The CB$_2$ Cannabinoid Receptor Controls Myeloid Progenitor Trafficking

IN VolvE IN THE PATHOGENESIS OF AN ANIMAL MODEL OF MULTIPLE SCLEROSIS

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Cannabinoids are potential agents for the development of therapeutic strategies against multiple sclerosis. Here we analyzed the role of the peripheral CB$_2$ cannabinoid receptor in the control of myeloid progenitor cell trafficking toward the inflamed spinal cord and their contribution to microglial activation in an animal model of multiple sclerosis (experimental autoimmune encephalomyelitis, EAE). CB$_2$ receptor knock-out mice showed an exacerbated clinical score of the disease when compared with their wild-type littermates, and this occurred in concert with extended axonal loss, T-lymphocyte (CD4$^+$) infiltration, and microglial (CD11b$^+$) activation. Immature bone marrow-derived CD34$^+$ myeloid progenitor cells, which play a role in neuroinflammatory pathologies, were shown to express CB$_2$ receptors and to be abundantly recruited toward the spinal cords of CB$_2$ knock-out EAE mice. Bone marrow-derived cell transfer experiments further evidenced the increased contribution of these cells to microglial replenishment in the spinal cords of CB$_2$-deficient animals. In line with these observations, selective pharmacological CB$_2$ activation markedly reduced EAE symptoms, axonal loss, and microglial activation. CB$_2$ receptor manipulation altered the expression pattern of different chemokines (CCL2, CCL3, CCL5) and their receptors (CCR1, CCR2), thus providing a mechanistic explanation for its role in myeloid progenitor recruitment during neuroinflammation. These findings demonstrate the protective role of CB$_2$ receptors in EAE pathology; provide evidence for a new site of CB$_2$ receptor action, namely the targeting of myeloid progenitor trafficking and its contribution to microglial activation; and support the potential use of non-psychoactive CB$_2$ agonists in therapeutic strategies for multiple sclerosis and other neuroinflammatory disorders.

During the last years, it has been shown that the endocannabinoid (eCB)$^7$ system, the endogenous system targeted by active ingredients of the hemp plant Cannabis sativa L (1), is altered in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS) (2–4). In addition, cannabinoid receptor agonist administration or modulation of the eCB tone with eCB reuptake/degradation inhibitors improves pathological signs of the disease, notably spasticity and tremor (3–7). Moreover, cannabinoids have been shown to be effective not only in palliating EAE symptoms but also as neuroprotective agents that, by promoting oligodendrocyte survival (8), reducing demyelinated lesions (9), and attenuating neuronal loss (10, 11), contribute to delayed progression of the disease. This protective role of the eCB system during neuroinflammation is exerted, at least in part, by decreasing immune cell activation and infiltration (12, 13).

Cannabinoid actions are mediated by the activation of two different G-protein-coupled receptors, namely CB$_1$ and CB$_2$ receptors (1). Alleviation of EAE symptoms by cannabinoid intervention is mostly attributed to the engagement of neuronal CB$_1$ receptors (4, 14), whereas CB$_2$ receptor expression by infiltrating T-cells and monocytes is involved in the control of neuroinflammation (4). Likewise, CB$_2$ receptors are functional in microglial cells, in which they inhibit the production of proinflammatory cytokines and oxygen and nitrogen reactive species (12, 15), and their expression is up-regulated upon cell activation (16). The involvement of microglial cell activation in the...
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Evolution and progression of different neurological disorders (MS, stroke, Alzheimer disease, amyotrophic lateral sclerosis, human immunodeficiency virus-associated dementia) has been put forward (17, 18), and it is becoming evident that, besides central nervous system-resident microglial cells, bone marrow-derived cells infiltrate the inflamed central nervous system and differentiate into functional microglia (19–21). Among these cells, CD34⁺ myeloid progenitors expand in the blood of EAE mice, target the inflamed central nervous system, and display differentiation potential toward microglia (22). The present study was therefore aimed at elucidating the role and action mechanism of the CB₂ cannabinoid receptor in EAE. In particular, we focused on the trafficking of myeloid progenitors toward the inflamed spinal cord and their contribution to microglial activation during EAE pathogenesis.

