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The immunopotentiating effects of shark-derived protein hydrolysate

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ABSTRACT

Objective: Peptides derived from natural sources can act as immunomodulating agents and prevent infections. The aim of this study was to investigate the immunopotentiating and protective effects of a shark-derived protein hydrolysate (SPH) against an enterotoxigenic Escherichia coli H10407 infection in a murine model.
Methods: Mice were fed an aqueous solution of SPH for 7 days before being inoculated with an experimental enterotoxigenic Escherichia coli H10407 infection. After euthanasia, small intestines were removed for histological study and the number of IgA and IgG producing cells was determined by direct immunofluorescence. Cytokines were measured in the serum and the intestinal fluid.
Results: The oral administration of SPH enhanced the gut barrier function via up-regulation of immunoglobulin A-producing cells and intestinal cytokines production, including interleukin-6 and tumor necrosis factor-α. The increase of transforming growth factor-β and interleukin-10 contribute to the down-regulation of uncontrolled-inflammatory reaction induced by *E. coli* infection. From these results, the anti-inflammatory properties of SPH may be caused by regulation and priming mechanisms of the immune system.
Conclusion: Enzymatic protein degradation confers immunomodulating and protective potentials to shark proteins and the resulted peptides could be used as an alternative therapy to reduce the risk of bacterial infections and inflammatory-related diseases.

Introduction

The immune response induced by bioactive peptides is thought to be one of the oldest forms of immunoregulation that has been conserved through evolution, as opioid-related peptides have been found in invertebrates [1]. These peptides are involved in antithrombotic, antioxidative, antibacterial, antifungal, and sensory physiological activities; they also improve the nutritional value of food [2]. Food-derived bioactive peptides are one source of these health-enhancing components. They can be released during gastrointestinal digestion or processing of a multitude of plant and animal proteins, especially milk, soy, or fish proteins [3]. Fish proteins are a particularly rich source of bioactive peptides [4–8]. Bioactive peptides have been isolated from fish tissues such as sardine muscle, tuna muscle, pacific hake [9], and bonito; many of these have antihypertensive properties because they inhibit the angiotensin I-converting enzyme. Marine-derived protein hydrolysates (MPH) are also sources of multifunctional peptides because antihypertensive biopeptides can enhance the activity of bradykinin, which stimulates macrophages, enhances lymphocyte migration, and increases the secretion of lymphokines [10–12].

Accordingly, an orally administered MPH from yeast-derived fermentations affected both the systemic (phagocytosis) and the small intestine mucosal (immunoglobulin [Ig]A) immune systems in a murine model [13,14]. Additionally, when analyzed in a variety of injury and repair models, some MPH have demonstrated biological activity and may offer a novel, less

JFM participated in the in vivo experiment and the drafting of the manuscript. JD conducted the experiments and revised the manuscript. GV conceived part of the study. CM conceived the study and drafted the manuscript. RA provided technical support. MB revised the manuscript. JFM, JD, GV, and CM have no conflicts of interest to report. RA and MB are affiliated with innoVactiv inc. They participated in the development of the shark-derived protein hydrolysate and the design of the experiment, but were not involved in obtaining the results of the animal experiments.

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expensive approach to preventing and treating the injurious effects of nonsteroidal anti-inflammatory drugs and other ulcerative conditions of the bowel [15]. Thus, it is clear that marine resources constitute a potential reservoir of powerful immunomodulators.

The immune regulatory response is a major cell event involved in the maintenance of immune homeostasis and the control of inflammation. Priming the immune response is key to this process and could increase the immunoregulatory response against mucosal infections, such as *Escherichia coli*. It is well known that priming the immune response induces a type of physiological inflammation that triggers immunoprotection in the mucosa-associated lymphoid tissue [16,17]. Shark is commonly used in traditional medicine in China with no scientific evidence so far. We hypothesized that peptides would exert a mild priming effect on the immune system by decreasing the expression of proinflammatory cytokines and helping to prevent exaggerated inflammatory responses when mice were challenged with *E. coli*. The aim of this study was to evaluate the effects of a shark-derived protein hydrolysate (SPH) on host immunity and to study their immunoprotection potential against enterotoxigenic *E. coli* (ETEC).

Methods

Animals and feeding procedures

Six- to 8-wk-old BALB/c mice weighing 18 to 20 g were obtained from Charles River (Montreal, Canada). Each experimental group included five mice that were housed together in plastic cages in a controlled atmosphere (temperature: 22 ± 2°C; humidity: 55 ± 2%) on a 12-h light–dark cycle. The experiment was repeated three times. The mice were treated in accordance with the guidelines of the Canadian Council on Animal Care and the experimental design was approved by the Animal Care Committee of the University of Ottawa.

