

CHAPTER VII

Leishmania donovani Induces Interferon
Regulatory Factor (IRF) In Murine
Macrophages: A Host Defense Response

1.0 Introduction

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 (Chan, et al., 1989; Descoteaux, 1991; Desjardins et al., 1997; McNeely and Turco, 1990; Piedrafita et al., 1999; Proudfoot et al., 1996; Puentes et al., 1988). *Leishmania donovani* promastigotes have been reported to evade the activation of mitogen-activated protein kinases during infection of naïve macrophages (Prive and Descoteaux, 2000).

Down stream cascade of these kinases are the ubiquitous transcription factors such as activating protein 1 (AP-1), NF- κ B and IFN regulatory factor (IRF). IFN regulatory factor (IRF) and NF- κ B activation has been shown to play a key role in the induction of macrophage effector molecules (Scharton-Kersten et al., 1997; Taki et al., 1997; Murphy et

al., 1995; Griscavage, Wilk and Ignarro, 1996) and are involved in 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 (Fujita et al., 1998). Molecular cloning revealed it to be a DNA-binding protein with a basic NH₂-terminal domain linked to an acidic COOH-terminal region, which is highly conserved between mice and humans (Miyamoto et al., 1988). The NH₂ terminus is responsible for DNA binding, whereas sequences within the COOH-terminal domain mediate transactivation (Lin et al., 1994). 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 (Miyamoto et al., 1988; Fujita et al., 1989). In addition, IRF-1 production is induced by other cytokines, such as IL-1, IL-6 and TNF- α (Fujita et al., 1989), viral infection (Miyamoto et al., 1988), and prolactin (Yu Lee et al., 1990). 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) (Tanaka, Kawakami and Taniguchi, 1993). This motif has been found in the upstream promoter elements of a number of IFN-inducible genes such as IFN- β (Ohno and Taniguchi, 1983), major histocompatibility complex class 1 (Johnson and Pober, 1994), and 2-5-oligo (A) synthetase (Benech et al., 1987). A number of studies have shown the importance of IRF-1 in mediating transcriptional up-regulation of these and other IFN-induced genes (Tanaka, Kawakami and Taniguchi, 1993). Earlier studies have shown that the gene for inducible nitric-oxide synthase (iNOS) is regulated by IRF-1 (Martin, Nathan and Xie, 1994; Kamijo et al., 1994). 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) (Martin, Nathan and Xie, 1994; Kamijo et al., 1994; Feng et al., 1999). IRF-1 is an IRF-E binding protein (Martin, Nathan and Xie, 1994; Kamijo et al., 1994; Feng et al., 1999).

It has been reported earlier that induction of IRF-1 is functionally related to the host defense response in *Mycobacterium tuberculosis* infection (Qiao et al., 2002). The role of LPS in inducing IRF-1 in macrophages has been demonstrated earlier (Barber et al., 1995). 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.

2.0 Materials and methods

2.1 Chemicals and antibodies

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 Co. (St. Louis, MO). [γ -³²P] ATP was purchased from Amersham Pharmacia Biotech Asia Pacific (Hongkong). Polynucleotide kinase was purchased from Promega (Madison, USA). PD98059 and SB203580 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Polymixin B, cytochalasin B, LPS (lipopolysaccharide) from *Escherichia coli* (serotype 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO). IRF-1 (M-20) antibody used in supershift assay was procured from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). All other reagents used were obtained from Sigma Chemical Co. (St. Louis, MO) unless indicated otherwise.

2.2 Parasite Culture

Leishmania donovani AG83 (MHOM/TN/1983/AG83) promastigotes were cultured at 24°C in modified M199 medium (Sigma, St. Louis, MO) supplemented with 100 units/ml penicillin (Sigma, St. Louis, MO), 100 µg/ml streptomycin (Sigma, St. Louis, MO), and 10% heat inactivated fetal calf serum (FCS) (Gibco-BRL Ltd., USA). 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 M-199 medium containing 30% fetal calf serum (Hart et al., 1981). Freshly transformed promastigotes were maintained at 22°C in M199 with 10% fetal calf serum.

2.3 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 CO₂ incubator (5% CO₂). The macrophages were seeded in tissue culture plates (90 mm) at a density of 1x10⁶ 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*.

2.4 Isolation and quantitation of lipophosphoglycan (LPG)

Purification, isolation and quantitation of LPG from *L. donovani* promastigotes, at the late log phase of growth, were carried out as previously described (Orlandi Jr and Turco, 1987). 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 (Rao and Pattabiraman, 1989).

