Activation of cannabinoid 2 receptors protects against cerebral ischemia by inhibiting neutrophil recruitment

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ABSTRACT **Activation of the cannabinoid 2 receptor (CB_2) reduces ischemic injury in several organs. However, the mechanisms underlying this protective action are unclear. In a mouse model of ischemic stroke, we show that the CB_2 agonist JWH-133 (1 $mg \cdot kg^{-1} \cdot d^{-1}$) decreases the infarct size measured 3 d after onset of ischemia. The neuroprotective effect of JWH-133 was lost in CB_2-deficient mice, confirming the specificity of JWH-133. Analysis of bone marrow chimeric mice revealed that bone marrow-derived cells mediate the CB_2 effect on ischemic brain injury. CB_2 activation reduced the number of neutrophils in the ischemic brain as shown by FACS analysis and by measuring the levels of the neutrophil marker enzyme myeloperoxidase. Indeed, we found *in vitro* that CB_2 activation inhibits adherence of neutrophils to brain endothelial cells. JWH-133 (1 μM) also interfered with the migration of neutrophils induced by the endogenous chemokine CXCL2 (30 ng/ml) through activation of the MAP kinase p38. This effect on neutrophils is likely responsible for the neuroprotection mediated by JWH-133 because JWH-133 was no longer protective when neutrophils were depleted. In conclusion, our data demonstrate that by activating p38 in neutrophils, CB_2 agonists inhibit neutrophil recruitment to the brain and protect against ischemic brain injury.—Murikinati, S., Jüttler, E., Keinert, T., Ridder, D. A., Muhammad, S., Waibler, Z., Ledent, C., Zimmer, A., Kalinke, U., Schwaninger, M. Activation of cannabinoid 2 receptors protects against cerebral ischemia by inhibiting neutrophil recruitment. *FASEB J.* 24, 788–798 (2010). www.fasebj.org**

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THE CONSEQUENCES OF ISCHEMIC INJURY in liver, heart, and brain can be ameliorated by cannabinoids (1), a group of diverse compounds that include constituents of the plant *Cannabis sativa* (phytocannabinoids), endogenous lipids (endocannabinoids), and synthetic substances. Most of the effects of cannabinoids are mediated by the G-protein-coupled receptors cannabinoid receptor 1 (CB_1) and cannabinoid receptor 2 (CB_2; ref. 2). In addition, cannabinoid modulation of ion channels and putative new receptors likely explain some apparently contradictory findings in this area of research (3–5).

Cannabinoids protect against ischemic stroke (6), although the molecular mechanisms underlying this finding are not entirely clear. Primary interest has focused initially on the CB_1, the predominant cannabinoid receptor in the brain. Although infarcts are larger in $CB_1^{-/-}$ mice than in control mice (7), the protective role of CB_1 receptors has been challenged by the findings that CB_1 antagonists reduce rather than increase ischemic brain damage (8, 9). More recently, pharmacological data have indicated that CB_2 agonists are neuroprotective in cerebral ischemia (10). CB_2 receptors thus seem to be involved in the modulation of different neurological disorders because agonists have a beneficial effect in experimental autoimmune encephalomyelitis, a model of multiple sclerosis (11), in a mouse model of amyotrophic lateral sclerosis (12), in amyloid Aβ-induced neurotoxicity (13), and in axotomy-induced apoptosis (14). The CB_2 receptor is expressed in some brainstem neurons; in activated microglia, astrocytes, and endothelial cells; and in peripheral immune cells (14–20), allowing for several

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potential mechanisms by which CB_2 agonists may protect against nerve cell injury. Neurons in precerebellar nuclei express CB_2 receptors, which mediate a neuroprotective effect through PI3K/Akt signaling (14). In a model of hepatic ischemia-reperfusion injury, however, CB_2 receptors decreased the induction of inflammatory mediators and infiltration of inflammatory cells (21). Inhibition of leukocyte rolling and adhesion to pial venules in the ischemic hemisphere have also been reported in cerebral ischemia (10, 22). However, the anti-inflammatory mechanisms and the contribution of neuronal or other parenchymal effects to CB_2-induced neuroprotection are not yet understood.

Using the selective CB_2 agonist JWH-133 and $CB_2^{-/-}$ mice, we present data confirming the protective effect of CB_2 receptor activation. Bone marrow transplantation demonstrated that the protection depended on CB_2 receptors in bone marrow-derived cells. Neutrophils most likely mediate the effect because JWH-133 reduced the number of neutrophils in the ischemic brain and was no longer protective if neutrophils were depleted. Indeed, CB_2 receptor activation reduced the adherence of neutrophils to brain endothelial cells and the chemotaxis of neutrophils.

MATERIALS AND METHODS

Model of cerebral ischemia

Male C57BL/6 mice and $CB_1^{-/-}$ and $CB_2^{-/-}$ mice (23, 24) were investigated at the age of 2–3 mo. Microosmotic pumps (1003D; Alzet, Cupertino, CA, USA) were filled with 100 μl of JWH-133 (Tocris, Ellisville, MO, USA) in PBS containing BSA (5 mg/ml) and 20% DMSO or with 100 μl of vehicle only. These pumps release 1 μl/h, which is equivalent to 1 or 0.5 $mg \cdot kg^{-1} \cdot d^{-1}$ of JWH-133 as indicated. For intraperitoneal implantation of the pumps, mice were anesthetized with isoflurane. Four hours later, the mice were again anesthetized by intraperitoneal injection of 150 μl 2.5% tribromoethanol/10 g body weight. To occlude the middle cerebral artery (MCA) distally, a skin incision was made between the ear and the orbit on the left side. The temporal muscle was removed by electrical coagulation. The stem of the MCA was exposed through a burr hole and occluded by microbipolar coagulation (Erbe, Tübingen, Germany). Surgery was performed under a microscope (Hund, Wetzlar, Germany). A body temperature of 37°C was maintained by using a heating pad that was controlled by the rectal body temperature. After the indicated time, mice were deeply reanesthetized with tribromoethanol and perfused intracardially with Ringer's solution. Coronal cryosections of the brains (20 μm in thickness) were cut every 400 μm and stained with a silver technique (25). Infarct volumes were corrected for brain edema as has been described previously (25). Surgery was performed and ischemic damage was measured by an investigator who had no knowledge of the treatment group or the genotype. To determine blood pressure, glucose, and blood gases that may influence infarct volume, the femoral artery was cannulated in a separate cohort of animals. A blood sample of 100 μl was drawn 10 min before and 10 min after MCA occlusion (MCAO). For laser Doppler measurements, the probe (P415-205; Perimed) was placed 3 mm lateral and 6 mm posterior to the Bregma. Relative perfusion units were determined (Periflux 4001; Perimed, Jarfalla, Sweden).

