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# Cannabinoid Type 2 Receptor Activation Downregulates Stroke-Induced Classic and Alternative Brain Macrophage/Microglial Activation Concomitant to Neuroprotection

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**Background and Purpose**—Ischemic stroke continues to be one of the main causes of death worldwide. Inflammation accounts for a large part of damage in this pathology. The cannabinoid type 2 receptor (CB2R) has been proposed to have neuroprotective properties in neurological diseases. Therefore, our aim was to determine the effects of the activation of CB2R on infarct outcome and on ischemia-induced brain expression of classic and alternative markers of macrophage/microglial activation.

**Methods**—Swiss wild-type and CB2R knockout male mice were subjected to a permanent middle cerebral artery occlusion. Mice were treated with either a CB2R agonist (JWH-133), with or without a CB2R antagonist (SR144528) or vehicle. Infarct outcome was determined by measuring infarct volume and neurological outcome. An additional group of animals was used to assess mRNA and protein expression of CB2R, interleukin (IL)-1 $\beta$ , IL-6, tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory peptide (MIP)-1 $\alpha$ , RANTES, inducible nitric oxide synthase (iNOS), cyclooxygenase-2, IL-4, IL-10, transforming growth factor  $\beta$  (TGF- $\beta$ ), arginase I, and Ym1.

**Results**—Administration of JWH-133 significantly improved infarct outcome, as shown by a reduction in brain infarction and neurological impairment. This effect was reversed by the CB2R antagonist and was absent in CB2R knockout mice. Concomitantly, administration of JWH-133 led to a lower intensity of Iba1+ microglia/macrophages and a decrease in middle cerebral artery occlusion-induced gene expression of both classic (IL-6, TNF- $\alpha$ , MCP-1, MIP-1 $\alpha$ , RANTES, and iNOS) and alternative mediators/markers (IL-10, TGF- $\beta$ , and Ym1) of microglial/macrophage activation after permanent middle cerebral artery occlusion.

**Conclusions**—The inhibitory effect of CB2R on the activation of different subpopulations of microglia/macrophages may account for the protective effect of the selective CB2R agonist JWH-133 after stroke. (*Stroke*. 2012;43:211-219.)

**Key Words:** ischemic stroke ■ MCAO ■ mouse ■ cannabinoid ■ microglia ■ alternative phenotype

The endocannabinoid system, integrated by endogenous ligands, cannabinoid receptors, and degrading enzymes, has been proposed as an important pharmacological target in several neurological diseases.<sup>1,2</sup> The most-studied cannabinoid receptors are cannabinoid receptor type 1 (CB1R)<sup>3,4</sup> and cannabinoid receptor type 2 (CB2R).<sup>5</sup> Whereas CB1R is predominantly expressed by neurons (reviewed in<sup>6</sup>), CB2R is mainly expressed by immune cells, regulating migration, cytokine production, and antigen presentation.<sup>7,8</sup>

Indeed, increasing evidence demonstrates the role of CB2R in cannabinoid-mediated regulation of the immune system (reviewed in<sup>1,2,7,8</sup>). Stimulation of CB2R has been shown to inhibit cytokine release,<sup>9,10</sup> to regulate B and T cell differentiation balance,<sup>11</sup> and to modulate macrophage/microglial migration and proliferation.<sup>12–14</sup>

Considering the important role of inflammation in ischemic stroke pathophysiology,<sup>15,16</sup> modulation of immune cells by CB2R activation has arisen as an interesting

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pharmacological approach toward neuroprotection after brain ischemia. In this context, the protective effect of selective CB2R agonists has been tested in different animal models of focal brain ischemia.<sup>17–20</sup> In these studies, CB2R activation resulted in neuroprotection concomitant to a decreased peripheral response as shown by inhibition of leukocyte rolling and adhesion in venules and arterioles<sup>18,20</sup> and inhibition of neutrophil recruitment<sup>17</sup>; attenuation of blood-brain barrier disruption has been also observed.<sup>20</sup> However, local brain effects of CB2R activation have not been explored.

In the present study, we decided to investigate effects of the administration of a single dose of the selective CB2R agonist JWH-133 on immune cell activation, infarct size, and functional outcome after permanent middle cerebral artery occlusion (pMCAO).

### Materials and Methods

The selective CB2R agonist (6aR,10aR)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran (JWH133) was purchased from Tocris Bioscience. JWH-133 has a very high affinity for the CB2R ( $K_i=3.4$  nmol/L), but low affinity for the CB1R ( $K_i=677$  nmol/L).<sup>21</sup> *N*-[(1*S*)-endo-1,3,3-trimethyl bicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR144528), a selective CB2R antagonist, was a kind gift from Dr. Francis Barth, Sanofi-Synthelabo (Montpellier, France). JWH-133 and SR144528 were dissolved in DMSO:Tween:PBS (1:1:18) and the total volume injected intraperitoneally into each animal was 200  $\mu$ L. The vehicle was DMSO:Tween:PBS (1:1:18) and was also injected in a total volume of 200  $\mu$ L.

### Animals

Adult male Swiss mice (age 8–10 weeks) were used. All animals were kept in a room with controlled temperature, a 12-hour dark/light cycle and fed with standard food and water ad libitum. Male CB2R knockout mice (CB2R-KO) on a Swiss congenic background were used.<sup>22</sup> All experimental protocols adhered to the guidelines of the Animal Welfare Committee of the Universidad Complutense (EU directives 86/609/CEE and 2003/65/CE).

### Induction of Permanent Focal Ischemia

Surgery leading to focal cerebral ischemia was performed as described in Supplemental Materials and Methods (<http://stroke.ahajournals.org>).

### Experimental Groups

All the groups were performed and quantified in a randomized fashion by investigators blinded to treatment groups for determination of infarct outcome; all treatments were administered 10 minutes after occlusion. Animals were allocated by randomization (coin toss) to 4 different groups. One group received an intraperitoneal injection of JWH-133 (1.5 mg/kg;  $n=12$ ). A second and third group were treated with SR144528 (3–5 mg/kg) and 3 minutes later with JWH-133 (1.5 mg/kg) intraperitoneally ( $n=12$  in each group); a fourth group of animals was injected with vehicle (DMSO:Tween:PBS) and was considered the control group ( $n=12$ ). In addition, a group of CB2R-KO mice was subjected to pMCAO and treated either with vehicle ( $n=12$ ) or with JWH-133 1.5 mg/kg ( $n=12$ ). An additional group of animals was treated 3 hours after pMCAO with either JWH-133 (1.5 mg/kg;  $n=8$ ) or vehicle.

### Infarct Outcome Determination

Infarct volume determination and neurological score were used as measures of infarct outcome as described in Supplemental Materials and Methods.

### Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from samples collected either 15 or 5 to 18 hours after pMCAO ( $n=5$  for each group) for inflammatory mediators or cannabinoid receptors expression, respectively, using TRIzol reagent (Invitrogen). Sample preparation and quantitative real-time polymerase chain reaction were performed as described in Supplemental Materials and Methods.

### Cytometric Bead Array and Western Blotting

Protein homogenates from brain peri-infarct tissue obtained 24 hours after pMCAO ( $n=4-8$  in each group) were used to measure the protein levels of IL-1 $\beta$ , tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), IL-6, macrophage inflammatory peptide (MIP) -1 $\alpha$ , MCP-1, RANTES, IL12/IL-23p40, and IL-10 by a cytometric bead array (Beckton Dickinson), and of iNOS and COX-2 by western blotting, as described in Supplemental Materials and Methods.

### Immunofluorescence and Confocal Microscopy

Free-floating coronal brain slices (30 mm) were processed 24 hours after pMCAO ( $n=4$  in each group) and immunofluorescence, confocal microscopy, and image analysis were performed as described in Supplemental Materials and Methods.

### Statistical Analysis

Student *t* test was used to compare 2 groups, and 1-way ANOVA with Newman-Keuls or Bonferroni post hoc tests were used to compare more than 2 groups. Data are expressed as mean  $\pm$  SD, and a  $P<0.05$  was assumed as statistically significant difference.

## Results

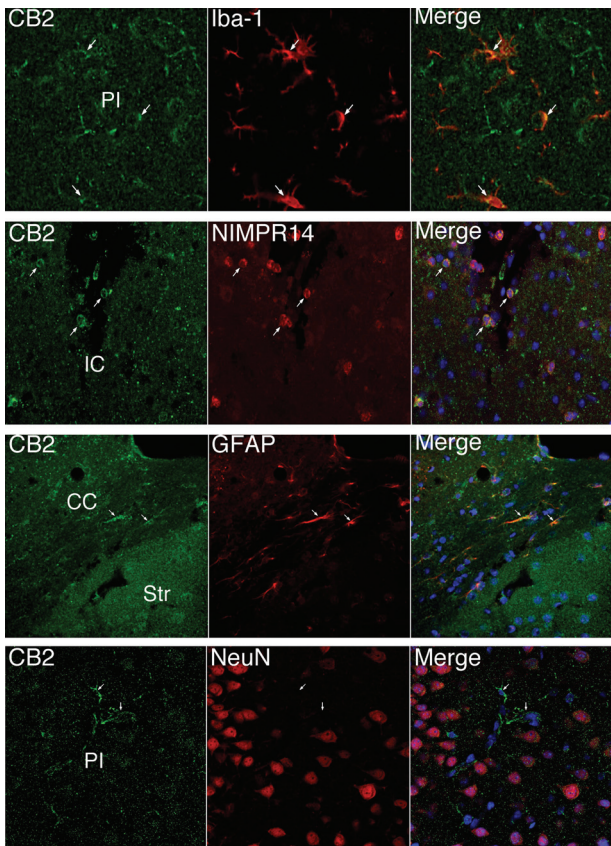
### Expression of CB1R and CB2R After pMCAO

pMCAO increased CB2R mRNA expression in the peri-infarct area 18 hours after injury and decreased CB1R mRNA levels at 5 hours (Supplemental Figure IA). The CB2R agonist JWH-133 decreased ischemia-induced mRNA expression of CB2R 15 hours after pMCAO, whereas CB1R mRNA expression was not affected by this treatment (Supplemental Figure IB).

