CD200-CD200R1 Interaction Contributes to Neuroprotective Effects of Anandamide on Experimentally Induced Inflammation

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ABSTRACT
The endocannabinoid anandamide (AEA) is released by macrophages and microglia on pathological neuroinflammatory conditions such as multiple sclerosis (MS). CD200 is a membrane glycoprotein expressed in neurons that suppresses immune activity via its receptor (CD200R) mainly located in macrophages/microglia. CD200-CD200R interactions contribute to the brain immune privileged status. In this study, we show that AEA protects neurons from microglia-induced neurotoxicity via CD200-CD200R interaction. AEA increases the expression of CD200R1 in LPS/IFN-γ activated microglia through the activation of CB_2 receptors. The neuroprotective effect of AEA disappears when microglial cells derive from CD200R1$^{-/-}$ mice. We also show that engagement of CD200R1 by CD200Fc decreased the production of the proinflammatory cytokines IL-1β and IL-6, but increased IL-10 in activated microglia. In the chronic phases of Theiler's virus-induced demyelinating disease (TMEV-IDD) the expression of CD200 and CD200R1 was reduced in the spinal cord. AEA-treated animals up-regulated the expression of CD200 and CD200R1, restoring levels found in sham animals together with increased expression of IL-10 and reduced expression of IL-1β and IL-6. Treated animals also improved their motor behavior. Because AEA up-regulated the expression of CD200R1 in microglia, but failed to enhance CD200 in neurons we suggest that AEA-induced up-regulation of CD200 in TMEV-IDD is likely due to IL-10 as this cytokine increases CD200 in neurons. Our findings provide a new mechanism of action of AEA to limit immune response in the inflamed brain.

INTRODUCTION

Immune responses are restricted in the central nervous system (CNS). The finely balanced anti-inflammatory microenvironment within the CNS contributes to its immune privilege status. CD200 is a membrane glycoprotein that suppresses immune activity via its receptor CD200R (Wright et al., 2000). CD200-CD200R interaction have been found to be down-regulated in neuroinflammatory diseases such as multiple sclerosis (MS) (Koning et al., 2007, 2009), but also in Alzheimer disease (Walker et al., 2009) as well as in the aging brain (Frank et al., 2006).

Microglia, the resident antigen presenting cells of the CNS is known to display diverse reactions associated with both protective and deleterious effects (Hannish and Kettenmann, 2007). In the CNS, microglia is rapidly activated in response to injury or pathological events while regulatory immune inhibitory molecules contribute to avoid its detrimental effects. CD200-CD200R interaction provides a cell-cell contact negative regulatory signal for microglia. Deletion of CD200 resulted in myeloid cell dysregulation and enhanced susceptibility to autoimmune inflammation (Gorzcynski et al., 2001, Simelyte et al., 2008; Hoek et al., 2000). CD200 is highly expressed in neurons of rodents and humans whereas CD200R is mainly expressed on cells of myeloid origin, such as residing microglia and invading macrophages (Koning et al., 2009). CD200R has been also found on a subset of T and B cells in humans (Rijkers et al., 2008). CD200R is closely structurally related to CD200, but it has longer cytoplasmic tail that delivers an inhibitory signal after ligation by CD200 (Jenmalm et al., 2006). Mice with increased neuronal levels of CD200 by spontaneous mutation in the *Wld* gene have less activated and infiltrating monocytes in EAE, with decreased disease severity (Chitnis et al., 2007). Changes in CD200 expression provide a mechanism for locally regulating myeloid cellular activity at appropriate sites such as inflamed tissue. Within the CNS the anti-inflammatory cytokine IL-4 has been shown to up-regulate the expression of CD200 in neurons during neuroinflammation (Lyons et al., 2007). The CD200-CD200R pathway is an attractive target for immunomodulation as its manipulation

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can induce either immune tolerance or autoimmune diseases.

The endocannabinoid system (ECS) has arisen as a promising new therapeutic target for the treatment of MS (Arévalo-Martín et al., 2008; Lambert and Fowler, 2005). In animal models of MS, exogenous cannabinoid agonists attenuate the pathological features of the disease (Arévalo-Martín et al., 2003; Croxford and Miller, 2003), an effect also observed by pharmacological interventions aimed at increasing the levels of endocannabinoids (Cabranes et al., 2005; Ligresti et al., 2006; Mestre et al., 2005; Ortega-Gutiérrez et al., 2005). Uncontrolled innate immune responses within the CNS are recognized as playing a major role in the development of autoimmune disorders and neurodegeneration, with MS and Alzheimer's diseases (AD) being primary examples. However, the role of the ECS in the regulation of inhibitory immunoregulatory proteins has not been elucidated.

During the past years, several *in vitro* and *in vivo* studies have suggested that ECS participates in the control of brain immune responses as well as in the protection of the CNS against injury (rev Scotter et al., 2010). AEA has been described to protect neurons from inflammatory damage (Eljaschewitsch et al., 2006). During immune-mediated attack of the brain, it has been hypothesized that the activation of endocannabinoids represents a protective mechanism, aimed at reducing both neurodegenerative and inflammatory damage through various and partially converging mechanisms that involve neuronal and immune cells. Here, we studied the role played by the endocannabinoid, AEA, in the interaction of CD200 and its receptor in an *in vitro* model of neuronal damage induced by microglia and in Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD), a well-characterized viral model of MS. In this study we reveal an unknown function of AEA as modulator of the CD200-CD200R interaction. We show that AEA protects neurons from inflammatory damage via CD200-CD200R interaction by up-regulating CD200R1 in microglia through the activation of CB_2 receptors. Our study also shows that the expression of CD200 and CD200R is reduced in the spinal cord of TMEV-infected mice at chronic phases of the disease. The treatment with an inhibitor of FAAH or implantation of miniosmotic pumps delivering AEA induces a recovery in the levels of CD200 and CD200R accompanied with improved motor behavior. In this study we have also assessed the effects of pharmacological modulation of AEA on the quantitative gene expression of IL-10, IL-1β, and IL-6 in the spinal cord of TMEV-infected mice to explore whether changes of CD200 and its receptor CD200R1 might influence the inflammatory environment.

MATERIAL AND METHODS

Mice

For cell cultures stock 8-week-old C57BL/6 mice were purchased from Harlan Europe and maintained in our in-house colony (Instituto Cajal, CSIC, Madrid, Spain). For neuronal cultures, embryos E17-18 and for microglia cultures, P0-P1 mice were used. In some experiments CD200R1$^{-/-}$ mice (BL/6 background) (P0-P1) and C57BL/6 WT mice (P0-P1) and embryos (E-17-18) from Kennedy Institute of Rheumathology, Imperial College (London, England) were used. IL-10 knock-out mice (IL-10$^{-/-}$ mice, BL/6 background) and the corresponding wild-type (WT) C57BL/6 controls were purchased from Jackson Laboratory (Ben Harbour Maine). For *in vivo* experiments, female SJL/J mice from Harlan Europe susceptible to TMEV-IDD development, were maintained in our in-house colony (Instituto Cajal, CSIC Madrid, Spain), on food and water *ad libitum* in a 12-h light/dark cycle. Four- to 6-week-old mice were inoculated intracerebrally in the right cerebral hemisphere with 10^6 pfu of Daniel's TMEV strain in 30 μL of DMEM supplemented with 10% FCS as previously described (Arévalo-Martín et al., 2003). Sham animals were inoculated with vehicle without virus. 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; BOE1201/2005) on the use and care of laboratory animals, and approved by the local Animal Care and Ethics Committee of the CSIC (RB00007851). In the case of CD200R1$^{-/-}$ mice the experiments were approved by the local Ethics Review Process Committee and the UK Home Office.

Reagents

Culture media and fetal calf serum (FCS) were obtained from Invitrogen (Barcelona, Spain). A soluble form of CD200, the mouse fusion protein CD200Fc and control murine IgG2a were provided by Genentech (UK). Rat antimouse CD200 from Abd Serotec (MCA1958; clone OX-90; Germany). Mouse anti-CD200R was obtained from Abcam (Cambridge, UK) and from AbD Serotec (MCA2281; clone OX-110; Germany). Anti-CD200 blocking antibody was from AbD Serotec, (Germany). For Western blots, CD200 (R-17) and CD200R (M-21) goat polyclonal antibodies were obtained from Santa Cruz Biotechnology Inc, CA. Anandamide (AEA) and lipopolysaccharide (LPS) from *Escherichia coli* serotype O127:B8 were from Sigma (Madrid, Spain). Cannabinoid antagonists SR141716A (SR1) and SR144528 (SR2) were supplied by Sanofi Recherche (Montpellier, France). The fatty acid amide hydrolase (FAAH) inhibitor, *N*-arachidonoylserotonin (AA-5-HT) was synthesized in Dr. Di Marzo's lab as previously described (Bisogno et al., 1998). Murine recombinant interferon-γ (IFN-γ) and Interleukine-10 (IL-10) were from PreproTech (London, UK). Miniosmotic pumps were purchased from Alzet (Cupertino, CA). All other reagents were obtained from standard suppliers.

