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Disruption of TGF-β signaling in smooth muscle cell prevents elastase-induced abdominal aortic aneurysm

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ABSTRACT

Transforming growth factor-β (TGF-β) signaling has been significantly implicated in the pathogenesis of aneurysm, prominently the initiation and progression of abdominal aortic aneurysm (AAA). Vascular smooth muscle cell (SMC) is the principal resident cell in aortic wall and is essential for its structure and function. However, the role of TGF-β pathway in SMC for the formation of AAA remains unknown. Therefore, the goal of the present study was to investigate the effect of TGF-β pathway in SMC for AAA pathogenesis, by using a genetical smooth muscle-specific (SM-specific) TGF-βtype II receptor (Tgfbr2) disruption animal model. Mice deficient in the expression of Tgfbr2 (MyhCre.Tgfbr2$^{f/f}$ and MyhCre.Tgfbr2$^{WT/f}$) and their corresponding wild-type background mice (MyhCre.Tgfbr2$^{WT/WT}$) underwent AAA induction by infrarenal peri-adventitial application of elastase. Fourteen days after elastase treatment, the aortas were analyzed and indicated that disruption of 1 or 2 alleles of Tgfbr2 in SMC provided markedly step-wise protection from AAA formation. And elastin degradation, medial SMC loss, macrophage infiltration, and matrix metalloproteinases (MMP) expression were all significantly reduced in Tgfbr2 deletion mice. Our study demonstrated, for the first time, that the TGF-β signaling pathway in SMC plays a critical role in AAA and disruption can prevent the aneurysm formation.

1. Introduction

Abdominal aortic aneurysm (AAA) is responsible for a significant number of death, and contributes to >15,000 operations a year in the United States [1]. At present, the incidence of AAA is 4–8% in men over 60 years old [2]. And with the possible introduction of more advanced screening techniques for AAA, more small AAA will be diagnosed. Surgery is currently considered the only appropriate therapy when AAA diameter exceeds 55 mm [3]. However, no pharmacology treatments have been proven effective in limiting AAA progression or reducing risk of rupture [4]. Insufficient understanding of the mechanisms underlying its pathogenesis limits the prevention and treatment of this disease.

TGF-β signaling has been implicated in many aspects of cellular performance with respect to aneurysm initiation and progression. Under defined circumstances, TGF-β signaling regulates innate immune responses [5–8], vascular inflammation, SMC growth or apoptosis [9,10], cellular differentiation or activation [11,12], and MMP-dependent proteolysis [13], and all of these activities are implicated in the pathogenesis of aneurysms. Despite decades of intensive study in TGF-β pathway, the net effects of this signaling in aneurysm pathogenesis and, perhaps more importantly, the application of its antagonism into therapeutic strategies, have engendered confusion and controversy.

Abbreviations: TGF-β, transforming growth factor-β; AAA, abdominal aortic aneurysm; MMP, matrix metalloproteinases; PPE, porcine pancreatic elastase; SMC, smooth muscle cell; Tgfbr2, TGF-β type II receptor; MCP1, monocyte chemoattractant protein 1; MIP1α, macrophage inflammatory protein 1α; SM, smooth muscle; ECM, extracellular matrix; TAA, thoracic aortic aneurysm.

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SMCs are the principal resident cells in the media of normal aorta and aneurysm, and are essential in maintaining aortic structure and functions through controlled proliferation and by secreting and maintaining a dynamic extracellular matrix (ECM) [14]. Furthermore SMC-derived factors are critical for recruiting and activating inflammatory cells in the initiation and progression of AAA [15–17].

However, as a critical factor for the formation of AAA, the role of TGF-β pathway in SMC remains unknown. Therefore, the goal of the present study was to investigate the effect of TGF-β pathway in SMC for AAA pathogenesis.

2. Methods

2.1. Mice

All animal experiments were approved by the Peking University Animal Care and Use Committee. The conditional SM-specific Tgfbr2-deletion mice on a C57BL/6 background were generously provided by George Tellides (Yale University School of Medicine, New Haven, CT). The genetically modified mice has been used successfully in our previous study to demonstrate SM-specific knockout of Tgfbr2 after tamoxifen injections [18]. All male mice (MyhCre.Tgfbr2$^{f/f}$, MyhCre.Tgfbr2$^{WT/f}$, and MyhCre.Tgfbr2$^{WT/WT}$) were treated by tamoxifen (Sigma–Aldrich) at 1 mg/d i.p. for 5 days starting at 5 weeks of age.

