Activation of Raf-1 and Mitogen-activated Protein Kinase in Murine Macrophages Partially Mimics Lipopolysaccharide-induced Signaling Events

By Julie Hambleton,[*][‡] Martin McMahon,[‖] and Anthony L. DeFranco[‡][§]

From the [*]Cancer Research Institute, Department of Medicine, [‡]George Williams Hooper Foundation, and [§]Department of Microbiology and Immunology, University of California, San Francisco, San Francisco, California 94143; and [‖]DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, California 94304

Summary

Lipopolysaccharide (LPS), a highly conserved component of the outer membrane of gram-negative bacteria, stimulates macrophages to release various cytokine and eicosanoid mediators of the immune response. The mechanism by which LPS stimulates these cells is poorly characterized. One of the most rapid LPS-stimulated events is the phosphorylation and activation of the p42 and p44 isoforms of mitogen-activated protein (MAP) kinase. We wished to examine the role of MAP kinase in LPS-induced signaling in murine macrophages by activating MAP kinase independently of LPS. An expression vector encoding a Raf-1:estrogen receptor (ER) chimeric protein was transfected into the murine macrophage cell line RAW 264.7. Activation of this chimeric protein (ΔRaf-1:ER) by estradiol resulted in rapid and prolonged activation of MAP kinase, as expected from previous results implicating Raf-1 as an upstream activator of this signaling cascade. LPS stimulation induced accumulation of MAP kinase phosphatase 1 messenger RNA, whereas ΔRaf-1:ER activation did not, perhaps accounting for the more prolonged activation of MAP kinase seen in response to ΔRaf-1:ER activation. Similarly, activation of DNA binding by the transcription factor, nuclear factor (NF) κB, as assessed by electrophoretic mobility shift assay, occurred in response to LPS stimulation but not in response to ΔRaf-1:ER activation or phorbol myristate acetate (PMA) stimulation. Using an enzyme-linked immunosorbent assay for murine tumor necrosis factor α (TNF-α), we found that LPS and PMA stimulation and ΔRaf-1:ER activation induced secretion of TNF-α, although the amount of TNF-α secreted in response to ΔRaf-1:ER activation and PMA stimulation was ~20-fold less than that secreted in response to LPS. Correspondingly, accumulation of TNF-α messenger RNA was weakly induced by ΔRaf-1:ER activation or PMA stimulation, whereas strong induction was noted in response to LPS. These results suggest that Raf-1 or PMA activation of MAP kinase in murine macrophages is sufficient for a small amount of TNF-α production and secretion in the absence of NF-κB activation, but LPS stimulation involves additional signaling events, such as NF-κB activation, that augment the response seen with activation of MAP kinase alone.

The host's response to infection with gram-negative bacteria is initiated by exposure to LPS, a major component of the bacterium's outer membrane and a potent activator of macrophages (1, 2). Subsequent production and release of large numbers of immunoregulatory molecules, including TNF-α, IL-1, IL-6, and arachidonic acid metabolites, then recruit and activate other immune cells to help fight the infection (3, 4). Additionally, LPS stimulation of IFN-γ–primed macrophages induces them to acquire bactericidal and tumoricidal activity (5, 6). Systemic stimulation of these immune reactions may culminate in septic shock, a major cause of morbidity and mortality each year.

The mechanism by which LPS activates macrophages is still poorly understood. At physiological concentrations, LPS binds to the serum protein LPS-binding protein. This complex then interacts with CD14 on macrophages or neutrophils (7–11). As CD14 is a glycosyl phosphatidylinositol–linked protein, it is unclear how LPS binding to it leads to intracellular signaling events. Nonetheless, phosphorylation of various proteins on tyrosyl residues is the most rapid intracel-

lular event induced by LPS in macrophages known thus far (12, 13). Inhibitors of protein tyrosine phosphorylation block the secretion of cytokines and the generation of eicosanoids (13–17), arguing for the importance of this signaling event. Among the most prominent tyrosine-phosphorylated proteins in LPS-stimulated macrophages are the p42 and p44 isoforms of mitogen-activated protein (MAP)[1] kinase (13, 18, 19). MAP kinases are a family of serine/threonine protein kinases that participates in signaling pathways initiated by many extracellular stimuli. In *Saccharomyces cerevisiae*, for example, distinct MAP kinase pathways are involved in mediating the pheromone response, cell wall biosynthesis, and osmoregulation (20, 21). In multicellular organisms, the MAP kinase cascade also plays an important role in mediating signaling events. MAP kinase activation has been implicated in the activation of the transcription factors p62^{TCF} and nuclear factor (NF)–IL-6 (22, 23) and of cytoplasmic phospholipase A$_2$, which catalyzes the release of arachidonic acid from membrane phospholipid (24).

To examine the role of MAP kinase activation in LPS signaling, we wished to determine whether activation of MAP kinase by a means independent of LPS would mimic the downstream biologic events seen with LPS stimulation. In this report, we show that activation of a regulatable form of Raf-1 in murine macrophages resulted in strong activation of p42 and p44^{MAPK} but only modest *TNF-α* messenger RNA (mRNA) accumulation and protein secretion. Moreover, LPS stimulated accumulation of *MAP kinase phosphatase 1 (MKP-1)* mRNA and activation of the transcription factor NF-κB, whereas Raf-1 did not. These results suggest that LPS induces additional signaling events, such as NF-κB activation, that augment the response seen with activation of MAP kinase alone.

Materials and Methods

Materials and Antibodies. LPS (*Salmonella minnesota* R595) was purchased from List Biological Laboratories, Inc. (Campbell, CA) and prepared as a 5-mg/ml suspension in pyrogen-free H$_2$O. β-Estradiol and myelin basic protein (MBP) were purchased from Sigma Chemical Co. (St. Louis, MO). ICI 164,384, which binds and inhibits the function of native estrogen receptors, was a gift of Alan Wakeling (Zeneca Pharmaceuticals, Cheshire, UK). Estradiol and ICI 164,384 were stored as 1-mM stock solutions in ethanol at −20°C. G418 was purchased from Gibco BRL (Gaithersburg, MD). Anti-p42^{MAPK} mAb (1B3B9) was a gift from Dr. Michael Weber (University of Virginia, Charlottesville, VA). Anti–TNF-α antibody was purchased from Genzyme Corp. (Cambridge, MA), and anti–IκB-α and anti-p44^{MAPK} antibodies were procured from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Sheep anti–mouse IgG conjugated to horseradish peroxidase and the enhanced chemiluminescent detection kit for immunoblotting were purchased from Amersham Corp. (Arlington Heights, IL).

