Postprint of Food Research International, Volume 126, December 2019, 108585
DOI: https://doi.org/10.1016/j.foodres.2019.108585

Lupine protein hydrolysates decrease the inflammatory response and improve the oxidative status in human peripheral lymphocytes

Ivan Cruz-Chamorro[a,b], Nuria Álvarez-Sánchez[a], María del Carmen Millán-Linares[c], María del Mar Yust[c], Justo Pedroche[c], Francisco Millán[c], Patricia Judith Lardone[a,b], Cecilio Carrera-Sánchez[d], Juan Miguel Guerrero[a,b,e], Antonio Carrillo-Vico[a,b,*]

[a] Instituto de Biomedicina de Sevilla, IBiS (Universidad de Sevilla, HUVR, Junta de Andalucía, CSIC), Spain.
[b] Departamento de Bioquímica Médica y Biología Molecular e Inmunología, Universidad de Sevilla, Spain.
[c] Plant Protein Group, Instituto de la Grasa, CSIC, Seville, Spain.
[d] Departamento de Ingeniería Química, Facultad de Química, Universidad de Sevilla, Spain.
[e] Departamento de Bioquímica Clínica, Unidad de Gestión de Laboratorios, Hospital Universitario Virgen del Rocío, Sevilla, Spain.

*Address correspondence and reprint requests to Antonio Carrillo-Vico, Instituto de Biomedicina de Sevilla, IBiS (Universidad de Sevilla, HUVR, Junta de Andalucía, CSIC), Spain. Avda. Manuel Siurot s/n, 41013 Seville, Spain; Tel: +34955923106; Fax: +34954907048; E-mail: vico@us.es

Abbreviations

CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, oxidized form of glutathione; IL, interleukin; LPHs, lupine protein hydrolysates; PBMCs, peripheral blood mononuclear cells; PHA, phytohaemagglutinin; ROS, reactive oxygen species; SOD, superoxide dismutase; TAC, total antioxidant capacity.

Declarations of interest: none.

Abstract

Although cell-free systems and immortalized cell lines have been used to demonstrate the potential health benefits of lupine proteins and peptides, no study has examined the effects of lupine protein hydrolysates (LPHs) on the immune and oxidative responses of non-immortalized human cells. Therefore, the aims of this study were to evaluate the effects of the *in vitro* administration of LPHs from *Lupinus angustifolius* on the immunological and oxidative statuses of human peripheral blood mononuclear cells (PBMCs) from 53 healthy donors. LPHs reduced PBMCs proliferation and the levels of Th1, Th9 and Th17 pro-inflammatory cytokines without being cytotoxic. LPHs also skewed the pro-/anti-inflammatory balance towards a Th2 protective response. Additionally, LPHs increased superoxide dismutase and catalase activities, and the total antioxidant capacity (TAC). This study is the first to show that LPHs reduce T cell inflammatory responses and improve the anti-inflammatory/pro-inflammatory cytokine balance and the TAC by PBMCs. Thus, LPHs may represent an effective option for developing nutritional strategies to prevent pathologies with underlying inflammation and oxidative stress.

Keywords: Lupine peptides, vegetable hydrolysates, bioactivity, peripheral blood mononuclear cells, cytokines, Th1 response, inflammation, anti-oxidant.

1 Introduction

Over the past few years, a growing body of scientific evidence has highlighted that proteins and peptides from food products might have beneficial biological effects beyond their established nutritional value on human health (Bougle & Bouhallab, 2015). Thus, the study of functional foods from natural sources is an emerging field in the area of food technology and health due to the increasing popularity of functional foods among consumers who continually demand healthier products that improve their quality of life and thus prevent or treat diseases. In this regard, inflammation and oxidative stress are key components in the progression of several chronic diseases that are considered principal causes of death in modern society (Dandekar, Mendez, & Zhang, 2015).

Cytokine production reflects the immunological state and characterizes different subpopulations of immune cells. Type 1 T helper cells (Th1) produce inflammatory cytokines such as interleukin (IL)-2, interferon-γ (IFN-γ) and tumor necrosis factor (TNF), which play a central role in T cell activation and proliferation; Th1 cells are also involved in macrophage activation, and they can evoke cell-mediated immunity and activate phagocytosis. Type 2 T helper cells (Th2) produces anti-inflammatory cytokines such as IL-4 and IL-13, and this subset is implicated in inducing antibody-mediated responses (Romagnani, 2000). Th9, Th17 and Th22 cells, which produce IL-9, IL-17, and IL-22, respectively, are involved in inflammatory and autoimmune diseases and in the development of allergic pathologies (Azizi, Yazdani, & Mirshafiey, 2015; Singh et al., 2014; Zbikowska-Gotz et al., 2016; Zhao, Xiao, Ghobrial, & Li, 2013). IL-10 is a classical anti-inflammatory cytokine and is principally synthesized by T regulatory cells, which play a key role in controlling inflammatory processes (Ouyang, Rutz, Crellin, Valdez, & Hymowitz, 2011).

In addition to cytokines, the immune system uses reactive oxygen species (ROS) to eliminate pathogens (Torres, Jones, & Dangl, 2006). At physiological levels, ROS are essential for cell survival; however, a considerable increase in their production could generate cell damage and death (Yang, Bazhin, Werner, & Karakhanova, 2013). To prevent irreversible cellular damage, antioxidant systems involved in restoring and maintaining cell redox homeostasis are activated (Sies, Berndt, & Jones, 2017). Many different enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR), are implicated in the maintenance of intracellular redox balance by using the same substrate and/or generating a product that will be a substrate for subsequent antioxidant enzyme (Sies et al., 2017).

The use of food bioactive peptides is a new frontier in the field of nutrition (Hayes, 2018); the objective of this field is to control biological functions and treat, prevent or reduce the risk of disease. Peptides become bioactive only when the proteins are hydrolyzed and the bioactive fragments are released; these bioactive peptides can be generated *in vivo* through digestive or microbial enzymes during digestion or *in vitro* through food processing (Moller, Scholz-Ahrens, Roos, & Schrezenmeir, 2008). Many different *in vitro* effects, such as immunomodulatory (Vernaza, Dia, de Mejia, & Chang, 2012), antioxidant (Lindmark-Mansson & Akesson, 2000) and antimicrobial actions (Farnaud & Evans, 2003) have already been described for bioactive peptides. A number of studies have shown that many peptides derived from animal sources, mostly dairy whey and egg, can influence the immune response and oxidative balance (Gauthier, Pouliot, & Saint-Sauveur, 2006; Yousr & Howell, 2015).

Moreover, different studies have shown the *in vitro* effects of plant protein hydrolysates on inflammatory markers and oxidative stress. For example, trypsin-digested rice protein improves immune function by promoting phagocytosis in human leukocytes (Gauthier et al., 2006), while *Vigna subterranea* protein hydrolysates can protect against several free radicals (Arise et al., 2016). *Glycine max*, commonly called soybean, is one of most studied vegetables due to its low cost, good nutritional value, functional properties and ability to yield different products (milk, oil, cheese and tofu) (Singh, Kumar, Sabapathy, & Bawa, 2008). Thus, several groups have shown that different soybean peptides obtained with different hydrolysis systems have antimicrobial (Vasconcellos, Woiciechowski, Soccol, Mantovani, & Soccol, 2014), antidiabetic (Yang, Kwon, Kim, Kang, & Park, 2012), antioxidant (Yang, Mau, Ko, & Huang, 2000) and oncostatic (Rayaprolu, Hettiarachchy, Chen, Kannan, & Mauromostakos, 2013) properties. Moreover, these peptides can inhibit angiotensin I converting enzyme (ACE) activity (Daskaya-Dikmen, Yucetepe, Karbancioglu-Guler, Daskaya, & Ozcelik, 2017; Shimakage, Shinbo, & Yamada, 2012), which is implicated in the regulation of blood pressure, and can act upon cholesterol metabolism (Cho, Juillerat, & Lee, 2007).

The same effects on ACE inhibition have been observed for a protein hydrolysate of *Lupinus angustifolius* obtained by pepsin digestion (Boschin, Scigliuolo, Resta, & Arnoldi, 2014). Moreover, total proteins and pepsin- or trypsin-hydrolyzed peptides from different species of the genus *Lupinus* have shown *in vitro* hypocholesterolemic activity (Lammi et al., 2016).

Lupine seeds belong to the legume family, which is one of the most important food families in the human nutrition around the world (soybeans, chickpeas, peas, beans, cowpeas, lentils, etc.). The production of lupine seeds in 2016 was closely to 1.3 million tonnes but well below the production of other legumes such as soybeans (335.0 MT), beans (26.8 MT) or chickpeas (12.1 MT) (FAOSTAT, 2018). Lupine beans were introduced into northern Europe as a means of improving soil quality (Van de Noort, 2016). They have a high protein composition, dietary fiber and antioxidants, and are very low in starch like soybean as well as being gluten-free. In addition, they possess the relevant advantage of being genetically modified food-free (Duranti, Consonni, Magni, Sessa, & Scarafoni, 2008). Although the introduction of lupine in human consumption is still limited, the presence of "sweet lupine" varieties with low alkaloid content, as those used in the present study, allows the opportunity to study the obtaining of high added-value compounds with a great potential in human nutrition such as the bioactive peptides found in this raw material. We have recently described that *Lupinus angustifolius* protein hydrolysates (LPHs) obtained by using a food-grade non-specific endopeptidases Alcalase 2.4 L, can exert anti-inflammatory effects on both cell-free *in vitro* systems and cultured macrophages derived from THP-1 human monocytic cell line (Millan-Linares, Bermudez, Yust, Millan, & Pedroche, 2014a; Millan-Linares, Yust, Alcaide-Hidalgo, Millan, & Pedroche, 2014b). Additionally, a peptide identified as Gly-Pro-Glu-Thr-Ala-Phe-Leu-Arg (GPETAFLR) (Patent number WO 2016051000A1), has been identified as a novel anti-inflammatory in THP-1 derived macrophages (Millan-Linares, Millan, Pedroche, & Yust, 2015). Therefore, we hypothesized that LPHs could improve the immune and antioxidant status in human peripheral blood mononuclear cells (PBMCs).

