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Target regulation of PI3K/Akt/mTOR pathway by cannabidiol in treatment of experimental multiple sclerosis

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ABSTRACT

This study was aimed to investigate whether treatment with purified cannabidiol (CBD) may counteract the development of experimental multiple sclerosis (MS), by targeting the PI3K/Akt/mTOR pathway. Although the PI3K/Akt/mTOR pathway was found to be activated by cannabinoids in several immune and non-immune cells, currently, there is no data about the effects of CBD in the PI3K/Akt/mTOR activity in MS.

Experimental Autoimmune Encephalomyelitis (EAE), the most common model of MS, was induced in C57BL/6 mice by immunization with myelin oligodendroglial glycoprotein peptide $(MOG)_{35–55}$. After EAE onset, which occurs approximately 14 days after disease induction, mice were daily intraperitoneally treated with CBD (10 mg/kg mouse) and observed for clinical signs of EAE. At 28 days from EAE-induction, mice were euthanized and spinal cord tissues were sampled to perform immunohistochemical evaluations and western blot analysis.

Our results showed a clear downregulation of the PI3K/Akt/mTOR pathway following EAE induction. CBD treatment was able to restore it, increasing significantly the phosphorylation of PI3K, Akt and mTOR. Also, an increased level of BNDF in CBD-treated mice seems to be involved in the activation of PI3K/Akt/mTOR pathway. In addition, our data demonstrated that therapeutic efficacy of CBD treatment is due to reduction of pro-inflammatory cytokines, like IFN-γ and IL-17 together with an up-regulation of PPARγ. Finally, CBD was found to promote neuronal survival by inhibiting JNK and p38 MAP kinases.

These results provide an interesting discovery about the regulation of the PI3K/Akt/mTOR pathway by cannabidiol administration, that could be a new potential therapeutic target for MS management.

1. Introduction

Cannabinoids, the secondary metabolites produced by the plant *Cannabis sativa*, are well known for their anti-inflammatory properties [1,2]. Among these, Cannabidiol (CBD), has drawn in the last years considerable interest in the treatment of a range of neurological disorders [3,4]. Recent reports show the effectiveness of CBD, for its anti-inflammatory as well as immunosuppressive properties, in several animal models of diseases with inflammatory background like multiple sclerosis (MS) [1,5–7].

MS is a neurodegenerative inflammatory disease of unknown trigger and complex pathology that involves myelin degradation and alteration in central nervous system (CNS) functions. Several lines of evidence recognize an aberrant autoimmune response in which T and B lymphocytes destroy myelin of neurons as the main etiopathogenetic event of MS development [8,9]. This causes also inflammatory lesions in the CNS and leads to the loss of oligodendroglia and axonal degeneration [10]. Sativex®(GW Pharma, LTd, Salisbury, Wiltshire, UK), a mixture of two cannabinoid extracts in approximately a 1:1 ratio (2.7 mg of Δ^9-THC and 2.5 mg of CBD in an alcoholic solution), is to date the only commercially available preparation containing cannabinoids introduced in clinical management of symptomatic treatment of chronic pain and spasticity in MS patients which did not show an appropriate response to other drugs during an initial trial period of therapy [11,12]. Although this drug has been approved in several countries, the limits regarding unavoidable psychotropic effects exhibited by Δ^9-THC remains to be overcome. Therefore, the interest of researchers is focused on non-psychotropic compound, like CBD. Recently, we and other research groups demonstrated that CBD is able to ameliorate CNS neuroinflammation and demyelination in mouse experimental autoimmune encephalomyelitis (EAE), an induced model of MS [5,13–17]. Moreover, it was established that CBD treatment modulates many intracellular pathways associated to EAE/MS etiopathology, by improving the severity of clinical signs of disease together with a decrease of pro-inflammatory cytokines production and by counteracting neuronal apoptosis [13,18]. Moreover, CBD has proved to inhibit immune cell proliferation, activation, maturation, migration and antigen presentation regulating in this

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way the immune cell functions [19]. However, the mechanisms of these beneficial protective activities of CBD are not yet completely understood. Some of effects exerted by cannabinoid compounds could be linked to the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B)/mammalian target of rapamycin (mTOR) pathway [20]. Cannabinoids indeed exert its anti-inflammatory function *via* endogenous receptors, such as cannabinoid receptor 1 (CB1), cannabinoid receptor 2 (CB2), transient receptor potential vanilloid receptor 1 (TRPV1) and G protein-coupled receptor, like GPR55, GPR18 and GPR119 [21]. As confirmed by *in vitro* studies cannabinoids can activate the PI3K/Akt/mTOR pathway by binding CB1 receptor, found on neurons and glial cells, and in less manner CB2 ones, found mainly in the body's immune system [22,23].

The PI3K/Akt/mTOR signaling is involved in a wide spectrum of cellular signaling pathways [24,25]. It plays a central role in regulating inflammation, and abnormalities in this pathway could be linked to the development of autoimmunity [26,27]. It is also involved in cellular proliferation, survival and differentiation [28]. Although PI3K/Akt/mTOR pathway has been proven to be target by cannabinoids in several immune and non-immune cells [1,29], there is currently almost no data about the effects of cannabinoids in the activity of this signaling pathway in inflammatory as well as autoimmune conditions such as MS [30]. In this regard, the present study was aimed to investigate whether treatment with purified CBD may counteract the development of experimental MS, by targeting the PI3K/Akt/mTOR pathway.

