Tumor Necrosis Factor Receptor–Associated Factor 2 Signaling Provokes Adverse Cardiac Remodeling in the Adult Mammalian Heart

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Background—Tumor necrosis factor superfamily ligands provoke a dilated cardiac phenotype through a common scaffolding protein termed tumor necrosis factor receptor–associated factor 2 (TRAF2); however, virtually nothing is known about TRAF2 signaling in the adult mammalian heart.

Methods and Results—We generated multiple founder lines of mice with cardiac-restricted overexpression of TRAF2 and characterized the phenotype of mice with higher expression levels of TRAF2 (myosin heavy chain [MHC]-TRAF2HC). MHC-TRAF2HC transgenic mice developed a time-dependent increase in cardiac hypertrophy, left ventricular dilatation, and adverse left ventricular remodeling, and a significant decrease in LV+dp/dt and LV−dp/dt when compared with littermate controls (P<0.05 compared with littermate). During the early phases of left ventricular remodeling, there was a significant increase in total matrix metalloproteinase activity that corresponded with a decrease in total myocardial fibrillar collagen content. As the MHC-TRAF2HC mice aged, there was a significant decrease in total matrix metalloproteinase activity accompanied by an increase in total fibrillar collagen content and an increase in myocardial tissue inhibitor of metalloproteinase-1 levels. There was a significant increase in nuclear factor–κB activation at 4 to 12 weeks and jun N-terminal kinases activation at 4 weeks in the MHC-TRAF2HC mice. Transcriptional profiling revealed that >95% of the hypertrophic/dilated cardiomyopathy–related genes that were significantly upregulated genes in the MHC-TRAF2HC hearts contained κB elements in their promoters.

Conclusions—These results show for the first time that targeted overexpression of TRAF2 is sufficient to mediate adverse cardiac remodeling in the heart. (Circ Heart Fail. 2013;6:535-543.)

Key Words: dilated cardiomyopathy • inflammation • TNF receptor–associated factor 2 • tumor necrosis factor superfamily

Sustained inflammatory signaling in the heart leads to the development of a cardiomyopathy that is characterized by left ventricular (LV) dilatation, LV dysfunction, and myocardial fibrosis. If the inciting cause for the inflammation is resolved (eg, viral myocarditis or sepsis), the cardiomyopathy is often fully reversible, even though the extent of LV dysfunction may have been severe. If, on the contrary, inflammation is sustained and the inciting agent is persistent (eg, Chagas disease), sustained inflammatory signaling frequently leads to the development of an irreversible cardiomyopathy. Although a number of proinflammatory cytokines have been implicated in this process, the cytokine that has been characterized most extensively thus far is tumor necrosis factor (TNF). Indeed, mice with cardiac-restricted overexpression of TNF in the heart demonstrate consistent morphological and structural changes in the myocardium, including LV wall thinning, LV dilatation, LV dysfunction, and increased myocardial fibrillar collagen content, thus overlapping the phenotype observed in inflammation-induced dilated cardiomyopathy.1-3

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TNF is the prototypical member of the TNF superfamily (TNFSF), which consists of 19 well-characterized ligands and 34 TNF superfamily receptors (TNFRSF). Members of the TNFSF of ligands and receptors are expressed in a broad variety of cell types, including myocardial cells.7 Without exception, all members of the TNFSF exhibit proinflammatory activity. Of note, recent studies have identified a potential role for TNFSF ligands/receptors in terms of mediating inflammatory responses in the heart, including FasL/Fas (TNFSF6/TNFRSF6), TWEAK (tumor necrosis factor superfamily (TNFSF12/TNFRSF12)).
factor-like weak inducer of apoptosis)/TWEAKR (TNFSF12/TNFRSF12), and RANKL (receptor activator of NF-κB ligand)/RANK (TNFSF11/TNFRSF11). Although cardiac-restricted overexpression of FasL does not lead to a dilated cardiomyopathy,\(^1\) overexpression of TNF, TWEAK, and RANKL are each sufficient to provoke a dilated cardiac phenotype. In contrast to FasL, TNF, TWEAK, and RANKL signal through TNFSF receptors that engage a common scaffolding protein termed TNF receptor-associated factor 2 (TRAF2) that is recruited to the TNFSF receptors after engagement of their cognate ligands. Importantly, TRAF2 engages downstream signal transduction pathways that have been implicated in the development of a dilated cardiac phenotype, including mitogen-activated protein kinases, NF-κB, and c-jun N-terminal kinases (JNK).\(^2\) Taken together, these observations suggested the interesting possibility that TRAF2 may coordinate the signaling events that contribute to the changes in LV structure and function that occur during inflammation-induced cardiomyopathy. To address this question, we generated lines of transgenic mice with cardiac-restricted overexpression of TRAF2. Here, we show for the first time that cardiac-restricted overexpression of TRAF2 is sufficient to mediate adverse cardiac remodeling in the heart and thus phenocopies many aspects of the deleterious effects of TNFSF ligand signaling.

**Methods**

**Transgenic Mice**

We generated founder lines of transgenic mice with cardiac-restricted expression of murine TRAF2, using the α-myosin heavy chain (MHC) promoter (a gift from Jeff Robbins) to target TRAF2 to the cardiac myocyte, as described.\(^3\) Mice expressing the highest copy number of TRAF2 (referred here to as MHC-TRAF2\(_{HC}\)) were selected for further study. The MHC-TRAF2\(_{HC}\) lines of mice were generated and maintained on an FVB background. Age-matched littermate mice (LM) that lack the transgene were used as controls in these experiments.

All experiments were approved by the Institutional Animal Care and Use Committees at the Baylor College of Medicine and Washington University School of Medicine and were conducted in accordance with the guidelines of the Baylor College of Medicine and Washington University School of Medicine, Animal Care and Research Advisory Committee and the rules governing animal use, as published by the National Institutes of Health.

