Chapter I

Fucoidan cures infection with both antimony-susceptible and -resistant strains of Leishmania donovani through Th1 response and macrophage-derived oxidants
Introduction

Visceral leishmaniasis (VL) is a progressive fatal infection caused by the protozoan parasite *Leishmania donovani*. The spectrum of leishmaniasis encompasses 12 million people worldwide with 0.5 million new cases per annum and the severity is further magnified by the emergence of HIV co-infection. Active VL is associated with the absence of parasite-specific cell-mediated immune response (Murray et al., 2005) resulting in clinical symptoms like fever, cachexia, hepatosplenomegaly, anemia and blood cytopenia (Murray et al., 2005). Current first-line chemotherapy to alleviate leishmaniasis relies on a rather limited arsenal of drugs including pentavalent antimonial, amphotericin B, and miltefosine, but these entail either problems of emerging resistance, high toxicity, serious side effects, or high costs (Murray, 2001; Sundar et al., 2000). In India, sodium antimony gluconate (SAG or SbV), the age-old conventional therapy for VL, is no longer useful as a drug because more than 65% of VL patients fail to respond or promptly relapse (Sundar et al., 2000). Thus, identification of new, safer and cheaper drugs that can be effective against both antimony-susceptible and resistant strains of *Leishmania* is of tremendous economic and medical importance. Immunomodulators are becoming popular as alternative to traditional medicine for the treatment of various infectious diseases since they correct immune systems that are out of balance (Barroso et al., 2007; Hernandez-Pando et al., 2005). Fucoidan, an immunomodulatory sulfated polysaccharide mainly composed of L-fucose, is extracted from marine brown algae (Berteau and Mulloy, 2003). It has been approved in Japan and Korea for years at a commercial level (Li et al., 2005; Yang et al., 2006) and has been ascribed with important biological functions including anti-viral, anti-malarial, contraceptive, anti-thrombotic and anti-coagulant activities (Barroso et al., 2008; Chen et al., 2009; Durig et al., 1997; Oehninger et al., 1992; Trinchero et al., 2009). As a ligand for the macrophage scavenger receptor (MSR), fucoidan increased the level of interleukin (IL)-1, tumour necrosis factor (TNF)-α and IL-12, in macrophages and dendritic cells (Kim and Joo, 2008; Yang et al., 2008). Moreover, inducible nitric oxide synthase (iNOS) promoter activation and NO generation in RAW 264.7 cells were also found to be induced by fucoidan (Nakamura et al., 2006).

Pro-inflammatory and modulatory cytokines have an essential role in generating and directing immune response to infectious microbes, including *Leishmania*. Leishmania infection results in impaired microbicidal machinery of macrophages as evidenced by modification of Th1/Th2 paradigm, resulting in parasite survival (Awasthi et al., 2004;
Control of *L. donovani* infection depends on IL-12-driven expansion of Th1 cells, macrophage activation through production of interferon (IFN)-γ and the subsequent generation of NO and ROS (Diefenbach et al., 1999; Sharma and Madhubala, 2009). Because fucoidan can induce Th1 cytokines and NO generation in macrophages, we tested its therapeutic efficacy in both *in vitro* and *in vivo* model of visceral leishmaniasis. Our data demonstrated that fucoidan could confer complete protection to both antimony-susceptible and -resistant *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.

**Materials and methods**

**Parasites, cell culture and infection**

*L. donovani* antimony-susceptible strain AG83 (MHOM/IN/1983/AG83) and antimony-resistant strain GE1F8R (MHOM/IN/89/GE1F8R) were grown as described earlier (Das et al., 2001; Sharma and Madhubala, 2009). Soluble leishmanial antigen (SLA) was prepared from promastigotes by freeze thawing the cell suspension as described earlier (Das et al., 2001). Macrophages were collected by peritoneal lavage from mice (BALB/c, 20-25 g) given i.p. injection of 0.5 mL of 4% thioglycollate broth 5 days before harvest and were used as described earlier (Das et al., 2001). *In vitro* infection experiments were carried out with macrophages using stationary phase promastigotes at a 10:1 parasite/macrophage ratio as described earlier (Das et al., 2001). Cell viability was assessed using an MTT-based colorimetric assay kit (Roche Applied science, Indianapolis, IN) according to the manufacturer’s instructions. Fucoidan was purchased from Sigma and dissolved in phosphate-buffered saline. Endotoxin level of 100 µg/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, MD) following the manufacturer’s manual.

