Cannabidiol prevents LPS-induced inflammation by inhibiting the NLRP3 inflammasome and iNOS activity in BV2 microglia cells via CB2 receptors and PPARγ

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ABSTRACT

Neuroinflammation stands as a critical player in the pathogenesis of diverse neurological disorders, with microglial cells playing a central role in orchestrating the inflammatory landscape within the central nervous system. Cannabidiol (CBD) has gained attention for its potential to elicit anti-inflammatory responses in microglia, offering promising perspectives for conditions associated with neuroinflammation. Here we investigated whether the NLRP3 inflammasome and inducible nitric oxide synthase (iNOS) are involved in the protective effects of CBD, and if their modulation is dependent on cannabinoid receptor 2 (CB2) and PPARγ signalling pathways. We found that treatment with CBD attenuated pro-inflammatory markers in lipopolysaccharide (LPS)-challenged BV2 microglia in a CB2- and PPARγ-dependent manner. At a molecular level, CBD inhibited the LPS-induced pro-inflammatory responses by suppressing iNOS and NLRP3/Caspase-1-dependent signalling cascades, resulting in reduced nitric oxide (NO), interleukin-1β (IL-1β), and tumour necrosis factor-alpha (TNF-α) concentrations. Notably, the protective effects of CBD on NLRP3 expression, Caspase-1 activity, and IL-1β concentration were partially hindered by the antagonism of both CB2 receptors and PPARγ, while iNOS expression and NO secretion were dependent exclusively on PPARγ activation, with no CB2 involvement. Interestingly, CBD exhibited a protective effect against TNF-α increase, regardless of CB2 or PPARγ activation. Altogether, these findings indicate that CB2 receptors and PPARγ mediate the anti-inflammatory effects of CBD on the NLRP3 inflammasome complex, iNOS activity and, ultimately, on microglial phenotype. Our results highlight the specific components responsible for the potential therapeutic applications of CBD on neuroinflammatory conditions.

Keywords: cannabidiol; microglia; neuroinflammation; NLRP3 inflammasome; iNOS.

Abbreviations

AM630 - CB2 receptor antagonist
ASC - Apoptosis-associated speck-like protein containing a CARD
BV2: Immortalised murine microglial cell line
cAMP: Cyclic AMP
CB2 - Cannabinoid receptor 2
CBD - Cannabidiol
CD86: Cluster of differentiation 86
COX2 - Cyclooxygenase-2
DAMPs - Damage-associated molecular patterns
DAPI - 4′,6-Diamidino-2-phenylindole
DMEM - Dulbecco's Modified Eagle Medium
ELISA - Enzyme-linked immunosorbent assay
ERK: Extracellular signal-regulated kinase
F4/80 - Epidermal growth factor (EGF)-like module-containing mucin-like hormone receptor-like 1
FAAH - Fatty acid amide hydrolase
FBS - Foetal bovine serum
GPR55 - G protein-coupled receptor 55
GW9662 - PPARγ antagonist
IL-1β - Interleukin 1β
iNOS - Inducible nitric oxide synthase
JNK - Jun N-terminal kinase
LPS - Lipopolysaccharide
MAPK: Mitogen-activated protein kinase
MTT - 3-(4,5-Dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide
NF-κB - Nuclear factor kappa B
NLRP3 - Nod-like receptor protein 3
NO - Nitric oxide
Nrf2 - Nuclear factor erythroid 2–related factor 2
P/S - Penicillin/streptomycin
PAMPs - Pathogen-associated molecular patterns
PCA - Principal Components Analysis
PKA: Protein kinase A
PBS - Phosphate-buffered saline
PPARγ - Nuclear peroxisome proliferator-activated receptor gamma
PRR - Pattern recognition receptors
ROS - Reactive oxygen species
RT - Room temperature
TLR4: Toll-like receptor 4
t-SNE - T-distributed stochastic neighbour embedding
TNF-α - tumour necrosis factor-alpha
TRPV - Transient Receptor Potential Vanilloid

1. Introduction

Neuroinflammation is defined as an inflammatory response within the central nervous system (CNS) and it is largely mediated by microglia, the primary innate immune cells of the CNS. They play a crucial role in maintaining the brain's homeostasis and responding to various challenges, such as infection and traumatic injury [33]). Microglia are dynamic cells that can adopt different phenotypes or functional states in response to their microenvironment. The main phenotypes are often described as pro-inflammatory and anti-inflammatory, depending on the physiological context [51, 75]. The pro-inflammatory state is mainly induced through the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRR), or damage-associated molecular patterns (DAMPs) on damaged host cells. PRR activation triggers an immune response via the secretion of pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, chemokines, reactive oxygen species (ROS) and nitric oxide (NO) [36].

One of the main PRRs in microglia involved in the pro-inflammatory state is the Nod-like receptor protein 3 (NLRP3). It is an intracellular PRR found in macrophages, neutrophils, lymphocytes, epithelial and dendritic cells, osteoblasts and neurons [9]. NLRP3 is activated as a result of nuclear factor kappa B (NFkB) transcription by a variety of stimuli, including infections, exogenous irritants or endogenous DAMPs. Upon recognising these signals, NLRP3 (sensor) recruits the apoptosis-associated speck-like protein containing a CARD (ASC, adaptor) and pro-Caspase-1 (effector) to form the large cytosolic NLRP3 inflammasome complex [65]. Activation of Caspase-1 triggers the maturation and release of pro-inflammatory cytokines IL-1β and IL-18 which function in both paracrine and autocrine manners to trigger innate and adaptive immune responses [46]. For experimental purposes, microglial inflammation via NLRP3 activation is often induced by treatment with lipopolysaccharide (LPS), a main

component of Gram-negative bacterial cell wall, as a direct connection between LPS-triggered inflammation and NLRP3 activation has been shown both *in vitro* and *in vivo* [55, 67].

Another well known downstream product of PRR signalling and NFkB activation is the inducible nitric oxide synthase (iNOS). iNOS is not constitutively expressed in most cells but can be induced in response to inflammatory signals. Unlike the other NOS isoforms (nNOS and eNOS), which produce NO in a regulated and localised manner for physiological signalling, iNOS generates large amounts of NO for extended periods in response to inflammatory stimuli [11, 44]. While iNOS-mediated NO production is crucial for host defence, excessive or prolonged activation of iNOS and the subsequent production of NO contributes to the inflammatory cascade which sustains chronic tissue damage [11, 21].

While crucial for maintaining CNS homeostasis, excessive or prolonged microglial activation can contribute to neuroinflammation and tissue damage, which is associated with several conditions, such as multiple sclerosis, Alzheimer's disease and autism spectrum disorder [6, 16, 17]. Therefore, the suppression of the excessive inflammatory responses from hyperactivated microglia is expected to limit the production of pro-inflammatory cytokines and ROS, which can be a promising strategy for the treatment of neurological disorders [5, 41].

