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Restraint of Proinflammatory Cytokine Biosynthesis by Mitogen-Activated Protein Kinase Phosphatase-1 in Lipopolysaccharide-Stimulated Macrophages

Pelli Chen, Ji Li, Janice Barnes, Gertrude C. Kokkonen, John C. Lee, and Yusen Liu

Exposure of macrophages to LPS elicits the production of proinflammatory cytokines, such as TNF-α, through complex signaling mechanisms. Mitogen-activated protein (MAP) kinases play a critical role in this process. In the present study, we have addressed the role of MAP kinase phosphatase-1 (MKP-1) in regulating proinflammatory cytokine production using RAW264.7 macrophages. Analysis of MAP kinase activity revealed a transient activation of c-Jun N-terminal kinase (JNK) and p38 after LPS stimulation. Interestingly, MKP-1 was induced concurrently with the inactivation of JNK and p38, whereas blocking MKP-1 induction by triptolide prevented this inactivation. Ectopic expression of MKP-1 accelerated JNK and p38 inactivation and substantially inhibited the production of TNF-α and IL-6. Induction of MKP-1 by LPS was found to be extracellular signal-regulated kinase dependent and involved enhanced gene expression and increased protein stability. Finally, MKP-1 expression was also induced by glucocorticoids as well as cholera toxin B subunit, an agent capable of preventing autoimmune diseases in animal models. These findings highlight MKP-1 as a critical negative regulator of the macrophage inflammatory response, underscoring its premise as a potential target for developing novel anti-inflammatory drugs. The Journal of Immunology, 2002, 169: 6408–6416.

Macrophages serve as the first line defense against pathogenic microbial insult (1, 2). Among their many antimicrobial arsenals, macrophages produce potent proinflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-8, which induce inflammation and recruit other immune cells, e.g., neutrophils and T lymphocytes (3). Although these proinflammatory cytokines are beneficial to the host defense, they can also trigger pathological conditions when expressed in excess (4). For example, massive stimulation of macrophages after a severe Gram-negative bacterial infection leads to excessive production of proinflammatory cytokines, including TNF-α and IL-1, and the development of fatal septic shock syndrome, characterized by fever, disseminated intravascular coagulation, and multiple organ failure (4, 5). In addition, higher levels of proinflammatory cytokines are also implicated in a variety of chronic inflammatory diseases including rheumatoid arthritis, psoriasis, and Crohn’s disease (4). Moreover, recent studies suggest that macrophage hyperactivity and the overproduction of TNF-α contribute to autologous cell destruction and T cell-mediated autoimmune diseases (6).

In macrophages, the biosynthesis of cytokines, especially TNF-α, is regulated at multiple levels and involves a multitude of signal transduction pathways (2). It has been shown that LPS, a component of Gram-negative bacterial cell walls, binds to LPS binding protein (LBP), CD14, a macrophage/monocyte-specific receptor protein, presents the LPS-LBP complexes to Toll-like receptor 4 (2). Interaction of Toll-like receptor 4 with the LPS-LBP-CD14 complexes triggers a multitude of signaling events, including those that culminate in the activation of both the mitogen-activated protein (MAP) kinases and the transcription factor NF-κB, which ultimately leads to increased TNF-α expression via both transcriptional and posttranscriptional mechanisms (2, 7).

Recently, it was shown that glucocorticoids can target this ARE to increase TNF-α biosynthesis in response to LPS stimulation (9). The critical role of p38 in TNF-α biosynthesis has been well established (10). Selective inhibition of p38 using specific imidazole compounds such as SB230580 substantially decreases the translation of TNF-α in LPS-stimulated macrophages. Furthermore, inactivation of the gene for MAP kinase-activated protein kinase-2 (MAPKAPK-2) (11), a downstream target of p38, abolishes LPS-triggered TNF-α production and renders mice resistant to endotoxin-induced septic shock (12). Moreover, deletion of the TNF-α ARE bypasses the requirement of p38/MAPKAPK-2 for LPS-induced TNF-α production (13, 14). JNK also appears to play a role in relieving the ARE-mediated translational silencing of TNF-α mRNA, because glucocorticoids have been shown to inhibit LPS-induced JNK activation and to reduce TNF-α production by macrophages (7, 13, 15, 16). More recently, using transgenic mice, Dumitru et al. (8) demonstrated that overexpression of MAPKAPK-2 decreased TNF-α expression and protected mice from LPS-induced septic shock.