2.5 Electrophoretic mobility shift assay (EMSA)

For the preparation of nuclear extracts (Schreiber et al., 1989; Chaturvedi, La Pushin, and Aggarwal, 1994), the treated cells were washed twice with ice-cold phosphate-buffered saline (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 MgCl₂, 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, 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 MgCl₂, 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 13,000 × 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 (Bradford, 1976). Binding reactions were initiated by incubating nuclear extract (4 to 6 µg protein) with double stranded poly (dI-dC) (1µg/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 MgCl₂, 1.0 mM DTT) and 1.0 ng/µl of 5' end ³²P-labeled dsDNA oligonucleotide in a total volume of 60 µl. Double stranded DNA (dsDNA, 10 ng) was labeled with [γ -³²P] 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. 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.

3.0 Results

3.1 *Leishmania donovani* activates IRF-transcription factor in J774A.1 murine macrophages

Infection by *Leishmania donovani* results in dose-and time-dependent activation of IRF-E DNA-binding activity in macrophages. In order to find out if the host immune response is dependent on the activation of nuclear transcription factor IRF, the effect of both *Leishmania donovani* and *Leishmania* surface molecule, lipophosphoglycan (LPG) on IRF-E DNA binding in J774A.1 macrophage cell line was evaluated. 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 were performed. 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 it was decided to check the specificity of these complexes using IRF-1 specific antibody (Martin, Nathan and Xie, 1994; Kamijo et al., 1994; Feng et al., 1999). The identity of the *L. donovani*, LPG-induced IRF-like complexes is unclear since the antibody supershift studies showed that they do not contain IRF-1 (Fig. 2A and B). Similarly, the antibody supershift studies with LPS-induced IRF-like complexes did not contain IRF-1 (Fig. 2C). 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 (Feng et al., 1999).

Activation was dependent on the concentration of *Leishmania 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 (Fig. 3A and B). IRF-E binding was also activated in cells treated with lipophosphoglycan (LPG). LPG (10 $\mu\text{g/ml}$) resulted in 4.7 fold induction whereas 20 $\mu\text{g/ml}$ of LPG resulted in 18.2-fold induction (Fig. 4A and B) when compared with the untreated macrophages. LPG (10 $\mu\text{g/ml}$) 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 (Fig. 5A and B).

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 (Fig. 6A 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, reached its peak at 120 min (Fig. 7A and B). A significant decline in IRF-E-binding activity was observed at 180 min.

3.2 Inhibitor of LPS does not abrogate IRF-E activation

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 (Barber et al., 1995). Polymyxin B (PB) has been shown to bind to LPS and abrogate its effect. In order 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. 8). 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.

3.3 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. 9. Cytochalasin B plays a role in inhibition of invasion and phagocytosis (Grassme et al., 1996; Naumann et al., 1997). 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. 9B) when compared to untreated cells (Fig. 9A). 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 μ M) on the intracellular parasite uptake. The concentration of cytochalasin used inhibited *Leishmania* internalization but did not prevent induction of IRF activation.

3.4 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 kinase (MAPK) 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. 10). Nuclear extracts were prepared from cells treated with *L. donovani* for 60 min and pretreated for 60 min with PD98059 (20 μ M), a specific inhibitor of ERK1/2 pathway or SB203580 (5 μ M), a specific inhibitor of the p38 pathway and were analyzed for IRF-DNA complex by EMSA. Pre-incubation of cells with SB203580 (5 μ M) or PD98059 (20 μ M) 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.

