Evaluation of The Immunomodulatory *In Vivo* Activity of *Laminaria Hyperborea* Fucoidan Relative to Commercial (1,3/1,6)-B-D-Glucans from Yeast and Mushrooms

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Abstract

A fucoidan with high ester sulfate content (38-40% by weight) has been isolated in a commercial scale process from *Laminaria hyperborea* harvested from the western coast of Norway. This is the first characterization of a fucoidan from this species of macroalgae. Ultra purified samples with negligible endotoxin levels were evaluated for cytokine expression in cultured human mononuclear cells. Initiators of the acute phase response were only moderately elevated while the chemotactic cytokine interleukin-8 increased significantly over baseline. Following oral dosing of mice with fucoidan, splenocytes showed significant increases in interleukin-2 expression and in phagocytic activity similar to effects by equivalent doses of yeast and mushroom derived (1,3/1,6)-β-D-glucans. Adaptive immunity was also affected by fucoidan as evidenced by significant increases in antibody titers in mice vaccinated with ovalbumin. Combination treatments of fucoidan together with a yeast beta-glucan showed additive effects, but gave no indication of synergy. To our knowledge, this is the first report of the effects of combinations of fucoidan with a beta-glucan in *in vivo* evaluations.

Keywords: Fucoidan; Beta-glucan; Immunomodulation; Phagocytosis; Cytokines

Introduction

Fucoidans are natural polysaccharides comprised primarily of L-fucose and ester sulfate groups and are found in brown seaweeds (*Phaeophyceae*) and certain marine invertebrates such as sea urchins and sea cucumbers. Each seaweed species comprises a unique fucoidan and though these structures are complex, there are several compositional constants such as the ester sulfate residues always being located on a polyfucan backbone. The number and distribution of the sulfate groups vary by species of macroalgae, by geographical location, and by season [1-3]. Secondary components can include other neutral monosaccharides, uronic acids, cations and protein. Additionally, some fucoidans are partially acetylated [4-6]. While the molecular backbone of fucoidan molecules is linear L-fucose, the overall structure of fucoidans has extensive branching, which may include additional L-fucose units with the other components mentioned above. The combination of a high degree of branching and the significant charge density has a profound effect on the rheological properties of fucoidans which show low apparent viscosities relative to their weight-average molecular weight (Mw) and display Newtonian behavior distinguishing them from linear seaweed polysaccharides [7]. Further details on fucoidin structure can be found in [8]. The primary brown seaweed species used to make commercial-scale fucoidan extracts for human consumption are *Laminaria japonica, Cladosiphon okamuranus, Saccharina sculpera (Kjellmaniella crassifolia), Undaria pinnatifida*, and *Fucus vesiculosus.*

Polysaccharides with high Mw such as the (1,3/1,6)-β-D-glucans (beta-glucans), zymosan and laminarin, contain epitopes that appear to be conformational mimics of motifs found in pathogen-associated molecular patterns (PAMPs). PAMPs are structurally associated with extracellular components of microorganisms such as lipopolysaccharide (LPS), lipoteichoic acid and lipoarabinomannan; enabling them to bind to pattern-recognition receptors (PRRs) such as CD14, Dectin-1 and the toll-like receptor family (TLRs) found on immune competent cells [9-11]. Fucoidans also appear to contain PAMPs as they serve as ligands to human TLR-2 and TLR-4 expressed in HEK-293 cells as demonstrated by the ability of fucoidans derived from *L. japonica, L. cichorioides*, and *F. evanescens* to activate transcription nuclear factor NF-*K*B following binding to recombinant TLR receptors [12]. Stimulation of innate and acquired immunity by fucoidans has also been observed in animal models; analyses of phagocytosis by peritoneal macrophages isolated from mice in which a fucoidan extracted from *Sargassum thunbergii* was administered intraperitoneally, showed a significant increase [13]. Oral administration of a fucoidan from *U. pinnatifida* significantly increased antibody titers in pathogen challenged mice [14].

Perhaps the best characterized of the immunomodulating polysaccharides are the beta-glucans derived from various sources including mushrooms and yeast. Some of the structure activity relations for the beta-glucans have been determined; the frequency of (1,6)-linkages, molecular weight and solution conformation have been found to significantly affect binding affinities to macrophage PRRs [15,16]. In the present study, a fucoidan isolated from *L. hyperborea* has been characterized in terms of structure and biological activity for the first time and was compared directly with beta-glucans for immune modulating activities. This is the first direct comparison of these bioactive polysaccharides as orally administered treatments as well as including the first evaluation of a combination oral treatment of beta-glucan plus fucoidan.

Materials and Methods

Production of fucoidan from *Laminaria hyperborea*

Fresh *Laminaria hyperborea* was harvested as whole plants including the holdfast, from the western coastline of Norway in a region from the county of Rogaland in the south to the county of Sor-Trondelag in the north. The seaweed was stored at ambient temperature in concrete enclosures with only that amount of seawater that accompanied the harvested material. The physiological response of the seaweed to exposure to air is to exude fucoidan and laminaran which runs off the harvested biomass as the incidental seawater (leachwater) which drains down the sloped floor of the storage bin. The leachwater was collected through stainless steel grates into a collection trench and pumped into a stainless steel holding container. The dry matter of the exudate solution after drying overnight at 105 °C was 5.7% w/v. Fucoidan purification was conducted in a batch process of 3000 liters per production run. The exudate solution is filtered through a Plate and Frame pressure filter in three cycles (about 500 liters/cycle with each cycle requiring one hour) with help of a filter aid (heated stone weighing 22.5 kg) to remove large solid debris from the exudate. The solution is then concentrated by ultra-filtration over 100 kDa Molecular Weight Cut-Off (MWCO) filters (spiral membrane from Alfa Laval). The retentate is dialyzed four times by adding equal volumes of potable water while continuing the ultra-filtration. The retentate is then subjected to a microbial 'kill' step by passage through an Ultra-High Temperature (UHT) unit. Finally, the retentate, which has been reduced in total volume about 5-fold, is spray dried in a co-current dryer with a high pressure nozzle at 180 bar with 238 °C inlets and about 105 °C outlets on the air. The process is conducted under Good Manufacturing Practices (cGMP) as specified for food products. The development of this process has made fucoidan from this species of macroalgae, commercially available for the first time. Because exudate from freshly harvested biomass, rather than a whole plant extract, is used as starting material, the only macromolecules present are fucoidan and laminarans. The laminarans are removed during the dialysis process resulting in a fucoidan from *Laminaria hyperborea* (LhF) that is routinely of approximately 90% purity.

