ORIGINAL RESEARCH

c-Jun Is Required for Nuclear Factor-κB–Dependent, LPS-Stimulated Fos-Related Antigen-1 Transcription in Alveolar Macrophages

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Abstract

Previously, we have reported that Fos-related antigen-1 (Fra-1) transcription factor promotes LPS-induced acute lung injury and mortality, and that LPS-induced *Fra-1* expression in the lung occurs predominantly in alveolar macrophages. Nuclear factor-κB (NF-κB) and c-Jun transcription factors play key roles in modulating inflammatory and immune responses induced by infectious and non-infectious insults. Here, we report that NF-κB and c-Jun coregulate *Fra-1* induction by LPS in alveolar macrophages and that this regulation occurs through both the NF-κB and the extracellular signal-regulated protein kinase (ERK) signaling pathways. Transient transfections with *Fra-1* promoter–reporter constructs and inhibitor studies revealed that the transcriptional activation of *Fra-1* by LPS in alveolar macrophages is mediated by NF-κB and ERK1/2 signaling. Importantly, chromatin immunoprecipitation assays revealed the recruitment of c-Jun and NF-κB to the endogenous *Fra-1* promoter after LPS stimulation. We found that inhibition of ERK1/2 signaling reduced LPS-stimulated c-Jun and NF-κB recruitment to the promoter. Likewise, NF-κB inhibitor blocked LPS-induced NF-κB and c-Jun binding to the promoter. ERK1/2 inhibition had no effect on c-Jun activation but suppressed LPS-stimulated NF-κB phosphorylation. Finally, functional assays showed reduced levels of LPS-stimulated NF-κB regulated proinflammatory IL-1β and macrophage inflammatory protein-1α expression and increased antiinflammatory IL-10 expression in lung alveolar macrophages of *Fra-1*–null mice *in vivo*. Thus, our studies indicate that NF-κB and c-Jun coregulate LPS-induced *Fra-1* transcription and that Fra-1 selectively modulates LPS-stimulated inflammatory cytokine expression in lung alveolar macrophages during inflammatory lung injury.

Keywords: Fos-related antigen-1; activator protein-1; inflammation; macrophage; lung

Clinical Relevance

Heightened levels of lung inflammation caused by infectious and noninfectious insults can lead to pathogenesis, and alveolar macrophages play an important role in this process. Fos-related antigen-1 (Fra-1) transcription factor promotes LPS-induced inflammatory lung injury and sepsis *in vivo*. This study indicates that nuclear factor-κB and c-Jun coregulate LPS-induced *Fra-1* transcription and that Fra-1 selectively upregulates LPS-induced nuclear factor-κB–dependent proinflammatory cytokine expression and dampens antiinflammatory response in alveolar macrophages during inflammatory lung injury.

Lung inflammation is one of the important host defense mechanisms to combat both infectious and noninfectious insults. However, impaired resolution of lung inflammation after acute lung injury caused by these insults can lead to pathogenesis. Alveolar macrophages play an important role in both innate and adaptive immune responses elicited by LPS (endotoxin) and prooxidant stimuli in the lung. In response to LPS, Toll-like receptor-4 (TLR-4) initiates inflammatory and injury responses in the lung by simultaneously activating nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), leading to the transcriptional

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induction of genes encoding cytoprotective proteins, inflammatory cytokines and chemokines, adhesion molecules, and growth factors (1). By stimulating IκBα degradation, LPS promotes NF-κB nuclear accumulation and its target gene expression. In addition, LPS-induced TLR-4 signaling, via tumor progression locus-2 (TPL-2) kinase (also known as mitogen-activated protein kinase [MAPK] 3K8 or COT), stimulates the extracellular signal-regulated protein kinase (ERK) 1/2 pathway, which is crucial for the activation of the members of the AP-1 family of proteins (2, 3). LPS-stimulated MAPK signaling also regulates IκBα degradation and NF-κB phosphorylation and the activation of ternary complex factors such as Ets-like protein-1 (ELK-1) (2, 3).

The AP-1 transcription factor composed of the JUN and FOS families of proteins regulates both inflammatory and immune responses (4, 5). The conditional deletion of *c-Jun* and *Jun-B* in the epidermis leads to psoriasis in mice, accompanied by the enhanced expression of inflammatory mediators (6). Genetic disruption of c-*Fos* leads to increased production of inflammatory cytokines in response to LPS and enhances susceptibility to experimental colitis, in part because of NF-κB activation (7). We have shown that mice lacking Fos-related antigen-1 (*Fra-1*) gene are less susceptible to LPS-induced lung inflammation and mortality (8). On the contrary, mice that overexpress *Fra-1* showed increased susceptibility to LPS-induced mortality (9). Increased expression of Fra-1 in alveolar macrophages and epithelial cells has been reported in the lungs of patients with adult respiratory distress syndrome (10) and in human lungs infected with bacteria *ex vivo* (11), as well as in alveolar macrophages of the lungs of mice treated with LPS (11). Although these studies demonstrate that Fra-1 plays key roles in inflammatory lung injury and sepsis, the mechanisms underlying transcriptional activation of *Fra-1* by LPS in alveolar macrophages are poorly understood. In this study, we examined the mechanisms regulating *Fra-1* induction by LPS in mouse alveolar macrophages, especially the roles of NF-κB and c-Jun in mediating this process. Here, we report that NF-κB and c-Jun are required for *Fra-1* induction by LPS, and that ERK1/2 and NF-κB signaling mutually promote NF-κB and c-Jun binding to the promoter in alveolar macrophages. Furthermore, we show that Fra-1 distinctly up-regulates NF-κB–dependent LPS-stimulated proinflammatory cytokine expression in alveolar macrophages *in vivo*.

Materials and Methods

Cell Culture and Reagents

The murine alveolar macrophage cell line, MH-S, kindly provided by Irena Levitan (University of Illinois at Chicago) was cultured in RPMI 1640 medium containing 10% fetal bovine serum and antibiotics. Phospho-c-Jun (SC-822, serine 65), Fra-1 (SC-183) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and phospho-NF-κB (p65, serine 536) antibodies (catalog 3033) and U0126 were obtained from Cell Signaling Technology (Beverly, MA). SP600125, SB203580, and BAY 11-7082 were purchased from EMD Millipore (Billerica, MA). Cells were incubated with inhibitors at 10 μM, with the exception of SB203580, which was incubated at 20 μM.

