Increased Brain Injury and Worsened Neurological Outcome in Interleukin-4 Knockout Mice After Transient Focal Cerebral Ischemia

Xiaoxing Xiong, MD; George E. Barreto, PhD; Lijun Xu, MD; Yi Bing Ouyang, PhD; Xinmin Xie, PhD, MD; Rona G. Giffard, PhD, MD

Background and Purpose—Stroke causes brain injury with activation of an inflammatory response that can contribute to injury. We tested the hypothesis that the anti-inflammatory cytokine interleukin-4 (IL-4) reduces injury after stroke using IL-4 knockout (KO) adult male mice.

Methods—IL-4 KO and wild-type mice were subjected to transient middle cerebral artery occlusion. Outcome was assessed by triphenyltetrazolium chloride staining for infarct volume, neuroscore and spontaneous activity for behavioral outcome, and immunostaining and stereological counting for cellular response.

Results—Infarction volume at 24 hours was significantly larger in IL-4 KO mice, neurological score was significantly worse, and spontaneous activity was reduced compared with wild-type mice. Increased macrophage/microglial infiltration, increased numbers of myeloperoxidase-positive cells, and increased Th1/Th2 ratio were observed in the infarct core in IL-4 KO mice. Reduced astrocyte activation was observed in the cortical penumbra in IL-4 KO mice. Recombinant IL-4 administered intracerebroventricularly before middle cerebral artery occlusion significantly reduced infarct volume, improved neurological score, reduced macrophages/microglia, and lowered the Th1/Th2 ratio in IL-4 KO mice, but not in wild-type.

Conclusions—Loss of IL-4 signaling in KO mice was associated with worse outcome, and this was reversed by giving exogenous IL-4. Worsened outcome was associated with increased inflammation in the core, which was reversed in IL-4 KO but not significantly changed in wild-type mice by exogenous IL-4. This is consistent with IL-4 signaling leading to reduced inflammation in the core and a possible beneficial role for activated astrocytes in the penumbra. (Stroke. 2011;42:2026-2032.)

Key Words: astrocyte ■ inflammation ■ microglia ■ stroke ■ Th1

Stroke causes brain injury and infarction of tissue with activation of an inflammatory response that includes activation of microglia and astrocytes.1–4 Ischemia induces transformation of resting microglia into reactive, proliferating, hypertrophied microglia. Activated microglia express major histocompatibility complex Class II and adhesion/costimulation molecules in response to neuronal damage, suggesting a role in T cell activation.5 Initially, reactive microglia accumulate in the boundary zone or penumbra,1,5 subsequently accumulating in the ischemic core. Although activated microglia are often considered harmful, microglia can also release beneficial neurotrophic factors.6 Astrocytes also respond to and produce inflammatory signals7 and through their interaction with microglia and neurons help determine the outcome from injury. T lymphocytes enter the brain parenchyma and accumulate in the ischemic core within 24 hours after stroke.8,9 They can produce damage by releasing proinflammatory mediators.10,11 Interleukin-4 (IL-4) plays a central role in the differentiation of antigen-stimulated naïve T cells into Th2 cells that produce anti-inflammatory cytokines IL-4, IL-10, and IL-13 at the same time as suppressing generation of Th1 cells, which produce proinflammation cytokines IL-1 and interferon-γ.12 The anti-inflammatory cytokine IL-10 was shown to be protective in stroke,13 but IL-4 has not yet been studied. Serum levels of IL-4 from Th2 cells were elevated significantly in patients with cerebral infarction.14 In vitro, astrocytes express IL-4 receptors, which can regulate astrocyte activation. IL-4 induces astrocytes to secrete nerve growth factor,15 suggesting a protective function of IL-4-stimulated astrocytes. Although IL-4 can induce microglial proliferation, the cells display more of a resting phenotype with downregulated phagocytic and antigen-presenting functions.16 Despite the likely importance of IL-4 signaling in stroke, the effects of endogenous IL-4 are not clear. We used IL-4 knockout (KO) mice to assess the role of IL-4 on infarct volume, neurological
outcome, and glial activation 24 hours after middle cerebral artery occlusion (MCAO).

