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## Sexual dimorphism in the inflammatory response to traumatic brain injury

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### Abstract

The activation of resident microglial cells, alongside the infiltration of peripheral macrophages, are key neuroinflammatory responses to traumatic brain injury (TBI) that are directly associated with neuronal death. Sexual disparities in response to TBI have been previously reported, however it is unclear whether a sex difference exists in neuroinflammatory progression after TBI. We exposed male and female mice to moderate-to-severe controlled cortical impact injury and studied glial cell activation in the acute and chronic stages of TBI using immunofluorescence and *in situ* hybridization analysis. We found that the sex response was completely divergent up to 7 days post-injury. TBI caused a rapid and pronounced cortical microglia/macrophage activation in male mice with a prominent activated phenotype that produced both pro- (IL-1 $\beta$  and TNF $\alpha$ ) and anti-inflammatory (Arg1 and TGF $\beta$ ) cytokines with a single-phase, sustained peak from 1 to 7 days. In contrast, TBI caused a less robust microglia/macrophage phenotype in females with biphasic pro-inflammatory response peaks at 4 hours and 7 days, and a delayed anti-inflammatory mRNA peak at 30 days. We further report that female mice were protected against acute cell loss after TBI, with male mice demonstrating enhanced astrogliosis, neuronal death, and increased lesion volume through 7 days post-TBI. Collectively, these findings indicate that TBI leads to a more aggressive neuroinflammatory profile in male compared to female mice during the acute and sub-acute phases post-injury. Understanding how sex affects the course of neuroinflammation following brain injury is a vital step toward developing personalized and effective treatments for TBI.

### Keywords

trauma; inflammation; microglia; sex-differences; and cytokine

### INTRODUCTION

The activation of resident microglial cells, alongside the infiltration of peripheral macrophages, are key neuroinflammatory responses to traumatic brain injury (TBI). These inflammatory responses are a classic component of the secondary injury cascade and are

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associated with neuronal death (Corps et al. 2015). This neuroinflammation begins in the acute phase but can last for years post-injury in both humans and animals (Johnson et al. 2013; Kumar et al. 2013; Rice et al. 2017; Smith et al. 2013). Microglial/macrophages (MG/M $\phi$ ) are the main players in propagating inflammation to tissues neighboring the core site of injury. They perform this task through the production of inflammatory mediators including cytokines and chemokines. The activation of MG/M $\phi$  can be observed experimentally by examining time specific changes in morphology, and the production profiles of pro- and anti-inflammatory cytokines, nitric oxide, prostaglandins, or chemokines (Harry and Kraft 2008; Kettenmann et al. 2011; Ransohoff and Perry 2009).

Microglia are resident immune cells in the brain that are activated in response to injury (Saijo and Glass 2011). CNS injury also induces macrophage recruitment from the periphery, whose role is to rapidly access the injury site as a first line of defense against infection (Corps et al. 2015; Mishra et al. 2016). Comprehensively differentiating these two cell populations is difficult, but they do differ in their morphological and phenotypical classification. Typically, activated microglial cells appear hypertrophic/bushy with robust prolongations, while peripheral macrophages display an amoeboid morphology containing numerous vacuoles (Kettenmann et al. 2011). Depending on the grade of activation after brain injury, the MG/M $\phi$  population can exhibit different functions and distinct metabolic stages. Studies have shown a “pro-inflammatory” MG/M $\phi$  phenotype associated with the production of cytokines such as interleukin 1 beta (IL-1 $\beta$ ), tumor necrosis factor alpha (TNF $\alpha$ ), interferon gamma (IFN $\gamma$ ), and cluster of differentiation 40 (CD40) receptors; an “alternative activation” phenotype associated to the production of Arginase-1 (Arg1), interleukins 4 and 13 (IL-4, IL-13); and a “deactivated” phenotype associated with interleukin 10 (IL-10) or transforming growth factor beta 1 (TGF $\beta$ ) which facilitate wound healing (Hsieh et al. 2013; Jin et al. 2012; Kumar et al. 2016a; Kumar et al. 2016b; Martinez and Gordon 2014; Tang and Le 2016). While a coordinated transition from a pro-inflammatory phenotype to an alternative or deactivated phenotype is hypothesized to facilitate the transition from acute brain injury response to neuron repair, emerging studies suggest that the switch between these stages has not been adequately established, and that cytokines classically defined as pro-inflammatory can also have anti-inflammatory properties, and vice versa (Durafourt et al. 2012; Martinez and Gordon 2014; Orihuela et al. 2016; Selenica et al. 2013; Tang and Le 2016). Indeed, following experimental TBI there is an overlapping expression of phenotypic markers in MG/M $\phi$  (Kim et al. 2016; Kumar et al. 2016a; Morganti et al. 2016).

A recent review highlights the sparse and controversial background literature on sex-differences in mortality and functional outcome after TBI (Caplan et al. 2017), and it is clear that more studies are required to understand how sex affects neuroinflammatory mechanisms and outcomes after injury. However, the vast majority of the experimental preclinical TBI studies looking at the inflammatory response to TBI have been conducted using male animals. While it is known that females respond different to brain injury due to hormonal changes, the specific mechanisms underlying these sex differences remains elusive. Due to these challenges, the National Institutes of Health (NIH) are encouraging sexual diversity in pre-clinical research (McCullough et al. 2014). As far we are aware, this is the first study demonstrating the temporal and spatial differences in MG/M $\phi$  activation following TBI, with

a focus on morphological changes and cytokine mRNA production. Here, we report that the male/female MG/M $\phi$  response shows complete divergence in the sub-acute phase post-injury with male mice displaying faster and more pronounced MG/M $\phi$  activation and astrogliosis following moderate-to-severe TBI compared to female mice. *In situ* hybridization also reveals a divergent cytokine mRNA profile in MG/M $\phi$  cells after injury. This is associated with early neuronal cell death and larger lesion volume in the first days post-injury in male compared to female mice. Our data suggest that pharmacological interventions that target the inflammatory cascade should consider sex differences as a significant variable following TBI.

## MATERIALS AND METHODS

### Animals and Controlled Cortical impact injury

We used young adult (9–14 weeks) male and female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME), housed under standard 12h light:dark cycle, 5 mice per cage, with open access to food and water. We used random cycling of female mice to better reflect clinical applicability. We housed 5 females per cage, isolated from male mice. To neutralize weight as a variable, we matched male and female mice for each group by weight. The average body weight at the time of the injury was  $20.84 \pm 0.33$  g for males and  $20.28 \pm 0.20$  g for females. Mice were also weighed at time of euthanasia, and there were no significant differences between males or females at all time-points (data not shown).

Mice were anesthetized with isoflurane (induction at 3% and maintained at 1.5%) evaporated in oxygen and administered through a nose mask. The mice were placed on a custom-made stereotaxic frame with a built-in heating bed that maintains body temperature at 37°C. A 10-mm midline incision was made over the skull to allow a 4 mm craniotomy on the left parietal bone. We performed moderate-to-severe controlled cortical impact (CCI) injury in the right somatosensory cortex target area (interaural 2 mm and bregma 2 mm) using a Leica Impact One Stereotaxic Impactor device (Leica Biosystems) to deliver the cortical impact, with a 3 mm diameter flat impact tip, impact velocity of 5.25 m/sec, impact depth of 1.5 mm, and dwell time of 0.1 sec. After injury, the incision was closed with staples and the mice were allowed to recover fully from anesthesia before transferring to their home cages. Sham injury consisted of exposure to anesthesia; stereotaxic mounting, skin and fascia reflection and incision closed using staples. All procedures were performed in accordance with protocols approved by the Georgetown University Animal Care and Use Committee. Mice were euthanized with CO<sub>2</sub> and transcardially perfused with phosphate buffer and decapitated at 4 hours (h), 1, 3, 7 and 30 days post-injury (dpi). After post-fixation of the brains in 4% paraformaldehyde overnight, brains were stored in a 30% sucrose solution for 48 h at 4°C. A sliding microtome (Microm HM 430, Thermo Fisher Scientific, Tusin, CA) was used to section the brains at 20  $\mu$ m-thick in coronal orientation. The brain sections were then cryoprotected in an antifreeze solution (30% glycerol + 30% ethylene glycol + 40% 0.01 M PBS) for storage at – 20°C.

## Immunohistochemical techniques

Free-floating parallel sections were washed three times with phosphate-buffered saline (PBS), before staining and incubated with blocking buffer (5% normal goat serum in PBS) and 0.3% Triton X-100 (PBST) for 2 h at room temperature. For the histological analysis of glial activation, brain sections were incubated overnight at 4°C with either one of the following primary antibodies: 1) rabbit polyclonal anti-Iba-1 antibody (1:500, Wako, Chemicals, Richmond, VA) for microglia/macrophages cells and 2) mouse monoclonal anti-GFAP antibody (1:1000, Millipore, Temecula, CA) for astrocytes. After three 5 min washes in PBS, sections were incubated with a corresponding anti-rabbit or anti-mouse Alexa Fluor 568-conjugated IgG secondary antibodies (1:1000, Invitrogen, Carlsbad, CA) for 2 h at room temperature. For double labeling, sections were processed with polyclonal anti-rabbit P2Y12 (1:1000, Anaspec, San Jose, CA) and polyclonal anti-rat F4/80 (1:200, R&D Systems, Minneapolis, MN) overnight at 4°C. As negative controls, sections were incubated in media lacking the primary antibody. After three 5 min washes in PBS, sections were incubated with a corresponding anti-rabbit Alexa Fluor 568-conjugated IgG and anti-rat Alexa Fluor 488-conjugated IgG secondary antibodies (1:1000, Invitrogen, Carlsbad, CA) for 2 h at room temperature. Sections were rinsed with PBS three times and incubated in PBS with DAPI solution (1:50,000) for counterstained nuclei. The sections were rinsed with distilled water and coverslipped with Fluoro-Gel with Tris Buffer mounting medium (Electron Microscopy Sciences, Hatfield, PA).

