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## Toll-Like Receptor 4 Is Involved in Brain Damage and Inflammation After Experimental Stroke

Javier R. Caso, BSc\*; Jesús M. Pradillo, BSc\*; Olivia Hurtado, PhD; Pedro Lorenzo, MD, PhD; María A. Moro, PhD; Ignacio Lizasoain, MD, PhD

**Background**—Stroke is the second to third leading cause of death. Toll-like receptor 4 (TLR4) is a signaling receptor in innate immunity that is a specific immunologic response to systemic bacterial infection and cerebral injury. The role of TLR4 in brain ischemia has not been examined yet. We have therefore investigated whether cerebral ischemia and inflammation produced by permanent occlusion of the middle cerebral artery differ in mice that lack a functional TLR4 signaling pathway.

**Methods and Results**—Permanent occlusion of the middle cerebral artery was performed on 2 strains of TLR4-deficient mice (C3H/HeJ and C57BL/10ScNJ) and respective controls (C3H/HeN and C57BL/10ScSn). Stroke outcome was evaluated by determination of infarct volume and assessment of neurological scores. Brains were collected 24 hours and 7 days after stroke. When compared with control mice, TLR4-deficient mice had lower infarct volumes and better outcomes in neurological and behavioral tests. Mice that lacked TLR4 had minor expression of stroke-induced interferon regulatory factor-1, inducible nitric oxide synthase, and cyclooxygenase-2, mediators implicated in brain damage. The levels of interferon- $\beta$  and of the lipid peroxidation marker malondialdehyde were also lower in brains from TLR4-deficient mice than in those from control mice. In addition, the expression of matrix metalloproteinase-9, which is induced and mediates brain damage, was also reduced in TLR4-deficient mice after experimental stroke.

**Conclusions**—TLR4-deficient mice have minor infarctions and less inflammatory response after an ischemic insult. These data demonstrate that TLR4 signaling and innate immunity are involved in brain damage and in inflammation triggered by ischemic injury. (*Circulation*. 2007;115:1599-1608.)

**Key Words** cerebral ischemia ■ immune system ■ infection ■ inflammation ■ metalloproteinases  
■ nitric oxide synthase ■ stroke

Stroke is the second to third leading cause of death and the main cause of severe long-term disability in adults. In spite of this, treatment is often limited to fibrinolysis, a therapy useful to a very low percentage of patients. Therefore, the need for new therapeutic strategies is imperative.

### Clinical Perspective p 1608

It has been mentioned that inflammation and infection processes are important in the pathology of stroke. First, cerebral ischemia evokes a strong inflammatory response characterized by activation and release of cytokines, chemokines, adhesion molecules, and proteolytic enzymes that exacerbate tissue damage. This inflammatory response is associated with an active gene expression of inflammatory-related genes.<sup>1,2</sup> In humans, it has been demonstrated that increased levels of proinflammatory cytokines are related to a greater extent of cerebral infarct and poorer clinical outcome in patients with ischemic

stroke<sup>3</sup> and that inflammatory markers (leukocytes, fibrinogen, C-reactive protein) are independent predictors of ischemic stroke.<sup>4</sup> In addition, ischemic tissue damage can be reduced in experimental models by a variety of antiinflammatory agents. Such antiinflammatory strategies have proven disappointing in clinical practice, however. Furthermore, previous studies have demonstrated that chronic infections (mainly infection with *Chlamydia pneumoniae* or *Helicobacter pylori*) might increase the risk of stroke; however, the precise role of infection remains unclear.<sup>4,5</sup>

In this context, innate immunity is a specific immunologic response not only to systemic bacterial infection but also to cerebral injury.<sup>6,7</sup> Activation of this innate immunity takes place in the brain and utilizes toll-like receptors (TLRs).<sup>6</sup> Human TLR4 was the first characterized mammalian TLR,<sup>8</sup> but there are at least 10 distinct TLR family members.<sup>6,7,9</sup>

A role for TLR4 in stroke is suggested by several observations. First, TLR4 is expressed in microglia and

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astrocytes after inflammation in the central nervous system.<sup>10,11</sup> Second, exogenous and endogenous mediators that have been isolated after brain ischemia have been identified as ligands of TLR4.<sup>9</sup> Third, TLR4 activates nuclear factor  $\kappa$ -B signaling pathways linked to the transcription of many proinflammatory genes that encode cytokines, chemokines, proteins of the complement system, enzymes such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), matrix metalloproteinase-9 (MMP-9) and adhesion molecules,<sup>8</sup> events that have been also demonstrated in brain ischemia.<sup>1</sup> Finally, mutations in the TLR4 gene have been associated with risk of ischemic stroke and atherothrombosis among some populations.<sup>12,13</sup>

We have therefore investigated whether cerebral ischemia and inflammation produced by permanent occlusion of the middle cerebral artery (pMCAO) differed in mice that lack a functional TLR4 signaling pathway.

## Methods

### Animals

Adult male C3H and C57BL mice that weighed 28 to 30 g were used. C3H/HeN, C3H/HeJ, C57BL/10ScNJ (former name C57BL/10ScCr), and C57BL/10ScSn mice were from The Jackson Laboratory (Bar Harbor, Me). The murine strains C3H/HeJ and C57BL/10ScNJ do not express functional TLR4; whereas C3H/HeJ mouse represents a point mutation, C57BL/10ScNJ represents a deletion of the TLR4 gene.<sup>14</sup> C3H/HeN and C57BL/10ScSn substrains do not express the mutation and are considered as control groups. All experimental protocols adhered to the guidelines of the Animal Welfare Committee of the Universidad Complutense (in accordance with DC 86/609/EU). Mice were housed under standard conditions of temperature and humidity, with a 12-hour light/dark cycle and free access to food and water.

### Induction of Focal Cerebral Ischemia

Mice were anesthetized with 5% isoflurane (in 70%N<sub>2</sub>O, 30%O<sub>2</sub>) for induction and 1.5% isoflurane for maintenance. The rectal temperature was maintained with a heating pad at 37°C. The middle cerebral artery (MCA) was exposed and occluded permanently by electrocoagulation.<sup>15</sup> Two groups of C3H/HeN mice were treated with 20 and 40 mg/kg N-(3-(aminomethyl)benzyl) acetamide (1400W, a specific iNOS inhibitor, donated by GlaxoSmithKline, Stevenage, Herts, UK) at 8-hour intervals by an intraperitoneal injection volume of 0.25 mL/100 g body weight during 1 day after MCAO (n=6), according to previous data.<sup>16</sup> A group of C3H/HeN mice was also treated with 10 mg/kg NS398 (a COX-2 inhibitor). NS398 was administered intraperitoneally 3 times; 1 hour before, 1 hour after, and 6 hours after MCAO (n=6), as previously described.<sup>17</sup>

### Infarct Size

Brains were removed 24 hours and 7 days after MCAO, and cut into 7 coronal brain slices 1 mm thick (Brain Matrix, WPI-Europe, Aston, Stevenage, UK), which were stained in 1% TTC (2,3,5-triphenyltetrazolium chloride) in 0.1 mol/L phosphate buffer, and infarct size and volumes (mm<sup>3</sup>) were determined as we have previously described.<sup>16</sup>

### Neurological and Behavioral Characterization

Before euthanization, neurological and behavioral deficits were measured as previously described<sup>18,19</sup> according to a graded scoring system. Each animal was scored for each of the outcomes for approximately 1 minute, and assessment was repeated a further 3 times for consistency. Scores of 0 correspond to a normal neurological status, and lower scores correspond to a behavioral deficit.

### Lipid Peroxidation

Lipid peroxidation was measured by the thiobarbituric acid test for malondialdehyde in accordance with the method described by Das and Ratty.<sup>20</sup> The pink chromogen was measured at 532 nm in a Beckman DU-7500 spectrophotometer.

