c-Fos suppresses systemic inflammatory response to endotoxin

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Abstract

We explored the role of the transcription factor c-Fos in lipopolysaccharide (LPS)-induced cytokine response using mice lacking c-Fos (Fos−/− mice). Compared with wild-type controls, Fos−/− macrophages and mice showed significantly enhanced production of tumour necrosis factor (TNF-α), interleukin (IL)-6 and IL-12 p40, but reduced production of the anti-inflammatory cytokine IL-10. Bandshift analysis revealed that LPS-induced NF-κB binding activity to a functional site in the TNF-α promoter was significantly higher in Fos−/− than in wild-type macrophages. Using telemetry, we monitored body temperature and heart rate after LPS injection and found that Fos−/− mice undergo more severe hypothermia and bradycardia than wild-type mice. Such shock responses in Fos−/− mice were significantly reversed by neutralizing TNF-α. These data reveal a novel in vivo role for c-Fos as an anti-inflammatory transcription factor acting through suppression of NF-κB activity.

Introduction

Septic shock is a complex clinical syndrome that results from a deleterious host response to infection (1). The profound pro-inflammatory response that occurs in sepsis is manifested by changes in body temperature (BT), heart rate (HR) and lung function, and in severe cases by organ dysfunction and death. In mice, exposure to LPS, or endotoxin, an outer cell-wall component of Gram-negative bacteria, has been used as a model for endotoxemia (2–4), although involvement of lipopolysaccharide (LPS) per se in human sepsis is controversial (5). Toll-like receptor (TLR) 4 recognizes LPS with the help of LPS-binding protein, CD14 and MD-2 (6, 7). Signalling downstream of TLR4 reaches the nucleus and activates, in particular, the two dimeric transcription factors NF-κB and AP-1 (activator protein-1), causing production of pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-12 and tumour necrosis factor (TNF)-α as well as the anti-inflammatory mediator IL-10 (6, 8).

c-Fos belongs to the Fos family of proteins that, together with the Jun family, composes AP-1 (9, 10). Fos and Jun family proteins all contain the highly conserved ‘basic leucine zipper’ structure. The basic region is responsible for DNA binding, and the leucine zipper mediates dimerization. c-Fos is induced by a variety of stimuli in diverse cell types including keratinocytes and neurons. The most prominent phenotype of Fos−/− mice is osteopetrosis due to failure of osteoclast differentiation (11–13). In contrast, c-Fos is not essential for differentiation of macrophages, which are derived from the same myeloid precursors as osteoclasts (12, 14).

While AP-1 generally acts as an activator of pro-inflammatory genes (15–17), AP-1 containing c-Fos suppresses production of both IL-12 (18–22) and inducible NO synthase (23) in macrophages and dendritic cells. Here, we provide experimental evidence that endotoxin shock is more severe in Fos−/− mice mainly due to enhanced production of TNF-α. Furthermore, we demonstrate that NF-κB activity is elevated in these mice. We propose that c-Fos acts as anti-inflammatory transcription factor in vivo.

Methods

Mice

Fos−/− mice of 129 × C57BL/6 mixed-background (11) were bred and maintained under specific pathogen-free conditions.

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Powder diet was provided to *Fos*⁻/⁻ mice. Wild-type littermates were used as controls. All experiments were conducted in accordance with institutional review board-approved protocols.

**Peritoneal macrophages**

Peritoneal cells were harvested from mice by flushing peritoneum with DMEM containing 10% FBS and penicillin-streptomycin. The cell suspension was passed through a 70-μm cell strainer (Falcon) to remove debris. Cells were plated at a density of 1 × 10⁶ cells per well in 24-well tissue culture plates (Falcon). Cultures were kept overnight, washed with PBS to remove non-adherent cells and fresh medium was added. These adherent cells were used as peritoneal macrophages (PMs).

