Chapter II

Curative effect of fucoidan on visceral leishmaniasis is mediated by protein kinase C isoforms: involvement of p38, ERK1/2 and NF-κB.
Introduction

Visceral leishmaniasis (VL) is a progressive fatal infection caused by the protozoan parasite *Leishmania donovani*. *Leishmania* parasites survive the hostile environment of macrophages by hindering their microbicidal mechanisms, like generation of nitric oxide (NO) and reactive oxygen species (ROS) (Gantt et al., 2001; Olivier et al., 1992b). Multiple intracellular signaling pathways stringently regulate macrophage effector response and protein kinase C (PKC) signaling pathway is one of the most ancient and conserved pathways, which plays an important role in generation of ROS and NO (Descoteaux et al., 1992; Mookerjee Basu et al., 2006; Olivier et al., 1992b). One of the major controlling tactics by which *Leishmania* can promote its survival within the host is through dysregulation of PKC signaling. Lipophosphoglycan (LPG), a leishmanial surface molecule negatively regulates typical PKC isoforms by inhibiting their membrane insertion (Descoteaux et al., 1992). Moreover, the expression of atypical PKC isoforms (PKC-ε and ζ) increases while Ca²⁺ dependent PKC-β expression decreases during infection (Kar et al., 2010). Deactivation of PKC-β is co-related with increased IL-10 production, a hallmark Th2 cytokine associated with disease progression. Additionally, PKC signaling is known to activate NF-κB, a major transcription factor involved in enhancing the expression of molecules that may assist in host defence (Chen and Lin, 2001). The importance of NF-κB in host defence against leishmaniasis was shown by gene deletion studies as both c-Rel and p52 null mice were unable to effectively clear the infection due to impaired immune response (Grigoriadis et al., 1996; Speirs et al., 2002). *Leishmania* parasites were able to inhibit induction of MAPK's, which is a major pathway for the control of infection, in response to a variety of agonists, thus helping the parasite to grow and survive within the host (Martiny et al., 1999; Nandan et al., 1999). Studies revealed that ERK1/2 and p38 MAPK play a key role in regulation of iNOS (Ajizian et al., 1999). Activation of p38 by anisomycin enhanced macrophage-dependent leishmanicidal effects whereas its inhibition by *Leishmania* co-relates well with impaired generation of iNOS and production of NO (Junghe and Raynes, 2002). Activation of MAPK signaling pathways by various stimuli induced NF-κB activation either through the phosphorylation of its inhibitor, IκB or by direct post-transcriptional modification of its p65 subunit (Goebeler et al., 2001). Thus, *Leishmania* successfully evades the macrophage microbicidal machinery and thrives in, through deregulating production of NO and ROS. Establishment of an effective immune response therefore may depend on stimulation of PKC...
signaling as well as MAPK-mediated activation of NF-κB, which may prove a potential advancement in therapy of leishmaniasis.

Fucoidan, a sulphated polysaccharide has been extensively used in traditional medicine. Accumulating evidences suggest that fucoidan exhibits a variety of pharmacological effects including anti-thrombotic, anti-malarial and immunomodulatory activities (Barroso et al., 2008; Chen et al., 2009; Kim and Joo, 2008). Fucoidan mediated immunomodulatory effect may be associated with favorable expression of proinflammatory cytokines along with generation of NO and ROS (Nakamura et al., 2006; Zhang et al., 2011). Our previous study has established fucoidan as a novel antileishmanial agent (Kar et al., 2011). The anti-leishmanial effect of fucoidan was associated with shift from disease promoting Th2 to disease resolving Th1 cytokine response in conjunction with generation of NO and ROS. However, the intricate signaling events associated with such response are not known. The present investigation was aimed towards exploring the cellular mechanisms underlying the antileishmanial effect of fucoidan in macrophage culture as well as in animal model of visceral leishmaniasis.

