

Themed Issue: Cannabinoids in Biology and Medicine, Part I

RESEARCH PAPER

Cannabidiol inhibits pathogenic T cells, decreases spinal microglial activation and ameliorates multiple sclerosis-like disease in C57BL/6 mice

Ewa Kozela^{1*}, Nirit Lev^{2*}, Nathali Kaushansky³, Raya Eilam⁴, Neta Rimmerman⁵, Rivka Levy⁵, Avraham Ben-Nun³, Ana Juknat^{1*} and Zvi Vogel^{1,5*}

¹The Dr. Miriam and Sheldon G. Adelson Center for the Biology of Addictive Diseases, Physiology and Pharmacology Department, Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel, ²Neurology Department, Rabin Medical Center, Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel, ³Immunology Department, ⁴Histology Department, ⁵Neurobiology Department, Weizmann Institute of Science, Rehovot, Israel

BACKGROUND AND PURPOSE

Cannabis extracts and several cannabinoids have been shown to exert broad anti-inflammatory activities in experimental models of inflammatory CNS degenerative diseases. Clinical use of many cannabinoids is limited by their psychotropic effects. However, phytocannabinoids like cannabidiol (CBD), devoid of psychoactive activity, are, potentially, safe and effective alternatives for alleviating neuroinflammation and neurodegeneration.

EXPERIMENTAL APPROACH

We used experimental autoimmune encephalomyelitis (EAE) induced by myelin oligodendrocyte glycoprotein (MOG) in C57BL/6 mice, as a model of multiple sclerosis. Using immunocytochemistry and cell proliferation assays we evaluated the effects of CBD on microglial activation in MOG-immunized animals and on MOG-specific T-cell proliferation.

KEY RESULTS

Treatment with CBD during disease onset ameliorated the severity of the clinical signs of EAE. This effect of CBD was accompanied by diminished axonal damage and inflammation as well as microglial activation and T-cell recruitment in the spinal cord of MOG-injected mice. Moreover, CBD inhibited MOG-induced T-cell proliferation *in vitro* at both low and high concentrations of the myelin antigen. This effect was not mediated via the known cannabinoid CB₁ and CB₂ receptors.

CONCLUSIONS AND IMPLICATIONS

CBD, a non-psychoactive cannabinoid, ameliorates clinical signs of EAE in mice, immunized against MOG. Suppression of microglial activity and T-cell proliferation by CBD appeared to contribute to these beneficial effects.

LINKED ARTICLES

This article is part of a themed issue on Cannabinoids in Biology and Medicine. To view the other articles in this issue visit <http://dx.doi.org/10.1111/bph.2011.163.issue-7>

Abbreviations

CBD, cannabidiol; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein

Correspondence

Zvi Vogel, Neurobiology Department, Weizmann Institute of Science, Rehovot 76100, Israel and Physiology and Pharmacology Department, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel. E-mail: zvi.vogel@weizmann.ac.il

*Authors contributed equally to this work.

Keywords

cannabinoids; cannabidiol; microglia; T cells; EAE; MOG

Received

5 December 2010

Revised

10 March 2011

Accepted

10 March 2011

Introduction

The anti-inflammatory properties of cannabinoids, constituents of the *Cannabis sativa* plant, have been appreciated since ancient times and have been supported experimentally, in models of inflammation *in vitro* and *in vivo* (Tanasescu and Constantinescu, 2010). Indeed, immune cells express the elements of the cannabinoid system including endocannabinoid ligands, endocannabinoid enzymatic machinery and cannabinoid CB receptors, mostly of the CB₂ type and many fewer of the CB₁ type (Mackie, 2005; nomenclature follows Alexander *et al.* (2009). Moreover, expression of CB₁ and CB₂ receptors and the activity of the endocannabinoid system are regulated in response to inflammation, a finding that further confirms the involvement of the cannabinoid system in immune processes (Croxford and Yamamura, 2005; Maresz *et al.*, 2005; Pietr *et al.*, 2009).

The clear and promising therapeutic potential of cannabinoids (Klein and Newton, 2007) is limited due to central, CB₁-mediated, psychotropic effects of many of these materials. A good example is Δ^9 -tetrahydrocannabinol (THC) as the most studied psychoactive cannabinoid, activating both CB₁ and CB₂ receptors (Bayewitch *et al.*, 1995). The preferential CB₂ receptor expression on immune cells offers an attractive opportunity to regulate the function of the immune system with CB₂ receptor ligands. Studies on CB₁/CB₂ receptor knock-down mice revealed the existence of other possible, receptor and non-receptor, cannabinoid targets (Járai *et al.*, 1999), confirmed to be immunomodulatory (Kaplan *et al.*, 2003; 2008). Thus, cannabinoid compounds not acting on CB₁ or CB₂ receptors seem to offer new tools to manipulate inflammation. Cannabidiol (CBD) is one of these compounds and is the major *Cannabis*-derived non-CB₁/CB₂ receptor ligand (Showalter *et al.*, 1996). This compound is not only devoid of psychotropic effects but also is able to inhibit many central effects of CB₁ receptor ligands, for example, the anxiogenic and psychotogenic activities of THC (Zuardi, 2008). Interestingly, despite different pharmacological and behavioural effects, CBD shares with 'classic' psychocannabinoids many beneficial effects, including its capacity to act as an immunomodulator.

Indeed, CBD exerts a wide range of anti-inflammatory properties and regulates cell cycle and function of various immune cells. These effects include suppression of humoral responses, such as release of cytokines, chemokines, growth factors, as well as suppression of immune cell proliferation, activation, maturation, migration and antigen presentation (Mechoulam *et al.*, 2007). In an earlier publication, we showed that CBD inhibited production of the cytokine IL-6 and the chemokine CCL-2 by activated microglial cells and in parallel activated intracellular anti-inflammatory pathways (Kozela *et al.*, 2010).

Among the many types of neurodegenerative diseases in which inflammation is involved, multiple sclerosis (MS) is one of those clearly induced and driven by dysfunctional immune system activity. In MS, myelin autoreactive peripheral T cells migrate into the CNS and initiate cytotoxic, degenerative processes that include demyelination, oligodendrocyte cell death and axonal degeneration. These effects lead to neurological deficits such as visual and sensory disturbances, motor weakness, tremor, ataxia and progressive dis-

ability as the main clinical symptoms (Compston and Coles, 2008). Infiltrating T cells are constantly reactivated within the CNS parenchyma by microglia. Microglial cells via chemokine and cytokine release and constant antigen presentation potentiate T-cell recruitment to the CNS and facilitate their polarization into cytotoxic phenotypes (Th1 or Th17). Moreover, via released chemokines such as CCL-2, microglial cells recruit other immune cells of myeloid origin, specialized in epitope spreading and phagocytosis of myelin including monocytes, macrophages, B cells and dendritic cells (Jack *et al.*, 2005; Koning *et al.*, 2009). Depletion of microglia or impairment of their function can attenuate disease progression in experimental animal models of MS supporting their role in initiation and development of this disease (Huitinga *et al.*, 1990; Heppner *et al.*, 2005). Thus, suppression of microglia will potentially reduce inflammatory lesions and limit demyelination within the CNS.

One of the best described and commonly used animal models of MS is experimental autoimmune encephalomyelitis (EAE) which is induced when the animals are immunized with myelin components, for example, myelin basic protein, proteolipid protein or myelin oligodendrocyte glycoprotein (MOG) or by passive transfer of autoreactive myelin specific T cells to produce demyelination and MS-like neurological and clinical signs (Shevach, 1999). Using the MOG-induced EAE mouse model, we investigated if systemically given CBD at the time of symptomatic disease onset could affect the progression of the disease. We observed that CBD ameliorated the severity of the EAE in MOG-injected mice. Moreover, CBD attenuated microglia activation in MOG-immunized animals and inhibited MOG-induced proliferation of encephalitogenic T cells.

Methods

Animals and experimental design

All animal care and experimental procedures complied with and were approved under the guidance and regulations of the Weizmann Institute of Science and Tel Aviv University. EAE was induced in 30 8-week-old female C57BL/6 mice (Harlan Laboratories, Rehovot, Israel) by two subcutaneous injections of MOG35-55 fragment (encompassing amino acids 35–55 of MOG) on days 1 and 8, injected in the left and right flanks respectively. Each injection contained 300 μ g MOG in 200 μ L of complete Freund's adjuvant (CFA) containing *Mycobacterium tuberculosis* at 200 μ g (Sigma, Israel). Control mice received only CFA without MOG (Ctrl, $n = 10$).

CBD (5 mg·kg⁻¹; CBD + EAE group) or its vehicle (cremophor, ethanol and PBS, v:v:v 1:1:18; EAE group) was injected (i.p.) into 15 MOG-immunized mice immediately at the onset of disease signs for 3 consecutive days (on days 19, 20 and 21). Clinical disease scores were recorded daily until 30 days after first immunization. At this time point, spinal cords were collected for further pathological and immunological studies. The signs of EAE were scored as follows: 0, no clinical signs; 1, loss of tail tonicity; 2, partial hind limb paralysis; 3, complete hind limb paralysis; 4, partial frontal limb paralysis; 5, total paralysis; 6, death (Lev *et al.*, 2004). In parallel, we used control mice which received only CFA (Ctrl)

and were also injected with CBD on days 19, 20 and 21 (Ctrl + CBD group, each group $n = 10$). The days of EAE onset and respective CBD injections were chosen based on our previous experience with this model (Lev *et al.*, 2004).

