

# The CB<sub>2</sub> Cannabinoid Receptor Controls Myeloid Progenitor Trafficking

## INVOLVEMENT IN THE PATHOGENESIS OF AN ANIMAL MODEL OF MULTIPLE SCLEROSIS<sup>\*§</sup>

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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<sub>2</sub> 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<sub>2</sub> 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<sup>+</sup>) infiltration, and microglial (CD11b<sup>+</sup>) activation. Immature bone marrow-derived CD34<sup>+</sup> myeloid progenitor cells, which play a role in neuroinflammatory pathologies, were shown to express CB<sub>2</sub> receptors and to be abundantly recruited toward the spinal cords of CB<sub>2</sub> 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<sub>2</sub>-deficient animals. In line with these observations, selective pharmacological CB<sub>2</sub> activation markedly reduced EAE symptoms, axonal loss, and microglial activation. CB<sub>2</sub> 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 find-

ings demonstrate the protective role of CB<sub>2</sub> receptors in EAE pathology; provide evidence for a new site of CB<sub>2</sub> receptor action, namely the targeting of myeloid progenitor trafficking and its contribution to microglial activation; and support the potential use of non-psychoactive CB<sub>2</sub> agonists in therapeutic strategies for multiple sclerosis and other neuroinflammatory disorders.

During the last years, it has been shown that the endocannabinoid (eCB)<sup>7</sup> 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<sub>1</sub> and CB<sub>2</sub> receptors (1). Alleviation of EAE symptoms by cannabinoid intervention is mostly attributed to the engagement of neuronal CB<sub>1</sub> receptors (4, 14), whereas CB<sub>2</sub> receptor expression by infiltrating T-cells and monocytes is involved in the control of neuroinflammation (4). Likewise, CB<sub>2</sub> 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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<sup>7</sup> 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, monoacylglycerol lipase.

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),<sup>8</sup> 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<sup>+</sup> 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 CB<sub>1</sub> receptors, the use of CB<sub>2</sub>-selective agonists is an attractive therapeutic possibility (15, 23). The present study was therefore aimed at elucidating the role and action mechanism of the CB<sub>2</sub> 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: CB<sub>2</sub> 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-CB<sub>2</sub> 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 Flt-3 were from PreproTech Inc. (London, UK).

**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 CB<sub>2</sub> 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 7 by subcutaneous injection of 150 μg of myelin oligodendrocyte glycoprotein 35–55 peptide emulsified in complete Freund's adjuvant, as described previously (25). In addition, at days 0 and 2, mice were administered intraperitoneally with 500 ng of pertussis toxin (Sigma). Clinical scores 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 hemiparalysis; 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 CB<sub>2</sub> receptor genetic ablation or HU-308 administration, respectively. Cell transfer experiments were performed by intracardiac injection of 5 × 10<sup>6</sup> bone marrow cells derived from healthy C57BL/6 (ACTβ-EGFP) mice into EAE-induced wild-type (WT) and CB<sub>2</sub><sup>-/-</sup> syngenic mice at day 9 before symptom appearance. Recipient mice were sacrificed and analyzed 10 days after engraftment.

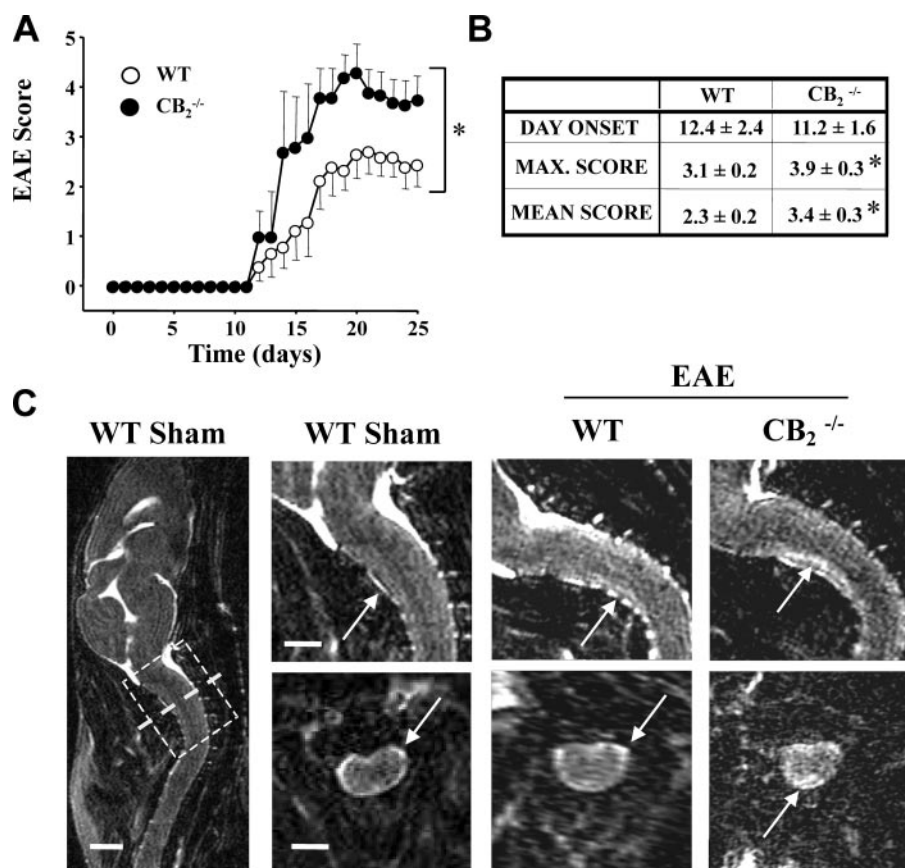
Magnetic resonance imaging was performed in EAE mice (*n* = 4 each group) the day before sacrifice at the Nuclear Magnetic Resonance Center of Complutense University (Madrid, Spain). Anesthetized mice were placed in a Biospec 47/40 (Bruker, Ettlingen, Germany) operating at 4.7 teslas, equipped with a 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 recovery = 2226 ms, effective time echo = 117 ms, field of view = 2.5 × 1.6 × 1.0 cm<sup>3</sup>, 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, 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 CB<sub>2</sub> receptor immunoreactivity was corroborated by using CB<sub>2</sub><sup>-/-</sup> mouse sections, in which no immunoreactivity was observed, and allowed to adjust optimal confocal microscope settings. CB<sub>2</sub> receptor expression was analyzed with anti-CB<sub>2</sub> 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 CB<sub>2</sub> 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

