Remote Ischemic Preconditioning-Mediated Neuroprotection against Stroke is Associated with Significant Alterations in Peripheral Immune Responses

Zong-Jian Liu,^{1,2,3} Chen Chen,^{2,3} Xiao-Rong Li,^{2,3} Yuan-Yuan Ran,^{2,3} Tao Xu,^{2,3} Ying Zhang,^{1,3} Xiao-Kun Geng,^{2,3} Yu Zhang,¹ Hui-Shan Du,^{2,3} Rehana K. Leak,⁴ Xun-Ming Ji^{1,2,3} & Xiao-Ming Hu²

1 Institute of Hypoxia Medicine, Xuanwu Hospital, Capital Medical University, Beijing, China

2 China-America Institute of Neuroscience, Beijing Luhe Hospital, Capital Medical University, Beijing, China

3 Beijing Key Laboratory of Hypoxic Conditioning Translational Medicine, Beijing, China

4 Division of Pharmaceutical Sciences, Duquesne University, Pittsburgh, PA, USA

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Correspondence

Xun-Ming Ji, Institute of Hypoxia Medicine, Xuanwu Hospital, Capital Medical University, Beijing 100053, China.

Tel.: +86-10-83198931;

Fax: +1-4126924636;

E-mail: jixm@ccmu.edu.cn

and

Xiao-Ming Hu, China-America Institute of Neuroscience, Beijing Luhe Hospital, Capital Medical University, Beijing 101100, China.

Tel.: +1-412-6488991;

Fax: +86-10-83194754;

E-mail: hux2@upmc.edu

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SUMMARY

Aims: Remote ischemic preconditioning (RIPC) of a limb is a clinically feasible strategy to protect against ischemia–reperfusion injury after stroke. However, the mechanism underlying RIPC remains elusive. **Methods:** We generated a rat model of noninvasive RIPC by four repeated cycles of brief blood flow constriction (5 min) in the hindlimbs using a tourniquet. Blood was collected 1 h after preconditioning and 3 days after brain reperfusion. The impact of RIPC on immune cell and cytokine profiles prior to and after transient middle cerebral artery occlusion (MCAO) was assessed. **Results:** Remote ischemic preconditioning protects against focal ischemia and preserves neurological functions 3 days after stroke. Flow cytometry analysis demonstrated that RIPC ameliorates the post-MCAO reduction of CD3⁺CD8⁺ T cells and abolishes the reduction of CD3⁺/CD161a⁺ NKT cells in the blood. In addition, RIPC robustly elevates the percentage of B cells in peripheral blood, thereby reversing the reduction in the B-cell population after stroke. RIPC also markedly elevates the percentage of CD43⁺/CD172a⁺ noninflammatory resident monocytes, without any impact on the percentage of CD43⁺/CD172a⁺ inflammatory monocytes. Finally, RIPC induces IL-6 expression and enhances the elevation of TNF- α after stroke. **Conclusion:** Our results reveal dramatic immune changes during RIPC-afforded neuroprotection against cerebral ischemia.

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Introduction

Stroke is a leading cause of death and a major cause of permanent disability among adults worldwide. Ischemic pre-/per-/postconditioning is an efficient protective strategy against ischemia–reperfusion injury after stroke [1,2]. In ischemic preconditioning, one or more short episodes of sublethal ischemia protect the brain against subsequent severe ischemic attacks [3]. Ischemic preconditioning protocols vary greatly in terms of location, timing, and duration. Remote ischemic preconditioning (RIPC) of the limbs elicits tolerance against brain ischemia through repeated cycles of brief blood flow constriction [4–8]. Specifically, noninvasive bilateral limb occlusion by tourniquet has been shown to ameliorate brain damage in rats after cerebral ischemic challenges [9]. Compared to ischemic preconditioning by direct artery occlusion, noninvasive

RIPC procedures can be applied more readily to humans and have therefore garnered much support for clinical translation. Indeed, we have reported that brief repetitive bilateral upper arm ischemic preconditioning improves cerebral perfusion and reduces recurrent strokes in patients with intracranial arterial stenosis [10]. RIPC may also be applied to other patients whose stroke occurrence is predictable, such as (1) patients whose cerebral vascular surgery may result in ischemia, (2) patients who have high risk factors for developing stroke, or (3) patients with hemorrhagic stroke who may develop ischemic stroke within a predictable timeframe.

