N2 Neutrophils, Novel Players in Brain Inflammation After Stroke

Modulation by the PPARγ Agonist Rosiglitazone

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Background and Purpose—Neutrophils have been traditionally recognized as major mediators of a deleterious inflammatory response in acute ischemic stroke, but their potential as a therapeutic target remains unexplored. Recent evidence indicates that neutrophils may acquire different phenotypes and contribute to resolution of inflammation through the release of anti-inflammatory mediators. Thus, similar to M2 macrophages, neutrophils have been proposed to shift toward an N2 phenotype, a polarization that is peroxisome proliferator-activated receptor-γ dependent in macrophages. We hypothesize that peroxisome proliferator-activated receptor-γ activation with rosiglitazone induces changes in neutrophilic mobilization and phenotype that might influence stroke outcome.

Methods—Brain sections and cell suspensions were prepared from mice exposed to permanent distal middle cerebral artery occlusion. Double immunostaining with stereological counting of brain sections and flow-cytometry analysis of brain cell suspensions were performed.

Results—Rosiglitazone accelerated neutrophil infiltration to the ischemic core, concomitantly to neuroprotection. Some neutrophils (≈31%) expressed M2 markers, namely Ym1 and CD206 (mannose receptor). After treatment with the peroxisome proliferator-activated receptor-γ agonist rosiglitazone, most neutrophils (≈77%) acquired an N2 phenotype. Interestingly, rosiglitazone increased neutrophil engulfment by microglia/macrophages, a clearance that preferentially affected the N2 subset.

Conclusions—We present the first evidence of neutrophil reprogramming toward an N2 phenotype in brain inflammation, which can be modulated by activation of the peroxisome proliferator-activated receptor-γ nuclear receptor. We also show that N2 polarization is associated with an increased neutrophil clearance, thus suggesting that this switch is a crucial event for resolution of inflammation that may participate in neuroprotection. **(*Stroke*. 2013;44:3498-3508.)**

Key Words: immunomodulation ■ inflammation ■ phagocytosis

Early after stroke onset, ischemic injury is exacerbated by a robust inflammatory response that involves a local reaction as well as an influx of blood-borne cells recruited by cytokines, adhesion molecules, and chemokines.[1,2] Among these cells, neutrophils are rapidly mobilized from the bone marrow to provide an effective innate immune response; they rapidly infiltrate into the ischemic brain (a few hours after occlusion), reach maximal levels at early time points (days 1–3), and then decrease over time.[1,3] Because of several inflammatory mechanisms (adhesion to endothelium, reactive oxygen species generation, protease secretion, etc), neutrophil infiltration into the ischemic brain has been associated with increased tissue injury[4] but its exact role in stroke pathogenesis remains under debate.

Neutrophils have functional heterogeneity in vivo and a capacity to change their phenotype after in vitro cytokine exposure,[5] a function that makes them plastic cells, capable of responding to extracellular stimuli in a context-dependent manner.[6–9] Such plasticity has been largely studied in macrophages,[10–13] and similar to M2 macrophages, neutrophils have been proposed to polarize to an N2 phenotype.[9]

In this context, peroxisome proliferator-activated receptor-γ (PPARγ), a ligand-activated transcription factor belonging to

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the nuclear receptor superfamily, has been shown to orchestrate the macrophage phenotype switch,[14,15] from the classically activated/proinflammatory M1 to the alternatively activated or M2 phenotype, thus leading to inhibition of inflammation and tissue repair.[12,16] To date, the influence of PPARγ activation on the neutrophil phenotype has not been explored.

Several groups, including ours, have demonstrated the neuroprotective properties of PPARγ agonists in stroke models.[17–22] On activation, this receptor has been reported to decrease both markers of microglia/macrophage activation and neutrophil infiltration in brain 2 to 3 days after experimental stroke.[22–24] The aim of the present study was to explore whether PPARγ agonists are able to induce changes in neutrophil mobilization and phenotypes that might influence stroke outcome after exposure of mice to middle cerebral artery occlusion.

Materials and Methods

Materials

Rosiglitazone (RSG) maleate was from Enzo Life Sciences (Farmingdale, NY) or from Selleck Chemicals (Houston, TX). Other reagents were obtained from Sigma (Madrid, Spain) or as indicated in the text.

Animals

C57BL mice (8–10 weeks) were obtained from The Jackson Laboratory. All experimental protocols adhered to the guidelines of the Animal Welfare Committee of the Universidad Complutense (following European Union directives 86/609/CEE and 2003/65/CE). Animals were housed individually under standard conditions of temperature and humidity and a 12-hour light/dark cycle (lights on at 8:00 AM) with free access to food and water.

Experimental Groups

All groups were performed and quantified in a randomized fashion by investigators blinded to specific treatment. Mice were subjected to a distal permanent middle cerebral artery occlusion (pMCAO) through ligation (Material in the online-only Data Supplement). Animals received an intraperitoneal administration of either saline (vehicle [VEH]-treated group, n=6) or 3 mg/kg of RSG (RSG-treated group, n=6) 10 minutes after pMCAO. Two additional groups consisted of control animals that received an intraperitoneal injection of either saline or RSG. An additional set of animals was treated for neutrophil depletion as described below. Experimental groups for molecular determinations were used as indicated in the text.

Brain Infarct Determination

Infarct volume determination was calculated as described in Material and Methods in the online-only Data Supplement.

Immunofluorescence

Free-floating coronal brain slices (30 μm) were processed 24 or 48 hours after pMCAO (n=4–6 per group), and immunofluorescence, confocal microscopy, and image analyses were performed as described in Materials and Methods in the online-only Data Supplement.

Brain Dissociation and Cell Suspensions Analysis by Flow Cytometry

Twenty-four hours after pMCAO, mice brain were removed, and infarct and peri-infarct tissue were dissociated in cell suspensions that were further processed for subsequent analysis by flow cytometry as described in Materials and Methods in the online-only Data Supplement.

Stereological Analysis

To estimate the number of NIMP-R14$^+$ or double NIMP-R14$^+$/Ym1$^+$ cells in the infarct core, 7 coronal sections between −1.94 and −2.46 mm posterior to bregma (30 μm per section, 600 μm apart) were quantified with the optical fractionator approach, an unbiased cell counting method that is not affected by either the volume of reference or the size of the counted elements[25] (Materials and Methods in the online-only Data Supplement).

Quantification of Brain Neutrophil Clearance by Phagocytosis

To analyze phagocytosis, simultaneous visualization of Iba1, NIMP-R14, and Ym1 staining[26] was analyzed using a laser-scanning confocal imaging system (Zeiss LSM710) as described in Materials and Methods in the online-only Data Supplement.

Neutrophil Depletion

To deplete neutrophils, 2 groups of mice (n=8 per group) were injected with either rabbit anti–polymorphonuclear leukocyte antibody (Accurate Chemical & Scientific, Westbury, NY; AIAG31140; 20 mg/kg IV) or control rabbit IgG isotype for 5 days (n=8 each). On the third day of injection, mice were subjected to pMCAO. Two days after pMCAO, the number of peripheral blood neutrophils (May-Grunwald Giemsa)[27] and neutrophils infiltrated into the brain parenchyma (NIMP-R14$^+$ cells) were counted and the infarct volume determined in Nissl-stained sections.

