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Title: ANTI-INFLAMMATORY ACTIVITY OF LUPINE (*Lupinus angustifolius* L.) PROTEIN HYDROLYSATES IN THP-1-DERIVED MACROPHAGES

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**Abstract:** The effect of two different lupine protein hydrolysates (LPHs) on in vitro macrophage activation in a THP-1-derived macrophage model was investigated. THP-1-derived macrophages were exposed to RPMI medium containing two LPHs obtained by enzymatic hydrolysis using two different proteases: Izyme AL and Alcalase 2.4 L. Cytokine's expression was measured by quantitative PCR. THP-1-derived macrophages exhibited attenuated expression of proinflammatory cytokines (tumour necrosis factor (TNF), IL-6, IL-1 $\beta$ ) and increased expression of anti-inflammatory marker genes (chemokine (C-C motif) ligand 18 (CCL18)) relative to control without LPH. The anti-inflammatory effect of both hydrolysates favoured M2 polarization by quenching C-C chemokine receptor type 2 (CCR2) expression and migratory capacity. Furthermore, LPHs significantly decreased nitric oxide production. Moreover, LPHs promoted the survival of human THP-1-derived macrophages. Therefore, inclusion of LPHs in foods may help to prevent chronic diseases associated with chronic inflammation.

## HIGHLIGHTS

- Lupine protein hydrolysates attenuate expression of proinflammatory cytokines
- Lupine protein hydrolysates decrease migration capability of macrophages
- Lupine hydrolysates may help to prevent diseases related to chronic inflammation

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**ANTI-INFLAMMATORY ACTIVITY OF LUPINE (*Lupinus angustifolius* L.)  
PROTEIN HYDROLYSATES IN THP-1-DERIVED MACROPHAGES**

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4 **Abstract**  
5

6 The effect of two different lupine protein hydrolysates (LPHs) on *in vitro* macrophage  
7 activation in a THP-1-derived macrophage model was investigated. THP-1-derived  
8 macrophages were exposed to RPMI medium containing two LPHs obtained by  
9 enzymatic hydrolysis using two different proteases: Izyme AL and Alcalase 2.4 L.  
10 Cytokine's expression was measured by quantitative PCR. THP-1-derived macrophages  
11 exhibited attenuated expression of proinflammatory cytokines (tumour necrosis factor  
12 (TNF), IL-6, IL-1 $\beta$ ) and increased expression of anti-inflammatory marker genes  
13 (chemokine (C-C motif) ligand 18 (CCL18)) relative to control without LPH. The anti-  
14 inflammatory effect of both hydrolysates favoured M2 polarization by quenching C-C  
15 chemokine receptor type 2 (CCR2) expression and migratory capacity. Furthermore,  
16 LPHs significantly decreased nitric oxide production. Moreover, LPHs promoted the  
17 survival of human THP-1-derived macrophages. Therefore, inclusion of LPHs in foods  
18 may help to prevent chronic diseases associated with chronic inflammation.  
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38 **Keywords**  
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40 Anti-inflammatory activity, bioactive peptides, lupine protein hydrolysates, macrophage  
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## 1. Introduction

The consumption of dietary protein drives many fundamental metabolic processes and is particularly important in nutrient-based biological functions (Gersh, Sliwa, Mayosi & Yusuf, 2010). In addition to providing essential amino acids to various systemic modulatory pathways, proteins and peptides produced by the hydrolysis of food proteins may also elicit potent anticancer, antimicrobial, hypocholesterolaemic, antihypertensive, antithrombotic, and anti-inflammatory effects (Möller, Scholz-Ahrens, Roos & Schrezenmeir, 2008). Bioactive peptides can be cleaved from polypeptide chains through gastrointestinal digestion, by fermentation or ripening during food processing, and by controlled hydrolytic processes using exogenous proteases (Pedroche et al., 2007). Many of these biologically active peptides are derived from both plant and animal sources, with most potentially stemming from milk-based products and legumes, such as soybean. Many reports have shown the potential health benefits of enzymatic hydrolysates prepared from milk, egg, and soy proteins (Möller et al., 2008; Shahidi & Zhong, 2008). Much research has focused on hydrolysates with angiotensin-converting enzyme inhibitory and antioxidant activities whereas other bioactive properties such as anti-inflammatory effect are less studied. In fact, very few studies report anti-inflammatory properties of protein hydrolysates (Vo, Ryu & Kim, 2008; Xu, Yang, Yin, Liu & Mine, 2012). Regarding the bioactive properties of lupine, it was previously reported that protein extracts from white lupine can lower plasma cholesterol (Weiße et al., 2010) and triacylglycerol concentrations (Spielmann et al., 2007) in hypercholesterolaemic animal models. Furthermore, lupine has shown anti-atherogenic effects in laboratory animals due to its lipid-lowering properties. In this study, Marchesi et al. (2008) demonstrated that a

