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Mitogen-activated protein kinase phosphatase-1 inhibits myocardial TNF-α expression and improves cardiac function during endotoxemia

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Aims Myocardial tumour necrosis factor-α (TNF-α) expression induces cardiac dysfunction in endotoxemia. The aim of this study was to investigate the role of mitogen-activated protein kinase phosphatase-1 (MKP1) pathway in myocardial TNF-α expression and cardiac function during endotoxemia.

Methods and results Lipopolysaccharide (LPS) increased MKP1 expression in the myocardium *in vivo* and in cultured neonatal cardiomyocytes *in vitro*. LPS-induced extracellular signal-regulated kinase (ERK) 1/2 and p38 phosphorylation in the myocardium was prolonged in MKP1$^{-/-}$ mice. Myocardial TNF-α mRNA and protein levels were enhanced in MKP1$^{-/-}$ compared with wild-type (WT) mice in endotoxemia, leading to a further decrease in cardiac function. To study if Rac1/p21-activated kinase 1 (PAK1) signalling regulates MKP1 expression, cardiomyocytes were treated with LPS. Inhibition of Rac1 and PAK1 by a dominant negative Rac1 adenovirus (Ad-Rac1N17) and *PAK1* siRNA, respectively, blocked LPS-induced MKP1 expression in cardiomyocytes. *PAK1* siRNA also decreased p38 and c-Jun N-terminal kinase (JNK) activation, and TNF-α expression induced by LPS. Furthermore, deficiency in either Rac1 or JNK1 decreased myocardial MKP1 expression in endotoxemic mice.

Conclusion LPS activates the Rac1/PAK1 pathway, which increases myocardial MKP1 expression via JNK1. MKP1 attenuates ERK1/2 and p38 activation, inhibits myocardial TNF-α expression, and improves cardiac function in endotoxemia. Thus, MKP1 represents an important negative feedback mechanism limiting pro-inflammatory response in the heart during sepsis.

Keywords Lipopolysaccharide • MKP1 • TNF-α • Cardiac function • Sepsis

1. Introduction

Sepsis is the 10th most common cause of death in the USA.[1] Cardiac dysfunction frequently accompanies severe sepsis and septic shock, and is associated with a significant increase in mortality rate (70%) compared with septic patients without cardiovascular impairment (20%).[2] Tumour necrosis factor-α (TNF-α) produced by cardiomyocytes is a major contributing factor to cardiac dysfunction.[2,3] However, the underlying molecular mechanisms regulating myocardial TNF-α production during sepsis remain elusive.

Mitogen-activated protein kinases (MAPKs), including p38, extracellular signal-regulated kinase (ERK) 1/2, and c-Jun N-terminal kinase (JNK) 1/2 are key signalling molecules regulating inflammatory responses and the expression of pro-inflammatory cytokines.[4,5] It has been demonstrated that these MAPKs exhibit different effects on myocardial TNF-α expression during endotoxemia.[6–10] Indeed, activation of ERK1/2 and p38 promotes TNF-α expression.[6–8,10] In contrast, we recently demonstrated that JNK1 decreases TNF-α expression and improves cardiac function through inhibition of ERK1/2 and p38 activity.[9] However, the molecular mechanisms by which JNK inhibits ERK1/2 and p38 are not fully understood.

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Inactivation of MAPKs is achieved primarily by MAPK phosphatases (MKPs) that dephosphorylate phosphothreonine and phosphotyrosine residues of MAPKs.[11,12] Studies have shown that mitogen-activated protein kinase phosphatase-1 (MKP1) negatively regulates the inflammatory response of the innate immune system by accelerating MAPK inactivation and attenuating the production of pro-inflammatory cytokines, including TNF-α, interleukin (IL)-1β, and IL-6 in macrophages following lipopolysaccharide (LPS) stimulation.[13,14] Furthermore, deficiency in MKP1 results in a significantly higher incidence of mortality during endotoxemia, suggesting a protective role of MKP1 in sepsis.[14–16] MKP1 is also expressed in cardiomyocytes and is involved in cardiomyocyte apoptosis and cardiac hypertrophy.[17–19] However, the role of MKP1 in myocardial TNF-α expression and cardiac dysfunction in endotoxemia remains unknown.

MKP1 has intrinsic phosphatase activity and is inducible in response to extracellular stimuli. The mechanisms responsible for regulating MKP1 expression are cell type specific.[20–23] For example, ERK1/2[20] and JNK[21] are responsible for MKP1 induction in fibroblasts, while ERK, JNK1, and p38 are required for MKP1 expression in macrophages.[13,22,24,25] Rac is necessary for cyclic strain stress-induced MKP1 expression in smooth muscle cells.[23] We have demonstrated that Rac1 is a critical regulator of TNF-α expression and cardiac dysfunction in endotoxemia.[12] Interestingly, p21-activated kinase (PAK), a serine-threonine protein kinase, acts as a downstream effector of Rac.[26] Furthermore, activation of the Rac1/PAK pathway increases pro-inflammatory factor expression in macrophages through JNK and p38.[27] PAK1 is the main PAK isoform in cardiomyocytes.[28] The role of PAK1 in MKP1 and TNF-α expression in cardiomyocytes has not been elucidated.

In the present study, we hypothesized that MKP1 is induced during endotoxemia via the Rac1/PAK1/JNK pathway in cardiomyocytes, leading to inhibition of TNF-α expression and improvement of cardiac function in endotoxemia. To test this hypothesis, cultured cardiomyocytes and MKP1$^{-/-}$ mice were employed. Our results demonstrated for the first time that MKP1 represents an important negative feedback mechanism in limiting pro-inflammatory response in the heart during endotoxemia.

2. Methods

2.1 Animals and preparation of neonatal mouse cardiomyocytes

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication #85-23, revised 1996) and the experimental protocols were approved by Animal Use Subcommittee at the University of Western Ontario. C57BL/6 wild-type (WT), *Rac1* floxed (Rac1$^{f/f}$), and JNK1$^{-/-}$ mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). *Cre* transgenic mice (Cre$^{TG/+}$), which overexpressed Cre recombinase under the control of α-myosin heavy-chain (MHC) promoter, were provided by Dr E. Dale Abel (University of Utah, UT, USA). The generation of cardiomyocyte-specific *Rac1* knockout mice (Rac1^{CKO}) was achieved by breeding Rac1$^{f/f}$ mice with Cre$^{TG/+}$ mice as previously described.[29] MKP1$^{-/-}$ mice were kindly provided by Bristol-Myers Squibb Pharmaceutical Research.[30] Neonatal mice were sacrificed by cervical dislocation without anaesthesia. Hearts were removed, and cardiomyocytes were isolated and cultured according to methods we have previously described.[31,32] Cells were treated with LPS (Sigma, Oakville, Ontario, Canada), p21-activated kinases inhibitor III (IPA-3, EMD Biosciences, San Diego, CA, USA) and SP600125 (Enzo Life Sciences, Plymouth Meeting, PA, USA), infected with adenoviruses or transfected with small-interfering RNAs (siRNAs).

2.2 Adenoviral infection of neonatal cardiomyocytes

Cardiomyocytes were infected with adenoviruses carrying a dominant-negative form of Rac1 (Ad-Rac1N17, Vector Biolabs, Philadelphia, PA, USA), MKP1 (Ad-MKP1), or LacZ (Ad-LacZ, Vector Biolabs, Philadelphia, PA, USA) at a multiplicity of infection of 10 plaque forming units/cell. Adenovirus-mediated gene transfer was applied as previously described.[10] Experiments were performed after 48 h of adenoviral infection.

