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Epigenetic regulation by oleacein mitigates IL-1 β -induced inflammation in human SW982 synovial cells

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Inflammatory arthritis is a term used to describe a diverse group of rheumatic disorders involving the inflammation and hyperproliferation of synovial joints and systemic manifestations. Oleacein (OLA) is one of the most abundant secoiridoids in extra virgin olive oil, the principal source of fat in the Mediterranean diet, which has been shown to exhibit beneficial effects. The objective of the study was to explore the anti-oxidant and anti-inflammatory effects induced by OLA in a human cell line of synovial cells (SW982), as well as to evaluate its possible role as an epigenetic modulator through the regulation of DNA methylation. Sulforhodamine B assay was utilised to assess cell viability. The levels of inflammatory marker production (MMP-1, MMP-3, TNF- α , IL-1 β , IL-6, and PGE₂) were evaluated by ELISA, and IL-8 gene expression was analysed by RT-qPCR. The expression of pro-inflammatory enzymes, including COX-2 and mPGES-1, and signaling pathways (MAPK, NF- κ B, Keap1/Nrf-2/HO-1 and inflammasome) were evaluated by western blotting. In addition, global DNA methylation was analysed by ELISA, and we studied the gene expression of DNMT1/3A enzymes by RT-qPCR. OLA exhibited anti-inflammatory and antioxidant effects through the regulation of key inflammatory signaling pathways such as inflammasome, MAPK, NF- κ B, and the Keap1/Nrf-2/HO-1 axis. In addition, it reduced the production and expression of pro-inflammatory markers (COX-2, mPGES-1, MMP-1, MMP-3, IL-8, IL-6, TNF- α and PGE₂) and regulated IL-1 β -induced changes in DNA methylation modulating DNMT1 and DNMT3 gene expression and global DNA methylation. These results show OLA as a promising epigenetic regulator of the inflammatory response in rheumatic diseases.

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1. Introduction

Psoriatic arthritis (PsA) is a chronic immune-mediated inflammatory disease that affects the musculoskeletal system and belongs to the seronegative spondyloarthritis family. PsA affects men and women equally, with a prevalence of 0.3–1% in the general population.¹ PsA diagnosis is usually complex since there are no clear biomarkers.² Some authors have described PsA as a form of arthritis with negative serum rheumatoid factor (RF), which affects psoriatic patient.³ In fact, about 25% of patients diagnosed with psoriasis develop PsA.⁴ Rheumatoid arthritis (RA) is also a chronic inflammatory-autoimmune disorder that affects the joints, with a prevalence of 0.4%–1.3% and affecting more women than men. Unlike PsA, RA involves symmetrical inflam-

mation of the affected joints and approximately 80% of patients have been found to be seropositive.^{5–7} In addition, osteoarthritis (OA) is a widespread inflammatory and degenerative joint disease with a complex pathogenesis involving the synovial membrane, cartilage, bone, tendon, ligament, and meniscus, affecting hundreds of millions globally with increasing prevalence, particularly in aging populations, causing pain and physical disabilities.^{8,9} Although the etiopathogenesis of these diseases has yet to be fully described, and each exhibits its own idiosyncrasies, several points of convergence exist. Their development involves inflammation, altered cellular mechanisms in synovial fibroblasts (SFs) and chondrocytes, aberrant activation of the immune response accompanied by an excessive production of pro-inflammatory markers (*e.g.*, cyclooxygenase (COX)-2 and pro-inflammatory cytokines), cartilage degradation caused by the overproduction of matrix metalloproteinases (MMPs), and the activation of several signaling pathways implicated in the expression of pro-inflammatory genes in addition to epigenetic modifications.^{5,6,9–11}

SFs are primary stromal cells residing in the lining and sub-lining layers of the joint synovium. In healthy joints, they maintain the homeostasis between the synovial fluid, the

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extracellular matrix and the synovial tissue.¹² However, in arthritis, including RA, PsA, and OA, these cells exhibit a marked phenotypic shift, transitioning from a protective role to adopting a destructive phenotype, actively participating in the transition of inflammation from the acute to the chronic state¹³ and in joint destruction.¹⁴ Indeed, in the context of arthritic conditions, the proliferation of SFs is accelerated, accompanied by the production of growth factors and pro-angiogenic factors, which contribute to the increase of local vascularisation and the enhancement of migratory capacity. Their ability to adhere to articular cartilage, secrete enzymes that degrade the cartilaginous matrix, and, in certain arthritic processes, invade the underlying bone to promote bone loss underscores their detrimental potential, establishing them as crucial players in the pathogenesis of arthritis. Consequently, SFs represent a central focus for investigating the mechanisms of arthropathies and developing therapeutic interventions.¹⁵

Regarding therapy, novel antirheumatic and antiarthritic drugs have been introduced and are currently undergoing clinical use. Nevertheless, the present limitations in the efficacy of long-term treatment and the occurrence of toxic side effects mean that the availability of effective medications is currently restricted. It is therefore essential to develop innovative alternative strategies and therapeutic agents for the treatment of these pathologies.^{7,8,16}

Nutritional therapy can exert direct effects through the provision of anti-inflammatory foods and indirect effects *via* the management of comorbidity. Dietary intervention has demonstrated the capacity to ameliorate disease activity by mitigating oxidative stress and inflammation in addition to its salutary effects on the gut microbiota. Moreover, manipulating dietary intake has been observed to effectively regulate gene expression patterns through various epigenetic modulation mechanisms. In this line, dietary nutrients could regulate pathological and physiological mechanisms *via* critical epigenetic strategies, promoting modifications of gene expression without alteration of the genetic code.^{17,18} Although nowadays the scope of epigenetic modifications is not completely known, it has been shown that they are fundamental factors in the differentiation of immunocompetent cells, inflammatory response regulation and cell growth.^{19,20}

Recent research has indicated that nutritional therapy for inflammatory arthritis, as an adjunct to medical treatment, should be based on the Mediterranean diet (MD) using extra virgin olive oil (EVOO) as a primary lipid source. EVOO's beneficial properties are attributed both to its major fraction of monounsaturated fatty acids, mainly oleic acid and to its additional minor constituents, notably secoiridoids. The secoiridoids present in EVOO are characterized by the presence of a phenolic hydroxytyrosol motif in their molecular structure; therefore, they could be considered polyphenols. However, not all secoiridoids contain this group in their molecular structure.^{21–23} Essentially, the bioactivities of these phenolic compounds have been related to their potential to induce epigenetic modifications including DNA methylation, histone modification and microRNAs (miRNAs) expression.^{24,25}

Secoiridoids are active compounds derived from the secondary metabolism of terpenes,^{26,27} ligustroside, and oleuropein (OL), together with its corresponding aglycones, oleocanthal (OLE), and oleacein (OLA) are some examples of secoiridoids with phenolic moieties. OLA is one of the most abundant secoiridoids in EVOO and corresponds to the dialdehyde form of the decarboxymethyl aglycone of OL (3,4-DHPEA-EDA).²⁸ In fact, during olive milling and extraction of EVOO, OL is converted to OLA by internal glycosylases and decarboxylation reactions. OLA has been documented to exhibit cardioprotective and anticancer effects^{29–31} and recent research has demonstrated the antioxidant, anti-inflammatory and immunomodulatory activities of OLA in immune cells^{32,33} as well as in *in vivo* models of autoimmune encephalomyelitis,^{34,35} RA³⁶ and SLE³⁷ where OLA was able to attenuate disease progression. Furthermore, OLA was able to regulate epigenetic changes in *in vitro* and *in vivo* models by modulating DNA methylation,³⁷ as well as post-translational modifications of histones^{30,33} and the expression of several microRNAs.^{37,38}

Given this background, the current study aimed to explore the possible beneficial effects of OLA in interleukin (IL)-1 β -stimulated SW982 cells. This cell line of human synovial sarcoma cells is a valuable *in vitro* model for the study of inflammatory arthritis. In response to IL-1 β , SW982 human synovial cells are characterized by the overproduction of pro-inflammatory markers as cytokines or MMPs.^{39,40} Our study also provides in-depth insight into the signaling pathways involved in its anti-inflammatory effects. Moreover, epigenetic changes were also determined.

