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.

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. 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. 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 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, a function that makes them plastic cells, capable of responding to extracellular stimuli in a context-dependent manner. Such plasticity has been largely studied in macrophages and similar to M2 macrophages, neutrophils have been proposed to polarize to an N2 phenotype.

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,\textsuperscript{14,15} from the classically activated/proinflammatory M1 to the alternatively activated or M2 phenotype, thus leading to inhibition of inflammation and tissue repair.\textsuperscript{12,16} To date, the influence of PPAR\(\gamma\) activation on the neutrophil phenotype has not been explored.

Several groups, including ours, have demonstrated the neuroprotective properties of PPAR\(\gamma\) agonists in stroke models.\textsuperscript{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.\textsuperscript{22–24} The aim of the present study was to explore whether PPAR\(\gamma\) 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 ligature (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 \(\mu m\)) 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 intact 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 \(\mu m\) per section, 600 \(\mu m\) 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.\textsuperscript{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 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)\textsuperscript{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**

**PPAR\(\gamma\) Agonist RSG Reduces Infarct Volume Concomitantly With an Accelerated Increase in Brain Neutrophil Number in the Ischemic Core**

The PPAR\(\gamma\) 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.\textsuperscript{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 perivascular neutrophils at 24 hours was further confirmed as an increase in CD11b\(^+\), Gr-1\(^+\) 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...
ischemia showed that most neutrophils were found infiltrated into the brain parenchyma and not within the lumen of blood vessels as recently reported28 (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 (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,14 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 Mantovani30), 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; 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.
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 ischemia (core, p<i>i</i>); 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+, CD45hi, Gr-1hi, Ym1+) was also confirmed 24 hours after ischemia by flow cytometry characterization (Figure 1D in the online-only Data Supplement).

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

**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).

**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. 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
confirmed at 24 hours after pMCAO by flow cytometry quantification of CD11b+, Gr-1hi, 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 5B, middle). 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, right), 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, and 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.
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 al5). 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...
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+, CD45hi, Gr-1hi 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 at 2 or 3 days after the occlusion. However, myeloperoxidase expression has been detected not only in neutrophils, but also in monocytes/macrophages, microglia, and even in neurons. 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, and indeed, our findings show that neutrophil depletion causes a significant infarct volume reduction in VEH-treated animals, in agreement with previous data. 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 neutrophils37 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 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 al4 and Soehnlein and Lindbom46), 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.

References

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL MATERIAL AND METHODS

Induction of focal ischaemia
Mice were subjected to a distal pMCAO (permanent Middle Cerebral Artery Occlusion) through ligation of the MCA with a 9–0 suture just before its bifurcation into the frontal and parietal branches. The ipsilateral common carotid artery was also permanently occluded. These experimental conditions led to moderately sized cortical infarcts. Mice were anesthetized with isoflurane 1.5-2% in a mixture of 80% air/20% oxygen, and body temperature was maintained at physiological levels with a heating pad during the surgical procedure and anaesthesia recovery. Following surgery, individual animals were returned to their cages with free access to water and food. Animals were sacrificed by an overdose of sodium pentobarbital. Physiological parameters were not significantly different among the different groups studied. No spontaneous mortality was found after pMCAO with this model, and this was unaffected by the different experimental treatments.

Brain infarct determination
Magnetic resonance examination was performed 24 hours after pMCAO using a BIOSPEC BMT 47/40 (Bruker, Ettlingen, Germany). T2-weighted images were acquired and infarct volume was calculated using the MRI analysis calculator application from ImageJ software (NIH, USA). To calculate infarct volume as the percentage of hemisphere that is infarcted (% of infarcted hemisphere), we estimated the volume of the contralateral hemisphere (CH) and that of the non-lesioned ipsilateral hemisphere (NLH) in 17 coronal sections between -1.78 and -3.64mm posterior to bregma (450µm apart). The % of infarcted hemisphere was then calculated using the formula = (CH-NLH/CH) X 100. Volume was normalized by edema index, which is the ratio between the volume of the contralateral and ipsilateral hemisphere.

In addition, infarct volume was determined by Nissl staining: animals subjected to pMCAO were killed 24-48h after surgery and subjected to perfusion fixation with 4% p-formaldehyde in phosphate buffer (pH 7.4); brains were then frozen, serially sectioned at 30µm and stained with conventional histological Nissl (0.1% cresyl violet). Contralateral and healthy ipsilateral areas were delimited at 10x magnification using Stereoinvestigator (MicroBright Fields, Inc). Volumes were estimated using the Cavalieri probe as previously described. Infarct size was expressed as % of injured ipsilateral hemisphere by the formula (CH-NLH/CH) X 100.

