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*J Immunol* 2014; 192:4783-4794; Prepublished online 11 April 2014;
doi: 10.4049/jimmunol.1303108

[http://www.jimmunol.org/content/192/10/4783](http://www.jimmunol.org/content/192/10/4783)
Polyinosinic-Polycytidylic Acid Has Therapeutic Effects against Cerebral Ischemia/Reperfusion Injury through the Downregulation of TLR4 Signaling via TLR3

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Recent reports have shown that preconditioning with the TLR3 ligand polyinosinic-polycytidylic acid (poly(I:C)) protects against cerebral ischemia/reperfusion (I/R) injury. However, it is unclear whether poly(I:C) treatment after cerebral I/R injury is also effective. We used mouse/rat middle cerebral artery occlusion and cell oxygen-glucose deprivation models to evaluate the therapeutic effects and mechanisms of poly(I:C) treatment. Poly(I:C) was i.p. injected 3 h after ischemia (treatment group). Cerebral infarct volumes and brain edemas were significantly reduced, and neurologic scores were significantly increased. TNF-α and IL-1β levels were markedly decreased, whereas IFN-β levels were greatly increased, in the ischemic brain tissues, cerebral spinal fluid, and serum. Injuries to hippocampal neurons and mitochondria were greatly reduced. The numbers of TUNEL-positive and Fluoro-Jade B+ cells also decreased significantly in the ischemic brain tissues. Poly(I:C) treatment increased the levels of Hsp27, Hsp70, and Bcl2 and decreased the level of Bax in the ischemic brain tissues. Moreover, poly(I:C) treatment attenuated the levels of TNF-α and IL-1β in serum and cerebral spinal fluid of mice stimulated by LPS. However, the protective effects of poly(I:C) against cerebral ischemia were abolished in TLR3−/− and TLR4−/− mice. Poly(I:C) downregulated TLR4 signaling via TLR3. Poly(I:C) treatment exhibited obvious protective effects 14 d after ischemia and was also effective in the rat permanent middle cerebral artery occlusion model. The results suggest that poly(I:C) exerts therapeutic effects against cerebral I/R injury through the downregulation of TLR4 signaling via TLR3. Poly(I:C) is a promising new drug candidate for the treatment of cerebral infarcts. The Journal of Immunology, 2014, 192: 4783–4794.

Stroke is the most common cause of severe disability and the second most common cause of death in the world (1). Currently, recombinant tissue plasminogen activator (rt-PA) thrombolytic therapy is the only effective treatment for ischemic stroke (2); this paucity of treatment options is related to the fact that the pathophysiological mechanisms of ischemic injury have not been clearly elucidated. Recent research has revealed that inflammation plays an important role in secondary brain insult following cerebral ischemia (3, 4).

TLRs have been demonstrated to play critical roles in the induction of immune and inflammatory responses. Patten-associated molecular patterns and damage-associated molecular patterns (DAMPs) activate TLRs and induce the expression of NF-κB–dependent proinflammatory cytokines and/or type I IFN, and these molecules participate in pathophysiological processes (5). DAMPs released from ischemic neurons can activate the TLR4/MyD88/NF-κB signaling pathway in the neighboring microglia and induce the release of proinflammatory cytokines, which gives rise to the degeneration and apoptosis of neurons (6–8). Recent studies have shown that modification of the TLR-mediated NF-κB signaling pathway significantly attenuates ischemic injuries to the organs. Our group and others have demonstrated that cerebral tissue injury is alleviated in TLR4-deficient mice after cerebral ischemia/reperfusion (I/R) (9, 10), and preconditioning with TLR 2, 4, 7, and 9 ligands can increase tolerance to subsequent ischemia; furthermore, significant reductions in neurologic impairments have also been reported (11–14).

Upon activation by dsRNA viruses, TLR3 recruits the regulatory protein Toll/IL-1R domain–containing adapter inducing IFN-β (TRIF) to activate IFN regulatory factors (IRFs) and NF-κB via a TRIF-dependent signaling pathway (15). TLR3-deficient mice do not exhibit reduced cerebral infarct volumes after cerebral I/R compared with wild-type mice (16). However, recent reports have shown that polyinosinic-polycytidylic acid (poly(I:C)) preconditioning protects against cerebral I/R injury (17, 18). However, it is unclear whether poly(I:C) treatment after cerebral I/R injury is also effective. Additionally, if treatment is effective, the protective mechanisms remain unknown. It has been demonstrated that

References


poly(I:C) attenuates LPS-induced liver injury by downregulating TLR4 expression in macrophages and reducing the levels of TNF-α (19). Therefore, we proposed that postischemia treatment with the TLR3 ligand poly(I:C) would also have protective effects against cerebral I/R injury, which could be mediated by activation of TLR3 and subsequent downregulation of the TLR4 signaling pathway. In this study, we used the mouse/rat middle cerebral artery occlusion (MCAO) and cell oxygen-glucose deprivation (OGD) models to evaluate the therapeutic effects and mechanisms of postischemia poly(I:C) treatment. The results suggested that poly(I:C) treatment exerts therapeutic effects against cerebral I/R injury through the downregulation of TLR4 signaling via TLR3. Poly(I:C) is a promising new drug candidate for the treatment of cerebral infarcts.

