The effect of matrix metalloproteinase 2 and matrix metalloproteinase 2/9 deletion in experimental post-thrombotic vein wall remodeling

Kristopher B. Deatrick, MD,[a] Catherine E. Luke, LVT,[a] Megan A. Elfline, BS,[a] Vikram Sood, BS,[a] Joseph Baldwin, BS,[a] Gilbert R. Upchurch, Jr, MD,[a] Farouc A. Jaffer, MD, PhD,[b] Thomas W. Wakefield, MD,[a] and **Peter K. Henke, MD,[a]** *Ann Arbor, Mich; and Boston, Mass*

Background: **Vein wall fibrotic injury following deep venous thrombosis (VT) is associated with elevated matrix metalloproteinases (MMPs). Whether and by what mechanism MMP2 contributes to vein wall remodeling after VT is unknown.**

Methods: **Stasis VT was produced by ligation of the inferior vena cava and tissue was harvested at 2, 8, and 21 days in MMP2 -/- and genetic wild type (WT) mice. Tissue analysis by immunohistochemistry, enzyme-linked immunosorbent assay, real-time polymerase chain reaction, and zymography was performed.**

Results: **Thrombus resolution was less at 8 days in MMP2 -/- compared with WT, evidenced by a 51% increase in VT size ($P < .01$), and threefold fewer von Willebrand's factor positive channels ($P < .05$). In MMP2 -/- mice, the main phenotypic fibrotic differences occurred at 8 days post-VT, with significantly less vein wall collagen content ($P = .013$), fourfold lower procollagen III gene expression ($P < .01$), but no difference in procollagen I compared with WT. Decreased inflammation in MMP2 -/- vein walls was suggested by ~ threefold reduced TNFα and IL-1β at 2 days and 8 days post-VT ($P < .05$). A fourfold increase in vein wall monocytes ($P = .03$) with threefold decreased apoptosis ($P < .05$), but no difference in cellular proliferation at 8 days was found in MMP2 -/- compared with WT. As increased compensatory MMP9 activity was observed in the MMP2 -/-mice, MMP2/9 double null mice had thrombus induced with VT harvest at 8 days. Consistently, twofold larger VT, a threefold decrease in vein wall collagen, and a threefold increase in monocytes were found (all $P < .05$). Similar findings were observed in MMP9 -/- mice administered an exogenous MMP2 inhibitor.**

Conclusions: **In stasis VT, deletion of MMP2 was associated with less midterm vein wall fibrosis and inflammation, despite an increase in monocytes. Consideration that VT resolution was impaired with MMP2 (and MMP2/9) deletion suggests direct inhibition will likely also require anticoagulant therapy. (J Vasc Surg 2013;58:1375-84.)**

Clinical Relevance: **Post-thrombotic syndrome has no direct therapies and causes significant morbidity. Anticoagulation limits thrombosis but does not clinically impact directly the vein wall response to injury as well as has bleeding risks. In this experimental study, we show that matrix metalloproteinase 2 genetic deletion lessens fibrotic injury and inflammation at the midterm timepoint, yet is also important for thrombus resolution. Future therapies that positively impact vein wall remodeling will need to account for how the thrombus responds as well.**

Deep venous thrombosis (DVT) is a significant health care problem in this country, with over 250,000 patients affected yearly, although these figures may be conservative.[1] An additional 200,000 patients are affected by the late sequelae of post-thrombotic syndrome (PTS), characterized by leg pain, sensations of heaviness, limb edema, discoloration, and occasionally ulceration.[2] The end result after DVT is the conversion of a compliant, thin walled vein with functional valves to a thick walled vessel, often with nonfunctioning valves.[3] The physiological result of damaged veins is pooling of blood in the dependent extremities, producing venous hypertension, fluid transudation, edema, and potentially ulceration.

Despite effective anticoagulant therapy treatments for DVT, there are no therapies that specifically target the PTS remodeling processes.[4] Although surgical and interventional therapy may be beneficial for selected patients, treatment for PTS is mainly supportive, consisting of compression and wound care. Although largely effective, compression does not correct the fundamental changes that occur in the venous system and compliance with such ongoing regimens is often difficult.

Rodent studies have suggested that the mechanism and duration of thrombosis affects the magnitude of

From the Conrad Jobst Vascular Research Laboratory, Section of Vascular Surgery, Department of Surgery, University of Michigan Medical School, Ann Arbor[a]; and the Cardiovascular Research Center and Cardiology Division, Massachusetts General Hospital, Harvard Medical School, Boston.[b]

Supported by HL083918 and HL092129 (P.K.H.) and T32HL092129 (T.W.).

Author conflict of interest: none.

Additional material for this article may be found online at www.jvascsurg.org.

Reprint requests: Peter K. Henke, MD, University of Michigan Health System, 1500 E. Medical Center Dr, Cardiovascular Center-5463, Ann Arbor, MI 48109-5867 (e-mail: henke@umich.edu).

The editors and reviewers of this article have no relevant financial relationships to disclose per the JVS policy that requires reviewers to decline review of any manuscript for which they may have a conflict of interest.

0741-5214/$36.00

http://dx.doi.org/10.1016/j.jvs.2012.11.088

post-thrombotic vein wall damage seen. For example, a stasis thrombus is more damaging than a nonstatic thrombus and negatively affects collagen structure and venous compliance.[5,6] In addition to the intense inflammatory reaction seen following DVT, there is a phenotypic alteration in the extracellular matrix, associated with activation of matrix metalloproteinases (MMPs), including MMP2 and 9.[6-9] Of these, MMP2 is most significantly elevated in the vein wall after stasis venous thrombosis (VT) at the mid and later timepoints,[6] and is when PTS occurs in humans. Although broad MMP inhibition may decrease some measures of injury,[10,11] their specific role has not been defined in the vein wall after VT. The additive effects of MMP2/9 genetic deletions have been highlighted with experimental lung injury.[12] Moreover, recent studies suggest certain biomarkers may be predictive of PTS well as associated with DVT resolution in humans, including MMPs.[4,13]

We hypothesized that abrogation of matrix remodeling via genetic deletion of MMP2 and combined MMP2/9 would lessen the damage seen in the vein wall following stasis VT.

