LIST OF PUBLICATIONS

Kapoor, P., Raj, S.V., Saxena, S., **Balaraman, S.** and Madhubala, R. 2001 Effect of *Leishmania donovani* lipophosphoglycan on ornithine decarboxylase activity in macrophages. **J. Parasitol.** 87(5): 1071-1076.

Balaraman, S., Tewary, P., Singh, K.V. and Madhubala, R. 2004 *Leishmania donovani* induces interferon regulatory factor in murine macrophages: a host defense response. **Biochem. Biophys. Res. Comm.** 317: 639-647.

Balaraman, S., Singh, K.V., Tewary, P. and Madhubala, R. *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. **(Manuscript in preparation).**

Singh, K.V., **Balaraman, S.,** Tewary, P. and Madhubala, R. Host Defense Response to Infection by *Leishmania donovani:* Induction of Nuclear Transcription Factor-κB. **(Manuscript in preparation).**

Leishmania donovani **induces interferon regulatory factor in murine macrophages: a host defense response**

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Abstract

Macrophages play a key role in directing the host immune response to infection. Interaction of *Leishmania donovani* with macrophages results in the antagonization of host defense mechanisms by interfering with a cascade of cell signaling processes in the macrophages. Macrophages secrete interferon (IFN), as well as other cytokines, following lipopolysaccharide (LPS) stimulation. The interferon regulatory factors (IRFs) comprise a family of DNA-binding proteins that have been implicated in the transcriptional regulation of IFN and certain IFN-inducible genes. IRF-1 is a transcription factor, which regulates induction of several macrophage effectors and is known to bind to IRF-E site in the inducible nitric oxide synthase (iNOS) promoter. We for the first time report that *L. donovani* and its surface molecule lipophosphoglycan (LPG) result in a dose- and time-dependent activation of IRF–DNA-binding activity in macrophages. The components of this novel LPG-stimulated IRF-like complex are unclear. The interaction of parasite with the macrophages and not the cellular uptake was important for IRF activation. The use of inhibitors selective for ERK (PD98059) and p38 (SB203580) mitogen-activated protein (MAP) kinase pathway showed that preincubation of cells with either SB203580 or PD98059 did not affect the binding activity of IRF-E, suggesting that both p38 and ERK MAP kinase activation are not necessary for IRF-E activation. It is likely that induction of IRF in response to infection by *L. donovani* represents a host defense mechanism.

Article Outline

Leishmania donovani is an obligate intracellular parasite that survives inside the macrophages of the vertebrate host, resulting in visceral leishmaniasis in humans. The parasite exists in two forms, a motile intracellular promastigote form within the gut of the sandfly vector and a non-motile amastigote within the macrophages of the mammalian host. Macrophages have to become activated in order to kill infectious organisms indicating the need for the signal to the nucleus. Since *L. donovani* is known to survive within the macrophages, this could be due to the impairment of several macrophage accessory functions responsible for microbicidal action.

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 nitric oxide synthase (iNOS), and cytokines, resistance to oxygen radicals, resistance to complement-mediated lysis, and inhibition of phagosomal maturation [1, 2, 3, 4, 5, 6 and 7]. *L. donovani* promastigotes have been reported to evade the activation of mitogen-activated protein kinases during infection of naïve macrophages [8].

Downstream cascade of these kinases are the ubiquitous transcription factors such as activating protein 1 (AP-1), NF-κB, and IFN regulatory factor (IRF). IFN regulatory factor (IRF) and NF-κB activation has been shown to play a key role in the induction of macrophage effector molecules [9, 10, 11 and 12] and is involved in the regulation of functions involved in host defense and inflammation.

Interferon regulatory factor-1 (IRF-1) is a transcription factor that was first identified to be important in the virus-induced activation of the IFN-β gene [13]. Molecular cloning revealed it to be a DNA-binding protein with a basic NH_2-terminal domain linked to an acidic COOH-terminal region, which is highly conserved between mice and humans [14]. The NH_2 terminus is responsible for DNA binding, whereas sequences within the COOH-terminal domain mediate transactivation [15]. IRF-1 is generally present only at low levels within resting cells; but after treatment with either type I or type II IFN, transcription of the IRF-1 gene is increased, and levels of IRF-1 protein rise dramatically [14 and 16]. In addition, IRF-1 production is induced by other cytokines, such as IL-1, IL-6, and TNF-α [16], viral infection [14], and prolactin [17]. IRF-1 enters the nucleus rapidly after synthesis, where it binds to a DNA motif with the sequence G(A)AAA(G/C)(T/C)-

GAAA(G/C)(T/C) [18]. This motif has been found in the upstream promoter elements of a number of IFN-inducible genes such as IFN-β [19], major histocompatibility complex class 1 [20], and 2-5-oligo (A) synthetase [21]. A number of studies have shown the importance of IRF-1 in mediating transcriptional up-regulation of these and other IFN-induced genes [18]. Earlier studies have shown that the gene for inducible nitric oxide synthase (iNOS) is regulated by IRF-1 [22 and 23]. Within the mouse iNOS promoter sequence there are four copies of an IFN-γ response element, two copies of γ-activated site, and two copies of the IFN-stimulated response element (ISRE). The complementary nucleotide sequence of one of these ISRE core closely matches a consensus sequence termed IFN regulatory factor element (IRF-E) [22, 23 and 24]. IRF-1 is an IRF-E-binding protein [22, 23 and 24].