### Protein Expression in Brain Homogenates and in Cytosolic and Nuclear Extracts

Brain cortical tissue was collected from the infarcted and surrounding areas. For determination of iNOS, COX-2, and interferon regulatory factor 1 (IRF-1) protein expression levels, mice were euthanized 24 hours after MCAO. Brain areas that corresponded to the infarct and surrounding area were collected and homogenized as previously described.<sup>21</sup> IRF-1 was determined in cytosolic and nuclear extracts that were prepared as described<sup>21</sup> and obtained from brains of mice euthanized 24 hours after MCAO.

### Western Blot Analysis

Samples that contained 40  $\mu$ g of protein were loaded, and the proteins were size-separated in 7% to 10% SDS-polyacrylamide gel electrophoresis (110 mA). Proteins were blotted onto a PVDF membrane (Hybond-P, Amersham Biosciences Europe GmbH, Freiburg, Germany) and incubated with specific primary antibodies against iNOS (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif; 1:500), COX-2, and IRF-1 (Santa Cruz Biotechnology, Inc, 1:1000). Proteins recognized by the antibody were revealed by the ECL kit according to manufacturer instructions (Amersham Biosciences). The results reflect data from 6 to 8 different blots from 3 to 4 different animals.  $\beta$ -actin and Sp1 levels were used as loading controls for cytosolic and nuclear protein expression, respectively.

### Brain NO<sub>x</sub><sup>-</sup> (NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup>) Levels

NO production was estimated from the amounts of its stable metabolites nitrite (NO<sub>2</sub><sup>-</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>) in brain tissue, as found by a colorimetric assay based on the Griess reaction<sup>22</sup> in a Thermo-max microplate reader (Molecular Devices, Sunnyvale, Calif).

### Brain Prostaglandin E<sub>2</sub> Levels

Brain prostaglandin E<sub>2</sub> levels were determined in cortical homogenates by an EIA kit (Amersham Biosciences) after sample purification (Amprep minicolumns, Amersham Biosciences), according to manufacturer instructions.

### Gelatin Zymography

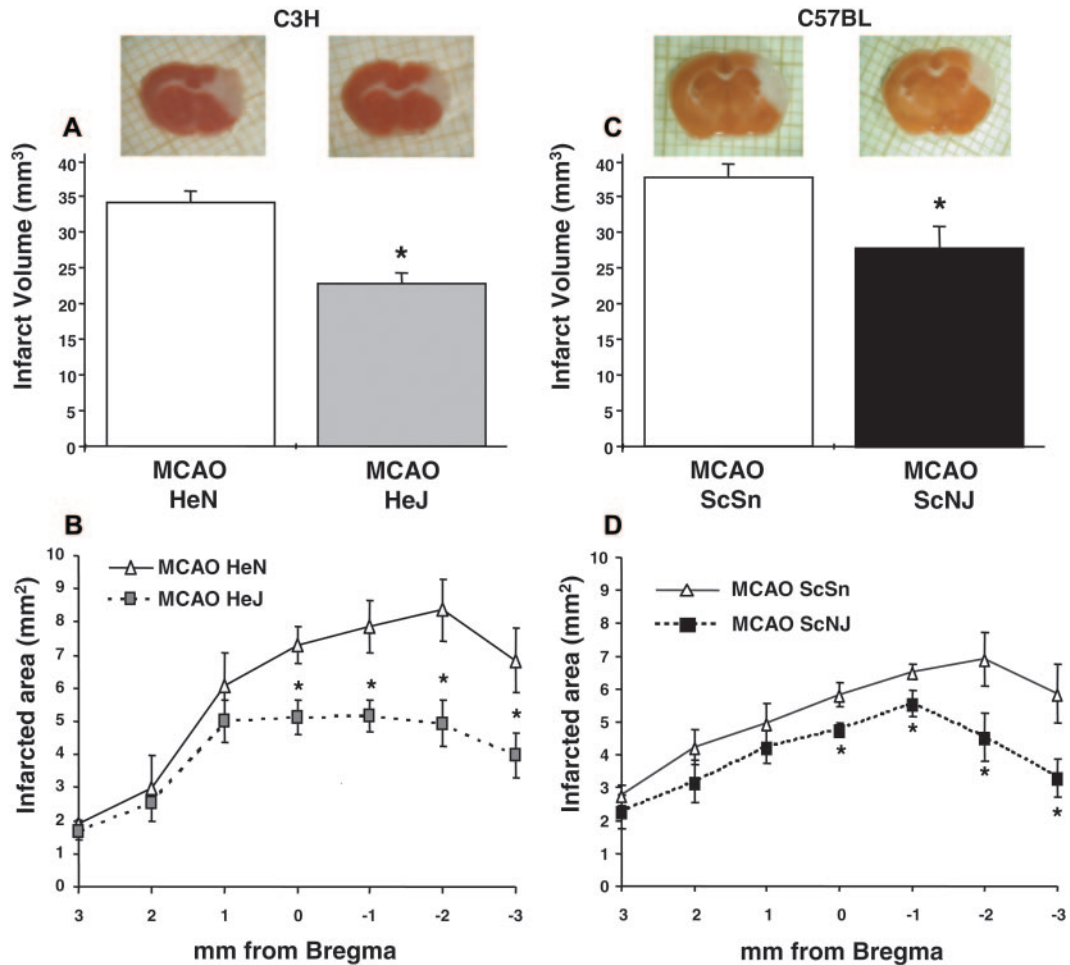
Gelatin zymography was performed as previously described.<sup>21</sup> The gelatinolytic activities were detected as transparent bands against the background of Coomassie blue-stained gelatin. MMP-9 was identified by its molecular weight when compared with standards.

### Brain Concentrations of Interleukin-1 $\beta$ , Tumor Necrosis Factor- $\alpha$ , Interferon- $\alpha$ , Interferon- $\beta$ , and Interferon- $\gamma$

Supernatants from brain homogenates were used for determination with commercially available mouse ELISA kits (Biotrak ELISA System, Amersham Biosciences, for interleukin-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$ ; R&D Systems, Minneapolis, Minn, for IFN- $\alpha$  and IFN- $\beta$ ), according to the manufacturer instructions.

### Immunohistochemistry

Mice were anesthetized 24 hours after MCAO with sodium pentobarbital and perfused through the left ventricle with 25 mL of 0.2 mol/L sodium phosphate buffer as a vascular rinse followed by 50 mL of fixative solution that contained 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4. The brains were removed, postfixed for 4 hours in the same solution at room temperature, and then cryoprotected by immersion overnight at 4°C in a 0.1 mol/L phosphate buffer that contained 30% sucrose. Brains were frozen,



**Figure 1.** Infarct outcome in TLR4-deficient mice after pMCAO performed on C3H/HeJ (HeJ) and C57BL/10ScNJ (ScNJ) mice, which lack expression of TLR4, and on C3H/HeN (HeN) and C57BL/10ScSn (ScSn) mice, which express TLR4 normally (control mice). Infarct volume (A and C) and area (B and D) were determined 24 hour after the insult. Data are mean $\pm$ SEM; n=14; \* $P$ <0.05 versus HeN or ScSn, respectively (see Methods). Photographs of brain slices from representative experiments.

and serial frontal sections were cut (8  $\mu$ m thick) with a Leitz sledge microtome. Sections were stained with hematoxylin and eosin to assess abnormal brain morphology. For double immunofluorescence, sections were acetone-permeabilized for 10 minutes at 4°C, and washed in PBS that contained 3% BSA and 0.1% Triton X-100 for 30 minutes. Then sections were incubated in the primary antibody for 1 hour at room temperature for each double-staining: anti-TLR4 (Santa Cruz Biotechnology, Inc; 1:10 dilution) and a mouse antineuron nuclei antibody (NeuN, Chemicon, Temecula, Calif; 1:10 dilution) to identify neurons, a mouse anti-glial fibrillary acidic protein antibody (Chemicon; 1:20 dilution) to identify astrocytes, and a fluorescein-labeled tomato lectin (agglutinin from *Lycopersicon esculentum*; 1:150 dilution) to characterize microglia, macrophages, and endothelium. After sections were washed in PBS, they were incubated in each respective secondary antibody for 1 hour. For TLR4, Cy3-labeled goat anti-rabbit immunoglobulin G was used (Amersham; 1:10 dilution; red color with fluorescence maximum at 670 nm); for NeuN and glial fibrillary acidic protein, the sections were incubated with Cy2-labeled goat anti-mouse immunoglobulin G (Amersham; 1:10 dilution; green color with fluorescence maximum at 506 nm). Visualization was performed under a fluorescence microscope (Eclipse TE300, Nikon Corp., Tokyo, Japan) with Plan Fluor  $\times$ 20/0.45 or  $\times$ 40/0.6 objectives and phase optics, a Nikon B2A filter for fluorescein isothiocyanate, and Cy2 fluorescence or a Nikon G2A filter for Cy3 fluorescence. The areas selected corresponded to regions in the proximity to the occluded vessel from both cortex and striatum. Each experiment was performed in duplicate

and repeated 3 times. Image acquisition was performed with a laser-scanning confocal imaging system (MRC1024, BioRad, Hempstead, UK).

