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A role for CB2 receptors in anandamide signalling pathways involved in the regulation of IL-12 and IL-23 in microglial cells

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ABSTRACT

The endocannabinoid system represents a novel therapeutic target for autoimmune and chronic inflammatory diseases. IL-12 and IL-23 are functionally related heterodimeric cytokines that play a crucial role in the pathogenesis of multiple sclerosis (MS). In the present study we investigated the effects of the endocannabinoid anandamide (AEA) on the inducible expression of the biologically active cytokines IL-12p70 and IL-23, and their forming subunits, in activated microglial cells. We also studied the signalling pathways involved in the regulation of IL-12p70/IL-23 expression and addressed the possible interactions of AEA with these pathways. Here, we show that AEA was capable to inhibit the production of biologically active IL-12p70 and IL-23, and their subunits, by activated human and murine microglial cultures. Treatment of activated microglial cells with inhibitors of several mitogen-activated protein kinase (MAPK) reveals that AEA acts through the ERK1/2 and JNK pathways to down-regulate IL-12p70 and IL-23. These effects were partially mediated by CB2 receptor activation. Together, our results provide the first demonstration of a role of AEA in inhibiting IL-12p70/IL-23 axis in human and murine microglial cells via the CB2 receptor and suggest that the pharmacological manipulation of the endocannabinoid system is a potential tool for treating brain inflammatory and autoimmune diseases, like MS.

1. Introduction

The production of interleukin-12 by cells of myeloid lineage, such as antigen-presenting cells (APCs), is critical for host defence against pathogens. IL-12 is a heterodimeric cytokine composed of p35 and p40 subunits, and of special importance since its expression regulates innate immunity and determines the type and duration of adaptive immune response [1]. Besides forming IL-12p70 heterodimer, p35 and p40 may dimerize with alternate partners to form distinct bimolecular complexes. For example, the recently identified p19 subunit, which lacks biological activity *per se*, combines to the p40 subunit to give the cytokine IL-23. Since both IL-12p70 and IL-23 share the common subunit p40, they have overlapping, but

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also distinct, effects on their targets [2,3]. One of the major differences is that while IL-12 preferentially acts by favouring the development of Th1 cells, IL-23 is involved in the maintenance of Th17 cells, which have been related to multiple sclerosis (MS) pathogenesis in a specific way [4,5].

Microglia, the brain-resident immune cells are activated in most pathological conditions of the central nervous system (CNS) and participate in the regulation of innate and adaptive immune responses [6]. Human, mouse, and rat microglial cells mainly express the cannabinoid receptor type 2, CB2 [7,8], and its expression level depends on the activation state of these cells as occurs for other immune cells [9,10]. The endogenous ligands, anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are also synthesized by microglia that produce approximately 20-fold more endocannabinoids than neurons and astrocytes [11]. The endocannabinoid system is highly activated during brain inflammation and AEA, but not 2-AG, has been shown to be increased in active lesions of MS patients and to protect neurons from inflammatory damage [12]. Studies on the role played by the cannabinoid system during neuroinflammation support the interest of this system as a novel target for therapeutic approach [13,14]. In animal models of MS, cannabinoids attenuate the pathological features of the disease [15,16], an effect also observed by pharmacological interventions aimed at increasing the levels of the endocannabinoid AEA [17–21]. Results from different groups show that the cannabinoid system also plays a role in regulating microglial function such as proliferation [22], cellular migration [11] or the generation of inflammatory mediators [23,24].

Theiler's murine encephalomyelitis virus (TMEV) infects glial cells and causes demyelinating disease in certain mouse strains [25]. In our previous studies, we showed that macrophages infected with TMEV increased their production of AEA and that both AEA and the inhibitors of AEA cellular re-uptake, OMDM1 and OMDM2, inhibited IL-12p40 release [20]. In addition, AEA regulated the transcriptional activity of p40 promoter by activating the repressor site GA-12, likely through prostamide E_2 generation [26]. The identification of human microglia as one of the major sources of IL-23 in brain sections from MS patients [27] points out the importance of microglial cells as cellular targets to down-regulate this family of heterodimeric cytokines. To date, however, the mechanisms by which AEA may regulate the IL-12p70/IL-23 axis in microglial cells are unknown. Cannabinoid receptors have been shown to signal through mitogen-activated protein kinase (MAPK) signalling pathways [14]. The MAPK signal transduction pathways are highly conserved cascades implicated in several aspects of the immune response [28]. The family of protein kinases includes Jun N-terminal protein kinase (JNK), ERK1/2 and p38. The MAP kinases phosphorylate transcription factors that are involved in the process of activation of macrophages for the generation of IL-12p70 and other family-related cytokines [29,30]. The importance of the MAPK signal transduction pathway in controlling many aspects of immune-mediated inflammatory responses has made them a priority for research related to many neuroinflammatory diseases. Here we showed that AEA inhibits the expression of the three subunits p19, p35 and p40 subunits as well as the release of IL-12p70 and IL-23 by LPS/IFN-γ activated human and murine microglia. Our results also indicate that AEA down-regulates IL-12p70 and IL-23 production acting through ERK1/2 and JNK by a mechanism that involves the activation of CB2 receptors.

2. Materials and methods

2.1. Reagents and antibodies

The following materials were used: lipopolysaccharide from *E. coli* serotype O127:B8 and anandamide were purchased from Sigma (Madrid, Spain). Murine recombinant interferon-γ (IFNγ) was from PreproTech (London, UK). Cannabinoid antagonists SR141716A (*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazol-carboxamide) (SR1) and SR144528 (*N*-[1S)-endo-1,3,3,-trimethylbicyclo[2.2.1]heptan-2-yil-5-(4-chloro-3methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide) (SR2) were supplied by Sanofi Recherche (Montpellier, France). JWH-133 was purchased from Tocris Cookson Ltd. (UK). SP600125, PD98059 and SB203580 were purchased from Cell Signalling Technology (Beverly, USA). Anti-phospho-SAPK/JNK, anti-total-SAPK/JNK, anti-phospho-p44/p42 MAPK (phospho-ERK1/2), anti-total-p44/p42 MAPK (total ERK1/2), anti-phospho-p38 MAPK and anti-total-p38 MAPK antibodies were purchased from New England Biolabs (Beverly, USA). Anti-tubulin antibody was obtained from Sigma (Madrid, Spain). Peroxidase-conjugated anti-rabbit secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, USA) and anti-mouse secondary antibody was from Bio-Rad (Hercules, USA). All other reagents were obtained from standard supplies.

2.2. Animals

Balb/c mice from our in-house colony (Cajal Institute, Madrid) were used. Animals were housed in cages with filter tops in a laminar flow hood and maintained on food and water *ad libitum* in a 12-h dark–light cycle. Handling of animals was performed in compliance with the guidelines of animal care set by the European Union (86/609/EEC) and the Spanish regulations (BOE67/8509-12; BOE 1201/2005) on the use and care of laboratory animals, and approved by the local Animal Care and Ethics Committee of the Consejo Superior de Investigaciones Científicas (CSIC).

2.3. Isolation of murine microglia and cell culture

Primary mix glial cultures were prepared as described previously [50]. Briefly, after decapitation, forebrains of newborn Balb/c mice were dissociated mechanically, filtered through a 150-μm nylon mesh, resuspended in DMEM containing 10% heat-inactivated FBS, 10% heat-inactivated horse-serum and 1% penicillin/streptomycin and plated on poly-L-lysine (15 μg/mL) 75 cm^2 flasks (Falcon; Le Pont de Claix, France). After 15 days in culture the flasks were shaken at 230 rpm at 37 °C for 3 h to remove loosely adherent microglia. The supernatant was plated on multiwell culture plates for 2 h. After this, medium was changed to remove non-adherent cells. Cells were grown in a humidified environment containing 5% CO_2 and held at a constant temperature of 37 °C. The

purity of microglial cultures was assessed by examining the characteristic cell morphologies under phase-contrast microscopy and was confirmed by immunostaining with Mac-1 anti-CD11b antibody (Serotec Ltd., Oxford, UK).

2.4. Isolation of human microglia and cell culture

Brain tissues from 14 to 16 weeks old foetuses were obtained following protocols according to the French Institutional and National Guidelines. Foetal brain tissues were dissociated mechanically, filtered through a 150-μm nylon mesh, resuspended in DMEM containing 10% heat-inactivated FBS, 10% heat-inactivated horse-serum and 1% penicillin/streptomycin and plated on poly-L-lysine (15 μg/mL) 75 cm^2 flasks (Falcon; Le Pont de Claix, France). After 10–14 days in incubation, flasks were placed on an orbital shaker and rotated 200 rpm for 3 h. Detached cells were harvested from the supernatant and reseeded in 24-well plates at a density of 1×10^5 cells/mL per well. Cells were grown in a humidified environment containing 5% CO_2 and held at a constant temperature of 37 °C. The purity of microglial cultures was assessed by examining the characteristic cell morphologies under phase-contrast microscopy and was confirmed by immunostaining with anti-CD68 antibody for human microglia.

2.5. Exposure of microglia to cannabinoids and treatment with LPS/IFNγ

One hour before each experiment, media containing serum were removed and replaced with serum-free media. Microglial cells then were maintained for the indicated times in serum-free DMEM containing cannabinoids or vehicle, LPS/IFNγ mix was added directly to the microglial cultures. Cells were harvested at the indicated times after treatment with LPS/IFNγ. AEA was used at 10 μM, LPS/IFNγ at 50 ng/mL and 100 U/mL, respectively, SP600125 and PD98059 at 10 μM and SB203580 at 25 μM according to each case.