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3 Abbreviations used in this paper: LBP, LPS binding protein; MAP, mitogen-activated protein; ARE, AU-rich element; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPKAPK-2, MAP kinase-activated protein kinase-2; MKP, MAP kinase phosphatase; CTB, cholera toxin B subunit; CHX, cycloheximide; MEK, MAP/ERK kinase.
that LPS-triggered ERK activation is required for the nucleocytoplasmic transport of TNF-α mRNA via a mechanism that involves the TNF-α ARE.

The activities of all MAP kinases are regulated via reversible phosphorylation of the conserved threonine and tyrosine residues in their tripeptide TXY signature motifs (17). In mammalian cells, inactivation of MAP kinases is primarily conducted by a family of dual-specificity MAP kinase phosphatases (MKPs), with MKP-1 being the archetype (18). Because the expression of MKPs can be regulated by MAP kinases, it has been suggested that MKPs play an important role in the feedback control of MAP kinase signaling (18, 19). The results from the present study strongly suggest that MKP-1 is a critical negative regulator in macrophage signaling in response to inflammatory stimuli and is responsible for switching off the production of proinflammatory cytokines. Finally, we demonstrate that MKP-1 is induced by certain anti-inflammatory drugs/agents and propose that MKP-1 could be a target for developing novel anti-inflammatory drugs.

Materials and Methods

Cell culture, transfection, and treatment

RAW264.7 cells were cultured in DMEM (Invitrogen, San Diego, CA) supplemented with 10% FCS (HyClone Laboratories, Logan, UT) at 37°C in a humidified atmosphere containing 5% CO₂. Cells were transfected with MKP-1 expression constructs together with pcDNA3 (Invitrogen) using FuGENE6 transfection reagent (Roche, Indianapolis, IN) or Lipofectin (Invitrogen) according to the manufacturer’s specifications. Cells were selected in medium containing G418, and resistant clones were isolated. LPS (Escherichia coli 055:B5; Calbiochem, La Jolla, CA) and cholera toxin B subunit (CTB; Sigma-Aldrich, St. Louis, MO) were dissolved in serum-free medium and added to the medium at the indicated concentrations. In experiments determining MKP-1 stability, 10 μg/ml cycloheximide (CHX; Calbiochem) was added to the culture medium, either alone or in combination with LPS. Dexamethasone (Sigma-Aldrich) and triptolide (Calbiochem) were dissolved in DMSO and added to the culture at the concentrations indicated. The MAP/ERK kinase (MEK) inhibitor U0126 (Promega, Madison, WI), and the p38 inhibitor SB203580 (Calbiochem) were dissolved in DMSO and added to the medium at a final concentration of 10 μM 30 min before the addition of LPS. To isolate proteins, cells were harvested in a lysis buffer containing 10 mM HEPES (pH 7.4), 50 mM β-glycerophosphate, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 10 mM NaF, 1 mM Na₃VO₄, 20 mM microcystin-LR, 2 μM leupeptin, 2 μM aprotinin, and 1 mM PMSF.

Expression vectors

pSRα-Flag-MKP-1, which expresses full-length MKP-1 tagged with three Flag epitopes at its amino terminus, has been previously described (20). pSRa-Flag-MKP-1 ΔC was created by introducing a premature stop codon in the open reading frame of MKP-1 through site-directed mutagenesis (QuickChange; Stratagene, La Jolla, CA) to delete the last 54 aa from the C terminus. The bacterial expression vector for GST-tagged p38 has been described previously (21).

Western blotting and ELISA

Western blot analysis was conducted essentially as previously described using ECL reagent (Amersham Pharma Biotech, Piscataway, NJ) (22). MKP-1 was detected using a rabbit polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA). Phosphorylated ERK and p38 were detected using rabbit polyclonal Abs from Cell Signaling Technology (Beverly, MA). Phosphorylated JNK was detected using either a monoclonal MAb (BD Transduction Laboratories) or a polyclonal Ab specifically recognizing phosphorylated JNK1 and JNK2 (BD Pharmingen, San Diego, CA). Total p38 was detected using a mAb against Flag (M2) (Berkeley Antibody, Richmond, CA). In the experiments for determining the stability of MKP-1, Western blot films were scanned using a Scanner 5 (MicroTec, Carson, CA) and the images were analyzed using ImageMaster 1D Elite software (Phar machia Biotech, Uppsala, Sweden). TNN-α and IL-6 in the culture medium were determined using ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s recommendations.