Cannabidiol (CBD), a non-psychotropic terpenophenol derived from Cannabis sativa, is known for having anti-inflammatory and antioxidant properties that have been suggested to benefit a range of immunomediated conditions [4]. Studies indicate that CBD can attenuate symptoms associated with epilepsy, anxiety, chronic pain, and cognitive dysfunctions [56, 70]. CBD exerts anti-inflammatory effects by suppressing the production of pro-inflammatory cytokines, inhibiting the activation and proliferation

of immune cells, and reducing reactive oxygen species [4, 19, 23]. However, although the anti-inflammatory effects of CBD have been well documented, the signalling pathways through which such effects take place are still not well understood. In addition to its interactions with the cannabinoid receptors-1 (CB1) and 2 (CB2), CBD engages with other systems and receptors crucial for immunological response. These include the G protein-coupled receptor 55 (GPR55), Transient Receptor Potential Vanilloid (TRVP) channel, and nuclear peroxisome proliferator-activated receptors (PPARs) [38]. Previous studies have pointed towards CB2 and PPARγ as promising targets for CBD in macrophages [54], intestinal epithelial cells [12], sensory neurons [62] and myeloid-derived suppressor cells [35]. However, in microglia, the role of CB2 receptors and PPARγ on the effects of CBD is under explored.

In the present study, we aimed to investigate whether CBD exerts its protective effects on BV2 microglia cells via NLRP3 inflammasome and iNOS and if such modulation is dependent on CB2 receptors and/or PPARγ activation.

2. Materials and methods

2.1. Chemicals and reagents

Cannabidiol (CBD) (ab120448), anti-NLRP3 (ab4207), anti-inducible nitric oxide synthase (iNOS) (ab204017), Donkey Anti-Rabbit IgG H&L Alexa Fluor 647 (ab150075), Goat Anti-Rabbit IgG H&L Alexa Fluor 488 (ab150077), Goat Anti-Rat IgG H&L Alexa Fluor 555 (ab150158), Donkey Anti-Goat IgG H&L Alexa Fluor 488 (ab150129) and Mounting medium with 4′ ,6-diamidino-2-phenylindole (DAPI) (ab104135) were purchased from Abcam (Cambridge, UK). AM630 (1120) and GW9662 (1508) were purchased from Tocris (Bristol, UK). Mouse tumour necrosis factor alpha (TNF-α) (88-7324-86) and mouse Interleukin- 1β (IL-1 β) (88-7013A-88) ELISA Kits, anti-CD86 (14-0862-82), Goat anti-Rat IgG H&L Alexa Fluor 647 (A-

21247), eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (00-5523-00) and Fixable Viability Dye eFluor 450 (65-0863-14) were purchased from Invitrogen (Waltham, MA, USA). High glucose Dulbecco's modified Eagle medium + GlutaMAX (DMEM + GlutaMAX) (10569010), Fetal bovine serum (FBS) (A5256801), Penicillin-Streptomycin (15140122), and Trypsin-EDTA 0.05% Cell Dissociation Reagent (25300062) were purchased from Gibco (Carlsbad, CA, USA). Lipopolysaccharides from Escherichia coli O111:B4 (L4391), N-(1-Naphthyl)ethylenediamine dihydrochloride (33461), Sulfanilamide (S9251), Sodium Nitrite (S2252), Phosphoric Acid (695017) Ammonium Chloride (213330), Triton X-100 (X100), 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) and anti-α-Tubulin (MAB1864) were obtained from Sigma-Aldrich (Saint Louis, MO, USA). PE/Cyanine7 anti-F4/80 (123114) was obtained from BioLegend (San Diego, CA, USA). Anti-ASC/TMS1 (67824) was purchased from Cell signalling Technology Inc. (Danvers, MA, USA). FAM-FLICA® Intracellular Caspase-1 Detection Probe (YVAD) was purchased from Immunochemistry Technologies (Davis, California, USA).

2.2. Cell culture and treatments

BV2 microglia cells were provided by Oswaldo Cruz Foundation (FIOCRUZ - Brazil) (ICLC Cat# ATL03001, RRID:CVCL_0182) and were cultured in DMEM+GlutaMAX supplemented with 10% heat-inactivated FBS and 1% Penicillin-Streptomycin at 37◦C in a constant temperature incubator with an atmosphere of 95% humidity, and 5% CO_2. Cells were cultured at a density of 1×10^5 cells/mL in T75 flasks until 80% confluence was achieved and were used between 15 to 25 passages. Cultures were tested for mycoplasma contamination regularly.

Drugs were freshly diluted in Dimethyl sulfoxide (DMSO) and then further diluted into serum-free cell culture media to the established doses. Final concentrations of DMSO in culture wells were equal to or below 0.01%. Cells were changed to serum-free media and treated with either 1μM AM630 (CB2R inverse agonist) [49] or 10μM GW9662 (PPARγ antagonist) [78, 79] for 30 minutes before treatment with 1μM CBD [19, 40] for 1 hour, and then stimulated with 1 μg/mL of LPS for 24 h in serum-free cell culture media (Fig. 1). Selection of doses and treatment time was done based on previous studies on similar models. Additionally, we have tested CBD concentrations of 1, 5 and 10 μM, with 1 μM presenting the best results without inducing cell toxicity (data not shown). Control groups for the treatments received DMSO in the same volume used for the drugs and control groups for LPS received deionized water in the same volume used for LPS.

2.3. Cytotoxicity assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay was used to determine BV2 cells proliferation and survival in response to LPS and the drugs (Mosmann, 1983). For that, BV2 cells were seeded in a 96-well plate (1,5 x 10^4 cells/well) and cultured overnight. After treatment with the drugs and/or CBD and stimulation with LPS for 24 h, 10 μl of MTT stock solution in PBS (5 mg/mL) was added to the wells and incubated at 37◦C for 2h. Subsequently, the cells were lysed, and the dark blue crystals were solubilized in 100 μl of acidified isopropanol (2% HCl 2M). The optical density of each well was determined using a Tecan Infinite M200 Pro® plate reader at 570 nm.

