Interleukin-4 Is Essential for Microglia/Macrophage M2 Polarization and Long-Term Recovery After Cerebral Ischemia

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Background and Purpose—Interleukin-4 (IL-4) is a unique cytokine that may contribute to brain repair by regulating microglia/macrophage functions. Thus, we examined the effect of IL-4 on long-term recovery and microglia/macrophage polarization in 2 well-established stroke models.

Methods—Transient middle cerebral artery occlusion or permanent distal middle cerebral artery occlusion was induced in wild-type and IL-4 knockout C57/BL6 mice. In a separate cohort of wild-type animals, IL-4 (60 ng/d for 7 days) or vehicle was infused into the cerebroventricle after transient middle cerebral artery occlusion. Behavioral outcomes were assessed by the Rotarod, corner, foot fault, and Morris water maze tests. Neuronal tissue loss was verified by 2 independent neuron markers. Markers of classically activated (M1) and alternatively activated (M2) microglia were assessed by real-time polymerase chain reaction, immunofluorescence, and flow cytometry.

Results—Loss of IL-4 exacerbated sensorimotor deficits and impaired cognitive functions ≤21 days post injury. In contrast to the delayed deterioration of neurological functions, IL-4 deficiency increased neuronal tissue loss only in the acute phase (5 days) after stroke and had no impact on neuronal tissue loss 14 or 21 days post injury. Loss of IL-4 promoted expression of M1 microglia/macrophage markers and impaired expression of M2 markers at 5 and 14 days post injury. Administration of IL-4 into the ischemic brain also enhanced long-term functional recovery.

Conclusions—The cytokine IL-4 improves long-term neurological outcomes after stroke, perhaps through M2 phenotype induction in microglia/macrophages. These results are the first to suggest that immunomodulation with IL-4 is a promising approach to promote long-term functional recovery after stroke. (Stroke. 2016;47:498-504. DOI: 10.1161/STROKEAHA.115.012079.)

Key Words: flow cytometry  ■ interleukin-4  ■ macrophages  ■ neurological function  ■ stroke

Cerebral ischemia launches multiple rapid immune responses, characterized by the activation of resident microglia, the infiltration of peripheral immune cells such as macrophages and lymphocytes, and the accumulation of immune mediators in the lesioned brain. Remarkably, emerging evidence suggests that activated microglia/macrophages polarize into distinct phenotypes to differentially regulate the microenvironment in the injured brain.1–3 Specifically, classically activated M1 microglia release destructive proinflammatory mediators and exacerbate brain damage.1–4 In contrast, the alternatively activated M2 phenotype is essential for tissue preservation and brain repair because M2 cells resolve local inflammation, clear cell debris, and provide trophic factors.1,4–6 We recently discovered a M2-to-M1 phenotype shift in the subacute stages of stroke,2 resulting in an unfavorable environment for brain recovery. Further elucidating the endogenous factors that regulate microglia/macrophage polarization after stroke may hasten the identification of novel therapeutic targets to improve stroke outcomes.

Interleukin-4 (IL-4) is a multifunctional cytokine secreted mainly by T-helper 2 cells, mast cells, eosinophils, basophils, and stromal cells.7 IL-4 is well-known to regulate a variety of immune responses, including T-cell differentiation and IgE class switch in B cells. It is also thus far the best-characterized promoter of M2 polarization in microglia and macrophages. Accumulating evidence indicates that IL-4 plays critical roles...
in brain function under both physiological and pathological conditions. For example, T cell–derived IL-4 is important in learning and memory in the normal brain. IL-4 levels in the brain decline with age, perhaps contributing to cognitive decline in aged populations and increasing the risk for development of Alzheimer disease. Recent results from clinical and animal studies support the importance of IL-4 in the acute stages of stroke. In patients, serum levels of IL-4 increase significantly several hours after stroke onset. IL-4 deficiency exacerbates brain injury and worsens neurological outcomes 24 hours after transient middle cerebral artery occlusion (tMCAO) in animal models of stroke, suggesting that IL-4 serves as an early endogenous neuroprotective mechanism soon after stroke onset. The effect of IL-4 on long-term recovery after stroke has not yet been explored although persistent recovery is more important for clinical translation.

