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Phenotypic changes in immune cell subsets reflect increased infarct volume in male vs. female mice

Anirban Banerjee^{1,2,*}, Jianming Wang^{3,*}, Sheetal Bodhankar^{1,2}, Arthur A Vandenbark^{1,2,4}, Stephanie Murphy³, and Halina Offner^{1,2,3}

¹Neuroimmunology Research, Portland VA Medical Center, Portland, OR, USA

²Department of Neurology, Oregon Health & Science University, Portland, OR, USA

³Department of Anesthesiology and Perioperative Medicine, Oregon Health & Science University, Portland, OR, USA

⁴Department of Molecular Microbiology & Immunology, Oregon Health & Science University, Portland, OR, USA

Abstract

Inflammatory responses in brain after cerebral ischemia have been studied extensively in male but not female mice, thus potentially giving a less-than-accurate view of gender-based pathological processes. In humans, cerebral infarcts are typically smaller in premenopausal females than age-matched males. In the current study, we confirmed smaller infarcts in female vs. male mice after middle cerebral artery occlusion and 96 hours of reperfusion. Moreover, we explored immunological alterations related to this difference and found that the percentage of CD4⁺ T lymphocytes was significantly higher in males than females in spleens with increased expression of the activation markers, CD69 and CD44. In contrast, the percentage of CD8⁺ T lymphocytes was significantly higher in females than males in spleens, leading to the identification of a small but distinct population of IL-10-secreting CD8⁺CD122⁺ T-suppressor cells that were also increased in females. Finally, we observed that males have a greater percentage of activated macrophages/microglia in brain than females, as well as increased expression of the VLA-4 adhesion molecule in both brain and spleen. This new information suggesting gender-dependent immunological mechanisms in stroke implies that effective treatments for human stroke may also be gender specific.

Keywords

Experimental stroke; gender bias; immune markers; activated T-cells; T-suppressor cells; ischemia

Corresponding author: Dr. Halina Offner, Neuroimmunology Research, R&D-31, Portland Veterans Affairs Medical Center, 3710 SW US Veterans Hospital Rd., Portland, OR 97239, USA. Fax: +1-503-721-7975; offnerva@ohsu.edu.

*Contributed equally to this work.

Compliance with Ethics Requirements

Conflict of Interest

Anirban Banerjee declares that he has no conflict of interest. Jianming Wang declares that he has no conflict of interest. Sheetal Bodhankar declares that she has no conflict of interest. Arthur A Vandenbark declares that he has no conflict of interest. Stephanie Murphy declares that she has no conflict of interest. Halina Offner declares that she has no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed. This article does not contain any studies with human subjects.

Introduction

Ischemic stroke is a leading cause of death and disability in the United States, yet relatively little is known about gender-associated differences that might contribute to stroke outcome in females versus males. Previous studies have reported that although premenopausal females have smaller cerebral infarct volumes than age-matched males after ischemic stroke [1,2], female stroke patients have a worse prognosis with time [3]. Although male sex is considered a stroke risk factor, the lifetime risk of stroke is higher in women [4]. Clearly, stroke risk and outcomes are not identical in both sexes. For example, stroke incidence is higher in men vs. women across nations and ethnic backgrounds [5]. This sexually dimorphic epidemiology persists until well beyond the menopause, suggesting that estrogen does not fully account for the sex effect [6]. In the infrequent experimental study that stratifies by sex, outcome and pathophysiology of brain ischemia are clearly different [7–9]. A better understanding of such differences is crucial for development of effective therapies for stroke patients of both genders.

The inflammatory response in brain after cerebral ischemia has been extensively studied in male but not female mice, thus potentially giving a less-than-accurate view of the pathological picture of stroke when this research is translated to human trials and applied to treatment of both genders. Clinical stroke and experimental cerebral ischemia induced by middle cerebral artery occlusion (MCAO) manifests local inflammatory processes that undeniably contribute to total cerebral injury [10,11]. There have been several reports on sex differences in stroke related to pathophysiology and hormonal effects, epidemiological work, treatments and outcomes [12,13]. Although these differences are not fully understood, recognition of gender differences in brain inflammation may help to establish more appropriate treatments and improve outcomes.

