JNK1/c-fos inhibits cardiomyocyte TNF-α expression via a negative crosstalk with ERK and p38 MAPK in endotoxaemia

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Aims Myocardial tumour necrosis factor-alpha (TNF-α) production plays an important role in cardiac dysfunction during sepsis. The aim of this study was to investigate the role of c-Jun NH_2-terminal kinases (JNK) signalling in cardiomyocyte TNF-α expression during lipopolysaccharide (LPS) stimulation and myocardial function in endotoxaemic mice.

Methods and results In cultured neonatal mouse cardiomyocytes, deficiency of JNK1 or selective inhibition of JNK1 signalling by over-expression of a dominant negative mutant of JNK1 enhanced LPS-induced TNF-α expression, which was associated with elevations in phosphorylation of extracellular signal-regulated kinase (ERK)1/2 and p38 mitogen-activated protein kinase (MAPK). At the organ level, LPS-induced TNF-α expression was significantly increased in $JNK1^{-/-}$ compared with wild-type hearts. JNK1 activation by LPS also induced immediate c-fos expression in cardiomyocytes, which was blocked by inhibition of JNK1 signalling. The role of c-fos expression in LPS-induced TNF-α expression was investigated in both cultured $c\text{-}fos^{-/-}$ cardiomyocytes and isolated $c\text{-}fos^{-/-}$ hearts. Deficiency of c-fos significantly enhanced LPS-induced TNF-α expression in cardiomyocytes and isolated hearts. Over-expression of c-fos decreased TNF-α expression in LPS-stimulated cardiomyocytes, which was associated with a decrease in phosphorylation of ERK1/2 and p38. In mice with endotoxaemia, deficiency of either JNK1 or c-fos further decreased cardiac function compared with corresponding wild-type controls.

Conclusion JNK1/c-fos inhibits ERK1/2 and p38 MAPK signalling, leading to decreased cardiomyocyte TNF-α expression and improvements in cardiac function during endotoxaemia.

1. Introduction

Lipopolysaccharide (LPS), a pathogen released by Gram-negative bacteria, induces tumour necrosis factor-α (TNF-α) production in cardiomyocytes leading to cardiac depression.[1–3] It is well established that TNF-α is a major cytokine responsible for myocardial depression in sepsis.[1,3] Studies to date have demonstrated a number of signalling pathways leading to TNF-α expression in response to LPS. For example, LPS activation of the toll-like receptor-4/MyD88 complex results in the expression of TNF-α through NF-κB signalling in cardiomyocytes.[4,5] We and others have shown that LPS activates mitogen-activated protein kinases (MAPK), the extracellular signal-regulated kinase (ERK1/2) and p38 kinase, both of which are required for TNF-α expression induced by LPS in cardiomyocytes.[1,2]

Furthermore, we also demonstrated that NADH oxidase plays an important role in LPS-induced TNF-α expression in cardiomyocytes and the effect of NADH oxidase is mediated through O_2^- generation and subsequent activation of ERK1/2 and p38 MAPK.[6] However, signalling pathways that limit TNF-α expression are poorly understood.

The c-Jun NH_2-terminal kinase (JNK), also known as stress-activated protein kinase, is a member of the MAPK family. There are three isoforms in JNK family: JNK1, JNK2, and JNK3. JNK is activated by protein synthesis inhibitors, oxidative stress, and LPS, in addition to pro-inflammatory cytokines such as TNF-α and interleukin-1β.[7] In response to LPS, JNK activation promotes TNF-α expression in macrophages and monocytes.[8,9] JNK1 and JNK2 are detectable in the heart.[10] We recently showed that LPS increases phosphorylation of JNK in cardiomyocytes.[11] However, whether JNK interacts with ERK1/2 and p38 MAPK and modulates cardiomyocyte TNF-α expression and cardiac function during sepsis is not known.

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MAPK activation induces expression and phosphorylation of c-fos, a member of the fos family.[12–14] The induction of c-fos is implicated in cell proliferation, growth, and apoptosis.[15,16] It is well known that c-fos dimerizes with c-jun to form AP-1 complex, a transcription factor, which induces target gene expression by binding the AP-1 consensus site(s) in the promoter region. In response to LPS, c-fos is induced and promotes cytokines expression through AP-1 in various cells such as monocytic cells[17] and endothelial cells.[18] In contrast, an inhibitory effect of c-fos on LPS-induced TNF-α and interleukin-12 expression has been shown in macrophages.[19,20] LPS upregulates c-fos in the heart.[21] However, the role of c-fos in cardiomyocyte TNF-α expression and myocardial dysfunction during sepsis is unknown.

The aim of the present study was to investigate the role of JNK1/c-fos signalling in cardiomyocyte TNF-α expression and myocardial dysfunction during LPS stimulation. We demonstrated that deficiency of JNK1 enhanced cardiomyocyte TNF-α expression during LPS stimulation and further decreased myocardial function in endotoxaemia. The effect of JNK1 was mediated through c-fos induction in the heart. Our results suggest that JNK1/c-fos inhibits ERK1/2 and p38 MAPK signalling, leading to decreased myocardial TNF-α expression and improvements in cardiac function during endotoxaemia.

2. Experimental procedures

2.1 Animals and neonatal mouse cardiomyocyte culture

The investigation confirms with the 'Guide for the Care and Use of Laboratory Animals' published by the US National Institute of health (NIH Publication no. 85–23, revised 1996). Animal handling was approved by Animal Use Sub-committee at the University of Western Ontario (protocol no. 2002-115-12). The breeding pairs of C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). JNK1$^{-/-}$ mice were generously provided by Dr Richard Flavell, Yale University, CT, USA,[22] and crossed back to C57BL/6 background for more than 8 generations before experiments. Mice bearing the modified c-fos gene containing loxP sites and transgenic mice with cardiomyocyte-specific expression of Cre recombinase were generously provided by Dr Ming Xu (University of Cincinnati, OH, USA) and Dr E. Dale Abel (University of Utah, UT, USA), respectively.[16,23] The generation of mice with cardiomyocyte specific c-fos$^{-/-}$ was achieved by crossing the floxed c-fos mice with mice over-expressing Cre recombinase under the control of α-myosin heavy chain (Cre+) as we recently described.[24] Neonatal cardiomyocytes were prepared and cultured according to methods we previously described.[2]

2.2 Adenoviral infection of neonatal mouse cardiomyocytes

Cardiomyocytes were infected with adenoviral vectors containing a dominant negative mutant of JNK1 (Ad-dnJNK1, a gift from Dr Keith Webster, University of Miami School of Medicine), a dominant negative mutant of c-fos (Ad-Afos, a gift from Dr Charles Vinson, National Cancer Institute, Bethesda, MD, USA), c-fos (Ad-fos), Cre recombinase

(Ad-Cre, purchased from Vector Biolabs), or green fluorescence protein (Ad-GFP, a gift from Dr J. Lipp, Medical University of Vienna, Austria) as a control at a multiplicity of infection of 100 pfu/cell. Adenovirus-mediated gene transfer was implemented as previously described.[2,11] All experiments were performed after 24 h of adenoviral infection.

