Extracellular Signal-Related Kinase (ERK) and p38 Mitogen-Activated Protein (MAP) Kinases Differentially Regulate the Lipopolysaccharide-Mediated Induction of Inducible Nitric Oxide Synthase and IL-12 in Macrophages: Leishmania Phosphoglycans Subvert Macrophage IL-12 Production by Targeting ERK MAP Kinase

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Extracellular Signal-Related Kinase (ERK) and p38 Mitogen-Activated Protein (MAP) Kinases Differentially Regulate the Lipopolysaccharide-Mediated Induction of Inducible Nitric Oxide Synthase and IL-12 in Macrophages: Leishmania Phosphoglycans Subvert Macrophage IL-12 Production by Targeting ERK MAP Kinase

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Macrophage activation by cytokines or microbial products such as LPS results in the induction and release of several key immune effector molecules including NO and IL-12. These have been shown to play crucial roles in the development of immunity to intracellular pathogens such as Leishmania. The molecular mechanisms underlying the induction of these effector molecules are not fully understood. We now show that the extracellular signal-related kinase (ERK) and p38 mitogen-activated protein (MAP) kinases play differential roles in the regulation of LPS-stimulated inducible NO synthase and IL-12 gene expression. In macrophages, LPS stimulates the simultaneous activation of all three classes of MAP kinases, ERK, c-jun N-terminal kinase, and p38, albeit with differential activation kinetics. However, studies using inhibitors selective for ERK (PD98059) and p38 (SB203580) show that while p38 plays an essential role in the induction of inducible NO synthase, ERK MAP kinases play only a minor role in promoting NO generation. In contrast, while p38 promotes induction of IL-12 (p40) mRNA, ERK activation suppresses LPS-mediated IL-12 transcription. The biological relevance of these regulatory signals is demonstrated by our finding that Leishmania lipophosphoglycans, which promote parasite survival, act by stimulating ERK MAP kinase to inhibit macrophage IL-12 production. Thus, as ERK and p38 MAP kinases differentially regulate the induction of the macrophage effector molecules, inducible NO synthase and IL-12, these kinases are potential targets not only for the development of novel strategies to combat intracellular pathogens but also for therapeutic immunomodulation. The Journal of Immunology, 1999, 163: 6403–6412.

Macrophages play a key role in directing the host immune response to infection. Recruitment and stimulation of macrophages by cytokines and/or microbial products such as LPS results in the induction and release of several key immune effector molecules such as NO and IL-12. These play crucial roles in the development of immunity against a number of intracellular pathogens, including Leishmania major, Mycobacterium tuberculosis, Listeria monocytogenes, and Toxoplasma gondii (1–7). Indeed, survival of the Leishmania parasite has been shown to be associated with the ability of lipophosphoglycan glycoconjugates expressed on the cell surface of Leishmania promastigotes to regulate production of both NO (8) and IL-12 (9) in macrophages.

NO, which mediates many of the nonspecific cytotoxic and inflammatory responses of macrophages following infection by pathogens, is generated following the up-regulation of expression of the inducible form of NO synthase (iNOS). Similarly, the biological activity of IL-12 (a heterodimeric cytokine comprising p35 and p40 subunits), which is the key cytokine driving Th1 cell development (3, 10), is regulated by the induction of the p40 subunit (11, 12). Expression of these immunomodulatory proteins appears to be regulated primarily at the level of transcription (13–18), and, indeed, NF-κB activation has been shown to be a key factor in the regulation of induction in both cases (12, 19). Similarly, IFN regulatory factors (IRFs) also appear to play a key role in the induction of both of these macrophage effectors (Refs. 15, 20, and 21 and our unpublished data) via the IFN-stimulating response element in iNOS (13) and the nuclear complex termed F1, which includes multiple nuclear factors (IRF-1, c-Rel, and GLP109) and acts at the Ets site in the IL-12 promoter (17). However, little is known about the early signaling events underlying the up-regulation of iNOS or IL-12 (p40) expression by LPS in macrophages.

LPS has been reported to stimulate a variety of signal transduction elements such as the Src-related protein tyrosine kinases (22, 23), phospholipases (24, 25), protein kinase C (26, 27), raf (28, 29), phospholipase A2 (29, 30), phosphatidylinositol 3-kinase (31), protein kinase D (32), and JNK (33) and p38 mitogen-activated protein (MAP) kinases (34–37), which appear to be key components of the signaling circuitry responsible for the regulation of iNOS and IL-12 (p40) expression. However, while p38 plays an essential role in the induction of inducible NO synthase, ERK MAP kinases play only a minor role in promoting NO generation. In contrast, while p38 promotes induction of IL-12 (p40) mRNA, ERK activation suppresses LPS-mediated IL-12 transcription. The biological relevance of these regulatory signals is demonstrated by our finding that Leishmania lipophosphoglycans, which promote parasite survival, act by stimulating ERK MAP kinase to inhibit macrophage IL-12 production. Thus, as ERK and p38 MAP kinases differentially regulate the induction of the macrophage effector molecules, inducible NO synthase and IL-12, these kinases are potential targets not only for the development of novel strategies to combat intracellular pathogens but also for therapeutic immunomodulation.