### Statistical Analysis

Results are expressed as mean $\pm$ SEM of the indicated number of experiments; statistical analysis involved one-way ANOVA (or the Kruskal-Wallis test when the data were not normally distributed) followed by individual comparisons of means (Student-Newman-Keuls, or the Dunn method when the data were not normally distributed).  $P$ <0.05 was considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

## Results

### Infarct Outcome in TLR4-Deficient Mice After pMCAO

Permanent MCAO was performed on C3H/HeJ (HeJ) and C57BL/10ScNJ (ScNJ) mice, which lack expression of TLR4, and on C3H/HeN (HeN) and C57BL/10ScSn (ScSn) mice, which express TLR4 normally (control mice). TLR4-deficient mice had significantly smaller infarct volume (Figure 1A and 1C) and infarct area (Figure 1B and 1D) 24 hours

**Neurological and Behavioral Status of TLR4-Deficient Mice [C3H/HeJ (HeJ); C57BL/10ScNJ (ScNJ)] Exposed to pMCAO**

	Control (HeN=HeJ) (ScSn=ScNJ)	MCAO C3H HeN	MCAO C3H HeJ	MCAO C57BL ScSn	MCAO C57BL ScNJ
Neurological score (points)					
No deficit (0)	12 of 14	0 of 14	1 of 14	0 of 7	0 of 7
Flexion (1)	2 of 14	3 of 14	11 of 14	2 of 7	7 of 7
Circling (2)	0 of 14	11 of 14	2 of 14	5 of 7	0 of 7
Spontaneous circling (3)	0 of 14	0 of 14	0 of 14	0 of 7	0 of 7
No activity (4)	0 of 14	0 of 14	0 of 14	0 of 7	0 of 7
Mean score	0.1	1.78	1.07*	1.7	1.0*
Behavior (points)					
Body position (0 to 4)	4	2	2.66	2.66	3.16
Spontaneous activity (0 to 3)	3	2.16	2.5	1.5	2.33
Transfer arousal (0 to 5)	2.83	2.5	3	3.16	3.5
Gait (0 to 3)	3	3	3	3	3
Touch escape (0 to 3)	2.83	2	2.33	1.5	2.33
Positional passivity (0 to 4)	4	3	3	2.5	3
Mean score	19.66	14.66	16.50*	14.33	17.33*

Data are shown as the number of animals that showed each neurological score vs total number of animals per group, or as mean score per behavior.

\* $P < 0.05$  vs HeN or ScSn, respectively (n=7 to 14). See Methods for details.

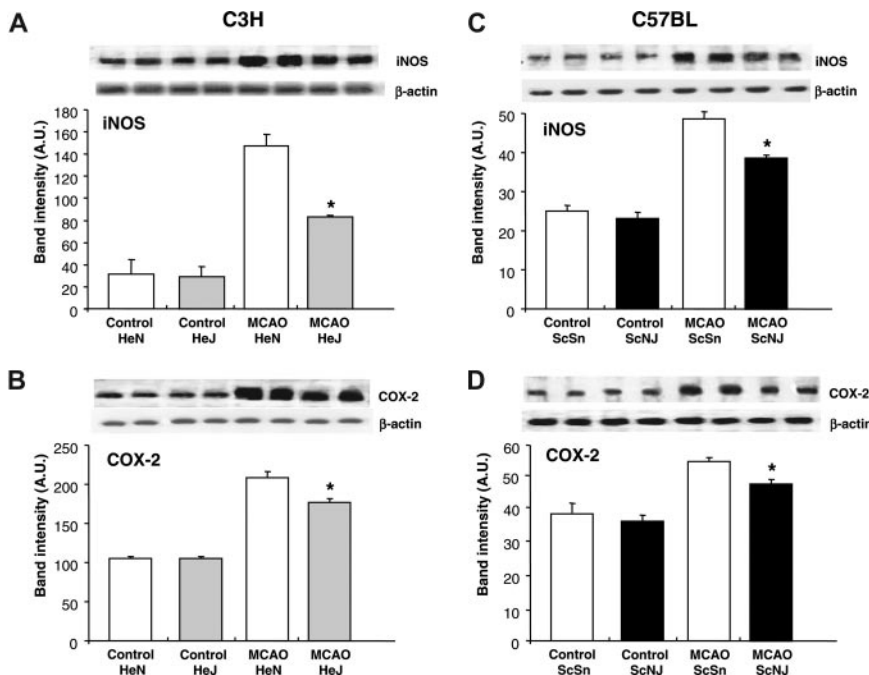
after ischemic injury when compared with control mice (25% and 23% reductions, respectively;  $P < 0.05$ ). When infarct volume was measured 7 days after occlusion, the decrease in infarct size persisted (HeN:  $33.1 \pm 3.0$  versus HeJ:  $24.5 \pm 0.4$  mm<sup>3</sup>; ScSn:  $29.8 \pm 0.6$  versus ScNJ:  $24.1 \pm 1.1$  mm<sup>3</sup>; 26% and 19.5% reductions, respectively;  $P < 0.05$ ).

In addition, TLR4-deficient mice showed an improved neurological and behavioral outcome (Table 1) after MCAO when compared with control mice ( $P < 0.05$ ). When the tests were determined 7 days after the insult, the improvement

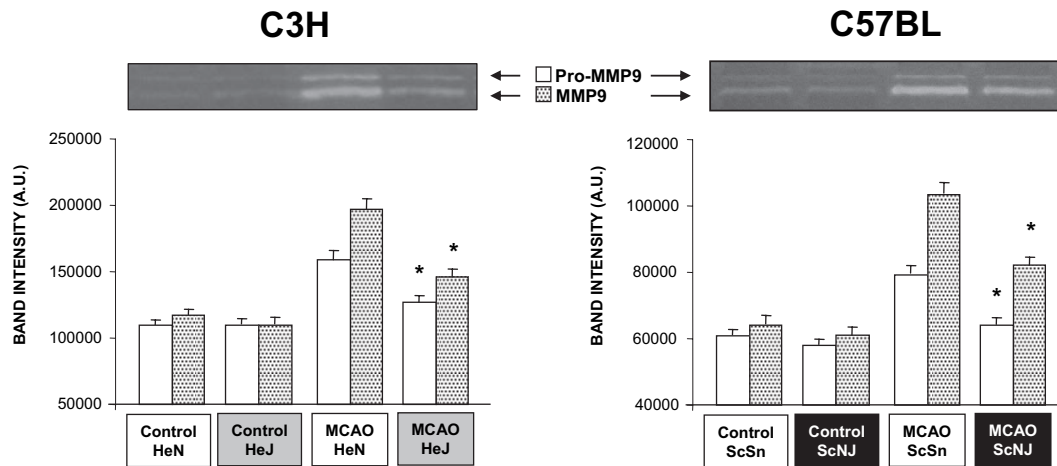
persisted in both neurological (HeN=ScSn: 1.7 versus HeJ=ScNJ: 1.0;  $P < 0.05$ ) and behavioral outcomes (HeN: 15.0 versus HeJ: 17.5; ScSn: 15.5 versus ScNJ: 17.5;  $P < 0.05$ ).

