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Lipopolysaccharide-Induced Desensitization of *junB* Gene Expression in a Mouse Macrophage-Like Cell Line, P388D1

Mitsuhiro Fujihara,[1]* Kenji Ikebuchi,* Takami L. Maekawa,[2] Shinobu Wakamoto,* Chikayo Ogiso,* Takatoshi Ito,* Tsuneo A. Takahashi,[3] Tsuneo Suzuki,† and Sadayoshi Sekiguchi*

Treatment of a mouse macrophage cell line, P388D1, for 1 h with bacterial LPS caused a transient increase in the level of *junB* mRNA expression. These cells became refractory in terms of the *junB* gene response to exposure to a second round of LPS or lipid A, but not to PMA. The LPS-induced desensitized state was not due to the shortening of the half-life of *junB* mRNA, but was suggested, by nuclear run-on analysis, to be caused by reduction of *junB* gene transcription. Pretreating cells with herbimycin A, a tyrosine kinase inhibitor, substantially inhibited LPS-induced expression of *junB* mRNA and decreased tyrosine phosphorylation of 38- to 42-kDa proteins, which comigrated with p38 and p42 mitogen-activated protein (MAP) kinases. Parallel to down-regulation of *junB* mRNA expression, activation of the p38 MAP kinase was markedly reduced in LPS-tolerant cells, whereas activation of p42 MAP kinase was relatively constant. The specific p38 MAP kinase inhibitor, SB202190, potently inhibited LPS-induced *junB* mRNA expression. These results suggest that the LPS-induced desensitization of *junB* gene expression occurs at or upstream of the level of gene transcription and may be involved in a defective LPS-induced p38 MAP kinase pathway. ***The Journal of Immunology*, 1998, 161: 3659–3665.**

The product of the *junB* gene is a member of the activating protein-1 (AP-1)[4] family consisting of Fos (c-Fos, FosB, Fra-1, and Fra-2) and Jun (c-Jun, JunB, and JunD). AP-1 proteins in various combinations transactivate target genes that possess 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-responsive elements (TRE) in their promoters (1–3). A wide variety of extracellular stimuli, such as serum, growth factors (3, 4), cytokines (5–8), tumor promoters (9, 10), and intracellular cAMP-elevating agents (9, 11), or cross-linking of B cell surface Igs (12, 13) was shown to rapidly and transiently induce transcription of the *junB* gene in various cell types. JunB failed to activate single TRE site and, in fact, inhibited the activation of such a site by c-Jun (9, 14). On the other hand, like c-Jun, JunB has been shown to activate efficiently constructs containing multimeric TREs (9) and thus potentially can activate genes containing multiple TRE sites in the promoters or enhancers.

LPS is a major component of the outer membrane of Gram-negative bacteria and is a potent activator of macrophage functions (15, 16). LPS is also a potent stimulant of *junB* expression, resulting in the up-regulation of AP-1 element binding activity (17–19). One of the initial events in LPS-triggered macrophage activation, which was suggested to mediate some of the downstream responses to LPS (20–22), is protein tyrosine phosphorylation. Since the members of the mitogen-activated protein (MAP) kinase family (p38, p42/extracellular signal-regulated kinase-2 (ERK2), p44/ERK1, and c-Jun NH_2-terminal kinase (JNK)) are predominantly tyrosine phosphorylated in LPS-stimulated macrophages (23–26), a MAP kinase pathway has been implied to be involved in LPS-induced cellular responses (19, 23, 27).

Initial exposure of various monocytes, macrophages, or their cell lines to a submaximal dose of LPS resulted in a state of refractoriness to the secondary maximal dose of LPS exposure (28–34). The macrophages in an LPS-tolerant state typically respond to a secondary LPS stimulation with a much lesser level of response (such as LPS-induced cytokine production) than the level induced by an initial stimulation. In vitro models for studying endotoxin tolerance in macrophage or monocytic cell lines have been developed (28–35), although none of these models completely mimicks in vivo endotoxin tolerance. However, studying such systems provides valuable information regarding the biochemical signal pathway of endotoxin response (28–35). In this paper the molecular basis of in vitro LPS tolerance was examined using LPS-induced expression of the *junB* gene in a mouse macrophage cell line, P388D1, because the LPS-triggered *junB* gene expression in P388D1 cells was found to be transient (17). The results presented show that LPS-induced desensitization of *junB* gene expression 1) occurs at or upstream of the level of gene transcription, and 2) may be involved in the defective LPS-induced p38 MAP kinase pathway.

Materials and Methods

Cell culture and reagents

The mouse macrophage-like cell line, P388D1, which was isolated from a methylcholanthrene-induced lymphoid neoplasm of a DBA/2 mouse and

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[4] Abbreviations used in this paper: AP-1, transcription factor activating protein-1; TRE, 12-*O*-tetradecanoylphorbol 13-acetate response element; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; CRE, cyclic adenosine 3′,5′-monophosphate response element.

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has been shown to possess characteristics typical of macrophages, was obtained from the Japanese Cancer Research Resources Bank. P388D1 cells were grown in RPMI 1640 medium containing 10% heat-inactivated FBS supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Herbimycin A and PB202190 were purchased from Calbiochem-Novabiochem (La Jolla, CA).

