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Anti-inflammatory properties of cannabidiol, a non-psychotropic cannabinoid, in experimental allergic contact dermatitis

Stefania Petrosino, Roberta Verde, Massimo Vaia, Marco Allarà, Teresa Iuvone, Vincenzo Di Marzo*

Endocannabinoid Research Group, Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Pozzuoli, Napoli, Italy (S.P., R.V., M.A., V.M.). Epitech Group SpA, Saccolongo, Padova, Italy (S.P., M.A.). Dipartimento di Farmacologia Sperimentale, Università di Napoli "Federico II", Napoli, Italy (M.V., T.I.).

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Running title: Cannabidiol and allergic contact dermatitis

* To whom correspondence should be addressed:

Dr. Vincenzo Di Marzo, PhD, Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Via Campi Flegrei 34, 80078 Pozzuoli, Napoli, Italy.

Phone: ++39 081 867 5018, Fax: ++39 081 867 5340, Email: vdimarzo@icb.cnr.it

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List of non-standard abbreviations:

2-AG - 2-arachidonoyl-glycerol

ACD - allergic contact dermatitis

AEA - anandamide (*N*-arachidonoyl-ethanolamine)

CB_1 and CB_2 - cannabinoid receptors of type-1 and -2

CBC - cannabichromene

CBD - cannabidiol

CBDA - cannabidiol acid

CBDV - Cannabidivarin

CBDVA - cannabidivarinic acid

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CBG - cannabigerol

CBGA - cannabigerolic acid

CBGV - cannabigevarin

DMSO - dimethyl sulfoxide

DNFB - dinitrofluorobenzene

G-CSF - granulocyte colony stimulating factor (G-CSF)

GM-CSF - granulocyte-macrophage colony-stimulating factor

HaCaT - human keratinocytes

IL - interleukin

INF-γ - interferon-γ

I-RTX - 5'-iodio-resiniferatoxin

LC-APCI-MS - liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry

MCP-1 and MCP-2 - monocyte chemotactic protein-1 and -2

MIP-1β - macrophage inflammatory protein-1β

MTT - 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide

OEA - oleoylethanolamide

PEA - palmitoylethanolamide

poly-(I:C) - polyinosinic-polycytidylic acid

THC - Δ^9-tetrahydrocannabinol

THCV - tetrahydrocannabivarin

THCVA - tetrahydrocannabivarinic acid

TNF-α - tumor necrosis factor-α

TRPV1 - transient receptor potential vanilloid type-1 channel

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Abstract

Phytocannabinoids modulate inflammatory responses by regulating the production of cytokines in several experimental models of inflammation. Cannabinoid type-2 (CB_2) receptor activation was shown to reduce the production of the monocyte chemotactic protein-2 (MCP-2) chemokine in polyinosinic-polycytidylic acid [poly-(I:C)]-stimulated human keratinocyte (HaCaT) cells, an *in vitro* model of allergic contact dermatitis (ACD). We investigated if non-psychotropic cannabinoids like cannabidiol (CBD) produced similar effects in this experimental model of ACD. HaCaT cells were stimulated with poly-(I:C) and the release of chemokines and cytokines was measured in the presence of CBD or other phytocannabinoids (such as CBDA, CBDV, CBDVA, CBC, CBG, CBGA, CBGV, THCV, THCVA) and antagonists of cannabinoid type-1 (CB_1), CB_2 or transient receptor potential vanilloid type 1 (TRPV1) receptors. HaCaT cell viability following phytocannabinoid treatment was also measured. The cellular levels of endocannabinoids [anandamide (AEA), 2-arachidonoylglycerol (2-AG)] and related molecules [palmitoylethanolamide (PEA), oleoylethanolamide (OEA)] were quantified in poly-(I:C)-stimulated HaCaT cells treated with CBD. We showed that in poly-(I:C)-stimulated HaCaT cells, CBD elevated the levels of AEA and dose-dependently inhibited poly-(I:C)-induced release of MCP-2, IL-6, IL-8 and TNF-α in a manner reversed by CB_2 and TRPV1 antagonists, AM630 and I-RTX, respectively, with no cytotoxic effect. This is the first demonstration of the anti-inflammatory properties of CBD in an experimental model of ACD.

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Introduction

Allergic contact dermatitis (ACD), a form of delayed type hypersensitivity, is a typical T-cell-mediated skin inflammatory response that occurs after cutaneous exposure to an allergen. In particular, following first application to the skin, epidermal Langerhans cells (LCs) take up allergen, process it and migrate towards the regional lymph nodes, where the antigen is presented to naïve T cells which, once activated, migrate towards peripheral tissues. During this process, known as "the sensitization phase", LCs convert from a "quiescent" into an "activated" functional state. This activation of LCs is initiated by keratinocytes, which secrete inflammatory cytokines such as interleukin (IL), IL-6, IL-2, tumor necrosis factor (TNF)-α and granulocyte-macrophage colony-stimulating factor (GM-CSF), which in turn contribute to LC activation and migration (Enk, et al., 1992; Becker and Knop, 1993). The subsequent allergen application induces "the elicitation phase" of ACD that involves the degranulation of mast cells, vasodilatation and influx of neutrophils, followed by substantial leukocyte infiltration into tissue and oedema formation peaking between 24 and 48 hours. This late-phase response has the same direct effects on the skin as the first allergen contact during sensitization (i.e. pro-inflammatory effects, LC activation), but T cell activation is subjected to the release of cytokines produced by T lymphocytes, which amplify the inflammatory response by generating a process that leads to further accumulation of infiltrating cells and resulting in clinically manifested ACD (van Loveren, et al., 1983; Watanabe, et al., 2002). However, the production of cytokines (such as IL-1, IL-6 and IL-8) from keratinocytes, as well as the induction of adhesion molecules (such as intercellular adhesion molecule (ICAM)) in keratinocytes, are also required for T cell activation, chemotactic activity and adhesion in the epidermis, indicating that these cells have a crucial role in ACD (Barker, 1992).

Although many studies report anti-inflammatory properties for two major cannabinoids present in marijuana such as the psychoactive compound Δ^9-tetrahydrocannabinol (THC) and the non-psychoactive compound cannabidiol (CBD) (Burstein, 2015; Burstein and Zurier, 2009), the first evidence of the anti-inflammatory effects of cannabinoids in an animal model of ACD has been

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reported by Oka et al., (2006), and soon thereafter by Karsak, et al., (2007) in collaboration with our group (Karsak, et al. 2007). In particular, it was demonstrated that both subcutaneous and topical application of THC attenuated ACD in 2,4-dinitrofluorobenzene (DNFB)-treated wild-type mice (Karsak, et al., 2007). THC significantly decreased ear swelling and reduced the recruitment of Gr positive granulocytes in comparison to untreated mice (Karsak, et al., 2007). Intriguingly, the cannabinoid type-2 (CB_2) receptor antagonist, SR144528, was reported, on the one hand, to counteract DNFB-induced (Ueda, et al., 2005) and oxazoline-induced (Oka, et al., 2006) ACD in mice, and, on the other hand, to inhibit the anti-inflammatory effect of THC on this condition (Karsak, et al., 2007). Nevertheless, Karsak and collaborators also demonstrated that the synthetic cannabinoid agonist, HU-210, was able to reduce the production of the monocyte chemotactic protein-2 (MCP-2) chemokine in polyinosinic-polycytidylic acid [poly-(I:C)]-stimulated human keratinocytes (HaCaT) cells, an *in vitro* model of the first phase of ACD (Karsak, et al., 2007). More recently, THC was suggested to inhibit DNFB-induced dermatitis also via non-CB_1, non-CB_2-mediated pathways (Gaffal, et al., 2013). Indeed, we reported that also the anti-inflammatory compound, palmitoylethanolamide (PEA), which belongs to the same chemical class as the endocannabinoid anandamide (AEA), but is unable to directly activate cannabinoid receptors (Petrosino and Di Marzo, 2017), reduced the production of MCP-2 in poly-(I:C)-stimulated HaCaT cells, as well as the DNFB-induced ear skin oedema in mice (Petrosino, et al., 2010). Moreover, we also demonstrated that, although PEA is known to directly activate the peroxisome proliferator-activated receptor-α (PPAR-α) (Lo Verme, et al., 2005), only the selective antagonism of transient receptor potential vanilloid type 1 (TRPV1) channels reversed the effects of PEA on MCP-2 production in poly-(I:C)-stimulated HaCaT cells, and on the first, keratinocyte-mediated, stage of DNFB-induced ear skin oedema in mice, respectively (Petrosino, et al., 2010), whereas CB_2 receptors were involved in PEA effects in the late, mast cell-mediated, stage of this *in vivo* model of ACD (Vaia, et al., 2016). These previous data are in agreement with the indirect stimulatory actions

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of PEA on TRPV1 and CB_2 and with the role of TRPV1 and CB_2 in ACD (De Petrocellis, et al., 2001; Petrosino, et al., 2016).