2. Materials and methods

2.1. Chemicals

OLA was obtained from (-)-oleuropein following the isolation, purification and identification methods described by us in ref. 33 and 37.

2.2. Cell culture

The SW982 human synovial sarcoma cell line was procured from CLS Cell Lines Service GmbH (CLS®, Eppelheim, Germany, Cat. Number: 300404; Batch Number 300404-714). Dulbecco's modified Eagle's medium (DMEM) containing 1% penicillin-streptomycin, 1 mM pyruvate, 4 mM L-glutamine and 10% FBS was used to culture synovial cells (37 °C/5% CO₂). Cells were regularly subcultured in T-75 flasks every 3–4 days, with the addition of fresh medium.

2.3. Sulforhodamine B (SRB): cell viability assay

For the assessment of cell viability, SW982 cells were plated in ninety-six well plates at a density of 2.5×10^4 cells per well. Synovial cells were then incubated for 48 h in the absence or presence of OLA (200–1.56 μ M). After the incubation period, cell viability was evaluated using the SRB assay. 50 μ L of 50% (w/v) cold trichloroacetic acid (Sigma-Aldrich®, St Louis, MO,



USA) was added to fix the cells. The cells were then incubated for 60 min at 4 °C. This was followed by five rinses with distilled water and air drying. After washing, we added 50 µL of a solution of SRB (Sigma-Aldrich®, St Louis, MO, USA) at a concentration of 0.4% w/v in 1% acetic acid (Panreac®, Barcelona, Spain). SW982 cells were incubated at room temperature for 30 min, protected from light. After that, a 1% acetic acid solution was used to wash the plates (5 times). Finally, 100 µL of Tris base (10 mmol L⁻¹, pH 10.5) (Sigma-Aldrich®, St Louis, MO, USA) was added to each well in order to dissolve SRB. Finally, a multiwell plate reader (Biotek®, Bad Friedrichshall, Germany) was used to read the absorbance at 492 nm. The viability of the SW982 cells was determined as the percentage of absorbance compared to untreated cells (control cells). The toxicity of dimethylsulfoxide (DMSO), the vehicle used to dissolve OLA, was also studied.

2.4. Evaluation of pro-inflammatory cytokine, prostaglandin (PG)E₂ and MMPs levels in SW982 cells

SW982 cells were seeded in twenty-four well plates (2.5 × 10⁵ cells per well) in supplemented DMEM. Cells were incubated for 48 h at 37 °C under a humidified atmosphere of 5% CO₂ to allow cell adhesion. Then, the synovial cells were pretreated for 24 h with OLA (25 and 12.5 µM). After the incubation period, IL-1β (5 ng mL⁻¹) was added as a stimulus and left to incubate for another 24 h for the detection of tumor necrosis factor (TNF)-α, IL-6, PGE₂, and MMPs (MMP-1 and MMP-3) levels. On the other hand, in order to assess IL-1β production, the SW982 cells were pretreated for 24 h with OLA (25 and 12.5 µM) and stimulated with bacterial lipopolysaccharide (LPS) (5 µg mL⁻¹, 24 h). Then adenosine triphosphate (ATP) (5 mM) was added, and the cells were incubated for 30 min. Finally, culture supernatants were harvested and stored at -80 °C. The production of pro-inflammatory cytokines and MMPs was quantified in cell supernatants using ELISA kits for IL-6, IL-1β, TNF-α (Diaclone®, Besancon Cedex, France), prostaglandin (PG)E₂ (Abcam®, Cambridge, UK), MMP-1 (RayBiotech®, Atlanta, GA, USA) and MMP-3 (R&D System®, Minneapolis, Canada, USA), following the manufacturer's instructions.

2.5. Western blot analysis

As previously described, the cells were cultured in 24-well plates (2.5 × 10⁵ cells per well) in supplemented DMEM and incubated at 37 °C for 48 h to allow cell adhesion. The SW982 synovial cells were then pretreated with OLA (25 and 12.5 µM) for 24 h and stimulated with IL-1β (5 ng mL⁻¹) for different time points depending on the pathway (Table 1). In addition, to study the inflammasome pathway, the cells were pretreated with OLA (25 and 12.5 µM) for 24 h and activated with LPS (5 µg mL⁻¹, 24 h) and subsequently with ATP (5 mM, 30 min).

After the incubation period, proteins were isolated using ice-cold lysis buffer (1 mmol L⁻¹ phenylmethylsulfonyl fluoride (PMSF), 8 mmol L⁻¹ MgCl₂, 5 mmol L⁻¹ ethylene glycol bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA),

Table 1 Antibody timepoints and stimulus used in western blotting

Antibody	Dilution	Identification	Timepoint	Stimulus
Anti-rabbit IgG	1 : 1000	Cell Signaling Technology Cat# 7074, RRID: AB_2099233		
Anti-mouse IgG	1 : 1000	Cell Signaling Technology Cat# 7076, RRID: AB_330924		
β-Actin	1 : 10 000	Abcam Cat# ab49900, RRID: AB_867494	24 h	IL-1β 5 (ng mL ⁻¹)
COX-2	1 : 1000	Cell Signaling Technology Cat# 12282, RRID: AB_2571729	24 h	IL-1β 5 (ng mL ⁻¹)
Microsomal prostaglandin E synthase-1 (mPGES-1)	1 : 1000	Abcam Cat# ab62050, RRID: AB_2269175	24 h + 30 min	LPS (5 µg mL ⁻¹) + ATP (5 mM)
Nod-like receptor family pyrin domain containing 3 (NLRP3)	1 : 1000	Cell Signaling Technology Cat# 15101, RRID: AB_2722591	24 h + 30 min	LPS (5 µg mL ⁻¹) + ATP (5 mM)
Caspase 1	1 : 1000	Cell Signaling Technology Cat# 3866, RRID: AB_2069051	24 h + 30 min	LPS (5 µg mL ⁻¹) + ATP (5 mM)
Inhibitor of nuclear factor kappa B (IκB-α)	1 : 1000	Cell Signaling Technology Cat# 13833, RRID: AB_2798325	24 h + 30 min	LPS (5 µg mL ⁻¹) + ATP (5 mM)
pp65	1 : 1000	Cell Signaling Technology Cat#4812, RRID: AB_10694416	6 h	IL-1β 5 (ng mL ⁻¹)
pJNK	1 : 1000	Cell Signaling Technology Cat# 3033, RRID: AB_331284	6 h	IL-1β 5 (ng mL ⁻¹)
pERK	1 : 1000	Cell Signaling Technology Cat#4668, RRID: AB_823588	30 min	IL-1β 5 (ng mL ⁻¹)
pp38	1 : 1000	Cell Signaling Technology Cat#4370, RRID: AB_2315112	30 min	IL-1β 5 (ng mL ⁻¹)
JNK	1 : 1000	Cell Signaling Technology Cat#4511, RRID: AB_2139682	30 min	IL-1β 5 (ng mL ⁻¹)
ERK	1 : 1000	Cell Signaling Technology Cat#9252, RRID: AB_2250373	30 min	IL-1β 5 (ng mL ⁻¹)
P38	1 : 1000	Cell Signaling Technology Cat#9107, RRID: AB_10695739	30 min	IL-1β 5 (ng mL ⁻¹)
Kelch-like ECH-associated protein 1 (Keap1)	1 : 1000	Cell Signaling Technology Cat#8690, RRID: AB_10999090	30 min	IL-1β 5 (ng mL ⁻¹)
Nuclear factor-erythroid 2 related factor 2 (Nrf-2)	1 : 1000	Cell Signaling Technology Cat# 8047, RRID: AB_10860776	24 h	IL-1β 5 (ng mL ⁻¹)
Heme-oxygenase 1 (HO-1)	1 : 1000	Cell Signaling Technology Cat#12721, RRID: AB_2715528	24 h	IL-1β 5 (ng mL ⁻¹)
	1 : 1000	Enzo Life Sciences Cat#ADI-SPA-896-D, RRID: AB_2039233	24 h	IL-1β 5 (ng mL ⁻¹)



