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The impact of sex and age on T cell immunity and ischemic stroke outcomes

Hilda Ahnstedt, **Louise D McCullough**

Department of Neurology, McGovern Medical School at The University of Texas Health Science Center at Houston (UTHealth), Houston, Texas, USA

Abstract

Sex differences are well-recognized in ischemic stroke, a disease mainly affecting the elderly. Stroke results in robust activation of central and peripheral immune responses which contributes to functional outcome. Aging is associated with increased low-grade chronic inflammation known as "inflammaging" that renders aged males and females more susceptible to poor outcomes after ischemic stroke. Despite that sex differences are well-documented in immunity and inflammation, few studies have focused on sex differences in inflammatory responses after ischemic stroke and even fewer in the context of aging. The role of T cell responses in ischemic stroke have gained increasing attention over the past decade as data suggest a major role in the pathophysiology/recovery. T cells offer an attractive therapeutic target due to their relatively delayed infiltration into the ischemic brain. This review will focus on T cell immune responses in ischemic stroke, highlighting studies examining the effects of aging and biological sex.

Keywords

T cells; ischemic stroke; inflammation; sex differences; aging; sex; sex hormones; immunity

Introduction

Ischemic stroke is a leading cause of mortality and morbidity in the elderly. There are considerable sex differences in stroke incidence across the lifespan, with earlier onset in men [1]. However stroke risk in middle-aged females and females above 85 years of age is higher than in men [1,2]. Hormonal effects due to early life exposure to sex hormones (organizational effects) and acute effects of circulating hormones during the reproductive years are thought to play a role in this sexual dimorphism. There has been increasing recognition of the contribution of the sex chromosomes (XX vs. XY) and X-linked genes to ischemic sensitivity, especially after reproductive senescence [3–5]. Using the four core genotype (FCG) mouse model, studies indicate that the ischemic phenotype, and whether female neuroprotection is present, is hormone-dependent in young mice while it is chromosomal-dependent in aged mice [4,5]. Aging is associated with chronic low-grade

inflammation termed “inflammaging” and an overall decline in the function of the immune system. Males and females show different immunological responses to foreign and self-antigens. This contributes to sex differences in autoimmune diseases, susceptibility to infectious diseases and vaccine efficacy, and in age-related diseases such as Alzheimer’s Disease [6,7]. Despite these well-known sex differences in immune responses (see Table 1), immunology is ranked lowest out of the biological disciplines when it comes to reporting the sex of the animal/human subject, with fewer than ten percent of articles analyzing their data by sex [6]. The majority of pre-clinical studies have not included a sex-specific design or analysis [8], and are often underpowered to detect sex differences. Historically, female subjects have also been underrepresented in clinical trials [9] but due to policy changes by the FDA in 1993 that required efficacy and safety of new drugs to be evaluated in both sexes, female numbers have increased and the overall participation of men and women is comparable [10,11]. Women are still underrepresented in phase I clinical trials and in certain areas such as cardiovascular trials, however this was correlated to age of enrollment as age-sex differences in cardiovascular disease prevalence are well-known [12]. Differences in drug efficacy and side-effects led to the withdrawal of eight out of ten prescription drugs in the US between 1997 and 2001 because they posed greater risks for women than men [13]. In ischemic stroke, the anti-inflammatory drug minocycline affords neuroprotection in male mice but not in females [14]. Sex differences in ischemic stroke have also been reported in clinical trials , e.g. uric acid was shown to improve the rate of excellent outcome at 90 days (modified Rankin score 0-1) in women, but not in men [15]. The inflammatory cascade that is activated acutely after stroke continues for weeks and months after the injury, and contributes to outcome and recovery [16,17]. An increasing number of studies highlight an important role for T lymphocytes in the pathophysiology and recovery after ischemic stroke, yet our understanding of sex differences in these responses are limited. In this review, we highlight recent work on sex differences in T cell immune responses and how they are affected by aging and ischemic stroke.

Sex differences in T cell immunity

It is well-documented that immune responses differ by sex (Table 1), which is emphasized by the higher proportion of females with multiple autoimmune diseases in which female to male ratios can approach 11:1 [18]. Differential responses to infection and vaccines exist in that females mount a stronger antigenic response, are less susceptible to a wide variety of pathogens, and have superior clearance of pathogens [3,19]. T cells are part of the adaptive immune system and have two major phenotypes; $CD4^+$ helper/inducer cells and $CD8^+$ cytotoxic/suppressor cells, which compromise approximately 95% of T cells and are characterized by their distinct surface markers [20]. Naive $CD4^+$ T cells differentiate into specific T helper (T_H) cells depending on the cytokine microenvironment, namely T_H1, T_H2, T_H17 and induced T regulatory cells (iTregs) [21]. iTregs and naturally induced Tregs in the thymus (nTregs) are responsible for maintaining immune homeostasis and act in an anti-inflammatory fashion to suppress effector T cells [22]. Classical $CD4^+$ and $CD8^+$ T cells carry the αβ T cell receptor (TCR) that are expressed on their surface. Another type of T cell expresses a γδ TCR instead of the classical αβ TCR and comprises a small subset of T cells called γδ T cells that are enriched in epithelial and mucosal tissues such as the skin and gut

[23]. Sex differences and sex hormone effects on these different T cell subsets have been described [6], and they have been implicated in pathophysiology and recovery after stroke [24–26], which will be discussed in later sections.