2.3 Measurement of TNF-α protein

TNF-α levels in the culture media and heart perfusates were determined using a mouse TNF-α ELISA Kit (ALPCO Diagnostics, USA) as our previous reports.[1,2,6] The values were expressed as pg/mL media or pg/50 μL perfusates.

2.4 Real-time RT–PCR

Total RNA was extracted from cardiomyocytes using the Trizol Reagent (Gibco-BRL) following the manufacturer's instructions. Real-time RT–PCR analysis for TNF-α and c-fos mRNA levels was performed using the same primers as previously described.[2,16]

2.5 Western blot analysis

c-fos protein and phosphorylation of ERK1/2, JNK1/2 and p38 were analysed by western blot using specific antibodies against c-fos, ERK1/2 and phospho-ERK1/2, p38 and phospho-p38, and JNK1/2 and phospho-JNK1/2 (Cell Signalling), respectively, as previously described.[2]

2.6 Reactive oxygen species

The formation of reactive oxygen species (ROS) was measured by using the ROS sensitive dye, 2,7-dichlorodihydro-fluorescein diacetate (DCF-DA), as an indicator. The assay was performed on freshly dissected heart tissues. Samples (50 μg proteins) were incubated with 10 μL of DCF-DA (10 μM) for 3 h at 37°C. The fluorescent product formed was quantified by spectrofluorometer at the 485/525 nm. Changes in fluorescence were expressed as an arbitrary unit.

2.7 Isolated mouse heart preparations

Adult mouse (male aged 2.5 months) hearts were isolated and perfused in a Langendorff system with Krebs–Henseleit buffer at 2 mL/min constant flow. The perfusion buffer was maintained at 37°C and bubbled continuously with a mixture of 95% O_2 and 5% CO_2. Myocardial function was assessed as we recently described.[6] Briefly, a 6-0 silk suture was placed through the apex of the left ventricle and threaded through a light-weight coupling rod, which was in direct continuity with a force–displacement transducer (FT03) to record tension and heart rate. The heart work was calculated by multiplying the force (g) and heart rate (beats/min). Maximal and minimal first derivative of force ($+\mathrm{d}F/\mathrm{d}t_{\mathrm{max}}$ and $-\mathrm{d}F/\mathrm{d}t_{\mathrm{min}}$) as the rate of contraction and relaxation were analysed by a PowerLab Chart program (ADInstruments, Mountain View, CA, USA).

2.8 *In vivo* LV pressure–volume measurements

Mice were anaesthetized with ketamine/xylazine cocktail and a Millar pressure conductance catheter (SPR-839, 1.4F) was inserted into the LV via the right carotid artery to measure LV pressures, volumes, and heart rate. Data were

recorded by a PowerLab Chart program. All haemodynamic parameters were analysed by a PVAN software (Millar Instruments, Houston, TX, USA) as we recently described.[25,26]

2.9 Statistical analysis

All data were given as mean ± SD or SEM from at least three independent experiments. Measurements for all *in vitro* experiments were made in duplicate or triplicate. Differences between the two groups were compared using the unpaired Student's *t*-test. For multigroup comparisons, ANOVA followed by the Newman–Keuls test was performed. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1 Enhanced TNF-α expression by LPS in JNK1$^{-/-}$ cardiomyocytes

Cultured cardiomyocytes express TNF-α in response to LPS.[2,6] We recently showed that LPS increases phosphorylation of JNK1/2 in cardiomyocytes.[11] To investigate whether LPS-stimulated TNF-α expression is modulated by JNK1, TNF-α mRNA and protein expression were measured in wild-type and JNK1$^{-/-}$ cardiomyocytes after LPS treatment. JNK1 deficiency did not have any effect on basal levels of TNF-α expression in cardiomyocytes. In response to LPS, TNF-α protein levels were significantly enhanced by 106% ($P < 0.05$) in JNK1$^{-/-}$ compared with wild-type cardiomyocytes (*Figure 1A*). The enhanced TNF-α protein levels were associated with elevations in TNF-α mRNA expression (*Figure 1B*). To further confirm the role of JNK1 in LPS-induced TNF-α expression, an adenoviral vector containing a dominant negative mutant of JNK1, Ad-dnJNK1, was employed. The inhibitory effect of Ad-dnJNK1 on JNK1 activation has been well characterized in cardiomyocytes by Andreka *et al.*[27] and our recent study.[11] Ad-dnJNK1 infection increased TNF-α production by 86% ($P < 0.05$, *Figure 1C*) and augmented mRNA expression by 67% (*Figure 1D*) in response to LPS in wild-type cardiomyocytes. These data support the notion that JNK1 activation suppresses LPS-induced TNF-α production in cardiomyocytes. However, treatment with cycloheximide, an inhibitor of protein synthesis, showed no difference in LPS-induced TNF-α mRNA levels between WT and JNK$^{-/-}$ cardiomyocytes (TNF-α/GAPDH mRNA ratio: 0.59 ± 0.26 vs. 0.60 ± 0.11, $n = 4$ independent experiments). The data indicate that the effect of JNK1 deficiency on LPS-induced TNF-α expression requires *de novo* protein synthesis.

3.2 Enhanced TNF-α expression induced by LPS in JNK1$^{-/-}$ hearts

To confirm JNK activation during LPS stimulation, wild-type mice were treated with LPS (4 mg/kg, i.p.) for 30 min and JNK1/2 phosphorylation was determined by western blot analysis. Data showed that JNK1/2 phosphorylation in the heart was significantly increased after LPS treatment ($P < 0.05$, *Figure 2A* and *B*). To demonstrate the role of JNK1 in LPS-induced TNF-α expression at the organ level, hearts isolated from JNK1$^{-/-}$ and wild-type mice were perfused with LPS (5 μg/mL) for 1 h in a Langendorff preparation. The perfusates were collected for TNF-α production. As shown in *Figure 2C*, TNF-α protein levels were significantly enhanced

by 114% in JNK1$^{-/-}$ compared with wild-type heart perfusates ($P < 0.05$). This indicates that JNK1 also exerts an inhibitory effect on LPS-induced TNF-α production in the adult hearts.