Materials and Methods

Animals

Age- and weight-matched male C57BL/6 mice (8–12 wk old, 20–23 g), neonatal C57BL/6 mice born within 24 h, and male SD rats (8–12 wk old, 220–250 g) were obtained from the laboratory animal center of the Third Military Medical University (Chongqing, China). The TLR3+/− mice (8–12 wk old, 21–23 g) and TLR4+/− mice (8–12 wk old, 20–23 g) were purchased from The Jackson Laboratory (Bar Harbor, ME). The TLR3+/− and TLR4+/− mice have been backcrossed to C57BL/6 mice for more than eight generations. All protocols involving the use of animals were approved by the Institutional Animal Care and Use Committee of the Third Military Medical University.

MCAO model

The MCAO model was employed as previously described (20). Briefly, the mice were first anesthetized using 5.0% isoflurane. Then an incision in the skin was made, and the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were carefully exposed. Microvascular aneurysm clips were applied to the right CCA and ICA. A coated 6-0 filament (Doccol, Redlands, CA) was introduced into an arteriotomy hole, fed distally into the ICA, and advanced a predetermined distance 8 mm from the carotid bifurcation toward the MCA. After a 60-min period of focal cerebral ischemia, the filament was gently removed (Fig. 1B). Rectal temperature was monitored and maintained at 36.5˚C during the procedure. The pO2, pCO2, and pH of umbilical blood and the MABP were measured (data not shown). Therefore, we chose the 1.25 μg/g dose of poly(I:C) for subsequent experiments. In vitro, 20 μg/ml poly(I:C) was added after 3 h of OGD, and the cells were collected 24 h later for use in different assays.

Intracerebral ventricular injection of IFN-β after MCAO

Recombinant mouse IFN-β (rmIFN-β; Cell Sciences, Canton, MA) or vehicle (artificial CSF) was injected into the mice right lateral ventricle as described previously (23). Injections (1 μl) of either rmIFN-β (200 U) or artificial CSF were administered at 3 h after MCAO. Infarct volume was measured 48 h after stroke.

Assessment of cerebral infarct volume

The cerebral infarct volume was determined as previously described (9). Forty-eight hours after MCAO, animals were sacrificed and perfused with ice-cold PBS via the ascending aorta. The brains were removed and sectioned coronally (2-mm-thick slices for mice, 5-mm-thick slices for rats). The slices were stained with 2% triphenyl tetrazolium chloride (TTC) solution (Sigma-Aldrich, St. Louis, MO) at 37˚C for 15 min. Normal brain tissue was stained bright red whereas the infarcted areas were pale white. We measured the entire area of the proenkephalin and the cerebral infarct with Image-Pro Plus 5.0 image processing software (Media Cybernetics, Rockville, MD). These areas were calculated using the formula V = l(A1 + A2 + ... + An), where V is the volume of the infarct or proenkephalin, l is the thickness of the slice, and A is the infarct size and the volume ratio of the cerebral infarct (cerebral infarct volume/proenkephalin volume).

Evaluation of neurologic score

Animals were scored as previously described (20). Briefly, the mouse scoring system included performance measurements in five principal tasks: spontaneous activity during a 3-min period (0–3), symmetry of movement (0–3), open-field path linearity (0–3), beam walking on a 3 × 1-cm beam (0–3), and response to vibrissae touch (0–3). The total scores obtained using this system thus ranged from 0 to 15, where 15 represents a perfect score and 0 represents death due to cerebral I/R injury.

For the rats, we performed the postural reflex test, which assesses the upper body posture while the animal is suspended by the tail, and the forelimb-placing test, which examines sensorimotor integration during forelimb-placing responses to visual, tactile, and proprioceptive stimuli. Neurological function was graded on a scale ranging from 0 to 12, with higher scores indicating greater neurologic deficit.

Scoring was performed by two trained investigators who were blinded to the animal group designation, and the mean score of the subscales was used as the final score for each animal.

Assessment of brain edema

Brain water content was measured as previously described (24). At 48 h after cerebral ischemia, the right hemispheres were dissected, immediately weighed (wet weight), dried at 110˚C for 48 h, and reweighed (dry weight). The brain water content was calculated with the formula [(wet weight − dry weight)/wet weight] × 100%.

Nissl staining

Nissl staining was performed as previously described (20). Paraffin sections were cut at a thickness of 5 μm. The sections were deparaffinized and subsequently stained with 0.1% cresyl violet (Bayoette, Beijing, China) for 2 min. Shrinkage of cell bodies and the nuclear condensation of neurons were evaluated using light microscopy (Olympus BX60; Olympus, Tokyo, Japan).
Electron microscopic examination

Forty-eight hours after MCAO, mice were perfused with 2% glutaraldehyde through the ascending aorta. Blocks (1 mm²) of ischemic cerebral tissue were fixed and stored in 0.25% glutaraldehyde. The mitochondrial structure was observed using a transmission electron microscope (Hitachi, Tokyo, Japan). Normal mitochondria were classified by compacted matrix cristae and a continuous outer membrane. In contrast, damaged mitochondria were classified into mild and severe injury depending on mitochondrial shape, matrix density, crystal structure, disrupted outer membrane, and appearance of any abnormal structures [25]. Randomly selected noncontiguous, nonoverlapping, digitized images of each cerebral tissue pellet were captured, and these images were evaluated by an observer blinded to the source of mitochondria.

