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

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Objectives: The aim of this study was to evaluate and characterize the antileishmanial efficacy of fucoidan, a polyanionic sulphated polysaccharide from brown algae, in experimental infections of BALB/c mice with antimony-susceptible (AG83) and -resistant (GE18ER) *Leishmania donovani*.

Methods: The effect of fucoidan was assessed against intracellular parasites in cultured macrophages and in suppressing splenic and liver parasite burdens in a BALB/c mouse model of visceral leishmaniasis by microscopic evaluation of surviving intracellular amastigotes stained with Giemsa. To evaluate the type of immunological responses, real-time PCR and ELISA were performed for various Th1 and Th2 cytokines in both *in vitro* and *in vivo* infected conditions. To determine the effector mechanism, reactive oxygen species (ROS) and NO were measured in fucoidan-treated animals by H_2DCFDA-based fluorometric analysis and Griess reaction, respectively.

Results: In addition to having appreciable inhibitory effect on amastigote multiplication within macrophages (>93% inhibition at 50 μg/mL), complete elimination of liver and spleen parasite burden was achieved by fucoidan at a dose of 200 mg/kg/day given orally, 3 times weekly, in a 6-week mouse model of both antimony-susceptible and -resistant strains. This curative effect is associated with switching of T cell differentiation from Th2 to Th1 mode. Further, splenocytes of fucoidan-treated infected (AG83 and GE18FR) mice generated significantly enhanced levels of superoxide and NO. Not only was this treatment curative when administered orally 15 days post-infection, but it also imparted resistance to reinfection.

Conclusions: These results suggest the effectiveness of fucoidan as potent immunomodulator for controlling both antimony-susceptible and -resistant visceral leishmaniasis.

Keywords: visceral leishmaniasis, T cell response, nitric oxide, reactive oxygen species

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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 a parasite-specific cell-mediated immune response[1] resulting in clinical symptoms like fever, cachexia, hepatosplenomegaly, anaemia and blood cytopenia.[2] Current first-line chemotherapy to alleviate leishmaniasis relies on a rather limited arsenal of drugs, including pentavalent antimonials, amphotericin B, and miltefosine, but these entail either problems of emerging resistance, high toxicity, serious side effects, or high costs.[3,4] 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.[4] 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 an alternative to traditional medicine for the treatment of various infectious diseases since they correct immune systems that are out of balance.[5,6] Fucoidan, an immunomodulatory sulphated polysaccharide mainly composed of L-fucose, is extracted from marine brown algae.[7] It has been approved in Japan and Korea for years at a commercial level[8,9] and has been ascribed with important biological functions

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including antiviral, anti-malarial, contraceptive, anti-thrombotic and anti-coagulant activities.[10–14] As a ligand for the macrophage scavenger receptor (MSR), fucoidan increases the level of interleukin (IL)-1, tumour necrosis factor (TNF)-α and IL-12 in macrophages and dendritic cells.[15,16] Moreover, inducible nitric oxide synthase (iNOS) promoter activation and NO generation in RAW 264.7 cells were also found to be induced by fucoidan.[17]

Pro-inflammatory and modulatory cytokines have an essential role in generating and directing the 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.[18,19] 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 reactive oxygen species (ROS).[20,21] Because fucoidan can induce Th1 cytokines and NO generation in macrophages, we tested its therapeutic efficacy in both *in vitro* and *in vivo* models 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 previously.[21,22] Soluble leishmanial antigen (SLA) was prepared from promastigotes by freeze thawing the cell suspension as described previously.[22] Macrophages were collected by peritoneal lavage from mice (BALB/c, 20–25 g) given intraperitoneal (ip) injection of 0.5 mL of 4% thioglycollate broth 5 days before harvest and were used as described previously.[22] *In vitro* infection experiments were carried out with macrophages using stationary phase promastigotes at a 10:1 parasite/macrophage ratio as described previously.[22] Cell viability was assessed using an MTT-based colorimetric assay kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions. Fucoidan was purchased from Sigma and dissolved in PBS. The 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, USA) 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, the same number of promastigotes were 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 on the 15th day after infection. Visceral infection was assessed by removing liver and spleen from 6-week-infected mice; multiple impression smears were prepared and stained with Giemsa. Spleen or liver parasite burdens, expressed as Leishman–Donovan units (LDU), were calculated as the number of parasites per 1000 nucleated cells×organ weight (in grams).[22] The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23 revised 1996) and had the approval of the Institutional Animal Care and Use Committee.