CBD (National Institute on Drug Abuse, Rockville, MD, USA) solution was prepared freshly before each of the treatments. The dose of CBD was chosen based on previous studies with systemic administration of the drug in which CBD at $5 \text{ mg}\cdot\text{kg}^{-1}$ exhibited maximal efficiency at relieving rheumatoid signs in collagen-induced arthritis (Malfait *et al.*, 2000). MOG synthesis was carried out by the Weizmann Institute Synthesis Unit, using a solid-phase technique on a peptide synthesizer (Applied Biosystems Inc., Foster City, CA, USA).

Histology and immunocytochemistry

Histological analysis of spinal cord sections was used to define severity of inflammation and demyelination and the effect of CBD treatment on these parameters. Spinal cords were dissected 30 days after first immunization of mice, fixed in 10% buffered formalin and embedded in paraffin. The presence of axonal pathology was supported by immunohistochemistry with anti-non-phosphorylated neurofilament H (SMI-32, Sternberg Antibodies, Emeryville, CA, USA) on $8 \mu\text{m}$ thick paraffin sections. Subsequently, $4 \mu\text{m}$ thick sections were stained with haematoxylin and eosin (H&E) and assessed for inflamed areas. The assessment of immune cell infiltration was performed using immunocytochemistry on $4 \mu\text{m}$ spinal cord sections and included staining for T cells (CD3+, Serotec, Kidlington, Oxford, UK), microglia/macrophage number and their activation state (Iba-1, Wako, Richmond, VA, USA; and Mac-2/Galectin 3, Cedarlane, Burlington, Ontario, Canada).

It should be noted that the proteins, ionizing calcium-binding adaptor molecule 1 (Iba-1) and Mac-2/Galectin-3, do not discriminate between microglia, resident macrophages within the CNS, and invasive macrophage-like cells from peripheral sites that crossed the blood-brain barrier. Thus, in further description of the stainings we refer to microglia/macrophage populations. The Iba-1 protein is specifically expressed in microglia/macrophages and becomes up-regulated during the activation of these cells. Mac-2/Galectin-3 is another microglial/macrophage marker whose expression reflects specifically the activation state of these cells. For example, Mac-2/Galectin-3 expression is known to be increased in these cells following phagocytosis of damaged myelin and cell particles (Reichert and Rotshenker, 1999).

The immunostainings were visualized with respective secondary antibodies conjugated to Cy2 or Cy3 fluorochromes. Spinal cords from control, healthy mice that received 3 daily injections of either CBD or its vehicle were dissected and stained in parallel. Assessments of intensity and differences in immunofluorescence staining for Iba-1 and Mac-2/Galectin-3 were performed using the Image Pro Plus analysis software (Media cybernetics, Bethesda, MD, USA) and collected as arbitrary units representing optical density per area. The statistics was carried out on per cent values calculated from optical density values and is given in the appropriate figure legend. In the case of T cells, CD3+ cells were counted (three areas per

mouse, three to four mice per treatment) and the cell numbers were subjected to statistical analysis.

Encephalitogenic T-cell line

The MOG35-55-specific T-cell line was established from lymph node cells of C57BL/6 female mice that had been primed 10 days earlier with MOG35-55 emulsified in CFA as previously described (Kaushansky *et al.*, 2006). The T-cell line was maintained *in vitro* in RPMI-1640 (Biological Industries, Kibbutz Beit HaEmek, Israel) containing 5% fetal calf serum and supplemented with $10 \text{ U}\cdot\text{mL}^{-1}$ IL-2 (Peprotech Inc, Rocky Hill, NJ, USA), L-glutamine, $100 \mu\text{g}\cdot\text{mL}^{-1}$ of streptomycin, $100 \text{ U}\cdot\text{mL}^{-1}$ of penicillin, $50 \mu\text{M}$ β -mercaptoethanol, 1 mM non-essential amino acids and 1 mM sodium pyruvate with alternate stimulation with MOG every 14 days as previously described (Ben-Nun and Lando, 1983; Kaushansky *et al.*, 2006).

Encephalitogenic T-cell proliferation

The MOG35-55-reactive line of T cells raised from primed mice was cultured in 96-well plates (1.5×10^4 cells-per well) together with irradiated (25Gy) splenic antigen presenting cells (APCs; 5×10^5 cells per well). APCs were isolated from spleens of naïve C57BL/6 mice. Lysis of erythrocytes was performed using ACK solution ($150 \text{ mM NH}_4\text{Cl}$, 10 mM KHCO_3 and 0.1 mM EDTA). The cells were cultured in 0.2 mL RPMI-1640 containing 2.5% fetal calf serum and supplemented with L-glutamine, $50 \mu\text{M}$ β -mercaptoethanol, and in the presence of MOG35-55 peptide (1 or $2.5 \mu\text{g}\cdot\text{mL}^{-1}$). CBD at final concentrations of 1 , 5 or $10 \mu\text{M}$ or its vehicle (0.1% ethanol in RPMI-1640) was added to the cells together with the MOG. The cells were then incubated for 72 h at 37°C in 5% CO_2 humidified air (Ben-Nun and Lando, 1983; Kaushansky *et al.*, 2006). Cell proliferation was measured by pulsing the cells with [^3H]Thymidine ($0.5 \mu\text{Ci}\cdot\text{well}^{-1}$) for the last 16 h of the incubation period and the cells were then harvested and counted using a Matrix 96 Direct β counter (Packard Instr., Meriden, CT, USA). To evaluate if CB_1 or CB_2 receptors were involved in the effects of CBD on T-cell proliferation, SR141716 (CB_1 receptor antagonist; RTI International, NC, USA) and SR144528 (CB_2 receptor antagonist; RTI International, NC, USA) at $1 \mu\text{M}$ concentration were applied 30 min before the application of CBD (5 or $10 \mu\text{M}$) and MOG at $1 \mu\text{g}\cdot\text{mL}^{-1}$. The proliferative responses of the cell microcultures were converted to per cent values with MOG effect expressed as 100%. Per cent values were calculated from Stimulation Index (SI) values which are the fold changes in mean counts per minute (cpm) of MOG cultures over mean cpm of cultures without MOG (spontaneous proliferation). Statistical analysis was performed on per cent values. Each experiment was carried out in triplicates and repeated three or four times.

Statistical analysis

EAE clinical scores were reported as average \pm SD. Repeated measure analysis of variance (ANOVA) was used for statistical analysis of these data. The fluorescence intensity and T-cell proliferation data were expressed as the mean \pm SEM and analysed for statistical significance using one-way ANOVA, followed by Bonferroni *post hoc* tests. $P < 0.05$ was considered

significant. *Graph Pad Prism* program (La Jolla, CA, USA) was used for statistical analysis of the data.

Results

CBD ameliorates clinical signs of MOG-induced EAE and disease progression

C57BL/6 mice immunized with MOG35-55 developed severe EAE disease with complete hind limb paralysis [EAE mice, average clinical score 2.52 ± 0.54 ; Figure 1; with 13 out of 15 mice exhibiting clinical signs of EAE (EAE ratio 13/15)]. As shown in Figure 1, three injections (i.p.) of CBD (5 mg·kg⁻¹, one a day) during the onset of clinical disease resulted in amelioration of the disease signs during the days of injections as well as markedly delaying disease progression. On day 21, MOG-induced EAE mice exhibited limp tail and partial paresis of the hind limbs (clinical score 1.03 ± 0.29 ; EAE ratio 9/15). However, MOG-induced EAE mice that received CBD during days 19–21 exhibited minimal clinical signs of EAE (average clinical score 0.13 ± 0.09 , $P < 0.05$; Figure 1; EAE ratio 1/15). Interestingly, 2 days after the CBD injections (day 23), the clinical score of CBD-treated mice increased slowly, reaching mild disease severity at the end of the study (average clinical score 1 and EAE ratio 6/15 vs. average clinical score of 2.54 ± 0.54 in untreated EAE mice, $P < 0.05$; EAE ratio 13/15).

CBD effect on spinal cord of healthy, non-EAE mice

Neither mice that received CFA alone nor those that received CFA with 5 mg·kg⁻¹ of CBD exhibited any clinical signs of EAE

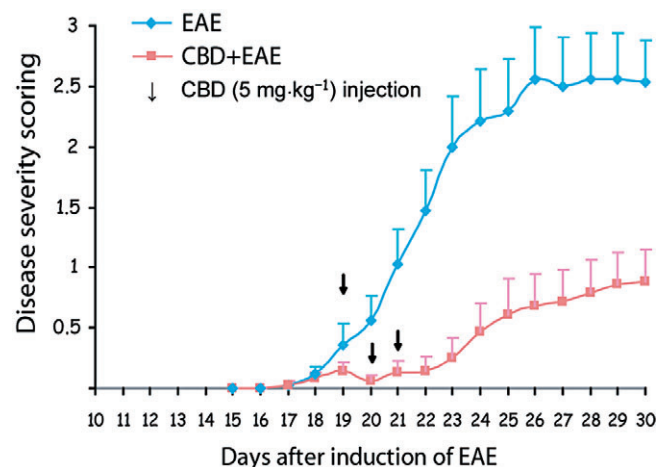


Figure 1

Cannabidiol (CBD) ameliorates the clinical signs and disease progression of EAE induced by myelin oligodendrocyte glycoprotein (MOG). EAE was induced in C57BL/6 mice by flank s.c. immunization with MOG35-55. Clinical disease scores were recorded daily until day 30 after induction. CBD (CBD + EAE) or its vehicle (EAE group), was injected i.p. during the onset of the disease for 3 consecutive days (days 19–21; indicated by arrows). The mean clinical scores \pm SD are shown. Each group consisted of 15 mice. Repeated measure ANOVA showed significant differences between the EAE and CBD + EAE groups ($P < 0.001$).

