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Tollip attenuated the hypertrophic response of cardiomyocytes induced by IL-1beta

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1. ABSTRACT

We examined the role of Tollip in the hypertrophic response of cardiomyocytes. C57BL/6 mice were subjected to transverse aortic constriction (TAC) for 2 weeks and age-matched sham surgical operated mice served as control. TAC significantly reduced the association of Tollip with IRAK-1 by 66.4 percent and increased NF-kappaB binding activity by 86.5 percent and the levels of phospho-p38 by 114.6 percent in the myocardium compared with sham control, respectively. *In vitro* experiments showed that IL-1beta stimulation also significantly reduced the association of Tollip with IRAK-1 and increased NF-kappaB binding activity in neonatal cardiomyocytes. Tollip overexpression by transfection of cardiac myocytes significantly attenuated the IL-1beta-induced hypertrophic response of cardiac myocytes as evidenced by reduced cell size (16.4 percent) and decreased ANP expression (33.3 percent). Overexpression of Tollip also reduced NF-kappaB binding activity by 30.7 percent and phospho-p38 by 47.1 percent, respectively. The results suggest that Tollip could be a negative regulator during the development of cardiac hypertrophy. The negative regulation of cardiac hypertrophy by Tollip may involve downregulation of the MyD88-dependent NF-kappaB activation pathway.

2. INTRODUCTION

Recent studies have highlighted the role of Toll-like receptors (TLRs) and Interleukin-1 receptor (TLRs/IL-1R)-mediated signaling pathways in the induction of innate and adaptive immune responses. TLRs/IL-1R signals predominantly activate the nuclear factor kappaB (NF-kappaB) pathway through the adaptor protein myeloid differentiation factor 88 (MyD88).

TLR4 has been characterized to specifically recognize Gram-negative bacterial lipopolysaccharide (LPS) and endogenous ligands such as heat shock proteins, fibronectin, and hyaluronic acid, etc. After recognition of ligands by TLR4, MyD88 recruits IL-1 receptor-associated kinase (IRAK), which stimulates TNF-alpha receptor association factor-6 (TRAF6), leading to activation of inhibitor of nuclear factor-kappaB-kinase (IkappaKs) and the mitogen-activated protein kinase (MAPK) family, resulting in NF-kappaB translocation to the nucleus, binding to target DNA sequences, and stimulation of gene expression. In particular, this pathway is involved in several cardiovascular diseases, including atherosclerosis, ischemia/reperfusion injury, congestive heart failure and cardiac hypertrophy (1, 2). We have reported that the TLR4-mediated MyD88 dependent NF-kappaB pathway

plays a role in cardiac hypertrophy (3). Since Tollip is a negative regulator in the TLR mediated signaling pathway, published data indicate that negative regulation of the MyD88-dependent NF-kappaB activation pathway could be an effective approach for preventing the development of cardiac hypertrophy.

Toll-interacting protein (Tollip) was identified as an important negative regulator of the MyD88-dependent NF-kappaB activation pathway (4). When cells are in the resting state, Tollip binds to IL-1R-associated kinase 1 (IRAK-1) as a complex that prevents IRAK-1 phosphorylation and activation. Following stimulation, the Tollip/IRAK-1 complex forms an active IL-1R complex that brings the death domains of IRAK-1 and MyD88 into close contact, resulting in the dissociation of Tollip. Thus IRAK-1 is autophosphorylated and initiates downstream signaling. Increased Tollip levels positively correlated with the decreased cellular innate immune response (5-8) and overexpression of Tollip suppressed TLR-mediated NF-kappaB activation, suggesting that Tollip could negatively regulate TLR signaling via suppression of IRAK-1 activity. We have previously shown that the TLR4-mediated MyD88-dependent NF-kappaB activation pathway plays a critical role in the development of cardiac hypertrophy (3). Specifically, deficiency of TLR4 resulted in significant resistance to pressure overload-induced cardiac hypertrophy (9). Blockade of either MyD88 or NF-kappaB activation significantly attenuated pressure overload-induced cardiac hypertrophy (3). Collectively, these data suggested that TLR4-mediated MyD88-dependent NF-kappaB activation is a novel signaling pathway that contributes to the development of cardiac hypertrophy. It is unclear, however, whether negative regulators in the TLR-mediated MyD88-dependent signaling pathway will play a role in attenuation of the development of cardiac hypertrophy.

In the present study, we examined the role of Tollip in the regulation of the hypertrophic response of cardiac myocytes. We observed that pressure overload resulted in a significant decrease in the association of Tollip with IRAK, resulting in increased NF-kappaB binding activity. Overexpression of Tollip significantly attenuated the hypertrophic response of cardiac myocytes induced by IL-1beta. Mechanisms by which Tollip attenuated the cardiac hypertrophic response may involve blunting the activation of the MyD88-dependent NF-kappaB signaling pathway.

3. MATERIALS AND METHODS

3.1 Experimental animals

Male C57Bl/6 mice (8-10 weeks, 18-20g) were provided by the Model Animal Research Center (MARC) of Nanjing University and maintained in the Animal Laboratory Resource Facility at Nanjing Medical University. All experiments were done in accordance with the guidelines for the "Principles of Laboratory Animal Care" and the "Guide for the care and use of laboratory animals" published by NIH (NIH Publication No. 85-23, revised 1996). The Nanjing Medical University Committee on Animal Care approved all aspects of the animal care and experimental protocols.

