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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

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-1β) 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.

Keywords

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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 (Weiβe 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

protein isolate from lupine reduces focal plaque development in the common carotid arteries in a rabbit model of atherosclerosis. However, no information has been reported about the anti-inflammatory properties of lupine proteins and peptides.

Inflammation is an important normal immune response during lesions and infections. However, an excessive inflammation can contribute to several acute and chronic diseases characterised by uncontrolled production of pro-inflammatory cytokines, eicosanoids derived from arachidonic acid, reactive oxygen species (ROS) and adhesion molecules (Calder, 2006). Therefore, inhibitors of the pro-inflammatory cytokines have been considered as a candidate of anti-inflammatory drugs. Chronic inflammation is a hallmark of several pathologies, such as rheumatoid arthritis, inflammatory bowel disease, atherosclerosis and cancer. The macrophage is the key player of the chronic inflammatory response, which the monocytic cell line THP-1 represents an appropriate model system to study immune responses (Weldon, Mullen, Loscher, Hurley, & Roche, 2007). It is well known that macrophages are key players during inflammatory responses and their phenotype determines the cytokine secretion profile. Thus, classically activated M1 macrophages contribute to the development and enhancement of inflammatory and immunity processes and are, therefore, associated with high microbicidal activity, supporting the activity of Th1 cells (Mills, Kincaid, Alt, Heilman, & Hill, 2000). This type of activation is associated with an elevated production of pro-inflammatory cytokines, such as tumour necrosis factor (TNF), IL-6, and IL-1; ROS; and nitrogen intermediates (Cathcart, 2004). Alternative macrophage activation (M2) is triggered in response to IL-4 or IL-13 and is associated with tissue remodeling and immunoregulation (Gordon & Martinez, 2010). M2 macrophages produce anti-inflammatory cytokines, such

as IL-10, chemokine (C-C motif) ligand 18 (CCL18), and IL-1 receptor antagonist. Due to their anti-inflammatory profile, M2 macrophages are frequently associated with all types of activation triggered by anti-inflammatory stimuli.

The increase in the incidence of inflammation related disorders has led to the search of proteins and peptides with anti-inflammatory properties (Ndiaye, Vuong, Duarte, Aluko, & Matar, 2012). There is evidence of the ability of distinct food compounds, including proteins, to modulate inflammation in experimental models involving macrophages (Boesch-Saadatmandi et al., 2011; Hämäläinen et al., 2011; Yu, Correll, & Vanden Heuvel, 2002). Indeed some peptides with anti-inflammatory activity have been purified from plants (Dia, Wang, Oh, de Lumen, & Gonzalez de Mejia, 2009).

In a previous paper, we have described that blue lupine protein hydrolysates (LPHs) inhibited some enzymes involved in the inflammatory pathway, such as phospholipase A2 and cyclooxygenase-2 (Millán-Linares, Yust, Alcaide-Hidalgo, Millán, & Pedroche, 2014). In this work, we investigated the potential anti-inflammatory activity of two lupine protein hydrolysates (LPHs) in a THP-1-derived macrophage model. LPHs were obtained by hydrolysis of lupine protein isolate (LPI) with Izyme AL and Alcalase 2.4 L, two food-grade proteases produced by Novozymes. Izyme AL has trypsin-like activity whereas Alcalase is a non-specific endoprotease. Both trypsin and Alcalase have previously been used for the generation of bioactive peptides (Korhonen & Pihlanto, 2006).

2. Materials and methods

2.1 Materials

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

2.2 Preparation of LPHs

Hydrolysis was performed in a bioreactor while stirring at a controlled pH and temperature. LPI was suspended in distilled water (10% w/v), and two types of hydrolysis were performed: one with Izyme AL followed by Alcalase and one using only Alcalase. The following conditions were used:

Hydrolysis with Izyme AL and Alcalase: First, LPI was hydrolysed with Izyme AL for 1 h at pH 10, 50ºC, E/S = 100 EU/g protein. A second step of hydrolysis with Alcalase at pH 8, 50ºC, E/S = 0.3 AU/g protein, was then performed for 15 min.

Hydrolysis with Alcalase: pH 8, 50ºC, E/S = 0.3 AU/g protein, and duration of hydrolysis of 15 min.

Enzymes were inactivated by heating at 85ºC for 15 min, centrifuged at 8000 rpm for 15 min, and the supernatants constituted LPHs. LPH obtained with Izyme AL followed by Alcalase was designated I+15A, and LPH obtained using only Alcalase was designated 15A.