2.6. Immunocytochemistry in cultured cells

Immunostaining was done directly on cells seeded on glass coverslips. Microglia plated onto poly-D-lysine-coated (PLD; 5 μg/mL) coverslips were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature (RT). Coverslips were then rinsed in PBS and incubated for 2 h at RT with the Mac-1 anti-CD11b antibody at a dilution 1:500 (Serotec, Oxford, UK) and anti-p35 (1:500), anti-p40 (1:500) or anti-p19 (1:500) antibodies (Santa Cruz, Inc. CA, USA). Immunostaining was visualized with Alexa-conjugated secondary (1:1000) anti-mouse IgG-Alexa 488 and anti-rabbit IgG Alexa antibodies (Molecular Probes, Eugene, Oregon, USA). Nonspecific interactions of secondary antibodies were confirmed by omitting primary antibodies. At least three independent cultures were examined.

2.7. RNA isolation and quantitative polymerase chain reaction (qPCR)

Total RNAs from microglial primary culture were prepared using the RNAeasy extraction columns (Qiagen; Hilden, Germany). Samples were digested with DNAse (Sigma) to avoid genomic DNA contamination. Samples (1 μg) of total mRNA were transcribed into cDNA using the Reverse Transcription System kit (Promega) according to manufacture's instructions. Primers and probes for IL-12p35, IL-12p40, IL-23p19, 80S and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were supplied by Applied Biosystems (Warrington, UK). Probes were labelled at the 5′-end with a 6′-carboxyfluorescerin (FAM) reporter dye and at the 3′-end with a 6′-carboxy-tetramethyl rhodamine (TAMRA) quencher dye. TaqMan PCR was performed from 1 μL of cDNA using Universal TaqMan Mastermix with 100 nM primers and 50 nM probe. Cycling conditions were: 50 °C for 2 min, 95 °C for 10 min, followed by 50 cycles of amplification (95 °C for 15 s, 60 °C for 1 min). Samples were assayed on the Applied Biosystems PRISM 7000 Sequence detection system. Samples were assayed in duplicate and a five-point standard curve was run in parallel. To ensure the absence of genomic DNA contamination, a control sample of non-reverse-transcribed RNA was run for each set of RNA extractions. Relative quantification was obtained by calculating the ratio between the values obtained for each gene of interest and the murine house-keeping gene GAPDH or human 18S.

2.8. Western blot analysis

After treatments, microglial cells were washed with ice-cold PBS and lysed in Tris-buffered saline pH 7.6 (TBS), containing 10% glycerol, 1% Nonidet P-40, EDTA 1 mM, EGTA 1 mM plus complete protease inhibitors cocktail. Cell lysates were mixed with 5X Laemmli sample buffer and boiled for 5 min. Then equal amount of protein (30 μg) were resolved on 10% SDS-PAGE and electroblotted at 100 V for 70 min at 4 °C to nitrocellulose (Amershan Biosciences; Barcelona, Spain). The membranes were blocked for 1 h at RT in 5% (w/v) dry skim milk (Sveltesse, Nestlé; Barcelona, Spain) in TBS with 0.1% Tween 20 (TBST). Then, the membranes were incubated overnight at 4 °C with the corresponding primary antibodies in 5% milk–TBST, extensively washed with 5% milk–TBST solution and incubated with the correspondent secondary antibodies for 1 h at RT. Finally, the blots were rinsed and the peroxidase reaction was developed by enhanced chemiluminescence (Amershan Biosciences, Barcelona, Spain). The blots were stripped in 62.5 mM Tris–HCl, pH 6.8, containing 2% SDS and 0.7% β-mercaptoethanol and were reprobed sequentially.

2.9. ELISA analysis

Levels of IL-12p70 in murine and human microglia culture supernatants were quantified using specific ELISA kits purchased from Biosource Int. (CA, USA) with a sensitivity of 4 pg/mL and 2 pg/mL, respectively. Levels of IL-23 in murine and human microglia culture supernatants were measured with specific IL-23 ELISA kit with a sensitivity of 10 pg/mL and 15 pg/mL, respectively, and they were obtained from eBioscience (Boston, USA) following manufacturer's recommendations.

2.10. Statistical analysis

Results are presented as means ± S.E.M. of at least three experiments performed in triplicate with different cell

preparations. Analysis of variance followed by the Tukey test for multiple comparison were used to determine statistical significance (95%; $p < 0.05$).

3. Results

3.1. *The endocannabinoid anandamide abrogates LPS/IFN-γ-induced IL-12p70 and IL-23 in human and murine microglial cells through CB2 receptor activation*

A previous study [27] described that IL-23 p19 expression in MS patient's brain tissue was associated with markers of macrophages/microglial cells. Therefore, we first investigated whether murine microglial cells, when activated by inflammatory stimuli like LPS/IFN-γ, express the different subunits p35, p40 and p19 that compose the biologically active cytokines IL-12p70 (p35/p40) and IL-23 (p19/p40). Fig. 1A–C show that LPS/IFNγ stimulation for 24 h induces the expression of the three subunits in microglial cells. Next, we analysed the temporal course of both IL-12p70 (Fig. 1D) and IL-23 (Fig. 1E) expression by measuring the levels of these cytokines in the supernatant of primary microglial cultures. We found that the maximum levels of these cytokines are produced at 18 h post-stimulation, since there was no statistical difference between IL-12p70 and IL-23 levels at 18 h and 24 h post-LPS/IFNγ treatment. The effects of different doses of AEA on the production of the biologically active proteins IL-12p70 and IL-23 after LPS/IFN-γ stimulation of murine primary microglial cells are shown in Fig. 2A and B, respectively. The dose of AEA (10 μM) was chosen since it was at this dose that the effects of AEA on IL-12p70 and IL-23 were higher. These observations are in agreement with previous studies [20,26,31–33]. The production of both, IL-12p70 (Fig. 2A) and IL-23 (Fig. 2B) was down-regulated in the presence of AEA, as reflected by the ELISA results in the cell free supernatants after 18 h of incubation time. To clarify whether the down-regulation of IL-12p70 and IL-23 production was due to specific

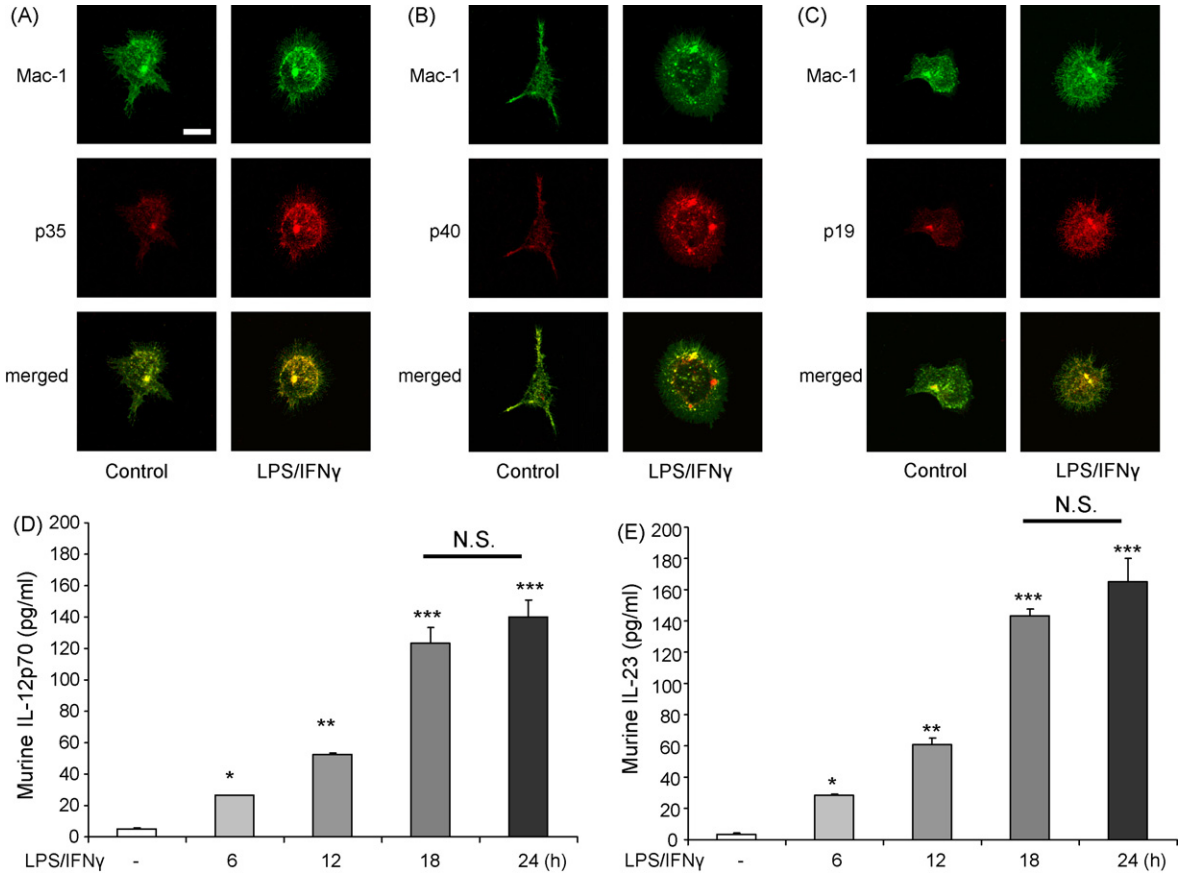

Fig. 1 – IL-12p70 and IL-23 production by microglial cells. Immunofluorescence staining of microglial cells in culture with anti-p35 (A), anti-p40 (B) and anti-p19 (C) antibodies in the presence or absence of a stimulus with LPS/IFNγ (50 ng/mL and 100 U/mL, respectively) for 24 h. Cells were co-immunostained with the specific marker Mac-1. A representative experiment of three independent experiments is shown. Scale bar: 20 μm. Temporal course of IL-12p70 production by microglia stimulated with LPS/INFγ (50 ng/mL and 100 U/mL, respectively). There was no statistical difference between IL-12p70 levels at 18 h and 24 h post-stimulus. Statistics: $^{*}p < 0.05$ vs. control; $^{**}p < 0.01$ vs. control; $^{***}p < 0.001$ vs. control (mean ± S.E.M.; $n = 6$); NS = non significance (D). Temporal course of IL-23 production by microglia stimulated with LPS/INFγ (50 ng/mL and 100 U/mL, respectively). There was no statistical difference between IL-23 levels at 18 h and 24 h post-stimulus (E). Statistics: $^{*}p < 0.05$ vs. control; $^{**}p < 0.01$ vs. control; $^{***}p < 0.001$ vs. control (mean ± S.E.M.; $n = 6$); NS = non significance.