2.2. The mouse model of elastase-induced AAA

The mouse model of elastase-induced AAA has been previously described [19]. After tamoxifen induction, 8-week-old male MyhCre.Tgfbr2$^{WT/WT}$, MyhCre.Tgfbr2$^{WT/f}$ and MyhCre.Tgfbr2$^{f/f}$ mice were assigned randomly to two groups, one for porcine pancreatic elastase (PPE) and another for control, respectively. After anesthesia, a laparotomy was performed, and the abdominal aorta was isolated in situ. Then the infrarenal aorta (from just below the left renal vein to the iliac bifurcation) was bathed in either 10 μL of 100% elastase (Sigma–Aldrich) or heat-inactivated elastase (control) for 10 min. After elastase exposure, the wound was closed with 6–0 prolene. The maximal aortic diameters were measured in situ before elastase application and at harvest using a stereomicroscope (Carl Zeiss). The standard of AAA was defined as a 100% increase in maximal aortic diameter compared to baseline diameter before elastase treatment [20]. The abdominal aortas were harvested at 7 or 14 days after elastase treatment ($n = 20$ per treatment group per day) for analysis by histology, immunohistochemistry, and quantitative RT-PCR.

2.3. Histology and immunohistochemistry

Mice were euthanized by an overdose of pentobarbital and the aortas were perfusion-fixed with 4% paraformaldehyde via the left ventricle at physiological pressure. Then further fixed in 4% paraformaldehyde at 4 °C by overnight incubation followed by paraffin embedding and transversely sectioning at 5 μm. For histology, the sections were stained with hematoxylin-eosin staining (H&E), elastin–Van Gieson (EVG), and Masson's trichrome staining using standard techniques. For Immunohistochemistry, the sections were performed with primary antibodies to PCNA (ZSGB-BIO, China), CD68 (Proteintech, USA),α-actin (Sigma–Aldrich, USA), CD3 (Sigma–Aldrich, USA), MMP9 (Proteintech, USA) or isotype-matched, irrelevant IgG. Antibody binding was detected with GTVision Detection System (Gene Tech, China), counterstained with hematoxylin. And imaged using an Leica DFC DM4000B microscope (Leica Microsystems, Germany). The histological sections were analyzed by ImageJ software (http://rsbweb.nih.gov/ij/). Quantitation of immunohistochemistry was determined by the ratio of the number of positive cells to total number of hematoxylin positive nuclei in a defined field (400× magnification) on multiple slides (⩾10 slides per mouse) [21]. Measurements were conducted by two trained, independent observers blinded to genotype and treatment conditions.

In situ cell death detection was performed using an In Situ Cell Death Detection kit (Roche Biochemicals), according to the manufacturer's instructions.

2.4. RT-PCR

mRNA was extracted from frozen aortic tissue with TRIzol reagent (Invitrogen, USA) as previously described [22,23]. cDNA was synthesized with iScript cDNA synthesis kit (Bio-Rad, USA). RT-PCR was performed using Sensifast SYBR Supermix (Bioline, USA) with primers described previously [12,20]. Levels of mRNA were normalized to GAPDH. The experiments were performed in triplicate.

2.5. Statistical analysis

Data represent mean ± SEM when appropriate. Statistical tests included Fisher's exact tests, ANOVA, and Bonferroni's multiple comparison tests. P values ⩽0.05 were considered statistically significant. Data were analyzed using Prism 6.0 software for Mac (GraphPad Software).

