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ORIGINAL CONTRIBUTION

Anti-oxidant, anti-inflammatory and immunomodulating properties of an enzymatic protein hydrolysate from yellow field pea seeds

Fatou Ndiaye · Tri Vuong · Jairo Duarte · Rotimi E. Aluko · Chantal Matar

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Abstract
Purpose Enzymatic protein hydrolysates of yellow pea seed have been shown to possess high anti-oxidant and anti-bacterial activities. The aim of this work was to confirm the anti-oxidant, anti-inflammatory and immunomodulating activities of an enzymatic protein hydrolysate of yellow field pea seeds.
Methods The anti-oxidant and anti-inflammatory properties of peptides from yellow field pea proteins (*Pisum sativum* L.) were investigated in LPS/IFN-γ-activated RAW 264.7 NO(−) macrophages. The immunomodulating potential of pea protein hydrolysate (PPH) was then studied in a murine model.
Results Pea protein hydrolysate, after a 12 h pre-treatment, showed significant inhibition of NO production by activated macrophages up to 20%. Moreover, PPH significantly inhibited their secretion of pro-inflammatory cytokines, TNF-α- and IL-6, up to 35 and 80%, respectively. Oral administration of PPH in mice enhanced the phagocytic activity of their peritoneal macrophages and stimulated the gut mucosa immune response. The number of IgA+ cells was elevated in the small intestine lamina propria, accompanied by an increase in the number of IL-4+, IL-10+ and IFN-γ+ cells. This was correlated to up-regulation of IL-6 secretion by small intestine epithelial cells (IEC), probably responsible for B-cell terminal differentiation to IgA-secreting cells. Moreover, PPH might have increased IL-6 production in IECs via the stimulation of toll-like receptors (TLRs) family, especially TLR2 and TLR4 since either anti-TLR2 or anti-TLR4 was able to completely abolish PPH-induced IL-6 secretion.
Conclusions Enzymatic protein degradation confers anti-oxidant, anti-inflammatory and immunomodulating potentials to pea proteins, and the resulted peptides could be used as an alternative therapy for the prevention of inflammatory-related diseases.

Keywords Anti-oxidant · Anti-inflammatory · Immunomodulating · Pea proteins · Cytokines

Introduction

A growing body of evidence has shown that peptides from milk and soy proteins can prevent cancer due to their anti-oxidant and anti-inflammatory effects [1, 2]. Pea proteins are of great nutritional importance being one of the major food legumes grown in different parts of the world [3]. Therefore, enzymatic digestion of pea proteins may bring some improvement in the matter of their digestibility as well as lower allergy symptom occurrence and thus have a positive influence on human health. Indeed, anti-oxidative, anti-tumour and immunomodulating effects have been demonstrated in enzymatic protein hydrolysates of whey

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[4], rapeseed [5] and fish [6]. Recently, enzymatic protein hydrolysates of yellow pea seed have been shown to possess high anti-oxidant [7] and anti-bacterial activities [8]. Such hydrolysates may be used as food components in order to beneficially influence human health such as chemoprevention.

Moreover, peptides from milk and fish proteins could also increase parameters of the immune response, such as immunoglobulin A (IgA)-producing cells, macrophage activity and anti-tumour activity [6, 9–11]. It has been demonstrated that the presence of these dietary antigens on Peyer's patches or in the immune cells associated with the villi of small intestine is the main pathway of gut mucosal immunostimulation [12]. However, to interact with the gut-associated immune cells, dietary antigens must first interact with the cells lining the intestine. Previous study indicated that peptides from milk stimulated members of toll-like receptors family, TLR-2 and TLR-4, and induced an enhanced ex vivo production of the cytokine IL-6 by isolated small intestine epithelial cells (IECs) [11, 13, 14].

Macrophages are ubiquitous cells that secrete a number of potent bioactive inflammatory mediators, including free radicals and cytokines, which normally promote tissue healing at the site of injury [15]. However, these mediators also stimulate recruitment of additional macrophages at the inflammatory site and propagate inflammation [16]. Nitric oxide (NO) is probably the most studied free radical because of its crucial role in normal physiological function and inflammation [17]. Production of large amounts of NO is considered cytotoxic and related to various diseases, especially cancer [18]. Indeed, the persistent inflammatory cells recruitment, repeated generation of ROS and pro-inflammatory mediators, and continued proliferation of genomically unstable cells are associated with neoplastic transformation and ultimately result in tumour invasion and metastasis [19]. Therefore, restoration of improperly working anti-oxidant machinery or suppression of abnormally amplified inflammatory signalling can provide important strategies for chemoprevention. Consequently, determination of anti-inflammatory and/or anti-oxidant properties has been proposed as a good indicator for screening anti-cancer agents [20, 21].

