

## Role of TLR4 (Toll-Like Receptor 4) in N1/N2 Neutrophil Programming After Stroke

Alicia García-Culebras, PhD\*; Violeta Durán-Laforet, BPharm\*; Carolina Peña-Martínez, MSc\*; Ana Moraga, PhD; Ivan Ballesteros, PhD; Maria I. Cuartero, PhD; Juan de la Parra, PhD; Sara Palma-Tortosa, PhD; Andres Hidalgo, PhD; Angel L. Corbí, PhD; Maria A. Moro, PhD; Ignacio Lizasoain, MD, PhD

**Background and Purpose**—After stroke, the population of infiltrated neutrophils in the brain is heterogeneous, including a population of alternative neutrophils (N2) that express M2 phenotype markers. We explored the role of TLR4 (toll-like receptor 4) on neutrophil infiltration and polarization in this setting.

**Methods**—Focal cerebral ischemia was induced by occlusion of the middle cerebral artery occlusion in TLR4-KO and WT (wild type) mice. Infarct size was measured by Nissl staining and magnetic resonance imaging. Leukocyte infiltration was quantified 48 hours after middle cerebral artery occlusion by immunofluorescence and flow cytometry. To elucidate mechanisms underlying TLR4-mediated N2 phenotype, a cDNA microarray analysis was performed in neutrophils isolated from blood 48 hours after stroke in WT and TLR4-KO mice.

**Results**—As demonstrated previously, TLR4-deficient mice presented lesser infarct volumes than WT mice. TLR4-deficient mice showed higher density of infiltrated neutrophils 48 hours after stroke compared with WT mice, concomitantly to neuroprotection. Furthermore, cytometric and stereological analyses revealed an increased number of N2 neutrophils (YM1<sup>+</sup> cells) into the ischemic core in TLR4-deficient mice, suggesting a protective effect of this neutrophil subset that was corroborated by depleting peripheral neutrophils or using mice with TLR4 genetically ablated in the myeloid lineage. Finally, cDNA microarray analysis in neutrophils, confirmed by quantitative polymerase chain reaction, showed that TLR4 modulates several pathways associated with ischemia-induced inflammation, migration of neutrophils into the parenchyma, and their functional priming, which might explain the opposite effect on outcome of the different neutrophil subsets.

**Conclusions**—TLR4 deficiency increased the levels of alternative neutrophils (N2)—an effect associated with neuroprotection after stroke—supporting that modulation of neutrophil polarization is a major target of TLR4 and highlighting the crucial role of TLR4 at the peripheral level after stroke.

**Visual Overview**—An online [visual overview](#) is available for this article. (*Stroke*. 2019;50:00-00. DOI: 10.1161/STROKEAHA.119.025085.)

**Key Words:** animals ■ brain ■ inflammation ■ mice ■ neuroprotection

Stroke is the leading cause of death and disability worldwide, its overall incidence remaining high because of the aging population and the high prevalence of smoking and hypertension.<sup>1</sup> Stroke elicits an extensive inflammatory response in brain with both proliferation of resident microglia and recruitment of circulating leukocytes.<sup>2</sup> Among these, neutrophils are rapidly mobilized from the bone marrow (BM) to the injury site to provide an effective innate immune response, where they rapidly infiltrate into the ischemic brain, reaching

maximal levels at early time points and eventually decreasing over time.<sup>3,4</sup>

TLR4 (toll-like receptor 4) plays an important role in inducing inflammation and contributing to tissue damage after stroke<sup>5</sup>; indeed, experimental evidence shows that TLR4 deficiency significantly attenuates ischemic brain damage,<sup>5-7</sup> intracerebral hemorrhage,<sup>8</sup> and hemorrhagic transformation,<sup>9</sup> at least, in part, by decreasing brain inflammatory response.<sup>5</sup> The brain responds to ischemic injury

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From the Unidad de Investigación Neurovascular, Departamento de Farmacología y Toxicología, Facultad de Medicina and Instituto Universitario de Investigación en Neuroquímica, Unidad de Investigación Neurovascular, Universidad Complutense Madrid, Spain (A.G.-C., V.D.-L., C.P.-M., A.M., I.B., M.I.C., J.d.l.P., S.P.-T., M.A.M., I.L.); Area of Cell and Developmental Biology, Fundación Centro Nacional Investigaciones Cardiovasculares (CNIC), Madrid, Spain (A.H.); and Departamento de Biología Celular, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Madrid, Spain (A.L.C.).

\*Dr García-Culebras, V. Durán-Laforet, and C. Peña-Martínez contributed equally as co-first authors.

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Correspondence to María A. Moro, PhD, Unidad de Investigación Neurovascular, Departamento de Farmacología y Toxicología, Facultad de Medicina and Instituto Universitario de Investigación en Neuroquímica, Universidad Complutense Madrid, 28040 Madrid, Spain, Email [neurona@ucm.es](mailto:neurona@ucm.es) or Ignacio Lizasoain, MD, PhD, Unidad de Investigación Neurovascular, Departamento de Farmacología y Toxicología, Facultad de Medicina and Instituto Universitario de Investigación en Neuroquímica, Universidad Complutense Madrid, 28040 Madrid, Spain, Email [ignacio.lizasoain@med.ucm.es](mailto:ignacio.lizasoain@med.ucm.es)

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with an acute and prolonged inflammatory process, characterized by the rapid activation of microglial resident cells and the infiltration of inflammatory cells into the ischemic tissue. Interestingly, TLR4 expression in peripheral immune cells has been linked to ischemic damage in experimental stroke models and humans.<sup>10,11</sup>

The view of neutrophils as a homogeneous population is changing, and nowadays, it is believed that there are neutrophil subpopulations that exhibit different characteristics.<sup>12</sup> One of the first studies to describe neutrophil heterogeneity showed the existence of tumor-associated neutrophils, a subset of protumorigenic neutrophils named by analogy to the M1/M2 macrophage paradigm as N2 neutrophils,<sup>13</sup> as opposed to a proinflammatory neutrophil subset within the tumor that was named N1. A similar phenotypic neutrophil heterogeneity was described by our group in the setting of stroke, where we found that PPAR $\gamma$  (peroxisome proliferator-activated receptor gamma) activation skewed neutrophils toward an M2-like or N2 phenotype, which was associated with neuroprotection and resolution of inflammation after experimental stroke.<sup>14</sup> Supporting this ability of neutrophils to change their phenotype and display functional heterogeneity, one of the most accepted views proposes that there are different populations of neutrophils depending on their cellular age, whereby newly produced neutrophils, recently released from the BM, display a set of surface markers that progressively change over time as they acquire higher proinflammatory activity.<sup>15,16</sup>

Because the full range of mechanisms and factors responsible for neutrophil reprogramming toward an alternative phenotype in brain ischemia has not yet been elucidated, given its potential importance in stroke outcome, the aim of the present study was to explore the role of TLR4 in this setting.

## Methods

Details of materials and experimental procedures are available in the [online-only Data Supplement](#). The data that support the findings of this study are available from the corresponding author on reasonable request.

### Animals

Adult male WT (wild type) mice expressing TLR4 (B6.C57BL/6J), TLR4-KO mice lacking functional TLR4 (B6.B10ScN-Tlr4<sup>lps-del/JthJ</sup>), and mice lacking TLR4 in myeloid cells were used as are described in the [online-only Data Supplement](#). All procedures were performed in accordance with the European Parliament and of the Council Directive 2010/63/EU and Spanish legislation (RD 53/2013) and were approved by the Ethics Committee on Animal Welfare of University Complutense (PROEX number 016/18) and are reported according to ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments). Further details are provided in the [online-only Data Supplement](#).