To the best of our knowledge, there are no studies regarding the bioactive effects of LPHs on non-immortalized human cells based-models. Thus, in the present study we use an *ex vivo* system of cultured PBMCs from healthy donors to explore a wide spectrum of LPHs immunomodulatory and antioxidant properties.

2 Material and methods

2.1 Characterization and preparation of LPHs

Lupine protein isolate was obtained at the Instituto de la Grasa (Consejo Superior de Investigaciones Científicas, Seville, Spain) as described elsewhere (Millan-Linares et al., 2018; Millan-Linares et al., 2015; Millan-Linares et al., 2014b). Hydrolysis was performed in a bioreactor under stirring at a controlled pH and temperature. Lupine protein isolate

was suspended in distilled water (10% w/v), and hydrolyzed with Alcalase 2.4 (2.4 AU/g; Novozymes, Bagsvaerd, Denmark) for 15 min at pH 8, 50 °C and E/S = 0.3 AU/g protein. Enzyme was inactivated by heating at 85 °C for 15 min. Finally, the supernatant obtained after centrifugation at 8000 rpm for 15 min constituted the LPHs that were lyophilized and stored at room temperature. The chemical composition of the LPHs in dry matter and the degree of hydrolysis are shown in supplemental Table 1.

The protein concentrations were determined by elemental microanalysis as a percentage of nitrogen content × 6.25 using a Leco CHNS-932 analyser (St. Joseph, MI, USA). Ash content was determined by the direct ignition method (550ºC for 25 h). Total dietary fiber was determined according to (Lee, Prosky, & de Vries, 1974). Oil content was measured using the AOAC method 945.16. Soluble sugars and polyphenols were measured using standard glucose curves (Moores, Demott, & Wood, 1948) and chlorogenic acid (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), respectively.

The degree of hydrolysis, defined as the percentage of peptide bonds cleaved, was determined by the TNBS method according to (Adler-Nissen, 1979). The total number of amino groups was determined in a sample that had been 100% hydrolyzed at 110 C for 24 h in 6 N HCl.

The molecular profile of the LPHs (supplemental Figure 1) was determined by molecular exclusion chromatography. The estimation of the molecular weights was held by filtration gel chromatography in a Akta Purifier system (GE Healthcare) equipped with a Superose 12 HR 10/30 column with an exclusion limit around 2 x 10^6 Da and an optimal range of separation of 1000-3 x 10^5 Da. The elution was developed in a 50 mM sodium phosphate 0.5 M NaCl, 0.02% (w/v) sodium azide buffer and monitored at 280 nm. The analytical flow was 1.0 ml/min and the protein concentrations of the samples were 1 mg protein/ mL, with an injection volume of 0.5 mL.

Finally, lyophilized LPHs were freshly dissolved in incomplete RPMI 1640 medium (BioWest, Nuaillé, France) at a final concentration of 4 mg/mL before each experiment. When completely dissolved, the solution was passed through a sterile membrane filter with a 0.2 µm pore size and then autoclaved.

2.2 Cell culture

Human peripheral blood cells were obtained from the buffy coats of blood samples provided anonymously from 53 healthy adults donated to the Biobank of the Andalusian Public Health System at the Virgen del Rocío University Hospital (Seville, Spain). The

study followed the Helsinki Declaration for medical research involving human subjects; it was approved by the Virgen del Rocío University Hospital ethical review board (reference number 2012PI/200) and a written informed consent was obtained from all study participants. PBMCs were obtained by density gradient separation using Histopaque-1077 solution (specific density 1.077; Sigma-Aldrich, MO, USA). The cells were then cultured at $1x10^6$ cells/mL in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 U/mL streptomycin (BioWest) in the presence or absence of the proliferation stimulator phytohemagglutinin-P (PHA; 8 µg/mL; Sigma-Aldrich) and different concentrations of LPHs and incubated at 37°C in a 5% CO_2 humidified atmosphere.

2.3 Cell viability and proliferation assays

Cell viability and proliferation were determined in non-stimulated (cell viability) or PHA-stimulated (cell proliferation) cells cultured for 72 hours in 96 well plates (100,000 cells/well). Cell viability was assayed by adding the cell proliferation reagent WST-1 (Roche Diagnostic, Bassel, Switzerland) during the last 5 hours of culture according to the manufacturer's instructions, whereas cell proliferation was determined by using a 5-bromo-2-deoxyuridin (BrdU) Cell Proliferation ELISA (Roche) following the manufacturer's instructions. Absorbance values were measured for both assays at 450 nm (reference wavelength: 620 nm) with a Multiskan™ FC Microplate Photometer (Thermo Scientific, Vantaa, Finland). The background absorbance value from a blank control was subtracted from all the samples.

2.4 RNA extraction, reverse transcription and real-time PCR

After non-stimulated cells were incubated overnight with/without LPHs in 6 well plates ($6x10^6$ cells/well), RNA was extracted using Tripure Isolation Reagent (Roche) according to the manufacturer's instructions. The dried RNA pellets were resuspended in RNase-free water, and RNA quantity and purity were analyzed using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). Single-strand cDNA was synthesized from 3 µg of RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche). Real-time PCR was performed with a LightCycler 480 (Roche) and LightCycler® 480 SYBR Green I Master Mix (Roche). The primer sequences are listed in supplemental Table 2. All amplification reactions were performed in duplicate. The expression levels of each gene were

normalized to that of β-actin, and the relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.5 Cytokine determination

Representative Th1, Th2, Th9 and Th17 cytokine levels were determined in the 48-hour cell culture supernatants of PHA-stimulated PBMCs (100,000 cells/well/96 well plate) treated with different concentrations LPHs using a Human 13plex Kit FlowCytomix (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. The cytokines were then measured with a BD FACSCanto II Flow Cytometer (BD Biosciences, San Jose, CA, USA).

2.6 Antioxidant activity assays

Supernatants from the RNA extraction cell cultures were aliquoted for each antioxidant activity assay and stored at -20°C. The enzymatic activities of GR (BioVision, CA, USA), CAT, GPx (Cayman Chemical, MI, USA), and SOD (Arbor Assays, MI, USA) and the total antioxidant capacity (TAC) (Cell Biolabs, CA, USA) were determined by colorimetric assays according to the manufacturers' instructions. The TAC Assay Kit (OxiSelect™) measures the total antioxidant capacity within a sample through a single electron transfer mechanism based on copper reduction (Cu^{2+} to Cu^{+}). Samples are compared to a known concentration of uric acid standards. Samples and standards are diluted with a reaction reagent and, upon the addition of copper, the reaction proceeds for a few minutes. The reaction is stopped and the absorbance is recorded at 490 nm. The absorbances were measured with a Tecan Infinite 200 PRO microplate reader (Tecan, Männedorf, Switzerland).

2.7 Statistical analysis

The results are expressed as the mean and standard error of the mean (SEM). All variables studied were continuous. Variables did not follow a normal distribution, assessed by the Shapiro–Wilk normality test. Due to human inter-subject variability, we considered untreated- and LPHs treated PBMCs from the same subject as paired variables. Thus, we used the non-parametric Wilcoxon test, a statistical test for non-normal and paired variables that allowed us to assess the effect of both concentrations of LPHs (0.5 and 0.75 mg/mL) in comparison with untreated cells from the same subject. Additionally, Bonferroni´s correction was applied to counteract the problem of multiple comparisons.

Correlations were analyzed by the non-parametric Spearman's correlation. P values of ≤0.05 were considered statically significant. The data were analyzed with SPSS® v24 software (IBM, Armonk, NY, USA).

3 Results

3.1 LPHs reduce cellular proliferation in PHA-stimulated PBMCs without causing cytotoxicity

The effects of LPHs on cellular proliferation showed that the hydrolysates caused a significant dose-dependent reduction in PHA-induced cell proliferation compared with that in the control group (Fig. 1A). To determine whether the anti-proliferative effects of LPHs were due to cytotoxicity, WST-1 viability assays were performed on PBMCs treated with LPHs under the same culture conditions as the proliferation study. No effects of LPHs on cell viability were observed when compared to the control group (Fig. 1B).

3.2 LPHs skew the pro-/anti-inflammatory cytokine balance towards a protective phenotype

To analyze the possible immunological effects of the hydrolysates, pro- and anti-inflammatory cytokine levels were determined in cell culture supernatants from PHA-stimulated PBMCs treated with LPHs at the indicated concentrations. As shown in Table 1, both of the tested LPHs concentrations significantly decreased the levels of the pro-inflammatory Th1 cytokines IL-2, IL-12, IFN-γ and TNF. IL-17 levels were also significantly decreased after PBMCs were treated with LPHs. Moreover, LPHs significantly diminished IL-9 production. Th2 cytokines IL-4 and IL-13 were also analyzed. Although IL-4 levels were not altered, the production of IL-13 was decreased after LPHs treatment. Finally, we studied the effect of LPHs on the production of IL-10, a classical anti-inflammatory cytokine. LPHs caused a significant decrease in IL-10 production. Despite the fact that IL-4 production was not affected by LPHs treatment and that IL-10 and IL-13 levels were reduced, a significant increase in the ratios of IL-4 (Figs. 2A-E) or IL-13 (Figs. 2F-J) and Th1 cytokines (IL-12, IFNγ, and TNF) was observed upon LPHs treatment. Additionally, LPHs also increased the ratios of IL-10/IFN-γ and IL-10/IL-12 (Figs. 2L and N). According to these data, LPHs downregulated the mRNA expression of Th1 cytokines (supplemental Figure 2A and B) whereas LPHs treatment significantly increased the levels of IL-10 mRNA (supplemental Figure 2C).

