Macrophages proliferate in the presence of their growth factor, macrophage colony-stimulating factor (M-CSF), in a process that is dependent on early and short ERK activation. Lipopolysaccharide (LPS) induces macrophage activation, stops proliferation, and delays ERK phosphorylation, thereby triggering an inflammatory response. Proliferating or activating responses are balanced by the kinetics of ERK phosphorylation, the inactivation of which correlates with Mkp1 induction. Here we show that the transcriptional induction of this phosphatase by M-CSF or LPS depends on JNK but not on the other MAPKs, ERK and p38. The lack of Mkp1 induction caused by JNK inhibition prolonged ERK-1/2 and p38 phosphorylation. The two JNK genes, jnk1 and jnk2, are constitutively expressed in macrophages. However, only the JNK1 isoform was phosphorylated and, as determined in single knock-out mice, was necessary for Mkp1 induction by M-CSF or LPS. JNK1 was also required for pro-inflammatory cytokine biosynthesis (tumor necrosis factor-α, interleukin-1β, and interleukin-6) and LPS-induced NO production. This requirement is independent of Mkp1 expression, as shown in Mkp1 knock-out mice. Our results demonstrate a critical role for JNK1 in the regulation of Mkp1 induction and in LPS-dependent macrophage activation.

The serine/threonine mitogen-activated protein kinase (MAPK) family includes extracellular signal-regulated protein kinase (ERK-1/2), stress-activated protein kinases (SAPK), p38, and c-Jun NH2-terminal protein kinases (JNK). These MAPKs are responsible for transmitting extracellular signals from the membrane to the nucleus, which leads to the phosphorylation of several transcription factors and the regulation of genes involved in the control of a number of fundamental cellular processes, including proliferation, survival, differentiation, apoptosis, motility, and metabolism (1, 2). Although MAPKs are conserved evolutionary pathways present in eukaryotic cells, the kinetics of activation and their subcellular compartmentalization is cell type-specific, and they orchestrate differential cellular responses. For example, in neuronal cells, sustained MAPK activation of even days is required for cellular activation or differentiation, whereas in fibroblasts activation of this kinase family is very short. In contrast, to achieve proliferation, extended activation of MAPKs is required in these cells (2, 3).

Macrophages differentiate and proliferate in the presence of macrophage colony-stimulating factor (M-CSF). However, the proliferation of these cells is blocked when they are activated by Gram-negative lipopolysaccharide (LPS) or by IFN-γ (4). Activation causes many biochemical and morphological modifications, such as in the expression of inducible nitric-oxide synthase (NOS2) and the biosynthesis and release of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin 1 (IL-1β), and IL-6. Macrophage response to M-CSF and LPS involves the phosphorylation of the three members of the MAPK family (5).

The correct spatiotemporal regulation of MAPK signaling activation is crucial in determining cellular responses to growth or activating factors (6, 7). In our cellular model, an early peak of ERK activity (5 min) correlated with cellular proliferation, whereas a later peak (15 min) was associated with the activation program (7). Also, the length of ERK activation is critical. For proliferation, ERK must be dephosphorylated ~15 min after activation (7). A number of conditions that elongate ERK activity, for example extracellular matrix proteins such as decorin or fibrinogen or treatment with cyclosporin A or FK506, reduce proliferation (8, 9).

MAPK phosphatases (MKPs or DUSP) are responsible for dephosphorylating tyrosine and threonine residues of MAPKs. Eleven MKP family members have been identified thus far, these differing in tissue-specific expression, subcellular localization, post-translational regulation, and substrate specificity within the MAPK family (10). Of these, nuclear MKP1, also termed DUSP1, is encoded by an immediate-early response gene induced in macrophages upon stimulation with M-CSF (11) or LPS (12). Although MKP1 was initially identified as an in vitro ERK-specific phosphatase, depending on the cell type it...
also dephosphorylates other members of the MAPK family such as JNK and p38, thus suppressing signaling downstream of these kinases (10). Genetic ablation of Mkp1 has shown that this phosphatase is a pivotal feedback control regulator of the innate immune response (13–16).

