Membrane-Bound Thrombomodulin Regulates Macrophage Inflammation in Abdominal Aortic Aneurysm

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Objective—Thrombomodulin (TM), a glycoprotein constitutively expressed in the endothelium, is well known for its anticoagulant and anti-inflammatory properties. Paradoxically, we recently found that monocytic membrane-bound TM (ie, endogenous TM expression in monocytes) triggers lipopolysaccharide- and gram-negative bacteria–induced inflammatory responses. However, the significance of membrane-bound TM in chronic sterile vascular inflammation and the development of abdominal aortic aneurysm (AAA) remains undetermined.

Approach and Results—Implicating a potential role for membrane-bound TM in AAA, we found that TM signals were predominantly localized to macrophages and vascular smooth muscle cells in human aneurysm specimens. Characterization of the CaCl₂-induced AAA in mice revealed that during aneurysm development, TM expression was mainly localized in infiltrating macrophages and vascular smooth muscle cells. To investigate the function of membrane-bound TM in vivo, transgenic mice with myeloid- (LysMcre/TMflox/flox) and vascular smooth muscle cell–specific (SM22-cre/¹/²/TMflox/flox) TM ablation and their respective wild-type controls (TMflox/flox and SM22-cre/¹/²/TMflox/flox) were generated. In the mouse CaCl₂-induced AAA model, deficiency of myeloid TM, but not vascular smooth muscle cell TM, inhibited macrophage accumulation, attenuated proinflammatory cytokine and matrix metalloproteinase-9 production, and finally mitigated elastin destruction and aortic dilatation. In vitro TM-deficient monocytes/macrophages, versus TM wild-type counterparts, exhibited attenuation of proinflammatory mediator expression, adhesion to endothelial cells, and generation of reactive oxygen species. Consistently, myeloid TM–deficient hyperlipidemic mice (ApoE²⁻/⁻/LysMcre/TMflox/flox) were resistant to AAA formation induced by angiotensin II infusion, along with reduced macrophage infiltration, suppressed matrix metalloproteinase activities, and diminished oxidative stress.

Conclusions—Membrane-bound TM in macrophages plays an essential role in the development of AAA by enhancing proinflammatory mediator elaboration, macrophage recruitment, and oxidative stress. (Arterioscler Thromb Vasc Biol. 2015;35:00-00. DOI: 10.1161/ATVBAHA.115.305529.)

Key Words: abdominal aortic aneurysm ■ monocytes ■ macrophages ■ myeloid cells ■ thrombomodulin

Abdominal aortic aneurysm (AAA), a chronic degenerative process of the abdominal aorta, is a life-threatening disease in the elderly population.¹,² Open surgery and endovascular repair have been the only 2 established therapies for the treatment of AAA. These 2 treatment modalities are currently recommended only when the aorta has reached 5.5 cm in diameter, the threshold leading to an upsurge in the risk of aneurysm rupture.¹,² Nevertheless, physicians remain incapable of modifying the natural history of AAA progression.¹,³ Understanding the underlying events promoting aneurysm formation may help develop potential therapeutic strategies that alter the disease course of AAA.

Macrophages are derived from monocytes that represent circulating members of the myeloid cell lineage.³ Recruitment of monocytes/macrophages and other inflammatory cells has been one of the key pathogenic features in AAA.¹,⁵ These macrophages may sustain vascular inflammation and extracellular matrix degradation through release of proinflammatory cytokines and matrix-degrading proteinases; enhancement of oxidative stress, leading to extensive infiltration of...
inflammatory cells; depletion of vascular smooth muscle cells (VSMCs); and destruction of elastin lamellae, all of which contribute to aneurysm development. Genetic modification of macrophage mediators or surface receptors, such as angio- poietin-like protein-2, neurofibrin, and CD14, leads to changes in proinflammatory cytokines, matrix metalloproteinases (MMPs), reactive oxygen species (ROS), and adhesive properties of macrophages, thereby modulating the phenotype of experimental AAA. Therefore, targeting vascular inflammation mediated by macrophages, the major inflammatory cells in AAA, may be a potential therapeutic approach for aneurysm pathologies.

Thrombomodulin (TM), constitutively expressed in endothelial cells (ECs) of the vasculature, was originally identified as a receptor of thrombin. TM is a membrane-bound glycoprotein structurally consisting of 5 domains, including an N-terminal C-type lectin-like domain, a domain with 6 epidermal growth factor-like structures, a serine- and threonine-rich domain, a transmembrane domain, and a cytoplasmic domain. The epidermal growth factor-like domain of TM provides the binding site for thrombin. The thrombin–TM complex may accelerate activation of protein C, a natural anticoagulant that inactivates coagulation factors Va and VIIIa, thereby inhibiting propagation of the coagulation cascades. Activated protein C exerts a broad range of anti-inflammatory effects, such as reduction of proinflammatory cytokines and attenuation of leukocyte–EC adhesion. In addition, TM also exhibits anti-inflammatory activities independent of activated protein C. Transgenic mice lacking the lectin-like domain of TM exhibited an increased sensitivity to tissue damage in sepsis and other experimental inflammatory models. Moreover, recombinant soluble forms of TM domains can suppress inflammatory responses via inactivation of complement or neutralization of high-mobility group box 1 and Lewis Y carbohydrate. Our previous works have demonstrated that recombinant soluble-form TM domains exert therapeutic effects in amelioration of various inflammatory disorders, including atherosclerosis, AAA, sepsis, and diabetic nephropathy.

Despite well-documented anti-inflammatory properties of soluble-form TM, the function and significance of membrane-bound TM in inflammatory responses remain largely undetermined. Studies have identified TM expression by monocytes and macrophages. The expression of membrane-bound TM in circulating monocytes in response to lipopolysaccharide stimulation or systemic inflammatory responses, such as cardiopulmonary bypass used for coronary artery bypass graft surgery and disseminated intravascular coagulopathy, has been evaluated, but the results and subsequent inferences were controversial. Using lineage-restricted transgenic mice, however, we recently showed that loss of monocytic membrane-bound TM, which triggers lipopolysaccharide- and gram-negative bacteria–induced inflammatory responses, may prolong survival in experimental acute sepsis. This discovery, inconsistent with the general understanding regarding anti-inflammatory functions of TM, raises the suspicion that membrane-bound TM in macrophages might positively regulate the molecular mechanisms underpinning the development of AAA and other macrophage-predominant chronic sterile inflammatory disorders.

In studies reported here, we attempted to gain insight into the significance of membrane-bound TM in AAA. We have investigated the hypothesis that membrane-bound TM in macrophages might be crucial in enhancing inflammatory responses during the development of aneurysm pathologies. Human abdominal aortic specimens were inspected for the potential relevance of membrane-bound TM in AAA. Also, the expression of TM was examined during the progression of experimental AAA in mice. Different cell lineage–restricted transgenic mice were generated to explore the impact of membrane-bound TM in mouse AAA models. In vitro studies were performed to explore the effects of membrane-bound TM deletion in macrophages on proinflammatory mediator production, cell adhesion, and ROS generation. These studies may help understand the functional significance of membrane-bound TM during the development of AAA.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
TM Is Expressed by VSMCs and Infiltrating Macrophages in Human AAA
First, we sought to investigate whether membrane-bound TM is relevant to human AAA. Double-immunofluorescent staining revealed that TM expression was highly restricted to intimal CD31-positive ECs but not in α-smooth muscle actin–positive VSMCs and CD68–positive macrophages in normal human abdominal aortic specimens (Figure 1A–1I). In human AAA specimens, however, no TM signal was found in intimal ECs. In contrast, TM expression was localized mainly to VSMCs and macrophages (Figure 1J–1R). These findings implicated a role for membrane-bound TM in AAA formation.

