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Anti-inflammatory effects of oleic acid and the anthocyanin keracyanin alone and in combination: effects on monocyte and macrophage responses and the NF-κB pathway

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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Monocyte recruitment and activation of macrophages are essential for homeostasis but are also related to the development and progression of cardiometabolic diseases. The management of inflammation with dietary components has been widely investigated. Two components that may influence inflammation are unsaturated fatty acids such as oleic acid (OA; 18:1cis-9) and antioxidant compounds like anthocyanins. Molecular and metabolic effects of such bioactive compounds are usually investigated in isolation, whereas they may be present in combination in foods or the diet. Considering this, we aimed to analyze the effects of OA and the anthocyanin keracyanin (AC) alone and in combination on toll-like receptor-mediated inflammatory responses in monocytes and macrophages. For this, THP-1-derived macrophages and monocytes were exposed to 3 treatments: OA, AC, or the combination (OAAC) and then stimulated with lipopolysaccharide. Inflammation-related gene expression and protein concentrations of IL-1β, TNF-α, IL-6, MCP-1, and IL-10 were assessed. Also, NFκBp65, IκBα, and PPAR-γ protein expression were determined. OA, AC, and OAAC decreased pNFκBp65, PPARγ, IκBα, TNF-α, IL-1β, IL-6, and MCP-1 and increased IL-10. MCP-1 protein expression was lower with OAAC than with either OA and AC alone. Compared to control, OAAC decreased mRNA for TLR4, IκKα, IκBα, NFκB1, MCP-1, TNF-α, IL-6, and IL-1β more than OA or AC did alone. Also, IL-10 mRNA was increased by OAAC compared with control, OA, and AC. In summary, OA and AC have anti-inflammatory effects individually but their combination (OAAC) exerts a greater effect.

Introduction

Inflammation is essentially a protective mechanism, representing the first biological response to harmful agents and damaged tissues, and contributing to the maintenance of homeostasis [1]. However, inflammation itself can become pathological [1]. Furthermore, several metabolic diseases result from long-term inflammatory processes becoming detrimental [2–4]. An important event in initiating pro-inflammatory responses is the recruitment of monocytes and their activation and differentiation to macrophages [5]. A common mechanism of activation of monocytes and macrophages is through toll-like receptors (TLRs), a family of transmembrane type I receptors that are essential for the recognition of, and response to, pathogens [6]. TLR4 signaling through nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) culminates in altered patterns of gene expression and secretion of several pro-inflammatory cytokines [7–9]. These cytokines have been shown to play critical roles in both classic inflammatory diseases [1] and inflammation-associated metabolic diseases such as obesity, diabetes, and metabolic syndrome [3,4,10]. Another family of transcription factors, the peroxisome proliferator-activated receptors (PPARs) act to inhibit TLR4 signaling and NFκB activation, therefore exhibiting anti-inflammatory effects [11].

Dietary patterns and specific dietary components can influence the inflammatory response. In this sense, a Western diet pattern is recognized for its deleterious and pro-inflammatory effects. On the other hand, the Mediterranean diet is recognized for its anti-inflammatory and health-promoting properties [12]. The beneficial effects of the Mediterranean diet are attributed, at least in part, to moderate consumption of polyphenols like anthocyanins (AC) and a major contribution to dietary fat from the monounsaturated fatty acid oleic acid (OA)

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[13–15]. However, it is unlikely that a single component of the Mediterranean diet is capable of exerting the same effects as the complete dietary pattern, suggesting that the presence of multiple nutrients, which might interact synergistically, is likely responsible for the health benefits of the diet [15,16].

Although access to the Mediterranean diet is limited to a specific population and geographic region, these same considerations will apply to other healthy diets patterns and indeed to individual foods found in different cultures around the world like avocado, açai berry, and nuts that contain both bioactive fatty acids like OA and polyphenolic compounds [17,18].

Bioactive compounds from foods may exert pro- or anti-inflammatory actions and the latter effect is relevant to the prevention and reduction of inflammatory responses and processes [19]. OA has been reported to have anti-inflammatory effects and to protect against insulin resistance and cardiovascular diseases [20–23]. Anthocyanins and related molecules (e.g., AC) have been reported to modulate lipid accumulation in THP-1 cell-derived macrophages, preventing their conversion into foam cells [24–26]. *In vitro* molecular investigations of such compounds in isolation ignore that they may be found together in individual foods or a meal and there is a lack of studies of combining such bioactive compounds. Therefore, this study aims to investigate the anti-inflammatory effects of OA and AC individually and in combination by assessing TLR-mediated inflammatory responses in monocytes and macrophages after lipopolysaccharide (LPS) challenge.

Material and Methods

THP-1 cell culture

Human leukemia-derived THP-1 monocytic cells (ATCC®, LGC standards, Middlesex, UK) were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), L-glutamine (2 mM), and penicillin/streptomycin (100 U/ml/100 µg/ml), purchased from Sigma-Aldrich (Gillingham, UK). Cells were kept in a humidified environment at 37°C and 5 % CO_2 in polystyrene culture flasks. THP-1 cells were cultured during the exponential growth phase keeping cell number between 2×10^5 and 1×10^6/mL of medium and transferring to new medium every 3 days for no longer than subculture passage 20, according to the standard protocol provided by the American Type Culture Collection (ATCC®; LGC Standards, Middlesex, UK).

THP-1 macrophages-like differentiation

The differentiation of monocytes into a macrophage-like phenotype was induced by exposure to 25 nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich; Gillingham, UK) for 72 h followed by a resting period of 24 h before experimental procedures. The differentiation protocol used was previously optimized by Lund [27].

Concentrations of oleic acid and anthocyanin used

Considering the monounsaturated fatty acid OA and moderate polyphenol (e.g., anthocyanins) consumption as components of an anti-inflammatory and antioxidant diet, this study investigated the effect of treating THP-1 cells with OA and AC in isolation or in combination at a molar ratio of 100:0.8 µM of OA to AC. These concentrations were based on preliminary experiments where THP-1 cells remained viable after exposure up to 100 mM OA and 0.8 mM AC (section Preliminary experiments with OA and AC), and in accordance with previous studies of such compounds reporting non-toxic concentrations [25,28].

OA (C18:1n-9) and AC (keracyanin - cyanidin-3-rutinoside) (Cambridge Bioscience Ltd.; Cambridge, UK) were reconstituted as stock solutions in 100% ethanol and stored at –80°C until use. Before each experiment, the stock solutions were diluted in complete RPMI culture medium to yield three different final concentrations of OA (10 µM, 50 µM, 100 µM), AC (0.08 µM, 0.4 µM, 0.8 µM) or OAAC (10 µM+0.08 µM, 50 µM+0.4 µM, 100 µM+0.8 µM) in 0.1% ethanol. The corresponding control was a 0.1% ethanol solution diluted in complete RPMI medium.

THP-1 lipopolysaccharide stimulus

To determine the best concentration and exposure time for LPS (*E. coli* O111: B4; Sigma-Aldrich; Gillingham, UK) as a pro-inflammatory stimulus, dose-response and time-course experiments were conducted. The LPS doses tested were between 0.01 and 10 µg/mL and time points were between 0 and 48 h. Cytokine production by both THP-1 cells and THP-1-derived macrophages was very low in the absence of LPS stimulation and was not influenced by OA or AC in the absence of LPS (data provided in supplementary material in Fig. S1 and S2). An LPS concentration of 0.1 µg/mL was selected for use based upon these preliminary experiments.

