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Squalene targets pro- and anti-inflammatory mediators and pathways to modulate over-activation of neutrophils, monocytes and macrophages

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HIGHLIGHTS

1. Squalene has anti-inflammatory activities on LPS-mediated inflammatory response.

2. Squalene targets pro-inflammatory mediators such as TLR4, iNOS, COX-2 or MPO.

3. Squalene enhanced expression of anti-inflammatory enzymes (HO-1).

4. Squalene modulated Nrf2, NF-κB, MAPKs, MMPs and PPARγ signalling pathways.

5. Squalene prevents the over activation of neutrophils/monocytes/macrophages.

ABSTRACT

Squalene is a natural triterpene consumed as an integral part of the human diet. Increasing evidence demonstrates that squalene has antioxidant, cardioprotective and anti-carcinogenic activities. Nevertheless, its anti-inflammatory properties remain unclear. The effects of squalene on lipopolysaccharide (LPS)-mediated inflammatory response in murine macrophages and human monocytes and neutrophils were investigated. Squalene reduced intracellular levels of ROS, nitrites, cytokines (TNF-α, IL-1β, IL-6 and IFN-γ) and pro-inflammatory enzymes (iNOS, COX-2 and MPO), including a decreased expression of TLR4 and key proteins for signalling pathways mediated by NF-κB (IκBα), MAPKs (JNK) and MMPs (1, 3 and 9). In addition, squalene enhanced expression levels of anti-inflammatory enzymes (HO-1) and transcription factors (Nrf2 and PPARγ). This study establishes that squalene has significant potential for management of inflammatory conditions characterized by an over-activation of neutrophils/monocytes/macrophages and thereby for the efficient termination of the inflammatory response.

Keywords:

Squalene

Inflammation

Macrophages

Monocytes

Neutrophils

1. Introduction

Squalene (2,6,10,15,19,23-hexamethyl tetracosahexaene) is a natural lipid belonging to the terpenoid family, containing six isoprene units, and a biochemical precursor of cholesterol biosynthesis and other biological substances. Besides, to be synthesized in the liver and the skin of human, squalene is widely present in nature, especially in wheat germ, rice bran, shark liver and olive oils. Therefore, it is consumed as an integral part of the human diet. Up to date, anticancer, antioxidant, drug carrier, detoxifier, skin hydrating and emollient activities of squalene have been reported both in animal models and *in vitro* environments (Kim & Karadeniz, 2012; Reddy & Couvreur, 2009). In fact, squalene is a highly effective antioxidant acting as a direct reactive oxygen species (ROS) scavenging agent reducing intracellular oxidant stress and also protects human skin surfaces from lipid peroxidation as a quencher of singlet oxygen (Kabuto, Yamanushi, Janjua, Takayama, & Mankura, 2013). Moreover, this natural isoprenoid has been shown to ameliorate atherosclerotic lesions through the reduction of CD36 scavenger receptor expression in macrophages (Granados-Principal et al., 2012), in addition to important cardioprotective effects in several experimental models mainly by blocking lipid peroxidation (Lou-Bonafonte, Arnal, Navarro, & Osada, 2012; Sabeena Farvin et al., 2004). However, although the importance as a powerful antioxidant agent of squalene has been largely described (Kim & Karadeniz, 2012), its anti-inflammatory properties still remain unclear.

Monocytes and macrophages are closely related to phagocytic cells that cooperate during the onset, progression and resolution of inflammation (Soehnlein & Lindbom, 2010). Pro-inflammatory, metabolic and immune stimuli increase recruitment of monocytes from blood vessels to peripheral sites where the differentiation into macrophages and dendritic cells occurs contributing to host defence, tissue remodelling

and repair (Gordon & Taylor, 2005; Shi & Pamer, 2011). Furthermore, neutrophils, as a key component of the inflammatory response, generate chemotactic signals that attract monocytes and dendritic cells, and influence whether macrophages differentiate to a predominantly pro- or anti-inflammatory state (Nathan, 2006). In these cell types, lipopolysaccharide (LPS) acts as an endotoxin by its binding to the CD14/TLR4/MD2 receptor complex, which promotes the secretion of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α), interleukin (IL)-8, IL-6 or interferon gamma (IFN)-γ among others (Chanput, Mes, Vreeburg, Savelkoul, & Wichers, 2010). Moreover, some transcription factors such as the peroxisome proliferator-activated receptor gamma (PPARγ), nuclear transcription factor (NF)-kB, nuclear factor-E2-related factor-2 (Nrf2) or mitogen-activated protein kinase (MAPK) family (Jung et al., 2010; Kang & Kim, 2013) have been shown to be major effectors in the LPS-induced inflammatory response through the induction of several pro-inflammatory enzymes including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX)-2 (Takahashi, Kozaki, Yatabe, Achiwa, & Hida, 2002) or antioxidant enzymes, such as heme oxygenase 1 (HO-1) (Alexander, Mathie, & Peters, 2011). Importantly, matrix metalloproteinases (MMPs) regulate aspects of immune cell development, effector function, migration and ligand–receptor interactions and activate signal transduction pathways that control cytokine biosynthesis and direct systemic inflammation or barrier immunity. For this reason, the stimulation with LPS constitutes an excellent model for the screening and subsequent evaluation of the effects of candidate drugs on the inflammatory pathway (Sanchez Miranda, Perez Ramos, Fresan Orozco, Zavala Sanchez, & Perez Gutierrez, 2013).

Therefore, we investigated the anti-inflammatory activity of squalene on LPS-stimulated murine peritoneal macrophages and human blood monocytes and

neutrophils. In this model, redox changes, myeloperoxidase (MPO) activity, pro-inflammatory (IL-1β, IL-6, TNF-α and IFN-γ) and anti-inflammatory (IL-10) cytokines production, gene/protein expression of pro-inflammatory (iNOS and COX-2) and anti-inflammatory (HO-1) enzymes were measured. NF-kB, Nrf2, MAPKs, MMPs (MMP-1, MMP-3 and MMP-9) and PPARγ signalling pathways were also explored to understand the underlying mechanisms by which squalene may prevent inflammation.

2. Materials and methods

2.1. Murine peritoneal macrophage isolation

Thirty 8–10 weeks-old male Swiss mice (Harlan Interfauna Ibérica, Barcelone, Spain) weighing 20-30 g were injected intraperitoneally with 1 ml of sterile thioglycollate medium (10 g w/v) (Scharlau, Barcelone, Spain). After 3 days, murine peritoneal macrophages were isolated as described previously (Cardeno, Sanchez-Hidalgo, Aparicio-Soto, & Alarcon-de-la-Lastra, 2014). Cells were treated with 5 μg/ml LPS from *E. coli* (Sigma-Aldrich, Madrid, Spain) in presence or absence of squalene (12.5, 25 and 50 μM) for 18 h. All animal care and experimental procedures complied with the Guidelines of the European Union regarding animal experimentation (Directive of the European Counsel 86/609/EC), followed a protocol observed by the Animal Ethics Committee of the University of Seville and reported in accordance with the ARRIVE guidelines for reporting experiments involving animals.