### Induction of Focal Ischemia and Brain Infarct Determination

All groups were performed and quantified in a randomized fashion (coin toss) by investigators blinded to each specific condition. Mice were subjected to a permanent middle cerebral artery occlusion (pMCAO). Magnetic resonance examination was performed 24 hours after pMCAO. Infarct volume was also determined by Nissl staining 24 to 48 hours after surgery. Further details are provided in the [online-only Data Supplement](#).

### Neutrophil Depletion and BM Transplant for Chimeric Mice

Neutrophil depletion and BM transplants are described in the [online-only Data Supplement](#).

### Blood and BM Collection and Determinations

Details for determination of brain cell suspension, microglia isolation, TLR4 polymerase chain reaction, immunofluorescence, stereological analysis, flow cytometry, cell sorting, RNA isolation, and microarray analysis are provided in the [online-only Data Supplement](#).

### Statistical Analysis

Results are expressed as mean $\pm$ SEM for the indicated number of experiments. Statistical significance was determined by *t* test, or 1- 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 were considered statistically significant. Further details are provided in the [online-only Data Supplement](#).

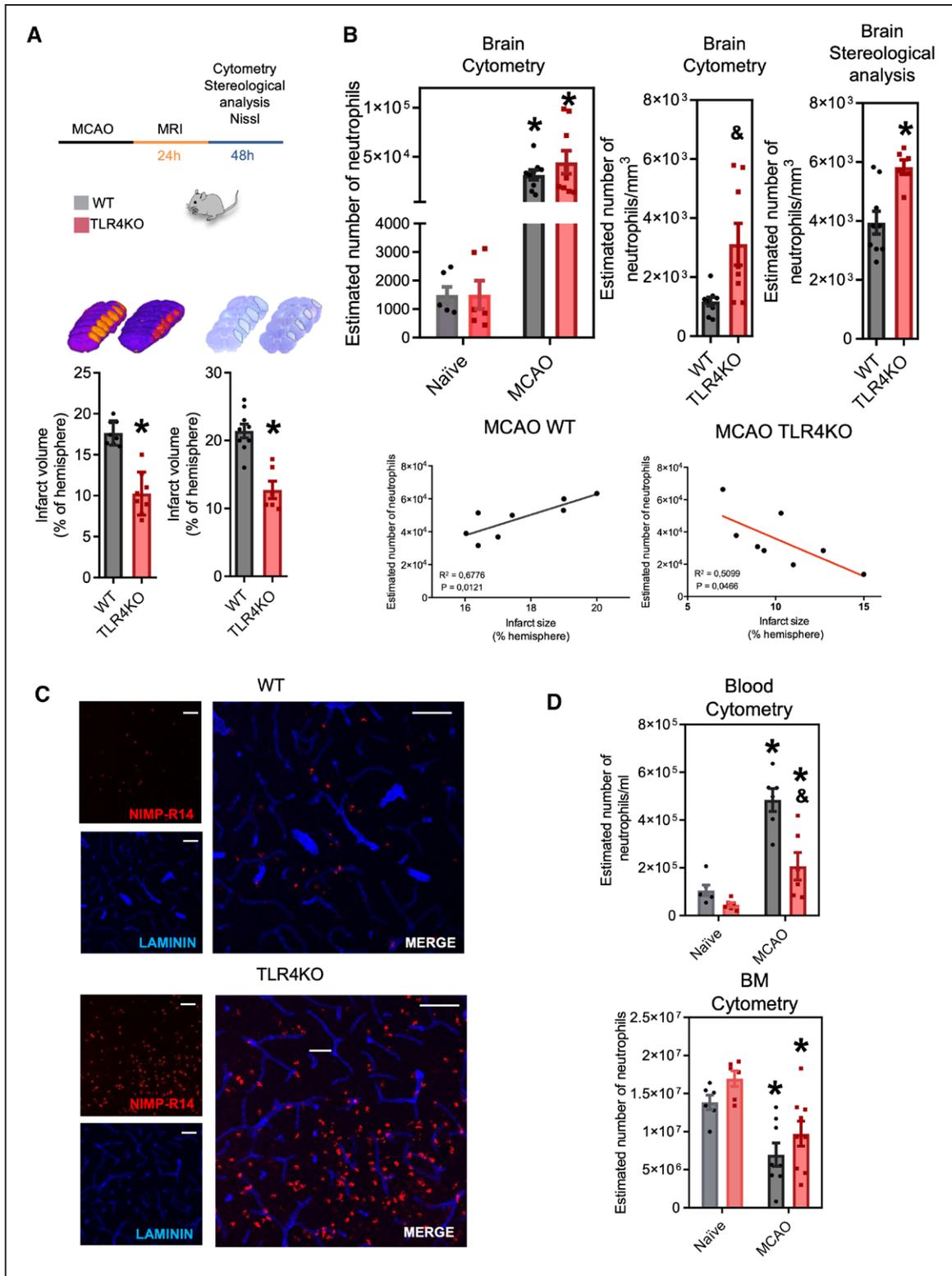
## Results

### TLR4 Absence Increases Neutrophil Infiltration Concomitant to Neuroprotection

To assess the role of TLR4 on neutrophil function after cerebral ischemia, both WT and TLR4-KO mice were subjected to pMCAO, and isolated brain myeloid cells were analyzed 48 hours by flow cytometry using specific markers (CD11b<sup>+</sup>Ly6G<sup>+</sup>; Figure 1A). As reported previously,<sup>5,9</sup> TLR4-deficient mice showed reduced infarct volume determined by magnetic resonance imaging and Nissl staining 24 and 48 hours after middle cerebral artery occlusion (MCAO), respectively (Figure 1A, bottom). Flow cytometry analysis of brain myeloid cells showed an almost 60-fold increase in the total number of brain neutrophils (Figure 1B, left; *P*<0.05) in ischemic mice compared with naive animals. Interestingly, the number of infiltrated neutrophils was not significantly affected by the reduced infarct volume observed in TLR4-KO mice. In fact, when the number of infiltrated cells was normalized by infarct volume in each genotype, we found an increased number in the reduced infarcted area of TLR4-KO mice (Figure 1B, center; *P*<0.05), indicating a higher density of neutrophils in these mice. These results were confirmed by immunofluorescence studies and stereological quantification of infiltrated neutrophils (Figure 1B, right; Figure 1C).

In agreement with previous studies indicating that neutrophil infiltration is associated with brain damage,<sup>17</sup> we found a positive correlation between the number of neutrophils and infarct volume in WT animals (Figure 1B, bottom, left; *P*<0.05). Remarkably, in TLR4-KO mice, an inverse correlation was found (Figure 1B, bottom, right; *P*<0.05), indicating that higher numbers of infiltrated neutrophils were associated with lesser cerebral damage after stroke, thus supporting a differential role of neutrophils in the absence of TLR4.