2.4. Flow Cytometry

BV2 cells were seeded in a 96-well plate (2 x10^5 cells/well) and cultured overnight. After treatment with the drugs and/or CBD and stimulation with LPS for 24 h, the plates were centrifuged at 1000 rpm for 5 min and the supernatant was removed. For regular immunodetection, cells were marked with a fixable viability dye (eFluor 450) for 30 min at 4◦C, then fixed and permeabilized using a Transcription Factor Staining Buffer Set according to the manufacturer's instructions. Cells were then incubated with primary antibodies against CD86 (1:250), NLRP3 (1:100), ASC (1:250) and iNOS (1:250) for 30 min at 4◦C, followed by incubation with secondary antibodies marked with Alexa Fluor 488 and 647 (1:500) for 30 min at 4◦C. Lastly, cells were incubated with a PE/Cyanine7 anti-F4/80 conjugated antibody (1:250) for another 30 min at 4◦C. All the washes in between were performed at 400g for 5 min using a Blocking solution (Permeabilization buffer + 2% FBS).

For Caspase-1 activity assessment, after treatments the plates were centrifuged at 1000 rpm for 5 min and the supernatant was removed. Intracellular Caspase-1 detection probe was added to the wells (1:100 in PBS) and incubated for 30 min at 37◦. The cells were washed once with PBS then resuspended in PBS for analysis.

All the flow cytometry analyses were performed at a Beckman Coulter CytoFlex® and data were analysed using FlowJo software. Data were expressed as Mean Fluorescence Intensity (MFI) for cell surface markers and as Stimulation Index (SI) (frequency of each well/average frequency of control wells within each experiment) for the intracellular markers.

2.4.1. Clustering and dimensionality analysis

Mean Fluorescence Intensity (MFI) data obtained from flow cytometry were utilised for dimensionality reduction and clustering analysis. These data were normalised per

experimental day. The clustering and dimensionality reduction analyses were conducted in R (version 4.2.2). Principal Components Analysis (PCA) and k-means analysis were performed using the base R package. T-distributed stochastic neighbour embedding (t-SNE) analysis was carried out using the "Rtsne" package (version 0.16).

2.5. Immunofluorescence

BV2 cells were seeded in 12-well plates on glass coverslips (10^5 cells/well) and cultured overnight. After treatment with the drugs and/or CBD and stimulation with LPS for 24 h, media was removed and the cells were washed with PBS 2 times and fixed with 4% paraformaldehyde at room temperature (RT) for 15 min. Next, the cells were washed again three times with PBS, permeabilized with Ammonium Chloride 50mM for 10min, and 0.2% Triton X-100 for 5 min at RT, then washed again another three times with PBS, and 5% goat serum was added to block the non-specific binding at RT for 30 min. The cells were incubated with primary antibodies against CD86 (1:250) and α-Tubulin (1:1000) at RT for 2 hours, followed by incubation with secondary antibodies labelled with Alexa Fluor 555 and 647 at RT for 1 hour. Finally, the coverslips were transferred to glass slides with a mounting medium containing DAPI and imaged by confocal microscopy (ZEISS LSM 880®). At least one hundred cells were imaged from each slide. Images were analysed using Fiji ImageJ software.

2.6. Determination of NO in cell supernatant

NO concentration on cell supernatant was assessed by Griess assay. BV2 cells were seeded in a 96-well plate (1,5 x 10^4 cells/well) and cultured overnight. After treatment with the drugs and/or CBD and stimulation with LPS for 24 h, the plates were centrifuged at 1000 rpm for 5 min and the supernatant was collected. Griess reagent was prepared mixing 1,45 mL Phosphoric acid, 0,25g Sulfanilamide and 0,025g N-(1-

Naphthyl)ethylenediamine dihydrochloride to a total of 25 mL in dH2O. Standard curve was generated by serial dilution of Sodium Nitrite 50mM for 8 points starting at 200uM in cell culture medium. In a 96-well plate, equal volumes of cell supernatant and Griess reagent were added to each well. The plate was incubated at room temperature for 5 min and absorbance was measured using a Tecan Infinite M200 Pro® plate reader at 540 nm.

2.7. Determination of TNF-α and IL-1β concentrations

Concentrations of TNF-α and IL-1β were measured by enzyme-linked immunosorbent assay (ELISA) kits according to instructions from the manufacturer. Briefly, BV2 cells were seeded in a 96-well plate (1,5 x 10^4 cells/well) and cultured overnight. After treatment with the drugs and/or CBD and stimulation with LPS for 24 h, the plates were centrifuged at 1000 rpm for 5 min and the supernatant was collected for detection of the aforementioned inflammatory cytokines. Absorbance was measured using a Tecan Infinite Pro ® plate reader at 450 nm.

2.8. Data analysis and statistics

Data were analysed using Graphpad Prism 9 statistical software (GraphPad Software, San Diego, CA, USA), submitted to the Kolmogorov–Smirnov test to assess normality and then to one-way ANOVA with Bonferroni post hoc. The results were expressed as the mean ± standard error of the mean (SEM). All analyses were based on three to four experiments consisting of an independent cell culture preparation, with duplicates or triplicates within each experiment. Each point represents the one replicate within each individual experiment. Outliers were removed using the ROUT test, and statistically significant differences were considered at $p < 0.05$.

3. Results

3.1. Cannabidiol does not prevent cellular viability loss caused by LPS in BV2 microglia cells

We firstly assessed cell viability by MTT assay to evaluate the effects of LPS and the treatments on BV2 microglial cells (Fig. 2a). We confirmed the viability by the Fixable viability dye (FVD) by flow cytometry (Fig. 2b). LPS exposure significantly reduced cell viability after 24 hours compared with CT on both MTT (ANOVA: $F_{7,102}$ = 28.41; P<0.0001. LPS vs CT: P<0.0001) and FVD (ANOVA: $F_{5,83}$ = 17.59; P<0.0001. LPS vs CT: P<0.0001). Pre-treatment with CBD and/or AM630 and GW9662 did not exert any effects on cell survival after LPS stimulation. These findings indicate a lack of protective effect of CBD against LPS-induced cell death. Additionally, GW9662 and AM630 by themselves or as CBD co-treatments showed no effects on control cells (data not shown).

3.2. Cannabidiol attenuates microglial polarisation towards a pro-inflammatory phenotype induced by LPS through CB2 and PPARγ activation

Microglial polarisation in BV2 cells was assessed by CD86 and F4/80 markers - both constitutive microglial markers whose increased expression is associated with a pro-inflammatory phenotype. LPS induced a significantly increased expression of CD86, seen on both immunofluorescence (ANOVA: $F_{5,3380}$ = 25.31; P<0.0001. CT vs LPS: P<0.0001) (Fig. 3a and 3b) and flow cytometry (ANOVA: $F_{5,45}$ = 17.71; P<0.0001. CT vs LPS: P<0.0001) (Fig. 3c), presenting cells with a more activated morphology as well as higher fluorescence intensity. However, CBD was able to significantly attenuate this overexpression (LPS vs. CBD-LPS: P=0.0351 and 0.0208, respectively). Interestingly, CB2 receptor antagonism completely inhibited CBD's effect on CD86 expression as seen on immunofluorescence (CBD-LPS vs

AM630/CBD-LPS: P<0.0001). However, on flow cytometry, it only marginally blunted this effect, since the group was different from neither LPS nor CBD-LPS. PPARγ antagonism partially attenuated CBD's effect on CD86 expression as well.