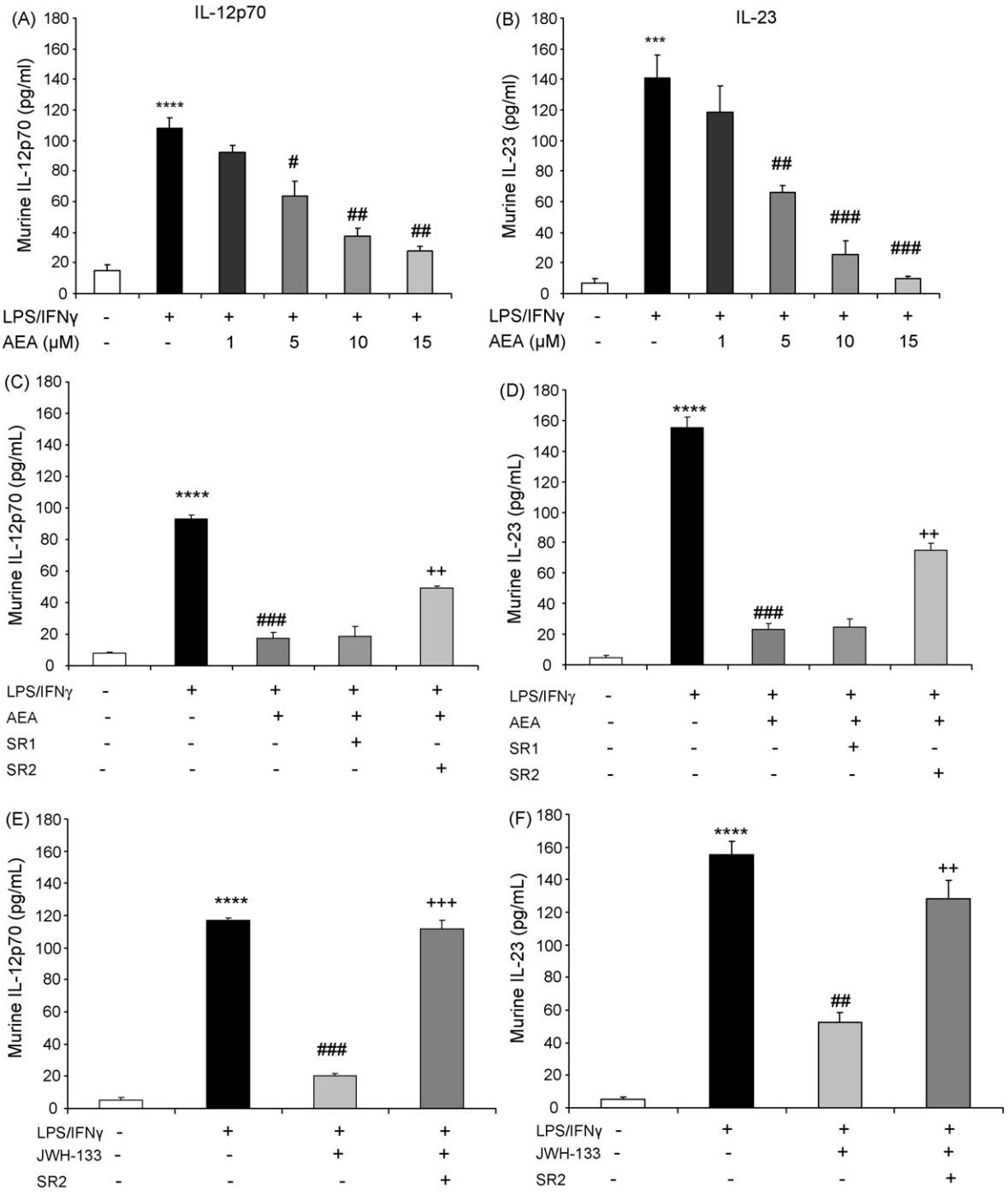

Fig. 2 – Production of biologically active IL-12p70 and IL-23 by LPS/IFNγ-activated microglia is regulated by AEA by a mechanism partially dependent of CB2 receptor. AEA reduced IL-12p70 (A) and IL-23 (B) production by microglial cells induced by treatment with LPS/IFNγ (50 ng/mL and 100 U/mL, respectively, 18 h) in a dose-dependent fashion. Statistic: $^{***}p < 0.005$ vs. control; $^{****}p < 0.001$ vs. control; $^{\#}p < 0.05$ vs. LPS/IFNγ; $^{\#\#}p < 0.01$ vs. LPS/IFNγ; $^{\#\#\#}p < 0.005$ vs. LPS/IFNγ (mean ± S.E.M., $n = 8$). Murine microglial cells stimulated with LPS/INFγ (50 ng/mL and 100 U/mL, respectively, 18 h) showed an induction of the expression of IL-12p70 and IL-23. This induction was reversed by the co-treatment with AEA (10 μM). CB2 receptor is involved in AEA effects since SR2 (1 μM) partially reversed AEA effects on both IL-12p70 and IL-23 production. Again, CB1 antagonist, SR1 (1 μM) showed no effect in reversing AEA actions. Statistics: $^{****}p < 0.001$ vs. control; $^{\#\#\#}p < 0.005$ vs. LPS/IFNγ; $^{++}p < 0.01$ vs. LPS/IFNγ + AEA (mean ± S.E.M., $n = 8$). The specific CB2 agonist, JWH-133 (100 nM) reduced IL-12p70 (E) and IL-23 (F) production. These effects were completely reversed by CB2 blockade with SR2 (1 μM). Statistics: $^{****}p < 0.001$ vs. control; $^{\#\#}p < 0.01$ vs. LPS/IFNγ; $^{\#\#\#}p < 0.005$ vs. LPS/IFNγ; $^{++}p < 0.01$ vs. LPS/IFNγ + JWH-133; $^{+++}p < 0.005$ vs. LPS/IFNγ + JWH-133 (mean ± S.E.M., $n = 8$).