**Materials and Methods**

**Reagents, cell culture and infections**

All antibodies were purchased from Cell Signaling Technology and Santa Cruz Biotechnology. BAY 11-7085, BAY 11-7082, PKC-β inhibitor and \(^{25}\)Ser-substituted peptide from the pseudosubstrate region of PKC was acquired from Calbiochem. Fucoidan was obtained from Sigma (St. Louis, MO) and dissolved in PBS. All other chemicals were from (Sigma), unless indicated otherwise. The murine macrophage cell line RAW 264.7 was maintained in RPMI 1640 supplemented with 10 % fetal bovine serum with 100 μg/ml streptomycin and 100 units/ml penicillin (Invitrogen). Bone marrow derived macrophages (BMM) was cultured as described previously (Kar et al., 2010). *L. donovani* promastigotes (MHOM/IN/1983/AG83) were grown as described previously (Ukil et al., 2005). For *in vitro* infections, macrophages were infected with stationary phase *L. donovani* promastigotes at 10:1 parasite/macrophage ratio. Infection was allowed to progress for 4 h, after which unphagocytized parasites were removed by washing the plates three times with media, and the cells were allowed to grow for 20 h. Soluble leishmanial antigen (SLA) was prepared by freeze thawing the cell as described previously (Das et al., 2001). For in vivo experiments, 4-
6 weeks old female BALB/c mice were injected with $10^7$ *L. donovani* promastigotes through tail vein. Fucoidan (25-200 mg/kg/day, given 3 times weekly) was given orally for a period of 4 weeks starting from 14 days after infection. Infection was determined by removing spleen and liver from infected mice at 6 weeks, and parasite burden was calculated as LDU (Ukil et al., 2005) after staining with Giemsa. The investigation was in accordance with the Guide for the Care and Use of Laboratory Animals published by US National Institute of Health (NIH Publication No. 85-23 revised 1996) along with agreement of the Institutional Animal Care and Use Committee.

**Immunoblot analysis and ELISA**

Immunoblot was performed as described previously (Ukil et al., 2005). Levels of IL-12 and TNF-α were measured by sandwich ELISA kit (Quantikine M; R&D systems) in culture supernatants obtained from BMM, splenocytes or RAW 264.7 cells as described previously (Kar et al., 2010). Spleen cells were stimulated with 20 μg/mL SLA for 48 h before analysis. Detection limit of these assays are $> 5.1$, $> 2.5$ for TNF-α and IL-12 p70.

**Real-time PCR**

Total RNA was isolated from BMM, RAW 264.7 or splenocytes using RNeasy Mini kit (Qiagen) and Real Time PCR was performed as described previously (Kar et al., 2010).

**PKC activity assay**

Immunoprecipitation of PKC isoforms (α, βI, βII, ζ and ε) was done as described earlier (Nitti et al., 2002). For PKC α and β kinase assay, beads were washed twice with PKC buffer (10mM Tris-HCl, 150mM NaCl, 10mM MgCl₂, and 0.5mM DTT). Kinase reaction was performed by adding 15 μl buffer containing 5 μCi $^32$ATP, 0.5 μg phosphatidylserine, 0.2 μg diglycerol, 0.5 mM CaCl₂ and histone H1 (10 μg) as substrate. Reaction was allowed to progress for 10 min at 30°C and Laemmli’s buffer was used to stop it. 12.5% SDS-polyacrylamide gels were used to load reaction mixtures, dried and then exposed to autoradiographic film at 80°C for 24 h. Densitometric values of autoradiographs represents relative intensity of phosphorylated substrates. PKC ζ and ε kinase assay were performed as described earlier (Kar et al., 2010).
Transient transfection and reporter assay

RAW 264.7 (2 x 10^6 cells) were transfected with appropriate constructs in serum free media using Lipofectamine (Invitrogen) as described earlier (Kar et al., 2009). Three hours after transfection, cells were washed and replaced with RPMI medium and 10% FBS. Cells were processed after 24 h, as indicated in figure legend. NF-κB or AP-1 luciferase activity was measured using luminometer, after cells were harvested using reporter lysis buffer (Promega) and luciferase activity was normalized with co-transfected β-galactosidase expression vector.

For siRNA mediated inhibition, transfection of cells were carried out with 1 μg commercial available siRNA constructs (Santa Cruz Biotechnology) for 24 h according to manufacturer’s protocol and then stimulated with fucoidan for indicated time periods.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from RAW 264.7 or spleen cells were isolated and EMSA for NF-κB was performed as mentioned earlier (Ukil et al., 2005).

Statistical Analysis

Experiments were performed three times each. Triplicate macrophage cultures were set and 5-6 mice per group were used for animal experiments. Student's t-test was performed to evaluate the statistical significances between a pair of data sets, p value < 0.05 was considered to be significant.