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3 Abbreviations used in this paper: iNOS, inducible NO synthase; IRF, IFN regulatory factor; MAP, mitogen-activated protein; ERK, extracellular signal-related kinase; JNK, c-jun N-terminal kinase; sPG, synthetic phosphoglycan; SAPK, stress-activated protein kinase; MEK, ERK-specific MAP kinase.
cultured in 96-well plates and incubated with or without the MEK inhibitor, transcription of IL-12 (p40).

Through stimulation of ERK MAP kinase, which acts to suppress its inhibitory effects on the production of IL-12 by macrophages, kinase regulation of macrophage effector function is demonstrated in macrophages. The physiological relevance of such MAP kinase activation plays differential roles in the regulation of LPS-stimulated induction of IL-12 (p40) and iNOS gene expression in macrophages. Although LPS has previously been reported to activate all three types of MAP kinases, the physiological relevance of such MAP kinase signaling to macrophage function remains unclear.

In this study, we show that LPS stimulates, with differential kinetics, the activation of ERK, JNK, and p38 MAP kinases in J774 macrophages. Moreover, we demonstrate, by the use of cell-permeable inhibitors selective for the ERK (PD98059) (40) and p38 (SB203580) (41) signaling cascades, that such ERK and p38 MAP kinase activation plays differential roles in the regulation of LPS-stimulated induction of IL-12 (p40) and iNOS gene expression in macrophages. The physiological relevance of such MAP kinase regulation of macrophage effector function is demonstrated by our finding that synthetic Leishmania lipophosphoglycans exerts its inhibitory effects on the production of IL-12 by macrophages through stimulation of ERK MAP kinase, which acts to suppress transcription of IL-12 (p40).

Materials and Methods

Reagents and Abs

Reagents used were obtained from Sigma (Poole, U.K.) unless indicated otherwise. PD98059 and SB203580 were obtained from Alexis (Nottingham, U.K.). Abs used were obtained from the following sources: monoclone anti-macNOS (Transduction Laboratories, Affiniti, Exeter, U.K.); monoclonal anti-IL-12 (p40) (Genzyme, Cambridge, U.K.); polyclonal anti-IL-12 (p70) Ab (raised in our laboratory using rIL-12 (p70)). Abs against all three MAP kinase family members were obtained from New England Biolabs (Hertfordshire, U.K.). Abs against IRF family proteins, NF-κB family proteins, and Ets1/2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated anti-mouse IgG and anti-rabbit IgG abs were obtained from the Scottish Ab Production Unit (Lanarkshire, U.K.).

Purification of murine peritoneal macrophages, cell culture, and cytokine measurement

Thioglycollate-elicited peritoneal macrophages were removed from BALB/c or 129 mice by peritoneal washing and enriched by plastic adherence. Adherent peritoneal macrophages or the murine macrophage cell line, J774 (American Type Culture Collection, Manassas, VA), were cultured at 37°C in 95% air/5% CO2 using DMEM (Life Technologies, Paisley, U.K.) supplemented with 10% heat-inactivated FCS (Life Technologies), 2 mM L-glutamine, 50 U/mL of penicillin, and 50 μg/mL of streptomycin. Cells were cultured in 96-well plates and incubated with or without the MEK inhibitor, PD98059, or the p38 inhibitor, SB203580, at the indicated concentrations for 1 h before stimulation with LPS or LPS plus IFN-γ. Culture supernatants were collected 24 h after LPS stimulation, and IL-12 (p40) was assayed by ELISA using paired Abs (Genzyme). IL-12 (p70) was measured using an ELISA kit from PharMingen (San Diego, CA).

Synthetic phosphoglycan (sPG)

Synthetic fragments ([Gal(1→4)Man(1→6)P-OH])2NH2, NH2) of Leishmania lipophosphoglycans, which have previously been shown to mimic the actions of the natural parasite molecules (8, 9), were synthesized as described previously (42).