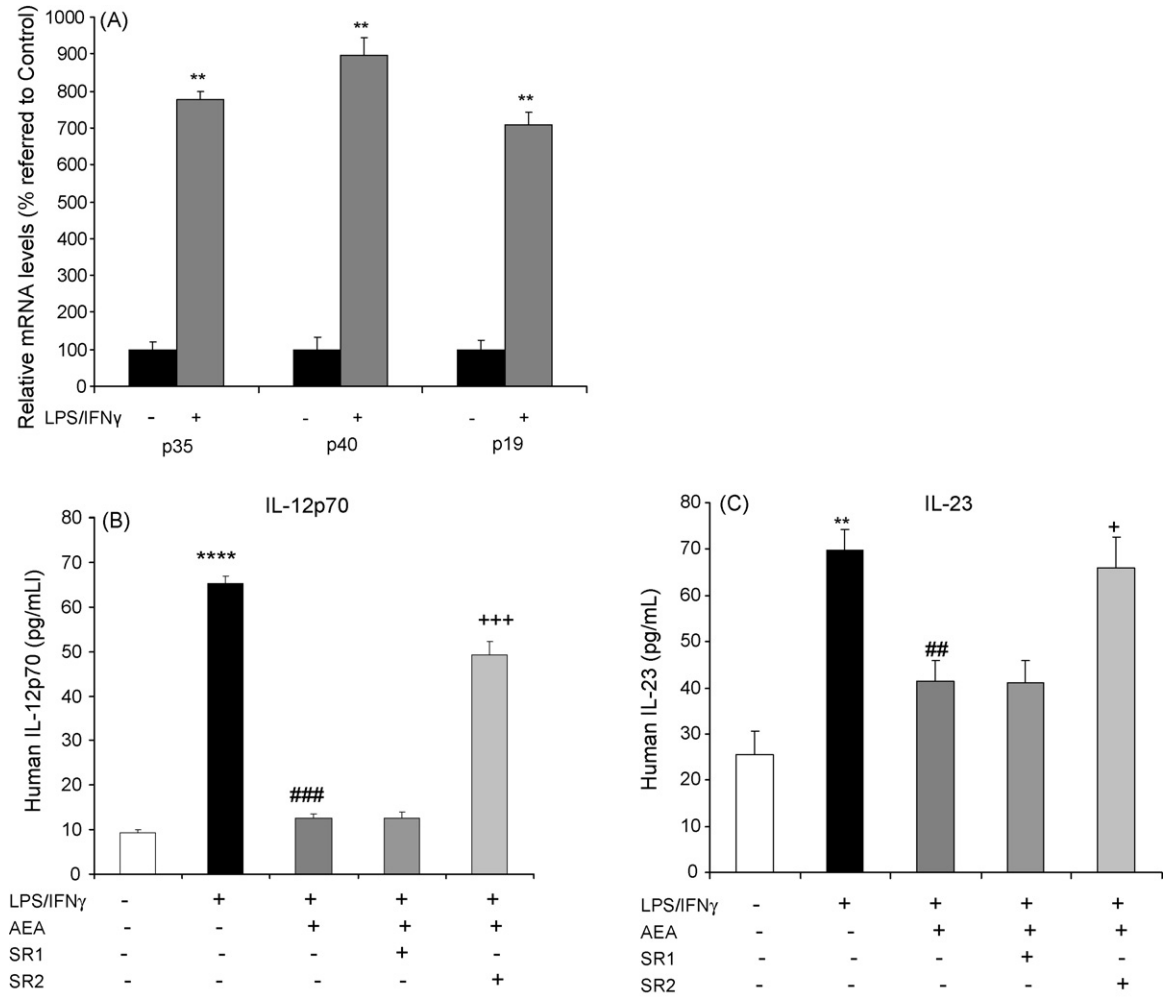

Fig. 3 – AEA reduced the production of both IL-12p70 and IL-23 induced by LPS/IFNγ in human microglial cells. LPS/IFNγ (50 ng/mL and 100 U/mL, respectively, 18 h) treatment induced IL-12p35 (p35), IL-12p40 (p40) and IL-23p19 (p19) mRNA expression in human microglial cells. mRNA levels were measured by real-time RT-PCR and are expressed as ratios between p35, p40 or p19 and 18S mRNA levels. Data are plotted as percentage of the p35/18S, p40/18S or p19/18S ratio obtained in untreated conditions. Statistics: $^{**}p < 0.01$ vs. control (mean ± S.E.M.; $n = 5$) (A) Human microglial cells stimulated with LPS/INFγ (50 ng/mL and 100 U/mL, respectively, 18 h) showed an induction of the expression of biologically active IL-12p70 (B) and IL-23 (C). This induction was reversed by the co-treatment with AEA (10 μM). The pre-treatment with the CB2 receptor antagonist, SR2 (1 μM) partially reversed the effects of AEA, suggesting an involvement of CB2 receptor in AEA regulation of IL-12p70 and IL-23 production. However, the CB1 receptor antagonist, SR1 (1 μM) showed no effect in reversing AEA actions. Statistics: $^{**}p < 0.01$ vs. control; $^{****}p < 0.001$ vs. control; $^{\#\#}p < 0.01$ vs. LPS/IFNγ; $^{\#\#\#}p < 0.005$ vs. LPS/IFNγ; $^{+}p < 0.05$ vs. LPS/IFNγ + AEA; $^{+++}p < 0.005$ vs. LPS/IFNγ + AEA; (mean ± S.E.M., $n = 6$).

interactions between AEA and CB1 or CB2 receptors, we treated murine microglial cells with the pharmacological antagonists SR141617A (SR1), for CB1 and SR144528 (SR2), for CB2 receptors prior to the exposure to the endocannabinoid. In these experiments it was observed that AEA effects were reversed by CB2, but not CB1 antagonist (Fig. 2C and D). To further confirm the involvement of CB2 receptors on AEA effects on IL-12p70 and IL-23 production, we used the specific CB2 agonist, JWH-133 (100 nM). In both cases, the treatment with JHW-133 strongly reduced the production of IL-12p70 (Fig. 2E) and IL-23 (Fig. 2F). These effects were completely reversed by the blockade of CB2 receptors with SR2, confirming the involvement of this type of cannabinoid receptor in the regulation of these heterodimeric cytokines. Interestingly, while CB2 receptor blockade completely reversed JWH-133 effects, it only partially reversed those of AEA. These observations are suggestive of the existence of alternative mechanisms for AEA actions and are in agreement with previous reports that have shown that AEA may exert its biological activity by mechanisms independent on cannabinoid receptors activation [26,34].

Next, we investigated whether human microglial cells, when activated by inflammatory stimuli like LPS/IFN-γ, express the different subunits p35, p40 and p19. Fig. 3A shows LPS/IFNγ stimulation for 18 h induces the mRNA expression of the three subunits in microglial cells. The effects of AEA on the production of the biologically active proteins IL-12p70 (p35/p40) and IL-23 (p19/p40) after LPS/IFN-γ stimulation of human

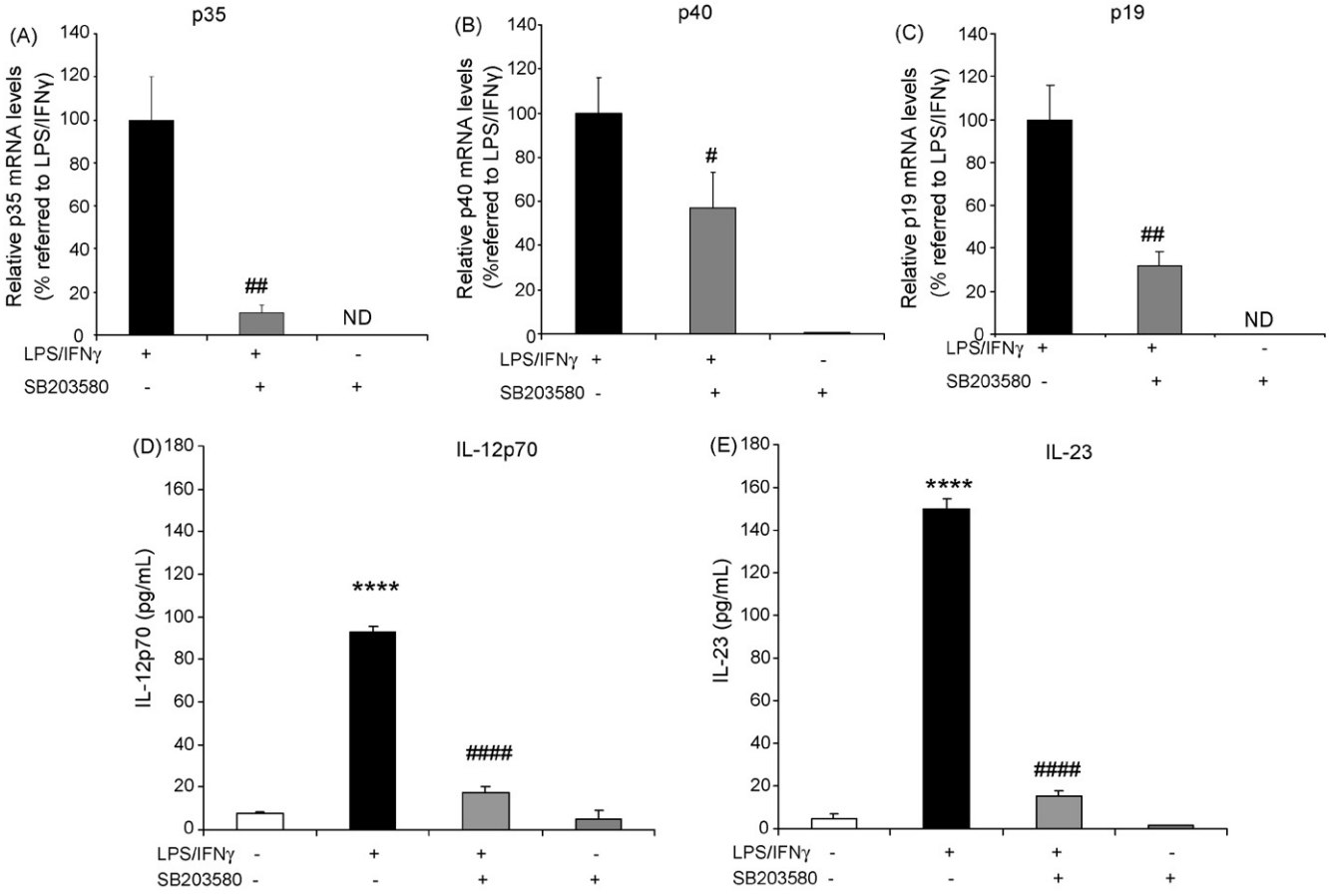

Fig. 4 – p38 MAPK is involved in the regulation of IL-12 and IL-23 subunits as well as the biologically active cytokines. LPS/IFNγ-induced upregulation of p35 (A), p40 (B) and p19 (C) subunits mRNA was reversed by the p38 MAPK specific inhibitor, SB203580 (25 μM), in murine microglial cells. mRNA levels were measured by real-time PCR and are expressed as ratios between p35, p40 or p19 and GAPDH mRNA levels. Data are plotted as percentage of the p35/GAPDH, p40/GAPDH or p19/GAPDH ratio obtained in LPS/IFNγ-stimulated conditions. Statistics: $^{\#}p < 0.05$ vs. LPS/IFNγ; $^{\#\#}p < 0.01$ vs. LPS/IFNγ; ND: non detectable (mean ± S.E.M., $n = 6$). p38 MAPK inhibition with SB203580 (25 μM) almost completely reversed the LPS/IFNγ upregulation of the biologically active cytokines IL-12p70 (D) and IL-23 (E). Protein levels were measured by ELISA in the culture supernatants at 18 h after stimulation. Statistics: $^{****}p < 0.001$ vs. control; $^{\#\#\#\#}p < 0.001$ vs. LPS/IFNγ (mean ± S.E.M., $n = 6$).

primary microglial cells are shown in Fig. 3B and C. Primary human microglial cells show the same profile of response than human microglial cells, therefore we concluded that in both cell types, AEA is capable of suppressing IL-12p70 and IL-23 via interaction with CB2 receptors.