The observation that ischemic preconditioning in a remote organ can lead to brain protection has led to the novel concept that peripheral elements, such as humoral, cellular, and neural factors, are involved in establishing brain tolerance [11–13]. The identifi-

cation of these peripheral elements may provide novel therapeutic strategies for stroke. In particular, emerging evidence supports the view that ischemic preconditioning dramatically alters the immune system. As many peripheral immune cells, such as T lymphocytes, B lymphocytes, nature killer (NK) cells, neutrophils, monocytes, and macrophages, are known to be critical for the progression of stroke and poststroke recovery [14], an intervention that modifies immune responses may directly impact stroke outcomes. For example, repetitive hypoxic preconditioning induces an immunosuppressive B-cell phenotype prior to stroke onset, thereby preventing the infiltration of other immune cells into the brain after stroke and protecting the brain against ischemic attack [15]. A limited number of human studies have attempted to explore alterations in circulating immune cells following RIPC. These studies demonstrate that RIPC via brief forearm ischemia reduces the expression of proinflammatory genes in leukocytes [16] and alters neutrophil functions [17]. As peripheral immune cells are early responders after stroke and quite accessible for therapeutic interventions, further elucidation of the immune cell populations that participate in RIPC-induced neuroprotection might lead to the development of novel therapies for stroke patients.

In this study, we used a model of noninvasive RIPC by tourniquet to study the impact of RIPC on immune cell and cytokine profiles after stroke and to explore the immune mechanisms associated with RIPC-afforded neuroprotection. Our data indicate that RIPC results in dramatic changes in many immune cell populations, including CD8 T cells, B cells, NKT cells, and monocytes after stroke. We also discovered that RIPC induces IL-6 expression and boosts the elevation in TNF- α after stroke. Collectively, our results reveal potential immune mechanisms underlying RIPC-afforded protection that warrant further mechanistic investigations.

Materials and Methods

Subjects

Male Sprague-Dawley (SD) rats weighing 280–320 g were purchased from the Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). Animal care was carried out in accordance with guidelines approved by Capital Medical University. All efforts were made to minimize any suffering and to reduce the number of animals sacrificed.

Remote Ischemic Preconditioning

Noninvasive RIPC of the limbs was performed as described previously [9,18]. Rats were anesthetized with intraperitoneal injections of pentobarbital sodium salt (50 mg/kg, Sigma-Aldrich, San Jose, CA, USA) and maintained spontaneous breathing during the preconditioning procedure. Limb RIPC was achieved with four cycles of bilateral hindlimb ischemia (5 min/cycle, 40 min total). For each cycle, the proximal part of the hindlimbs was tied with gauze ropes (13 cm $\times$ 13 cm) for 5 min, and this was followed by 5 min of reperfusion with the gauze ropes untied (Figure 1A). Two-dimensional laser speckle imaging (Perimed AB, Järfälla, Sweden) was used to reveal the blood flow to the hindlimbs. The blood flow was reduced to about 25% of baseline during ischemia and that reperfusion was fully reestablished after 5-min ischemia

(Figure 1B,C). Non-RIPC animals were exposed to the same anesthesia with pentobarbital sodium salt, and untied gauze ropes were placed on both their hindlimbs for 40 min. The preconditioning procedure was performed 1 h before middle cerebral artery occlusion (MCAO) or sham surgery.