Preparation of cell lysates

Stimulated cells (1–2 × 10^6/sample) were washed twice with ice-cold TBS (25 mM Tris·Cl, pH 7.4, 150 mM NaCl, and 100 mM sodium orthovanadate) and harvested with a plastic scraper. The cells were lysed in lysis buffer (25 mM Tris buffer, pH 7.4, containing 150 mM NaCl, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM EDTA, 2 mM EGTA, 10 mM NaF, 1 mM DTT, 50 μg/mL each of leupeptin, aprotonin, and PMSF) by incubation on ice for 30 min. Lysates were then centrifuged at 13,000 × g for 4°C for 10 min, and the supernatants were transferred to fresh tubes and stored at −70°C until required. Protein concentrations of the lysates were determined using the Coomassie protein assay reagent (Pierce, Rockford, IL).

Western blot analysis

Cell lysates were resolved by SDS-PAGE before transfer to nitrocellulose using a transblot system (Hoffman Scientific SE600, San Francisco, CA). Nitrocellulose filters were then incubated with washing buffer (0.1 M Tris·HCl buffer, pH 7.5, containing 0.1 M NaCl and 0.1% Tween 20) containing 2% BSA for at least 1 h to block nonspecific protein binding. Primary Abs were diluted in washing buffer containing 1% BSA and applied to the filter for 1 h at room temperature or overnight at 4°C. Following washing, the blots were incubated with the appropriate HRP-conjugated secondary Ab (diluted up to 1:5000 in wash buffer containing 1% BSA) for 1 h at room temperature. Immunoactive bands were visualized by the enhanced chemiluminescence system (Amersham, Buckinghamshire, U.K.).

Northern blot analysis

IL-12 (p40) and β-actin cDNA fragments ampliﬁed from J774 cells by RT-PCR were cloned into TA vector (Novagen, Abingdon, U.K.), conﬁrmed by DNA sequencing. These cDNA probes were labeled with [α–32P]dATP using a commercial random priming kit (Life Technologies). Macrophage total cellular RNA was puriﬁed using the Bio/RNA-Xcell method (Biogenesis, Cambridge, U.K.), and the concentration of RNA in samples was estimated by UV spectroscopy. Northern hybridization analysis was conducted using radiolabeled IL-12 (p40) cDNAs as specific probes, and the β-actin probe was used for reblotting as a loading control.

EMSA

Nuclear extracts were prepared essentially as described previously (44– 46). Briefly, treated cells were washed twice with ice-cold TBS before resuspending in 400 μl cold buffer A (10 mM HEPES buffer, pH 7.9, containing 10 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 100 μM sodium orthovanadate). Cells were allowed to swell on ice for 15 min, after which 25 μl of a 10% solution of Nonidet P-40 (Fluka, Poole, U.K.) was added and the cells were vortexed vigorously for 15 s. The homogenate was centrifuged at 13,000 × g for 30 s, and the resulting nuclear pellet was resuspended in 100 μl ice-cold buffer B (20 mM HEPES buffer, pH 7.9, containing 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 100 μM sodium orthovanadate) and rocked vigorously at 4°C for 15 min. The nuclear extract was centrifuged for 5 min at 13,000 × g at 4°C, and the supernatant was collected and stored in aliquots at −70°C until use. Binding reactions were initiated by preincubation of nuclear extract protein (10 μg) with double-stranded poly(dI·dC) (200 ng, Pharmacia Biotech, St. Albans, U.K.) in a reaction buffer (20 mM HEPES buffer, pH 7.9, containing 40 mM KCl, 1 mM MgCl2, 0.1 mM EDTA, 0.5 mM DTT, 10% glycerol, and 0.1% Nonidet P-40) for 20 min on ice in a volume of 30 μl. The appropriate radiolabeled synthetic oligonucleotide DNA probe (0.5 ng in 1 μl) was then added, and the mixture was incubated on ice for a further 20 min. For Ab suppression assays, 1 μl of Abs (1 μl) was added during the preincubation period. The resultant DNA-protein complexes were resolved by nondenaturing 6% PAGE and the gels dried and visualized by autoradiography. The oligonucleotide probes used in this work are: probe D (5'-CCCAACGTGGGGACTCTT CCGTGG-3'), NF-κB site on murine iNOS promoter region; probe E (5'-CAAAACAGCCTTGGGGAAATTTAAGA-3'), NF-κB site on murine iNOS (p40) promoter region; and probe G (5'-CAGTGTGCCCACATTGC TACATTCTGATA-3'), an IRE·E in iNOS promoter; probe K (5'-CAGACTCTAATAATCTGTGATCCATGCTT AAGGGT-3'), a sequence that binds to F1 in the murine IL-12(p40) promoter.

NO2 measurement

Nitrite concentrations in culture supernatants were determined by Griess reaction as described previously (43).