3.2. Induction of cardiac hypertrophy by transverse aortic constriction (TAC)

Mice (n equal 8) were anesthetized, intubated, and ventilated using a rodent ventilator as described previously (10). The hearts were exposed through a left thoracotomy in the third intercostal space. After the transverse aorta was isolated from between the carotid arteries, a 7-0 silk suture was drawn under the transverse aorta and tied against a 26-gauge needle that was promptly removed (10). For the age-matched sham operation (n equal 8), the identical procedure was performed except a suture was not tied around the aorta. Two weeks after TAC, hearts were harvested and the ratios of heart weight/body weight (HW/BW) and left ventricular weight/tibia length (LVW/TL) were calculated. The heart samples were frozen in liquid nitrogen and stored at -80°C.

3.3. Echocardiography

Cardiac functions in TAC and sham control mice were evaluated by echocardiography (GE Vivid 7 equipped with a 14-Mhz phase array linear transducer, S12, allowing a 150 maximal sweep rate). The mice were anesthetized using a mixture of isoflurane (1.5 percent) and oxygen (0.5 l/min). The body temperature was maintained at 37°C using a heating pad. M-mode tracings were used to measure left ventricle (LV) wall thickness, LV end-systolic diameter (LVESD), and LV end-diastolic diameter (LVEDD). Percent fractional shortening (percent FS) was calculated as described previously (11). The same investigator performed all echocardiographic assessments. All measurements were averaged over five consecutive cardiac cycles.

3.4. Cell culture and pCMV-Tollip plasmid transfection

Primary cultures of ventricular myocytes were prepared from 1-2 day old neonatal Sprague-Dawley rats as described previously (12). Cells were plated at a density of 1×10^6 cells/ml and cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen Corporation, USA) supplemented with 10 percent bovine calf serum, 100 units/ml penicillin and 100 units/ml streptomycin. After 24 hrs of incubation, the medium was replaced by serum-free DMEM containing 10 micrograms/ml transferrin and 10 micrograms/ml insulin. Plasmid pCMV-Tollip was constructed by inserting PCR amplified Tollip coding sequence in frame into the BamH1/Xho1 site of pCMV TAG-3A vector. Cells were transfected with pCMV-Tollip using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer and described previously (8, 13). Thirty-six hrs after transfection, the cells were treated with IL-1beta (10 ng/ml, Sigma) for 24 hrs. Untreated cells served as control. Cell size and ANP mRNA expression were analyzed as described previously (14). The expression of Tollip and Myc protein were examined by Western blot with cellular protein preparations.

3.5. Image analysis of cardiomyocytes:

Neonatal cardiac myocytes were cultured on cover-slips. Cell size was determined by staining

membranes with anti-sarcomeric alpha-actinin antibody (Sigma) as described previously (15). Cardiomyocytes were visualized through a fluorescence microscope (Olympus) and 150 myocytes for each set of data were analyzed with the image software (NIH).

3.6. Western blot analysis

Cytoplasmic proteins were prepared from heart tissues and cardiomyocytes, respectively, and immunoblots were performed as described previously (9, 16). Briefly, the cytoplasmic proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, USA). The PVDF membranes were incubated with the appropriate primary antibodies anti-phospho-p38, anti-p38, anti-beta-actin, (Cell Signaling Technology), anti-IRAK-1, (Santa Cruz) and anti-Tollip (Alexis Biochemicals)) followed by incubation with peroxidase-conjugated secondary antibodies. The signals were detected with the ECL system (Amersham). The same membranes were probed with anti-beta-actin (Sigma) after being washed with stripping buffer. The signals were quantified by scanning densitometry with the Image J analysis system. The results from each experimental group were expressed as relative integrated intensity compared with the control group measured at the same time.

3.7. Immunoprecipitation (IP)

Immunoprecipitation was performed for analysis of the association of IRAK-1 with Tollip. Briefly, 300 micrograms of tissue cytoplasmic proteins or 150 micrograms of cardiomyocyte proteins were immunoprecipitated with 2 micrograms of anti-IRAK-1 (Santa Cruz) at 4°C for 2 hrs followed by the addition of 15 microliters of protein A/G Agarose beads (Santa Cruz) overnight at 4°C. After being washed three times with cold wash buffer and one time with lysis buffer, the immunoprecipitates were resuspended in 20 microliters of lysis buffer and subjected to Western blot for examination of IRAK-1 and Tollip proteins.

3.8. RT-PCR assay

Total cell RNA was isolated from cultured cardiomyocytes using RNAiso reagent (Takara Biotechnology), and RT-PCR assays were performed with an RNA PCR kit (Promega) as previously described (17). RT-PCR primers for atrial natriuretic peptide (ANP) were as follows: forward, 5'-GTGACGGCTGAGTTGTTTT-3', reverse, 5'-TTTGTGCTGGAAGATAAGA AA-3'. beta-Actin primers were as follows: forward, 5'-GTCGTACCACAGGCATTGTGATG G-3', reverse, 5'-GCAATGCCTGGGT ACATGGTGG-3'. The PCR products of ANP and beta-actin were analyzed by electrophoresis on 1 percent agarose gels, which were stained with ethidium bromide and scanned. All values obtained with the ANP primers were normalized to the values obtained with the beta-actin primers.