TUNEL and Fluoro-Jade B staining

TUNEL and Fluoro-Jade B (FJB) staining were performed according to the manufacturers’ protocols. The cells were deeply anesthetized and perfused through the ascending aorta with cold PBS followed by 4% paraformaldehyde, and the brains were sectioned coronally into 20-μm-thick frozen slices. TUNEL staining was performed using an in situ cell death detection kit (Roche, Indianapolis, IN). Cell degeneration was detected using an FJB staining kit (Millipore, Billerica, MA). Both assays were performed according to the manufacturers’ protocols. The stained sections were photographed with a confocal fluorescence microscope (Leica, Wetzlar, Germany). The nuclei were stained with DAPI (blue), and the apoptotic cells were TUNEL+ (green). We calculated the number of DAPI+ cells (blue), and TUNEL-positive cells (green). The percentage of TUNEL+ cells was calculated using the formula green/blue

Cell cultures and OGD

Primary hippocampal neuron cultures, microglial cell cultures, mixed neuronal-glial cell cultures, and OGD were performed as previously described [14, 26]. In brief, the cell cultures were prepared from neonatal mice, and the hippocampal neurons were dissected and dissociated with 0.25% trypsin-EDTA (Life Technologies, Burlington, MI). Primary hippocampal neurons were cultured in neurobasal media (containing 4.5 g/l glucose, supplemented with 10% FBS, 1% penicillin/streptomycin, and 2% B27; Life Technologies) for 14 d. OGD was performed by removing the culture medium and replacing it with glucose-free DMEM (Life Technologies; Birmingham, MI). Neurons were cultured in neurobasal media (containing 4.5 g/l glucose, supplemented with 10% FBS (Sigma-Aldrich) for 14 d, and the mixed neuronal-glial cell cultures were cultured in 5% FBS (Sigma-Aldrich) and 5% horse serum (Sigma-Aldrich) supplemented with 15 mM glucose for 14 d. OGD was performed by removing the culture medium and replacing it with glucose-free DMEM (Life Technologies); the cultures were then incubated in an anaerobic atmosphere of 85% N₂, 10% CO₂, and 5% H₂ at 37°C for 3 h. OGD was terminated by replacing the glucose-free DMEM with normal culture medium and returning the cells to a normoxic incubator. Control plates were kept in the normoxic incubator during the OGD interval. For in vitro experiments, cells were treated with lipopolysaccharide (LPS; Escherichia coli, 011: B4; Sigma-Aldrich, 100 ng/ml), poly I:C (20 μg/ml), or saline.

Transwell coculture experiment

Cocultures of neurons and microglial cells were established using a transwell system (Millipore). Microglia were plated onto the transwell inserts at a density of 1 × 10⁷/well. Hippocampal neurons were plated onto the cell plate at a density of 1 × 10⁵ cells/well and were cultured with 10% FBS in an atmosphere of 5% CO₂ at 37°C for 48 h. Following 3 h of OGD, the neuron cultures (20 μg/ml polyICL:C or vehicle was added to the transwell insert, and immunofluorescence staining of the hippocampal neurons was performed 24 h after stimulation. The hippocampal neurons were stained with a class III β-tubulin Ab (green; 1:200; Abcam, Cambridge, MA), and the nuclei were stained with DAPI (blue). We determined the number of DAPI+ cells (blue) and β-tubulin+ cells (green). The hippocampal neuron survival rate was calculated with the formula green×blue × 100%. Four quadrants were selected from each section. The numbers of positive cells in each quadrant were counted, and the average values were calculated.

Western blotting

Western blotting was performed as previously described [20]. At 48 h after cerebral ischemia, the mice were sacrificed and the brains were removed. Following 3 h of cellular OGD, 20 μg/ml polyIC (C) or vehicle was added and the cells were collected after an additional 24 h. The proteins were electroblotted through an SDS–polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Amersham Pharmacia, Piscataway, NJ). The polyvinylidene difluoride membranes were first incubated with primary Abs (anti-Bcl-2, 1:200; anti-Bax, 1:200; anti–NF-κB [p65], 1:200; anti–IFN-γ, 1:200; anti-Hsp70, 1:200; anti–IRF-3 [1:200; Santa Cruz Biotechnology, Santa Cruz, CA]; anti–TRIF, 1:1000; anti–MyD88, 1:1000 [Abcam]; and anti–TLR3, 1:200 [E Bioscience, San Diego, CA]) and subsequently incubated with peroxidase-conjugated secondary Abs (Cell Signaling Technology, Danvers, MA). The signals were detected using an ECL system, and the membranes were reprobed with anti-GAPDH (Santa Cruz Biotechnology). The signals were quantitated with scanning densitometry using a bioimaging analysis system (LabWorks analysis software; UVP, Upland, CA).

Real-time RT-PCR

Ischemic brain tissue was harvested quickly at 24 and 48 h after cerebral I/R. Total RNA was extracted with TRIZol (Invitrogen, Gaithersburg, MD) according to the manufacturer’s instructions. cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) and real-time RT-PCR was carried out on a Bio-Rad iCycler with the iQ SYBR Green Supermix (Bio-Rad) in 96-well plates. Primers were purchased from Shanghai Sangon Biological Engineering (Shanghai, China). The primer sequences were as follows: TLR2, forward, 5′-GAA AGATGCG TCT TCC TGA AC-3′; reverse, 5′-CCG CTA AGA GCA GGA GTA ACA-3′. To quantify measurements of gene expression, a threshold cycle value (Ct) was calculated using the ΔΔCt method as previously described [27].

