Original Contribution

IL-4 Is Required for Sex Differences in Vulnerability to Focal Ischemia in Mice

Xiaoxing Xiong, MD, PhD; Lijun Xu, MD; Liang Wei, MD; Robin E. White, PhD; Yi-Bing Ouyang, PhD; Rona G. Giffard, MD, PhD

Background and Purpose—Interleukin (IL)-4 protects from middle cerebral artery occlusion in male mice. Females generally show less injury in response to the same ischemic challenge, but the underlying mechanisms are not fully understood. We tested the importance of IL-4 in female protection using IL-4 knockout (KO) mice.

Methods—IL-4 KO and wild-type (WT) mice of both sexes were subjected to middle cerebral artery occlusion. Infarct volume was assessed by triphenyltetrazolium chloride staining and neurobehavioral outcome by neuroscore. T cell proliferation was assessed after Concanavalin A exposure. Ischemic brain immune cell populations were analyzed by fluorescence-activated cell sorting and immunostaining.

Results—Infarction in WT females during estrus and proestrus phases was significantly smaller than in males; neurological score was better. Infarct volume was larger and neurological score worse in IL-4 KO compared with WT in both sexes, with no sex difference. Proliferation of T cells was inhibited in WT females with higher proliferation and no sex difference in IL-4 KO. Macrophage numbers and total T cells in the ischemic hemisphere were lower in WT females, and monocytes increased markedly in IL-4 KOs with no sex difference. The reduced macrophage infiltration in WT-females was predominantly M2. Loss of IL-4 increased CD68+ and iNOS+ cells and reduced YM1+ and Arg1+ cells in both sexes.

Conclusions—IL-4 is required for female neuroprotection during the estrus phase of the estrus cycle. Protected WT females show a predominance of M2-activated microglia/macrophages and reduced inflammatory infiltration. Increasing macrophage M2 polarization, with or without added inhibition of infiltration, may be a new approach to stroke treatment. (Stroke. 2015;46:00-00. DOI: 10.1161/STROKEAHA.115.008897.)

Key Words: estrus ■ estrus cycle ■ IL-4 ■ macrophage ■ microglia ■ stroke ■ T cells

Stroke is one of the leading causes of disability and death worldwide, yet treatment options are limited to recombinant tissue plasminogen activator in nonhemorrhagic stroke within the first few hours.1 Thus, alternative strategies for brain protection are still urgently needed. Stroke is sexually dimorphic. Women have a reduced incidence of stroke compared with men until well into old age, but poorer functional outcomes after stroke.2,3 The phases of the estrus cycle also affect stroke outcome.4 Female sex hormones, especially estrogen,5 reduce the consequences of ischemia by multiple mechanisms6 and moderate inflammation.7,8 In addition, male and female brain cells differ in the apoptotic cell death pathways invoked, even in the absence of added hormones, with the male more dependent on poly ADP ribose polymerase activation, leading to apoptosis inducing factor translocation. In the female, poly ADP ribose polymerase is protective against middle cerebral artery occlusion (MCAO), and inhibition leads to worsened outcome, the opposite of the result in male mice.9 Sex disparate effects were also seen in studies of a g-protein–coupled estrogen receptor agonist.10 Inflammation poststroke is a critical determinant of outcome, with both detrimental effects, such as causing secondary injury, and beneficial effects, including phagocytosis of debris, contributing to healing and recovery.11,12 The protective effects of estrogen in cardiovascular disease have been suggested to favorably influence the balance of M1/M2 macrophage activation.13 After stroke, the resident microglia are activated rapidly, and peripheral immune cells, including monocytes, neutrophils, and T and B cells, infiltrate the ischemic brain, interacting with each other and with brain cells, though the roles of these different cell types and their different activation patterns remain poorly understood.
Microglia/macrophages are highly plastic cells that can assume diverse phenotypes and engage different functional programs in response to specific microenvironmental signals. Though classically macrophages were described as having 2 distinct activation states, classically activated/proinflammatory macrophages (M1) and alternatively activated/resolving, regenerating macrophages (M2), it is now clear that there is a spectrum of phenotypes.14 Although the timecourse of M1 and M2 macrophages has been studied, and M2 suggested to be beneficial,15 it is clear that we do not fully understand the different roles of these different phenotypes because treatment with M2 macrophages after stroke did not improve outcome.16 In our previous study, the absence of interleukin (IL)-4 resulted in larger infarct related to greater Th1 cell and microglia/macrophage infiltration into the ischemic brain in male IL-4 knockout (IL-4 KO) mice compared with wild-type (WT).17

Despite the importance of IL-4 signaling in male stroke,17 the importance of IL-4 in female stroke is unknown, and the mechanism(s) remains unclear.