2.3 siRNA transfection of neonatal cardiomyocytes

Cardiomyocytes were treated with *PAK1* siRNA (2.5 μM) to knockdown PAK1 expression (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). A scrambled siRNA (Santa Cruz Biotechnology Inc.) was used as a control. The transfection was performed with transfection reagent (Santa Cruz Biotechnology Inc.) according to the manufacturer's instructions. After transfection, cells were maintained in normal culture medium for additional 48 h before LPS treatment.

2.4 Measurement of TNF-α and MKP1 mRNA

Total RNA was extracted from neonatal cardiomyocytes and myocardium using the Trizol Reagent (Invitrogen, Burlington, ON, Canada), following the manufacturer's instructions. The MKP1 primers were: upstream 5′-GGA GAT CCT GTC CTT CCT GTA-3′ and downstream 5′-CTG ATG TCT GCC TTG TGG TTG-3′. TNF-α and 28S primers were same as in our previous report.[10] MKP1 and TNF-α mRNA levels were determined by real-time reverse transcriptase–polymerase chain reaction (RT–PCR).[10]

2.5 Measurement of TNF-α protein

TNF-α protein levels were measured using a mouse TNF-α ELISA kit (eBioscience, San Diego, CA, USA), according to the manufacturer's instructions. The measurements were standardized with cell numbers.

2.6 Western blot analysis

Ten to 20 μg of protein lysates were subjected to separation on a 10% SDS–PAGE gel, followed by electrotransfer to nitrocellulose membranes. Blots were probed with specific antibodies against ERK1/2, phospho-ERK1/2, p38, phospho-p38, JNK1/2, phospho-JNK1/2, PAK1 (1:1000), and phospho-PAK1 (1:500, Cell Signaling Technology, Danvers, MA, USA), MKP1 (1:1000, Upstate, Lake Placid, NY, USA), α-actinin, and GAPDH (Santa Cruz Biotechnology Inc.) respectively, as previously reported.[10] Signals were detected by the chemiluminescence and quantified by densitometry.

2.7 Isolated heart preparations

Adult WT and MKP1$^{-/-}$ mice (male, 10 weeks old) were treated with LPS (10 mg/kg, i.p.) or saline. After 4 h, mice were heparinized (5000 U/kg, i.p.) and sacrificed by cervical dislocation under ketamine (50 mg/kg, i.p.) and xylazine (12.5 mg/kg, i.p.) anaesthesia. The adequacy of anaesthesia was monitored by the absence of withdrawal reflex to tail pinch. Hearts were isolated and perfused in a Langendorff system with Kreb's-Henseleit buffer at 3 mL/min constant flow. The Kreb's-Henseleit buffer was kept at 37°C and consistently bubbled with a mixture of 95% O_2 and 5% CO_2. Myocardial function was detected as previously described.[12]

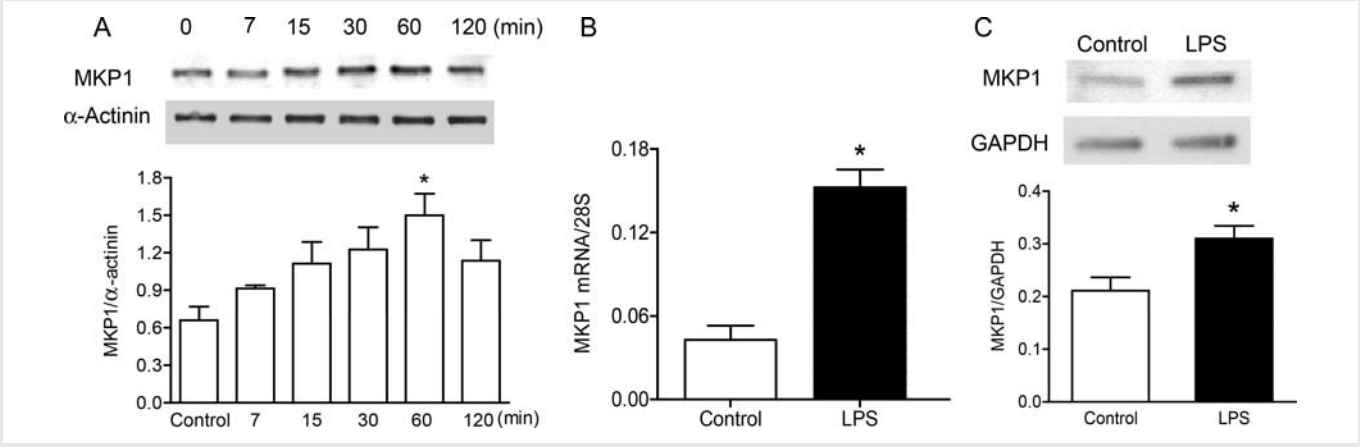

Figure 1 LPS induces MKP1 expression in cultured cardiomyocytes and in the adult myocardium. (*A*) WT cardiomyocytes were cultured in serum-free medium for 24 h and then treated with vehicle or LPS (1 μg/mL) for 7, 15, 30, 60, and 120 min. The MKP1 protein was detected by western blotting. Adult male WT mice were treated with LPS (4 mg/kg, i.p.) for 1 or 1.5 h. MKP1 mRNA (*B*) and protein (*C*) levels in the LV myocardium were measured by real-time RT–PCR and western blotting, respectively. Data are means ± SEM from three to four mice or replicates of cell cultures. $^*P < 0.05$ vs. control.

2.8 Echocardiography

Adult male WT and MKP1$^{-/-}$ mice (8–10 weeks old) were treated with LPS (1.5 mg/kg, i.p.) or saline. After 4 and 24 h, mice were anaesthetized with 0.5–1% halothane USP inhalation in a mixture of 95% O_2 and 5% CO_2. Echocardiography (Sonos 5500, Hewlett-Packard, Andover, MA, USA) was performed as previously described.[33,34] A 15 MHz paediatric probe (Agilent Technologies, Santa Clara, CA, USA) was used and M-mode data were captured for each animal. Parameters were determined using the American Society for Echocardiography leading-edge technique in a blinded fashion.[33,34]

2.9 Statistical analysis

Results are presented as mean ± SEM. Differences between two groups were analysed by a standard Student *t*-test. For multi-group comparisons, one or two-way ANOVA followed by Student–Newman–Keuls or Bonferroni post-test was performed. $P < 0.05$ was considered statistically significant.

3. Results

3.1 LPS increases myocardial MKP1 expression

To examine whether LPS regulates myocardial MKP1 expression, we first measured MKP1 protein levels in cultured cardiomyocytes and found that the MKP1 protein was increased after 1 h of LPS stimulation (*Figure 1A*). To verify these *in vitro* results, WT mice were treated with LPS or saline for 1 and 1.5 h. Hearts were then harvested and myocardial MKP1 mRNA and protein levels were detected. Myocardial MKP1 mRNA and protein levels were markedly increased after LPS stimulation (*Figure 1B* and *C*). These data show that LPS promotes MKP1 expression in cardiomyocytes *in vitro* and in the myocardium *in vivo*.