2.2. Human monocyte and neutrophil isolation

Study subjects were recruited at Virgen del Rocio University Hospital, Seville, Spain. Venous blood were obtained from healthy adult volunteers (<35 years old) non-smokers and not taking any medication. Donors were recognised as healthy, according to medical history and routine laboratory test. Neutrophils were isolated by dextran

sedimentation (2% dextran/0.9% NaCl) coupled to differential centrifugation over Histopaque 1077 (Sigma-Aldrich, Madrid, Spain). Residual erythrocytes were removed using hypotonic lysis with 0.2% and 1.6% saline solutions. The purity of neutrophils preparation was >97% by trypan blue exclusion. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over Ficoll Histopaque (Sigma-Aldrich, Madrid, Spain) gradient and monocytes isolated from PBMCs using positive selection with CD14 MicroBeads according to the manufacturer's instructions (MACS, Myltenyi Biotec, Madrid, Spain). Monocytes were tested for purity by CD14 fluorescein isothiocyanate labeling and fluorescence-activated cell sorter analysis using a FACScanto II flow cytometer and FACSDiva software (BD) (Varela et al., 2011). Following isolation, the cells were suspended in a RPMI 1640 medium supplemented with L-glutamine, penicillin, streptomycin and 1% heat-inactivated fetal bovine serum. Neutrophils were seeded at a density of $3x10^6$ cells/ml and monocytes at $5x10^5$ cells/ml. Cells were treated with 0.1 μg/ml LPS in presence or absence of squalene (50 μM) for 18 h. The study conformed to the principles outlined in the Helsinki Declaration of the World Medical Association.

2.3. Reagents

Squalene was purchased by Sigma-Aldrich and was always freshly prepared as stock solutions in dimethyl sulphoxide (DMSO) (Panreac, Barcelona, Spain) and diluted to desired concentration in the culture medium. The assayed concentrations of squalene in murine macrophages were 12.5, 25 and 50 μM. Squalene concentration in human monocytes and neutrophils (50 μM) was selected based on the previous result obtained in murine macrophages. The final concentration of DMSO in the culture medium was ≤1% in all experiments and it had not significantly influence cell response (data not shown).

2.4. Cell viability

Cells seeded in 96-well plates ($1x10^5$ cells/well) were incubated in presence or absence of different squalene concentrations for 18 h. At the end of the exposure time, the effect on cell growth/viability was analysed by sulphorhodamine B (SRB) (Sigma-Aldrich) assay (Skehan et al., 1990). Cell survival was measured as the percentage of absorbance compared with that obtained in control cells (non-treated cells).

2.5. Measurement of intracellular ROS

Intracellular ROS production was measured using 2',7'-dichlorfluorescein-diacetate (DCFH-DA) (Sigma-Aldrich, St Louis, MO, USA). DCFH-DA penetrates into the cells and is hydrolysed by intracellular esterases to the non-fluorescent 2,7-dichlorofluorescein (DCFH), which can be rapidly oxidized to the highly fluorescent 2,7-dichlorofluorescein (DCF) in the presence of ROS. Peritoneal macrophages were seeded at $1x10^6$ cells/well in 24-well plates, incubated with or without squalene and after 30 min treated with LPS for 18 h. The fluorescence intensity was measured as described previously (Cardeno et al., 2014). Results were expressed as intracellular ROS production percentage compared with LPS control cells (stimulated LPS-treated cells). H_2O_2 (100 μM) (30% pure) (Panreac, Barcelona, Spain) was used as pro-oxidant positive control.

2.6. Measurement of nitrite production

Peritoneal macrophages in 24-well plates were incubated in presence or absence of squalene (12.5, 25 or 50 μM), and 30 min later treated with LPS for 18 h. The culture supernatants (100 μl) were transferred to a 96-well assay plate mixed with Griess reagent (Sigma, St Louis, MO, USA). The amount of nitrite, as an index of NO generation, was determined by a spectrophotometric method using the Griess reaction (Moorcroft, Davis, & Compton, 2001) and obtained by extrapolation from a standard

curve with sodium nitrite. The absorbance at 540 nm was measured by an enzyme-linked immunosorbent assay reader (BioTek, Bad Friedrichshall, Germany). Results were expressed as the nitrite production percentage compared with LPS control cells (stimulated untreated cells). Dexamethasone 1 μM (Sigma-Aldrich) was used as a positive control (data not shown).

2.7. Isolation and immunoblotting detection of cytoplasmic and nuclear proteins

Peritoneal macrophages (1×10^6 cell/ml) were incubated in presence or absence of squalene and treated with LPS for 18 h. Then, cells were rinsed, scraped off and collected in ice-cold PBS containing a cocktail of protease and phosphatase inhibitors and processed as described (Sanchez-Hidalgo, Martin, Villegas, & Alarcon De La Lastra, 2005) in order to isolate cytoplasmic or nuclear proteins. Protein concentration was measured for each sample using a protein assay reagent (BioRad, Hercules, CA, USA) according to the Bradford´s method and using γ-globulin as a standard (Bradford, 1976). Aliquots of supernatant contains equal amount of protein (20 μg) were separated on 10% acrylamide gel by sodium dodecyl sulphate polyacrylamide gel electrophoresis. In the next step, the proteins were electrophoretically transferred into a nitrocellulose membrane and incubated with specific primary antibodies: rabbit anti-COX-2 and rabbit anti-iNOS (Cayman, Ann Arbor, MI, USA) (1:2500 and 1:1000, respectively), rabbit anti-inhibitory NF-κB protein alpha (IκBα) (Cell Signalling, Danvers, MA, USA) (1:1000), rabbit anti-p65, mouse anti-phosphorylated c-Jun N-terminal kinases (JNK), rabbit anti-JNK, mouse anti-phosphorylated p38, rabbit anti-p38 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:1000) and rabbit anti-Nrf2 (Santa Cruz Biotechnology) (1:500), overnight at 4ºC. After rinsing, the membranes were incubated with a horseradish peroxidase-labelled (HRP) secondary antibody anti-rabbit (Cayman Chemical, Ann Arbor, MI, USA) (1:50000) or anti-mouse (Dako, Atlanta, GA, USA)

(1:2000) containing blocking solution for 1-2 h at room temperature. To prove equal loading, the blots were analysed for β-actin expression using an anti-β-actin antibody (Sigma-Aldrich). Immunodetection was performed using enhanced chemiluminescence light-detecting kit (Pierce, Rockford, IL, USA). The signals were captured using LAS-3000 Imaging System from Fujifilm Image Reader (Stamford, CT, USA) and densitometry data were studied following normalization to the housekeeping loading control. The signals were analysed and quantified by an Image Processing and Analysis in Java (Image J, Softonic) and expressed in relation to the LPS-treated cells.