THP-1 cells and THP-1-derived macrophages were exposed to treatment with OA or AC or their combination for 48 h, followed by exposure to 0.1 µg/mL of LPS (*E. coli* O111: B4; Sigma-Aldrich; Gillingham, UK) for up to 48 h. At the end of the incubation, the cell pellets and supernatants were collected for further analyses. Each set of experiments was performed in triplicate and on three independents occasions. Fig. 1 summarizes the experimental protocol.

Cell Viability - MTT Assay

For the assessment of cell viability, THP-1 cells or THP-1-derived macrophages were seeded in 96 well plates (5×10^4/ well). At the end of the culture period, 100 µL of medium containing 0.05 mg/mL of MTT [3-(4, 5-dimethylthiahiazol-2-y1)-2, 5-diphenyl tetrazolium bromide)] from Sigma-Aldrich (Gillingham, UK) was added to each well, and the cells cultured for 4 h. After this, the supernatant was removed (75 µL) and replaced by 85 µL dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Gillingham, UK). Absorbance was measured at 550 nm on a microplate spectrophotometer. Data for each treatment were normalized to the control cultures (no OA or AC added) set at 100%. A cut-off point of < 75% was chosen to indicate loss of viability.

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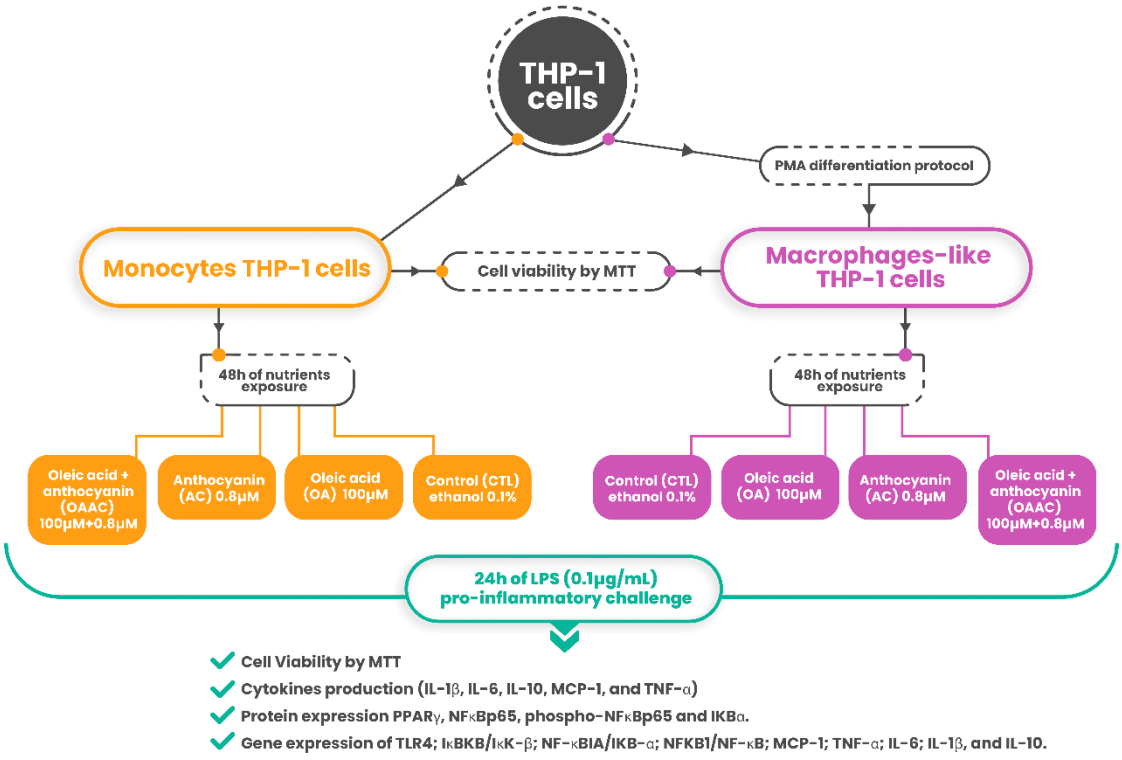

Fig. 1. Experimental protocol description. THP-1 cells were differentiated into a macrophage-like phenotype. Monocytes and macrophages were exposed to oleic acid (100 µM), keracyanin (0.8 µM) or oleic acid + keracyanin (100 µM+0.8 µM) for 48 h, followed by a pro-inflammatory challenge with LPS (0.1 µg/mL) for 24 h. Multiple outcomes were assessed.

Western blotting

The expression of inflammatory pathway proteins was determined by Western blotting. Cells were seeded in 6 well plates at a density of 2 x 10^6/well. After treatment, cells were incubated for 30 minutes in 200 µL of RIPA buffer, vortexing every 10 minutes. Then samples were centrifuged at 20,800 × g for 10 min at 4◦C, and the supernatant was collected. The protein concentration was measured by the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Loughborough, UK) and normalized to 30 µg of protein per sample. The 10% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad Laboratories Inc., Hercules, CA, USA) were used for electrophoretic separation of proteins which were then transferred to PVDF membranes (Bio-Rad Laboratories Inc., Hercules, CA, USA). Membranes were blocked for 1 h with 5% skimmed milk (Marvel, UK) solution at room temperature. The membranes were then incubated overnight in a cold room at 4ºC with the following primary antibodies: PPARγ (ab191407), NFκBp65 (ab32536), phospho-NFκBp65 (ab86299), IKBα (ab32518). Aiming to match the real-time qPCR data and considering its widespread use, GAPDH (ab181602) was chosen as the housekeeping protein. Primary antibodies were supplied by Abcam (Abcam, Cambridge, UK). Specific horseradish peroxidase-conjugated secondary antibodies were incubated with the membranes for 1 h at room temperature. Bands were visualized using enhanced chemiluminescence in a Syngene GeneGnome XRQ (Cambridge, UK) after adding ECL reagent (Thermo Fisher Scientific, Loughborough, UK). Scion Image (Scion Image-Release Beta 3b; NIH, Frederick, MD, USA) was used to quantify the band intensity.

RNA isolation, reverse transcription, and real-time qPCR

Cells were seeded in 24 well plates at a density of 5 x 10^5/well and treated as previously described. After treatment, RNA was extracted using the ReliaPrep™ RNA cell miniprep system (Promega, Southampton, UK) following the supplier's recommendations. RNA was quantified in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA), and RNA purity was checked by the 260/280 nm ratio, requiring values between 1.7 and 2.1. One microgram of total

RNA was reverse transcribed using a GoScript™ Reverse Transcription System kit (Promega, Southampton, UK) to synthesize complementary DNA (cDNA). Levels of mRNA for inflammation-related proteins were assessed by RT-qPCR with the TaqMan® fluorophore marker (Thermo Fisher Scientific, Loughborough, UK) using StepOne 7500 fast equipment (Thermo Fisher Scientific, Loughborough, UK). The gene expression of TLR4 (Hs00152939_m1); IκBKB/IκK-β (Hs00233287_m1); NF-κBIA/IKB-α (Hs00355671_g1); NFKB1/NF-κB (Hs00765730); MCP-1 (Hs00234140_m1); TNF-α (Hs00174128_m1); IL-6 (Hs00174131_m1); IL-1β (Hs01555410_m1), and IL-10 (Hs0096162_m1) was quantified by the comparative method using the expression of peptidylprolyl isomerase B (PPIB; Hs00168719_m1) and phosphoglycerate kinase 1 (PGK1; Hs00943178_g1) as the control genes. Housekeeping genes were chosen based on a previous study with THP-1 cells [29]. Results were expressed as the relative increase, using the 2−ΔΔCt method previously described by Livak & Schmittgen [30].