Blood and Bone Marrow Cells Characterization by Flow Cytometry

Twenty-four hours after pMCAO, a single-cell suspension from peripheral blood or bone marrow was prepared for flow cytometry and analyzed as described in Material and Methods in the online-only Data Supplement. Granulocytes were identified by forward and side scatter analysis and confirmed by their expression of CD11b and Gr-1.

Statistical Analysis

Results are expressed as mean±SEM for the indicated number of experiments. Prism4 (GraphPad Software, Inc, La Jolla, CA) was used for statistical analysis. Unpaired Student *t* test was used to compare 2 groups. One- or 2-way ANOVA was used to compare >2 groups or parameters with the Tukey and Bonferroni post hoc tests, respectively. Values of $P<0.05$ was considered statistically significant.

Results

PPARγ Agonist RSG Reduces Infarct Volume Concomitantly With an Accelerated Increase in Brain Neutrophil Number in the Ischemic Core

The PPARγ agonist RSG (3 mg/kg) decreased infarct volume 24 hours after pMCAO in mice (Figure 1A; $P<0.05$; n=6) as previously demonstrated by histological staining 48 hours after the occlusion.[21] In contrast, we found that RSG-induced neuroprotection was concomitant with an accelerated increase in the number of neutrophils in the infarcted area (core), as shown by a higher number of NIMP-R14$^+$ cells at 24 hours ($P<0.05$; Figure 1B and 1C), but not at 48 hours ($P>0.05$; Figure 1B and 1C), after pMCAO. The increase in parenchymal neutrophils at 24 hours was further confirmed as an increase in CD11b$^+$, Gr-1^{hi} cells in the RSG-treated animals after brain dissociation and flow cytometry characterization (Figure I in the online-only Data Supplement). In addition, double immunofluorescence studies of the neutrophilic marker Ly-6G with the pan-laminin antibody 24 hours after

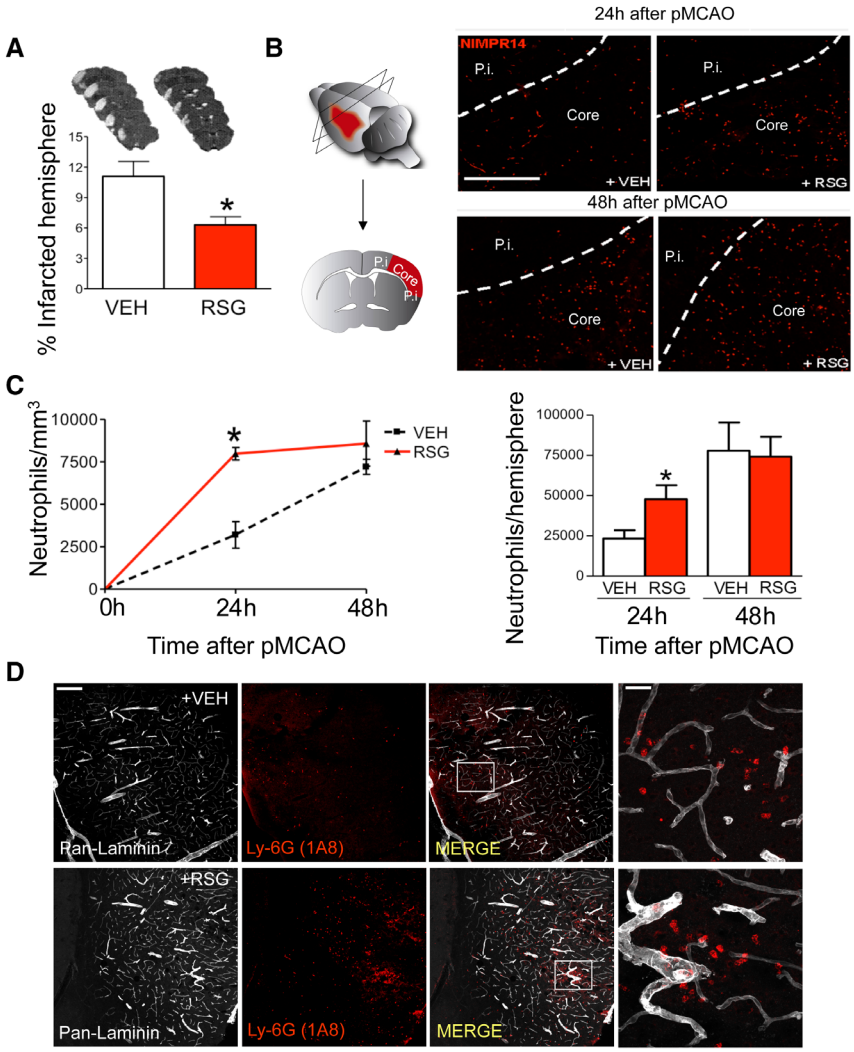

Figure 1. The peroxisome proliferator-activated receptor-γ agonist rosiglitazone (RSG) reduces infarct volume concomitantly to an accelerated infiltration on neutrophils into the ischemic core. **A**, Infarct volume assessed by MRI 24 hours after permanent middle cerebral artery occlusion (pMCAO) in vehicle (VEH)- and RSG-treated mice (n=6; **P*<0.05 vs VEH). **B**, Representative images of neutrophilic (NIMP-R14$^+$ cells infiltration into the ischemic core 24 and 48 hours after pMCAO in VEH- and RSG-treated mice (P.i. indicates peri-infarct; bar=300 μm). **C**, Quantification of neutrophil infiltration, performed by sampling NIMP-R14$^+$ cells in 7 coronal sections (optical fractionator technique) and expressed normalized by infarct volume (Cavalieri, cubic millimeter; **left**) or as the total number of NIMP-R14$^+$ cells (**right**; n=4–6; **P*<0.05 vs VEH. **D**, Double immunofluorescence staining of neutrophils (Ly-6G$^+$, red) and the endothelial marker laminin (white) in the infarct core of VEH- or RSG-treated mice 24 hours after MCAO.

ischemia showed that most neutrophils were found infiltrated into the brain parenchyma and not within the lumen of blood vessels as recently reported[28] (Figure 1D). This infiltration was increased by RSG at the time studied (Figure 1D).

Neuroprotective Role of the PPARγ Agonist RSG Is Abolished After Depletion of Neutrophils

To assess whether RSG-induced increase in neutrophils in the ischemic core was involved in subsequent stroke outcome, we determined infarct volume after the in vivo depletion of the peripheral neutrophilic population with an anti–polymorphonuclear leukocyte antibody antibody (Figure 2A). Consistent with previous data,[29] such treatment caused a reduction in circulating neutrophils (>70%; $P<0.05$; n=8; Figure 2B), as well as a decrease in the number of neutrophils in the ischemic core ($P<0.05$; n=8; Figure 2C). In accordance with the deleterious role of neutrophils in cerebral ischemia,[1,4] infarct volume in mice treated with anti–polymorphonuclear leukocyte antibody was significantly smaller than that found in the IgG isotype-treated group ($P<0.05$; n=8; Figure 2D). In contrast, the administration of the PPARγ agonist RSG to neutrophil-depleted animals did not further reduce infarct volume ($P>0.05$; n=8; Figure 2D).

