Chapter III

Therapeutic effect of fucoidan on phosphatase-dependent modulation of MAPK during experimental visceral leishmaniasis: regulatory effect of reactive oxygen species.
Introduction

Visceral Leishmaniasis or kalazar, caused by the protozoan parasite *Leishmania donovani*, is the most severe form of Leishmaniasis and is associated with irregular fever, weight loss, enlargement of liver and spleen and anemia (Murray et al., 2005). The disease is of increasing concern due to worldwide occurrence of 0.5 million new cases per annum, increase in resistance to standard antimony based drugs, and HIV *Leishmania* coinfection. Currently pentavalent antimony is the mainstay of the therapy of this infection, but treatment with antimonial agents has been unsuccessful due to high toxicity and resistance (Sundar et al., 2000). Liposomal Amphotericin B has limited efficacy due to high reoccurrence and requirement of sustained therapy. *Leishmania donovani* infection is accompanied by parasite specific immune depression mediated by T cells and macrophages, thereby preventing spontaneous cure and development of protective immunity (Awasthi et al., 2004; Rogers et al., 2002). Pro-inflammatory and modulatory cytokines have an essential role in host defense against *Leishmania donovani* infection. Control of *Leishmania donovani* parasitism during infection is considered to be critically dependent on direct macrophage activation by cytokines. Macrophages activated by IFN-γ and tumor necrosis factor-α (TNF-α) synthesize nitric oxide (NO) (Diefenbach et al., 1999). Studies performed on mice infected with *Leishmania* demonstrated that host defense against this infection depends on IL-12 driven expansion of the Th1 subset, with production of cytokines such as IFN-γ that activate macrophages for parasite killing through the release of NO (Diefenbach et al., 1999). NO, which mediates many of the non-specific cytotoxic and inflammatory responses of macrophages after infection by pathogens, is generated after the up-regulation of inducible nitric oxide synthase (iNOS). Similarly, the biological activity of IL-12 which is the key cytokine driving Th1 cell development is regulated by the induction of the p40 subunit. *Leishmania* species was observed which is accompanied with induction of Protein Tyrosine phosphatases (PTP) (Verma et al., 1995). Induction of PTP and MAPK directed phosphatases is one of the major manipulative strategies by which *Leishmania* induces immune silencing and alters the normal host signaling mechanisms. The induction of Src homology 2 domain containing tyrosine phosphatase (SHP-1), a PTP member, induction has been shown to be vital for survival of *Leishmania* (Olivier et al., 2005). Moreover, persistence and propagation of the parasite within the host is also dependent upon induction of MKPs and PP2A which disrupts many cell signaling pathways including MAPK and PKC (Descoteaux et al., 1992; Ghosh et al., 2002). Thus, *Leishmania* successfully evades the macrophage microbicidal...
machinery and thrives in, through dysregulating 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.

Our previous studies had revealed that that fucoidan could confer complete protection to both antimony-susceptible and -resistant \textit{L. donovani} strains by switching the functional differentiation of Th2-type CD4\(^+\) cells to Th1-type as well as up regulation of NO and ROS. Moreover it revealed that differential activation of PKC isoforms along with induction of p38 and ERK1/2 MAPK are essential for maintaining host favourable immune response. The present investigation was aimed towards exploring whether ROS has any effect on the kinase phosphatase balance maintained by fucoidan mediated antileishmanial effect in macrophage culture as well as in animal model of visceral leishmaniasis.

**Materials and Methods**

**Reagents**

All the antibodies were from Santa Cruz Biotechnology. All other chemicals were from Sigma unless indicated otherwise. Fucoidan was purchased from Sigma and dissolved in PBS. The endotoxin level of 100 \(\mu\)g/mL fucoidan preparation was less than 0.1 endotoxin units (EU)/mL as measured by chromogenic Limulus amoebocyte lysate (‘LAL’) endpoint assay (QCL-1000; BioWhittaker) following the manufacturer’s manual.

**Cell culture, infection and fucoidan treatment**

Bone marrow derived macrophages (BMM) was cultured as described previously (Kar et al., 2010). The murine macrophage cell line RAW 264.7 was maintained in RPMI 1640 supplemented with 10% fetal bovine serum with 100 \(\mu\)g/ml streptomycin and 100 units/ml penicillin (Invitrogen). \textit{L. donovani} strain AG83 (MHOM/IN/1983/AG83), isolated from an Indian patient with kala-azar (14 years), was maintained in inbred BALB/c mice by i.v. passage every 6 weeks. \textit{L. donovani} promastigotes were obtained by allowing isolated splenic amastigotes to transform in parasite growth medium for 72 h at 22°C. The growth medium consisted of medium 199 (Invitrogen Life Technologies) supplemented with 10% (v/v) heat-inactivated FCS. For \textit{in vitro} infections, macrophages were infected with stationary phase \textit{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 before stimulation with fucoidan. For \textit{in vivo} BALB/c mice were infected and treated as described previously (Kar et al., 2011).