Luminex Assay

The concentrations of IL-1β, IL-6, IL-10, MCP-1, and TNF-α were measured simultaneously in cell culture supernatants (5 x 10^5 cells/well in 1 mL culture medium) using a Human Magnetic Luminex Screening Assay ELISA (R&D Systems, Minneapolis, MN, USA), performed according to the manufacturer's instructions. Samples were read on a Bio-plex 200 System (Bio-Rad Laboratories Inc., Hercules, CA, USA). Lower limits of detection were: IL-1β (4.2 pg/mL), IL-6 (1.1 pg/mL), IL-10 (1.0 pg/mL) MCP-1(7.3 pg/mL) and TNF-α (1.9 pg/mL).

Data Analysis

Data were submitted to Grubb's test to remove significant outliers. Analysis of variance (two-way or one-way ANOVA) followed by Bonferroni post-hoc test was used. The level of significance was set at $p < 0.05$. Data are described as mean ± S.E.M. Statistical analysis was performed using the PASW Statistics software version 22.0 (IBM Corp., Armonk, NY, USA). All data shown refer to three independent experiments each performed in triplicate.

Results

Viability of THP-1 monocytes and THP-1-derived macrophages incubated with LPS, OA, AC, and OAAC

In comparison to control cultures, higher concentrations of LPS (1.0 and 10.0 μg/mL) reduced viability of THP-1 monocytes at 24 h ($p < 0.01$ and $p < 0.001$, respectively) and at 48 h (both $p < 0.0001$), and reduced viability of THP-1-derived macrophages (both $p < 0.0001$ at 24 h and 48 h) as shown in Fig. 2. Based on these observations, the LPS concentration of 0.1 μg/mL and the maximum incubation time of 24 h was selected for further experiments aiming to maintain optimum cell viability.

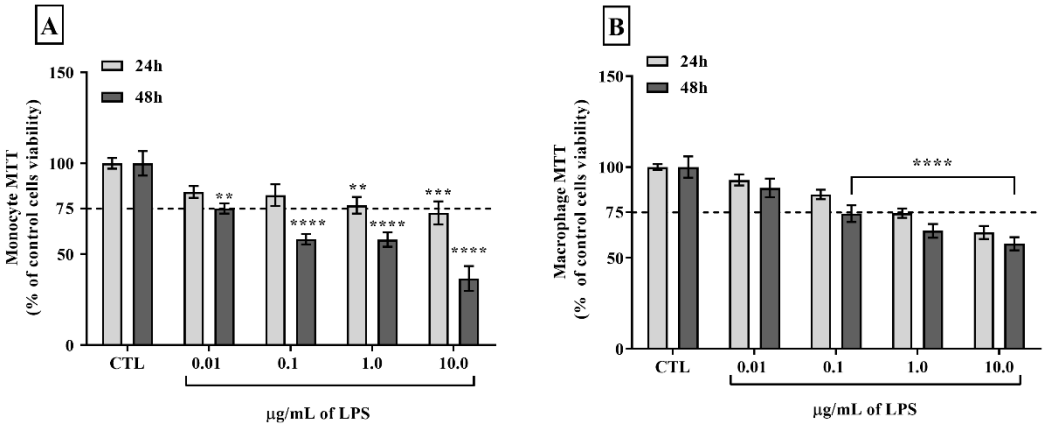

Fig. 2 Cell viability after incubation with different concentrations of LPS for [A] THP-1 monocytes and [B] THP-1-derived macrophages. The cutoff point of 75% was selected as the threshold for viability. Data are mean ± SEM. (n = 3, two-way ANOVA with Bonferroni post hoc test (**p < 0.01; ***p < 0.001; ****p < 0.0001 versus CTL).

Neither OA nor AC affected the viability of THP-1 monocytes or THP-1-derived macrophages at the different concentrations tested (Fig. 3).

Preliminary experiments with OA and AC

Most of the cytokines were undetectable or at the lower boundary of the detection limit in the medium of THP-1 monocytes or THP-1-derived macrophages incubated with OA, AC, or their combination but without LPS (Fig. S1 and S2). However, cells stimulated with LPS had markedly increased cytokine concentrations in their supernatant (Fig. S1 and S2). Additionally, higher concentrations of OA (100 μM) and AC (0.8 μM) exerted stronger anti-inflammatory effects on cytokine production after LPS exposure of both monocytes (Supplementary material Fig. S1) and macrophages (Supplementary material Fig. S2). Thus, further experiments used the concentrations of 100 μM OA and 0.8 μM AC.

Effects of OA, AC, and OAAC on signaling protein expression in THP-1 monocytes and THP-1-derived macrophages stimulated with LPS

A time course for inflammatory signaling protein expression was performed (Fig. S3). The cells were exposed to LPS (0.1 μg/mL) for up to 3 h aiming to evaluate the changes in NFκBp65 and PPARγ protein expression assessed by Western blotting. LPS decreased NFκBp65 and PPARγ in both cell types (Supplementary material Fig. S3). Based on these findings, an incubation time of 2 h was used in further experiments.

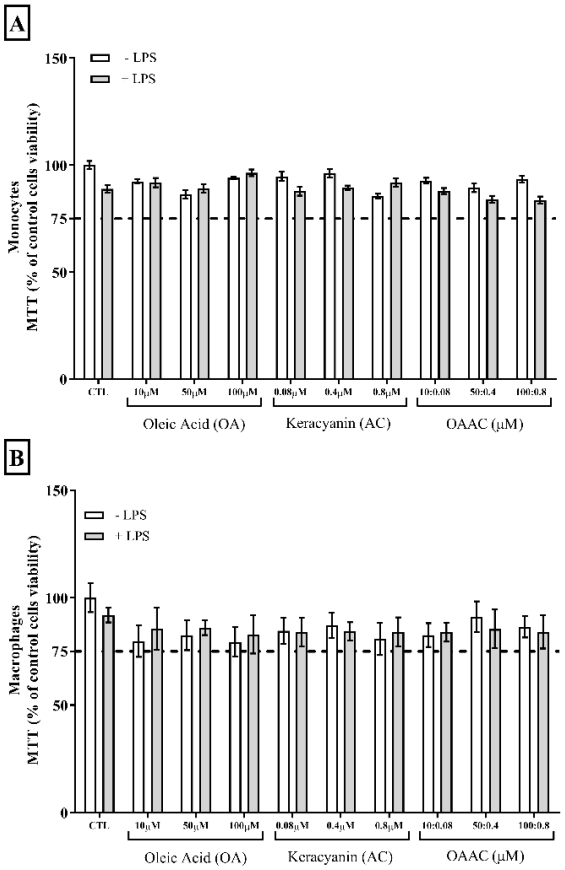

Fig. 3 Cell viability after incubation with oleic acid and keracyanin at different concentrations for [A] THP-1 monocytes and [B] THP-1-derived macrophages (n = 3, two-way ANOVA with Bonferroni post hoc test: no significant differences). Cells were cultured for 48 h with RPMI (0.1% of ethanol, CTL) or different concentrations of oleic acid (10 μM, 50 μM, 100 μM), keracyanin (0.08 μM, 0.4 μM, 0.8 μM) or oleic acid + keracyanin (10 μM+0.08 μM, 50 μM+0.4 μM, 100 μM+0.8 μM), followed by incubation with (+) or without (−) LPS (0.1 μg/mL) for 24 h. The cutoff point of 75% was selected to indicate viability. Data are mean ± SEM.

