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Research Paper

Sex differences in regulatory cells in experimental stroke

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ABSTRACT

Stroke is the leading cause of disability in the United States. Sex differences, including smaller infarcts in females and greater involvement of immune-mediated inflammation in males may affect the efficacy of immune-modulating interventions. To address these differences, we sought to identify distinct stroke-modifying mechanisms in female vs. male mice. The current study demonstrated smaller infarcts and increased levels of regulatory $CD19^+CD5^+CD1d^{hi}$ B10 cells as well as anti-inflammatory $CD11b^+CD206^+$ microglia/macrophages in the ipsilateral vs. contralateral hemisphere of female but not male mice undergoing 60 min middle cerebral artery occlusion followed by 96 h of reperfusion. Moreover, female mice with MCAO had increased total spleen cell numbers but lower B10 levels in spleens. These results elucidate differing sex-dependent regulatory mechanisms that account for diminished stroke severity in females and underscore the need to test immune-modulating therapies for stroke in both males and females.

1. Introduction

Stroke is the 5th leading cause of death and the leading cause of disability in the United States. An estimated 795,000 people will have a stroke each year resulting in nearly $33 billion each year in health care costs and missed work days [1]. It has become clear there are differences in stroke outcomes in men and women. Men have a significantly higher mortality from stroke compared to women in the age group of 45–74 years old. However, after age 85 the mortality from stroke significantly increases in women compared to men [2]. In addition to differences in stroke severity between the sexes it has also become apparent the immune system plays a pivotal role in increasing neural injury following stroke. Differences have also been observed in the immune responses in male and female rodents following experimental stroke induced by middle cerebral artery occlusion (MCAO).

One clear immunologic difference between male and female mice is that splenectomy two weeks prior to experimental stroke significantly decreases infarct volumes in male but not in female mice [3]. All prior studies used only male animals but consistently demonstrated significant neural protection for male animals that underwent splenectomy two weeks prior to permanent [4,5] or transient ischemia [6] and intracerebral hemorrhage (ICH) [7] and even traumatic brain injury (TBI) [8]. Inclusion of female mice in the same study showed that splenectomy reduced the number of circulating activated T cells and monocytes/macrophages in male but not in female mice. There was also a significant decrease in circulating regulatory T cells (Treg) in female mice that underwent splenectomy but not in male mice [3]. Additionally, splenectomy in male mice decreased the frequency of activated microglia/macrophages to levels not significantly different from spleen-intact female mice [3,9]. Splenectomy in male rodents decreased the levels of interferon gamma (IFNγ) in the brain after MCAO [5,6].

One immune cell type that has consistently been shown to be important in reducing infarct size in males is B cells, particularly interleukin 10 (IL-10) producing B cells. Male B cell knock-out mice, $\mu MT^{-/-}$, had increased infarct volumes compared to wild type male mice [10]. Treating $\mu MT^{-/-}$ male mice with IL-10 secreting B cells reduced infarct size [11] in contrast to IL-$10^{-/-}$ B cells that had no effect [12]. IL-10 secreting B cells (Breg) also reduced infarct size in wild type male mice [13]. While these studies were all done using only male mice several studies in experimental autoimmune encephalomyelitis (EAE) showed that IL-10 producing Breg cells were important, with estrogen treatment, in protecting female mice from disease progression. Regulatory B cells (Breg) are important immune modulators in neurological diseases like experimental autoimmune encephalomyelitis [14] through the secretion of IL-10. These cells provide protection that is partially

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mediated by estrogen. Female mice are protected from disease progression in EAE when treated with estrogen [15]. Female B cell knockout mice, $\mu MT^{-/-}$, are not protected from EAE progression even when treated with estrogen, suggesting B cells are important in protecting the CNS from EAE induced neural damage [16]. Restoring B cells in female $\mu MT^{-/-}$ mice with IL-10 producing B cells restored the protection from EAE with estrogen treatment to levels seen in wild type female mice [15]. B10 cells, a subset of Breg ($CD19^{+}CD5^{+}CD1d^{hi}$) are known to secrete IL-10 and modulate the function of T cells and monocytes [17–19].

This study sets out to address gaps in the current knowledge base on how the female immune system responds to ischemic stroke and how this response differs from the male immune response. The immune response to stroke has been documented in male animals with few studies comparing male and female animals. Females have smaller infarcts than males and this protection is thought to be largely mediated by sex hormones, but some of the protection seen in females could be due to an altered immune response to stroke. Some previous work has shown immune modulating therapies that are protective in male animals that are not protective in female animals. This study looks at the role of regulatory lymphocyte populations in the spleen and brain in males and females after experimental ischemic stroke, and how these regulatory populations could be modulating other immune cell functions in the spleen and brain after stroke.

2. Materials and methods

2.1. Ethics statement

The study was conducted in accordance with National Institutes of Health guidelines for the use of experimental animals, and the protocols were approved by the Portland Veteran Affairs Medical Center Institutional Animal Care and Use Committee, protocol #2840-12, and the Oregon Health and Science University Animal Care and Use Committee, protocol #IS00003885.

2.2. Animals

Male and female C57BL/6J mice, age 8–10 weeks, were purchased from The Jackson Laboratory (Sacramento, CA). Mice were given food and water *ad libitum* and kept on a 12 h light/dark cycle in climate controlled housing. Animals were cared for according to institutional guidelines in the animal resource facility at the Oregon Health and Science University, Portland, OR.