Northern blot analysis

Northern blot analysis was conducted as previously described (17). P388D1 cells (1×10^7/experiment) were treated as indicated in the text and were then washed with cold PBS, quickly frozen, and stored at −80°C until use. Total cellular RNA was extracted from the cells using the guanidinium thiocyanate procedure (36). The isolated RNA (10 μg) was electrophoresed in 1% agarose gels containing 2.2 M formaldehyde and then transferred to Nytran nylon membranes (Schleicher and Schuell, Dassel, Germany) by capillary action using 10× SSC. The membranes were prehybridized for at least 2 h at 42°C in a solution containing 50% (v/v) formamide, 5× SSPE, 5× Denhardt's solution, 0.5% SDS, 0.01 M EDTA, and 100 μg/ml salmon sperm DNA. Membranes were then incubated for 20 h at 42°C in the same solution with ^{32}P-labeled cDNA probes specific for *junB* (3) previously labeled by the random hexamer priming method using [α-^{32}P]dCTP (Amersham, Aylesbury, U.K.). Membranes were then washed twice at 25°C in 0.1% SDS and 2× SSPE for 30 min and twice at 42°C in 0.1% SDS and 0.1× SSPE, and autoradiographed on Kodak XAR-5 film (Eastman Kodak, Rochester, NY) at −80°C with intensifying screens. Membranes were then stripped and reprobed with ^{32}P-labeled β-actin cDNA (37).

Nuclear run-on assay

P388D1 cells (~5×10^7/experiment) were treated with or without LPS (1 μg/ml) for 1 h. After washing twice with LPS-free medium, cells were incubated for 2 h in LPS-free medium, then stimulated with or without LPS (1 μg/ml) for 1 h at 37°C. Isolation of nuclei and nuclear run-on transcription assays were performed as previously described (38). The plasmids used in these experiments included pGEM-2 vector alone; pGEM-2, a vector containing a 1.0-kb cDNA insert of mouse *junB* (3), pHA4.1, a vector containing a 3.5-kb cDNA insert of human β-actin (37); pG3PDH, a vector containing a 1.2-kb cDNA insert of human glyceraldehyde-3-phosphate dehydrogenase (39); and pMuTNF, a vector containing a 1.0-kb cDNA insert of mouse TNF-α.

mRNA stability analysis

P388D1 cells (1×10^7/experiment) were treated with or without LPS (1 μg/ml) for 1 h. After washing twice with LPS-free medium, cells were first incubated for 2 h in LPS-free medium, then stimulated with LPS (1 μg/ml) for 1 h at 37°C. At the end of the incubation period, actinomycin D was added at a final concentration of 5 μg/ml. At various times after the addition of actinomycin D, total RNA was isolated, and 10 μg of each sample was subjected to Northern blot analysis.

Western blot analysis

The cells treated with LPS were rapidly chilled on ice, washed twice with ice-cold PBS containing 2 mM sodium orthovanadate (Na_3VO_4), and then lysed in 1 ml of lysis buffer (1% Triton X-100, 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM Na_3VO_4, 10 mM NaF, 2 mM PMSF, 100 μg/ml leupeptin, and 100 μg/ml aprotinin) for 15 min on ice. The lysate was centrifuged ($10{,}000 \times g$, 15 min, 4°C), and the clear supernatant was subjected to electrophoresis in 5 to 20% SDS-polyacrylamide gels. The proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane (Schleicher and Schuell) using an electrophoretic transfer cell. Subsequently, the nitrocellulose membrane was blocked with TBST (10 mM Tris, 150 mM NaCl, and 0.05% Tween-20, pH 8.0) containing 2% BSA overnight at room temperature. The membrane was then incubated with TBST containing the relevant Abs for 3 h. The primary Abs used were the monoclonal anti-phosphotyrosine Ab (clone 4G10, Upstate Biotechnology, Lake Placid, NY), the monoclonal anti-p42/44 MAP kinase (New England Biolabs, Beverly, MA), and the monoclonal anti-p38 MAP kinase (New England Biolabs) at a 1/1000 dilution. The membrane was then rinsed with three changes of TBST, once for 15 min and twice for 5 min, incubated with horseradish peroxidase-conjugated goat anti-mouse IgG at a 1/4000 dilution for 1 h at room temperature, washed three times, and developed using an enhanced chemiluminescence system (Amersham). Membranes were stripped of the primary Ab-secondary Ab complex by incubating them in stripping buffer (100 mM 2-ME, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7) for 30 min at 50°C. To detect activated forms of p42/44 MAP kinase and p38 MAP kinase, Abs specifically recognized p42/44 MAP kinase phosphorylated on Thr^{202} and Tyr^{204} or Abs that specifically recognize Thr^{180} and Tyr^{182} on p38 MAP kinase were purchased from New England Biolabs and used in immunoblot analyses. Dual phosphorylation on Thr^{202} and Tyr^{204} is diagnostic for activated p42/44 MAP kinase, and dual phosphorylation on Thr^{180} and Tyr^{182} is diagnostic for activated p38 MAP kinase. Detection of Ab binding was conducted as described in the manufacturer's instructions using the Photope chemiluminescent detection system (New England Biolabs).