Mice and LPS Treatment

Mice bearing "floxed" alleles of *Fra-1* (*Fra-1*$^{F/F}$) (kindly provided by Erwin Wagner, Spanish National Cancer Research Center, Madrid, Spain) and *Fra-1*-null (*Fra-1*$^{\Delta/\Delta}$) mice were generated by crossing *Fra-1*$^{F/F}$ mice with mesenchyme homeobox 2–Cre mice, as described previously (8). *Fra-1*$^{F/F}$ (designated as *Fra-1*$^{+/+}$) and *Fra-1*$^{\Delta/\Delta}$ mice (8–10 wk old, male) were treated intratracheally with sterile phosphate-buffered saline (PBS) (vehicle) or 10 micrograms of LPS (L4005; Sigma-Aldrich, St. Louis, MO) for 3 hours. Mice were killed according to the protocol approved by the animal care committee at the University of Illinois at Chicago.

Isolation of Mouse Lung Alveolar Macrophages

After LPS treatment and the mice were killed, lungs were collected and instilled with 1 ml of RPMI-1640 medium containing dispase II (1 mg/ml) (Roche Life Sciences, Indianapolis, IN) and collagenase type II (1 mg/ml) (Thermo-Fisher Scientific, Waltham, MA), and tubes with solution were incubated at 37°C for 20 minutes. Lungs were minced and digested further for 15 min, and then passed through a 100-μM strainer. The filtrate was plated on culture dishes and incubated at 37°C for 45 minutes, supernatant was removed, and attached macrophages (12) were lysed with TRIzol for RNA isolation. We found that ∼90% of the cells attached were macrophages. We chose this method to minimize the perturbations associated with cell sorting analysis.

Gene Expression Analyses

For mRNA analysis, total RNA (2 μg) was reverse transcribed using a complementary DNA synthesis kit (Quanta Biosciences, Gaithersburg, MD), and real-time quantitative reverse transcription (qRT)–polymerase chain reaction (PCR) was performed using SYBR Green gene expression assays using β-actin as a reference. For immunoblot analysis, total protein (∼50 μg) was separated on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, blotted on to membrane, and probed with antibodies.

Promoter Reporter Assays

DNA fragments bearing the −213/+132 (referred to as 213-Luc), −132/+132 (132-Luc) or −92/+132 (92-Luc) 5′-flanking region of *Fra-1* (mouse) were generated by PCR amplification of genomic DNA and cloned into the multiple cloning site of pGL-3 reporter (13). Cells were transfected with promoter reporter constructs together with the Renilla luciferase (pRL-TK) plasmid (Promega Corp., Madison, WI). After overnight transfection, cells were incubated with medium containing 2% fetal bovine serum for ∼3 hours and then treated with vehicle (dimethyl sulfoxide) or LPS (100 ng/ml) (L2637, Sigma-Aldrich), and promoter activity was analyzed (13).

Chromatin Immunoprecipitation Assays

Cells (∼1×10^7) were treated with LPS at different time points and were then incubated with formaldehyde and lysed, and chromatin was prepared using an EZ-ChIP kit (Upstate Biotechnology Inc., Lake Placid, NY). Chromatin immunoprecipitated with antibodies was purified, and real-time qRT-PCR was performed using *Fra-1* promoter-specific primers: Forward, (−206/−186), 5′-GATTTTGTTTCGCCCTG TGT-3′; Reverse, (+87/+68), 5′-GACCCACCTCTCCAGATGAA-3′, as detailed elsewhere (14).

Statistical Analysis

Data are presented as mean ± SEM/SD. Statistical significance between the untreated and treated groups was determined using *t* test. $P = \leqslant 0.05$ was considered significant.

Results

Transcriptional Induction of *Fra-1* by LPS in Alveolar Macrophages

To define the mechanisms of regulation of *Fra-1* expression by LPS, alveolar macrophages (MH-S cells) were stimulated with LPS for different time points, RNA was isolated, and qRT-PCR was performed. As shown in Figure 1A, LPS stimulated *Fra-1* mRNA expression by 3 hours, which remained elevated above the basal level up to 6 hours, and the mRNA expression was verified by Western blot analysis (Figure 1B). Studies with the transcriptional inhibitor actinomycin D revealed that LPS stimulates *Fra-1* expression at the transcriptional level (Figure 1C). These results are consistent with the reported transcriptional up-regulation of *Fra-1* expression by mitogenic and oxidant stimuli in different cell types (13–16).

To further elucidate the mechanisms controlling LPS-inducible *Fra-1* expression, we generated luciferase reporter constructs bearing by the −213/+132 (referred to as 213-Luc), −132/+132 (132-Luc), or −92/+132 (92-Luc) 5′-flanking region of *Fra-1* (Figure 1D). The positions of κB (NF-κB binding motif), TRE (AP-1 binding site), and serum response element (SRE) relative to the transcriptional site are shown. These reporter constructs bearing different lengths of *Fra-1* promoter were transfected into MH-S cells, and the levels of promoter activity stimulated by LPS were analyzed. Both the 132-Luc– and the 213-Luc–driven promoter activities in PBS-treated cells were approximately fourfold higher than those for the 92-Luc, suggesting that −132/−92 bp region drives the basal

