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RESEARCH ARTICLE

The two synthetic cannabinoid compounds 4'-F-CBD and HU-910 efficiently restrain inflammatory responses of brain microglia and astrocytes

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Abstract

To study the anti-inflammatory potential of the two synthetic cannabinoids 4'-F-CBD and HU-910, we used post-natal brain cultures of mouse microglial cells and astrocytes activated by reference inflammogens. We found that 4'-F-CBD and HU-910 efficiently curtailed the release of TNF- α , IL-6, and IL-1 β in microglia and astrocytes activated by the bacterial Toll-Like Receptor (TLR)4 ligand LPS. Upon LPS challenge, 4'-F-CBD and HU-910 also prevented the activation of phenotypic activation markers specific to microglia and astrocytes, that is, Iba-1 and GFAP, respectively. In microglial cells, the two test compounds also efficiently restrained LPS-stimulated release of glutamate, a non-cytokine inflammation marker for these cells. The immunosuppressive effects of the two cannabinoid compounds were concentration-dependent and observable between 1 and 10 μ M. These effects were not dependent on cannabinoid or cannabinoid-like receptors. Both 4'-F-CBD and HU-910 were also capable of restraining the inflammogenic activity of Pam3CSK4, a lipopeptide that activates TLR2, and of BzATP, a prototypic agonist of P2X7 purinergic receptors, suggesting that these two cannabinoids could exert immunosuppressive effects against a variety of inflammatory stimuli. Using LPS-stimulated microglia and astrocytes, we established that the immunosuppressive action of 4'-F-CBD and HU-910 resulted from the inhibition of ROS produced by NADPH oxidase and subsequent repression of NF- κ B-dependent signaling events. Our results suggest that 4'-F-CBD and

Abbreviations: 4'-F-CBD, 4'-fluoro-CBD; FCS, fetal calf serum; APO, apocynin; AUC, area under the curve; BzATP, 2'-(3')-O-(4-benzoylbenzoyl)-ATP; CB1, cannabinoid type 1 receptor; CB2, cannabinoid type 2 receptor; CBD, cannabidiol; Ct, cycle threshold; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GPR55, G protein-coupled receptor 55; HU-910, (1S,4R)-(2-(2,6-dimethoxy-4-(2-methyloctan-2-yl)phenyl)-7,7-dimethyl-bicyclo [2.2.1]hept-2-en-1-yl) methanol; Iba-1, ionized calcium-binding adaptor molecule-1; LPS, lipopolysaccharide; NOX, NADPH oxidase; PBS, Dulbecco's phosphate-buffered saline medium; PEI, polyethyleneimine; ROS, reactive oxygen species; TLR, toll-like receptor; TPCA-1, 2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide; Δ 9-THC, delta-9 tetrahydrocannabinol.

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HU-910 may have therapeutic utility in pathological conditions where neuroinflammatory processes are prominent.

KEYWORDS

astrocytes, microglia, neuroinflammation, oxidative stress, synthetic cannabinoids

1 | INTRODUCTION

Cannabis sativa is a plant that contains about 125 chemical entities collectively referred as cannabinoids, a family of compounds consisting of prenylated polyketides derived from fatty acid and isoprenoid precursors (Bow & Rimoldi, 2016; Hanus et al., 2016). Several cannabinoids including cannabidiol (CBD), cannabigerol, and delta-9-tetrahydrocannabinol (Δ^9 -THC) have been postulated to have medicinal value (Izzo et al., 2009; Tahir et al., 2021).

Among these compounds, CBD has attracted the most attention because it displays interesting pharmacological properties while having no psychotomimetic effects contrarily to Δ^9 -THC (Batalla et al., 2020). The speculation regarding its therapeutic potential has been broad and includes several applications for pain, inflammation, and psychiatric disorders, among others (Britch et al., 2021). In addition, CBD was described to attenuate brain damage in preclinical models of ischemia and chronic neurodegenerative disorders, suggesting that this cannabinoid might also possess neuroprotective properties (Campos et al., 2016; Garcia-Gutierrez et al., 2020). Importantly, CBD was also found to significantly reduce childhood epilepsy associated with severe intractable seizures, and it is now an FDA-approved drug for such neurological conditions (Britch et al., 2021). The mechanisms of CBD effects are still not entirely clear but seem to involve multiple targets that include cannabinoid type 1 receptors (CB1) and cannabinoid type 2 receptors (CB2), Peroxisome-Proliferator Activated Receptor- γ

(PPAR γ) as well as G-protein-coupled receptor 55 (GPR55) (Mechoulam & Parker, 2013; O'Sullivan, 2016).

Despite numerous positive reports describing the interesting pharmacological effects of CBD in a wide range of pathological conditions and disease states, a limitation to its clinical use is its low and variable oral availability in humans (Aguere et al., 1981). Besides, CBD effects follow an inverted U shape curve, and this compound has a limited effective dose range (Rock & Parker, 2017). To possibly circumvent these limitations, new synthetic cannabinoid compounds have been designed and developed in the hope in discovering new chemical entities with improved pharmacokinetics and a better activity profile (Bow & Rimoldi, 2016).

In this study, we were particularly interested in further characterizing the effects of two synthetic cannabinoids, 4'-fluoro-cannabidiol (4'-F-CBD) and HU-910, chemically designed as (1S,4R)-(2-(2,6-dimethoxy-4-(2-methyloctan-2-yl)phenyl)-7,7-dimethylbicyclo[2.2.1]hept-2-en-1-yl) methanol (Figure 1). 4'-F-CBD is a hemisynthetic derivative of CBD that is fluorinated at position 4' of the benzene ring moiety. 4'-F-CBD has more potent anxiolytic, antidepressant, antipsychotic, anti-compulsive activity in mice in comparison to its parent compound (Breuer et al., 2016). It was also reported more recently to prevent chemotherapy-induced neuropathic pain (Silva et al., 2022). HU-910 is still closely analogous to CBD but is more distant structurally from its parent compound and, consequently it is obtained through a total synthesis procedure (Horvath et al., 2012). Interestingly, HU-910 was found to be protective previously in a model of hepatic ischemia/reperfusion (Horvath et al., 2012).

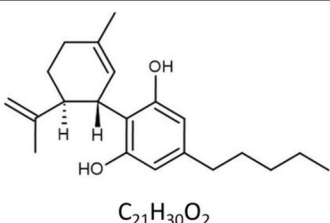
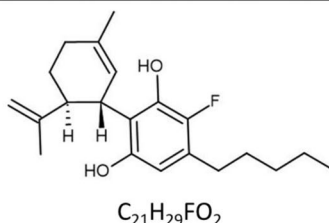
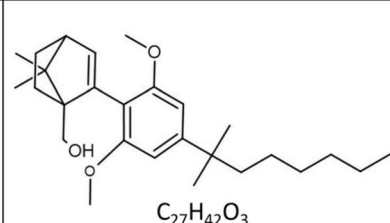
Molecular structure			
Abbreviation or code name	Cannabidiol (CBD)	4'-Fluorocannabidiol (4'-F-CBD); HUF-101; HU-474; PECS101	HU-910
IUPAC name	2-[[[(1R,6R)-3-methyl-6-prop-1-en-2-ylcyclohex-2-en-1-yl]-5-pentylbenzene-1,3-diol	4-Fluoro-2-[[[(1R,6R)-3-methyl-6-(prop-1-en-2-yl)cyclohex-2-en-1-yl]-5-pentylbenzene-1,3-diol	(1S,4R)-(2-(2,6-dimethoxy-4-(2-methyloctan-2-yl)phenyl)-7,7dimethylbicyclo[2.2.1] hept-2-en-1-yl)methanol
Molecular weight	314.5 g.mol ⁻¹	332.5 g.mol ⁻¹	414.61 g.mol ⁻¹

FIGURE 1 Chemical structures of 4'-F-CBD and HU-910 compared with that of CBD. Descriptive (IUPAC) and code names as well as molecular weights are also given for these compounds.

We wanted more specifically to explore the anti-inflammatory potential of 4'-F-CBD and HU-910 toward brain glial cells, and better understand the mechanism of action of these compounds in that context. For that, we established purified cultures of microglial cells and astrocytes isolated from postnatal mouse brain and implemented activation paradigms with reference inflammogens (Dos-Santos-Pereira et al., 2018; Dos-Santos-Pereira et al., 2020). Using cytokine and non-cytokine inflammation markers, we established that both 4'-F-CBD and HU-910 exert potent anti-inflammatory effects on microglial cells and astrocytes. These immunosuppressive effects required inhibition of reactive oxygen species (ROS)-dependent NF- κ B-dependent signaling. We also found that neither cannabinoid (CB1/CB2) nor cannabinoid-like (PPAR γ , GPR55) receptors contributed significantly to these effects.

2 | MATERIALS AND METHODS

2.1 | Pharmacological reagents

4'-F-CBD and HU-910 were generously given by Raphael Mechoulam (Hebrew University of Jerusalem, Jerusalem, Israel). Stock solutions of these two cannabinoid compounds were prepared at 2.5 and 5 mM, respectively, using distilled water with 10% of dimethyl sulfoxide. The antagonists/inverse agonists of CB1 (AM251) and CB2 (SCH336), and the antagonist of PPAR γ (GW9662) were from Tocris Bioscience (Bristol, UK). Lipopolysaccharide (LPS; Escherichia coli strain O26:B6; #L8274) and 2-[(Aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide (TPCA-1; #T1452) were purchased from Sigma Aldrich (L'Isle d'Abeau Chesnes, France). Apocynin (APO; #4663) was from R&D Systems Europe (Lille, France). Dulbecco's modified Eagle's medium (DMEM; #31885023), DMEM with Ham's F-12 component (DMEM/F-12; #21331046), the penicillin/streptomycin cocktail and the 0.05% Trypsin-EDTA solution used to produce subcultures of glial cells were obtained from Thermo-Fisher Scientific (Saint Aubin, France). Clodronate liposomes were provided by Liposoma BV (Amsterdam, The Netherlands). Fetal bovine serum (FCS) was from Biowest LLC (Eurobio, Les Ulis, France).