2. Material and methods

2.1. Plant material and treatment

Cannabis sativa L. was collected from greenhouse cultivation at CRA-CIN, Rovigo (Italy). The isolation and purification of cannabinoids was done according to their legal status (Authorization SP/106 23/05/2013 of the Ministry of Health, Rome, Italy). Pure CBD (>99%) was isolated from the tops of an Italian variety of industrial hemp (named Carmagnola) in accordance with to a standardized method [31] of the cannabinoid purification to avoid any trace of THC that could interfere in the trial or cause legal limitation.

CBD (10 mg/kg/mouse) was diluted in vehicle solution (ethanol, Tween-20 and saline at 1:1:8 ratio) and intraperitoneally administered in mice according body weight.

2.2. Animals

Male C57BL/6 mice (Harlan Milan, Italy) 12 weeks of age and weighing 20–25 g were housed in individually ventilated cages with food and water *ad libitum*. The room was maintained at a constant temperature and humidity on a 12 h/12 h light/dark cycle.

2.3. Ethics statement

Animals were cared in accordance with the European Organization Guidelines for Animal Welfare. The protocol was approved by the Ministry of Health "General Direction of animal health and veterinary drug" (Authorization 150/2014-B 28/03/2014). Particularly, animal care was in compliance with Italian regulations on the protection of animals used for experimental and other scientific purposes (D.lgs 26/2014). All efforts were made during experimental procedures, to minimize animal suffering and also to reduce the number of animal used.

2.4. Induction of experimental autoimmune encephalomyelitis (EAE)

For this study, male C57BL/6 have been chosen since several studies have demonstrated that gender does not influence the incidence and disease course of EAE [32,33]. After anesthesia, induced with an anesthetic cocktail composed of tiletamine plus xylazine (10 ml/kg, ip), EAE was actively induced using Myelin Oligodendrocyte Glycoprotein peptide $(MOG)_{35-55}$ (MEVGWYRSPFSRVVHLYRNGK; % peak area by HPLC ≥ 95, AnaSpec, EGT Corporate Headquarters, Fremont, *CA*, USA), according to Paschalidis et al. [34]. Mice were immunized subcutaneously with 300 μl/flank of the emulsion consisting of 300 μg of $(MOG)_{35-55}$ in phosphate-buffered saline (PBS) mixed with an equal volume of Complete Freund's Adjuvant (CFA) containing 300 μg heat-killed *M. tubercolosis* H37Ra (Difco Laboratories Sparks, MD, USA). Immediately after $(MOG)_{35-55}$ injection, the animals received an ip injection of 100 μl of *B. pertussis* toxin (Sigma-Aldrich, Milan, Italy) (500 ng/100 μl, i.p), repeated 48 h later. Approximately 14 days after EAE induction, mice show the first signs of disease, characterized by loss of tail tonus and hind limb paralysis and body weight loss.

2.5. Experimental animal groups

Mice were randomly separated into the following groups (N = 25 total animals):

1. Naive group (N = 5): mice did not receive $(MOG)_{35-55}$ or other treatment;
2. EAE group (N = 10): mice subjected to EAE as described above;
3. EAE + CBD group (N = 10): mice subjected to EAE were treated with CBD (10 mg/kg dissolved in ethanol, Tween-20 and saline at 1:1:8 ratio).

CBD was daily injected (i.p.) into $(MOG)_{35-55}$-immunized mice immediately after the onset of disease signs (around 14 days after immunization) and the treatment was daily protracted until the sacrifice.

Here, animals of the naive + CBD vehicle group have not been provided because in our previous studies we did not found any effects due to vehicle solution injection alone by using another experimental set of mice [13]. For this reason and also to minimize the number of animals used for experiment, we have decided not include this group in experimental design.

At the end of the experiment, which occurred at the 28th day from EAE-induction, animals were euthanized with ip of Tanax (5 ml/kg body weight) and spinal cord tissues were sampled and processed in order to evaluate parameters of disease.

2.6. Clinical disease score

Mice were daily observed for signs of EAE. Disease severity was evaluated with a 0–6 scoring system, according to Rodrigues et al. [35] with 0 representing no disease and 6 representing death due to EAE. In detail, the signs of EAE were scored as follows: 0 = no signs; 1 = partial flaccid tail; 2 = complete flaccid tail; 3 = hind limb hypotonia; 4 = partial hind limb paralysis; 5 = complete hind limb paralysis; 6 = moribund or dead animal. Animals with a score ≥ 5 were sacrificed to avoid animal suffering. The measure of clinical disease score has been expressed compared to day of EAE induction (day zero). The value day has been expressed as mean ± SEM of all animals for each experimental group.

2.7. Light microscopy

At 28 days EAE-induction, spinal cord issues were sampled and fixed in 10% (w/v) in PBS-buffered formaldehyde, embedded in paraffin and then cut into sections 7 μm thick. The sections were deparaffinized with xylene, rehydrated, and stained with H&E to be studied by optical microscope (Leica microscope ICC50HD).

2.8. Immunohistochemistry on mice spinal cord tissues

Spinal cord tissues were fixed in 10% (w/v) PBS-buffered formaldehyde, and 6-μm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min.