Methods

Animals

BALB/cJ and IL-4 KO mice (BALB/c-IL-4tm2Nnt/J) from Jackson Laboratory (Bar Harbor, ME) were bred in our animal facility as homozygotes. Mice were housed in the Stanford Medical School Animal Care Facility, and all use of animals was according to the National Institute of Health (NIH) Guide for Care and Use of Laboratory Animals. A total of 241 mice survived and were analyzed out of a total of 318 mice. Breakdown of conditions using the Benjamini–Hochberg method for multiple-testing correction to control the false-discovery-rate. One-way analysis of variance was used to compare all female variances were used to assess contributions of genotype and sex, followed by Student’s <0.05 considered significant.

Statistical Analyses

Data are expressed as mean±SD with P<0.05 considered significant. Student’s t-tests were used to compare 2 groups, 2-way analysis of variances were used to assess contributions of genotype and sex, followed by Bonferroni post-tests using Prism 5 (GraphPad, San Diego, CA). One-way analysis of variance was used to compare all female WT to male WT for infarct and neuroscore. For the fluorescence-activated cell sorting data, 2-way analysis followed by Student’s t-test to compare pairs of conditions using the Benjamini–Hochberg method for multiple-testing correction to control the false-discovery-rate.

Immunofluorescence Staining

Mice were euthanized with isoflurane overdose, perfused with ice-cold phosphate-buffered saline (PBS; pH 7.4), followed by 4% paraformaldehyde in PBS as previously.17 After postfixation for 72 hours, 50 μm coronal brain sections were cut. Free-floating sections were incubated for 1 hour with blocking solution (0.1 mol/L PBS, 0.1% Triton X-100, and 5% normal goat serum), then for 48 hours at 4°C with anti-CD68 antibody (1:100, MCA1957GA, AbD Serotec, Kidlington, Oxford, UK),19 antimyeloperoxidase antibody (diluted 1:50; No. A0398; DAKO, Carpinteria, CA), anti-iNOS (1:100, No. 1 (1:100, No. 610708; BD Biosciences Pharmingen, San Jose, CA), and antimouse inducible nitric oxide synthase+ (iNOS;1:100, No. 610329; BD Biosciences Pharmingen).20 Sections were rinsed, incubated 2 hours at room temperature with Alexa Fluor 488-conjugated goat antirabbit (anti-myeloperoxidase), goat antirat (CD68), goat antimouse (iNOS and arginase 1), or Alexa Fluor 594-conjugated goat antirabbit (anti-myeloperoxidase), goat antirat (CD68), goat antimouse (iNOS and arginase 1), or Alexa Fluor 594-conjugated goat antirabbit (iNOS and arginase 1). Mice were euthanized with an isoflurane overdose. To isolate splenic mononuclear leukocytes, spleens were chopped into small pieces, homogenized, filtered through a 70 μm strainer, and centrifuged at 250g for 5 minutes. After red blood cells were lysed using ACK Lysing buffer (GIBCO; Invitrogen, Carlsbad, CA), cells were labeled with 1 μm carboxyfluorescein succinimidyl ester (Invitrogen) and resuspended in RPMI1640 medium with 10% FBS. Carboxyfluorescein succinimidyl ester-labeled splenocytes were incubated with a Zeiss Axiovert inverted epifluorescence microscope (Zeiss LSM510, Jena, Germany), covering 0.14 mm². Three sections (−1.70 to −2.18 relative to Bregma/mouse) were randomly chosen and the number of CD68 or antimyeloperoxidase expressing cells in the core was counted using Image J; counts for each animal were averaged. Counts were performed on coded sections by a blinded investigator.