2.2 | Use of animals

C57BL/6J mice provided by Janvier LABS (Le Genest St Isle, France) were housed, handled, and cared for in strict accordance with the European Union Council Directives (2010/63/EU). The Committee on the Ethics of Animal Experiments Charles Darwin no. 5 approved experimental protocols under authorization number Ce5/2017/005. About 250–280 mouse pups were required for completion of this study. Note that male and female pups were mixed to generate glial cell cultures.

2.3 | Microglial cell cultures

Microglial cell isolation was performed by taking advantage of the preferential adhesion of these cells onto Polyethyleneimine (PEI)

coating under specific culture conditions (Dos-Santos-Pereira et al., 2018; Sepulveda-Diaz et al., 2016). Briefly, mouse pups (postnatal day 1) were sacrificed, and the whole brain rapidly dissected out. The tissue was processed as described before (Dos-Santos-Pereira et al., 2018) and cells in suspension plated in PEI-coated T75 Corning culture flasks using an isolation medium made of DMEM supplemented with 10% FCS and 1% of a cocktail penicillin/streptomycin. We plated an equivalent of two newborn mouse brains per 75-cm² culture flask to generate approximately 5 million cells/flask 14–16 days later. No medium change was performed until completion of microglial cell isolation that was spontaneous.

To produce subcultures, microglial cells were trypsinized 5 min at 37°C and seeded in uncoated 48-multiwell plates at a density of about 10⁵ cells/cm² using DMEM supplemented with 1% FCS, and 1% of an antibiotic cocktail. These cultures were virtually free of astrocytes (Figure S1a).

2.4 | Astrocyte cultures

To generate purified astrocyte cultures, we used an isolation protocol initially comparable to that described for microglial cells. However, the isolation medium was a DMEM/F-12 nutrient mixture supplemented with 10% FCS and 1% antibiotics, and the cells were seeded in laminin-coated flasks (1 μ g/mL). We plated an equivalent of one newborn mouse brain per 75-cm² culture flask to obtain approximately 8 million cells/flask upon completion of isolation 10 days later. Importantly, cultures received 2 \times /week clodronate liposomes (40 μ g/mL) to eliminate residual microglial cells (Dos Santos Pereira et al., 2020).

To produce astrocyte subcultures, adherent cells were mechanically dislodged from laminin-coated culture flasks and seeded at a density of about 10⁵ cells into laminin-coated Nunc 48-multiwell plates containing DMEM/F-12 medium supplemented with 1% FCS and 1% antibiotics. Stimulation experiments were performed on cultures of confluent astrocytes. These cultures were virtually free of microglial cells (Figure S1b).

2.5 | p47-phox/NADPH oxidase immunodetection

Cultures were fixed with 4% paraformaldehyde in PBS (20 min, room temperature), washed three times with PBS, and then incubated overnight with a polyclonal antibody against p47-phox/NADPH oxidase (NOX)2 (#H-195; Cliniscience, Nanterre, France; 1:50 in PBS-0.05% triton X-100). An Alexa-Fluor 555 conjugated secondary antibody (1:250 in PBS, 2 h) was used to reveal the immunosignal. The nuclei of labeled cells were counterstained with Hoechst-33,342. Fluorescent images were acquired using a Nikon TE 2000 inverted microscope (Nikon, Tokyo, Japan) equipped with an ORCA-ER digital cooled camera and the HCI imaging software. Changes in immunofluorescence signal intensities were quantified at the cellular level using previously described protocols (Dos-Santos-Pereira et al., 2020).

2.6 | Western immunoblotting

After drug stimulation, cells were processed using an established protocol (Santa-Cecilia et al., 2016). Briefly, cells were scraped off culture wells with M-PER buffer (Invitrogen) containing a cocktail of protease and phosphatase inhibitors (Invitrogen) and aliquots thereof resuspended for protein quantification in a Nanodrop 8000 Spectrophotometer (Thermo Fisher Scientific). Protein samples were then resolved onto an SDS-PAGE 4–12% gel and transferred to PVDF membranes with the Transfer-Turbo apparatus (Biorad). Membranes incubated with primary antibodies against either Glial fibrillary acidic protein (GFAP; 1:1000, overnight; FUJIFILM Wako #019-19741), ionized calcium-binding adapter molecule 1 (Iba-1; 1:500, overnight; Agilent Dako #GA52461-2), phospho-NF- κ B p65 Ser536 (1:1000, overnight, Cell Signaling, #3033) or Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:2000, 2 h; Cell Signaling, #2118), were then washed with 0.1% Tris Tween-20 buffered saline before incubation with an IRDye secondary antibody (1:10000, LI-COR Biosciences, Lincoln, NE, USA). Bands were visualized using a LI-COR Odyssey near-infrared imaging system (LI-COR Biosciences) and quantitative analysis was performed using the ImageJ software.

2.7 | Determination of cytokine production

After 24 h of stimulation with each of the test inflammogens, the culture medium was recovered and frozen for later analysis. Colorimetric ELISA detection kits from Thermo Fisher Scientific were used to quantify TNF- α (#BMS607-3), IL-1 β (#BMS6002) and IL-6 (#BMS603-2) using the manufacturer's instructions. Quantitative assessment of the cytokines was carried out using a SpectraMax M4 spectrophotometer (Molecular Devices, Sunnyvale, CA). Standard ELISA curves were generated using a four-parameter logistic curve model (GraphPad Prism 8, GraphPad Software).

2.8 | Glutamate assay

Glutamate levels were measured using the Amplex Red Glutamic Acid/Glutamate Oxidase Kit (#A12221; Invitrogen) according to the manufacturer's instructions. The fluorescent reaction product resulting from glutamic acid oxidation was quantified using a SpectraMax M4 microplate reader.

2.9 | Measurement of NADPH oxidase activity

To estimate ROS production by NADPH oxidase, glial cells were seeded in 96-well Nunc multiplates, at a density of 3.10^4 cells/well. After seeding, the cultures were preincubated or not for 2 h with the test treatments before being activated with LPS. After 24 h, the culture medium was removed and the cultures washed with a Hank's Balanced Salt Solution. They were then incubated with 100 μ L of assay medium provided in the superoxide anion assay kit (CS1000; Sigma Aldrich) in the presence or in the absence of the test treatments. The enzymatic

reaction was then triggered by the addition of luminol, and the chemiluminescence signal monitored at 37°C using a SpectraMax M4 microplate reader (Molecular Devices, Sunnyvale, CA), with an acquisition every 2 min for a total of 300 min. Quantitative ROS measurements were performed by quantifying the area under the curve (AUC) within the 50–100 min interval of each chemiluminescence trace.

2.10 | Real-time PCR amplification

Total RNA from microglial and astroglial cultures was extracted using a NucleoSpin RNA XS Kit (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions and reverse transcription, carried out using 850–950 ng total RNA in 20 μ L of reverse transcription reaction mixture (Verso cDNA Synthesis Kit, ThermoFisher). Real-time PCR amplification was performed using a LightCycler 480 SYBR Green I Master Kit (Roche Diagnostics). The following sets of primers from were used: CB1 (Cnr1) forward: 5'-GTGCTGTTGCTGTTTCATTGTG-3', reverse: 5'-CTTGC CATCTTCTGAGGTGTG-3'; CB2 (Cnr2) forward: 5'-TCTCTCTTCGA GGGAGTGAACCTGAACG-3', reverse: 5'-CTCGGTTACAGAAACAGAG GCTGATG-3'; PPAR γ (Pparg) forward: 5'-CGGTTTCAGAAGTGCCTTG-3', reverse: 5'-GGTTCAGCTGGTCGATATCAC-3'; GPR55 (Gpr55) forward: 5'-AGGCTATCTTACCAAGCAGCAC-3', reverse: 5'-TGGTTCAGC TGTCTGCCATTTTC-3'; TLR4 (Tlr4) forward: 5'-GCAATGTCTCTGG-CAGGTGTA-3', reverse: 5'-CAAGGGATAAGAACGCTGAGA-3'; GAPDH (Gapdh) forward: 5'-TGTGTCCGTCGTGGATCTGA-3', reverse: 5'-TTGCTGTTGAAGTCGAGGAG-3' (Eurofins, Nantes, France). Cycle threshold (Ct) values, which indicate the number of cycles required to reach the threshold for amplification were used to estimate gene transcript expression levels.

2.11 | Data presentation and statistical analysis

Graphs and data statistical comparisons were performed using the GraphPad Prism program (v.8.0). When pertinent, we introduced on the graphs' y-axis, a break interval with different linear scales for the lower and upper segment to evaluate better, smaller differences of test parameters. Data values were derived from at least two independent experiments performed in duplicate or triplicate. Pairwise comparisons were performed by One-Way Analysis of Variance (ANOVA), followed by a Bonferroni post-hoc test. Ct values derived from real-time PCR amplification plots were presented as boxplots.

3 | RESULTS

3.1 | 4'-F-CBD and HU-910 inhibit the release of proinflammatory cytokines in cultured glial cells challenged with LPS

We used purified cultured microglia and astrocytes activated by the bacterial cell wall component LPS (10 ng/mL), a prototypical TLR4

agonist (Doring et al., 2017), to evaluate the anti-inflammatory potential of the two synthetic cannabinoid compounds 4'-F-CBD and HU-910. After 24 h of exposure to LPS, microglial cells released large amounts of the two pro-inflammatory cytokines TNF- α and IL-6 and, comparatively smaller quantities of IL-1 β , a cytokine whose production relies on NLRP3 inflammasome activation (Kelley et al., 2019). When LPS-treated microglial cultures were exposed concomitantly to 4'-F-CBD or HU-910, we observed that the release of all of the pro-inflammatory cytokines was reduced (Figure 2a-c). However, whereas HU-910 had a robust inhibitory effect, at both 1 and 10 μ M whatever the cytokine measured, 4'-F-CBD exhibited a significant effect at 1 μ M, only in the case of TNF- α . The glucocorticoid dexamethasone (DEX; 2.5 μ M) used as reference immunosuppressive drug, mimicked the inhibitory effects of the two synthetic cannabinoids on microglial cytokine release.