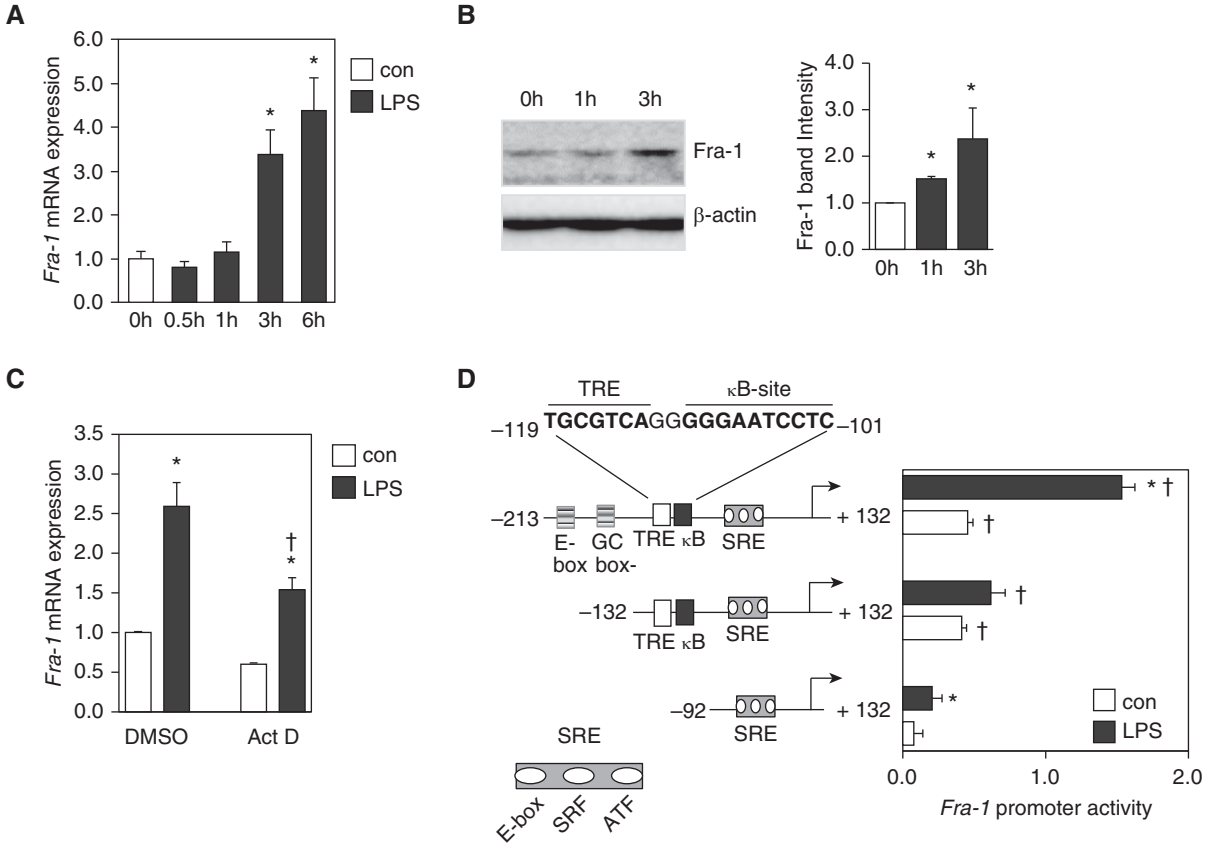

Figure 1. LPS induces Fra-1 expression in alveolar macrophages. (*A*) Cells were cultured with medium containing 2% FBS for 3 hours and then stimulated with LPS (100 ng/ml) for the indicated periods. Total RNA was extracted and *Fra-1* mRNA expression was analyzed by quantitative reverse transcription–polymerase chain reaction using β-actin as a reference. Data are presented as mean ± SEM ($n = 3$); *$P \leqslant 0.05$, PBS versus LPS. (*B*) Lysates from cells treated with LPS were isolated and immunoblotted with Fra-1 antibodies. Membrane was stripped and reprobed with β-actin antibodies. (*C*) Cells were incubated with actinomycin D (Act D), 5 μg/ml for 30 minutes and then stimulated with LPS for 3 hours; RNA was isolated, and *Fra-1* mRNA expression was analyzed. Data are presented as mean ± SEM ($n = 3$); *$P \leqslant 0.05$, PBS versus LPS; †$P \leqslant 0.05$, vehicle versus Act D. (*D*) The *Fra-1* promoter reporter constructs bearing major *cis*-acting regulatory elements shown are: TPA response element (TRE); serum response element (SRE); GC-box, Sp1-binding site; and ETS-binding motif. The SRE contains the ternary complex factor binding site, the serum response factor (SRF) binding CArG site, and the activating transcription factor (ATF) or cAMP response element binding protein site. The position of these *cis* elements relative to the transcription start site is assigned on the basis of a previous study (24). The κB element of *Fra-1* bears 90% homology with a consensus κB element (5′-GGGRNWYYCC-3′). Cells were transfected with the indicated promoter reporter construct together with a reference plasmid, pRL-TK. After 24 hours, cells were cultured with medium containing 2% FBS for 3 hours and then treated with LPS for 6 hours. The luciferase activity was calculated after normalizing with Renilla values. Data are presented as mean ± SEM ($n = 3$); *$P \leqslant 0.05$, PBS versus LPS; †$P \leqslant 0.05$, 92-Luc versus 132-Luc or 213-Luc. con, control; Fra-1, Fos-related antigen-1.

level *Fra-1* expression. However, no significant increase in promoter activity after LPS treatment was noted in cells transfected with either the 132-Luc or the 92-Luc construct. In contrast, the 213-Luc exhibited a higher level (~2.5-fold) of luciferase activity after LPS treatment. These results indicate that LPS stimulates *Fra-1* expression mainly at the transcriptional level, and that the −213/+132 bp region contains *cis* elements in mediating this response.

MAPK Pathway Is Essential for LPS-Induced *Fra-1* Expression

Previously, we showed that MAPK signaling regulates both TPA- and tumor necrosis factor (TNF)-α–stimulated *FRA-1* transcription in human lung epithelial cells (13–15). Therefore, we next analyzed the effects of MAPK pathway inhibition on LPS-stimulated *Fra-1* expression. As shown in Figure 2A, treatment of cells with the mitogen-activated ERK kinase 1/2 (MEK1/2)-ERK1/2 signaling inhibitor (U0126), the c-Jun N-terminal kinase 1/2 (JNK1/2) inhibitor (SP600125), or the p38 MAPK inhibitor (SB203580) significantly attenuated LPS-stimulated *Fra-1* mRNA expression. To assess the importance of MAPK signaling in the regulation of *Fra-1* transcription, cells were transfected with the 213-Luc *Fra-1* promoter reporter construct (Figure 1), and LPS-stimulated *Fra-1* promoter activity was analyzed in the presence or absence of the inhibitor (Figure 2B). As anticipated, LPS-induced *Fra-1* promoter activity was markedly attenuated in cells treated with either U0126, SP600125, or SB203580, further supporting a critical role for MAPK signaling in controlling *Fra-1* induction by LPS.

