Journal of Neurochemistry, 2005, **92**, 1327–1339 doi:10.1111/j.1471-4159.2004.02979.x

Pharmacological modulation of the endocannabinoid system in a viral model of multiple sclerosis

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Abstract

Theiler's virus infection of the central nervous system (CNS) induces an immune-mediated demyelinating disease in susceptible mouse strains and serves as a relevant infection model for human multiple sclerosis (MS). Cannabinoids have been shown to exert beneficial effects on animal models of MS and evidence suggests that the endocannabinoid system plays a role in the tonic control of spasticity. In this study we show that OMDM1 [(*R*)-*N*-oleoyl-(1′-hydroxybenzyl)-2′-ethanolamine] and OMDM2 [(*S*)-*N*-oleoyl-(1′-hydroxybenzyl)-2′-ethanolamine], two selective inhibitors of the putative endocannabinoid transporter and hence of endocannabinoid inactivation, provide an effective therapy for Theiler murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD). Treatment of TMEV-infected mice with OMDM1 and OMDM2 enhanced anandamide levels in the spinal cord and ameliorated motor symptoms. This was associated with a down-regulation of inflammatory responses in the spinal cord. In addition we show that OMDM1 and OMDM2 down-regulate macrophage function by (i) decreasing the surface expression of major histocompatibility complex (MHC) class II molecules, (ii) inhibiting nitric oxide synthase-2 (NOS-2) expression and (iii) reducing the production of the pro-inflammatory cytokines interleukin-1beta (IL-1β) and interleukin-12 (IL-12p40). Taken together, these results point to the manipulation of the endocannabinoid system as a possible strategy to develop future MS therapeutic drugs.

Keywords: endocannabinoid inactivation, experimental multiple sclerosis, macrophages.

J. Neurochem. (2005) **92,** 1327–1339.

Macrophages/microglia are activated early in response to infection or injury and are major players in both innate and immune-mediated central nervous system (CNS) inflammatory responses, by performing both scavenger and antigen presenting cell functions. Multiple sclerosis (MS) is an immune-mediated inflammatory disease in humans that is characterized by peripheral T-cell responses to myelin proteins and demyelinating lesions in the brain and spinal cord associated with the presence of both CD4 T cells and activated microglia/macrophages (Noseworthy *et al*. 2000). Epidemiological evidence suggests that virus infection may trigger the development of MS (Johnson 1994). Theiler's

Received May 12, 2004; revised manuscript received October 29, 2004; accepted November 1, 2004.

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Abbreviations used: AEA, anandamide; 2-AG, 2-arachydonoyl glycerol; CB, cannabinoid; CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2; CNS, central nervous sytem; CREAE, chronic relapsing remitting experimental allergic encephalomyelitis; DA, Daniel's strain; FACS, fluorescence-activated cell sorting; IFN-γ, interferon-γ; IL-1β, interleukin-1β; IL-12, interleukin-12; IL-12p40, interleukin-12 subunit p40; LPS, lipopolysaccharide; MHC, major histocompatibility complex; MOI, multiplicity of infection; MS, multiple sclerosis; NO, nitric oxide; NOS-2, nitric oxide synthase-2; OMDM1, (*R*)-*N*-oleoyl-(1′-hydroxybenzyl)-2′-ethanolamine; OMDM2, (*S*)-*N*-oleoyl-(1′-hydroxybenzyl)-2′-ethanolamine; SR1, antagonist CB1 receptors; SR2, antagonist CB2 receptors; TMEV, Theiler's murine encephalomyelitis virus; TMEV-IDD, TMEV-induced demyelinating disease.

murine encephalomyelitis virus (TMEV) is a common enteric mouse picornavirus, but when injected into the CNS of susceptible strains of mice induces a demyelinating disease that serves as a highly relevant virus-induced model for human MS. Infection of SJL/J mice with TMEV results in a life-long persistent infection of macrophages, microglia, and astrocytes (Pena-Rossi *et al*. 1997; Zheng *et al*. 2001). A chronic progressive demyelinating disease is observed with the onset of symptoms around 30–60 post-infection days. Initial myelin damage is mediated by a bystander mechanism, wherein the primary effector cells are mononuclear phagocytes (microglia-macrophages) activated by inflammatory cytokines produced from TMEV-specific Th1 cells responding to viral epitopes that persist in the CNS. In the chronic phases of the disease, epitope spreading leads to T cell responses to myelin autoepitopes (Miller *et al*. 1997). Mice exhibit progressive impaired motor coordination, incontinence and paralysis associated with axonal loss and spinal cord atrophy (McGavern *et al*. 2000).

Cannabinoids, the bioactive components of *Cannabis sativa*, as well as synthetic cannabinoid-related compounds, owe the majority of their activities to activation of the known cannabinoids receptors, CB1 and CB2 (Matsuda *et al*. 1990; Howlett *et al*. 2002) as well as of possible yet to be identified cannabinoid-like receptors (Di Marzo *et al*. 2000; Breivogel *et al*. 2001). Furthermore, fatty acid-derived endogenous ligands of cannabinoid receptors, the endocannabinoids anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) (Devane *et al*. 1992; Mechoulam *et al*. 1995), and a degradation system including a re-uptake mechanism and hydrolytic enzymes for these compounds, have been identified (Di Marzo *et al*. 1998). Cannabinoids have been reported to alleviate spasticity and tremor in mice with chronic relapsing experimental allergic encephalitis (CREAE) (Baker *et al*. 2000), in agreement with their inhibitory effects on pain and motor behaviour. Of interest is that spastic mice showed increased levels of endocannabinoids in brain and spinal cord, suggesting a tonic control of spasticity by the endocannabinoid system, at least in the case of CREAE mice (Baker *et al*. 2001).

Most of the current therapeutic treatments for MS involve strategies related to immune modulation (Compston and Coles 2002). In this line, several recent reports described beneficial effects of cannabinoids, based on their anti-inflammatory and immunomodulatory properties, on viral models of MS such as TMEV encephalomyelitis. Thus, amelioration of clinical scores by treatment with a cannabinoid receptor agonist WIN 55212 was associated with decreased viral and myelin specific Th1 effector functions (Croxford and Miller 2003). Besides, our group showed recovery of motor function and enhancement of remyelination in the spinal cord of TMEV-infected mice, possibly through the attenuation of inflammation and/or promoting the survival of oligodendroglial progenitors following the treatment with CB1 and CB2 cannabinoid receptor agonists (Molina-Holgado *et al*. 2002; Arévalo-Martín *et al*. 2003). Because results from the first large-scale randomized trial to assess the potential benefit of cannabis in the treatment of MS suggested some improvement in patients' mobility and pain perception (Zajicek *et al*. 2003), it is necessary to gain further insights into new therapeutic targets related to the pharmacological manipulation of the endocannabinoid network. The present study investigated the potential protective effects in the TMEV-induced demyelinating disease (TMEV-IDD) model of MS of selective inhibitors of endocannabinoid cellular uptake (Ortar *et al*. 2003), which by preventing the degradation of endocannabinoids enhance their half-life *in vivo*. The effects of these inhibitors on the expression of activation markers of macrophages, was also investigated.

Materials and methods

Animals and Theiler's virus inoculation

We used female SJL/J mice, susceptible to TMEV-IDD development, from our in-house colony (Cajal Institute, Madrid), maintained on food and water *ad libitum* in a 12-h dark–light cycle. Four-week-old mice were inoculated intracerebrally in the right cerebral hemisphere with 10^6 plaque forming units (PFU) of Daniel's (DA) TMEV strain in 30 μL of Dulbecco's modified Eagle's medium supplemented with 10% of fetal calf serum as previously described (Arévalo-Martín *et al*. 2003). Handling of animals was performed in compliance with the guidelines of animal care set by the European Union (86/609/EEC).

Evaluation of motor function

To evaluate neurological deficits of mice, we used the rotarod test, which measures balance, coordination and motor control. The rotarod apparatus (Ugo Basile, Milan, Italy) consists of a suspended rod able to run at constant or at accelerating speed. All mice were exposed to a training period at constant speed to familiarize them with the apparatus prior to treatments and at accelerating speed while the test was being performed. Data were collected from mouse rotarod performance 1 day before the beginning of treatment, and 1 day after the end of the treatment. The trial was terminated when mice fell from the apparatus or after a maximum of 5 min. The screening for locomotor activity was performed using an activity cage (Activity Monitor System Omnitech Electronics, Inc., Columbus, OH, USA) coupled to a Digiscan Analyser. Data for the following variables of locomotor activity for a session of 10 min were collected every 5 min (1–5 min: 1; 5–10 min: 2): (i) horizontal activity, as the duration time (s) travelled by the animal, and (ii) vertical activity, as the total number of beam interruptions in the vertical sensor. Data were analysed using analysis of variance (ANOVA) and by a *post hoc* Tukey multiple comparison test, when appropriate. For multiple data obtained by the repeated measurement from one subject, we used ANOVA for repeated measures.