In this report, we used IL-4 knockout (KO) mice to investigate the natural role of endogenous IL-4 in long-term neurobehavioral and neuropathological outcomes after focal cerebral ischemia. We discovered that IL-4 deficiency exacerbates sensorimotor and cognitive deficits ≤21 days after tMCAO or distal MCAO (dMCAO), and that this persistent effect is accompanied by impaired microglia/macrophage polarization toward the M2 phenotype. Conversely, the administration of IL-4 directly into the ischemic brain enhances functional recovery after stroke. Therefore, IL-4 is an important immune modulator serving to improve long-term outcomes after stroke, perhaps by regulating microglia/macrophage polarization and promoting an immune milieu that is highly permissive for behavioral recovery.

### Materials and Methods

#### Animal Models

All animal experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed in accordance with the principles outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Wild-type (WT) or IL-4 KO male C57/BL6 mice (8–10 w, 25–30 g body weight; Jackson Laboratory, Bar Harbor, ME) were randomly assigned to sham or cerebral ischemia groups using a lottery drawing box. Transient focal ischemia was induced by MCAO for 60 minutes as previously described. Sham-operated animals underwent anesthesia and exposure of the arteries without MCAO induction. Rectal temperature was maintained at 37.0±0.5°C during and after surgery with a temperature-controlled heating pad. Physiological parameters were maintained within normal ranges. Regional cerebral blood flow was monitored in all stroke animals using laser Doppler flowmetry. Animals that died or failed to show at least 70% regional cerebral blood flow reduction were excluded from further analyses. Mice in both genotypes were subjected to repeated measurements of CBF before ischemia, 5 minutes after tMCAO and 5 minutes after reperfusion using a laser speckle contrast imager (LDF, PeriFlux System 5000, Perimed). In all experiments, the tMCAO or sham surgeries were performed by an investigator blinded to genotype.

#### Intracerebroventricular IL-4 Administration

WT mice subjected to 60-minute MCAO or sham operation were randomly assigned to vehicle or IL-4 groups. An intracerebroventricular catheter was stereotaxically implanted into the ventricle contralateral to the lesion site (coordinates: −0.20 mm anterior and 1.00 mm lateral to bregma). An ALZET osmotic minipump containing IL-4 (Peprotech, 60 ng/d) or phosphate-buffered saline was implanted subcutaneously above the spine and connected to the intracerebroventricular catheter by an investigator blinded to experimental groups. The pump was programmed to achieve a constant infusion rate of 0.5 µL/h, starting from 6 hours after tMCAO. The pump was removed at 7 days after tMCAO.

#### Statistical Analysis

All data were reported as means±SEM. Significant differences between means were assessed by 1- or 2-way ANOVA and post hoc least significant difference tests for multiple comparisons. The Student’s t test was used for single comparisons. A P value <0.05 was deemed statistically significant. See the online-only Data Supplement for full description of experimental procedures.

#### Results

### Increased Expression of the IL-4 Receptor in the Ischemic Brain

Previous studies report an early increase of IL-4 in the serum of patients with stroke. We found that the expression of IL-4 mRNA in the mouse brain increased slightly but not significantly between 1 and 7 days after tMCAO and then collapsed on day 14 (Figure 1A). Interestingly, the expression of the...
IL-4 receptor (IL-4Rα) was significantly elevated within 1 day after stroke, and this elevation was maintained for at least 14 days after stroke (Figure 1B). These findings reveal compensatory changes in the IL-4 pathway after stroke injury in vivo.