Results

Therapeutic effects of fucoidan

We have earlier demonstrated that curative effect of fucoidan on experimental visceral leishmaniasis was associated with host favourable T cell response and generation of NO (Kar et al., 2011). In order to evaluate the potential contribution of each pro-inflammatory cytokine as well as NO in fucoidan-mediated supression of parasite, L. donovani-infected mice were orally administered 200 mg/kg/day of fucoidan alone or in combination with 200 μg of anti-IFN-γ, anti-TNF-α, anti-IL-12 or control IgG (given i.p., 3 times weekly) 14 days post infection. In another set of expermentin, mice were infected and treated with fucoidan as stated above along with 5 mg/kg/day of AMT. Spleen parasite
burden was then determined 45 days after infection. During the experiment no marked difference on body weight was noted in any of the experimental groups. In fucoidan treated infected mice, dose related inhibition was seen for both liver and spleen. Inhibition was almost complete (>99% parasite suppression) for both spleen and liver at a dose of 200 mg/kg/day (Figure 1A). Anticytokine mAbs reactive against IFN-γ, TNF-α or IL-12 greatly reduced fucoidan-mediated protection (57%, 52% and 63% parasite suppression for IFN-γ, TNF-α and IL-12 respectively, compared to >95% suppression in fucoidan-treated mice, Table 1). No significant difference was found between the parasite load in mice treated with fucoidan plus control Ab or with fucoidan alone. Moreover, mice treated with AMT also showed much less parasite suppression (53%) compared to >95% in fucoidan treated mice. Consistent with these, the mRNA levels of iNOS, which is the main enzyme that facilitates the formation of NO, is remarkably higher in fucoidan treated infected mice (9.15-fold induction) at 4-week post infection, which remained high even after 6-week post infection (Figure1B). These data collectively suggest that fucoidan can not only confer protection against leishmania, but also helps in activation of favorable host immune response.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Spleen parasite burden (LDU)</th>
<th>% Parasite Suppression</th>
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<tbody>
<tr>
<td>L. donovani + PBS</td>
<td>380 ±41.2</td>
<td>0</td>
</tr>
<tr>
<td>L. donovani + Fucoidan</td>
<td>12 ±1.5</td>
<td>97</td>
</tr>
<tr>
<td>L. donovani + anti-IFN-γ</td>
<td>163 ±17.2</td>
<td>57</td>
</tr>
<tr>
<td>L. donovani + anti-IL-12</td>
<td>141 ±15.6</td>
<td>63</td>
</tr>
<tr>
<td>L. donovani + anti-TNF-α</td>
<td>198 ±20.2</td>
<td>52</td>
</tr>
<tr>
<td>L. donovani + AMT</td>
<td>176 ±18.7</td>
<td>53</td>
</tr>
<tr>
<td>L. donovani + Control IgG</td>
<td>18 ±1.4</td>
<td>95</td>
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Table 1. Mice were infected and treated and described in the legend of Figure 6A. On day 14 mice were treated with 200 µg of mAbs of TNF-α, IFN-γ, IL-12 or control IgG. In another set of experiment AMT (5mg/kg/day) was administered along with fucoidan three times weekly. Spleen parasite burden was evaluated 45 days after infection. Results are representative of three independent experiments done in triplicate ± SD, n = 3.
Involvement of MAPK pathway in fucoidan mediated immune response