To assess whether differences in neutrophil infiltration in the presence or absence of TLR4 depend on stroke-induced mobilization, we checked neutrophil numbers in blood circulation and in the BM by flow cytometry 48 hours after MCAO (Figure 1D). As expected, MCAO induced an increase in the number of neutrophils in blood and a reduction in the BM



**Figure 1.** Effect of TLR4 (toll-like receptor 4) on neutrophil infiltration after focal cerebral ischemia. **A, Top**, Experimental design: mice were subjected to middle cerebral artery occlusion (MCAO), and infarct volumes were determined 24 or 48 h later by magnetic resonance imaging (MRI) or Nissl, respectively. Flow cytometry was performed in brain, blood, and bone marrow (BM) from MCAO WT (wild type), MCAO TLR4-knockout (KO), and naive WT and TLR4-KO animals (n=6–9) as controls. Neutrophil infiltration was quantified 48 h after MCAO. **Bottom**, Infarct volume assessed by MRI 24 h after MCAO in WT and TLR4-KO mice (n=8; \*P<0.05 vs MCAO WT; **left**) or by Nissl 48 h after MCAO in WT and TLR4-KO mice (n=6–9; \*P<0.05 vs MCAO WT; **right**). **B, Top**, Total estimated number of brains infiltrated neutrophils by flow cytometry in MCAO and naive animals (n=6–9; \*P<0.05 vs naive; **left**). Estimated number of infiltrated neutrophils in the ischemic core after MCAO normalized by infarct volume in mm<sup>3</sup> (n=8; &P<0.05 vs MCAO WT; **middle**). Quantification of neutrophil infiltration performed by sampling NIMP-R14<sup>+</sup> (rat monoclonal neutrophil antibody) cells in 7 coronal sections (optical fractionator technique) and expressed normalized by infarct volume in mm<sup>3</sup> (n=8–9; \*P<0.05 vs MCAO WT; **right**). **Bottom**, Linear correlation between neutrophil infiltration 48 h after MCAO by flow cytometry (Continued)

**Figure 1 Continued.** and infarct volume determined by MRI 24 h after ischemia (MCAO WT and TLR4-KO group, n=8). **C**, Double immunofluorescence staining of neutrophils (NIMP-R14<sup>+</sup>; red) and endothelial cells (laminin; blue) in the infarct core of WT and TLR4-KO mice 48 h after MCAO (scale bar=50  $\mu$ m). **D**, Estimated number of neutrophils in blood and BM determined by flow cytometry in MCAO and naive animals (n=6–8; \* $P$ <0.05 vs naive; & $P$ <0.05 vs MCAO WT). Data are mean $\pm$ SEM. Data were compared by using  $t$  test or using the 1-way ANOVA and Tukey post hoc multiple comparisons method.

following stroke (Figure 1D;  $P$ <0.05). However, blood neutrophilia was attenuated in TLR4-KO-deficient mice (Figure 1D;  $P$ <0.05) despite their increased infiltration into the ischemic tissue when normalized by ischemic area (Figure 1B;  $P$ <0.05). Of note, double immunofluorescence studies in both WT and TLR4-KO mice 48 hours after ischemia showed that most neutrophils infiltrated the brain parenchyma (Figure 1C) as observed previously<sup>14</sup> and were not retained within the vascular lumen as described in the transient ischemic model.<sup>18</sup> These results support that TLR4-deficient neutrophils infiltrate more efficiently into the damaged brain than WT neutrophils (Figure 1C).

Because TLR4 absence was associated with an increased neutrophil infiltration, we examined the role of TLR4 in this setting by generating chimeric mice by transplanting BM from GFP<sup>loxP/Lyz-cre</sup> (that express GFP [green fluorescent protein] in TLR4-expressing myeloid cells) and TLR4<sup>loxP/Lyz-cre</sup> (that do not express TLR4 in the myeloid lineage) into irradiated TLR4<sup>loxP/loxP</sup> recipients. When chimeric mice were exposed to MCAO and brains analyzed by immunofluorescence after 48 hours, the percentage of TLR4-expressing myeloid cells (GFP<sup>+</sup>/NIMP-R14<sup>+</sup>) into brain parenchyma was significantly lower than that of TLR4-KO myeloid cells (GFP<sup>-</sup>/NIMP-R14<sup>+</sup>), whereas at the luminal level, no significant changes were observed between both groups (Figure 2A through 2C), strongly supporting that TLR4-deficient neutrophils have a higher ability to infiltrate into the ischemic brain tissue than TLR4-expressing ones.

### Depletion of Neutrophils Abolishes Neuroprotection Afforded by TLR4 Deletion

All these aforementioned results suggest a differential role of neutrophils depending on the presence or absence of TLR4. To determine whether the increased neutrophil infiltration into the ischemic core of TLR4-deficient mice was involved in the lesion reduction seen in these mice, we eliminated neutrophils *in vivo* using an anti-Ly6G antibody treatment (Figure 3A, top).

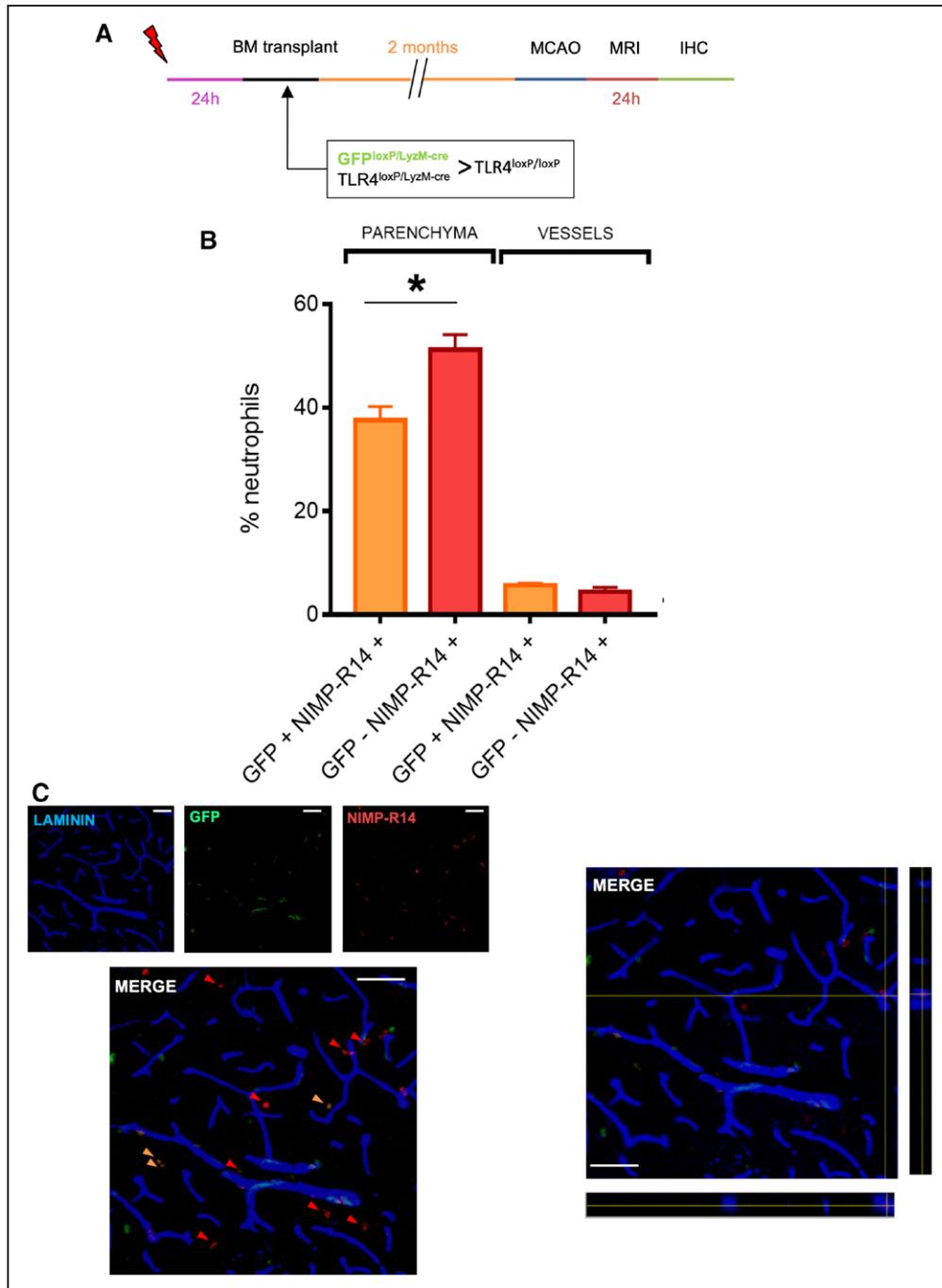
Anti-Ly6G treatment caused a reduction in the circulating neutrophil population compared with the isotype group (from 21.08% to 4.01% of total blood cells; Figure 3B;  $P$ <0.05) but not in the monocyte population (from 4.96% to 7.02%; Figure 3B;  $P$ >0.05). In accordance with the known deleterious role of neutrophils in cerebral ischemia,<sup>3,19,20</sup> WT mice treated with anti-Ly6G leukocyte antibody presented a significantly smaller infarct volume than those treated with the IgG isotype (Figure 3A, bottom;  $P$ <0.05). Remarkably, and confirming our hypothesis, neutrophil-depleted TLR4-KO animals had a significantly higher infarct volume (Figure 3A, bottom;  $P$ <0.05), strongly supporting that TLR4-deficient neutrophils display neuroprotective properties. Altogether, our data support the notion that neutrophil infiltration into the ischemic tissue has functional and opposite consequences in the presence or absence of TLR4.