2.8. RNA isolation and quantitative real-time PCR analysis

Total RNA was extracted from human neutrophils and monocytes by using Trisure Reagent (Bioline, London, UK), as instructed by the manufacturer. RNA quality was assessed by A_{260}/A_{280} ratio in a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington DE, USA). RNA (1 μg) was subjected to reverse transcription according to the manufacturers' protocol (iScript cDNA synthesis kit, BioRad). An amount of 20 ng of the resulting cDNA was used as a template for real-time PCR amplifications. The mRNA levels for specific genes were determined by real-time PCR in a MX3000P system (Stratagene, Madrid, Spain). For each PCR reaction, cDNA template was added to Brilliant SYBR green QPCR Supermix (BioRad) containing the primer pairs for either gene or for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase (HPRT) as housekeeping genes. The sequence and information for the primers used in this study are in Supporting Information Table 1S. All amplification reactions were performed in triplicate and average threshold cycle (Ct) numbers of the triplicates were used to calculate the relative mRNA expression of candidate genes. The magnitude of change of mRNA expression for candidate genes was calculated by using the standard $2^{-(\Delta\Delta Ct)}$ method. All

data were normalized to endogenous reference (GAPDH and HPRT) gene content and expressed as percentage of controls.

2.9. Statistical evaluation

All values in the figures and text are expressed as arithmetic means ± standard error (S.E.M). Experiments were carried out in triplicate. Data were evaluated with Graph Pad Prism Version 5.01 software (San Diego, CA, USA). The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA); using Tukey multiple comparisons test as post hoc test. *P* values of <0.05 were considered statistically significant. In the experiments involving densitometry, the figures shown are representative of at least three different experiments performed on different days.

3. Results

3.1. Effect of squalene on cell viability of murine peritoneal macrophages and human monocytes and neutrophils

After 18 h of treatment, squalene at concentrations up to 50 μM had no significant effect on viability of murine peritoneal macrophages (by means of SRB assay) and human monocytes or neutrophils (by means of Trypan Blue exclusion test) (data not shown).

3.2. Squalene functions as antioxidant in LPS-treated murine peritoneal macrophages

ROS are suggested to be signalling messengers in LPS-mediated inflammatory response (Brune et al., 2013). Thus, we tested the effects of squalene on LPS-induced intracellular ROS production in murine peritoneal macrophages using the fluorescent probe DCFH-DA, which can be oxidized to the highly fluorescent compound DCF. As

shown in Fig. 1A, cells incubated with 50 μM of squalene for 18 h caused a significant decrease ($P < 0.05$ vs. LPS-control) in intracellular ROS.

To deep insight into the intracellular mechanisms for squalene antioxidant activity, we investigated the protein expression of Nrf2, which is a key transcription factor that regulates the cellular antioxidant response (Jaiswal, 2004). As shown in Fig. 1B, LPS induced a reduction of Nrf2 protein expression in murine peritoneal macrophages; however, the incubation with 50 μM of squalene for 18 h caused a significant increase of Nrf2 protein expression in LPS-treated cells ($P < 0.01$ vs. LPS-control), reaching a level comparable with that of untreated cells.

3.3. Squalene counteracts nitrite production and suppresses iNOS and COX-2 protein expression in LPS-treated murine peritoneal macrophages

It is known that LPS may induce NO synthesis and release in murine macrophages (Gordon & Taylor, 2005). Our study demonstrated a marked increase of nitrites as an indicator of NO production in the medium of LPS-treated murine peritoneal macrophages; however, this effect was almost abrogated by squalene ($P < 0.001$ vs. LPS-control, Fig. 1C). Accordingly, the incubation with 25 and 50 μM of squalene for 18 h elicited a significant reduction of iNOS protein expression in LPS-treated cells ($P < 0.05$ and $P < 0.01$ vs. LPS-control, respectively; Fig. 1D). We also investigated the potential effect of squalene on COX-2. As shown in Fig. 1D, LPS induced an increase of COX-2 protein expression in murine peritoneal macrophages; however, the incubation with 25 and 50 μM of squalene for 18 h caused a significant reduction of COX-2 protein expression in LPS-treated cells ($P < 0.05$ and $P < 0.01$ vs. LPS-control, respectively) with a very similar pattern to that observed for iNOS protein expression.

3.4. Squalene modulates MAPK and NF-κB signalling pathways in LPS-treated murine peritoneal macrophages

The expression of many inflammatory mediators such as iNOS and COX-2 is known to be mediated by MAPKs (Kaminska, 2005). Therefore, to further explore the molecular mechanism underlying the ability of squalene to minimize pro-inflammatory signals, we next investigated whether squalene can target the activity of MAPK pathways. As shown in Fig. 2A, LPS induced an increase of phosphorylated JNK and p38 MAPK expression in murine peritoneal macrophages; however, the incubation with 25 and 50 μM of squalene for 18 h caused a significant decrease of only phosphorylated JNK ($P < 0.05$ vs. LPS-control) but not p38 MAPK expression in LPS-treated cells, reaching a level comparable with that of untreated cells. Besides, NF-κB is one of the key regulators of the cellular responses to oxidative stress in mammalian cells (Helenius, Kyrylenko, Vehviläinen, & Salminen, 2001). Analogously to MAPKs, the NF-κB transcriptional system is a major effector pathway involved in inflammation and its activation may be mediated by LPS (Andreakos et al., 2004). As shown in Fig. 2B, LPS induced a decrease of the inhibitory protein IκBα in the cytoplasm but an increase of p65 NF-κB protein in the nucleus of murine peritoneal macrophages; however, the incubation with 25 and 50 μM of squalene for 18 h caused a significant increase of IκBα ($P < 0.05$ vs. LPS-control), but a significant decrease of p65 in LPS-treated cells ($P < 0.05$ vs. LPS-control), reaching levels comparable with those of untreated cells. Squalene also reduced mRNA levels of NF-κB downstream genes such as TNF-α (-78.5%, $P < 0.001$) and IL-1β (-88.1%, $P < 0.001$) in LPS-treated cells.

3.5. Squalene down-regulates MPO and up-regulates HO-1 gene expression in LPS-treated human monocytes and neutrophils

MPO is a human protein stored in the azurophilic granules of neutrophils and in the lysosomes of monocytes (de Araujo et al., 2013). MPO is released upon LPS-activation to catalyse the formation of a powerful oxidant such as hypochlorous acid, which is a

reactive chlorine species that accumulates at sites of chronic inflammation (M. Liu et al., 2014). As shown in Fig. 3A, the mRNA level of MPO was down-regulated in LPS-treated human monocytes and neutrophils after the incubation with 50 μM of squalene for 18 h ($P < 0.001$ vs. LPS-control). On the contrary, the anti-inflammatory gene HO-1 was up-regulated by squalene both in LPS-treated human monocytes and in LPS-treated human neutrophils ($P < 0.05$ and $P < 0.001$ vs. LPS-control, respectively; Fig. 3B).