It has been reported earlier that induction of IRF-1 is functionally related to the host defense response in *Mycobacterium tuberculosis* infection [25]. The role of LPS in inducing IRF-1 in macrophages has been demonstrated earlier [26]. In the present study, we examined changes in IRF–DNA-binding activity after *L. donovani* infection in macrophages. We suggest the possibility that the IRF family of nuclear transactivating factors is involved in the response to *L. donovani* and this may be a mechanism for host defense.

Materials and methods

Chemicals and reagents. The tissue culture chemicals like M-199, RPMI-1640, sodium bicarbonate, streptomycin, penicillin-G, poly (dI:dC), leupeptin, and aprotinin were procured from Sigma Chemical (St. Louis, MO). [γ-^{32}P]ATP was purchased from Amersham Pharmacia Biotech Asia Pacific (Hong kong). Polynucleotide kinase was purchased from Promega (Madison, USA). PD98059 and SB203580 were obtained from

Santa Cruz Biotechnology (Santa Cruz, CA). Reagents used were obtained from Sigma Chemical (St. Louis, MO) unless indicated otherwise.

Parasite culture. *Leishmania donovani* AG83 (MHOM/IN/1983/AG83) promastigotes were cultured at 24 °C in modified M199 medium (Sigma, St. Louis, MO) supplemented with 100 U/ml penicillin (Sigma, St. Louis, MO), 100 μg/ml 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. Amastigotes were isolated from spleens as previously described and transformed to promastigotes in M199 medium containing 30% FCS [27]. Freshly transformed promastigotes were maintained at 22 °C in M199 with 10% FCS.

Cell culture, treatment, and infection. A murine macrophage cell line J774A.1 (American Type Culture Collection, Rockville, Maryland) was used in this study. The macrophages were maintained at 37 °C in RPMI-1640 medium with 10% FCS in a CO_2 incubator (5% CO_2). The macrophages were seeded in tissue culture plates (90 mm) at a density of 1×10^6 cells/plate and incubated for 24 h before being used for the requisite assay. In studies involving MAP kinase inhibitor PD98059 (20 μM), a specific inhibitor of ERK1/2 pathway, or SB203580 (5 μM), a specific inhibitor of the p38 pathway, cells were pretreated for 60 min followed by infection with *L. donovani*.

Isolation and quantitation of lipophosphoglycan. Purification, isolation, and quantitation of lipophosphoglycan (LPG) from *L. donovani* promastigotes, at the late log phase of growth, were carried out as previously described [28 and 29]. 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 [30].

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Electrophoretic mobility shift assay. For the preparation of nuclear extracts [31 and 32],

the treated cells were washed twice with ice-cold PBS 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

leupeptin, 1 μg/ml aprotinin, and 0.2 mM DTT). The cells were allowed to swell on ice for

20 min and then centrifuged at 1000g 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 leupeptin, 0.5 μg/ml aprotinin, and 0.1 mM DTT) by

occasionally tapping the nuclear pellet on ice for 1 h. The nuclear extract was centrifuged at

13,000g 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

[33]. Binding reactions were initiated by incubating nuclear extract (4–6 μg protein) with

double-stranded poly (dI-dC) (1 μg/μl) (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

$MgCl_2$, and 1.0 mM DTT) and 1.0 ng/μl 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 radiolabeled probe was an oligonucleotide (5'-

CACTGTCAATATTTCACTTTCATAAT-3'), an IRF-E in the iNOS promoter. The

specificity of binding was also examined by competition with 2- to 20-fold molar excess of

the unlabeled dsDNA IRF-E oligonucleotide by adding simultaneously with the labeled

probe. For supershift analysis 1 μg of anti-IRF-1-antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the reaction mixture. 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.

Results

Leishmania donovani activates IRF-transcription factor-binding activity in J774A.1 murine macrophages

Infection by *L. donovani* results in dose- and time-dependent activation of IRF-E DNA-binding activity in macrophages. To find out if the host immune response is dependent on the activation of nuclear transcription factor IRF, we studied the effect of both *L. donovani* and *Leishmania* surface molecule, LPG, on IRF-E DNA binding in J774A.1 macrophage cell line. We performed electrophoretic mobility shift assays (EMSAs) with extracts of nuclei isolated from J774A.1 cells after incubation with either *L. donovani* or different concentrations of LPG for 1 h at 37 °C. With a probe containing IRF-E-binding site for IFN-activated transcription factor, two constitutive complexes were found in untreated cells (designated a and b). A low level of predominant IRF-E consensus probe-binding complex a was present in untreated nuclei (Fig. 1). Following treatment with LPG at 10 μg/ml, a noticeable increase of this constitutive form of IRF was observed by 1 h (Fig. 1). However, no change was observed in complex b after treatment with LPG. To demonstrate that the DNA oligomer–protein complexes formed in the EMSA represent specific molecular interactions, unlabeled probe competition experiments were performed (