Phenotypic Characterization of Neutrophils Present in the Ischemic Core

The finding of a neuroprotective effect associated with an increased neutrophilic infiltration rate in RSG-treated animals, together with the lack of effect of RSG on infarct volume after neutrophil depletion, suggested the existence of different neutrophil subsets in the ischemic brain. In this regard, macrophages are now known to exhibit context-dependent effector functions, with alternative M2 macrophages associated with anti-inflammatory and tissue repair functions.[12,16] Because similar functional plasticity has been recently reported in neutrophils (reviewed in Mantovani[30]), we examined whether neutrophils expressing M2 markers (such as Ym1, CD206, or arginase I) were present in brain after pMCAO. Double immunofluorescence studies showed the presence of the chitinase Ym1 and of the endocytic and phagocytic receptor CD206 (mannose receptor) located mainly in neutrophils (NIMP-R14$^+$ or Ly-6G$^+$ cells; Figure 3A and 3B) but not in other cell types, such as microglia/macrophages, neurons, and astrocytes of the ischemic core (Figure IIA and IIB in the online-only Data Supplement). The neutrophilic nature of Ym1$^+$ and CD206$^+$ cells present in the ischemic tissue was further confirmed

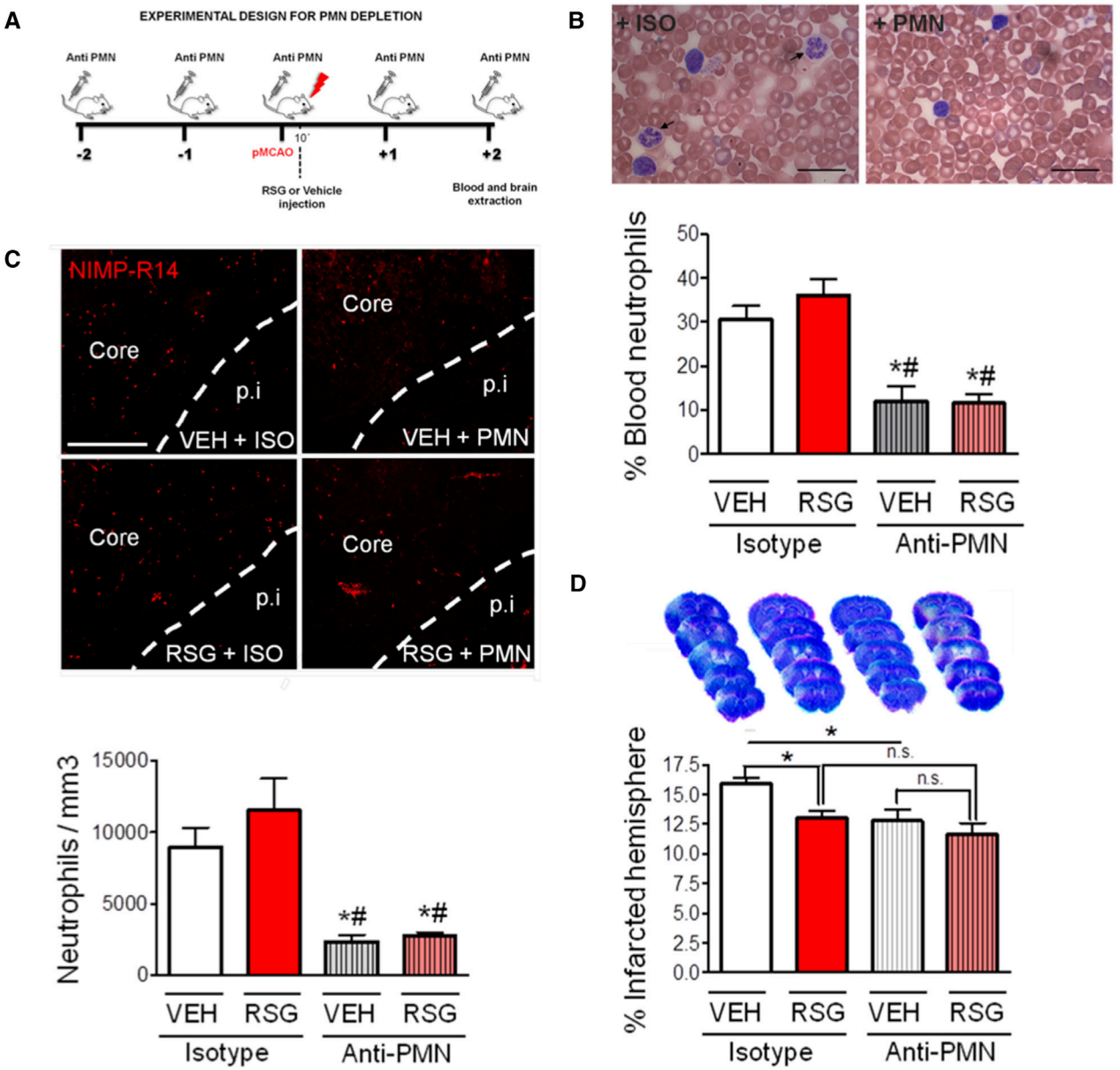

Figure 2. Effect of neutrophil depletion on infarct outcome. **A**, Experimental design of the experiment (see Materials and Methods). **B**, Quantification of blood neutrophils. Percentage of blood neutrophils determined by May-Grunwald Giemsa staining of peripheral blood smears 48 hours after permanent middle cerebral artery occlusion (pMCAO) followed by quantification of neutrophils based on their nuclear hypersegmentation and pale pink cytoplasm (n=8; *P<0.05 vs isotype vehicle [VEH]; #P<0.05 vs isotype rosiglitazone [RSG]). **C**, Representative micrographs of neutrophil infiltration with or without anti–polymorphonuclear leukocyte antibody (anti-PMN treatment; p.i indicates peri-infarct; bar=300 μm). Bottom graph shows the effect of anti-PMN administration on the number of neutrophils in the ischemic core after pMCAO (n=8; *P<0.05 vs isotype VEH; #P<0.05 vs isotype RSG) in VEH- and RSG-treated mice. **D**, Effect of anti-PMN on infarct volume in VEH- and RSG-treated mice (n=8; *P<0.05; n.s. indicates not significant). Data are shown as percentage of infarcted hemisphere after Nissl staining 48 hours after pMCAO.

by flow cytometric analysis of brain-dissociated cells showing that 2 M2 markers are expressed by neutrophils, characterized as CD11b$^+$, Ly-6G$^+$ cells, 48 hours after ischemia (Ym1$^+$ cells=33.8±4.7% of CD11b$^+$/Ly-6G$^+$ cells; CD206$^+$ cells=21.4±3.9% of CD11b$^+$ Ly-6G$^+$ cells; Figure 3B). In addition, we also performed a stereological quantification of the number of neutrophils (as NIMP-R14$^+$ cells) expressing Ym1 in brain sections 48 hours after pMCAO; our data confirm the values obtained by flow cytometry of CD11b$^+$/Ly-6G$^+$ cells, as 27.7±2.4% of NIMP-R14$^+$ cells were Ym1$^+$, showing a good agreement between NIMP-R14 and Ly-6G as neutrophil cell markers in this setting.