Experimental procedure

At 60 days after TMEV infection, motor dysfunction was tested by both, the rotarod assay and the activity cage. Sham-mice (control,

$n = 40$) and TMEV-infected mice ($n = 40$) were assigned to five groups ($n = 8$), with initially no significant differences between them in their ability to perform the rotarod test. Mice from these groups were injected intraperitoneally once a day for 10 days with OMDM1 [(*R*)-*N*-oleoyl-(1′-hydroxybenzyl)-2′-ethanolamine] or OMDM2 [(*S*)-*N*-oleoyl-(1′-hydroxybenzyl)-2′-ethanolamine] (2 mg/kg or 7 mg/kg) or vehicle (5% bovine serum albumin and 0.2% dimethylsulfoxide in phosphate-buffered saline). Doses were calculated based on their cannabinoid receptor binding affinities (Ortar *et al*. 2003) and on their activity *in vivo* in rodents (de Lago *et al*. 2004). We also evaluated locomotor activity in control or TMEV-infected mice subjected to the same treatment with OMDM1 and OMDM2, or the appropriate vehicle using the activity cage for a 10-min session. To evaluate the effects of OMDM1 and OMDM2, motor behavioural tests were performed 1 day after the corresponding treatments. Sham OMDM1 and OMDM2-treated mice displayed similar locomotor responses than vehicle treated mice.

Tissue processing and immunohistochemistry

Mice were anaesthetized by intraperitoneal pentobarbital administration (50 mg/kg body weight) and perfused transcardially with 4% paraformaldehyde in 0.1 mol/L phosphate buffer. Spinal cords were collected, the tissue was post-fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer, cryoprotected with a 30% solution of sucrose in 0.1 mol/L phosphate buffer and frozen in dry ice. We then obtained 35 μm-thick coronal cryostat sections and processed them to visualize microglia/macrophages using Mac-1 anti-CD11b antibody (Serotec Ltd, Oxford, UK). Immunostaining was visualized with Alexa-conjugated secondary anti-mouse (Molecular Probes, Eugene, Oregon, USA). Specificity of staining was confirmed by omitting the primary antibody. Five randomly selected spinal cord images per mouse at different levels were obtained by confocal microscopy, with constant laser beam intensity and photodetector sensitivity. Images were analysed with NIH Image software fixed to detect Mac-1^+ cells larger than 25 μm^2, a size we consider as reactive microglia. To confirm that this was a correct size threshold, we analysed spinal cords from sham mice (control animals), and the software detected no activated cells in spinal cord**.** For routine histological examination, adjacent spinal cord sections were stained with Tomato lectin (biotinylated lectin from *Lycopersicon esculetum*, Tomato, L-9389, Sigma, Madrid, Spain) for the visualization of microglial cells. Briefly, after endogenous peroxidase blocking with 2% hydrogen peroxide in 100% methanol, sections were incubated with the biotinylated lectin. After incubation, sections were washed in Tris-buffered saline and incubated with avidin-peroxidase (A-3151, Sigma) followed by counterstaining with toluidine blue. The peroxidase reaction product was visualized using 3,3′-diaminobenzidine method.

Macrophage cultures

Peritoneal exudate macrophages were harvested by peritoneal lavage of SJL/J female mice with ice-cold sterile Hank's balanced salt solution (Sigma) 3 days after intraperitoneal injection of mice with 2 mL of 5% thioglycollate broth (Sigma). Cells were centrifuged at low speed 10 min at room temperature (22–24°C) and resuspended in Gey's red cells lysis buffer. After 20 min of incubation at room temperature, cells were centrifuged and resuspended in fresh Dulbecco's modified Eagle's medium supplemented with 10% inactivated fetal bovine serum (Gibco BRL; Life Tec. Ltd Ger), 100 U/mL penicilin and 100 μg/mL streptomycin (Gibco BRL, Life Technologies Ltd, Karlsruhe, Germany). Cells were seeded in 12-well plates (0.5×10^6 cells/well) and incubated overnight at 37°C in a humified atmosphere containing 5% CO_2. One hour prior to stimulation, non-adherent cells were removed by washing twice with Dulbecco's modified Eagle's medium and macrophages were resuspended in fresh culture medium supplemented with antibiotics and 5% fetal bovine serum. Five minutes previous to lipopolysaccharide (LPS; *Escherichia coli*, Serotype 026:B6, Sigma) and interferon-γ (IFN-γ; PeproTech, London, UK) stimulation, macrophages were exposed to 1, 5, 10 or 15 μM AEA (Sigma) and/or cannabinoid-related compounds added at final concentrations of 5 and 10 μM in the case of OMDM1 and OMDM2. CB1 and CB2 receptor antagonists, SR141716A [*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide] and SR144528 [(*N*-[1*S*]-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl)-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide], respectively, were gifts from Sanofi (Montpellier, France), and added at doses of 1 μM. LPS and IFN-γ were diluted in Dulbecco's modified Eagle's medium and, based in our previous experiments (Molina-Holgado *et al*. 2001), added to each well at a final concentration of 50 ng/mL and 100 U/mL, respectively. Stimulated cells were incubated for 18 h at 37°C in a humidified atmosphere with 5% CO_2. After this time, cells were harvested for protein measurement and supernatants were collected for cytokine determination. Trypan dye exclusion testing or the 3,4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide thiazol blue test indicated that the cannabinoid-related compounds did not affect cell viability.

Infection of macrophage cultures

Macrophage cultures from SJL/J mice were infected with the DA strain of TMEV at a multiplicity of infection (MOI) of 5 PFU per cell. Cell cultures were washed twice to remove serum components and 0.25 mL of appropriately diluted virus stock solution was added to each well. After adsorption of the virus for 2 h at 37°C cells, we added 0.75 mL of new medium containing only 2% fetal bovine serum. The supernatants from TMEV-infected and non-infected cultures were collected in parallel for cytokine determination at 24 h post-infection. Infected macrophages did not exhibit significant cytopathic effects 24 h after infection.

Western blot analysis

To determine nitric oxide synthase-2 (NOS-2) expression, macrophages were washed with ice-cold phosphate-buffered saline and lysed in 200 μL of Tris-buffered saline pH 7.6 containing 10% glycerol, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulphonyl fluoride, 50 μg/mL leupeptin, 10 μg/mL aprotinin, 5 mM benzamidine, 1 mM sodium orthovanadate, 2 mM NaF and 5 mM dithiothreitol. Whole-cell extracts were mixed with 5 × Laemmli sample buffer and boiled for 5 min. Equal amounts of protein (30 μg) were resolved on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted for 1 h at 4°C to nitrocellulose (Amersham Biosciences). The membrane blots were blocked for 1 h at room temperature in 5% (w/v) dry skim milk (Sveltese, Nestlé, Barcelona, Spain) in Tris-buffered saline containing 0.1% Tween-20 and then incubated with primary antibodies anti-NOS-2 (Signal Transduction, Calbiochem La Jolla, CA; dilution 1 : 2000) for 1.5 h. After extensive washing in 5%

milk–Tris-buffered saline plus Tween solution, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno-Research Laboratories, West Grove, PA, USA) and visualized by chemiluminescence using an enhanced chemiluminiscence western Blotting Detection Kit (Amersham Biosciences Europe, Barcelona, Spain). The signals were quantified by densitometry. To normalize for equal loading and protein transfer, the membranes were stripped in 62.5 mM Tris-HCl buffer pH 6.8 containing 2% sodium dodecyl sulfate and 0.7% β-mercaptoethanol and were reprobed by incubating with an antibody for α-tubulin (1 : 40000, Sigma).

Immunocytochemistry in cultured cells

Immunostaining was done directly on cells seeded on glass coverslips. Macrophages plated onto poly D-lysine-coated (5 μg/mL) coverslips were fixed in 4% paraformaldehyde in phosphate-buffered saline for 20 min at room temperature. Coverslips were then rinsed in phosphate-buffered saline and incubated for 2 h at room temperature with the Mac-1 anti-CD11b antibody at a dilution 1 : 500 (Serotec) or anti I-A class II antibody (1 : 1000, clone OX-6, Serotec). Immunostaining was visualized with Alexa-conjugated secondary (1 : 1000) anti-mouse IgG-Alexa 488 and anti-rat IgG Alexa antibodies (Molecular Probes). Non-specific interactions of secondary antibodies were confirmed by omitting primary antibodies. At least three independent cultures were examined.