Carbohydrate analysis

Mono-saccharides were released from the fucoidan polysaccharide by acid hydrolysis at 100 °C followed by derivitization through extensive methanolysis. Quantification and identification was then carried out by HPLC on reverse phase C18 columns against standards including D-Glucose, D-Galactose, D-Mannose, D-Xylose, L-Fucose and D-Glucuronic acid [17].

Molecular weight

Molecular weight determinations of LhF were made by Gel Permeation/Size Exclusion Chromatography (GPC/SEC) on a Viscotek TDAmax system with two ViscoGEL α-M columns. The mobile phase (eluent) was 0.1 mol/L $LiNO_3$ + 1 g/L EDTA at pH 7 at 70 °C. The eluent was monitored with a triple detection system utilizing refractive index, laser light scattering and intrinsic viscosity detectors.

Total sulfate

Sulfate determinations were made gravimetrically, essentially as described by Lugg [18]. Briefly the extract was digested in boiling nitric acid; following cooling excess nitric acid is reduced by the addition of formaldehyde. The sample is brought back to a boil with hydrochloric acid and barium chloride and the resulting barium sulfate precipitate is allowed to accumulate overnight. The precipitated material is then washed, oven dried and quantified by weight.

Endotoxin removal from fucoidan

Ultra-purified fucoidan (UP-LhF) was generated from LhF used as starting material by FMC NovaMatrix (Sandvika, Norway). A proprietary column purification procedure conducted on recirculating activated carbon columns was adapted from processes used to generate ultra-purified alginates for human implantation yielding fucoidan with endotoxin levels < 1000 EU/g [19].

HPLC analysis

Chromatographic traces of LhF were generated using an Agilent 1100 liquid chromatograph coupled to charged aerosol detection (CAD). Twenty ul samples solubilized in deionized water (dH_2O) at a concentration of 120 mg/ml were loaded onto a Phenomenex Bio Sep-SEC-S®, 300 × 7.80 mm column of 5 μm particle size. An isocratic mobile phase was made of 9 parts deionized water (pH 3.0) and 1 part 0.2% (v/v) acetic acid in methanol and run at a flow rate of 0.8 ml/min, for an 18 min run time/sample while the column temperature was held at 30 °C.

FTIR spectral analysis

Films containing the LhF were formed by dissolving 200 mg of the test material in 50 mL of dH_2O at room temperature. A 4.5 ml sample was placed in a weighing dish and dried in an oven for 3 h at 50 °C. The resulting film was scanned from 2000 to 750 cm^{-1} with a Shimadzu IRAFFINITY-1-FT-IR spectrophotometer using an ATR

Degree of acetylation by ^{1}H NMR

The frequency of acetyl groups was determined from the ^{1}H NMR spectrum using the methyl hydrogen resonance of O-acetyl groups and the fucose methyl resonance to determine the relative proportion of the acetyl and fucose groups [20].

Purification of fucoidan

Fucoidan from *Laminaria hyperborea* was purified by complexation with a quaternary salt. A 5% (w/v) sample of the dialyzed, spray-dried commercial product was dissolved in dH_2O and adjusted to pH 5.0 with dilute acetic acid. Fucoidan was then precipitated from solution by the slow addition of a 20% solution of cetyl-trimethyl-ammonium-bromide (CATB) to a final concentration of 6% CATB (w/v). The precipitated fibers were washed three times by alternately centrifuging (2500 rpm for 15 min) and resuspending the pellet in 200 ml dH_2O after discarding the supernatant. The final pellet was dissolved in 600 ml of a saturated NaCl solution which was then slowly added to 3 volumes of 100% isopropyl alcohol (IPA) with vigorous stirring until the fucoidan fibers came out of solution. The fucoidan fibers and salt solids were recovered by centrifugation as before and washed with 500 ml of 85% IPA followed by washing with 100% IPA. Fucoidan was separated from the salts by resuspending the final washed pellet in 600 ml dH_2O, then two volumes of 100% IPA were added slowly with stirring until the fucoidan fibers came out of solution, these were washed as before, first with 85% and finally with 100% IPA. The final pellet was oven dried at 60 °C recoveries were about 72% by weight.

Quantitative metachromatic dye analysis

The dried, CATB purified fucoidan was serially diluted to concentrations of 1 to 10 ug/ml in distilled H_2O prior to adding 0.001% w/v toluidene blue. The dye solutions were thoroughly mixed then absorbance at 630 nm was determined in 1 cm cells on a spectrophotometer as described by Beattie et al. [21].

Beta-glucans

The (1,3/1,6)-β-D-glucans included Wellmune WGP® derived from Saccharomyces cerevisiae, manufactured by Biothera (Eagan, MN) and Maitake Gold 404, a mushroom sourced β-D-glucan which is produced by Mushroom Science of Eugene OR. Wellmune WGP® was purchased through Immune Health Basics (Eagan, MN). Maitake Gold 404 samples were purchased from Tradeworks (Brattleboro, VT).

Animals

Balb/c mice purchased from the Jackson Laboratory (Bar Harbor, ME) of both sexes were used, aged 5-6 weeks at the initiation of the trial. The mice received doses of the experimental immunostimulants as oral treatments through the drinking water for 14 days prior to being sacrificed and analyzed for immunological markers. Treatment, care and sacrifice of the animals were conducted under the guidelines approved by the University of Louisville Institutional Animal Care and Use Committee (IACUC).

Cytokine expression profiling

Cytokine expression in cultured human peripheral blood mononuclear cells (PBMC) was determined in assays conducted by Ricerca Biosciences (Concord, OH) as their ImmunoSignal - Inflammation Tox-Screen [22].

Cell Cytometry

Briefly, determinations of membrane surface receptor expression were carried out by quantification of fluorescence staining of cells isolated from the spleen with anti-mouse CD4, CD8 and CD19 conjugated with Fluorescein isothiocyanate (FITC). Following incubation with the antibodies, the cells were washed; stained cells were then counted by flow cytometry [23].

Phagocytic activity

Stimulation of phagocytosis was determined by incubating peripheral blood cells of treated and untreated mice with 2-hydroxyethyl methylacrylate (HEMA) synthetic, polymeric microspheres at a concentration of $5x10^8$/ml. These particles carry only a slight negative charge, limiting non-specific adherence to cell surfaces. Cell suspensions were incubated at 37 °C for 60 min with intermittent shaking. Smears were developed with Wright stain, cells that had taken up three or more HEMA particles were considered positive [24].