Immunofluorescence characterization of brain sections 24 hours after MCAO and subsequent confocal analysis were performed to elucidate the nature of the CB2R-expressing cells. A large proportion of microglial cells (Iba-1+) in the peri-infarct area coexpressed CB2R (Figure 1 and Supplemental Figure IIB). In both peri-infarct (Figure 1) and ischemic core (Supplemental Figure IIB), all neurons (NeuN+ cells) were negative for CB2R. Some isolated astrocytes (GFAP+ cells) present in the corpus callosum showed CB2R expression (Figure 1 and Supplemental Figure IIB). In addition, the ischemic core appeared infiltrated with neutrophils (NIMP-R14+ cells), many of them expressing CB2R (Figure 1 and Supplemental Figure IIB). In contrast, we could not detect a significant CB2R expression in brain sections from sham-operated animals (Supplemental Figure IIA).

### Effects of the CB2R Agonist JWH-133 on Infarct Outcome

Administration of the CB2R agonist JWH-133 (1.5 mg/kg) 10 minutes after pMCAO caused a decrease in the infarct



**Figure 1.** Cannabinoid type 2 receptor (CB2R) expression in different cell types: Representative confocal microscopy analyses of CB2R (green) in brain sections of middle cerebral artery occlusion animals at 24 hours were performed with anti-NeuN (neurons), anti-Iba-1 (microglia/macrophages), anti-GFAP (astrocytes), and anti-NIMP-R14 (neutrophils) antibodies. Colocalization is shown in Iba-1+, GFAP+, and NIMP-R14+ cells. CB2R expression does not colocalize with anti-NeuN neuronal cells. IC indicates ischemic core; PI, peri-infarct; CC, corpus callosum; Str, striatum.

size determined 48 hours after the ischemic injury when compared with the vehicle group ( $n=12$ ;  $P<0.05$ ). Lower and higher doses of the agonist (0.5 mg/kg and 5 mg/kg) did not significantly affect infarct volume ( $P>0.05$ ; Figure 2A). Similarly, animals treated with the CB2R agonist (1.5 mg/kg) had a lower score in the modified Neurological Severity Score (mNSS) 48 hours after the pMCAO (Figure 2D). The effect of JWH-133 on infarct volume was reversed in a dose-dependent manner by the administration of the CB2R selective antagonist SR144528 (3–5 mg/kg; Figure 2B,D). Likewise, the administration of JWH-133 (1.5 mg/kg), 3 hours after pMCAO, also decreased the infarct volume compared with the vehicle-treated group (Figure 2C).

To test further the selectivity of JWH-133 on CB2R, a group of CB2R-KO mice ( $n=12$ ) were subjected to pMCAO and treated either with vehicle or the CB2R agonist. Our results show that there are no differences in infarct outcome when wild-type and CB2R-KO mice are compared. Furthermore, the CB2R agonist did not affect either infarct size or neurological impairment in the CB2R-KO mice (Figure 2B, D).

## Effects of JWH-133 on Gene and Protein Expression of Inflammatory Molecules

Exposure to pMCAO caused an increase in mRNA expression of the proinflammatory cytokines and chemokines IL-1 $\beta$ , IL-6, MCP-1, MIP-1 $\alpha$ , RANTES, and IL-12/IL-23p40 as well as expression of the inflammatory enzymes myeloperoxidase (MPO), iNOS, and COX-2 in peri-infarct tissue 15 hours after the insult (Figure 3). Treatment with the CB2R agonist significantly decreased MCAO-induced mRNA expression of IL-6, IL-12/IL-23p40, MCP-1, MIP-1 $\alpha$ , RANTES, and iNOS. No significant differences were observed in TNF- $\alpha$ , IL-1 $\beta$ , and MPO mRNA expression between the groups at the time studied (Figure 3).

Ischemia also increased brain protein expression of MCP-1, IL-6, TNF- $\alpha$ , MIP-1 $\alpha$ , RANTES, IL-12/IL-23p40, and iNOS, but not of COX-2, in the cortical peri-infarct brain tissue of vehicle-treated mice 24 hours after pMCAO (Figure 4). Treatment with the CB2R agonist JWH-133 significantly reduced iNOS, IL-6, TNF- $\alpha$ , MIP-1 $\alpha$ , and RANTES, but not IL-12 or MCP-1 protein expression 24 hours after the ischemic insult (Figure 4). Protein expression of IL-1 $\beta$  was not detectable with the assay used.

## Effects of JWH-133 on the Gene Expression of Alternative Microglia/Macrophage Phenotype Markers

Ischemia induced mRNA expression of the alternative microglia/macrophage phenotype markers TGF- $\beta$ , Ym1, and IL-10 (but not of arginase I and IL-4) in samples obtained from the peri-infarct area 15 hours after pMCAO. This effect was decreased by the CB2R agonist JWH-133 (Figure 5).

## Effects of JWH-133 on Microglial Activation in the Ischemic Boundary

Ischemia induced the activation of microglia/macrophages in the injured cortex, as demonstrated by a slight hypertrophy of the processes of Iba1-positive cells and an increased intensity of Iba1 expression in the peri-infarct microglia. Moreover, the group treated with the CB2R agonist had a lower expression of Iba1 in the ischemic boundary when compared with the vehicle-treated group (Figure 6A–B).

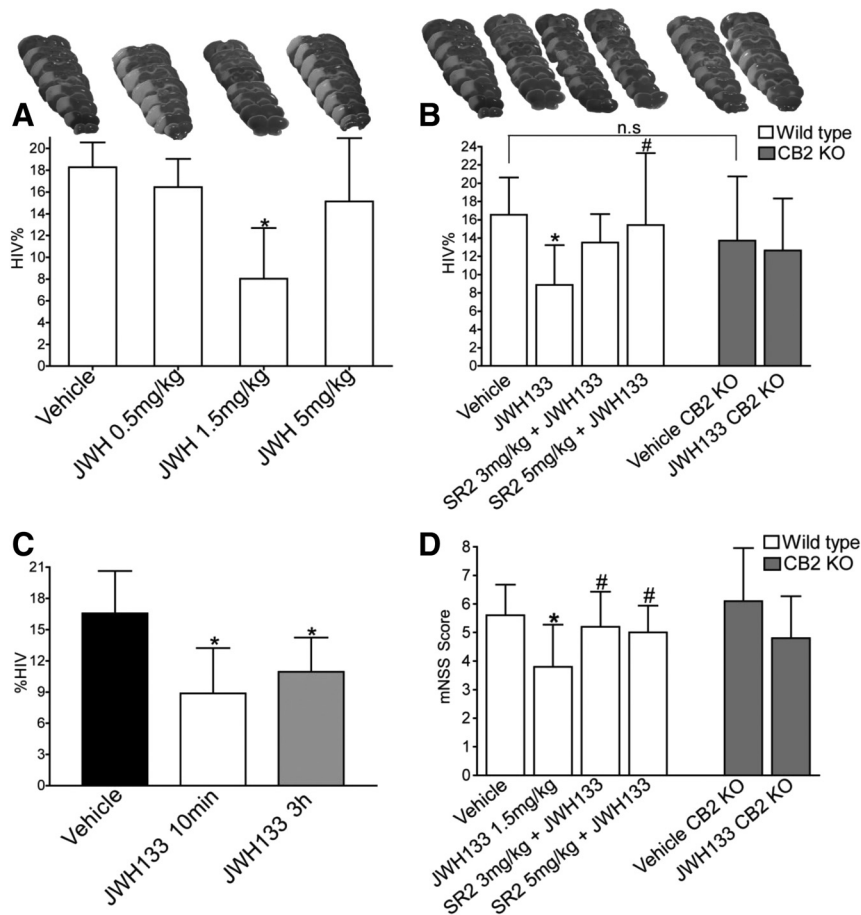
## Effects of JWH-133 on Peripheral Blood Cells and on Neutrophil Infiltration

Ischemia did not change the percentage of neutrophils, monocytes, and lymphocytes in peripheral blood 4 hours after pMCAO compared with the sham control group. Treatment with JWH-133 (1.5 mg/kg) did not alter the number of blood immune cells at the time observed (Supplemental Figure IV).

Similarly, treatment with JWH-133 (1.5 mg/kg) did not affect the number of neutrophils (NIMP-R14+ cells) infiltrated into the ischemic core 24 hours after MCAO (Supplemental Figure V).

## Discussion

In this study, we have explored the effects of CB2R activation in experimental stroke by using a permanent model of focal ischemia in mice. Our data show that a



**Figure 2.** Neuroprotective effect of JWH-133 on infarct outcome. **A**, Dose-response effect of the CB2R agonist JWH-133 (0.5–5 mg/kg) on % of hemisphere-infarcted volume (%HIV) 48 hours after permanent middle cerebral artery occlusion. **B**, Lack of effect of JWH-133 in the presence of the selective CB2R antagonist SR144528 (5 mg/kg) or in CB2R<sup>-/-</sup> animals. **C**, Effect of the administration of JWH-133 10 minutes or 3 hours after pMCAO on %HIV. **D**, Modified Neurological Severity Score (mNSS) 48 hours after pMCAO. Data are expressed as mean ± SD, n=8–12, \**P*<0.05 vs vehicle, #*P*<0.05 vs JWH-133-treated group. ANOVA and Newman-Keuls post hoc test.