The host response to intracellular infections highly relies on $CD8^+$ T cells. Mon et al. showed that upon viral or bacterial infection, females elicited a larger $CD8^+$ T cell response that consisted mostly of short-lived effector cells, whereas males induced a higher proportion of memory precursor effector cells [27]. Short-lived effector cells are more terminally differentiated, express high levels of cytokines and cytolytic molecules, hence phenotypically are more apoptotic and largely responsible for elimination of infected cells during an infection [28]. Memory precursor cells on the other hand, are less responsive during infection but transition into the long-lived memory pool and respond to repeat infections. To exclude differences in host environment and to focus on cell-intrinsic sex differences, an equal number of $CD8^+$ T cells that expressed an identical TCR were adoptively transferred into male recipient mice followed by bacterial infection with *L. monocytogenes* [27]. Even in the same host environment there was a consistently higher rise in short-lived effector cells along with higher levels of granzyme B and interferon-γ (IFN-γ) by female $CD8^+$ T cells, indicating intrinsic sex differences at the cellular level [27]. This was not due to a lower antigen threshold in females, but rather to an enhanced propensity of female $CD8^+$ T cells to respond to IL-12, facilitating effector cell differentiation. Interestingly, some pro-inflammatory genes including IFN-γ and IL-12 are known to be more responsive to estrogen as these immune genes contain estrogen response elements (ERE) in their promoters, which will be discussed below [29,30].

Sex differences in T cells are evident also in the absence of stimuli like vaccines and pathogens. Flow cytometry analysis of baseline differences in peripheral blood mononuclear cells showed that women have a higher fraction of $CD4^+$ T cells than men, which has been shown by multiple investigators [31,32]. The proportion of individuals with an inverted CD4/CD8 ratio either through targeted cell death of $CD4^+$ T cells, expansion of $CD8^+$ T cells, or a combination of both, was found to be significantly higher in men [31]. A low or inverted CD4/CD8 ratio is an immune risk profile indicative of altered immune function, immune senescence and chronic inflammation in both HIV and in uninfected populations [33]. The prevalence of an inverted CD4/CD8 ratio increases with age, but is lower in women across all age groups, a phenomenon that may partly be due to hormonal influences. Estrogen deficiency in women with premature ovarian failure, or in mice with natural menopause or by ovariectomy, have low or inverted CD4/CD8 ratios which clearly suggest an important role for estrogen in T cell immunity [34,35].

Effect of sex hormones

Estrogens, progesterones and androgens are the major gonadal hormones. All have numerous well-documented effects on the immune system that mediate many of the known sex differences in immunity (reviewed in detail in [6]). Upon binding to their respective hormone receptors, the estrogen receptors (ER) ERα and ERβ. the progesterone receptor (PR) and the androgen receptor (AR) complex serves as a hormone-induced transcription factor that in turn binds to hormone response elements in the promoter region of target genes

[36]. The IFN-γ gene is one of the target genes that is activated upon binding of estrogen to ERs [29,30]. While ERα is expressed on almost all immune cells, ERβ expression is more restricted [37]. $CD4^+$ T cells have high levels of ERα compared to ERβ. whereas $CD8^+$ T cells have low level of both ERs. Estrogens promote the expansion of Tregs in mice and healthy women [38], induce T_H2-type responses and decrease production of interleukin-17 by T_H17 cells [39]. In response to estrogen fluctuations throughout the course of the menstrual cycle, the number of Tregs undergo significant changes and will affect overall immunity accordingly as Tregs regulate the peripheral T cell pool and the response to infections [40]. Interestingly, the impact of ischemic stroke in young cycling female mice vary throughout the estrous cycle and neuroprotection is seen primarily during proestrus when estradiol levels are high [41]. However, Treg and effector T cell levels throughout the estrous cycle have not been investigated in relation to neuroprotection in ischemic stroke.