Flow cytometric analysis

Cells (10^6/mL) were scraped and, after centrifugation, resuspended and incubated in flow cytometric analysis (FACS) blocking buffer (phosphate-buffered saline, 0,1% sodium azide, 5% fetal calf serum) at 4°C for 30 min. After three washes with FACS staining buffer (phosphate-buffered saline, 0,1% sodium azide, 2% fetal calf serum), the cells were resuspended and incubated with anti-mouse major histocompatibility complex class II (MHC II) (I-Ak):FITC (Clon 14 V18, Serotec) at 4°C for 30 min in the dark. After three washes with FACS buffer staining, cells were incubated with IgG:FITC (isotype control) at 4°C for 30 min in the dark. Cells were washed twice with FCAS staining buffer, resuspended in cold buffer and fixed with 1% paraformaldehyde. Samples were run on an EPICS XL FACScan instrument (Beckman Coulter Inc., Fullerton, CA, USA).

Cytokine enzyme-linked immunosorbent assay

IL-1β content in macrophage culture supernatants was measured by solid phase sandwich ELISA, using a monoclonal antibody specific for murine IL-1β (Biosource Int., Camarillo, CA, USA). The minimum detectable dose of mouse IL-1β was less than 7 pg/mL and the intra and inter coefficient of variation were 4.4 ± 0.5 and 8.9 ± 0.9, respectively. Levels of IL-12 subunit p40 (IL-12p40) in macrophage supernatants were quantified using specific ELISA kits purchased from Biosource Int., according to the manufacturer's instructions. The assay detected >2 pg/mL and the intra and inter assay coefficients of variations were 3.3–4.5% and 5.6–6.4%, respectively.

Effect of endocannabinoid uptake inhibitors on endocannabinoid levels

The levels of endocannabinoids in the spinal cords of vehicle- and inhibitor-treated TMEV-infected mice, and in vehicle- and TMEV-infected macrophages, were measured by means of isotope-dilution liquid chromatography coupled to atmospheric pressure chemical ionization mass spectrometry. Lipid extracts from tissues (obtained from TMEV and sham mice 1 day after the treatment with the endocannabinoid uptake inhibitors) and cells, in the presence of 50 pmol each of deuterated (d8) AEA and 2-AG, were obtained and pre-purified by means of open bed silica chromatography as previously described (Fontana *et al.* 1995), whereas the endogenous levels of the two endocannabinoids were measured by isotope dilution as described previously (Di Marzo *et al.* 2000; Marsicano *et al.* 2003). Data are reported as pmol (AEA) or nmol (2-AG) per g of wet tissue weight, in the case of the spinal cords, or as pmol (for both endocannabinoids) per mg of lipid extract, in the case of macrophages, ± SEM, and were compared by ANOVA followed by the Bonferroni's test. In the case of the spinal cord, treatments with OMDM-1 and OMDM-2 (at the 7 mg/kg dose) were carried out using the same conditions used to assess motor behaviour.

Results

Treatment with OMDM1 or OMDM2 improves motor function during established neurological symptomatology

Previous studies described poor ability to perform the rotarod test after demyelination induced by TMEV infection (McGavern *et al.* 1999, 2000). Accordingly, TMEV-infected mice exhibited significantly reduced ($p < 0.01$) ability in rotarod performance compared to control mice (Fig. 1a). To evaluate the effects of OMDM1 and OMDM2 we used two schedule doses, 2 mg/kg and 7 mg/kg. Both doses were effective in improving rotarod performance in the case of OMDM1. Figure 1(a), shows the results corresponding to the dose of 7 mg/kg 1 day after a 10-day treatment protocol with OMDM1 and OMDM2. As can be seen, mice showed increased ability to perform the test correctly after the administration of OMDM1 for 10 consecutive days ($p < 0.001$). In the case of OMDM2 the improvement in test performance did not achieve statistical significance. To further confirm the motor behavioural effects of OMDM1 and OMDM2 treatment, sham-mice (controls) and TMEV-infected mice were tested for spontaneous motor activity. The administration of OMDM1 and OMDM2 (schedule doses 2 and 7 mg/kg) did not induce significant changes in horizontal and vertical activity in sham-mice (Fig. 1b shows the results corresponding to the dose of 7 mg/kg) in agreement with previous findings in rats (de Lago *et al.* 2004). The spontaneous motor behavioural responses of OMDM1 and OMDM2 (schedule dose of 7 mg/kg) in TMEV-treated mice are shown in Fig. 1(c). The hypoactivity, as reflected by the horizontal distance (HACTV) travelled by TMEV-infected mice, was statistically counteracted by the treatment with both OMDM1 ($p < 0.001$) and OMDM2 ($p < 0.02$) for the first 5 min [HACTV(1)].

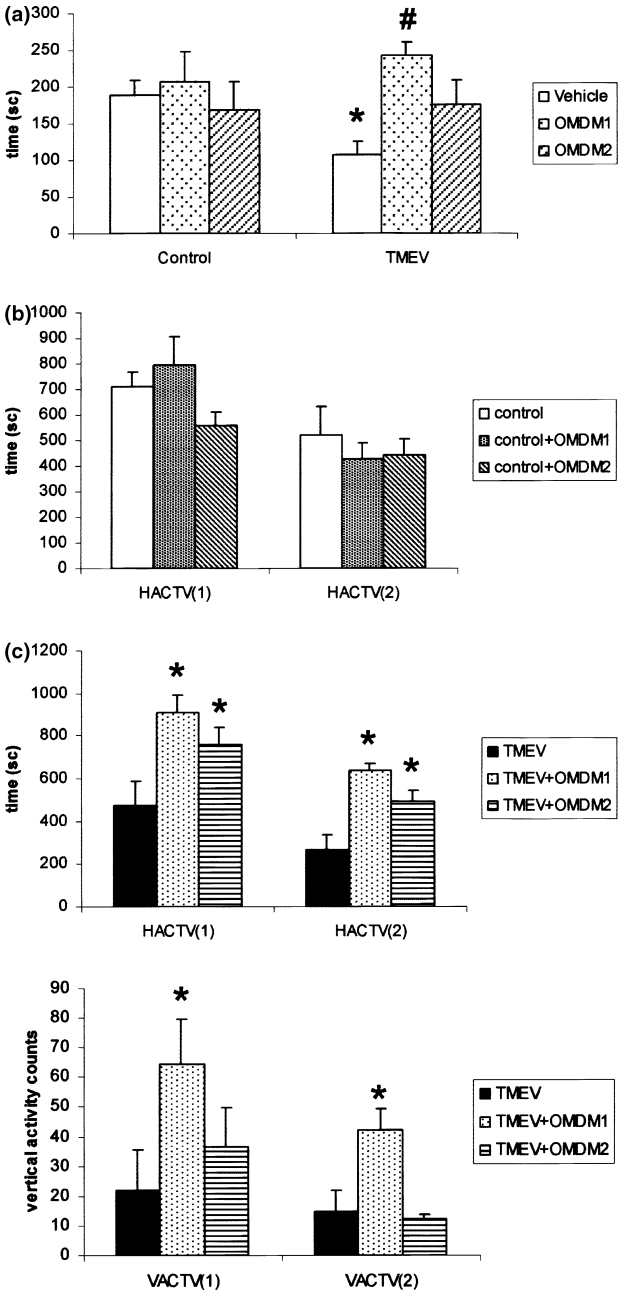

Fig. 1 The treatment with the selective inhibitors of endocannabinoids re-uptake, OMDM1 and OMDM2 (dose schedule: 7 mg/kg daily for 10 consecutive days) induce improvement of motor function 1 day after the cessation of the treatment. Theiler's murine encephalomyelitis virus (TMEV)-infected mice exhibited reduced ability in rotarod performance before OMDM1 and OMDM2 treatment compared with sham animals (control). (a) Data from the rotarod assay show a significant increase in motor function of OMDM1-treated mice 1 day after the end of the 10-day treatment protocol (*$p < 0.001$ vs. control mice; #$p < 0.01$ vs. TMEV-infected mice), OMDM2 administration to TMEV-infected mice increased latencies in rotarod test that did not reach statistical significance in comparison to vehicle TMEV-infected mice ($p = 0.447$); (b) Horizontal activity displayed in the activity cage by non-infected mice (control) 1 day after the treatment for 10 consecutive days with vehicle, OMDM1 or OMDM2. (c) Horizontal (HACTV) and vertical (VACTV) activity displayed in the activity cage by TMEV-infected mice 1 day after the treatment for 10 consecutive days with vehicle, OMDM1 or OMDM2 (*$p < 0.001$ vs. vehicle-TMEV-infected mice). The activity parameters were recorded every 5 min for a session of 10 min: 0–5 min (1); 5–10 min (2). Note that OMDM2 treatment increased deambulation for the session 5–10 (2), although the increments are in the limit of significance. Results are the mean ± SEM of seven to eight mice per group.