Consequently, the mechanisms responsible for regulating Mkp1 expression are determinant for controlling the length of MAPK responses in macrophages. In some cell types, such as fibroblasts, Mkp1 induction is dependent on ERK-1/2 activation (17, 18) or SAPK (19). Results from our group show that the activation of the MEK/ERK-1/2 cascade is not required for the induction of Mkp1 in macrophages (11, 12). In those studies, we also found that of all the protein kinase C isoforms expressed in macrophages, only PKCe was involved in Mkp1 transcriptional induction by M-CSF or LPS and, consequently, in the negative control of ERK activity (11, 12, 20). Recently, we reported that Raf-1 activation is required for control of ERK activity (11, 12, 20). In those studies, we also found that of all the protein kinase C isoforms expressed in macrophages, only PKCe was involved in Mkp1 transcriptional induction by both M-CSF and LPS, thus regulating the activation profile of the other MAPKs. In addition, we demonstrate that JNK1 mediates the expression of NOS2 and pro-inflammation profile of the other MAPKs. In some cell types, such as JNK and p38, thus suppressing signaling downstream of these kinases (10). Genetic ablation of Mkp1 has shown that this phosphatase is a pivotal feedback control regulator of the innate immune response (13–16).

Here we studied the role of all MAPK family members in the regulation of Mkp1 expression in primary cultures of macrophages. Only JNK, and specifically the JNK1 isoform, was involved in Mkp1 induction by both M-CSF and LPS, thus regulating the activation profile of the other MAPKs. In addition, we demonstrate that JNK1 mediates the expression of NOS2 and pro-inflammatory cytokines during LPS and TNF-α activation. Our results show that the JNK1 isoform plays a crucial role in macrophage biology.

**EXPERIMENTAL PROCEDURES**

**Reagents**—LPS, actinomycin D, propidium iodide, wortmannin, LY294002, anti-ERK Thr-183/Tyr-185, and anti-β-actin antibodies (Abs) were from Sigma. In several experiments, the results obtained with commercial LPS were compared with highly purified LPS from *Salmonella abortus equi*, kindly donated by Dr. C. Galanos (Max Planck Institute, Freiburg, Germany (22)) and no differences were found. Recombinant M-CSF and IFN-γ were from R&D Systems Inc. (Minneapolis, MN). Murine TNF-α was obtained from Peptotech (London, UK). The p38MAPK Ab was obtained from Cell Signaling Technology (Beverly, MA). The anti-JNK1, JNK, and MKP1 Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PD98059, GF109203X, SB203580, SP600125, JNK inhibitor II, and NOS2 Abs were from Calbiochem. Secondary horseradish peroxidase anti-mouse (MP Biologicals, Irvine, CA) and anti-rabbit (Sigma) Abs were also used.

**Cell Culture**—Bone marrow macrophages were obtained from 6-week-old BALB/c mice (Harlan Ibérica, Barcelona, Spain) and cultured as described previously (23). Macrophages were cultured for 6 days in Dulbecco’s modified Eagle’s medium (BioWhittaker-Cambrex, Emerainville, France), supplemented with 20% fetal calf serum (Sigma-Aldrich) and 30% L-cell conditioned media. Macrophages (80–90% confluent) were synchronized by culture with 10% fetal calf serum for 18 h (23). Macrophages from Jnk1, Jnk2, and Mkp1 knock-out (KO) mice (24, 25) were obtained in the same way. For the experiments with these mice, we used the corresponding background mouse controls. Animal use was approved by the Animal Research Committee of the University of Barcelona (Approval Number 2523).