In this report, we tested the hypothesis that the evolution of post-ischemic inflammatory cycling between the brain and peripheral immune system is strongly influenced by biological sex, including sexually dimorphic immune cell subsets and key inflammatory mechanisms that affect brain-spleen-brain cycling of inflammatory cells after focal cerebral ischemia.

Materials and Methods

Ethics Statement

The study was conducted in accordance with National Institutes of Health guidelines for the use of experimental animals, and the protocols were approved by the Portland Veteran Affairs Medical Center's Institutional Animal Care and Use Committee, protocol # 2840-12, local database ID # 2840.

Experimental Animals

The Oregon Health and Science University Animal Care and Use Committee approved all experiments. Male and female C57BL/6J wild-type (WT) mice (Jackson Laboratories, Bar Harbor, ME, USA) weighing 20–25 g were housed in a climate-controlled room on a 12-hour light/dark cycle. Female mice were not subjected to estrous cycle synchronization and were allowed to cycle naturally. Food and water were provided ad libitum. The experimental groups for this study were male and female mice subjected to MCAO. The animal number for each experiment is given in table 1 and is denoted in the respective figures.

Middle Cerebral Artery Occlusion Model

Transient focal cerebral ischemia was induced in male and female mice for 1 hour by reversible right MCAO under isoflurane anesthesia followed by 96 hours of reperfusion as described previously [14]. Head and body temperature were controlled at $37.0 \pm 1.0^\circ\text{C}$ throughout MCAO surgery with warm water pads and a heating lamp. Occlusion and reperfusion were verified in each animal by laser Doppler flowmetry (LDF) (Model DRT4, Moor Instruments Ltd., Oxford, England). The common carotid artery was exposed and the external carotid artery was ligated and cauterized. Unilateral MCAO was accomplished by inserting a 6-0 nylon monofilament surgical suture (ETHICON, Inc., Somerville, NJ, USA) with a heat-rounded and silicone-coated (Xantopren comfort light, Heraeus, Germany) tip into the internal carotid artery via the external carotid artery stump. Adequacy of MCAO was confirmed by monitoring cortical blood flow at the onset of the occlusion with a LDF probe affixed to the skull. Animals were excluded if mean intra-ischemic LDF was greater than 30% pre-ischemic baseline. At 1 hour of occlusion, the occluding filament was withdrawn to allow for reperfusion. Mice were then allowed to recover from anesthesia and survived for 96 hours following initiation of reperfusion. In sham-treated mice, the filament is placed but not advanced to achieve MCAO. 16 male and 16 female mice were used (8 for MCAO and 8 for sham MCAO) for this study.

Neurological Deficit Score

Neurological function was evaluated at baseline (before MCAO), just after reperfusion, 24, 48, 72, and 96 hours after reperfusion using a 0 to 5 point neurological deficit score as follows: 0, no neurological dysfunction; 1, failure to extend left forelimb fully when lifted by tail; 2, circling to the contralateral side; 3, falling to the left; 4, no spontaneous movement or in a comatose state; 5, death [14].

Infarct Volume Analysis

The brains were harvested after 96 hours of reperfusion and sliced into five 2-mm-thick coronal sections for staining with 1.2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO, USA) in saline as described previously [14]. The 2-mm brain sections were incubated in 1.2% TTC for 15 min at 37°C , and then fixed in 10% formalin for 24 hours. Infarction volume was measured using digital imaging and image analysis software (Systat, Inc., Point Richmond, CA, USA). To control for edema, infarct volume (cortex, striatum, and hemisphere) was determined by subtraction of the ipsilateral noninfarcted regional volume from the contralateral regional volume. This value was then divided by the contralateral regional volume and multiplied by 100 to yield regional infarction volume as a percent of the contralateral region.

