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Leishmania lipophosphoglycan activates the transcription factor activating protein 1 in J774A.1 macrophages through the extracellular signal-related kinase (ERK) and p38 mitogen-activated protein kinase

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Abstract

Leishmania donovani is an obligatory intracellular pathogen that resides and multiplies in the phagolysosomes of macrophages. The outcome of this infection depends on the balance between the host ability to activate macrophage killing and the parasite ability to suppress or evade this host immune response. Lipophosphoglycan (LPG) glycoconjugate, the surface molecule of the protozoan parasite is a virulence determinant and a major parasite molecule involved in this process. In this study, we examined the ability of *Leishmania* and its surface molecule, lipophosphoglycan to activate activating protein 1 (AP-1) through the mitogen-activated protein kinase (MAPK) cascade. We report here that the *Leishmania* surface molecule, lipophosphoglycan stimulates the simultaneous activation of all three classes of MAP kinases, extracellular signal-related kinases (ERKs), the *c-jun* amino-terminal kinase (JNK) and the p38 MAP kinase with differential kinetics in J774A.1 macrophage cell line. Furthermore, both *L. donovani* and its surface molecule lipophosphoglycan resulted in a dose- and time-dependent induction of AP-1 DNA-binding activity. We have also shown a dose-dependent increase of AP-1 binding activity in both low and high virulent strains of parasite. The use of inhibitors selective for ERK (PD98059) and p38 (SB203580) pathway showed that pre-incubation of cells with either SB203580 or PD98059 affected the binding activity of AP-1 suggesting that both p38 and ERK MAP kinase activation appear to be necessary for AP-1 activation by LPG. Lipophosphoglycan induced IL-12 production and generation of nitric oxide in murine macrophages. These results demonstrate that *L. donovani* LPG activates pro-inflammatory, endotoxin-like response pathway in J774A.1 macrophages and the interaction may play a pivotal role in the elimination of the parasite.
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Keywords: *Leishmania donovani*; Macrophages; Mitogen-activated protein kinase; AP-1 transcription factor; Lipophosphoglycan

1. Introduction

Leishmania are protozoan parasites that are responsible for severe morbidity and mortality in infected people in several parts of the world. *Leishmania* species multiply within the mammalian macrophages. Interaction between *Leishmania* and macrophage molecules ultimately affects the pathological and immunological responses to *Leishmania* infection. The outcome of infection with *Leishmania major* is to a large extent dependent on the genetics of the host [1]. Interaction with *Leishmania* induces Th1 cell-mediated immune responses in C57BL/6 resistant mice and Th2-type responses in susceptible BALB/c mice leading to intracellular clearance or survival of the parasite, respectively [1]. The signal transduction mechanisms that determine these polarized responses are still poorly understood.

Leishmania surface molecule lipophosphoglycan (LPG) glycoconjugate is a virulence factor that binds to macrophages and has significant effects on macrophage function

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[2]. *Leishmania* LPG has been reported to activate innate immune signaling pathways in macrophages through Toll-like receptors (TLR) leading to activation of pro-inflammatory cytokine synthesis thereby increasing the effective destruction of the parasite [3]. On the other hand, the survival of the parasite has been shown to be associated with the ability of its surface molecule, lipophosphoglycan glycoconjugate to regulate production of both NO [4] and IL-12 in macrophages [5]. *Leishmania* surface molecule lipophosphoglycan has been reported to participate in a variety of processes during the establishment of infection within the mammalian cells, like impairment of macrophage signal transduction pathways, modulation of immunomodulatory effector molecules like inducible form of NO synthase (iNOS) and cytokines, resistance to oxygen radicals, resistance to complement mediated lysis and inhibition of phagosomal maturation [4–11]. The early recognition of the parasite-derived molecules by the innate immune system and further relay of the signal to the adaptive immune system is a crucial step towards polarized response.

Leishmania donovani promastigotes have been reported to evade the activation of mitogen-activated protein kinases (MAPK) during infection of naive macrophages [12]. The MAP kinases play an important role as signal kinases and cause phosphorylation and thereby control the activation status of transcription factors. These kinases form a link between transmembrane signaling and gene induction in the nucleus. Three major subgroups of MAP kinases in mammalian cells are extracellular signal-regulated kinases 1 and 2, the *c-jun* amino-terminal kinases (JNKs) and the p38 MAP kinase [13,14]. These MAP kinases are an important group of serine/threonine signaling kinases.

Down stream cascade of these kinases are the ubiquitous transcription factors such as activating protein 1 (AP-1), NF-κB and IFN regulatory factors (IRFs). IFN regulatory factor and NF-κB activation has been shown to play a key role in the induction of macrophage effector molecules [15–18] and are involved in regulation of functions involved in host defense and inflammation. The role of AP-1 family members in transcriptional regulation is controlled by a number of well-characterized mechanisms [19–24]. The genes coding AP-1 proteins (Fos and Jun) are often among the first genes to be transcribed after stimulation of a cell and are usually transiently expressed. The AP-1 protein function is regulated primarily by phosphoregulation [25–27] The AP-1 family members differ in their ability to repress transcription [28,29]. In the present work, we report that the interaction of J774A.1 macrophages with *Leishmania* LPG resulted in the activation of MAP kinase cascade and this activation was necessary for the induction of activated transcription factor-1. These results suggest that activation of J774A.1 macrophages by *Leishmania* LPG may lead to effective destruction of the parasite and may therefore be significant for the regulation of parasitological and immunological responses to *Leishmania* infection.

2. Materials and methods

2.1. Reagents and antibodies

Reagents used were obtained from Sigma Chemical Co. (St. Louis, MO) unless indicated otherwise. Antibodies used against all MAP kinase family members were obtained from Santa Cruz Biotechnology Inc., (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG antibodies were obtained from Bio-Rad (Hercules, CA). PD98059 and SB203580 were obtained from Santa Cruz Biotechnology Inc., (Santa Cruz, CA).

2.2. Parasite culture

L. donovani AG83 (MHOM/IN/1983/AG83) promastigotes were cultured at 22 °C in modified M199 medium (Sigma, St. Louis, MO) supplemented with 100 U ml^{-1} penicillin (Sigma, St. Louis, MO), 100 μg ml^{-1} streptomycin (Sigma, St. Louis, MO), and 10% heat inactivated fetal calf serum (FCS) (Biological Industries, Kibbutz Beit Haemek, Israel). All the infectivity assays were done using virulent parasites (except where indicated otherwise) that were maintained in BALB/c mice. All parasites used in this study were taken from stationary phase cultures. Amastigotes were isolated from spleens as previously described and transformed to promastigotes in M199 medium containing 30% fetal calf serum. Freshly transformed promastigotes were maintained at 22 °C in M199 with 10% fetal calf serum [30]. Animals were used in accordance with the institutional guidelines. The relevant committee duly approved the use of animals for this work.

2.3. Macrophage culture

A murine macrophage cell line J774A.1 (American Type Culture Collection, Rockville, MD) was used in this study. The macrophages were maintained at 37 °C in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10% FCS in an atmosphere of 5% CO_2 in air. The macrophages were seeded onto tissue culture plates (60 mm) at a density of 1×10^6 cells/plate and incubated for 24 h before being used for the requisite assay.