On the basis of this background, and in view of the fact that it has been demonstrated that CBD also stimulates and desensitizes TRPV1 channels (De Petrocellis, et al., 2011; Iannotti, et al., 2014), the aim of the present study was to investigate the pharmacological effects of this and other non-psychotropic phytocannabinoids in an *in vitro* model of ACD, as well as to identify the molecular target(s) for its actions in the *in vitro* model.

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Materials and methods

Drugs and Reagents

Cell culture media, antibiotics and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Milano, Italy). Poly-(I:C) was purchased from InvivoGen (Aurogene Srl, Roma, Italy). Botanical CBD, cannabidiol acid (CBDA), cannabidivarin (CBDV), cannabidivarinic acid (CBDVA), cannabichromene (CBC), cannabigerol (CBG), cannabigerolic acid (CBGA), cannabigevarin (CBGV), tetrahydrocannabivarin (THCV) and tetrahydrocannabivarinic acid (THCVA) (> 99.9% purity) were provided by GW Research Ltd (Cambridge, UK). AM251, AM630, 5'-iodio-resiniferatoxin (I-RTX), AEA and URB597 were purchased from Tocris Bioscience (Space Import-Export Srl, Milano, Italy). The human MCP-2 ELISA kit was purchased from RayBiotech, Inc (Tebu-Bio Srl, Milano, Italy). The Bio-Plex Pro human cytokine assay was purchased from Biorad (Life Science, Segrate, Milano, Italy). Deuterated standards – $[^2H]_8$-AEA, $[^2H]_5$-2-AG, $[^2H]_4$-PEA and $[^2H]_2$-OEA – were purchased from Cayman Chemical (Cabru SAS, Arcore, Italy).

Cell culture

HaCaT cell line (Item number: 300493; Mycoplasma specific PCR: negative) was purchased from CLS Cell Lines Service (Eppelheim, Germany), and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with glutamine (2 mM), penicillin (400 U/ml), streptomycin (50 mg/ml) and 10% Fetal Bovine Serum (FBS) at 37 °C in humidified 5% CO_2.

Poly-(I:C)-induced ACD in HaCaT cells

HaCaT cells were plated into twenty-four-well culture plates at a cell density of 2 x 10^5 cells per well, and after 1 day were stimulated with poly-(I:C) (100 μg/ml) (Petrosino, et al., 2010) or vehicle (water) and incubated for 6, 12 and 24 h at 37 ºC in 5% CO_2. To study the effect of CBD, poly-(I:C)-stimulated HaCaT cells were treated with CBD (1, 5, 10 and 20 μM) or vehicle (methanol) for

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the indicated times. To study the effect of CB_1, CB_2 and TRPV1 antagonists, poly-(I:C)-stimulated HaCaT cells were treated with AM251 (1, 2.5 and 5 μM), AM630 (0.01, 0.1 and 1 μM), I-RTX (0.01, 0.1 and 1 μM), respectively, in the presence or absence of CBD (20 μM) for the indicated times. AM251 was dissolved in methanol, AM630 and I-RTX in dimethyl sulfoxide (DMSO). The effects of the other phytocannabinoids (all dissolved in DMSO) such as CBDA, CBDV, CBDVA, CBC, CBG, CBGA, CBGV, THCV and THCVA (all tested at 5, 10 and 20 μM) on MCP-2 production in poly-(I:C)-stimulated HaCaT cells were also investigated. Finally, the effects of the endocannabinoid AEA (dissolved in methanol), and of URB597 (dissolved in methanol) [a selective inhibitor of the major enzyme responsible for AEA inactivation, fatty acid amide hydrolase (FAAH)] on MCP-2 production in poly-(I:C)-stimulated HaCaT cells were also studied. After 6, 12 and 24 h the supernatants were used for MCP-2 ELISA assay and for Bio-Plex Pro assay [IL-1β, IL-2, IL-6, IL-8, granulocyte colony stimulating factor (G-CSF), GM-CSF, TNF-α], according to the manufacturer's instructions. Results are expressed as pg/ml of released MCP-2 and cytokines.

Cell viability

Cell viability was measured after 6, 12, and 24 h in HaCaT cells treated with CBD, CBC, CBG, THCV, CBGV (all tested at 10 and 20 μM), or vehicle by using the MTT colorimetric assay. Briefly, after 6, 12 and 24 h HaCaT cells were incubated with MTT (5 mg/ml) for 3 h at 37 °C in 5% CO_2. After 3 h HaCaT cells were lysed with DMSO and absorbance was measured at 630 nm. Results are expressed as % of cell viability, where optical density values from vehicle-treated cells were defined as 100% of cell viability.

Analysis of endocannabinoids and related *N*-acylethanolamines

HaCaT cells were plated into six-well culture plates at a cell density of 9 x10^5 cells per well, and after 1 day were stimulated with poly-(I:C) (100 μg/ml) and treated with CBD (20 μM) or vehicle,

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and incubated for 6, 12 and 24 h at 37 ºC in 5% CO_2. After the indicates times the resulting cells and supernatants were subjected to measurement of endocannabinoids such as AEA and 2-arachidonoyl-glycerol (2-AG), and *N*-acylethanolamines related to AEA, i.e. PEA and *N*-oleoylethanolamine (OEA).

Cells and supernatants were homogenized in a solution of chloroform/methanol/Tris-HCl 50 mM pH 7.4 (2:1:1 by vol.) containing 10 pmol of $[^2H]_8$-AEA, and 5 pmol of $[^2H]_5$-2-AG, $[^2H]_4$-PEA and $[^2H]_2$-OEA as internal deuterated standards. The lipid-containing organic phase was pre-purified by open-bed chromatography on silica gel (Bisogno, et al., 1997; Di Marzo, et al., 2001), and fractions obtained by eluting the column with a solution of chloroform/methanol (90:10 by vol.) were analyzed by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) by using a Shimadzu HPLC apparatus (LC10ADVP) coupled to a Shimadzu (LCMS-2020) quadrupole MS via a Shimadzu APCI interface. LC-APCI-MS analyses of AEA, 2-AG, PEA and OEA were carried out in the selected ion monitoring (SIM) mode (Marsicano, et al., 2002), using m/z values of molecular ions +1 for deuterated and undeuterated compounds, respectively as follows: 356 and 348 (AEA), 384.35 and 379.35 (2-AG), 304 and 300 (PEA), 328 and 326 (OEA). AEA, 2-AG, PEA and OEA levels were calculated on the basis of their area ratio with the internal deuterated standard signal areas, and their amounts (pmol) were normalized per ml of volume.

Statistics

Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA). The data are expressed as means ± standard error of the mean (SEM). Student's *t*-test or one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test were used for analysis. P values < 0.05 were considered statistically significant.

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Results

CBD reduces MCP-2 protein levels in poly-(I:C)-stimulated HaCaT cells

We investigated the effects of CBD, CBDA, CBDV, CBDVA, CBC, CBG, CBGA, CBGV, THCV and THCVA on MCP-2 protein levels in poly-(I:C)-stimulated HaCaT cells. HaCaT cells stimulated for 6, 12 and 24 h with poly-(I:C) (100 μg/ml) and treated with the vehicle of the phytocannabinoids produced significantly higher levels of the MCP-2 chemokine as compared to vehicle-stimulated HaCaT cells (Fig. 1). When HaCaT cells were co-stimulated with poly-(I:C) and CBD (1, 5, 10 and 20 μM) for 6 h, we observed a strong concentration-dependent reduction of MCP-2 protein levels as compared to poly-(I:C)-stimulated HaCaT cells treated with the vehicle of CBD (Fig. 1A). The maximum effect was observed at the highest concentration tested of CBD (20 μM), as compared to poly-(I:C)-stimulated HaCaT cells treated with the vehicle of CBD (Fig. 1A). Likewise, CBD (1, 5, 10 and 20 μM) in a concentration-dependent manner was also able to strongly reduce MCP-2 production in poly-(I:C)-stimulated HaCaT cells after 12 and 24 h, and the maximum effect was also observed with CBD 20 μM (Fig. 1B-C). On the contrary, when HaCaT cells were co-stimulated with poly-(I:C) and CBC or CBG no effect was observed at low concentrations (5 and 10 μM), although at the highest concentration tested (20 μM), these two phytocannabinoids were able to reduce MCP-2 production (Fig. 2A-B). Likewise, when HaCaT cells were co-stimulated with poly-(I:C), THCV had no effect at the lowest concentration tested (5 μM), but at 10 μM and 20 μM it was able to reduce MCP-2 production (Fig. 2C). CBGV was able to reduce MCP-2 production only at 10 μM (Fig. 2D). No effect was observed on MCP-2 protein levels after treatment of poly-(I:C)-stimulated HaCaT cells with CBDA, CBDV, CBDVA, CBGA and THCVA as compared to poly-(I:C)-stimulated HaCaT cells treated with the respective vehicles (data not shown). Likewise, no significant variation was observed on MCP-2 protein levels after that HaCaT cells were treated with CBD or the other phytocannabinoids alone (at highest

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concentration tested, 20 μM), i.e. in the absence of poly-(I:C), as compared to vehicle-treated HaCaT cells (data not shown), indicating that this concentration of CBD was not cytotoxic.