Isolation of leukocytes from spleen and brain

Spleens from individual sham- and MCAO-treated mice (8 mice in each group) were removed and a single-cell suspension was prepared by passing the tissue through a 100 μm nylon mesh (BD Falcon, Bedford, MA). The cells were washed using RPMI 1640 and the red cells lysed using $1\times$ red cell lysis buffer (eBioscience, Inc., San Diego, CA) and incubated for 3 min. The cells were then washed twice with RPMI 1640, counted, and resuspended in stimulation medium (RPMI, containing 10% FBS, 1% sodium pyruvate, 1% L-glutamine, 0.4% ME). The brain was divided into the ischemic (right) and nonischemic (left) hemisphere, digested for 60 min with 1 mg/ml Type IV collagenase (Sigma Aldrich, ST. Louis, MO) and DNase I (50 mg/ml, Roche diagnostics, Indianapolis, IN) at 37°C with shaking at 200 rpm. Samples were mixed with a 1 ml pipette every 15 min. The suspension was washed $1\times$ in RPMI, resuspended in 80% Percoll overlaid with 40% Percoll and

centrifuged for 30 min at 1600 RPM. The cells were then washed twice with RPMI 1640, counted, and resuspended in stimulation medium.

Analysis of cell populations by fluorescence-activated cell sorting (FACS)

All antibodies were purchased (BD Biosciences, San Jose, CA or eBioscience, Inc., San Diego, CA) as published. Four-color (FITC, PE, APC and PerCP) fluorescence flow cytometry analyses were performed to determine the phenotypes of splenocytes and brain cells, as previously published [15]. One million cells were washed with staining medium (PBS containing 0.1% NaN₃ and 1% bovine serum albumin (Sigma, Illinois) and incubated with the combinations of the following monoclonal antibodies: CD4 (GK1.5), CD8 (53-6.7), CD11b (MAC-1), CD45 (Ly-5), CD11c (HL-3), CD19 (1D3), CD69 (H1.2F3), CD44 (IM7), and CD49d (VLA-4) for 20 min at 4°C. One ml of staining buffer was added to wash the cells. Propidium iodide was added to identify dead cells.

Intracellular staining

Intracellular IL-10 expression was visualized by modification of a previously published immunofluorescence staining protocol [16] which was reported by our laboratory [17]. According to the protocol, isolated leukocytes were resuspended (2×10^6 cells/mL) in stimulation media (RPMI 1640 media containing 10 % FCS, 1 mM pyruvate, 200 µg/mL penicillin, 200 U/mL streptomycin, 4 mM L-Glutamine, and 5×10^{-5} M 2-beta-ME with LPS [10 µg/mL], PMA [50 ng/mL], ionomycin [500 ng/mL], and Brefeldin A [10 µg/mL] [all reagents from Sigma-Aldrich]) for 4 h. For IL-10 detection, Fc receptors were blocked with mouse Fc receptor-specific mAb (2.3G2; BD PharMingen) before cell-surface staining and then fixed and permeabilized using a Cytotfix/Cytoperm kit (BD Biosciences), according to the manufacturer's instructions. Permeabilized cells were washed with $1 \times$ Permeabilization Buffer (BD Bioscience) and stained with APC-conjugated anti-IL-10 mAb (JES5-16E3; eBioscience). Isotype matched mAb served as negative controls to demonstrate specificity and to establish background IL-10-staining levels. Data were collected with CELLQUEST (BD Biosciences, San Jose, CA) and FCS EXPRESS (De Novo Software, Los Angeles, CA) software on a FACSCalibur (BD Biosciences).

Statistical Analysis

Data are presented as mean $\pm$ SEM. Differences in cortical, striatal, and total (hemispheric) infarct volume were determined with Student's t-test. Functional outcomes for neurological deficit scores were analyzed by Mann-Whitney U test. Statistical significance was $p < 0.05$. Statistical analyses were performed using SigmaStat Statistical Software, Version 3.1 (SPSS, Inc., Chicago, IL, USA). Spleen and brain cell counts and percentages of cellular subtypes for FACS analysis were analyzed by Student's t test. The criterion for statistical significance was $p = 0.05$.