TM Is Localized in Macrophages and VSMCs During AAA Development in Mice
Transient application of CaCl₂ periaortically on the infrarenal aorta has been a common experimental mouse AAA model, with a consistent development of an aneurysm-like dilatation during a 28-day period. ECs, VSMCs, and macrophages are essential cellular components in the arterial and aneurysm wall. Herein we used double-immunofluorescent staining to determine which cell type is the major source of membrane-bound TM at different timings during CaCl₂-induced AAA formation.
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In wild-type C57BL/6J mice. In the normal abdominal aorta, TM expression was predominantly detected in CD31-positive ECs (Figure 2A–2C). No TM signal was observed in the media and adventitia (Figure I in the online-only Data Supplement). On day 7 after AAA induction, diminished TM expression was observed in intimal ECs (Figure 2D–2F). The expression of TM shifted to neoformed vessels in the adventitia (Figure 2F), α-smooth muscle actin–positive VSMCs in the media (Figure 2G–2I), and TM with CD68–positive macrophages that were mostly in the adventitia (Figure 2J–2L). On day 28, the expression of TM was rarely observed in ECs (Figure 2M–2O). The expression of TM in VSMCs was greatly reduced (Figure 2P–2R), and TM expression was mostly found in macrophages (Figure 2S–2U). In summary, TM expression was mainly localized in VSMCs and infiltrating macrophages during AAA formation in the CaCl₂-induced model.

Deficiency of Myeloid TM, not VSMC TM, Attenuates CaCl₂-Induced AAA Formation in Mice

A complete loss of TM function in mice inevitably causes embryonic lethality within 10 days post coitum before establishment of a functional cardiovascular system. Therefore, it is necessary to obtain transgenic mice with tissue-specific loss of TM to explore the role of membrane-bound TM in vivo. Transgenic mice with TM specifically ablated in myeloid cells (LysMcre/TMfloxflox mice, Mut) and their wild-type controls (TMfloxflox mice, WT) were generated to study whether membrane-bound TM in macrophages might contribute to CaCl₂-induced AAA formation. Immunoblotting confirmed that TM was eliminated from macrophages in LysMcre/TMfloxflox mice (Figure II in the online-only Data Supplement).

The aortic diameters in TMfloxflox and LysMcre/TMfloxflox mice on day 0 before CaCl₂ injury were similar (0.52±0.01 mm versus 0.53±0.01 mm, n=12; P>0.05). However, on day 28 after AAA induction, the aortic diameter was significantly greater in TMfloxflox mice than in LysMcre/TMfloxflox mice (0.97±0.06 mm versus 0.67±0.03 mm, n=12; P<0.001; Figure 3A). High concentrations of proinflammatory cytokines and increased infiltration of macrophages are prominent features in human AAA and the CaCl₂-induced model. At 3 days, no differences existed in the aortic levels of tumor necrosis factor-α (TNF-α), interleukin-6, and monocyte chemotactic protein-1 between TMfloxflox and LysMcre/TMfloxflox mice. At 7 days and 28 days after AAA induction, the levels of
these proinflammatory cytokines were increased in TM^floxed/flox^ mice. However, the increases in proinflammatory cytokines were suppressed in LysMcre/TM^floxed/flox^ mice (Figure 3B–3D). Histological analysis revealed that the MOMA-2–positive macrophage numbers in LysMcre/TM^floxed/flox^ and TM^floxed/flox^ mice were not different at 3 days (7.0±0.3 versus 8.6±1.0 per HPF, n=5; P>0.05; Figure 2E). At 7 days, the macrophage number in LysMcre/TM^floxed/flox^ mice was lower than that in TM^floxed/flox^ mice (7.2±1.0 versus 13.0±1.5 per HPF, n=5; P<0.05; Figure 3E). At 28 days, the MOMA-2–positive macrophages were detected abundantly in TM^floxed/flox^ mice, and significantly fewer macrophages were observed in LysMcre/TM^floxed/flox^ mice (30.5±4.7 versus 10.3±1.6 per HPF, n=6; P<0.01; Figure 3E and 3F). In summary, analysis of the aortic samples obtained at different time points revealed that the progression of proinflammatory cytokine levels and macrophage recruitment accompanying with aneurysm development was greatly retarded in myeloid TM–deficient LysMcre/TM^floxed/flox^ mice, suggesting that vascular inflammatory status was alleviated by myeloid TM deficiency.

During CaCl₂-induced AAA formation, MMP-9 and MMP-2 are both essential proteolytic enzymes for extracellular matrix degradation. Analysis of the aortic samples at 28 days showed that the levels of total MMP-9 and MMP-2 were lower in LysMcre/TM^floxed/flox^ mice than in TM^floxed/flox^ mice, although the difference in MMP-2 did not reach significance (Figure 3G). In parallel to the results in MMPs, Verhoeff–Van Gieson staining for aortic samples of TM^floxed/flox^ mice at 28 days after AAA induction (n=5 per time point). Nuclei are counterstained with DAPI (insets B, E, H, K, N, Q, and T). Arrow indicates a neoformed vessel. L indicates lumen. Scale bar, 50 μm.
in LysMcre/TM^lox/lox mice was mostly preserved (Figure III in the online-only Data Supplement). In summary, myeloid-specific TM-deficient LysMcre/TM^lox/lox mice were resistant to CaCl\textsubscript{2}-induced AAA formation, probably in association with reduction of macrophage accumulation, suppression of cytokine and MMP production, and attenuation of elastin degradation.

TM expression was found in VSMCs in human AAA and in the CaCl\textsubscript{2}-induced model. Subsequently, transgenic mice with TM specifically ablated in VSMCs (SM22-cre/TM^floxfloxflox mice, Mut\textsubscript{1}), and their wild-type controls (SM22-cre/+/+ mice, WT\textsubscript{2}) were also yielded to study whether membrane-bound TM in VSMCs might also be crucial in CaCl\textsubscript{2}-induced AAA formation. To determine the knockout efficiency of TM in VSMCs, aortic VSMCs from SM22-cretg/TM^floxfloxflox and SM22-cretg/TM^+/+ mice on day 3, day 7, and day 28 by ELISA (n=6–8 per time point), respectively. E. Macrophage numbers in WT, and Mut1 on day 3, day 7, and day 28 (n=6 per time point). F. Representative microscopic images of immunofluorescent staining for macrophages on day 28. Arrows indicate MOMA-2–positive cells. Nuclei are counterstained with DAPI. G. The levels of total matrix metalloproteinase (MMP)-9 and MMP-2 on day 28 by ELISA (n=5). H. Aortic diameter in SM22-cre/+/+(WT) and SM22-cre/+/+ (WT) mice on day 0 and day 28 after AAA induction. WT1=TM^floxfloxflox, Mut1=LysMcre/TM^floxfloxflox, Mut2=SM22-cretg/TM^floxfloxflox, n.s. P>0.05, *P<0.05, **P<0.01 compared WT1. Days after AAA induction. L indicates lumen. Scale bar, 50 μm.