Results corresponding to the 5–10 min session [HACTV(2)] indicate that OMDM1 significantly improves horizontal activity ($p < 0.001$), whereas OMDM2 induced increases in deambulation that are in the limit of statistical significance. The vertical activity (VACTV) was also increased after the treatment with OMDM1 ($p < 0.05$) for the first 5 min session [VACTV(1)] as well as for the following 5 min [VACTV(2)], whereas OMDM2 administration did not modify this parameter. Therefore, in the activity cage test, again OMDM1 was more efficacious than OMDM2, in particular in relation with the vertical activity measurement.

Endocannabinoid levels in Theiler's murine encephalomyelitis virus-infected mice and macrophages

As shown in Table 1, treatment with OMDM-1 and, to a lesser extent, OMDM-2, significantly increased ($p < 0.05$) the levels of AEA, but not 2-AG, in the spinal cords of TMEV-infected mice. Interestingly, TMEV-infection caused a significant ($p < 0.05$) almost twofold enhancement of AEA levels in TMEV-infected macrophages (with only a trend towards enhancement for 2-AG), but not in the spinal cords from TMEV-infected mice (Table 1).

Actions of OMDM1 and OMDM2 on macrophage/microglial activation

TMEV-IDD is characterized by microglial activation in spinal cord, and cytotoxic factors produced by activated microglia have been associated with the ongoing demyelination. As shown in Fig. 2, microglial cells in the spinal cord of TMEV-infected mice show a reactive morphology in white and grey matter as assessed by immunolabelling with Mac-1. Interestingly, treatment with either OMDM1 or OMDM2 (schedule dose of 7 mg/kg) abrogated, in a similar way, microglial activation in spinal cords of TMEV-infected mice. Quantification of reactive microglia within the spinal cord showed that OMDM1 and OMDM2 induced a significant reduction in the number of reactive microglial cells 1 day after the end of the treatment. It is important to note that the schedule dose of 2 mg/kg was less effective in diminishing microglial activation (not shown).

Macrophage/microglial cells are potent antigen presenting cells within the CNS and express CB1 and CB2 receptors (Walter and Stella 2004), suggesting that these cells can

Table 1 Levels of anandamide (AEA) and 2-arachydonoyl glycerol (2-AG) in spinal cords from Theiler's murine encephalomyelitis virus (TMEV)-infected mice or in TMEV-infected macrophages (Daniel's strain, multiplicity of infection of 5 PFU per cell)

	AEA	2-AG
Spinal cord + vehicle	22.8 ± 3.7 pmol/g tissue	4.8 ± 0.8 nmol/g tissue
Spinal cord + TMEV + vehicle	28.7 ± 1.0 pmol/g tissue	3.5 ± 0.5 nmol/g tissue
Spinal cord + TMEV + OMDM1 (7 mg/kg)	45.6 ± 3.0 pmol/g tissue*,†	2.8 ± 0.3 nmol/g tissue
Spinal cord + TMEV + OMDM2 (7 mg/kg)	33.6 ± 0.4 pmol/g tissue*	2.9 ± 0.1 nmol/g tissue
Macrophages + vehicle	3.4 ± 0.8 pmol/mg lipid extract	4.2 ± 0.3 pmol/mg lipid extract
Macrophages + TMEV	6.2 ± 0.9 pmol/mg lipid extract*	6.0 ± 1.9 pmol/mg lipid extract

Data are means ± SEM of $n = 4$–6 determinations (for spinal cords) and $n = 3$ determinations (for macrophages).
*$p < 0.05$ vs. corresponding vehicle.
†$p < 0.05$ vs. OMDM2.

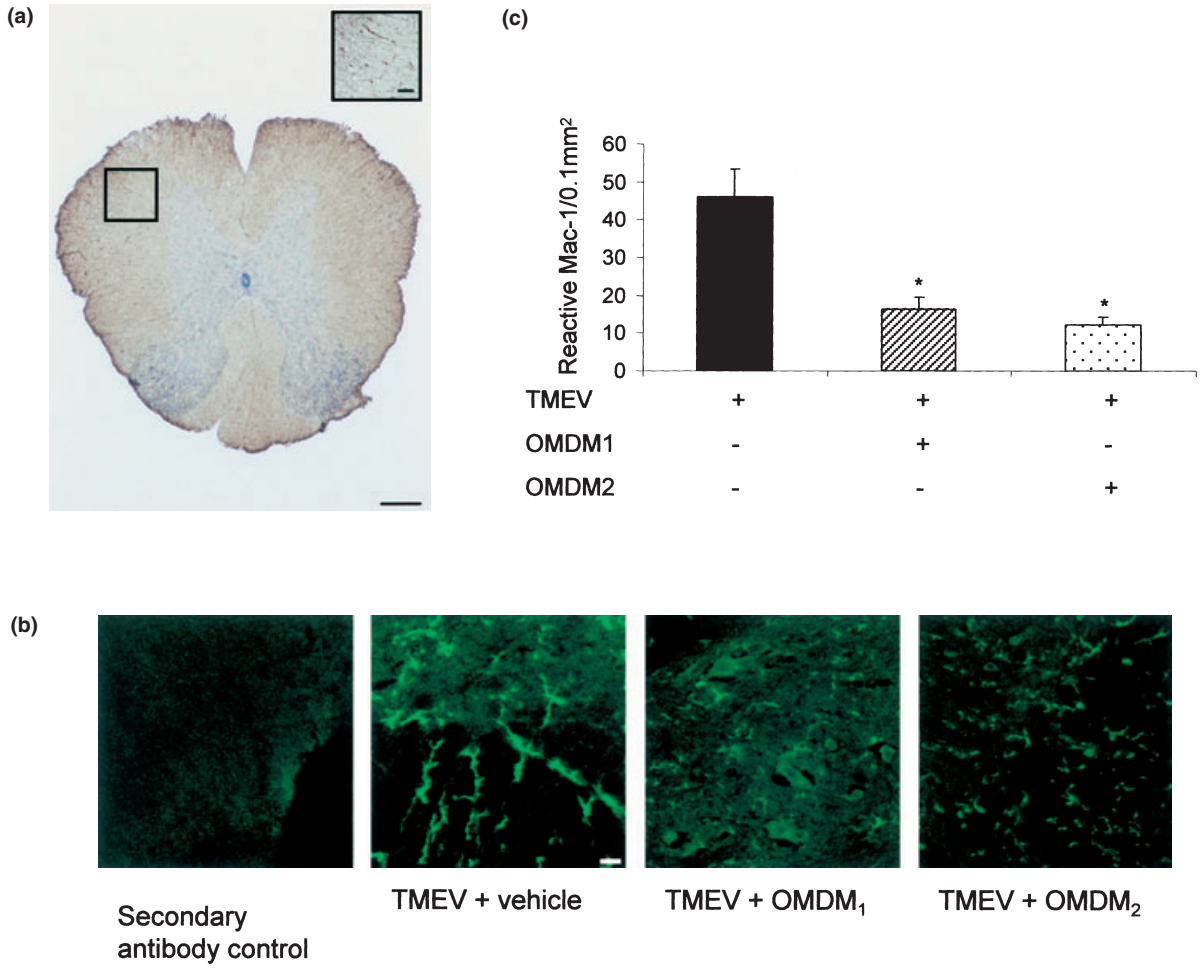

Fig. 2 (a) Representative photomicrograph of a spinal cord section at the thoracic level from a Theiler's murine encephalomyelitis virus (TMEV)-infected mouse showing microglia/macrophages (tomato-lectin binding) and counterstained with toluidin blue. Scale bar, 50 μm, magnification 4 ×, Scale bar in insert: 50 μm, magnification 20 ×. Note that blood vessels were also stained by tomato lectin. (b) Confocal images with constant laser beam and photodetector sensitivity of microglia/macrophages ($CD11b^+$ cells) in representative sections of ventral columns of thoracic spinal cord of TMEV-infected mice subjected to vehicle or OMDM1 and OMDM2 treatment (dose-schedule: 7 mg/kg daily for 10 consecutive days) 1 day after the cessation of the treatment. Scale bar, 50 μm. (c) Quantification of the $CD11b^+$ cells (reactive Mac-1) in the spinal cord sections from TMEV-infected mice subjected to the different treatments. Statistical analysis: *$p < 0{,}0001$ vs. TMEV.