**Loss of IL-4 Disrupts Long-Term Neurological Functions After Ischemic/Reperfusion Injury**

Next, we used IL-4 KO mice to investigate the effect of IL-4 on long-term neurological outcomes after stroke. Transient focal ischemia was induced by 60-minute tMCAO. Physiological parameters, including blood pressure, blood gases, and glucose levels, exhibited no significant differences between WT and KO mice (not shown). Regional cerebral blood flow, as determined with a laser speckle contrast imager, did not differ by genotype before ischemia, 5 minutes after ischemia, or 5 minutes after reperfusion (Figure 1C). These findings verify that WT and IL-4 KO mice were subjected to the same ischemic insult during tMCAO and that any differences across genotypes cannot be attributed to variations in blood flow.

To determine the impact of IL-4 on functional outcomes after stroke, a battery of neurobehavioral tests were administered during both the acute and the long-term recovery stages after tMCAO. Although the sensorimotor outcomes were comparable between IL-4 KO and WT mice early after stroke, the KO mice exhibited much slower recovery over time, resulting in significant deterioration of behavioral performance at late stages after stroke. As shown in Figure 1D, the latency to fall off the Rotarod was much shorter in IL-4 KO mice than in WT mice at 5 to 9 days after stroke. Similarly, a protracted impairment in the corner test was observed in IL-4 KO mice at 9 to 14 days after tMCAO.

We further determined whether IL-4 deficiency impaired long-term cognitive functions using the Morris water maze test. The IL-4 KO and WT mice exhibited comparable cognitive functions after sham surgery under physiological conditions. However, long-term learning and memory deficits were significantly exacerbated in IL-4 KO mice after tMCAO, as manifested by an increased latency to find the hidden platform (impaired spatial learning ability) and reduced time spent in the target quadrant when the platform was removed (impaired memory) compared with WT mice (Figure 2A–2C). Both the genotypes exhibited similar swim speeds (Figure 2D), suggesting equivalent swimming skills. Taken together, the results of the behavioral assays demonstrate a stable or persistent exacerbation of neurological dysfunction after stroke in the absence of IL-4.

**IL-4 Deficiency Results in Early, Transient Enlargement of Infarct Size After Stroke**

IL-4 KO mice exhibited greater infarct volumes than WT mice at 5 days after tMCAO, as measured by loss of microtubule-associated protein 2 (MAP2) (marker of neuronal dendrites and somas, Figure 3) and NeuN (marker of neuronal nuclei, Figure 1 in the online-only Data Supplement). In contrast to the delayed deterioration of neurological functions shown in Figures 1 and 2, the neuronal tissue loss in IL-4 KO mice was not significantly different from WT mice at 14 and 21 days after tMCAO (Figure 3; Figure 1 in the online-only Data Supplement). These results suggest that endogenous IL-4 may delay neuronal death but cannot rescue neurons or reduce neuronal tissue loss over the long term.

**Loss of IL-4 Shifts Microglia/Macrophage Polarization Toward M1**

Recent studies have highlighted the importance of microglia/macrophage polarity in the progression of brain injury and in brain repair. IL-4 is known to induce microglia/macrophage polarization toward the beneficial M2 phenotype. Therefore, we explored whether the poor outcomes in IL-4 KO mice were associated with altered microglia/macrophage polarization after stroke. Real-time polymerase chain reaction measurements revealed the elevation of multiple M1-like markers, including CD16, tumor necrosis factor α, and inducible nitric oxide synthase (iNOS), at both 5 and 14 days after tMCAO in IL-4 KO mice (Figure 4A). Double immunostaining for CD16 and the microglia/macrophage marker Iba-1 confirmed that the number of CD16+ M1 microglia/macrophages was significantly increased in the striatum, but not in the cortex, in IL-4 KO mice after tMCAO (Figure 4B–4D). These results are consistent with an enhancement in M1 polarization and cerebral inflammation that extends into the late stages after stroke in IL-4 KO mice. Consistent with these findings, IL-4 KO mice exhibited reduced expression of M2-like markers (CD206 and IL-10) in the ischemic brain at both 5 and 14 days after tMCAO (Figure 5A). As shown by
immunostaining, the number of CD206+ M2 microglia/macrophages was significantly decreased in IL-4 KO mice in both cortex and striatum at 14 days after stroke (Figure 5B). We then used flow cytometry to further characterize microglia (CD45intermediateCD11b+) and macrophages (CD45highCD11b+) during IL-4–induced M2 phenotype shifts. We found that IL-4 deficiency reduced the number of CD206+ macrophages in the ischemic brain (Figure 5C). The majority of resting microglia in the brain was CD206+, although the expression level of CD206 per cell was relatively weak. Interestingly, the MCAO insult enhanced the mean fluorescence intensity of CD206 staining in each cell without increasing the total number of CD206+ microglia. These results suggest that resting microglia may already possess M2 properties that might be enhanced on stimulation. This notion is consistent with previous reports that M2 microglia show a greater tendency to maintain their M2 status.14,15 Compared with the flow cytometry results, the immunostaining revealed a more dramatic drop in the number of CD206+ microglia/macrophages in IL-4 KO stroke mice. This finding might be attributed to the relatively lower sensitivity and higher threshold for immunohistochemical detection of CD206+, such that weakly positive cells in flow cytometry may be identified as negative by immunohistochemical staining. Taken together, our data suggest that endogenous IL-4 plays an important role in M2 microglia/macrophage polarization after stroke, which would be expected to facilitate long-term neurobehavioral recovery by creating an anti-inflammatory milieu.