Materials and Methods

Animals
BALB/c and IL-4 KO mice (BALB/c-IL-4tm2Nnt/J) were purchased from Jackson Laboratory (Bar Harbor, ME) and then bred in our animal facility as homozygotes. Mice were housed in the Stanford Medical School Animal Care Facilities and all use of animals was according to protocols approved by the Stanford Institutional Animal Care and Use Committee and were conducted according to the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Model of Focal Cerebral Ischemia
Male mice, 10 to 12 weeks old, were anesthetized with 2% isoflurane in 70% N₂O and balanced O₂ by face mask. Cerebral ischemia was 1 hour of MCAO with a silicone-coated 6-0 monofilament (Doccol Corp, Redlands, CA) followed by reperfusion.²⁷ Sham-operated mice underwent an identical procedure without inserting the suture.

Mice underwent an identical procedure without inserting the suture. Recombinant human IL-4 (R&D Systems, Minneapolis, MN) was administered 1 hour in blocking solution (0.1 mol/L PBS, 0.3% Triton X-100, and 5% equine serum), washed several times, then incubated overnight at 4°C with an antitryptic fibrillar acidic protein antibody (GFAP, diluted 1:5, #22522; Immunostar, Hudson, WI) for reactive and resting astrocytes, antibody to CD68 (diluted 1:200; MCA1957GA; Serotec, Kidlington, UK), for reactive macrophages/microglia, or antimmunoperoxidase (MPO, diluted 1:50, #A0398; DAKO, Glostrup, Denmark) for leukocytes. Sections were rinsed, incubated for 2 hours at room temperature with Alexa 594-conjugated secondary antibody (for GFAP²⁷ cells or MPO⁺ cells) or Alexa 488-conjugated secondary antibody (for CD68⁺ cells, diluted 1:200; Inovitrogen), washed, and mounted on glass slides using Vectashield mounting medium with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Immunostaining was absent when the first antibody was omitted.

Ischemic or sham-operated mice were anesthetized and perfused with 0.9% saline followed by paraformaldehyde in PBS (pH 7.4) as previously described.¹⁸ After 48 hours in 4% paraformaldehyde in PBS (pH 7.4), 50-μm sections were cut. Immunohistochemistry was carried out on free-floating sections. Washes and incubations were in 0.1 mol/L PBS (pH 7.4) containing 0.3% Triton X-100. Sections were incubated for 1 hour with blocking solution (0.1 mol/L PBS, 0.3% Triton X-100, and 5% equine serum), washed several times, then incubated overnight at 4°C with an antitryptic fibrillar acidic protein antibody (GFAP, diluted 1:5, #22522; Immunostar, Hudson, WI) for reactive and resting astrocytes, antibody to CD68 (diluted 1:200; MCA1957GA; Serotec, Kidlington, UK), for reactive macrophages/microglia, or antimmunoperoxidase (MPO, diluted 1:50, #A0398; DAKO, Glostrup, Denmark) for leukocytes. Sections were rinsed, incubated for 2 hours at room temperature with Alexa 594-conjugated secondary antibody (for GFAP²⁷ cells or MPO⁺ cells) or Alexa 488-conjugated secondary antibody (for CD68⁺ cells, diluted 1:200; Inovitrogen), washed, and mounted on glass slides using Vectashield mounting medium with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Immunostaining was absent when the first antibody was omitted.