Nonspecific adsorption was minimized by incubating sections in 2% (v/v) normal goat serum in PBS for 20 min.

Sections were incubated overnight with:

- anti-IFN-γ monoclonal antibody (1:100 in PBS v/v; Santa Cruz Biotechnology, Inc);
- anti-IL-17 polyclonal antibody (1:100 in PBS v/v; Santa Cruz Biotechnology, Inc).

Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with biotin and avidin (DBA, Milan, Italy), respectively. Tissue sections were rinsed with PBS and incubated with secondary antibody. Specific labeling was performed using a biotin-conjugated anti-rabbit IgG and avidin–biotin peroxidase complex (Vectastain ABC kit, Denmark). Then, the tissue sections were stained with DAB peroxidase substrate kit (Vector Laboratories, USA) followed by hematoxylin counterstaining. In addition, tissue sections were incubated with either primary or secondary antibody to assess antibody specificity. In these cases, no positive staining was observed in the tissue sections, indicating that the immunoreactions were positive in all the experiments carried out. Immunohistochemical staining was evaluated using light microscopy (LEICA DM 2000 combined with LEICA ICC50 HD camera) and images were acquired by Leica Application Suite V4.2.0 software to perform densitometric analysis. All images are representative of three independent experiments.

2.9. Western blot analysis

All the extraction procedures were performed on ice using ice-cold reagents. In brief, spinal cord tissues were suspended in extraction buffer containing 0.32 M sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 2 mM EDTA, 5 mM NaN3, 10 mM 2-mercaptoethanol, 50 mM NaF, protease inhibitor tablets (Roche Applied Science, Monza, Italy), and they were homogenized at the highest setting for 2 min. The homogenates were chilled on ice for 15 min and then centrifuged at 1000*g* for 10 min at 4 °C, and the supernatant was collected to evaluate content of cytoplasmic proteins. The pellets were suspended in the supplied complete lysis buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA protease inhibitors (Roche), and then were centrifuged for 30 min at 15,000 *g* at 4 °C. Then, supernatant containing nuclear extract was collected to evaluate the content of nuclear proteins. Supernatants were stored at −80 °C until use. Protein concentration in homogenate was estimated by Bio-Rad Protein Assay (Bio-Rad, Segrate, Italy) using BSA as standard, and 20 μg of cytosol and nuclear extract from each sample were analyzed. Proteins were separated on sodium dodecyl sulfate-polyacrylamide minigels and transferred onto PVDF membranes (Immobilon-P Transfer membrane, Millipore), blocked with PBS containing 5% nonfat dried milk (PBS-milk (PM)) for 45 min at room temperature, and subsequently probed at 4 °C overnight with specific antibodies, phospho-PI3Kinase (1:750 Cell Signaling Technology); PI3Kinase (1:1000; Cell Signaling Technology); phospho-Akt (1:750 Cell Signaling Technology); Akt (1:1000; Cell Signaling Technology); phospho-mTOR (1:750 Cell Signaling Technology), mTOR (1:1000; Cell Signaling Technology); phospho-S6 Ribosomal protein kinase (1:2000; Cell Signaling Technology); S6 Ribosomal protein kinase (1:1000; Cell Signaling Technology); JNK (1:250 Santa Cruz Biotechnology, USA); phospho-p38 MAP kinase (1:750; Cell Signaling Technology); p38 MAPK (1:1000; Cell Signaling Technology); PPARγ (1:250 Santa Cruz Biotechnology, USA); BDNF (1:250 Santa Cruz Biotechnology, USA) in 1× PBS, 5% (w/v) non-fat dried milk, 0.1% Tween-20 (PMT). HRP-conjugated goat anti-mouse IgG, HRP-conjugated goat anti-rabbit IgG or HRP-conjugated chicken anti-rat were incubated as secondary antibody (1:2000; Santa Cruz Biotechnology Inc) for 1 h at room temperature. To ascertain that blots were loaded with equal amounts of protein lysates, they were also incubated with antibody for GAPDH HRP Conjugated (1:1000; Cell Signaling