(data not shown). Next, we evaluated if CBD injections in these healthy mice affected the spinal cords. As shown on Figure 2A, CBD injections did not affect the pattern of H&E histological staining, compared with the group of healthy mice, treated only with CFA. Moreover, Iba-1 microglial expression in Ctrl + CBD group treated with CBD and CFA did not differ from that in the mice given only CFA (Figure 2B). CD3+ T cells were absent in spinal cords of mice given CFA, with or without CBD (Figure 2C). The amount of staining is presented in Figures 5C and 6C.

CBD slows down MOG-induced axonal damage and inflammation in spinal cord of EAE mice

Mice were killed 30 days after EAE induction and spinal cord sections were analysed for the degree of damage to the white matter tissue, using the SMI-32 antibody which stains damaged axons. SMI-32 staining revealed extensive axonal degeneration in the MOG-immunized EAE mice. In contrast, CBD-treated EAE mice showed much lesser axonal damage (Figure 3). Next, we performed H&E histological staining to visualize the inflamed areas in the spinal cord. Results presented in Figure 4 show markedly weaker inflammation in sections of spinal cord derived from CBD-treated EAE mice, compared with untreated EAE mice. These results were further confirmed by immunocytochemical staining for CD3+ T lymphocytes and markers of macrophages/microglial cells. Spinal cords derived from the EAE mice were rich in CD3+ T-cell infiltrates (Figure 5A,C), while treatment with CBD profoundly and significantly decreased CD3+ infiltrates into the white matter (Figure 5B,C).

CBD decreases MOG-induced microglial/macrophage activation

As shown above, immunohistochemical staining for the presence of Iba-1 microglial marker showed that Iba-1 is expressed in the spinal cord of control mice at a very low level and that CBD injections to control naïve mice did not affect this basal expression (Figure 2B). Iba-1 expression was, however, dramatically increased on day 30 post MOG immunization (Figure 6A,C). In contrast, a profoundly reduced number of Iba-1 stained cells was present after CBD treatment of these mice (Figure 6B,C). The Iba-1 expression in CBD-treated MOG-induced EAE group remained slightly higher than that observed in naïve control animals showing that CBD treatment did not completely eliminate microglial activation. This result is in agreement with the mild but still present clinical signs as shown in Figure 1.

Mac-2/Galectin-3 is a microglial/macrophage marker whose expression reflects the activation state of these cells. Staining of spinal cord of healthy, naïve animals for Mac-2/Galectin-3 shows absence (or presence of very low number) of activated microglial cells (Figure 7C). CBD injections did not change Mac-2/Galectin-3 expression in these naïve control animals. However, staining of spinal cords derived from EAE mice resulted in dramatically increased number of Mac-2/Galectin-3 positive cells in the spinal cord (Figure 7A,C). This increase was significantly reduced by CBD injections during the onset of clinical EAE (Figure 7B,C).

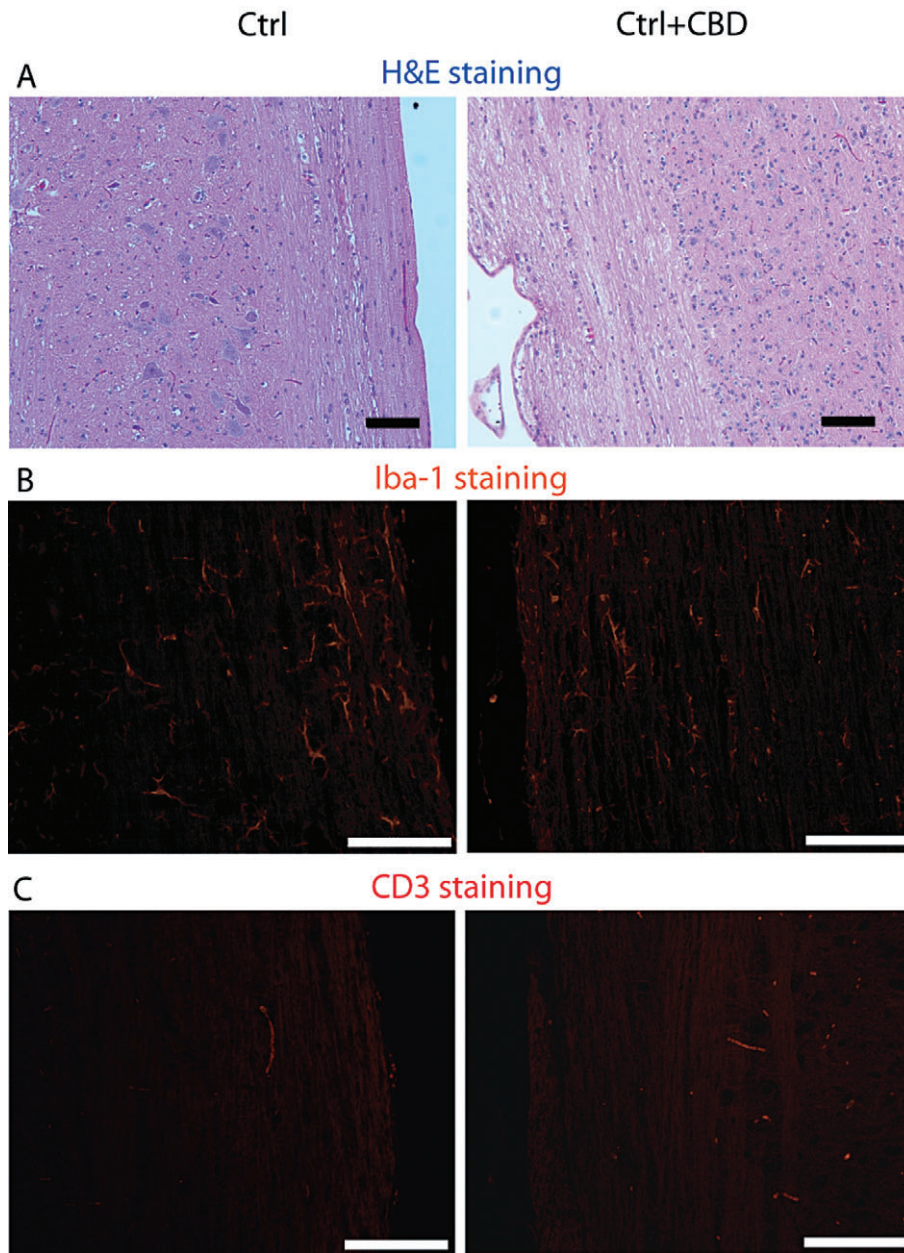


Figure 2

Cannabidiol (CBD) treatment of control, naïve C57BL/6 mice does not affect spinal cord integrity, basal microglial and T-cell number. The Figure shows representative stainings of spinal cord sections prepared from control healthy mice receiving complete Freund adjuvant either without CBD (Ctrl) or with CBD (Ctrl + CBD) treatment. (A) Haematoxylin and eosin (H&E) staining; (B) Iba-1 staining for the presence of microglial/macrophages; and (C) CD3⁺ staining for presence of T-cells. Scale bars represent 100 μm . Statistical analysis of the CD3⁺ and Iba-1 expression is presented in the bar graphs of Figures 5C (CD3⁺) and 6C (Iba-1).

CBD inhibits the MOG-induced proliferation of encephalitogenic T cells

Encephalitogenic MOG35-55 T cells were co-cultured with APCs in the presence of MOG at two concentrations (Ben-Nun and Lando, 1983; Kaushansky *et al.*, 2006). We found that 1 $\mu\text{g}\cdot\text{mL}^{-1}$ of MOG induced 24 ± 11 -fold increase in [³H]Thymidine incorporation and 2.5 $\mu\text{g}\cdot\text{mL}^{-1}$ produced a 42 ± 10 -fold increase in T-cell proliferation. We assayed the effect of CBD on this MOG-induced proliferation. The results

presented in Figure 8 show the inhibitory effect of CBD on the proliferation of these encephalitogenic MOG-specific T cells. We found that the proliferation of these cells stimulated with 1 $\mu\text{g}\cdot\text{mL}^{-1}$ (Figure 8A) or 2.5 $\mu\text{g}\cdot\text{mL}^{-1}$ (Figure 8B) of MOG35-55 was significantly down-regulated in the presence of 1–10 μM CBD. Neither SR141716 (a CB₁ receptor antagonist) nor SR144528 (a CB₂ receptor antagonist), both at 1 μM , affected the proliferative induction observed with MOG35-55 or the antiproliferative effect of CBD (Figure 8 Inset).