**Infection and fucoidan treatment**

Female BALB/c mice (20-25 g) were injected via the tail vein with 10^7* L. donovani* promastigotes. For reinfection experiments same number of promastigotes was injected 60 days after the first infection. Fucoidan (25-250 mg/kg/day, 3 times weekly), was administered orally for a period of 4 weeks starting at 15th day after infection. Visceral infection was assessed by removing liver and spleen from 6-week infected mice, multiple impression
Cytokine analysis by Real time PCR

To detect the mRNA profile of various cytokines and iNOS, total RNA was extracted from the peritoneal macrophages or splenocytes, isolated as described earlier (Das et al., 2001), using an RNeasy kit (Qiagen, Valencia, CA) (Kar et al., 2010). 1 µg of RNA was used as template for cDNA synthesis and quantitative Real time PCR (ABI 7500 Fast Real-Time PCR system; Applied Biosystems, Foster City, CA) analyses were performed using TaqMan Fast Universal PCR Master Mix (Applied Biosystems) as described earlier (Kar et al., 2010). Taqman probes for various cytokines and iNOS were obtained from Applied Biosystems. Relative quantitation was performed using the comparative ΔΔCt method; data for each sample was normalized to β-actin mRNA levels and expressed as a -fold change compared with respective controls.

Immunoblot analysis

Immunoblot analysis was performed for iNOS as described earlier (Kar et al., 2009).

Cytokine analysis by ELISA

Various cytokines levels in the splenocytes or peritoneal macrophages was measured using a sandwich ELISA Kit (Quantikine M; R&D systems, Minneapolis, MN) as described earlier (Kar et al., 2010). Spleen cells were stimulated with 20 µg/mL SLA for 48 h. The detection limit of these assays was <5.1, <2.5, <4 and <4.6 pg/mL for TNF-α, IL12p70, IL-10 and transforming growth factor (TGF)-β respectively.

Measurement of ROS

To measure the level of ROS, the cell permeable probe H$_2$DCFDA (Sigma, St. Louis, MO) was used as described earlier (Sharma and Madhubala, 2009). Splenocytes from different groups of BALB/c mice were stimulated with SLA (50 µg/mL) for 48 h or left
without SLA stimulation, resuspended in DMEM, and incubated with H2DCFDA (2 μg/mL) at room temperature for 20 min in the dark. Relative fluorescence was measured in a Perkin–Elmer LS50B Spectrofluorometer at an excitation wavelength of 510 nm and emission wavelength of 525 nm. Fluorometric measurements were made in triplicate and expressed as mean fluorescence intensity units.

Quantification of NO

NO is quantified by the accumulation of nitrite in macrophage culture supernatants and nitrite was detected by the Griess reaction as previously described (Kar et al., 2009). For \textit{in vivo} experiment, splenocytes (2 × 10⁵/mL) from different groups of experimental BALB/c mice were stimulated with or without 50 μg/mL SLA for 48 h before nitrite assay.

T cell proliferation assay

The T cell proliferation assay was performed as described elsewhere (Sharma and Madhubala, 2009). Splenocytes (10⁵ cells/well in 96-well plates) were allowed to proliferate for 3 days at 37°C in a 5% CO₂ incubator in the presence or absence of SLA (5 μg/mL). At 18 h before harvesting, cells were pulsed with 1 μCi [³H]-thymidine/well. Incorporation of [³H]-thymidine, as an index of proliferation, was measured using a liquid scintillation counter (Tri-Carb 2100TR; Packard Instrument).