RT-PCR

Cells or homogenized tissues were triturated several times with 1 ml RNApure reagent/100 mg tissue (Peqlab, Erlangen, Germany). The lysate was mixed with chloroform (0.2 ml) and centrifuged at 13,000 *g* for 20 min at 4°C. The aqueous layer was transferred to another tube containing 0.5 ml isopropanol. After a 1 h incubation at −80°C, the samples were centrifuged, and the RNA pellet was washed with 75% ethanol and dissolved in dH_2O. RNA was transcribed with Moloney murine leukemia virus reverse transcriptase and random primers. The mouse CB_2 gene uses 2 transcription start sites, resulting in 2 mRNA species, CB2A and CB2B, that differ in the untranslated exon 1 (26, 27). For qualitative PCR, the following primers were used: CB2A-F, 5′-CATCTGCGAAAGTGTGAGAGC-3′; CB2B-F, 5′-AGCTGTGCCTGAATGAGCAGA-3′; CB2AB-R, 5′-GTGCAGGGAACCAGCATATG-3′, resulting in PCR products of 954 bp for CB2A and of 845 bp for CB2B; GAPDH-F, 5′-ATCCTGCACCACCAACTGCTTA-3′; and GAPDH-R, 5′-TTCAAGAGAGTAGGGAGGGCT-3′ (PCR product, 645 bp). For quantitative real-time PCR the Absolute Blue QPCR SYBR Green Mix (Thermo Scientific, Waltham, MA, USA) and the following primers were used: CXCL2-F, 5′-CCAACCACCAGGCTACAGG-3′; CXCL2-R, 5′-GCGTCACACTCAAGCTCT G-3′ (PCR product, 108 bp); TNF-F, 5′-TGTAGCCCACGTCGTAGCAAA-3′; TNF-R, 5′-GCTGGCACCACTAGTTGGTTGT-3′ (PCR product, 120 bp); IL1β-F, 5′-CGAGGCCTAATAGGCTCATCT G-3′; IL1β-R, 5′-CACTGTCAAAAGGTGGCATTTC-3′ (PCR product, 117 bp); cyclophilin forward, 5′-AGGTCCTGGCATCTTGTCCAT-3′; and cylophilin reverse, 5′-GAACCGTTTGTGTTTGGTCCA-3′ (PCR product, 51 bp). The results were normalized to cyclophilin. The purity of the amplified products was checked by the dissociation curve.

Bone marrow transplantation

Bone marrow was obtained aseptically from femurs and tibiae of wild-type, ACTB-EGFP (28), or $CB_2^{-/-}$ mice (24) after the animals were euthanized by cervical dislocation. Unfractionated bone marrow cells were resuspended in 0.25 ml sterile PBS and injected retro-orbitally into 10- to 13-wk-old C57BL/6 or $CB_2^{-/-}$ mice that had been lethally irradiated (10 Gy) 1 d before. Six weeks after reconstituting the bone marrow, we confirmed that engraftment was successful by FACS analysis of GFP^+ cells in the blood. In ACTB-EGFP mice, 93 ± 2% of $CD45^+$ cells were GFP^+, whereas in C57BL/6 mice transplanted with ACTB-EGFP bone marrow, 87 ± 2% of $CD45^+$ cells were GFP^+, indicating that the transplantation efficiency was 94%.

FACS analysis

FACS analysis of brain cells after MCAO was performed as described previously (29). Briefly, 48 h after MCAO mice were anesthetized by intraperitoneal injection of 200 μl tribromoethanol/10 g and perfused intracardially with Ringer's solution, the brain was freed from meninges, and the olfactory bulb and cerebellum were discarded. The separated hemispheres were homogenized in 1× PBS containing BSA (0.2%), EDTA (0.01 M), and DNase 1 (10 mg/ml; Roche, Mannheim, Germany) and filtered through a 40-μm nylon cell strainer (BD PharMingen, Erembodegem, Belgium). After centrifugation, cells from 1 hemisphere were resuspended in 5 ml of isotonic Percoll brought to a density of 1.030 g/ml. This solution was underlayered with 2.5 ml of Percoll (1.095 g/ml), overlayered with 2.5 ml of HBSS, and centrifuged for 20 min at 1000 *g* at room temperature. Cells

were collected from the top of the 1.095 g/ml layer, washed in 10 ml HBSS containing 10% FBS, and counted in the Neubauer chamber. After treatment with purified rat anti-mouse CD16/32 (Fc Block, 553141; BD Pharmingen) for 10 min on ice, cells were incubated with the following immunoglobulins (BD PharMingen) for 45 min on ice: PerCP-labeled rat anti-CD45 antibody (557235), PerCP-labeled rat IgG2b,κ isotype control (552991), PE-labeled rat anti-CD11b antibody (557397), PE-labeled rat IgG2b,κ isotype control (553989), PE-labeled rat anti-Ly-6G (551461), PE-labeled rat isotype control IgG2a,κ (553930), PE-labeled rat anti-CD3 (555275), PE-labeled mouse anti-NK-1.1 (557391), and PE-labeled mouse IgG2a,κ isotype control (553457).

To investigate CXCR2 expression, isolated neutrophils were pretreated for 30 min with vehicle or JWH-133 in Roswell Park Memorial Institute (RPMI) medium followed by a 1 h incubation period with CXCL2 as indicated. After treatment with purified rat anti-mouse CD16/32 for 10 min, cells were incubated with PerCP/Cy5.5-labeled anti-mouse CXCR2 or PerCP/Cy5.5-labeled IgG2a,κ (Biolegend, San Diego, CA, USA) for 45 min on ice.

Myeloperoxidase assay

Mice were euthanized 48 h after MCAO. Myeoloperoxidase activity was measured as described previously (30). Briefly, the ischemic cortex and the corresponding cortical area on the contralateral side were dissected and homogenized with a tissue homogenizer in phosphate buffer (5 mM; pH 6; 1:20, w/v). After centrifugation at 30,000 *g* for 30 min (4°C), the supernatant was discarded, and the pellet was washed again in phosphate buffer. Then, the pellet was extracted in 50 mM potassium phosphate buffer (pH 6; 25°C) containing 0.5% hexadecyltrimethylammonium bromide (Sigma, Steinheim, Germany) at an original tissue weight to volume ratio of 1:10. After 3 freeze-thaw cycles with sonications in between, the lysates were incubated at 4°C for 20 min. After centrifugation (12,500 *g* for 15 min), 6.7 μl of the supernatant was mixed with 186.6 μl of phosphate buffer (50 mM, pH 6) containing *o*-dianisidine dihydrochloride (0.167 mg/ml; Sigma) and 1% hydrogen peroxide. OD_{460} was measured every 15 s for 3 min. Optical density units were converted into units of concentration using the molar absorptivity coefficient for oxidized *o*-dianisidine [$\varepsilon=10{,}062\times(M\times cm)^{-1}$] (31). One unit of myeloperoxidase degrades 1 μmol hydrogen peroxide/min at 37°C.

Isolation of neutrohils

Neutrophils were isolated from bone marrow by using a discontinuous Percoll (Pharmacia Biotech, Uppsala, Sweden) gradient as described previously (32). Briefly, bone marrow cells from 1 mouse were resuspended in 3 ml 45% Percoll (diluted in HBSS containing 10 mM glucose) and overlaid on 4 layers of Percoll (3 ml 66%, 2 ml 60%, 2 ml 55%, and 2 ml 50%). After centrifugation (1800 *g*, 30 min, room temperature), cells were collected and washed with HBSS. Neutrophil purity was higher than 85% as shown by FACS.