Experimental Procedures

Materials—The following materials were kindly donated: CB₂ receptor knock-out mice by Nancy Buckley (National Institutes of Health, Bethesda, MD) and HU-308 by Pharmos (Rehovot, Israel). The following antibodies were used: polyclonal anti-200-kDa neurofilament heavy protein and anti-human CD11b (clone M1/70) from AbCam (Cambridge, UK); monoclonal rat anti-mouse CD45R/B220 (clone RA3–6B2) fluorescein isothiocyanate-conjugated antibody, rat anti-mouse CD11b (clone M1/70), and anti-CD4 from BD Biosciences; monoclonal anti-mouse CD34-biotin (clone RAM34) from eBioscience (San Diego, CA); polyclonal anti-CB₂ receptor from Affinity BioReagents (Golden, CO); monoclonal anti-human CD45RB (clone PD7/26) from Dako (Glostrup, Denmark); polyclonal anti-GFP from Invitrogen; and rabbit monoclonal anti-200-kDa neurofilament heavy protein and anti-human CD45RB (clone PD7/26) from Dako (Glostrup, Denmark); polyclonal anti-GFP from Invitrogen; and rabbit monoclonal anti-Ki-67 (SP6) from LabVision (Fremont, CA). Macrophage-colony stimulating factor and Fli-3 were from PreproTech Inc. (London, UK).

Animal Procedures and EAE Induction—Animal procedures were performed according to the European Union guidelines (86/669/EU) for the use of laboratory animals. Adult CB₂ receptor-deficient mice (8 weeks old) (24) and their respective wild-type littermates, housed in a temperature-controlled room with 50% humidity, were monitored using the following scale: 0, lack of clinical signs; 1, tail weakness; 2, hind limb paraparesis, hemiparesis, or ataxia; 3, hind limb paralysis or hemiparesis; 4, complete paralysis; 5, moribund; 6, death. In pharmacological experiments, HU-308 (15 mg/kg) was administered intraperitoneally starting at the day of maximal score and thereafter daily until sacrifice. Control animals received the corresponding vehicle injections (100 μl of phosphate-buffered saline (PBS) supplemented with 0.5 mg/ml defatted bovine serum albumin and 4% dimethyl sulfoxide). Each clinical score value was obtained from a representative experiment of four or three independent experiments aimed at elucidating the effect of CB₂ receptor genetic ablation or HU-308 administration, respectively. Cell transfer experiments were performed by intracardiac injection of 5 × 10⁶ bone marrow cells derived from healthy C57BL/6 (ACTβ-EGFP) mice into EAE-induced wild-type (WT) and CB₂⁻/⁻ syngenic mice at day 9 before symptom appearance. Recipient mice were sacrificed and analyzed 10 days after engraftment.

Magnetic resonance imaging was performed in EAE mice (n = 4 each group) the day before sacrifice at the Nuclear Magnetic Resonance Center of Complutense University (Madrid, Spain). Anesthetized mice were placed in a Biospec 47/40 (Bruker, Ettlingen, Germany) operating at 4.7 teslas, equipped with a 12-cm gradient set and using a 4-cm radio frequency surface coil. Three-dimensional T2-weighted spin-echo images were acquired with a fast spin-echo sequence. The acquisition parameters were: time resolution = 2226 ms, effective time echo = 117 ms, field of view = 2.5 × 1.6 × 1.0 cm³, and two averages. The acquisition matrix size was 256 × 128 × 32, which was zero-filled to get a reconstructed matrix size of 256 × 256 × 32. The total acquisition time was 19 min. Diffusion water images delineated the area of neuroinflammation evidenced as hyperintense signals.

Immunofluorescence and Confocal Microscopy—EAE mice were sacrificed, and isolated spinal cords were frozen on dry ice. Immunofluorescence analysis (26) was performed on ethanol-fixed 14-μm-thick cryostat sections. Spinal cord sections were rinsed and blocked for 30 min in PBS supplemented with 10% goat serum and 4% bovine serum albumin and, after washing, incubated with the indicated primary antibodies. Secondary antibody incubation (1 h at room temperature) was performed with the appropriate mouse, rat, and rabbit highly cross-adsorbed Alexa Fluor 488, Alexa Fluor 594, and streptavidin conjugates (Invitrogen). Washed sections were incubated with Hoechst 33342 (5 μg/ml) in PBS prior to mounting.

