Combination of TNFα Ablation and MMP Inhibition Prevents Heart Failure After Pressure Overload in TIMP-3 Mutant Mice

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Abstract—Cytokine and extracellular matrix (ECM) homeostasis are distinct systems that are each dysregulated in heart failure. Here we show that tissue inhibitor of metalloproteinase (TIMP)-3 is a critical regulator of both systems in a mouse model of left ventricular (LV) dilation and dysfunction. Timp-3$^{-/-}$ mice develop precipitous LV dilation and dysfunction reminiscent of dilated cardiomyopathy (DCM), culminating in early onset of heart failure by 6 weeks, compared with wild-type aortic-banding (AB). Timp-3 deficiency resulted in increased (TNFα converting enzyme) TACE activity within 6 hours after AB leading to enhanced tumor necrosis factor-α (TNFα) processing. In addition, TNFα production increased in timp-3$^{-/-}$-AB myocardium. A significant elevation in gelatinase and collagenase activities was observed 1 week after AB, with localized ECM degradation in timp-3$^{-/-}$-AB myocardium. Timp-3$^{-/-}$/tnf$\alpha^{-/-}$ mice were generated and subjected to AB for comparative analyses with timp-3$^{-/-}$-AB mice. This revealed the critical role of TNFα in the early phase of LV remodeling, de novo expression of Matrix metalloproteinases (MMP)-8 in the absence of TNFα, and highlighted the importance of interstitial collagenases (MMP-2, MMP-13, and MT1-MMP) for cardiac ECM degradation. Ablation of TNFα, or limiting MMP activity with a synthetic MMP inhibitor (PD166793), each partially attenuated LV dilation and cardiac dysfunction in timp-3$^{-/-}$-AB mice. Notably, combining TNFα ablation with MMP inhibition completely rescued heart disease in timp-3$^{-/-}$-AB mice. This study provides a basis for anti-TNFα and MMP inhibitor combination therapy in heart disease. (***Circ Res*****. 2005;97:0-0.)**

Key Words: left ventricular dilation and dysfunction ■ extracellular matrix ■ tissue inhibitor of metalloproteinase-3 ■ matrix metalloproteinase ■ tumor necrosis factor-α

Cardiovascular disease is the major cause of death in the Western world and is predicted to be the leading cause of mortality worldwide by 2020.[1] A close relationship between the severity of cardiac dysfunction, development of heart failure, and cardiac expression of tumor necrosis factor-α (TNFα) has been demonstrated.[2,3] TNFα is a pleiotropic cytokine and is found elevated in patients with dilated cardiomyopathy (DCM),[4,5] ischemic heart disease, and congestive heart failure (CHF).[3] Based on the potential importance of TNFα in heart disease, anti-TNFα therapy has been attempted in patients with heart failure although significant benefits of this therapy remain to be demonstrated.[6,7] This suggests that other factors play key roles in the progression of heart failure. Maladaptive extracellular matrix (ECM) remodeling is a common feature of ventricular remodeling in patients with DCM and CHF.[8] Matrix metalloproteinases (MMPs) are the primary ECM remodeling enzymes,[9] and another metalloproteinase, ADAM-17/TACE (TNFα converting enzyme) converts membrane bound TNFα to its soluble form.[10,11] Furthermore, TNFα signaling is known to induce the transcription of metalloproteinases,[9,12] evoking a potentially important but overlooked interaction between TNFα signaling and ECM remodeling. Whether a direct relation between cytokine and ECM homeostasis operates during the progression of heart failure is currently unknown. Such an interaction could help explain the lack of efficacy of TNFα-targeted therapy in heart disease. The discovery of factors that regulate both these systems is critical to designing novel treatments for heart disease.

Among tissue inhibitor of metalloproteinases (TIMPs), TIMP-3 is the only ECM-bound and the most expressed TIMP in the heart.[13] The classical role of TIMP-3, as with the other TIMPs, is inhibition of a broad spectrum of MMPs.[14] We have recently reported that TIMP-3 is a physiological inhibitor of TACE/ADAM-17, and regulates TNFα levels in liver homeostasis and injury.[15] Patients with DCM have

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significantly reduced levels of TIMP-3 levels,[16] and elevated levels of TACE and TNFα.[4,5,17] We have reported that mice deficient in timp-3 develop spontaneous DCM at 21 months of age.[18] In the current study, we investigated the consequence of TIMP-3 deletion in progression of heart disease following pressure overload in young mice, and dissected the relationship between cardiac TIMP-3, TACE, TNFα, and MMPs. Our study uncovers the concurrent regulation of cytokine bioactivity and matrix remodeling by TIMP-3, as well as the mechanistic integration between these systems that have so far been studied independently in cardiovascular disease. We also demonstrate that deterioration of cardiac structure and function is prevented by simultaneously targeting both TNFα and MMP activities. This study forms the basis for anti-TNFα and MMPi combination therapy for human heart disease, which individually provide only a partial rescue.

Materials and Methods

Pressure Overload and Cardiac Function

Eight-week-old wild-type (WT), timp-3$^{-/-}$, or TNF$\alpha^{-/-}$/timp-3$^{-/-}$ mice were subjected to pressure overload by constriction of descending aorta. Sham-operated mice from each group served as controls. In vivo Cardiac function was monitored by echocardiographic imaging, and confirmed by hemodynamic measurements. All animals were cared for in accordance with the Toronto Community Care Assess Centre (CCAC) guidelines. Complete Materials and Methods may be found in the online data supplement at http://circres.ahajournals.org.

Confocal and Electron Microscopy of Matrix Structure and Immunohistochemistry

Picocirius red-stained sections were visualized using 2-photon confocal microscopy. Electron microscopy was performed with a FEI CM100 Biotwin electron microscope. Apoptosis was assessed by TUNEL. Neutrophils were stained using rat anti-neutrophil.

Protein Analysis and Enzymatic Activity

Total gelatinase or collagenase activity was measured in myocardial homogenates using EnzChek assay kit using collagen type I as the substrate for collagenase activity. Pro- and active MMP-2 and MMP-9 were detected by gelatin zymography. TACE activity and Western blotting for TNFα was performed as described.[15]

TaqMan RT-PCR Analysis

RNA levels for the indicated genes were quantified by Real-time TaqMan RT-PCR.[13] 18S rRNA was used as an endogenous control. Details of sequences are in the online Materials and Methods.

In Vivo MMPi Treatment

An MMP-specific inhibitor, PD166793[19] (Pfizer Inc) was administered daily by gavage. PD166793 treatment (15 mg/kg/d) began 1 week before aortic-banding and continued until mice were euthanized.

An expanded Materials and Methods section is provided in the online data supplement.

Statistical Analysis

Survival between groups was compared by Kaplan-Meier survival analysis. All other comparisons were performed by ANOVA followed by multiple comparison testing (Student-Neuman Keuls). Values are reported as Mean±SEM. Statistical significance is recognized at $P<0.05$.

