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Disruption of Tumor Necrosis Factor Receptor Associated Factor 5 Exacerbates Pressure Overload Cardiac Hypertrophy and Fibrosis

Zhouyan Bian,[1,2] Jia Dai,[1,2] Nakano Hiroyasu,[3,4] Hongjing Guan,[1] Yuan Yuan,[1] Lihua Gan,[2] Heng Zhou,[1,2] Jing Zong,[1,2] Yan Zhang,[1] Fangfang Li,[1,2] Ling Yan,[1] Difei Shen,[1] Hongliang Li,[1,2] and Qizhu Tang[1,2*]

[1]*Department of Cardiology, Renmin Hospital of Wuhan University, Wuhan 430060, PR China*

[2]*Cardiovascular Research Institute of Wuhan University, Wuhan 430060, PR China*

[3]*Department of Immunology, Juntendo University, School of Medicine, Tokyo, Japan*

[4]*Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation, Tokyo, Japan*

ABSTRACT

The cytoplasmic signaling protein tumor necrosis factor (TNF) receptor-associated factor 5 (TRAF5), which was identified as a signal transducer for members of the TNF receptor super-family, has been implicated in several biological functions in T/B lymphocytes and the innate immune response against viral infection. However, the role of TRAF5 in cardiac hypertrophy has not been reported. In the present study, we investigated the effect of TRAF5 on the development of pathological cardiac hypertrophy induced by transthoracic aorta constriction (TAC) and further explored the underlying molecular mechanisms. Cardiac hypertrophy and function were evaluated with echocardiography, hemodynamic measurements, pathological and molecular analyses. For the first time, we found that TRAF5 deficiency substantially aggravated cardiac hypertrophy, cardiac dysfunction and fibrosis in response to pressure overload after 4 weeks of TAC compared to wild-type (WT) mice. Moreover, the mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK)-extracellular signal-regulated kinases 1/2 (ERK1/2) signaling pathway was more activated in TRAF5-deficient mice than WT mice. In conclusion, our results suggest that as an intrinsic cardioprotective factor, TRAF5 plays a crucial role in the development of cardiac hypertrophy through the negative regulation of the MEK-ERK1/2 pathway. J. Cell. Biochem. 115: 349–358, 2014.

KEY WORDS: TRAF5; CARDIAC HYPERTROPHY; FIBROSIS

Cardiac hypertrophy is a response of the myocardium to increased workload. It is characterized by an increase in myocardial mass and the accumulation of extracellular matrix, leading to left ventricular (LV) dilatation, fibrosis and impaired systolic function [Berk et al., 2007; Gupta et al., 2007; Swynghedauw et al., 2010]. Despite an initially beneficial adaptive response, prolonged hypertrophy is a risk factor for arrhythmias, sudden death, and heart failure [Dorn et al., 2003; Dorn, 2007; Rohini et al., 2010]. Although much is known about the pathways that regulate hypertrophic responses, the pivotal regulatory factors that participate in antagonizing these pathways have not been clearly defined. Therefore, understanding the underlying molecular mechanism that mediates cardiac remodeling will allow us to identify specific new targets to prevent the development of heart failure resulting from cardiac hypertrophy.

The cytoplasmic signaling protein TNF receptor-associated factor 5 (TRAF5) is a member of the tumor necrosis factor (TNF) receptor-associated factor (TRAF) family, which was identified as a family of

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*Correspondence to: Qizhu Tang, MD, Department of Cardiology, Renmin Hospital of Wuhan University, Cardiovascular Research Institute of Wuhan University, JieFang Road 238, Wuhan 430060, PR China. E-mail: qztang@whu.edu.cn

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signal transducers for members of the TNF receptor superfamily. TRAFs are composed of an N-terminal cysteine/histidine-rich region containing a zinc RING and/or zinc finger motifs, a coiled-coil (leucine zipper) motif, and a homologous region in the C-terminus that defines the TRAF domain. TRAFs have been reported to play a fundamental role in cellular signaling responses through the tumor necrosis factor receptor (TNFR) superfamily members and the interleukin-1 (IL-1) receptor [Nakano et al., 1996, 1997]. Ishida et al. also found that the TRAF family proteins, including TRAF5, could translocate into the nucleus, where they might directly interact with DNA, and the coiled-coil domain could function as a protein–protein interaction domain [Ishida et al., 1996]. TRAF5 is considered to be a putative positive regulator of a number of TNFR super-family receptors, and it may be a key molecule in the innate response to viral infection. TRAF5 plays a critical role in LMP1-mediated c-Jun kinase activation and induced the activation of p38 MAPK (mitogen-activated protein kinases) in HeLa cells [Shirakata et al., 2001; Kraus et al., 2009]. Many previous studies have suggested that TRAFs interact with nuclear factor-κB (NF-κB)-inducing kinase (NIK), MAP kinase/ERK kinase kinase 1 (MEKK1), and transforming growth factor β-activated kinase and that these kinases phosphorylate IKKs, resulting in NF-κB activation [Malinin et al., 1997; Ninomiya-Tsuji et al., 1999; Sanz et al., 2000]. The innate immune response and signaling pathways, such as the MAPK and NF-κB pathways, are important mechanisms that participate in the development of cardiac hypertrophy [Tada et al., 2001; Li et al., 2004; Zelarayan et al., 2009; Kehat and Molkentin, 2010; Rose et al., 2010]. However, the exact contribution of TRAF5 to cardiac hypertrophy, especially with respect to the signaling pathways that regulate cardiac hypertrophy, is not yet clear. Thus, we used the mice deficient in the TRAF5 gene to evaluate the exact roles of TRAF5 in the development of cardiac hypertrophy. For the first time, we show that TRAF5 is an intrinsic suppressor of cardiac hypertrophy that blocks MEK/ERK signaling.

METHODS

ETHICAL APPROVAL

All of the studies were performed in accordance with the guidelines of the NIH (Guide for the Care and Use of Laboratory Animals, 1996) and approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University. TRAF5 knockout mice and their wild-type littermates subjected to sham or transthoracic aorta constriction operation were divided into the following four groups ($n = 10$ for each group): (1) wild-type littermates subjected to sham operation (WT Sham); (2) TRAF5 knockout subjected to sham operation (KO Sham); (3) wild-type littermates subjected to transthoracic aorta constriction (WT TAC); and (4) TRAF5 knockout subjected to transthoracic aorta constriction (KO TAC). All of the mice used in the experiments were male, aged 8–10 weeks, and had a body weight of 24–27 g. Mice were anaesthetized with sodium pentobarbital (P3761, Sigma, 80 mg/kg, i.p.).

ANIMAL MODELS

Transthoracic aorta constriction (TAC) was performed as described previously [Bian et al., 2010]. Mice were anaesthetized and horizontal skin incision was made at the level of 2–3 intercostals space. The descending aorta was isolated, and a 7-0 silk suture was snared and pulled back around the aorta. A blunt 26-gauge needle was then placed next to the aorta, and the suture was tied around the needle and the aorta. After ligation, the needle was quickly removed, the chest and skin were closed, and the mice were allowed to recover. To serve as controls, the mice of sham groups underwent the same procedure without ligation. Buprenorphine (0.1 mg/kg, s.c.) was administered for post-operative analgesia. Doppler analysis was performed to ensure that the aorta was adequately constricted. The hearts and lungs of the sacrificed mice were harvested and weighed, and the tibial lengths were measured to compare the heart weight/body weight (HW/BW, mg/g), lung weight/body weight (LW/BW, mg/g), and heart weight/tibial length (HW/TL, mg/cm) ratios in the different groups. All of the surgeries and analyses were performed in a blinded fashion.