Besides the classical signaling of hormone and hormone receptors, non-classical direct signaling occur in immune cells between ERs and ERE-independent transcription factors including nuclear kappa beta and activator protein 1. In addition, estrogens can bind to the membrane associated estrogen G protein-coupled receptor 30 (GPR30) to provide more rapid signaling [42]. Similar to estradiol, a GPR30-specific agonist G1 was shown to be neuroprotective in ischemic stroke and improved immunosuppression by partially restoring splenocyte numbers in female mice [43].

Progesterone receptors are found on many different immune cells, including T cells and Natural Killer (NK) cells [44]. Progesterone has broad anti-inflammatory effects including the capacity to increase Treg frequency and decrease the activity of $CD8^+$ T cells, T_H17 cells and T_H1 cells [45,46]. The androgens, testosterone and dihydrotestosterone, are generally suppressive of immune cell activity and are known to reduce T/B cell proliferation, decrease $CD8^+$ T cell numbers and increase Treg numbers [47]. The hormonal milieu in males and females change as we age, with a rapid decline in females and a more gradual decrease in males which parallels a functional decline in the immune system of both sexes.

Effects of sex chromosomes

Hormones are not the only contributors to sex differences in immunity; sex chromosome effects are also important modulators [48]. This is exemplified by Klinefelter and Turner syndromes, where males have an extra X chromosome (Klinefelter syndrome) and women have only one X chromosome (or major X chromosome deletions, Turner syndrome). Males with Klinefelter's mount a similarly strong immunological response as seen in XX females, including elevated $CD4^+$ T cell numbers and CD4/CD8 ratios [49]. On the other hand, women with Turner syndrome have lower immunoglobulin and T cell levels and are less susceptible to systemic lupus erythematosus (SLE) [50,51]. Both patients with Klinefelter syndrome and patients with Turner syndrome show increased susceptibility to autoimmune disorders speaking for an important role for the X chromosome in influencing autoimmunity [52]. Several immune genes are located on the X chromosome, including the Forkhead box protein P3 (FoxP3) specific for Tregs, and CD40L, which is critical for T cell activation [48]. Genes on the X chromosome have to be inactivated to ensure only one copy functions in each sex, a process initiated by the X-inactive specific transcript (XIST) gene. In humans,

approximately 15% of X genes escape inactivation and are found in higher copy number in females, the equivalent number in mice being 3% [6]. The impact of sex chromosomes can be studied using the FCG mouse model where the sex determining gene SRY is placed on an autosome [53]. In this model, gonadal males with XY or XX chromosomes, and gonadal females with XX or XY chromosomes can be generated. Gonadectomy in these mice unmask sex chromosome effects including susceptibility to autoimmune diseases and viral infections [54]. In ischemic stroke, aged mice with a second X chromosome (male or female) had significantly larger infarct volumes and a larger population of infiltrating lymphocytes compared to XY-females or XY-males [4]. However, changes in specific T cell populations and phenotypes after stroke using this model have yet to be studied.

Effects of aging

Aging is associated with a decreased function of the adaptive immune system and profound changes in T cell function have been reported. The thymus is critical for maturation and development of a diverse T cell repertoire. As we age thymic involution occurs, a process that is shaped by sex hormones [55]. The number of naïve lymphocytes are reduced in parallel to an increased proportion of memory and memory-like lymphocytes from a life-long exposure to a variety of pathogens and antigens [56]. Aged naïve $CD4^+$ T cells have a reduced ability to respond to antigens and antigen presenting cells and thereby the immune response is less intense. As a consequence, $CD4^+$ T cells from aged mice do not expand, produce cytokines or differentiate as well as those from young mice [57]. When it comes to $CD8^+$ T cells, the most striking feature of aging is decreased TCR repertoire. This is detrimental as a diverse TCR is vital for protection from viral infections, and explains, in part, why older individuals are more susceptible to infection [58]. For example, $CD8^+$ TCR repertoire usage in a mouse influenza virus model is significantly decreased in aged animals compared to young [58]. Not all T cell subtypes becomes impaired as we age, for instance mouse and human Tregs increase in number and function with aging, and might be higher in males [59–62]. Although aging affects the adaptive immune system in both males and females, aging may be occurring at an accelerated rate in males. For example, during the process of aging, females better maintain the proliferative capacity of T cells [63]. Males experience a larger loss of naïve T cells and a larger increase in senescent $CD8^+$ effector memory cells, which is also evident in the aging brain [64]. These sex differences in the aging immune system are likely to affect age-related diseases such as ischemic stroke. In fact, Ritzel et al. demonstrated that the aged mouse brain accumulated effector memory $CD8^+$ T cells that were primed to potentiate inflammation and leukocyte recruitment following ischemic injury [64]. In the next section we will first give an overview of known T cell responses after ischemic stroke, unfortunately most are from studies performed exclusively in young male animals. Thereafter we describe studies that have included, sex and/or age-specific analysis.