Morphometric Analysis
The expression of GFAP, CD68, or MPO was analyzed with the optical fractionator method on epifluorescence photomicrographs taken with a Zeiss Axiosvert inverted epifluorescence microscope (Zeiss, Jena, Germany) covering a total of 0.16 mm². The expression of T-bet or GATA3 was observed with a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan) covering a total of 0.78 mm². For each animal, the number of GFAP⁺ or CD68⁺/H9262 immunoreactive cells in the cortical penumbra or ischemic core (for CD68 and MPO, −1.70 to −2.18 mm relative to bregma) was counted, and a total of 48 counting frames of 100×100 μm was assessed per animal using Image J software. Representative micrographs showing glial response to injury were photographed at 20x or 40x magnification using a digital camera attached to a Zeiss LSM 510 META inverted laser scanning confocal microscope (Zeiss).

Assessment of Spontaneous Activity
The SmartCage system (AfaSci, Inc, Burlingame, CA) was used for automated analysis of spontaneous animal activity. Data sampled at 4 Hz in each sensor is gathered by 2 rows of infrared photobeams mounted on a Plexiglas box into which a standard mouse cage fits. Automated data analysis used CageScore software (AfaSci, Inc). The home cage activity variables assessed were beam breaks (x, y, and z photonbeam break counts) and locomotion by distance traveled and velocity. Distance traveled in centimeters was obtained from the lower horizontal sensors (x and y) taking into account the path taken. Average velocity was distance traveled per second in the forward direction averaged over the monitoring period. All mice were assessed continuously for 24 hours beginning after 3 hours reperfusion.

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Table. Physiological Measurements

<table>
<thead>
<tr>
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<th>WT (n=40)</th>
<th>IL-4 KO (n=40)</th>
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<tbody>
<tr>
<td>Before MCAO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>450.80±7.30</td>
<td>465.90±9.91</td>
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<tr>
<td>SpO₂, %</td>
<td>95.80±0.20</td>
<td>94.65±0.18</td>
</tr>
<tr>
<td>Temperature, °C</td>
<td>37.03±0.02</td>
<td>36.94±0.06</td>
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<tr>
<td>During MCAO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>456.40±9.02</td>
<td>438.70±9.47</td>
</tr>
<tr>
<td>SpO₂, %</td>
<td>95.70±0.16</td>
<td>96.10±0.18</td>
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<tr>
<td>Temperature, °C</td>
<td>36.61±0.09</td>
<td>36.83±0.11</td>
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<tr>
<td>After 24 h reperfusion</td>
<td></td>
<td></td>
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<tr>
<td>Heart rate, beats/min</td>
<td>439.15±7.60</td>
<td>405.45±10.26*</td>
</tr>
<tr>
<td>SpO₂, %</td>
<td>94.50±0.22</td>
<td>94.90±0.16</td>
</tr>
<tr>
<td>Temperature, °C</td>
<td>36.09±0.42</td>
<td>35.52±0.37</td>
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</tbody>
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Values are mean±SEM. Before MCAO=5 min before MCAO; During MCAO=at the end of 60 min MCAO.

WT indicates wild-type; IL-4, interleukin-4; KO, knockout; MCAO, middle cerebral artery occlusion; SpO₂, oxygen saturation.

*P<0.01.

Western Blotting

The ipsilateral hemisphere was harvested 24 hours after ischemia, homogenized in cold lysis buffer (10 mmol/L 4 to 2-hydroxyethyl-1-piperazine-ethanesulfonic acid [pH 7.9], 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 1 mmol/L dithiothreitol) plus protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined using bichinonic acid (Pierce, Rockford, IL). Equal amounts (30 µg) of protein were separated on 4% to 15% polyacrylamide gels (Bio-Rad, Hercules, CA) and electrotransferred to Immobilon polyvinylidene fluoride membranes (Millipore Corp, Bedford, MA) as previously described. Membranes were blocked with 5% nonfat dry milk in PBS with 0.1% Tween 20 for 1 hour, incubated overnight with rat anti-CD68 antibody (1:1000 dilution; Serotec) or anti-GFAP (1:1500; Cell Signaling, Danvers, MA), washed 3 times with 0.1% Tween in PBS, and incubated with 1:2000 secondary antibody (Cell Signaling) in 5% milk, 0.1% Tween in PBS for 90 minutes. Enhanced chemiluminescence reagent (Amer sham, Piscataway, NJ) and film were used for detection. Equal protein loading was confirmed by blotting with anti-β-actin antibody (1:1000; Santa Cruz Biotechnology). Densitometric analysis of bands was performed using Image J software.

