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# Detailed characterization of the endocannabinoid system in human macrophages and foam cells, and anti-inflammatory role of type-2 cannabinoid receptor

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ABSTRACT

*Objective:* Cannabinoid receptors are activated in murine macrophages upon exposure to oxidized low-density lipoproteins (oxLDL), and type-1 cannabinoid receptor ($CB_1R$) is considered as a risk factor in atherosclerosis, because it promotes cholesterol accumulation and release of inflammatory mediators. Conversely, accumulated evidence suggests a protective role for type-2 cannabinoid receptor ($CB_2R$). Here, we sought to ascertain whether different elements of the endocannabinoid system (ECS) were activated in human lipid-laden macrophages, and whether $CB_2R$ played any role in atherogenesis and inflammation of these cells.

*Methods and results:* Human macrophages were exposed to oxLDL in order to obtain lipid-laden foam cells. Liquid chromatography/mass spectrometry (LC/MS) was used to measure the production of the endocannabinoids in both macrophages and foam cells, and radiometric assays were performed to measure cannabinoid receptor binding and activity of endocannabinoid metabolizing enzymes. OxLDL accumulation was investigated by confocal imaging, and cytokine production and release were measured by means of flow cytometry and ELISA. The results showed that human macrophages possess a fully functional ECS, which was modulated by oxLDL. Selective $CB_2R$ activation reduced cellular oxLDL accumulation, which was associated with decreased expression of CD36 scavenger receptor, and decreased production of TNFα, IL-12 and IL-10. These anti-atherogenic and anti-inflammatory effects were reverted by the selective $CB_2R$ antagonist SR144528.

*Conclusions:* A fully active ECS is present in human macrophages and macrophage-derived foam cells. Selective activation of $CB_2R$ reduces CD36-dependent oxLDL accumulation and modulates production of inflammatory cytokines, thus representing a potential therapeutic strategy to combat atherosclerosis.



## 1. Introduction

Atherosclerosis is a chronic inflammatory disease, which is the primary cause of morbidity and mortality in the Western world. The appearance of oxidized low-density lipoproteins (oxLDL) and of lipid-laden macrophages is one of the first hallmarks of atherogenesis [1]. The transformation of these macrophages into foam cells is mediated by a dysregulated uptake of oxLDL through several types of scavenger receptors, most notably CD36 [1,2]. It is now widely accepted that atherosclerosis is an inflammatory disease, whose progression and consequences are determined by dynamic interactions between inflammatory cells, recruited in response to sub-endothelial lipid accumulation, and the local reparative "wound-healing" response of surrounding vascular smooth muscle cells [3–5]. Although understanding the pathogenesis of atherosclerosis has made a significant progress, there is still a strong need to develop novel anti-atherosclerotic therapeutics, able to avoid the manifold adverse effects of statins [6]. In this context,

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the endocannabinoid system (ECS) has emerged as a promising group of molecules endowed with anti-inflammatory effects potentially useful to treat different chronic inflammatory disorders, from neurodegenerative to liver diseases and, remarkably, atherosclerosis [7–9].

The ECS comprises type-1 ($CB_1R$) and type-2 ($CB_2R$) cannabinoid receptors, their endogenous ligands (in particular anandamide [*N*-arachidonoylethanolamine, AEA], and 2-arachidonoylglycerol [2-AG]), and the enzymes that synthesize (*N*-acyl-phosphatidyl-ethanolamine-hydrolyzing phospholipase D [NAPE-PLD] and diacylglycerol lipase [DAGL]) or degrade (fatty acid amide hydrolase [FAAH] and monoacylglycerol lipase [MAGL]) AEA and 2-AG, respectively [10,11]. AEA, unlike 2-AG, binds to and activates also transient receptor potential vanilloid 1 (TRPV1) channels, thus being considered a true "endovanilloid" [12].

Recent evidence has documented that $CB_1R$ and $CB_2R$ expression increases in murine foam cells, and so do the levels of AEA and 2-AG [13]. Moreover, $CB_1R$ has been clearly associated with cardiometabolic risk factors, including obesity and increased serum lipid production [14,15]. In particular, pre-clinical murine models of atherosclerosis indicate that pharmacological inhibition of $CB_1R$ reduces oxLDL accumulation in macrophages, limits the vascular inflammation and associated disease progression, and also decreases smooth muscle cells proliferation [14,15]. Conversely, $CB_1R$ activation induces reactive oxygen species and cell death in human coronary artery endothelial cells [16], supporting beneficial effects of $CB_1R$ antagonists in humans with obesity and/or diabetes and metabolic syndrome [17,18]. In addition, a protective role for $CB_2R$ was suggested in a relevant mice model of atherosclerosis, where the plant-derived $CB_1R/CB_2R$ agonist Δ [9]-tetrahydrocannabinol (THC) was shown to inhibit atherosclerotic plaque progression [19,20]. Such an effect of THC occurred mainly by inhibition of macrophage recruitment, and was reversed by the selective $CB_2R$ antagonist SR144528 [19,20]. As yet, the majority of the studies on ECS involvement in atherosclerosis have been performed in mouse models, therefore the actual role of endocannabinoid signaling in diseased human subjects remains largely unknown. In the present study, we sought to investigate presence and activity of the major ECS elements in human macrophages and foam cells, and their possible effect on CD36-mediated oxLDL accumulation. Additionally, we sought to clarify whether $CB_2R$ could have a protective and anti-inflammatory role in human foam cells, thus opening the avenue to the therapeutic exploitation of drugs targeted towards this specific receptor subtype.