3.3 Myocardial TNF-α expression and cardiac dysfunction in endotoxaemia

To further study the effect of myocardial TNF-α expression on cardiac function, JNK1$^{-/-}$ and wild-type mice were treated with LPS (4 mg/kg, i.p.) for 2 h. Both *in vivo* cardiac function and myocardial TNF-α protein levels were determined in the same mice using a Millar pressure-conductance catheter and by ELISA, respectively. Data showed that myocardial TNF-α protein levels were increased, whereas LV dP/dt and ESPVR were decreased in JNK1$^{-/-}$ compared with wild-type mice (*Table 1, Figure 3A*–C, $P < 0.05$). The results suggest that increased myocardial TNF-α expression contributes to cardiac dysfunction in JNK1$^{-/-}$ mice during endotoxaemia.

3.4 Role of JNK1 in ROS production during endotoxaemia

To investigate if JNK1$^{-/-}$ also enhances ROS production in the heart, JNK1$^{-/-}$ and wild-type mice were treated with LPS (4 mg/kg, i.p.) for 2 h. ROS production was determined in myocardial tissues. Deficiency of JNK1 did not affect basal ROS levels in the heart. In response to LPS, ROS production was significantly enhanced by 138% in JNK1$^{-/-}$ compared with wild-type hearts (*Figure 3D*). These data suggest an inhibitory role of JNK1 in ROS generation during endotoxaemia.
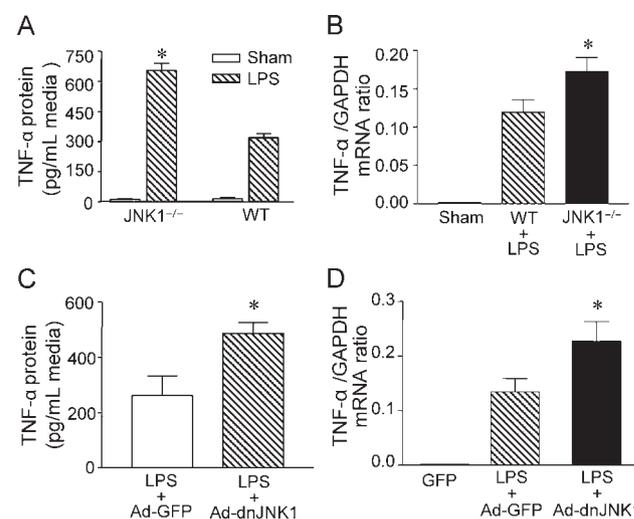

Figure 1 Lipopolysaccharide-induced tumour necrosis factor-alpha expression in JNK1$^{-/-}$ and JNK1 knockdown cardiomyocytes. (*A* and *B*) Lipopolysaccharide-induced tumour necrosis factor-alpha expression wild-type (WT) and JNK1$^{-/-}$ cardiomyocytes. After 72 h of culture, cardiomyocytes were treated with lipopolysaccharide (10 μg/mL) or vehicle (sham) for 4 h. Tumour necrosis factor-alpha protein (*A*) and mRNA (*B*) were determined by ELISA and real-time RT–PCR, respectively. (*C* and *D*) Effects of Ad-dnJNK1 on lipopolysaccharide-induced tumour necrosis factor-alpha expression. After 48 h of culture, cardiomyocytes were infected with Ad-dnJNK1 or Ad-GFP for 24 h, followed by lipopolysaccharide (10 μg/mL) treatment for 4 h. Ad-dnJNK1 infection increased tumour necrosis factor-alpha protein (*C*) and mRNA (*D*) levels in WT cardiomyocytes. Data are mean ± SD from three to four independent experiments. *$P < 0.05$ vs. WT or corresponding controls.

3.5 Role of JNK1 in myocardial dysfunction during endotoxaemia

To study the role of JNK1 in myocardial depression induced by endotoxaemia, JNK1$^{-/-}$ and wild-type mice were
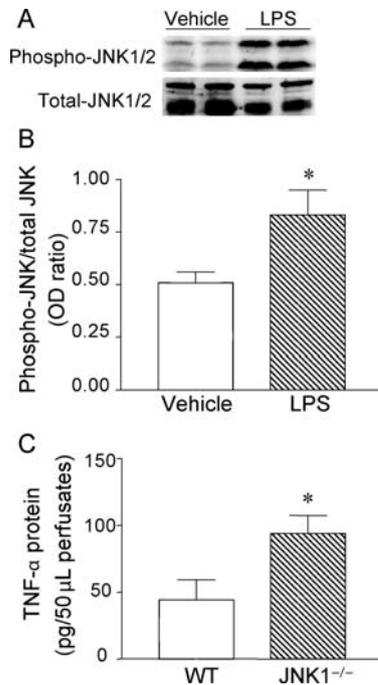

Figure 2 c-Jun NH$_2$-terminal kinase activation and tumour necrosis factor-alpha expression in the heart during lipopolysaccharide stimulation. Wild-type (WT) mice were treated with lipopolysaccharide (4 mg/kg, i.p.) for 30 min. Hearts were isolated and western blot analysis was performed to detect JNK1/2 phosphorylation. A representative blot (*A*) and quantitative data (*B*) show significant JNK1/2 phosphorylation in the heart after lipopolysaccharide treatment *in vivo*. (*C*) Hearts isolated from WT and JNK1$^{-/-}$ mice were perfused with lipopolysaccharide (5 μg/mL) for 1 h. The perfusates were collected for tumour necrosis factor-alpha protein measurement. Data are mean ± SD, *n*=5–6 per group. *$P < 0.05$ vs. vehicle or WT.

treated with LPS (4 mg/kg, i.p.) for 2 h. Mice were then sacrificed and cardiac function was assessed in a Langendorff preparation as our recent report.[6] Heart work and rate of contraction were significantly reduced in endotoxaemic mice compared with sham animals ($P < 0.05$, *Figure 4*). Decreases in heart work and rate of contraction during endotoxaemia were significantly pronounced in JNK1$^{-/-}$ compared with wild-type mice ($P < 0.05$, *Figure 4*). These results suggest that JNK1 activation improves myocardial function during endotoxaemia in mice.

3.6 JNK1 signalling induces c-fos expression in LPS-stimulated cardiomyocytes

LPS treatment has been shown to induce c-fos expression in the myocardium.[21] In the present study, LPS (10 μg/mL) increased c-fos expression in cardiomyocytes (*Figure 5A* and *B*). Up-regulation of c-fos mRNA and protein expression peaked around 0.5 and 4 h, respectively, in parallel with an increase in AP-1 activity (data not shown). Based on this time course, 0.5 and 4 h were chosen for subsequent c-fos mRNA and protein experiments, respectively. To investigate if JNK1 plays a role in c-fos expression in LPS-stimulated cardiomyocytes, Ad-dnJNK1 was employed to block JNK1 signalling. Ad-dnJNK1 infection inhibited LPS-induced c-fos mRNA and protein expression in cardiomyocytes (*Figure 5C* and *D*), indicating that JNK1 signalling is required for LPS-induced c-fos expression. This result was confirmed by a pharmacological inhibitor of JNK, SP600125, and using JNK1$^{-/-}$ cardiomyocytes. Incubation with SP600125 blocked c-fos expression (*Figure 5C* and *D*) and deficiency of JNK1 abrogated c-fos mRNA expression (data not shown) in LPS-stimulated cardiomyocytes.