Cell Culture, Electroporation, Stimulation, and Cell Lysis. The Abelson murine leukemia virus–transformed macrophage cell line

RAW 264.7 (American Type Culture Collection, Rockville, MD) was cultured in DME containing 10% heat-inactivated fetal bovine serum (containing <0.6 endotoxin U/ml) and 2 mM glutamine at 37°C in a 5% CO$_2$/air mixture. A truncated, oncogenic form of human p74^{Raf-1} fused to the hormone-binding domain of the HE14 allele of the human estrogen receptor (ΔRaf-1:ER) was cloned into the replication-defective retrovirus pLNCX vector that encodes G418 resistance, as previously described (25). This expression vector was transfected into RAW 264.7 cells by electroporation, using a Gene Pulser (Bio-Rad Laboratories, Richmond, CA) at settings of 350 V and 500 μF. G418-resistant clones were screened for expression of the ΔRaf-1:ER protein by immunoblotting, using a polyclonal antiserum that recognizes the hormone-binding domain of the human estrogen receptor (gift of Dr. S. Robbins, G. W. Hooper Foundation, University of California, San Francisco, CA). Transfectants were maintained in the above medium supplemented with 1.5 mg/ml G418.

To examine cellular responses, 1–2 × 10^6 cells were seeded onto six-well plates (Costar Corp., Cambridge, MA) in 1.5 ml of medium. Parental RAW 264.7 cells were cultured ∼16 h to reach 80% confluency. RAW:ΔRaf-1:ER cells, which grow more slowly than the parental cells when maintained in G418 at 1.5 mg/ml, were cultured for 2 d to reach ∼80% confluency. After stimulation, cells were washed in situ with ice-cold PBS containing 1 mM sodium orthovanadate (Na$_3$VO$_4$) and then lysed on ice in 0.2 ml of lysis buffer containing 20 mM Tris (pH 7.9), 137 mM NaCl, 5 mM Na$_2$ EDTA, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 1 mM PMSF, 1 mM aprotinin, 1 mM leupeptin, 1 mM Na$_3$VO$_4$, 1 mM EGTA, 10 mM sodium fluoride, 1 mM tetrasodium pyrophosphate, and 100 μM β-glycerophosphate. Insoluble material was removed by centrifugation at 12,000 g, and soluble protein was stored at −80°C or used immediately. Protein concentrations of the lysates were determined by using the BCA protein assay kit (Pierce, Rockford, IL).

Electrophoresis and Immunoblotting. Protein samples were prepared for electrophoresis as previously described (25). Samples were separated on 12.5% SDS-PAGE containing an acrylamide/bis-acrylamide ratio of 120:1 and transferred to nitrocellulose. The nitrocellulose membranes were blocked by incubation in Tris-buffered saline containing 0.5% (vol/vol) NP-40 and 5% nonfat dry milk. Subsequently, the membranes were incubated with anti–MAP kinase antibody and washed in Tris-buffered saline containing 0.5% (vol/vol) NP-40. Antigen–antibody complexes were visualized by incubating the membranes with 1:10,000 diluted sheep anti–mouse IgG antibody coupled to horseradish peroxidase and using the enhanced chemiluminescent detection system.

In-gel MAP Kinase Assay. MAP kinase activity was assessed by the in-gel kinase assay, wherein MBP, an MAP kinase substrate, was copolymerized into the polyacrylamide gel (25, 26). After resolution of lysate proteins by SDS-PAGE through the MBP-containing gel, the gel was subjected to a denaturation/renaturation procedure, after which it was incubated in the presence of γ-[^{32}P]ATP to allow phosphorylation of MBP, washed extensively, dried, and exposed to x-ray film (25, 26).

RNA Isolation and Northern Blot Analysis. Total RNA was extracted from cells by the acid phenol method as described (27), with the exception that only one isopropanol precipitation was performed. RNA (2.0–2.5 μg/lane) was resolved by electrophoresis through 1.2% agarose gels containing 2 M formaldehyde, buffered with 5 mM Hepes, pH 7.0, transferred to membranes (GeneScreen; New England Nuclear, Boston, MA), and cross-linked to the membranes by UV irradiation with an irradiator (Stratalinker 1800; Stratagene, La Jolla, CA). Specific RNA was detected by probing the mem-

[1] *Abbreviations used in this paper:* EMSA, electrophoretic mobility shift assay; ER, estrogen receptor; MAP, mitogen-activated protein; MBP, myelin basic protein; MKP-1, MAP kinase phosphatase 1; mRNA, messenger RNA; NF, nuclear factor.

branes with random oligonucleotide-primed (Boehringer Mann-heim Corp., Indianapolis, IN) ^{32}P-labeled cDNA probes. Hybrid-ization was performed at 42°C in buffer containing 50% formamide, 1 M NaCl, 1% SDS, 10% dextran sulfate, and 125 μg/ml dena-tured salmon sperm DNA. Filters were washed in 0.1 × SSC and 1% SDS at 65°C to remove nonspecific binding of the probe. To strip off probe for subsequent reprobing, blots were boiled for 10 min in 0.1% SDS. To ensure that similar amounts of RNA were loaded in each lane, the stripped blots were reprobed with the cDNA of the constitutively expressed gene *GAPDH* (28).

The probe for *MKP-1 (3CH134)* was made from the EcoRI frag-ment of *3CH134* BALB/c mouse DNA, obtained from Dr. D. Na-thans (Johns Hopkins University, Baltimore, MD) (29). The probe for the murine *TNF-α* gene was a gift from Dr. S. Reiner (Univer-sity of California, San Francisco) (30).

NF-κB Assay. For the assay of NF-κB activation, ∼2–4 × 10^{6} cells were seeded onto 100-mm plates and grown to 70–80% confluency. Preparation of nuclear extracts for measurement of NF-κB DNA binding activity by electrophoretic mobility shift assay (EMSA) were performed as described by Kitchens et al. (31). The NF-κB oligonucleotide containing the murine immunoglobulin κB enhancer sequence (32) 5' TCAGAGGGGACTTTCCGAGAGGT 3'; 3' TCCCCTGAAAGGCTCTCCAGCT 5' was prepared by an-nealing complementary synthetic oligonucleotides. This binding site was labeled with α-[32P]dCTP using the Klenow fragment of *Escherichia coli* DNA polymerase I (New England Biolabs, Inc., Beverly, MA). Competition experiments were performed with var-ious amounts of unlabeled double-stranded oligonucleotide or with an oligonucleotide that contains mutations in the binding motif and subsequently does not bind NF-κB (33). The mutant κB oli-gonucleotide sequence was as follows: 5' TCAGAGCTCACTTT-CCGAGAGGT 3'. Protein–DNA complexes were then analyzed by PAGE (4%) in 0.5× tris-borate EDTA (34).