2.4. Preparation of cell lysates

Stimulated cells (1×10^6 per sample) were washed twice with ice-cold TBS (50 mM Tris–HCl (pH 7.4), 400 mM NaCl and 1 mM sodium orthovanadate) and harvested with a plastic scraper. The cells were lysed in lysis buffer (50 mM Tris–HCl (pH 7.4), 400 mM NaCl, 1 mM sodium orthovanadate, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 μg ml^{-1} each of leupeptin and aprotinin) by incubation on ice for 30 min. Lysate was then centrifuged at

(15,000 × *g*) at 4 °C for 10 min, and supernatant was transferred to fresh tubes and stored at −80 °C till required. Protein concentration of the lysates was determined using a colorometric assay against a BSA standard [31].

2.5. Western blot analysis

Cell lysates were resolved by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE) before transferring to nitrocellulose membrane using a transblot system (Bio-Rad). Nitrocellulose filter was then incubated with TBST (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20) containing 5% skimmed milk for at least 2 h to block nonspecific protein binding. Primary antibodies were diluted in TBST and applied to the filter for at least 2 h at room temperature. The blots were washed with TBST thrice and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (diluted up to 1:5000 in TBST) for 1 h at room temperature. Immunoreactive bands were visualized by the enhanced chemiluminescence system from Santa Cruz Biotechnology Inc., (Santa Cruz, CA). Autoradiograms of the phosphorylated proteins were analyzed by using model FLA 5000 imaging densitometer (Fuji, Japan). The results shown are from a single experiment typical of at least two giving identical results.

2.6. Isolation and quantitation of lipophosphoglycan (LPG)

Purified LPG from *L. donovani* promastigotes, at the stationary phase of growth, was isolated as previously described [32,33]. The LPG, thus, obtained was dissolved in phosphate-buffered saline (PBS) or complete medium and the concentration was estimated by determining the amount of hexose in the extract [34].

2.7. Electrophoretic mobility shift assays (EMSA)

For the preparation of nuclear extracts [35,36], the treated cells were washed twice with ice-cold phosphate-buffered saline before resuspending in 1 ml cold hypotonic "low salt buffer" buffer A (20 mM HEPES buffer (pH 7.9), 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 0.2% Triton X-100, 0.2 mM PMSF, 0.4 μg ml^{-1} leupeptin, 1 μg ml^{-1} aprotinin, and 0.2 mM DTT). The cells were allowed to swell on ice for 20 min, then centrifuged at 1000 × *g* at 4 °C for 15 min and the resulting nuclear pellet was resuspended in 30 μl ice-cold "high salt buffer" buffer B (20 mM HEPES buffer (pH 7.9), 20% glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 0.2% Triton X-100, 0.1 mM PMSF, 0.2 μg ml^{-1} leupeptin, 0.5 μg ml^{-1} aprotinin, and 0.1 mM DTT) by occasionally tapping the nuclear pellet on ice for 1 h. The nuclear extract was centrifuged at 13,000 × *g* for 10 min at 4 °C and the supernatant was collected and stored in aliquots at −80 °C until use. The protein content of the extract was measured by the method of Bradford [31]. Binding reactions were initiated by incubating nuclear extract (4–6 μg protein) with double stranded poly (dI-dC) (1 μg μl^{-1}) (Pharmacia Biotech, St. Albans, UK), under specific salt/pH conditions in a binding buffer (20 mM HEPES (pH 7.9), 3.4% glycerol, 1.5 mM $MgCl_2$, 1.0 mM DTT) and 1.0 ng μl^{-1} of 5′ end ^{32}P-labeled dsDNA oligonucleotide in a total volume of 60 μl. Double stranded DNA (dsDNA, 10 ng) was labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase in a kinase buffer (New England Biolabs, Beverley, MA). This mixture was incubated for 30 min at 37 °C, and the reaction was stopped with 5 μl of 0.2 M EDTA. The labeled oligonucleotide was extracted with phenol/chloroform and passed through G-50 sephadex column. The dsDNA oligonucleotide, which was used as a probe contained the AP-1 consensus sequence (5′CGCTTGATGAGTCAGCCGGAA3′). Oligonucleotide was kindly gifted by Dr. Shekhar Reddy, John Hopkins University, Baltimore, USA. The specificity of binding was also examined by competition with 10–100-fold molar excess of the unlabeled dsDNA AP-1 oligonucleotide by adding simultaneously with the labeled probe. The resultant DNA-protein complexes were resolved by non-denaturing 8% (w/v) polyacrylamide gels. The gels were subsequently dried and autoradiographed. Visualization and quantitation of radioactive bands were conducted by PhosphorImager (Fuji film BAS-1800, Japan) using Image Quant software. The results shown are from a single experiment typical of at least two or three giving identical results.

2.8. Nitrite assay

Macrophages (5×10^5 cells/well) were cultured in a 96-well tissue culture plate in the presence of different concentrations of LPG or LPS. The release of nitrite by LPG was assayed from the cultured supernatants at 24 h using spectrophotometric method based on the Griess reaction [37].

2.9. Cytokine measurement

J774A.1 cells were plated in 100 μl of the media at 5×10^5 cells/well in a 96-well plate. Cells were allowed to adhere overnight, and then stimulated with LPG (10 μg ml^{-1}) or LPS (1 μg ml^{-1}). The concentration of IL-12p40 in culture supernatants was determined by the enzyme linked immunosorbent assay (ELISA). The assay was performed using Opt EIA kit (Pharmingen, SanDiego, CA) according to manufacturer's instructions. Cytokine concentration was determined using a standard curve using recombinant murine IL-12p40.

3. Results

3.1. Effect of LPG on ERK, JNK and p38 MAP kinase in murine macrophages

To verify whether LPG stimulates the ERK1, ERK2, p38 and JNK MAP kinase signal transduction pathway, J774A.1