2.3 Cell culture and treatments

The human monocytic THP-1 cell line, was cultured in suspension in RPMI 1640 medium supplemented with 1% P/S and 10% heat-inactivated FBS. To induce monocyte-macrophage differentiation; THP-1 cells were cultured in the presence of PMA (100 nmol/L) for 4 days (Weldon et al., 2007). PMA-stimulated THP-1 cells (referred to as THP-1-derived macrophages) were exposed to RPMI medium (supplemented with 1% FBS) for 24 h and then treated with the LPH I+15A or the LPH 15A at a concentration of 500 µg/mL RPMI medium (1% FBS) for 6 h.

2.4 Measurement of cell proliferation by the MTT method

THP-1-derived macrophage proliferation was evaluated by measuring optical density at different concentrations of treatments in a 96-well plate. Cells were incubated at 37ºC with the LPH I+15A or the LPH 15A at final concentrations of 100, 300, 500, 700, or 2000 µg/mL for 18 h. An aliquot of 20 µL of MTT (5 mg/mL) was added to each well and incubated at 37°C for 6 h. MTT is reduced to purple formazan in living cells

(Carmicheal, DeGraff, Gazdar, Minna, & Mitchell, 1987). The supernatant was removed, and 200 μL of DMSO were added to each well to dissolve the insoluble purple formazan product into a colored solution, followed by shaking for 10 min. Absorbance was measured at 570 nm using a microplate reader, and cell proliferation was calculated. RPMI medium (1% FBS) was used as control.

2.5 Analysis of cellular DNA content

Cellular DNA content was assessed by propidium iodide (PI) staining and FACS analysis as previously described, with modifications (Mills et al., 2000). The cells were exposed to RPMI medium (1% FBS) containing the LPH I+15A or the LPH 15A at a concentration of 500 μg/mL for 24 h of treatment. The cells were then fixed in 1% paraformaldehyde in PBS containing 0.5% saponin for 5 min at 4ºC. After centrifugation, the cells were incubated in buffer containing 5 μg/mL PI and 1 mg/mL ribonuclease A for 10 min at 4ºC. The cells were analyzed using a BD FACSCanto II flow cytometer and BD FACSCanto II Software (BD Biosciences, San Jose, CA, USA). FBS 1% and FBS 10% were used as negative and positive control, respectively. Cells that were hypodiploid due to DNA fragmentation were regarded as apoptotic cells.

2.6 Cell viability

Cells were exposed to the LPH I+15A or the LPH 15A at 100, 300, 500, 700, 1000, or 2000 μg/mL for 24 h, rinsed with PBS, and evaluated for live/dead cells using the trypan blue exclusion test. Viable cells excluded the dye, whereas dead cells were stained an intense blue. At least 200 cells were scored to assess live/dead cells. The number of viable cells was quantified by confocal microscopy (Olympus IX81, Tokyo, Japan).

RPMI medium (1% FBS) was used as control. Cells were tested in triplicate, and the results were averaged.

2.7 Measurement of apoptotic cells

Early events associated with apoptosis were evaluated using the binding of annexin V to detect the translocation of phosphatidylserine from the inner side to the outer leaflet of the plasma membrane of apoptotic cells and using PI to detect the DNA of necrotic cells, as described in the Annexin V-FITC Kit. An analysis of stained cells was performed by measuring fluorescence emission using a BD FACSCanto II flow cytometer at 530 nm and 585 nm for fluorescein isothiocyanate and PI, respectively, and using BD FACSCanto II Software. RPMI medium (1% FBS) was used as control and staurosporine (pro-apoptotic) as positive control.

2.8 mRNA extraction and analysis of PCR products

Total RNA was extracted from the THP-1-derived macrophages using NucleoSpin® RNA II. RNA quality was assessed using the OD260:OD280 ratio determined by a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). One microgram of total RNA was subjected to RT-PCR to obtain cDNA according to the manufacturer's protocol.

The mRNA levels for specific genes were determined using an Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA, USA). For each QPCR, 10 ng of cDNA template was added to Brilliant SYBR Green QPCR Master Mix containing primer pairs for TNF, IL-6, IL-1β, CCL18, C-C chemokine receptor type 2 (CCR2), and chemokine (C-C motif) ligand 2 (CCL2). The reference genes HPRT and GAPDH were used to correct for RNA concentration differences between the samples.