3.2 MKP1 inhibits LPS-induced myocardial ERK1/2 and p38 activation

MKP1 inactivates MAPKs by dephosphorylating phosphothreonine and phosphotyrosine residues. To determine the role of MKP1 in cardiac MAPK inactivation, we examined myocardial ERK1/2 and p38 phosphorylation in WT and MKP1$^{-/-}$ mice after LPS (4 mg/kg, i.p.) treatment for 1, 1.5, 2, 2.5, and 3 h. ERK1/2 and p38 phosphorylation was induced by LPS in both WT and MKP1$^{-/-}$ hearts (*Figure 2A* and *B*). Phosphorylation of ERK1/2 was diminished in the WT myocardium between 2 and 3 h after LPS stimulation, but was sustained in the MKP1$^{-/-}$ myocardium (*Figure 2A*). Similarly, myocardial p38 phosphorylation peaked at 1.5 h and then declined between 2 and 3 h after LPS stimulation. Importantly, p38 phosphorylation was significantly higher in MKP1$^{-/-}$ than the WT myocardium (*Figure 2B*). Thus, MKP1 deficiency enhanced ERK1/2 and p38 phosphorylation, demonstrating an important role for MKP1 in reducing the levels of myocardial ERK1/2 and p38 activation after LPS stimulation.

3.3 MKP1 inhibits myocardial TNF-α expression in endotoxemia

To investigate the role of MKP1 in myocardial TNF-α expression during endotoxemia *in vivo*, WT and MKP1$^{-/-}$ mice were treated with vehicle or LPS. Our data showed that LPS increased myocardial TNF-α expression in both WT and MKP1$^{-/-}$ mice. Compared with the WT group, MKP1$^{-/-}$ mice exhibited significantly higher TNF-α mRNA and protein levels (*Figure 2C* and *D*). In addition, neonatal cardiomyocytes were infected with Ad-MKP1. Consistently, MKP1 overexpression in neonatal cardiomyocytes inhibited LPS-induced TNF-α mRNA and protein levels (*Figure 2E* and *F*). These results suggest that MKP1 limits myocardial TNF-α expression in response to LPS.

3.4 MKP1 improves cardiac function during endotoxemia

Cardiac function was determined using the Langendorff preparation after 4 h of LPS *in vivo* treatment. The rates of contraction ($+dF/dt_{max}$) and relaxation ($-dF/dt_{min}$), and heart work were reduced in both WT and MKP1$^{-/-}$ mice relative to vehicle-treated mice (*Figure 3*). Furthermore, compared with WT mice, rate of contraction and relaxation and heart work were significantly lower in MKP1$^{-/-}$ mice (*Figure 3A–C*). To confirm these *ex vivo* data, *in vivo* cardiac

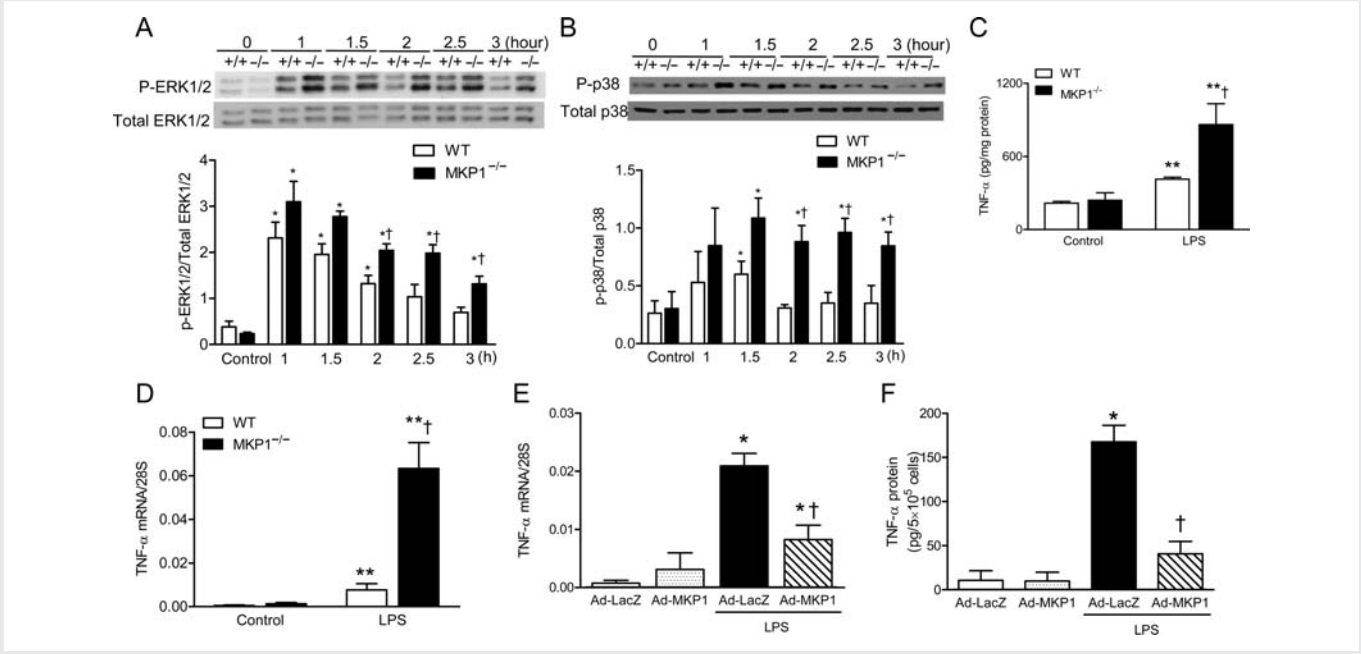

Figure 2 MKP1 inhibits myocardial ERK1/2 and p38 activation and TNF-α expression in endotoxemia. Adult male WT and MKP1$^{-/-}$ mice were treated with LPS (4 mg/kg, i.p.) for 1, 1.5, 2, 2.5, and 3 h. Myocardial ERK1/2 (*A*) and p38 (*B*) phosphorylation was determined. TNF-α mRNA (*C*) and protein (*D*) levels in the WT and MKP1$^{-/-}$ myocardium were measured after 1 and 2 h of LPS treatment by real-time RT–PCR and western blotting, respectively. (*E* and *F*) Neonatal cardiomyocytes were infected with Ad-MKP1. After 48 h, cells were treated with LPS (1 μg/mL) for 3 or 5 h. TNF-α mRNA (*E*) and protein (*F*) levels were measured. Data are means ± SEM from three to five mice or three to four replicates of cell cultures. $^{*}P < 0.05$ vs. control, $^{**}P < 0.01$ vs. control, $^{\dagger}P < 0.05$ vs. WT+LPS.