IL-4 Deficiency Exacerbates Long-Term Neurological Functions and Impairs Microglia/Macrophage M2 Polarization After Permanent dMCAO

Effects that can be generalized across different stroke models are more likely to translate to clinical studies. Thus, we also tested the effect of IL-4 in a model of permanent distal MCAO (dMCAO). The corner test revealed that dMCAO resulted in transient sensorimotor deficits early after surgery with a recovery by 9 days after dMCAO in WT mice. IL-4 deficiency significantly prolonged the sensorimotor deficits (Figure IIA in the online-only Data Supplement). Consistent with the results in the tMCAO model, NeuN immunostaining revealed similar neuronal tissue loss in WT and IL-4 KO mice at 21 days after dMCAO (Figure IIB in the online-only Data Supplement). Real-time polymerase chain reaction measurements revealed increased mRNA expression of M1 markers (tumor necrosis factor α) and reduced expression of M2 markers (CD206 and IL-10) at 21 days after dMCAO in IL-4 KO mice (Figure IIC in the online-only Data Supplement). These results verify that IL-4 deficiency impairs M2 polarization and worsens neurological outcomes in 2 independent stroke models.

Figure 3. Loss of interleukin-4 (IL-4) increases brain tissue loss at early but not late stages after ischemia. A, Representative brain slices with MAP2-negative infarcts in wild-type (WT) mice and IL-4 knockout (KO) mice at 5, 14, and 21 days after ischemia. B–D, Quantification of infarct size by loss of MAP2 immunostaining in the cortex (B), striatum (C), and total brain (D); n=6 mice per group. Data are expressed as mean±SEM. *P<0.05; **P<0.01 compared with WT mice.