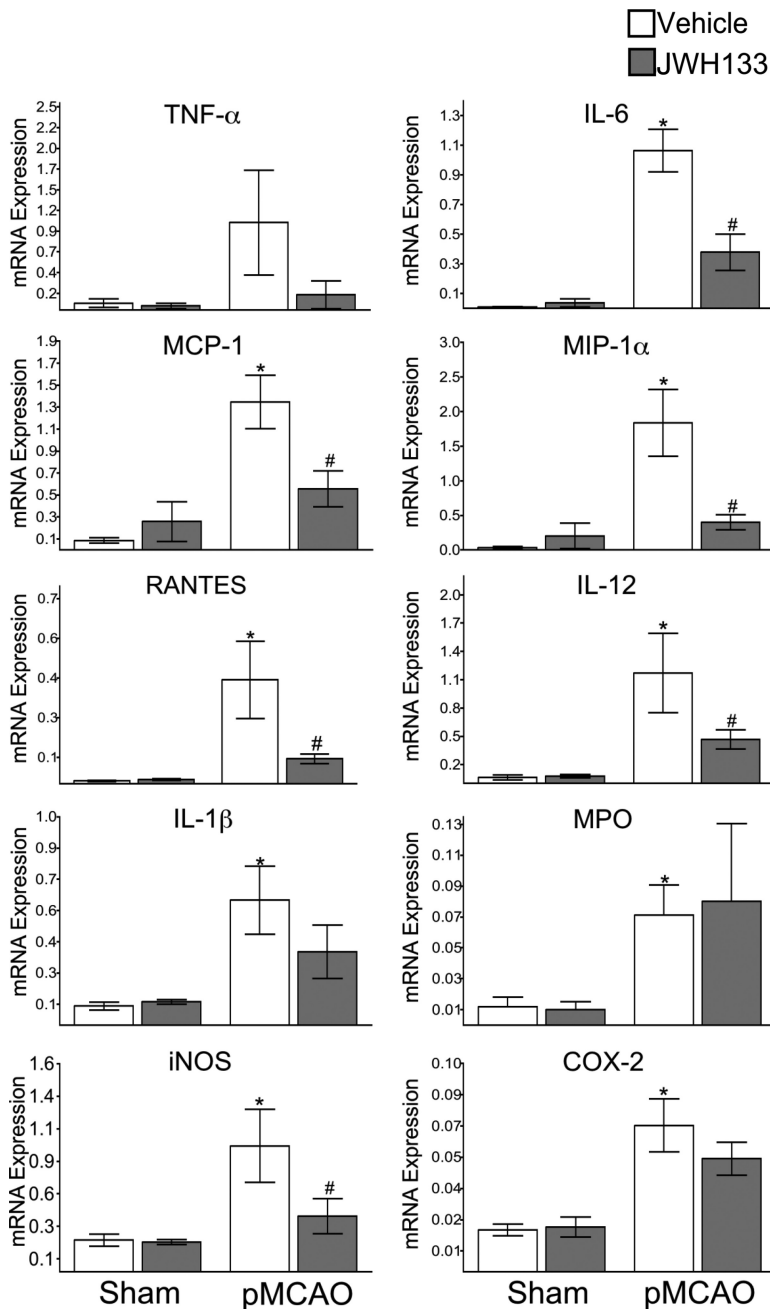
single administration of a CB2R receptor agonist after the onset of ischemic injury improves stroke outcome concomitant to reduced classic and alternative microglial activation in the injured cortex.

Indeed, our experiments show that a single acute dose of the CB2R selective agonist JWH-133 administered after permanent focal ischemia (10 minutes or 3 hours) is neuroprotective by reducing infarct volume and improving neurological outcome. This effect was reversed by the CB2R selective antagonist SR144528 and was absent in CB2R-KO mice, thus demonstrating that JWH-133-induced neuroprotection is selectively dependent on CB2R receptor activation. In this vein, the existing literature shows a neuroprotective effect in different protocols of administration of CB2R agonists in animal models of transient and permanent brain ischemia.<sup>17–20</sup> Here we have used a model of permanent occlusion of the middle cerebral artery (MCA), the clinical relevance of which is supported by the fact that the only approved treatment for stroke to date is thrombolysis with recombinant tissue-type plasminogen activator<sup>23</sup>; this is available only to 2% to 5% of the patients and only 32% of those patients undergo a complete recanalization.<sup>24</sup> Thus, stroke models based on permanent vessel occlusions are the ones that more closely represent the human patient situation, at least in the first 24 hours after stroke onset.<sup>25,26</sup> Furthermore, our data demonstrate a neuroprotective effect of a CB2R agonist administered postischemia (10 minutes or 3 hours), suggesting its utility in the clinical situation.

As shown in previous studies,<sup>17,20</sup> the neuroprotective effect of the CB2R agonist was lost at the highest doses used. Although the exact mechanisms of the bell-shaped dose-response curves remain to be elucidated, it could be speculated that interactions with other cannabinoid receptors might be involved.<sup>17,20,27,28</sup>

As previously reported,<sup>17</sup> lack of CB2R expression did not affect infarct outcome. It has been reported that no significant changes in N-arachidonylethanolamine, one of the main endogenous cannabinoid agonists, was seen after 4, 12, or 24 hours of pMCAO in mice (reviewed in<sup>6</sup>). As to the other major endocannabinoid, 2-arachidonoylglycerol, its content did increase in the ipsilateral hemisphere after 4, but not after 12 or 24 hours of MCAO. Then, it is plausible that a poor endogenous CB2R activation caused by low endocannabinoid production might explain why CB2R deletion does not correlate with a clear effect in neuroprotection.

Our results illustrate the time-expression profile of cannabinoid receptors in mouse brains after pMCAO injury, showing an increased expression of CB2R that corroborates the presence of the drug target. A previous study reported that CB1R and CB2R are induced 6 and 24 hours, respectively, after an ischemia/reperfusion model in mice.<sup>19</sup> In this context, we show that CB2R mRNA expression occurs earlier (18 hours after pMCAO) after a permanent occlusion in mice. Our data are consistent with several reports that demonstrate a brain upregulation of CB2R in animal models of different neurological diseases in which inflammation is involved.<sup>29</sup>



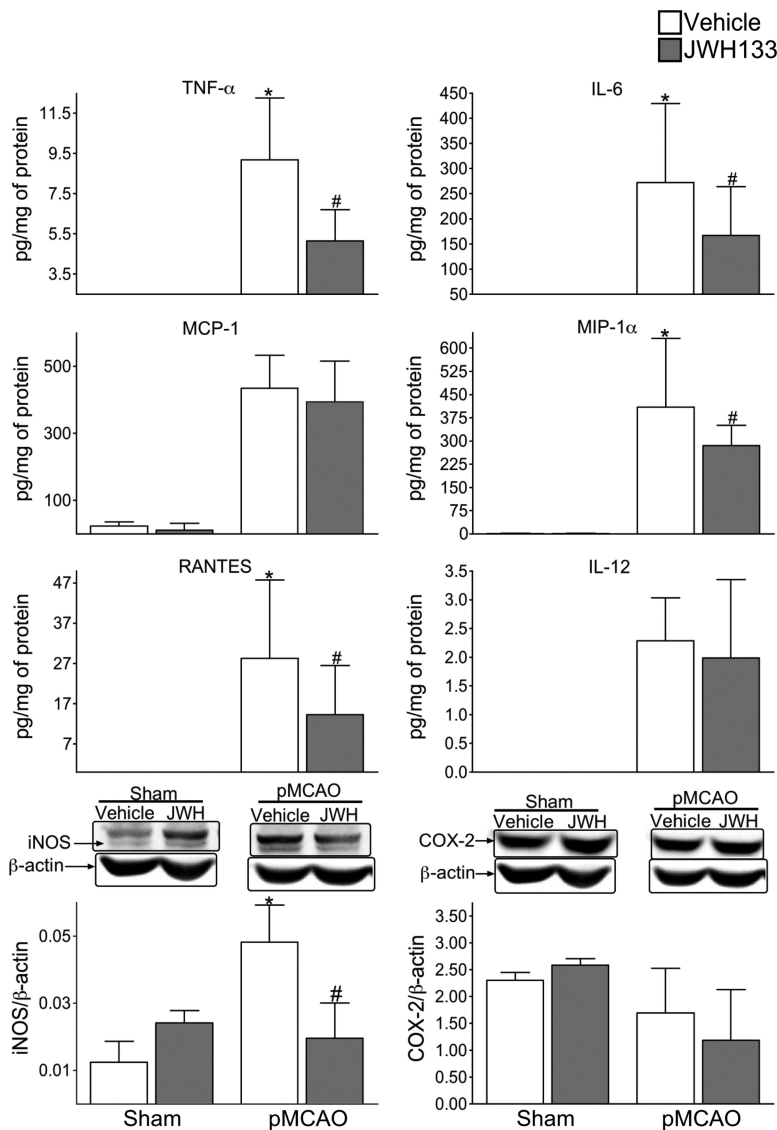
**Figure 3.** Effects of JWH-133 on brain mRNA expression of inflammatory molecules after permanent middle cerebral artery occlusion. mRNA expression of TNF- $\alpha$ , IL-6, MCP-1, MIP-1 $\alpha$ , RANTES, IL-12/IL-23p40, IL-1 $\beta$ , MPO (myeloperoxidase), iNOS, and COX-2 15 hours after sham or pMCAO surgery. Samples were tested in triplicate,  $n=5$  in each group. Data expressed as mean $\pm$ SD, \* $P<0.05$  vs sham+vehicle, # $P<0.05$  vs pMCAO+vehicle. ANOVA and Newman-Keuls post hoc test.

Conversely, CB1R is transiently downregulated, possibly because of neuronal damage, but levels are subsequently recovered, an effect that might be caused by increased CB1R expression in astrocytes because of gliosis and/or in surviving neurons in the ischemic boundary (additional details are discussed in Supplemental Discussion).

Regarding the mechanisms involved in the neuroprotective effect of CB2R, we have found that JWH-133 decreases activation of microglia after pMCAO, as shown by the reduced expression of Iba1 and the predominance of a resting morphology in microglial cells located within the ischemic boundary.<sup>30,31</sup> This is in agreement with previous reports,<sup>29,32</sup> pointing to a central inactivation of microglia/macrophage cells<sup>30</sup> as the main mechanism by which CB2R mediates protection after stroke. Because we have shown that CB2R

expression is induced in reactive microglia after pMCAO, downregulation of ischemia-induced CB2R expression after JWH-133 treatment further supports lower microglial activation. In addition, our results showed that disperse astrocytes at the corpus callosum displayed immunoreactivity for CB2R, the activation of which might also be involved in JWH-133-induced neuroprotection.