Results

Mortality Attributable to Heart Failure in TIMP-3–Deficient Mice

Timp-3$^{-/-}$ mice were subjected to cardiac pressure overload by aortic-banding (AB). These mice were compared with 3 groups of control littermates throughout the study, aortic-banded WT, sham-operated WT, and sham-operated timp-3$^{-/-}$ mice. Aortic-banding generated comparable pressure gradient (59 to 64 mm Hg) independent of the genotype, but caused significantly higher mortality in timp-3$^{-/-}$ mice compared with WT (Figure 1A). Six weeks following AB timp-3$^{-/-}$ mice exhibited significant morbidity and had to be euthanized. Timp-3$^{-/--}$ hearts were grossly enlarged within 1 week after AB and had increased left ventricular (LV) chamber size (Figure 1B).

To characterize the effects of pressure overload on WT and timp-3$^{-/-}$ hearts, we analyzed the cardiac function by echocardiography (Table). In WT-AB mice, significant cardiac dysfunction was seen at 6 weeks. In contrast, as early as 1 week after AB we observed dramatic LV dilation, reduced aortic outflow velocity, cardiac contractility, and velocity of circumferential fiber shortening in timp-3$^{-/-}$-AB mice. These parameters deteriorated progressively over 6 weeks (Figure 1C and 1D; Table). Notably, the extent of cardiac dysfunction in timp-3$^{-/-}$ mice after 6 weeks was comparable to WT mice after 12 weeks of aortic-banding (Table). Cardiac dysfunction was confirmed by in vivo hemodynamic measurements. Timp-3$^{-/-}$-AB mice had lower LV developed pressure (LVDP), higher end-diastolic pressure (LVEDP), and markedly suppressed LV peak rates of pressure-rise and pressure-fall ($\pm dp/dt$) compared with WT-AB (Figure 1E). Reduced $-dp/dt$ and increased LVEDP suggest diastolic dysfunction in timp-3$^{-/-}$ mice. Terminal heart failure in patients is associated with pulmonary congestion. We also observed pulmonary edema and fibrosis in timp-3$^{-/-}$-AB mice at 6 weeks (online Figure I). Altogether, advanced cardiac dysfunction as indicated by the extreme LV dilation and suppressed function in combination with pulmonary congestion demonstrates that unlike the WT mice, timp-3$^{-/-}$ mice develop rapid CHF 6 weeks after pressure overload. These features of cardiac dysfunction are reminiscent of DCM in patients.

Excess Hypertrophy with Timp-3 Loss

Next, we examined cardiomyocyte apoptosis that generally underlies LV dilatation. TIMP-3 deletion and overexpression are both linked to apoptosis.[20,21] Apoptosis in timp-3$^{-/-}$-AB LV was significantly higher than WT-AB at 6 weeks, suggesting that it is not responsible for the onset of LV dilation in these mice (Figure 2A and 2B). Baseline apoptosis was not different between sham groups. We investigated if myocyte hypertrophy occurred as a compensatory response to biomechanical stress. Heart weight-to-tibial length ratio was far greater in timp-3$^{-/-}$-AB than in WT-AB mice. We also measured myocyte cross-sectional area, length, and expression of prototypic markers of hypertrophy and heart disease (atrial natriuretic factor, ANF; brain natriuretic peptide, BNP). Although WT mice developed significant hypertrophy

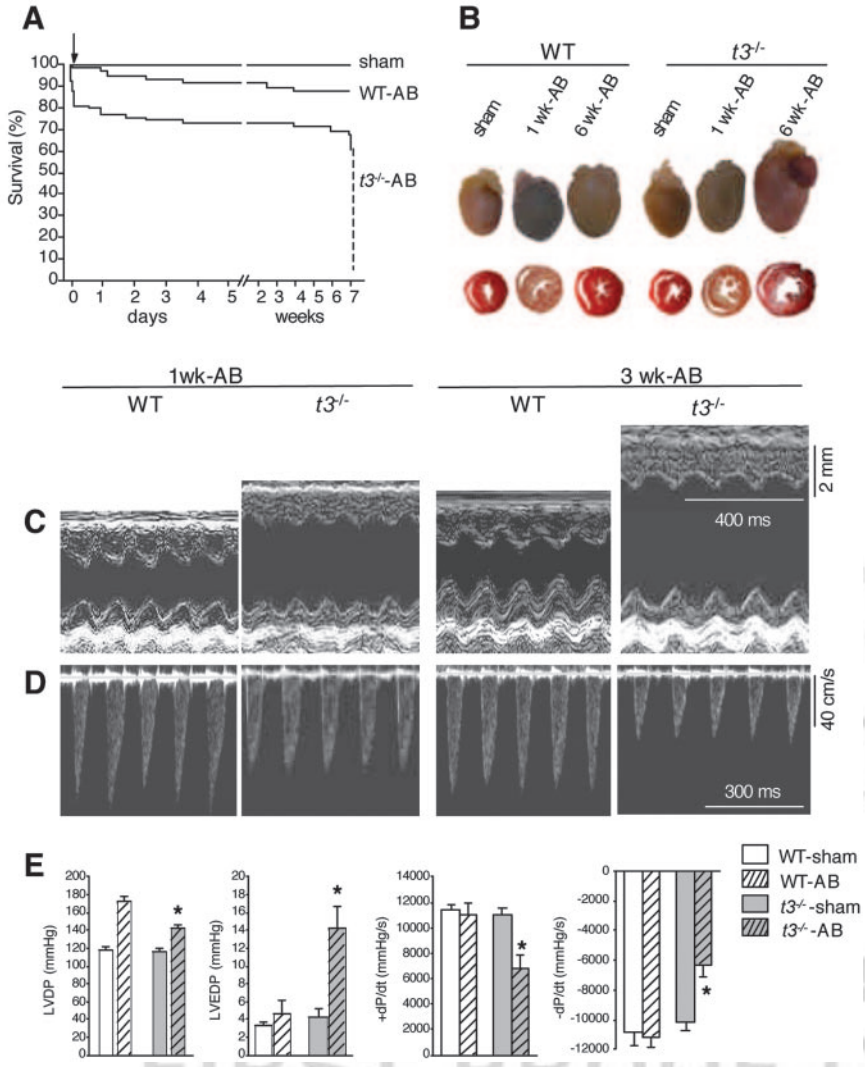

Figure 1. Severe LV dilation and dysfunction in timp-3$^{-/-}$ mice following pressure overload. A, Survival analysis of WT (n=56) and timp-3$^{-/-}$ (n=76) mice after aortic-banding (AB) or sham-operation (n=25/genotype). The arrow shows when aortic-banding was induced. The dash line indicates when aortic-banded timp-3$^{-/-}$ mice had to be euthanized ($P<0.05$ timp-3$^{-/-}$-AB vs WT-AB). B, Representative gross morphology of whole hearts and corresponding transverse cross-sections at mid-ventricle level. C and D, Representative M-mode LV echocardiographs (C) and aortic velocity profiles (D) after AB in WT and timp-3$^{-/-}$ hearts. F, In vivo hemodynamic measurements of LV developed pressure (LVDP=LVESP − LVEDP), LV end-diastolic pressure (LVEDP), and the peak rates of pressure-rise (+dP/dt) and pressure-fall (−dP/dt) after 3 weeks of sham (n=6) or AB (n=8) in WT and timp-3$^{-/-}$ mice. *$P<0.05$ compared with all other groups. t3 indicates timp-3.

over the 6-week period, timp-3$^{-/-}$-AB mice showed markedly greater hypertrophy as determined by higher levels of all hypertrophy parameters compared with WT-AB (Figure 2C through 2G). Increased myocyte cross-sectional area and length indicate a combination of eccentric and concentric hypertrophy in timp-3$^{-/-}$-AB hearts, whereas WT-AB hearts exhibit only concentric hypertrophy. This can explain LV dilation in timp-3$^{-/-}$-AB hearts in the absence of reduced LV wall thickness.