T cell responses after ischemic stroke

Ischemic stroke triggers multiple inflammatory cascades in the brain and periphery. T cells are integral to the pathophysiology of stroke and are a key component of the adaptive immune system [16,65,66]. The time-course and extent of T cell infiltration after ischemic

stroke vary depending on the experimental stroke model used (permanent vs transient) and the age of the animals. In general the adaptive immune system is viewed as the delayed/slower response occurring 1-7 days to weeks after ischemic stroke in contrast to the early innate response that occurs within hours after stroke, however an important role for T cells early after ischemic stroke has also been suggested [17,24,26,66]. The distinction between early and late T cell responses after stroke is likely related to antigen-independent and antigen-dependent mechanisms which is a subject of debate discussed in a later section. Nevertheless, the delayed profile of T cell migration and its potential for an extended time-window for ischemic stroke offer an attractive therapeutic target.

The importance of T cells in the deleterious processes after ischemic stroke have been demonstrated by the use of transgenic animals and/or antibody-depletion strategies [20,24,26,67–69]. $Rag^{-/-}$ mice devoid of T- and B-cells exhibited smaller brain injuries and decreased neurological deficits 24 hours after ischemic stroke [24,26,69]. The protection was lost when $CD3^+$ T cells were reconstituted in these mice, while replenishment of B cells did not affect the infarct volume, nor did systemic B cell deficiency suggesting a less important role for B cells in acute injury [26,69]. In contrast, existing data point to a regulatory and protective role for B cells through interleukin-10 (IL-10) secretion thereby limiting neuroinflammation and neurological deficits 48 hours post-stroke [70]. It has been suggested that delayed infiltration of B cells contribute to cognitive impairment 7 weeks post-stroke [71]. In summary, while overall T cell responses and the role of specific T cell subsets (discussed in the next section) after ischemic stroke are fairly well-studied, further studies are needed to determine the beneficial/detrimental effect of B cells.

Role of specific T cell populations in ischemic stroke

Further evidence for the importance of T cells in the evolution of ischemic brain injury has been shown after treatment with an immunomodulatory drug, FTY720 (fingolimod), used in multiple sclerosis patients, that inhibits migration of T cells into inflamed tissues [24]. T lymphocytes were essential for infarct growth and FTY720 significantly reduced the infarct volume and the number of infiltrating T cells, including γδ T cells to the brain, without affecting macrophage infiltration [24]. Depletion of γδ T cells through a TCRγδ-specific antibody (either administered pre-stroke or post-stroke), or in TCRγδKO mice, resulted in significantly smaller brain injury at day 7 post-stroke [24], suggesting an essential role of T cell entry in the early and sub-acute phase of stroke.

Liesz et al. elegantly showed that invading T cells, through secretion of IFN-γ and cytotoxic actions (perforin), are important to ischemia-induced injury [20]. Overall inhibition of leukocyte infiltration through blockade of very late antigen-4 (VLA-4, CD49d) reduced stroke size 7 days post-stroke in both a permanent and transient model of ischemic stroke. The protection was shown to be lymphocyte dependent, and $CD4^+$ and $CD8^+$ T cell dependent in particular, since $Rag2^{-/-}$ mice, and mice depleted of $CD4^+$ T cells or cytotoxic $CD8^+$ T cells, were not further protected by anti-CD49d treatment [20]. Depletion of $CD8^+$ T cells, NK or NK T cells prior to stroke has also been shown to be protective after ischemic stroke [67,72]. Astrocytic over-expression of interleukin-15 has been shown to exacerbate the ischemic injury, but this effect was abolished after $CD8^+$ T cell and NK cell depletions

[67,72]. In contrast, another study showed that depletion of cytotoxic $CD8^+$ T cells decreased infarct volumes and reduced neurological deficits in two different models of ischemia, while NK cell depletion had no effect [73]. The reduced infarct volume was observed in treatment regimens where the CD8α-antibody was given either prior to stroke or 5 hours after, however a significant reduction was not seen until day 7 [73]. These data indicate that $CD8^+$ T cells may have an important role in delayed and secondary injury to ischemic stroke. Additional experiments demonstrated that the neurotoxic action of $CD8^+$ T cells were mediated by perforin, likely in an antigen-dependent manner as $CD8^+$ T cells derived from transgenic mice with an ovalbumin-specific T cell receptor infiltrated to the ischemic brain in fewer numbers than from wild-type mice [73].