The sequence of and information about the primers that were used in this study are as follows: TNF (NM_000594.3): 5′-TCCTTCAGACACCCTCAACC-3′ and 5′-AGGCCCCAGTTTGAATTCTT-3′ (reverse); IL-6 (NM_001001928): 5′-GTTTGAGGGGGTAACAGCAA-3′ and 5′-GCTAACTGCAGAGGGTGAGG-3′; IL-1β (NM_138712): 5′-GCTGTGCAGGAGATCACAGA-3′ and 5′-GGGCTCCATAAAGTCACCAA-3′; CCL18 (NM_002957): 5′-GGGTTTTCTTCCCTTTCGAG-3′ and 5′-GCGTGTTCCTTTTCCACAAT-3′; CCR2 (NM_001002): 5′-TCGACAATGGCAGCATCTAC-3′ and 5′-ATCCGTCTCCACAGACAAGG-3′; CCL2 (NM_002982.3): 5′-CCCCAGTCACCTGCTGTTAT-3′ and 5′-TGGAATCCTGAACCCACTTC-3′; HPRT (NM_000194.2): 5′-ACCCCACGAAGTGTTGGATA-3′ and 5′-AAGCAGATGGCCACAGAACT-3′; and GAPDH (NM_002046.4): 5′-GAGTCAACGGATTTGGTCGT-3′ and 5′-TTGATTTTGGAGGGATCTCG-3′.

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

2.9 Enzyme-linked immunosorbent assay (ELISA).

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

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

As an indicator of NO production, nitrite (NO_2^-) concentration was measured in the cell culture supernatants. Equal volumes of culture supernatants and Griess reagent were

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

2.11 Migration assays

THP-1-derived macrophages were collected in RPMI-1640 medium containing 10% FBS, and 1% P/S and seeded in 24-well culture plates at 5×10^5 per well to allow high-density adhesion of the macrophages. After removing the floating cells, the adherent cells were incubated at 37°C in 5% CO_2 for 24 h to form a confluent monolayer. The macrophage monolayer was wounded by scratching with a thin pipette tip. The cells were treated with each LPH (I+15A and 15A) and allowed to migrate for 24 h. The migration of THP-1-derived macrophages was examined and quantified by confocal microscopy. Images were captured at the beginning and at 24 h of cell migration to close the wound and compared to quantify the migration rates of the cells.

2.12 Statistical analysis

The data are presented as the mean ± SEM of three independent determinations. Group-wise statistical comparisons were performed by a one-way ANOVA with a post-hoc Bonferroni test. Differences were considered to be significant at $P < 0.05$.

3. Results and discussion

3.1 LPHs does not alter the cellular integrity of THP-1-derived macrophages

Before assessing the anti-inflammatory activity, cell viability and the potential cytotoxicity of the LPHs were evaluated (Gülden & Seibert, 2003).

To investigate whether LPHs may cause a cytotoxic effect, an MTT assay was performed on THP-1-derived macrophages after separately adding each LPHs at increasing

concentrations to conditioned RPMI culture medium for 6 h. The LPHs I+15A and 15A had no significant effects (Fig. 1A and 1B) compared with the untreated control group. Moreover, using flow cytometric analysis to study the DNA distribution in the cell cycle (Sawai & Domae, 2008), we observed that the percentage of S population among THP-1-derived macrophages treated with the LPH I+15A or the LPH 15A was slightly increased (Fig. 1C), but did not reach significance. For the quiescent phase (G_0/G_1) and G_2/M population no differences were found in relation to the control (1% FBS). For all samples, even though a degree of proliferative activity, which was not significant, was observed, the cells remained in the quiescent phase for a longer period. The addition of 10% FBS was used as a positive control.

The contribution of LPHs to the activation of programmed cell death (apoptosis) was also investigated. Interestingly, apoptosis was reduced in the presence of the LPH 60I+15A or the LPH 15A (29 and 35%, respectively) compared with the control (Fig. 1D). This effect seems to endow both LPHs with a protective effect against apoptosis. To complete the feasibility studies of the LPHs, cell viability was assayed by trypan blue exclusion in THP-1-derived macrophages treated with increasing concentrations of the LPH I+15A or the LPH 15A for 24 h. As expected, there was no differences in cell viability after 24 h of incubation in the presence of higher concentrations of the LPH I+15A, which ranged from 100 to 2000 µg/mL, when compared with the control (Fig. 2A). The same pattern was observed after treatment with the LPH 15A (Fig. 2B).

Taken together, these results suggest that in general, LPHs do not compromise the integrity of THP-1-derived macrophages. Although cell viability was decreased upon exposure to higher concentrations, this decrease did not reach 30% cell loss for either of

the LPHs tested. Hence, LPHs did not have any major effect on membrane integrity in the selected cell model.