Neutrophil adhesion to brain endothelial cells

The mouse brain endothelial cell line bEnd.3 was obtained from American Type Culture Collection (Manassas, VA, USA) and grown in DMEM (Life Technologies, Inc., Karlsruhe, Germany) containing glucose (4.5 g/L), 10% FBS, penicillin (100 IU/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM). bEnd.3 cells (5×10^5/well) were seeded on coverslips in 24-well plates. When they reached confluence after 2–3 d, we treated them with mouse TNF (10 ng/ml; Sigma) for 6 h as indicated. Neutrophils were labeled by incubation with calcein-AM (5 μg/ml; Molecular Biotechnology, Göttingen, Germany) for 30 min. Then, neutrophils (5×10^5/well) were added to the bEnd.3 monolayer together with JWH-133 as indicated. After a 20 min incubation period, the cells were washed 3 times with PBS containing $MgCl_2$ (1 mM) and $CaCl_2$ (1 mM) and mounted with Mowiol. Labeled neutrophils were counted in 2 random fields/coverslip. The results are expressed as the mean number of neutrophils per field.

Chemotaxis assay

Neutrophils were preincubated with SB 203580 (10 μM; Calbiochem, San Diego, CA, USA), PD 184352 (1 μM; Alexis Biochemicals, Lörrach, Germany), SP 600125 (10 μM; Alexis Biochemicals), or vehicle for 10 min in RPMI medium containing 0.01% BSA. JWH-133 (1 μM) was added for 30 min as indicated. Subsequently, neutrophils (10^6 in 0.2 ml) were added on top of a Transwell filter (ThinCert, pore size 3 μm; Greiner, Frickenhausen, Germany) that was inserted into a 24-well plate filled with 300 μl RPMI containing 0.01% BSA. The chemoattractants recombinant murine CXCL2 (452-M2; R&D Systems, Wiesbaden, Germany) or N-formyl-Met-Leu-Phe (fMLP; Sigma, F3506) were added to the lower chamber. We added the test compounds SB 203580, PD 184352, SP 600125, and JWH-133 to both the upper and lower chamber to avoid a concentration gradient. After the Transwell plates were incubated for 1 h at 37°C in 5% CO_2, the upper side of membrane was wiped with cotton buds, and the insert was washed with PBS. Then, cells were stained on the membrane with the Diff-Quick staining set (130832; Medion Diagnostics, Düdingen, Germany). Neutrophils were counted in 3 random fields. The results are expressed as the mean number of neutrophils per field.

Neutrophil depletion

To deplete neutrophils, mice were injected with rabbit anti-polymorphonuclear leukocyte antibody (anti-PMN; Accurate Chemical & Scientific, Westbury, NY, USA; AIAG31140; 2 mg/kg i.p.) or control rabbit IgG for 4 d. On the third day of injection, the mice were subjected to MCAO. Two days after MCAO, we counted neutrophil numbers in blood and determined the infarct volume.

Immunoblotting

Neutrophils were pretreated for 10 min with the p38 inhibitor SB 203580 (10 μM) or vehicle. Then, JWH-133 (1 μM) or vehicle was added for 1 h. Neutrophil lysates were resolved by 12% SDS-polyacrylamide gel electrophoresis as described previously (33). Then, phospho-p38 and p38 were detected with rabbit anti-phospho-p38 (Thr 180/Tyr182) and anti-p38 antibodies (Cell Signaling, Danvers, MA).

Statistical analysis

Student's *t* test was used to compare 2 groups and 1-way ANOVA to compare >2 groups. Data are expressed as means ± SE.

RESULTS

Activation of CB_2 receptors protects against cerebral ischemia

The CB_2 agonist JWH-133 was administered by implanting microosmotic pumps 4 h before MCAO. The

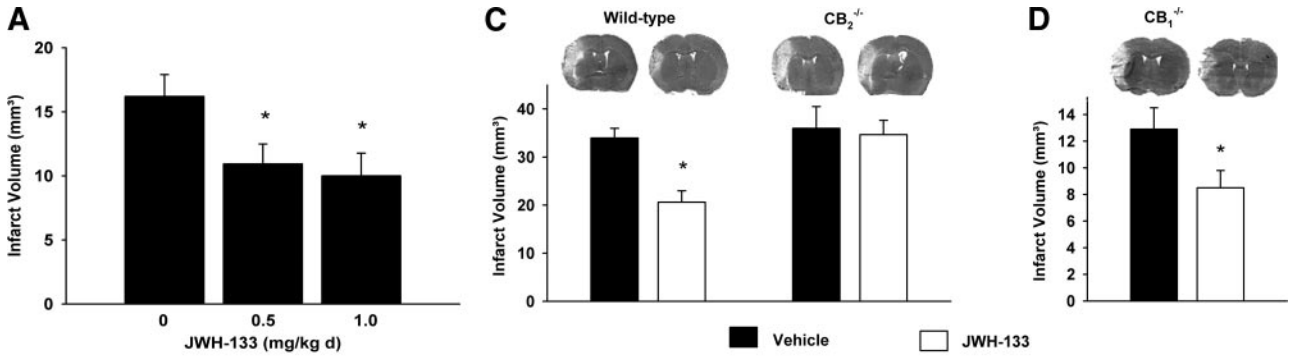

Figure 1. Activation of CB_2 receptors by JWH-133 is neuroprotective in distal MCAO. *A*) CB_2 agonist JWH-133 reduced infarct volumes that were determined 3 d after distal MCAO. [ANOVA, $F(2/30)=3.752$, $P=0.035$]. $*P < 0.05$ *vs.* vehicle-treated group; Fisher least significant difference (LSD) method. *B*) Protective effect was lost if JWH-133 was administered in a dose of 8 $mg \cdot kg^{-1} \cdot d^{-1}$. [ANOVA, $F(2/30)=5.927$, $P=0.007$]. $*P < 0.005$; Fisher LSD method. *C*) JWH-133 (1 $mg \cdot kg^{-1} \cdot d^{-1}$) reduced infarct volumes in wild-type mice but not in $CB_2^{-/-}$ animals. Infarct volumes were determined 2 d after distal MCAO. [ANOVA, $F(3/32)=5.312$, $P=0.004$]. $*P = 0.005$ *vs.* vehicle-treated wild-type group; Fisher LSD method. *D*) JWH-133 (1 $mg \cdot kg^{-1} \cdot d^{-1}$) reduced infarct volumes in $CB_1^{-/-}$ mice. Infarct volumes were determined 2 d after distal MCAO. Because CB_1 is deleted on a genetic CD1 background, the infarct volumes of $CB_1^{-/-}$ mice should not be compared with wild-type and $CB_2^{-/-}$ mice, which both have a C57BL/6 background. We did not find statistically significant differences in infarct volumes of $CB_1^{-/-}$ and wild-type CD1 mice (data not shown). $*P < 0.05$; unpaired *t* test.

pumps release JWH-133 for ≥3 d. In the model of permanent distal MCAO that we used, infarcts are mainly restricted to the cortex. JWH-133 at doses of 0.5 and 1 $mg \cdot kg^{-1} \cdot d^{-1}$ reduced the infarct volume by 30–40% (**Fig. 1*A***). The effect size compares favorably with those we have obtained with other anti-inflammatory strategies in the same MCAO model (25, 29). JWH-133 lost its efficacy at a dose of 8 $mg \cdot kg^{-1} \cdot d^{-1}$ (Fig. 1*B*). For consecutive experiments, we used 1 $mg \cdot kg^{-1} \cdot d^{-1}$. Treatment of mice with JWH-133 did not affect cardiovascular parameters that are known to modulate ischemic brain damage (**Table 1**). To test the specificity of JWH-133 for CB_2 receptors in cerebral ischemia, we administered it to wild-type and $CB_2^{-/-}$ mice. The protective effect was abrogated in $CB_2^{-/-}$ animals (Fig. 1*C*). The infarct volume of untreated $CB_2^{-/-}$ mice did not differ from that in wild-type mice, which argues against a potential neuroprotective function of endocannabinoids through activation of CB_2 receptors. JWH-133 is a weak CB_1 agonist (2). As expected, the protective effect of JWH-133 was preserved in $CB_1^{-/-}$ mice (Fig. 1*D*), demonstrating that the protective effect of JWH-133 is independent of CB_1 receptors. In summary, activation of the CB_2 receptor by JWH-133 reduces the infarct volume after focal cerebral ischemia.