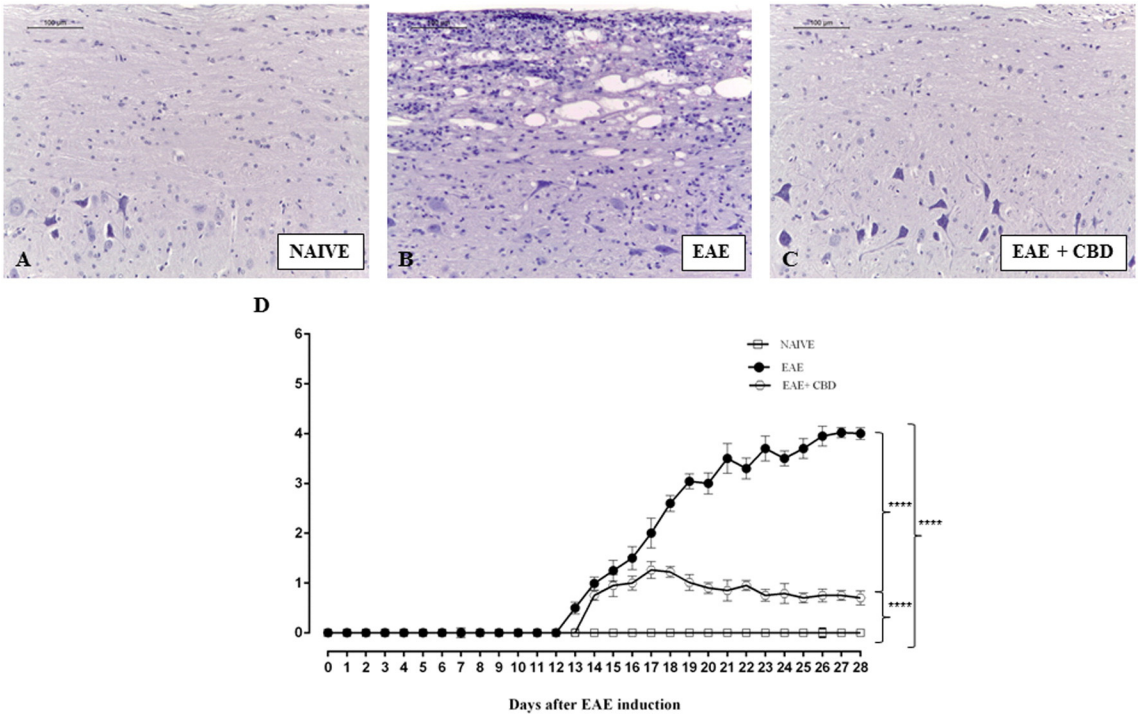

Fig. 1. Eosin/hematoxylin (E/H) staining. Naive mice (A) did not show histological alterations in the spinal cord tissues, whereas EAE mice (B) showed a wide area of infiltrating cells in the white matter of spinal cord. Conversely, treatment with CBD (C) led to a complete resolution of inflammatory cells infiltration. Clinical score (D). After EAE induction, mice were daily monitored for clinical disease score. Naive mice did not display motor deficit. EAE mice exhibited an high score of disease with a mean of 4.02, whereas mice treated with CBD displayed a lower grade of disease severity with a mean score of 0.75. The measure of clinical disease score is expressed as mean ± SEM of all measurements of each experimental group. One way-analysis of variance with Bonferroni test were used to determine the statistical significance of differences. Naive *vs* EAE $^{****}p < 0.0001$; Naive *vs* EAE + CBD $^{****}p < 0.0001$; EAE *vs* EAE + CBD $^{****}p < 0.0001$.

Technology). The relative expression of protein bands, was visualized using an enhanced chemiluminescence system (Luminata Western HRP Substrates, Millipore) and protein bands were acquired and quantified with ChemiDoc™ MP System (Bio-Rad) and a computer program (ImageJ software) respectively. Blots are representative of three separate and reproducible experiments. The statistical analysis was performed on three repeated blots obtained from separate experiments.

2.10. Statistical evaluation

Data were analyzed with GraphPad Prism version 6.0 program (GraphPad Software, La Jolla, *CA*). The results were statistically analyzed using one-way ANOVA followed by a Bonferroni *post hoc* test for multiple comparisons. A p value less than or equal to 0.05 was considered significant. Results are expressed N ± SEM of n experiments.

3. Results

3.1. CBD treatment improves the characteristic signs of disease in EAE mice

EAE is a well-documented animal model of MS, which reproduces similar clinical (*i.e.* paralysis and body weight loss) and pathologic (*i.e.* demyelination, infiltration of inflammatory cells into the CNS) features to human MS [36,37]. This model allows to easily evaluate the typical inflammatory frame occurring mainly in the spinal cord, which in turn resulted in chronic demyelination. In this context, histological evaluation of spinal cord sections showed evident differences in experimental groups. More in detail, no histologic changes were found in the tissues of the spinal cord taken from naive mice (Fig. 1 A), whereas a wide area of infiltrating inflammatory cells was observed in the white matter of the spinal cord of EAE mice (Fig. 1 B). Thus, CBD treatment improved the histological EAE score by attenuating infiltration of inflammatory cells, suggesting a protective effect in CNS tissues (Fig. 1 C). In parallel, the improvement of histological damage coincides with an improvement of the clinical score, assessed as parameters of disease. Indeed, $(MOG)_{35-55}$-immunized mice exhibit paralysis of the limbs resulting in loss of muscle mass, as proven by an high score of disease (mean 4.02 ± 0.351). On the contrary significant reduction in the clinical score was observed in EAE mice treated with CBD (mean 0.75 ± 0.123) (Fig. 1 D).

3.2. CBD treatment mediates activation of PI3K/Akt/mTOR pathway

Western blot analysis was performed in order to investigate the modulation of the PI3K/Akt/mTOR signaling pathway at 28 days after EAE induction in mice spinal cord. Specifically, we focused on the phosphorylation status of PI3K/Akt/mTOR as its activation is mediated by phosphorylation of the proteins involved. Achieved results clearly showed a downregulation of the PI3K/Akt/mTOR pathway in EAE mice. In detail, a lower expression of p-PI3K (Fig. 2 A), p-AKT (Fig. 2 B), and p-mTOR (Fig. 2 C) was found in spinal cord tissues taken from EAE mice compared to naive ones. On the contrary, a significant increase of p-PI3K, p-AKT, and p-mTOR was observed in EAE + CBD group. Non-phosphorylated PI3K, Akt and TOR proteins were not different between naive, EAE and EAE + CBD. In addition, to confirm activation of p-mTOR, we investigated the status of critical downstream mTOR substrates, particularly ribosomal protein S6 kinase (S6 K) (Fig. 2 D). It was found