Focal ischemia induced mRNA and/or protein expression in brain parenchyma of several proinflammatory and inflammatory mediators, which include IL-1 $\beta$ , IL6, iNOS, COX-2, RANTES, MCP-1, MIP-1 $\alpha$ , TNF- $\alpha$ , IL-12/IL-23p40, and MPO, consistent with neural injury, macrophage/microglial classic activation, and infiltration of immune cells (lymphocytes, neutrophils, macrophages); these are events that have detrimental results in the acute phase of stroke.<sup>15,16,33</sup> Acti-

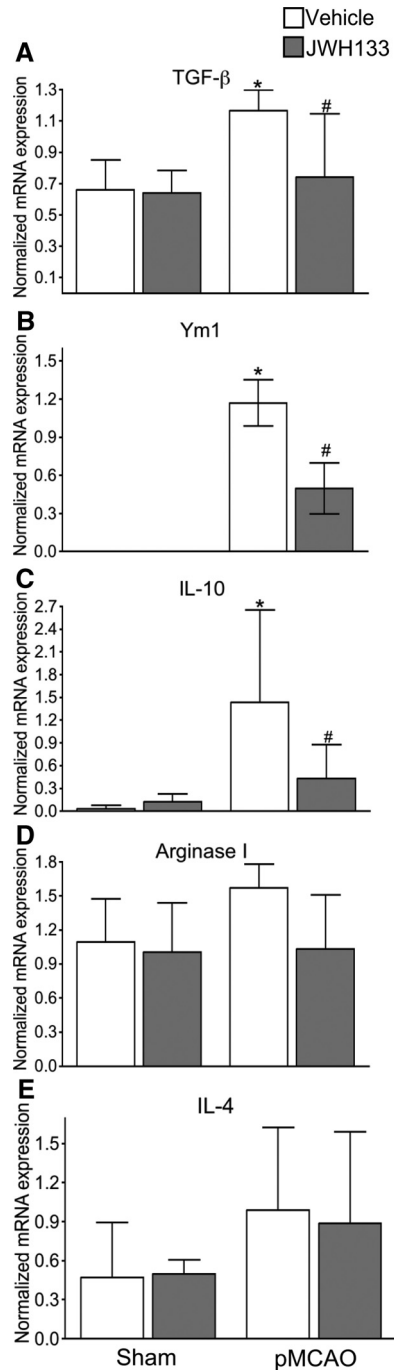


**Figure 4.** Effects of JWH-133 on brain protein expression of inflammatory molecules after permanent middle cerebral artery occlusion (pMCAO). Protein levels were determined 24 hours after pMCAO by cytometric bead array (TNF- $\alpha$ , IL-6, MCP-1, MIP-1 $\alpha$ , RANTES, and IL-12) or western blot (iNOS, COX-2). Samples were tested in duplicate,  $n=4$  to 8 in each group. Data are expressed as mean  $\pm$  SD, \* $P < 0.05$  vs sham+vehicle, # $P < 0.05$  vs pMCAO+vehicle. ANOVA and Newman-Keuls post hoc test.

vation of CB2R decreased this effect, as demonstrated in other settings of central nervous system injury.<sup>34,35</sup> This effect differed among the mediators studied, a fact likely caused by the specific time profiles of induction of each molecule following pMCAO. A recent study in mice subjected to permanent ischemia (MCA electrocoagulation) did not find any effect of JWH-133 on TNF- $\alpha$ , IL-6, and CXCL2 mRNA cortical expression, thus claiming a peripheral effect of the CB2R agonist because of reduced neutrophil infiltration.<sup>17</sup> Our results are the first to demonstrate that a unique dose of JWH-133 (1.5 mg/kg), administered after the onset of ischemic injury, diminishes the expression of proinflammatory molecules in the cortical peri-infarct tissue. Moreover, we did not observe any effect of the treatment with JWH-133 either on the gene expression of the neutrophil marker MPO 15 hours after pMCAO, or in the number of neutrophils present in the ischemic core 24 hours after the ischemic occlusion. The apparent controversy with the aforementioned study could be explained by differences in the ischemia models, drug administration protocols, and doses used in the 2 studies. Whereas the acute inflammatory response is associated with

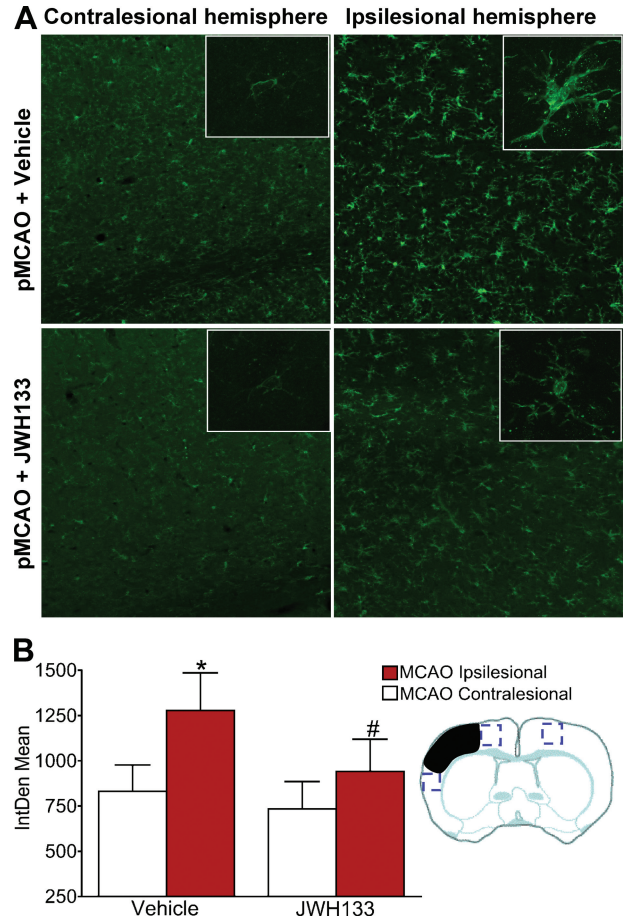
molecules that contribute to the demise of neurons in the penumbra, inflammation may be also instrumental for lesion containment and repair (for review, see<sup>36,37</sup>). In this context, additional studies are needed to elucidate the role of CB2R-induced modulation of inflammation in the later, chronic phase of stroke.

As commented on above, these inflammatory mediators are characteristic features of the classic activation of macrophages/microglial cells (reviewed in<sup>38,39</sup>). This classic, or "M1," phenotype arises as a rapid response to tissue injury and is associated with expression of molecules that will contribute to the demise of neurons in the penumbra; therefore, this inflammatory response is detrimental to neurological outcome. Consequently, its inhibition by CB2R activation is likely to contribute to the neuroprotective effect reported here. Interestingly, microglial cells and macrophages are able to acquire other states to arrest the killing phase and to restore tissue homeostasis. In this context, the switch to an alternative phenotype, characterized by anti-inflammatory and resolute mediators, provides the scenario for repair and tissue reconstruction. Mirroring the Th1/Th2 nomenclature of T



**Figure 5.** Effects of JWH-133 on brain mRNA expression of alternative activation mediators/markers after permanent middle cerebral artery occlusion (pMCAO). **A–E:** mRNA expression of TGF- $\beta$ , Ym1, IL-10, Arginase I, and IL-4 15 hours after sham or pMCAO surgery. Samples were tested as triplicates,  $n=5$  in each group. Data are expressed as mean  $\pm$  SD, \* $P < 0.05$  vs Sham+vehicle, # $P < 0.05$  vs pMCAO+vehicle. ANOVA and Newman-Keuls post hoc test.

helper cells, the term “M2” was proposed, given that its polarization is caused by anti-inflammatory mediators such as Th2-derived IL-4 and IL-13 (toward M2a, alternative, or wound-healing macrophages/microglia), or Treg-derived IL-10 and TGF- $\beta$  (toward M2c, regulatory, or deactivated macrophages/microglia). A third subgroup, M2b, includes regulatory macrophages activated by immune complexes+Toll-



**Figure 6.** Effects of JWH-133 on microglial activation after pMCAO. **A,** Representative images of Iba1+ cells in the contralateral healthy and the ipsilateral peri-infarct hemispheres in untreated or JWH-133-treated animals. **B,** Densitometry of peri-infarct microglia in pMCAO mice treated either with vehicle or JWH-133. Sq dashed lines show the ROIs (Regions of Interest) where the images were taken.  $n=4$  in each group. Data were expressed as mean  $\pm$  SD, \* $P < 0.05$  vs MCAO contralateral+vehicle, # $P < 0.05$  vs MCAO+vehicle. ANOVA and Bonferroni post hoc test.

like receptors/IL-1R ligands (reviewed in<sup>38–41</sup>). Because the CB2R receptor regulates the balance of Th1-proinflammatory to Th2-anti-inflammatory cytokines,<sup>11</sup> as well as enhances the expression of IL-10 in vitro,<sup>32,42</sup> we decided to explore the effects of JWH-133 on anti-inflammatory and M2 markers. Interestingly, our data show that brain ischemia causes the expression of the M2-polarizing cytokines TGF- $\beta$  and IL-10, which are in turn required for M2c activation, and also of Ym1, a secretory chitinase-like protein marker of the M2a state<sup>38,41,43</sup>; this is consistent with the coexistence of different subsets of macrophage/microglial cells in the ipsilesional hemisphere and suggests an endogenous protective response. Surprisingly, acute administration of the CB2R agonist decreased pMCAO-induced expression of these mediators at the time studied, 15 hours after the ischemic insult. To our knowledge, this is the first report of the induction of the M2a or alternative marker Ym1 in experimental stroke, and the inhibitory effect of CB2R activation on anti-inflammatory mediators in this setting. It is known that IL-10 and TGF- $\beta$  are induced in focal brain ischemia, where they mediate

neuroprotection.<sup>16,44</sup> The reason for which CB2R causes the downregulation of these molecules deserves additional study, but it might be caused by an action on microglia toward an inactivated, quiescent state; this is in agreement with the immunofluorescence studies showing decreased microglial activation, with features close to the cells present in the contralesional healthy hemisphere. Our results open an interesting line for future studies aimed at the study of the role of CB2R activation on microglial cell phenotypes in the chronic phase of ischemia, and specifically on its involvement on neurorepair processes at this late stage.

In summary, the results of the present study demonstrate the important role of the CB2R receptor in neuroprotection after focal brain ischemia, which appears to be caused by central microglia/macrophage inactivation and subsequently an anti-inflammatory mechanism with the inhibition of TNF- $\alpha$ , IL-6, IL-12/IL-23p40, MCP-1, MIP-1 $\alpha$ , and RANTES mediated by the CB2R receptor. Finally, the effects of CB2R activation on the expression of both mediators or markers of alternatively activated macrophages indicate a general inactivation of different subpopulations of microglia/macrophages.

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### Disclosures

None.