Fig. 1). The corresponding unlabeled probe strongly competes for complex formation with complex a. However, unlabeled probe did not compete with complex b. These data indicate that the proteins activated by LPG and detected by EMSA were highly specific for IRF (complex a). Since IRF-1 is an IRF-E-binding protein we decided to check the specificity of these complexes using IRF-1 specific antibody [22, 23 and 24]. The identity of the LPG-induced IRF-like complexes is unclear since the antibody supershift studies showed that they do not contain IRF-1 (data not shown). Similarly, the antibody supershift studies with LPS-induced IRF-like complexes did not contain IRF-1 (data not shown). Earlier studies have ruled out a role of any of the well-characterized factors (IRF-1, IRF-2, ICSBP (IFN consensus sequence-binding protein), or ICSAT) in LPS-stimulated IRF-like complex [24].


(9K)
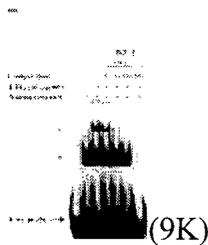
Fig. 1. LPG-mediated nuclear translocation of IRF-like complex. J774A.1 cells were incubated for 1 h in the absence or the presence of LPG (10 μg/ml). Six micrograms of each nuclear extract was incubated with γ^{32}P-end-labeled synthetic double-stranded probe containing the IRF family protein-binding sequence in the iNOS promoter. Lane 1, Free probe; lane 2, untreated J774A.1 cells; lane 3, J774A.1 cells incubated with 10 μg/ml LPG; and lanes 4–7, the IRF-E specific band is competed away by 2-, 5-, 10-, and 20-fold molar excess of an unlabeled synthetic wild-type (wt) IRF-E cold competitor. The position of specific complexes is indicated by a and b on the left side. Data are representative of three experiments.

Activation was dependent on the concentration of *L. donovani* with parasite/macrophage ratio of 10:1 (corresponding to 1×10^7 parasites) resulting in 7.2-fold activation of IRF-E-binding activity whereas higher parasite/macrophage ratio, i.e., 20:1 (corresponding to 2×10^7 parasites) resulted in 21.3-fold induction of IRF-E-binding activity (Figs. 2A and B). IRF-E binding was also activated in cells treated with LPG. LPG (10 μg/ml) resulted in 4.7-fold induction whereas 20 μg/ml of LPG resulted in 18.2-fold induction (Figs. 3A and B) when compared with the untreated macrophages. LPG (10 μg/ml) 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 (11.5-fold) in IRF-E-binding activity over the unstimulated controls (Figs. 4A and B).

(5K)
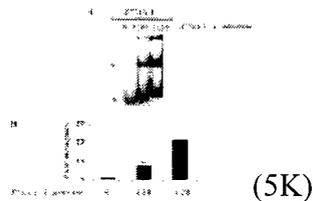
Fig. 2. Induction of IRF–DNA complex by *L. donovani* in macrophage cell line, J774A.1 (A and B). Macrophages (1×10^6 cells/ml) were incubated with indicated concentrations of *L. donovani* at 37 °C for 1 h. Nuclear extracts were prepared as described under Materials and methods and analyzed for the IRF-E-DNA-binding activity by EMSA. Only the relevant part of EMSA with nuclear IRF activity in J774A.1 cells is shown. (A) Dose-dependent activation of nuclear IRF-E-DNA-binding activity with *L. donovani*. (B) PhosphorImager quantitation of resultant DNA–Protein complexes with *L. donovani*.. The data shown are from one of the three independent experiments that yielded similar results. Values in the histogram represent means and standard deviation.

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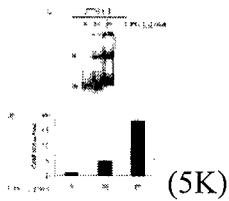
Fig. 3. Induction of IRF–DNA complex with LPG in macrophage cell line, J774A.1 (A and B). J774A.1 cells (1 × 10^6 cells/ml) were stimulated for 1 h at 37 °C with indicated concentrations of LPG. Nuclear extracts were prepared as described under Materials and methods and analyzed for the IRF-E-DNA-binding activity by EMSA. Only the relevant part of EMSA with nuclear IRF activity in J774A.1 cells is shown. (A) Dose-dependent activation of IRF-E-DNA binding activity with LPG. (B) PhosphorImager quantitation of resultant DNA–Protein complex with LPG. The data shown are from one of the three independent experiments that yielded similar results. Values in the histogram represent mean and standard deviation.


(6K)
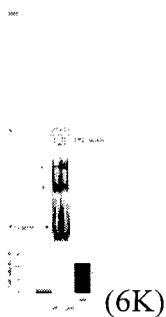
Fig. 4. Induction of IRF–DNA complex with LPS in macrophage cell line, J774A.1 (A and B). J774A.1 cells (1 × 10^6 cells/ml) were stimulated for 1 h at 37 °C with indicated concentration of LPS. Nuclear extracts were prepared as described under Materials and methods and analyzed for the IRF-E-DNA-binding activity by EMSA. (A) Dose-dependent activation of IRF-E-DNA-binding activity with LPS. (B) PhosphorImager quantitation of resultant DNA–Protein complex with LPS. The data shown are from one of the two

independent experiments that yielded similar results. Values in the histograms represent the average.