Statistical analysis

The \textit{in vitro} cultures were set in triplicates and the animal experiments were carried out with 5-6 mice per group. Data shown are representative of at least three independent experiments and are expressed as mean ± SD. Student’s t test was employed to assess the statistical significances of differences among pair of data sets with a \( P \) value <0.05 considered to be significant.
**Results**

*Fucoidan induced host protective cytokine response and NO generation in infected macrophages*

We first investigated the ability of fucoidan to induce NO production as well as Th1 response, which imparts a protective immunity against infection. Fucoidan could upregulate NO generation in peritoneal macrophages in a concentration- and time-dependent manner (data not shown) which was found to be maximum at 24 h (15.7 ± 2.1 μM/10^6 cells) at a dose of 50 μg/mL fucoidan (Figure 1A). We then checked whether fucoidan treatment could enhance the generation of NO in infected macrophages. Treatment of fucoidan (50 μg/mL) in *L. donovani*-infected peritoneal macrophages led to a marked generation of NO (13.3 ± 1.4 μM and 11.6 ± 1.1 μM/10^6 cells for AG83 and GE1F8R respectively) at 24 h post-treatment (Figure 1A). As far as iNOS is concerned, 50 μg/mL of fucoidan elicited an 9.75-fold and 8.9-fold (*P*<0.001) increase in iNOS transcripts in peritoneal macrophages infected with AG83 and GE1F8R respectively (Figure 1B). Consistent with the Real Time PCR, the levels of iNOS protein also displayed a significant up regulation (3.6- and 5.1-fold for AG83 and GE1F8R respectively) (Figure 1C). During leishmaniasis, the protective immune response is intricately associated with skewing from anti-inflammatory to pro-inflammatory cytokine response along with generation of NO. Similar to NO, fucoidan treatment increased the levels of IL-12 and TNF-α in a dose- and time-dependent manner with a maximum induction of IL-12 (470 ± 48 pg/mL) and TNF-α (525 ± 53 pg/mL) at 24 h post-treatment (Figure 1D and E) with a dose of 50 μg/mL. We then evaluated the effect of fucoidan on modulating pro- and anti-inflammatory cytokine synthesis in macrophages infected with AG83 and GE1F8R. But the level of IL-12 (61 ± 6.2 pg/mL) and TNF-α (70 ± 7.1 pg/mL) did not appreciably change in control macrophages following infection with AG83 and GE1F8R (Figure 1F). However, both these strains showed a robust surge of IL-10 (8.7- and 8.9-fold increase for AG83 and GE1F8R respectively, *P*<0.001) and TGF-β (9.6- and 9.8-fold increase for AG83 and GE1F8R respectively, *P*<0.001) following infection (Figure 1G). In contrast, fucoidan (50 μg/mL) treatment significantly enhanced the production of IL-12 (7.5- and 6.7-fold increase in AG83- and GE1F8R-infected macrophages respectively, *P*<0.001) and TNF-α (7.1- and 6.4-fold increase in AG83- and GE1F8R-infected macrophages respectively, *P*<0.001) (Figure 1F). In case of anti-inflammatory cytokines, fucoidan could markedly attenuate the level of IL-10 (79% and 75% decrease in AG83- and GE1F8R-infected macrophages
respectively, \( P<0.001 \) and TGF-β (77% and 73% decrease in AG83- and GE1F8R-infected macrophages respectively, \( P<0.001 \)) (Figure 1G). Collectively, these results suggest that fucoidan treatment increases the ability of macrophages to mount an effective Th1 response and NO generation thereby inhibiting intracellular multiplication of amastigotes.