## Densitometry and quantitative analysis of immunohistochemistry

For quantitative analysis of immunolabeled sections, we implemented unbiased standardized sampling techniques to measure tissue areas corresponding to the cortex, hippocampal dentate gyrus, and thalamus showing positive immunoreactivity (Villapol et al. 2015; Villapol et al. 2014) (Fig. 1). To quantify the number of Iba-1 positive cells, an average of five single plane sections from the lesion epicenter (−1.34 to −2.30 mm from bregma) were analyzed blind for each animal for each brain region. Within each brain region, every Iba-1-positive cell in each of 5 (cortex), 2 (dentate gyrus) or 3 (dentate gyrus and thalamus) fields (x20, 151.894 mm<sup>2</sup>) around the impact area (n=5–6 mice/group at each time-point) (Fig. 1B). Data is presented as the mean number of cells per mm<sup>2</sup>. For proportional area measurements, the magnitude of the individual reaction for microglial and astroglial cells was reported as the proportional area of tissue occupied by immunohistochemical stained cellular profiles within a defined target area. Proportional area measurements do not necessarily reflect changes in actual cell numbers. Fluorescent images were acquired on a Axioplan 2 microscope (Zeiss, Thorwood, NY) with a Photometrics camera. Images were transferred to ImageJ64 software (National Institutes of Health, Bethesda, MD, USA), for inversion and thresholding, and densitometric analysis was performed as previously described (Villapol et al. 2015; Villapol et al. 2014). The thresholding function is used to set a black and white threshold corresponding to the imaged field, with the averaged background subtracted out. Once a threshold is set, the “Analyze Particles” function can be used to sum up the total area of positive staining, and to calculate the fraction of the total area that is positive for the stain as previously was described (Villapol et al. 2015; Villapol et al. 2014). Data are shown as the percentage of Iba-1 or GFAP positive immunoreactivity per the total area that occupied on the field studied.

### Microglia and astrocyte morphologic assessment

Under physiological conditions, microglia exist in the “surveying” stage. In response to TBI, ramified microglia transform into activated non-phagocytic cells commonly referred to as “activated” microglia (Nimmerjahn et al. 2005). To characterize the morphology of Iba-1 positive cells, we counted and classified the number of cortical microglial cells in each of the five MG/M $\phi$  morphologic phenotypes (Ayoub and Salm 2003; Doyle et al. 2017; Glenn et al. 1992; Kettenmann et al. 2011) in sham brains and injured brains at 4 h, 1, 3, 7 and 30 days after TBI. Morphological classification was based on a visual assessment of the MG/M $\phi$  population. We used the following classifications 1) *ramified* (small cell body and long finely branched processes that extend in all directions from the perinuclear cytoplasm); 2) *ramified with rough prolongations* (first stage of microglia activation, phagocytic abilities); 3) *hypertrophy/bushy microglia with pseudopodia* (activated microglia demonstrate modest hypertrophy, and have limited, cytoplasmic extensions); 4) *amoeboid with phagocytosed cells* (macrophages or phagocytic microglia with fully retracted processes and clearly evident phagocytized debris); 5) *amoeboid round* (macrophages or microglial-derived brain macrophages round in shape). An investigator that was blinded to the identity of the experimental groups performed morphometric analysis (n=5–6 mice/group at each time-point).

### Fluorescent *In Situ* Hybridization combined with immunohistochemical labeling for microglia/macrophages population

Coronal brain sections were mounted on gelatin-coated glass slides (Superfrost Plus, Thermo Fisher Scientific) and stored at – 80°C until use. Fluorescent in situ hybridization (FISH) was performed as per the manufacturer’s instructions using RNAscope® Technology 2.0 Red Fluorescent kit for Fresh Frozen Tissue (Advanced Cell Diagnostics, Inc. (ACD), Hayward, CA). All target probes consisted of 20 short double-Z oligonucleotide probe pairs that are gene specific and were obtained from ACD. For amplification and visualization, both Z-probes must bind to the mRNA of interest. Following probe hybridization, sections underwent a series of probe signal amplification steps followed by incubation of fluorescently labeled probes designed to target the Red channel associated (Fig. 1H). Brain tissue sections were dehydrated by 50%, 70%, and 100% ethanol gradually for 5 min; boiled during 10 min with pretreatment 2 solution (citrate buffer), then they were incubated with pretreatment 3 solution (protease buffer) for 30 min before hybridization. Sections were then incubated at 40°C for 2 h with the following target probes for mouse: *Mus musculus interleukin 1 beta* (IL-1 $\beta$ ) mRNA, (accession number NM\_008361.3, target region 2–950); *Mus musculus Tumor necrosis factor alpha* (TNF $\alpha$ ) mRNA, (accession number NM\_013693.2, target region 41–1587); *Mus musculus transforming growth factor, beta 1* (TGF $\beta$ 1) mRNA, (accession number NM\_011577.1, target region 588–1913); *Mus musculus arginase* (Arg1) mRNA, (accession number NM\_007482.3, target region 2–1114). A dapB probe targeting a bacterial gene was used as a negative control and Ppib (*Mus musculus* peptidylprolyl isomerase B mRNA; accession number NM\_011149.2, target region 98–856) was used as a positive control. Positive hybridization consisted of a punctate signal representing a single mRNA target molecule, the color label was assigned to either FAR RED (Excitation 647 nm; Emission 690  $\pm$  10 nm). After FISH, slides were washed three times with PBS and blocking with PBST and 5% normal goat serum for 1 h.

Immunofluorescence was performed using a mixture of primary antibodies to visualize the total MG/M $\phi$  population; polyclonal anti-rabbit Iba-1 (1:500), polyclonal anti-rabbit P2Y12 (1:1000) and polyclonal anti-rat F4/80 (1:200) overnight at 4°C. Alexa Fluor 488-conjugated goat anti-rabbit, and anti-rat IgG (1:1000, Invitrogen, Carlsbad, CA) were applied for 1 h at room temperature. Sections were rinsed with PBS three times and incubated in PBS with DAPI solution (1:50,000) for counterstained nuclei. The sections were washed three times with distilled water and coverslipped with Fluoro-Gel with Tris Buffer mounting medium (Electron Microscopy Sciences).

### Quantification of immunofluorescence and FISH

Investigators blinded to the experimental conditions quantified FISH signal in three fields ( $\times 10$ , 514.560 mm<sup>2</sup>) within the primary somatosensory cortex of 4 non-contiguous slices of the ipsilateral cortex (or cortical layer III–IV for sham mice) in a range of bregma –1.34 to –2.30 mm (Fig. 1A). The number of mRNA-positive cells that colocalized with DAPI nuclei were manually counted for IL-1 $\beta$ , TNF $\alpha$ , TGF $\beta$ , and Arg1. The negative probe used as a control did not contain any stained cells. The number of mRNA-positive/MG/M $\phi$ -positive cells, mRNA-positive/MG/M $\phi$ -negative cells, and the total number of MG/M $\phi$  cells were also quantified in four to six microscopic fields ( $\times 20$ , 151.894 mm<sup>2</sup>) of view. A total of 15–20 counting frames were assessed per animal, and cells were evaluated for the presence of a labeled nucleus and expected cellular morphology as was previously described (Villapol et al. 2013). In addition, the percentage of MG/M $\phi$  immunoreactive cells with different mRNA probes was also assessed (n=5–6 mice/group at each time-point). The images were analyzed using AxionVision software and quantified using ImageJ64 software. Images were cropped and resized (if necessary) using Adobe Photoshop CS5, any changes to brightness and contrast settings were applied equally to the entire image, and to all corresponding images within the same dataset. A confocal microscope (Leica SP8) was used to take representative images from FISH-ISH brain sections using  $\times 20$ ,  $\times 40$  and  $\times 63$  objectives.

### Cell Death Assay

Brain sections were processed for Terminal deoxynucleotidyl transferase-mediated biotinylated-dUTP nick-end labeling (TUNEL) staining was performed by using the *In situ* Cell Death Detection kit, Fluorescein (Roche, IL), according to the manufacturer's instructions. Coronal brain sections were mounted on gelatin-coated glass slides (Superfrost Plus). Briefly, cryosections were rinsed with PBS and were incubated with kit-supplied TUNEL reaction mixture at 37°C for 1 h. For double-staining, cryosections were incubated with primary antibody against anti-Neurofilament 200 (NF200) (1:200, Abcam) overnight at 4°C, Alexa Fluor 568-conjugated goat anti-mouse IgG (1:1000, Invitrogen, Carlsbad, CA) were applied for 1 h at room temperature. Sections were rinsed with PBS three times and incubated in PBS with DAPI solution for counterstained nuclei. The sections were washed three times with distilled water and coverslipped with Fluoro-Gel with Tris Buffer mounting medium (Electron Microscopy Sciences). TUNEL-positive nuclei were counted in cortical regions ( $\times 20$ ) in the three to five coronal sections for each animal (n=5–6 mice/group at each time-point).



## Histological Nissl stain and lesion volume measurements

Nissl staining was assessed on an average of 13 brain sections spaced equidistant apart, between 0 to – 2.70 mm from bregma corresponding to the injured area. Brain slices were mounted on gelatin-coated glass slides (Superfrost Plus) and stained for 5 min with 1% Cresyl-violet (Sigma-Aldrich, St. Louis, MO) dissolved in distilled water and filtered. Slides stained were dehydrated for 2 min using 100, 95, 70 and 50% ethanol, cleared in xylene for 2 min, covered with Permount mounting medium (Thermo Fisher Scientific) and coverslipped. The lesion volume was calculated by subtracting the ipsilateral hemisphere area from the contralateral hemisphere area, and multiplying by the distance between sections. Lesion percentage was calculated by dividing the ipsilateral hemisphere by the contralateral hemisphere (Villapol et al. 2012).

## Statistical analyses

Data were analyzed using two-way analysis of variance (ANOVA) with Bonferroni post-hoc test using GraphPad Prism software v. 5.0 (Graphpad). A p-value < 0.05 was considered statistically significant. Quantitative data for all figures and tables are expressed as mean  $\pm$  S.E.M.