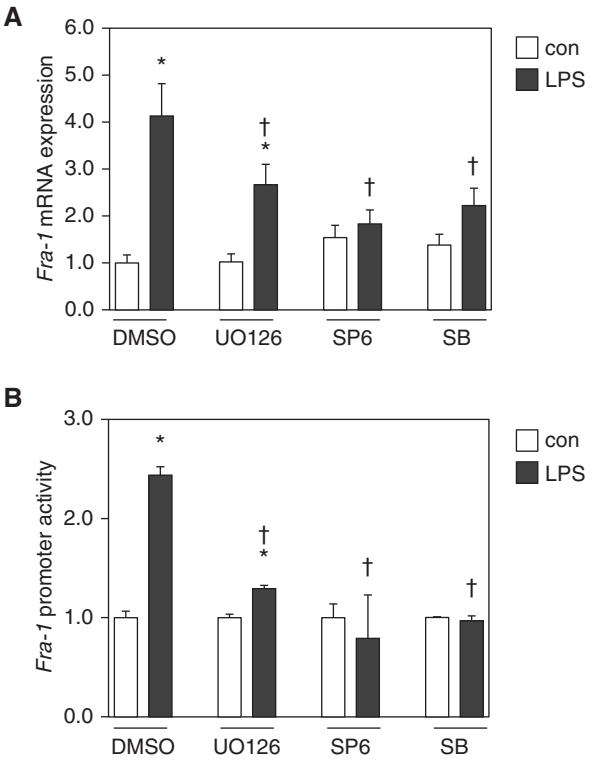

Figure 2. The mitogen-activated protein kinase pathway is essential for LPS-induced *Fra-1*expression in macrophages. (*A*) MH-S cells were incubated with U0126, SP600125 (SP6), or SB202190 (SB) for 30 minutes and then treated with LPS for 6 hours. *Fra-1* mRNA expression was analyzed by real-time polymerase chain reaction. Data are presented as mean ± SEM ($n = 3$); $^{*}P \leq 0.05$, PBS versus LPS; ($n = 3$). $^{\dagger}P \leq 0.05$, vehicle versus inhibitor. (*B*) MH-S cells were transfected with −213/+132 bp *Fra-1* promoter (213-Luc) reporter construct. After 24 hours, cells were incubated with inhibitors as indicated for 30 minutes and then treated with LPS for 6 hours. The fold-activation of reporter activity by LPS was calculated using the values obtained from vehicle (PBS)-treated cells as 1.0. Data are presented as mean ± SEM ($n = 3$). Experiment was repeated to obtain reproducible result.

ERK1/2 Inhibition Blocks c-Jun Recruitment to the *Fra-1* Promoter

Previously, we demonstrated c-Jun binding to the *FRA-1* (human) promoter after mitogenic (e.g., phorbol 12-myristate 13-acetate and epidermal growth factor) and cytokine (e.g., TNF-α) stimuli, and this recruitment occurs mainly through ERK1/2-mediated signaling in lung epithelial cells (13–15). To assess whether or not ERK1/2 signaling also regulates c-Jun recruitment to the endogenous *Fra-1* promoter in response to LPS, we performed chromatin immunoprecipitation (ChIP) assays to determine its binding, using *Fra-1* promoter-specific primers spanning the −213/+132 region (Figure 3A). Cross-linked chromatin fragments were prepared from control and LPS-stimulated cells with and without the ERK1/2 inhibitor, U0126. As shown in Figure 3B, LPS stimulated c-Jun recruitment to the *Fra-1* promoter in as early as 30 minutes, and the binding remained elevated above the basal level at 60 minutes. However, LPS-stimulated c-Jun recruitment to the *Fra-1* promoter was significantly lower in cells treated with U1026 at both 30 and 60 minutes as compared with their vehicle-treated counterparts. These results support an important role for ERK1/2 signaling in regulating c-Jun binding to the *Fra-1* promoter in alveolar macrophages after LPS stimulation.

NF-κB Regulates LPS-Induced *Fra-1* Promoter Activation

The DNA sequence analysis revealed the presence of a κB-like site adjacent to the AP-1 binding site, TRE (Figure 1A). Thus, to determine whether or not NF-κB regulates LPS-induced *Fra-1* transcription, cells transfected with the *Fra-1* promoter reporter (213-Luc) construct were incubated with either vehicle or NF-κB inhibitor, BAY 11-7082, for 30 minutes and then were treated with LPS, and promoter activity was analyzed. As shown in Figure 4A, LPS-stimulated *Fra-1* promoter activity was markedly lower in cells treated with BAY 11-7082 than in vehicle-treated counterparts. In agreement with this result, LPS-stimulated *Fra-1* mRNA expression (Figure 4B) was significantly lower in cells treated with NF-κB inhibitor. We next performed ChIP assays to determine whether or not NF-κB binds directly to the promoter and up-regulates LPS-stimulated *Fra-1* transcription (Figure 4B). Soluble cross-linked chromatin was prepared from vehicle- and LPS-treated cells incubated with BAY 11-7082 and immunoprecipitated with NF-κB antibodies, and ChIP assays were performed, as detailed above. ChIP assays