Interleukin-2

IL-2 production was measured from purified spleen cells cultured for 72 h at 37 °C and 5% CO_2. Positive controls were generated by the addition of Concanavalin A to 1 mg/ml. Supernatants were then collected and IL-2 quantified with a Quantikine mouse IL-2 kit (R&D Systems, Minneapolis, MN) [25].

Adjuvant Activity of Polysaccharides

The production of antibodies to ovalbumin was determined as described previously, except that experimental immunomodulators were administered orally rather than by intraperitoneal (ip) injection [26]. Briefly, mice were inoculated with 100 μg of ovalbumin as antigen following ten days of oral dosing with beta-glucans or fucoidan. With continued feeding of the immunomodulators, mice received a booster inoculation of 100 μg of ovalbumin two weeks after the initial injection. Serum was collected one week after the final injection of antigen and the titer of specific antibodies to ovalbumin was determined by ELISA.

Statistical Analysis

Data was analyzed by two-way ANOVA using the Data Analysis plug-in module for Microsoft Excel. On the dose response figures, variability is indicated graphically as the Least Significant Difference at the 0.05% probability level ($LSD_{0.05}$) calculated from the within treatment error from the ANOVA. P values from the F ratios are also displayed for each experiment. Differences between treatment means within an experiment were analyzed by Duncan's multiple range test.

Results

The carbohydrate composition of the fucoidan from *L. hyperborea* (LhF) was 41% by weight and shown to be predominantly fucose (36%) with an additional, small contribution by galactose (3-4%) and trace amounts of glucose (<1%) and mannose (<0.5%). Other neutral monosaccharides such as arabinose, xylose, mannose and rhamnose, were below the limits of detection (<0.2%). There was no evidence for the presence of uronic acids (glucuronic and galacturonic). In addition, no traces of guluronic or mannuronic acid were detected indicating there was no contaminating alginate present. Relative to previous reports for fucoidans from *L. japonica*, the LhF is distinguished by a lack of glucuronic acid and a slightly higher percentage of fucose [27,28]. The frequency of sulfation on fucose was determined to be 1.7, yielding a percentage by weight of 38-40% for ester sulfate (calculated as SO_4--). This is among the highest values for sulfation reported for a fucoidan with those from other species typically ranging from 15-30% with the notable exception of one report of 45% sulfation in a fucoidan from *F. vesiculosus* [4,20, 27-32]. The degree of acetylation as a molar percent of the fucose content was calculated at 1:0.31 somewhat less than the 1:0.41 ratio reported for fucoidan from *U pinnatifida* [20].

The Mw determined for LhF by gel permeation chromatography (GPC) analysis was slightly over 1200 kDa (Table 1). This is greater than the values most typically observed for fucoidans from other species which usually fall between 100 to 500 kDa, but is not unprecedented as isolates from *U. pinnatifida* and *A. nodosum* have been reported with molecular weights of 1246 and 1323 kDa respectively [5,7,20,27,33,34].

Sample	Mw[a]	Mn[b]	Mw/Mn[c]	IV[d]	Rh[e]	M-Ha[f]	Recovery %
Prep 1 analysis 1	1.22E+06	337210	3.62	1.06	22.9	0.55	83.1
Prep 1 analysis 2	1.21E+06	345062	3.50	1.01	22.67	0.55	83.9
Prep 2 analysis 1	1.22E+06	364724	3.36	1.07	22.83	0.65	78.4
Prep 2 analysis 2	1.22E+06	368059	3.30	1.06	22.98	0.63	78.7

Mw[a]	Weight average molecular weight
Mn[b]	Number average molecular weight
Mw/Mn[c]	Polydispersity index
IV[d]	Intrinsic viscosity
Rh[e]	Hydrodynamic radius
M-Ha[f]	The Mark-Houwink Slope

Table 1: Molecular weight determinations of the fucoidan isolate from *L. hyperborea* were undertaken by Gel Permeation/Size Exclusion Chromatography. Two different experimental samples were prepared from the same fucoidan manufacturing lot (Prep 1 and Prep 2) each sample was then subjected to two consecutive analyses

The size of any given fucoidan polymer appears not to be a function of the species of origin as much as the isolation method with harsher chemical extractions made from dried, rather than freshly harvested materials potentially leading to degradation of the polymer. A number of reports have demonstrated that fucoidan polymers from various species can be readily hydrolyzed to shorter chain fragments under relatively mild acidic conditions [35-37]. In this case, the isolation process did not include any mechanical maceration or solvent extraction of the biomass, but consisted only of dialyzing and concentrating exudate collected from freshly harvested seaweed, conditions that might be expected to contribute to preservation of intact, high molecular weight polymers.

A qualitative visualization of the purity of the spray-dried product was made by analyzing samples by HPLC. The fucoidan eluted as a single peak when monitored by charged aerosol detection (CAD) with a retention time of 6.4 min under the conditions described in the experimental section with no additional peaks or deflections not also observed in the blank vehicle sample (Figure 1).
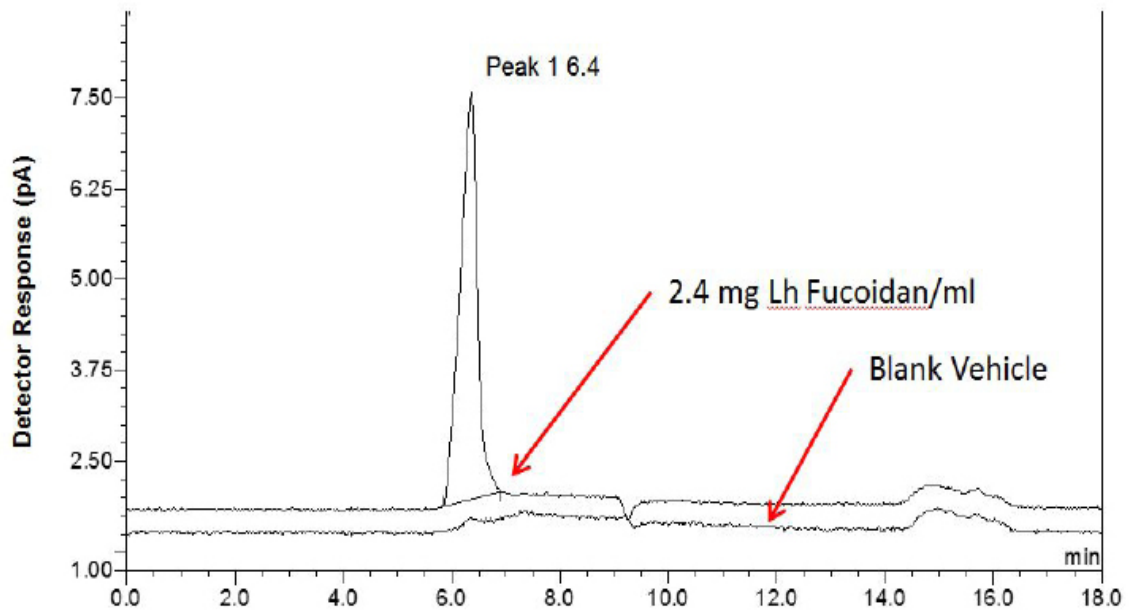
Figure 1: Chromatographic traces of fucoidan from *Laminaria hyperborea* were obtained by charged aerosol detection monitoring 20 ul samples solubilized in deionized water at a concentration of 120 mg/ml. Sample trace is shown overlaid on baseline trace from a blank vehicle sample