### Specific Myeloid Ablation of TLR4 Also Induces Neuroprotection After Stroke

To determine whether TLR4 deficiency was relevant in specific cell subsets, we obtained mice with TLR4-deficient myeloid lineage by using the TLR4<sup>loxP/LyzM-cre</sup> genetic construction<sup>21</sup> (Figure 3C). First, in TLR4<sup>loxP/LyzM-cre</sup> mice, microglial cells expressed TLR4, thus discarding any bias arising from the absence of TLR4 in these cells (Figure 1A and 1B in the [online-only Data Supplement](#)). Second, TLR4<sup>loxP/LyzM-cre</sup> mice showed dramatically decreased lesion volumes compared with their respective controls (TLR4<sup>loxP/loxP</sup>; Figure 3C;  $P$ <0.05). Confirming our hypothesis, specific depletion of neutrophils in TLR4<sup>loxP/LyzM-cre</sup> animals increased the infarct volume as opposed to the reduction found in control TLR4<sup>loxP/loxP</sup> mice (Figure 3D;  $P$ <0.05). These data support that myeloid-specific TLR4 deficiency is involved in neuroprotection in these mice after pMCAO.

### TLR4 Modulates Neutrophil Polarization After Focal Cerebral Ischemia

Chitinase-like protein (also named Chil3 protein or YM1) is considered a reference marker of M2 macrophage polarization (alternative activation) in mouse.<sup>22</sup> Our group has described a population of neutrophils, so-called N2, that expressed YM1 after rosiglitazone (PPAR $\gamma$  agonist) treatment in experimental ischemia.<sup>14</sup> Thus, we selected this marker to explore whether TLR4 presence affects the relative proportions of classical (N1) versus N2 neutrophil populations in the ischemic brain. First, we determined the percentage of N1 (Ly6G<sup>+</sup>/YM1<sup>-</sup>) and N2 (Ly6G<sup>+</sup>/YM1<sup>+</sup>) neutrophils in blood. In naive conditions, there were no differences between WT (50.40% N1 and 49.60% N2) and TLR4-KO mice (46.05% N1 and 53.95% N2). However, 48 hours after MCAO, the percentage of N2 neutrophils was 53.7 $\pm$ 10.5% in WT mice and 81.1 $\pm$ 5.9% in TLR4-KO mice ( $P$ <0.05). We then quantified by flow cytometry the number of N2 neutrophils in brains of naive and MCAO-exposed WT and TLR4-KO mice (Figure 4A). MCAO increased the number of infiltrated YM1<sup>+</sup> neutrophils in brain of both WT and TLR4-KO mice (Figure 4A, center;  $P$ <0.05). Interestingly, this effect was not significantly affected by the reduced infarct volume observed in TLR4-KO mice. In fact, when we normalized the number of infiltrated YM1<sup>+</sup> (N2) neutrophils by the infarct volume (YM1<sup>+</sup> neutrophils/mm<sup>3</sup>) of each genotype, we found an increased density of these cells in the infarcted area of TLR4-KO mice (Figure 4A, right;  $P$ <0.05). These results were confirmed by stereological analysis, measuring the number of N2 neutrophils as YM1<sup>+</sup> NIMP-R14<sup>+</sup> cells in the ischemic core in WT and TLR4-KO mice (Figure 4B, left). At 48 hours, 73.6% and 26.4% of NIMP-R14<sup>+</sup> neutrophils in brain sections were N1 and N2 neutrophils in the WT group, in contrast to the TLR4-KO group in which 54.7% and 45.3% of neutrophils were N1 and N2, respectively (Figure 4B, right; Figure 4C;  $P$ <0.05). These results might indicate that TLR4 absence increases the number of infiltrated N2 neutrophils into the ischemic brain.