Regarding F4/80, LPS insult increased its expression (ANOVA: $F_{5,48}$ = 11.69; P<0.0001. CT vs LPS: P=0.0410), while CBD also attenuated this effect (LPS vs CBD-LPS: P=0.0107) (Fig. 3d and 3e). Antagonism of both CB2 receptors and PPARγ before CBD partially prevented this effect, suggesting that the protective effect exerted by CBD is dependent on these pathways at least to some extent.

3.3. Cannabidiol reduces inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) concentration in a PPARγ-dependent manner

Expression of iNOS in BV2 microglial cells was assessed by flow cytometry and NO concentration in cell supernatant was quantified by the Griess assay. LPS induced an increased expression of iNOS (ANOVA: $F_{5,50}$ = 16.30; P<0.0001. CT vs LPS: P<0.0001) (Fig. 4a) along with elevated concentration of secreted NO (ANOVA: $F_{5,17}$ = 8.428; P=0.0004. LPS vs CT: P=0.0088) (Fig. 4b), while CBD was able to significantly reduce this effect on both ends (LPS vs CBD-LPS: P=0.0046 and 0.0060, respectively). However, the antagonism of PPARγ before CBD prevented these effects (CBD-LPS vs GW9662/CBD-LPS: P=0.0039 and 0.0211), suggesting that the protective effect of CBD on the production of NO is dependent on this signalling pathway. On the other hand, antagonism of CB2 receptors partially interfered with the effects of CBD on iNOS expression and did not influence its effects on NO concentrations (LPS vs AM630/CBD-LPS: P=0.0045).

3.4. Cannabidiol reduces Inflammasome activation and Caspase-1 activity in a CB2 and PPARγ-dependent manner

To assess if CBD anti-inflammatory effects were mediated by the NLRP3 Inflammasome in BV2 microglial cells, we measured NLRP3 and ASC expression and Caspase-1 activity by flow cytometry (Fig. 5). As expected, LPS induced an increase in all the aforementioned markers (NLRP3 - ANOVA: $F_{5,60}$ = 11.04; P<0.0001. CT vs LPS: P=0.0002. ASC - ANOVA: $F_{5,40}$ = 6.141; P=0.0003. CT vs LPS: P=0.0003. Caspase-1 - ANOVA: $F_{5,52}$ = 7.874; P<0.0001. CT vs LPS: P=0.0002), while CBD significantly reduced this effect on NLRP3 (LPS vs CBD-LPS: P = 0.0181) and Caspase-1 (LPS vs CBD-LPS: P = 0.0439). The antagonism of both CB2 receptors and PPARγ slightly impaired CBD effects on the expression of NLRP3 (Fig. 5a) as well as the percentage of active Caspase-1 (Fig. 5c and 5d), since AM630/CBD-LPS and GW9662/CBD-LPS groups are not different from LPS nor CBD-LPS. Interestingly, the effect of CBD on ASC expression was not significant, once CBD-LPS is not different from LPS (P = 0.0813), however CBD-LPS is not different from CT as well and an overall tendency similar to NLRP3 can be observed (Fig. 5b). These results suggest that the activation of the NLRP3 Inflammasome by LPS can be prevented by CBD and it is partly mediated by CB2 receptors and PPARγ.

3.5. Cannabidiol modulates overall clustering and dimensionality away from LPS in a CB2 and PPARγ-dependent manner

PCA and t-SNE analysis were conducted to explore the underlying structure and patterns within the groups using the MFI from all the markers analysed by flow cytometry. The goal was to visualise high-dimensional data in a lower-dimensional space while preserving local structures. Each point in the plot represents an observation, and the proximity of points reflects their similarity. Both PCA (Fig. 6a) and t-SNE (Fig. 6b) visualisations reveal distinct clusters and patterns within the data, consistent with experimental groups. For instance, the cluster representing the CBD-

LPS group overlaps with the one for the CT group, suggesting tight inter-cluster similarities. However, when CB2 receptors (AM630/CBD-LPS) and PPARγ (GW9662/CBD-LPS) are inhibited, the samples tend to cluster closer to the LPS group.

3.6. Cannabidiol differentially attenuates the effects of LPS on TNF-α and IL-1β concentrations

Lastly, to assess the effects of CBD on the secretion of pro-inflammatory cytokines in response to LPS, we dosed the concentrations of TNF-α and IL-1β on the BV2 cells' supernatants by ELISA (Fig. 7). LPS exposure induced an increase in the concentrations of both cytokines (TNF-α - ANOVA: $F_{5,36}$ = 13.27; P<0.0001. CT vs LPS: P<0.0001. IL-1β - ANOVA: $F_{5,42}$ = 2.626; P=0.0370. CT vs LPS: P=0.0241), while CBD was able to reduce this effect (LPS vs CBD-LPS: P = 0.0057 and 0.0432, respectively). The antagonism of both CB2 receptors and PPARγ marginally impaired CBD effects on IL-1β (Fig. 7a), however, neither CB2 nor PPARγ interfered with the effects of CBD on TNF-α concentration (LPS vs AM630/CBD-LPS: P=0.0052; LPS vs. GW9662/CBD-LPS: P=0.0171) (Fig. 7b). These results suggest that the secretion of IL-1β induced by LPS can be attenuated by CBD partially through its activation of CB2 receptors and PPARγ, but its effects on TNF-α do not seem to be mediated by these signalling pathways.

4. Discussion

Neuroinflammation has emerged as a focal point when delving into the intricate cellular dynamics underlying various neurological disorders. Among the diverse cell populations contributing to the neuroinflammatory milieu, microglial cells have drawn significant attention due to their central role in immune surveillance within the central

nervous system. Cannabidiol (CBD) has been shown to promote anti-inflammatory responses in these cells, which could be beneficial for a series of conditions stemming from neuroinflammation [19, 37, 38, 73]. Here we have studied the signalling pathways of the anti-inflammatory potential of CBD on LPS-triggered BV2 microglial cells through the NLRP3 inflammasome and iNOS activity.