Statistical Analysis

Data are expressed as mean±SEM. Differences were considered statistically significant for a probability value<0.05. Student t test was used when only 2 groups were compared. Two-way analysis of variance was used when both genotype and treatment were taken into account followed by Bonferroni post-tests using Prism 4 (GraphPad Software for Science, San Diego, CA).

Results

Infarction Volume and Neurological Deficit Are Increased in IL-4 KO Mice

Physiological variables were not significantly different between WT and IL-4 KO mice before MCAO, during MCAO, or at 24 hours reperfusion for respiratory rate or O₂ saturation (Table). Heart rate was lower in IL-4 KO mice than in WT (Table) at 24 hours reperfusion. Infarction volume at 24 hours was significantly larger in IL-4 KO mice (48.8%±4.3%) compared with WT (36%±3.2%; n=10/group, P=0.032;

Spontaneous Motor Activity Is Reduced More in IL-4 KO Mice After MCAO

After surgery, animals were allowed to recover for 3 hours on a heating pad and then while still in their home cage placed within the SmartCage to analyze spontaneous motor activity for 24 hours. Overall motor activity indicated by photobeam breaks and total travel distance were not significantly different between IL-4 KO mice and WT mice in either the naïve or sham surgery groups. After MCAO, beam breaks and travel distance, but not velocity, were significantly decreased in IL-4 KO mice compared with WT and sham controls (Figure 2A–D). These results are consistent with the difference in neurological scores and infarct volume.

Increased Macrophage/Microglial Infiltration Reduced Astrocyte Activation in IL-4 KO

Glial activation was prominent in animals after MCAO. A panoramic low-power view is shown (Figure 3A). Morphometric analysis (Figure 3B–C) shows a greater increase in the number of GFAP-positive cells in the cortical penumbra in
WT animals compared with IL-4 KO animals at 24 hours reperfusion. Almost no GFAP+ astrocytes are observed in the ischemic core in either genotype. No significant differences in astrocyte activation were observed in sham-operated animals. The total number of activated macrophages/microglia increased to a significantly greater extent in the ischemic core of IL-4 KO compared with WT animals (Figure 3C). This contrasts with very low numbers of GFAP-positive cells in the ischemic core (data not shown). This increased number of activated macrophages/microglia in the ischemic core is associated with significantly increased infiltration of leukocytes, detected as MPO-positive cells in the ischemic core (Figure 3C). Consistent with the increased numbers of activated microglia/macrophages, CD68 protein levels were significantly higher in IL-4 KO mice compared with WT mice 24 hours after MCAO in the ischemic hemisphere (Figure 4A). In contrast, GFAP expression did not differ by genotype (Figure 4B).

Recombinant Human IL-4 Reduces Infarction Volume, Neurological Score, and Inflammation in IL-4 KO Mice
Recombinant human IL-4 administered intracerebroventricularly before MCAO significantly reduced infarct volume from 50.6±1.9 (n=9) to 33.4±3.3 (n=8, P=0.0006; Figure 5A) and reduced neurological score from (2.80±0.20 n=5) to 1.78±0.22 (n=9, P=0.01; Figure 5B). Recombinant human IL-4 administration decreased CD68+ cells in the core from 35.75±2.25 to 22.00±1.09 (P=0.001, n=4/group; Figure 5C) and penumbra from 24.25±1.11 to 18.00±2.58 (P=0.068, n=4/group; Figure 5D). Staining for the Th1 marker T-bet and the Th2 marker GATA3 (Figure 5E–H) showed decreased Th1/Th2 ratio from 1.67±0.21 to 0.79±0.10 (P=0.008, n=4/group; Figure 5F) in IL-4 KO mice but no change in WT mice.

