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Understanding microglial responses in large animal models of traumatic brain injury: an underutilized resource for preclinical and translational research

Michael R. Grovola[1,2], Catherine von Reyn[3,4], David J. Loane[5,6] and D. Kacy Cullen[1,2,7*]

Abstract

Traumatic brain injury (TBI) often results in prolonged or permanent brain dysfunction with over 2.8 million affected annually in the U.S., including over 56,000 deaths, with over 5 million total survivors exhibiting chronic deficits. Mild TBI (also known as concussion) accounts for over 75% of all TBIs every year. Mild TBI is a heterogeneous disorder, and long-term outcomes are dependent on the type and severity of the initial physical event and compounded by secondary pathophysiological consequences, such as reactive astrocytosis, edema, hypoxia, excitotoxicity, and neuroinflammation. Neuroinflammation has gained increasing attention for its role in secondary injury as inflammatory pathways can have both detrimental and beneficial roles. For example, microglia—resident immune cells of the central nervous system (CNS)—influence cell death pathways and may contribute to progressive neurodegeneration but also aid in debris clearance and neuroplasticity. In this review, we will discuss the acute and chronic role of microglia after mild TBI, including critical protective responses, deleterious effects, and how these processes vary over time. These descriptions are contextualized based on interspecies variation, sex differences, and prospects for therapy. We also highlight recent work from our lab that was the first to describe microglial responses out to chronic timepoints after diffuse mild TBI in a clinically relevant large animal model. The scaled head rotational acceleration of our large animal model, paired with the gyrencephalic architecture and appropriate white:gray matter ratio, allows us to produce pathology with the same anatomical patterns and distribution of human TBI, and serves as an exemplary model to examine complex neuroimmune response post-TBI. An improved understanding of microglial influences in TBI could aid in the development of targeted therapeutics to accentuate positive effects while attenuating detrimental post-injury responses over time.

Keywords Mild TBI, Neuroinflammation, Microglia, Large animal models, Preclinical models

*Correspondence:
D. Kacy Cullen
dkacy@pennmedicine.upenn.edu
Full list of author information is available at the end of the article

Background

Traumatic brain injury (TBI) is a serious health concern and a major contributor to morbidity and mortality in the United States each year. The most recent surveillance report from the Centers for Disease Control and Prevention described estimates of the national incidence of TBI from 2006 to 2014: the CDC reported 2.87 million patients diagnosed with TBI and 56,800 TBI-related deaths in 2014 alone [1]. TBI is typically caused by a mechanical insult, such as a fall or motor vehicle crash, which impacts and/or rapidly accelerates the head (typically angular rotation) producing focal contusions, hematomas, and/or shear deformation of white matter tracts [2]. This primary physical insult may induce a number of secondary injuries, such as blood–brain barrier (BBB) permeability, neuronal dysfunction, dendritic/synaptic disruption, astrocyte reactivity, leukocyte infiltration, axonal degeneration, cell death, and neuroinflammation; all of which are responsible for neurological impairments following TBI [2–6].

In particular, neuroinflammation has gained increasing attention for its role in secondary injury as neuroinflammatory responses can have both detrimental and beneficial roles. Microglia—the resident immune cell of the central nervous system (CNS)—mediate cell death and may contribute to progressive neurodegeneration but also aid in debris clearance and neuroplasticity [7]. Further complicating the role of microglia, their activity is influenced by patient age, sex, mechanism of injury, degree of injury, co-morbidities, and genetic factors [8]. Insight into these inflammatory profiles and their evolution over time is crucial to the development of treatment strategies to manage the neuroinflammatory deficits produced by TBI. Here, we will review the current understanding of the roles of microglia following TBI based on experimental and clinical reports, with a particular focus on recently described chronic neuroinflammatory responses in an established porcine model of closed-head diffuse TBI.

Development and role of microglia

Microglia are parenchymal immune cells that are distinct from perivascular, meningeal, and choroid plexus macrophages in the CNS due to their cell lineage. Microglia are formed during primitive hematopoiesis, where macrophages derived from the yolk sac migrate down the neural tube and eventually become microglia [9]. In contrast, other CNS macrophages are derived from hematopoietic stem cells in the bone marrow and require the Myb transcription factor for proper development [10]. As such, microglia are genetically distinct from other CNS macrophages, which would in turn imply functional differences between the two cell populations. Indeed, microglia have been shown to play crucial and distinct functional roles in the brain. Numerous studies have demonstrated that microglia have noninflammatory, homeostatic functions in the healthy brain. Microglia have sentinel-like activity; they continually scan their local environment and utilize their processes to contact and monitor neurons [11, 12]. This continuous surveillance covers the entire brain over the course of several hours, possibly enabling microglia to clear the parenchyma of accumulated metabolic products and deteriorated tissue components [12].

