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Toll-like receptor 2-dependent activation of monocytes by *Spirulina* polysaccharide and its immune enhancing action in mice

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Abstract

We reported previously that a high molecular weight polysaccharide fraction (Immulina) from *Spirulina* was a potent activator of NF-kappa B and induced both IL-1 β and TNF-α mRNAs in THP-1 human monocytes. In the present study, we show that NF-kappa B activation by Immulina is suppressed by antibodies to CD14 and TLR2 but not by antibodies to TLR4. Similarly, NF-kappa B directed luciferase expression was enhanced by Immulina treatment when cells were co-transfected with vectors expressing proteins supporting TLR2- (CD14 and TLR2) but not TLR4-(CD14, TLR4, and MD-2) dependent activation. Mice that consumed a chemically defined chow mixed with an extract containing Immulina exhibited changes in several immune parameters. The *ex vivo* production of IgA and IL-6 from Peyer's patch cells was enhanced 2-fold and interferon-gamma production from spleen cells was increased 4-fold in Immulina-treated mice. The enhanced production of these factors was most notable with mice that had consumed this extract for 4 or 5 days. These studies shed light on how Immulina activates cells of the innate immune system and suggests that oral consumption of this polysaccharide can enhance components within both the mucosal and systemic immune systems.

Keywords: Spriulina extract; Immulina; Toll-like receptor; Mucosal immune; Systemic immune

1. Introduction

Spirulina is a microalgae rich in protein (60–70%) and other nutrients and has been consumed by humans for hundreds of years as food, health drinks or as nutritional supplements. Although early interest in commercial production of *Spirulina* was focused mainly on its nutrient content, recent attention has been given to its therapeutic properties such as antioxidant effects, immunomodulation, anticancer potency, antiviral and cholesterol regulatory properties [1].

The immune enhancing properties of *Spirulina* were first reported in mice in 1994 [2]. Subsequently, other studies have also demonstrated the immunomodulatory action of *Spirulina* in various animal models such as in chickens [3,4], cats [5], dogs [6] and also in humans [7,8]. The immunomodulatory action of *Spirulina* has been suggested by some researchers to be mediated through the innate immune system [7].

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In most of the above-mentioned reports, the investigators have either used *Spirulina* powder or its hot water extract to study immune effects. Bioactive phycocyanin and water soluble polysaccharides of *Spirulina* may be responsible for its enhanced biological defense activity against infectious diseases and reduction of allergic inflammation through sustaining the functions of the mucosal immune system. These components were reported to cause immune modulation via increased proliferation of erythrocytes, granulocyte–monocyte, and fibroblast lineage cells derived from bone marrow cells of mice [8].

We reported previously [9] that a high molecular weight polysaccharide fraction (Immulina) from *Spirulina* was a potent activator of NF-kappa B and induced both IL-1 β and TNF-α mRNAs in THP-1 human monocytes. Although this Immulina preparation exhibited more potent activity than *Escherichia coli* LPS for *in vitro* activation of NF-kappa B in monocytes, its *in vivo* effects after oral administration remain undefined. In the present study, we identify some immunological parameters affected by this preparation and in addition show that a member of the Toll-like receptor (TLR) family at least partially mediates the cellular response to these polysaccharides.

2. Materials and methods

2.1. Preparation of Spirulina extract

A crude extract was prepared from *Spirulina platensis* (obtained from Cyanotech Corporation) using a patent-pending procedure [10]. Raw material was extracted two times with 50% ethanol at 70 °C, 45 min each time. Supernatants from both extractions were combined following centrifugation for 5 min at 1500×*g*. The ethanol concentration of the extract was adjusted to 75% by addition of 1 volume of cold ethanol. Following incubation for several hours at −20 °C, precipitable material was collected by centrifugation at 1500×*g* and subsequently washed with cold ethanol. The final extract material was dried and represented a 15% yield of raw material dry weight.