CBD reduces IL-6, IL-8 and TNF-α protein levels in poly-(I:C)-stimulated HaCaT cells

We also investigated the effects of CBD, CBC, CBG, THCV and CBGV on the production of different cytokines (IL-1β, IL-2, IL-6, IL-8, G-CSF, GM-CSF, TNF-α) in poly-(I:C)-stimulated HaCaT cells. HaCaT cells stimulated for 6 h with poly-(I:C) (100 μg/ml) and treated with the vehicle of the phytocannabinoids produced significantly higher levels only of IL-6, IL-8 and TNF-α, as compared to vehicle-stimulated HaCaT cells (Fig. 3). CBD (1, 5, 10 and 20 μM) in a concentration-dependent manner was able to strongly reduce IL-6 and TNF-α protein levels in poly-(I:C)-stimulated HaCaT cells, as compared to poly-(I:C)-stimulated HaCaT cells treated with the vehicle of CBD (Fig. 3A, C). IL-8 protein levels were strongly reduced only by the two highest concentrations tested of CBD (10 and 20 μM) (Fig. 3B). When HaCaT cells were stimulated for 12 h with poly-(I:C) and treated with the vehicle of the phytocannabinoids, we only observed a strong increase of TNF-α protein levels (Fig. 4A), which was significantly reduced by the highest concentration tested of CBD (20 μM) (Fig. 4A). When HaCaT cells were stimulated for 24 h with poly-(I:C) and treated with the vehicle of the phytocannabinoids, we observed a strong increase in both IL-6 (Fig. 4B) and TNF-α (Fig. 4C) protein levels, which were again strongly reduced by treatment with CBD 20 μM (Fig. 4B, C). On the contrary, the other phytocannabinoids found here to produce anti-inflammatory effects on MCP-2 levels, were able to down-regulate only some of these cytokines after 6 h, i.e.: i) CBC (20 μM) was able to reduce only IL-6 and IL-8 levels (Fig. 5A-B); ii) CBG (10 μM) was able to reduce only IL-6 and IL-8 levels (Fig. 5B-C); and iii) THCV (20 μM) was able to reduce only IL-6 levels (Fig. 5D). No effect was observed on the levels of other cytokines, i.e. IL-1β, IL-2, G-CSF and GM-CSF, after stimulation of HaCaT cells with poly-(I :C) in the presence or absence of CBD or the other phytocannabinoids (data not shown).

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CBD and other phytocannabinoids are not cytotoxic to HaCaT cells

No cytotoxicity was observed after treatment of HaCaT cells for 6 h with CBD, CBC, CBG, THCV or CBGV at the highest concentrations tested, 10 and 20 μM (Fig. 6A). Likewise, no cytotoxicity was observed after treatment of HaCaT cells for 12 and 24 h with CBD 10 and 20 μM (Fig. 6B). These results indicate that the decreased MCP-2 and or cytokine levels in poly-(I:C)-stimulated HaCaT cells were due to the anti-inflammatory effects of these compounds.

CB_1 receptors do not mediate the action of CBD in poly-(I:C)-stimulated HaCaT cells

We investigated the effect of a CB_1 receptor antagonist (AM251 1, 2.5 and 5 μM) on MCP-2 protein levels in poly-(I:C)-stimulated HaCaT cells, in the presence or absence of CBD (20 μM). Our results show that when HaCaT cells were co-stimulated for 6 h with poly-(I:C) and low concentrations of AM251 (1 or 2.5 μM), MCP-2 protein levels were comparable to those found in the absence of the antagonist (Fig. 7A). On the contrary, when HaCaT cells were co-stimulated with poly-(I:C) and the highest concentration tested of AM251 (5 μM), MCP-2 protein levels were comparable to those observed in the presence of CBD 20 μM (Fig. 7A). In addition, when HaCaT cells were co-stimulated with poly-(I:C), CBD 20 μM and *per se* inactive concentration of AM251 (2.5 μM), MCP-2 production was comparable to that observed in poly-(I:C)-stimulated HaCaT cells treated with CBD 20 μM alone (Fig. 7A). No effect was observed on MCP-2 protein levels after treatment of HaCaT cells with the antagonist alone, AM251 (at the highest concentration tested, 5 μM), i.e. in the absence of poly-(I:C), as compared to vehicle-treated HaCaT cells (data not shown). Likewise, no additive effect was found on MCP-2 protein levels after that poly-(I:C)-stimulated HaCaT cells were treated with CBD 20 μM and the highest concentration tested of AM251 (5 μM), as compared to poly-(I:C)-stimulated HaCaT cells treated with CBD 20 μM only (data not shown).

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CB_2 and TRPV1 receptors mediate the action of CBD in poly-(I:C)-stimulated HaCaT cells

We investigated the effect of a CB_2 receptor antagonist (AM630 0.01, 0.1 and 1 μM) and a TRPV1 receptor antagonist (I-RTX 0.01, 0.1 and 1 μM) on MCP-2 protein levels in poly-(I:C)-stimulated HaCaT cells, in the presence or absence of CBD (20 μM). Our results show that when HaCaT cells were co-stimulated for 6 h with poly-(I:C) and low concentrations of AM630 (0.01 or 0.1μM) or high concentration of I-RTX (1 μM), MCP-2 protein levels were comparable to those observed in the absence of the antagonists (Fig. 7B). On the contrary, when HaCaT cells were co-stimulated with poly-(I:C) and a higher concentration of AM630 (1 μM) or lower concentrations of I-RTX (0.01 or 0.1 μM), MCP-2 protein levels were comparable to those observed in the presence of CBD 20 μM (Fig. 7B). Importantly, when HaCaT cells were co-stimulated with poly-(I:C), CBD 20 μM and the highest *per se* inactive concentrations of AM630 or IRTX (0.1 and 1 μM, respectively), MCP-2 chemokine production was comparable to that observed in poly-(I:C)-stimulated HaCaT cells treated with the vehicle (Fig. 7B). However, no effects of AM630 or IRTX (0.1 and 1 μM, respectively) on MCP-2 protein levels were observed after 12 and 24 h in poly-(I:C)-stimulated HaCaT cells treated with CBD 20 μM, as compared to poly-(I:C)-stimulated HaCaT cells treated with CBD 20 μM (data not shown). In addition, no effect was observed on MCP-2 protein levels after that HaCaT cells were treated with the antagonists alone, AM630 or I-RTX (at highest concentrations tested, 1 μM), i.e. in the absence of poly-(I:C), as compared to vehicle-treated HaCaT cells (data not shown). Likewise, no additive effects were found on MCP-2 protein levels after poly-(I:C)-stimulated HaCaT cells were treated with CBD 20 μM and the highest concentration tested of AM630 (1 μM), or the lowest concentrations tested of IRTX (0.01 and 0.1 μM), as compared to poly-(I:C)-stimulated HaCaT cells treated with CBD 20 μM (data not shown). On the basis of these results, we also investigated the effects of AM630 (0.1 μM) and I-RTX (1 μM) on the production of cytokines, such as IL-6, IL-8 and TNF-α, in poly-(I:C)-stimulated HaCaT cells, in the presence or absence of CBD (20 μM). Our results show that no effect was found with

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AM630 (0.1 μM) or I-RTX (1 μM) on the inhibitory action of CBD (20 μM) on the levels of the other 3 cytokines that were elevated after poly-(I:C)- stimulation of HaCaT cells (data not shown).

CBD elevates AEA levels in poly-(I:C)-stimulated HaCaT cells

We measured the effect of CBD (20 μM) on AEA, 2-AG, PEA and OEA levels in poly-(I:C)-stimulated HaCaT cells. We observed that when HaCaT cells were stimulated for 6 h with poly-(I:C), AEA levels were significantly increased by 3-fold compared to vehicle-treated HaCaT cells, and a nearly statistically significant trend towards elevation of PEA levels (P = 0.0633) was also observed (Fig. 8A, C). When poly-(I:C)-stimulated HaCaT cells were treated with CBD (20 μM), AEA levels were increased by -8-fold compared to vehicle-treated HaCaT cells, and by -2.7-fold compared to poly-(I:C)-stimulated HaCaT cells (Fig. 8A). These increase were only observed during the early sensitization phase of ACD, i.e. after 6 h (Fig. 8A). No consistent effect of CBD was observed on 2-AG and OEA levels after 6 h (Fig. 8B, D), as well as on AEA levels after 12 and 24 h (data not shown), in poly-(I:C)-stimulated HaCaT cells.

AEA and URB597 reduce MCP-2, IL-6 and IL-8 protein levels in poly-(I:C)-stimulated HaCaT cells

We investigated the effects of AEA and a selective FAAH inhibitor, URB597, on the production of MCP-2 and other different cytokines (IL-6, IL-8 and TNF-α) in poly-(I:C)-stimulated HaCaT cells. Our results show that when HaCaT cells were co-stimulated with poly-(I:C) and AEA (10 μM), the levels of MCP-2 and IL-8 proteins were reduced as compared to poly-(I:C)-stimulated HaCaT cells treated with the vehicle (Fig. 9A, C). When HaCaT cells were co-stimulated with poly-(I:C) and URB597 (10 μM), the levels of MCP-2, IL-6 and IL-8 proteins were also reduced as compared to poly-(I:C)-stimulated HaCaT cells treated with the vehicle (Fig. 9A-C). No effect was observed on IL-6 levels after treatment of poly-(I:C)-stimulated HaCaT cells with AEA (10 μM) (Fig. 9B).