Though the precise role of microglia process motility and surveillance is unclear, one of microglia's most likely tasks is to influence synaptic function in both healthy and diseased states through the process of synaptic pruning. During early neuronal development, microglia engulf axons and synaptic components to shape neuronal circuits [13, 14]. Super resolution microscopy in the mouse hippocampus has shown colocalization of microglia with both PSD95, an excitatory postsynaptic marker, and SNAP25, a presynaptic protein, during the period of synaptic maturation. In addition, delaying this synaptic pruning resulted in an excess of dendritic spines and electrophysiologically immature brain circuitry [14]. Interestingly, in the mouse visual system, synaptic pruning by microglia is regulated by neural activity, as significantly more synaptic inputs were engulfed after treatment with Tetrodotoxin, an action potential inhibitor, while significantly less synaptic inputs were engulfed after treatment with forskolin, which increases cAMP and neural activity. The microglia-specific phagocytic complement pathway may underlie some synaptic changes, yet knocking out complement receptors (CR3) only reduced pruning by 50% suggesting that other phagocytic mechanisms are also involved [13]. Challenging the extent of synaptic remodeling, Weinhard et al. directly tested this role of microglia in phagocytic synapse elimination in organotypic mouse hippocampal cultures. Employing time-lapse light sheet microscopy on microglia-synapse interactions, they found partial elimination of presynaptic boutons and axons by microglia, but no elimination of postsynaptic material. Instead, microglia reorganized postsynaptic sites by inducing spine filopodia formation [15]. In addition, microglia may alter activity-dependent visual circuitry; for instance, the prolonged closure of one eye changes connections reserved for binocular vision yet disrupting the P2Y12 purinergic receptor expressed in microglia while closing one eye negates these changes [16]. Future research will be needed to examine this more nuanced role for microglia in synapse remodeling as well

as assess how specific synapses are recognized and targeted for synaptic pruning.

While changes to microglia can impact neural circuits during development, learning and memory can also be impaired due to microglial changes in adulthood. Indeed, in the healthy adult CNS microglia appear to be involved in activity-dependent long-term synaptic plasticity. One recent study found that experience-driven accumulation of neuronal interleukin(IL)-33 directs microglia to engulf the extracellular matrix, and loss of IL-33 leads to reduced plasticity and diminished fear memory integration [17]. Moreover, knocking out the fractalkine receptor, a necessary receptor for neuron–microglia communication, has led to deficits in contextual fear conditioning, spatial learning and memory, and long-term potentiation (LTP) [18].

Microglia's impact on learning and memory is finely interwoven with microglia's regulation of neurogenesis. In the adult hippocampus, a structure essential for memory formation, neuroprogenitor cells produce neuroblasts in the dentate gyrus. However, the majority of these cells do not integrate into hippocampal circuitry as many cells undergo apoptosis and are phagocytosed by microglia [19]. In fact, during both early development and ongoing adult neurogenesis, microglia phagocytose neurons that die as a result of programmed cell death (for a review see [20]). These phagocytic functions are regulated by TAM receptor tyrosine kinases Mer and Axl, as mice deficient in these kinases exhibit an accumulation of apoptotic cells [21]. Yet microglia also play an active role in apoptosis by releasing superoxide ions or tumor necrosis factor (TNF), indicating that microglia can also drive programmed cell death and further diversifying their physiological roles in the brain [22–24].

Phenotypes of microglia

Microglia are dynamic cells with a wide repertoire of functions that respond to changes in their microenvironment. Traditional microglial evaluation attempted to classify them into a pro-inflammatory (M1) or anti-inflammatory (M2) bimodal arrangement, with each having their own phenotypic markers. M2 microglia were thought to be responsible for neurogenesis regulation and synaptic plasticity, and express IL-10, TGFβ, CD206, Arg-1, whereas M1 were thought to be stimulated by DAMPs, responsible for long-term inflammation and neurodegeneration, and express IL-1β, TNF, IL-6 [8]. However, transcriptomic profiling does not support this hypothesis and has led to a rejection of a M1 vs M2 dichotomy. For instance, acutely isolated microglial cultures from injury models have cells which co-express both sets of these "signature" genes or express none of these genes [25, 26]. Based on these studies, the M1 vs M2 classification appears to be overly simplistic and insufficient to classify heterogenous sets of microglia. Indeed, a 2022 white paper written by leading microglial researchers discard this M1 vs M2 labeling and provide a series of recommendations for diverse microglial nomenclature [27].

Accordingly, in an effort to distinguish microglia subpopulations in different states, one study examined more than 76,000 individual mouse microglia during development, old age, and after brain injury. Their analysis detected at least nine transcriptionally distinct microglia states defined by unique markers [28]. In 2022, Shih et al. conducted the first study to define the pig microglial transcriptome and conduct interspecies comparisons. They found 239 core microglial genes as well as 150 genes that varied based on brain region. In addition, normalized gene expression was compared to humans and rodents, and demonstrated that core microglial genes are conserved across species, while species-specific expression also exists [29]. By highlighting the complexity of transcriptional microglia signatures, the field is gradually taking steps towards elucidating the function of numerous microglial states. Future studies will need to test each putative state individually, possibly using genetic manipulation to alter each subpopulation to eventually determine their function and phenotype.