An aqueous solution of SPH was administered by gavage (4.5 mg/kg) to the animals in lieu of water for 5 or 7 d consecutively. The control group was given water instead of SPH. Both groups were fed ad libitum with a conventional balanced diet (2018 Teklad Global 18% Protein Rodent diet, Harlan Laboratories Inc, Madison, WI, USA).

At the end of each feeding period, the animals were anesthetized with intraperitoneal injections of ketamine (100 mg/mL), xylazine (20 mg/mL), and acepromazine (10 mg/mL) and sacrificed by cervical dislocation to obtain the various tissues to be used in the immunologic studies.

Shark-derived protein hydrolysate

The SPH preparation studied, PeptiBal™, was provided by innoVactiv Inc. The product is a patent-pending blend of small peptides generated by a trypsin-chymotrypsin hydrolysis process of purified shark proteins. Protein extracts from shark flesh (Waitaki Biosciences, New Zealand) were used as raw material for the studies described here. Marine hydrolysates were produced using enzymatic digestion by trypsin from porcine pancreas (Sigma-Aldrich) and type II α-chymotrypsin from bovine pancreas (Sigma-Aldrich), using a concentration 5% (w/vol) of protein extract in water. The hydrolysis reaction was performed at pH 8.0 and 33°C to 37°C. The pH-stat technique was used to maintain the enzyme preparation at its optimal pH [18]. The enzymatic hydrolysate was ultrafiltered using a 10-kDa cutoff membrane to remove the protease and the non-hydrolyzed proteins. The retentate or reaction mixture was discarded, whereas permeate, so-called total hydrolysate was further characterized and used for evaluation of its functional properties. The enzymatic hydrolysate was clarified by filtration and centrifugation to remove insoluble material (i.e., non-hydrolysated protein). The filtrate was then ultrafiltered using two subsequent cutoff membranes to remove high-molecular-weight polypeptides including residual enzymes. After ultrafiltration, the retentate was discarded, whereas permeate, so-called total hydrolysate, was pasteurized to inactivate residual enzymes. Spray drying was performed on the pasteurized total hydrolysate.

SPH has been tested for endotoxins with the LAL Chromogenic Endotoxin Quantitation Kit by Pierce Protein (Thermo Fisher, Burlington, ON, Canada).

Bacterial translocation assay

The livers were removed in sterile conditions and homogenized using the Omni TH homogenizer with a 7-mm generator probe (Omni International, Kennesaw, GA, USA) in 5 mL 0.1% sterile peptone water. One mL of each liver homogenate was plated on MacConkey agar (for enterobacteria). Translocation was considered to have occurred when colonies were observed on the agar plates because the liver is an organ normally devoid of bacteria [19].

Immunofluorescence test to identify the B-cell population (IgA+ and IgG+ cells)

The small intestines were removed for histologic preparation using the Sainte-Marie technique for paraffin inclusion. The paraffin blocks were cut in 4-μm sections. The number of IgA-producing (IgA+) cells on histologic sections of the samples from the ileum region near Peyer's patch was determined using a direct immunofluorescence method. The number of IgG-producing (IgG+) cells on histologic sections of the small intestine was also determined. The immunofluorescence test was performed using fluorescein isothiocyanate (FITC)-conjugated goat (α-chain specific) polyclonal antimouse IgA or FITC-conjugated goat (γ-chain specific) polyclonal antimouse IgG (Sigma-Aldrich, St. Louis, MO, USA). The histologic sections were deparaffinized and rehydrated in a graded series of ethanol (from 95% to 40%). The deparaffinized histologic samples were incubated with the appropriate antibody dilution (1/100 for IgA or 1/50 for IgG) in phosphate-buffered saline (PBS) solution for 30 min at 37°C. The samples were then washed twice with PBS solution and examined using a fluorescent light microscope. Results were expressed as the number of IgA+ or IgG+ cells (positive: Fluorescent cell) per 10 fields (magnification: 1000×). The data represent the mean of three histologic sections from each animal for each feeding period.

ELISA of serum samples

Blood was collected from anesthetized mice by cardiac puncture. The blood was coagulated by allowing it to rest for 30 min at room temperature and then was centrifuged for 10 min at 1000 *g*. The serum was stored at −20°C until it was analyzed. The level of transforming growth factor (TGF)-β in the serum was analyzed by enzyme-linked immunosorbent assay (ELISA; eBioscience, Inc., San Diego, CA, USA). The serum level of IgA was analyzed by DAS-ELISA using affinity-purified goat antimouse IgA antibodies (Sigma Chemical Co., St Louis, MI, USA).