3. Results

3.1. SM-specific deletion of Tgfbr2 prevents elastase-induced AAA formation

The goal of the present study was to investigate the effect of TGF-β pathway in SMC for AAA pathogenesis, by using the conditional SM-specific Tgfbr2 disruption mice in the elastase-induced model [19,24]. The genetically modified mice have been used successfully in our previous study to demonstrate SM-specific knockout of Tgfbr2 after tamoxifen injections (Fig. 1A and B) [18]. Deletion of SM-specific Tgfbr2 did not show any discernable differences in lamellar structure or aortic diameter of abdominal aorta. The maximal aortic diameter of MyhCre.Tgfbr2$^{WT/WT}$ mice at 14 days after elastase treatment was 1169 ± 26.57 μm. Whereas the maximal aortic diameter of MyhCre.Tgfbr2$^{f/f}$ mice was 652.5 ± 20.99 μm (Fig. 1C and D). Furthermore deletion of SM-specific Tgfbr2 markedly reduced the incidence of elastase-induced AAA, compared to MyhCre.Tgfbr2$^{WT/WT}$ mice (AAA incidence: 100% for MyhCre.Tgfbr2$^{WT/WT}$ versus 5% for MyhCre.Tgfbr2$^{f/f}$) (Fig. 1E). All MyhCre.Tgfbr2$^{WT/WT}$ or MyhCre.Tgfbr2$^{f/f}$ mice did not exhibit AAA when treated with inactivated elastase.

Aortas from inactivated elastase-treated MyhCre.Tgfbr2$^{WT/WT}$ mice were histologically indistinguishable from aortas from inactivated elastase-treated MyhCre.Tgfbr2$^{f/f}$ mice (data not shown). H&E staining showed substantial deterioration of aortic walls in the elastase-treated aortas of MyhCre.Tgfbr2$^{WT/WT}$ mice (Fig. 2A). Furthermore, in accordance with AAA phenotype, there were significant breakdown of elastin fibers and collagens in the aortas of the MyhCre.Tgfbr2$^{WT/WT}$ mice (Figs. 2B and S1). In contrast, the abdominal aortas from MyhCre.Tgfbr2$^{f/f}$ mice showed significantly reduced disintegration of elastin fibers and collagens and seemed to be histologically indistinguishable from those of inactivated elastase-treated mice (Figs. 2A, B and S1).