3.3 LPHs increase SOD and GR mRNA expression

To evaluate possible LPHs effects on cellular oxidative stress, we analyzed the mRNA expression levels of CAT, GPx, SOD and GR, key antioxidant enzymes participating in the maintenance of redox homeostasis. Significant increases in the mRNA expression levels of SOD and GR were observed at the highest dose of LPHs (0.75 mg/mL) (Fig. 3).

3.4 LPHs increase the enzymatic activities of SOD and CAT

SOD activity was significantly increased by both of the tested LPHs concentrations (Fig. 4A), while CAT activity was increased at the 0.75 mg/mL LPHs concentration (Fig. 4B). Neither GPx activity (Fig. 4C) nor GR activity (Fig. 4D) was affected by LPHs treatment. We also studied the correlation between different antioxidant enzyme activities by non-parametric Spearman's correlation (Table 2). Significant positive correlations between GPx and GR activities and between SOD and CAT activities were detected for 0.75 mg/mL LPHs treatment. Moreover, negative correlations among the GPx, SOD and CAT activities were also observed.

3.5 LPHs increase the total antioxidant capacity and protect PBMCs from the H_2O_2 induced oxidative stress

To determine the overall effect of LPHs on the antioxidant status, a TAC assay was performed. A significant dose-dependent increase in the TAC was detected for both the LPHs concentrations tested (Fig. 4E). Positive correlations between the TAC and SOD and CAT activities were detected in the presence of 0.75 mg/mL LPHs, while negative correlations between the TAC and GR and GPx activities were found (Table 2). LPHs also significantly protected from cell death mediated by oxidative stress induced by H_2O_2 (supplemental Figure 3).

4 Discussion

This study shows the potential beneficial effects of LPHs obtained by hydrolysis with Alcalase 2.4L, a food-grade non-specific endopeptidases, on the immunological and oxidative statuses of *in vitro* cultured human PBMCs from healthy donors. This endopeptidase was selected as previous studies have shown simple and more efficient designs of protein hydrolysis through enzymatic hydrolysis with Alcalase regarding another peptidase (Millan-Linares et al., 2014b). Moreover, Alcalase 2.4 L is a well-known protease in terms of pH, temperature activity and stability and has a very broad substrate specificity.

It is a food grade enzyme that has been widely used to prepare soluble protein hydrolysates from several sources such as soy (Hanafi et al., 2018), fish (Liu et al., 2014) and lupine (Millan-Linares et al., 2014b) as well as producing bioactive peptides (Zhang, Olsen, Grossi, & Otte, 2013) and is currently in use in food industry. LPHs showed to have two mechanisms of action: first, LPHs treatment decreased PBMCs proliferation and Th1, Th17 and Th9 cytokine levels to skew the pro-/anti-inflammatory cytokine balance towards a protective phenotype; second, LPHs treatment increased both SOD and CAT activities to yield a higher global antioxidant capacity. To the best of our knowledge, these effects have not been previously described.

The lack of effects on cell viability after LPHs treatment indicates that the inhibitory effects on cell proliferation and cytokine production are not related to toxic effects. These results are in concordance with a previous study in which we described non-cytotoxic LPHs effects on macrophages derived from THP-1 human monocytic cell line (Millan-Linares et al., 2014a).

Some food-derived peptides can influence the immune response (Bougle & Bouhallab, 2015; Gauthier et al., 2006). For example, inhibitory effects of recombinant β1-, β3-, and β6-conglutin proteins from *Lupinus angustifolius* have been shown on IL-1β and inducible nitric oxide synthase (iNOS) levels in *in vitro* cultured PBMCs from type 2 diabetes patients (Lima-Cabello et al., 2017). We have also previously described the inhibitory effects of LPHs on TNF production in macrophages derived from THP-1 human monocytic cell line and their upregulation of the mRNA expression of CCL18 (Millan-Linares et al., 2014a), a Th2-associated chemokine (Bellinghausen et al., 2012). Our present results support the anti-inflammatory effects of LPHs, which occur through not only a decrease in TNF levels but also the inhibition of additional Th1 cytokines, such as IL-2, IL-12, and IFNγ. Some authors have demonstrated that hydrolysates from animal or vegetable sources can decrease TNF and/or IFN-γ production. For example, ethanol extracts of *Salvia plebeian* and soybean protein hydrolysates inhibit inflammatory markers, such as nitric oxide and TNF, produced by RAW 264.7 macrophages (Jang et al., 2016; Vernaza et al., 2012). Marine red seaweed (*Porphyra columbina*) hydrolysates also show anti-inflammatory effects of down-regulating TNF and IFN-γ production and up-regulating IL-10 production in Concanavalin A-stimulated and LPS-stimulated rat splenocytes (Fan, Bai, Zhu, Yang, & Zhang, 2014). In contrast, the simulated gastrointestinal digestion product of milk supplemented with a protein-rich fraction of brewers' spent grain had no effects on IL-2,

IFN-γ or TNF production in Jurkat T lymphocytes or RAW 264.7 macrophages (Crowley et al., 2015).

Notably, although LPHs decreased IL-10 and IL-13 levels but had no effects on IL-4 levels in this study, LPHs significantly increased anti-/pro-inflammatory balance. Specifically, these hydrolysates increased the ratios of IL-4/IL-12, IFNγ, and TNF, as well as the ratios of IL-13/IFNγ, and TNF and IL-10/IFNγ. Thus, these ratios indicate that PBMCs produced more anti-inflammatory cytokine (IL-4, IL-13, and IL-10) respect to pro-inflammatory cytokine (IL-12, IFNγ, and TNF).

IL-4 provides positive feedback in maintaining the Th2 subset of cells, and it is implicated in the activation of B-cells and their differentiation into plasma cells; IL-13 has an activity similar to IL-4, but it cannot drive Th2 differentiation (Chomarat & Banchereau, 1998). IL-10 is implicated in the blockade of cytokine synthesis and in the suppression of inflammatory responses, thus limiting the potential tissue damage caused by inflammation (Ouyang et al., 2011). Although some studies have shown that casein (Lahart et al., 2011) or seaweed (Cian, López-Posadas, Drago, Sánchez de Medina, & Martínez-Augustín, 2012) hydrolysates can increase IL-10 production in a human Jurkat T cell line and rat lymphocytes, our previous study did not detect any variations in IL-10 mRNA or protein levels in LPHs-treated macrophages derived from THP-1 human monocytic cell line (Millan-Linares et al., 2014a). However, GPETAFLR, an octapeptide isolated from LPHs, increased the IL-10 gene expression in primary human monocytes (Montserrat-de la Paz et al., 2019).

In the present study, the observed decrease in IL-10 levels could be associated with the LPHs-mediated reduction in Th2 and Th9 responses since those cell subsets can express IL-10 (Ouyang et al., 2011). Interestingly, LPHs treatment significantly increased the levels of IL-10 mRNA. Consequently, we suggest that the decreased levels of IL-10 in the PBMCs supernatant treated with LPHs is due to its anti-proliferative effect and not to a direct action on mRNA gene expression, as happens in the Th1 cytokines. This is also supported by the increase in the ratio of IL-10/Th1 cytokines in the LPHs-treated PBMCs.

Therefore, to the best of our knowledge, the present study is the first to describe that LPHs skew the Th1 (IL-12, IFNγ, and TNF)/Th2 (IL-4, IL-13, and IL-10) cytokine balance towards a Th2 protective phenotype in a non-immortalized human cell-based system.

Since Th17 cells are implicated in inflammatory and autoimmune responses (Singh et al., 2014) and are involved in the pathogenesis of food allergies (Zbikowska-Gotz et al., 2016), we explored the possible effects of LPHs on IL-17 production. As in the case of Th1 cells,

LPHs diminished the Th17 response by reducing IL-17 production. A positive relationship between IL-17 serum levels and the severity of allergic inflammation has been shown (Ciprandi et al., 2009). Additionally, IL-17 serum levels are higher in patients with food allergy hypersensitivity than in control subjects (Zbikowska-Gotz et al., 2016). Thus, a decrease in the levels of this cytokine can reduce the allergic inflammation response and counteract exacerbated inflammatory responses.

Furthermore, LPHs diminished the Th9 response, which is also implicated in the development of allergic diseases (Zhao et al., 2013). It has also been suggested that IL-9 plays an important role in food allergies. In fact, IL-9-deficient mice failed to develop experimental oral antigen-induced intestinal anaphylaxis, while the overexpression of IL-9 induced an intestinal anaphylaxis phenotype (Forbes et al., 2008).

By and large, our data show that LPHs have anti-inflammatory activity and that this effect is due to the inhibition of pro-inflammatory cytokines from Th1, Th17, and Th9 cells rather than the increase in the production of anti-inflammatory mediators, such as IL-10. Moreover, Th2 cytokines, which are higher in proportion to Th1 cytokines, could be implicated in promoting or maintaining the Th2 subset and thus inhibiting Th1 cell differentiation.