**Cell Surface Staining**—Analysis of cell surface receptors was performed as described (26). 1 × 10⁶ cells/ml were incubated with primary antibodies against Ly-71 (F4/80) and CD11b (Mac-1) fluorescein isothiocyanate Abs (eBioscience, San Diego) after blocking Fc receptors with anti-CD16/CD32 (FcγRIII/II receptor) Ab (Pharmingen). Detection was done directly or after incubation with fluorescein isothiocyanate anti-rabbit IgG (Sigma). Cells were fixed with paraformaldehyde solution before flow cytometry analysis (Epics XL, Coulter Corp., Hialeah, FL). Each figure is representative of three independent experiments with triplicates expressed as the mean ± S.D. Dead cells, detected through low forward and side light scatter, were excluded. Blocking and direct incubation with secondary Ab was used as negative control.

**Apoptosis Assay**—Cell viability was assessed by particle counting using FACS (Coulter Multisizer II, Midland, Canada) and confirmed by trypan blue exclusion. Cell death was also assessed by FACS analysis using the rAnnex V-FITC kit (Bender MedSystems, Burlingame, CA) following the manufacturer’s instructions. Actinomycin D was used as a positive control of apoptosis. Each point was performed in triplicate and the results were expressed as the mean value ± S.D.

**Proliferation Assay**—After 24 h of treatment, cells were pulsed with [³H]Thd (1 μCi/ml) (Amersham Biosciences) for 6 h as described previously (27). Each point was performed in triplicate, and the results are expressed as the mean ± S.D.

**Cell Cycle Analysis**—Cell cycle was analyzed as described (4). Cells were fixed with EtOH 95%, incubated with propidium iodide plus RNase A, and then analyzed by FACS. Cell cycle distributions were analyzed with the Multicycle program (Phoenix Flow Systems, Inc., San Diego).

**Western Blot Analysis**—Total cytoplasmic extracts were made by lysing cells as described previously (4). SDS-PAGE was performed and proteins transferred to nitrocellulose membranes (Hybond-C, Amersham Biosciences). After blocking, incubation with primary Abs and secondary Ab was performed. The detection of the bands was done using the EZ-ECL kit (Biological Industries, Kibbutz Beit Haemek, Israel) and by exposure to x-ray films (Agfa, Mortsel, Belgium). β-Actin was used as a loading control. Analysis of maximal expression was determined with a molecular analyser system (Bio-Rad).

**In-gel Kinase Assay**—ERK activity was analyzed as described previously using 50–100 μg of total protein obtained as described above and separated by 12.5% SPD-PAGE containing 0.1 mg/ml myelin basic protein (Sigma) as substrate co-polymerized in the gel (7). After several washes, denaturing, and renaturing, a phosphorylation assay was performed with 50 μM ATP and 100 μCi of [γ-³²P]ATP (Amersham Biosciences).

**JNK Activity Assay**—JNK activity was measured as described previously (28). Briefly, cells were lysed and immunoprecipitated with protein A-Sepharose and anti-JNK1 or anti-JNK2 Ab. After several washes, the reaction was performed with 1 μg of GST-c-Jun-(1–169) (MBL, Woburn, MA) as JNK substrate, 20 μM ATP and 1 μCi of [γ-³²P]ATP. SDS-PAGE was performed and the gel exposed to Agfa x-ray films.
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RNA Extraction and Northern Blot Analysis—Total RNA was extracted with the RNA kit EZ-RNA (Biological Industries). 10–15 μg of the RNA extract was separated in agarose gel containing formaldehyde and then transferred to nitrocellulose membrane (Amersham Biosciences). Probes for Mkp1, IL-6, IL-1β, Tnf-α, and 18S were labeled with [α-32P]dCTP (Amersham Biosciences) using the random prime labeling system (Amersham Biosciences). 18S was used as the loading control.