250 mmol L⁻¹ NaCl, 0.5 mmol L⁻¹ EDTA, 50 mmol L⁻¹ Tris-HCl (pH 7.5), 0.01 mg mL⁻¹ pepstatin, 0.01 mg mL⁻¹ aprotinin and 0.01 mg mL⁻¹ leupeptin). The protein content was evaluated using the Bradford assay. Aliquots comprising 25 µg of total protein were separated *via* sodium dodecyl sulfate polyacrylamide gel electrophoresis, with the percentage of gel used being either 15% or 10%. Subsequently, the proteins were transferred to a nitrocellulose membrane (BioRad®, Hercules, CA, USA) and then subjected to overnight incubation with specific primary antibodies at 4 °C (Table 1).

Following the washing step, blocking buffer (comprising 1% casein (Sigma-Aldrich®, St Louis, MO, USA) and 0.5% Nonident-P40 substitute (Sigma-Aldrich®, St Louis, MO, USA) in PBS buffer) containing anti-rabbit horseradish peroxidase-labeled (HRP) secondary antibody (Cell Signaling®, Danvers, MA, USA) or anti-mouse HRP secondary antibody (Cell Signaling®, Danvers, MA, USA) were added to membranes for 1.5–2 h. ERK, P38, JNK (Cell Signaling®, Danvers, MA, USA) and β-actin (Abcam®, Cambridge, UK) were used as the loading control (housekeeping gene). Finally, immunodetection was conducted with an enhanced chemiluminescence light detection kit (Biorad®, Hercules, CA, USA). The immunosignals were captured using an Amersham Imager 600 from GE Healthcare® (Buckinghamshire, UK) and quantified using Java (ImageJ®, Softonic). Finally, the results were normalized with the IL-1β or LPS + ATP group cells. The used antibodies, the designated time points, and the stimulants employed for the evaluation of protein expression by western blotting are outlined in Table 1.

2.6. Real-time quantitative reverse transcription PCR (RT-qPCR)

For nucleic acid extraction, cells were seeded in 6-well plates at a density of 2×10^6 cells per well and pretreated with OLA (25 and 12.5 µM) for 24 h. After the incubation period, IL-1β (5 ng mL⁻¹) was added as a stimulus and left to incubate for another 24 h. The extraction of total RNA was conducted in accordance with the protocol of the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen®, Hilden, Germany). A Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific®, Waltham, MA, USA) was used to measure total RNA. Then, cDNA was obtained using the iScript™ cDNA Synthesis Kit (BioRad®, Hercules, CA, USA); 1000 ng of total RNA was employed per cDNA reaction.

Next, in order to perform real time PCR (qPCR), the cDNA samples were aliquoted to a final concentration of 20 ng µL⁻¹. Then, 4 µL (100 ng) of cDNA were mixed with 10 µL of iTaq Universal SYBR Green Supermix (BioRad®, Hercules, CA, USA), 5 µL of nuclease-free water and 1 µL of PrimePCR™ SYBR® Green Assay (BioRad®, Hercules, CA, USA) for a final reaction volume of 20 µL. qPCR analysis was performed using a CFX Connect Real-Time PCR Detection System (BioRad®, Hercules, CA, USA). Cycling conditions are described in Table 2. The GAPDH gene was used as the housekeeping gene. Data were normalized to the IL-1β control group. The delta-delta cycle threshold (ΔΔCt) method was used to calculate the fold

Table 2 Cycling conditions

Step	Temperature (°C)	Time	Number of cycles
Activation	95	2 min	1
Denaturation	95	5 s	40
Annealing/extension	60	30 s	40
Melt curve	65–95 (0.5 increments)	5 s per step	1

Table 3 Target gene details (BioRad®, Hercules, CA, USA)

Gene	Assay ID
IL-8	qHsaCED0046633
DNA methyltransferase (DNMT)1	qHsaCID0020933
DNMT3A	qHsaCID0010863
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	qHsaCED0038674

expression levels for each target gene.⁴¹ All reactions were performed in duplicate and a negative control without cDNA was included. Details of the genes studied are shown in Table 3.

2.7. Quantification of global DNA methylation

Cells were seeded in 6-well plates and cultured as described in section 2.6. Total DNA was isolated from the SW982 cells following the manufacturer's instructions of the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen®, Hilden, Germany) and quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific®, Waltham, MA, USA) as described below. Then, the MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit (Epigentek Inc.®, Farmingdale, NY, USA) was used in accordance with the manufacturer's methodology to quantify the % of 5-methylcytosines (5Mc). Results are expressed as % of 5-mC present in total DNA.

3. Results

3.1. Effect of OLA on SW982 synovial fibroblast survival

The impact of OLA on SW982 cell viability was initially assessed *via* the SRB assay. As depicted in Fig. 1, 48 hour of incubation with varying OLA concentrations (1.56–200 µM) revealed no significant alterations in cell viability below 100 µM, where SW982 cell survival remained above 80%. Furthermore, evaluation of the vehicle control (DMSO) indicated no adverse effects on cell viability.

3.2. OLA treatment reduced MMP-1 and MMP-3 levels in SW982 cells

Given the role of MMPs in collagen degradation and the infiltration of endothelial cells and synovial fibroblasts,⁴² we explored the effects of OLA treatment on their production. Specifically, we measured MMP-1 and MMP-3 levels in the supernatant of IL-1β-stimulated SW982 cells. IL-1β stimulation



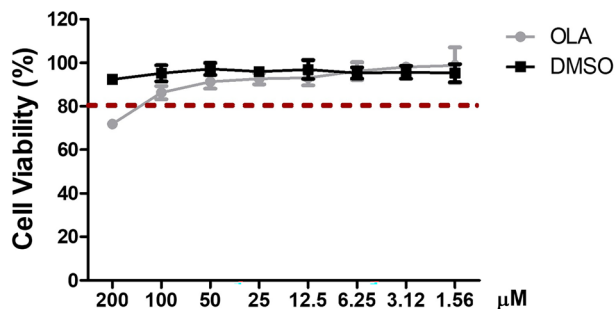


Fig. 1 SW982 cell survival in the presence of OLA and DMSO. Cell viability was measured using SRB after 48 h of incubation with OLA and DMSO (200–1.56 μM). A cell viability above 80% was considered not toxic (OLA 100–1.56 μM). Data are expressed as means \pm SEM ($n \geq 5$).