Bone marrow-derived cells mediate the protective effect of CB_2 activation

By RT-PCR, we detected transcripts of the mouse CB_2 gene in spleen, neutrophils, and brain (**Fig. 2*A***). This finding is in line with reports that the CB_2 receptor is expressed in the brain and in peripheral immune cells (15–19). To determine whether central or peripheral CB_2 receptors are responsible for the protective effect

TABLE 1. *Physiological parameters of vehicle- and JWH-133-treated mice 10 min before and 10 min after MCAO*

	Vehicle		JWH-133 (1 $mg \cdot kg^{-1} \cdot d^{-1}$)	
Parameter	Before MCAO (n=5)	After MCAO (n=5)	Before MCAO (n=5)	After MCAO (n=5)
Body weight (g)	22.6 ± 0.2		22.9 ± 0.2	
MABP (mmHg)	70.0 ± 1.6	56.2 ± 1.3	71.0 ± 1.8	49.5 ± 6.5
Body temperature (°C)	37.0 ± 0.0	37.0 ± 0.0	37.0 ± 0.0	37.1 ± 0.1
Glucose (mg/dl)	181.0 ± 15.4	173.0 ± 19.9	168.8 ± 15.3	172.5 ± 25.1
PO_2 (mmHg)	94.5 ± 6.5	87.3 ± 3.5	93.7 ± 3.6	84.6 ± 4.4
pCO_2 (mmHg)	40.0 ± 3.1	43.2 ± 2.7	44.0 ± 3.0	44.0 ± 3.0
pH	7.30 ± 0.02	7.21 ± 0.02	7.30 ± 0.01	7.21 ± 0.02
Drop in Doppler signal (%)		77.4 ± 2.8		79.6 ± 2.5

Data are means ± SE. There was no significant difference between the treatment groups (*t* test). MABP, mean arterial blood pressure.

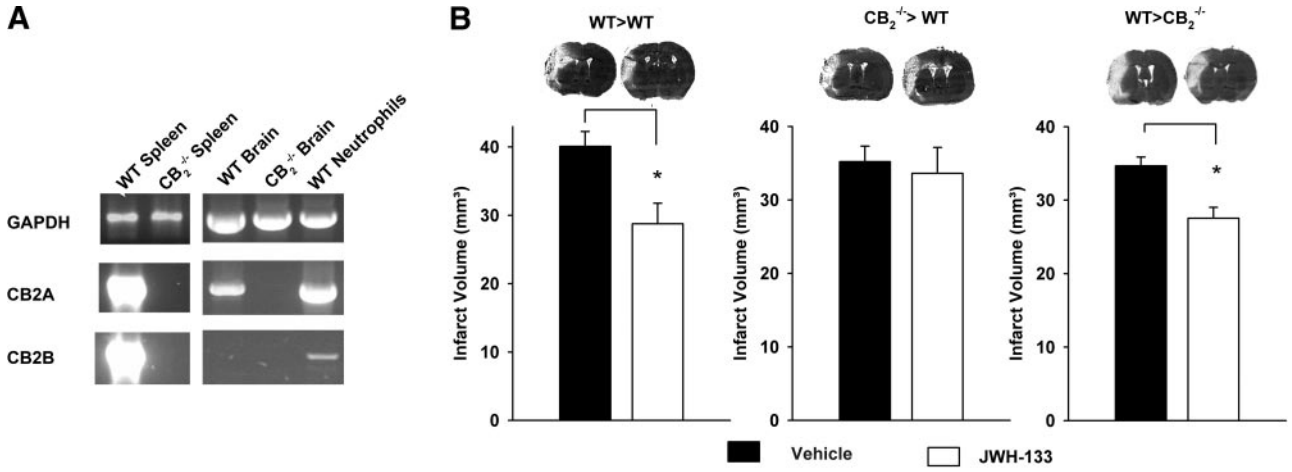

Figure 2. Bone marrow-derived cells mediate the protective effect of JWH-133 in cerebral ischemia. *A*) CB_2 receptor is expressed in the spleen, brain, and isolated neutrophils. CB2A and CB2B are 2 transcripts of the CB_2 receptor gene; see text for details. *B*) JWH-133 ($1\ mg \cdot kg^{-1} \cdot d^{-1}$) reduced infarct volumes if wild-type bone marrow was transplanted to wild-type mice (WT>WT) or to $CB_2^{-/-}$ mice (WT>$CB_2^{-/-}$). However, the effect of JWH-133 was lost if $CB_2^{-/-}$ bone marrow was transplanted to wild-type mice ($CB_2^{-/-}$>WT). Six weeks after transplantation, MCAO was performed. Infarct volumes were determined 2 d after distal MCAO. [ANOVA, $F(5/48)=3.365$, $P=0.011$]. $^*P < 0.05$ *vs.* vehicle-treated group; Fisher LSD method.

of JWH-133, we generated bone marrow chimeric mice and subjected them to MCAO 6 wk later. In a control group of wild-type mice transplanted with wild-type bone marrow (WT>WT), JWH-133 treatment significantly reduced the infarct volume (Fig. 2*B*). This effect was lost if $CB_2^{-/-}$ bone marrow was transplanted instead ($CB_2^{-/-}$>WT), showing that bone marrow-derived cells are required for the neuroprotective effect of JWH-133. Transplantation of wild-type bone marrow to $CB_2^{-/-}$ mice (WT>$CB_2^{-/-}$) restored the responsiveness to the CB_2 agonist JWH-133 (Fig. 2*B*), which demonstrates that bone marrow-derived cells are sufficient to mediate the effect of CB_2 receptor activation on ischemic brain damage.

CB_2 activation interferes with the recruitment of neutrophils to the ischemic brain

Bone marrow-derived cells are recruited to the ischemic brain and modulate ischemic brain damage (34,