Formation of a tautomeric imino-base between the sulfate groups on the polysaccharide and a metachromatic dye such as toluidine blue cause a shift in absorbance from blue to red-violet, thus higher concentrations of fucoidan lead to a lower absorbance at 630 nm and this relation was used to generate standard concentration curves for the CATB purified LhF [38]. The relation between absorbance and concentration was highly linear from 1 to 6 ug/ml (R^2 = 0.9988). The purity of the commercial, spray dried material was then calculated on the basis of toluidine blue titrations relative to the CATB purified reference sample and was determined to be 88.7% with a moisture content of 5.6% or a purity of 94.3% on an anhydrous basis if the water retained following spray drying is not considered an impurity. The primary contaminants by weight were KCl (1%) and protein (4.7%).

The mid-range infrared spectrum (FT-IR) revealed a broad ester sulfate peak from about 1300 to 1200 cm^{-1}; this would not be seen in alginate, the other major, high molecular weight polysaccharide that might be expected to be retained following dialysis. A sharper equatorial ester sulfate peak was also observed from about 980 to 950 cm^{-1} (Figure 2).
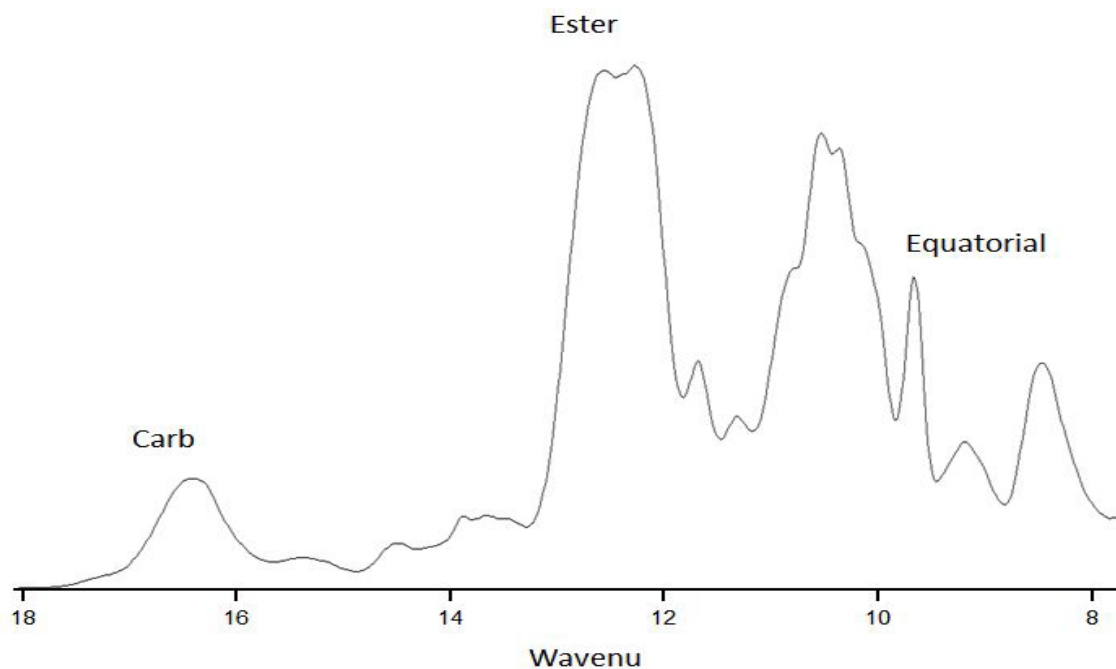
Figure 2: FT-IR traces of a film formed from oven-dried fucoidan from *Laminaria hyperborea*. Sample was analyzed using an ATR accessory focusing on the wavelength range from 2000 to 750 cm^{-1}