Figure 3. Deficiency of myeloid thrombomodulin (TM), not vascular smooth muscle cells (VSMC) TM, attenuates CaCl\textsubscript{2}-induced mouse abdominal aortic aneurysm (AAA) formation. A. Aortic diameter in TM^floxfloxflox (WT1) and LysMcre/TM^floxfloxflox (Mut1) mice on day 0 and day 28 after AAA induction (n=12). B–D, The levels of tumor necrosis factor (TNF-α), interleukin (IL)-6 (C), and monocyte chemotactic protein-1 (MCP-1; D) in WT, and Mut, on day 3, day 7, and day 28 by ELISA (n=6–8 per time point), respectively.

In summary, deficiency of myeloid TM, but not VSMC TM, may confer protection against aortic dilatation induced by CaCl\textsubscript{2}, in agreement with our hypothesis that membrane-bound TM in macrophages is essential in the pathogenesis of AAA.

Genetic Blockade of TM in Macrophages Suppresses Production of Proinflammatory Mediators, Adhesion to ECs, and Generation of ROS

Infiltrating macrophages in AAA lesions have been identified as inflammatory macrophages and an important and primary source of proinflammatory mediators and oxidative stress. Thioglycollate (TG)-induced peritoneal macrophages are bone marrow–derived inflammatory cells that produce proinflammatory cytokines, suggesting that their behaviors may resemble those of infiltrating macrophages in AAA. Because membrane-bound TM in infiltrating macrophages was critical in CaCl\textsubscript{2}-induced AAA formation, TM
expression in inflammatory macrophages and in noninflammatory resident macrophages was assessed. As shown in Figure VI in the online-only Data Supplement, TG-induced peritoneal macrophages showed significantly increased TM expression compared with noninflammatory resident macrophages, suggesting that TG-induced peritoneal macrophages were a significant source of membrane-bound TM. We therefore used TG-induced peritoneal macrophages to explore possible mechanisms by which membrane-bound TM in macrophages mediates vascular inflammation during AAA formation.

TG-induced peritoneal macrophages were obtained from TM^lox/lox^ and LysMcre/TM^lox/lox^ mice to determine whether membrane-bound TM in macrophages might modulate production of proinflammatory cytokines and MMPs. As measured using quantitative reverse transcriptase real-time polymerase chain reaction, a significant reduction in mRNA levels of proinflammatory cytokines (tnf-α, il-6, and mcp-1) was observed in macrophages from LysMcre/TM^lox/lox^ mice compared with those from TM^lox/lox^ mice (Figure 4A). Although there was no difference in MMP-9 expression in peritoneal macrophages from the 2 genotypes, the expression of MMP-9 after stimulation with TNF-α was significantly lower in TM-deficient macrophages (Figure 4B). These data suggested that membrane-bound TM may regulate proinflammatory mediator production in macrophages.

Adhesion to ECs is the first and critical step in monocyte/macrophage recruitment. Whether membrane-bound TM in monocytes/macrophages might affect adhesion to ECs was then evaluated. In the cell adhesion assay, TG-induced TM-deficient peritoneal macrophages from LysMcre/TM^lox/lox^ mice exhibited a reduction in adhesion to the confluent EC monolayers composed of SVEC4-10 cells, a murine cell line of ECs, as compared with TM wild-type macrophages from WT1 mice. This difference was more apparent in monocyte/macrophage adhesion to ECs (without or with tumor necrosis factor [TNF]-α stimulation). The representative images are shown in C, and adhered macrophage numbers are counted (D, n=5). The fluorescence intensity of adhered cells was measured by an automated microplate reader (E, n=5). F, Representative flow cytometry profiles showing ROS levels in TG-induced peritoneal macrophages (n=4). WT, TM^lox/lox^; Mut, LysMcre/TM^lox/lox^ versus THP-1 cells carrying luciferase-specific short hairpin RNA, exhibiting a reduction in adhesion to the confluent EC monolayers composed of human umbilical vein ECs (Figure VII in the online-only Data Supplement). Taken together, these data suggested that membrane-bound TM mediates monocyte/macrophage adhesion to ECs.

Oxidative stress with ROS accumulation has been crucial in aneurysm formation. ROS, mainly produced from activated macrophages, are increased in human AAA and in the angiotensin II (AngII)–infused mouse model. Therefore, ROS production in TG-induced peritoneal macrophages from
LysMcre/TMfloxflox mice was analyzed by flow cytometry. A decrease in intracellular ROS production was found in TM-deficient macrophages from LysMcre/TMfloxflox mice as compared with TM wild-type macrophages from TMfloxflox mice (Figure 4F).

In summary, these findings suggested that membrane-bound TM in monocytes/macrophages may regulate proinflammatory cytokine and MMP-9 production, adhesion to ECs, and intracellular ROS generation.

Deletion of Myeloid TM Inhibits AngII-Infused AAA in ApoE−/− Mice

In addition to the locally created CaCl2-induced model, we used AngII-infused AAA model to determine whether deficiency of membrane-bound TM in macrophages might attenuate AAA formation. Therefore, myeloid TM–deficient mice on an ApoE−/− background (ApoE−/−/LysMcre/TMfloxflox mice, Mut3) and their TM wild-type controls (ApoE−/−/TMfloxflox mice, WT3) were generated. Immunoblotting confirmed that TM in macrophages was deleted in ApoE−/−/LysMcre/TMfloxflox mice (Figure VIII in the online-only Data Supplement).

Increase of arterial stiffness has been reported in the AngII-infused model.35 Before AngII infusion, arterial stiffness measured by pulse wave velocity was not different between ApoE−/−/TMfloxflox and ApoE−/−/LysMcre/TMfloxflox mice. After AngII infusion, ApoE−/−/TMfloxflox mice displayed higher pulse wave velocity values, whereas ApoE−/−/LysMcre/TMfloxflox mice showed no increase in pulse wave velocity values in relation to their respective basal pulse wave velocity values (Figure IX in the online-only Data Supplement). However, the systolic blood pressure and other physiological parameters in ApoE−/−/LysMcre/TMfloxflox and ApoE−/−/TMfloxflox mice were not different after AngII infusion (Table I in the online-only Data Supplement). The incidences of rupture-associated mortality in ApoE−/−/TMfloxflox and ApoE−/−/LysMcre/TMfloxflox mice (4 of 17 [24%] versus 2 of 13 [15%]; P>0.05) were not different during the 28-day period of AngII infusion. Notably, after 28-day AngII infusion, the maximal external diameter of the suprarenal aorta was significantly smaller in ApoE−/−/LysMcre/TMfloxflox mice than in ApoE−/−/TMfloxflox mice (1.29±0.09 mm versus 1.9±0.16 mm, n=11–13; P<0.01; Figure 5A). AngII infusion enhances vascular inflammation, MMP production, and oxidative stress in ApoE−/− mice.8,32,36 Histological analysis of the suprarenal aortic samples after 28-day AngII infusion revealed that MOMA-2–positive macrophages were abundant in the medial and adventitial layers in ApoE−/−/TMfloxflox mice. However, the macrophage number was markedly reduced in ApoE−/−/LysMcre/TMfloxflox mice (30.0±1.9 versus 10.4±2.5 per HPF, n=5; P<0.01; Figure 5B). The aortic levels of proinflammatory cytokines were diminished in ApoE−/−/LysMcre/TMfloxflox mice versus ApoE−/−/TMfloxflox mice (Figure XA in the online-only Data Supplement). The MMP production and activities in the AngII-infused aorta were determined using ELISA and in situ zymography. The aortic levels of total MMP-9 and MMP-2 in ApoE−/−/LysMcre/TMfloxflox mice were reduced in comparison with those in ApoE−/−/TMfloxflox mice (Figure XB in the online-only Data Supplement).
Supplement). The MMP activities in aortic sections were considerably high in ApoE−/−/TMflox/flox mice but barely detectable in ApoE−/−/LysMcrt/TMflox/flox mice (Figure SC; Figure XIA in the online-only Data Supplement). After the aortic sections were incubated with dihydroethidium, an oxidant-sensitive dye, the fluorescent signal was markedly higher in the ApoE−/−/TMflox/flox mice than in ApoE−/−/LysMcrt/TMflox/flox mice, suggesting deletion of myeloid TM–diminished aortic oxidative stress (Figure 5D; Figure XIB in the online-only Data Supplement). Likewise, the Verhoef–Van Gieson–stained aortic sections from AngII-infused ApoE−/−/TMflox/flox mice showed medial elastin degradation, but those from ApoE−/−/LysMcrt/TMflox/flox mice displayed well-defined elastic laminae (Figure XIA in the online-only Data Supplement). Consistently, the number of elastin breaks per aortic section was higher in ApoE−/−/TMflox/flox mice than in myeloid TM–deficient ApoE−/−/LysMcrt/TMflox/flox mice (Figure XIB in the online-only Data Supplement). Taken together, these findings, consistent with in vitro results, suggested that myeloid TM in macrophages promotes AngII-infused AAA in hyperlipidemic mice, possibly through its action on macrophage recruitment, proinflammatory mediator production, and ROS generation.

