Key Role of CD36 in Toll-Like Receptor 2 Signaling in Cerebral Ischemia

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Background and Purpose—Toll-like receptors (TLRs) and the scavenger receptor CD36 are key molecular sensors for the innate immune response to invading pathogens. However, these receptors may also recognize endogenous “danger signals” generated during brain injury, such as cerebral ischemia, and trigger a maladaptive inflammatory reaction. Indeed, CD36 and TLR2 and 4 are involved in the inflammation and related tissue damage caused by brain ischemia. Because CD36 may act as a coreceptor for TLR2 heterodimers (TLR2/1 or TLR2/6), we tested whether such interaction plays a role in ischemic brain injury.

Methods—The TLR activators FSL-1 (TLR2/6), Pam3 (TLR2/1), or lipopolysaccharide (TLR4) were injected intracerebroventricularly into wild-type or CD36-null mice, and inflammatory gene expression was assessed in the brain. The effect of TLR activators on the infarct produced by transient middle cerebral artery occlusion was also studied.

Results—The inflammatory response induced by TLR2/1 activation, but not TLR2/6 or TLR4 activation, was suppressed in CD36-null mice. Similarly, TLR2/1 activation failed to increase infarct volume in CD36-null mice, whereas TLR2/6 or TLR4 activation exacerbated postischemic inflammation and increased infarct volume. In contrast, the systemic inflammatory response evoked by TLR2/6 activation, but not by TLR2/1 activation, was suppressed in CD36-null mice.

Conclusions—In the brain, TLR2/1 signaling requires CD36. The cooperative signaling of TLR2/1 and CD36 is a critical factor in the inflammatory response and tissue damage evoked by cerebral ischemia. Thus, suppression of CD36-TLR2/1 signaling could be a valuable approach to minimize postischemic inflammation and the attendant brain injury. (Stroke. 2010;41:898-904.)

Key Words: inflammation ■ middle cerebral artery occlusion ■ cyclooxygenase-2 ■ stroke ■ microglia

Ischemic brain injury results from the concerted action of multiple pathogenic effectors.1 Although ATP depletion, excitotoxicity, and oxidative stress are key determinants of tissue damage in the early stages, inflammation and apoptosis are involved in the progression of the injury in the late postischemic period.1 Considering that many stroke patients reach medical attention several hours after the onset of symptoms, targeting these late pathogenic events may be of therapeutic value.2 Inflammation is a particularly attractive target because attenuation of the inflammatory response to cerebral ischemia reduces experimental brain injury with a wide therapeutic window.2 However, the signaling pathways initiating postischemic inflammation and mediating the resulting tissue damage are poorly understood.

Toll-like receptors (TLRs), type I membrane proteins that include at least 12 members (TLR1–12), are key players in innate immunity and are involved in the recognition of molecular components of invading bacteria, viruses, and parasites.3 TLRs have also been implicated in ischemic brain injury. In particular, TLR2 and TLR4 are upregulated after middle cerebral artery (MCA) occlusion and contribute to tissue damage by triggering the expression of inflammatory and apoptotic genes via the transcription factors nuclear factor-κB and activator protein-1.4 Thus, in addition to their role in innate immunity, TLRs may also recognize endogenous “danger signals” generated by brain ischemia and initiate a maladaptive immune response that contributes to tissue injury.5 TLR2 has attracted particular interest because of the multiplicity of ligands that it recognizes, a feature attributed to its unique ability to form heterodimers with TLR1 or 6.5 TLR2 signaling also requires CD36, a class B scavenger receptor involved in diverse cellular functions, including inflammatory signaling.6 In monocytes, TLR2/6 heterodimers assemble with CD36 within lipid “rafts,” and the resulting complex is essential for triggering the inflammatory response.7 Interestingly, CD36 is also involved in focal ischemic brain injury, an effect related to suppression of postischemic nuclear factor-κB activation and free-radical production.8,9 However, it is not known whether CD36 is also involved in TLR2 signaling in the setting of postischemic inflammation and, if so, whether the effects of CD36 depend on TRL2/6 or 2/1.
We used specific activators of TLR2/1, TLR2/6, and TLR4 to investigate the role of CD36 in TLR-induced inflammatory signaling in the brain. We found that CD36 is required for the neuroinflammation induced by TLR2/1 activation, but not by TLR2/6 activation, as in the case of systemic inflammation. CD36 is also required for the component of ischemic injury attributable to the inflammatory response triggered by TLR2/1 activation. The findings raise the possibility that the TLR2/1-CD36 complex is a sensor of ischemia-induced danger signals and is critical for the attendant inflammatory response. Thus, the interaction between TLR2/1 and CD36 could be targeted for the development of new stroke therapies.