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 and isolated as described previously,[22] using an RNeasy kit (Qiagen, Valencia, CA, USA).[23] RNA (1 μg) was used as a template for cDNA synthesis and quantitative real-time PCR (ABI 7500 Fast Real-Time PCR System; Applied Biosystems, Foster City, CA, USA) analyses were performed using TaqMan Fast Universal PCR Master Mix (Applied Biosystems) as described previously.[23] Taqman probes for various cytokines and iNOS were obtained from Applied Biosystems. Relative quantification was performed using the comparative ΔΔCt method; data for each sample were 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 previously.[24]

Cytokine analysis by ELISA

Various cytokine levels in the splenocytes or peritoneal macrophages were measured using a sandwich ELISA Kit (Quantikine M; R&D Systems, Minneapolis, MN, USA) as described previously.[23] 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, IL-12p70, IL-10 and transforming growth factor (TGF)-β, respectively.

Measurement of ROS

To measure the level of ROS, the cell permeable probe H_2DCFDA (Sigma, St Louis, MO, USA) was used as described previously.[21] 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 H_2DCFDA (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 was quantified by the accumulation of nitrite in macrophage culture supernatants and nitrite was detected by the Griess reaction as previously described.[24] For *in vivo* experiments, splenocytes (2×10^6/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.[21] Splenocytes (10^5 cells/well in 96-well plates) were allowed to proliferate for 3 days at 37°C in a 5% CO_2 incubator in the presence or absence of SLA (5 μg/mL). At 18 h before harvesting, cells were pulsed with 1 μCi of [^{3}H]thymidine/well. Incorporation of [^{3}H]thymidine, as an index of proliferation, was measured using a liquid scintillation counter (Tri-Carb 2100TR; Packard Instrument).

Statistical analysis

The *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 pairs of datasets with a *P* value <0.05 considered to be significant.

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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 up-regulate NO generation in peritoneal macrophages in a concentration- and time-dependent manner (data not shown) that was found to be maximal 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 was concerned, 50 μg/mL fucoidan elicited a 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