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FIGURE 1. Time course of LPS-induced phosphorylation of MAP kinases. J774 cells (A, B, D, E, G, and H) or peritoneal macrophages (C, F, and I) were stimulated with LPS (1 μg/ml) and harvested at the indicated times (0–60 min). Whole-cell lysates (30 μg) were resolved by SDS-PAGE (10%), followed by immunoblotting using a set of Abs that recognized either activated (dually phosphorylated on Tyr/Thr) or total MAP kinase expression. A, Anti-phospho-ERK-2 (upper panel) and reprobed with anti-total ERK-2 (lower panel). B, Densitometer scan of phospho-ERKs/ERKs ratio. C, Dual phosphorylation of ERK-1 and ERK-2 resulting from stimulation of peritoneal macrophages with LPS (1 μg/ml) and/or IFN-γ (100 U/ml) for 10 min. D, Whole-cell lysates of J774 cells blotted with anti-phospho-p38 MAP kinase (upper panel, pp38) and reprobed with anti-total p38 MAP kinase. E, Densitometer scan of pp38/p38 ratio. F, Dually phosphorylated p38 in peritoneal macrophages stimulated with LPS (1 μg/ml) and/or IFN-γ (100 U/ml) for 10 min. G, Whole-cell lysates of J774 cells blotted with anti-phospho-SAPK/JNK (upper panel) and reprobed with anti-total SAPK/JNK. H, Densitometer scan of phospho-SAPK/JNK:SAPK/JNK ratio. I, Dually phosphorylated p46 and p54 JNK in peritoneal macrophages stimulated with LPS (1 μg/ml) and/or IFN-γ (100 U/ml) for 10 min. Equivalent loading of peritoneal macrophage samples was determined by reprobing the appropriate blots with anti-total ERK, anti-total p38, and anti-total JNK MAP kinase Abs.
Statistical analysis

Statistical significance (p < 0.05 or p < 0.01) of differences between treatment groups was assessed by the Student’s t test.

Results

LPS activates ERK, JNK, and p38 MAP kinases in murine macrophages

To investigate whether the ERK, JNK, and p38 MAP kinase pathways are involved in LPS signal transduction in macrophages, we examined the activation of the three MAP kinases by detecting their dually phosphorylated (Tyr/Thr) forms by Western blotting using specific anti-phosphokinase abs. LPS strongly stimulated a rapid (within 1 min) and transient increase in the levels of activation of both ERK-1 (3.7-fold) and ERK-2 (3.2-fold) MAP kinase activities, which peaked at 10 min but remained elevated for at least 60 min above the low basal levels of ERK activation observed in unstimulated J774 cells (Fig. 1, A and B). Although there was no basal activity of ERK MAP kinases in murine peritoneal macrophages, LPS strongly stimulated both ERK-1 and ERK-2 activation within 10 min. In contrast, IFN-g only weakly stimulated ERK activity and appeared to suppress LPS-stimulated ERK phosphorylation (Fig. 1C). Little or no activation of p38 MAP kinase could be detected in unstimulated J774 cells. LPS strongly activated p38, which was maximal at 10 min and sustained for up to 60 min (Fig. 1, D and E). Likewise, p38 was activated following stimulation of murine peritoneal macrophages with either LPS or LPS plus IFN-g for 10 min (Fig. 1F). Similarly, although stress-activated protein kinase (SAPK)/JNK MAP kinases were found not to be significantly activated in either unstimulated J774 cells or murine macrophages, LPS, following a lag period of up to 10 min, strongly stimulated both p46 and p54 JNK MAP kinases with maximum activity of both isoforms achieved at 30 min before declining toward basal levels within 60 min in J774 cells (Fig. 1, G and H). Moreover, p46 and p54 JNK were both stimulated in murine peritoneal macrophages by either LPS or LPS plus IFN-g within 10 min (Fig. 1I). These results clearly show that LPS stimulates all three classes of MAP kinase in macrophages, but with differential kinetics of activation.

Effect of selective inhibitors of the ERK and p38 signaling cassettes on LPS-stimulated MAP kinase activity

A selective and potent inhibitor of the ERK MAP kinase cascade, PD98059, mediates its effects by binding to and inactivating the ERK-specific MAP kinase kinase, MEK. It has no effect on any of the components of the JNK or p38 MAP kinase cascades (40). Similarly, the compound SB203580 is a selective and potent inhibitor of p38 MAP kinase, which does not affect either ERK or JNK MAP kinases (41). Therefore, these reagents are useful pharmacological tools to identify the functional activities mediated by p38 and ERK MAP kinases. To ensure that these inhibitors are specific for p38 and MEK in murine macrophages, we examined the effects of these reagents on the dual phosphorylation of their targets and heterologous MAP kinases in J774 cells and BALB/c peritoneal macrophages (Fig. 2 and results not shown). As expected, PD98059 (20 μM) profoundly inhibited both the basal and stimulated ERK MAP kinase activation, but had no effect on phosphorylation of p38 or SAPK/JNK (Fig. 2). Indeed, higher concentrations of PD98059 completely abrogated ERK MAP kinase activation (results not shown). Conversely, SB203580 inhibited p38, but not ERK or JNK phosphorylation (Fig. 2). Preincubation of cells with SB203580 leads to an increase in both basal and stimulated ERK (particularly ERK-1) activation, suggesting that p38 may act, at least in part, to exert inhibitory effects on ERK signaling in J774 cells. This finding is consistent with the proposed regulatory cross-talk postulated to occur among the different MAP kinase signaling cassettes (47).