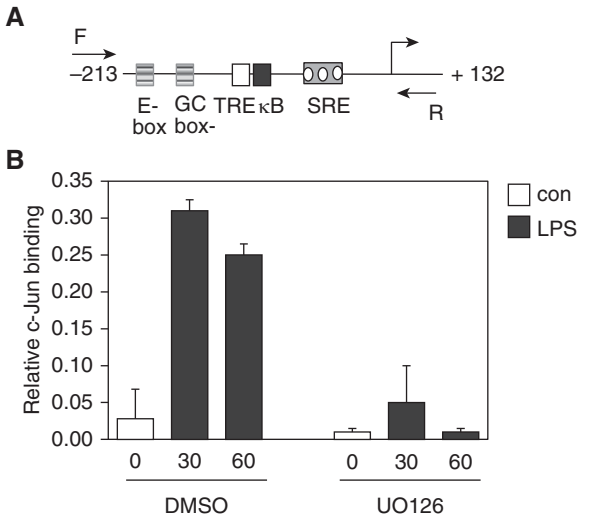

Figure 3. Extracellular signal-regulated protein kinase1/2 signaling regulates c-Jun recruitment to the *Fra-1* promoter. (*A*) *Arrows* indicate the positions of the primers used for chromatin immunoprecipitation assays to analyze protein binding to the endogenous *Fra-1* promoter. (*B*) Cells were incubated with U0126 for 30 minutes and then treated with LPS for 0, 30, or 60 minutes, and chromatin protein–DNA complexes were cross-linked with formaldehyde. The purified nucleoprotein complexes were immunoprecipitated with c-Jun antibody or nonimmune IgG, and precipitated DNA was amplified by quantitative reverse transcription–polymerase chain reaction as detailed in METHODS. Data are presented as mean ± SD ($n = 2$). Experiment was repeated to obtain reproducible results. F, forward; R, Reverse.

revealed that the binding of NF-κB at the *Fra-1* promoter was very low or undetectable in unstimulated cells (Figure 4C). However, LPS-stimulated NF-κB recruitment to the *Fra-1* promoter was detectable in as early 30 minutes, and it remained above the basal level for up to 60 minutes. As anticipated, the level of LPS-induced NF-κB binding to the *Fra-1* promoter was reduced in cells treated with BAY 11-7082. These results support a direct role for NF-κB in LPS-inducible Fra-1 expression in alveolar macrophages.

Inhibition of NF-κB or ERK1/2 Signaling Blocks LPS-Induced c-Jun and NF-κB Recruitment to the *Fra-1* Promoter

The data above demonstrated the involvement of NF-κB and c-Jun in the regulation of *Fra-1* transcription. We performed ChIP assays to determine whether ERK1/2 signaling modulates LPS-stimulated NF-κB binding to the *Fra-1* promoter and whether inhibition of NF-κB signaling affects the c-Jun binding. Soluble cross-linked chromatin isolated from the BAY 11-7082 and U0126-treated cells were immunoprecipitated with anti–c-Jun or anti–NF-κB antibodies, respectively, and ChIP assays were performed. As shown in Figure 5, LPS-stimulated c-Jun binding at the Fra-1 promoter was reduced in cells treated with BAY 11-7082 (Figure 5A). Likewise, the NF-κB recruitment to the *Fra-1* promoter induced by LPS was decreased in cells treated with U0126, as compared with vehicle-treated counterparts (Figure 5B). These results indicate that LPS-stimulated NF-κB and c-Jun binding to the *Fra-1* promoter are mutually regulated by NF-κB and ERK1/2 signaling in alveolar macrophages.

Inhibition of ERK1/2 Signaling Affects NF-κB Phosphorylation but not c-Jun Activation

To determine whether ERK1/2 signaling by regulating c-Jun activation modulates *Fra-1* expression in response to LPS in alveolar macrophages, cells were treated with LPS for 0–90 minutes with and without UO126. Cell lysates were prepared, blotted onto the membranes, and then probed with phospho-specific and native c-Jun antibodies. As shown in Figure 6, LPS-stimulated c-Jun phosphorylation can be detectable in as early as 15 minutes, but ERK1/2 inhibition had no

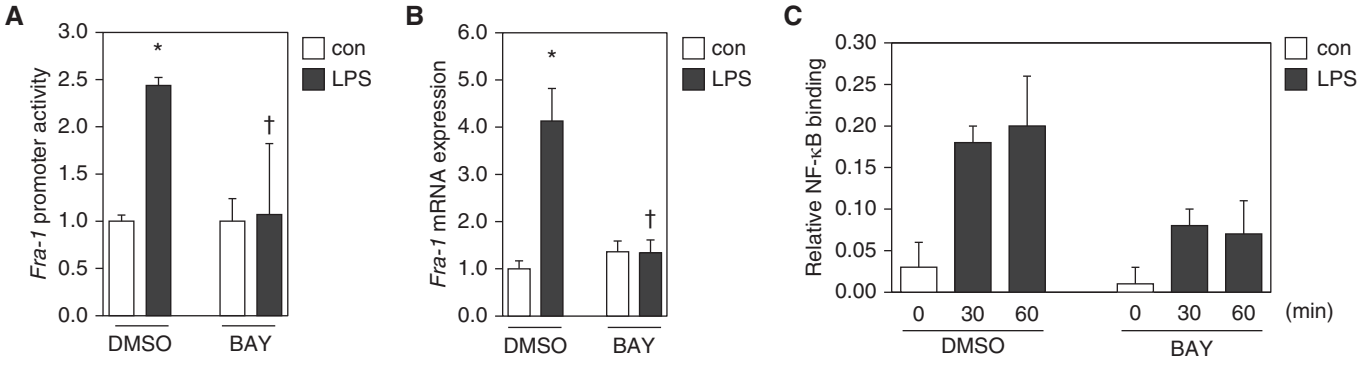

Figure 4. Nuclear factor-κB (NF-κB) regulates LPS-induced *Fra-1* transcription in alveolar macrophages. (*A*) Cells were transfected with −213/+132 bp *Fra-1* promoter reporter construct together with the Renilla luciferase (pRL-TK) plasmid. After 24 hours of transfection, cells were incubated with BAY 11-7082 (BAY) for 30 minutes and then treated with LPS for 6 hours. The activation of reporter activity by LPS was calculated on the basis of the values obtained with vehicle (PBS)-treated cells as 1.0. Data are presented as mean ± SD ($n = 2$). Experiment was repeated to obtain reproducible results. (*B*) MH-S cells were incubated with BAY for 30 minutes and then treated with LPS for 6 hours, and *Fra-1* mRNA expression was analyzed by real-time polymerase chain reaction. Data are presented as mean ±SEM ($n = 3$); $^{*}P \leq 0.05$, PBS versus LPS; $^{\dagger}P \leq 0.05$, vehicle versus BAY. (*C*) Cells were incubated with BAY for 30 minutes and then treated with LPS for 0, 30, or 60 minutes; chromatin was cross-linked and immunoprecipitated with NF-κB antibodies, and chromatin immunoprecipitation assays were performed as in Figure 3. Data are presented as mean ± SD ($n = 2$). Experiment was repeated to obtain reproducible results.