The aim of this work was to confirm the anti-oxidant and anti-inflammatory activities of enzymatic protein hydrolysates of yellow field pea seeds in LPS/IFN-γ-activated RAW 264.7 NO(−) macrophages. Then, their immunomodulating capacity was investigated in a murine model previously used for the study of the immunomodulating properties of fermented milks [11], kefir [22] and fish protein hydrolysates [6]. Finally, their effects on IL-6 production from the intestinal epithelium were conducted in isolated small intestine epithelial cells [14].

Materials and methods

Preparation of pea protein hydrolysate (PPH)

Pea protein isolate (80% protein, dry weight basis) was a gift from Nutri-Pea Ltd. (Portage La Prairie, MB, Canada). Low molecular weight (1,000 Da) protein hydrolysates were obtained from enzymatic hydrolysis of pea protein isolate as previously described [7]. Briefly, pea protein isolate was dispersed in distilled water to obtain 6.0% (w/v) protein slurry. Under stirring with amagnetic stirrer, the slurry was heated to 55 °C and adjusted to pH 8.0 using dilute NaOH solution. Thermolysin (Sigma Chemicals, St. Louis, MO) was added to initiate hydrolysis at a ratio of 0.5% (on the basis of protein weight, w/w). The temperature and pH of the slurry were maintained constant for 3 h, after which the hydrolysis was stopped by heating the slurry to 95 °C and held for 15 min. The hydrolysate was cooled to room temperature and centrifuged at 10,000*g* for 25 min at 4 °C. The clear supernatant was collected and passed through a stirred ultrafiltration cell using a 3 kDa molecular weight (MW) cut-off membrane (Sartorius Co., Germany). The resulting permeates containing peptides with MW <3 kDa were collected, freeze-dried and stored at −20 °C for further use as the PPH. The protein content (86%) and amino acid composition of the PPH have been reported elsewhere [7].

Cell culture

Murine RAW 264.7 NO(−) cell line was obtained from American Type Cell Collection (ATCC; Chicago, IL). Cells were cultured in RPMI-1640 media containing FBS (10%, v/v) (Sigma–Aldrich, Oakville, ON, Canada), penicillin/streptomycin (0.05 mg/mL) at 37 °C in a humidified atmosphere with 5% CO_2. At 90% confluence, cells were gently detached using a scraper (Fisher Scientific, Ottawa, ON) and plated at a density of 6×10^5 cells/well in 24-well plates. Cells were grown for 1 h, and unhydrolyzed pea protein isolate (PPI) or hydrolysated pea proteins (PPH) were added to obtain a series of final concentrations with a dilution factor of 2 from 25 to 1.56 µg/mL. After 12 h pre-treatment, cells were stimulated with 10 ng/mL LPS and 10 units/mL IFN-γ. The activated cells were further incubated for 24 h, and then, supernatants were collected to determine nitrite, TNF-α and IL-6 concentration.

Cell viability

Cell viability was assessed by WST-1 assay (Roche, Laval, QC, Canada). After treatment, WST-1 was added (10%, v/v) and incubated at 37 °C for 2 h. The absorbance was

measured at 450 nm in a μ-Quant plate reader (Bio-Tek, Winooski, VT).

NO determination

Nitric oxide production was assessed by Griess reaction [23]. Briefly, 100 µL of supernatant was placed in a 96-well plate. A volume of 50 µL of 1% (w/v) *N*-(1-naphtyl)-ethylenediamine dihydrochlorid was added, followed by 50 µL of 1% (w/v) sulphanilamide in 5% (v/v) phosphoric acid. After 20 min at room temperature, the absorbance at 540 nm was measured by a μ-Quant plate reader.

TNF-α and IL-6 determination

BD OptEIA Mouse TNF-α or IL-6 ELISA sets (BD Biosciences, Mississauga, ON) were used to measure TNF-α and IL-6 production following the manufacturer's instructions.

Animals

Six- to 8-week-old BALB/c female mice weighing 18–20 g (Charles River, Montreal, QC) were randomly distributed into 6 groups: a PPH-fed group and a control group with three different feeding periods (2, 5 or 7 days). Each experimental group consisted of 6 mice housed in a controlled atmosphere (temperature 22 ± 2 °C; humidity 55 ± 2%) with a 12-h light/dark cycle. Mice were maintained and treated in accordance with the guidelines of the Canadian Council on Animal Care.

Since PPI did not show any effect in vitro, in vivo experiments in this study were mainly assessed on PPH. PPH were dissolved in water. Treated mice received by gavage 100 µg/day of PPH for 2, 5 or 7 consecutive days. Control mice of each treatment period received the same volume of water instead. All groups of mice, and a control group, received simultaneously a conventional balanced diet ad libitum and water. All animals were sacrificed after feeding periods, and the small intestines were removed for histological preparation following the Sainte-Marie technique [24] for paraffin inclusion. Histological slices (4 µm) were stained with haematoxylin–eosin followed by light microscopy examination (magnification 400×).