**Characterization of MHC-TRAF2\(_{HC}\) Mice**

MHC-TRAF2\(_{HC}\) and LM control mice were evaluated at 4, 8, and 12 weeks age using standard morphometric and histological analyses.\(^4\) Cardiac hypertrophy was evaluated by determining the heart weight/body weight ratio, as described.\(^5\) Cardiac-restricted expression of murine TRAF2 was found to mediate adverse cardiac remodeling in the heart and thus phenocopies many aspects of the deleterious effects of TNFSF ligand signaling.

**LV Structure and Function**

LV structure and function were assessed using two dimension-direct- ed M-mode echocardiography as described previously (see online-only Data Supplement for details).\(^6\)

**Extracellular Matrix**

Deparaffinized sections of perfusion fixed hearts from 4-, 8-, and 12-week-old MHC-TRAF2\(_{HC}\) and littermate control mice were stained using the picrosirius red technique, as described (see online-only Data Supplement for details).\(^7\) Matrix metalloproteinases (MMP) activity from 4-, 8-, and 12-week-old MHC-TRAF2\(_{HC}\) and LM control mouse hearts was obtained using gelatin zymography, as previously described (see online-only Data Supplement for details).\(^8\) The intensity of the zymographic bands was quantified by image analysis software (ImageJ, National Institutes of Health, Bethesda, MD). Levels of tissue inhibitors of metalloproteinase (TIMP-1) were measured in myocardial extracts from MHC-TRAF2\(_{HC}\) mice and littermate controls at 4, 8, and 12 weeks by ELISA (Amersham RPN 2611), according to manufacturer’s recommendations.\(^9\)

**Cell Death**

Cardiac myocyte apoptosis was assessed in the hearts of 4-, 8-, and 12-week LM and MHC-TRAF2\(_{HC}\) mice by terminal deoxynucleotidyl transferase (TUNEL), using a commercially available kit (in situ Cell Death Detection Kit; Roche Applied Science, Mannheim, Germany), according to the manufacturer’s suggestions. Cardiac myocytes were distinguished from nonmyocyte cell types within the myocardium, as described.\(^10\) Sections were counterstained with the nucleic acid binding dye, 4′,6′-diamidino-2-phenylindole hydrochloride (Vector Labs) to visualize the total number of myocyte nuclei in each myocardial section. The number of TUNEL positive nuclei per high-power field (×400) was determined in 8 randomly selected fields by an investigator blinded to the experimental group being studied. We also examined for the presence or absence of random cell necrosis at 4 weeks by Evans Blue dye staining. In brief, mice were injected intraperitoneally with 1% Evans Blue dye solution. Hearts were excised, and nuclei were stained with 4′,6′-diamidino-2-phenylindole hydrochloride (Vector Labs) to visualize the total number of myocyte nuclei in each myocardial section.

**NF-κB Activation and JNK Activity**

NF-κB activation was assessed using electrophoretic mobility-shift assays, using an NF-κB oligonucleotide consensus sequence (5′-AGT TGA GGC TTT CCC AGG C-3′) (Santa Cruz Biotechnology, Santa Cruz, CA), as described.\(^11\) The specificity of binding was determined by competition with a 20× magnification molecular excess of the respective unlabeled oligonucleotide. NF-κB activation was also assessed at 12 weeks in nuclear extracts obtained from 12-week MHC-TRAF2\(_{HC}\) and LM control hearts using a NF-κB factor family (p65 [RelA], p50, p52, RelB) ELISA (Active Motif, Carlsbad, CA), which was performed exactly according to manufacturer’s instructions. Data represent the average of duplicate samples from each heart. NF-κB activity was determined in littermate control and TRAF2 hearts at 4, 8, and 12 weeks of age, using the SAPK (stress-activated protein kinase)/JNK Assay kit (Cat no. 9810 from Cell Signaling Technology, Inc, Danvers, MA), according to the manufacturer’s instructions.

**Gene Expression Profiling in MHC-TRAF2\(_{HC}\) Hearts**

To identify potential molecular pathways responsible for the phenotype of the MHC-TRAF2\(_{HC}\) mice, gene expression profiling was performed. In brief, total RNA was extracted from the 4 LM control hearts and 4 MHC-TRAF2\(_{HC}\) hearts using TRIzol reagent (Invitrogen, Carlsbad, CA) as per the manufacturer’s instructions. RNA was further processed and hybridized to a Mouse Ref-8 Illumina BeadChip by the Genome Technology Access Center at Washington University School of Medicine and scanned with the BeadStation system from Illumina.
Illumina, Inc (San Diego, CA). Quality standards for hybridization, labeling, staining, background signal, and basal level of housekeeping gene expression for each chip were verified. After scanning the probe array, the resulting image was analyzed using the GenomeStudio software (Illumina, Inc, San Diego, CA). The background was subtracted, and log transformation and quantile normalization were performed. Differentially expressed genes between MHC-TRAF2\(_{HC}\) and LM control mice were determined using ANOVA testing with contrasts using Partek GS (Partek, St. Louis, MO), using an unadjusted \(P\) value <0.05 and \(≥\)2-fold change. Changes in gene expression were analyzed by the statistical analysis of microarray program and plotted.\(^{16}\) Expected differentially expressed genes are reported on the x axis, whereas observed genes that were differentially expressed are displayed in the y axis. A false-discovery rate <5% was used for the statistical analysis of microarray plots. Functional analysis and pathway analysis were performed using database for annotation visualization and integrated discovery.\(^{17}\) Lists of genes that were significantly different in the MHC-TRAF2\(_{HC}\) lines with targeted overexpression of murine TRAF2, referred to herein as MHC-TRAF2\(_{HC}\) mice.