Figure 4. Loss of interleukin-4 (IL-4) promotes the M1 phenotype in microglia/macrophages after ischemic stroke. A, M1 marker (CD16, tumor necrosis factor α, and iNOS) mRNA levels. Real-time polymerase chain reaction was performed using total RNA extracted from ischemic brains at 5 and 14 days after transient middle cerebral artery occlusion (tMCAO) or from sham-operated brains. Data are expressed as % of sham-operated controls; n=4 to 6 per group. B, Quantification of the number of CD16+ microglia/macrophages at the inner boundary of the infarct in wild-type (WT) and IL-4 knockout (KO) mice at 14 days after tMCAO or sham operation; n=4 to 5 mice per group. Data are expressed as means±SEM. *P<0.05, **P<0.01, ***P<0.001 vs WT. C and D, Representative dual immunofluorescent staining of CD16 and Iba1 in the cortex (C) and striatum (D) in brain sections obtained from ischemic mice at 14 days after MCAO. Scale bar, 50 μm. DAPI indicates 4′,6-diamidino-2-phenylindole; and iNOS, inducible nitric oxide synthase.
If loss of endogenous IL-4 leads to long-term neurological dysfunction, administration of exogenous IL-4 should have the converse effect and lead to stable recovery of function. Furthermore, if IL-4 protein levels are low 1 to 7 days after stroke, IL-4 supplementation to be extremely effective at this time point. Thus, to further confirm the importance of IL-4 in persistent stroke recovery, recombinant IL-4 (60 ng/d) was continuously infused through an osmotic minipump into the brain of C57/BL6 mice beginning 6 hours after tMCAO or sham operation and lasting for 1 week. Neurological functions were then assessed at various time points after surgery. IL-4 post treatment failed to improve neurobehavioral performance 1 to 7 days after tMCAO. However, at late stages after tMCAO, IL-4–treated mice showed improved performance in the Rotarod (14–35 days) and foot fault tests (21–35 days; Figure 6A–6B). Moreover, IL-4–treated mice exhibited improvements in cognitive functions, as manifested by improved spatial learning and memory in the Morris water maze (Figure 6C). Overall neuronal tissue loss was measured on NeuN-stained sections at 14 and 35 days after tMCAO but revealed no significant effect of IL-4 treatment (Figure 6D), entirely consistent with the IL-4 KO data.

**Discussion**

IL-4 is an important anti-inflammatory cytokine that is already known to protect the brain against acute ischemic injury.11 A recent study further suggests that IL-4 is essential for the resistance to acute brain ischemia observed during estrus in female animals.13 However, the long-term influence of IL-4 on the pathophysiology of ischemic stroke remains elusive. In this study, we found a prolonged increase in mRNA levels of the IL-4R in the ischemic brain, suggesting that there is an increased response to IL-4 in both early and late stages after stroke. In contrast, IL-4 expression increases only slightly in the brain 1 to 7 days after stroke, followed by a

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dramatic decline at 14 days post injury. This transient IL-4 elevation might be attributed, at least partly, to the infiltration and accumulation of T cells, the major cellular source of IL-4, and other immune cells into the ischemic brain. In addition, a recent study demonstrated that injured neurons may release IL-4 at acute stages of stroke. These results suggest that increased signaling through the IL-4R may mitigate the inflammatory environment after stroke and lay the groundwork for recovery of neural function. Consistent with this hypothesis, we demonstrated that IL-4 deficiency significantly impairs sensorimotor and cognitive performance in 2 models of stroke, whereas IL-4 supplementation improves long-term functional outcomes after brain injury. These data strongly support a beneficial role for IL-4 in the recovery phase after stroke. Consistent with the present findings, population-based data demonstrate an association between IL-4 gene polymorphisms and functional outcomes after stroke and the incidence of poststroke depression. Therefore, boosting IL-4 levels is a promising therapeutic strategy to promote persistent and stable neurological recovery after stroke.

The importance of IL-4 in cognitive function under physiological conditions has been disputed. One early report suggested that IL-4 KO mice exhibit defects in spatial learning and memory capacities in the Morris water maze test. However, a recent study using the same approach demonstrated comparable performance between IL-4–deficient mice and WT controls. In addition, IL-4 KO mice performed normally in object location recognition tests (spatial memory) and novel object recognition tests (nonspatial memory), suggesting equivalent baseline cognitive functions between WT and IL-4 KO genotypes. The present results confirm that sham IL-4 KO mice experience no changes in cognitive function in the Morris water maze. However, IL-4 deficiency significantly exacerbated cognitive dysfunction after stroke, demonstrating the importance of IL-4 in maintaining or restoring cognitive capacities under pathological conditions.

IL-4–specific receptors are ubiquitously expressed in glial cells (microglia, astrocytes, and oligodendrocytes) as well as a variety of peripheral immune cells. However, it is not known whether IL-4 targets cells in the central nervous system directly or exerts central protection indirectly by modifying peripheral immune responses after stroke. The increase in IL-4R in ischemic brain cells in this study is consistent with the view that IL-4 exerts at least some effects on the central nervous system. Furthermore, intraventricular IL-4 infusions improve long-term recovery after stroke, which is also consistent with the hypothesis that IL-4 elevation within the central nervous system directly acts on cells in the brain to mitigate the long-term neurological sequelae of stroke.