Together with the observations in the experimental AAA models in mice, these data suggest that macrophage inflammation mediated by membrane-bound TM might be considered a potential target to develop future pharmacotherapies for AAA.

**Discussion**

TM is originally considered a marker of normal ECs and may be dramatically downregulated in response to various inflammatory stimuli.

However, the expression or upregulation of TM in other cell types can be observed in different cardiovascular diseases. TM is upregulated in cardiomyocytes in patients with congestive heart failure and in mice after prolonged pressure overload.

The expression of TM in cultured VSMCs may be provoked by cyclooxygenase-2–derived prostaglandins, platelet-derived growth factor-BB, or several other substances.

In human atherosclerotic lesions, TM expression is increased in macrophages and VSMCs but is reduced in ECs overlaying the atherosclerotic plaque.

In addition, upregulation of TM has been observed in VSMCs in mouse carotid arteries after carotid ligation and transverse aortic constriction, implicating a role of TM in the process of arterial remodeling.

In studies presented here, we attempt to investigate the spatiotemporal expression patterns of membrane-bound TM during aneurysm formation. Because human specimens obtained from surgical repair usually represent the end stage disease of AAA, established mouse models that will recapitulate different aspects of cellular and biochemical characteristics observed in human pathologies have been considered to be a platform to recognize the sequence of biological events during AAA development.

As might be expected, TM was predominantly expressed in resting ECs in the normal aorta, and endothelial TM was reduced in response to the CaCl2 insult. Loss of VSMCs is a prominent feature in human AAA and in the CaCl2-induced model.

At the early stage, however, increased TM expression was observed in medial VSMCs. The short-term appearance of TM in VSMCs was followed by a lessening, probably in association with the quantitative reduction of VSMCs during the late stage of aneurysm formation. In contrast, TM expression in infiltrating macrophages sustained throughout the 28-day period of CaCl2-induced AAA development, compatible with the finding in human specimens. Circulating levels of soluble-form TM have been correlated to homocysteine levels and AAA sizes in a human study. Our observations on human and mouse AAs corroborate the participation of TM in AAA, as observed in other vascular disease entities, and provide a histological basis to study the functional significance of membrane-bound TM in VSMCs and macrophages, the 2 essential cell types involved in aneurysm development.

For this purpose, we created cell lineage–restricted transgenic mice to elucidate the exact cell type expressing TM that is critical for AAA. Although increased TM expression in medial VSMCs has been transiently observed after CaCl2-induced vascular inflammation, mice deficient of VSMC TM exhibited no significant effects on experimental AAA formation. In addition, the levels of proinflammatory cytokines, MMPs, and elastin destruction in the aortic wall were not attenuated in VSMC-specific TM-deficient mice. These data suggested that membrane-bound TM in VSMCs may not play a decisive role in aneurysm formation. Conversely, histological analysis for the mouse model and human specimens demonstrated that increased TM signals were predominantly located in infiltrating macrophages in the aneurysm lesion. Also, upregulation of TM was found in TG-induced inflammatory peritoneal macrophages versus noninflammatory resident counterparts. Most important, the mice lacking membrane-bound TM expression in the myeloid lineage displayed an aneurysm-resistant phenotype.

Macrophage-mediated inflammation during aneurysm development is featured with recruitment of monocytes/macrophages and production of a wide range of proinflammatory cytokines and proteases that may sustain chronic vascular inflammation and extracellular matrix destruction. Through 2 different mouse AAA models, we demonstrated that myeloid membrane-bound TM may participate and play a decisive role in vascular inflammation during AAA forma
tion. Taken together, these in vivo and in vitro findings suggested that, at several levels, membrane-bound TM in macrophages is likely to positively regulate macrophage inflammation. First, in vitro studies showed that deficiency of membrane-bound TM attenuated macrophage secretory products, including proinflammatory cytokines (TNF-α, interleukin-6, and monocyte chemotactic protein-1) and MMP-9. These mediators have been critical in macrophage chemotaxis and elastolysis during the development of AAA.

Consistently, the aortic levels of proinflammatory cytokines and MMPs were suppressed in myeloid TM–deficient mice, resulting in abolishment of macrophage recruitment and maintenance of vascular integrity. Membrane-bound TM in macrophages is critical in regulating chronic inflammation and proteolytic degradation of extracellular matrix, the major pathological features in AAA. Second, myeloid cell recruitment and infiltration of the aortic wall to initiate elastin destruction are important steps in aneurysm formation, and adhesion is the first and critical step in recruiting circulating leukocytes to specific sites of injury.

Blockade of membrane-bound TM in sterile inflammatory...
peritoneal macrophages and in THP-1 cells impeded monocyte/macrophage adhesion to quiescent and activated ECs. In vivo macrophage accumulation was reduced in mice deficient of membrane-bound TM, suggesting that membrane-bound TM in monocytes/macrophages may be involved in the macrophage recruitment into the aneurysm lesion. Third, deficiency of membrane-bound TM in macrophages suppressed intracellular ROS generation in vitro. In the AngII-infused model, myeloid TM deletion reduced the production of proinflammatory cytokines and ROS in vivo. Overproduction of ROS has been detected in human AAA lesions and in the mouse AAA model induced by AngII infusion. It has been reported that ROS may induce proinflammatory cytokine release in macrophages, and in turn, proinflammatory cytokines may activate macrophages to increase intracellular ROS. Membrane-bound TM may be critical in the production of both proinflammatory cytokines and ROS in macrophages, leading to the synergism that may accelerate AAA development. Based on these in vivo and in vitro observations, we propose potential mechanisms by which membrane-bound TM in macrophages may modulate macrophage inflammation during aneurysm formation and thus plays a pivotal role in AAA.

In a recent study, Schultz et al investigated whether deficiency of thrombin activatable fibrinolysis inhibitor (TAFI, or plasma procarboxypeptidase B), a natural fibrinolytic inhibitor mainly produced by the liver and activated by thrombin/TM complex, contributes to elastase-perfused AAA formation in mice. Using mice with whole-body knockout of TAFI, the authors demonstrated that TAFI deficiency enhances AAA as the result of increased plasmin generation. Based on their observations and the well-known relationship between thrombin and TM, it is tempting to speculate that TM is a cofactor to activate TAFI that confers protection against AAA. In the present study, however, we demonstrate the significance of membrane-bound TM that is specifically located in macrophages and VSMCs on aneurysm formation and proteolysis, thereby conferring protection against AAA development. Interestingly, exogenous administration of soluble recombinant TMD1 enhances lipopolysaccharides and bacterial clearance, suppresses inflammatory responses, and reduces the mortality rate in Klebsiella pneumoniae–challenged mice. However, in another study, mice lacking endogenous TMD1 are protected from sepsis caused by melioidosis, implicating a detrimental role for TMD1 in the host response to sepsis. Based on these observations, it is plausible that soluble recombinant TM domains at least in part act as decoy receptors, whereas membrane-bound TM could be a mediator in response to inflammatory stimuli. Our recent study has demonstrated that monocytic membrane-bound TM interacts with TLR4/CD14 complex and triggers gram-negative bacteria-induced inflammatory responses. Further studies are warranted to provide molecular mechanisms by which membrane-bound TM in macrophages mediates sterile inflammatory responses in detail.

In conclusion, this study demonstrates a novel concept that membrane-bound TM positively regulates macrophage inflammation during AAA formation. Through targeted deletions of membrane-bound TM, we have understood the differential impact of membrane-bound TM in macrophages and VSMCs on aneurysm development in this study. Membrane-bound TM is predominantly expressed in macrophages in AAA, and myeloid TM deficiency confers protection against aneurysm formation in different mouse AAA models. Membrane-bound TM mediates macrophage-driven vascular inflammation in AAA, implicating the potential therapeutic application in macrophage-predominant chronic disorders.

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Disclosures

None.

References


In this study, we focused on the significance of membrane-bound thrombomodulin (TM) in abdominal aortic aneurysm (AAA). We found that membrane-bound TM was localized to vascular smooth muscle cells and infiltrating macrophages in human AAA and in mouse AAA model. We thus generated lineage-restricted transgenic mice to show that TM deficiency in myeloid cells, not vascular smooth muscle cells, ameliorated CaCl2-induced AAA formation. In vitro studies showed that genetic ablation of TM in macrophages reduced proinflammatory mediator production, monocyte/macrophage adhesion to endothelial cells, and reactive oxygen species generation. Consistently, in the angiotensin II infusion mouse AAA model, myeloid TM–deficient hyperlipidemic mice displayed an aneurysm-resistant phenotype, along with diminished matrix metalloproteinase activities and oxidative stress. Our study demonstrates the differential impact of membrane-bound TM in macrophages and vascular smooth muscle cells in AAA and supports the concept that membrane-bound TM in macrophages tightly modulates macrophage inflammation during aneurysm formation, implicating the therapeutic potential in macrophage-predominant chronic disorders.
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Materials and Methods

Membrane-Bound Thrombomodulin Regulates Macrophage Inflammation in Abdominal Aortic Aneurysm

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Generation of LysMcre/TM\textsuperscript{flox/flox}, SM22-cre\textsuperscript{tg}/TM\textsuperscript{flox/flox} and ApoE\textsuperscript{-/-}/LysMcre/TM\textsuperscript{flox/flox} Mice

Myeloid- and VSMC-specific TM-deficient mice using the Cre-loxP system were generated as the following procedures. In brief, to obtain myeloid-specific TM-deficient mouse, TM\textsuperscript{flox/flox} mice were crossbred to LysMcre mice (The Jackson laboratory, stock number: 004781) to generate LysMcre/TM\textsuperscript{flox/+} mice,\textsuperscript{1} which were subsequently interbred to yield LysMcre/TM\textsuperscript{flox/flox} mice (Mut\textsubscript{1}) and their wild-type controls, TM\textsuperscript{flox/flox} mice (WT\textsubscript{1}).\textsuperscript{2} To obtain VSMC-specific TM-deficient mice, TM\textsuperscript{flox/flox} mice were crossbred to transgenic mice carrying a SM22-cre transgene (SM22-cre\textsuperscript{tg}, The Jackson Laboratory, stock number: 004746) to generate SM22-cre\textsuperscript{tg}/TM\textsuperscript{flox/+} mice, which were subsequently interbred to yield SM22-cre\textsuperscript{tg}/TM\textsuperscript{flox/flox} mice (Mut\textsubscript{2}) and their wild-type controls, SM22-cre\textsuperscript{tg}/TM\textsuperscript{flox/+} mice (WT\textsubscript{2}). LysMcre/TM\textsuperscript{flox/flox} mice, SM22-cre\textsuperscript{tg}/TM\textsuperscript{flox/flox} mice and their respective wild-type counterparts (TM\textsuperscript{flox/flox}, SM22-cre\textsuperscript{tg}/TM\textsuperscript{flox/+} mice) were used for the CaCl\textsubscript{2}-induced AAA model.

Myeloid-specific TM-deficient hyperlipidemic mice were generated as the following procedures. ApoE\textsuperscript{-/-} mice (The Jackson Laboratory, stock number: 002052) in a C57BL/6J background and LysMcre/TM\textsuperscript{flox/flox} mice were crossbred to generate ApoE\textsuperscript{-/-}/LysMcre/TM\textsuperscript{flox/+} mice, which were subsequently interbred to obtain ApoE\textsuperscript{-/-}/LysMcre/TM\textsuperscript{flox/flox} (Mut\textsubscript{3}) and their TM wild-type controls, ApoE\textsuperscript{-/-}/TM\textsuperscript{flox/flox} mice (WT\textsubscript{3}). These mice were used for the angiotensin II (AngII)-infused AAA model.

The sequences of primers used in genotyping for genetically engineered mice are listed in Supplemental Table II. Animal care conditions and animal experiments were approved by the Institutional Animal Care and Use Committee, National Cheng Kung University (approval number: 100245) and conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication #85-23, revised 1996).

CaCl\textsubscript{2}-induced and AngII-infused AAA Models

In the CaCl\textsubscript{2}-induced AAA model, male mice aged 10 to 12 weeks were anesthetized using intraperitoneal injection of sodium pentobarbital and underwent periaortic application with 0.5 M CaCl\textsubscript{2} on the infrarenal aorta for 15 minutes.\textsuperscript{3} At indicated time points, the aortic diameter was measured using a digital caliper before aortic harvest (from left renal artery to aortic bifurcation) for further analysis.

To establish the AngII-infused model, 6-month-old male ApoE\textsuperscript{-/-}/TM\textsuperscript{flox/flox} and ApoE\textsuperscript{-/-}/LysMcre/TM\textsuperscript{flox/flox} mice were subcutaneously infused with AngII (1000 ng/kg/min; Sigma-Aldrich, St. Louis, MO) via osmotic pumps (Alzet 2004; Durect, Cupertino, CA) and were fed a Western diet (0.15% cholesterol and 21% milk fat, 57BD; TestDiet, Richmond, IN) for 28 days.\textsuperscript{4} At 28 days, the maximum diameter of the suprarenal aorta was measured before aortic harvest.