Neutrophil depletion
To deplete neutrophils, animals were injected with either rabbit anti-polymorphonuclear leukocyte antibody (anti-PMN; Accurate Chemical & Scientific, Westbury, NY, USA; AIAG31140; 20mg/kg i.v.) or control rabbit IgG isotype for 5 days. The anti-PMN antibody used has been previously demonstrated to induce neutropenia with minimal changes in another blood cell populations.

Immunofluorescence
Animals (n=4-6 for each group) were sacrificed 24 or 48h after pMCAO by pentobarbital overdose followed by transcardiac perfusion with 0.1M phosphate buffer and 4% p-formaldehyde in 0.1M phosphate buffer (pH 7.4). Brains were removed, post-fixed overnight and placed in 30% sucrose for 48h. Coronal serial sections (30µm) were cut on a freezing microtome (Leica SM2000R, Leica Microsystems GmbH, Wetzlar, Germany) and stored in cryoprotective solution. Double-label immunofluorescence was performed on free-floating sections and incubated overnight at 4°C with the following primary antibodies: rabbit anti-mouse Ym1 (Stem Cell Tech. Inc.), rabbit anti-mouse CD206 (Santa Cruz B.T.), mouse anti-mouse arginase I (BD
Biosciences), mouse anti-mouse NeuN (Chemicon), mouse anti-mouse GFAP (BD Biosciences), rabbit anti-mouse Iba1 (Wako), rat anti-mouse Ly-6G (clone 1A8; BD Biosciences), rat anti-mouse NIMP-R14 (Abcam) and anti-laminin biotin-conjugated (Novus Biological). Secondary antibodies used were goat anti-rabbit biotin or goat anti-mouse biotin (Vector laboratories) in combination with Alexa488 streptavidin (Molecular Probes), donkey Cy3 anti-rat (Jackson Immunoresearch) and donkey Cy3 anti-mouse (Vector Laboratories). Controls performed in parallel without primary antibodies showed very low levels of nonspecific staining.

Image acquisition was performed with a laser-scanning confocal imaging system (Zeiss LSM710) and image analysis was performed with the ZEN 2009 software (Zeiss). All colocalisation images shown were confirmed by orthogonal projection of z-stack files.

**Stereological analysis**

The stereology system consists of a Nikon Eclipse TE300 microscope fitted with a XYZ motorized computer stage and controller (Ludl Electronics Products, Hawthorne, NY, USA) and with the StereoInvestigator system (Microbrightfield version 7.003 software). The quantification of positive cells for the different markers was performed with the oil immersion 100X objective in the outlined area of the infarct, using the optical fractionator method with the following sampling parameters: (1) a counting frame area of 1600 µm², (2) a dissector height of 14 µm², (3) a guard zone of 2 µm². The estimated total positive cell number (N) was calculated using the equation: N= ∑Q- x 1/ssf x 1/asf x 1/tsf, where ∑Q- is the total number of cells counted with the fractionator, ssf is the section sampling fraction, asf is the sampling fraction area, and tsf is the sampling fraction thickness. The reliability of the sampling scheme was confirmed by the calculation of the Schmitz-Hof coefficient of error (CE), which was <0.1.

**Phagocytosis analysis**

Phagocytosis analysis was performed as previously described. To analyse phagocytosis, simultaneous visualisation of Iba1 and NIMP-R14 or Iba1, NIMP-R14 and Ym1 was analysed using a laser-scanning confocal imaging system (Zeiss LSM710). Two photographs of the ischemic core per section using the 20x magnification objective were taken in 7 coronal sections of each animal (n=4). Quantification of double or triple labelled cells in the orthogonal projection of z-stack files was performed using the cell counter tool of the software ImageJ (NIH). Analysis of phagocytosis was performed in both infarcted and peri-infarct areas of the brain 48h after ischemia. For the analysis, microglial morphology was carefully examined in order to exclude false positives resulting from phagocytic neutrophils engulfing necrotic microglial debris, and only undamaged, “healthy” Iba1+ cells were counted.

