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Sex differences in the inflammatory response to stroke

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Abstract

Ischemic stroke is a leading cause of morbidity and mortality and disproportionally affects women, in part due to their higher longevity. Older women have poorer outcomes after stroke with high rates of cognitive deficits, depression, and reduced quality of life. Post-stroke inflammatory responses are also sexually dimorphic and drive differences in infarct size and recovery. Factors that influence sex-specific immune responses can be both intrinsic and extrinsic. Differences in gonadal hormone exposure, sex chromosome compliment, and environmental/social factors can drive changes in transcriptional and metabolic profiles. In addition, how these variables interact, changes across the lifespan. After the onset of ischemic injury, necrosis and apoptosis occur, which activate microglia and other glial cells within the central nervous system, promoting the release of cytokines and chemokines and neuroinflammation. Cells involved in innate and adaptive immune responses also have dual functions after stroke as they can enhance inflammation acutely, but also contribute to suppression of the inflammatory cascade and later repair. In this review, we provide an overview of the current literature on sex-specific inflammatory responses to ischemic stroke. Understanding these differences is critical to identifying therapeutic options for both men and women.

Keywords

Ischemic stroke; Sex differences; Neuroinflammation; Sex hormones

Background

Stroke is a major cause of morbidity and mortality worldwide. Stroke prevalence increases with age and is expected to increase significantly in the next decade as the population ages further [1, 2]. While the mortality from stroke has decreased by over 7% over the last decade, due to improvements in acute care, the number of patients left disabled after a stroke remains high, and there are currently over a million stroke survivors living in the USA [1, 3]. Similarly, compared to 2010, the cost of stroke care is expected to triple to $184.0 billion by 2030 [2].

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Females have a higher lifetime risk of having an ischemic stroke, in large part due to their longer life expectancy [4, 5]. Stroke risk is also higher in younger females (≤ 35 years of age) compared with men in the same age group [6]. In middle age, men have a higher risk of stroke. With increasing age, these differences diminish, and the incidence climbs in both sexes [5, 7, 8]. Importantly, the incidence of stroke in women did not decrease significantly over a 25-year period in the Greater Cincinnati/Northern Kentucky Stroke Study, whereas the incidence in men did [9]. Similarly in the Framingham study, the 30-day mortality decreased significantly for men, but not for women over a 50-year period [10]. Females are also more likely to be severely disabled, less likely to be discharged home and more likely to die after their stroke [11]. However, when adjusted for age and other confounding factors, men have similar to higher mortality [11].

There are multiple reasons for these sex differences. Women have a higher incidence of stroke when < 35 years old, and pregnancy and pregnancy-associated hypertension are important risk factors [12]. Other risk factors include use of oral contraceptive pills (OCPs) and a higher incidence of migraines and autoimmune conditions [13]. Older women are also almost twice as likely to have stroke when in atrial fibrillation (AF) compared to men, which is reflected in the CHA_2DS_2-VASc that allocates an additional point for female sex [14–17]. The CHA_2DS_2-VASc is routinely used in clinical practice to help physicians risk stratify patients who are at high risk for embolic stroke and should be started on anticoagulation for stroke prevention (Tables 1 and 2). The reason for the higher embolic risk in women withAF is unknown, but recent trials have found higher baseline levels of endogenous factor Xa in women, which may lead to enhanced thrombosis[18]. The lower incidence of stroke in females between the ages of 35 and 75 years old may partially be explained by exposure to sex hormones as discussed later. However, this neuroprotection is lost after menopause [19].

There are also sex differences in the inflammatory response to an acute stroke, which include disruption of the blood–brain barrier (BBB) and resultant secondary ischemic, hemorrhagic, and edema-related damage. These differences may account for post-stroke morbidity, and hence, identification of these disparities is important to stroke management and drug development [20]. These differences may be secondary to variations in hormones, sex chromosomal effects, and their differential effects with aging. In this review, we summarize the known sex differences in the inflammatory response to stroke.

Neuroinflammatory responses after stroke

Innate immune responses in stroke

Following arterial occlusion, the innate immune system detects injury with pattern recognition receptors (PRR). PRRs such as toll-like receptors (TLRs) respond to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), which are passively released from cells that have died after the induction of cell death pathways and contribute to activation of the complement system and the innate immune system [21–23]. Experimental studies have found that the activation and response to cell death pathways diverge between the sexes, even in cell culture models, which likely contributes to the subsequent immune response [24–27]. Activation of the complement system in the intravascular and intraparenchymal compartments is associated

with worse outcomes [28–30]. Microglia migrate towards the area of ischemic damage [31], and macrophages appear at the site of infarction within minutes to hours and peak 1–4 days after stroke [32]. Microglia are brain-resident innate immune cells that are present throughout the CNS and respond quickly to inflammatory stimuli. Previously, microglial activation states were discretely classified into “M1” (inflammatory) and “M2” (reparative) based on a limited number of cell-surface and cytokine markers. More recently, single-cell transcriptomic studies have demonstrated that this simplistic classification does not accurately capture the diverse multifunctional states of microglia, which exist along a continuous transcriptomic trajectory [33]. After ischemic injury, microglia undergo alternative gene transcription that results in the release of pro- or anti-inflammatory cytokines depending on their activation states. Commonly used pro-inflammatory markers include CD16/32, CD86, MHCII, COX-2, NOS2, interleukin (IL)-1β, and tumor necrosis factor (TNF)-α, while CD206, arginase 1, FIZZ1, and YM1 are considered as anti-inflammatory markers [34, 35]. Caution should be taken when using these markers in clinical studies as human microglia show have limited overlap with mice models [35]. Microglia in the nearby area and perivascular macrophages mediate early neurotoxicity as they release reactive oxygen species (ROS); cytokines such as such as IL-6, IL-1β, and TNF-α; nitric oxide (NO); and matrix metalloproteinases (MMPs) [36–38]. Huang et al. demonstrated that the proinflammatory phenotype of microglia and their associated inflammatory cytokines remained elevated up to 28 days after initial ischemic injury in rats [39]. With time, microglia phagocytose dead cells and debris, helping reduce inflammation and clearing the way for reparative processes [40]. Multiple different cytokines, immunocomplexes, apoptotic cells, fatty acids, oxysterols, and 9-cis retinoic acid then regulate transcription factors to induce genes with anti-inflammatory effects [41, 42]. This transition of microglia results in a cascade of anti-inflammatory pathways including IL-4, shown to decrease infarct size [38]. Microglia with a defined antiinflammatory phenotype release IL-10, chitinase-3-like protein 3, and transforming growth factor-β (TGF-β), which promotes angiogenesis and BBB repair [43] and promotes neural stem/progenitor cells differentiation by upregulating TGF-α [44].

As neurons die, they release extracellular RNA, and microglia release cytokines including IL-1α, TNF, and C1q which induces astrocytes to rapidly kill additional neurons and oligodendrocytes [32, 45]. This is mediated by activation of the transcription factor NF-κB, which leads to increased IL-6 and TNF-α levels [45]. Astrocytes also release IL-15 which recruits peripheral immune cells into the brain and CXCL1 which recruits neutrophils to the brain [46, 47]. In parallel to the activation of the inflammatory cascade, the coagulation cascade is also activated resulting in release and transcription of adhesion molecules, which enhance leukocyte adhesion and transmigration into the brain, and provides a chemotactic stimulus for monocytes and neutrophils [48–50].