4.0 Discussion

The transcriptional regulation of IFN and IFN-inducible gene expression has been an area of active research over the past several years that have led to the identification of a novel family of DNA-binding proteins referred to as Interferon Regulatory Factors (IRFs) (Miyamoto et al., 1988). 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-3 γ (p48) (Nguyen, Hiscott and Pitha, 1997). 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 (Miyamoto et al., 1988). 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 (Kamijo et al., 1994). It is also possible that IRF-1 might have a role independent of the IFN-gamma 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 (Miyamoto et al., 1988). Recent studies have shown that the mycobacterium infection leads to alteration of IRF-1 transcription factor complex (Qiao et al., 2002). 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 antiviral 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 (Qiao et al., 2002; Pine, 2002). 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- κ B (Barber et al., 1995). LPS has been reported to stimulate two nuclear complexes to the IRF-site in the iNOS promoter in macrophages (Martin, Nathan and Xie, 1994; Feng et al., 1999). The components of this novel LPS-stimulated IRF-1 like complex have not yet been identified. The role of IRF-1, IRF-2, ICSBP (IFN consensus sequence-binding protein) or ICSAT do not appear to be involved (Martin, Nathan and Xie, 1994; Feng et al., 1999). 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 study 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 IRF-E-binding activity was seen with *L. donovani*. IRF-E-binding activity was also induced in cells treated with *Leishmania* surface molecule lipophosphoglycan (LPG). A time dependent increase in IRF-E-binding activity was observed with lipophosphoglycan. Identification of the components of this novel LPG-stimulated IRF-like complex is not yet clear. Role for well-characterized factor IRF-1 has been ruled out. 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 (Martin, Nathan and Xie, 1994; Feng et al., 1999). 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 (Grassme et al., 1996; Naumann et al., 1997). 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.

It has also been reported 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 (Lohoff et al., 1997; Feng et al., 1999). 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.

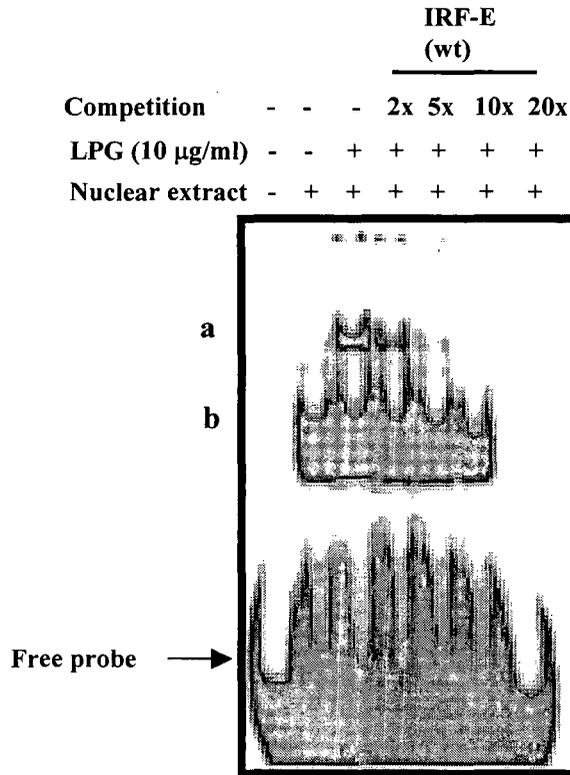


Fig. 1: LPG-mediated nuclear translocation of IRF-like complex. J774A.1 cells were incubated for 1 h in the absence or the presence of LPG (10 µg/ml). Six micrograms of each nuclear extract was incubated with $\gamma^{32}\text{P}$ -end-labeled synthetic double-stranded probe containing the IRF family 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 of LPG; 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 is representative of three experiments.