3.9. Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were prepared from heart samples and cultured cardiomyocytes, respectively, as described previously (10). NF-kappaB binding activity was examined using an EMSA kit (Pierce Biotech Inc., USA), according to the manufacturer. In brief, a biotin end-labeled DNA duplex of sequences containing the NF-kappaB binding site (3'-TCA ACT CCC CTG AAA GGG TCC G-5', 5'-AGT TGA GGGGAC TTT CCC AGG C-3') was incubated with nuclear proteins at room temperature for 20 min. The reaction mixture was separated on 6 percent native polyacrylamide gel electrophoresis, and transferred to a nylon membrane (Amersham). The membranes were subjected to UV light to cross-link proteins for 1 min followed by incubation with conjugate/blocking buffer containing stabilized streptavidin-horseradish peroxidase conjugate. The signal on the membranes was detected with the ECL system. The membranes were then exposed to X-ray film for 2-5 min. The NF-kappaB binding bands were scanned and the relative intensities were analyzed with Image J software.

3.10. Statistical analysis

Results were expressed as means +/- SE. For analysis of significance between the groups, one-way analysis of variance (ANOVA) and Tukey's procedure for multiple range tests were performed. A value of P less than 0.05 was considered to be significant.

4. RESULTS

4.1. TAC induced cardiac hypertrophy

Figure 1A shows that pressure overload induced by transverse aortic constriction (TAC) significantly increased the ratios of HW/BW and LVW/TL by 20.0 percent (5.93 +/- 0.27 vs 4.94 +/- 0.13, n equal 8, P less than 0.05) and 40.6 percent (5.6 +/- 0.38 vs 4.0 +/- 0.13, n equal 8, P less than 0.01), respectively, compared with the age-matched sham control group. There was no significant difference in the body weight between TAC mice and sham control mice (23.3 +/- 0.42g vs 23.1 +/- 0.60g). Ventricular wall thickness was examined by two-dimensional ultracardiography. As shown in Figure 1B, pressure overload significantly increased the interventricular septum systolic dimension (IVSS) by 18.2 percent (1.75 +/- 0.10 vs 1.48 +/- 0.22, n equal 8, P less than 0.05) and left ventricular posterior wall systolic dimension (LVPWS) by 14.5 percent (1.58 +/- 0.15 vs 1.38 +/- 0.13, n equal 8, P less than 0.05), respectively, compared with the age-matched sham control group (Table 1).

4.2. TAC reduced the association of IRAK with Tollip in the myocardium

Tollip is one of the negative regulator proteins in the MyD88-dependent signaling pathway (4). Following stimulation of TLR/IL-1R-mediated MyD88 signaling, Tollip is dissociated from IRAK-1, resulting in activation of IRAK-1 (5). To examine whether pressure overload will result in dissociation of Tollip from IRAK-1, we performed immunoprecipitation with specific antibody against IRAK-1 in the heart tissues followed by immunoblotting with specific antibody against Tollip. As shown in Figure 2, TAC significantly reduced the association of Tollip with IRAK-1 by 66.4 percent compared with the age-matched sham control group (0.44 +/- 0.03 vs 1.31 +/- 0.06, n equal 5, P less than 0.01).