3.2. *Role of p38MAP kinase on the production of IL-12p70 and IL-23 in LPS/IFN-γ-stimulated microglia: effects of anandamide*

Next, we addressed the question of which signalling pathway could be responsible for AEA-suppressed microglia production of IL-12p70 and IL-23 by LPS/IFN-γ. Activation of MAPK family members is known to play an important role in the transduction of LPS signals [29]. Therefore, using a pharmacological approach we first studied whether the specific inhibition of p38MAPK by the treatment with SB203580 modified the expression of mRNA for p19, p35 and p40 stimulated by LPS/IFN-γ as well as the production of IL-12p70 and IL-23 from murine microglia. For that purpose, cells were pre-treated with SB203580 (25 μM) for 30 min and then stimulated by LPS/IFN-γ. The pre-treatment with SB203580, significantly decreased the expression of p35 ($p < 0.001$), p40 ($p < 0.01$) and p19 ($p < 0.001$) mRNAs induced by LPS/IFNγ (Fig. 4A–C) as determined by real-time PCR. Accordingly, the biologically active cytokines IL-12p70 and IL-23 were dramatically reduced in the presence of the inhibitor of p38MAPK (Fig. 4D and E). Therefore, the activation of p38 MAPK appears to be critical for the LPS/IFNγ induction of both IL-12p70 and IL-23 production by microglial cells. To investigate whether AEA treatment may influence the above pathway and thus diminish IL-12/IL-23 cytokine production, we studied the time course of p38 MAPK phosphorylation after LPS/IFN-γ in the absence or presence of AEA. As shown in Fig. 5A, the maximum level of phosphorylation induced by LPS/IFN-γ, as studied by western immunobloting, was observed at 30–60 min before declining at 120 min. In the presence of AEA, phosphorylation of p38 MAPK was only slightly, but significantly,

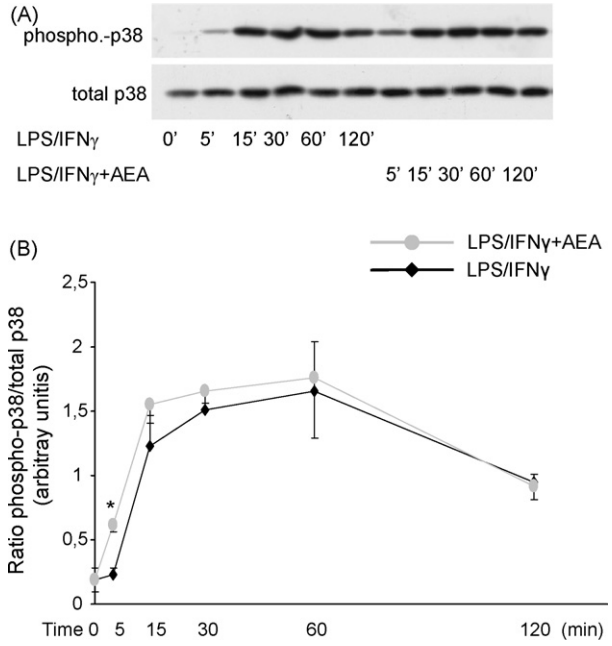

Fig. 5 – AEA does not interfere with LPS/IFNγ-induced p38 activation. Time-dependent p38 MAPK phosphorylation induced by LPS/IFNγ (50 ng/mL and 100 U/mL, respectively) and LPS/IFNγ + AEA (10 μM) after 0, 5, 15, 30, 60 and 120 min. AEA co-treatment did not modify p38 MAPK temporal course of phosphorylation nor its intensity. The amount of phosphorylated and total p38 MAPK was evaluated by immunoblotting. Immunoreactivity for phosphorylated p38 MAPK levels increased after 5 min of treatment and remained elevated at 120 min of treatment. The photograph shows a representative picture of 3 blots from three independent experiments (A). Densitometric analysis of p38 MAPK activation in murine microglia. Cells were treated with LPS/IFNγ and LPS/IFNγ + AEA after 0, 5, 15, 30, 60 and 120 min. Immunoreactivity for phosphorylated and total p38 MAPK, evidenced by immunoblotting, was quantified by densitometry. p38 MAPK phosphorylation was expressed as ratios between phosphorylated and total p38 MAPK intensity (Pp38/p38) (B). Data are plotted as ratio of Pp38/p38 obtained in each condition. Statistics: $^{*}p < 0.05$ vs. LPS/IFNγ (mean ± S.E.M., $n = 5$).

enhanced at 5 min ($p < 0.05$) (Fig. 5B). Therefore, our results suggest that AEA-induced suppressed IL-12p70 and IL-23 is not likely mediated through the p38 MAPK pathway.

3.3. *Role of JNK on the production of IL-12p70 and IL-23 in LPS/IFN-γ–activated microglia: effects of anandamide*

JNK MAPK pathway is strongly activated during cellular stress situations [35,36]. Thus, to determine whether the effect of LPS/IFN-γ on IL-12p70 and IL-23 production was due to JNK phosphorylation, we used the specific inhibitor SP600125 at a dose (10 μM) known to impair the phosphorylation of JNK [36]. The blockade of this pathway in activated cells increases mRNAs level expression for p35 ($p < 0.05$) and p19 ($p < 0.05$) subunits without modifying the p40 subunit expression (Fig. 6A–C). We evaluated the synthesis of the biological proteins and we observed that both cytokines, IL-12p70 and IL-23 were significantly increased by the treatment with SP600125 in the bathing medium of activated microglial cells (Fig. 6D and E). Together these results suggest that JNK activation negatively regulates IL-12p70 and IL-23 production in microglia. In this case, AEA co-treatment significantly potentiated LPS/IFNγ-induced phosphorylation of JNK at 15 min ($p < 0.01$) (Fig. 7A and B). This increase, in terms of an earlier activation of JNK, may contribute to the effects of AEA on IL-12/IL-23 regulation. Then, we studied whether activation of CB2 receptors was involved in AEA-mediated enhancement of JNK phosphorylation in LPS/IFN-γ activated microglial cells. Fig. 7C shows that the blockade of CB2 receptors by the selective antagonist SR2, significantly reverses AEA-induced enhancement of JNK phosphorylation at 15 min. To confirm the involvement of this signalling pathway on AEA effects on IL-12p70 and IL-23 production, we used the pharmacological inhibitor SP600125 in the presence of the endocannabinoid. As shown in Fig. 7D and E, when JNK MAPK signalling pathway is inhibited, AEA effects on IL-12p70 and IL-23 production is partially reversed ($p < 0.05$ and $p < 0.01$, respectively). These results suggest that JNK MAPK signalling is important for AEA regulation of heterodimeric cytokines.

3.4. *Role of MEK (ERK1/2 kinases) on the production of IL-12p70 and IL-23 in LPS/IFN-γ stimulated microglial cells: effects of anandamide*

Previous studies have shown that activation of ERK1/2 targets IL-12p40 transcription [36]. Here, we first investigated the effect of PD98059, a specific inhibitor of MEK, the upstream regulator of the ERK1/2 phosphorylation. Microglial cultures were pre-treated with PD98059 30 min before the addition of LPS/IFN-γ and mRNA levels for p35, p40 and p19 were evaluated by RT-PCR. The blockade of ERK1/2 promotes the expression of p35 and p40 subunits in activated cells in a significant level ($p < 0.01$) while p19 expression was significantly ($p < 0.05$) decreased (Fig. 8A–C). The level of production of IL-12p70 showed a similar pattern to their mRNA expression subunits, p35 and p40, and thus, significantly incremented after PD98059 treatment (Fig. 8D; $p < 0.05$). However, there was no a significant change in IL-23 protein production in PD98059-treated cells compared with LPS/IFN-γ-stimulated microglia (Fig. 8E). These results indicate that IL-12p70 and IL-23 are differentially regulated by ERK1/2 phosphorylation. As shown in Fig. 9A and B LPS/IFN-γ stimulation of microglial cells resulted in a rapid (at 5 min) increase of ERK1/2 phosphorylation, which was maximal at 30 min and declined toward basal levels within 60 min. In the presence of AEA, ERK1/2 phosphorylation was significantly higher and long-lasting than after LPS/IFN-γ alone. Activation of ERK1/2 remained significantly elevated at 60 min returning to basal levels at 120 min (Fig. 9B). By differentially regulating p35, p40 and p19 subunits, ERK1/2 pathway regulates in a negative way IL-12p70 production without modifying IL-23 synthesis. These findings might explain the regulatory effects of AEA on IL-12p70 production according to the idea that ERK acts as a negative