![Figure 1. Effect of fucoidan on NO generation and cytokine response. Peritoneal macrophages from BALB/c mice were infected with either AG83 or GE1F8R promastigotes (macrophage:parasite, 1:10) for 4 h. Non ingested promastigotes were removed by washing, and macrophages were cultered for another 20 h. Infected macrophages were then treated with fucoidan (50 µg/mL) for 24 h. (A) NO production was determined by measuring the accumulation of nitrite in the culture medium by Griess reagent. iNOS expressions at mRNA (B) and protein (C) levels 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. (D and E) Peritoneal macrophages were treated with various concentrations of fucoidan for different time periods. Levels of IL-12 (D) and TNF-α (E) in the culture supernatants were determined by ELISA as described in Materials and Methods. (F and G) Infected macrophages were treated with fucoidan (50 µg/mL) for 24 h and levels of IL-12, TNF-α (F) and IL-10, TGF-β (G) in culture supernatants were determined by ELISA. Bands were analyzed densitometrically. Error bars represent mean ± SD. The data shown are representative from three independent experiments. ** \( P<0.001 \) versus corresponding infected control.

**In vitro leishmanicidal effect of fucoidan**

We then examined the efficacy of fucoidan on intracellular growth of *L. donovani* strains. Fucoidan inhibited amastigote multiplication of both the strains within peritoneal macrophages in a concentration- and time-dependent manner. At a lower dose (25 µg/mL), parasite killing varied between antimonial-sensitive (62%) and -resistant (45%) strains. However, at 50 µg/mL for 24 h the inhibition was almost complete (96% and 93% for AG83
and GE1F8R respectively) (Figure 2A and B). To ascertain the involvement of NO in the inhibition of intracellular amastigote multiplication by fucoidan, the infection index was measured in the presence of an iNOS inhibitor, 2-amino-5,6-dihydro-6- methyl-4H-1,3-thiazine (AMT). The *in vitro* inhibitory effect of fucoidan was markedly reduced in presence of 10 μM AMT (86% and 77% reduction in parasite killing for AG83 and GE1F8R respectively) at 24 h (Figure 2A and B). Fucoidan did not have any direct effect on the viability of either AG83 or GE1F8R promastigotes as judged by MTT assay at a dose of 25 and 50 μg/mL. However, very little inhibition (9% and 7% for AG83 and GE1F8R respectively) was found at a dose of 100 μg/mL for 48 h (Figure 2C and D). To further evaluate the safety index of fucoidan, its effect on viability of murine peritoneal macrophages was evaluated. Macrophage viability remained unaffected up to 150 μg/mL of fucoidan (Figure 2E). 50 μg/mL of fucoidan was chosen for subsequent experiments, as it showed no cytotoxicity and maximum generation of NO and Th1 response.
Figure 2. Effect of fucoidan on in vitro proliferation of antimony-susceptible and -resistant *L. donovani* strains.