## 2. Methods

### 2.1. Cell cultures

The human monocytic cell line THP-1 was obtained from the American Type Culture Collection (ATCC, Rockville, MD), and was cultured in Falcon flasks with RPMI-1640 medium supplemented with 10% fetal bovine serum, L-glutamine (2 mM), sodium pyruvate (100 μg/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were cultured at 37 °C in a humidified 5% $CO_2$ atmosphere. THP-1 monocytes were differentiated into macrophages in the presence of 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma, Italy) for 72 h, as reported [21–23]. Peripheral blood mononuclear cells (PBMCs) were obtained from four healthy donors and were isolated by Ficoll-hystopaque gradient centrifugation, as reported [24,25]. Using the monocyte isolation kit (Miltenyi Biotec Gmbh, Germany), highly purified human CD14+-cells were isolated and differentiated into macrophages by incubation with 20 ng/ml M-CSF and 10 ng/ml GM-CSF (Invitrogen Molecular Probes, Italy) for 5 days.

### 2.2. LDL oxidation and foam cell formation

LDL (Sigma) were reconstituted in deionized water (1 mg LDL protein/ml), and were oxidized at 37 °C by incubation with 5 μM $Cu_2SO_4$ for 24 h. Lipoprotein oxidation was assessed by changes in absorbance at 234 nm (due to conjugated dienes), and $Cu^{2+}$ ions were removed by extensive dialysis. PMA-differentiated THP-1 macrophages were incubated with 100 μg/ml of oxLDL for 18 h, in order to allow lipoproteins uptake, and hence conversion of macrophages into foam cells as reported [24].

### 2.3. qRT-PCR analysis of ECS

RNA was extracted from human control or lipid-laden THP-1 macrophages, using the RNeasy extraction kit (Qiagen, Crawley, UK), as suggested by the manufacturer. Total RNA was used for reverse transcriptase (RT) reaction, performed by means of the iScript TM cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA), as reported [25]. The following program was used for the quantitativeRT-PCR: 25 °C for 10 min, 42 °C for 50 min, 85 °C for 5 min, then after addition of 0.1 unit/ml of *Escherichia coli* RNase H, the product was incubated at 37 °C for 20 min. The target transcripts within the ECS were amplified by means of an ABI PRISM 7700 sequence detector system (Applied Biosystems, Foster City, CA), using the following specific primers for CB1R, CB2R, TRPV1, NAPE-PLD, FAAH, DAGL, and MAGL, as reported [26].

Human $CB_1R$ F1 (5′-CCTTTTGCTGCCTAAATCCAC-3′) and R1 (5′-CCACTGCTCAAACATCTGAC-3′); human $CB_2R$ F1 (5′-TCAACCCTGTCATCTATGCTC-3′) and R1 (5′-AGTCAGTCCCAACACTCATC-3′); human TRPV1 F1 (5′-TCACCTACATCCTCCTGCTC-3′) and R1 (5′-AAGTTCTTCCAGTGTCTGCC-3′); human NAPE-PLD F1 (5′-TTGTGAATCCGTGGCCAACATGG-3′) and R1 (5′-TACTGCGATGGTGAAGCACG-3′); human FAAH F1 (5′-CCCAATGGCTTAAAGGACTG-3′) and R1 (5′-ATGAACCGCAGACACAAC-3′); human DAGL F1 (5′-TTCCAAGGAGTTCGTGACTGC-3′) and R1 (5′-TTGAAGGCCTTGTTGTCGCC-3′); human MAGL F1 (5′-ATGCAGAAAGACTACCCTGGGC-3′) and R1 (5′-TTATTCCGAGAGAGCACGC-3′); and β-actin F1 (5′-TGACCCAGATCATGTTTGAG-3′) and R1 (5′-TTAATGTCACGCACGATTTCC-3′).

β-Actin was used as housekeeping gene for quantity normalization. One μl of the first strand of cDNA product was used for amplification (in triplicate) in a 25 μl reaction solution, containing 12.5 μl of Platinum SYBRGreen qPCR Super-Mix UDG (Invitrogen) and 10 pmol of each primer. The following PCR program was used: 95 °C for 10 min, 40 amplification cycles at 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s [26].

### 2.4. Immunoblotting of ECS

Control THP-1 macrophages or THP-1-derived foam cells, seeded at $3 \times 10^6$ cells/well, were washed 3 times with cold phosphate buffer saline (PBS), incubated for 3 min with accutase and collected for cell lysis. Then, cells were lysed with RIPA buffer, and protein expression was assessed by Western blotting, as reported [26]. Cell homogenates were subjected to 10% SDS-PAGE (50 μg/lane) under reducing conditions, then gels were electroblotted onto 0.45-mm nitrocellulose filters and were immunoreacted with anti-$CB_1R$ (1:250), anti-$CB_2R$ (1:500), anti-TRPV1 (1:400), anti NAPE-PLD (1:200), anti-FAAH (1:200), anti-DAGL (1:200), or anti-MAGL (1:200) polyclonal antibodies (Cayman Chemical Co., Ann Arbor, MI), or with anti-β-actin monoclonal antibody (1:5000, Bio-Rad) [26]. Proteins were detected by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) and by exposure to X-ray film (Hyper ECL; Amersham Pharmacia Biotech), quantitating band intensity by densitometry in a ChemiImager 4400 apparatus (Alpha Innotech, San Leandro, CA) [26].