Excess TNFα and TACE Activity in Timp-3$^{-/-}$-AB Mice

In aged timp-3$^{-/-}$ mice, we proposed involvement of TNFα in the development of spontaneous DCM.[18] In a liver injury model, we have shown TIMP-3 is a physiological regulator of TNFα processing through inhibition of ADAM-17/TACE.[15] In pursuit of the mechanism underlying the early onset of severe heart disease following pressure overload in young timp-3$^{-/-}$ mice, we asked whether cardiac expression of the TIMP3-TACE-TNFα axis was affected. In WT hearts, TIMP-3 and TACE mRNA levels underwent parallel temporal changes after aortic-banding peaking at 3 weeks (Figure 3A and 3B). In contrast, in timp-3$^{-/-}$ hearts, TACE expression increased immediately after AB (within 6 hours, ≈1.5-fold, Figure 3B) concomitant with a 4-fold transient rise in TNFα mRNA (Figure 3C). TACE activity also increased within 6 hours (1.7-fold) in timp-3$^{-/-}$-AB hearts, whereas it rose much later (3 weeks) in WT-AB hearts (Figure 3D), temporally consistent with the increase in TACE expression. Addition of recombinant TIMP-3 (rTIMP-3), but not rTIMP-1 or a synthetic MMP-specific inhibitor (PD166793), inhibited the enhanced cardiac TACE activity in timp-3$^{-/-}$ mice (Figure 3E). TIMP-3-specific inhibition is considered signature of TACE.[22] TACE processes membrane-bound 26-kDa TNFα to its soluble 17-kDa species. Membrane-bound TNFα levels were higher in timp-3$^{-/--}$ compared with WT sham hearts. Following pressure overload, elevated membrane-bound and processed TNFα proteins were detected in timp-3$^{-/-}$ hearts (Figure 3F). Overall, along with a rapid increase in TACE activity, TACE and TNFα productions were accelerated and enhanced in timp-3$^{-/-}$-AB compared with WT-AB hearts, leading to amplified activity of this pathway.

Activation of the MMP Axis Perturbs ECM in Timp-3$^{-/-}$ Mice

TIMP-3 is known to inhibit a broad range of MMPs.[14] We determined whether TIMP-3 loss allowed for altered MMP

Echocardiographic and Morphometric Parameters in WT and t3^{-}/$^{-}$ Mice After Aortic-Banding

		WT-Sham	t3^{-}/$^{-}$ -Sham	WT-AB	t3$^{-/-}$ -AB
1 week	HR	533±11.4	527±836	534±10	543±7.8
	PW	0.55±0.01	0.54±0.02	0.71±0.02	0.68±0.03
	LVEDD	3.87±0.05	3.85±0.05	3.97±0.06	4.28±0.05*†
	LVESD	1.82±0.05	1.8±0.05	1.93±0.05	2.81±0.08*†
	FS	53.1±1.8	53.2±1.2	51.3±1.2	34.3±1.7*†
	VCF_c	10.8±0.3	10.9±0.31	10.2±0.3	6.59±0.24*†
	PAV_c	90.8±1.4	92.8±1.5	92.1±1.8	87.6±2.6
	HW/TL	5.8±0.1	5.4±0.1	7.8±0.4*	7.1±0.3*
	LVM/BW	0.32±0.01	0.34±0.01	0.45±0.04*	0.43±0.03*
3 weeks	HR	527±11.2	532±8.5	531±5.1	524±8.2
	PW	0.59±0.01	0.58±0.02	0.85±0.02‡*	0.85±0.03*
	LVEDD	3.91±0.06	3.92±0.05	3.78±0.07	4.79±0.08*†
	LVESD	1.9±0.05	1.88±0.04	1.83±0.05	3.58±0.17*†
	FS	51.5±0.9	52.2±1.1	50.8±1.3	25.5±2.6*†
	VCF_c	10.1±0.3	10.2±0.31	9.72±0.26	4.58±0.46*†
	PAV_c	95.7±1.2	95.4±1.7	94.5±1.3	72.2±2.1*†
	HW/TL	5.8±0.2	5.7±0.3	8.6±0.5*	10.4±0.7*†
	LVM/BW	0.31±0.02	0.33±0.02	0.48±0.05‡*	0.66±0.06*†
6 weeks	HR	545±8	538±11.5	542±9.1	535±15
	PW	0.63±0.01	0.62±0.02	0.81±0.03‡*	0.88±0.05‡*
	LVEDD	3.90±0.05	3.94±0.06	4.35±0.08‡*	5.38±0.11*†
	LVESD	1.86±0.05	1.87±0.05	2.57±0.08‡	4.31±0.21*†
	FS	52.2±1.1	52.5±0.7	40.8±1.6‡**	19.5±2.3*†
	VCF_c	10.3±0.26	10.4±0.38	7.81±0.24‡*	3.9±0.3*†
	PAV_c	96.4±2.6	95.9±1.8	84.8±2*	67.5±3.3*†
	HW/TL	6.6±0.1	5.4±0.3	9.2±0.5‡*	14.3±1.0‡*†
	LVM/BW	0.32±0.02	0.36±0.01	0.49±0.03*	0.81±0.10*†
12 weeks	HR	540±7	N/A	539±10.2	N/A
	PW	0.65±0.02		0.72±0.04	
	LVEDD	3.95±0.04		5.43±0.15‡*	
	LVESD	1.89±0.06		4.39±0.08‡	
	FS	52.6±1.5		18.8±2.9‡*	
	VCF_c	10.8±0.3		3.78±0.23‡*	
	PAV_c	97.0±2.8		74.2±2.‡9‡*	
	HW/TL	6.45±0.05		10.6±1.1‡*	
	LVM/BW	0.37±0.03		0.51±0.04*	

t3$^{-/-}$ indicates timp-3$^{-/-}$; HR, heart rate (bpm); PW, posterior wall thickness (mm); LVEDD and LVESD, left ventricular end diastolic and systolic dimension (mm); FS, fractional shortening (%); VCF_c, velocity of circumferential fiber shortening (circ/s); PAV_c, peak aortic velocity corrected for HR (cm/s); HW/TL, heart weight-to-tibial length ratio (mg/mm); LVM/BW, LV mass-to-body wt ratio (%). n=6 per sham group, n=8 per AB group. *$P<0.01$ vs sham groups. †$P<0.01$ vs WT-AB group. ‡$P<0.05$ vs 1 week within WT. N/A: data not available as timp-3$^{-/-}$ -AB mice do not survive beyond 6 weeks.