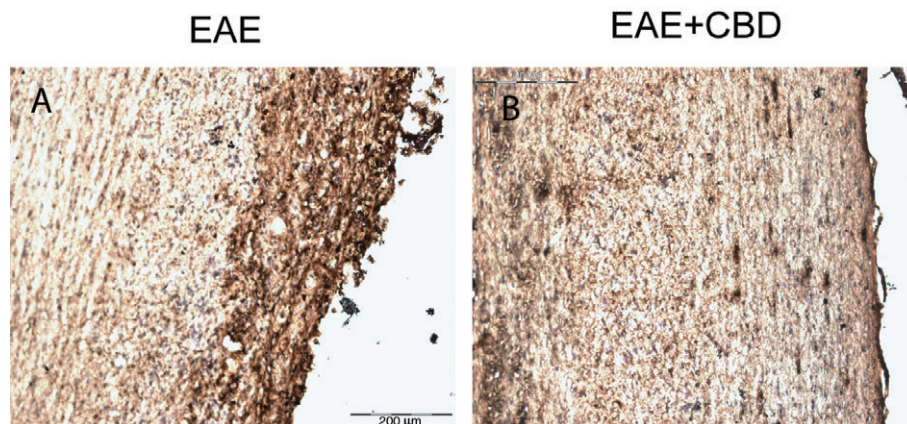


Figure 3

Cannabidiol (CBD) reduces the MOG-induced axonal damage in spinal cords of EAE mice. Mice were killed 30 days after EAE disease induction and sections of spinal cord were analysed for damaged axons using SMI-32 antibody. (A) MOG-induced EAE mice; (B) MOG-induced EAE mice treated with CBD (as described in Figure 1). Scale bar represents 200 μm .

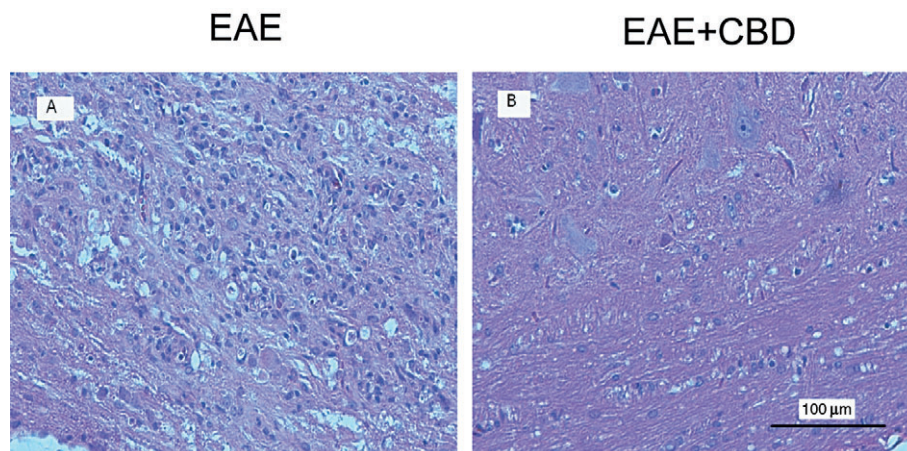


Figure 4

Cannabidiol (CBD) treatment attenuates EAE-induced immune cell infiltration into spinal cord of mice. Haematoxylin and eosin staining revealed that CBD treatment reduces immune cell infiltration into the white matter of the spinal cords of EAE mice. (A) EAE mice and (B) EAE mice treated with CBD. Scale bar represents 100 μm .

In parallel, we evaluated the effect of CBD on the spontaneous proliferation of T cells (without MOG activation). As described above, this spontaneous proliferation was very low compared with the MOG-induced proliferation. We found that neither 0.15 μM ($\text{SI} = 1.1 \pm 0.0$), 5 μM ($\text{SI} = 1.1 \pm 0.0$) nor 10 μM ($\text{SI} = 0.9 \pm 0.0$) of CBD affected the basal level of proliferation of the T cells in the absence of MOG. Altogether, these results show that CBD selectively inhibited antigen-induced cell proliferation but did not affect basal (non-antigen-induced) proliferation.

Discussion

Our data showed that CBD, when administered systemically for 3 days during clinical EAE disease onset, ameliorated the

EAE clinical signs and slowed its progression. The attenuation of EAE progression was accompanied by diminished axonal damage and immune cell infiltration (including T cells and microglial cells) and activation. Using a highly specific autoreactive T-cell line, we observed that CBD decreased the MOG-induced proliferation of these cells.

The effectiveness of cannabinoids in ameliorating the clinical signs of EAE in rodents had been previously shown using mostly mixed affinity CB_1/CB_2 receptor ligands (THC, WIN552122) in either myelin peptide or virus-induced models (Lyman *et al.*, 1989; Arévalo-Martín *et al.*, 2003; Croxford and Miller, 2003). It should be noted that the immunosuppressive effects of CB_1 or mixed CB_1/CB_2 receptor ligands are mostly evident at doses inducing central effects like sedation and hypothermia (Lyman *et al.*, 1989; Croxford *et al.*, 2008). CBD and its derivatives are devoid of such psychoac-

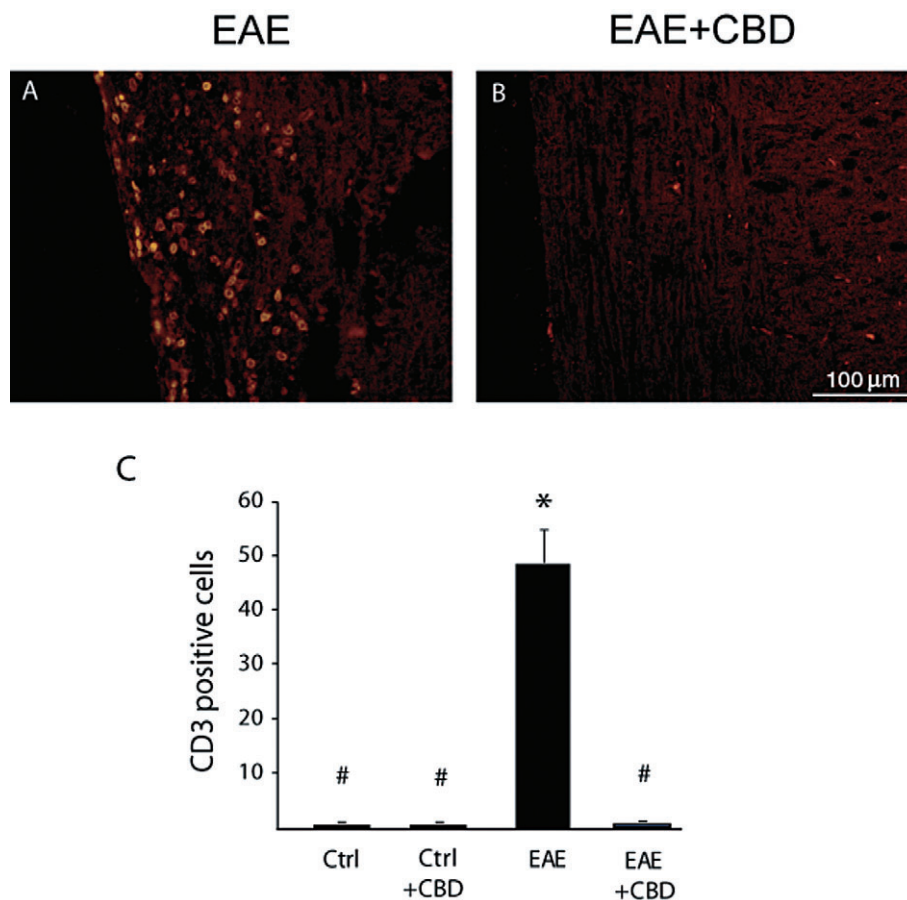


Figure 5

Cannabidiol (CBD) treatment attenuates EAE-induced T-cell infiltration into spinal cord of mice. CD3+ staining reveals that CBD treatment reduces the infiltration of T cells into the white matter of the spinal cords of EAE mice. (A) EAE mice. (B) EAE mice treated with CBD. Scale bar represents 100 μm. The results are presented as mean ± SEM of CD3+ cells from 9–12 spinal fragments from three to four mice per each experimental group. One-way ANOVA $F(3,43) = 45.3$, $P < 0.0001$, followed by Bonferroni *post hoc* test. * $P < 0.001$ versus Ctrl. # $P < 0.001$ versus EAE mice.

tive effects and offer a plethora of beneficial activities. Among them, the immunosuppressive, anti-oxidative and neuroprotective properties of CBD are the most relevant for the complex pathology of MS. CBD was reported to be well tolerated when chronically administered to humans for its anxiolytic and antipsychotic activities (Cunha *et al.*, 1980; Consroe *et al.*, 1991). Moreover, CBD (in a mixture with THC, as in *Sativex*) has been already approved for the treatment of pain and spasticity associated with MS (Zajicek *et al.*, 2003). These features suggest that CBD and CBD derivatives are relatively safe and effective alternatives for the treatment of autoimmune and/or neurodegenerative diseases, such as MS.