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Likewise, no effect was observed on TNF-α production after treatment of poly-(I:C)-stimulated HaCaT cells with AEA (10 μM) or URB597 (10 μM) (data not shown). Thus, the effects of AEA and URB597 in poly-(I:C)-stimulated HaCaT cells were comparable although not identical to those observed with CBD (20 μM) (Fig. 9).

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Discussion

In this study, we demonstrated for the first time that a non-psychotropic plant cannabinoid, CBD, inhibits the production of the MCP-2 chemokine and other pro-inflammatory cytokines (i.e. IL-6, IL-8 and TNF-α) in poly-(I:C)-stimulated keratinocytes more potently/efficaciously/dose-dependently than other non-psychotropic phytocannabinoids tested here (CBDA, CBDV, CBDVA, CBC, CBG, CBGA, CBGV, THCV and THCVA). Importantly, we also identified the molecular targets for some of the actions of CBD on keratinocytes.

First, we demonstrated that when HaCaT cells are stimulated for 6 h and up to 24 h with an agonist of the toll-like receptor 3, i.e. poly-(I:C), they produce higher levels of MCP-2. This chemokine is a pro-inflammatory mediator involved in the recruitment of macrophages and mast cells into inflammatory sites (Taub, et al., 1995; de Paulis, et al., 2001), and is down-regulated by CB_1/CB_2 agonists, such as THC and HU-210, in ear keratinocytes of mice with DNFB-induced ACD, as well as in poly-(I:C)-stimulated HaCaT cells *in vitro* (Karsak, et al., 2007). We previously demonstrated that this chemokine can be also down-regulated by an endogenous lipid mediator, namely PEA, which is known for its important anti-inflammatory property and is currently used in the clinic against neuropathic and inflammatory pain, but, unlike AEA, is not capable of directly activating CB_1 or CB_2 receptors (Iannotti, et al., 2016). In particular, we showed that PEA at the concentration of 10 μM was able to reduce, through a TRPV1-mediated mechanism, both the expression and the production of MCP-2 in poly-(I:C)-stimulated HaCaT cells *in vitro* (Petrosino, et al., 2010). On the contrary, neither the antagonism of CB_2 receptors nor the antagonism of PPAR-α receptors reversed the anti-inflammatory effect of PEA in poly-(I:C)-stimulated HaCaT cells (Petrosino, et al., 2010). Therefore, we here investigated: a) whether also CBD, which, like PEA and unlike THC, is unable to directly activate CB_1/CB_2 receptors, can nevertheless reduce MCP-2 protein levels in poly-(I:C)-stimulated HaCaT cells, and b) the molecular target(s) through which CBD exerts this putative anti-inflammatory action. We found that CBD, in a concentration-

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dependent manner, strongly reduced MCP-2 protein levels in HaCaT cells stimulated for 6 h and up to 24 h with poly-(I:C), with the maximum effect being observed at the highest concentration tested of 20 μM, and no significant differences in the extent of the effect being noted at different time points. This concentration is 2-fold higher than the efficacious (and maximally soluble in the same vehicle) concentration of PEA (10 μM), in the same *in vitro* model of ACD (Petrosino, et al., 2010). Here we also demonstrated for the first time that when HaCaT cells are stimulated for 6 h and up to 24 h with poly-(I:C) they produce higher levels of other pro-inflammatory cytokines (i.e. IL-6, IL-8 and TNF-α), which, like MCP-2, are produced from keratinocytes during the sensitization phase (Enk, et al., 1992; Becker and Knop, 1993; Barker, 1992), and that CBD reduces also these effects of poly-(I:C). However, of these three cytokines, only TNF-α exhibited significant increases at all time points, suggesting their potentially different roles in different phases of inflammation, at least in this *in vitro* model.

Since it is well known that: 1) the pharmacological blockade of CB_2 receptor attenuates the oxazoline-induced contact dermatitis in mice (Oka, et al., 2006); 2) the activation of CB_2, but not CB_1 receptor, is involved in THC-induced anti-inflammatory effects (Yang, et al., 2015; Xie, et al., 2016; Shang, et al., 2016; 3) CB_1 or CB_2 receptor activation and the activation/desensitization of TRPV1 channels contribute to the *in vitro* and *in vivo* pharmacological actions of CBD (Costa, et al., 2004; Arnold, et al., 2012; Pazos, et al., 2013; Stanley, et al., 2015); 4) CBD can directly activate and desensitize TRPV1 channels (Iannotti, et al., 2014); and 5) CB_1 and CB_2 receptors as well as TRPV1 are targets for the treatment of the early phase of ACD (Karsak, et al., 2007; Petrosino, et al., 2010), we investigated whether the anti-inflammatory action of CBD could be mediated by one or more of these targets in the *in vitro* model of ACD. Although it is also known that CBD weakly activates the human 5-hydroxytryptamine (serotonin) receptor 1A (5-HT1A) (Russo, et al., 2005), we did not investigate this mechanism because it has been demonstrated that HaCaT cells do not express such receptor (Slominski, et al., 2003). Thus, we studied the effects of

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CB_1, CB_2 and TRPV1 antagonists on MCP-2 and cytokine (i.e. IL-6, IL-8 and TNF-α) production in poly-(I:C)-stimulated HaCaT cells *in vitro*, in the presence or absence of CBD 20 μM. Our results show that, similar to CBD, the CB_1 antagonist, AM251, at the highest concentration tested (5 μM), was able to only reduce MCP-2 protein levels after 6 h in poly-(I:C)-stimulated HaCaT cells. The inhibitory effect of CBD on MCP-2 production was not influenced by the highest *per se* inactive concentration of AM251 (2.5 μM). Like AM251 and CBD, the CB_2 antagonist, AM630, at the highest concentration tested (1 μM), was able to only reduce MCP-2 protein levels after 6 h in poly-(I:C)-stimulated HaCaT cells, but unlike AM251, the highest *per se* inactive tested concentration of AM630 (0.1 μM) was able to reverse the inhibitory effect of CBD on MCP-2 levels. On the other hand, low concentrations of the TRPV1 antagonist, I-RTX, were able to only reduce MCP-2 protein levels after 6 h in poly-(I:C)-stimulated HaCaT cells, whereas the highest *per se* inactive concentration tested of this compound (1 μM) was able to reverse the inhibitory effect of CBD on MCP-2 levels. These results : 1) suggest that, since the effects of CB_2 and TRPV1 antagonists, *per se* or on the effects of CBD, are only observed on MCP-2 production and only at 6h, this chemokine and these receptors are mostly involved in the early sensitization phase of ACD and in the inhibitory actions thereupon of the phytocannabinoid; the effect of the highest concentrations of AM251 *per se* may be non-CB_1-mediated, since this compound at high concentrations acts as an agonist on a potential anti-inflammatory off-target such as GPR55 (Ryberg, et al., 2007; Cantarella, et al., 2011; Montecucco, et al., 2016), 2) confirm that CB_2 receptors may play also a facilitatory role in ACD (Ueda, et al., 2005), as indicated here by the anti-inflammatory effect of the highest concentration of AM630; and 3) support the previous finding that TRPV1 may play both facilitatory and inhibitory roles against ACD (Banvolgyi, et al., 2005), as shown here by the fact that low, but not high, concentrations of the I-RTX counteracted poly-(I:C)-induced MCP-2 production in HaCaT keratinocytes. Thus, the anti-inflammatory effect of CBD might be due to both CB_2 activation, as previously shown for THC (Karsak, et al., 2007; Yang, et

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al., 2015; Xie, et al., 2016; Shang, et al., 2016), and TRPV1 activation/desensitization, as suggested here by the fact that this effect was reversed by both a low concentration of a CB_2 receptor antagonist (AM630) and by a high concentration of a TRPV1 antagonist (IRTX). Whilst this latter effect is not surprising due to the aforementioned mentioned capability of CBD to stimulate and desensitize TRPV1 (Iannotti, et al., 2014), this phytocannabinoid exhibits only low affinity for CB_2 (Pertwee, 2008). Therefore we hypothesized that endogenous ligands could mediate the anti-inflammatory effect of CBD at this receptor and explain why such effect was antagonised by AM630. Indeed, CBD, at a ~20 μM concentration ($IC_{50} = 27.5$ μM), inhibits both AEA cellular uptake and enzymatic hydrolysis (Bisogno, et al., 2001), and these effects could both explain the present finding of its stimulatory action on AEA levels and indirect activation of CB2 receptors. Consequently, our hypothesis that CBD acted via elevation of AEA levels in HaCaT cells was supported by our present finding that both AEA and a synthetic inhibitor of its degradation, URB597, like CBD, were able to reduce the production both of MCP-2 and other pro-inflammatory cytokines (i.e. IL-6 and IL-8) produced by poly-(I:C)-stimulated keratinocytes. Interestingly, the stimulatory action of CBD on AEA levels was only observed after 6 h, and this could explain why the CB_2 antagonist did not attenuate here the anti-inflammatory effects of CBD after 12 and 24 h. Importantly, while the effects of AEA and URB597 on MCP-2 production were comparable to those of CBD, those on IL-6 and IL-8 were statistically significant but less efficacious. This observation supports the above suggestion that these cytokines plays different roles in the sensitization phase of ACD, at least in the *in vitro* model used here, thus possibly explaining why CB2 and TRPV1 antagonists did not attenuate the effect of CBD on these inflammatory mediators.

