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The colonic polyphenol catabolite dihydroferulic acid (DHFA) regulates macrophages activated by oxidized LDL, 7-ketocholesterol, and LPS switching from pro- to anti-inflammatory mediators†

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Macrophage activation plays a central role in the development of atherosclerotic plaques. Interaction with oxidized low-density lipoprotein (oxLDL) leads to macrophage differentiation into foam cells and oxylipin production, contributing to plaque formation. 7-Ketocholesterol (7KC) is an oxidative byproduct of cholesterol found in oxLDL particles and is considered a factor contributing to plaque progression. During atherosclerotic lesion regression or stabilization, macrophages undergo a transformation from a pro-inflammatory phenotype to a reparative anti-inflammatory state. Interleukin-10 (IL-10) and PGE_1 appear to be crucial in resolving both acute and chronic inflammatory processes. After coffee consumption, the gut microbiota processes non-absorbed chlorogenic acids producing various lower size phenolic acids. These colonic catabolites, including dihydroferulic acid (DHFA), may exert various local and systemic effects. We focused on DHFA's impact on inflammation and oxidative stress in THP-1 macrophages exposed to oxLDL, 7KC, and lipopolysaccharides (LPS). Our findings reveal that DHFA inhibits the release of several pro-inflammatory mediators induced by LPS in macrophages, such as CCL-2, CCL-3, CCL-5, TNF-α, IL-6, and IL-17. Furthermore, DHFA reduces IL-18 and IL-1β secretion in an inflammasome-like model. DHFA demonstrated additional benefits: it decreased oxLDL uptake and CD36 expression induced by oxLDL, regulated reactive oxygen species (ROS) and 8-isoprostane secretion (indicating oxidative stress modulation), and selectively increased IL-10 and PGE_1 levels in the presence of inflammatory stimuli (LPS and 7KC). Finally, our study highlights the pivotal role of PGE_1 in foam cell inhibition and inflammation regulation within activated macrophages. This study highlights DHFA's potential as an antioxidant and anti-inflammatory agent, particularly due to its ability to induce PGE_1 and IL-10.

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Introduction

Atherosclerosis is characterized by the accumulation of immune cells like macrophages and oxidized LDL (oxLDL) in arterial walls, leading to plaque formation.[1] During this process, macrophages contribute to plaque destabilization and rupture by secreting inflammatory cytokines and matrix metalloproteinases (MMPs).[2] Additionally, macrophage surface overexpression and activation of scavenger receptors such as CD36 and pathogen recognition receptors such as Toll-like receptors (*i.e.*, TLR2, -4, and -6) are key activating inflammatory and oxidative stress pathways that promote plaque development.[3,4] Ligands of these receptors such as lipopolysaccharides (LPS) activate NF-κB and lead to pro-inflammatory gene expression, and in combination with CD36 ligands, promote sterile inflammation and coordinate the activation of the NLRP3 inflammasome.[5]

In contrast, the expression of immunosuppressive mediators (*e.g.*, IL-10) promotes atheroma resolution by stimulating angiogenesis and phagocytosis and reducing inflammation. Several studies indicate that IL-10 has a strong anti-inflammatory effect on macrophages and its role in atherogenesis may be related to the deactivation of inflammatory cells.[6] In transgenic mice, IL-10 blocks atherosclerotic lesion formation.[7,8] In peripheral blood mononuclear cells (PBMCs) from patients with acute coronary syndrome, IL-10 inhibits the

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release of inflammatory cytokines and MMPs, promoting plaque stabilization,[9] and in oxLDL-stimulated THP-1 cells and monocyte-derived macrophages from acute coronary syndrome patients, IL-10 contributed to plaque stabilization in part by counteracting apoptosis.[10] Prostaglandins are also crucial in immune system regulation, and PGs modulate immune cell function and response orientation.[11] Among prostaglandins, PGE_1 is a powerful vasodilator and platelet aggregation inhibitor, with therapeutic effects in advanced human atherosclerosis[12] and peripheral vascular disease. Dihomo-gamma-linolenic acid, which is mainly metabolized to PGE_1, displays an anti-atherosclerotic effect in apoE-deficient mice.[13] PGE_1 has been shown to reduce cholesterol and decrease the LDL entry into the rabbit arterial wall;[14,15] it also suppresses TNFα production in LPS-stimulated PBMCs, while leaving IL-6 unchanged and increasing IL-10,[16] and prevents liver damage caused by *Escherichia coli* infection in mice by raising IL-10 levels, decreasing circulating IL-12, and inducing a dominant Th2-like response.[17] These findings suggest that PGE_1 plays a role in anti-inflammatory regulation, potentially through the induction of IL-10. However, the precise mechanisms by which PGE_1 influences macrophage shift and modulates inflammatory responses *via* IL-10 require further elucidation, particularly in defining its impact on the macrophage phenotype and the broader inflammatory landscape.

Recent studies show that oxysterols, natural oxidation products released from cholesterol by oxidative stress, can promote a pro-inflammatory macrophage phenotype in atherosclerotic plaques.[18] Among oxysterols, 7-ketocholesterol (7KC) is the most abundant and a key player in this switch.[19] It has been shown in *in vitro* models that 7KC induces inflammatory pathways, including activation of cell proliferation through the epidermal growth factor receptor, endoplasmic reticulum stress, stimulation of Akt, TLR4, as well as NF-κB-mediated cytokine production, reactive oxygen species (ROS) induction, and inflammasome activation.[19,20] One of the most well-studied inflammasome complexes sensing metabolic imbalances is NOD-like receptor-family pyrin domain containing 3 (NLRP3).[21] Its activation requires two signals; the first signal involves the interaction between TLRs and their ligands, leading to the activation of NF-κB and the synthesis of NLRP3, pro-IL-1β and pro-IL-18. Then, a second signal triggers caspase-1 autoactivation, the cleavage of pro-cytokines, and the release of mature IL-1β and IL-18.[21] LPS have been confirmed to provide both activation signals in human monocytes and macrophages, leading to IL-1β and IL-18 secretion.[22,23] Studies show that various dietary phenolic compounds, including flavonoids (*e.g.*, quercetin, epigallocatechin, morin, baicalin, apigenin, anthocyanins), stilbenes (*e.g.*, resveratrol), and phenolic acids like ferulic acid can target the NLRP3 inflammasome[24–27] through various molecular mechanisms, such as decreasing caspase-1 activity, inhibiting TLR4-IκB-NFκB and TLR-MyD88-NFκB pathways, and hindering NLRP3 assembly. Consequently, these compounds lead to a reduction in production and release of IL-1β and IL-18.

Despite its therapeutic potential, physiological effects of dietary bioactive components are often limited by their bioavailability and biotransformation in the organism. In particular, chlorogenic acids (CGAs) from coffee are poorly absorbed and the circulating fraction hydrolyzed into caffeic and ferulic acids, reaching the maximum levels in plasma close to 0.1 μM.[28] The low bioavailability of CGAs (~30%) means that unabsorbed compounds reach the large intestine, where the colon microbiota produces several small phenolic acids, in which dihydrocaffeic (DHCA) and dihydroferulic (DHFA) can reach physiologically relevant concentrations in the intestine and are found in plasma at levels of up to 0.7 and 1 μM, respectively, 5–10 hours after the intake of 400 ml of coffee containing approximately 400 mg CGAs.[29] These compounds exhibit diverse activities. Specifically, DHFA, a dietary compound with potential anti-inflammatory properties, has been shown to have diverse biological activities, including antioxidant and anti-inflammatory effects, protection of neuronal cells from ischemic injury, inhibition of *in vitro* platelet activation, and induction of antioxidant genes.[30–33] In a previous study, we observed that pre-treatment with DHFA, DHCA, and several CGAs mitigated the *in vitro* production of isoprostanes (IsoPs) and prostaglandins induced by oxLDL in foam cells.[34] These findings demonstrate the dual antioxidant and anti-inflammatory activities of these compounds.