Ex vivo phagocytosis assay in peritoneal macrophages

The assay was performed according to [25]. Briefly, peritoneal macrophages were harvested (in sterile conditions) by washing the peritoneal cavity with 5 mL of PBS containing 10 U/mL of Heparin (Sigma–Aldrich, St. Louis, MO, USA) and 0.1% Bovine Serum Albumin (Jackson ImmunoResearch, West Grove, PA, USA). The macrophage suspension was washed twice with the same buffer, and it was adjusted to a concentration of 10^6 cells/mL. A heat-killed (100 °C, 15 min) *Candida albicans* suspension (10^7 cell/mL) was opsonized with mouse autologus serum (10%) for 15 min at 37 °C. Opsonized yeasts (0.15 mL) were added to 0.15 mL of each macrophage suspension. The mixture was incubated for 30 min at 37 °C. The phagocytosis was measured as the % of activated (with at least one cell of yeasts phagocyted) macrophages after a 100-cell count using an optical microscope.

Immunofluorescence test for B population (IgA+ and IgG+ cells) identification

The number of IgA- and IgG-producing (IgA+ and IgG+) cells was determined on histological slices from the small intestine by a direct immunofluorescence method. The immunofluorescence test was performed using (α-chain specific) anti-mouse IgA FITC conjugate or (γ-chain specific) anti-mouse IgG FITC conjugate (Sigma–Aldrich, St. Louis, MO, USA). Histological slices were deparaffinized and rehydrated in a graded series of ethanol. Deparaffinized histological samples were incubated with the appropriate antibody dilution (1/100 for IgA or 1/50 for IgG) in PBS solution for 30 min at 37 °C. Then, samples were washed 2 times with PBS solution and examined using a fluorescent light microscope. The results were expressed as the number of IgA+ or IgG+ cells (positive = fluorescent cell) per 10 fields (magnification 100×). Data represent the mean of three histological slices for each animal, for each sampling day (three animals per group).

Cytokine determination in lamina propria of small intestine

IL-4, IL-10, IL-12 and IFN-γ were studied by an indirect immunofluorescence method on histological slices from the small and large intestine lamina propria. Histological slices were deparaffinized and rehydrated in a graded series of ethanol and then, incubated for 30 min in a 1% blocking solution of bovine serum albumin (BSA) (Jackson Immuno Research, West Grove, PA, USA) at room temperature. Histological slices were then incubated for 60 min at 37 °C with rabbit anti-mouse IL-4, IL-10 and IFN-γ or goat anti-mouse IL-12 (Peprotech, Inc., Rocky Hill, NJ, USA) polyclonal antibodies (diluted 1/100 in PBS solution). The incubation was followed by two washes with PBS solution. Histological slices were treated for 45 min at 37 °C with a dilution of a goat anti-rabbit antibody conjugated with FITC (Jackson Immuno Research) diluted 1/100 in PBS solution, washed twice with PBS and examined using a

fluorescent light microscope. The results were expressed as the number of IL-4+, IL-10+, IL-12+ or IFN-γ+ cells (positive = fluorescent cell) per 10 fields (magnification 100×). Data represent the mean of three histological slices for each animal, for each sampling day (three animals/sampling day).

Primary culture of small intestine epithelial cells from mice

Preparation of primary cultures of enterocytes was performed as previously described [26]. After each feeding period, the small intestine was removed and placed in Hank's balanced salt solution (HBSS; Sigma–Aldrich) containing 2% glucose (Sigma–Aldrich), 100 U/mL penicillin (Sigma–Aldrich) and 0.1 mg/mL streptomycin (Sigma–Aldrich) on ice. Intestines were flushed 6 times with 10 mL of the same buffer, cut into 2- to 3-mm fragments and collected in HBSS. Then, the small intestines were digested in 20 mL of HBSS containing 300 U/mL collagenase (Sigma–Aldrich C-7657) and 0.1 mg/mL dispase (Gibco, Grand Island, NY, USA) at 25 °C, with agitation at 150 rpm for 45 min. Digestion was stopped by the addition of 20 mL of Dulbecco's Modified Eagle Medium (DMEM) without phenol red (Gibco) supplemented with 10% heat-inactivated foetal bovine serum (ATCC, Manassas, VA, USA), epidermal growth factor at 10 ng/mL (US Biological, Swampscott, MA, USA), Insulin–Transferrin–Selenium-A at 2.50, 0.55 µg/mL and 1.68 pg/mL, respectively, from a 100 x-ready-to-use solution (Gibco), penicillin at 100 U/mL and streptomycin at 0.1 mg/mL. The largest fragments were allowed to settle at the bottom of the flask for 2 min. The supernatant was then transferred to centrifuge tubes and centrifuged for 3 min at 100*g*. The pellet was washed twice with the culture medium and finally resuspended in the same medium at a concentration of 4–6 × 10^5 single cells or clusters of intestinal epithelial cell (IEC)/mL. A 200 µL portion of each organoid suspension was then transferred to a well in each of once 96-well cell culture plates. The plates were incubated for 8 h at 37 °C under an atmosphere containing 5% CO_2. An in vitro toxicology assay kit (Sigma–Aldrich) based on 3(4, 5 dimethylthiazol-2 YL)-2, 5 diphenyltetrazolium bromide (MTT) and trypan blue (0.4%) exclusion were used to confirm cell viability. Supernatants were collected for IL-6 determination as indicated above.