<sup>8</sup>N. Davoust, C. Vuillat, G. Androdias, and S. Nataf, manuscript in preparation.

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**FIGURE 1. EAE is exacerbated in CB<sub>2</sub>-deficient mice.** *A*, EAE score was determined daily after induction of the disease in WT (open circles) and CB<sub>2</sub><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 CB<sub>2</sub><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 sagittal projections (bottom panels), 1.7 mm.

activation, neurofilament H and CD11b staining was quantified with Metamorph-Offline software (Universal Imaging, Downingtown, PA) and referred to the total white matter area of spinal cord sections. T-cells, CD34<sup>+</sup> cells, and bone marrow GFP infiltration were quantified by immunoreactive positive cell counting, and results are given as mean cell number per mm<sup>2</sup>. CD34 and CD31 double immunostaining was routinely performed to confirm that CD34 immunoreactivity included, in addition to CD31<sup>+</sup> vascular endothelial cells, cells with microglial features. Only CD34<sup>+</sup> cells displaying a rounded morphology (similar to amoeboid microglia) or extending short processes (similar to reactive microglia) were counted. Both perivascular and parenchymal CD34<sup>+</sup> 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<sup>2</sup>. *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)

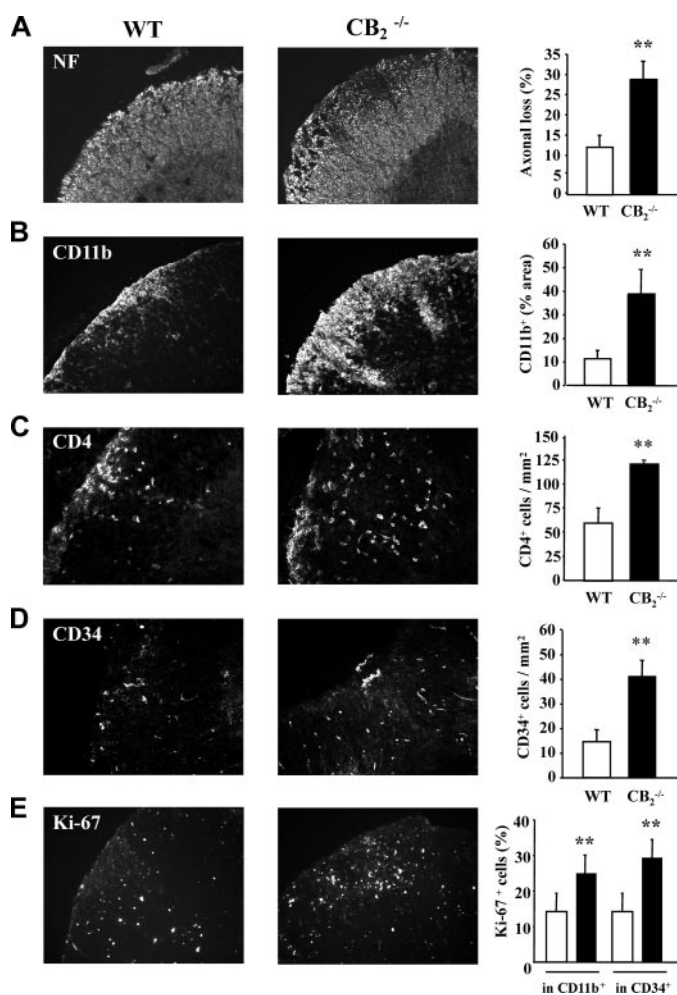
and acquired with a CDD camera (F-View II; Soft Imaging System). Images in Figs. 4–6 and 9 were examined using Leica TCS-SP2 software (Wetzlar, Germany) and SP2 microscope with two passes by Kalman filter and a 1024 × 1024 collection box.