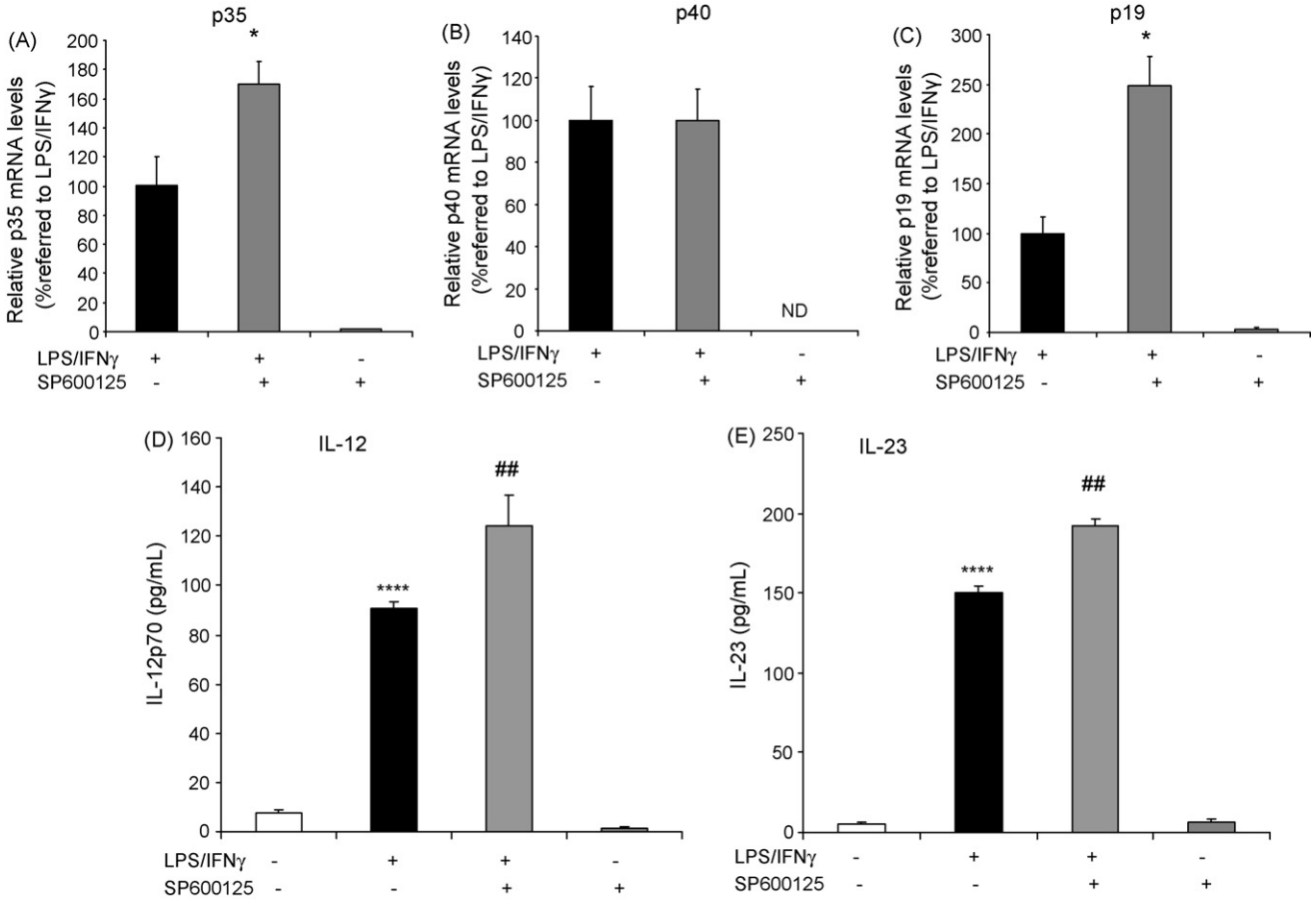

Fig. 6 – JNK MAPK is implicated in the regulation of p35 and p19 but not IL-p40 subunit mRNAs. Involvement of this signalling pathway in the production of biologically active IL-12p70 and IL-23. LPS/IFNγ-induced upregulation of p35 mRNA was potentiated by the JNK MAPK specific inhibitor, SP600125 (10 μM) in murine microglial cells. mRNA levels were measured by real-time PCR and are expressed as ratios between p35 and GAPDH. Data are plotted as percentage of the p35/GAPDH ratio obtained in LPS/IFNγ-stimulated conditions (A). Statistics: $^{*}p < 0.05$ vs. LPS/IFNγ (mean ± S.E.M., $n = 6$). Pharmacological inhibition of JNK MAPK pathway did not alter p40 mRNA expression in murine microglia. mRNA levels were measured by real-time RT-PCR and are expressed as ratios between p40 and GAPDH (B). Data are plotted as percentage of the p40/GAPDH ratio obtained in LPS/IFNγ-stimulated conditions (mean ± S.E.M.; $n = 6$). LPS/IFNγ-induced upregulation of p19 mRNA was potentiated by the JNK MAPK specific inhibitor, SP600125 (10 μM) in murine microglial cells. mRNA levels were measured by real-time RT-PCR and are expressed as ratios between p19 and GAPDH (C). Data are plotted as percentage of the p19/GAPDH ratio obtained in LPS/IFNγ-stimulated conditions. Statistics: $^{*}p < 0.05$ vs. LPS/IFNγ (mean ± S.E.M.; $n = 6$). Pharmacological inhibition of the JNK MAPK signalling pathway with SP600125 (10 μM) potentiated the production of IL-12p70 (D) and IL-23 (E) in activated microglia, measured by ELISA in the culture supernatants. Statistics: $^{****}p < 0.001$ vs. control; $^{\#\#}p < 0.01$ vs. LPS/IFNγ (mean ± S.E.M.; $n = 6$).

feedback regulator of p40 [23,37]. Next, we evaluated whether activation of CB2 receptors was involved in AEA-mediated enhancement of ERK1/2 phosphorylation in LPS/IFN-γ activated microglial cells. As shown in Fig. 9C, CB2 receptor blockade by the antagonist SR2 reverses the effects of AEA on the activation of ERK1/2 indicating that this pathway is activated following the interaction of AEA with the CB2 receptor. To further confirm the involvement of this signalling pathway on AEA effects on IL-12p70, we used the pharmacological inhibitor PD98059 in the presence of the endocannabinoid. As shown in Fig. 9D, when ERK1/2 MAPK signalling pathway is inhibited, AEA effects on IL-12p70 production is partially reversed ($p < 0.05$), indicating that, through the activation of ERK1/2, AEA differentially regulates the expression of heterodimeric cytokines.

4. Discussion

In the present study, we show that AEA inhibits LPS/IFNγ-induced production of the bioactive cytokines IL-12 (p35/p40) and IL-23 (p19/p40) in microglial cells by a mechanism that is partially dependent on CB2 receptor activation. Cells of the innate immune system such as dendritic cells, macrophages and microglial cells after pro-inflammatory stimulation, such as LPS treatment, are able to produce endocannabinoids