As previously shown for Ly-6G$^+$ cells (Figure 1D), neutrophils expressing N2 markers (NIMP-R14$^+$, Ym1$^+$ cells) were also found within the brain parenchyma (Figure 3D). In addition, the presence of N2 neutrophils (CD11b$^+$, CD45^{hi}, Gr-1^{hi}, Ym1$^+$) was also confirmed 24 hours after ischemia by flow cytometry characterization (Figure ID in the online-only Data Supplement).

Interestingly, although CD206 was mostly simultaneously coexpressed with Ym1 in neutrophils of the ischemic core,

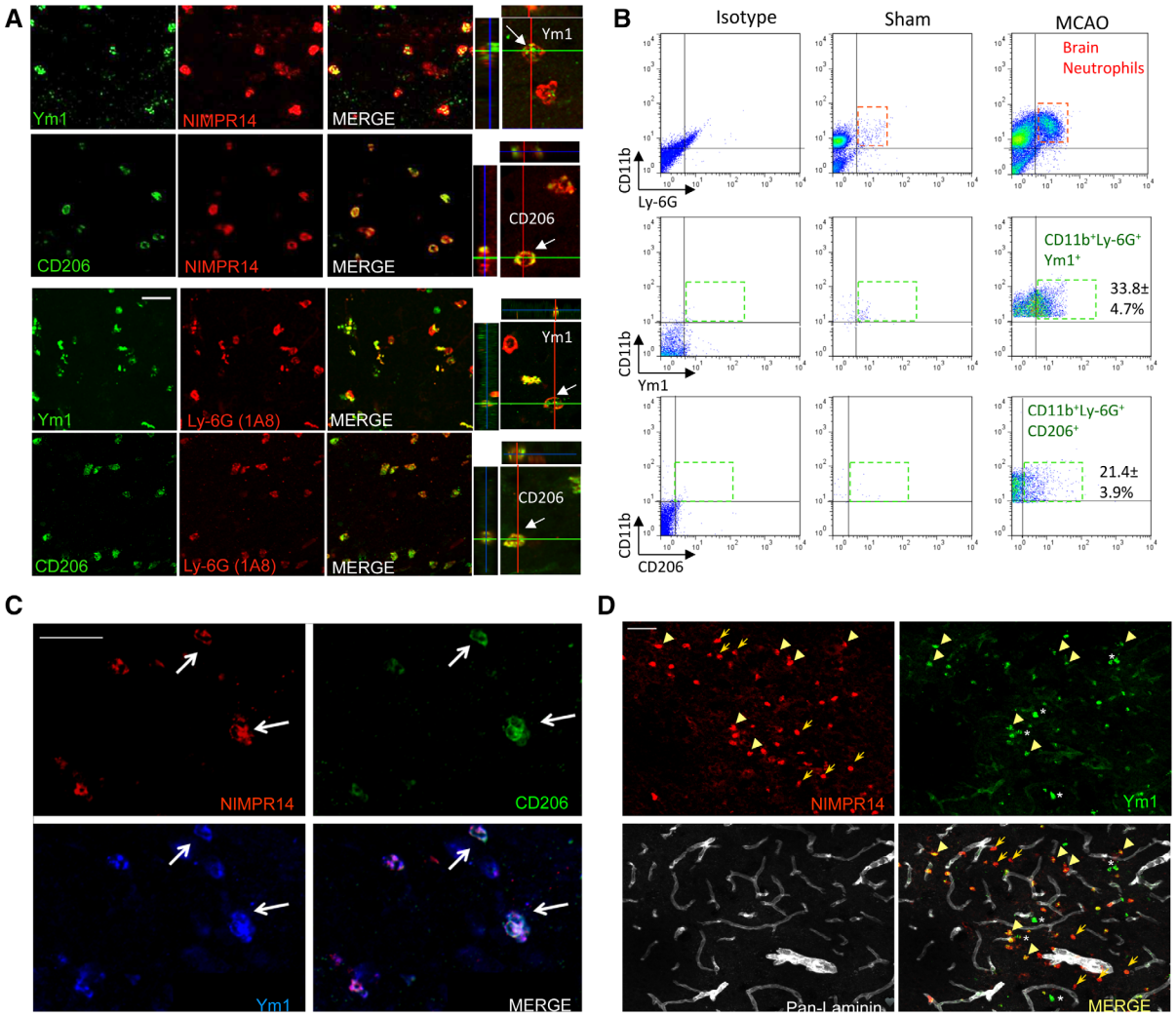

Figure 3. Cellular localization of Ym1 and CD206 in mouse brain neutrophils. **A**, Double immunofluorescence of Ym1 and CD206 (green) in neutrophils (NIMP-R14⁺ or Ly-6G⁺ cells; red) located at the infarct core in brain sections of ischemic animals. **Right**, Orthogonal projection of CD206⁺ and Ym1⁺ (green) colocalization in neutrophils (red) showing a membrane (CD206) or a vesicular staining (Ym1). Arrows indicate colocalization (yellow) of Ym1 and CD206 (green) with neutrophils (red; bar=25 μm). **B**, Flow cytometric analysis of brain neutrophils in sham or ischemic mice brain 48 hours after ischemia. **Upper**, Gated cells were analyzed for the expression of CD11b and Ly-6G to identify neutrophils (CD11b⁺, Ly-6G⁺ cells; red box). Brain neutrophils gate was further analyzed to confirm the expression of Ym1 (**center**; green box) or CD206 (**bottom**; green box). **C**, Triple immunofluorescence showing double colocalization of CD206 (green) and Ym1 (blue) in some neutrophils (red; bar=25 μm). **D**, Triple immunofluorescence of Ym1 (green), NIMP-R14 (red), and laminin (white) showing NIMP-R14⁺ (red; yellow arrows), Ym1⁺, NIMP-R14⁺ (yellow; yellow arrow heads), and Ym1⁺, NIMP-R14⁻ cells (green, asterisks) in the brain parenchyma (bar=50 μm).

some Ym1⁺, CD206⁻ cells, could be found (Figure 3C). In this context, although no detectable expression of Ym1⁺ was found in microglia/macrophages (Iba1⁺, Figure IIA in the online-only Data Supplement), a Ym1⁺/NIMP-R14⁻/Ly-6G⁻ cell subpopulation was detected in the ischemic brain (Figure 3D, white asterisks; Figure IIC in the online-only Data Supplement).

Finally, arginase I was only scarcely detected in neutrophils at the time studied (Figure IID in the online-only Data Supplement).

PPARγ Agonist RSG Increases the Number of M2-Like Neutrophils (N2) in the Ischemic Brain

Ym1, a chitinase-like protein, is considered a bona fide marker of M2 macrophage polarization (alternative activation) in mouse.[12] Because Ym1 immunoreactivity was mainly found in neutrophils, we selected this marker to explore whether PPARγ activation affects the relative proportions of classic and N2 neutrophil populations in the ischemic brain.

