# TGF-β inhibits lipopolysaccharide-stimulated activity of c-Jun N-terminal kinase in mouse macrophages

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**Abstract Transforming growth factor-β (TGF-β) is a potent anti-inflammatory cytokine. Although this cytokine inhibits lipopolysaccharide (LPS)-mediated septic shock, the molecular mechanism of TGF-β is not well known. Since recent studies showed that c-Jun N-terminal kinase (JNK), one of the mitogen-activated protein kinases, plays an important role in LPS signalling, we focused here on the inhibitory action of TGF-β1 on LPS-stimulated JNK activity in mouse macrophages. TGF-β1 inhibited LPS-stimulation of phosphorylated JNK1 and JNK2 and consequently of JNK activity in the cells. This JNK activity resulted in a decreased level of phosphorylated c-Jun protein. Using Western blotting, we also observed TGF-β1 inhibition of newly synthesized c-Jun protein in LPS-stimulated cells. These results demonstrate that TGF-β1 inhibits LPS-stimulated JNK activity in mouse macrophages. Also, our present study suggests a possible inhibitory mechanism of TGF-β in signalling of LPS-induced inflammatory responses.**



*Key words:* Transforming growth factor-β; Lipopolysaccharide; c-Jun N-terminal kinase; Macrophage

## 1. Introduction

Transforming growth factor-β (TGF-β) plays important roles in growth and development, inflammation and repair and host immunity [1–3]. Many studies [4–8] have demonstrated inhibitory actions of this cytokine in immunological and inflammatory responses. In fact, various interesting studies [9–12] have shown that the progressive inflammation found in TGF-β1-deficient mice is associated with several manifestations of autoimmunity, including circulating antibodies to nuclear antigens and immune complex deposition. These findings strongly demonstrate that TGF-β1 is a potent anti-inflammatory cytokine. However, the molecular mechanism of anti-inflammatory action of TGF-β1 has still not been investigated.

LPS stimulates inflammatory responses via production of cytokines such as interleukin (IL)-1, IL-6 and TNF-α by macrophages and neutrophils that express predominantly the membrane receptor CD14 [13–20]. Therefore, it is very important to demonstrate the intracellular mechanism of signal transduction in LPS-stimulated inflammatory responses. Recently, several investigations [21–25] have suggested that especially c-Jun N-terminal kinase (JNK), one of the mitogen-activated protein (MAP) kinases, plays a predominant role in lipopolysaccharide (LPS) signalling.

In light of these facts and to demonstrate the molecular mechanism of the anti-inflammatory action of TGF-β1, it is of interest to investigate whether TGF-β1 is able to inhibit LPS-stimulated JNK activity in mouse macrophages. Our present study demonstrates TGF-β1 inhibition of LPS-stimulated JNK activity in mouse macrophages.

## 2. Materials and methods

### 2.1. Reagents

TGF-β1 was purified from human platelets to homogeneity (>98%, determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and HPLC analyses: PEPROTECH, London, UK). RPMI 1640 was obtained from Nissui Pharmaceutical (Tokyo, Japan) and fetal calf serum from Hyclone (Logan, UT, USA). *Escherichia coli* 0111 B4-derived LPS was from Sigma Chemical (St. Louis, MO, USA). [γ-$^{32}$P]ATP was purchased from Amersham Pharmacia Biotech (Tokyo, Japan). The phospho-specific SAPK/JNK antibody (Thr-183/Tyr-185), control anti-SAPK/JNK antibody, phospho-specific c-Jun(Ser-63) II antibody, phospho-specific c-Jun(Ser-73) antibody and control c-Jun antibody were all rabbit polyclonal antibodies purchased from New England Biolabs (Beverly, MA, USA).

### 2.2. Preparation of mouse peritoneal macrophages

BALB/c mice, 7 weeks of age, were injected intraperitoneally with 3 ml of thioglycollate medium (Difco, Detroit, MI, USA). Peritoneal macrophages were prepared from the mouse peritoneal exudate cells as described earlier [26]. The prepared macrophages were treated for selected times with test samples.