Cytokine assay

At the end of each treatment period, the small intestines were removed and processed for histologic preparation as described previously. Interleukin (IL)-4, IL-6, IL-10, tumor necrosis factor (TNF)-α, cytotoxic T-lymphocyte antigen 4 (CTLA-4), and interferon (IFN)-γ were analyzed using an indirect immunofluorescence method. The histologic sections were deparaffinized and rehydrated in a graded series of ethanol (from 95% to 40%) and then incubated for 30 min in a 1% blocking solution of bovine serum albumin (Jackson Immuno Research, West Grove, PA, USA) at room temperature. The histologic sections were then incubated for 60 min at 37 °C with affinity purified rabbit antimouse IL-4, IL-6, IL-10, TNF-α, IFN-γ (Peprotech, Inc., Rocky Hill, NJ, USA) or CTLA-4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) polyclonal antibodies. After incubation, the samples were washed twice with PBS solution, and finally, the sections were treated for 45 min at 37°C with a diluted FITC-conjugated goat antirabbit antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). The data represent the mean of three histologic sections from each animal for each feeding period.

Primary culture of mouse small intestine epithelial cells

The primary cultures of the enterocytes were prepared as described previously [20], with slight modifications as described in this section. At the end of each period of orally administering the test substances (SPH or water) the animals were euthanized as previously described. The small intestines were removed and placed in a digestion buffer of Hanks' balanced salt solution (Sigma-Aldrich, St. Louis, MO, USA) with 2% glucose (Sigma-Aldrich), 100 U/mL penicillin (Sigma-Aldrich), and 0.1 mg/mL streptomycin (Sigma-Aldrich). The intestines were flushed six times with 10 mL of the digestion buffer. They were then cut into 2- to 3-mm fragments and digested in 20 mL of digestion buffer supplemented with 300 U/mL Type XI collagenase (Sigma-Aldrich) and 0.1 mg/mL dispase (Gibco, Grand Island, NY, USA) at 25°C at 150 *g* for 45 min. Digestion was stopped by adding 20 mL of Dulbecco's modified Eagle medium (DMEM) without phenol red (Gibco), supplemented with 10% heat-inactivated fetal bovine serum (ATCC, Manassas, VA, USA), 10 ng/mL epidermal growth factor (U.S. Biological, Swampscott, MA, USA), insulin-transferrin-selenium-A (2.50 μg/mL, 0.55 μg/mL, and 1.68 pg/mL, respectively) from a 100× ready-to-use solution (Gibco), 100 U/mL penicillin (Sigma-Aldrich) and 0.1 mg/mL streptomycin (Sigma-Aldrich). Large fragments were removed by allowing them to settle for 2 min at the bottom of the flask. The supernatant was centrifuged for 3 min at 300 *g*. The pellet was washed twice with the DMEM solution and then resuspended in the same culture medium at a concentration of 4×10^5 to 6×10^5 organoids (single cells or intestinal epithelial cells [IEC]) per mL. The IEC suspensions were then transferred to 96-well cell culture plates (200 μL/well) and

incubated for 8 h (37°C; 5% CO_2). An in vitro toxicology assay kit, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based assay (Sigma-Aldrich), and trypan blue (0.4%) exclusion were used to assess cell viability. The supernatants were recovered for cytokine determination. IL-6 was determined using the corresponding mouse IL-6 ELISA set (BD OptEIA; BD Biosciences Pharmingen, San Diego, CA, USA).

Blocking IEC with anti-TLR4 or anti-TLR2 antibodies and a challenge with test substances

The blocking and challenge experiments were performed primarily to determine IL-6 values. IEC suspensions were obtained from untreated mice as described previously and incubated for 30 min at room temperature in the presence of 40 μg/mL (10 times the blocking concentration suggested by the manufacturer) of functional grade purified antimouse toll-like receptor (TLR)4/MD2, antimouse TLR2 (eBioscience), or DMEM (control). After the blocking period using the anti-TLRs, a functional dose of SPH suspension or unhydrolyzed fish proteins were added to the supernatant to a concentration of 6 μg/mL. Lipopolysaccharide (LPS; final concentration 0.1 μg/mL), a potent inducer of IL-6, was used as a control to indicate an inflammatory level of IL-6. The IEC suspensions were incubated for 8 h (37°C; 5% CO_2), and the supernatants were recovered and frozen until IL-6 levels were quantified by ELISA.