In conclusion, in the present study we demonstrated that in an *in vitro* model of ACD: 1) CBD inhibits the production of MCP-2 as well as of IL-6, IL-8 and TNF-α; 2) the endogenous levels of AEA are increased after CBD treatment; and 3) the anti-inflammatory effect of CBD during the early sensitization phase (i.e. after 6 h) is antagonized both by a selective CB_2 antagonist - and hence potentially mediated by the endogenous agonist for CB_2 receptors, AEA, - and a

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selective TRPV1 antagonist - likely because the phytocannabinoid can directly activate and desensitize the TRPV1 channel. Given the established safety profile of CBD in humans (Leweke, et al., 2012; Pertwee, 2015), these data warrant further experiments on the preclinical testing of this compound in animal models of ACD.

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Declaration of interest

S.P. and M.A. are employees of Epitech Group SpA. V.D. is the recipient of research grants, provides consultancy services and performs sponsored research for GW Research Ltd. No other authors have conflict of interests.

Authorship Contributions:

Participated in research design: S.P., T.I., V.D.

Conducted experiments: S.P., R.V, M.A.

Performed data analysis: S.P., V.D.

Performed *in vivo* experiments (not reported in this paper): M.V., T.I.

Wrote or contributed to the writing of the manuscript: S.P., V.D.

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Footnotes

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Legend to figures:

Figure 1. CBD reduces MCP-2 levels in poly-(I:C)-stimulated HaCaT cells.

ELISA assay for MCP-2 release in the supernatants of poly-(I:C)-stimulated HaCaT cells (100 μg/ml) in the presence of vehicle or CBD (1, 5, 10, 20 μM) for 6 h (A), 12 h (B) and 24 h (C), at 37 °C in 5% CO_2. Data represent mean ± SEM of three independent experiments performed in triplicate. §§§ $P < 0.001$ *vs* vehicle; *** $P < 0.001$ *vs* poly-(I:C). Assay range for MCP-2, 0.8-200 pg/ml.

Fig. 2. CBC, CBG, THCV and CBGV reduce MCP-2 levels in poly-(I:C)-stimulated HaCaT cells.

ELISA assay for MCP-2 release in the supernatants of poly-(I:C)-stimulated HaCaT cells (100 μg/ml, 6 h, 37 °C) in the presence of vehicle or CBC (A), CBG (B), THCV (C), CBGV (D) (all tested at 5, 10, 20 μM). Data represent mean ± SEM of three independent experiments performed in triplicate. §§§ $P < 0.001$ *vs* vehicle; ** $P < 0.01$ and *** $P < 0.001$ *vs* poly-(I:C). Assay range for MCP-2, 0.8-200 pg/ml.

Fig. 3. CBD reduces IL-6, IL-8 and TNF-α levels in poly-(I:C)-stimulated HaCaT cells after 6 h.

Bio-Plex Pro assay for IL-6 (A), IL-8 (B) and TNF-α (C) release in the supernatants of poly-(I:C)-stimulated HaCaT cells (100 μg/ml, 6 h, 37 °C) in the presence of vehicle or CBD (1, 5, 10 and 20 μM). Data represent mean ± SEM of three independent experiments performed in triplicate. §§§ $P < 0.001$ *vs* vehicle; * $P < 0.05$ and *** $P < 0.001$ *vs* poly-(I:C). Assay range for: IL-6, 37.68 pg/ml; IL-8, 42.15 pg/ml; TNF-α, 64.80 pg/ml.

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Fig. 4. CBD reduces IL-6 and TNF-α levels in poly-(I:C)-stimulated HaCaT cells after 12 and 24 h.

(A) Bio-Plex Pro assay for TNF-α release in the supernatants of poly-(I:C)-stimulated HaCaT cells (100 μg/ml, 12 h, 37 °C) in the presence of vehicle or CBD (20 μM). (B) Bio-Plex Pro assay for IL-6 (B) and TNF-α (C) release in the supernatants of poly-(I:C)-stimulated HaCaT cells (100 μg/ml, 24 h, 37 °C) in the presence of vehicle or CBD (20 μM). Data represent mean ± SEM of three independent experiments performed in triplicate. §§§ $P < 0.001$ *vs* vehicle; *** $P < 0.001$ *vs* poly-(I:C). Assay range for: IL-6, 37.68 pg/ml; TNF-α, 64.80 pg/ml.

Fig. 5. CBC, CBG and THCV reduce IL-6 and IL-8 levels in poly-(I:C)-stimulated HaCaT cells after 6 h.

Bio-Plex Pro assay for IL-6 (A, C, E) and IL-8 (B, D) release in the supernatants of poly-(I:C)-stimulated HaCaT cells (100 μg/ml, 6 h, 37 °C) in the presence of vehicle or CBC, CBG and THCV (all tested at 5, 10 and 20 μM). Data represent mean ± SEM of three independent experiments performed in triplicate. §§§ $P < 0.001$ *vs* vehicle; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ *vs* poly-(I:C). Assay range for: IL-6, 37.68 pg/ml; IL-8, 42.15 pg/ml.

Fig. 6. CBD and other phytocannabinoids are not cytotoxic in HaCaT cells.

(A) MTT assay in HaCaT cells treated with vehicle (white histogram) or CBD, CBC, CBG, THCV, CBGV (all tested at 10, 20 μM) for 6 h at 37 °C in 5% CO_2. (B) MTT assay in HaCaT cells treated with vehicle or CBD (10, 20 μM) for 12 and 24 h at 37 °C in 5% CO_2. Data represent mean ± SEM of three independent experiments performed in triplicate.

Fig. 7. CB_2 and TRPV1 receptors mediate the action of CBD in poly-(I:C)-stimulated HaCaT cells only after 6 h.

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(A) ELISA assay for MCP-2 release in the supernatants of poly-(I:C)-stimulated HaCaT cells (100 μg/ml, 6 h, 37 °C) treated with AM251 (1, 2.5, 5 μM) in the presence or absence of CBD (20 μM). (B) ELISA assay for MCP-2 release in the supernatants of poly-(I:C)-stimulated HaCaT cells (100 μg/ml, 6 h, 37 °C) treated with AM630 (0.01, 0.1 μM) or I-RTX (0.01, 0.1 μM) in the presence or absence of CBD (20 μM). Data represent mean ± SEM of three independent experiments performed in triplicate. §§§ $P < 0.001$ *vs* vehicle. *** $P < 0.001$ and ** $P < 0.01$ *vs* poly-(I:C). °° $P < 0.01$ and °°° $P < 0.001$ *vs* poly-(I:C) + CBD 20 μM. Assay range for MCP-2, 0.8-200 pg/ml.

Fig. 8. CBD elevates AEA levels in poly-(I:C)-stimulated HaCaT cells after 6 h.

Concentrations of AEA (A), 2-AG (B), PEA (C) and OEA (D) in poly-(I:C)-stimulated HaCaT cells (100 μg/ml, 6 h, 37 °C) in the presence of vehicle or CBD (20 μM). Data represent mean ± SEM of three independent experiments performed in triplicate. *** $P < 0.001$ *vs* vehicle; °°° $P < 0.001$ *vs* poly-(I:C) + vehicle.

Fig. 9. AEA and URB597 reduce MCP-2, IL-6 and IL-8 levels in poly-(I:C)-stimulated HaCaT cells after 6 h.

ELISA assay for MCP-2 (A) and Bio-Plex Pro assay for IL-6 (B) and IL-8 (C) release in the supernatants of poly-(I:C)-stimulated HaCaT cells (100 μg/ml, 6 h, 37 °C) in the presence of vehicle or CBD (20 μM), AEA (10 μM), URB597 (10 μM). Data represent mean ± SEM of three independent experiments performed in triplicate. §§§ $P < 0.001$ *vs* vehicle; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ *vs* poly-(I:C). Assay range for: MCP-2, 0.8-200 pg/ml; IL-6, 37.68 pg/ml; IL-8, 42.15 pg/ml.

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Figure 1.