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4 protein isolate from lupine reduces focal plaque development in the common carotid  
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6 arteries in a rabbit model of atherosclerosis. However, no information has been reported  
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8 about the anti-inflammatory properties of lupine proteins and peptides.  
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10  
11 Inflammation is an important normal immune response during lesions and infections.  
12  
13 However, an excessive inflammation can contribute to several acute and chronic diseases  
14  
15 characterised by uncontrolled production of pro-inflammatory cytokines, eicosanoids  
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17 derived from arachidonic acid, reactive oxygen species (ROS) and adhesion molecules  
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19 (Calder, 2006). Therefore, inhibitors of the pro-inflammatory cytokines have been  
20  
21 considered as a candidate of anti-inflammatory drugs. Chronic inflammation is a  
22  
23 hallmark of several pathologies, such as rheumatoid arthritis, inflammatory bowel  
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25 disease, atherosclerosis and cancer. The macrophage is the key player of the chronic  
26  
27 inflammatory response, which the monocytic cell line THP-1 represents an appropriate  
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29 model system to study immune responses (Weldon, Mullen, Loscher, Hurley, & Roche,  
30  
31 2007). It is well known that macrophages are key players during inflammatory responses  
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33 and their phenotype determines the cytokine secretion profile. Thus, classically activated  
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35 M1 macrophages contribute to the development and enhancement of inflammatory and  
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37 immunity processes and are, therefore, associated with high microbicidal activity,  
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39 supporting the activity of Th1 cells (Mills, Kincaid, Alt, Heilman, & Hill, 2000). This  
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41 type of activation is associated with an elevated production of pro-inflammatory  
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43 cytokines, such as tumour necrosis factor (TNF), IL-6, and IL-1; ROS; and nitrogen  
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45 intermediates (Cathcart, 2004). Alternative macrophage activation (M2) is triggered in  
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47 response to IL-4 or IL-13 and is associated with tissue remodeling and immunoregulation  
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49 (Gordon & Martinez, 2010). M2 macrophages produce anti-inflammatory cytokines, such  
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4 as IL-10, chemokine (C-C motif) ligand 18 (CCL18), and IL-1 receptor antagonist. Due  
5  
6 to their anti-inflammatory profile, M2 macrophages are frequently associated with all  
7  
8 types of activation triggered by anti-inflammatory stimuli.  
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10  
11 The increase in the incidence of inflammation related disorders has led to the search of  
12  
13 proteins and peptides with anti-inflammatory properties (Ndiaye, Vuong, Duarte, Aluko,  
14  
15 & Matar, 2012). There is evidence of the ability of distinct food compounds, including  
16  
17 proteins, to modulate inflammation in experimental models involving macrophages  
18  
19 (Boesch-Saadatmandi et al., 2011; Hämäläinen et al., 2011; Yu, Correll, & Vanden  
20  
21 Heuvel, 2002). Indeed some peptides with anti-inflammatory activity have been purified  
22  
23 from plants (Dia, Wang, Oh, de Lumen, & Gonzalez de Mejia, 2009).  
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26  
27 In a previous paper, we have described that blue lupine protein hydrolysates (LPHs)  
28  
29 inhibited some enzymes involved in the inflammatory pathway, such as phospholipase  
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31 A2 and cyclooxygenase-2 (Millán-Linares, Yust, Alcaide-Hidalgo, Millán, & Pedroche,  
32  
33 2014). In this work, we investigated the potential anti-inflammatory activity of two lupine  
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35 protein hydrolysates (LPHs) in a THP-1-derived macrophage model. LPHs were obtained  
36  
37 by hydrolysis of lupine protein isolate (LPI) with Izyme AL and Alcalase 2.4 L, two  
38  
39 food-grade proteases produced by Novozymes. Izyme AL has trypsin-like activity  
40  
41 whereas Alcalase is a non-specific endoprotease. Both trypsin and Alcalase have  
42  
43 previously been used for the generation of bioactive peptides (Korhonen & Pihlanto,  
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45 2006).  
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## 52 **2. Materials and methods**

### 53 **2.1 Materials**

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4 LPI was prepared according to Yust, Pedroche, Millán-Linares, Alcaide-Hidalgo and  
5  
6 Millán (2010). Izyme AL and Alcalase 2.4 L were provided by Novozymes (Bagsvaerd,  
7  
8 Denmark). The cell type used was THP-1 monocytes, ATCC® Number TIB-202™. The  
9  
10 medium for this line was Gibco® RPMI 1640 (Life Technologies SA, Alcobendas,  
11  
12 Spain). PBS, foetal bovine serum (FBS), and penicillin/streptomycin (P/S) solution were  
13  
14 obtained from Gibco® as well. Dimethyl sulphoxide (DMSO), formyl-methionyl-leucyl-  
15  
16 phenylalanine (fMLP), phorbol 12-myristate 13-acetate (PMA), trypan blue solution, and  
17  
18 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), and Griess  
19  
20 reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% N-naphthylenediamine-  
21  
22 HCl) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ribonuclease A  
23  
24 was obtained from Nacalai Tesque (Kyoto, Japan). The iScript cDNA Synthesis Kit was  
25  
26 from Bio-Rad Laboratories (Hercules, CA, USA). The Annexin V-FITC Kit was obtained  
27  
28 from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Brilliant II Syber® Green  
29  
30 QPCR Master Mix was purchased from Agilent Technologies (Santa Clara, CA, USA).  
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32 Primers were purchased from Eurofins Biolab S.L.U. (Barcelona, Spain). NucleoSpin  
33  
34 RNA II was obtained from Macherey-Nagel GmbH & Co. KG (Düren, Germany).  
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36 Human TNF and IL-10 ELISA Sets were from Bionova Científica (Madrid, Spain).  
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## 45 **2.2 Preparation of LPHs**

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48 Hydrolysis was performed in a bioreactor while stirring at a controlled pH and  
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50 temperature. LPI was suspended in distilled water (10% w/v), and two types of  
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52 hydrolysis were performed: one with Izyme AL followed by Alcalase and one using only  
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54 Alcalase. The following conditions were used:  
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4 Hydrolysis with Izyme AL and Alcalase: First, LPI was hydrolysed with Izyme AL for 1  
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6 h at pH 10, 50°C, E/S = 100 EU/g protein. A second step of hydrolysis with Alcalase at  
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8 pH 8, 50°C, E/S = 0.3 AU/g protein, was then performed for 15 min.  
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10  
11 Hydrolysis with Alcalase: pH 8, 50°C, E/S = 0.3 AU/g protein, and duration of hydrolysis  
12  
13 of 15 min.  
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15  
16 Enzymes were inactivated by heating at 85°C for 15 min, centrifuged at 8000 rpm for 15  
17  
18 min, and the supernatants constituted LPHs. LPH obtained with Izyme AL followed by  
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20 Alcalase was designated I+15A, and LPH obtained using only Alcalase was designated  
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22 15A.  
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### 25 26 **2.3 Cell culture and treatments**

27  
28 The human monocytic THP-1 cell line, was cultured in suspension in RPMI 1640  
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30 medium supplemented with 1% P/S and 10% heat-inactivated FBS. To induce monocyte-  
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32 macrophage differentiation; THP-1 cells were cultured in the presence of PMA (100  
33  
34 nmol/L) for 4 days (Weldon et al., 2007). PMA-stimulated THP-1 cells (referred to as  
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36 THP-1-derived macrophages) were exposed to RPMI medium (supplemented with 1%  
37  
38 FBS) for 24 h and then treated with the LPH I+15A or the LPH 15A at a concentration of  
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40 500 µg/mL RPMI medium (1% FBS) for 6 h.  
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### 45 46 **2.4 Measurement of cell proliferation by the MTT method**

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48 THP-1-derived macrophage proliferation was evaluated by measuring optical density at  
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50 different concentrations of treatments in a 96-well plate. Cells were incubated at 37°C  
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52 with the LPH I+15A or the LPH 15A at final concentrations of 100, 300, 500, 700, or  
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54 2000 µg/mL for 18 h. An aliquot of 20 µL of MTT (5 mg/mL) was added to each well  
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56 and incubated at 37°C for 6 h. MTT is reduced to purple formazan in living cells  
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4 (Carmicheal, DeGraff, Gazdar, Minna, & Mitchell, 1987). The supernatant was removed,  
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6 and 200  $\mu$ L of DMSO were added to each well to dissolve the insoluble purple formazan  
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8 product into a colored solution, followed by shaking for 10 min. Absorbance was  
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10 measured at 570 nm using a microplate reader, and cell proliferation was calculated.  
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14 RPMI medium (1% FBS) was used as control.  
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## 16 **2.5 Analysis of cellular DNA content**