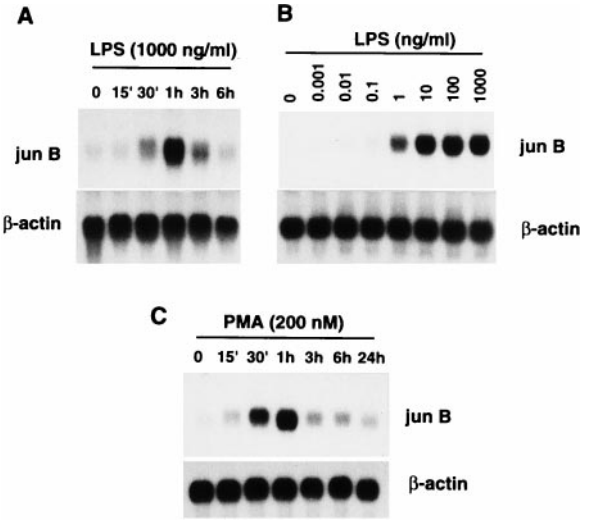

FIGURE 1. Induction of *junB* mRNA expression in P388D1 cells exposed to LPS or PMA. P388D1 cells (1×10^7 cells) were treated with LPS (1 μg/ml; *A*) or PMA (200 nM; *C*) for varying periods of time (0–6 h) or were treated for 1 h with LPS at various doses (0–1000 ng/ml; *B*). Following each treatment, total RNA extracted from the cells was analyzed by Northern blotting for the expression of *junB* mRNA. The same membrane was stripped and reprobed with cDNA specific for β-actin mRNA to verify the level of RNA in each lane. Additional experiments gave similar results.

Results

Treatment with LPS or PMA of P388D1 cells transiently increases junB *mRNA expression*

The kinetics and dose-response characteristics of junB mRNA expression in P388D1 cells in response to LPS treatment were first examined by Northern analysis. P388D1 cells (1×10^7) were incubated with LPS (1 μg/ml) for varying periods of time (0–6 h). Northern blot analysis of the total RNAs extracted from the cells (Fig. 1*A*) showed that the expression of *junB* mRNA was increased as early as 30 min after LPS treatment, peaked at 60 min, and had declined substantially by 3 h. The degree of *junB* mRNA expression 1 h after LPS exposure was also dependent on the dose of LPS; the minimum LPS concentration necessary to induce the detectable *junB* mRNA level was about 1 ng/ml (Fig. 1*B*). The exposure of cells to PMA also markedly increased the expression of *junB* mRNA by 30 min, further increased expression by 1 h, then decreased expression almost to the constitutive level by 3 h (Fig. 1*C*).

Prior exposure to LPS reduces junB *gene expression in response to secondary LPS treatment*

Next, the question of how repeated exposure of P388D1 cells to LPS would affect *junB* gene expression was investigated. To this end, P388D1 cells were exposed for 1 h at 37°C to varying doses of LPS (10 pg/ml to 1 μg/ml). The cells were then washed, incubated for an additional 2 h in LPS-free medium, and restimulated with 1 μg/ml of LPS for 1 h. Northern analysis of total RNAs extracted from the cells (Fig. 2*A*) showed that the cells first exposed to >1 ng/ml LPS did not respond to the secondary LPS

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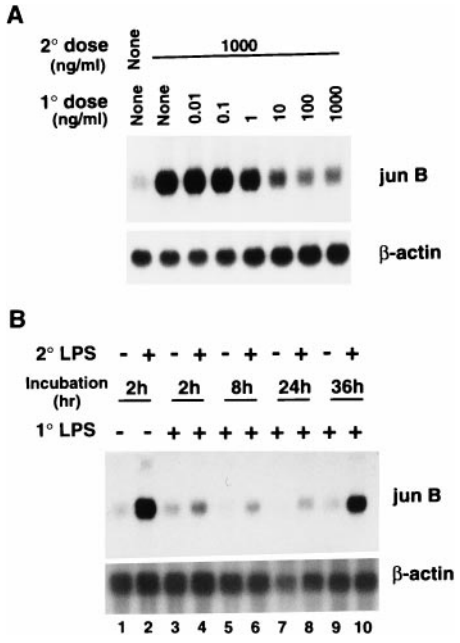

FIGURE 2. *A*, Effect of pretreatment with LPS on *junB* mRNA expression in response to secondary exposure to LPS. P388D1 cells (1×10^7 cells) were treated for 1 h with varying concentrations (10 pg/ml to 1 μg/ml) of LPS. The cells were then washed twice in PBS, incubated for 2 h in LPS-free medium, and restimulated with 1 μg/ml LPS for 1 h. *B*, Recovery of junB expression following pretreatment with LPS. P388D1 cells (1×10^7 cells) were untreated (*lanes 1* and *2*) or treated (*lanes 3–10*) for 1 h with 1 μg/ml LPS. The cells were washed twice in PBS, incubated in LPS-free medium for 2 h (*lanes 1–4*), 8 h (*lanes 5* and *6*), 24 h (*lanes 7* and *8*), and 36 h (*lanes 9* and *10*). Cells were then treated with (*lanes 2*, *4*, *6*, *8*, and *10*) or without (*lanes 1*, *3*, *5*, *7*, and *9*) 1 μg/ml LPS for 1 h. Total RNA extracted from the cells was subjected to Northern analysis using the *junB* cDNA probe. The same membrane was stripped and rehybridized with β-actin cDNA. Additional experiments gave similar results.

stimulation by *junB* gene activation, whereas the initial exposure of cells to <1 ng/ml LPS did not substantially alter *junB* mRNA expression in response to the secondary exposure. Next, we examine how long after washing is required for recovery of junB expression by restimulation. Figure 2*B* shows that responsiveness was almost completely recovered by 36 h after the initial stimulus. To examine whether this observed refractory response is restricted to LPS, the capacities of PMA and lipid A as secondary inducers of *junB* mRNA were tested by exposing the control and LPS-primed cells to LPS, PMA, or lipid A. Northern analysis of total RNA extracted from these cells (Fig. 3) showed that the cells exposed to a primary LPS treatment displayed the expected hyporesponsiveness to secondary LPS challenge (compare *lane 3* to *lane 4*). The initial exposure of cells to LPS did not change the response of the *junB* gene to PMA (*lane 6* vs *lane 5*). In contrast, LPS-tolerant cells showed marked reduction in response to treatment with lipid A, as observed for LPS (*lane 8* vs *lane 7*).