2.2. Preparation of high molecular weight polysaccharide fraction (Immulina)

The high molecular weight Immulina polysaccharide fraction was purified from the crude *Spirulina* extract. The crude dried extract was dissolved in water and partitioned against water-saturated *n*-butanol (1:1) two times. The water layer was then passed through an ultrafiltration device with a 100,000 molecular weight cut-off polyethersulfone membrane (Centricon Plus-20 from Millipore). The high molecular weight retentate was freeze-dried and represented 25–30% of crude *Spirulina* extract dry weight.

2.3. Monocyte activation assay

The THP-1 human monocyte cell line was transfected with a luciferase reporter gene construct containing two copies of NF-kappa B motif from HIV/IgK as described previously [11]. Monoclonal antibodies to TLR2 (clone TL2.1) and TLR4 (clone HTA125) and control antibody IgG_{2a} (clone eBM_{2a}) were obtained from *e*Bioscience and the monoclonal antibody to human CD14 (MY4) and control antibody ($MsIgG_{2b}$) was

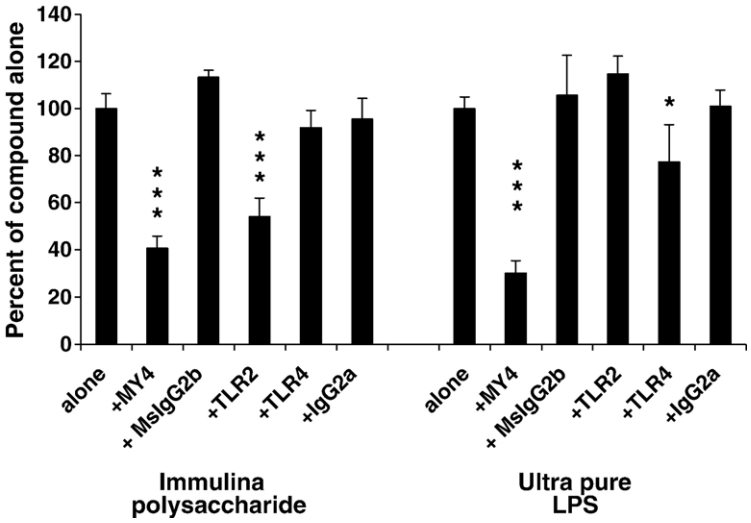


Fig. 1. Effect of anti-CD14, anti-TLR2 and anti-TLR4 antibodies on Immulina polysaccharide induced NF-kappa B activation. THP-1 cells were transfected with the NF-kappa B luciferase reporter plasmid and incubated at 37 °C for 24 h. Cells were then treated with antibodies to CD14 (MY4), TLR2, TLR4 or control IgG fractions for these antibodies ($MsIgG_{2b}$, IgG_{2a}) for 30 min (all at 10 μg/ml) prior to addition of Immulina polysaccharide (0.05 μg/ml) or ultrapure LPS (0.01 μg/ml). Four hours later, cells were harvested for luciferase assay. Results are the average±S.D. of two experiments with each sample performed in duplicate. Statistical differences between treatments with antibody and control antibody were determined by unpaired two-tailed Student's *t*-test (*$P<0.05$ and ***$P<0.001$).

purchased from Coulter. Ultrapure *Salmonella minnesota* LPS was from List Biological Laboratories, Inc.

2.4. TLR expression vector experiments

HEK 293 cells were cultured in DMEM/F12 medium supplemented with fetal bovine serum (10%) and 1% penicillin-streptomycin at 37 °C, under 5% CO_2. Actively growing cells were transiently transfected with the appropriate plasmid(s) using electroporation (at 150 V and one 70 ms pulse) based on a published method [12]. Following electroporation, cells were plated at a density of 5×10^4 cells in 200 μl/well of culture medium. After 48 h, agents to be tested were added to transfected cells. Six hours after addition of samples, cells were harvested and luciferase activity was measured. The NF-kappa B plasmid construct (pBIIXLUC) was a gift from Dr. Riccardo Dalla-Favera and contains two copies of NF-kappa B motif from HIV/IgK [13]. Plasmids co-expressing human CD14 and human TLR2 (pDUO-hCD14/TLR2), human CD14 and human TLR4 (pDUO-hCD14/TLR4A), and expressing human MD-2 (pUNO-hMD2) were purchased from InvivoGen. Zymosan was purchased from Sigma, and *Staphylococcus aureus* peptidoglycan from Fluka.