Peritoneal macrophages were infected with either AG83 or GE1F8R promastigotes as described in the legend of figure 1. Infected macrophages were then treated with various concentrations of fucoidan for 24 and 48 h. In another set of experiments, AMT (10 μM) was given along with fucoidan. Intracellular parasite number for AG83 (A) and GE1F8R (B) was determined by Giemsa staining. The bar diagrams show the number of parasites per 100 peritoneal macrophages. Exponential-phase promastigotes (2*10^5/200 μL per well) of AG83 (C) and GE1F8R (D) were incubated with increasing concentrations of fucoidan (25-100 μg/mL) for 24 and 48 h and cell viability was assessed using a MTT-based colorimetric assay. (E) Peritoneal macrophages were incubated with varying concentrations of fucoidan (25-150 μg/mL) for different time periods. Cell viability was assessed by MTT method. Results are representative of three independent experiments and data shown are mean ± SD at each time point. * P<0.01, ** P<0.001 versus respective infected control.
Effective intramacrophage parasite suppression and apparent immunomodulatory effect of fucoidan paved the way for its evaluation as a candidate antileishmanial agent in murine model of visceral leishmaniasis. BALB/c mice (6 weeks old, ~25 g) were infected intravenously with *L. donovani* AG83 or GE1F8R promastigotes, as described in Materials and Methods. Fucoidan was administered orally with a dose range of 25–250 mg/kg/day, given three times weekly, starting at 15th day of infection. After 6 weeks, antileishmanial potency was assessed in terms of liver and spleen parasite burden. During the experiment, all the animals remained healthy and no marked effect on body weight was noted in any of the experimental groups. The dose titration experiment suggested very little control of infection (10% and 12% suppression in splenic parasite burden for AG-83- and GE1F8R-infected mice respectively) at a lower dose of fucoidan (25 mg/kg/day). However, a dose-related inhibition was noted at higher doses, and at 150 mg/kg/day there was 75% and 83% suppression in splenic parasite burden for AG83- and GE1F8R-infected mice respectively (Figure 3A and B). The inhibition was almost complete at 200 mg/kg/day of fucoidan, with almost complete suppression of liver and spleen parasite burden for both the strains (Figure 3A and B). Absence of parasites in the spleen was further confirmed by culturing spleen specimens in transformation medium at 22°C for 96 h. To further ascertain whether fucoidan had conferred long-standing immunity, cured mice were later reinfected i.v. 60 days after primary infection. Spleen parasite burden in the reinfected animals progressed prominently in placebo-treated BALB/c mice, whereas fucoidan (200 mg/kg/ day)-treated mice were largely resistant, as observed up to 7 weeks (Figure 3C). Thus, fucoidan therapy might exert an acquired protective immunity against both antimony-sensitive and -resistant strains in vivo.
Effect of fucoidan on proliferation of splenocytes and T cell immune response in vivo

Visceral leishmaniasis is associated with impaired T cell proliferation and cell-mediated immunity which is reflected by marked T cell anergy specific to *Leishmania* antigens (Carvalho et al., 1994; Gifawesen and Farrell, 1989). We then investigated whether this could be reversed by fucoidan. As observed, splenocytes of mice infected with either AG83 or GE1F8R failed to mount antileishmanial T cell response to SLA (5 μg/mL). In contrast, at a similar antigen concentration, 12.8- and 11.2-fold increase in splenic T cell proliferation were observed after fucoidan treatment in AG83- and GE1F8R-infected mice at
4 weeks post infection (Figure 4A). To further evaluate the type of immunological response, a detailed splenic cytokine analysis was performed by ELISA in *L. donovani*-infected mice after fucoidan treatment. Comparative cytokine profile showed that in both groups of infected (AG83 and GE1F8R)-mice, at 4 weeks, IFN-γ, IL-12 and TNF-α showed 7.5-, 6.4- and 5.9-fold increases (*P* < 0.001) in AG-83-infected fucoidan-treated animals (Figure 4B) whereas those were 6.5-5.4- and 5.8-fold (*P* < 0.001) in GE1F8R-infected animals (Figure 4D). On the other hand, fucoidan-treated AG83-infected mice showed 79% and 75% decrease (*P* < 0.001) in IL-10 and TGF-β protein synthesis (Figure 4C) whereas those were 72% and 70% (*P* < 0.001) for GE1F8R-infected animals (Figure 4E). Consistent with our ELISA studies, transcript levels of various pro- and anti-inflammatory cytokines in spleen cells of fucoidan-treated infected animals showed similar results as assessed by real time PCR (data not shown). Collectively, these results suggest that in *in vivo* situation, fucoidan could confer protection against both antimony-sensitive and-resistant strains by inducing lympho-proliferation of splenocytes as well as by switching the cytokine balance towards host protective Th1 mode.