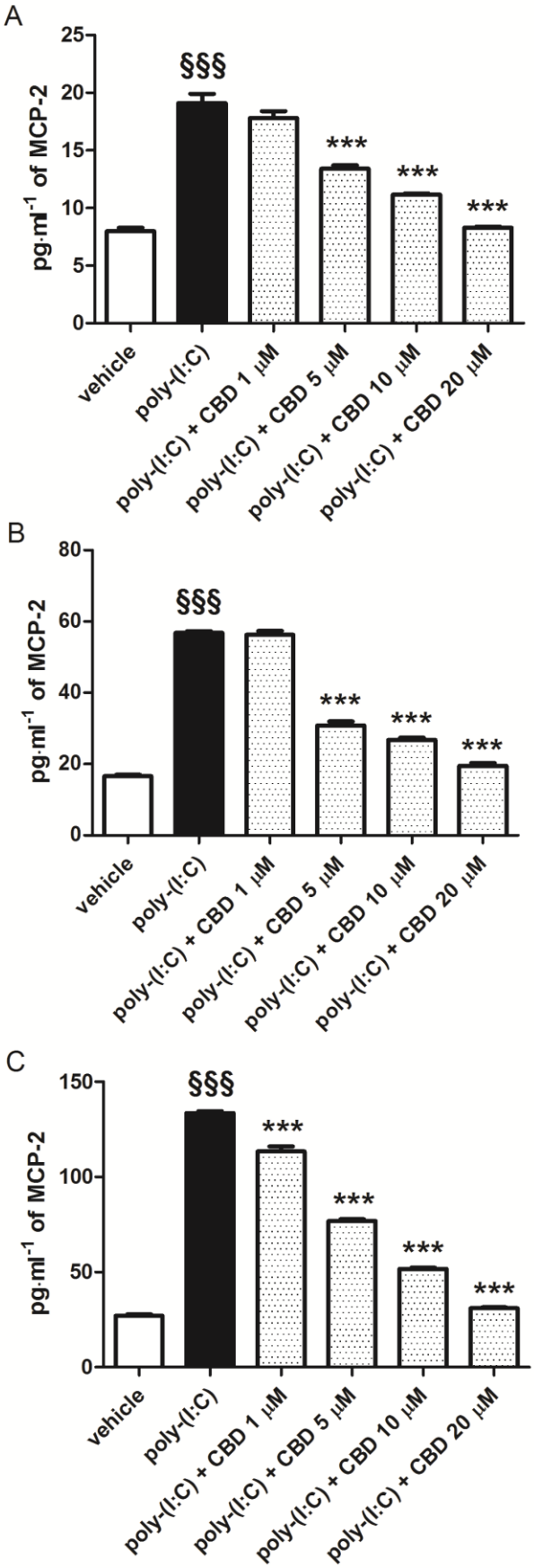

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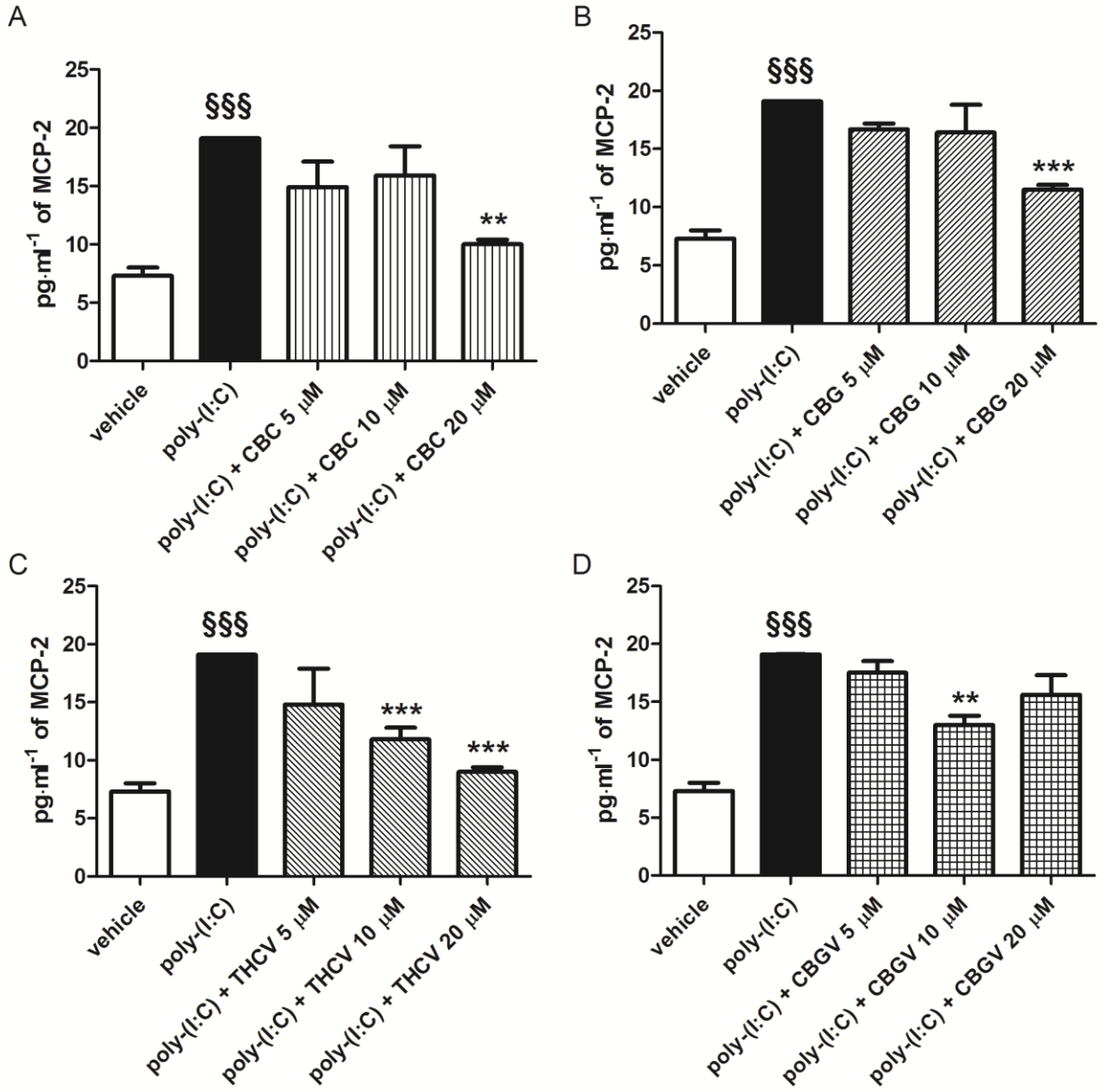
Figure 2.



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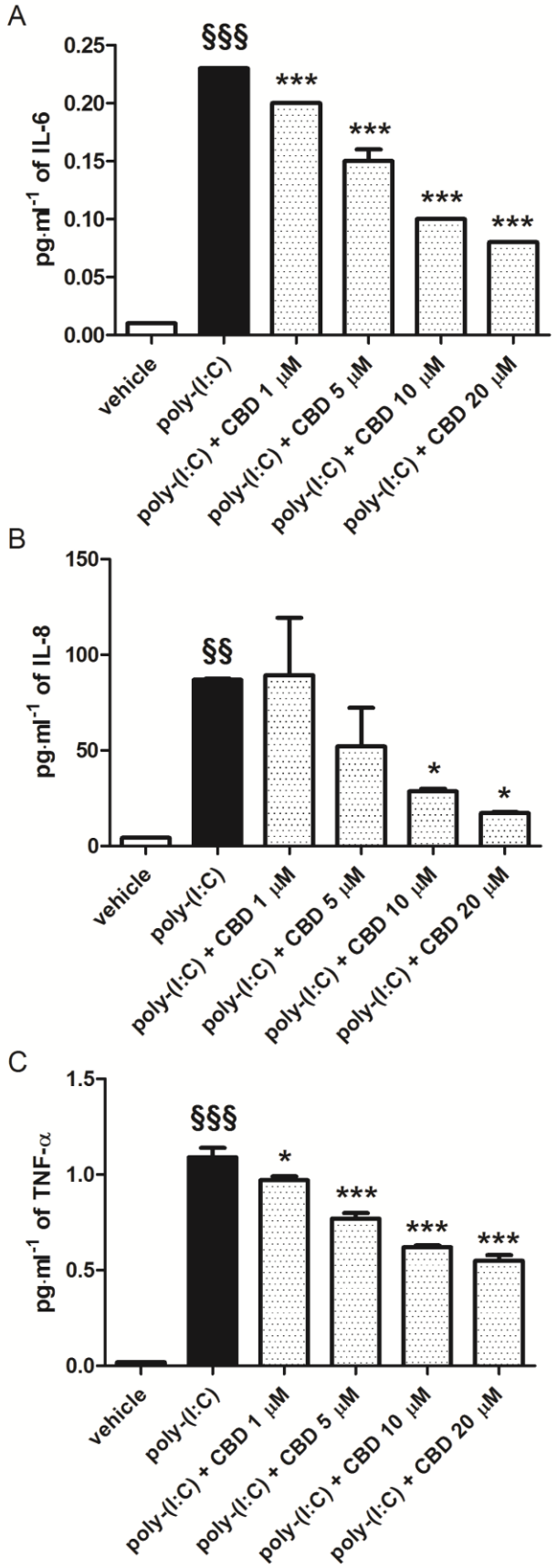