The kinetics of IRF-E-binding activity 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. A time-dependent increase in IRF-E-binding activity was observed with a gradual increase in DNA-binding activity with peak activity by 90 min and remained steady for up to 180 min (Figs. 5A and B). A similar time-dependent activation of DNA-binding activity of IRF-E was observed with LPG (10 μg/ml). The DNA-binding activity gradually increased, reaching its peak at 120 min (Figs. 6A and B). A significant decline in IRF-E-binding activity was observed at 180 min.


(13K)
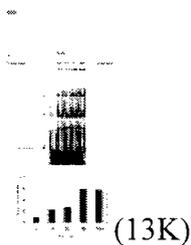
Fig. 5. Kinetics of IRF activation by *L. donovani* in macrophage cell line, J774A.1 (A and B). J774A.1 cells were infected with *L. donovani*. The nuclear extracts prepared from these cells were analyzed by EMSA for the IRF-E-DNA-binding activity. (A) Time-dependent activation of IRF-E DNA-binding activity. (B) Represents resultant DNA–Protein complexes with *L. donovani*, quantitated by PhosphorImager. The data shown are from one of the two independent experiments that yielded similar results. Values in the histogram represent the average.

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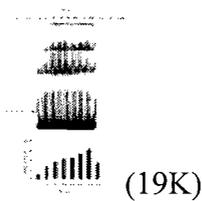
Fig. 6. Kinetics of IRF activation lipophosphoglycan in macrophage cell line, J774A.1. J774A.1 cells were treated with LPG for the indicated time. The nuclear extracts prepared from these cells were analyzed by EMSA for the IRF-E-DNA-binding activity. (A) Time-dependent activation of IRF-E-DNA-binding activity. (B) Represents resultant DNA–Protein complexes with LPG, quantitated by PhosphorImager. The data shown are from one of the two independent experiments that yielded similar results. Values in the histogram represent the average.

Inhibitor of LPS does not abrogate IRF-E-binding activity

Inhibitor of LPS, polymyxin B (PB), does not abrogate IRF-E activation by either *L. donovani* or LPG. Bacterial surface structures like LPS have been demonstrated to activate IRF-1 [26]. PB has been shown to bind to LPS and abrogate its effect. To determine whether the IRF-E-binding activated by *L. donovani* was not due to LPS, we incubated the parasite for 30 min with PB (5 μg/ml) and then used these parasites to infect J774A.1 macrophages. PB (5 μg/ml) by itself did not activate IRF-E binding (data not shown). Preincubation of *L. donovani* with PB had no effect on the IRF-E binding activity induced by *L. donovani* (Fig. 7). In addition, PB had no effect on LPG-induced IRF-E-binding activity. However, preincubation of LPS with PB for 30 min abrogated the ability of LPS to activate IRF-E. These results clearly demonstrate that the effect of *L. donovani* and LPG on IRF-E-binding activity was not due to LPS.

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(15K)
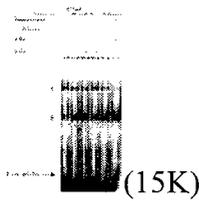
Fig. 7. Treatment of polymyxin B did not inhibit *L. donovani* or LPG-activated IRF–DNA complex. *L. donovani* pretreated with polymyxin B for 30 min was used to infect macrophages. Nuclear extracts from the treated cells were analyzed for IRF-E DNA-binding activity. Polymyxin B-pretreated LPG was also used to stimulate activation of IRF. Polymyxin B-pretreated LPS was used to stimulate activation of IRF in J774A.1 macrophages for comparison. The data shown are from one of the three independent experiments that yielded similar results.

Effect of cytochalasin B, an actin-depolymerizing drug, on L. donovani-induced IRF activation

Effect of cytochalasin B, an actin-depolymerizing drug, on *L. donovani*-induced IRF activation is shown in Fig. 8. Cytochalasin B plays a role in inhibition of invasion and phagocytosis [34 and 35]. We investigated if cellular uptake of *L. donovani* is required for IRF activation. We examined the effect of cytochalasin B on *Leishmania*-induced IRF activation. The data show that 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 binding activity of IRF-E by *L. donovani* (Fig. 8B) when compared to untreated cells (Fig. 8A). The results clearly indicate that the activation of IRF does not require infectivity of *L. donovani* in the cells and its cellular uptake. We further evaluated the role of cytochalasin B (10 μM) in the intracellular parasite uptake.

The concentration of cytochalasin used inhibited *Leishmania* internalization (data not shown) but did not prevent induction of IRF activation.