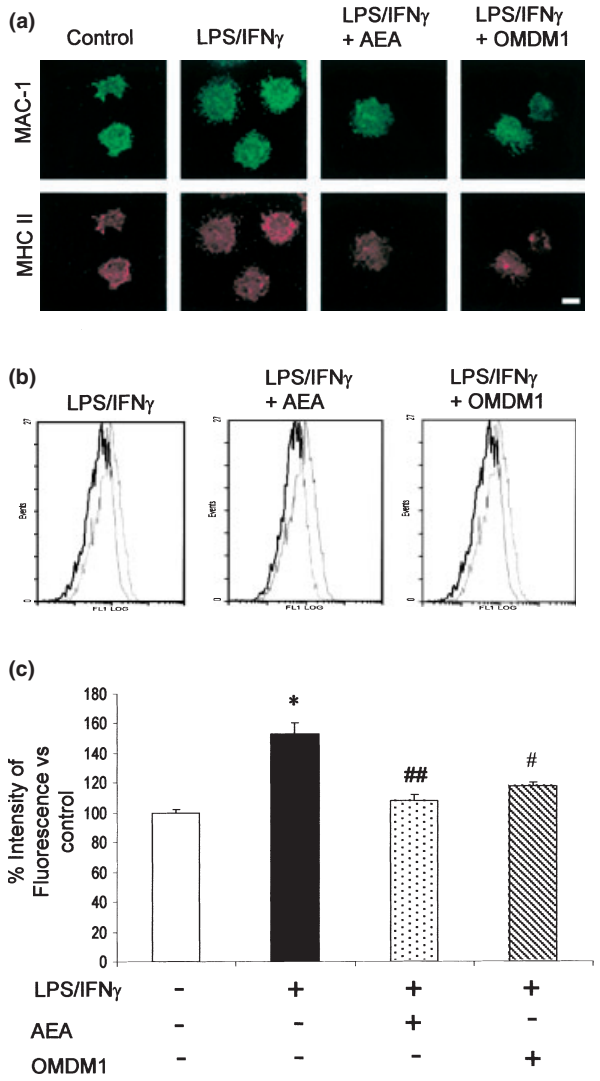

Fig. 3 OMDM1 abrogrates murine macrophage major histocompatibility complex (MHC) class II expression induced by lipopolysaccharide (LPS)/interferon-γ (IFN-γ) activated cells. (a) Representative immunofluorescence confocal laser scanning micrographs showing the suppression of MHC class II antigen (red) expression by Mac-1 (green) positive cells in macrophage cultures activated by LPS/IFN-γ and treated with anandamide (AEA, 10 μM), OMDM1 (5 μM) as described in Materials and Methods. Scale bar, 20 μm. (b) Representative histograms generated from flow cytometric analysis of cells after the different treatments. (c) Quantification of MHC Class II molecule expression by flow cytometric analysis after the different treatments, expressed as mean values ± SEM from three independent experiments performed in duplicate. *$p < 0.002$ vs. control; ##$p < 0.005$; #$p < 0.01$ vs. LPS/IFN-γ activated macrophages.

respond to endocannabinoids. MHC class II molecules on macrophage cell surface play a critical role in the initiation of acquired immune response against TMEV (Olson *et al*. 2001). Recently, an increase in AEA production by LPS-activated macrophages has been described (Liu *et al*. 2003), and we have found here that TMEV infection also increases AEA levels in macrophages. Therefore, we next investigated whether the AEA transporter inhibitor, OMDM1 at the dose of 5 μM, affect the expression of MHC class II molecules in activated macrophages (Fig. 3). As expected, LPS/IFN-γ treatment induced the expression of H-2A antigens in Mac positive staining cells. Exposure to AEA, as well as the treatment with OMDM1, prevented MHC class II expression, as indicated by the decreased immunoreactivity for this antigen (Fig. 3a). These results were confirmed by flow cytometric analysis, as we measured MHC class II antigen expression by direct staining in LPS/IFN-γ activated cells in the presence or absence of AEA or OMDM1. As shown in Fig. 3b, there was a reduction in the expression of MHC class II antigen in cells treated with AEA or OMDM1. Quantification data (Fig. 3c) revealed a significant decreased expression of MHC class II antigen by AEA ($p < 0.005$) or OMDM1 ($p < 0.01$).

OMDM1 and OMDM2 inhibit nitric oxide synthase-2 expression in activated macrophages

Activation of macrophages by LPS/IFN-γ resulted in the expression of NOS-2 as measured by western blotting. However, this stimulatory effect was abolished by pre-treatment of cell cultures with AEA ($p < 0.001$). The addition of the inhibitor of AEA transporter, OMDM1, at the dose of 10 μM also reduced NOS-2 expression ($p < 0.001$), but to a lesser extent than AEA. When OMDM1 was added to activated macrophages treated with AEA, an enhancement of AEA effects were observed ($p < 0.001$) (Fig. 4a). We next examined whether the cannabinoid receptors are involved in the inhibition of NOS-2 expression in macrophages. Exposure of cells to SR141716A or SR144528 alone at doses of 1 μM did not modify significantly NOS-2 expression induced by LPS/IFN-γ, although SR141716A caused a slight increase of the protein (not shown). However, we found that the addition of both SR141716A, a CB1 receptor antagonist, or SR144528, a CB2 receptor antagonist, partially prevented ($p < 0.05$) the inhibition of NOS-2 expression elicited by OMDM1 (Fig. 4a). These results indicate that the actions of OMDM1 on NOS-2 expression may involve both CB1 and CB2 receptors. In order to discard putative direct effects of OMDM1 and OMDM2 on cannabinoid receptors we performed a set of experiments using the above transporter inhibitors at a concentration of 5 μM. This dose, which is inactive on both CB1 and CB2 receptors (Ortar *et al*. 2003), was still effective ($p < 0.05$), for both compounds, at decreasing the expression of NOS-2, and OMDM1 also exhibited a trend towards the enhancement of AEA effects (Fig. 4b).

OMDM1 and OMDM2 decrease interleukin-1β production by activated macrophages

OMDM1 and OMDM2 were next compared for their ability to affect critical cytokine production by LPS/IFN-γ

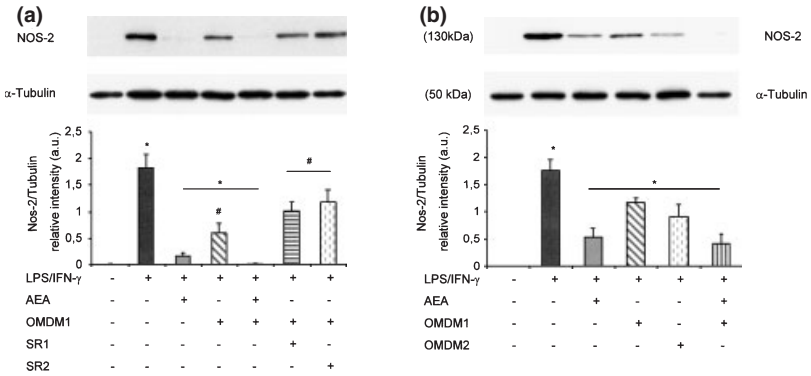

Fig. 4 Inhibition of nitric oxide synthase (NOS-2) expression by OMDM1 and OMDM2 in lipopolysaccharide (LPS)/interferon-γ (IFN-γ)-treated murine macrophages. (a) Representative western blot of NOS-2 in macrophage cultures in basal and LPS/IFNγ activated conditions in the absence or presence of anandamide (AEA, 10 μM), OMDM1 (10 μM), AEA + OMDM1, OMDM1 + SR1 (antagonist CB1 receptors: 1 μM) or OMDM1 + SR2 (antagonist CB2 receptors: 1 μM). Cells were stimulated with LPS/IFN-γ for 18 h and incubated in the presence of the different compounds as described in Materials and Methods. After treatments, cells were washed and lysed in buffer lysis. Proteins in cell lysates were resolved on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subjected to immunoblot analysis (30 μg protein/lane) using antibodies against the indicated proteins (NOS-2, dilution, 1 : 2000 and α-tubulin, dilution, 1 : 40000). Densitometric data represent the mean ± SEM of three independent experiments performed in separate cell preparations and in triplicate (statistical significances: $^{*}p < 0.001$ vs. control or LPS/IFN-γ; $\#p < 0.05$ vs. LPS/IFNγ + AEA or LPS/IFNγ + OMDM1; $^{+}p < 0.001$ vs. LPS/IFN-γ + AEA. (b) Representative western blot of NOS-2 in macrophage cultures in the absence or presence of AEA (1 μM) and OMDM1 or OMDM2 at the dose of 5 μM. Densitometric analysis of two independent experiments in triplicate indicated a significant effect of OMDM1 and OMDM2 in suppressing NOS-2 expression ($^{*}p < 0.0001$ vs. control; $^{*}p < 0.001$ vs. LPS/IFN-γ). Immunoblotting for α-tubulin was carried out to ensure equal loading of protein and data are expressed by density ratios to α-tubulin bands.