Extracellular injury signals that drive microglia

After TBI, extracellular signals trigger microglia to perform a variety of both neurodegenerative and regenerative functions. TBI may induce membrane permeabilization and/or blood–brain barrier disruption, which can release proteins, nucleic acids, or other molecules collectively known as damage-associated molecular patterns (DAMPs) [8, 30]. One classic example of DAMP release is high mobility group protein B1 (HMGB1); HMGB1 stabilizes nucleosomes under normal conditions but is greatly upregulated after injury and associated with elevated intracranial pressure in TBI patients as well as cerebral edema after moderate TBI in mice [31]. In addition, adenosine 5′-triphosphate (ATP) may be released from damaged neurons into the extracellular environment; ATP triggers a rapid convergence of microglial processes towards the site of injury [11, 32]. Moreover, astrocytes can actively release ATP into the extracellular environment to potentially amplify the immune cell response [11, 33].

Detecting post-traumatic DAMP release, cell surface toll-like receptors (TLR) activate microglia and astrocytes, and rapidly upregulate major drivers of neuroinflammation: TNF, IL-6, and IL-1β [34]. Alternatively, ATP can activate NOD-like receptors (NLR), a central part of the inflammasome. Recent studies indicate moderate/

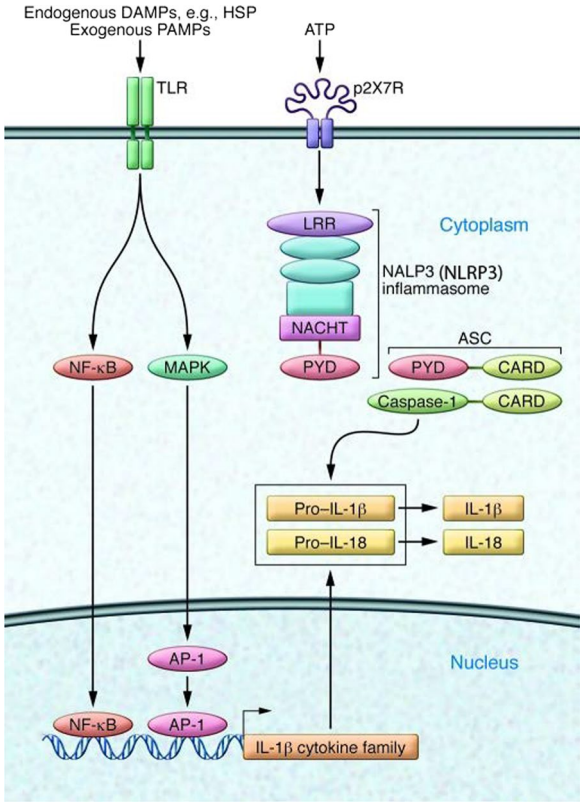


Fig. 1 Innate recognition of tissue injury. DAMPs activate cell surface toll-like receptors (TLRs) and ligation of TLRs initiates an intracellular signaling cascade that drive the production of IL-1 family cytokine precursor proteins. ATP activates NOD-like receptors (NLRs), which signals the cleavage and maturation of IL-1 family cytokines (Adapted from Ransohoff and Brown [34])

severe TBI upregulates the NLRP3 inflammasome, which is increasingly studied as its inhibition improved outcomes in rodent TBI and may serve as a biomarker of inflammatory conditions [35, 36]. Finally, activation of NLRP3 autoactivates caspase-1 and catalyzes the active form of IL-1 family cytokines (Fig. 1) [34].

In parallel with DAMP activity, excessive extracellular glutamate, the major excitatory neurotransmitter of the CNS, may occur. In the healthy brain, astrocytes can uptake excess glutamate released from neurons; however, after TBI, glutamate uptake by astrocytes can be transiently impaired following the injury [37, 38]. This excess glutamate can lead to excitotoxicity via overactivation of *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Activation of these receptors allows an influx of calcium through receptor-gated channels, voltage-gated channels, or intracellular calcium stores, which can produce proteases, lipases, oxygen radicals, mitochondrial damage, DNA degradation, and ultimately lead to cell death [39].