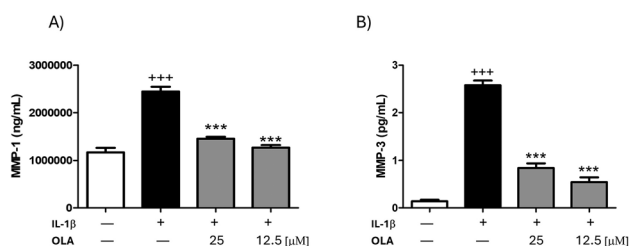


Fig. 2 OLA suppresses MMP-1 (A) and MMP-3 (B) overproduction in IL-1 β stimulated synovial fibroblasts. Cells were stimulated for 24 h with IL-1 β (5 ng mL $^{-1}$) after 24 h of pretreatment with OLA (25 and 12.5 μM). Control cells were also treated with IL-1 β or nontreated. Data are expressed as means \pm SEM ($n \geq 4$). Statistical analysis was performed by ANOVA followed by Bonferroni's multiple comparisons test: +++ $P < 0.001$ vs. control cells (not stimulated); *** $P < 0.001$ vs. Control-IL-1 β cells.

led to a significant upregulation of MMP-1 (Fig. 2A) and MMP-3 (Fig. 2B) production in SW982 cells (+++ $P < 0.001$ vs. control cells without stimulation). Conversely, pretreatment with OLA (25 and 12.5 μM) effectively reduced these elevated MMP levels (*** $P < 0.001$ vs. control cells stimulated with IL-1 β).

3.3. OLA inhibited the production and expression of pro-inflammatory cytokines in the SW982 cell line

The levels of IL-6 and TNF- α pro-inflammatory cytokines were measured by ELISA. IL-6 (Fig. 3A) and TNF- α (Fig. 3B) production was significantly increased in the cell culture supernatant of IL-1 β stimulated SW982 cells compared to non-stimulated fibroblasts (+++ $P < 0.001$ vs. Control (not stimulated) cells) (Fig. 3). Nevertheless, OLA pretreatment resulted in a marked down-regulation of both pro-inflammatory cytokine levels assayed (** $P < 0.01$, *** $P < 0.001$ vs. Control-IL-1 β cells). On the other hand, the gene expression of the cytokine IL-8 was also analysed by RT-qPCR. As shown in Fig. 3C, treatment with OLA at both concentrations (25 and 12.5 μM) down-regulated the gene expression of this cytokine, counteracting the effect of IL-1 β (** $P < 0.01$ vs. Control-IL-1 β cells).

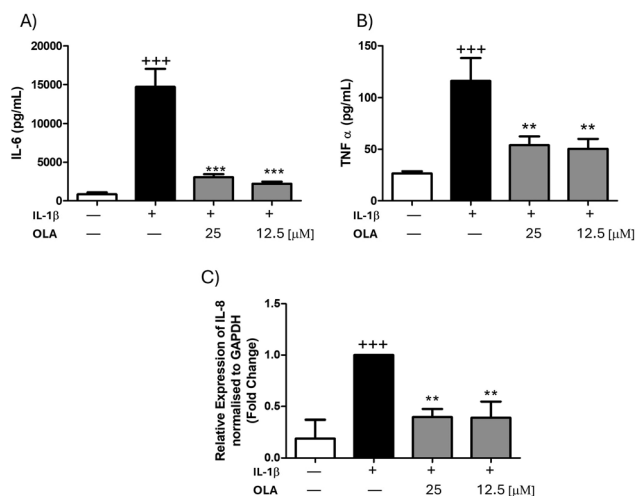


Fig. 3 Effect of OLA pre-treatment in IL-6 (A), TNF- α (B) production and IL-8 (C) gene expression. Cells were cultured in the presence/absence of OLA (25 and 12.5 μM) for 24 h and stimulated with IL-1 β for another 24 h. IL-6 and TNF- α secretion was evaluated by ELISA ($n \geq 6$) and IL-8 gene expression was evaluated by RT-qPCR ($n \geq 5$). Control cells were also treated with IL-1 β or nontreated. Data are expressed as means \pm SEM. +++ $P < 0.001$ vs. control cells (not stimulated); ** $P < 0.01$, *** $P < 0.001$ vs. Control-IL-1 β cells. Statistical analysis was performed by ANOVA followed by Bonferroni's multiple comparisons test.

3.4. OLA pretreatment modulated COX-2/mPGES-1 protein expression and PGE₂ production

A pivotal process in the inflammatory response is the upregulation of COX-2 and mPGES-1 expression, which results in an increase in PGE₂ production.⁴³ As anticipated, in SW982 cells stimulated with IL-1 β , increased protein expression of COX-2 (Fig. 4A) and mPGES-1 (Fig. 4B), and oversecretion of PGE₂ (Fig. 4C) can be observed (++ $P < 0.01$, +++ $P < 0.001$ vs. Control (not stimulated) cells). This effect was significantly counteracted by pretreatment with OLA (Fig. 4) (* $P < 0.05$ vs. Control-IL-1 β cells).

3.5. Effects of OLA pretreatment on NLRP3 inflammasome activation

Moreover, we assessed the effects of OLA on the NLRP3 inflammasome by western blotting in order to explore possible signaling pathways that may underlie the anti-inflammatory activity of OLA. The inflammasome is a cytosolic multimeric complex whose activation leads to caspase 1 release and IL-1 β and IL-18 maturation, also leading to an increase in K⁺ efflux and pyroptosis.⁴⁴ Therefore, we studied NLRP3 inflammasome protein expression by western blotting in LPS + ATP activated SW982 cells. LPS + ATP co-stimulation increased NLRP3 and ASC protein expression in comparison with control cells (Fig. 5A); in the same way, the expression of caspase 1 was also regulated in LPS + ATP activated SW982 (Fig. 5C) (++ $P < 0.01$, +++ $P < 0.001$ vs. Control (not stimulated) cells). However, OLA pretreatment was able to modulate the activation of this signaling pathway, resulting in a reduction in ASC, NLRP3 and



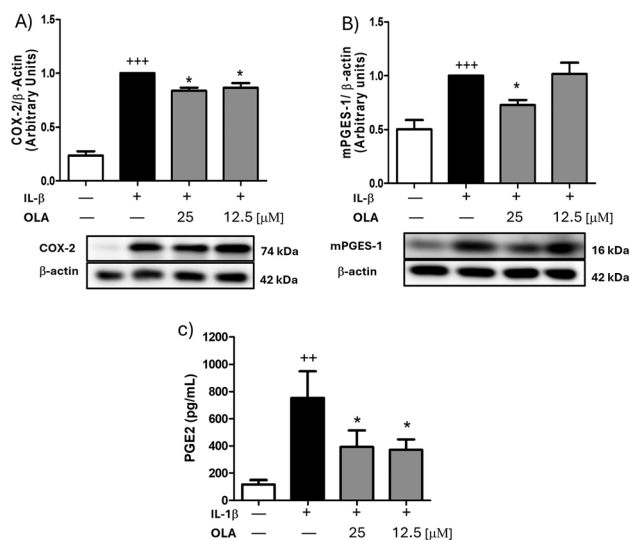


Fig. 4 COX-2/mPGES-1 overexpression and PGE₂ production were regulated by OLA pretreatment in synovial fibroblasts activated with IL-1 β . A, (B) western blot analysis of COX-2/mPGES-1 protein expression; and (C) PGE₂ secretion. Cells were pretreated with OLA (25 and 12.5 μ M) for 24 h and then stimulated with IL-1 β (5 ng mL⁻¹) for 24 h. Densitometry was performed after normalization to the control (β -actin housekeeping gene). Data are represented as mean \pm SEM ($n \geq 6$). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test: ++ $P < 0.01$ +++ $P < 0.001$ vs. control cells (not stimulated); * $P < 0.05$ vs. Control-IL-1 β cells.

