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IL-4 is required for sex differences in vulnerability to focal ischemia in mice

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Abstract

Background and Purpose—IL-4 protects from middle cerebral artery occlusion (MCAO) 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-4KO and wild-type mice of both sexes were subjected to MCAO. Infarct volume was assessed by triphenyltetrazolium chloride staining and neurobehavioral outcome by neuroscore. T cell proliferation was assessed after ConA exposure. Ischemic brain immune cell populations were analyzed by FACS and immunostaining.

Results—Infarction in WT-females during estrus and proestrus phases was significantly smaller than in males; neurological score was better. Infarction volume was larger and neurological score worse in IL-4KO 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-4KO. Macrophage numbers and total T cells in the ischemic hemisphere were lower in WT-females, and monocytes increased markedly in IL-4KOs 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.

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Keywords

IL-4; stroke; estrus cycle; 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 non-hemorrhagic 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 to men until well into old age, but poorer functional outcomes following 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 (PARP) activation leading to apoptosis inducing factor (AIF) translocation. In the female PARP is protective against 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 post stroke is a critical determinant of outcome, with both detrimental effects such as causing secondary injury, and beneficial effects including phagocytosis of debris and 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 two 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 While 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, as treatment with M2 macrophages after stroke did not improve outcome.¹⁶ In our previous study, the absence of IL-4 resulted in larger infarcts related to greater Th1 cell and microglia/macrophage infiltration into the ischemic brain in male IL-4 knockout (IL-4 KO) mice compared to 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-4^{tm2Nnt/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 protocols approved by the Stanford Institutional Animal Care and Use Committee and conducted according to the 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 by sex and genotype is in the supplemental table.

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 HEMA3 (Fisher Scientific, Kalamazoo, MI), see supplemental Figure I.¹⁸

Transient Focal Cerebral Ischemia, Neurological Evaluation

Male and female mice 10-12 weeks old, were randomized to treatment groups, then anesthetized with 2% isoflurane in O₂ by face mask. The middle cerebral artery was occluded (MCAO) for 1 hour with a silicone-coated 6-0 monofilament (Doccol Corp, Redlands, CA) as previously.¹⁷ The suture was not inserted in sham-operated mice. Neurological status was scored¹⁷ by a blinded investigator at 48 hours, with 0, no observable deficit to 4 unable to walk spontaneously and depressed level of consciousness. Mice that showed no neurological deficit and brains with evidence of hemorrhage were excluded from analysis.

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% 2,3,5-triphenyltetrazolium chloride (TTC) 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

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 post-fixation for 72 hours 50µm coronal brain sections were cut. Free-floating sections were incubated for 1 hour with blocking solution (0.1M PBS, 0.1% Triton X-100 and 5% normal goat serum), then 48 hours at 4°C with anti-CD68 antibody (1:100; MCA1957GA, AbD Serotec, Kidlington, Oxford, UK),¹⁹ anti-myeloperoxidase antibody (MPO, diluted 1:50, #A0398, DAKO, Carpinteria, CA), anti-Ym1 (1:100, #01404, Stem Cell Technologies, Vancouver, Canada), anti-arginase 1 (1:100, #610708, BD Biosciences Pharmingen, San Jose, CA) and anti-mouse iNOS/NOSII (1:100, #610329, BD Biosciences Pharmingen).²⁰

Sections were rinsed, incubated 2 hours at room temperature with Alexa Fluor 488-conjugated goat anti-rabbit (MPO), goat anti-rat (CD68), goat anti-mouse (iNOS and arginase 1) or Alexa Fluor 594-conjugated goat anti-rabbit (Ym1), (all diluted 1:400 (Invitrogen, Carlsbad, CA), then washed and mounted on glass slides with Vectashield with 4', 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Negative controls without primary antibodies were performed in parallel and showed no staining. Fluorescence micrographs were made 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 MPO 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.