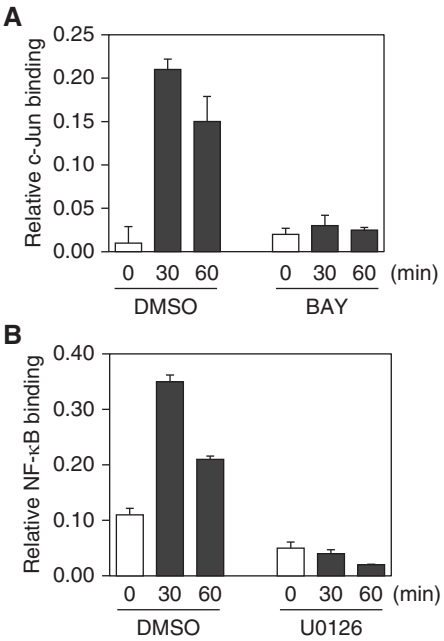

Figure 5. NF-κB inhibition blocks c-Jun recruitment to the *Fra-1* promoter, and extracellular signal-regulated protein kinase 1/2 inhibition blocks NF-κB binding. Cells incubated with (*A*) BAY or (*B*) U0126 were stimulated with LPS for 0–60 minutes; chromatin was cross-linked, immunoprecipitated with either anti–NF-κB or anti–c-Jun antibodies, and amplified by quantitative reverse transcription–polymerase chain reaction as in Figure 3. Data are presented as mean ± SD ($n = 2$). Experiment was repeated to obtain reproducible results.

effect on c-Jun activation. In contrast, inhibition of ERK1/2 signaling reduced LPS-stimulated NF-κB phosphorylation. These results indicate that ERK1/2 signaling by modulating NF-κB, and not c-Jun activation (phosphorylation), regulates Fra-1 induction by LPS in alveolar macrophages.

Reduced Levels of LPS-Induced NF-κB Regulated Inflammatory Cytokine Expression in Fra-1–Deficient Alveolar Macrophages *In Vivo*

To define the role of Fra-1 in regulating LPS-stimulated NF-κB–dependent cytokine expression in alveolar macrophages *in vivo*, *Fra-1*$^{+/+}$ and *Fra-1*$^{\Delta/\Delta}$ mice were treated intratracheally with LPS for 3 hours, and alveolar macrophages from their lungs were isolated for gene expression analysis, as outlined in schema (Figure 7A). We chose this time point to minimize inflammatory cell recruitment into the lung in our experimental conditions (11). NF-κB–regulated proinflammatory cytokine expression levels were quantitated by qRT-PCR (Figure 7B). We choose IL-1β, IL-6, macrophage inflammatory protein (MIP)-1α, and TNF-α for our analysis because they are putative transcriptional targets of NF-κB, and they are known to modulate inflammatory responses in the lung. As expected, LPS stimulated IL-1β, MIP-1α, and TNF-α, but not IL-6, expression in alveolar macrophages of *Fra-1*$^{+/+}$ mice. However, IL-1β and MIP-1α expression stimulated by LPS was significantly lower in alveolar macrophages of *Fra-1*$^{\Delta/\Delta}$ mice, whereas TNF-α expression was modestly decreased (Figure 7A). In contrast, antiinflammatory IL-10, but not transforming growth factor-β, expression stimulated by LPS was significantly increased in *Fra-1*$^{\Delta/\Delta}$ macrophages but not in wild-type counterparts (Figure 7B). Increased expression of *Fra-1* in alveolar macrophages was verified in LPS-treated *Fra-1*$^{+/+}$ mice (Figure 7C). These results suggest that Fra-1 selectively regulates LPS-induced NF-κB–regulated inflammatory cytokine expression in alveolar macrophages.

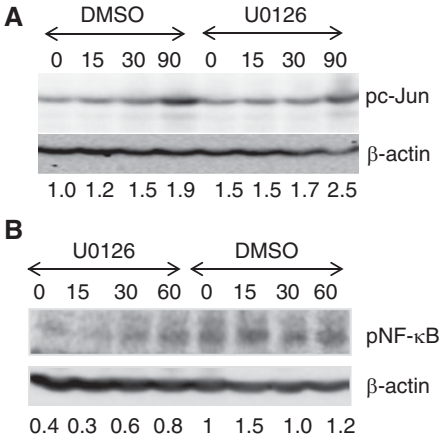

Figure 6. Extracellular signal-regulated protein kinase 1/2 inhibition blocks LPS-stimulated NF-κB phosphorylation. MH-S cells were incubated with U0126 for 30 minutes and then treated with LPS for 0–90 minutes. Total lysates were isolated, blotted onto membrane, and probed with phospho-c-Jun (serine 65) (*A*) or phospho-NF-κB (pNF-κB) (p65, serine 536) (*B*) antibodies. Membranes were stripped and probed with β-actin antibody to demonstrate equal protein loading. Relative band intensities from a representative blot ($n = 2$) are shown.