Microglia themselves express both ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). Following acute CNS injury, local glutamate activity can rapidly alter microglial functional responses. For example, microglia express both AMPA and ainite iGluRs, which upon receptor activation, triggers the release of TNF-α in cultured microglia [40]. Moreover, depending on the different subtypes of mGluRs expressed, microglia may adopt a more neuroprotective or a more neurotoxic phenotype, via the activation of group III and group II mGluRs, respectively [41–43]. The direct activation of group II mGluRs via mGluR2 stimulation induces metabolic stress leading to release of TNF-α and Fas ligand, which triggers neuronal caspase-3 activation via TNFR1 and Fas receptor, leading to neuronal cell death [43]. Furthermore, the link between glutamate toxicity and inflammatory cytokines has been outlined in many studies. For example, excitotoxic brain damage increases TNF-α and IL-1β expression, which in turn increases NMDA receptor-induced neuronal death (for a review see [44]). Further solidifying this connection, NMDA receptor antagonists reduce levels of IL-1β and TNF-α, and IL-1 receptor antagonists reduce excitotoxicity [44]. Unfortunately, NMDA receptor antagonists have failed clinical trials for treatment of TBI patients [45].

Effects of chronic inflammation

After the acute phase of injury, the optimal outcome is resolution of the inflammatory response and release of trophic factors [7]. However, a subset of patients or animals experience persistent inflammation out to chronic timepoints [46]. One rodent study subjected adult mice to a single moderate controlled cortical impact injury and detected highly reactive microglia up to 1-year post-injury with microglia expressing major histocompatibility complex class II (CR3/43), CD68, and NADPH oxidase (NOX2). Moreover, these biochemical markers were associated with progressive lesion expansion, hippocampal degeneration, and loss of myelin, supporting a link between chronic microglia activation and neurodegeneration [47]. Indeed, there is evidence of persistent inflammation and degeneration after single moderate/severe TBI in humans. Johnson et al. [5], found cases up to 18-years post-injury with CR3/43 and/or CD68 immunoreactive pathology paired with white matter degeneration. Neuroimaging studies (TSPO - Translocator Protein 18kDa) and serum biomarker (TNFα) analysis corroborate this neuropathology of chronically elevated cytokines and inflammation [48, 49].

These analyses have been complimented by RNA sequencing techniques, yielding rich data sets on chronic

inflammatory markers, revealing the complexity of microglial heterogeneity after TBI, and enabling more nuanced classification schemes beyond simplistic M1 vs M2 markers. After 1.2 atmosphere midline FPI, Witcher et al. [50] employed single-cell RNA sequencing in mouse cortex at 7 days-post injury (DPI), a critical point in the transition of acute to chronic pathology. They found 10 distinct microglial subclusters determined by each cell's transcriptome along with increased type-1 interferon and neurodegenerative-related genes. In addition, Witcher et al. utilized nanoString mRNA panels on 30 DPI mouse cortex and found an increase in several transcripts related to innate immunity (CD14, CD68, GPR84, Itgax, TLR4, and TREM2) compared to controls. Todd et al. [51] conducted bulk RNA sequencing on FACS sorted microglia and astrocytes at 7 DPI following severe fluid percussion injury in mice. They identified 518 differentially expressed genes between TBI and sham microglia, with many of the top differentially expressed genes being a part of the type I interferon pathway. Finally, Lipponen et al. [52] examined chronically altered gene expression at 3-months post-injury after 3.3 atmosphere lateral fluid percussion in rats. Using gene ontology analysis, they found immunity and inflammatory genes sets upregulated in the perilesional cortex and thalamus, but not the hippocampus, indicating functional gene sets remain activated over a wide post-TBI time window. Overall, the field is still unsure of the genetic susceptibilities, molecular triggers, and pathways that contribute to chronic inflammation, yet additional factors such as injury severity, sex, and model species are thought to influence inflammation.