EMSA

EMSA was performed as previously described [28]. At 48 h after cerebral ischemia, the mice were sacrificed and the brains were removed. Nucleoprotein was extracted from ischemic brain tissue. Ten micrograms nucleoprotein of each sample was incubated with the reaction buffer at room temperature for 15 min and then [³²P]-labeled double-stranded oligonucleotide containing the binding motif of NF-κB (5′-AGTTGAGGCGGATTT CCCAGGC-3′; Promega, Madison, WI) or a [³²P]-labeled double-stranded oligonucleotide corresponding to the IFN-stimulated response element of the IFN-inducible Isig1 gene (5′-GATCGGAAAGGGAAACCGAAGG- GCCG-3′; Cell Signaling Technology, Boston, MA) for IRF3 binding was added to the reaction buffer and incubated for 15 min. After incubation for 20 min at 25°C, the reaction mixture was subjected to 6% nondenaturing polyacrylamide gel electrophoresis. Autoradiography was performed at room temperature. Finally, the images were analyzed using a Bio-Rad image analyzer and the results were expressed as OD.

Flow cytometry

Flow cytometry was employed to detect TLR3 and TLR4+ microglial cells. In brief, following 3 h of microglial OGD, 20 μg/ml polyIC (C) or vehicle was added. The cells were collected 24 h after stimulation, and mAbs, including slgphycocyanin-conjugated anti-TLR4 (1:200, eBioscience), PerCP-Cy–conjugated anti-CD11b (1:200; eBioscience), and FITC-conjugated anti-TLR3 (1:200; Novus Biologicals, Littleton, CO) were added. The cells were incubated in the dark at 4°C for 30 min and washed twice. Anti-mouse TLR3 (1:100; Abcam) was used to block the TLR3 signal. A BD FACsVerse flow cytometer (Becton Dickinson, Franklin Lakes, NJ) was used for detection. The percentage of positive cells and the average fluorescence intensities were analyzed using BD FACSuite software.

ELISA

Samples of the supernatants from the ischemic brain tissues, the cultured cells, the CSF, and serum were obtained, and the inflammatory factors TNF-α, IL-1β, and IFN-β were quantified with an ELISA kit (Dakewe, Beijing, China) according to the manufacturer’s protocol.

Statistical analysis

All data are expressed as the means ± SEM. Differences between multiple groups were examined using one-way ANOVA and post hoc Bonferroni tests, and independent sample t tests were used for comparisons of two groups. When the data were not normally distributed, the nonparametric Kruskal–Wallis and Mann–Whitney U tests were used. A p value < 0.05 was considered statistically significant.

Results

Poly(IC) reduced focal cerebral I/R injury

To evaluate the protective effects of poly(IC) against cerebral I/R injury, mice were i.p. injected with poly(IC) (1.25 μg/g) 3 h after cerebral ischemia. Poly(IC) treatment significantly reduced the cerebral infarct volume compared with the I/R group (Fig. 1A; p < 0.01). However, poly(IC) treatment 6 h after cerebral ischemia did not reduce cerebral infarct volume (data not shown).
The regional CBF decreased by 80% in mice after MCAO, and CBF recovered completely after the occlusion was removed (Fig. 1B). Additionally, no remarkable differences were found in pH, pO2, pCO2, MABP, or rectal temperature between the poly(I:C)-treated and vehicle groups (Supplemental Table I). Compared with the I/R group, the brain water content of the right
hemisphere was significantly decreased and the neurologic scores were increased markedly in the poly(I:C) group (Fig. 1C; \( p < 0.05 \)). Moreover, the IFN-\( \beta \) content was increased markedly, and the levels of the inflammatory factors TNF-\( \alpha \) and IL-1\( \beta \) were decreased significantly in the ischemic brain tissue (Fig. 1D; \( p < 0.01 \)), CSF (Fig. 1E; \( p < 0.01 \)), and serum (Fig. 1F; \( p < 0.01 \)).

Similar results were observed in mixed neuronal-glial cell cultures after OGD treated with poly(I:C) (Supplemental Fig. 1A). Nissl staining showed neuronal damage in the cornu ammonis 1 and dentate gyrus fields of hippocampal formation characterized by shrunken cell bodies accompanied by shrunken and pyknotic nuclei in the I/R mice for 48 h (Fig. 1G). Hippocampal neuron damage was significantly reduced in the poly(I:C) treatment group (Fig. 1G).

Transmission electron microscopy demonstrated that mitochondrial membranes were severely damaged, resulting in irregularly shaped organelles with poor membrane integrity in the I/R group; in contrast, in the poly(I:C) group, mitochondria demonstrated evidence of edema with matrix, but with intact surrounding membranes (Fig. 1H).