**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 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<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.

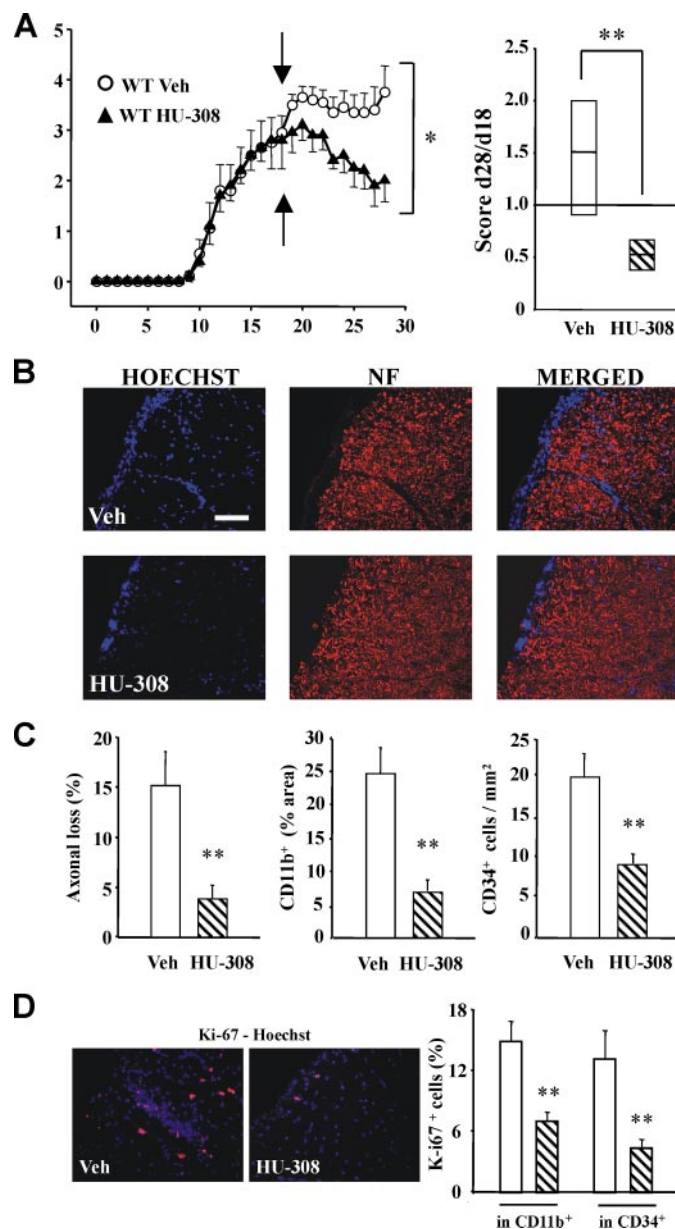
**mRNA Detection and Quantification**—RNA was obtained with the RNeasy Protect kit (Qiagen, Valencia, CA) using the RNase-free DNase kit. cDNA was subsequently obtained using the SuperScript first-strand cDNA synthesis kit (Roche Applied Science, Welwyn Garden City, UK) and amplified with the primers 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



**FIGURE 2. Characterization of spinal cord sections in CB<sub>2</sub>-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 CB<sub>2</sub><sup>-/-</sup> 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<sup>+</sup> area referred to the total area examined. C and D, the number of infiltrating CD4<sup>+</sup> (C) and CD34<sup>+</sup> (D) cells were also determined and expressed as the mean cell number per mm<sup>2</sup>. 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.

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.

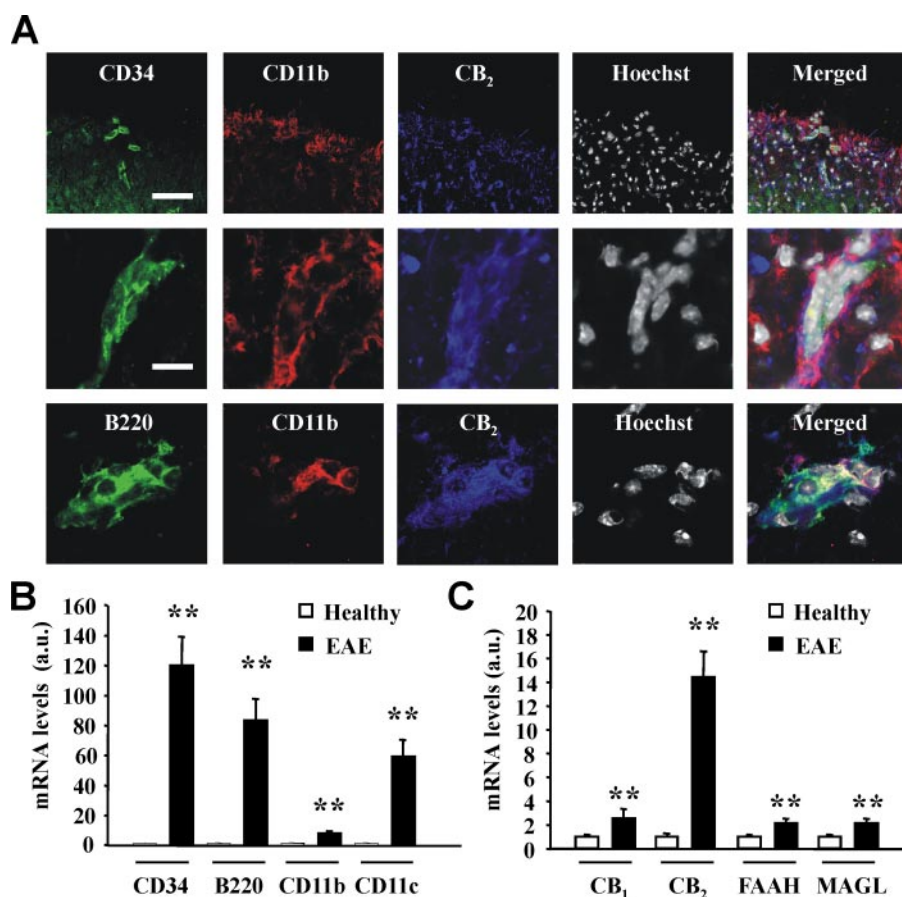
**Multiple Sclerosis Human Tissue Samples**—Tissue samples were supplied by the UK Multiple Sclerosis Tissue Bank, funded by the Multiple Sclerosis Society of Great Britain and Northern Ireland, Registered Charity 207495. Cortical brain samples fixed in formalin, embedded, and cut in 4-μm-thick sections 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-hu-