**Expression of iNOS and COX-2 in TLR4-Deficient Mice After pMCAO**

Expression of the enzymes iNOS (Figure 2A and 2C) and COX-2 (Figure 2B and 2D) was studied, as both are described to mediate inflammatory damage after stroke.<sup>1</sup> MCAO caused



**Figure 2.** Expression of iNOS (A and C) and COX-2 (B and D) enzymes in TLR4-deficient mice after pMCAO were analyzed in brains from TLR4-deficient mice [C3H/HeJ (HeJ); C57BL/10ScNJ (ScNJ)] and from mice that express TLR4 normally [C3H/HeN (HeN); C57BL/10ScSn (ScSn)] under normal conditions (control) and 24 hours after ischemia (MCAO). Blots show Western blot analyses in brain homogenates, and the corresponding lower panels show the densitometric analysis of bands. Data are mean  $\pm$  SEM; n=6 to 8; \* $P < 0.05$  versus MCAO HeN or MCAO ScSn, respectively (see Methods). AU indicates arbitrary units.



**Figure 3.** Expression of the proform and the active form of MMP9 enzyme in TLR4-deficient mice after pMCAO was analyzed in brains from TLR4-deficient mice [C3H/HeJ (HeJ); C57BL/10ScSn (ScSn)] and from mice that express TLR4 normally [C3H/HeN (HeN); C57BL/10ScSn (ScSn)] under normal conditions (control) and 24 hours after ischemia (MCAO). Figure shows a representative zymogram from brain homogenates. Data are mean  $\pm$  SEM;  $n=6$  to  $8$ ; \* $P<0.05$  versus MCAO HeN or MCAO ScSn, respectively (see Methods).

expression of the inflammatory enzymes iNOS and COX-2 in brains from all substrains studied, as shown by their levels 24 hours after the ischemic insult (Figure 2). TLR4 deficient mice had significantly lower expression of both enzymes at the time examined when compared with control mice (Figure 2).

#### Effect of iNOS or COX-2 Inhibition in C3H/HeN Mice After pMCAO

To explore the implication of iNOS in TLR4 signaling, 2 groups of C3H/HeN mice were treated with 20 and 40 mg/kg 1400W at 8-hour intervals during the first day after MCAO. Treatment with 1400W induced partial reductions in the total volume of brain infarct (HeN+1400W 20 mg/kg:  $29.1 \pm 1.0$  and HeN+1400W 40 mg/kg:  $22.5 \pm 2.2$  mm<sup>3</sup>; 17% and 35% reductions versus HeN:  $35.0 \pm 2.1$ , respectively;  $P<0.05$ ), and produced an improvement in both neurological tests (data not shown). Levels of brain NO<sub>x</sub><sup>-</sup> after MCAO were determined as an indicator of NO synthesis. C3H/HeN mice ( $n=8$ ) treated with 1400W at 40 mg/kg had lower levels of NO<sub>x</sub><sup>-</sup> (HeN:  $111.4 \pm 9.2$  versus HeN+1400W 40 mg/kg:  $34.6 \pm 8.1$  pmol/mg protein;  $P<0.05$ ).

To study the role of COX-2 in TLR4 signaling, a group of C3H/HeN mice was treated with 10 mg/kg NS398, administered 1 hour before, 1 hour after, and 6 hours after MCAO. Mice treated with NS398 had smaller infarct volumes (HeN:  $36.0 \pm 2.0$  versus HeN+NS398:  $24.5 \pm 1.2$  mm<sup>3</sup>; 32% reduction;  $P<0.05$ ) and better neurological and behavioral outcomes than control mice (data not shown). Mice treated with NS398 ( $n=6$ ) had also lower levels of brain prostaglandin E<sub>2</sub>, an indicator of COX-2 activity (HeN:  $24.8 \pm 2.1$  versus HeN+NS398 10 mg/kg:  $8.1 \pm 1.3$  pg/mg protein;  $P<0.05$ ).

#### Nitrosative and Oxidative Parameters in TLR4-Deficient Mice After pMCAO

Permanent MCAO caused an increase in brain NO<sub>x</sub><sup>-</sup> in both groups, but C3H/HeJ mice ( $n=8$ ) had lower levels of NO<sub>x</sub><sup>-</sup> than the strain with a normal expression of TLR4 (control HeN=control HeJ:  $22 \pm 15$ ; MCAO HeN:  $108 \pm 10$  versus MCAO HeJ:  $66 \pm 11$  pmol/mg protein;  $P<0.05$ ).

MCAO caused the accumulation of the lipid peroxidation product malondialdehyde in cortex from both substrains of C3H mice at 24 hours after the ischemic insult. TLR4-deficient mice (C3H/HeJ;  $n=8$ ) had less accumulation of malondialdehyde than control mice (control HeN=control HeJ:  $0.08 \pm 0.01$ ; MCAO HeN:  $0.74 \pm 0.01$  versus MCAO HeJ:  $0.61 \pm 0.01$  nmol/mg protein;  $P<0.05$ ).

#### Expression of MMP-9 in TLR4-Deficient Mice After pMCAO

In all the substrains studied, MCAO caused an increase in the levels of the proform and the active form of MMP-9, a MMP that is induced and mediates damage caused by proinflammatory stimuli such as cerebral ischemia.<sup>23</sup> Again, TLR4 deficient mice had significantly reduced expression of both forms of MMP-9 at the time examined when compared with control groups (Figure 3).

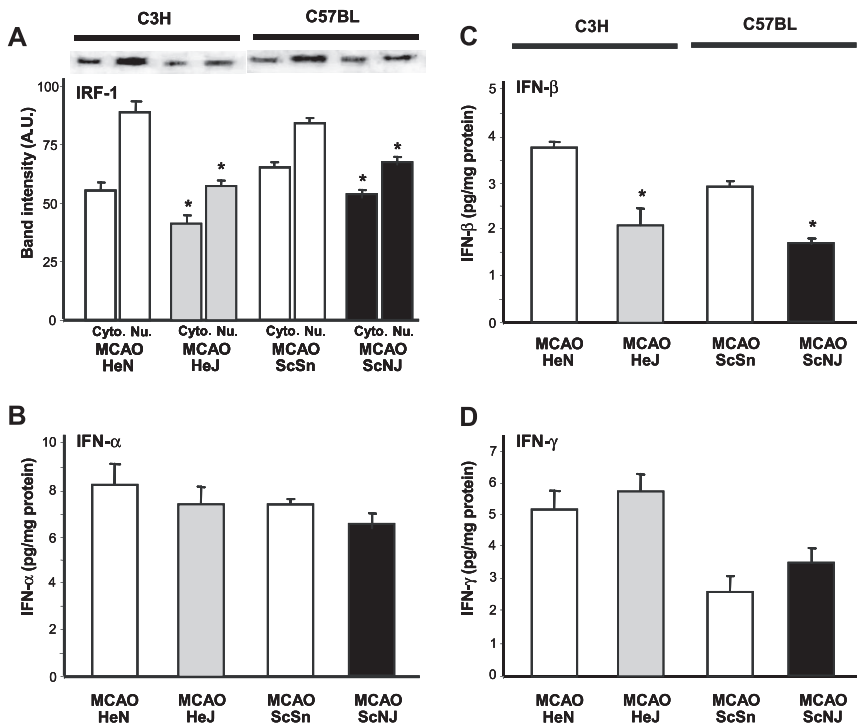
#### Levels of IRF-1, IFN- $\alpha$ , IFN- $\beta$ , and IFN- $\gamma$ in TLR4-Deficient Mice After pMCAO

Experimental ischemia caused activation of IRF-1, as revealed by the augmented nuclear expression of IRF-1 in all mice strains studied (Figure 4A). IRF-1 is a transcription factor that plays a role in IFN gene expression, and contributes to cerebral ischemic damage.<sup>24</sup> TLR4 deficient mice had significantly reduced expression of IRF-1 at the time examined when compared with control mice (Figure 4A).