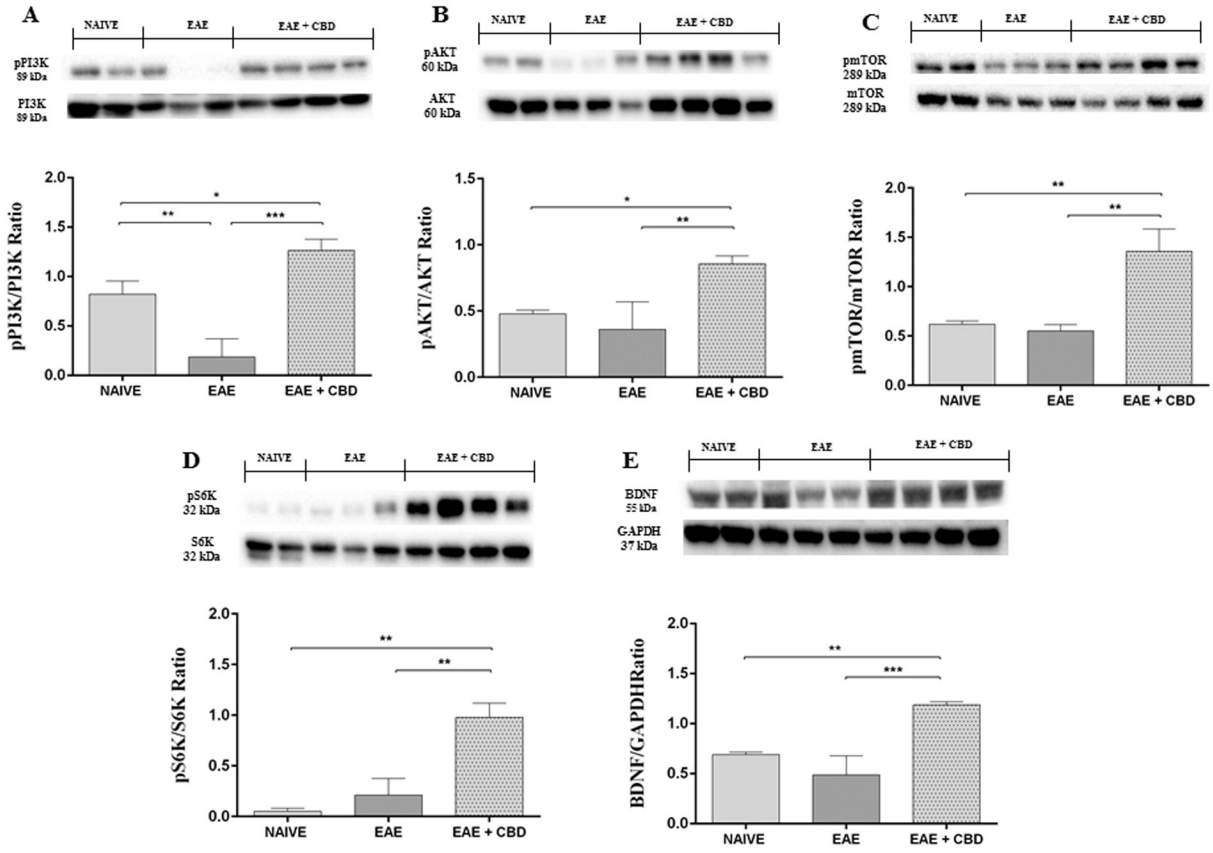

Fig. 2. Western blot analysis for pPI3K (A). Naive *vs* EAE, $^{**}p = 0.0008$; Naive *vs* EAE + CBD, $^{*}p = 0.0374$; EAE *vs* EAE + CBD, $^{***}p = 0.0002$. Western blot for pAKT (B). Naive *vs* EAE + CBD, $^{*}p = 0.0404$; EAE *vs* EAE + CBD, $^{**}p = 0.0065$. Western blot for pmTOR (C). Naive *vs* EAE + CBD, $^{**}p = 0.0059$; EAE *vs* EAE + CBD, $^{**}p = 0.0020$. Western blot for pS6 K (D). Naive *vs* EAE + CBD, $^{**}p = 0.0021$; EAE *vs* EAE + CBD, $^{**}p = 0.0030$. Western blot for BDNF (E). Naive *vs* EAE + CBD, $^{**}p = 0.0065$; EAE *vs* EAE + CBD, $^{***}p = 0.0005$. All Western blot analyses were performed on spinal cord tissues sampled at 28 days from EAE induction. Blots are representative of three separate and reproducible experiments. Statistical analysis was carried out on three repeated blots performed on separate experiments. Data are expressed as mean ± SEM.

decreased expression of pS6 K in EAE mice, increased instead by CBD administration.

3.3. CBD treatment increases BDNF expression levels in EAE mice

By western blot analysis we have detected BDNF expression in order to demonstrate whether activation of PI3K/Akt/mTOR pathway could be correlated to BDNF expression. As known, BDNF is an important growth factor, beneficial for neuronal function following neuronal damage. BDNF *via* binding to its specific receptors, tyrosine kinase B (TrkB) and p75 [38,39] triggers intracellular signaling cascades, including PI3K/Akt/mTOR pathway [40]. Our results showed that EAE induction caused a decreased BDNF expression, while, CBD treatment increased BDNF levels. Naive mice showed a basal level of BDNF expression (Fig. 2 E).