**FIGURE 3. Administration of the CB<sub>2</sub>-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.

man CD45RB and rat anti-human CD11b monoclonal antibodies together with rabbit polyclonal anti-CB<sub>2</sub> receptor antibody.

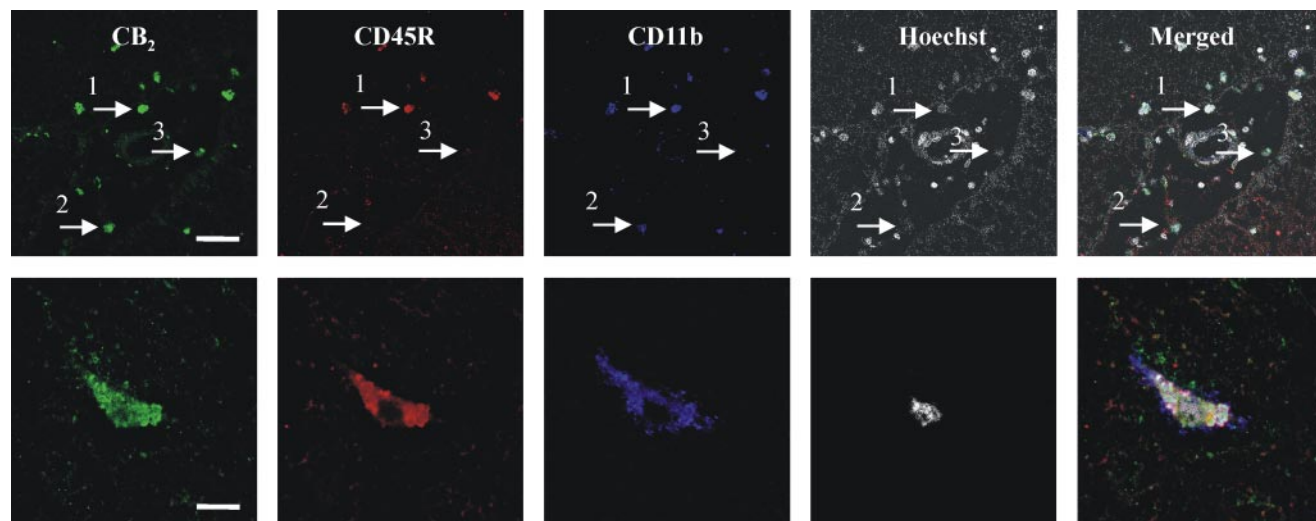
**Statistical Analysis**—Results shown represent the means ± S.E. of the number of experiments indicated in every case. Statistical analysis was performed by analysis of variance. A *post hoc* analysis was made by the Student-Neuman-Keuls test. *In vivo* data were analyzed by an unpaired Student's *t*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