Statistical analysis

Statistical analysis of the data by ANOVA and Fisher post hoc tests were performed using StatView software (Cary, NC, USA). Statistical significance was set at $p \leq 0.05$. Data are reported as mean ± SD.

Results

PPH inhibited nitric oxide, IL-6 and TNF-α production from LPS/INF-γ-activated macrophages

Anti-oxidant activities of PPI and PPH were assessed in LPS/IFN-γ-activated RAW 264.7 NO(−) macrophages. Results of the cytotoxicity WST-1 study showed that after 24-h incubation with either PPI or PPH, there was no variation in cell viability at all concentrations tested (0–25 µg/mL, data not shown). While PPI did not show any significant effect ($p > 0.05$), PPH significantly ($p < 0.05$) inhibited NO production of activated macrophages after 12 h pre-treatment in a dose-dependent manner (Fig. 1).

To evaluate the anti-inflammatory action, inhibitory effects of PPI and PPH on pro-inflammatory cytokines, such as TNF-α and IL-6, were investigated. LPS/IFN-γ-activated RAW 264.7 NO(−) macrophages showed a marked up-regulation of TNF-α and IL-6 production. This increase was inhibited in a dose-dependent manner by PPH at concentrations higher than 3.1 µg/mL ($p < 0.05$). Treatment with 25 µg/mL of PPH reduced TNF-α and IL-6 levels in activated macrophages by 35 and 80%, respectively ($p < 0.0001$). No effects on TNF-α and IL-6 production were found when PPI was added to LPS/IFN-γ-stimulated cells (Fig. 2, panel a and b).

PPH enhanced phagocytic activity of peritoneal macrophages

Macrophages are first line of defence in immune response to foreign invaders. Phagocytic activities of peritoneal

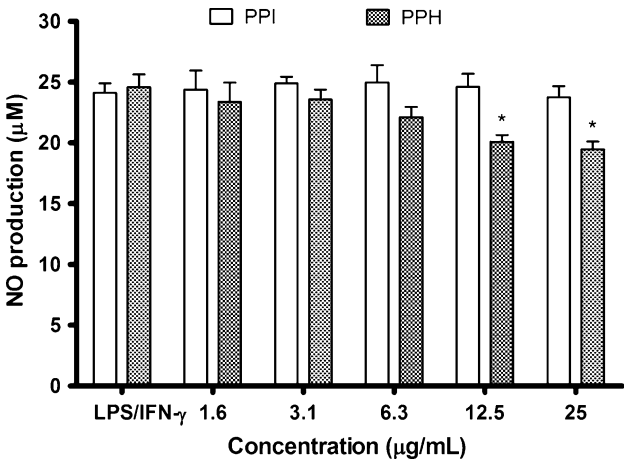

Fig. 1 Effects of unhydrolyzed pea protein isolate (*PPI*) and enzymatic pea protein hydrolysate (*PPH*) on NO production of RAW 264.7 NO(−) macrophages after 24 h LPS/IFN-γ-activation. All values are means of four separated experiments ±SD. *Denotes statistical significance at $p < 0.05$ versus control