ROS participate in defense mechanisms and play an important role in immunity regulation (Yang et al., 2013). In this regard, the immune system uses the superoxide radical (O_2^-) to kill invading microorganisms. Due to the high toxicity of O_2^-, nearly all organisms living in presence of oxygen have SOD, which catalyzes the dismutation of the O_2^- radical into ordinary molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). The latter is a dangerous substance as it is the precursor of the highly toxic hydroxyl radical (•OH), which is produced via the Fenton reaction; thus, H_2O_2 must be quickly removed. Two enzymes are implicated in the H_2O_2 detoxification process. On one hand, the enzyme CAT can convert millions of H_2O_2 molecules into water (H_2O) and O_2 every second; on the other hand, the enzyme GPx reduces H_2O_2 to H_2O using the reduced form of glutathione (GSH). GSH plays an important role in the antioxidant defense system and in many metabolic processes, such as the detoxification of xenobiotics and some endogenous compounds (Pisoschi & Pop, 2015). For this reason, the maintenance of GSH levels is an extremely important biological process; GSH levels are maintained by the GR enzyme, which catalyzes the transformation of the oxidized form of glutathione (GSSG) to GSH.

It is widely known that antioxidant molecules are present in many foods (Carlsen et al., 2010). Several authors have reported that vegetable protein hydrolysates have a strong

antioxidant capacity in *in vitro* cell-free systems. *Brassica napus* (rapeseed) protein hydrolysates exhibit dose-dependent antioxidant functions according to various *in vitro* tests (Pan, Jiang, & Pan, 2011). Antioxidant effects were also described for corn gluten meal hydrolysates (Li, Han, & Chen, 2008). Also, were showed that *Cicer arietinum* (chickpea) protein hydrolysates exert antioxidant activity (Torres-Fuentes, del Mar Contreras, Recio, Alaiz, & Vioque, 2015), and this bioactivity could be increased by selecting the type of proteases and the hydrolysis duration (Yust, Millan-Linares, Alcaide-Hidalgo, Millan, & Pedroche, 2012). Additionally, the antioxidant activity of lupine seed phenolic compounds has already been reported (Siger et al., 2012). Therefore, we examined the possible antioxidant effects of LPHs on *in vitro* cultured PBMCs. LPHs increased SOD and CAT activities without affecting GPx and GR activities. SOD and CAT enzymes are implicated in the direct and subsequent elimination of O_2^- and H_2O_2, respectively (Fig. 5); H_2O_2 elimination reduces the frequency of Fenton reactions whose product is •OH, the most devastating ROS to biological macromolecules because it causes nucleic acid mutation, lipid peroxidation and protein oxidation (Pisoschi & Pop, 2015). As the SOD-CAT pathway is glutathione-independent, we hypothesize that in the presence of LPHs, the GSH/GSSG ratio remains invariant. This is in accordance with our results that show no effects of LPHs on GPx and GR activities. Although the SOD-GPx-GR pathway is not affected by LPHs, the total antioxidant capacity was significantly improved in the presence of LPHs, suggesting that the SOD-CAT pathway is responsible for ameliorating the total cellular antioxidant status. In accordance, correlation analyses established that the augmented SOD activity in LPHs-treated cells is accompanied by an increase in CAT activity and a decrease in GPx activity. The latter was also negatively correlated with CAT activity. As expected, GPx and GR activities were positively correlated, whereas we observed robust positive correlations between TAC and both SOD and CAT and negative correlations with both GPx and GR. These findings also support that LPHs increase ROS scavenging through the SOD-CAT pathway instead of SOD-GPx-GR (Fig. 5). LPHs not only induced a higher anti-oxidant capacity but was also able to protect PBMCs from cell death induced by oxidative stress.

Although we cannot rule out that the small amount of flavonoids contained in the LPHs fraction after hydrolyzation (0.06%) can slightly contributes to the biological effect of LPHs, a previous study using a synthetic peptide from the LPHs, lacking polyphenols, has shown anti-inflammatory effects similar to those described in the present paper using the whole LPHs. Moreover, we have also described that not all LPHs fractions have anti-

inflammatory properties, even those, which due to their size, should contain flavonoids (Millan-Linares et al., 2015), which reinforces the fact that the observed LPHs biological effects are mainly due to the peptides fraction rather than to their phenolic components.

The non-characterization of the individual peptides produced after Alcalase treatment could be considered a limitation of the study. However, the main objective of this work resides in the examination of the whole hydrolysate bioactivity in a more physiological system (human *ex vivo* cells) than those previously performed (immortalized cellular line) with the final purpose of minimizing production costs at the pilot plant level and thus increasing the possible applicability of this product in the food market.

Inflammation and oxidative stress are also associated with the aging process. In fact, the loss of the ability to control stress agents and the simultaneous increase in the pro-inflammatory status is a key feature of aging; this process is called “inflamm-aging” (Franceschi et al., 2000) and is highly correlated with many diseases, such as Alzheimer's disease, atherosclerosis, heart disease, type II diabetes, and cancer (Xia et al., 2016). Although the positive health benefits of lupine protein derivatives have been shown to affect pathophysiological conditions, such as hypercholesterolemia, diabetes, and hypertension (Arnoldi, Boschin, Zanoni, & Lammi, 2015; Lima-Cabello et al., 2017), to the best of our knowledge this is the first report of the clear action of LPHs on immune and oxidative responses in non-immortalized human cells.

5 Conclusions

This study is the first to show that LPHs reduce Th1, Th17, and Th9 pro-inflammatory responses and increase the anti-inflammatory (Th2)/pro-inflammatory (Th1) ratio in human peripheral leukocytes; LPHs were also shown to increase the total antioxidant capacity through enhancing SOD and CAT activities. Therefore, these and previous results from our group and others show that LPHs may represent an effective option in the generation of functional foods; this research thus provides new opportunities for developing nutritional strategies with *Lupinus angustifolius* as a dietary source of vegetable protein. Therefore, our next goal is to generate a functional food and evaluate the safety and efficacy to modulate the immune and oxidative status in a clinical food trial.

Acknowledgments

We thank the blood donors for providing us with their blood samples. We are grateful to María José Castro from the IBiS Flow Cytometry Facility and to Dr. Juan Manuel Praena-Fernández from the Statistics and Methodology Unit for their valuable assistance. The authors also thank Mr. John Leslie Brown for correcting the language of the manuscript.

The following are the authors' contributions: Research design: AC-V, PJL, JMG, JP, FM, and CC-S. Research conduct: IC-C, NA-S, MCM-L, and MMY. Carry out the experiments: IC-C, NA-S, MCM-L, and MMY. Data analysis and interpretation: IC-C, NA-S, HS-S, MCM-L, MMY, and AC-V. Drafting of the manuscript: IC-C, AC-V, JM-G, PJ-L, JP, FM and CC-S. Reading and approving the final version of the manuscript: all authors.

Funding sources

This work was supported by the Spanish Government, Ministerio de Economía y Competitividad [AGL2012-40247-C02-01, AGL2012-40247-C02-02]; the Andalusian Government Ministry of Health [PC-0111-2016-0111]; and the PAIDI Program from the Andalusian Government [CTS160]. IC-C was supported by an FPU grant from the Spanish Ministerio de Educación, Cultura y Deporte [FPU13/01210].

References

Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *Journal of Agricultural and Food Chemistry, 27*(6), 1256-1262. doi: 10.1021/jf60226a042.

Arise, A. K., Alashi, A. M., Nwachukwu, I. D., Ijabadeniyi, O. A., Aluko, R. E., & Amonsou, E. O. (2016). Antioxidant activities of bambara groundnut (Vigna subterranea) protein hydrolysates and their membrane ultrafiltration fractions. *Food & Function, 7*(5), 2431-2437. doi: 10.1039/c6fo00057f.

Arnoldi, A., Boschin, G., Zanoni, C., & Lammi, C. (2015). The health benefits of sweet lupin seed flours and isolated proteins. *Journal of Functional Foods, 18*(Part A), 550-563. doi: 10.1016/j.jff.2015.08.012.

Azizi, G., Yazdani, R., & Mirshafiey, A. (2015). Th22 cells in autoimmunity: a review of current knowledge. *European Annals of Allergy and Clinical Immunology, 47*(4), 108-117.

Bellinghausen, I., Reuter, S., Martin, H., Maxeiner, J., Luxemburger, U., Tureci, O., . . . Saloga, J. (2012). Enhanced production of CCL18 by tolerogenic dendritic cells is associated with inhibition of allergic airway reactivity. *Journal of Allergy and Clinical Immunology, 130*(6), 1384-1393. doi: 10.1016/j.jaci.2012.08.039.

Boschin, G., Scigliuolo, G. M., Resta, D., & Arnoldi, A. (2014). ACE-inhibitory activity of enzymatic protein hydrolysates from lupin and other legumes. *Food Chemistry, 145*, 34-40. doi: 10.1016/j.foodchem.2013.07.076.

Bougle, D., & Bouhallab, S. (2015). Dietary Bioactive Peptides: Human Studies. *Critical Reviews in Food Science and Nutrition*. doi: 10.1080/10408398.2013.873766.

Carlsen, M. H., Halvorsen, B. L., Holte, K., Bohn, S. K., Dragland, S., Sampson, L., . . . Blomhoff, R. (2010). The total antioxidant content of more than 3100 foods, beverages, spices, herbs and supplements used worldwide. *Nutrition Journal, 9*, 3. doi: 10.1186/1475-2891-9-3.