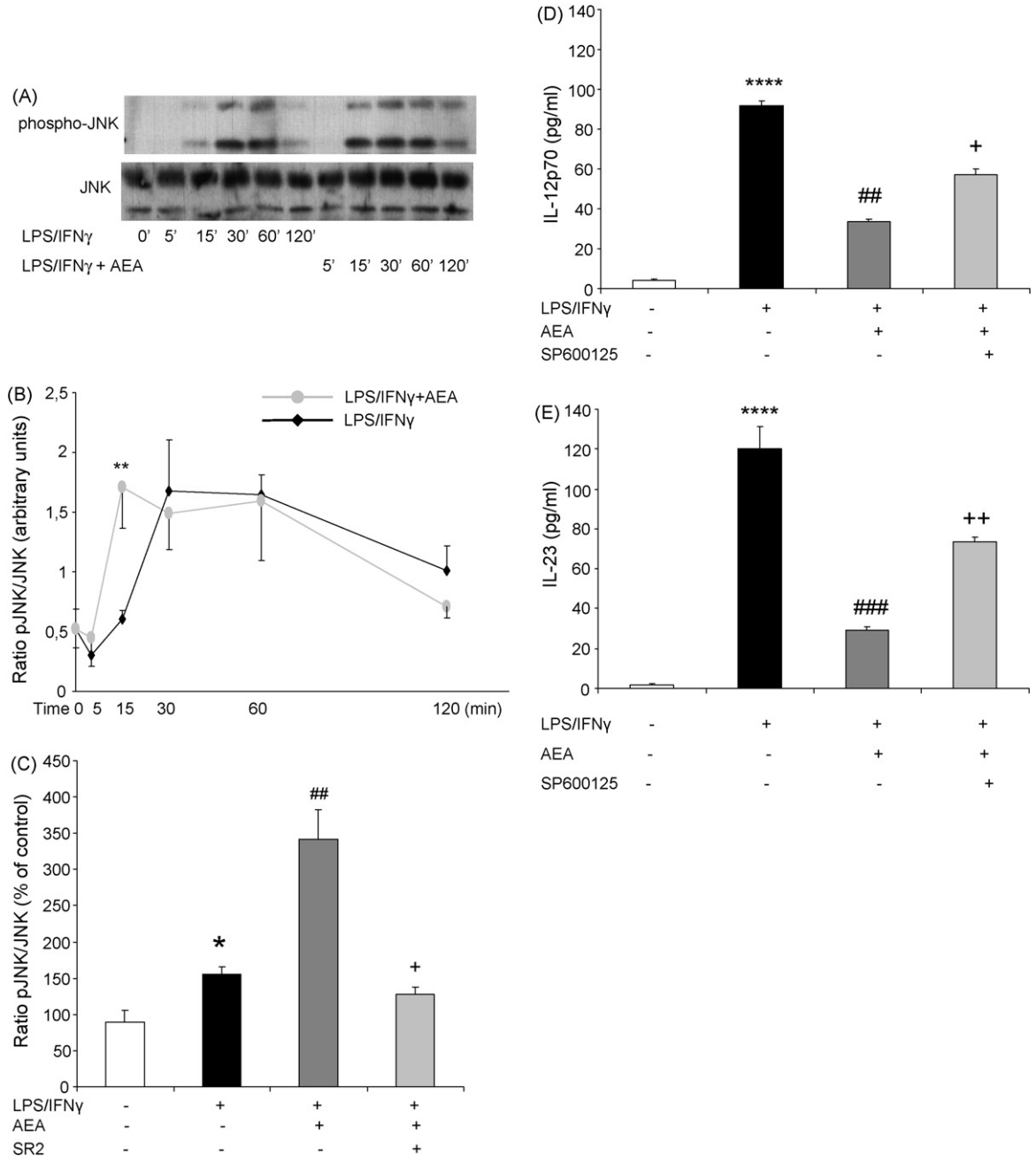

Fig. 7 – AEA co-treatment induces an earlier and longer activation of the JNK MAPK signalling pathway. Time-dependent pJNK MAPK phosphorylation induced by LPS/IFNγ (50 ng/mL and 100 U/mL, respectively) and LPS/IFNγ + AEA (10 μM) after 0, 5, 15, 30, 60 and 120 min. AEA co-treatment induced an earlier (15 min) and longer activation of the JNK MAPK pathway as evaluated by immunoblotting. Immunoreactivity for phosphorylated JNK MAPK levels increased after 15 min of treatment and remained elevated at 120 min of treatment. The photograph shows a representative picture of 3 blots from three independent experiments (A). Densitometric analysis of JNK MAPK activation in murine microglia. JNK MAPK phosphorylation was expressed as ratios between phosphorylated and total pJNK MAPK intensity (pJNK/JNK). Statistics: $^{**}p < 0.01$ vs. LPS/IFNγ 15 min (mean ± S.E.M.; $n = 5$) (B). Densitometric analysis of JNK MAPK activation in murine microglia. Cells were treated for 15 min with LPS/IFNγ (50 ng/mL and 100 U/mL, respectively), LPS/IFNγ + AEA (10 μM) or with LPS/IFNγ + AEA + SR2 (1 μM), a CB2 receptor antagonist. Immunoreactivity for phosphorylated and total JNK MAPK, evidenced by immunoblotting, was quantified by densitometry. Data are plotted as percentage of the pJNK/JNK ratio obtained in untreated conditions. CB2 receptor antagonism reversed AEA-induced potentiated phosphorylation of JNK MAPK. Statistics: $^{*}p < 0.05$ vs. control; $^{\#\#}p < 0.01$ vs. LPS/IFNγ; $^{+}p < 0.05$ vs. LPS/IFNγ + AEA (mean ± S.E.M.; $n = 5$) (C). Inhibition of JNK MAPK signalling pathway with SP600125 (10 μM) partially reversed AEA effects on IL-12p70 (D) and IL-23 (E) production in LPS/IFNγ stimulated microglia. Statistics: $^{****}p < 0.001$ vs. control; $^{\#\#}p < 0.01$ vs. LPS/IFNγ; $^{\#\#\#}p < 0.005$ vs. LPS/IFNγ; $^{+}p < 0.05$ vs. LPS/IFNγ + AEA; $^{++}p < 0.01$ vs. LPS/IFNγ + AEA (mean ± S.E.M.; $n = 6$).

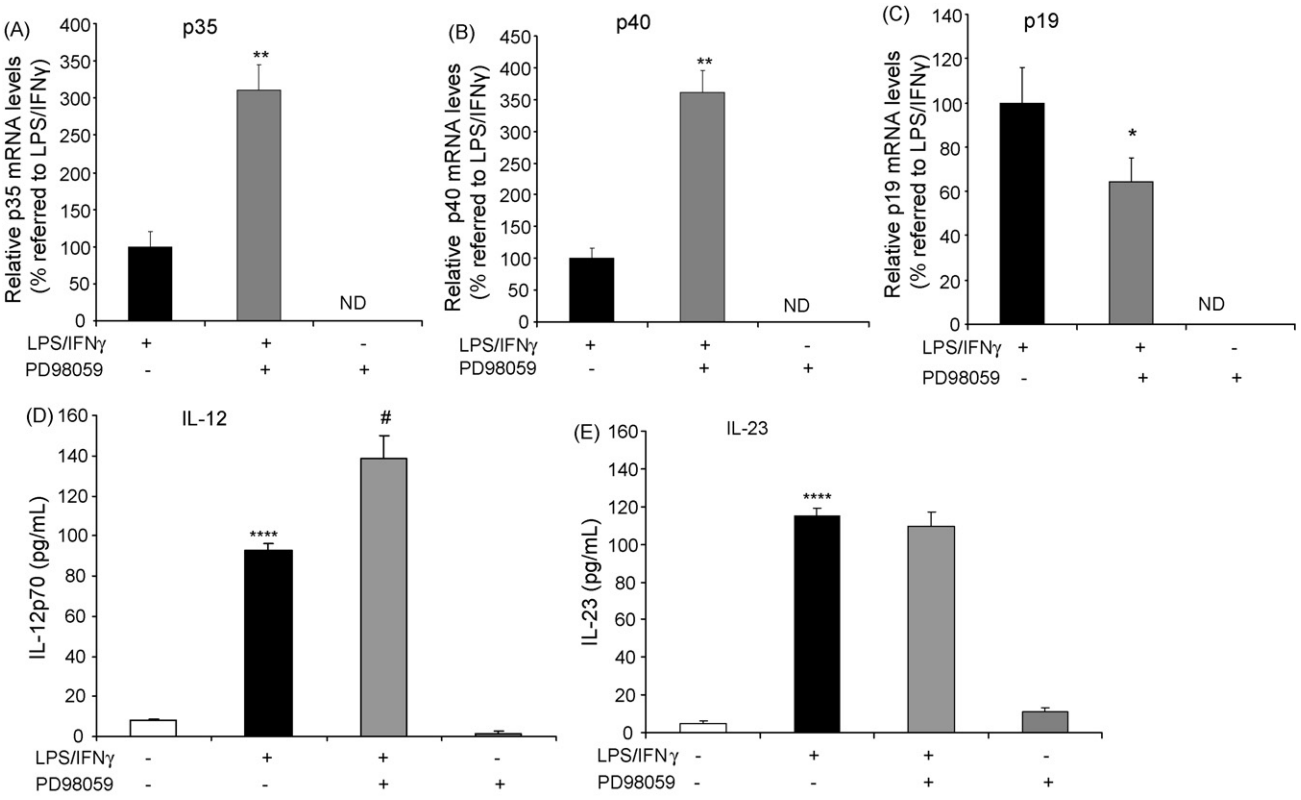

Fig. 8 – ERK1/2 MAPK negatively regulates p35 and p40 subunit mRNA expression and positively regulates p19 subunit mRNA expression. Implications of this signalling pathway in the production of the biologically active cytokines IL-12p70 and IL-23. LPS/IFNγ-induced upregulation of p35 mRNA expression was potentiated by the inhibition of ERK1/2 signalling pathway with PD98059 (10 μM) in murine microglial cells. mRNA levels were measured by real-time RT-PCR and are expressed as ratios between p35 and GAPDH (A). Data are plotted as percentage of the p35/GAPDH ratio obtained in LPS/IFNγ-stimulated conditions. Statistics: $^{**}p < 0.01$ vs. LPS/IFNγ; ND: non detectable (mean ± S.E.M.; $n = 6$). Pharmacological inhibition of ERK1/2 MAPK pathway with PD98059 (10 μM) potentiated LPS/IFNγ-induced upregulation of p40 mRNA expression in murine microglia. mRNA levels were measured by real-time PCR and are expressed as ratios between p40 and GAPDH (B). Data are plotted as percentage of the p40/GAPDH ratio obtained in LPS/IFNγ-stimulated conditions. Statistics: $^{**}p < 0.01$ vs. LPS/IFNγ; ND: non detectable (mean ± S.E.M.; $n = 6$). LPS/IFNγ-induced upregulation of p19 mRNA expression was reversed by the pharmacological inhibition of ERK1/2 signalling pathway with PD98059 (10 μM) in murine microglial cells. mRNA levels were measured by real-time PCR and are expressed as ratios between p19 and GAPDH (C). Data are plotted as percentage of the p19/GAPDH ratio obtained in LPS/IFNγ-stimulated conditions. Statistics: $^{*}p < 0.05$ vs. LPS/IFNγ; ND: non detectable (mean ± S.E.M.; $n = 6$). Pharmacological inhibition of ERK1/2 phosphorylation resulted in a potentiation of the LPS/IFNγ-induced IL-12p70 production, measured by ELISA in culture supernatants after 18 h of stimulation (D). Statistics: $^{****}p < 0.001$ vs. control; $^{\#}p < 0.05$ vs. LPS/IFNγ (mean ± S.E.M.; $n = 6$). Pharmacological inhibition of ERK1/2 phosphorylation did not modify LPS/IFNγ-induced IL-23 production, measured by ELISA in the culture supernatants 18 h post-stimulation (E). Statistics: $^{****}p < 0.001$ vs. control (mean ± S.E.M.; $n = 6$).

[20,38,39]. Within the CNS the endocannabinoid AEA has been shown to be increased in inflammatory lesions of patients with active MS [12]. AEA was also increased in the CSF of relapsing MS patients as well as in peripheral lymphocytes of these patients [40] whereas the endocannabinoid 2-AG remained unchanged. Therefore, AEA seems to be preferentially engaged during CNS inflammatory diseases and cumulative evidence highlight its role as a modulator of the immune response during both MS and animal models of MS [12,20,21]. Microglia can activate naïve T cells or reactivate myelin-specific T cells. Microglia cells also express functional CB receptors and the activation of CB2 receptors has been related to the inhibition of several pro-inflammatory genes which are critical for the maintenance of CNS inflammation [41], such as we observed in the case of IL-12p70 and IL-23. The heterodimeric cytokine IL-12p70 has long been considered important in the pathogenesis of MS [42]. The first study about the subunit p40 described that it was produced by macrophages/microglia in MS plaques and that its levels correlate with disease severity [43]. However, more recent results support a critical role of IL-23 in the development of EAE model of MS [4,5,44]. In line with these observations, a recent study reported the expression of IL-23 in active MS lesions and identified activated macrophages/microglia as the cellular sources of IL-23p19 in the lesions [27]. In the present study, we have characterized the reduction of IL-12p70 and IL-23 production in activated microglia by AEA that