Building on this knowledge, we aimed to study the activity of DHFA as a molecule with anti-inflammatory/antioxidant activities in an *in vitro* experimental model of activated human macrophages. Here, differentiated THP-1 macrophages were treated with pathological concentrations of oxLDL, 7KC, or LPS, which can stimulate inflammatory or oxidative stress responses, and were challenged with DHFA at concentrations from 1 μM. Since DHCA is also one of the most common catabolites from CGAs produced in the colon, we compared the biological activity of DHFA with DHCA and also evaluated the mixture of DHFA/DHCA. We focused in particular on the ROS production and release of 8-isoprostane (8-IsoP), inflammasome activation by the release of IL-1β and IL-18, the modulation of pro-inflammatory cytokines (TNF-α, IL-6, IL-8, IL-12, IL-17, and INFγ), chemokines (CCL-2, -3 and -5), vascular cell adhesion molecule (VCAM-1), and metalloproteinases (MMP-1, -2, -3, -7, -9, and -13), the impact on regulatory cytokines (IL-4, IL-10, and IL-13), inhibition of the atherogenic receptor CD36 and the foam cell formation, and prostaglandin E1 (PGE_1) induction, a mediator of immune regulation.

Materials and methods

Chemicals and materials

The *E coli* O111:B4 LPS was provided by Sigma-Aldrich (St Louis, Missouri, USA). Chloromethyl 2′,7′-dichlorofluorescein diacetate (CM-H2DCFDA) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) were obtained from Invitrogen Life Technologies (Carlsbad, California, USA). The 7-ketocholesterol (5-cholesten-3β-ol-7-one) was purchased from

Steraloids Inc. (Newport, Rhode Island, USA). Prostaglandin E1 (PGE_1) was from Cayman Chemicals (Ann Arbor, Michigan, USA). The dihydroferulic acid (DHFA; 96%; CAS number 1135-23-5) was purchased from Sigma-Aldrich (St Louis, Missouri, USA) and dihydrocaffeic acid (DHCA; 95% CAS number 1078-61-1) was from Extrasynthese S.A. (Lyon, Auvernia-Ródano-Alpes, FR); Benzoic (>98%; CAS number 65-85-0), 3- phenyl propionic (>98%; CAS number 501-52-0), 3-hydroxybenzoic (>98%; CAS number 99-06-9), 4- hydroxybenzoic (>98%; CAS number 99-96-7), 4-hydroxyphenyl acetic (>98%; CAS number 156-38-7), 3-hydroxyphenyl acetic (>98%; CAS number 621-37-4), and 3-(4-hydroxyphenyl) propionic (>98%; CAS number 501-97-3) acids were purchased from TCI America, Ltd (Tokyo, Kanto, JP). The 3,4-dihydroxybenzoic (>98%; CAS number 99-50-3), and 3-(3-hydroxyphenyl) propionic acids (ND%; CAS number 621-54-5) were obtained from Pfaltz Bauer, Inc. (Waterbury, Connecticut, USA). Phenolic acids stock solutions were prepared at 35 mM in DMSO and stored at −80 °C until further use. The work solutions were prepared at 1 mM in PBS, and sterilized by filtration with 0.22 μm acrodisc syringe filters.

LDL isolation, oxidation and DiI labelling

LDL was purified from human plasma by using a discontinuous density gradient using a Beckman XL-100 ultracentrifuge (Brea, California, USA) according to methods previously described.[35] Briefly, the LDL fraction was collected and desalted by ultrafiltration with ultra-centrifugal filters (0.5 ml, Ultracel 3K; Amicon; Tullagreen, Cork, IRL) against five-fold PBS at 16 000*g* for 10 min at 4 °C. Desalted LDL was diluted in PBS, filtered (0.22 μm, sterile) and stored at 4 °C until use. The protein fraction of LDL was quantified using the bicinchoninic acid assay (Pierce, Rockford, Illinois, USA). The oxLDL used in all experiments was obtained by oxidation of LDL with 5 μM $CuSO_4$ at 37 °C for 6 h.[35] For uptake experiments, LDL (1 mg ml^{-1}) was labeled with 0.1 μg ml^{-1} DiI for 12 h at 37 °C under a nitrogen atmosphere in the dark,[34] and then the DiI–LDL complex (0.5 mg ml^{-1}) was oxidized as described above. The unbound DiI and copper ions were removed from the oxLDL–DiI particles by ultrafiltration using Ultracel 3K ultra-centrifugal filters.

Macrophage culture and treatments

The inductive effect of oxLDL, 7KC, and LPS, as well as the regulatory response to PGE_1 and DHFA were assessed on THP-1 monocyte-derived macrophages (ATCC TIB-202) cultured and differentiated as described by Lara-Guzmán.[34,35] Cells were seeded and treated according to their experimental setup: 5.0×10^4 cells per well in 96-well plates for cell viability; 1×10^5 cells per well in 96-well black plates for ROS, and 2×10^5 cells per well in 24-well plates for flow cytometry, ELISA and Luminex immunoassays. Before activation with oxLDL, macrophages were exposed to 1 or 10 μM of each phenolic acid, based on the maximum levels reported in plasma,[28,29] and an equivalent volume of solvent (<0.05% DMSO) was used as the negative control. The exposure times of cells to DHFA and PGE_1 varied according to the assay as follows: 1 h for ROS, 4–12 h for CD36 expression, for oxLDL uptake, for 8-IsoP and cytokine secretion, and 24–48 h for cell viability; the time of exposure to inductors for macrophage activation was: 36 h for of cell viability and oxLDL receptor assays; 6 h for oxLDL uptake; 1 h for ROS production; and 24 h for ELISA and Luminex immunoassays. For the inflammasome-like model, macrophages were primed with 20 μM 7KC for 4 hours, and then, activated with 10 ng ml^{-1} LPS for additional 20 hours.

DiI-oxLDL (DiI) uptake, surface CD36 expression (FITC), and ROS production (DCF) in macrophages were assessed by flow cytometry using LSRFortessa™ apparatus (Beckton Dickinson; San Jose, California, USA), acquiring at least 10 000 events. Cytometric analysis was performed with FlowJo software. The data were calculated by subtracting cell's autofluorescence and/or isotype control fluorescence from the fluorescence of the treated samples. The results are expressed as the mean fluorescence intensity (MFI).

DiI-oxLDL uptake

After treatments, the cells in microplates were washed three times with PBS and harvested with 0.25% trypsin and 0.02% EDTA. Then, the macrophages were centrifuged at 300*g* for 5 min, washed once with RPMI-1640 and 10% FBS at 37 °C and twice with PBS at 300*g* for 5 min at 4 °C. Finally, the cells were suspended in cold PBS and analyzed using a flow cytometer.

CD36 expression

After treatments, the macrophage cultures were washed with PBS and harvested from the wells with cold PBS supplemented with EDTA (0.2%) with the aid of cell scrapers. Nonspecific binding of antibodies was blocked by incubating cells in 10% FBS-PBS. Cells were incubated with 1 μL per 100 000 cells of purified antihuman monoclonal antibodies (R&D Systems; Minneapolis, Minnesota, USA), CD36/SR-B3-FITC (clone 255606; Cat# FAB19551F, RRID: AB 1026194) or the isotype control, rat IgG2B-FITC (clone 141945; Cat# IC013F, RRID: AB_357258). Finally, the cells were washed and suspended in cold PBS and analyzed using a flow cytometer.

Macrophage ROS production

Cells were incubated with a 2 μM fluorescent ROS-sensitive probe CM-H_2DCFDA for 30 minutes. Then, the cells were washed twice, suspended in PBS, and analyzed by either flow cytometry or spectrofluorometry at 488 nm excitation and 525 nm emission wavelengths.

Cell viability

Lactate dehydrogenase (LDH) was quantified in the supernatants collected after the treatments (LDH assay kit; Promega, Madison, USA) at 340 nm using a synergy HT multimode microplate reader (BioTek Instruments Inc.; Winooski, Vermont, USA). The samples and controls were evaluated in

triplicate, and the results are expressed as the percent of control.