Experimental murine model of diarrhea caused by ETEC

We followed the mouse diet restrictions and antibiotic treatments described previously [21]. Briefly, 6- to 8-wk-old CD-1 mice were obtained from Charles River. After 4 d of acclimatization, the mice were separated into three experimental groups: Mice that received water before ETEC infection, mice that received SPH before ETEC infection, and a control group. Mice were housed in groups of three in plastic cages in a controlled atmosphere (temperature: 22 ± 2°C; humidity: 55 ± 2%) on a 12-h light–dark cycle. The mice were treated in accordance with the guidelines of the Canadian Council on Animal Care and the experimental design was approved by the Animal Care Committee of the University of Ottawa. The mice were given water or SPH by gavage for 7 d. At the end of 7 d, their drinking water was replaced with a solution of 0.5% (w/v) streptomycin with 7% fructose in water for 48 h. This solution was replaced with sterile water 3 h before infection. Twelve h before infection, food was removed, and was replaced 3 h after infection. Mice received cimetidine (50 mg/kg) intraperitoneally 3 h before infection to diminish their stomach acidity and to allow the passage of ETEC through the stomach. The mice in the two experimental groups were inoculated with 10^9 colony-forming units/mouse of a 4-h culture (exponential growth phase) of ETEC ATCC 10407. Mice were sacrificed at 3, 24, and 48 h after infection, as per the same procedures used for the BALB/c mice. Serum was analyzed using the Millipore Magnetic Mouse Cytokine Kit (EMD Millipore, Bedford, MA, USA) on a MagPix platform (Luminex Corporation, Austin, TX, USA).

Statistical analysis

The data were analyzed using a one-way analysis of variance procedure, using SPSS software. The differences among the means were detected with Duncan's multiple-range test in IBM SPSS Statistics, Version 19.0 (IBM Corporation, Armonk, NY). A statistically significant difference was defined by *P*-value <0.05.

Results

SPH increased IL-6 production from IEC

SPH has been tested for endotoxins and was found to contain less than the minimal detection level of our assay (<0.01 ng/mL).

The ex vivo experiment was conducted by culturing IEC with different concentrations of SPH, aiming to determine the functional dose (data not shown) and to compare the production of IL6 by IEC stimulated by SPH with LPS. The experiment also served to determine the effect of blocking TLR2 and TLR4 on the production of IL-6 by IEC in the presence of the SPH and a control substance (i.e., unhydrolyzed proteins).

Compared with LPS-treated and controls, the SPH stimulated a lower level of IL-6 production than the inflammatory level of IL-6 produced by LPS. Unhydrolyzed fish proteins had no effect on the production of IL-6. A pretreatment with anti-TLR2 and anti-TLR4 prevented the stimulating effect of the SPH. As expected, the anti-TLR treatment had no effect on the cells challenged with unhydrolyzed fish proteins (Fig. 1).

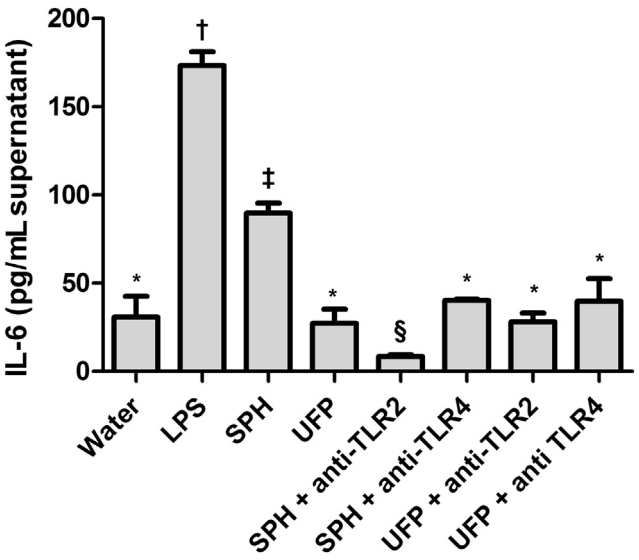

Fig. 1. The effect of SPH on IL-6 production, regulated by TLR2 and TLR4. The secretion of IL-6 by cultured IEC isolated from naive mice after treatment with water, LPS (0.1 μg/mL), SPH (6 μg/mL), UFP (6 μg/mL), SPH (6 μg/mL) + anti-TLR2, SPH (6 μg/mL) + anti-TLR4, UFP (6 μg/mL) + anti-TLR2, UFPs (6 μg/mL) + anti TLR4. Data are presented as the mean ± SD. IEC, intestinal epithelial cells; IL, interleukin; LPS, lipopolysaccharide; SPH, shark-derived protein hydrolysate; TLR, toll-like receptor; UFP, unhydrolyzed fish proteins. Means without a common letter differ significantly ($P < 0.05$).

SPH enhances immunosurveillance without compromising the gut barrier

The administration of SPH did not induce bacterial translocation to the liver (data not shown).