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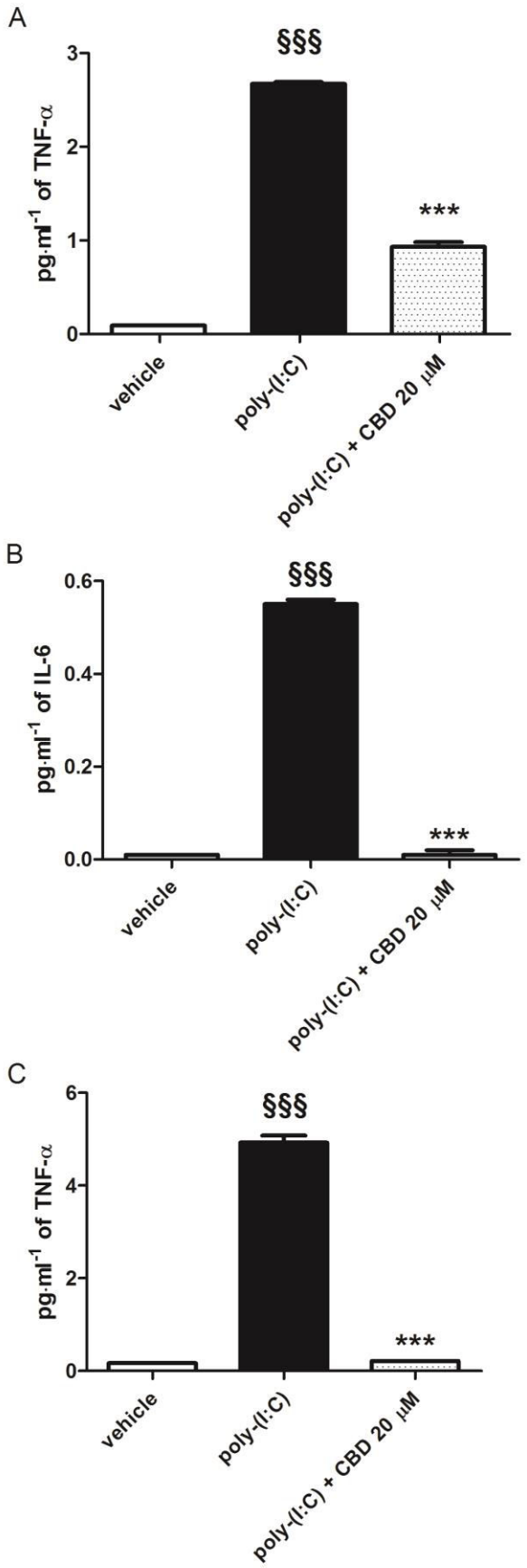

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Figure 5.

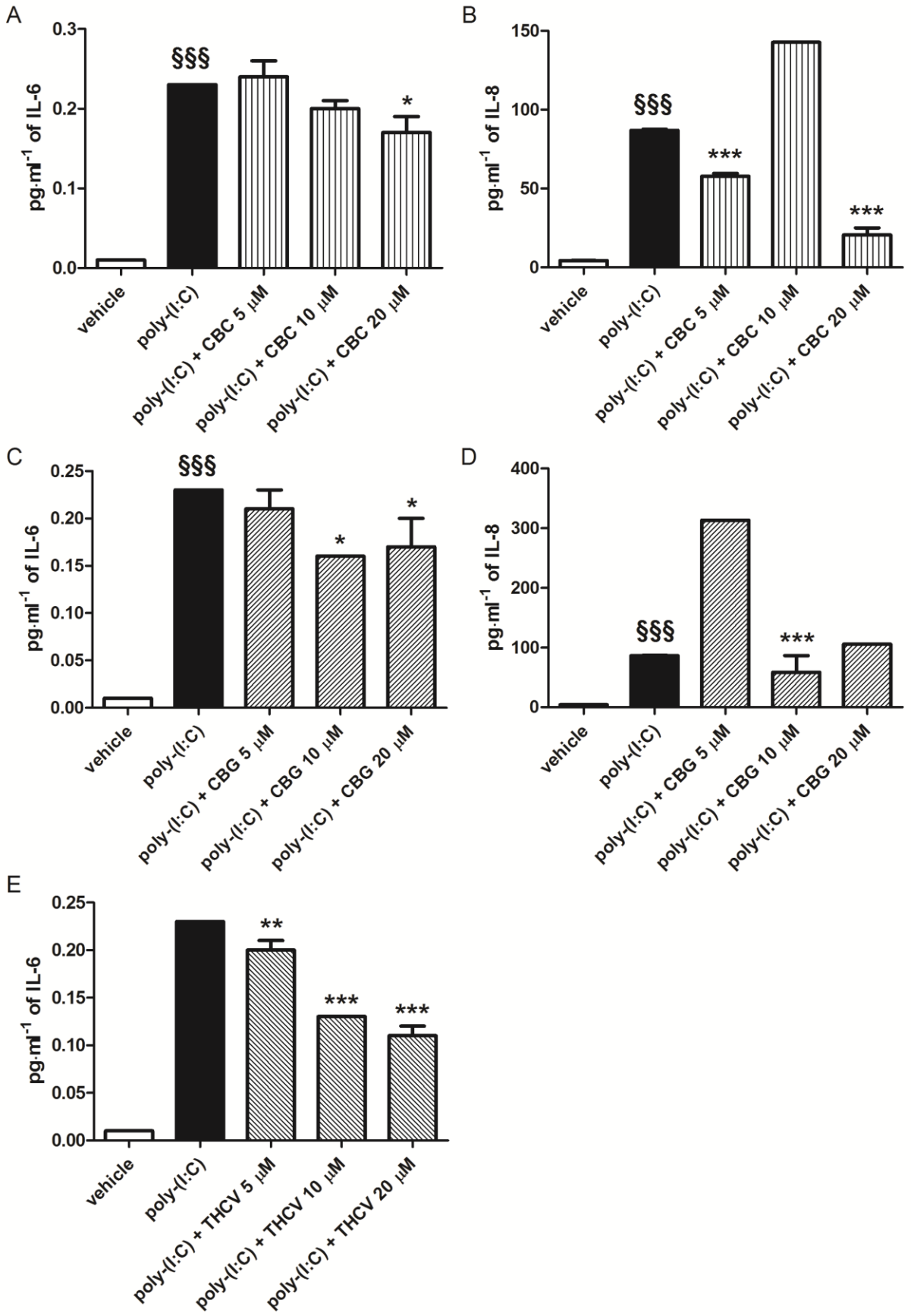

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Figure 6.