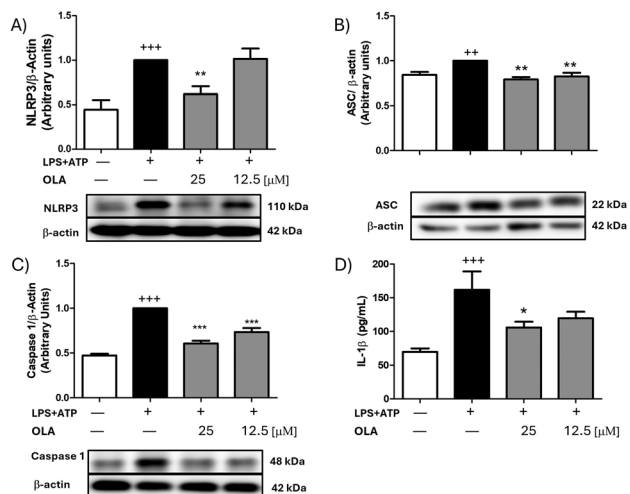


Fig. 5 Effects of OLA treatment in the canonical inflammasome signaling pathway in SW982 cells. (A) NLRP3, (B) ASC, (C) Caspase 1 protein expression; and (D) IL-1 β secretion. SW982 cells were treated with OLA (25 and 12.5 μ M) for 24 h and then stimulated with LPS (5 μ g mL⁻¹, 24 h) and ATP (5 mM, 30 min). Densitometry was performed after normalization to β -actin as a housekeeping gene. Data are expressed as mean \pm SEM ($n \geq 6$). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test: ++ $P < 0.01$, +++ $P < 0.001$ vs. control cells (not stimulated); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. Control-IL-1 β cells.

Caspase 1 protein expression (Fig. 5A–C) (** $P < 0.01$, *** $P < 0.001$ vs. Control-LPS + ATP cells). Consequently, a reduction in IL-1 β levels was also detected in cells treated with OLA (25 μ M) (Fig. 5D), which would be in line with the results mentioned above (* $P < 0.05$ vs. Control-LPS + ATP cells).

3.6. OLA pretreatment downregulated NF- κ B induction in activated SW982 cells

It is widely known that the transcription factor NF- κ B is a key regulator of genes involved in the immune-inflammatory response; therefore, an aberrant activation of this complex could be closely related to the development of immune-mediated inflammatory diseases like RA.⁴⁵ Inactive NF- κ B is found in the cytoplasm, where it is controlled by the inhibitory protein I κ B. However, after a pro-inflammatory stimulus, I κ B is phosphorylated by I κ B kinases, leading to its degradation and the release of NF- κ B and its translocation to the nucleus to induce the expression of pro-inflammatory genes as mentioned above.⁴⁶ In order to assess the role of OLA treatment in the NF- κ B pathway, we evaluated the protein expression of I κ B- α and pp65 in 24 h OLA-pretreated SW982 cells stimulated with IL-1 β (5 ng mL⁻¹) for 6 h. As shown in Fig. 6, the addition of IL-1 β led to the activation of the NF- κ B signaling pathway with consequent degradation of I κ B- α and increased pp65 protein expression (++ $P < 0.01$ vs. Control (not stimulated) cells). In contrast, after pretreatment with OLA at its highest concentration (25 μ M), I κ B- α levels were significantly restored, inhibiting NF- κ B (*** $P < 0.001$ vs. Control-IL-1 β cells). At 12.5 μ M, an increase in I κ B- α expression was observed but the results were not significant (Fig. 6). Regarding pp65 protein expression, a significant decrease in pp65 protein expression can be observed at 25 μ M (* $P < 0.05$ vs. Control-IL-1 β cells), in line with the results obtained for I κ B- α .

3.7. Mitogen-activated protein kinase (MAPK) family induction was modulated by OLA in IL-1 β stimulated synovial fibroblasts

Another key signaling pathway in the inflammatory process, activated in the presence of an inflammatory stimulus or cellular stress, is the MAPK family, which through a cascade of phosphorylation and subsequent translocation to the nucleus leads to the expression of pro-inflammatory genes.⁴⁷ Therefore, the levels of JNK, p38 and ERK MAPK phosphorylation after 30 min of IL-1 β stimulation were analyzed by western blotting. IL-1 β induction led to an increase in JNK, ERK and p38 MAPK phosphorylation (+ $P < 0.05$, +++ $P < 0.001$ vs. Control (not stimulated) cells), as expected (Fig. 7). However, OLA was able to decrease phosphorylation and, consequently, activation of JNK and ERK at 25 μ M (* $P < 0.05$, ** $P < 0.01$ vs. Control-IL-1 β cells). On the other hand, no alterations were observed in p38 phosphorylation in OLA-pretreated cells (Fig. 7).



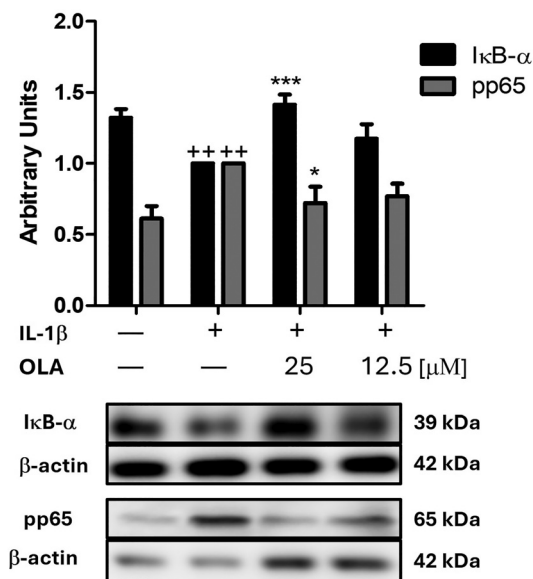


Fig. 6 OLA pretreatment modulated the activation of the NF- κ B signaling pathway in IL-1 β activated synovial cells. Cells were pretreated with OLA (25 and 12.5 μ M) for 24 h and then stimulated with IL-1 β (5 ng mL $^{-1}$) for 6 h. Densitometry was performed after normalization to the control (β -actin housekeeping gene). Data are represented as mean \pm SEM ($n \geq 6$). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test: ++ $P < 0.01$ vs. control cells (not stimulated); * $P < 0.05$, *** $P < 0.001$ vs. Control-IL-1 β cells.