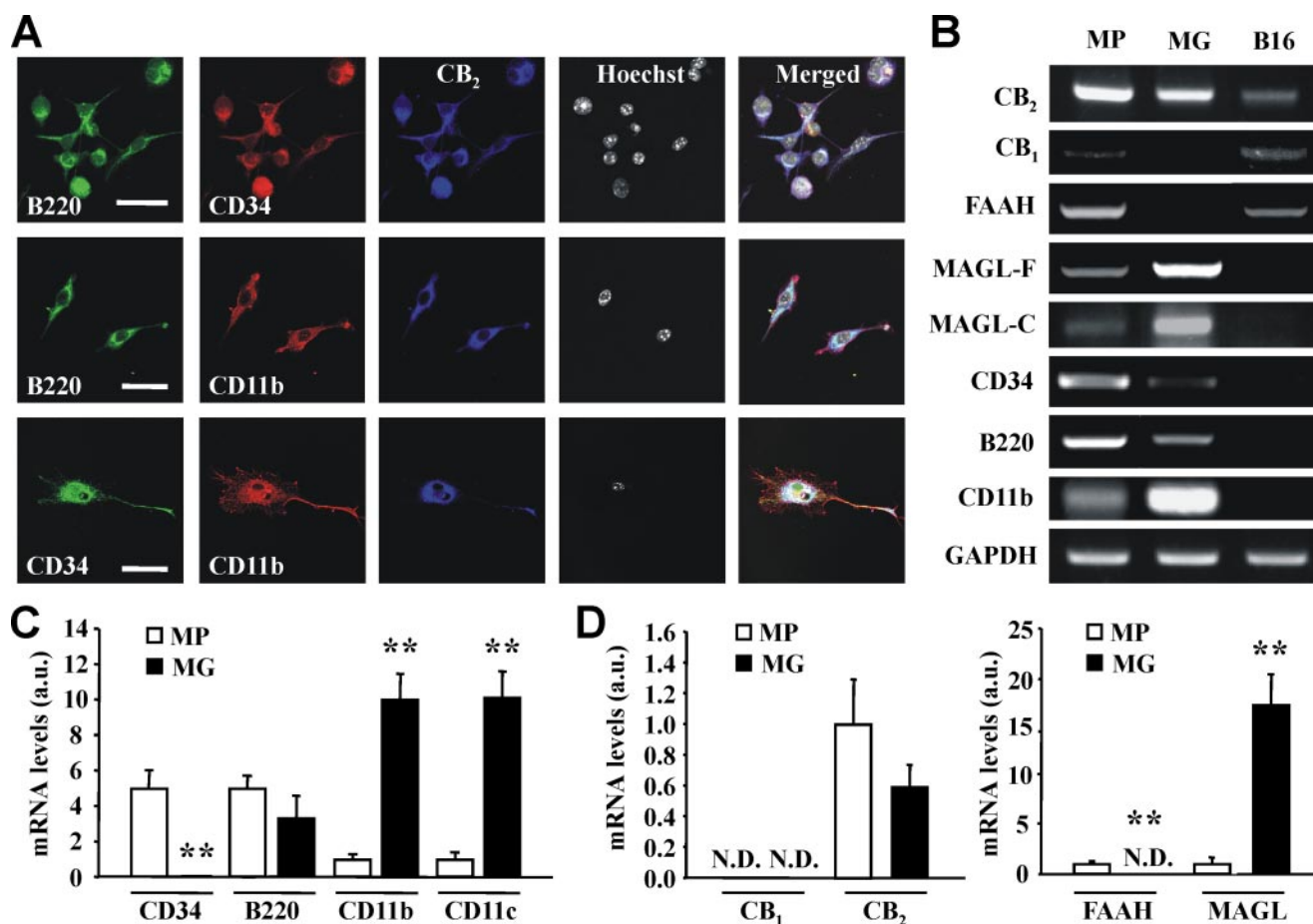
**FIGURE 4. Expression of the CB<sub>2</sub> cannabinoid receptor in spinal cord myeloid-derived microglial cells.** A, confocal microscopy images of EAE spinal cord sections were obtained with CD34 (green), CD11b (red), and CB<sub>2</sub> receptor (blue) antibodies. The CB<sub>2</sub> receptor was also present in CD45R/B220<sup>+</sup> (green) and CD11b<sup>+</sup> (red) cells. Total cell nuclei were counterstained with Hoechst 33342 (gray). The right column shows the merged images. Scale bars, 50 and 10  $\mu$ m 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.

## RESULTS

**CB<sub>2</sub> 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  $12.4 \pm 4.2$  and, after reaching a maximal stage at day  $20.2 \pm 2.7$  (Fig. 1A), entered a chronic clinical phase (22, 25). The involvement of the CB<sub>2</sub> receptor in the appearance of EAE symptoms was investigated by comparing wild-type and CB<sub>2</sub><sup>-/-</sup> 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<sub>2</sub><sup>-/-</sup> 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<sup>+</sup>) activation and T-lymphocyte (CD4<sup>+</sup>) infiltration in CB<sub>2</sub>-deficient mice (Fig. 2, B and C), thus providing further evidence for the exacerbated phenotype of these animals. As CB<sub>2</sub> receptors are highly



**FIGURE 5. Expression of the CB<sub>2</sub> cannabinoid receptor in CD45R<sup>+</sup> microglial cells in cortical plaques of multiple sclerosis patients.** Confocal microscopy analyses were performed by using CB<sub>2</sub> receptor (green), CD45R (red), and CD11b (blue) antibodies. Images show CB<sub>2</sub> receptor expression in CD45<sup>+</sup> CD11b<sup>+</sup> microglial cells (denoted by arrows) (1), CD11b<sup>+</sup> CD45R<sup>+</sup> microglial cells (2), and CD11b<sup>-</sup> CD45R<sup>+</sup> cells (3) located in the vicinity of blood vessels. Cell nuclei were counterstained with Hoechst 33342 (gray). Scale bars, 35 and 10  $\mu$ m in upper and lower panels, respectively.