Neutrophils are recruited early into the infarcted brain and contribute to increased infarct volume stroke severity and hemorrhagic transformation [51, 52]. Interestingly, depletion of microglia with a colony-stimulating factor 1 receptor (CSF1R) inhibitor increased neutrophil numbers and enlarged the ischemic lesion, suggesting that microglial are an important first line of defense against neutrophil entry after stroke [53]. Neutrophils increase BBB disruption by releasing MMPs including MMP-9, which increases cerebral edema

secondary to inflammation [54] and induces thrombosis secondary to the formation of neutrophil extracellular traps (NETs) [55, 56]. Similar to microglia, external stimuli can alter gene transcription in neutrophils, so they produce more anti-inflammatory cytokines and chemokines which are associated with reduced infarct volume and improved outcomes [57]. Dendritic cells are also an important component of the innate immune response to stroke and function as antigen presenting cells. They migrate to the infarct core and express MHCII which contributes to activation of lymphocytes [36].

Adaptive immune responses in stroke

T cells are a critical component of adaptive immunity. With BBB compromise, several subsets of T cells enter the brain parenchyma. $CD8^+$ T cells exert a direct cytotoxic effect by releasing granzymes and perforins in target cells which results in apoptosis of these cells [58]. $CD4^+$ T cells release IL-21 which binds to IL-21 receptors on neurons and induces autophagy [59]. Th17 and γδT cells produce IFN-γ and IL-17 which leads to neutrophil recruitment, BBB disruption, neurotoxicity, and microvascular dysfunction. In addition, previous studies have shown that γδT cell depletion improves stroke outcomes [60, 61]. Regulatory T (Treg) cells release IL-10 which inhibits proinflammatory cytokines IL-1β and TNF-α [62], and these cells also inhibit MMP activity, which improves BBB integrity [63]. Tregs also inhibit astrogliosis through amphiregulin, an epidermal growth factor receptor ligand, promote oligodendrogenesis by secreting osteopontin and inducing reparative microglia, and repair the injury by secreting IL-10 which promote neural stem cell proliferation [64–66]. After the acute phase of inflammation, T cells and microglia release growth factors that aid in recovery after stroke [67, 68]. IL-10-secreting $CD19^+$ B cells limit infarct volume by inhibiting activation and recruitment of inflammatory T cells, macrophages, and microglia [69] (Fig. 1).

Systemic responses to stroke

In addition to these local responses, other peripheral organs are also affected by ischemic stroke. Sahota et al. showed that spleen size decreases over the first few days after an ischemic stroke, which is likely due to the exodus of leukocytes [70]. Similarly, the number of monocytes in the spleen decreases and increases in the brain following stroke [71], and monocyte numbers are associated with poorer outcomes [72]. Conversely, splenectomy 2 weeks prior to middle cerebral artery occlusion (MCAO) surgery in rats reduced infarct volumes by 80% and decreased the numbers of macrophages and neutrophils in the brain [73]. However, determining causality can be difficult, as differences in infarct size may mediate the subsequent immune response. In mice, males had larger strokes than females, but after splenectomy, infarct volumes in males were comparable to that seen in females [74]. This may be due to intrinsic differences in splenic T cell composition. More regulatory lymphocytes were found in the spleens of females, whereas male mice had more activated splenic T cells which may contribute to the differential response to splenectomy [75–77]. Although splenectomy decreases infarct volume in animal models of stroke, this is not a practical approach for patients. The contribution of peripheral tissues to post-stroke neuroinflammation (e.g., vagal signals) needs to be investigated as a more translationally relevant target.

Non-splenic immune organs contributing to stroke outcome: gut and bone marrow

The bidirectional communication between the brain, the gut, and the microbiome (often termed the microbiota-gut-brain axis) has been increasingly recognized in neurological diseases including stroke [78]. Stroke-induced dysbiosis (imbalance of the gut microbiota composition) led to infiltration of both anti- and proinflammatory cells (e.g., T cells) into the brain. In addition, post-stroke impairment of intestinal barrier function may lead to the translocation of luminal antigens (e.g., bacteria) evoking inflammatory responses mediated by the immune cells in lamina propria that subsequently exacerbate both systemic and neuroinflammation. In terms of sex differences in the gut response to stroke, it was previously reported that male rats (5–7 months of age) had increased gut permeability and higher systemic proinflammatory cytokines (e.g., IL-17A, MCP-1, and IL-5) and worse neurological outcomes after stroke as compared with females [79]. Likewise, these changes in gut permeability were seen in aged mice and led to an increased risk of hemorrhagic transformation in males [80]. Sex differences in gut dysbiosis have been reported in stroke and other neurodegenerative diseases (reviewed in Korf et al. [81]).

In addition to the involvement of the gut, the hematopoietic stem cell niche in the bone marrow also plays a crucial role in post-stroke inflammation. Courties et al. demonstrated that ischemic stroke causes an efflux of inflammatory monocytes and neutrophils from the bone marrow into the circulation, regulated by sympathetic nerve signaling [82]. Future investigations examining potential sex differences in the role of the bone marrow and other peripheral tissues in post-stroke immune responses are needed.

Thromboinflammation

Thromboinflammation refers to the interaction between thrombotic and inflammatory cascades activated by stroke [83, 84]. Blood components, such as platelets along with innate and adaptive immune cells, play a central role in the pathogenesis of thromboinflammation. Platelets adhere to injured endothelium via glycoprotein (GP) VI, integrin α2β1, and GPIbα subunit binding to collagen and von Willebrand factor (VWF) after which they become activated [84, 85]. After activation, platelets release adenosine diphosphate and thromboxane A_2 and platelet aggregation ensues. Platelets activate Factor XII which then initiates coagulation and triggers the kallikrein-kinin system, producing bradykinin. Activation of the bradykinin receptor results in BBB disruption and cerebral edema [86]. T cells interact with platelets through CD40/CD40L which enhances transendothelial migration [83]. Similarly, neutrophils also interact with platelets with MAC-1/GP1ba connections activating neutrophil elastase which induces the production of MMP9, which further disrupts the BBB and triggers the release of DAMPs and ROS.

Epoxyeicosatrienoic (EETs) acid are vasodilators and protect the endothelium [87]. Soluble epoxide hydrolase (sEH) is responsible for the metabolism of EETs, reducing their function. sEH levels are higher in males [88], but these sex differences are lost in sEH knockout mice and after ovariectomy [85]. Data demonstrating differences in thrombus size and composition is conflicting. Male rats had larger thrombus size, and administration of testosterone increased both mortality and thrombus size [89]. In porcine models, ovariectomy resulted in enhanced platelet aggregation and MMP levels [90]. This suggests

that estrogen plays a protective role in platelet aggregation and loss of estrogen with menopause may explain the increased incidence of thrombotic events in women, but this has not been recapitulated in humans [91]. While thromboinflammation has been well studied, sex differences in thromboinflammation have been less so. Additional work is needed to elucidate these sex differences.

Sex differences in immunity

Multiple studies have demonstrated sex differences in inflammatory response, which have been reviewed previously [92]. These sex differences vary with age and hormonal exposure. Differences in innate immunity are seen early in the inflammatory cascade, for example, recognition of PRRs. Females have higher levels of TLR7, which results in increased production of IFN-α [93]. Male macrophages express higher levels of TLR4 which results in increased CXC-chemokine ligand 10 production, a proinflammatory chemokine [94]. Adaptive immunity also varies based on age. As women transition from childhood to adulthood and then into old age, their $CD4^+$ T cell count, B cell count, and CD4/CD8 ratio increase in comparison to men [95–97]. Females also show stronger antibody responses and have higher immunoglobulin levels [92]. In addition to hormonal differences, there are also genetic differences between the sexes that influence the transcriptional profile and lead to differential protein expression after ischemic injury, which will be discussed below.