Additionally, MCAO caused an increase in brain IFN- $\alpha$ , IFN- $\beta$ , and IFN- $\gamma$  concentrations in all groups when measured 24 hours after the ischemic insult (Figure 4). C3H/HeJ and C57BL/10ScNJ mice had lower levels of IFN- $\beta$  than the strains with a normal expression of TLR4 (Figure 4C). However, no differences in IFN- $\alpha$  and IFN- $\gamma$  concentrations were found in TLR4-deficient mice (Figure 4B and 4D).

#### Levels of Interleukin-1 $\beta$ and TNF- $\alpha$ in TLR4-Deficient Mice After pMCAO

MCAO caused an increase in brain interleukin-1 $\beta$  and TNF- $\alpha$  concentrations in all groups when measured 24 hours after the



**Figure 4.** Levels of IRF-1, IFN- $\alpha$ , IFN- $\beta$ , and IFN- $\gamma$  in TLR4-deficient mice after pMCAO. Expression of IRF-1 was analyzed in cytosolic and nuclear extracts (A) and levels of IFN- $\alpha$  (B), IFN- $\beta$  (C), and IFN- $\gamma$  (D) were studied in supernatants. All were obtained from brains of TLR4-deficient mice [C3H/HeJ (HeJ); C57BL/10ScNJ (ScNJ)] and from mice that express TLR4 normally [C3H/HeN (HeN); C57BL/10ScSn (ScSn)] 24 hours after ischemia (MCAO). Data are mean  $\pm$  SEM; n=6; \* $P$ <0.05 versus MCAO HeN or MCAO ScSn, respectively (see Methods). cyto indicates cytosolic; nu, nuclear.

ischemic insult. No differences were found in TLR4-deficient mice when compared with mice with a normal expression of TLR4 (Figure 5).

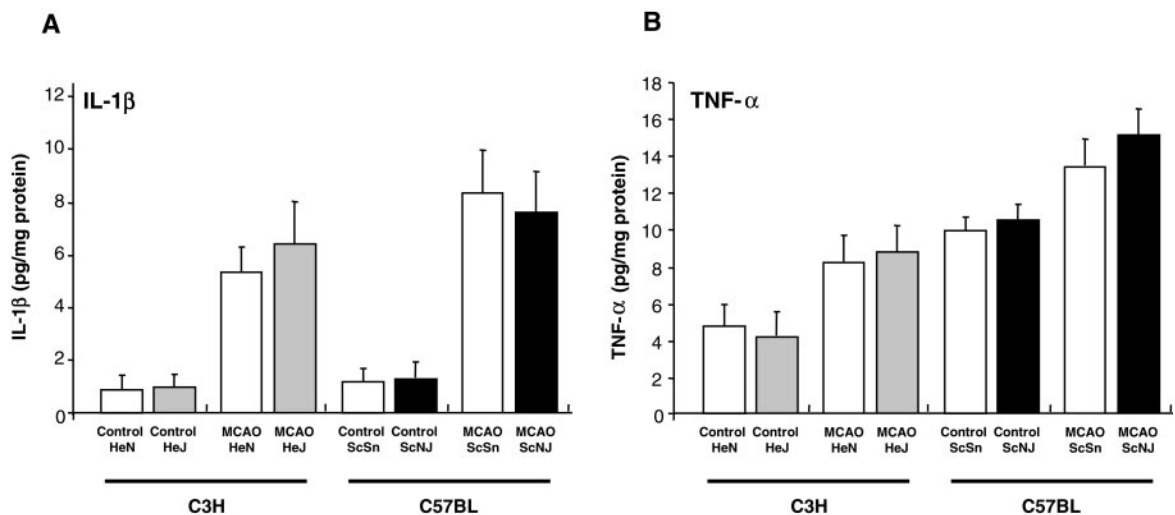
### Brain Morphology and Cellular Expression of TLR4

Both mouse strains (C3H and C57BL) showed a normal brain morphology without any signs of neural defects after staining with hematoxylin and eosin (Figure 6). TLR4 localization was examined by double immunofluorescence staining in mouse brains after MCAO. In C3H/HeN and C57BL/10ScSn mice, double immunostaining showed TLR4 immunoreactivity in astrocytes (glial fibrillary acidic protein-positive cells)

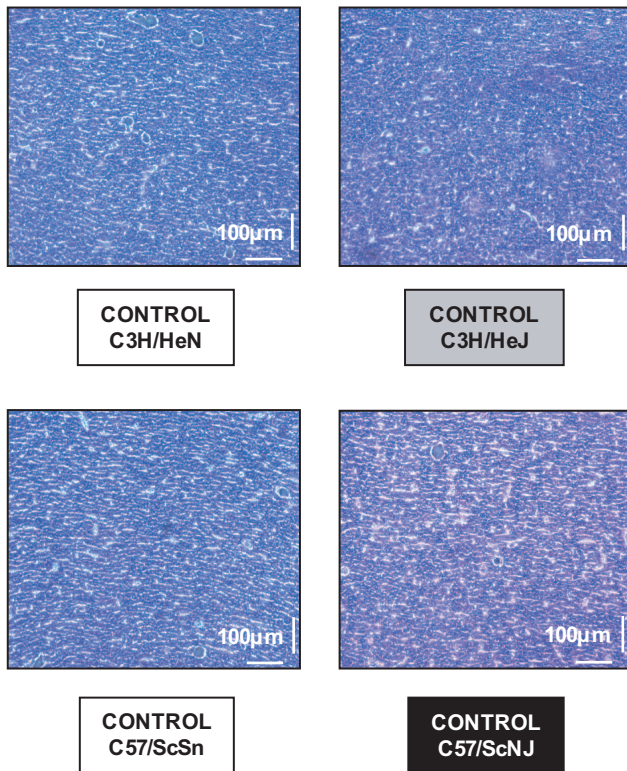
and in cells positive for tomato lectin (microglia, as well as macrophages and blood vessels) 24 hours after the occlusion (Figure 7). However, only C3H/HeJ but not C57BL/10ScNJ showed expression of TLR4 after MCAO, and, once again, TLR4 localization was similar (astrocytes and microglia) to that found in wild-type mice (Figure 7).

### Discussion

This study shows that TLR4-deficient mice have smaller cerebral infarctions and less inflammatory response after an experimental stroke. These data demonstrate that TLR4 signaling is involved in brain damage and in inflammation triggered by ischemic injury.



**Figure 5.** Levels of interleukin-1 $\beta$  (A) and TNF- $\alpha$  (B) in TLR4-deficient mice after pMCAO were analyzed in brains from TLR4-deficient mice [C3H/HeN (HeN); C57BL/10ScSn (ScSn)] and from mice that express TLR4 normally [C3H/HeN (HeN); C57BL/10ScSn (ScSn)] under normal conditions (control) and 24 hours after ischemia (MCAO). Data are mean  $\pm$  SEM; n=6 to 8 (see Methods).



**Figure 6.** Brain morphology in TLR4 mice. Sections obtained from brains of TLR4-deficient mice [C3H/HeJ (HeJ); C57BL/10ScNJ ScNJ] and from mice that express TLR4 normally [C3H/HeN (HeN); C57BL/10ScSn (ScSn)] were stained with hematoxylin and eosin to assess possible abnormal brain morphology. Figure shows a representative photograph from brain sections (see Methods).