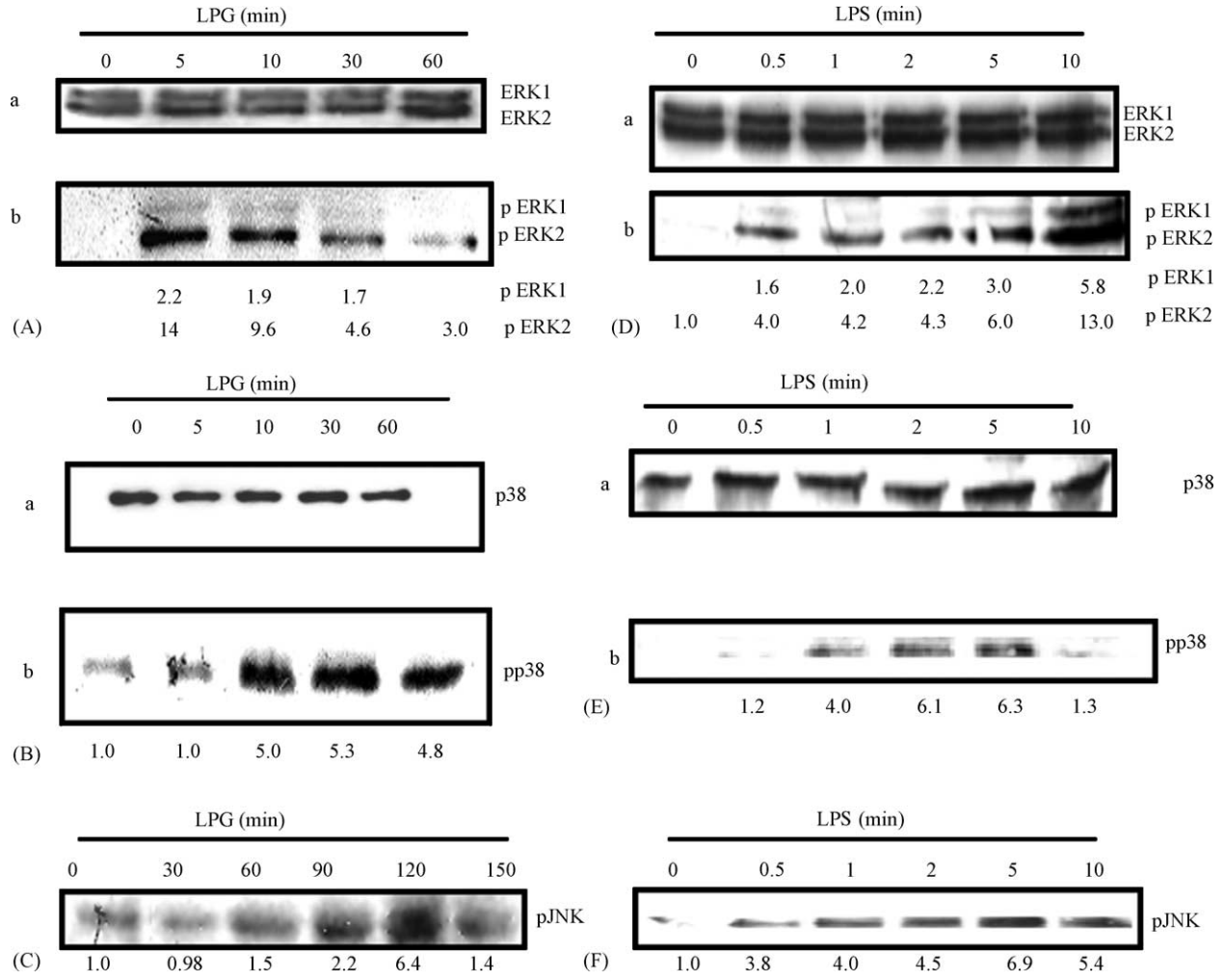
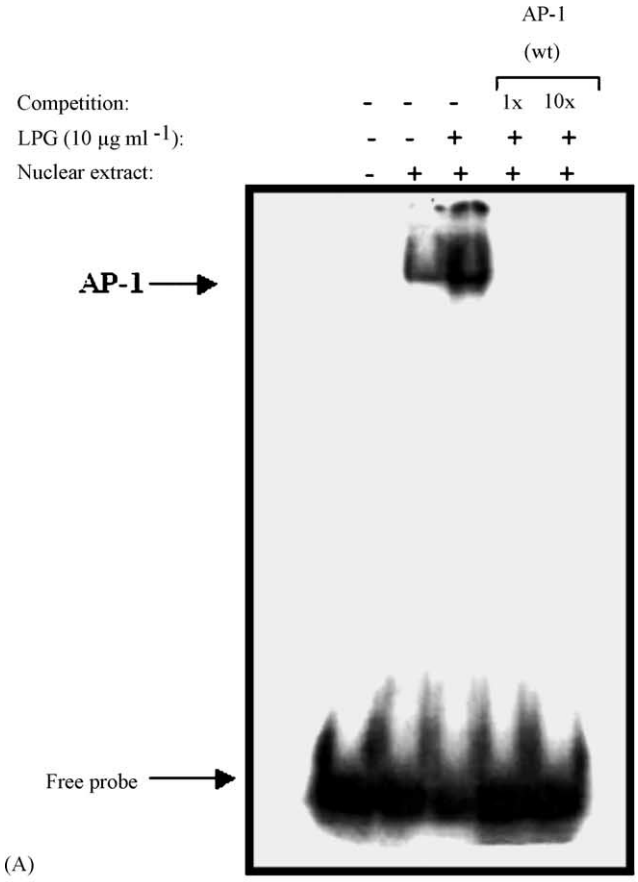


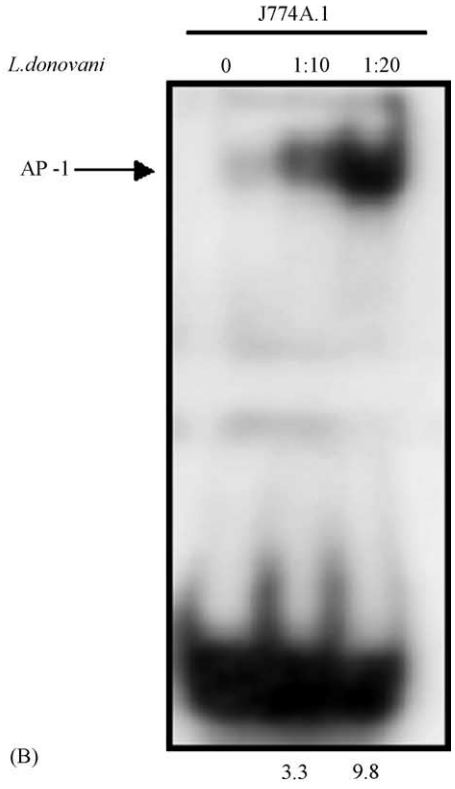
Fig. 1. Time course of LPG and LPS induced phosphorylation of MAP kinases. J774A.1 cells were stimulated with LPG (10 μg ml^{-1}) or LPS (1 μg ml^{-1}) and harvested at the indicated times (0–150 min). Whole cell lysates were resolved by SDS-PAGE (10%), followed by immunoblotting using a set of antibodies that recognized either activated (dually phosphorylated on Tyr/Thr) or total MAP kinase expression. (A): (a) anti-total ERK1 and ERK2 (b) anti-phospho-ERK1 and ERK2. (B): (a) anti-total p38 MAP kinase and (b) anti-phospho-p38 MAP kinase. (C): anti-phospho-JNK. Effect of LPS on ERK1 and ERK2 (D), p38 (E), and JNK (F) activation after different stimulation times is also shown. Fold increases in ERK1, ERK2, P38 and JNK activation are shown below each lane for each blot. The data shown are from two experiments that yielded similar results.