OA, AC, or their combination did not significantly affect phosphorylation of NFκBp65, total NFκBp65, or their ratio (although this tended to be decreased by about 50% with both compounds and their combination) or IκBα in THP-1 monocytes (Fig. 4). However, OAAC significantly decreased PPARγ protein compared to the CTL group ($p < 0.05$), while the individual compounds tended to decrease PPARγ in monocytes (OA $p = 0.063$ and AC $p = 0.072$) (Fig. 4).

OA, AC and their combination significantly decreased phosphorylation of NFκBp65 ($p < 0.001$ for OA and $p < 0.0001$ for AC and OAAC) with a tendency to decrease total NFκBp65 ($p = 0.083$ for AC and $p = 0.084$ for OAAC) in THP-1-derived macrophages (Fig. 5C, D). OAAC significantly decreased the ratio of phosphoNFκBp65/NFκBp65 compared to CTL ($p < 0.01$) and to OA ($p < 0.05$) (Fig. 5E). IκBα expression was decreased by AC ($p< 0.001$), OA and OAAC ($p < 0.0001$) versus the CTL THP-1-derived macrophages (Fig. 5A). OA, AC and OAAC all significantly decreased PPARγ protein compared to the CTL cells (all $p < 0.0001$) (Fig. 5B). No significant differences were observed among OA, AC or the combination (Fig. 5A, B, C, D).

Effects of OA, AC, and OAAC on inflammatory gene expression in THP-1 monocytes and THP-1-derived macrophages stimulated with LPS

A time course for gene expression in response to LPS was performed (data provided in supplementary material Fig. S4 and S5) considering that changes in gene expression are the primary response to a stimulus. Thus, LPS induced marked time-dependent increases in mRNA for IL-6, IL-1β, MCP-1, TNF-α, and IL-10 in both cell types (Fig. S4 and S5). Based on these findings, an optimum incubation time of 6 h was used in further experiments.

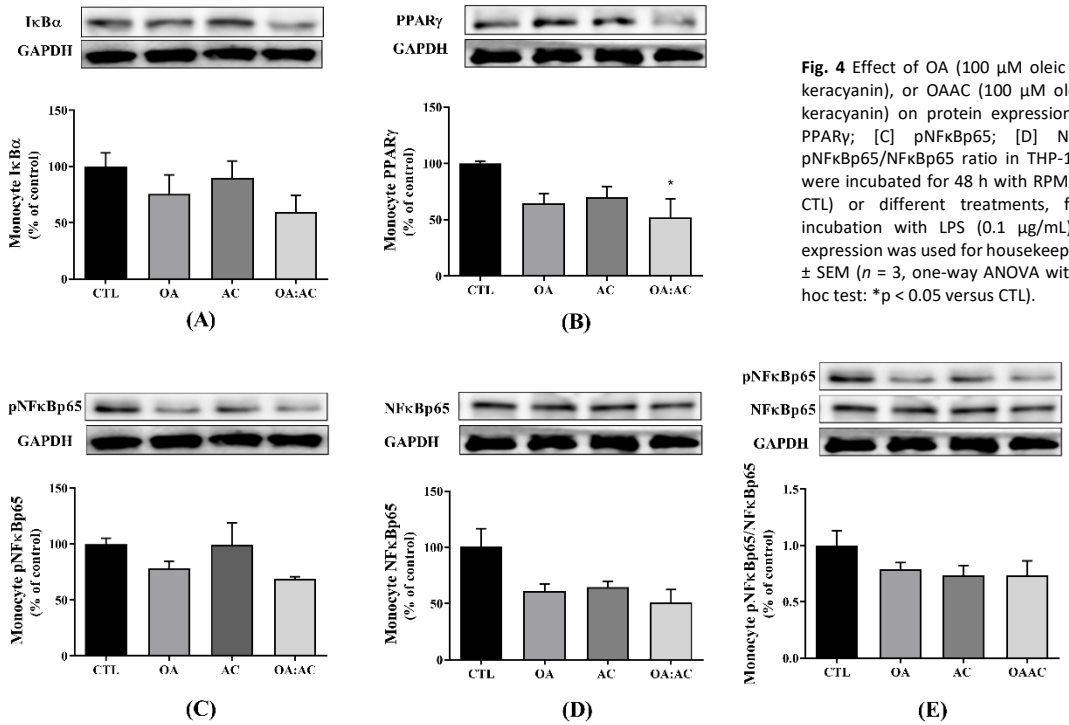

Fig. 4 Effect of OA (100 μM oleic acid), AC (0.8 μM keracyanin), or OAAC (100 μM oleic acid + 0.8 μM keracyanin) on protein expression of [A] IκB-α; [B] PPARγ; [C] pNFκBp65; [D] NFκBp65 and [E] pNFκBp65/NFκBp65 ratio in THP-1 monocytes. Cells were incubated for 48 h with RPMI (0.1% of ethanol, CTL) or different treatments, followed by 2 h incubation with LPS (0.1 μg/mL). GAPDH protein expression was used for housekeeping. Data are mean ± SEM (*n* = 3, one-way ANOVA with Bonferroni post-hoc test: *$p < 0.05$ versus CTL).