**FIGURE 6. The CB<sub>2</sub> cannabinoid receptor is expressed in bone-marrow myeloid progenitors.** *A*, immunofluorescence of cultured myeloid progenitors was performed with the indicated combinations of antibodies against CD34, CD45R/B220, CD11b, and CB<sub>2</sub> receptor. Total cell nuclei were counterstained with Hoechst 33342 (gray), and the right column shows the merged images. Scale bars, 25  $\mu$ m in upper and middle panels and 45  $\mu$ m in lower panels. *B*, reverse transcription-PCR expression analysis of the eCB system receptors (CB<sub>1</sub>, CB<sub>2</sub>) 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 18S RNA expression and are provided in arbitrary units (a.u.). \*\*,  $p < 0.01$  versus myeloid progenitors; N.D., non-detectable.

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<sup>+</sup> cells was evident in CB<sub>2</sub>-deficient spinal cords (Fig. 2D). Increased proliferation (Ki-67<sup>+</sup> cells) of CD11b and CD34 cell populations was also observed in CB<sub>2</sub> knock-out mice (Fig. 2E).

**Selective CB<sub>2</sub> Cannabinoid Receptor Activation Palliates EAE Symptoms and Pathogenesis**—As CB<sub>2</sub> receptor genetic ablation exacerbates EAE pathogenesis, we assessed the impact of CB<sub>2</sub> receptor selective activation on disease progression. Daily injections of the CB<sub>2</sub>-selective agonist HU-308 (29) were performed starting at the day of maximal disease score. In agreement with the observations from CB<sub>2</sub>-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 CB<sub>2</sub><sup>-/-</sup> 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<sup>+</sup> 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 CB<sub>2</sub> receptor activation, we examined spinal cord sections of EAE mice. Confocal microscopy confirmed the expression of CB<sub>2</sub> receptors in CD34<sup>+</sup> 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, CB<sub>2</sub> receptors were expressed in CD11b<sup>+</sup> 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 CB<sub>2</sub>

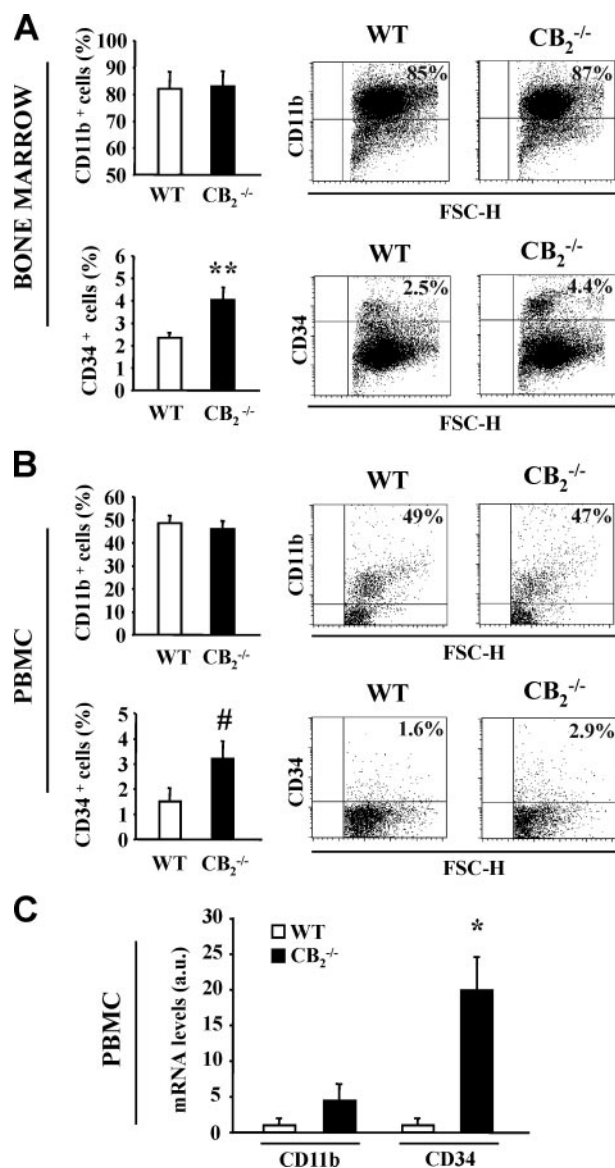
## Control of Myeloid Progenitor Recruitment by CB<sub>2</sub> Receptors