Materials and Methods

Mice
All procedures were approved by the institutional animal care and use committee of Weill Cornell Medical College. Experiments were performed in 2- to 3-month-old male CD36-/-, cyclooxygenase (COX)-2-/-, or TLR2-null mice (weight, 20 to 22 g). Mice were obtained from in-house colonies and were on a C57BL/6 background.10 C57BL/6 mice were used as wild-type controls.

Intracerebroventricular Injection of TLR Ligands
Mice were anesthetized and placed in a stereotaxic apparatus. After a small hole was drilled in the left parietal bone (coordinates: 0.5 mm posterior to bregma and 1.0 mm lateral from the midline), a glass micropipette was lowered 2.0 mm below the dural surface, and the TLR2/6 ligand FSL-1 (a synthetic lipopeptide corresponding to the N-terminal domain of the lipoprotein LP44 of Mycoplasma salivarium; 0.1 mg/mL in 4 μL; Invivogen),7,11 the TLR2/1 ligand palmitoyl-S-dipalmitoylglycerol (Pam3; 0.1 mg/mL in 4 μL; Invivogen),7,11 the TLR4 ligand lipopolysaccharide (LPS from Salmonella typhimurium; 20 ng in 4 μL; Sigma-Aldrich),11 or vehicle (endotoxin-free saline) was injected. Mice were allowed to recover and then returned to their cages.

Real-Time Polymerase Chain Reaction
The mRNA for proinflammatory genes was examined by real-time polymerase chain reaction in nonischemic mice injected once intracerebroventricularly (ICV) with vehicle or TLR activators, as previously described.9 The following genes were studied: endothelial leukocyte adhesion molecule-1, intercellular adhesion molecule-1, interleukin-6, and monocyte chemotactic protein-1, genes whose expression depends on the transcription factor nuclear factor-kB in the postischemic brain.9,10 Mice were killed 6 hours after intracerebroventricular injection of each TLR ligand or vehicle, and their brains were removed. This time point was selected on the basis of pilot experiments in which the time course of mRNA upregulation was studied (data not shown). The right hemisphere was frozen and then expressed relative to the respective control condition.9

MCA Occlusion
Procedures for transient MCA occlusion were identical to those previously described and are only summarized.5–10,12 Mice were anesthetized with isoflurane (1.5% to 2%). A fiberoptic probe was glued to the parietal bone and connected to a laser Doppler flowmeter (Periflux System 5010) for cerebral blood flow monitoring. A 6-0 monofilament surgical suture was inserted into the external carotid artery, advanced into the internal carotid artery, and wedged into the Circle of Willis to obstruct the origin of the MCA. The filament was left in place for 25 minutes and then withdrawn. Only animals that exhibited a 85% reduction in cerebral blood flow during MCA occlusion and in which cerebral blood flow recovered by 80% after 10 minutes of reperfusion were included in the study (see supplemental Figure I, available online at http://stroke.ahajournals.org).8–10,12 Twelve percent of the mice failed the inclusion criteria. In MCA occlusion experiments, TLR ligands or vehicle was administered intracerebroventricularly once 5 to 10 minutes after reperfusion. In all mice, rectal temperature was kept at 37.0±0.5°C during surgery and in the recovery period until the animals regained consciousness. Overall mortality was 11%. To test forepaw strength, animals were made to grasp with the forepaws a thin wire suspended above a padded surface, and the latency to fall was recorded (hanging wire test). The test was repeated 3 times for each animal with a 5-minute rest between trials, and the scores from 3 trials were averaged.9