The specificity of CB₂ receptor immunoreactivity was corroborated by using CB₂⁻/⁻ mouse sections, in which no immunoreactivity was observed, and allowed to adjust optimal confocal microscope settings. CB₂ receptor expression was analyzed with anti-CB₂ receptor antibody together with anti-CD11b and anti-CD34-biotinilated antibodies (overnight incubation at 4 °C) followed by secondary staining for rabbit and mouse IgGs with highly cross-adsorbed Alexa Fluor 647, Alexa Fluor 594, and streptavidin-Alexa Fluor 488 secondary antibodies, respectively. In addition, triple immunostaining was performed with a combination of anti-CD34, CD45R/B220, and CB₂ primary antibodies and their appropriate secondary fluorescent antibodies. All immunofluorescence data were obtained in a blinded manner by two independent observers in a minimum of 5–7 adjacent slices of two different samples from the lumbo-thoracic spinal cord of the same animal. To determine the loss of axonal surface and the extent of microglial...
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FIGURE 1. EAE is exacerbated in CB2-deficient mice. A, EAE score was determined daily after induction of the disease in WT (open circles) and CB2<sup>−/−</sup> littermates (closed circles; n = 8 each group). Scores were compared between the two groups day by day. *, p < 0.05 versus WT from day 17 on. B, the mean values of the day of clinical onset, maximum score, and mean score from symptom appearance are shown. *, p < 0.05 versus WT. C, representative MRI images at day 24 of spinal cords from WT and CB2<sup>−/−</sup> mice after EAE induction are shown. The bright signal of low water diffusion areas corresponds to inflamed tissue. Scale bars: general axial projection (left panel), 2.0 mm; magnified axial projections (top panels), 1.3 mm; and magnified sagital projections (bottom panels), 1.7 mm.

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on Ficoll (Histopaque 1077; Sigma) from blood samples obtained from the cave vein. Cells were washed with PBS and resuspended in PBS supplemented with 2% goat calf serum for flow cytometry analysis or frozen for RNA extraction. Flow cytometry was performed with 0.5 × 10<sup>6</sup> cells per condition fixed in 1% paraformaldehyde at 4 °C. Antibodies and their corresponding controls were incubated for 30 min at 4 °C in 2% goat serum-PBS and, after washing, samples were subjected to secondary antibody incubation. Ten thousand cells per recording were analyzed using a FACSCalibur flow cytometer.

mRNA Detection and Quantification—RNA was obtained with the RNeasy Protect kit (Qiagen, Valencia, CA) using the RNase-free DNase kit. cDNA was subsequently obtained using the SuperScript first-strand cDNA synthesis kit (Roche Applied Science, Welwyn Garden City, UK) and amplified with the primers indicated in supplemental Table 1A. CB<sub>1</sub> and CB<sub>2</sub> PCR amplifications were performed using the following conditions: 93 °C for 1 min, two rounds (30 s at 59 °C, 1 min at 72 °C, and 30 s at 93 °C), two rounds (30 s at 57 °C, 1 min at 72 °C, and 30 s at 93 °C), and 35 cycles (30 s at 55 °C, 1 min at 72 °C, and 30 s at 93 °C).
93 °C). Finally, after a final extension step at 72 °C for 5 min, PCR products were separated on 1.5% agarose gels. Real-time quantitative PCR was performed with Universal probe system (Roche Applied Science, Basel, Switzerland) using the primers indicated in supplemental Table 1B. Amplifications were run in a 7900-HT Fast Real-time PCR system, and obtained values were adjusted using 18 S RNA levels as reference.