3.6. Squalene down-regulates gene expression of TLR4 and pro-inflammatory cytokines in LPS-treated human monocytes and neutrophils

The stimulation of TLR4 by LPS induces the release of critical pro-inflammatory and immunoregulatory cytokines that are crucial to potently activate the innate immune response (Chang, Kim, & Chang, 2014). Our study shows a down-regulation of TLR4 gene expression in LPS-treated human leukocytes, but more markedly in monocytes after the incubation with 50 μM of squalene for 18 h ($P < 0.001$ vs. LPS-control, Fig. 4A). The expression of the pro-inflammatory cytokine genes TNF-α and IL-1β but not IL-6 or IL-10 was significantly reduced by squalene (50 μM, 18 h) in LPS-treated human monocytes ($P < 0.001$ and $P < 0.01$, respectively vs. LPS-control; Fig. 4B). Moreover, the expression of cytokine genes TNF-α, IL-1β, IL-6 and IFN-γ was significantly reduced by squalene (50 μM, 18 h) in LPS-treated human neutrophils ($P < 0.01$, $P < 0.001$, $P < 0.01$, $P < 0.001$ vs. LPS-control, respectively; Fig. 4B).

3.7. Squalene down-regulates overexpressed iNOS and COX-2 enzymes in LPS-treated human monocytes and neutrophils

We also investigated the influence of squalene on gene expression of enzymes related to LPS-induced inflammatory response in human monocytes and neutrophils. As shown in Fig. 5, the incubation with 50 μM of squalene for 18 h caused a marked down-regulation of iNOS and COX-2 gene expression in LPS-treated human monocytes ($P <$

0.001 vs. LPS-control), whereas only iNOS ($P < 0.001$ vs. LPS-control) but not COX-2 gene expression was significantly down-regulated in LPS-treated human neutrophils.

3.8. Squalene down-regulates MMPs and up-regulates PPARγ gene expression in LPS-treated human monocytes and neutrophils

MMPs regulate aspects of immune cell development, effector function, migration and ligand–receptor interactions, which are all relevant for persistent inflammatory response (Khokha, Murthy, & Weiss, 2013). Therefore, we investigated the influence of squalene on gene expression of three major MMPs (MMP-1, MMP-3 and MMP-9). As PPARγ agonists have been shown to inhibit LPS-mediated MMPs and pro-inflammatory cytokines (Renga et al., 2011; Wang et al., 2011), our study also undertook to explore whether squalene can affect PPARγ gene expression in LPS-treated cells. As shown in Fig. 6A, the incubation with 50 μM of squalene for 18 h caused a significant down-regulation of MMP-1 and MMP-9 gene expression in LPS-treated human monocytes ($P < 0.001$ and $P < 0.01$ vs. LPS-control, respectively), and of MMP-1 and MMP-3 gene expression in LPS-treated human neutrophils ($P < 0.001$ vs. LPS-control). These findings were also supported by a significant up-regulation of PPARγ gene expression in LPS-treated human monocytes and neutrophils after the incubation with 50 μM of squalene for 18 h ($P < 0.001$ vs. LPS-control, Fig. 6B).

4. Discussion

Herein, we elucidate for first time the underlying mechanisms by which the natural isoprenoid squalene exhibits antioxidant and anti-inflammatory effects on LPS-activated murine peritoneal macrophages, human monocytes and neutrophils.

Macrophages play a central role in inflammatory response controlling key events in the initiation and resolution processes. LPS-stimulated macrophages have disrupted the balance of the intracellular reduction-oxidation state, which is usually accompanied by a

ROS-mediated damage that mediates the progression of inflammatory disorders (Kang & Lee, 2012). Therefore, modulators of ROS production and ROS-induced signalling pathways could represent potential targets for anti-inflammatory intervention (K. J. Kim, Yoon, & Lee, 2012). Our study demonstrated that squalene has the capacity to inhibit ROS intracellular generation and to activate Nrf2 signalling by the increase of total Nrf2 protein expression (Gjyshi et al. 2014); Nrf2 is a master regulator of cellular antioxidant processes against ROS that has a cytoprotective role against acute inflammatory response (Jung et al., 2010). Activated macrophages are also known to stimulate the expression of a number of inflammatory mediators that include iNOS and COX-2 (Epelman, Lavine, & Randolph, 2014). NO acts as an intracellular messenger in modulating the formation of endogenous ROS such as hydrogen peroxide and peroxynitrite that orchestrate inflammatory responses (Li, Xue, Geng, & Chen, 2012). Moreover, COX-2 activity also leads to ROS production inducing tissue damage (O'Connor, Lapointe, Beck, & Buret, 2010). We found that LPS-mediated increase of iNOS and COX-2 activity was markedly attenuated by squalene in murine peritoneal macrophages. Collectively, these data suggest that squalene may dampen intracellular oxidant stress in response to inflammatory stimulus via blocking iNOS, COX-2 and Nrf2 signalling pathways.

MAPKs are essential for both induction and propagation of LPS-mediated inflammatory response in macrophages (Radnai et al., 2009). MAPKs include extracellular signal-regulated kinases (ERK-1 and -2), JNKs and p38 (Gurgis, Ziaziaris, & Munoz, 2014). Previous studies have recognized that JNK and p38 MAPK are primarily involved in LPS-induced expression of iNOS and COX-2 in macrophages (Kyriakis & Avruch, 2012; Kwon et al. 2013). In the present study, we found that LPS-mediated phosphorylation of JNK, but not p38 MAPK, was markedly attenuated by squalene in

murine peritoneal macrophages. Taking NF-κB as a dimeric redox-sensitive transcription factor that is downstream of MAPKs signalling, binds to the promoter of a vast collection of inducible pro-inflammatory genes including iNOS and COX-2 (Lee & Surh, 2012; Tak & Firestein, 2001) and coordinates its activity with Nrf2 (Cardozo et al., 2013), we also analysed the effect of squalene on phosphorylation of IκB proteins. IκBs are a family of related proteins required for cytoplasmic sequestration of five NF-κB member subunits [RelA/p65, RelB, c-Rel, NF-kB1 (p50/p105) and NF-kB2 (p52/p100)] as an inactive complex in unstimulated cells; the major IκB protein is IκBα (Tak & Firestein, 2001). Consistently, our data show that squalene induced a reduction of IκBα phosphorylation and degradation, decreasing p65-NF-κB levels and gene expression of NF-κB target genes (TNF-α and IL-1β) in LPS-activated murine peritoneal macrophages. Therefore, squalene emerges as a potentially important molecule for modulating inflammatory processes involving JNK and NF-κB signalling pathways.

Recent evidence suggests that the heme-enzyme MPO, whose expression remains restricted to neutrophils and monocytes, is a critical determinant for the course of inflammation (Liu et al., 2014; Nussbaum, Klinke, Adam, Baldus, & Sperandio, 2013). MPO catalyses via the MPO-hydrogen peroxide-halide system the production of highly reactive intermediates such as hypochlorous acid that interfere with various cell functions; MPO also has extracatalytic properties that contribute to injury in inflamed tissues. The present study demonstrates that LPS-mediated increase of MPO expression was markedly attenuated by squalene in human neutrophils and monocytes. This finding is in accordance with the above described antioxidant and anti-inflammatory effects of squalene in murine peritoneal macrophages. Consistent with this activity, squalene also induced HO-1 up-regulation in LPS-activated human neutrophils and monocytes. HO-1

is an Nrf2-regulated gene and its inducible expression has been described as a host defence mechanism that protects against oxidative stress and contributes to the anti-inflammatory activity (Araujo, Zhang & Yin, 2012).