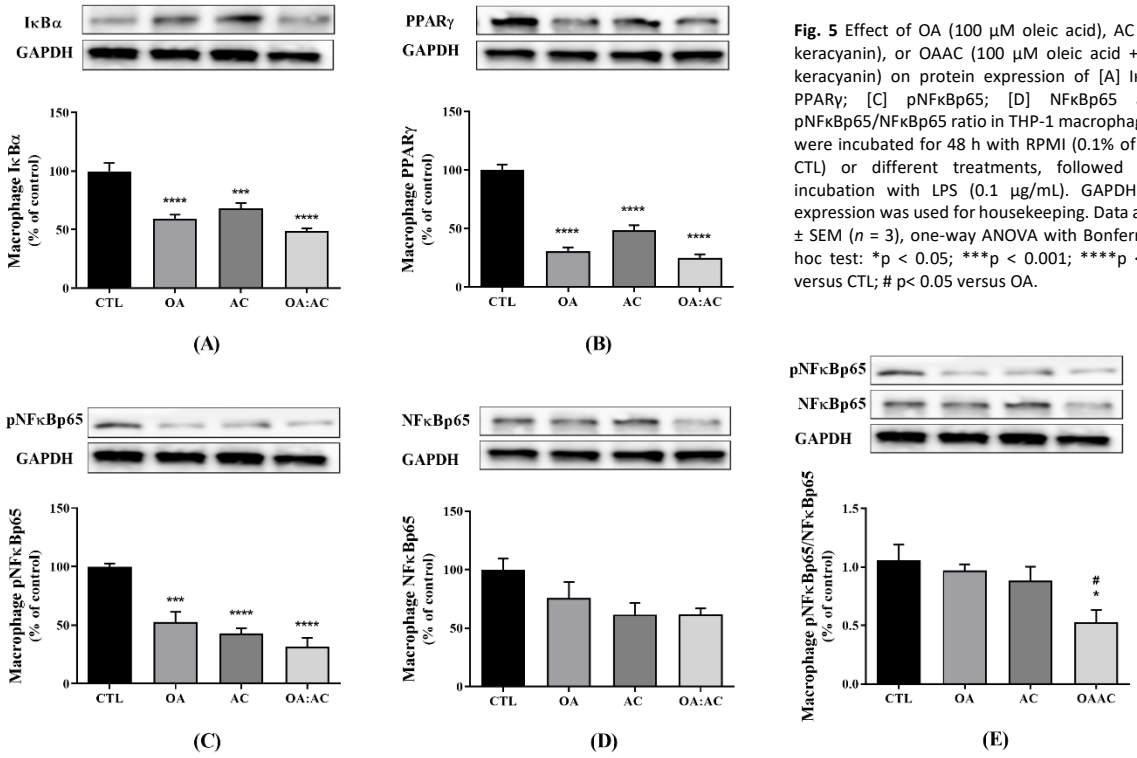

Fig. 5 Effect of OA (100 μM oleic acid), AC (0.8 μM keracyanin), or OAAC (100 μM oleic acid + 0.8 μM keracyanin) on protein expression of [A] IκB-α; [B] PPARγ; [C] pNFκBp65; [D] NFκBp65 and [E] pNFκBp65/NFκBp65 ratio in THP-1 macrophages. Cells were incubated for 48 h with RPMI (0.1% of ethanol, CTL) or different treatments, followed by 2 h incubation with LPS (0.1 μg/mL). GAPDH protein expression was used for housekeeping. Data are mean ± SEM (*n* = 3), one-way ANOVA with Bonferroni post hoc test: *p < 0.05; ***p < 0.001; ****p < 0.0001 versus CTL; # p< 0.05 versus OA.

Compared to CTL, OA decreased mRNA for IκBα ($p < 0.01$), MCP-1 ($p < 0.001$) and IL-6 ($p < 0.0001$) and tended to decrease mRNA for TNF-α ($p = 0.056$) in THP-1 cells (Fig. 6). AC decreased mRNA for IL-6 ($p < 0.01$) and MCP-1 ($p < 0.05$) and tended to decrease mRNA for IκBα ($p = 0.069$) and TNF-α ($p = 0.059$) (Fig. 6). The combination of OA and AC decreased mRNA for TLR4 ($p < 0.01$), IκBα ($p < 0.001$), MCP-1 ($p < 0.0001$), TNF-α ($p < 0.01$), IL-6 ($p < 0.0001$) and IL-1β ($p <0.05$). The combination also decreased IL-1β mRNA compared to OA alone and IL-6 and IL-1β mRNA compared to AC alone (Fig. 6).

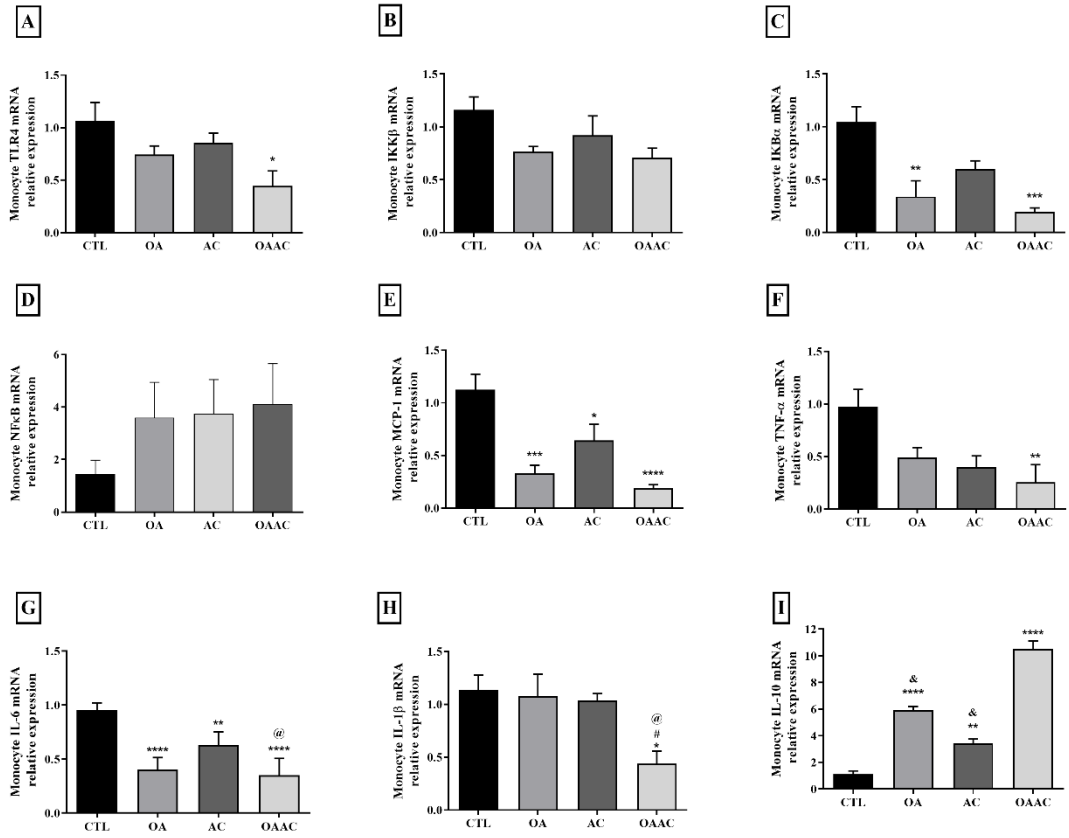

Fig. 6 Effect of OA (100 μM oleic acid), AC (0.8 μM keracyanin), or OAAC (100 μM oleic acid + 0.8 μM keracyanin) in gene expression of [A] TLR4; [B] IκKβ; [C] IκBα; [D] NFκB1; [E] MCP-1; [F] TNF-α; [G] IL-6; [H] IL-1β; [I] IL-10 in LPS-stimulated THP-1 monocytes. Cells were incubated for 48 h with RPMI (0.1% of ethanol, CTL) or different treatments, followed by 6 h incubation with LPS (0.1 μg/mL). Housekeeping genes used were PPIB and PGKI. Data are mean ± SEM (*n* = 3), one-way ANOVA with Bonferroni post-hoc test: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 versus CTL; # p< 0.05 versus OA; @ p< 0.05 versus AC; & p < 0.0001 versus OAAC.