The effects of orally administered SPH on the production of antibodies were assessed. As shown in Figure 2A, the number of IgA+ cells was significantly greater in the small intestine lamina propria of the mice fed SPH (92 ± 2 cells/10 fields of vision at a magnification of 1000×), compared with the mice in the control group (75 ± 2). The number of IgG+ cells remained at a normal level in both groups (control: 38 ± 1; SPH: 36 ± 1). The augmentation of the IgA-producing cells was confirmed in serum using an IgA-specific DAS-ELISA. The SPH increased the serum IgA levels of female mice fed the SPH for 5 d (11 974 ± 214 pg/mL) and 7 d (11 102 ± 543 pg/mL) compared with mice not receiving the SPH (8513 ± 391 pg/mL). In comparison to control mice (8832 ± 61 pg/mL), male mice fed the SPH for 5 d had significantly more IgA circulating in their serum (12 112 ± 287 pg/mL), but this production was not maintained after 7 d of feeding (8631 ± 61 pg/mL) (Fig. 2B).

The effect of orally administered SPH on cytokine-positive cells in the small intestine mucosa was assessed by immunofluorescence. The number of IL-4–, IL-6–, TNF-α–, and IFN-γ–positive cells in the small intestines were significantly greater after administering the SPH to the mice for 7 d compared with the corresponding values in the control group (Fig. 2A). The number of anti-inflammatory IL-10–positive and the immune system down-regulator CTLA-4-positive cells in the small intestines were significantly greater after administering the SPH to the mice for 7 d compared with the corresponding values in the control group (Fig. 3A). A threefold increase in the serum level of TGF-β was observed among mice fed the SPH compared with control group mice (Fig. 3B)

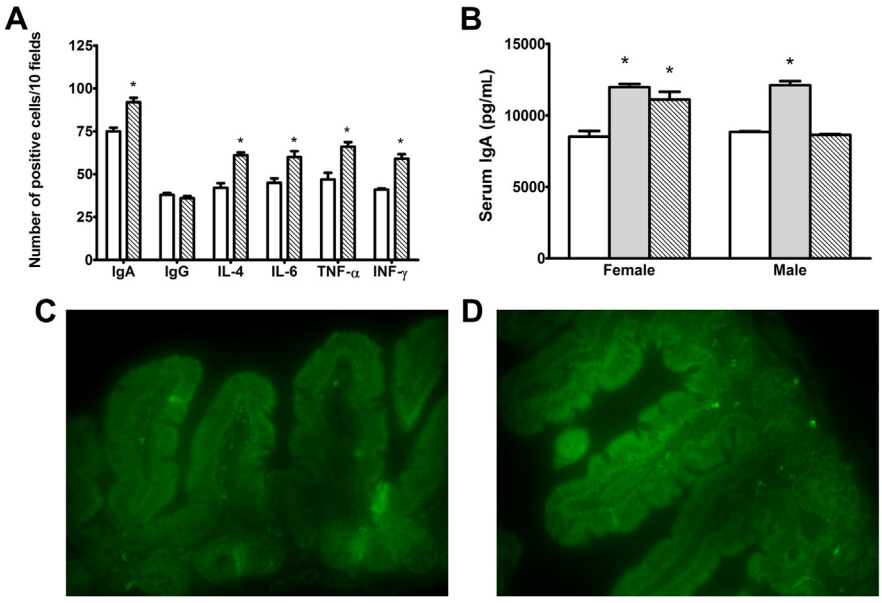

Fig. 2. Effects of orally administered SPH on the small intestines of mice. (A) The number of positive cells in histologic sections from the small intestines of mice that received SPH (4.5 mg/kg) for 7 d compared with control. (B) Effect of SPH on the serum IgA levels of unfed control mice and mice that were administered SPH (4.5 mg/kg) for 5 and 7 d. Images of IgA+ B cells in the small intestine of (C) the mice that received SPH (4.5 mg/kg) for 7 d compared with (D) the control. Data are presented as the mean ± SD. Ig, immunoglobulin; SPH, shark-derived protein hydrolysate. *$P < 0.05$ versus the control.