GST pull down

GST-p38 was produced in Escherichia coli as previously described (21). RAW264.7 cell lysates containing 200 μg of total protein were incubated with 4 μg of GST-p38 prebound to glutathione-Sepharose beads (Amersham Pharma Biotech) for 1 h at 4°C. The beads were washed twice with 1 ml of lysis buffer and twice with 1 ml of lysis buffer supplemented with 200 mM NaCl. The proteins were separated by electrophoresis and analyzed by Western blotting using the anti-MKP-1 Ab (Santa Cruz Biotechnology).

Northern blot analysis

Total RNA was isolated with STAT-60 (Tel-Test, Friendswood, TX). Northern blot analysis was performed using mouse MKP-1 cDNA as a probe as described previously (23, 24). The membrane was stripped and reprobed with an oligonucleotide corresponding to 18S rRNA (22).

Immune complex kinase assays

MAPKAPK-2 activity was measured by immune complex kinase assay as previously described (20). Briefly, endogenous MAPKAPK-2 was immunoprecipitated from 500 μg of RAW264.7 cell lysate using 3 μg of the rabbit polyclonal antisera (kindly provided by J. Huot, l’Université Laval, Quebec, Canada) and protein A-Sepharose (Amersham Pharmacia Biotech). The kinase activity in the MAPKAPK-2 immune complexes was assayed using [γ-32P]ATP and recombinant heat shock protein 25 (StressGen Biotechnologies, Victoria, BC, Canada) as a substrate.

Results

Induction of MKP-1 correlates with the inactivation of JNK and p38 MAP kinases in LPS-stimulated RAW264.7 cells

The kinetics of MAP kinase activation in subconfluent RAW264.7 cells stimulated with 100 ng/ml LPS were examined by Western blotting using Abs specifically recognizing phosphorylated ERK, JNK, and p38 (Fig. 1A). JNK and p38 were rapidly activated by LPS, reaching their maximal activities within 15 min. Their activities then decreased and plunged to nearly basal levels by 60 min. ERK was also potently activated. In contrast to JNK and p38, ERK activity decreased only modestly after achieving maximal activation but was sustained at relatively high levels throughout the time period examined. MAPKAPK-2, the downstream target of p38, was also activated in response to LPS with kinetics similar to those seen for p38 MAP kinase (Fig. 1B). These observations raised the possibility that a MKP(s) that preferentially inactivates JNK and p38 could be induced in response to LPS and prompted us to directly examine the role of MKPs in this process.

MKP-1, which is expressed as a 39-kDa protein in a wide variety of cell types, has been shown to preferentially dephosphorylate p38 and JNK as compared with ERK (23, 25, 26). MKP-1 protein in RAW264.7 cells was examined by Western blotting (Fig. 1C). In unstimulated cells, the MKP-1 protein level was very low, essentially below the limit of detection. In response to LPS stimulation, MKP-1 protein increased dramatically, reaching its maximal level between 1 and 2 h, and then decreased slightly. The reciprocal relationship between MKP-1 and the two stress-activated MAP kinases, p38 and JNK, supports the notion that MKP-1 may play a critical role in the inactivation of these MAP kinases.

Blocking LPS-triggered MKP-1 induction with diterpenoid trioxipoxide triptolide prevents the inactivation of MAP kinases

To further understand the relationship between MKP-1 induction and MAP kinase inactivation, we screened various chemical compounds for agents that block the induction of MKP-1 by LPS. We found that triptolide, a diterpenoid trioxipoxide (27), potently blocked MKP-1 induction by LPS in a dose-dependent manner (Fig. 2A). This dose-dependent blockade of MKP-1 protein accumulation was associated with a reciprocal increase in the levels of

Inactivation of MAP kinases was associated with a reciprocal increase in the levels of...
phosphorylated/active JNK and p38. Triptolide pretreatment also prevented the modest decrease in ERK phosphorylation that normally occurred after ~60 min after LPS stimulation (Fig. 2A). To ascertain that triptolide did not act by neutralizing LPS or by merely slowing down the signal transduction process, we examined the activation kinetics of these kinases over a 2-h time period (Fig. 2B). LPS stimulated MAP kinase activation in both control and the triptolide-pretreated cells within 15 min. Triptolide completely blocked MKP-1 induction and prevented the inactivation of MAP kinases (Fig. 2B). These observations strengthen the link between MKP-1 induction and the inactivation of MAP kinases, especially JNK and p38.