Cell Cultures

Primary mix glial cultures were prepared as previously described (Molina-Holgado et al., 2002). Briefly,

after decapitation, forebrains of newborn (P0-P1) C57BL/6 mice (WT, CD200R1$^{-/-}$ or IL-10$^{-/-}$ mice) were dissociated mechanically, filtered through a 150-μm nylon mesh, resuspended in DMEM (Lonza Ibérica S.A. Barcelona, Spain) containing 10% heat-inactivated FBS, 10% heat-inactivated horse serum and 1% penicillin/streptomycin and plated on poly-L-lysine (5 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 seeded on multiwell culture plates for 2 h. After this, medium was changed to remove nonadherent 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). Microglial cultures were subjected to LPS/IFN-γ for 2 h and CD200R1 expression was evaluated by Western blot, conventional PCR, and real-time PCR, in the presence or absence of AEA (10 μM). In some experiments the effects of CB_1 and CB_2 antagonists were assessed.

Cortical neuronal cultures were prepared from embryos (E17-E18) of C57BL/6 mice as previously described (Loría et al., 2010). After the dissection, cortices were stripped from meningeal tissue and dissociated in PBS containing trypsin (0.25 % Gibco-Invitrogen S.A., Barcelona, Spain) and DNAse I (1 mg/mL; Roche Diagnostics, S.L., Manheim, Germany) at 37°C for 15 min. A single cell suspension was prepared by triturating tissue, and the pellet was resuspended in DMEM (Lonza Ibérica S.A. Barcelona, Spain) supplemented with heat-inactivated horse serum (10 %v/v) and cells (5×10^5) were seeded on poli-L-lysine (5 μg/mL; Sigma-Aldrich Química, S.A., Madrid, Spain) 12 multiwell culture plates for 3 h. After this, medium was changed to remove nonadherent cells and added the new medium containing Neurobasal medium supplemented with penicillin/streptomycin (1 %v/v; Gibco-Invitrogen S.A., Barcelona, Spain), glutamax (2 mM; Gibco-Invitrogen S.A., Barcelona, Spain) and the antioxidant B-27 supplement (Invitrogen S.A; Cedex, France). After 3 days *in vitro* (DIV), non-neuronal cell division was halted by exposure to 10 μM cytosine-D-arabinoforanoside (Ara C, Sigma-Aldrich Química, S.A, Madrid, Spain). Cells were grown in a humidified environment containing 5% CO_2 and held at a constant temperature of 37°C for about 7 to 8 days. Neurons are labeled using anti-MAP-2 (Calbiochem, La Jolla, CA). The amount of cells other than neurons were quantified using GFAP (Calbiochem, La Jolla, CA) for astrocytes, Mac-1, anti-CD11b antibody (Serotec, Oxford, UK) for microglia and PDFGRα (Pharmingen, BD Biosciences, Europe) for oligodendrocyte progenitors, and was estimated to a total of less than 2% of the number of neuronal cells. In a set of experiments neuronal cultures were evaluated for CD200 expression after 2 h of LPS/IFN-γ exposure in the presence or absence of AEA (10 μM). The effect of IL-10 (20 ng/mL) in the expression of CD200 by neuronal cultures was also evaluated.

Neuronal-microglia co-cultures

Primary cortical neuron cultures and primary microglia cultures (from C57BL/6 WT or CD200R1$^{-/-}$ or IL-10$^{-/-}$ mice) were isolated and prepared as described above. In order to establish the adequate co-culture conditions we performed preliminary experiments varying the density of microglial cells. Primary microglial cells were seeded with neuronal cultures at a density of 1.5×10^4; 30×10^4, or 60×10^4 cells per well in 12-well plates containing 5×10^5 neurons per well. The optimized co-culture conditions were achieved by adding 1.5×10^4 primary microglial onto the neuronal cultures that had been cultured for 7 to 8 days. Microglial cells were stimulated with LPS (50 ng/mL) and IFN-γ (100 U) for 24 h. In one set of experiments we added CD200-Fc fusion protein (5 μg/mL; Genentech, UK), a soluble ligand of CD200R1. In another set of experiments AEA at a dose of 10 μM on the basis of our previous studies (Correa et al., 2010) was added in the absence or presence of LPS/IFN-γ. In all cases neuronal death was followed by examination of the co-cultures under bright-field microscopy and in time lapse microscopy. Neuronal dead was quantitatively assessed by measurement of lactate dehydrogenase (LDH) release into the bathing medium 24 h after adding microglial cells as we previously described (Loría et al., 2010).

In Vivo Treatments

TMEV-infected mice were injected intraperitoneally, in the chronic phase of the disease at 78 days postinfection (pi) with the FAAH inhibitor *N*-arachidonoylserotonin (AA-5HT), at a dose of 5 mg/kg on the basis of our previous work (Correa et al., 2011) or subjected to vehicle administration (Tween 80:PBS; 1:14 or Tocrisolve^{TM}). Mice were sacrificed at 90 days pi. In another set of experiments mice were subjected to a chronic infusion of AEA (3.5 μg/μL; 1 μL/h) at 83 days pi through miniosmotic pumps (Alzet, model 2001, Durect Corporation, Cupertino, CA) implanted subcutaneously. The pumps were filled with either vehicle or AEA (3.5 mg/kg) and mice were sacrificed 7 days after implantation of the miniosmotic pumps (90 days pi). All the protocols were used during established disease as evaluated by motor function deficits. Spontaneous motor activity was evaluated by using an Activity Monitor System (Omnitech Electronics, Columbus, OH).

Tissue Collection and RNA Extraction

Mice were anaesthetized by intraperitoneal administration of EuTalender (Normon Lab., Madrid, Spain) and after saline perfusion, tissue for RT-PCR studies

was rapidly removed at the indicated days pi (21, 35, 60, and 90 days) and after the different treatments. Spinal cord tissue samples were frozen in dry ice and stored at −70°C until required. Total RNA was extracted using RNeasy mini columns (Qiagen, UK). Contaminating genomic DNA was degraded by a treatment with DNaseI (Qiagen, UK). The yield of RNA was determined using a Nanodrop® spectrophotometer (Nanodrop technologies).

Reverse Transcription (RT) and TaqMan Polymerase Chain Reaction (PCR)

Total RNA (1 μg in 20 μL) was reverse transcribed into cDNA using the Promega reverse transcription kit (Promega, Spain) with poly-dT primers. TaqMan primers and probes for IL-1β, IL-6 IL-10, CD200, and CD200R1 were supplied by Applied Biosystems, UK. Probes were labeled 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. Primers and probes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were supplied by Applied Biosystems (Warrington, UK). TaqMan PCR was performed from 1 μL of cDNA (corresponding to 50 ng RNA input) using Universal TaqMan Mastermix with 100 nM primers and a 50 nM probe. Cycling conditions were: 50°C for 2 min, 95°C for 10 min, followed by 40 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. Each sample was assayed in triplicate and a 6-point standard curve run in parallel. To ensure the absence of genomic DNA contamination, a control sample of nonreverse-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 house-keeping gene GAPDH. Results are expressed as a percentage of sham animals for each time point.

Immunocytochemistry in Cultured Cells

Immunostaining was done directly on cells seeded on glass coverslips. Microglia plated onto poly-L-lysine-coated (5 μg/mL) coverslips was 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 anti-CD200R1 antibody at a dilution 1:100 (AbD Serotec (MCA2281; clone OX-110; Germany)) and Mac-1 anti-CD11b antibody (1:100, Serotec Ltd., Oxford, UK). In the case of neuronal cultures the antibodies used were mouse monoclonal anti-MAP-2 (1:250; Calbiochem, La Jolla, CA), and anti-CD200 (1:200; Abcam, Cambridge, UK). Immunostaining was visualized with Alexa-conjugated secondary (1:1,000) anti-mouse IgG-Alexa 488 and anti-goat Alexa antibodies (Molecular Probes, Eugene, OR). Nonspecific interactions of secondary antibodies were confirmed by omitting primary antibodies. At least three independent cultures were examined.

Cytokine Assays

The concentration of IL-10, IL-1β, or IL-6 in the harvested supernatants from microglial cultures was determined using enzyme-linked immunoadsorbent assays (Bender MedSystems Inc, Burlingame, CA). The detection limits were 5 pg/mL for IL-10, 1.2 pg/mL for IL-1β, and 7.9 pg/mL for IL-6. Reproducibility within the assay was evaluated in three independent experiments for each cytokine and calculated intra-assay coefficients of variation were <5%. Coefficients of inter-assay variation were always <10%.