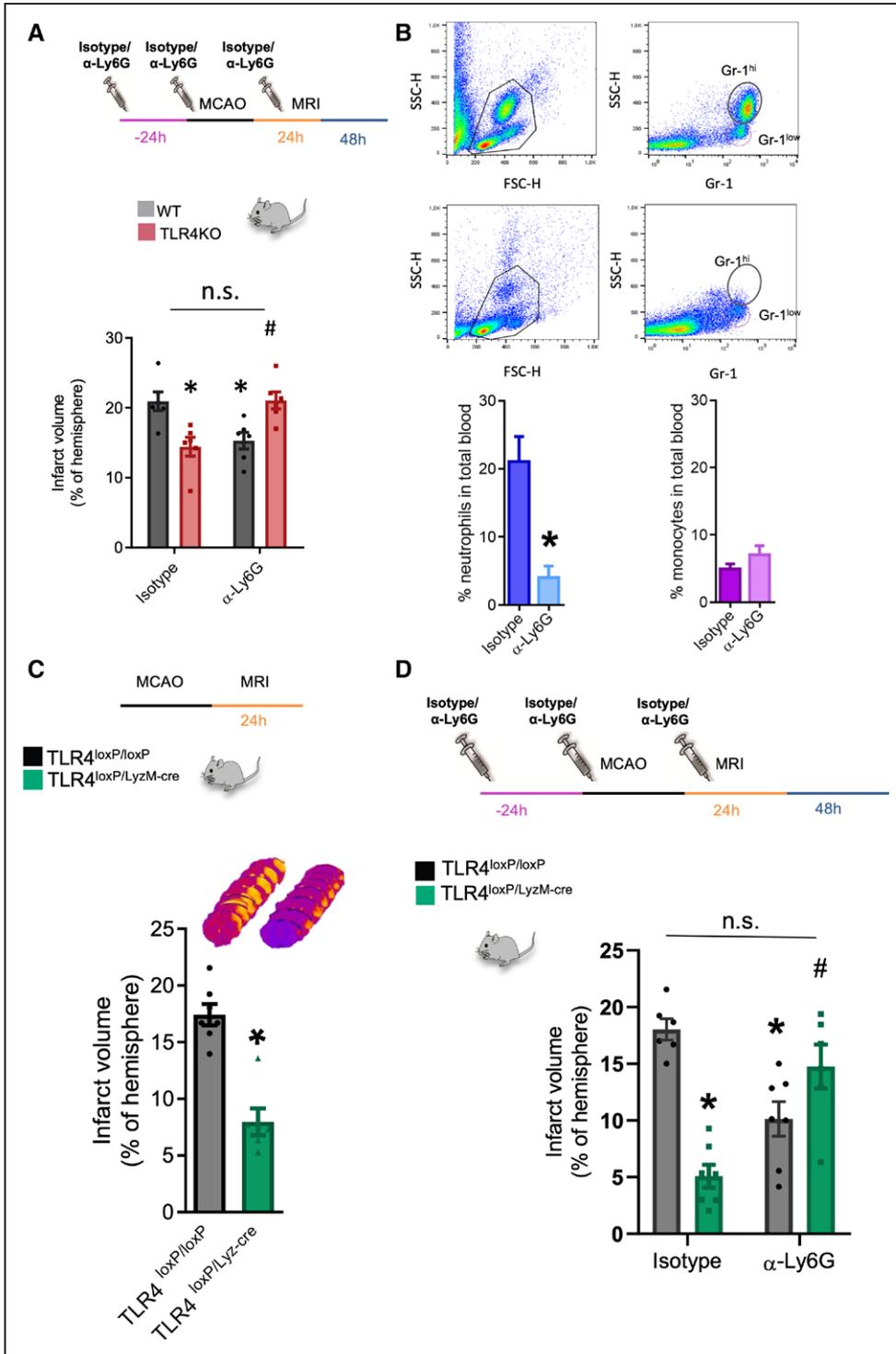


**Figure 2.** Differential myeloid cell infiltration in chimeric mice after stroke. **A**, Experimental design: recipient mice from TLR4<sup>loxP/loxP</sup> (n=3) were lethally irradiated 24 h before reconstitution with donor bone marrow (BM). Donor BM was harvested from mice of GFP<sup>loxP/LyzM-cre</sup> and TLR4<sup>loxP/LyzM-cre</sup>, and equal number of cells from each genotype were mixed and intravenously injected into irradiated mice. Two months later, mice were subjected to middle cerebral artery occlusion (MCAO) and their brains collected for immunofluorescence studies 48 h later. **B**, Percentage of myeloid cells expressing (GFP<sup>+</sup>/NIMP-R14<sup>+</sup> [rat monoclonal neutrophil antibody]) or not expressing TLR4 (GFP<sup>-</sup>/NIMP-R14<sup>-</sup>) into the parenchyma and inside the vessels within the ischemic core (n=3; \*P<0.05). **C**, Representative photomicrographs showing GFP (green), neutrophils (red), and laminin (blue) immunostaining. Arrowheads indicate colocalization of GFP<sup>+</sup>/NIMP-R14<sup>+</sup> cells (orange) and GFP<sup>-</sup>/NIMP-R14<sup>-</sup> cells (red; scale bar=50  $\mu$ m). Representative photomicrographs showing orthogonal colocalization of a neutrophil inside a blood vessel (neutrophils, red; laminin, blue) immunostaining (scale bar=50  $\mu$ m). IHC indicates immunohistochemistry; MRI, magnetic resonance imaging; and TLR4, toll-like receptor 4.

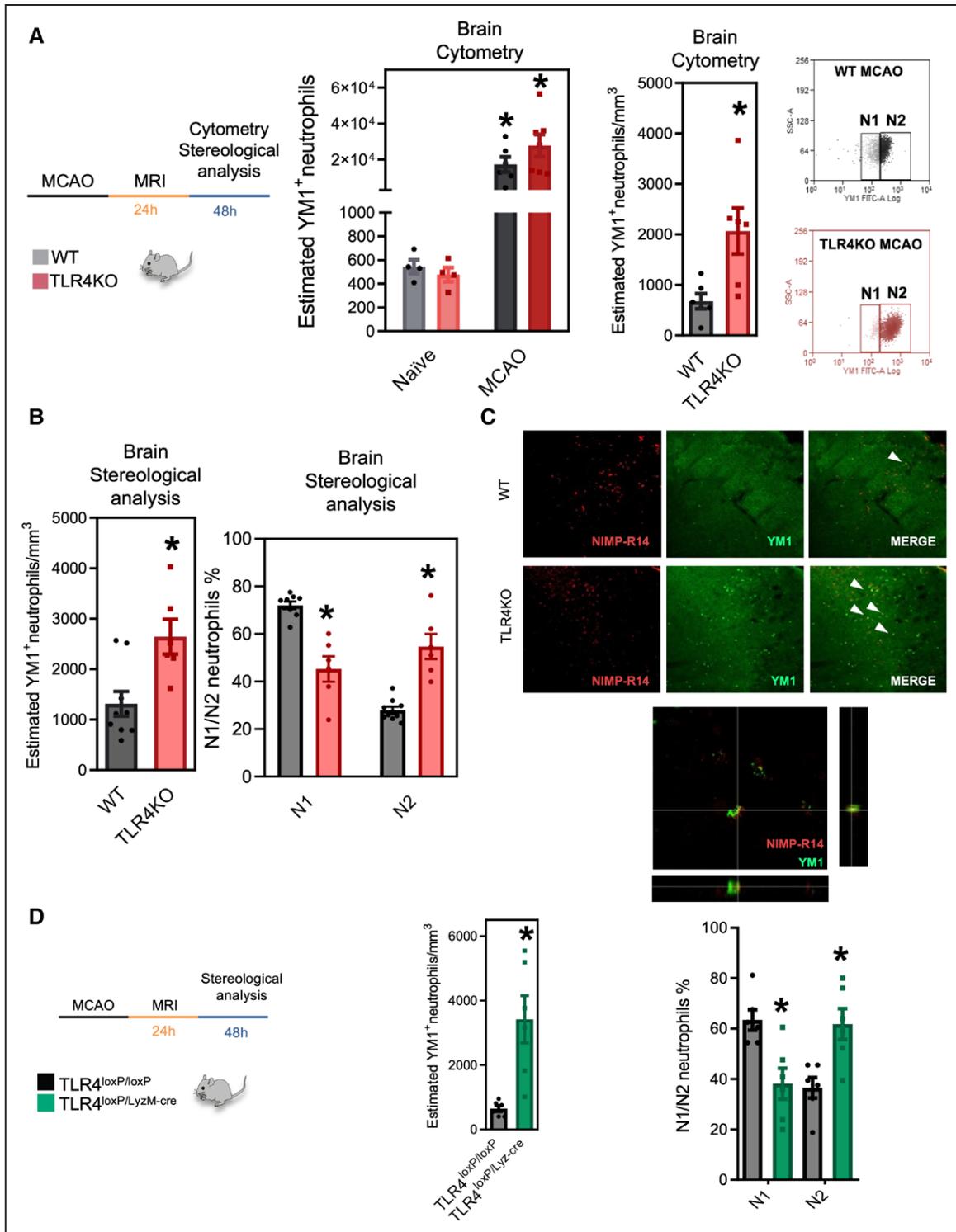
These results were further confirmed in mice with TLR4-deficient myeloid lineage (TLR4<sup>loxP/LyzM-cre</sup> mice), where we found a marked increase in N2 neutrophils (YMI<sup>+</sup>, NIMP-R14<sup>+</sup> cells; Figure 4D, center; P<0.05) and an inverted N1/N2 ratio in TLR4<sup>loxP/LyzM-cre</sup> mice (Figure 4D, right; P<0.05) after stereological analysis 48 hours after pMCAO (Figure 4D).