**Statistical Analysis**

Data are expressed as means±SEM. Two-way repeated ANOVA was used to test for differences between MHC-TRAF2\(_{HC}\) and littermate controls at 4, 8, and 12 weeks of age. Post hoc analysis of variance testing was performed between groups at individual time points where appropriate, using the Tukey test. Differences in LV dimension, \(+dP/dt, −dP/dt,\) and necropsy data between the MHC-TRAF2 HC mice and littermate controls at 4, 8, and 12 weeks of age. As shown, the MHC-TRAF2\(_{HC}\) mice develop a dilated cardiac phenotype (Figure 1A) characterized by an increased heart weight/body ratio (Figure 1C). The increased heart weight/body ratio was secondary to a significant increase in heart weight (mg) in the MHC-TRAF2\(_{HC}\) mice at 12 weeks (130.5±5.5 versus 112.9±5.4; \(P<0.05\)), insofar as the body weight (gm) was not different in the MHC-TRAF2\(_{HC}\) and LM control mice, respectively (25.5±0.8 versus 24.1±0.8; \(P>0.05\)), at 12 weeks. Light microscopic evaluation of the 12-week hearts from MHC-TRAF2\(_{HC}\) mice revealed that the histological appearance was similar to LM controls. Notably, there was no discernible inflammatory infiltrate by hematoxylin and eosin staining (Figure 1B). Transmission electron microscopic evaluation of the 12-week LM hearts revealed a characteristic linear array of sarcomeres and myofibrils (Figure 1D and 1E). In contrast, the myofibrils in the 12-week-old MHC-TRAF2\(_{HC}\) mice were less organized, with loss of sarcomere registration, effacement of the Z line, loss of the M line, and accumulation of protein aggregates, consistent with the ultrastructural changes previously reported in MHC-TNF mice.\(^{1,18}\)

**Results**

**Characterization of MHC-TRAF2\(_{HC}\) Mice**

The generation and characterization of the transgenic founder lines with targeted overexpression of murine TRAF2 is reported in the online-only Data Supplement (see Figure 1 and Table I). Founder lines that expressed >10 copies of the transgene developed a dilated phenotype by 12 weeks of age that was similar to the phenotype observed in lines of mice with targeted overexpression of TNF.\(^{1,2}\) For the studies reported herein, we selected mice that expressed 24 copies of the TRAF2 transgene, referred to herein as MHC-TRAF2\(_{HC}\) mice.

**Morphology and Ultrastructure**

Figure 1 depicts the characterization of the MHC-TRAF2\(_{HC}\) mouse hearts in comparison with LM controls at 12 weeks of age. As shown, the MHC-TRAF2\(_{HC}\) mice develop a dilated cardiac phenotype (Figure 1A) characterized by an increased heart weight/body ratio (Figure 1C). The increased heart weight/body ratio was secondary to a significant increase in heart weight (mg) in the MHC-TRAF2\(_{HC}\) mice at 12 weeks (130.5±5.5 versus 112.9±5.4; \(P<0.05\)), insofar as the body weight (gm) was not different in the MHC-TRAF2\(_{HC}\) and LM control mice, respectively (25.5±0.8 versus 24.1±0.8; \(P>0.05\)), at 12 weeks. Light microscopic evaluation of the 12-week hearts from MHC-TRAF2\(_{HC}\) mice revealed that the histological appearance was similar to LM controls. Notably, there was no discernible inflammatory infiltrate by hematoxylin and eosin staining (Figure 1B). Transmission electron microscopic evaluation of the 12-week LM hearts revealed a characteristic linear array of sarcomeres and myofibrils (Figure 1D and 1E). In contrast, the myofibrils in the 12-week-old MHC-TRAF2\(_{HC}\) mice were less organized, with loss of sarcomere registration, effacement of the Z line, loss of the M line, and accumulation of protein aggregates, consistent with the ultrastructural changes previously reported in MHC-TNF mice.\(^{1,18}\)

**LV Structure and Function**

As shown in Figure 2A, there was a progressive increase in LV end-diastolic dimension in the MHC-TRAF2\(_{HC}\) mice that was significantly (\(P<0.05\)) different from LM controls by 8 weeks.
and 12 weeks. There was also a corresponding significant \( (P<0.05) \) increase in the \( r/h \) ratio in the MHC-TRAF2HC mouse hearts at 12 weeks of age when compared with LM, consistent with adverse cardiac remodeling (Figure 2B). Importantly, the increase in \( r/h \) ratio was not secondary to decreased wall thickness in the MHC-TRAF2HC mice, because the LV wall thickness was similar in the LM and MHC-TRAF2HC mice at 12 weeks of age (1.19±0.08 versus 1.16±0.07, respectively; \( P>0.05) \). The increase in heart weight/body ratio in the MHC-TRAF2HC mice at 12 weeks was accompanied by a \( \approx 24\% \) increase in myocyte length (276.2±3.5 versus 222.5±3.4 μm; \( P<0.001 \)) and a corresponding \( \approx 11\% \) decrease in myocyte width (50.9±1.1 versus 56.9±1.8; \( P<0.01) \) compared with age-matched LMs, consistent with cardiac myocyte elongation observed in eccentric remodeling of the heart. To determine whether targeted expression of TRAF2 resulted in LV dysfunction, as we have observed in lines of mice with targeted overexpression of TNF,19 we measured LV +dP/dt and LV−dP/dt ex vivo in a buffer-perfused Langendorff apparatus. As shown in Figure 2C and 2D, there was a significant \( (P<0.01) \) decrease in peak+dP/dt and peak−dP/dt in 12-week MHC-TRAF2HC mice when compared with LM controls.