3.4. CBD treatment inhibits cytokines production in EAE mice

Several studies performed by using EAE model have provided convincing evidence that T cells specific for self-antigens mediate pathology in this disease. Two distinct subsets of autoreactive T cells have been primarily involved in the pathogenesis of both EAE and MS: the IFN-γ producing CD4+ T helper (Th) 1 and interleukin (IL)-17 producing Th17 cells [41–43]. Immunohistochemical analysis performed in spinal cord sections, showed a negative staining for IFN-γ as well as for IL-17 in naive mice (Fig. 3 A and D, respectively). A positive staining for these pro-inflammatory mediators was observed in EAE mice (Fig. 3 B and E, respectively). Conversely no positive staining for IFN-γ as well as for IL-17 was obtained in mice treated with CBD (Fig. 3 C and F, respectively). Also, by western blot analysis we investigated the role of peroxisome proliferator activated receptor γ (PPARγ), showing a mild increase in PPARγ expression in EAE mice which might have resulted from innate anti-inflammatory response, while administration of CBD markedly increased PPARγ levels (Fig. 4 A).

3.5. Effects of CBD treatment on MAPK signal-transduction pathway

PI3K/Akt/mTOR pathway leads to trigger a variety of intracellular pathways, including the mitogen-activated protein kinase (MAPK) pathway [44,45], which plays a pivotal role in regulating many cell functions in different cell types, including survival, proliferation and apoptosis [46,47]. Western blot analysis for c-Jun. N-terminal protein kinase (JNK) revealed that MAPK signaling pathway is strongly activated following EAE-induction while CBD treatment reduces the expression levels of this marker in spinal cord tissue taken from EAE mice (Fig. 4 B). In addition, it was found that EAE upregulated the levels of p-p38 in spinal cord tissue (Fig. 4 C). On the contrary, the treatment with CBD reduced significantly reduced the phospho-p38 expression. A basal expression of JNK and p-p38 was detected in spinal cord samples from naive animals.

4. Discussion

The PI3K/Akt/mTOR pathway is an essential cellular signaling implicated in a wide range of fundamental physiological functions, including cell growth, proliferation, metabolism, protein synthesis and autophagy [25,28,48,49]. Dysregulation in PI3K/Akt/mTOR pathway was found in many diseases, and especially its aberrant activation is associated to cancer development [24,49–52]. However, this pathway was widely investigated in the pathogenesis of cognitive dysfunction and neurologic diseases. In particular, the inhibition of PI3K/Akt/mTOR signaling is