2.3. Middle cerebral artery occlusion Model

Transient focal ischemia was induced in male and female mice for 60 min by reversible middle cerebral artery occlusion (MCAO) under isoflurane anesthesia followed by 96 h of reperfusion, as previously described [3,13]. Briefly, mice were anesthetized with isoflurane, 5% induction and 2% maintenance, then the right common carotid artery (CCA) and external carotid artery (ECA) were exposed and the ECA was ligated. A 6-0 nylon monofilament (ETHICON, Inc., Somerville, NJ) was advanced into the ECA and up the internal carotid artery (ICA) to the base of the middle cerebral artery (MCA) occluding blood flow to the MCA territory. The filament was left in place for 60 min and then withdrawn to allow reperfusion of the MCA. After reperfusion the mice were survived for 96 h. To ensure adequate drops in blood flow during occlusion and increases in flow during reperfusion, cerebral blood flow was monitored by Laser Doppler Flowmetry (LDF) (Model DRT4, Moor Instruments Ltd., Wilmington, DE) with a probe fixed to the skull during the surgery. Mice were excluded from the study if the mean intra-ischemic LDF was greater than 30% of the pre-ischemic baseline LDF. No mice were excluded from the study. During the surgery mouse body and head temperature was maintained at 37 ± 1.0 °C with a warm water blanket and a heating lamp.

2.4. Infarct volume quantification

Brains were harvested for infarct volume quantification after 96 h of reperfusion. Brains were sectioned into five 2 mm thick coronal sections and stained with 1.2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, St. Louis, MO) for 15 min at 37 °C, as described previously (Hurn 2007). The sections were then fixed in 10% formalin for 24 h. Infarct was measured using digital imaging and image analysis software (Systat, Inc., Point Richmond, CA). Infarct volume was calculated for the cortex, striatum, and hemisphere by subtracting the non-infarcted regional volume of the ipsilateral side from the contralateral regional volume. This value was divided by the contralateral regional volume and multiplied by 100 to obtain a regional infarct volume as a percentage of the contralateral region to control for edema.

2.5. Leukocyte Isolation from brain and spleen

Spleens were processed into single cell suspensions by passing the spleen through a 100 μm nylon mesh (BD Falcon, Bedford, MA) into RPMI 1640. Red blood cells were lysed with 1x red cell lysis buffer (eBioscience, Inc., San Diego, CA). After being washed with RPMI 1640 the cells were counted on a Cellometer Auto T4 cell counter (Nexcelom, Lawrence, MA). Cells were centrifuged and resuspended in staining buffer (PBS with 0.1% NaN_3 and 1% bovine serum albumin) for staining. Brains were split into right (ipsilateral) and left (contralateral) hemispheres and placed in a solution of 1 mg/ml of Type IV collagenase (Sigma Aldrich, St. Louis, MO) and 50 mg/ml of DNase I (Roche Diagnostics, Indianapolis, IN) for 1 h at 37 °C with intermittent shaking. Cells were then washed in RPMI 1640 and centrifuged. Cells were resuspended in 80% Percoll, overlaid with 40% Percoll then centrifuged at 1600 rpm for 30 min. Cells were removed from the 80/40 interface and resuspended in RPMI 1640 to remove any remaining Percoll. Cells were resuspended in staining buffer for staining and counted on a hemocytometer.

2.6. Flow cytometry analysis

For flow cytometric analysis, cells were placed in staining buffer at a concentration of 1×10^6 cells/ml for splenocytes and 2×10^5 cell/ml for brain and blood cells. Cells were blocked with rat anti-mouse CD16/CD32 Mouse BD Fc Block™ (BD Bioscience, San Jose, CA) and then incubated, protected from light, with various combinations of fluorescently tagged antibodies. To assess cell survival, 7-aminoactinomycin D (7AAD) viability dye was used in some samples. For intracellular and FoxP3 staining, the samples were fixed with 4% paraformaldehyde and washed. FoxP3 staining was done using the fixation/permeabilization reagents per the manufacturer's instructions (eBioscience). For intracellular staining, cells were resuspended in 1× permeabilization buffer (BD Bioscience) and incubated with antibodies or isotype controls. The samples were then run on a BD Accuri™ C6 (BD Bioscience) under a four color (FITC, PE, PerCP/PECy5.5, and APC) fluorescence flow cytometry analysis.

The following antibodies were used: CD4 (GK1.5), PDL1 (M1H5), CD11b (M1/70), CD19 (1D3), CD1d (1B1), CD23 (B3B4), TIM1 (RMT1-4), CD138 (281-2), CD25 (PC61), CD86 (GL1), CD21 (7G6), IA/IE (2G9), TNFα (18139A), NOS2 (CXNFT), CD206 (CO68C2) (BD Biosciences), CD44 (1M7), FoxP3 (FJK-16s), IRF4 (3E4), IFNγ (XMG1.2) (eBiosciece), CD9 (MZ3), CD5 (53-7.3) (Biolegend), and PE-ARG1 (R & D Systems, Minneapolis, MN).

2.7. Statistics

All analyses were performed blinded. Data were analyzed using Prism (GraphPad Software, La Jolla, CA) using the Student's *t*-test with