**Extracellular Matrix**

To characterize the alterations in the extracellular matrix associated with LV remodeling in the MHC-TRAF2HC mice, we evaluated time-dependent changes in myocardial fibrillar collagen content using picrosirius red staining. Figure 3A shows representative histological sections of myocardium at 12 weeks, and Figure 3B summarizes the result of group data. As shown, LV myocardial fibrillar collagen content was similar \( (P>0.05) \) in MHC-TRAF2HC and LM control hearts at 4 weeks. However, as the mice aged, there was a significant (2.1-fold) increase in collagen content in the MHC-TRAF2HC mice at 8 weeks \( (P=0.002) \) and a significant (4.4-fold) increase at 12 weeks \( (P=0.019) \) compared with LM controls, consistent with our previous observations in mice with targeted overexpression of TNF.1

To determine whether the increased fibrillar collagen content in the MHC-TRAF2HC mice was secondary to changes in MMP activity and myocardial levels of TIMP activity, we examined MMP activity and TIMP levels. Figure 3C and 3D show 2 important findings. First, there was an \( \approx 40\% \) increase in MMP zymographic activity in the MHC-TRAF2HC mice at 4 weeks of age compared with LM controls \( (P<0.05). \) However, at 8 and 12 weeks of age, the MMP activity in the MHC-TRAF2HC mice declined significantly and was not significantly different \( (P>0.05) \) in MHC-TRAF2HC and LM hearts at 8 and 12 weeks. The decrease in MMP activity was accompanied by an increase in TIMP-1 levels, which were significantly greater \( (P<0.05) \) in the MHC-TRAF2HC mice by 12 weeks of age. Taken together, the decrease in MMP activity and increase in TIMP levels seen at 12 weeks of age is consistent with the increase in fibrosis observed in the MHC-TRAF2HC mice at 12 weeks of age.

**Cell Death**

Previously, we have reported that lines of transgenic mice with targeted overexpression of TNF develop progressive cardiac myocyte apoptosis that contributes to adverse cardiac remodeling.18,20 Figure 4A illustrates representative fluorescent micrographs of TUNEL staining in MHC-TRAF2HC at 12 weeks, and Figure 4B summarizes the results of group data at 4, 8, and 12 weeks of age. As shown, the number of TUNEL positive nuclei was significantly increased \( (P<0.05) \) in the MHC-TRAF2HC hearts when compared with LM controls, whereas the prevalence of TUNEL positive nuclei was similar \( (P>0.05) \) in MHC-TRAF2HC and LM hearts at 8 and 12 weeks of age, suggesting that progressive cardiac myocyte apoptosis
was unlikely to contribute to the progressive cardiac dilation observed in the MHC-TRAF2HC mice at 8 and 12 weeks of age (Figure 1D). There was no evidence of myocyte necrosis in the MHC-TRAF2LC or LM control hearts (Figure II in the online-only Data Supplement), consistent with our observations in the MHC-TNF mice.14

**NF-κB Activation and JNK Activity**

Previous studies suggest that the effects of TRAF2 in nonmyocytes are mediated by activation of NF-κB and JNK.21 To determine whether these pathways were activated in the MHC-TRAF2HC transgenic mouse hearts, we assessed NF-κB activation by electrophoretic mobility-shift assay and ELISA, as well as JNK activity. Figure 5A shows a representative electrophoretic mobility-shift assay at 12 weeks of age, whereas Figure 5B summarizes the results of group data at 4 to 12 weeks. The salient finding shown by Figure 5B is that NF-κB activation was significantly increased at 4, 8, and 12 weeks in the MHC-TRAF2HC mice compared with LM control mice. The specificity of the DNA–protein interaction was determined by cold-chase experiments, which showed that the labeled NF-κB–DNA complexes were disrupted by a 20-fold excess of unlabeled oligonucleotide. As shown in Figure III in the online-only Data Supplement, there was a significant increase in p50, RelB, and p52 subunits in the MHC-TRAF2HC hearts when compared with LM controls. Moreover, there was a significant increase in p50, RelB, and p52 subunits in the MHC-TRAF2HC hearts when compared with MHC-TRAF2LC hearts, consistent with a gene dosage effect of TRAF2 on activation of canonical and noncanonical signaling in the MHC-TRAF2HC mice. The important finding shown by Figure 5C is that although JNK activity was increased in the hearts of the MHC-TRAF2HC mice at 4 weeks compared with LM, there was no significant difference in JNK activity at 8 and 12 weeks, consistent with our findings in the MHC-TRAF2LC mouse hearts.3

**Transcriptional Profiling in MHC-TRAF2HC Mice**

To determine the mechanisms responsible for the dilated cardiac phenotype in the MHC-TRAF2HC mouse hearts, we performed transcriptional profiling at 12 weeks of age. As shown by the statistical analysis of microarray plot in Figure IVA in the online-only Data Supplement, 1136 genes were significantly upregulated, and 823 genes were significantly downregulated in the 12-week MHC-TRAF2HC mouse hearts compared with LM controls. Kyoto encyclopedia of genes and genomes functional analysis (Figure IVB in the online-only Data Supplement) identified significant changes in gene expression in pathways implicated in both hypertrophic (P<0.005) and dilated cardiomyopathy (P<0.03) in the MHC-TRAF2HC compared with LM control hearts. Figure 6 displays a diagram of the cardiac hypertrophy/dilated cardiomyopathy–related cardiac myocyte genes that were either upregulated or downregulated in the MHC-TRAF2HC hearts compared with LM controls. Notably, genes that were involved with altered cytoskeletal linkage (α7 integrin subunit, cytосolic actin, desmin, α- and γ-sarcoglycan), decreased muscle contraction (α- and β-myosin heavy chain, troponin I), impaired excitation contraction coupling (β-1,4 subunits of the dihydropyridine receptor, ryanodine receptor, and SERCA2A) and increased myocardial fibrosis (TGF-β1) were altered in the transgenic mouse hearts (see Table II in the online-only Data Supplement). Of the 37 cardiac hypertrophy/dilated cardiomyopathy pathway, genes identified in the Kyoto encyclopedia of genes and genomes analysis, 35 genes (94.6%) had predicted κB sites when analyzed by the UCSC_TFBS option in database for annotation visualization and integrated discovery, consistent with the significant increase in NF-κB activity observed in the MHC-TRAF2HC hearts.