### mRNA Microarray Assessment of Global Gene Expression in WT and TLR4-KO Blood Neutrophils After Stroke

To elucidate the molecular mechanisms underlying TLR4-mediated neutrophil phenotype, we performed a cDNA microarray analysis of neutrophils isolated by cell sorting from



**Figure 3.** Effect of neutrophil depletion on infarct outcome. **A**, Experimental design: WT (wild type) and TLR4 (toll-like receptor 4)-KO mice (n=6) were injected with either mouse antineutrophil antibody or control isotype daily for 4 d, starting 24 h before middle cerebral artery occlusion (MCAO). All groups were subjected to footprint test at baseline, 24 h, and 48 h after MCAO (top). Infarct volumes from the different genotypes with their treatments. A significant interaction between treatment and genotype was found for infarct volume (F[1,18]=18.89;  $P<0.0004$ ; n=6; Bonferroni post hoc: \* $P<0.05$  vs MCAO WT isotype; # $P<0.05$  vs MCAO TLR4-KO isotype; bottom). **B**, Top, Flow cytometry analysis of blood samples from animals treated with isotype (upper plot) or anti-Ly6G antibody (lower plot). Neutrophil population was characterized as Gr-1<sup>+</sup> and CD11b<sup>+</sup>. Bottom, Percentage of total neutrophil and monocyte populations in blood (lower graphs) with the isotype and anti-Ly6G antibody (\* $P<0.05$  vs isotype). **C**, Experimental design: TLR4<sup>loxP/loxP</sup> and TLR4<sup>loxP/LyzM-cre</sup> mice were subjected to MCAO; infarct volumes were determined by magnetic resonance imaging (MRI) 24 h after ischemic insult in both groups (top). Infarct volumes in TLR4<sup>loxP/loxP</sup> and TLR4<sup>loxP/LyzM-cre</sup> mice (n=6–7; \* $P<0.05$  vs TLR4<sup>loxP/loxP</sup>; bottom). **D**, Experimental design: TLR4<sup>loxP/loxP</sup> and TLR4<sup>loxP/LyzM-cre</sup> mice were injected with either mouse antineutrophil antibody or control isotype as in **A** (top). Infarct volumes from the different genotypes with their respective treatments. A significant interaction between treatment and genotype was found for infarct volume (F[1,22]=40.11;  $P<0.0001$ ; n=6–7; Bonferroni post hoc: \* $P<0.05$  vs MCAO TLR4<sup>loxP/loxP</sup> isotype; # $P<0.05$  vs MCAO TLR4<sup>loxP/LyzM-cre</sup> isotype). Data are mean $\pm$ SEM. Data were compared by using 2-way ANOVA followed by Bonferroni post hoc testing. CD11b indicates neutrophil marker, antibody; FSC-H, forward scatter - height; Gr-1, neutrophil marker, antibody; SSC-H, side scatter - height; TLR4, toll-like receptor 4; and TLR4-KO, toll-like receptor 4 - knockout.



**Figure 4.** Effect of the absence of TLR4 (toll-like receptor 4) on neutrophil polarization after focal cerebral ischemia. **A**, WT (wild type) and TLR4-KO mice were subjected to middle cerebral artery occlusion (MCAO); infarct volumes were determined 24 h later by magnetic resonance imaging (MRI); and flow cytometry and stereological quantification were performed at 48 h (left). Total  $Ym1^+$  infiltrated neutrophils in brain of MCAO and naive animals by flow cytometry ( $n=4-8$ ;  $*P<0.05$  vs naive; middle). Estimated number of  $Ym1^+$  neutrophils normalized by infarct volume in  $mm^3$  in the ischemic core of both groups ( $n=8$ ;  $*P<0.05$  vs MCAO WT) and representative flow cytometric analysis of  $Ym1^+$  neutrophils in WT (top) and TLR4-KO (bottom) mice brain (right). **B**, Quantification of  $Ym1^+$  neutrophil infiltration, performed by sampling  $Ym1^+$ , NIMP-R14<sup>+</sup> cells in 7 coronal sections (optical fractionator technique) and expressed normalized by infarct volume in  $mm^3$  ( $n=6-9$ ;  $*P<0.05$  vs MCAO WT; left). Percentage of N1 neutrophils ( $Ym1^+$ , NIMP-R14<sup>+</sup>) vs N2 ( $Ym1^+$ , NIMP-R14<sup>-</sup>) in the ischemic core of both groups ( $n=6-9$ ;  $*P<0.05$  vs MCAO WT; right). **C**, Representative photomicrographs showing  $Ym1$  (green) and neutrophil (red) double immunostaining after MCAO in WT (top) or TLR4-KO mice (bottom). Arrows indicate colocalization (yellow) of  $Ym1$  (green) with neutrophils (red); scale bar=50  $\mu m$ . Orthogonal projection of NIMP-R14<sup>+</sup> (red) and  $Ym1^+$  (green) colocalization in neutrophils (inset). **D**, Experimental design: TLR4<sup>loxP/loxP</sup> and TLR4<sup>loxP/LyzM-cre</sup> mice were subjected to MCAO; infarct volumes were determined 24 h later by MRI; and stereological quantification was performed (left). Quantification of  $Ym1^+$  neutrophil (Continued)

**Figure 4 Continued.** infiltration, performed by sampling YM1<sup>+</sup>, NIMP-R14<sup>+</sup> cells in 7 coronal sections (optical fractionator technique) and expressed normalized by infarct volume in mm<sup>3</sup> (n=6; \**P*<0.05 vs MCAO TLR4<sup>loxP/loxP</sup>; **middle**). Percentage of neutrophils N1 (YM1<sup>-</sup>, NIMP-R14<sup>-</sup>) vs N2 (YM1<sup>+</sup>, NIMP-R14<sup>+</sup>) in the ischemic core of both groups (n=6; \**P*<0.05 vs MCAO TLR4<sup>loxP/loxP</sup>; **right**). Data are mean±SEM. Data were compared by using *t* test, using the 1-way ANOVA and Tukey post hoc multiple comparisons method, or by using 2-way ANOVA followed by Bonferroni post hoc testing. NIMP-R14 indicates rat monoclonal Neutrophil antibody; TLR4, toll-like receptor 4; and TLR4-KO, toll-like receptor 4-knockout.

blood 48 hours after ischemia from WT and TLR4-KO by using Affymetrix platform and posterior Gene Set Enrichment Analysis (MIT Broad Institute). The heat map of normalized expression for over 1500 selected genes (*P*<0.05) between WT and TLR4-KO shows a clearly different signature in these 2 groups of cells (Figure 5A). To link genes to specific pathways, we evaluated key pathways and gene groups using bioinformatic approaches. Gene Set Enrichment Analysis of the pathways enriched for the 500 genes more differentially expressed in TLR4-KO neutrophils showed 3 major groups of upregulated pathways, related to inflammation, circadian rhythms, and cell death. Interestingly, it also revealed an alteration in pathways related to neutrophil migration such as the IL (interleukin)-6–related pathways, involved in neutrophil trafficking and positive regulation of locomotion and cell surface interactions at the vascular wall (Figure 5B). These results suggest that neutrophil TLR4 modulates the contribution of these cells to ischemia-induced inflammation, their migration into the parenchyma, and their functional priming. We conducted quantitative polymerase chain reaction validation of some of the gene expression changes in WT versus TLR4-KO. The results were highly concordant between the quantitative polymerase chain reaction and microarray data, especially regarding the change in expression. Given the TLR4-mediated neutrophil phenotype, we looked for genes involved in N2 neutrophil characterization, and we found higher levels of mRNA for *Chil3* and *Arg1* in TLR4-KO than in WT. Expression of NF-κB (nuclear factor κB)–related genes, such as *Tnfaip3* (tumor necrosis factor alpha induced protein 3; negative regulation of IκB kinase and NF-κB signaling), increased as well in TLR4-KO. We also studied one gene of the molecular clock (*Per1* [period circadian protein homolog 1]), observing that its expression was again increased in TLR4-KO (Figure 5C).