3.7 Role of JNK1/c-fos on LPS-induced TNF-α expression

To investigate if JNK1 activation suppresses LPS-induced TNF-α expression through c-fos induction, an *in vitro* study was performed on c-fos$^{-/-}$ cardiomyocytes using a Cre-loxP approach. Cultured cardiomyocytes from

Table 1 *In vivo* hemodynamic measurements in wild-type and JNK1$^{-/-}$ mice

Parameters	Wild-type	JNK1$^{-/-}$	*P*-value
n	5	6	
Heart rate, b.p.m.	378 ± 16	383 ± 22	0.853
Mean arterial pressure, mmHg	61 ± 5	57 ± 4	0.568
LV systolic pressure, mmHg	94 ± 2	82 ± 3	0.012*
LV diastolic volume, μL	23.1 ± 1.5	25.6 ± 6.2	0.717
LV systolic volume, μL	13.8 ± 1.4	14.7 ± 3.9	0.827
Stroke volume, μL	9.3 ± 1.1	10.8 ± 3.1	0.665
Cardiac output, μL/min	3586 ± 573	3943 ± 958	0.759
Ejection fraction, %	40.5 ± 4.6	43.1 ± 6.5	0.752
LV + dP/dt_{max}, mmHg/s	5339 ± 437	3498 ± 134	0.011*
LV − dP/dt_{min}, mmHg/s	5687 ± 217	3525 ± 207	0.001*
τ, ms	9.0 ± 0.5	12.1 ± 1.1	0.033*
ESPVR (mmHg/μL)	3.9 ± 0.9	1.0 ± 0.6	0.029*
EDPVR (mmHg/μL)	0.2 ± 0.5	0.8 ± 0.7	0.548

Mice were treated with LPS (4 mg/kg, i.p.) for 2 h. Cardiac function was determined using a Millar pressure-conductance catheter. LV, left ventricle; τ, time constant of isovolumic LV pressure fall in milliseconds (ms); ventricular pressure ESPVR, end systolic pressure volume relationship; EDPVR, end diastolic pressure volume relationship. Data are mean ± SEM. *Statistically significant.

homozygous c-fos floxed mice were infected with Ad-Cre or Ad-GFP for 24 h and then incubated with LPS for 4 h. In response to LPS, TNF-α protein and mRNA were increased by 35 and 65% in c-fos$^{-/-}$ compared with Ad-GFP-infected cardiomyocytes, respectively ($P < 0.05$, *Figure 6A* and *B*). However, Ad-Cre infection did not significantly change TNF-α expression in wild-type cardiomyocytes compared with Ad-GFP (data not shown). In a separate experiment, WT cardiomyocytes were infected with Ad-Fos or Ad-GFP for 24 h, followed by incubation with LPS for 4 h. In response to LPS, TNF-α mRNA were decreased by 41% in Ad-Fos compared with Ad-GFP-infected cardiomyocytes ($P < 0.05$, *Figure 6C*). Thus, the results strongly support that c-fos suppresses TNF-α expression in LPS-stimulated cardiomyocytes.

To study the relationship between JNK1 and c-fos following LPS stimulation, c-fos$^{-/-}$ cardiomyocytes were treated with Ad-dnJNK1. *Figure 6B* shows that blocking JNK1 by Ad-dnJNK1 had no effect on TNF-α expression in c-fos$^{-/-}$

cardiomyocytes, suggesting JNK1 is upstream of c-fos in inhibiting LPS-induced TNF-α expression.

To test if AP-1 formation is involved in the action of c-fos on TNF-α expression, cardiomyocytes were infected with Ad-Afos, an adenoviral vector expressing a dominant negative mutant c-fos which blocks the formation of c-fos/c-jun AP-1 complex.[28] Over-expression of the c-fos mutant did not alter TNF-α expression (*Figure 6D*). These data suggest that AP-1 is not involved in LPS-induced TNF-α expression. This result was further confirmed by using decoy AP-1 oligodeoxynucleotide. Pre-incubation with decoy AP-1 oligodeoxynucleotide inhibited LPS-induced AP-1 DNA binding activity (data not shown) but had no effect on TNF-α protein expression in LPS-stimulated cardiomyocytes (*Figure 6E*). Thus, the inhibitory role of c-fos in TNF-α expression is independent of the AP-1 pathway.

To demonstrate the role of c-fos in LPS-induced TNF-α expression at the organ level, we generated mice with cardiomyocyte-specific c-fos$^{-/-}$ mice as described in our recent study.[24] Hearts were isolated from c-fos$^{-/-}$ and wild-type littermates and perfused with LPS for 1 h in a Langendorff system. There was no significant difference in TNF-α production in the perfusates between wild-type, c-fos floxed, and Cre+ hearts. Consistent with the results from JNK1$^{-/-}$ hearts, TNF-α protein levels in the perfusates were significantly enhanced by 104% in c-fos$^{-/-}$ compared with wild-type hearts ($P < 0.05$, *Figure 6F*). These data indicate that c-fos exerts an inhibitory effect on TNF-α production induced by LPS in the adult hearts. Thus, our study suggests that JNK1 suppresses LPS-induced cardiac TNF-α expression through c-fos induction.
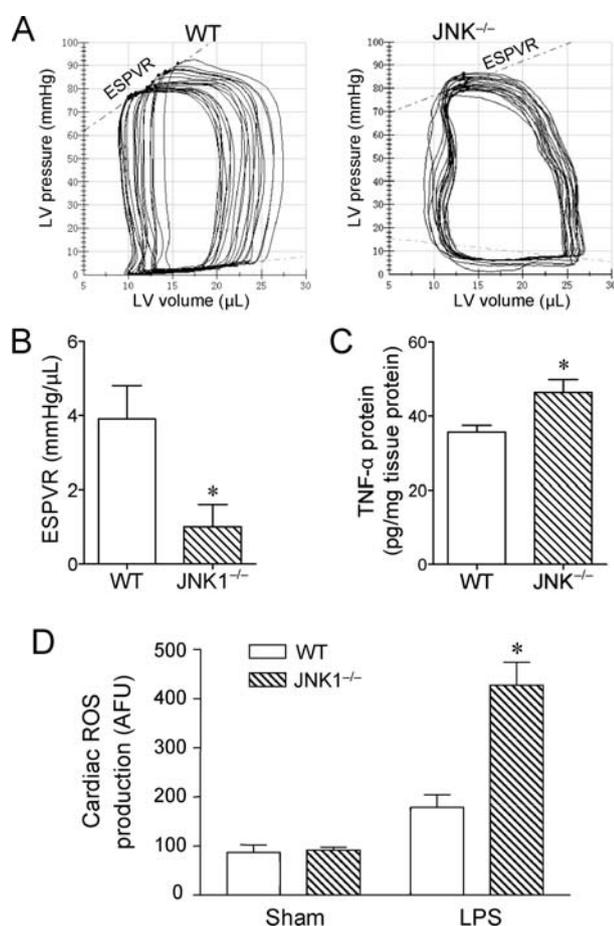

Figure 3 *In vivo* cardiac function, myocardial tumour necrosis factor-alpha, and reactive oxygen species (ROS) production during endotoxaemia. Wild-type (WT) and JNK1$^{-/-}$ mice were treated with lipopolysaccharide (4 mg/kg, i.p.) for 2 h. Cardiac function was determined using a Millar pressure-conductance catheter. (*A*) Representative tracings of LV pressure–volume loops in WT and JNK1$^{-/-}$ mice. (*B*) End systolic pressure volume relationship (ESPVR) was significantly decreased in JNK1$^{-/-}$ ($n=6$) compared with WT mice ($n=5$, *$P < 0.05$). (*C*) Myocardial tumour necrosis factor-alpha protein levels were determined from the same mice as in (*B*) (*$P < 0.05$ vs. WT). Data are mean $\pm$ SEM for (*B* and *C*). (*D*) Myocardial ROS production after 2 h of lipopolysaccharide treatment (4 mg/kg, i.p.) in separate groups of mice. Data are mean $\pm$ SD, $n=3$–5 per group. *$P < 0.05$ vs. WT. AFU, arbitrary fluorescence units.
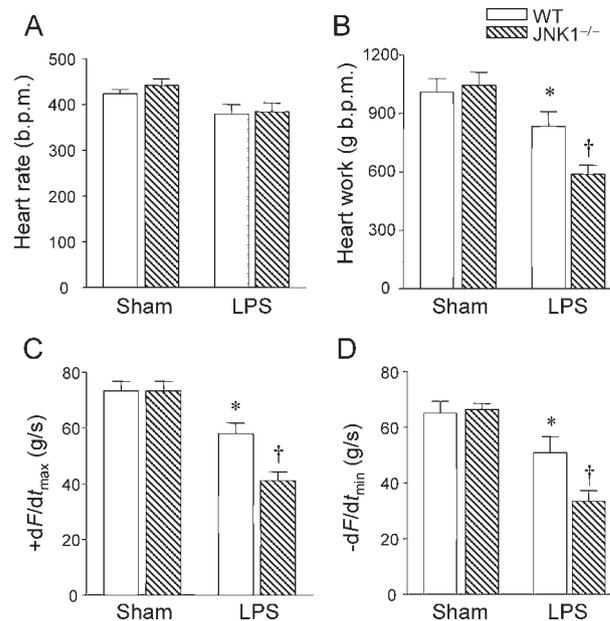

Figure 4 Cardiac function in JNK1$^{-/-}$ and wild-type (WT) mice with endotoxaemia. JNK1$^{-/-}$ and WT mice were treated with lipopolysaccharide (4 mg/kg, i.p.) for 2 h. Hearts were then isolated and perfused in a Langendorff system. Contractile function of the heart was determined. Changes in heart rate (*A*), heart work (*B*), rate of contraction (+dF/dt_{max}) (*C*), and relaxation (−dF/dt_{min}) (*D*) are presented. Data are mean $\pm$ SEM, $n=6$-9 per group. *$P < 0.05$ vs. Sham in WT; †$P < 0.05$ vs. lipopolysaccharide in WT. b.m.p., beats/min.

3.8 c-fos deficiency and myocardial dysfunction during endotoxaemia

To study the role of c-fos in myocardial depression induced by endotoxaemia, c-fos$^{-/-}$ and corresponding littermates including wild-type, c-fos floxed, and Cre+ mice were treated with LPS (4 mg/kg, i.p.) for 2 h. Cardiac function was assessed in a Langendorff preparation. Under basal conditions, HR, heart work, and rate of contraction were not significantly different between c-fos$^{-/-}$ and relevant wild-type littermates including wild-type, c-fos floxed, and

Cre+ mice. In response to LPS, changes in cardiac function were similar between wild-type, c-fos floxed, and Cre+ mice (data not shown). Therefore, data from c-fos floxed mice were chosen as littermate controls for c-fos$^{-/-}$ mice in the present study. Heart work and rate of contraction were significantly decreased in endotoxaemic mice compared with sham animals ($P < 0.05$, *Figure 7*). Decreases in heart work and rate of contraction during endotoxaemia were significantly pronounced in c-fos$^{-/-}$ compared with wild-type mice ($P < 0.05$, *Figure 7*). These results demonstrated that c-fos deficiency decreases myocardial function during endotoxaemia.

3.9 JNK1/c-fos signalling inhibits ERK1/2 and p38 activation during LPS stimulation

We have previously shown that LPS activates ERK1/2 and p38 in cardiomyocytes and that both ERK1/2 and p38 are required for LPS-induced TNF-α expression in cardiomyocytes.[1,2,6] We therefore examined the role of JNK1/c-fos signalling in ERK1/2 and p38 activation in LPS-stimulated
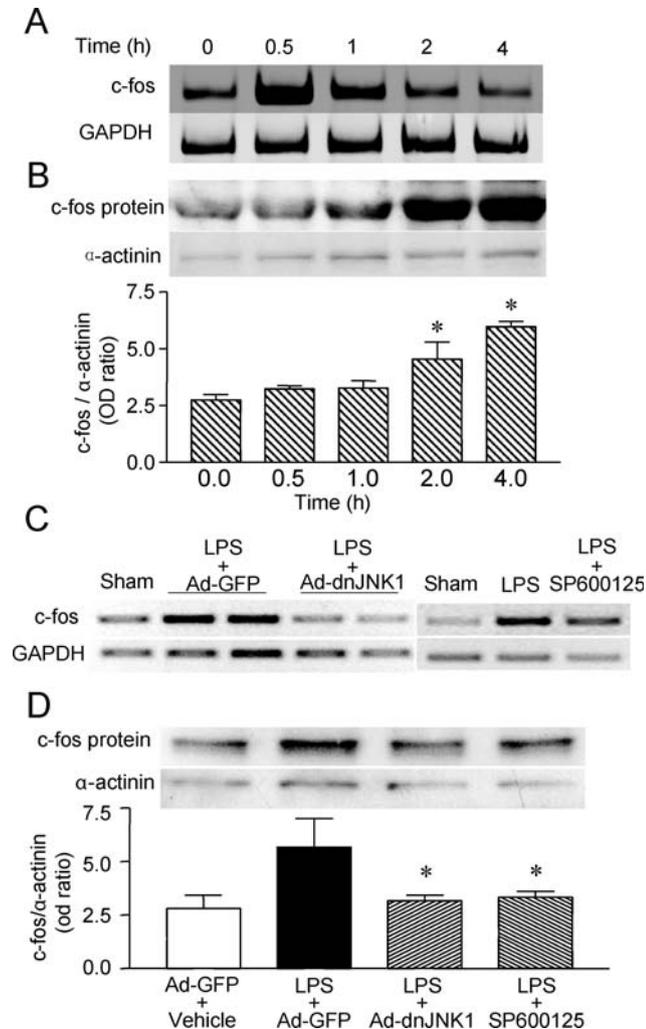