Immunofluorescent Staining and Histological Analysis for Human Aortic Specimens

Human AAA specimens obtained retrospectively from patients undergoing conventional surgical repair. The paraffin-embedded tissue sections were retrieved from the tissue bank in National Cheng Kung University Hospital. Normal human abdominal aorta specimens obtained from autopsy (Pantomics, Richmond, CA; US Biomax, Rockville, MD; OriGene Technologies, Rockville, MD) were used for comparison. None of these individuals had connective tissue disorders. Sections (4
μm thick) were deparaffinized in xylene and rehydrated through a series of ethanol. For double-immunofluorescent staining, the sections were stained for anti-human TM (1:50; Atlas Antibodies, Stockholm, Sweden) with anti-human CD31 (1:50; GeneTex, Irvine, CA), anti-α-SMA (1:400; Sigma-Aldrich, St. Louis, MO) or anti-human CD68 (1:100; Dako, Carpinteria, CA) antibodies, followed by incubation with appropriate fluorescent secondary antibodies (1:200; Invitrogen, Carlsbad, CA). Nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI). Isotype IgG control (Calbiochem, San Diego, CA) slides were provided as negative controls to confirm the specificity of respective primary antibodies in human aortic specimens (Supplemental Figure XIII).

Collection of human tissue was approved by the Institutional Review Board of National Cheng Kung University Hospital (IRB approval number: A-ER-103-043).

**Immunofluorescent Staining and Histological Analysis for Mouse Aortas**

Abdominal aortas isolated from mice were embedded in optimum cutting temperature compound. Frozen sections (5 μm thick) were fixed with ice-cold acetone for 5 minutes. Sections were blocked using 5% goat serum in phosphate buffered saline (PBS) for 1 hour, followed by incubation in rabbit anti-mouse TM antibody with another primary antibodies against CD31 (1:50; Abcam, Cambridge, MA) for endothelial cells, α-SMA (1:400; Sigma-Aldrich) for vascular smooth muscle cells (VSMCs), MOMA-2 (1:200; Abcam) for macrophages, or respective isotype IgG controls (Calbiochem) overnight at 4°C. The rabbit anti-mouse TM antibody was generated as described previously.5 Alexa 488 and 546 conjugated secondary antibodies (1:200; Invitrogen) were applied to sections for 1.5 hours at room temperature. After repeated washes with PBS, nuclei were counterstained with DAPI. For in situ zymography, unfixed aortic cryosections were incubated with 40 μg/mL dye-quenched gelatin (DQ-gelatin; Invitrogen) as a substrate for 1 hour at 37°C. Serial sections that were co-incubated with 10 mM EDTA served as negative controls. To measure in situ oxidative stress, unfixed aortic cryosections were incubated with 5 μM dihydroethidium (DHE; Invitrogen) for 30 min at 37°C. Serial sections that were co-incubated with 20 mM ROS scavenger N-acetyl cysteine (NAC) served as negative controls. The fluorescent signal was captured using a fluorescence microscope (DP73; Olympus, Tokyo, Japan). Negative controls were shown in Supplemental Figure XIII.

Frozen sections were used for immunostaining or for macrophages (MOMA-2), elastin degradation [Verhoeff-Van Gieson (VVG) staining; Sigma-Aldrich]. In the angiotensin II (AngII)-infused AAA model, suprarenal aortic specimens were harvested from the ascending aorta to the iliac bifurcation, elastin breaks were counted at the maximal expansion of the suprarenal aorta (i.e., the segment between the last pair of intercostal arteries and the right renal branch). For each mouse, elastin break numbers were counted in 3 serial 5-μm sections and were then averaged. The mean value of elastin fragmentation from six mice in each group was presented.6 7 Digital images were analyzed using Image J software (version 1.43u). The macrophage numbers and elastin degradation were evaluated blindly by two independent observers.

**Quantification for Proinflammatory Cytokines and Matrix Metalloproteinases (MMPs) in Mouse Aortas**
Abdominal aortas isolated from mice were homogenized in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl and 1% NP-40) supplemented with 1X protease inhibitor (Roche, Madison, WI) and 1mM PMSF (Sigma-Aldrich). The bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) was used to determine the protein concentration of aortic extracts. A 2.5-μg homogenate from each aortic extract was applied to measure the concentration of tumor necrotic factor-α (TNF-α), interleukin-6 and monocyte chemotactic protein-1 (R&D Systems, Minneapolis, MN), MMP-2 and MMP-9 (Abnova, Taipei, Taiwan) using the enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions.

Isolation of Mouse Aortic VSMCs and Peritoneal Macrophages

Primary mouse SMCs from aortas of both SM22-cre<sup>tg</sup>/TM<sup>flox/flox</sup> mice and wild-type mice at the age of 10 to 12 weeks were isolated separately as described previously with minor modifications. Briefly, the aorta was perfused with PBS and was harvested from the aortic arch down to the aortic bifurcation. The aorta was rinsed with HBSS buffer (GIBCO BRL, Grand Island, NY) supplemented with 1% penicillin streptomycin (P/S; Sigma-Aldrich) and 0.25 μg/mL fungizone (Sigma-Aldrich) and was incubated in digestion buffer [(DMEM supplemented with 1.5 mg/mL type II collagenase (Worthington Biochemical, Freehold, NJ) and 50 mg/mL elastase (Sigma-Aldrich)] for 10 minutes at 37°C. The adventitial layer was pulled away from the medial layer, and the residual aortic tissue was further digested for 4 hours at 37°C. Cell suspension was harvested by centrifugation and was seeded into 12-well plate with 10% fetal bovine serum (FBS) and 1% P/S containing DMEM medium. The plate was left undisturbed for 96 hours at 37°C and the medium were changed every two days. On day 9 after isolation, TM expression in primary SMCs was detected by immunoblotting.

For isolation of inflammatory mouse peritoneal macrophages, mice at 8 to 12 weeks of age were intraperitoneally injected with 1 mL of 4% thioglycollate. Peritoneal macrophages were obtained from abdominal lavages with PBS at 4 days. Non-inflammatory resident peritoneal macrophages without thioglycollate stimulation from wild-type mice were also isolated. For purification of macrophages, isolated cells were seeded in culture dishes with 10% FBS and 1% P/S containing RPMI 1640 medium (GIBCO BRL) for 1 hour at 37°C. Non-adherent cells were removed by washing with serum-free medium, and adherent cells were used for further experiments. For detection of mmp-9 expression, thioglycollate-induced peritoneal macrophages from TM<sup>floxflox</sup> and LysMcre/TM<sup>flox/flox</sup> mice were treated with 30 ng/mL TNF-α or PBS for another 24 hours.