Recognition of LPS by mainly TLR4 initiates several signalling cascades leading to the activation of NF-κB and MAPK pathways that mediate the expression of inflammatory cytokines and transactivation of pro-inflammatory enzymes such as iNOS and COX-2 in cells of innate system (Chang et al., 2014; Murad, 2014). Our study shows that squalene abrogated gene expression of TLR4, TNF-α, IL-1β, IL-6, IFN-γ, iNOS and COX-2 in LPS-activated human neutrophils and monocytes. All of these effects are likely contributing to the idea of squalene as a disruptor of TLR mediated pro-inflammatory responses in several types of inflammatory cells.

MMPs, particularly LPS-induced interstitial collagenase-1 (MMP-1), stromelysin-1 (MMP-3) and gelatinase B (MMP-9), have been reported to be involved in inflammation by degrading components of the extracellular matrix and regulating cytokine signalling through coupling with COX-2 and NF-κB/MAPK pathways (Lai, Zhou, Shankavaram, Peng, & Wahl, 2003; Nissinen & Kahari, 2014; Steenport et al., 2009). Our results demonstrate that squalene repressed gene expression of MMP-1 and MMP-3 in LPS-activated human neutrophils but MMP-1 and MMP-9 in LPS-activated human monocytes, revealing another mechanism by which squalene can supress inflammation probably at the level of the transmigration, recruitment or influx of inflammatory cell subsets (Nissinen & Kahari, 2014). These findings are in accordance with the above-described effects of squalene on COX-2 gene expression in human monocytes, and COX-2 protein expression and NF-κB/MAPK pathways in murine peritoneal macrophages. We further demonstrate that squalene induced PPARγ up-regulation in LPS-activated human neutrophils and monocytes. PPARγ is known to

modulate oxidative stress-sensitive and NF-κB pathways (Bordet, Gele, Duriez, & Fruchart, 2006), and plays a pivotal role in the dynamic balance among overall matrix synthesis, deposition and degradation by inhibiting MMP expression in inflamed tissues (Jeon et al., 2014).

While the cardioprotective effects of squalene have been largely described and discussed (M. Liu et al., 2014; Y. Liu et al., 2009; Ostlund, Racette, & Stenson, 2002; Scolastici, Ong, & Moreno, 2004; Zhang, Yeung, Huang, & Chen, 2002) the hypothesis of squalene as anti-inflammatory compound remains almost unexplored. However, it has been recorded that the unsaponifiable fraction of virgin olive oil, which contains 40% of squalene, has anti-inflammatory effects in vitro in murine macrophages (Cardeno et al., 2014) and prevented in vivo acute colitis in mice (Sanchez-Fidalgo et al., 2012). It is therefore important to investigate the possible beneficial effects of squalene as a non-synthetic anti-inflammatory dietary complement. Although, the major weakness of this study is that only *in vitro* effects of squalene in murine and human inflammatory cells were addressed, it does provide the first evidence that squalene has antioxidant and anti-inflammatory activities by mechanisms targeting pro-inflammatory (iNOS, COX-2, NF-κB, MAPKs, MPO, TLR4, MMPs) and anti-inflammatory (Nrf2, HO-1, PPARγ) mediators and pathways in closely related phagocytic cells that cooperate during the onset, progression and resolution of inflammation.

5. Conclusion

Taken together, our results suggest that squalene has significant potential for management of inflammatory conditions characterized by an over-activation of neutrophils/monocytes/macrophages and thereby for the efficient termination of the inflammatory response.

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Conflicts of interest

The authors state no conflict of interest.

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Figure Legends

Fig. 1 - Squalene functions as antioxidant in LPS-treated murine peritoneal macrophages. Cells were incubated with squalene (SQ) (12.5, 25 or 50 μM) for 30 min and then treated with LPS for 18 h; U, untreated cells; C, LPS-treated control cells. (A) Intracellular ROS production. DCF fluorescence intensity values are expressed as percentage of LPS-control. H_2O_2 was used as pro-oxidant positive control. Data shown are means ± S.E.M. (n = 3). *P < 0.05 vs. LPS-control. (B) Densitometry analysis of Nrf2 protein expression. β-actin housekeeping protein was served for normalization. The plot represents band intensities measured by Image J software. Data shown are means ± SEM (n = 3). *P < 0.01 vs. LPS-control. (C) Nitrite generation. Values are expressed as percentage of LPS-control. ***P < 0.001 vs. LPS-control. (D) Densitometry analysis of iNOS and COX-2 protein expression. β-actin housekeeping protein was served for normalization. The plot represents band intensities measured by Image J software. Data shown are means ± SEM (n = 3). *P < 0.05 and **P < 0.01 vs. LPS-control.

Fig. 2 - Squalene modulates MAPK and NF-κB signalling pathways in LPS-treated murine peritoneal macrophages. Cells were incubated with squalene (SQ) (12.5, 25 or 50 μM) for 30 min and then treated with LPS for 18 h; U, untreated cells; C, LPS-treated control cells. (A) Densitometry analysis of phosphorylated-JNK (pJNK) vs. JNK and of phosphorylated-p38 (pp38) vs. p38 protein expression. (B) Densitometry analysis of nuclear p65 and cytoplasmic IκBα protein expression. β-actin housekeeping protein was served for normalization. The plots represent band intensities measured by Image J software. Data shown are means ± SEM (n = 3). *P < 0.05 vs. LPS-control.

Fig. 3 - Squalene down-regulates MPO and up-regulates HO-1 gene expression in LPS-treated human monocytes and neutrophils. Cells were treated with squalene (SQ) and LPS as indicated for 18 h. (A) qRT-PCR analysis of MPO mRNA expression. (B) qRT-PCR analysis of HO-1 mRNA expression. GAPDH and HPRT housekeeping genes were served for normalization. Values are expressed as percentage of LPS-control. Data shown are means ± SEM (n = 3). *P < 0.05 and ***P < 0.001 vs. LPS-control.

Fig. 4 - Squalene down-regulates gene expression of TLR4 and pro-inflammatory cytokines in LPS-treated human monocytes and neutrophils. Cells were treated with squalene (SQ) and LPS as indicated for 18 h. (A) qRT-PCR analysis of TLR4 mRNA expression. (B) qRT-PCR analysis of TNF-α, IL-1β, IL-6, IL-10 and IFN-γ mRNA expression. GAPDH and HPRT housekeeping genes were served for normalization. Values are expressed as percentage of LPS-control. Data shown are means ± SEM (n = 3). **P < 0.01 and ***P < 0.001 vs. LPS-control.

Fig. 5 - Squalene down-regulates gene expression of iNOS and COX-2 in LPS-treated human monocytes and neutrophils. Cells were treated with squalene (SQ) and LPS as indicated for 18 h and qRT-PCR analysis of iNOS and COX-2 mRNA expression was evaluated. GAPDH and HPRT housekeeping genes were served for normalization. Values are expressed as percentage of LPS-control. Data shown are means ± SEM (n = 3). ***P < 0.001 vs. LPS-control.