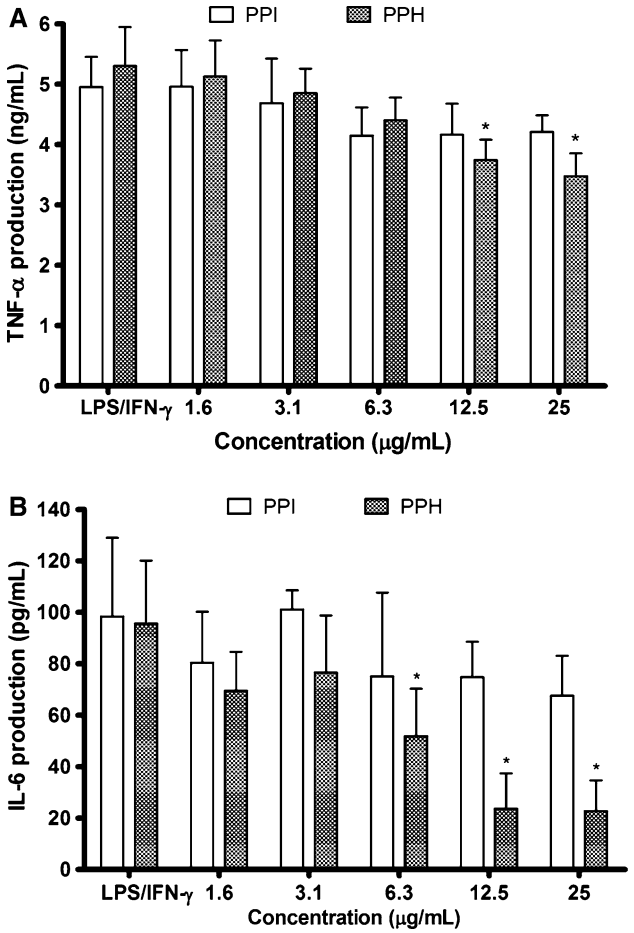

Fig. 2 Effects of unhydrolyzed pea protein isolate (*PPI*) and enzymatic pea protein hydrolysate (*PPH*) on TNF-α (*panel* **a**) and IL-6 (*panel* **b**) production of RAW 264.7 NO(−) macrophages after 24 h LPS/IFN-γ-activation. All values are means of four separated experiments ±SD. *Denotes statistical significance at $p < 0.05$ versus control

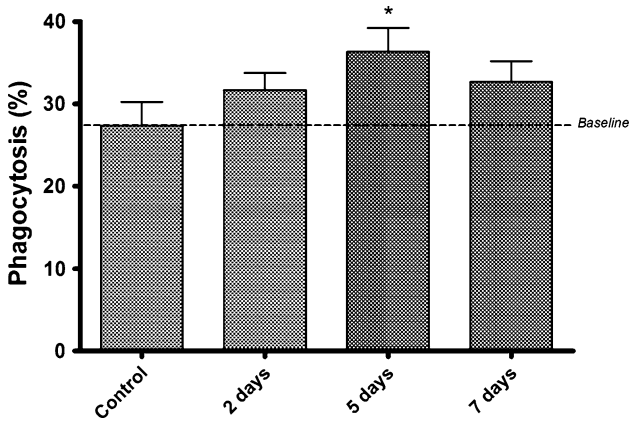

Fig. 3 Phagocytic activity of peritoneal macrophages from mice treated with enzymatic pea protein hydrolysate (*PPH*) for 2, 5 and 7 days. All values are means ± SD ($n = 6$). *Denotes statistical significance at $p < 0.05$ versus control

macrophages from PPH-fed mice for either 2, 5 and 7 days were measured. Macrophages from 5-day PPH-fed mice showed a significant increased phagocytic activity as compared with cells from control mice while those from either 2- or 7-day PPH-fed mice showed a slight but not significant ($p > 0.05$) increase (Fig. 3).

PPH activated mucosal immune response

The histological study of the small intestine (haematoxylin–eosin) revealed no differences in the morphological architecture between control mice and PPH-fed animals. No lymphocyte infiltrates, or the presence of oedema or mucosal atrophy was observed.

Effects of the oral administration of PPH on the B-cell population and on cytokine+ cells in the small intestine mucosa were assessed. The number of IgA+ cells was significantly increased for 5- and 7-day PPH-fed mice (Fig. 4a), while the number of IgG+ cells did not change when compared with control values (Fig. 4b). Similarly, except for IL-12+ cells (Fig. 5d), the number of IL-4+ (Fig. 5a), IL-10+ (Fig. 5b), IFN-γ+ (Fig. 5c) cells associated with the small intestine was significantly increased as compared with their respective controls, after 5- and 7-day administration periods assayed.

PPH increased IL-6 production from intestinal epithelial cells via TLRs stimulation

A primary culture of small intestine epithelial cells was used to study the effects of PPH on IL-6 production from enterocytes. Only epithelial cells isolated from 7-day PPH-fed mice showed a significant increase ($p < 0.05$) of IL-6 secretion as compared with cells from control animals (Fig. 6).

In parallel, epithelial cells isolated from non-treated mice that were challenged with different PPH concentrations in vitro. At 25 μg/mL, PPH significantly increased ($p < 0.05$) the IL-6 secretion by 20% as compared with unchallenged cells. However, this stimulation was totally abolished in the presence of either anti-TLR2 or anti-TLR4 (Fig. 7).

Discussion

Enzymatic protein hydrolysates from yellow field pea seeds exerted potent inhibitory activity of pro-inflammatory mediators' production by LPS/IFN-γ-stimulated RAW264.7 NO(−) macrophages such as NO, TNF-α and IL-6, without any effect on cell viability. Similar inhibition was not found with natural pea proteins. Production of large amounts of free radical NO is considered cytotoxic