Carboxyfluorescein Succinimidyl Ester–Labeled Splenocytes, Mitogen-Induced Proliferation

Mice were euthanized with isoflurane overdose. To isolate splenic mononuclear leukocytes, spleens were chopped into small pieces, homogenized, filtered through a 70 μm strainer, and centrifuged at 250g for 5 minutes. After red blood cells were lysed using ACK Lysing buffer (GIBCO; Invitrogen, Carlsbad, CA), cells were labeled with 1 μm carboxyfluorescein succinimidyl ester (Invitrogen) and resuspended in RPMI1640 medium with 10% FBS. Carboxyfluorescein succinimidyl ester-labeled splenocytes at 2x10⁵ cells/well in 96 well flat-bottom tissue culture plates were incubated at 37°C and 5% CO₂ for 5 hours. 50 μl carboxyfluorescein succinimidyl ester dilution was quantified by flow cytometry.21

Fluorescence-Activated Cell Sorting Analyses for Immune Cells

Mice were deeply anesthetized with isoflurane 48 hours after reperfusion, transcardially perfused with cold PBS, and the ischemic hemisphere was analyzed. Brain microglia and mononuclear leukocytes were collected as previously.22 Cells were stained with fluorochrome-labeled mAbs against CD45 (FITC), CD11b (APC), CD3 (PECy7), CD4 (APCCy7), CD8 (Pacific Blue; BioLegend Inc, San Diego, CA) on ice for 30 minutes. Data on stained samples were acquired on a BD LSR II flow cytometer using Diva software (v6.1.2; Becton Dickinson, San Jose, CA) and analyzed using FlowJo software (v7.6.2; Tree Star, Ashland, OR).

Vaginal Smears for Estrus Cycle Stage Determination

Vaginal cells from female mice were evaluated for the presence of white blood cells and morphology of epithelial cells to determine the stage of the estrus cycle after staining with HEMAs (Fisher Scientific, Kalamazoo, MI), see Figure I in the online-only Data Supplement.

Measurement of Cerebral Infarction Area

Forty-eight hours after MCAO, mice were anesthetized with isoflurane, decapitated, and brains sectioned coronally with a rodent brain slicer matrix (Zivic Instruments, Pittsburgh, PA). Sections were incubated in 2% 3,3-diaminobenzidine chloride and infarction volume determined by a blinded observer using 4 slices per mouse and corrected for edema using NIH Image J (Image J 1.37v, Wayne Rasband, available from NIH) as previously.17

Transplant Focal Cerebral Ischemia, Neurological Evaluation

Male and female mice 10 to 12 weeks old were randomized to treatment groups, then anesthetized with 2% isoflurane in O ᵒ by face mask. The middle cerebral artery was occluded for 1 hour with a silicone-coated 6-0 monofilament (Doccol Corp, Redlands, CA), see Figure I in the online-only Data Supplement.

Data were analyzed out of a total of 318 mice. Breakdown of conditions using the Benjamini–Hochberg method for multiple-testing correction to control the false-discovery-rate.
Results

Female IL-4 KO Mice Fail to Show Protection

The 4 stages of the estrus cycle were determined by histological analysis of vaginal smears (Figure 1 in the online-only Data Supplement). Infarction volumes 48 hours post-MCAO were measured in males and in all 4 phases of the estrus cycle for females for WT and IL-4 KO mice (Figure 1A and 1B).

Infarction volumes in WT female mice during estrus and proestrus but not during diestrus and metestrus were significantly smaller than in WT male mice, and the neurological score showed significantly better function (Figure 1C). Estrus and proestrus are the phases of the cycle with higher estrogen levels. As previously reported, infarction volume was larger in IL-4 KO male mice compared with WT. Similarly, the female IL-4 KO mice had larger infarcts and the neurological score was significantly worse in both sexes. Loss of IL-4 abolished the sex difference in infarct size, neurological deficit, and the variation with phase of the estrus cycle (Figure 1A–1C).

Reduced Concanavalin A Stimulated Splenocyte Proliferation in WT Females During Estrus

For the rest of these studies, we evaluated male mice and female mice in the estrus phase of the estrus cycle, one of the 2 phases during which they show significant protection (Figure 1) and elevated estrogen levels. We evaluated the proliferative ability of splenocytes stimulated by Concanavalin A from WT and IL-4 KO mice. Proliferation of T cells was significantly lower in WT females during the estrus phase of the cycle compared with male mice, proliferation increased significantly in IL-4 KO mice (Figure 2A and 2B) with loss of sex differences with loss of IL-4 (Figure 2A and 2B).