Transcriptional induction of MKP-1 is mediated primarily by the ERK and modulated by p38

To understand the mechanism(s) mediating MKP-1 induction by LPS, Northern blot analysis was performed. MKP-1 mRNA levels were virtually undetectable in unstimulated control cells. In response to LPS treatment, MKP-1 mRNA was potently induced within 30 min, reaching its maximal levels after 60 min (Fig. 3A). Similar to what was observed at the protein level (Fig. 1A), a significant decrease in MKP-1 mRNA level was observed at later time points. To examine the role of ERK and p38 in MKP-1 induction by LPS, we exploited the pharmacological inhibitors for these pathways. Pretreatment of cells with either U0126, an inhibitor specific for MEK1/2, or SB203580, a well-established inhibitor for p38, did not alter basal MKP-1 mRNA levels (Fig. 3B). U0126 (10 μM) substantially attenuated MKP-1 induction, decreasing MKP-1 mRNA by 65%, whereas SB203580 (10 μM) had little effect on MKP-1 induction by LPS. Surprisingly, compared with MKP-1 mRNA in cells pretreated with U0126 alone, MKP-1 mRNA levels in cells pretreated with both U0126 and SB203580 were significantly lower (Fig. 3B). Similar to what was observed at the MKP-1 mRNA levels, U0126 also potently inhibited the MKP-1 increase at the protein level, resulting in an ~72% reduction in MKP-1 protein (Fig. 3C). Pretreatment of cells with SB203580 also moderately inhibited the MKP-1 protein increase (33% reduction at the MKP-1 protein levels). Pretreatment of cells with a combination of U0126 and SB203580 almost abolished the LPS-triggered MKP-1 protein induction. Consistent with the notion

FIGURE 1. MKP-1 induction is associated with inactivation of JNK and p38 in LPS-stimulated RAW264.7 cells. A, Activation kinetics of ERK, JNK, and p38 after LPS stimulation. Cells were treated with 100 ng/ml LPS for the indicated times, and lysates were analyzed by Western blot analysis using Abs specifically recognizing phospho-ERK, phospho-JNK, and phospho-p38. Western blots were also performed using Abs that recognize total ERK, JNK, and p38, which further served to control for sample loading. B, Time course of MAPKAPK-2 activation. MAPKAPK-2 activity was measured by immune complex kinase assay using [γ-32P]ATP and heat shock protein 25 as a substrate. C, Time course of MKP-1 induction by LPS. MKP-1 was detected by Western blotting using an MKP-1 Ab. Numbers on the right are size markers in kilodaltons.

FIGURE 2. Blocking MKP-1 induction by triptolide inhibits the dephosphorylation of MAP kinases. RAW264.7 cells were pretreated with or without the indicated concentration of triptolide for 30 min before the addition of LPS (100 ng/ml). Samples (each containing 20 μg of protein) were analyzed by Western blotting using Abs for MKP-1, phospho-ERK, phospho-JNK, and phospho-p38. A, Dose response of the effect of triptolide on MKP-1 expression and on MAP kinase activity. B, Effect of triptolide on the kinetics of MAP kinase activation and MKP-1 expression.
that MKP-1 is responsible for the dephosphorylation of p38 in these cells, blockage of MKP-1 induction by either U0126 alone or a combination of U0126 and SB203580 resulted in a significant increase in phosphorylated p38 (Fig. 3C). These results indicate that MKP-1 induction in response to LPS is primarily regulated by the ERK pathway with p38 playing only a minor role.

Ectopic expression of MKP-1 accelerates inactivation of p38 and JNK and inhibits the production of TNF-α and IL-6