## RESULTS

### Sex dimorphism in MG/M $\phi$ activation after TBI

Previous reports have consistently demonstrated a role for microglial activation in the pathophysiology of TBI, however, the impact of sex differences on this response is unknown. Using immunohistochemistry, we detected Iba-1 positive cells and determined their number, area occupied and morphology in several injured brain regions of adult male and female mice at several time-points after TBI. We report that the total Iba-1-positive area was significantly higher in male mice in the primary somatosensory cortex (306.2 and 172.90% increase in male vs. female mice at 1 and 3 dpi respectively;  $p < 0.001$ ; Fig. 2A–C), in the dentate gyrus (240.4, 225.3 and 129.8% increase in male vs. female mice at 1, 3 and 7 dpi respectively;  $p < 0.001$ ; Fig. 2D–F), and in the thalamus (158.4 and 149.4% increase in male vs. female mice at 1 and 3 dpi respectively;  $p < 0.05$ ; Fig. 2G–I). For quantitative analysis, we focused on the primary somatosensory cortex region, located immediately adjacent to the injury site (Fig. 1B). We found that TBI in male mice caused a rapid increase in the number of Iba-1 cells beginning at 1 dpi, but female mice did not increase Iba-1 cells until 7 dpi – suggesting decreased MG/M $\phi$  infiltration/proliferation at the acute phase post-trauma (Fig. 2J). Male and female mice had a similar number of MG/M $\phi$  cells at 30 dpi in all brain regions. Next, we determined the morphological status of the MG/M $\phi$  into a) ramified, b) ramified with thick, rough prolongations, c) hypertrophic/bushy with pseudopodia, d) amoeboid with phagocytosed cells, or d) amoeboid round cells (Fig. 2K–O). Following sham-injury, 100% of the MG/M $\phi$  were classified as ramified, displaying small cell bodies with thin, long, and highly branched processes. In both sexes, TBI caused a time-dependent change in MG/M $\phi$  morphology with the number of ramified microglia in the primary somatosensory cortex being completely abolished by 3 dpi (Fig. 2K). Female mice had higher numbers of ramified MG/M $\phi$  with thick rough prolongations at 4 h and 1 dpi ( $p < 0.01$ ; Fig. 2L). At 1 and 3 dpi, male mice had significantly higher numbers of hypertrophy/

bushy MG/M $\phi$  with pseudopodia ( $p < 0.001$ ; Fig. 2M), and significantly higher amoeboid MG/M $\phi$  with phagocytosed cells at 7 dpi ( $p < 0.05$ ; Fig. 2N), and significantly higher amoeboid round MG/M $\phi$  ( $p < 0.01$ ; Fig. 2O).

### **Rapid activation and infiltration of MG/M $\phi$ to the injured cortex occurs in male, but not female mice**

TBI induces the rapid infiltration of blood-derived peripheral immune cells into the brain. We used a combination of membrane-specific markers and morphological changes to determine if the Iba-1-labeled cells with large phagocytic vacuoles/vesicles could be infiltrating cells. F4/80 is a well-characterized membrane protein highly expressed on macrophages, and F4/80-positive macrophages have been shown to rapidly infiltrate the injured cortex following TBI (Hsieh et al. 2013; Kumar et al. 2016a; Morganti et al. 2015). To discern the total MG/M $\phi$  population, we also used the P2Y12 maker, a known membrane marker that distinguishes microglia from other myeloid lineage cells; its expression is limited to the ramified processes of microglia (Moore et al. 2015), however, P2Y12 was also identified in activated microglia (Ohsawa et al. 2010; Ohsawa and Kohsaka 2011). Sham-injured male and female brains both had P2Y12-positive microglia, with no evidence of F4/80-positive cells in the somatosensory cortex (Fig. 3A–A2, B–B2, K). Within 4 h post-injury, F4/80 positive macrophages remained rare (Fig. 3C–D) in the injured cortex; however, changes in the morphology of P2Y12 cells were evident (Fig. 3C–C2, D–D2, K). Male mice displayed a significant increase in F4/80-positive cells at 1 and 3 dpi that did not occur in female mice ( $p < 0.01$ ; Fig. 3E–H, K). Infiltrating macrophages were evident in female mice at 7 dpi and were not significantly different to F4/80 cell number in male mice at this time-point (Fig. 3I–J, K). By 30 dpi there were no F4/80 cells in either male or female mice (data not shown). We also performed immunofluorescence to detect CD68, a marker of highly activated and phagocytic myeloid cells that include both microglia and macrophages (Fig. 3L–N). We again found that male mice had significantly higher numbers of CD68-positive cells at 1, 3 and 7 dpi ( $p < 0.001$ ; Fig. 3L) compared females (Fig. 3L–N).

### **Sex differences in pro-inflammatory cytokine expression after TBI**

RNAscope FISH in combination with immunofluorescence was performed on brain sections collected from male and female mice. Females had significantly higher levels of IL-1 $\beta$  cytokines at 4 h post-TBI, compared to males (Fig. 4A), suggesting that the IL-1 $\beta$  expression in response to injury within the male and female brain may be functionally, or biochemically, distinct. There was no detectable IL-1 $\beta$  expression in sham animals, while approximately 50% of cells expressing IL-1 $\beta$  were detected in MG/M $\phi$  (see plots in Fig. 4B). Collectively, these data show that IL-1 $\beta$  mRNA expression within the male and female brains is not only produced by the MG/M $\phi$  population, but also by other glial cell types or neurons (Choi and Friedman 2009; Srinivasan et al. 2004). Additionally, mRNA IL-1 $\beta$  expression intensity was sex-specific in IL-1 $\beta$  positive cells, within high levels in MG/M $\phi$  of females at 4 h compared to males, and an inverse mRNA IL-1 $\beta$  MG/M $\phi$  expression profile at 1 and 3 dpi (Fig. 4C). In accordance with this data, females had increased mRNA TNF $\alpha$  expression compared to males at 4 h post-TBI, whereas males had significantly increased mRNA TNF $\alpha$  expression at 3 dpi compared to females (Fig. 4L). There was no detectable TNF $\alpha$  expression in sham-operated animals. Approximately 70% of TNF $\alpha$  positive cells



colocalized with MG/M $\phi$  (see plots in Fig. 4M). Additionally, we determined the collective intensity levels of mRNA TNF $\alpha$  expression showed no sex discrepancies. MG/M $\phi$  localization and TNF $\alpha$  positive cell distribution in the ipsilateral cortex cells in males and females is shown in Fig. 4(O, P, S, T, t1–t3) and (Q, R, U, V, v1–v3), respectively. By 7 dpi, male and female TBI mice had similar expression profiles of both pro-inflammatory IL-1 $\beta$  and TNF $\alpha$  mRNAs (Fig. 4A, L).

### **Anti-inflammatory cytokines are predominantly expressed in males after brain injury**

The expression profiles of TGF $\beta$ , a marker of immunoregulatory cells, and Arg1, a marker of alternatively activated cells, were analyzed in the cortex of males and female mice. Following TBI we observed an increase in the production of TGF $\beta$  in males, but not in females, at 1 dpi compared to sham mice. Peak expression of TGF $\beta$  was observed at 7 dpi, but no sex-dependent differences were observed (Fig. 5A). Approximately 80% of TGF $\beta$  positive cells colocalized with MG/M $\phi$  (Fig. 5M). These results demonstrated that a high percentage of MG/M $\phi$  cells express TGF $\beta$  and that the remainder of cells correspond to astrocytes and/or vessels (data not shown). Furthermore, TGF $\beta$  expression levels demonstrate a strong correlation in the pattern of TGF $\beta$  expression per mm<sup>2</sup> in both male and female animals. An increase in the Arg1 levels was found only in the ipsilateral cortex of male mice at 3 dpi, continuing up to 7 dpi. Female mice did not show similarly robust increased expression levels (Fig. 5L). In addition, the observed sex difference was not found in sham-injured mice. Approximately 70–90% of Arg1 positive cells were detected in MG/M $\phi$ , across all time-points (Fig. 5M). With respect to Arg1 expression, mRNA Arg1 was predominantly produced by MG/M $\phi$  cells that displayed an amoeboid morphology (Fig. 5t1–t3). Furthermore, mRNA Arg1 expression levels were significantly higher in male compared to female mice at 3 and 7 dpi ( $p < 0.001$ ; Fig. 5N), respectively.

### **Cell death is time and sex dependent following TBI**

We examined TBI-induced neuronal cell death in the pericontusional cortex, adjacent to the impact. At 4 h after injury, we observed 38% more TUNEL positive cells in female compared with male mice ( $p < 0.001$ ; Fig. 6A). There were no sex-dependent differences in TUNEL cell number at 1 dpi, however, at 3 dpi, there was a 358% increase in TUNEL positive neurons in males compared to females in the pericontusional cortical regions ( $p < 0.05$ ; Fig. 6A–C). This translated into significantly larger infarct area in male mice compared to female mice at 3 dpi (Fig. 6D, E). Overall, lesion was 64% larger in male mice at 3 dpi ( $P < 0.001$ ) and 36% larger at 7 dpi ( $P < 0.01$ ) compared to female mice (Fig. 6F, G). Lesion volume at 30 dpi was similar between male and female mice (Fig. 6F, G).

### **Acute astrogliosis occurs in male, but not female, mice after TBI**

We used GFAP immunohistochemistry to assess the temporal and spatial distribution of astrogliosis in males and female brains following TBI. We found that the immunoreactive area occupied by GFAP positive astrocytes was higher in males compared to females at 1 and 7 dpi in the ipsilateral cortex (833 and 196% respectively) (Fig. 7A–C), at 1 and 3 dpi in the ipsilateral hippocampal dentate gyrus (560 and 163% respectively) (Fig. 7D–F), and in the ipsilateral thalamus at 1, 3 and 7 dpi (297, 629 and 140% respectively) (Fig. 7G–I). For

all areas studied, astrogliosis was not significantly different between male and female mice at 30 dpi.