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

Several diseases, such as obesity-associated insulin resistance, diabetes, and metabolic syndrome, are sustained by chronic subclinical inflammation (Faloia et al., 2012). Elevated levels of cytokines, such as leptin, TNF, IL-1, and IL-6, are generally increased during inflammatory diseases (Hajer, Van Haeften, & Visseren, 2008). A growing body of evidence has shown that biologically active peptides derived from plants can prevent many inflammatory disorders due to the peptides' antioxidant and anti-inflammatory effects (Politis, Theodorou, Lampidonis, Chronopoulou, & Baldi, 2012; Vernaza, Día, & González de Mejía, 2012). Classically activated macrophages (with an M1 phenotype) mediate tissue damage and initiate inflammatory responses by releasing pro-inflammatory mediators (Olefsky & Glass, 2010). These mediators recruit additional macrophages, establishing a feed-forward process that further increases leukocytes content and propagates the chronic inflammatory state (Shen, Lu, Duan, & Duan, 2011). Herein, we first evaluated the ability of LPHs (I+15A and 15A) to modulate the expression of the pro-inflammatory cytokines TNF, IL-1β, and IL-6 in THP-1-derived macrophages (Fig. 3A-C). TNF showed significant inhibition after 6 h of incubation with either hydrolysate. This reduction was markedly pronounced after I+15A treatment (TNF: -45%, IL-1: -32%, and IL-6: -43%). The LPH 15A induced the downregulation of TNF and IL-1 in activated macrophages by 30 and 35%, respectively, whereas IL-6 levels were blunted (70%). No significant differences were observed between the treatments. These results indicate that LPHs tend to decrease the pro-inflammatory

capacity of activated M1 macrophages by diminishing cytokines expression, setting a trend of regulation of the inflammatory process. Macrophage polarization dramatically alters the immune properties of these cells, as evidenced by the potent anti-microbial properties of M1 macrophages compared with the prominent anti-inflammatory tissue repair properties of M2 macrophages (Joshi et al., 2010). Depending on the cytokine microenvironment, the M2 macrophage phenotype is characterized by the expression of cell surface proteins such as CD206 and CD163 and of soluble factors such as CCL18 (Bellón et al., 2011). To corroborate the hypothesis that LPHs have potential anti-inflammatory role, the expression of CCL18 and IL10 (an M2 markers) after 6 h of incubation with the LPHs 15A or the LPH I+15A were evaluated. Surprisingly, both LPHs highly increased CCL18 expression, doubling the value compared with the control (Fig. 3D). However, no significant differences were observed in the IL10 expression In THP-1-derived macrophages after treatment with LPHs (Fig. 3E).

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

3.3 Effect of LPHs on cytokines production

To corroborate the effect of LPHs on cytokines mRNA expression, the concentration of TNF and IL-10 was measured in cell culture supernatants. The production of TNF was decreased by both LPHs (Fig. 4A). This inhibition was higher in LPH I+15A, which inhibited more than 80% TNF production. Regarding IL-10, significant differences were not observed among LPHs and control (Fig. 4B). These data coincided with the ones obtained by quantitative PCR, where THP-1-derived macrophages exhibited attenuated

expression of proinflammatory cytokine TNF, and showed no significant differences in the expression of IL-10.

3.4 Effect of LPHs on the nitrite production

Nitric oxide (NO) has been shown to play a central role in inflammatory and immune reaction activities and macrophages appear to be the main cellular source of NO (Montserrat-de la Paz, Fernández-Arche, Ángel-Martín & García-Giménez, 2012). The effect of LPHs on the release of this inflammatory mediator is depicted in Fig. 5. Both LPHs inhibited approximately 50% of NO production. Other legume protein hydrolysates have shown inhibition of NO production by activated macrophages (Ndiaye et al., 2012).

3.5 LPHs impair the chemotactic capacity of human THP-1-derived macrophages

Previously, Fontanari, Batistuti, da Cruz, Hilario, and Saldiva (2012) investigated the potential hypolipidaemic effect of a total protein extract from *Lupinus albus*, which is associated with a mechanism shared with soya proteins (Duranti et al., 2004; Lovati et al., 2000; Lovati, Manzoni, Gianazza & Sirtori, 1998). Furthermore, soy-based diets have been shown to reduce atherosclerotic lesions through downregulation of the expression levels of monocyte chemokines essential for the initiating events in atherosclerosis, such as monocyte chemoattractant protein-1 (MCP-1) or CCL2. CCL2 have a systemic role in the regulation of metabolism, and particularly in controlling leukocyte extravasation and chemotaxis toward inflamed tissues. For instance, Weisberg et al. (2006) reported a significant reduction in plaque macrophage content in mice lacking CCR2 ($CCR2^{-/-}$ mice). Furthermore, certain evidence has indicated an increase in M1 and decrease in M2 macrophages in obese adipose tissue (Lumeng, Bodzin, & Saltiel, 2007). Interestingly, such a phenotypic switch was not observed in $CCR2^{-/-}$ mice, suggesting that the MCP-

1/CCR2 pathway could contribute to M2 macrophage polarization.