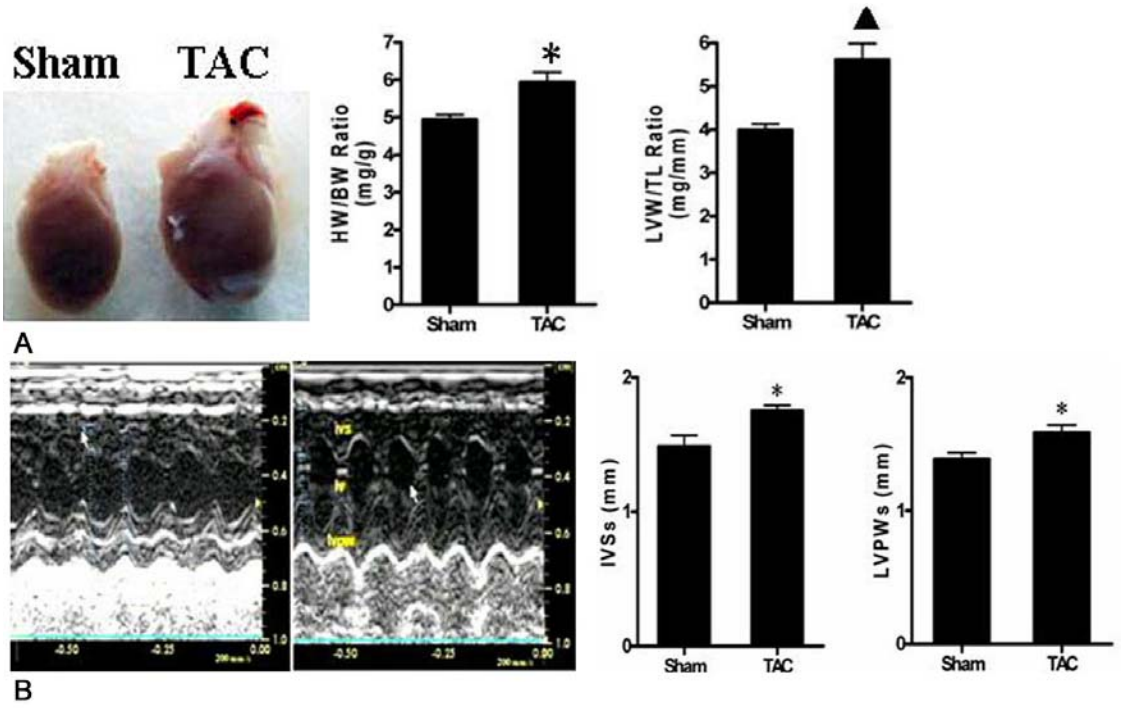

Figure 1. TAC induced cardiac hypertrophy. Mice were subjected to TAC for 2 wk (n equal 8) and the age-matched sham surgical operated mice served as the control group (n equal 8). (A) The hearts were harvested and the HW/BW and LVW/TL ratios were calculated. The photos on the top are representative hearts showing that aortic banding induced cardiac hypertrophy. (B) M-mode images of parasternal short-axis view at the papillary muscle levels in TAC and sham control mice (n equal 8). LVESD: left ventricular end-systolic diameter, LVEDD: left ventricular end-diastolic diameter. Ejection fraction (EF) and fractional shortening (FS) changes following TAC are shown in the left. * P less than 0.05, compared with the sham control group, ▲P less than 0.01, compared with the sham control group.

4.3. TAC increased NF-kappaB binding activity and p38 phosphorylation in the myocardium

NF-kappaB is an important transcription factor downstream in the MyD88-dependent signaling pathway (17). p38 MAPK activity can also be induced following activation of the MyD88-dependent signaling pathway (18). Therefore, we examined NF-kappaB binding activity using EMSA and the levels of phospho-p38/p38 using Western blot. Figure 3A shows that pressure overload significantly increased NF-kappaB binding activity by 86.5 percent compared with the sham control group (P less than 0.01, n equal 5). The levels of phospho-p38/p38 were also significantly increased by 114.6 percent in the myocardium following TAC compared with the sham control group (P less than 0.01, n equal 5) (Figure 3B).

4.4. Overexpression of Tollip attenuated the IL-1beta-stimulated hypertrophic response of neonatal cardiac myocytes

To further investigate the role of Tollip in negative regulation of the cardiac hypertrophic response of cardiomyocytes, we performed *in vitro* experiments using neonatal cardiac myocytes. We transfected neonatal cardiac myocytes with pCMV-Tollip 36 hrs before the cells were stimulated with IL-1beta (13). The pCMV empty vector served as control. pCMV-Tollip contains a Myc epitope, therefore, we examined the expression of both Tollip and Myc-Tollip in transfected cells with specific antibodies. Figure 4A shows that high levels of Tollip were observed in pCMV-Tollip transfected neonatal cardiac myocytes and Myc expression was only observed in Tollip transfected cells but was not detected in pMCV empty vector control cells (Figure 4A).

IL-1beta can induce a hypertrophic response by cardiac myocytes (19, 20) through a MyD88-dependent signaling pathway (21). We employed IL-1beta to stimulate neonatal cardiac myocytes that were transfected with or without Tollip. The hypertrophic response of neonatal cardiac myocytes was determined by measuring the cell size and the levels of ANP mRNA expression. As shown in Figure 4B, IL-1beta administration induced a significant hypertrophic response by neonatal cardiac myocytes as evidenced by increased cell size (24.4 percent) compared with untreated control cells. The levels of ANP mRNA were increased by 106.3 percent following IL-1beta stimulation compared with unstimulated cells. However, transfection of pCMV-Tollip into cardiomyocytes significantly attenuated the IL-1beta-induced hypertrophic response of cardiomyocytes. The cell size of Tollip transfected cells was significantly reduced by 16.4 percent compared with pCMV empty vector control cells (Figure 4B). The level of ANP mRNA in Tollip transfected cells was significantly reduced by 33.3 percent compared with IL-1beta stimulated cells that were transfected with pMCV empty vector (Figure 4C).

Table 1. Two-dimensional ultracardiography examination

	Sham (n equal 8)	TAC (n equal 8)
IVSD, mm	0.94 +/- 0.11	1.05 +/- 0.10
IVSS, mm	1.48 +/- 0.22	1.75 +/- 0.10[1]
LVIDD, mm	3.01 +/- 0.64	2.85 +/- 0.38
LVIDS, mm	1.41 +/- 0.50	1.50 +/- 0.17
LVPWD, mm	1.07 +/- 0.17	1.02 +/- 0.08
LVPWS, mm	1.38 +/- 0.13	1.58 +/- 0.15[1]
EDV, ml	0.11 +/- 0.02	0.07 +/- 0.04
ESV, ml	0.01 +/- 0.01	0.01 +/- 0.00
EF (percent)	89.63 +/- 4.44	85.37 +/- 4.80
SV, ml	0.10 +/- 0.02	0.07 +/- 0.03
FS (percent)	51.49 +/- 6.38	48.76 +/- 5.73

IVSD: Interventricular Septum Diastolic Dimension, IVSS: Interventricular Septum Systolic Dimension, LVIDD: LV end-Diastolic Dimension, LVIDS: LV end-Systolic Dimension, LVPWD: LV Posterior Wall Diastolic Dimension, LVPWS: LV Posterior Wall Systolic Dimension, EDV: LV end-Diastolic Volume, ESV: LV end-Systolic Volume, EF: Ejection Fraction, SV: Stroke Volume, FS: Fractional Shortening. [1]P less than 0.05, compared with the sham control group.