Fig. 6 - Squalene down-regulates MMPs and up-regulates PPARγ gene expression in LPS-treated human monocytes and neutrophils. Cells were treated with squalene (SQ) and LPS as indicated for 18 h. (A) qRT-PCR analysis of MMP-1, MMP-3 and MMP-9 mRNA expression. (B) qRT-PCR analysis of PPARγ mRNA expression. GAPDH and HPRT housekeeping genes were served for normalization. Values are expressed as

percentage of LPS-control. Data shown are means ± SEM (n = 3). *P < 0.05 and ***P < 0.001 vs. LPS-control.

Table 1S. Sequences of RT-PCR primers for gene expression analysis

Target	GenBank accession number	Direction	Sequence (5'→3')
MPO	NM_000250	Forward Reverse	CAGCCCAGATATACCCCTCA GACAACACAGGCATCACCAC
HO	NM_002133	Forward Reverse	AGCTCCTGCAACTCCTCAAA GGGTGATAGAAGAGGCCAAGA
TLR4	NM_138554	Forward Reverse	CTGCCACATGTCAGGCCTTAT AATGCCCACCTGGAAGACTCT
iNOS	NM_000625	Forward Reverse	ACCCAGACTTACCCCTTTGG GCCTGGGGTCTAGGAGAGAC
COX-2	NM_000963	Forward Reverse	TTCAAATGAGATTGTGGAAAAAT AGATCATCTCTGCCTGAGTATCTT
IL-1beta	NM_000576	Forward Reverse	GGGCCTCAAGGAAAAGAATC TTCTGCTTGAGAGGTGCTGA
TNF-alpha	NM_000594	Forward Reverse	TCCTTCAGACACCCTCAACC AGGCCCCAGTTTGAATTCTT
IL-6	NM_000600	Forward Reverse	TACCCCCAGGAGAAGATTCC TTTTCTGCCAGTGCCTCTTT
IL-10	NM_000572	Forward Reverse	GCCTAACATGCTTCGAGATC TGATGTCTGGGTCTTGGTTC
IFN-gamma	NM_000619	Forward Reverse	CAGGCAGGACAACCATTACTGGGATGCTC TGAACTCATCCAAGTGATGGCTGAACTGTCG
MMP-1	NM_ 001145938	Forward Reverse	CTGCTTGACCCTCAGAGACC ATGCTGAAACCCTGAAGGTG
MMP3	NM_002422	Forward Reverse	GAGTGTCGGAGTCCAGCTTC GCAGTTTGCTCAGCCTATCC
MMP9	NM_004994	Forward Reverse	CAGGGATCTCCCCTCCTTAG GTCTTGTGGAGGCTTTGAGC
PPAR-gamma	NM_005037	Forward Reverse	GCTGTGCAGGAGATCACAGA GGGCTCCATAAAGTCACCAA

GAPDH	NM_001289746	Forward Reverse	CACATGGCCTCCAAGGAGTAAG CCAGCAGTGAGGGTCTCTCT
HPRT	NM_000194	Forward Reverse	ACCCCACGAAGTGTTGGATA AAGCAGATGGCCACAGAACT

Figure 1
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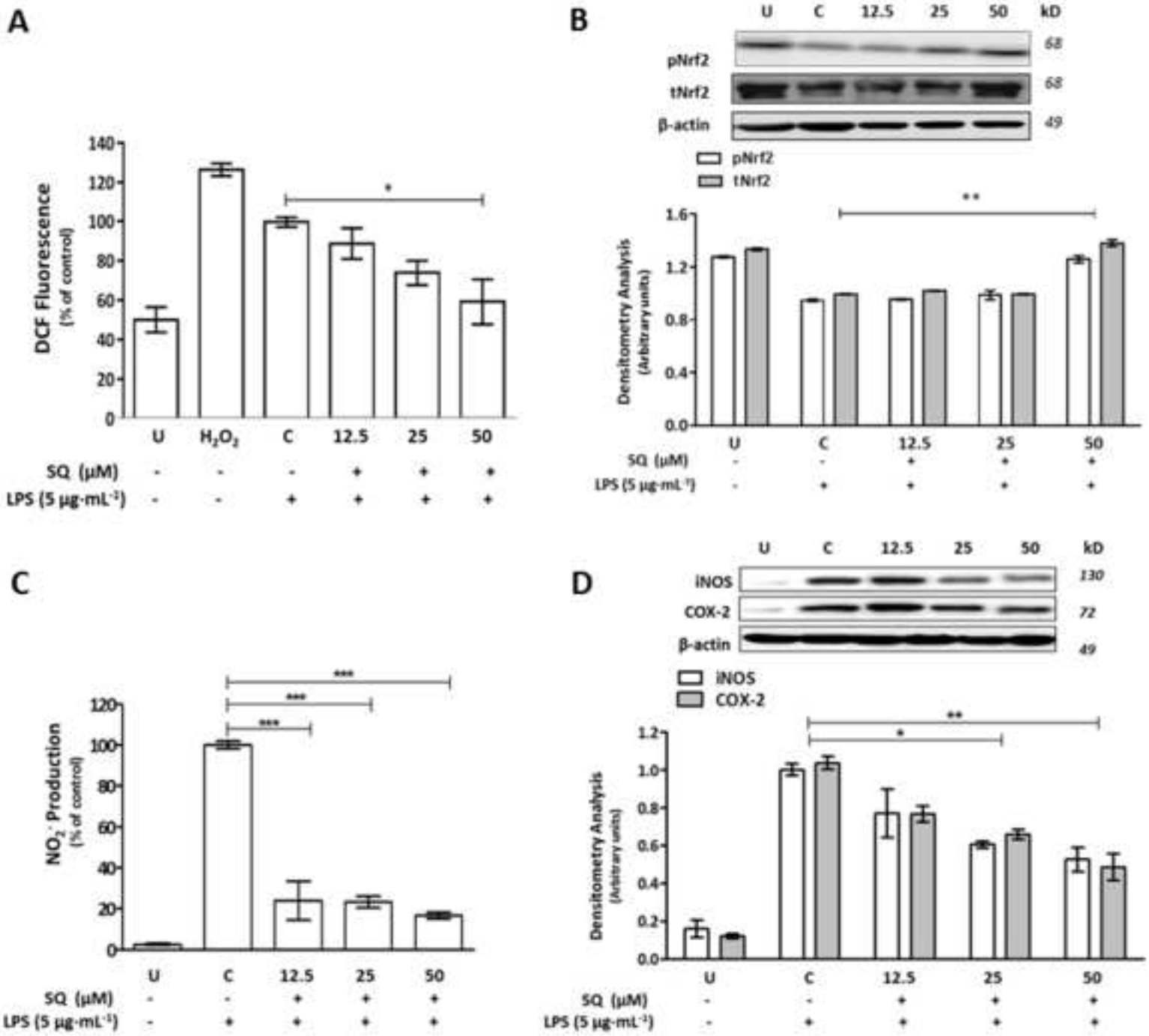
Fig. 1