We hypothesized that as occurs with soy proteins, lupine hydrolysates may exert a beneficial effect on the chemotaxis mechanism by modulating the CCR2/CCL2 axis. To examine this issue, the expression of CCR2 and CCL2 in human THP-1-derived macrophages was evaluated after 6 h of incubation with either LPH. As expected, the CCL2/CCR2 axis was noticeably modulated by both LPHs. Fig. 4A shows a dramatic reduction in CCR2 expression by the LPH I+15A (-62%), whereas this expression was more blunted by the LPH 15A (-84%). Despite the significant reduction in CCR2, we could not find any significant difference in the expression of its ligand, CCL2 (Fig. 4B), but did note a decreasing tendency. Accordingly, the migration index was reduced after 24 h of treatment with the LPH I+15A (-44%) or the LPH 15A (-58%). A potent chemotactic agent, fMLP, was used as positive control (Fig. 4C).

Thus far, the results indicate that the addition of LPHs to fully differentiated THP-1 macrophages results in the decreased expression of both CCR2 and CCL2 and, consequently, a decrease in the cells' migration capability. These findings suggest that infiltrating activated macrophages exposed to LPHs may lose their chemotactic ability, which ameliorates the inflammatory state.

In conclusion, after LPH treatment, THP-1-derived-macrophages showed attenuated expression of TNF, IL-6, IL-1β (proinflammatoty cytokines) and increased expression of CCL18 (antiinflammatory). Moreover, NO production was inhibited. Thereby, LPHs displayed hyporeactivity to M1-type ligands and polarization to the M2 phenotype. Furthermore, our data demonstrates that both LPHs attenuate the macrophage migratory response, which is partly mediated by skewing THP-1-derived macrophages toward

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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FIGURE CAPTIONS

Figure 1. Effect of LPHs (500–2000 μg/mL) I+15A (A) and 15A (B) on cell proliferation of human THP-1-derived macrophages determined by MTT assay after 6 h. Study of the DNA distribution in the cell cycle (% G0/ G1, % S and % G2/M) in THP-1- derived macrophages treated with LPH I+15A or LPH 15A (C). Percentage of apoptotic cell death in THP-1-derived macrophages after exposure to LPHs at 500 μg/mL for 24 h (D). Values marked with different letter are significantly different ($P < 0.05$).

Figure 2. Cell viability (%), determined by trypan blue exclusion assay, in presence of LPHs I+15A (A) and 15A (B), after 24 h of treatment and different concentrations (100-2000 μg/mL). Some images obtained with confocal microscopy are presented.

Figure 3. TNF (A), IL-1β (B), IL-6 (C), CCL18 (D), and IL10 (E) mRNA expression in THP-1-derived macrophages after 6 h of treatment with LPHs (I+15A, and 15A). Values marked with different letter are significantly different ($P < 0.05$).

Figure 4. Effect on TNF (A) and IL-10 (B) production of THP-1-derived macrophages. after 48 h of treatment with LPHs (I+15A, and 15A). Values marked with different letter are significantly different ($P < 0.05$).

Figure 5. NO production (%) in THP-1-derived macrophages after 48 h of treatment with LPHs (I+15A, and 15A). Values marked with different letter are significantly different ($P < 0.05$).

Figure 6. CCR2 (A) and CCL2 (B) mRNA expression in THP-1-derived macrophages after 6 h of treatment with LPHs (I+15A, and 15A). Migration Index (C) of THP-1-derived macrophage after 24 h of incubation. Values marked with different letter are significantly different ($P < 0.05$).