Discussion

We showed recently that increased expression of *Fra-1* occurs largely in alveolar macrophages in human lungs infected with bacteria *ex vivo* (11) and in the lungs of mice treated with LPS (11). Moreover, using a transgenic mouse model bearing human *Fra-1* promoter, we showed that LPS-stimulated *Fra-1* transcriptional induction occurs predominantly in alveolar macrophages *in vivo* (11). This study demonstrates that both NF-κB and c-Jun coregulate LPS-stimulated *Fra-1* transcription in alveolar macrophages. Importantly, we showed that inhibition of either NF-κB signaling or ERK1/2 pathway blocks the recruitment of both c-Jun and NF-κB transcription factors to the endogenous *Fra-1* promoter after LPS stimulation. These results suggest a requirement for both NF-κB and ERK1/2 signaling for LPS-stimulated *Fra-1* induction mediated by NF-κB and c-Jun in alveolar macrophages. In earlier studies, we reported that mice lacking *Fra-1* showed reduced expression levels of several inflammatory cytokines in the lung (8). Consistent with these earlier observations, reduced levels of NF-κB–regulated IL-1β and MIP-1α expression and increased expression of IL-10 in alveolar macrophages of *Fra-1*–null mice treated with LPS were found (Figure 7), suggesting that Fra-1 selectively up-regulates LPS-induced NF-κB–dependent proinflammatory cytokine expression and dampens the antiinflammatory response in alveolar macrophages during inflammatory lung injury.

Deletion analysis of the *Fra-1* promoter revealed that the −92/+132 bp (92-Luc) and −132/+132 bp (132-Luc) regions mediate minimal and basal promoter activities, respectively. The −92/+132 bp promoter containing the SRE lacks the necessary elements required for LPS inducibility, because its activity in LPS-stimulated cells is similar to that in unstimulated cells (Figure 1). The −132/+132 bp promoter harboring TRE and κB motifs, in addition to the SRE, showed a modest response to LPS, suggesting additional *cis*-acting elements are required for LPS inducibility. The −213/+132 bp promoter (213-Luc), which bears the GC box (Sp1 site), in addition to TRE, κB, and SRE motifs, showed a stronger response to LPS compared with the 132-Luc reporter, although both constructs displayed similar levels of promoter activity in the unstimulated state (Figure 1). Thus, a cooperative interaction

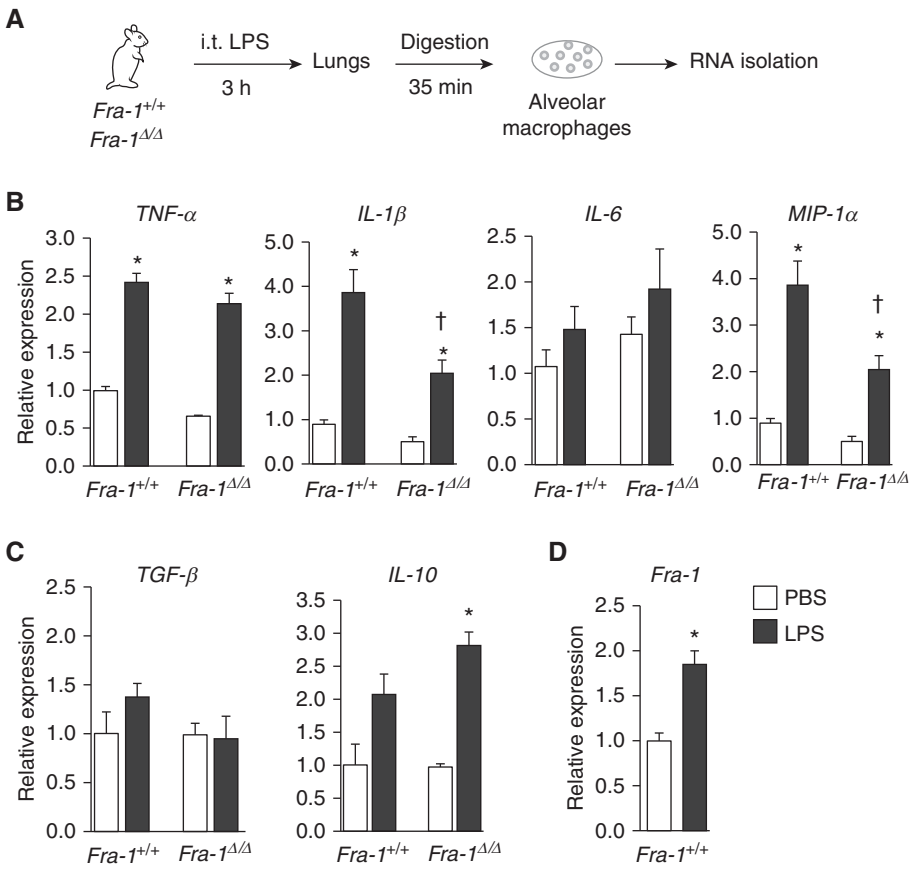

Figure 7. The effects of Fra-1 deficiency on LPS-stimulated NF-κB–dependent cytokine expression in lung alveolar macrophages *in vivo*. (*A*) *Fra-1*$^{+/+}$ and *Fra-1*$^{\Delta/\Delta}$ mice were intratracheally (i.t.) instilled with PBS or LPS (10 μg/mouse) ($n = 4$) and killed after 3 hours, and alveolar macrophages from their lungs were isolated as outlined in the schema *A*. Analysis of LPS-stimulated (*B*) proinflammatory and (*C*) antiinflammatory cytokine expression in alveolar macrophages of *Fra-1*$^{+/+}$ and *Fra-1*$^{\Delta/\Delta}$ mice treated with LPS. (*D*) *Fra-1* mRNA expression in alveolar macrophages of *Fra-1*$^{+/+}$ mice treated with LPS. Data are presented as mean ± SEM ($n = 3$–4). *$P < 0.05$, PBS versus LPS; †$P < 0.05$, *Fra-1*$^{+/+}$ versus *Fra-1*$^{\Delta/\Delta}$ genotype. MIP-1α, macrophage inflammatory protein 1-α; TGF-β, transforming growth factor-β.

between proteins binding to multiple *cis* elements of the −213/+132 bp promoter is involved in the regulation of LPS-induced *Fra-1* transcription in alveolar macrophages. These results are in agreement with those of our previous studies demonstrating the requirement of multiple regulatory elements, such as ETS-binding motif, GC box, TRE, and SRE for mitogen (e.g., phorbol 12-myristate 13-acetate and epidermal growth factor)- or cytokine (e.g., TNF-α)-inducible *FRA-1* transcription in human lung epithelial cells (13–15). The *FRA-1* (human) promoter also contains the NF-κB binding site adjacent to the TRE (*see* Figure E1 in the online supplement), in a manner identical to that of the *Fra-1* (mouse) promoter (Figure 1). Promoter activation studies (Figure E1) revealed an important role of NF-κB and ERK1/2 signaling in the regulation of LPS-stimulated *FRA-1* transcription in alveolar macrophages.