It is important to note that while estrogen has been shown to have protective effects in animal models, hormone therapy (HT) after menopause for primary or secondary prevention of stroke has not been shown to be beneficial. In the WEST trial, post-menopausal women with a recent ischemic stroke or transient ischemic attack (TIA) were randomized to estrogen therapy (1 mg of estradiol-17β per day) or placebo. The incidence of nonfatal stroke and nonfatal ischemic stroke was similar in the two treatment groups although there was a non-significant increase in both risk of death and for more severe stroke in women treated with estrogen (RR; 2.9; 95 percent CI; 0.9 to 9.0) [98]. Other randomized trials confirmed an increase in the risk of ischemic stroke in healthy postmenopausal patients taking HT [99–101]. The reasons for the conflicting results in animal models versus clinical trials have been discussed elsewhere but likely involve the timing of HT and dosing regimens [102]. In the WEST trial, women had established vascular disease, and the average age of enrolled patients was 71. In the WHI, the average age on enrolled subjects was 63, over a decade post-menopause. Animal studies have implicated that extended period of hypoestrogenicity leads to a loss of estrogen-mediated neuroprotection and suppresses its anti-inflammatory actions [103, 104]. Ongoing clinical trials that enrolled women at the time of menopause are currently ongoing [105, 106].

Sex differences in the blood–brain barrier (BBB)

The BBB consists of endothelial cells, astrocytes, and pericytes and with neurons and microglia, forming the neurovascular unit [107]. These cells efficiently provide nutrients to the brain parenchyma while preventing harmful antigens from entering the brain. Endothelial cell tight junctions strictly regulate paracellular and transcellular transport of antigens, with pericytes providing direct contact to the endothelium and lending additional

integrity to the BBB [108, 109]. Limited data is available on sex differences in the integrity of BBB, but there is some evidence that females have decreased permeability and hence have a more intact BBB as reviewed by Weber et al. [110]. This may due to protective effects of estrogen as ovariectomy increased the permeability of BBB in mice [111]. Similarly, rats had a higher astrocytic aquaporin-4 expression after ovariectomy which led to vasogenic edema. Estrogen replacement decreased the BBB disruption and restored the expression of aquaporin-4 in adult female rats, further demonstrating the protective role of estrogen [112]. Testosterone is also linked with BBB integrity, and male mice who were castrated showed loss of tight junction protein and increased BBB permeability, which could be reversed with testosterone supplementation [113].

Ischemia further disrupts the BBB barrier. Liu et al. demonstrated that aging female mice have higher BBB permeability compared to young female mice, while aging male mice had decreased permeability compared to young male mice [114]. The difference seen in females with aging is likely secondary to estrogen as mice with ovariectomy had the highest BBB permeability, while male mice continue to metabolize testosterone to estrogen (via aromatase). One small clinical study in stroke patients found no sex differences in MMP-9 levels, a protein that has been shown to disrupt the BBB by degrading tight junctions and the basement membrane, or its inhibitor, tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) [115]. MMP-9-induced disruption of the BBB is associated with hemorrhagic transformation, but sex differences in hemorrhagic transformation incidence remain unclear [116]. While BBB disruption is associated with post-ischemic injury, the role of sex differences in BBB integrity and its implications on infarct size and outcomes is not yet clear.

Cell-specific sex differences in post-stroke immune responses

Microglia

Sex differences in microglia in the healthy brain: Microglia are brain-specific macrophages derived from the fetal yolk sac which account for 5–12% of all brain cells [117]. They are involved in the development and maturation of synapses [118, 119], participate in brain masculinization [120], clear debris, and are involved in regeneration [121]. There is well-documented heterogeneity in the location and functional states of microglia between the sexes [122–126]. Along with differences in density, studies have also shown varying morphologies between males and females. Guneykaya et al. demonstrated that microglia have similar soma sizes in male and female mice at 3 weeks, but at 13 weeks, male microglia were larger [125]. Schwartz et al. showed that at postnatal day 4, male rats had rounder microglia with thick long processes, but this reversed by day 30 [126]. In the prelimbic cortex, female rats had a higher primed/ramified ratio of microglia when compared to males. However, with stress, this ratio increased in males and decreased in females [124]. Another study showed that males had more complex microglia in the dorsal hippocampus, while females had a more complex microglia morphology in the prefrontal cortex [127]. These differences are important to note as they suggest microglia in male rodents are already in a more activated state and are more responsive to neuroinflammatory stimuli, which may play an important role in the response to stroke.

Transcriptomic analysis has shown that there are considerable differences in microglia in males and females, and these differences vary based on brain region and age. In 13-week-old mice, there were 1109 differentially expressed genes in the hippocampus and 55 in the cortex. The genes that were more highly transcribed in males included "transcription factor activity" and "histone demethylation and deacetylation" in the cortex and "regulation of defense response to bacteria," "insulin receptor pathway," "glia cell differentiation," and "ATP binding" in the hypothalamus. Females had overrepresentation of "ubiquitin protein activity" and "magnesium ion transport" in their gene set [125]. Similarly, Villa et al. identified 546 differentially expressed genes in mice with increased expression of transcription factors associated with 79% of the 95 inflammatory genes that were more expressed in males. These included genes involved in the inflammatory response, leucocyte migration, regulation of response to wounding, chemotaxis, and regulation of cytokine production. In contrast, microglia from females had increased expression of genes regulated by transcription factors that inhibit inflammation [128]. Other studies demonstrated differential expression of genes at all age groups but showed that as female mice aged, the expression of their inflammatory genes increased [129] including genes involved with the APOE network [130], an important risk factor for neurodegenerative disease. These transcriptomic analyses suggest that genes are differentially expressed in male and female rodents and that males have enhanced expression of transcription factors that induce proinflammatory genes and females have a higher number of transcription factors that induce anti-inflammatory genes. One important caveat is that these studies were performed in young animals, so how the microglial transcriptome changes with aging remains relatively unexplored.

Sex differences in microglial response to stroke: Microglia are highly plastic cells that assume diverse roles in response to different signals [131] and play a central role in the neuroinflammatory response to stroke. As mentioned in previous sections, transcription, phenotype, and location of microglia vary based on sex. Transcriptomics analysis shows that of the differentially expressed genes, proinflammatory genes are more common in males. Similarly, proinflammatory phenotypes of microglia are more dominant in males [131], whereas anti-inflammatory phenotypes of microglia increase significantly in females post-stroke [75] (Fig. 2).

One important inducer of the anti-inflammatory phenotype of microglia is IL-4, which is more highly expressed in female mice. Deletion of IL-4 using knockout mice led to a loss of sex differences in infarct volume and outcome [131]. In contrast, male-derived microglia have higher expression of S100a8, a TLR4-binding protein that is involved in proinflammatory cytokine regulation [125, 132–134]. With aging, females have an increase in the expression of inflammatory microglial complement proteins, which is consistent with the higher levels of inflammation in this population [55].