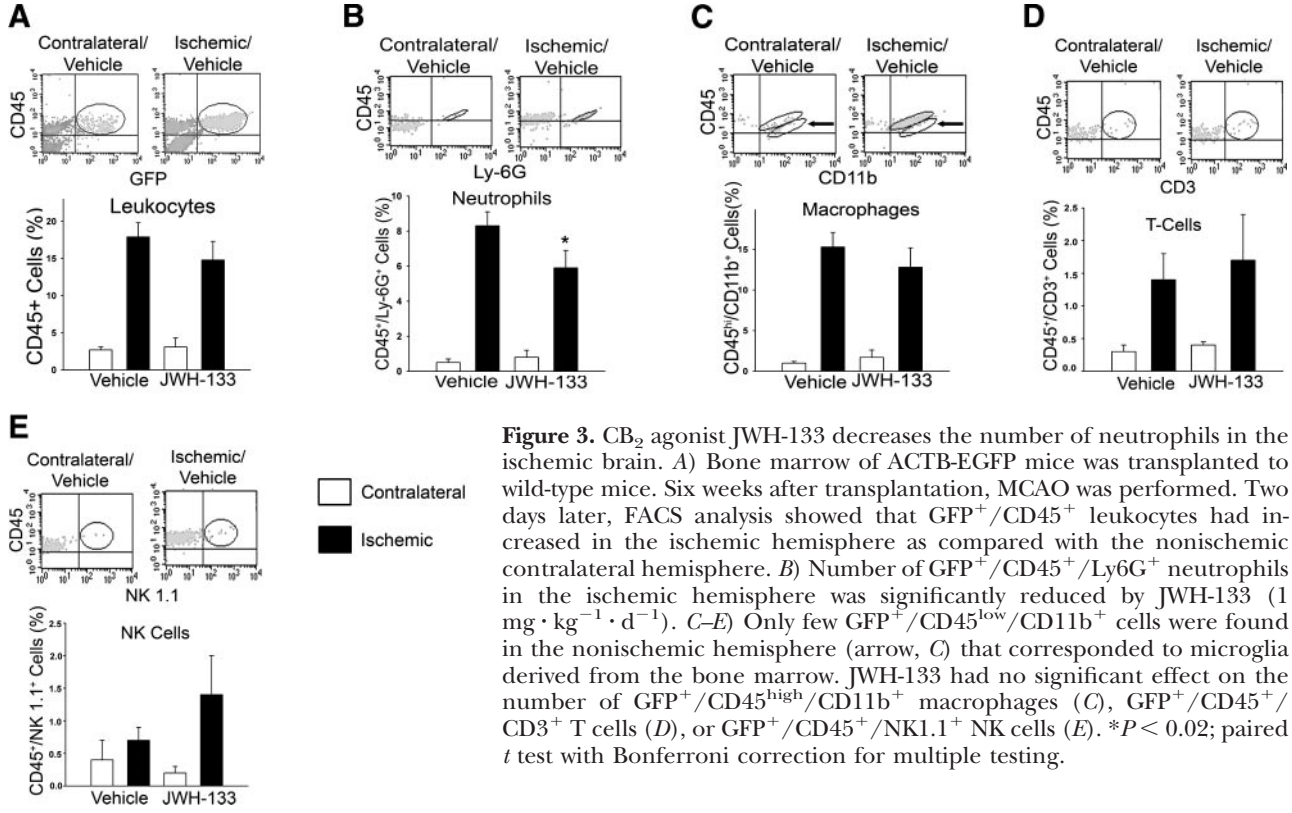

Figure 3. CB_2 agonist JWH-133 decreases the number of neutrophils in the ischemic brain. *A*) Bone marrow of ACTB-EGFP mice was transplanted to wild-type mice. Six weeks after transplantation, MCAO was performed. Two days later, FACS analysis showed that $GFP^+/CD45^+$ leukocytes had increased in the ischemic hemisphere as compared with the nonischemic contralateral hemisphere. *B*) Number of $GFP^+/CD45^+/Ly6G^+$ neutrophils in the ischemic hemisphere was significantly reduced by JWH-133 ($1\ mg \cdot kg^{-1} \cdot d^{-1}$). *C–E*) Only few $GFP^+/CD45^{low}/CD11b^+$ cells were found in the nonischemic hemisphere (arrow, *C*) that corresponded to microglia derived from the bone marrow. JWH-133 had no significant effect on the number of $GFP^+/CD45^{high}/CD11b^+$ macrophages (*C*), $GFP^+/CD45^+/CD3^+$ T cells (*D*), or $GFP^+/CD45^+/NK1.1^+$ NK cells (*E*). $^*P < 0.02$; paired *t* test with Bonferroni correction for multiple testing.

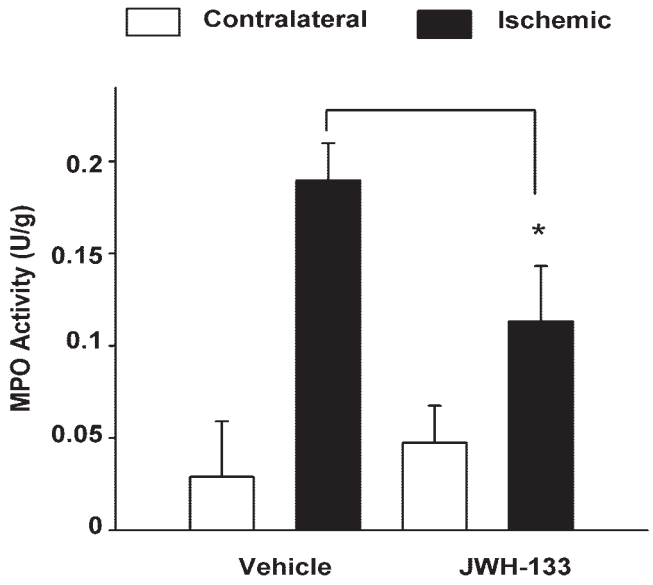

Figure 4. CB_2 agonist JWH-133 reduces myeloperoxidase (MPO) activity, a marker of neutrophils, in the ischemic brain. MPO activity was determined 2 d after distal MCAO. [ANOVA, $F(3/20)=8.329$, $P<0.001$]. $*P < 0.05$ *vs.* vehicle-treated group; Fisher LSD method.

35). To track bone marrow-derived cells in the ischemic brain, we transplanted ACTB-EGFP bone marrow to wild-type mice. Six weeks after transplantation, we performed MCAO and characterized brain cells by FACS 48 h later. In the nonischemic contralateral hemisphere, only few cells were $GFP^+/CD45^+$, indicating that they were derived from the transplanted bone marrow. The number of $GFP^+/CD45^+$ cells markedly increased in the ischemic hemisphere (**Fig. 3*A***). Further characterization of $GFP^+/CD45^+$ cells disclosed that the number of $GFP^+/CD45^+/Ly6G^+$ neutrophils, $GFP^+/CD45^{high}/CD11b^+$ macrophages, and $GFP^+/CD45^+/CD3^+$ T lymphocytes was higher in the ischemic hemisphere than in the nonischemic contralateral hemisphere. In contrast, the number of $GFP^+/CD45^+/NK1.1^+$ natural killer cells was not elevated in the ischemic hemisphere. JWH-133 treatment of mice significantly lowered the number of neutrophils in the ischemic hemisphere but had no statistically significant effect on the other leukocyte populations (Fig. 3*B–E*).

The activity of myeloperoxidase, a marker of neutrophils, increased in the ischemic cortex as compared with the nonischemic contralateral side 48 h after MCAO (**Fig. 4**). This increase was significantly reduced by JWH-133 treatment, confirming that activation of CB_2 receptors by JWH-133 limits the recruitment of neutrophils to the ischemic brain. The reduced number of neutrophils in the ischemic brain was not due to neutropenia, because JWH-133 treatment ($1\ mg \cdot kg^{-1} \cdot d^{-1}$) did not affect the number of $CD45^+/GFP^+$ cells in the blood of ACTB>WT mice (data not shown) or the neutrophil counts in blood in wild-type mice (**Fig. 6*A***).

To elucidate how CB_2 activation interferes with neutrophil recruitment, we quantified the expression of cytokines that contribute to this process. Tumor necrosis factor (TNF), interleukin-1β (IL-1β), and CXCL2 (MIP-2) are known to be involved in neutrophil recruitment to ischemic tissues (36, 37). Quantitative real-time RT-PCR showed that the expression of TNF, IL-1β, and CXCL2 in the ischemic brain was enhanced 24 h after the onset of MCAO (**Fig. 5*A***). However, JWH-133 treatment did not affect induction. This finding is in line with a direct effect of CB_2 activation on bone marrow-derived cells (Fig. 2*B*).