TNF-α ELISA. Murine TNF-α ELISA kits were purchased from PerSeptive Diagnostics (Cambridge, MA) and BioSource Inter-national (Camarillo, CA). 50-μl aliquots of cell medium from un-treated cells or from cells stimulated with LPS, estradiol, ICI 164,384, or PMA were analyzed according to the instructions that accompanied the kits. Each sample was incubated for the same length of time and tested in duplicate.

Results

ΔRaf-1:ER Activation Results in Phosphorylation and Activa-tion of MAP Kinase. Stimulation of macrophages with LPS rapidly results in the tyrosine phosphorylation of several pro-teins, including the p42 and p44 isoforms of MAP kinase (13, 18, 19). In addition, the activity of these MAP kinases is greatly increased by LPS treatment. To gain insight into the role of MAP kinase in mediating the response to LPS in macrophages, we sought to activate MAP kinase indepen-dently of LPS. One mechanism by which receptors activate MAP kinase is through stimulation of Ras, which in turn activates the serine/threonine–specific protein kinase Raf-1. Therefore, we made use of an estradiol-dependent form of Raf-1, ΔRaf-1:ER. Addition of estradiol to 3T3 cells expressing ΔRaf-1:ER leads to rapid activation of the fusion protein, MEK, and p42/p44 MAP kinases, and ultimately to onco-genic transformation (25). The RAW 264.7 macrophage cell line, which responds well to LPS, was stably transfected with the expression vector, and transfectants were screened for ex-pression of the ΔRaf-1:ER protein.

Transfectant cells (RAW:ΔRaf-1:ER) and parental RAW 264.7 cells were stimulated for 15 min with estradiol (1 μM), ICI 164,384 (1 μM), LPS (1 μg/ml), or a buffer control (0.1% ethanol vol/vol). Triton X-100–soluble proteins were obtained from the cells, resolved by SDS-PAGE, and immunoblotted with an anti-p42[MAPK] antibody. Phosphorylation of p42[MAPK] is accompanied by reduced electrophoretic mobility upon SDS-PAGE. The electrophoretic shift of p42[MAPK] occurred in re-sponse to LPS stimulation in the parental cells and in response to estradiol, ICI 164,384, or LPS stimulation in RAW:ΔRaf-1:ER cells (Fig. 1 and additional data not shown). This du-ration of LPS stimulation led to the phosphorylation of vir-tually 100% of p42[MAPK]. Activation of ΔRaf-1:ER for the same duration led to phosphorylation of ∼60% of p42[MAPK]. Tyrosine phosphorylation of the shifted form of p42[MAPK] was verified by immunoblotting with the 4G10 antiphos-photyrosine antibody (data not shown). A similar elec-trophoretic shift of p44[MAPK] was seen with blotting with anti-p44 antibody (data not shown). Transfected RAW 264.7 cells that were resistant to G418 but did not express detect-able levels of the ΔRaf-1:ER chimera showed no activation of p42[MAPK] in response to estradiol or ICI 164,384 (data not shown).

To verify that the shifted form of p42/p44 MAP kinase correlated with activation of the enzyme and to analyze the kinetics of this activation, RAW:ΔRaf-1:ER cells were treated with LPS, estradiol, or ICI 164,384 for various lengths of time. Triton X-100–soluble proteins were analyzed by an in-gel MAP kinase activity assay (Fig. 2). In this assay, the ac-tivity of the p42 and p44 MAP kinase isoforms is reflected by the amount of phosphorylation of MBP, an MAP kinase substrate that is incorporated into the gel, at the position in the gel where these MAP kinases migrate. As previously demonstrated for the parental RAW 264.7 cells (13), LPS stimulation of RAW:ΔRaf-1:ER cells, as well as stimulation of ΔRaf-1:ER by estradiol or ICI 164,384, resulted in activa-tion of the p42 and p44 isoforms of MAP kinase. LPS stimu-lation of p42/p44 was noted after 10 min of stimulation, was maximal by 15 to 20 min, and declined between 1 and 4 h (Fig. 2 *A* and additional data not shown). Stimulation of ΔRaf-1:ER by estradiol or ICI 164,384 resulted in a similar onset of activation, but this activation was prolonged and persisted for at least 30 h (Fig. 2, *A* and *B*).
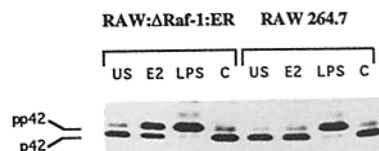

Figure 1. Shift in electrophoretic mobility of p42[MAPK] upon ΔRaf-1:ER activation or LPS stimulation. 50 μg of detergent-soluble proteins from unstimulated (*US*) RAW:ΔRaf-1:ER or parental RAW 264.7 cells or from cells treated for 15 min with estradiol (*E2*; 1 μM), LPS (1 μg/ml), and control (*C*; ethanol 0.1% vol/vol) were separated on 12.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with 1B3B9 anti-p42[MAPK] antibody. p42 refers to p42[MAPK], and pp42 indicates the phos-phorylated form of p42[MAPK], which is a more slowly migrating form of p42[MAPK].
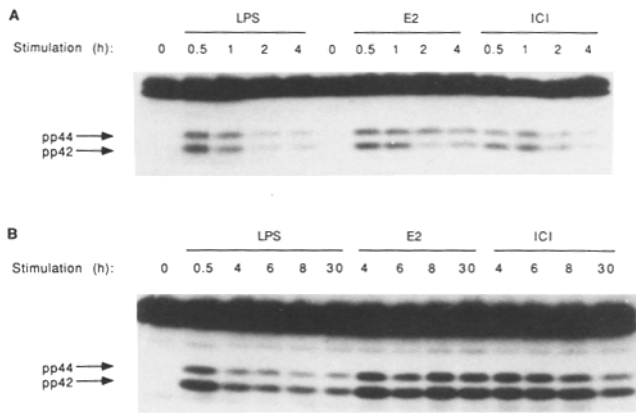
Figure 2. Enzymatic activation of p42 and p44 isoforms of MAP kinase in response to LPS, estradiol, or ICI 164,384. 50 μg of detergent-soluble protein from unstimulated (time 0) RAW:ΔRaf-1:ER cells or from these cells treated with LPS (1 μg/ml), estradiol (E2; 1 μM), or ICI 164,384 (1 μM) for varying lengths of time were electrophoresed into SDS-polyacrylamide gels containing MBP. After denaturation and renaturation of proteins, the kinase activity was determined in situ by phosphorylation of the incorporated MBP. pp42 and pp44 indicate the activated p42 and p44 isoforms of MAP kinase, respectively. Please note that B is a longer exposure than A.