Microglia residing in the central nervous system, as well as centrally infiltrating macrophages, are established cellular targets of IL-4. Although IL-4 is well-known to induce microglia/macroglia polarization in vitro and in vivo in models of other neurological diseases, the effect of endogenous IL-4 on microglia/macroglia polarization status after stroke has not previously been investigated. Our data clearly show reductions in M2 microglia/macrophage polarization in both the striatum and the cortex of IL-4 KO mice, strongly supporting the importance of IL-4 in determining the M1/M2 equilibrium at both early and late stages after stroke. Because of the well-known protective or restorative effects of M2 microglia/macrophages in the injured brain, the M2 phenotype shift observed in this study may be critical for long-term behavioral protection after stroke. Further studies are warranted to test the novel hypothesis that M2 induction by IL-4 after stroke injury serves as a natural brake on the progression of neurological dysfunction in vivo. Other M2-inducing cytokines, such as IL-10, have also been demonstrated to be protective in models of stroke. Our data show that loss of IL-4 reduces the expression of IL-10 in the ischemic brain. Whether IL-4 works independently or in concert with IL-10 to modulate microglia/macrophage M2 polarization after stroke also deserves further investigation.

We have shown that the M1 phenotype exacerbates, whereas the M2 phenotype mitigates neuronal death under ischemic conditions. Although IL-4 loss promoted the M1 phenotype and reduced M2 induction in this study, there was no significant long-term effect on neuronal loss, suggesting that microglia/macrophage polarization is probably necessary but not sufficient for neuroprotection. Because IL-4 deficiency increased neuronal loss at acute phases of stroke but did not seem to affect neuronal loss at late stages, it is possible that neuron-independent mechanisms contribute to the long-term beneficial role of IL-4 in neurological functions. For example, recent evidence highlights the importance of white matter injury in long-term sensorimotor and cognitive deficits after stroke. Indeed, some compounds have been shown to protect white matter integrity after stroke without any significant influence on gray matter, suggesting that white matter protection may be achieved independent of neuronal protection. Therefore, white matter components may represent potential targets for IL-4 treatment in stroke victims. Consistent with these hypotheses, a protective role of IL-4 against white matter injury has been shown in a model of multiple sclerosis, in which IL-4 KO mice exhibited greater demyelination and functional deficits. Furthermore, experiments using selective depletion of M1 or M2 phenotypic cells have confirmed that the M2 phenotype promotes, whereas the M1 phenotype impairs, white matter recovery after demyelination. Further studies are warranted to confirm the effect of IL-4 on white matter integrity after stroke and further elucidate the cellular targets of IL-4.

Our current study focused on the long-term effect of IL-4 on stroke in males. Interestingly, recent studies support the importance of IL-4 in sex differences in stroke models. IL-4 is critical for neuroprotection during the estrus phase of the estrous cycle in females. Furthermore, microglia isolated from female mice after MCAO express higher levels of the IL-4R on the M2 phenotype. Further studies are warranted to investigate the effect of IL-4 on long-term outcomes after stroke in female mice.
immunomodulatory approaches to improve functional recovery after stroke.

**Sources of Funding**

This work was supported by the National Institutes of Health grants NS092618 (to Dr Hu), NS05671 and NS089534 (to Dr Chen), grant 13SDG14570025 from the American Heart Association (to Dr Hu), the Chinese Natural Science Foundation grants 81471209 (to Dr Liu), 81171149, 81371306 (to Dr Gao), and 81228008 (to Dr Chen). Dr Chen is also supported by a Veteran’s Affairs research career scientist award.

**Disclosures**

None.