METHODS

Animal model. Male mice 6 to 8 weeks old, MMP2 -/- (kindly donated by Dr Eric Choi) and C57BL/6 wild type (WT) were used for the experiments. With all surgical procedures, the mice were anesthetized using inhalational isoflurane and O_2, and continuously monitored. Animal studies were approved by the University of Michigan Committee on Use and Care of Animals.

Experimental VT was created in the mouse using infrarenal inferior vena cava (IVC) ligation as previously described.[6,14-16] Briefly, mice were anesthetized, and underwent midline laparotomy. The IVC was ligated with a 7-0 prolene suture immediately below the renal veins. Back (lumbar) branches were ablated with cautery, and all visible side branches were interrupted with 7-0 prolene suture. Mice were sacrificed on days 2, 8, and 21 days post-ligation. At sacrifice, the thrombosed IVC segment was carefully dissected and removed for formalin fixation and paraffin embedding (for histology/immunohistochemistry) or immediately snap frozen (−70°C) to preserve for tissue processing.

At days 2 and 8, the thrombus was separated from the vein wall for processing; however, at 21days, the thrombus and vein wall form a segment of scar tissue, which cannot be separated without tissue disruption, and those segments were processed together. In other experiments, a specific MMP2 inhibitor[17,18] (OA-Hy; EMD Biosciences, Gibbstown, NJ) 6.25 mcg per mouse in 1 mg/mL diluted dimethyl sulfoxide or the vehicle, was administered intraperitoneal to MMP9 -/- mice immediately after IVC ligation and at 4 days post-VT, with harvest at 8 days. Separate C57BL/6 MMP9 -/- were crossed with C57BL/6 MMP-2 -/- mice to generate MMP2 -/- double null mice.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis gelatin zymography. Activity of the gelatinases (MMP2 and MMP9, active and latent forms) was determined by gelatin zymography on 10% sodium dodecyl sulfate-polyacrylamide gels, as previously described.[5,15,18] Activity was visualized as light staining bands on a dark background and normalized to the total amount of protein present in each sample as previously described.

Histology/immunohistochemical/apoptosis/collagen staining. Tissue samples were formalin fixed, paraffin embedded, and cut into 5 μm sections as described.[14,15] Nonspecific sites were blocked with normal serum, and sections were incubated with primary antibodies to Mac2 (1:200; Cedarlane Laboratories, Burlington, NC), von Willebrand's factor (vWF; 1:500; Abcam, Cambridge, Mass), and Ki-67 (1:100; Abcam). A species-specific ABC peroxidase kit for either rabbit or rat (Vector Laboratories Inc, Burlingame, Calif) was used according to the manufacturer's instructions for the corresponding secondary antibody and subsequent steps. The slides were counterstained with hematoxylin. In a blinded fashion, positive cells in five high power fields (×1000) radially around the IVC were counted and totaled.

The presence of apoptotic cell death was assessed in the vein wall by using a commercially available kit to determine the presence of characteristic DNA breaks by the TUNEL method (Trevigen Inc, Gaithersburg, Md).

Picrosirius red staining to quantify collagen content was performed as described.[19,20] These sections were then analyzed in crossed-plane polarized light from a monochromatic source to assess cross-linked collagen. Two images for each were obtained using a Zeiss Axio M1 scope and Zeiss AxioVision software (Carl Zeiss Microimaging GmbH, Göttingen, Germany) at 0 and 90 degrees to the plane of polarization, to capture the birefringence of fibers extinguished in one direction. The images were analyzed blindly utilizing NIH Image J software. The area corresponding to the vein wall was selected as a region-of-interest, and then the image underwent threshold segmentation to differentiate collagen from other (mainly cellular and empty space) components of the vein wall. A vein wall collagen score was assigned by the formula [(% birefringent area) × (measured vein wall area)]/(total specimen area).[21]

To account for noncollagen vein wall changes, intimal thickness scoring was assessed from H and E sections as described.[22] A consistent midsection thrombosed IVC segment was used for all histologic analysis.[16,18,23]

Antigen analysis by enzyme-linked immunosorbent assay. Vein wall tissue was homogenized, subjected to ultrasonic sonication, followed by centrifugation samples centrifuged at ×10,000 g for 5 minutes, and the supernatant was collected for analysis. Quantification of the analyzed antigens was normalized to the total protein present in the sample, using a modified Bradford assay (Pierce Inc, Rockford, Ill). Enzyme-linked immunosorbent assay for mouse tumor necrosis factor-alpha (TNFα), interleukin-1 beta (IL-1β), transforming growth factor beta (TGFβ), and monocyte chemotactic protein-1 (MCP-1) was performed according to manufacturer's instructions (all from R and D, Minneapolis, Minn).[5,14]

Quantitative real-time polymerase chain reaction. The levels of expression for genes of interest were determined by isolating total RNA via Trizol extraction as previously described.[15,16] Then, the RNA underwent reverse transcription. The complementary DNA was then subjected to a real-time reverse transcriptase reaction using Taq polymerase (Promega, Madison, Wisc) in a SmartCycler quantitative polymerase chain reaction system (Cepheid, Sunnyvale, Calif). SYBR green intercalating dye (Roche, Indianapolis, Ind) was used to monitor levels of complementary DNA amplification for each gene. β-actin was used as an internal control for reference in each sample. The sequence numbers were: Col1a2 - RefSeq# NM_007743.3; Col3a1 - RefSeq# NM_009930.2; tropoelastin - RefSeq # NM_0036834.3; beta Actin - RefSeq# NM_007393.3.

Statistical analysis. All data are presented as mean ± SE. Comparisons were made using an unpaired Student *t*-test or analysis of variance with Dunnett or Bonferroni multiple comparison tests as appropriate, using GraphPad Prism v. 4.0 for Windows (GraphPad Software, San Diego, Calif).

RESULTS

MMP2 deletion impairs midpoint VT resolution. The weight:length ratio of a resolving thrombus provides a simple but reliable measure of thrombus resolution.[6,14-16] Given that the inflammatory response is crucial for resolving VT, interventions that affect inflammation might impair thrombus resolution. At 8 days, MMP2 -/- mice had a 51% increase in VT size compared with WT (n = 15-20; $P < .001$) (Fig 1, *A*). These data are consistent with prior investigation of MMP2 activity in VT resolution between 4 and 8 days and this timepoint was not repeated.[15,18] No differences were observed in VT size at 2 days (n = 12-14; $P = .6$) or 21 days (14 ± 1 vs 17 ± 2 mg/cm; n = 20-22; $P = .16$) in MMP2 -/- mice compared with WT controls.

Resolution of a VT is conferred in part by development of vWF + vascular channels in the thrombus.[14,16] Correlating with impaired thrombus resolution in MMP2 -/- mice at 8 days were threefold less vWF + channels (n = 5; $P = .025$) (Fig 1, *B-D*).

Post-thrombotic vein wall collagen metabolism is altered in MMP2 -/- mice. The 8- and 21-day timepoints were chosen to evaluate the loss of MMP2 as these mimic the post-thrombotic syndrome time frame, with a significantly thicker and collagen dense vein wall, as well as when MMP2 activity is present.[5,6] Vein wall collagen as assessed by Picosirius red increases over time following thrombosis (Supplementary Fig 1, online only). The collagen content was ∼35% less in the MMP2 -/- mice compared with the WT (n = 4-5; $P = .013$) (Fig 2, *A-C*), with no differences in intimal thickness at 8 days (Supplementary Fig 2, online only). No difference in collagen was found at 21 days in MMP2 -/- compared with WT (6.5 ± 1.3 vs 6.0 ± 1.4% vein wall section; n = 4-5; $P = .9$).

Experimental VT is associated with an upregulation of procollagen messenger RNA in the vein wall,[13] specifically procollagen I and procollagen III. This was analyzed at 2 and 8 days. In the MMP2 -/- mice, procollagen I gene expression was not significantly different at 2 or 8 days (2-day comparison $P = .26$; 8-day comparison $P = .55$; n = 4-5 each group) (Fig 2, *D*). Procollagen III gene expression was decreased ∼fourfold in the MMP2 -/- mice compared with the WT at 8 days (n = 5; $P < .01$), with no differences at 2 days (not shown) (Fig 2, *E*). As a precursor to elastin, tropoelastin gene expression was not altered at 2 days, but was decreased ∼ 2.5-fold at 8 days in MMP2 -/- compared with WT mice (Fig 2, *F*).

Vein wall inflammation and apoptosis is decreased in MMP2 -/- mice at 8 days. Inflammatory mediators are present in the thrombus and vein wall after VT, including TNFα, IL-1β, and MCP-1.[5,14,15] In the MMP2 -/- mice, TGFβ protein levels were not significantly different at 8 days (3.9 ± 0.9 vs 2.5 ± 0.08 ng/mg protein; n = 5; P = NS), compared with WT. TNFα was reduced three- to fourfold at 2 and 8 days in the MMP2 -/- mice compared with WT (n = 4-5; $P = .03$) (Fig 3, *A*). IL-1β was reduced 2.5-fold in the MMP2 -/- mice compared with WT at 2 days (n = 4-5; $P = .01$) (Fig 3, *B*) but not at 8 days (517 ± 300 vs 361 ± 71 pg/mg protein; n = 5; $P > .05$). No significant differences were found in MCP-1 levels at 2 or 8 days (not shown). The MMP2 -/- mice had ∼threefold reciprocally increased MMP9 activity by zymography (n = 5; $P \leq .01$) at day 8 only (Fig 3, *C*).

Since MMP2 is both produced by leukocytes in the early inflammatory response, and in part mediates their migration across collagen-rich matrices such as the basement membrane,[24] we investigated whether the loss of MMP2 would have an effect on the number of inflammatory cells present in the vein wall. In the MMP2 -/- vein wall, monocytes were increased ∼fourfold in MMP2 -/- mice compared with WT (n = 4-5; $P = .03$) (Fig 3, *D* and Supplementary Fig 3, online only).

Medial cellular proliferation and apoptosis contributes to postvascular injury fibrosis.[25,26] To evaluate for the contribution of cellular proliferation, Ki67 antigen staining was evaluated in the medial vein layer.[5] Ki67 positive cells staining showed no significant difference in MMP2 -/- compared with WT sections (7 ± 1 vs 5 ± 1 cells/5hpf; n = 6-7; $P = .3$). However, fewer apoptotic nuclei by TUNEL + staining were present in the MMP2 -/- mice, with ∼fourfold less apoptosis at 8 days compared with WT (n = 6; $P = .045$) (Fig 3, *E-G*).

Combined MMP2 and MMP9 deletion on vein wall response. For the previous experiments with the single MMP2 deleted mice, we found reciprocally elevated MMP9 activity at 8 days. This was also the point where the collagen metabolism was affected most significantly by the MMP2 deletion. Thus, we evaluated MMP2 -/- MMP9 -/- double null mice as well as MMP9 -/- mice given a specific MMP2 inhibitor.[18] Stasis VT was induced with harvest at 8 days. We found that thrombus resolution was impaired in the MMP9 -/- + MMP2 inhibitor with

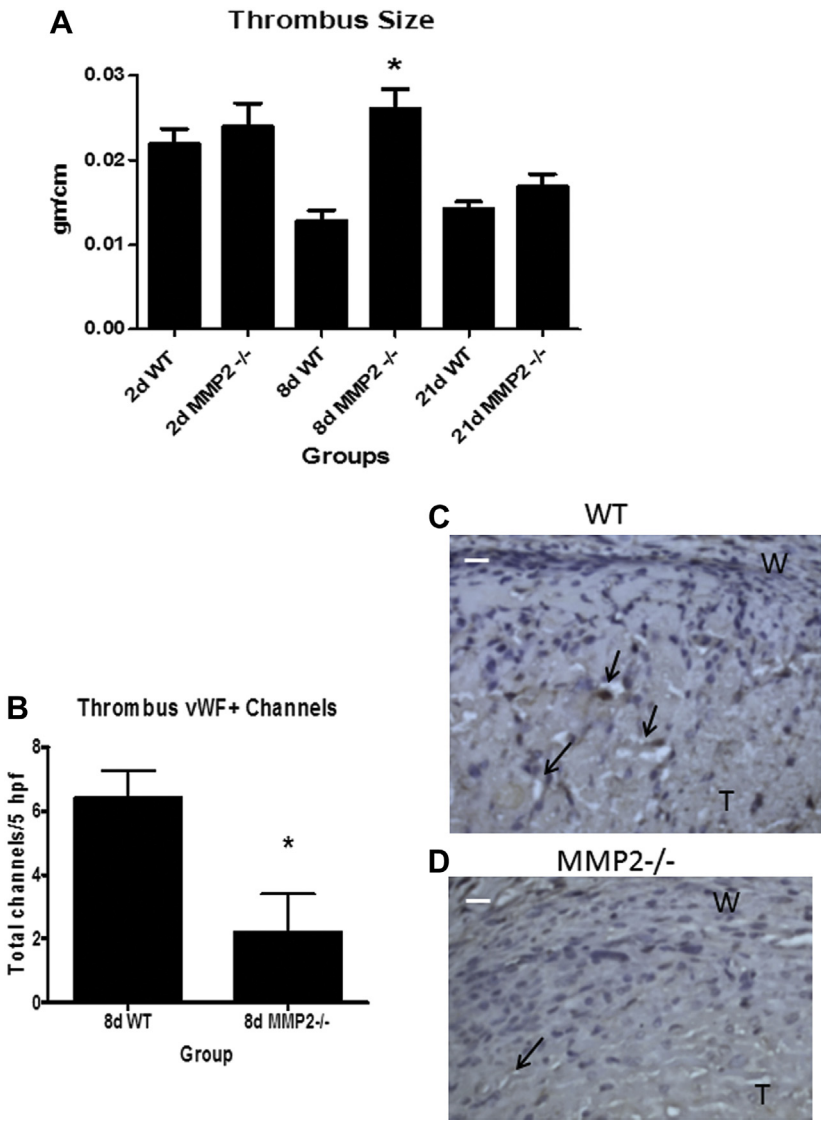

Fig 1. Thrombus size as measured by weight:length ratio showed larger venous thrombosis (VT) at day 8 in matrix metalloproteinase (*MMP*)2 -/- mice **(A)**. Thrombus channels were fewer in MMP2 -/- mice compared with wild type (*WT*) **(B)**. Photomicrographs in WT **(C)** show numerous channels in periphery of the thrombus. Fewer von Willebrand's factor (*vWF*) + channels noted in MMP2 -/- VT **(D)**. At ×400, *arrows* mark channels. *T*, Thrombus; *W*, wall. *White bar* = 10 μm.

2.5-fold increase (n = 14; $P < .05$), and 1.6-fold increase in VT size in the MMP2/9 double null mice compared with WT (n = 5; $P < .05$) (Fig 4, *A*). Evaluating the vein wall collagen by Sirius red analysis, we found that MMP9 -/- + MMP2 inhibitor and MMP2/9 double null mice had three- to sixfold reduced collagen content in the vein wall (n = 4-5; $P < .05$) (Fig 4, *B*, *D*, *E*, and *F*). Consistent with the single MMP9 -/- or MMP2 -/- phenotypes, we found a ~fivefold increase in vein wall monocytes with MMP9 -/- + MMP2 inhibitor and a fourfold increase in monocytes in the MMP2/9 double null mice compared with WT (n = 4; $P < .05$) (Fig 4, *C*).

We performed a second analysis using only MMP2 -/- as the control for the MMP9 -/- + MMP2 inhibitor and MMP2/9 -/- (Fig 4). We found that comparison using analysis of variance with Bonferroni correction that the VT size was only significantly larger in the MMP9 -/- + MMP2 inhibitor group ($P < .05$) compared with the MMP2 -/- group. Vein wall collagen content was significantly less in both MMP9 -/- + MMP2 inhibitor and MMP2/9 -/- compared with MMP2 -/- ($P < .001$). Finally, vein wall monocytes were significantly greater in the MMP9 -/- + MMP2 -/- inhibitor group ($P = .03$), but not greater in the MMP2/9 -/- group.

DISCUSSION

Although anticoagulation is effective treatment for DVT, no specific therapies are available to target the vascular

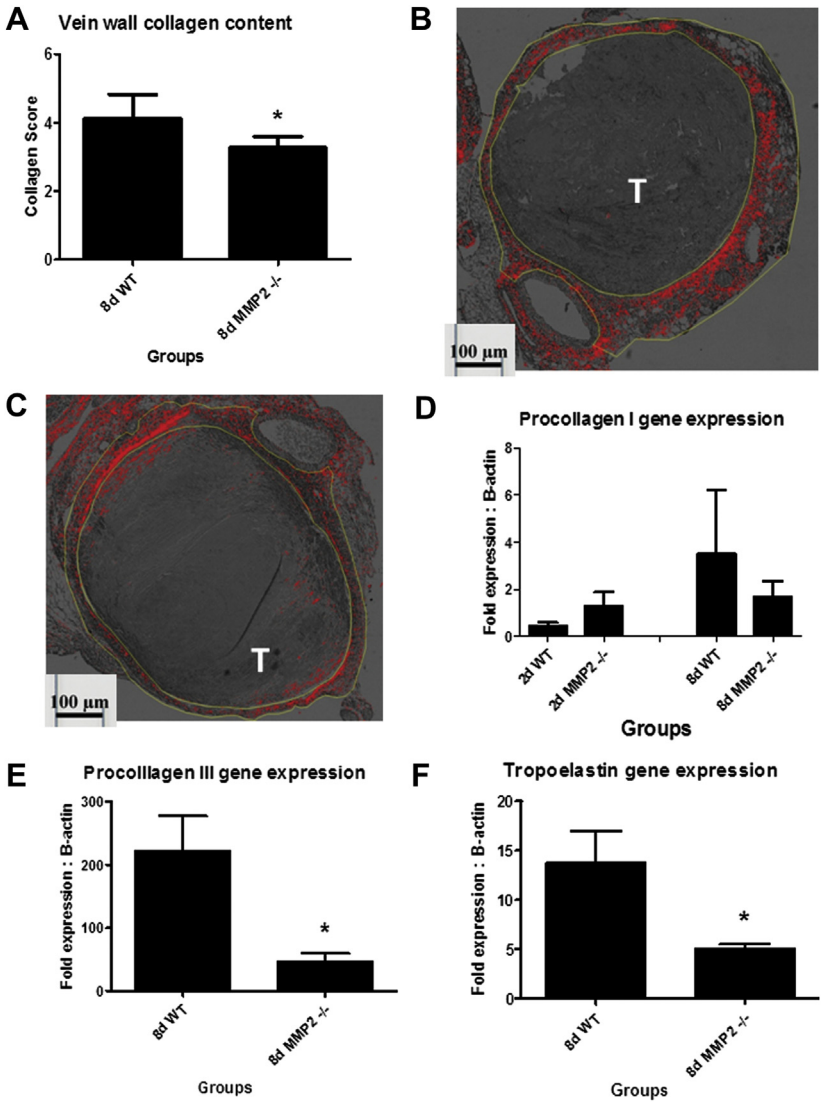

Fig 2. A, Vein wall collagen content was less in matrix metalloproteinase (*MMP*)2 -/- compared with wild type (*WT*). Sirius *red* stained vein wall section of WT **(B)**, contrasting with the thinner and less dense collagen (*red*) in the MMP2 -/- vein walls **(C)**. Histologic images at ×100. Procollagen I was not altered in the MMP2 -/- vein wall compared with WT **(D)**, while procollagen III was reduced at 8 days **(E)**. Tropoelastin gene expression was reduced at 8 days in the MMP2 -/- compared with WT **(F)**. *T*, Thrombus. **P* < .05.

inflammatory and fibrotic processes that result in PTS.[2,4] Remodeling of the extracellular matrix in vessels is mediated in part by the MMPs,[25] and activity has been visualized in vivo in arterial injury[27] and veins after VT.[9] Resolving VT and the vein wall response are associated with MMP2 and MMP9 time dependent activity changes, but their direct role had not been assessed.[6,7] The MMP2 seems most likely involved with vein wall metabolism given its kinetics and activities[6,25] and thus we focused on this MMP type. In this study, we demonstrate VT resolution was impaired in MMP2 -/- mice, and that the post-thrombotic vein is characterized by temporal changes in collagen content, dependent on both MMP2 with associated genetic, cellular, and inflammatory changes. These findings also suggest a complex interplay of factors that is likely not simply related to collagen or matrix turnover.[24,25]

Although not the primary focus of these experiments, VT resolution was impaired at the mid-timepoint (8 days) in MMP2 -/- mice. Our data suggest VT resolution is in part dependent on MMP2 activity, as VTs were significantly larger in MMP2 -/- mice at 8 days. We have also observed a correlation between concentration of MMP activity and thrombus resolution in real time imaging.[9] Prior work in our laboratory has also shown that deletion or inhibition of MMP2 is associated with impaired thrombus resolution at 4 days,[15,18] independent

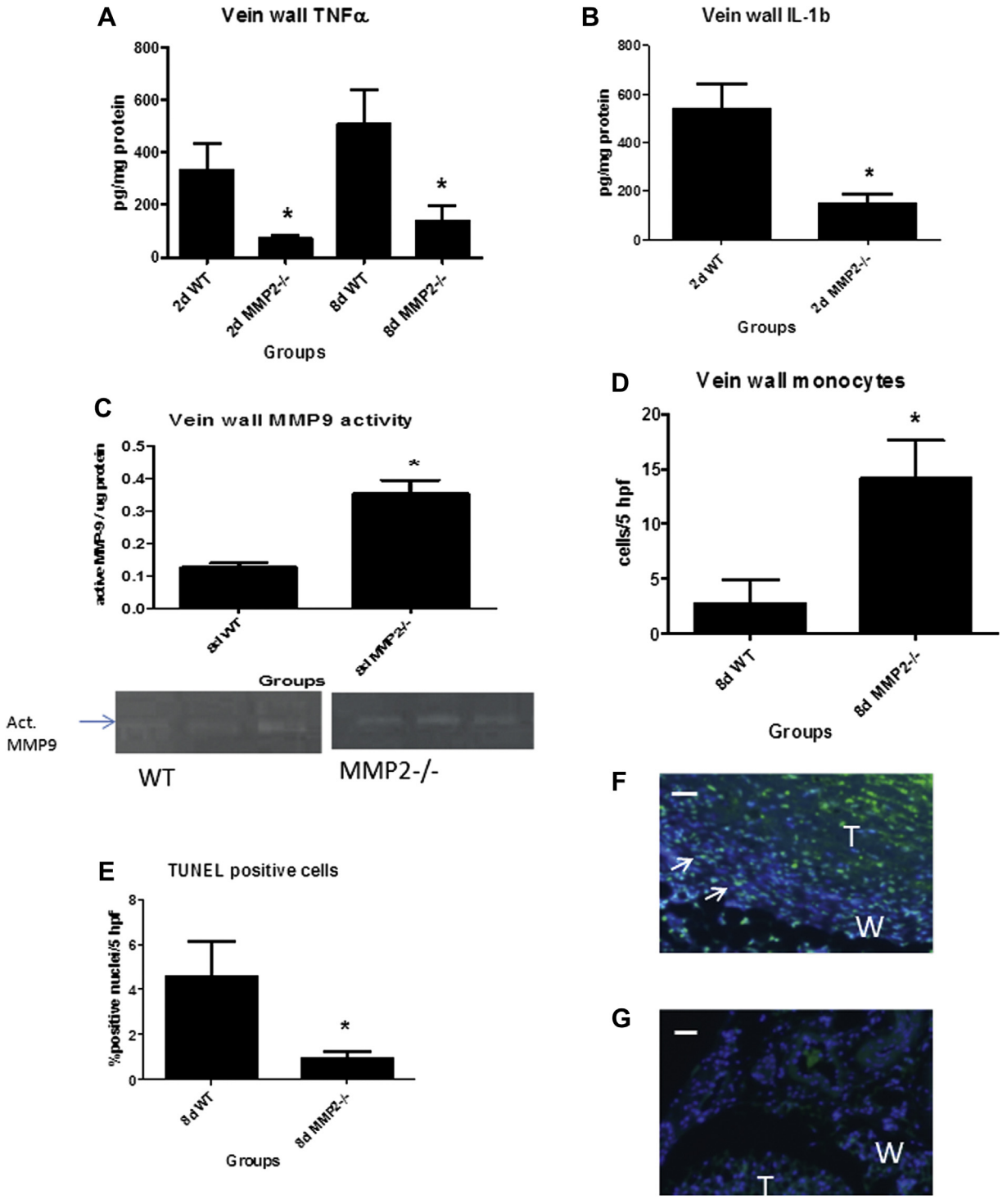

Fig 3. Vein wall tumor necrosis factor-alpha (*TNFα*) in matrix metalloproteinase (*MMP*)2 -/- was less at 2 and 8 days **(A)**, while interleukin (*IL*)-1β was reduced at 2 days **(B)**. **C,** Vein wall active MMP9 activity was increased in MMP2 -/- mice at 8 days by zymography. **D,** Vein wall monocytes were increased at 8 days in MMP2 -/- mice compared with wild type (*WT*). **E-G,** Apoptosis as marked by terminal deoxynucleotidyl transferase end labeling (*TUNEL*) + *green* colocalized with 4′,6-diamidino-2-phenylindole *blue* staining nuclei showed less medial + cells at 8 days in the MMP2 -/- vein wall compared with WT. At ×400. *T,* Thrombus; *W,* wall; *arrows* mark + cells; **P* < .05. *White measurement bar* = 10 μm.

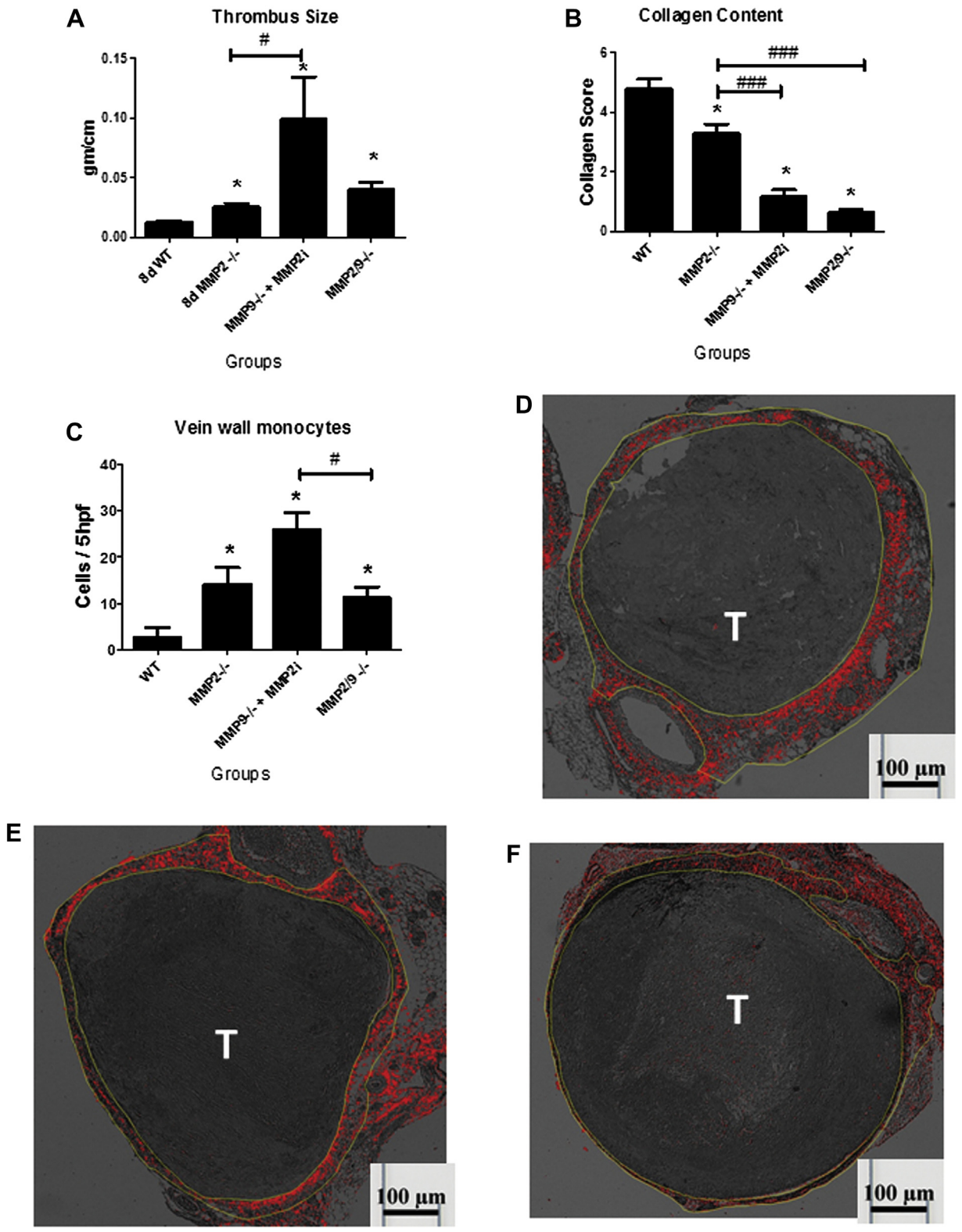

Fig 4. A, Thrombus size was increased at 8 days in matrix metalloproteinase (*MMP*)9 -/- + MMP2 inhibitor as well as MMP2/9 -/- double null mice. **B,** Collagen content was reduced in the MMP9 -/- + MMP2 inhibitor and the MMP2/9 -/- double null compared with wild type (*WT*). **C,** Monocytes were elevated in MMP9 -/- + MMP2 inhibition and MMP2/9 double null -/- mice. **D-F,** Representative photomicrographs of WT, MMP9 -/- + MMP2 inhibition, and MMP2/9 -/- mice. Histologic images at ×100. *T*, Thrombus; **P* < .05 by analysis of variance with Dunnett multiple comparison test. #*P* < .05; ###*P* < .001 by analysis of variance with Bonferonni correction.

of plasminogen activation. Thrombi resolve in part by neovascularization.[8] MMP2 is critical for neovascularization[28] and consistent with impaired VT resolution, significantly fewer vWF + channels in MMP2 -/- thrombi were found. The lack of MMP2 activity did not affect early thrombus formation or resolution, probably because the stasis model mechanism predominates, as well as cellular mediated resolution may be more important at mid to later timepoints.[15,29] Other cellular mechanisms independent from MMP2 exist as the VT sizes were similar at 21 days, suggesting compensatory accelerated resolution in the MMP2 -/- mice. This observation also underscores the temporal importance of MMP2 in VT resolution.

Numerous studies have highlighted the role of MMPs in the response to vascular injury, including VT resolution.[6,25] Matrix metalloproteinases are zinc containing endoproteinases with multiple targets, including matrix and nonmatrix substrates.[24] In multiple models of tissue injury, early activation of both MMP2 and MMP9 occurs prior to the end stage-fibrotic process,[25] including our own model.[6] In this study, we demonstrate that loss of MMP2 is associated with less vein wall collagen at midterm after the stasis thrombosis injury. Moreover, the addition of MMP9 deletion was additive, as the MMP2/9 -/- double null mice or MMP9 -/- with an exogenous MMP2 inhibitor had markedly less post-thrombotic vein wall collagen than the single MMP2 deletion (Fig 4). Consistent with our findings is that both MMP2 and/or MMP9 gene deletions are associated with less constrictive fibrosis in direct and flow mediated arterial injury models.[26,30-32] Although we did not specifically investigate the MMP2 -/- venous vascular smooth muscle cell (VSMC) migration potential, these reports suggest significant migration impairment in vitro. These studies provide an explanation for our observed phenotype; namely, less collagen production after injury occurs, possibly because of less VSMC migration and activation. We acknowledge that we did not specifically colocalize collagen production to VSMC and other medial cells may be important. Lastly, the protection from late vein wall fibrosis in MMP2 -/- mice was not observed at 21 days post-thrombosis. We have observed this in other experiments with different genetic deletions and genetic backgrounds.[21] This suggests that other processes that are not monocyte and MMP dependent mechanisms of injury predominate at this late timepoint.

In general, the MMPs regulate vessel collagen by degradation as well as cellular influx and function.[24,25] Deletion of MMP2 was associated with decreased gene expression of procollagen III and tropoelastin in response to vein wall injury, suggesting less vein wall metabolic turnover. Specifically, the decrease in vein wall collagen in the MMP2 -/- mice may be directly related to decreased collagen gene expression and protein translation. Moreover, increased TNFα is associated with fibrosis,[33,34] and was significantly decreased in the MMP2 -/- mice. We did not directly address if any of the cytokines were mechanistically involved with vein wall responses, however. It is unlikely that VSMC were directly affected, as medial cell proliferation was not affected by loss of MMP2, consistent with some, but not all reports.[25,31] However, medial vein wall cellular apoptosis was significantly decreased in MMP2 -/- compared with WT. This may be related to the relative increase in MMP9 activity in the MMP2 -/- mice, and greater clearance of apoptotic cells, as suggested in other models.[12] Other MMPs that we did not assess may also play a role in response to injury, such as MMP3 and MMP12.[32] Besides the proinflammatory cytokine induction of MMP activation, vessel wall strain may contribute as has been highlighted by arterial experimental data.[35] Certainly, stasis thrombosis distends the vein wall and likely directly promotes MMP activation, possibly accounting for the upregulation of MMP9 activity in the MMP2 -/- mice.

Although TIMPs may play a major role in certain disease process such as atherosclerosis[36] and hepatic fibrosis,[37] it did not appear these were altered significantly in our prior report after stasis VT.[6] Preliminary experiments with TIMP1 -/- mice, which have significantly increased MMP2 and 9 activities at 8 and 21 days, did not show a significant increase in vein wall collagen (Henke PK, unpublished data). This suggests that increased MMP2 and 9 activity itself does not worsen parameters of vein wall healing in the stasis VT model, contrasting with solid organ injury models.[37]

Most of the phenotype differences were observed 8 days post-VT, which is when monocytes predominate in the vein wall.[5,14,15] While part of the normal inflammatory response, monocytes may or may not promote fibrotic tissue repair.[38] Consistent with the current report is that *P*-selectin inhibition after stasis VT is associated with increased vein wall monocytes, but less vein wall fibrosis.[22,39] Recent investigations suggest that monocyte subtypes confer differing inflammatory or anti-inflammatory responses (eg, M2) depending on the environment and time frame.[38] Interestingly, both MMP2 -/-, the MMP9 -/- + MMP2 inhibitor, and the double null mice had increased vein wall monocyte influx, suggesting these proteinases are not essential for monocyte influx following injury but may be important for cellular egress. Indeed, in a pulmonary inflammatory model with MMP2/9 double deletion, a significant increase in inflammatory cells was observed after injury because of inhibited trafficking.[12] Another mechanism accounting for increased monocytes may involve MMP chemokine processing.[24] However, since MCP-1 was not significantly altered with MMP2 deletion, other chemokines such as MCP 2-5 may be responsible for the increase in vein wall monocytes observed. Interestingly, the MMP9 -/- + MMP2 inhibitor had a more exaggerated response with larger thrombi and more vein wall monocytes than the MMP2/9 -/- compared with MMP2 -/- alone. This may be due to the fact that the MMP2 inhibitor, while specific,[17] may have other effects on the vein wall injury as it is a long chain fatty acid with fibronectin binding sites, and may mimic the basement membrane of the vessel wall with alteration in monocyte influx and thrombus resolution.[40]

Targeting of MMPs has been evaluated in various models of cardiovascular injury and VT.[11,25,41] Although the deletion of MMP2 in these experiments correlates with diminished collagen at 8 days, the significance of this effect is lost by 21 days. Thus, the role of MMP2 in the fibrotic response is temporally related to the duration of thrombus contact. Translationally, the timing and duration of MMP2 inhibition needs to guide future studies in treatment of PTS, and a practical means to reduce up-regulation of MMPs may be by limiting the thrombus-vein wall contact time via anticoagulation and pharmaco-mechanical therapies.[4] More importantly, the cost of decreased fibrosis may be a larger thrombus.

AUTHOR CONTRIBUTIONS

Conception and design: KD, CL, GU, TW, FJ, PH
Analysis and interpretation: KD, JB, VS
Data collection: KD, CL, ME, VS, JB
Writing the article: KD, PH
Critical revision of the article: KD, PH
Final approval of the article: all
Statistical analysis: KD, PH
Obtained funding: PH
Overall responsibility: PH

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Submitted Jul 13, 2012; accepted Nov 21, 2012.

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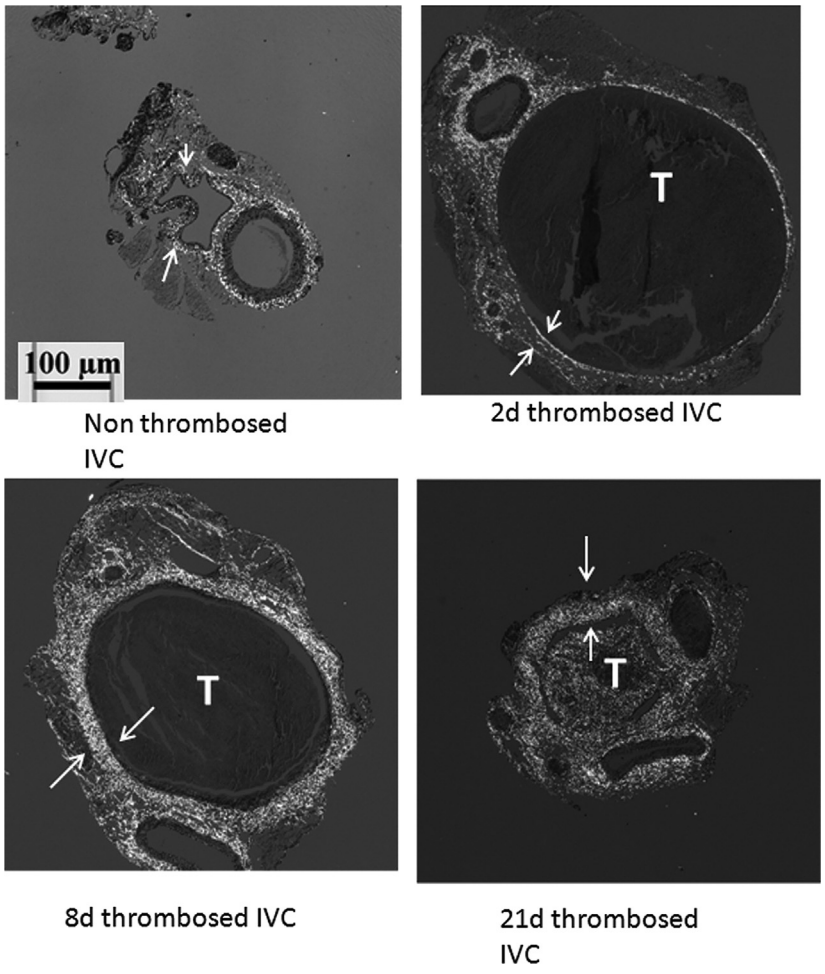
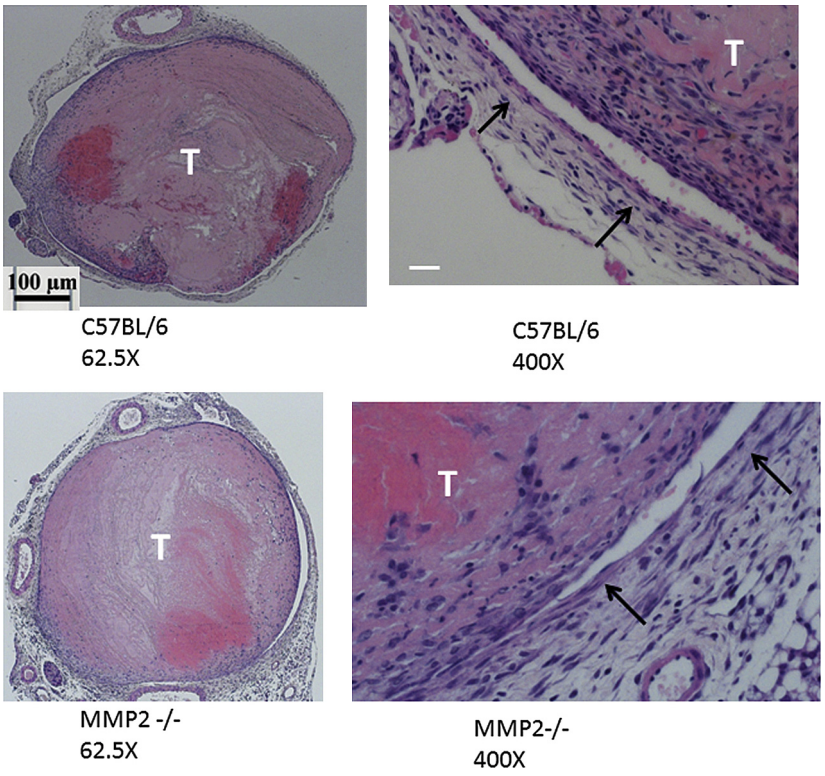
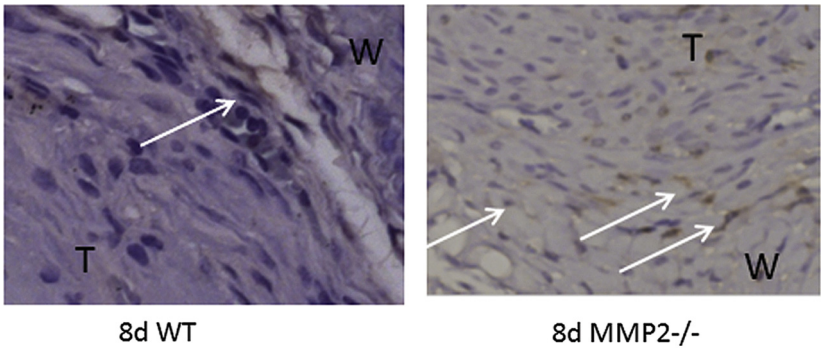


Supplementary Fig 1 (online only). Representative polarized photomicrographs of Sirius red stained nonthrombosed vein, and 2, 8, and 21 days thrombosed inferior vena cava (*IVC*) sections. Collagen is *white*. Note vein wall thickening over time after thrombosis. The *arrows* demarcate the vein wall. *T,* Thrombus.



Supplementary Fig 2 (online only). Photomicrographs of 8 days hematoxylin and eosin (H&E) stained thrombosed inferior vena cava (IVC) sections in wild type (WT) and in matrix metalloproteinase (*MMP*)2 -/- mice. Higher-power views show medial and intimal layers of the vein wall. Note no difference in intimal layers as denoted by the *arrow*. *T*, Thrombus.



Supplementary Fig 3 (online only). Representative panels of wild type (WT) C57 and C57 in matrix metalloproteinase (*MMP*)2 -/- at ×400. *Arrows* denote + Mac2 cells. Fewer are present in the WT vein wall. *T*, Thrombus; *W*, wall.