Figure 5 Effects of JNK1 inhibition on lipopolysaccharide-induced c-fos expression in cardiomyocytes. (*A* and *B*) Time course of lipopolysaccharide-induced c-fos expression in cardiomyocytes. Neonatal cardiomyocytes were incubated with lipopolysaccharide (10 μg/mL) for 0.5, 1, 2, and 4 h. (*A*) Representative RT–PCR amplification for c-fos and GAPDH mRNA expression. (*B*) Upper panel shows a representative western blot of c-fos protein and lower panel its quantification. (*C*) Representative gels of c-fos and GAPDH amplification by RT–PCR. Cultured cardiomyocytes were infected with Ad-dnJNK1 or Ad-GFP for 24 h, followed by lipopolysaccharide treatment (10 μg/mL) for 0.5 h. In addition, cardiomyocytes were treated with lipopolysaccharide in the presence or absence of SP600125 (10 μM), a c-Jun NH$_2$-terminal kinase inhibitor for 0.5 h. (*D*) Cardiomyocytes were infected with Ad-dnJNK1 or Ad-GFP for 24 h, followed by lipopolysaccharide or vehicle in the presence or absence of SP600125 (10 μM) for 4 h. Upper panel shows a representative blot of c-fos protein and lower panel its quantification. Data are mean ± SD, n=3–4 per group. Blots and gels are representatives from three to four independent experiments. *$P < 0.05$ vs. time 0 or lipopolysaccharide + Ad-GFP.
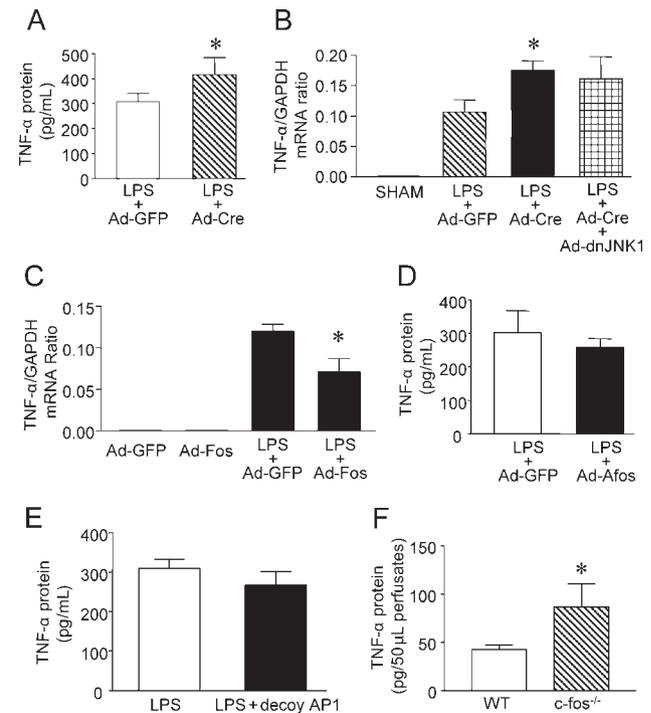

Figure 6 Role of c-fos in lipopolysaccharide-induced tumour necrosis factor-alpha expression in cardiomyocytes and isolated hearts. For experiments using adenovirus, cardiomyocytes were infected with adenoviral vectors for 24 h followed by 4 h of lipopolysaccharide treatment (10 μg/mL). (*A* and *B*) Effects of Ad-Cre, Ad-GFP, or Ad-Cre plus Ad-dnJNK1 on tumour necrosis factor-alpha protein (*A*) and mRNA (*B*) in c-fos floxed cardiomyocytes. *$P < 0.05$ vs. lipopolysaccharide + Ad-GFP. (*C*) Effects of Ad-Fos or Ad-GFP on tumour necrosis factor-alpha mRNA in WT cardiomyocytes. *$P < 0.05$ vs. lipopolysaccharide + Ad-GFP. (*D*) Effects of Ad-Afos or Ad-GFP on tumour necrosis factor-alpha protein in the culture medium of WT cardiomyocytes. (*E*) Cardiomyocytes were incubated with decoy AP-1 oligodeoxynucleotide (5 μM) for 1 h and then with lipopolysaccharide for 4 h. Tumour necrosis factor-alpha protein in the culture medium was quantified. (*F*) Hearts isolated from WT and c-fos$^{-/-}$ mice were perfused with lipopolysaccharide (5 μg/mL) for 1 h. The perfusates were collected and tumour necrosis factor-alpha protein was determined. *$P < 0.05$ vs. WT. For (*A–F*), tumour necrosis factor-alpha protein and mRNA were determined by real-time PCR and ELISA, respectively. Data are mean ± SD from three independent experiments (*A–E*) or n = 5 per group (*F*).
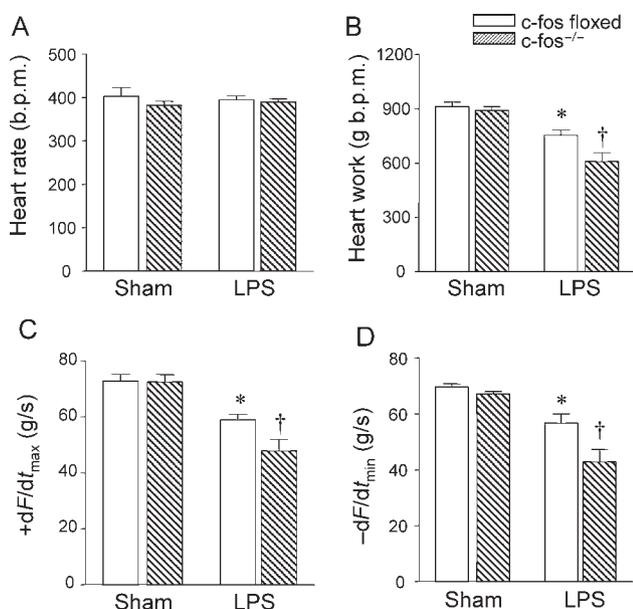
Figure 7 Cardiac function in c-fos$^{-/-}$ and c-fos floxed (littermate) mice with endotoxaemia. Mice were treated with lipopolysaccharide (4 mg/kg, i.p.). Two hours later, hearts were isolated and perfused in a Langendorff system. Contractile function of the heart was determined. Changes in heart rate (A), heart work (B), rate of contraction ($+dF/dt_{max}$) (C) and relaxation ($-dF/dt_{min}$) (D) are presented. Data are mean $\pm$ SEM, $n = 6$–9 per group, *$P < 0.05$ vs. Sham in c-fos floxed; $^{\dagger}P < 0.05$ vs. lipopolysaccharide in c-fos floxed. b.p.m., beats per min.