The availability of a sample of ultrapurified fucoidan from *L. hyperborea* (UP-LhF) ensured that evaluations of cell-based cytokine expression could be undertaken without confounding from contaminating endotoxins. The effect of UP-LhF, the inflammatory endotoxin lipopolysaccharide (LPS) and a yeast derived beta-glucan (YbG) on expression of the following cytokines was monitored in PBMC cultured cells: IL-1β, IL-6, IL-8, MIP-1α and TNF-α. Both the profile and potency of cytokine expression differed significantly between the inflammatory LPS and the eukaryotic polysaccharides UP-LhF and YbG (Figure 3).
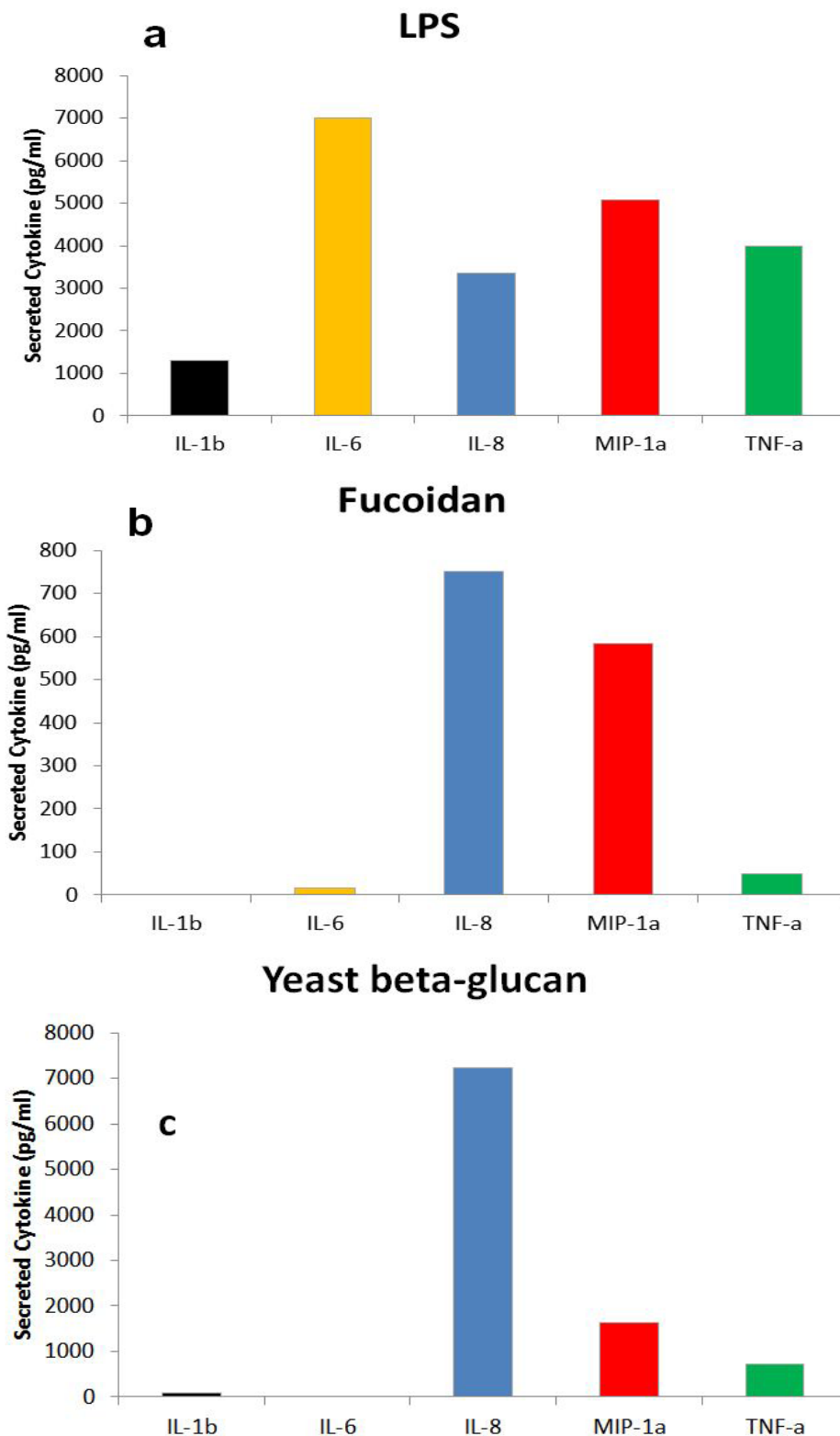

Figure 3: Cytokine expression in cultured Human peripheral blood mononuclear cells (PBMC). Cryo-preserved cells were seeded to a density of $10e^4$ cells/140 ul in 96 well plates and held at 37 °C for 1 h prior to treatment with test articles. Cells were then held for 24 h before centrifugation and collection of the supernatants for determination of cytokine expression. The bars represent the mean of two replicates. Dose response curves for both the fucoidan and beta-glucan were parabolic, i.e. the highest concentrations reduced cytokine expression. Therefore data is shown for the dose for each treatment that elicited the maximum expression levels. a) The endotoxin Lipopolysaccharide (LPS) at 50 pg/ml; b) Fucoidan from *Laminaria hyperborea*, at 10 mg/ml c) beta-glucans from yeast at 0.3 mg/ml. All values represent significant difference from control levels

Whereas the primary initiators of the acute phase response IL-1, IL-6 and TNF- α were strongly responsive to 50 pg/ml LPS, 10 mg/ml UP-LhF had no effect on IL-1β expression and IL-6 was increased slightly, but not significantly over baseline. Although TNF-α expression increased measurably in response to 10 mg/ml UP-LhF, the signal was 79-fold less than that elicited by a 2×10^8 lower concentration of LPS.

In the first mouse trial, LhF and beta-glucans extracted from mushroom (MbG) stimulated the phagocytic activity of spleen cells isolated after two weeks of oral dosing (Figure 4a). Relative to the controls, the stimulation induced by both treatments was highly significant at both doses (p < 0.01); however, though the LhF treatment showed a trend towards greater efficacy, there was no statistically significant difference in activity between the two polysaccharides at either dose. In a second trial, the response to the 1.7 mg/kg body weight (bw) dose of LhF was identical to that observed in the first experiment (42% versus 43% stimulation) indicating significant consistency in this *in vivo* experimental model, but a dose response for LhF was not evident in the second trial (Figure 4b). In the second trial it was again observed that each of the immunomodulatory treatments induced significant stimulation over the control response. In this case, the difference in response observed between LhF and the YbG at the 5 mg/kg bw dose just reached statistical significance, which was not the case at the lower dose. The magnitude of the effect observed for a combination treatment made up of equal weights of LhF plus YbG suggested an additive effect for the two compounds with no indication of synergy (Figure 4b).
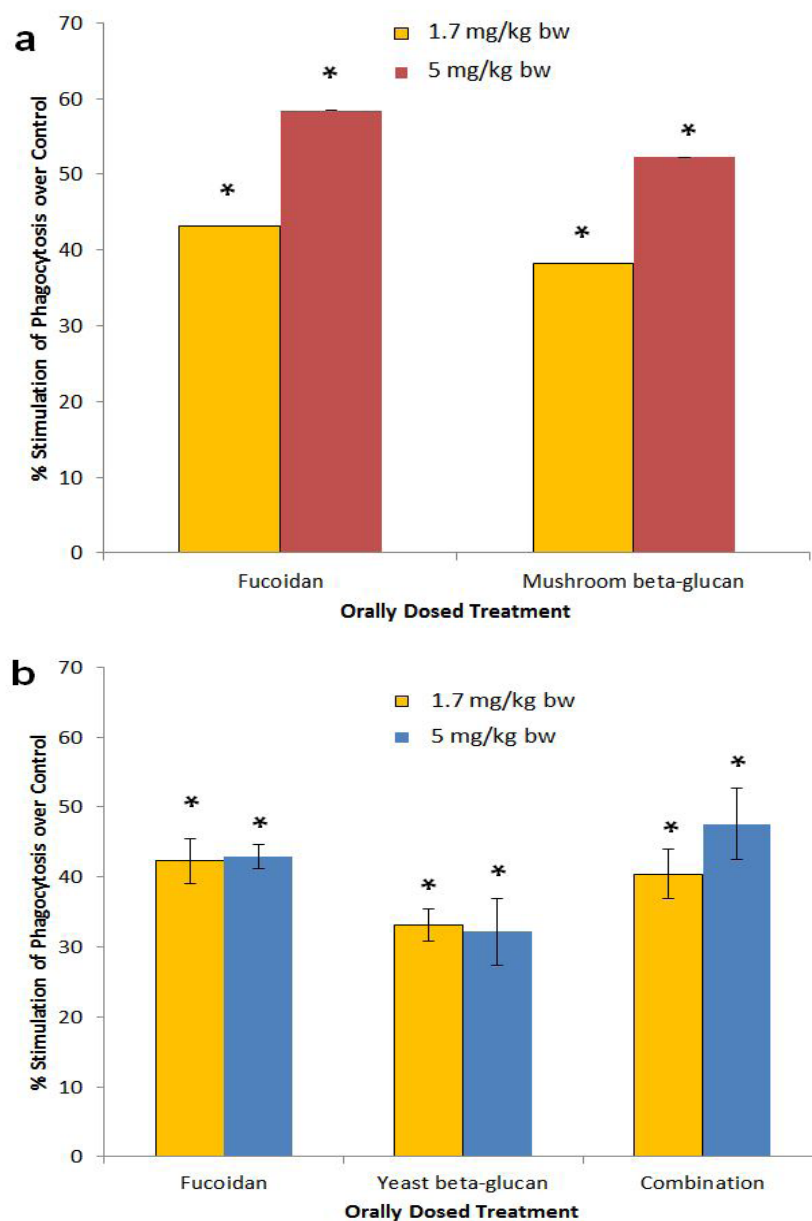
Figure 4: Uptake of 2-hydroxyethyl methylacrylate particles (5×10^8/ml) by neutrophils isolated from mice orally dosed with experimental immunomodulators for two weeks, relative to controls. The "Combination" treatment was formulated by combining equal weights of fucoidan from *L. hyperborea* with yeast beta-glucan; the blended test article was then administered at the doses indicated. Each point is the mean of three replicates; error bars indicate the standard error of the mean. The Least Significant Difference (LSD) is calculated from the within treatment error from the Two-Way ANOVA for p value = 0.05. **a)** Fucoidan compared to mushroom derived beta-glucans; **b)** Fucoidan compared to yeast derived beta-glucans and a combination treatment of fucoidan blended with yeast beta-glucans.*Significant difference from control at P = 0.05 level