One of the first telltale signs of microglial immune response is the shift towards a pro-inflammatory phenotype, characterised by higher production and release of pro-inflammatory cytokines, ROS and NO [53, 72]. Here we observed an increase in CD86 and F4/80 surface markers in BV2 cells exposed to LPS, indicating a pro-inflammatory polarisation. Pre-treatment with CBD was able to prevent this transition. Furthermore, its effects on CD86 expression seem to be highly CB2-dependent and partially PPARγ-dependent, while the effects on F4/80 seem to be only marginally blunted by CB2 and PPARγ antagonism. Reflecting the surface findings on an internal level, we found that LPS stimulation caused a substantial increase in NLRP3 and ASC speck expression, indicative of inflammasome assembly. The mechanisms of NLRP3 inflammasome activation in macrophages have been extensively investigated, showing a strong correlation with microglia-mediated neuroinflammation in response to various insults, such as LPS [3, 24, 69], amyloid beta peptides [29, 30] and mitochondrial complex inhibitors [1]. Furthermore, LPS-stimulated cells presented a higher percentage of active Caspase-1, with a consequential increase in IL-1β concentration in cell supernatant. CBD treatment attenuated the upregulation of NLRP3 expression, Caspase-1 activity and IL-1β concentration, while the antagonism of CB2 receptors or PPARγ partially hindered this effect. Similarly, corroborating with previous studies [2, 39, 71], we have also shown that CBD is able to reduce iNOS overexpression and subsequent NO secretion through PPARγ activation, but not CB2.

CB2 receptors are coupled primarily to inhibitory G proteins, leading to the suppression of adenylate cyclase and subsequent reduction in cyclic AMP (cAMP) levels. This signalling route can modulate various downstream effectors, including protein kinase A (PKA) and mitogen-activated protein kinase (MAPK) pathways, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. These pathways play crucial roles in regulating inflammatory responses, cell proliferation, and cell survival in microglia, therefore, CB2 activation is known to result in an anti-inflammatory shift in microglial cells [42, 43, 66, 77]. Likewise, PPARγ is a ligand-activated transcription factor that regulates the expression of genes involved in inflammation, lipid metabolism, and cell differentiation. Its upregulation has been shown to exert anti-inflammatory effects, upregulating nuclear factor erythroid 2–related factor 2 (Nrf2) transcription, inhibiting nuclear factor kappa B (NF-κB) activity, and promoting a shift towards the M2 phenotype [15, 25, 26, 28, 68]. Accordingly, the effects of CBD on different models of neuroinflammatory conditions have been shown to be at least partially dependent on its activation of CB2 receptors and/or PPARγ [10, 18, 47, 52], resulting in downregulation of JNK and p38 MAPK [39], reduced the expression of cyclooxygenase-2 (COX-2) [57], and suppression of production of pro-inflammatory cytokines [48].

It is worth noting that one study by dos-Santos-Pereira and collaborators has demonstrated protective effects of CBD similar to the ones seen here on primary microglia culture, however, such effects were only marginally blunted by CB2 receptor antagonism and completely independent of PPARγ activation. Nevertheless in the present study we have used an immortalised microglial cell line, which may present distinct responses from primary cells [19].

Interestingly, CBD also exerted a protective effect against the increase in TNF-α

concentration, however, the blockage of neither CB2 nor PPARγ reverted these results. While the most known targets for CBD are cannabinoid receptors 1 (CB1) and 2 (CB2), some of the biological effects of CBD are mediated through other putative candidates, including serotonergic, adenosine, α1 adrenergic, and μ-opioid receptors, and transient-receptor-potential channels [7, 13]. CBD was also found to operate as an antagonist to the noncanonical G protein-coupled receptor 55 (GPR55) [32, 58, 61]. Evidence suggests GPR55 activation may aid effective TLR4 activation and/or signalling to induce inflammatory responses [60, 74]. Accordingly, GPR55 blockade can reduce activation of the arachidonic acid cascade in LPS-activated microglial cells, suggesting that CBD suppressive effects may also occur through inhibition of GPR55 [32, 59, 60]. Additionally, certain effects attributed to CBD through cannabinoid mediation might stem from its capacity to inhibit the degradation of endocannabinoids by the FAAH enzyme. Consequently, this leads to elevated concentrations of endocannabinoids, ultimately triggering receptor activation, mainly by anandamide [8, 13, 22]. Lastly, CBD has been found to regulate mitochondrial energy metabolism, calcium concentrations, and oxidative stress in microglial cells [39, 50, 76], which would explain the partial effects seen in the presence of the antagonists, as well as persistence of its effects on TNF-α concentration even when CB2 or PPARγ were inactive.

Conversely, while NLRP3/Caspase-1 signalling is known for regulating apoptotic responses and, therefore, its downregulation should reflect on decreased cell death in response to LPS, we have not seen a recovery in viability on the CBD-LPS group. This corroborates with similar findings by Li et al, which showed that CBD improved inflammatory profile without rescuing cell viability on BV2 cells [39]. This raises the hypothesis that although CBD does not protect against LPS-triggered cell death, it may

push the remaining cells towards an anti-inflammatory state, which can result in improved tissue repair over time, as shown in tissue regeneration studies [31, 34, 45].

In summary, these findings bolster the anti-inflammatory role of CBD in BV2 microglial cells, primarily mediated through the inhibition of the NLRP3/Caspase-1 inflammasome complex and iNOS activity, with subsequent reduction of pro-inflammatory hallmarks. This knowledge can help narrow down targets for the treatment of neuroinflammatory conditions stemming from microglial hyperactivation.

5. Conclusions

Altogether, these findings indicate that CB2 receptors and PPARγ mediate the anti-inflammatory effects of CBD on the NLRP3 inflammasome complex, iNOS activity and, ultimately, on microglial phenotype. Our results highlight the specific components responsible for the potential therapeutic applications of CBD on neuroinflammatory conditions related to microglia function. More studies should be conducted on primary cultured cells and animal models in order to better explore these signalling pathways.

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Statements and declarations

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Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Conceptualization was performed by Fernanda da Silva Rodrigues, Karen Wright, Renada Padilha Guedes and Victorio Bambini-Jr. Experimental procedures and sample analysis were conducted by Fernanda da Silva Rodrigues, William Robert Newton, Isadora D'Ávila Tassinari, Felipe Henrique da Cunha Xavier and Adél Marx. Statistical analysis was conducted by Fernanda da Silva Rodrigues and Felipe Henrique da Cunha Xavier. Critical revision of the manuscript was performed by Luciano Stürmer de Fraga, Karen Wright, Renata Padilha Guedes and Victorio Bambini-Jr. All authors have written, read, edited, and approved the final version of the manuscript.

Data availability

The raw data supporting the conclusions of this article will be made available by the authors upon reasonable request.

Ethical approval

This is an in vitro study using immortalised cell lines. Lancaster University Research Ethics Committee has confirmed that no ethical approval is required.