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Discussion

The results of this study, in which we generated and characterized lines of transgenic mice with cardiac-restricted overexpression of TRAF2 (MHC-TRAF2HC), demonstrate that TRAF2-mediated signaling leads to a dilated cardiac phenotype that overlaps the phenotype observed in transgenic mice overexpressing TNFSF superfamily ligands.1–3,5 The following lines of evidence support this statement. First, MHC-TRAF2HC transgenic mice develop progressive LV dilation, cardiac hypertrophy, and adverse cardiac remodeling (Figures 2A, 2B, and 1C). Second, the 12-week MHC-TRAF2HC mouse hearts had significantly decreased LV+dP/dt and LV−dP/dt when compared with LM controls (Figure 2C and 2D). Analysis of myocardial ultrastructure in 12-week-old MHC-TRAF2HC disclosed sarcomere disarray, with effacement of the Z line and loss of the M line, thus providing a potential ultrastructural explanation for the observed decline in LV systolic and diastolic function (Figure 1F and 1G). Third, LV dilation in the MHC-TRAF2HC was accompanied by increased MMP activation at 4 weeks, followed by increased TIMP expression and increased myocardial fibrosis at 8 and 12 weeks, consistent with previous observations in the MHC-TNF transgenic mice.10,14 The reasons for the differences in the prevalence of myocyte apoptosis in the 2 models are not clear, but may be related to the complexity of TRAF2-mediated signaling, which can transmit prodeath or prolife signals depending on the context of TRAF2 activation.23 Finally, we observed increased NF-κB activation from 4 to 12 weeks (Figure 5B) and increased JNK activity at 4 weeks of age (Figure 5C) in the MHC-TRAF2HC mice, consistent with the known signaling pathways downstream from TRAF2.21 Although this study was not designed to delineate the specific signaling pathways that are responsible for heart failure phenotype in the MHC-TRAF2HC mice, our results suggest an important role for NF-κB signaling. As shown in Figure 6, the transcriptional profiling studies show that TRAF2 signaling leads to alterations in cardiac myocyte gene expression that have been associated with the development of heart failure, including alterations in the cytoskeleton genes, activation of fetal gene programs, changes in excitation contraction coupling,
as well as alterations in genes, that regulate remodeling of the extracellular matrix. The observation that NF-κB activation persisted from 4 to 12 weeks and occurred pari passu with the development and progression of cardiac remodeling, coupled with the observation that >95% of the genes identified in the Kyoto encyclopedia of genes and genomes cardiac hypertrophy/dilated cardiomyopathy functional pathway analysis (Figure 6 and Table II in the online-only Data Supplement) contain predicted κB binding sites, suggesting an important mechanistic role for TRAF2-mediated activation of NF-κB. Moreover, this point of view is consistent with a previous report which demonstrated that backcrossing TNF transgenic mice (TNF 1.6) with a transgenic mice expressing a cardiac-restricted dominant negative IκB transgene (3M) resulted in improved fractional shortening, decreased cardiac hypertrophy, and improved survival.²⁴ Data from the present study do not support a critical role for TRAF2-mediated JNK activation in the development of a dilated cardiac phenotype, insofar as JNK activation was not significant for >4 weeks of age in the MHC-TRAF2HC mice. The mechanism for the decrease in JNK signaling in the MHC-TRAF2HC from 8 to 12 weeks is not known, but is consistent with the known effects of NF-κB–mediated dampening of JNK activation through GADD45β.²⁵

TRAF-Mediated Signaling in the Heart

TNF and its superfamily of ligands and receptors represent a double-edged sword for the cardiovascular system. That is, members of the TNFSF play an important role in mediating homeostatic response in the heart, as well as deleterious effects, when activated either inappropriately or in a sustained manner.²⁶ Relevant to this discussion, many of the TNF receptor superfamily members (eg, type 1 TNF receptor [TNFR1, TNFRSF1A] and FAS) contain death domains that initiate signal transduction pathways that lead to caspase activation and apoptotic cell death, consistent with the known deleterious effects of TNFSF/TNFRSF signaling. Recently, a family of cytoplasmic proteins has been identified that is capable of both negatively and positively regulating apoptotic pathways, as well as inducing the expression of genes, that promote cell survival. Members of this family of signal transduction molecules were first described because of their ability to bind to the type 2 TNF receptor (TNFR2, TNFRSF1B) and, therefore, were given the name TNF receptor–associated factors. Subsequent studies have demonstrated that TRAFs serve as adapter proteins for a wide variety of innate immune cell surface receptors and play important roles in regulating not only apoptosis, but also in mediating stress responses. TRAF proteins are cytoplasmic adapter proteins
that can interact directly with the intracellular domains of cell surface receptors (e.g., TNFR2) or can be recruited indirectly through adapter proteins that bind directly to the cytoplasmic tail of the TNFRSF member (e.g., TRADD-binding TNFRI). Recruitment of TRAF2 to the cytoplasmic domains of receptors leads to the assembly of signaling complexes that activate mitogen-activated kinases that converge on JNK and NF-kB signaling. To date, 7 mammalian TRAFs (TRAF1-7) have been identified, of which TRAF 2, 3, and 6 are known to be expressed in the heart. With the exception of TRAF2, which is capable of upregulating cytoprotective pathways and is cardioprotective when expressed at low levels, virtually nothing is known about the role of TRAFs in the mammalian heart. Here, we show that overexpression of TRAF2 in the heart phenocopies the diluted cardiomyopathic phenotype observed in TNF and sTWEAK transgenic mice. Although the precise reasons for the beneficial and deleterious cardiac phenotypes in mice that express, respectively, low and high levels of TRAF2 is not known, our data suggest that these differences are related to a gene dosage effect, insofar as there was a significantly greater degree of activation of canonical and noncanonical NF-κB subunits in the MHC-TRAF2HC mouse hearts when compared with MHC-TRAF2LC mouse hearts (Figure III in the online-only Data Supplement). Nonetheless, we cannot exclude the formal possibility that the effects observed in the MHC-TRAF2HC mice were nonspecific and were secondary to high levels of expression of a transgene, as has been reported for invertebrate proteins that have been overexpressed in the heart.