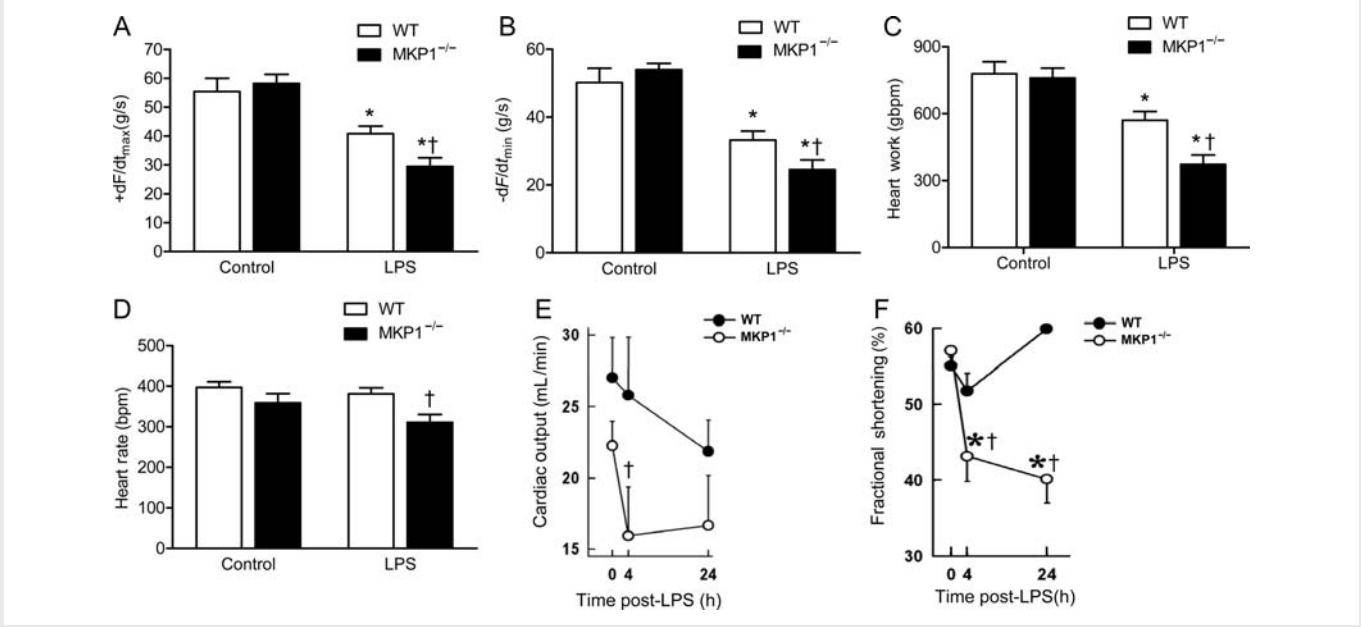

Figure 3 Cardiac function in WT and MKP1$^{-/-}$ mice with endotoxemia. After 4 h of *in vivo* LPS treatment (10 mg/kg, i.p.), mouse hearts were isolated and perfused using the Langendorff system. Changes in contraction ($+dF/dt_{max}$, *A*), relaxation ($-dF/dt_{min}$, *B*) heart work (*C*), and heart rate (*D*) are presented. (*E* and *F*) Echocardiography of adult WT and MKP1$^{-/-}$ mice after 4 and 24 h of *in vivo* LPS treatment (1.5 mg/kg, i.p.). Cardiac output (*E*) and fractional shortening (*F*) were measured. Data are means ± SEM, $n = 6–9$ mice per group. $^{*}P < 0.05$ vs. control, $^{\dagger}P < 0.05$ vs. WT+LPS.

function was determined using echocardiography in WT and MKP1$^{-/-}$ mice at baseline and at 4 and 24 h after LPS treatment. Basal LV dimension, wall thickness, and heart function showed no difference between MKP1$^{-/-}$ and WT mice (*Table 1*). In response to LPS, cardiac output and fractional shortening showed a trend but not significant decrease in WT mice (*Figure 3E* and *F*). However, fractional shortening was significantly decreased in MKP1$^{-/-}$ mice after LPS stimulation ($P < 0.05$). Furthermore, both cardiac output and fractional shortening were significantly decreased after LPS treatment in MKP1$^{-/-}$ compared with WT mice ($P < 0.05$). Taken together, these data indicate that MKP1 expression improves cardiac function during endotoxemia.

Table 1 Echocardiographic measurements of cardiac function in adult male WT and MKP1$^{-/-}$ mice at baseline

Parameters	WT	MKP1$^{-/-}$
n	6	6
LVIDd (mm)	3.46 ± 0.07	3.40 ± 0.09
LVIDs (mm)	2.18 ± 0.04	2.07 ± 0.07
LV ejection fraction (%)	68.4 ± 2.0	70.6 ± 1.6
LV fractional shortening (%)	37.5 ± 1.6	39.1 ± 1.3
Diastolic left ventricular volume (μL)	49.7 ± 2.3	47.8 ± 3.0
Systolic left ventricular volume (μL)	15.7 ± 1.0	14.1 ± 1.3
Diastolic posterior wall thickness (mm)	0.92 ± 0.04	0.98 ± 0.05
Systolic posterior wall thickness (mm)	1.35 ± 0.03	1.41 ± 0.06

LVIDd, left ventricular internal dimension in diastole; LVIDs, left ventricular internal dimension in systole. Measurements were made under ketamine and xylazine anaesthesia using the Vevo 2100 system with a 40 MHz probe (VisualSonics, Toronto, Canada). Data are mean ± SEM. There was no statistical difference in any of the parameters between WT and MKP1$^{-/-}$ mice (P = n.s.).

3.5 LPS activates PAK1 in cardiomyocytes

To assess PAK1 activation, cultured cardiomyocytes from WT mice were treated with LPS (1 μg/mL) for 7, 15, 30, and 60 min. PAK1 phosphorylation in these cells peaked at 15 min and then returned to control levels at 60 min (*Figure 4A*), indicating that LPS activates PAK1 in cardiomyocytes.

3.6 PAK1 increases LPS-induced TNF-α expression

To elucidate the role of PAK1 in LPS-induced TNF-α expression, cardiomyocytes were treated with IPA-3, an inhibitor of PAKs. IPA-3 (1–10 μM) decreased LPS-induced TNF-α mRNA and protein levels in a dose-dependent manner (*Figure 4B* and *C*). These results were further verified by using *PAK1* siRNA. As shown in *Figure 4D*, PAK1 mRNA expression was decreased by 56% after *PAK1* siRNA treatment. The inhibition of PAK1 expression was associated with decreased TNF-α mRNA and protein levels (*Figure 4E* and *F*). These data suggest that the activation of PAK1 promotes cardiac TNF-α expression in response to LPS.