To ascertain the molecular mechanisms underlying the leishmanicidal effects of fucoidan, we examined the induction of MAPK, in response to fucoidan (50 μg/ml) treatment. Time course analysis (0-3 h) by Western blot demonstrated a time-dependent increase in phosphorylation status of p38 and ERK1/2, in bone marrow derived macrophages (BMM) with a maximum induction of 4.67 and 5.75-fold at 2 h post treatment, as compared to untreated control (Figure 2A). No significant induction was observed for p46 and p54 JNK (Figure 2A). In *L. donovani*-infected BMM also, treatment of fucoidan elicited a time dependent increase in phosphorylation status of p38 and ERK1/2 although to a lesser extent (maximum induction of 3.0- and 2.76-fold respectively, for p-p38 and p-ERK1/2, at 2 h post treatment). Preincubation of infected cells with ERK1/2 or p38 inhibitors, apigenin or SB203580 or both for 1 h prior to stimulation with 50 μg/ml of fucoidan for 24 h resulted in significant decrease in levels of both IL-12 and TNF-α (Figure 2C), however the consequent decrease in the level of TNF-α was minimal in the case of ERK1/2 inhibitor. A comparative
cytokine profile showed that in cells treated with apigenin, IL-12 and TNF-α showed a decrease of 67% and 41% respectively, whereas a decrease of 73% and 64% was observed in case of SB203580 (Figure 2C). However, co-treatment of apigenin and SB203580 rendered a decrease of 88% and 83% for IL-12 and TNF-α respectively in fucoidan-treated infected macrophages (Figure 2C). iNOS gene expression in phagocytic cells is under the control of MAPK signaling cascades and increase in iNOS levels is intimately associated with the protective immune response in experimental visceral leishmaniasis. We therefore investigated whether fucoidan-induced expression of iNOS is under the control of MAPK pathway and for that the effect of ERK1/2 and p38 inhibitors on mRNA levels of iNOS was checked. Similar to proinflammatory cytokines, iNOS transcript level also showed a decrease of 57% and 33% (Figure 2D), when infected BMM were preincubated with either SB203580 or apigenin for 1 h, before stimulation with fucoidan for 24 h. However, co-treatment of ERK1/2 and p38 inhibitors showed a 73% decrease in iNOS transcript levels, showing that p38, and to a lesser extent ERK1/2 is involved in the protective immune response associated with fucoidan.
Involvement of MAPKs in fucoidan-dependent pro-inflammatory cytokine response and iNOS expression in BMM. BMM were left alone or infected with *L. donovani* for 4 h, non-ingested promastigotes were removed by washing and cells were further cultured for 24 h. Normal or infected macrophages were then treated with 50 μg/ml fucoidan for different time periods. (A and B) Activation of MAPKs in fucoidan treated normal (A) and infected macrophages (B) was detected by Western blotting. (C and D) *L. donovani* infected BMMs were incubated with either SB203580 (30 μM) or apigenin (40 μM) or both for 1 h followed by treatment with 50 μg/ml fucoidan for 24 h. Levels of IL-12 and TNF-α(C) in culture supernatants by ELISA and iNOS expression (D) by Real-time PCR were measured. Densitometries are shown as bar graphs on the right-hand side of each panel. The data shown are mean ± SD, n = 3. Results are representative of three separate experiments, *p<.05, **p<0.001, ***p<0.0001; Student's t-test.