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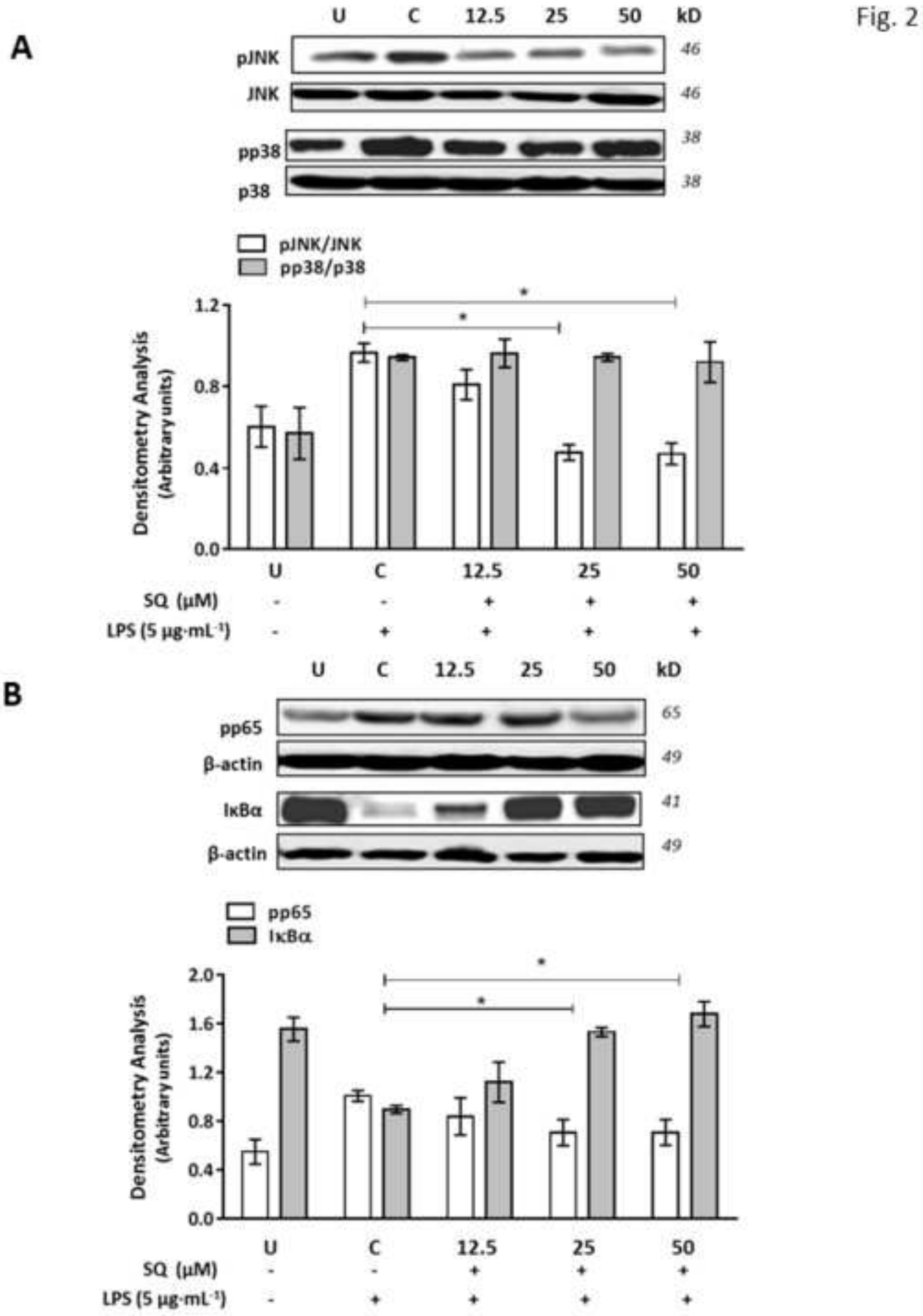

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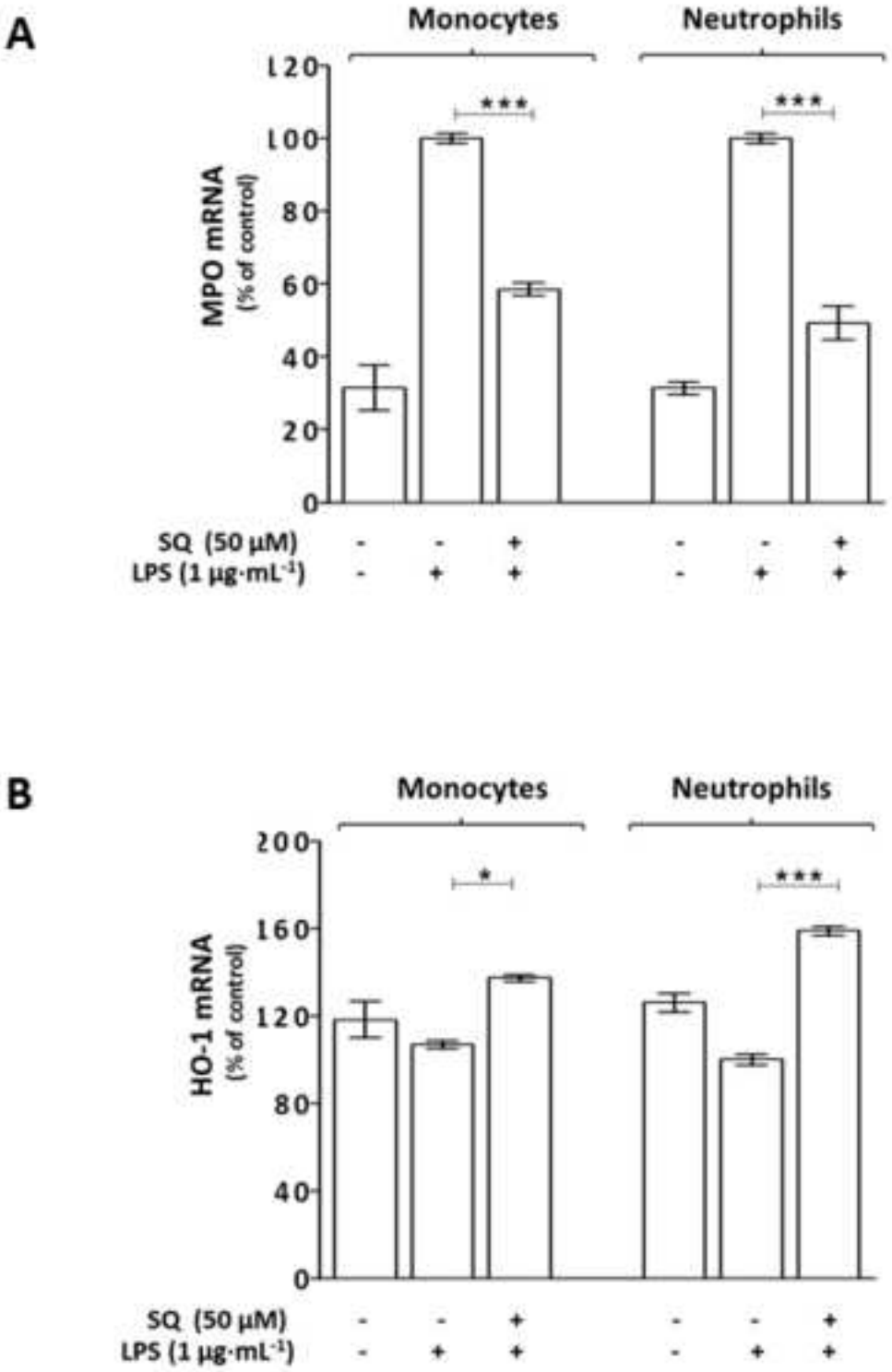