To examine the effect of MKP-1 on the production of proinflammatory cytokines, a mammalian vector expressing Flag-tagged MKP-1 was transfected into RAW264.7 cells by Fugene6 or Lipofectin to establish cells stably expressing Flag-MKP-1. After selection in medium containing G418, the colonies were pooled (hereafter referred to as pools F and N for pools established using Fugene6 or Lipofectin, respectively). Western blotting using an Ab against the Flag tag indicated that MKP-1 expression levels were higher in pool F than in pool N (Fig. 4A). The ectopically expressed MKP-1 was compared with the endogenous protein levels through Western blotting using an Ab that recognizes MKP-1. Even in pool F, the absolute level of ectopically expressed MKP-1 was moderate at best, which was estimated to be less than 30% of the endogenous protein found in LPS-stimulated cells (Fig. 4A). The effect of elevated MKP-1 expression on MAP kinase activities was examined by Western blotting. As indicated in Fig. 4B, LPS stimulation at a dose of 50 ng/ml potently activated all three MAP kinases within 15 min. At the 15-min time point, there was little difference in either the phospho-JNK or the phospho-p38 levels between the cell pool stably transfected with an empty vector and the two MKP-1-expressing cell pools. However, by 60 min, the levels of both phospho-JNK and phospho-p38 were only slightly reduced in the cell pool carrying the empty vector. In contrast, the amounts of both phospho-JNK and phospho-p38 were reduced to almost basal levels in pool F. Although less prominent than in pool F, the level of phospho-JNK was also considerably lower in pool N than in the cells carrying the empty vector (Fig. 4B). However, there was no significant difference in the phospho-ERK levels between the cells carrying the vector and any of the stable MKP-1-expressing
pools (data not shown). Taken together, these results indicate that a moderate amount of ectopically expressed MKP-1 accelerates the inactivation of p38 and JNK in a dose-dependent manner.

The amounts of both endogenous and ectopically expressed MKP-1 protein were also examined by Western blotting using the MKP-1 Ab (Fig. 4B, bottom panel). Interestingly, the ectopically expressed MKP-1 increased substantially after LPS stimulation in all stable MKP-1-expressing pools. It should be pointed out that Flag-tagged MKP-1 was expressed under a heterologous promoter in an expression cassette that contains only the MKP-1 open reading frame and lacks both the 3’ and 5’ untranslated regions (20). The marked increase in Flag-MKP-1 protein after LPS stimulation in pool F and pool N strongly suggests that MKP-1 protein is also regulated posttranslationally.

The effect of increased MKP-1 expression on the production of TNF-α and IL-6 was examined by ELISA. Compared with cells transfected with an empty vector or the cell pools (pools F and N) expressing different levels of Flag-MKP-1, IL-6 production by the stable pools after LPS stimulation. Data represent the mean ± SE from three independent experiments. Values represent the concentration of cytokine secreted by 10^6 cells.

LPS enhances the stability of MKP-1 through mechanisms primarily mediated by ERK

To examine whether the stability of MKP-1 protein was influenced by LPS, cells from pool F were treated with either CHX alone or CHX together with LPS and were harvested at different time points (Fig. 6A). The decay of Flag-MKP-1 was studied by Western blotting using Flag Ab. In the absence of LPS, Flag-MKP-1 degraded rapidly with a half-life of ~50 min. LPS treatment resulted in a significant increase in MKP-1 stability, extending its half-life to ~200 min. Pretreatment with U0126 substantially accelerated the degradation of Flag-MKP-1 in LPS-stimulated cells, reducing the half-life to close to 60 min (Fig. 6B). In contrast, SB203580 had little effect on MKP-1 stability. These results indicate that posttranslational stabilization of MKP-1 plays a significant role in
MKP-1 induction by LPS and that ERK plays an important role in mediating this stabilization process.

Previously, it has been shown that MKP-1 stability could be enhanced by ERK-dependent MKP-1 phosphorylation (28). Furthermore, Brondello et al. (28) have identified two serine residues (serine 359 and serine 364) in the C terminus of MKP-1 to be phosphorylation sites. To examine whether the C-terminal region of MKP-1 is involved in its LPS-induced stabilization, we established cell lines constitutively expressing a C-terminal truncated MKP-1 mutant (hereafter referred to as MKP-1ΔC). MKP-1ΔC lacks the last 54 aa in its C terminus, which includes the two serine residues shown to be phosphorylated by ERK and a putative docking motif for ERK binding. The stability of MKP-1ΔC in control and LPS-stimulated cells was examined by Western blotting (Fig. 7). In unstimulated cells, the half-life of MKP-1ΔC was comparable to that of the full-length protein. In response to LPS stimulation, its half-life was increased by less than twofold, from ~47 to ~78 min, indicating that the C-terminal domain of MKP-1 plays an important role in mediating its LPS-induced stabilization. This result also suggests that in addition to the phosphorylation sites targeted by ERK in the MKP-1 C terminus, other domains of MKP-1 may also play a role in LPS-stimulated stabilization.