Figure 1

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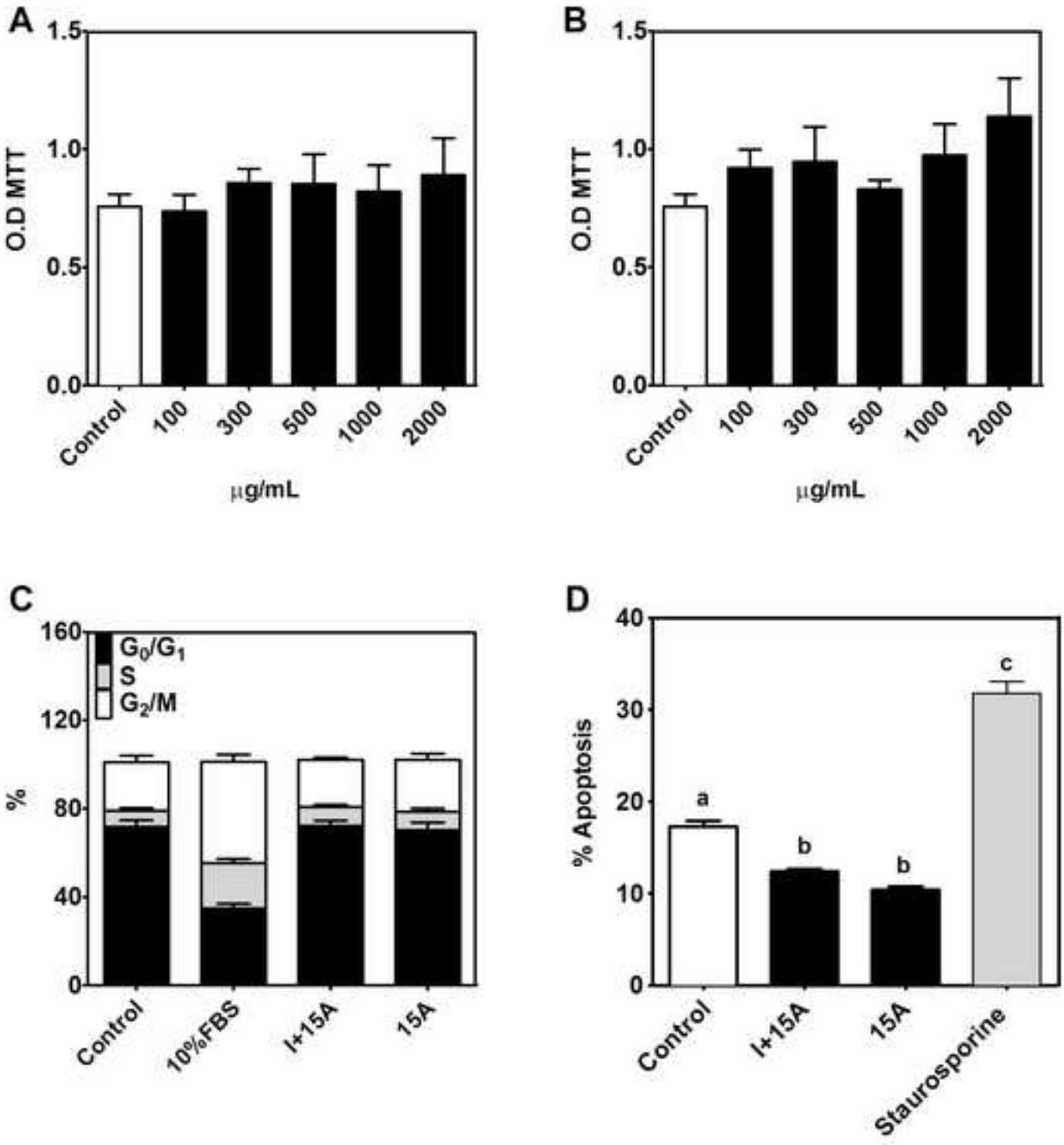