### 2.3. Western blot analysis

Macrophage monolayers in 9 cm diameter dishes ($5\times10^6$ peritoneal exudate cells) were incubated in the presence or absence of test samples at various concentrations. Thereafter, the cells were solubilized with lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% (v/v) Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM $Na_3VO_4$, 1 mM β-glycerolphosphate, 1 μg/ml leupeptin, 1 mM PMSF). The samples (20 μg protein) were electrophoresed on 10% polyacrylamide gels by SDS-PAGE using a Tris-glycine buffer system (0.025 M Tris, 0.192 M glycine, 0.1% SDS). The protein was transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) by making use of the semidry transblot system (ATTO, Tokyo, Japan). Blots were blocked with 5% skin milk in Tris-buffered saline including 0.1% Tween-20 (TBS-T) for 1 h at room temperature and washed with TBS-T. Then, the membrane was incubated with the primary antibody diluted 1:1000 in 5% bovine albumin in TBS-T, overnight at 4°C. Protein was detected with a Phototope-HRP Western blot detection kit (New England Biolabs). The blots were exposed to X-omat film (Eastman Kodak, Rochester, NY, USA)

### 2.4. JNK activity assay

The activity of JNK was measured using the SAPK/JNK assay kit (New England Biolabs) according to the instructions from the manufacturer. Briefly, macrophage monolayers in 9 cm diameter dishes ($5\times10^6$ peritoneal exudate cells) were incubated in the presence or absence of test samples at various concentrations. Thereafter, the cells were solubilized with lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM $Na_3VO_4$, 1 mM β-glycerolphosphate, 1 μg/ml leupeptin, 1 mM PMSF). Then, the cell lysates were incubated

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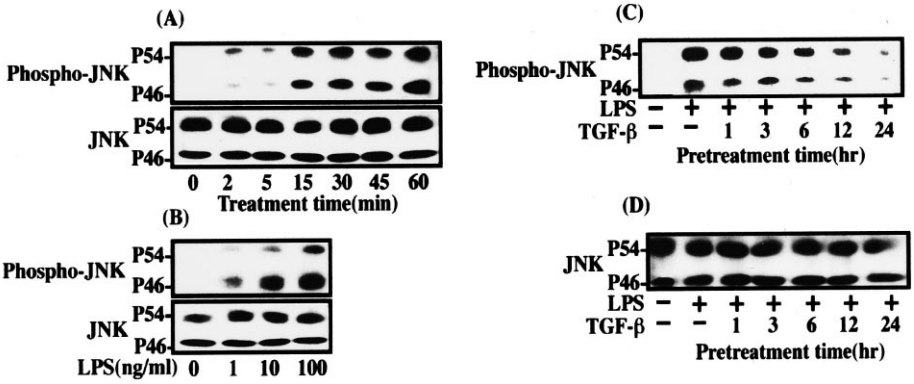


Fig. 1. TGF-β1 inhibits LPS-stimulated phosphorylation of JNK1 and JNK2 in mouse peritoneal macrophages. (A) The cells from BALB/c mice were treated or not for the selected periods with LPS at 100 ng/ml. Thereafter, phosphorylation of JNK1 and JNK2 in equal amounts of cell lysates was analyzed after SDS-PAGE and immunoblotting with antibody against phosphorylated JNK1 and JNK2. (B) The cells from BALB/c mice were treated or not for 1 h with LPS at various doses. Thereafter, phosphorylation of JNK1 and JNK2 was detected as described in 'A'. (C) The cells from BALB/c mice were pretreated or not for the selected times with TGF-β1 at 1 ng/ml and then treated or not for 1 h with LPS at 100 ng/ml. Thereafter, phosphorylation of JNK1 and JNK2 was detected as described in 'A'. (D) The experimental conditions were the same as in 'C'. Then, JNK in equal amounts of cell lysates was analyzed after SDS-PAGE and immunoblotting with anti-JNK antibody. An identical experiment independently performed gave similar results.

with GST-c-Jun coupled to GSH-Sepharose beads, overnight at 4°C, to precipitate JNK. After the beads were washed, the solid-phase kinase reaction was carried out at 30°C for 30 min in kinase buffer (25 mM Tris-HCl (pH 7.5, 150 mM NaCl, 2 mM dithiothreitol, 0.1 mM $Na_3VO_4$, 5 mM β-glycerolphosphate, 10 mM $MgCl_2$, 100 mM ATP). The reaction was terminated by adding sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 50 mM DTT, 0.1% (w/v) bromophenol blue). Phosphorylation of GST-c-Jun on Ser-63 was analyzed after SDS-PAGE and immunoblotting with phospho-specific c-Jun(Ser-63) antibody.

## 3. Results

### 3.1. TGF-β1 inhibits LPS stimulation of phosphorylation of JNK1 and JNK2 in mouse macrophages

Since JNK is one of the major regulatory molecules in signal transducing mechanisms in macrophages stimulated by LPS, firstly, we investigated whether TGF-β1 modulates phosphorylation of Thr-183 and Tyr-185 of JNK in macrophages stimulated with LPS. The cells were treated or not with TGF-β1 at various times prior to LPS addition at 100 ng/ml and then, two apparent bands, p46 (JNK1) and p54 (JNK2), were analyzed by an immunoblot assay with phospho-specific JNK antibody. As shown in Fig. 1A and B, although LPS stimulated in a treatment time- and dose-dependent manner the phosphorylation of the two JNKs in the cells, TGF-β1 inhibited this stimulation dramatically. The TGF-β1 inhibition of JNK1 and JNK2 was observed to be significant after at least 3 h of pretreatment and was pretreatment time-dependent (Fig. 1C). However, stimulation of non-phosphorylated JNK protein production was not observed in LPS-treated cells and TGF-β1 did not reduce the level of non-phosphorylated JNK protein (Fig. 1D). These results show that TGF-β1 inhibited LPS-induced phosphorylation of JNK1 and JNK2 in mouse macrophages.