Cian, R. E., López-Posadas, R., Drago, S. R., Sánchez de Medina, F., & Martínez-Augustín, O. (2012). A Porphyra columbina hydrolysate upregulates IL-10 production in rat macrophages and lymphocytes through an NF-kappaB, and p38 and JNK dependent mechanism. *Food Chemistry, 134*(4), 1982-1990. doi: 10.1016/j.foodchem.2012.03.134.

Ciprandi, G., De Amici, M., Murdaca, G., Fenoglio, D., Ricciardolo, F., Marseglia, G., & Tosca, M. (2009). Serum interleukin-17 levels are related to clinical severity in allergic rhinitis. *Allergy, 64*(9), 1375-1378. doi: 10.1111/j.1398-9995.2009.02010.x.

Crowley, D., O'Callaghan, Y., McCarthy, A., Connolly, A., Piggott, C. O., FitzGerald, R. J., & O'Brien, N. M. (2015). Immunomodulatory potential of a brewers' spent grain protein hydrolysate incorporated into low-fat milk following in vitro gastrointestinal digestion. *International Journal of Food Sciences and Nutrition, 66*(6), 672-676. doi: 10.3109/09637486.2015.1077788.

Cho, S. J., Juillerat, M. A., & Lee, C. H. (2007). Cholesterol lowering mechanism of soybean protein hydrolysate. *Journal of Agricultural and Food Chemistry, 55*(26), 10599-10604. doi: 10.1021/jf071903f.

Chomarat, P., & Banchereau, J. (1998). Interleukin-4 and interleukin-13: their similarities and discrepancies. *International Reviews of Immunology, 17*(1-4), 1-52. doi: 10.3109/08830189809084486.

Dandekar, A., Mendez, R., & Zhang, K. (2015). Cross talk between ER stress, oxidative stress, and inflammation in health and disease. *Methods in Molecular Biology, 1292*, 205-214. doi: 10.1007/978-1-4939-2522-3_15.

Daskaya-Dikmen, C., Yucetepe, A., Karbancioglu-Guler, F., Daskaya, H., & Ozcelik, B. (2017). Angiotensin-I-converting enzyme (ACE)-inhibitory peptides from plants. *Nutrients, 9*(4), 316. doi: 10.3390/nu9040316.

Dubois, M., Gilles, K., Hamilton, J., Rebers, P., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry, 28*, 7. doi: 10.1021/ac60111a017.

Duranti, M., Consonni, A., Magni, C., Sessa, F., & Scarafoni, A. (2008). The major proteins of lupin seed: characterisation and molecular properties for use as functional and nutraceutical ingredients. *Trends in Food Science & Technology, 19*(12), 624-633. doi: 10.1016/j.tifs.2008.07.002.

Fan, X., Bai, L., Zhu, L., Yang, L., & Zhang, X. (2014). Marine algae-derived bioactive peptides for human nutrition and health. *Journal of Agricultural and Food Chemistry, 62*(38), 9211-9222. doi: 10.1021/jf502420h.

Farnaud, S., & Evans, R. W. (2003). Lactoferrin--a multifunctional protein with antimicrobial properties. *Molecular Immunology, 40*(7), 395-405. doi: 10.1016/S0161-5890(03)00152-4.

Forbes, E. E., Groschwitz, K., Abonia, J. P., Brandt, E. B., Cohen, E., Blanchard, C., . . . Hogan, S. P. (2008). IL-9- and mast cell-mediated intestinal permeability predisposes to oral antigen hypersensitivity. *Journal of Experimental Medicine, 205*(4), 897-913. doi: 10.1084/jem.20071046.

Franceschi, C., Bonafe, M., Valensin, S., Olivieri, F., De Luca, M., Ottaviani, E., & De Benedictis, G. (2000). Inflamm-aging. An evolutionary perspective on immunosenescence. *Annals of the New York Academy of Sciences, 908*, 244-254. doi: 10.1111/j.1749-6632.2000.tb06651.x.

Gauthier, S. F., Pouliot, Y., & Saint-Sauveur, D. (2006). Immunomodulatory peptides obtained by the enzymatic hydrolysis of whey proteins. *International Dairy Journal, 16*(11), 1315-1323. doi: 10.1016/j.idairyj.2006.06.014.

Hanafi, M. A., Hashim, S. N., Chay, S. Y., Ebrahimpour, A., Zarei, M., Muhammad, K., . . . Saari, N. (2018). High angiotensin-I converting enzyme (ACE) inhibitory activity of Alcalase-digested green soybean (Glycine max) hydrolysates. *Food Research International, 106*, 589-597. doi: 10.1016/j.foodres.2018.01.030.

Hayes, M. (2018). Food Proteins and Bioactive Peptides: New and Novel Sources, Characterisation Strategies and Applications. *Foods, 14*(3). doi: 10.3390/foods7030038.

Jang, H. H., Cho, S. Y., Kim, M. J., Kim, J. B., Lee, S. H., Lee, M. Y., & Lee, Y. M. (2016). Anti-inflammatory effects of Salvia plebeia R. Br extract in vitro and in ovalbumin-induced mouse model. *Biological Research, 49*(1), 41. doi: 10.1186/s40659-016-0102-7.

Lahart, N., O'Callaghan, Y., Aherne, S., O'Sullivan, D., FitzGerald, R. J., & O'Brien, N. M. (2011). Extent of hydrolysis effects on casein hydrolysate bioactivity: evaluation using the human jurkat T cell line. *International Dairy Journal, 21*(10), 777-782. doi: 10.1016/j.idairyj.2011.03.014.

Lammi, C., Zanoni, C., Ferruzza, S., Ranaldi, G., Sambuy, Y., & Arnoldi, A. (2016). Hypocholesterolaemic Activity of Lupin Peptides: Investigation on the Crosstalk between Human Enterocytes and Hepatocytes Using a Co-Culture System Including Caco-2 and HepG2 Cells. *Nutrients, 8*(7). doi: 10.3390/nu8070437.

Lee, S., Prosky, L., & de Vries, J. W. (1974). Determination of total, soluble and insoluble dietary fiber in food-enzymatic-gravimetric method, MESTRIS buffer: Collaborative study. *Journal of AOAC INTERNATIONAL, 75*, 22.

Li, X., Han, L., & Chen, L. (2008). In vitro antioxidant activity of protein hydrolysates prepared from corn gluten meal. *Journal of the Science of Food and Agriculture, 88*(9), 1660-1666. doi: 10.1002/jsfa.3264.

Lima-Cabello, E., Alche, V., Foley, R. C., Andrikopoulos, S., Morahan, G., Singh, K. B., . . . Jimenez-Lopez, J. C. (2017). Narrow-leafed lupin (Lupinus angustifolius L.) beta-conglutin proteins modulate the insulin signaling pathway as potential type 2 diabetes treatment and

inflammatory-related disease amelioration. *Molecular Nutrition & Food Research, 61*(5). doi: 10.1002/mnfr.201600819.

Lindmark-Mansson, H., & Akesson, B. (2000). Antioxidative factors in milk. *British Journal of Nutrition, 84 Suppl 1*, S103-110. doi: 10.1017/S0007114500002324.

Liu, Y., Li, X., Chen, Z., Yu, J., Wang, F., & Wang, J. (2014). Characterization of structural and functional properties of fish protein hydrolysates from surimi processing by-products. *Food Chemistry, 151*, 459-465. doi: 10.1016/j.foodchem.2013.11.089.

Millan-Linares, M. C., Bermudez, B., Yust, M. M., Millan, F., & Pedroche, J. (2014a). Anti-inflammatory activity of lupine (Lupinus angustifolius L.) protein hydrolysates in THP-1-derived macrophages. *Journal of Functional Foods, 8*, 224-233. doi: 10.1016/j.jff.2014.03.020.

Millan-Linares, M. C., Lemus-Conejo, A., Yust, M. M., Pedroche, J., Carrillo-Vico, A., Millan, F., & Montserrat-de la Paz, S. (2018). GPETAFLR, a novel bioactive peptide from Lupinus angustifolius L. protein hydrolysate, reduces osteoclastogenesis. *Journal of Functional Foods, 47*, 299-303. doi: 10.1016/j.jff.2018.05.069.

Millan-Linares, M. C., Millan, F., Pedroche, J., & Yust, M. M. (2015). GPETAFLR: A new anti-inflammatory peptide from Lupinus angustifolius L. protein hydrolysate. *Journal of Functional Foods, 18*, 358-367. doi: 10.1016/j.jff.2015.07.016.

Millan-Linares, M. C., Yust, M. M., Alcaide-Hidalgo, J. M., Millan, F., & Pedroche, J. (2014b). Lupine protein hydrolysates inhibit enzymes involved in the inflammatory pathway. *Food Chemistry, 151*, 141-147. doi: 10.1016/j.foodchem.2013.11.053.

Moller, N. P., Scholz-Ahrens, K. E., Roos, N., & Schrezenmeir, J. (2008). Bioactive peptides and proteins from foods: indication for health effects. *European Journal of Nutrition, 47*(4), 171-182. doi: 10.1007/s00394-008-0710-2.

Montserrat-de la Paz, S., Lemus-Conejo, A., Toscano, R., Pedroche, J., Millan, F., & Millan-Linares, M. C. (2019). GPETAFLR, an octapeptide isolated from Lupinus angustifolius L. protein hydrolysate, promotes the skewing to the M2 phenotype in human primary monocytes. *Food & Function*. doi: 10.1039/c9fo00115h.