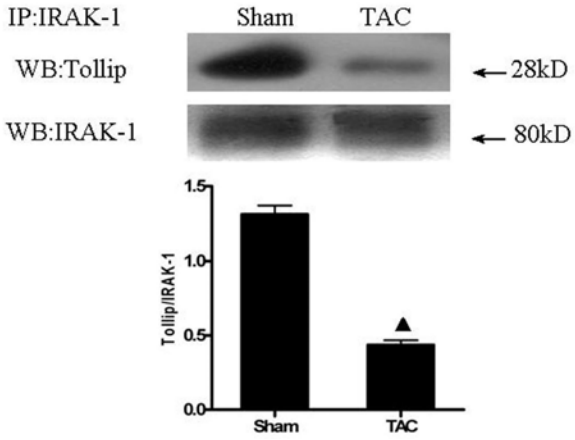

Figure 2. Pressure overload reduced the association of Tollip with IRAK-1 in the myocardium. Cellular proteins were isolated from TAC and sham control mice and subjected to immunoprecipitation with specific anti-IRAK-antibody. The immunoprecipitates were probed with specific anti-Tollip antibody. There were 5 mice in each group. ▲P less than 0.01, compared with the sham control group.

4.5. Overexpression of Tollip prevented the dissociation of Tollip from IRAK-1 following IL-1beta stimulation

To examine the effect of overexpression of Tollip on the association of IRAK-1 with Tollip following a hypertrophic stimulator challenge, we transfected neonatal cardiac myocytes with Tollip plasmid and 36 hrs after transfection, the cells were stimulated with IL-1beta for 24 hrs. The association of IRAK-1 with Tollip was examined by immunoprecipitation with specific antibody against IRAK-1 followed by immunoblot with specific antibody against Tollip. Figure 5 shows that IL-1beta stimulation significantly reduced the association of IRAK-1 with Tollip by 75.0 percent compared with unstimulated cells (P less than 0.01, n equal 3). In Tollip transfected cells, the decreased association of IRAK-1 with Tollip following IL-1beta stimulation was significantly attenuated. The levels of association of IRAK-1 with Tollip in Tollip-transfected cells following IL-1beta stimulation were significantly higher than IL-1beta stimulated cells and there was no significant difference compared with unstimulated control cells.

4.6. Overexpression of Tollip attenuated IL-1beta-increased NF-kappaB binding activity and p38 phosphorylation

To investigate whether attenuation of the disassociation of Tollip from IRAK-1 could inhibit IL-1beta-stimulated NF-kappaB activity and p38 phosphorylation, we transfected cells with Tollip plasmid 36 hrs before the cells were stimulated with IL-1beta for 24 hrs. NF-kappaB binding activity was assessed by EMSA and the levels of p38 phosphorylation were examined by immunoblot. As shown in Figure 6, IL-1beta stimulation significantly increased NF-kappaB binding activity by 127.4 percent compared with unstimulated cells. The levels of phospho-p38/p38 were significantly increased by 125.8 percent following IL-1beta administration compared with unstimulated cells. In Tollip transfected cells, IL-1beta-stimulated NF-kappaB binding activity was significantly attenuated. NF-kappaB binding activity was significantly blunted by 30.7 percent compared with IL-1beta stimulated cells. In addition, overexpression of Tollip also significantly blunted the levels of phospho-p38/p38 in IL-1beta stimulated cells by 47.1 percent compared with IL-1beta stimulated cells (Figure 6).

5. DISCUSSION

A significant finding of the present study was that pressure overload resulted in a significant decrease in the association of Tollip with IRAK-1 and an increase in the activation of the MyD88-dependent NF-kappaB signaling pathway. Overexpression of Tollip significantly attenuated the IL-1beta-induced hypertrophic response of neonatal cardiac myocytes. Overexpression of Tollip also significantly blunted activation of the MyD88-dependent NF-kappaB pathway. The data suggest that Tollip is an important negative regulator in the MyD88-dependent signaling pathway and that maintaining the association of Tollip with IRAK-1 could be important for preventing the development of cardiac hypertrophy.

Tollip was originally cloned as a protein that interacts with the IL-1R accessory protein (5). Subsequently, Tollip was found to interact directly with the cytoplasmic TIR domains of TLRs/IL-1R and to inhibit TLRs/IL-1R-mediated cellular responses by suppressing the phosphorylation and activation of IRAK-1 (6). When cells are in a quiescent condition, Tollip binds to the N-terminal of the IRAK-1 death domain, preventing IRAK-1 from forming a dimer. Following TLRs/IL-1R activation, the Tollip/IRAK-1 complex shifts to the MyD88-TIR complex. Tollip then dissociates from the complex, leading to association of the death domains of MyD88 with IRAK-1 and formation of an IRAK-1 dimer, resulting in activation of IRAK-1 (5). Therefore, Tollip functions as a negative regulator in the IL-1R/TLR-mediated signaling pathway