(19K)
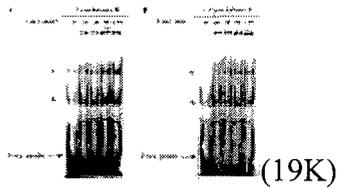
Fig. 8. Cytochalasin B treatment conferred no protection from *L. donovani*-induced IRF-E-DNA activation. J774A.1 cells were incubated at 37 °C with 10 μM cytochalasin B for 15 min before infection with virulent *L. donovani* (10:1 parasite/macrophage ratio). Nuclear extracts from the treated cells were analyzed for IRF-E-DNA-binding activity (A and B). The data shown are from one of the three independent experiments that yielded similar results.

Role of ERK1/2 and p38 MAP kinases in regulation of IRF-E triggered by L. donovani

To further investigate the role of mitogen-activated protein (MAP) kinase intermediates in the signal transduction pathway resulting in the IRF activation, the effects of specific inhibitors on the activation of this DNA-binding factor were analyzed (Fig. 9). Nuclear extracts were prepared from cells treated with *L. donovani* for 60 min and pretreated for 60 min with PD98059 (20 μM), a specific inhibitor of ERK1/2 pathway, or SB203580 (5 μM), a specific inhibitor of the p38 pathway, and were analyzed for IRF–DNA complex by EMSA. Preincubation of cells with SB203580 (5 μM) or PD98059 (20 μM) did not affect the binding activity of IRF-E, suggesting that both p38 and ERK MAP kinase activation are not involved in the IRF activation. At these concentrations the inhibitors did not cause cellular damage.

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(8K)
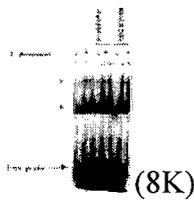
Fig. 9. Effects of PD98059 and SB203580 on IRF-E-binding activity induced by *L. donovani.* J774A.1 cells pretreated with PD98059 (20 μM), SB203580 (5 μM), or medium for 1 h were stimulated with *L. donovani* for further 1 h as described in Materials and methods. The nuclear proteins were extracted. EMSA analysis of these nuclear extracts was conducted using the γ^{32}P-end-labeled synthetic double-stranded IRF-E probe. The data shown are from one of the three independent experiments that yielded similar results.

Discussion

The transcriptional regulation of IFN and IFN-inducible gene expression has been an area of active research over the past several years that has led to the identification of a novel family of DNA-binding proteins referred to as interferon regulatory factors (IRFs) [14]. The IRF family of transcription factors is cellular DNA-binding proteins that act as activators or repressors of promoters containing variations on the IRF binding sequence. There are currently nine members in this family of related proteins, including IRF-1 (ISGF-2), IRF-2, IRF-3, IRF-4 (lymphocyte specific IRF), ICSBP, and ISGF-3γ (p48) [36]. The IRF-1 gene is transcriptionally activated by type I interferon (IFN-α/β), type II interferon (IFN-γ), tumor necrosis factor $\alpha$and other cytokines, and LPS [14]. However, type II IFN (IFN-γ) is more potent than IFN-α as an inducer of IRF-1. Disruption of the IRF-1 gene prevents induction of some IFN-γ-regulated genes, including inducible nitric oxide synthase [23]. It is also possible that IRF-1 might have a role independent of the IFN-γ

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system. Therefore, study of this transcription factor provides opportunities to examine the possible interplay among several physiologically distinct signal transduction pathways. Previous studies have shown that IRF-1 protein is responsible for part of the anti-viral state induced by IFNs [14]. Recent studies have shown that the mycobacterium infection leads to alteration of IRF-1 transcription factor complex [25]. Induction of IRF-1 by *M. tuberculosis* infection of monocytes and macrophages has been reported to be a possible host-defense mechanism, since the pleiotropic functions of IRF-1 in the immune system, such as anti-viral and anti-bacterial phenotypes or involvement in production of NK cells and development of Th1 cell-mediated responses all contribute to host defense against infectious diseases [25 and 37]. It has also been reported earlier that LPS induces IRF-1, IRF-2, and ICSBP genes thus adding these IRFs to the list of LPS-inducible transcription factors that currently induce *c-fos*, *c-jun*, *c-myc*, and NF-κB [26]. LPS has been reported to stimulate two nuclear complexes to the IRF-site in the iNOS promoter in macrophages [22 and 24]. The components of this novel LPS-stimulated IRF-1like complex have not yet been identified. The role of IRF-1, IRF-2, ICSBP (IFN consensus sequence-binding protein) or ICSAT does not appear to be involved [22 and 24]. In the present study, LPS was used as a positive control and was found to stimulate the IRF-site in the iNOS promoter.

The results presented in this paper show that infection of J774A.1 macrophage with *L. donovani*-induced IRF-binding activity; the response was dependent on the concentration of *L. donovani*. A dose-dependent increase in IRF-E-binding activity was seen with *L. donovani*. IRF-E-binding activity was also induced in cells treated with *Leishmania* surface molecule LPG. A time-dependent increase in IRF-E-binding activity was observed with lipophosphoglycan. We have not yet identified the components of this novel LPG-

stimulated IRF-like complex, we have ruled out a role for well-characterized factor IRF-1. It has been reported earlier that the LPS-induced two nuclear complexes to the IRF-site in the iNOS promoter in the macrophages are unique and different from the reported IRF-complexes [22 and 24]. Further studies are required to identify the components of this LPG and *L. donovani*-induced IRF-like complexes.