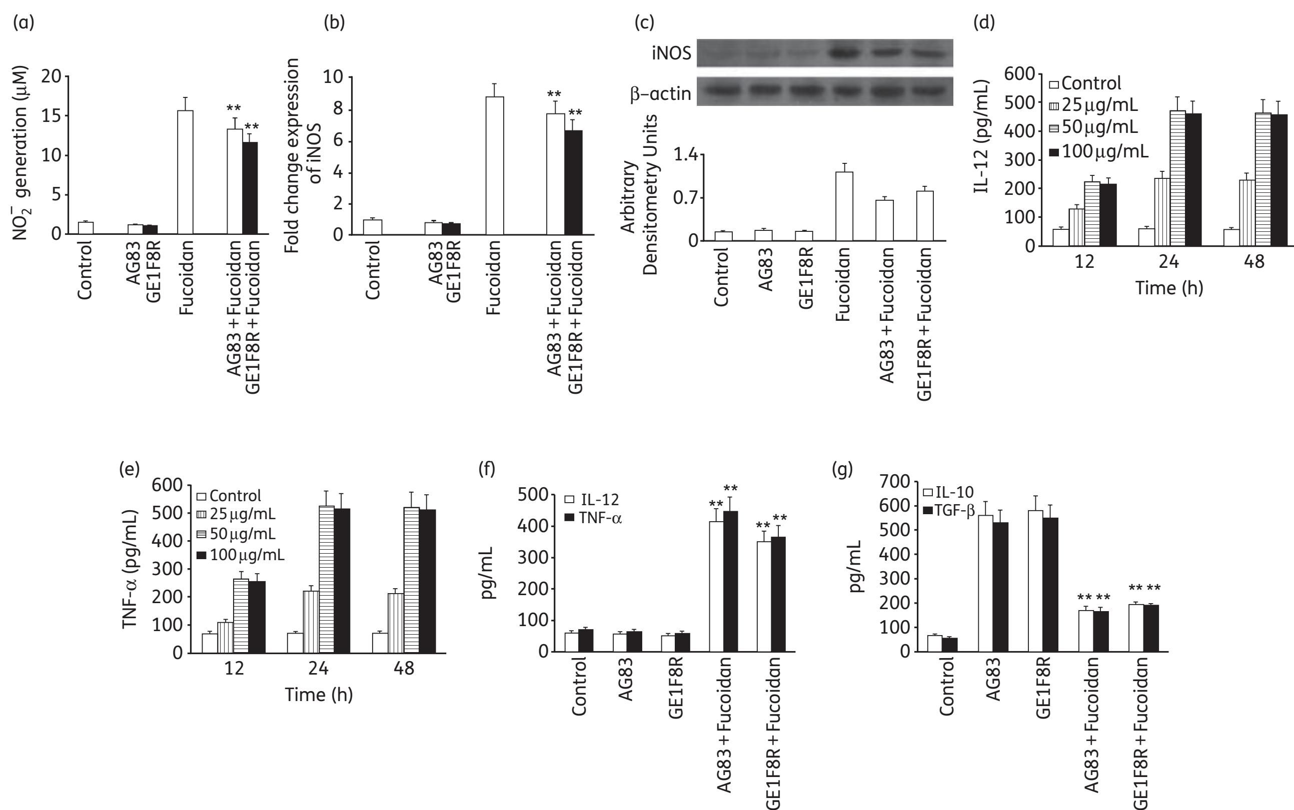

Figure 1. Effect of fucoidan on NO generation and cytokine response. Peritoneal macrophages from BALB/c mice were infected with either AG83 or GE1F8R promastigotes (macrophage/parasite, 1:10) for 4 h. Non-ingested promastigotes were removed by washing, and macrophages were cultured for another 20 h. 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 the Materials and methods section. (f and g) Infected macrophages were treated with fucoidan (50 μg/mL) for 24 h and levels of IL-12 and TNF-α (f) and IL-10 and TGF-β (g) in culture supernatants were determined by ELISA. Bands were analysed densitometrically. Error bars represent means ± SD. The data shown are representative of three independent experiments. **$P<0.001$ versus corresponding infected control.

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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 markedly attenuated 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.

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-susceptible (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-4*H*-1,3-thiazine (AMT). The *in vitro* inhibitory effect of fucoidan was markedly reduced in the 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 the viability of murine peritoneal macrophages was evaluated. Macrophage viability remained unaffected up to 150 μg/mL fucoidan (Figure 2e); 50 μg/mL fucoidan was chosen for subsequent experiments, as it showed no cytotoxicity and maximum generation of NO and Th1 response.

In vivo antileishmanial efficacy of fucoidan against antimony-susceptible and -resistant strains

Effective intramacrophage parasite suppression and the apparent immunomodulatory effect of fucoidan paved the way for its evaluation as a candidate antileishmanial agent in a 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 the Materials and methods section. Fucoidan was administered orally with a dose range of 25–250 mg/kg/day, given 3 times weekly, starting on the 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