In contrast to the focus on $CD4^+$ helper and $CD8^+$ effector T cells in most of previous stroke studies, a recent study has implicated $CD3^+CD4^-CD8^-$ T cells (double-negative T cells; DNTs) in neuroinflammation after ischemic stroke [74]. DNTs increased in brain and blood of stroke patients and mice subjected to experimental stroke, and were found in close proximity to microglia. Although DNTs account for a small subset of mature peripheral T cells (1 to 5%), $Rag^{-/-}$ mice that received wildtype DNTs prior to the induction of stroke had exacerbated ischemic injury, suggesting they are an important contributor to inflammation after stroke [74]. The authors suggested that DNTs are critical for microglia-induced neuroinflammation after ischemic stroke. The concept of “crosstalk” between T cells and other immune cells, including microglia, in ischemic stroke is gaining attention, and both pro-inflammatory anti-inflammatory effects have been documented [75]. Pro-inflammatory microglia and T_H1/T_H17 T cells can promote immune responses that exacerbates brain injury after stroke, while interactions between anti-inflammatory microglia and T_H2/Tregs contribute to brain recovery [75].

Autoreactive T cell responses after stroke

Autoimmune responses can occur when T cells react to brain antigens, either within the brain itself or systemically, as the blood brain barrier becomes compromised after ischemic stroke allowing for lymphocyte entry and leakage of brain antigens from injured neurons and glia. Development of autoimmunity to brain antigens such as myelin basic protein and related peptides in stroke remains controversial in regards to whether it is beneficial or detrimental [16,65,76]. Brain-derived antigens are found in lymphoid tissues of stroke patients, as well as elevated levels of $CD69^+$ T cells in lymphoid tissues, indicative of T cell activation [77]. Interestingly, elevated immunoreactivity to neuronal-derived antigens was associated with smaller infarcts and better long-term outcome, whereas greater reactivity to myelin basic protein correlated to worse stroke severity (NIHSS at admission), larger infarcts and worse functional outcomes at 3 months in stroke patients [77]. Adoptive transfer of lymphocytes specific for myelin proteins in rats and mice worsens stroke outcomes indicating the presence of autoreactive T cells is merely not just a consequence of worse outcome [78,79]. Clonal T cell expansion can be detected in the mouse brain at day 7 and 14 after stroke, making it unlikely that early T cell responses after stroke is antigen-dependent [80,81]. Ortega et al. showed that autoimmune $CD4^+$ and $CD8^+$ T cells, as well as autoreactive $CD19^+$ B cells in spleen and cervical lymph nodes increase at 4 to 10 days post-stroke [82]. This highlights the importance of considering the time-course of immune cell

infiltration when distinguishing between antigen-independent and antigen-dependent mechanisms. Early deleterious effects of T cells in ischemic stroke are most likely antigen-independent. Whether brain antigen specific T cells are protective or not could also depend on the time-course of the concomitant activation of regulatory T cells. Work by Becker and others have demonstrated that increased tolerance to myelin basic protein characterized by a Treg responses improves outcomes after stroke in pre-clinical models [83–86].

Regulatory T cells in ischemic stroke

Tregs are a subpopulation of $CD4^+$ T cells characterized by nuclear expression of the transcription factor FoxP3. These cells are pro-homeostatic during healthy conditions and upon injury act to limit extensive inflammation. They act through direct interaction with other cells as well as by secreting IL-10 and transforming growth factor-β. In ischemic stroke, Tregs have been suggested to be major neuroprotective modulators through IL-10 signaling [25]. In these experiments, *in vivo* depletion of Tregs with a CD25-specific antibody administered 48 hours prior to stroke resulted in increased delayed brain injury at 7 days and worsened sensorimotor function. Treg depletion led to elevated production of IFN-γ in brain invading T cells at day 5 post-stroke [25]. In contrast, Kleinschnitz et al. reported reduced infarct size and improved neurological function 24 post-stroke when Tregs were selectively depleted before stroke using the DEREG mouse model [68]. A large number of Tregs accumulate in the brain by day 14 after stroke and continue to increase thereafter [87,88]. Post-stroke depletion of Tregs using DEREG mice and application of diphtheria toxin on day 7, 9 and 11 resulted in worsened neurological recovery without affecting the infarct volume on day 14 [87]. In this study Ito et al. suggested that Tregs potentiate neurological recovery by suppressing astrogliosis during the chronic phase of stroke. Overall, the majority of studies point to a neuroprotective role of Tregs in ischemic stroke.

Sex differences in T cell immune responses after ischemic stroke

While it has become evident that T cell responses are major determinants of acute and long-term stroke outcome in males, the contributions of age and sex to T cell-mediated inflammation after ischemic stroke remains mainly unknown. Similar to observations in human stroke patients, sex differences are apparent in experimental stroke [89]. Young female animals sustain smaller ischemic brain injury and have less neurological deficits than age-matched males, which is largely due to sex hormones, primarily estrogens, as ovariectomy (OVX) abolishes the sex differences and estrogen-replacement restores the female protection (reviewed in detail by Ahnstedt et al. [89]). Some of the protective effects of estrogens have been linked to its immunomodulatory actions; here we will focus on the effect of sex (hormonal and chromosomal) on T cell immune responses after ischemic stroke (see Table 2).