**MKP-1 is induced by the immune-modulatory agents dexamethasone and CTB**

Glucocorticoids are well-known anti-inflammatory and immune-suppressant agents that can block the production of TNF-α, at least in part, by inhibiting JNK activity (13, 15). In mast cells, glucocorticoids have been shown to inhibit ERK activity by increasing MKP-1 transcription and decreasing MKP-1 protein degradation (29). To investigate whether in macrophages enhanced MKP-1 expression is involved in the anti-inflammatory activity of glucocorticoids, RAW264.7 cells were treated with dexamethasone over a 2-day period. MKP-1 protein was indicated time points and analyzed by Western blotting using an Ab that recognizes an anti-Flag epitope. Graph depicts the quantitation of Flag-MKP-1ΔC mRNA in RAW264.7 cells (Fig. 8B).

CTB is a component of the cholera toxin produced by the Gram-negative bacterium *Vibrio cholerae*. It has been shown that in addition to functioning as a carrier for the cholera toxin A subunit to target adenylate cyclase, CTB can exert immune-modulating effects independent of the A subunit (30, 31). CTB has been reported to suppress the onset of T cell-dependent autoimmune diseases and to potentiate tolerance of the adaptive immune response (32–34). Pretreatment of macrophages with CTB has been reported to suppress the LPS-induced production of TNF-α and IL-6 (30, 31). However, the signaling mechanism(s) involved are not fully understood. We examined the effect of CTB on MKP-1 protein expression. Treatment of RAW264.7 cells with CTB potently increased MKP-1 protein levels within 2 h, and the effect lasted up to 8 h (Fig. 9A). Pretreatment of cells with CTB significantly increased the MKP-1 protein levels and attenuated the LPS-stimulated activation of all three major MAP kinases (Fig. 9B). Interestingly, compared with the cells pretreated with CTB, cells that did not receive CTB pretreatment accumulated a higher level of MKP-1 at the 45-min time point (Fig. 9B, bottom panel), likely due to the more robust ERK activation in these cells. This inhibitory effect of CTB on MAP kinases was also reflected in the production of both TNF-α and IL-6. CTB pretreatment substantially inhibited TNF-α production (>75%) and completely abolished IL-6 biosynthesis in response to LPS stimulation, although CTB alone slightly increased the biosynthesis of TNF-α and had no effect on IL-6 (Fig. 9C). These findings suggest that MKP-1 induction could be a potential mechanism accounting for the immune-modulating properties of CTB. The findings may also be relevant to the pathogenic property of *V. cholerae*.

**Discussion**

This study was undertaken to address the role of MKP-1 in the inflammatory response of macrophages, particularly its influence on the production of proinflammatory cytokines. MKP-1 was potently induced in response to LPS stimulation in RAW264.7 cells.
Induction of MKP-1 correlated with inactivation of both JNK and p38 (Fig. 1). MKP-1 was found to interact both in vitro and in vivo with representative members of all three major MAP kinase subfamilies (data not shown). More importantly, a block in MKP-1 induction by the pharmacological inhibitor triptolide prevented the inactivation of both JNK and p38 that normally occurs at ~60 min after LPS stimulation (Fig. 2). An increase of MKP-1 expression, even to a modest level, significantly accelerated the inactivation of JNK and p38 (Fig. 4), resulting in a substantial decrease in TNF-α production and a complete inhibition of IL-6 biosynthesis in LPS-stimulated cells (Fig. 5). Taken together, these results strongly support a critical role for MKP-1 in the termination of signals that govern the biosynthesis of the proinflammatory cytokines.

Despite the fact that MKP-1 can play a pivotal role in the dephosphorylation of ERK MAP kinases in some cell systems (18, 19), the primary targets of MKP-1 in terminating proinflammatory cytokine biosynthesis do not appear to be the ERK MAP kinases in the present model system. This notion is supported by two observations. First, maximal MKP-1 induction did not correlate with a substantial dephosphorylation of ERK in LPS-stimulated cells. MKP-1 expression was maximally induced by LPS after ~60 min, a time when JNK and p38 were almost completely inactivated, but a substantial amount of ERK activity still remained (Fig. 1, A and C). Second, ectopic expression of MKP-1 did not significantly alter the time course of LPS-triggered ERK activation (data not shown), but significantly attenuated proinflammatory cytokine biosynthesis (Fig. 5). Our data are consistent with the conclusion by Franklin and Kraft (25) that among all three major MAP kinase subfamilies, MKP-1 prefers members of the p38 and JNK subfamilies as substrates. However, we neither exclude a role for MKP-1 in the inactivation of ERK MAP kinases nor rule out the possibility that other MKPs, such as phosphatase of activated cells-1, may also participate in the inactivation of p38 and JNK MAP kinases.