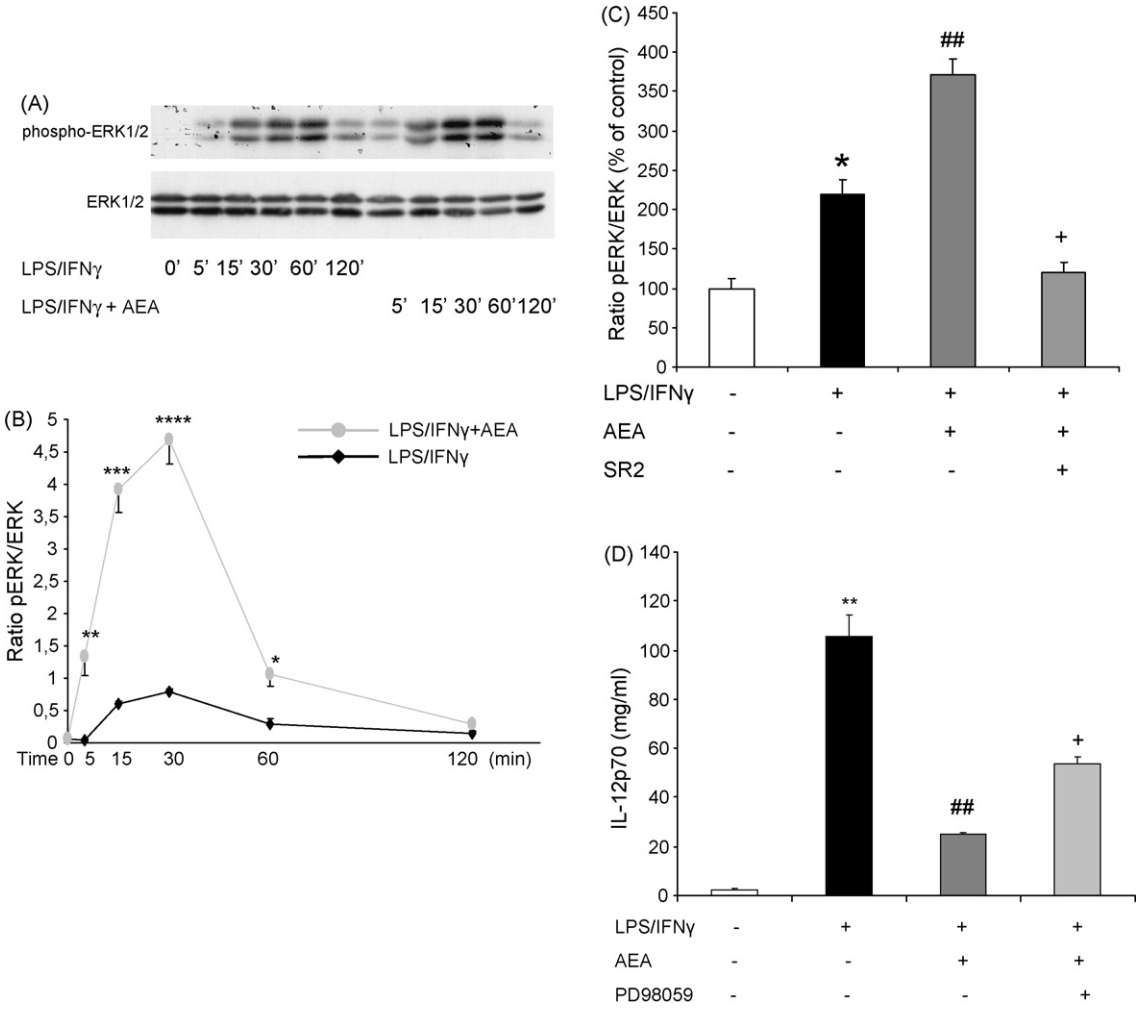

Fig. 9 – AEA co-treatment induces a greater phosphorylation of ERK1/2 MAPK pathway maintaining the same temporal pattern of activation. (A) Time-dependent pERK1/2 MAPK phosphorylation induced by LPS/IFNγ (50 ng/mL and 100 U/mL, respectively) and LPS/IFNγ + AEA (10 μM) after 0, 5, 15, 30, 60 and 120 min. AEA co-treatment induced a greater phosphorylation of the ERK1/2 MAPK pathway with the same temporal pattern of activation, as evaluated by immunobloting. Immunoreactivity for phosphorylated ERK MAPK levels increased after 15 min of treatment and returned to basal levels at 120 min of treatment. The photograph shows a representative picture of 3 blots from three independent experiments. (B) Densitometric analysis of ERK1/2 MAPK activation in murine microglia. ERK1/2 MAPK phosphorylation was expressed as ratios between phosphorylated and total ERK1/2 MAPK intensity (pERK1/2/ERK1/2). Statistics: $^{*}p < 0.05$ vs. LPS/IFNγ 60 min; $^{**}p < 0.01$ vs. LPS/IFNγ 5 min; $^{***}p < 0.005$ vs. LPS/IFNγ 15 min; $^{****}p < 0.001$vs.LPS/IFNγ 30 min (mean ± S.E.M.; $n = 6$). (C) Densitometric analysis of ERK1/2 MAPK activation in murine microglia. Cells were treated for 30 min with LPS/IFNγ (50 ng/mL and 100 U/mL, respectively), LPS/IFNγ + AEA (10 μM) or with LPS/IFNγ + AEA + SR2 (1 μM), a CB2 receptor antagonist. Immunoreactivity for phosphorylated and total ERK1/2 MAPK, evidenced by immunoblotting, was quantified by densitometry. ERK1/2 MAPK phosphorylation was expressed as ratios between phosphorylated and total ERK1/2 MAPK intensity (pERK1/2/ERK1/2). Data are plotted as percentage of the pERK1/2/ERK1/2 ratio obtained in untreated conditions. CB2 receptor antagonism reversed AEA-induced potentiated phosphorylation of ERK1/2 MAPK. Statistics: $^{*}p < 0.05$ vs. control; $^{\#\#}p < 0.01$ vs. LPS/IFNγ; $^{+}p < 0.05$ vs. LPS/IFNγ + AEA (mean ± S.E.M.; $n = 6$). (D) Inhibition of ERK1/2 MAPK signalling pathway with PD98059 (10 μM) partially reversed the effects of AEA on IL-12p70 production in LPS/IFNγ stimulated microglia. Statistics: $^{**}p < 0.01$ vs. control; $^{\#\#}p < 0.01$ vs. LPS/IFNγ; $^{+}p < 0.05$ vs. LPS/IFNγ + AEA (mean ± S.E.M.; $n = 6$).

involves the activation of CB2 receptors. MAPK signalling cascades are important in both innate and acquired immune response [28] and the three families of protein kinases have been previously involved in the process of activation of macrophages for the generation of IL-12 and other family-related cytokines [36,45,46].

Using a specific inhibitor of p38 MAP kinase we showed that blocking this signalling pathway significantly reduced p19, p35 and p40 expression in activated microglia, and consequently the generation of the biological active cytokines, IL-12p70 and IL-23. The results herein are consistent with a previous study which demonstrated that blocking p38 MAP kinase reduced

p19 expression in microglia [47] as well as p40 subunit mRNA expression in LPS-stimulated macrophages and monocytes [30], suggesting that p38 activity promotes IL-12p70 and IL-23 production. However, AEA does not seem to interfere with this pathway since time-courses of p38 phosphorylation showed similar profiles of activation in the presence or absence of AEA. Several studies have assessed the inhibitory roles of ERK1/2 and JNK in the IL-12p70 regulation, mainly by determining their effects on p40 subunit gene transcription in macrophages, monocytes and dendritic cells [29,35–37]. Our results suggest that the selective blockade of the JNK pathway promoted the expression of the subunits p19 and p35 without modifying p40, and this led to significant increments in IL-12p70 and IL-23 by activated microglia. This finding means that the JNK pathway is implicated in the negative regulation of both cytokines, IL-12p70 and IL-23. By contrast, activation of ERK1/2 signalling differentially affected mRNA expression of the units forming IL-12p70 and IL-23, as the pharmacological blockade of ERK1/2 phosphorylation enhanced the expression of subunits, p40 and p35, but decreased p19 mRNA levels. Analyzing protein levels in activated microglia, ERK1/2 inhibition drives an increase of IL-12p70 whereas IL-23 levels were unmodified. Therefore, the level of induction of the individual cytokine subunits is dependent on the activation of differential regulatory pathways and this would play a key role in determining the precise phenotype of the resulting immune response and/or disease pathogenesis. In this context, it is important to highlight that the inhibitory effects of AEA on IL-12p70 and IL-23 induction involves both ERK 1/2 and JNK MAPK pathways. Here, we have found that AEA enhanced early JNK phosphorylation (10–15 min) and potentiated ERK1/2 phosphorylation in a more lasting way (5–60 min) in activated microglial cells. Cannabinoid receptors, mainly CB1, have been shown to be coupled to the activation of ERK1/2 [48], of c-JNK and p38 MAPK [49,50] as well as to the activation of PI3K/Akt pathway [51]. Transduction systems responsible for CB2 receptor signalling are less known. In a previous study [12] AEA was shown to protect neurons from microglial inflammatory damage by CB1 and CB2 receptor-mediated induction of mitogen-activated-protein kinase phosphatase-1 (MKP-1). In this study we demonstrated that activation of CB2 receptors mediated phosphorylation of ERK1/2 and JNK and that this was involved in the inhibition of IL-12p70 and IL-23 in activated microglia. Therefore, AEA targeting microglia leads to the blockade of essential cytokines that favor Th1 responses in the case of IL-12p70 or Th-17 cell expansion/maintenance in the case of IL-23 [52]. This is especially relevant since microglia are the major APCs in the brain and are involved in the reactivation of infiltrating T cells [53]. Taken together, the findings of the present study may contribute to a better understanding of the events involved in the reduction of inflammatory damage that is observed by activating the endocannabinoid system in animal models of MS.

Acknowledgments

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