3.8. OLA enhanced the Keap1/Nrf-2/HO-1 axis in SW982 synovial fibroblasts activated by IL-1 β

To investigate the possible antioxidant role of OLA, the protein expression of the Keap1/Nrf-2/HO-1 pathway was measured, whose activation has a protective effect under oxidant conditions and cellular stress. Keap1 keeps Nrf-2 inactive in the cytoplasm, preventing it from translocating to the nucleus and interacting with antioxidant response elements (ARE), inducing the expression of antioxidant enzymes such as HO-1. Under oxidative stress conditions, Keap1 is degraded, releasing Nrf-2 and activating this antioxidant axis.⁴⁸ In cells pretreated at both concentrations of OLA (25 and 12.5 μ M), we noticed a tendency towards a reduction in the protein expression of Keap1, which was slightly increased in the stimulated control cells, although the results are not significant (Fig. 8). According to these findings, Nrf-2 levels were reduced in the presence of IL-1 β and significantly restored in cells treated with OLA at 25 μ M OLA (* $P < 0.05$ vs. Control-IL-1 β cells). Furthermore, an increase in Nrf-2 expression was observed at the lowest concentration. In a similar manner, HO-1 protein expression was also up-regulated after OLA pretreatment (* $P < 0.05$ vs. Control-IL-1 β cells) (Fig. 8).

3.9. Effects of OLA pre-treatment on DNA methylation

Finally, we investigated the ability of OLA to regulate DNA methylation in the human synovial fibroblast cell line. Global DNA hypomethylation has been reported in immuno-inflam-

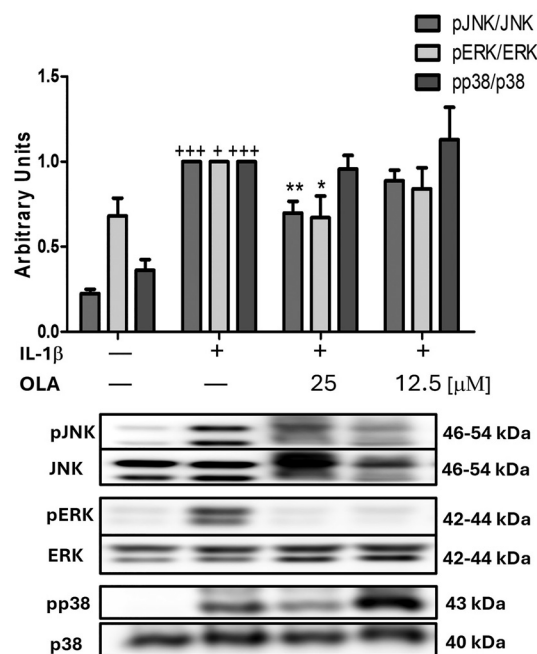


Fig. 7 Effects of OLA pretreatment on MAPK activation in SW982 synovial fibroblasts. pJNK, pERK, and pp38 protein expression was evaluated by western blotting. Cells were pretreated with OLA (25 and 12.5 μ M) for 24 h and then incubated in the absence or presence of IL-1 β (5 ng mL $^{-1}$) for 30 min. Densitometry was performed after normalization to the control (JNK, ERK, and p38 housekeeping gene). Results are represented as means \pm SEM ($n \geq 6$). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test: + $P < 0.05$, +++ $P < 0.001$ vs. control cells (not stimulated); * $P < 0.05$, ** $P < 0.01$ vs. Control-IL-1 β cells.

matory diseases such as RA and PsA, which may be involved in altering the immune response and fibroblast functionality.⁴⁸ Therefore, we assessed the gene expression of the enzymes responsible for DNA methylation (DNMT1 and DNMT3A) and the global % of DNA methylation. As shown in Fig. 9B, treatment with OLA at its highest concentration (25 μ M) was able to significantly induce DNA methylation, increasing the expression of the enzyme DNMT3A (* $P < 0.05$ vs. Control-IL-1 β cells) which controls *de novo* methylation and, consequently, an increase in global DNA methylation is also observed (Fig. 9A) (* $P < 0.05$ vs. Control-IL-1 β cells). However, DNMT1 gene expression was upregulated in IL-1 β -stimulated cells and OLA treatment restored it to levels similar to control cells (Fig. 9C) (* $P < 0.01$, ** $P < 0.01$ vs. Control-IL-1 β cells).

4. Discussion

Notably, our findings revealed, for the first time, that OLA, one of the most abundant secoiridoids in EVOO, is able to mitigate the inflammatory response induced by IL-1 β in SW982 human synovial cells. This cell line, of sarcomatous origin, constitutes a valuable *in vitro* model for investigating inflammation in arthritis. Its utility is predicated on its ability to mimic the



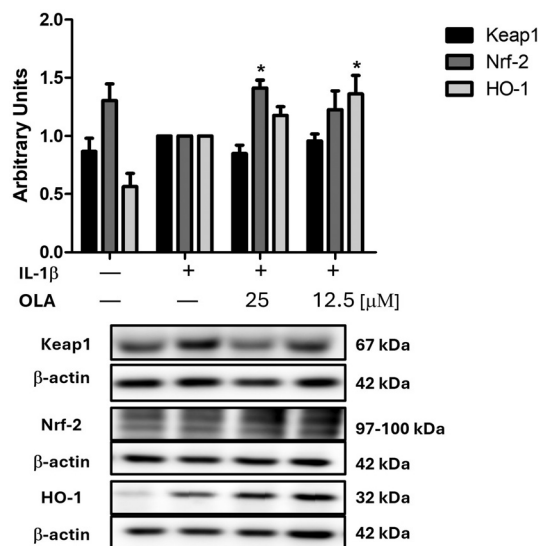


Fig. 8 OLA promoted Keap1/Nrf-2/HO-1 axis activation in IL-1 β activated SW982 cells. Protein expression was evaluated by western blotting. SW982 cells were pretreated with OLA (25 and 12.5 μ M) for 24 h and stimulated with IL-1 β (5 ng mL $^{-1}$) for 24 h. Densitometry was performed after normalization to the control (β -actin housekeeping gene). Results are represented as means \pm SEM ($n \geq 6$). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test: * $P < 0.05$, vs. Control-IL-1 β cells.