The following parameters were analysed: 1) % of microglia/macrophages engulfing neutrophils (% of Iba1+ cells containing NIMP-R14+ particles) was calculated as the ratio between the number of Iba1+ cells engulfing NIMP-R14+ particles divided by the total number of Iba1+ cells found in the field; 2) % of cleared neutrophils (% of neutrophils engulfed by microglia/macrophages) was calculated as the ratio between the number of NIMP-R14+ cells engulfed by Iba1+ cells divided by the total number of NIMP-R14+ cells found in the field; 3) % of cleared Ym1+ neutrophils (% of Ym1+ neutrophils engulfed by microglia/macrophages) was calculated as the ratio between the number of Ym1+ NIMP-R14+ cells engulfed by Iba1+ cells divided by the total number of Ym1+ NIMP-R14+ cells found in the field; 4) % of cleared Ym1- neutrophils (% of Ym1- neutrophils engulfed by microglia/macrophages) was calculated as the ratio between the number of Ym1- NIMP-R14+ cells engulfed by Iba1+ cells divided by the total number of Ym1- NIMP-R14+ cells found in the field.
Blood and bone-marrow cells characterisation by flow cytometry

Peripheral blood or bone marrow cells from VEH or RSG treated-animals were incubated for 10 minutes with a standard ammonium chloride lysing solution. Samples were washed twice with phosphate-albumin buffer (PAB; 0.0455% sodium azide, and 0.1% bovine serum albumin) and resuspended in PAB and mouse Fc Block (1:500; BD Pharmingen). 200 μl of cell suspension was incubated with conjugated antibodies CD11b-FITC clone M1/70 and Gr1-APC (BD Pharmingen). After membrane staining, cells were washed and fixed in 2% PFA and permeabilised with PAB and 0.5% of Tween20 prior to staining of intracellular Ym1 (1:100; StemCell Technologies Inc.,) followed by the incubation of immunofluorescent anti-rabbit-PERCP (BD Pharmingen). The stained cells were washed and resuspended in 300 μl of FACS Flow (BD Pharmingen). 15000 events of total gated cells were acquired using a FACS Calibur flow cytometer with CellQuest software (BD Pharmingen, San Jose, CA). Granulocytes were identified by forward and side scatter analysis and confirmed by their expression of CD11b and Gr1.

Brain dissociation and cell suspensions analysis by flow cytometry

Brain cell suspensions were prepared as described11,12. Briefly, 24 and 48 h after pMCAO, mice brain is rapidly removed, and hemispheres are separated and freed from meninges; then, cortex from the ischemic hemisphere is microdissected using a fine scalpel, ensuring its correct isolation from other brain areas, placed into 15 mL of ice-cold PBS and dissociated in a single cell suspension using a gentleMACSTM Dissociator (Miltenyi) according to manufacturer’s instructions. Cell suspension was filtered on 50μm nylon mesh strainers (BD Biosciences) and centrifuged at 300g for 10 minutes. Pellets were resuspended in 3 mL of 50% Percoll and overlaid on the top of a gradient containing 3 mL of 30% of Percoll. The gradient was centrifuged at 500g for 20 minutes at room temperature. Cells were collected from the 30% to 50% interface and resuspended on 200 μl of 2.5% BSA in PBS with Fc Block reagent (Miltenyi). Cell suspensions were incubated with conjugated antibodies CD11b-FITC, CD45-PE, Gr1-APC (Miltenyi), Ly-6G-PercP and CD206-APC (Biolegend). After membrane staining, cells were washed and fixed in 2% PFA and permeabilised with PBS and 0.5% of Tween20 to proceed to the staining of intracellular Ym1 (1:100; StemCell Technologies Inc.) followed by incubation with immunofluorescent anti-rabbit-PERCP or anti-rabbit-APC (BD Pharmingen). Stained cells were washed and resuspended in 300 μl of FACS Flow (BD Pharmingen) and the whole suspension was acquired using a FACS Calibur flow cytometer with CellQuest software (BD Pharmingen, San Jose, CA). Isotype controls (Miltenyi) were used in parallel.