Moores, R. M. C., Demott, D., & Wood, T. (1948). Determination of chlorogenic acid in coffee. *Analytical Chemistry, 28*, 5. doi: 10.1021/ac60019a007.

Ouyang, W., Rutz, S., Crellin, N. K., Valdez, P. A., & Hymowitz, S. G. (2011). Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annual Review of Immunology 29*, 71-109. doi: 10.1146/annurev-immunol-031210-101312.

Pan, M., Jiang, T. S., & Pan, J. L. (2011). Antioxidant activities of rapeseed protein hydrolysates. *Food and Bioprocess Technology, 4*(7), 1144-1152. doi: 10.1007/s11947-009-0206-y.

Pisoschi, A. M., & Pop, A. (2015). The role of antioxidants in the chemistry of oxidative stress: A review. *European Journal of Medicinal Chemistry, 97*, 55-74. doi: 10.1016/j.ejmech.2015.04.040.

Rayaprolu, S. J., Hettiarachchy, N. S., Chen, P., Kannan, A., & Mauromostakos, A. (2013). Peptides derived from high oleic acid soybean meals inhibit colon, liver and lung cancer cell growth. *Food Research International, 50*(1), 282-288. doi: 10.1016/j.foodres.2012.10.021.

Romagnani, S. (2000). T-cell subsets (Th1 versus Th2). *Annals of Allergy, Asthma and Immunology, 85*(1), 9-18. doi: 10.1016/S1081-1206(10)62426-X.

Shimakage, A., Shinbo, M., & Yamada, S. (2012). ACE inhibitory substances derived from soy foods. *International Journal of Biological Macromolecules, 12*(3), 72-80. doi: 10.14533/jbm.12.72.

Sies, H., Berndt, C., & Jones, D. P. (2017). Oxidative Stress. *Annual Review of Biochemistry, 86*, 715-748. doi: 10.1146/annurev-biochem-061516-045037.

Siger, A., Czubinski, J., Kachlicki, P., Dwiecki, K., Lampart-Szczapa, E., & Nogala-Kalucka, M. (2012). Antioxidant activity and phenolic content in three lupin species. *Journal of Food Composition and Analysis, 25*(2), 190-197. doi: 10.1016/j.jfca.2011.10.002.

Singh, P., Kumar, R., Sabapathy, S. N., & Bawa, A. S. (2008). Functional and edible uses of soy protein products. *Comprehensive Reviews in Food Science and Food Safety, 7*(1), 14-28. doi: 10.1111/j.1541-4337.2007.00025.x.

Singh, R. P., Hasan, S., Sharma, S., Nagra, S., Yamaguchi, D. T., Wong, D. T., . . . Hossain, A. (2014). Th17 cells in inflammation and autoimmunity. *Autoimmunity Reviews, 13*(12), 1174-1181. doi: 10.1016/j.autrev.2014.08.019.

Torres-Fuentes, C., del Mar Contreras, M., Recio, I., Alaiz, M., & Vioque, J. (2015). Identification and characterization of antioxidant peptides from chickpea protein hydrolysates. *Food Chemistry, 180*, 194-202. doi: 10.1016/j.foodchem.2015.02.046.

Torres, M. A., Jones, J. D. G., & Dangl, J. L. (2006). Reactive oxygen species signaling in response to pathogens. *Plant Physiology, 141*(2), 373-378. doi: 10.1104/pp.106.079467.

Van de Noort, M. (2016). Lupin: An Important Protein and Nutrient Source. *Sustainable Protein Sources* (pp. 165-183). Elsevier.

Vasconcellos, F. C. S., Woiciechowski, A. L., Soccol, V. T., Mantovani, D., & Soccol, C. R. (2014). Antimicrobial and antioxidant properties of-conglycinin and glycinin from soy protein isolate. *International Journal of Current Microbiology and Applied Sciences, 3*, 144-157.

Vernaza, M. G., Dia, V. P., de Mejia, E. G., & Chang, Y. K. (2012). Antioxidant and antiinflammatory properties of germinated and hydrolysed Brazilian soybean flours. *Food Chemistry, 134*(4), 2217-2225. doi: 10.1016/j.foodchem.2012.04.037.

Xia, S., Zhang, X., Zheng, S., Khanabdali, R., Kalionis, B., Wu, J., . . . Tai, X. (2016). An Update on Inflamm-Aging: Mechanisms, Prevention, and Treatment. *Journal of Immunology Research, 2016*, 8426874. doi: 10.1155/2016/8426874.

Yang, H. J., Kwon, D. Y., Kim, M. J., Kang, S., & Park, S. (2012). Meju, unsalted soybeans fermented with Bacillus subtilis and Aspergilus oryzae, potentiates insulinotropic actions and improves hepatic insulin sensitivity in diabetic rats. *Nutrition & Metabolism, 9*(1), 37. doi: 10.1186/1743-7075-9-37.

Yang, J. H., Mau, J. L., Ko, P. T., & Huang, L. C. (2000). Antioxidant properties of fermented soybean broth. *Food Chemistry, 71*(2), 249-254. doi: 10.1016/S0308-8146(00)00165-5.

Yang, Y., Bazhin, A. V., Werner, J., & Karakhanova, S. (2013). Reactive oxygen species in the immune system. *International Reviews of Immunology, 32*(3), 249-270. doi: 10.3109/08830185.2012.755176.

Yousr, M., & Howell, N. (2015). Antioxidant and ACE Inhibitory Bioactive Peptides Purified from Egg Yolk Proteins. *International Journal of Molecular Sciences, 16*(12), 29161-29178. doi: 10.3390/ijms161226155.

Yust, M. M., Millan-Linares, M. C., Alcaide-Hidalgo, J. M., Millan, F., & Pedroche, J. (2012). Hypocholesterolaemic and antioxidant activities of chickpea (Cicer arietinum L.) protein hydrolysates. *Journal of the Science of Food and Agriculture, 92*(9), 1994-2001. doi: 10.1002/jsfa.5573.

Zbikowska-Gotz, M., Palgan, K., Gawronska-Ukleja, E., Kuzminski, A., Przybyszewski, M., Socha, E., & Bartuzi, Z. (2016). Expression of IL-17A concentration and effector functions of peripheral blood neutrophils in food allergy hypersensitivity patients. *International Journal of Immunopathology and Pharmacology, 29*(1), 90-98. doi: 10.1177/0394632015617069.

Zhang, Y., Olsen, K., Grossi, A., & Otte, J. (2013). Effect of pretreatment on enzymatic hydrolysis of bovine collagen and formation of ACE-inhibitory peptides. *Food Chemistry, 141*(3), 2343-2354. doi: 10.1016/j.foodchem.2013.05.058.

Zhao, P., Xiao, X., Ghobrial, R. M., & Li, X. C. (2013). IL-9 and Th9 cells: progress and challenges. *International Immunology, 25*(10), 547-551. doi: 10.1093/intimm/dxt039.

Table 1. Cytokine production levels

	LPHs		
	0 mg/mL	**0.5 mg/mL**	**0.75 mg/mL**
IL-2 (pg/mL)	207.3±30.98	140.3±14.86***	137±14.84**
IL-12 (pg/mL)	88.87±12.01	43.59±5.45***	40.91±5.75***
IFN-γ (ng/mL)	7.70±0.87	5.22±0.79***	4.28±0.74***
TNF (ng/mL)	14.84±2.0	10.96±1.44***	10.87±1.47***
IL-17 (pg/mL)	337.9±49.55	242.6±38.7***	233.1±39.3*
IL-9 (pg/mL)	215.2±20.78	155.3±15.84***	141.2±13.42***
IL-4 (pg/mL)	63.29±6.1	57.24±6.14	56.04±7.07
IL-13 (pg/mL)	976.8±113.1	762.9±84.12***	714.5±78.3***
IL-10 (pg/mL)	2111±242.9	1690±204.2***	1592±199.6***

Cytokine production in PHA-stimulated PBMCs after 48 h of treatment with different concentrations of LPHs (0, 0.5, and 0.75 mg/mL). The data represent the mean and standard error of the mean of each group (n= 30). * p≤0.05; ** p≤0.01; *** p≤0.001 with respect to the control group (LPHs 0 mg/mL). IFN-γ: interferon-γ; IL: interleukin; LPHs: lupine protein hydrolysates; PBMCs: peripheral blood mononuclear cells; PHA: phytohemagglutinin-P; TNF: tumor necrosis factor.

Table 2. Non-parametric correlations for antioxidant enzymes activities

	CAT		GPx		GR		TAC	
	r	***P***	**r**	***P***	**r**	***P***	**r**	***P***
SOD	0.500	*0.006*	-0.615	*0.007*	-0.049	*0.805*	0.658	*0.001*
CAT			-0.622	*0.006*	-0.230	*0.239*	0.490	*0.021*
GPx					0.682	*0.002*	-0.888	*<0.001*
GR							-0.622	*0.002*

Nonparametric correlations between antioxidant activities at 0.75 mg/mL LPHs (n= 17). CAT: catalase; GPx: glutathione peroxidase; GR: glutathione reductase; LPHs: lupine protein hydrolysates, SOD: superoxide dismutase; TAC: total antioxidant capacity. *P*: P value.