**References**


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*Stroke.* 2016;47:498-504; originally published online January 5, 2016;
doi: 10.1161/STROKEAHA.115.012079
*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/47/2/498

Data Supplement (unedited) at:
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Supplemental Methods:

Murine model of permanent focal ischemia: All animals were randomly assigned to sham and distal MCAO groups through the use of a lottery-drawing box. Focal cerebral ischemia was produced by permanent distal MCAO, as previously reported, plus ipsilateral common carotid artery (CCA) occlusion. Briefly, mice were anesthetized with 1.5% isoflurane in a 30% O2/68.5% N2O mixture under spontaneous breathing conditions. Rectal temperature was maintained at 37.0°C±0.5°C during surgery with a temperature-regulated heating pad. First, a skin incision was made at the midline of the neck. After being separated from the vagal nerve, the left CCA was exposed and occluded by ligation. The skin was sutured and another skin incision was made between the left eye and the ear. The temporal muscle was dissected by bipolar electrocautery (Bipolar Coagulator, Codman & Shurtleff Inc., Randolph, MA, USA). The temporal muscle temperature was maintained at 37.0°C±0.5°C by a heat lamp. After opening the burr hole and performing a craniotomy, the distal part of the MCA was exposed. The dura mater was then cut and the distal MCAO was completed with low-intensity bipolar electrocautery at the immediate lateral part of the rhinal fissure. Regional cerebral blood flow (CBF) was measured in all stroke animals using laser Doppler flowmetry. Animals that did not show a regional CBF reduction to <30% of pre-ischemia baseline levels were excluded from further experimentation. Sham-operated animals underwent the same anesthesia and surgical procedures but were not subjected to CCAO and MCAO. All behavioral (sensorimotor and memory tests) and histological (immunostaining and cell counting) assessments were performed by investigators blinded to experimental group assignments.

Measurement of tissue loss: Animals were euthanized and transcardially perfused with saline followed by 4% paraformaldehyde in PBS (pH 7.4). Frozen serial coronal sections (25 μm) were sliced beginning +1.42 mm from bregma. Slices were immunohistochemically stained with rabbit anti-MAP2 (Santa Cruz Biotechnology) or rabbit anti-NeuN (Millipore) antibodies. Tissue loss was determined using Image J software by an observer blinded to group assignments. The area of tissue loss was measured by subtracting the nonlesioned area of the ipsilateral hemisphere from that of the contralateral hemisphere. The volume of tissue loss was calculated from the lesion areas in seven sections spanning the rostrocaudal extent of the ischemic injury.

Rotarod test: The Rotarod test was performed with the Rotamex 5 apparatus (Columbus Instruments). Animals were placed on an accelerating rotating rod and the speed was increased from 4 rpm (start speed) to 40 rpm (final speed) within 5 minutes. Mice were tested 3 times daily with a break of at least 5 minutes between tests. The latency to fall off the rotating rod was recorded by a blinded investigator. Data were expressed as the mean value from three trials.

Corner test: Two black boards with dimensions of 30×20×1 cm³ were placed together at a 30° angle. Mice were placed at the middle of the open side and trained to walk toward the corner. When the mouse entered deep into the corner, it would turn backwards to leave the corner. The non-ischemic mouse turned without side preference, whereas the ischemic mouse preferentially
turned toward the left side. Ten trials were performed for each mouse and the number of left turns was recorded over the course of ten trials.

Foot fault test The foot fault test was performed to assess forelimb function. Mice gripped the wire with their paws while moving on an elevated grid surface. A foot fault was recorded when the paw fell or slipped between the wires. Every animal was tested by a blinded investigator for three trials lasting 1 min each. The data were expressed as the number of errors made by the limbs contralateral to the injured hemisphere as a percentage of total steps.

Morris water maze test A circular platform (11 cm diameter) was submerged in one quadrant of a circular pool (109 cm diameter). The aim of the place navigation test is to assess the ability of the mice to find the submerged platform by memorizing external spatial cues. During the test, each mouse was put in the water in one of four quadrants and was allowed 60 seconds to find the platform. When each trial ended, the mouse was placed on the platform for another 30 seconds to help it remember the external spatial cues displayed around the room. Three trials were performed per day with the platform present. Data from those trials are shown as the latency to locate the hidden platform on each day and are in indirect proportion to spatial learning abilities.