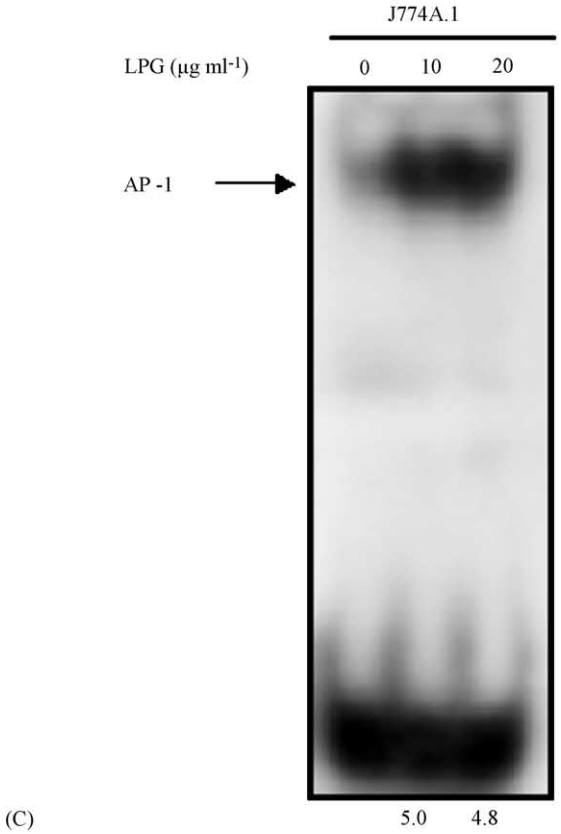
cells (1×10^6 cells/ml) were treated with 10 μg ml^{-1} of LPG. Cell lysates were prepared at different time points after the beginning of the stimulation.

The role of ERK, JNK and p38 MAP kinase pathways in LPG signal transduction in macrophages is shown in Fig. 1. The activation of the three MAP kinases was investigated by detecting their phosphorylated forms by Western blotting using specific anti-phosphokinase antibodies. LPG stimulated (within 5 min) increase in the levels of activation of both ERK1 and ERK2 MAP kinase activities in J774A.1 cells. ERK2 remained elevated even after 60 min above the low basal levels of ERK activation in unstimulated J774A.1 cells. A gradual decline in the levels of activation of ERK2 was observed after 5 min (Fig. 1A-b). Significant but low levels

Fig. 2. Induction of AP-1-DNA complex by *L. donovani* and its surface molecule lipophosphoglycan in macrophage cell line, J774A.1. Macrophages (1×10^6 cells/ml) were incubated with indicated concentrations of *L. donovani* at 37 °C for 1 h. Nuclear extracts were prepared as described under Section 2 and analyzed for the AP-1-DNA-binding activity by EMSA. (A): binding reactions with unlabeled excesses of AP-1 consensus oligonucleotide. J774A.1 cells were incubated for 1 h in the absence or the presence of LPG (10 μg ml^{-1}). Six microgram of each nuclear extract were incubated with γ-^{32}P-end-labeled synthetic double-stranded AP-1 probe. Lane 1: free probe; lane 2: untreated J774A.1 cells; lane 3: J774A.1 cells incubated with LPG; Lanes 4 and 5: the AP-1 specific band is competed away by 1- and 10-fold molar excess of an unlabeled synthetic wild-type (wt) AP-1 cold competitor. An arrow on the left side indicates the position of the specific complex. (B) Dose-dependent activation of nuclear AP-1-DNA binding activity with *L. donovani*. (C) Dose-dependent activation of nuclear AP-1-DNA binding activity with LPG. (D) Dose-dependent activation of AP-1-DNA binding activity with LPS. Fold increases in AP-1 activation are shown below each lane for each blot The data shown are from one of the three independent experiments that yielded similar results.


AP-1
(wt)
Competition: - - - 1x 10x
LPG (10 μg ml -1): - - + + +
Nuclear extract: - + + + +
AP-1
Free probe
(A)


J774A.1
L.donovani 0 1:10 1:20
AP -1
(B)
3.3 9.8


J774A.1
LPG (μg ml-1) 0 10 20
AP -1
(C)
5.0 4.8


J774A.1
LPS (ng ml -1) 0 100 200
AP -1
(D)
2.2 2.7

of activation of p38 MAP kinase was observed in unstimulated J774A.1 cells. This slight activation is possibly due to the presence of serum in the medium. A small difference was detected between cells cultured in the presence and absence of serum (data not shown). LPG stimulated phosphorylation of p38, within 10 min and was sustained for up to 60 min (Fig. 1B-b). LPG strongly stimulated JNK MAP kinase following a lag period of 30 min before reaching peak with maximum activity at 120 min (Fig. 1C). These data indicate that LPG stimulates all three classes of MAP kinase in macrophages, but with differential kinetics of activation. Lipopolysaccharide (LPS; 1 $\mu g\,ml^{-1}$), known to activate ERK, JNK and p38 MAP kinases [9] induced the phosphorylation of ERK1, ERK2, p38 and JNK MAP kinases in J774A.1 macrophages (Fig. 1D–F). However, the kinetics of induction was different from that with LPG.

3.2. *L. donovani activates AP-1 transcription factor in J774A.1 murine macrophages*

We studied the effect of both *L. donovani* and *Leishmania* surface molecule, lipophosphoglycan on AP-1 transcription factor in J774A.1 macrophage cell line. For AP-1 consensus oligomer, a predominant DNA-protein complex was revealed by EMSA. A low level of predominant AP-1 consensus probe-binding complex was present in untreated nuclei. Following treatment with LPG at 10 $\mu g\,ml^{-1}$, a noticeable increase of this constitutive form of AP-1 was observed by 1 h (Fig. 2A). To demonstrate that the DNA oligomer-protein complexes formed in the EMSA represent specific molecular interactions, unlabeled probe competition experiments were performed (Fig. 2A). The corresponding unlabeled probe strongly competes for complex formation. These data indicate that the proteins activated by LPG and detected by EMSA were highly specific for AP-1.

Incubation of J774A.1 cells with macrophage/parasite ratio of 1:10 or 1:20 for 1 h at 37 °C activated AP-1 (Fig. 2B). Activation was dependent on the concentration of *L. donovani* with parasite/macrophage ratio of 10:1 (corresponding to 1×10^7 parasites) resulting in 3.3-fold activation of AP-1 binding activity whereas higher parasite/macrophage ratio, i.e., 20:1 (corresponding to 2×10^7 parasites) resulted in 9.8-fold induction of AP-1 binding activity (Fig. 2B). AP-1 was also activated in cells treated with lipophosphoglycan. LPG (10 $\mu g\,ml^{-1}$) resulted in 5.0-fold induction where as 20 $\mu g\,ml^{-1}$ of LPG resulted in 4.8-fold induction (Fig. 2C) when compared with the untreated macrophages. LPG (10 $\mu g\,ml^{-1}$) used to stimulate macrophages corresponded to 2×10^7 parasites/ml or equal to 1:20 macrophage/parasite ratio. LPS was used as a positive control. LPS-treated J774A.1 macrophages exhibited a significant increase in AP-1 activation over the unstimulated controls (Fig. 2D). In order to demonstrate that the AP-1 activated by *L. donovani* and LPG was not due to LPS, we pretreated *L. donovani* and LPG for 30 min with polymyxin B (PB) (5 $\mu g\,ml^{-1}$). Pretreatment of LPG and *L. donovani* with PB had no effect on the AP-1 activation induced by LPG or *L. donovani* in J774A.1 macrophages (data not shown). These data clearly demonstrate that the effect of *L. donovani* and LPG were not due to LPS.