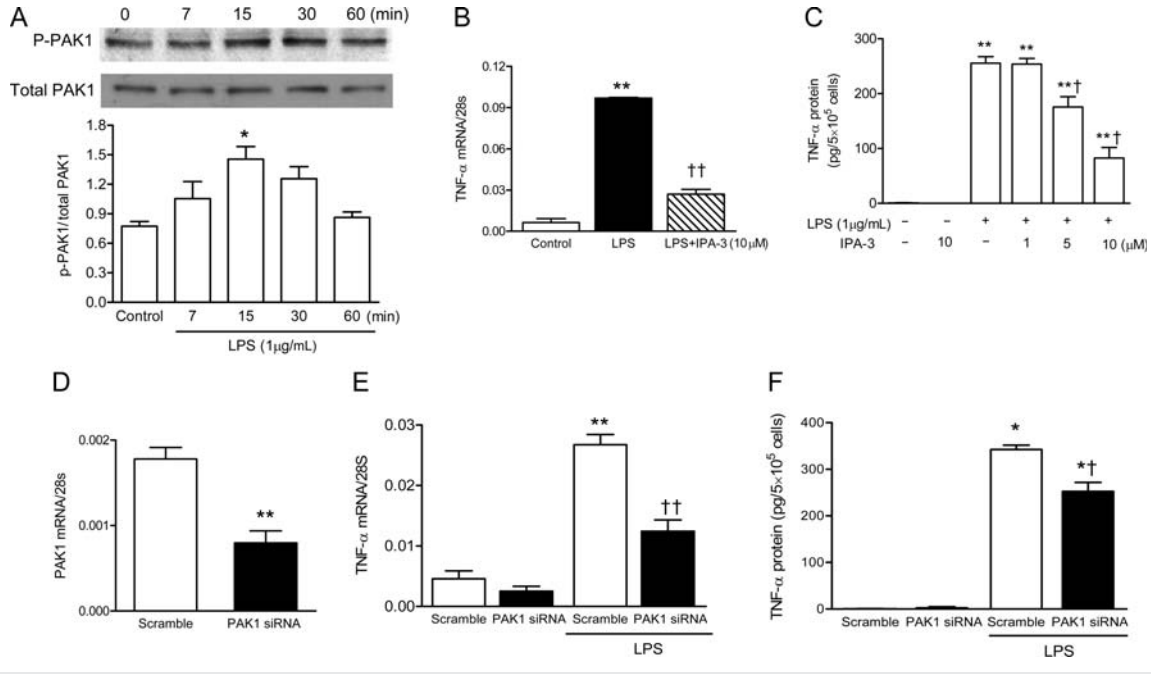

Figure 4 PAK1 activation promotes LPS-induced TNF-α expression in cultured cardiomyocytes. (*A*) Cardiomyocytes isolated from WT mice were treated with vehicle or LPS (1 μg/mL) for 7, 15, 30, and 60 min. PAK1 phosphorylation was measured by western blotting. (*B* and *C*) Cells were treated with IPA-3 (10 μM) with or without LPS (1 μg/mL) for 3 or 5 h. TNF-α mRNA (*B*) and TNF-α protein in the culture medium (*C*) were measured by real-time RT–PCR and ELISA, respectively. (*D*) WT cardiomyocytes were treated with scrambled or *PAK1* siRNA (2.5 μM). *PAK1* mRNA levels were determined. (*E* and *F*) WT cardiomyocytes were transfected with scrambled or *PAK1* siRNA (2.5 μM) followed by LPS (1 μg/mL) treatment for 3 or 5 h. TNF-α mRNA (*E*) and protein (*F*) levels were measured. Data are means ± SEM from three to four replicates of cell cultures. *$P < 0.05$, **$P < 0.01$ vs. control; †$P < 0.05$, ††$P < 0.01$ vs. LPS and Scramble+LPS.

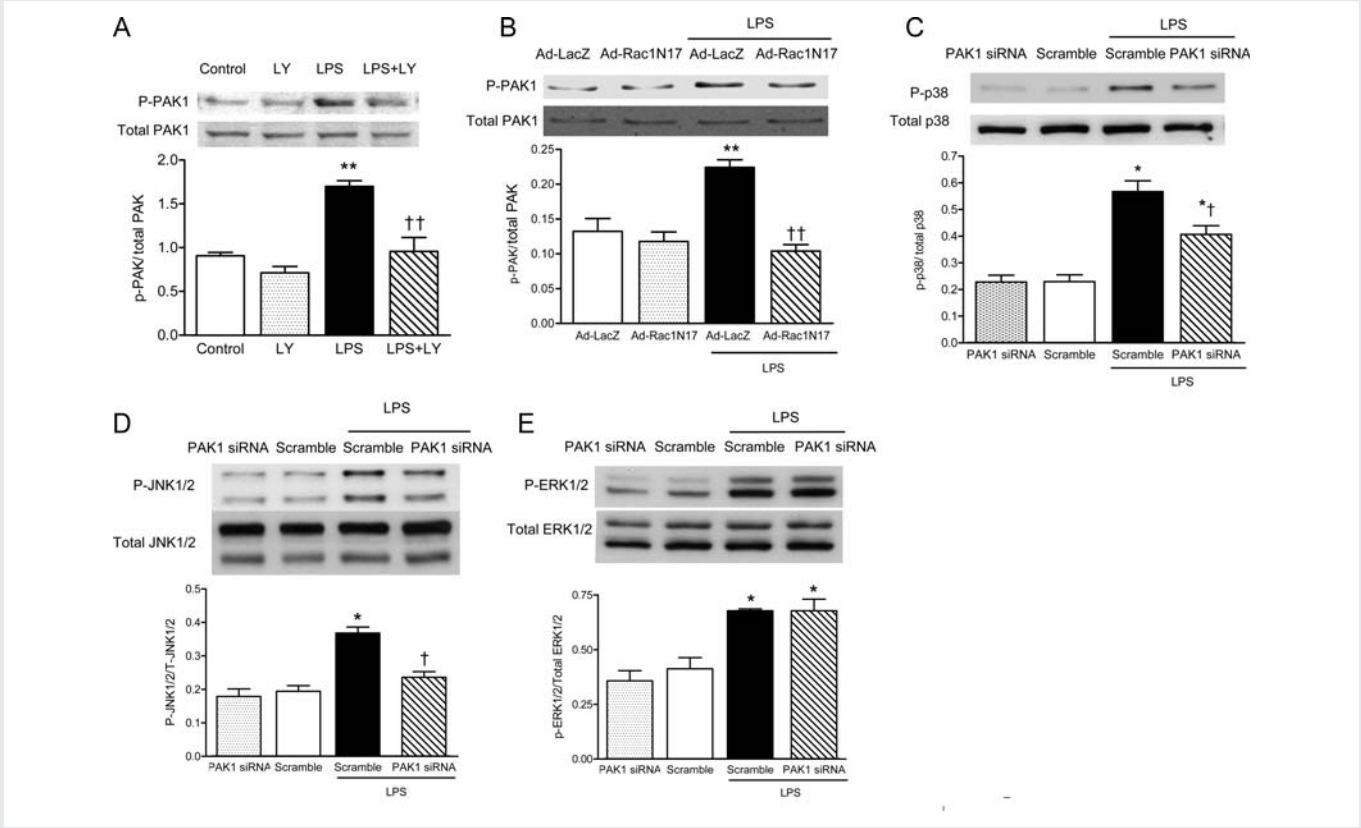

Figure 5 PI3K/Rac1/PAK1 signalling activates p38 and JNK1/2 but not ERK1/2 during LPS stimulation. (*A*) WT cardiomyocytes were treated with LPS (1 μg/mL, 15 min) with or without LY294002 (LY, 10 μM). (*B*) Cells were infected with Ad-Rac1N17 followed by LPS treatment (1 μg/mL) for 15 min. PAK1 phosphorylation was determined. WT cardiomyocytes were transfected with scrambled or *PAK1* siRNA (2.5 μM) followed by LPS (1 μg/mL) treatment for 1 h. Phosphorylation of p38 (*C*), JNK1/2 (*D*), and ERK1/2 (*E*) was determined. Data are mean ± SEM from three replicates of cell cultures. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. LPS, Ad-LacZ+LPS or Scramble+LPS.