Quantification for TM in Cell Lysates

Lysates extracted from peritoneal primary macrophages, primary VSMCs or THP-1 cells with lysis buffer (Cell Signaling Technology, Danvers, MA) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, followed by electrophoretically onto a polyvinyl difluoride membrane. Membranes were blocked with 5% non-fat milk and were subsequently probed with specific antibodies against human TM (Santa Cruz Biotechnology, Santa Cruz, CA), mouse TM (Santa Cruz Biotechnology) and GAPDH (Santa Cruz Biotechnology).
RNA Isolation and Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA extraction of macrophages from TM^{flox/flox} and LysMcre/TM^{flox/flox} mice was performed with TRIzol reagent (Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. cDNA was synthesized by reverse transcription reaction for 60 minutes at 42°C. The reaction mixture contained 1.5 μg RNA, 5X M-MLV reverse transcriptase reaction buffer (Promega, Madison, WI), 1 mM dNTPs (Viogene, New Taipei City, Taiwan), 400 ng oligo(dT)_{15} primer (Promega), 20 units RNase inhibitor (Promega) and 200 units M-MLV reverse transcriptase (Promega).

The qRT-PCR mixture was prepared using 40-ng cDNA, 500 nM forward and reverse primers and SYBR Green Master Mix (Promega). The reactions were performed by ABI7500 sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Waltham, MA).

The abundance of mRNA was calculated using the ΔΔCt method with normalization to β-actin. The relative mRNA expression level of TM^{flox/flox} macrophages was arbitrarily set at 1 for normalization of that of LysMcre/TM^{flox/flox} macrophages. The sequences of primers used in qRT-PCR are listed in Supplemental Table III.9, 10

Transient Knockdown of TM in THP-1 Cells

Preparation of lentivirus-delivered short hairpin RNA and transfection were established as described previously.2 Briefly, pLKO.1-puro vector containing human TM-specific short hairpin RNA (shTM) was used for TM knockdown and luciferase-specific short hairpin RNA (shLuc) as a negative control. All lentiviral shRNA constructs were obtained from the National RNAi Core Facility in Taiwan. Human leukemia monocytic THP-1 cells were purchased from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan) and were maintained in 10% FBS and 1% P/S containing RPMI 1640 medium. Cells were transfected with medium containing recombinant lentiviruses with constructs and polybrene (8 μg/mL) overnight at 37°C, followed by selection using puromycin (2 μg/mL) for 3 days. The knockdown efficiency of TM in THP-1 cells was evaluated using immunoblotting.

Cell Adhesion Assay

SVEC4-10 cells, obtained from BCRC, are a simian virus 40-transformed mouse endothelial cell line. In the macrophage adhesion assay, SVEC4-10 cells, at a density of 10^5 cells/mL, were seeded in the 96-well plate (Nunc, Roskilde, Denmark) with 10% FBS and 1% P/S containing DMEM medium. After 16 hours, cells were activated with or without TNF-α (50 ng/mL) for 24 hours. Thioglycollate-induced peritoneal macrophages were labeled with calcein AM (Invitrogen) at a density of 10^6 cells/mL and were subsequently added to the confluent monolayers of SVEC4-10 cells. After 30 minutes, the plate was washed with serum-free DMEM medium for five times. The fluorescence intensity was measured by an automated microplate reader (SpectroMax M2e; Molecular Devices, Sunnyvale, CA) and adherent macrophage numbers was counted under the microscope. The average cell number was calculated from numbers obtained from four random fields.

The monocyte adhesion assay was performed as described above using THP-1 cells and human umbilical vein endothelial cells (HUVECs; Invitrogen) between passages 2 and 4. HUVECs were cultured in M199 medium (GIBCO BRL) supplemented with 20% FBS, 1% endothelial cell growth supplement (Sigma-Aldrich),
1% heparin and 1% P/S.

**Detection of Reactive Oxygen Species (ROS) by Flow Cytometry**

Mouse primary peritoneal macrophages were labeled with 5(6)-carboxyfluorescein diacetate (CFDA) according to the manufacturer’s protocol. Cells were washed with PBS for 3 times and intracellular levels of ROS were detected using flow cytometry. The geometric mean fluorescence intensity (MFI) was measured using WinMDI 2.9 software.

**Measurement of Systolic Blood Pressure**

The blood pressure in ApoE<sup>−/−</sup>/TM<sup>flox/flox</sup> mice and ApoE<sup>−/−</sup>/LysMcre/ TM<sup>flox/flox</sup> mice was recorded using the non-invasive tail-cuff device (BP-2000 System, Visitech Systems, Apex, NC). Mice were adapted to the device for 5 days. The measurements were performed before and at 3 weeks following AngII infusion for 3 consecutive days. Ten measurements of systolic blood pressure per day were averaged to yield a daily value in each mouse. Daily values were used for statistical comparisons.

**Pulse Wave Velocity (PWV) Measurement in Mice**

Mice infused with AngII for 27 days (one day before sacrifice and aortic harvest) were anesthetized with Tribromoethanol and were placed in supine position on a temperature-controlled electrocardiogram board (Indus Instruments, Houston, TX). A 20-MHz pulsed Doppler probe (Indus Instruments, Houston, TX) was noninvasively used to detect Doppler signals of blood flow at the aortic arch and abdominal aortic site. Aortic PWV in each mouse was calculated by dividing the distance between two measurement locations (4 cm apart) by the time difference between pulse arrivals relative to the R-wave of the electrocardiogram.\(^{11,12}\)

**Statistics Analysis**

The incidences of rupture-associated morality between two groups were compared using Fisher’s exact test. In this study, data were expressed as mean ± SEM. For comparisons between two groups, a Student’s t-test was used in data that passed both normality (Kolmogorov-Smirnov test) and equal variance (F-test); otherwise, a non-parametric Mann-Whitney U test was performed. For comparisons among multiple groups, one-way analysis of variance followed by post hoc analysis (Bonferroni test) was used in data that passed both normality and equal variance (Bartlett’s test); otherwise, a non-parametric Kruskal–Wallis test followed by Dunn’s post hoc analysis was used. The data with two variables examined (Figures 3B-E) were analyzed using two-way analysis of variance as these data passed normality and equal variance. Statistical analyses were performed using Prism 5 (GraphPad Software; San Diego, CA) and a \(P<0.05\) was considered statistically significant.
References


Supplemental Figure I. TM expression in the normal aorta in mice. Representative microscopic images of double-immunofluorescent staining (A-C) for TM with α-SMA (a marker for VSMCs) and (D-F) TM with MOMA-2 (a marker for macrophages) in the normal infrarenal aorta in mice (n=5). Nuclei are counterstained with DAPI (inserts B and E). L, lumen. Scale bar, 50 μm.
Supplemental Figure II. Characterization of macrophage-specific TM deletion. TM expression in isolated resident peritoneal macrophages from TM^{flox/flox} and LysMcre/TM^{flox/flox} mice was measured using immunoblotting. GAPDH was used as the loading control. The result is typical of those obtained in 3 independent experiments.
Supplemental Figure III. Elastin destruction in TM$^{\text{flox/flox}}$ (WT$_1$) and LysMcre/TM$^{\text{flox/flox}}$ (Mut$_1$) mice on day 28 after CaCl$_2$ injury. Representative microscopic images of VVG staining in WT$_1$ and Mut$_1$. Arrows indicate disrupted elastic lamella. WT$_1$=TM$^{\text{flox/flox}}$, Mut$_1$=LysMcre/TM$^{\text{flox/flox}}$. L, lumen. Scale bar, 50 μm.
Supplemental Figure IV. Validation of TM deletion in VSMCs. TM expression in cultured VSMCs from SM22-cre^{lg}/TM^{+/+} and SM22-cre^{lg}/TM^{flox/flox} mice was measured using immunoblotting. GAPDH was used as the loading control. The result is typical of those obtained in 3 independent experiments.
Supplemental Figure V