Figure captions

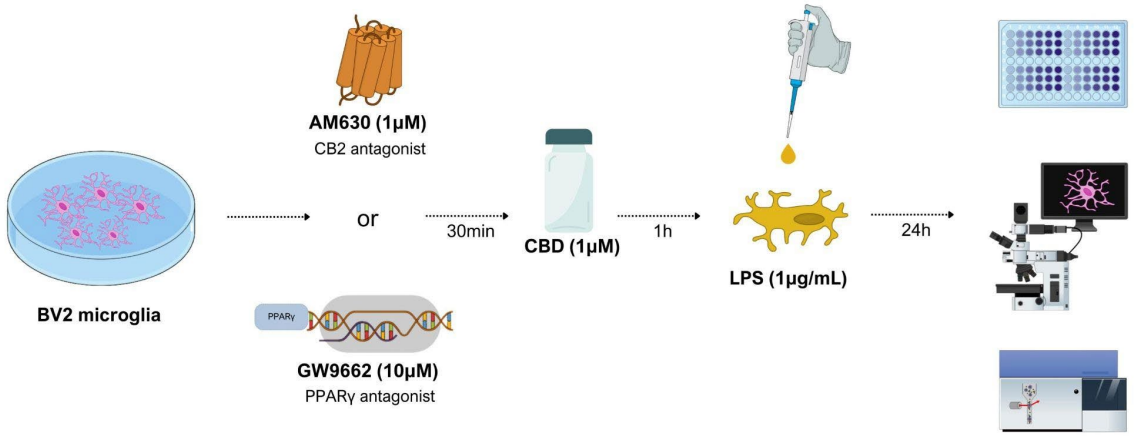

Figure 1. A schematic description of the experimental design and groups. BV2 microglial cells were treated with CB2 antagonist (AM630) or PPARγ antagonist (GW9662) for 30 minutes. Then, Cannabidiol (CBD) was added to the cell culture medium for 1 hour. Lastly, lipopolysaccharide (LPS) was added and cells were incubated for 24 hours. Assays, flow cytometry and confocal microscopy were performed after the 24 hour incubation period.

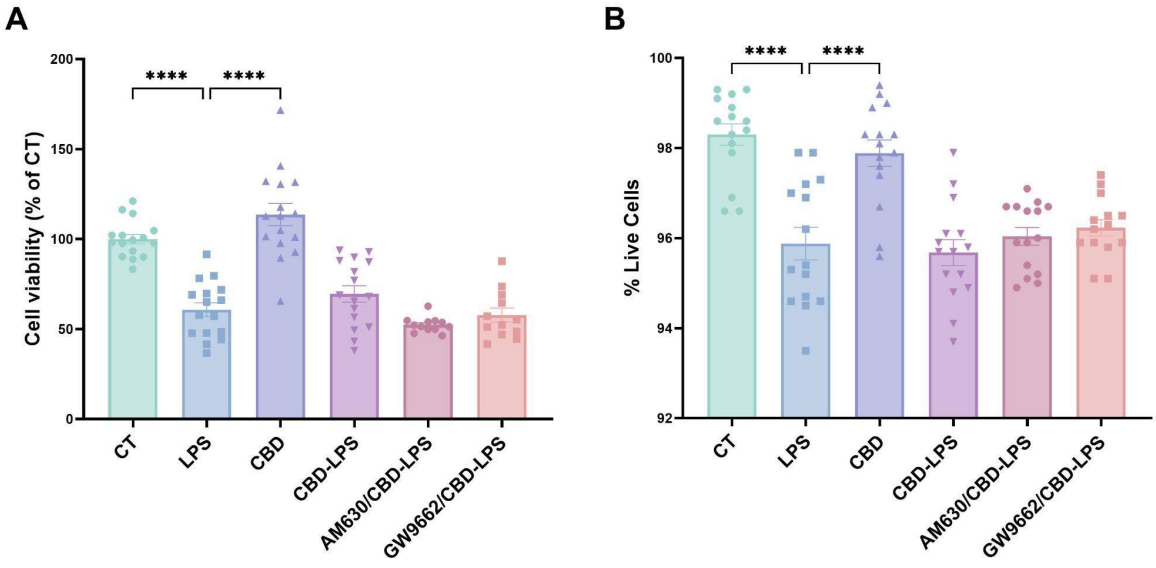

Figure 2. Effects of CBD, AM630 and GW9662 on BV2 microglial cells viability assessed by MTT (a) and Fixable viability dye (b). LPS reduced cell viability

compared to CT regardless of previous treatment. CBD, AM630 or GW9662 did not exert any effects on cell viability. ****P<0.0001