\textbf{Real Time PCR, Immunoblot analysis and ELISA}

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). Levels of IL-12, TNF-\(\alpha\) and IL-10 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., 2011). Spleen cells were stimulated with 20 \(\mu\)g/mL SLA for 48 h before analysis. Immunoblot was performed as described previously (Ukil et al., 2005).

\textbf{PTP and PTK activity assay}

PTP activity was measured using the PTP assay kit (Sigma-Aldrich), according to the manufacturer’s instructions. Briefly, cells were lysed in lysis buffer (50 mM Hpes, pH 7.4, containing 0.5% Triton X-100, 10% glycerol, 1 mM benzamidine, 10 \(\mu\)g/ml aprotinin, 10 \(\mu\)g/ml leupeptin, and 2 \(\mu\)g/ml pepstatin A), incubated on ice for 30 min, and centrifuged at 10,000 g for 15 min at 4°C. PTP activity in clear supernatants was determined using a monophosphorylated phosphotyrosine peptide as substrate. Free inorganic phosphate was detected with malachite green (Sigma), and OD was taken at 620 nm. PTK assay was performed as described previously (Sodhi and Pandey, 2011).

\textbf{Results}

\textbf{Negative regulation of PTP and MAPK directed phosphatases by fucoidan}

Kinase phosphorylation induces macrophage signal transduction events during infection which are generally countered by phophatases, by the parasite for its survival. To understand whether MAP kinase activation by fucoidan could be an after-effect of down regulation of phosphatase activity, we first checked the total PTP activity in infected and infected fucoidan-treated macrophages. \textit{L. donovani} infection induced a time dependent increase in PTP activity (4.52-fold at 2 h after infection), which was significantly decreased by fucoidan treatment (maximum reduction of 62.8\% at 4 h) (Figure 4A). Several studies...
suggest that oxidation by ROS renders PTP inactivated. (12, JI wrote), so we evaluated the effect of ROS inhibition of infected macrophages treated with NAC (a ROS quencher). Infected macrophages were preincubated with NAC for 2 h and then stimulated with fucoidan. As depicted in Figure 4A, pretreatment of with NAC (5 mM) markedly increased PTP activity and the maximum activity was observed at 2 h posttreatment (4.28-fold). Next we shifted our attention to MKP1, MKP3 and PP2A (MAPK directed phosphatases), which recently we have demonstrated to play a vital role during visceral leishmaniasis. Real time PCR showed that at 90 mins post-infection the levels of MKP1, MKP3 and PP2A showed 6.8-, 6.2- and 6.1-fold increase while fucoidan treatment brought down the levels of MKP1, MKP3 and SHP-1 by 52%, 58% and 61% respectively (Figure 4B and D). Similarly immunoblot analysis also showed significant decrease in the levels of all the MAPK phosphatases (Figure 4C). The expression of SHP-1 remained unchanged both at mRNA and protein level after *L. donovani* infection and/or fucoidan treatment (Figure 4C and 4D). These data indicate that fucoidan can significantly downregulate PTPs and MAPK phosphatases and this is dependent upon generation of ROS.
Figure 1. Effect of fucoidan on PTP and MKPs 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. In another set or experiment BMM were pretreated with 5μM NAC 2 h before stimulation with fucoidan. (A) Induction of macrophage PTP activity during *L. donovani* infection was measured using a PTP assay kit. Results are expressed as the relative increase (n-fold) over PTP activity in control cells. (B, C and D) MKP1 and MKP3 (B), PP2A and SHP-1 (D) expressions at mRNA and protein levels (C) were evaluated by real-time PCR and western blotting, respectively. mRNA levels were normalized to β-actin and expressed as a fold change compared with control. Error bars represent means ± SD. The data shown are representative of three independent experiments. **P < 0.01, ***P < .001 versus corresponding infected control.

Effect of inhibition of ROS on immunostimulatory properties of fucoidan:

We investigated the effect of fucoidan on Protein tyrosine Kinase (PTK) activity and whether the inhibition of ROS generation would have any effect on that. In this line of thought we found that fucoidan increased the PTK activity by 6.2-fold 3 h post treatment in infected macrophages (Figure 2A). Pre-treatment of NAC significantly downregulated the PTK activity by 66% (Figure 2A). Concomitant with the decrease in the levels of PTK, phosphorylation levels of both ERK1/2 and p38 decreased significantly (53% and 71%
reduction) in cells pre-treated with NAC (Figure 2B). ROS have been demonstrated as one of the key regulators in signal transduction pathways (28), and because of pro-inflammatory cytokines released from macrophages are a result of NF-κB activation (15), we measured the expression of IL-12 and TNF-α along with NF-κB. Use of ROS quencher (NAC) in macrophages resulted in significant reduction of IL-12 and TNF-α expression in infected-treated macrophages (69.5% and 75.1% reduction in IL-12 and TNF-α, respectively, as compared with infected-treated macrophages) (Figure 2C). Since several proinflammatory genes is regulated by transcription factor NF-κB (Ghosh et al., 2002), we employed luciferase reporter assay to determine the effect of fucoidan on the activation of these transcription factors. Fucoidan implicated a 6.2-fold increase in the reporter activity of NF-κB, which was significantly down regulated after treatment with NAC (72% decrease) (Figure 2D). Collectively, these results suggest that enhanced ROS generation is essential for protective immunity conferred by fucoidan against visceral leishmaniasis.
Role of ROS in in vivo anti-leishmanial effect of fucoidan