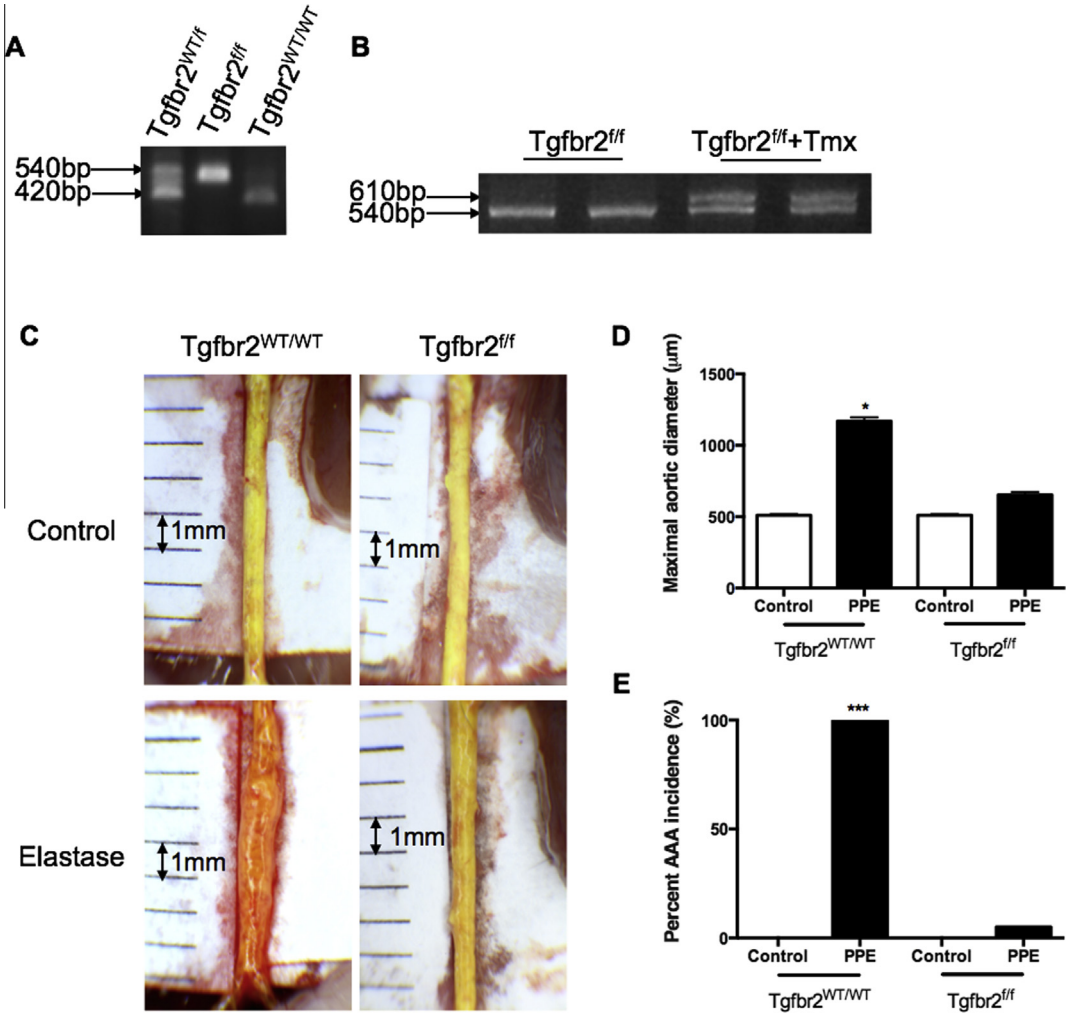

Fig. 1. Deletion of Tgfbr2 in smooth muscle cell prevents elastase-induced AAA formation. (A) Genotyping for Tgfbr2-loxP allele, the 420 bp band represents the wild-type allele, the 540 bp band represents the loxP allele, the presence of both bands indicates a mouse that is heterozygous for the loxP allele. (B) After tamoxifen treatment, successful disruption of Tgfbr2 is confirmed by the presence of a 610 bp band. (C) Gross appearance of abdominal aortas of MyhCre.Tgfbr2$^{WT/WT}$ and MyhCre.Tgfbr2$^{f/f}$ mice at day 14 after elastase or inactivated elastase treatment. (D) Maximal aortic diameter is shown for MyhCre.Tgfbr2$^{WT/WT}$ and MyhCre.Tgfbr2$^{f/f}$ mice at day 14 after elastase treatment. $^{*}P < 0.05$, $n \geqslant 10$ per group. (E) Percentage of AAA incidence at day 14 after elastase treatment. $^{***}P < 0.001$, $n \geqslant 10$ per group.

3.2. SM-specific deletion of Tgfbr2 attenuates proliferation and apoptosis of SMC

As the principal resident cells in the media of normal aorta and aneurysm, SMCs are essential in maintaining aortic structure and functions. Loss of SMCs together with macrophage infiltration, promotes extensive structural remodeling of abdominal aortic wall, characterized by downregulation of SMC marker expression and upregulation of apoptosis [25–28]. To determine the changes of SMC after Tgfbr2 disruption that may provide protection from AAA formation, we evaluated the proliferation and apoptosis of SMC via α-actin, PCNA and TUNEL expressions, respectively.

As determined by means of immunohistochemistry, α-actin staining was significantly reduced in the elastase-treated aortas of MyhCre.Tgfbr2$^{WT/WT}$ mice compared to that in MyhCre.Tgfbr2$^{f/f}$ mice (Fig. 2C). And aortas from inactivated elastase-treated MyhCre.Tgfbr2$^{WT/WT}$ mice were histologically indistinguishable from aortas from inactivated elastase-treated MyhCre.Tgfbr2$^{f/f}$ mice (data not shown).

In concert with the loss of SMC, proliferation and apoptosis were both enhanced in MyhCre.Tgfbr2$^{WT/WT}$ mice relative to that in MyhCre.Tgfbr2$^{f/f}$ mice, as shown by PCNA and TUNEL stainings (Fig. S2).