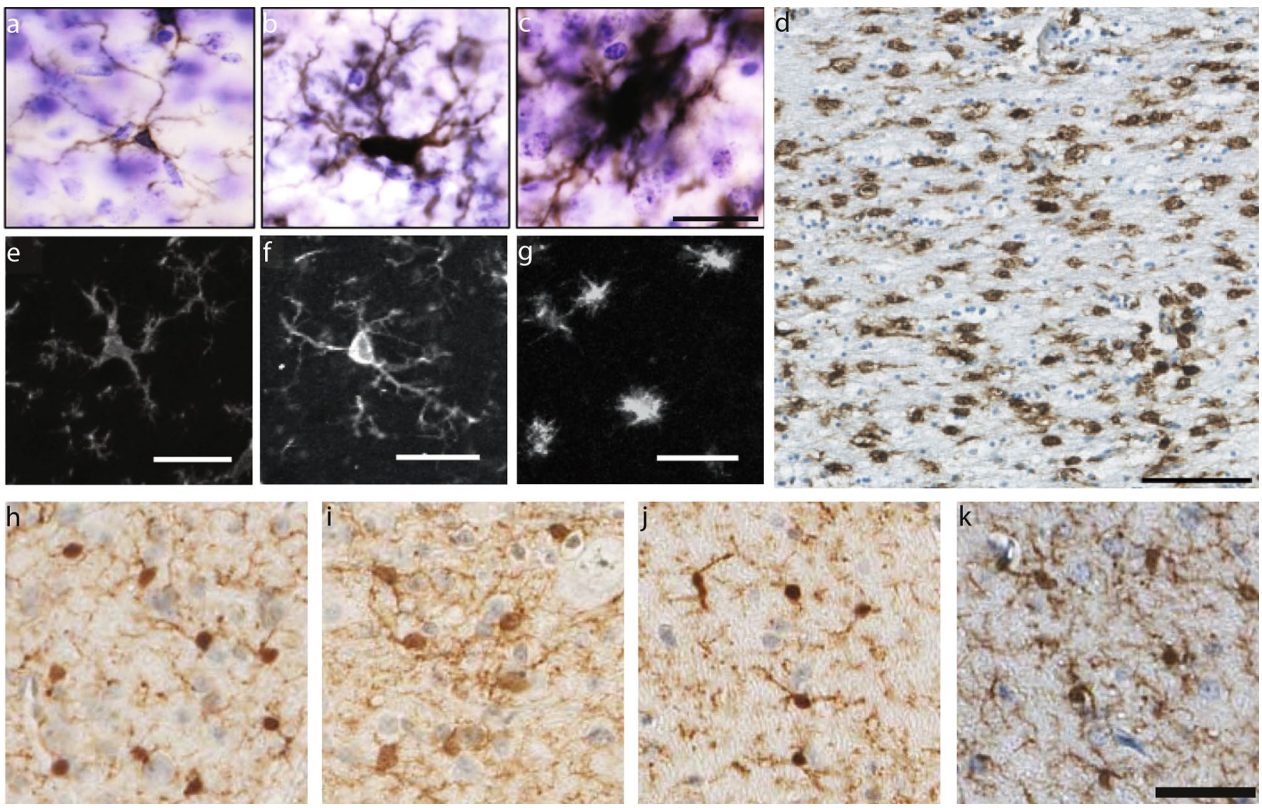


Fig. 2 Microglial phenotypes in post-mortem TBI immunochemistry. The pattern and extent of microglial reactivity may vary according to species and injury severity. Representative images of Iba-1+ microglia display ramified (**a**), hypertrophic (**b**), and bushy (**c**) morphologies after moderate-level controlled cortical impact in rodents (scale = 25 µm; images adapted from Henry et al. [62]). Human post-mortem studies demonstrate morphologically altered CR3/43+ microglia after a single moderate-to-severe TBI (**d**) (scale = 100 µm; images adapted from Johnson et al. [5]). In a closed-head rotational acceleration model of TBI in pigs, Iba-1+ microglia display ramified morphologies in sham conditions (**e**) and after acute mild TBI absent permeabilized neurons (**f**) yet display ameboid morphology after acute mild TBI localized with permeabilized neurons (**g**) (scale = 25 µm; images adapted from Wofford et al. [59]). In this same model, Iba-1+ microglia have been shown to undertake a more ramified phenotype after single mild TBI at longer timepoints post-injury. Representative images of Iba-1+ microglia compare morphology in sham (**h**) and 7-day postinjury (**i**) in the hippocampal hilus, and sham (**j**) and 1-year post-injury (**k**) in the hippocampal molecular layer (scale = 50 µm; images adapted from Grovola et al. [67])

Effects of injury severity and interspecies variation on inflammation

Following injury, microglial activation can persist in the brain for weeks, years, or even decades, as demonstrated in chronic assessments of mild TBI in rodents, chronic assessments of moderate-to-severe TBI in humans, and acute assessments of mild TBI in pigs. However, until recently, the microglial activation dynamics after mild TBI had not been assessed at chronic timepoints in a large animal model. While several large animal models of TBI—most notably porcine models—are in use in the field, such carefully controlled laboratory models may ultimately inform future treatment for humans [53–59].

Accordingly, a survey of previous work shows that the mechanism(s) and severity of traumatic loading as well as the species being assessed can impact the pattern and extent of neuroinflammation (Fig. 2). Rodent studies have shown a range of chronically altered microglia—with morphologies described as ramified, hypertrophic, or bushy (Fig. 2a–c)—and inflammatory gene expression [2, 47, 60–62]. For example, in a weight drop model of mild TBI, MAPT, GFAP, and TNF genes increased in the cortex at acute time points, while AIF1, CCL11, and TARDBP genes were upregulated in the cortex at chronic timepoints [61]. Comparatively, mice subjected to moderate controlled cortical impact displayed an upregulation of genes in the cortex related to the NLRP3 inflammasome and NADPH oxidase pathways at chronic timepoints [62]. After closed-head rotational acceleration TBI in pigs, microglia display ameboid morphologies around permeabilized neurons, while microglia display more ramified morphologies absent permeabilized neurons and in shams (Fig. 2e–g) [59]. Moreover, microglia reactivity has been shown to vary across species when all subjects are injured using the same model. Gorse and Lafrenaye [63] investigated thalamic microglial–axonal interaction differences between pigs and rats after central fluid percussion injury. They noted a decrease in microglial interactions with injured axons in rats but an increase in microglial interactions with injured axons in pigs suggesting that the species used for in vivo preclinical studies influences post-TBI microglial response.