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## **SUPPLEMENTAL DATA**

### **SUPPLEMENTARY MATERIALS AND METHODS**

#### **Induction of permanent focal ischemia**

Surgery leading to focal cerebral ischemia was conducted under anesthesia with isoflurane in a mix of O<sub>2</sub> and N<sub>2</sub>O (0.3/0.7 L/min). During surgery, body temperature was maintained at 37.0±0.5 °C using a servo-controlled rectal probe–heating pad. The surgical procedure was a variant of that described by Chen et al. (1986)<sup>1</sup> and Liu et al. (1989)<sup>2</sup>. A small craniotomy was made over the trunk of the left middle cerebral artery and above the rhinal fissure. The permanent middle cerebral artery (MCA) occlusion (pMCAO) was done by ligature of the trunk just before its bifurcation between the frontal and parietal branches with a 9-0 suture. Complete interruption of blood flow was confirmed under an operating microscope. Additionally, the left common carotid artery was then occluded. Mice in which the MCA was exposed but not occluded served as sham-operated controls (sham). After surgery, individual animals were returned to their cages with free access to water and food.

Physiological parameters (rectal temperature, mean arterial pressure, pO<sub>2</sub>, pCO<sub>2</sub>, pH) were not significantly different between all studied groups (Supplementary table 1). No spontaneous mortality was found after MCAO with this model, and this was unaffected by the different experimental treatments.

#### **Infarct outcome determination**

For infarct volume determination, brains were removed 48 h after pMCAO, and cut into eight coronal brain slices of 1-mm (Brain Matrix, WPI, UK), which were stained in 2% TTC (2,3,5-triphenyl-tetrazolium chloride, Merck, Madrid, Spain) in 0.2 mol/L phosphate buffer. Infarct size was determined as follows: infarct volumes were measured by sampling stained sections with a digital camera (Nikon Coolpix 990, Nikon Corporation, Tokyo, Japan), and the image of each

section was analyzed using ImageJ 1.44I (NIH, Bethesda, MD, USA). The digitalized image was displayed on a video monitor. With the observer masked to the experimental conditions, the ratio between the volume of the spared cortex in the damaged hemisphere ( $L_N$ ) and that in the whole neocortex of the contralateral hemisphere ( $R$ ) was calculated, and used to detect differences in the amount of cortex that was damaged by the infarct in each animal. To calculate the percent of hemisphere infarcted volume (HIV%) we used the formula:  $HIV\% = [1 - (L_N/R)] \times 100$ .

To determine neurological impairment, the modified Neurological Severity Score (mNSS) was applied to every animal 24 and 48 hours after pMCAO as previously described<sup>3</sup>. This neurological score evaluates motor symptoms (hemiparesis and gait), balance and reflexes (Supplementary Table 2).

### **Tissue dissection for mRNA and protein levels studies**

Tissue samples for mRNA and protein determination were dissected out from both the peri-infarct area and the homologous area in the sham group and contralateral healthy hemisphere. For this, the brain was placed in a brain matrix (Brain Matrix, WPI, UK) and cut into 2-mm coronal slices. The most rostral and caudal coronal slices (1-mm thick) were excluded to avoid the chance of presence or lack of infarct due to inter-animal variability. Then, the left MCA was identified, the territory around this vessel was excluded (core) and 2 mm of tissue around the ischemic core in each slice was dissected and immediately frozen in liquid N<sub>2</sub>. For mRNA, samples were taken at 15h (inflammatory mediators) or at 5 and 18h (for CBRs expression) (n=5 for each group). For determination of protein levels, tissue was collected (n=4 for each group) 24 h after MCAO.

### **mRNA determination by quantitative RT-PCR**

RNA quantity in tissue extracts was determined spectrophotometrically with a Nanodrop (ND-1000) spectrophotometer, and the purity was confirmed by the relative absorbance at 260 nm

versus 280 nm. 1 µg of RNA was reverse-transcribed with iScript cDNA Synthesis kit (Bio-Rad). Quantitative real-time PCR was performed using a Bio-Rad iQ5 Thermocycler with triplicate samples. The mRNA expression was normalized to *actin* gene expression. For all genes, denaturation at 95°C for 5 min was followed by 45 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 40 s. Melt curve analysis was included to assure a single PCR product was formed. Specific primers for mice genes were designed using PubMed library (Supplementary Table 3).

Regarding CB1R mRNA levels (Supplementary Figure 1), a decrease was found at early times (5h) that might be due to neuronal damage; however, levels were recovered up to sham values 18h after the pMCAO. CB1 receptors are expressed at high level throughout the brain by many different classes of neurons, but are also expressed at lower levels by glial cells (see, for instance, rev. in <sup>4</sup>). A possibility to explain recovery of CB1R mRNA levels is an increased CB1R expression in areas where gliosis takes place, as it is the case of the ischemic boundary and adjacent peri-infarct. In addition, it has been reported that CB1 receptor-like immunoreactivity is up-regulated in neuronal-type cells in the ischemic boundary zone as early as 2 hours after reperfusion in rats (rev. in <sup>5</sup>). However, this effect was found after transient but not permanent MCA, being additional studies needed to elucidate the mechanisms that govern brain CB1R expression after pMCAO in mice.

### **Protein determination by cytometric bead array (CBA)**

Protein homogenates from brain infarcted tissue obtained 24h after pMCAO were used to measure the protein levels of IL-1β, TNF-α, IL-6, MIP-1α, MCP-1, RANTES, IL12/IL-23p40 and IL-10 by a BD™ Cytometric Bead Array (CBA). This assay allows for the discrimination of different particles on the basis of size and fluorescence. Capture Antibodies (Abs) were covalently coupled to microspheres (beads) according to the manufacturer's instructions (BD Bioscience). Samples or standards were added to 75mm tubes containing 50µl of mixed capture beads following a 1-hour

incubation at room temperature. Then, 50µl of phycoerythrin (PE) detection reagent (BD Bioscience) was added to each sample or standard tube and left 1 hour at RT. After the incubation, samples and standards were washed with the kit's buffer and centrifuged 5' at 200g. Finally, the supernatant was discarded and another 300µl of wash buffer were added to each tube. Four-color flow cytometric analysis was performed using a FACSCalibur® flow cytometer [Becton Dickinson S.A]. Data were acquired with the BD Cellquest™ PRO and analyzed using the FCAP Array™ software. FSC vs. SSC gating was employed to exclude any sample particles other than the 7.5-µm polystyrene beads. Data were displayed as two-color dot plots (FL-2 vs. FL-4) such that the eight discrete FL-4 microparticle dye intensities were distributed along the Y-axis. Standard curves were plotted [cytokine calibrator concentration vs. FL-2 mean fluorescence intensity (MFI)] using a four-parameter logistic curve-fitting model (Supplementary Figure 3). Cytokine concentrations were determined from these standard curves. When a sample had a cytokine concentration below the detection limit for the assay, a value of 0 was assigned for that particular cytokine concentration.

### **Protein determination by Western blotting**

Protein concentration was determined in tissue homogenates with the Bradford protein assay. Equal amounts of total protein (24.5 µg) were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Immunodetection was performed by standard procedures. The membranes were blocked with 5% nonfat milk in TBS-T (0.05% Tween 20 in TBS) and probed with mouse rabbit anti-NOS2 (1:100; Santa Cruz Biotechnology) and goat anti-COX-2 (1:200; Santa Cruz Biotechnology). Mouse anti-β-actin (1:10000; Sigma) was included to ensure equal protein loading. Membranes were incubated with the corresponding secondary antibodies coupled to horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnology) and subsequent enhanced chemiluminescence detection (PerkinElmer Life and Analytical Sciences). Immunoreactive bands

were visualized using the GeneSnap Image Acquisition Software (SynGene; Version 7.08). Specific signals were quantified using GeneTools Gel Analysis software (Syngene version 4.01).

### **Immunofluorescence and confocal microscopy**

Free-floating coronal brain slices (30  $\mu$ m) were processed as described previously<sup>4</sup>. In brief, brain sections were blocked with 5% goat serum and incubated with rabbit polyclonal anti-CB2R receptor (Ab3561; Abcam), rabbit polyclonal anti-IBA1 receptor (WAKO Pure Chemical Industries Ltd. #019-19741), purified mouse anti-GFAP (BD Pharmingen), monoclonal rat anti-NIMPR14 (ab2557; Abcam) and mouse anti-NeuN (MAB 377; Millipore) over 3 nights at 4°C, followed by the appropriate rabbit secondary antibody Alexa 488 (Invitrogen A-11008), anti-mouse Cy3 (Jackson Immuno Research; 715-165-151), anti-rat Cy3 (Jackson Immuno Research; 712-165-150) Alexa 647 (Invitrogen A-21245) secondary antibody incubation (1h, RT). For double immunofluorescence with anti-Iba-1 and anti-CB2R antibodies, a sequential immunofluorescence protocol was used with the appropriate controls. In brief, free-floating slices were incubated with the primary CB2R antibody over 3 nights followed by Alexa 488 (Invitrogen A-11008) secondary antibody incubation (1 h, RT) and then fixed with formalin 10% for 15min, washed and blocked with 5% normal goat serum for 1h. Then, an overnight incubation with anti-IBA1 antibody followed by Alexa 647 (Invitrogen A-21245) secondary antibody incubation (1 h at room temperature) was performed. To avoid unspecific labeling with mouse antibodies, a KIT-MOM (BMK-2202; Vector laboratories) was used following manufacturer's instructions.

All immunofluorescence images were obtained in a blinded manner from seven correlative slices of each brain. Stacks at 10X of the upper and lower part of the peri-infarcted tissue and one from the contralateral healthy hemisphere were obtained (Figure 7e). With the ImageJ v. 1.44i software (NIH, Bethesda, MD, USA), each image was converted into a binary image and the Integrated Density (IntDen) was calculated. The IntDen is a calculus of the mean stained area times the

intensity of stain in each pixel in the area, and indicates the total amount of staining material in that area.