Western Blot Analysis

After treatments, neuronal cell cultures or microglial cell cultures were washed with ice-cold PBS and lysed in Tris-buffered saline (TBS), pH = 7.6, containing 10% glycerol, 1% Nonidet P-40, EDTA 1 mM, EGTA 1 mM plus complete protease inhibitors cocktail (Roche Diagnostics, Mannheim, Germany). Neuronal and microglia cell lysates were mixed with 5× Laemmli sample buffer and boiled for 5 min. Then equal amount of protein (30 μg) were resolved on 10% SDS-PAGE and electroblotted at 90 V for 70 min at 4°C to nitrocellulose (Amersham Biosciences). The membranes were blocked for 1 h at RT in 5% (w/v) dry skim milk (Sveltese, 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 the case of neuronal cell lysates (anti-CD200, 1:50, Santa Cruz Biotechnology, Inc; CA); and for CD200R in the case of microglia cell lysates (anti-CD200R, 1:50, Santa Cruz Biotechnology Inc; CA) in 5% milk-TBST, extensively washed with 5% milk-TBST solution and incubated with horseradish peroxidase-conjugated anti-goat (1:8,000) secondary antibody (Bio-Rad, Hercules, CA) for 1 h at RT. Finally, the blots were rinsed and the peroxidase reaction was developed by enhanced chemiluminescence (Amersham Biosciences). 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 for monoclonal anti-α-Tubulin (1:40,000, Sigma, Madrid, Spain).

Statistical Analysis

Results are presented as means ± SEM. For *in vitro* experiments, means ± SEM of at least three experiments performed in triplicate were analyzed by one-way analysis of variance (ANOVA) and the appropriate *post hoc* test of significance. For *in vivo* experiments ANOVA followed by the Tukey test for multiple comparison were used to determine statistical significance (95%; $P < 0.05$).

RESULTS

AEA Protects Neurons from LPS/IFN-γ Activated Microglia-Induced Toxicity via CD200-CD200R Interaction

To investigate the relevance of CD200-CD200R interaction in microglia-induced neurotoxicity, we first analyzed the effect of the treatment with the soluble form of CD200 (CD200Fc) on neuronal death using a neuron/microglia co-culture system. CD200Fc binds to murine and human CD200R1 but not to other murine isoforms (Liu et al., 2010). As showed in Fig. 1A,B, LPS/IFN-γ activated microglia resulted in significant neuronal death ($P < 0.001$). The addition of CD200Fc at 5 μg/mL (Gorczynski et al., 2008) elicited a significant decrease in neuronal death induced by LPS/IFN-γ activated microglia ($P < 0.001$; Fig. 1A) which was completely abrogated when microglia were from CD200R1$^{-/-}$ mice (Fig. 1B). The exposure to control mouse IgG2a did not modify neuronal death (data not shown). In all cases, neuronal death after microglia addition was exacerbated when microglia were from CD200R1$^{-/-}$ mice as the level of neuronal death in control conditions varies from 10% (WT mice) to approximately 35% (CD200R1$^{-/-}$). Under inflammatory conditions neuronal death was between 50 and 60% in the WT co-culture model while in the CD200R1$^{-/-}$ model was around 80 to 90%. As AEA is accumulated in inflamed brain tissue (Eljaschewitsch et al., 2006), in this study we asked whether AEA might protect neurons from inflammatory damage via CD200-CD200R interaction. Fig. 1C, shows that AEA was able to rescue neurons from death induced by LPS/IFN-γ activated microglia ($P < 0.001$). The involvement of CD200-CD200R interaction in AEA-induced neuroprotection was examined using microglia from CD200R1$^{-/-}$ mice. In the absence of CD200R1, AEA did not improve neuronal survival as shown in Fig. 1D. Because activated microglia from CD200R1$^{-/-}$ mice increased notably the neuronal death, we asked whether AEA was not capable of protecting neurons due to the high level of neuronal death in this situation. Therefore, we induced the same level of neuronal death by increasing the dose of LPS to 1 μg/mL in WT co-cultures and observed that AEA protected neurons even in the case of 80% of neuronal death (data not shown).

Downregulation of CD200R1 Expression on Microglial Cells by Inflammatory Stimuli: Anandamide Treatment Prevents this Effect by Activation of CB_2 Receptors

As previously described (Chitnis et al., 2007; Meuth et al., 2008) immunofluorescence staining of CD200 and the neuronal marker MAP-2 in neuronal cultures showed that CD200 expression occurred mainly in the soma (Fig. 2A). As expected, microglia cultures constitutively expressed CD200R1 evaluated by immunocytochemistry by double staining with anti CD200R1 and the microglia marker CD11b (Fig. 2B).

In order to study whether AEA might modify the expression of the ligand CD200 in neurons under inflammatory stimuli, we performed experiments using primary neuronal cultures. Western blot analysis showed that CD200 expression remained without changes after LPS/IFN-γ. Furthermore, the treatment with AEA (10 μM) did not change the level of expression of CD200 in neurons (Fig. 3A).

We next focused on the regulation of microglial CD200R1 by AEA. The expression of CD200R1 by murine microglia was examined under inflammatory stimuli. Western blot analysis demonstrated that CD200R1 was down-regulated 2 h after LPS/IFN-γ activation ($P < 0.001$; Fig. 3B). To assess the effects of AEA on microglial CD200R1 we co-treated the cells with 10 μM AEA, in the presence of LPS/IFN-γ. The treatment with AEA increased the level of expression of CD200R1 as evaluated by immunoblotting ($P < 0.001$; Fig. 3B). This also occurred when we assessed mRNA CD200R1 expression by conventional RT-PCR. Thus, microglia subjected to LPS/IFN-γ stimulation showed reduced CD200R1 expression ($P < 0.001$; Fig. 3C) that was recovered by AEA treatment ($P < 0.001$). To clarify whether the up-regulation of CD200R1 was due to specific interactions between AEA and CB_1 or CB_2 receptors, we perform experiments with real-time PCR under the same conditions as above, but incubating microglia with the pharmacological antagonists SR1 for CB_1 and SR2 for CB_2 receptors. In these experiments, we found that CB_2 blockade was effective in preventing AEA effects on CD200R1 gene expression ($P < 0.05$; Fig. 3D) while CB_1 blockade did not modify CD200R1. The addition of both antagonists SR1 and SR2 simultaneously did not result in an enhanced reversion of AEA effects, suggesting that only CB_2 receptor is involved in the up-regulation of CD200R1. In all cases, CD200R1 expression did not change when the two antagonists were used alone without AEA (data not shown). In summary, the overexpression of CD200R1 induced by AEA involves the activation of CB_2 receptors.

We then explored whether the stimulation of CD200R1 by CD200Fc affected microglial responses to LPS/IFN-γ. Cytokines production was evaluated in LPS/IFN-γ −stimulated microglia after the treatment with CD200Fc (5 μg/mL) on the basis of previous studies (Simelyte et al., 2008). We found that CD200Fc inhibited the production of proinflammatory cytokines such as IL-6 and IL-1β induced by LPS/IFN-γ ($P < 0.001$), (Fig. 4A,B). In contrast, the secretion of the anti-inflammatory cytokine IL-10 was stimulated after the treatment with CD200Fc ($P < 0.001$, Fig. 3C). Treatment with mouse control IgG2a did not change cytokine responses to LPS/IFN-γ in microglia (data not shown).

IL-10 Exerts Neuroprotective Effects in the Model of Microglia Mediated Cytotoxicity and Enhances the Expression of CD200 in Neurons

Because triggering of CD200R1 signaling stimulates IL-10 production and our previous results showed that