Transcriptomic analysis did not demonstrate a difference between male and female expression of estrogen receptors. This suggests that the circulating levels of hormones, rather than the number of estrogen receptors, are responsible for any hormone-related differences [34, 135]. In adult female mice, 17β-estradiol levels are high and induce an anti-inflammatory phenotype in microglia which results in downregulation of IL-1β,

IL-6, and TNF-α and upregulation of the anti-inflammatory cytokine, IL-10 (Fig. 2) [135]. Progesterone also induces microglial transition into an antiinflammatory phenotype, decreasing the inflammatory response [136].

Interestingly, with aging and gonadal senescence, the sex chromosome compliment (XX vs. XY) plays an increasingly important role in the response to ischemic injury. Although the second X chromosome in females is "deactivated" for genetic balancing between the sexes, females express a high degree of mosaicism, depending on which X chromosome is silenced and what tissue is examined. It is becoming increasingly clear that many X-linked genes escape X-chromosome inactivation, and this is more common with aging and in the brain [137]. The four-core genotype (FCG) model can be used to dissociate gonadal and chromosomal sex. In this model, the testis determining gene *Sry* was spontaneously deleted from its normal position on the Y chromosome. A transgenic *Sry* gene is placed on an autosome (chromosome 3). Breeding XX gonadal female mice with XY $^{-}$*Sry* gonadal male mice produces XX females and XY females (with no *sry* on the Y chromosome), both of which have ovaries, and XX*Sry*-males (with *sry* on chromosome 3) and XY*Sry* + males (with *sry* on chromosome 3) that have the testis [138]. In young mice, Manwani et al. found that young FCG mice with ovaries had smaller infarcts than mice with testes. However, this difference was lost after gonadectomy, after which all four genotypes had equivalent infarct size. This suggests that estrogen is the main protective factor in this age group [139]. However, in reproductively senescent aged mice with similar levels of gonadal hormones (18–20 months of age), mice with two X chromosomes had worse outcomes regardless of gonadal sex. All mice with an XX chromosome compliment had enhanced "proinflammatory" microglia, and a downstream increase in proinflammatory cytokines [135, 140]. This was due to X chromosome genes that escape X chromosome activation in a low hormone milieu [140] and was mediated by interferon regulatory factors (IRF). Expression of KDM6A, a gene on the X chromosome that escapes inactivation, led to H3K27me3 demethylation and higher levels of IRF5, which promotes the proinflammatory microglia phenotype. The KDM6A-IRF5 pathway was increased in females, confirming epigenetic involvement in cytokine production [141]. Microglia in aged females also show an increased expression of MHCII and increased proinflammatory cytokine production [140]. Multiple factors influence microglial activation, and the resultant production of cytokines and chemokines (Table 3). Additional studies are needed to further investigate the sex-specific modulation of microglia.

Astrocytes

Sex differences in astrocytes

Astrocytes form tight junctions that contribute the integrity of the BBB, use calcium ion signaling to regulate blood flow, and express aquaporin 4 to regulate water balance [142]. They also serve as a source of estradiol, progesterone, and testosterone in the CNS [143]. Female astrocytes also produce a positive feedback loop, increasing estrogen levels [144]. Astroglial transcriptomic analysis in mice showed 20 to a 100 differentially expressed genes in males compared to females. This varied with age with the peak difference seen at

postnatal day 14, with few differences in adulthood. These differences were mostly found in genes related to cell cycle and neurite, dendritic, and synaptic development [145].

Sex differences in the astrocytic response to stroke

Multiple identified sex differences are seen in the astrocytic response to ischemia (Table 3). Female astrocytes release increased amounts of ionized calcium which is neuroprotective after stroke secondary to a reduction in brain edema from decreased water uptake [146, 147]. Aquaporin-4 supports paravascular flow of CSF. Increased aquaporin-4 polarization is indicated by higher aquaporin-4 presence in perivascular endfeet than in the parenchyma [148]. Aquaporin-4 polarity is associated with increased interstitial solute clearance, and lack of polarity is associated with heightened neuroinflammation [147]. Morrison et al. demonstrated that while astrocytes are equally polarized in males and females at baseline, after stroke, male astrocytes are more likely to have a change in polarity, which could also account for increased inflammation [147]. In the same study, S100β, an astrocyte Ca^{2+} binding protein that acts as a DAMP and promotes the neuroinflammatory response was found to increase more in males than in females [147].

Similar to what is seen in microglia, estrogen decreases the expression of the transcription factor NF-κB, leading to a reduction in the activation of proinflammatory genes and, thus, a decrease in proinflammatory cytokine levels [149]. Estrogen also blocks oxygen–glucose deprivation-induced astrocyte mitochondrial dysfunction and cell death which results in less ROS release [150]. Estrogen also induces glutamate transporter-1 and glutamate-aspartate transporter expression, which decreases excitotoxicity by removing glutamate and hence decreases the inflammatory response [151]. Further studies are needed to investigate sex differences in astrocyte after stroke.

Monocytes and macrophages

Sex differences in monocytes and macrophages

Monocytes and macrophages are central components of the innate immune response. Transcriptomic analysis showed that in human monocytes, there are 428 differentially expressed genes by sex, but the female-male fold change value was low [152]. Another study on the blood of humans with chronic inflammation showed that the expression of genes involved in the production of Fcγ receptors was increased in females, which are involved in monocyte activation (153). Similarly, females had a stronger IFN-γ response [153]. In murine bone marrow, Fcγ receptors genes were also differentially expressed in macrophages. Female mice also had a higher expression of genes that are stimulated by IFN [152]. On the other hand, analysis of human blood has shown that men have a higher expression of genes involved in phagocytosis and extracellular anti-microbial response [154].

Sex difference in monocytic and macrophagic response to stroke

After ischemic injury, monocytes (e.g., $Ly6C^{high}CD43^{low}$ cells) are recruited to the brain within a few hours and differentiate into macrophages, releasing IL-1β and TNF-α [155, 156]. Similar to other cells, mononuclear macrophages also show sex differences. After

stroke, higher numbers of activated macrophages are seen in the brain of male mice, along with increased percentage of activated microglia and cells expressing the homing marker, VLA-4, that have transmigrated into the infarct [76]. These cells might contribute to larger infarcts size in males but may also be secondary to the increased ischemic damage seen in young male vs. female animals. Female mice exhibited anti-inflammatory phenotypes in microglia/macrophage with higher expression of CD206 compared with males after stroke [75]. Female mice also had more robust anti-inflammatory responses from IL-10-producing CD8 + CD122 + T cells [76]. Notably, this female-specific neuroprotection was not observed in IL-4 KO mice (131).

The spleen exhibits sex differences in stroke outcomes. Dotson et al. showed that splenectomy reduces infarct volumes in stroke males, and infarct volumes between splenectomized males and females were comparable. Interestingly, spleen-intact males had higher levels of circulating CD11b + monocytes compared to male mice with splenectomy, which was not seen in females (74). Implication of sex differences in monocyte function in inflammation after stroke needs additional study.

Neutrophils

Sex differences in neutrophils

Neutrophilic transcriptome analysis identified 106 genes which were upregulated and 128 genes which were down-regulated in women compared to men [157]. Women also had more activated and mature neutrophils (e.g., enhanced type I interferon), which have a lower activation threshold in inflammation [157, 158]. These IFN-primed neutrophils increase ROS production, migration, NET formation, and adhesion molecule expression [157]. In addition, male neutrophils had elevated mitochondrial metabolism compared with female neutrophils. Interestingly, estradiol-treated male neutrophils had lower mitochondrial metabolism, which was similar with female neutrophils, indicating that the sex-specific maturation of neutrophils might be modulated by sex hormones [157].