Human post-mortem studies have described persistent neuroinflammation with morphologically altered microglia after a single moderate-to-severe TBI (Fig. 2d) or after repetitive mild TBIs [5, 64, 65]. These pathological studies often note neuroinflammation in the corpus callosum; however, a neuroimaging study using positron emission tomography (PET) reported inflammation-related ligand binding differences in the thalamus and putamen, but not the corpus callosum after single moderate-to-severe TBI [66]. Interestingly, neuropathological analysis from our lab did not observe microglial reactivity in the corpus callosum after single mild closed-head TBI [67]. Moreover, NFL players with a history of TBI displayed increased TSPO binding (expression of which increases in activated microglia) in the hippocampus, left entorhinal cortex, parahippocampal cortex, supramarginal gyrus, and left temporal pole [48]. Yet, drawing conclusions from these human studies is difficult as these are not able to be as carefully controlled as preclinical studies. Collectively, TBI studies to date have reaffirmed that microglia are exquisitely sensitive to their microenvironment and complex with many fluctuating subpopulations, and that their reactivity can change based on the species, degree of injury, and over time.

Sex differences in TBI

The overall TBI rate is greater in males compared to females. Among patients seen in Emergency Departments, 547.6 per 100,000 persons are males who experienced any-severity TBI, while 385.9 per 100,000 persons are female [68]. Interestingly, studies on collegiate athletes indicate that women experience more concussions than men when playing the same sport [69]. Moreover, a growing number of women are choosing to serve in active military duty, increasing their risk of combat-related injury [70].

To assess whether TBI outcomes are different in males vs females, Gupte et al. [71] performed an extensive review on sex differences in TBI in both human and animal research. Overall, the authors found that human studies have generally reported worse outcomes in women than men, whereas animal studies have generally reported better outcomes in females than males; however, the authors were careful to note that injury severity, genetic factors, and age may all interact with biological sex to determine TBI outcome [71]. Unfortunately, fewer women than men are enrolled in clinical trials for TBI, and male rodents have been predominately used in preclinical research [71]. The paucity of female subjects in brain injury research may impede the development of TBI treatments as biological sex can affect neuroanatomy, cellular pathways, and drug pharmacokinetics [72, 73]. In addition, sex hormones are an important aspect of effective TBI therapy as estrogen and progesterone have been examined for potential neuroprotective effects. Unfortunately, while preclinical studies have indicated a decrease in inflammation from progesterone treatment post-TBI, clinical trials have failed to demonstrate improved outcomes between progesterone treatment and placebo [74, 75].

Sex differences also exist in microglia quantity and phenotype according to recent rodent studies [75]. In early postnatal stages, males have more microglia in

specific neuroanatomical subregions (preoptic area, parietal cortex, hippocampus, and amygdala) and generally have more ameboid microglia in the amygdala, while females have more ameboid microglia in the hippocampus (for a review see [76]). One study found that microglia from female brains at age 3 days expressed higher levels of inflammatory cytokines compared to male brains, yet differences did not exist at later timepoints [77]. Therefore, it has been postulated that microglia may have different roles and responses between males and females after TBI [75]. While the data are limited, several studies have examined differing microglia responses based on sex. After LPS stimulation, microglia in male mouse neonates expressed greater IL-1β compared to female neonates [78]. After penetrating brain injury, COX-2 expression (an enzyme which stimulates inflammation) and apoptotic cell death measured by TUNEL staining increased in male rats at 24 h post-injury, yet astrogliosis and microgliosis did not differ [79]. After a cortical stab wound, male mice had greater microglial density only at the lesion border [80]. Finally, after controlled cortical impact in adult mice, males showed a greater influx of peripheral myeloid cells followed by proliferation of microglia compared to females [81]. The data for sex differences in human microglia after TBI are lacking but the limited preclinical data underscores the importance of understanding TBI-related sex differences to develop effective clinical therapies.

Unfortunately, there is little data concerning sex differences after TBI in pigs [82]. One notable study conducted by Missios et al. [83] examined the effect of age and sex on lesion volume after cortical impact in piglets. Lesion volumes were significantly larger in male 1-month-old piglets and infant males had higher levels of circulating sex steroids compared to females. Future porcine TBI studies must enroll male and female subjects to understand sex differences in neuropathology and neuroinflammation, and to better serve female patients who have experienced TBI.