Results

Males have larger infarct volumes than females

Evaluation of brain infarct after MCAO showed that males had significantly larger infarct volumes compared to females as shown in Figure 1A. Cortical infarct volume was $60.9 \pm 3.6\%$ in males compared to $35.7 \pm 5.5\%$ in females ($p = 0.003$). Similarly, striatal infarct volume was $81.1 \pm 6.4\%$ vs. $43.0 \pm 10.4\%$ respectively in males vs. females ($p = 0.007$), whereas the volume in hemisphere was $54.2 \pm 5.0\%$ vs. $30.8 \pm 4.7\%$ ($p = 0.003$). Quantitative assay of TTC stained cerebral sections after 96 hours of reperfusion illustrated the larger infarct area in males compared to females (Figure 1B). There were no significant differences

in neurological deficit scores before initiation of MCAO or during reperfusion between males and females (data not shown).

Gender differences in total splenocytes and brain cells after MCAO

Our previous studies established that the spleens of male mice undergoing MCAO became severely atrophied compared to those after sham treatment and that there were increased numbers of cells in the ischemic brain hemisphere after MCAO. A similar pattern of splenic atrophy and increased cell numbers in brain was observed in the current study for both genders, although the spleens of female mice were significantly less atrophied than males after MCAO, consistent with the smaller infarct size in females (Table 1A and B).

Gender differences in T-cell subtypes in periphery after MCAO

To identify immune cell phenotypes that might account for the difference in infarct size in males vs. females, we evaluated immunocyte subsets in spleen from males and females with MCAO by flow cytometry. As shown in Table 1 and Figure 2, the percentage of CD4⁺ T-cells was significantly higher in males compared to females in spleens ($20.7 \pm 2.7\%$ vs. $15.8 \pm 2.8\%$, $p = 0.05$, Fig. 2). Although the percentage of CD4⁺ T-cells was significantly higher in males, the opposite was found for CD8⁺ T-cells, with a higher percentage present in female spleens ($20.1 \pm 1.9\%$ vs. $16.3 \pm 2.7\%$, $p = 0.05$, Fig. 2).

IL-10-secreting CD8⁺CD122⁺ suppressor T-cells in the ischemic brain are increased in females after MCAO

The smaller infarcts coupled with increased levels of CD8⁺ T-cells in spleens of females suggested a role for CD8⁺ T-suppressor cells. We thus quantified CD8⁺ T-cells expressing the CD122 marker, a small but distinct subpopulation known for its ability to release IL-10. As is shown in Figure 3A, there was no significant difference in the percentage of CD8⁺CD122⁺ cells in females vs. males in the ischemic (right) brain (Fig. 3A). However, there was a highly significant difference in IL-10-secreting CD8⁺CD122⁺ T-suppressor cells in females ($28.82 \pm 11.7\%$ vs. $18.82 \pm 6.5\%$, $p = 0.05$, Fig. 3B), thus supporting the contention that smaller infarct volumes in females could be due to enhanced IL-10 release by CD8⁺ suppressors.

Activated CD4⁺ T-effector cells from spleen are increased in males after MCAO

The larger infarcts in male mice suggested the possibility that there might be an increased frequency of activated inflammatory CD4⁺ T-effector cells. Thus, we evaluated expression of CD69, a cell surface glycoprotein involved signaling and lymphocyte proliferation, and CD44, a marker for effector-memory T-cells. As shown in Fig. 4, both CD69 ($14.47 \pm 3.15\%$ vs. $8.48 \pm 3.22\%$) and CD44 ($13.76 \pm 1.88\%$ vs. $9.05 \pm 1.89\%$) were significantly increased on CD4⁺ splenic T-cells from males vs. females after MCAO ($p = 0.05$).

VLA-4⁺ cells from spleen and brain are increased in males after MCAO

VLA-4 (very late antigen 4) is expressed in all leukocytes and binds to VCAM1 upon activation. The involvement of VLA-4 in transmigration across the blood-brain barrier is well established and likely contributes to infarct development by infiltrating inflammatory cells. As is shown in Figure 5, we found that after 60 min MCAO followed by 96 h reperfusion, cells from both spleens ($6.9 \pm 0.9\%$ vs. $2.2 \pm 1.0\%$) and brains ($73.7 \pm 6.0\%$ vs. $34.9 \pm 2.9\%$) showed a significantly higher percentage of VLA-4 (CD49d) expression in males vs. females ($p = 0.01$). In contrast, there was no significant difference in VLA-4 expression on brain cells from sham-treated males vs. females ($24.5 \pm 9.8\%$ vs. $21.1 \pm 4.6\%$, Table 1).