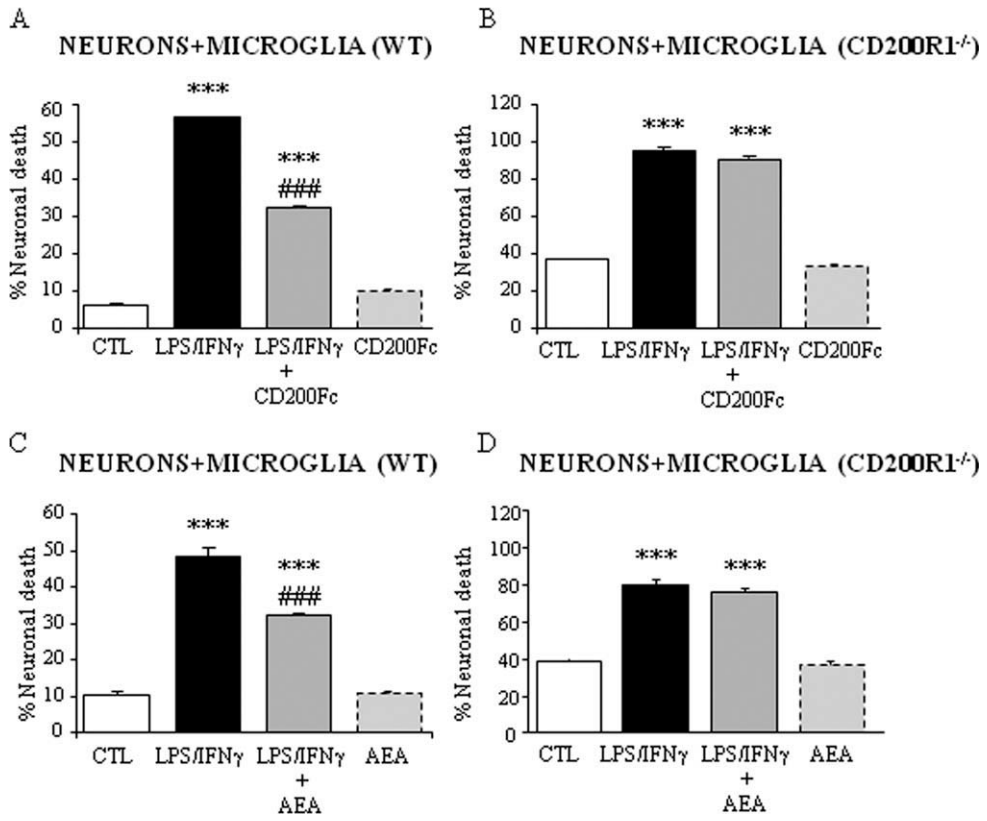

Fig. 1. AEA promotes neuroprotection from activated microglia-induced toxicity: Role of CD200-CD200R interaction. We used an *in vitro* model of microglia mediated neurotoxicity. For this, resting or LPS/IFN-γ stimulated microglial cells were cultured with neurons (8D) in the presence or absence of CD200Fc (5 μg/mL) and in the presence or absence of AEA (10 μM). Microglial cells were from wild type or CD200R1$^{-/-}$ mice. (**A**) Cortical neurons were co-cultured with microglial cells for up to 24 h and neuronal death was estimated by LDH measurement in the media (mean ± SEM, $n = 12$). The increased neuronal death by LPS/IFN-γ (50 ng/100 U) stimulated microglia ($P < 0.001$) was significantly reduced ($P < 0.001$) by the treatment with CD200Fc. (**B**) Please note, that neuron-microglia co-cultures in which microglial cells were from CD200R1$^{-/-}$ mice show higher LDH release to the medium both, in control or stimulated (LPS/IFN-γ) conditions in comparison with co-cultures in which microglia were from wild type mice. The lack of CD200R1 completely prevented the neuroprotective effect of CD200Fc on LPS/IFN-γ stimulated conditions. Results are the mean ± SEM from three independent experiments in triplicate, $n = 9$. (**C**) AEA (10 μM) treatment of neuron-microglia co-cultures significantly decreased neuronal death induced by LPS/IFN-γ activated microglial cells ($P < 0.001$). Results are the mean ± SEM from four independent experiments in triplicate, $n = 12$. (**D**) The lack of CD200R1 prevented the neuroprotective action of AEA in the co-culture model under LPS/IFN-γ activated conditions ($P < 0.001$). Results are the mean ± SEM from three independent experiments in triplicate, $n = 9$. ***$P < 0.001$ *versus* CTL; ###$P < 0.001$ *versus* LPS/IFN-γ.

AEA increases the production of IL-10 in microglia (Correa et al., 2010, 2011), we then evaluated whether IL-10 (20 ng/mL) increases neuronal survival in the neuron-microglia co-culture model using microglia from WT or CD200R1$^{-/-}$ mice. It is emphasized that separate experiments were done for the two sources of microglia. We observed (Fig. 5A,B) that IL-10 was able to protect neurons from microglia mediated damage in the both cases studied ($P < 0.001$). However, the percentage of reduction of neuronal death induced by IL-10 was significantly higher ($P < 0.001$) when microglia were from WT (47.04%) in comparison with microglia from CD200R1$^{-/-}$ mice (21.69%). Therefore, the lack of CD200R1 in microglia diminished the capability of IL-10 to protect neurons from inflammatory damage. This suggests that the neuroprotection induced by IL-10 implies, at least in part, the interaction CD200-CD200R1. Next, we addressed whether IL-10 regulate the expression of CD200 in neurons. We found that CD200 was significantly enhanced in primary neurons treated with IL-10 (20 ng/mL) ($P < 0.05$; Fig. 5C). Thus, the possibility that IL-10 might contribute to the neuroprotective effects of AEA through the CD200-CD200R interaction cannot be ruled out. Next, we addressed the role played by IL-10 in the neuroprotective effect of AEA by using microglia derived from IL-10$^{-/-}$ mice in our co-culture system. As it is shown in Fig. 5D the lack of IL-10 is critical for AEA-induced neuroprotection. Thus, AEA did not modify the level of neuronal death induced by activated microglia from IL-10$^{-/-}$ mice ($P < 0.001$; Fig. 5D). These findings point out a pivotal role of IL-10 in the regulation and control of neuronal survival by AEA under CNS inflammation.

Temporal Pattern of Expression of CD200 and CD200R1 in TMEV-Induced Demyelinating Disease

Previous studies have showed decreased expression of CD200 in active chronic lesions in postmortem tissue from MS patients (Koning et al., 2007). However, little

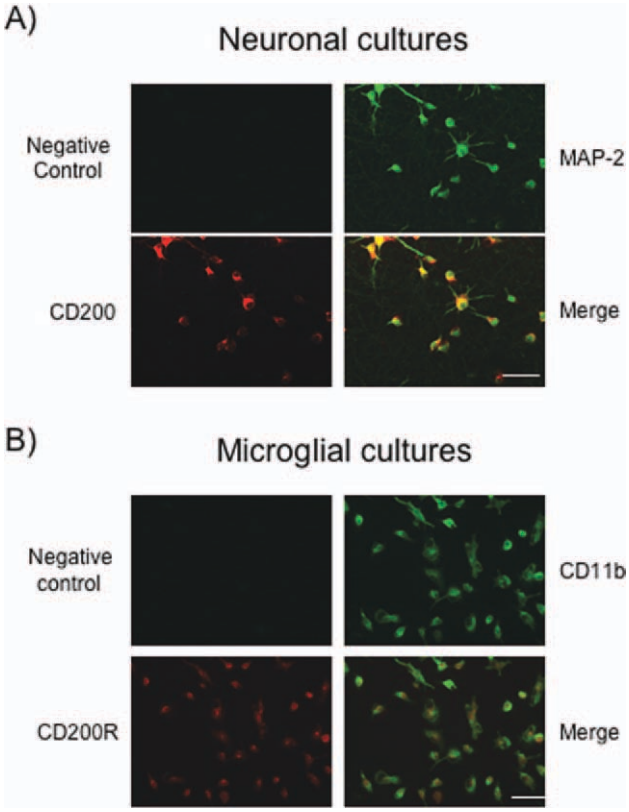

Fig. 2. (**A**) Confocal micrographs of neuronal cultures showing colocalization of CD200 (red) immunoreactivity with cells demonstrating reactivity to neuronal marker MAP-2 (green) (see merge). Original magnification ×40. (**B**) Confocal micrographs of microglia cultures showing colocalization of CD200R (red) with cells showing reactivity to microglial marker CD11b (green) (merge). Original magnification ×40.

is known about the temporal pattern of expression of CD200 and its receptor in TMEV-IDD. To address this question, we collected the spinal cords of sham and TMEV-infected mice at different time-points and analyzed mRNA expression of CD200 (Fig. 6A), and CD200R1 (Fig. 6B). On the basis of previous studies (Loria et al., 2008; Mc Mahon et al., 2005) the timing of gene study was chosen to differentiate the expression of these molecules on the spinal cord when viral antigens start to be detected in the spinal cord, 21 days pi *versus* their expression on ongoing demyelinating disease, at 35 days pi (asymptomatic phase), at 60 days pi (early chronic symptomatic phase), and at 90 days pi (late chronic phase). As shown in Fig. 6A, CD200 mRNA followed a biphasic pattern of expression, with a peak at day 21 pi ($P < 0.01$) in TMEV-infected mice when compared with the level of expression in the sham ones, then the expression of CD200 started to decrease at 35 days and at 60 and 90 days pi reached statistical significance ($P < 0.05$). A similar pattern was observed for CD200R1 at 60 and 90 days pi as we found a significant reduction of mRNA CD200R1 expression (Fig. 6B; $P < 0.05$). However, at 21 and 35 days pi, mRNA CD200R1 changes did not reach statistical significance in comparison to mRNA levels found in time matched sham animals.