Real-time PCR—cDNA was obtained from 1 μg of total RNA using Moloney murine leukemia virus reverse transcriptase transcript (M-MLV, Promega, Madison, WI) as described (29). The primer sequences used for Mkp1, Tnf-α, IL-1β, IL-6, and NOS2 were designed with the Primer Express software (Applied Biosystems). The primers were the following: for β-actin, 5′-ACTATTTGGGCAACGACCGTTT-3′ and 5′-AAGGAA-GGCTGGGAAAAAGAGGG-3′; for NOS2, 5′-GCCACCAACA-ATGGCAACA-3′ and 5′-CTGACCGTAGACTGTGATT-3′; for c-jun, 5′-TGAAGGCGAAACACTCCGAG-3′ and 5′-GACCCCACTGTTAAGTGTTTCC-3′; for Tnf-α, 5′-GCTTTGTGCTCTCCCTTTTGC-3′ and 5′-TCAGTGA-TGTAAGGCAAGCCTG-3′; for IL-1β, 5′-CTCTGTTTCTCCTTGCCT-3′ and 5′-GCTAATGTCCTCCTGGTAA-3′; for IL-6, 5′-CAGAGAAATGGCTAAAGGACCA-3′ and 5′-ACGACTATGGTTTCCGAGTAG-3′; for Jnk1, 5′-GATTGTTGACTGCGGAGACT-3′ and 5′-TAGCCCATG-CGGAATA-3′; for Jnk2, 5′-TGAATGATCCAGACAAG-CGG-3′ and 5′-AAATTGAGGTTGTTGCC-3′; for Jnk-3, 5′-AAACTACGTTGGAATGGCC-3′ and 5′-TGCCCTTG-GCTGGCTTTAAGT-3′ and for Mkp1, 5′-GGCAACACCA-AGGCAGACATC-3′ and 5′-GGCTGGAAAAGAGGG-3′. Real-time PCR was carried out with 2× SYBR Green PCR Master Mix using the ABI Prism 7900 detection system (Applied Biosystems, Foster city, CA). Thermal cycling conditions were 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s for 35 cycles. Data were expressed as relative mRNA values normalized to β-actin expression levels in each sample.

Statistical Analysis—To calculate the statistical differences between control and treated samples, we used the Student’s paired t test. Values of p < 0.05 or lower were considered significant.

RESULTS

JNK Is Required for the Induction of Mkp1 Expression by M-CSF or LPS—Although ERK-1/2 activation is required for both macrophage proliferation and activation, the differential kinetics of their phosphorylation/dephosphorylation correlates with specific macrophage responses (7). In macrophages stimulated with M-CSF or LPS, the induction of Mkp1 correlates with the dephosphorylation of ERK-1/2 (11, 12). Because of inefficient transfection of primary cultures of macrophages (30), we studied the effect of MAPK activation on Mkp1 induction by means of specific inhibitors. PD98095, SB203580, and SP600125 have been used extensively as selective inhibitors of the activation of MEK/ERK, p38, and all JNK isoforms, respectively (31–33). These compounds also inhibited MAPKs in our macrophage model (data not shown).

To test the effect of MAPKs on Mkp1 induction by M-CSF or LPS, we inhibited each MAPK separately. No effect was found when p38 or ERK-1/2 was inhibited (Fig. 1, A and B). Interestingly, JNK activation was required for Mkp1 protein expression (Fig. 1C and D). Northern blot analysis showed that the inhibition of JNK blocked the induction of Mkp1 mRNA (Fig. 1, E and F). Similar results were obtained by real-time PCR (data not shown). Because the half-life of Mkp1 mRNA is short (34), we examined whether the decrease in the level of mRNA was due to inhibition of mRNA production or to an increase in its degradation. For this purpose, we measured the rate of mRNA degradation in the presence or absence of JNK inhibitors. Cells were treated for 30 min with M-CSF or LPS, and actinomycin D was then added at a concentration (5 μg/ml) sufficient to block all further RNA synthesis as determined by [3H]UTP incorporation (35). RNA was isolated at 15, 30, and 60 min after the addition of actinomycin D, which allowed us to estimate the half-life of Mkp1. These mRNAs are highly unstable, and JNK inhibition did not alter the half-life (Fig. 1, G and H), which indicates that the reduction in mRNA levels was due to a
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**FIGURE 2.** Inhibition of JNK activation causes an elongation of phosphorylation of other MAPKs. A, JNK inhibition delays M-CSF-induced ERK deactivation. Macrophages were treated with SP600125 (SP) (25 μM) and stimulated with M-CSF (1200 units/ml) for the indicated times, and ERK activity was determined by an in-gel kinase assay using myelin basic protein as substrate. B, elongated ERK-1/2 activation in macrophages stimulated with LPS (10 ng/ml) and with SP600125. Shown is Western blot analysis of ERK-1/2 phosphorylation of kinase assay using myelin basic protein as substrate. C and D, p38 phosphorylation is extended in JNK-inhibited macrophages. Phosphorylated p38 was determined by Western blot analysis of macrophages treated with SP600125 and stimulated with M-CSF or LPS. E and F, JNK activation is independent of ERK and p38. JNK activity was determined in macrophages stimulated with M-CSF or LPS in the presence of SB203580 (SB) (5 μM) or PD98059 (PD) (50 μM). The results shown are representative of independent experiments that were repeated 3–5 times with similar results.

**FIGURE 3.** JNK is required for LPS-dependent activation. A, JNK is involved in LPS-dependent pro-inflammatory cytokine production. Macrophages were treated with LPS (10 ng/ml) with or without SP600125 (SP) (25 μM) for 24 h, and IL-6, Tnf-α, and IL-1β gene expression was then determined by Northern blot analysis. B, JNK is required for LPS-dependent expression of Nos2. Western blot of Nos2 from total cell lysates of LPS-stimulated cells in the presence of SP600125 is shown. C, Mkp1 KO macrophages present intact LPS-induced JNK activity. JNK activation was determined by JNK assay of LPS-stimulated macrophages at the indicated times. D, the absence of Mkp1 increases LPS-induced cytokine expression. Shown is Northern blot analysis of Tnf-α, IL-1β, and IL-6 gene expression of LPS-stimulated macrophages derived from wild-type (WT) and Mkp1 KO mice. Results are representative of independent experiments that were repeated 3–5 times with similar results.

decrease at the transcriptional level. To determine the nonselective effects of SP600125, we used the inactive analogue JNK inhibitor II (33). At the same concentration (25 μM) at which SP600125 inhibited the expression of Mkp1, no effect was observed when we used the JNK inhibitor II (data not shown).

**Inhibition of JNK Activation Causes an Elongation of Phosphorylation of other MAPKs**—In macrophages treated with either M-CSF or LPS, SP600125 did not modify the induction of ERK-1/2 activation, as determined by in-gel kinase assay (Fig. 2A) and by Western blot using specific antibodies against the phosphorylated form (Fig. 2B). Although the initial activation of these kinases was not modified, the length of activation was prolonged, thereby showing an effect of JNK inhibition on the other MAPKs. Similar effects were observed on p38 phosphorylation induced by M-CSF (Fig. 2C). For LPS, the SP600125 treatment modified the kinetics of activation by slowly delaying and prolonging activation of p38 (Fig. 2D). Cell incubation with SP600125 alone did not induce activation of ERK or p38 (data not shown). However, when we tested the involvement of ERK or p38 in JNK activation, no modifications were detected (Fig. 2, E and F). Taken together, these results indicate that JNK activity is not required for the initial activation of the other MAPKs, but it does control the duration of their activity through induction of Mkp1.

**JNK Is Required for LPS-dependent Activation**—We also studied the role of JNK during LPS activation. Total RNA of macrophages pretreated with SP600125 and then stimulated with LPS was analyzed for pro-inflammatory cytokine expression (Fig. 3A). The expression of Tnf-α, IL-1β, and IL-6 was severely compromised when JNK activity was inhibited. These results were confirmed using real-time PCR (data not show). Furthermore, SP600125 treatment caused a substantial reduction of LPS-induced Nos2 protein expression (Fig. 3B). These data indicate that JNK activation mediates several events in the macrophage inflammatory response to LPS.