Advantages and limitations of large animal models of mild TBI

Mild TBI accounts for 75% of all TBI incidents in the US each year and these injuries may cause long-term impairments or disabilities [84]. Mild TBI is generally distinguished from moderate and severe through diagnostic criteria; mild TBI can have any period of transient confusion and memory dysfunction around the time of injury, loss of consciousness lasting less than 30 min, a Glasgow coma scale score of 13–15 after 30 min, and computed tomography or magnetic resonance imaging may be normal [85, 86]. Therefore, preclinical TBI studies need to consider these diagnostic criteria as well as the biomechanics of human TBI to establish a viable translational model.

Prime candidates for recreating biomechanical parameters known to be injurious in humans are species with a large brain mass and gyrencephalic brain architecture. In particular, pigs are similar to humans in that they possess a relatively large brain mass and gyrencephalic convolutions with a 60:40 white:gray matter ratio—similar to the ratio found in the human brain [87]. This is in stark contrast to mice and rats typically used in TBI studies; mice and rats have smaller brain masses, smooth lissencephalic brains, and 10:90 and 14:86 white:gray matter ratios, respectively [87–89]. This is particularly important as white matter injury has been described as the hallmark pathology of closed-head diffuse TBI in humans [90].

To replicate the biomechanics of human TBI, our group conducts closed-head rotational injuries, the most common form of mild TBI and typically induced through rapid angular acceleration and deceleration of the head [1, 91, 92]. Pigs experienced peak angular acceleration (corresponding to maximum angular deceleration) ranging from 66,000 to 186,000 rad/s^2 with an average minimum angular acceleration of 128,000 rad/s^2. By scaling these angular accelerations according to the brain masses of pigs vs humans via Holburn's scaling equation, we estimate that these injuries would be approximately equivalent to 10,000 rad/s^2 to 29,000 rad/s^2 (mean of approximately 23,000 rad/s^2) in the human brain [93, 94]. These acceleration levels are similar to levels experienced by humans during TBI; on-field head impact data from high school and collegiate football players measure head angular acceleration $\leq$ 15,000 rad/s^{2,} while motor vehicle crash simulations predict head angular acceleration at approximately 25,000 rad/s^2 [95–97].

Finally, many rodent studies use a contusion model that produces cortical tissue loss and cavitation that is not observed even in severe human TBI. Conversely, our pig model employs closed-head rotational acceleration TBI, which produces loss of consciousness from mild to severe levels and emulates the clinical criteria outlined for human mild TBI. The lack of rotational injury is a limitation in other large animal models of TBI as head rotation is critical in inducing loss of consciousness [98, 99]. Furthermore, our group has used these injury kinematics to examine acute recovery outcomes; compared to controls, pigs experiencing mild TBI were more likely to have apnea, shorter time to extubation, and longer time from extubation to standing [94]. Some rodent studies have tried to incorporate head acceleration, but rodent brains are simply too small to produce the appropriate rotation acceleration-induced forces and, therefore, cannot reach the scaled thresholds of injury [93, 100]. The scaled head