### 3.2. TGF-β1 inhibits LPS-stimulated JNK activity in mouse macrophages

The TGF-β1 inhibition of LPS-stimulated phosphorylation of JNKs suggested that TGF-β1 may be able to inhibit the toxin-stimulated JNK activity in mouse macrophages. Therefore, we examined TGF-β1 inhibition of LPS-stimulated JNK activity in LPS-stimulated cells. The cells were treated or not for 24 h with TGF-β1 prior to LPS addition and then, JNK activity in the cell lysates was analyzed at 1 h after the addition of LPS. Fig. 2A shows that LPS stimulation of JNK activity in the cells was dose-dependent. Importantly, TGF-β1 clearly inhibited LPS-stimulated JNK activity in the cells (Fig. 2B).

### 3.3. TGF-β1 inhibits LPS stimulation of c-Jun phosphorylation in mouse macrophages

Since TGF-β1 inhibition of LPS-stimulated JNK activity in macrophages suggested that this cytokine may inhibit LPS-induced phosphorylation of c-Jun, we examined this point by conducting an immunoblot assay with two antibodies

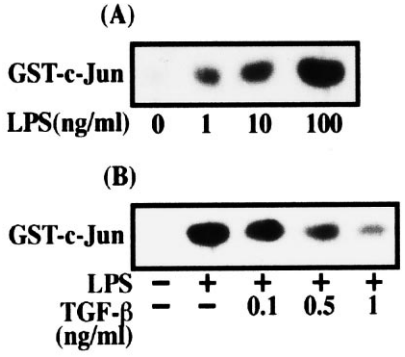


Fig. 2. TGF-β1 inhibits LPS-stimulated JNK activity in mouse peritoneal macrophages. (A) The cells from BALB/c mice were treated or not for 1 h with LPS at various doses. Thereafter, equal amounts of the cell lysates were incubated with GST-c-Jun coupled to GSH-Sepharose beads to precipitate JNK. After the beads were washed, the solid phase kinase reaction was carried out at 30°C for 30 min. Phosphorylation of GST-c-Jun on Ser-63 was analyzed after SDS-PAGE and immunoblotting with phospho-specific c-Jun (Ser-63) antibody. (B) The cells were pretreated or not for 24 h with TGF-β1 at the selected doses and then treated or not for 1 h with LPS at 100 ng/ml. The JNK activity was determined as described in 'A'. An identical experiment independently performed gave similar results.

that recognize c-Jun phosphorylated at Ser-63 and Ser-73, respectively. The cells were treated or not for 24 h with TGF-β1 at 1 ng/ml and then treated or not for various times with LPS at 100 ng/ml. Then, phosphorylation of c-Jun on Ser-63 and Ser-73 in cell lysates was analyzed after SDS-PAGE and immunoblotting with each antibody. As shown in Fig. 3, LPS stimulation of phosphorylation of c-Jun on Ser-63 and Ser-73 in the cells was observed to be marked after at least 15 min of LPS treatment. Also, as expected, TGF-β1 strongly inhibited LPS-stimulated phosphorylation of the oncogene product.

### 3.4. TGF-β1 inhibits LPS-stimulated synthesis of c-Jun protein in mouse macrophages

Finally, we addressed by a Western blot assay whether TGF-β1 is able to inhibit newly synthesized c-Jun protein in LPS-treated macrophages. As shown in Fig. 4A, this cytokine inhibited LPS-stimulated production of c-Jun protein by the cells in a dose-dependent manner and the inhibitory effect was observed as early as 5 min after the initiation of the LPS stimulation (Fig. 4B). These results clearly showed that TGF-β1 is able to inhibit LPS-stimulated synthesis of c-Jun protein in the macrophages.

## 4. Discussion

LPS activates three separate MAP kinases, i.e. JNK, extracellular signal-regulated kinase and p38, in a macrophage cell line (34-41). Especially, it is well known that JNK regulates the transcription of many genes by activating AP-1 with a relative high specificity. In fact, a recent study [27] showed the direct involvement of JNK in the biosynthesis of TNF-α, a major mediator of septic shock, in macrophages. Therefore, we focused our present study on the regulatory action of TGF-β1 on LPS-stimulated JNK activity in murine macrophages. We showed here that TGF-β1 acts as a potent inhibitor of LPS-stimulated activity of JNK in the macrophages.