Roles of ERK and p38 MAP kinases in the LPS-mediated induction of iNOS expression

To investigate whether ERK or p38 MAP kinases are involved in the LPS-stimulated induction of iNOS in macrophages, we determined whether PD98059 or SB203580 affects LPS-stimulated NO2- production by J774 cells and murine peritoneal macrophages. Both PD98059 (0–50 μM) and SB203580 (0–20 μM) inhibited LPS-stimulated NO2- induction in a dose-dependent manner (Fig. 3 and results not shown). At the concentrations used, these compounds did not affect cell viability (results not shown). Whilst treatment with PD98059 (up to 50 μM) only partially inhibited NO2- generation in J774 cells (Fig. 3A), NO2- production was almost completely abrogated by incubation with 5 μM of SB203580 (Fig. 3B). These results were confirmed by Western blot analysis of iNOS expression, which showed that whilst PD98059 (20 μM) only partially blocked the induction of iNOS protein expression (41%), SB203580 (5 μM) almost completely
abrogated LPS-induction of iNOS expression (Fig. 3C). LPS-stimulated TNF-α production (Northern blot analysis of mRNA and ELISA measurement of secreted protein) from J774 cells was similarly inhibited by PD98059 and SB203580 (results not shown). Taken together, these results suggest that while both p38 and ERK MAP kinases appear to play a positive role in the LPS-mediated induction of iNOS and TNF-α production, p38 activation is more critical and appears to be necessary and sufficient for effector induction. The physiological relevance of these findings was underlined by studies which showed that PD98059 (20 μM) and SB203580 (5 μM) similarly inhibited NO2− production in murine peritoneal macrophages resulting from stimulation with either LPS or LPS plus IFN-γ. Suppression of NO2− generation by these MAP kinase inhibitors was not restricted to a particular haplotype as essentially identical results were obtained with macrophages derived from BALB/C (Fig. 3D) and 129 mice (results not shown).

Roles of ERK and p38 MAP kinases in the LPS-mediated induction of IL-12 (p40) expression

Stimulation of macrophages with LPS leads to the generation of IL-12 (p40), which forms the biologically active heterodimeric form of IL-12 (p70) with the constitutively expressed IL-12 (p35). The transcription factor NF-κB plays a key role in the regulation of IL-12 gene expression. NF-κB is involved in the regulation of both iNOS and IL-12 gene induction. Therefore, we investigated whether the ERK or p38 MAP kinases mediate their effects on NO2− and IL-12 synthesis by modulating NF-κB activity. LPS-stimulated binding of NF-κB to the iNOS and IL-12 promoters (Fig. 6). Ab supershift assays showed that these binding complexes comprised p65-p50, c-Rel-p50 complexes, and p50/p50 homodimers (Fig. 6). Preincubation of the cells with SB203580 or PD98059 did not affect the binding of these complexes (Fig. 6), suggesting that neither p38 nor ERK MAP kinase activation appears to be necessary or sufficient for NF-κB binding to the NF-κB binding sites of either the iNOS or IL-12 promoters.

FIGURE 3. Effects of PD98059 and SB203580 on LPS-induced iNOS expression. A and B, Triplicate samples were cultured in 96-well plates and incubated with the indicated concentrations of PD98059, SB203580, or medium alone for 1 h before stimulation with LPS (1 μg/ml). After a further 24 h incubation, culture supernatants were collected, and NO2− was measured by Griess reaction. Data are mean (±SD) obtained from triplicate samples. C, Cells were preincubated with or without PD98059 (20 μM) or SB203580 (5 μM) for 1 h before stimulated with LPS for 16 h. Cell lysates were analyzed by Western blotting using anti-mouse iNOS Ab. Data are representative of three experiments. The effects of PD98059 and SB203580 on NO2− generation resulting from stimulation of BALB/c peritoneal macrophages (D), with LPS (1 μg/ml), or LPS plus IFN-γ (100 U/ml) for 24 h was determined by the Griess reaction. Data are means (±SD) obtained from triplicate samples and are representative of three experiments. *p < 0.05; **p < 0.01 compared with LPS alone or LPS plus IFN-γ.
Roles of ERK and p38 MAP kinases in regulation of IRF binding to iNOS and IL-12 promoters