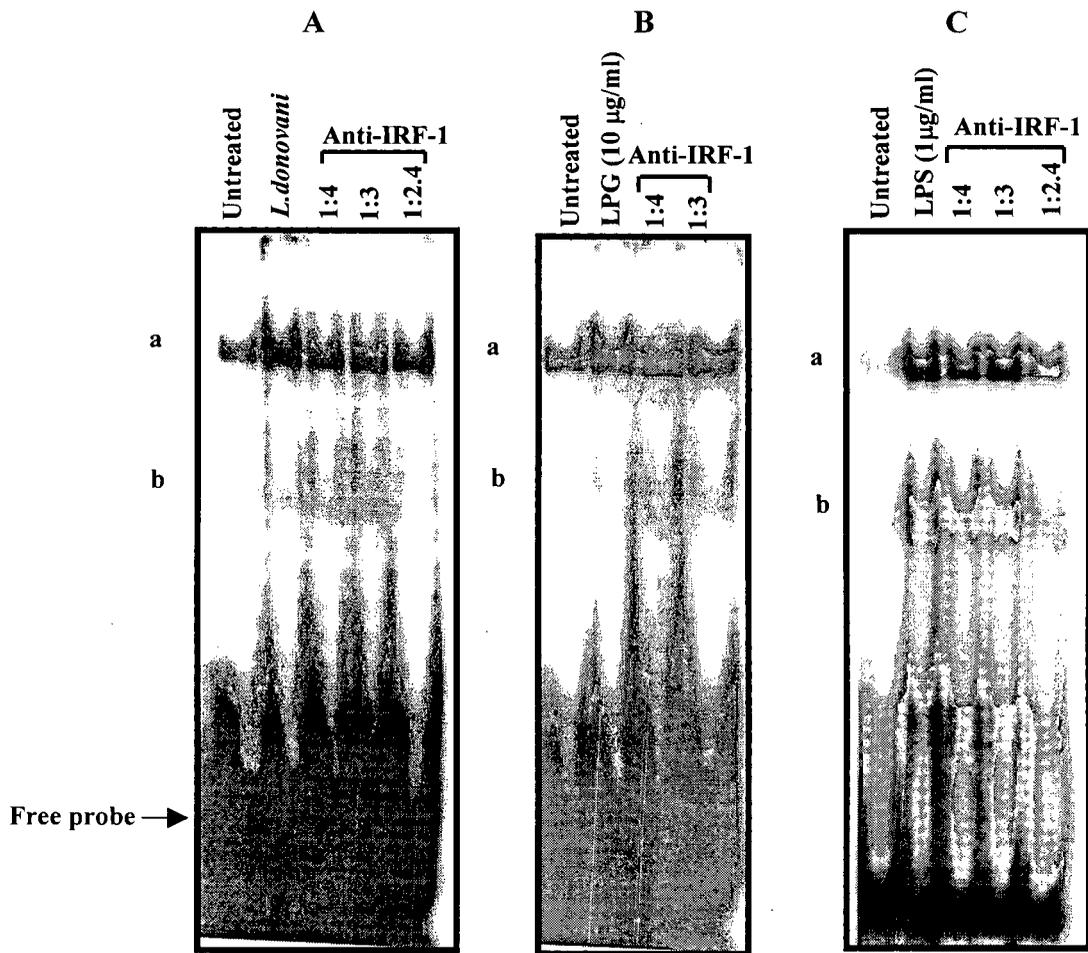


Fig. 2A, B and C: Supershift assay using specific polyclonal antibody for IRF-1. The nuclear extracts from *L. donovani* (A), LPG (B) and LPS (C) treated J774A.1 cells were incubated with specific polyclonal antibody for IRF-1 before addition of radiolabelled IRF-E oligonucleotide. However, the result confirms that the identity of the *L. donovani*, LPG and LPS-induced IRF-like complexes is unclear, since the antibody supershift studies showed that they do not contain IRF-1. Antibody were diluted from the stock concentration of 200 µg/1000 µl used in the experiment in a ratio having a concentration corresponding to 1:4 (3 µg/60 µl), 1:3 (4 µg/60 µl) and 1:2.4 (5 µg/60 µl) respectively.

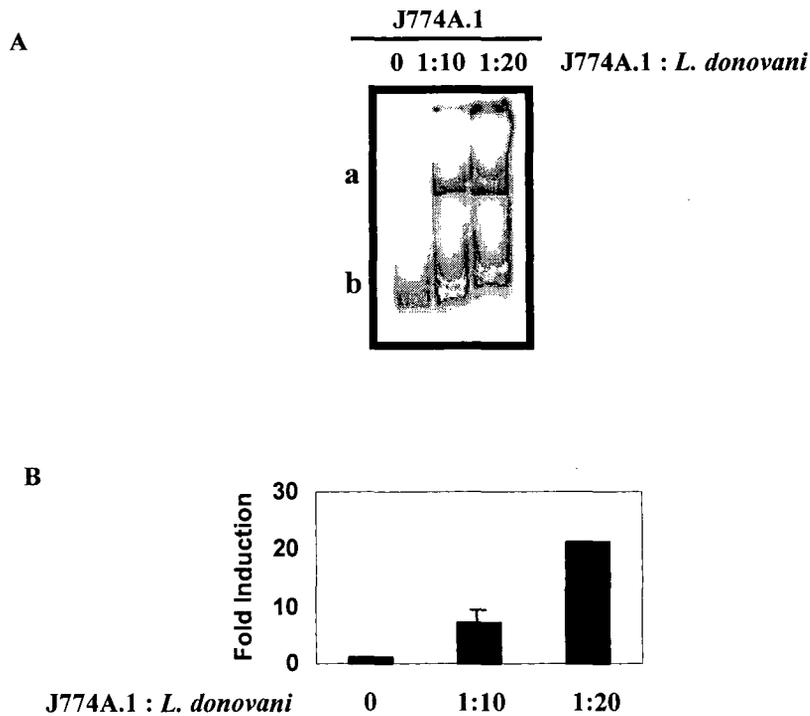


Fig. 3A and B: Induction of IRF-DNA complex by *L. donovani* in macrophage cell line, J774A.1 (A and B). Macrophages (1×10^6 cells/ml) were incubated with indicated concentrations of *L. donovani* at 37°C for 1 h. Nuclear extracts were prepared as described under Materials and Methods and analyzed for 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: Phosphor-Imager 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 mean and standard deviation.