receptor expression, which was accompanied by increased expression of the CB<sub>1</sub> receptor and the degrading enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL; Fig. 4C). Next, we analyzed the expression of CB<sub>2</sub> receptors in brain sections of MS patients. CB<sub>2</sub> 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 CB<sub>2</sub> receptors in myeloid progenitors from bone marrow-derived cultures. Immunofluorescence analysis showed the co-expression of CB<sub>2</sub> receptors with CD34 and CD45R/B220 (Fig. 6A). CB<sub>2</sub> 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. CB<sub>2</sub> receptor transcripts were highly expressed in myeloid progenitors and were also evident in differentiated microglial-like cells (Fig. 6, B and D). In contrast, CB<sub>1</sub> 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 CB<sub>2</sub> Cannabinoid Receptor Controls Bone Marrow-derived Myeloid Cell Trafficking**—The mechanism of CB<sub>2</sub> 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<sup>+</sup> cells was not altered in CB<sub>2</sub><sup>-/-</sup> mice when compared with wild-type mice (Fig. 7A, *upper panel*). In contrast, CD34<sup>+</sup> cells were increased in mice lacking the CB<sub>2</sub> receptor (Fig. 7A, *lower panel*), whereas the opposite was observed upon HU-308 administration (CD34<sup>+</sup> 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<sup>+</sup> but not CD11b<sup>+</sup> cells were expanded into circulating blood cells in mice lacking the CB<sub>2</sub> receptor. These findings are in line with the aforementioned higher number of CD34<sup>+</sup> and CD11b<sup>+</sup> cells found in spinal cords of CB<sub>2</sub><sup>-/-</sup> mice, suggesting that the CB<sub>2</sub> receptor can control myeloid progenitor cell trafficking toward neuroinflamed tissue.

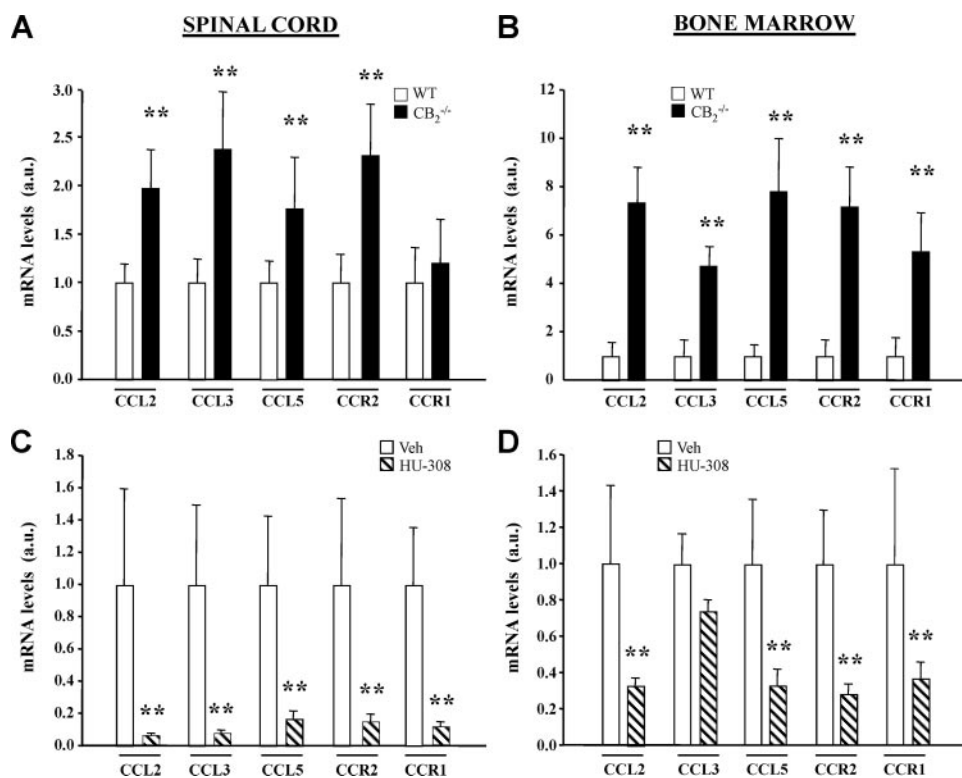
An analysis of the *in vivo* expression of chemokine ligands and receptors was performed in EAE spinal cord and bone marrow. CB<sub>2</sub> receptor ablation up-regulated chemokines and receptors known to be important in microglial recruitment to inflammatory lesions (30). Thus, CCL2, CCL3, and CCL5 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



**FIGURE 7. The CB<sub>2</sub> cannabinoid receptor regulates myeloid progenitor cell trafficking.** A, flow cytometry analysis of CD11b<sup>+</sup> and CD34<sup>+</sup> cells within bone marrow cells obtained from WT and CB<sub>2</sub><sup>-/-</sup> EAE mice at the end of the experiment (*n* = 8 each group). Representative histograms are shown (*right panels*). B, flow cytometry analysis of CD11b<sup>+</sup> and CD34<sup>+</sup> cells of PBMCs from WT and CB<sub>2</sub><sup>-/-</sup> 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.