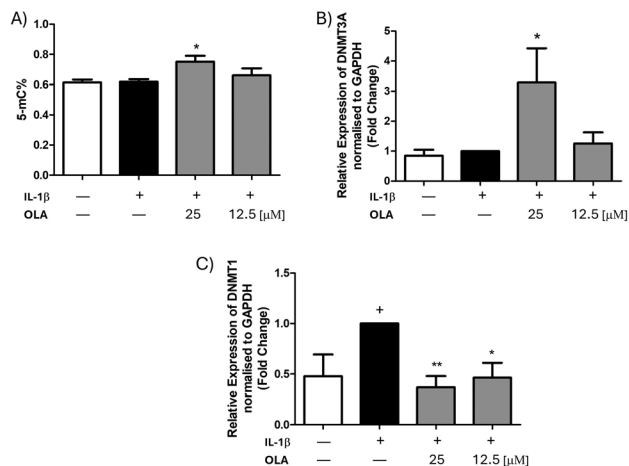


Fig. 9 OLA treatment regulated DNA global methylation in the synovial fibroblast SW982 cell line. (A) Global 5-mC%, (B and C) DNMT3A and DNMT1 gene expression. SW982 cells were pre-treated with OLA (25–12.5 μ M) for 24 h and stimulated with IL-1 β (5 ng mL $^{-1}$) for 24 h. Gene expression was evaluated by RT-qPCR and normalized with the GAPDH housekeeping gene. Results are represented as means \pm SEM ($n \geq 5$). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test: + $P < 0.05$ vs. control cells (not stimulated); * $P < 0.05$, ** $P < 0.01$ vs. Control-IL-1 β cells.

behaviour of human fibroblast-like synoviocytes, critical in the pathogenesis of this condition. As a result, it provides a reproducible alternative to primary fibroblasts derived from arthritis

patients, the isolation of which can be challenging and exhibits inter-individual variability.^{49,50}

OLA is one of the most abundant secoiridoids found in olive oil. Nevertheless, the presence of this phenolic compound in EVOO is dependent on various factors, including the variety of olive, the degree of ripeness, and the extraction conditions. However, research concerning its bioavailability and metabolism remains limited. A study employing the single-pass intestinal perfusion (SPIP) method demonstrated that OLA exhibited high intestinal absorption, comparable to that of naproxen, and a substantial presence of OLA metabolites.⁵¹ These observations suggest a potential correlation with the first-pass effect. Consequently, further research is required to elucidate the bioavailability and metabolism of OLA, thereby enabling the estimation of the amount of OLA that reaches the bloodstream following the ingestion of EVOO.

Synovial fibroblasts have been shown to play a significant role in the degradation of the joint matrix in arthritis. This process is driven by the secretion of inflammatory mediators, primarily cytokines like IL-8, TNF- α and IL-6. These cytokines contribute to the sustained activation of regulatory feedback loops, which in turn lead to the production of enzymes, including MMPs (MMP-1, MMP-3, and MMP-13), via the activation of cellular signaling pathways involving MAPKs.¹¹ These cytokines drive a self-perpetuating inflammatory cycle, leading to synovial hyperplasia, lymphocyte influx, and the production of effector proteins that participate in synovitis development and bone destruction. Macrophages are the primary source of IL-1 β and TNF- α , while intimal lining SFs are the main producers of IL-6.⁵²

IL-6 constitutes a further pivotal factor secreted by SFs with the capacity to modulate B cell functions. Beyond its diverse pleiotropic effects, IL-6 activation plays critical roles in the ontogeny of B cells, specifically in their survival, expansion, and maturation, as well as in their differentiation towards the T follicular helper (Tfh) cell lineage through the induction of the transcription factor BCL-6. As a consequence, SFs support B cell function, encompassing the production of autoantibodies, and contribute to the pathogenesis of RA.⁴⁹ Moreover, IL-6 oversecretion is linked to increased MMPs synthesis, modifications in the subchondral bone layer and reduced production of type II collagen.⁵³ Additionally, IL-8 facilitates the recruitment of neutrophils, monocytes, and lymphocytes to inflammatory sites to mitigate the inflammatory response.⁵⁴ Conversely, TNF- α is implicated in early joint swelling and chronic inflammation associated with erosive joint changes.⁵⁵ Furthermore, TNF- α regulates IL-6 and IL-8 production in SFs through the activation of NF- κ B. Therefore, the inhibition of the production of inflammatory cytokines and the prevention of migration, proliferation and invasion of synovial cells could provide an effective solution to the management of arthritic diseases. In this study, we demonstrated that OLA treatment significantly inhibited IL-1 β -induced overproduction of IL-8, IL-6 and TNF- α , in SW982 cells. These findings provide evidence that OLA has the capacity to regulate pro-inflammatory cytokines in human synovial cells.



As zinc-dependent endopeptidases, MMPs such as MMP-1 (collagenase 1) and MMP-3 (stromelysin) have been shown to be released by SFs, which can be directly stimulated by IL-1 β *via* the activation of signal transduction pathways. MMP-1 is a key neutral proteinase that degrades native fibrillar collagens in the extracellular matrix, while MMP-3 is responsible for the degradation of various extracellular matrix components, including proteoglycan and several collagen types, and is crucial for the full activation of collagenases. Furthermore, the ability of MMPs to degrade synovial collagen within the extracellular matrix facilitates the migration and invasion of SFs.^{56,57} The present data demonstrated that IL-1 β stimulation resulted in a significant upregulation of MMP-1 and MMP-3 production in human SW982 cells. Conversely, OLA pretreatment effectively downregulated the levels of both MMPs in these stimulated cells.

COX-2 and mPGES-1 are the principal enzymes mediating the sequential conversion of arachidonic acid to prostaglandins. These enzymes, driving the excessive production of PGE₂ in inflammation, are upregulated by EP4 receptor activation and are critically involved in the inflammatory response.⁵⁸ Our data indicated that IL-1 β stimulation significantly increased the levels of these biomarkers; however, we have demonstrated that OLA effectively decreased the expression of COX-2 and mPGES-1, leading to reduced PGE₂ levels in IL-1 β -stimulated SW982 cells. The present data are consistent with findings reported by Castejón *et al.*, who demonstrated that OL, the most prevalent secoiridoid in olive leaves and unripe olives, decreased the levels of MMP-1 and MMP-3, the cytokines TNF- α and IL-6, and the overexpression of COX-2 and mPGES-1.¹¹

There is growing evidence that shows that multiple stress pathways, including MAPKs and NF- κ B, are involved in the pathogenic mechanisms of inflammation and joint destruction in arthritic diseases.⁵⁹ In fact, NF- κ B is implicated in cellular proliferation, increased cytokine production, inhibition of apoptosis and numerous processes related to arthritis. NF- κ B is also a key factor in the activation and development of Th-1 responses and, along with MAPKs, induces COX-2 and iNOS upregulation. Suppression of NF- κ B signaling has been shown to induce apoptosis and inhibit the proliferation of SFs.⁶⁰ As a dimeric transcription factor, NF- κ B exists in the cytoplasm as an inactive complex bound to the inhibitory protein I κ B- α . An inflammatory signal, such as IL-1 β , induces the activation of the I κ B- α kinase complex, leading to the phosphorylation of I κ B family members. Phosphorylated I κ B becomes ubiquitinated and is subsequently targeted for proteasomal degradation. The resulting NF- κ B dimers can then translocate to the nucleus and modulate gene transcription.⁶¹ The MAPK signaling pathway is pivotal in the pathogenesis of arthritis by modulating key cellular processes; its overactivation is associated with articular cartilage destruction and synovial inflammation. This pathway, encompassing the ERK1/2 subfamilies (implicated in cellular differentiation, proliferation, and survival in response to stress), JNK (primarily involved in cartilage destruction *via* MMPs), and p38 MAPK (central to the inflammatory response, regulating the production of cytokines and

chemokines that contribute to synovial thickening, and inhibiting apoptosis), is considered a significant therapeutic target for rheumatoid arthritis and other chronic inflammatory diseases.⁵⁹ The IL-1 β stimulus was related to increased I κ B α degradation and the activation and phosphorylation of JNK, p38 and ERK, demonstrating the activation of the MAPK and NF- κ B signaling pathways in SW982 cells.^{11,39} On the other hand, pre-treatment with OLA (25 μ M) regulated the inflammatory process *via* the NF- κ B and MAPK pathways inhibiting the degradation of I κ B α , decreasing pp65 protein expression and decreasing the phosphorylation of JNK and ERK MAPKs.