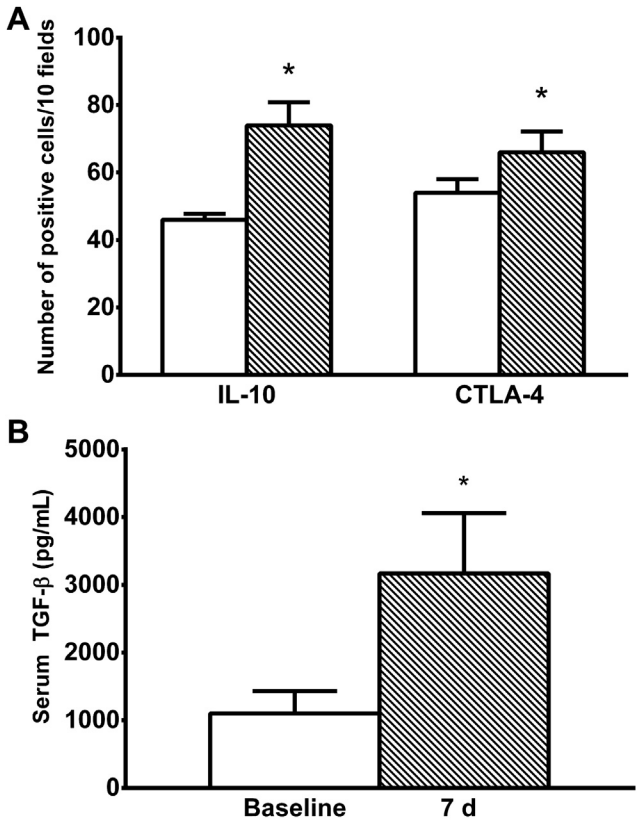

Fig. 3. Effects of orally administered SPH on the small intestines of mice implicating the regulatory immune network. (A) The number of IL-10-positive cells and CTLA-4-positive cells in the small intestines and (B) the serum levels of TGF-β of the mice that received SPH (4.5 mg/kg) for 7 d compared with the control. Data are presented as the mean ± SD. CTLA, cytotoxic T-lymphocyte antigen; IL, interleukin; SPH, shark-derived protein hydrolysate; TGF, transforming growth factor. *$P < 0.05$ versus the control.

SPH prevents ETEC pathogenicity

Similar to the BALB/c mice, the CD-1 mice that were fed the SPH before the *E. coli* infection had a higher number of IgA+ cells in their small intestines (Fig. 4A) across all time points compared with the control group. However, this change was not observed in the groups of mice that were infected with *E. coli* but did not receive the SPH. Additionally, in this experiment, SPH did not significantly alter the number of IgG+ cells (Fig. 4B) after 3, 24, or 48 h.

The SPH did increase the concentration of TGF-β in serum at 24 h after infection in the prefed mice compared with the lower concentration in the *E. coli*-exposed group; however, there were no significant differences observed at 3 or 48 h after infection (Fig. 4C).

At 24 h postinfection, the IL-17 levels (Fig. 4D) in the intestinal fluid reached significantly lower levels than the *E. coli*-treated group.

At 3 h postinfection, the group that received the pretreatment with the SPH had a significantly higher level of IL-1 α (Fig. 4E) in the intestinal fluid compared with the corresponding *E. coli*-treated group, but a significantly lower level at 48 h post-infection. No significant difference was observed at 24 h post-infection. No significant differences in IL-1 β (Fig. 4F) were measured at 3 and 24 h after infection. However, the groups of mice that did receive SPH had significantly lower IL-1 β levels at 48 h postinfection.

Discussion

It has been well documented that bioactive peptides derived from food can interact with host cells, subsequently altering intracellular signal transduction pathways [22–25]. Additionally, food-derived peptides have shown the ability to prevent and repair the damage caused by oxidative stress and inflammatory reactions [26].