Down-regulation of junB *gene expression in LPS-tolerant cells occurs at or upstream of the* junB *gene transcription level*

We speculated that the decreased steady state level of *junB* mRNA in LPS-tolerant cells may have been due to an increased rate of degradation of *junB* mRNA. To test this possibility, LPS-tolerant and control cells were first incubated for 1 h with 1 μg/ml LPS. At the end of the incubation period, actinomycin D was added at 5 μg/ml. Total RNA was extracted from the cells at 0, 15, 30, 60, and 90 min after the addition of actinomycin D. As shown in

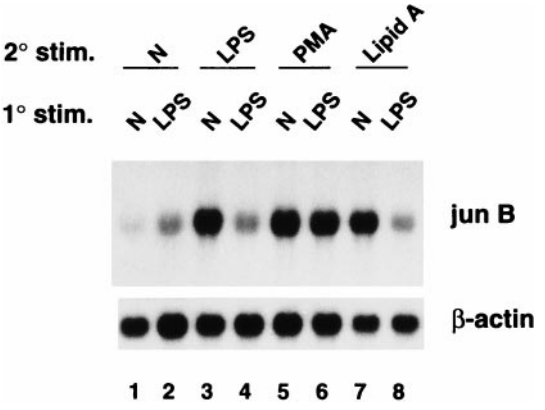

FIGURE 3. Effect of pretreatment with LPS on *junB* mRNA expression in response to LPS, PMA, or lipid A. P388D1 cells were incubated for 1 h in the absence (*lanes 1*, *3*, *5*, and *7*) or the presence (*lanes 2*, *4*, *6*, and *8*) of LPS (1 μg/ml), washed twice, and incubated for 2 h in LPS-free medium. Then, cells were left unstimulated (*lanes 1* and *2*) or were stimulated with 1 μg/ml LPS (*lanes 3* and *4*), 200 nM PMA (*lanes 5* and *6*), or 1 μg/ml lipid A (*lanes 7* and *8*) for an additional 1 h. Total RNA extracted from the cells was analyzed by Northern blotting using *junB* cDNA. The same membrane was stripped and reprobed with β-actin cDNA. Results are representative of five experiments.

Figure 4, *junB* mRNA expression was reduced 30 min after the addition of actinomycin D in both LPS-tolerant and control cells, suggesting no significant difference in *junB* mRNA half-life between control and LPS-tolerant cells.

Next, the question of whether the reduced level of steady state *junB* mRNA observed in LPS-tolerant cells is due to a decrease in transcription was examined by nuclear run-on analysis, as follows. P388D1 cells were incubated for 1 h with or without 1 μg/ml LPS. Cells were then washed, incubated for 2 h in LPS-free medium, and incubated for 0, 15, 30, and 60 min with 1 μg/ml LPS. Nuclei were isolated from the cells and incubated with [α-^{32}P]UTP. Figure 5 shows that treatment of control cells with LPS markedly enhanced transcription of the *junB* gene over the constitutive level, with a peak at 30 min and a decline thereafter (*lanes 1–4*). In

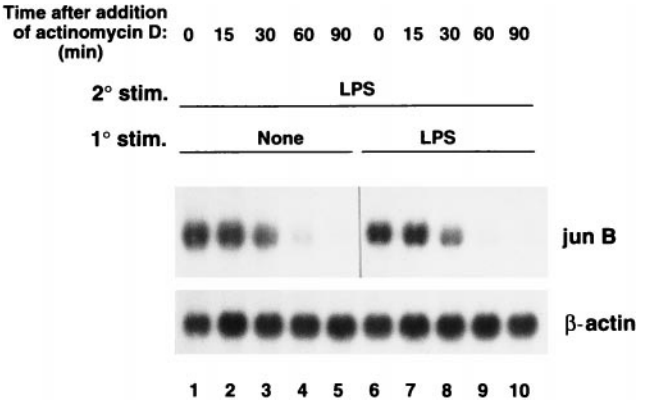

FIGURE 4. Stability of *junB* mRNA. P388D1 cells were incubated for 1 h in the absence (*lanes 1–5*) or the presence (*lanes 6–10*) of 1 μg/ml LPS, washed twice, incubated for 2 h in LPS-free medium, then incubated for an additional 1 h without (*lanes 1–5*) or with 1 μg/ml LPS (*lanes 6–10*). Cells were further incubated with actinomycin D (5 μg/ml) for varying periods (0–90 min). Total RNA isolated at various times after addition of actinomycin D was successively probed with cDNAs specific for *junB* and β-actin, respectively. *Lanes 6* to *10* were exposed longer to allow visualization of the weak *junB* gene signal. Results are representative of three experiments.