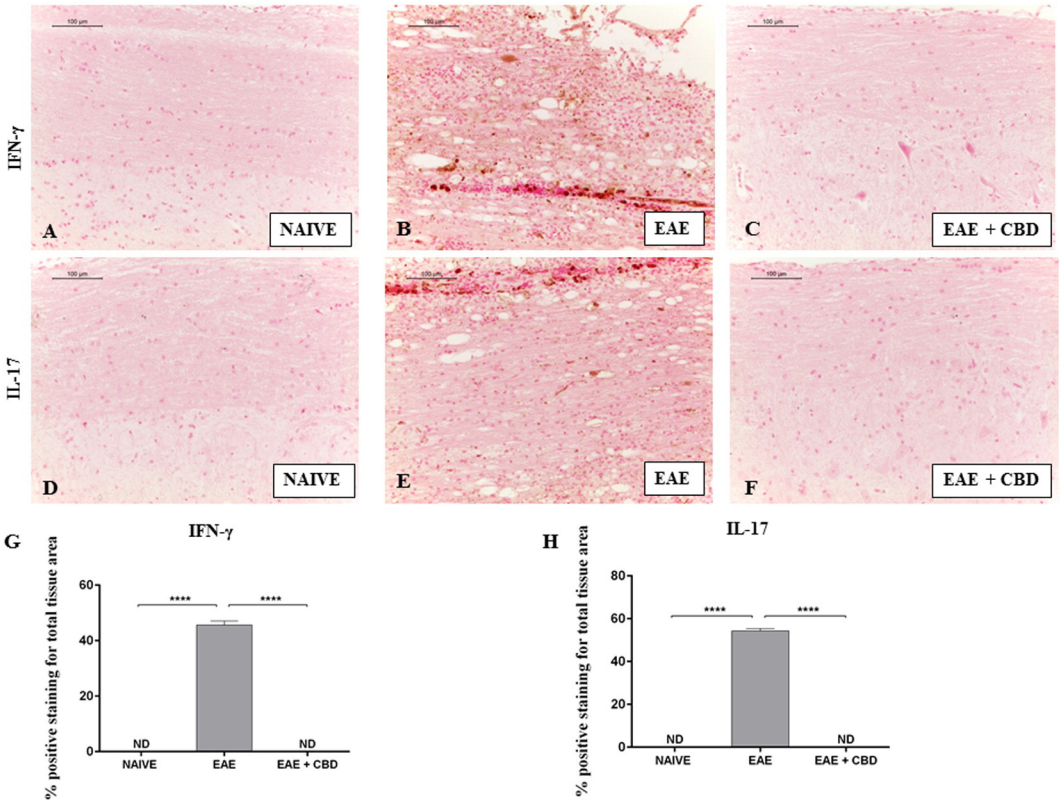

Fig. 3. Immunohistochemical analysis for INF-γ and IL-17. A negative staining for INF-γ and IL-17 was observed in spinal cord tissues obtained from naive mice (A and D, respectively). Increased INF-γ and IL-17 tissue localization in EAE mice was found (B and E, respectively). Specifically, positive staining for inflammatory cells in vascular endothelium of spinal cord tissues was observed. On the contrary, negative staining for INF-γ and IL-17 was observed in mice treated with CBD (C and F, respectively). All sections were obtained using light microscopy (LEICA DM 2000 combined with LEICA ICC50 HD camera). Leica Application Suite V4.2.0 software was used as the image computer program to acquire immunohistochemical pictures. Densitometric analysis for INF-γ (G) and IL-17 (H). Results were analyzed by one-way ANOVA followed by a Bonferroni test for multiple comparisons. $^{****}p < 0.0001$. ND: not detectable.

known to arrest the pro-survival system leading to degeneration in Parkinson disease, schizophrenia and brain ischemia [53–56].

Several line of evidences have identified this pathway as a major regulator of developmental myelination, which is the primary target of the immune attack during MS. Particularly, mTOR, the key downstream mediator of PI3K/Akt, is involved in oligodendrocyte differentiation *in vitro* and in developmental myelination [57–59]. Moreover, the importance of PI3K/Akt/mTOR activation pathway for oligodendrocyte survival and axon myelination was also demonstrated in EAE model [60]. Due to the numerous cellular functions in which this pathway is involved, a growing interest is direct to identify pharmacological modulators of PI3K/Akt/mTOR activity as potential therapeutic target for the treatment of many diseases. In this context, it was found that cannabinoids, by involving CB1 receptor, activate the PI3K/Akt/mTOR pathway and promote cellular survival in astrocytes and oligodendrocytes [22,23,61]. Moreover, Molina-Holgrado et al. reported that use of the synthetic cannabinoid HU-210 stimulates the PI3K/Akt/mTOR pathway exerting neuroprotective effects in primary cortical neurons [62].

As there is no data regarding the effects of CBD in the PI3K/Akt/mTOR activity in MS, the present study was aimed to we investigate the involvement of the PI3K/Akt/mTOR signaling after treatment with purified CBD in EAE model.

In agreement with previous study [17], we demonstrated that CBD treatment reduces clinical disease score as well as inflammatory cell infiltrates as showed by histological evaluations.

Our results showed an evident downregulation of the PI3K/Akt/mTOR pathway following EAE induction. On the contrary, treatment with CBD was able to restore it, increasing significantly the phosphorylation of PI3K, Akt and mTOR when compared to EAE group.

mTOR represents the critical downstream target of the PI3K/Akt pathway as it regulates during protein synthesis the translational rate and the initiation step in several cellular processes [63,64]. The inhibition of mTOR *in vivo* was found to limit myelination during development [58], representing thus one of the causes that leads to a myelin loss in MS. Both *in vivo* and *in vitro* studies showed effectively that mTOR plays a key role in CNS myelination. It was found that mTOR inhibition of oligodendrocyte progenitor cells (OPCs) *in vitro* blocked the transition from the OPC to the immature oligodendrocyte and reduced cellular morphological complexity, myelin protein expression, and myelination [65]. In addition, transgenic mice overexpressing constitutively active Akt in oligodendrocytes show increased expression of mTOR and increased myelination [57]. Therefore, it is likely to believe that restoring of the PI3K/Akt/mTOR pathway and in particular the increased expression of mTOR following treatment with CBD could be the cause of the improvement in the progression of disease probably due to the arrest of myelin loss.

mTOR in turn induces phosphorylation of a positive regulator of protein synthesis, pS6K, that promotes assembly of the ribosome complex and activates translation by phosphorylating ribosomal protein S6 [66,67]. The phosphorylation status of S6K is indeed commonly used as a marker of mTOR activity [66,68]. We noticed an enhanced expression of pS6 K in EAE mice treated with CBD compared to EAE untreated ones.

Moreover, assuming that CBD activates the PI3K/Akt/mTOR pathway, we examined the expression of BDNF, since by binding its specific