REAGENTS

The anti-TRAF5 (SC7220) (reactive with mouse) antibody was purchased from Santa Cruz Biotechnology. The primary antibodies against phospho-MEK1/2$^{ser217/221}$ (9154), T-MEK1/2 (9122), T-JNK1/2 (c-Jun N-terminal Kinase) (9258), phospho-JNK1/2$^{Thr183/Tyr185}$ (4688), phospho-ERK1/2$^{Thr202/Tyr204}$ (4370), T-ERK1/2 (4695), phospho-p38$^{Thr180/Tyr182}$ (4511), T-p38 (9212), phospho-NFκB^{ser536} (3033) and T-NFκB (4764) were purchased from Cell Signaling Technology. The antibody against GAPDH (MB001) was purchased from Bioworld Technology. IRDye® 800CW-conjugated secondary antibodies (LI-COR Biosciences) were used for visualization. The BCA protein assay kit was purchased from Thermo Technology (23227, Thermo). All of the other reagents were obtained from Sigma.

ECHOCARDIOGRAPHY AND HEMODYNAMICS

The internal diameter and the wall thickness of the left ventricle (LV) were assessed with echocardiography. Echocardiography was performed with a MyLab30CV ultrasound (Biosound ESAOTE, Inc.) with a 10-MHz linear array ultrasound transducer. The LV end-systolic diameter (LVESD) and LV end-diastolic diameter (LVEDD) were measured in parasternal short-axis views from the LV M-mode tracing with a sweep speed of 50 mm/s at the level of the mid-papillary muscle [Tang et al., 2009].

For hemodynamic measurements, a 1.4 F microtip catheter transducer (SPR-839, Millar Instruments, Inc., USA) was inserted through the right carotid artery and advanced into the left ventricle. The pressure, volume signals and heart rate were recorded continuously using a pressure-volume conductance system (MPVS-300 Signal Conditioner, Millar Instruments, Inc.) after stabilization for 10–15 min, and the results were analyzed with Chart 5.0 software as described previously [Bian et al., 2010].

HISTOLOGICAL ANALYSIS AND IMMUNOHISTOCHEMISTRY

The hearts were excised, washed in phosphate buffer saline (PBS), arrested in diastole with 10% KCl (Potassium chloride), weighed, fixed with 10% paraformaldehyde, and embedded in paraffin or snap-frozen in liquid nitrogen for protein and RNA analysis. The paraffin sections were cut transversely into 5-μm-thick sections and stained and visualized with light microscopy for histopathology. For the

myocyte cross-sectional area, the sections were stained with hematoxylin/eosin. A single myocyte was measured with a quantitative digital image analysis system (Image Pro-Plus, 6.0). The outlines of 100 myocytes were traced in each group. The tissue sections were stained with picrosirius red in a saturated picric acid solution to determine collagen deposition. Fibrillar collagen was identified through its red appearance in the sections. These sections were analyzed morphometrically with an image analysis system (Image Pro-Plus, 6.0).

To observe the Traf5 expression after TAC, immunofluorescence staining with the Traf5 antibody was performed in the mouse heart sections. The sections were later stained with anti-α-actinin and DAPI to observe the myocyte structure and the nuclei. The images were merged to observe the expression of Traf5 under the fluorescence microscope.

WESTERN BLOTTING

Cardiac tissue and cardiomyocytes from the different groups was lysed in RIPA lysis buffer, and the protein concentration was measured with a BCA protein assay kit. The protein extracts (50 μg) were resolved through polyacrylamide gel electrophoresis, then transferred onto Immobilon-FL transfer membranes (IPFL00010, Millipore) and probed with various primary antibodies. After incubation with a secondary IRDye® 800CW-conjugated antibody, the blots were scanned and visualized with an Odyssey Infrared Imaging System (Odyssey, LI-COR). The specific protein expression levels on the same membrane were normalized to GAPDH levels.

QUANTITATIVE REAL-TIME PCR

Total RNA was extracted from frozen cardiac tissue with TRIzol reagent (7950567275, Roche) according to the manufacturer's instructions to detect the mRNA expression levels of hypertrophic and fibrotic markers. The RNA purities were estimated according to the OD260/OD280 ratios detected with the SmartSpec Plus Spectrophotometer (Bio-Rad). The RNA (2 μg of each sample) was reverse-transcribed into cDNA using oligo (dT) primers and the Transcriptor First Strand cDNA Synthesis Kit (04896866001, Roche). The PCR amplifications were quantified using the LightCycler 480 SYBR Green I Master kit (04887352001, Roche), and the results were normalized against GAPDH expression.

CELL CULTURE AND SURFACE AREA

The cultures of H9c2 rat cardiomyocytes (ATCC, Rockville, MD) were prepared as described previously with a minor revision [Dai et al., 2013]. The cells were seeded at a density of 1×10^6/well onto six-well culture plates in Dulbecco's modified Eagle's medium (DMEM)/F12 with 10% fetal bovine serum (FBS; Gibco, 1133067) and penicillin/streptomycin. After 48 h, the H9c2 cells were serum-starved for 24 h in F10 medium containing 0.1% FBS. On culture day 3, the cardiac myocytes were then transfected with pCMV-SPORT6-Traf5 or pCMV-SPORT6-GFP for 24 h and subsequently stimulated with 1 μM angiotonin II (Ang II) for the indicated times. The recombinant pCMV-SPORT6-TRAF5 expression vector was constructed with the Traf5 cDNA (openbiosystem, Clone ID: 4218625), and pCMV-SPORT6-GFP was used as a parallel control during gene transfection. For surface area measurements, the cells were fixed with RCL2 (ALPHELYS, RCL2-CS24L), permeabilized in 0.1% Triton X-100 in PBS, and stained with anti-α-actinin (Millipore, 05-384) at a dilution of 1:100. The secondary antibody was Alexa FluorH 488 goat antimouse IgG (A11004, Invitrogen). The myocytes on coverslips were mounted by SlowFade Gold antifade reagent with DAPI (S36939, Invitrogen).

STATISTICAL ANALYSIS

All of the data are expressed as the mean ± SEM. The differences in the data between two groups were determined with Student's *t* test. Comparisons among the groups were assessed with a two-way ANOVA followed by a post hoc Tukey's test. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

EXPRESSION OF TRAF5 IN RESPONSE TO TAC

To determine the potential role of TRAF5 in hypertrophic stress, we first analyzed the protein levels of TRAF5 in the heart of WT mice after inducing the well-established model of cardiac hypertrophy through TAC for different durations. Intriguingly, the protein expression of TRAF5 was gradually and significantly increased during the first 2 weeks (including 1 days, 3 days, 1 week, and 2 weeks after TAC) but markedly decreased after 4 weeks of TAC (Fig. 1A). These findings imply a compensatory increase in TRAF5 expression during adaptive cardiac hypertrophy and a subsequent decompensatory decrease during maladaptive remodeling. The immunostaining were further performed to detect the cellular localization of TRAF5. Immunofluorescence on heart sections of C57BL/6 mice subjected to TAC showed pronounced cardiac TRAF5 protein expression compared to sham treatment (Fig. 1B). These results demonstrate that TRAF5 was also expressed in the cardiomyocytes, and the increase of the expression of TRAF5 was induced in hypertrophic hearts following TAC.

DEFICIENCY IN TRAF5 PROMOTES CARDIAC HYPERTROPHY IN RESPONSE TO TAC

In order to explore whether TRAF5 is involved in the regulation of cardiac hypertrophy, we used TRAF5 gene knockout (KO) mice to perform the loss-of-function experiments. Under basal conditions, TRAF5 deficiency had no significant effect on HW/BW, LW/BW or LV dimensions, wall thickness, or LV function compared to the WT mice (Table I). The results were consistent for 24–26 weeks in the TRAF5 gene KO mice (Table II).