Our finding that CBD treatment ameliorates EAE differs from the results by Maresz *et al.* (2007) which showed that CBD had no beneficial effect in their experimental system. The discrepancy between our results and those of Maresz may result from species differences (C57BL/6 vs. ABH mice) and the antigen used (MOG35-55 peptide vs. spinal cord homogenate) as it is well known that susceptibility, severity and pathology of antigen-induced EAE is highly species and antigen dependent (Shevach, 1999). Moreover, even THC, used in their experiments, had to be given at very high doses

of 20 mg·kg⁻¹ and had little or no effect at 5 and 2.5 mg·kg⁻¹. CBD was, however, effective in alleviating signs of other autoimmune inflammatory diseases such as in collagen-induced arthritis (Malfait *et al.*, 2000) and in autoimmune diabetes (Weiss *et al.*, 2008), showing its potential in ameliorating this class of diseases.

Cannabinoids and immune effects

There are many ways in which the cannabinoid system affects immune processes. Cannabinoids were reported to affect the number and function of various immune cells, including T cells, B cells, natural killer cells, dendritic cells, microglia and macrophages in rodents and humans (Nahas *et al.*, 1977; Croxford and Yamamura, 2005; Tanasescu and Constantinescu, 2010). Cannabinoids suppress the production of proinflammatory cytokines including TNFα, IL-1β, IL-2, IL-6, IL-12 and IFN-γ, as well as mitogen-induced cell proliferation, migration, antigen presentation and trafficking into inflamed tissues (Croxford and Yamamura, 2005; Rieder *et al.*, 2010; Tanasescu and Constantinescu, 2010). Because the CB₂ receptor is robustly expressed on immune cells (Mackie, 2005), many of these effects were assumed or have

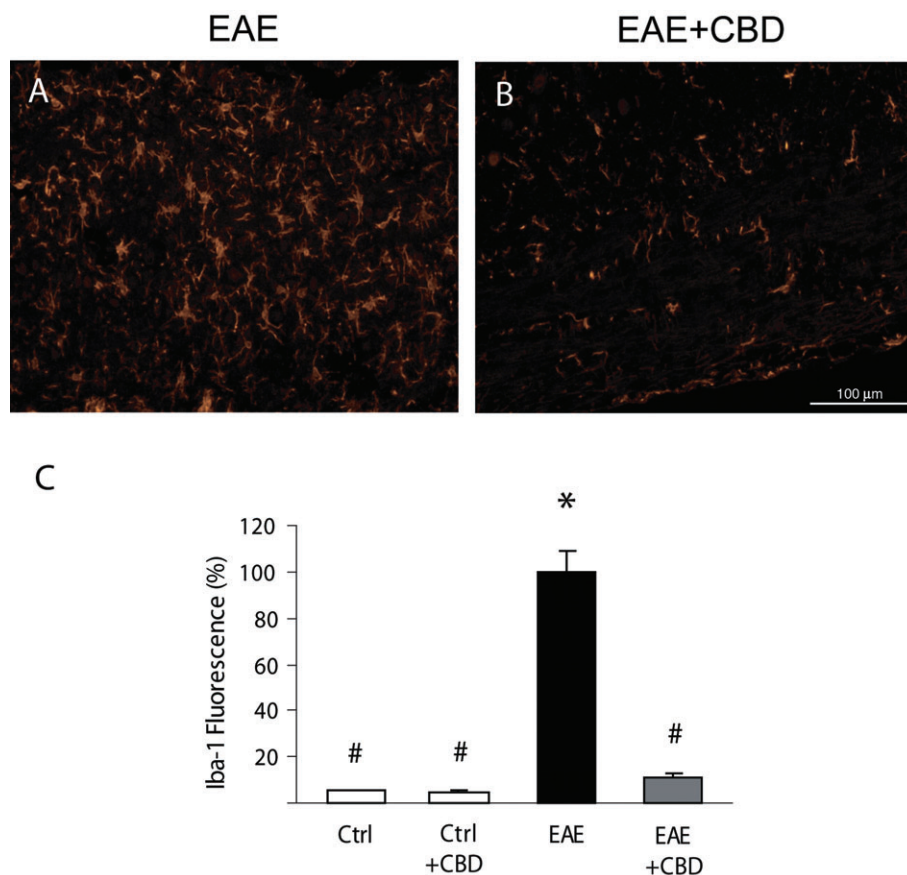


Figure 6

Cannabidiol (CBD) reduces the MOG-up-regulated Iba-1 expression in spinal cords of mice. Representative pictures showing that (A) MOG injections increase Iba-1 staining in spinal cord, and (B) that CBD injections significantly decrease the level of Iba-1 staining. (C) Quantification of Iba-1 fluorescence in sections of control (Ctrl) and of EAE mice with and without CBD treatment. The results are presented as mean \pm SEM of total fluorescence from 8–11 spinal fragments from three to four mice per each experimental group. One-way ANOVA $F(3,37) = 30.23$, $P < 0.0001$, followed by Bonferroni *post hoc* test. * $P < 0.001$ versus Ctrl. # $P < 0.001$ versus EAE mice. Scale bar represents 100 μm .

been shown to be mediated via the CB₂ receptor (Buckley *et al.*, 2000). CB₁ receptor-dependent immunosuppressive effects were also observed, despite their moderate expression in immune cells (Cabral *et al.*, 2001).

However, studies with CB₁ and CB₂ receptor knockout mice as well as the use of specific CB₁ or CB₂ receptor antagonists and of non-CB₁/CB₂ receptor ligands, revealed that many anti-inflammatory effects of cannabinoids do not involve the classic CB₁/CB₂ receptor pathways (Puffenbarger *et al.*, 2000; Kaplan *et al.*, 2003; 2008; Walter *et al.*, 2003; Kozela *et al.*, 2010). Indeed, it is known that non-CB₁/CB₂ receptor immunomodulatory effects exist and include control of cytokine release (Kaplan *et al.*, 2003; Kozela *et al.*, 2010) and migratory activity of microglial cells (Walter *et al.*, 2003; McHugh *et al.*, 2010). Interestingly, some of the effects of known CB₁/CB₂ receptor agonists also seem to involve non-CB₁/CB₂ targets and the effects of non-CB₁/CB₂ receptor ligands were slightly diminished in cells from CB₁/CB₂ receptor knockout mice (Kaplan *et al.*, 2003; 2008) suggesting that several types of cannabinoid targets may complement or interact with each other.

Consequently, CBD, a compound active on non-CB₁ or CB₂ receptors (Showalter *et al.*, 1996), has been shown to

possess a wide anti-inflammatory profile. CBD was shown to decrease TNF α , IL-2 and IFN γ release from activated splenocytes and macrophages (Malfait *et al.*, 2000; Jan *et al.*, 2007; Kaplan *et al.*, 2008). It also suppresses concavalin A and collagen induced T-cell proliferation (Malfait *et al.*, 2000; Jan *et al.*, 2007), microglial migration (Walter *et al.*, 2003) and cytokine release (Kozela *et al.*, 2010). It suppresses antigen-specific antibody production in splenocytes (Jan *et al.*, 2007), as well as attenuates endothelial inflammation and barrier disruption (Rajesh *et al.*, 2007). These activities of CBD may contribute to its beneficial effects in EAE, as observed in our hands, because many of these immune processes were reported to be involved in EAE pathology at different stages of the disease model.

Role of microglial cells in EAE

It is well established that microglia become rapidly activated when the integrity of the CNS is disturbed as a consequence of lesions, neurotoxicity, infections and autoimmune diseases (Hanisch and Kettenmann, 2007). This activation includes the release of a variety of cytotoxic agents, for example, cytokines and chemokines, and of trophic factors that recruit other specialized cells from the periphery into the brain