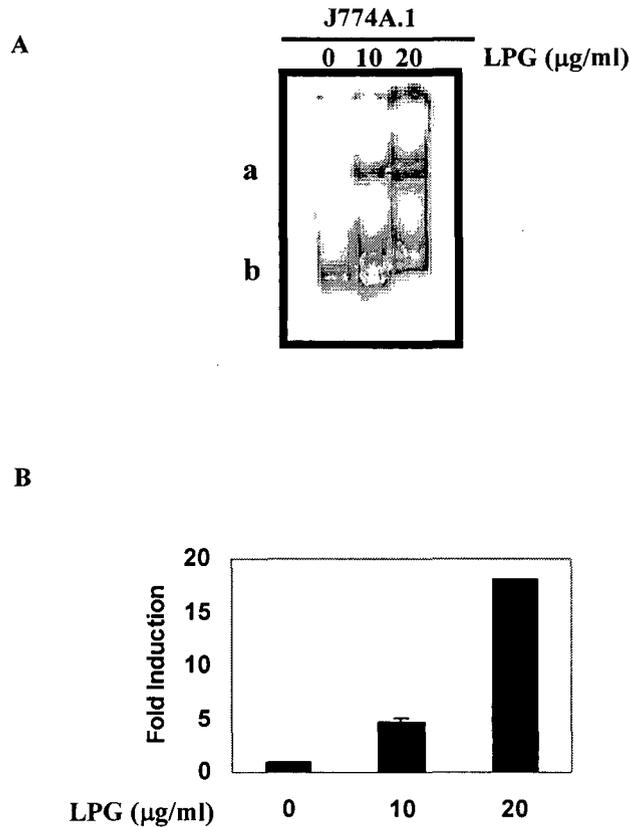


Fig. 4A and B: Induction of IRF-DNA complex with LPG in macrophage cell line, J774A.1 (A and B). J774A.1 cells (1×10^6 cells/ml) were stimulated for 1 h at 37°C with indicated concentrations of LPG. Nuclear extracts were prepared as described under Materials and Methods and analyzed for the IRF-E-DNA-binding 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 is from one of the three independent experiments that yielded similar results. Values in the histogram represent mean and standard deviation.