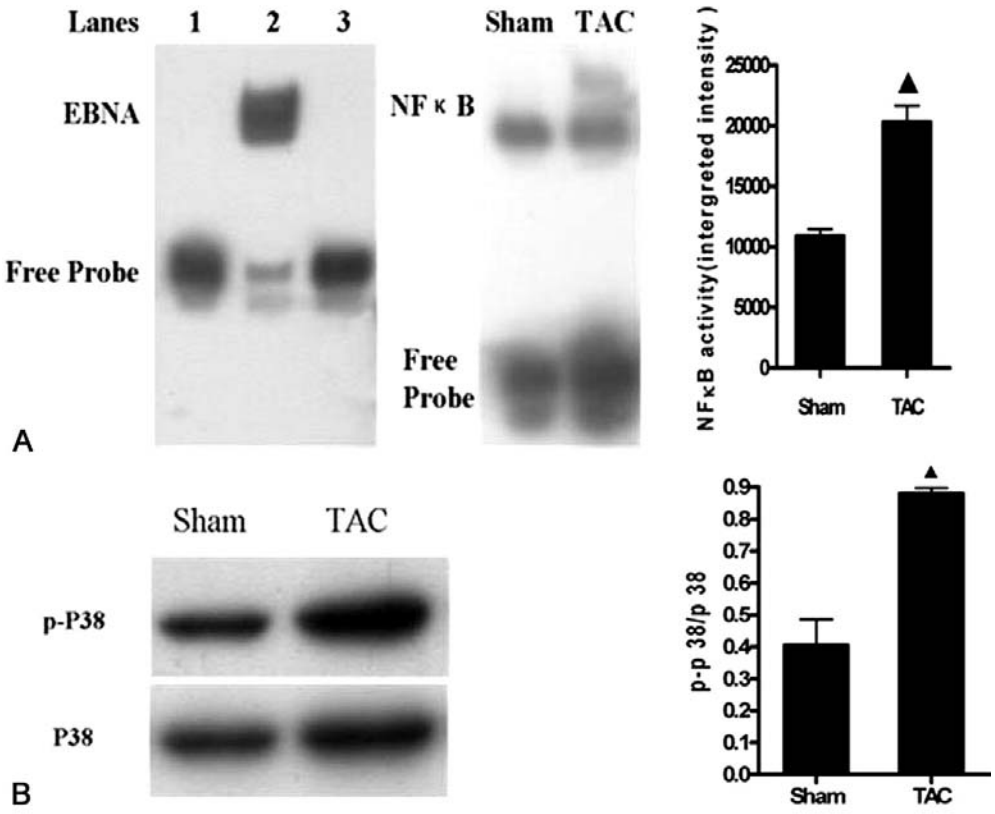

Figure 3. Pressure overload increased NF-kappaB binding activity (A) and p38 MAPK phosphorylation (B). Nuclear proteins were isolated from TAC (n equal 5) and sham control mice (n equal 5) and subjected to EMSA for NF-kappaB binding activity (A). Representative EMSA results are shown on the left. Lane 1: Biotin-EBNA control DNA (without EBNA extract), Lane 2: Biotin-EBNA control DNA+EBNA extract, Lane 3: Biotin-EBNA competition control (with 200-fold of unlabeled EBNA). ▲P less than 0.01, compared with the sham control group. (B) The levels of phospho-p38/p38 MAPK were measured by Western blot with cytoplasmic protein preparations (n equal 5/group). Representative results of p38 MAPK Western blots are shown on the left. ▲P less than 0.01, compared with the sham group control.

through inhibiting IRAK activity, which prevents activation of NF-kappaB, Jun N-terminal protein kinase, and p38 MAPK (22, 23). The contribution of NF-kappaB activation to the development of cardiac hypertrophy has been well documented (3, 10, 24, 25). We have previously demonstrated that inhibiting NF-kappaB activation attenuated pressure overload-induced cardiac hypertrophy *in vivo* (10). In the present study, we observed that *in vivo* pressure overload significantly reduced association of Tollip with IRAK-1, resulting in increased NF-kappaB binding activity. IL-1beta stimulation significantly induced a hypertrophic response in cultured neonatal cardiac myocytes and increased NF-kappaB binding activity and the levels of phospho-p38 that were positively correlated with decreased association of Tollip with IRAK-1. Overexpression of Tollip attenuated the IL-1beta-stimulated hypertrophic response of neonatal cardiac myocytes, which was positively correlated with reduced NF-kappaB binding activity and reduced levels of phospho-p38. The data suggests that Tollip could be an important negative regulator in the development of cardiac hypertrophy through blocking activation of the MyD88-dependent signaling pathway.

Sustained hemodynamic overload on the myocardium results in cardiac hypertrophy (26). It has been shown that inflammatory responses, including macrophage/neutrophil infiltration and secretion of inflammatory cytokines, such as IL-1beta and TNFalpha (27) are involved in the development of cardiac hypertrophy (28, 29). Recent studies have reported that IL-1beta plays a role in the induction of the hypertrophic response of cardiac myocytes. For example, the expression of IL-1beta mRNA was significantly increased in the myocardium subjected to pressure overload (20). IL-1beta administration can induce a hypertrophic response in cultured primary cardiac myocytes (30). Importantly, IL-1beta stimulates IL-1R/TLR mediated MyD88-dependent NF-kappaB activation pathways. We observed in the present study that IL-1beta administration significantly increased the expression of ANP mRNA and the size of cultured neonatal cardiomyocytes. Overexpression of Tollip attenuated the IL-1beta-induced hypertrophic response of cardiomyocytes, suggesting that the IL-1beta-induced hypertrophic response of cardiomyocytes is through activation of IL-1R/TLR mediated MyD88-dependent signaling pathways. Indeed, we have previously