NF-κB is one of the major effectors of TLR-4 mediated signaling activated by LPS. It binds to the κB site and up-regulates the expression of several inflammatory cytokines and adhesion molecules (17, 18). Several studies have shown the requirement for NF-κB activity in the regulation of AP-1 activation by mitogenic and cytokine stimuli. For example, decreased AP-1 family member expression (e.g., c-Fos and Fos-B) in response to mitogen and cytokine stimuli was reported in mouse embryonic fibroblast cells lacking *IKK1* or *IKK2*, the upstream kinases essential for NF-κB activation (19). Recently, it was shown that *c-Fos* promoter contains a functional κB site, NF-κB and ERK1/2 signaling positively regulates *c-Fos* expression (20). Our results showed that inhibition of NF-κB signaling blocks LPS-stimulated *Fra-1* transcription. Furthermore, ChIP assays demonstrated the recruitment of NF-κB to the *Fra-1* promoter after LPS treatment but not in the presence of NF-κB inhibitor, supporting a direct role of NF-κB in mediating LPS-stimulated *Fra-1* transcription. Likewise, we also found that c-Jun regulates LPS-stimulated *Fra-1* transcription in alveolar macrophages. Previously, we reported enhanced recruitment of c-Jun to the *FRA-1* promoter in human lung epithelial cells treated with mitogenic and cytokine stimuli (13–16). Thus, it appears that c-Jun plays a larger role in regulating both LPS-induced NF-κB–dependent *Fra-1* transcription in alveolar macrophages (current study) and mitogen- and cytokine-stimulated *FRA-1* expression in lung epithelial cells (14, 15).

ERK1/2 signaling regulates both innate and adaptive immune responses, including the regulation of LPS-induced NF-κB–dependent gene expression (21). Our results showed that MEK1/2-ERK1/2 inhibitor blocks LPS-stimulated *Fra-1* promoter activity and mRNA expression, suggesting a role for the ERK1/2 pathway, in addition to NF-κB signaling, in the regulation of *Fra-1* induction by LPS. Although ERK1/2 inhibition blocked LPS-stimulated c-Jun recruitment to the *Fra-1* promoter (Figure 3), it had no effect on c-Jun activation levels (phosphorylation) (Figure 6). This result suggests that ERK1/2 signaling regulates c-Jun recruitment indirectly by affecting the activation of other factors. We showed previously, using ChIP assays, that ERK1/2 signaling plays a crucial role in the activation of ELK-1, serum response factor (SRF), and cAMP response element binding protein (CREB)/activating transcription factors, which are constitutively bound to the functional SRE of the *FRA-1* promoter in pulmonary epithelial cells in response to mitogen (e.g., TPA), toxicant (e.g., cigarette smoke), and cytokine (e.g., TNF-α) stimuli (13–16). But ChIP assays revealed increased c-Jun binding to the *FRA-1* promoter after mitogen or cytokine stimulation. Thus, it is likely that the ERK1/2 pathway controls LPS-stimulated *Fra-1* transcription by activating ELK-1, SRF, and CREB proteins that are constitutively bound

to the SRE, thereby facilitating the c-Jun recruitment to the promoter.

TPL-2 is a major MAPK kinase that is required for LPS/TLR-4–induced MEK1/2-ERK1/2 kinase pathway activation. The activation of TPL-2 in turn occurs through IKK2-mediated phosphorylation (21). Our studies revealed that the ERK1/2 pathway is also required for LPS-stimulated NF-κB recruitment to the *Fra-1* promoter (Figure 5). Inhibition of either ERK1/2 or the NF-κB pathway limits both NF-κB and c-Jun recruitment to the *Fra-1* promoter in LPS-stimulated cells. Previous studies have shown that IKK/NF-κB proteins are putative substrates for ERK1/2 kinases (22). Indeed, inhibition of ERK1/2 caused an attenuation of LPS-stimulated NF-κB phosphorylation (Figure 7). The inability of NF-κB to bind to the *Fra-1* promoter in LPS-treated cells incubated with U0126 suggests that ERK1/2 signaling by modulating NF-κB phosphorylation, in addition to the activation of ELK-1, SRF, and CREB proteins, may facilitate the recruitment of c-Jun to the *Fra-1* promoter. The NF-κB binding-like site is located adjacent to the c-Jun binding site, TRE, of both mouse (Figure 1) and human (Figure E1) *Fra-1* promoters. Previously, it was shown that c-Jun can interact physically with NF-κB, and that this interaction potentiates their DNA binding and functional activities (23). Thus, it is possible that c-Jun binding may facilitate the recruitment of NF-κB or vice versa, enabling mutually inclusive interactions with the other transcription factors binding to the SRE and leading to enhanced transcription. Whether or not such cross-talk exits in regulating the transcriptional induction of *Fra-1* by LPS in alveolar macrophages remains to be investigated.

Conclusions

In summary, to the best of our knowledge, we have provided the first evidence that both NF-κB and c-Jun play a prominent role in regulating LPS-stimulated *Fra-1* transcription in alveolar macrophages. The induction of the *Fra-1* by LPS occurs through the NF-κB and ERK1/2 signaling pathways, which cooperatively recruit NF-κB and c-Jun to the promoter and up-regulate the transcription. Furthermore, our studies suggest that Fra-1 promotes inflammatory lung injury by selectively up-regulating LPS-induced NF-κB–dependent proinflammatory cytokine expression and suppressing the antiinflammatory response in alveolar macrophages. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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