The kinetics of AP-1 activation was determined by exposing the J774A.1 cells to *L. donovani* (macrophage/parasite ratio of 1:10) for different time intervals and the samples were then analyzed by EMSA. Increase in AP-1 DNA binding activity was observed as early as 10 min and remained steady for up to 120 min (Fig. 3A). However, a significant decline in AP-1 binding activity was observed at 180 min. A similar time dependent activation of DNA binding activity of AP-1 was observed with LPG (10 $\mu g\,ml^{-1}$). The DNA binding activity increased at 10 min and remained stable for up to 120 min (Fig. 3B). A significant decline in AP-1 binding activity was observed at 180 min.

We investigated if cellular uptake of *L. donovani* is required for AP-1 activation. Cytochalasin B is an actin-depolymerizing drug that inhibits bacterial invasion and phagocytosis [38,39]. We examined the effect of cytochalasin B on *Leishmania* induced AP-1 activation. Inhibition of cellular invasion by pretreating the cells with cytochalasin B for 15 min followed by incubation with *L. donovani* for different time intervals did not prevent activation of AP-1 by *L. donovani* when compared to untreated cells (data not shown). The results clearly indicated that the activation of AP-1 does not require infectivity of *L. donovani* in the cells and its cellular uptake.

3.3. *Relationship of AP-1 activation to L. donovani virulence*

In order to check if there is any relationship between the virulence of the parasite and AP-1 activation, J774A.1 cells were incubated with different concentrations of either low virulent or high virulent strain of *L. donovani* for 1 h. The low virulent strain was passaged in the laboratory over several months and is therefore not infective in the mice model and the high virulent strain was maintained in BALB/c mice. The low and high virulent strains differed in their uptake by J774A.1 macrophages. At 24 h, the number of low virulent amastigotes in the infected macrophages was 109 ± 10 per 100 macrophages where as the number of high virulent parasites per 100 macrophages was 450 ± 25. As shown in Fig. 4, both high (Fig. 4A) and low virulent strains (Fig. 4B) of *L. donovani* resulted in a dose-dependent increase in AP-1-DNA-binding activity. The ability of *L. donovani* to activate AP-1 was independent of its virulence.

3.4. *Role of ERK1, ERK2 and p38 MAP kinases in regulation of AP-1 triggered by lipophosphoglycan*

To further investigate the role of MAP kinase intermediates in the signal transduction pathway resulting in the AP-1

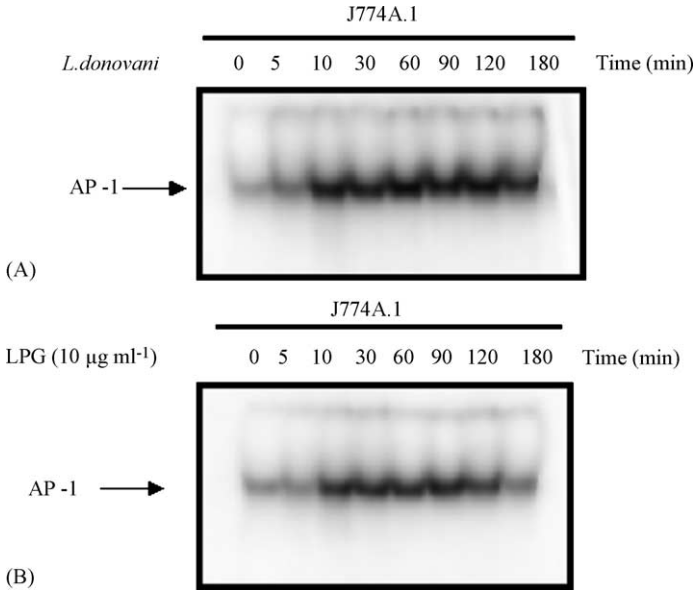


Fig. 3. Kinetics of AP-1 activation by *L. donovani* and its surface molecule lipophosphoglycan in J774A.1 macrophage cell line. J774A.1 cells were infected with *L. donovani* (A) or treated with lipophosphoglycan (B) for the indicated times. The nuclear extracts prepared from these cells were analyzed by EMSA for the AP-1-DNA-binding activity. Only the relevant part of EMSA with nuclear AP-1 activity J774A.1 cells is shown. The data shown are from one of the three independent experiments that yielded similar results.

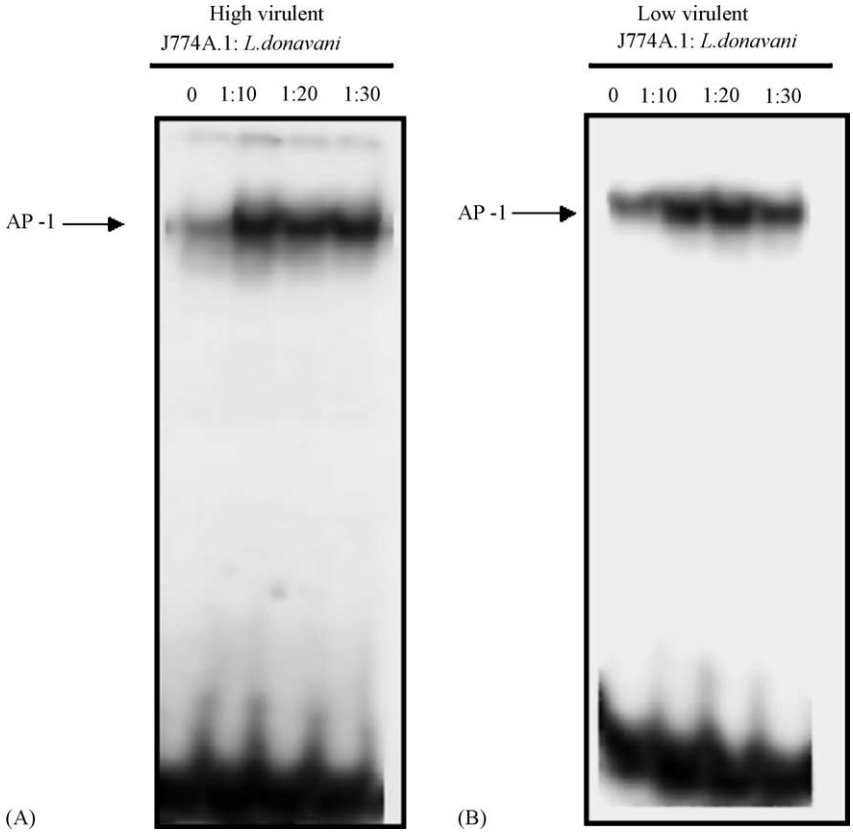


Fig. 4. Both low- and high-virulent *L. donovani* activated AP-1. J774A.1 cells were infected with high-virulent (A) and low-virulent *L. donovani* (B) at the indicated concentrations for 1 h. The nuclear extracts were prepared from the infected cells and analyzed for the AP-1-DNA-binding activity by EMSA. The data shown are from one of the two independent experiments that yielded similar results.