It has been established from work by Offner and Hurn that stroke induces a massive, rapid activation of the peripheral immune system [90]. Stroke leads to splenic atrophy characterized by a reduction in organ size and in the number of splenocytes. In OVX females, estradiol administration partly restored the drastic reduction in splenocytes after ischemic stroke [43]. *In vitro,* estradiol increased splenocyte proliferation upon stimulation

with CD3/CD28 designed to activate and expand T cells. Furthermore, estradiol normalized (decreased) the percentage of $CD4^+CD25^+FoxP^+$ Tregs in the spleen compared what was seen in OVX females, although the changes were minor [43].

Focusing on sex differences, female mice had significantly more splenocytes at 96 hours post-stroke than male mice, including higher levels of Tregs and IL-10 producing regulatory B cells paralleled with higher levels of anti-inflammatory microglia/macrophages ($CD11b^+$ $CD206^+$) in the ischemic hemisphere [91]. In male mice, adoptive transfer of these $IL\text{-}10^+$ B cells 24 hours post-stroke resulted in significantly smaller infarcts, less splenic atrophy, reduced number of activated T cells and decreased T cell infiltration to the brain at 96 hours [92]. Interestingly, adoptive transfer of $IL\text{-}10^+$ B cells increased another regulatory subpopulation of T cells, namely $CD8^+CD122^+$ suppressor cells, in the ischemic hemisphere [92]. These $CD8^+CD122^+$ T cells are naturally occurring Tregs, resembling a memory T cell phenotype with the potential to inhibit T cell responses mainly by IL-10 [93]. In stroke patients, higher serum IL-10 levels correlated with poor acute (24 hours) and long-term outcomes (3, 12 months) in women but not in men [94]. However, after controlling for cofounders, IL-10 was not an independent predictor of functional outcome suggesting IL-10 levels are related to other factors such as age and stroke severity.

Previously it has been shown that while there were no sex differences in the number of $CD8^+CD122^+$ suppressor cells, females had more suppressor cells that expressed IL-10 [95]. In parallel to larger splenic atrophy, males had higher levels of activated splenic T cells. However, brain injury is greater in young males compared to age-matched cycling females, making it difficult to tease apart baseline sex differences in peripheral immune responses to stroke, from sex differences merely as a consequence of the larger injury seen in males versus females. To this end, splenectomy two weeks prior to stroke was shown to be protective in young males and not in females, and abolished the sex differences in infarct size and stroke-induced peripheral/central immune responses [96]. These studies indicate that sex differences in the peripheral inflammation after stroke is a major contributor to male-female differences in ischemic brain injury. Recently it was shown that splenectomy also is protective in aged mice, however whether splenectomy is beneficial to aged female mice has not been investigated [97].

Besides IL-10, another important anti-inflammatory cytokine, IL-4, has been implicated in the neuroprotection seen in young female mice [98]. In support of previous studies, a smaller infarct size was seen in young females compared to males, in particular during the estrus and proestrus phases of the estrous cycle when estradiol levels are high [41]. Loss of IL-4 in IL-4 KO mice resulted in larger infarcts in males and females with no sex differences in either infarct size or neurological deficits. Wildtype female mice had fewer total number of T cells, including $CD4^+$ and $CD8^+$ T cells, in the ischemic hemisphere after stroke which was exacerbated in IL-4 deficient female mice. The data suggest that IL-4 is a key factor in sex-specific neuroprotection after stroke with effects on leukocyte/T cell infiltration to the brain.

Interestingly, data from Brait et al. suggest that sex differences after experimental stroke is dependent on reperfusion, and on Nox2 signaling [99]. In a model of ischemia and reperfusion achieved by the transient intraluminal middle cerebral artery occlusion model

(MCAO), the classical sex differences in infarct were observed; female mice had less brain injury. However, using a permanent model in which the filament remained in the internal carotid artery preventing reperfusion; no sex differences in infarct size were seen 24 hours post-stroke. Infarct development in males was dependent on Nox2, predominantly expressed on T cells. In the transient MCAO model, $CD3^+$ T cells in blood from male mice were capable of producing more superoxide upon stimulation, suggesting a possible underlying mechanism for the greater brain injury in young males versus young females [99]. It is apparent that studies of sex differences in T cell responses after stroke are very limited and the majority have addressed peripheral T cell responses rather than those in the brain. Our knowledge on T cell responses after stroke in aged animals is even more limited which is critical as sex and age interactions are well-known in stroke [89,100].