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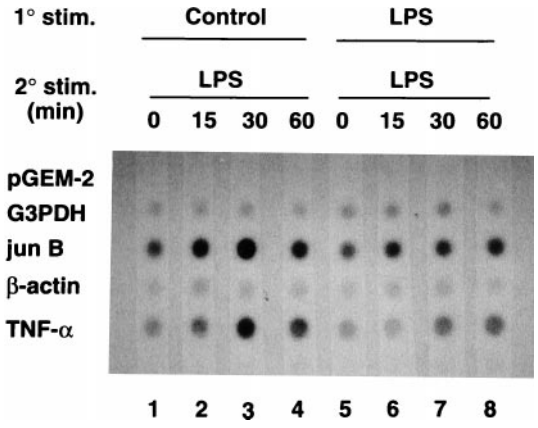

FIGURE 5. Nuclear run-on transcription analysis. P388D1 cells ($\sim 5 \times 10^7$ cells) were incubated in the absence (*lanes 1–4*) or the presence (*lanes 5–8*) of LPS (1 μg/ml), washed twice, and incubated for 2 h in LPS-free medium. Cells were then incubated for 0 min (*lanes 1* and *5*), 15 min (*lanes 2* and *6*), 30 min (*lanes 3* and *7*), or 60 min (*lanes 4* and *8*) with LPS (1 μg/ml). Nuclei were isolated, and nuclear RNA was radiolabeled by incubation with [α-^{32}P]UTP. Radiolabeled RNA was purified and used to probe equal amounts (5 μg/dot) of denatured plasmids blotted onto Nytran membranes. Additional experiments gave similar results.

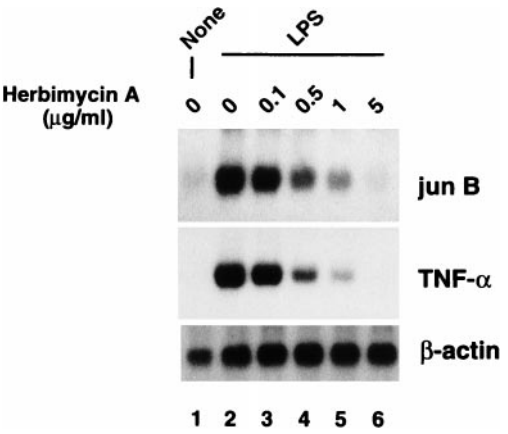

FIGURE 6. Inhibition of LPS-induced *junB* expression by pretreatment with herbimycin A. P388D1 cells were pretreated with the indicated concentrations of herbimycin A for 4 h. Then cells were left unstimulated or were treated with LPS (1 μg/ml) for 1 h. Total RNA extracted from the cells was analyzed by Northern blotting.

contrast, the increase in *junB* gene transcriptional activity in LPS-tolerant cells after secondary stimulation with LPS was very small (*lanes 5–8*). A similar transcriptional pattern was observed for TNF-α. In fact, in this cell line, TNF-α expression occurred in response to LPS with a peak at 1 h, and then quickly declined to the basal level. A similar desensitization phenomenon was detected for TNF-α (data not shown). The transcriptional levels of housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase and β-actin were almost unchanged in response to LPS. These results suggest that the decreased levels of LPS-induced steady state *junB* mRNA in these cells were primarily due to decreased transcription.

Effect of herbimycin A on LPS-triggered junB *gene expression*

The tyrosine phosphorylation of several proteins catalyzed by tyrosine kinases has been shown to be involved in LPS-induced cytokine gene activation and cytokine production (20–22). It follows that one of the upstream events associated with LPS-induced *junB* gene transcription may require tyrosine phosphorylation through tyrosine kinases. To test this possibility, the effect of herbimycin A, a potent tyrosine kinase inhibitor (40), on the LPS-induced expression of *junB* mRNA in P388D1 cells was examined, as follows. P388D1 cells were incubated for 4 h with varying concentrations (0.1–5 μg/ml) of herbimycin A and then were incubated for 1 h with or without LPS. The total RNA extracted from the cells was analyzed by Northern blotting. As shown in Figure 6, pretreatment of P388D1 cells with herbimycin for 4 h resulted in a significant inhibition of *junB* gene expression in a dose-dependent manner, suggesting the involvement of tyrosine phosphorylation through tyrosine kinases during LPS-triggered *junB* mRNA expression. In addition to *junB*, herbimycin A inhibited TNF-α expression in response to LPS. Thus, a tyrosine kinase-sensitive step(s) is not a *junB*-specific phenomenon.

Differential effect of p38 MAP kinase and p42/44 MAP kinase in response to LPS in LPS-tolerant cells

The p38 MAP kinase, p42/p44 MAP kinase, and JNK are the most prominent tyrosine-phosphorylated proteins in response to LPS (20–22). Therefore, we tested whether the tyrosine phosphorylation of these proteins would be suppressed in LPS-tolerant cells. First, to examine the effect of LPS on cellular tyrosine phosphorylation in P388D1 cells, the total cell lysates were prepared from P388D1 cells exposed to LPS for 0, 7, 15, or 30 min or to PMA for 15 min. Then, cell lysates were subjected to SDS-PAGE. The proteins separated were electrophoretically transferred to a nitrocellulose membrane and blotted with an anti-phosphotyrosine Ab (4G10). As shown in Figure 7*A* (*left panel*), LPS treatment of cells (*lanes 1–4*) resulted in a time-dependent enhancement of tyrosine phosphorylation of two protein bands of approximately 38 to 42 kDa. LPS-induced tyrosine phosphorylation of these bands was significantly inhibited after pretreatment of P388D1 cells with herbimycin A at doses (0.5–5 μg/ml) that effectively inhibited LPS-induced *junB* gene expression (Fig. 7*B*). To establish whether the 38- to 42-kDa tyrosine-phosphorylated polypeptide was a member of the MAP kinase family, the membrane shown in the *left panel* of Figure 7*A* was reprobed after being stripped of 4G10 Ab using Abs that recognize 42/44 MAP kinase (independent of phosphorylation) or p38 MAP kinase (independent of phosphorylation). The *middle* and *right panels* of Figure 7*A* show that the electrophoretic mobility of the tyrosine-phosphorylated 38- to 42-kDa protein appeared to coincide with those of p38 MAP kinase and p42 MAP kinase, respectively. Although the p42/44 MAP kinase Ab used in this study recognize both p42 and p44 MAP kinases, the expression of p44 MAP kinase was below the detection level in this cell line. In contrast, we observed the tyrosine phosphorylation of three distinct bands of p38, p42, and p44 MAPKs in response to LPS in the mouse macrophage cell line RAW 264.7 (data not shown). The levels of p38 MAP kinase and p42 MAP kinase were relatively unaffected by exposure to LPS or PMA.