Activated CD45^{hi}CD11b⁺ microglial cells/macrophages from the ischemic brain hemisphere are increased in males after MCAO

The pronounced increase in VLA-4⁺ cells in the ischemic brain hemisphere of males and females represented a much greater percentage of cells (~35% for females and >70% for males) than could be accounted for by the CD4⁺ and CD8⁺ T-cells (only 4–5%). We thus evaluated activated microglia and infiltrating macrophages, well known for their role in localized inflammation contributing to increased infarct size, that characteristically express the CD45^{hi} and CD11b⁺ markers (Figure 6A&B). We found that both the number (~60,800 for males vs. ~22,200 for females, from Table 1) and the percentage (47.1±9.0% versus 20.1±5.3%, Figure 6C and Table 1B) of CD45^{hi}CD11b⁺ cells were significantly higher in the ischemic right brain in males vs. females ($p = 0.01$). In contrast, no difference was observed in the number (~9,000 for both males and females) or frequency (15.1±3.2% vs. 15.6±3.9%, Table 1B) of CD45^{hi}CD11b⁺ cells in the right brain hemisphere of sham-treated males vs. females. These data implicate the infiltrating macrophages and activated microglia as major contributors to the increased infarct size in males compared to females at 96 hours post MCAO.

Discussion

Consistent with the literature, the current study verified that infarct size was indeed significantly smaller in females than males after 60 min MCAO and 96 hours of reperfusion. In order to explain this difference, we hypothesized that the larger infarct size in males occurs due to increased numbers of activated immunocytes migrating into the region of infarct and producing further localized damage. We thus carried out an evaluation of phenotypic markers on immunocytes from the spleen and brain 4 days after MCAO. The results demonstrate clearly that the larger infarct volumes in male mice are associated with increased percentages of activated CD69⁺ and CD44⁺ CD4⁺ T-cells in the spleen, as well as vastly increased numbers of activated macrophages, microglia and other as-yet unidentified cells expressing the homing marker, VLA-4, that have transmigrated into the area of the developing brain infarct. In contrast, the smaller infarct volumes in female mice are reflected by fewer activated cells but an increased percentage of CD8⁺ T-cells in spleens, including a small subset of IL-10-secreting CD122⁺ regulatory CD8⁺ T-cells in the ischemic brain that conceivably could limit stroke severity in females.

The contribution of the spleen to brain injury after MCAO has been well described [18, 19]. In our previous studies [10], we demonstrated that stroke induces massive early activation of splenocytes, leading to release of proinflammatory cytokines at the 6–22 hour time-points post MCAO, followed by a drastic reduction in proinflammatory cytokines and severe splenic atrophy by 96 hours post occlusion. Several proinflammatory pathways such as COX-2 and NOX-2 have also been shown to be important in determining infarct size after stroke [1], although these data were based on earlier time points (24h–72h). Our aim in the current study was to focus on the activated T-lymphocytes and macrophages that were perhaps transmigrating into the brain post MCAO to promote further localized brain injury even after a decline in the inflammatory phase in the periphery, which was at its peak at earlier time-points.

Our study clearly distinguishes key immunological differences in females vs. males that provide crucial insights into the development of their respective infarct sizes. Our initial finding was that splenic atrophy after MCAO was less severe in females than males (73% vs. 87% reduction of splenocytes, from Table 1A). It is well established that stroke leads to splenic atrophy characterized by a reduction of organ size, drastic loss of splenocyte counts along with activation of apoptotic markers [15]. Recent publications support the contention that splenic alterations in stroke result from a proinflammatory response [20]. Thus, in

keeping with the smaller infarct in females and consistent with our previous studies showing that splenic atrophy and resulting immunosuppression are a function of stroke severity [15], it can be predicted that less atrophy of spleen in females compared to males is indicative of reduced proinflammatory activation and consequent lesser infarct size.