The increased phagocytic activity induced by all three polysaccharides was accompanied by dramatic increases in the interleukin-2 (IL-2) expression by the spleen cells isolated from the treated animals (Figure 5).
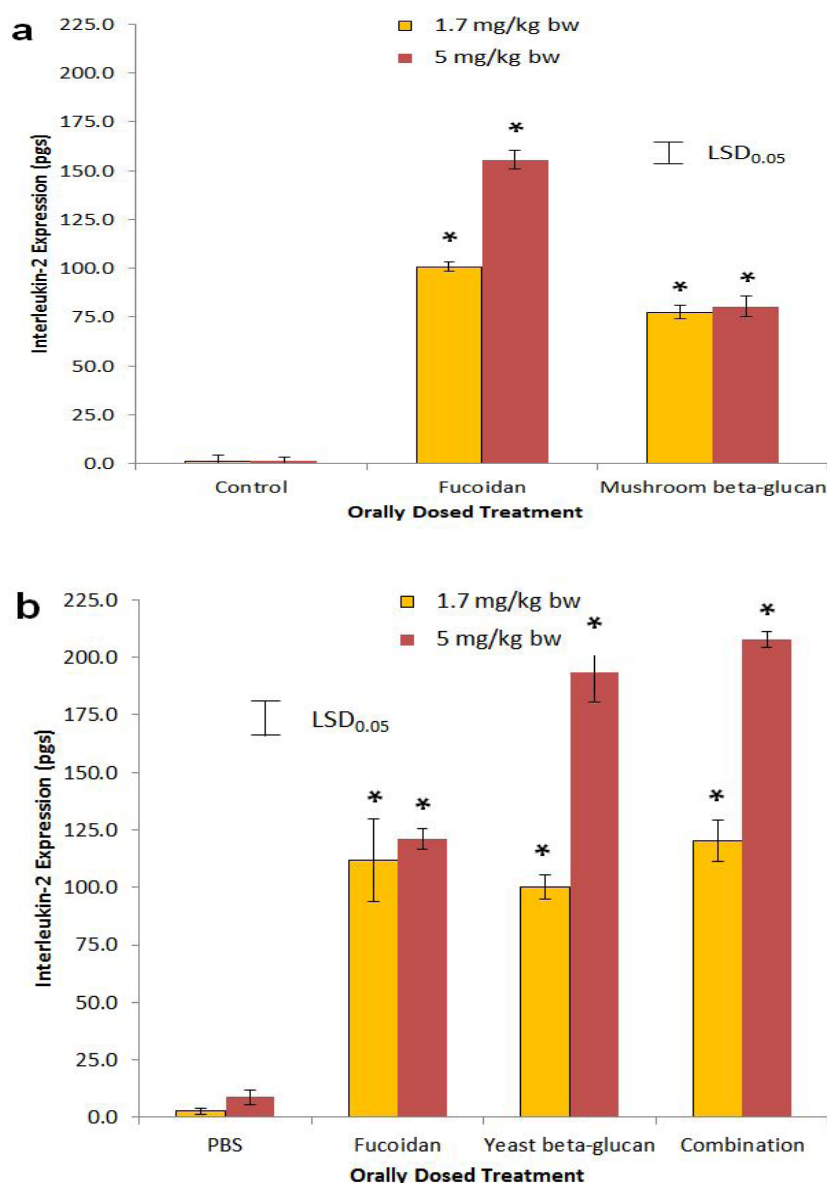
Figure 5: IL-2 production in purified spleen cells from mice orally dosed with experimental immunomodulators for two weeks. Each point is the mean of three replicates; error bars indicate the standard error of the mean. The "Combination" treatment was formulated by combining equal weights of fucoidan from *L. hyperborea* with yeast beta-glucan; the blended test article was then administered at the doses indicated. The Least Significant Difference (LSD) is calculated from the within treatment error from the Two-Way ANOVA for p value = 0.05. **a)** Fucoidan compared to mushroom derived beta-glucans; **b)** Fucoidan compared to yeast derived beta-glucans and a combination treatment of fucoidan blended with yeast beta-glucans. *Significant difference from control at P = 0.05 level

The elevations in levels of IL-2 are consistent with the stimulation in phagocytosis that was observed as this T helper cytokine activates the proliferation and differentiation of phagocytes [39]. Fucoidan has been reported to bind IL-2 directly, but it is not apparent how this would stimulate IL-2 expression [40]. IL-2 increases were also observed in a human clinical trial of Maritech extract, a treatment of mixed fucoidans from *Laminaria* species, *Undaria pinnatifida* and *Fucus vesiculosus* [41]. As in the case for the stimulation of phagocytosis, the expression of IL-2 induced by a combination treatment made up of equal weights of LhF plus YbG suggested an additive effect for the two compounds with no indication of synergy (Figure 5b).