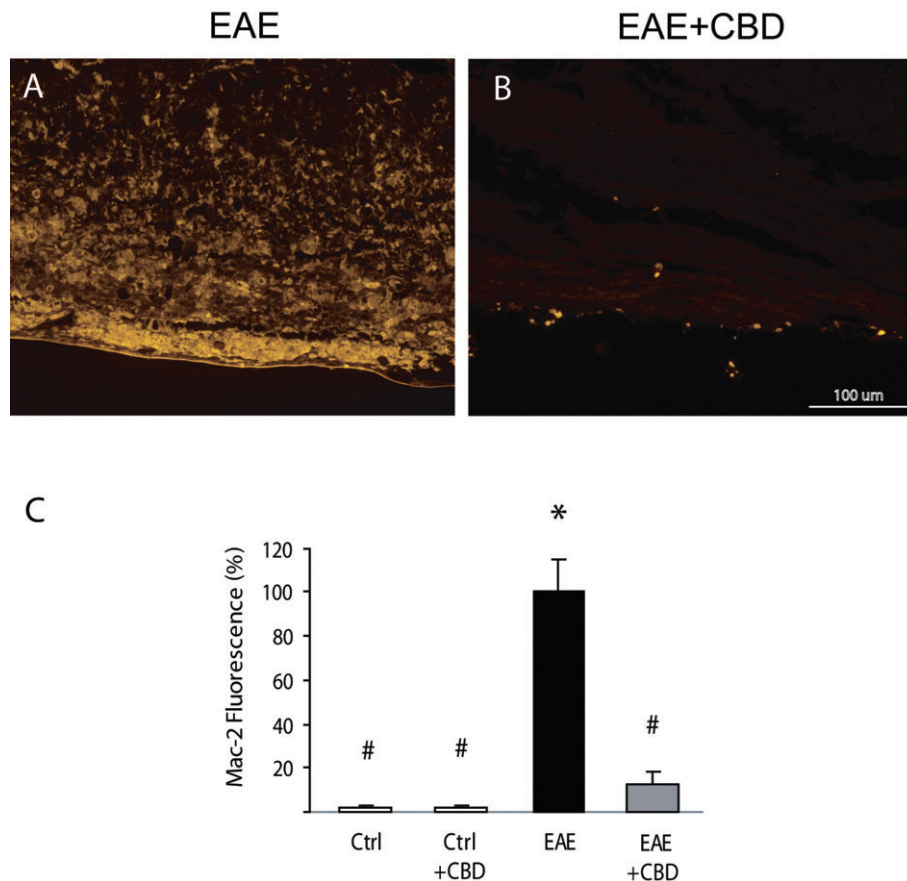


Figure 7

Cannabidiol (CBD) reduces the increased Mac-2/Galectin-3 expression in the spinal cords of EAE mice. Representative pictures showing that (A) MOG injections increase Mac-2 expression and (B) that CBD injections during the onset of EAE significantly decrease the level of Mac-2 staining. (C) Quantification of Mac-2 fluorescence in spinal cord sections of control and EAE mice with and without CBD treatment. The results are presented as mean \pm SEM of total fluorescence from 8–11 spinal fragments from three to four mice per each experimental group. One-way ANOVA $F(3,34) = 63.25$, $P < 0.0001$, followed by Bonferroni *post hoc* test. * $P < 0.001$ versus Ctrl. # $P < 0.001$ versus EAE mice. Scale bar represents 100 μ m.

parenchyma. Microglial activation is accompanied by morphological changes and surface expression of various molecules that serve as adhesion molecules, enzymes, complement and immunoglobulin receptors and molecules related to T-cell activation (Hanisch and Kettenmann, 2007). For example, the Iba-1 protein, located within a segment of the major histocompatibility complex class III region, is specifically expressed in macrophages/microglia and its expression is up-regulated during the activation of these cells. Mac-2 is a member of the Galectin-3 family of galactose binding lectins, is another microglia differentiation and activation marker. In this regard, microglial cells expressing Mac-2 are capable of phagocytosis of myelin, while resting microglia are not (Reichert and Rotshenker, 1999).

Microglial cells are considered as key players in MS at various stages of the disease (Ponomarev *et al.*, 2005; Koning *et al.*, 2009). Depletion or blockade of microglia reduces inflammatory lesions, demyelination and EAE disease progression (Huitinga *et al.*, 1990; Heppner *et al.*, 2005). Thus, arrest of microglial function is postulated to be beneficial in EAE/MS treatment. Indeed, we showed here that CBD injections diminished the activation of microglia as measured by

Iba-1 and Mac-2 expression in the spinal cord of MOG-injected mice. As mentioned above, although Mac-2 and particularly Iba-1 are generally accepted expression markers for microglia (Reichert and Rotshenker, 1999; Heppner *et al.*, 2005), these proteins are also expressed on some perivascular macrophages and macrophages infiltrating the CNS during pathological conditions. Thus, the inhibitory activity of CBD may apply to microglia as well as to several other macrophage-like cells. Previously, we and others have shown that CBD *in vitro* decreases the production of TNF α , IL-1 β , IL-6 and ROS species in LPS-activated microglia (El-Remessy *et al.*, 2006; Liou *et al.*, 2008; Kozela *et al.*, 2010). Moreover, the microglial production of one of the most potent T-cell attracting chemokines, CCL-2, was also reduced following CBD treatment (Kozela *et al.*, 2010; Juknat *et al.*, 2011). CBD also reduces the migration of microglial cells (Walter *et al.*, 2003). These data suggest that CBD is a potent inhibitor of various functions of microglial cells. Thus, by silencing microglial cells, CBD may be able to prevent further inflammatory processes within the CNS parenchyma, that is, myelin antigen exposure, T-cell infiltration, cytokine/chemokine release and recruitment of other immune cells.

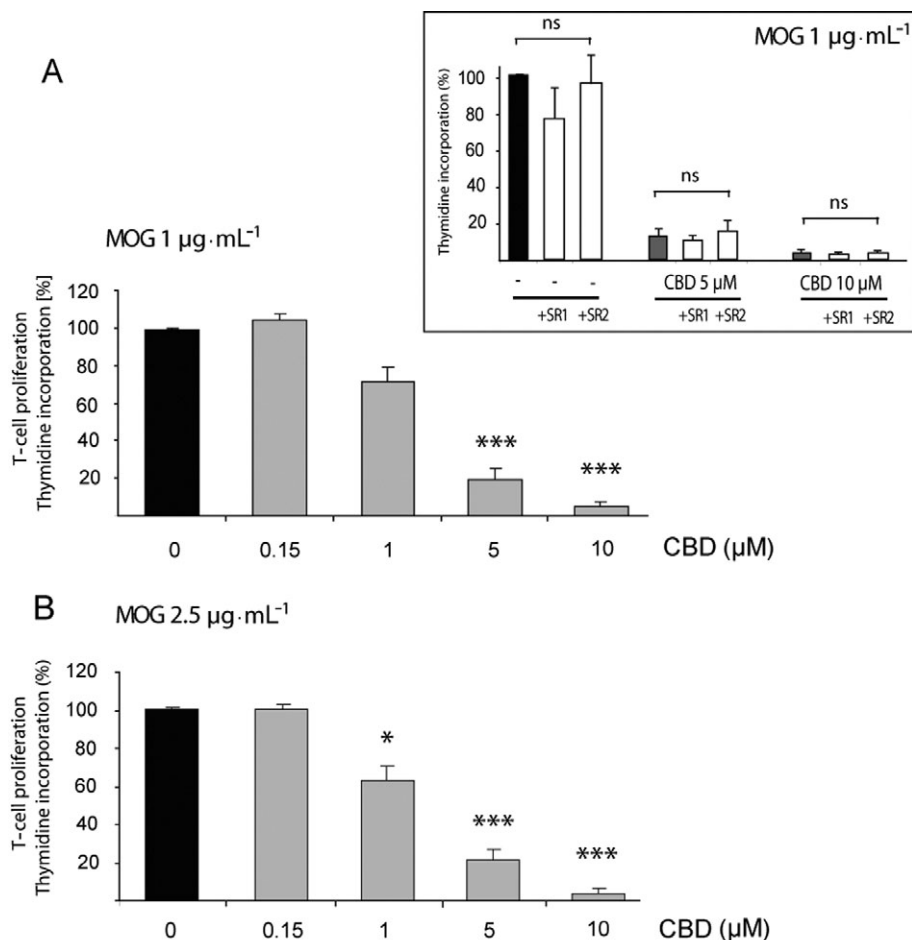


Figure 8

Cannabidiol (CBD) inhibits MOG-induced T-cell proliferation. MOG-induced T-cell proliferation was determined by [³H]thymidine incorporation. Encephalitogenic T cells were co-cultured with antigen presenting cells and exposed to MOG35-55 at 1 (A) and 2.5 µg·mL⁻¹ (B). CBD was added 5 min before MOG. Assays were carried out each time in triplicate and mean per cent values ± SEM are shown based on four independent experiments. (A) One-way ANOVA $F(4,19) = 124.7$, $P < 0.0001$; (B) One-way ANOVA $F(4,19) = 31.1$, $P < 0.0001$, followed by Bonferroni *post hoc* test. * $P < 0.05$, *** $P < 0.001$ versus MOG alone at the respective concentration. *Inset*: neither the CB₁ receptor antagonist (1 µM of SR141716; SR1) nor the CB₂ receptor antagonist (1 µM of SR144528; SR2) applied 30 min before CBD and MOG affected MOG35-55 (1 µg·mL⁻¹) – induced T-cell proliferation either in the absence or presence of CBD; one-way ANOVA $F(8,26) = 26.8$, $P < 0.05$; ns, non-significant.

Antiproliferative effects of CBD

We showed that CBD decreased MOG-induced encephalitogenic T-cell proliferation. This antiproliferative effect of CBD was not mediated via CB₁ or CB₂ receptors. High proliferation rate is a core response of immune cells (including T cells) following various stimuli, such as cytokines, mitogens and antigens presented by APCs. With regard to EAE or MS, it was shown that myelin-specific T-cell clones penetrate the blood-brain barrier and the CNS parenchyma initiating demyelination. We observed that CBD inhibited the MOG-induced proliferation of encephalitogenic T cells isolated from mice, previously immunized against MOG. This potent antiproliferative activity of CBD probably contributes significantly to the EAE-ameliorating effects of CBD and is in agreement with the lower number of T cells present in the spinal cords of CBD-treated EAE mice. Indeed, CBD has been reported to induce apoptosis in transformed T-cell lines (McKallip *et al.*, 2006; Lee *et al.*, 2008) as well as in primary splenic lympho-

cytes including CD4⁺, CD8⁺ and B220⁺ subsets (Lee *et al.*, 2008; Wu *et al.*, 2008). It had a similar apoptotic activity towards lymphocytes in peripheral blood (Ignatowska-Jankowska *et al.*, 2009). Moreover, concavalin A-induced (Jan *et al.*, 2007) and collagen-induced stimulation of T cells (Malfait *et al.*, 2000) was inhibited by CBD. On the other hand, primary monocytes were observed to be resistant to the apoptotic effects of CBD (Gallily *et al.*, 2003). This difference in apoptotic susceptibility of various immune cell types to CBD is of considerable significance but its mechanism is still unknown.