To that aim, we quantified stereologically the number of N2 neutrophils as Ym1⁺, NIMP-R14⁺ cells in the ischemic core, 24 and 48 hours after pMCAO, in mice treated with VEH or RSG (Figure 4A–4D). At 24 hours, 31% of infiltrated neutrophils in both groups were Ym1⁺ (N2; Figure 4B–4D). At 48 hours, 72% of NIMP-R14⁺ neutrophils in brain sections were Ym1⁻ in the VEH-treated group, in contrast to the RSG-treated group in which 77% of neutrophils were N2 (Ym1⁺; Figure 4B–4D). Furthermore, the absolute number of N2 (Ym1⁺) neutrophils, determined as either number of cells per cubic millimeter (Figure 4C; $P<0.05$; n=4–6) or total number (Figure 4D; $P<0.05$; n=4–6), was remarkably higher in RSG-treated animals at both times studied. These values were

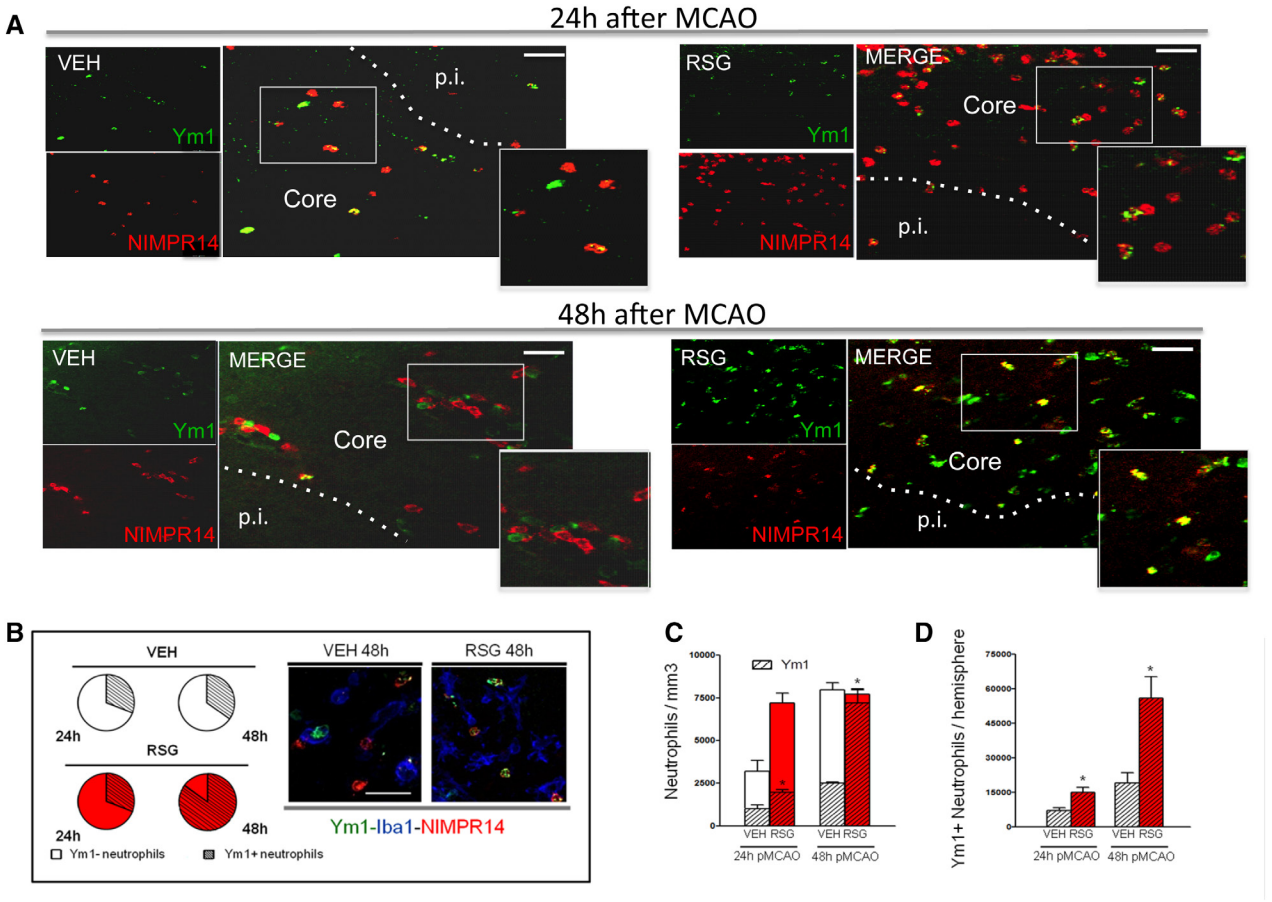

Figure 4. Effect of rosiglitazone (RSG) treatment on neutrophil shift toward an N2 phenotype. **A**, Representative photomicrographs showing Ym1 (green) and neutrophils (red) double immunostaining 24 (**upper**) and 48 hours (**lower**) after permanent middle cerebral artery occlusion (pMCAO) in vehicle (VEH)- or RSG-treated mice (bar=50 μm). **B**, Pie charts showing percentage of neutrophils that colocalize with the M2 marker Ym1 (striped sectors) 24 and 48 hours after pMCAO in VEH- or RSG-treated animals. **Left**, Representative images of Ym1 (green) immunoreactive neutrophils (red) in combination with Iba1 staining (blue) in the ischemic core 48 hours after pMCAO of VEH- vs RSG-treated animals (bar=25 μm). **C** and **D**, Ym1$^+$ neutrophils (striped bars) per cubic millimeter (**C**) or per hemisphere (**D**) 24 and 48 hours after ischemia in VEH- (white bar) or RSG-treated mice (red bar). Ym1$^+$ and NIMP-R14$^+$ cells were sampled in 7 coronal sections by the fractionator technique and normalized by infarct volume (n=4–6; *P<0.05 vs pMCAO+VEH). p.i. indicates peri-infarct.

confirmed at 24 hours after pMCAO by flow cytometry quantification of CD11b$^+$, Gr-1^{hi}, Ym1$^+$ (Figure ID in the online-only Data Supplement). These results indicate that RSG increases the number of N2 neutrophils (N2) in the ischemic brain.

Effect of the PPARγ Agonist RSG on Blood and Bone Marrow N1 and N2 Neutrophil Subsets After pMCAO

Twenty-four hours after the occlusion, the total number of neutrophils in peripheral blood, determined by flow cytometry, was increased when compared with control animals, an effect not modified by the PPARγ agonist RSG (n=4–6; P<0.05; Figure 5A, upper, and 5C, left). Consistently, at this time, pMCAO decreased the total number of neutrophils in bone marrow when compared with control animals, with no difference between VEH- and RSG-treated mice (n=4–6; P<0.05; Figure 5B, upper, and 5C, right), suggesting that the ischemic insult increases overall neutrophil mobilization from the bone marrow in a PPARγ-independent manner.