## DISCUSSION

There are limited studies on the effect of sex on inflammation after CNS injury, and in this study, we examine the spatial and time-dependent neuroinflammatory changes that occur after experimental TBI. We report that after brain injury, male mice have a faster and more robust MG/M $\phi$  activation and peripheral macrophage recruitment compared to females (Fig. 8). This leads to increased cell death and lesion volume in the first week post-injury. However, beyond the first week post-injury, the female inflammatory response accelerates to become indistinguishable from that of males. While we can detect sex-differences in lesion volume acutely after injury, we observe identical brain tissue loss at 1-month post-injury. Previous clinical and animal studies have demonstrated that sex-differences in mortality and functional outcomes exist after TBI (Caplan et al. 2017), and the use of hormone-based therapies in preclinical studies clearly show that female sex hormones, estrogen, and progesterone, are protective after TBI (Engler-Chiurazzi et al. 2016; Stein 2015). One of the major targets of estrogen and progesterone is the inflammatory response, and *in vitro* and *in vivo* studies demonstrate that these hormones can attenuate microglial activation and pro-inflammatory cytokine release (Arevalo et al. 2013; Belcredito et al. 2001; Bruce-Keller et al. 2007; Bruce-Keller et al. 2000; Crain et al. 2013; Dimayuga et al. 2005; Goodman et al. 1996; Habib and Beyer 2015; Sawada et al. 2000; Vegeto et al. 2001). Here we studied how sex altered the inflammatory response following moderate-to-severe TBI with a focus on MG/M $\phi$  number, morphology, and cytokine mRNA profile.

Microglia are dynamic immune cells whose number and morphological changes are closely associated with their functional activity (Ohsawa and Kohsaka 2011; Stence et al. 2001; Tam and Ma 2014). We found that TBI caused a rapid increase in the total number of Iba-1 immunopositive cells in male mice that was significantly faster than the response in female mice. These cells were also much larger on average, with male Iba-1 immunopositive cells having a larger area than female cells through 7 dpi. These data are in agreement with previous results studying stab injury, where an injury induced a greater density of Iba-1 cells in the lesion border in male mice at 7d post-TBI compared to female mice (Acaz-Fonseca et al. 2015).

Morphologically, microglia in the un-injured brain have numerous, thin, ramified processes that survey the local tissue microenvironment, and following acute CNS insult microglia rapidly transform into an activated phenotype with distinct hypertrophic or bushy morphologies (Faden et al. 2016; Jin et al. 2012; Schnell et al. 1999). We observed sexual dimorphism in the morphological changes that occurred after TBI, with hypertrophic/amoeboid/phagocytic MG/M $\phi$  being more predominant in males compared to females. Morphological changes in MG/M $\phi$  phenotypes could be a determinant factor in activation of the inflammatory cascade, involving the expression of a broad range of surface receptors that allows them to sense subtle changes in the microenvironment and help to identify the degree of activation (Castellano et al. 2016; Hsieh et al. 2013; Perego et al. 2011). We used a CD68 marker to identify activated MG/M $\phi$ , with a preference for phagocytic cells (Perego et al.

2011). We found a significant elevation in CD68 cells by 1 dpi in male mice, which continued to increase through 7 dpi. In female mice we did not see significant increases in CD68 positive cells until 7 dpi, again demonstrating that female MG/M $\phi$  have a blunted response to TBI compared to male MG/M $\phi$ .

P2Y12 has been identified as a receptor that distinguishes CNS-resident ramified microglia from other blood-derived myeloid lineage cells (Butovsky et al. 2014; Kumar et al. 2016b; Moore et al. 2015), and loss of P2Y12 expression in microglia results in a decreased number of processes and cell migration following focal injury (Haynes et al. 2006). We found a dense population of microglial cells expressing P2Y12 marker in sham brains that remained after injury in both sexes. P2Y12 expression in microglial cells has been reported to diminish as the microglial cells become fully ramified (Haynes et al. 2006), however, we clearly observed ramified and activated P2Y12-positive microglial cells up to 7 dpi in both male and female mice. Previous reports also demonstrate that P2Y12 is preferentially expressed in CD68+/CD163+ (anti-inflammatory) MG/M $\phi$  during parasitic brain infection (Moore et al. 2015), however in our study we did not observe any evidence that P2Y12 positive cells were better associated with specific cytokines after brain injury.

Rapid infiltration of peripheral cells plays a critical role in mediating sex differences in the brain response to experimental stroke, with splenectomy prior to ischemia eliminating the sex differences in infarct volume and activated brain MG/M $\phi$  in mice (Dotson et al. 2015). Similarly, TBI also triggers infiltration of circulating blood cells (Hsieh et al. 2013; Jin et al. 2012), and we used the presence of the F4/80 protein as an indicator of macrophage recruitment to the CNS, as has been previously described (Hsieh et al. 2013; Kumar et al. 2016b; Morganti et al. 2014). We observed that male mice had a high expression of F4/80 positive cells by 1 dpi that were highly concentrated at the lesion border. Female TBI brain had very low levels of F4/80 positive MG/M $\phi$  until 7 dpi when these cells became apparent around the lesion border. Very few F4/80-positive cells overlapped with P2Y12-positive cells in mouse brain after injury indicating distinct populations. Our data is in agreement with findings from the ischemia field where male mice have more peripheral leukocytes 3 days after hypoxia-ischemia compared to females (Mirza et al. 2015), causing increased secondary neuronal damage. Together these data suggest that female mice have reduced peripheral inflammatory cell infiltration to the injured CNS, which appears to protect against acute neuronal injury.

Morphological changes have been associated with the grade of activation and production of specific cytokines (Cao et al. 2012; Tam and Ma 2014), and the persistent production of inflammatory cytokines by microglia results in chronic neuroinflammation after TBI (Loane and Byrnes 2010). To analyze the cytokine expression in MG/M $\phi$  cells following TBI, we used a novel *in situ* hybridization technique for labeling specific RNA sequences in brain slices, combined with immunofluorescence using a cocktail of antibodies to detect the entire MG/M $\phi$  population. We used FISH as the use of antibodies against specific cytokines can produce variable results that may be due to the detection limit of immunohistochemistry for low levels of cytokines. The use of FISH improves specificity and sensitivity when examining cytokine mRNAs in distinct cells confirmed by antibody co-immunolabeling, which allows us to identify the cellular source of cytokine production following TBI with

greater confidence. Our data demonstrates that a mixed pattern of both pro-inflammatory and anti-inflammatory cytokine expression occurred in MG/M $\phi$  in the first week after TBI. In male mice, both pro-and anti-inflammatory cytokines appeared at the same time-points. This is consistent with previous reports that there is an overlapping expression of phenotypic markers in MG/M $\phi$  following experimental TBI in male mice (Kim et al. 2016; Kumar et al. 2016a; Morganti et al. 2016). IL1 $\beta$  and TNF $\alpha$  are two important pro-inflammatory cytokines produced after TBI (Kumar et al. 2016a) that are known to induce neuronal cell death. While MG/M $\phi$  cells were the primary source of TNF $\alpha$  mRNA, the IL1 $\beta$  mRNA signal was as much as 68% from non-microglial cells sources after TBI. There were clear differences in the male and female IL1 $\beta$  and TNF $\alpha$  cytokine mRNA expression levels. While male mice presented with a sustained cytokine peak between 1–7 dpi, there was an unusual biphasic peak in pro-inflammatory cytokines that occurred at 4 h post-TBI in female mice that retreated and peaked again at 7 dpi. The mechanism and purpose of this biphasic peak are not known, but the reduced expression of IL1 $\beta$  and TNF $\alpha$  at 1 and 3 dpi in female mice may explain the acute differences in neuroprotection between sexes.

One of the best characterized alternative-activation markers is Arg1, which contributes to wound healing and matrix deposition, and outcompetes iNOS to downregulate production of nitric oxide (Bansal and Ochoa 2003). We found a strong Arg1 signal at the lesion border at 3 and 7 dpi in males. This is consistent with previous reports that show a sustained increase of Arg1 up 7 days post-TBI, with most of the Arg1 positive MG/M $\phi$  being hypertrophic/ amoeboid and located near the cortical lesion (Kumar et al. 2016a). However, in our study, only male mice showed this strong Arg1-positive signal at the lesion border. In fact, we only detected a significant increase in Arg1 cells in female mouse brain at 30 dpi. We also studied the expression of TGF $\beta$  which is thought to be an anti-inflammatory cytokine, but with chronic exposure can also induce pro-inflammatory responses (Cohen et al. 2014). Again we report a sexual dimorphism in TGF $\beta$  mRNA at 1 dpi, but this is resolved by 3 dpi.

Multiple reports have noted that females rodents appear protected against the effects of CNS injury compared to males (Dang et al. 2011; Fleiss et al. 2012; Igarashi et al. 2001) but conversely a study examining the effect of sex on TBI outcome in mice report no effect on lesion volume (Bruce-Keller et al. 2007; Tucker et al. 2017; Tucker et al. 2016; Xiong et al. 2007). Most of these studies have examined a single time-point post-injury, but in the current study, we found that female mice had a faster resolution of TUNEL staining compared to male mice, which correlated with smaller lesion volume at 3 and 7 dpi. The increased expression of pro-inflammatory cytokines in male mice occurred in the acute phase and could explain why male mice have increased cell death and larger lesion volume. Lesion in male mice followed a classical pattern that has previously been reported (Villapol et al. 2014). However, this pattern did not occur in female mice. When we continued to study lesion size over time and report that by 30 dpi, we do not detect any difference in lesion volume between male and female mice. A limitation of our study is that our chronic outcome measures were restricted to histological lesion volume. We have previously shown that the moderate-to-severe CCI used in this study, can be difficult to distinguish from severe CCI when using lesion volume alone (Washington et al. 2012). Other groups have used in-depth behavioral studies to study sex-differences after preclinical TBI. Similar to our findings, Tucker et al demonstrated that female mice had a similar lesion volume to male

mice 30 d after CCI, and presented with similar behavioral deficits in the Morris Water maze and rotarod test, although female mice display fewer anxiety-like behaviors after CCI (Tucker et al. 2017; Tucker et al. 2016). Other studies have found that female mice have better motor ability following TBI compared to males (Roof et al. 1993; Wagner et al. 2004). These confounding data may be explained by a study where the motor and cognitive function were assessed alongside lesion volume in male and female TBI mice. The authors found no difference in lesion volume or cognitive ability, but still report a significant reduction in foot-faults in female mice after TBI (Xiong et al. 2007). More studies are required to establish whether the differences in acute inflammatory responses can be harnessed to improve outcome in female mice after TBI.