Cultured astrocytes also released large amounts of TNF- α and IL-6, and comparatively smaller quantities of IL-1 β upon LPS stimulation (Figure 2d-f). These effects were efficiently prevented by 10 μ M of the two synthetic cannabinoids, 4'-F-CBD and HU-910. HU-910 remained highly effective at 1 μ M whereas 4'-F-CBD demonstrated inhibitory effects at this concentration but only against the release of IL-6 and IL-1 β .

3.2 | Impact of 4'-F-CBD and HU-910 on non-cytokine inflammation markers in LPS-activated microglial cells and astrocytes

3.2.1 | Phenotypic activation markers: Iba-1 and GFAP

Visualization of Western immunoblots from LPS-treated microglial cultures exposed or not to 10 μ M 4'-F-CBD or HU-910, revealed that the expression of the phenotypic activation marker Iba-1 was robustly repressed by each of the two cannabinoid compounds (Figure 3a). This was confirmed by quantitative analysis of immunoblot signals showing that 10 ng/mL LPS caused about a 3-fold increase in Iba-1 expression levels and that both 4'-F-CBD and HU-910 were able to reverse this effect (Figure 3a). Comparable observations were made by visual inspection of LPS-treated microglial cell cultures, exposed or not to the two cannabinoid compounds and then processed sequentially for CD11b and Iba-1 fluorescence immunodetection (Figure 3b).

The same approach was used to estimate the impact of the two cannabinoid compounds on GFAP, a phenotypic activation marker for astrocytes. We established that in astrocyte cultures, LPS increased the intensity of a predominant 50-KDa GFAP band and induced a weaker upper band at 52 KDa (Figure 3c). This led us to pool GFAP immunoblot signals from these two bands for measuring changes in GFAP expression. We found that treatments with either 10 μ M of 4'-F-CBD or HU-910 reduced GFAP induction by LPS, HU-910 being, however, in that case slightly less effective than 4'-F-CBD (Figure 3c). Likewise, the two cannabinoid compounds repressed LPS-mediated induction of GFAP expression when the protein was visualized by

fluorescence immunodetection (Figure 3d). The original immunoblots for Iba-1 and GFAP are presented in Figure S2.

3.2.2 | Extracellular glutamate levels

We also measured to what extent 4'-F-CBD and HU-910 could prevent LPS-stimulated release of glutamate in microglia, as this excitatory neurotransmitter is considered to be a non-cytokine inflammation marker for these cells (Dos-Santos-Pereira et al., 2018; Takeuchi et al., 2006). As expected, 10 ng/mL LPS strongly stimulated the release of glutamate in cultured microglia (Figure 3e), an effect requiring the activation of the Xc⁻ antiporter in these cells (Dos-Santos-Pereira et al., 2018). This effect was efficiently antagonized by 10 μ M of 4'-F-CBD or HU-910. In fact, 10 μ M HU-910 was so effective, that it put the glutamate release level to control level. At 1 μ M, HU-910 but not 4'-F-CBD significantly blunted glutamate release in LPS-treated microglia. Note that DEX was found totally ineffective in decreasing LPS-induced glutamate release.

LPS did not stimulate glutamate release in astrocytes. On the contrary, glutamate was accumulated and presumably detoxified by astrocytes (Mahmoud et al., 2019; Ye & Sontheimer, 1998) whatever the treatment applied to the cultures (Figure 3f). DEX was without effect in this paradigm.

Note that the dashed lines in Figure 3e,f represent glutamate concentrations inherently present in serum-supplemented media used for microglial and astrocyte cultures. These concentrations were estimated at 7.9 and 55.8 μ M in the two culture media, respectively.

3.3 | Contribution of canonical and non-canonical cannabinoid receptors to the anti-inflammatory action of 4'-F-CBD and HU-910 in cultured microglial cells and astrocytes

First, we wished to determine whether candidate cannabinoid (CB1, CB2) and cannabinoid-like (GPR55, PPAR γ) receptors susceptible to mediate 4'-F-CBD and HU-910 anti-inflammatory effects, were expressed in control microglial (Figure 4a) and astroglial (Figure 4b) cultures using real-time PCR amplification assays with GAPDH as housekeeping gene. Using Ct values derived from the amplification plots, we found that CB1, CB2, GPR55, and PPAR γ transcripts were expressed under control conditions whatever the type of culture. CB2 and PPAR γ showed the highest transcript abundance in microglial cultures with Ct values between 27 and 29 cycles. CB1 and GPR55 transcripts were, however, much less abundant in microglial cultures. CB1, CB2, GPR55, and PPAR γ transcripts were all moderately abundant in astrocyte cultures with Ct values between 29 and 31 cycles. The abundance of TLR4 transcripts was also estimated comparatively to that of cannabinoid and cannabinoid-like receptors. As expected TLR4 transcripts were abundantly expressed in microglial cultures with Ct values of about 25 cycles. Ct values for TLR4 transcripts were of the same order in pure astrocyte cultures. This is not necessarily expected

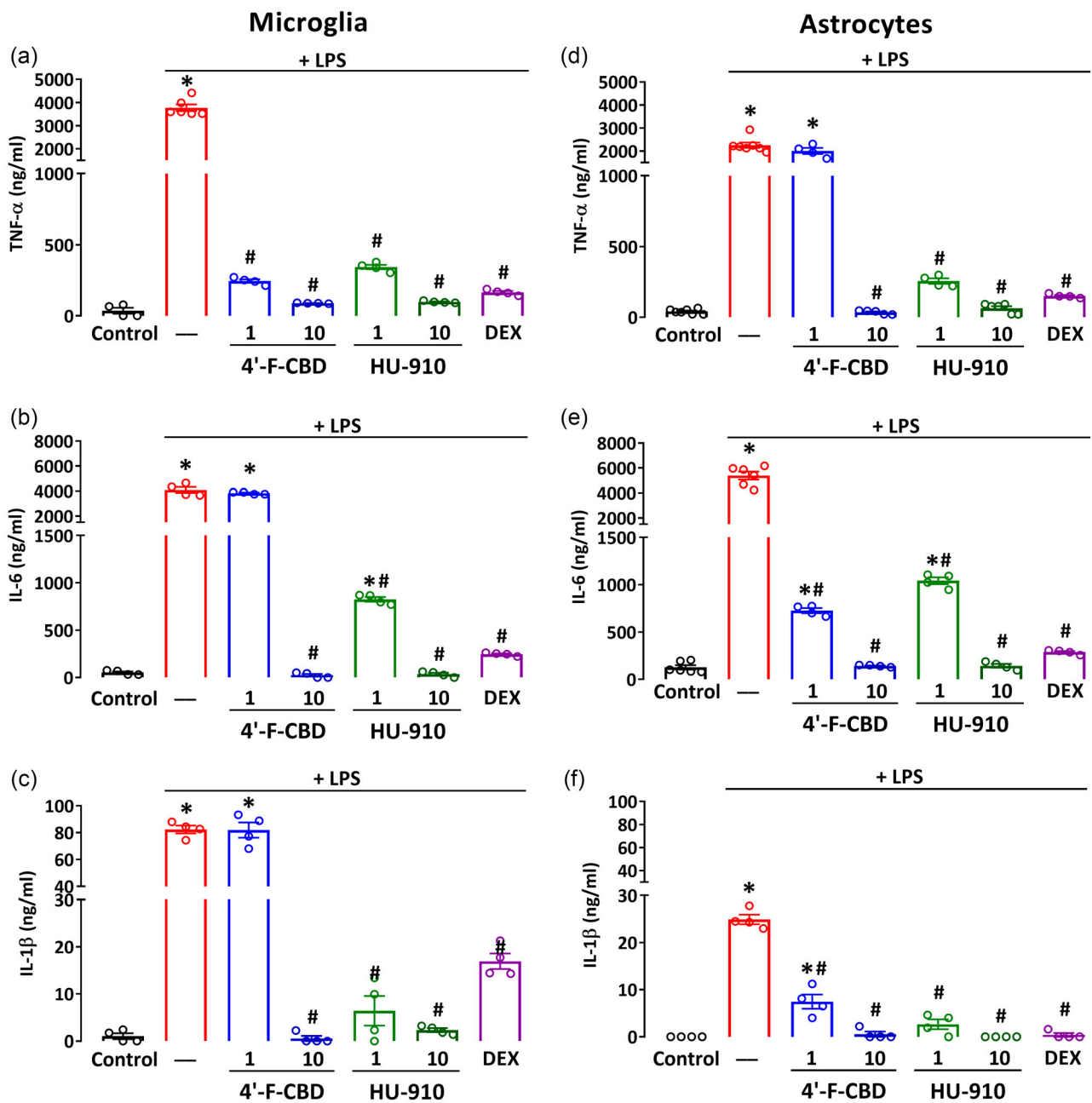


FIGURE 2 Anti-inflammatory effects of 4'-F-CBD and HU-910 in cultured microglia and astrocytes challenged with LPS. (a–c) Release of (a) TNF- α , (b) IL-6, and (c) IL-1 β in microglial cell cultures treated with LPS (10 ng/mL) for 24 h in the presence or the absence of 4'-F-CBD and HU-910, at 1 and 10 μ M. DEX (2.5 μ M) was used as reference immunosuppressive drug in this paradigm. (d–f) Release of (d) TNF- α , (e) IL-6, and (f) IL-1 β in astrocyte cultures exposed to the same treatments. Data expressed in ng/mL are presented as mean \pm SEM ($n = 4$ –6). * $p < .05$ versus control. # $p < .05$ versus LPS. One-way ANOVA followed by Bonferroni post-hoc test.

if we refer to data showing that cultured astrocytes are resistant to TLR4-dependent activation by LPS in the absence of microglia (Liddel et al., 2017) but consistent with present results and other reports showing that pure astrocyte cultures are responsive to LPS challenge (Li et al., 2021; Rodgers et al., 2020; Tarassishin et al., 2014). Finally, Ct values for the housekeeping gene GAPDH were always between 19 and 20 cycles whatever the type of culture.