Poly(I:C) increased the expression of Bcl2, Hsp27, and Hsp70, decreased Bax expression, and reduced cellular degeneration and apoptosis

Neuron apoptosis plays an important role in cerebral ischemia injury. Bcl-2 is important for cell survival owing to its antiapoptotic effects, whereas Bax can promote apoptosis. We examined the effect of poly(I:C) treatment on the levels of Bcl-2 and Bax after cerebral I/R. The results showed that poly(I:C) treatment significantly increased Bcl-2 expression (Fig. 2A; \( p < 0.01 \)) and significantly decreased Bax expression (Fig. 2B; \( p < 0.01 \)).
decreased Bax expression (Fig. 2A; \( p < 0.05 \)) in the ischemic brain tissues compared with the I/R group; similar results were obtained in the mixed neuronal-glial cell OGD model (Supplemental Fig. 1B). Recent studies have shown that Hsp27 and Hsp70 have protective effects against cerebral I/R injury (29, 30). Our results also showed that poly(I:C) significantly increased the levels of Hsp27 (Fig. 2B; \( p < 0.05 \)) and Hsp70 (Fig. 2B; \( p < 0.01 \)) in the ischemic brain tissues compared with the I/R group.

TUNEL staining is a molecular biological-histochemical system for sensitive and specific staining of DNA fragmentation (31). Neuronal degeneration was detected with FJB staining. In this study, compared with I/R group, the numbers of TUNEL+ and FJB+ cells in ischemic brain tissues were markedly decreased in the poly(I:C) group (Fig. 2C, 2D; \( p < 0.01 \)). In the transwell coculture model, the hippocampal neuronal survival rate was markedly increased with poly(I:C) treatment (Supplemental Fig. 1C).

**Poly(I:C) protected against cerebral ischemia via TLR3**

We investigated whether the protective effects of poly(I:C) are exerted via TLR3. First, Western blotting showed that the expression of TLR3 in the ischemic brain tissues was examined by Western blotting (\( n = 3 \)). (A) Expression of TLR3 in the ischemic brain tissues was examined by Western blotting (\( n = 3 \)). (B) Infarction volumes and neurologic scores in TLR3−/− mice (\( n = 8 \)). Original magnification \( \times 1.1 \). The numbers of TUNEL+ cells (C) and FJB+ cells in ischemic cerebral tissues (D) were not significantly different between the two groups after 48 h of MCAO (\( n = 9 \)). Following 3 h of cell OGD, 20 \( \mu \)g/ml poly(I:C) was added and the cells were detected 24 h later. (E) The hippocampal neuronal survival rates were not different in the OGD plus poly(I:C) group compared with the OGD plus vehicle group after 24 h of OGD (\( n = 3 \)). **\( p < 0.01 \). Data are representative of at least three independent experiments.
pression of TLR3 in the poly(I:C) group was increased significantly (Fig. 3A; \( p < 0.01 \)), and similar results were observed in the microglia OGD model (Supplemental Fig. 2A, 2B). Second, in the TLR3−/− mice, the cerebral infarct volumes and neurologic scores did not differ in the poly(I:C) group compared with the I/R group (Fig. 3B). The TUNEL+ and FJB+ cell counts in the ischemic cerebral tissues with poly(I:C) treatment were not decreased (Fig. 3C, 3D), and the hippocampal neuron survival rate was not increased in the poly(I:C) treatment group (Fig. 3E).