To establish whether the role of JNK in cytokine induction was a direct effect or was mediated through MKP1 regulation, we used macrophages from mice with disrupted Mkp1 (24). In these cells, JNK activation in response to LPS was not impaired (Fig. 3C). The LPS-induced expression of pro-inflammatory cytokines was analyzed using Northern blots (Fig. 3D). An increased expression of Tnf-α was detected in Mkp1 KO macrophages, thereby confirming the results of other authors (13–16). This observation demonstrates that the effects of JNK during LPS activation are not due to impaired Mkp1 regulation.

**Jnk1 Is Required for Mkp1 Induction by LPS or M-CSF**—To date, 10 isoforms resulting from three encoding genes of Jnk, namely Jnk1, Jnk2, and Jnk3, have been characterized (36–38). Jnk1 and Jnk2 are expressed ubiquitously, whereas Jnk3 is restricted to brain, testis, and heart (37, 39). We studied the JNK isoforms predominantly expressed when macrophages proliferate or become activated by LPS. Using PCR with specific primer pairs, the mRNA levels of each isoform were determined. Jnk1 and Jnk2 were constitutively expressed (Fig. 4A). However, Jnk3 was not detected in macrophages even after treatment with M-CSF (Fig. 4A) or LPS (data not shown). Therefore, we focused our studies on the activation of these isoforms. The specificity of the antibodies was tested using the KO mice for Jnk1, Jnk2, and Jnk3. For Jnk1, two distinct protein products were detected. Although p46 was constitutively pres-
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ent, p54 was detected preferentially after stimulation with M-CSF (Fig. 4B). For JNK2, only p54 was detected, and this protein was not modified by treatments with M-CSF, LPS, or TNF-α (Fig. 4C).

To evaluate the JNK isoform that is active during proliferation or activation, we immunoprecipitated the anti-JNK isoform and then performed phosphorylation assays using c-Jun as substrate. Both stimuli, LPS and M-CSF, induced strong activation of the JNK1 isoform with a similar time course (Fig. 4D). Under these experimental conditions no JNK2 activity was detected.

The results thus far have suggested that the JNK1 isoform is involved in the transcription of *Mkp1* induced by M-CSF and LPS. To corroborate these results, we performed studies with Jnk1 and Jnk2 single KO mice. First, the maturation of bone marrow-derived macrophages was characterized measuring F4/80 and Mac-1 surface expression as markers of macrophage terminal differentiation (40, 41). Cytometric analysis did not reveal significant differences in the differentiation state of macrophages from single isoform-specific Jnk KO mice and wild-type animals (Fig. 5A), and neither were differences found when we measured M-CSF-dependent proliferation (Fig. 5B), the cell cycle (Fig. 5C), or the induction of apoptosis by deprivation of the growth factor M-CSF (Fig. 5D).

Using macrophages from Jnk2 KO mice, LPS-dependent JNK1 activity was detected (Fig. 5E). Although in macrophages from wild-type mice, no JNK2 activity was observed, in Jnk1 KO macrophages we detected activity for this isoform (Fig. 5F). As expected, neither JNK1 nor JNK2 activity was detected in Jnk1 or Jnk2 KO macrophages, respectively (data not shown).

The absence of JNK1 caused a decrease in the induction of *Mkp1* expression similar to the effect obtained after JNK inhibition with SP600125 (Fig. 6, A and B), suggesting that the effect of the inhibitor is mediated directly on JNK and not on other kinases. Similar results were obtained when we determined the protein levels of MKP1 (data not shown). However, no effect on *Mkp1* expression was observed in the absence of JNK2 (Fig. 6, C and D). In Jnk2 KO macrophages, SP600125 inhibited the expression of the phosphatase, thereby confirming that a JNK isoform other than JNK2 was involved in *Mkp1* transcription.