**Involvement of distinct PKC isoforms in fucoidan mediated immune response**

One of the vital strategies applied by *Leishmania* to survive in the hostile environment of macrophages is differential modulation of PKC isoforms (Bhattacharyya et al., 2001; Giorgione et al., 1996; Kar et al., 2010). We, therefore, investigated the specific PKC isoforms that might be affected by infection and whether fucoidan has any role in maintaining the PKC balance within the macrophages. Since all the isoforms of PKC are not present in BMM, we therefore used the murine macrophage cell line RAW 264.7 for our subsequent experiments. Immunoblot analysis of all eight PKC isoforms (α, βI, βII, δ, ε, μ, λ and ζ) showed that in infected macrophages PKC ε and ζ expressions were upregulated (4.48- and 4.36-fold increase as compared to control macrophages, respectively) whereas levels of PKC α, βI and βII were markedly attenuated (59.6%, 54.9% and 42.6% decrease respectively as compared to uninfected control) (Figure 3A). However, no appreciable change was observed in expression levels PKC δ, μ and λ (Figure 3A). Interestingly, treatment with fucoidan caused a marked upregulation of PKC α, βI and βII (2.21, 3.75 and 3.35-fold respectively) associated with significant downregulation of PKC ε (50.4% decrease) and ζ (59.4% decrease) at 12 h post treatment (Figure 3A). To further ascertain the role of PKC α, βI, βII, ζ and ε, immunocomplex kinase assays were performed. Treatment of infected macrophages with fucoidan induced a time dependent increase in kinase activities of PKC α, βI and βII that was found to be maximum at 90 min post treatment (2.1-, 2.7- and 2.67-fold induction as compared to uninfected control respectively) (Figure 3B, C and D). In contrast, fucoidan treatment markedly attenuated kinase activity of PKC ε and ζ (71.4% and 76.1% decrease as compared to infected control respectively) at 90 min post treatment in infected cells (Figure 3E and F). We next sought to identify whether PKC signaling has any role in fucoidan
mediated macrophage inflammatory response. As depicted in Figure 3A, pretreatment of RAW 264.7 cells with a PKC β-specific inhibitor markedly attenuated fucoidan-induced IL-12 synthesis (74.1% decrease as compared to infected and fucoidan-treated cells) and to a lesser extent TNF-α synthesis (47.6% decrease), while treatment with PKC-α siRNA caused a decrease of 79.5% and and 46.1% for IL-12 and TNF-α respectively (Figure 3J). To further determine whether increase in PKC α and β upregulation correlated well with increased phosphorylation of p38 and ERK1/2 in response to fucoidan, we evaluated the effect of inhibition of PKC-α and β. Preincubation of PKC-β specific inhibitor peptide effectively blocked phosphorylation of p38 (67.5% reduction) and to a lesser extent the phosphorylation of ERK1/2 (42.5% decrease) (Figure 3G). Similarly, PKC-α inhibition by siRNA resulted in 67.5% decrease in phosphorylation levels of p38 along with 50% decrease in p-ERK1/2 (Figure 3H). The specificity of siRNA targeted against PKC-α was measured by Western blotting. PKC-α specific siRNA transfection caused a significant reduction in protein levels of PKC-α (65.9% decrease) (Figure 3K). Taken together these results show a functional correlation between differential regulation of PKC isotypes, coupled with the increased phosphorylation of p38 and ERK1/2, in response to fucoidan treatment.
allowed to grow for another 12 h. Macrophages were then treated with 50 μg/ml fucoidan for indicated time periods and immunoblot analysis for different PKC isoforms were performed. Macrophages are infected as stated above, treated with 50 μg/ml fucoidan for indicated time periods, lysed and immunoprecipitates were obtained using respective PKC antibodies and activity of PKC-βI (B), PKC-βII (C), PKC-α (D), PKC-ε (E) and PKC-ζ (F) were measured as stated in Materials and Methods. (G, H, I and J) RAW 264.7 cells were infected and with *L. donovani* for 4 h, allowed to grow for 12 h, treated with PKC-β inhibitor for 2 h (G and I) or PKC-α siRNA for 24 h (H and J) and then treated with 50 μg/ml fucoidan. Phosphorylation levels of p38 and ERK1/2 were measured after 3 h (G and H) and levels of IL-12 and TNF-α were measured 24 h after treatment with fucoidan (I and J). (K) PKC-α siRNA specificity was ascertained in RAW 264.7 cell lysates expressing PKC-α or control siRNA by Western blotting. Densitometries are shown as bar graphs. The error bar represents mean ± SD, n =3. The data shown are representative of three independent experiments. **p<0.001, ***p<0.0001; Student’s t-test.

**Role of NF-κB and AP-1 modulation in fucoidan induced protective immunity**

Since several proinflammatory genes as well as iNOS expression is regulated by transcription factor NF-κB and AP1 (Ghosh et al., 2002), we employed luciferase reporter assay to determine the effect of fucoidan on the activation of these transcription factors. RAW 264.7 cells were transiently transfected with a plasmid containing either NF-κB or AP-1-binding sites, and the luciferase activities were measured. Treatment of fucoidan at 50 μg/ml significantly increased NF-κB dependent luciferase activity in a time dependent manner with maximum induction of 9.7-fold at 8 h post treatment (Figure 4A). Fucoidan also induced the luciferase activity of AP-1 (2.8-fold as compared to untreated controls at 8 h post treatment) (Figure 4B). We then checked whether fucoidan treatment could also modulate the luciferase activity of NF-κB and AP-1 in infected macrophages. Fucoidan elicited a time-dependent increase in NF-κB dependent luciferase activity with a maximum induction of 6.4-fold at 12 h post treatment in *L. donovani*-infected macrophages (Figure 4C). However, no significant change in luciferase activity of AP-1 was observed in infected macrophages (1.4-fold as compared to untreated control at 12 h) (Figure 4D). Moreover EMSA analysis revealed that, similar to reporter assay, fucoidan treatment elicited a marked induction in NF-κB binding (3.6-fold at 8 h post treatment) as compared to infected control (Figure 4E). To further characterize the subunits of NF-κB that are involved in fucoidan mediated NF-κB activation, super shift assay was performed using specific antibodies against p50, p52, p65, c-Rel and Rel B. While anti-c-Rel, anti-p52 and anti-Rel B antibodies has no effect, anti-p65 and anti-p50 antibodies markedly caused a shift of entire signal (Figure 4F), culminating in the fact that fucoidan-mediated induction of NF-κB is regulated through p50 and p65 subunits.
Figure 4. Effect of fucoidan on modulation of NF-κB and AP-1. (A and B) RAW 264.7 cells were transfected with 1 μg of NF-κB or AP-1 luciferase reporter vector along with 0.5 μg pCMV-β-galactosidase. After 24 h, cells were treated with 50 μg/ml of fucoidan for different time periods (0-12 h), lysed and luciferase activity of NF-κB (A) and AP-1 (B) was measured. (C and D) Infected RAW 264.7 cells (24 h) were transfected with NF-κB or AP-1 luciferase reporter vector along with pCMV-β-galactosidase as described above, followed by treatment with 50 μg/ml of fucoidan for indicated time periods. Cells were lysed and luciferase activity of NF-κB (C) and AP-1 (D) was measured. (E and F) RAW 264.7 cells were infected with L. donovani promastigotes as described in Fig. 3A and treated with 50 μg/ml of fucoidan for indicated time periods.
Involvement of NF-κB in fucoidan-mediated regulation of iNOS and pro-inflammatory cytokines