A general trend was observed, across the experiments and polysaccharide treatments, for an increase in CD4 expression with an accompanying decrease in CD8 and with smaller or no changes observed in CD 19 (Table 2). Small increases in CD4 levels (T-lymphocyte helper cells), accompanying no increase or even slight reductions in CD8 (T-lymphocyte suppressor cells) suggests a slight stimulation of Th1 over Th2 responses. But the fact that no dramatic changes in CD4, CD8 or CD19 (B-cells) levels were observed, lends additional support to the hypothesis that these polysaccharides are serving as immunomodulators rather than as inflammatory agents.

Experiment 1			
Fucoidan	14	-13	3
Mushroom beta-glucan	9	3	1
Experiment 2			
Fucoidan	11	6	10
Yeast beta-glucan	24	-5	16
Combination (fucoidan+Yeast beta- glucan)	30	-1	5

Table 2: Relative increases in the fluorescence signal of FITC tagged antibodies to Cluster of Differentiation (CD) receptors on mouse spleen cells, isolated from mice receiving 5 mg/kg test article orally, as detected under flow cytometry. Data (n = 3) is expressed as the percent increase or decrease in signal relative to untreated controls

The influence of LhF and YbG on adaptive immunity was assessed by vaccinating mice with ovalbumin following an oral course of treatments with the polysaccharides initiated at the time of the first vaccination and which was continued through the booster inoculation two weeks later. Serum collected from animals treated with LhF, YbG and with a combination of LhF + YbG all showed significantly higher levels of anti-ovalbumin than the controls (ANOVA for treatment effects $p < 3.34 E^{-11}$) (Figure 6). Antibody titers were higher in sera collected from the 1.7 mg/kg LhF treated animals than those receiving the equivalent dose of YbG, but at the higher dose, the difference between the two polysaccharide treatments was not statistically significant. This was the one case where the combination of YbG and LhF appeared to be antagonistic as the 1.7 mg/kg combined treatment was significantly less effective than either polysaccharide administered alone at the same concentration.
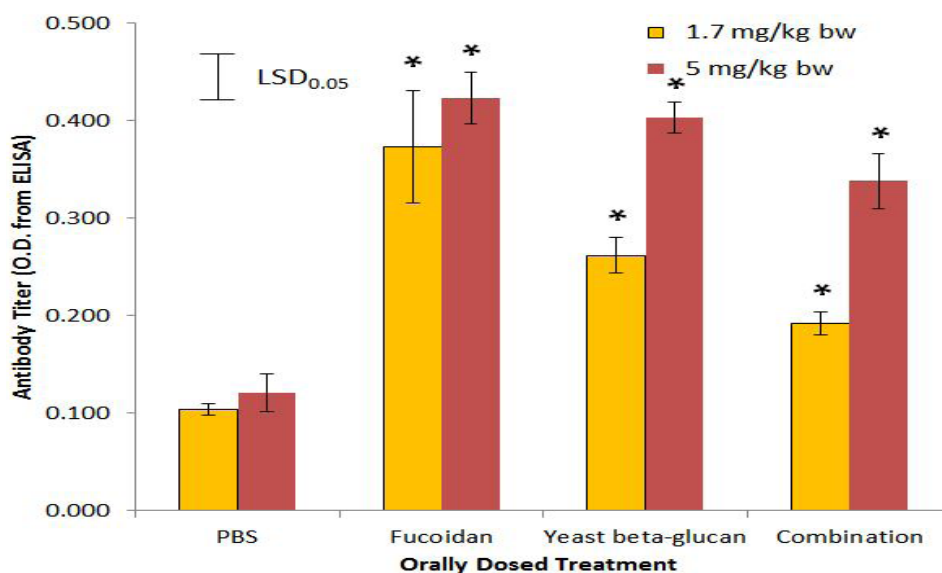
Figure 6: Relative titers of antibodies raised to ovalbumin in treated mice. Mice were administered yeast beta-glucans or fucoidan from *L. hyperborea* by oral dosing for ten days and then inoculated with ovalbumin as antigen, followed by a booster inoculation two weeks after the initial injection. Serum was collected one week later and the titers of specific antibodies to ovalbumin were determined by ELISA. Each point is the mean of three replicates; error bars indicate the standard error of the mean. The Least Significant Difference (LSD) is calculated from the within treatment error from the Two-Way ANOVA for p value = 0.05. *Significant difference from control at P = 0.05 level

Discussion

Fucoidans have been variously reported to have effects on TNF-α expression ranging from no effect to potent stimulation to being inhibitors of LPS stimulation of TNF-α release. This may have been due to differences in experimental conditions or the cell line used [42-44]. When direct comparisons were conducted in peritoneal macrophages, variations in activity appear to have been due to pharmacological differences intrinsic to fucoidans from different macroalgal species, at least in the case of *Fucus vesiculosus* versus *Cladosiphon okamuranus* [45]. However, in evaluating these reports the possibility of contamination by endotoxins needs to be taken into account particularly since polysaccharides can themselves interfere with certain versions of the *Limulus amebocyte* lysate assay commonly used for endotoxin detection [46].

Although LPS contains a polysaccharide moiety it is distinguished from fucoidan and beta-glucans by an additional component – Lipid A – a phosphorylated glucosamine disaccharide conjugated to various fatty acids. This domain elicits the hyper-response from cells of immune origin [47]. Although the YbG treatment was significantly more potent at inducing cytokine expression, the stimulation profile was strikingly similar for the YbG and LhF treatments; and markedly different from the pattern observed for LPS. The acute phase response cytokines IL-1 and IL-6 were refractory to both YbG and LhF with only the chemotactic effectors IL-8 and MIP-1α, responding strongly to both materials. IL-8 is also known as 'neutrophil chemotactic factor' inducing chemotaxis

primarily in neutrophils, and increasing phagocytosis at the site of infection as well as stimulating a respiratory burst. IL-8 has previously been shown to increase in response to beta-glucan treatments [48,49]. Stimulation of MIP-1α expression suggests activation of TLRs [50]. Agonism of TLRs has also been reported for fucoidans from a number of other macroalgae species [12].