activated macrophages (Fig. 5). Unstimulated macrophages expressed low levels of IL-1β, but the stimulation with LPS/IFN-γ significantly up-regulated its biosynthesis ($p < 0.001$). LPS/IFN-γ-stimulated production of IL-1β was down-regulated by the presence of AEA (1–15 μM) in a dose–response manner (Fig. 5a). The lowest effective dose of AEA was 5 μM ($p < 0.05$), but AEA at 10 μM and 15 μM induced a marked inhibition ($p < 0.001$) of IL-1β generation by activated macrophages, as revealed by the low amounts of IL-1β measured in the culture media. A significantly decreased IL-1β production ($p < 0.001$) was also observed following exposure of cells to OMDM1 at a 10 μM concentration (Fig. 5b). Incubation of cells with 1 μM of SR141716A caused a small increase in LPS-induced IL-1β production, whereas incubation with SR144528 did not affect IL-1β content (LPS/IFN-γ: 312.26 ± 5.9 pg/mL; SR141716A + LPS/IFN-γ: 364.38 ± 7.5 pg/mL; SR144528 + LPS/IFN-γ: 327.50 ± 5.2 pg/mL). The results obtained with OMDM1 in the presence of CB1 (SR141716A) or CB2 (SR144528) receptor antagonists indicate a partial blockade ($p < 0.05$) of OMDM1 effects, suggesting the participation of both types of receptors. The combined exposure of SR141716A and SR144528 confirmed the involvement of both receptors, as a much greater blockade was observed ($p < 0.001$). Addition of SR141716A or SR144528 partially reversed the inhibitory effects of AEA 5 μM on IL-1β secretion by stimulated macrophages (LPS/IFN-γ: 114.25 ± 16.4 pg/mL; AEA + LPS/IFN-γ: 71.75 ± 7.02 pg/mL, $p < 0.05$; SR141716A + AEA + LPS/IFN-γ: 90.5 ± 4.44 pg/mL, $p < 0.05$), whereas, the combined treatment with both antagonists completely blocked AEA effects (SR141716A + SR144528 + AEA + LPS/IFN-γ: 104.75 ± 7.36 pg/mL). Figure 5(c) shows the results obtained when using a concentration of 5 μM for either OMDM1 or OMDM2. At this dose, both compounds were still able to inhibit IL-1β biosynthesis ($p < 0.001$). Interestingly, OMDM1 and OMDM2 also potentiated AEA-induced IL-1β inhibition ($p < 0.05$). This observation is consistent with the hypothesis that the OMDM compounds act by inhibiting the inactivation of AEA, be it endogenously produced, as in TMEV-infected macrophages, or exogenous. We then assessed whether infection of macrophages from SJL/J mice with TMEV, under the same conditions leading to increased AEA levels (see above), would result in increased production of IL-1β, and if so, what was the effect of AEA and OMDM1. As expected, TMEV-infected macrophages contain high levels of IL-1β compared with uninfected cells (TMEV: 465.0 ± 86.7 pg/mL vs. control: 12.7 ± 4.2 pg/mL; $p < 0.0001$). The treatment with AEA (10 μM) elicited an important decrease of IL-1β content in the cell supernatants (AEA + TMEV: 192.3 ± 38.9 pg/mL; $p < 0.001$ vs. TMEV), whereas the addition of OMDM1 (10 μM) decreased IL-β levels to a lesser extent (OMDM1 + TMEV: 338.3 ± 57.5 pg/mL; $p < 0.05$ vs. TMEV).

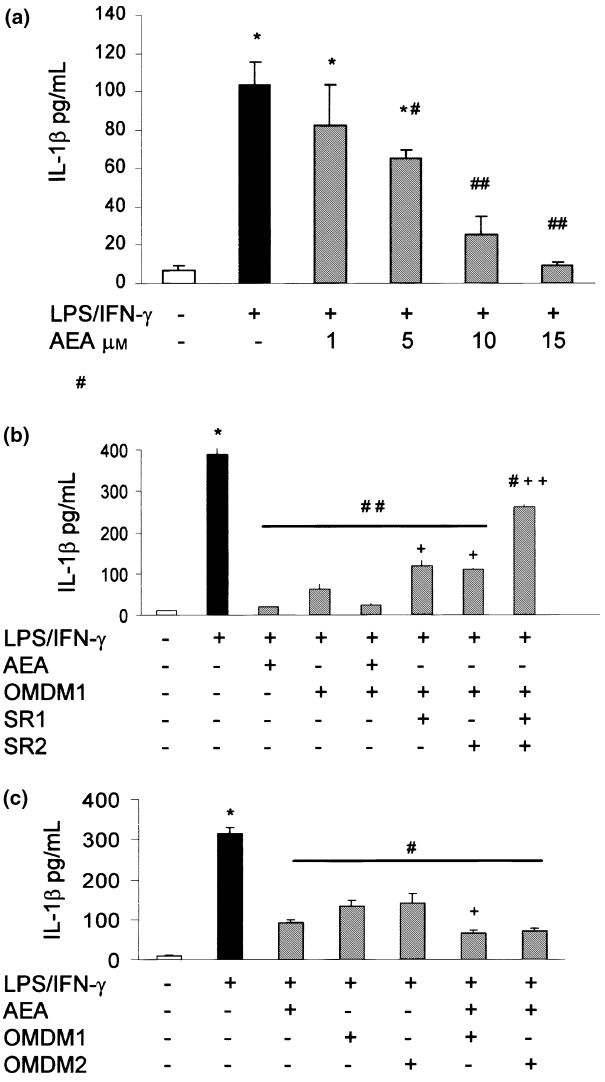

Fig. 5 Effects of OMDM1 and OMDM2 on interleukin-1β (IL-1β) production by lipopolysaccharide (LPS)/interferon-γ (IFN-γ) activated macrophages. (a) Effects of increasing doses of anandamide (AEA, 1–15 μM) on IL-1β production by macrophages stimulated with LPS/IFN-γ as described in Methods. Incubation was continued for 18 h. Supernatants were assayed for IL-1β release by ELISA. Statistics: $^{*}p < 0.001$ vs. control; $\#p < 0.05$, $\#\#p < 0.001$ vs. LPS/IFNγ. (b) Macrophage cultures production of interleukin-1β (IL-1β) were treated with different doses of anandamide (AEA, 10 μM), OMDM1 (10 μM), AEA + OMDM1, OMDM1 + SR1 (antagonist CB1 receptors), OMDM1 + SR2 (antagonist CB2 receptors), and OMDM1 + SR1 + SR2, before stimulation with LPS/IFN-γ, as described in Methods. Incubation was continued for 18 h. Supernatants were assayed for IL-1β release by ELISA. Statistics: $^{*}p < 0.001$ vs. control; $\#\#p < 0.001$ vs. LPS/IFNγ; $\#p < 0.05$ vs. LPS/IFN-γ; $^{+}p < 0,05$; $^{++}p < 0.001$ vs. LPS/IFNγ + OMDM1. (c) Macrophage cultures were treated with AEA (1 μM), OMDM1 (5 μM), OMDM2 (5 μM), or AEA + OMDM1, before stimulation with LPS/IFN-γ. Incubation was continued for 18 h. Supernatants were assayed for IL-1β release by ELISA. Statistics: $^{*}p < 0.001$ vs. control; $\#p < 0001$ vs. LPS/IFN-γ; $^{+}p < 0.05$ vs. LPS/IFN-γ + AEA.