We have demonstrated that mice (C3H/HeJ and C57BL/10ScNJ) that lack expression of TLR4 present a significantly better stroke outcome, as shown by a remarkable reduction of the infarct volume and a substantial recovery in the neurological deficits induced by MCAO 24 hours and 7 days after the occlusion compared with mice (C3H/HeN and C57BL/10ScSn) that express TLR4 normally. The improvement in the neurological function was studied with 2 different assessments: a simple neurological test widely used,<sup>18</sup> and a behavioral test that includes the study of sensorimotor deficits.<sup>19</sup>

In the search for mechanisms involved in this effect, we found that MCAO-induced iNOS expression is significantly lower in brains from TLR4-deficient mice than in those from control mice. We have mentioned that TLRs activate nuclear factor  $\kappa$ -B signaling pathways that produce the transcription of many proinflammatory genes and enzymes such as iNOS.<sup>8</sup> It has been described recently that TLR4 mediates iNOS expression in macrophages in a MyD88-independent (TRIF-dependent) manner.<sup>25</sup> Because iNOS mediates cytotoxicity in many cell systems such as the ischemic brain,<sup>1,26</sup> the reduction of the infarct volume found in TLR4-deficient mice might be explained at least in part by the reduced expression of this isoform. In fact, we have demonstrated that mice that express TLR4 normally (C3H/HeN) and received a treatment with 1400W, a specific iNOS inhibitor, after the ischemic insult show a partial protective effect, as indicated by a

recovery in the neurological deficit and a reduction in the infarct volume, which is quantitatively similar to previously reported data.<sup>16,27</sup>

Similar to iNOS, TLR4 is also able to trigger the expression of COX-2 via nuclear factor  $\kappa$ -B translocation.<sup>8,28,29</sup> This enzyme also participates in the ischemic inflammatory cascade and in the subsequent ischemic brain damage.<sup>30–32</sup> In this context, we have found that TLR4-deficient mice have significantly lower expression of COX-2 compared with control mice, which suggests that increased expression of COX-2 may mediate brain damage after ischemia through TLR4-dependent signaling. Indeed, we also demonstrate that treatment with the COX-2 inhibitor NS398 causes a partial neuroprotective effect in mice that express TLR4 normally (C3H/HeN), in agreement with previous results.<sup>17</sup>

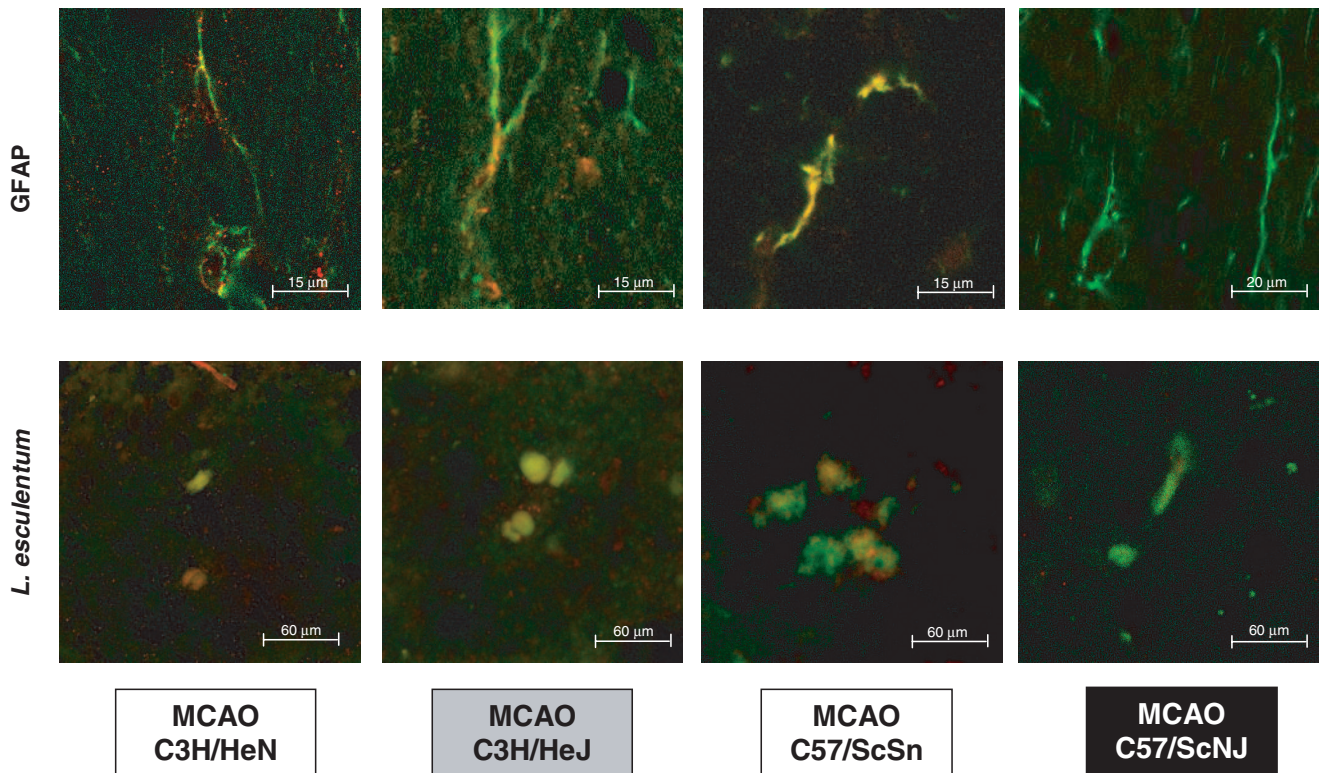
Once iNOS and COX-2 are expressed, the formation of large amounts of oxygen and nitrogen-reactive species might account for the oxidation of cellular components such as membrane lipid peroxidation, which results in the formation of several cytotoxic products such as malondialdehyde.<sup>33</sup> The fact that TLR4-deficient mice present lower levels of malondialdehyde after MCAO compared with control mice supports the hypothesis that TLR4 signaling is involved in inflammation, and subsequent oxidative stress brain damage after ischemic injury.

MMP-9 (gelatinase B) is another inflammatory mediator that contributes to ischemic cerebral damage<sup>23</sup> as it participates in extracellular matrix degradation.<sup>34</sup> It has been shown that MMP-9 participates in the hemorrhagic transformation in acute ischemic stroke in humans.<sup>35</sup> Our data confirm that experimental stroke increases the expression of this MMP in mice in both precursor and active forms. More importantly, TLR4-deficient mice present a lower expression of MMP-9 compared with control mice. It has been reported that increased NO production is necessary for MMP-9 activation,<sup>36–38</sup> which might constitute a potential extracellular proteolysis pathway to neuronal cell death in cerebral ischemia. Given the relevance of both interrelated signaling for cell damage, the dual reduction in iNOS and MMP-9 expression found after MCAO in TLR4-deficient mice is likely to be one of the main mechanisms responsible for the protective effect of the deletion of TLR4-mediated signaling.

The transcription factor IRF-1 is involved in the molecular mechanisms of inflammation and apoptosis, and contributes directly to cerebral ischemia.<sup>24</sup> Our data confirm that experimental stroke increases the expression of IRF-1, and, more interestingly, that TLR4-deficient mice present a lower expression of IRF-1 when compared with control mice. As, it has been reported that the volume of brain injury produced by MCAO is markedly reduced in mice with a null mutation of the IRF-1 gene,<sup>24</sup> our results indicate that the reduction of the infarct volume found in TLR4-deficient mice might be also explained at least in part by the reduced expression of IRF-1. In addition, we have also found that TLR4-deficient mice present lower levels of IFN- $\beta$  but not of IFN- $\alpha$  and IFN- $\gamma$ , which suggests that other members of the IRF family could be also involved.<sup>39</sup>

Our data do not show any differences in the levels of the cytokines interleukin-1 $\beta$  and TNF- $\alpha$  24 hours after MCAO in





**Figure 7.** Cellular localization of TLR4 after pMCAO. Double immunofluorescence staining of brain sections from TLR4-deficient mice [C3H/HeJ (HeJ); C57BL/10ScNj (ScNj)] and from mice that express TLR4 normally [C3H/HeN (HeN); C57BL/10ScSn (ScSn)] of TLR4 (red) and cellular markers (green) GFAP (astrocytes) and *L. esculentum* (microglia). GFAP indicates glial fibrillary acidic protein.

TLR4-deficient mice compared with wild-type substrains. These results suggest that the TLR4 signaling does not affect the activation of these cytokines, although we cannot discard changes at other time points.