Then, the TRAF5 KO mice and WT littermate control mice were subjected to TAC surgery or sham surgery for 4 weeks. The results suggested that TRAF5-deficient animals displayed a marked increase in cardiac hypertrophy. The TAC-induced increases in the HW/BW, LW/BW, and HW/TL were higher in the TRAF5-deficient mice than the WT controls (Fig. 2A). Likewise, histological analyses based on gross and whole-heart examinations and HE staining revealed the enhanced effect of TRAF5 deficiency on cardiac hypertrophy in response to TAC (Fig. 2B). Further analysis of the cardiomyocyte cross-sectional area (CSA) of the histological sections revealed a significantly larger cell size in the KO mice than the WT controls after TAC (Fig. 2A). Moreover, cardiac hypertrophy and the function of the left ventricle in response to TAC were evaluated with

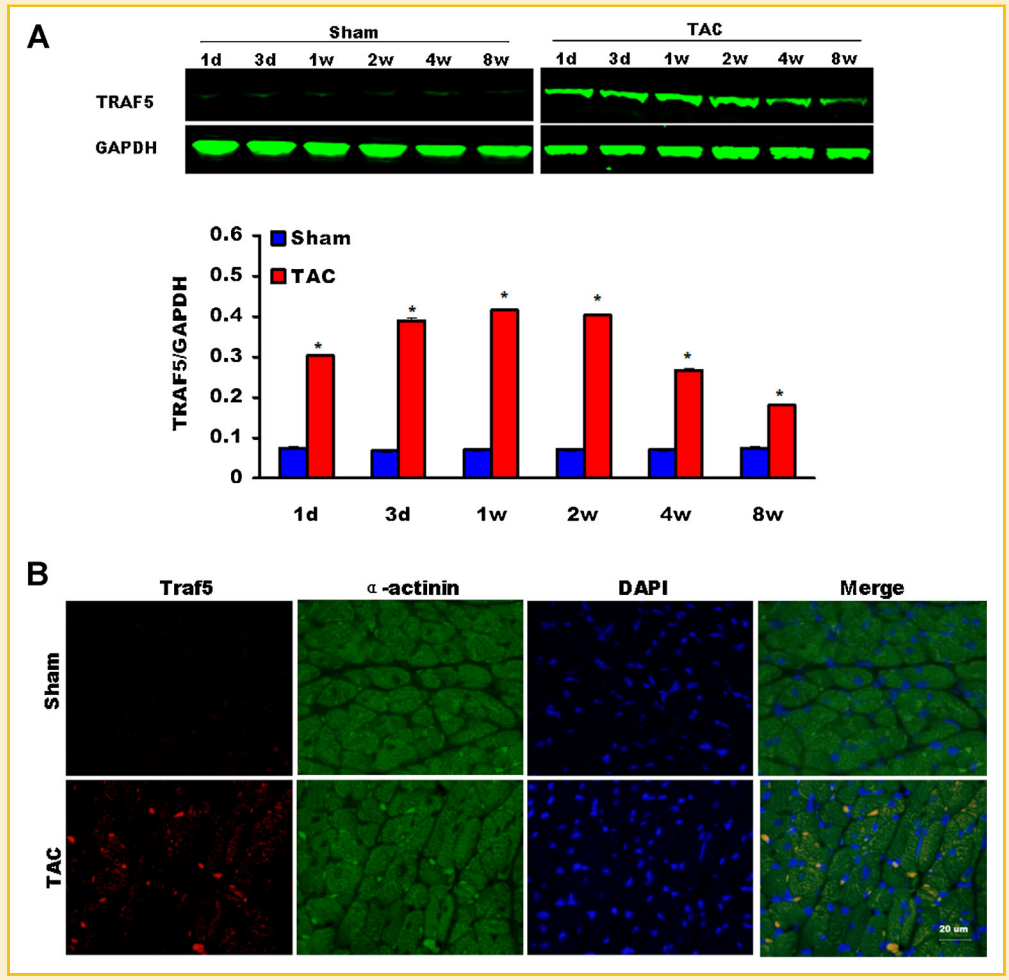

Fig. 1. Expression of TRAF5 in response to cardiac hypertrophy. A, Top: Representative Western blots of TRAF5 in hypertrophic hearts from WT mice induced with TAC at the indicated time points. Bottom: Quantitative results of the protein levels of TRAF5 in heart tissue after TAC. $^{*}P < 0.05$ versus sham. B: Immunofluorescence showing the cardiac TRAF5 protein expression in WT hearts after aortic banding or sham treatment.

echocardiography and hemodynamics 4 weeks after surgery. Although the LV dimensions and wall thickness were significantly increased in both the KO and WT mice after TAC, the TRAF5 KO mice displayed substantially enlarged LV chambers compared with their WT littermates, according to echocardiographic parameters, such as LVEDD, LVESD, and interventricular septum depth (IVSD) (Fig. 2C,D). The significant decrease in the fractional shortening (FS), the ejection fraction (EF), and the dP/dt max (mm Hg/s) modulus in the KO mice after 4 weeks of TAC showed an exacerbation of the LV function in the TRAF5 KO mice compared with the WT controls (Fig. 2C,E). In addition, we detected the expression of cardiac hypertrophy markers in response to pressure overload with quantitative real-time PCR. The mRNA levels of atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and β-myosin heavy chain (β-MHC) were markedly augmented in both the TRAF5 KO mice and WT controls after TAC. What's more, these increases were more prominent in the TRAF5 KO mice than the WT controls 4 weeks after TAC (Fig. 2F), and they were also accompanied with a significant down-regulation of α-myosin heavy chain (α-MHC) and sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA2α) (Fig. 2F).

TABLE I. Anatomic and Echocardiographic Analysis in TRAF5 KO Mice and WT Controls at 4 Weeks After Sham Operation or TAC

Parameter, number	Sham WT mice, n = 6	Sham TRAF5 KO mice, n = 6	TAC WT mice, n = 6	TAC TRAF5 KO mice, n = 6
BW (g)	26.10 ± 0.23	25.52 ± 0.27	26.73 ± 0.57	26.10 ± 0.28
HW/BW (mg/g)	4.36 ± 0.04	4.33 ± 0.05	6.92 ± 0.15*	8.94 ± 0.19*,#
LW/BW (mg/g)	5.48 ± 0.10	5.34 ± 0.08	5.54 ± 0.12	8.85 ± 0.78*,#
HW/TL (mg/cm)	6.26 ± 0.06	6.08 ± 0.09	10.22 ± 0.12*	12.86 ± 0.35*,#
HR (beats/min)	490 ± 13	526 ± 22	505 ± 13	481 ± 17
LVEDD (mm)	3.58 ± 0.04	3.57 ± 0.06	4.29 ± 0.13*	4.75 ± 0.12*,#
LVESD (mm)	2.11 ± 0.06	2.16 ± 0.06	2.93 ± 0.15*	3.56 ± 0.15*,#
LVPWD (mm)	0.69 ± 0.01	0.70 ± 0.01	0.77 ± 0.01*	0.77 ± 0.01*
IVSD (mm)	0.71 ± 0.01	0.69 ± 0.02	0.78 ± 0.01*	0.83 ± 0.01*,#
FS (%)	40.67 ± 1.29	39.22 ± 0.87	32.19 ± 1.70*	25.17 ± 1.43*,#

BW, body weight; HW/BW, heart weight/body weight; LW/BW, lung weight/body weight; HW/TL, heart weight/tibial length; HR, heart rate; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LVPWD, left ventricular posterior wall, diastolic; IVSD, left ventricular septum, diastolic; FS, fractional shortening. All values are mean ± SEM.

$^{*}P < 0.05$ versus sham operation.

$^{\#}P < 0.05$ versus WT TAC.

TABLE II. Anatomic and Echocardiographic Analysis in 24–26 Week Old Mice of TRAF5 KO Mice and WT Controls

Parameter, number	WT mice, n = 6	TRAF5 KO mice, n = 6
BW (g)	30.45 ± 0.65	32.30 ± 1.17
HW/BW (mg/g)	4.20 ± 0.10	3.99 ± 0.10
HW/TL (mg/cm)	6.82 ± 0.19	7.17 ± 0.14
HR (beats/min)	502 ± 13	515 ± 17
LVEDD (mm)	3.71 ± 0.10	3.83 ± 0.04
LVESD (mm)	2.26 ± 0.08	2.44 ± 0.02
LVPWD (mm)	0.71 ± 0.01	0.72 ± 0.01
FS (%)	38.89 ± 0.85	37.17 ± 0.58

BW, body weight; HW/BW, heart weight/body weight; LW/BW, lung weight/body weight; HW/TL, heart weight/tibial length; HR, heart rate; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LVPWD, left ventricular posterior wall, diastolic; FS, fractional shortening.