Transient Focal Cerebral Ischemia and Reperfusion

Transient focal cerebral ischemia was induced by 90-min occlusion of the right MCA as described previously [19,20]. In brief, anesthesia was induced by inhalation of 5% isoflurane (Lunan Pharmaceutical Group Corporation; Shandong, China) in a 30% O₂/68.5% N₂O mixture and maintained with 2% isoflurane inhalation. Rectal temperature was maintained at $37 \pm 0.5^\circ\text{C}$ with a heating pad during surgical procedures. Laser Doppler flowmetry was used to measure regional cerebral blood flow, and only animals with cerebral blood flow reduction $>70\%$ of preischemia baseline levels during MCAO were used for further investigations. Sham-operated animals underwent anesthesia and exposure of the right MCA without occlusion. RIPC rats were randomly assigned to MCAO or sham operation groups. Three groups of animals were included in this study (Figure 1A): non-RIPC+MCAO (designated as MCAO), RIPC+Sham, and RIPC+MCAO. All outcomes were assessed by investigators blinded to experimental group assignments.

2, 3, 5-Triphenyltetrazolium Chloride Staining

For 2, 3, 5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich) staining, brains were removed rapidly on ice and sliced into seven coronal sections (2 mm thick). The sections were immersed in 2% TTC at 37°C for 20 min and then fixed in 4% paraformaldehyde. The infarct volume with edema correction was calculated as the volume of the contralateral hemisphere minus the noninfarcted volume of the ipsilateral hemisphere.

Neurological Deficit Assessments

Neurological deficits were assessed before surgery and 0.5, 24, 48, and 72 h after brain reperfusion by an investigator blinded to experimental groups. Two scoring systems were used: (1) the 12-scale scoring system proposed by Belayev *et al.* [21,22] (higher scores indicate worse deficits) and (2) the Longa scoring system [23] (0 = no deficit, 1 = failure to extend left forepaw, 2 = circling to the left, 3 = falling to the left, 4 = failure to walk spontaneously and loss of consciousness, 5 = death).

Leukocyte and Serum Harvest

Leukocytes and sera were collected immediately before and 3 days after MCAO. Briefly, blood (0.5–1 mL) was harvested from the left or right caudal vein into tubes containing heparin–saline anticoagulant. After centrifugation (500 *g*, 4 min at 4°C), the supernatant (plasma) was collected and frozen at -80°C for cytokine analyses. Cell pellets were treated with red blood cell lysis buffer (Beyotime Biotechnology Co. Ltd., Jiangsu, China) to remove red blood cells. Leukocytes were then collected for flow cytometry.

Figure 1 Experimental protocols and model of remote limb ischemic preconditioning. **A.** Three groups of animals were generated: remote ischemic preconditioning (RIPC)+Sham; non-RIPC+ middle cerebral artery occlusion (MCAO) (designated as MCAO); and RIPC+MCAO. Limb RIPC was conducted by four cycles (5 min/cycle, 40 min total) of bilateral hindlimb ischemia under pentobarbital anesthesia. Non-RIPC animals were exposed to the same anesthesia for 40 min. Brain ischemia was induced by 90 min middle cerebral artery occlusion (MCAO). Sham-operated animals underwent anesthesia and surgical exposure of the right MCA without occlusion. Blood was collected from the caudal tail vein at 1 h after preconditioning and 3 days after brain reperfusion. ① blood sample taken from rats in MCAO group before stroke; ② blood sample taken from rats in MCAO group after 3 days of reperfusion; ③ blood sample taken from rats in RIPC+MCAO group 1 h after preconditioning; ④ blood sample taken from rats in RIPC+MCAO group 3 days after reperfusion. Animals were sacrificed 3 days after reperfusion. **B.** Representative images of blood flow in the left thigh using a laser speckle contrast imager before RIPC, 3 min after one cycle of RIPC, and 1 min after reperfusion. **C.** Representative recordings of blood flow before RIPC, 3 min after one cycle of RIPC, and 1 min after reperfusion.