3.7 PI3K/Rac1 pathway mediates LPS-induced PAK1 activation

PAK1 is activated by small GTPases, such as Rac.[26] We have demonstrated that LPS activates the PI3K/Rac1 pathway, which is critical for cardiac TNF-α expression during endotoxemia.[12] To determine whether PI3K acts as an upstream regulator of PAK1, the PI3K inhibitor, LY294002, was employed. LY294002 significantly decreased PAK1 phosphorylation induced by LPS (*Figure 5A*). To determine the role of Rac1 on PAK1 activity, cardiomyocytes were treated with an adenovirus encoding a dominant negative form of the *Rac1* gene (Ad-Rac1N17), which specifically inhibits Rac1 activity. Overexpression of Rac1N17 inhibited PAK1 activity in response to LPS (*Figure 5B*). Taken together, these data imply that LPS activates PAK1 via PI3K/Rac1 in cardiomyocytes.

3.8 PAK1 mediates LPS-induced p38 and JNK phosphorylation

To determine whether PAK1 regulates p38, ERK1/2, and JNK phosphorylation, cultured cardiomyocytes were treated with *PAK1* siRNA. Interestingly, *PAK1* siRNA blocked p38 and JNK phosphorylation in response to LPS, but had no significant effect on ERK1/2 phosphorylation (*Figure 5C–E*).

3.9 Rac1/PAK1/JNK pathway mediates LPS-induced MKP1 expression

To further explore the downstream effectors of the Rac1/PAK1/JNK pathway, MKP1 expression was studied. Overexpression of Rac1N17 in cardiomyocytes significantly decreased MKP1 protein levels (*Figure 6A*). This result was further confirmed *in vivo*. As shown in *Figure 6B*, LPS enhanced MKP1 protein levels in $Rac1^{f/f}$ but not in $Rac1^{CKO}$ hearts. To examine whether PAK1 mediates MKP1 expression, cultured cardiomyocytes were treated with IPA-3 or *PAK1* siRNA. LPS-induced MKP1 protein expression was blocked by either IPA-3 or *PAK1* siRNA (*Figure 6C* and *D*). To determine whether JNK regulates MKP1 expression, cardiomyocytes were treated with SP600125, an inhibitor of JNK. LPS-induced MKP1 protein expression was significantly reduced by SP600125 (*Figure 6E*). To verify this result *in vivo*, WT and $JNK1^{-/-}$ mice were treated with LPS (4 mg/kg, i.p.) or saline for 1.5 h. LPS significantly increased myocardial MKP1 protein levels in WT mice but had no apparent effect in $JNK1^{-/-}$ mice ($P < 0.05$, *Figure 6F*). Taken together, these data indicate that the Rac1/PAK1/JNK pathway mediates LPS-induced MKP1 expression.

4. Discussion

The present study demonstrated for the first time that myocardial MKP1 is induced by LPS via the PI3K/Rac1/PAK1/JNK pathway.

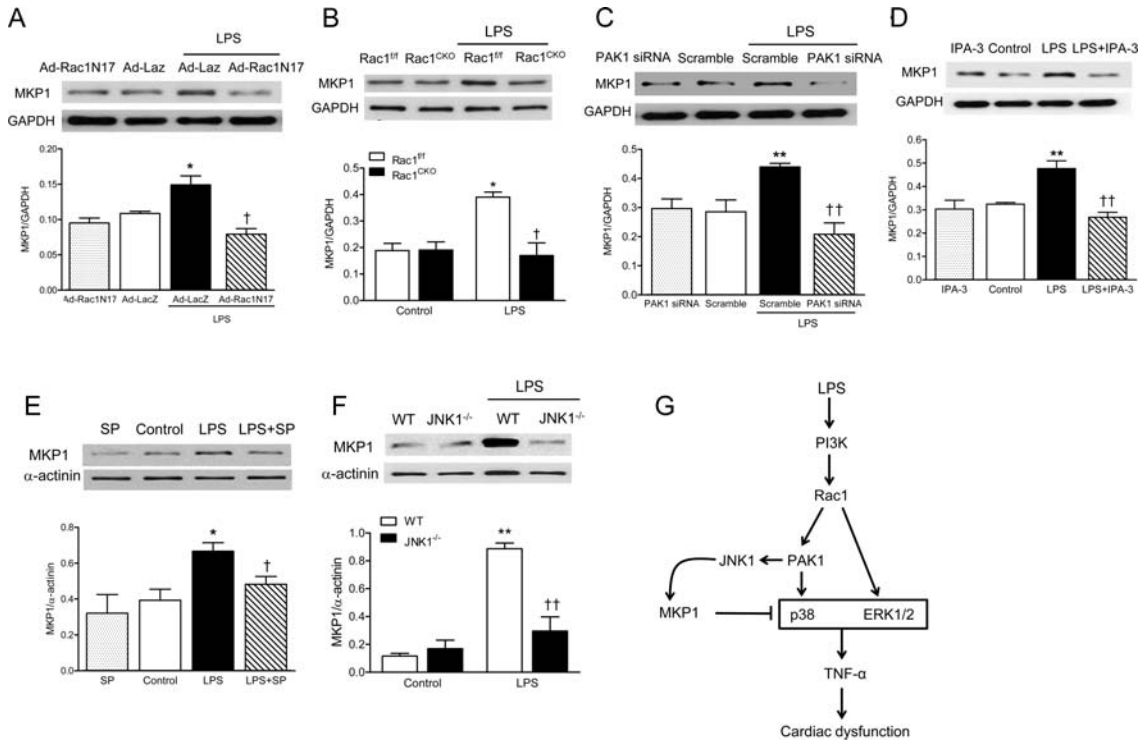

Figure 6 Rac1/PAK1/JNK signalling upregulates MKP1 protein expression during LPS stimulation. (*A*) WT cardiomyocytes were treated with Ad-Rac1N17 followed by LPS treatment (1 μg/mL) for 1 h. MKP1 protein levels were detected by western blotting. (*B*) Adult male $Rac1^{f/f}$ and $Rac1^{CKO}$ mice were treated with LPS (4 mg/kg, i.p. injection) for 1.5 h. Myocardial MKP1 protein levels were determined. (*C*) WT cardiomyocytes were treated with *PAK1* siRNA (2.5 μM) followed by LPS treatment for 1 h. (*D* and *E*) WT cardiomyocytes were treated with LPS for 1 h with or without IPA-3 (10 μM, *D*) and SP600125 (SP, 10 μM, *E*). MKP1 protein levels in these cells were determined. (*F*) Adult male WT and $JNK1^{-/-}$ mice were treated with LPS (4 mg/kg, i.p. injection) for 1.5 h. Myocardial MKP1 protein levels were determined. Data are mean ± SEM from four to six mice or three to four replicates of cell cultures. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. LPS, Ad-LacZ +LPS, Scramble+LPS. (*G*) Schematic of the MKP1 signalling regulating TNF-α expression and cardiac function during LPS stimulation. PAK1 is activated by the PI3K/Rac1 pathway and increases p38 and JNK activity. PAK1 increases TNF-α expression via p38 activation in response to LPS. On the contrary, the Rac1/PAK1/JNK pathway enhances MKP1 expression, which inactivates ERK1/2 and p38, limits TNF-α expression, and improves cardiac function during endotoxemia.

Enhanced MKP1 expression attenuates ERK1/2 and p38 phosphorylation, leading to inhibition of myocardial TNF-α expression and improvement of cardiac function during endotoxemia (*Figure 6G*).

