The CB$_2$ Cannabinoid Receptor Controls Myeloid Progenitor Trafficking

IN Volvement in the Pathogenesis of an Animal Model of multiple sclerosis

Javier Palazuelos$^1$, Nathalie Davoust$^2$, Boris Julien$^3$, Eric Hatterer$^5$, Tania Aguado$^4$, Raphael Mechoulam$^6$, Cristina Benito$^1$, Julian Romero$^2$, Augusto Silva$^*$$^*$, Manuel Guzmán$^1$, Serge Nataf$^5$, and Ismael Galve-Roper$^5,6$

From the $^1$Department of Biochemistry and Molecular Biology I, School of Biology, and Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Complutense University, 28040 Madrid, Spain, the $^2$INSERM U433, Institut Fédéral Recherche (IFR) des Neurosciences de Lyon, Faculté de Médecine Laënnec, 69372 Lyon, France, the $^3$Department of Medicinal Chemistry and Natural Products, School of Pharmacy, The Hebrew University, 91120 Jerusalem, Israel, the $^4$Laboratorio de Apoyo a la Investigación, Fundación Hospital Alcorcón, 28922 Madrid, Spain, and the $^5$Centro de Investigaciones Biológicas (CIB-CSIC), Ramiro de Maeztu 9, 28040 Madrid, Spain.

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

---

$^*\text{This work was supported by grants from the Picasso Program (Grant} 
HF2005-0017), \text{Comunidad Autónoma de Madrid (Grants S-SAL/0261/2006 and 950344), Santander Complutense (Grant PR27/05-13988), Fundación de Investigación Médica Mutua Madrileña Automovilística, Ministerio de Educación y Ciencia (Grants SAF2004/00237), the French Embassy in Spain, and Association pour la Recherche sur la Sclérose en Plaques. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.}$

$^\text{1}$Complutense University, 28040 Madrid, Spain.

$^2$INSERM U433, Institut Fédéral Recherche (IFR) des Neurosciences de Lyon, Faculté de Médecine Laënnec, 69372 Lyon, France.

$^3$Department of Medicinal Chemistry and Natural Products, School of Pharmacy, The Hebrew University, 91120 Jerusalem, Israel.

$^4$Laboratorio de Apoyo a la Investigación, Fundación Hospital Alcorcón, 28922 Madrid, Spain.

$^5$Centro de Investigaciones Biológicas (CIB-CSIC), Ramiro de Maeztu 9, 28040 Madrid, Spain.

$^6$This on-line version of this article (available at http://www.jbc.org) contains a supplemental table.

$^\text{1}$Supported by the Ministerio de Educación y Ciencia (Formación de Personal Investigador program; Spain).

$^\text{2}$Present address: INSERM U851, Université de Lyon, 69365 Lyon Cedex 07, France.

$^\text{3}$Supported by the Fondation pour Recherche Médicale (France).

$^\text{4}$Supported by the Comunidad Autónoma de Madrid (Spain).

$^\text{5}$Both authors contributed equally to this work.

$^\text{6}$To whom correspondence should be addressed. Tel.: 34-913944668; Fax: 34-913944672; E-mail: igri@quim.ucm.es.

---

$^\text{7}$The abbreviations used are: eCB, endocannabinoid; EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; PBS, phosphate-buffered saline; GFP, green fluorescent protein; EGFP, enhanced GFP; WT, wild-type; PBMC, peripheral blood mononuclear cell; FAAM, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase.
Control of Myeloid Progenitor Recruitment by \( \text{CB}_2 \) Receptors

**Materials**—The following materials were kindly donated: \( \text{CB}_2 \) 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 RA3–8B2) 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-\( \text{CB}_2 \) 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-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 \( \text{CB}_2 \) receptor-deficient mice (8 weeks old) (24) and their respective wild-type littermates, housed in a temperature-controlled room with a fixed 12-h light/12-h dark cycle, were obtained from het-500tertype littermates, housed in a temperature-controlled room with a fixed 12-h light/12-h dark cycle, were obtained from heterotyphetal mating partners, and their respective wild-type littermates, housed in a temperature-controlled room with a fixed 12-h light/12-h dark cycle, were obtained from het-200te type littermates, housed in a temperature-controlled room with a fixed 12-h light/12-h dark cycle, were obtained from het-

A fixed 12-h light/12-h dark cycle, were obtained from het-

It is becoming evident that, besides human immunodeficiency virus-associated dementia (MS, stroke, Alzheimer disease, amyotrophic lateral sclerosis, and selective atrophies), CB2 receptor engagement with cannabinoid components is an attractive therapeutic possibility (15, 23). 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**

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 those cells, CD34+ myeloid progenitors expand in the blood of EAE mice, target the inflamed central nervous system, and display differentiation potential toward microglia (22). As modulation of cannabinoid signaling may result in unwanted psychoactive actions mediated by neuronal CB1 receptors, the use of \( \text{CB}_2 \)-selective agonists is an attractive therapeutic possibility (15, 23). The present study was therefore aimed at elucidating the role and action mechanism of the \( \text{CB}_2 \) 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.