**M-CSF-dependent macrophages**

Bone marrow from wild-type tibiae and femora or splenocytes from *Fos*⁻/⁻ spleens were harvested by flushing with DMEM containing 10% FBS and penicillin-streptomycin. After passage through a 70-μm cell strainer, cells were cultured overnight in 10-cm tissue culture dishes (Falcon). Floating cells were then collected and expanded in the presence of 5 ng ml⁻¹ M-CSF (R&D), for up to 6 days. Cultures were washed with PBS, and the adherent cells were re-plated at a density of 1 × 10⁵ cells per well in 48-well tissue culture plates (Falcon). These cells were used as M-CSF-dependent macrophages (MDMs).

**Stimulation with bacterial components**

PMs or MDMs were stimulated with 100 ng ml⁻¹ or 1 μg ml⁻¹ LPS from *Salmonella enterica* serovar Typhimurium (S. Minnesota Re595, Sigma), 4 μg ml⁻¹ peptidoglycan from *Staphylococcus aureus* (Fluka), 0.1 nM flagellin from *Salmonella muenchen* (Calbiochem), 100 nM phosphorothioate CpG (5'-TCCATgACg-TTCTgATgCT'-3') or control GpG (5'-TCCATgAgCTTCT-gATgCT'-3') oligonucleotides (Proligo) in medium. Where specified, PMs were serum-starved in DMEM without FCS for 2 h and then LPS was added to the starvation medium. Mice were injected i.p. with 0.1, 1, or 4 mg kg⁻¹ of LPS in PBS as indicated.

**RT-PCR and ELISA analysis**

For RT-PCR, PMs or spleens were collected and homogenized in 1 ml isogen reagent (Nippon Gene), and total RNA was purified. cDNA was synthesized using the Enhanced HS RT-PCR kit (Sigma-Aldrich). Quantitative PCR was performed on an ABI PRISM 7000 using TaqMan Assay-on-Demand kits for IL-1β, IL-6, IL-10, IL-12 p40, TNF-α, and GAPDH (Applied Biosystems). Macrophage culture supernatants and mouse serum were collected, and protein levels of the above cytokines measured using ELISA sets (BD Pharmingen).

**Electrophoretic mobility shift assay (EMSA)**

To assess NF-κB activation, EMSA was performed as described previously (24). Briefly, 3 μg nuclear extracts prepared from untreated or LPS-treated MDMs were incubated with [³²P]-end-labelled 27-mer double-stranded κB3 oligonucleotide, containing the κB3 binding site of mouse TNF-α promoter (5'-AGCTGAGGAGGAAGCCCCCTGTTG-3') (25), octamer-binding protein (Oct-1) consensus oligonucleotide (5'-ATGAAATCATAGAA-3') (26) or AP-1 binding site (14) for 30 min at 37°C. The DNA–protein complex formed was then separated from free oligonucleotide on 6.6% native polyacrylamide gels. For supershift assays, 2 μg nuclear extracts prepared from LPS-treated cells were incubated with antibodies (Santa Cruz) against the p50 or p65 subunit of NF-κB or control mouse IgG for 15 min at 37°C and the complex was then analysed by EMSA on 5% native polyacrylamide gels. Binding specificity was examined by competition with 100-fold excess unlabelled κB3 oligonucleotide and 100-fold excess mutated oligonucleotide (5'-AGCTGAGGAGGAAAGCCTGTTG-3'; boldface indicates mutated sequence). Dried gels were visualized using a BAS5000 imaging analyzer (FujiFilm), and bands were quantified using MultiGauge software (FujiFilm).

**BT and HR measurement**

A telemetric radio transmitter (TA10ETA-F20, Data Sciences) for measuring BT and HR was implanted subcutaneously on the back of each mouse under pentobarbital sodium anaesthesia (30 mg kg⁻¹, i.p.) as described (27). To avoid effects due to extreme size differences with wild-type mice, only *Fos*⁻/⁻ mice weighing 20 g or greater were used. Mice were individually housed unrestrained in cages inside a chamber (MIR-553, Sanyo), wherein a light–dark cycle (LD 12:12; light on at 8:00) was maintained, and temperature was kept at 24 ± 1°C. Food and water were supplied ad libitum. Mice were allowed at least 10 days to recover from surgery before data collection. BT and HR were monitored continuously by receivers (CTR-86, Data Sciences) below the cages, and recorded every 5 min by a Data Quest analysing system (Data Sciences). LPS was administered at 13:00 for each experiment in consideration of circadian rhythm. When specified, anti-TNF-α antibody (1.0 mg kg⁻¹) (Genzyme) was injected i.p. as described (28).