JNK mediates its effects through induction and activation of the transcription factor c-Jun (39, 42). In
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Induction of Mkp1 by LPS or M-CSF requires JNK1. A number of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, are dependent on the transcriptional activator AP-1 complex (43, 44). Consequently, we studied the effect of JNK isoforms on the pro-inflammatory response of macrophages by means of real-time PCR. The absence of JNK1 was critical for TNF-α, IL-1β, or NOS2 induction by LPS (Fig. 6A). Similar results were found when we used TNF-α as activator. The involvement of JNK2 was also examined but no defects were observed in cytokine production or NOS2 expression (Fig. 7, B and C). The inhibition of JNK or ERK in macrophages from Jnk2 KO mice blocked LPS-dependent cytokine production. These data corroborate the critical role of JNK1 as a mediator of LPS-induced macrophage activation.

In summary, our results demonstrate that JNK1 is involved in two critical mechanisms for macrophage function, namely the induction of the MKP1 phosphatase, responsible for deactivating MAPKs, and the pro-inflammatory response induced by LPS.

DISCUSSION

In bone marrow-derived macrophages, inactivation of ERK-1/2 tightly follows the induction of MKP1 (7). Interestingly, a number of conditions that inhibit Mkp1 expression in macrophages, for example extracellular matrix proteins, such as decorin or fibrinogen or treatment with cyclosporin A or FK506, elongate ERK activity and reduce proliferation (8, 9). These observations indicate that the extent of ERK activation contributes to determining macrophage responses. Recently, the inhibition of Mkp1 by gene targeting shows that this phosphatase has little effect on ERK inactivation (16, 24). However, under these conditions, other members of the phosphatase family are induced that have profiles that correlate with ERK inactivation.3

In several cell types, Mkp1 expression is regulated directly by ERK-1/2 (17, 18), thus providing a direct negative feedback mechanism. However, in macrophages, Mkp1 induction by M-CSF and LPS is not mediated by ERK itself (11, 12). In this report, we demonstrate that another member of the MAPK family, JNK1, is required for the induction of Mkp1 during macrophage response to LPS or M-CSF. This regulation has also been proposed in serum-treated NIH3T3 cells (19). In our hands, blockage of JNK activity resulted in extended ERK activity, which confirms that JNK1 affects the activation profile of ERK-1/2 in macrophages through the control of Mkp1 expression. These data are in agreement with other observations in a number of cellular models. For example, Cos-7 cells transfected with an active form of Jun exhibit attenuated ERK activation, thus establishing a point of cross-talk between the JNK and ERK pathways (45). Transcriptional regulation of Mkp1 expression by JNK1 is probably exerted through the phosphorylation of c-Jun. In fact, the promoter of Mkp1 contains an AP-1 box, responsible for the transcriptional induction by M-CSF or by LPS.4 As shown in Xenopus oocytes, JNK may also decrease the stability of MKP1 protein (46), thereby regulating this phosphatase at two distinct levels.

Here we also observed extended activation of p38 upon JNK inhibition, which suggests that MKP1 regulation also serves as a point of cross-talk between the JNK and p38 pathways. Although we are uncertain of the functional consequences of changes in the activation profile of p38 in macrophages, JNK1 acts as a master regulator of the MAPK family in macrophages. Although JNK has been considered mainly a stress-responsive protein kinase, a role in differentiation, proliferation, and cell death has also been proposed (47). Here we have shown that

3 A. F. Valledor, E. Sánchez-Tilló, Ll. Arpa, M. Comalada, C. Casals, J. Xaus, C. Caelles, J. Lloberas, and A. Celada, manuscript in preparation.