Host cell invasion has been found to be a prerequisite for the activation of some transcription factors in several intracellular pathogens [34 and 35]. The possibility that the induction of IRF-transcription factors was contact-dependent or required internalization of the parasites was also determined. The macrophages were pretreated with cytochalasin B, an inhibitor of phagocytosis. The results clearly indicated that the activation of IRF-E binding does not require infectivity of *L. donovani* in the cells and its cellular uptake.

We have also shown that both ERK1/2 and p38 MAP kinases are not involved in the induction of IRF. This is the first report showing induction of IRF-binding activity in macrophages due to *L. donovani* infection. This is likely to be a defensive host response to *L. donovani* infection. Role of IRF-1-transcription factor in the Th1/Th2 differentiation decision and the bias to Th2 in *L. major* infected IRF-1$^{-/-}$ mice has been shown to have important functional consequences for the outcome of the parasite infections which normally is healed with the help of Th1 cells [38 and 39]. An understanding of the role of IRF in this signaling cascade, interacting partners and importantly, their cross-talk will pave the way for the design of new preventive and therapeutic modalities.

Acknowledgements

References

1. J. Chan, T. Fujiwara, P. Brennan, M. McNeil, S.J. Turco, J.C. Sibille, M. Snapper, P. Aisen and B.R. Bloom, Microbial glycolipids: possible virulence factors that scavenge oxygen radicals. *Proc. Natl. Acad. Sci. USA* **86** (1989), pp. 2453–2457. Abstract-MEDLINE | Abstract-EMBASE | $Order Document

2. A. Descoteaux, S.J. Turco, D.L. Sacks and G. Matlashewski, *Leishmania donovani* lipophosphoglycan selectively inhibits signal transduction in macrophages. *J. Immunol.* **146** (1991), pp. 2747–2753. Abstract-MEDLINE | Abstract-EMBASE | $Order Document

3. M. Desjardins and A. Descoteaux, Inhibition of phagolysosomal biogenesis by the *Leishmania* lipophosphoglycan. *J. Exp. Med.* **185** (1997), pp. 2061–2068. Abstract-MEDLINE | Abstract-EMBASE | $Order Document | **Full Text** via CrossRef

4. T.B. McNeely and S.J. Turco, Requirement of lipophosphoglycan for intracellular survival of *Leishmania donovani* within human monocytes. *J. Immunol.* **144** (1990), pp. 2745–2750. Abstract-MEDLINE | Abstract-EMBASE | $Order Document

5. D. Piedrafita, L. Proudfoot, A.V. Nikolaev, D. Xu, W. Sands, G.J. Feng, E. Thomas, J. Brewer, M.A. Ferguson, J. Alexander and F.Y. Liew, Regulation of macrophage IL-12 synthesis by *Leishmania* phosphoglycans. *Eur. J. Immunol.* **29** (1999), pp. 235–244. Abstract-MEDLINE | Abstract-EMBASE | $Order Document | **Full Text** via CrossRef

6. L. Proudfoot, A.V. Nikolaev, G.J. Feng, W.Q. Wei, M.A. Ferguson, J.S. Brimacombe and F.Y. Liew, Regulation of the expression of nitric oxide synthase and leishmanicidal activity by glycoconjugates of *Leishmania* lipophosphoglycan in murine macrophages.

Proc. Natl. Acad. Sci. USA **93** (1996), pp. 10984–10989. Abstract-Elsevier BIOBASE | Abstract-MEDLINE | $Order Document | **Full Text** via CrossRef

7. S.M. Puentes, D.L. Sacks, R.P. da Silva and K.A. Joiner, Complement binding by two developmental stages of *Leishmania major* promastigotes varying in expression of a surface lipophosphoglycan. *J. Exp. Med.* **167** (1988), pp. 887–902. Abstract-MEDLINE | Abstract-EMBASE | $Order Document

8. C. Prive and A. Descoteaux, *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 naïve macrophages. *Eur. J. Immunol.* **30** (2000), pp. 2235–2244. Abstract-Elsevier BIOBASE | Abstract-MEDLINE | Abstract-EMBASE | $Order Document | **Full Text** via CrossRef

9. T. Scharton-Kersten, C. Contursi, A. Masumi, A. Sher and K. Ozato, Interferon consensus sequence binding protein-deficient mice display impaired resistance to intracellular infection due to primary defect in interleukin 12 p40 induction. *J. Exp. Med.* **186** (1997), pp. 1523–1534. Abstract-MEDLINE | Abstract-EMBASE | $Order Document | **Full Text** via CrossRef

10. S. Taki, T. Sato, K. Ogasawara, T. Fukuda, M. Sato, S. Hida, G. Suzuki, M. Mitsuyama, E.H. Shin, S. Kojima, T. Taniguchi and Y. Asano, Multistage regulation of Th1-type immune responses by the transcription factor IRF-1. *Immunity* **6** (1997), pp. 673–679. Abstract | **Full Text + Links** | PDF (125 K)