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18 Cellular DNA content was assessed by propidium iodide (PI) staining and FACS analysis  
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20 as previously described, with modifications (Mills et al., 2000). The cells were exposed to  
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22 RPMI medium (1% FBS) containing the LPH I+15A or the LPH 15A at a concentration  
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24 of 500  $\mu$ g/mL for 24 h of treatment. The cells were then fixed in 1% paraformaldehyde in  
25  
26 PBS containing 0.5% saponin for 5 min at 4°C. After centrifugation, the cells were  
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28 incubated in buffer containing 5  $\mu$ g/mL PI and 1 mg/mL ribonuclease A for 10 min at  
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30 4°C. The cells were analyzed using a BD FACSCanto II flow cytometer and BD  
31  
32 FACSCanto II Software (BD Biosciences, San Jose, CA, USA). FBS 1% and FBS 10%  
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34 were used as negative and positive control, respectively. Cells that were hypodiploid due  
35  
36 to DNA fragmentation were regarded as apoptotic cells.  
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## 43 **2.6 Cell viability**

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45 Cells were exposed to the LPH I+15A or the LPH 15A at 100, 300, 500, 700, 1000, or  
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47 2000  $\mu$ g/mL for 24 h, rinsed with PBS, and evaluated for live/dead cells using the trypan  
48  
49 blue exclusion test. Viable cells excluded the dye, whereas dead cells were stained an  
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51 intense blue. At least 200 cells were scored to assess live/dead cells. The number of  
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53 viable cells was quantified by confocal microscopy (Olympus IX81, Tokyo, Japan).  
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4 RPMI medium (1% FBS) was used as control. Cells were tested in triplicate, and the  
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6 results were averaged.  
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## 8 9 **2.7 Measurement of apoptotic cells**

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11 Early events associated with apoptosis were evaluated using the binding of annexin V to  
12  
13 detect the translocation of phosphatidylserine from the inner side to the outer leaflet of  
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15 the plasma membrane of apoptotic cells and using PI to detect the DNA of necrotic cells,  
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17 as described in the Annexin V-FITC Kit. An analysis of stained cells was performed by  
18  
19 measuring fluorescence emission using a BD FACSCanto II flow cytometer at 530 nm  
20  
21 and 585 nm for fluorescein isothiocyanate and PI, respectively, and using BD  
22  
23 FACSCanto II Software. RPMI medium (1% FBS) was used as control and staurosporine  
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25 (pro-apoptotic) as positive control.  
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## 30 31 **2.8 mRNA extraction and analysis of PCR products**

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33 Total RNA was extracted from the THP-1-derived macrophages using NucleoSpin<sup>®</sup> RNA  
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35 II. RNA quality was assessed using the OD260:OD280 ratio determined by a NanoDrop  
36  
37 ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). One microgram  
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39 of total RNA was subjected to RT-PCR to obtain cDNA according to the manufacturer's  
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41 protocol.  
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45 The mRNA levels for specific genes were determined using an Mx3000P Real-Time PCR  
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47 System (Stratagene, La Jolla, CA, USA). For each QPCR, 10 ng of cDNA template was  
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49 added to Brilliant SYBR Green QPCR Master Mix containing primer pairs for TNF, IL-  
50  
51 6, IL-1 $\beta$ , CCL18, C-C chemokine receptor type 2 (CCR2), and chemokine (C-C motif)  
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53 ligand 2 (CCL2). The reference genes HPRT and GAPDH were used to correct for RNA  
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55 concentration differences between the samples.  
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4 The sequence of and information about the primers that were used in this study are as  
5 follows: TNF (NM\_000594.3): 5'-TCCTTCAGACACCCTCAACC-3' and 5'-  
6 AGGCCCCAGTTTGAATTCTT-3' (reverse); IL-6 (NM\_001001928): 5'-  
7 GTTTGAGGGGGTAACAGCAA-3' and 5'-GCTAACTGCAGAGGGTGAGG-3'; IL-1 $\beta$   
8 (NM\_138712): 5'-GCTGTGCAGGAGATCACAGA-3' and 5'-  
9 GGGCTCCATAAAGTCACCAA-3'; CCL18 (NM\_002957): 5'-  
10 GGGTTTTCTTCCCTTTCGAG-3' and 5'-GCGTGTTCCTTTTCCACAAT-3'; CCR2  
11 (NM\_001002): 5'-TCGACAATGGCAGCATCTAC-3' and 5'-  
12 ATCCGTCTCCACAGACAAGG-3'; CCL2 (NM\_002982.3): 5'-  
13 CCCAGTCACCTGCTGTTAT-3' and 5'-TGGAATCCTGAACCCACTTC-3'; HPRT  
14 (NM\_000194.2): 5'-ACCCCACGAAGTGTGGATA-3' and 5'-  
15 AAGCAGATGGCCACAGAACT-3'; and GAPDH (NM\_002046.4): 5'-  
16 GAGTCAACGGATTTGGTCGT-3' and 5'-TTGATTTTGGAGGGATCTCG-3'.

17 All amplification reactions were performed in triplicate. The magnitude of the change in  
18 mRNA expression for the candidate genes was calculated using the standard  $2^{-(\Delta\Delta Ct)}$   
19 method. All data were normalized to endogenous reference genes (HPRT and GAPDH)  
20 levels and expressed as a percentage of the control.

## 2.9 Enzyme-linked immunosorbent assay (ELISA).

21 TNF and IL10 concentrations in cell culture supernatants were quantified by commercial  
22 ELISA kits according to manufacturer's instructions.