### Multiple Sclerosis Human Tissue Samples

Tissue samples were supplied by the UK Multiple Sclerosis Tissue Bank, funded by the Multiple Sclerosis Society of Great Britain and Northern Ireland, Registered Charity 207495. Cortical brain samples fixed in formalin, embedded, and cut in 4-μm-thick sections were employed for immunohistochemical study as described previously (28). Briefly, sections were deparaflinized and, after washing, subjected to antigen retrieval procedure. Tissue samples were incubated with mouse anti-human CD45RB and rat anti-human CD11b monoclonal antibodies together with rabbit polyclonal anti-CB2 receptor antibody.

### Statistical Analysis

Results shown represent the means ± S.E. of the number of experiments indicated in every case. Statistical analysis was performed by analysis of variance. A post hoc analysis was made by the Student-Neuman-Keuls test. In vivo data were analyzed by an unpaired Student’s t test.
RESULTS

CB$_2$ Cannabinoid Receptor Deficiency Exacerbates EAE Pathogenesis—Wild-type mice immunized by myelin oligodendrocyte glycoprotein injection developed EAE with the appearance of symptoms starting at day 12.4 ± 4.2 and, after reaching a maximal stage at day 20.2 ± 2.7 (Fig. 1A), entered a chronic clinical phase (22, 25) The involvement of the CB$_2$ receptor in the appearance of EAE symptoms was investigated by comparing wild-type and CB$_2^{-/-}$ littermates, which showed that the latter developed a notably higher symptomatic EAE score (Fig. 1, A and B). The day before sacrifice, magnetic resonance imaging analysis was performed, confirming the existence of an exacerbated neuroinflammatory process in the dorsal spinal cords of CB$_2^{-/-}$ animals as evidenced by the bright signal of low water diffusion areas (Fig. 1C). Spinal cord lesions were characterized in further detail by histological analysis. Quantification of neurofilament H immunofluorescence showed an increased axonal loss (Fig. 2A) together with extended microglial-cell (CD11b) activation and T-lymphocyte (CD4) infiltration in CB$_2$-deficient mice (Fig. 2, B and C), thus providing further evidence for the exacerbated phenotype of these animals.

As CB$_2$ receptors are highly expressed in various disease conditions, their role in the pathogenesis of these conditions is of particular interest. To investigate this further, we examined the expression of CB$_2$ receptors in spinal cord myeloid-derived microglial cells and in CD45R microglial cells in cortical plaques of multiple sclerosis patients. Confocal microscopy analyses were performed by using CB$_2$ receptor (green), CD45R (red), and CD11b (blue) antibodies. Images show CB$_2$ receptor expression in CD45R$^+$ microglial cells (denoted by arrows) (1), CD11b$^+$ CD45R$^+$ microglial cells (2), and CD11b$^+$ CD45R$^-$ cells (3) located in the vicinity of blood vessels. Cell nuclei were counterstained with Hoechst 33342 (gray). Scale bars, 35 and 10 µm in upper and lower panels, respectively.

FIGURE 4. Expression of the CB$_2$ cannabinoid receptor in spinal cord myeloid-derived microglial cells. A, confocal microscopy images of EAE spinal cord sections were obtained with CD34 (green), CD11b (red), and CB$_2$ receptor (blue) antibodies. The CB$_2$ receptor was also present in CD45RB220$^+$ (green) and CD11b$^+$ (red) cells. Total cell nuclei were counterstained with Hoechst 33342 (gray). The right column shows the merged images. Scale bars, 50 and 10 µm in upper and middle panels, respectively. B, transcript levels of the indicated elements of the eCB system. Transcript levels were normalized to 18 S RNA expression, referred to healthy mice levels, and provided in arbitrary units (a.u.). **, p < 0.01 versus control.