TRAF5 OVEREXPRESSION ATTENUATES HYPERTROPHY OF CARDIOMYOCYTES IN VITRO

In order to specifically investigate the role of TRAF5 in cardiomyocytes, we performed gain-of-function studies using cultured H9c2 rat cardiomyocytes. We treated the cultured cardiomyocytes transfected with pCMV-SPORT6-Traf5 or pCMV-SPORT6-GFP with 1 μM Ang II for the indicated times to observe the role of TRAF5 on myocyte hypertrophy. The pCMV-SPORT6-Traf5 vector transfection led to an overexpression of the TRAF5 protein in H9c2 rat cardiomyocytes. The hypertrophic response in cardiomyocytes was evaluated by measuring the cardiac myocyte area and upregulation of hypertrophy marker expression. Staining of these cardiomyocytes for α-actinin demonstrated that the Ang II-induced increase in cardiomyocyte area was significantly intercepted in pCMV-SPORT6-Traf5 transfected

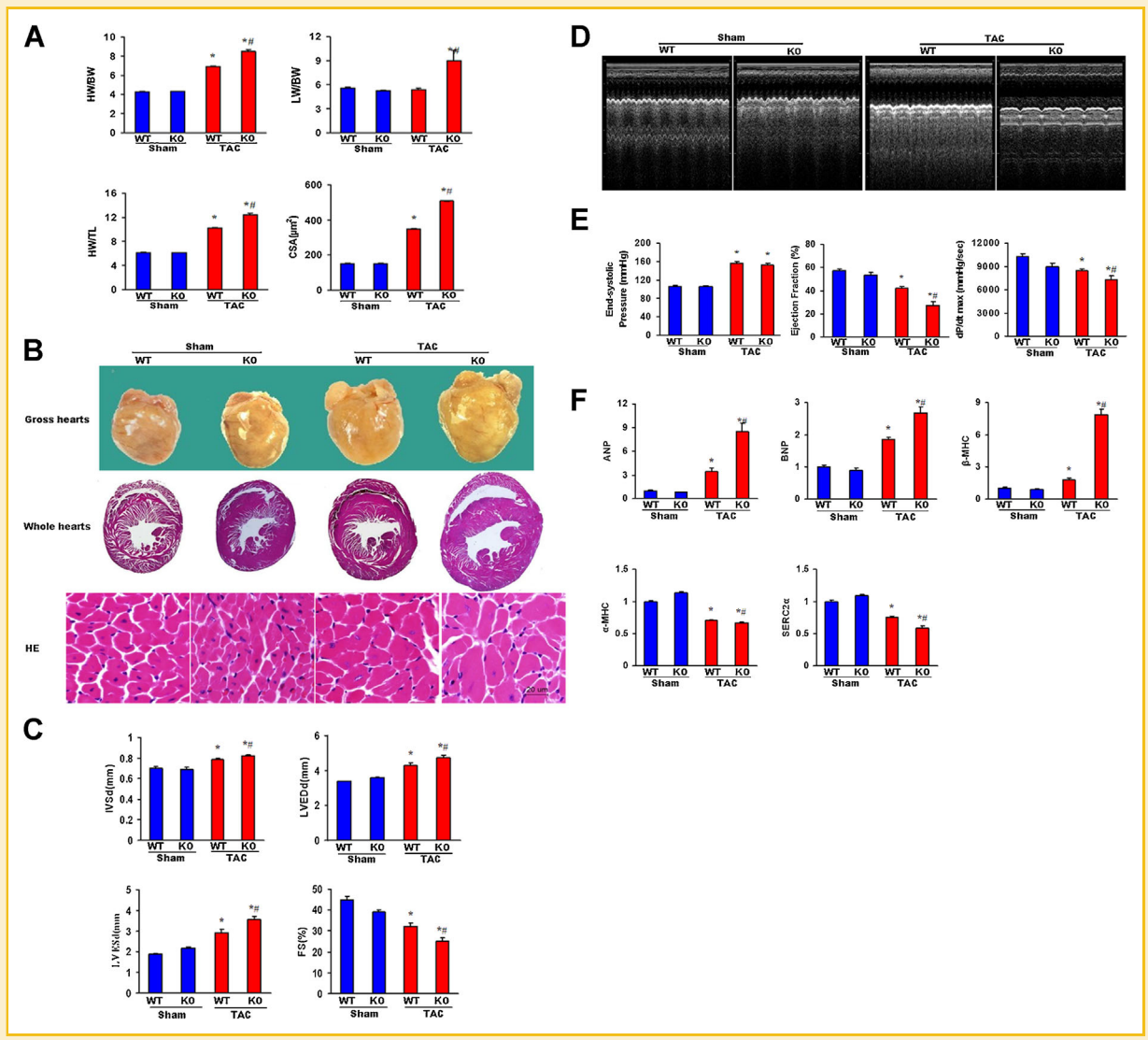

Fig. 2. Deficiency in TRAF5 promotes cardiac hypertrophy in response to TAC. A: Statistical results of the HW/BW, LW/BW, and HW/TL ratios (n = 10) and myocyte cross-sectional areas of the indicated groups (n = 100 cells per group). B: Gross hearts, whole hearts and HE staining in WT and TRAF5 KO mice 4 weeks after TAC or sham surgery (Scale bars: 20 mm). C,D: Echocardiography measurements and representative images in the indicated groups (n = 6). E: Hemodynamic measurements in the indicated groups (n = 6). F: The mRNA expression levels of ANP, BNP, β-MHC, α-MHC, and SERCA2α detected with RT-PCR 4 weeks after TAC or sham surgery (n = 4). $^{*}P < 0.05$ versus WT/sham, $^{\#}P < 0.05$ versus WT/TAC.

cardiomyocytes compared with controls (Fig. 3A,B). Moreover, real-time PCR showed that the overexpression of Traf5 in cardiomyocytes obviously reduced the mRNA levels of hypertrophy markers ANP and BNP induced by Ang II (Fig. 3C). These in vitro data confirm the inhibitory effect of Traf5 on hypertrophy of cardiomyocytes.

DEFICIENCY IN TRAF5 PROMOTES FIBROSIS IN RESPONSE TO TAC

The cardiac hypertrophy induced by pressure overload was always accompanied with extensive fibrosis. We therefore further evaluated the degree of fibrosis in the mice after TAC through picrosirius red staining and the detection of fibrosis markers. As shown in Figure 4A, the collagen deposition in the myocardial interstitium and vascular periphery was enhanced in both the TRAF5 KO mice and the WT controls subjected to TAC, but this effect was much more significant in the TRAF5 KO mice. A quantitative analysis also showed increased collagen volume in the TRAF5 KO mice compared to the WT controls (Fig. 4B). The mRNA levels of known markers of fibrosis, including connective tissue growth factor (CTGF), collagen I, collagen III, fibronectin and transforming growth factor (TGF)-β2 were significantly higher in the TRAF5 KO mice compared with the WT controls after the TAC procedure (Fig. 4C).