Sex difference in the neutrophilic response to stroke

Neutrophils are the predominate leukocyte in the blood and one of the first blood-borne immune cell to arrive in the brain following stroke, with possible contributions from the skull bone marrow and the blood via the leptomeningeal vessels [159–161]. They release proinflammatory factors, ROS, proteases, and MMPs, which leads to increased BBB damage, hemorrhagic transformation, and post-stroke edema. This results in increased infarct volumes, higher stroke severity, and poorer outcomes [36, 55]. It is important to note that neutrophils also have beneficial effects on inflammation as they promote angiogenesis, produce MMPs which can break down proinflammatory DAMPs leading to reduced inflammation, and help with recruitment of beneficial cells [162]. Estrogen plays a significant inhibitory role in neutrophil recruitment to the stroke as it inhibits CINC-2, a chemokine associated with neutrophilic chemotaxis [163]. In older mice, males had a significantly higher number of neutrophils in the brain and higher levels of MCP-1 and G-CSF in plasma when compared to females. This, along with elevated $CD8^+$ T and

regulatory T cells, resulted in a 25% higher mortality and 55% higher risk of hemorrhagic transformation in male mice (Table 4) [164].

Neutrophil extracellular traps (NETs)

Activated neutrophils release fragile fibers composed of chromatin with granular proteins called NETs [165]. NET formation peaks 3–5 days after ischemic injury [166]. NETs can form through nuclear delobulation and envelope disassembly which leads to chromatin decondensation and cellular depolarization induced by cell death. Subsequently the plasma membrane ruptures and NETs are released. NETs can also form via degranulation and expulsion of chromatin followed by extracellular NET assembly [167]. These NETs form primarily in the vessel lumen and act as a scaffold for platelet binding and further thrombosis following stroke [160, 168]. Three clinical studies which cumulatively enrolled more than 150 patients undergoing mechanical thrombectomy that had clots available for histological evaluation had increased markers of NET formation (citrullinated histone and extracellular DNA) [56, 169, 170]. Higher levels of NET markers were associated with worse discharge disposition and poorer outcomes at 1 year after the index stroke [171]. Two studies found no association between sex and the presence of NETs. A higher number of NETs was associated with poorer recanalization times, increased recanalization attempts, and contributed to worse outcomes by stabilizing the thrombus leading to less complete recanalization [169].

Following stroke, NETs release cytotoxic proteases including elastase myeloperoxidase and histones which disrupt the blood–brain barrier and reduce neovascularization [166]. NETs have also been implicated in a reduction of T cell activation threshold increasing the release of the proinflammatory cytokines IL-17 and IFN-γ [172, 173]. These findings suggest that NETs are key targets for improving recanalization with thrombolytics and thrombectomy and to reduce post-stroke inflammation and improve neovascularization.

Few studies have assessed sex differences in NETs, and most of these are confined to pregnant women as aberrant neutrophil activation is known to be involved in complications of pregnancy [174, 175]. Estrogen, granulocyte colony-stimulating factors (G-CSF), and human chorionic gonadotropin promote NETosis by increasing expression of peptidyl arginine deaminase 4 expression, neutrophil elastase, and myeloperoxidase [174]. Neutrophils from cells of healthy women underwent more NETosis after ex vivo calcium stimulation compared those from men [176]. While this suggests that women might have higher NETosis, further studies are needed to see if this impacts neuroinflammation after stroke. While DNAse-I and NET inhibitory peptides are effective in clearing NETs in vitro and ex vivo, further investigations into the role of NETs and stroke are needed with a focus on sex differences in NET pathophysiology [177].

Lymphocytes

Sex differences in T cells

Naïve $CD4^+$ T cells differentiate into T helper cells, including Th1, Th2, Th17, and Tregs. In adults, sex differences in T cell subsets exist; women having a higher $CD4^+$ T cell count

and CD4/CD8 ratio in blood [92, 95–97, 178]. These $CD4^+$ T cells also have higher levels of estrogen receptors than $CD8^+$ T cells. Estrogen promotes differentiation of these $CD4^+$ T cells to Treg cells which promotes anti-inflammatory cytokines and to Th2 cells which decreases the production of proinflammatory IL-17 by Th17 cells [179].

The X chromosome also contains the Forkhead box P3 gene which is important in Treg differentiation and the gene for CD40 ligand, which is needed for T cell activation [180]. As discussed previously, some X chromosome genes can escape inactivation in females and can result in a more robust immune response [180]. With age, $CD4^+$ and $CD8^+$ T cell function decreases, but this decline is accelerated in men and leads to a poor inflammatory response [181, 182].

Sex differences in T cell responses to stroke

$CD4^+$ and $CD8^+$ T cells contribute to the inflammatory response, especially in delayed ischemic injury. Ahnstedt et al. showed that aged male mice had higher $CD8^+$ T cells in the brain 15 days after stroke and higher mortality than age-matched females (20–22 months of age) [80]. In both human and murine studies, females have increased serum levels of IL10, which dampens inflammatory responses but led to an increased risk of post-stroke infection and delayed recovery in females but not in males [79]. However, these elevated levels of IL-10 did not independently predict better outcomes in stroke patients (Table 5) [183]. Similar to IL-10, females also have higher levels of IL-4, which is associated with decreased recruitment of $CD4^+$ and $CD8^+$ T cells and, consequently, smaller infarcts [182]. In transient ischemia models, males had higher Nox2-derived superoxide, Cox-2, and VCAM-1 which are proinflammatory proteins. Nox2-containing NADPH oxidase contributes to infarct development, whereas VCAM assists with leukocyte infiltration into the brain [184]. Females also have higher numbers of regulatory T cells, which are protective in ischemic stroke by releasing anti-inflammatory cytokines including IL-10 [74]. Estrogen plays a major role in the sexual differences in Treg number and function [185]. Despite the enhanced release of anti-inflammatory cytokines, women have worse outcomes after ischemic stroke, which may be due in part to a weaker suppressive response in Tregs to T cell activation in women, leading to enhanced inflammation [186]. Beckmann et al. recently demonstrated sex differences in the Treg response after neonatal hypoxic ischemia in male and female mice, at a time point where levels of sex hormones are similar between [187]. Female mice had increased cerebral Treg infiltration, coinciding with elevated chemokine receptor expression. Treg depletion in females aggravated HI-induced brain tissue injury which paralleled an increase in microglia and endothelial activation and leukocyte infiltration. Surprisingly, Treg depletion in male mice reduced HI-induced brain injury and behavioral deficits. Isolated female Tregs had an increased immunosuppressive activity on effector T cell proliferation ex vivo. This is important as it demonstrates that sex differences in Treg function are not only due to differences in sex hormone levels, but also to differential gene expression (Table 4) [187]. Females also have upregulation of genes mediating the IL-12 cytokine signaling pathways which promotes natural killer cell toxicity, T cell proliferation, and Th1 cell differentiation (Fig. 3) [188, 189]. The full extent of regulatory T cell involvement in post-stroke outcomes remains unclear in aged subjects [190].