11. T.L. Murphy, M.G. Cleveland, P. Kulesza, J. Magram and K.M. Murphy, Regulation of interleukin 12 p40 expression through an NF-kappa B half-site. *Mol. Cell. Biol.* **15** (1995), pp. 5258–5267. Abstract-MEDLINE | Abstract-Elsevier BIOBASE | Abstract-EMBASE | $Order Document

12. J.M. Griscavage, S. Wilk and L.J. Ignarro, Inhibitors of the proteasome pathway interfere with induction of nitric oxide synthase in macrophages by blocking activation of transcription factor NF-kappa B. *Proc. Natl. Acad. Sci. USA* **93** (1996), pp. 3308–3312. Abstract-Elsevier BIOBASE | Abstract-MEDLINE | Abstract-EMBASE | $Order Document | **Full Text via CrossRef**

13. T. Fujita, J. Sakakibara, Y. Sudo, M. Miyamoto, Y. Kimura and T. Taniguchi, Evidence for a nuclear factor(s), IRF-1, mediating induction and silencing properties to human IFN-beta gene regulatory elements. *EMBO J.* **7** (1998), pp. 3397–3405.

14. N. Miyamoto, T. Fujita, Y. Kimura, M. Maruyama, H. Harada, Y. Sudo, T. Miyata and T. Taniguchi, Regulated expression of a gene encoding a nuclear factor, IRF-1 that specifically binds to IFN-beta gene regulatory elements. *Cell* **54** (1988), pp. 903–913.

15. R. Lin, A. Mustafa, H. Nguyen, D. Gewert and J. Hiscott, Mutational analysis of interferon (IFN) regulatory factors 1 and 2. Effects on the induction of IFN-beta gene expression. *J. Biol. Chem.* **269** (1994), pp. 17542–17549. Abstract-MEDLINE | Abstract-Elsevier BIOBASE | Abstract-EMBASE | $Order Document

16. T. Fujita, L.F. Reis, N. Watanabe, Y. Kimura, T. Taniguchi and J. Vilcek, Induction of the transcription factor IRF-1 and interferon-beta mRNAs by cytokines and activators of second-messenger pathways. *Proc. Natl. Acad. Sci. USA* **86** (1989), pp. 9936–9940. Abstract-MEDLINE | Abstract-EMBASE | $Order Document

17. L.Y. Yu Lee, J.A. Hrachovy, A.M. Stevens and L.A. Schwarz, Interferon-regulatory factor 1 is an immediate-early gene under transcriptional regulation by prolactin in Nb2 T cells. *Mol. Cell. Biol.* **10** (1990), pp. 3087–3094. Abstract-MEDLINE | Abstract-EMBASE | $Order Document

18. N. Tanaka, T. Kawakami and T. Taniguchi, Recognition DNA sequences of interferon regulatory factor 1 (IRF-1) and IRF-2, regulators of cell growth and the interferon system. *Mol. Cell. Biol.* **13** (1993), pp. 4531–4538. Abstract-MEDLINE | Abstract-EMBASE | $Order Document

19. S. Ohno and T. Taniguchi, The 5'-flanking sequence of human interferon-beta 1 gene is responsible for viral induction of transcription. *Nucleic Acids Res.* **11** (1983), pp. 5403–5412. Abstract-MEDLINE | $Order Document

20. D.R. Johnson and J.S. Pober, HLA class I heavy-chain gene promoter elements mediating synergy between tumor necrosis factor and interferons. *Mol. Cell. Biol.* **14** (1994), pp. 1322–1332. Abstract-MEDLINE | Abstract-Elsevier BIOBASE | Abstract-EMBASE | $Order Document

21. P. Benech, M. Vigneron, D. Peretz, M. Revel and J. Chebath, Interferon-responsive regulatory elements in the promoter of the human 25 oligo (A) synthetase gene. *Mol. Cell. Biol.* **7** (1987), pp. 4498–4504. Abstract-MEDLINE | $Order Document

22. E. Martin, C. Nathan and Q.W. Xie, Role of interferon regulatory factor 1 in induction of nitric oxide synthase. *J. Exp. Med.* **180** (1994), pp. 977–984. Abstract-MEDLINE | Abstract-Elsevier BIOBASE | Abstract-EMBASE | $Order Document

23. R. Kamijo, H. Harada, T. Matsuyama, M. Bosland, J. Gerecitano, D. Shapiro, J. Le, S.J. Koh, T.W. Mak, T. Taniguchi and J. Vilcek, Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. *Science* **263** (1994), pp. 1612–1615. Abstract-MEDLINE | Abstract-EMBASE | Abstract-Elsevier BIOBASE | $Order Document

24. G.J. Feng, H.S. Goodridge, M.M. Harnett, X.Q. Wei, A.V. Nikolaev, A.P. Higson and F.Y. Liew, 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 macrophage: *Leishmania* phosphoglycans subvert macrophages IL-12 production by targeting ERK MAP kinase. *J. Immunol.* **163** (1999), pp. 6403–6412. Abstract-MEDLINE | Abstract-EMBASE | Abstract-Elsevier BIOBASE | $Order Document