**Statistical analysis**

Data are expressed as the mean ± SEM. All other data were analysed using the Student’s t test, with the exception of BT data for which analysis of variance was used.

**Results**

**Enhanced TNF-α production in *Fos*⁻/⁻ macrophages**

To examine whether cytokine response is affected by lack of c-Fos, we stimulated wild-type and *Fos*⁻/⁻ PMs with LPS. Cells and culture supernatants were harvested over a course of 9 h after stimulation. IL-1β, IL-6, IL-10, IL-12 p40 and TNF-α mRNAs were measured by quantitative RT-PCR, and supernatant proteins by ELISA (Fig. 1). We observed that mRNAs of TNF-α, IL-6 and IL-12 p40 peaked more rapidly in *Fos*⁻/⁻ PMs, and that supernatant cytokine levels were higher in *Fos*⁻/⁻ PMs than in wild-type PMs. The enhanced induction of pro-inflammatory cytokines in *Fos*⁻/⁻ PMs became dramatic within 3 h after LPS stimulation. In contrast, the anti-inflammatory
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Cytokine IL-10 was less efficiently transcribed in Fos−/− cells as previously reported (21). Differences in IL-1β production between the two genotypes were not prominent. These data suggest that the LPS-induced transcription of TNF-α and other pro-inflammatory cytokine genes is enhanced in Fos−/− PMs. Dysregulated cytokine response in Fos−/− macrophages was not limited to LPS, but was also observed with other PAMPs such as flagellin (Supplementary Figure S1, available at International Immunology Online), and with Salmonella typhi- murium infection (our unpublished observations).

Dysregulated serum cytokines in Fos−/− mice

We next asked whether the enhanced production of pro-inflammatory cytokines could be observed in Fos−/− mice in vivo. We injected wild-type and Fos−/− mice i.p. with either PBS or LPS. Three hours after injection, blood and spleens were collected from each mouse and measured mRNAs and proteins for IL-1β, IL-6, IL-10, IL-12 p40 and TNF-α. While differences in splenic mRNA were marginal, serum TNF-α, IL-6 and IL-12 p40 levels were significantly higher but IL-10 lower in Fos−/− mice compared with wild-type controls (Fig. 2). In this experiment, serum protein of IL-1β was below detection levels. Therefore, both in vitro and in vivo, absence of c-Fos enhances production of TNF-α and other pro-inflammatory cytokines in response to LPS.

Elevated NF-κB levels in Fos−/− macrophages

NF-κB is the major transcription factor which activates TNF-α and other pro-inflammatory cytokine genes in response to LPS (29). Functional NF-κB binding sites have been identified in the TNF-α, IL-1, IL-6 and IL-12 p40 promoters. We asked whether DNA-binding activity of NF-κB to the κB3 site of the

![Graph](https://example.com/graph.png)

**Fig. 1.** In vitro LPS stimulation of PMs. Cytokine profiles of wild-type (closed box) and Fos−/− (open box) PMs cultured in duplicate were measured by quantitative RT-PCR and ELISA. Cells were stimulated with 1 μg ml−1 LPS, and culture supernatant and cells were collected over a course of 9 h.

![Graph](https://example.com/graph.png)

**Fig. 2.** LPS-induced cytokine response in mice. Levels of cytokine splenic mRNA were measured by quantitative RT-PCR and serum protein, by ELISA. Wild-type (closed bar) and Fos−/− (open bar) mice (n = 3–5 mice per bar) were stimulated for 3 h by i.p. injection of PBS or 4 mg kg−1 LPS. *P < 0.05 compared with wild-type mice. N.D., not detected.
TNF-α promoter (25) is altered in Fos−/− macrophages. We stimulated MDMs derived from Fos−/− and wild-type mice with LPS, and analysed NF-κB binding activity in the nuclear extracts (Fig. 3A, upper). Even in unstimulated control samples (0 min), NF-κB binding activity was 3.4-fold higher in Fos−/− than in wild-type MDMs (Fig. 3A, upper). LPS treatment induced NF-κB in both wild-type and Fos−/− MDMs. However, fold induction of NF-κB binding activity was significantly higher in Fos−/− MDMs than wild-type MDMs at 30 min (7.3- versus 5.9-fold) and especially at 60 min (9.7-versus 5.8-fold) (Fig. 3A). In contrast, both basal and induced AP-1 binding activities were lower in Fos−/− MDMs (Fig. 3A, middle). The binding activities to an Oct-1 binding site were unaffected by LPS treatment or the absence of c-Fos.