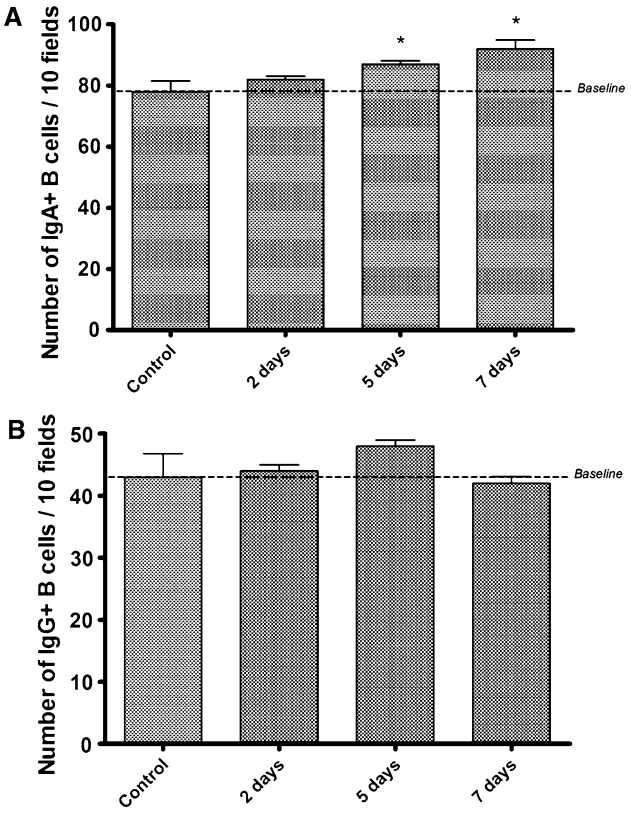

Fig. 4 Production of IgA (*panel* **a**) and IgG (*panel* **b**) of gut-associated immune cells from mice treated with enzymatic pea protein hydrolysate (*PPH*) for 2, 5 and 7 days. All values are means ± SD ($n = 6$). *Denotes statistical significance at $p < 0.05$ versus control

and related to various diseases, including cancer [18]. Therefore, inhibition of its secretion by activated macrophages can attenuate inflammation and reduce cancer risk. These results confirmed the previously demonstrated anti-oxidant activities of pea protein hydrolysates [7]. Moreover, PPH also inhibited the production of two important pro-inflammatory cytokines, TNF-α and IL-6, whose up-regulation is linked to the pathogenesis of many infectious and inflammatory diseases and cancer [27]. The mechanisms through which PPH inhibited pro-inflammatory mediators' production are unknown. However, since the inhibitory effects in PPH-treated macrophages required at least 12 h to be fully expressed, PPH might act via both transcriptional and translational mechanisms. Recently, peptides from soybean were reported to inhibit inflammation through suppression of NF-kB pathways [28] and to reduce risk of cancer via inhibition of histone acetylation and retinoblastoma protein phosphorylation [29]. Moreover, most of recent studies attributed anti-oxidant, anti-inflammatory and chemopreventive properties of peptides from legumes seeds to lunasin and lunasin-like peptides [30, 31]. Therefore, enzymatic digestion of pea proteins might increase its lunasin-like peptide levels, and future studies should address these possibilities.

Oral administration of dietary antigens could stimulate the host non-specific immunity by enhancing the systemic immune response or by modulating the functions of immunocompetent cells [25]. In mice, oral administration of PPH enhanced the phagocytic activity of their peritoneal macrophages, signalling the stimulation of the innate immune system. The mechanism by which peritoneal macrophages were activated is still unclear. However, it was demonstrated that this stimulation might be partially induced via modulation the gut mucosa immune response [6]. Indeed, there was an increase in the number of IgA+ cells in the small intestine lamina propria of PPH-fed mice. The specific secretory IgA against PPH was not investigated. Nevertheless, the induction of IgA+ B cells could mean an enhanced immune surveillance to prevent intestinal infections or other intestinal pathologies because the main function of secretory IgA in the gut is to exert immune exclusion by intimate cooperation with the innate non-specific defence mechanisms [32]. The number of IgG+ cells in the small intestine did not differ from control mice in animals that received PPH. This would suggest that no inflammatory immune response was induced by PPH. Results of the histological studies (data not shown) of the gut by haematoxylin–eosin stain confirmed no damage at the intestinal mucosal level.

B-cell immunoglobulin switching and differentiation to plasmocyte-secreting IgA occur in an environment rich in IL-4, IL-5 and TGFβ, while IL-6 promotes terminal differentiation of B cells into plasma cells [33]. In this study, the increase in the number of IgA+ cells was accompanied by an increase in the number of IL-4+, IL-10+ and IFN-γ+ cells. It is known that macrophages and dendritic cells of the lamina propria, among other cell types, are producers of IL-6, IL-10, IL-12, IFN-γ and TNF-α [22]. Additionally, mast cells are a potential source of early-response cytokines, such as TNF-α and IL-4, which are decisive in initiating the immune and inflammatory response [34]. These cell populations from the innate immune system could have been then the source of the enhanced number of cytokines+ cells observed in the small intestine lamina propria. In fact, it was demonstrated that dendritic cells in the intestinal mucosa are able to open up junctional complexes and send dendrites into the lumen for the internalization of luminal antigens, which can be directly presented to B cells in the gut lamina propria to undergo a T-cell-independent IgA isotype switch [35].

These results indicated that the increase in the number of cytokine+ cells observed in the gut lamina propria was probably associated with an effect of PPH mainly on cells of the innate immune system, as previously demonstrated for fermented milk [12, 13] or fish protein hydrolysates [6].