FIGURE 5. Expression of the CB$_2$ cannabinoid receptor in CD45R$^+$ microglial cells in cortical plaques of multiple sclerosis patients. Confocal microscopy analyses were performed by using CB$_2$ receptor (green), CD45R (red), and CD11b (blue) antibodies. Images show CB$_2$ receptor expression in CD45R$^+$ CD11b$^+$ microglial cells (denoted by arrows) (1), CD11b$^+$ CD45R$^+$ microglial cells (2), and CD11b$^+$ CD45R$^-$ cells (3) located in the vicinity of blood vessels. Cell nuclei were counterstained with Hoechst 33342 (gray). Scale bars, 35 and 10 µm in upper and lower panels, respectively.
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expressed in bone marrow immune cells (13, 15), and myeloid progenitor cells can be recruited into the neuroinflamed central nervous system (19–22), we analyzed the presence of cells expressing CD34, a marker for primitive myeloid progenitors. Of interest, an increased number of CD34⁺ cells was evident in CB₂-deficient spinal cords (Fig. 2D). Increased proliferation (Ki-67⁺ cells) of CD11b and CD34 cell populations was also observed in CB₂ knock-out mice (Fig. 2E).

Selective CB₂ Cannabinoid Receptor Activation Palliates EAE Symptoms and Pathogenesis—As CB₂ receptor genetic ablation exacerbates EAE pathogenesis, we assessed the impact of CB₂ receptor selective activation on disease progression. Daily injections of the CB₂-selective agonist HU-308 (29) were performed starting at the day of maximal disease score. In agreement with the observations from CB₂-deficient mice, HU-308 treatment improved EAE score and the evolution of the disease when compared with vehicle administration (Fig. 3A). The specificity of HU-308 action was confirmed by the use of CB₂⁻/⁻ mice, in which this agonist was unable to decrease EAE score (data not shown). The analysis of HU-308 administration on tissue histology revealed a strong reduction of spinal cord infiltrates (Fig. 3B) that correlated with reduced axonal loss (Fig. 3C, left panel), microglial activation (Fig. 3C, middle panel), and myeloid CD34⁺ cell infiltration (Fig. 3C, right panel). Of importance, HU-308 administration decreased microglial and infiltrating myeloid cell proliferation (Fig. 3D).

Expression of the eCB System in Myeloid Progenitor Cells—To confirm whether myeloid progenitors with the ability to generate microglial cells may be directly targeted by CB₂ receptor activation, we examined spinal cord sections of EAE mice. Confocal microscopy confirmed the expression of CB₂ receptors in CD34⁺ myeloid progenitor cells (Fig. 4A). Importantly, these cells were also positive for CD11b, supporting that recruited cells are prone to microglial differentiation (19–22). In addition, CB₂ receptors were expressed in CD11b⁺ cells that co-express CD45R/B220 (Fig. 4A), a marker that has been shown to identify myeloid progenitors with the potential to differentiate to microglial cells (22). Real-time PCR quantification analysis revealed that in EAE mice transcript levels of CD34, CD45R/B220, CD11b, and CD11c were increased in spinal cord extracts (Fig. 4B). The eCB system elements were also up-regulated during EAE, and the maximal induction was observed for CB₂.
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receptor expression, which was accompanied by increased expression of the CB1 receptor and the degrading enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL; Fig. 4C). Next, we analyzed the expression of CB2 receptors in brain sections of MS patients. CB2 receptors were expressed in microglial cells of plaques located in the vicinity of blood vessels (28), and importantly, a subpopulation of these cells was shown to co-express the myeloid marker CD45RB (Fig. 5).

Finally, we verified the expression of CB2 receptors in myeloid progenitors from bone marrow-derived cultures. Immunofluorescence analysis showed the co-expression of CB2 receptors with CD34 and CD45R/B220 (Fig. 6A). CB2 receptors were also present in committed cells that express CD11b together with CD45R/B220 or CD34 (Fig. 6A). Gene expression analysis by reverse transcription-PCR (Fig. 6B) and quantitative PCR (Fig. 6, C and D, left panel) of myeloid progenitor cell cultures confirmed the expression of CD34, CD45R/B220, and CD11b. Microglial-like cell differentiation reduced the expression of CD34, whereas increased expression of CD11b and CD11c was observed. CB2 receptor transcripts were highly expressed in myeloid progenitors and were also evident in differentiated microglial-like cells (Fig. 6, B and D). In contrast, CB2 receptor mRNA was hardly detectable in myeloid progenitors and was below detection limits after differentiation. The eCB degrading enzymes showed opposite patterns of expression with decreased FAAH and up-regulated MAGL transcripts upon differentiation (Fig. 6D, right panel).