Anti-oxidant and neuroprotective properties of CBD

Several cannabinoids including THC and CBD exert anti-oxidative and neuroprotective properties (Mechoulam *et al.*, 2007). Most of the current MS therapies are directed against various immune cells to achieve immunosuppressive effects.

However, increasing evidence shows that immunosuppression alone was not sufficient for therapeutic effect especially in late, secondary progressive MS (Bennett and Stüve, 2009; Jones and Coles, 2010). In these cases, the neurodegenerative processes become resistant to immunomodulation. Indeed, neurodegeneration that consists of neuronal and axonal loss can result from oxidative stress and excitotoxicity and is driven by activated microglial cells and macrophagic/monocytic infiltrates (Hanisch and Kettenmann, 2007). It appears that the cannabinoid system could provide a rescue mechanism in such conditions. Accordingly, the ameliorating activity of THC-like cannabinoids combines CB₂ receptor-mediated inhibition of autoreactive T cells and CB₁ receptor-mediated neuroprotective activity on neurons (Maresz *et al.*, 2007). Similarly, Croxford *et al.* (2008) pointed out that cannabinoid-mediated neuroprotection rather than immunosuppression, was relevant for the recovery process at the later, remissive stages of MS.

The main source of reactive oxygen and nitrogen species within the CNS under neurodegenerative conditions are reactive microglia and astrocytes (Hanisch and Kettenmann, 2007). Oxidative signalling negatively affects neuronal and axonal survival, especially of axons lacking a myelin sheath, and results in irreversible damage. Accordingly, deletion of nuclear factor-erythroid 2-related 2 (Nrf2), a redox-sensitive transcription factor that regulates expression of many protective antioxidant and detoxication enzymes and transporters (Kobayashi and Yamamoto, 2005), resulted in exacerbation of EAE (Johnson *et al.*, 2010). CBD has been frequently described as a potent neuroprotective and anti-oxidant agent. CBD reduced glutamate excitotoxicity and hydroperoxide-induced oxidative neuronal damage (Hampson *et al.*, 1998) and provided neuroprotection against 6-hydroxy-dopamine (Lastres-Becker *et al.*, 2005), β -amyloid (Iuvone *et al.*, 2004) and prion toxicities to neurons (Dirikoc *et al.*, 2007) and against neuroinflammation (Esposito *et al.*, 2007; Zuardi, 2008). In this regard, we have shown that exposure of microglial cells in culture to CBD up-regulates a number of anti-oxidative genes including genes that are involved in glutathione synthesis. Many of these genes are under the control of the Nrf2 transcription factor (Juknat *et al.*, 2011). Thus, the anti-oxidative properties of CBD may significantly contribute to the alleviation of EAE pathology and accompany its anti-inflammatory beneficial properties.

In summary, we have shown that CBD administered to MOG-immunized C57BL/6 mice, at the onset of EAE disease, reduced the severity of the clinical signs of EAE. CBD treatment was accompanied by diminished axonal loss and inflammation (infiltration of T cells and microglial activation). Moreover, CBD prevented proliferation of myelin-specific T cells *in vitro*. These observations suggest that CBD may have potential for alleviating MS-like pathology.

Acknowledgements

This work was supported by the Dr Miriam and Sheldon G. Adelson Center for the Biology of Addictive Diseases and by the Dr Miriam and Sheldon G. Adelson Medical Research

Foundation. A. J. and N. R. were supported by the Center for Absorption in Science in Israel.

Conflict of interest

The authors state no conflict of interest.

References

- Alexander SPH, Mathie A, Peters JA (2009). Guide to Receptors and Channels (GRAC), 4th edn. *Br J Pharmacol* 158: S1–S254.
- Arévalo-Martín A, Vela JM, Molina-Holgado E, Borrell J, Guaza C (2003). Therapeutic action of cannabinoids in a murine model of multiple sclerosis. *J Neurosci* 23: 2511–2516.
- Bayewitch M, Avidor-Reiss T, Levy R, Barg J, Mechoulam R, Vogel Z (1995). The peripheral cannabinoid receptor: adenylate cyclase inhibition and G protein coupling. *FEBS Lett* 375: 143–147.
- Bennett JL, Stüve O (2009). Update on inflammation, neurodegeneration, and immunoregulation in multiple sclerosis: therapeutic implications. *Clin Neuropharmacol* 32: 121–132.
- Ben-Nun A, Lando Z (1983). Detection of autoimmune cells proliferating to myelin basic protein and selection of T cell lines that mediate experimental autoimmune encephalomyelitis (EAE) in mice. *J Immunol* 130: 1205–1209.
- Buckley NE, McCoy KL, Mezey E, Bonner T, Zimmer A, Felder CC *et al.* (2000). Immunomodulation by cannabinoids is absent in mice deficient for the cannabinoid CB(2) receptor. *Eur J Pharmacol* 396: 141–149.
- Cabral GA, Harmon KN, Carlisle SJ (2001). Cannabinoid-mediated inhibition of inducible nitric oxide production by rat microglial cells: evidence for CB₁ receptor participation. *Adv Exp Med Biol* 493: 207–214.
- Compston A, Coles A (2008). Multiple sclerosis. *Lancet* 372: 1502–1517.
- Consroe P, Laguna J, Allender J, Snider S, Stern L, Sandyk R *et al.* (1991). Controlled clinical trial of cannabidiol in Huntington's disease. *Pharmacol Biochem Behav* 40: 701–708.
- Croxford JL, Miller SD (2003). Immunoregulation of a viral model of multiple sclerosis using the synthetic cannabinoid R+WIN55,212. *J Clin Invest* 111: 1231–1240.
- Croxford JL, Yamamura T (2005). Cannabinoids and the immune system: potential for the treatment of inflammatory diseases? *J Neuroimmunol* 166: 3–18.
- Croxford JL, Pryce G, Jackson SJ, Ledent C, Giovannoni G, Pertwee RG *et al.* (2008). Cannabinoid-mediated neuroprotection, not immunosuppression, may be more relevant to multiple sclerosis. *J Neuroimmunol* 193: 120–129.
- Cunha JM, Carlini EA, Pereira AE, Ramos OL, Pimentel C, Gagliardi R *et al.* (1980). Chronic administration of cannabidiol to healthy volunteers and epileptic patients. *Pharmacology* 21: 175–185.
- Dirikoc S, Priola SA, Marella M, Zsürger N, Chabry J (2007). Nonpsychoactive cannabidiol prevents prion accumulation and protects neurons against prion toxicity. *J Neurosci* 27: 9537–9544.