## 2.10 Measurements of nitrite in THP-1-derived macrophages cells.

23 As an indicator of NO production, nitrite (NO<sub>2</sub><sup>-</sup>) concentration was measured in the cell  
24 culture supernatants. Equal volumes of culture supernatants and Griess reagent were

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4 mixed and the absorbance was read at 540 nm in the microplate reader (Green et al.,  
5  
6 1981). The amount of nitrite was obtained by an extrapolation from a standard curve with  
7  
8 sodium nitrite.  
9

## 10 11 **2.11 Migration assays**

12  
13 THP-1-derived macrophages were collected in RPMI-1640 medium containing 10%  
14  
15 FBS, and 1% P/S and seeded in 24-well culture plates at  $5 \times 10^5$  per well to allow high-  
16  
17 density adhesion of the macrophages. After removing the floating cells, the adherent cells  
18  
19 were incubated at 37°C in 5% CO<sub>2</sub> for 24 h to form a confluent monolayer. The  
20  
21 macrophage monolayer was wounded by scratching with a thin pipette tip. The cells were  
22  
23 treated with each LPH (I+15A and 15A) and allowed to migrate for 24 h. The migration  
24  
25 of THP-1-derived macrophages was examined and quantified by confocal microscopy.  
26  
27 Images were captured at the beginning and at 24 h of cell migration to close the wound  
28  
29 and compared to quantify the migration rates of the cells.  
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## 35 36 **2.12 Statistical analysis**

37  
38 The data are presented as the mean  $\pm$  SEM of three independent determinations. Group-  
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40 wise statistical comparisons were performed by a one-way ANOVA with a post-hoc  
41  
42 Bonferroni test. Differences were considered to be significant at  $P < 0.05$ .  
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## 46 47 **3. Results and discussion**

### 48 49 **3.1 LPHs does not alter the cellular integrity of THP-1-derived macrophages**

50  
51 Before assessing the anti-inflammatory activity, cell viability and the potential  
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53 cytotoxicity of the LPHs were evaluated (Gülden & Seibert, 2003).  
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56 To investigate whether LPHs may cause a cytotoxic effect, an MTT assay was performed  
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58 on THP-1-derived macrophages after separately adding each LPHs at increasing  
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4 concentrations to conditioned RPMI culture medium for 6 h. The LPHs I+15A and 15A  
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6 had no significant effects (Fig. 1A and 1B) compared with the untreated control group.  
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8 Moreover, using flow cytometric analysis to study the DNA distribution in the cell cycle  
9  
10 (Sawai & Domae, 2008), we observed that the percentage of S population among THP-1-  
11  
12 derived macrophages treated with the LPH I+15A or the LPH 15A was slightly increased  
13  
14 (Fig. 1C), but did not reach significance. For the quiescent phase ( $G_0/G_1$ ) and  $G_2/M$   
15  
16 population no differences were found in relation to the control (1% FBS). For all  
17  
18 samples, even though a degree of proliferative activity, which was not significant, was  
19  
20 observed, the cells remained in the quiescent phase for a longer period. The addition of  
21  
22 10% FBS was used as a positive control.  
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28 The contribution of LPHs to the activation of programmed cell death (apoptosis) was also  
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30 investigated. Interestingly, apoptosis was reduced in the presence of the LPH 60I+15A or  
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32 the LPH 15A (29 and 35%, respectively) compared with the control (Fig. 1D). This effect  
33  
34 seems to endow both LPHs with a protective effect against apoptosis. To complete the  
35  
36 feasibility studies of the LPHs, cell viability was assayed by trypan blue exclusion in  
37  
38 THP-1-derived macrophages treated with increasing concentrations of the LPH I+15A or  
39  
40 the LPH 15A for 24 h. As expected, there was no differences in cell viability after 24 h of  
41  
42 incubation in the presence of higher concentrations of the LPH I+15A, which ranged  
43  
44 from 100 to 2000  $\mu\text{g}/\text{mL}$ , when compared with the control (Fig. 2A). The same pattern  
45  
46 was observed after treatment with the LPH 15A (Fig. 2B).  
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53 Taken together, these results suggest that in general, LPHs do not compromise the  
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55 integrity of THP-1-derived macrophages. Although cell viability was decreased upon  
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57 exposure to higher concentrations, this decrease did not reach 30% cell loss for either of  
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4 the LPHs tested. Hence, LPHs did not have any major effect on membrane integrity in  
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6  
7 the selected cell model.

### 9 **3.2 Effect of LPHs on the expression of pro and anti-inflammatory cytokines**

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11 Several diseases, such as obesity-associated insulin resistance, diabetes, and metabolic  
12  
13 syndrome, are sustained by chronic subclinical inflammation (Faloia et al., 2012).

14  
15 Elevated levels of cytokines, such as leptin, TNF, IL-1, and IL-6, are generally increased  
16  
17 during inflammatory diseases (Hajer, Van Haeften, & Visseren, 2008). A growing body  
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19 of evidence has shown that biologically active peptides derived from plants can prevent  
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21 many inflammatory disorders due to the peptides' antioxidant and anti-inflammatory  
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23 effects (Politis, Theodorou, Lampidonis, Chronopoulou, & Baldi, 2012; Vernaza, D  a, &  
24  
25 Gonz  lez de Mej  a, 2012). Classically activated macrophages (with an M1 phenotype)  
26  
27 mediate tissue damage and initiate inflammatory responses by releasing pro-  
28  
29 inflammatory mediators (Olefsky & Glass, 2010). These mediators recruit additional  
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31 macrophages, establishing a feed-forward process that further increases leukocytes  
32  
33 content and propagates the chronic inflammatory state (Shen, Lu, Duan, & Duan, 2011).  
34  
35 Herein, we first evaluated the ability of LPHs (I+15A and 15A) to modulate the  
36  
37 expression of the pro-inflammatory cytokines TNF, IL-1 $\beta$ , and IL-6 in THP-1-derived  
38  
39 macrophages (Fig. 3A-C). TNF showed significant inhibition after 6 h of incubation with  
40  
41 either hydrolysate. This reduction was markedly pronounced after I+15A treatment  
42  
43 (TNF: -45%, IL-1: -32%, and IL-6: -43%). The LPH 15A induced the downregulation of  
44  
45 TNF and IL-1 in activated macrophages by 30 and 35%, respectively, whereas IL-6  
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47 levels were blunted (70%). No significant differences were observed between the  
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49 treatments. These results indicate that LPHs tend to decrease the pro-inflammatory  
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4 capacity of activated M1 macrophages by diminishing cytokines expression, setting a  
5 trend of regulation of the inflammatory process. Macrophage polarization dramatically  
6 alters the immune properties of these cells, as evidenced by the potent anti-microbial  
7 properties of M1 macrophages compared with the prominent anti-inflammatory tissue  
8 repair properties of M2 macrophages (Joshi et al., 2010). Depending on the cytokine  
9 microenvironment, the M2 macrophage phenotype is characterized by the expression of  
10 cell surface proteins such as CD206 and CD163 and of soluble factors such as CCL18  
11 (Bellón et al., 2011). To corroborate the hypothesis that LPHs have potential anti-  
12 inflammatory role, the expression of CCL18 and IL10 (an M2 markers) after 6 h of  
13 incubation with the LPHs 15A or the LPH I+15A were evaluated. Surprisingly, both  
14 LPHs highly increased CCL18 expression, doubling the value compared with the control  
15 (Fig. 3D). However, no significant differences were observed in the IL10 expression In  
16 THP-1-derived macrophages after treatment with LPHs (Fig. 3E).