Flow Cytometry Analyses

Leukocytes were resuspended in phosphate-buffered saline (PBS) at a concentration of $2 \times 10^5/\text{mL}$ and then stained with fluorochrome-conjugated antibodies for 30 min at room temperature in the dark. All antibodies were purchased from BD Pharmingen (San Jose, CA, USA) or eBioscience (San Diego, CA, USA). Antibodies were directly labeled with one of the following fluorescent tags: fluorescein isothiocyanate (FITC), phycoerythrin (PE), or allophycocyanin (APC). Appropriate isotype controls matching the host species and isotype of each primary antibody were used. Cells were analyzed on a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson, San Jose, CA, USA). The population 1 (P1) of interest was gated on the scatter plots of forward scatter (FSC-H) and side scatter (SSC-H), excluding debris or cell aggregates. T-cell subsets, B-cell subsets, NK cells subsets, and mononuclear cell subsets were further gated in the P1 population based on the expression of specific markers. The cell populations that were examined here include CD4⁺ T cells (CD3⁺/CD4⁺), CD8⁺ T cells (CD3⁺/CD8⁺), B lymphocytes (CD3⁺/CD45RA⁺), NKT cells (CD3⁺/CD161a⁺), NK cells (CD3⁺/CD161a⁺), and monocytes (CD43^{dim}/CD172a⁺ and CD43⁺/

CD172a⁺). Results were expressed as percentages of total number of targeted cells.

Cytokine Assay by ELISA

Concentrations of IL-6, TNF- α , IL-1 β , and IL-10 in plasma were measured with commercially available enzyme-linked immunosorbent assay (ELISA) kits from Dakewe Biotech Company (Shenzhen, China) according to the manufacturer's instructions, as previously described [24].

Statistical Analyses

The number of rats in each experimental group was determined by power analyses, according to our past experience with similar measurements ($\alpha = 0.05$ and $\beta = 0.20$). All data are expressed as mean $\pm$ standard error of mean. Multiple comparisons were performed using a one-way ANOVA followed by the Bonferroni *post hoc* test. The Student's *t*-test was used for comparisons of only two groups. Normality of the distribution and homogeneity of variance were assessed by the F-test or Bartlett's test before the Student *t*-test and the ANOVA, respectively. Differences were deemed significant only when $P \leq 0.05$.

Results

RIPC Reduces Infarct Volumes and Improves Neurological Scores after Stroke

Rats subjected to RIPC demonstrated significant reductions in infarct volumes compared to non-RIPC animals, as revealed by TTC staining at 3 days after MCAO (Figure 2A). Consistent with the reductions in brain infarct sizes, RIPC rats also exhibited superior neurological function at 72 h after reperfusion (Table 1). Although body weights were comparable between RIPC and non-RIPC rats within 2 days after stroke, the RIPC rats weighed slightly more than non-RIPC rats by 72 h after stroke (Figure 2B). Taken together, these results suggest that RIPC protects against focal ischemia and preserves neurological functions after stroke.

The Effect of RIPC on T-cell Populations before and after Stroke

Remote ischemic preconditioning procedures alone significantly decreased the percentage of CD8 T cells ($CD3^+/CD8^+$)

and NKT cells ($CD3^+/CD161a^+$) in peripheral blood (① vs. ③ in Figure 3D–E and Figure 3G–H, respectively), whereas levels of CD4 T cells ($CD3^+/CD4^+$) (① vs. ③ in Figure 3A,B, respectively) remained unchanged. The numbers of CD4 and CD8 T lymphocytes dramatically decreased in peripheral blood at 3 days after stroke, both in animals without RIPC (① vs. ② in Figure 3A,B,D–E) and in animals with RIPC (③ vs. ④ in Figure 3A,B,D–E). Notably, RIPC significantly ameliorated the post-MCAO reduction of blood CD8 T cells (②–① vs. ④–③ in Figure 3F), but not CD4 T cells (②–① vs. ④–③ in Figure 3C).