activity in response to pressure overload. Total gelatinase and collagenase activities markedly increased 1 week post-AB in timp-3$^{-/-}$ cardiac extracts compared with WT that displayed only collagenase induction (Figure 4A). The increased gelatinase activity in the timp-3$^{-/-}$-AB group was reduced by rTIMP-3 and PD166793, although collagenase activity was additionally lowered by rTIMP-1 (Figure 4A). In search of specific candidates for the increased MMP activity, we detected active MMP-2 in timp-3$^{-/-}$ hearts beginning 1 week after AB, with both latent and active MMP-2 increasing over 6-week period (Figure 4B). Next, we investigated the trimolecular complex, comprised of MMP-2, TIMP-2, and MMP-14/MT1-MMP, typically required for MMP-2 activation.[23] mRNA levels of MMP-2 and MT1-MMP were elevated in timp-3$^{-/-}$-AB hearts (Figure 4C). In addition, we found a striking increase of MMP-13 in timp-3$^{-/-}$-AB myocardium

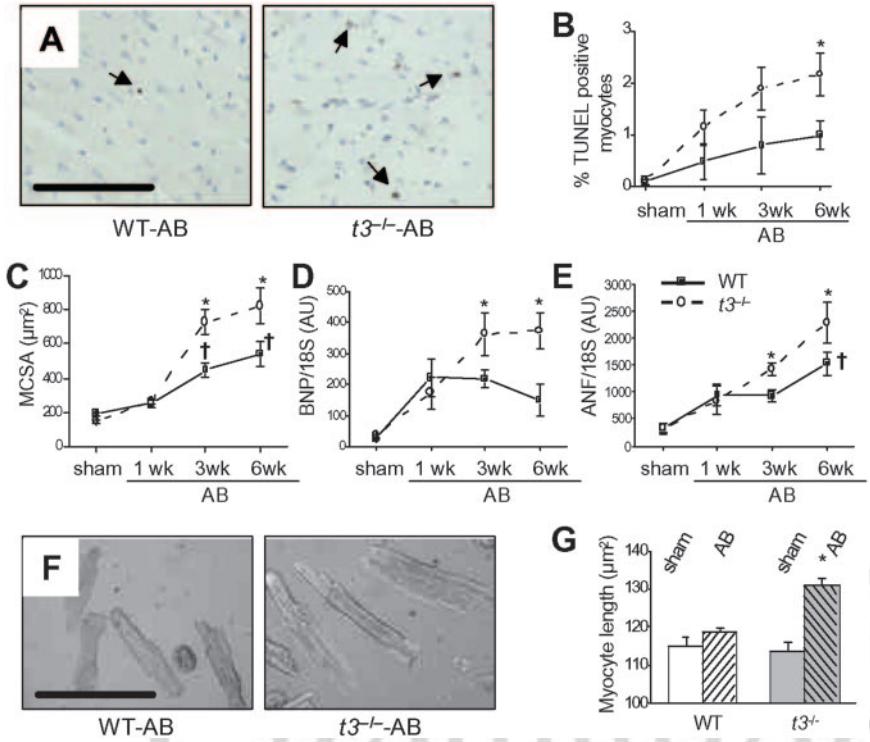

Figure 2. A, Representative TUNEL-stained WT and timp-3$^{-/-}$ LV 6 weeks after AB. B, Percent TUNEL-positive myocytes in WT and timp-3$^{-/-}$ LV myocardium at indicated weeks after AB (n=4 hearts, 900 to 1100 cells/group). C, Myocyte cross-sectional area (MCSA) in LV from WT-AB (n=75 cells), timp-3$^{-/-}$-AB (n=100 cells), or sham groups (n=50/genotype). D and E, mRNA levels of ANF, and BNP normalized to 18S RNA in WT and timp-3$^{-/-}$ hearts following sham (n=5) or AB (n=8). F, Representative isolated LV single myocytes. G, Averaged myocyte length in WT (n=120 cells) and timp-3$^{-/-}$ hearts (n=170 cells) before and after AB. *P<0.05 compared with WT group, †P<0.05 compared with 1 week within WT group. AU indicates arbitrary units. Scale bar=50 μm in A, 100 μm in F.

(Figure 4C). MMP-7 and MMP-8 were expressed minimally and remained unchanged post-AB (data not shown). MMP-9 mRNA increased over 6 weeks with no significant difference between WT and timp-3$^{-/-}$ hearts. Hence, 3 key interstitial collagenases,[19,24,25] MMP-2, MMP-13, and MT1-MMP, were elevated in response to pressure overload in timp-3$^{-/-}$ mice. Further, soluble myocardial TIMPs (TIMP-1, TIMP-2, or TIMP-4) were not elevated to compensate for the absence of ECM-bound TIMP-3 in sham-operated timp-3$^{-/-}$ hearts, although timp-1 expression increased significantly at 6 weeks post-AB (Figure 4C). Altogether, these data indicate that cardiac pressure overload in the absence of timp-3 leads to parallel but temporally differential activation of the 2 metalloproteinase axes, MMPs and ADAMs.