To further evaluate the role of NF-κB in fucoidan-induced cytokine balance and iNOS expression, we used pharmacological inhibitor of NF-κB. To this end, infected cells were treated with increasing concentrations of BAY 11-7082 (0.5 μM–5 μM), a potent inhibitor of NF-κB, followed by stimulation with fucoidan for 24 h and levels of IL-12 and TNF-α were measured. ELISA studies revealed that BAY 11-7082 markedly reduced levels of IL-12 and TNF-α in a concentration dependent manner. At higher doses (5 μM), there was 71.6% and 65.6% suppression in levels of IL-12 and TNF-α was observed (Figure 5B). Consistent with our ELISA studies, BAY 11-7082 treatment significantly decreased the level of iNOS transcript in a dose dependent manner with maximum inhibition of 85.2% at a dose of 5 μM (Figure 5A). This observation prompted us to look into the role of p38 and ERK1/2 MAPK, which are essential for activation of NF-κB, leading to generation of downstream antileishmanial molecules. Preincubation of p38 inhibitor SB203580 (30 mM) resulted in 64.8% decrease in fucoidan-induced NF-κB luciferase reporter activity whereas 44.5% decrease was found in case of preincubation with 40 μM apigenin (Figure 5C). However, combination of SB203580 and apigenin markedly inhibited NF-κB luciferase activation (84.2%), suggesting that both p38 and ERK1/2 MAPK are required in maintaining fucoidan-mediated NF-κB activation. Taken together, these results suggest a functional corelation between activation of p38, NF-κB and to a lesser extent that of ERK1/2 in induction of iNOS and Th1 cytokine expression.
Effect of fucoidan on modulation of NF-κB in vivo

In order to ascertain the role of fucoidan on modulation of NF-κB pathway in vivo, fucoidan (200 mg/kg/day) was administered orally in L. donovani-infected BALB/c mice for 3 times weekly for 4 wk starting at 14 days post-infection and splenocytes were isolated. Effect of fucoidan on NF-κB binding activity was examined by EMSA. Consistent with our in vitro finding, fucoidan treatment greatly increased NF-κB binding in infected treated mice as compared to untreated mice being maximum at 4-weeks post infection (3.3-fold) (Figure 6A). To further substantiate the effect of NF-κB pathways in fucoidan-mediated curative response, separate groups of infected mice were treated with NF-κB inhibitor BAY 11-7085 (5 mg/kg/day, given thrice weekly) over a 4-week period along with fucoidan. In all the groups (infected, infected fucoidan-treated, infected fucoidan-treated as well as NF-κB inhibitor-treated), spleen parasite burden and levels of IL-12 and TNF-α were measured. No
marked effect on body weight was noted in any of the experimental groups during the experiment. Similar to our in vitro findings, fucoidan treatment elicited a robust increase in both IL-12 and TNF-α (6.1-fold and 5.5-fold, respectively) in infected mice at 4 weeks, which remained high even after 6 weeks (Figure 6C and D). Moreover, complete inhibition of spleen parasite burden was observed in fucoidan treated animals at 6 weeks (Figure 6B). Interestingly, in vivo pharmacological inhibition of NF-κB by BAY 11-7085 markedly attenuated fucoidan induced TNF-α and IL-12 protein synthesis (71.6% and 73.2% decrease at 4 weeks post infection) (Figure 6C and D), which remained low even after 6 weeks. This decrease in proinflammatory cytokine synthesis has further been reflected in in vivo parasitemia. Fucoidan-mediated suppression of spleen parasite burden was significantly reversed in NF-κB inhibitor treated mice (67.3% reduction at 6 weeks post treatment). Collectively these results suggest that NF-κB activation plays an important role in fucoidan mediated antileishmanial response and is vital for generation of protective immunity.

![Figure 6. Effect of fucoidan on NF-κB mediated anti-leishmanial response.](image)

(A) Splenocytes from infected and infected-treated mice was isolated at indicated time points, as described in legend of Figure 1B. EMSA of NF-κB was performed with nuclear extract of splenocytes after incubating it with NF-κB labeled probe. Bands were analyzed densitometrically, and fold changes are indicated at the bottom. (B, C and D) In another set of experiment, mice were infected with L. donovani, after 14 days of infection mice were administered fucoidan (200 mg/kg/day) orally (3 times weekly) for a period of 4 weeks or
Discussion

The outcome of *Leishmania* infection depends on successful suppression of Th1 response along with induction of Th2 response (Awasthi et al., 2004; Rogers et al., 2002). Control of visceral leishmaniasis basically depends on stimulation of host-signaling cascades leading to the activation of phagocytic cells which in turn successfully control and largely determines the clearance of parasitemia (Awasthi et al., 2004). Thus exploration of molecular player(s) mediating activation of macrophages could possibly provide a better insight into the role of immune cells in control of visceral leishmaniasis. Natural dietary components are gaining immense importance as potent immunomodulators with multifarious roles and targets, as they not only boost the immune system to produce parasiticidal molecules like NO and ROS, but also modify Th1/Th2 paradigm in favor of the host (Bhattacharjee et al., 2009; Hernandez-Pando et al., 2005). Fucoidan, a sulfated polysaccharide, mainly found in marine brown algae, has been known to induce the expression and activity of PKC, along with induction of iNOS through NF-κB activation in macrophages (Hsu et al., 2001; Nakamura et al., 2006). Our previous study demonstrated the role of fucoidan as a potent antileishmanial agent against antimony-sensitive and -resistant *L. donovani*, both *in vitro* and *in vivo*, through upregulation of NO and ROS as well as induction of proinflammatory cytokines (Kar et al., 2011). The present study demonstrated that fucoidan mediated induction of iNOS and pro-inflammatory cytokines were dependent upon activation of p38 and ERK1/2 MAPK, which in turn activated NF-κB and the antileishmanial response associated with it. Interestingly, further investigations illuminated the functional involvement of distinct PKC isoforms in fucoidan-mediated MAPK activation. Moreover, the present investigation provides the first *in vivo* evidence for the fact that activation of NF-κB by fucoidan may be a prerequisite for shifting the cytokine balance toward disease-resolving Th1 mode for complete elimination of organ parasite burden.

Infection of macrophages by *Leishmania* along with significant downregulation of MAPK/NF-κB pathway, perhaps signify a survival mechanism of parasites by which they can inhibit harmful inflammatory response of host directed against them (Martiny et al., 1999; Speirs et al., 2002). Growing body of evidence suggest that production of leishmanicidal
molecules such as ROS and NO by many drugs including sodium antimony gluconate, the first-line antileishmanial drug, act through activation of p38 and ERK1/2 MAPK (Mookerjee Basu et al., 2006). The present study also indicated that fucoidan mediated increase in levels of iNOS is primarily dependent upon activation of p38 and ERK1/2. Interestingly, levels of IL-12 were found to be primarily controlled by p38 while those of TNF-α were controlled by ERK1/2. However combination of both p38 and ERK1/2 inhibitors was much more effective than either one of them indicating that both p38 and ERK1/2 are involved in the proinflammatory response induced by fucoidan. Microbicidal functions of macrophages is dependent upon normal PKC signaling, as pathogenic microbes tend to dysregulate PKC dependent signaling to survive in hostile environment of macrophages (Descoteaux et al., 1992; Olivier et al., 1992b). Leishmania parasites are known to differentially regulate PKC-dependent signaling to survive in hostile environment of macrophages (Bhattacharyya et al., 2001; Kar et al., 2010). Inhibition of Ca\(^{2+}\) dependent isoforms of PKC, in particular PKC-α and β has already been established (Bhattacharyya et al., 2001), however accumulating evidences also indicate a role of induction of Ca\(^{2+}\) independent PKC isoforms such as PKC-ε and ζ (Kar et al., 2010). The present study demonstrated that disease progressing PKC-cand ζ levels were decreased while there was significant increase in protein levels of host-protective PKC-α, βI and II. This was further confirmed by in vitro kinase activities which correlated well with increased expression of PKC-α, βI, βII and inhibition of PKC-ε and ζ. Moreover functional inhibition of PKC-α and β markedly abrogated fucoidan-induced phosphorylation of p38 and to a lesser extent of ERK1/2, along with induction of IL-12 and TNF-α which suggests that fucoidan-mediated leishmanicidal effect may be dependent upon differential regulation of PKC isoforms. A recent study showed that in L. major high dose of CD40 stimulated PKC α, βI, βII and ε, which in turn activated p38 and IL-12 and helped in clearance of parasites. In contrast, lower doses of CD40 stimulated PKC ζ, δ and λ along with concomitant increase in p-ERK and disease progressing cytokine like IL-10 (Sudan et al., 2012).

Intricate balance between kinases and phosphatases is a prerequisite for optimal macrophage response against invading pathogens. A number of studies emphasized the importance of dephosphorylation and thus deactivation of host PTP (protein tyrosine phosphatases) superfamily for normal activation of disease resolving PKC isoforms (Kar et al., 2010; Shio and Olivier, 2010; Ukil et al., 2011). Increasing evidence suggest that inhibition of host PTP resulted in suppression of visceral as well as cutaneous leishmaniasis both in vitro and in vivo (Olivier et al., 1998). It may be mentioned that by reducing the
expression and activity of PKC-ε and ζ, fucoidan might help in shifting of Th1/Th2 balance in favor of host along with induction of NF-κB and iNOS. Recent evidence suggest a crucial role of NF-κB family of transcription factors in encoding proinflammatory genes and NO which are pivotal for controlling *Leishmania* infection (Mise-Omata et al., 2009; Speirs et al., 2002). Some studies suggest that transcription factor AP-1 may also be involved in activation of NO (Nakamura et al., 2006). We found significant upregulation of NF-κB luciferase activity in infected macrophages but not AP-1. The most prominent complex of NF-κB present is p50/p65 heterodimer which play an essential role in activation of classical pathway, and many anti-leishmanial agents are reported to act through induction of p50/p65 complex (Kar et al., 2009; Ukil et al., 2005). By supershift assay, our studies suggested that p65 and p50 subunits of NF-κB are mainly responsible for fucoidan-mediated activation of NF-κB DNA binding, which possibly contributed to the induction of host-protective inflammatory gene expression. Recent evidence suggest a direct corelation between MAPK activation and upregulation of NF-κB leading to upregulation in disease resolving Th1 response along with generation of NO (Baeuerle and Henkel, 1994). Our results further indicated that fucoidan mediated induction of NF-κB as well as cellular and immunological response is dependent on both p38 and ERK1/2 as cotreatment with fucoidan and p38 and ERK inhibitors resulted in much more significant decrease in NF-κB luciferase activity as compared to either p38 or ERK inhibitor alone. Moreover, fucoidan activates NF-κB not only through classical pathway but may also act through MSK1, a downstream kinase of both p38 and ERK1/2, which activates NF-κB through phosphorylation of its p65 subunit, since pharmacological inhibition of p38 or ERK1/2 or both were able to significantly reduce NF-κB DNA binding activity. Using pharmacological inhibitor of NF-κB we demonstrated that it is essential for the generation of IL-12 and TNF-α culminating in induction of iNOS both *in vitro* and *in vivo*. Fucoidan mediated suppression of spleen parasite burden was significantly attenuated by inhibiting NF-κB, which suggests a functional significance of NF-κB in suppression of parasitemia. In line with our previous observation which suggested that fucoidan mediated induction of proinflammatory cytokines as well as generation of NO is maximum at 4 weeks post infection (Kar et al., 2011), we found time-dependent increase in DNA binding activity of NF-κB *in vivo* which was maximum at 4 weeks. However clearance of organ parasite burden was found to be maximum at 6 weeks after fucoidan treatment, which was quite obvious as this was the after effect of all the cellular defense machinery.

Overall, results of the present study have shed light on the immunomodulatory and leishmanicidal effect of fucoidan, which is mediated via differential contribution of PKC-ε and ζ. 
isoforms. Furthermore sequential activation of p38, ERK1/2 and modulation of its target transcription factor NF-κB promoted positive signal transduction, culminating in activation of effector macrophage functions for fucoidan-mediated leishmanicidal effect. However, further in-depth study is required for better understanding the role of fucoidan not only for the treatment of non healing leishmaniasis but also for other chronic infections.