To examine the effect of LPS refractoriness on the activation of p38 and p42 MAP kinases, we used Abs that are specific to the activated forms of p38 MAP kinase and p42/44 MAP kinase. Net levels of each MAP kinase were assessed by mAbs that recognize p38 MAP kinase or p42/44 MAP kinase independently of phosphorylation. LPS-tolerant and control cells were separately exposed to LPS for 0, 7, 15, or 30 min. Samples were separated on 12.5% SDS-PAGE. As shown in Figure 8, the activation of p42 MAP kinase in LPS-tolerant cells appeared to increase with time. In contrast, the activation of p38 MAP kinase in response to LPS in LPS-tolerant cells was much less than that in control cells. In each case, the total amount of p38 MAP kinase as well as that of

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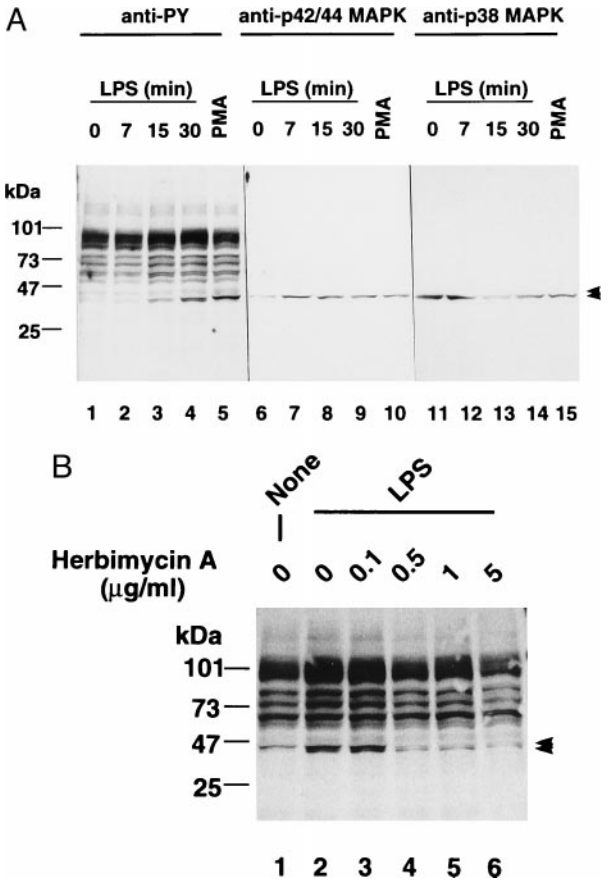

FIGURE 7. Increased tyrosine phosphorylation of 38- to 42-kDa protein in response to LPS (*A*) and effect of pretreatment of herbimycin A (*B*). *A*, *Left panel*, P388D1 cells (1×10^7 cells) were exposed to 1 μg/ml LPS for 0, 7, 15, or 30 min or to 200 nM PMA for 15 min. Proteins from cell lysates were subjected to SDS-PAGE, electrophoretically transferred to membrane, and probed with a monoclonal anti-phosphotyrosine Ab (4G10) followed by detection by enhanced chemiluminescence. *Middle panel*, The same blot as that shown in the *left panel* was stripped and reprobed with an anti-p42/44 MAP kinase mAb. *Right panel*, The same blot as that shown in the *left panel* was stripped and reprobed with an anti-p38 MAP kinase mAb. Arrowheads show p42 MAP kinase and p38 MAP kinase, respectively. *B*, P388D1 cells were pretreated with the indicated concentrations of herbimycin A for 4 h. Then cells were left unstimulated or were treated with 1 μg/ml LPS for 30 min. Arrowheads show p38 and p42/44kDa proteins, respectively.