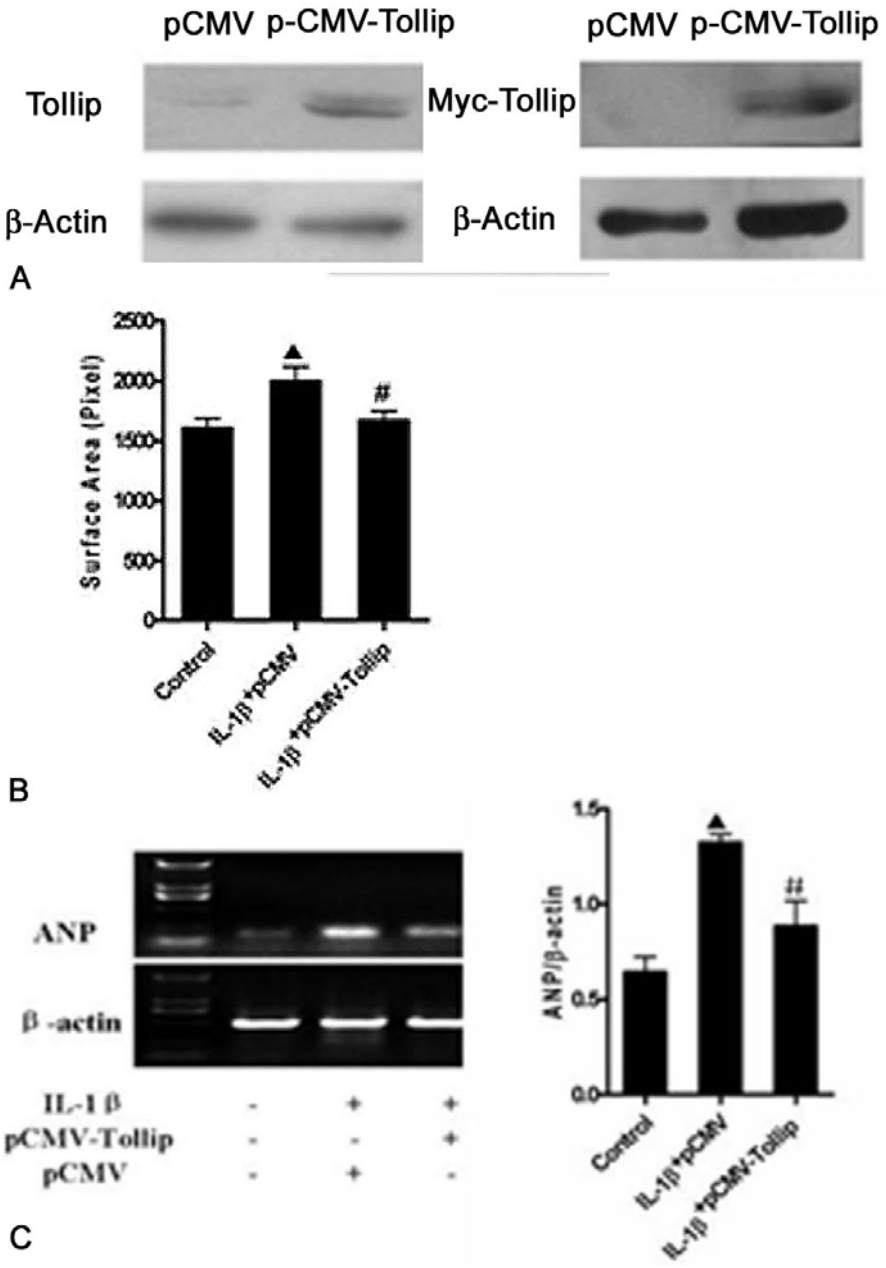

Figure 4. Overexpression of Tollip attenuated the IL-1beta-induced hypertrophic response of neonatal cardiac myocytes. Tollip plasmid was transfected into neonatal cardiac myocytes for 36 hrs. Expression of Tollip and Myc-Tollip were examined by Western blot with specific antibodies. Increased expression of Tollip and Myc-Tollip (A) was observed in the cells transfected with Tollip plasmid. Overexpression of Tollip attenuated the IL-1beta-increased cell size (cell area) (B) and the levels of ANP mRNA expression (C). ▲ P less than 0.01, compared with the control group, # P less than 0.05, compared with the IL-1beta stimulated group (n equal 3).

shown that either TLR4 deficiency or blockade of MyD88 by transfection of Ad5-dnMyD88 into the myocardium significantly attenuated pressure overload-induced cardiac hypertrophy *in vivo* (3). Therefore, modulation of the expression of negative regulators in the TLR4-mediated MyD88 dependent NF-kappaB signaling pathway could be an important approach to prevent the development of cardiac hypertrophy.