Figure 4. NF-κB activation in response to LPS or estradiol. EMSA was performed on 5 μg of nuclear extracts from parental or RAW:ΔRaf-1:ER cells (pLNC2). The parental RAW 264.7 cells were either unstimulated (US) or treated with LPS (1 μg/ml) for 1 h. EMSA was then performed using the ³²P-labeled κB oligonucleotide. Competition experiments were done with the addition of unlabeled wild type (WT κB) or mutated oligonucleotide (mut κB) as indicated. RAW:ΔRaf-1:ER cells were either unstimulated (US) or stimulated with LPS (1 μg/ml) or estradiol (E2; 1 μM) for 1 h. The positions of the oligonucleotide complex and the free oligonucleotide are indicated.

Sustained Activation of MAP Kinase by ΔRaf-1:ER May Be Due to the Absence of Phosphatase Induction. The prolonged phosphorylation and activation of p42^MAPK observed in estradiol-treated RAW:ΔRaf-1:ER cells suggested that the normal inactivation of the MAP kinase pathway seen in LPS-treated cells is not occurring. It is thought that inactivation of MAP kinase occurs by removal of the activating phosphates by phosphatases. *MKP-1* or *3CH134* is a growth factor–inducible gene that encodes a dual specificity phosphatase that can dephosphorylate and inactivate MAP kinase in vitro and in vivo (35, 36). In RAW:ΔRaf-1:ER cells, *MKP-1* mRNA was induced in response to LPS stimulation (Fig. 3). LPS stimulation of *MKP-1* mRNA was detected at 30 min, peaked at 2 h, and declined thereafter. In contrast, activation of the ΔRaf-1:ER fusion protein did not induce the *MKP-1* gene in these cells. Similarly, the *MKP-1* gene was not induced in response to PMA stimulation. Activation of ΔRaf-1:ER in 3T3 cells also does not lead to induction of *MKP-1* mRNA expression (McCarthy, S., and M. McMahon, unpublished
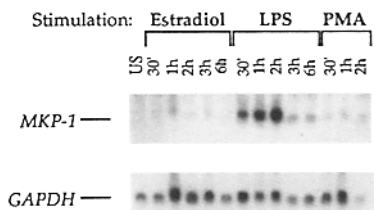

Figure 3. Induced expression of the *MKP-1* gene in RAW:ΔRaf-1:ER cells. Total RNA was isolated from unstimulated (US) RAW:ΔRaf-1:ER cells or from cells stimulated for varying lengths of time with estradiol (1 μM), LPS (1 μg/ml), or PMA (100 nM), and then subjected to Northern blot analysis (~2.0–2.5 μg RNA per lane). The filter was probed with an *MKP-1* cDNA clone, stripped, and then reprobed with *GAPDH* to verify similar loading of RNA in each lane.
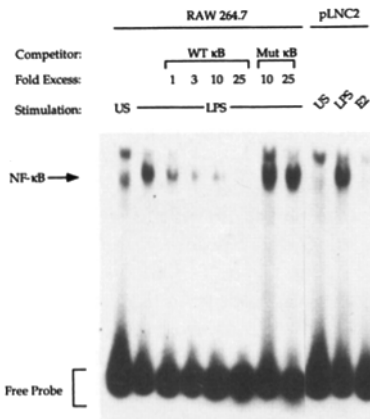
observations). The failure of the ΔRaf-1:ER protein to induce *MKP-1* expression may contribute to the prolonged activation of MAP kinases seen upon activation of this protein.

Stimulation of ΔRaf-1:ER Does Not Result in NF-κB Activation. Since activation of ΔRaf-1:ER in RAW 264.7 cells resulted in the rapid and near-complete activation of MAP kinase, it was of interest to assess whether activation of p42^MAPK and p44^MAPK by the ΔRaf-1:ER fusion protein mimics LPS with respect to downstream events. NF-κB is thought to play a central role in LPS-mediated transcriptional regulation. In RAW 264.7 cells, LPS has been shown to induce nuclear translocation of the NF-κB 50-kD subunit and activation of reporter constructs using NF-κB–binding sites (33, 37, 38). Moreover, there is one report that p74^Raf-1 may promote NF-κB activity via phosphorylation and inactivation of IκB-α (39). We therefore wished to test whether activation of ΔRaf-1:ER would lead to activation of NF-κB. RAW:ΔRaf-1:ER cells were stimulated for up to 2 h with estradiol, LPS, or PMA, and nuclear extracts were prepared. These extracts were tested for the presence of NF-κB DNA binding activity by EMSA. As shown in Fig. 4, LPS activated NF-κB binding activity in these cells, whereas estradiol did not. PMA stimulation of RAW 264.7 and RAW:ΔRaf-1:ER cells also did not lead to an increase in NF-κB DNA binding activity (data not shown). The LPS-inducible complex was specifically competed away by an unlabeled oligonucleotide containing the κB-binding site, but not by an unlabeled oligonucleotide with mutations in this κB site. These results demonstrate that the LPS-inducible complex is specific for κB-like DNA sites. Moreover, LPS stimulation resulted in the transient decrease in IκB-α protein that is noted with NF-κB activation (40), whereas no change in IκB-α protein level was seen with ΔRaf-1:ER activation by estradiol (data not shown).

In separate experiments conducted in 3T3 cells expressing ΔRaf-1:ER, we found no evidence that activation of ΔRaf-1:ER had any effect on NF-κB binding activity or transcriptional activation (Samuels, M. L., and M. McMahon, unpublished observations). Furthermore, immune complexes of ΔRaf-1:ER, which phosphorylated MEK, did not phosphorylate a purified GST-IκB-α fusion protein (Samuels, M. L., S. Gerondakis, and M. McMahon, unpublished observations).