2.5. Animals

The animal experiments adhered to the University of Mississippi guide for the care and use of laboratory animals and were approved by the animal welfare committee. Male C3H/He mice (age 6 to 7 weeks) were purchased from Harlan Sprague/Dawley. Upon arrival, they were acclimated for 7 days and fed laboratory rodent diet (Lab diets 5001).

2.6. Mouse treatment

Mice were housed one per cage and food consumption (Research Diets AIN-76A) per day was monitored by weight. In the first experiment, mice (4 per treatment) were fed for 4 days with either AIN-76A alone or AIN-76A mixed with crude *Spirulina* extract to result in intakes of ~10 mg/day/mouse. Average consumption per day over the 4-day period for all mice was 10.8 mg/day (range 10.1 to 12.2 mg). In the second experiment, mice were fed AIN-76A for 21 days prior to treatment with crude *Spirulina* extract for 3, 4 or 5 days. The average consumption was 10.6 mg/day (range 9.1–11.8), 11 mg/day (range 9.8–11.5), and 9.7 mg/day (range 9–10.3) for all mice for the 3-, 4- and 5-day treatment groups respectively.

Following the specified treatment period, mice were sacrificed by CO_2 asphyxiation. Spleens and Peyer's patches were removed aseptically from the animals and cells were isolated and cultured as described previously [14]. The culture supernatants from Peyer's patch cells were used for the determination of total IgA (Bethyl Laboratories Inc.) and IL-6 (BD Biosciences) by ELISA. Spleen cell culture supernatants were used for the analysis of IFN-γ (R&D systems) by ELISA according to manufacturer's instructions.

3. Results

3.1. Immulina polysaccharide activates NF-kappa B through a CD14- and TLR2-dependent pathway

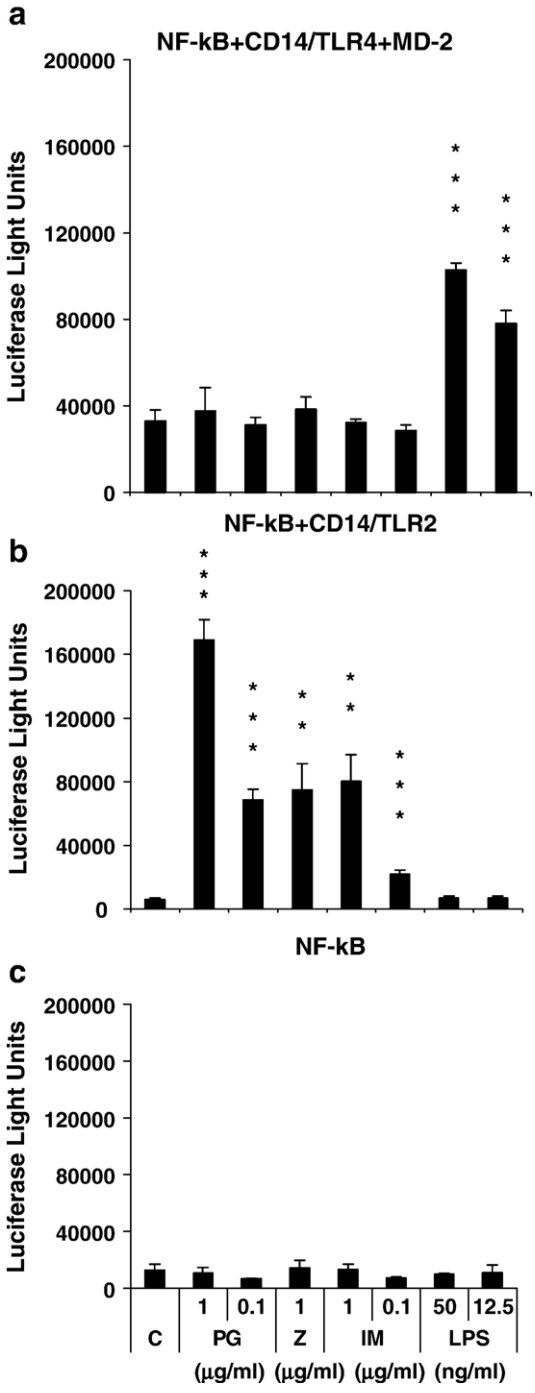
The experiment presented in Fig. 1 suggests that TLR2 is involved in the activation of NF-kappa B in THP-1 monocytes by Immulina polysaccharide purified from the *Spirulina* crude extract. This polysaccharide fraction represents 25% to 30% of the dry weight of the crude extract. Consistent with its role in mediating the action of TLR2 [15], antibodies to CD14 also inhibited activation by Immulina polysaccharide. In contrast, antibodies to TLR4 did not inhibit Immulina polysaccharide-dependent activation. The control IgG fractions for these antibodies ($MsIgG_{2b}$ and IgG_{2a}) were not effective at inhibiting activation. Specificity of antibodies was demonstrated in that activation by ultrapure *S. minnesota* LPS, a known TLR4 ligand [16], and was suppressed by TLR4 antibody, but not by TLR2 antibody.