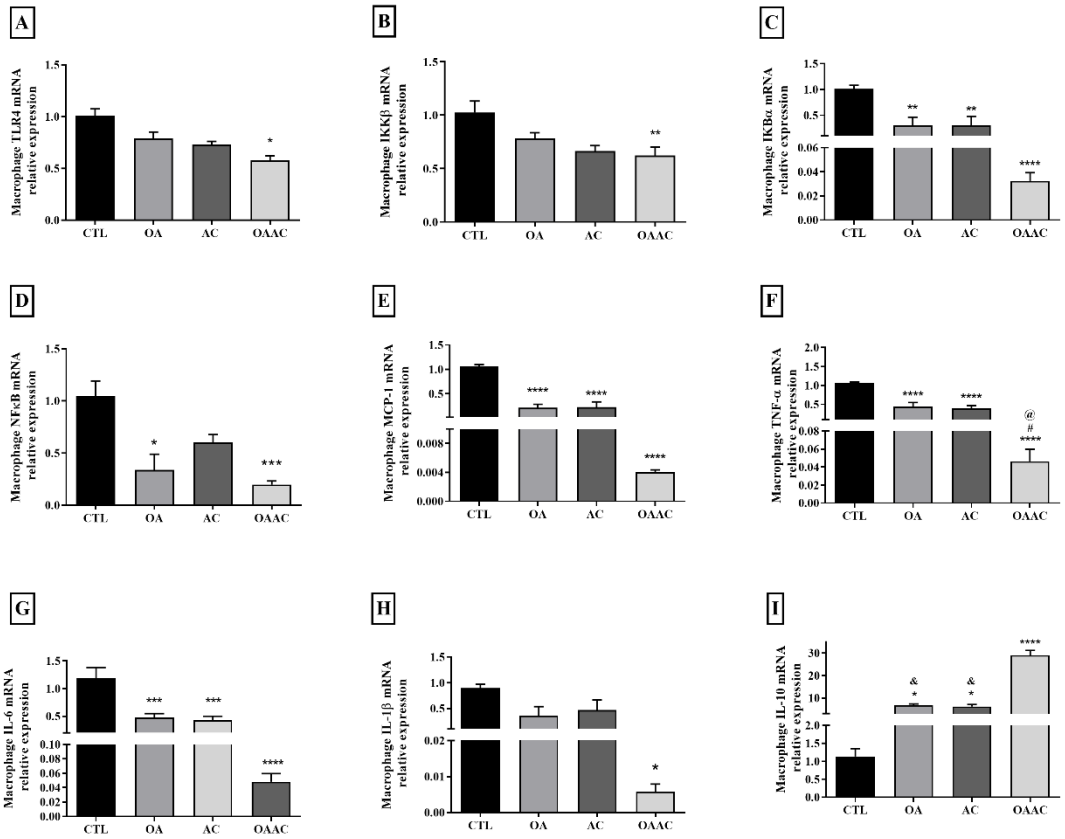

Fig. 7 Effect of OA (100 μM oleic acid), AC (0.8 μM keracyanin), or OAAC (100 μM oleic acid + 0.8 μM keracyanin) on mRNA expression of [A] TLR4; [B] IκKβ; [C] IκBα; [D] NFκB1; [E] MCP-1; [F] TNF-α; [G] IL-6; [H] IL-1β; [I] IL-10 in THP-1-derived macrophages stimulated with LPS. Cells were incubated for 48 h with RPMI (0.1% of ethanol, CTL) or different treatments, followed by 6 h incubation with LPS (0.1 μg/mL). The housekeeping genes were PPIB and PGKI. Data are mean ± SEM (*n* = 3), one-way ANOVA with Bonferroni post-hoc test: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 versus CTL; $ p< 0.05 versus OA; # p< 0.01 versus OA; @ p< 0.01 versus AC; & p < 0.0001 versus OAAC.

Finally, IL-10 mRNA was increased by OA ($p < 0.0001$), AC ($p < 0.01$) and OAAC ($p < 0.0001$) compared to CTL. Furthermore, the combination (OAAC) had higher IL-10 mRNA compared to OA or AC alone (both $p < 0.0001$) (Fig. 6).

Compared to CTL, AC decreased mRNA for IκBα ($p < 0.01$), OA decreased mRNA for NFκB ($p < 0,.05$), and both OA and AC decreased mRNA for IκBα (both $p < 0.01$), MCP-1 ($p < 0.0001$), TNF-α (both $p < 0.0001$) and IL-6 (both $p < 0.001$) in THP-1-derived macrophages (Fig. 7). Compared to CTL, the combination of OA and AC decreased mRNA for TLR4 ($p < 0.05$), IκKα ($p < 0.01$), IκBα, NFκB1, MCP-1, TNF-α, IL-6 (all $p < 0.0001$) and IL-1β ($p < 0.05$). The combination of OA and AC resulted in lower mRNA for TNF-α than either OA or AC alone (both $p < 0.05$).

Finally, IL-10 mRNA was increased by OA ($p < 0.05$), AC ($p < 0.05$) and OAAC ($p < 0.0001$) compared with CTL. Furthermore, the combination (OAAC) had higher IL-10 mRNA compared to OA or AC alone (both $p < 0.05$) (Fig. 7).

Effects of OA, AC, and OAAC on cytokine production by THP-1 monocytes and THP-1-derived macrophages stimulated with LPS

Cytokine production was assessed by measuring cytokine concentrations in the supernatants of LPS-stimulated THP-1 cells or THP-1-derived macrophages (Fig. 8). Compared to CTL, OA decreased MCP-1 production by THP-1 monocytes ($p < 0.05$), while AC decreased production of TNF-α ($p < 0.05$), IL-1β ($p < 0.01$), IL-6 ($p < 0.01$) and MCP-1 ($p < 0.001$). Compared to CTL, the combination of OA and AC decreased production of TNF-α ($p < 0.01$), IL-1β ($p < 0.001$), IL-6 ($p < 0.0001$) and MCP-1 ($p < 0.0001$). Production of IL-1β and IL-6 after treatment of THP-1 cells with AC or OAAC was different from when the cells were treated with OA alone (all $p < 0.05$). Neither OA nor AC affected IL-10 production by THP-1 monocytes.

In THP-1-derived macrophages, compared to CTL, OA, AC and the combination of OA and AC decreased production of TNF-α (all $p < 0.0001$), IL-1β ($p < 0.05$, < 0.01, < 0.001, respectively), IL-6 ($p < 0.001$, 0.0001, 0.0001, respectively), and MCP-1 ($p < 0.001$, < 0.001, < 0.0001, respectively) (Fig. 8). MCP-1 production was lower after the combination of OAAC than after either OA or AC alone (both $p < 0.05$). In contrast to THP-1 monocytes OA, AC and their combination increased IL-10 production by THP-1-derived macrophages (all $p < 0001$), with the combination having a greater effect than either OA or AC alone (both $p < 0.05$) (Fig. 8).