Measurement of Infarct Volume
As described in detail elsewhere,8–10,12 3 days after ischemia, brains were removed, frozen, and sectioned (30-μm thickness) in a cryostat. Brain sections were collected at 600-μm intervals and stained with cresyl violet. Infarct volume was determined with an image analyzer (MCID, Imaging Research Inc) and corrected for swelling, as previously described.5–10,12

Immunohistochemistry
Three days after MCA occlusion, mice were euthanized with an overdose of sodium pentobarbital (120 mg/kg IP) and perfused transcardially with saline. Brain sections (14-μm thickness) were cut in a cryostat, collected at the same rostrocaudal levels as used for determination of infarct volume, and fixed in methanol or Carnoy fixative. Sections processed for myeloperoxidase (MPO) immunocytochemistry were treated with H2O2 to inhibit endogenous peroxidases. Sections were incubated overnight with primary antibodies (MPO; 1:100, EMD Biosciences; glial fibrillary acidic protein (GFAP), 1:1000, Sigma-Aldrich; F4/80, 1:200, AbD Serotec), followed by biotinylated secondary antibodies (MPO, 1:500, Santa Cruz Biotechnology; GFAP and F4/80, 1:200, AbD Serotec), then by streptavidin–horseradish peroxidase (1:1000, Vector Laboratories) and finally visualized with diaminobenzidine (Vector Laboratories). Sections were examined under a microscope, and MPO- or F4/80-positive cells were counted in the ischemic hemisphere in all rostrocaudal sections, as previously described.5–10,12

Plasma Measurement of TNF-α
Saline, FSL-1 (0.1 mg/kg), Pam3 (2.5 mg/kg), or LPS (0.5 mg/kg IP) was administrated to C57BL6 or CD36-null mice in which the MCA was not occluded. Two hours later, blood was collected by cardiac puncture, and plasma was assayed for tumor necrosis factor (TNF)-α by ELISA (eBioscience).
Statistical Analysis
Data are presented as mean±SEM. Comparisons between 2 groups were statistically evaluated by the Student *t* test. Multiple comparisons were evaluated by ANOVA followed by a Newman-Keuls multiple comparison test. Differences were considered significant at *P*<0.05.

Results

**TLR2/1 Ligand Pam3 Does Not Induce Inflammatory Gene Expression in CD36-Null Mice**

We used the TLR2/1 ligand Pam3, the TLR2/6 ligand FSL-1, and the TLR4 ligand LPS (Figure 1) to examine the role of CD36 in the inflammatory signaling mediated by these TLR complexes in mice without MCA occlusion. In wild-type mice, intracerebroventricular injection of Pam3, FSL-1, or LPS upregulated intercellular adhesion molecule-1, endothelial leukocyte adhesion molecule-1, interleukin-6, and monocyte chemotactic protein-1 mRNAs (Figure 2). In CD36-null mice, Pam3 failed to upregulate these genes, whereas FSL-1 elicited a response comparable to that in wild-type mice (Figure 2). However, in TLR2-null mice, both Pam3 and FSL-1 failed to upregulate these genes (Figure 2). LPS induced a normal response in both CD36- and TLR2-null mice (Figure 2). Therefore, the inflammatory signaling induced by TLR2/1 activation in the normal brain requires CD36.