Additional evidence that TLR2 mediates the activation of NF-kappa B by this polysaccharide is shown in Fig. 2. The NF-kappa B luciferase reporter plasmid was introduced into HEK 293 cells (cell line with minimal TLR expression) either alone or co-transfected with vectors expressing proteins supporting either TLR2- (CD14 and TLR2) or TLR4- (CD14, TLR4, and MD-2) dependent NF-kappa B activation. Neither the TLR2 specific (peptidoglycan or zymosan) [17] or TLR4 specific (LPS) ligands nor the Immulina polysaccharide activated NF-kappa B-dependent luciferase expression in HEK 293 cells transfected with the reporter plasmid alone. Consistent with the blocking antibody experiments presented in Fig. 1, the Immulina polysaccharide activated NF-kappa B in cells supporting TLR2-dependent (expressing CD14 and TLR2) but not TLR4-dependent (expressing CD14, TLR4, and MD-2) activation. Peptidoglycan and zymosan activated NF-kappa B only in cells expressing TLR2 and CD14 while LPS activated NF-kappa B only in cells expressing CD14, TLR4 and MD-2.

3.2. Oral intake of Spirulina extract enhances immune parameters in mice

We have previously shown that several immune parameters in mice were impacted by the oral intake of an immunostimulatory melanin preparation extracted from alfalfa sprouts or American ginseng [14]. These same immune parameters were examined in the present study since these melanins, like the *Spirulina* polysaccharides, also activated THP-1 cells in a CD14- and TLR2-dependent fashion. Dried *Spirulina* extract was mixed with chemically defined chow (AIN-76A), pressed into pellets, and food consumption monitored by weight. Fig. 3a shows that IgA production was enhanced in Peyer's patch cells isolated from mice that had consumed this extract for 4 days. Similarly, IFN-γ production was enhanced in cells isolated from the spleens of mice consuming the *Spirulina* extract (Fig. 3b).

We next determined the time required for the above changes to occur in mouse Peyer's patch and spleen cells after commencing *Spirulina* extract ingestion. For these experiments, mice were fed a chemically defined diet (AIN-76A) for 21 days to reduce the immunostimulatory influence of commonly used laboratory rodent chow (Lab Diets 5001). The immunostimulatory activity of 5001 chow is observable *in vitro* in the THP-1/NF-kappa B assay system (EC_{50} = 200 μg/ml for finely ground material). In contrast, AIN-76A is 25 times less active in this assay.