Then, to functionally assess the contribution of these receptors to 4'-F-CBD or HU-910 anti-inflammatory effects, we exposed LPS-treated

microglial and astrocyte cultures to each cannabinoid compound (10 μ M) in the presence or the absence of an antagonist/inverse agonist of CB1 (AM251) (Pertwee, 2009) or CB2 (SCH336) (Lunn et al., 2006), or of an antagonist of PPAR γ (GW9662) (Seargent et al., 2004). Two concentrations (0.1 and 1 μ M) of AM251, SCH336, or GW9662 were used. Using TNF- α release as a marker of the activation state of LPS-treated glial cells, we found that none of the test antagonists could reduce the anti-inflammatory effects provided by the two synthetic cannabinoid compounds in either microglial (Figure 4c) or astrocyte (Figure 4d) cultures.

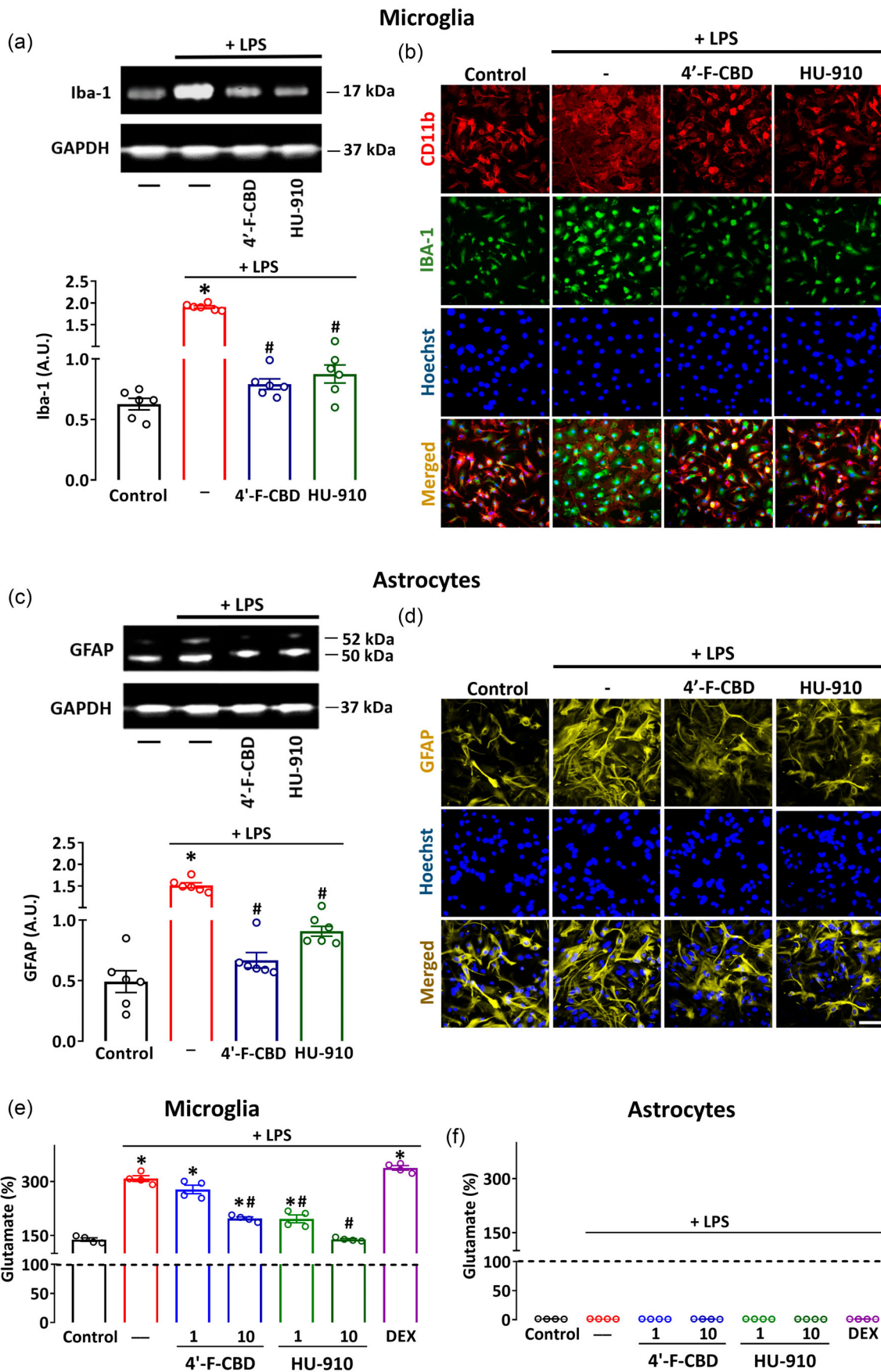


FIGURE 3 Legend on next page.

These antagonists were also found ineffective in reducing microglial glutamate release evoked by LPS (not shown).

The fact that the CB1 antagonist/inverse agonist AM251, which is also an agonist at the cannabinoid-like receptor GPR55, did not limit the anti-inflammatory activity of 4'-F-CBD or HU-910 in glial cell cultures treated with LPS, also demonstrates that these two compounds do not operate by functional antagonism of GPR55.

3.4 | The anti-inflammatory effect of 4'-F-CBD and HU-910 are not restricted to the paradigm of glial cell activation by LPS

At this stage, we found that it could be relevant to determine whether the anti-inflammatory effects of 4'-F-CBD and HU-910 could be observed against other pro-inflammatory stimuli. Specifically, we used a synthetic triacylated lipopeptide Pam3CSK4 (1 $\mu\text{g}/\text{mL}$) which is an agonist at TLR2, and BzATP (500 μM), a prototypic P2X7 purinergic receptor agonist, to activate microglial cells and astrocytes.

We found that Pam3CSK4 (1 $\mu\text{g}/\text{mL}$) leads to a robust increase in TNF- α release in cultures of microglial cells (Figure 5a). Even if the pro-inflammatory effect of the lipopeptide was comparatively of smaller intensity in astrocyte cultures, it was still very robust (Figure 5b). At a concentration of 10 μM , both 4'-F-CBD and HU-910 largely reduced TNF- α release upon Pam3CSK4 stimulation in both types of glial cultures (Figure 5a,b). Both compounds seemed a bit more efficacious in astrocyte cultures.

In comparison to Pam3CSK4 (1 $\mu\text{g}/\text{mL}$), BzATP (500 μM) led to a much smaller stimulation of TNF- α release in both microglial and astrocyte cultures (Figure 5c,d). Under these conditions, 4'-F-CBD and HU-910 remained highly effective in restraining TNF- α release (Figure 5c,d).

3.5 | Both 4'-F-CBD and HU-910 prevent oxidative burst in LPS-activated microglia and astrocytes

We used a quantitative cell-based chemiluminescence assay for the measurement of ROS in LPS-stimulated glial cells. Our results

show that LPS (10 ng/mL) caused a robust increase of ROS in LPS-activated microglia (Figure 6a). This increase peaked 70 min post-exposure to LPS and declined thereafter to become negligible 150 min after exposure to the bacterial toxin. Strikingly, both 4'-F-CBD and HU-910 strongly reduced the chemiluminescent signal when applied to the cultures at 10 μM . These inhibitory effects were mimicked by APO (300 μM), a specific inhibitor of NOX, an enzyme responsible for ROS emission in activated glial cells (Bedard & Krause, 2007). Comparing the area under the chemiluminescence emission curve for each treatment condition provided a quantitative evaluation of the inhibitory effects of 4'-F-CBD, HU-910 and APO against LPS-induced ROS emission (Figure 6b).