Interestingly, although the total number of cells present in the ischemic hemisphere of both male and female mice was increased compared to sham-treated mice, the numbers in female vs. male mice were not significantly different from each other, suggesting differences in the composition of the infiltrating cells. Indeed, we found significant differences in T-cell subsets and macrophages in the spleen and CNS in females vs. males after MCAO that were not present in sham-treated mice (Table 1A & 1B). The contribution of the immune mediated inflammatory cascade to MCAO has already received considerable attention in male mice, due likely to their larger infarcts. It has been reported that expression of the adhesion molecule VLA-4 increases rapidly 96 hours post MCAO among circulating macrophages [15]. Activation of resident cells (mainly microglia) and infiltration of monocyte/macrophages into the ischemic brain tissue have also been reported [21], as well as the profound recruitment of T cells to the ischemic brain after stroke [19] that exacerbates brain damage caused by the initial insult [14,22]. T-lymphocytes are believed to migrate to the brain exacerbating neurodegeneration within 48 hours after stroke [20] and produce damage through the generation of proinflammatory cytokines, chemokines and other mediators [23] and superoxides [24,25]. Although recent studies suggest causal relationships between T lymphocytes and brain outcome [26–28,22], comparable studies on the contribution of activated immune cells to MCAO have not been reported in female mice after MCAO. This is surprising as there are known sex differences in T lymphocyte-mediated autoimmunity [29–31].

Regarding T-cells, our major findings were a significant increase in CD4⁺ cells in spleens males, but a significant increase in CD8⁺ T-cells in spleens of females after MCAO. Although the increased level of CD8⁺ T cells could contribute to CNS injury in female stroke, we also considered a possible role for CD8⁺ suppressor T-cells, noted previously by others to mediate immunosuppression after CNS injury [32]. We did not find any significant differences between males and females in the adaptive CD8⁺ T-suppressor population (CD8⁺CD25⁺FoxP3⁺) (data not shown) or the less discussed, naturally occurring CD8⁺ T-suppressors (CD8⁺CD122⁺). However, we did find a robust increase in the frequency of IL-10-secreting CD8⁺CD122⁺ T-suppressor subset in the brains of female vs. male mice after MCAO (Fig. 3). The functional ability of this population of CD8⁺CD122⁺ T-cells to release IL-10 has been described [33–35], as has their ability to inhibit the expansion and release of inflammatory cytokines by CD4⁺ T-cells in EAE [36]. Both of these regulatory functions of CD8⁺CD122⁺ T-cells could play an important role in limiting infarct size in female mice after MCAO. However, implication of IL-10 as a regulatory cytokine produced by these cells does not exclude a contribution by other anti-inflammatory cytokines.

While not the focus of this study, estrogen could be contributing to the differences in immune cell subsets we observed in male and female mice. For example, not addressed in our study are the potential effects of estrogen on the induction of T-suppressor cells. Dysfunction of regulatory T-cells in estrogen-deficient mice may contribute to an acceleration of organ-specific autoimmune lesions [37]. Estrogen in combination with progesterone has been found to enhance regulatory responses to HPV in healthy women [38] and likely provides greater neuroprotection in females after MCAO. In fact, exogenous administration of both hormones has been shown to improve outcome after cerebral ischemia and traumatic brain injury in experimental models [39]. This is also in accordance with our previous report that estradiol deficiency exacerbates immunosuppression after focal stroke in females [40]. Therefore the significant increase in IL-10 production by CD8⁺

suppressor T-cells in females after MCAO could be attributed to increased levels of estrogen and progesterone. Finally, it is worth noting that the majority of CD8⁺ T-cells in females were not T-suppressor cells and these CD8⁺ T-cells could well be responsible for the worse prognosis over time in females with cerebral ischemia [3].

One limitation of our study is that female mice were not subjected to estrous cycle synchronization and were allowed to cycle naturally. This was done in order to determine if sex differences in infarct volume and immune cell subsets were present even in the face of varying estrogen levels, as these levels normally fluctuate in both female animals and humans depending on age, stress, diurnal variation, and reproductive cycle. Future studies will explore the contribution of estrogen and other sex steroids to phenotypic changes in immune cell subsets after focal cerebral ischemia by controlling sex steroid levels through estrous cycle synchronization, reduction of endogenous hormone levels via gonadectomy, and exogenous hormone replacement in gonadectomized mice.