TNF stimulates the adherence of neutrophils to brain endothelial cells (38). Indeed, treatment of the mouse brain endothelial cell line bEnd.3 with TNF (10 ng/ml) enhanced the adherence of neutrophils (Fig. 5*B*). Treatment of wild-type neutrophils with JWH-133 significantly reduced adherence to TNF-stimulated endothelial cells. However, the inhibitory effect of JWH-133 was completely lost when the adherence of $CB_2^{-/-}$ neutrophils to wild-type bEnd.3 was investigated, thus demonstrating that CB_2 receptors mediate the effect. This finding is also in line with the conclusion that the CB_2 agonist acts directly on neutrophils.

After adherence to brain endothelial cells, migration toward a chemoattractant is required for recruitment of neutrophils to the ischemic brain. As the neutrophil chemoattractant CXCL2 is induced in the ischemic brain (Fig. 5*A*), we used recombinant CXCL2 *in vitro* to test the effect of CB_2 activation. In a Transwell assay, neutrophils migrated toward CXCL2 in a concentration-dependent manner (Fig. 5*C*). In the presence of JWH-133 (1 μM) in both the upper and lower chamber, chemotaxis was significantly reduced (Fig. 5*C*). $CB_2^{-/-}$ neutrophils migrated toward CXCL2, like wild-type cells. However, JWH-133 did not inhibit the migration of $CB_2^{-/-}$ neutrophils (Fig. 5*D*), confirming that JWH-133 acts through CB_2 receptors to inhibit neutrophil chemotaxis.

To test whether the effect of JWH-133 is specific for CXCL2-induced chemotaxis, we investigated whether JWH-133 also inhibits migration toward the bacterial neutrophil chemoattractant fMLP. While JWH-133 reduced CXCL2-induced migration, it had no effect on fMLP-induced migration (Fig. 5*E*). Because the selective effect of JWH-133 on CXCL2-stimulated chemotaxis could be due to down-regulation of the CXCL2 receptor CXCR2, we determined the CXCR2 levels on neutrophils by FACS. As has been shown previously, activation of CXCR2 induced its down-regulation (Fig. 5*F*) (39). However, JWH-133 treatment did not further enhance this down-regulation.

Alternatively, it is possible that JWH-133 selectively interferes with the intracellular signaling of CXCL2 but not of fMLP. Notably, the MAP kinase p38 has been reported to inhibit CXCL2- but not fMLP-induced chemotaxis of neutrophils (40, 41). Indeed, JWH-133 activated the MAP kinase p38 in neutrophils as shown by the phosphorylation status of p38 (Fig. 5*G*). In contrast, JWH-133 had no effect on the phosphorylation of the other MAP kinases JNK and p44/p42 (data not shown). Inhibition of p38 by the selective p38