3.3. SM-specific deletion of Tgfbr2 reduces macrophage infiltration and MMP9 upregulation

Macrophage infiltration is an important event for AAA formation [29–31]. Therefore, in our study we examined the role of TGF-β signaling in SMC for macrophage infiltration during elastase-induced AAA formation. Immunohistochemical staining for the macrophage marker CD68 in the abdominal aortas from elastase-treated MyhCre.Tgfbr2$^{WT/WT}$ mice showed a significant infiltration of macrophages in the media and adventitia (Fig. 3A and B). However, CD68 staining was markedly reduced in the abdominal aortas of MyhCre.Tgfbr2$^{f/f}$ mice (Fig. 3A and B). And aortas from inactivated elastase-treated MyhCre.Tgfbr2$^{WT/WT}$ mice were histologically indistinguishable from aortas from inactivated elastase-treated MyhCre.Tgfbr2$^{f/f}$ mice (data not shown). In addition, we observed a significant increase in the mRNA expression for monocyte chemoattractant protein 1 (MCP1) and macrophage inflammatory protein 1α (MIP1α), that are known to be highly critical for macrophage infiltration [22], in the aortas of MyhCre.Tgfbr2$^{WT/WT}$ mice after elastase-treatment (Fig. 3C and D). In contrast, elastase treatment in MyhCre.Tgfbr2$^{f/f}$ mice did not significantly increase MCP1 and MIP1α expressions (Fig. 3C and D).

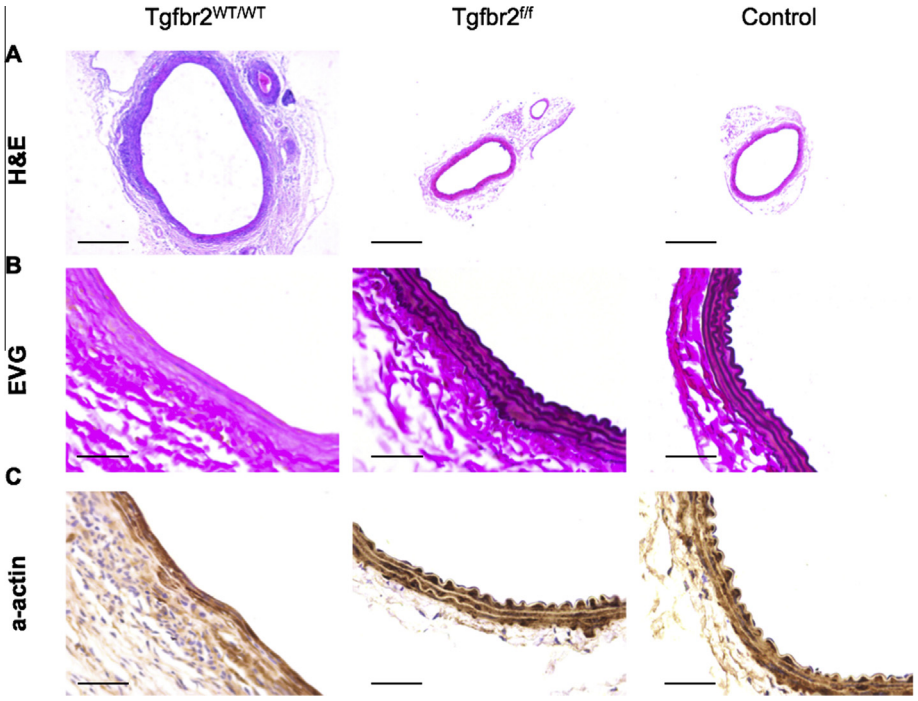

Fig. 2. Deletion of Tgfbr2 in smooth muscle cell prevents elastase-induced AAA formation. Representative photomicrographs of abdominal aortic segments from elastase-treated MyhCre.Tgfbr2$^{WT/WT}$ mice, elastase-treated MyhCre.Tgfbr2$^{f/f}$ mice and inactivated elastase-treated MyhCre.Tgfbr2$^{WT/WT}$ mice at day 14. (A) H&E staining (scale bars: 100 μm). (B) EVG staining (scale bars: 25 μm). Black staining indicates elastin. (C) Smooth muscle cell shown by SM α-actin staining (scale bars: 25 μm). Brown staining shows α-actin expression, and sections are counterstained with hematoxylin (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Along with macrophage infiltration, T lymphocyte is involved in AAA pathogenesis [32,33]. The infiltration of T lymphocyte was significantly increased in the elastase-treated aortas of MyhCre.Tgfbr2$^{WT/WT}$ mice (Fig. S3). In contrast, the aortas from MyhCre.Tgfbr2$^{f/f}$ mice showed markedly reduced T lymphocyte infiltration (Fig. S3).

MMP has been mechanistically involved in the pathogenesis of AAA. Furthermore MMP2 and MMP9, particularly, are demonstrated to have a critical role for AAA initiation and progression [34–36].