Consistent with previous reports, NKT cells, a subpopulation of T cells that share properties of both T cells and natural killer (NK) cells, were significantly reduced after stroke in non-RIPC rats (① vs. ② in Figure 3G–H). RIPC also significantly reduced blood NKT cells (① vs. ③ in Figure 3G–H) whereas the reduction of blood NKT cells after stroke was completely abolished (③ vs. ④ in Figure 3G–H). Thus, RIPC resulted in significant attenuation of NKT cell loss in the blood after stroke (②–① vs. ④–③ in Figure 3I).

Figure 2 Remote limb preconditioning reduces infarct size and improves neurological scores after stroke. **A.** Representative TTC images from animals treated with or without remote ischemic preconditioning (RIPC) followed by middle cerebral artery occlusion (MCAO) or sham operation (left), and quantification of infarct volumes (right). **B.** Body weight in all groups. $n = 12$ rats per group. Data are presented as means $\pm$ SEM, $*P \leq 0.05$, $**P \leq 0.01$ versus MCAO.

Table 1 Neurological function scores after middle cerebral artery occlusion (MCAO) surgery

Groups	NFS 0.5-h median (IQR)	NFS 24-h median (IQR)	NFS 48-h median (IQR)	NFS 72-h median (IQR)
12 Scale Score				
MCAO	8 (2)	9 (4)	8 (2.25)	8.5 (2.5)
RIPC+MCAO	6.5 (2.25)	10 (3.25)	7 (1.5)	6 (1.25)*
Longa Score				
MCAO	3 (1)	3 (1)	3 (0.25)	3 (0.25)
RIPC+MCAO	3 (1)	2.5 (1)	2 (1)	2 (2)*

NFS, indicates neurological function score; IQR, the interquartile range, is calculated as Quartile 3 - Quartile 1. * $P \leq 0.05$ vs. MCAO group at the same time point.

Preconditioning Results in Robust Increases in B-Cell Populations after Stroke

The percentage of B cells ($CD3^-/CD45RA^+$) in peripheral blood was slightly reduced in rats subjected to RIPC alone (① vs. ③, Figure 4A,B) and in non-RIPC rats after stroke (① vs. ② in Figure 4A,B). Strikingly, in the RIPC+MCAO group, the B-cell ratio was elevated after stroke (③ vs. ④, Figure 4A,B), which reversed the reduction in the peripheral B-cell population otherwise observed after stroke (②-① vs. ④-③, Figure 4C).

Preconditioning Increases the Monocyte Population before Stroke

Among rat monocytes, the $CD43^-/CD172a^+$ subpopulation is classically associated with extravasation and inflammation, whereas the $CD43^+/CD172a^+$ subpopulation is thought to replenish resident tissue macrophages and dendritic cells [25]. In this study, we analyzed both subsets of monocytes. RIPC and MCAO exerted minimal effects on the percentage of inflammatory $CD43^-/CD172a^+$ monocytes. In contrast, the percentage of $CD43^+/CD172a^+$ noninflammatory resident monocytes in the blood exhibited a trend toward a decrease after stroke (① vs. ② and ③ vs. ④, Figure 5A and 5B). Interestingly, RIPC significantly elevated the percentage of $CD43^+/CD172a^+$ monocytes, resulting in an increase in the percentage of total monocytes in the blood (① vs. ③, Figure 5A and 5B). Moreover, animals subjected to RIPC exhibited a trend toward greater reduction of $CD43^+/CD172a^+$ monocytes in the blood after stroke (②-① vs. ④-③, Figure 5C). Finally, RIPC exerted minimal effects on the NK-cell population prior to and after stroke (Figure 6A).

Preconditioning alters Cytokine Profiles after Stroke

Using ELISAs, we found that plasma IL-6 was significantly increased 3 days after stroke, whereas the levels of TNF- α and IL-10 remained unchanged (① vs. ②, Figure 7A). RIPC alone significantly increased IL-6 expression, but did not influence the concentration of TNF- α and IL-10 (① vs. ③, Figure 7A). The ischemic challenge in rats subjected to RIPC further increased

plasma IL-6 levels (③ vs. ④, Figure 7A). Interestingly, previous exposure to RIPC greatly elevated TNF- α level in the plasma after stroke (②-① vs. ④-③, Figure 7B). The abovementioned data demonstrate that RIPC magnifies the poststroke upregulation of IL-6 and TNF- α in the plasma.