activation, the effects of specific inhibitors on the activation of this DNA binding factor were analyzed (Fig. 5A). Nuclear extracts were prepared from cells pretreated for 60 min with either PD98059 (20 μM), a specific inhibitor of ERK1 and ERK2 pathway, or SB203580 (5 μM), a specific inhibitor of the p38 pathway followed by treatment with LPG (10 μg ml^{-1}) for 60 min. A decrease in the binding activity of AP-1 was observed when compared with controls stimulated with LPG and not treated with the inhibitors. Pre-incubation of cells with PD98059 (20 μM) or SB203580 (5 μM) inhib-

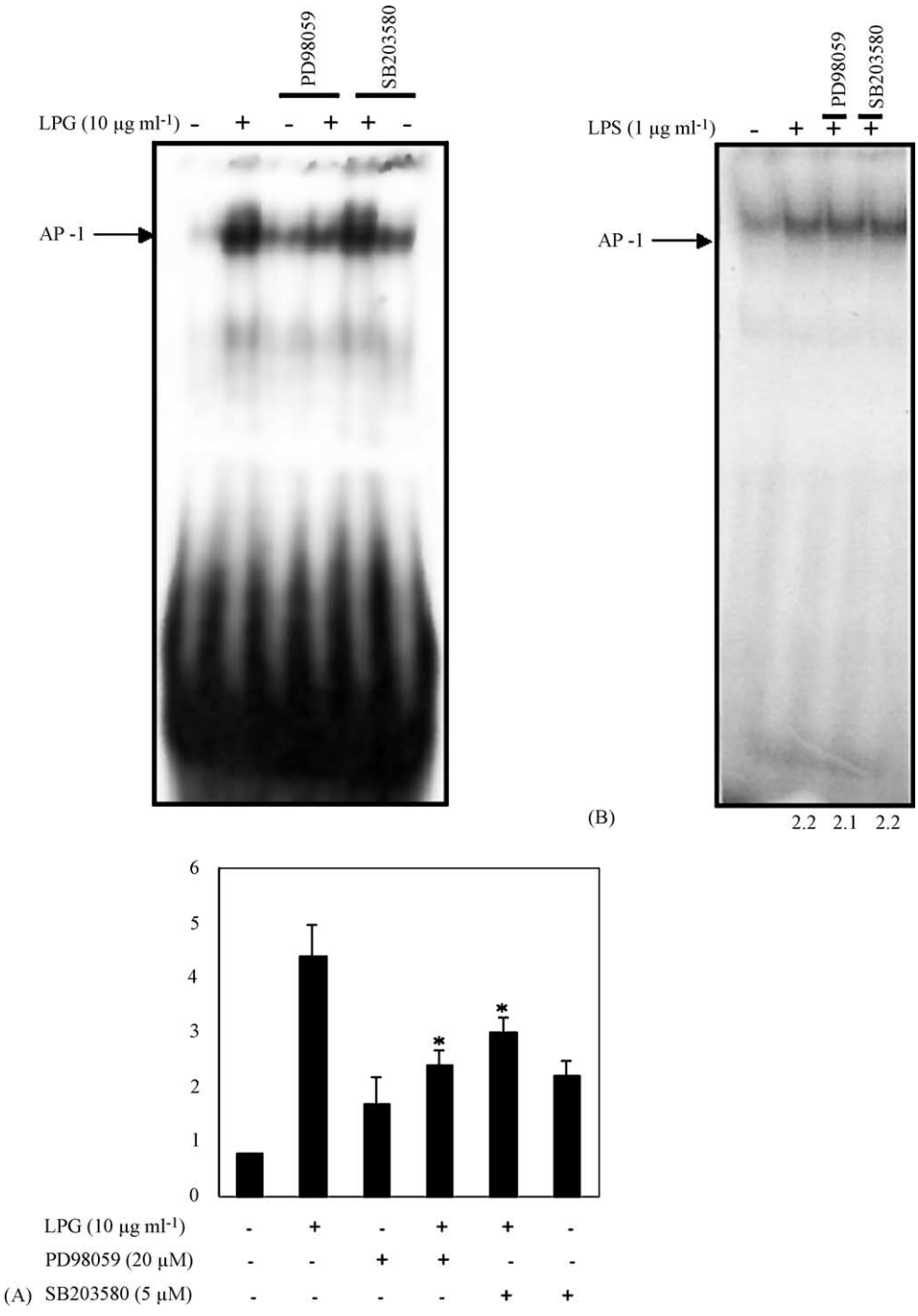


Fig. 5. Effects of PD98059 and SB203580 on AP-1 binding activity induced by LPG. J774A.1 cells pretreated with PD98059 (20 μM), SB203580 (5 μM), or medium for 1 h were stimulated with either LPG (10 μg ml^{-1}) (A) or LPS (1 μg ml^{-1}) (B) for further 1 h, and the nuclear protein were extracted. EMSA analysis of these nuclear extracts was conducted using the γ-^{32}P-end-labeled synthetic double-stranded AP-1 probe. The data shown are from one of the two independent experiments that yielded similar results. Values in the histogram represent the average of two determinations from independent experiments. Means, which differ significantly from that of the corresponding LPG-treated control are indicated by ($^{*}P < 0.05$). Fold increases in AP-1 activation by LPS are shown below each lane.

ited the binding activity of AP-1 by 54% and 68%, respectively, over LPG-treated group suggesting that both ERK and p38 MAP kinase activation appear to be necessary for AP-1 activation. At these concentrations the inhibitors did not cause cellular damage. Pretreatment of macrophages with PD98059 (20 μM) and SB203580 (5 μM) followed by treatment with LPS (1 μg ml^{-1}) did not effect the AP-1 binding activity when compared with LPS stimulated cells that were not treated with the inhibitors (Fig. 5B). This indicates that p38 and ERK MAP kinase activation is not necessary for AP-1 activation by LPS in J774A.1 macrophage cell line.

3.5. LPG induced nitric oxide in J774A.1 macrophages

Generation of NO by activated macrophages has been correlated directly with the leishmanicidal capacity of the cells [4]. *L. major* LPG has been reported to promote as well as inhibit NO synthesis by the murine macrophages, thereby playing an important role in the host–parasite relationship [4]. LPG resulted in the present study, a dose-dependent increase in the NO levels in J774A.1 macrophages (Fig. 6A). LPS alone, as expected induced significant NO synthesis (Fig. 6B). Polymyxin B had no effect on NO synthesis by macrophages induced by LPG, whereas, it completely inhibited NO synthesis by LPS (data not shown).