Conclusions
This study shows for the first time that TRAF2-mediated signaling is sufficient to confer a heart failure phenotype in the adult mammalian heart. Given that TRAF2 associates directly or indirectly with the majority of TNFSF receptor members expressed in the heart (TNFRI, TNFR2, RANK, and TWEAKR) and that the respective cognate ligands for these receptors, including TNF, RANKL, and TWEAK, provoke a heart failure phenotype, the results of this study raise the interesting possibility that TRAF2 may serve as a nodal convergence point that orchestrates inflammatory responses in the heart after cardiac injury. Given that TRAF2-mediated signaling can be targeted through activation of GPR120 with ω-3 fatty acids, which have been shown to be beneficial in heart failure, or possibly through disruption of E3 ligases which are essential for TRAF2 signaling, the current observations may have therapeutic importance as well.

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Disclosures
None.

References
Sustained inflammatory signaling in the heart leads to the development of a cardiomyopathy that is characterized by left ventricular dilation, left ventricular dysfunction, and myocardial fibrosis. Although a number of proinflammatory cytokines have been implicated in this process, the cytokine that has been characterized most extensively thus far is tumor necrosis factor. All tumor necrosis factor superfamily ligands that provoke a dilated cardiac phenotype signal through a common scaffolding protein termed tumor necrosis factor receptor–associated factor 2 (TRAF2); however, virtually nothing is known about TRAF2 signaling in the adult mammalian heart. We generated transgenic mice with cardiac-restricted overexpression of TRAF2 and characterized the phenotype of mice with high levels of TRAF2 (myosin heavy chain [MHC]-TRAF2_{HC}) expression. MHC-TRAF2_{HC} transgenic mice developed a time-dependent increase in cardiac hypertrophy, LV dilation, and adverse LV remodeling with myocardial fibrosis, and a significant decrease in LV+dP/dt and LV−dP/dt when compared with littermate controls. These changes were accompanied by a significant increase in NF-κB activation at 4 to 12 weeks and c-jun N-terminal kinases activation at 4 weeks in the MHC-TRAF2_{HC} mice. Transcriptional profiling revealed that >95% of the hypertrophic/dilated cardiomyopathy–related genes that were significantly upregulated in the MHC-TRAF2_{HC} hearts contained κB elements in their promoters. Viewed together, these results suggest that TRAF2 is sufficient to mediate adverse cardiac remodeling in the heart and raise the interesting possibility that TRAF2 may serve as a nodal convergence point that orchestrates inflammatory responses in the heart after cardiac injury.
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DATA SUPPLEMENT TO:

Tumor Necrosis Factor Receptor Associated Factor 2 Signaling Provokes Adverse Cardiac Remodeling in the Adult Mammalian Heart

Methods/Results

Characterization of Founder Lines of MHC-TRAF2 Mice

The TRAF2 cDNA used for to generate the MHC-TRAF2 construct was the generous gift of Dr. Yongwon Choi. To facilitate separation of endogenous TRAF2 from the TRAF2 that was driven by the alpha-myosin heavy chain promoter, we FLAG-tagged the TRAF-2 construct using an EcoRV cleavage site with the primer sequence: CTA GGA TAT CCG TTT AGT GAA CCG TCA GAA TTG ATC TAC CAT GGA CTA CAA AGACGA TGA CGA CAA GGC TGC AGC CAG TGT GAC. The FLAG-tag was inserted at the N-terminus using a NotI cleavage site with the primer sequence: ACG TGC GGC CGC CTA GAG TCC TGT C. The transgene construct was injected into single cell embryos of FVB mice at the Baylor College of Medicine transgenic core facility. Founder lines of MHC-TRAF2 transgenic mice (FVB background) were identified by PCR and confirmed by Southern blotting using a 286 bp probe targeted to exon 8 of the TRAF2 gene (supplemental Figure IA). Western blotting was performed to confirm the expression of TRAF2 protein in the hearts of 12 week MHC-TRAF2HC mouse hearts (supplemental Figure IB) using an anti-TRAF2 antibody (sc-877, Santa Cruz Biotechnology, Santa Cruz, CA). Immunohistochemical staining of founder lines showed that TRAF2 protein was localized diffusely throughout the cytoplasm in MHC-TRAF2 mouse hearts compared to littermate control mice (supplemental Figure IC). We obtained three founder lines: 329W (7 copies of TRAF2 transgene); 330W (10 copies of transgene); 335W (24 copies of transgene).

Supplemental Table I displays the morphometrics of the MHC-TRAF2 founder lines of mice. The 329W line, with the lowest copy of the transgene, referred to as MHC-TRAF2LC, has been characterized in detail and reported previously. Mice with 10 and 24 copies of the TRAF2 transgene developed an increased heart-weight-to-body weight ratio and a dilated cardiac phenotype by 12 weeks of age. Lines of mice that
expressed 24 copies of the transgene (referred to here as MHC-TRAF2_{HC} mice) were found to express ~ two-fold higher levels of TRAF2 protein compared to mice that expressed 7 or 10 copies by Western blot analysis (supplemental Figure 1B). Mice expressing 24 copies of the TRAF2 transgene (referred to here as MHC-TRAF2_{HC}) were selected for further study.