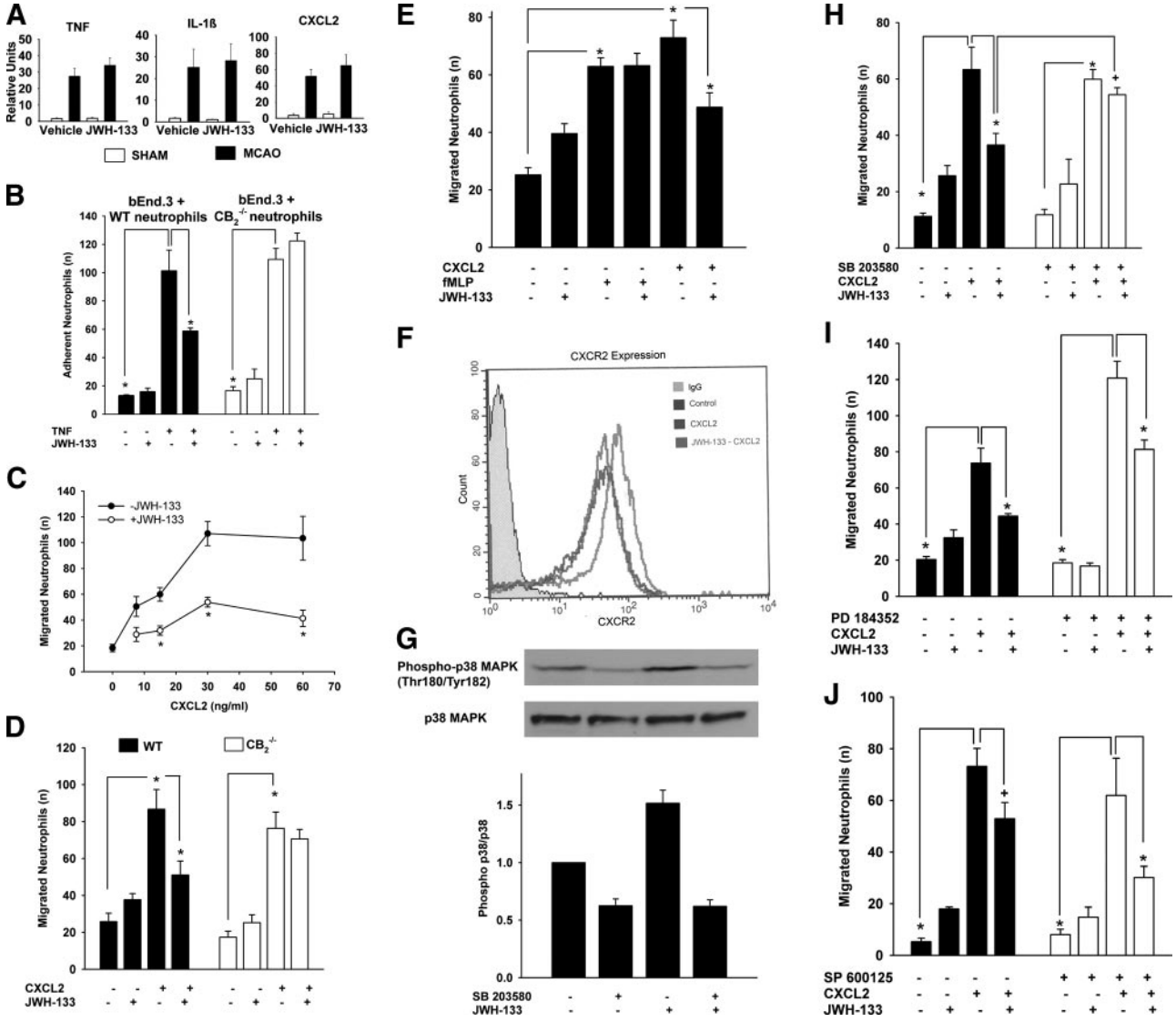

Figure 5. CB_2 activation does not interfere with expression of TNF, IL-1β, and CXCL2 in the ischemic brain but inhibits neutrophil adhesion and migration toward CXCL2. *A*) Induction of TNF, IL-1β, and CXCL2 24 h after distal MCAO was not reduced by JWH-133 treatment (1 mg · kg^{-1} · d^{-1}). mRNA levels were quantified by real-time RT-PCR. $n = 6–8$. *B*) Adherence of wild-type neutrophils to TNF-activated bEnd.3 was inhibited by JWH-133 (1 μM). In $CB_2{}^{-/-}$ neutrophils, this effect was not observed. bEnd.3 cells were pretreated with TNF (10 ng/ml) for 6 h before neutrophils were added for 20 min. Average numbers of neutrophils per random field are depicted. [ANOVA, $F(7/16)=47.343$, $P<0.001$]. $^*P < 0.001$; Fisher LSD method. *C*) Chemotaxis of wild-type neutrophils toward increasing concentrations of CXCL2 was inhibited by JWH-133 (1 μM). JWH-133 was used to pretreat neutrophils for 30 min and was present in the upper and lower chamber of Transwells. Average numbers of neutrophils per random field are depicted. $^*P < 0.05$ *vs.* −JWH-133 group; unpaired *t* test with Bonferroni correction for multiple testing. *D*) Inhibition of CXCL2-induced chemotaxis by JWH-133 depended on CB_2 receptors. JWH-133 (1 μM) was used to pretreat neutrophils for 30 min and was present in the upper and lower chamber of Transwells. Average numbers of neutrophils per random field are depicted. CXCL2, 30 ng/ml. [ANOVA, $F(7/64)=20.032$, $P<0.001$]. $^*P < 0.001$; Fisher LSD method. *E*) JWH-133 (1 μM) inhibited CXCL2-induced chemotaxis of wild-type neutrophils but not fMLP-induced chemotaxis. JWH-133 was used to pretreat neutrophils for 30 min and was present in the upper and lower chamber of Transwells. CXCL2, 30 ng/ml; fMLP, 1 ng/ml. [ANOVA, $F(5/18)=17.276$, $P<0.001$]. $^*P < 0.001$; Fisher LSD method. *F*) Representative histogram of CXCR2 expression (flow cytometric analysis) of neutrophils pretreated with JWH-133 (1 μM) for 30 min followed by a 1-h incubation period with CXCL2 (60 ng/ml) as indicated. Controls were treated with vehicle. *G*) Treatment of neutrophils with JWH-133 (1 μM) for 1 h increased the level of phospho-p38. This effect was blocked by pretreatment with the p38 inhibitor SB 203580 (10 μM). Top panel: typical immunoblot. Bottom panel: quantification of 3 independent experiments. Values are mean ± SE phospho-p38 signal normalized to p38. *H*) Inhibition of CXCL2-induced chemotaxis by JWH-133 (1 μM) was reversed by the p38 inhibitor SB 203580 (10 μM). SB 203580 was added 10 min before JWH-133. CXCL2, 30 ng/ml. [ANOVA, $F(7/16)=18.497$, $P<0.001$]. $^*P < 0.001$, $^+P < 0.05$; Fisher LSD method. *I*) PD 184352 (1 μM), an inhibitor of p44/p42 signaling, did not block the inhibition of CXCL2-induced chemotaxis by JWH-133 (1 μM). PD 184352 was added 10 min before JWH-133. CXCL2, 30 ng/ml. [ANOVA, $F(7/40)=54.088$, $P<0.001$]. $^*P < 0.001$; Fisher LSD method. *J*) SP 600125 (10 μM), a JNK inhibitor, did not interfere with the inhibition of CXCL2-induced chemotaxis by JWH-133 (1 μM). SP 600125 was added 10 min before JWH-133. CXCL2, 30 ng/ml. [ANOVA, $F(7/40)=16.267$, $P<0.001$]. $^*P < 0.001$, $^+P < 0.05$; Fisher LSD method.

blocker SB 203580 reversed the inhibitory effect of JWH-133 (Fig. 5*H*). However, blockade of p44/p42 signaling by PD 184352 or of JNK by SP 600125 did not block the inhibitory effect of JWH-133 (Fig. 5*I, J*). Therefore, CB_2 activation inhibits neutrophil migration through p38 activation.

Protective effect of CB_2 activation is mediated by neutrophils

Neutrophils contribute to ischemic brain damage (36, 42–45). To investigate whether the protective effect of CB_2 activation is due to lower neutrophil recruitment, we ablated neutrophils in mice by injecting an anti-neutrophil antibody. This treatment largely reduced neutrophil counts in peripheral blood (Fig. 6*A*). When neutrophils were ablated, JWH-133 lost its protective effect (Fig. 6*B*), indicating that neutrophils are required for the effect of JWH-133 on ischemic brain damage.

DISCUSSION

Cannabinoid analogues protect against ischemia and other cell-damaging insults. While the role of CB_1 receptors has been somewhat controversial, recent evidence (10, 21, 46, 47) has indicated that CB_2 agonists ameliorate the consequences of hepatic, cerebral, and myocardial ischemia. The data presented here show that activation of CB_2 receptors has a neuroprotective effect in cerebral ischemia and confirm previous studies in which only pharmacological tools were used (10, 48). The effect of JWH-133 was lost in $CB_2^{-/-}$ mice but not in $CB_1^{-/-}$ mice, underscoring that JWH-133 is a specific CB_2 agonist (21). Higher doses of JWH-133 were less effective, as has been reported previously for another CB_2 agonist (22). The bell-shaped dose-response curve may be due to unspecific effects at higher doses. JWH-133 was originally reported to have a 200-fold selectivity for CB_2 over CB_1 receptors (49). However, recent studies (50, 51) using functional and binding assays found a 35- to 40-fold selectivity for CB_2 over CB_1 receptors. These values are close to the ratio of 16 that we observed between an effective dose ($0.5\ mg \cdot kg^{-1} \cdot d^{-1}$) and an ineffective dose ($8\ mg \cdot kg^{-1} \cdot d^{-1}$), suggesting that the lack of efficacy of higher doses might be due to activation of CB_1 receptors.

CB_2 receptors are primarily expressed by immune cells but also by neurons, activated astrocytes and microglia, as well as endothelial cells (14–20). Therefore, the localization of CB_2 receptors involved in the protection against ischemic damage is not self-evident. *In vitro* studies reported that CB_2 receptors on neural cells protect against excitotoxicity (52) and hypoxic-ischemic damage (53). In organotypic cultures, inhibition of inflammatory responses in microglia has been identified as a mechanism by which CB_2 activation may protect against brain injury (54). However, both approaches neglect the effect of blood-borne cells that express CB_2 receptors. Using adoptive transfer of T cells, Maresz *et al.* (11) showed that CB_2 receptors on T cells regulate the response to experimental autoimmune encephalomyelitis. Here, we used a chimeric technique to distinguish between effects on neural cells and bone marrow-derived cells. Bone marrow transplantation targets peripheral leukocyte populations but only a minority of resident microglia in the brain (55).