Oxidative stress markers in cell culture supernatants

The 8-isoprostane and PGE_1 levels were measured using commercially available ELISA kits from Cayman and Novus biologicals, respectively (Cayman, Ann Arbor, Michigan, USA; Novus biologicals, Denver, Colorado, USA), following the manufacturer's instructions.

Inflammation markers in cell culture supernatants

The cytokines IL-1β, IL-18, TNF-α, IL-6, IL-8, MMP-9 and IL-10 were measured using Human DuoSet ELISA kits (R&D Systems, Minneapolis, MN), following the manufacturer's instructions. The levels of CCL2/JE/MCP-1, CCL3/MIP-1α, CCL5/RANTES, IL-1β/IL-1F2, IL-4, IL-12p70, IL-13, IL-17/IL-17A, MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-13, myeloperoxidase/MPO and VCAM-1/CD106 were measured using a customized human magnetic premixed multiplex (17-plex) assay kit (R&D Systems, Minneapolis, MN, USA) for Magpix™ (Luminex Corp, Austin, TX, USA). Briefly, 50 μL of mixed magnetic microspheres were added to the sample and standards at the indicated dilutions. The plate was incubated for 2 h at room temperature, kept away from light. After incubation, samples were washed with wash buffer by applying a magnet to the bottom of the microplate. Then, a biotin-cocktail detection antibody (50 μL) was added to all assay wells and incubated at room temperature for one hour. Finally, a streptavidin–PE (50 μL) conjugate was added and incubated for 30 min. After this period, the microparticles in each well were washed and suspended in wash buffer (100 μL). The plate was incubated for two minutes on a shaker (800 ± 50 rpm) before data acquisition using the Magpix system. Data were analyzed with the Luminex Xponent program v4.3. The results were expressed as pg ml^{-1} culture supernatants. The limits of detection for CCL2/MCP-1, CCL3/MIP-1α, CCL5/RANTES, IL-1β, IL-4, IL-12p70, IL-13, IL-17, MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-13, myeloperoxidase/MPO, VCAM-1/CD106, and INFγ were 9.9, 16.2, 1.8, 0.8, 9.3, 20.2, 36.6, 1.8, 2.7, 108, 5.3, 23.2, 13.6, 19, 26.2, 238, and 0.22 pg ml^{-1}, respectively.

Statistical analysis

Data are reported as the means ± SD. The normality of the continuous variables in each group was evaluated by the Shapiro–Wilk test. To evaluate the effect of treatments, an unpaired *t*-test or one-way analysis of variance (ANOVA) was performed using GraphPad Prism version 10 for Windows (GraphPad Software, Inc., San Diego, California, USA). The raw data for statistical analyses are available on Github (**https://github.com/vidarium/Lipidox**). *P* values ≤ 0.05 were considered statistically significant. When differences were observed in ANOVA, *post hoc* Dunnett's multiple comparison tests were performed.

Results

THP-1 macrophages are activated with 7KC, oxLDL, and LPS stimuli, and release of pro-inflammatory cytokines, proteases and ROS

Macrophages are important players in the progression of atherosclerosis and promote inflammation through the release of pro-inflammatory cytokines and proteases in response to danger signals. First, we examined whether LPS, 7KC and oxLDL affected the macrophage viability and then the effects of 7KC, oxLDL, and LPS on macrophages' inflammatory profile were compared. After being treated with LPS for 24 hours, about 20% of the cells were dead. In contrast, neither 7KC nor oxLDL or their combination induced macrophage death after 36 h of treatment (ESI Fig. 1†). In contrast to cells under the basal conditions, LPS strongly activated macrophages, causing them to release primarily pro-inflammatory cytokines (*e.g.*, TNF-α, IL-1β, IL-4, IL-6, IL-8, IL-17, IL-18, and INF-γ), MPO, MMP-13, VCAM-1, and the chemokines CCL-2, CCL-3, and CCL-5 but not PGE_1 and IL-10 (Fig. 1A). The 7KC treatment significantly induced the secretion of PGE_1, and the two prominent pro-inflammatory cytokines generated by inflammasome (IL-1β and IL-18), while reduced the release of MPO, MMP-13, IL-4 and IL-10 ($p < 0.01$). Remarkably, the treatment with oxLDL only induced macrophages to produce PGE_1.

Two signals are needed for inflammasome assembly. In the inflammasome-like model, as depicted in Fig. 1B, THP-1 macrophages primed with 7KC (a TLR ligand) released significant amounts of IL-1β ($p < 0.05$) and IL-18 ($p < 0.01$), after the second signal with LPS.[20,22,23] In this model, when comparing the response of macrophages treated solely with 7KC or LPS, there is a synergistic increase in the release of IL-18 when cells were treated with a combination of LPS and 7KC.

Finally, because ROS are important mediators for the activation of pro-inflammatory signaling pathways, the ROS responses of macrophages treated with 7KC, oxLDL, and LPS were also evaluated. oxLDL treatment induced a potent ROS response in spite of its limited ability to induce macrophages to release pro-inflammatory cytokines and chemokines (Fig. 1C). When macrophages were exposed to 20 μM 7KC, they also produced ROS ($p < 0.01$), although the activation was less than that of oxLDL or LPS at physiologically relevant concentrations.

7KC enhances macrophage atherogenicity

Oxidation of the cholesterol in LDL generates several oxidation products including 7KC, which is the most abundant oxysterol present in oxLDL. To investigate the role of 7KC in ROS production, we compared the ability of oxLDL and that of free 7KC to trigger ROS production by macrophages using flow cytometry. Although a significant ROS production was found with the exposure to 7KC and oxLDL ($p < 0.001$) (Fig. 1C), the 24 h exposure to free 7KC alone was associated with the macrophage secretion of 8-isoprostane (8-IsoP) in culture supernatants (Fig. 1D), and not with the combination of 7KC and