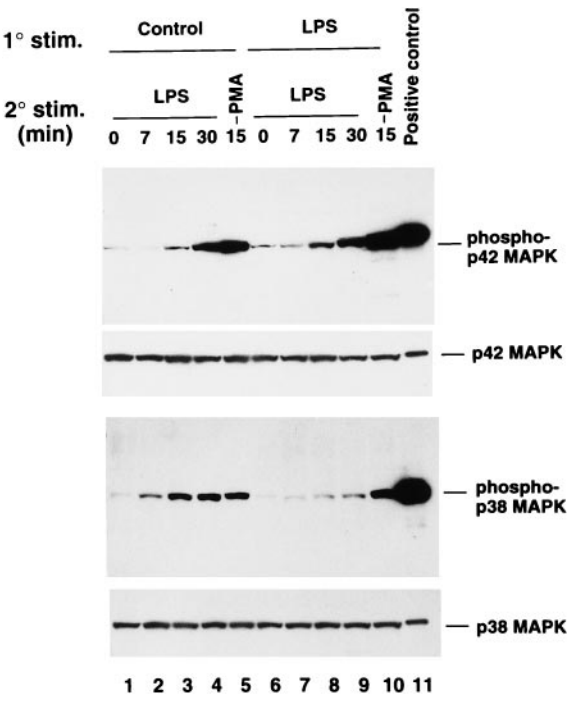

FIGURE 8. Effect of pretreatment with LPS on the activation of p38 MAP kinase and p42/44 MAP kinase in response to subsequent stimulation with LPS or PMA. P388D1 cells (1×10^7 cells) were incubated for 1 h in the absence (*lanes 1–5*) or the presence (*lanes 6–10*) of 1 μg/ml LPS. After washing twice and incubating for 2 h in LPS-free medium, cells were exposed to 1 μg/ml LPS for 0, 7, 15, and 30 min or to 200 nM PMA for 15 min. Cell lysates were subjected to 12.5% SDS-PAGE and blotted. Activation of p42/44 MAP kinase and p38 MAP kinase was determined by dual phosphorylation using phospho-p42/44 MAP kinase- or phospho-p38 MAP kinase-specific Ab. The blots were stripped and reprobed with an anti-42/44 MAP kinase or an anti-p38 MAP kinase Ab and showed similar amounts of all proteins in all lanes. Bacterially expressed, fully phosphorylated ERK2 (p42 MAP kinase) served as the positive control. Cell lysate prepared from the LPS-treated mouse macrophage cell line RAW 264.7 was used as the positive control for phospho-p38 MAP kinase. All experiments were repeated three times.

p42 MAP kinase were unaffected in response to stimulation. Thus, the LPS-tolerant state appeared to differentially affect the activation of p38 MAP kinase and p42 MAP kinase.

Effect of PB202190 on LPS-triggered junB *gene expression*

The above findings indicated that the down-regulated *junB* expression in response to LPS may be associated with reduced activation of p38 MAP kinase in LPS-tolerant cells. Since the role of p38 MAP kinase in the up-regulation of the *junB* gene in response to LPS had not been established, we evaluated whether PB202190, a potent p38 MAP kinase inhibitor (19), inhibits the LPS-triggered *junB* mRNA expression in P388D1 cells. P388D1 cells were incubated for 1 h with varying concentrations (0.1–10 μM) of PB202190 and then incubated for 1 h with or without LPS. As shown in Figure 9, inhibition of p38 MAP kinase by PB202190 markedly suppressed LPS-induced *junB* mRNA expression, suggesting that the p38 MAP kinase pathway is required for LPS-triggered *junB* mRNA expression. These results indicate that the reduced response to LPS in the *junB* gene expression may be due at least in part to the diminished p38 MAP kinase activity in LPS-tolerant cells.

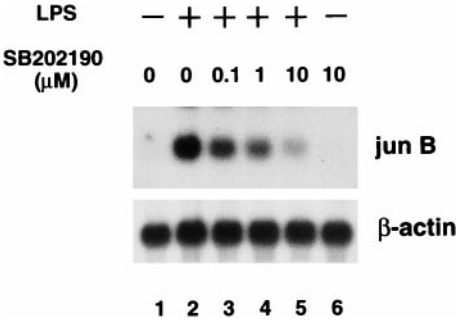

FIGURE 9. Inhibition of LPS-induced *junB* expression by pretreatment with SB202190. P388D1 cells were pretreated with the indicated concentrations of SB202190 for 1 h. Then cells were left unstimulated or were treated with LPS (1 μg/ml) for 1 h. Total RNA extracted from the cells was analyzed by Northern blotting. Results are representative of three experiments.

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Discussion

LPS treatment of macrophages has been shown to lead to the activation of the genes for several cytokines, such as TNF-α, IL-1α IL-1β, IL-6, and IFN-β, as well as proto-oncogenes, such as c-*fos*, c-*jun*, and *junB* (17, 41–43). LPS is also known to cause desensitization of the expression of LPS-inducible genes, such as TNF-α and IL-1β (28–35), and proto-oncogenes, such as c-*fos* (44). Our present data demonstrate that *junB* gene expression in P388D1 cells is also desensitized after exposure of cells to >1 ng/ml LPS (Figs. 1 and 2). In general, endotoxin tolerance is regarded as unresponsiveness to the maximal dose of LPS dose due to previous exposure to a submaximal dose. In this regard, our result does not satisfy this definition, because 1 ng/ml LPS partially turned on *junB* message, but this dose only slightly decreased *junB* mRNA expression after subsequent exposure to LPS. This may be due to our experimental protocol involving a 2-h incubation period after extensive washing with LPS-free medium before challenge. Such a low dose of LPS and subsequent washing may have caused dilution of the potential negative signal for shutting off *junB* gene expression by a maximal stimulation. Alternatively, this may be characteristic of this cell line. Indeed, a submaximal dose (10 ng/ml) of LPS for IL-1β expression in THP-1 cells was not able to prevent subsequent stimulation by a maximal stimulatory dose (1000 ng/ml) (35). At least our result showed that the degree of tolerance became more pronounced when the primary dose was increased. Both c-Fos and JunB are members of the AP-1 family, which is known to regulate the expression of genes containing the TRE motif in their promoters. LPS induced changes in AP-1 and AP-1-like element binding activities in monocytic cells (17–19). Thus, LPS-induced down-regulation of *junB* and c-*fos* expression can potentially affect the expressions of various AP-1-regulated genes in LPS-tolerant macrophages/monocytes. Such a scenario may occur for infection with multiple exposures to microbial pathogens such as LPS.