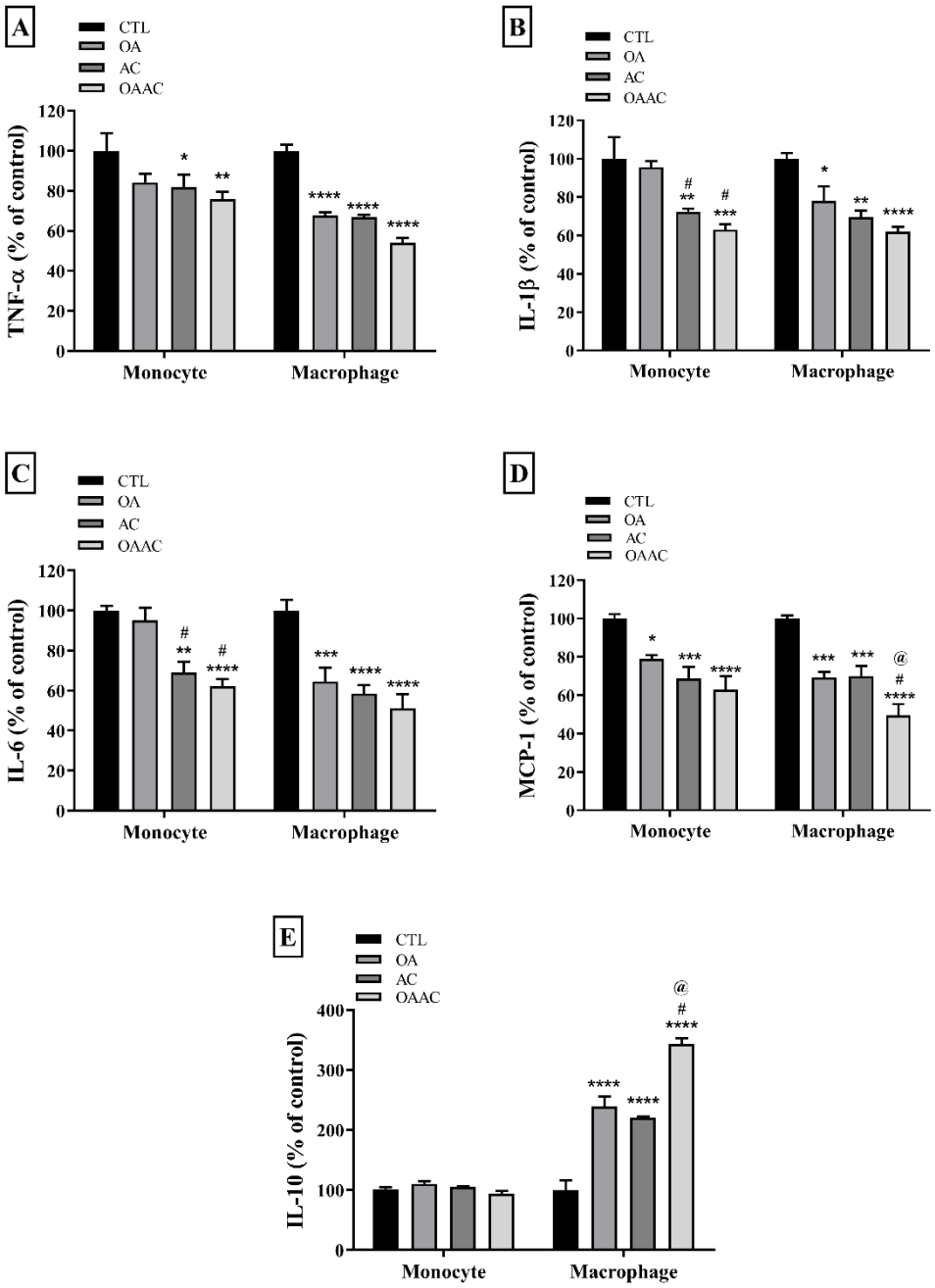

Fig. 8 Effect of OA (100 µM oleic acid), AC (0.8 µM keracyanin), or OAAC (100 µM oleic acid + 0.8 µM keracyanin) on supernatant concentrations of [A] TNF-α; [B] IL-1β; [C] IL-6; [D] MCP-1; [E] IL-10 after LPS stimulation of THP-1 monocytes and THP-1-derived macrophages. Cells were incubated for 48 h with RPMI (0.1% of ethanol, CTL) or different treatments, followed by 24 h incubation with LPS (0.1 µg/mL). Data are mean ± SEM (*n* = 3), two-way ANOVA with Bonferroni post-hoc test: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 versus CTL; # p< 0.01 versus OA and @ p< 0.01 versus AC.

Discussion

This study confirms that OA and AC individually and combined have anti-inflammatory properties, acting to decrease the TLR4-NFκB signaling pathway resulting in reduced production of pro-inflammatory cytokines, an effect especially evident in macrophages. One component of this anti-inflammatory effect of OAAC on macrophages is the increased production of IL-10. The findings are in general accordance with previous reports [25,26,31,32]. This study also demonstrates that the combination of OA and AC displayed a significantly stronger anti-inflammatory effect on monocytes and macrophages compared with the individual components. This may suggest independent actions of OA and AC or that there is an interaction between them.

Even though neither OA nor AC are essential nutrients, it is noteworthy that both are able to exert biologically positive effects [12]. OA is found in many plant foods such as avocado and açai berry pulp, and in many plant oils, particularly olive oil. Several studies have shown that OA has anti-inflammatory effects in diverse experimental

models [32–34]. AC is a polyphenolic pigment widespread in the red-orange to blue-violet plant food sources such as berries and grapes. AC has also been demonstrated to have anti-inflammatory effects [24,25,35]. Although the anti-inflammatory actions of OA and AC have been described, and are confirmed in the current study, the effects of their combination (OAAC), as found in the Mediterranean diet, have not been explored previously in molecular *in vitro* studies.

In general, THP-1-derived macrophages showed higher responsiveness to LPS than THP-1 monocytic cells, and the macrophages were more sensitive to OA, AC, and their combination than the monocytes. Physiologically, monocytes circulate in the bloodstream and are attracted to sites of infection or inflammatory activity where they differentiate into macrophages [5,36]. Macrophages are strong effectors of the inflammatory response within tissues. The difference in monocyte and macrophage responsiveness to LPS, as seen in the current study, fits with these different roles of the two related cell types. Here we studied several components of the response to LPS including the transcription factor system, inflammatory gene expression, and the production of inflammatory cytokines. All components were affected by OA and AC or their combination in both monocytes and macrophages but to differing extents.

We anticipated that the NFκB pathway would be inhibited by OA and AC. Thus, the lower level of phosphorylated NFκBp65 in macrophages incubated with OA and AC was expected. However, an important finding is that OAAC significantly decreased the ratio of phosphoNFκBp65/NFκBp65 compared to CTL and to OA alone, demonstrating a stronger effect of modulating the pro-inflammatory pathway in competent cells like macrophages. Also, IκBα expression was significantly decreased in macrophages by AC, OA, and OAAC compared to CTL, and despite the lack of statistical difference among the isolated or combined treatments, the OAAC combination showed the lowest expression of IκBα. Interestingly, the mRNA data for Ikkβ and NFκB, did not differ among the isolated or combined compound treatments. This suggests that the anti-inflammatory effects seen could be due to post-transcriptional effects with a reduction in synthesis of these proteins [39]. In fact, the Mediterranean diet has been recently related to modulating circulating exosomal non-coding RNAs, affecting a higher number of miRNAs. [56]. An epigenetic action in particular on miRNAs has also been shown, and this could lead to modulation of gene expression by binding to the complementary regions of the 3'UTR sequence of specific mRNA targets[,57]. The modulation of non-coding RNAs such as miRNAs could help to explain some of the effects observed here and requires further investigation.

Another expected effect was an enhancement of the PPARγ system. However, the lower level of PPARγ found after treatment with OA or AC, especially in macrophages, was not expected. Nevertheless, down-regulation of PPARγ could be beneficial in some settings since it has been shown that genes activated by PPARγ stimulate cellular lipid uptake, and PPARγ is highly expressed in lipid-accumulating macrophages where it modulates macrophage lipid metabolism which might contribute to atherogenesis [37,38].

The inhibition of pNFκBp65 expression leads to a reduction of gene expression of pro-inflammatory cytokines [39]. Therefore, modifications of gene expression would be expected upon treatment with OA and AC, especially in macrophages. Indeed, genes related to the TLR4-NFκB signaling system and a range of inflammatory cytokines were modulated by OA and AC, more prominently in macrophages than in monocytes. Specifically, the expression of the TLR4 and TNF-α genes in macrophages and the IL-1β gene in monocytes responded to OAAC with a reduction in these pro-inflammatory mRNAs, indicating promotion of an anti-inflammatory state by OAAC. These observations suggest that reduced activation of NFκB in macrophages (less phosphorylated NFκB) in the presence of OA, AC, and OAAC results in reduced expression of genes encoding pro-inflammatory cytokines.