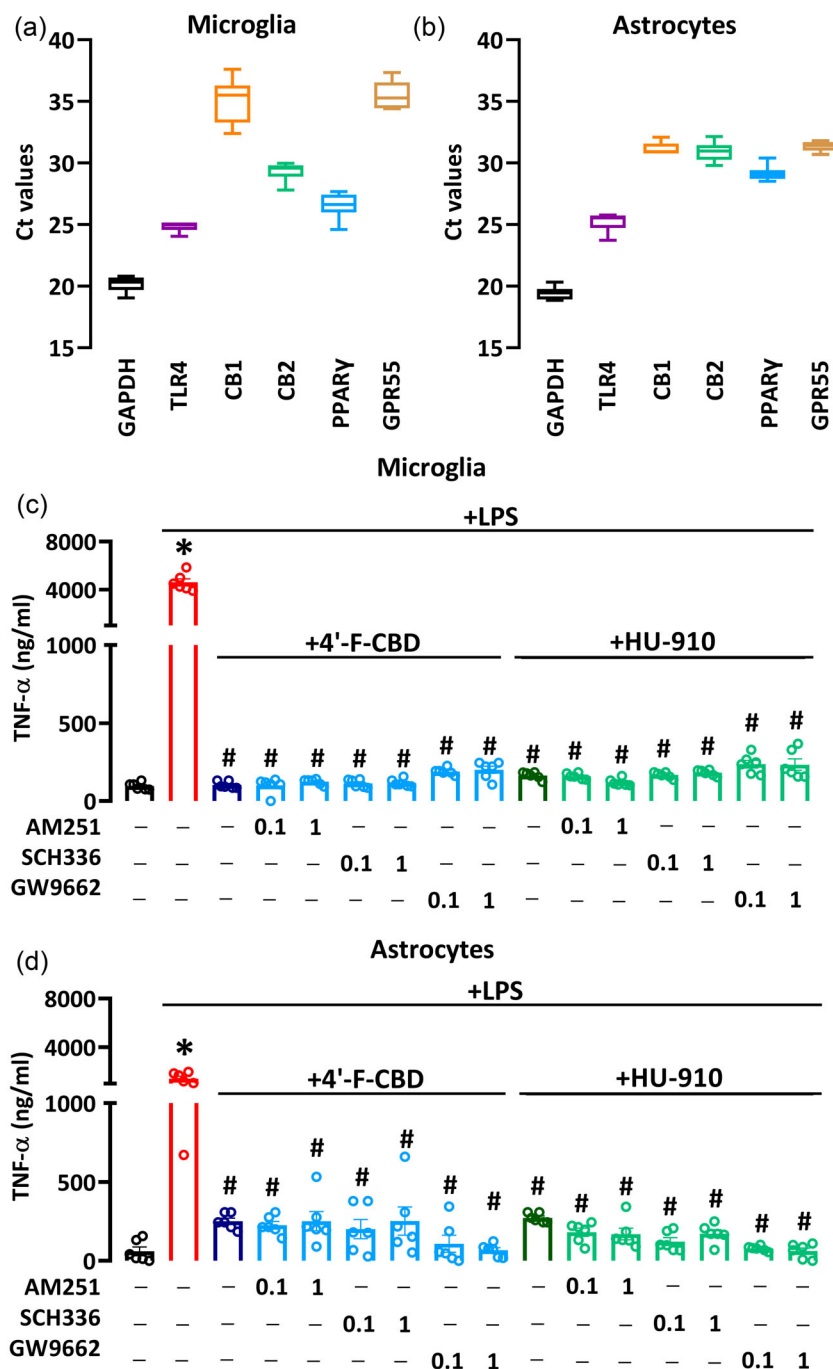
In astrocyte cultures, the ROS burst occurred after 70 min of exposure to LPS and plateaued up to 100 min to decline then abruptly (Figure 6c). Similar to what was observed in LPS-treated microglia, ROS emission was strongly reduced by 10 μM of either 4'-F-CBD or HU-910. APO (300 μM) also mimicked the effects of the two cannabinoid compounds. Quantitative interpretation of the ROS emission curves obtained under the different conditions of treatment, confirmed the potential of the two cannabinoid compounds as well as that of APO to repress ROS production in astrocyte cultures (Figure 6d).

To further explore the mechanism by which 4'-F-CBD and HU-910 could exert their antioxidant effects, we evaluated cell expression levels of p47phox, a subunit of NOX2, an isoform of NOX abundantly expressed in microglia and astrocytes (Bedard & Krause, 2007).

Fluorescent microphotographs of microglial cells (Figure 6e) and astrocytes (Figure 6f) treated with LPS in the presence or not of 4'-F-CBD or HU-910 and then immunostained for p47phox are provided as illustration of the impact of test treatments. Quantification of the immunofluorescent signal revealed an increase of about 30% and 25% of p47phox in LPS-treated microglial (Figure 6e) and astrocyte (Figure 6f) cultures, respectively. The NOX2 p47phox subunit, however, remained elevated if LPS treatment was performed in the presence of 10 μM of 4'-F-CBD or HU-910, indicating that ROS inhibition by these two drugs is not due to a repressive effect on NOX2 expression.

FIGURE 3 Inhibitory effects of 4'-F-CBD and HU-910 on non-cytokine inflammation markers induced by LPS in microglial and astrocyte cultures. (a) Upper panel: Western immunoblot showing variations of Iba-1 expression in LPS (10 ng/mL)-treated microglial cultures exposed or not exposed to 10 μM 4'-F-CBD or 10 μM HU-910. Lower panel: Quantitative immunoblotting analysis of Iba-1 expression in the same conditions as above. (b) Visualization of CD11b (red) and Iba-1 (green) immunolabeled microglial cells with Hoechst-33,342 counterstaining of nuclei (blue) in the same conditions of treatment as in (a). Scale bar: 50 μm . (c) Upper panel: Western immunoblot showing variations in GFAP expression in LPS (10 ng/mL)-treated astrocyte cultures exposed or not exposed to 10 μM 4'-F-CBD or 10 μM HU-910. Lower panel: Quantitative immunoblotting analysis of GFAP expression in the same conditions as above. (d) Immunodetection of GFAP⁺ (green) astrocytes with Hoechst-33,342 counterstaining of nuclei (blue) under the same conditions as in (c). Scale bar: 50 μm . In (a) and (c) GAPDH expression is used for normalization of Western blot immunosignals. Data expressed in arbitrary units (AU) are presented as mean \pm SEM ($n = 6$). * $p < .05$ versus control. # $p < .05$ versus LPS. One-way ANOVA followed by Bonferroni post-hoc test. Microphotographs of Hoechst-33,342 stained nuclei in (b) and (d) indicate that neither 4'-F-CBD nor HU-910 could act primarily by repressing cell proliferation in LPS-treated cultures. (e, f) Extracellular glutamate levels in microglial (e) and astroglial (f) cell cultures exposed to the same treatments as in (a). Data expressed in % of basal glutamate levels in complete (serum-supplemented) culture medium (dashed lines) are presented as mean \pm SEM ($n = 6$). * $p < .05$ versus control. # $p < .05$ versus LPS. One-way ANOVA followed by Bonferroni post-hoc test. Actual concentrations of glutamate were estimated at 7.9 and 55.8 μM in complete culture media used for microglial and astrocyte cultures, respectively.

FIGURE 4 Anti-inflammatory effects of 4'-F-CBD and HU-910 in glial cultures are neither mediated by CB1/2, PPAR γ nor GPR55. (a, b) Box plot representation of transcript abundance of cannabinoid and cannabinoid-like receptors in control microglial (a) and astroglial (b) cultures based on corresponding Ct values ($n = 6$) derived from real-time PCR amplification plots. The abundance of TLR4 transcripts is also estimated comparatively to that of cannabinoid and cannabinoid-like receptors. GAPDH is used as the housekeeping gene. Lines across the boxes show median values and box spans, interquartile ranges. (c) TNF- α release in microglial cell cultures challenged with LPS (10 ng/mL) and treated or not with 10 μ M 4'-F-CBD or HU-910 in the presence or absence of AM251 (CB1 antagonist/inverse agonist; GPR55 agonist), SCH336 (CB2 antagonist/inverse agonist), or GW9662 (PPAR γ antagonist). The concentrations (0.1, 1 μ M) are indicated directly under bars. (d) TNF- α release in astrocyte cultures exposed to the same treatments as in (c). The fact that the CB1 antagonist/inverse agonist AM251 operates as an agonist at GPR55, suggests that anti-inflammatory effects of 4'-F-CBD or HU-910 do not occur by functional antagonism, at this receptor. Data expressed in ng/mL are presented as mean \pm SEM ($n = 6$). * $p < .05$ versus control. # $p < .05$ versus LPS. One-way ANOVA followed by Bonferroni post-hoc test.



3.6 | 4'-F-CBD and HU-910 operate by inhibiting ROS-dependent NF- κ B signaling

To determine to what extent inhibition of NF- κ B signaling contributes to the anti-inflammatory effects of 4'-F-CBD or HU-910, we monitored and quantified expression levels of phosphorylated (Ser536) NF- κ B p65 in microglial (Figure 7a) and astrocyte (Figure 7b) cultures under various treatment conditions. Quantitative analysis of Western immunoblots showed that the increase of the phosphorylated (activated) form of NF- κ B p65 by LPS was totally abolished by treatment with 10 μ M of 4'-F-CBD or HU-910 in either microglial or astrocyte

cultures (Figure 7a,b). APO (300 μ M) and TPCA-1 (1.5 μ M), a blocker of I κ B kinase-2, a kinase required for NF- κ B activation mimicked the repressive action of 4'-F-CBD and HU-910 on LPS-dependent NF- κ B activation in microglial (Figure S3a) and astrocyte (Figure S3b) cultures.

Finally, we also established that inhibitory effects of 4'-F-CBD (10 μ M) and HU-910 (10 μ M) on LPS-induced TNF- α release were reproduced by APO (300 μ M) and TPCA-1 (1.5 μ M) in either microglial (Figure 7c) or astrocyte (Figure 7d) cultures. This suggested that 4'-F-CBD and HU-910 operated by repressing ROS-dependent NF- κ B signaling in this setting.