Fig. 1. Cellular proliferation and viability analysis. Cell proliferation in PHA-stimulated cells incubated for 72 hours with LPHs (0, 0.5 and 0.75 mg/mL; n= 39) (**A**). Cell viability in non-stimulated PBMCs cultured for 72 hours with LPHs (0, 0.5 and 0.75 mg/mL; n = 45) (**B**). The data are expressed as the percentage of the control group (LPHs at 0 mg/mL) and represent the mean and standard error of the mean. * p≤0.05; ** p≤0.01; *** p≤0.001. LPHs: lupine protein hydrolysates; PBMCs: peripheral blood mononuclear cells; PHA: phytohemagglutinin-P.

Fig. 2. Anti-/pro-inflammatory cytokine production balance. Ratios of IL-4 (**2A-E**), IL-13 (**2F-J**) IL-10 (**2K-O**) and pro-inflammatory cytokines (IL-2, IFN-γ, TNF, IL-12 and IL-17). The data are expressed as the mean and standard error of the mean (n= 27). * p≤0.05; ** p≤0.01; *** p≤0.001 with respect to the control group. IFN-γ: interferon-γ; IL: interleukin; TNF: tumor necrosis factor.

Fig. 3. Antioxidant enzyme mRNA expression levels. Relative expression levels of SOD (**A**), CAT (**B**), GPx (**C**) and GR (**D**) in non-stimulated PBMCs after overnight treatment with LPHs at different concentrations (0, 0.5, and 0.75 mg/mL). *CAT*: catalase; *GPx*: glutathione peroxidase; *GR*: glutathione reductase; LPHs: lupine protein hydrolysates; PBMCs: peripheral blood mononuclear cells; *SOD*: superoxide dismutase. The data represent the mean of the value calculated with the $2^{-\Delta\Delta Ct}$ method and the standard error of the mean of each group (n= 18). ** p≤0.01, *** p≤0.001 with respect to the control group.

Fig. 4. Antioxidant enzymes activities. Enzyme activities of SOD (**A**), CAT (**B**), GPx (**C**), GR (**D**) and TAC (**E**) in the supernatants from non-stimulated PBMCs cultured overnight with LPHs at different concentrations (0, 0.5, and 0.75 mg/mL). The activities are expressed as a percentage of the controls. CAT: catalase; GPx: glutathione peroxidase; GR: glutathione reductase; LPHs: lupine protein hydrolysates; PBMCs: peripheral blood mononuclear cells; SOD: superoxide dismutase; TAC: total antioxidant capacity. The data represent the mean and standard error of the mean of each group (n= 18). * p≤0.05; *** p≤0.001 with respect to the control group.

Fig. 5. General description of the LPHs effects. Representation of the reactions involved in the detoxification of the superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2).

LPHs augment SOD and CAT activities without modifying GPx and GR activities. Overall, the LPHs effects increase the TAC. CAT: catalase; GPx: glutathione peroxidase; GR: glutathione reductase; GSH: glutathione; GSSG: glutathione disulfide; LPHs: lupine protein hydrolysates; SOD: superoxide dismutase; TAC: total antioxidant capacity.

Declarations of interest: none.

Highlights

- LPHs reduce human PBMCs proliferation.
- Th1, Th9 and Th17 inflammatory responses are decreased in LPHs treated PBMCs.
- LPHs skew the pro-/anti-inflammatory balance towards a protective phenotype.
- The total antioxidant capacity is improved in LPHs-treated PBMCs.

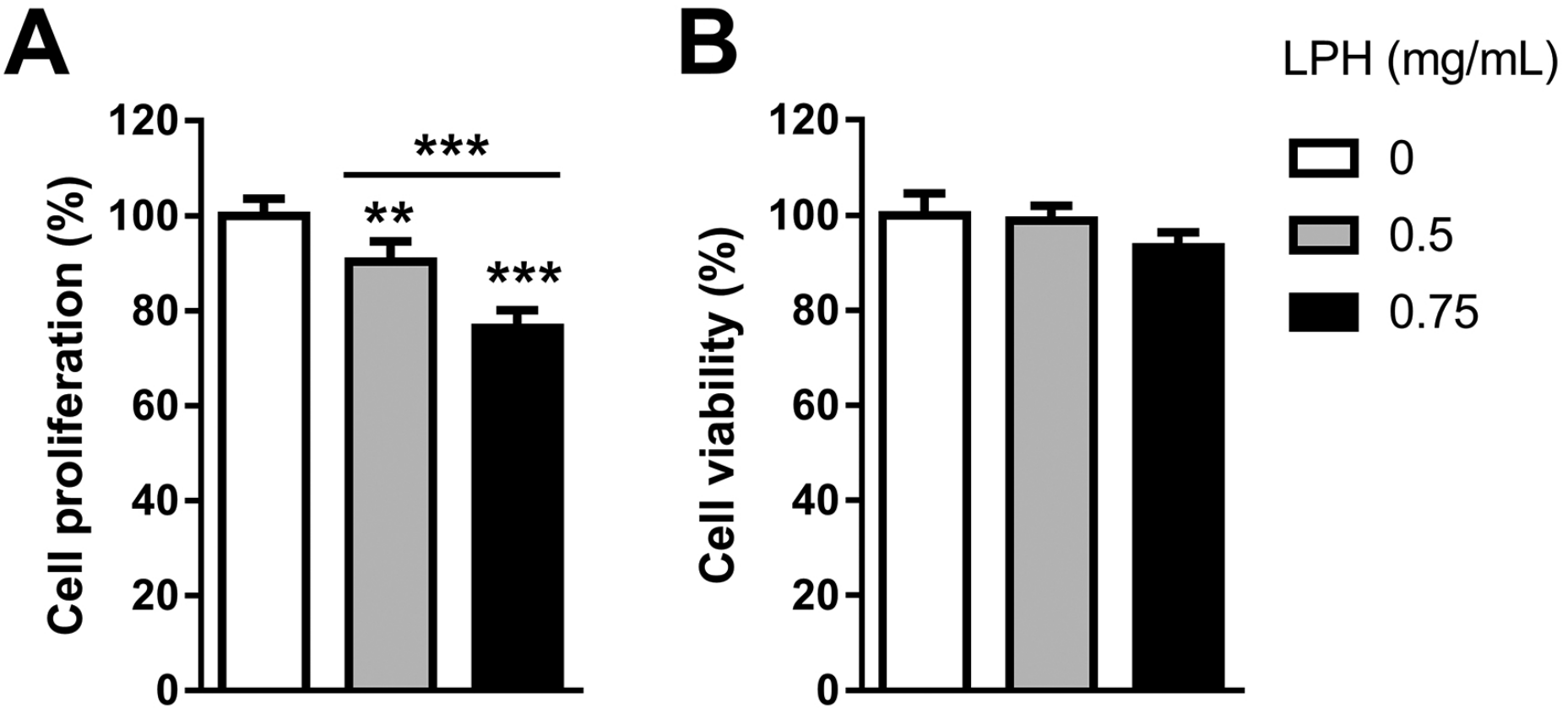
A
Cell proliferation (%)
120
100
80
60
40
20
0
***
**
***
B
Cell viability (%)
120
100
80
60
40
20
0
LPH (mg/mL)
0
0.5
0.75

Figure 1

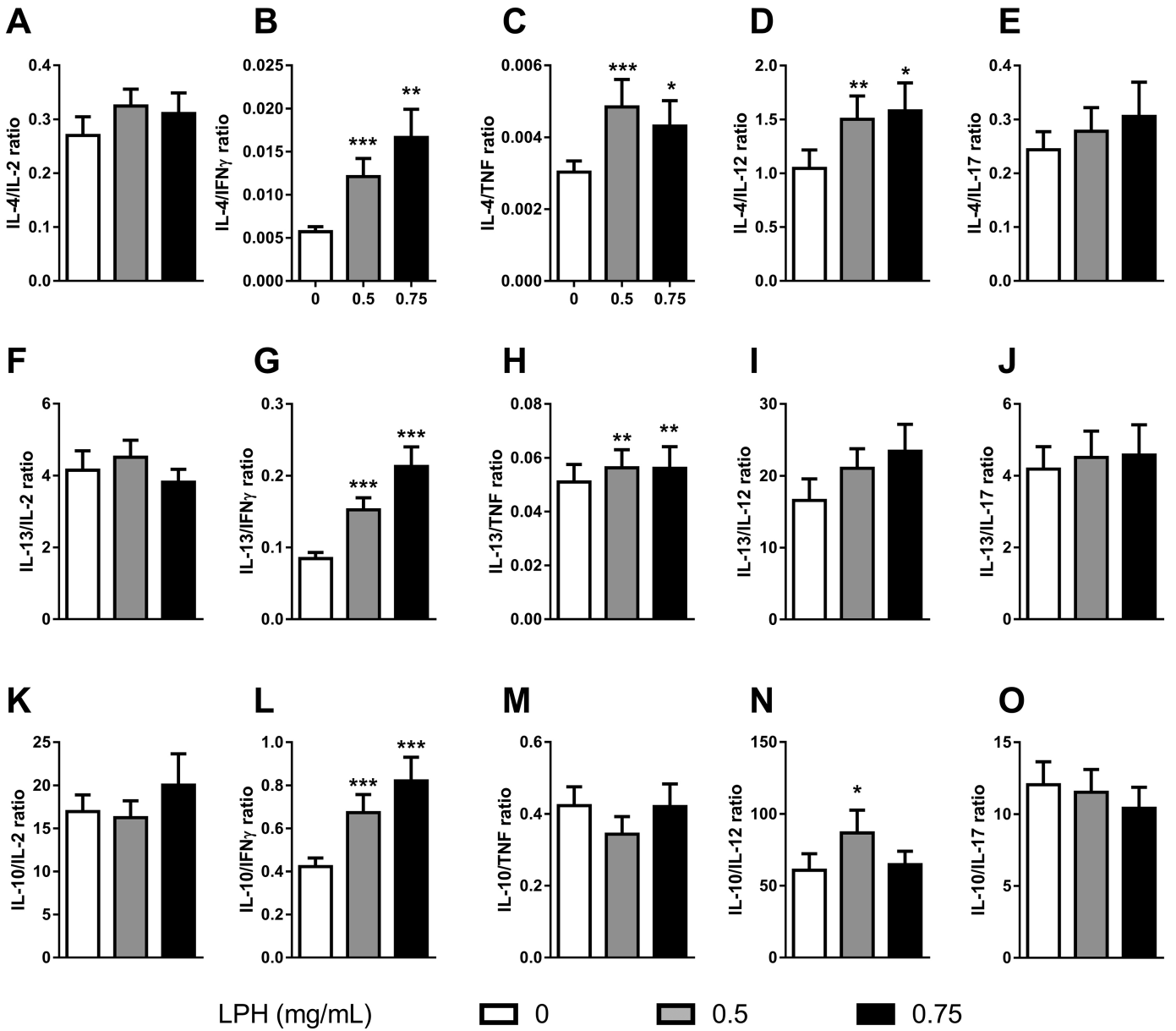
A
B
C
D
E
IL-4/IL-2 ratio
IL-4/IFNγ ratio
IL-4/TNF ratio
IL-4/IL-12 ratio
IL-4/IL-17 ratio
F
G
H
I
J
IL-13/IL-2 ratio
IL-13/IFNγ ratio
IL-13/TNF ratio
IL-13/IL-12 ratio
IL-13/IL-17 ratio
K
L
M
N
O
IL-10/IL-2 ratio
IL-10/IFNγ ratio
IL-10/TNF ratio
IL-10/IL-12 ratio
IL-10/IL-17 ratio
0
0.5
0.75
LPH (mg/mL)