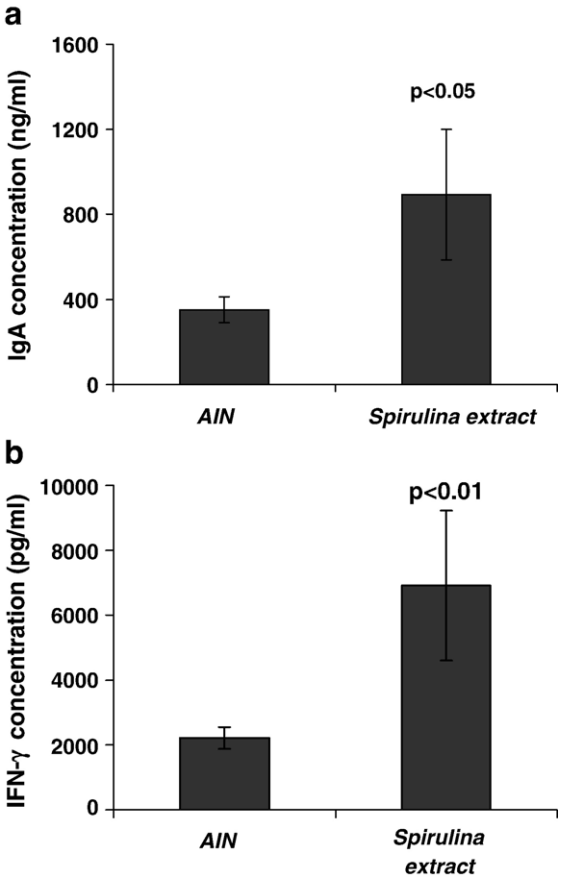
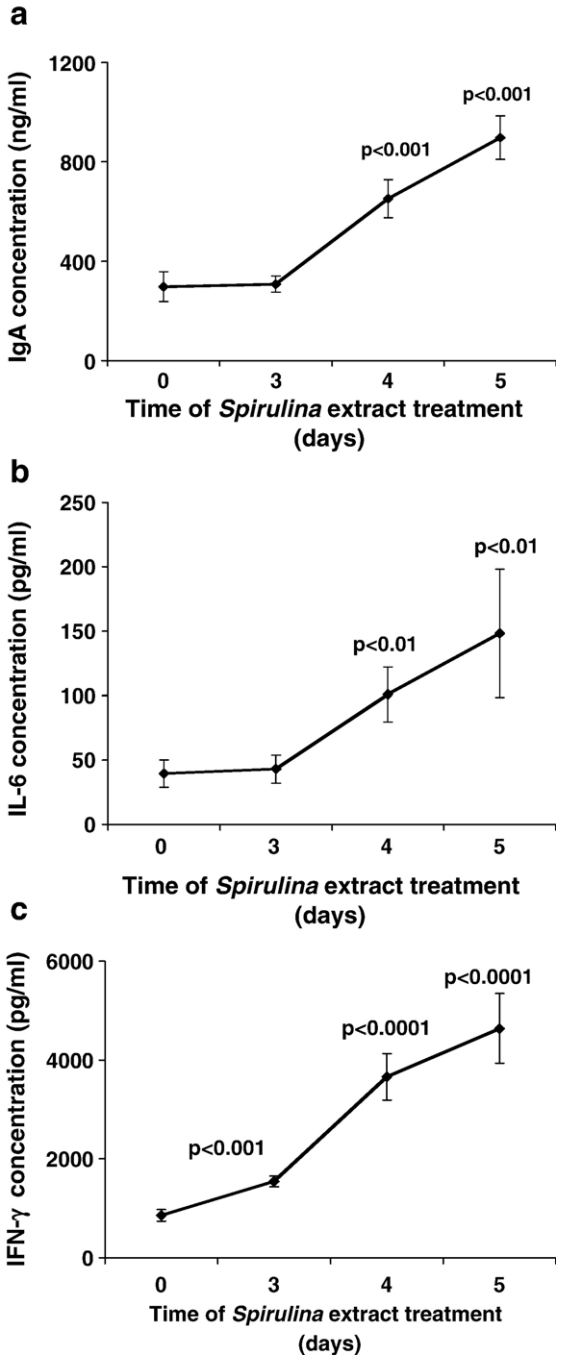


Fig. 3. Effect of crude *Spirulina* extract on mouse immune parameters. C3H/He mice (4 per treatment group) were treated for 4 days with either *Spirulina* extract (10 mg/day) mixed with animal diet (AIN-76A pellets) or AIN-76A pellets alone. Peyer's patch cells isolated from these mice were cultured for 2 days and the culture medium analyzed for IgA (a) by ELISA. Spleen cells isolated from these mice were cultured for 2 days and the culture medium analyzed for IFN-γ (b) by ELISA. Values are average±S.D. and statistical analysis was by a Student's *t*-test.

