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 phagoctic 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-4<sup>tm2Nnt/J</sup>) 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% <sub>N</sub>2O and balanced O<sub>2</sub> by face mask. Cerebral ischemia was 1 hour of MCAO with a silicone-coated 6-0 monofilament (Doccol Corp, Redlands, CA) followed by reperfusion. Sham-operated mice underwent an identical procedure without inserting the suture. Rectal temperature was maintained at 37°C with a heating pad/H11006. Either vehicle (0.1% bovine serum albumin in 0.9% saline) or recombinant human IL-4 (R&D Systems, Minneapolis, MN) was infused in 5 g of recombinant human IL-4 (over 10 minutes) into the bone wound was closed with bone wax and the mouse prepared to undergo surgical incision. The incision was closed with 2-0 sutures. In pilot experiments, we tested 20.5°C with a heating pad/H11006.

Infarction volume is the sum of infarction areas multiplied by slice thickness.

Measurement of Cerebral Infarction Area
Twenty-four hours after MCAO and assessment of neurological score, mice were anesthetized with isoflurane and decapitated. Brains were removed and sectioned coronally with a rodent brain slicer matrix (Zivic Instruments, Pittsburgh, PA). Sections were incubated in 2% 2,3,5-triphenyletrazolium chloride and infarction volume determined using 4 slices per mouse analyzed by a blinded observer and corrected for edema using the National Institutes of Health Image program (Image J 1.37v, Wayne Rasband, available through the National Institutes of Health) as described previously. Infarction volume is the sum of infarction areas multiplied by slice thickness.

Evaluation of Neurological Status
Neurological status was evaluated at 24 hours according to a neurological grading score, from 0, no observable neurological deficit to 4, unable to walk spontaneously and a depressed level of consciousness. The evaluator was blinded to experimental treatment.

Treatment With Recombinant Human IL-4
Recombinant human IL-4 (R&D Systems, Minneapolis, MN) was injected intracerebroventricually. IL-4 KO mice and WT mice (25 to 30 g) were anesthetized with 2% isoflurane in 70% <sub>N</sub>2O balanced O<sub>2</sub> by face mask and placed in a stereotaxic frame with a mouse head holder. Either vehicle (0.1% bovine serum albumin in 0.9% phosphate-buffered saline (PBS) or 1 µg of recombinant human IL-4 dissolved in vehicle was infused in 5 µL over 10 minutes) into the right lateral ventricle through a burr hole once 30 minutes before MCAO based on a prior study. In pilot experiments, we tested 2 injections of IL-4 but observed almost 50% mortality. Due to this unexplained toxicity, we chose a single injection protocol. After that, the bone wound was closed with bone wax and the mouse prepared for MCAO. After 24 hours of reperfusion, the animals were euthanized and the brains removed for 2,3,5-triphenyletriazolium chloride staining.

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 photobeam 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.

Immunohistochemistry
Ischemic or sham-operated mice were anesthetized and perfused with 0.9% saline followed by paraformaldehyde in PBS (pH 7.4) as previously described. 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 antineurofilament 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 antitrymloperoxidase (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 GFAP<sup>+</sup> cells or MPO<sup>+</sup> cells) or Alexa 488-conjugated secondary (for CD68<sup>+</sup> cells, diluted 1:200; Invitrogen), 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.

For stereological evaluation of Th1 and Th2 cells, dianiomobenzidine staining was used. All washes and incubations were 0.1 mol/L PBS, 0.3% bovine serum albumin, and 0.3% Triton X-100 buffer. After 1 hour in blocking solution, endogenous peroxidase activity was quenched for 10 minutes in 3% hydrogen peroxide in 30% methanol. After several washes, sections were incubated for 1 day at 4°C with an antibody for T-bet (diluted 1:100, rabbit antimouse T-bet, clone Ch-210; Santa Cruz Biotechnology Inc, Santa Cruz, CA) or for GATA-3 (diluted 1:100, mouse anti-GATA-3, clone L50-823; BD Pharmingen, San Diego, CA). After incubating with primary antibody, sections were rinsed and incubated with biotinylated goat anti-rabbit immunoglobulin G (BA-1000; Vector Laboratories, Burlingame, CA; diluted 1:300 in wash buffer) or biotinylated horse antimouse immunoglobulin G (BA-2000; Vector Laboratories; diluted 1:300 in buffer). After several washes, sections were incubated for 90 minutes with avidin–biotin–peroxidase complex (Immunopure ABC peroxidase staining kit; Pierce, Rockford, IL; diluted 1:250), then incubated with 2 µg/mL 3,3'-dianiomobenzidine (Sigma-Aldrich, St Louis, MO) and 0.01% hydrogen peroxide in 0.1 mol/L phosphate buffer. Sections were dehydrated and mounted on gelatinized slides.

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 mm<sup>2</sup>. The expression of T-bet or GATA3 was observed with a Nikon Eclipse TE300 microscope (Nikon, Tokyo, Japan) covering a total of 0.78 mm<sup>2</sup>. For each animal, the number of GFAP<sup>+</sup> or CD68-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).
**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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<tr>
<td><strong>Before MCAO</strong></td>
<td></td>
<td></td>
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<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>
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<tr>
<td>Temperature, °C</td>
<td>37.03±0.02</td>
<td>36.94±0.06</td>
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<tr>
<td><strong>During MCAO</strong></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><strong>After 24 h reperfusion</strong></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>
</tr>
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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 (50 μg) of protein were separated on 4% to 15% gradient polyacrylamide gels (Bio-Rad, Hercules, CA) and electrotransferred to Immobilon polyvinylidene fluoride membranes (Millipore Corp, Bedford, MA) as previously described.17 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 (Amersham, 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...
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/microglia 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.
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

Disclosures
None.


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