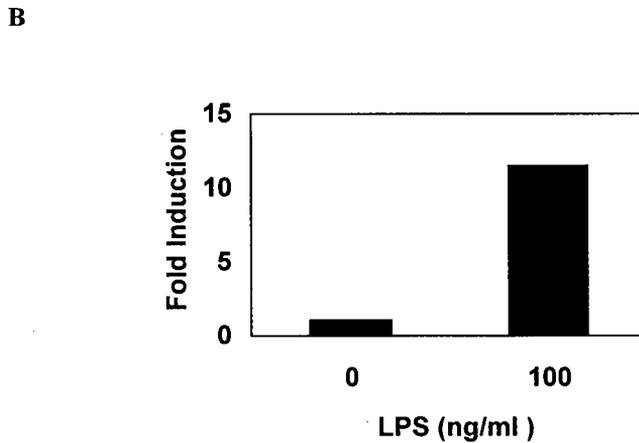
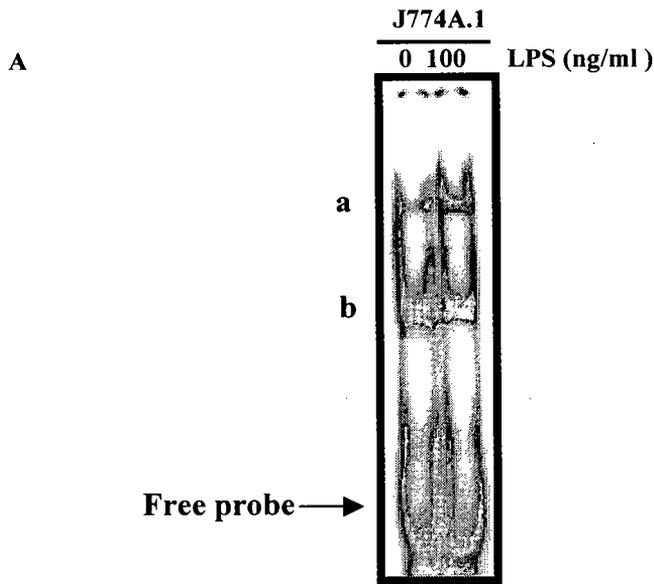
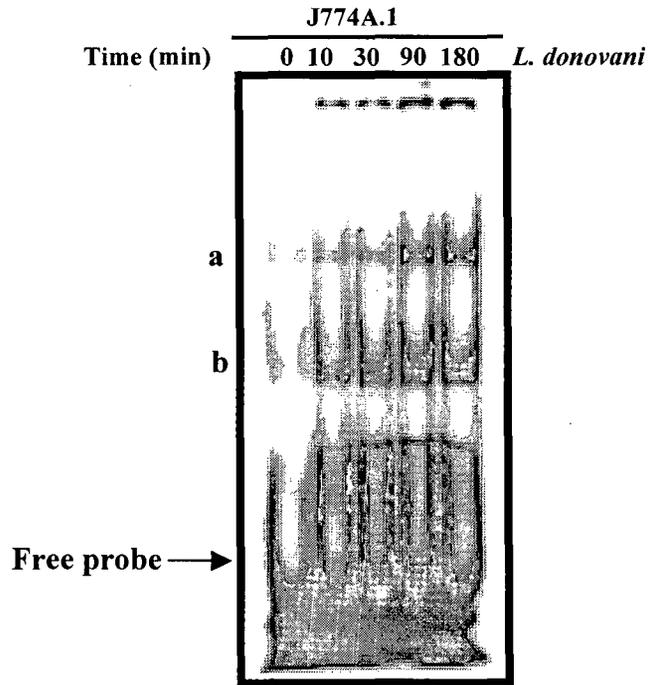


Fig. 5A and B: Induction of IRF-DNA complex with LPS in macrophage cell line, J774A.1 (A and B). J774A.1 cells (1×10^6 cells/ml) were stimulated for 1 h at 37°C with indicated concentration of LPS. Nuclear extracts were prepared as described under Materials and Methods and analyzed for the IRF-E-DNA-binding 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 is from one of the two independent experiments that yielded similar results. Values in the histograms represent the average.

A



B

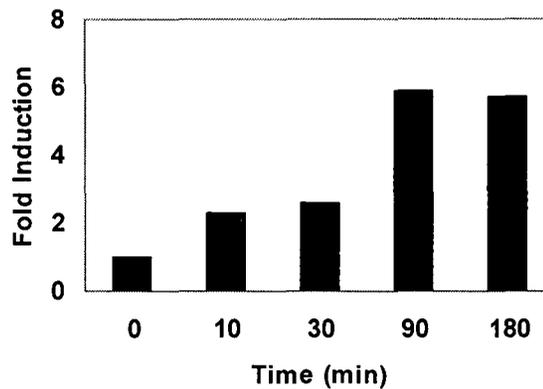
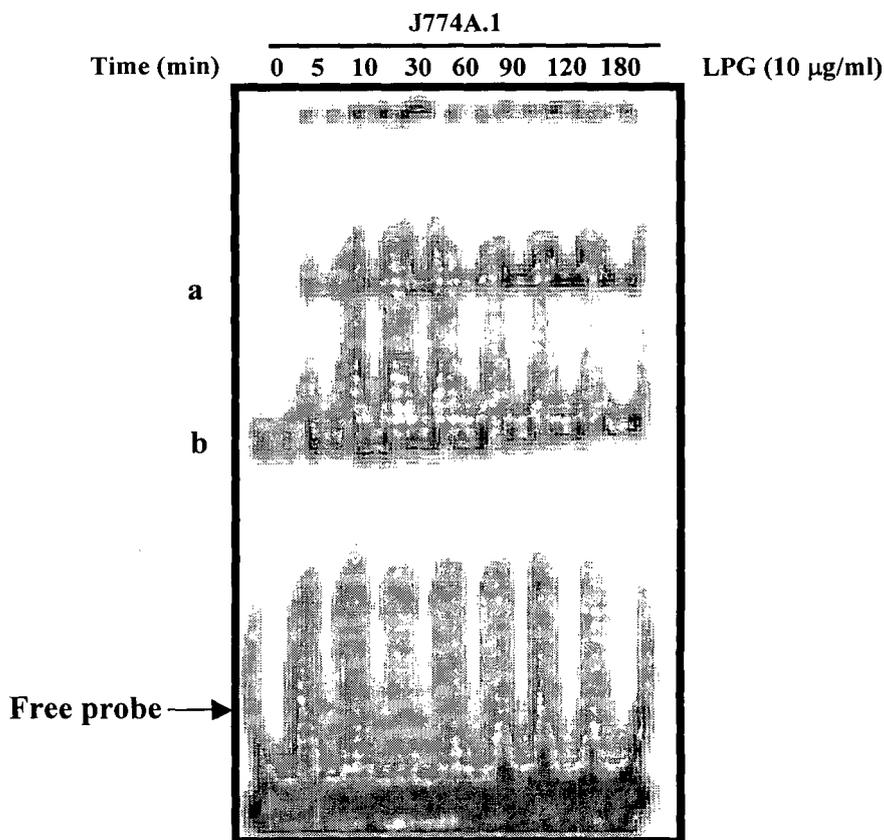