The Inhibition of FAAH as well as AEA Delivery by Miniosmotic Pumps Increase CD200mRNA and CD200R1mRNA Expression in the Spinal Cord of TMEV-Infected Mice and Improve their Motor Behavior

We next examined the effects of manipulating AEA activity on CD200 and CD200R1 gene expression in late chronic phases in TMEV-IDD. Then, CD200 and CD200R mRNAs were analyzed in spinal cord samples from TMEV-infected mice subjected to a subchronic treatment with an inhibitor of FAAH (AA-5HT), the main enzyme involved in AEA hydrolysis (Cravatt et al., 2001) (Fig. 7A,B). The level of expression of CD200 that was significantly reduced in TMEV-infected mice ($P < 0.05$) at 90 days pi (Fig. 7A) was increased ($P < 0.05$) in the mice subjected to the treatment with AA-5HT. The same profile of response was reflected in the case of CD200R1 as we found that the reduced expression of CD200R1 in TMEV-infected mice ($P < 0.01$; 90 days pi) was counteracted and even increased in AA-5HT treated mice ($P < 0.01$). To confirm the specific role of AEA we addressed whether AEA treatment can reproduce the effects observed with AA-5HT. For this, we performed a set of experiments in which TMEV-infected mice were implanted with miniosmotic pumps to produce a continuous deliver of AEA for 7 days. AEA treatment also led to increased expression of CD200 (Fig. 7C) and of CD200R1 (Fig. 7D) in the spinal cord of TMEV-infected mice. Therefore, different approaches involving increased AEA activity were capable to up-regulate the expression of CD200 and CD200R1 in a viral model of MS. We also examined the effects of the above treatments on the motor behavior displayed by TMEV-infected mice. As expected, TMEV-infected mice exhibited a lower spontaneous motor activity as shown in Fig. 7E,F when compared with sham mice. The parameters of vertical activity (VACT) and horizontal activity (HACT) were discriminated and analyzed separately. After the treatment with the inhibitor of FAAH, TMEV infected mice showed a recovery in their ambulatory capacity ($P < 0.05$) and the VACT counts also increased when compared with their littermates treated with vehicle but without reaching statistical difference (Fig. 7E). Similarly, infected mice treated with AEA showed higher spontaneous HACT when compared to vehicle treated mice (Fig. 7F; $P < 0.001$) and there was a tendency to increase their VACT.

The Inhibition of FAAH as well as AEA Delivery by Miniosmotic Pumps Increase mRNA Expression of IL-10 Whereas IL-1β and IL-6 mRNA Levels are Decreased in the Spinal Cord of TMEV-Infected Mice

In an attempt to evaluate if increased AEA might affect cytokine levels in the spinal cord of TMEV-infected mice we performed a set of experiments in which gene expression of IL-10, IL-1β, and IL-6 were

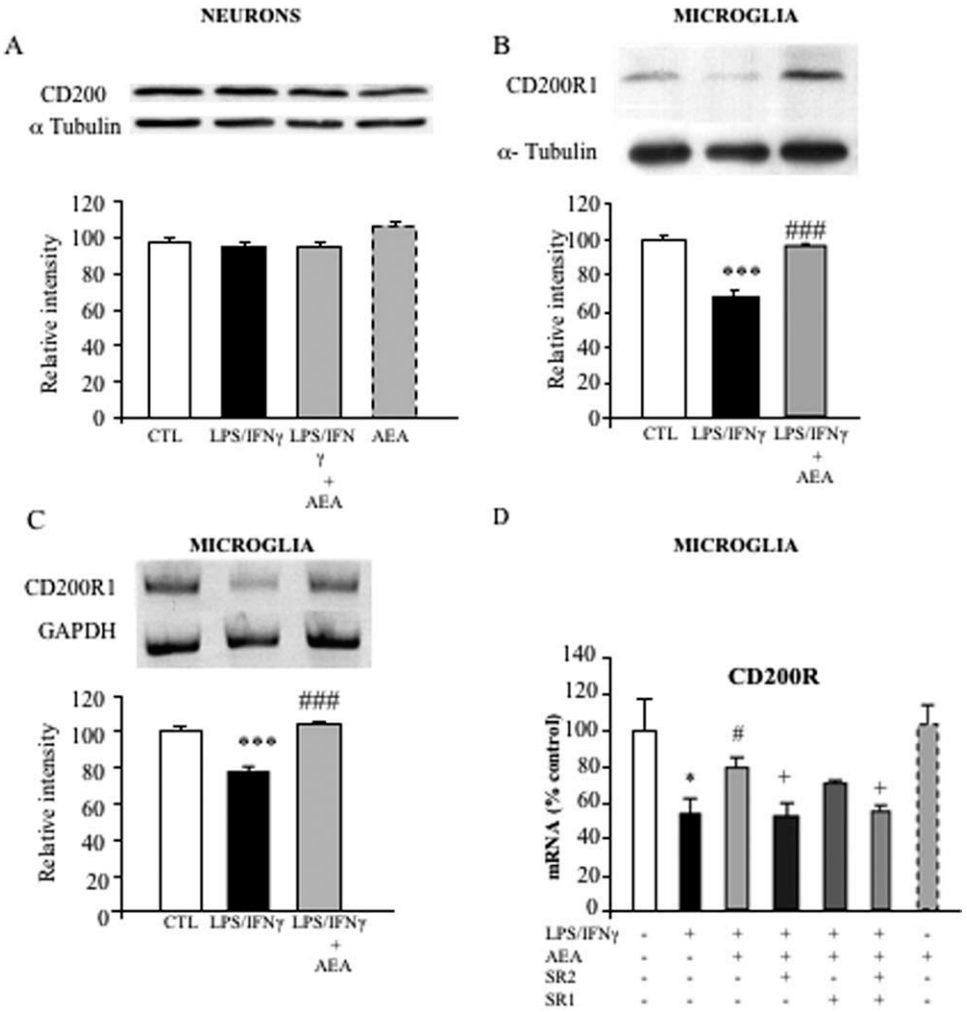

Fig. 3. AEA increases CD200R1 expression in LPS/IFN-γ activated microglial cells: involvement of CB_2 receptors. (**A**) Expression of CD200 on murine neuronal cultures was not modified after 2 h LPS/IFN-γ exposure in the presence or absence of AEA (10 μM). Results are representative of four independent experiments in triplicate. (**B**) Expression of CD200R1 on murine microglial cells 2 h after LPS/IFN-γ exposure in the presence or absence of AEA (10 μM). One representative blot is shown. Densitometric analysis shows a decrease in CD200R1 by LPS/IFN-γ ($P < 0.001$). Treatment with AEA (10 μM) increases CD200R1 ($P < 0.001$). Results are the mean ± SEM of four independent experiments in triplicate. (**C**) Expression of CD200R1 mRNA in murine microglial cells as evaluated by conventional PCR. Levels of mRNA CD200R1 were decreased by LPS/IFN-γ ($P < 0.001$) and AEA induces the recovery of its expression ($P < 0.001$). Results are the mean of four independent experiments in triplicate. (**D**) Results of real time PCR analyses for CD200R1 mRNA expression in LPS/IFN-γ activated microglia in the presence or absence of AEA (10 μM). Results also show the effects of pretreatment with the CB_1 receptor antagonist, SR1 (1 μM), the CB_2 receptor antagonist, SR2 (1 μM) or the combined treatment of both, SR1 + SR2. AEA induces a recovery of CD200R1 mRNA expression by activation of CB_2 receptors. Data are the mean ± SEM of three independent experiments in triplicate. Statistics: $^{*}P < 0.05$ *versus* CTL; $^{***}P < 0.001$ *versus* CTL; $\#P < 0.05$ *versus* LPS/IFN-γ; $\#\#\#P < 0.001$ *versus* LPS/IFN-γ; $+P < 0.05$ *versus* LPS/IFN-γ + AEA.

assessed by real-time PCR. Interestingly, animals treated with AA-5HT or with AEA showed an up-regulation of transcripts for IL-10 ($P < 0.05$ and $P < 0.01$ respectively), that were decreased in TMEV-infected mice ($P < 0.05$) as it is shown in Fig. 8A,B. In an opposite way IL-1β (Fig. 8C,D) and IL-6 (Fig. 8E,F) mRNA levels were drastically reduced in TMEV-infected mice subjected to inhibition of FAAH ($P < 0.01$) or that received AEA ($P < 0.01$). As expected, TMEV-infected mice expressed higher levels of IL-1 β ($P < 0.001$) and IL-6 ($P < 0.05$) when compared with sham mice (Fig. 8C–F).

DISCUSSION

In the past years, several *in vitro* and *in vivo* studies suggested that the ECS participates in the control of immune responses and in CNS protection. In this study, we have focused on the effects of AEA on neuron microglia crosstalk via CD200-CD200R interaction and its role on neuronal survival. Our results show that AEA, by enhancing CD200-CD200R interaction, plays a critical role in neuronal protection in the setting of inflammation-mediated neurodegeneration. We also pointed out the possible participation of IL-10 in AEA effects.