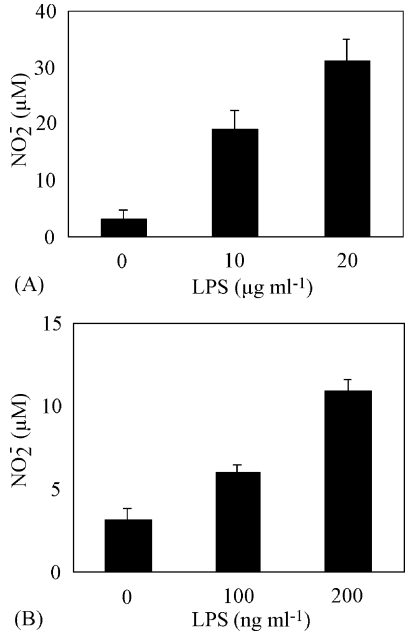


Fig. 6. Effect of LPG on nitrite levels in J774A.1 macrophages. J774A.1 macrophages (5×10^5 cells/well) were seeded onto 96-well tissue culture plates. Cells were then treated with different concentrations of LPG (A) or LPS (B) and again incubated at 37 °C for 24 h. Nitrite levels present in the supernatants were quantified by Griess reagent. Data are mean ± S.D. of three separate treatments.

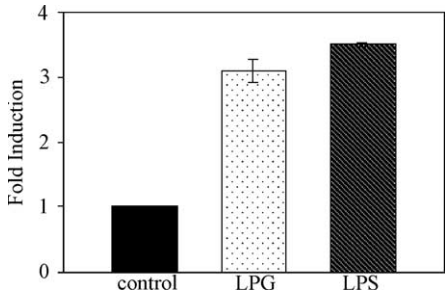


Fig. 7. LPG induces IL-12 levels in J774A.1 macrophages. Macrophages were treated with either LPG (10 μg/ml) or LPS (1 μg ml^{-1}) for 24 h before supernatants were assayed for IL-12p40 production. All values represent the mean ± S.D. of average fold induction of triplicates samples. The data shown is from one of the two independent experiments that yielded similar results.

3.6. LPG activated IL-12 protein production in macrophages

J774A.1 macrophages were treated with LPG (10 μg ml^{-1}) or LPS (1 μg ml^{-1}). Supernatants were collected 24 h later and assayed for IL-12p40 production by ELISA. As shown in Fig. 7, both LPS and LPG resulted in a modest 3.1- and 3.5-fold increase, respectively, in IL-12p40 production over that of unstimulated cells.

4. Discussion

In this study, we examined the effects of leishmanial surface molecule lipophosphoglycan on the activation of MAP kinase pathway and AP-1 cascades in a murine macrophage cell line J774A.1. It is well established that AP-1 activation, resulting from the engagement of several different surface receptors with their extracellular ligands, usually involves the MAP kinase intermediates [20,23]. Our major finding is that all three classes of MAP kinase, ERK, p38 and JNK are simultaneously activated by LPG in macrophages with maximal activation occurring between 10 and 150 min post-stimulation in J774A.1 cell line.

Earlier reports have shown that *Leishmania* fails to induce phosphorylation of ERK1, ERK2, p38 MAP kinase and *c-jun* N-terminal kinase in naive macrophages [12]. However, in IFN-gamma primed bone marrow derived macrophages, *L. donovani* promastigotes strongly induced the phosphorylation of ERK1 and ERK2 and p38 kinase [12]. The use of *L. donovani* mutants defective in the biosynthesis of LPG resulted in induction of MAP kinases in naïve bone marrow derived macrophages [12]. It has also been reported earlier that synthetic phosphoglycan (SPG) stimulated ERK activity, both ERK1 and ERK2, in J774 cells and such activation was sustained for at least 30 min [9]. Purified lipophopshoglycan from *L. major* has also been reported to activate both p38 and extracellular signal-regulated kinase (ERK) in RAW-ELAM macrophages [3]. Our data show that purified LPG stimulated all the three classes of MAP kinases, ERK, JNK and

p38, simultaneously, in J774A.1 macrophages. It is possible that MAP kinases transduce differential regulatory effects that vary in a receptor- and cell type-dependent manner, perhaps reflecting cell-lineage-restricted expression of different classes of MAP kinases [9]. Mitogen-activated protein kinases are known to play a central role in the regulation of innate response including the production of proinflammatory cytokines and NO [13,14]. Induction of MAP kinases by LPG in this study may therefore represent a key step in the elimination of the parasite.

Our present data show that infection of J774A.1 macrophages with *L. donovani* activated AP-1. The response was dependent on the concentration of *L. donovani*. AP-1 was also activated in cells treated with *Leishmania* surface molecule lipophosphoglycan. Our results also indicate that in J774A.1 cells, LPG involved both p38 and ERK1 and ERK2 pathway in AP-1 activation. Quantitative Western blot analysis of whole cell lysate using c-Fos and c-Jun specific antibodies showed that these proteins that are capable of forming AP-1 dimers were up regulated in macrophages treated with LPG (data not shown).

Data presented here further demonstrates that the major surface molecule of *Leishmania* promastigotes can induce NO synthesis by macrophages, thereby playing an important role in the host–parasite relationship. The leishmanicidal capacity of these cells showed a significant decrease in the parasite burden at 48 h after infection with *L. donovani* strain AG83 (data not shown).

Effective immunity against *Leishmania* infection is dependent on the induction of parasite-specific Th-1 cells [1]. IL-12 production is critical for the development of Th-1 responses [40]. In the present study, purified LPG resulted in a modest but significant increase in IL-12 production thereby inducing the Th-1 amplification circuit.

Recent reports show that *L. donovani* infection in macrophages from susceptible mice resulted in down regulation of extracellular signal-related kinase dependent AP-1 and NF-κB transactivation [41]. On the contrary, *L. donovani* infection in macrophages from the leishmaniasis-resistant C.D2 mice induced MAP kinase phosphorylation and AP-1 and NF-κB DNA-binding activity [41]. Interaction of *Leishmania* with the macrophages resulted in the present study enhanced mitogen-activated protein kinase phosphorylation, AP-1 DNA-binding activity, and elevated levels of nitric oxide and IL-12. Thus, the response that we have observed is analogous to the response in the Leishmaniasis-resistant C.D2 mice. The differences in the signal transduction cascade between susceptible and resistant mouse model at the transcription factor level needs to be further explored. These results suggest that *Leishmania* LPG plays an important role in the analysis of host–parasite interactions and may prove to be significant for the regulation of pathological and immunological responses to *Leishmania* infection. The present results also show that this transcription factor could therefore be a potential target for development of novel strategies and therapeutic modalities to combat the intracellular pathogen.