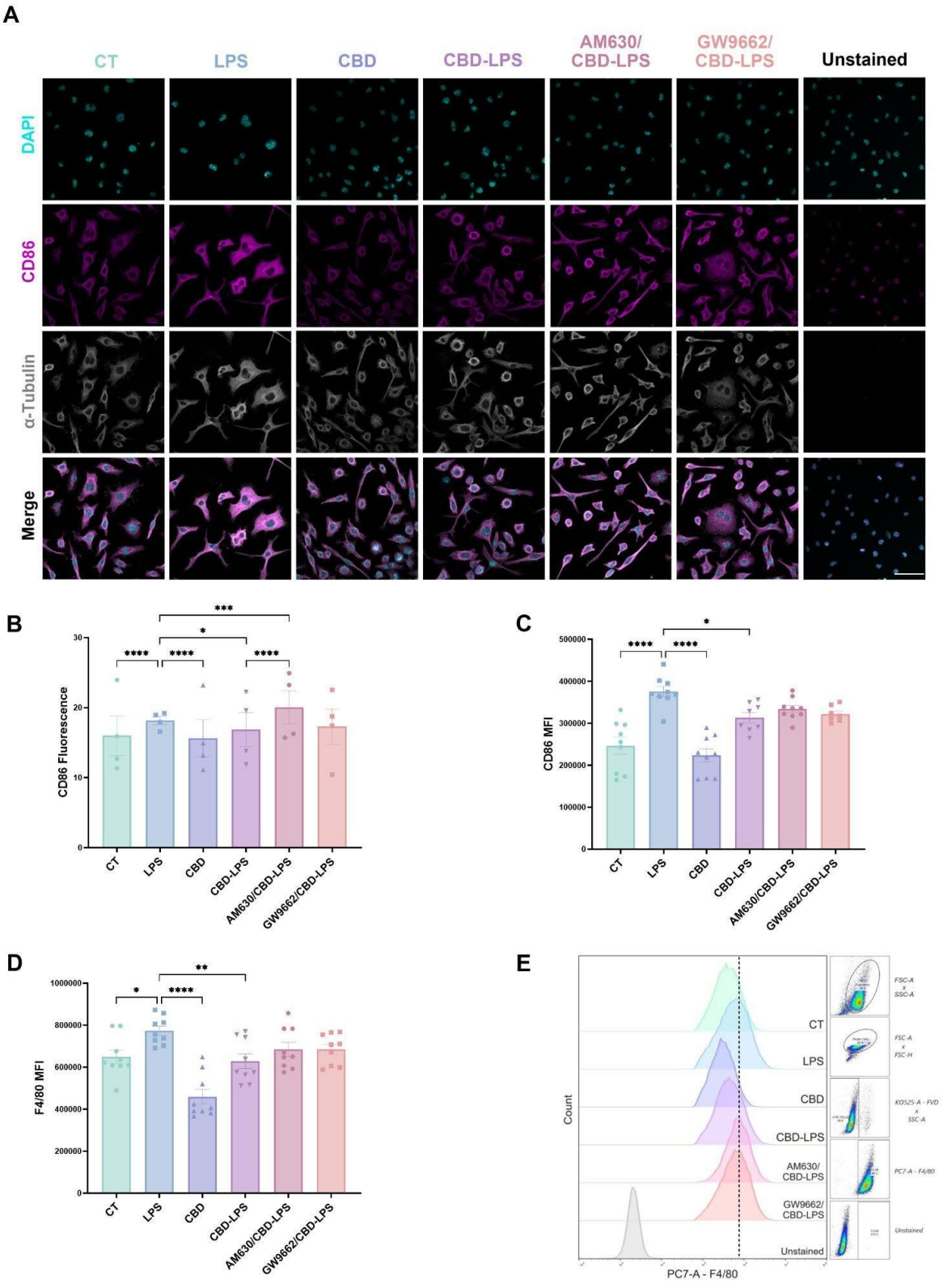

Figure 3. Effects of CBD on microglial polarisation in response to LPS in BV2 cells. CBD suppressed the pro-inflammatory phenotype induced by LPS as shown by CD86 (a, b and c) and F4/80 (d and e) expression. CB2 and PPARγ antagonism by AM630 and GW9662, respectively, partially attenuated the effects of CBD. Gating strategy (E). Scale bar is 50μm. *P<0.05, **P<0.01, ****P<0.0001

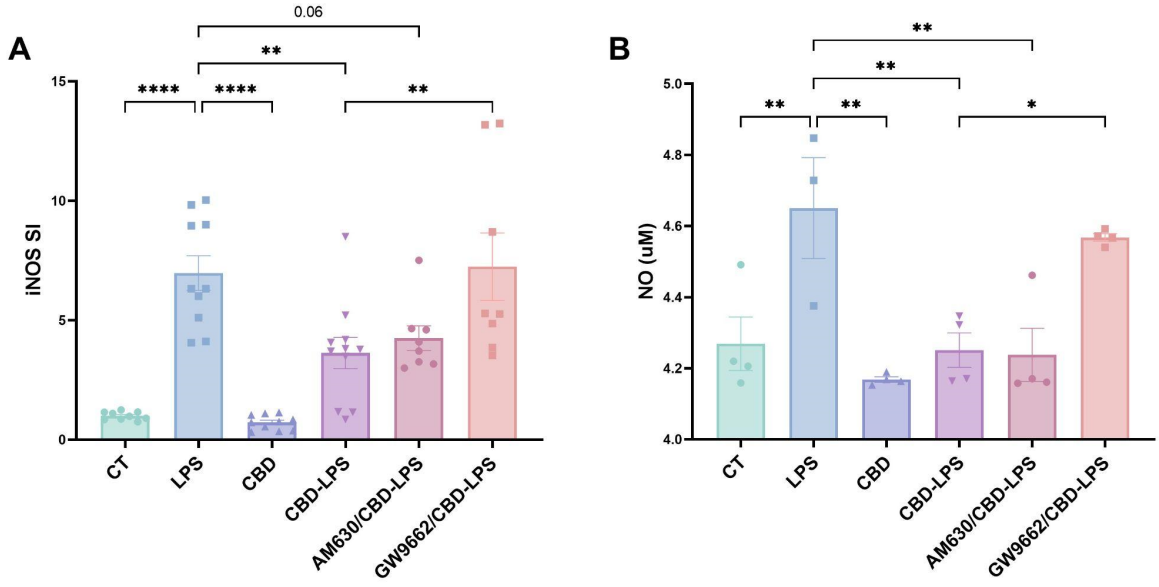

Figure 4. Effects of CBD on iNOS expression and NO concentration in BV2 microglial cells in response to LPS. CBD suppressed the overexpression of iNOS (a) and the elevated concentration of NO in the cell supernatant (b) induced by LPS. PPARγ antagonism by GW9662 hampers the effects of CBD, but blockage of CB2 receptors by AM630 exerts no effects. The percentage of cells expressing iNOS was calculated as a relative value to an average percentage of CT. *P<0.05, **P<0.01, ****P<0.0001