### **FACS analysis of peripheral blood cells**

Blood was extracted by cardiac puncture 4h after pMCAO and kept in ice and protected from light until its use. Each sample was lysed for 5 minutes at room temperature with 10ml of lysis buffer (4.15g  $\text{NH}_4\text{Cl}$ , 0.5g  $\text{NaHCO}_3$ , 0.18g of disodium EDTA in 200ml  $\text{H}_2\text{O}$ ). Samples were centrifuged for 5 min at 1500 rpm, supernatant was removed and cold PBS was added (10ml). After this, samples were centrifuged again and the supernatant was discarded and 700  $\mu\text{l}$  of cold PBS-BSA 0.1% were added with 2  $\mu\text{l}$  of blocking solution (CD16/CD32 mouse BD Fc Block, BD Pharmingen). Each sample was then incubated for 1 h at  $4^\circ\text{C}$  with 150  $\mu\text{l}$  of the following direct cytometry antibodies: APC-conjugated anti-mGr1/Ly6G (RyD Systems), PERCP-conjugated anti-mCD3 (RyD Systems) and FITC-conjugated anti-CD11b (Miltenyi Biotec). Finally, samples were washed with 1ml of cold PBS, centrifuged (5 minutes at 1500 rpm), the supernatant was discarded and 300  $\mu\text{l}$  of FACS-FLOW (BD Bioscience) were added.

Using a FACSCalibur® (Becton Dickinson S.A.) cytometer and the BD Cellquest™ PRO software, fluorescence intensity was analyzed for each marker. 15,000 events of blood polymorphonuclear and mononuclear cells were acquired and defined by size and cell complexity (SSC/FSC). Lymphocytes were defined as cells  $\text{CD3}^+/\text{CD11b}^-$  located in the mononuclear cell region (SSC<sub>low</sub>). The percentage of monocytes was defined as the number of total cell events  $\text{CD11b}^+/\text{CD3}^-$  SSC<sub>low</sub> and with a variable fluorescence of GR1. Finally, polymorphonuclear cells were defined as  $\text{CD11b}^+/\text{Gr1}^{\text{high}}$  SSC<sub>high</sub>. Positive fluorescence was defined using isotopic controls for each antibody.

**Supplementary Table 1.** Parameters were measured in mice before and after MCAO in vehicle and JWH-133-treated animals. MABP indicates mean arterial blood pressure; Hb: hemoglobin; Ht: hematocrit. Values are mean $\pm$ SD, (n=4).

| Physiological Parameters |                  |                   |                  |
|--------------------------|------------------|-------------------|------------------|
|                          | Pre-pMCAO        | Post-pMCAO        |                  |
|                          |                  | Vehicle           | JWH133           |
| pH                       | 7,34 $\pm$ 0,034 | 7,3 $\pm$ 0,03    | 7,3 $\pm$ 0,07   |
| pCO <sub>2</sub>         | 33,73 $\pm$ 7,03 | 39,4 $\pm$ 6,6    | 31,3 $\pm$ 5,4   |
| pO <sub>2</sub>          | 177,2 $\pm$ 6,27 | 184,7 $\pm$ 10,87 | 176,3 $\pm$ 7,37 |
| Ht                       | 37 $\pm$ 1,73    | 39,3 $\pm$ 2,6    | 41 $\pm$ 2,6     |
| Hb                       | 12,73 $\pm$ 0,71 | 13,37 $\pm$ 1,06  | 13,93 $\pm$ 0,90 |
| MABP                     | 73.1 $\pm$ 8.01  | 75.35 $\pm$ 22.25 | 62.73 $\pm$ 7.72 |

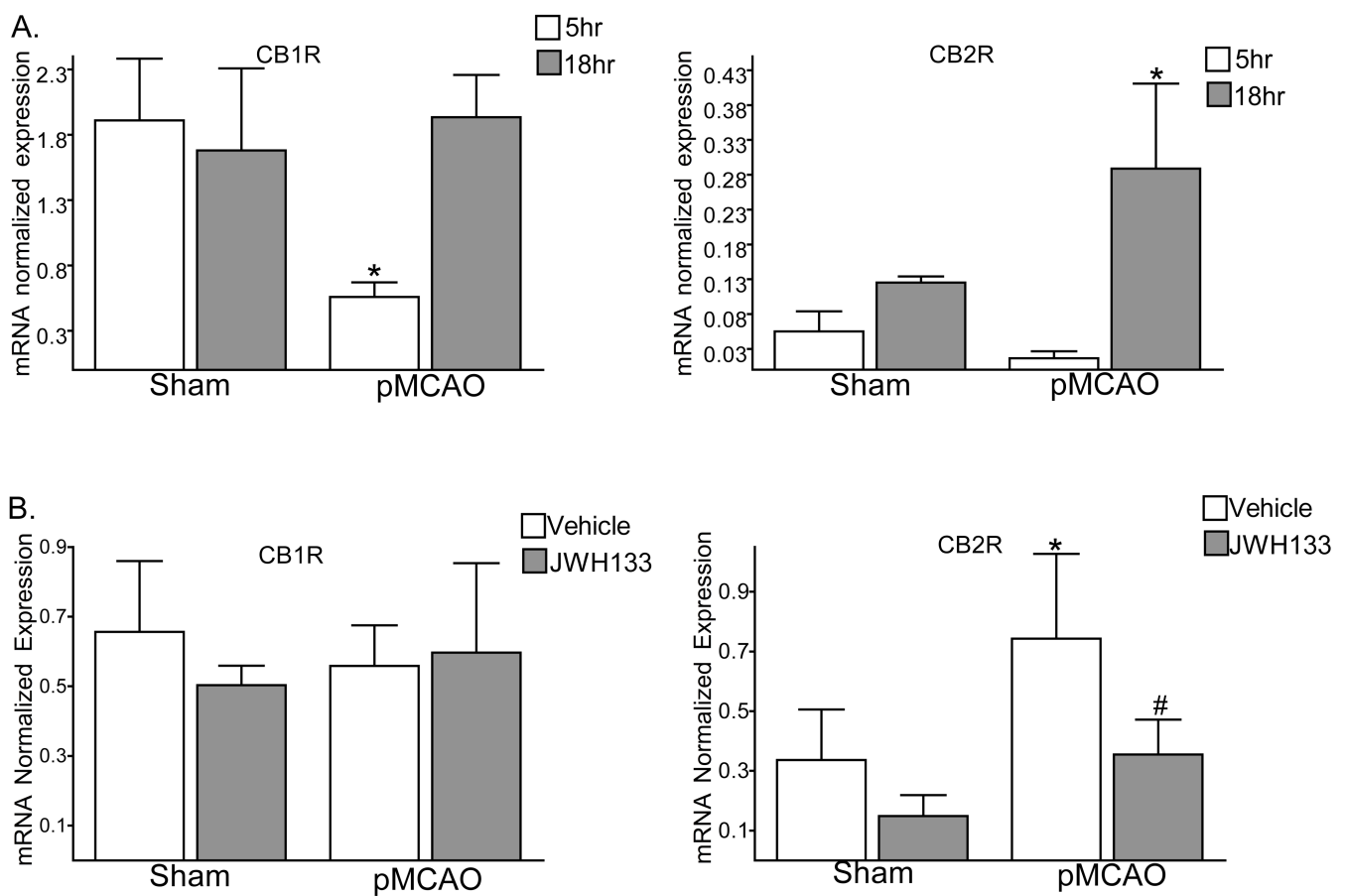
**Supplementary Table 2. Modified Neurological Severity Scores (mNSS).** One point is awarded for inability to perform the tasks or for lack of a tested reflex. 10 to 14 indicate severe injury; 5 to 9, moderate injury; 1 to 4, mild injury. \* An accumulative score is given.

| Modified Neurological Severity Scores (mNSS)                            |              | Score      |
|-------------------------------------------------------------------------|--------------|------------|
| Motor tests                                                             |              | <b>0-6</b> |
| Raising mice by the tail                                                | <b>TOTAL</b> | <b>0-3</b> |
| Flexion of forelimb                                                     |              | 1          |
| Flexion of hind limb                                                    |              | 1          |
| Head moved .10° to vertical axis within 30 s                            |              | 1          |
| Placing mice on the floor (0=normal; maximum=3) *                       | <b>TOTAL</b> | <b>0-3</b> |
| Normal walk                                                             |              | 0          |
| Inability to walk straight                                              |              | 1          |
| Circling toward the paretic side                                        |              | 2          |
| Fall down to the paretic side                                           |              | 3          |
| Beam balance tests *                                                    | <b>TOTAL</b> | <b>0-6</b> |
| Balances with steady posture                                            |              | 0          |
| Grasps side of beam                                                     |              | 1          |
| Hugs the beam and one limb falls down from the beam                     |              | 2          |
| Hugs the beam and two limbs fall down, or spins (>30 for mice)          |              | 3          |
| Attempts to balance on the beam but falls off (>20 for mice)            |              | 4          |
| Attempts to balance on the beam but falls off (>10 for mice)            |              | 5          |
| Falls off: No attempt to balance or hang on to the beam (<10 for mice)  |              | 6          |
| Reflexes absent <b>TOTAL</b>                                            |              | <b>0-2</b> |
| Pinna reflex (head shake when touching the auditory meatus)             |              | 1          |
| Corneal reflex (eye blink when lightly touching the cornea with cotton) |              | 1          |
|                                                                         | <b>TOTAL</b> | <b>14</b>  |