REFERENCES


Supplemental Table I
Characterization of founder lines of mice with targeted overexpression of TRAF2

<table>
<thead>
<tr>
<th>Mice</th>
<th>Copy #</th>
<th>HW (mg)</th>
<th>BW (g)</th>
<th>HW/BW (mg/g)</th>
<th>Cardiac Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC-TRAF2_{LC}</td>
<td>7</td>
<td>132.0±7.3*</td>
<td>28.9±1.6</td>
<td>4.6±0.2*</td>
<td>Hypertrophy</td>
</tr>
<tr>
<td>LM</td>
<td>-</td>
<td>120.7±4.4</td>
<td>30.1±1.5</td>
<td>4.0±0.1</td>
<td></td>
</tr>
<tr>
<td>MHC-TRAF2_{IC}</td>
<td>10</td>
<td>184.3±0.01*</td>
<td>25.7±1.2</td>
<td>7.2±0.5*</td>
<td>Dilated</td>
</tr>
<tr>
<td>LM</td>
<td>-</td>
<td>117.7±0.01</td>
<td>27.9±2.6</td>
<td>4.2±0.1</td>
<td></td>
</tr>
<tr>
<td>MHC-TRAF2_{HC}</td>
<td>24</td>
<td>130.5±5.5*</td>
<td>25.5±0.8</td>
<td>5.3±0.1*</td>
<td>Dilated</td>
</tr>
<tr>
<td>LM</td>
<td>-</td>
<td>112.9±5.4</td>
<td>24.1±0.8</td>
<td>4.5±0.1</td>
<td></td>
</tr>
</tbody>
</table>

(Data are expressed as mean ± SEM. Necropsy data for heart weight (HW), body weight (BW), heart-weight-to-body-weight-ratio (HW/BW) and phenotype of 12 week old mice with varying copies of the TRAF2 transgene and 12 week littermate (LM) controls. (n ≥ 5 mice per group; * = p <= 0.05 vs. littermate control)
### Supplemental Table II: Changes in Gene Expression in MHC-TRAF2<sub>HC</sub> Mice