Sex differences in the B cell response to stroke

B cells, and in particular IL-10 producing B cells, are associated with decreased infarct volume. Female mice have increased regulatory B (Breg) cells ($CD19^{+}CD5^{+}CD1d^{high}$ cells) in the ischemic hemisphere of the brain after stroke, which corresponds to fewer Breg cells in the spleen of females [75]. B cells are also implicated in the increase of anti-inflammatory microglia seen in the female brain post-stroke. IL-10 producing Breg cells promote the anti-inflammatory phenotype in microglial cells, in both males and females. Female mice have higher levels of microglial IL-10 receptor expression, which responds to the IL-10 released by Bregs. This results in sex differences with higher levels of anti-inflammatory microglia in females [191].

Conclusion

Neuroinflammation plays a vital role in post-stroke injury and recovery. Neuronal damage induced by inflammation occurs both acutely and chronically following stroke, hence providing a larger window for intervention as compared to thrombolytics and thrombectomy. The identification of sex differences in post-stroke neuroinflammation is important. There is an urgent need for sex-stratified clinical and preclinical analyses to identify and investigate these differences and implement this knowledge to develop novel sex-specific treatments for both men and women.

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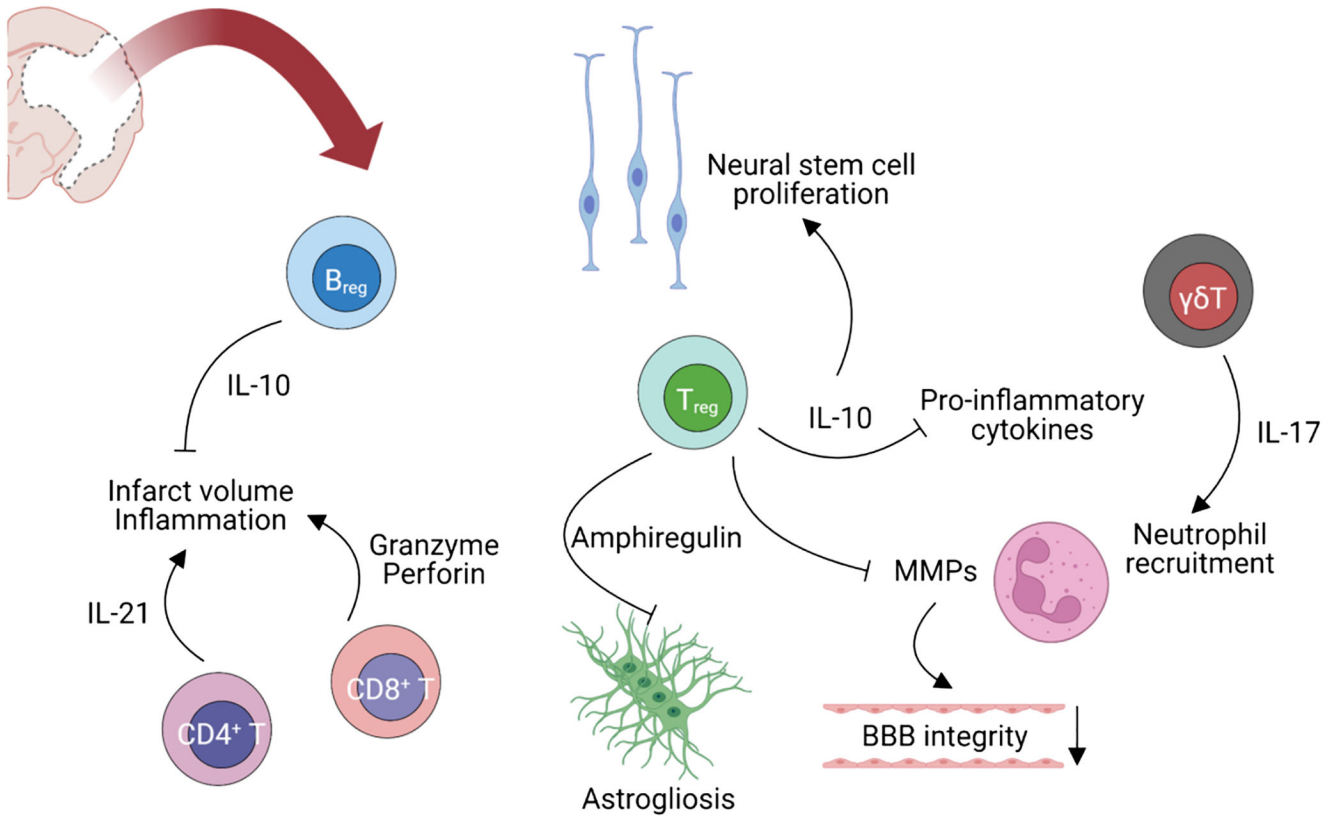

Fig. 1.

Adaptive immune responses regulated by T and B cells in stroke. (created with BioRender.com)

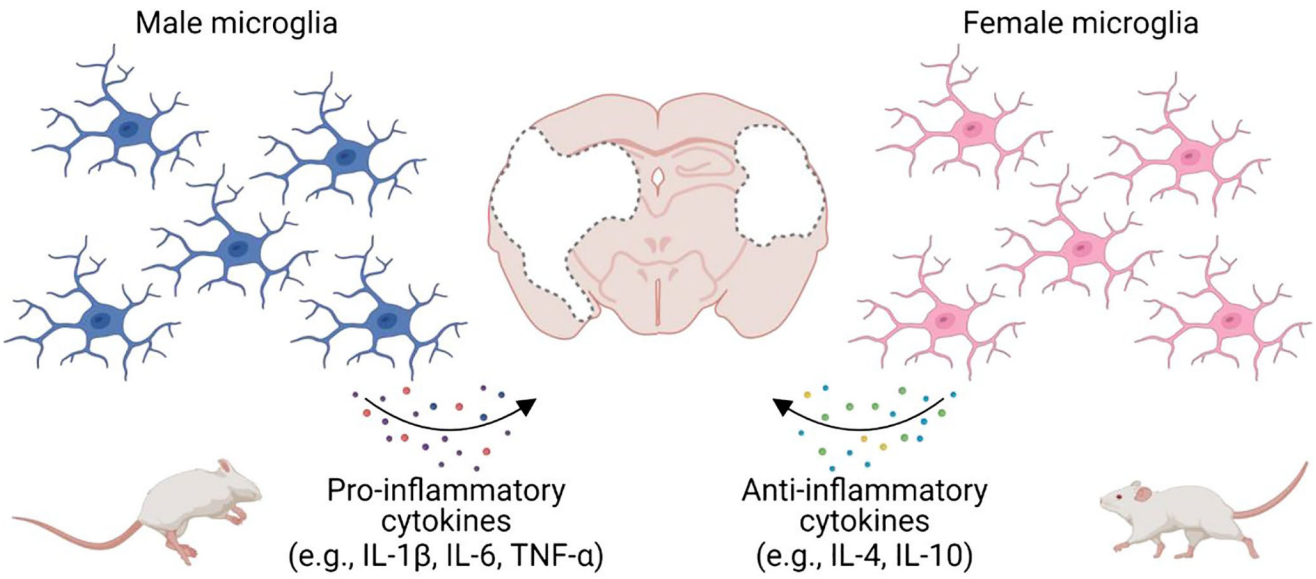

Fig. 2.