MKP1 dephosphorylates MAPKs and impedes their cellular functions. Recent studies have shown that MKP1 expression is up-regulated in macrophages in response to LPS.[13] Deficiency of MKP1 enhances p38 and JNK phosphorylation and pro-inflammatory cytokine production, including TNF-α, IL-1β, and IL-6 in macrophages.[13,14] Consistent with this notion, $MKP1^{-/-}$ mice exhibit significantly higher serum cytokine concentrations and higher mortality rate after LPS stimulation.[14–16] In the present study, we showed that LPS induced myocardial MKP1 expression. Furthermore, overexpression of MKP1 decreased TNF-α expression induced by LPS. MKP1 deficiency enhanced myocardial ERK1/2 and p38 phosphorylation and increased cardiac TNF-α expression. Most importantly, MKP1 deficiency further decreased cardiac function during endotoxemia. Thus, both *in vitro* and *in vivo* evidence demonstrated that LPS induces MKP1 expression, which inhibits ERK1/2 and p38 activation and myocardial TNF-α expression, and improves cardiac function during endotoxemia.

Rac is one of the factors that are responsible for MKP1 expression.[23] We recently demonstrated that PI3K-mediated Rac1 activation promotes TNF-α expression and cardiac dysfunction in endotoxemia.[12] PAK proteins are downstream effectors of Rac and regulate many cellular events, including cell motility, survival, proliferation, and gene expression.[26] PAK1 is the main isoform of this enzyme in the myocardium and plays an important role in cardiac contractility and hypertrophy.[35,36] Studies have shown that the PAK protein is activated and contributes to IL-1 expression through MAPKs upon ligand stimulation in macrophages.[27] In the present study, LPS enhanced PAK1 activity in cardiomyocytes. Inhibition of PAK1 activity by the pharmacological inhibitor, IPA-3, or PAK1 expression by *PAK1* siRNA decreased LPS-induced TNF-α expression in cardiomyocytes. Furthermore, inhibition of PI3K and Rac1 decreased PAK1 activity in response to LPS in cardiomyocytes. These results indicate that PI3K/Rac1-mediated PAK1 activation plays a key role in LPS-induced TNF-α expression in cardiomyocytes.

ERK1/2, p38, and JNK MAPKs are important signalling molecules regulating TNF-α expression in cardiomyocytes.[6–10] ERK1/2 and p38 are positive regulators of TNF-α expression.[6–8,10] In contrast, JNK1 inhibits TNF-α expression via inactivating LPS-induced ERK1/2

and p38 MAPKs.[9] In the present study, inhibition of PAK1 activity blocked LPS-induced p38 and JNK phosphorylation. These results suggest that PAK1 may have both stimulatory and inhibitory effects on LPS-induced TNF-α expression in cardiomyocytes. Its activation on p38 promotes TNF-α expression. On the other hand, PAK1-mediated JNK activation inhibits LPS-induced TNF-α expression.

To address the molecular mechanisms by which JNK inhibits LPS-induced TNF-α expression, the role of MKP1 was examined in the present investigation. It has been shown that MAPKs are involved in regulating MKP1 expression. For example, ERK1/2 and JNK activation is responsible for MKP1 induction in fibroblasts.[20,21] However, the effects of ERK1/2, p38, and JNK on MKP1 expression in macrophages are controversial. Several studies have shown that ERK1/2 and p38 are required for MKP1 expression in macrophages. [13,24,25] On the other hand, Sanchez-Tillo *et al.*[22] reported that JNK1 is necessary for MKP1 expression in macrophages which consequently decreases ERK1/2 and p38 phosphorylation levels. In the present study, we demonstrated that LPS-induced MKP1 expression was blocked by the inhibition of Rac1, PAK1, and JNK1 in cardiomyocytes, suggesting that the Rac1/PAK1/JNK1 pathway is required for LPS-induced MKP1 expression in cardiomyocytes. MKP1 mediates the inhibitory effect of JNK1 on ERK1/2 and p38 activity. Therefore, Rac1/PAK1/JNK1-mediated MKP1 expression provides a key negative feedback mechanism to limit myocardial TNF-α production and improve cardiac function during endotoxemia. These results do not exclude other pathways through which Rac1 promotes MKP1 expression. For example, we have previously shown that Rac1 activation leads to TNF-α production through reactive oxygen species (ROS), which is also a regulator of MKP1.[12,37] Thus, the Rac1/ROS pathway could be an alternative mechanism responsible for MKP1 expression.

In conclusion, the present study showed that PAK1 is activated by the PI3K/Rac1 pathway and increases p38 and JNK activity. PAK1 increases TNF-α expression via p38 activation in response to LPS. On the other hand, the Rac1/PAK1/JNK1 pathway enhances MKP1 expression, which inactivates ERK1/2 and p38, limits TNF-α expression, and improves cardiac function during endotoxemia (*Figure 6G*). Thus, the Rac1/PAK1/JNK1/MKP1 signalling pathway represents a novel negative feedback mechanism in regulating TNF-α expression and cardiac function in endotoxemia. Our study suggests that MKP1 may have therapeutic potential in the clinical treatment of sepsis. Further studies are required to assess its efficacy and safety in large animal models and in patients with sepsis.