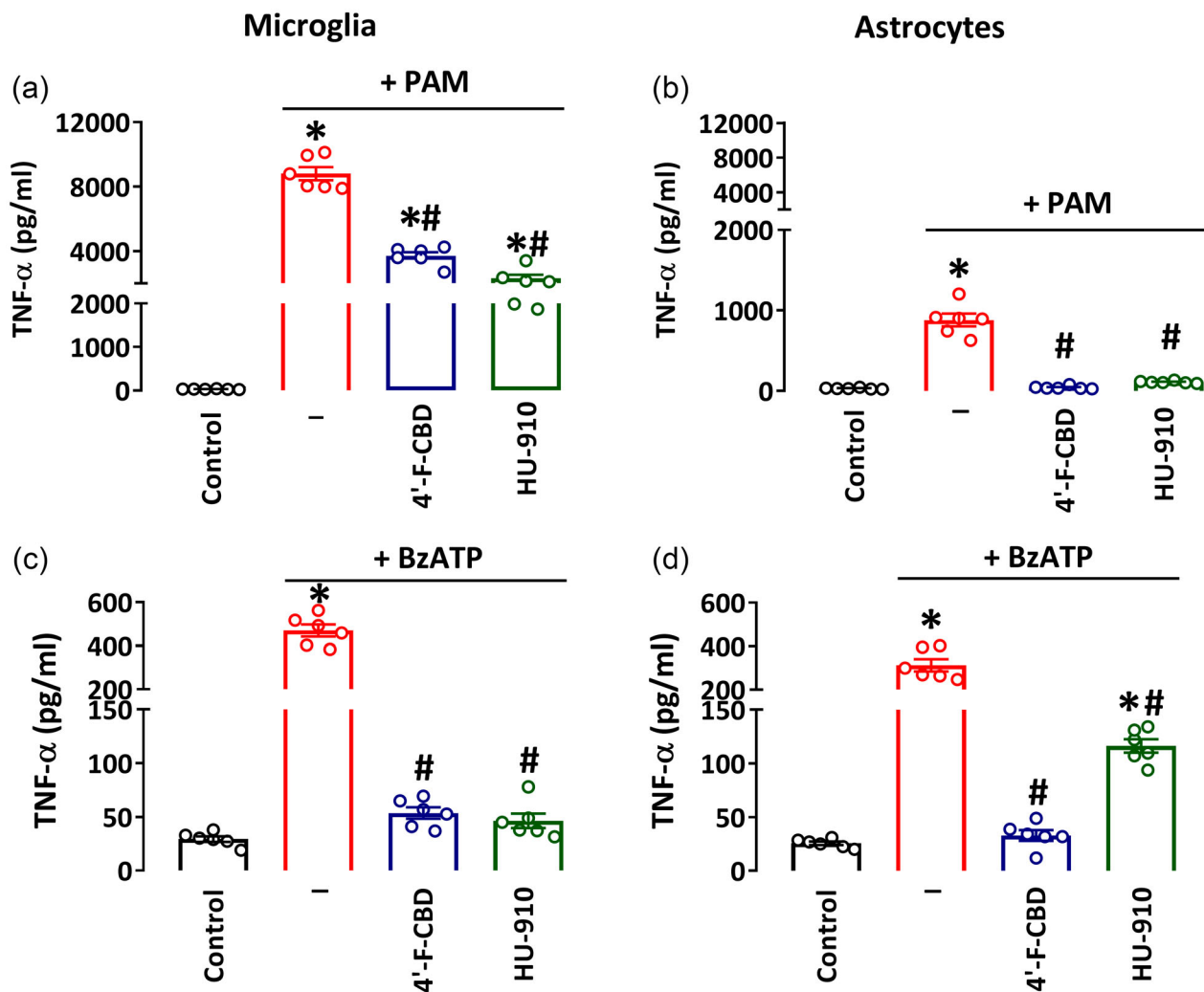


FIGURE 5 Inhibitory effects of 4'-F-CBD and HU-910 on TNF- α release evoked by agonists of TLR2 or P2X7 receptors in microglial and astrocyte cultures. (a, b) Impact of 4'-F-CBD (10 μ M) and HU-910 (10 μ M) on the release of TNF- α evoked by an agonist of TLR2 receptors Pam3CSK4 (PAM; 1 μ g/mL) in microglial (a) and astrocyte (b) cultures. (c, d) Impact of 4'-F-CBD (10 μ M) and HU-910 (10 μ M) on the release of TNF- α evoked by an agonist of P2X7 purinergic receptors BzATP (500 μ M) in microglial (c) and astrocyte (d) cultures. Data expressed in ng/mL are presented as mean \pm SEM ($n = 6$). * $p < .05$ versus control. # $p < .05$ versus corresponding inflammogen. One-way ANOVA followed by Bonferroni post-hoc test.

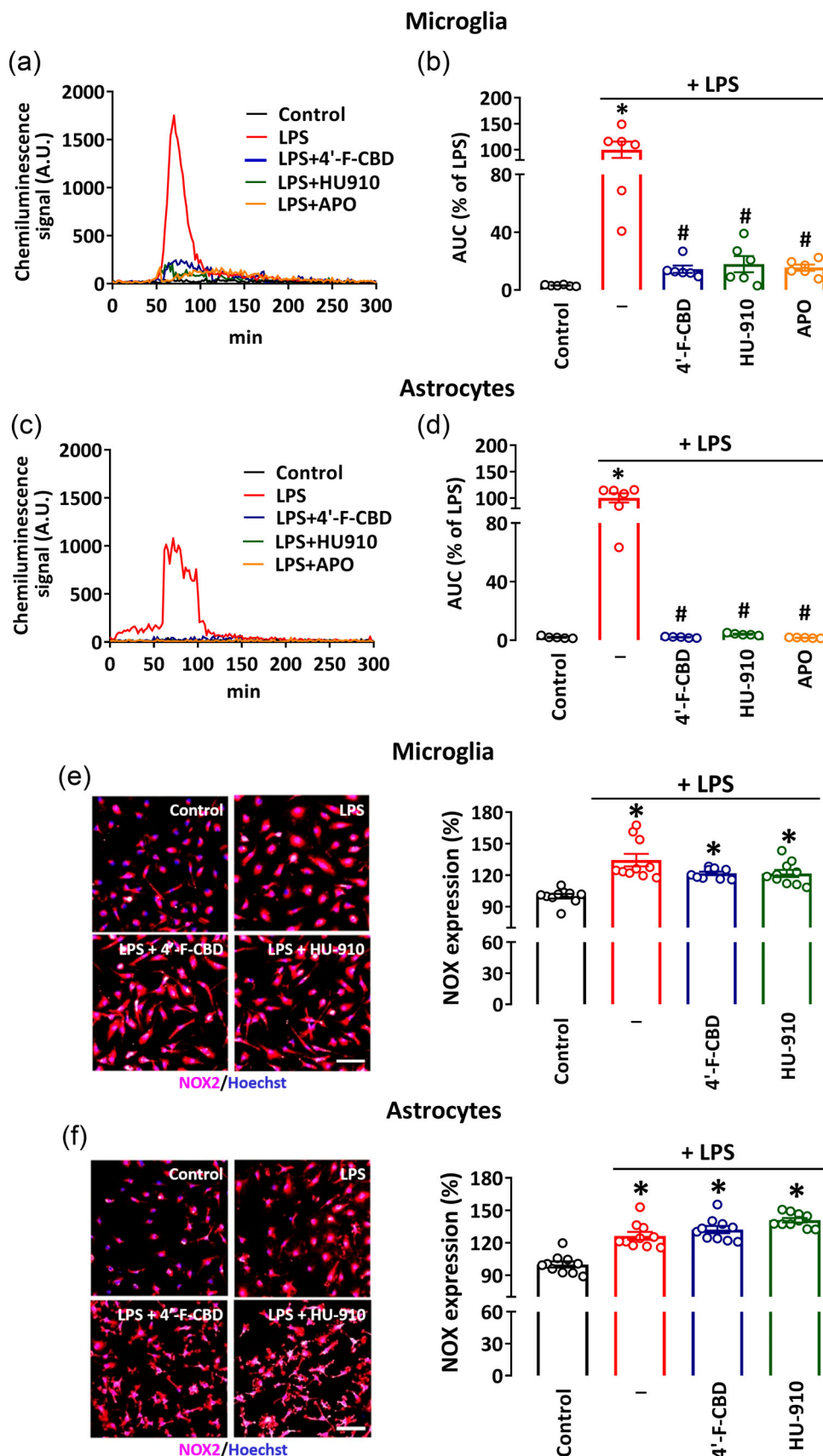
4 | DISCUSSION

We established here that the two synthetic cannabinoid compounds 4'-F-CBD and HU-910 could efficiently restrain the inflammatory response of brain microglia and astrocytes activated by the TLR4 ligand LPS. These immunosuppressive effects observed on cytokine and non-cytokine inflammation markers, were not antagonized by canonical or non-canonical cannabinoid receptor antagonists. Both 4'-F-CBD and HU-910 were also capable to limit the inflammatory response of glial cells exposed to ligands of TLR2 or purinergic P2X7 receptors, suggesting that these two compounds could provide immunosuppressive effects in different inflammatory pathological contexts. Interestingly, we established that 4'-F-CBD and HU-910 exert their immunosuppressive action toward LPS by inhibiting sequentially NOX-mediated ROS production and NF- κ B-mediated signaling events.

4.1 | 4'-F-CBD and HU-910 exert potent anti-inflammatory effects in LPS-activated microglial cells and astrocytes

The naturally occurring cannabinoid CBD has been reported to possess robust anti-inflammatory properties (Dos-Santos-Pereira et al., 2020; Henshaw et al., 2021) that are illustrated for instance in animal models of pulmonary hypertension (Krzyszewska et al., 2022), rheumatoid arthritis (Lowin et al., 2020), interstitial cystitis/bladder pain (Kuret et al., 2023) and brain degeneration (Dos Santos Pereira et al., 2020; Dos-Santos-Pereira et al., 2016; Meyer et al., 2022). Here, we wished to evaluate the anti-inflammatory potential of two synthetic structural analogs of CBD, 4'-F-CBD (Breuer et al., 2016; Li et al., 2020) and HU-910 (Horvath et al., 2012) using brain glial cells in culture. Our specific

FIGURE 6 ROS burst prevention by 4'-F-CBD and HU-910 in microglial cells and astrocytes activated with LPS. (a) Representative kinetic profile of the chemiluminescence signal that reflects ROS emission in LPS-treated cultured microglial cells exposed or not to 4'-F-CBD (10 μ M), HU-910 (10 μ M), or APO (300 μ M). (b) Quantification of the ROS signal emitted by cultured microglial cells is performed by integrating the AUC within the interval 50–100 min for each treatment condition described in (a). (c) Representative kinetic profile of ROS emission in LPS-treated cultured astrocytes exposed or not to 4'-F-CBD or HU-910 (10 μ M) or APO (300 μ M). (d) Quantitative assessment of ROS emitted by cultured astrocytes receiving the same treatments as in (c). In (a, c) chemiluminescence intensity was continuously monitored for 300 min and data are expressed in arbitrary units (AU). In (b, d) AUCs are expressed as mean-values percentages of LPS treatment \pm SEM ($n = 6$). One-way ANOVA followed by Bonferroni post-hoc test. * $p < .05$ versus control. # $p < .05$ versus LPS. (e) Visualization and quantification of the p47phox NOX2 subunit immunosignal in LPS-treated microglial cultures, exposed or not, to 4'-F-CBD (10 μ M), HU-910 (10 μ M) or APO (300 μ M). (f) Visualization and quantification of the p47phox immunosignal in astrocyte cultures receiving the same treatments as in (e). In (e, f) data are presented as mean-values percentages \pm SEM of controls ($n = 10$). One-way ANOVA followed by Bonferroni post-hoc test. * $p < .05$ versus control. Scale bars: 120 μ m.



interest in 4'-F-CBD results from the fact it has a better pharmacological profile than CBD (Morales et al., 2017; Silva et al., 2017). HU-910 is more distant structurally from CBD but still highly

analogous to its natural parent compound (Horvath et al., 2012), suggesting that it could also possess interesting anti-inflammatory properties.