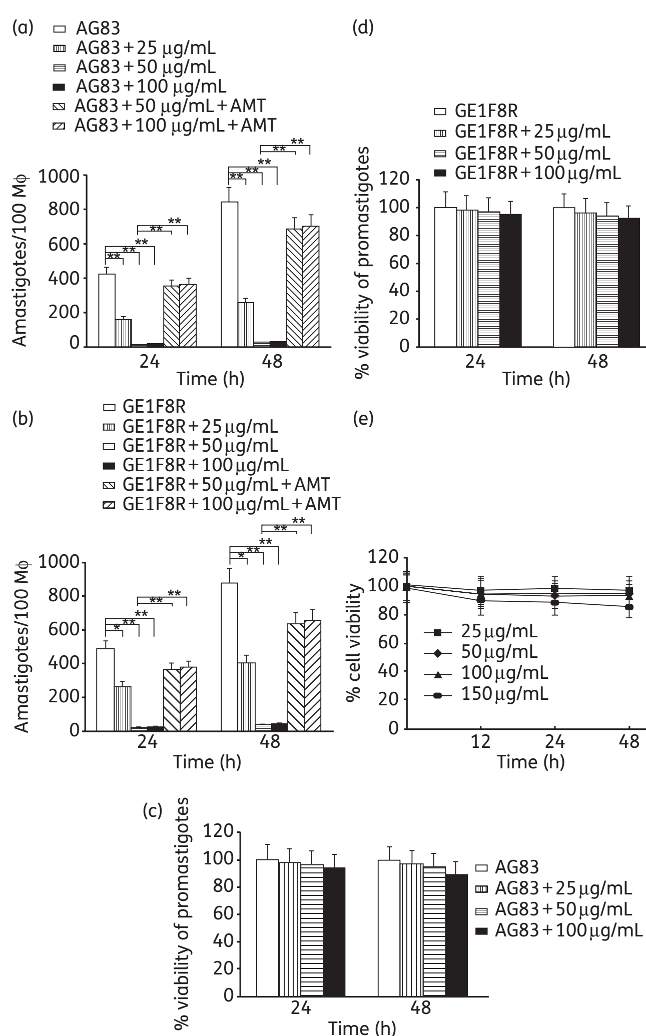

Figure 2. Effect of fucoidan on *in vitro* proliferation of antimony-susceptible and -resistant *L. donovani* strains. Peritoneal macrophages were infected with either AG83 or GE1F8R promastigotes as described in the legend of Figure 1. Infected macrophages were then treated with various concentrations 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 an MTT-based colorimetric assay. (e) Peritoneal macrophages were incubated with various concentrations of fucoidan (25–150 μg/mL) for different time periods. Cell viability was assessed by the MTT method. Results are representative of three independent experiments and data shown are means±SD at each timepoint. *$P<0.01$ and **$P<0.001$ versus respective infected control.

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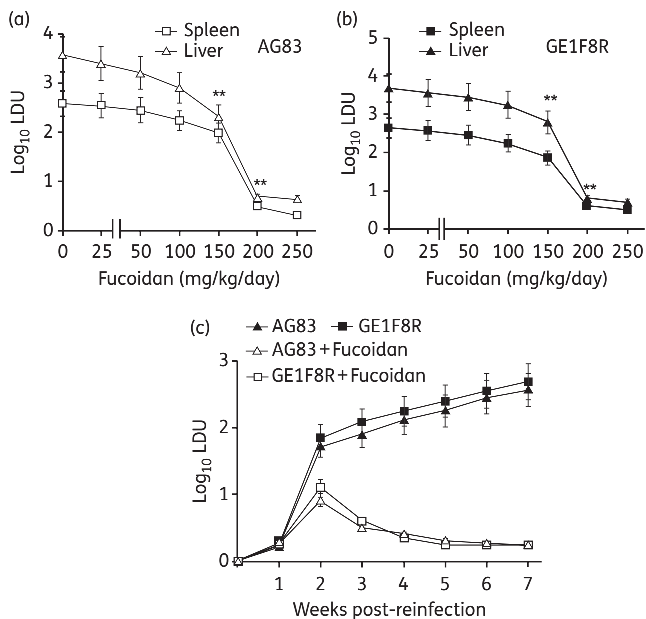

Figure 3. Effect of fucoidan therapy on visceral infection in BALB/c mice. Various doses of fucoidan (25–250 mg/kg/day) were given orally (3 times weekly) for a period of 4 weeks starting on the 15th day after infection. The parasite burdens in liver and spleen for AG83-infected (a) and GE1F8R-infected (b) mice were then determined at 6 weeks after infection. (c) The course of visceral reinfection was studied by intravenous administration of 1×10^7 *L. donovani* (AG83 or GE1F8R) promastigotes into naive, age-matched BALB/c mice and cured (200 mg/kg/day fucoidan-treated) mice. The progression of infection was monitored by determining the spleen parasite burden, expressed as LDU, up to 7 weeks after reinfection. The results are representative of three independent experiments and data shown are means±SD at each timepoint. **P*<0.01 and ***P*<0.001 versus corresponding infected control.

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 intravenously 60 days after primary infection. Spleen parasite burden in the reinfected animals progressed rapidly 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-susceptible 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.[25,26] 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. A 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 AG83-infected fucoidan-treated animals (Figure 4b), whereas these 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 a 79% and 75% decrease (*P*<0.001) in IL-10 and TGF-β protein synthesis (Figure 4c), whereas these 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* situations, fucoidan could confer protection against both antimony-susceptible and -resistant strains by inducing lymphoproliferation of splenocytes as well as by switching the cytokine balance towards host protective Th1 mode.