Discussion
The main finding of the present study is that IL-4 reduces ischemic injury, microglial activation, and Th1/Th2 ratio after stroke. The studies were conducted with mice bred homozygously. Although the effect of replacing IL-4 suggests that the loss of IL-4 accounts for the main effects observed, to rule out another genetic contribution, the studies should be confirmed using mice bred from heterozygous crosses. We observed a greater reduction of spontaneous locomotion after MCAO in IL-4 KO mice. Clinical studies
have shown that early neurological functional deficit is a major predictor of stroke outcome. In rodents, reduction of infarct volume and neuroscore are widely used as primary outcomes; neuroscore correlates well with infarct volume in acute stroke but less well with mild neurological deficits. The assessment of spontaneous motor activity with the SmartCage system provides detailed quantitative assessment, which here was consistent with infarct and neuroscore. Additional studies are needed to test this correlation over a range of injuries, but this may be a useful addition to outcome assessment. Additional studies are also needed for long-term outcome.

The absence of IL-4 was associated with decreased numbers of GFAP-immunoreactive astrocytes in the penumbra. Astrocyte activation encompasses a range of changes in gene expression and activity, so it is not clear whether this indicates fewer activated astrocytes or an alteration in activation pattern with reduced induction of GFAP. It is currently unknown whether the observed change in astrocyte activation in the penumbra in the absence of IL-4 contributes to increased inflammation. Prior work demonstrated a protective role for activated astrocytes after stroke and brain injury, so reduced astrocyte activation acutely may contribute to injury. Prior work has shown that astrocytes are as efficient as microglia at inducing IL-4 secretion from Th2 cells but less

**Figure 3.** GFAP-, CD68-, and MPO-immunoreactive cells in the cortical penumbra and ischemic core were assessed at 24 hours reperfusion. The upper picture shows a whole brain section in which the cortical penumbra (CP) and ischemic core (IC) areas are indicated. A, Glial activation in the CP and IC from WT and IL-4 KO animals (20×). B, Images and quantitation of GFAP + astrocytes or CD68 + microglia/macrophages at 40× magnification in CP. Increase in GFAP + cells is greater in WT with no statistical difference in CD68 + between genotypes. C, CD68 + cells and MPO + leukocytes (20×) were counted in the IC after MCAO. CD68 + and MPO + cells were greater in the IL-4 KO group. Scale bars, 50 μm. *P<0.05 vs sham of the same genotype; #P<0.05 vs WT MCAO. n=4 for MCAO IL-4 KO, n=5 MCAO WT, n=3 both sham groups. GFAP indicates glial fibrillary acidic protein; MPO, myeloperoxidase; WT, wild-type; IL-4, interleukin-4; KO, knockout.

**Figure 4.** CD68 (A) and GFAP (B) expression in the ischemic hemisphere assessed by Western. A, CD68 protein level increases after MCAO are greater in IL-4 KO, P=0.037. n=3 sham, n=6 WT MCAO, n=7 IL-4 KO MCAO. B, GFAP protein levels increased similarly in IL-4 KO and WT after MCAO. *P<0.05 vs sham same genotype; #P<0.05 vs WT MCAO; n=3 all groups. GFAP indicates glial fibrillary acidic protein; MCAO, middle cerebral artery occlusion; IL-4, interleukin-4; KO, knockout; WT, wild-type.

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efficient at stimulating Th1 responses. Future studies should examine interactions among astrocytes, microglia, and T lymphocytes.