MKP-1 is potently induced after LPS stimulation through mechanisms mediated, in a large part, by the ERK pathway. This is an intriguing result, considering that MKP-1 preferentially acts on p38 and JNK. The induction of MKP-1 involves both increased MKP-1 mRNA levels, presumably due to elevated gene transcription (Fig. 3), and enhanced protein stability (Fig. 6). LPS-induced increases in MKP-1 mRNA are substantially inhibited by the MEK inhibitor U0126 (Fig. 3), illustrating the importance of the ERK pathway in MKP-1 induction. The role of p38 in MKP-1 induction is less clear. Although the p38 inhibitor SB203580 alone did not show a significant inhibitory effect on MKP-1 mRNA induction, in combination with the MEK inhibitor U0126 it did attenuate MKP-1 mRNA induction (Fig. 3B), and by itself it significantly reduced the LPS-triggered increase in MKP-1 protein (Fig. 3C).

These observations suggest that p38 plays a minor role in mediating MKP-1 mRNA induction by LPS. The fact that the induction of MKP-1 expression is mediated primarily by the ERK pathway raises a very interesting possibility that MKP-1, and potentially also other MKPs, may serve as a critical mediator for interplay and cross-talk between the various MAP kinase pathways (Fig. 4C). A finding that also illustrates this possibility has been reported for MKP-M, another MKP family member isolated from macrophages (35). MKP-M has been shown to primarily act on JNK, although its induction is primarily mediated by p38. Our finding that MKP-1 induction by LPS is primarily mediated by the ERK pathway is different from a previous observation made by Valledor et al. (36) using mouse bone macrophages. Although LPS also potently induced MKP-1 in those cells, MKP-1 induction was resistant to PD98051, another MEK inhibitor (36). It is unclear whether this discrepancy may be due to the differences between primary bone marrow macrophages and the established macrophage cell line used here or due to differences in experimental methodologies. Future studies using primary macrophages will address this issue.

In addition to up-regulating MKP-1 mRNA levels, LPS also substantially stabilizes the MKP-1 protein. Presumably, enhanced MKP-1 protein stability in addition to increased MKP-1 transcription offers more leverage for the ERK pathway to dominate the biological program and prevent overproduction of proinflammatory cytokines. Previously, in fibroblasts as well as in Xenopus oocytes (28), MKP-1 has been shown to be stabilized through phosphorylation mediated by ERK MAP kinases. Our experiments using stable MKP-1-expressing cells provide unequivocal support for this conclusion. In the stable cell pools, MKP-1 expression was driven by a heterologous expression cassette. Because this cassette only contains an open reading frame from MKP-1, the increase in ectopically expressed MKP-1 after LPS stimulation is very likely due to an increase in protein stability. This is supported by an approximately fourfold increase in MKP-1 half-life after LPS stimulation (Fig. 6A). Such an increase in half-life after LPS stimulation explains the accelerated inactivation of JNK and p38 in the two cell pools that express moderate amounts of MKP-1 (Fig. 4B).
The fact that U0126 significantly shortens the half-life of MKP-1 confirms the conclusion that ERK plays an important role in the MKP-1 protein increase seen after LPS treatment. However, ERK MAP kinases are unlikely to be the only mediators involved in MKP-1 stabilization, because U0126 did not completely prevent the LPS-triggered increase in Flag-MKP-1 stability (Fig. 6B). Another interesting finding from this study is that LPS-induced stabilization of MKP-1 is not solely mediated by the C terminus domain of MKP-1 (Fig. 7). The involvement of the MKP-1 C-terminal domain in enhancing its stability is not surprising because two serine residues, serine 359 and serine 364, have been shown to be phosphorylated by ERK (28). The fact that a C-terminal truncated MKP-1 mutant can undergo a compromised (compared with the full-length protein) but nevertheless significant increase in protein half-life suggests that other domains of MKP-1 contribute to its stabilization (Fig. 7), possibly via phosphorylation by ERK or other protein kinases. Recently, threonine 168 has been identified as a third phosphorylation site targeted by ERK (37). Whether threonine 168 plays a role in LPS-stimulated MKP-1 stabilization remains to be examined.