CSFE labeled splenocytes, mitogen-induced proliferation

Mice were sacrificed 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 250 × g for 5 minutes. After red blood cells were lysed using ACK Lysing buffer (GIBCO, Invitrogen, Carlsbad, CA), cells were labeled with 2µM CFSE (Invitrogen) and re-suspended in RPMI1640 medium with 10% FBS. CFSE labeled splenocytes at 2 × 10⁵ cells/well in 96 well flat-bottom tissue culture plates were incubated at 37°C and 5% CO₂ for 66 hours in the presence of 1 µg/ml T cell mitogen Concanavalin A (ConA, Sigma-Aldrich, Santa Clara, CA), then CFSE dilution was quantified by flow cytometry.²¹

FACS 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, USA) on ice for 30 min. 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).

Statistical analyses

Data is expressed as mean ± SD with $P < 0.05$ considered significant. Student's t-tests were used to compare two groups, two-way ANOVAs were used to assess contributions of genotype and sex, followed by Bonferroni post-tests using Prism 5 (GraphPAD, San Diego, CA). One way ANOVA was used to compare all female wild type to male wild type for infarct and neuroscore. For the FACS data two-way ANOVA was performed on log transformed data, 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.

Results

Female IL-4 KO mice fail to show protection

The four stages of the estrus cycle were determined by histological analysis of vaginal smears (Supplemental Figure I). Infarction volumes 48 hours post-MCAO were measured in males and in all four phases of the estrus cycle for females for WT and IL-4 KO mice (Figure 1A,B). 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,¹⁷ 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-C).

Reduced ConA 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 two phases during which they show significant protection (Figure 1) and elevated estrogen levels. We evaluated the proliferative ability of splenocytes stimulated by ConA 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, B) with loss of sex differences with loss of IL-4 (Figure 2A, B).

WT female mice have fewer mononuclear cells in ischemic brain

We assessed microglia (CD45^{inter}CD11b⁺), monocytes/macrophages (CD45^{high}CD11b⁺), total T cells (CD45^{high}CD3⁺), CD8 (CD45^{high}CD3⁺CD8⁺) and CD4 T cells (CD45^{high}CD3⁺CD4⁺), in the ischemic hemisphere 48 hours post-MCAO by FACS (Figure 3A, B). There were significantly fewer T cells in the ischemic hemisphere of WT female mice during the estrus phase compared to 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 post-ischemic 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⁺ cells were significantly fewer in WT females during estrus compared to 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, while IL-4 KO reduced numbers of YM1 and Arg-1 positive cells in both sexes (Figure 5A,B). In a reciprocal fashion, numbers of NOS2 (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 MPO for neutrophils showed significantly fewer infiltrating cells in the

ischemic core of female mice in estrus than 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,^{23,24} 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 appears to play a central role in female protection.

A picture is beginning to emerge of the changes in immune cells in the brain following stroke,^{15,25} though the data in female rodents is much less than in males. Banerjee and colleagues²⁶ compared male and female mice after MCAO and reported fewer CD45^{hi}/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 CD45^{hi} from CD45^{inter} but did find more CD45^{hi} 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 CD14^{dim}CD16⁺ phenotype proposed to promote repair and associated with better outcome.²⁷

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 pro-inflammatory 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.¹⁷ Microglia/macrophages respond dynamically with M2 phenotype detected early after stroke then diminishing, while M1 phenotype increased with time and persisted.¹⁵ An earlier study found macrophage brain infiltration peaked at 3 days, but did not evaluate M1 vs M2 markers.²⁵ Here we found fewer Ym1⁺ and Arg-1⁺ cells in males, and fewer activated microglia/macrophages and neutrophils in females. M2 cells decreased while 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 appears central to sex differences and female protection from stroke. While it is impossible to conclusively state that increased numbers of M2 activated cells causes reduced infarct size, since reduced infarct size from any cause may be associated with reduced inflammatory infiltration, our results are consistent with those of others

suggesting that a balance of activation shifted towards the M2 phenotype is associated with smaller infarct size.¹⁹

Estrogen is thought to play a key role in ischemic protection of female rodents and people. A recent study by Manwani and colleagues²⁸ demonstrated the central role of gonadal hormones in brain ischemic susceptibility. Earlier work has suggested multiple mechanisms for protection by estrogen, including anti-oxidant effects, immune regulatory effects, and effects on cell death pathways (for review).⁸

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 wildtype, 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 (Supplemental Figure II).