Effects of OMDM1 on interleukin-12 p40 protein production by macrophages stimulated with lipopolysaccharide/interferon-γ or infected with Theiler's murine encephalomyelitis virus

IL-12 is a cytokine primarily produced by monocytes and macrophages, and plays an essential role in the development of cell mediated immunity by stimulating T helper type I immune responses (Hsieh *et al*. 1993). This cytokine is a heterodimer of p35 and p40 subunits, and secretion of p40 is limited to cells of macrophage/monocyte lineage and occurs only after activation of these cells. Here, we examined the effect of AEA and OMDM1 on the production of IL-12 subunit p40 by LPS/IFN-γ stimulated macrophages. As shown in Fig. 6(a), LPS/IFN-γ significantly stimulated the production of IL-12p40, as indicated by the increased levels of this cytokine in cell supernatants. Treatment with AEA resulted in a strong decrease of LPS/IFN-γ-induced IL-12p40 ($p < 0.0001$), whereas the exposure to OMDM1 (5 μM) decreased IL-12p40 release, albeit to a lesser degree ($p < 0.003$). The combination of AEA/OMDM1 induced a significant synergistic suppression of IL-12p40 ($p < 0.001$). To examine whether a cannabinoid receptor may participate in the IL-12 p40 inhibition, selective antagonists were again used. The inhibitory effect of OMDM1 was not abrogated by SR141716A, but a partial blockade by SR144528 alone or the combination of both antagonists was observed without reaching statistical significance ($p = 0.065$), although it is noteworthy that the combination of OMDM1 with either SR144528 alone or the two antagonists together was no longer significantly different from LPS/IFN-γ-treated cells. To further confirm the importance of IL-12 in the immune responses to TMEV, we performed experiments using macrophages subjected to TMEV infection, which we have shown here to over-produce AEA, in the presence of AEA and/or OMDM1. Figure 6(b) shows that viral infected cells release a marked amount of IL-12p40 ($p < 0.001$), which was significantly blunted by treatment with AEA ($p < 0.001$). Treatment with OMDM1 (10 μM) also reduced IL-12p40 production by TMEV-infected macrophages, but again to a lesser extent. Figure 6(c), shows the percentage of IL-12p40 inhibition following AEA (70%), OMDM1 (38%) and AEA + OMDM1 (90%) by LPS/IFN-γ stimulated macrophages. Interestingly, in the case of TMEV infection the extent of IL-12 inhibition elicited by the cannabinoid compounds are similar to those observed following LPS/IFN-γ stimulation (Fig. 6d).

Discussion

In this study we show that two selective inhibitors of the putative endocannabinoid transporter, OMDM1 and OMDM2, provide an effective protection against the symptoms and, possibly, the progress of TMEV-IDD, a viral murine experimental model for MS. Treatment of

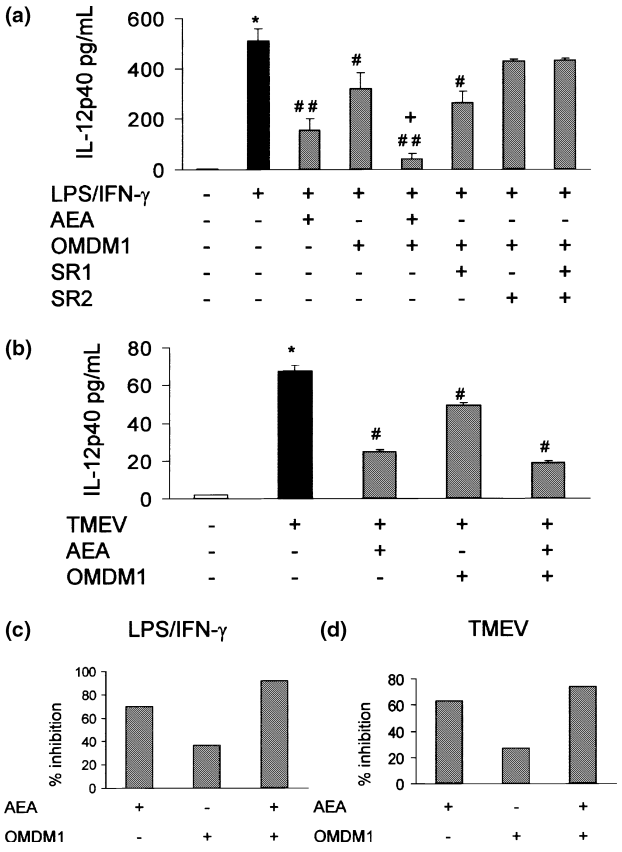

Fig. 6 Effects of OMDM1 and OMDM2 on interleukin-12 subunit p40 (IL-12p40) production by activated macrophages (a) Effect of anandamide (AEA), OMDM1, AEA + OMDM1, OMDM1 + SR1 (antagonist CB1 receptors); OMDM1 + SR2 (antagonist CB2 receptors) and OMDM1 + SR1 + SR2 on IL-12p40 protein production in primary mouse macrophages stimulated with lipopolysaccharide (LPS)/interferon-γ (IFN-γ) for 18 h. Supernatants were collected and assayed for IL-12p40 content by ELISA. Statistics: $^{*}p < 0.001$ vs. control; $\#\#p < 0.001$ vs. LPS/IFNγ; $\#p < 0003$ vs. LPS/IFNγ; $^{+}p < 0.05$ vs. LPS + AEA; (b) Effect of AEA, OMDM1 and AEA + OMDM1 on IL-12p40 protein production in primary mouse macrophages after Theiler's murine encephalomyelitis virus (TMEV) infection (Daniel's strain, multiplicity of infection of 5 PFU per cell). Supernatants were harvested 24 h post-infection and assayed for IL-12p40 by ELISA. Statistics: $^{*}p < 0.001$ vs. uninfected cultures; $\#p < 0.001$ vs. TMEV. (c) Percentage of IL-12 p40 inhibition elicited by AEA, OMDM1, and AEA + OMDM1 in LPS/IFN-γ stimulated macrophages. (d) Percentage of IL-12 p40 inhibition elicited by AEA, OMDM1 and AEA + OMDM1 in macrophage cultures infected with TMEV.

TMEV-infected mice with OMDM2 and, particularly, OMDM1 ameliorated motor symptoms. This was associated with: (i) an up-regulation of AEA levels, which was more efficacious with OMDM1, and (ii) a down-regulation of inflammatory responses in the spinal cord of TMEV-infected mice. Several pieces of evidence indicate that exogenous and endogenous cannabinoids modulate the functions of immune cells in experimental *in vitro* and *in vivo* models (Berdyshev 2000; Klein *et al.* 2003). In particular, a diminished macrophage activity by cannabinoids has been postulated (McCoy *et al*. 1999). Here we showed that AEA levels are significantly increased in macrophages infected with TMEV, and that OMDM1 and OMDM2 down-regulate macrophage function by: (i) decreasing the surface expression of MHC class II molecules, (ii) inhibiting NOS-2 expression, and (iii) reducing the production of pro-inflammatory cytokines, such as IL-1β and IL-12p40 by activated macrophages.

Previous studies have demonstrated that CB1 and CB2 cannabinoid agonists ameliorate spasticity and tremors in the CREAE model of MS (Baker *et al*. 2000). The increased levels of AEA and 2-AG in spastic mice suggest an involvement of endocannabinoid system, which may participate as an endogenous protective mechanism (Baker *et al*. 2001). This hypothesis is supported by the finding that CB1-deficient CREAE mice exhibit a higher degree of neuronal damage than they wild-type littermates (Pryce *et al*. 2003). Here we found that TMEV infection, which does not result in chronic-relapsing spasticity as in the CREAE model, does not cause a significant enhancement of endocannabinoid levels in the spinal cord of the diseased mice. This is in agreement with the previous finding of enhanced endocannabinoid levels being associated with spasticity rather than with the disorder itself (Baker *et al*. 2001). Instead, TMEV-infection does seem to produce an enhancement of endocannabinoid tone in macrophages, thus suggesting that increased endocannabinoid levels might be an adaptive reaction aimed at inhibiting the inflammatory responses. Indeed, the results of the present study, in which the treatment with inhibitors of AEA cellular re-uptake both improved motor function and reduced inflammatory responses in microglia/macrophages, support the concept that a potentiation of the endocannabinoid tone has beneficial effects on the severity of both neurological and inflammatory MS-like symptoms (Baker *et al*. 2001). The stimulation of endocannabinoid action on neural circuits involved in motor control is probably one of the causes of the amelioration of motor deficits observed in TMEV-infected mice. In support of this hypothesis is the observation that, although both OMDM1 and OMDM2 improve motor behaviour, OMDM1 [which of the two compounds is the one completely inactive as a 'direct' CB1 agonist (Ortar *et al*. 2003)] was more effective in the two motor behavioural tests used, and was also the one that more efficiently elevated AEA levels in the spinal cord of TMEV-infected mice. Furthermore, previous work from our group (Arévalo-Martín *et al*. 2003) showed that cannabinoid receptor agonists reduced the number of reactive microglia, suppressed microglial MHC class II expression and diminished $CD4^{+}$ T cell infiltration within the spinal cord of TMEV-infected mice, and led to a clear motor function recovery. In support of the beneficial immunoregulatory properties of cannabinoids in the TMEV-IDD model, Croxford and Miller (2003) described a diminished clinical