Our data strongly support that TLR4 signaling is implicated in ischemic brain damage through the expression of iNOS, COX-2, IRF-1, and MMP9. The decreased expression of inflammatory mediators in TLR4-deficient animals as a neuroprotective mechanism could be reinforced in future studies with the use of other ischemic models and time points with a more pronounced inflammatory response.

We have also showed that TLR4 is expressed in microglia and astrocytes after MCAO, in agreement with previous data that show that this receptor is expressed in microglia<sup>10</sup> and astrocytes<sup>11</sup> after inflammatory stimuli; however, it is not known how ischemia activates TLR. In this context, several reports in the literature have described endogenous and exogenous ligands that activate TLR4 to produce an inflammatory response:<sup>6,9</sup> TLR4 is activated by endogenous ligands from necrotic cells such as heat-shock proteins (HSP60 and HSP70),<sup>40,41</sup> from extracellular matrix components as hyaluronic acid<sup>42</sup> or fibronectin,<sup>43</sup> and from damaged blood vessel such as fibrin and fibrinogen.<sup>44</sup> All these ligands have been described to play a role in stroke and atherosclerosis, and some of them have been found to be independent stroke risk factors.<sup>45</sup>

On the other hand, TLR4 is also activated by exogenous ligands such as lipopolysaccharide from Gram-negative bacteria<sup>14,46,47</sup> and the fusion protein of respiratory syncytial

virus.<sup>48</sup> In this context, it has been reported that chronic infections caused mainly by Gram-negative bacteria might increase the risk of stroke and atherosclerosis.<sup>4,5</sup> Although the precise role of infection is still controversial, *Chlamydia pneumoniae* has been the bacteria most frequently implicated as a risk factor of stroke and also of atherosclerosis, the main cause of ischemic stroke. In fact, a positive association between elevated *C pneumoniae* antibodies indicative of chronic infection and stroke has been reported in several studies.<sup>2,4</sup> Interestingly, our new data suggest that this Gram-negative bacteria would play a role not only as a risk factor for ischemic stroke, but also as a causative agent of worse outcome caused by higher inflammation and brain damage. This would result from overactivation of TLR4 receptor by both exogenous ligand such as lipopolysaccharide and endogenous ligand such as chlamydial HSP-60. Indeed, it has been demonstrated that chlamydial HSP-60 induces the production of TNF- $\alpha$  and MMPs by macrophages,<sup>49</sup> which are mechanisms that might enhance the inflammation and damage found in stroke.

Taking into account that the brain and heart share some cell death pathways after ischemia, the fact that TLR4 signaling has been implicated in myocardial ischemia-reperfusion injury and inflammation<sup>50</sup> also supports its role in the brain after stroke. Finally, the described mutations in the TLR4 gene (Asp299Gly, C119A), which are associated with risk of atherothrombosis, myocardial infarctions, and ischemic stroke in some populations,<sup>12,13</sup> also remark the importance of this signaling pathway in all these pathologies and particularly in stroke.

In summary, to our knowledge, this is the first report to show a direct implication of TLR4 signaling in brain injury and inflammation caused by stroke. Our data confirm that innate immunity participates in the brain damage after stroke. Considering that the immunity response takes place early after the injury, it would be useful to develop new therapies to inhibit TLR4 signal through the use of neutralizing antibodies or drugs with antagonist characteristics to produce a neuroprotective effect.

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### Disclosures

None.

### References

- del Zoppo G, Ginis I, Hallenbeck JM, Iadecola C, Wang X, Feuerstein GZ. Inflammation and stroke: putative role for cytokines, adhesion molecules and iNOS in brain response to ischemia. *Brain Pathol.* 2000;10:95–112.
- Emsley HC, Tyrrell PJ. Inflammation and infection in clinical stroke. *J Cereb Blood Flow Metab.* 2002;22:1399–1419.
- Vila N, Castillo J, Davalos A, Chamorro A. Proinflammatory cytokines and early neurological worsening in ischemic stroke. *Stroke.* 2000;31:2325–2329.
- Lindsberg PJ, Grau AJ. Inflammation and infections as risk factors for ischemic stroke. *Stroke.* 2003;34:2518–2532.
- Paganini-Hill A, Lozano E, Fischberg G, Perez Barreto M, Rajamani K, Ameriso SF, Heseltine PN, Fisher M. Infection and risk of ischemic stroke: differences among stroke subtypes. *Stroke.* 2003;34:452–457.
- Medzhitov R. Toll-like receptors and innate immunity. *Nat Rev Immunol.* 2001;1:135–145.
- Nguyen MD, Julien J-P, Rivest S. Innate immunity: the missing link in neuroprotection and neurodegeneration? *Nat Rev Neurosci.* 2002;3:216–227.
- Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature.* 1997;388:394–397.
- Zuany-Amorim C, Hastewell J, Walker C. Toll-like receptors as potential therapeutic targets for multiple diseases. *Nat Rev Drug Discov.* 2002;1:797–807.
- Lehnardt S, Lachance C, Patrizi S, Lefebvre S, Follett PL, Jensen FE, Rosenberg PA, Volpe JJ, Vartanian T. The toll-like receptor TLR4 is necessary for lipopolysaccharide-induced oligodendrocyte injury in the CNS. *J Neurosci.* 2002;22:2478–2486.
- Bowman CC, Rasley A, Tranguch SL, Marriott I. Cultured astrocytes express toll-like receptors for bacterial products. *Glia.* 2003;43:281–291.
- Zee RY, Hegener HH, Gould J, Ridker PM. Toll-like receptor 4 Asp299Gly gene polymorphism and risk of atherothrombosis. *Stroke.* 2005;36:154–157.
- Li YC, Chang YM, Yu JM, Yen JH, Chang JG, Hu CJ. Toll-like receptor 4 gene C119A but not Asp299Gly polymorphism is associated with ischemic stroke among ethnic Chinese in Taiwan. *Atherosclerosis.* 2005;180:305–309.
- Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in TLR4 gene. *Science.* 1998;282:2085–2088.
- Lamberts KL, Meldgaard M, Ladeby R, Finsen B. A quantitative study of microglial–macrophage synthesis of tumor necrosis factor during acute and late focal cerebral ischemia in mice. *J Cereb Blood Flow Metab.* 2005;25:119–135.
- Pérez-Asensio FJ, Hurtado O, Burguete MC, Moro MA, Salom JB, Lizasoain I, Torregrosa G, Leza JC, Alborch E, Castillo J, Knowles RG, Lorenzo P. Inhibition of iNOS activity by 1400W decreases glutamate release and ameliorates stroke outcome after experimental ischemia. *Neurobiol Dis.* 2005;18:375–384.
- Sugimoto K, Iadecola C. Delayed effect of administration of COX-2 inhibitor in mice with acute cerebral ischemia. *Brain Res.* 2003;960:273–276.
- Hatcher JP, Virley D, Hadingham SJ, Roberts J, Hunter AJ, Parsons AA. The behavioural effect of middle cerebral artery occlusion on apolipoprotein-E deficient mice. *Behav Brain Res.* 2002;131:139–149.
- Hunter AJ, Hatcher J, Virley D, Nelson P, Irving E, Hadingham SJ, Parsons AA. Functional assessments in mice and rats after focal stroke. *Neuropharmacology.* 2000;39:806–816.
- Das NP, Ratty AK. Studies on the effects of the narcotic alkaloids, cocaine, morphine, and codeine on nonenzymatic lipid peroxidation in rat brain mitochondria. *Biochem Med Metab Biol.* 1987;37:258–264.
- Pereira MP, Hurtado O, Cárdenas A, Alonso-Escolano D, Bosca L, Vivancos J, Nombela F, Leza JC, Lorenzo P, Lizasoain I, Moro MA. The non-thiazolidinedione PPAR-gamma agonist L-796,449 is neuroprotective in experimental stroke. *J Neuropathol Exp Neurol.* 2005;64:797–805.
- Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannerbaum SR. Analysis of nitrate, nitrite and [15N] in biological fluids. *Anal Biochem.* 1982;126:131–138.
- Mun-Bryce S, Rosenberg GA. Matrix metalloproteinases in cerebrovascular disease. *J Cereb Blood Flow Metab.* 1998;18:1163–1172.
- Iadecola C, Salkowski CA, Zhang F, Aber T, Nagayama M, Vogel SN, Ross ME. The transcription factor interferon regulatory factor 1 is expressed after cerebral ischemia and contributes to ischemic brain injury. *J Exp Med.* 1999;189:719–727.
- Lee JY, Lowell CA, Lemay DG, Youn HS, Rhee SH, Sohn KH, Jang B, Ye J, Chung JH, Hwang DH. The regulation of the expression of inducible nitric oxide synthase by Src-family tyrosine kinases mediated through MyD88-independent signaling pathways of Toll-like receptor 4. *Biochem Pharmacol.* 2005;70:1231–1240.
- Iadecola C, Zhang F, Xu S, Casey R, Ross ME. Inducible nitric oxide synthase gene expression in brain following cerebral ischemia. *J Cereb Blood Flow Metab.* 1995;15:378–384.
- Parmentier S, Böhme GA, Lerouet D, Damour D, Stutzmann J-M, Margail I, Plotkine M. Selective inhibition of inducible nitric oxide synthase prevents ischaemic brain injury. *Br J Pharmacol.* 1999;127:546–552.
- Rhee SH, Hwang D. Murine Toll-like receptor 4 confers lipopolysaccharide responsiveness as determined by activation of NF-kappa B and expression of the inducible cyclooxygenase. *J Biol Chem.* 2000;275:34035–34040.
- Lee JY, Sohn KH, Rhee SH, Hwang D. Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. *J Biol Chem.* 2001;276:16683–16689.
- Planas AM, Soriano MA, Rodríguez-Farre E, Ferrer I. Induction of cyclooxygenase-2 mRNA and protein following transient focal ischemia in rat brain. *Neurosci Lett.* 1995;200:187–190.
- Collaco-Moraes Y, Aspey B, Harrison M, de Bellerocche J. Cyclo-oxygenase-2 messenger RNA induction in focal cerebral ischemia. *J Cereb Blood Flow Metab.* 1996;16:1366–1372.
- Nogawa S, Zhang F, Ross E, Iadecola C. Cyclo-oxygenase-2 gene expression in neurons contributes to ischemic brain damage. *J Neurosci.* 1997;17:2746–2755.
- Mattson MP. Modification of ion homeostasis by lipid peroxidation: roles in neuronal degeneration and adaptive plasticity. *Trends Neurosci.* 1998;21:53–57.
- Asahi M, Wang X, Mori T, Sumii T, Jung JC, Moskowitz MA, Fini ME, Lo EH. Effects of matrix metalloproteinase-9 gene knockout on the proteolysis of blood-brain barrier and white matter components after cerebral ischemia. *J Neurosci.* 2001;21:7724–7732.
- Castellanos M, Leira R, Serena J, Pumar JM, Lizasoain I, Castillo J, Davalos A. Plasma metalloproteinase-9 concentration predicts hemorrhagic transformation in acute ischemic stroke. *Stroke.* 2003;34:40–46.
- Gu Z, Kaul M, Yan B, Kridel SJ, Cui J, Strongin A, Smith JW, Liddington RC, Lipton SA. S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. *Science.* 2002;297:1186–1190.
- Marcet-Palacios M, Graham K, Cass C, Befus AD, Mayers I, Radomski MW. Nitric oxide and cyclic GMP increase the expression of matrix metalloproteinase-9 in vascular smooth muscle. *J Pharmacol Exp Ther.* 2003;307:429–436.
- Mayers I, Hurst T, Radomski A, Johnson D, Fricker S, Bridger G, Cameron B, Darkes M, Radomski MW. Increased matrix metallopro-