Reverse transcription polymerase chain reaction (RT-qPCR) for mRNA quantification Total RNA was extracted from cerebral tissues (ischemic hemisphere) using the RNeasy Mini Kit (Qiagen) and reverse transcribed into cDNA using the Superscript III rev transcript kit (Invitrogen) according to the manufacturers’ protocols. The resulting cDNA was used for PCR on a real-time PCR System (Bio-Rad). Primers for qRT-PCR were as follows: IL-4 forward primer: 5'-GGTCTCAACCCCCAGCTAGT-3', reverse primer: 5'-GCCGATGATCTCTCTCAAGTGAT-3'; IL-4R forward primer: 5'-TGCCCTTTATTTACTTTCCG-3', reverse primer: 5'-ACCCAGTCACCTCCTTTG-3'; CD16 forward primer: 5'-TTTGGACACCAGATGTTCAG-3', reverse primer: 5'-GTCTTCTTGGAGACCTGGATC-3'; TNFa forward primer: 5'-AGAAGTTCCAAAATGGCCTC-3', reverse primer: 5'-TTTTACAGGAGGAATCG-3'; iNOS forward primer: 5'-CAAGCACCTTGGAAAGAGAG-3', reverse primer: 5'-AAGGCCAACACAGCATACC-3'; CD206 forward primer: 5'-CAAGGAAGGTGGCATTTG-3', reverse primer: 5'-CCTTACAGCTCTTTTGGCAAGC-3'; IL-10 forward primer: 5'-CCAAGCCCTTATCGGAAATGA-3', reverse primer: 5'-TTTTACAGGAGGAAATCG-3'; GAPDH forward primer: 5'-GTGAAGTCCGTTGAACGG-3', reverse primer: 5'-GTTTCCCCGTTGATGACCAG-3'. All data were normalized to GAPDH mRNA levels as an internal control.

Immunofluorescence staining Immunohistochemistry was performed on 25-μm free-floating brain sections. Primary antibodies included the following: rabbit anti-NeuN (Millipore), goat anti-CD206 (R&D Systems), rat anti-CD16 (BD Bioscience), and rabbit anti-Iba1 (Wako).
Immunostaining was analyzed with confocal microscopy (Olympus). All images were processed with Image J for the unbiased counting of automatically recognized cells by a blinded observer. Means were calculated from three randomly selected microscopic fields in the cortex and striatum of each section, and three consecutive sections were analyzed for each brain. Data are expressed as mean numbers of cells per square millimeter.

**Flow cytometry** Single cell suspensions were prepared from sham or MCAO brains using the neural tissue dissociation kit (Miltenyi Biotec), according to the manufacturer’s instructions. Cells were stained with anti-mouse CD206, CD11b, CD45 and the appropriate isotype controls (eBioscience). Flow cytometric analysis was performed using a FACS flow cytometer (BD Biosciences).

**Supplemental Figures:**

**Supplemental Figure I.** Loss of IL-4 increases neuronal tissue loss at early but not late stages after ischemia. **A**, Representative brain slices with dotted white lines depicting NeuN-negative infarct zones in wild-type mice and IL-4 KO mice at 5 d, 14 d, and 21 d after ischemia. **B**, Quantification of tissue injury by loss of NeuN immunostaining in the brain. n=4-6 mice/group. Data were expressed as mean ± SEM. **p≤0.01 compared to WT mice.
Supplementary Figure II. IL-4 deficiency disrupts long-term neurological function and impairs microglia/macrophage M2 polarization after permanent dMCAO. A, Sensorimotor function was evaluated up to 21d after dMCAO by the corner test in IL-4 KO and WT mice. n=8 for WT, n=10 for IL-4 KO. B, Brain tissue loss was measured on NeuN-stained coronal sections 21d after dMCAO, n=6 for each group. C, mRNA levels for M1 and M2 markers. RT-PCR was performed using total RNA extracted from ischemic brains at 21 days after dMCAO. Data are expressed as % of sham-operated controls. n=4 per group.

References: