The CB₂ Cannabinoid Receptor Controls Myeloid Progenitor Trafficking

INVolvement in the pathogenesis of an animal model of multiple sclerosis™

Received for publication, September 24, 2007, and in revised form, February 11, 2008. Published, JBC Papers in Press, March 11, 2008, DOI 10.1074/jbc.M707960200

Javier Palazuelos 1, Nathalie Davoust ², Boris Julien ³, Eric Hatterer ⁵, Tania Aguado ⁶, Raphael Mechoulam ⁶, Cristina Benito ⁴, Julian Romero ⁴, Augusto Silva ⁵, Manuel Guzmán ⁴, Serge Nata ⁵, Ismael Galve-Roperh ²,§, and Iisnales Nataf ¹, Julian Romero ², Serge Nata ³, Tania Aguado ³, Eric Hatterer ³, Tania Aguado ⁵, Manuel Guzmán ⁵, and Ismael Galve-Roperh ²,§.

From the ¹Department 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 ²INSERM U433, Institut Fédératif Recherche (IFR) des Neurosciences de Lyon, Faculté de Médecine Laennec, 69372 Lyon, France, the ³Department of Medicinal Chemistry and Natural Products, School of Pharmacy, The Hebrew University, 91120 Jerusalem, Israel, the ⁴Department of Biochemistry and Molecular Biology I, School of Biology, and 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 CB₂ 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). CB₂ 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 CB₂ receptors and to be abundantly recruited toward the spinal cords of CB₂ 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 CB₂-deficient animals. In line with these observations, selective pharmacological CB₂ activation markedly reduced EAE symptoms, axonal loss, and microglial activation. CB₂ 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 CB₂ receptors in EAE pathology; provide evidence for a new site of CB₂ receptor action, namely the targeting of myeloid progenitor trafficking and its contribution to microglial activation; and support the potential use of non-psychoactive CB₂ 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 CB₁ and CB₂ receptors (1). Alleviation of EAE symptoms by cannabinoid intervention is mostly attributed to the engagement of neuronal CB₁ receptors (4, 14), whereas CB₂ receptor expression by infiltrating T-cells and monocytes is involved in the control of neuroinflammation (4). Likewise, CB₂ 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

*This work was supported by grants from the Picasso Program (Grant HF2005-0017), 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 Sclerose 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.

† The on-line version of this article (available at http://www.jbc.org) contains a supplemental table.

‡ Supported by the Ministerio de Educación y Ciencia (Formación de Personal Investigador program; Spain).

§ Present address: INSERM U851, Université de Lyon, 69365 Lyon Cedex 07, France.

‡ Supported by the Fondation pour Recherche Medicale (France).

⁴ Supported by the Comunidad Autónoma de Madrid (Spain).

⁵ Both authors contributed equally to this work.

⁶ To whom correspondence should be addressed. Tel.: 34-913944668; Fax: 34-913944672; E-mail:igr@quim.ucm.es.

* 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; FAAH, fatty acid amide hydrolase; MAGL, monoyacylglycerol lipase.

© 2008 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
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 CB2-selective agonists 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**

*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 antibody incubation (1 h at room temperature) was performed with the indicated primary antibodies. Secondary antibody incubation (1 h at room temperature) was performed with the appropriate mouse, rat, and rabbit highly cross-absorbed Alexa Fluor 488, Alexa Fluor 594, Alexa Fluor 647 secondary antibodies or with Alexa Fluor 488 or Alexa Fluor 594 streptavidin conjugates (Invitrogen). Washed sections were incubated with Hoechst 33342 (5 μg/ml) in PBS prior to mounting.

