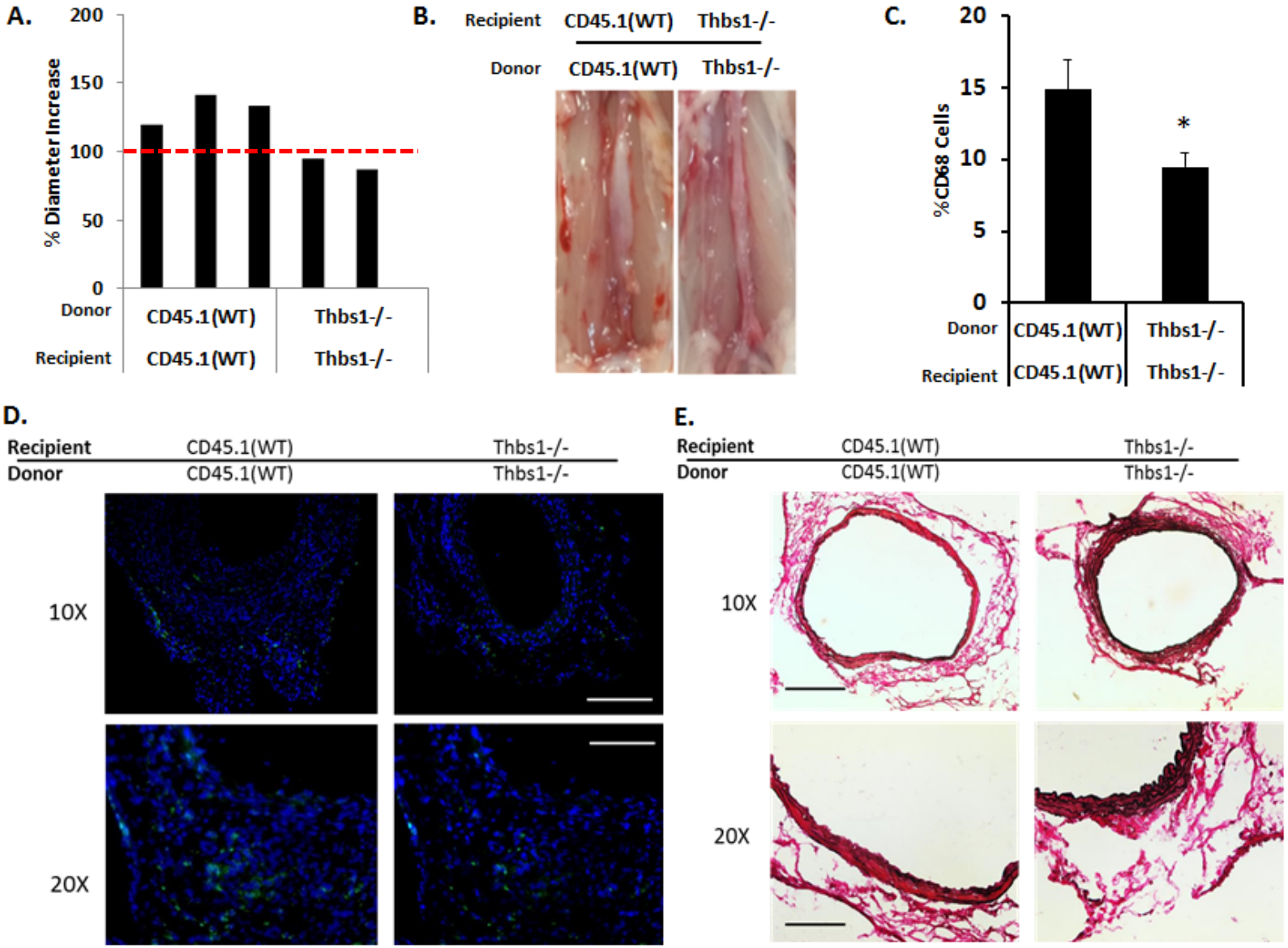
Supplemental Figure VIII. TGFβ activation is not changed in *Thbs1*$^{-/-}$ arteries. (A) Representative immunohistochemical stains for phosphorylated Smad3 in *Thbs1*$^{+/+}$ and *Thbs1*$^{-/-}$ mice 7 days after treatment with inactive elastase (control) or elastase. Scale bar 100µm; inlay 10x scale 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.