We next analysed composition of the NF-κB binding activity in wild-type and Fos−/− MDMs after LPS stimulation using anti-p50 and anti-p65 antibodies. Supershift revealed that the LPS-induced NF-κB contains the p50 and p65 subunits in both genotypes (Fig. 3B). Specificity of binding was confirmed because labelled oligonucleotide was efficiently competed out by excess cold κB3 oligonucleotide, but not cold mutant oligonucleotide (Fig. 3B).

These results indicate that nuclear levels of NF-κB in Fos−/− macrophages are not only higher than wild-type, but also contain the p65 subunit, which is known to function as a transcriptional activator. Elevated NF-κB binding activity may be the molecular basis for the elevated production of TNF-α and other pro-inflammatory cytokines in Fos−/− macrophages and mice.

**Profound endotoxic shock response in Fos−/− mice**

Drastic changes in BT, whether fever or hypothermia, are a hallmark sign of endotoxemia in laboratory animals (30). Fatal septic shock is also associated with haemodynamic changes such as bradycardia (31). To examine whether increased pro-inflammatory cytokine production in Fos−/− mice makes them more sensitive to LPS-induced shock, we measured changes in BT after stimulating wild-type and Fos−/− mice i.p. with increasing doses of LPS. Maximum BT drop in Fos−/− mice showed a clear LPS dose dependence, and at each dose, hypothermia in Fos−/− mice exceeded that in wild-type mice (Fig. 4A). Fig. 4B depicts the time course of BT changes after administration of LPS. Following transient fever of 1–2°C, hypothermia was observed in both genotypes (Fig. 4B). The severity of hypothermic response was much greater in Fos−/− mice, with BT reaching an average lowest value of 28°C, versus only 32°C in control mice.

We also compared HR in these mice after LPS stimulation. In both genotypes, following administration of LPS we observed HR was elevated for ~2 h and then began to drop (Fig. 4C). Strikingly, Fos−/− mice showed intense bradycardia with HR dropping as low as 447 beats per min (bpm), versus the lowest wild-type value of ~524 bpm (Fig. 4C). These BT and HR data

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**Fig. 3.** NF-κB binding activity. (A) Wild-type and Fos−/− MDMs were treated with 1 μg ml−1 LPS. Nuclear extracts were prepared and analysed for binding activities to NF-κB site (upper), AP-1 site (middle) and Oct-1 site (lower) by EMSA. (B) Nuclear extracts from untreated (−) and 1 h LPS-treated (+) wild-type or Fos−/− MDMs were incubated with the indicated antibodies, non-specific mouse IgG, a cold κB3 oligonucleotide or a cold κB3 mutant oligonucleotide and analysed for NF-κB DNA-binding activity by EMSA. NF-κB bandshift is indicated by the arrowhead and supershift by asterisk.
indicate that lack of c-Fos results in increased severity of LPS-induced shock.

Neutralizing TNF-α attenuates shock in Fos<sup>−/−</sup> mice

TNF-α is known to induce hypothermia in mice (28), and has been implicated as a factor depressing cardiac function in septic shock (32). We attempted to counteract the hypothermia and bradycardia by pre-treating mice with anti-TNF-α antibody 2 h prior to LPS stimulation. Anti-TNF-α antibody was effective in reducing responses in wild-type mice (data not shown). Fig. 4D shows hypothermic response in Fos<sup>−/−</sup> mice with and without anti-TNF-α antibody pre-treatment. Pre-treated Fos<sup>−/−</sup> mice showed less profound hypothermia (drop to 31°C) than non-pre-treated (drop to 29°C). The rate of recovery back to baseline was also more rapid in pre-treated mice.