Quantitative RT-PCR

Total RNA was extracted using TRIzol® reagent (Invitrogen, USA) from brain homogenates of sham or ischemic mice treated with vehicle or RSG 5 hours after the surgical process (n=5 per group). RNA (1 μg) was reverse-transcribed using the iScript cDNA Synthesis kit (BioRad, Germany) and quantitative real-time PCR was performed in triplicate using a BioRad IQ5 Thermocycler. The mRNA expression was normalized to that of actin and expressed as the fold difference relative to the controls. Specific primers were designed using Primer Express software and are as followed: Actin (mus musculus): F: 5’-TGAGCGCAAGTACTCTGTGGAT-3’ R: 5’-TAGAAGCATTTCGCGT-GACAGTG-3’; TGF-B (Mus musculus): F: 5’-GGAGCCACAAACCCCGCTC-3; R: 5’-GCCAGCAGGTCCGAGGGAGA.
SUPPLEMENTAL FIGURE LEGENDS

Supplementary Figure I. Flow cytometric analysis after brain dissociation confirms the accelerated infiltration of neutrophils into the ischemic core and the co-labelling of a subset with Ym1. Brain isolated cells were stained for four-color flow cytometry with mAbs against CD11b, Gr-1, CD45 and Ym1 24h after pMCAO. **A**) Representative dot-plot scatter analysis of brain isolated cells after Percoll gradient obtained in control and pMCAO animals after VEH or RSG-treatment. A gate was drawn to exclude dead cells from further analysis. **B**) Gated cells in (A) were analysed for the expression of CD11b and Gr-1 to identify neutrophil population in control (left dot plots) and pMCAO (right dot plots) groups after VEH or RSG-treatment. Lower graph shows the number of brain isolated neutrophils in pMCAO animals after VEH or RSG-treatment normalized by infarct volume (volume represented in mm$^3$) (n=4-6; *P<0.05 vs. VEH). **C**) Representative dot plots show that a subset of CD11b+, Gr-1$^\text{hi}$, CD45$^\text{hi}$ brain neutrophils express Ym1 in both pMCAO groups 24h after ischemia. **D**) Graph shows the number of brain isolated total and Ym1$^+$ neutrophils (striped bars) in pMCAO animals after VEH or RSG-treatment normalized by infarct volume (represented in mm$^3$) (n=4-6; *P<0.05 vs. VEH).

Supplementary Figure II. Heterogeneity of the neutrophil population in the ischemic core. **A,B**) Double immunofluorescence of Ym1 (A) or CD206 (B) and cellular markers (red) of neurons (NeuN), astrocytes (GFAP) or microglia/macrophages (Iba1) in infarct core of brain sections of ischemic mice. Scale bar=25 µm. **C**) CD11b$^+$ cell subpopulation of the ischemic brain was gated and further analysed for the expression of Ly-6G and Ym1 (central plot) or Ly-6G and CD206 (right plot). Plots show that CD11b$^+$Ym1$^+$ cells can be differentiated in two subpopulations based on Ly-6G expression, whereas the CD11b$^+$CD206$^+$ population is mainly composed by Ly-6G$^+$ cells. **D**) Some NIMP-R14 (red) positive cells express arginase I (green) (white arrow). Scale bar=25 µm.

Supplementary Figure III. Phagocytosis of Ym1$^+$ neutrophils by macrophages/microglia. Effect of RSG on neutrophil membrane integrity. **A**) Representative serial confocal images along the z-axis of a Ym1$^+$ (gray) neutrophil (red) engulfed by a macrophage/microglia (Iba1$^+$). Lower panel shows the z-stack image of the N2 neutrophil phagocytotic process. Scale bar=10 µm. **B**) Representative images (left panel) of NIMP-R14$^+$ (red) neutrophils in vehicle and RSG-treated mice showing an increased loss of membrane integrity in RSG-treated animals. Scale bar=25 µm. Representative serial confocal images along the z-axis of NIMP-R14$^+$ cells are shown in the middle panel in VEH or RSG-treated mice; right panel shows the z-stack image.

Supplementary Figure IV. TGF-β mRNA levels in sham and pMCAO animals. Effect of rosiglitazone. TGF-β mRNA expression in vehicle and RSG-treated sham and pMCAO animals, 5 hours after the surgery (n=5/group; *P<0.05 vs. Sham).
Supplementary Figure 1
Supplementary Figure II
Supplementary Figure IV

This image shows a bar graph comparing TGF-Beta mRNA relative ratios between different groups: Veh Sham, RSG Sham, Veh pMCAO, and RSG pMCAO. The graph indicates a statistically significant increase in TGF-Beta mRNA levels in the RSG pMCAO group compared to the other groups.
SUPPLEMENTAL REFERENCES

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