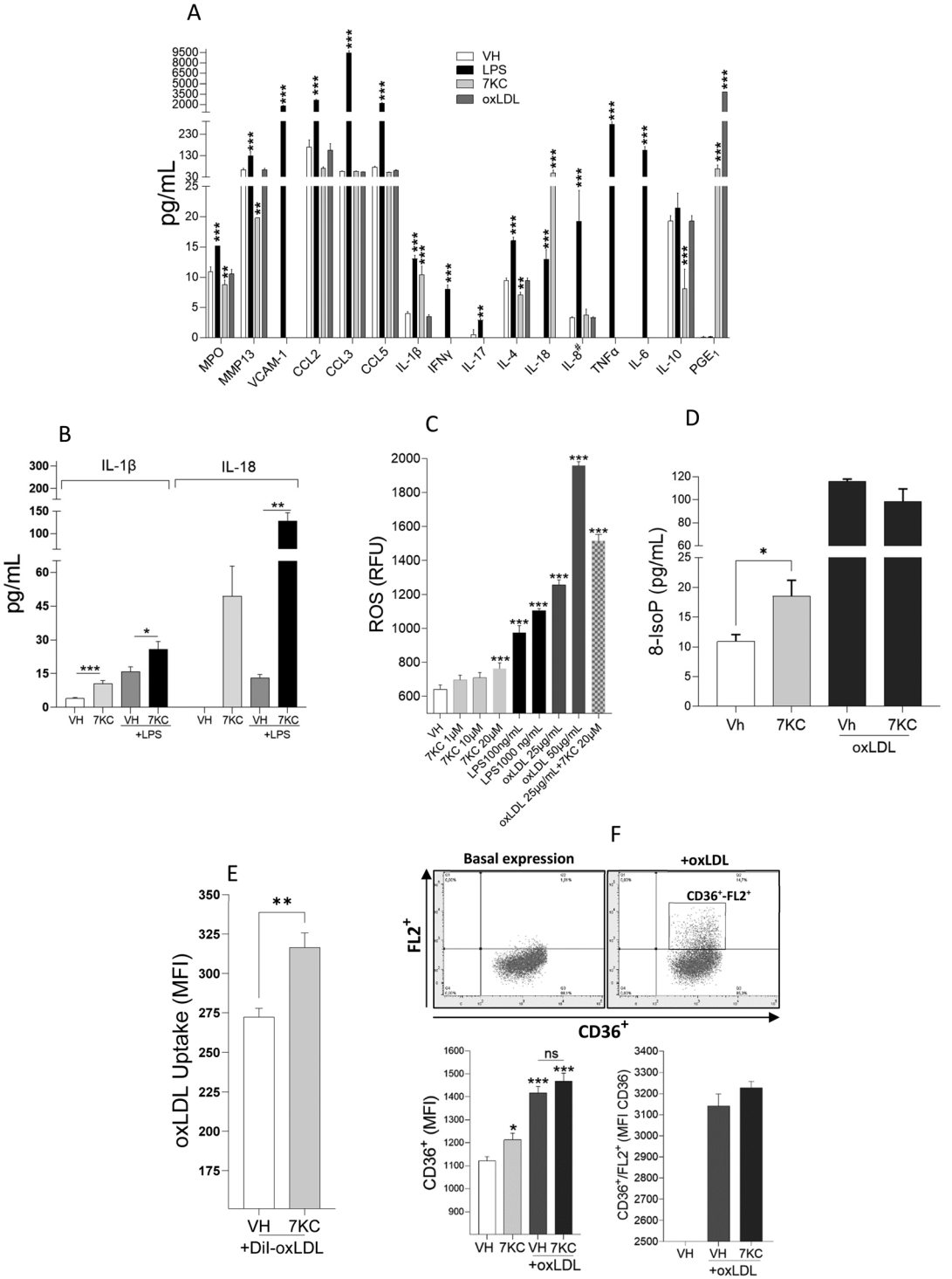

Fig. 1 Effects of 7KC, oxLDL, and LPS on inflammatory mediators and ROS generation, in resting THP-1 macrophages. (A) Inflammatory profile in the culture supernatants; cells were exposed to 20 μM 7KC, 50 μg ml^{-1} oxLDL and 10 ng ml^{-1} LPS for 24 h. (B) IL-1β and IL-18 released into macrophages in culture supernatants in the inflammasome-like model established with 20 μM 7KC for 4 h, and then 10 ng ml^{-1} LPS as an additional treatment for 20 h. (C) ROS production comparison by spectrofluorometry at 1 h after induction. (D) The 8-isoprostane (IsoP) production in supernatants evaluated at 24 h. (E) DiI-oxLDL uptake by flow cytometry; DiI-oxLDL is reported as the MFI. (F) Flow cytometry analysis of the CD36 expression levels in macrophages at the basal level and compared with treatment with oxLDL (up; dot plots). The levels of CD36 are expressed as the MFI of $CD36^+$ cells, and MFI of $CD36^+/FL2^+$ cells. Error bars represent the SD. The *p* values in (A) and (C) represent ANOVA and * denotes statistical differences according to the Dunnett test with the VH (vehicle). For (B), and (D–F), * differs significantly from their controls (VH) after an unpaired two-tailed Student's *t*-test; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. # in IL-8 indicates ×1000. RFU, relative fluorescence units. MFI, mean fluorescence intensity. Data shown are representative of at least two independent experiments.

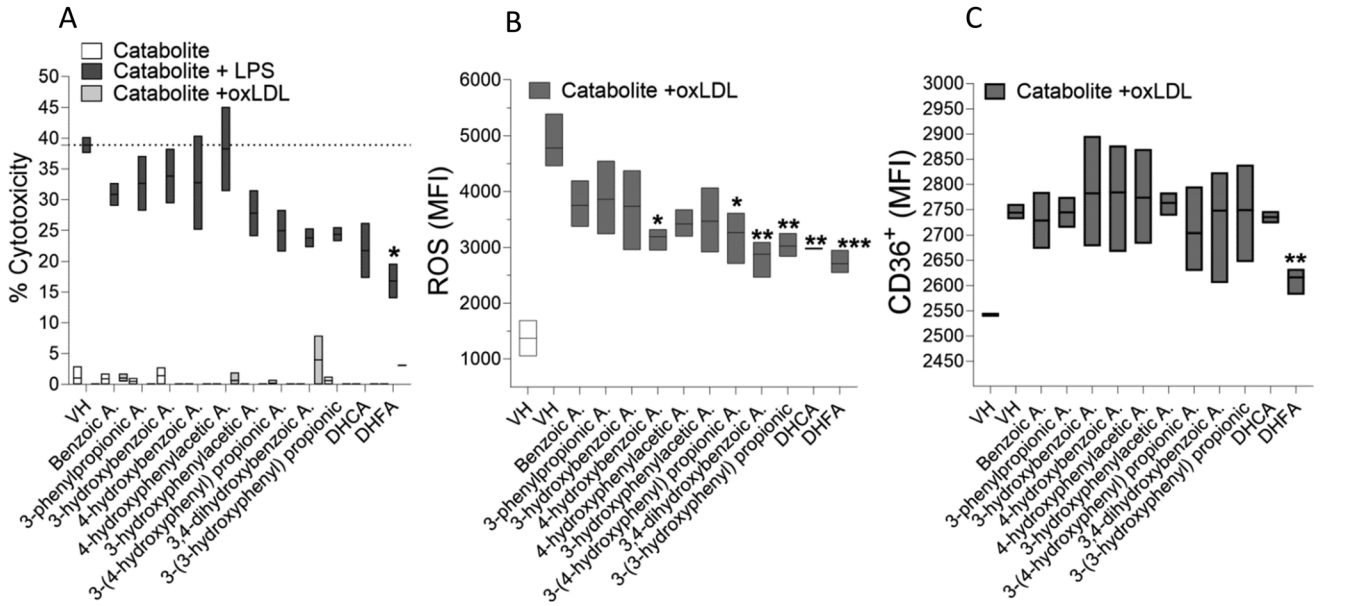

Fig. 2 Effects of coffee colonic catabolites (1 μM) under oxidative stress and CD36 surface expression in macrophages activated by oxLDL. (A) LDH release into culture supernatants measured after 48 h of pre-treatment with chlorogenic acid catabolites and/or 50 μg ml^{-1} oxLDL or 10 ng ml^{-1} LPS; cytotoxicity results are expressed as percentage over LDH release of lysed cells. (B) Intracellular ROS production monitored with a ROS-sensitive fluorescent dye in macrophages pre-treated for 1 h with catabolites, followed by 25 μg ml^{-1} oxLDL for 1 h. (C) CD36 expression determined by flow cytometry in macrophages pre-treated with catabolites for 24 h, followed by exposure to 50 μg ml^{-1} oxLDL for 36 h. Error bars represent the SD. One-way ANOVA: bars labeled with * differ significantly compared with the positive control (LPS or oxLDL); Dunnett's multiple comparison test, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$; data shown are representative of two or more independent experiments.

oxLDL. This might be due to the strong ROS production by oxLDL itself, masking any additional effect from 7KC.

OxLDL uptake by macrophages *via* scavenger receptors such as CD36 and their subsequent transformation into foam cells, which accumulate in the artery wall, are essential factors underlying atherogenesis. Oil red O staining confirmed the presence of lipid droplets following oxLDL uptake by macrophages (ESI Fig. 2;† Fig. 1E), adding to phenotypic changes observed in flow cytometry dot plots, which included an increase in surface CD36 expression and the appearance of an additional, more autofluorescence cell subset (14–20%) with a higher internal complexity (more granular due to lipid droplet accumulation) ($CD36^+/FL2^+$) characteristic of foam cells (Fig. 1F). Following a 36 hour pre-treatment with 7KC, macrophages exhibited enhanced uptake of DiI-oxLDL (Fig. 1E; $p < 0.01$), along with increased CD36 expression on their surface ($CD36^+$) and in the $CD36^+/FL2^+$ cell population (Fig. 1F). However, compared with macrophages exposed to oxLDL alone, neither the CD36 surface expression nor the percentage of $CD36^+/FL2^+$ cells were significantly increased by the combination of 7KC and oxLDL ($p > 0.05$).