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References

[1] Sacks D, Noben-Trauth N. The immunology of susceptibility and resistance to *Leishmania major* in mice. Nat Rev Immunol 2002;2:845–8.
[2] Beverley SM, Turco SJ. Lipophosphoglycan and the identification of virulence genes in the protozoan parasite *Leishmania*. Trends Microbiol 1998;6:35–40.
[3] De Veer MJ, Curtis JM, Baldwin TM, et al. MyD88 is essential for clearance of *Leishmania major*: possible role for lipophosphoglycan and Toll-like receptor signaling. Eur J Immunol 2003;33:2822–31.
[4] Proudfoot L, Nikolaev AV, Feng GJ, et al. Regulation of the expression of nitric oxide synthase and leishmanicidal activity by glycoconjugates of *Leishmania* lipophosphoglycan in murine macrophages. Proc Natl Acad Sci USA 1996;93:10984–9.
[5] Piedrafita D, Proudfoot L, Nikolaev AV, et al. Regulation of macrophage IL-12 synthesis by *Leishmania* phosphoglycans. Eur J Immunol 1999;29:235–44.
[6] Chan J, Fujiwara T, Brennan P, et al. Microbial glycolipids: possible virulence factors that scavenge oxygen radicals. Proc Natl Acad Sci USA 1989;86:2453–7.
[7] Descoteaux A, Turco SJ, Sacks DL, Matlashewski G. *Leishmania donovani* lipophosphoglycan selectively inhibits signal transduction in macrophages. J Immunol 1991;146:2747–53.
[8] Desjardins M, Descoteaux A. Inhibition of phagolysosomal biogenesis by the *Leishmania* lipophosphoglycan. J Exp Med 1997;185:2061–8.
[9] Feng GJ, Goodridge HS, Harnett MM, et al. 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* phosphogylcan subvert macrophage IL-12 production by targeting ERK MAP kinase. J Immunol 1999;163:6403–12.
[10] McNeely TB, Turco SJ. Requirement of lipophosphoglycan for intracellular survival of *Leishmania donovani* within human monocytes. J Immunol 1990;144:2745–50.
[11] Puentes SM, Sacks DL, da Silva RP, Joiner KA. Complement binding by two development stages of *Leishmania major* promastigotes varying in expression of surface lipophosphoglycan. J Exp Med 1988;167:887–902.
[12] Prive C, Descoteaux A. *Leishmania donovani* promastigotes evade the activation of mitogen-activated protein kinases p38, c-Jun N-terminal kinase, and extracellular signal-regulated kinase-1/2 during infection of naive macrophages. Eur J Immunol 2000;30:2235–44.
[13] Ip YT, Davis RJ. Signal transduction by the c-Jun N-terminal kinase (JNK) – from inflammation to development. Curr Opin Cell Biol 1998;10:205–19.

[14] Robinson MJ, Cobb MH. Mitogen-activated protein kinase pathways. Curr Opin Cell Biol 1997;9:180–6.
[15] Griscavag JM, Wilk S, Ignarro LJ. Inhibitors of the proteasome pathway interfere with induction of nitric oxide synthase in macrophages by blocking activation of transcription factor NF-kappaB. Proc Natl Acad Sci USA 1996;93:3308–12.
[16] Kamijo R, Harada H, Matsuyama T, et al. Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. Science 1994;263:1612–5.
[17] Murphy TL, Cleveland MG, Kulesza P, Magram J, Murphy KM. Regulation of interleukin 12 p40 expression through an NF-kappaB half-site. Mol Cell Biol 1995;15:5258–67.
[18] Taki S, Sato T, Ogasawara K, et al. Multistage regulation of Th1-type immune responses by the transcription factor IRF-1. Immunity 1997;6:673–9.
[19] Angel P, Karin M. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim Biophys Acta 1991;1072:129–57.
[20] Karin M. The regulation of AP-1 activity by mitogen-activated protein kinases. Philos Trans R Soc Lond B Biol Sci 1996;351:127–34.
[21] Karin M, Liu Z, Zandi E. AP-1 function and regulation. Curr Opin Cell Biol 1997;9:240–6.
[22] Kaminska B, Pyrzynska B, Ciechomska I, Wisniewska M. Modulation of the composition of AP-1 complex and its impact on transcriptional activity. Acta Neurobiol Exp 2000;60:395–402.
[23] Whitmarsh AJ, Davis RJ. Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. J Mol Med 1996;74:589–607.
[24] Wisdom R. AP-1: one switch for many signals. Exp Cell Res 1999;253:180–5.
[25] Cohen DR, Ferreira PC, Gentz R, Franza Jr BR, Curran T. The product of a fos-related gene, fra-1, binds cooperatively to the AP-1 site with Jun: transcription factor AP-1 is comprised of multiple protein complexes. Genes Dev 1989;3:173–84.
[26] Cook SJ, Aziz N, McMahon M. The repertoire of fos and jun proteins expressed during the G_1 phase of the cell cycle is determined by the duration of mitogen-activated protein kinase activation. Mol Cell Biol 1999;19:330–41.
[27] Derijard B, Hibi M, Wu IH, et al. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell 1994;76:1025–37.
[28] Ryseck RP, Bravo R. c-JUN, JUN B and JUN D differ in their binding affinities to AP-1 and CRE consensus sequences: effect of FOS proteins. Oncogene 1991;6:533–42.
[29] Vandel L, Pfarr CM, Huguier S, Loiseau L, Sergeant A, Castellazzi M. Increased transforming activity of JunB and JunD by introduction of a heterologous homodimerisation domain. Oncogene 1995;10:495–507.
[30] Hart DT, Vickerman K, Coombs GH. A quick, simple method for purifying *Leishmania mexicana* amastigotes in large numbers. Parasitology 1981;82:345–55.
[31] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[32] Orlandi Jr PA, Turco SJ. Structure of the lipid moiety of the *Leishmania donovani* lipophosphoglycan. J Biol Chem 1987;262:10384–91.
[33] Russo DM, Turco SJ, Burns Jr JM, Reed SG. Stimulation of human T lymphocytes by *Leishmania* lipophosphoglycan associated proteins. J Immunol 1992;148:202–7.
[34] Rao P, Pattabiraman TN. Reevaluation of the phenol-sulfuric acid reaction for the estimation of hexoses and pentoses. Anal Biochem 1989;181:18–22.
[35] Chaturvedi MM, LaPushin R, Aggarwal BB. Tumor necrosis factor and lymphotoxin. Qualitative and quantitative differences in the mediation of early and late cellular response. J Biol Chem 1994;269:14575–83.
[36] Schreiber E, Matthias P, Muller MM, Schaffner W. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res 1989;17:6419.
[37] Green LC, Wagmer DA, Glogowski J, et al. Analysis of nitrate, nitrite and [^{15}N] nitrate in biological fluids. Anal Biochem 1982;126:131–8.
[38] Grassme HU, Ireland RM, van Putten JP. Gonococcal opacity protein promotes bacterial entry-associated rearrangements of the epithelial cell actin cytoskeleton. Infect Immun 1996;64:1621–30.
[39] Naumann M, Wessler S, Bartsch C, Weiland B, Meyer TF. *Neisseria gonorrhoeae* epithelial cell interaction leads to the activation of the transcription factors nuclear factor kappaB and activator protein 1 and the induction of inflammatory cytokines. J Exp Med 1997;186:247–58.
[40] Trinchieri G. Interleukin-12- a proinflammatory cytokine with immunoregulatory function that bridge innate resistance and antigen-specific adaptive immunity. Ann Rev Immunol 1995;13:251–76.
[41] Ghosh S, Bhattacharyya S, Sirkar M, et al. *Leishmania donovani* suppresses activated protein 1 and NF-kappaB activation in host macrophages via ceramide generation: involvement of extracellular signal-regulated kinase. Infect Immun 2002;70:6828–38.