Discussion

It is becoming increasingly accepted that the cross-talk between the central nervous system and the immune system plays a pivotal role in the progression of ischemic stroke. In this study, we discovered that RIPC, which protects the brain against subsequent ischemic strokes and preserves neurological function, dramatically alters the levels of multiple immune cell populations and circulating cytokines. These results suggest that immunomodulation might be an important mechanism underlying remote limb-mediated preconditioning of the ischemic brain.

Lymphocytes are a major class of immune cells that are actively involved in poststroke neuroinflammation and brain damage. Severe combined immunodeficiency (SCID) mice [26] or $Rag1^{-/-}$ mice that lack mature B and T lymphocytes [27,28] exhibit smaller infarcts and superior neurological function shortly after transient cerebral ischemia, in support of a destructive role for lymphocytes after stroke. Further studies have confirmed that the detrimental effects of lymphocytes are largely attributed to T lymphocytes, because the neuroprotection observed in $Rag1^{-/-}$ mice can be attenuated by T-cell, but not B-cell, reconstitution. Selective depletion of either CD4 T cells or CD8 T cell subpopulations reveals that both T-cell types damage the ischemic brain. [27,29] Consistent with previous reports [30], we found a dramatic reduction in the percentage of T cells (both CD4 T cells and CD8 T cells) after stroke, which may reflect their infiltration into the ischemic brain from the circulation [31]. Interestingly, previous exposure to RIPC, while having no obvious effect on CD4 T cells, dramatically reduced the number of CD8 T cells in the blood prior to stroke and ameliorated the reduction in blood CD8 T cells after stroke. These results are consistent with the view that RIPC reduces the infiltration of cytotoxic CD8 $^+$ T cells into the ischemic brain, which may at least partially contribute to neuroprotective effects of RIPC after stroke.

In contrast to the detrimental effects of CD8 T cells, recent studies highlight a protective role for B lymphocytes, specifically IL-10-secreting B cells, in stroke models. B-cell deficiency contributes to further brain damage and exacerbates functional outcomes after transient cerebral ischemia, while adoptive transfer of B cells into B-cell deficient mice reduces infarct sizes and improves neurological function [32]. Intriguingly, IL-10-secreting B cells exert both central and peripheral protective effects after ischemia [33,34]. Our data reveal that the percentage of B cells in RIPC animals was even higher after MCAO than before MCAO, resulting in a robust increase in the B-cell population after MCAO, in contrast to a reduction in the B-cell population in non-RIPC animals after MCAO. These results suggest that the upregulation of B cells in the blood might be due to increased B-cell generation and/or release from storage places such as spleen or bone marrow.[35] Such an elevation in circulating B cells, even without central penetration, might provide indirect protection to the ischemic brain [34]. In support of the importance of B cells in preconditioning, a

Figure 3 The effect of preconditioning on T-cell populations before and after middle cerebral artery occlusion (MCAO). Blood was collected from the caudal tail vein at 1 h after preconditioning and 3 days after brain reperfusion. **A–C.** Flow cytometry analysis of CD3⁺CD4⁺ T cells. **D–F.** Flow cytometry analysis of CD3⁺CD8⁺ T cells. **G–I.** Flow cytometry analysis of CD3⁺CD161⁺ NKT cells. ②–① reflects the change in T-cell populations prior to and after MCAO without preconditioning; ④–③ reflects the change in T-cell populations prior to and after MCAO in the remote ischemic preconditioning (RIPC)+MCAO group. Data are expressed as means ± SEM for 12 independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$ versus ① or ②–①; # $P \leq 0.05$, ## $P \leq 0.01$ versus ③.

recent study reported that a group of immunosuppressive B cells is important in hypoxic preconditioning-mediated long-term tolerance of brain ischemia [15]. Further studies are warranted to identify which subpopulation of B cells is elevated after stroke in RIPC animals and confirm whether such a change in B-cell populations is essential for RIPC-afforded protection against ischemia.