Activated microglia not only synthesize numerous soluble and membrane-bound inflammatory mediators, including proinflammatory cytokines IL-1β, tumor necrosis factor-α, chemokines macrophage inflammatory protein-1α, monocyte chemoattractant protein-1, macrophage inflammatory protein-2, reactive oxygen, and nitrogen species, but can also present antigen to activate T cells. We observed an increased Th1/Th2 ratio and increased numbers of macrophages/macrophages in IL-4 KO mice, both of which reversed with exogenous IL-4. This suggests that activated microglia/macrophages and increased Th1/Th2 ratio may contribute to ischemic damage in IL-4 KO mice.

T and B cell-deficient mice have smaller infarcts. Both CD4⁺ and CD8⁺ T cells increase stroke injury. The increased Th1/Th2 ratio in IL-4 KO mice is consistent with a detrimental role for Th1CD4⁺ cells. Prior work suggests that the balance between Th1 and Th2 polarization may critically contribute to stroke with Th2 cells being protective but potentially also contributing to infectious complications. T cells express NADPH oxidase 2 in stroke, and recruitment is greater in males suggesting that greater oxidative stress may also contribute to worsened injury.

IL-4 is a major negative regulator of proinflammatory cytokine production by both brain cells and T lymphocytes and appears to play a key role in controlling neuroinflammation. On the other hand, microglia are normal brain constituents, defend against infection and toxic substances released from dying cells, and are beneficial in some settings. Therefore, fine-tuning the inflammatory response by the integrated signaling of IL-4 and other factors is likely critical under both physiological and pathological conditions. Here, absence of IL-4 was associated with increased infiltration of MPO-positive cells into the core. Prior studies have shown that inhibiting adhesion molecules reduced stroke volume, consistent with the idea that excessive infiltration of leukocytes worsens injury.

Figure 5. Administration of recombinant human interleukin-4 (rhIL-4) protects only in IL-4 KO mice. A, Infarct volume in WT was unchanged with rhIL-4 (n=7) but significantly decreased in IL-4 KO rhIL-4 (n=9) compared with IL-4 KO vehicle (P=0.0006, n=8). B, Neurological score was unchanged in WT rhIL-4 (P=0.28, n=4) but significantly decreased in IL-4 KO rhIL-4 (n=9) compared with vehicle-treated (n=5, P=0.01); (C) rhIL-4 did not change CD68⁺ cells in the ischemic core in WT but significantly decreased CD68⁺ cells in IL-4 KO rhIL-4 compared with IL-4 KO vehicle (P=0.002, n=4/group). D, IL-4 treatment did not change CD68⁺ cells in penumbra of WT (n=4) or IL-4 KO (P=0.068, n=4/group). E, Images of T-bet⁺ cells (Th1) or GATA-3⁺ (Th2) at 20× magnification in ischemic core (IC). Scale bar, 50 μm. F, Th1/Th2 ratio was greater in IL-4 KO vehicle compared with WT vehicle (P=0.008) or IL-4 KO rhIL-4 (P=0.008, n=4/group). Th1/Th2 ratio was unchanged in WT with/without rhIL-4 (n=4/group). G, RhIL-4 significantly decreased T-bet⁺ cells in the core of IL-4 KO compared with IL-4 KO vehicle, P=0.029. H, Fewer GATA3⁺ cells were in the ischemic core in IL-4 KO vehicle compared with WT vehicle (P=0.007, n=4/group). *P<0.05 vs vehicle injected same genotype; #P<0.05 vs WT vehicle. KO indicates knockout; WT, wild-type.
In conclusion, increased injury after MCAO in IL-4 KO mice was associated with increased CD68+ cells, MPO+ cells, and Th1/Th2 ratio in the core and decreased GFAP+ cells in the penumbra. The greater inflammation observed in the IL-4 KO mice suggests increased Th1 polarization is associated with greater injury and a possible beneficial role for activated astrocytes. Interestingly, intracerebroventricular injection of IL-4 provided no additional benefit in WT, suggesting that the beneficial effects in WT are already optimal.

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References
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