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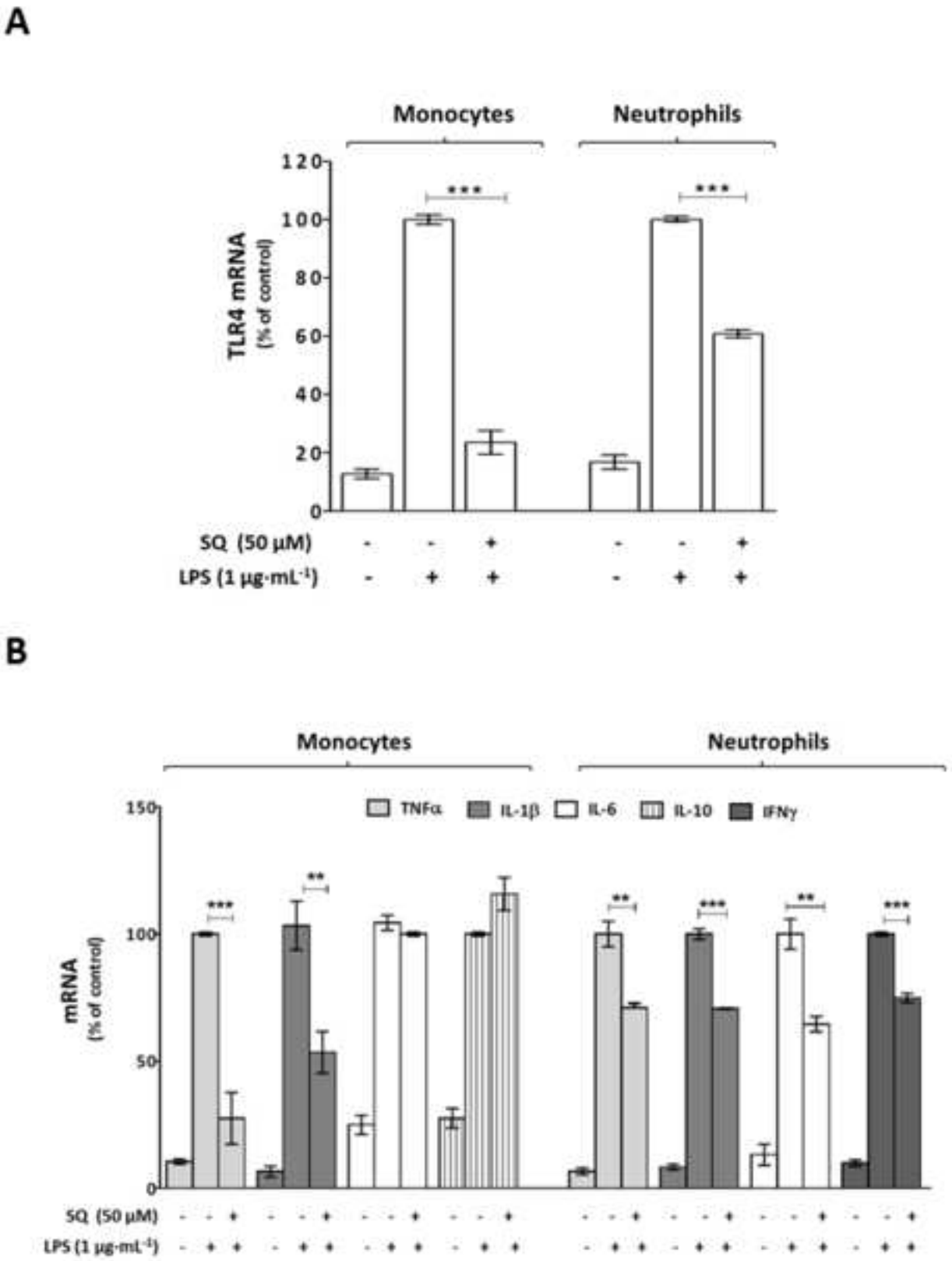

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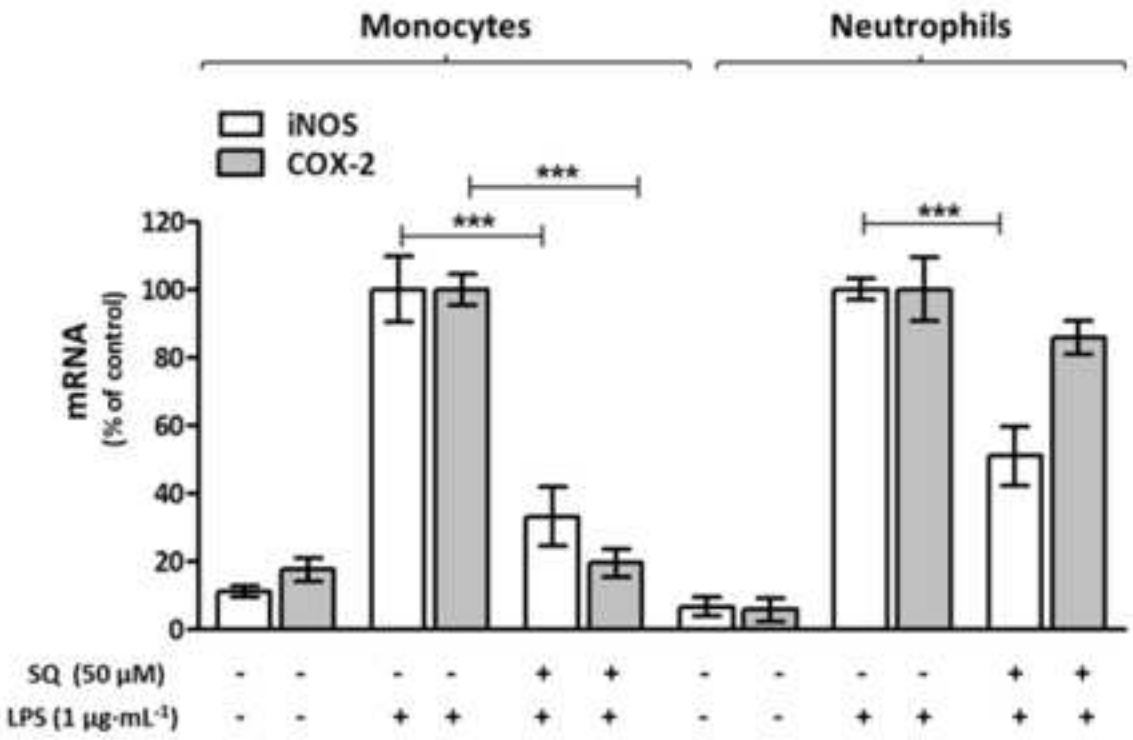

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