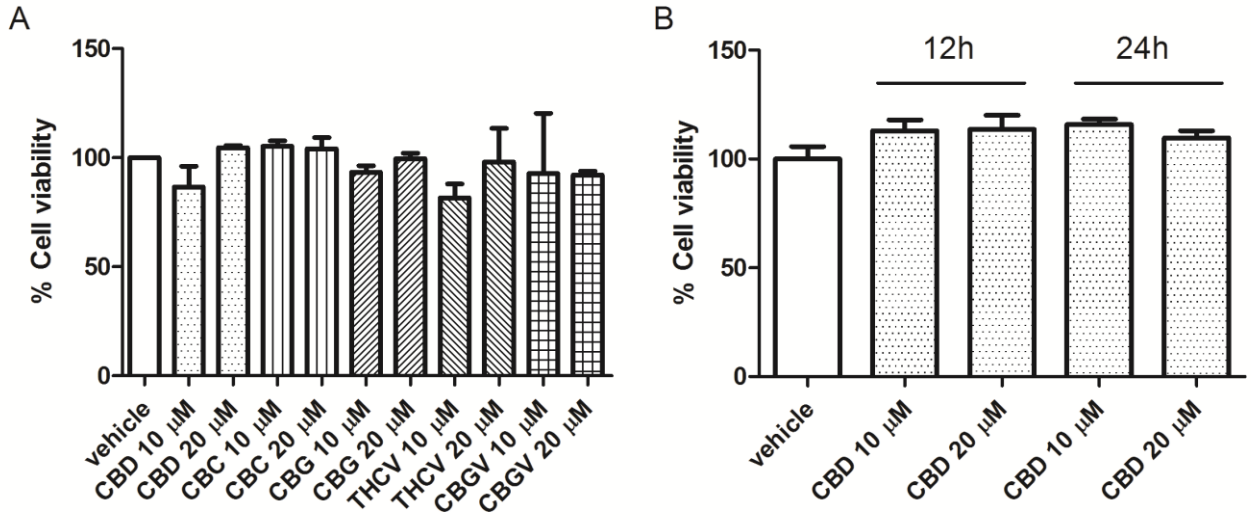

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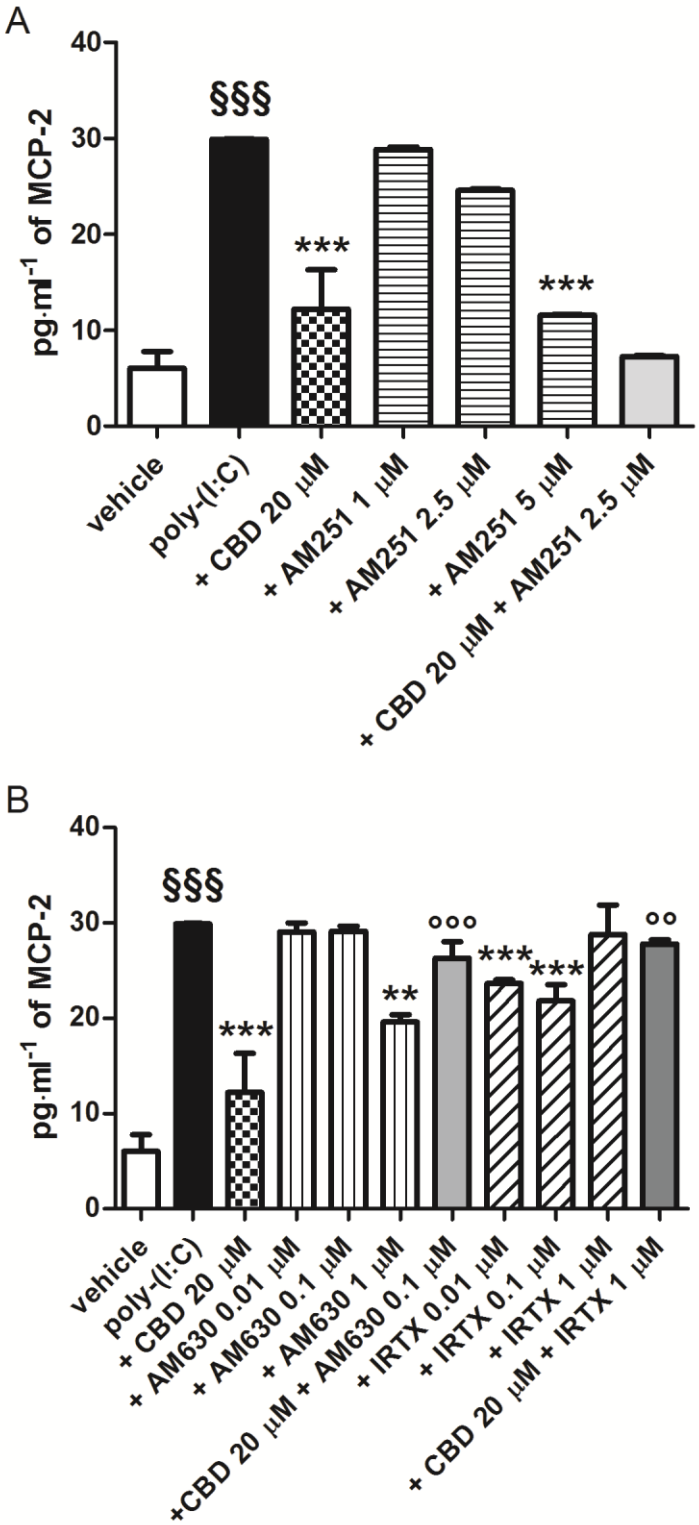
Figure 7.



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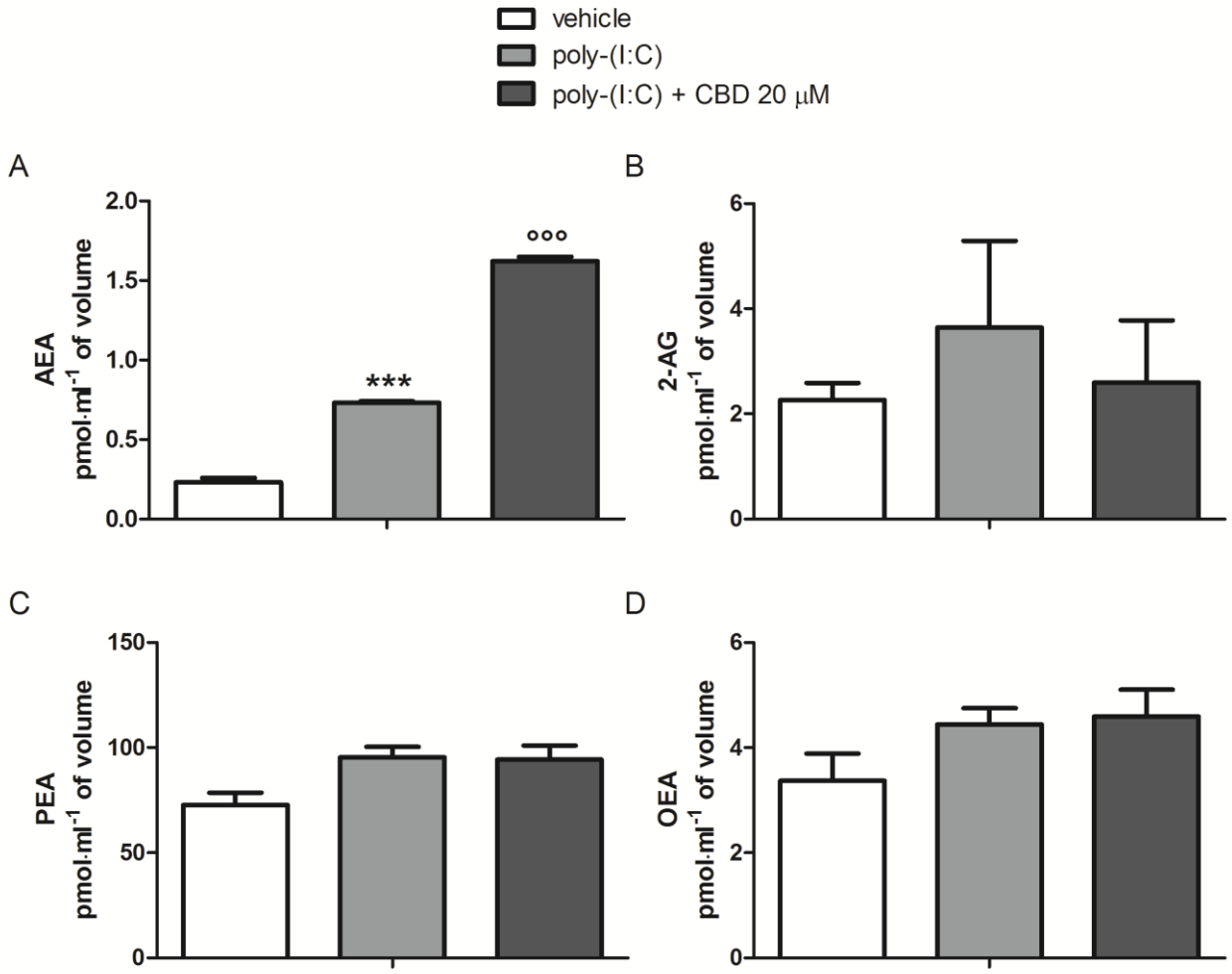

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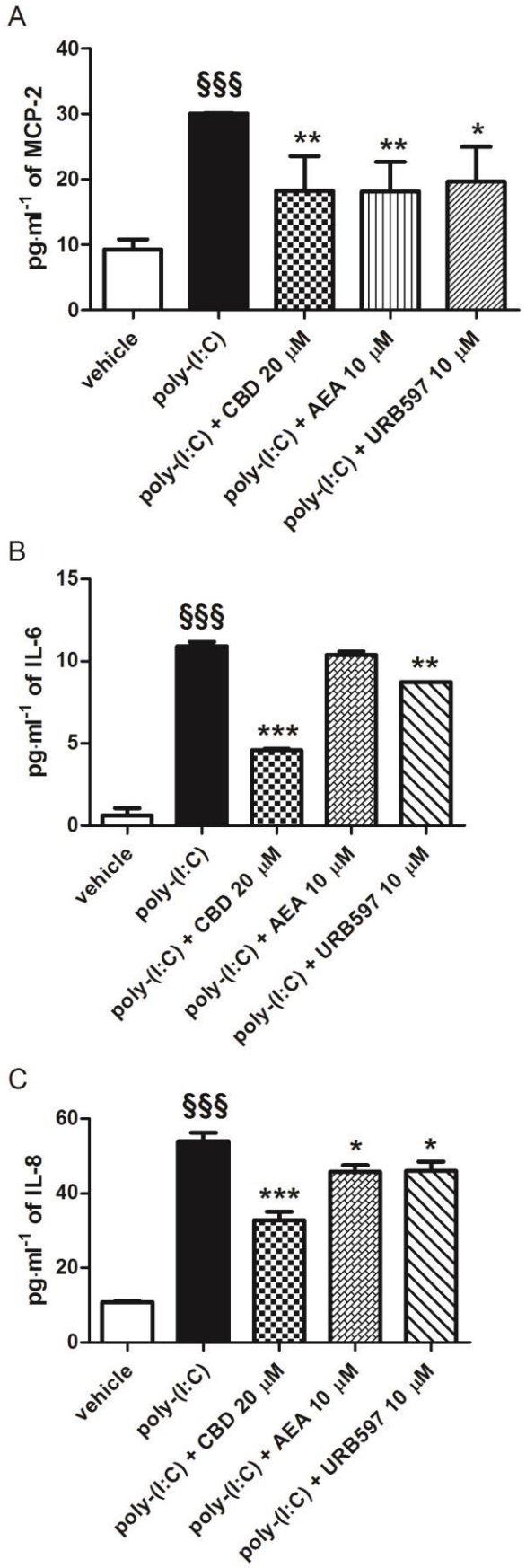

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