A second limitation of our study is that we did not monitor the estrous cycle of our mice at the time of injury. We used random cycling in our mice to better resemble the clinical setting of TBI in cycling females. Previous studies have revealed that the estrous cycle stage at the time of the injury has no effect on post-injury neuroprotection of female mice and rats (Bramlett and Dietrich 2001; Monaco et al. 2013). In addition, when TBI was induced at random stages of the estrous cycle the performance variability was similar to that reported in male mice (Tucker et al. 2017; Tucker et al. 2016).

In summary, in this work, we have demonstrated that female mice are acutely more resistant to moderate-to-severe TBI compared to male mice, primarily due to reduced neuroinflammation. However, this may not result in chronic brain sparing as female and male mice have similar brain volume loss at 30d post-TBI. It is clear that further research aimed at investigating the effects of sex, microglial function, and cell death are needed to elucidate the molecular and cellular mechanisms underlying hormonal and sex-related differences in the incidence and clinical course of brain injuries. Future studies will be essential for exploring personalized therapeutic treatments for TBI patients of both sexes.

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## Abbreviations used

<b>CCI</b>	Cortical controlled impact
<b>DAPI</b>	40,6-diamidino-2-phenylindole
<b>GFAP</b>	glial fibrillary astrocytic protein
<b>dpi</b>	days post-injury
<b>hpi</b>	hours post-injury
<b>Iba-1</b>	ionized calcium binding adaptor molecule 1

<b>PBS</b>	phosphate-buffered saline
<b>TBI</b>	traumatic brain injury
<b>TUNEL</b>	terminal deoxynucleotidyl transperase-mediated biotinylated UTP nick end labeling

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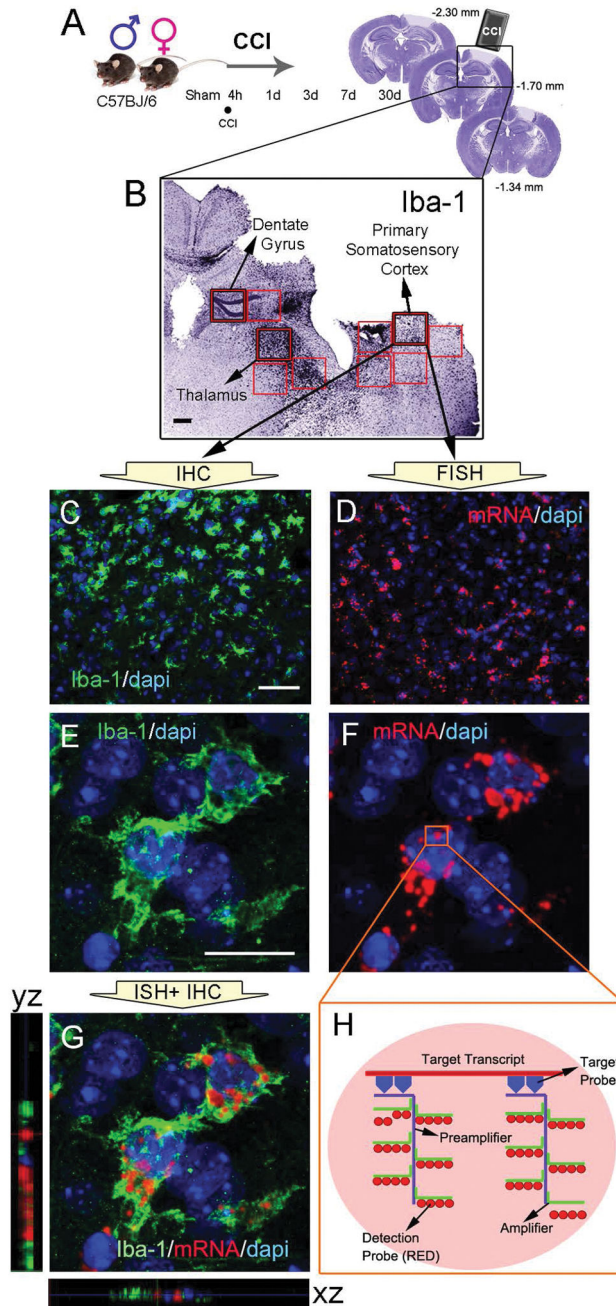
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**Main Points**

1. After brain damage, male mice show increased microglial activation, macrophage infiltration, astrogliosis and cell death compared to female mice.
2. Traumatic brain injury induces sex and timing-dependent cytokine production in microglia/macrophages cells.

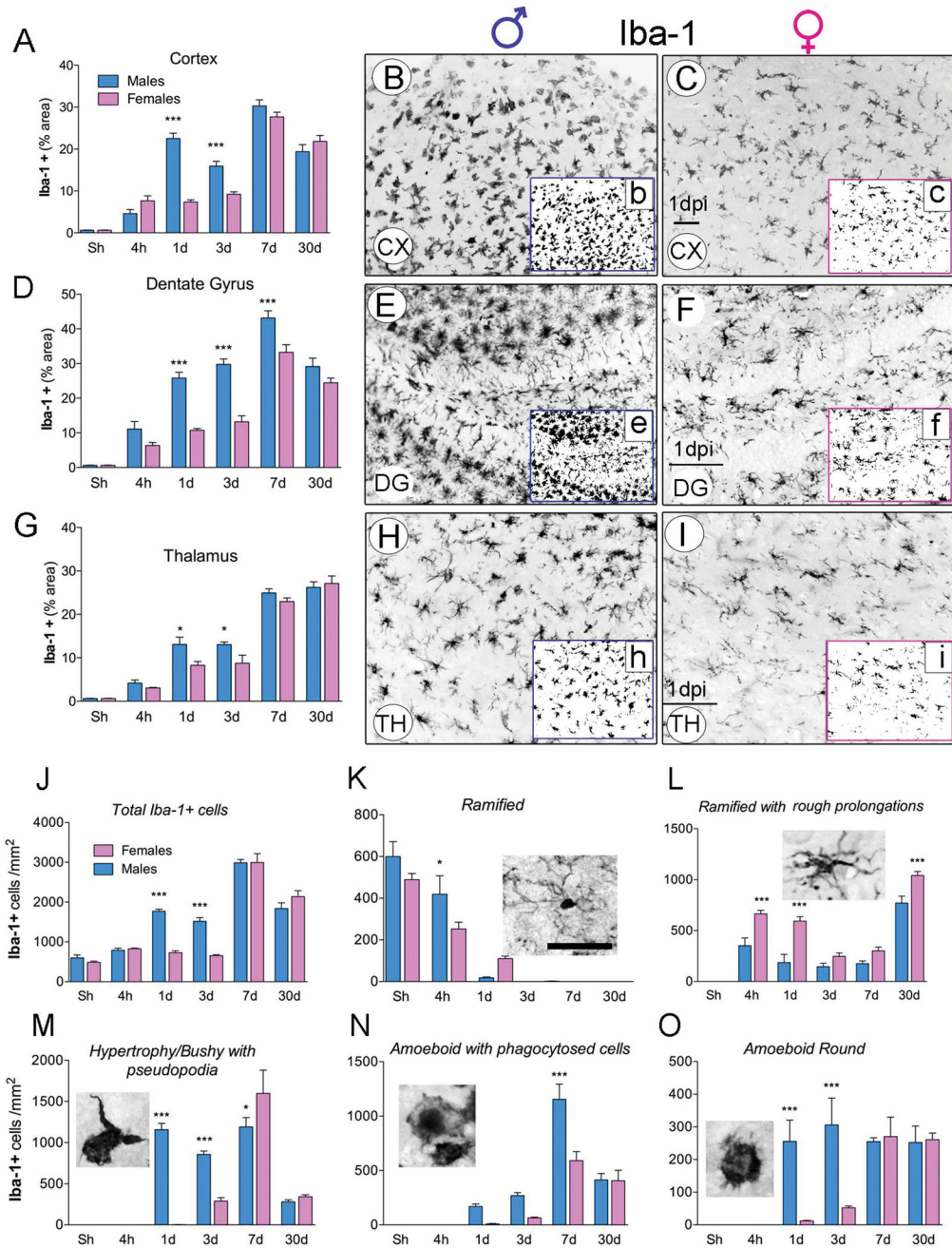


**Figure 1. In situ hybridization combined with immunohistochemistry for analysis of the immune response in injured mouse brains**

**A.** Male and female C57BL/6J mice received a controlled cortical impact (CCI) injury and were sacrificed at 4 hours, 1, 3, 7, or 30 days after CCI. Coronal mouse brains sections corresponding to the lesion area from  $-1.34$  mm to  $-2.30$  mm posterior to Bregma, Nissl stained, were selected for analysis. **B.** Tissue was probed for microglia/macrophages cells (Iba-1, darker staining) and counterstained with nuclei (dapi, blue), Brain regions (primary somatosensory cortex, hippocampal dentate gyrus, and thalamus) used for quantification are outlined in this brain section micrograph. **C.** Representative image field of