The CB2 Cannabinoid Receptor Controls Bone Marrow-derived Myeloid Cell Trafficking—The mechanism of CB2 receptor regulation on myeloid cell recruitment and microglial replenishment during EAE pathogenesis was further investigated. Bone marrow cells from EAE mice were obtained at the end of the experiment and analyzed by flow cytometry. The number of CD11b+/− cells was not altered in CB2−/− mice when compared with wild-type mice (Fig. 7A, upper panel). In contrast, CD34+ cells were increased in mice lacking the CB2 receptor (Fig. 7A, lower panel), whereas the opposite was observed upon HU-308 administration (CD34+ cells 2.4 ± 0.2 and 1.4 ± 0.3% in vehicle- and HU-308-treated mice, respectively; p < 0.01). Flow cytometry (Fig. 7B) and real-time PCR quantification analysis (Fig. 7C) of PBMCs obtained from the same EAE mice supported that, in parallel with bone marrow cell profiles, CD34+ but not CD11b+ cells were expanded into circulating blood cells in mice lacking the CB2 receptor. These findings are in line with the aforementioned higher number of CD34+ and CD11b+ cells found in spinal cords of CB2−/− mice, suggesting that the CB2 receptor can control myeloid progenitor cell trafficking toward neuroinflamed tissue.

An analysis of the in vivo expression of chemokine ligands and receptors was performed in EAE spinal cord and bone marrow. CB2 receptor ablation up-regulated chemokines and receptors known to be important in microglial recruitment to inflammatory lesions (30). Thus, CCL2, CCL3, and CCL5 transcript levels were increased, and similarly, their principal receptors CCR2 and CCR1 were also induced in bone marrow (Fig. 8, A and B). On the other hand, HU-308 administration resulted in an overall reduction of these chemoattractant ligands and receptors (Fig. 8, C and D).

Increased Bone Marrow-derived Cell Recruitment in Spinal Cords of CB2 Receptor-deficient EAE Mice—To confirm the involvement of the CB2 receptor in microglial replenishment from myeloid progenitors, bone marrow GFP-labeled cells derived from healthy EGFP transgenic mice were transferred into EAE-induced wild-type and CB2−/− mice before symptom appearance (Fig. 9A). At the end of the experiment, flow cytometry analysis revealed the presence of a significant population of grafted cells in the PBMC fraction obtained from the blood of recipient animals (9.6 ± 1.1%), of which the majority (96.1 ±
Genetic and pharmacological studies support that neuronal CB₁ receptor expression is required for cannabinoid-mediated suppression of EAE symptoms (4, 14, 32). In contrast, other reports have pointed to the involvement of both CB₁ and CB₂ receptors in the beneficial effects of cannabinoids in MS models (4, 7, 9, 11, 33). Thus, the precise role of CB₂ receptors in EAE pathology is still a matter of debate.

Infiltrating T-cells and resident microglia make a major contribution to the ethiopathology of neuroinflammation and neurodegeneration in MS patients, as well as in animal models of the disease (17). In addition, recent research has shown that brain-resident microglia can be replenished by grafted bone marrow-derived myeloid progenitors (19–21, 34). In particular, under neuroinflammatory conditions such as EAE, bone marrow-derived CD34⁺ myeloid progenitors mobilize through the blood and target the inflamed brain, allowing the recruitment of new microglial cells (22). By using various experimental approaches, here we demonstrate that CB₂ receptors play an important role in the control of EAE pathology and provide evidence for the mechanism of CB₂ action, namely the targeting of myeloid progenitor cells. Thus, the absence of CB₂ receptors significantly exacerbates EAE symptoms and myeloid cell recruitment into the inflamed central nervous system, whereas the opposite is observed upon CB₂-selective agonist administration. Control of microglial recruitment by CB₂ receptors involves the regulation of important mediators of cell trafficking such as chemokines and their receptors. Genetic and pharmacological models of CB₂ receptor manipulation elicit changes in the expression of chemokines that promote myeloid cell recruitment to neuroinflamed tissue, allowing the recruit-ment of new microglial cells (22).