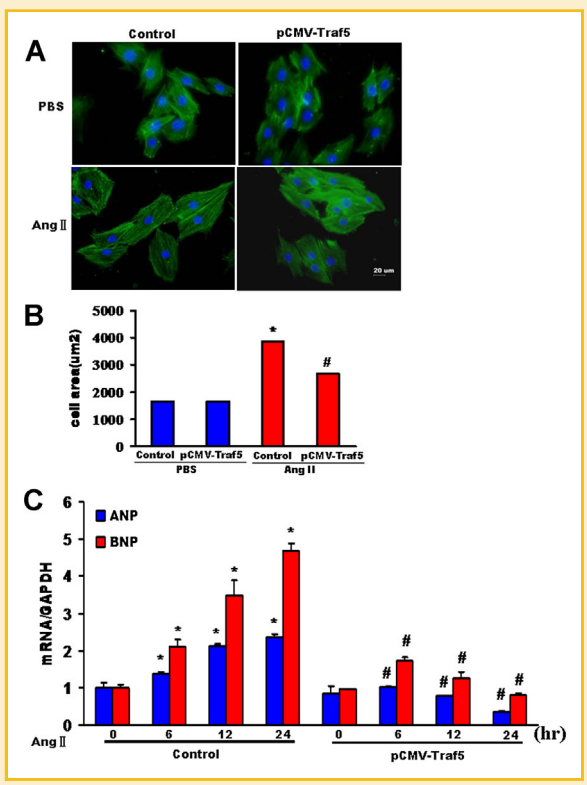

Fig. 3. TRAF5 overexpression attenuates cardiocyte hypertrophy in vitro. A: Representative images of the cardiomyocytes showed the inhibitory effect of TRAF5 overexpression on the enlargement of cardiomyocytes in response to Ang II for 24 h. B: Quantification of cell cross-sectional area by measuring 100 random cells. C: Real-time PCR analysis of the mRNA levels of the hypertrophy markers ANP and BNP in response to Ang II at the time points indicated. $^{*}P < 0.05$ versus Control at the 0 time point, $^{\#}P < 0.05$ versus Control at the same time point.

THE EFFECT OF TRAF5 ON INFLAMMATION IN VIVO AND IN VITRO

Activation of inflammatory signaling pathways promotes cardiac hypertrophy and fibrosis [Li et al., 2007a,b]. We therefore examined the expression of the representative cytokines interleukin 6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) and TNF-α after pressure overload. Western blots suggested the protein expression of IL-6, MCP-1, and TNF-α were significantly elevated after TAC in both the TRAF5 KO mice and WT controls. And quantitative analysis indicated the expression were higher in the TRAF5 KO mice compared to the WT controls (Fig. 5A,B). We further examined the phosphorylation levels of NF-κB and found that the TRAF5 deficiency increased the phosphorylation of NF-κB in response to TAC (Fig. 6A,B). To validate the effects of TRAF5 on NF-κB signaling, we cultured rat cardiac myocytes for in vitro analyses. Overexpression of TRAF5 in H9c2 cardiomyocytes attenuated the phosphorylation of NF-κB induced by Ang II (Fig. 6C,D). These results indicate that TRAF5 blocks NF-κB dependent inflammatory responses in response to hypertrophic stimuli both in vivo and in vitro.

THE EFFECT OF TRAF5 ON MEK/ERK SIGNALING IN VIVO AND IN VITRO

We have previously shown that MAPK signaling is a crucial pathway in cardiac hypertrophy [Bian et al., 2009; Cai et al., 2009; Li et al., 2010]. To explore the molecular mechanisms through which TRAF5 regulated the hypertrophic response, we first examined the phosphorylated levels of ERK1/2, JNK1/2 and p38 in the TRAF5 KO mice and WT controls. The Western blot data suggested that the TRAF5 deficiency markedly enhanced the phosphorylation levels of ERK1/2 compared with the WT mice after TAC. However, the phosphorylation of JNK1/2 and p38 showed no significant difference between the TRAF5 KO mice and WT controls. To identify the mechanism through which ERK1/2 was activated, we further examined the upstream components of the MAPK signaling pathway and found that the TRAF5 deficiency enhanced the phosphorylation of MEK compared with the WT mice after TAC (Fig. 6A,B). Therefore, the TRAF5 deficiency augmented the activation of MEK/ERK signaling in vivo, but it had no effect on JNK1/2 or p38.

To further confirm the effect of TRAF5 on MEK-ERK1/2 signaling, we exposed the TRAF5 overexpressed H9c2 cardiomyocytes transfected with pCMV-SPORT6-Traf5 to AngII for indicated times to substantiate our in vivo findings. As shown in Figure 6C,D, AngII-stimulated the phosphorylation of MEK-ERK1/2 were significantly attenuated in Traf5 overexpression cardiomyocytes. Therefore, the TRAF5 overexpression suppressed MEK/ERK signaling in vitro.

DISCUSSION

In the present study, we got a novel finding that the expression of TRAF5 was markedly induced in the hypertrophic heart. We further investigated the role of TRAF5 in the development of pathological cardiac hypertrophy and explored the molecular mechanisms. The loss-of-function researches showed that TRAF5 deficiency significantly aggravated cardiac hypertrophy, fibrosis and inflammation in an experimental model of TAC, and TRAF5 deficiency lead to a