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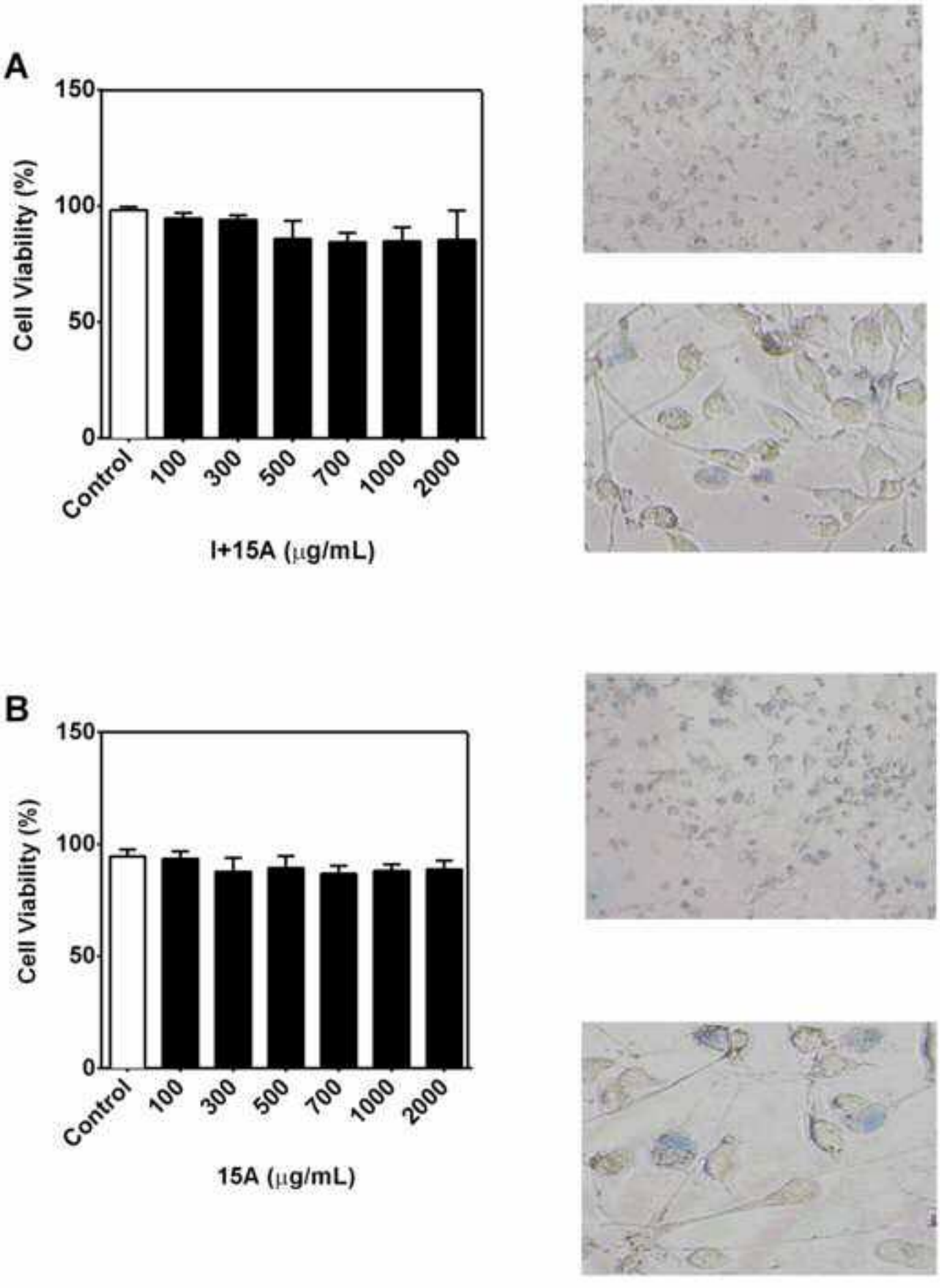

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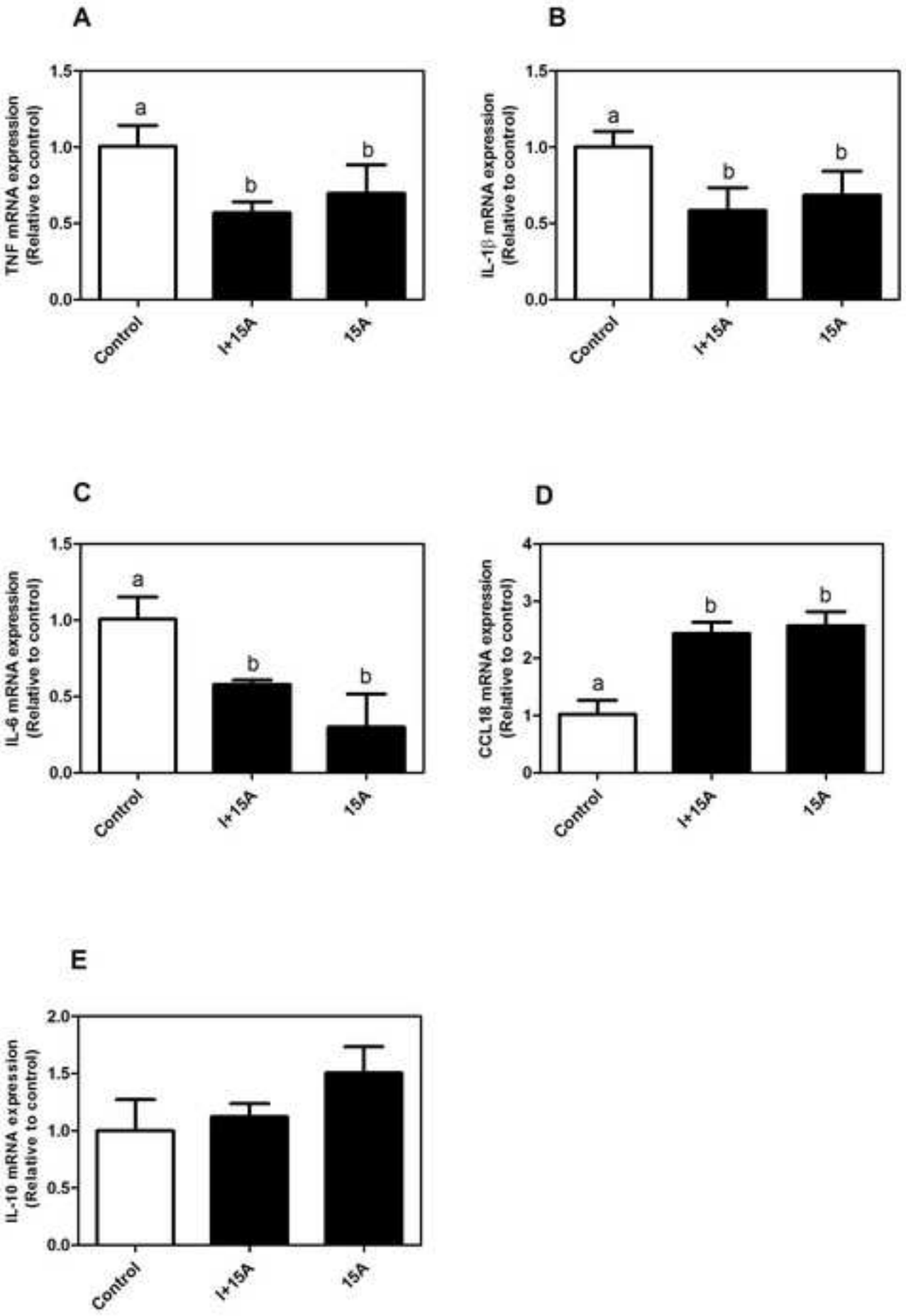