Despite effects on signalling proteins and gene expression, it is the extracellular secretion of cytokines that is of biological relevance since they act to influence the functional activity of other cells. In accordance with the effects on cytokine gene expression, concentrations of pro-inflammatory cytokines in the culture medium were decreased by OA, AC and the OAAC combination. The effects of OA and AC on pro-inflammatory cytokine gene expression and on extracellular release of those cytokines were mostly parallel to one another. In this sense, another striking finding was the enhanced gene and protein expression of the anti-inflammatory cytokine IL-10 promoted by OAAC. OA and AC also enhanced extracellular IL-10 release by macrophages, but this effect was not seen for monocytes. The effect of the combination of OAAC on IL-10 gene expression in macrophages and extracellular protein concentration was greater than the effect of OA or AC alone. These observations indicate that OA and AC have anti-inflammatory effects, particularly in macrophages, and that the combination of OAAC is superior to either compound alone. These observations suggest that the combination of OA and AC may promote macrophage polarization to the M2 phenotype. This interpretation is consistent with reports in the literature that both OA and AC (separately) are able to promote macrophages to display the features of the M2 phenotype [32,33,40,41].

Although there were no significant differences between the OAAC combination and the individual compounds with regard to expression of mRNA for IL-6, MCP-1, and IL-1β, TNF-α mRNA was decreased by OAAC in macrophages compared to OA and AC alone. On the other hand, the protein secretion results showed reduced MCP-1 production by OAAC compared to OA and AC alone. The secretion of TNFα by macrophages treated with OAAC - although reduced - was not statistically different from the isolated treatments. These inconsistencies between gene expression and protein secretion may be attributed to post-transcriptional modulation promoted by OAAC through modulation of miRNAs [56,57].

The combination of compounds (OAAC) demonstrated an effect on production of MCP1 (decreased) and IL-10 (increased), which was significantly greater than the effects of the isolated compounds (OA or AC). In addition, the production of TNF-α, IL-1β and IL-6 was numerically lower with OAAC than with OA or AC, although the difference was not significant. These observations strongly suggest that the OAAC combination has a stronger anti-inflammatory effect than the individual compounds. TNF-α is a key cytokine that is rapidly released after exposure to an inflammatory stimulant like LPS (Fig. S4 and S5). It is secreted by several cell types, including monocytes, macrophages, adipocytes, and myocytes. In metabolic diseases, there is an increased expression of TNF-α by resident monocytes and

macrophages aiming to minimize the hypertrophy of adipose tissue [42]. One action of TNF-α is related to the reduction of lipoprotein lipase (LPL) activity and increased lipolysis in adipose tissue [43]. Furthermore, TNF-α is involved in decreasing insulin signaling through phosphorylation of serine and threonine residues of the type 1 insulin receptor substrate (IRS-1), generating resistance to the action of this hormone [44]. IL-1β is a cytokine largely produced by macrophages and closely related to pro-inflammatory effects and insulin resistance in metabolic diseases. Additionally, IL-1β secretion is associated with apoptosis mechanisms such as caspase-1 and inflammasome activation by mitochondrial damage contributing to pathogenesis of metabolic disease [45,46]. IL-6 is an immunomodulatory cytokine with pro-inflammatory and endocrine actions. IL-6 is secreted by macrophages, stimulating the differentiation of new monocytes into macrophages [47]. There is also secretion of IL-6 at the hypothalamic level where it plays a role in regulating appetite and energy expenditure. Like TNF-α, it is involved in the metabolism of lipids and glucose [48,49]. MCP-1 is a potent chemoattractant, which can regulate monocyte and macrophage migration and infiltration into tissues. Due to its strong chemotactic activity, MCP-1 is one of the earliest contributors to atherosclerosis [50]. Thus, our observations of reduced production of a range of pro-inflammatory cytokines and chemokines with OAAC are relevant to both inflammatory and metabolic diseases. Furthermore, IL-10 is an anti-inflammatory cytokine that acts on energy homeostasis, insulin sensitivity, and the immune response [51,52]. In the innate immune system, IL-10 plays a role in inhibiting activated macrophages, acting as a TNF-α antagonist [52]. Its levels are typically low in an inflammatory state, which favors the emergence of the pro-inflammatory process through the activation of the NFκB pathway mediated by TLRs and TNFR1 (TNF type 1 receptor) [53]. Hence, the observations of increased IL-10 gene expression and protein secretion with OAAC are again highly relevant to both inflammatory and metabolic diseases.

One strength of the current study is the examination of the effects of the combination of OA and AC in comparison with each compound alone. In general, the combination was more effective than the individual compounds. Both OA and AC target the NFκB-inflammatory cytokine cascade, but each compound might exert other unique anti-inflammatory mechanisms not investigated here. Thus, when the two compounds are combined, multiple mechanisms of action may come into play resulting in a stronger biological effect. It will be important to examine the possible mechanisms in further detail to fully understand the biological actions of this combination of bioactive nutrients. Nevertheless, we suggest that the combination of these two compounds might be generating a lipid-phenolic interaction as previously demonstrated for the components of extra virgin olive oil. For example, phenolic compounds such as AC could protect unsaturated fatty acids like OA from oxidative damage so favoring or promoting their biological effects [54,55]. Another strength is that we assessed multiple points of the TLR-NFκB signaling pathway. However, it is important to note that THP-1 cells are a model and may not replicate all features of primary human monocytes or macrophages. It is also important to note that we have studied only two bioactive molecules (OA and AC) and that individual foods and entire diets may contain multiple bioactives and also that the food matrix is important in determining the availability of such bioactive molecules. Nevertheless, the findings reported herein suggest a novel interaction worthy of future research.

Conclusions

In conclusion, OA, AC and their combination (OAAC) exert anti-inflammatory actions in monocytes and especially in macrophages, acting to inhibit the NFκB activation pathway, inflammatory gene expression, and production of pro-inflammatory cytokines. There was also increased expression of the gene for the anti-inflammatory cytokine IL-10 and increased IL-10 production especially with thev combination (OAAC). The combination of OAAC has greater effects than each compound individually, highlighting that the compounds present in a healthy diet such as Mediterranean Diet can interact to improve particular outcomes. These effects may be relevant to inflammatory and metabolic diseases. The nature of the interaction between OA and AC requires further investigation. The findings of this study indicate anti-inflammatory effects of OA and AC, which are enhanced when the two bioactives are combined. Since these effects may act to promote human heath and well-being through modulation of inflammatory processes, there is a need for deeper investigation of these molecule's action and interaction mechanisms. The current study was limited to *in vitro* investigations and it will be important to translate the findings to relevant *in vivo* models and ultimately to humans.

Author Contributions

PCC and LPP conceived the research; ABS, EAM, and PCC designed the experiments; ABS, EJB, ADM, and CAV performed the experiments and analyzed the data, under the supervision of EAM and PCC; ABS drafted the manuscript; LPP and PCC made critical revisions to the manuscript; all authors approved the final version of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

The authors acknowledge financial support from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 2018/23623-7; 2015/13875-0). LPP thanks CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) fellowship.

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