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene Name</th>
<th>Expression level (relative to LM)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Increased</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MYH7</td>
<td>myosin, heavy chain 7, cardiac muscle, beta</td>
<td>14.92</td>
<td>7.61x10&lt;sup&gt;-05&lt;/sup&gt;</td>
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<tr>
<td>TGBF3</td>
<td>transforming growth factor, beta 3</td>
<td>4.00</td>
<td>9.82x10&lt;sup&gt;-06&lt;/sup&gt;</td>
</tr>
<tr>
<td>TIMP1</td>
<td>Tissue inhibitor of metalloproteinase 1</td>
<td>3.39</td>
<td>2.82x10&lt;sup&gt;-03&lt;/sup&gt;</td>
</tr>
<tr>
<td>MMP23</td>
<td>Matrix metallopeptidase 23</td>
<td>2.88</td>
<td>5.252x10&lt;sup&gt;-03&lt;/sup&gt;</td>
</tr>
<tr>
<td>CACNB1</td>
<td>calcium channel, voltage-dependent, beta 1 subunit</td>
<td>2.60</td>
<td>8.49x10&lt;sup&gt;-04&lt;/sup&gt;</td>
</tr>
<tr>
<td>TGBF2</td>
<td>transforming growth factor, beta 2</td>
<td>2.29</td>
<td>3.88x10&lt;sup&gt;-02&lt;/sup&gt;</td>
</tr>
<tr>
<td>DES</td>
<td>Desmin</td>
<td>2.22</td>
<td>9.84x10&lt;sup&gt;-05&lt;/sup&gt;</td>
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<tr>
<td>ITGA11</td>
<td>integrin, alpha 11</td>
<td>2.05</td>
<td>2.53x10&lt;sup&gt;-02&lt;/sup&gt;</td>
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<tr>
<td>PRKB2</td>
<td>protein kinase, AMP-activated, beta 2 non-catalytic subunit</td>
<td>1.81</td>
<td>7.88x10&lt;sup&gt;-03&lt;/sup&gt;</td>
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<tr>
<td>COL1A1</td>
<td>collagen, type I, alpha 1</td>
<td>1.77</td>
<td>1.70x10&lt;sup&gt;-02&lt;/sup&gt;</td>
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<tr>
<td>COL3A1</td>
<td>collagen, type III, alpha 1</td>
<td>1.77</td>
<td>3.24x10&lt;sup&gt;-02&lt;/sup&gt;</td>
</tr>
<tr>
<td>MMP14</td>
<td>matrix metallopeptidase 14</td>
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<td>2.96x10&lt;sup&gt;-02&lt;/sup&gt;</td>
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<tr>
<td>MMO2</td>
<td>matrix metallopeptidase 2</td>
<td>1.60</td>
<td>2.66x10&lt;sup&gt;-02&lt;/sup&gt;</td>
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<tr>
<td>ITGB5</td>
<td>integrin, beta 5</td>
<td>1.51</td>
<td>4.16x10&lt;sup&gt;-02&lt;/sup&gt;</td>
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<tr>
<td>LMNA</td>
<td>lamin A/C</td>
<td>1.50</td>
<td>9.95x10&lt;sup&gt;-04&lt;/sup&gt;</td>
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<tr>
<td>ACTB</td>
<td>actin, beta</td>
<td>1.48</td>
<td>7.37x10&lt;sup&gt;-03&lt;/sup&gt;</td>
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<tr>
<td>TIMP2</td>
<td>Tissue inhibitor of metalloproteinase 2</td>
<td>1.38</td>
<td>1.44x10&lt;sup&gt;-02&lt;/sup&gt;</td>
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<tr>
<td>IGF1</td>
<td>insulin-like growth factor 1 (somatomedin C)</td>
<td>1.37</td>
<td>1.77x10&lt;sup&gt;-03&lt;/sup&gt;</td>
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<tr>
<td>MMP7</td>
<td>Matrix metalloproteinase 7</td>
<td>1.34</td>
<td>6.34x10&lt;sup&gt;-03&lt;/sup&gt;</td>
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<tr>
<td>MYBP3</td>
<td>myosin binding protein C, cardiac</td>
<td>1.29</td>
<td>2.93x10&lt;sup&gt;-02&lt;/sup&gt;</td>
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<tr>
<td>ITGA5</td>
<td>integrin, alpha 5 (fibronectin receptor, alpha polypeptide)</td>
<td>1.28</td>
<td>3.42x10&lt;sup&gt;-02&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Decreased</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>SGCA</td>
<td>sarcoglycan, alpha (50kDa dystrophin-associated glycoprotein)</td>
<td>-1.94</td>
<td>8.27x10&lt;sup&gt;-04&lt;/sup&gt;</td>
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<tr>
<td>TNNI3</td>
<td>troponin I type 3 (cardiac)</td>
<td>-1.66</td>
<td>2.39x10&lt;sup&gt;-03&lt;/sup&gt;</td>
</tr>
<tr>
<td>ITGA7</td>
<td>integrin, alpha 7</td>
<td>-1.65</td>
<td>6.78x10&lt;sup&gt;-03&lt;/sup&gt;</td>
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<tr>
<td>SGCG</td>
<td>sarcoglycan, gamma (35kDa dystrophin-associated glycoprotein)</td>
<td>-1.62</td>
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<tr>
<td>ADCY6</td>
<td>adenylate cyclase 6</td>
<td>-1.59</td>
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<tr>
<td>MMP15</td>
<td>matrix metalloproteinase 15</td>
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<td>PRKAB1</td>
<td>protein kinase, AMP-activated, beta 1 non-catalytic subunit</td>
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<td>1.18x10&lt;sup&gt;-02&lt;/sup&gt;</td>
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<tr>
<td>ATP2A2</td>
<td>ATPase, Ca++ transporting, cardiac muscle, slow twitch 2</td>
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<tr>
<td>ITGB6</td>
<td>integrin, beta 6</td>
<td>-1.41</td>
<td>3.48x10&lt;sup&gt;-03&lt;/sup&gt;</td>
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<tr>
<td>TGBF1</td>
<td>transforming growth factor, beta 1</td>
<td>-1.33</td>
<td>2.41x10&lt;sup&gt;-02&lt;/sup&gt;</td>
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<tr>
<td>ADCY4</td>
<td>adenylate cyclase 4</td>
<td>-1.31</td>
<td>3.09x10&lt;sup&gt;-03&lt;/sup&gt;</td>
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<tr>
<td>MYH6</td>
<td>myosin, heavy chain 6, cardiac muscle, alpha</td>
<td>-1.26</td>
<td>2.40x10&lt;sup&gt;-02&lt;/sup&gt;</td>
</tr>
<tr>
<td>RYR2</td>
<td>ryanodine receptor 2 (cardiac)</td>
<td>-1.25</td>
<td>2.84x10&lt;sup&gt;-02&lt;/sup&gt;</td>
</tr>
<tr>
<td>CACNG4</td>
<td>calcium channel, voltage-dependent, gamma subunit 4</td>
<td>-1.24</td>
<td>4.40x10&lt;sup&gt;-02&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Changes in gene expression in MHC-TRAF2<sub>HC</sub> mice vs. LM control mice at 12 weeks of age identified by the KEGG functional pathway for cardiac hypertrophy/dilated cardiomyopathy were modified to include changes in extracellular matrix gene expression that have been linked to the development of dilated cardiomyopathy. Changes in gene expression are displayed visually in Figure 6 in the manuscript.
Supplemental Figure I: Generation of transgenic mice overexpressing TRAF2 in the heart. (A) Representative southern blot showing three lines of mice overexpressing 7, 10 and 24 copies of the TRAF2 transgene in comparison to controls. (B) Western blot of TRAF2 protein expression in hearts of MHC-TRAF2 mice expressing 7, 10 and 24 copies of the TRAF2 transgene. (C) Representative immunohistochemical staining of littermate and MHC-TRAF2HC mouse hearts (100x).

Supplemental Figure II: Evans Blue dye uptake in littermate control and MHC-TRAF2HC mouse hearts. To determine the presence of absence of myocyte cell necrosis, 4 week littermate control and MHC-TRAF2HC mice were injected intraperitoneally with Evans Blue dye, the hearts excised, fixed and examined by fluorescence microscopy (200x). As a positive control, littermate control mouse hearts were subjected to ischemia reperfusion injury ex vivo, and stained with Evan Blue dye as described.\(^5\)
Supplemental Figure III: NF-κB subunits. An NF-κB ELISA was performed on nuclear extracts from obtained from 12 week littermate control and MHC-TRAF2HC mouse hearts (n = 6-9 hearts/group).

Supplemental Figure IV: Transcriptional profiling. (A) SAM plot of changes in gene expression in the 12 week MHC-TRAF2HC mouse hearts compared to LM controls (n = 4 hearts/group). Genes that were significantly increased are denoted by red circles; genes that were significantly decreased are denoted by green circles. A false discovery rate (FDR) less than 5% was used for the SAM plots. (B) KEGG analysis of functional pathways that were significantly different in the MHC-TRAF2HC compared to LM control hearts at 12 weeks of age.