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References

1. Dellinger RP, Levy MM, Carlet JM, Bion J, Parker MM, Jaeschke R *et al.* Surviving Sepsis Campaign: International guidelines for management of severe sepsis and septic shock 2008. *Crit Care Med* 2008;**36**:296–327.
2. Merx MW, Weber C. Sepsis and the heart. *Circulation* 2007;**116**:793–802.
3. Parrillo JE, Burch C, Shelhamer JH, Parker MM, Natanson C, Schuette W. A circulating myocardial depressant substance in humans with septic shock. Septic shock patients with a reduced ejection fraction have a circulating factor that depresses *in vitro* myocardial cell performance. *J Clin Invest* 1985;**76**:1539–1553.
4. Dumitru CD, Ceci JD, Tsatsanis C, Kontoyiannis D, Stamatakis K, Lin JH *et al.* TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. *Cell* 2000;**103**:1071–1083.
5. Kotlyarov A, Neininger A, Schubert C, Eckert R, Birchmeier C, Volk HD *et al.* MAPKAP kinase 2 is essential for LPS-induced TNF-alpha biosynthesis. *Nat Cell Biol* 1999;**1**:94–97.
6. Thakur V, Pritchard MT, McMullen MR, Wang Q, Nagy LE. Chronic ethanol feeding increases activation of NADPH oxidase by lipopolysaccharide in rat Kupffer cells: role of increased reactive oxygen in LPS-stimulated ERK1/2 activation and TNF-alpha production. *J Leukoc Biol* 2006;**79**:1348–1356.
7. Rosengart MR, Arbabi S, Garcia I, Maier RV. Interactions of calcium/calmodulin-dependent protein kinases (CaMK) and extracellular-regulated kinase (ERK) in monocyte adherence and TNF-alpha production. *Shock* 2000;**13**:183–189.
8. Peng T, Lu X, Feng Q. Pivotal role of gp91phox-containing NADH oxidase in lipopolysaccharide-induced tumor necrosis factor-alpha expression and myocardial depression. *Circulation* 2005;**111**:1637–1644.
9. Peng T, Zhang T, Lu X, Feng Q. JNK1/c-fos inhibits cardiomyocyte TNF-alpha expression via a negative crosstalk with ERK and p38 MAPK in endotoxaemia. *Cardiovasc Res* 2009;**81**:733–741.
10. Peng T, Lu X, Lei M, Moe GW, Feng Q. Inhibition of p38 MAPK decreases myocardial TNF-alpha expression and improves myocardial function and survival in endotoxemia. *Cardiovasc Res* 2003;**59**:893–900.
11. Liu Y, Shepherd EG, Nelin LD. MAPK phosphatases—regulating the immune response. *Nat Rev Immunol* 2007;**7**:202–212.
12. Zhang T, Lu X, Beier F, Feng Q. Rac1 activation induces tumor necrosis factor-alpha expression and cardiac dysfunction in endotoxemia. *J Cell Mol Med* 2011;**15**:1109–1121.
13. Chen P, Li J, Barnes J, Kokkonen GC, Lee JC, Liu Y. Restraint of proinflammatory cytokine biosynthesis by mitogen-activated protein kinase phosphatase-1 in lipopolysaccharide-stimulated macrophages. *J Immunol* 2002;**169**:6408–6416.
14. Zhao Q, Wang X, Nelin LD, Yao Y, Matta R, Manson ME *et al.* MAP kinase phosphatase 1 controls innate immune responses and suppresses endotoxic shock. *J Exp Med* 2006;**203**:131–140.
15. Salojin KV, Owusu IB, Millerchip KA, Potter M, Platt KA, Oravecz T. Essential role of MAPK phosphatase-1 in the negative control of innate immune responses. *J Immunol* 2006;**176**:1899–1907.
16. Hammer M, Mages J, Dietrich H, Servatius A, Howells N, Cato AC *et al.* Dual specificity phosphatase 1 (DUSP1) regulates a subset of LPS-induced genes and protects mice from lethal endotoxin shock. *J Exp Med* 2006;**203**:15–20.
17. Fischer TA, Singh K, O'Hara DS, Kaye DM, Kelly RA. Role of AT1 and AT2 receptors in regulation of MAPKs and MKP-1 by ANG II in adult cardiac myocytes. *Am J Physiol* 1998;**275**:H906–H916.
18. Palm-Leis A, Singh US, Herbelin BS, Olsovsky GD, Baker KM, Pan J. Mitogen-activated protein kinases and mitogen-activated protein kinase phosphatases mediate the inhibitory effects of all-trans retinoic acid on the hypertrophic growth of cardiomyocytes. *J Biol Chem* 2004;**279**:54905–54917.
19. Kaiser RA, Bueno OF, Lips DJ, Doevendans PA, Jones F, Kimball TF *et al.* Targeted inhibition of p38 mitogen-activated protein kinase antagonizes cardiac injury and cell death following ischemia-reperfusion *in vivo*. *J Biol Chem* 2004;**279**:15524–15530.
20. Brondello JM, Brunet A, Pouyssegur J, McKenzie FR. The dual specificity mitogen-activated protein kinase phosphatase-1 and -2 are induced by the p42/p44MAPK cascade. *J Biol Chem* 1997;**272**:1368–1376.
21. Bokemeyer D, Sorokin A, Yan M, Ahn NG, Templeton DJ, Dunn MJ. Induction of mitogen-activated protein kinase phosphatase 1 by the stress-activated protein kinase signaling pathway but not by extracellular signal-regulated kinase in fibroblasts. *J Biol Chem* 1996;**271**:639–642.
22. Sanchez-Tillo E, Comalada M, Xaus J, Farrera C, Valledor AF, Caelles C *et al.* JNK1 is required for the induction of Mkp1 expression in macrophages during proliferation and lipopolysaccharide-dependent activation. *J Biol Chem* 2007;**282**:12566–12573.
23. Li C, Hu Y, Mayr M, Xu Q. Cyclic strain stress-induced mitogen-activated protein kinase (MAPK) phosphatase 1 expression in vascular smooth muscle cells is regulated by Ras/Rac-MAPK pathways. *J Biol Chem* 1999;**274**:25273–25280.
24. Kim C, Sano Y, Todorova K, Carlson BA, Arpa L, Celada A *et al.* The kinase p38 alpha serves cell type-specific inflammatory functions in skin injury and coordinates pro- and anti-inflammatory gene expression. *Nat Immunol* 2008;**9**:1019–1027.
25. Ananieva O, Darragh J, Johansen C, Carr JM, McIlrath J, Park JM *et al.* The kinases MSK1 and MSK2 act as negative regulators of Toll-like receptor signaling. *Nat Immunol* 2008;**9**:1028–1036.
26. Molli PR, Li DQ, Murray BW, Rayala SK, Kumar R. PAK signaling in oncogenesis. *Oncogene* 2009;**28**:2545–2555.
27. Hsu HY, Chiu SL, Wen MH, Chen KY, Hua KF. Ligands of macrophage scavenger receptor induce cytokine expression via differential modulation of protein kinase signaling pathways. *J Biol Chem* 2001;**276**:28719–28730.
28. Sheehan KA, Ke Y, Solaro RJ. p21-Activated kinase-1 and its role in integrated regulation of cardiac contractility. *Am J Physiol Regul Integr Comp Physiol* 2007;**293**:R963–R973.

29. Rui T, Feng Q, Lei M, Peng T, Zhang J, Xu M *et al*. Erythropoietin prevents the acute myocardial inflammatory response induced by ischemia/reperfusion via induction of AP-1. *Cardiovasc Res* 2005;**65**:719–727.
30. Dorfman K, Carrasco D, Gruda M, Ryan C, Lira SA, Bravo R. Disruption of the erp/mkp-1 gene does not affect mouse development: normal MAP kinase activity in ERP/MKP-1-deficient fibroblasts. *Oncogene* 1996;**13**:925–931.
31. Song W, Lu X, Feng Q. Tumor necrosis factor-alpha induces apoptosis via inducible nitric oxide synthase in neonatal mouse cardiomyocytes. *Cardiovasc Res* 2000;**45**:595–602.
32. Peng T, Lu X, Feng Q. NADH oxidase signaling induces cyclooxygenase-2 expression during lipopolysaccharide stimulation in cardiomyocytes. *FASEB J* 2005;**19**:293–295.
33. Han B, Baliga R, Huang H, Giannone PJ, Bauer JA. Decreased cardiac expression of vascular endothelial growth factor and redox imbalance in murine diabetic cardiomyopathy. *Am J Physiol Heart Circ Physiol* 2009;**297**:H829–H835.
34. Chaves AA, Baliga RS, Mihm MJ, Schanbacher BL, Basuray A, Liu C *et al*. Bacterial lipopolysaccharide enhances cardiac dysfunction but not retroviral replication in murine AIDS: roles of macrophage infiltration and toll-like receptor 4 expression. *Am J Pathol* 2006;**168**:727–735.
35. Sheehan KA, Ke Y, Wolska BM, Solaro RJ. Expression of active p21-activated kinase-1 induces Ca^{2+} flux modification with altered regulatory protein phosphorylation in cardiac myocytes. *Am J Physiol Cell Physiol* 2009;**296**:C47–C58.
36. Sussman MA, Welch S, Walker A, Klevitsky R, Hewett TE, Price RL *et al*. Altered focal adhesion regulation correlates with cardiomyopathy in mice expressing constitutively active rac1. *J Clin Invest* 2000;**105**:875–886.
37. Hou N, Torii S, Saito N, Hosaka M, Takeuchi T. Reactive oxygen species-mediated pancreatic beta-cell death is regulated by interactions between stress-activated protein kinases, p38 and c-Jun N-terminal kinase, and mitogen-activated protein kinase phosphatases. *Endocrinology* 2008;**149**:1654–1665.