Colonic coffee catabolite DHFA decreases the cell atherogenicity on macrophages activated by oxLDL

Natural compounds, including phenolic acids, have the ability to shift macrophages from pro- to anti-inflammatory phenotypes, potentially aiding in the resolution of pre-existing inflammatory processes. Thus, ROS and CD36 assays were used to characterize the antiatherogenic potential of major colonic catabolites from chlorogenic acid at 1 μM in oxLDL-induced macrophages, as shown in Fig. 2. After 48 hours of exposure, neither the oxLDL and chlorogenic acid catabolites nor their combination caused any toxicity in macrophages; in contrast, lipopolysaccharide (LPS)-induced significant *in vitro* macrophage cytotoxicity (40%; Fig. 2A), a phenomenon widely reported to be mitigated by the exposure to DHFA. Notably, the reduction of intracellular ROS levels was most effectively achieved by using DHFA and DHCA among all catabolites (Fig. 2B, $p < 0.001$), with DHFA being the only one decreasing the CD36 expression (Fig. 2C, $p < 0.01$).

Regulatory effect of DHFA on inflammatory markers in macrophages activated with LPS, oxLDL and 7-KC

It is already established that DHFA functions upstream by preventing LPS from activating MAPK p38, ERK, and AKT kinase, which in turn reduces and inhibits NF-κB activation affecting the expression of genes including those encoding cytokines and chemokines, and inflammasome regulators *in vitro*. However, it is unknown if DHFA can modulate the release of anti-inflammatory mediators in activated macrophages. To investigate this, macrophages were pretreated with DHFA prior to LPS, 7KC or oxLDL stimulation. DHFA treatment inhibited the LPS-induced macrophage release of chemokines (*i.e.*, CCL-2, CCL-3, and CCL-5; $p < 0.001$), and cytokines IL-17 ($p < 0.05$), IL-18 ($p < 0.05$), TNF-α ($p < 0.001$), and IL-6 ($p < 0.05$) (Fig. 3A and B). Notably, DHFA treatment increased

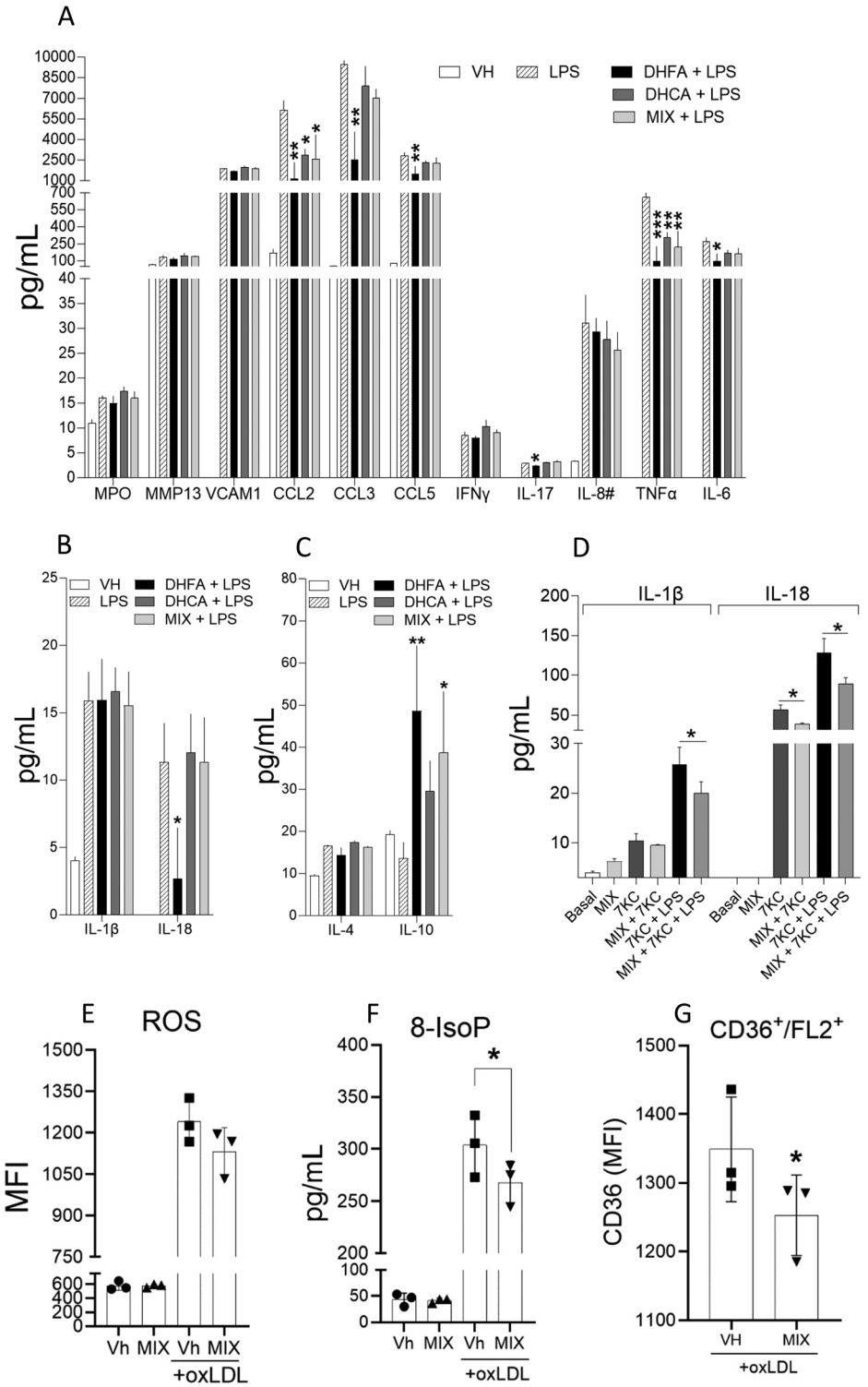

Fig. 3 Inflammatory regulator effect of DHFA on the activated macrophages. Macrophages were pre-treated with DFHA, DHCA, or a DHFA/DHCA mixture at 1 μM each or vehicle (DMSO < 0.01%) for 4 h, and was then stimulated with 10 ng ml^{-1} LPS for 24 h; the concentration of inflammatory markers in the culture supernatant was measured by specific immunoassays for Luminex and ELISA. (A) Pro-inflammatory mediators. (B) Cytokines related with inflammasome. (C) Regulatory cytokines. (D) IL-1β and IL-18 secretion in macrophages primed for 4 h with 20 μM 7KC (signal 1) and then stimulated with 10 ng ml^{-1} LPS (signal 2) for 20 h in the inflammasome like assay. Modulatory effects of DHFA/DHCA mixture on: (E) ROS production; (F) 8-isoprostane (8-IsoP) secretion; and (G) foam cell development from macrophages stimulated with oxLDL. The levels of ROS production and receptor expression were expressed as the mean fluorescence intensity (MFI). Error bars represent the SD. The *p* values in (A–C) represent ANOVA and * denotes statistical differences according to the Dunnett test with the VH (vehicle). For (D–G), * differs significantly from their controls (VH) after an unpaired two-tailed Student's *t*-test: (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). # in IL-8 indicates x1000. Data shown are representative of two or more independent experiments.

the macrophage release of the regulatory cytokine IL-10 ($p < 0.001$), but had no effect on IL-4 secretion (Fig. 3C), suggesting that DHFA can inhibit the release of pro-inflammatory mediators and also stimulate regulatory pathways. On the other hand, DHCA pretreatment or the combination of DHCA/DHFA (by mixing 1 μM each) only significantly reduced the release of TNF-α ($p < 0.001$) and CCL-2 ($p < 0.05$) by macrophages, but the combination maintained the effect on IL-10 ($p < 0.05$). These data suggested that DHFA, present in the mixture with DHCA, is the compound contributing to the increased secretion of the anti-inflammatory cytokine IL-10.