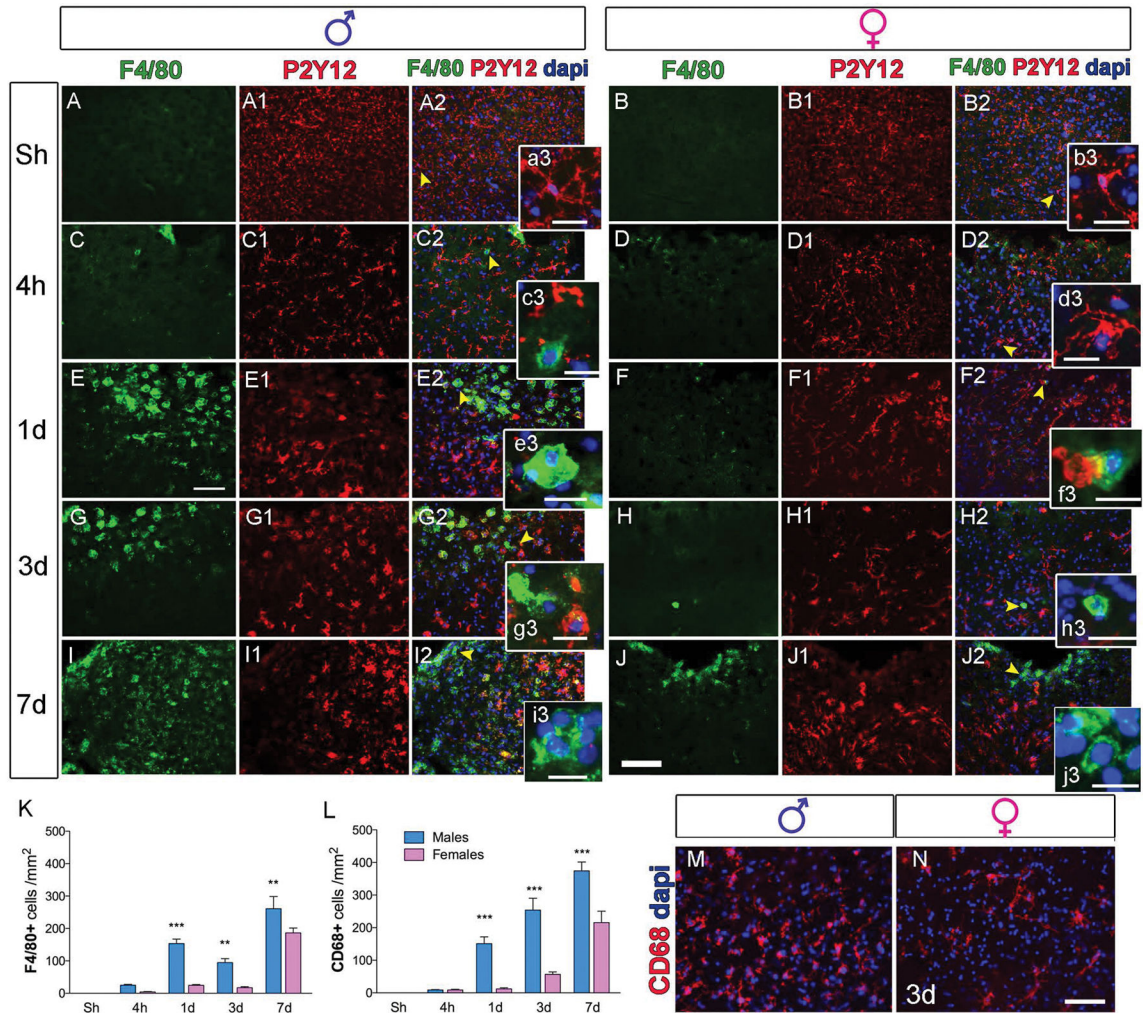
immunohistochemistry (IHC) of microglia/macrophages cells (Iba-1, green) and nuclei (dapi, blue), and **(D)** an example of mRNA expression (probe, red) corresponding to fluorescent *in situ* hybridization (FISH) using RNAscope (scale bar; 50  $\mu\text{m}$ ). **(E–G)** Microglia/Macrophage cells detected by IHC analysis with Iba-1 antibody **(E)**, green) in the lesioned cortex exhibited strong FISH for a representative fluorescence probe (red) counterstained with nuclei (dapi, blue) (scale bar; 20  $\mu\text{m}$ ). RNAscope FISH produces puncta spike in signal (circle red) that represents a single mRNA transcript. The red square in **F** shows an image of a puncta signal, and the illustration depicted in **H** demonstrates the process of “Z” probes hybridizing with the target transcript, and subsequent fluorophore binding.



**Figure 2. Sex-dependent differences in microglia/macrophage morphology and activation after TBI**

A–C TBI causes an increase in Iba-1 positive cell area in the cortex (CX) of male mice that is stronger than the response in female mice. Representative images of Iba-1 immunohistochemical staining show an increase in microglial/macrophages cells in male mice compared to female mice at 1-day post-injury (dpi) (CX). Sex-differences are also observed in the hippocampal dentate gyrus (DG; D–F), and thalamus (TH; G–I), (Scale bar; 50  $\mu$ m). Inset images (b, c, e, f, h, i) are corresponding thresholded images used to quantify the percentage area occupied by Iba-1 positive cells. (J–O) A number of Iba-1 positive cells

is classified according to microglial morphology in the primary somatosensory cortex located in the perilesional brain regions. Representative photos in each graph are depicting the five classifications of microglia/macrophage morphology (ramified; ramified with thick, rough prolongations; hypertrophy/bushy with pseudopodia; amoeboid with phagocytized cells; and amoeboid round). The total number of Iba-1 positive cells per mm<sup>2</sup> (**J**) is higher in males compared to females at 1 and 3 dpi. We observed ramified morphology (**K**, inset representative image) in sham brains with long processes at 4 hours post-injury (hpi), absent at later times; the ramified morphology with rough prolongations (**L**, inset representative image) is higher in females at 4 hpi and 1 dpi compared to male injured brains, decreasing at 3 and 7 dpi, reaching a peak at 30 dpi but without sex differences. We found hypertrophy/bushy Iba-1 positive cells with pseudopodia (**M**, inset representative image) at 1 and 3 dpi in male brains but rarely in females until 7 dpi, microglia retract most of their processes and show enlargement of cell bodies. Amoeboid cells with phagocytosed cells (**N**, inset representative image) were found mainly in male brains, reaching a peak at 7 dpi, compared to female brains. Amoeboid round morphology (**O**, inset representative image) was typically present in males at 1 and 3 dpi and absent in females, with sex equivalence at 7 and 30 dpi. In chronic stages, the majority of the microglia/macrophages still had increased cell body size and fewer processes when compared to sham mice. Scale bar for inset representative images 20  $\mu\text{m}$ . \* =  $p < 0.05$ ; \*\*\* =  $p < 0.001$  vs. females at the same time-point. n = 5/group.



**Figure 3. Male, but not female, TBI mice have rapid infiltration of macrophages into the injured brain**

Representative images indicate cortical brain sections stained with P2Y12 (resident microglial cells, red) and F4/80 (macrophages, green) markers, and nuclei counterstained with dapi (blue). No F4/80 positive cells were observed in sham (Sh) male mice (A, A1, A2, a3), sham female mice (B, B1, B2, b3), 4h TBI male mice (C, C1, C2, c3), or 4h TBI female mice (D, D1, D2, d3). F4/80 positive macrophages with amoeboid morphology predominantly appear at 1 dpi in the perilesional cortex of male mice (E, E1, E2, e3) but remain absent in female brains (F, F1, F2, f3). F4/80 positive macrophages persist at 3 dpi in male TBI brain (G, G1, G2, g3) but still remain absent in female TBI brain (H, H1, H2). By 7 dpi F4/80 cells are widespread in the injured cortex of male mice (I, I1, I2, i3), and are apparent around the lesion in female TBI brain (J, J1, J2, j3). Quantitative graphs indicate an increase in F4/80 positive macrophages (K) and CD68 positive activated microglia/macrophages cells (L) in males compared to females at all time-points. Representative images of CD68 positive cells (red) staining and nuclei (dapi, blue) are showing for males (M) and females (N) at 1 dpi. Scale bars for images 50  $\mu$ m, and for high-power

magnification boxes 20 $\mu$ m. \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$  vs. females at the same time-point  $n = 5$ /group.