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 CB<sub>2</sub> Receptor-deficient EAE Mice**—To confirm the involvement of the CB<sub>2</sub> 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 CB<sub>2</sub><sup>-/-</sup> 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 ±



**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 ( $91.2 \pm 3.6\%$ ), whereas in some GFP<sup>+</sup> cells ( $20.2 \pm 3.6\%$ ), 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

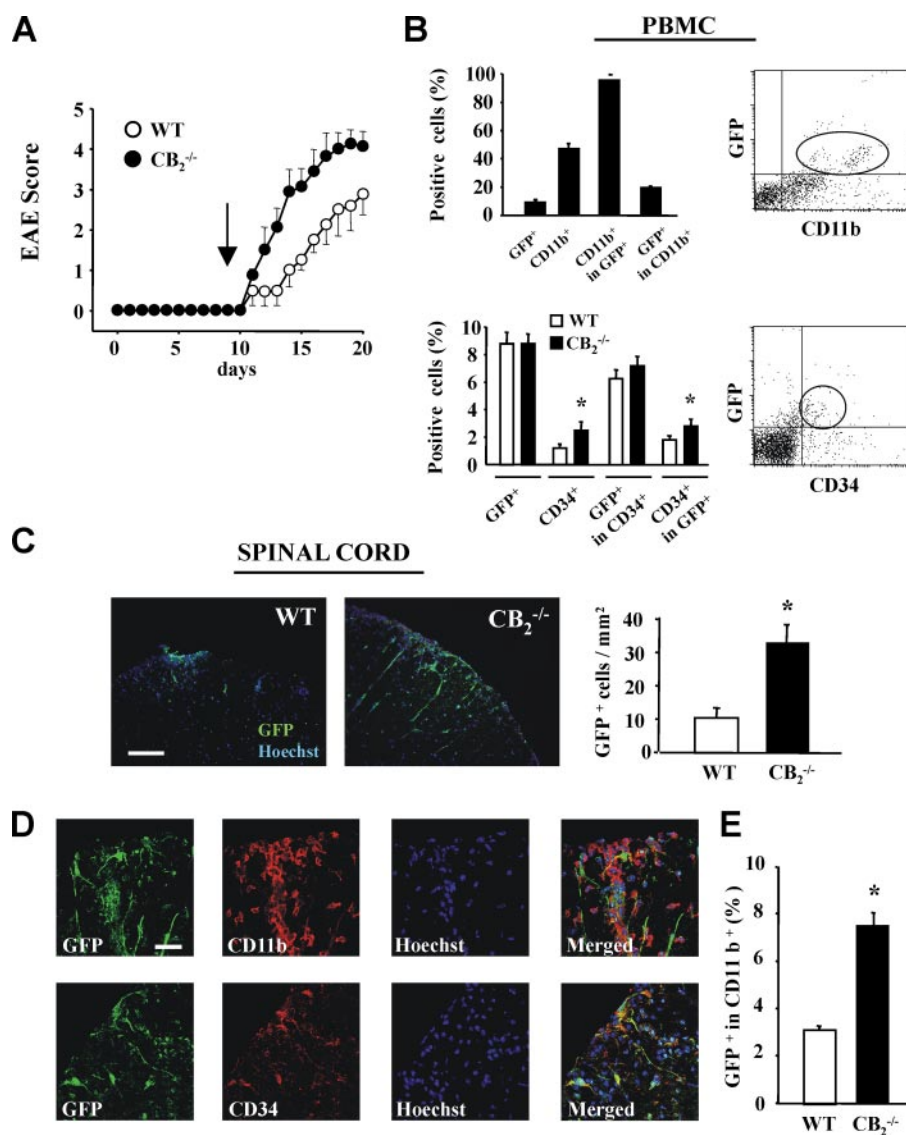
Alterations of the eCB system have been implicated in the pathogenesis of several neurodegenerative disorders, and activation of cannabinoid receptors exerts neuroprotection in various models of brain damage including excitotoxicity, traumatic brain injury, and stroke (1, 31). Moreover, cannabinoid administration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

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).<sup>8</sup> By using various experimental approaches, here we demonstrate that CB<sub>2</sub> receptors play an impor-

tant 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 9. Bone marrow-derived GFP-labeled cell transfer experiments in EAE mice.** *A*, EAE score progression of WT (open circles) and CB<sub>2</sub><sup>-/-</sup> mice (closed circles; *n* = 6 each group) subjected to bone marrow GFP-cell injection at day 9 prior to symptom appearance. *B*, at the end of the experiment (day 19), circulating GFP<sup>+</sup> bone marrow-derived monocytic cells in the blood of recipient animals were detected by flow cytometry and immunostained with CD11b and CD34 antibodies. *C*, double staining with GFP antibody (green) and Hoechst 33342 (red) in EAE spinal cord sections (left panel). GFP-positive immunoreactive cells were quantified (right panel). *D*, high magnification immunofluorescence images obtained with GFP (green) and either CD34 or CD11b (red) antibodies are shown. Total cell nuclei were counterstained with Hoechst 33342 (gray), and the right column shows the merged images. *E*, spinal cord-infiltrating GFP<sup>+</sup>CD11b<sup>+</sup> cells were quantified and referred to the total number of CD11b<sup>+</sup> cells. Scale bars, 100 and 30 μm in *C* and *D*, respectively. \*, *p* < 0.05 versus WT.

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 CB<sub>2</sub> 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 CB<sub>2</sub> receptor-regulated myeloid progenitor trafficking.

Our data agree with the current notion that microglial cells express CB<sub>2</sub> 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 CB<sub>2</sub> 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 CB<sub>2</sub> 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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