![Figure 4](image-url)
incorporation of [3H] thymidine. (b-e) Splenocytes (2 × 10^6 cells) from L. donovani (AG83 or GE1F8R)-infected and fucoidan (200 mg/kg/day)-treated mice were isolated at various time periods and incubated with 5 μg/mL SLA for 48 h. Levels of cytokines in culture supernatants were determined by ELISA. (B) IFN-γ, TNF-α, IL-12 and (C) IL-10, TGF-β levels of AG83-infected and (D) IFN-γ, TNF-α, IL-12 and (E) IL-10, TGF-β levels of GE1F8R-infected. Results are representative of one of three individual experiments. Data represent mean ± SD. **P<0.001 versus as indicated.

Effect of fucoidan on NO and ROS generation ex vivo

ROS and NO are two potent macrophage-derived microbicidal molecules that are critical in controlling Leishmania infection (Basu et al., 2005; Sharma and Madhubala, 2009). We, therefore, estimated the generation of ROS and NO in the culture supernatants of splenocytes isolated from L. donovani-infected (AG83 and GE1F8R) and fucoidan-treated mice up to 6 weeks of infection. After stimulation by SLA (50 μg/mL), splenocytes from both AG83- and GE1F8R-infected fucoidan (200 mg/kg/day)-treated mice showed 5.7- and 4.9-fold induction of ROS generation respectively, compared to corresponding infected controls after 4 weeks of infection (Figure 5B). Similarly, markedly increased nitrite generation was detected in fucoidan-treated mice. SLA stimulation resulted in only 3.5 μM/10^6 cells and 3.3 μM/10^6 cells nitrite production in the splenocytes of AG83- and GE1F8R-infected mice after 4 weeks of infection whereas those were 28.4 μM/10^6 cells and 24.5 μM/10^6 cells in fucoidan-treated mice (Figure 5A). Significantly enhanced generation of ROS and NO in fucoidan-treated mice further suggests the overall activated state of spleen cells for successful elimination of both antimony-sensitive and -resistant parasites in vivo.

**Figure 5.** In vivo generation of ROS and NO from infected fucoidan-treated mice. BALB/c mice were infected with L. donovani (AG83 or GE1F8R) promastigotes followed by treatment with fucoidan (200 mg/kg/day) as described in the legend of Figure 4. Splenocytes (2 × 10^6 cells) from different groups of experimental mice were isolated at various time periods as indicated and incubated with 50 μg/mL SLA for 48 h in a 5% CO2 incubator at 37 °C. (A) The culture supernatant of splenocytes was used to evaluate NO generation by Griess method. (B) ROS generation was measured by H2DCFDA probe staining of the splenocytes from different groups of mice. Results are representative of one of three individual experiments and the error bars represent mean ± SD. **P < 0.001 versus corresponding infected control.
Discussion

Fucoidan, a sulfated polysaccharide, naturally found in cell wall matrix of brown algae has been endowed with a number of immunomodulatory activities and medicinal properties (Berteau and Mulloy, 2003; Chen et al., 2009; Kim and Joo, 2008; Trinchero et al., 2009). In the present study, we have demonstrated the superior efficacy of fucoidan in eliminating intracellular amastigotes of both antimony-sensitive and -resistant *L. donovani* in *in vitro* macrophage model and *in vivo* mouse model of visceral leishmaniasis. This is associated with induction of disease-resolving Th1 cytokine response as well as generation of ROS and NO. Moreover, reinfection of cured animals resulted in only a slight and transient increase in organ parasite burden suggesting thereby that fucoidan therapy might also have exerted a long-term protective immunity. Although mouse model for *L. donovani* has been widely studied but it suffers from the limitation that it does not reproduce all the features of active human VL. In mouse model there is an early increase in parasite burden but after 8 weeks the infected animal is able to mount antileishmanial cellular immune response that control parasite replication. Hamster model for VL is perhaps the best model as it closely mimics active human disease. Although clinicopathologic features and immunopathologic mechanisms of VL in the hamster model are remarkably similar to the human disease, the non-availability of immunological tools for hamster model restricts researchers to use BALB/c mice for most of the immunological studies. Even though BALB/c mice differ from symptomatic human subjects with *L. donovani* infection, it is a good experimental model for early parasite replication with similar immunopathological features of human VL.