In order to ascertain the role of fucoidan on modulation of immunity in favour of host in BALB/c mice, 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, along with NAC, which was administered i.p. starting 2 days before the infection, daily at a dose of 300mg/kg/day. Quenching of ROS significantly increased the parasite survival for both liver and spleen (64% and 66% for liver and spleen respectively), while only fucoidan treatment has conferred complete elimination of liver and spleen parasite burden (Figure 3A and B). Similarly the levels of protective Th1 cytokines decreased in mice treated with NAC along with fucoidan (55% and 63% decrease for IL-12 and TNF-α, respectively) (Figure 3C and D). Moreover the levels of host-suppressive cytokine IL-10 levels significantly increased in mice receiving co-treatment of NAC and fucoidan (Figure 3E). To evaluate the role of phosphatases in the modulation of disease progression PTP activity was assayed. Similar to the in vitro activity of PTP decreased after treatment with fucoidan (72% decrease as compared to fucoidan treated mice), but in mice which received co-treatment the activity of PTP was restored (Figure 3E), emphasizing the fact that ROS generation is essential for the protective effects of fucoidan both in vitro and in vivo.
Figure 3. Involvement of ROS in in vivo spleen and liver parasite burden, Th1 cytokine stimulation and PTP activity modulated by fucoidan. *L. donovani* infected mice were given various doses of fucoidan (200 mg/kg/day) orally (3 times weekly), after 14 days of infection, for a duration of 4 weeks and NAC was given i.p. at a dose of 300mg/kg daily starting 2 days before infection. (A and B) The parasite burdens in liver (A) and spleen (B) were then determined at 6 weeks after infection. (C, D and E) Splenocytes (2×10⁵ cells) from *L. donovani*-infected, NAC and/or fucoidan (200 mg/kg/day)-treated mice were isolated at various time periods and incubated with 5 pg/mL SLA for 48 h. (C) IL-12, (D) TNF-α and (E) IL-10 levels were determined in culture supernatants by ELISA. (F) PTP activity was assayed in infected, NAC and/or fucoidan treated mice. Results are expressed as the relative increase (n-fold) over PTP activity in control cells. Results shown are representative of three individual experiments. Data represent mean ± SD, n =3. **p<0.01; Student’s t-test.

Discussion

Leishmania parasites survive within the macrophages by suppression of ROS (Olivier et al., 1992a; Olivier et al., 1992b). *Leishmania* parasites are well equipped with the machinery to avoid triggering the oxidative burst by actively inhibiting PKC-mediated NADPH activation. While we have already shown that fucoidan can cure both antimony-susceptible and resistant strains of *L. donovani* via PKC/MAPK/NF-κB pathway, the role of ROS has not been studied previously. *Leishmania* parasite ability to alter the host-signalling leads to functional inhibition macrophages, and this in turn has been shown to involve the host PTP and MAPK phosphatase (Kar et al., 2010). Previously we have already established
that MAPK phosphatase MKP1, MKP3 and PP2A play an important role in deactivation of p38 and ERK MAPK (Kar et al., 2010).

To our knowledge, this is the first study to examine the role of ROS in fucoidan mediated anti-leishmanial effect and their downstream effect on, deactivation of PTPs and MKP1, MKP3 and PP2A. Moreover, we found that ROS quencher NAC can revert the beneficial effects mediated by fucoidan as Th1 cytokine production, activation of p38 and ERK MAPK. The activation of MAPK may be an after effect of neutralization of host PTPs, as we observed that the maximum inhibition of PTP by fucoidan was at 90 min post-treatment, while the optimum activation of MAPK took place at 180 min post-treatment. We have validated our observation further by using fucoidan, a curing agent for experimental visceral leishmaniasis, and showed that inhibition of PTP activity in vivo by fucoidan may be necessary for shifting cytokine balance toward a Th1 mode, and subsequent elimination of organ parasite burden of infected mice. However after using NAC in vivo situations, we found that the protective effects of fucoidan is reverted back, suggesting that deactivation of PTPs by ROS is necessary for maintaining the anti-leishmanial effect of fucoidan.