**TLR2/1 Ligand Pam3 Does Not Increase Stroke Volume in CD36-Null Mice**

Next, we sought to determine whether activation of TLR2/1 (Pam3), TLR2/6 (FSL-1), or TLR4 (LPS) after cerebral ischemia enhances the volume of the infarct in CD36- or TLR2-null mice. In wild-type mice, Pam3, FSL-1, or LPS did not increase infarct volume (Figure 3A). To determine whether TLR ligands accelerate the pace of brain injury,13 we investigated the effect of LPS on infarct volume assessed 8 hours after ischemia. LPS did not increase infarct volume at this early time point (supplemental Figure II, available online at http://stroke.ahajournals.org), suggesting that LPS does not accelerate injury development. In contrast to wild-type mice, FSL-1 and LPS, but not Pam3, increased infarct volume and motor deficits in CD36-null mice (Figure 3B, 3E, and 3F), but only LPS increased injury in TLR2-null mice (Figure 3C). In contrast, Pam3 increased stroke volume in COX-2-null mice (Figure 3D), providing a positive control for the lack of effect of this ligand in CD36- and TLR2-null mice.

**TLR2/1 Ligand Pam3 Does Not Enhance the Cellular Inflammatory Response to Ischemia in CD36-Null Mice**

These experiments sought to establish whether TLR2/1 or 2/6 activation enhances the cellular inflammatory response to ischemia.
cerebral ischemia in CD36-null mice. In wild-type mice, MCA occlusion upregulated the astrocytic marker GFAP, induced microglial activation (assessed by the microglial marker F4/80), and induced infiltration of MPO-positive neutrophils (Figure 4A through 4C). This cellular inflammatory response was attenuated in CD36-null mice (Figure 4A through 4C). However, FSL-1 administration increased these inflammatory markers in CD36-null mice, whereas Pam3 had no effect (Figure 4A through 4C).

In Systemic Inflammation, TLR2/6 Signaling Depends on CD36

Finally, we sought to confirm that CD36 signaling relies on TLR2/1 or 2/6 in systemic inflammation. To achieve this goal, we administered TLR ligands intraperitoneally in normal mice and assessed TNF-α production in plasma.14 In this model, TNF-α is secreted mainly by monocytic cells in peripheral organs.11 In wild-type mice, FLS-1 (TLR2/6), Pam3 (TLR2/1), and LPS (TLR4) increased plasma TNF-α (Figure 5). However, in CD36-null mice, FSL-1 failed to increase TNF-α, whereas Pam3 and LPS elicited a strong TNF-α response (Figure 5).

Discussion

We have demonstrated that activation of TLR2/1, 2/6, and 4 induces inflammatory gene expression in the brains of wild-type mice. In contrast, TLR2/2 activation failed to induce an inflammatory response in CD36-null mice. Furthermore, TLR2/1 activation did not increase infarct volume after MCA occlusion, whereas activation of TLR2/6 or TLR4 was able to do so. CD36-null mice exhibited reduced microglial activation and neutrophil infiltration after MCA occlusion, which was increased by TLR2/6, but not by TLR2/1, activation. In contrast to brain inflammation and consistent with previous reports,7,11 in systemic inflammation CD36 was involved in TLR2/6, but not TLR2/1, signaling. These observations suggest that in the brain, unlike the periphery, TLR2/1 signaling requires CD36 and the association between TLR2/1 and CD36 is a major factor in postischemic inflammation and the related brain damage.

The findings of the present study cannot be attributed to differences in the background strains of the null mice because CD36-, TLR2-, and COX-2–null mice were congenic with the C57BL/6 strain. Furthermore, the results cannot be attributed to a lack of specificity of the TLR2/1 and 2/6 ligands, because Pam3 and FSL-1 did not induce inflammatory signaling in TLR2/2-null mice. This observation also rules out that our findings are related to contamination of the TLR activators with pathogen-associated molecules, as suggested for heat shock proteins.15 Finally, compensatory changes resulting in reductions in TLR4 expression or function in CD36- and TLR2-null mice are unlikely to play a role, because the TLR4 activator LPS also induced inflammatory signaling in these mice. The observation that the TLR2/1 activator Pam3 did not exacerbate ischemic injury in CD36- and TLR2-null mice, but was effective in COX-2–null mice, also attests to the specificity of the effect of this activator.
The endogenous molecules that activate TLR2/1 after cerebral ischemia remain to be identified. Cerebral ischemia produces an extraordinarily large number of TLR ligands, including modified lipids, heat shock proteins, the nuclear protein high-mobility group box 1, and amyloid-β. In particular, triacylated lipoproteins are specific ligands for the TLR2/1 complex, which, as shown here, requires CD36 to initiate postischemic inflammatory signaling and could be produced after cerebral ischemia. On the other hand, specific CD36 ligands, such as oxidized LDL, are also upregulated after ischemia and could initiate inflammatory signaling independently of TLRs, as demonstrated in transfected HEK293 cells.