**Poly(I:C) conferred protection against cerebral I/R injury through the downregulation of TLR4 signaling via TLR3**

It has been reported that pretreatment with the TLR3 ligand poly(I:C) downregulates the expression of TLR4 on macrophages and decreases TNF-\( \alpha \) levels to attenuate LPS-induced liver injury (19). We hypothesized that the induction of cerebral ischemic protective effects by poly(I:C) might also be mediated by down-regulation of TLR4 signaling. In the MCAO model, Western blot analysis showed that the expression levels of TRIF, IRF3, and IFN-\( \beta \) were significantly increased in the poly(I:C) treated group (Fig. 4A; \( p < 0.01 \)). EMSA showed that poly(I:C) significantly increased IRF3 activity (Fig. 4B; \( p < 0.01 \)). Similar results were obtained in the microglia OGD model (Fig. 4C, 4D; \( p < 0.01 \)). In contrast, the expression levels of TLR4, MyD88, and NF-\( \kappa \)B p65 were decreased significantly in the poly(I:C) group (Fig. 5A; \( p < 0.05 \)). NF-\( \kappa \)B activity also decreased in the poly(I:C) group (Fig. 5B; \( p < 0.01 \)), and similar results were also obtained in the microglia OGD model (Fig. 5C, 5D). Flow cytometry further verified that poly(I:C) reduced the proportion of TLR4+ microglia (Fig. 5E; \( p < 0.05 \)). To verify that TLR4 is indeed downregulated via TLR3 treated with poly(I:C), we detected the proinflammatory cytokine levels of mice responses to subsequent LPS treatment following poly(I:C) pretreatment. The results showed that poly(I:C) pretreatment indeed downregulated the levels of TNF-\( \alpha \) and IL-1\( \beta \) in serum and CSF of mice stimulated by the LPS (Fig. 5F, 5G; \( p < 0.01 \)), and similar results were also obtained in vitro model (Fig. 5H, \( p < 0.01 \)). Because TLR2 plays important roles in cerebral I/R injury (32), we assessed whether TLR2 had been downregulated in ischemic brain tissues after administration of poly(I:C) with the real-time RT-PCR. The results showed that the TLR2 mRNA was not downregulated in poly(I:C) group at 24 and 48 h after mice MCAO compared with the I/R group (Supplemental Fig. 2C). Next, we explored whether poly(I:C) could alleviate cerebral I/R injury in the TLR4−/− MCAO model. The results showed that poly(I:C) did not reduce cerebral infarction volumes or brain water content and did not improve neurologic scores in the TLR4−/− MCAO mice compared with the I/R group (Fig. 6A). We further investigated the effect of TLR3 on TLR4 signaling in the OGD model treated with poly(I:C). In the TLR3−/− microglia OGD model, the poly(I:C)-induced downregulation of TLR4 signaling and NF-\( \kappa \)B activity were abolished (Fig. 6B, 6C). Moreover, treatment with the anti-mouse TLR3 Ab yielded similar results (Supplemental Fig. 2D, 2E). Flow cytometry revealed that poly(I:C) significantly reduced the proportion of TLR4+ microglia (Fig. 6D). To confirm that the TLR4+ microglia, whereas treatment with TLR3 Abs diminished the effect of TLR3 on TLR4 signaling in the OGD model treated with poly(I:C), we detected the proinflammatory cytokine levels of mice responses to subsequent LPS treatment following poly(I:C) pretreatment. The results showed that poly(I:C) pretreatment indeed downregulated the levels of TNF-\( \alpha \) and IL-1\( \beta \) in serum and CSF of mice stimulated by the LPS (Fig. 5F, 5G; \( p < 0.01 \)), and similar results were also obtained in vitro model (Fig. 5H, \( p < 0.01 \)). Because TLR2 plays important roles in cerebral I/R injury (32), we assessed whether TLR2 had been downregulated in ischemic brain tissues after administration of poly(I:C) with the real-time RT-PCR. The results showed that the TLR2 mRNA was not downregulated in poly(I:C) group at 24 and 48 h after mice MCAO compared with the I/R group (Supplemental Fig. 2C). Next, we explored whether poly(I:C) could alleviate cerebral I/R injury in the TLR4−/− MCAO model. The results showed that poly(I:C) did not reduce cerebral infarction volumes or brain water content and did not improve neurologic scores in the TLR4−/− MCAO mice compared with the I/R group (Fig. 6A). We further investigated the effect of TLR3 on TLR4 signaling in the OGD model treated with poly(I:C). In the TLR3−/− microglia OGD model, the poly(I:C)-induced downregulation of TLR4 signaling and NF-\( \kappa \)B activity were abolished (Fig. 6B, 6C). Moreover, treatment with the anti-mouse TLR3 Ab yielded similar results (Supplemental Fig. 2D, 2E). Flow cytometry revealed that poly(I:C) significantly reduced the proportion of TLR4+ microglia (Fig. 6D). Finally, in the microglia OGD model, IFN-\( \beta \) significantly downregulated the expression levels of TLR4, MyD88, NF-\( \kappa \)B p65, and NF-\( \kappa \)B activity by Western blotting and EMSA data are representative of three independent experiments. **\( p < 0.01 \), CI, competitive inhibition; NC, negative control; SS, supershift.

**FIGURE 4.** Poly(I:C) activated TLR3 signaling. (A) Expression of TRIF, IRF3, and IFN-\( \beta \) were examined by Western blotting in the brain tissues after 48 h of MCAO (\( n = 3 \)). (B) IRF3 activity was determined by EMSA (\( n = 3 \)). (C) Expression of TRIF, IRF3, and IFN-\( \beta \) were examined by Western blotting in microglia after 24 h of OGD (\( n = 3 \)). (D) IRF3 activity was determined by EMSA in microglia (\( n = 3 \)). Western blotting and EMSA data are representative of three independent experiments. **\( p < 0.01 \), CI, competitive inhibition; NC, negative control; SS, supershift.
expressions of TLR4, MyD88, and NF-κB p65 (Fig. 7E; \( p, 0.05 \)), and NF-κB activity (Fig. 7F; \( p, 0.01 \)). These results suggest that poly(I:C) confers protection against cerebral I/R injury through the downregulation of TLR4 signaling via TLR3.

**Poly(I:C) exerted protective effects after cerebral I/R injury in mice and rats**

The long-term protective effects of poly(I:C) after cerebral I/R injury were evaluated by i.p. injection of poly(I:C) (1.25 μg/g) at 3 and 24 h and once again 48 h after cerebral ischemia (we chose to administer poly(I:C) at 3, 24, and 48 h after cerebral ischemia because the inflammatory response mediated by the TLR4/MyD88 pathway occurs \( \sim 24–48 \) h following cerebral I/R; Ref. 33). The neurologic scores of the poly(I:C) treatment group were significantly higher than those of the I/R group at 1, 3, 10, and 14 d after ischemia (Fig. 8A; \( p < 0.05 \)), and the cerebral infarct volumes were significantly reduced at 14 d (Fig. 8B; \( p < 0.01 \)).