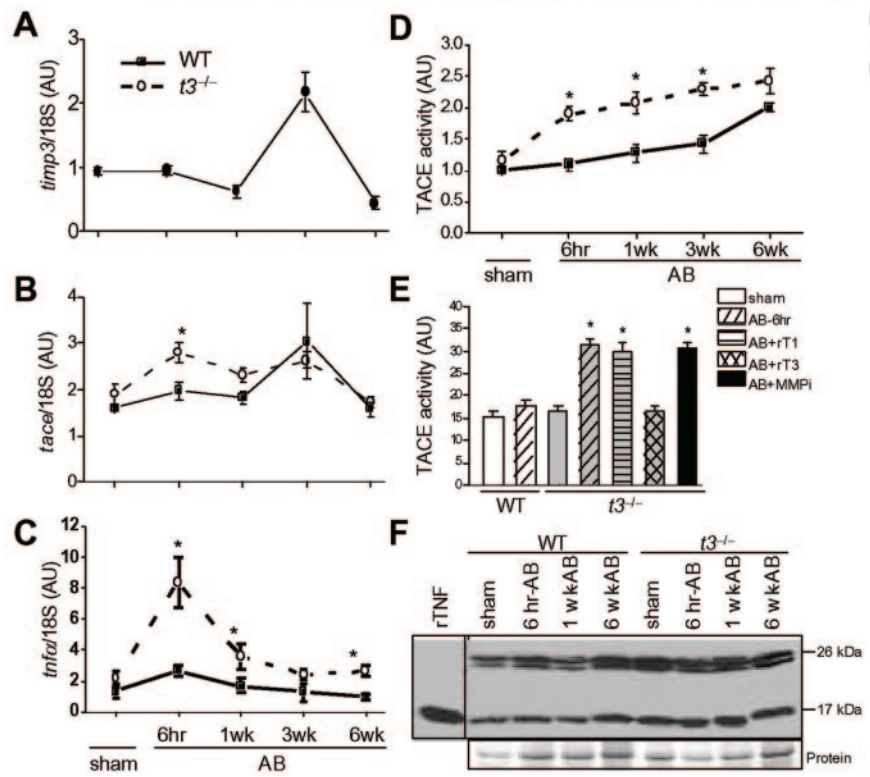

Figure 3. Absence of TIMP-3 results in increased activity of TACE/ADAM-17 and TNFα processing. A through C, mRNA levels of TIMP-3, TACE, and TNFα normalized to 18S post-AB in WT and timp-3$^{-/-}$ heart (n=8). D, Enzymatic activity of TACE in WT and timp-3$^{-/-}$ myocardium (n=5). E, Enhanced TACE activity in timp-3$^{-/-}$ hearts is blocked with 5 μmol/L recombinant TIMP-3 (rTIMP-3), but not rTIMP-1 or 100 μmol/L PD166793, a MMP-specific inhibitor. Gray columns represent the timp-3$^{-/-}$ group. F, Western blot showing membrane bound (26kDa) and cleaved (17kDa) TNFα protein before and after AB in WT and timp-3$^{-/-}$ hearts. rTNFα provided a positive control for cleaved TNFα, and protein loading was controlled for by silver-staining (bottom).

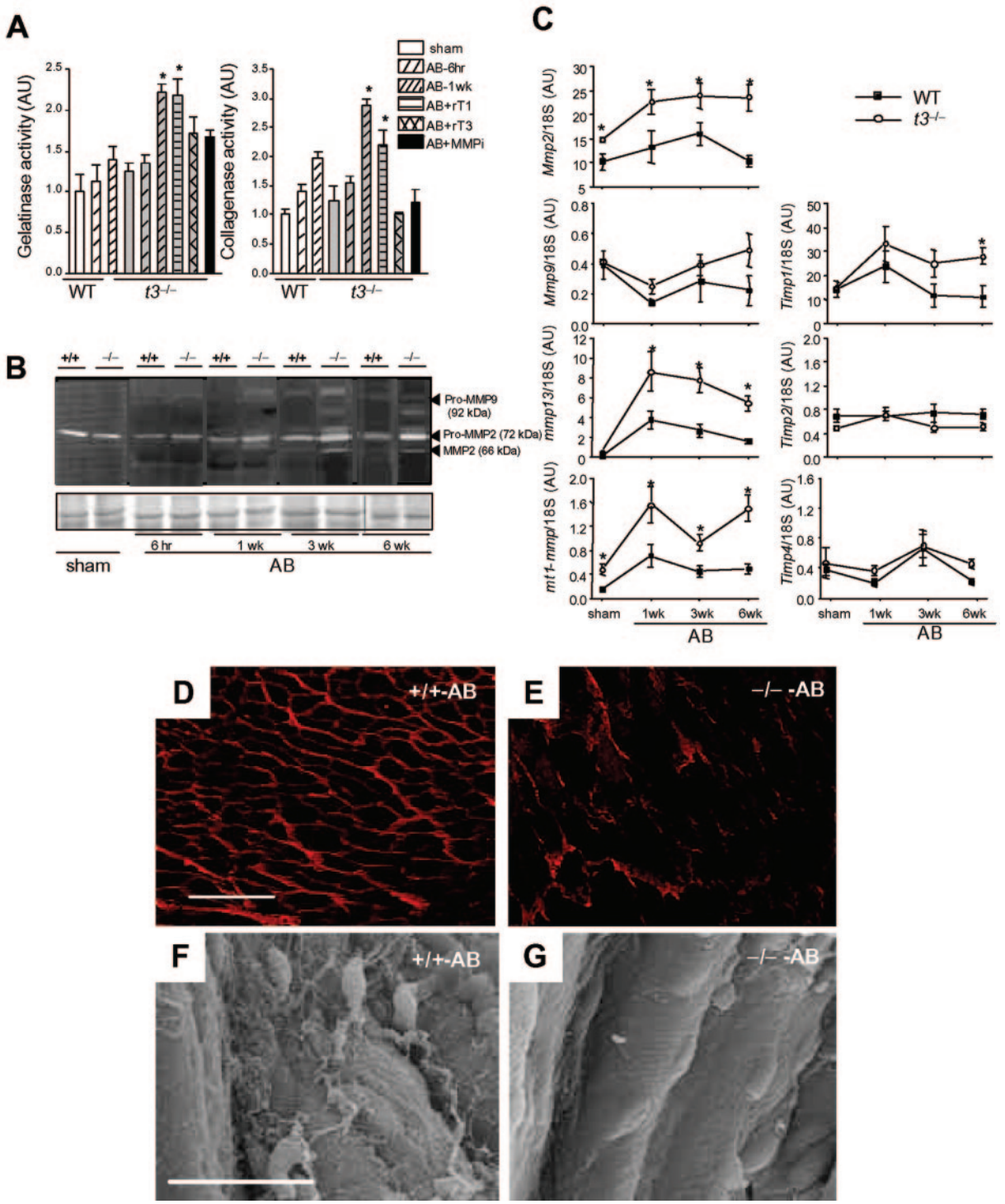

Figure 4. Enhanced MMP activity and ECM perturbation in timp-3$^{-/-}$-AB hearts. A, Total gelatinase and collagenase activity in WT and timp-3$^{-/-}$ hearts 6 hours and 1 week after AB. The enhanced gelatinase activity in timp-3$^{-/-}$-AB at 1 week was blocked by 5 μmol/L rTIMP-3 and 100 μmol/L PD166793, but not 5 μmol/L rTIMP-1, whereas the enhanced collagenase activity decreased significantly by all 3 interventions Gray columns represent the timp-3$^{-/-}$ group. B, Gelatin zymogram shows active MMP-2 levels in timp-3$^{-/-}$ hearts beginning at 1 week. Silver staining (bottom) was used as the protein loading control. C, Alterations in mRNA levels of MMPs and TIMPs normalized to 18S in WT and timp-3$^{-/-}$ hearts post-AB, n=5/sham, n=8/AB. *$P<0.05$ compared with WT. D and E, Confocal microscopy of Picosirius red-stained sections visualize fibrillar collagen in the WT-AB (D) and timp-3$^{-/-}$-AB LV (E). F and G, Representative images of the ECM network obtained by scanning electron microscopy in WT (F) and timp-3$^{-/-}$ LV (G) 3 weeks after AB. Scale bars=50 μm in D and E, 20 μm in F and G.

Next, we determined whether the enhanced MMP activity affected the myocardial matrix. Using confocal microscopy on Picocirius red-stained sections and scanning electron microscopy, we examined the integrity of the fibrillar component of myocardial ECM that provides normal structural support. It is primarily comprised of collagen types I and III.[26] Intact ECM

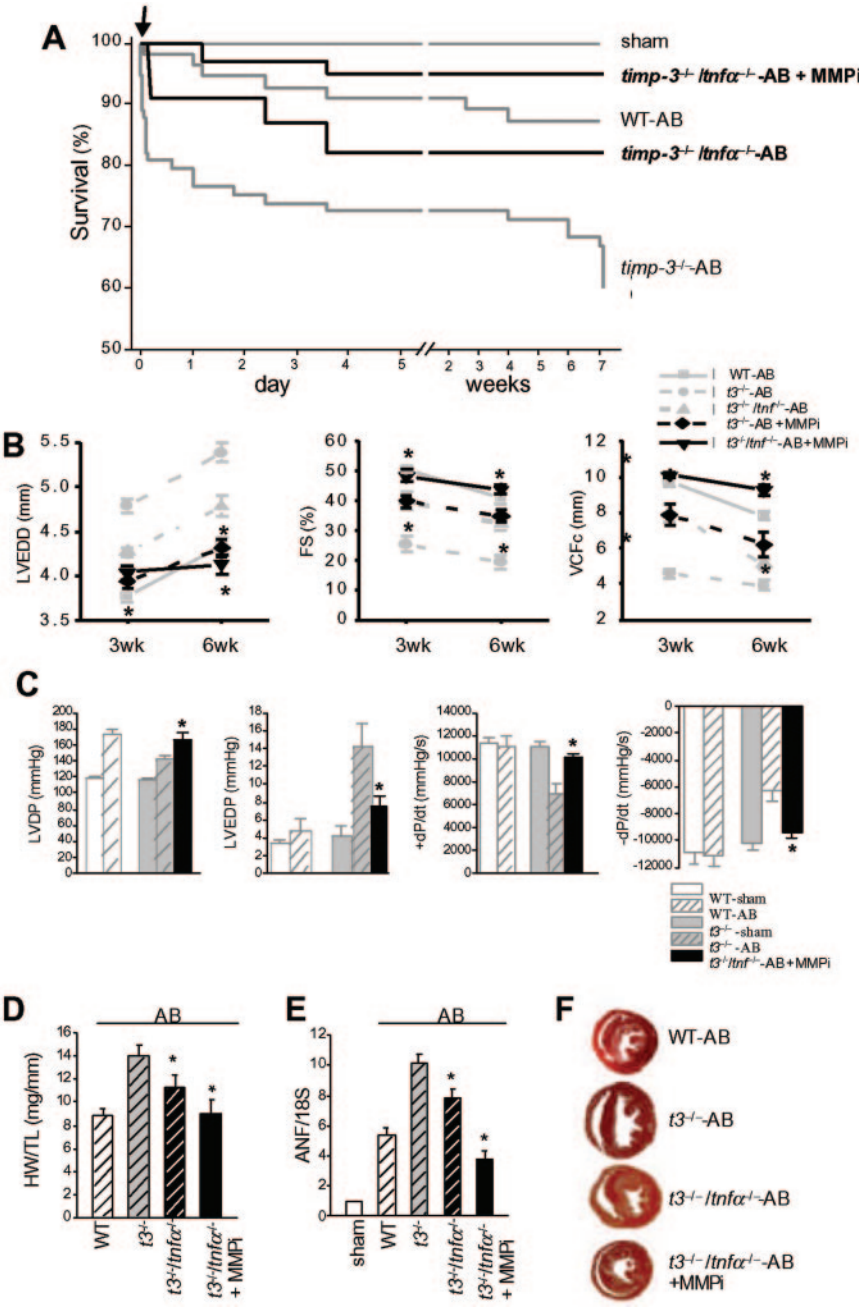

Figure 6. Cardiac structure and function is rescued when tnfα ablation is combined with MMP inhibition. A, Survival analysis of timp-3$^{-/-}$/tnfα$^{-/-}$-AB (n=30) and timp-3$^{-/-}$/tnfα$^{-/-}$-AB+MMPi (n=25) mice compared with all other groups ($P<0.05$ compared with timp-3$^{-/-}$-AB). B, Echocardiographic measurements of LV dilation (LVEDD), and cardiac contractility (FS and VCFc) in timp-3$^{-/-}$-AB mice following MMPi treatment alone (black broken line), and following combination of TNFα loss and MMPi treatment (timp-3$^{-/-}$/tnfα$^{-/-}$-AB+MMPi, black solid line), n=8/group. C, Hemodynamic measurements showing LVDP, LVEDP, and ±dp/dt in timp-3$^{-/-}$/tnfα$^{-/-}$-AB +MMPi treatment (black columns) compared with WT-AB and timp-3$^{-/-}$-AB groups. Gray lines and columns indicate data presented in earlier figures and are included here to allow comparison. D and E, heart weight-to-tibial length ratio and ANF mRNA levels in the indicated groups 6 weeks after AB. F, Representative gross morphology of transverse cross-sections of hearts 6 weeks after AB showing complete prevention of DCM when tnfα ablation was combined with MMPi treatment. *$P<0.05$ compared with timp-3$^{-/-}$-AB group.

network and uniform collagen distribution were seen in WT-AB hearts (Figure 4D and 4F), whereas we found areas devoid of or with disrupted fibrillar collagens in timp-3$^{-/-}$-AB myocardium (Figure 4E and 4G). Although WT-AB hearts showed interstitial fibrosis 6 weeks after AB, localized fibrotic areas were noticeably increased in timp-3$^{-/-}$-AB myocardium (data not shown). These findings are consistent with the maladaptive ECM remodeling found in patients with LV dilation.[27]

Genetic Deletion of TNFα Attenuates Cardiac Disease in Timp-3$^{-/-}$-AB Hearts

To determine whether TNFα is a critical molecule for LV dilation and dysfunction in timp-3$^{-/-}$ mice, we generated timp-3$^{-/-}$/tnfα$^{-/-}$ double mutants. After AB, survival was improved markedly in the double mutants compared with timp-3$^{-/-}$ mice (Figure 6A), although disease was delayed and partially rescued (Figure 5). Specifically, after only 3 weeks LV chamber size increased and cardiac contractility declined in timp-3$^{-/-}$/tnfα$^{-/-}$-AB mice, but was significantly attenuated versus timp-3$^{-/-}$-AB mice (Figure 5A through 5C). Cardiac hypertrophy in the double mutants also diverged from WT-AB group at 3 weeks, but remained far less pronounced than timp-3$^{-/-}$-AB hearts until 6 weeks (Figure 5D and 5E). No myocyte apoptosis (Figure 5H) or pulmonary congestion (Figure 5F and 5G) was detected in double mutants. Hence, genetically coupling TNFα ablation with TIMP-3 loss prevented CHF and attenuated heart disease in timp-3$^{-/-}$-AB mice.