Fig. 6A and B: Kinetics of IRF activation by *L. donovani* in macrophage cell line, J774A.1 (A and B). J774A.1 cells were infected with *L. donovani*. The nuclear extracts prepared from these cells were analyzed by EMSA for the IRF-E-DNA-binding activity. A: time dependent activation of IRF-E DNA binding 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.

A



B

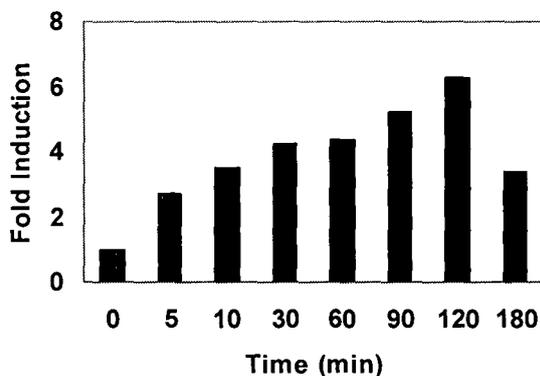


Fig. 7A and B: Kinetics of IRF activation lipophosphoglycan in macrophage cell line, J774A.1. J774A.1 cells were treated with LPG for the indicated time. The nuclear extracts prepared from these cells were analyzed by EMSA for the IRF-E-DNA-binding activity. A: Time-dependent activation of IRF-E-DNA 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.

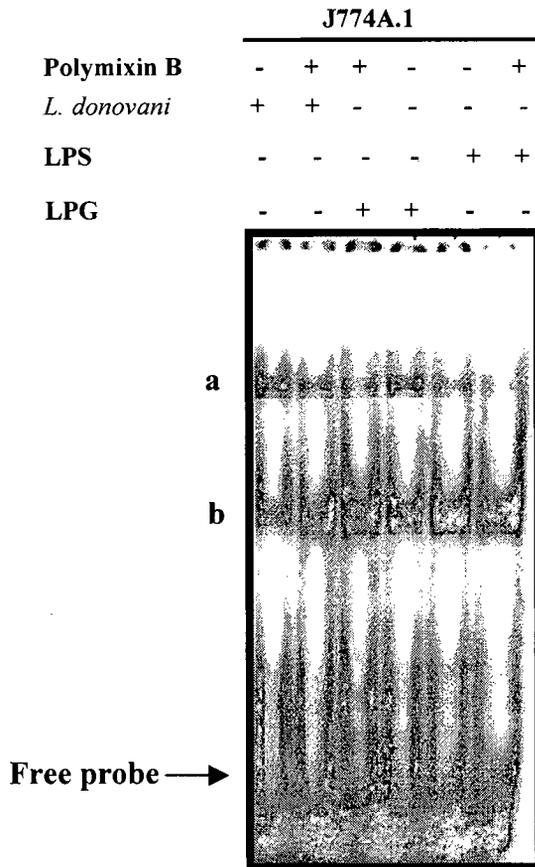


Fig. 8: Treatment of polymyxin B did not inhibit *L. donovani* or LPG-activated IRF-DNA complex. *L. donovani* pretreated with polymyxin B for 30 min was used to infect macrophages. Nuclear extracts from the treated cells were analyzed for IRF-E DNA-binding activity. Polymyxin B-pretreated LPG was also 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.