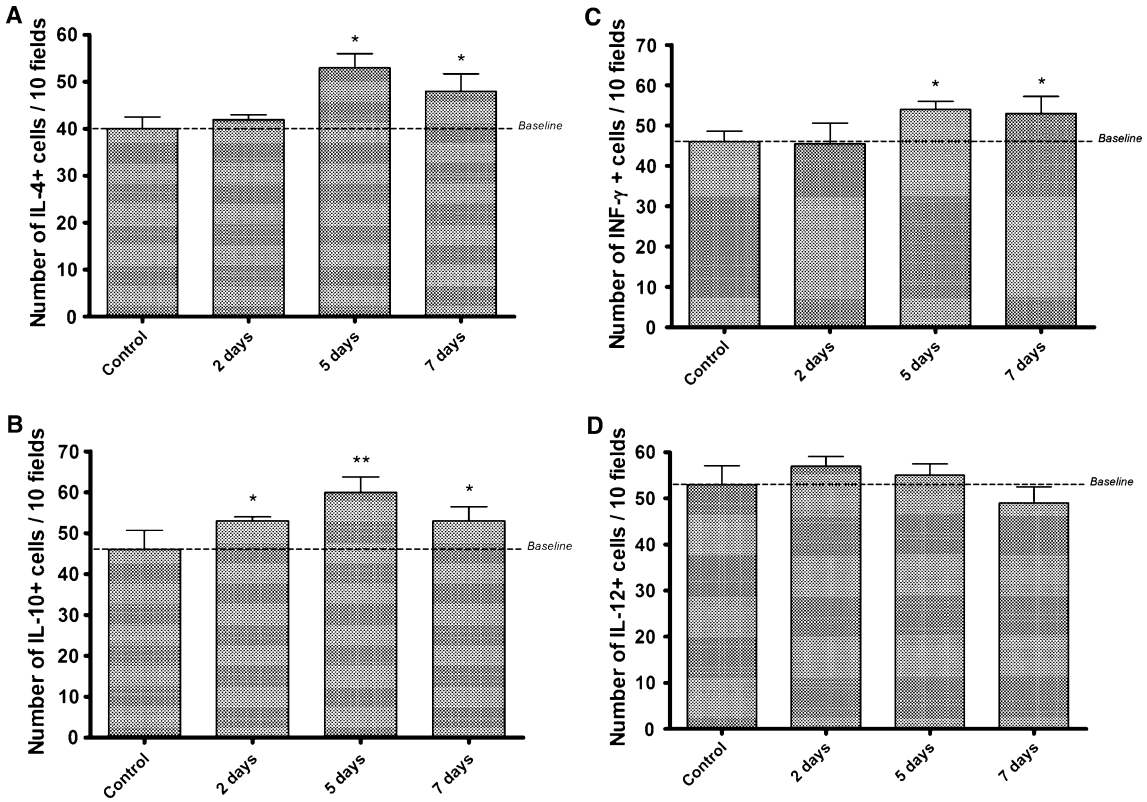

Fig. 5 Production of IL-4 (*panel* **a**), IL-10 (*panel* **b**), IFN-γ (*panel* **c**) and IL-12 (*panel* **d**) of gut-associated immune cells from mice treated with enzymatic pea protein hydrolysate (*PPH*) for 2, 5 and 7 days. All values are means ± SD ($n = 6$). *Denotes statistical significance at $p < 0.05$ versus control

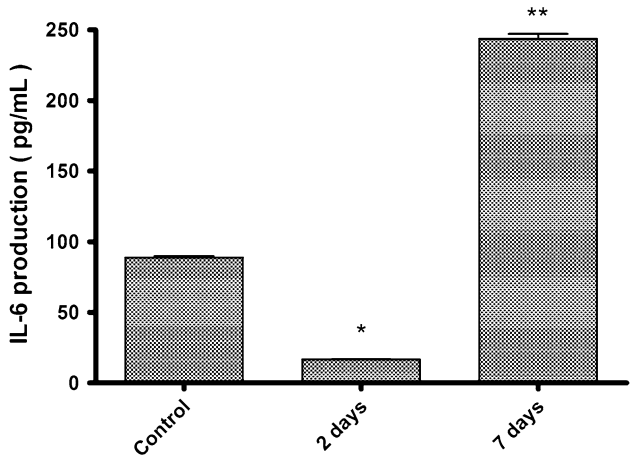

Fig. 6 IL-6 production of isolated small intestine epithelial cells from 2- and 7-days enzymatic pea protein hydrolysate (*PPH*)-fed mice. All values are means of two separated experiments ±SD ($n = 6$). *Denotes statistical significance at $p < 0.05$ versus control