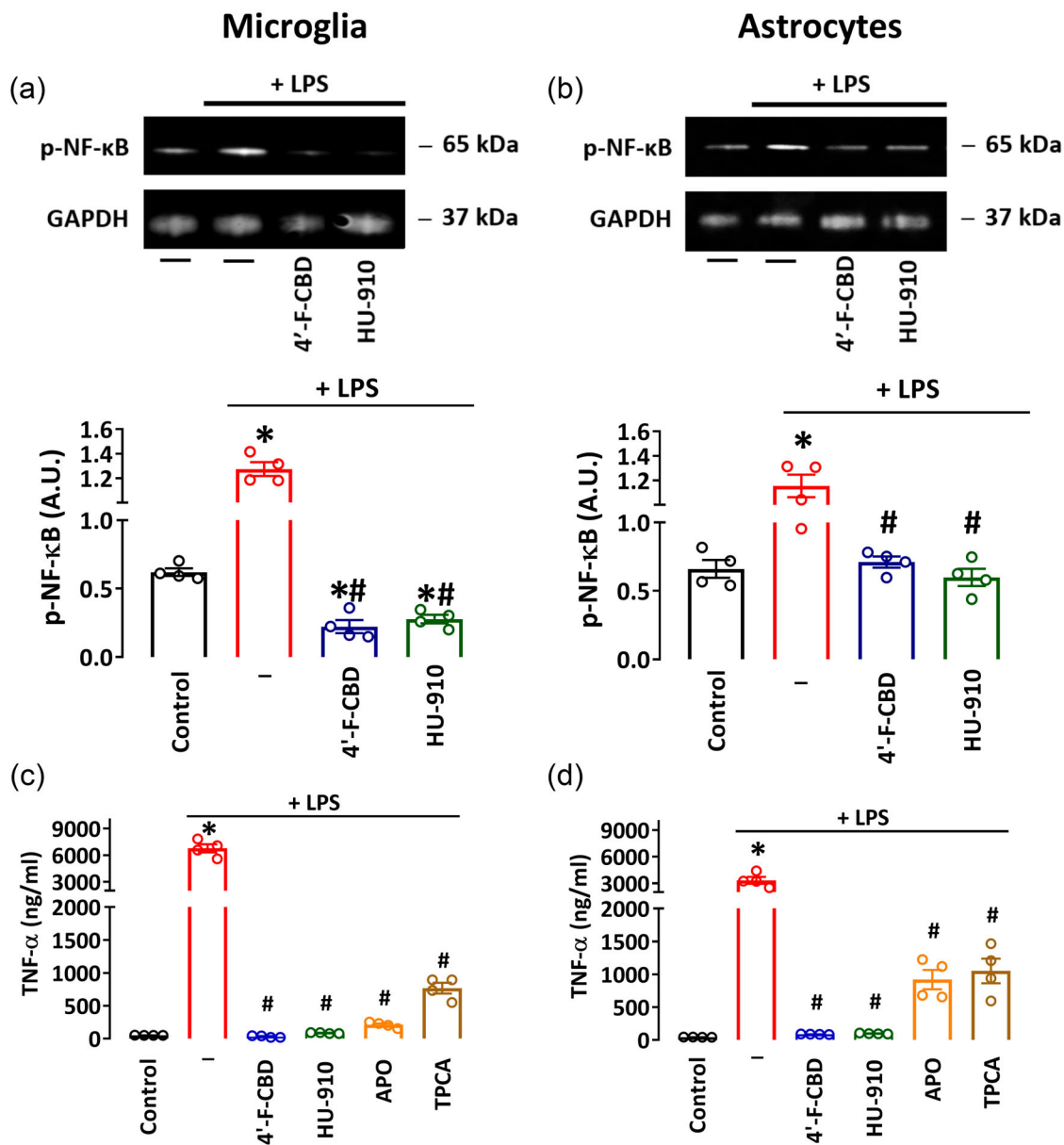


FIGURE 7 4'-F-CBD and HU-910 repress glial inflammatory processes by repressing ROS-dependent activation of NF-κB. (a, b) Western blot visualization and quantification of p-NF-κB p65 expression in cultured microglial cells (a) and astrocytes (b) exposed to 10 ng/mL LPS for 30 min in the presence or absence of 4'-F-CBD (10 μM) or HU-910 (10 μM). GAPDH expression was used for normalization of the immunosignal. Data expressed in arbitrary units (A.U.) are presented as mean ± SEM (n = 4). **p* < .05 versus control. #*p* < .05 versus LPS. One-way ANOVA followed by Bonferroni. (c, d) Release of TNF-α in microglial and astrocyte cultures challenged with LPS (10 ng/mL) for 24 h in the presence or the absence of 4'-F-CBD (10 μM), HU-910 (10 μM), TPCA-1 (1.5 μM), or APO (300 μM). Data expressed in ng/mL are presented as mean ± SEM (n = 4). **p* < .05 versus control. #*p* < .05 versus LPS. One-way ANOVA followed by Bonferroni post-hoc test.

After 24 h of exposure to LPS, pure microglia and astrocyte cultures released large amounts of the two pro-inflammatory cytokines TNF-α and IL-6 and, comparatively smaller quantities of IL-1β. The astrocyte response was not necessarily expected since LPS-mediated astrogliosis is believed to depend on soluble factors released by microglial cells (Liddel et al., 2017). Still, a number of studies showed that LPS-stimulated astrocytes are capable of releasing cytokines in a microglial cell-free environment, suggesting that the inflammation response of astrocytes might be context-dependent and

possibly determined by cell culture conditions (Rodgers et al., 2020; Tarassishin et al., 2014; Zhang et al., 2016).

We found that 4'-F-CBD exerts potent inhibitory effects against LPS-mediated cytokine release in both culture models, which is coherent with previous reports describing that 4'-F-CBD efficiently reduces reactive gliosis following neonatal sciatic nerve axotomy (Perez et al., 2018) and prevents upregulation of pro-nociceptive cytokines in a neuropathic pain model (Silva et al., 2022). HU-910 was also highly effective in limiting the inflammatory cytokine response of

cultured microglial cells and astrocytes exposed to LPS. Overall, HU-910 was generally more potent than 4'-F-CBD in repressing the release of cytokines. Present observations are coherent with a previous study showing that HU-910 restrains inflammatory-type reactions and cell death associated with hepatic ischemia/reperfusion injury (Horvath et al., 2012).

Noticeably, 4'-F-CBD and HU-910 were also capable of potently inhibiting the release of the non-cytokine inflammation mediator glutamate in LPS-treated microglial cultures, an effect requiring activation of the Xc⁻ antiporter in these cells (Dos-Santos-Pereira et al., 2018). Neither compound had such an effect in astrocyte cultures, as glutamate was accumulated and presumably detoxified by astrocytes (Mahmoud et al., 2019; Ye & Sontheimer, 1998) whatever the treatment applied to the cultures.

4.2 | 4'-F-CBD and HU-910 anti-inflammatory effects on glial cells are observed against diverse inflammatory stimuli

We showed that 4'-F-CBD and HU-910 could efficiently curtail the inflammatory responses elicited by LPS whose effects are exclusively mediated by TLR4 (Doring et al., 2017). However, we also demonstrate that both cannabinoid compounds are highly effective under conditions where glial inflammatory responses are elicited by either the triacylated lipopeptide Pam3CSK4 or the benzoyl ester of ATP, BzATP, that is, activation signals operating through TLR2 (Kaur et al., 2022) and purinergic P2X7 receptors (Young et al., 2007), respectively. This signifies that 4'-F-CBD and HU-910 might be potentially effective against activation signals promoting neuroinflammation in a variety of pathological states. Note that the effective concentrations of 4'-F-CBD and HU-910 were comprised between 1 and 10 μ M in all present test paradigms. This is very similar to what has been reported previously with CBD in different models of microglial inflammation (Dos-Santos-Pereira et al., 2020; Landucci et al., 2022; Yang et al., 2022).

4.3 | The anti-inflammatory effects of 4'-F-CBD and HU-910 are not sensitive to antagonists of canonical and non-canonical cannabinoid receptors

Cannabinoid (CB1, CB2) and cannabinoid-related (GPR55, PPAR γ) receptors were reported to be expressed by microglia and astrocytes not only *in vivo* but also under various cell culture conditions (Aguirre-Rueda et al., 2015; Egana-Huguet et al., 2021; Eraso-Pichot et al., 2023; Facchinetti et al., 2003; Lively & Schlichter, 2018; Pietri et al., 2009; Stella, 2010). We established, here, that CB1, CB2, GPR55, and PPAR γ are also expressed under present culture conditions.