Effect of age and sex on T cell immune responses in stroke

Aging is associated with vast changes in the immune system. While young female mice sustain smaller infarcts than young males, aged animals of both sexes exhibit smaller infarcts after stroke yet suffer from high mortality and morbidity, a paradox that is also evident in humans [100–103]. Although stroke is a disease of the elderly, and despite the apparent sex differences, a limited number of studies on ischemic stroke have included aged animals of both sexes. An original paper by Manwani et al. characterized the differential effects of aging and sex on stroke-induced inflammation across the lifespan [100]. A significant sex by age interaction on histological brain damage was seen; neuroprotection was evident in young females versus age-matched males, while greater injury was seen in middle-aged female mice compared to middle-aged males (14-15 months). Aged male mice (18-20 months) had smaller infarcts than young males, but had significantly worse neurological deficits and mortality. In females, infarct size also varied over the lifespan, with small infarcts in young (due to the protective effects of estrogens), large infarcts in middle age, and small infarcts in aged. Old females, similar to what was seen in old males, had the poorest neurological outcomes and the highest mortality after stroke. Aging was associated with greater accumulation of T cells in the brain 24 hours post-stroke with no sex-specific effects. Aging resulted in decreased number of T cells in the spleens of male and female mice, supporting the overall view that aging is associated with a reduction of the naïve T cell pool in spleen and thymus [3].

Sex differences in T cell responses and risk factors for stroke

The immune system, including T cells, have also been implicated in the development and progression of common risk factors for stroke including hypertension and obesity [104–106]. Hypertension occurs earlier in men than in women and in the majority of experimental models of hypertension, arterial pressure is higher in males than in females [107]. T cells are important modulators of inflammation and are associated with hypertension as male $Rag^{-/-}$ mice deficient in T and B cells have reduced elevation of mean arterial pressure (MAP) in angiotensin II (Ang II)-induced hypertension. Primary work by Ji et al. showed that $Rag^{-/-}$ mice no longer exhibit the “female protected” sex difference in Ang II-induced hypertension [104]. Furthermore, adoptive transfer of male $CD3^+$ T cells into male $Rag^{-/-}$ mice resulted in a greater MAP response to Ang II compared to when the donor T cells were from female

mice. Interestingly, T cell infiltration into perivascular adipose tissue and kidney was greater if the donor T cells were from male mice versus female mice.

We investigated sex differences in a natural model of obesity-induced inflammation that occurs with aging, as obesity is an important risk factor for stroke and may be more detrimental in women [105]. Obesity has been shown to be up to ten times more common in women and is more strongly associated with increased risk of stroke compared to men [108,109]. The menopausal transition in middle-aged women correlates with increased body weight and abdominal fat, and a spike in stroke risk is seen in this age group [2,110,111]. We observed increased adipose tissue mass in middle-aged mice (15-16 months) of both sexes and age-associated increases in adipose cytotoxic $CD8^+$ T cells, and this was augmented in females [105]. In addition, a greater number of activated adipose $CD8^+CD69^+$ T cells were observed in females. Upon stimulation *ex vivo,* female $CD8^+$ T cells produced higher levels of IFN-γ, TNF-α and granzyme B. In parallel, females had lower levels of adipose Tregs than age-matched males. We proposed that this imbalance in pro/anti-inflammatory T cell milieu contribute to a “primed” pro-inflammatory environment in middle-aged females, and may underlie the greater brain injury upon ischemic stroke that is seen in middle-aged females [100].

Conclusions and future perspectives

While our knowledge of T cell immune responses after ischemic stroke is expanding in males, from this review it is clear that most pre-clinical studies do not include female subjects [89]. Work by Offner et al. has contributed to our understanding on peripheral immune responses after stroke, mainly in the spleen, but less is known about the brain. This is a critical aspect as the immune system and inflammatory responses both at baseline, in response to infections/vaccines, as well as in age-associated diseases such as Alzheimer’s disease differ significantly in males and females [6,7]. Female sex is an important variable in the development of therapeutics which is evident from pre-clinical and clinical trials including the minocycline and uric acid studies [14,15], where differential effects were seen in males and females. FDA mandated the inclusion of women in clinical trials in 1993 which significantly increased the inclusion of female subjects [10,11]. Age and sex interact to generate different ischemic phenotypes throughout life [100]. While men suffer from strokes earlier in life, middle-aged women, coinciding with sex-specific events such as pregnancy and menopause, and elderly women have an increased risk of stroke [1]. Since women are older at the time of their stroke, mortality and functional outcomes tend to be worse. Gonadal hormones and sex chromosomes also affect ischemic stroke outcome and inflammatory responses differently depending on age [5,89], an area that also have not been well studied. These sex and age-specific effects are likely introducing irreversible epigenetic modifications that continue to contribute to sex differences in immunity throughout the remaining lifespan. Similar to the FDA mandate of including female subjects in clinical trials, in 2016 NIH introduced their policy of factoring in sex as a biological variable in research studies and requires scientists to provide strong scientific justification if studies were to include only one sex. It is about time sex and age are considered important biological variables in ischemic stroke studies.