WT Female Mice Have Fewer Mononuclear Cells in Ischemic Brain

We assessed microglia (CD45<sub>int</sub>CD11b<sup>+</sup>), monocytes/macrophages (CD45<sub>high</sub>CD11b<sup>+</sup>), total T cells (CD45<sub>high</sub>CD3<sup>+</sup>D<sub>3</sub>3+CD<sub>4</sub>3+D<sub>8</sub>3+), CD8<sub>+</sub> T cells (CD45<sub>high</sub>CD3<sup>+</sup>CD<sub>4</sub>8<sub>+</sub>), and CD4<sub>+</sub> T cells (CD45<sub>high</sub>CD3<sup>+</sup>CD<sub>4</sub>4<sub>+</sub>) in the ischemic hemisphere 48 hours post-MCAO by fluorescence-activated cell sorting (Figure 3A and 3B). There were significantly fewer T cells in the ischemic hemisphere of WT female mice during the estrus phase compared with WT male mice. In IL-4 KO mice, the numbers of these immune cells were sharply increased for all cell types relative to WT, and there were no longer significant sex differences (Figure 3B).

Predominant M2 Phenotype in Postischemic WT Female Brains

We evaluated sex differences in macrophage activation patterns in the ischemic core 48 hours post-MCAO (Figure 4A) by immunostaining for expression of CD68 (reactive microglia/macrophages; Figure 4B). CD68<sup>+</sup> cells were significantly fewer in WT females during estrus compared with WT males, but increased in both sexes of IL-4 KO mice such that there was no significant difference (Figure 4B). The number of cells expressing the M2 polarization markers, YM1 or Arg-1, was significantly higher in WT females than males, whereas IL-4 KO reduced numbers of YM1 and Arg-1-positive cells in both sexes (Figure 5A and 5B). In a reciprocal fashion, numbers of iNOS (an M1-associated marker)-expressing cells were increased by loss of IL-4 in both sexes (Figure 4C) and did not differ by sex. Staining for antitymoleperoxidase for neutrophils showed significantly fewer infiltrating cells in the ischemic core of female mice in estrus than in male mice. The number of cells increased markedly and the sex difference was abolished with loss of IL-4 (Figure 5C).
Discussion

Inflammation plays a critical role in the pathophysiology of ischemia-induced brain injury; however, the mechanisms by which inflammation contributes to sex differences in stroke largely remain to be elucidated. Here loss of IL-4 abolished sex differences in infarct volume, neuroscore, and infiltrating immune cells. Protected WT female mice had more cells expressing the M2 markers YM1 and Arg-1 and fewer T cells and neutrophils. These differences were lost in the absence of IL-4. Thus, IL-4 seems to play a central role in female protection.

A picture is beginning to emerge of the changes in immune cells in the brain after stroke, though the data in female rodents is much less than in males. Banerjee and colleagues compared male and female mice after MCAO and reported fewer CD45hi/CD11b+ cells in brains of females than in males, and a trend to more CD4+ cells in males, consistent with our results. They did not distinguish CD45hi from CD45int but did find more CD45hi in male brains and pointed out an increase in a subset of CD8+ T cells that secrete IL-10 in females after stroke. Similar to our data, this indicates females have a more anti-inflammatory activation with increased IL-10 and increased IL-4 effects. Circulating monocyte subtypes have been shown to predict clinical course in human stroke with a CD14dimCD16+ phenotype proposed to promote repair and associated with better outcome.

IL-4 plays a central role in the differentiation of Th2 cells that produce anti-inflammatory cytokines and in suppressing generation of Th1 cells, which produce proinflammatory cytokines. M1 and M2 macrophages are inducers and effectors in Th1 and Th2 immunity. We previously found IL-4 KO resulted in greater Th1 and microglia/macrophage infiltration and increased Th1/Th2 ratio in males. Microglia/macrophages respond dynamically with M2 phenotype detected early after stroke.
stroke then diminishing, whereas M1 phenotype increased with time and persisted. An earlier study found macrophage brain infiltration peaked at 3 days, but did not evaluate M1 versus M2 markers. Here we found fewer Ym1+ and Arg-1+ cells in males and fewer activated microglia/macrophages and neutrophils in females. M2 cells decreased, whereas the others cells all markedly increased with IL-4 KO, abolishing sex differences.

Although there was no difference in iNOS-positive cells (an M1 marker) between male and female WT, the ratio of M1 to M2 is different by sex in WT, and this is lost with loss of IL-4, with the change greater in female mice. Consistent with the literature, we find that IL-4 is a key factor in determining the M1/M2 balance, and this balance in macrophage/microglial activation seems central to sex differences and female protection from stroke. Although it is impossible to conclusively state that increased numbers of M2 activated cells causes reduced infarct size because reduced infarct size from any cause may be associated with reduced inflammatory infiltration, our results are consist with those of others suggesting that a balance of activation shifted toward the M2 phenotype is associated with smaller infarct size.