Regarding each specific subset, the number of N2 neutrophils in VEH and RSG groups was not significantly affected in blood after pMCAO versus nonischemic mice (n=4–6; P>0.05; Figure 5A, lower, and 5B, middle), but they decreased in the bone marrow (n=4–6; P<0.05; Figure 5B, lower, and 5D, middle); of note, after pMCAO, RSG decreased blood N2 number when compared with VEH-treated mice (n=6; P<0.05; Figure 5A, lower, 5B, middle). That the decrease in bone marrow neutrophils is not reflected by an increase in blood is consistent with a preferential infiltration of N2 into the ischemic brain parenchyma, an effect enhanced by PPARγ activation. In agreement with this, Ym1$^+$ neutrophils were often found adjacent to blood vessel walls (Figure II in the online-only Data Supplement). On the contrary, the number of blood Ym1$^-$ neutrophils was increased after pMCAO, regardless of the treatment (n=4–6; P<0.05; Figure 5A, lower, and 5B, right), concomitant with their decrease in the bone marrow (n=4–6; P<0.05; Figure 5C, lower, and 5D, right). Therefore, Ym1$^-$ neutrophils seem to be equally mobilized by the ischemic insult, but in contrast to Ym1$^+$ (N2), their increase in blood might suggest that they infiltrate the injured tissue to a lesser extent than their Ym1$^+$ counterparts.

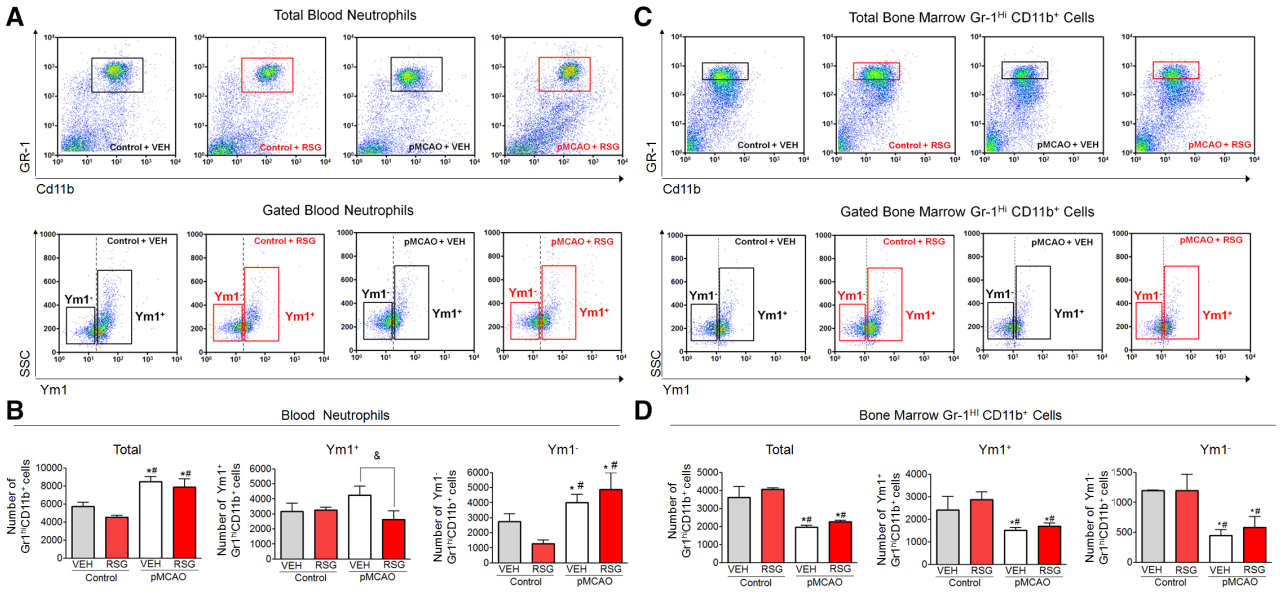

Figure 5. Effect of permanent middle cerebral artery occlusion (pMCAO) with or without rosiglitazone (RSG) treatment in peripheral blood and bone marrow neutrophils. Blood (**A** and **B**) and bone marrow cells (**C** and **D**) were stained for 3-color flow cytometry with monoclonal antibodies against CD11b, Gr-1, and Ym1 24 hours after pMCAO. Dot plots show the characterization of the GR-1^{hi}/CD11b^{+} neutrophil population in blood (**A**; **right upper**) and bone marrow (**C**; **left upper**). The Ym1^{+}/GR-1^{hi}/CD11b^{+} blood (**A**; **bottom**) and bone marrow (**C**; **bottom**) neutrophilic population in control and pMCAO animals after vehicle (VEH) or RSG treatment is also shown. Quantification of total (**left**), Ym1^{+} or N2 (**middle**) and N1 or Ym1^{-} (**right**) neutrophils in blood (**B**) and bone marrow (**D**) in control and pMCAO animals after VEH or RSG treatment (n=4–6; **P*<0.05 vs control+VEH; #*P*<0.05 vs control+RSG; &*P*<0.05 vs pMCAO+VEH).

N2 Neutrophils Are Preferentially Phagocytosed by Microglia/Macrophages in the Ischemic Core

To decrease damage associated to their inflammatory activity and therefore contribute to an early and efficient resolution process, neutrophils should be cleared away as soon as they have performed their function as scavengers of injurious stimuli. To determine the possible role of the N2 neutrophil subset in this process, we measured neutrophil clearance by microglial/macrophage phagocytosis in the ischemic core with simultaneous visualization of Iba1, NIMP-R14, and Ym1. Confocal micrographs show that, in some cases, the cytoplasm of mononuclear brain phagocytes (Iba1^{+}) contained NIMP-R14^{+} cells/particles, with or without Ym1 immunoreactivity (Figure 6A and 6B and Figure IIIA in the online-only Data Supplement). For quantification, we measured phagocytosis as percent of microglia/macrophages (Iba1^{+} cells) engulfing neutrophils (Figure 6A–6C and Figure IIIA in the online-only Data Supplement), and neutrophil phagocytic clearance as percent of total, Ym1^{+}, or Ym1^{-} neutrophils being engulfed by microglia/macrophages (Figure 6A, 6B, and 6D). RSG-treated brains showed an increased phagocytosis of total neutrophils (n=4–6; *P*<0.05; Figure 6C) as well as an increased neutrophil clearance (n=4–6; *P*<0.05; Figure 6D) when compared with sections from VEH-treated animals. Specific evaluation of Ym1 staining shows, in the VEH-treated group, the presence of numerous Ym1^{-} neutrophils that do not undergo phagocytosis, whereas in the RSG-treated group, most neutrophils are Ym1^{+} (N2) and are being engulfed by microglia/macrophages. Quantification shows that the N2 subset of neutrophils is the one that is preferentially cleared by microglia/macrophages at the ischemic core in both groups when compared with the Ym1^{-} neutrophil subpopulation (n=4–6; **P*<0.05; Figure 6D). In agreement with these results, neutrophil morphology in RSG-treated animals showed increased impairment of membrane integrity when compared with VEH-treated animals 48 hours after pMCAO (Figure IIIB in the online-only Data Supplement).

Discussion

We show here that PPARγ activation with the agonist RSG induces a polarization of neutrophils toward an M2-like or N2 phenotype, which is associated with neuroprotection and resolution of inflammation after experimental stroke, induced by pMCAO. Lymphocytes and macrophages have been shown to possess the capacity to switch phenotypes, and recent data show that neutrophils share this ability (reviewed in Mantovani et al[5]). We presented above the first evidence of neutrophil reprogramming in brain inflammation, with modulation by activation of the PPARγ nuclear receptor concomitantly with an improvement in stroke outcome.