disease score associated with down-regulation of Th1-mediated viral and myelin responses, and thus decreased expression of mRNA encoding for proinflammatory cytokines. In our case, however, because improvement of motor activity was achieved with the two schedule doses treatment of OMDM1 and OMDM2, and down-regulation of microglial reactivity was only significantly after the higher dose schedule treatment, it is reasonable to postulate that the effects on motor behaviour induced by OMDM1 and OMDM2 are dependent only in part from inflammatory conditions within the spinal cord. Because the intermediacy of cannabinoid CB1 and CB2 receptors in the actions of the two inhibitors was only demonstrated *in vitro*, we cannot rule out the possibility that part of the effects observed *in vivo* are unrelated to the ones observed *in vitro* and are not mediated by indirect activation of cannabinoid receptors by OMDM1 and OMDM2, particularly as AEA has been shown to activate other targets beside CB1 and CB2 receptors (Di Marzo *et al*. 2000).

In an attempt to elucidate the possible mechanism(s) underlying the inhibition of macrophage/microglial reactivity by the endocannabinoid transporter inhibitors, we performed experiments *in vitro* with macrophage cultures. One of the most important finding was that OMDM1 and OMDM2 suppressed the expression of MHC class II molecules induced by IFN-γ/LPS. These effects are particularly interesting if one considers the role of macrophages in TMEV-IDD. Macrophages are an important reservoir of TMEV and depletion of macrophages prevents chronic demyelination from commencing (Pena-Rossi *et al*. 1997). Macrophages/microglia express CB1 and CB2 receptors, indicating that these cells are sensitive to alteration in endocannabinoid levels during neuroinflammation (Walter and Stella 2004). Although recent studies postulated that 2-AG is the main endocannabinoid involved in microglial cell migration and proliferation (Walter *et al*. 2003; Carrier *et al*. 2004), the fact that inflammatory stimuli such as LPS increase the production of AEA by macrophages (Wagner *et al*. 1997; Di Marzo *et al*. 1999) indicates the possibility of a regulatory role of this endocannabinoid on macrophage function. The results of the present study support this notion, because macrophages infected with TMEV overproduced AEA selectively over 2-AG, and macrophage exposure to AEA, OMDM1 or OMDM2 was able to inhibit NOS-II expression. Activated macrophages/microglia produce important amounts of nitric oxide (NO), which may act as a cytotoxic effector molecule in demyelinating diseases. Both endogenous NO released by macrophages/microglia and NO generated from exogenous donors induce oligodendrocyte death (Mitrovic *et al*. 1995; Boullerne *et al*. 1999; Molina-Holgado *et al*. 2001). Cannabinoids have been reported to increase NO production from monocytic cells (Stefano *et al*. 1996) and to decrease NO generation by activated macrophages (Jeon *et al*. 1996), microglial cells (Waksman *et al*. 1999) and TMEV-infected astrocytes (Molina-Holgado *et al*. 1997). Because, in our study, AEA per se induced the suppression of NOS-2 expression and OMDM1 enhanced this effect, we propose that the increase in availability of AEA caused by the inhibition of its reuptake is responsible for diminished NOS-2 expression. In agreement with this finding, macrophages are known to contain a putative endocannabinoid transporter (Bisogno *et al*. 1997), and the effect of OMDM1 in our study was partially antagonized by the CB1 and CB2 antagonists, SR141716A or SR144528. Furthermore, we found that OMDM1, which in previous studies was inactive on CB1 and CB2 receptors up to a 10 μM concentration (Ortar *et al*. 2003), was still capable of producing these effects at a 5 μM concentration.

We have also provided data showing that AEA, OMDM1 and OMDM2 diminish IL-1β production by LPS/IFN-γ activated macrophages. The potentiation of the AEA-induced inhibition of IL-1β by OMDM1 is in good agreement with the idea of a common mechanism of action in the suppression of IL-1β production. IL-1β has been considered as a mediator of experimentally induced brain damage, and is expressed initially by microglia/macrophages and later by astrocytes (Davies *et al*. 1999). Interestingly, the endogenous IL-1 receptor antagonist, which limits IL-1β action, has been reported to mediate the neuroprotective and anti-inflammatory actions of CBs in neurones and glia. (Molina-Holgado *et al*. 2003). Other studies have described that exogenous and endogenous cannabinoids inhibit the expression of mRNA for several cytokines including IL-1β by macrophages and microglial cells (Klein *et al*. 2000; Puffenbarger *et al*. 2000), but the type of cannabinoid receptors involved remained to be established. In the present study, our results suggest the involvement of both CB1 and CB2 receptors, although we do not exclude the participation of other putative cannabinoid-like receptors. Furthermore, the decreased IL-12p40 release from TMEV-infected infected macrophages after AEA or OMDM1 is consistent with the immunomodulatory role proposed for the endocannabinoids. Production of IL-12p40 occurs during both innate and acquired immunity, and this cytokine brings about the development of Th1 $CD4^+$ T cells from naïve $CD4^+$ T cells. Therefore, the up-regulation of IL-12p40 biosynthesis after infection of macrophages with TMEV would contribute to the activation and differentiation of Th1 cells, which constitutes the major immune response in the CNS of TMEV (Pope *et al*. 1996). The blunted IL-12p40 release by AEA and OMDM1 may have important implications for the initial inflammatory response as well as for the ensuing adaptative immune response to viral antigens and subsequently to TMEV-induced demyelination. We have also shown that IL-12p40 production by LPS/IFN-γ activated macrophages was blocked by AEA, and that OMDM1 significantly potentiated this effect. Collectively, these results

indicate that OMDM1 and OMDM2, by retarding the inactivation of endogenous cannabinoid receptor agonists, exhibit important anti-inflammatory properties in macrophages that might be useful for the therapeutic treatment of chronic inflammatory disorders. This conclusion seems to be further supported by our own unpublished observations showing an increased IL-10 production by AEA under inflammatory conditions using different cell preparations (Raw 264.7, macrophages and primary microglial cells, C. Guaza, unpublished data).

In conclusion, we have shown that, in a widely employed murine model of MS, OMDM1 and OMDM2 can efficaciously: (i) alleviate motor deficits, and (ii) afford protection from neuroinflammation by targeting macrophage/microglial function. Four types of observations indicate that OMDM1 and OMDM2 act by inhibiting endocannabinoid inactivation and, subsequently, by inducing/strengthening an endocannabinoid tone: (i) the rank of efficacy of the two compounds (which differ only by their stereochemistry) in this study, and the fact that one of them is active *in vitro* at a concentration totally inactive on cannabinoid receptors, rules out their action via direct activation of these receptors; (ii) the fact that enhanced endocannabinoid synthesis has been previously demonstrated both *in vivo*, in an animal model of MS, and *in vitro*, in LPS-stimulated and, in the present study, in TMEV-infected macrophages; (iii) the present finding that the effects of OMDM1 and OMDM2 on macrophages are attenuated by cannabinoid receptor antagonists; and (iv) the present finding that both compounds enhance AEA levels in the spinal cord of diseased mice, and do so with relative efficacies that are identical to their relative efficacies at contrasting MS-like motor symptoms. Further studies are therefore warranted to establish the endogenous cannabinoid system as a therapeutic target for the treatment of demyelinating diseases.

Acknowledgements

We gratefully appreciated Dr Moses Rodriguez (Department of Immunology and Neurology, Mayo Clinic/Foundation, Rochester, Minnesota, USA) for gentile delivery of Theiler's virus DA strain. This work was supported by grants from the Ministerio de Ciencia y Tecnología (MCYT, Spain, SAF-2001/1246) and Ministero Italiano per l'Universita′ e la Ricerca (MIUR-FIRB, to VDM). We express our gratitude to R. Fernandez-Senso, C. Bailón, C. Hernandez and S. Petrosino for their technical assistance. We also thank Pedro Lastres for his help in cytometric analysis (CIB; CSIC). FC was supported by a fellowship from the Fundación Carolina (MEC).

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