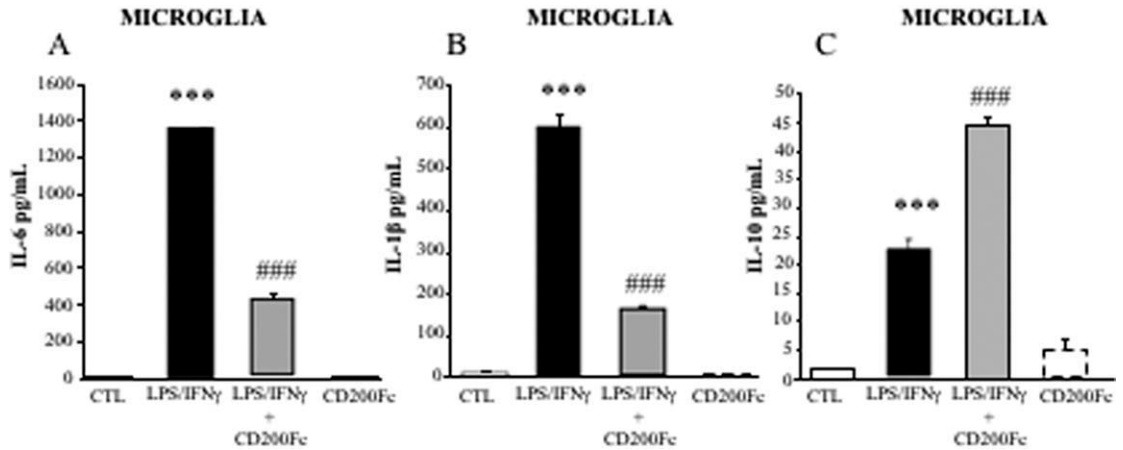

Fig. 4. CD200Fc treatment regulated microglia responses to LPS/IFN-γ. Cytokine secretion (IL-6, IL-1β, and IL-10) were determined by ELISA in supernatants from microglia cultures exposed to LPS/IFN-γ (50 ng/100 U) and/or CD200Fc (5 μg/mL). LPS/IFN-γ- induced IL-6 (**A**) and IL-1β (**B**) secretion ($P < 0.001$) was significantly ($P < 0.001$) inhibited when CD200R1 was activated by CD200Fc. (**C**) In contrast, LPS/IFN-γ- induced IL-10 secretion ($P < 0.001$) was significantly enhanced when CD200R1 was activated by CD200Fc ($P < 0.001$). Results are the mean ± SEM of four independent experiments in triplicate. Statistics: $^{***}P < 0.001$ *versus* CTL; $^{\#\#\#}P < 0.001$ *versus* LPS/IFN-γ.

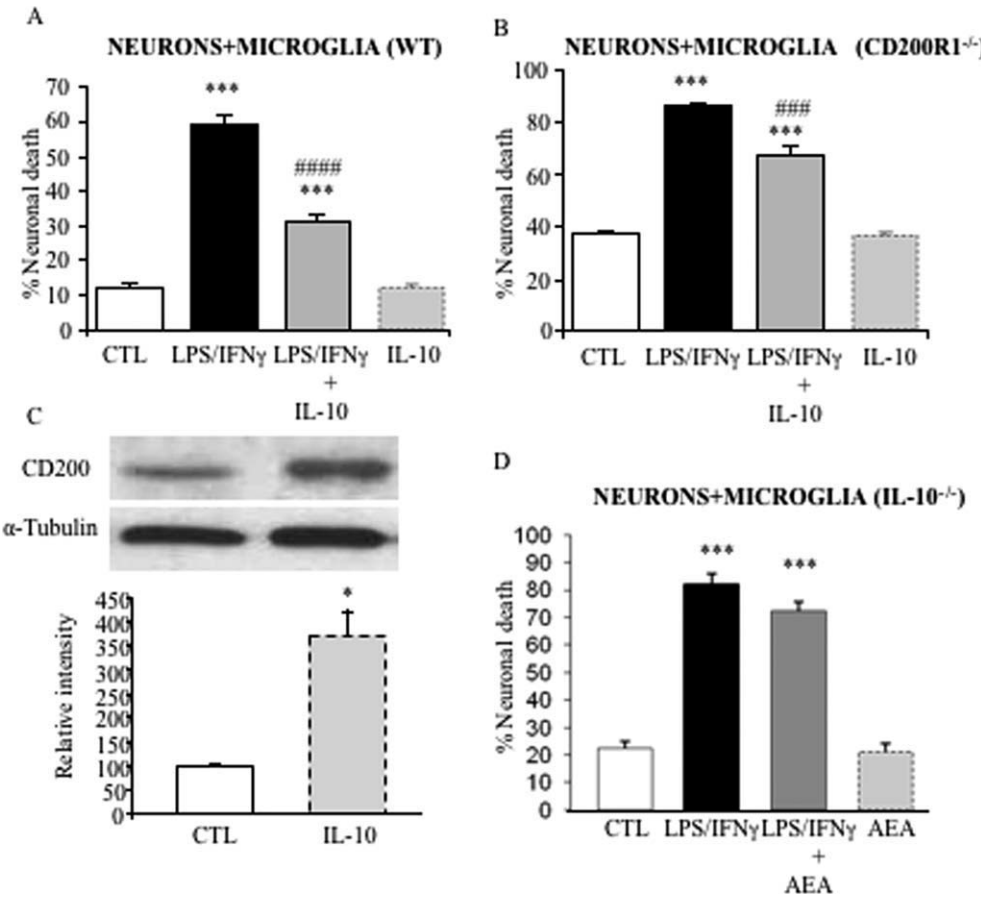

Fig. 5. IL-10 exerts neuroprotective effects in the microglia mediated cytotoxicity model and enhances the expression of CD200 in neurons. (**A**) IL-10 (20 ng/mL) protects neurons ($P < 0.0001$) from microglia mediated inflammatory damage in the co-culture model when microglial cells were from WT mice. Results are the mean ± SEM of three independent experiments in triplicate. Statistics: $^{***}P<0.001$ *versus* CTL; $^{\#\#\#\#}P < 0.0001$ *versus* LPS/IFN-γ; (**B**) IL-10 (20 ng/mL) reduces neuronal death induced by activated microglia from CD200R1$^{-/-}$ mice, but the lack of CD200R1 in microglia diminishes the neuroprotective effect of IL-10 in comparison with the results obtained when microglia cells were from WT mice. Results are the mean ± SEM of three independent experiments in triplicate. Statistics: $^{***}P < 0.001$ *versus* CTL; $^{\#\#\#}P < 0.001$ *versus* LPS/IFN-γ. (**C**) IL-10 (20 ng/mL) increases CD200 protein expression in neuronal cultures ($P < 0.05$ *vs*. CTL). It is shown one representative blot. Densitometric analyses are the mean ± SEM from three independent experiments in triplicate. (**D**) Neuron-microglia co-cultures were performed by using microglia from IL-10$^{-/-}$ mice. LPS/IFN-γ induces a high level of neuronal death and AEA (10 μM) was not capable to exert neuroprotection under the conditions of lacking IL-10. Results are the mean ± SEM of three independent experiments in triplicate. Statistics: $^{***}P < 0.001$ *versus* CTL.

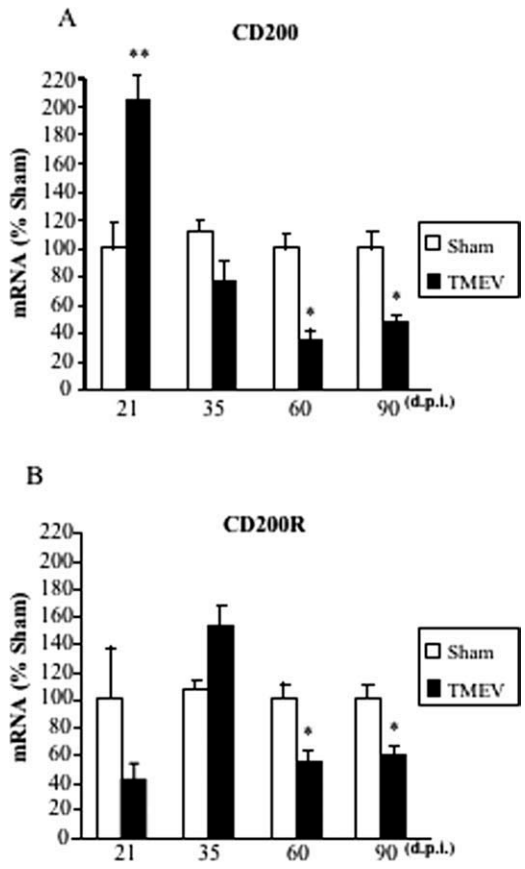

Fig. 6. Temporal course of expression of CD200 and CD200R1 mRNA in the spinal cord of TMEV infected mice. SJL/J mice were infected with TMEV (10^6 PFU) and evaluated for spinal cord expression of CD200 and CD200R1 by real-time PCR at different periods of time pi: 21, 35, 60, and 90 days. Sham uninfected mice were inoculated with the viral vehicle solution. (**A**) CD200 mRNA is up-regulated at 21 pi, assessed by TaqMan RT-PCR, and significantly downregulated at 60 and 90 days pi in TMEV-infected mice (black bars) when compared with sham mice (white bars). Relative quantification was obtained by calculation the ratio between the values for CD200 and the house-keeping gene GAPDH. Results are expressed as a percentage of sham for each time (n = 6–10). Statistics: $^*P < 0.05$ *versus* sham animals at the same time point. (**B**) CD200R1mRNA assessed by TaqMan RT-PCR is significantly down-regulated at 60 and 90 days pi in TMEV-infected mice (black bars) when compared with sham mice (white bars). Relative quantification was obtained by calculation the ratio between the values for CD200R1 and the house-keeping gene GAPDH. Results are expressed as a percentage of sham for each time (n = 6–10). Statistics: $^*P < 0.05$ *versus* sham animals at the same time point.

Finally, we described how AEA drives a recovery of CD200 and CD200R1 mRNA levels that were reduced at late chronic phases in TMEV-IDD.