LPS stimulates the binding of two nuclear complexes (Fig. 7A, bands a and b) to the IRF-site in the iNOS promoter. Experiments using SB203580 or PD98059 to probe the role of p38 and ERK MAP kinases in promoting such complex formation showed that abrogation of p38 MAP kinase, but not ERK MAP kinase, prevented binding of complex b to the IRF site on the iNOS promoter (Fig. 7). This is consistent both with our results above showing a key role for p38 MAP kinase in promoting iNOS induction (Fig. 3) and with previous reports that IRFs are major regulatory elements in the induction of iNOS (13–15). At present, the components of the LPS-induced IRF-like complexes are unclear, but Ab supershift studies showed that they do not contain IRF-1, IRF-2, ICSBP (IFN consensus sequence-binding protein), or ICSAT (a homologue of mouse Pip) (data not shown).

LPS similarly stimulated the formation of multiple complexes at the Ets site (which comprises complexes containing at least Ets, IRF-1, c-Rel, and GLp109) in the IL-12 promoter (17). However, neither SB203580 nor PD98059 were able to block any of these complexes (Fig. 7B, lanes 1 and 2). This is consistent with the report that p38 MAP kinase does not affect the LPS-induced promoter activity of the IL-12 promoter (17).

FIGURE 4. Effects of PD98059 and SB203580 on LPS-induced expression of IL-12 (p40). Cells were cultured with or without the indicated concentrations of PD98059 (A) or SB203580 (B) for 1 h before stimulation with LPS (1 μg/ml). Data are representative of three experiments. C. Peritoneal macrophages from BALB/c mice were stimulated with LPS and IFN-γ. Culture supernatants were collected at 24 h, and IL-12 (p40) was measured by ELISA. Data are means (±SD) obtained from triplicate samples and are representative of four experiments. * p < 0.05 compared with LPS plus IFN-γ.

FIGURE 5. Northern blot analysis of the effects of PD98059 and SB203580 on LPS-induced IL-12 (p40) mRNA expression. J774 cells pretreated with PD98059 (20 μM), SB203580 (5 μM), or medium alone for 1 h were stimulated with LPS (1 μg/ml) for 0–6 h, and total RNA was extracted. Total RNA was hybridized with a cloned murine IL-12 (p40) cDNA probe (A and C, upper panels), and, after stripping, a cloned murine β-actin probe was used as a loading control (A and C, lower panels). B and D, Densitometric analysis of the IL-12 (p40):β-actin mRNA ratios derived from the data presented in A and C, respectively. Data are representative of two experiments.
of IL-12 could be mimicked by sPG containing the galactose(β1–4)mannose(α1)-PO₄ repeating units of the parasite molecule (9). Moreover, inhibition of IL-12 could be mimicked by sPG containing the galactose(β1–4)mannose(α1)-PO₄ repeating units of the parasite molecule (9). Although this inhibition was at the transcriptional level, it was not mediated through modulation of NF-κB binding and hence was reminiscent of the ERK MAP kinase-mediated suppression of IL-12 production. Therefore, we investigated whether sPG modulates IL-12 production by stimulating ERK MAP kinase in J774 macrophages (Fig. 9). We found that sPG stimulated ERK activity as evidenced by Western blotting studies using Abs that recognize the dually (Thr/Tyr)-phosphorylated forms of ERK-1 and -2. sPG stimulated both ERK-1 and -2, and such activation was sustained for at least 30 min (Fig. 9A). Furthermore, we found that sPG enhanced LPS-stimulated ERK MAP kinase activity in J774 cells (Fig. 9B), supporting our proposal that sPG may inhibit IL-12 (p40) production in J774 cells by increasing ERK-mediated suppression of transcription. Evidence that ERK MAP kinase does indeed mediate the inhibitory effects of sPG was provided by experiments in which the MEK inhibitor, PD98059, prevented sPG-inhibition of IL-12 production (Fig. 9C).

The physiological relevance of these findings was explored in murine peritoneal macrophages. In these primary macrophages, stimulation with LPS alone only induces low levels of IL-12, and hence we focused on the sPG-mediated suppression of the synergistic induction of IL-12 resulting from stimulation with LPS plus IFN-γ (Fig. 10A), which is also subject to the opposing regulatory signals mediated by ERK and p38 MAP kinases (Fig. 4C). sPG-mediated inhibition of IL-12 (p40) production was found to be biologically relevant, as it was reflected by a similar inhibition of the generation of the biologically active p70 form of IL-12 (Fig. 10B). Moreover, such sPG-mediated inhibition of IL-12 production was blocked by the MEK inhibitors PD98059 or U0126 (Fig. 10A and results not shown). Western blot analysis of the dually phosphorylated forms of ERK-1 and ERK-2 in murine peritoneal macrophages confirmed that sPG stimulated ERK-1 and ERK-2 activation under physiological conditions, and the suppressive effects of sPG on IL-12 production correlated with a substantial enhancement of the ERK activity observed in response to either LPS or LPS plus IFN-γ (Fig. 10C). Interestingly, these data also suggested that IFN-γ may exert at least some of its synergistic effects on LPS-stimulated IL-12 production by suppressing the levels of ERK MAP kinase activation observed in response to LPS alone. However, sPG-mediated suppression of IL-12 production is not responsible for conferring resistance or susceptibility of mice to Leishmania infection as essentially identical results were observed in BALB/c (susceptible; Fig. 10) and 129 (resistant) mice (results not shown). Moreover, this suppression of IL-12 does not reflect stimulation of IL-10 by sPG (results not shown).