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.[21,27] 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 after 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 with 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 $\mu M/10^6$ cells and 3.3 $\mu M/10^6$ cells nitrite production in the splenocytes of AG83- and GE1F8R-infected mice after 4 weeks of infection, whereas these were 28.4 $\mu M/10^6$ cells and 24.5 $\mu M/10^6$ 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-susceptible and -resistant parasites *in vivo*.

Discussion

Fucoidan, a sulphated polysaccharide, naturally found in the cell wall matrix of brown algae has been endowed with a number of immunomodulatory activities and medicinal properties.[7,10,11,16] In the present study, we demonstrated the superior efficacy of fucoidan in eliminating intracellular amastigotes of both antimony-susceptible and -resistant *L. donovani* in *in vitro* macrophage models and *in vivo* mouse models of visceral leishmaniasis. This is associated with induction of disease-resolving

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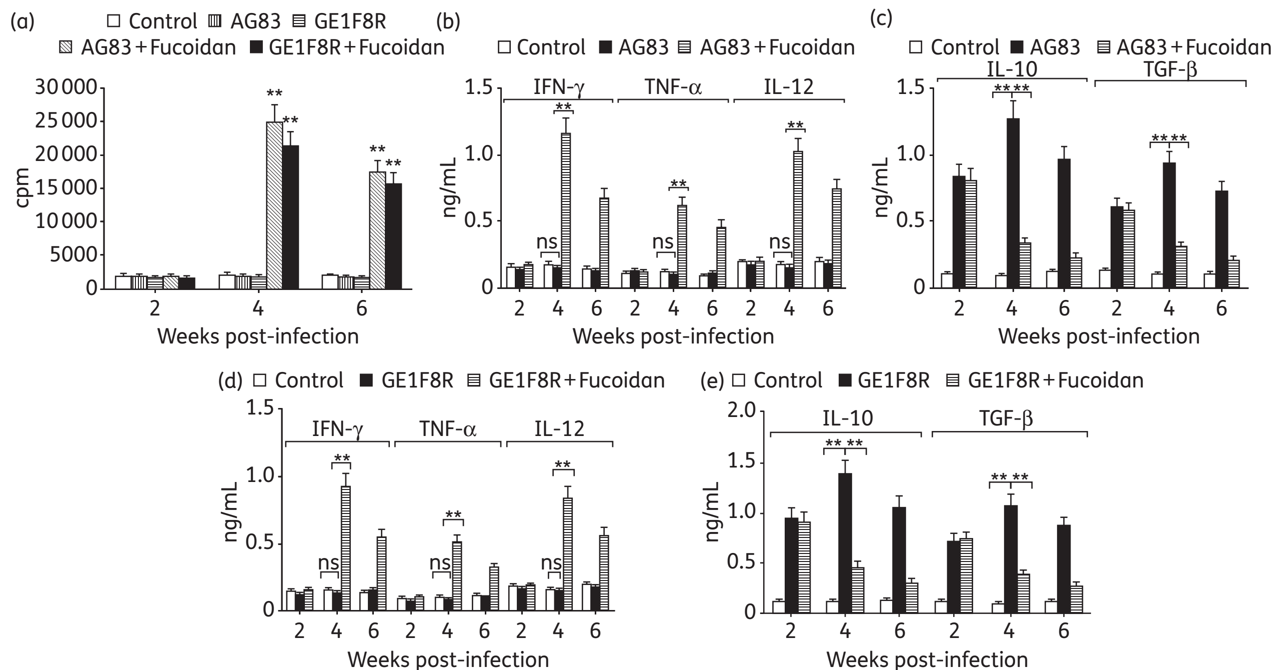

Figure 4. Effect of fucoidan on T cell response of *L. donovani*–infected mice. (a) BALB/c mice were infected with *L. donovani* (AG83 or GE1F8R) promastigotes followed by treatment with fucoidan (200 mg/kg/day) as described in the legend of Figure 3. Splenocytes (2×10^6 cells) from different groups of experimental mice were isolated at various time periods as indicated and incubated with 5 μg/mL SLA in a 5% CO_2 incubator at 37°C. T cell proliferation was measured by incorporation of [^{3}H]thymidine. (b–e) Splenocytes (2×10^5 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 and IL-12; (c) IL-10 and TGF-β levels of AG83-infected mice; (d) IFN-γ, TNF-α and IL-12; and (e) IL-10 and TGF-β levels of GE1F8R-infected mice. Results are representative of one of three individual experiments. Data represent means ± SD. $^{**}P<0.001$ versus as indicated.