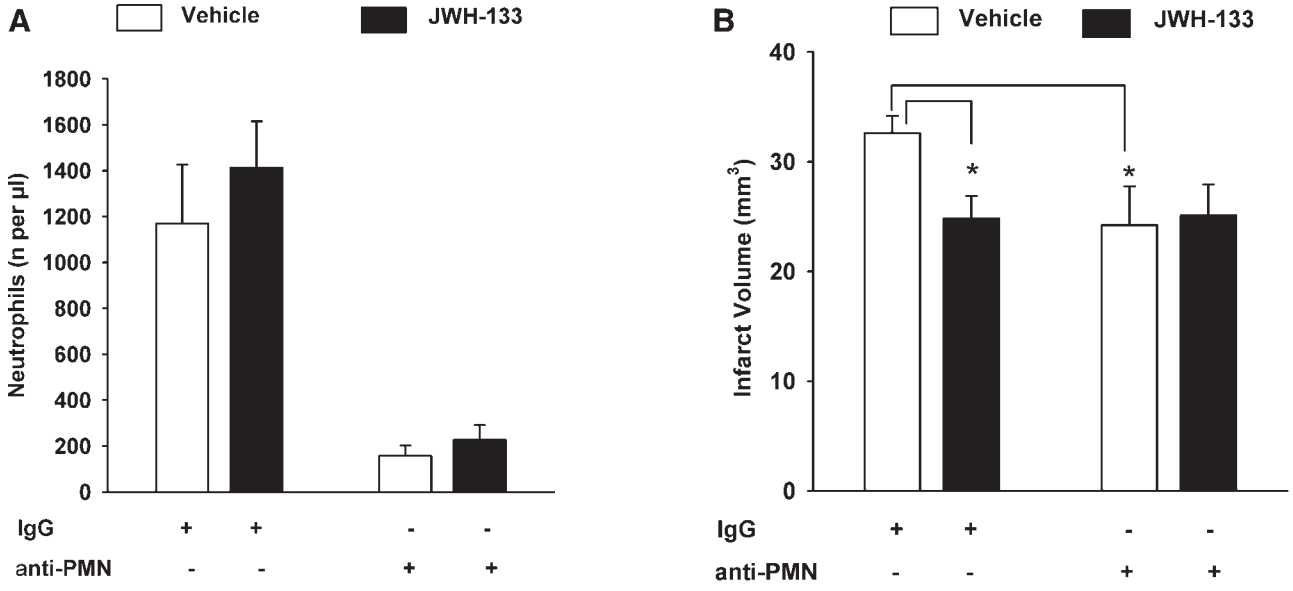

Figure 6. Neuroprotective effect of JWH-133 is mediated by neutrophils. Mice were injected with an anti-neutrophil antibody (anti-PMN) or unspecific IgG for 3 d and subjected to distal MCAO. *A*) Neutrophil counts in blood 2 d after MCAO were significantly reduced by anti-neutrophil treatment but not by JWH-133 ($1\ mg \cdot kg^{-1} \cdot d^{-1}$). *B*) Infarct volumes were reduced by JWH-133 ($1\ mg \cdot kg^{-1} \cdot d^{-1}$). However, this protective effect was lost if neutrophils were depleted. Infarct volumes were determined 2 d after distal MCAO. [ANOVA, $F(3/49)=2.848$, $P<0.05$]. $^*P < 0.05$; Fisher LSD method.

Indeed, we found only few bone marrow-derived cells with the microglial markers $CD45^{low}$ and $CD11b^{+}$ in the nonischemic hemisphere. Interestingly, transplantation of CB_2-deficient bone marrow abrogated the protective effect of the CB_2 agonist JWH-133. Conversely, transplantation of wild-type bone marrow reconstituted the JWH-133 effect in $CB_2^{-/-}$ mice, demonstrating that neuroprotection is mediated by CB_2 receptors in bone marrow-derived cells. Considering the low number of microglia derived from bone marrow cells, it is unlikely that microglia are responsible for the CB_2 effect. To further define which bone marrow-derived cell population mediates the effect of JWH-133, we investigated whether JWH-133 interferes with recruitment of leukocytes. While there was a trend toward lower recruitment of macrophages to the ischemic brain territory, JWH-133 significantly reduced the number of neutrophils. In accordance with a selective effect on bone marrow-derived cells, CB_2 activation did not reduce the neural expression of the neutrophil chemoattractants TNF, IL-1β, and CXCL2, but it directly inhibited the adhesion of neutrophils to brain endothelial cells and neutrophil chemotaxis toward CXCL2. Neutrophils likely mediate the effect of JWH-133 because JWH-133 was ineffective if neutrophils were ablated.

Interestingly, JWH-133 specifically inhibited chemotaxis triggered by CXCL2 but had no effect on chemotaxis triggered by the bacterial component fMLP. CXCL2 and fMLP use different signaling pathways to attract neutrophils (40), which offers a possible explanation for the selectivity of JWH-133. CB_2 receptors stimulate p38 activity in neutrophils (Fig. 5*F*), which inhibits the chemoattractant effect of CXCL2 on neutrophils but not that of fMLP (41, 56). P38 affects the cellular localization of the phosphatase PTEN and phosphatidylinositol-3,4,5-triphosphate, which determine migration of neutrophils toward CXCL2. We demonstrate that CB_2 activation inhibits neutrophil recruitment by activating p38 (Fig. 5*G*). Because this mechanism does not inhibit fMLP-induced chemotaxis, defense against bacterial infections may be unaffected by CB_2 agonists. Indeed, a recent report (57) demonstrated that CB_2 agonists are beneficial in a mouse model of sepsis. This selectivity may prove to be an important advantage in clinical applications because bacterial infections represent a major cause of death in acute stroke (58, 59).

Interestingly, the infarct size did not differ between wild-type and $CB_2^{-/-}$ mice, which argues against an endocannabinoid tone through CB_2 receptors in cerebral ischemia (Fig. 1*B*). This is in contrast to experimental animal models of multiple sclerosis and sepsis in which $CB_2^{-/-}$ mice exhibited a more severe pathology than wild-type animals (11, 57). Cerebral ischemia leads to increased brain endocannabinoid levels (6, 60). However, there is some controversy as to whether levels of 2-AG, the endogenous CB_2 agonist, increase in cerebral ischemia (6). In accordance with the report that CB_2 agonists both stimulate and inhibit cell migration (19), we found that JWH-133 not only inhibits CXCL2-induced chemotaxis of neutrophils but also exerts a chemoattractant effect itself (data not shown). *In vivo*, the source of cannabinoids probably determines their effect. In this concept, peripheral administration would inhibit neutrophil recruitment, whereas endocannabinoid production in the ischemic brain may even enhance recruitment providing a possible explanation why we found no enlargement of infarcts in $CB_2^{-/-}$ mice.

Neutrophil recruitment to the ischemic brain has been recognized as an important pathogenic factor for several years already (36, 42–45). Initial evidence suggested that neutrophils are involved in transient but not in permanent cerebral ischemia (61) because they would not reach the ischemic brain tissue without reperfusion of the MCA. However, recent studies (62) have shown that leukocytes infiltrate the ischemic brain after permanent occlusion of the MCA, at least in the penumbra. Our data support this view showing a marked increase in the number of neutrophils in the ischemic hemisphere after permanent occlusion of the MCA. Indeed, inhibition of neutrophil chemotaxis or neutrophil depletion was neuroprotective in a model of permanent cerebral ischemia (63). We confirmed that neutrophils are involved in our model of permanent cerebral ischemia by demonstrating that neutrophil depletion reduced the infarct size significantly (Fig. 6). Attempts to block neutrophil recruitment by anti-ICAM-1 antibodies failed in the clinic, probably due to an immune response to the antibody (64). This problem could be avoided by employing small molecules such as JWH-133. CB_2 agonists are also free of adverse psychotropic effects mediated by CB_1 receptors. Indeed, CB_2 activation may represent a therapeutic strategy against ischemic injury of several organs because CB_2 agonists were also shown to protect against ischemia of the liver and heart (21, 46). At present, it is unclear whether protection in these territories stems from a direct effect on neutrophils or whether other mechanisms are operating. FJ

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