Pretreatment of mouse macrophages with TGF-β1 inhibited LPS-stimulated phosphorylation of JNK1 and JNK2 and also that of c-Jun on Ser-63 and Ser-73 stimulated in the cells. In addition, we showed that TGF-β1 blocked LPS-stimulated JNK activity in the cells. In this regard, a recent study [27] demonstrated that glucocorticoid, an another potent inhibitor of inflammation, also inhibits JNK activity in a LPS-stimulated RAW macrophage cell line. However, the mechanism of this inhibition is unknown. Also, although the inhibitory mechanism of TGF-β1 with respect to JNK activity has not yet been demonstrated, two possible inhibitory mechanisms may be postulated: (1) TGF-β1 might inhibit JNK by inducing phosphatase activity against TGF-β-activated kinase (TAK1), a strong activator of JNK, or (2) the TAK1 → MKK4/SEK1 → JNK kinase cascade is consumed by TGF-β1 pretreatment and consequently, LPS is unable to use JNK that is required in its signalling. In this regard, several investigators have shown that TGF-β actually initiates a signalling cascade leading to SAPK/JNK activation [28,29]. Therefore, in further studies, it is very important to elucidate the mode of action of upstream compounds of JNK induced by TGF-β1. In this study, although we focused on the inhibitory action of TGF-β1 in LPS-induced JNK, it also remains of interest to explore this cytokine action toward other MAP kinases that are activated in the LPS response.

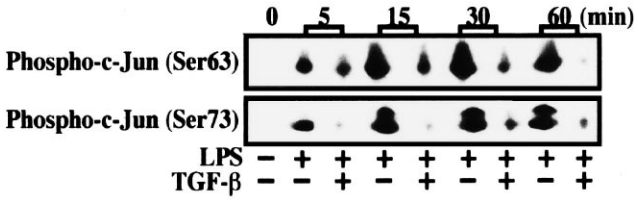


Fig. 3. TGF-β1 inhibits LPS-stimulated c-Jun protein phosphorylation at Ser-63 and Ser-73 in mouse peritoneal macrophages. The cells from BALB/c mice were pretreated or not for 24 h with TGF-β1 at 1 ng/ml and were then treated or not for various times with LPS at 100 ng/ml. Thereafter, phosphorylation of c-Jun on Ser-63 and Ser-73 in equal amounts of cell lysates was analyzed after SDS-PAGE and immunoblotting with each phospho-specific antibody against c-Jun Ser-63 or Ser-73. An identical experiment independently performed gave similar results.

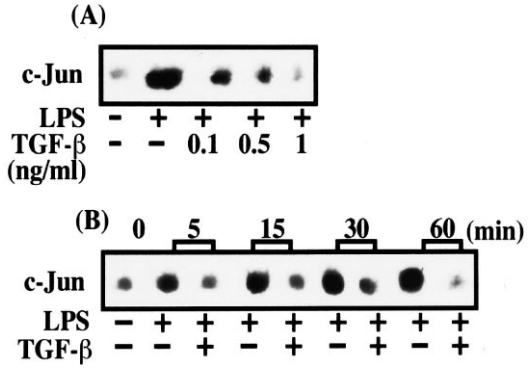


Fig. 4. TGF-β1 inhibits LPS-stimulated production of c-Jun by mouse peritoneal macrophages. (A) The cells from BALB/c mice were pretreated or not for 24 h with TGF-β1 at various doses and then treated or not for 1 h with LPS at 100 ng/ml. Thereafter, c-Jun in cell lysates was analyzed after SDS-PAGE and immunoblotting with anti-c-Jun antibody. (B) The cells from BALB/c mice were pretreated or not for 24 h with TGF-β1 at 1 ng/ml and then treated or not for the selected times with LPS at 100 ng/ml. Thereafter, c-Jun in cell lysates was analyzed as described in (A). An identical experiment independently performed gave similar results.

Inducible NOS is a potent mediator of LPS-induced septic shock. However, since TNF-α is a strong stimulator of iNOS expression in several kinds of cells, we considered that it is very important to examine the inhibitory action of TGF-β1 against LPS-stimulated expression of TNF-α in septic shock in mice. Our preliminary study shows that TGF-β1 is able to inhibit LPS-stimulated expression of TNF-α in mouse macrophages and that the TGF-β1 inhibitory effect was also observed in the lungs, kidneys and spleen of LPS-injected mice (unpublished data). However, at present, we do not know whether this inhibitory action in vivo is dependent on down-regulation of JNK activity as observed in vitro.

In conclusion, the results of our present study have allowed us to propose a possible molecular mechanism for the anti-inflammatory action of TGF-β1 in Gram-negative bacterial infections.

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