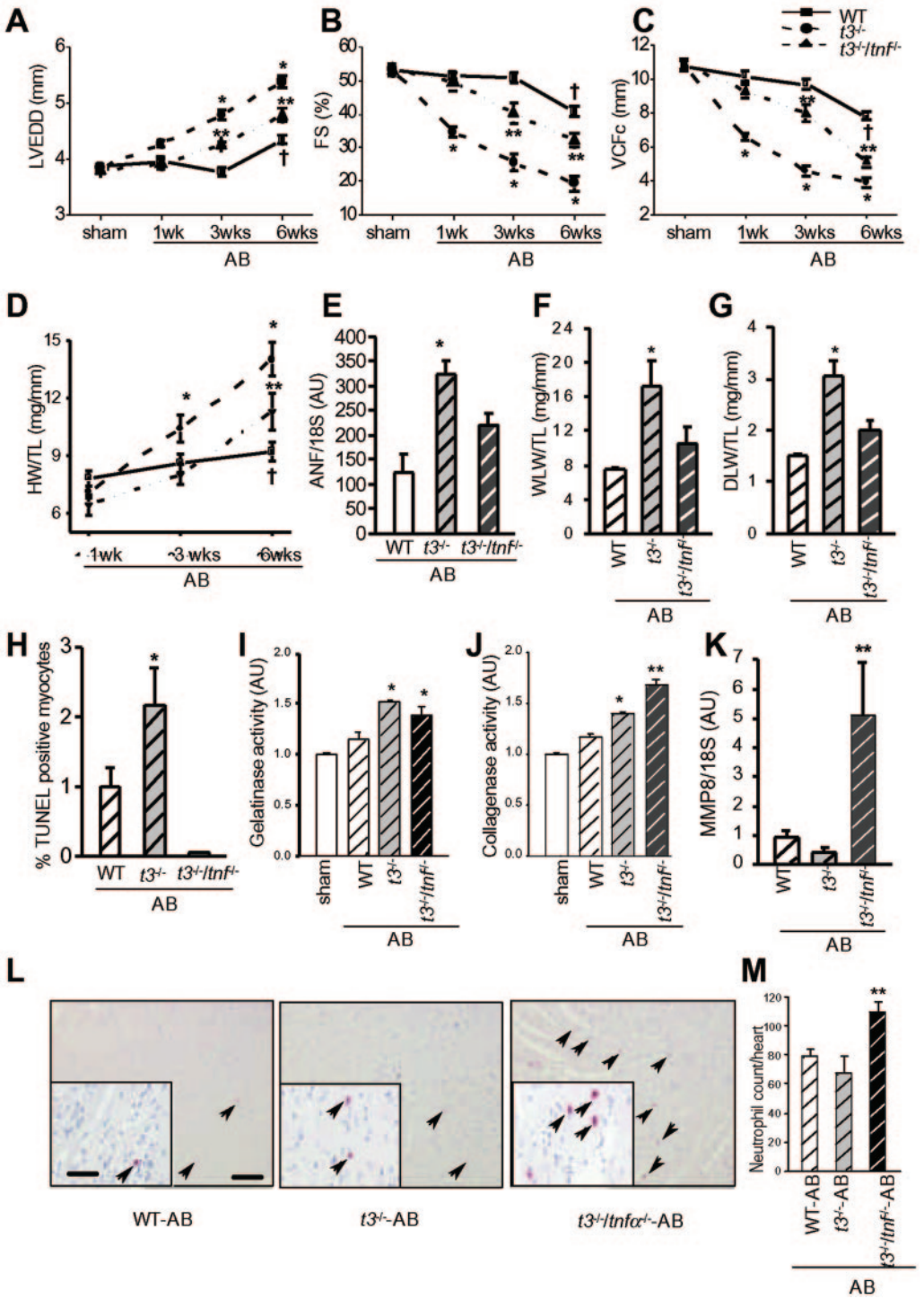

Figure 5. Ablation of TNFα partially rescues the cardiac disease in timp-3$^{-/-}$-AB mice. A through C, Echocardiographic measurements of LV dilation (LVEDD) and function (FS and VCFc) in timp-3$^{-/-}$/tnf$\alpha^{-/-}$, timp-3$^{-/-}$, and WT mice after AB. D through H, Heart weight-to-tibial length ratio (D), mRNA levels of ANF/18S (E), wet and dry lung weight-to-tibital length ratio (F and G), and myocardial apoptosis (H) 6 weeks after AB in the indicated groups. I and J, Total gelatinase and collagenase activities 3 weeks after AB in the indicated groups. K, MMP-8 mRNA levels 3 weeks after AB. L, Immunostaining for neutrophils in LV myocardium (arrowheads indicate positive staining). M, Averaged neutrophil count/heart. *$P<0.05$ compared with WT, **$P<0.05$ compared with all other groups, †$P<0.05$ compared with 1 week within WT group. Scale bar=100 μm, and 10 μm (inset).

Dependency of MMP Activity on TNFα

Because TNFα is a transcriptional inducer of MMPs,[9,12,28] we explored the TNFα-dependency of MMP expression by comparing MMP activity and expression profiles between timp-3$^{-/-}$/tnf$\alpha^{-/-}$-AB and timp-3$^{-/-}$-AB myocardium. Surprisingly, collagenase but not gelatinase activity was significantly higher in timp-3$^{-/-}$/tnf$\alpha^{-/-}$-AB than in timp-3$^{-/-}$-AB hearts, with both activities remaining higher than WT-AB (Figure 5I and 5J). We further investigated contribution of specific MMPs in these mice (online Figure II). Overall,

MMP-2 activation was abrogated with loss of TNFα, but the expression of MMP-13 and MT1-MMP were only partially affected.

De Novo MMP-8 Induction in the Absence of TNFα and TIMP-3

Despite the reduced transcriptional induction of specific interstitial collagenases, total myocardial collagenase activity was still higher in timp-3$^{-/-}$/tnf$\alpha^{-/-}$-AB than timp-3$^{-/-}$-AB. Screening additional MMPs revealed that expression of MMP-8, another interstitial collagenase,[29] was elevated $\approx$5-fold in timp-3$^{-/-}$/tnf$\alpha^{-/-}$-AB compared with timp-3$^{-/-}$-AB and WT-AB hearts (Figure 5K). Timp-3$^{-/-}$/tnf$\alpha^{-/-}$-AB myocardium had significantly higher numbers of neutrophils scattered throughout the LV compared with timp-3$^{-/-}$-AB and WT-AB myocardium (Figure 5L and 5 mol/L). Neutrophils are the primary source of MMP-8.[29] The greater increase in MMP-8 RNA levels than in neutrophil numbers may be attributable to the activation state of the neutrophils present in the myocardium. Thus, neutrophil infiltration and de novo MMP-8 induction on loss of TNFα likely contributed to elevated collagenase activity in the double mutant mice following pressure overload. We found no increase in macrophage numbers in timp-3$^{-/-}$/tnf$\alpha^{-/-}$-AB hearts by immunostaining with Mac3 or F4/80 (data not shown).