To study the possible contribution of these receptors to the immunosuppressive action of 4'-F-CBD and HU-910 toward microglial cells and astrocytes, we used the paradigm of LPS stimulation

and TNF- α as an inflammation marker. We found that the anti-inflammatory effects of 4'-F-CBD and HU-910 were resistant to reference antagonists/inverse agonists of canonical CB1 (AM251) and CB2 (SCH336) (Lunn et al., 2006; Sink et al., 2010), and to an antagonist of PPAR γ (GW9662), a nuclear receptor operating as non-canonical receptor for cannabinoids (Esposito et al., 2011; O'Sullivan, 2016). These observations are quite surprising given previous reports showing that 4'-F-CBD could activate PPAR γ (Silva et al., 2022) and HU-910 CB2 (Horvath et al., 2012). Note that 4'-F-CBD and HU-910 could also possibly exert their effects by functionally antagonizing GPR55, a plasma membrane receptor identified previously as a putative cannabinoid-like receptor modulating inflammation (Gray & Whalley, 2020; Saliba et al., 2018; Saliba et al., 2021). The fact that the CB1 antagonist/inverse agonist AM251 failed to reduce the immunosuppressive activities of 4'-F-CBD and HU-910, argues against this possibility as this compound is also an agonist at GPR55 (Sharir & Abood, 2010).

Overall, present results suggest that the two test cannabinoid compounds repress glial inflammatory responses through a mechanism that is presumably receptor-independent, which is reminiscent of what we reported earlier for CBD (Dos-Santos-Pereira et al., 2020). That another putative receptor could possibly contribute to the anti-inflammatory effects of 4'-F-CBD and HU-910 cannot be, however, totally ruled out at this stage.

4.4 | 4'-F-CBD and HU-910 operate by inhibiting ROS emission

We also established that the inflammatory response to LPS was associated with a robust elevation of intracellular oxidative stress in both microglial cells and astrocytes. Interestingly, this response was prevented by concentrations of 4'-F-CBD and HU-910 that reduce microglial inflammatory-type responses, and by APO, an inhibitor of the superoxide-producing enzyme NOX (Stefanska & Pawliczak, 2008), indicating that ROS generated through NOX activation were probably crucially involved in the inflammatory response to LPS, and that 4'-F-CBD and HU-910 possibly interfered with this process. Note that 4'-F-CBD and HU-910 failed to repress the cellular expression of p47phox, a subunit of NOX2, an abundant isoform of NOX in glial cells, suggesting that the two test compounds may operate downstream to enzyme activation possibly by directly interfering with ROS production. Such a hypothesis would fit with the idea that the anti-inflammatory effects of the two synthetic cannabinoid compounds are receptor-independent in this experimental setting.

The antioxidant activity for 4'-F-CBD could be logically explained by its close homology to CBD, which has itself a strong intrinsic potential to neutralize free radical species (Borges et al., 2013; Dos-Santos-Pereira et al., 2020). This antioxidant potential could be largely explained by the two-hydroxyl groups present in the ortho position of the aromatic ring of 4'-F-CBD. The antioxidant potential of HU-910 is more difficult to explicate as the two ortho-hydroxy groups have been replaced by ortho-methoxy substituents having

significant but supposedly lower free radical scavenging capacity (Farhoosh et al., 2016). It should be noted, however, that the antioxidant capacity of these two compounds appears equivalent if we refer to our two cell-based assays.

To our knowledge, there was no previous report demonstrating that 4'-F-CBD possesses antioxidant properties. The fact that HU-910 could partly operate through an antioxidant effect is, however, supported by a report showing that this compound can restrain oxidative stress in a model of hepatic ischemia/reperfusion injury (Horvath et al., 2012). Overall, it appears that the immunosuppressive effects of 4'-F-CBD and HU-910 on glial cells are partly related to their antioxidant potential.

4.5 | 4'-F-CBD and HU-910 operate by preventing ROS-dependent activation of NFκB

The expression of the phosphorylated (activated) form of the p65 subunit of the NF-κB transcription factor was found to be elevated in

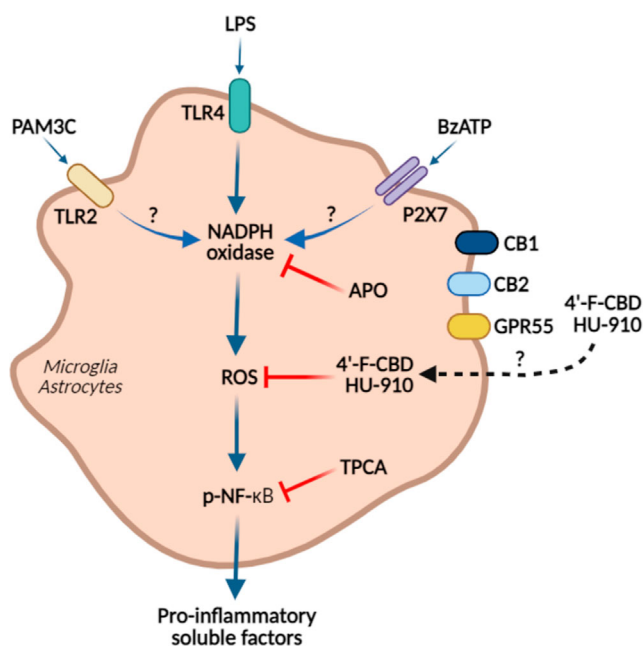


FIGURE 8 Schematic drawing representing the hypothetical mechanism by which 4'-F-CBD and HU-910 could curtail the proinflammatory response of activated microglia and astrocytes. The bacterial inflammogen LPS promotes TLR4-dependent activation of NADPH oxidase activity, which leads in turn to ROS production. ROS stimulate p-NF-κB translocation and as a consequence the release of proinflammatory mediators. The two cannabinoid compounds 4'-F-CBD and HU-910 operate independently of cannabinoid receptors, through an antioxidant mechanism that blocks LPS-induced ROS burst, and consequently NF-κB-dependent production of proinflammatory factors. Inhibition of NADPH oxidase by APO or IκB kinase 2 by TPCA-1 is sufficient to mimic the effects of 4'-F-CBD or HU-910. Note that 4'-F-CBD and HU-910 also efficiently curtail the inflammatory response of microglia and astrocytes evoked by the TLR2 agonist PAM and the P2X7 purinergic receptor ligand BzATP.

microglia and astrocyte cultures activated by LPS whereas the presence of 4'-F-CBD and HU-910 suppressed this effect, suggesting that these compounds blocked glial inflammatory processes by interfering with NF-κB-dependent signaling. The inhibitory effect of 4'-F-CBD and HU-910 on oxidative burst was most probably critical for this effect as ROS operate as a trigger for NF-κB activation (Gloire et al., 2006). Comforting this view, blocking the activity of the NOX enzyme with APO, led to a reduction of NF-κB activation upon LPS treatment. As expected, the suppressive action of 4'-F-CBD and HU-910 on p-NF-κB expression was also reproduced by TPCA-1, a compound that blocks IκB kinase-2, a kinase that is required for NF-κB activation.

In line with these observations, we found that inhibiting the NOX enzyme with APO or blocking the activation of NF-κB with TPCA-1, reproduced the inhibitory effects that the two synthetic cannabinoid compounds exert on the stimulation of TNF-α release by LPS. This confirms that 4'-F-CBD and HU-910 restrain the inflammatory response of brain glial cells by preventing ROS-dependent NF-κB signaling. These data are also coherent with previous reports showing that CBD exerts anti-inflammatory effects on microglial cells through a similar mechanism of action (Chen et al., 2023; Dos-Santos-Pereira et al., 2020).

Altogether, the present study demonstrates that the two synthetic cannabinoids 4'-F-CBD and HU-910 possess strong immunosuppressive properties toward inflamed glial cells. These anti-inflammatory effects appear independent of most common cannabinoid receptors, and probably due to the capacity of these two compounds to curtail ROS dependent NF-κB signaling (Figure 8). Present data also provide further confirmation that 4'-F-CBD and HU-910 may have a therapeutic utility in pathological conditions where neuroinflammatory processes are prominent; target pathologies may include chronic neurodegenerative disorders such as Alzheimer disease, Parkinson's disease and multiple sclerosis as well as other neurological conditions such as migraine, epilepsy and neuropathic pain (Gilhus & Deuschl, 2019).

AUTHOR CONTRIBUTIONS

Maurício dos Santos Pereira, Francisco Silveira Guimarães, Elaine Del-Bel, Rita Raisman-Vozari, and Patrick Pierre Michel conceived the project. Elaine Del-Bel and Patrick Pierre Michel received funding for the project. Maurício dos Santos Pereira carried out the experiments with the contribution of Bruna Maitan Santos and Rocio Gimenez. Francisco Silveira Guimarães provided pharmacological reagents. Maurício dos Santos Pereira and Patrick Pierre Michel analyzed the data and wrote the original draft. Maurício dos Santos Pereira, Elaine Del-Bel, Rita Raisman-Vozari, Francisco Silveira Guimarães, and Patrick Pierre Michel reviewed and edited the manuscript.

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CONFLICT OF INTEREST STATEMENT

Francisco S. Guimarães is a co-inventor of the patent “Fluorinated CBD compounds, compositions and uses thereof. Pub. No. WO/2014/108899. International Application No. PCT/IL2014/050023” Def. US no. Reg. 62193296; 29/07/2015; INPI on 19/08/2015 (BR1120150164927). The University of São Paulo has licensed the patent to Phytects Pharm (USP Resolution No. 15.1.130002.1.1). The University of São Paulo has an agreement with Prati-Donaduzzi (Toledo, Brazil) to “develop a pharmaceutical product containing synthetic cannabidiol and prove its safety and therapeutic efficacy in the treatment of epilepsy, schizophrenia, Parkinson's disease, and anxiety disorders.” The other authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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