cardiomyocytes. Cultured cardiomyocytes from wild-type and JNK1$^{-/-}$ mice were incubated with LPS for 30 min. Phosphorylation of ERK1/2 and p38 were determined by western blot analysis. As shown in *Figure 8A* and *B*, deficiency of JNK1 enhanced ERK1/2 and p38 phosphorylation by 27 and 45%, respectively. The data indicate that JNK1 activation decreases ERK1/2 and p38 activation in LPS-stimulated cardiomyocytes. To elucidate the role of c-fos induction in ERK1/2 and p38 activation, cultured cardiomyocytes from homozygous c-fos floxed mice were infected with Ad-Cre or Ad-GFP for 24 h and then incubated with LPS for 30 min. Similar to JNK1$^{-/-}$ cardiomyocytes, deficiency of c-fos enhanced ERK1/2 and p38 phosphorylation by 46 and 21% (*Figure 8C* and *D*), respectively. Likewise, over-expression of c-fos attenuated ERK1/2 and p38 phosphorylation by 34 and 47%, respectively, after LPS treatment in cardiomyocytes (*Figure 9*). Thus, our data indicated that JNK1/c-fos signalling inhibits ERK1/2 and p38 activation in response to LPS.

4. Discussion

TNF-α produced by cardiomyocytes plays an important role in myocardial depression during endotoxaemia.[1,3] Activation of TNF-α expression is controlled by multiple mechanisms, involving a balance between positive and negative signalling pathways. In recent years, much attention has been paid to signal transduction pathways that positively regulate TNF-α expression in LPS-stimulated cardiomyocytes.[1,2,5] However, little is known about the negative signalling on TNF-α expression in sepsis. The present study provided definitive evidence that JNK1 activation and c-fos induction decreased LPS-induced TNF-α expression in cardiomyocytes. JNK1 activation induced c-fos expression during LPS stimulation. The
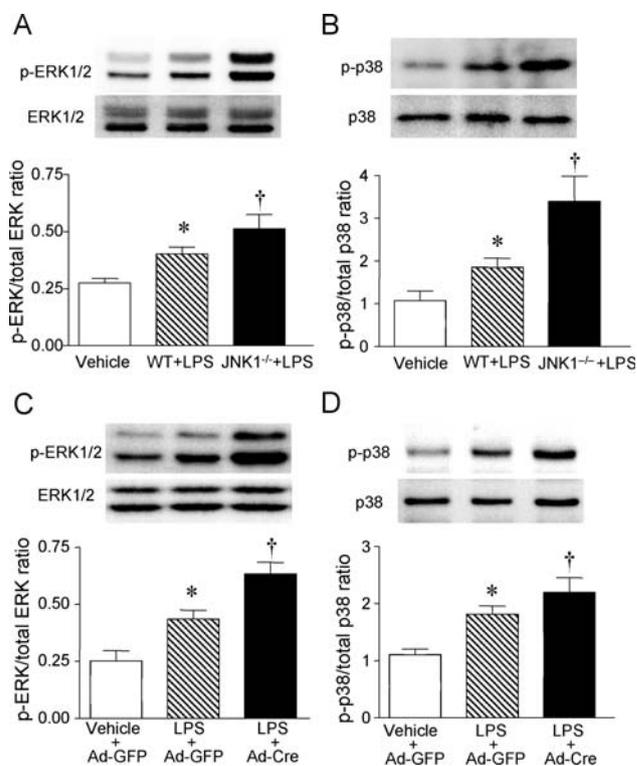

Figure 8 Deficiency in JNK1 or c-fos on lipopolysaccharide-induced ERK1/2 and p38 phosphorylation in cardiomyocytes. (A and B) Neonatal cardiomyocytes from wild-type (WT) and JNK1$^{-/-}$ mice were treated with lipopolysaccharide (10 μg/mL) or vehicle for 0.5 h. Phosphorylation of ERK1/2 (A) and p38 (B) was assessed by western blot analysis. (C and D) Neonatal cardiomyocytes from c-fos floxed mice were infected with Ad-Cre or Ad-GFP for 24 h, followed by lipopolysaccharide or vehicle treatment for 0.5 h. Phosphorylation of ERK1/2 (C) and p38 (D) were assessed by western blotting. Upper panel shows representative western blots for ERK1/2 (A and C) and p38 (B and D) phosphorylation. Lower panel is the quantification of ERK1/2 (A and C) and p38 (B and D) phosphorylation. Data are mean $\pm$ SD, $n = 4$ per group. *$P < 0.05$ vs. vehicle or vehicle + Ad-GFP, $^{\dagger}P < 0.05$ vs. WT + lipopolysaccharide or lipopolysaccharide + Ad-GFP.
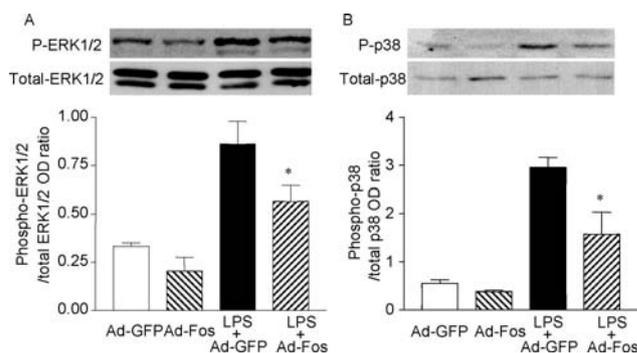