Figure 2

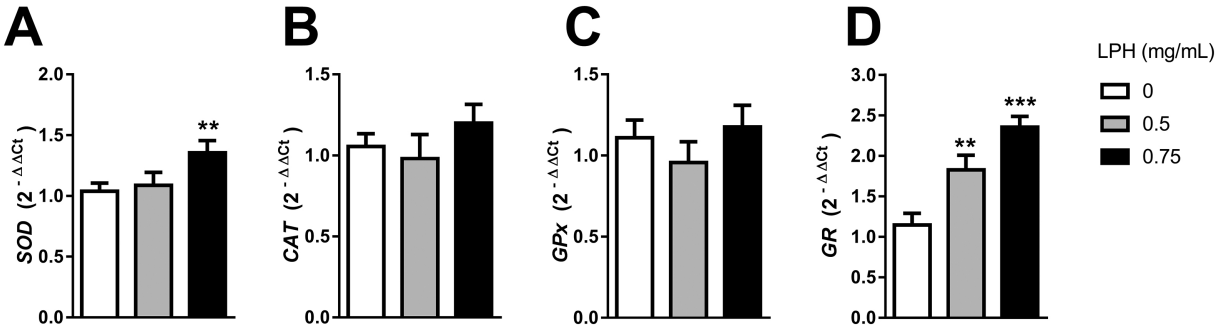
A
B
C
D
SOD (2 - ΔΔCt)
CAT (2 - ΔΔCt)
GPx (2 - ΔΔCt)
GR (2 - ΔΔCt)
2.0
1.5
1.0
0.5
0.0
3.0
2.5
**
***
LPH (mg/mL)
0
0.5
0.75

Figure 3

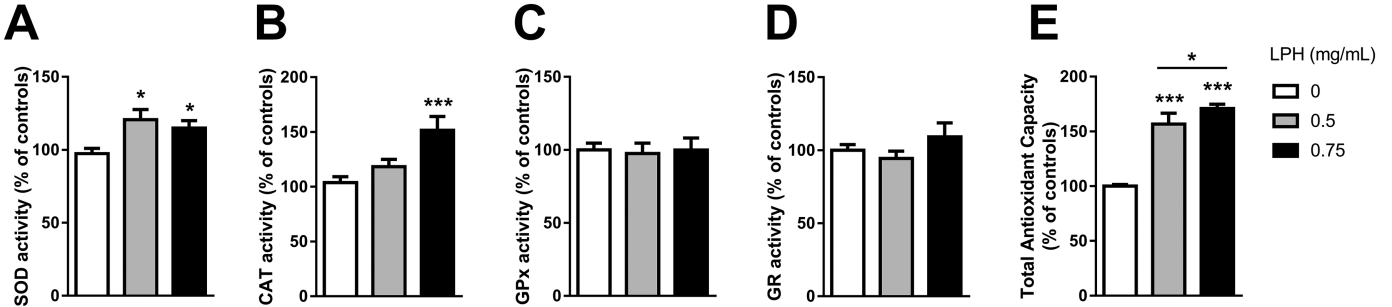
A
SOD activity (% of controls)
150
100
50
0
*
*
B
CAT activity (% of controls)
200
150
100
50
0
***
C
GPx activity (% of controls)
150
100
50
0
D
GR activity (% of controls)
150
100
50
0
E
Total Antioxidant Capacity (% of controls)
200
150
100
50
0
*
***
***
LPH (mg/mL)
0
0.5
0.75

Figure 4

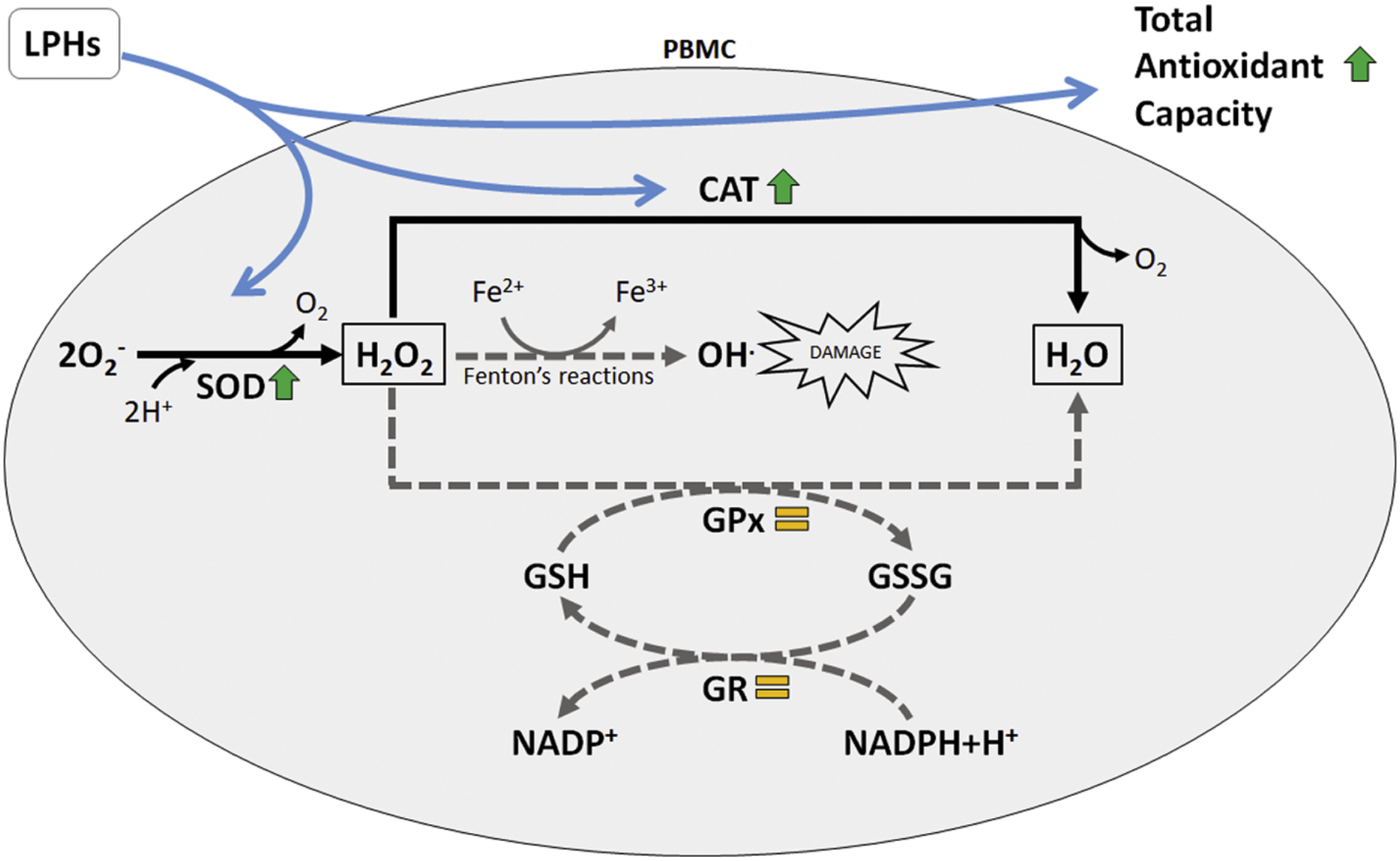
LPHs
PBMC
Total
Antioxidant
Capacity
CAT
O_2
Fe^{2+}
Fe^{3+}
$2O_2^-$
O_2
H_2O_2
$OH^{\cdot}$
DAMAGE
H_2O
$2H^+$
SOD
Fenton's reactions
GPx
GSH
GSSG
GR
$NADP^+$
$NADPH+H^+$

Figure 5