Similarly, in the two signal inflammasome-like experiment, in which macrophages were pretreated with the DHCA/DHFA mixture, and then primed with 7KC for 4 h and followed by LPS for additional 20 h, the mixture inhibited the secretion of IL-1β and IL-18 (Fig. 3D); this effect was more pronounced on the secretion of IL-18 than on that of IL-1β. These results indicated that DHFA alone, or in a mixture with DHCA could inhibit inflammasome activation by interfering with signal 1 or 2. Finally, the DHCA/DHFA mixture also considerably reduced ROS generation, 8-IsoP secretion and appearance of foam $CD36^+/FL2^+$ cells in response to oxLDL ($p < 0.05$) (Fig. 3E–G).

Effects of PGE_1 on inflammatory markers in activated macrophages

PGE_1 has been shown to induce immune regulation in a number of cell models and *in vivo* studies, at least in part by triggering the production of IL-10, a powerful anti-inflammatory mediator. Our results demonstrated that 7KC and oxLDL, as opposed to LPS treatment, caused PGE_1 secretion in macrophages (Fig. 1A). Moreover, DHFA demonstrated immunoregulatory properties by inhibiting pro-inflammatory mediators (Fig. 3A and B) and inducing IL-10 (Fig. 3C). Since DHFA can induce IL-10, we hypothesized that it might benefit macrophages in inflammatory or oxidative stress scenarios, even those induced by LPS. To test this hypothesis, we assessed DHFA's ability to induce PGE_1 in macrophages treated with oxLDL, 7KC, or LPS, either by itself or in conjunction with DHCA.

First, we observed an increased PGE_1 production in macrophages treated with both DHFA and the DHFA/DHCA mixture following LPS stimulation (Fig. 4A). Remarkably, PGE_1 production was 75 times higher after DHFA than after the DHFA/DHCA mixture treatment, reaching levels comparable to the oxLDL's effect (Fig. 4B). PGE_1 secretion induced by oxLDL was not inhibited by the DHFA/DHCA mixture (Fig. 4B). On the other hand, PGE_1 levels induced by 7KC alone were significantly reduced by the DHFA/DHCA mixture and PGE_1 increased by the presence of DHFA in the inflammasome-like model (7KC + LPS) (Fig. 4C). We then investigated whether PGE_1 could regulate macrophage activation by LPS, 7KC, and oxLDL. Interestingly, PGE_1 significantly increased macrophages' IL-10 secretion ($p < 0.001$), while highly suppressing the secretion of IL-1β and IL-18 induced by LPS (Fig. 4D). PGE_1 exhibited a similar suppressive effect on IL-1β and IL-18 production compared to both 7KC and 7KC + LPS stimulation (Fig. 4D). Notably, PGE_1 only induced IL-10 secretion when macrophages were exposed to LPS + 7KC, suggesting activation of this regulatory pathway specifically under these co-stimulatory conditions (Fig. 4D). These data support the regulatory roles of PGE_1 and DHFA during pro-inflammatory events.

Finally, although PGE_1 treatment did not regulate ROS production in oxLDL-activated macrophages (Fig. 4F), it significantly decreased both the uptake of DiI-oxLDL ($p < 0.001$) and the CD36 expression in the $CD36^+/FL2^+$ cell subset ($p < 0.05$) (Fig. 4G and H). Furthermore, PGE_1 significantly reduced the over-uptake of DiI-oxLDL induced by 7KC. Altogether, these results suggested that DHFA treatment in macrophages may be beneficial for preventing inflammation and oxidative stress in this atherosclerosis cell model by acting as an antioxidant and also by promoting anti-inflammatory regulation in macrophages by inducing PGE_1 and IL-10 secretion.

Discussion

Macrophages are critical components of the immune system. However, their function becomes a double-edged sword in chronic inflammatory diseases like atherosclerosis. Shifting the macrophage phenotype towards an anti-inflammatory one within plaques could stabilize atherosclerotic lesions and prevent complications, and inhibiting oxidative and pro-inflammatory signals that attract macrophages to the plaque could slow disease progression; these are two therapeutic approaches that could be used to target macrophage physiology in atherosclerosis.[36] The current study aimed to investigate the ability of colonic catabolites of chlorogenic acid, specially DHFA to modulate the overexpression of pro-inflammatory mediators, cytokines related to the inflammasome, to regulate the production of ROS and 8-IsoP, to affect the uptake of oxLDL and the expression of CD36, a receptor involved in oxLDL uptake, and to promote the secretion of regulatory mediators like IL-1 and PGE_1. THP-1 macrophages activated with LPS, 7KC, and oxLDL at pathological concentrations served as a model system to assess DHFA's bioactivity.[34,37,38]

The atherosclerotic plaque exhibits a high metabolic rate, with increased production of ROS and oxidized lipids such as oxylipins and oxysterols, including 7KC.[39] 7KC, a molecule generated from cholesterol by oxidative stress, has been linked to various inflammatory diseases, particularly those affecting the cardiovascular and nervous systems.[40] It accumulates in areas with high lipoprotein deposits, making it relevant to atherosclerosis. Studies have shown that 7KC primarily triggers inflammation through the TLR4 receptor, activating specific signaling pathways that lead to cytokine production such as AKT-PKC-NF-KB, p38 MAPK, and ER.[20] Therefore, interfering with 7KC's inflammatory mechanism holds promise for developing small-molecule therapies. Such therapies could potentially reduce inflammation and delay the onset of chronic diseases like atherosclerosis.