Sex differences in microglial response following stroke. Female microglia confer neuroprotection in stroke by secreting higher levels of anti-inflammatory cytokines including IL-4 and IL-10 and lower proinflammatory cytokines including IL-1β, IL-6, and TNF-α, compared with male microglia (created with BioRender.com)

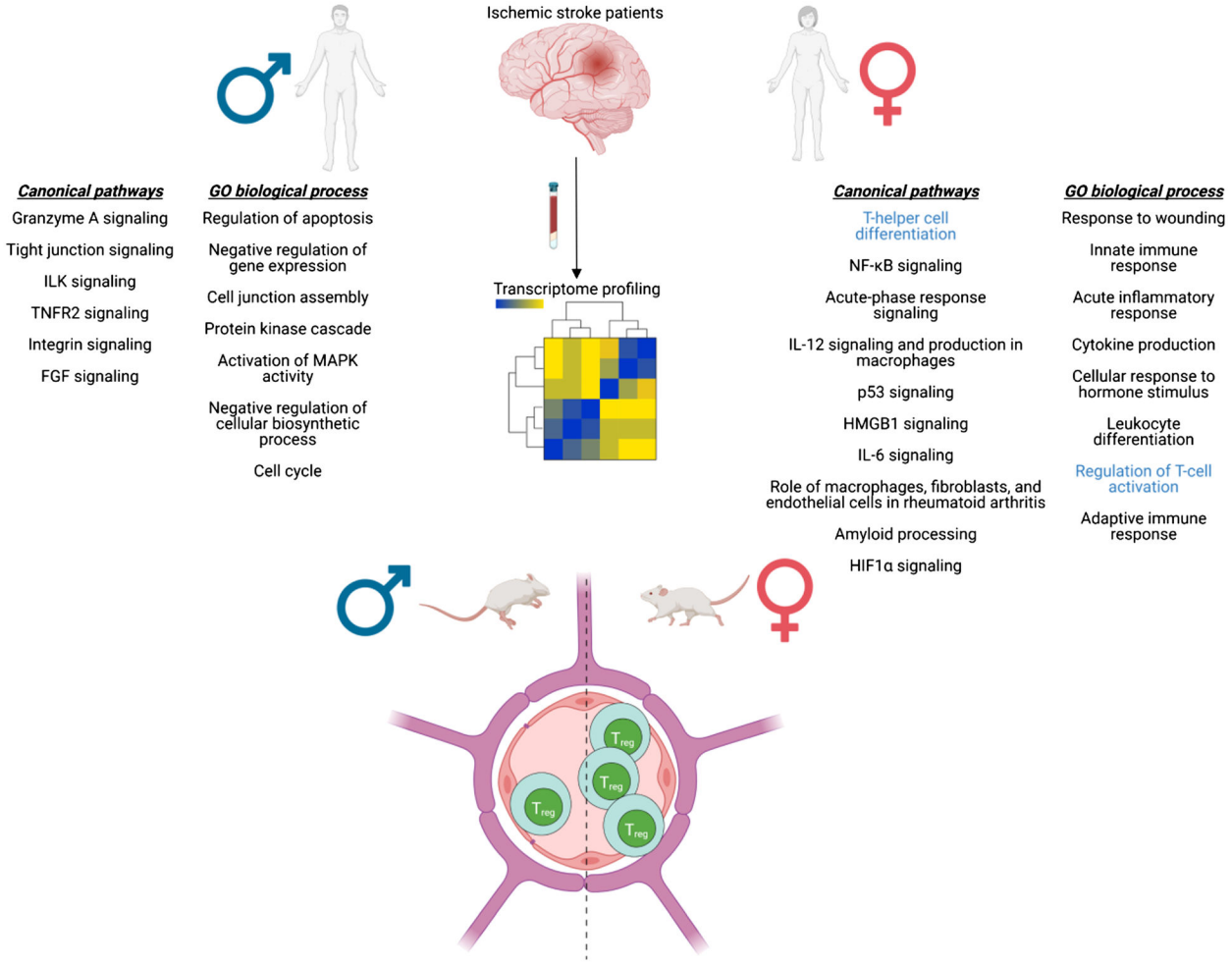

Fig. 3.

Sex differences in the systemic T cell response following stroke. Tian et al. [188] profiled gene expression in the blood from male and female ischemic stroke patients (≤ 3, 5, and 24 h) and performed functional analysis to identify pathways. Of note, female-specific pathways include T-helper cell differentiation and regulation of T cell activation. Canonical pathways and GO biological process for ≤ 3 h after stroke were shown here. In a mouse model of stroke, females had higher regulatory T cells in the blood compared with males [74] (created with BioRender.com)

Table 1

Components of the CHA_2DS_2VASc score

Letter	Risk factor	Score
C	History of congestive heart failure	**1**
H	Hypertension	**1**
A_2	Age 75 years or older	**2**
D	Diabetes	**1**
S_2	Stroke/TIA/thromboembolism history	**2**
V	Vascular disease history	**1**
A	Age 65–74 years	**1**
S	Sex, female	**1**

Table 2

Annual risk of embolic stroke, transient ischemic attack, and systemic embolism per year in patients with atrial fibrillation [192]

CHA_2DS_2-VASc score	Risk of ischemic stroke (%)	Risk of stroke/transient ischemic attack/systemic embolism (%)
0	0.2	0.3
1	0.6	0.9
2	2.2	2.9
3	3.2	4.6
4	4.8	6.7
5	7.2	10.0
6	9.7	13.6
7	11.2	15.7
8	10.8	15.2
9	12.2	17.4

Table 3

Preclinical studies examining sex-specific differences in microglia and astrocytes in the response to ischemic stroke

Study	Species and strain	Age	Key findings
Xiong et al. [131]	BALB/cJ and IL-4 KO (BALB/c-IL-4tm2Nnt/J) mice	10–12 weeks	IL-4 is neuroprotective in female mice. It prompts differentiation to the M2 (anti-inflammatory) microglia phenotype and reduced monocyte/macrophage infiltration into the ischemic brain
Seifert et al. [75]	C57BL/6 J mice	8–10 weeks	Post MCAO, female mice had smaller infarct volumes. This was likely due to higher frequency of anti-inflammatory microglia/macrophages, $CD11b^{+}CD206^{+}$ in infarcted tissue. Female mice also have decreased anti-inflammatory macrophages and IFNγ expression after MCAO
Bodhankar et al. [191]	Wild-type C57BL/6 J	10–12 weeks	Treatment with rIL-10 B cells inhibited proinflammatory cytokine production and differentiation into M2 microglia in both sexes. However, the inhibition was more pronounced in female mice. rIL-10 B cells also increase the production of the neuroprotectant IL-4 in females. Post-stroke, female mice have higher expression of IL-10R and IL-4Rα
Crain et al. [135]	C57Bl/6 mice	P3, P21,7–8 week, 4 months, 12 months	Microglia gene expression changes with age. The expression of inflammatory and anti-inflammatory cytokines is also sex dependent. Female mice had a more anti-inflammatory response at different age groups, but there was shift towards a more inflammatory phenotype in older mice
McCullough et al. [140]	C57BL/6 J XYM and wild-type C57BL/6 J mice bred to produce the following genotypes: XYM, XXM, XYF, XXF	18–20 months	Aged mice with a second X chromosome (XXF and XXM) had larger strokes volumes, with significantly more activated microglia and higher levels of inflammatory cytokines, when gonadal hormones are not present
Qi et al. [141]	C57BL/6 J XYM and wild-type C57BL/6 J mice bred to produce the following genotypes: XYM, XXM, XYF, XXF	18–22 months	The KDM-Histone-IRF pathway, which regulates microglia cytokine production, was more highly expressed in wild-type females and in FCG mice with 2 X chromosomes
Manwani et al. [193]	C57BL/6 mice	5–6 months, 14–15 months, and 20–22 months	Stroke leads to splenic contraction in male mice of all ages and aged female mice. Dendritic cells were recruited in higher numbers to the female compared to the male brain. No differences in the number of macrophages and neutrophils were identified
Dotson et al. [74]	C57BL/6 J mice	Not reported	Prior to splenectomy, male mice had larger stroke volumes than female mice. After splenectomy, male mice had reduced stroke volume such that there was no longer a difference in the infarct size between male and female mice. Activated microglia were reduced in females regardless of splenectomy and in males after splenectomy when compared to male mice without splenectomy
Morrison et al. [147]	C56BL6/J and C57BL/6 with $CX_3CR_1^{GFP/+}$	3 months	Female mice have a higher number of CD11b microglia at baseline, whereas these numbers increase in males after MCAO. Astrocyte AQP4 changes polarity in male mice
Banerjee et al. [76]	C57BL/6 J mice	Not reported	Post-stroke, male mice have higher numbers of $CD45^{hi}CD11b^{+}$ microglial cells and macrophages
Villapol et al. [194]	C57BL6/J mice	Postnatal day 9	Male mice had more proinflammatory microglia markers in the ischemic brain compared to female mice, 3 days after stroke. Higher levels of the proinflammatory cytokine, TNF α, was seen in male mice
CordeauJr et al. ([195]	Transgenic GFAP-luc mice	10–17 week	Astrocyte response (GFAP upregulation) results in increased infarct size in males but not females