In summary, we observed that the association of Tollip with IRAK was significantly reduced in the myocardium subjected to pressure overload *in vivo*. *In vitro* studies showed that overexpression of Tollip significantly attenuated the hypertrophic response of neonatal cardiac myocytes induced by IL-1beta, which was positively correlated with blunted NF-kappaB binding activity. Our results suggest that the MyD88-dependent NF-kappaB

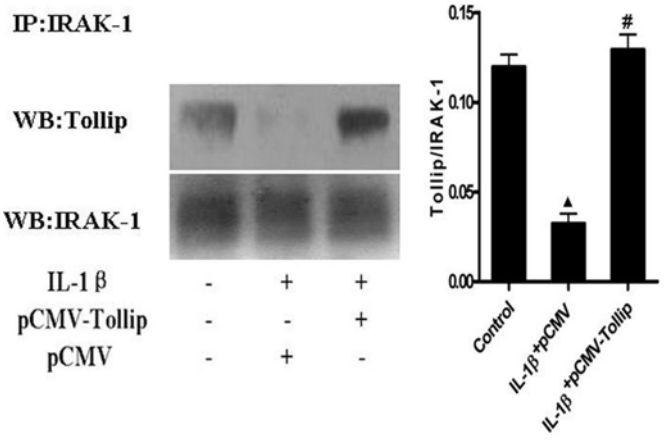

Figure 5. Overexpression of Tollip preserved the association of Tollip with IRAK-1 following IL-1beta stimulation. Neonatal cardiac myocytes were transfected with Tollip plasmid 36 hrs before the cells were subjected to IL-1beta stimulation. The association of Tollip with IRAK-1 was determined by immunoprecipitation followed by immunoblot. ▲ P less than 0.01, compared with the control group, # P less than 0.01, compared with the IL-1beta stimulated group (n equal 3).

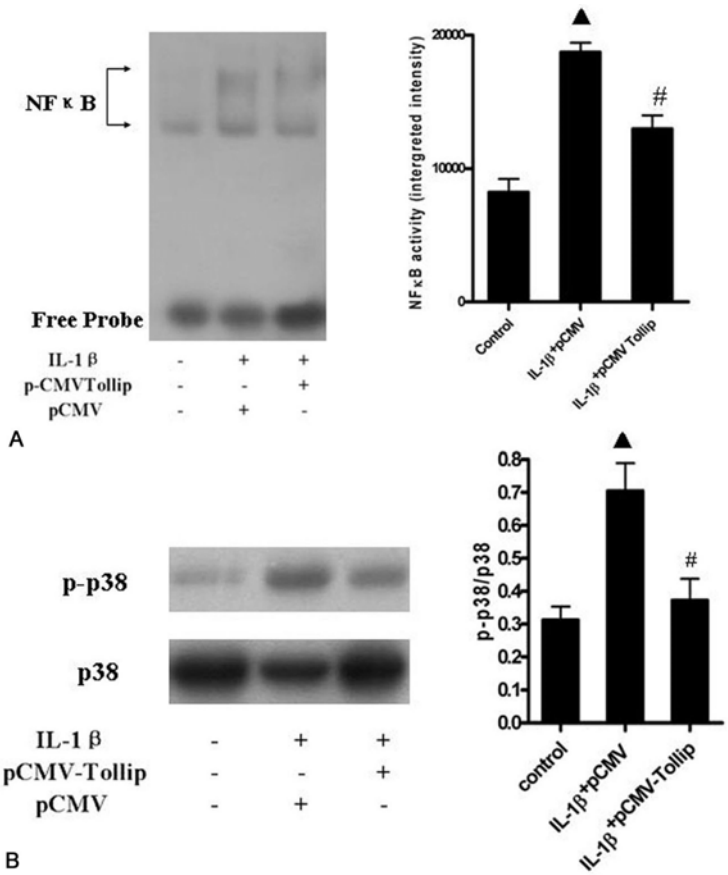

Figure 6. IL-1beta-stimulated NF-kappaB binding activity (A) and increased phospho-p38/p38 (B) were attenuated following overexpression of Tollip. Neonatal cardiac myocytes were transfected with Tollip plasmid 36 hrs before the cells were subjected to IL-1beta stimulation. (A) NF-kappaB binding activity was determined by EMSA. ▲ P less than 0.01, compared with control group, # P less than 0.01, compared with IL-1beta stimulated group (n equal 3). (B) The levels of phospho-p38/p38 were determined by Western blot. ▲P less than 0.05, compared with the control group, # P less than 0.05, compared with the IL-1beta stimulated group (n equal 3).

activation pathway plays an important role in the development of cardiac hypertrophy. Tollip, as a negative regulator protein in this signaling pathway could be a target for prevention of cardiac hypertrophy. Further studies are needed to elucidate the mechanisms by which Tollip negatively regulates cardiac hypertrophy.

6. ACKNOWLEDGMENT

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Abbreviations: ANP: Atrial Natriuretic Peptide, DMEM: Dulbecco's Modified Eagle Medium, ECL: Enhanced Chemiluminescent, EF: Ejection Fraction, EMSA: Electrophoretic Mobility Shift Assay, FS: Fractional Shortening, IL-1beta: Interleukin-1 beta, IP: Immunoprecipitation, IRAK-1: Interleukin-1 Receptor-Associated Kinase 1, LPS: lipopolysaccharide, LVEDD: Left Ventricle end-Diastolic Diameter, LVESD: Left Ventricle end-Systolic Diameter, MAPK: Mitogen-Activated Protein Kinase, MyD88: Myeloid differentiation primary response gene (88), NF-kappaB: Nuclear Factor kappaB, PVDF: Polyvinylidene Difluoride, RT-PCR: Reverse Transcriptase Polymerase Chain Reaction, SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis, TAC: Transverse Aortic Constriction, TLRs: Toll-like receptors, TNF-alpha: Tumor necrosis factor-alpha, Tollip: Toll-Interacting Protein, TRAF6: TNF-alpha Receptor Association Factor-6

Key Words: cardiac hypertrophy, signaling pathway, Tollip, IRAK-1, NF-kappaB , MAPK

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