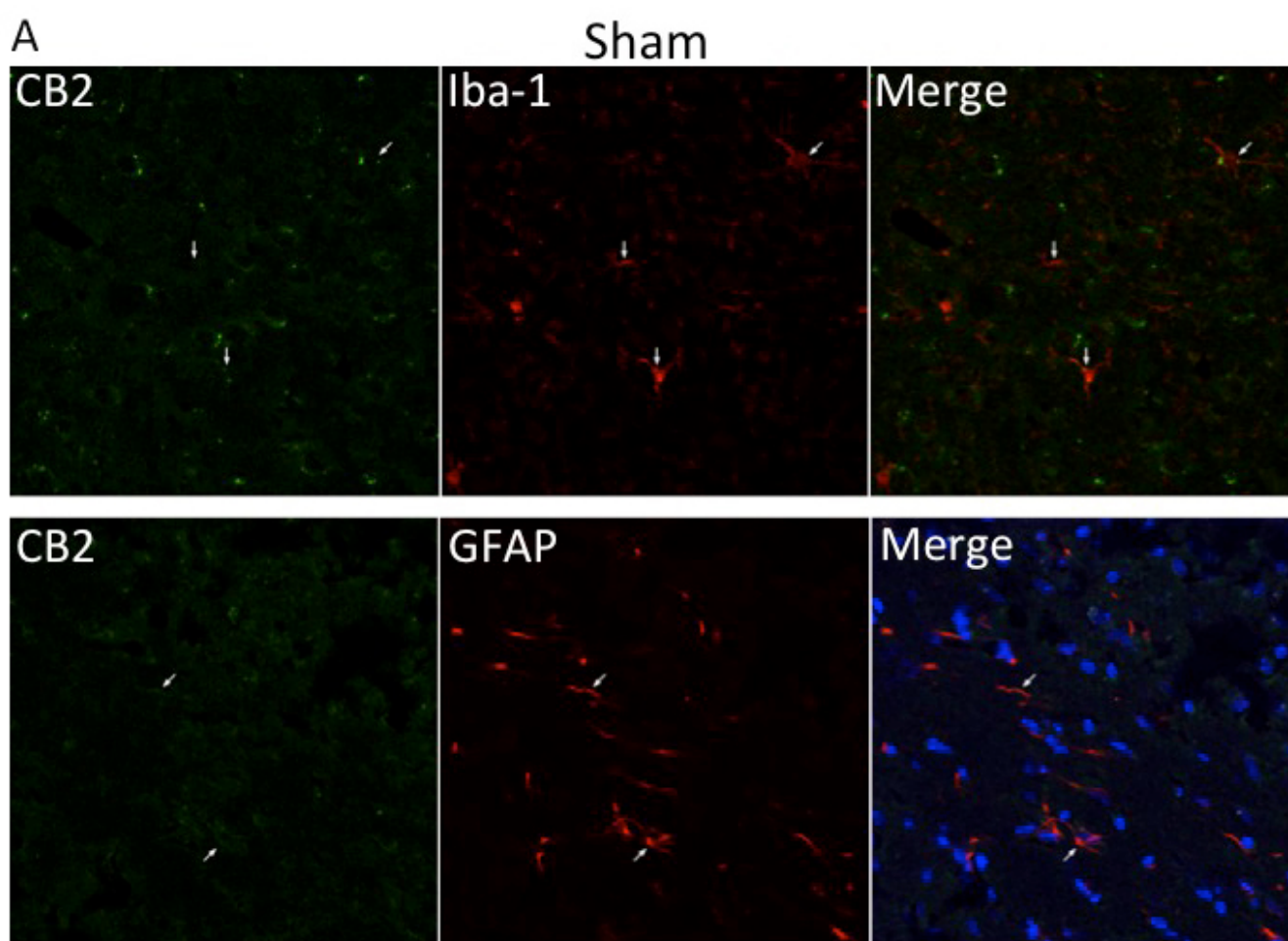
**Supplementary Table 3. Genes Primer sequences**

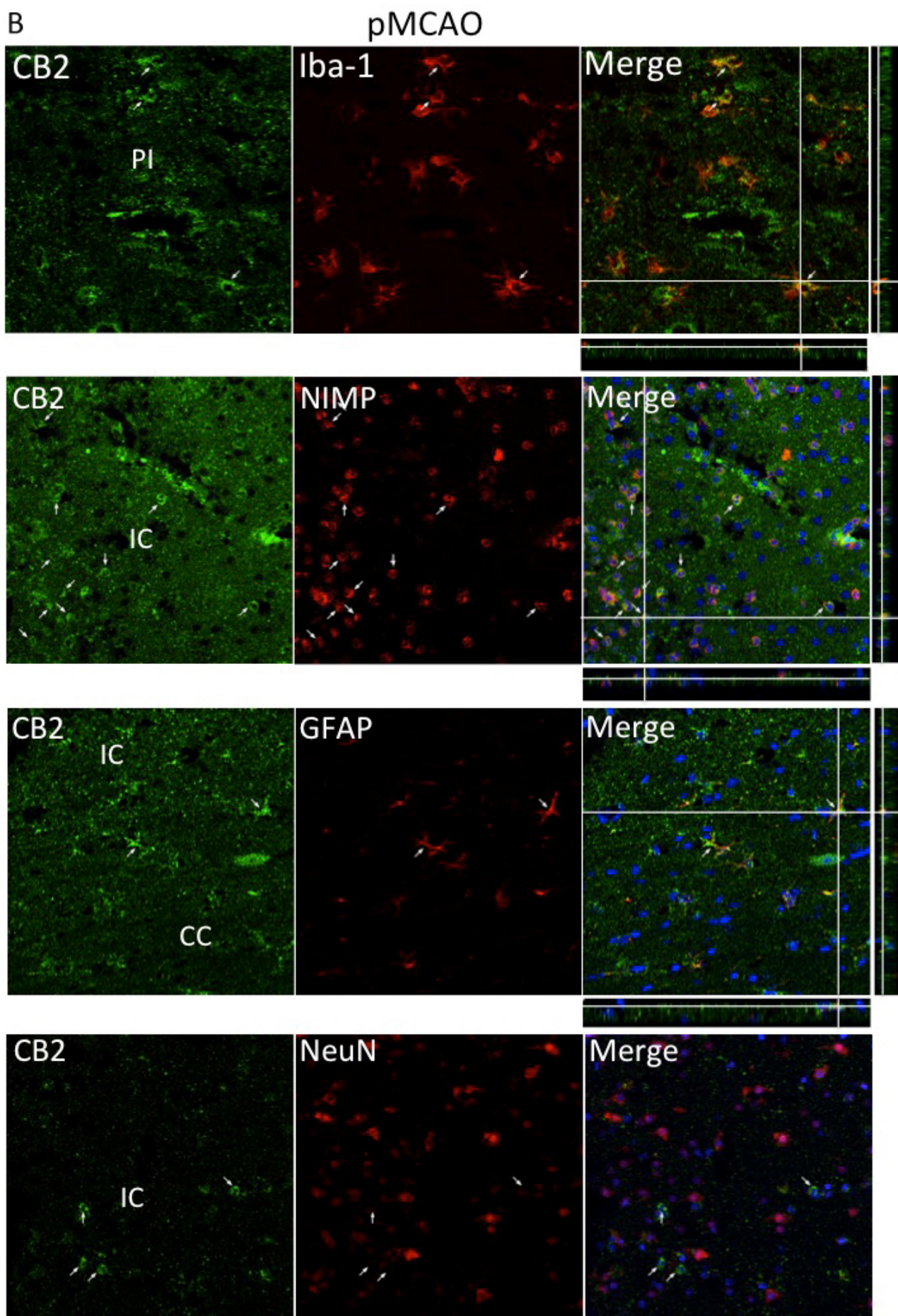
| Gene           | Forward Sequence          | Reverse Sequence          |
|----------------|---------------------------|---------------------------|
| TNF- $\alpha$  | TTCCGAATTCACCTGGAGCCTCGAA | TGCACCTCAGGGAAGAATCTGGAA  |
| IL-6           | TGGCTAAGGACCAAGACCATCCAA  | AACGCACTAGGTTTGCCGAGTAGA  |
| IL-1 $\beta$   | AAGGGCTGCTTCCAAACCTTTGAC  | ATACTGCCTGCCTGAAGCTCTTGT  |
| MIP-1 $\alpha$ | CGTTCCTCAACCCCCATC        | TGTCAGTTCATGACTTTGTCATCAT |
| MCP-1          | AGGTGTCCCAAAGAAGCTGTA     | ATGTCTGGACCCATTCTCTTCT    |
| RANTES         | GTGCCACGTCAAGGAGTAT       | CCCACTTCTTCTCTGGGTTG      |
| IL-12/IL-23p40 | CTCACATCTGCTGCTCCACAAG    | AATTTGGTGCTTCACACTTCAGG   |
| CB2R           | TGAAGATCGGCAGTGTGACCATGA  | AATGCTGAGAGGACCCACATGACA  |
| iNOS           | CTGCTGGTGGTGACAAGCACATTT  | ATGTCATGAGCAAAGGCGCAGAAC  |
| COX-2          | TTGCTGTACAAGCAGTGGCAAAGG  | TGCAGCCATTTCTTCTCTCCTGT   |
| IL-10          | CCAAGCCTTATCGGAAATGA      | TTTTCACAGGGGAGAAATCG      |
| IL-4           | ACAGGAGAAGGGACGCCAT       | GAAGCCCTACAGACGAGCTCA     |
| TGF- $\beta$   | GGAGCCACAAACCCGCCTC       | GCCAGCAGGTCCGAGGGGAGA     |
| Arginase I     | GGAAGACAGCAGAGGAGGTG      | TATGGTTACCCTCCCGTTGA      |
| Ym1            | ACAATTTAGGAGGTGCCGTG      | CCAGCTGGTACAGCAGACAA      |
| Actin          | TGTGATGGTGGGAATGGGTCAGAA  | TGTGGTGCCAGATCTTCTCCATGT  |

**Supplementary Figure 1. CBRs expression after pMCAO. (A)** Time profile of CB1R and CB2R mRNA expression after permanent middle cerebral artery occlusion (pMCAO) or sham. **(B)** Effect of the CB2R agonist JWH-133 on CB1R and CB2R mRNA expression 15h after pMCAO. Data are expressed as mean $\pm$ S.D., n=5, \*p<0.05 vs sham, #p<0.05 vs pMCAO vehicle. ANOVA and Newman-keuls post-hoc test.