A

Supplemental Figure V. Proinflammatory mediator production and elastin destruction in SM22-cre^{tg}/TM^{+/+} mice (WT\textsubscript{2}) and SM22-cre^{tg}/TM^{flox/flox} (Mut\textsubscript{2}) mice on day 28 after CaCl\textsubscript{2} injury. A and B, The levels of TNF-\alpha, IL-6, MCP-1 (A), MMP-9 and MMP-2 (B) by ELISA (n= 4 or 5). C, Representative microscopic images of VVG staining. Arrows indicate disrupted elastic lamella. WT\textsubscript{2}=SM22-cre^{tg}/TM^{+/+} mice, Mut\textsubscript{2}=SM22-cre^{tg}/TM^{flox/flox} mice. n.s. P>0.05 compared with WT\textsubscript{2}. L, lumen. Scale bar, 50 \textmu m.
Supplemental Figure VI

**Supplemental Figure VI. Increased TM expression in TG-induced peritoneal macrophages.** TM expression in TG-induced and non-inflammatory resident peritoneal macrophages was measured using immunoblotting. GAPDH was used as the loading control. The result is typical of those obtained in 3 independent experiments. Relative TM expression in TG-induced and non-inflammatory resident peritoneal macrophages is shown in the right panel. *P<0.05 compared with non-inflammatory resident peritoneal macrophages.
Supplemental Figure VII

A

Knockdown efficiency of TM in THP-1 cells was measured by immunoblotting (n=4). GAPDH was used as the loading control.

B

Adhesion of calcein AM-labeled THP-1 cells to human umbilical vein endothelial cells (stimulated with or without TNF-α). Representative images are shown (B) and adhered macrophage numbers are counted (C, n=5). The fluorescence intensity was measured by an automated microplate reader (D, n=5). shLuc, luciferase-specific short hairpin RNA. shTM, TM-specific short hairpin RNA. n.s. P>0.05, *P<0.05, **P<0.01, ***P<0.001 compared with shLuc. Scale bar, 50 μm.
Supplemental Figure VIII. Validation of TM deletion in macrophages from hyperlipidemic mice. TM expression in isolated resident peritoneal macrophages from ApoE^{-/-}/TM^{flox/flox} and ApoE^{-/-}/LysMcre/TM^{flox/flox} mice was measured using immunoblotting. GAPDH was used as the loading control. The result is typical of those obtained in 3 independent experiments.
Supplemental Figure IX.

Supplemental Figure IX. Measurement of aortic stiffness in ApoE\(^{-/-}\) /TM\(^{flox/flox}\) (WT\(_3\)) and ApoE\(^{-/-}\)/LysMcre/TM\(^{flox/flox}\) (Mut\(_3\)) mice. Pulse wave velocity (PWV) was measured one day before AAA induction and day 27 after AngII infusion in both genotypes (n=6-9). WT\(_3\)=ApoE\(^{-/-}\)/TM\(^{flox/flox}\), Mut\(_3\)=ApoE\(^{-/-}\)/LysMcre/TM\(^{flox/flox}\). *\(P<0.05\) compared with WT\(_3\) 27 days after AngII infusion. #\(P<0.05\) compared with WT\(_3\) one day before AngII infusion. n.s. \(P>0.05\) compared with Mut\(_3\) one day before AngII infusion.
Supplemental Figure X

Supplemental Figure X. Aortic concentrations of proinflammatory mediators in ApoE\(^{-/-}\)/TM\(^{flox/flox}\) (WT\(_3\)) and ApoE\(^{-/-}\)/LysMcre/TM\(^{flox/flox}\) (Mut\(_3\)) mice on day 28 after AngII infusion. A and B, The levels of TNF-\(\alpha\), IL-6, MCP-1 (A), MMP-9 and MMP-2 (B) by ELISA (n=5 per group). WT\(_3\)=ApoE\(^{-/-}\)/TM\(^{flox/flox}\), Mut\(_3\)=ApoE\(^{-/-}\)/LysMcre/TM\(^{flox/flox}\). **\(P<0.01\), ***\(P<0.001\) compared with WT\(_3\).
Supplemental Figure XI. In situ zymography and in situ dihydroethidium (DHE) staining. A and B, Microscopic images of in situ zymography (A) and in situ DHE staining (B) from two additional individual mice in each group. The images of animal 1 in each group are shown in Figure 5C and 5D. WT<sub>3</sub>=ApoE<sup>–/–</sup>/TM<sup>flox/flox</sup>, Mut<sub>3</sub>=ApoE<sup>–/–</sup>/LysMcre/TM<sup>flox/flox</sup>. L, lumen. Scale bar, 50 µm.
Supplemental Figure XII. Elastin fragmentation in ApoE<sup>-/-</sup>/TM<sup>flox/flox</sup> (WT<sub>3</sub>) and ApoE<sup>-/-</sup>/LysMcre/TM<sup>flox/flox</sup> (Mut<sub>3</sub>) mice on day 28 after AngII infusion.

A, Representative microscopic images of VVG staining. Arrows indicate elastin breaks. B, Elastin breaks per vessel (n=6). WT<sub>3</sub>=ApoE<sup>-/-</sup>/TM<sup>flox/flox</sup>, Mut<sub>3</sub>=ApoE<sup>-/-</sup>/LysMcre/TM<sup>flox/flox</sup>. **P<0.01 compared with WT<sub>3</sub>. L, lumen. Scale bar, 50 μm.
Supplemental Figure XIII. Negative controls of immunostaining, in situ zymography and in situ dihydroethidium (DHE) staining. A-D, Representative microscopic images of immunostaining with respective isotype-matched IgG controls in (A) the human AAA, (B) the normal mouse aorta (in comparison with serial sections shown in Figures 2A-2C and Supplemental Figure I in the online-only Data Supplement), (C) the mouse CaCl₂-induced AAA (in comparison with serial sections shown in Figures 2M-2U), and (D) the AngII-induced mouse AAA. Nuclei are counterstained with DAPI. E, DQ-gelatin was used as substrates for tissue MMP activities. The serial section at the right panel was co-incubated with EDTA and served as a negative control. F, DHE was used as sensitive indicators for tissue oxidative stress. The serial section at the right panel was co-incubated with N-acetyl cysteine (NAC) and served as a negative control. L, lumen. Scale bar, 50 μm.
### Supplemental Table I

**Supplemental Table I.** The Body weight and systolic blood pressure before and after AngII infusion and the level of plasma cholesterol in ApoE⁻/⁻/TM^{flox/flo} and ApoE⁻/⁻/LysMcre/TM^{flox/flo} mice.

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<th>Systolic blood pressure (mmHg)</th>
<th>Plasma cholesterol (mg/dL)</th>
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<td>Baseline</td>
<td>AngII infusion</td>
<td>Baseline</td>
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<tr>
<td>ApoE⁻/⁻/TM^{flox/flo}</td>
<td>29.2±0.5</td>
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*** P<0.001 compared with baseline data within the same genotype group, n=6-9 per group.
**Supplemental Table II.** List of primers used in genotyping for genetically engineered mice.

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<th>Primer type</th>
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Supplemental Table III. List of sequences of primers used in quantitative real-time reverse transcription-polymerase chain reaction.

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