We additionally compared the 500 genes most overexpressed in TLR4-KO neutrophils versus WT ones with the transcription changes observed in aged and nonaged neutrophils as reported by Frenette and cols.<sup>16</sup> Genes overexpressed in N2 neutrophils were also significantly enriched among those overexpressed in the nonaged group<sup>16</sup> (Normalized Enrichment Score, -1.38; false discovery rate *q*-value, 0.00229; Figure 5D, left). Interestingly, when performing an enrichment assay only for the genes at the right side of the leading edge (dotted red line) in the Gene Set Enrichment Analysis, 3 upregulated pathways were found, corresponding to regulation of inflammatory response, negative regulation of immune effector process, and negative regulation of IL-6 production (Figure 5D, right). These data demonstrate an alteration of both TLR4-KO and nonaged neutrophils in the ability to participate in the inflammatory process, strongly supporting the important role of TLR4 in neutrophil function.

## Discussion

We and others demonstrated that TLR4 plays an important role in inducing inflammation and tissue damage after stroke.<sup>5–7</sup>

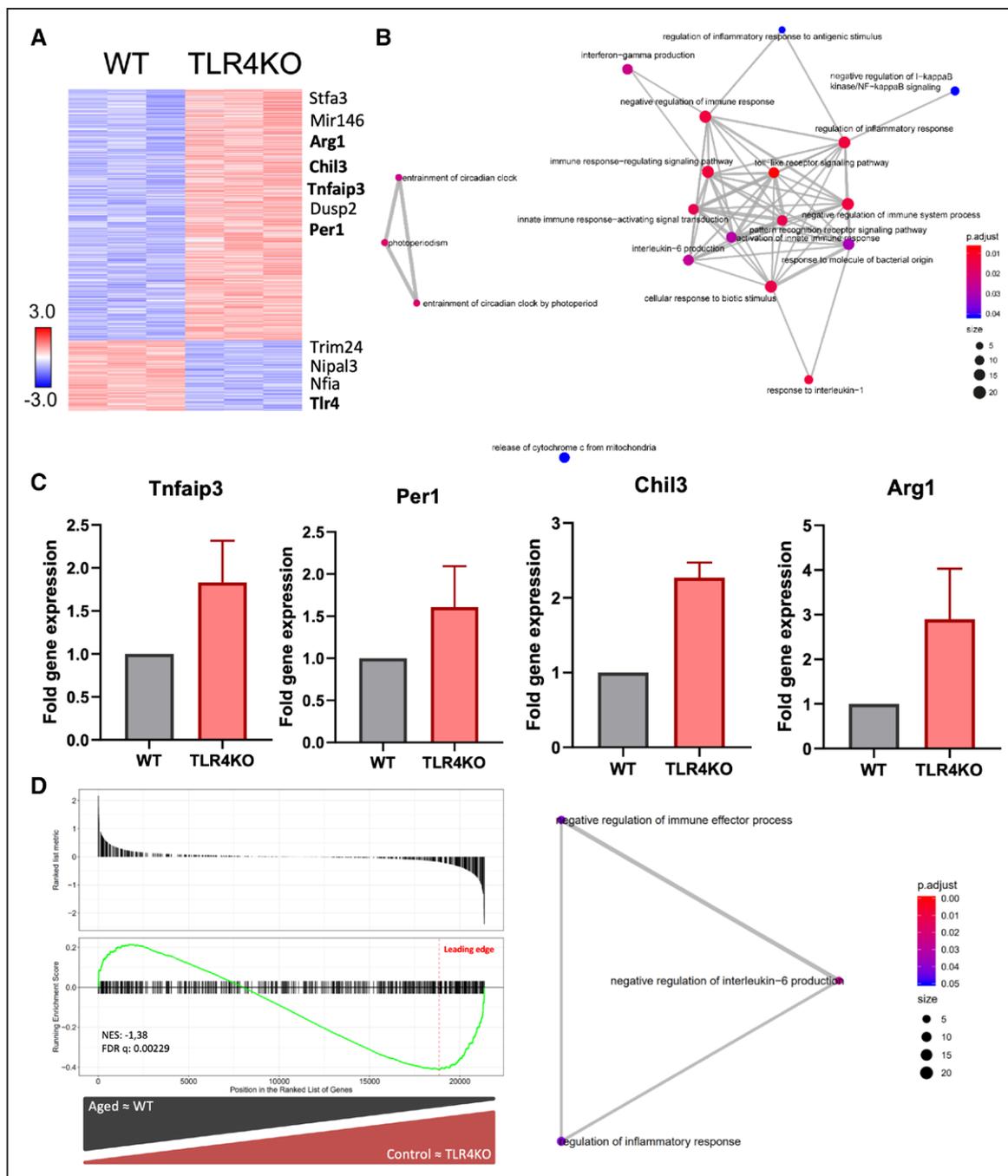
However, the specific mechanisms by which TLR4 is deleterious are not completely understood. We show here that TLR4 plays a crucial role in neutrophil polarization and function, in such a way that TLR4 absence increases neutrophil infiltration and induces a polarization of neutrophils toward an N2 phenotype, which is associated with neuroprotection. We have also found that neutrophil TLR4 absence modulates the induction of several pathways previously associated with the inflammatory process after ischemia, their migration into the parenchyma, and their maturation, all of which might underlie the functional outcome during stroke. To our knowledge, our data are the first evidence of neutrophil reprogramming in brain inflammation through the modulation of TLR4.

As stated above, the absence of TLR4 has been widely described to mediate a neuroprotective effect due to the inhibition of the inflammatory response after stroke.<sup>5–7</sup> Interestingly, and in contrast with the published data on the effects of neutrophil infiltration,<sup>3,4,19,23</sup> we describe that such neuroprotective effect is concomitant to an increased density of infiltrated neutrophils into the ischemic tissue. Importantly, Kilic et al<sup>24</sup> described that TLR4 deficiency goes along with increased neutrophil accumulation in the ischemic brain, pointing toward a futile inflammatory response as compensatory mechanism counteracting the TLR4 defect. Therefore, we show that TLR4 absence increases neutrophil accumulation associated with neuroprotection as described previously,<sup>24</sup> which is due to the polarization of neutrophils toward an N2, protective phenotype, as discussed below.