Activation of ΔRaf-1:ER Results in a Low Level of TNF-α Secretion. LPS stimulation of macrophages leads to secretion of many cytokines, among the most important of which is TNF-α. LPS regulates TNF-α expression at both the transcriptional and translational levels (41–44). LPS has been shown to induce TNF-α expression at the transcriptional level at least in part via the NF-κB–binding sites in the 5′ promoter region of the gene (41). Using an ELISA to measure the amount of TNF-α protein secreted into the cell medium, we found that estradiol, ICI 164,384, PMA, or LPS stimulation induced secretion of TNF-α by RAW:ΔRaf-1:ER cells, although the amount of TNF-α secreted in response to estradiol, ICI 164,384, or PMA stimulation was 20-fold less than that secreted in response to LPS stimulation (Fig. 5, *A* and *B*). The kinetics of TNF-α production was similar in each case, and secretion was seen as early as 30 min after stimulation. The levels of secreted TNF-α depicted in Fig. 5 are representative of at least two experiments for each stimulation condition. TNF-α secretion was not increased in response to estradiol or ICI 164,384 in either the RAW 264.7 cells or the G418-resistant transfected cells that did not express the ΔRaf-1:ER chimera (Fig. 5 *C*). In the RAW:ΔRaf-1:ER cells, ethanol (0.1%) did not induce TNF-α secretion (data not shown). LPS-induced secretion of TNF-α at 3 and 6 h was confirmed by immunoblotting with anti-TNF-α antibody (data not shown).

To test whether this difference in TNF-α secretion between LPS and estradiol or PMA stimulations was reflected at the mRNA level, Northern blot analysis of RNA from RAW:ΔRaf-1:ER cells was performed. A strong induction of the *TNF-α* gene in response to LPS stimulation was demonstrated, whereas only weak inductions in response to ΔRaf-1:ER activation or PMA stimulation were detected (Fig. 6). This weak induction over background was confirmed by quantitation on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and is representative of two experiments. Thus, activated Raf-1 was able to induce accumulation of *TNF-α* mRNA, but considerably less efficiently than LPS.

Discussion

LPS stimulation of macrophages results in a rapid increase in protein tyrosine phosphorylation and activation of the p42 and p44 isoforms of MAP kinase. We have introduced a regulatable form of Raf-1 that can activate MAP kinase independently of LPS into murine macrophages, allowing us to evaluate possible downstream events. In RAW 264.7 macrophage cells, as previously reported in fibroblasts, estradiol-induced activation of the ΔRaf-1:ER chimeric protein resulted in rapid and prolonged phosphorylation and activation of the
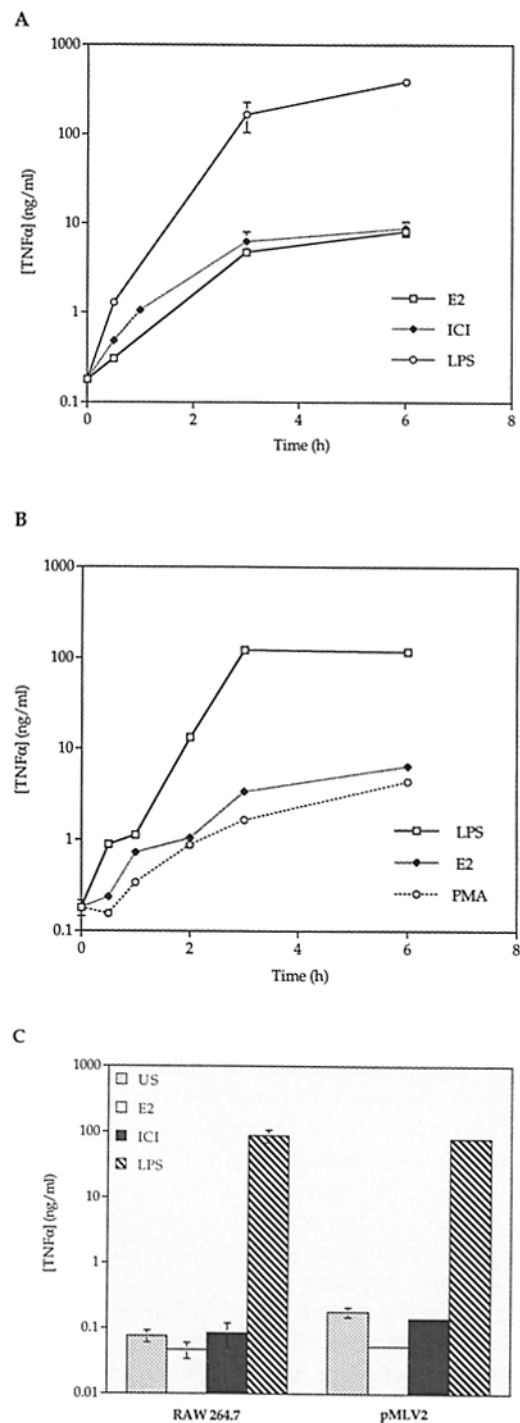
Figure 5. Induced production of secreted TNF-α in RAW:ΔRaf-1:ER cells. 50-μl aliquots of medium from untreated RAW:ΔRaf-1:ER cells (time 0) or from cells stimulated (*A*) with LPS (1 μg/ml), estradiol (*E2*; 1 μM), or ICI 164,384 (1 μM); or, in a different experiment (*B*), with LPS, estradiol, or PMA (100 nM); were analyzed for TNF-α secretion by ELISA. All samples were incubated for the same length of time, so that the 0 time point reflects an unstimulated control. The samples were tested in duplicate. (*C*) 50-μl aliquots of medium from unstimulated (*US*) parental RAW 264.7 cells or G418-resistant transfected cells that do not express ΔRaf-1:ER (*pMLV2*), or from cells stimulated with LPS (1 μg/ml), estradiol (*E2*; 1 μM), or ICI 164,384 (1 μM), were analyzed for TNF-α secretion by ELISA. The RAW 264.7 cells were stimulated for 3 h, and the pMLV2 cells were stimulated for 2 h.
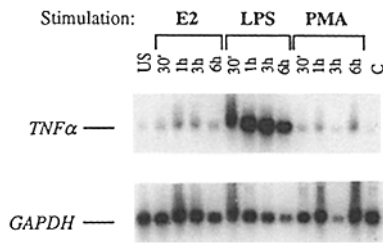
151   Hambleton et al.