As shown in Fig. 3E and F, elastase treatment significantly increased MMP9 expression in MyhCre.Tgfbr2$^{WT/WT}$ mice. In contrast, the deletion of Tgfbr2 in smooth muscle cells markedly attenuated the increase of MMP9.

3.4. Heterozygous SM-specific deletion of Tgfbr2 results in attenuated aneurysm formation

As we have demonstrated that deletion of 2 alleles of Tgfbr2 in SMC shows significant prevention of AAA formation, we hypothesized that TGF-β signaling in SMC plays a critical role in aneurysm formation, and there is an expression-dependent effect of TGF-β on abdominal aortic dilation following elastase application. To test this, we used heterozygous SM-specific Tgfbr2 deletion mice.

Heterozygous Tgfbr2 deletion mice had evident reduction in maximal aortic diameter and incidence of AAA compared with MyhCre.Tgfbr2$^{WT/WT}$ mice (Fig. 4). Given the significant difference in AAA formation between MyhCre.Tgfbr2$^{WT/f}$ mice and MyhCre.Tgfbr2$^{WT/WT}$ mice, we examined the degradation of elastin fibers and collagens at 14 days after elastase induction. H&E, EVG and Trichrome stainings demonstrated significantly decreased elastin and collagen fragmentations in heterozygous Tgfbr2 deletion mice compared with MyhCre.Tgfbr2$^{WT/WT}$ mice (Fig. S4).

The preceding data indicated that deletion of just even 1 allele of Tgfbr2 in smooth muscle cell can significantly attenuate AAA formation and deficiency of TGF-β signaling in SMC can provide markedly step-wise protection from AAA formation. And the results demonstrated that TGF-β signaling pathway in SMC plays a critical role in AAA formation and is essential for the pathogenesis of elastase-induced AAA.

4. Discussion

In the present study, we investigated the role of TGF-β signaling pathway in SMC for the pathogenesis of AAA, and found that it is essential for its formation. Furthermore we demonstrated that disruption of Tgfbr2 in SMC can prevent elastase-induced AAA formation.

Despite decades of intensive study in TGF-β pathway, there are paradoxical discoveries on the role of TGF-β signaling for the development of aneurysm. TGF-β signaling elevation results in thoracic aortic aneurysm (TAA) in Marfan syndrome, and by incorporation of its systemic pan-specific TGF-β-neutralizing antibody many critical phenotypes could be attenuated [37,38]. Furthermore several studies suggested that increased TGF-β signaling is the cause of AAA and TAA [39,40]. However in contrast, systemic abrogation of TGF-β signaling deteriorated (rather than attenuated) AAA progression in an angiotensin II infusion model [33]. These seemingly contradictory discoveries could be less surprising in light of that the TGF-β pathway comprises molecules with diverse and often opposing functions. TGF-β signaling seems to act as a master upstream modulator regulates both pro- and anti-inflammatory pathways depending upon aneurysm localization (thoracic versus abdominal) and the principal types of cells (SMC versus inflammatory cell) that modulated by TGF-β signaling [18]. In this study, we showed that, contrary to its protective role in the formation of AAA

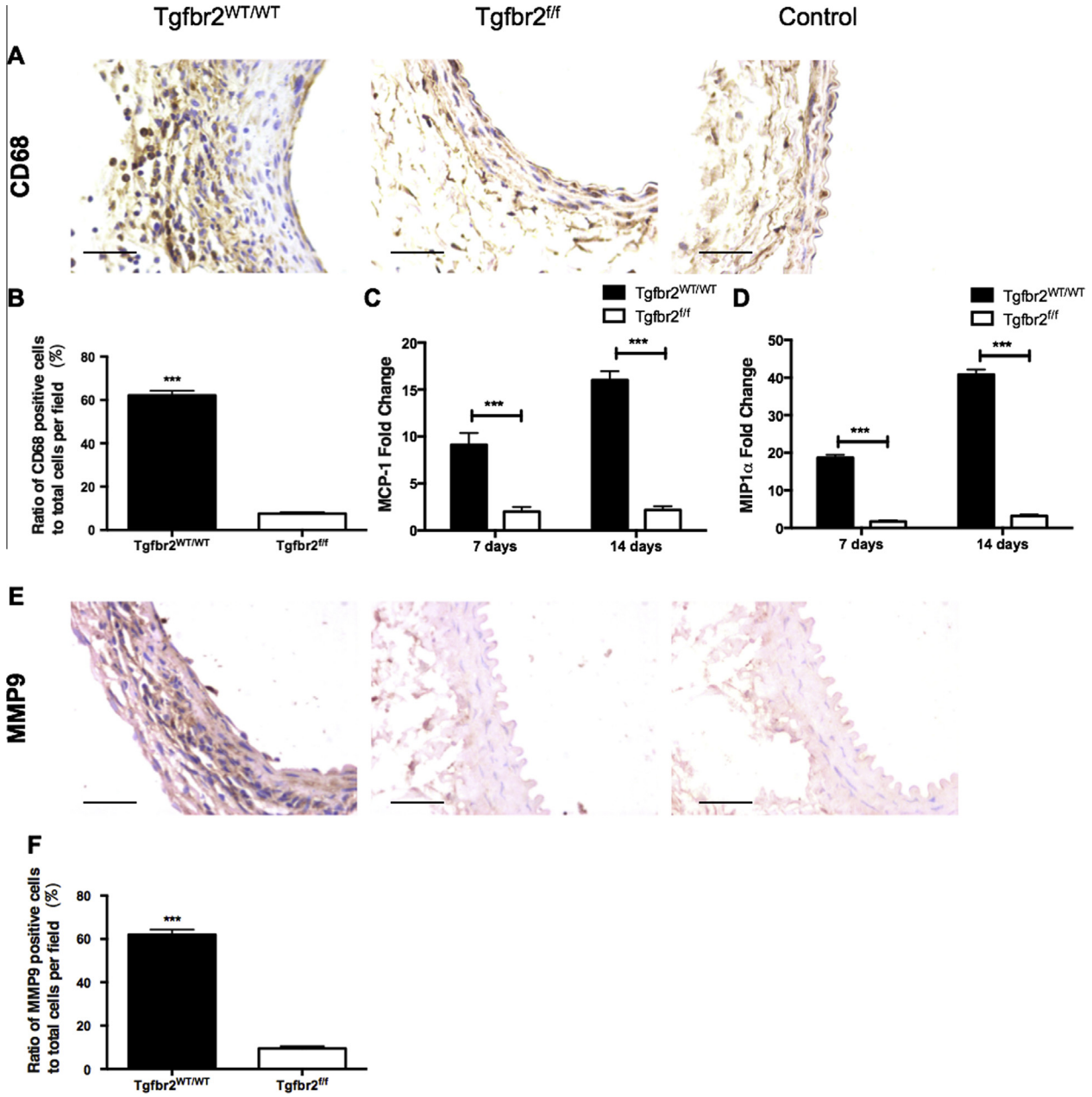

Fig. 3. SM-specific deletion of Tgfbr2 reduces macrophage infiltration and MMP9 upregulation. Representative photomicrographs of abdominal aortic segments from elastase-treated MyhCre.Tgfbr2$^{WT/WT}$ mice, elastase-treated MyhCre.Tgfbr2$^{f/f}$ mice and inactivated elastase-treated MyhCre.Tgfbr2$^{WT/WT}$ mice at day 14. (A) Brown staining indicates CD68 expression, and sections are counterstained with hematoxylin (blue) (scale bars: 25 μm). (B) Quantification of immunopositive cells. $^{***}P < 0.001$, $n \geqslant 10$ per group. (C) Quantitative PCR analysis of monocyte chemoattractant protein-1 (MCP-1) mRNA expression in the abdominal aortas of MyhCre.Tgfbr2$^{WT/WT}$ and MyhCre.Tgfbr2$^{f/f}$ mice at day 7 or 14 after elastase application. $^{***}P < 0.001$, $n \geqslant 10$ per group. (D) Quantitative PCR analysis of macrophage inflammatory protein1α (MIP1α) mRNA expression in the abdominal aortas of MyhCre.Tgfbr2$^{WT/WT}$ and MyhCre.Tgfbr2$^{f/f}$ mice at day 7 or 14 after elastase application. $^{***}P < 0.001$, $n \geqslant 10$ per group. (E) Brown staining indicates MMP9 expression, and sections are counterstained with hematoxylin (blue) (scale bars: 25 μm). (F) Quantification of MMP9 – immunopositive cells. $^{***}P < 0.001$, $n \geqslant 10$ per group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