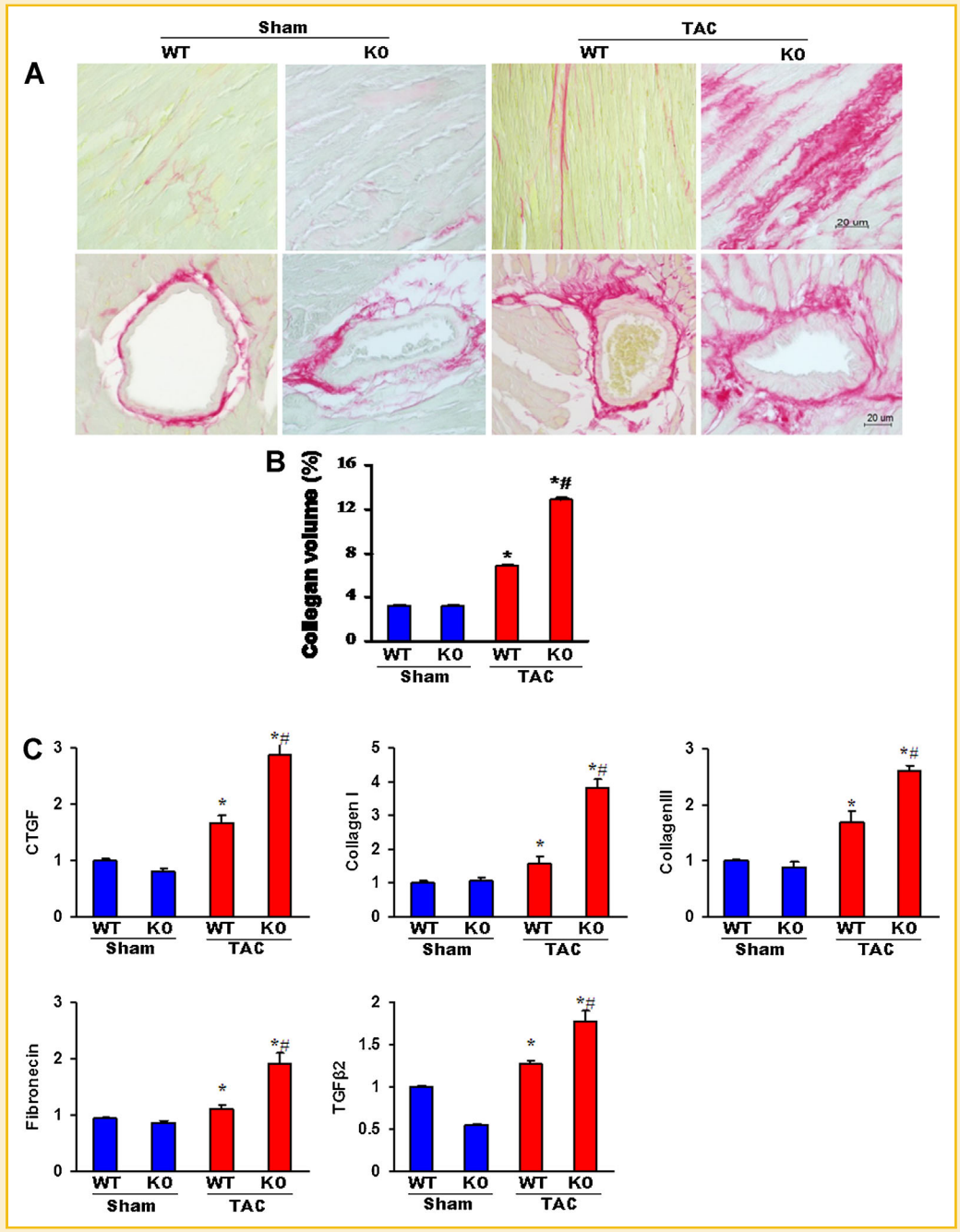

Fig. 4. Deficiency in TRAF5 promotes fibrosis in response to TAC. A: The picrosirius red staining of histological sections of the left ventricles was performed in the indicated groups (Scale bar: 20 μm). B: The fibrotic areas of the histological sections were quantified using an image analysis system. C: The mRNA expression of CTGF, collagenI, collagenIII, fibronectin and TGF-β2 in the myocardium were detected with RT-PCR 4 weeks after TAC or sham surgery (n = 4). $^{*}P < 0.05$ versus WT/sham, $^{\#}P < 0.05$ versus WT/TAC.

significant activation of the NF-κB and MEK/ERK signaling. Whereas the gain-of-function studies demonstrated that TRAF5 overexpression attenuated hypertrophy of cardiomyocytes induced by Ang II, and TRAF5 overexpression suppressed the NF-κB and MEK/ERK signaling in vitro. Therefore, our study for the first time suggests that targeting of TRAF5 might be a novel and promising strategy for the treatment of pathological cardiac hypertrophy.

As a cytoplasmic adaptor protein for the TNF/interleukin-1/Toll-like receptor superfamily [Missiou et al., 2010], TRAF5 were considered highly expressed in immune system such as adrenal gland, thyroid gland and spleen to implicate in several biological functions in T/B lymphocytes and the innate response against viral infection in previously research [Mizushima et al., 1998; Kraus et al., 2009; Tang and Wang, 2010]. However, we found that the

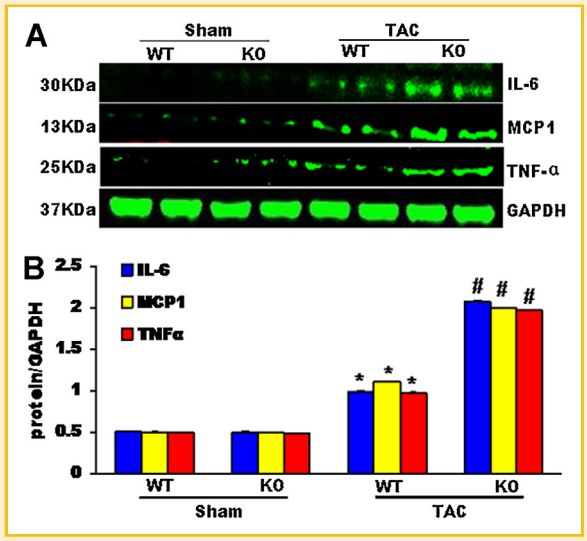

Fig. 5. Deficiency in TRAF5 promotes imflammation in response to TAC. A: Representative Western blots of IL-6, MCP-1, and TNF-α protein expressed in heart tissues obtained from indicated groups. B: Quantitative results of the protein levels of IL-6, MCP-1 and TNF-α in heart tissues obtained from indicated groups. $^{*}P < 0.05$ versus WT/sham, $^{\#}P < 0.05$ versus WT/TAC.

expression of TRAF5 in WT mice was increased during the first 2 weeks after hypertrophic stimuli and decreased beginning 4 weeks after TAC surgery. This induced increase of TRAF5 is likely a compensatory response of the heart in an effort to cope with increased cardiac hypertrophy. Nevertheless, the upregulation of TRAF5 might be inadequate to antagonize cardiac hypertrophy, and the compensatory hypertrophy evolves into a subsequent decompensated state under persistent stress. This result is consistent with a previous observation that most stress-dependent intrinsic antihypertrophic mediators tend to exhibit a two-phase change in their expression levels in response to hypertrophic stimuli [Kumarapeli et al., 2008; Oceandy et al., 2009]. To further determine the role of TRAF5 in the heart to hypertrophic stimuli, we subjected the TRAF5 KO mice and WT mice to sham and TAC surgery. Compared with WT mice, TRAF5 KO mice displayed higher HW/BW, LW/BW, HW/TL and CSA, greater reactivation of the cardiac hypertrophy marker genes and aggravation of the LV function. These findings suggested that the deficiency of TRAF5 exacerbated the pathological cardiac responses to TAC-induced LV pressure overload. And the in vitro data also confirmed the inhibitory effect of Traf5 on hypertrophy of cardiomyocytes. In addition, collagen deposition was obviously enhanced, and the expression of several fibrotic mediators was higher in the TRAF5 KO mice compared with the WT controls. These results indicated that a

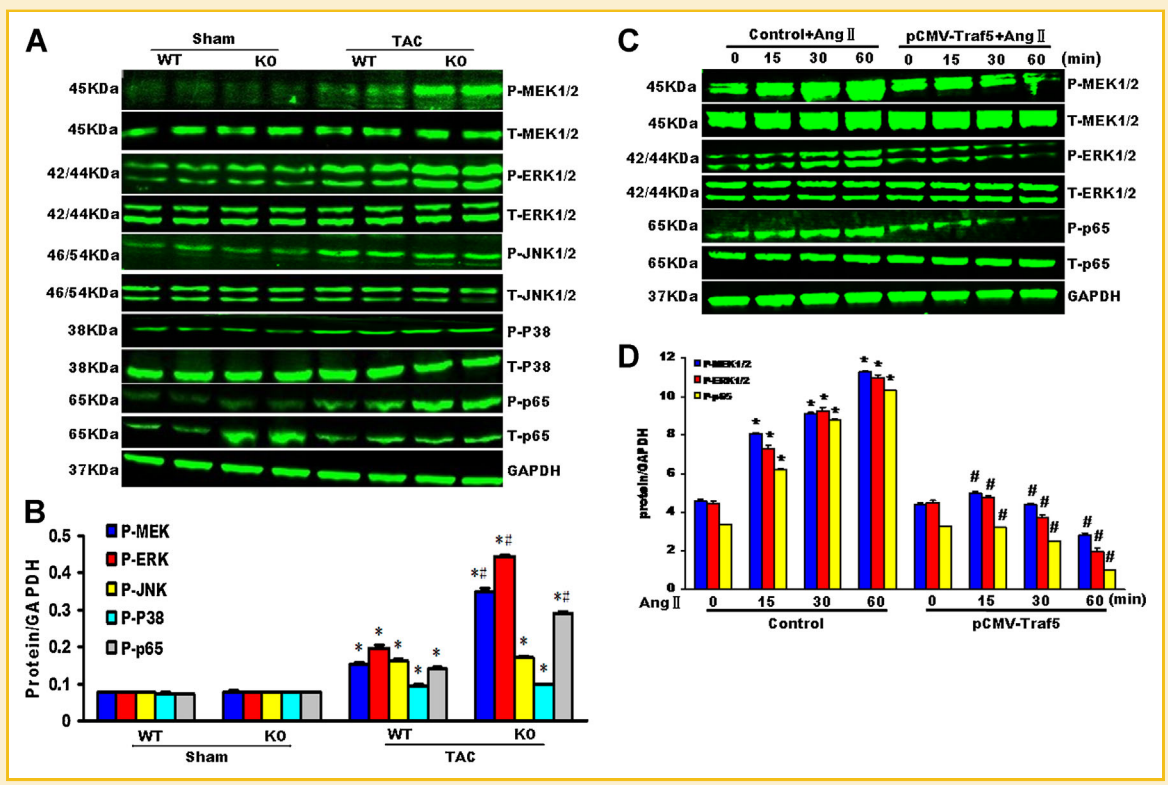

Fig. 6. Deficiency in TRAF5 augments the activation of MEK/ERK signaling. A,B: The phosphorylated and total protein expression levels of MEK1/2, ERK1/2, JNK1/2, p38 and NF-κB 4 weeks after TAC or sham surgery in WT and TRAF5 KO mice (n = 4). Deficiency in TRAF5 augments the activation of MEK/ERK signaling. A: Representative blots (the duplicate lanes represent two different heart samples); (B) quantitative results. $^{*}P < 0.05$ versus WT/sham, $^{\#}P < 0.05$ versus WT/TAC. C.D: Phosphorylation and total protein expression levels of MEK1/2, ERK1/2 and NF-κB after treatment with Ang II for the indicated times in H9c2 rat cardiomyocytes with or without TRAF5 overexpression. TRAF5 overexpression attenuates the activation of MEK/ERK signaling. C: Representative blots; (D) quantitative results. $^{*}P < 0.05$ versus Control at the 0 time point, $^{\#}P < 0.05$ versus Control at the same time point.