Peripheral monocyte/macrophages are known to infiltrate into the infarct areas soon after stroke and contribute to brain injury

and repair [36]. Shifting spleen monocytes to a less proinflammatory state can reduce infarct volume after transient focal ischemia, suggesting that the phenotype of peripheral monocytes may influence stroke outcome [37]. As shown in the present study, MCAO reduces noninflammatory CD43⁺/CD172a⁺ monocytes in the blood with no change in inflammatory CD43⁺/CD172a⁺ monocytes. This is consistent with the increase of M2 microglia/macrophages in the ischemic brain soon after MCAO [38]. RIPC

Figure 4 The effect of preconditioning on B cells before and after MCAO. Blood was collected from the caudal tail vein at 1 h after preconditioning and 3 days after brain reperfusion. B cells were labeled with CD3 and CD45RA antibodies and analyzed by flow cytometry. **A.** Representative flow cytometry graphs of CD3⁺CD45RA⁺ B cells prior to and after MCAO. **B.** Statistical analysis of B-cell percentages prior to and after MCAO. **C.** Statistical analysis of B-cell changes before and after MCAO. ②-① reflects the change in B cells before and after MCAO without preconditioning; ④-③ reflects the change in B cells before and after MCAO in the remote ischemic preconditioning (RIPC)+MCAO group. Data are expressed as means $\pm$ SEM for 12 independent experiments, ** $P \leq 0.01$ versus ②-①.

Figure 5 The effect of preconditioning on monocytes before and after middle cerebral artery occlusion (MCAO). Blood was collected from the caudal tail vein at 1 h after preconditioning and 3 days after brain reperfusion. **A.** Representative flow cytometry graphs of monocytes before and after MCAO. **B.** Statistical analysis of monocytes percentages before and after MCAO. **C.** Statistical analysis of monocyte changes before and after MCAO. ②-① reflects the change in monocytes before and after MCAO without preconditioning; ④-③ reflects the change in monocytes before and after MCAO in the remote ischemic preconditioning (RIPC)+MCAO group. Data are expressed as means $\pm$ SEM for 12 independent experiments.

increases noninflammatory monocytes in the circulation prior to stroke. This beneficial phenotype switch may also help to mitigate the effects of the ensuing ischemic attack.

The RIPC procedure itself resulted in the loss of NKT cells in the blood, although no further reduction in NKT cells was observed in these animals after MCAO. The functional impairments in hepatic NKT cells after stroke have recently been identified as crucial for poststroke immunosuppression and the establishment of subsequent infections [39]. Therefore, the reduction of blood NKT cells in RIPC rats—although it may not directly relate to the brain infarct—might impair stroke outcomes by weakening defenses against infectious complications after stroke. This limitation will have to be addressed before RIPC is translated into clinical use.

Notably, exposure to RIPC resulted in significant induction of TNF- α after MCAO. TNF- α is one of the first cytokines identified as

essential for the induction of ischemic tolerance. Some inflammatory preconditioning stimuli, including lipopolysaccharides and CpG oligodeoxynucleotides [40,41], are known to increase circulating TNF- α . Elevations in TNF- α help to establish neuroprotection against subsequent ischemia, as these preconditioning stimuli fail to protect TNF- α -deficient mice [42] or TNF- α -neutralized neuron–glia cultures [43] against ischemic insults. Furthermore, TNF- α itself serves as a preconditioning stimulus to protect against brain ischemia [44]. Therefore, the induction of TNF- α after RIPC might represent an important mechanism underlying remote limb preconditioning-mediated protection.