Stimulation: E2 LPS PMA

Figure 6. Accumulation of *TNF-α* mRNA in response to stimulation of RAW:ΔRaf-1:ER cells. Total RNA was isolated from unstimulated (*US*) RAW:ΔRaf-1:ER cells or from cells stimulated for varying lengths of time with estradiol (*E2*; 1 μM), LPS (1 μg/ml), PMA (100 nM), or ethanol control (*C*; 0.1% vol/vol; 1-h treatment) and then subjected to Northern blot analysis (~2.0–2.5 μg RNA per lane). The filter was probed with a *TNF-α* cDNA clone, stripped, and then reprobed with *GAPDH* to verify similar loading of RNA in each lane.

p42/p44 MAP kinases. This was evident by mobility shift on SDS-PAGE, which is characteristic for p42/p44^{MAPK} phosphorylation and activation, and by an increase in MAP kinase activity as assessed by an in-gel protein kinase activity assay.

One interesting feature of the activation of MAP kinase by ΔRaf-1:ER is the prolonged activation in comparison to that seen with LPS. The onset of p42/p44 MAP kinase activation by ΔRaf-1:ER was similar to LPS stimulation. However, LPS treatment led to a strong transient activation of MAP kinase followed by a low level of continued activation. In contrast, activation of the ΔRaf-1:ER chimeric protein resulted in a strong sustained activation of p42/p44 MAP kinase. There are many mechanisms that could shut down the LPS response, but one interesting possibility involves the regulation of phosphatases that dephosphorylate and thereby inactivate MAP kinases. In the RAW:ΔRaf-1:ER cells, the immediate early response gene *MKP-1* is induced in response to LPS stimulation, but not in response to activation of the ΔRaf-1:ER chimeric protein with estradiol, suggesting that *MKP-1* induction is not downstream of Raf-1 or MAP kinase activation. Thus, the expression of *MKP-1* in macrophages is not a result of direct feedback of MAP kinase action. Rather, LPS stimulation induces this MAP kinase inhibitor via a distinct signaling route.

After characterizing the activation of MAP kinase by ΔRaf-1:ER, we wished to examine its effect on downstream events. Activation of the transcription factor NF-κB is implicated in the induction of a number of genes by LPS. This transcription factor is composed of two subunits of 50 and 65 kD, which are sequestered in the cytoplasm in an inactive state by IκB. Upon activation, IκB undergoes phosphorylation and degradation, and the NF-κB heterodimer translocates into the nucleus, where it binds to DNA and activates transcription (45, 46). In agreement with other reports (38), LPS stimulated increased DNA binding by NF-κB in RAW 264.7 cells. In contrast, activation of the ΔRaf-1:ER chimera with estradiol did not activate NF-κB detectably. This observation is contrary to a recent report by Li and Sedivy, in which it was found that v-*raf* phosphorylated IκB and activated NF-κB nuclear translocation. Moreover, in their tran-

sient transfection experiments, v-*raf* induced transcription via an NF-κB–based promoter in fibroblasts (39). The failure of ΔRaf-1:ER to activate NF-κB in RAW 264.7 cells could reflect the different means of activating Raf-1 in our experiments versus the experiments of Li and Sedivy. Alternatively, there could be some difference in intracellular components between macrophages and fibroblasts. We also did not observe activation of NF-κB when RAW 264.7 cells were stimulated with PMA, in agreement with other reports (33, 37). As PMA is a potent activator of MAP kinase in RAW 264.7 cells (13), activation of MAP kinase does not correlate with NF-κB activation. Our results suggest that neither Raf-1 nor MAP kinase is responsible for activating NF-κB in macrophages, and that LPS does this via a different signaling pathway.

NF-κB activation is thought to be critical for *TNF-α* gene expression in macrophages stimulated by LPS (41, 42, 44). While LPS stimulates strong TNF-α production and *TNF-α* mRNA accumulation, activation of the ΔRaf-1:ER chimera or stimulation with PMA gave smaller (~20× less by ELISA) but clearly positive TNF-α responses. The most straightforward interpretation of these results is that activation of MAP kinase by Raf-1 or PMA in murine macrophages is sufficient for a small level of TNF-α secretion in the absence of NF-κB activation. In contrast, LPS activates both MAP kinase and NF-κB, which may cooperate to give the high level of TNF-α secretion seen upon stimulation in this way. Alternatively, other signaling events triggered by LPS may play critical roles instead of or in addition to NF-κB and MAP kinase. Similarly, Raf-1 could be activating other proteins, in addition to MAP kinase, that have not yet been identified but that mimic LPS and PMA stimulation with respect to downstream events.

The antiestrogen ICI 164,384 binds and inhibits the function of native estrogen receptors (47–49) and is a potent activator of the ΔRaf-1:ER chimeric protein. The ability of this compound to induce activation of MAP kinase and secretion of TNF-α in cells expressing ΔRaf-1:ER argues against the possibility that endogenous estrogen receptors play a role in these signaling processes, and it ascribes that function to the activation of Raf-1 in these cells.

The mechanism by which LPS activates MAP kinase is not yet completely established. In vertebrates, the p42 and p44 isoforms of MAP kinase can be activated by at least two separate pathways that converge at the level of MAP kinase kinase or MEK. One pathway involves ligand-activation of receptor tyrosine kinases with the sequential activation of Ras, Raf, MEK, and MAP kinase. This pathway has been established by a variety of biochemical experiments (20, 21) and by genetic studies of developmental mutants of *Drosophila melanogaster* and *Caenorhabditus elegans* (50). The existence of a second pathway was suggested by studies in yeast, and a vertebrate homologue of the yeast gene product STE 11, called MEK kinase, has been cloned and found capable of activating MEK (51).

In human monocytes, LPS stimulation increases Ras GTP/GDP exchange (52), and LPS treatment of BAC-1.25 macrophages leads to activation of Raf-1, MEK, and MAP kinase, as well as phosphorylation of the transcription factor

Elk-1 (53). Experiments with a dominant negative form of Ras also indicate that LPS may activate MAP kinase and TNF-α production via the Ras/Raf-1 pathway in RAW 264.7 cells (54). In one contrary report, LPS did not activate Ras, but it did seem to act via Raf-1 (55). Thus, evidence to date largely supports the possibility that LPS activates Raf-1 and subsequently MEK and MAP kinase. We have used the ΔRaf-1:ER chimera in macrophages to provide a method for ac-

tivating MAP kinase. These experiments have revealed that Raf-1 and MAP kinase activation stimulate only some down-stream events seen with LPS stimulation. These results suggest that LPS stimulation of macrophages activates at least two signal transduction pathways, one involving MAP kinase and a second that is independent of the MAP kinase cascade.