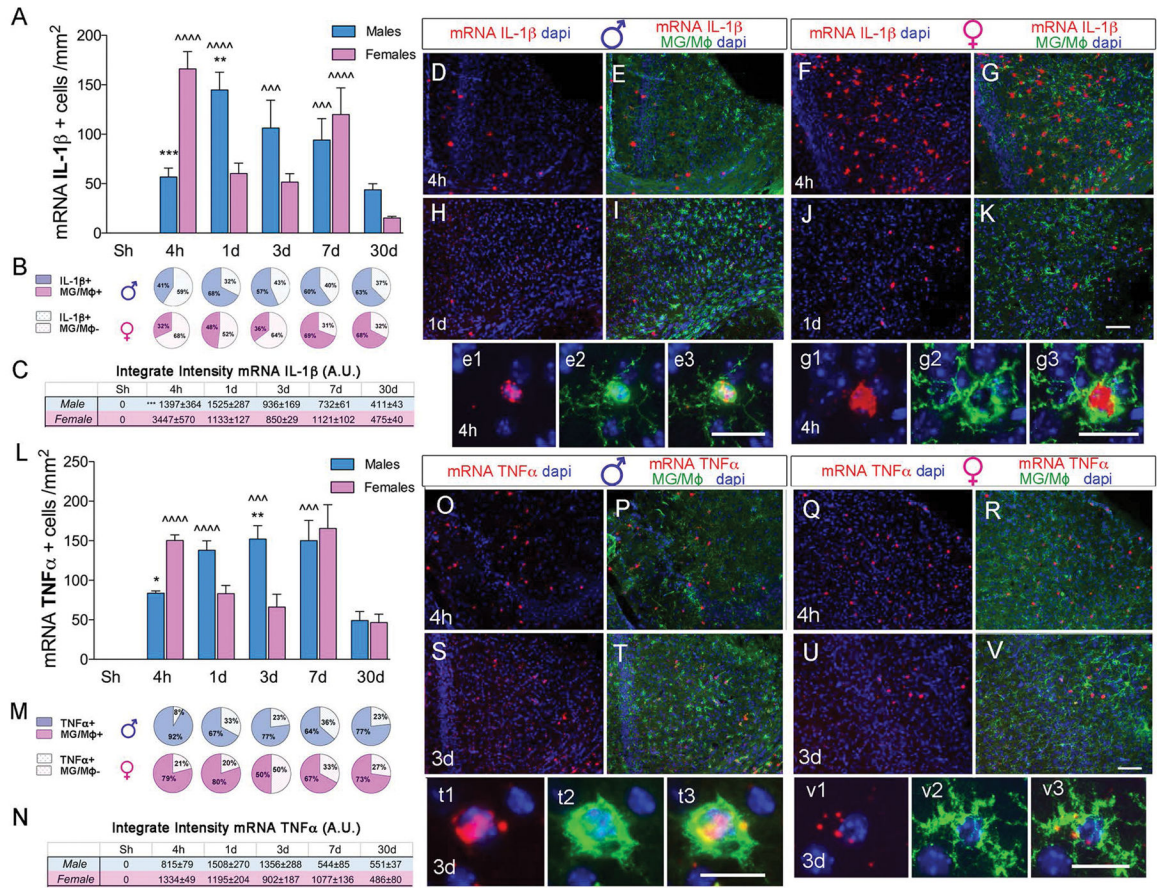
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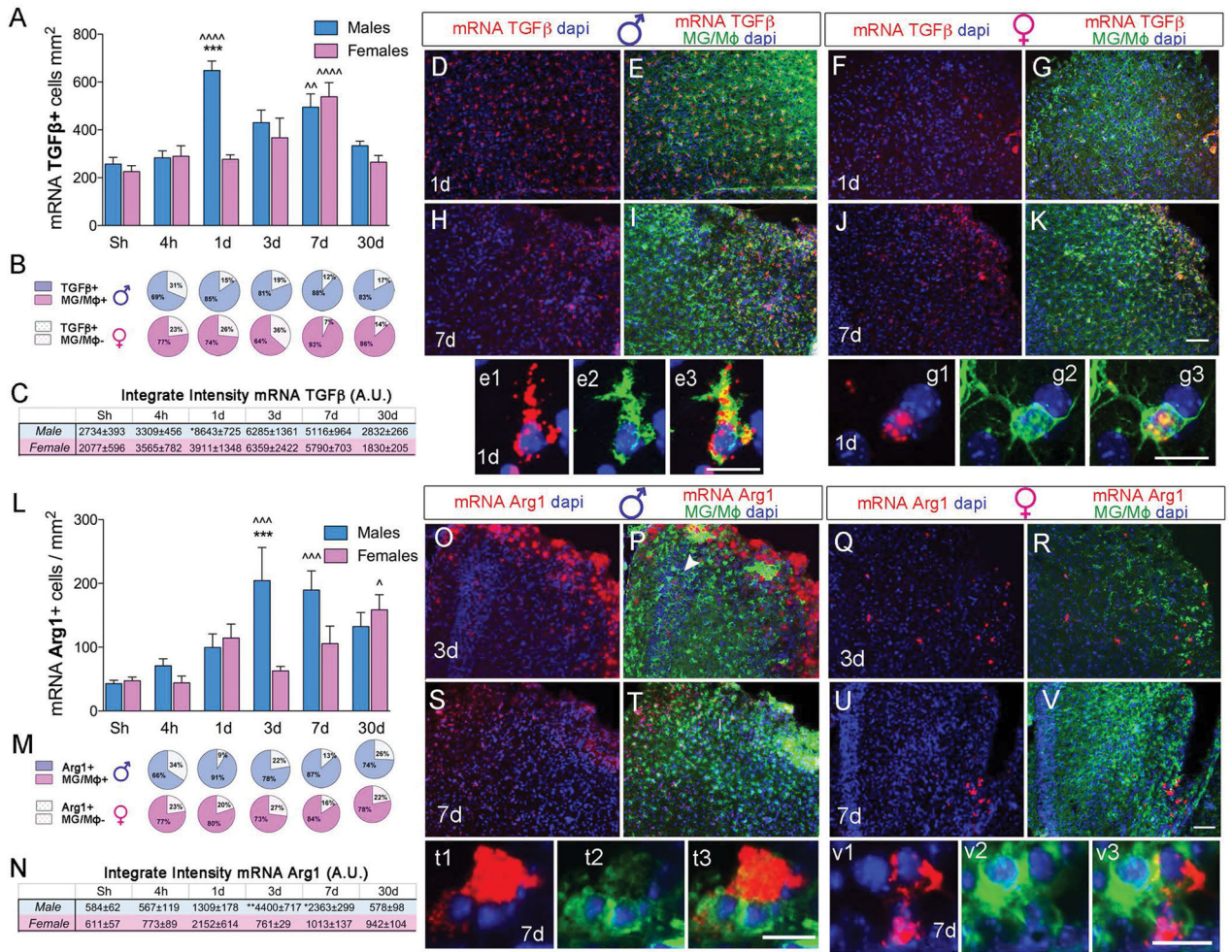




**Figure 4. Sex-specific IL-1 $\beta$  and TNF $\alpha$  cytokine expression after TBI**

**A.** Increased IL-1 $\beta$  positive cells in the injured cortex in female mice compared to males at 4-hours post-injury, but increased expression in males at 1 and 3 days post-injury. **B.** Total IL-1 $\beta$  positive cells expressed in microglia/macrophage cells (MG/M $\phi$ ) vs. non-MG/M $\phi$  in the cortex of male and female brain at each time point after TBI. **C.** Average mRNA IL-1 $\beta$  expression determined by fluorescent intensity in the cortex of males and females after TBI. **D–K.** Representative images for IL-1 $\beta$  positive cells (red), MG/M $\phi$  (green) and nuclei (dapi, blue) in the cortex of male (**D, E, e1, e2, e3**) and female mice (**F, G, g1, g2, g3**) at 4h, and male (**H, I**) and female mice (**J, K**) at 1d post-injury. Scale bar 50  $\mu$ m for D–K; and 20  $\mu$ m for e1–e3 and g1–g3. **L.** Female mice have more TNF $\alpha$  positive cells at increased levels in the injured cortex at 4h compared to male mice, but male mice have more TNF $\alpha$  positive cells at 1 and 3d compared to females. **M.** TNF $\alpha$  positive cells expressed in MG/M $\phi$  vs. non-MG/M $\phi$  in the cortex of male and female brain after TBI. **N.** Average mRNA TNF $\alpha$  expression determined by fluorescent intensity in the cortex of males and females after TBI. **O–V.** Representative images for TNF $\alpha$  positive cells (red), MG/M $\phi$  (green) and nuclei (dapi, blue) in the cortex of male (**O, P**) and female mice (**Q, R**) at 4 hpi, and male (**S, T, t1, t2, t3**) and females (**U, V, v1, v2, v3**) at 3 dpi. Scale bar 50  $\mu$ m for D–K; and 20  $\mu$ m for e1–e3 and g1–g3. \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$  vs. female mice at the same time-point. ^^^ =  $p < 0.001$ ; ^^^^ =  $p < 0.0001$  vs. sham mice of the same sex. n = 5/6 group.





**Figure 5. TGF $\beta$  and Arg1 cytokines expression by activated microglia/macrophages predominate in males after brain injury**

**A.** TGF $\beta$  positive cells in the injured cortex significantly increased in males compared to females at 1-day post-injury (dpi). **B.** Percentage of total TGF $\beta$  positive cells expressed in microglia/macrophage cells (MG/M $\phi$ ) vs. non-MG/M $\phi$  in the cortex of males and females at each time point after TBI. **C.** Table shows the integrate intensity of TGF $\beta$  mRNA expression in the cortex of males and females after TBI. **D–K.** Representative images for TGF $\beta$  positive cells (red), MG/M $\phi$  (green) and nuclei (dapi, blue) in the cortex of males (**D, E, e1, e2, e3**) and females (**F, G, g1, g2, g3**) at 1 dpi, and males (**H, I**) and females (**J, K**) at 7 dpi. Scale bar 50  $\mu$ m for D–K; and 20  $\mu$ m for e1–e3 and g1–g3. **L.** Arg1 positive cells in the injured cortex are significantly increased in males at 3 and 7 dpi compared to females. **M.** Percentage of total Arg1 positive cells expressed in MG/M $\phi$  vs. non-MG/M $\phi$  in the cortex of males and females brains per each time point after TBI. **N.** The table shows the integrate intensity of Arg1 mRNA expression in the cortex of males and females after TBI. **O–V.** Representative images for Arg1 positive cells (red), MG/M $\phi$  (green) and nuclei (dapi, blue) in the cortex of males (**O, P**) and females (**Q, R**) at 3 dpi, and males (**S, T, t1, t2, t3**) and females (**U, V, v1, v2, v3**) at 7 dpi. Scale bar 50  $\mu$ m for D–K; and 20  $\mu$ m for e1–e3 and g1–