The data shown in Figure 3 suggest a probable difference between signal transduction mechanisms of *junB* gene expression mediated by LPS and PMA, since LPS-tolerant cells were able to respond to PMA with full expression of the *junB* gene, but responded to secondary stimulation with LPS with a substantially reduced level of *junB* gene expression. Indeed, we have previously demonstrated that the activation of protein kinase C was important in PMA-induced *junB* gene expression, but was not required for LPS-triggered *junB* expression in a mouse macrophage cell line, J774 (17). The noted reduced response of LPS-tolerant cells to lipid A is expected because lipid A is a biologically active moiety of LPS.

The reduction in the steady state *junB* level in LPS-tolerant cells could have been due to accelerated degradation of the *junB* transcript. However, the data shown in Figure 4 rule out this possibility, because the half-life of the *junB* transcript of LPS-tolerant cells did not differ from that in control cells. The data in Figure 5 suggest, on the other hand, that the steady state *junB* gene expression in response to secondary LPS stimulation is down-regulated primarily at or upstream of the transcriptional level. A possibility is that a negatively acting factor(s) that is synthesized as part of the response to initial LPS treatment may repress *junB* mRNA expression at or upstream of *junB* transcription. A potential role for such a negatively acting factor in IFN-α-induced desensitization in fibroblasts (45) and in LPS-induced tolerance for IL-1β gene expression in a human promonocytic cell line, THP-1 (35), has been reported. Lae and McCall suggested that a labile protein inhibits the activity of transcription in a repressor manner, since CHX treatment of the LPS-primed cells recovered not only the steady state level of IL-1β but also the transcription of IL-1β (35). These authors further suggested that IκB, which is an inhibitor protein of NF-κB, may be a candidate for the labile repressor in their system (35). Studies of nonlymphoid cells suggested that the transcriptional activation of *junB* is regulated by a variety of stimuli, including IL-6 (46–49), tumor promoter (50), cAMP-elevating agent (50, 51), platelet-derived growth factor, and fibroblast growth factor (52, 53). Moreover, negative regulation of cAMP response element binding protein-targeted protein phosphatase-1 or -2A resulted in *junB* transcriptional activation in B lymphocytes in response to membrane Ig (54). Each of these stimuli was shown to use a unique signal transduction pathway that apparently targets distinct *cis*-acting DNA sequences and transcriptional factors (46–54). The identification of *cis*-acting DNA sequence and a transcriptional factor(s) important for LPS-triggered *junB* activation would assist in understanding the mechanism(s) by which LPS causes desensitization of *junB* gene expression.

The reduced *junB* gene response in LPS-tolerant cells could be due to an aberration in the signal transduction pathway from LPS-LPS receptor interaction to transcriptional activation. Protein tyrosine phosphorylation has been shown to be one of the initial events in macrophage activation, which may mediate some of the downstream responses to LPS (20–22). The data presented in Figures 6 and 7 support this idea, because herbimycin A, which is a potent inhibitor of tyrosine kinase, but neither protein kinase C nor protein kinase A, simultaneously inhibited the tyrosine phosphorylation of the probable p38 MAP kinase and p42 MAP kinase and *junB* expression during LPS stimulation.

Among proteins that are tyrosine phosphorylated in response to LPS stimulation, MAPKs, including p38, p42 (ERK-2)/p44 (ERK-1), and JNK, are known to play important roles in the signal transduction by LPS (23–26). Of interest is the differential effect of LPS tolerance on p38 MAP kinase and p42 MAP kinase activation (Fig. 8). The activation of p42 MAP kinase by a secondary exposure of LPS occurred in LPS-tolerant cells, whereas the activation of p38 MAP kinase was much less than that in control cells. The *junB* promoter was positively regulated by $p21^{ras}$ and ERK in association with an ETS transcription factor (55). Therefore, our results suggest that the site of LPS tolerance in *junB* expression may be downstream to and independent of p42 MAP kinase. In this regard, our results differ from Ag-dependent T cell anergy characterized by the inability to produce IL-2, another example of adaptation to continuous or repetitive stimulation. Clonal anergy in Th1 cells appeared to be associated with a block in signal transduction to ERK and JNK, resulting in defective JunB and/or c-Fos and FosB expression (56–58). In contrast to p42 MAP kinase, it is tempting to speculate that the diminished activation of p38 MAP kinase in response to LPS may contribute to the down-regulated *junB* gene expression in the LPS-tolerant state. This is supported by the finding that pretreatment with SB202190 inhibited the up-regulation of *junB* gene expression in response to LPS, indicating that the activation of p38 MAP kinase is required for LPS-induced *junB* gene expression (Fig. 9). In vitro studies have shown that p38 MAP kinase directly phosphorylates activation transcriptional factor-2 in response to LPS (59). The activation transcriptional factor family is known to bind the cAMP response element (CRE) motif. Indeed, the *junB* gene contains a CRE-like site at a region of the 5′ flanking sequence as well as a CRE site at the 3′ noncoding sequence (46, 49, 51, 52). Identification of downstream substrates may be helpful for a better understanding of the possible association between the defective LPS-induced p38 MAP kinase pathway and the down-regulation of *junB* gene expression. It also would be interesting to define the upstream element of p38 MAP kinase at which LPS-tolerant state causes aberration. Further study is needed to answer these questions.

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