Based on the suggestions of the Stroke Therapy Academic Industry Roundtable (34), the protective effects of poly(I:C) against cerebral I/R injury were evaluated in a rat model of permanent

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**FIGURE 5.** Poly(I:C) downregulated TLR4 signaling. (A) Expression of TLR4, MyD88, and NF-κB p65 were examined by Western blotting in the ischemic brain tissues after 48 h of MCAO (n = 3). (B) NF-κB activity was determined by EMSA (n = 3). (C) Expression of TLR4, MyD88, and NF-κB p65 were examined by Western blotting in microglia after 24 h of OGD (n = 3). (D) NF-κB activity was determined by EMSA in microglia (n = 3). (E) TLR4 expression in microglia after 24 h of OGD as determined by flow cytometry (red, OGD plus vehicle; green, OGD plus poly(I:C); n = 4). Poly(I:C) was i.p. injected into mice 3 h before LPS (2 μg/g) i.p. injection. Serum and CSF samples were collected after 24 h. The levels of TNF-α and IL-1β in the serum (F) and CSF (G) were detected with ELISA (n = 3–5). Microglia were treated with either LPS (100 ng/ml) or vehicle for 3 h, and then poly(I:C) was added for 24 h. The levels of TNF-α and IL-1β (H) were detected with ELISA. Data are representative of at least three independent experiments. *\( p < 0.05 \), **\( p < 0.01 \). CI, competitive inhibition; NC, negative control; SS, supershift.
cerebral ischemia. Poly(I:C) (1.25 mg/g) or vehicle was injected i.p. into rats 3 h after cerebral ischemia. Rats were subjected to cerebral ischemia (48 h). Cerebral infarct volumes (Fig. 8C; \( p < 0.01 \)) and the severity of neurologic deficits (Fig. 8D; \( p < 0.05 \)) were significantly reduced in the poly(I:C) treatment group at 48 h after cerebral ischemia.

**Discussion**

Ischemia preconditioning can reduce cerebral I/R injury (35), and recent reports have shown that poly(I:C) preconditioning protects against cerebral I/R injury (17, 18). However, it has not been determined whether posts ischemia poly(I:C) treatment would also be effective in protecting against cerebral I/R injury. Additionally, the mechanism of this potentially protective effect remains unknown. The results showed that poly(I:C) treatment 3 h after ischemia had therapeutic effects on cerebral I/R injury. Most importantly, posts ischemia poly(I:C) treatment ameliorated neurologic deficits and reduced the cerebral infarct volume up to 14 d after ischemia, indicating that poly(I:C) may have long-term protective effects. To our knowledge, this study is the first to provide evidence suggesting that poly(I:C) has therapeutic effects against cerebral I/R injury. Moreover, the protective effects of poly(I:C) were abolished in the TLR3−/− mouse MCAO model, suggesting that the protective effects of poly(I:C) are mediated via TLR3.

It has been reported that poly(I:C) preconditioning could reduce I/R injury (17, 18), and our results demonstrated that poly(I:C) treatment 3 h after ischemia had therapeutic effects on cerebral I/R injury. However, a recent study has reported that TRL3 activation increased the susceptibility of neonatal brain to hypoxia and ischemia (36). The precise reasons of the observed differences of these results were not completely clear, which may be related to different animals, models, and dose of poly(I:C). We used 1.25 µg/g poly(I:C) by i.p. injection, whereas Stridh et al. (36) used 10 mg/kg. Moreover, we adopted the MCAO model, whereas the hypoxic-ischemia model was used by Stridh et al. Moreover,
Puyal et al. (37) showed that the adult mice pathophysiological mechanism of cerebral I/R injury were different from the neonatal mice, and whose early autophagic death was prominent in the ischemic penumbra of neonatal mice; however, necrotic and apoptotic cell death in the early ischemic neurons play an important role in the adult mice (38). Therefore, we speculated that the different pathophysiological mechanisms may also be an important reason of the differing results.

Currently, rt-PA thrombolytic therapy administered ∼3–4.5 h following ischemic stroke is the only effective treatment (2, 39); however, revascularization contributes to I/R injury and can aggravate neurologic deficits. It is well known that inflammation plays an important role in cerebral I/R injury. Proinflammatory factors produced by microglia, such as TNF-α and IL-1β, are involved in ischemic neuronal injury and aggravate neurologic deficits (3). Furthermore, I/R can also injure the blood–brain barrier and cause increases in the expression levels of adhesion molecules and the infiltration of inflammatory cells from the circulation, both of which can aggravate inflammatory injury (35, 40). Our data demonstrated that poly(I:C) administered 3 h after ischemia can inhibit inflammatory responses and reduce cerebral I/R injury. These findings suggest that the application of rt-PA combined with poly(I:C) could attenuate I/R inflammation injuries and improve neurologic deficits. Research in this area is ongoing.

It is generally acknowledged that neuronal apoptosis is involved in the response of the brain to cerebral I/R injury (41). Bcl-2 is important for cell survival and antiapoptotic effects, whereas Bax
FIGURE 8. Poly(I:C) exerted protective effects against cerebral I/R injury in mice and rats. Poly(I:C) (1.25 μg/g) or vehicle was injected i.p. into mice at 3 and 24 h and once again 48 h after cerebral ischemia. (A) The neurologic scores were significantly different between the poly(I:C) group and the vehicle group (p < 0.001, repeated measures ANOVA on ranks; n = 8). When the days were analyzed individually, there were differences between the vehicle and poly(I:C) groups on days 1, 3, 10, and 14 (p < 0.05, n = 8). (B) TTC staining after 14 d of MCAO (n = 6). Poly(I:C) (1.25 μg/g) or vehicle was injected i.p. to rats 3 h after cerebral ischemia. Rats were subjected to cerebral ischemia (48 h). Original magnification ×1.1. (C) Cerebral infarct volumes after 48 h of MCAO in the rat model of permanent MCAO (n = 9). (D) Neurological scores after 48 h of MCAO in rats (normal score, 0; maximal score, 12; n = 9). *p < 0.05, **p < 0.01.