17  
18 Taken together, these data suggest that the LPHs I+15A and 15A may have a beneficial  
19 capability to skew activated M1 macrophages toward the anti-inflammatory M2  
20 phenotype.

### 21 **3.3 Effect of LPHs on cytokines production**

22  
23 To corroborate the effect of LPHs on cytokines mRNA expression, the concentration of  
24 TNF and IL-10 was measured in cell culture supernatants. The production of TNF was  
25 decreased by both LPHs (Fig. 4A). This inhibition was higher in LPH I+15A, which  
26 inhibited more than 80% TNF production. Regarding IL-10, significant differences were  
27 not observed among LPHs and control (Fig. 4B). These data coincided with the ones  
28 obtained by quantitative PCR, where THP-1-derived macrophages exhibited attenuated  
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4 expression of proinflammatory cytokine TNF, and showed no significant differences in  
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6 the expression of IL-10.  
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### 9 **3.4 Effect of LPHs on the nitrite production**

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11 Nitric oxide (NO) has been shown to play a central role in inflammatory and immune  
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13 reaction activities and macrophages appear to be the main cellular source of NO  
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15 (Montserrat-de la Paz, Fernández-Arche, Ángel-Martín & García-Giménez, 2012). The  
16  
17 effect of LPHs on the release of this inflammatory mediator is depicted in Fig. 5. Both  
18  
19 LPHs inhibited approximately 50% of NO production. Other legume protein hydrolysates  
20  
21 have shown inhibition of NO production by activated macrophages (Ndiaye et al., 2012).  
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### 24 **3.5 LPHs impair the chemotactic capacity of human THP-1-derived macrophages**

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26 Previously, Fontanari, Batistuti, da Cruz, Hilario, and Saldiva (2012) investigated the  
27  
28 potential hypolipidaemic effect of a total protein extract from *Lupinus albus*, which is  
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30 associated with a mechanism shared with soya proteins (Duranti et al., 2004; Lovati et al.,  
31  
32 2000; Lovati, Manzoni, Gianazza & Sirtori, 1998). Furthermore, soy-based diets have  
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34 been shown to reduce atherosclerotic lesions through downregulation of the expression  
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36 levels of monocyte chemokines essential for the initiating events in atherosclerosis, such  
37  
38 as monocyte chemoattractant protein-1 (MCP-1) or CCL2. CCL2 have a systemic role in  
39  
40 the regulation of metabolism, and particularly in controlling leukocyte extravasation and  
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42 chemotaxis toward inflamed tissues. For instance, Weisberg et al. (2006) reported a  
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44 significant reduction in plaque macrophage content in mice lacking CCR2 (CCR2<sup>-/-</sup>  
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46 mice). Furthermore, certain evidence has indicated an increase in M1 and decrease in M2  
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48 macrophages in obese adipose tissue (Lumeng, Bodzin, & Saltiel, 2007). Interestingly,  
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50 such a phenotypic switch was not observed in CCR2<sup>-/-</sup> mice, suggesting that the MCP-  
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4 1/CCR2 pathway could contribute to M2 macrophage polarization.  
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6 We hypothesized that as occurs with soy proteins, lupine hydrolysates may exert a  
7 beneficial effect on the chemotaxis mechanism by modulating the CCR2/CCL2 axis. To  
8 examine this issue, the expression of CCR2 and CCL2 in human THP-1-derived  
9 macrophages was evaluated after 6 h of incubation with either LPH. As expected, the  
10 CCL2/CCR2 axis was noticeably modulated by both LPHs. Fig. 4A shows a dramatic  
11 reduction in CCR2 expression by the LPH I+15A (-62%), whereas this expression was  
12 more blunted by the LPH 15A (-84%). Despite the significant reduction in CCR2, we  
13 could not find any significant difference in the expression of its ligand, CCL2 (Fig. 4B),  
14 but did note a decreasing tendency. Accordingly, the migration index was reduced after  
15 24 h of treatment with the LPH I+15A (-44%) or the LPH 15A (-58%). A potent  
16 chemotactic agent, fMLP, was used as positive control (Fig. 4C).  
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33 Thus far, the results indicate that the addition of LPHs to fully differentiated THP-1  
34 macrophages results in the decreased expression of both CCR2 and CCL2 and,  
35 consequently, a decrease in the cells' migration capability. These findings suggest that  
36 infiltrating activated macrophages exposed to LPHs may lose their chemotactic ability,  
37 which ameliorates the inflammatory state.  
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45 In conclusion, after LPH treatment, THP-1-derived-macrophages showed attenuated  
46 expression of TNF, IL-6, IL-1 $\beta$  (proinflammatory cytokines) and increased expression of  
47 CCL18 (antiinflammatory). Moreover, NO production was inhibited. Thereby, LPHs  
48 displayed hyporeactivity to M1-type ligands and polarization to the M2 phenotype.  
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55 Furthermore, our data demonstrates that both LPHs attenuate the macrophage migratory  
56 response, which is partly mediated by skewing THP-1-derived macrophages toward  
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alternatively activated M2 macrophages, which are equipped for repair and resolution of the inflammatory response. Thus, this study is the first to describe the anti-inflammatory effect of LPHs in THP-1-derived macrophages and the influence in the control of macrophage polarization in the context of inflammatory state.