Neurons and microglia are in close contact with each other and the outcome of neuronal death or survival is dependent on intercellular interactions directly or through released factors. We established a mixed culture system by adding activated microglia over a neuronal culture to address the effect of AEA in the communication between both types of cells through the CD200-CD200R interaction. It is important to note that both, AEA and the soluble form of CD200, the fusion protein CD200Fc, elicited a significant decrease in the level of neuronal death that was completely abrogated when microglia were obtained from CD200R1$^{-/-}$ mice. This pointed out the existence of a novel mechanism of neuroprotection exerted by AEA that involves CD200-CD200R interaction. The results of this study also show that microglial expression of CD200R1 was decreased by LPS/IFN-γ but restored after AEA treatment. Supporting this, AEA was unable to induce neuroprotection when microglia was from CD200R1$^{-/-}$ mice. These findings suggest the importance of microglial CD200R1 to control inflammatory damage. In human myeloid cells inhibition of cytokine production by CD200R engagement was positively correlated with CD200R expression levels (Jenmalm et al., 2006). Accordingly, *in vivo* studies have shown that systemic LPS induced decreases in brain transcript levels of CD200R which precede and contribute to microglia activation (Masocha, 2009). CB_1 and CB_2 receptors appear to be important for AEA-mediated neuroprotection from inflammatory damage (Eljaschewitsch et al., 2006); however, activation of CB_2 receptors has been more closely related to the inhibition of neuroinflammation in MS (Cabral et al., 2008; Docagne et al., 2008). Our results correlate well with this notion since we observed that CB_2 receptor activation was involved in AEA-mediated up-regulation of CD200R1 in microglia. Although there are several isoforms of CD200R in human and mouse, CD200R1 is the dominant ligand in both species (Wright et al., 2003). Signaling through inhibitory receptors can be essential for regulating microglia in the CNS (Gasque et al., 2000; Koning et al., 2009). In our study soluble CD200Fc diminished the production of IL-1β and IL-6 while increased IL-10 by activated microglia. This is an important point as CD200R1 engagement not only decreases the production of proinflammatory cytokines, but enhances the production of an anti-inflammatory cytokine. Interestingly, IL-10 protected neurons from microglia-induced cytotoxicity, but the lack of CD200R1 diminished its level of neuroprotection suggesting that CD200-CD200R interaction may be one of the mechanisms by which IL-10 protects neurons from inflammatory damage. Nevertheless, additional mechanisms are implicated in the ability of IL-10 to protect neurons as even in the absence of CD200R1, IL-10 was neuroprotective. Elevated neuronal expression of CD200 has been related to reduced macrophage/microglia responses and attenuated neurodegeneration *in vivo* and *in vitro* (Chitnis et al., 2007). In our study, AEA did not modify CD200 in neurons, but IL-10 increased the neuronal expression of CD200 in support of the hypothesis that enhanced microglial production of IL-10 by AEA (Correa et al., 2010, 2011) may contribute to AEA-induced neuroprotection. Confirming the above idea, AEA lose its ability to protect neurons from inflammatory damage when microglia was obtained from IL-10$^{-/-}$ mice. From the above findings we suggest that AEA contribute to neuronal protection via two main mechanisms: the up-regulation of CD200R1 and the elevated production of IL-10. As a consequence of AEA treatment, IL-10 released from microglia would increase the expression of CD200 in neurons, amplifying the interaction of CD200-

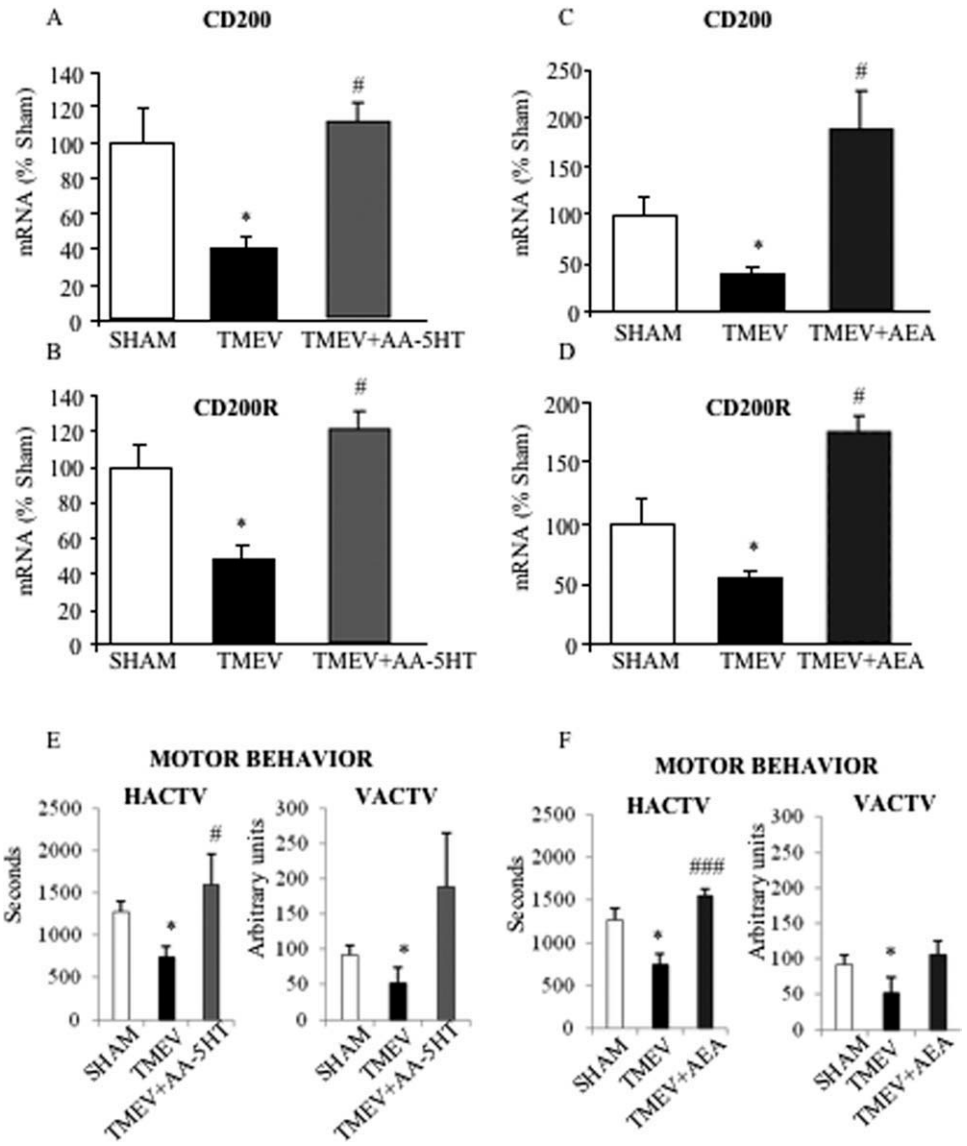

Fig. 7. The inhibition of FAAH or AEA administration increases CD200 mRNA and CD200R1 mRNA expression and improves motor behavior in TMEV-infected mice. TMEV-infected mice were subjected at 78 days pi to administration of AA-5HT (5 mg/kg; ip), for 12 consecutive days. Animals were sacrificed at 90 days pi. (**A**) TMEV-infected mice show decreased levels of CD200 mRNA as compared with sham mice ($P < 0.05$). AA-5HT treatment significantly increases CD200 mRNA levels in the spinal cord of TMEV-infected mice ($P < 0.05$). (**B**) The expression of mRNA CD200R1 is decreased in TMEV-infected mice ($P < 0.05$) and AA-5HT treatment significantly increased its expression ($P < 0.05$). (**C**) and (**D**) TMEV-infected mice were subjected at 83 days pi to the delivery of AEA (3.5 μg/μL) by miniosmotic pumps (1 μL/h) for 7 days and animals were sacrificed at 90 days pi. AEA treatment leads to increased levels of mRNA CD200 ($P < 0.05$; C) and CD200R1 ($P < 0.05$; D). (**E**) and (**F**) TMEV-infected mice exhibited a lower spontaneous motor activity when compared with sham mice. Inhibition of FAHH and AEA treatment improve spontaneous deambulatory activity ($P < 0.05$, E; $P < 0.001$, F). All values are the mean ± SEM from seven to nine mice per group. Statistics: *$P < 0.05$ *versus* sham; #$P < 0.05$ *versus* TMEV; ###$P < 0.001$ *versus* TMEV.

CD200R1. Because inappropriate macrophage or microglia activation is responsible for harmful inflammation, the up-regulation of CD200R1 and its ligand might be relevant in managing the inflammatory process in diseases like MS. In fact, CD200 deletion resulted in myeloid cell dysregulation and enhanced susceptibility to EAE and collagen-induced arthritis (CIA) indicating that CD200 normally induces immune suppression through CD200R1 (Hoek et al., 2000). In support of this, mice receiving soluble CD200 were resistant to CIA induction (Gorczynski et al., 2001; Simelyte et al, 2008) and CD200R1 agonists attenuated EAE symptomatology (Liu et al., 2010).