---

N. Davoust, C. Vuillat, G. Androdias, and S. Nataf, manuscript in preparation.
Control of Myeloid Progenitor Recruitment by CB$_2$ Receptors

![FIGURE 1. EAE is exacerbated in CB$_2$−/− mice. A, EAE score was determined daily after induction of the disease in WT (open circles) and CB$_2$−/− litters (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 CB$_2$−/− 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 sagittal projections (bottom panels), 1.7 mm.](image)

activation, neurofilament H and CD11b staining was quantified with Metamorph-Offline software (Universal Imaging, Downington, PA) and referred to the total white matter area of spinal cord sections. T-cells, CD34$^+$ cells, and bone marrow GFP infiltration were quantified by immunoreactive positive cell counting, and results are given as mean cell number per mm$^2$. CD34 and CD31 double immunostaining was routinely performed to confirm that CD34 immunoreactivity included, in addition to CD31$^+$ vascular endothelial cells, cells with microglial features. Only CD34$^+$ cells displaying a rounded morphology (similar to ameboid microglia) or extending short processes (similar to reactive microglia) were counted. Both perivascular and parenchymal CD34$^+$ cells were observed, and a significant fraction of those cells did not express the endothelial marker CD31 and co-expressed the microglial marker CD11b. Results are given as mean cell number per mm$^2$. In vitro immunofluorescence was performed with the indicated primary antibodies and the corresponding secondary fluorescent antibodies (as above). Immunofluorescence controls were routinely performed with incubations in which primary antibodies were not included.

Immunofluorescence images shown in Figs. 2 and 3 were obtained with a fluorescence microscope (Zeiss Axioplan II) for fluorescence-activated cell sorter analysis.

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 analysis. Flow cytometry was performed with 0.5 × 10$^6$ 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$_1$ and CB$_2$, 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). Images in Figs. 4–6 and 9 were examined using Leica TCS-SP2 software (Wetzlar, Germany) and SP2 microscope with two passes by Kalman filter and a 1024 × 1024 collection box.
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 18S 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 deparaffinized 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.

![FIGURE 2. Characterization of spinal cord sections in CB2-deficient mice during EAE. A, axonal loss was quantified (right panel) by neurofilament (NF) immunoreactivity in the white matter of spinal cord sections of WT and CB2-/- mice at the end of the experiment. Representative immunofluorescence images are shown (left panels). Phenotypic analysis of infiltrating cells in spinal cord sections was performed. B, quantification of microglial activation was determined as the CD11b+ area referred to the total area examined. C and D, the number of infiltrating CD4+ (C) and CD34+ (D) cells were also determined and expressed as the mean cell number per mm². E, proliferating cells were determined by double immunofluorescence with antibodies against Ki-67 and CD11b or CD34. Scale bar, 100 μm. **, p < 0.01 versus control.](image)

![FIGURE 3. Administration of the CB2-selective agonist HU-308 improves EAE symptoms and reduces spinal cord lesions and microglial activation. A, EAE score in WT mice treated with vehicle (Veh; open circles) or HU-308 (closed triangles; 15 mg/kg daily; n = 5 each group) at the indicated time point (arrow). *, p < 0.05 versus vehicle from day 24 on. The ratio of EAE score at day 28 to EAE score at day 18 is shown in the right panel. **, p < 0.01 versus vehicle. B, representative immunofluorescence images of neurofilament (NF) (red) and Hoechst 33342 nuclear infiltrates (blue) in vehicle- and HU-308-treated mice. Scale bar, 100 μm. C, quantification of axonal loss (NF immunoreactivity), microglial activation (CD11b), and CD34-positive cell infiltration. D, proliferating cells were determined by double immunofluorescence with antibodies against Ki-67 and CD11b or CD34. **, p < 0.01 versus vehicle.](image)
CB₂ 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 CB₂ receptor in the appearance of EAE symptoms was investigated by comparing wild-type and CB₂⁻/⁻ 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 CB₂⁻/⁻ 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) and T-lymphocyte (CD4) infiltration in CB₂-deficient mice (Fig. 2, B and C), thus providing further evidence for the exacerbated phenotype of these animals.

As CB₂ receptors are highly...
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+/H11001 cells was evident in CB2-deficient spinal cords (Fig. 2D). Increased proliferation (Ki-67+/H11001 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−/− 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).