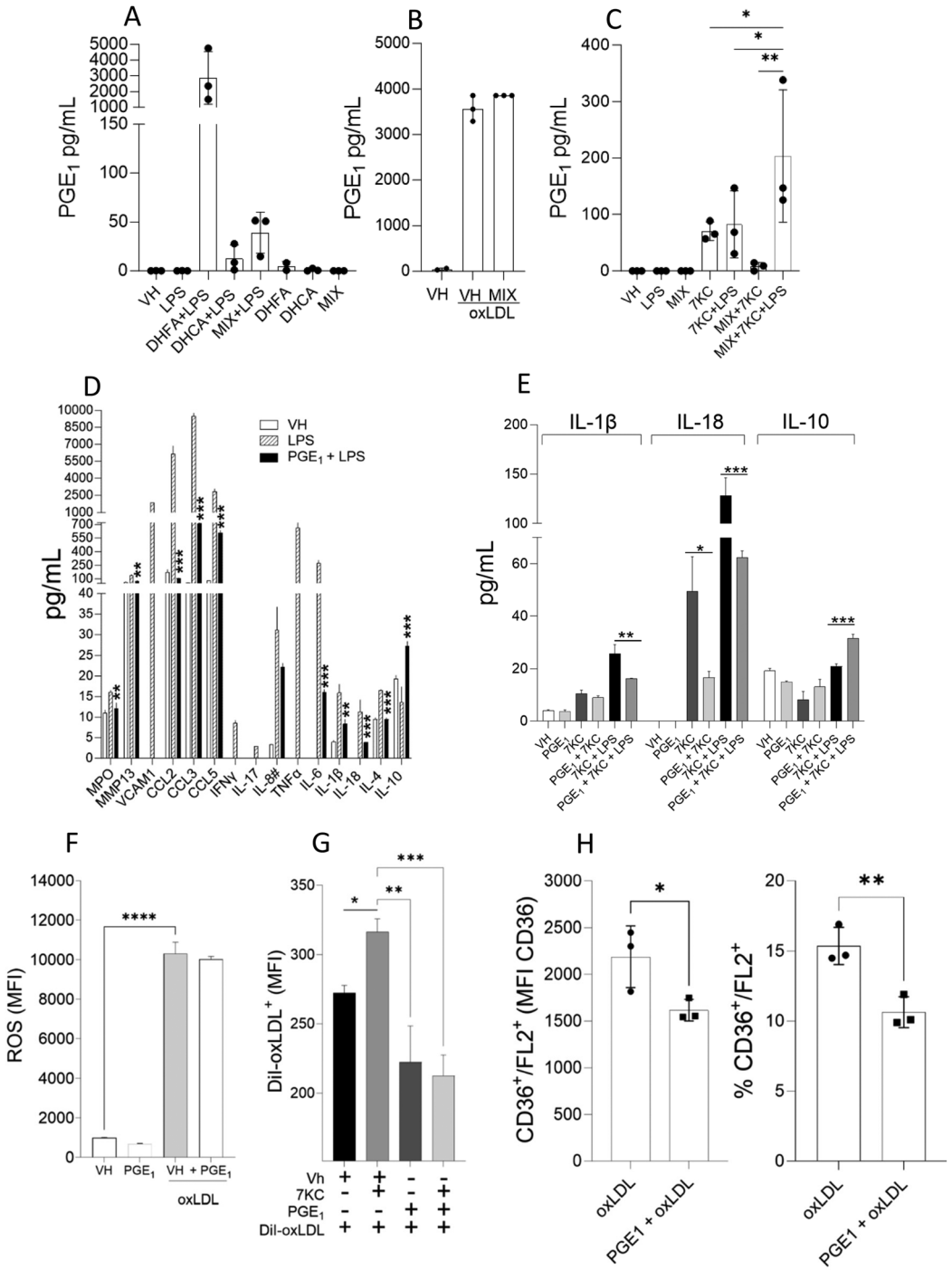

Fig. 4 Regulatory effects of PGE_1 on the activated macrophages. The induction of 20 μM PGE_1 was evaluated in macrophages pre-treated with the DFHA, DHCA or their mixture (DHFA/DHCA at 1 μM each), or vehicle (DMSO < 0.01%) for 4 h, and then stimulated with 10 ng ml^{-1} LPS (A), 20 μM 7KC (B) or 50 μg ml^{-1} oxLDL (C) for 24 h; each point represents an independent experiment. (D) For the inflammatory model, macrophages were pre-treated with PGE_1 for 24 h, and then stimulated with LPS (10 ng ml^{-1}) for 24 h; and the induction of the inflammasome cytokines (IL-1β and IL-18) and IL-10 (E) was also evaluated using 7KC (20 μM, 4 h; signal 1)-primed macrophages and then stimulated with LPS (10 ng ml^{-1}, 20 h; signal 2). (F) Intracellular ROS production was monitored with a ROS-sensitive fluorescent dye in macrophages pre-treated with PGE_1 for 1 h, and then 25 μg ml^{-1} oxLDL for 1 h. (G) The over uptake of Dil-oxLDL (25 μg ml^{-1}) by the pre-treatment of 7KC (20 μM) was decreased by PGE_1 and Dil-oxLDL and it was reported as the MFI. (H) CD36 expression was determined in macrophage pre-treated PGE_1 for 24 h, and then in 50 μg ml^{-1} oxLDL for 36 h. CD36 levels were measured as the mean fluorescence intensity (MFI) of $CD36^+/FL2^+$ cells and their percentage. Each data point represents a separate experiment. The levels of ROS production, Dil-oxLDL uptake, and receptor expression were evaluated by flow cytometry, and the values were expressed as the mean fluorescence intensity (MFI). Error bars represent the SD. For (A–E) and (H), * differs significantly after an unpaired two-tailed Student's *t*-test: (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). One-way ANOVA for (F and G): bars labeled with * differ significantly compared with the control (Dunnett's multiple comparison test, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$; data shown are representative of two or more independent experiments).

Recent research suggests that 7KC can activate the NLRP3 inflammasome pathway in various cell models, resulting in distinct levels of IL-1β and IL-18 production.[41–43] One significant finding of our study was that 7KC primarily increased the release of IL-18, a cytokine associated with the inflammasome complex. Interestingly, IL-1β, another inflammasome-related cytokine, had a weaker response, despite both being enhanced by LPS. Although we did not directly measure the complete inflammasome complex consisting of caspase-1, ASC, and NLRs,[41,42] it is well established that the inflammasome activation requires two independent signals: the first activates NF-κB, *via* TLR signaling, inducing the transcription of pro-IL-1β and pro-IL-18, and the second, often a damage or cellular stress associated signal, leads to the processing and secretion of mature IL 1β and IL-18.[41,44] 7KC signals through TLR-4[20] and may act in priming and activation signals required for inflammasome assembly. Further analysis revealed that 7KC also increased the expression of CD36 receptor and ROS production, potentially contributing to the inflammatory response, but most other tested inflammatory mediators, including cytokines like TNF-α and IL-6, chemokines, metalloproteases and adhesion molecules, did not seem to be significantly affected by 7KC. The selective over-production of IL-18 suggests that NLRP3 inflammasome is the primary responder to 7KC-induced inflammation.

LPS, in addition to being a major inductor of the inflammatory process on cell models, may also evoke changes in lipid homeostasis, elicit the inflammasome, and induce oxidative stress in macrophages.[45] To establish the present model of LPS-induced inflammation, we evaluated the effects on IL-1β and IL-18 and considered the production of inflammatory mediators cytokines (TNF-α, IL-4, IL-6, IL-8, IL-10, IL-12, IL13, IL-17, and INFγ), chemokines (CCL-2, -3 and -5), vascular cell adhesion molecule (VCAM-1), and MMPs (MMP-1, -2, -3, -7, -9, and -13), together with oxidative stress markers (ROS, and 8-IsoP), and PGE_1. Most of the mediators were induced by LPS, except for MMPs, IL-12 and IL-13, and the regulatory molecules such as IL-10 and PGE_1. We observed that the co-treatment with 7KC (signal 1) and LPS (signal 2) enhanced the secretion of IL-18 to a greater extent than that of IL-1β.

Results from earlier research on the inflammatory responses induced by oxLDL have been inconsistent and frequently contradictory.[46] In part because, the ability of minimally modified LDL (mmLDL) to bind to TLRs and activate TNF-α, IL-6, and IL-1β secretion *via* NF-kB signaling is superior to that of fully oxidized oxLDL, which causes primarily oxidative stress in macrophages, raising oxysterol accumulation and oxylipin secretion (*e.g.*, prostaglandins and isoprostanes).[35,47,48] Oxylipins and oxysterols may increase the expression of several inflammatory mediators in the atherosclerotic plaque and the vascular system by inducing oxidative stress.[49,50] In agreement with the current study, exposing macrophages to oxLDL or 7KC increased the production of ROS, CD36, and oxylipins such as 8-IsoP and PGE_1, and the co-treatment (oxLDL + 7KC) showed an additive response as well as an increase in oxLDL uptake. It is difficult to compare our findings to previous research because many studies examined inflammation in macrophages through cytokine expression; however, in our experiments, oxLDL did not induce cytokines, chemokines, MMPs, VCAM-1, or MPO.

Macrophages can be influenced by dietary components absorbed from the gut, including bioactive metabolites like DHFA. Studying these metabolites' effects on macrophages provides valuable insights into their potential health benefits.[51–53] Our previous research demonstrated that pre-treating macrophages with 1 μM DHFA before oxLDL exposure protected them from oxidative stress, prevented oxylipin production and decreased the levels of oxLDL receptors (CD36, SR-A, and LOX-1).[34] It is worthwhile to highlight the effects of DHFA, which was more active than DCHA, consistent with previous findings in inflammatory models.[32] To our knowledge, this is the first study on the effects of this colonic metabolite in cultured human macrophage cells activated by multiple inductors.