deficiency in TRAF5 augmented cardiac fibrosis, which is a classical feature of pathological hypertrophy [Leask, 2007]. Taken together, the present study reveals that TRAF5 is an important regulatory factor in protecting the heart from pressure overload.

Cardiac hypertrophy and fibrosis may be promoted by activation of inflammatory signaling pathways [Xu et al., 2004; Liehn et al., 2006; Schuh et al., 2008]. We observed that the induction of inflammatory mediators by hypertrophic stimuli was increased in TRAF5 KO mice, indicating an important role for TRAF5 in regulating inflammatory response in the heart. One possible mechanism is that TRAF5 deficiency increased the phosphorylation of NF-κB in response to TAC, resulting in increase of downstream cytokines, including IL-6, MCP-1 and TNF-α, each of which has a κB-binding domain in its promoter site. This was consistent with the previous study that TRAF5 deficiency enhances the expression of chemokines MCP-1 and accelerates atherogenesis in mice [Missiou et al., 2010]. Previous studies have suggested that TRAF5 participates in NF-κB activation [Nakano et al., 1996; Akiba et al., 1998; Mizushima et al., 1998]. In contrast, our data challenge this commonly held view by demonstrating that NF-κB is activated in the hearts of TRAF5-deficient animals under pressure overload. In accordance with our findings, Tada et al. found that TNF-induced NF-κB translocation was not significantly impaired in TRAF5 KO mouse embryo fibroblasts. They argued that these results suggested a redundant role for TRAF5 in TNF-induced NF-κB activation, and some molecule other than TRAF5 may mediate NF-κB activation [Tada et al., 2001]. Therefore, we proposed the activation of NF-κB in TRAF5 KO mice in response to TAC might be mediated by other signaling.

Our previously study and others have demonstrated that a number of signaling pathways are implicated in the regulation of cardiac hypertrophy, such as the MAPK, AKT/GSK3β and calcineurin/nuclear factor of activated T cells (NFAT) signaling cascades [Bian et al., 2010, 2012; Li et al., 2010]. Recent studies have demonstrated that the MAPK signaling pathway, which consists of the kinases p38, JNKs and ERKs, plays a key role in the progression of cardiac hypertrophy through the phosphorylation of intracellular targets, including numerous transcription factors, under stress stimuli [Das et al., 2006]. Our data demonstrate that the TRAF5 deficiency markedly promotes the activation of MEK-ERK1/2 signaling but has no effect on p38 or JNK activation in hearts subjected to TAC. Previous reports have suggested that JNK is more activated in macrophages, and the aortas of TRAF5-deficient animals [Missiou et al., 2010]. Nakano et al. [1999] also reported a similar activation of JNK in TRAF5-deficient and -competent fibroblasts. Interestingly, the present study reveals for the first time that MEK and ERK1/2 but not JNK and p38 were activated in the hearts of TRAF5-deficient animals under pressure overload. Our previous study has reported that NF-kB and NFAT activation are dependent on ERK1/2 signaling [Shen et al., 2010]. Therefore, the activation of ERK1/2-dependent NF-kB and MEK-ERK1/2 signaling likely accounted for the cardiac hypertrophy of the TRAF5 deficiency mice induced by pressure overload. Further studies are needed to establish the molecular signaling mechanism through which TRAF5 regulates MEK-ERK1/2 signaling.

In summary, our present work defines the role of TRAF5 in cardiac hypertrophy via regulating MEK-ERK1/2 signaling pathway for the first time. These findings support the concept that TRAF5 is a critical signaling molecule responsible for cardiac hypertrophy, and it could be an effective preventive and therapeutic target against cardiac hypertrophy and heart failure. Our study provides insights into the pathogenesis and molecular mechanism of cardiac hypertrophy, and it may has significant implications for the development of novel strategies for the treatment of cardiac hypertrophy through targeting TRAF5 signaling.

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REFERENCES

Akiba H, Nakano H, Nishinaka S, Shindo M, Kobata T, Atsuta M, Morimoto C, Ware CF, Malinin NL, Wallach D, Yagita H, Okumura K. 1998. CD27, a member of the tumor necrosis factor receptor superfamily, activates NF-kappaB and stress-activated protein kinase/c-Jun N-terminal kinase via TRAF2, TRAF5, and NF-kappaB-inducing kinase. J Biol Chem 273:13353–13358.

Berk BC, Fujiwara K, Lehoux S. 2007. ECM remodeling in hypertensive heart disease. J Clin Invest 117:568–575.

Bian Z, Cai J, Shen DF, Chen L, Yan L, Tang Q, Li H. 2009. Cellular repressor of E1A-stimulated genes attenuates cardiac hypertrophy and fibrosis. J Cell Mol Med 13:1302–1313.

Bian ZY, Huang H, Jiang H, Shen DF, Yan L, Zhu LH, Wang L, Cao F, Liu C, Tang QZ, Li H. 2010. LIM and cysteine-rich domains 1 regulates cardiac hypertrophy by targeting calcineurin/nuclear factor of activated T cells signaling. Hypertension 55:257–263.

Bian ZY, Wei X, Deng S, Tang QZ, Feng J, Zhang Y, Liu C, Jiang DS, Yan L, Zhang LF, Chen M, Fassett J, Chen Y, He YW, Yang Q, Liu PP, Li H. 2012. Disruption of mindin exacerbates cardiac hypertrophy and fibrosis. J Mol Med (Berl) 90:895–910.

Cai J, Yi FF, Bian ZY, Shen DF, Yang L, Yan L, Tang QZ, Yang XC, Li H. 2009. Crocetin protects against cardiac hypertrophy by blocking MEK-ERK1/2 signalling pathway. J Cell Mol Med 13:909–925.

Dai J, Shen DF, Bian ZY, Zhou H, Gan HW, Zong J, Deng W, Yuan Y, Li F, Wu QQ, Gao L, Zhang R, Ma ZG, Li HL, Tang QZ. 2013. IKKi deficiency promotes pressure overload-induced cardiac hypertrophy and fibrosis. PLoS ONE 8: e53412.

Das S, Otani H, Maulik N, Das DK. 2006. Redox regulation of angiotensin II preconditioning of the myocardium requires MAP kinase signaling. J Mol Cell Cardiol 41:248–24855.

Dorn GW, II. 2007. The fuzzy logic of physiological cardiac hypertrophy. Hypertension 49:962–970.

Dorn GW, II. Robbins J, Sugden PH. 2003. Phenotyping hypertrophy: Eschew obfuscation. Circ Res 92:1171–1175.

Gupta S, Das B, Sen S. 2007. Cardiac hypertrophy: Mechanisms and therapeutic opportunities. Antioxid Redox Signal 9:623–652.

Ishida TK, Tojo T, Aoki T, Kobayashi N, Ohishi T, Watanabe T, Yamamoto T, Inoue J. 1996. TRAF5, a novel tumor necrosis factor receptor-associated factor family protein, mediates CD40 signaling. Proc Natl Acad Sci USA 93:9437–9442.