To date, several groups have shown neuroprotective properties of PPARγ agonists in stroke models, attributable mainly to anti-inflammatory and antioxidant mechanisms.[17–22] In the present study, we decided to investigate the effects of PPARγ activation on the peripheral inflammatory response, specifically that orchestrated by infiltration of blood-borne neutrophils, a process that has been demonstrated to mediate tissue injury in stroke as in other diseases.[31,32] Although recent studies have reported the predominant association of infiltrated neutrophils at the lumen of the blood vessels after transient MCAO,[28] in our ischemia model most neutrophils were predominantly found infiltrated into the brain parenchyma. Intriguingly, we found that the PPARγ agonist RSG increased the number of infiltrated neutrophils at the earliest times, but

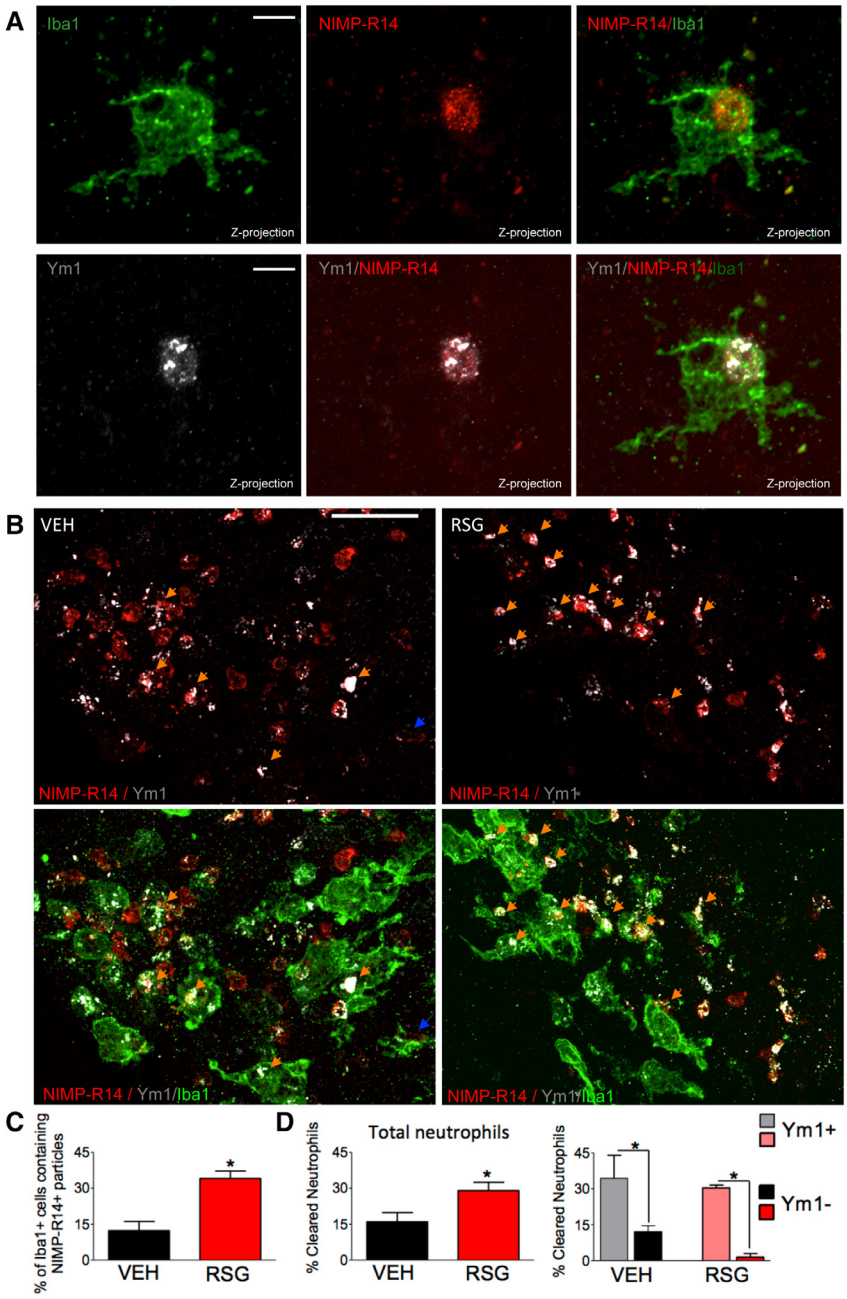

Figure 6. Preferential phagocytosis of N2 (Ym1⁺) neutrophils by macrophages/microglia. **A**, Representative phagocytosis micrograph showing a neutrophil (NIMP-R14⁺, red) of the ischemic core is engulfed by microglia/macrophage (Iba1⁺, green; **upper**). **Bottom**, The engulfed neutrophil is Ym1⁺ (gray; bar=10 μm). **B**, Double (**upper**) or triple immunofluorescence (**bottom**) of NIMP-R14 (red), Ym1 (gray), and Iba1 (green) 48 hours after permanent middle cerebral artery occlusion in vehicle (VEH)- vs rosiglitazone (RSG)-treated animals. Orange arrows show microglia/macrophages engulfing Ym1⁺ neutrophils, whereas blue arrows indicate microglia/macrophages engulfing Ym1⁻ neutrophils (bar=50 μm). **C**, Percentage of phagocytic microglia/macrophages (n=4–6; **P*<0.05 vs VEH). **D**, Quantification of clearance of total (**left** graph) or specific neutrophil population (Ym1⁺/Ym1⁻, **right** graph) determined as described in Material and Methods (n=4–6; **P*<0.05).

not at the latest time studied, namely 2 days after the ischemic injury. To our knowledge, this is the first stereological study, confirmed by flow cytometric analysis after brain dissociation, that evaluates the effect of PPARγ agonists on infiltration of neutrophils (assessed either as NIMP-R14⁺ or Ly-6G⁺ cells by immunofluorescence, or as CD11b⁺, CD45^{hi}, Gr-1^{hi} cells by flow cytometry) in brain ischemia. Other authors have described a decrease in neutrophil infiltration following PPARγ activation based on a reduced myeloperoxidase expression and activity[22–24] at 2 or 3 days after the occlusion. However, myeloperoxidase expression has been detected not only in neutrophils, but also in monocytes/macrophages,[33] microglia,[34] and even in neurons.[35] The different methods for identifying neutrophils, together with the different methodological approaches used, namely animal models (permanent versus transient MCAO) and time windows, may explain these differences.

Neutrophil infiltration into the ischemic brain has been recognized as an important pathogenic factor,[32,36] and indeed, our findings show that neutrophil depletion causes a significant infarct volume reduction in VEH-treated animals, in agreement with previous data.[29] However, treatment with the PPARγ agonist failed to induce neuroprotection after neutrophil depletion suggesting, first, that neutrophils do not play a deleterious role in the presence of RSG and, second, that the neuroprotective effect of RSG requires neutrophils.