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4 **FIGURE CAPTIONS**  
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6 **Figure 1.** Effect of LPHs (500–2000 µg/mL) I+15A (A) and 15A (B) on cell proliferation  
7 of human THP-1-derived macrophages determined by MTT assay after 6 h. Study of the  
8 DNA distribution in the cell cycle ( % G0/ G1, % S and % G2/M) in THP-1- derived  
9 macrophages treated with LPH I+15A or LPH 15A (C). Percentage of apoptotic cell  
10 death in THP-1-derived macrophages after exposure to LPHs at 500 µg/mL for 24 h (D).  
11 Values marked with different letter are significantly different (P < 0.05).  
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21 **Figure 2.** Cell viability (%), determined by trypan blue exclusion assay, in presence of  
22 LPHs I+15A (A) and 15A (B), after 24 h of treatment and different concentrations (100-  
23 2000 µg/mL). Some images obtained with confocal microscopy are presented.  
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28 **Figure 3.** TNF (A), IL-1β (B), IL-6 (C), CCL18 (D), and IL10 (E) mRNA expression in  
29 THP-1-derived macrophages after 6 h of treatment with LPHs (I+15A, and 15A). Values  
30 marked with different letter are significantly different (P < 0.05).  
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36 **Figure 4.** Effect on TNF (A) and IL-10 (B) production of THP-1-derived macrophages.  
37 after 48 h of treatment with LPHs (I+15A, and 15A). Values marked with different letter  
38 are significantly different (P < 0.05).  
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43 **Figure 5.** NO production (%) in THP-1-derived macrophages after 48 h of treatment with  
44 LPHs (I+15A, and 15A). Values marked with different letter are significantly different (P  
45 < 0.05).  
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50 **Figure 6.** CCR2 (A) and CCL2 (B) mRNA expression in THP-1-derived macrophages  
51 after 6 h of treatment with LPHs (I+15A, and 15A). Migration Index (C) of THP-1-  
52 derived macrophage after 24 h of incubation. Values marked with different letter are  
53 significantly different (P < 0.05).  
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Figure 1  
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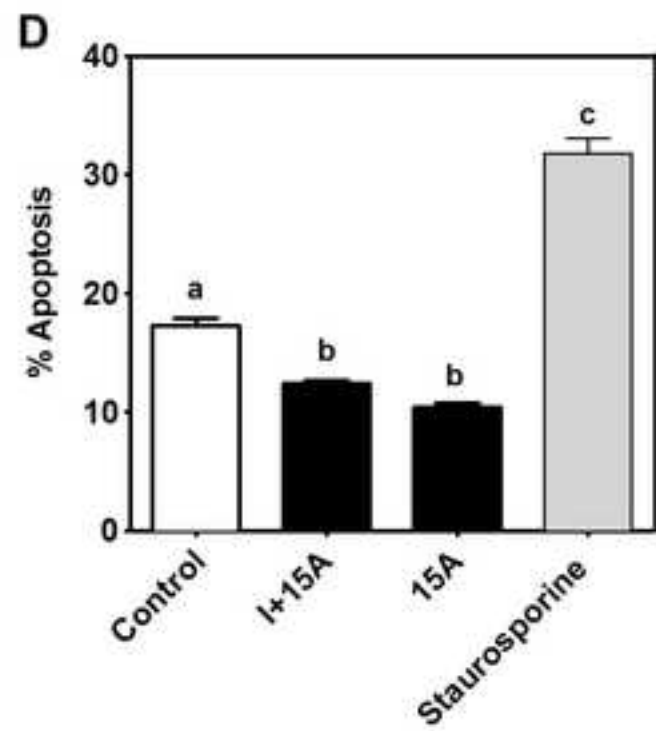
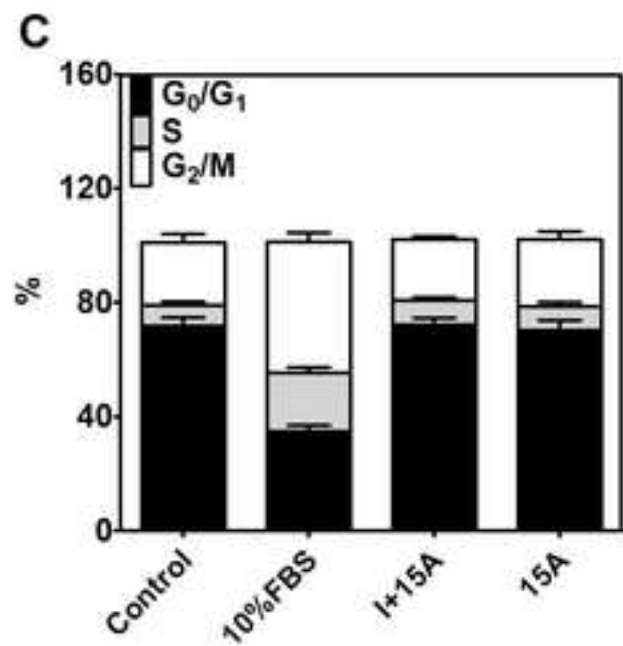
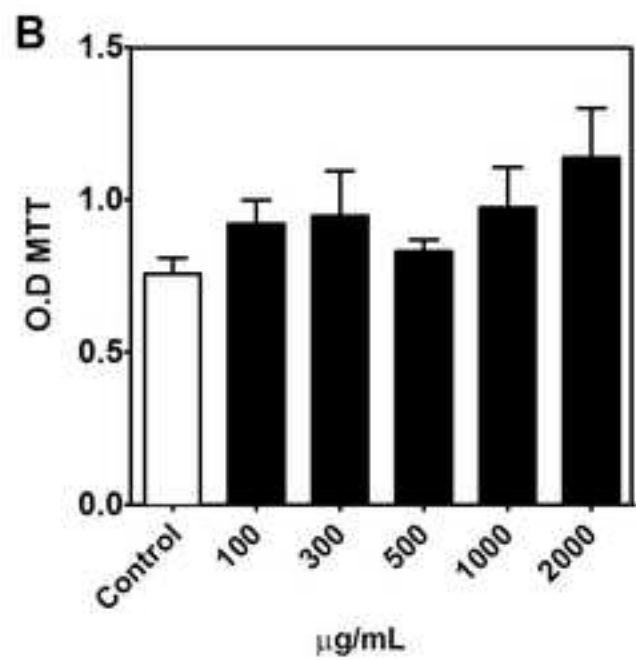
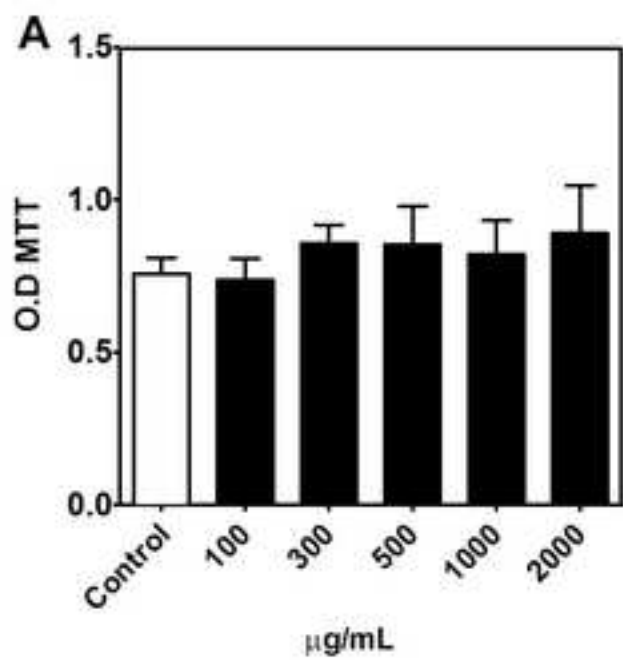
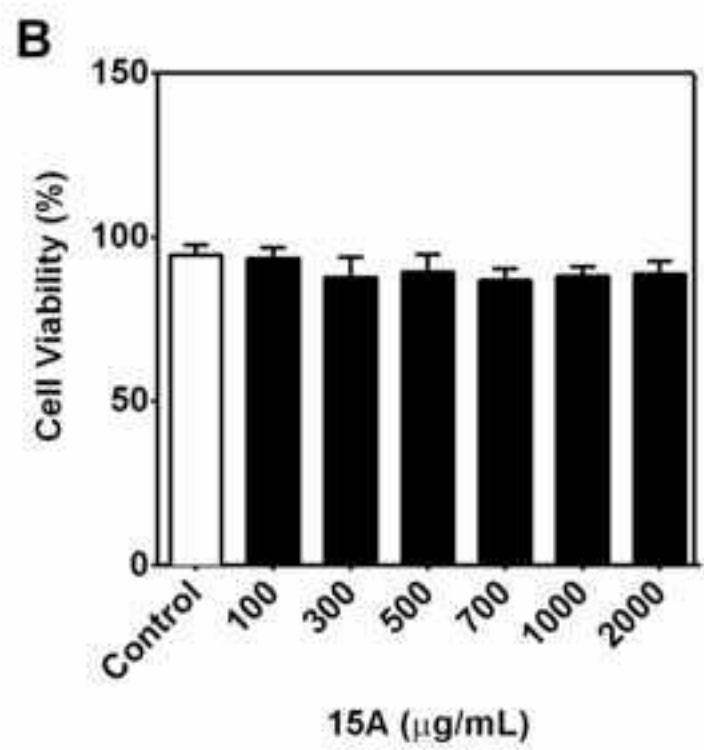
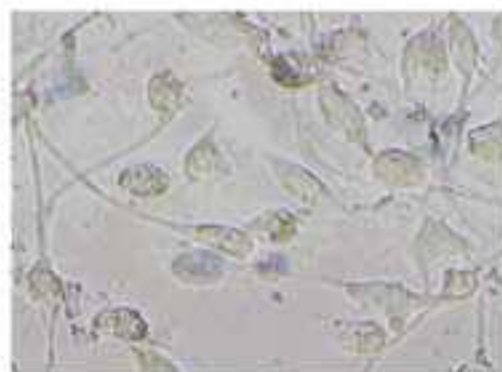
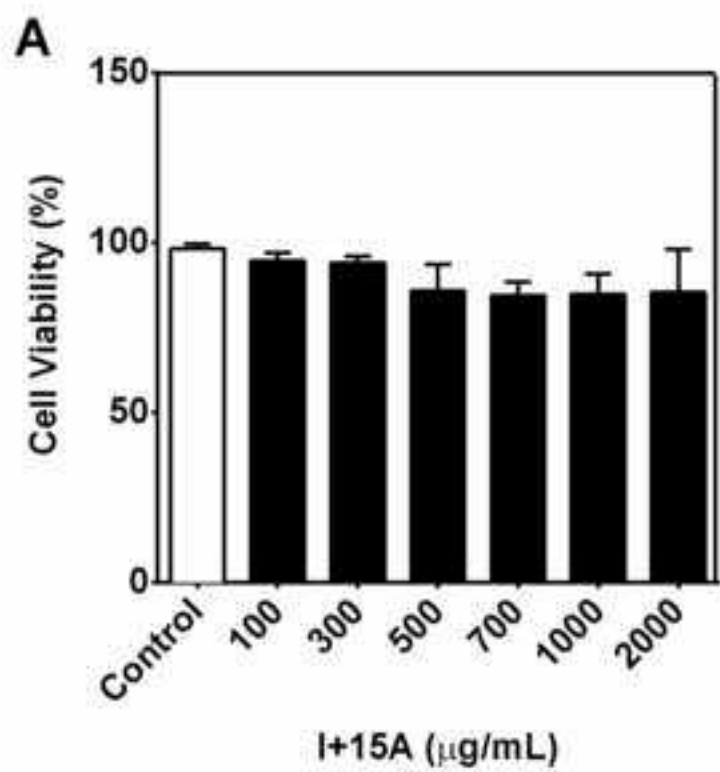