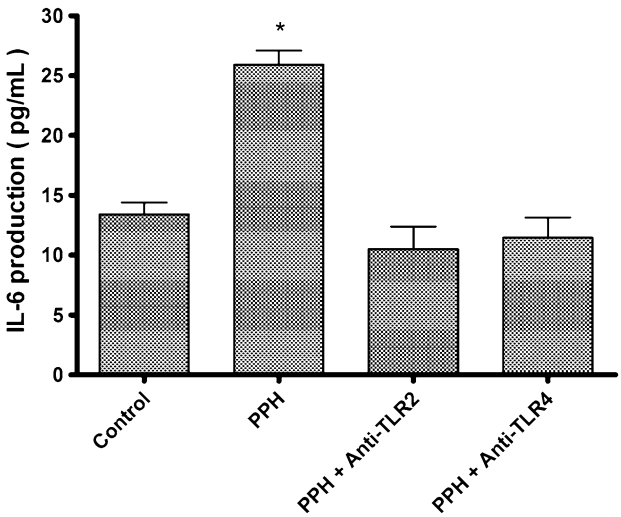

Fig. 7 IL-6 production of enzymatic pea protein hydrolysate (*PPH*)-treated epithelial cells 25 (μg/mL) in the absence/presence of anti-mouse TLR2 (200 pg/mL) and anti-mouse TLR4 (500 pg/mL). All values are means of two separated experiments ±SD. *Denotes statistical significance at $p < 0.05$ versus control

This further supported the chemopreventive potential of PPH since the stimulation of the innate immune system in the gut has been associated with anti-tumour activities in the same murine model, where the mice were further inoculated with breast cancer cells [1, 10, 36]. However, the participation of the adaptive immunity that could have also been induced should not be neglected, and further studies are needed to confirm this hypothesis.

The possibility that the epithelium may respond to luminal factors, signalling their presence to the mucosal immune system, was largely ignored. It is now recognized that enterocytes secrete cytokines, growth factors and their binding proteins and may directly present antigens to T cells [37]. Epithelial signalling enables luminal factors to alter immune responses while the integrity of the epithelial barrier is maintained [38]. In the present work, oral administration of PPH to mice was able to up-regulate IL-6 secretion by IECs, necessary, for example, to B-cell terminal differentiation to IgA-secreting cells in the gut lamina propria [39].

IL-6 is a multifunctional cytokine involved in diverse biological processes, such as host response to enteric pathogens, acute-phase reactions, hematopoiesis, growth factor for normal or neoplastic cells and terminal differentiation of B lymphocytes [40]. IL-6 has traditionally been considered the product of pro-inflammatory cells [41]. However, IL-6 is also known to possess several anti-inflammatory characteristics, such as its ability to down-regulate LPS-induced monocyte IL-1 and TNF-α mRNA expression [42]. IECs have been shown to secrete a variety of inflammatory cytokines and chemokines, including IL-6 after stimulation by bacteria [26] or dietary antigens [11]. In this study, PPH was able to stimulate IL-6 production in IECs. This stimulation was induced, at least partially, via members of the toll-like receptors (TLRs) family, TLR2 and TLR4, since either anti-TLR2 or anti-TLR4 was able to completely abolish PPH-induced IL-6 secretion. This suggests that PPH might contain peptides whose structures partially resemble ligands that bind TLR2 and TLR4. Indeed, TLR4 has been identified as surface membrane receptor for dietary antigens such as sulphated polysaccharide carrageenan [43]. Further studies are needed to clarify the crucial role of PPH in TLRs stimulation in IECs and thus the innate immune response. MHC class II molecules, in addition to their central role in adaptive immunity, were recently showed to synergize with TLR2 and TLR4 to produce the human-beta-defensin and inducing innate immune responses [44].

Conclusion

In conclusion, using LPS/INF-γ-stimulated RAW 264.7 macrophages as a model of inflammatory injury, PPH inhibited in a significant ($p < 0.05$) and dose-dependent manner, the release of pro-inflammatory cytokines TNF-α and IL-6, and NO production. Since oxidative stress and inflammation have been implicated as main causative factors in carcinogenesis, these results suggest that the antioxidant and anti-inflammatory properties of PPH might be beneficial in chemoprevention. Moreover, this is a first report showing the importance of the enzymatic pea protein hydrolysates on the gut mucosa immunostimulation in vivo. The enhanced IL-6 production by IECs helped to understand the increase in the number of IgA+ cells in the intestinal lamina propria observed in this study. It is clear that PPHs are able to deliver immune signals to IECs for the up-regulation of IL-6 production to an extent that would help B-cell differentiation into plasmocytes without reaching inflammatory levels that could cause intestinal damage. Further research would be needed to demonstrate the role of these peptides in prevention and/or treatment of other inflammatory-related disorders, such as cancer, neurodegenerative and cardiovascular diseases. Nevertheless, enzymatic protein degradation confers anti-oxidant, anti-inflammatory and immunomodulating potentials to pea proteins, and the resulting peptides could be used as an alternative therapy for the prevention of inflammatory-related diseases.

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