Discussion

The results presented in this paper show that ERK and p38 MAP kinases signal differential regulation of effector responses (iNOS and IL-12) in LPS-stimulated macrophages, suggesting that these
MAP kinases provide potential novel targets of therapeutic immunomodulation. The physiological relevance of such regulation is underlined by our findings that Leishmania phosphoglycans act to evade resistance to infection, resulting from IL-12-driven differentiation and expansion of Th1 cells and IFN-γ production, by subverting the host’s regulatory mechanism of ERK MAP kinase-mediated suppression of IL-12 production.

All three classes of MAP kinase, ERK, JNK, and p38, are simultaneously activated by LPS in macrophages with maximal activation occurring 10–30 min poststimulation in J774 cells (Fig. 1). However, the individual subtypes exhibit differential kinetics of activation in response to LPS. For example, while the activation of p46 and p54 JNK MAP kinases is relatively slow (detectable between 1–10 min) and returns almost to basal levels within 60 min, LPS-stimulation of ERK-1 and -2 or p38 activation is more rapid (within 1 min) and appears to be sustained for up to at least 60 min (Fig. 1). Interestingly, studies using the p38-selective inhibitor, SB203580, show that p38 MAP kinase acts to suppress ERK-1 and -2 activation in macrophages (Fig. 2), a finding consistent with an earlier report showing that p38 also negatively regulated basal and FceRI-stimulated activation of ERK-2 in mast cells (47). More importantly, use of this inhibitor showed that p38 plays an essential role in the LPS-mediated induction of iNOS (Fig. 3) and promotes the up-regulation of IL-12 (Figs. 4 and 5). In contrast, studies with PD98059, the MEK1 inhibitor that selectively targets the ERK MAP kinase, show that neither p38 nor ERK MAP kinases appear to play any role in the induction of iNOS and IL-12 production by targeting ERK MAP kinase. J774 cells were stimulated with LPS (1 μg/ml) and/or sPG (50 μM) for the indicated times, and total proteins were purified. Following preparation of cell lysates, samples (30 μg) were resolved by SDS-PAGE and immunoblotted using antibodies to p38 (Ab) and p21. Data are means (±SD) obtained from triplicate samples and are representative of three experiments. *p < 0.05 compared with LPS alone.

Previous studies on the role of individual MAP kinases in the induction of iNOS have produced conflicting results, suggesting that the regulation of such gene expression varies in a receptor- and cell type-dependent manner. For example, while both ERK and p38 are known to play positive, but nonessential, regulatory roles in the LPS-mediated induction of iNOS mRNA in microglia or astrocytes (33), ERK MAP kinases did not appear to be involved in the response of rat glioma cells to LPS plus IFN-γ (50). In addition, while ERK MAP kinases also appeared to play a positive regulatory role (51) in transducing the IL-1-mediated induction of iNOS in a number of systems, PD98059 had no effect on such NO production in other cell types (52). Similarly, while p38 activation had also been shown to promote IL-1 stimulation of iNOS mRNA induction in chondrocytes (53), astrocytes (52), and pancreatic islets (51), it served to suppress induction of iNOS mRNA in glomerular mesangial cells (54). Moreover, while Da Silva et al. (52) showed that SB203580 inhibited IL-1-stimulated NO production, they found that it had no effect on NF-κB activation in astrocytes, results consistent with our own findings in macrophages that p38 MAP kinase plays a key role in LPS-stimulated induction of iNOS without modulating NF-κB binding to the iNOS promoter. Interestingly, considering that NF-κB activation has been proposed to play a key role in iNOS induction (14), we find that neither p38 nor ERK MAP kinases appear to play any role in promoting NF-κB (p50/p50, p50/c-rel, and p50/p65 complexes) binding to the iNOS promoter (Fig. 6), despite ERK promoting and p38 MAP kinase being required for iNOS induction (Fig. 3). In contrast, experiments using SB203580 or PD98059 showed that abrogation of p38, but not ERK, MAP kinases prevented binding of an IRF-like complex to the iNOS promoter. Although we have not as yet identified the components of this novel LPS-stimulated IRF-like complex, we have ruled out a role for any of the well-characterized factors (IRF-1, IRF-2, ICSBP (IFN consensus sequence-binding protein), or ICSAT). Taken together, these results not only suggest that NF-κB binding may not be necessary or sufficient for induction of iNOS but rather support previous studies (including those using IRF knockout mice) proposing that IRFs are major regulatory elements in the induction of iNOS (13, 15). Moreover, they also suggest that p38 and ERK MAP kinases do not play universal roles in the regulation of iNOS induction but rather transduce differential regulatory effects that vary in a receptor- and cell type-dependent manner, perhaps reflecting cell lineage-restricted expression of isoforms of MAP kinase and/or transcription factors.