Fig. 2. Induction of NF-kappa B directed luciferase expression in HEK 293 cells by Immulina requires CD14/TLR2 but not CD14/TLR4/MD-2. HEK 293 cells were transiently transfected with a luciferase reporter gene construct containing two binding sites for NF-kappa B, alone (c), or in combination with the indicated expression plasmid(s) (pDUO-hCD14/TLR2 (b) or pDUO-hCD14/TLR4A and pUNO-hMD2 (a)). Forty-eight hours after transfection, cells were treated with either Immulina (IM) at 1.0 and 0.1 μg/ml, TLR2 agonists peptidoglycan (PG) or zymosan (Z), or TLR4 agonist ultrapure *S. minnesota* lipopolysaccharide (LPS) at the indicated concentrations. Luciferase activity was determined 6 h after addition of agents. Samples were run in triplicate in two separate experiments. Bars represent means±S.D. of one representative experiment. Statistical differences were determined by unpaired two-tailed Student's *t*-test (**$P<0.01$ and ***$P<0.001$).

Fig. 4a shows that Peyer's patch cells derived from *Spirulina* extract treated animals secreted more IgA *ex vivo* than AIN-76A fed mice at both 4 and 5 days but not at 3 days after the start of extract treatment. Interleukin-6 secretion by these cells followed a similar time course (Fig. 4b). Spleen cells from *Spirulina* extract treated animals also produced substantially more IFN-γ *ex vivo* on days four and five than on day three (Fig. 4c).



4. Discussion

The present study demonstrates that a high molecular weight polysaccharide fraction (Immulina) derived from *S. platensis* activates monocytes and NF-kappa B through a CD14- and TLR2-dependent process. This extends our previous work by identifying receptors within the monocyte cell membrane that are responsible for detecting these polysaccharides and signaling the activation of NF-kappa B. Other immune enhancing botanical supplements such as *Platycodon grandiflorum* [18] and safflower petals [19] have also been shown to contain polysaccharides that activate macrophages *in vitro* through a TLR-dependent pathway. We previously demonstrated that melanin, a high molecular weight component within botanicals such as *Echinacea*, alfalfa sprouts and American ginseng, activates monocytes *in vitro* through a TLR-dependent process [14]. The TLRs are a family of pattern-recognition receptors that are partially responsible for the detection of pathogens, and allow cells within the innate immune system to distinguish self-molecules from pathogen-associated non-self structures. In the gut, cells within the Peyer's patches such as macrophages and dendritic cells contain TLR and are thought to play a major role in the detection of pathogens within this mucosal inductive site.

One of the major factors influencing mucosal immunity is the alteration of gut micro-flora. The development of the germ free mouse most dramatically illustrated this relationship in that, compared to conventional animals, germ free mice exhibit suppressed immunological characteristics such as decreased lymph node, spleen and Peyer's patch size, reduced mucosal IgA production, decreased blood clearance of microorganisms and delayed immune response after antigenic challenge [20]. This suggests that the interaction of microbial components with TLRs present on certain immune cells within the gut at least partially maintains mucosal and systemic immune status. Ingested botanical components that interact with TLRs may "mimic" the influence that bacteria and fungi have on immune status. In this regard,