A, immunofluorescence of cultured myeloid progenitors was performed with the indicated combinations of antibodies against CD34, CD45R/B220, CD11b, and CB2 receptor. Total cell nuclei were counterstained with Hoechst 33342 (gray), and the right column shows the merged images. Scale bars, 25 μm in upper and middle panels and 45 μm in lower panels. B, reverse transcription-PCR expression analysis of the eCB system receptors (CB1, CB2) and de-activation enzymes FAAH; full-length and catalytic MAGL (MAGL-F and MAGL-C, respectively) in myeloid progenitors (MP), and microglial-like differentiated cells (MG). The B16 melanoma cell line was used as a control. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. C, real-time PCR quantification of the indicated transcripts in myeloid progenitors and microglial-like differentiated cells. D, transcript levels of the indicated elements of the eCB system including receptors (left panel) and de-activation enzymes (right panel). Transcript levels were normalized to 18 S RNA expression and are provided in arbitrary units (a.u.). **, p < 0.01 versus myeloid progenitors; N.D., non-detectable.
receptor expression, which was accompanied by increased expression of the CB₁ receptor and the degrading enzymes fatty acid amidase 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, CB₁ 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 SpinalCORDs 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 ±
Control of Myeloid Progenitor Recruitment by CB<sub>2</sub> Receptors

Genetic and pharmacological studies support that neuronal CB<sub>1</sub> 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 CB<sub>1</sub> and CB<sub>2</sub> receptors in the beneficial effects of cannabinoids in MS models (4, 7, 9, 11, 33). Thus, the precise role of CB<sub>2</sub> 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<sup>+</sup> 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 CB<sub>2</sub> receptors play an important role in the control of EAE pathology and provide evidence for the mechanism of CB<sub>2</sub> action, namely the targeting of myeloid progenitor cells. Thus, the absence of CB<sub>2</sub> receptors significantly exacerbates EAE symptoms and myeloid cell recruitment into the inflamed central nervous system, whereas the opposite is observed upon CB<sub>2</sub>-selective agonist administration. Control of microglial recruitment by CB<sub>2</sub> receptors involves the regulation of important mediators of cell trafficking such as chemokines and their receptors. Genetic and pharmacological models of CB<sub>2</sub> receptor manipulation elicit changes in the expression of chemokines that promote myeloid cell recruitment to neuroinflammatory lesions such as CCL2, CCL3, and CCL5. In addition, expression of their receptors CCR2 and CCR1 was also affected. Our results are in line with the role of the CCL2-CCR2 axis in microglial replenishment derived from inflammatory monocytes (35, 36). Likewise, CCR2 is required for bone marrow-derived microglial recruitment and the development of neuropathic pain (37). In this context, stimulation of human monocytes with CB<sub>2</sub>-selective agonists has been shown to inhibit CCL2- and CCL3-mediated chemotaxis by cross-desensitization of CCR2 and CCR1 (38).

CB<sub>2</sub> receptors participate in the control of cell proliferation, survival, and differentiation fate decisions (15). Thus, CB<sub>2</sub> receptor activation controls hematopoietic and neural progenitor cell proliferation and differentiation. An inverse relation between CB<sub>2</sub> receptor expression and stage of cell differentiation has been

FIGURE 8. The CB<sub>2</sub> cannabinoid receptor regulates the expression of chemokine ligands and receptors. Real-time PCR analysis of the indicated chemokines and their receptors normalized to 18S RNA transcript levels in spinal cord (A) and bone marrow (B) extracts obtained from WT and CB<sub>2</sub><sup>−/−</sup> EAE mice at the end of the experiment (n = 8 each group). a.u., arbitrary units. A similar analysis was performed in spinal cord (C) and bone marrow (D) of wild-type EAE mice treated with vehicle (Veh, white bars) or HU-308 (striped bars; 15 mg/kg daily; n = 5 each group). Results are provided in arbitrary units and referred to wild-type (A and B) or vehicle-treated mice (C and D). **, p < 0.01 versus WT or vehicle-treated mice.

2.7%) co-expressed CD11b (Fig. 9B, upper panel). CB<sub>2</sub><sup>−/−</sup> mice showed an increased number of total circulating CD34<sup>+</sup> cells, which was also evident within the GFP<sup>+</sup> population (Fig. 9B, lower panel). Spinal cord sections were also analyzed, and bone marrow-derived GFP<sup>+</sup> cells were observed (Fig. 9C). Quantification of GFP<sup>+</sup> cells showed a higher number of infiltrating myeloid cells in CB<sub>2</sub><sup>−/−</sup> mice than in wild-type littermates (Fig. 9C). Further immunofluorescence analysis evidenced that transferred GFP<sup>+</sup> cells constituted differentiated microglial infiltrates in the spinal cord as they expressed the CD11b marker (91.2 ± 3.6%), whereas in some GFP<sup>+</sup> cells (20.2 ± 3.6%), CD34 expression was still evident (Fig. 9D). Moreover, the fraction of GFP<sup>+</sup>CD11b<sup>+</sup> cells within the total CD11b<sup>+</sup> microglial cell pool was elevated in CB<sub>2</sub><sup>−/−</sup> mice (Fig. 9E). In summary, these results support the involvement of CB<sub>2</sub> 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).
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 have 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).

Acknowledgments—We are indebted to our laboratory colleagues for their support and encouragement, to I. del Valle and A. Sánchez for fruitful scientific discussions and collaboration, to A. Egia and E. Resel for technical support, and to M. Fernández and D. Castañón for excellent assistance in MRI imaging.