Figure 2

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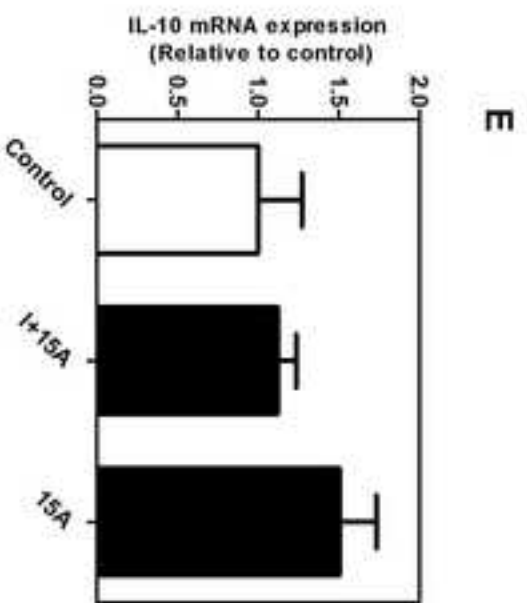
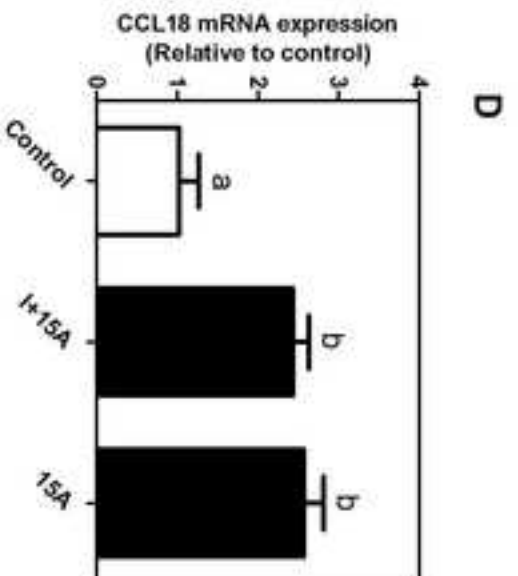
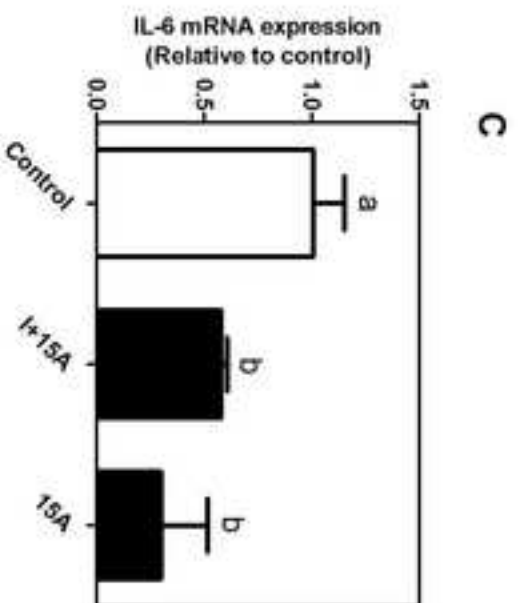
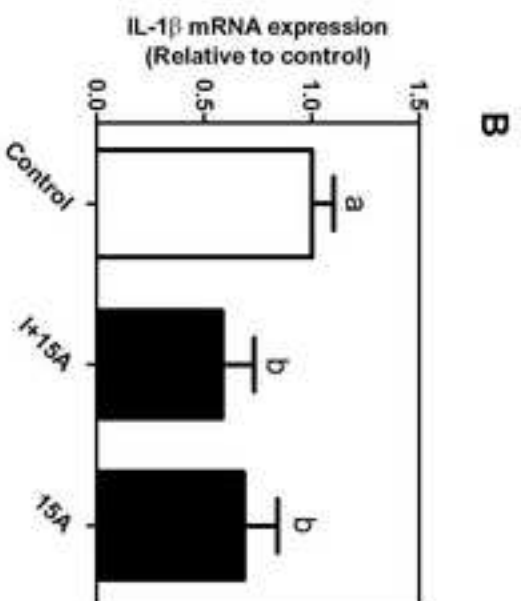
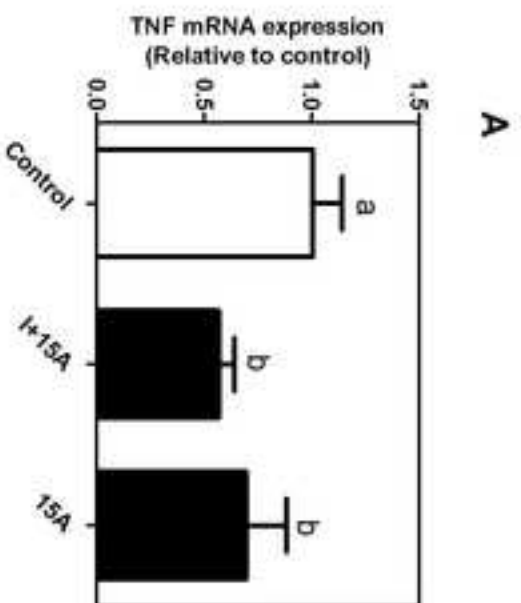


Figure 4  
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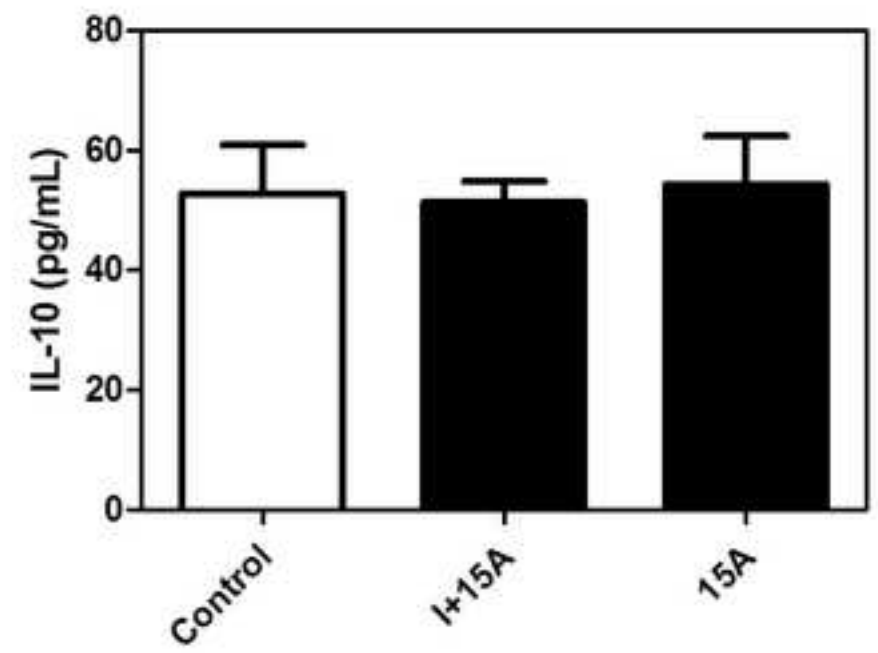
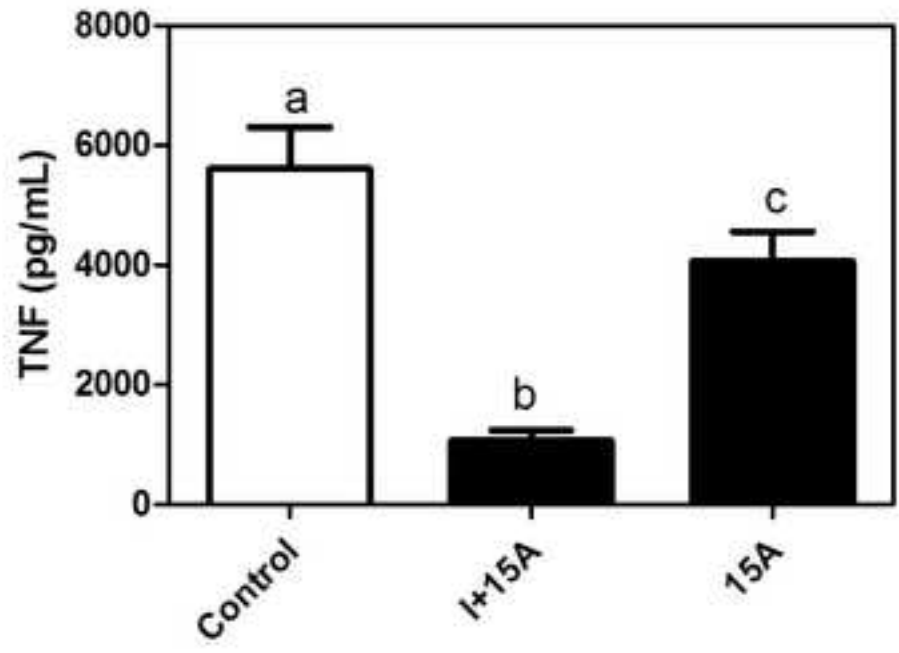


Figure 5  
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