In congruence with larger infarct size in males, we found that not only was the percentage of CD4⁺ T lymphocytes higher in males but the expression of activation markers such as CD69 and CD44 on CD4⁺ T lymphocytes from spleen was also higher in males compared to females. These differences were clearly due to MCAO as sham-treated males and females did not exhibit any significant differences in either CD69⁺ or CD44⁺ expression among CD4⁺ T-lymphocytes (Table 1A). It has been reported already that stroke patients had more CD69⁺ T-cells than controls, indicative of T cell activation [41], but ours is the first study to demonstrate that this increase occurs in males but not females after MCAO. Recruitment of bone marrow stromal cells in the brain by upregulation of CD44 after stroke has been reported [42] and it has also been discussed that CD4⁺ T lymphocytes may influence infarct size [14,22,1]. Therefore, our study showing that expression of these markers is gender dependent provides new insight into the mechanisms that distinguish the development of stroke lesions in males vs. females. One important conclusion is that the proposed hypothesis that MCAO activates spleen cells to transmigrate and to produce more damage in the injured brain cycle may largely be restricted to males.

Along with the recruitment of T-lymphocytes that has been discussed thus far, there is also a massive infiltration of monocytes/macrophages into the ischemic brain [21], aided by significant increases in the expression of adhesion molecules, including VLA-4 that was described previously after MCAO [15]. Upregulation of adhesion molecules by vascular endothelial cells allows infiltration into the brain of blood neutrophils, monocytes, macrophages, and T cells that promote further brain injury [43]. In our current study, we found that males had a higher expression of VLA-4 among all cells in both spleen and brain compared to females, which suggested a higher transmigration of activated cells in males which again corresponds to a larger infarct size in males.

Moreover, we found an increased frequency of CD45^{hi}CD11b⁺ cells, which represent infiltrating macrophages and activated microglia, in the ischemic right hemisphere in males vs. females (Table 1B). It is already well known that CD45^{hi}CD11b⁺ macrophages migrate into the brain as a result of injury or infection [44, 45], and the number of activated microglia and macrophages (CD45^{hi}CD11b⁺) in brain can serve to gauge the extent of inflammation and CNS injury [46]. Therefore, the increases in activated microglia and macrophages in the ischemic hemisphere in males would appear to be a major contributor to the greater infarct size in males.

In conclusion, our study provides new evidence that the extent of brain injury after stroke differs with gender and is influenced by different patterns of immunological responses. This new information suggesting gender-dependent mechanisms of brain damage after stroke

implies that effective treatments for stroke may also be gender specific. For example, males may respond better to anti-inflammatory therapies that target activated T-cells and macrophages or inhibit VLA-4 expression, whereas females may benefit from therapies that inhibit CD8⁺ T-effector cells and promote CD8⁺ T-suppressor cells. The key observations presented here clearly support the need to confirm and extend the general understanding of gender differences that contribute to stroke susceptibility and severity.

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Figure 1. Males have larger infarct volumes than females

(A) Infarct volumes were significantly decreased in female versus male mice after 60 min MCAO and 96 hours of reperfusion (**p < 0.01). (B) Representative 2,3,5-triphenyltetrazolium chloride stained cerebral sections illustrate differences between males and females.

Figure 2. Gender differences in T-cell subtypes in periphery after MCAO

The percentage of splenic CD4+ T-lymphocytes was higher in males, whereas the percentage of CD8+ T-lymphocytes was higher in females after 60 min MCAO and 96 hours of reperfusion. Mononuclear cells from 8 mice in each group were analyzed by FACS.