In summary, these results support the involvement of CB₂ receptors in bone marrow-derived myeloid cell recruitment toward neuroinflamed tissue.

**DISCUSSION**

Alterations of the eCB system have been implicated in the pathogenesis of several neurodegenerative disorders, and activation of cannabinoid receptors exerts neuroprotection in various models of brain damage including excitotoxicity, traumatic brain injury, and stroke (1, 31). Moreover, cannabinoid administration elicits an improvement of symptoms of different neuroinflammatory situations including EAE (2, 12).

2.7% co-expressed CD11b (Fig. 9B, upper panel). CB₂⁻/⁻ mice showed an increased number of total circulating CD34⁺ cells, which was also evident within the GFP⁺ population (Fig. 9B, lower panel). Spinal cord sections were also analyzed, and bone marrow-derived GFP⁺ cells were observed (Fig. 9C). Quantification of GFP⁺ cells showed a higher number of infiltrating myeloid cells in CB₂⁻/⁻ mice than in wild-type littermates (Fig. 9C). Further immunofluorescence analysis evidenced that transferred GFP⁺ cells constituted differentiated microglial infiltrates in the spinal cord as they expressed the CD11b marker (91.2 ± 3.6%), whereas in some GFP⁺ cells (20.2 ± 3.6%), CD34 expression was still evident (Fig. 9D). Moreover, the fraction of GFP⁺CD11b⁺ cells within the total CD11b⁺ microglial cell pool was elevated in CB₂⁻/⁻ mice (Fig. 9E). In summary, these results support the involvement of CB₂ receptors in bone marrow-derived myeloid cell recruitment toward neuroinflamed tissue.
shown in neural cells (from neural progenitors to mature neurons and neuroglial cells) (39) and B-cells (from virgin B-cells to centroblasts) (40), and CB2 receptor activation and overexpression has been reported to block neutrophil cell differentiation (41). It is therefore conceivable that changes in cell proliferation, as observed in our study, and differentiation may also contribute to CB2 receptor-regulated myeloid progenitor trafficking.

Our data agree with the current notion that microglial cells express CB2 receptors (42), which are strongly up-regulated in animal models of neuroinflammation (16) and in plaques of MS patients (28, 43). Microglial cells synthesize and degrade eCB ligands such as 2-arachidonoylglycerol (12), the levels of which are negatively controlled by the interferon-γ released by primed T-cells invading the central nervous system during EAE, thus indicating that impaired 2-arachidonoylglycerol production may be associated with neurodegeneration in EAE (44). In addition, cannabinoids down-regulate the production of proinflammatory cytokines (mostly interleukin-1β, interleukin-6, and tumor necrosis factor-α by microglial cells and interleukin-2, interferon-γ, and granulocyte-macrophage colony-stimulating factor by autoreactive T cells), as well as of nitrogen and oxygen reactive species (6, 12, 13). Overall eCBs via CB2 receptors appear to play a key neuroimmunomodulatory role in EAE not only by preventing T-cell-mediated neurodegeneration (2, 4) but also by inhibiting bone marrow-derived myeloid cell recruitment (present report) and microglial activation (11).

The preclinical studies evidencing the ability of cannabinoids to manage EAE symptoms have fostered the investigation for their potential translation to the clinic (2, 18, 45, 46). Beneficial cannabinoid actions in MS patients, supported by large scale phase III clinical trials, include alleviation of spasticity and tremor, neuropathic pain, and nocturia. Nonetheless, therapies for MS management should be able to prevent not only those symptoms but also neuroinflammation, demyelination, axonal loss, and neurodegeneration to exert a clinically relevant impact in the secondary phase of the disease (2, 17). In this context, cannabinoids constitute a very attractive possibility for therapeutic intervention as they are neuroprotective (31), prevent demyelination (9), and exert a wide array of anti-inflammatory actions (12, 13). The use of selective CB2 receptor agonists for the treatment of MS, and perhaps of other neuroinflammatory conditions, constitutes therefore an attractive possibility owing to the selective role of this cannabinoid receptor type in immune regulation and to the absence of marijuana-like psychoactive effects associated with its activation (15, 23).

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