has been demonstrated to promote apoptosis. It has been reported that LPS can induce the apoptosis of dental pulp cells and the expression of Bax (42), whereas TLR4-deficient mice exhibit attenuated doxorubicin-induced cardiac apoptosis, increased levels of Bcl-2, and decreased levels of Bax (43). Our results are in good agreement with these results. Poly(I:C) treatment increased Bcl-2 expression significantly and decreased Bax expression in both the MCAO model and the mixed neuronal-glial cerebral OGD model. The protective effects of poly(I:C) in cerebral I/R injury are apparently related to the increase in Bcl-2 expression, the reduction in Bax expression, and the attenuation of neuronal apoptosis.

Recent research has demonstrated that Hsp27 and Hsp70 have protective effects in cerebral I/R injury (29, 30). In the present study, we also observed that Hsp27 and Hsp70 levels were significantly increased in the poly(I:C) group. This finding suggests that the protective effects of poly(I:C) are related to the increased levels of Hsp27 and Hsp70. However, it remains unclear how poly(I:C), a TLR3 ligand, increases Hsp27 and Hsp70 levels in ischemic cerebral tissue. Recent reports have shown that poly(I:C) cannot induce Hsp70 expression but also promote the extracellular release of Hsp70 (44). It has also been reported that the signaling molecule IFN-β, which is downstream of TLR3, can upregulate the expression of Hsp70 in splenic dendritic cells (45). This finding suggests that the protective effects of the poly(I:C)-dependent upregulation of Hsp27 and Hsp70 may be related to the activation of the TLR3/TRIF signaling pathway. However, details regarding the molecular mechanisms of this process require further study.

We and other investigators have demonstrated that TLR4 signaling plays a critical role in the response to cerebral I/R injury (9, 10). The DAMPs act on TLR4 of microglia to activate the MyD88/NF-κB signaling pathway, which produces inflammatory factors that contribute to inflammatory injury and aggravate neurologic impairments (7). Thus, inhibition of TLR4 signaling strongly protects against cerebral ischemia (7, 15). It has been reported that pretreatment with the TLR3 ligand poly(I:C) downregulates the expression of TLR4 on macrophages and decreases TNF-α levels to attenuate LPS-induced liver injury (19). Our results also revealed that the levels of TLR4 and its downstream signaling molecules MyD88 and NF-κB p65 were downregulated, and TNF-α and IL-1β levels were decreased significantly after postischemia poly(I:C) treatment. Moreover, poly(I:C) did not further alleviate cerebral I/R injury in the mouse TLR4−/− MCAO model compared with the I/R group, suggesting that poly(I:C) protects against cerebral I/R injury via downregulation of TLR4 signaling. However, poly(I:C) is a ligand of TLR3, not TLR4; thus, we hypothesized that poly(I:C) provides therapeutic effects against cerebral I/R injury through the downregulation of TLR4/MyD88 signaling via TLR3. This view was confirmed based on the following evidence. First, in TLR3−/− mice, poly(I:C) did not downregulate TLR4/MyD88 signaling, and the protective effect of poly(I:C) against cerebral I/R injury was abolished. Second, after blocking with the TLR3 mAb, the expression levels of TLR4, MyD88, and NF-κB were not decreased. Third, IFN-β significantly downregulated the expression of TLR4, MyD88, and NF-κB in the wild-type microglial OGD model. Fourth, intraventricular injection of rmIFN-β after mice MCAO attenuated infarct volume and downregulated TLR4 signaling and NF-κB activity. Finally, poly(I:C) pretreatment attenuated the levels of TNF-α and IL-1β in serum and CSF of mice stimulated by the LPS. It has been reported that the inducible production of TRIM30-α (activated by IFR3) negatively regulates the TLR4/NF-κB signaling pathway (46). Although similar results were observed in the IFN-β group, the exact molecular mechanisms by which IFN-β mediates the downregulation of the TLR4/MyD88 pathway require further study.

The Stroke Therapy Academic Industry Roundtable has proposed some suggestions to facilitate the translation of drugs from the bench to clinical trials. First, ideal neuroprotective drugs should have short- and long-term effects. Second, the drugs should be effective in at least two animal models. Finally, the drugs should be protective against both transient and permanent focal ischemia (34). Thus, based on our results, poly(I:C) meets the requirements of the above suggestions. Poly(I:C) reduced the cerebral infarct volume and improved neurologic deficits 14 d after cerebral ischemia, and similar results were demonstrated in the rat model of permanent MCAO. Therefore, poly(I:C) is a promising drug for the treatment of cerebral I/R injury, and related clinical research trial is ongoing.

Collectively, our data show that poly(I:C) exerted therapeutic effects against cerebral I/R injury through the downregulation of TLR4/MyD88 signaling via TLR3. We suggest that the application of rt-PA in combination with poly(I:C) could ameliorate cerebral I/R inflammatory injury and improve neurologic deficits. Therefore, poly(I:C) is a promising new drug candidate for the treatment of cerebral infarcts.
Disclosures
The authors have no financial conflicts of interest.

References