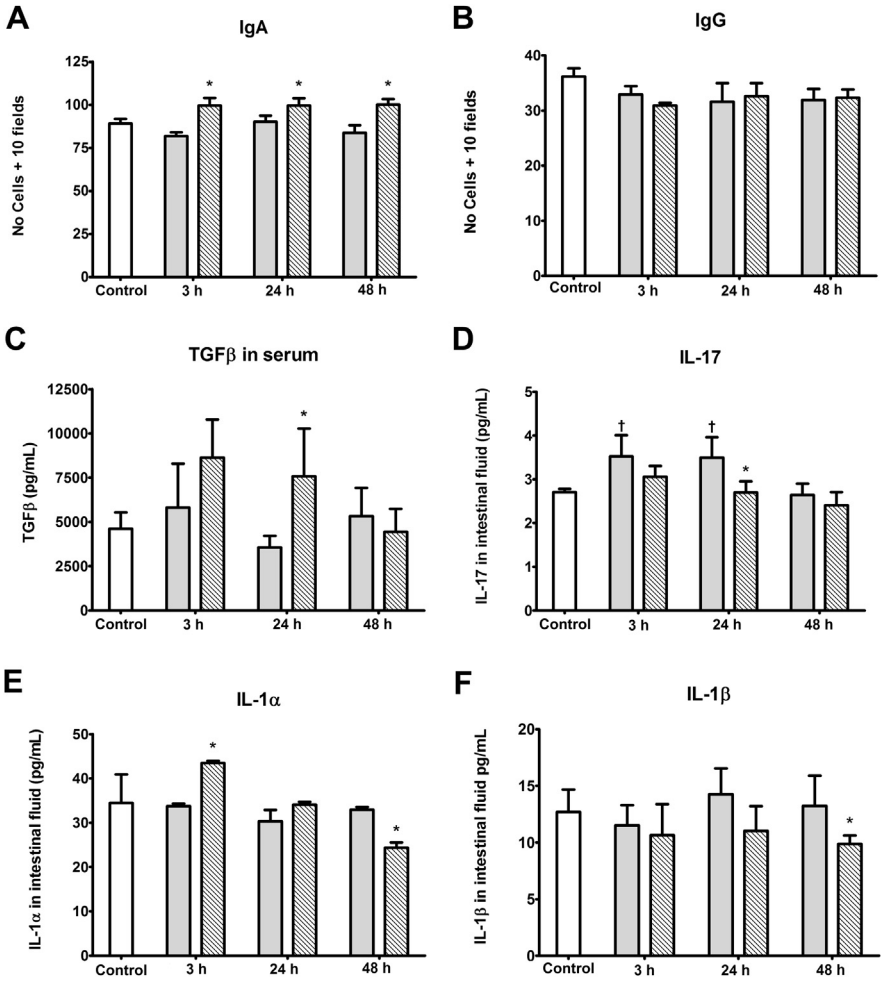

Fig. 4. Effects of orally administered SPH in an enterotoxigenic *Escherichia coli* pathogenesis model. (A, B) The number of IgA+ cells and IgG+ cells in the small intestines, (C) the serum levels of TGF-β, and (D–F) the levels of cytokines in the intestinal fluids of the mice that received SPH (4.5 mg/kg) for 7 d before infection compared with the control and *E. coli*-exposed groups. Data are presented as the mean ± SD. Ig, immunoglobulin; SPH, shark-derived protein hydrolysate; TGF, transforming growth factor. $^{*}P < 0.05$ versus corresponding *E. coli*-exposed group. $^{\dagger}P < 0.05$ versus control group.

The ability of peptides to modulate the immune response of a host also implies that peptides enhance the antibody production of secretory IgA [27], induce regulatory T cells [22,28], modulate apoptosis [29,30], block bacterial adhesion to epithelial cells and modulate TLR4 binding [23,31], enhance epithelial barrier function [32], and control cytokine production and proinflammatory mediators [13,28,33].

Indeed, in this study, the SPH had an effect on IgA-producing cells (Fig. 2A), IgA in serum (Fig. 2B), IL-10-positive, CTLA-4-positive cells in the small intestine mucosa (Fig. 3A), and TGF-β in serum (Fig. 3B), suggesting an immunomodulatory effect. Particularly, the increases in the production of IL-10 and TGF-β are strong indicators that the regulatory pathways are involved in immune homeostasis maintenance. The macrophages that produce IL-10 and TGF-β reduce inflammation and tissue damage [34]. Indeed, the secreted cytokines IL-10 and TGF-β and the surface molecules CTLA-4, PD-1, mTGF-β, mIL-10, TGF-βR, and IL-10 R [35] are known signals from the regulatory immune network. This SPH, a novel marine-source protein hydrolysate, has been shown to directly influence those parameters, which may lead to better regulatory effects on the host.

The primary biological role of enhanced IgA+ B cells is to increase immune surveillance to help prevent intestinal infections and other pathologies. IgA is also involved in the maintenance of intestinal homeostasis, and it may play an important role as a barrier against neoplasia in the mucosal territories [36]. As shown in Figures 2 and 3, the oral administration of SPH positively affected the milieu of the intestine, thereby enhancing the health status of the host.

The involvement of SPH in modulating the level of cytokine expression is reflected in the increase and modulation of TNF-α and IFN-γ. A recent study [37] reported a decrease in the percentage of TNF-α- and IL-12-producing monocytes and dendritic cells in patients with inflammatory bowel disease. In our experiment with SPH, the slight increase in TNF-α and IFN-γ levels (which are controlled by IL-10) might be associated with the priming of the immune response, achieved by increasing epithelial stimulation and initiating the cross-talk between the associated immune cells. The effect of SPH on TNF-α might be indirect and mediated by leptin, but this adipokine, which has a known relation with the immune system, was not measured in our experiments [38,39].