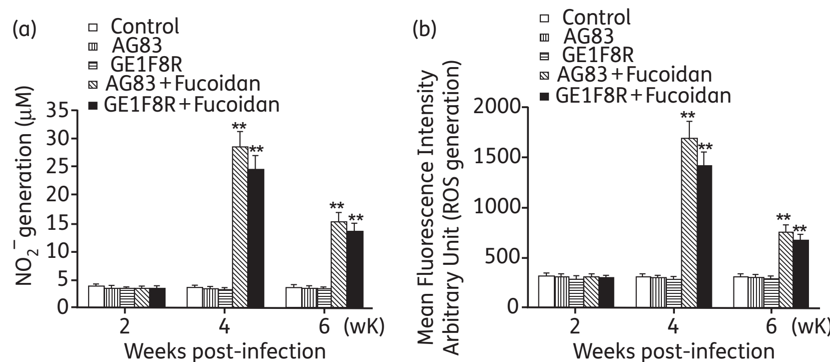

Figure 5. *In vivo* generation of ROS and NO from infected fucoidan-treated mice. BALB/c mice were infected with *L. donovani* (AG83 or GE1F8R) promastigotes followed by treatment with fucoidan (200 mg/kg/day) as described in the legend of Figure 4. Splenocytes (2×10^6 cells) from different groups of experimental mice were isolated at various time periods as indicated and incubated with 50 μg/mL SLA for 48 h in a 5% CO_2 incubator at 37°C. (a) The culture supernatant of splenocytes was used to evaluate NO generation by the Griess method. (b) ROS generation was measured by H_2DCFDA probe staining of the splenocytes from different groups of mice. Results are representative of one of three individual experiments. Data represent means ± SD. $^{**}P<0.001$ versus corresponding infected control.

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 that fucoidan therapy might also have exerted a long-term protective immunity. Although the mouse model for *L. donovani* has been widely studied, it suffers from the limitation that it does not reproduce all the features of active human VL. In the mouse model there is an early increase in parasite burden, but after 8 weeks the infected animal is able to mount an antileishmanial cellular immune response that controls parasite replication. The hamster model for VL is perhaps the best model, as it closely mimics active human disease. Although clinicopathological

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features and immunopathological mechanisms of VL in the hamster model are remarkably similar to the human disease, the non-availability of immunological tools for the hamster model restricts researchers to the use of 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 immunopathological features similar to human VL.

A growing body of evidence suggests that control of leishmaniasis in susceptible mice invariably promotes Th1 over Th2 response.[18,28] In the present study, *in vitro* treatment with fucoidan at a dose of 50 μg/mL resulted in acquired resistance to both antimony-susceptible 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.[29] In the present study also, IL-12 was markedly down-regulated in the AG83- and GE1F8R-infected mice, but significantly enhanced at both the 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 parasitaemia. 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.[30] Furthermore, IL-10 and TGF-β, both 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 with both groups of infected (AG83 and GE1F8R) controls. Induction of iNOS and subsequent release of nitrogen metabolites are vital for the cure of visceral leishmaniasis.[22,27] However, both reactive nitrogen and oxygen intermediates play an important role, as inhibition of either the reactive nitrogen intermediate or ROS pathway prevents macrophage-mediated killing of *L. donovani*.[30,31] An increased generation of ROS was also found in splenocytes from fucoidan-treated AG83- and GE1F8R-infected 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 with SAG and peroxovanadium compounds in infected BALB/c mice could suppress organ parasite burden by increased ROS and NO generation.[32]

In summary, successful parasite clearance and acquired resistance to reinfection by susceptible BALB/c mice during fucoidan therapy may be attributed to (i) the direct action of fucoidan for the induction of NO and ROS and (ii) 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 the obvious advantages 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.[33] Moreover, oral fucoidan in clinical trials has been shown to significantly amplify CXCR4-expressing CD34+ cells, leading to tumour suppression and metastasis.[33] 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 non-healing leishmaniasis, but also for the treatment of other chronic infectious diseases.

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Transparency declarations

None to declare.

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