25. Y. Qiao, S. Prabhakar, E.M. Coccia, M. Weiden, A. Canova, E. Giacomini and R. Pine, Host defense responses to infection by *Mycobacterium tuberculosis*. Induction of IRF-1 and a serine protease inhibitor. *J. Biol. Chem.* **277** (2002), pp. 22377–22385. Abstract-MEDLINE | Abstract-Elsevier BIOBASE | Abstract-EMBASE | $Order Document | **Full Text** via CrossRef

26. S.A. Barber, M.J. Fultz, C.A. Salkowski and S.N. Vogel, Differential expression of interferon regulatory factor 1 (IRF-1), IRF-2 and interferon consensus sequence binding protein genes in lipopolysaccharide (LPS)-responsive and LPS-hyporesponsive macrophages. *Infect. Immun.* **63** (1995), pp. 601–608. Abstract-MEDLINE | Abstract-Elsevier BIOBASE | Abstract-EMBASE | $Order Document

27. D.T. Hart, K. Vickerman and G.H. Coombs, A quick, simple method for purifying *Leishmania mexicana* amastigotes in large numbers. *Parasitology* **82** (1981), pp. 345–355. Abstract-EMBASE | Abstract-MEDLINE | $Order Document

28. P.A. Orlandi, Jr. and S.J. Turco, Structure of the lipid moiety of the *Leishmania donovani* lipophosphoglycan. *J. Biol. Chem.* **262** (1987), pp. 10384–10391. Abstract-MEDLINE | $Order Document

29. D.M. Russo, S.J. Turco, J.M. Burns, Jr. and S.G. Reed, Stimulation of human T lymphocytes by *Leishmania* lipophosphoglycan-associated proteins. *J. Immunol.* **148** (1992), pp. 202–207. Abstract-MEDLINE | Abstract-EMBASE | $Order Document

30. P. Rao and T.N. Pattabiraman, Reevaluation of the phenol-sulfuric acid reaction for the estimation of hexoses and pentoses. *Anal. Biochem.* **181** (1989), pp. 18–22. Abstract-MEDLINE | Abstract-EMBASE | $Order Document

31. E. Schreiber, P. Martthias, M.M. Muller and W. Schaffner, Rapid detection of octamer binding proteins with 'mini-extracts,' prepared from a small number of cells. *Nucleic Acids Res.* **17** (1989), p. 6419. Abstract-MEDLINE | Abstract-EMBASE | $Order Document

32. M.M. Chaturvedi, R. La Pushin and B.B. Aggarwal, Tumor necrosis factor and lymphotoxin. Qualitative and quantitative differences in the mediation of early and late cellular response. *J. Biol. Chem.* **269** (1994), pp. 14575–14583. Abstract-MEDLINE | Abstract-Elsevier BIOBASE | Abstract-EMBASE | $Order Document

33. M.M. Bradford, A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72** (1976), pp. 248–254. Abstract-EMBASE | Abstract-MEDLINE | $Order Document

34. H.U. Grassme, R.M. Ireland and J.P. van Putten, Gonococcal opacity protein promotes bacterial entry-associated rearrangements of the epithelial cell actin cytoskeleton. *Infect. Immun.* **64** (1996), pp. 1621–1630. Abstract-MEDLINE | Abstract-Elsevier BIOBASE | Abstract-EMBASE | $Order Document

35. M. Naumann, S. Weßler, C. Bartsch, B. Weiland and T.F. Meyer, *Neisseria gonorrhoeae* epithelial cell interaction leads to the activation of the transcription factors nuclear factor κB and activator protein 1 and the induction of inflammatory cytokines. *J. Exp. Med.* **186** (1997), pp. 247–258. Abstract-EMBASE | Abstract-MEDLINE | $Order Document | **Full Text** via CrossRef

36. H. Nguyen, J. Hiscott and P. Pitha, The growing family of interferon regulatory factors. *Cytokine Growth Factor Rev.* **8** (1997), pp. 293–312. SummaryPlus | **Full Text + Links** | PDF (2055 K)

37. R. Pine, IRF and tuberculosis. *J. Interferon Cytokine Res.* **22** (2002), pp. 15–25. Abstract-MEDLINE | Abstract-EMBASE | Abstract-Elsevier BIOBASE | $Order Document | **Full Text** via CrossRef

38. M. Lohoff, D. Ferrick, H.W. Mittrucker, G.S. Duncan, S. Bischof, M. Rollonghoft and T.W. Mak, Interferon regulatory factor-1 is required for T helper 1 immune response in vivo. *Immunity* **6** (1997), pp. 681–689. Abstract | **Full Text + Links** | PDF (129 K)

39. C. Feng, S. Watanabe, S. Maruyama, G. Suzuki, M. Sato, T. Furuta, S. Kojima, S. Taki and Y. Asano, An alternate pathway for type 1 T cell differentiation. *Int. Immunol.* **11** (1999), pp. 1185–1194. Abstract-MEDLINE | Abstract-EMBASE | Abstract-Elsevier BIOBASE | $Order Document | **Full Text** via CrossRef