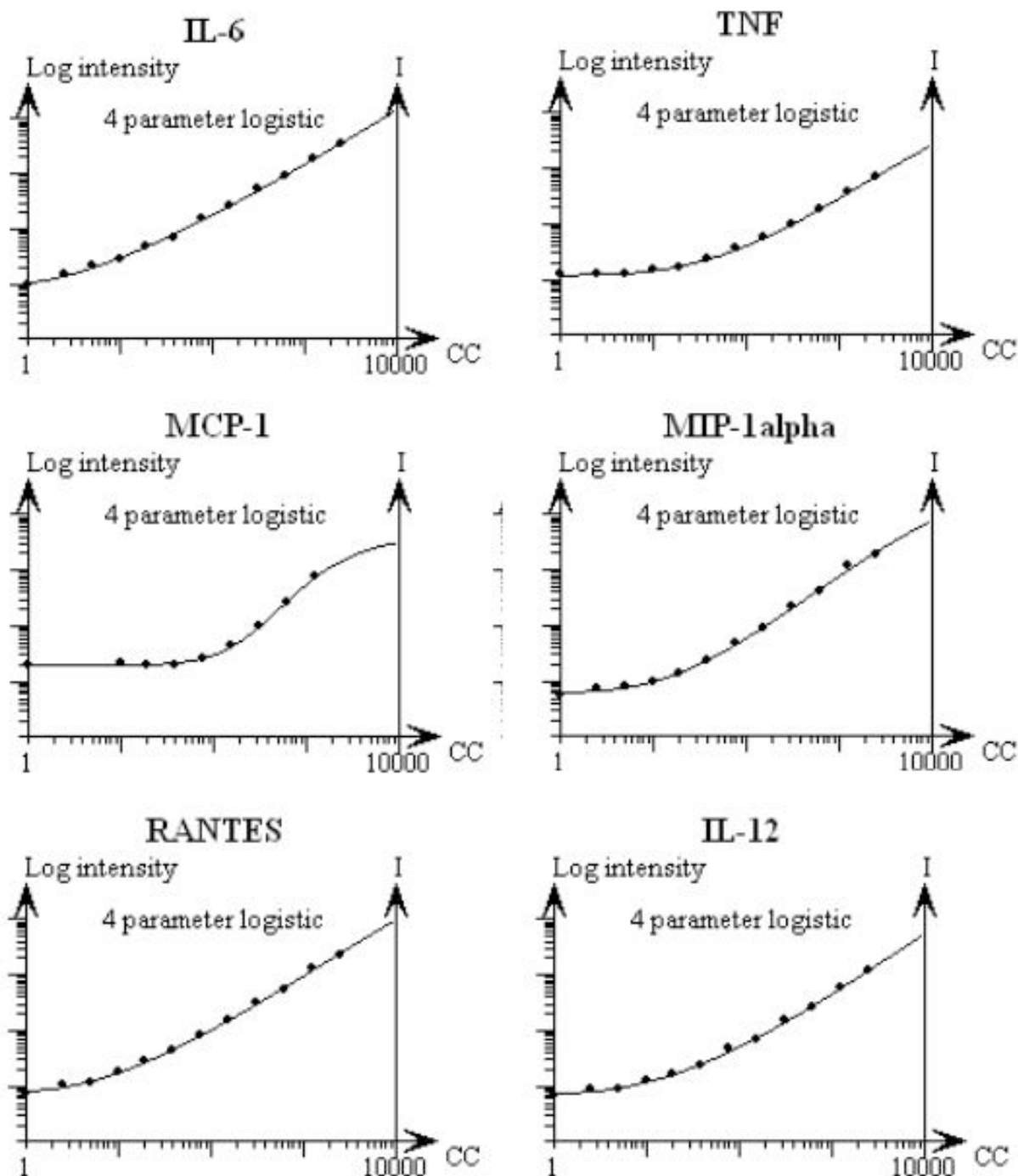


**Supplementary Figure 2. CB2R expression after pMCAO.** Representative confocal microscopy analyses of CB2R (green, Alexa 488) in brain sections of sham (A) and pMCAO (B) animals at 24h were performed with anti-NeuN (neurons; red, Cy3), anti-Iba-1 (microglia/macrophages; red, Alexa 647), anti-GFAP (astrocytes; red, Cy3), and anti-NIMP-R14 (neutrophils; red, Cy3) antibodies. Co-localization was shown by orthogonal images of CB2 receptor expression in Iba-1+, GFAP+ and NIMP-R14 cells. CB2R expression does not co-localize with anti-NeuN neuronal cells. IC: ischemic core; PI: peri-infarct; CC: corpus callosum.

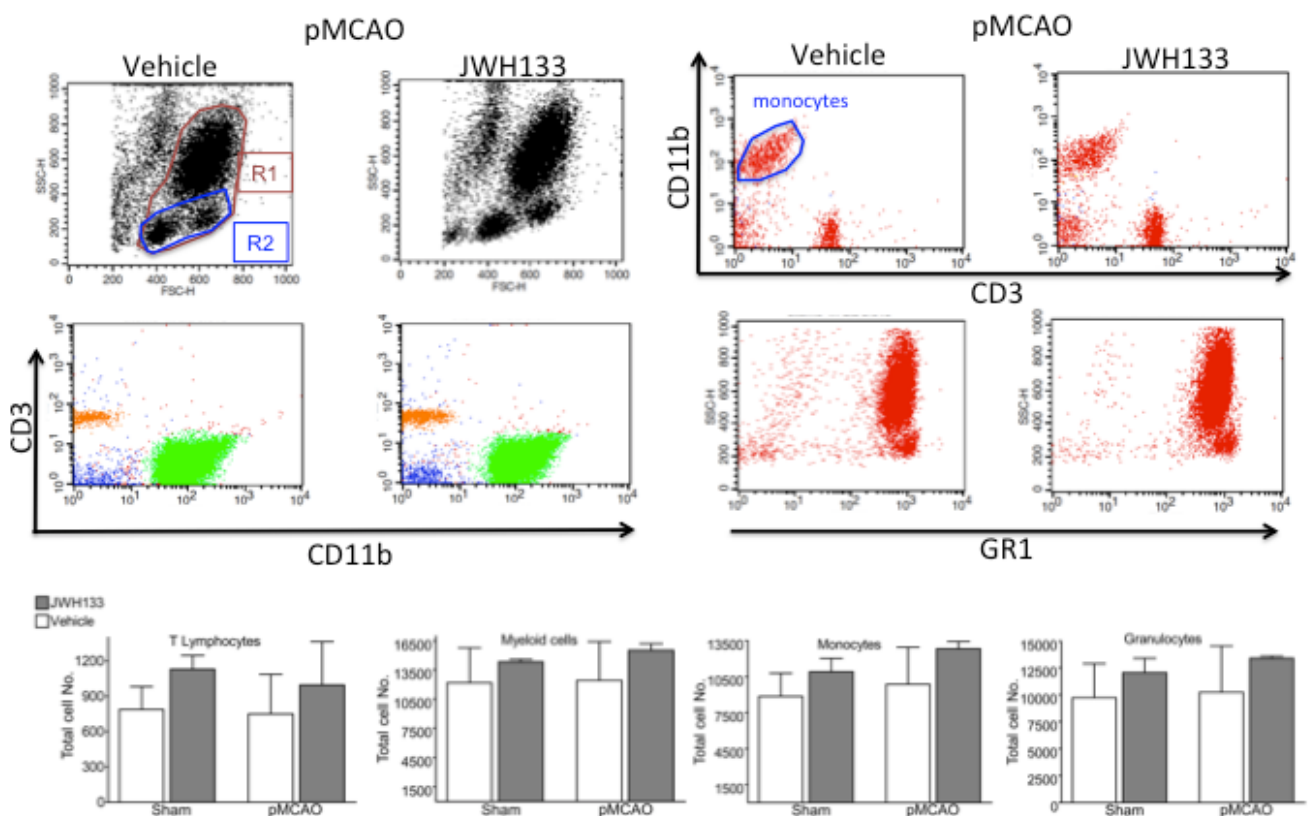




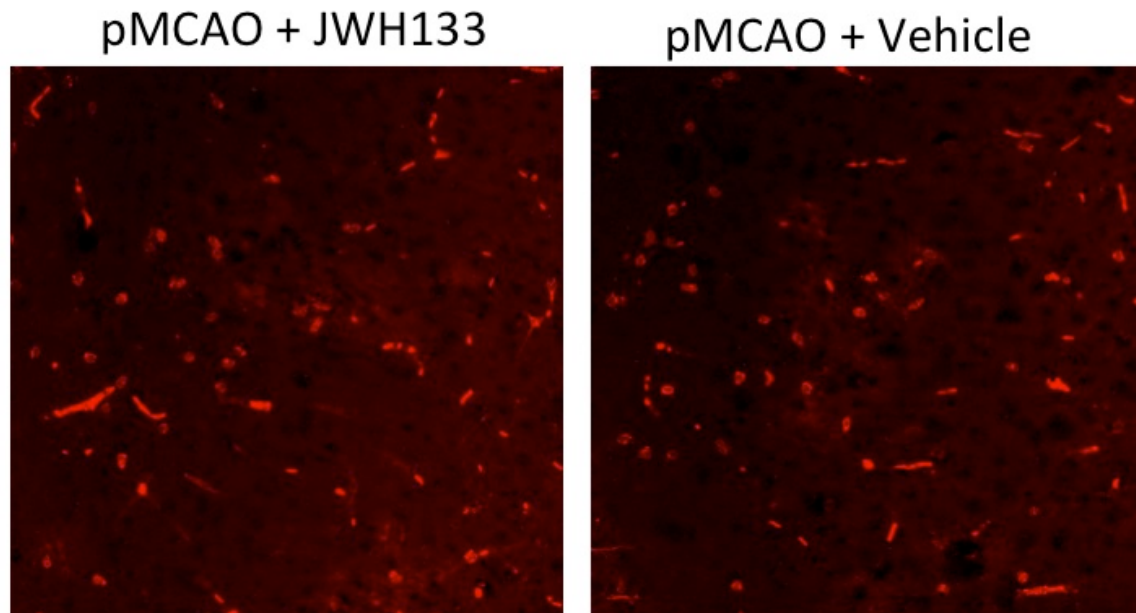
**Supplementary Figure 3.** Standard curves of each cytokine measured by CBA.



**Supplementary Figure 4.** Effect of the CB2R agonist JWH133 on peripheral blood cell populations. (A) Representative gating strategy for absolute cell count analysis of peripheral blood. Lymphocytes were determined as CD3<sup>+</sup>/CD11b<sup>-</sup> cells (yellow); myeloid cells were determined as CD11b<sup>+</sup>/CD3<sup>-</sup> (green); monocytes were determined as CD11b<sup>+</sup>/CD3<sup>-</sup> taken from the mononuclear cell population (R2) and granulocytes (neutrophils) were determined as the GR1<sup>+</sup> SSC-High. (B) Histograms showing the mean  $\pm$  SD of the different blood cell populations in sham and pMCAO mice treated with vehicle or JWH133. ANOVA and Newman-Keuls post-hoc test.



**Supplementary Figure 5.** Representative confocal microscopy analyses of ischemic core sections of MCAO and MCAO+JWH-133 animals at 24h were performed with anti-NIMP-R14 (neutrophils; red, Cy3) antibodies.



## SUPPLEMENTARY REFERENCES

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