In contrast to TNF- α , we discovered that the induction of IL-6 in RIPC animals occurred prior to the MCAO insult. IL-6 has been implicated in the pathophysiology of stroke. Several studies clearly demonstrate a neuroprotective role for IL-6 in stroke mod-

Figure 6 The effect of preconditioning on NK cells before and after middle cerebral artery occlusion (MCAO). Blood was collected from the caudal tail vein at 1 h after preconditioning and 3 days after brain reperfusion. **A.** Representative flow cytometry graphs of CD3⁺/CD161⁺ NK cells before and after MCAO. **B.** Statistical analysis of NK-cell percentages before and after MCAO. **C.** Statistical analysis of NK-cell changes before and after MCAO. ②-① reflects the change in NK cells before and after MCAO without preconditioning; ④-③ reflects the change in NK cells before and after MCAO in the remote ischemic preconditioning (RIPC)+MCAO group. Data are expressed as means $\pm$ SEM for 12 independent experiments.

Figure 7 Remote limb preconditioning alters cytokine expression before and after middle cerebral artery occlusion (MCAO). Blood was collected from the caudal tail vein at 1 h after preconditioning and 3 days after brain reperfusion. The levels of TNF- α , IL-6, and IL-10 were measured by ELISA. **A.** Statistical analysis of cytokines before and after MCAO, ** $P \leq 0.01$ versus ①, ## $P \leq 0.01$ versus ③. **B.** Statistical analysis of cytokine changes before and after MCAO. ②-① reflects plasma cytokine levels before and after MCAO without preconditioning; ④-③ reflects the change in cytokine levels before and after MCAO in the remote ischemic preconditioning (RIPC)+MCAO group. ** $P \leq 0.01$ versus ②-①. Data are expressed as means $\pm$ SEM for 12 independent experiments.

els [45,46], while others suggest a destructive role for IL-6 in stroke outcomes [47]. The reasons underlying this discrepancy remain uncertain, but may reflect the broad functions of IL-6 in both tissue damage and repair after stroke. Nevertheless, IL-6 has been shown to be important for hypoxic preconditioning-afforded protection against stroke [48]. Whether the RIPC-induced IL-6 is

essential for stroke protection in our model deserves further investigation. To this end, it would also be important to explore whether the alterations in circulating IL-6, as well as TNF- α , results in similar trend of changes in the brain parenchyma and whether there is a correlation between peripheral or central cytokine levels and the stroke outcome.

There are still questions remain to be answered regarding RIPC-induced immune responses. For example, how RIPC induces the alterations in peripheral immune system remains elusive. A previous study demonstrated that transfusion of the platelet-derived microparticles prepared from healthy rats subjected to hindlimb RIPC could protect the recipient mice against ischemic brain injury [13]. These microparticles are known to carry a rich set of cytokines, chemokines, enzymes, and signaling proteins [49]. It is therefore possible that the contents in these microparticles shed from the injured cells in the ischemic limb serve as messengers to exchange intercellular biological signals and activate immune cells in the circulation. Further study is needed to identify the exact trigger for immune responses after RIPC. In addition, in view of accumulating evidence showing the difference in poststroke injury and immune responses between male and female [50–53], it would be important to evaluate in the future study whether there is any difference in RIPC-induced immune changes in female rodents.

Our study provides considerable insight into the potential cross-talk between the central nervous system and the immune system in RIPC. Our results suggest that RIPC may provide protection against ischemia by mobilizing the peripheral immune system prior to an ischemic attack and enhancing host defenses. Further studies are warranted to (1) assess the temporal kinetics of the immune cell changes by including more time points of serum collection, (2) determine the signaling mechanism whereby RIPC influences circulating immune cells, (3) determine the contribution of each immune element in RIPC-afforded ischemic tolerance, and (4) elucidate the mechanism of action underlying the tolerance. Future studies of the effects of remote ischemic post-conditioning on peripheral immune cell changes following stroke are also highly warranted. These studies might lead to the devel-

opment of systemically administered immune therapies for stroke patients, because the serum is more accessible for therapeutics than the brain.

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Conflict of Interest

The authors declare no conflict of interest.

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