References

1. Morrison, D.C., and J.L. Ryan. 1979. Bacterial endotoxins and host immune responses. *Adv. Immunol.* 28:293–450.
2. Pabst, M.J., and R.B. Johnston. 1989. Bacterial lipopolysaccharide: a mediator of inflammation. *In* Handbook of Inflammation. P.M. Henson and R.C. Murphy, editors. Elsevier Science Publishing Co. Inc., New York. 361–393.
3. Adams, D.O., and T.A. Hamilton. 1984. The cell biology of macrophage activation. *Annu. Rev. Immunol.* 2:283–318.
4. Morrison, D.C., and J.L. Ryan. 1987. Endotoxins and disease mechanisms. *Annu. Rev. Med.* 38:417–432.
5. Hibbs, J.B., Jr., R.R. Taintor, H.A. Chapman, Jr., and J.B. Weinberg. 1977. Macrophage tumor cell killing: influence of the local environment. *Science (Wash. DC).* 197:279–282.
6. Pace, J.L., S.W. Russell, B.A. Torres, H.M. Johnson, and P.W. Gray. 1983. Recombinant mouse IFN-γ induces the priming step in macrophage activation for tumor cell killing. *J. Immunol.* 130:2011–2023.
7. Wright, S.D., R.A. Ramos, P.S. Tobias, R.J. Ulevitch, and J.C. Mathison. 1990. CD14, a receptor for complexes of LPS and LPS binding protein. *Science (Wash. DC).* 249:1431–1433.
8. Lee, J.-D., K. Kato, P.S. Tobias, T.N. Kirkland, and R.J. Ulevitch. 1992. Transfection of CD14 into 70Z/3 cells dramatically enhances the sensitivity to complexes of lipopolysaccharide (LPS) and LPS binding protein. *J. Exp. Med.* 175: 1697–1705.
9. Lee, J., V. Kravchenko, T.N. Kirkland, J. Han, N. Mackman, A. Moriarty, D. Leturcq, P.S. Tobias, and R.J. Ulevitch. 1993. Glycosyl-phosphatidylinositol-anchored or integral membrane forms of CD14 mediate identical cellular responses to endotoxin. *Proc. Natl. Acad. Sci. USA.* 90:9930–9934.
10. Golenbock, D.T., Y. Liu, F.H. Millham, M.W. Freeman, and R.A. Zoeller. 1993. Surface expression of human CD14 in chinese hamster ovary fibroblasts imparts macrophage-like responsiveness to bacterial endotoxin. *J. Biol. Chem.* 268:22055–22059.
11. Ulevitch, R.J., and P.S. Tobias. 1994. Recognition of endotoxin by cells leading to transmembrane signaling. *Curr. Opin. Immunol.* 6:125–130.
12. Weinstein, S.L., M.R. Gold, and A.L. DeFranco. 1991. Bac-
terial lipopolysaccharide stimulates protein tyrosine phosphorylation in macrophages. *Proc. Natl. Acad. Sci. USA.* 88:4148–4152.
13. Weinstein, S.L., J.S. Sanghera, K. Lemke, A.L. DeFranco, and S.L. Pelech. 1992. Bacterial lipopolysaccharide induces tyrosine phosphorylation and activation of mitogen-activated protein kinases in macrophages. *J. Biol. Chem.* 267:14955–14962.
14. Dong, Z., C.A. O'Brian, and I.J. Fidler. 1993. Activation of tumoricidal properties in macrophages by lipopolysaccharide requires protein-tyrosine kinase activity. *J. Leukocyte Biol.* 53:53–60.
15. Geng, Y., B. Zhang, and M. Lotz. 1993. Protein tyrosine kinase activation is required for lipopolysaccharide induction of cytokines in human blood monocytes. *J. Immunol.* 151:6692–6700.
16. Beaty, C.D., T.L. Franklin, Y. Uehara, and C.B. Wilson. 1994. Lipopolysaccharide-induced cytokine production in human monocytes: role of tyrosine phosphorylation in transmembrane signal transduction. *Eur. J. Immunol.* 24:1278–1284.
17. Novogrodsky, A., A. Vanichkin, M. Patya, A. Gazit, N. Osherov, and A. Levitzki. 1994. Prevention of lipopolysaccharide-induced lethal toxicity by tyrosine kinase inhibitors. *Science (Wash. DC).* 264:1319–1322.
18. Ding, A., E. Sanchez, and C.F. Nathan. 1993. Taxol shares the ability of bacterial lipopolysaccharide to induce tyrosine phosphorylation of microtubule-associated protein kinase. *J. Immunol.* 151:5596–5602.
19. Dong, A., X. Qi, and I.J. Fidler. 1993. Tyrosine phosphorylation of mitogen-activated protein kinases is necessary for activation of murine macrophages by natural and synthetic bacterial products. *J. Exp. Med.* 177:1071–1077.
20. Marshall, C.J. 1994. MAP kinase kinase kinase, MAP kinase kinase, and MAP kinase. *Curr. Opin. Genet. & Dev.* 4:82–89.
21. Blumer, K.J., and G.L. Johnson. 1994. Diversity in function and regulation of MAP kinase pathways. *Trends Biochem. Sci.* 19:236–239.
22. Gille, H., A. Sharrocks, and P.E. Shaw. 1992. Phosphorylation of transcription factor p62^{TCF} by MAP kinase stimulates ternary complex formation at c-fos promoter. *Nature (Lond.).* 358:414–417.

23. Nakajima, T., S. Kinoshita, T. Sasagawa, K. Sasaki, M. Naruto, T. Kishimoto, and S. Akira. 1993. Phosphorylation at threonine-235 by a *ras*-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. *Proc. Natl. Acad. Sci. USA.* 90:2207–2211.

24. Lin, L.-L., M. Wartmann, A.Y. Lin, J.L. Knopf, A. Seth, and R.J. Davis. 1993. cPLA$_2$ is phosphorylated and activated by MAP kinase. *Cell.* 72:269–278.

25. Samuels, M.L., M.J. Weber, J.M. Bishop, and M. McMahon. 1993. Conditional transformation of cells and rapid activation of the mitogen-activated protein kinase cascade by an estradiol-dependent human Raf-1 protein kinase. *Mol. Cell. Biol.* 13:6241–6252.

26. Gotoh, Y., E. Nishida, T. Yamashita, M. Hoshi, M. Kawakami, and H. Sukai. 1990. Microtubule-associated protein (MAP) kinase activated by nerve growth factor and epidermal growth factor in PC12 cells. Identity with the mitogen-activated MAP kinase of fibroblastic cells. *Eur. J. Biochem.* 193:661–669.

27. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA extraction by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.