The fact that PPARγ-induced neuroprotection is unexpectedly associated with an increased rate of neutrophil infiltration, together with the lack of effect of the PPARγ agonist on infarct volume after neutrophil depletion, led us to hypothesize

the existence of neutrophil subsets with different pathophysiological roles; in this context, PPARγ activation could be acting by favoring a reprogramming toward a phenotype with beneficial properties, such as those ascribed to the M2 macrophage phenotype, associated with inhibition of inflammation and promotion of tissue repair.[12,16] In this context, recent research in cancer shows that tumor-associated neutrophils can acquire a protumor phenotype (N2) characterized by the expression of arginase I, CCL2, and CCL5, a polarization largely driven by the transforming growth factor-β tumor microenvironment.[9] In the scenario of stroke, we observed that neutrophils formed a heterogeneous population, as evidenced by the finding of a subset of neutrophils expressing well-established M2 markers, namely the chitinase Ym1 and CD206 (mannose receptor). Some also expressed arginase I. To our knowledge, this is the first evidence of the presence of N2 neutrophils in the brain. Transforming growth factor-β mRNA expression increased 5 hours after pMCAO (Figure IV in the online-only Data Supplement), suggesting its participation in this setting.

Interestingly, PPARγ participates in the polarization of macrophages toward the M2 (alternative) phenotype,[14,15] which is associated with anti-inflammatory actions and tissue repair. We, therefore, postulated that PPARγ activation could induce a switch toward an N2 phenotype of neutrophils that could explain the neuroprotection found despite an increased number of these cells in the brain parenchyma. Among these molecules, Ym1 is a chitinase considered a bona fide marker of M2 polarization (alternative activation) of macrophages in the mouse, and its expression has also been reported in neutrophils[37] and in brain under inflammatory conditions.[38] Ym1 has been implicated in matrix reorganization, wound healing, downregulation of inflammation, and its brain expression has been associated with neuroprotection.[16,39–41] It could be that, because of its extracellular action on matrix reorganization, Ym1 expression and secretion by neutrophils are playing roles in neuroprotection after brain ischemia. Regarding CD206, little is known about its functions in neutrophils; however, it is also a distinctive antigen of the alternatively activated or M2 state on macrophages.[16] Furthermore, its expression has been associated to perivascular macrophages (Iba1$^-$ cells) in brain.[42] In agreement with this, we have found that CD206 protein is expressed in homogenates from control cortex of sham mice, likely by perivascular macrophages, being upregulated following ischemia (data not shown), an effect in which neutrophils are important contributors as indicated by our present findings. After RSG-induced PPARγ activation, we did not find an increased expression of transforming growth factor-β mRNA (Figure IV in the online-only Data Supplement); further studies at different times would be required to ascertain transforming growth factor-β implication in PPARγ-induced neutrophil polarization.

In addition, a Ym1$^+$/CD11b$^+$/NIMP-R14$^-$/Ly-6G$^-$ cell subpopulation was detected in the ischemic brain; because no detectable expression of Ym1$^+$ was found in Iba1$^+$ microglia/macrophages, these data suggest that this population might correspond to infiltrating monocytes, although more studies are required to ascertain this issue.

Because CD206 is shed by metalloproteases, especially under inflammatory conditions, its interstitial presence could hinder immunofluorescence analysis; therefore, we selected Ym1 as the N2 neutrophil marker for our study. To this aim, we performed a quantitative stereological quantification of the Ym1$^-$ (N1) and Ym1$^+$ (N2) neutrophilic subsets in the ischemic brain and we explored whether PPARγ activation was affecting their phenotypic balance. Counting of neutrophils showed that 24 hours after the occlusion, one third of the neutrophils in the ischemic core were Ym1$^+$, and that this percentage was not significantly affected by RSG, although its total number was remarkably increased, thus suggesting that the PPARγ agonist increases infiltration into the injured tissue independently of the neutrophilic phenotype. Interestingly, at 48 hours, the vast majority of brain neutrophils were positive for this chitinase in RSG-treated animals. The increased number of N2 neutrophils after PPARγ activation in the ischemic brain is likely attributable to an increased infiltration of blood-borne cells, as indicated by the observed reduction in blood N2 neutrophils after pMCAO in RSG-treated mice, despite its similar bone marrow mobilization. Interestingly, Ym1$^-$ neutrophils were similarly mobilized from bone marrow but, in this case, their number increased in blood, independently of the treatment, what could imply a lesser ability to infiltrate the tissue. However, further studies are needed to clarify whether this Ym1$^-$ population is homogeneous or whether, on the contrary, consists of different neutrophilic subsets as well. Other authors have shown that RSG increases the infiltration of M2 macrophages into adipose tissue,[43] suggesting that PPARγ may promote infiltration of certain cell types to inflamed tissue, in agreement with our data. Our findings represent the first demonstration of a PPARγ-induced reprogramming of neutrophils toward an N2 phenotype and their increased recruitment by the ischemic brain tissue concomitant with a neuroprotective effect.

Because disposal of apoptotic neutrophils is an important step in the resolution of inflammation and the restoration of tissue homeostasis (reviewed in Mantovani et al[5] and Soehnlein and Lindbom[44]), it could be argued that N2 reprogramming is designed to increase its own clearance by phagocytosis. In support of this idea, confocal microscopy examination reveals that neutrophil morphology in RSG-treated animals 48 hours after pMCAO shows an increased impairment of membrane integrity when compared with the VEH-treated group. Indeed, we have found that treatment with RSG increases the phagocytic clearance of neutrophils, shown by an increased number of total neutrophils being engulfed by macrophage/microglia. This is consistent with data in the literature showing that RSG increases phagocytosis in the ischemic brain.[45] Interestingly, our data demonstrate that it is the N2 population that is preferentially cleared by microglia/macrophages of the ischemic core, with a percentage almost 3-fold higher than that found for the Ym1$^-$ population. Although we cannot discard that microglial/macrophage phagocytic activity could be also be increased by RSG, it is noteworthy that our results showing that the percentage of Ym1$^+$ neutrophils does not change with the treatment indicates that RSG affects phagocytosis by modu-

lating the neutrophilic phenotype without affecting microglia/macrophage activity, at least at the times studied.

Taking into account that dying neutrophils ultimately disintegrate, release phlogistic cargo (eg, serine proteases and cationic proteins), and contribute to ongoing inflammation and tissue destruction, the effect of RSG on N2 polarization of neutrophils could be an important mechanism to reduce acute inflammation after stroke. Furthermore, phagocytosis promotes secretion of anti-inflammatory mediators, thus likely contributing to create an optimal scenario favorable for the process of brain recovery after stroke.[44]

In summary, our data strongly support the implication of PPARγ activation by RSG on myeloid modulation after stroke and demonstrate for the first time the existence of M2-like, N2, or alternatively activated neutrophils in brain ischemia. The heterogeneity of neutrophil populations and the ability of RSG to promote a selective entry of these cells to the ischemic tissue is a novel process for the modulation of acute inflammation. Moreover, we present the first demonstration of an association between N2 polarization of neutrophils and their increased ability to undergo phagocytosis, thereby increasing the removal of debris from the inflamed tissue, most likely contributing to the restoration of tissue homeostasis and ameliorating stroke outcome.

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Disclosures

None.

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