Estrogen is thought to play a key role in ischemic protection of female rodents and people. A recent study by Manwani and colleagues demonstrated the central role of gonadal hormones in brain ischemic susceptibility. Earlier work has suggested multiple mechanisms for protection by estrogen, including antioxidant effects, immune regulatory effects, and effects on cell death pathways (for review).

Our study has several limitations. We have conducted a preliminary study of T cell subtypes and characterized some markers that distinguish monocyte subtypes and activation pattern, but additional studies are needed to investigate the...
range of immune cell phenotypes and how they evolve over time, especially in females. The IL-4 KO mice used may have some compensatory changes; however, our prior study in male mice demonstrated that giving IL-4 restored infarct volume to that of WT, suggesting the phenotype largely reflects loss of IL-4.

We have repeated these studies with female mice and find similar results, administration of IL-4 to KO females reduces infarct size significantly, and IL-4 administration to WT does not alter infarct volume (Figure II in the online-only Data Supplement).

In conclusion, IL-4 is key in sex-dependent protection from stroke, affecting lymphocyte proliferation and infiltration, microglia/macrophage, and T cell activation and differentiation. Increasing M2 activation and reducing immune cell infiltration are potential targets for future stroke therapy.

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Disclosures
None.

References
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Full title: **IL-4 is required for sex differences in vulnerability to focal ischemia in mice**

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**Supplemental Table: Numbers of mice by genotype and sex**

<table>
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<th>WT male</th>
<th>IL-4 KO male</th>
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**WT= Wild Type**

**IL-4 KO= IL-4 Knock Out**

**Figure S I**

![Representative micrograph of vaginal smears shows the four phases of the estrus cycle. Estrus cycle phases were determined by histological evaluation of vaginal smears. The presence of white blood cells and the morphology of epithelial cells was assessed after staining with the HEMA 3 stain. Diestrous: polymorphonuclear lymphocytes (PMNs) present, proestrus: nucleated and cornified cells present, estrus: cornified epithelial cells present, metestrus: cornified epithelial cells and PMNs present.](image)

**Figure Legend Supplemental Figure I**

Representative micrograph of vaginal smears shows the four phases of the estrus cycle. Estrus cycle phases were determined by histological evaluation of vaginal smears. The presence of white blood cells and the morphology of epithelial cells was assessed after staining with the HEMA 3 stain. Diestrous: polymorphonuclear lymphocytes (PMNs) present, proestrus: nucleated and cornified cells present, estrus: cornified epithelial cells present, metestrus: cornified epithelial cells and PMNs present.

**Supplemental Figure II**

**Methods**

**Treatment with Recombinant Human IL-4**

Recombinant human IL-4 (R&D Systems, Minneapolis, MN) was injected intracerebroventricularly. Female WT and IL-4 KO mice were anesthetized with 2% isoflurane in O₂ 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 15 minutes) into the right lateral ventricle through a burr hole 60 minutes before MCAO, as previously described (1). The bone wound was closed with bone wax and the mouse prepared for MCAO. After 1 hr MCAO and 24 hours of reperfusion, the animals were assessed for neuroscore, and the brains removed for 2,3,5-triphenyltriazolium chloride (TTC) staining.
Figure S II

Figure Legend Supplemental Figure II

Effects of IL4 administration in WT and IL-4 KO female mice subjected to MCAO. Female mice of WT or IL-4 KO genotype were infused intracerebroventricularly with recombinant IL-4 or vehicle prior to undergoing MCAO. Neurological score was assessed 24 hr after MCAO. Brains were then collected for assessment of infarct volume.

A. Infarct volume was assessed by TTC staining. IL-4 KO mice had larger infarcts than WT when injected with vehicle, and this was significantly reduced by treatment with IL-4. * indicates a P < 0.05 compared to vehicle treated WT; # indicates p < 0.05 compared to vehicle injected IL-4 KO. Numbers of mice/group 9 WT + IL-4; 10 WT + vehicle; 9 IL-4 KO + IL-4; 6 IL4 KO + vehicle.

B. Neurological deficit score was assessed 24 hr after MCAO. * indicates p < 0.05 compared to WT with vehicle.

Values shown are mean + SD.

References