Growing body of evidences suggests that control of leishmaniasis in susceptible mice invariably promote Th1 over Th2 response (Awasthi et al., 2004; Kemp et al., 1993). In the present study, *in vitro* treatment with fucoidan at a dose of 50 μg/mL resulted in acquired resistance to both antimony-sensitive and -resistant strains of *L. donovani* by switching over the release of disease-promoting Th2 (IL-10, TGF-β) cytokines to disease-resolving Th1 (IL-12, TNF-α) cytokines. In line with our *in vitro* findings, mice treated with fucoidan were indeed cured as shown by the complete suppression of liver and spleen parasite burden and reversion of increased spleen size (data not shown). IFN-γ, a signature Th1 cytokine that has a dominant effect on macrophage microbicidal machinery, was found to be significantly elevated in fucoidan-treated infected mice. Th1 cell-mediated leishmanicidal events induced in IL-10-deficient mice are known to require IFN-γ that is largely induced by IL-12 (Murray, 2000). In the present study also, IL-12 was markedly down-regulated in the AG83 and...
GE1F8R-infected mice, but significantly enhanced at both RNA and protein level in both
groups of mice after fucoidan treatment. The synergistic induction of IL-12 with IFN-γ might
have a strong additive effect in clearing parasitemia. Fucoidan also increased the levels of
TNF-α, another inflammatory cytokine with well-defined antileishmanial effects that is
known to act either alone or with IFN-γ to induce the production of reactive nitrogen and
oxygen intermediates (Roach et al., 1991). Furthermore, IL-10 and TGF-β, Th1 suppressive
cytokines, were found to be profoundly down-regulated in infected fucoidan-treated mice.
TGF-β, a pleiotropic cytokine with diverse functions, is also known to inhibit the activities of
immune cells and was found to be significantly down-regulated in fucoidan-treated mice
compared to both groups of infected (AG83 and GE1F8R) control. Induction of iNOS and
subsequent release of nitrogen metabolites are vital for the cure of visceral leishmaniasis
(Basu et al., 2005; Das et al., 2001). However both reactive nitrogen or oxygen intermediates
play an important role as inhibition of either RNI or ROS pathway prevent macrophage-
mediated killing of *L. donovani* (Gantt et al., 2001; Roach et al., 1991). An increased
generation of ROS was also found in splenocytes from fucoidan-treated AG83- and GE1F8R-
infectected mice after stimulation with SLA. Increased ROS generation in response to SLA
might have additionally contributed to the efficiency of parasite killing. The pronounced
enhancement in NO and ROS in response to SLA stimulation is in agreement with a previous
study which showed that combined therapy of SAG and peroxovanadium compounds in
infected BALB/c mice could suppress organ parasite burden by increased ROS and NO
generation (Haldar et al., 2009).

In summary, successful parasite clearance and acquired resistance to reinfection by
susceptible BALB/c mice during fucoidan therapy may be attributed to 1) the direct action of
fucoidan for the induction of NO and ROS and 2) the switch of CD4+ T cell-mediated
immune responses from Th2 to Th1 mode. The existing antileishmanials for debilitating VL
are very few and are challenged by the widespread emergence of drug resistance. Attempts to
produce derivatives for oral treatment are of particular importance with obvious advantage of
ease of application and reduction of treatment cost. The pharmacological safety of fucoidan is
ensured by non-toxic consumption of up to 1 g/day in humans (Irhimeh et al., 2007).
Moreover, oral fucoidan in clinical trial has been shown to significantly amplify CXCR4-
expressing CD34+ cells leading to tumor suppression and metastasis (Irhimeh et al., 2007).
Establishment of an appropriate T cell response by fucoidan suggests that this natural product
could be used as a potential immunomodulator to generate the required immunity not only for
the treatment of nonhealing leishmaniasis, but also for the treatment of other chronic infectious diseases.