KO knock out, *IL* interleukin, *MCAO* middle cerebral artery occlusion, *IFN* interferon, *FCG* four core genotype, *AQP* aquaporin, *TNF* tissue necrosis factor, *GFAP* glial fibrillary acidic protein

Table 4

Preclinical studies examining sex-specific differences in neutrophil and lymphocyte responses in ischemic stroke

Study	Species and strain	Age	Key findings
Xiong et al. [131]	BALB/cJ and IL-4 KO (BALB/c-IL-4tm2Nnt/J) mice	10–12 weeks	During estrous, WT female mice have significantly lower T cells, but this difference was lost with deletion of IL4
Banerjee et al. [76]	C57BL/6 J mice	Not reported	Post-stroke, spleens of male mice become severely atrophic. Male spleens had higher $CD8^+CD122^+$ T-suppressor cells, whereas female spleens had higher IL-10 secreting $CD8^+CD122^+$ T-suppressor cells. There was a higher percentage of VLA-4 expression on male mice lymphocytes
Manwani et al. [193]	C57BL/6 mice	5–6 months, 14–15 months, and 20–22 months	Stroke leads to splenic contraction in male mice of all ages and aged female mice. Similarly, male mice with stroke had higher levels of T cells when compared with female mice, except in elderly mice, where the difference disappeared
Dotson et al. [74]	C57BL/6 J mice	-	Prior to splenectomy, male mice had larger stroke volumes than female mice. After splenectomy, male mice had reduced stroke volume, and there was no longer a difference in male and female mice stroke volume. Spleen intact females have lower CD4 + T cells and lower number of "activated" T cells than males, a difference lost with splenectomy
Brait et al. [184]	C57BL/6 J mice	6–8 weeks	Male mice have a higher $CD3^+$ T cell response with increased Nox2 expression resulting in larger infarcts than those in female mice
Seifert et al. [75]	C57BL/6 J mice	8–10 weeks	Post-MCAO, female mice had smaller infarct volumes. This was likely due to higher frequency of B10, $CD19^+CD5^+CD1d^{hi}$ cells. Female mice also have decreased Breg cells and increased Treg in their spleen after MCAO
Ahnstedt et al. [80]	C57BL/6 N mice	20–22 months	Increased $CD8^+$ T cells and Tregs in infarcts of aged males. No sex difference in $CD4^+$ T cells
Jackson et al. [196]	Wistar rats	12–15 weeks	After stroke, the blood of male mice had a higher percentage of circulating γδTCR and T_H1 cells, but lower Treg when compared to females In the brain, males had a higher percentage of T_H1 cells. Diabetes also had a sexually dimorphic effect on regulation of various T cell subtypes

IL interleukin, *VLA* very late antigen, *MCAO* middle cerebral artery occlusion

Table 5

Clinical studies examining sex-specific differences in the inflammatory response to ischemic stroke

Study	Number of study members	Age (years)	Key findings
Conway et al. [183]	178 patients (76 females and 102 males)	Female—72.9 Male—64.5	Women have higher levels of IL-10, but when controlled for other factors associated with post-stroke outcomes, elevated IL-10 level was not associated with outcome
Yan et al. [186]	77 stroke (31 female and 46 males) and 64 controls (31 females and 33 males)	Female controls—63 Female stroke patients—66 Male controls—63 Male stroke patients—65.5	Activated T cells were significantly elevated in patients with stroke. Tregs were increased in men. Tregs had a weaker suppressive response in women
Stamova et al. [197]	23 stroke (11 female and 12 males) and 23 controls (11 females and 12 males)	Female controls—59.1 Female stroke patients—71.3 Male controls—56.8 Male stroke patients—72.1	Women and men had differential expression of genes after stroke resulting in an earlier increase in inflammatory and anti-inflammatory pathways in women. Women also had higher expression of genes associated with cell death
Tian et al. [188]	Control Males: 28 and females: 24 Ischemic stroke Males: 27 and females: 24	Control Males: 60.3 and females: 60.6 Ischemic stroke Males: 63.6 and females: 65.1	Women and men had differential expression of genes after stroke. Women had increased expression of genes involved with inflammatory pathways and caspase-mediated cell death, while men had higher expression of genes involved with caspase-independent cell death pathway and vessel wall adhesion
Xu et al. [198]	Control: 20 Ischemic stroke Male: 10 and female 10	Not reported	Women had higher expression of CCL20, ICAM, and PTGS2 after ischemic stroke as compared to men. Both sexes had modulation of inflammatory responses
Ross et al. [199]	Males: 96 and females: 96	Males: 73.2 and females: 77.3	Women had an increase in monocytes which was not seen in men
Zhu et al. [200]	Control: 20 Ischemic stroke Male: 10 and female 10	Ischemic stroke: 60.2	IL1α, IL1β IL6, IL8, CXCL1, CXCL2, CXCL20, CCL4, ICAM1, and PTGS2 genes were down-regulated in women and upregulated in men
Trott et al. [201]	Ischemic stroke Male 70 and female 59	Ischemic stroke: 60.2	After thrombectomy, there was a positive association with NIHSS change and in WBC counts in women, a finding not seen in men
Stamova et al. [202]	Controls Males: 41 and females: 68 Ischemic stroke Males: 35 and females: 26	Controls Males: 50.2 and females: 49 Ischemic stroke Males: 67.1 and females: 67	Women and men had differential expression of genes after stroke

IL interleukin, *CCL* chemokine ligand, *ICAM* intercellular adhesion molecule, *PTGS2* prostaglandin-endoperoxide synthase, *CXCL* (C-X-C) motif ligand, *WBC* white blood cells, *NIHSS* National Institute of Health Stroke Scale