4 C. Casals, E. Alvarez, M. Serra, C. de la Torre, E. Sánchez-Tilló, C. Caelles, J. Lloberas, and A. Celada, manuscript in preparation.
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**A** JNK1 is required for the induction of pro-inflammatory cytokines and NOS2 by LPS and TNF-α. Macrophages from Jnk1 KO mice were stimulated with LPS (10 ng/ml) or TNF-α (10 ng/ml) for 6 h. The levels of mRNA were then measured by real-time PCR in relation to β-actin. Each point was performed in triplicate. B, JNK2 is not involved in cytokine production by LPS. The experiment was performed as in A. In some cases, SP600125 (SP) (25 μM) and PD98059 (PD) (50 μM) were used. C, JNK2 is not involved in NOS2 induction by LPS or by IFN-γ. Shown is Western blot analysis of NOS2 from wild-type (WT) and Jnk2 KO macrophages stimulated with LPS (10 ng/ml) or IFN-γ (1,200 units/ml) for the indicated times. Results are representative of independent experiments that were repeated 3–5 times with similar results. *p < 0.01 between the KO and the corresponding controls when we tested all of the experiments performed.

Recently it has been shown that glycosylphosphatidylinositol (GPI) from *Plasmodium falciparum* induces the production of inflammatory cytokines in macrophages (54). Their study of the requirement of MAPKs and NF-κB reveals that the production of TNF-α, IL-12, IL-6, and nitric oxide by macrophages stimulated with parasite GPIs is critically dependent on NF-κB and JNK pathways. In their results, JNK1 and JNK2 are functionally redundant for the expression of a proinflammatory response, whereas JNK2 but not JNK1 is essential for IL-12 production. However, there are two important differences that may explain the apparent discrepancy between these results and ours. First, although Zhu *et al.* (54) primed macrophages with IFN-γ, our results were obtained by stimulating nonprimed macrophages. Second, we stimulated macrophages with LPS at low concentrations (10 ng/ml) where only signaling through TLR4 is induced (55). This was confirmed because in macrophages from C3H/HeJ mice with the TLR4 disrupted, we found them unresponsive to the doses of LPS that we used (data not shown). In contrast to LPS, GPIs, are recognized mainly by TLR2 and to a lesser extent by TLR4. Therefore, the apparent discrepancy between the results of both groups is probably based on the different TLRs engaged as well as on the use of macrophages primed or not with IFN-γ.

In summary, our results provide experimental evidence that the JNK1 and JNK2 isoforms exert distinct functions in macrophage biology. JNK1 is involved in the induction of *Mkp1* expression under both M-CSF and LPS stimulation. Likewise, JNK1, but not JNK2, plays a predominant role in the induction of pro-inflammatory mediators in response to LPS and TNF-α activation, thus revealing a new insight into how to target abnormal inflammatory responses by activated macrophages.

Little is known about the role of JNK in human cancer. However, it has been proposed that activated JNK promotes a transformed phenotype (56, 57), and constitutively active JNK activity has been described in human tumor lines and inflammatory diseases (58, 59). In addition, *Mkp1* is overexpressed in many malignant human tumors (60, 61), which suggests a correlation between JNK and *Mkp1* expression. Therefore, we propose that JNK also should be considered for novel therapeutic strategies aimed at regulating *Mkp1* expression in tumors or in non-adapted inflammatory responses observed in several lung diseases such as asthma or acute respiratory distress syndrome.

**Acknowledgments**—We thank Bristol-Myers Squibb Co. and Dr. R. A. Flavell for the gift of *Mkp1* and *Jnk1*, *Jnk2*, and *Jnk3* KO mice. We also thank Tanya Yates for editing this manuscript.

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JNK1 Is Required for the Induction of Mkp1 Expression in Macrophages during Proliferation and Lipopolysaccharide-dependent Activation
Ester Sánchez-Tilló, Mónica Comalada, Jordi Xaus, Consol Farrera, Annabel F. Valledor, Carme Caelles, Jorge Lloberas and Antonio Celada

J. Biol. Chem. 2007, 282:12566-12573.
doi: 10.1074/jbc.M609662200 originally published online March 2, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M609662200

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