- teinase activity after canine cardiopulmonary bypass is suppressed by a nitric oxide scavenger. *J Thorac Cardiovasc Surg.* 2003;125:661–668.
39. Honda K, Taniguchi T. IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. *Nat Rev Immunol.* 2006;6:644–658.
  40. Ohashi K, Burkart V, Flohe S, Kolb H. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J Immunol.* 2000;164:558–561.
  41. Bulut Y, Faure E, Thomas L, Karahashi H, Michelsen KS, Equils O, Morrison SG, Morrison RP, Arditi M. Chlamydial heat shock protein 60 activates macrophages and endothelial cells through Toll-like receptor 4 and MD2 in a MyD88-dependent pathway. *J Immunol.* 2002;168:1435–1440.
  42. Termeer C, Benedix F, Sleeman J, Fieber C, Voith U, Ahrens T, Miyake K, Freudenberg M, Galanos C, Simon JC. Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. *J Exp Med.* 2002;195:99–111.
  43. Okamura Y, Watari M, Jerud ES, Young DW, Ishizaka ST, Rose J, Chow JC, Strauss JF 3rd. The extra domain A of fibronectin activates Toll-like receptor 4. *J Biol Chem.* 2001;276:10229–10233.
  44. Smiley ST, King JA, Hancock WW. Fibrinogen stimulates macrophage chemokine secretion through toll-like receptor 4. *J Immunol.* 2001;167:2887–2894.
  45. Wilhelmssen L, Svardsudd K, Korsan-Bengtson K, Larsson B, Welin L, Tibblin G. Fibrinogen as a risk factor for stroke and myocardial infarction. *N Eng J Med.* 1984;311:501–505.
  46. Qureshi ST, Lariviere L, Leveque G, Clermont S, Moore KJ, Gros P, Malo D. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4) [published correction appears in *J Exp Med.* 1999;189:1518]. *J Exp Med.* 1999;189:615–625.
  47. Laflamme N, Rivest S. Toll-like receptor 4: the missing link of the cerebral innate immune response triggered by circulating gram-negative bacterial cell wall components. *FASEB J.* 2001;15:155–163.
  48. Kurt-Jones EA, Popova L, Kwinn L, Haynes LM, Jones LP, Tripp RA, Walsh EE, Freeman MW, Golenbock DT, Anderson LJ, Finberg RW. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol.* 2000;1:398–401.
  49. Kol A, Sukhova GK, Lichtman AH, Libby P. Chlamydial heat shock protein 60 localizes in human atheroma and regulates macrophage tumor necrosis factor-alpha and matrix metalloproteinase expression. *Circulation.* 1998;98:300–307.
  50. Oyama J, Blais C Jr, Liu X, Pu M, Kobzik L, Kelly RA, Bourcier T. Reduced myocardial ischemia-reperfusion injury in toll-like receptor 4-deficient mice. *Circulation.* 2004;109:784–789.

### CLINICAL PERSPECTIVE

The innate immune system is able to recognize many pathogens, even without prior exposure, that trigger specific immune responses such as, among others, the synthesis of inflammatory cytokines and the production of reactive species that induce oxidative stress. In this context, toll-like receptors (TLRs) are key components of the innate immune system, and function as receptors that recognize ligands from microbial products, but also from damaged cells and tissues, and thus act as an endogenous danger signal. Innate immune response is largely mediated by leukocytes, such as neutrophils, macrophages, and dendritic cells. Interestingly, some evidence indicates that innate immunity and subsequent inflammatory mechanisms participate in several facets of brain injury. Taking into account that stroke is the one of the leading causes of death, and the main cause of severe long-term disability in adults, and that treatment is often limited to fibrinolysis, a therapy useful to a very low percentage of patients, immunomodulation strategies arise as a possible powerful approach for stroke treatment. Among TLRs, TLR4 is the most frequently observed and best-characterized receptor. Our data show that TLR4-deficient mice have minor infarctions and less inflammatory response after an ischemic insult. The present results also demonstrate that TLR4 signaling is involved in ischemic brain damage through the expression of the inflammatory mediators inducible nitric oxide synthase, cyclooxygenase-2, interferon-regulatory factor 1, and matrix metalloproteinase-9. Considering that the immune response takes place early after the injury, the development of therapies capable of TLR4 signal inhibition could provide a potent neuroprotective effect for acute stroke treatment.