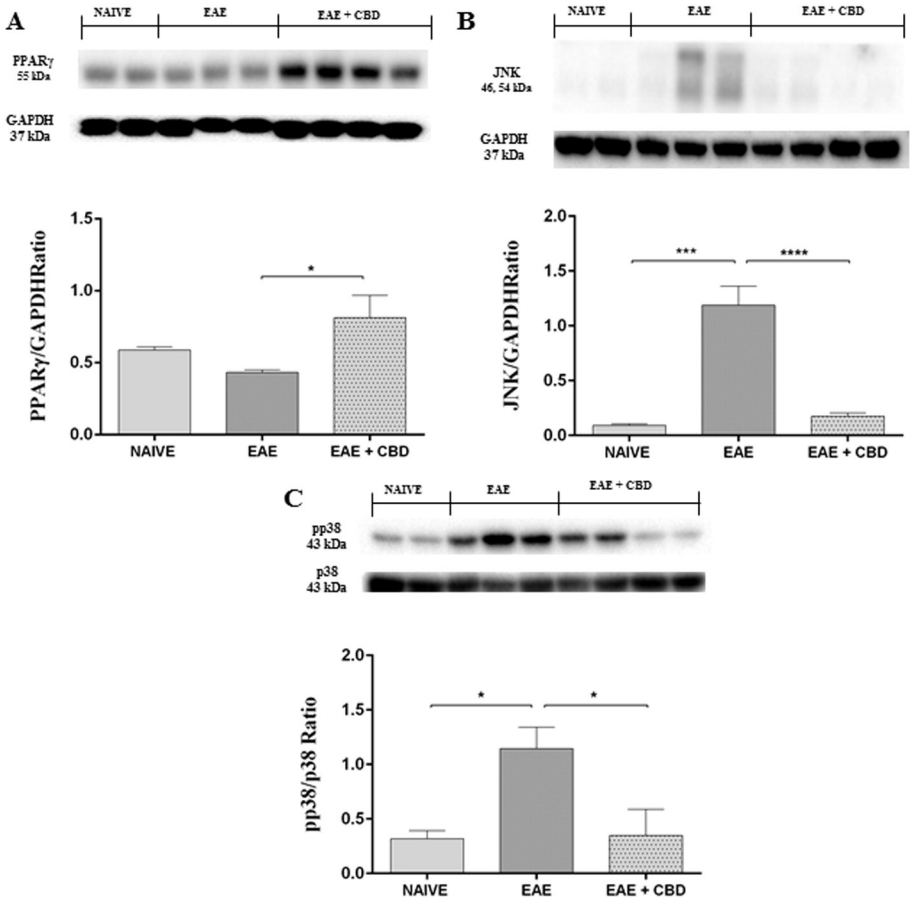

Fig. 4. Western blot analysis for PPARγ (A). EAE *vs* EAE + CBD, $^{*}p = 0.0120$. Western blot analysis for JNK (B). Naive *vs* EAE, $^{***}p = 0.0001$; EAE *vs* EAE + CBD, $^{****}p < 0.0001$. Western blot analysis for pp38 (C). Naive *vs* EAE, $^{*}p = 0.0176$; EAE *vs* EAE + CBD, $^{*}p = 0.0128$. All Western blot analyses were performed on spinal cord tissues sampled at 28 days from EAE induction. Blots are representative of three separate and reproducible experiments. Statistical analysis was carried out on three repeated blots performed on separate experiments. Data are expressed as mean ± SEM.

receptors, tyrosine kinase B (TrkB) and p75 [38,39], it triggers intracellular signaling cascades including PI3K/Akt/mTOR pathway [40]. Interestingly, our data indeed showed an increased level of BNDF in CBD-treated mice compared to EAE mice.

Current literature proves that the EAE model has a not specified involvement of the immune system [69,70]. A recent study emphasizes a clear engagement of T cells, including $CD4^+$, $CD25^+$ and Foxp3 in acute and chronic stages of EAE [71]. As already demonstrated, CBD repressed the EAE-associated Treg cells activation by diminishing $CD4^+$ and Foxp3 levels [17].

By immunohistochemical staining we found that CBD treatment reduced pro-inflammatory cytokines like INF-γ and IL-17 in spinal cord from EAE mice. Although the role of PI3K/Akt/mTOR pathway in modulating inflammation specifically in immune cells has been investigated, the results remain controversial. Some studies have shown activation of the PI3K/Akt/mTOR pathway as a negative regulator of inflammation [72,73], contrariwise others have reported that its activation promotes the inflammatory phenotype in immune cells [74]. It was found also that PI3K/Akt/mTOR pathway is involved in immunosuppression in autoimmune diseases progression [75,76]. Therefore, CBD, which has proved to exert immunomodulating activities [19] could induce immunosuppression through the PI3K/Akt/mTOR pathway modulation.

Therefore, we don't assess if the down-regulation of the pro-inflammatory mediators by CBD treatment is due to the up-regulation of PI3K/Akt/mTOR signaling. However, we suggest that anti-inflammatory effects of CBD are attributed to elevated level of PPARγ. Indeed, it was demonstrated the beneficial efficacy of PPARγ agonists in the treatment of MS and other neurodegenerative diseases to suppress inflammation [77].

Our results showed an increased expression of JNK and p-p38 in spinal cord from EAE mice, conversely, attenuated by CBD administration. These results are in agreement with literature that reported that phosphorylation of the PI3K/Akt/mTOR pathway leads in turn to inhibition of the MAPK pathway [44,45]. Therefore we suggest that CBD treatment avoiding the trigger of the MAPK signaling, may promote neuronal survival in EAE mice.

5. Conclusion

In light of the above findings, there is a valid rationale to suppose that therapeutic efficacy exhibited by CBD may be due to the activation of PI3K/Akt/mTOR signaling together with reduction of pro-inflammatory mediators and increase of PPARγ. CBD treatment is able also to promote neuronal survival by inhibiting MAPK pathway. Finally, looking to CBD activity on the PI3K/Akt/mTOR pathway, we strongly hope to have provided a short but important overview of evidences that are useful to better characterize the efficacy as well as the molecular pathways modulated by this molecule.

Declaration of interest

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The authors declare no conflicts of interest in relationship to performing this study.

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