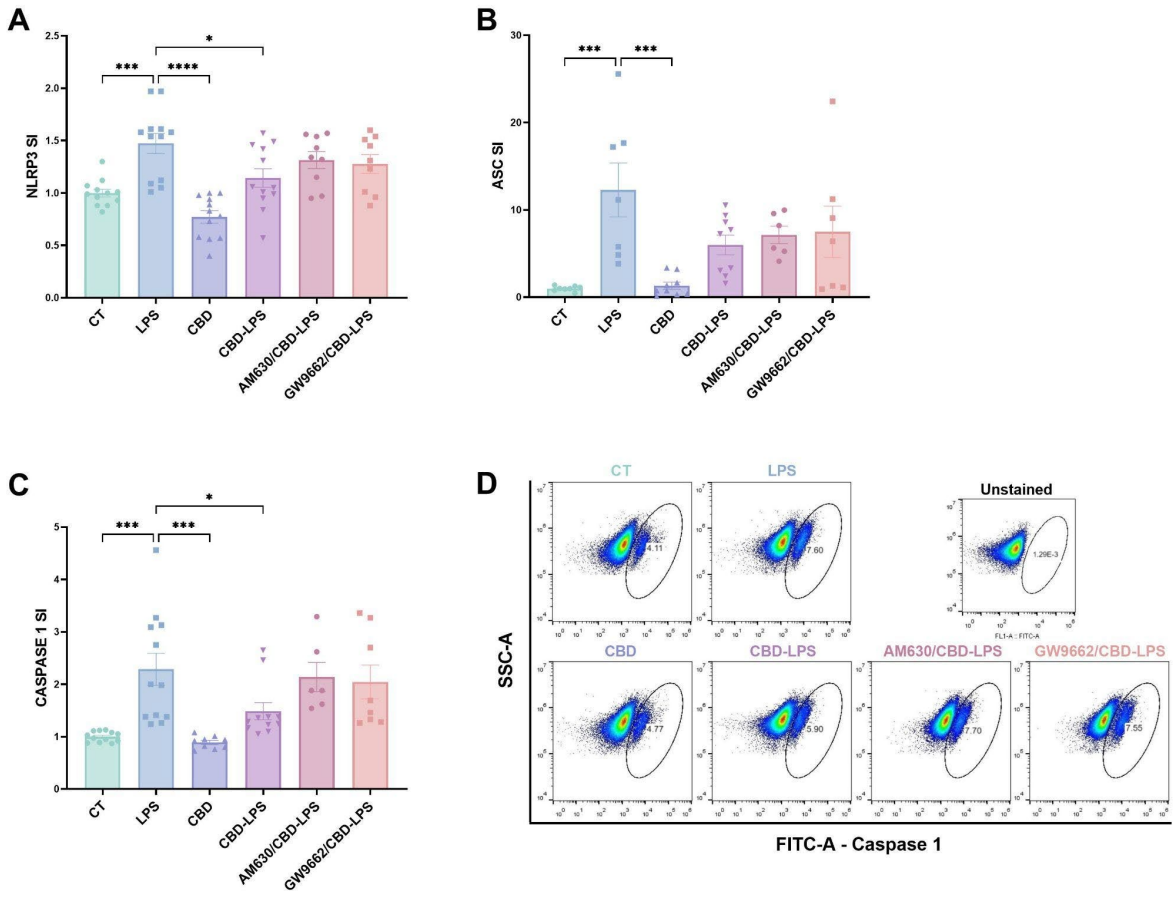

Figure 5. Effects of CBD on NLRP3 and ASC expression and Caspase-1 activity in response to LPS. CBD suppressed the overexpression of NLRP3 (a), and attenuated Caspase-1 activity (c) induced by LPS. CBD effects were partially blunted by CB2 and PPARγ antagonism by AM630 and GW9662, respectively. The percentage of cells expressing NLRP3 and ASC and with active Caspase-1 was calculated as a relative value to an average percentage of CT. *P<0.05, ***P<0.001 ****P<0.0001

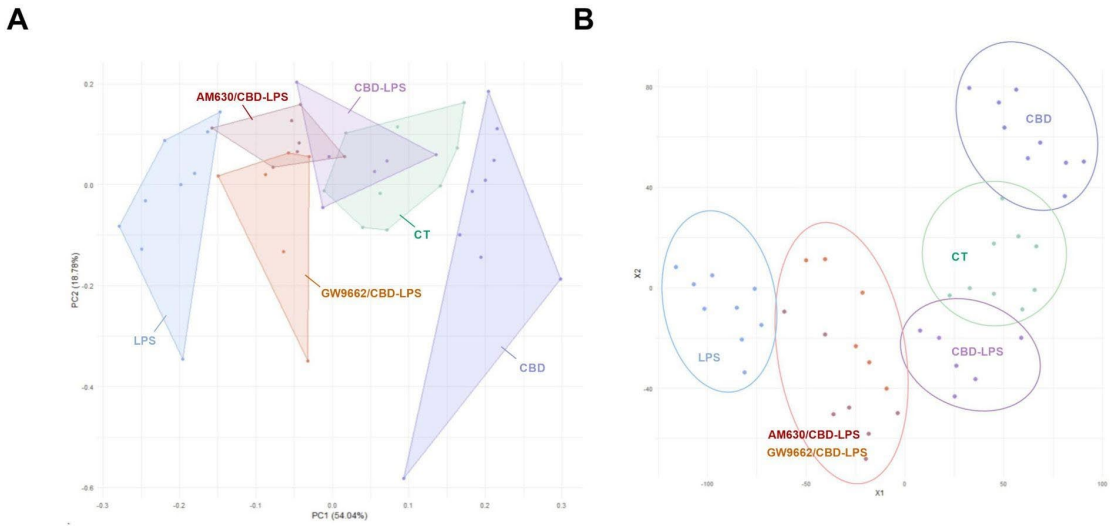

Figure 6. Effects of CBD on experimental clustering and dimensionality. CBD treatment deviates clustering towards CT and away from LPS, while CB2 and PPARγ antagonism deviates the groups towards LPS on both PCA (a) and t-SNE (b)

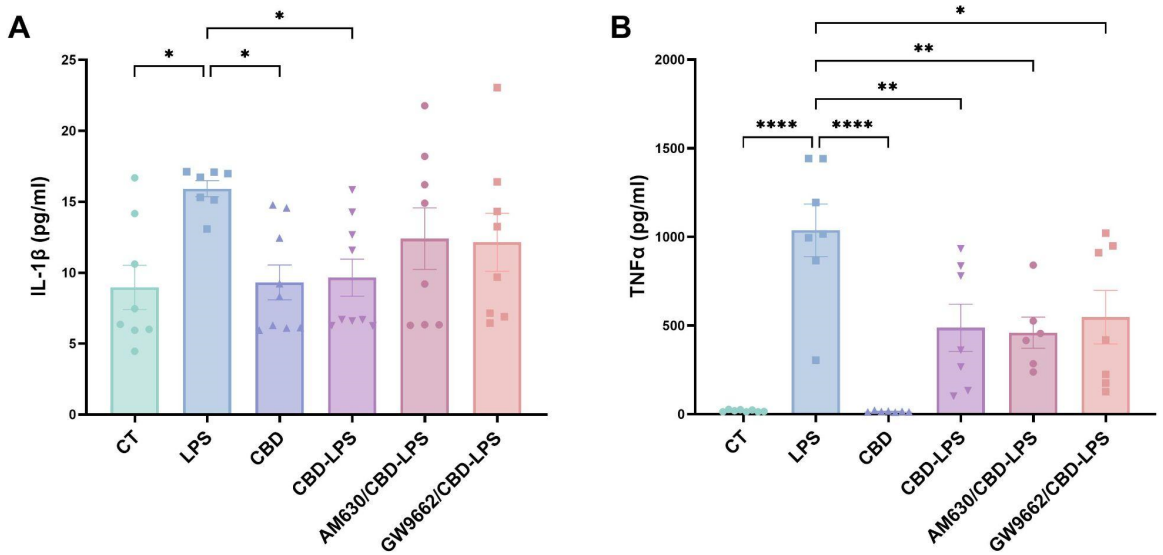

Figure 7. Effects of CBD on IL-1β and TNF-α secretion by BV2 cells in response to LPS. CBD suppresses the overproduction of both IL-1β and TNF-α induced by LPS. CBD effects on IL-1β were marginally blocked by AM630 and GW9662 (a), while the

effects on TNF-α were not affected by CB2 or PPARγ antagonism (b). *P<0.05, **P<0.01, ****P<0.0001.

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