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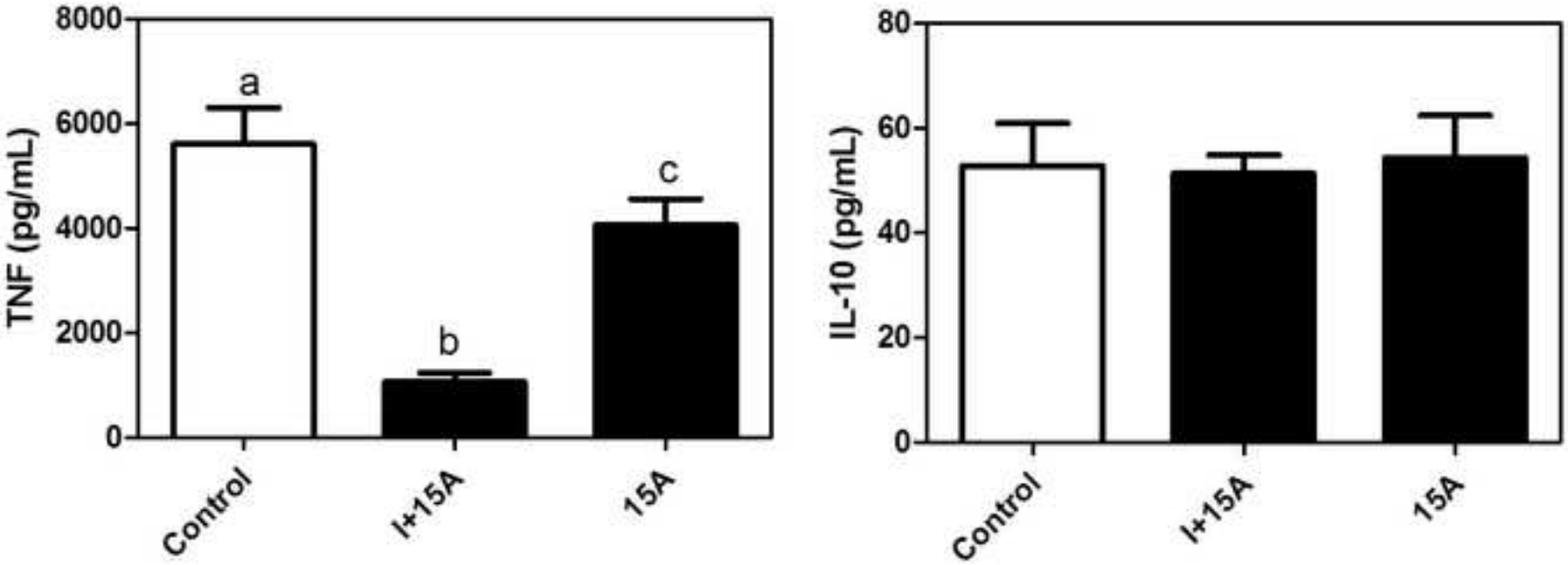

Figure 5

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