MMPi Treatment Attenuates LV Dilation and Dysfunction in Timp-3$^{-/-}$-AB Mice

Because total gelatinase and collagenase activities were elevated in timp-3$^{-/-}$ mice following pressure overload, we determined the contribution of this increased MMP activity toward DCM and heart failure, by using a broad-spectrum MMP-specific inhibitor (PD166793, MMPi) that does not inhibit TACE.[30] Oral administration of 15 mg/kg/d of PD166793 resulted in plasma drug concentration of 110$\pm$4 μmol/L, a level reported to be sufficient to inhibit MMP activity.[30] This level of PD166793 was sufficient to inhibit the excessive myocardial collagenase and gelatinase activities in timp-3$^{-/-}$-AB cardiac homogenates in vitro (Figure 4A). MMPi treatment of sham-operated WT mice did not affect cardiac function or structure, and no systemic toxicity was detected by histology of liver, kidney or lung (data not shown). During the course of 7-week MMPi treatment, mice in all groups remained active and showed no weight loss. Interestingly, treatment of timp-3$^{-/-}$-AB mice with MMPi abolished the interstitial fibrosis (data not shown), prevented the early heart failure and resulted in partial prevention of LV dilation and dysfunction (Figure 6B).

Combination of MMPi Treatment and TNFα Ablation Completely Prevents Heart Failure

We found that TNFα ablation or MMP inhibition individually resulted in partial prevention of DCM in timp-3$^{-/-}$-AB mice. Therefore, we reasoned that limiting MMP activity in addition to TNFα elimination should completely prevent heart disease in these mice. Treatment of timp-3$^{-/-}$/tnf$\alpha^{-/-}$-AB mice with 15 mg/kg/d of MMPi strikingly improved the survival (Figure 6A), completely rescued LV dilation and cardiac dysfunction as indicated by echocardiography and in vivo hemodynamics (Figure 6B and 6C), and hypertrophy (Figure 6D and 6E) up to 6 weeks after AB. These data demonstrate that excessive concomitant activity of both TNFα and MMPs are responsible for the cardiac dysfunction and heart failure in timp-3$^{-/-}$-AB mice.

Discussion

Dysregulations in proinflammatory cytokines and structural ECM underlie human heart disease.[8,26] TNFα has pleiotropic functions in cardiovascular diseases.[17,31] Maladaptive matrix remodeling is equally well recognized in promoting abnormal cardiac structure and function. These distinct disciplines have been studied extensively, but mostly independent of each other. We show here that timp-3 provides regulatory crossover for these distinct fields. TIMP-3 serves a dual inhibitory function in the heart against ADAM and MMP metalloproteinases to couple cytokine bioactivity with ECM homeostasis. Timp-3 deficiency results in severe LV dilation and dysfunction in response to cardiac pressure overload, which is partially rescued by TNFα deletion or by MMPi treatment, and completely prevented on combining TNFα ablation with MMP inhibition. These data demonstrate that TIMP-3 is essential in cardiac recovery from mechanical stress.

Our earlier study suggested involvement of TNFα in the development of spontaneous DCM in aged timp-3$^{-/-}$ mice as the soluble TNFα and its receptor (P75) levels were enhanced in coronary effluent from aged hearts.[18] The current study is an in depth investigation of the relationship between TIMP-3, TACE, TNFα, and MMPs in a pressure overload model of heart disease. TIMP-3 is the most highly expressed TIMP in the murine heart[13] and is the only known physiological inhibitor of ADAM-17/TACE.[15,22] TACE and TIMP-3 have parallel baseline expression patterns in murine organs during development.[32] We show that on pressure overload, TACE and TIMP-3 undergo parallel temporal inductions in WT hearts, a pattern suggesting that TIMP-3 normally serves to counteract increased TACE levels. In TIMP-3–deficient hearts, not only is there a dramatic immediate increase in the transcriptions of both TACE and TNFα, but their increased activities go unchecked in the absence of TIMP-3. TNFα feeds back positively on its own expression as well as that of TACE.[28] TNFα also induces TIMP-1 but downregulates TIMP-3 expression in myocyte cultures.[33,34] Such downregulation facilitates TNFα processing through increased TACE activity. Hence, the TIMP-3-TACE-TNFα system provides an inherent regulatory mechanism for controlling TNFα bioactivity following cardiac biomechanical stress.

TIMP-3 is classically known to inhibit MMP activity responsible for ECM turnover. Total gelatinase and collagenase activities rise markedly in *timp-3*$^{-/-}$ compared with WT hearts following aortic-banding. Screening of multiple MMPs reveals higher MMP-2, MMP-13 and MT1-MMP mRNA levels and higher MMP-2 activity. MMP-2 degrades several ECM proteins,[19,35] whereas MMP-2, MMP-13, and MT1-MMP are established interstitial collagenases.[19,25,36] MT1-MMP is also a key molecule for pro-MMP-2 and pro-MMP-13 activation.[24,37] MMP-2, MMP-13, and MT1-MMP are elevated in patients with heart disease,[38,39] and MMP-2

polymorphisms in human have been linked to CHF.[40] Thus, interstitial collagenases, MMP-2, MMP-13, and MT1-MMP appear to be intimately involved in myocardial ECM remodeling following biomechanical stress.

TNFα is well recognized for its pathophysiological importance in human heart disease.[4,17] It reduces cardiac contractility,[41] induces hypertrophy[42] and apoptosis.[43] In patients with DCM, both TACE and TNFα levels are significantly elevated[5] as in our aortic-banded timp-3$^{-/-}$ mice. Membrane-bound TNFα mediates cardiac hypertrophy whereas cleaved TNFα can induce DCM,[44] both detected in timp-3$^{-/-}$-AB mice. TNFα is also a key transcriptional inducer of several MMPs in vitro.[9,12] Our in vivo comparison of MMP expression in timp-3$^{-/-}$-AB and timp-3$^{-/-}$/tnfα$^{-/-}$-AB hearts provides evidence that TNFα induces MMP-2 and MMP-13, but minimally affects MMP-9 and MT1-MMP transcription. The de novo induction of MMP-8 in timp-3$^{-/-}$/tnfα$^{-/-}$-AB hearts is consistent with increased neutrophil infiltration in these mice, which intriguingly occurred without an increase in MMP-9, also known to be produced by neutrophils. MMP-8 is found decreased in human heart disease,[45] and its role as an interstitial collagenase in the myocardium needs to be further understood. Our data illustrate that TNFα bioactivity is intertwined with the amplification of MMP activity. The complete lack of myocardial apoptosis in double mutants suggests a proapoptotic role of TNFα in this model of heart disease. Understanding the multiple functions of TNFα in timp-3$^{-/-}$ myocardium will help us better understand its roles patients with DCM.

We demonstrate that treatment with MMPi in combination with TNFα loss completely prevents LV dilation and dysfunction, and heart failure in aortic-banded timp-3$^{-/-}$ mice, although only partial rescue is achieved with the individual interventions. This indicates that matrix degradation and cytokines are together responsible for heart disease. The anti-TNFα therapy in patients with class III and IV heart failure has so far proven unsuccessful.[6,7] Based on our findings, the lack of success with anti-TNFα therapy[6,7] may be attributable to application at too late a stage and/or the requirement for concomitant targeting of MMPs. Because TIMP-3 lies upstream of these 2 systems, and is commonly reduced in patients with cardiomyopathy, we propose that a therapeutic strategy that mimics TIMP-3 function in targeting both the MMPs and TNFα will be useful in producing an effective cardiac response to mechanical stress. Our findings provide a basis for anti-TNFα and MMPi combination therapy in heart disease.

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