28. Fort, P., L. Marty, M. Piechaczyk, S.E. Sabrouty, C. Dany, P. Jeantour, and J.M. Blanchard. 1985. Various rat adult tissues express only one major mRNA species from the glyceraldhyde-3-phosphate-dehydrogenase multigene family. *Nucleic Acids Res.* 13:1431–1442.

29. Lau, L.F., and D. Nathans. 1985. Identification of a set of genes expressed during the G0/G1 transition of cultured mouse cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:3145–3151.

30. Reiner, S.L., S. Zheng, D.P. Corry, and R.M. Locksley. 1993. Constructing polycompetitor cDNAs for quantitative PCR. *J. Immunol. Methods.* 165:37–46.

31. Kitchens, R.L., R.J. Ulevitch, and R.S. Munford. 1992. Lipopolysaccharide (LPS) partial structures inhibit responses to LPS in a human macrophage cell line without inhibiting LPS uptake by a CD14-mediated pathway. *J. Exp. Med.* 176:485–494.

32. Mackman, N., K. Brand, and T.S. Edgington. 1991. Lipopolysaccharide-mediated transcriptional activation of the human tissue factor gene in THP-1 monocytic cells requires both activator protein 1 and nuclear factor κB binding sites. *J. Exp. Med.* 174:1517–1526.

33. Pomerantz, R.J., M.B. Feinberg, D. Trono, and D. Baltimore. 1990. Lipopolysaccharide is a potent monocyte/macrophage-specific stimulator of human immunodeficiency virus type 1 expression. *J. Exp. Med.* 172:253–261.

34. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. B. 23.

35. Charles, C.H., H. Sun, L.F. Lau, and N.K. Tonks. 1993. The growth factor-inducible immediate-early gene *3CH134* encodes a protein-tyrosine-phosphatase. *Proc. Natl. Acad. Sci. USA.* 90:5292–5296.

36. Sun, H., C.H. Charles, L.F. Lau, and N.K. Tonks. 1993. MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo. *Cell.* 75:487–493.

37. Vincenti, M.P., T.A. Burrell, and S.M. Taffet. 1992. Regulation of NF-κB activity in murine macrophages: effect of bacterial lipopolysaccharide and phorbol ester. *J. Cell. Physiol.* 150:204–213.

38. Zheng, S., M.C. Brown, and S.M. Taffet. 1993. Lipopolysaccharide stimulates both nuclear localization of the nuclear factor κB 50 kDa subunit and loss of the 105 kDa precursor in RAW264 macrophage-like cells. *J. Biol. Chem.* 268:17233–

17239.

39. Li, S., and J.M. Sedivy. 1993. Raf-1 protein kinase activates the NF-κB transcription factor by dissociating the cytoplasmic NF-κB-IκB complex. *Proc. Natl. Acad. Sci. USA.* 90:9247–9251.

40. Henkel, T., T. Machleidt, I. Alkalay, M. Kronke, Y. Ben-Neriah, and P.A. Baeuerle. 1993. Rapid proteolysis of IκB-α is necessary for activation of transcription factor NF-κB. *Nature (Lond.).* 365:182–185.

41. Shakhov, A.N., M.A. Collart, P. Vassalli, S.A. Nedospasov, and C.V. Jongeneel. 1990. κB-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor α gene in primary macrophages. *J. Exp. Med.* 171:35–47.

42. Collart, M.A., P. Baeuerle, and P. Vassalli. 1990. Regulation of tumor necrosis factor alpha transcription in macrophages: involvement of four κB-like motifs and of constitutive and inducible forms of NF-κB. *Mol. Cell. Biol.* 10:1498–1506.

43. Han, J., T. Brown, and B. Beutler. 1990. Endotoxin-responsive sequences control cachectin/tumor necrosis factor biosynthesis at the translational level. *J. Exp. Med.* 171:465–475.

44. Drouet, C., A.N. Shakhov, and C.V. Jongeneel. 1991. Enhancers of transcription factors controlling the inducibility of the tumor necrosis factor-α promoter in primary macrophages. *J. Immunol.* 147:1694–1700.

45. Lenardo, M.J., and D. Baltimore. 1989. NF-κB: a pleiotropic mediator of inducible and tissue-specific gene control. *Cell.* 58:227–229.

46. Rice, N.R., and M.K. Ernst. 1993. In vivo control of NF-κB activation by IκB-α. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:4685–4695.

47. Thompson, E.W., D. Katz, T. Shima, A. Wakeling, M. Lippman, and R. Dickson. 1989. ICI 164,384, a pure antagonist of estrogen-stimulated MCF-7 cell proliferation and invasiveness. *Cancer Res.* 49:6929–6934.

48. Webster, N., S. Green, J. Jin, and P. Chambon. 1988. The hormone-binding domains of the estrogen and glucocorticoid receptors contain an inducible transcription activation function. *Cell.* 54:199–207.

49. Wiseman, L., A. Wakeling, F. May, and B. Westley. 1989. Effects of the antioestrogen, ICI 164,384, on oestrogen induced RNAs in MCF-7 cells. *J. Steroid Biochem.* 33:1–6.

50. Dickson, B., and E. Hafen. 1994. Genetics of signal transduction in invertebrates. *Curr. Opin. Genet. & Dev.* 4:64–70.

51. Lange-Carter, C.A., C.M. Pleiman, A.M. Gardner, K.J. Blumer, and G.L. Johnson. 1993. A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. *Science (Wash. DC).* 260:315–319.

52. Geng, Y., E. Gulbins, A. Altman, and M. Lotz. 1994. Monocyte deactivation by interleukin 10 via inhibition of tyrosine kinase activity and the ras signaling pathway. *Proc. Natl. Acad. Sci. USA.* 91:8602–8606.

53. Reimann, T., D. Buscher, R. Hipskind, S. Krautwald, M.-L. Lohmann-Matthes, and M. Baccarini. 1994. Lipopolysaccharide induces activation of the Raf-1/MAP kinase pathway: a putative role for Raf-1 in the induction of the IL-1β and the TNF-α genes. *J. Immunol.* 153:5740–5749.

54. Geppert, T.D., C.E. Whitehurst, P. Thompson, and B. Beutler. 1994. Lipopolysaccharide signals activation of tumor necrosis factor biosynthesis through the ras/raf-1/MEK/MAPK pathway. *Mol. Med.* 1:93–103.

55. Buscher, D., R.A. Hipskind, S. Krautwald, T. Reimann, and M. Baccarini. 1995. Ras-dependent and -independent pathways target the mitogen-activated protein kinase network in macrophages. *Mol. Cell. Biol.* 15:466–475.