Our findings that MKP-1 acts as a negative inhibitor of inflammatory cytokine production may have important implications in understanding the action of some anti-inflammatory drugs and immune-modulatory agents. Glucocorticoids are potent anti-inflammatory drugs that can inhibit the production of proinflammatory cytokines (7). It has been reported that suppression of TNF-α production in primary macrophages by glucocorticoids is mediated through the inhibition of JNK (8, 13, 15). Recently, it has been demonstrated that MKP-1 is potently induced by dexamethasone in mast cells (29). Our results indicate that MKP-1 expression can also be induced by dexamethasone in macrophages at both the protein and mRNA levels (Fig. 8). This is consistent with previous findings indicating that the MKP-1 promoter region harbors several putative glucocorticoid-response elements (29). Therefore, the results presented here strongly suggest that the induction of MKP-1 by glucocorticoids may be responsible for the inhibition of JNK and, at least in part, for the suppression of TNF-α production in macrophages. Very recently, TGF-β, a cytokine with anti-inflammatory activity, has also been shown to induce MKP-1 in RAW264.7 cells (38). Consistent with our findings, induction of MKP-1 by TGF-β is also mediated by ERK. It should be pointed out that MKP-1 induction is probably not the only mechanism via which the anti-inflammatory cytokines/drugs exert their effects. For example, the anti-inflammatory cytokine IL-10 has been shown to inhibit the p38 pathway and decrease TNF-α biosynthesis (39). It has been proposed that IL-10 may interfere with the MAP kinase kinase-6 signals to inhibit the p38 cascade (39). Consistent with such a hypothesis, preliminary results from our laboratory indicate that MKP-1 is not induced by IL-10 in our system (data not shown).

MKP-1 can also be induced by CTB (Fig. 9), an immune-modulatory agent (30, 31). Coupled to relevant auto antigens, CTB has been shown to effectively prevent the development of T cell-mediated autoimmune diseases, including encephalomyelitis, arthritis, and type I diabetes, in animal models (32–34). Recently, it has been reported that pretreatment of macrophages and monocytes with CTB diminishes the LPS-induced proinflammatory response, leading to a significant decrease in the production of TNF-α and IL-6 (30, 31). Because cytokines such as TNF-α and IL-6 are critical in the implementation of the T cell response, attenuating their production in macrophages by CTB could have a major impact on the Adaptive immune response. In this report, we have demonstrated that CTB potently induced MKP-1, abolished the LPS-elicted MAP kinase activation, and abrogated the secretion of both TNF-α and IL-6 (Fig. 9), thereby providing a mechanistic explanation for its inhibitory effect on the innate immune system. The higher MKP-1 level in cells treated with LPS alone in comparison with that in cells treated with CTB and LPS at the 45-min time point was likely due to the higher ERK activity in these cells (Fig. 9B). Thus, it is possible that, in addition to MKP-1, some ERK-selective phosphatases may also be induced by CTB. The relatively higher p38 activity in cells treated with LPS alone in comparison to cells treated with both CTB and LPS at the 45-min time point may be explained by the kinetic differences in MKP-1 protein accumulation between the two populations (Fig. 9B). In cells pretreated with CTB, a moderate amount of MKP-1 existed before LPS stimulation and the amount did not substantially increase after LPS stimulation (Fig. 9, A and B). In contrast, in cells that received no CTB pretreatment, the MKP-1 protein level was very low and only started to increase after 30 min (Fig. 1C). It is possible that in cells pretreated with CTB, the moderate amount of MKP-1 over a long period of time dephosphorylated more p38 than the MKP-1 acutely accumulated over a short period of time in cells that received no CTB pretreatment. Our results also indicate that CTB has immune-suppressive properties independent of the action of the cholera toxin A chain. Such an inhibitory effect of CTB on macrophages may offer V. cholerae protection against the host innate immunity and may be relevant to the pathophysiology of this infectious pathogen. In conclusion, the data presented here highlight MKP-1 as a negative regulator of the macrophage inflammatory response and as a potential target for the development of anti-inflammatory and anti-rheumatic drugs.

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