The pharmacological activation of cannabinoid system has been involved in the amelioration of neurological deficits in different models of MS (Arévalo-Martín et al., 2003; Baker, 2001; Croxford and Miller, 2003) as well as in other neurodegenerative diseases (Fernández-Ruiz et al., 2010; Romero and Orgado, 2009). The increased levels of 2-AG and AEA in different types of brain injury and in neuroinflammatory diseases has been suggested to be a protective physiological mechanism

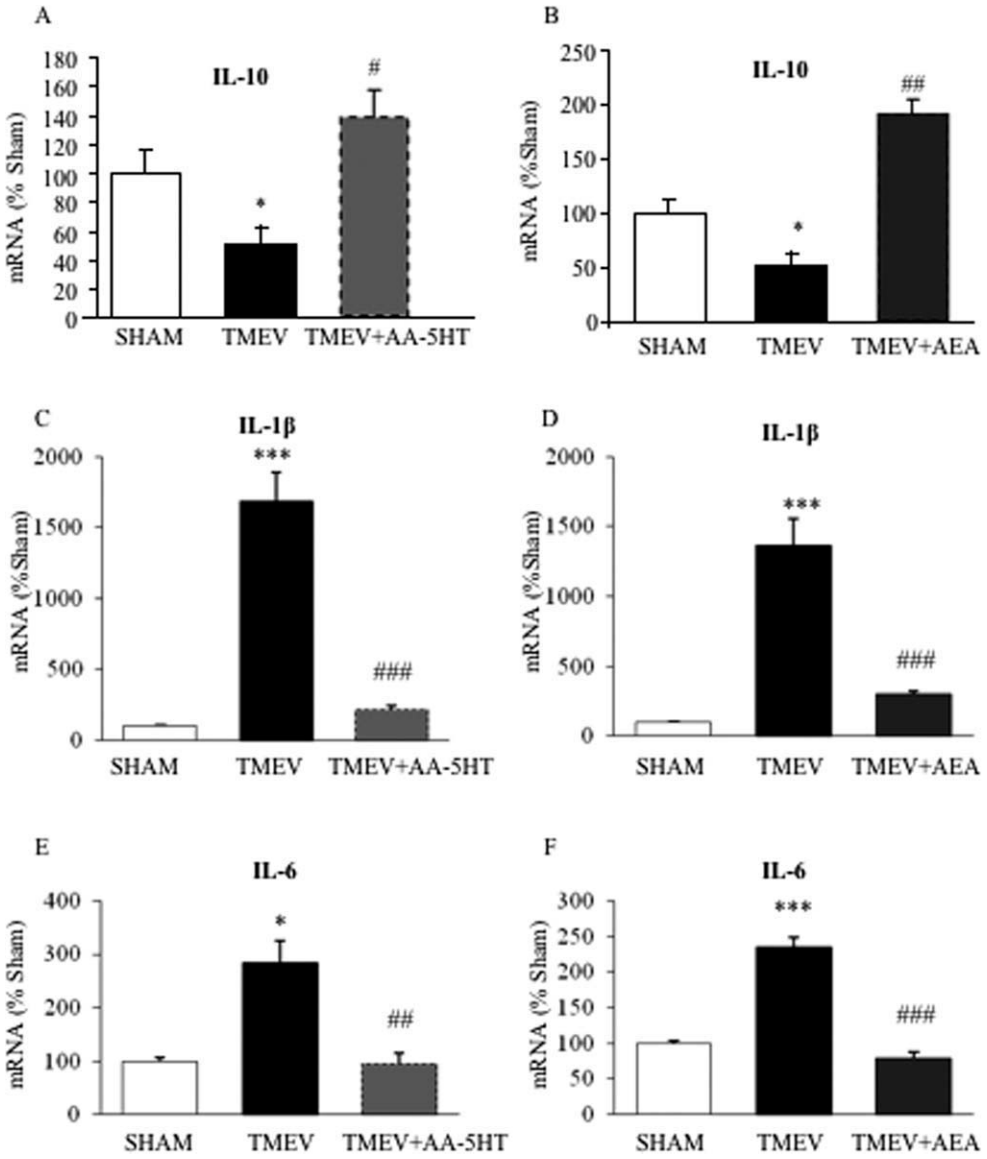

Fig. 8. The treatment with an inhibitor of FAAH or with AEA increases mRNA expression of IL-10 and decreases IL-1β and IL-6 mRNA levels in the spinal cord of TMEV-infected mice. Spinal cords were obtained from sham and TMEV-infected mice subjected or not to the corresponding treatments at 90 days pi. (**A**) and (**B**) TMEV infected mice show reduced levels of IL-10 mRNA ($P < 0.05$) and the treatment with AA-5HT (5 mg/kg for 12 consecutive days) or with AEA (3.5 μg/μL by miniosmotic pumps for 7 days) induces an up-regulation of transcripts for IL-10 ($P < 0.05$, A and $P < 0.01$, B). (**C**) and (**D**) TMEV infected mice show increases of mRNA IL-1β levels ($P < 0.001$, C, D) and mRNA IL-6 levels ($P < 0.05$ **E**; $P < 0.001$, **F**). IL-1β and of IL-6 mRNA levels were reduced in TMEV-infected mice subjected to the inhibition of FAAH ($P < 0.01$) or that received AEA ($P < 0.01$). Data are the mean ± SEM from seven to nine mice per group. Statistics: ***$P <$ 0.001; *$P < 0.05$ *versus* sham; ###$P < 0.001$ *versus*; TMEV; ##$P < 0.01$ *versus* TMEV #$P < 0.05$ *versus* TMEV.

(Eljaschewitsch et al., 2006; García-Ovejero et al., 2009; Loría et al., 2008). Cells of the innate immune system such as dendritic cells, macrophages, and microglia have been shown to produce endocannabinoids under inflammatory conditions (Matias et al., 2002; Mestre et al., 2005; Stella, 2009), likely to counteract overinflammation and so, to limit CNS damage. The endocannabinoid AEA was found to be increased in inflammatory lesions of patients with active MS (Eljaschewitsch et al., 2006). AEA was also elevated in the cerebrospinal fluid and in peripheral lymphocytes of relapsing MS patients (Centonze et al., 2007).

One possible mechanism through which AEA may control inflammation *in vivo* is via CD200-CD200R interaction. We have found in TMEV-IDD decreased expression of CD200 and CD200R1 in late chronic phases (60 and 90 days pi) accordingly with other studies that showed reduced expression of CD200 in active chronic lesions in MS patients (Koning et al., 2007). In Alzheimer disease it has been shown that CD200 and CD200R mRNAs were decreased in brain regions affected by the pathology (Walker et al., 2009) contributing to the maintenance of chronic inflammation. Although our study did not establish what cellular types are contributing to the

decreased gene expression of CD200 and CD200R1, it is likely that neurons and macrophages/microglia are the main contributors. However, we cannot excluded that astrocytes and oligodendrocytes might contribute to the changes of CD200 and CD200R as recent results have shown that astrocytes and oligodendrocytes express CD200R (Chitnis et al., 2007; Koning et al., 2009; Liu et al., 2010). In the present study the administration of AEA or increasing its endogenous level by inhibiting FAAH enhanced the expression of CD200 and CD200R1 in the spinal cord of TMEV-infected mice. Interestingly, the recovery of CD200 and CD200R1 in TMEV-IDD was accompanied by increases of IL-10 mRNA and reduced IL-1β and IL-6 mRNA levels.

Therefore, it is reasonable to assume that AEA targeting microglia/macrophages may limit local immune response by increasing CD200R1 and the anti-inflammatory cytokine IL-10. In this way, AEA favors an anti-inflammatory environment as IL-1β and IL-6 were dramatically reduced. In previous studies endocannabinoids attracted microglia to the lesion site by enhancing chemotaxis (Franklin and Stella, 2003) and there they help to control local immune response to prevent harmful overactivation. Here, we show that decreased expression pattern of CD200 and CD200R1 in TMEV-IDD was re-established following treatments involving an enhanced AEA activity that also contributed to disease improvement. All these data, considered together, suggest that the endocannabinoid system has a regulatory function in neuroinflammation, notion that is consistent with the fact that microglial cells, main players of inflammation events, have both the receptors and the molecular machinery to produce and degrade endocannabinoids (Stella, 2009).

In summary, we propose that the reduced expression of CD200 and CD200R1 is contributing to persistent chronic inflammation in TMEV-IDD. Augmentation or activation of this immune regulatory system has been shown to be protective in a number of animal models of chronic inflammatory diseases; this suggests that enhancement of CD200-CD200R interaction may have therapeutic value. As we showed that AEA increases CD200R1 on microglia and that activating CD200R1 promotes IL-10 release that, in turn increases neuronal expression of CD200, we propose that this molecular pathway may be underlying its anti-inflammatory actions. The results of the present study also support the interest of the pharmacological manipulation of the ECS as a promising therapeutic approach for the treatment of neurological deficits associated to the progression of MS.

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