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Highlights

- Sex differences are well-documented in ischemic stroke
- T cell responses are important modifiers of ischemic stroke outcome
- Sex differences in T cell immunity and inflammation are well-known
- Ischemic stroke and inflammation increase in prevalence as we age
- Few studies include sex and age as variables in ischemic stroke studies

Table 1

Sex differences in T cell immunity

Adults	Effects of Aging
♂<♀ Auto-immune diseases [112]	↓ Naïve T cell pool, ♂>♀ [63,113,114]
♂<♀ Antigenic response to infection and vaccines [115]	↑ Senescent $CD8^+$ effector memory cells, ♂>♀ [64]
♂>♀ More susceptible to pathogens [116]	↓ Proliferative capacity of T cells, ♂>♀ [63]
♂<♀ Rise in short-lived effector $CD8^+$ T cells upon viral/bacterial infection [27]	↑ Individuals with inverted CD4/CD8 ratio, ♂>♀ [33]
♂>♀ Rise in memory precursor cells $CD8^+$ T cells upon viral/bacterial infection [27]	↓ TCR repertoire and ↑ susceptibility to infections, ♂>♀ [58]
♂>♀ Individuals with inverted CD4/CD8 ratio (immune risk profde) [33]	↑ Treg numbers and function, ♂>♀ [59–62]
♂<♀ Higher fraction of $CD4^+$ T cells [31,32]	

Table 2

Sex differences in T cell responses after ischemic stroke

Species	Age	Genetics	Insult and end-point	Findings	Refs.
Mouse	6-8w	WT (C57BL/6J), Nox2$^{-/-}$	tMCAO/pMCAO, 24 and 72 h	♂>♀ Infarct size and brain CD3$^+$ cells ♂>♀ Superoxide production by blood CD3$^+$ cells ♂=♀ Infarct size after pMCAO or in Nox2$^{-/-}$ mice.	[99]
Human	M: 66y (28-88), F: 65.5y (27-87)		Only IS, 1d, 1w, 3w	↑ Tregs after stroke, ♂>♀ ↓Suppressor capacity of effector cells, ♂<♀	[117]
Mouse	5-6m, 14-15m, 20-22m	WT (C57BL/6J)	MCAO, 24h	♂>♀ Infarct size in young ♂<♀ Infarct size in middle-aged ♂=♀ Infarct size in aged mice ↑T cells in brain and neurological deficits after stroke in aged mice, no sex differences ↓T cells in spleen with aging, no sex differences ♂>♀ Splenic atrophy after stroke in all age groups	[100]
Mouse	20-25g	WT (C57BL/6J)	MCAO,96h	♂>♀ Infarct size ♂>♀ Activated (CD62, CD44) CD4$^+$ T cells in spleen after IS ♂<♀ IL-10 secreting CD8$^+$CD122$^+$ suppressor cells in brain after IS ♂>♀ VLA4 expression on brain leukocytes after stroke (essential for leukocytes to access the brain)	[95]
Mouse	20-25g	WT (C57BL/6J)	MCAO, 96h	Splx ↓ infarct size and neuroinflammation in males but NOT in females ♂<♀ Tregs and CD8$^+$CD122$^+$IL-10$^+$ in intact mice	[96]
Human	M: 65, F:73		IS, 24h	↓IL-10 levels correlated with poor acute and long-term outcome in females but NOT in males IL-10 was not an independent predictor of outcome after correcting for confounders such as age and severity	[94]
Mouse	10-12w	WT (BALB/cJ), IL-4$^{-/-}$	MCAO, 48h	♂>♀ Infarct size (only when compared to females in proestrus/estrus) ♂>♀ Brain CD4$^+$ and CD8$^+$ T cells and macrophages IL-4 KO ➔ no sex differences	[98]
Mouse	8-10w	WT (C57BL/6J)	MCAO, 96h	♂>♀ Infarct size and reduction in splenocyte numbers ♂<♀ Spleen Tregs ♂>♀ IFNγ^+ splenocyte intensity	[91]

IS-Ischemic stroke, tMCAO-transient middle cerebral artery occlusion, pMCAO-permanent MCAO, Splx-splenectomy, w-weeks, m-months, y-years, WT-Wild type