Kehat I, Molkentin JD. 2010. Extracellular signal-regulated kinase 1/2 (ERK1/2) signaling in cardiac hypertrophy. Ann NY Acad Sci 1188:96–102.

Kraus ZJ, Nakano H, Bishop GA. 2009. TRAF5 is a critical mediator of in vitro signals and in vivo functions of LMP1, the viral oncogenic mimic of CD40. Proc Natl Acad Sci USA 106:17140–17145.

Kumarapeli AR, Su H, Huang W, Tang M, Zheng H, Horak KM, Li M, Wang X. 2008. Alpha B-crystallin suppresses pressure overload cardiac hypertrophy. Circ Res 103:1473–1482.

Leask A. 2007. TGFbeta, cardiac fibroblasts, and the fibrotic response. Cardiovasc Res 74:207–212.

Li Y, Ha T, Gao X, Kelley J, Williams DL, Browder IW, Kao RL, Li C. 2004. NF-kappaB activation is required for the development of cardiac hypertrophy in vivo. Am J Physiol Heart Circ Physiol 287:H1712–H1720.

Li HL, She ZG, Li TB, Wang AB, Yang Q, Wei YS, Wang YG, Liu DP. 2007a. Overexpression of myofibrillogenesis regulator-1 aggravates cardiac hypertrophy induced by angiotensin II in mice. Hypertension 49:1399–1408.

Li HL, Zhuo ML, Wang D, Wang AB, Cai H, Sun LH, Yang Q, Huang Y, Wei YS, Liu PP, Liu DP, Liang CC. 2007b. Targeted cardiac overexpression of A20 improves left ventricular performance and reduces compensatory hypertrophy after myocardial infarction. Circulation 115:1885–1894.

Li H, He C, Feng J, Zhang Y, Tang Q, Bian Z, Bai X, Zhou H, Jiang H, Heximer SP, Qin M, Huang H, Liu PP, Huang C. 2010. Regulator of G protein signaling 5 protects against cardiac hypertrophy and fibrosis during biomechanical stress of pressure overload. Proc Natl Acad Sci USA 107:13818–13823.

Liehn EA, Zernecke A, Postea O, Weber C. 2006. Chemokines: Inflammatory mediators of atherosclerosis. Arch Physiol Biochem 112:229–238.

Malinin NL, Boldin MP, Kovalenko AV, Wallach D. 1997. MAP3K-related kinase involved in NF-kappaB induction by TNF, CD95 and IL-1. Nature 385:540–544.

Missiou A, Rudolf P, Stachon P, Wolf D, Varo N, Aichele P, Colberg C, Hoppe N, Ernst S, Munkel C, Walter C, Sommer B, Hilgendorf I, Nakano H, Bode C, Zirlik A. 2010. TRAF5 deficiency accelerates atherogenesis in mice by increasing inflammatory cell recruitment and foam cell formation. Circ Res 107:757–766.

Mizushima S, Fujita M, Ishida T, Azuma S, Kato K, Hirai M, Otsuka M, Yamamoto T, Inoue J. 1998. Cloning and characterization of a cDNA encoding the human homolog of tumor necrosis factor receptor-associated factor 5 (TRAF5). Gene 207:135–140.

Nakano H, Oshima H, Chung W, Williams-Abbott L, Ware CF, Yagita H, Okumura K. 1996. TRAF5, an activator of NF-kappaB and putative signal transducer for the lymphotoxin-beta receptor. J Biol Chem 271:14661–14664.

Nakano H, Shindo M, Yamada K, Yoshida MC, Santee SM, Ware CF, Jenkins NA, Gilbert DJ, Yagita H, Copeland NC, Okumura K. 1997. Human TNF receptor-associated factor 5 (TRAF5): cDNA cloning, expression and assignment of the TRAF5 gene to chromosome 1q32. Genomics 42:26–32.

Nakano H, Sakon S, Koseki H, Takemori T, Tada K, Matsumoto M, Munechika E, Sakai T, Shirasawa T, Akiba H, Kobata T, Santee SM, Ware CF, Rennert PD, Taniguchi M, Yagita H, Okumura K. 1999. Targeted disruption of Traf5 gene causes defects in CD40- and CD27-mediated lymphocyte activation. Proc Natl Acad Sci USA 96:9803–9808.

Ninomiya-Tsuji J, Kishimoto K, Hiyama A, Inoue J, Cao Z, Matsumoto K. 1999. The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway. Nature 398:252–256.

Oceandy D, Pickard A, Prehar S, Zi M, Mohamed TM, Stanley PJ, Baudoin-Stanley F, Nadif R, Tommasi S, Pfeifer GP, Armesilla AL, Cartwright EJ, Neyses L. 2009. Tumor suppressor Ras-association domain family 1 isoform A is a novel regulator of cardiac hypertrophy. Circulation 120:607–616.

Rohini A, Agrawal N, Koyani CN, Singh R. 2010. Molecular targets and regulators of cardiac hypertrophy. Pharmacol Res. 61:269–280.

Rose BA, Force T, Wang Y. 2010. Mitogen-activated protein kinase signaling in the heart: Angels versus demons in a heart-breaking tale. Physiol Rev 90:1507–1546.

Sanz L, Diaz-Meco MT, Nakano H, Moscat J. 2000. The atypical PKC-interacting protein p62 channels NF-kappaB activation by the IL-1-TRAF6 pathway. EMBO J 19:1576–1586.

Schuh A, Liehn EA, Sasse A, Hristov M, Sobota R, Kelm M, Merx MW, Weber C. 2008. Transplantation of endothelial progenitor cells improves neovascularization and left ventricular function after myocardial infarction in a rat model. Basic Res Cardiol 103:69–77.

Shen DF, Tang QZ, Yan L, Zhang Y, Zhu LH, Wang L, Liu C, Bian ZY, Li H. 2010. Tetrandrine blocks cardiac hypertrophy by disrupting reactive oxygen species-dependent ERK1/2 signalling. Br J Pharmacol 159:970–981.

Shirakata M, Imadome KI, Okazaki K, Hirai K. 2001. Activation of TRAF5 and TRAF6 signal cascades negatively regulates the latent replication origin of Epstein–Barr virus through p38 mitogen-activated protein kinase. J Virol 75:5059–5068.

Swynghedauw B, Delcayre C, Samuel JL, Mebazaa A, Cohen-Solal A. 2010. Molecular mechanisms in evolutionary cardiology failure. Ann NY Acad Sci 1188:58–67.

Tada K, Okazaki T, Sakon S, Kobarai T, Kurosawa K, Yamaoka S, Hashimoto H, Mak TW, Yagita H, Okumura K, Yeh WC, Nakano H. 2001. Critical roles of TRAF2 and TRAF5 in tumor necrosis factor-induced NF-kappa B activation and protection from cell death. J Biol Chem 276:36530–36534.

Tang ED, Wang CY. 2010. TRAF5 is a downstream target of MAVS in antiviral innate immune signaling. PLoS ONE 5:e9172.

Tang Q, Cai J, Shen D, Bian Z, Yan L, Wang YX, Lan J, Zhuang GQ, Ma WZ, Wang W. 2009. Lysosomal cysteine peptidase cathepsin L protects against cardiac hypertrophy through blocking AKT/GSK3beta signaling. J Mol Med (Berl) 87:249–260.

Xu YJ, Chapman D, Dixon IM, Sethi R, Guo X, Dhalla NS. 2004. Differential gene expression in infarct scar and viable myocardium from rat heart following coronary ligation. J Cell Mol Med 8:85–92.

Zelarayan L, Renger A, Noack C, Zafiriou MP, Gehrke C, van der Nagel R, Dietz R, de Windt L, Bergmann MW. 2009. NF-kappaB activation is required for adaptive cardiac hypertrophy. Cardiovasc Res 84:416–424.