*p 0.05

Figure 3. IL-10-secreting CD8⁺CD122⁺ suppressor T- cells in ischemic brain are increased in females after MCAO

(A) Percentage of CD8⁺ T-suppressor cells (CD8⁺/CD122⁺) was not significantly different between males and females, whereas (B) the percentage of IL-10⁺ CD8⁺CD122⁺ T-suppressor cells was significantly higher in females than males after 60 min MCAO and 96 hours of reperfusion. Data for ischemic hemisphere is represented as pooled data from 2 mice/group (n=4 in two separate experiments) analyzed by FACS. *p < 0.05

Figure 4. Activated CD4⁺ T-effector cells from spleen are increased in males after MCAO
(A) Spleen cells were isolated from females and males after 60 min MCAO and 96 hours of reperfusion and processed for FACS analysis by adding antibodies against CD4, CD69 and CD44. Cells were gated on CD4⁺ T-lymphocytes and **(A)** evaluated for expression of CD69 and CD44 among CD4⁺ T-cells as shown for representative samples. **(B)** Data demonstrate increased expression of both CD69 and CD44 activation markers among CD4⁺ T-lymphocytes in males vs. females after 60 min MCAO and 96 hours of reperfusion. Mononuclear cells from 6 mice in each group were analyzed by FACS. *p < 0.05

Figure 5. VLA-4⁺ cells from spleen and brain are increased in males after MCAO
(A) Representative FACS analysis of VLA-4⁺ leukocytes (inset) from spleen and brain from female and male mice after 60 min MCAO and 96 hours of reperfusion. **(B)** Data show increased expression of VLA-4⁺ cells in both spleen and brain in males vs. females. Data for ischemic hemisphere is represented as pooled data from 2 mice/group (n=4 in two separate experiments) analyzed by FACS. **p 0.01.

Figure 6. Activated CD45^{hi}CD11b⁺ microglial cells/macrophages from the ischemic brain hemisphere are increased in males after MCAO

(A) Cells isolated from the ischemic ipsilateral (Right) and contralateral (Left) hemispheres of brains from female and male mice after 60 min MCAO and 96 hours of reperfusion were stained with antibodies against CD11b and CD45 and analyzed by FACS. CD11b⁺ cells from each hemisphere of representative mice were gated and (B) evaluated for percentage of CD45^{hi} cells that represent activated microglia and infiltrating macrophages. (C) Data show increased percentages of CD45^{hi}CD11b⁺ cells in the ischemic brain hemisphere of males vs. females after MCAO. Data for ischemic hemisphere is represented as pooled data from 2 mice/group (n=4 in two separate experiments) analyzed by FACS. **p < 0.01.

Table 1A

Total splenocytes and percentage of different cell types in spleen

Cell Type	Sham females	MCAO females	Sham males	MCAO males
Total splenocytes (x10 ⁶) n=8	70.86±10.08	18.89±3.72	80.70±10.82	10.15±4.11*
CD4 % n=8	13.52±4.11	16.04±2.07	15.9±1.30	21.27±2.11*
CD8 % n=8	15.56±3.93	20.06±1.86*	12.8±1.64	16.26±2.71
VLA-4 % (all cells) n=8	1.23±0.34	2.17±0.98	1.33±0.38	6.85±0.72**
CD69/CD4 % n=6	4.66±1.17	8.47±3.22	5.47±2.21	14.47±3.15*
CD44/CD4 % n=6	4.41±1.06	9.00±1.88	5.07±1.08	13.76±1.88*

Table 1B

Percentage of different cell types in Brain

Ischemic (right) (n=8; data pooled from 2 mice/group in two separate experiments)	Sham females	MCAO females	Sham males	MCAO males
Total cells ($\times 10^6$)	0.58 $\pm$ 0.06	1.10 $\pm$ 0.12	0.61 $\pm$ 0.09	1.29 $\pm$ 0.16
CD4 %	1.34 $\pm$ 0.17	1.58 $\pm$ 0.18	1.46 $\pm$ 0.21	2.86 $\pm$ 0.15
CD8 %	1.35 $\pm$ 0.17	2.41 $\pm$ 0.33	1.18 $\pm$ 0.16	2.01 $\pm$ 0.13
VLA-4% (all cells)	21.05 $\pm$ 4.56	34.85 $\pm$ 2.86	24.45 $\pm$ 9.83	73.71 $\pm$ 6.01 ^{**}
CD45 ^{hi} CD11b ⁺ %	15.59 $\pm$ 3.86	20.14 $\pm$ 5.50	15.07 $\pm$ 3.21	47.11 $\pm$ 9.01 ^{**}

*
p 0.05;**
p 0.01 (comparison of MCAO females vs. MCAO males only)