In the systemic inflammation induced by TLR activators, CD36 has been shown to interact with TLR2/6, an observation confirmed in the present study. However, we found that in neuroinflammation, CD36 interacts exclusively with TLR2/1. Although this finding suggests that the presumed CD36 binding partners in the brain differ from those in peripheral organs, the basis for such differences is unclear. One possibility is that TLR2/1 is the predominant heterodimer in the brain, whereas TLR2/6 is more abundant in the cells initiating systemic inflammation. Alternatively, brain cells expressing CD36 could express TLR2/1 and not TLR2/6. The latter possibility seems unlikely, because in the brain, as in peripheral organs, CD36 is present in cells of monocytic lineage, that is, microglia, and endothelial cells. Therefore, the reasons for the difference in CD36 signaling partners in neuroinflammation and systemic inflammation remain unclear. Biochemical efforts to determine the TLR dimers expressed in brain cells and their association with CD36 would go a long way to address this issue. It would also be of interest to elucidate the intracellular signaling steps.
mediating the effect of CD36-TLR2/1, the MyD88 adaptor protein being a key target. However, the elucidation of the signaling pathway is complicated by the fact that both TLR2/1 and TLR2/6 use MyD88 as an adaptor protein. Nevertheless, it would be of interest to determine whether postischemic inflammatory signaling depends on MyD88 or is MyD88 independent, as suggested by a recent study.

We found that administration of TLR2 and 4 ligands after MCA occlusion does not increase infarct volume in wild-type mice, whereas TLR activators exacerbate the injury in CD36-, TLR2-, or COX-2–null mice. Others have also reported that TLR activators do not increase the volume of the completed infarct, but the reasons why remain unclear. One possibility is that in wild-type mice, the size of the infarct has reached a maximum and cannot become any larger. However, this possibility seems unlikely in our case, because in our model of temporary cerebral ischemia, the infarct was not maximal and could have been increased by 40% if the MCA were permanently occluded. Another possibility is that in wild-type mice, TLR ligands are already present at saturating concentrations and that administration of exogenous ligands cannot enhance inflammatory signaling further. In contrast, in CD36-, TLR2-, or COX-2–null mice, ischemic injury is attenuated, and the tissue damage is insufficient to generate saturating levels of TLR ligands. Consequently, exogenous TLR ligands are able to exacerbate the damage because endogenous levels are not maximal. The observations that the TLR4 ligand LPS increases brain injury in small, but not in larger, infarcts support this scenario. Intracallosal injection of LPS accelerates the development of inflammation are suppressed in CD36-null mice after middle cerebral artery occlusion.

In conclusion, we have demonstrated that TLR2/1 inflammatory signaling in the posts ischemic brain requires the scavenger receptor CD36. Thus, activators of TLR2/1 do not trigger inflammatory gene expression and do not exacerbate ischemic injury in CD36-null mice, whereas TLR2/6 and TLR4 ligands do. The link between CD36 and TLR2/1 is specific for brain inflammation, because in peripheral inflammation, CD36 is required for TLR2/6 signaling. The findings raise the possibility that the TLR2/1-CD36 complex is a critical sensor of danger signals produced by cerebral ischemia. The resulting maladaptive activation of the innate immune response contributes to the expansion of the infarct in the posts ischemic period. The findings provide further evidence that CD36 is a point of convergence of diverse signaling pathways that play a deleterious role in the posts ischemic brain, and, as such, could be a promising target for stroke therapy.

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Disclosures

None.

References


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