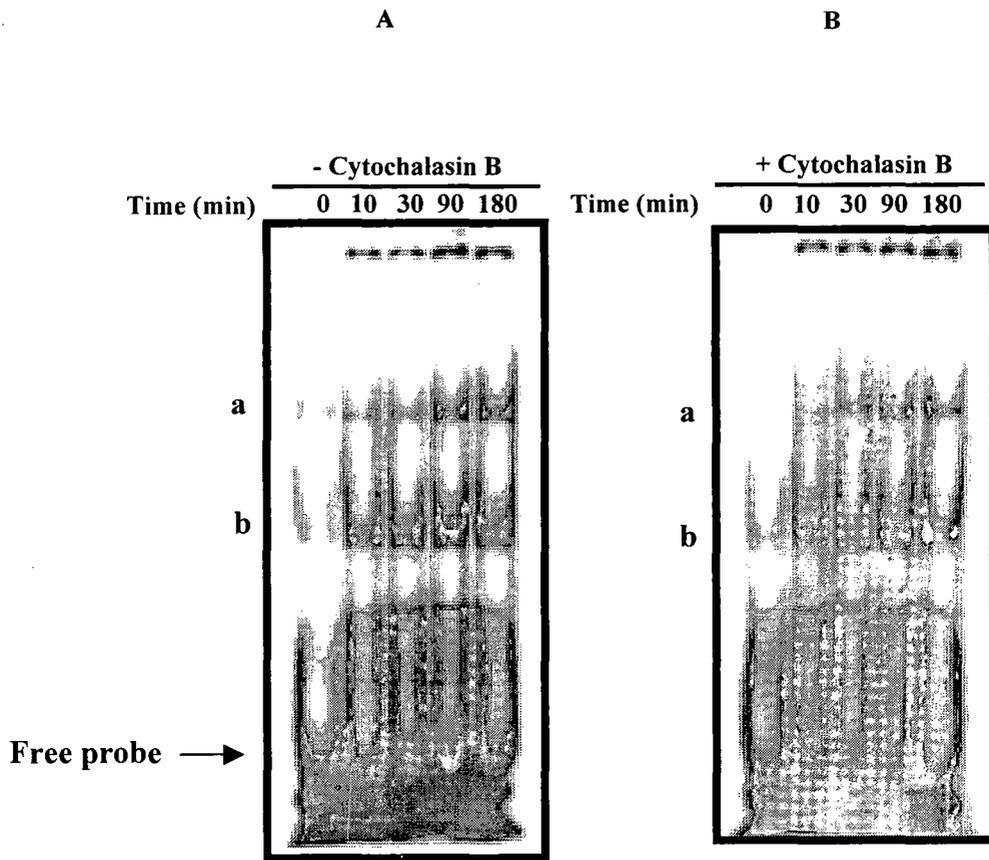


Fig. 9A and B: Cytochalasin B treatment conferred no protection from *L. donovani* induced IRF-E-DNA activation. J774A.1 cells were incubated at 37°C with 10 μ M cytochalasin B for 15 min before infection with virulent *L. donovani* (10:1 parasite/macrophage ratio). Nuclear extracts from the treated 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.

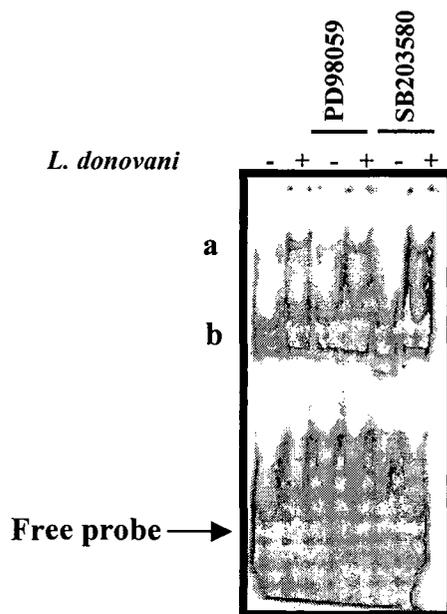


Fig. 10: Effects of PD98059 and SB203580 on IRF-E-binding activity induced by *L. donovani*. J774A.1 cells pretreated with PD98059 (20 μ M), SB203580 (5 μ M), or medium for 1 h were stimulated with *L. donovani* for further 1 h as described in Materials and Methods. The nuclear proteins were extracted. 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.