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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Female infarct volume varies with estrus cycle phase only in WT mice

A Representative TTC-stained coronal sections showing infarctions in WT and IL-4 KO male and female mice.

B Quantification of infarct volume as percent of hemispheric volume 48 h post stroke in male mice and female mice in each of the four phases of the estrus cycle. WT females in proestrus and estrus were significantly protected compared to WT male mice (* $P < 0.05$, ** $P < 0.01$). IL-4 KO increased injury in female mice of all phases such that none were

significantly different than male KO mice. IL-4 KO significantly increased infarct volume compared to WT ($\#P<0.01$; $\#\#P<0.001$).

C Neurological score was better in female WT mice (proestrus and estrus) after MCAO compared to WT male mice ($*P<0.05$). Loss of IL-4 worsened neurological outcome ($\#P<0.05$; $\#\#P<0.01$). N=9-11 mice/group, individual group numbers are shown on each bar.

Figure 2. WT estrous phase females show decreased splenocyte proliferation

Splenocyte proliferation in naive WT and IL-4 KO mice was quantitated by flow cytometry using CFSE. **A**, Representative FACS plots of spleen cells from WT and IL-4 KO mice after treatment with the T cell mitogen ConA. **B**, Quantification of T cell proliferation based on CFSE dilution expressed as percent of non-dividing cells. There is less proliferation in WT female mice compared to WT male (*** $P < 0.001$), but this difference is lost with IL-4 KO. Proliferation increased significantly without IL-4 (### $P < 0.001$). N=6mice/group

Figure 3. Effects of sex on monocyte distribution in ischemic brain of WT and IL-4 KO mice
 Total mononuclear leukocytes from ischemic hemispheres 2 days after stroke were analyzed. **A**, FACS plots showing the gating strategy for immune cell subpopulations **B**, Bar graphs show the quantitation of cells/ischemic hemisphere for each subpopulation. WT female brains (estrus phase) generally show fewer infiltrating cells than males (* $P < 0.05$, ** $P < 0.01$), while IL-4 KO increases the numbers of all cell types significantly (# $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$), and eliminates the male female difference. $N = 6/\text{group}$

Figure 4. CD68 and iNOS cells increase in the ischemic core in IL-4 KO mice

A, The box in the cresyl violet stained section indicates the region of the ischemic core where photomicrographs were taken to quantitate cell numbers, 48 hour after MCAO. **B**, The left hand panels show representative immunofluorescence images of CD68 stained brains, counterstained with 4',6-diamidino-2-phenylindole. Bar=50 μ m. The right hand graph shows the quantitation /0.14 mm² area within the core of the infarct. n=8 mice/group; 3 sections/mouse. * indicates comparison to WT male, * P <0.05; # indicates comparison between WT and IL-4 KO # P <0.05, ## P <0.01. **C**, Representative micrographs and quantitation of iNOS immunostaining cells is shown. ## P <0.01 compared to WT.

Figure 5. M2 markers are higher and infiltrating neutrophils lower in the female ischemic core
A, The left hand panels show representative immunofluorescence images of YM1 stained brains, counterstained with 4',6-diamidino-2-phenylindole. Bar=50μm. The graph shows the quantitation/ 0.14 mm² area within the core of the infarct. **B**, Representative micrographs and quantitation of Arg-1 immunostained cells. **C**, Representative micrographs and quantitation of MPO⁺ cells. * indicates comparison to WT male, **P*<0.05; ** *P*<0.01; # indicates comparison between WT and IL-4 KO #*P*<0.05, ##*P*<0.01, ###*P*<0.001. N=8 mice/group; 3 sections/mouse.