Figure 9 Over-expression of c-fos on lipopolysaccharide-induced ERK1/2 and p38 phosphorylation in cardiomyocytes. Neonatal cardiomyocytes from wild-type mice were infected with Ad-Fos or Ad-GFP for 24 h, followed by incubation with lipopolysaccharide (10 μg/mL) or vehicle for 0.5 h. Phosphorylation of ERK1/2 (A) and p38 (B) was assessed by western blot analysis. Upper panel shows representative western blots for ERK1/2 (A) and p38 (B) phosphorylation. Lower panel is the quantification of ERK1/2 (A) and p38 (B) phosphorylation. Data are mean $\pm$ SD, $n = 3$ per group. *$P < 0.05$ vs. lipopolysaccharide + Ad-GFP.

effect of JNK1/c-fos signalling was mediated, at least in part, by inhibition of ERK1/2 and p38 pathway. Furthermore, deficiency of either JNK1 or c-fos decreased myocardial function in endotoxaemic mice. To our knowledge, this is

the first demonstration that the JNK1/c-fos pathway inhibits LPS-induced TNF-α expression in cardiomyocytes and improves myocardial function during endotoxaemia. Thus, JNK1/c-fos signalling represents a novel mechanism that limits TNF-α induction in LPS-stimulated cardiomyocytes and improves myocardial function in endotoxaemia.

JNK is activated in both cultured cardiomyocytes and hearts under a variety of cellular stress, e.g. stretch, cytokines, ischaemia/ reperfusion, and G protein-coupled receptor activation.[29] Activation of JNK signal transduction cascades has been implicated in the regulation of hypertrophic and apoptotic responses in the myocardium.[30] However, the role of JNK has not been demonstrated in the heart during sepsis. We recently showed that LPS increases JNK activation in cultured cardiomyocytes.[11] This is also confirmed in the heart in the present study. Furthermore, we showed that JNK1 signalling inhibits LPS-induced TNF-α expression in cardiomyocytes. In addition to the up-regulation of TNF-α expression, we also demonstrated that deficiency of JNK1 enhanced ROS production in the heart during endotoxaemia. The increased ROS production may result from TNF-α up-regulation since TNF-α has been shown to induce ROS production.[31] Finally, our data showed that LPS-induced myocardial function was further decreased in JNK1$^{-/-}$ mice. Mechanisms by which deficiency of JNK1 enhanced myocardial dysfunction are not fully understood. However, data from the present study suggest that deficiency of JNK1 enhanced LPS-stimulated TNF-α expression, which may account for the increased ROS production in the heart. Thus, it is possible that ROS production may contribute to myocardial dysfunction during endotoxaemia.[1,6]

LPS has been shown to induce c-fos in the heart.[21] However, the role of c-fos in cardiomyocyte TNF-α expression and myocardial dysfunction in sepsis has not been demonstrated. The present study showed that LPS increased c-fos expression and deficiency of c-fos augmented LPS-induced TNF-α expression in cardiomyocytes and isolated hearts. Furthermore, direct evidence from over-expression of c-fos using Ad-Fos decreased LPS-induced TNF-α expression in cardiomyocytes. It is well known that c-fos dimerizes with c-jun to form AP-1 complex, which induces target gene expression by binding the AP-1 consensus site(s) in the promoter region. However, our data showed that LPS-induced cardiomyocyte TNF-α expression is independent of AP-1 activation since inhibition of AP-1 activation by either decoy AP-1 oligodeoxynucleotide or over-expression of a dominant negative mutant of c-fos had no effect on TNF-α expression. Furthermore, deficiency of c-fos further decreased myocardial function in endotoxaemic mice. These results demonstrated that c-fos induction (independent of AP-1) inhibits cardiomyocyte TNF-α expression and prevents further decrease in cardiac function during endotoxaemia. Our study suggests that c-fos acts as a signalling molecule but not a transcriptional factor against inflammatory response in sepsis.

In the present study, LPS not only activated JNK but also induced c-fos in cardiomyocytes. Inhibition of JNK1 signalling blocked LPS-induced c-fos expression in cardiomyocytes. In addition, inhibition of JNK1 had no effect on TNF-α expression in c-fos$^{-/-}$ cardiomyocytes during LPS stimulation. Thus, our results suggest that LPS activates JNK1, which in turn induces c-fos expression, leading to

the inhibition of TNF-α expression and improvement of myocardial function in endotoxaemia.

Crosstalk between various MAPK cascades (e.g. ERK1/2, JNK, and p38) has recently been recognized to play an important role in the regulation of MAPK signalling.[32,33] Although a negative crosstalk between JNK and ERK1/2 has been shown in COS-7 cells in response to activation of mixed lineage kinases,[33] it remains unknown if such crosstalk exists in cardiomyocytes during endotoxaemia. In addition, signalling molecules that mediate the crosstalk between JNK and ERK1/2, p38 MAPK are not clear. Our data showed that deficiency of JNK1 enhanced phosphorylation of ERK1/2 and p38 in LPS-stimulated cardiomyocytes, suggesting a negative crosstalk of JNK1 to ERK1/2 and p38 signalling pathways. More importantly, the present study demonstrated that this negative crosstalk is mediated by c-fos. This conclusion is supported by the following results. First, JNK1 signalling induced c-fos expression. Secondly, deficiency of c-fos enhanced LPS-stimulated phosphorylation of ERK1/2 and p38. Finally, over-expression of c-fos decreased ERK1/2 and p38 phosphorylation in LPS-stimulated cardiomyocytes. As ERK1/2 and p38 are required for LPS-induced TNF-α expression, our results suggest JNK1/c-fos signalling leads to a decreased cardiomyocyte TNF-α expression induced by LPS. It is not clear how c-fos inhibits ERK1/2 and p38 activation in cardiomyocytes. Phospholipase C (PLC) has been demonstrated to regulate ERK1/2 phosphorylation via Ca^{2+} and/or inositol triphosphate/protein kinase C pathways.[34] Recent studies have shown that c-fos modulates phospholipid synthesis and phospholipase activity.[35,36] Whether such mechanism is operating during LPS stimulation merits further investigation.

In summary, the present study has demonstrated a novel signalling pathway by which JNK1 suppresses TNF-α expression in LPS-stimulated cardiomyocytes. Inhibition of TNF-α expression by JNK1 signalling is mediated by c-fos induction and down-regulation of ERK1/2 and p38 phosphorylation. Activation of the JNK1/c-fos pathway improves myocardial function during endotoxaemia. This negative crosstalk between JNK1 and ERK1/2, p38 MAPK signalling mediated by c-fos is beneficial and may have therapeutic implications in sepsis.

Conflict of interest: none declared.

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