- El-Remessy AB, Al-Shabrawey M, Khalifa Y, Tsai NT, Caldwell RB, Liou GI (2006). Neuroprotective and blood-retinal barrier-preserving effects of cannabidiol in experimental diabetes. *Am J Pathol* 168: 235–244.
- Esposito G, Scuderi C, Savani C, Steardo L Jr, De Filippis D, Cottone P *et al.* (2007). Cannabidiol in vivo blunts β -amyloid induced neuroinflammation by suppressing IL-1 β and iNOS expression. *Br J Pharmacol* 151: 1272–1279.
- Gallily R, Even-Chena T, Katzavian G, Lehmann D, Dagan A, Mechoulam R (2003). γ -irradiation enhances apoptosis induced by cannabidiol, a non-psychoactive cannabinoid, in cultured HL-60 myeloblastic leukemia cells. *Leuk Lymphoma* 44: 1767–1773.
- Hampson AJ, Grimaldi M, Axelrod J, Wink D (1998). Cannabidiol and (-)Delta9-tetrahydrocannabinol are neuroprotective antioxidants. *Proc Natl Acad Sci U S A* 95: 8268–8273.
- Hanisch UK, Kettenmann H (2007). Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci* 10: 1387–1394.
- Heppner FL, Greter M, Marino D, Falsig J, Raivich G, Hovelmeyer N *et al.* (2005). Experimental autoimmune encephalomyelitis repressed by microglial paralysis. *Nat Med* 11: 146–152.
- Huitinga I, Van Rooijen N, De Groot CJ, Uitdehaag BM, Dijkstra CD (1990). Suppression of experimental allergic encephalomyelitis in Lewis rats after elimination of macrophages. *J Exp Med* 172: 1025–1033.
- Ignatowska-Jankowska B, Jankowski M, Glac W, Swiergel AH (2009). Cannabidiol-induced lymphopenia does not involve NKT and NK cells. *J Physiol Pharmacol* 3 (Suppl.): 99–103.
- Iuvone T, Esposito G, Esposito R, Santamaria R, Di Rosa M, Izzo AA (2004). Neuroprotective effect of cannabidiol, a non-psychoactive component from *Cannabis sativa*, on β -amyloid-induced toxicity in PC12 cells. *J Neurochem* 89: 134–141.
- Jack C, Ruffini F, Bar-Or A, Antel JP (2005). Microglia and multiple sclerosis. *J Neurosci Res* 81: 363–373.
- Jan TR, Su ST, Wu HY, Liao MH (2007). Suppressive effects of cannabidiol on antigen-specific antibody production and functional activity of splenocytes in ovalbumin-sensitized BALB/c mice. *Int Immunopharmacol* 7: 773–780.
- Járai Z, Wagner JA, Varga K, Lake KD, Compton DR, Martin BR *et al.* (1999). Cannabinoid-induced mesenteric vasodilation through an endothelial site distinct from CB₁ or CB₂ receptors. *Proc Natl Acad Sci U S A* 96: 14136–14141.
- Johnson DA, Amirahmadi S, Ward C, Fabry Z, Johnson JA (2010). The absence of the pro-antioxidant transcription factor Nrf2 exacerbates experimental autoimmune encephalomyelitis. *Toxicol Sci* 114: 237–246.
- Jones JL, Coles AJ (2010). New treatment strategies in multiple sclerosis. *Exp Neurol* 225: 34–39.
- Juknat A, Pietr M, Kozela E, Rimmerman N, Levy R, Coppola G *et al.* (2011). Differential transcriptional profiles mediated by exposure to the cannabinoids cannabidiol and Δ^9 -tetrahydrocannabinol in BV-2 microglial cells. *Br J Pharm* DOI: 10.1111/j.1476-5381.2011.01461.x.
- Kaplan BL, Rockwell CE, Kaminski NE (2003). Evidence for cannabinoid receptor-dependent and -independent mechanisms of action in leukocytes. *J Pharmacol Exp Ther* 306: 1077–1085.
- Kaplan BL, Springs AE, Kaminski NE (2008). The profile of immune modulation by cannabidiol (CBD) involves deregulation of nuclear factor of activated T cells (NFAT). *Biochem Pharmacol* 76: 726–737.
- Kaushansky N, Zhong MC, Kerlero de Rosbo N, Hoefftberger R, Lassmann H, Ben-Nun A (2006). Epitope specificity of autoreactive T and B cells associated with experimental autoimmune encephalomyelitis and optic neuritis induced by oligodendrocyte-specific protein in SJL/J mice. *J Immunol* 177: 7364–7376.
- Klein TW, Newton CA (2007). Therapeutic potential of cannabinoid-based drugs. *Adv Exp Med Biol* 601: 395–413.
- Kobayashi M, Yamamoto M (2005). Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. *Antioxid Redox Signal* 7: 385–394.
- Koning N, Uitdehaag BM, Huitinga I, Hoek RM (2009). Restoring immune suppression in the multiple sclerosis brain. *Prog Neurobiol* 89: 359–368.
- Kozela E, Pietr M, Juknat A, Rimmerman N, Levy R, Vogel Z (2010). Cannabinoids Delta(9)-tetrahydrocannabinol and cannabidiol differentially inhibit the lipopolysaccharide-activated NF-kappaB and interferon- β /STAT proinflammatory pathways in BV-2 microglial cells. *J Biol Chem* 285: 1616–1626.
- Lastres-Becker I, Molina-Holgado F, Ramos JA, Mechoulam R, Fernández-Ruiz J (2005). Cannabinoids provide neuroprotection against 6-hydroxydopamine toxicity in vivo and in vitro: relevance to Parkinson's disease. *Neurobiol Dis* 19: 96–107.
- Lee CY, Wey SP, Liao MH, Hsu WL, Wu HY, Jan TR (2008). A comparative study on cannabidiol-induced apoptosis in murine thymocytes and EL-4 thymoma cells. *Int Immunopharmacol* 8: 732–740.
- Lev N, Barhum Y, Melamed E, Offen D (2004). Bax-ablation attenuates experimental autoimmune encephalomyelitis in mice. *Neurosci Lett* 359: 139–142.
- Liou GI, Auchampach JA, Hillard CJ, Zhu G, Yousufzai B, Mian S *et al.* (2008). Mediation of cannabidiol anti-inflammation in the retina by equilibrative nucleoside transporter and A2A adenosine receptor. *Invest Ophthalmol Vis Sci* 49: 5526–5531.
- Lyman WD, Sonett JR, Brosnan CF, Elkin R, Bornstein MB (1989). Delta 9-tetrahydrocannabinol: a novel treatment for experimental autoimmune encephalomyelitis. *J Neuroimmunol* 23: 73–81.
- McHugh D, Hu SS, Rimmerman N, Juknat A, Vogel Z, Walker JM *et al.* (2010). N-arachidonoyl glycine, an abundant endogenous lipid, potently drives directed cellular migration through GPR18, the putative abnormal cannabidiol receptor. *BMC Neurosci* 11: 44.
- McKallip RJ, Jia W, Schlomer J, Warren JW, Nagarkatti PS, Nagarkatti M (2006). Cannabidiol-induced apoptosis in human leukemia cells: a novel role of cannabidiol in the regulation of p22phox and nox4 expression. *Mol Pharmacol* 70: 897–908.
- Mackie K (2005). Distribution of cannabinoid receptors in the central and peripheral nervous system. *Handb Exp Pharmacol* 168: 299–325.
- Malfait AM, Gallily R, Sumariwalla PF, Malik AS, Andreaskos E, Mechoulam R *et al.* (2000). The nonpsychoactive cannabis constituent cannabidiol is an oral anti-arthritis therapeutic in murine collagen-induced arthritis. *Proc Natl Acad Sci U S A* 97: 9561–9566.
- Maresz K, Carrier EJ, Ponomarev ED, Hillard CJ, Dittel BN (2005). Modulation of the cannabinoid CB₂ receptor in microglial cells in response to inflammatory stimuli. *J Neurochem* 95: 437–445.
- Maresz K, Pryce G, Ponomarev ED, Marsicano G, Croxford JL, Shriver LP *et al.* (2007). Direct suppression of CNS autoimmune inflammation via the cannabinoid receptor CB1 on neurons and CB2 on autoreactive T cells. *Nat Med* 13: 492–497.

- Mechoulam R, Peters M, Murillo-Rodriguez E, Hanus LO (2007). Cannabidiol—recent advances. *Chem Biodivers* 4: 1678–1692.
- Nahas GG, Morishima A, Desoize B (1977). Effects of cannabinoids on macromolecular synthesis and replication of cultured lymphocytes. *Fed Proc* 36: 1748–1752.
- Pietr M, Kozela E, Levy R, Rimmerman N, Lin YH, Stella N *et al.* (2009). Differential changes in GPR55 during microglial cell activation. *FEBS Lett* 583: 2071–2076.
- Ponomarev ED, Shriver LP, Maresz K, Dittel BN (2005). Microglial cell activation and proliferation precedes the onset of CNS autoimmunity. *J Neurosci Res* 81: 374–389.
- Puffenbarger RA, Boothe AC, Cabral GA (2000). Cannabinoids inhibit LPS-inducible cytokine mRNA expression in rat microglial cells. *Glia* 29: 58–69.
- Rajesh M, Pan H, Mukhopadhyay P, Ba' tkai S, Osei-Hyiaman D, Hasko G *et al.* (2007). Cannabidiol attenuates high glucose-induced endothelial cell inflammatory response and barrier disruption. *J Leukoc Biol* 82: 1382–1389.
- Reichert F, Rotshenker S (1999). Galectin-3/MAC-2 in experimental allergic encephalomyelitis. *Exp Neurol* 160: 508–514.
- Rieder SA, Chauhan A, Singh U, Nagarkatti M, Nagarkatti P (2010). Cannabinoid-induced apoptosis in immune cells as a pathway to immunosuppression. *Immunobiology* 215: 598–560.
- Shevach EM (1999). Animal models for autoimmune and inflammatory disease. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W (eds). *Current Protocols in Immunology*. John Wiley & Sons, Inc: New York, pp. 15.0.1–15.0.5.
- Showalter VM, Compton DR, Martin BR, Abood ME (1996). Evaluation of binding in a transfected cell line expressing a peripheral cannabinoid receptor (CB₂): identification of cannabinoid receptor subtype selective ligands. *J Pharmacol Exp Ther* 278: 989–999.
- Tanasescu R, Constantinescu CS (2010). Cannabinoids and the immune system: an overview. *Immunobiology* 215: 588–597.
- Walter L, Franklin A, Witting A, Wade C, Xie Y, Kunos G *et al.* (2003). Nonpsychotropic cannabinoid receptors regulate microglial cell migration. *J Neurosci* 23: 1398–1405.
- Weiss L, Zeira M, Reich S, Slavin S, Raz I, Mechoulam R *et al.* (2008). Cannabidiol arrests onset of autoimmune diabetes in NOD mice. *Neuropharmacology* 54: 244–249.
- Wu HY, Chu RM, Wang CC, Lee CY, Lin SH, Jan TR (2008). Cannabidiol-induced apoptosis in primary lymphocytes is associated with oxidative stress-dependent activation of caspase-8. *Toxicol Appl Pharmacol* 226: 260–270.
- Zajicek J, Fox P, Sanders H, Wright D, Vickery J, Nunn A *et al.* (2003). Cannabinoids for treatment of spasticity and others symptoms related to multiple sclerosis (CAMS study): multicenter randomized placebo-controlled trial. *Lancet* 362: 1517–1526.
- Zuardi AW (2008). Cannabidiol: from an inactive cannabinoid to a drug with wide spectrum of action. *Rev Bras Psiquiatr* 30: 271–280.