associating systemic inhibition of TGF-β activity in an angiotensin II infusion model [33], TGF-β signaling in SMC contributes to the pathogenesis of elastase-induced AAA and disruption can prevent the aneurysm formation. Systemic neutralization of TGF-β activity might affects multiple types of tissues and cells, particularly both SMC and inflammatory cell, potentially resulting in variable effects on aneurysm formation. And in that study TGF-β activity probably protected against AAA mainly via immune system, especially monocytes/macrophages. By contrast, a disruption that isolates SM-specific TGF-β pathway might be particularly more informative regarding the pathway contributing to AAA. Furthermore, the pathogenesis of aneurysm involves progressive cycles of inflammation, apoptosis, and ECM proteolysis, and the products of this process drives subsequent inflammation and proteolysis. And SMC-derived factors may be an initial catalyst and an aneurysmal initiator [34,41].

There are several limitations in our study that must also be considered in further work for the role of TGF-β signaling in aneurysm formation. First, elastase treatment of the abdominal aorta has been argued to be an artificial acute model of AAA formation that differs from human chronic aortic aneurysm. Nonetheless, terminal cellular and enzymatic effectors of the ECM degradation and

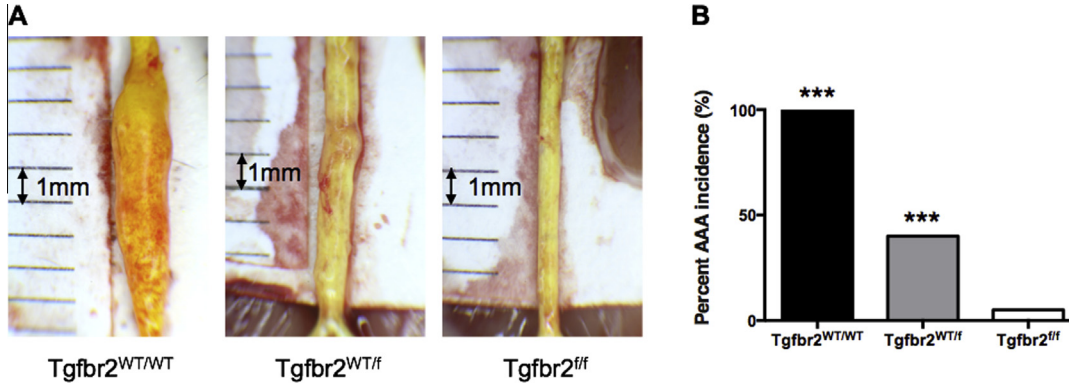

Fig. 4. Heterozygous SM-specific deletion of Tgfbr2 results in step-wise protection from AAA formation. (A) Representative aortas of MyhCre.Tgfbr2$^{WT/WT}$, MyhCre.Tgfbr2$^{WT/f}$ and MyhCre.Tgfbr2$^{f/f}$ mice at day 14 after elastase treatment. (B) Percentage of AAA incidence at day 14 after elastase treatment. $^{***}P < 0.001$, $n \geqslant 10$ per group.

macrophage infiltration appear to share similarities in this model and human AAAs, and this model recapitulates four of the key pathologic features of human AAA: intense local inflammatory infiltration and cytokine production [31], elevated expression of MMP [34], reduced SMCs [28], and ECM degradation. Second, this investigation was performed solely in vivo and therefore future experiments could seek to examine this hypothesis in vitro. Finally, the role of TGF-β signaling in inflammatory cell for AAA pathogenesis should also be explored in further studies.

5. Conclusion

In summary, our work addressed previously unanswered questions with respect to the role of TGF-β pathway in SMC for AAA pathogenesis and demonstrated that TGF-β signaling in SMC contributes to the pathogenesis of elastase-induced AAA and disruption can prevent its formation. Our study underscores the complex, controversial and context-dependent roles of TGF-β in aneurysm and clearly caution against the use of potent TGF-β agonists or antagonists for aortic aneurysms that may overenhance or oversuppress the signaling across multiple cell types. Furthermore the results suggest that targeting TGF-β signaling pathway in SMC may be a novel therapeutic strategy for preventing AAA.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.053.

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