Little is known regarding the molecular mechanisms underlying the induction of biologically active IL-12 apart from the fact that...
the transcription factors, NF-κB (18) and Ets (17, 55), appear to be important in the LPS-stimulated transcriptional activation of the IL-12 (p40) subunit. While p50/p65 and p50/c-Rel heterodimers of the NF-κB complexes have been reported to be involved (12), the Ets region interacts with a nuclear complex termed F1, which includes multiple nuclear factors (IRF-1, c-Rel, and GLp109) and appears to be highly inducible by either IFN-γ or LPS stimulation (17). We found that neither SB203580 nor PD98059 affect binding of NF-κB complexes (p50/p50, p50/c-rel, and p50/p65 dimers) to the IL-12 promoter (Fig. 6), suggesting that NF-κB complexes do not transduce either the p38 promotion of IL-12 induction or the ERK-mediated suppression of IL-12 (p40). This proposal is supported by our findings that while ERK-mediated suppression of IL-12 (p40) is not transduced by p38 domains of mice, our studies indicate that the IL-12 promoter (Fig. 6), suggesting that NF-κB complexes do not transduce either the p38 promotion of IL-12 induction or the ERK-mediated suppression of IL-12 (p40). This proposal is supported by our findings that while ERK-mediated suppression of IL-12 (p40) is not transduced by either IFN-γ or LPS stimulation (17). We found that neither SB203580 nor PD98059 affect binding of NF-κB complexes (p50/p50, p50/c-rel, and p50/p65 dimers) to the IL-12 promoter (Fig. 6), suggesting that NF-κB complexes do not transduce either the p38 promotion of IL-12 induction or the ERK-mediated suppression of IL-12 (p40). This proposal is supported by our findings that while Leishmania SPG inhibits IL-12 production by stimulating ERK MAP kinase activity (Figs. 9 and 10), it does not modulate NF-κB binding to the IL-12 promoter (9). Similarly, neither SB203580 nor PD98059 abrogates F1 complex formation (Fig. 7) or Ets nuclear translocation (Fig. 8), suggesting that assembly of the F1 complex also does not provide either the positive or negative regulatory target of MAP kinase action on the IL-12 promoter.

In conclusion, taken that ERK and p38 MAP kinases differentially regulate the LPS-mediated induction of iNOS and IL-12, together with the fact that ERK MAP kinases play opposing roles in modulating iNOS and IL-12 induction, our results suggest that such differential signaling of p38 and ERK MAP kinases may play an important role in determining the type of functional response resulting from stimulation of macrophages with LPS. Many immunomodulatory receptors on macrophages can also differentially signal via one or more MAP kinases. Our finding that distinct subtypes of these signaling cascades make qualitatively different contributions to induction of individual macrophage responses (such as iNOS and IL-12) suggests that macrophages can modify their response to pathogens depending on their environment. Similarly, pathogens may act to evade/modulate the host immune response by subverting host-MAP kinase regulation of macrophage effector responses. This is evidenced by our data demonstrating that Leishmania may suppress resistance to infection by “switching on” ERK MAP kinase-mediated negative regulation of IL-12 production and hence preventing generation of a protective Th1 immune response. That “susceptible” (BALB/c) and “resistant” (129) strains of mice are not differentially sensitive to sPG-mediated inhibition of IL-12 production following in vitro stimulation of macrophages suggests that sPG subversion of ERK MAP kinase-mediated down-regulation of IL-12 is not the molecular rationale underlying susceptibility. However, the ability of sPG to subvert such MAP kinase regulation of IL-12 production does provide the parasite with a mechanism to evade the generation of a protective Th1 immune response and promote parasite survival. Thus, the differential sensitivity of the macrophage effector responses of NO2− and IL-12 to pharmacological modulators of ERK and p38 activation suggests that such reagents could be used as potential immunomodulators to generate the required immunity to combat a particular pathogen or, alternatively, reduce inflammation/pathology associated with certain disease states.

References