Reduced ROS generation and decreasing 8-IsoP in cells treated with DHFA indicate that this compound acts as a primary antioxidant, mitigating the increased levels of ROS and oxidative damage caused by oxLDL-induced stress. These results are consistent with earlier studies conducted in hepatocytes[32] and enterocytes.[30] On the other hand, the high levels of 8-IsoP induced by oxLDL indicate a state that could result in irreversible oxidative damage to polyunsaturated fatty acids within the cell membranes. Pre-treatment with DFHA partially prevented this dangerous situation, which is consistent with the response observed for hydroxycinnamic acids from coffee.[34]

In the current study, DFHA was found to be effective in decreasing the majority of the inflammatory mediators tested while increasing IL-10 and PGE_1, confirming its anti-inflammatory potential on human macrophages. These findings are especially significant because DFHA is widely detected in human colon, plasma, and urine after consuming various phenolic compounds, including monomeric or oligomeric flavan-3-ols from cocoa,[54] anthocyanins from berries,[55] and citrus flavanones.[56] Previous studies have demonstrated that DHFA attenuated LPS-induced inflammatory response (nitric oxide and NF-κB) in enterocyte-like cells[30] and reduced the secretion of IL 6, IL-8, MCP-1, and MIP1α by TNFα-stimulated hepatic cells[32] and had a stronger inhibitory effect on P-selectin expression than their phenolic precursors (5-caffeoylquinic acid and 3,5-dicaffeoylquinic acid), suggesting an increased efficacy to modulate platelet activation with the catabolism of the phenolic compounds.[33]

Our findings suggest that DHFA-induced PGE_1 secretion may be a regulatory mechanism in LPS-activated macrophages. PGE_1 and DHFA inhibited the majority of pro-inflammatory mediators (cytokines, chemokines, VCAM-1, MPO, and MMP-13). Treatment with DHFA reduced IL-1β and IL-18 secretion in an inflammasome-like assay, similar to other polyphenols such as rutin, quercetin, naringenin, and silymarin that inhibited the NLRP3 inflammasome, suggesting a role in the regulation of the inflammasome multiprotein

complex.[24,57] Furthermore, in THP-1 macrophages, ferulic acid, one of the parental molecules of DHFA, increased autophagy, inhibited the activation of the NLRP3 inflammasome induced only by LPS, and reduced the expression and release of inflammatory mediators.[25] Our results suggest that DHFA + DHCA effectively inhibits IL-1β secretion only in the context of dual stimulation with LPS and 7KC, possibly by modulating inflammasome signaling. This differential response may reflect the distinct roles of LPS and 7KC in inflammasome activation, where LPS provides the secondary signal required for full inflammasome assembly.[24,57] Future studies will be essential to elucidate whether DHFA + DHCA specifically disrupts inflammasome complex formation and activation.

Similar to DHFA pre-treatment, PGE_1 pre-treatment allowed for the production of IL-10, a key anti-inflammatory cytokine that deactivates activated macrophages.[58] As previously reported, PGE is a potent immunomodulator, and the effects on cytokine production by human peripheral blood mononuclear cells activated with LPS are independent of the prostaglandin subtype (*i.e.* PGE_1, PGE_2, or PGE_3), but IL-10 production is guaranteed.[16] PGE differentially deactivated murine macrophages, and IL-10 was found to play a role. These findings suggest that the anti-inflammatory effect of PGE on mononuclear phagocytes is partially mediated by an autocrine feedback mechanism involving IL-10. PGE differentially deactivated murine macrophages, and IL-10 was found to play a role. These findings suggest that the anti-inflammatory effect of PGE on mononuclear phagocytes is partially mediated by an autocrine feedback mechanism involving IL-10.[59]

In vivo, PGE_1 administration protected mice from liver injury following *E. coli* infection by suppressing circulating IL-12 levels and increasing IL-10 production,[17] whereas patients who received PGE_1 during cardiac surgery reduced pro-inflammatory cytokine production and increased IL-10 in myocardial reperfusion injury compared to the control group.[60] The anti-inflammatory effects of IL-10 have been described in atherogenesis,[6] stabilizing the atherosclerotic plaque progression in mouse models, by inhibiting the release of pro-inflammatory cytokines and MMPs in PBMCs and macrophages from patients with acute coronary syndromes.[9,10] DHFA from diet is bioavailable after consuming phenolic-rich foods, such as green/roasted coffee,[61] whole-grain wheat[62] or *Cynara scolymus*,[63] and has been detected in the plasma at concentrations ranging from 0.1 to 1 μM. In our study, we employed DHFA concentrations of up to 1 μM, which, although in the upper limit of the concentrations found in plasma, are necessary to observe a biological effect in our *in vitro* model. Nevertheless, these concentrations provide valuable insights into the mechanism of action of DHFA, justifying further studies in more complex systems.[64] These levels are comparable to those used in *in vitro* studies, demonstrating the bioactivity of DHFA, including its ability to inhibit platelet activation[33] and counteract TNF-α-induced inflammation and oxidative stress in pre-adipocytes.[64] DHFA is a metabolite derived from the metabolism of phenolic compounds present in certain foods, particularly those rich in ferulic acid and its derivatives. Foods such as whole grains, coffee, and certain vegetables (*e.g.*, tomatoes, apples) are significant sources of these compounds.[65] Regular consumption of these foods has been associated with an increase in plasma DHFA levels, suggesting that dietary intake could modulate the inflammatory responses through DHFA, as demonstrated in our study.[62,66,67]

Our results have some limitations. First, we did not directly determine which signaling pathways DFHA may have an effect (such as MAPK p38, ERK, and Akt kinase or NLRP3 proteins). On the other hand, it is known that LPS also increases the production of PGE_2, affecting the release of TNF-α, IL-6, and IL-10 from macrophages *via* EP2 and EP4 receptors.[68,69] We did not evaluate PGE_2 in this study; however, our model with LPS-activated macrophages produced PGE_1, but only after pretreatment with DHFA and not with LPS alone. We hypothesize that DHFA might make dihomo-gamma-linolenic acid (DGLA) more readily metabolized by COX enzymes following LPS stimulation.[70] Our observation that DHFA induced PGE_1 production with LPS co-stimulation suggests a potential role for PGE_1 in the anti-inflammatory response mediated by IL-10. It is well-established that IL10 receptor (IL10R) and PGE receptor EP4 signaling synergize to elicit anti-inflammatory response.[71] These findings warrant further investigation using animal models or clinical trials to determine whether DHFA's ability to promote both PGE_1 and IL-10 production translates to clinically relevant improvements in chronic inflammatory states.

Conclusion

In conclusion, our findings demonstrate that DHFA possesses anti-inflammatory properties in macrophages under different inflammatory and oxidative stress conditions. DHFA treatment not only consistently increased the production of the anti-inflammatory cytokine IL-10, but also mitigated the inflammation induced by LPS and the oxidative stress atherogenic phenotype induced by 7KC and oxLDL. Notably, DHFA's effect appears to be mediated, at least in part, by the induction of PGE_1, as it seems to specifically promote the regulatory pathway involving IL-10 secretion. Interestingly, DHFA also reduced the uptake of oxidized LDL and CD36 expression, suggesting its potential role in diminishing atherogenic processes. Overall, these results suggest that DHFA may be a promising candidate for complementing future therapeutic strategies targeting macrophages to combat inflammatory diseases and atherosclerosis.

Author contributions

Muñoz-Durango, K., Sierra, J., and Lara-Guzmán, O. J.: conceptualization, investigation, writing – original draft, and writing – review and editing; Lara-Guzmán, O. J.: methodology, formal analysis and data curation. Arango-González, A. and Rivera,

D. A: methodology. Muñoz-Durango, K. and Sierra, J.: project supervision and administration.

Data availability

The raw data corresponding to the figures presented in this article are available at a Vidarium repository at **https://github.com/Vidarium/Lipidox**.

Conflicts of interest

KM-D, JAS, DAR, and OJL-G are researchers at the Vidarium, Nutrition, Health and Wellness Research Center – Nutresa Business Group. AA-G states that she has no conflicts of interest.

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