There is evidence that suggests orally administered marine hydrolysates are able to stimulate the mucosal immune system in the gut, including the increase in the number of IgA-producing cells, the modulation of the cytokine response, and the initiation of a cascade of immune responses. In the case of SPH, some preliminary research on humans found an

augmentation in serum IgA level in females but not in males. The serum IgA levels of male and female (Fig. 2B) mice might be indirectly related to the effect of IL-6 on IgA production. Female mice are known to mount stronger immune responses than male mice [40]. Estrogen may play a role, as it has been shown to enhance the production of IL-6 and antibodies [41]. The positive correlation between estrogen and the production of IgA in women was not seen in men [42]. The decrease in serum IgA levels observed in male mice could be explained by the down-regulation of the immune response caused by an oral tolerance that occurs faster in males then in females, which reinforces the need for a cyclic administration schedule to produce a beneficial effect [43]. A second explanation could be that there is a production cycle for IgA that our experiment is too short to observe [44].

The potential benefits of SPH may result from a direct effect on IEC, initiating the first communicative message to underlying immune cells that leads to a cascade of immune responses. The epithelial cells of the intestinal tract are thought to participate in the onset and the regulation of the mucosal immune response to exogenous antigens [32]. Distinguishing between exogenous and endogenous antigens is partially the responsibility of a sophisticated system of receptors, the TLRs. Although TLRs are very important in this process, it is likely that numerous other independent mechanisms also play a role in regulating the immune response.

The magnitude of the IEC response after challenging them with SPH is known to ensure that B cells will differentiate to plasmocytes without reaching inflammatory levels that could cause intestinal damage [32]. Moreover, when TLR2 and TLR4 were blocked by their respective anti-TLRs, the production of IL-6 by IEC challenged by SPH decreased, as shown in Figure 1. This fact suggests that the SPH has different epitopes that are involved in the interaction with IEC. Binding or partial binding to both TLRs might improve protection against enteric infections by creating competition with pathogens for adhesion sites. Moreover, TLR4 is an activator of nuclear factor (NF)-κB [45]. The modulation of this response is believed to play an important role in fighting inflammatory diseases.

To ascertain the immunomodulatory role of SPH, we used an infectious model of ETEC in mice. The results demonstrated that mice receiving SPH before *E. coli* administration were more resistant to infection, with a higher IgA response, higher levels of TGF-β, and modulated inflammatory markers (including IL-1 α and IL-1 β, as well as IL-17). Secretory IgA plays a key role in protecting and cross protecting against a variety of infectious agents, most importantly, against *E. coli* [46]. SPH has the important effect of down-regulating the inflammatory IL-17, which also controls the level of IL-1 β [47]. IL-17 also induces inflammatory responses by activating NF-κB. Studies have observed that IL-17 is expressed by patients suffering from rheumatoid arthritis, multiple sclerosis, asthma, and possibly *E. coli* infections [48,49].

Suppressing nonessential immune responses at environmental interfaces, such as the intestines and the lungs, is crucial to maintaining the proper functionality of these organs. Successful T-cell activation and homeostatic control require a delicate balance of positive and negative regulatory signals [50]. SPH, by inducing both proinflammatory and anti-inflammatory cytokine-producing cells, contribute to this balance. In fact, cytokines act in a network allowing optimal maintenance of the homeostasis, thus preparing the immune system to better respond to challenges including infection. This is of prime importance in human health and prevention of diseases.

Priming the immune cells may be another mechanism by which SPH controls the amplitude of the immune response. Priming the immune system by exposing it to biologically active peptides may prepare it to combat infectious challenges. Moreover, a recent study revealed that LPS priming or prestimulation of the udder protects against *E. coli*-elicited mastitis [51]. The mechanisms underlying this protection include production of antimicrobial factors, protection of the integrity of the epithelial cells by enforcing tissue stabilization, and wound healing [17].

Although our SPH demonstrated immunomodulatory effects in vivo, the mechanisms underlying its effects are not well understood. Biopeptides are able to modulate NF-κB and AP-1 pathways by controlling TNF-α and IL-6. Investigating these pathways could help us elucidate how SPH influences the immune system. Further investigation is needed to determine the specific anti-inflammatory pathways through which this SPH contribute to immunoregulation.

Conclusion

This SPH has the ability to enhance the gut barrier function by up-regulating IgA-producing cells and modulating inflammatory mediators. The systemic effects of the protein hydrolysate are potentially achieved through the maintenance of homeostasis and increased immunosurveillance. The modulation of the anti-inflammatory response may also be systemic, involving indirect effects produced at sites outside the gastrointestinal tract, including the mucosal territory, and external sites such as the joints and the skin. Thus, the potential medical applications of such hydrolysates extend beyond the gastrointestinal system.

Acknowledgments

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