Fig. 4. Time course of enhancement of immune parameters in mice by *Spirulina* extract. C3H/He mice (4 per treatment group) were fed with AIN-76A pellets for 21 days. They were then treated for 3, 4 or 5 days with either *Spirulina* extract (10 mg/day) mixed with animal diet (AIN-76A pellets) or AIN-76A pellets alone. Peyer's patch cells isolated from these mice were cultured for 2 days and the culture medium analyzed for IgA (a) and IL-6 (b) by ELISA. Spleen cells isolated from these mice were cultured for 2 days and the culture medium analyzed for IFN-γ by ELISA (c). Values are average ± S.D. and statistical analysis was by a Student's *t*-test. *P*-values were compared with 0 day treatment of *Spirulina* extract.

our data show that the oral consumption of a crude polysaccharide extract from *Spirulina* enhances the *ex vivo* production of IgA from Peyer's patch cells. Dendritic cells from Peyer's patches are sufficient to induce IgA production from B cells [21] and cytokines, especially IL-6, serve as a key factor for the development of IgA secreting B cells [22–24]. In mice, a specific subset of dendritic cells called CD11b(+) are able to induce high levels of IgA secretion from naive B cells by secreting higher levels of IL-6 [25]. In the present study, the crude *Spirulina* polysaccharide extract may have either increased the numbers of CD11b+ dendritic cells or stimulated them to produce higher levels of IL-6. The similar time course observed for the enhanced production of both IgA and IL-6 by Peyer's patch cells from mice that had consumed this extract suggests a causal relationship.

IgA secreted at mucosal surfaces functions to protect against various viral and bacterial pathogens by its unique nature of agglutination of microorganisms, neutralization of bacterial enzymes, toxins and inhibition of antigens [26]. Several investigators have observed an increased mucosal IgA response by long-term treatment with *Spirulina* or its components or after antigen stimulation. *Spirulina* ingestion enhanced IgA production in mouse Peyer's patches *ex vivo* after immunization with ovalbumin [27] and higher IgA levels in chicken serum on challenge with sheep red blood cells [28]. Treatment of mice with *Spirulina* for 4 weeks enhanced *ex vivo* production of IgA from Peyer's patch cells of antigen stimulated animals but not in animals fed *Spirulina* alone [29]. Ingestion of *S. platensis* by men for more than a year enhanced salivary S-IgA levels while treatment for less than a year had no effect [30]. In addition to the local effect that ingestion of this extract has on Peyer's patch cells, our results indicate that it can also influence immune cells systemically. Mice that ingested this extract exhibited enhanced spleen cell IFN-γ production *ex vivo*. Interestingly, the enhanced production of IFN-γ by spleen cells followed a time course similar to that exhibited for production of IL-6 and IgA by Peyer's patch cells in that enhanced production was seen at 4 and 5 days following the start of ingestion whereas at 3 days the effect was minimal. The similar time course may indicate that a common cell type is initially targeted by the extract and is mediating both the effects locally on Peyer's patches as well as systemically on spleen cells.

IFN-γ is a macrophage activating cytokine that promotes Th1 biased responses associated with cell mediated immunity [31]. The Th1/Th2 balance is critical in determining whether an immune response is to be dominated by macrophage activation or by antibody production. In the current study, increased spleen cell production of IFN-γ in mice fed *Spirulina* extract suggests a shift towards Th1 type cell mediated immunity. This result is consistent with earlier studies [1] that indicated a Th1 bias when *Spirulina* was fed to mice or [32] when added to cultures of human peripheral blood mononuclear cells (increased production of IFN-γ). In addition, consumption of a hot water extract of *Spirulina* for 2 months by human volunteers resulted in greatly enhanced production of IFN-γ by NK cells in response to IL-12 and IL-18 [7].

In conclusion, the results from the present study indicates that oral feeding of an extract from *Spirulina* (containing 25–30% Immulina polysaccharide) enhances several immunological functions in mice and that this polysaccharide may be a major contributing factor to the immune stimulating action observed in *Spirulina* studies. The fact that oral consumption of this extract can influence both mucosal inductive (Peyer's patch) and systemic (spleen) sites suggests that its immune stimulating action is not confined to the mucosal immune system. Future experiments using TLR2 knockout mice would determine if the observed *in vivo* effects require TLR2.

Acknowledgements

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