Fig. 4E shows HR in Fos<sup>−/−</sup> mice with and without anti-TNF-α antibody pre-treatment. Pre-treated mice showed attenuated bradycardia (lowest HR value of 528 bpm) compared with non-pre-treated mice (405 bpm). These data indicate that enhanced TNF-α production is at least in part, responsible for the severe hypothermia and bradycardia observed in Fos<sup>−/−</sup> mice.

Discussion

We present in vivo evidence that c-Fos suppresses the systemic inflammatory response. The telemetry analysis demonstrated that LPS injection into Fos<sup>−/−</sup> mice results in enhanced hypothermia and bradycardia, mainly through elevated production of TNF-α. We do not exclude the possibility that differences in body size or nervous system function may also contribute to the severity of response in Fos<sup>−/−</sup> mice. Interestingly, wild-type and Fos<sup>−/−</sup> mice showed no consistent differences in production of IL-1β, which is associated with thermoregulation and myocardial function during the inflammatory response (32, 33). Based on the neutralization experiment, it appears that c-Fos modulates pathophysiology during systemic inflammation especially by down-regulating TNF-α.
We demonstrated that pro-inflammatory cytokine production in response to LPS was elevated in the absence of c-Fos both in vitro and in vivo. Recently, we found that Fos\(^{-/-}\) mice are more susceptible to Salmonella infection, producing higher levels of pro-inflammatory cytokines (our unpublished observation). How does c-Fos suppress pro-inflammatory cytokines? Studies with gene deficient mice have identified various pathways regulating LPS response. Overproduction of pro-inflammatory cytokines and hyper-sensitivity to LPS have been observed in mice lacking IL-1 receptor-associated kinase (IRAK)-M, suppressor of cytokine signalling, tumour necrosis factor-related apoptosis inducing ligand (TRAIL) receptors, Dok-1, Dok-2 and A20 (3, 4, 34–36). None of these mechanisms have yet identified c-Fos as an important effector molecule. IRAK-M as well as Dok-1 and 2 inhibit Erk phosphorylation (3, 35). Since Erk is known to phosphorylate c-Fos, the relationship between Erk and c-Fos in these pathways needs to be further explored. In contrast, Erk phosphorylation is not involved in TRAIL-R and A20-mediated inhibition of NF-κB (34, 36).

NF-κB is known to drive transcription of pro-inflammatory cytokine genes. We showed that absence of c-Fos results in higher than normal nuclear levels of NF-κB, and that this NF-κB contains the activating p65 subunit. NF-κB hyperactivity in Fos\(^{-/-}\) macrophages suggests an antagonistic relationship between NF-κB and c-Fos. Indeed in Drosophila, LPS response of NF-κB and c-Fos/AP-1. In Drosophila, caspase-1 is essential for cytokine responses in inflammation. The possibility still remains that c-Fos may suppress inflammatory mediators through direct binding to the promoters, as has been suggested for IL-12 p40 (22) and inducible NO synthase (23). AP-1 binding sites exist also in the promoters of TNF-α and IL-6 (15, 16).

Finally, rheumatoid arthritis drugs which target c-Fos/AP-1 to suppress osteoclast-mediated pathological bone resorption are being developed, presumably inspired by the fact that Fos\(^{-/-}\) mice do not show inflammatory joint destruction in an experimental model of arthritis (38). However, care should be taken as such therapies may unmask beneficial immunosuppressive effects of c-Fos/AP-1. In conclusion, these data suggest that c-Fos acts as an anti-inflammatory transcription factor in vivo.

Supplementary data

Supplementary data are available at International Immunology Online.

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Abbreviations

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<th>Term</th>
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<tr>
<td>AP-1</td>
<td>activator protein-1</td>
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<tr>
<td>bpm</td>
<td>beats per min</td>
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<td>BT</td>
<td>body temperature</td>
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<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
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<td>IL-1 receptor-associated kinase-M</td>
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<td>TLR</td>
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References