Soluble beta-glucans are able to 'prime' CR3 effector immune cells by binding to the lectin domain of CR3 without triggering a full inflammatory response until cells (tumorous or microbial) presenting opsonized iC3b are encountered thus closing the immune response circuit through recognition of the membrane bound iC3b through the I domain of CR3. This ability of the soluble beta-glucans to place neutrophils, natural killer cells and mononuclear phagocytes, into a partially induced or poised state without fully initiating an inflammatory cascade *in vivo* is the rationale for categorizing the activity of beta-glucans as immunomodulation versus immune stimulation [51,52]. The pattern of highly specific cytokine stimulation observed in these experiments for the YbG treatment, in marked contrast to the global effect observed for LPS, is consistent with this concept of the beta-glucans as being immunomodulators and suggests that UP-LhF also belongs to this category of immune effectors. Taken together, the pattern of cytokine expression and the magnitude of the response indicate that UP-LhF does not possess the characteristics of a highly inflammatory agent thus allowing its effect on the immune status in mammals to be determined without expectation of inducing an acute phase inflammatory response.

Studies conducted previously using the identical mouse strain and evaluating phagocytic activity of isolated spleen cells under an identical protocol reported stimulation by yeast and mushroom beta-glucans that were remarkably similar to the activities reported in these trials even though the beta-glucans were administered IP in the previous study versus the oral dosing regimen reported here. This observation is consistent with experiments where the relative efficacies of oral administration of beta-glucans versus IP injections were compared directly, showing stimulation of phagocytic activity to be comparable under both formats [23].

The adjuvant effect of a fucoidan has also been evaluated in recent human clinical trials examining antibody production in Japanese subjects over the age of 60 years, a population with a reduced adaptive immune response relative to younger patients. Participants received 300 mg daily of a fucoidan isolated from *U. pinnatifida* or placebo for 4 weeks prior to receiving a trivalent seasonal influenza vaccine. The hemagglutination inhibition titer (CHMP evaluation criteria for seasonal influenza vaccine) and NK cell activity were determined from serum samples drawn 5 and 20 weeks following the vaccination. No allergic responses or signs of inflammation were observed, but the seroconversion rate and seroprotection at 5 weeks were higher for all vaccine strains in the fucoidan treatment group with the high titers maintained out to the 20 week period. The fucoidan group also showed increases in NK cell activity from baseline to 9 weeks following the initiation of the fucoidan treatments which was sustained after 24 weeks, while in the placebo group no significant increase was observed at 9 weeks over baseline with activity decreasing yet further after 24 weeks. In the fucoidan treatment group the response against the B antigen met the EU Licensure criteria for the geometric mean titer ratio (2.4) while the placebo group (1.7) failed to achieve these levels [53].

The *in vivo* activity reported here, begs the question of how such a highly charged, high Mw polymer as the fucoidan from *L. hyperborea* could possibly affect the immune system when administered orally. Certainly its physical/chemical properties are the very antithesis of what would be expected for effective absorption from the gut. A mechanism by which high molecular weight, indigestible beta-glucans might affect the immune system following oral ingestion has been suggested by a series of elegant, pharmokinetic experiments [54]. Following three days of daily oral administration, fluorescently tagged barley or yeast beta-glucans were observed in the gastrointestinal macrophages of mice, with uptake probably mediated by the Dectin-1 receptor. After four days the beta-glucans had been shuttled to the bone marrow where soluble, metabolized fragments were taken up by granulocytes leading to distribution to reticuloendothelial tissues as well as the bone marrow. Fucoidan was found to delay apoptosis and induce pro-inflammatory cytokines [55,56] to have anti-cancer activities [57], increase antibody production [58].

It is possible that fucoidan might also be fragmented and distributed following uptake by GI macrophages through TLR-4 or scavenger receptors [59]. In addition, the Kupffer cells were found responsible for the intestinal absorption of fucoidan [60]. However, an alternative mechanism has been identified by which fucoidan might be able to transduce signals from the gut to the bloodstream. These experiments by Ren et al., were initiated following the seemingly contradictory observations that following 5 weeks of daily oral doses of 400 mg of fucoidan from *L. japonica*, human subjects showed significant increases in the thrombolytic activity (platelet deaggregation) of their serum associated with an increase in the level of the eicosanoid prostacyclin; yet fucoidan could not be detected, by immunosorption, in those same serum samples. In follow-up rodent studies, when pure fucoidan was orally dosed at 5 mg/mouse, all of the fucoidan was recovered in the feces after 32 h and none could be detected in the serum at any time over the course of the experiment. The inability of fucoidan to be transported across intestinal epithelial cells was further demonstrated in *in vitro* experiments with cultured Caco-2 cells; however, 500 mg/L of fucoidan applied to the apical side of the cell layer did significantly increase expression of the H_2O_2 producing enzymes NOX1 and DUOX2 and concomitant increases in the concentration of H_2O_2 on the basolateral side were observed. A number of studies have now shown that reactive oxygen species (ROS) such as H_2O_2 can stimulate macrophage activity and cytokine expression directly through the transcription factor NF-*K*B. Production of TNF-α in Kupffer cells, macrophages residing in the liver, is regulated by a ROS activated NF-*K*B pathway [61]. ROS, produced by NADPH oxidase (NOX), is the main source of ROS required for LPS induced microglial phagocytosis. The PI3K and MAPK kinase pathways activated by LPS treatments are blocked by antioxidant neutralization of ROS or by inhibition of NOX leading to the prevention of phagocytic activation [62]. Therefore, it seems possible that fucoidan might through transduction affect the immune system, without leaving the gut lumen through the agency of NOX1 and DUOX2 stimulation in the intestinal epithelium thus raising systemic titers of ROS which in turn modulate immunity.

Conclusion

A fucoidan from *L. hyperborea* induced patterns of cytokine expression in cultured human immune cells that closely resembled the classic immunomodulators (1,3/1,6)-β-D-glucans from mushrooms and yeast. It is expected that a molecule such as fucoidan with a mean Mw of about 1000 kDa, carrying a significant net negative charge would not be readily absorbed from the gut. Especially not when administered in the doses used in this trial which would be equivalent to 100 or 300 mgs daily for a 60 kg human. Yet significant effects on primary acquired immunity were observed manifesting as significant simulation of phagocytosis, interleukin-2 expression and increases in specific antibody titers. We observed some additive effects, but no synergic effects were found, opening the way for simultaneous use of both substances. We offer the hypothesis that, as in the case of human clinical trials conducted with a fucoidan from *L. japonica* that indicated that fucoidan could transduce an activation signal across the intestinal epithelia to raise systemic concentrations of a prostacyclin, that fucoidan might also be able to activate both the innate and humoral arms of the immune system, *in vivo*, as a dietary fiber through the increased circulating titers of ROS.

Conflict of Interest

References

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