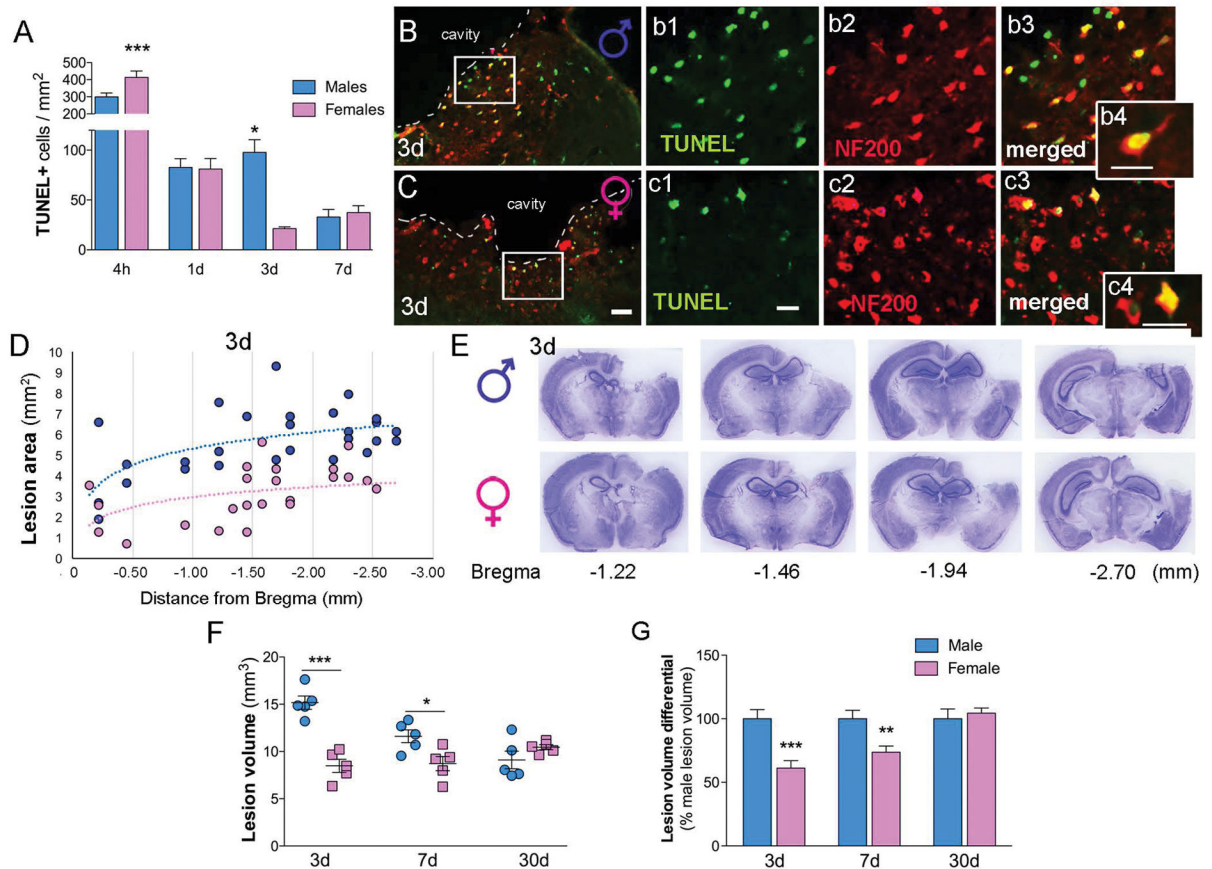
g3. \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$  vs. female mice at the same time-point. ^^^ =  $p < 0.001$ ; ^^^^ =  $p < 0.0001$  vs. sham mice of the same sex. n= 5–6/group.

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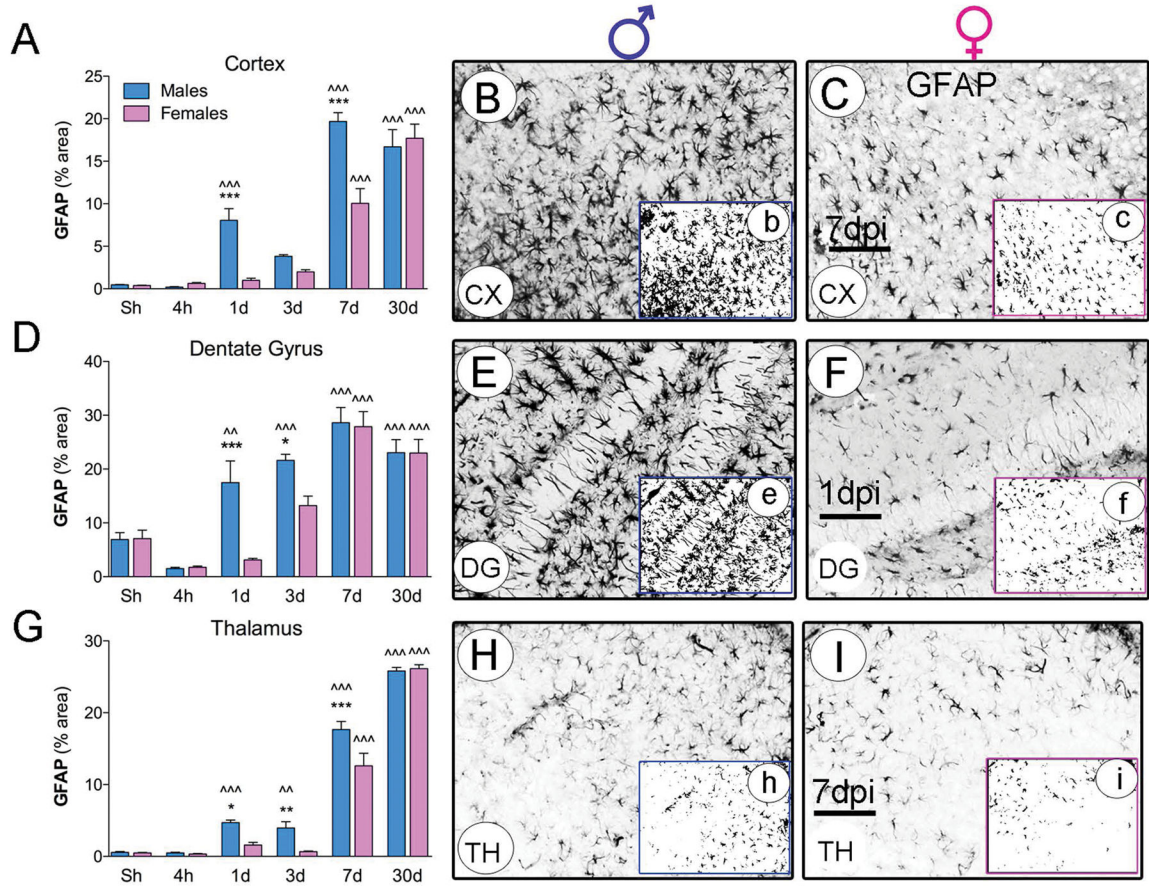
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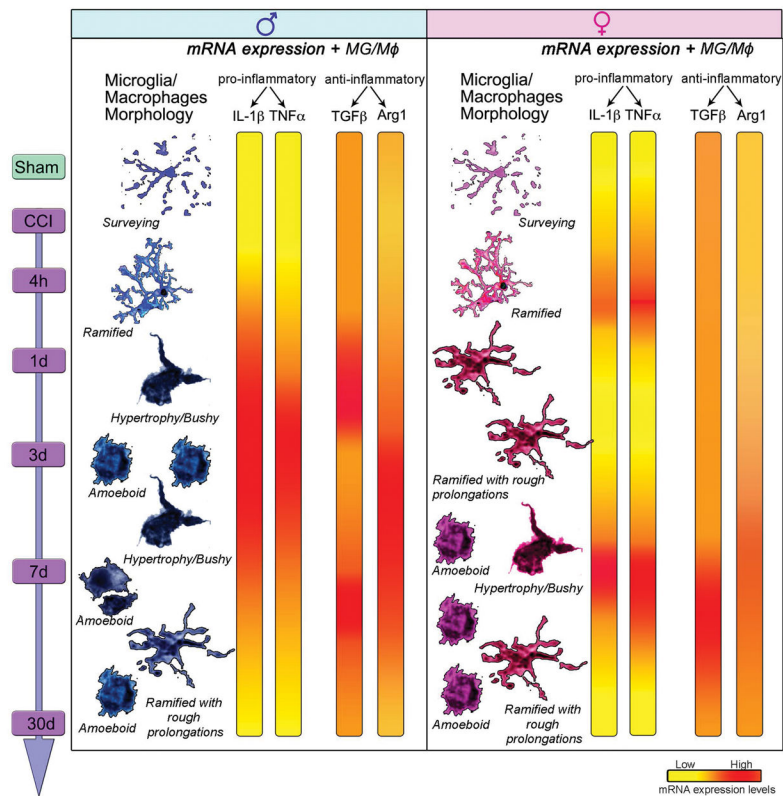
**Figure 6. Female mice have reduced cell death and lesion volume acutely after TBI compared to male mice**

**A.** A peak in cell death (apoptosis) was observed at 4 hours post-injury (hpi), which resolved in female mice quicker than male mice. At 3 days post-injury (dpi), male mice had significantly higher numbers of TUNEL-positive cells compared to females. Representative images for TUNEL (green), NF200 (neurons, red) and a merged image in male (**B**, **b1**, **b2**, **b3**) and female (**C**, **c1**, **c2**, **c3**) injured cortex, respectively. A white striped line is delineating the cavity and the perilesional cortex. Scale bars for **B** and **C**; 50  $\mu\text{m}$ , for **b1–b3** and **c1–c3**; 20  $\mu\text{m}$ , and high magnification insets 20  $\mu\text{m}$ . **D**, **E**. Detailed analysis of lesion volume in 3 dpi mice and representative Cresyl-violet images with their distance from Bregma. **F**. Lesion volume in male and female mice at 3, 7, and 30 dpi. **G**. Female lesion volume represented as percentage of male lesion volume at the same time-point. \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$  vs. female mice at the same time-point.  $n = 5/\text{group}$ .





**Figure 7. TBI induces a rapid astroglial response in male, but not female, mouse brain**  
 Immunohistochemical staining of GFAP reveals rapid astrocyte activation in male mouse brain mice compared to females in the primary somatosensory cortex (CX; **A–C**); hippocampal dentate gyrus (DG; **D–F**), and thalamus area (TH; **G–I**), (Scale bar; 50  $\mu$ m). Inset images (**b, c, e, f, h, i**) correspond to a thresholded image field to measure the percentage of area occupied by GFAP positive astrocytes. Scale bar; 50  $\mu$ m. \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$  vs. female mice at the same time-point. ^^^ =  $p < 0.001$ ; ^^^^ =  $p < 0.0001$  vs. sham mice of the same sex.  $n = 5/\text{group}$ .



**Figure 8. Sex differences in morphological and phenotypic diversity of microglia/macrophages following TBI**

Schematic diagram illustrating the characterization of M1/M2-like microglia/macrophage cells (MG/Mφ) in males and females. Both sham male and female brains present surveying microglia and basal levels of cytokines. Days after injury, male mice have increased levels of infiltrating macrophages and activated microglial cells with hypertrophied/bushy morphology. One week after injury, MG/Mφ predominate in both sexes. In the frame time where macrophage infiltration occurs, the peak of cytokine expression is increased in males, while reduced in females. Both phenotypes, pro-inflammatory (IL-1β and TNFα) or anti-inflammatory (TGFβ and Arg1) activation status, in response to injury are more prevalent in injured male brains compared to females.