Animal Procedures and EAE Induction—Animal procedures were performed according to the European Union guidelines (86/609/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 a fixed 12-h light/12-h dark cycle, were obtained from heterozygote crosses. For EAE induction, mice were immunized at days 0 and 2, mice were administered intraperitoneally 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 (ACT-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 using 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-absorbed Alexa Fluor 488, Alexa Fluor 594, Alexa Fluor 647 secondary antibodies or with Alexa Fluor 488 or Alexa Fluor 594 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-biotinylated 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

---

Control of Myeloid Progenitor Recruitment by CB₂ Receptors

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⁻/⁻ 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⁻/⁻ 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 with Metamorph-Offline software (Universal Imaging, Downingtown, 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 mm². CD34 and CD31 double immunostaining was routinely performed with incubations in which primary antibodies and their corresponding secondary fluorescent immunofluorescence was performed with the indicated primary antibodies and their 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 cultured as described (22, 27) with slight modifications. Briefly, 8-week-old female mice were sacrificed, and bone marrows were harvested by flushing out tibiae and femurs and culturing in Iscove’s modified Dulbecco’s medium supplemented with 4% N2 (Invitrogen), 15 ng/ml Flt-3, and macrophage colony-stimulating factor and penicillin and streptomycin. Six days after plating, non-adherent cells were harvested and, after washing, characterized by flow cytometry or replated for the generation of microglial-like cells. Myeloid progenitors were differentiated for 5 days in bone marrow culture medium (described above) supplemented with 1 volume (50%) of glial cell conditioned medium. Prior or after differentiation, adherent cells were fixed for immunofluorescence, frozen for RNA extraction, or detached for RNA analysis.

Cell Culture and Flow Cytometry Analysis—Bone marrow cells were cultured as described (22, 27) with slight modifications. Briefly, 8-week-old female mice were sacrificed, and bone marrows were harvested by flushing out tibiae and femurs and culturing in Iscove’s modified Dulbecco’s medium supplemented with 4% N2 (Invitrogen), 15 ng/ml Flt-3, and macrophage colony-stimulating factor and penicillin and streptomycin. Six days after plating, non-adherent cells were harvested and, after washing, characterized by flow cytometry or replated for the generation of microglial-like cells. Myeloid progenitors were differentiated for 5 days in bone marrow culture medium (described above) supplemented with 1 volume (50%) of glial cell conditioned medium. Prior or after differentiation, adherent cells were fixed for immunofluorescence, frozen for RNA extraction, or detached for RNA 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 × 10⁶ 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. CB₁, and CB₂ 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). 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 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.
RESULTS

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 (Fig. 4, A), confocal microscopy images of EAE spinal cord sections were obtained with CD34 (green), CD11b (red), and CB₂ receptor (blue) antibodies. The CB₂ receptor was also present in CD45R/B220 (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 myeloid and microglial markers were analyzed in spinal cord extracts from wild-type mice, either healthy or EAE-induced (clinical score ranging from 2.5 to 3.5). C, 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.

CB₂ receptors are highly expressed (Fig. 5, A), confocal microscopy analyses were performed by using CB₂ receptor (green), CD45R (red), and CD11b (blue) antibodies. Images show CB₂ 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.
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). 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.
Control of Myeloid Progenitor Recruitment by CB2 Receptors

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 CD45R/B220 and CD34 (Fig. 6). 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. 6C 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).

In contrast, CD34+ bone marrow cells obtained from WT and CB2−/− EAE mice at the end of the experiment (n = 8 each group). Representative histograms are shown (right panels). B, flow cytometry analysis of CD11b+ and CD34+ cells of PBMCs from WT and CB2−/− EAE mice. C, levels of CD11b and CD34 transcripts were determined by real-time quantitative PCR in PBMC extracts of the same animals. Transcript levels were normalized to 18 S RNA expression, referred to WT levels, and provided in arbitrary units (a.u.). #, p < 0.1, *, p < 0.05, **, p < 0.01 versus WT.
Control of Myeloid Progenitor Recruitment by CB₂ Receptors

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.

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).

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 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 CB₂-selective agonists has been shown to inhibit CCL2- and CCL3-mediated chemotaxis by cross-desensitization of CCR2 and CCR1 (38).

CB₂ receptors participate in the control of cell proliferation, survival, and differentiation fate decisions (15). Thus, CB₂ receptor activation controls hematopoietic and neural progenitor cell proliferation and differentiation. An inverse relation between CB₂ receptor expression and stage of cell differentiation has been
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).

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. Castejón for excellent assistance in MRI imaging.
Control of Myeloid Progenitor Recruitment by CB2 Receptors