Classically, neutrophils have been shown to be mainly detrimental, by mechanisms that will affect outcome, severity, and infarct volume by, among others, the induction of the no-reflow phenomenon, the release of elastase that might increase tissue damage, or the production of reactive oxygen species that contributes to blood-brain barrier disruption.<sup>25,26</sup> Consistently with the deleterious role of neutrophil infiltration, neutrophil depletion in WT animals caused a significant infarct lesion reduction, in agreement with previous data.<sup>3,4,19,23</sup> However, neutrophil depletion in TLR4 absence did not only fail to induce neuroprotection but increased cerebral damage after stroke.

Our data then indicate, first, that neutrophils do not play a deleterious role in the absence of TLR4 and, second, that TLR4 deletion–induced neuroprotective effect requires neutrophils. Reinforcing our results, TLR4-deficient myeloid lineage mice (TLR4<sup>loxP/LyzM-cre</sup>) displayed significantly decreased lesion volumes than their respective controls (TLR4<sup>loxP/loxP</sup>). Further confirming our hypothesis, neutrophil depletion in TLR4<sup>loxP/LyzM-cre</sup> animals increased infarct volume, clearly showing a cytoprotective effect of these cells. Therefore, our experiments using specific neutrophil depletion in both global TLR4-KO and TLR4<sup>loxP/LyzM-cre</sup> mice indicate that these cells are the relevant subset for the effect observed.

The fact that TLR4-KO–induced neuroprotection is unexpectedly associated with an increased rate of neutrophil



**Figure 5.** mRNA microarray assessment of global gene expression in WT (wild type) and TLR4-KO blood neutrophils after stroke. **A**, Heat map of normalized expression for 1499 selected genes ( $P < 0.05$ ) between WT and TLR4-KO neutrophils highlighting, among others, some selected genes validated. The color scale of the heat map indicates fold changes of expression for each gene; red, upregulation. **B**, Pathways enriched for the 500 genes that are more differentially expressed in TLR4-KO neutrophils. **C**, quantitative polymerase chain reaction for *Chil3*, *Arg1*, *Tnfaip3*, and *Per1* confirmed significant upregulation of expression in TLR4-KO mice 2 d after middle cerebral artery occlusion in comparison to WT-operated animals. **D**, Gene Set Enrichment Analysis (GSEA) analysis (left) showing that TLR4-KO neutrophils are similar to the control group by Frenette and cols.<sup>16</sup> Enrichment assay (right) only for the genes at the right side of the leading edge in the GSEA analysis showing the 3 altered pathways shared by control and TLR4-KO neutrophils. *Arg1* indicates arginase 1; *Chil3*, chitinase-like 3; FDR q, false discovery rate q-value; NES, Normalized Enrichment Score; *Per1*, period circadian protein homolog 1; qPCR, quantitative polymerase chain reaction; TLR4-KO, toll-like receptor 4 - knockout; and *Tnfaip3*: tumor necrosis factor alpha induced protein 3.

infiltration, together with the lack of effect of TLR4 absence on infarct volume after neutrophil depletion, led us to hypothesize the existence of neutrophil subsets with different pathophysiological roles. In this context, it has been demonstrated the existence of a subset with proinflammatory properties named as N1 by analogy to the macrophage M1/M2 paradigm

and another subpopulation with an anti-inflammatory or pro-resolutive profile or N2<sup>13, 14</sup>. Now, we demonstrate that TLR4 absence after experimental stroke skews neutrophils toward an N2 phenotype, which is associated with the observed neuroprotective effect. Indeed, we have found, by using stereological and cytometric analysis and after normalizing the

number of infiltrated cells by the infarct volume (YM1<sup>+</sup>/mm<sup>3</sup> neutrophils), a decreased number of N1 and an increased number of N2 neutrophils (as YM1<sup>+</sup> NIMP-R14<sup>+</sup> cells) in the infarcted area of TLR4-KO mice. These results were further confirmed when we used mice with TLR4-deficient myeloid lineage (TLR4<sup>loxP/LyzM-cre</sup> mice).

Importantly, whereas naive animals showed no differences in blood N1 and N2 neutrophil percentage regardless of the genotype (WT or TLR4-KO), after exposure to MCAO, TLR4-KO mice displayed a remarkable increase in the percentage of N2 neutrophils, which could be due to their polarization in the bloodstream or to an increased mobilization from the BM.

Further studies are required to elucidate the molecular signaling by which N1/N2 polarization is modulated. In this context, neutrophils might act in response to different molecules such as cytokines (TGF- $\beta$  [transforming growth factor- $\beta$ ], IL-27), damage-associated molecular patterns, or growth factors released after stroke, which are reported to be implicated in their polarization.<sup>27</sup> In addition, our present results are in agreement with our previous work demonstrating that N2 phenotype induced by PPAR $\gamma$  is associated with neuroprotection and resolution of inflammation after experimental stroke, as shown by an increased N2 neutrophil engulfment by microglia/macrophages.<sup>14</sup> Overall, our data highlight the important role of TLR4 on neutrophil phenotype and final fate after cerebral ischemia.

To explore further the molecular mechanisms underlying the protective effects of N2 phenotype after stroke in the absence of TLR4, we performed a cDNA microarray analysis of isolated neutrophils from WT and TLR4-KO mice. Gene Set Enrichment Analysis revealed that neutrophils from TLR4-KO mice differ from those from WT mice after pMCAO. According to our results, the absence of neutrophilic TLR4 might modulate the contribution of neutrophils to the inflammatory process after ischemia by regulating gene pathways, such as a negative regulation of signaling by I $\kappa$ B kinase/NF- $\kappa$ B, TNF $\alpha$  (tumor necrosis factor- $\alpha$ )/NF- $\kappa$ B, IL-6/Janus kinase/STAT3 (signal transducer and activator of transcription protein), IL-2/STAT5, and TGF- $\beta$ , as well as their migration into the parenchyma and their maturation, and by controlling diverse pathways related to circadian rhythms and to IL-6 signaling, which is crucial in neutrophil trafficking.

Regarding the capacity of neutrophils to change their phenotype, one of the most accepted views proposes that there are different populations of neutrophils depending on their age. In this context, we also compared the 500 genes more overexpressed in TLR4-KO neutrophils when compared with the WT ones, with the transcriptional changes observed in nonaged/aged neutrophils described by Frenette and cols.<sup>16</sup> Interestingly, we have found that genes overexpressed in neutrophils from our stroke-exposed TLR4-KO mice are significantly enriched in those overexpressed in the Frenette non-aged group. Consistently, Frenette aged neutrophils, similar to the predominant population in our stroke-exposed WT animals, exhibit a higher expression of TLR4 and an upregulation of several inflammatory pathways.<sup>16</sup> Furthermore, they also found a significant reduction of aged neutrophils in TLR4-KO mice<sup>16</sup> as we also observed.

## Conclusions

In summary, our data demonstrate the implication of TLR4 on neutrophil phenotypic polarization after stroke showing that, in this proinflammatory setting, the absence of TLR4 is associated with an increase in the levels of alternative N2 neutrophils, which might be involved in the neuroprotective effect observed in these mice. Our data support TLR4 inhibition or blockade as a promising therapeutic target for the treatment of stroke by reducing inflammation and ischemic damage,<sup>5</sup> by preventing tPA (tissue-type plasminogen activator)-induced hemorrhagic transformation,<sup>9</sup> and now by skewing neutrophils toward cytoprotective phenotypes. New drugs blocking TLR4, as we have recently described,<sup>28</sup> might be useful for the treatment of patients with stroke.

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## Disclosures

None.

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