The inflammasome is a multimeric complex key involved in the regulation of the innate immune-inflammatory response.³³ In this study, we focused on the NLRP3 inflammasome, which is up-regulated in patients with rheumatic diseases like RA or OA.⁶² The activation of the canonical NLRP3 inflammasome pathway usually requires two steps. The priming stimulus could be pathogen-associated molecular patterns, such as LPS, and the second step triggers these primed cells with danger signals, such as ATP.⁶³ Upon stimulation, NLRP3 becomes oligomerized, leading to the recruitment and activation of caspase-1 and the maturation of pro-IL-1 β to its bioactive form IL-1 β ,⁶⁴ as demonstrated by our findings. Our results suggest that the anti-inflammatory properties of OLA may be mediated by the inhibition of the NLRP3 inflammasome, due to a reduction in the expression of all components of the canonical inflammasome pathway (NLRP3, ASC and caspase 1) after pre-treatment with our study compound, as well as reduced IL-1 β levels. Furthermore, this study demonstrated for the first time the stimulation of the NLRP3 inflammasome with the dual stimulus LPS + ATP in the SW982 cell line.

In recent years, several lines of evidence have supported the notion that the Keap1/Nrf-2/HO-1 signaling pathway governs a wide array of cytoprotective genes, including those involved in redox regulation, and also in inflammation, immunity, and cartilage and bone metabolism.⁶⁵ Under basal conditions, Nrf-2 is sequestered in the cytoplasm by Keap1 and degraded by the ubiquitin-dependent 26S proteasome system, preventing its translocation to the nucleus. Under oxidative stress conditions, Keap1 is degraded, releasing Nrf-2, which interacts with antioxidant response elements (ARE), inducing the expression of antioxidant enzymes such as HO-1.⁶⁶ HO-1 protein expression could be part of an adaptive mechanism to limit cytotoxicity through the regulation of cell proliferation, the prevention of apoptosis and the scavenging of reactive species. Various *in vivo* and *in vitro* studies have shown that the activation of Nrf-2 can suppress inflammation and preclinical arthritis symptoms.^{67–69} Based on our results, an increase in the expression of the Keap1 repressor protein was observed in the stimulated control cells. According to these findings, Nrf-2 levels were reduced in the presence of IL-1 β and significantly restored in cells treated with OLA. Furthermore, an increase in Nrf-2 expression was observed at the lowest concentration. In a similar manner, HO-1 protein expression was also up-regulated after OLA pretreatment. These findings align with those observed for the secoiridoid



OL.¹¹ Overall, our data indicate that OLA treatment strongly enhanced Nrf-2/HO-1 expression, indicating the potential antioxidant effects of OLA in IL-1 β -stimulated SW982 cells.

Finally, we also investigated potential OLA-induced epigenetic modifications in IL-1 β -activated SW982 cells. Notwithstanding, the fact that the fundamental etiologies of inflammatory arthritis are yet to be fully elucidated, accumulating evidence posits a crucial role for epigenetics in the pathogenesis of these conditions.^{70–72} Specifically, this study focused on DNA methylation, the most studied epigenetic mechanism. DNA methylation functions as a regulatory mechanism that modulates gene expression by impeding the binding of transcription factors to DNA and obstructing the recruitment of proteins implicated in gene repression.^{73,74} This epigenetic alteration arises through the methylation of cytosine residues by the enzymes DNMT1 and DNMT3, whereby a methyl group is transferred to the 5'-carbon of cytosine. DNMT1 is responsible for the maintenance of methylation patterns during cell replication, whereas DNMT3 participates in *de novo* DNA methylation.⁷⁵ In mammals, 5-methylcytosines (5mC) are typically located within CpG islands, which are formed by cytosine–guanine dinucleotides. CpG islands are commonly situated near the promoter regions of genes; consequently, DNA methylation diminishes DNA accessibility, thereby contributing to genetic silencing.⁷⁶ Recent evidence noticed the presence of alterations in the DNA methylation pattern in patients with RA. However, these alterations are variable and manifest as patterns of both hypermethylation and hypomethylation of DNA, depending on the disease stage and the degree of inflammation.⁷⁷ Our results demonstrated global DNA hypomethylation and decreased DNMT3A gene expression in IL-1 β -activated SW982 SFs. These findings align with observations by Liu *et al.* and Nakano *et al.*, who reported reduced DNA hypomethylation and DNMT3 mRNA levels in synovial fibroblasts from patients with RA and in RA fibroblast-like synoviocytes stimulated with IL-1 β , respectively.^{78,79} Interestingly, pretreatment with OLA at its highest concentration (25 μ M) significantly induced the gene expression of DNMT3A, the enzyme responsible for *de novo* methylation. Concurrently, we detected an increase in DNA methylation following fibroblast exposure to OLA at this concentration. Conversely, we observed elevated DNMT1 gene expression in IL-1 β -activated SW982 human synovial cells. This may represent a cellular compensatory mechanism attempting to counteract DNA hypomethylation, given DNMT1's established role in maintaining basal methylation levels. This observation is consistent with previous studies reporting upregulated DNMT1 alongside DNA hypomethylation in CD4+ T cells from lupus patients.⁷⁹ Notably, OLA effectively reversed the effects of IL-1 β by increasing DNMT3A gene expression, upregulating DNA methylation, and downregulating DNMT1 gene expression. These results suggest that OLA holds promise as a methylome regulator, consistent with prior research highlighting its capacity to modulate the epigenome.^{30,33,37,80}

5. Conclusions

In conclusion, the present study has demonstrated the preventive effects of OLA in the oxidative stress and inflammatory response induced by IL-1 β and LPS + ATP in human synovial cells (SW982). These protective effects may be related to the downregulation of MAPKs, inflammasome and NF- κ B signaling pathways, as well as the induction of the Nrf-2/HO-1 axis. These pathways control the production of pro-inflammatory cytokines, MMPs, and PGE₂ levels, as well as the over-expression of COX-2 and mPGEs-1. Furthermore, OLA was also able to regulate epigenetic changes in DNA methylation, potentially acting as an epinutraceutical compound. Our results provide a new dietary strategy for preventing and managing arthritic diseases such as RA and PsA by regulating the epigenome.

Conflicts of interest

There are no conflicts to declare.

Author contributions

Conceptualization: C. A.-d.-l.-L. and M. S.-H.; data curation: R. M.-G., C. A.-d.-l.-L. and M. S.-H.; formal analysis: R. M.-G.; funding acquisition: C. A.-d.-l.-L. and M. S.-H.; investigation: R. M.-G.; methodology: R. M.-G., M. S.-H. and M. P.-S.; project administration: C. A.-d.-l.-L. and M. S.-H.; resources: C. A.-d.-l.-L. and M. S.-H.; software: R. M.-G.; supervision: C. A.-d.-l.-L. and M. S.-H.; validation: C. A.-d.-l.-L. and M. S.-H.; visualization: C. A.-d.-l.-L. and M. S.-H.; writing original draft: R. M.-G. and C. A.-d.-l.-L.; and manuscript revision: C. A.-d.-l.-L. and M. S.-H. All authors have read and agreed to the published version of the manuscript.

Data availability

The data supporting this study are available within the article. Other relevant data can be shared upon reasonable request.

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