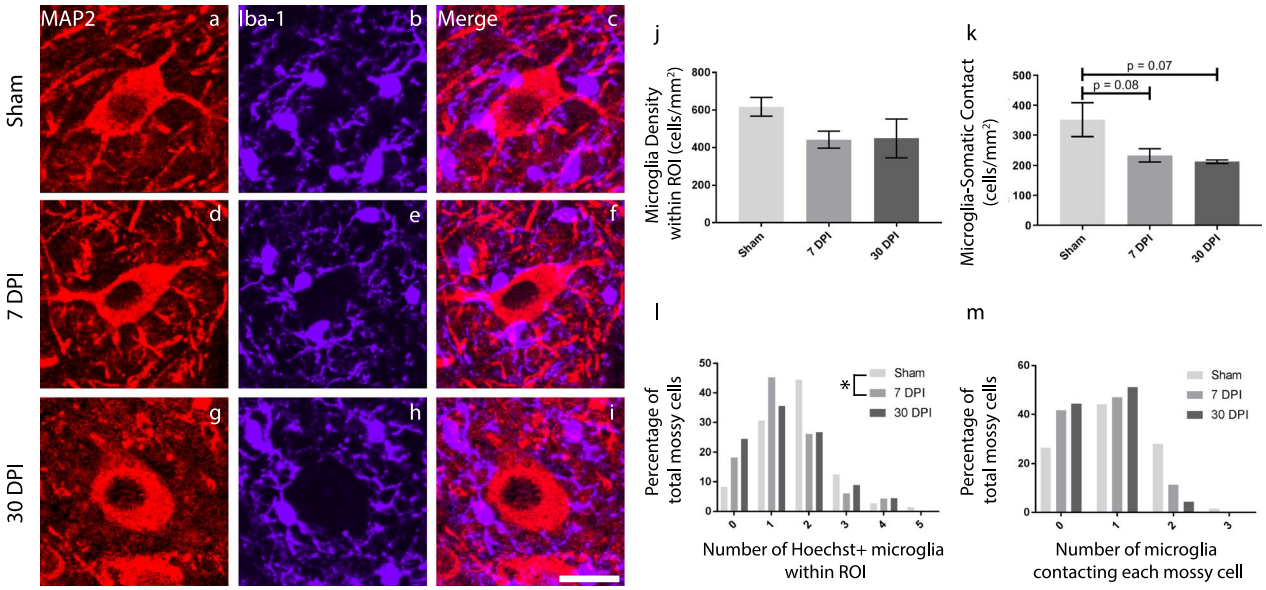
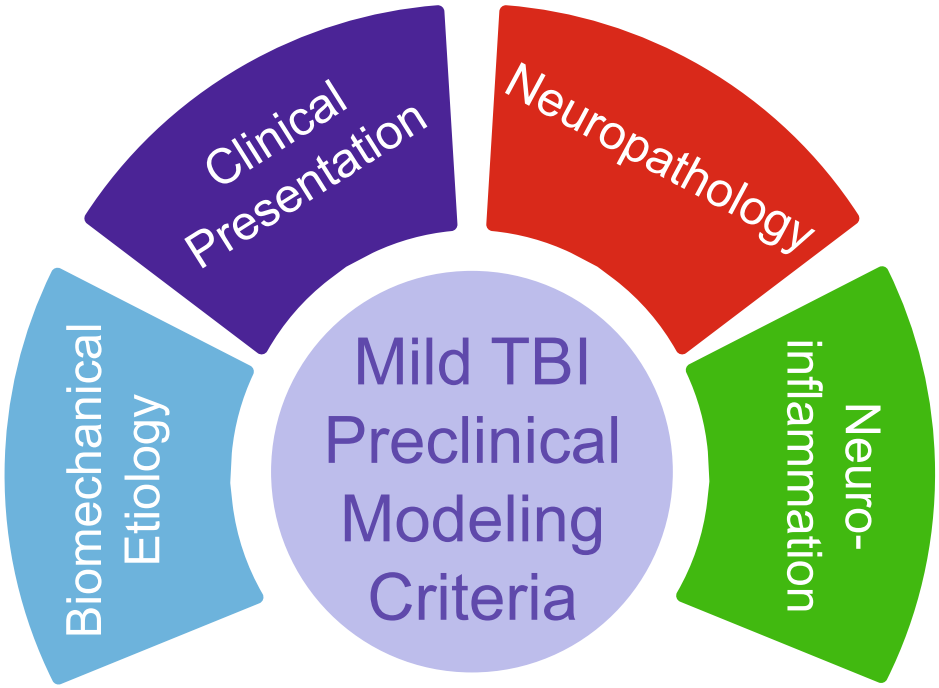


Fig. 3 Microglia density proximal to hilar mossy cells changes following mild TBI. Representative images of hilar mossy cells from sham (**a**), 7-days post-injury (DPI) (**d**), and 30 DPI (**g**), microglial activity from sham (**b**), 7 DPI (**e**), and 30 DPI (**h**), and corresponding merged images (**c**, **f**, **i**) are shown (scale = 25 µm). Microglia density did not change significantly around mossy cells (**j**) but a histogram of the number of microglia per mossy cell indicates there are less microglia more frequently at 7 DPI compared to sham (**l**). There was a non-significant decrease in the number of microglia contacting mossy cell somata (**k**) and a non-significant leftward shift of the data distribution at 7 DPI and 30 DPI compared to sham (**m**) (Images adapted from Grovola et al. [104])

rotational acceleration of our model, paired with the gyrencephalic architecture and proper white:gray matter ratio, allows us to produce pathology with the same anatomical patterns and distribution of human TBI, and, therefore, validates our large animal model of TBI according to biomechanical, clinical, and neuropathological criteria [56, 57, 101].

Using this large animal model of TBI, researchers have induced reproducible neurological and neuropathological deficits including loss of consciousness, mild edema, increased intracranial pressure, astrogliosis, diffuse axonal injury, and neuroinflammation [56, 57, 102, 103]. Our group has recently published a series of studies that examine neuropathology out to 1 year following a single mild TBI in male pigs. First, we analyzed synaptic changes and microglia activity in the hippocampus, where we found cell hypertrophy in hilar mossy cells—the primary glutamatergic neuron in the hilus—at 30 DPI, upregulation of synapsin labeling around mossy cells at 7 DPI, and an increase in microglia density at various subacute and chronic timepoints (Fig. 3) [104]. Then, to further characterize microglial phenotypes, we employed sensitive and quantitative morphological analysis of microglia using automated skeletal analysis. We observed increases to microglial ramification out to 1-year post-injury in various hippocampal sub-regions (Fig. 2h–k). We also noted an increase in microglial ramification in periventricular white matter at 3 DPI and 7 DPI, coinciding with an increase in axonal pathology in the periventricular white matter at these timepoints [67]. These data suggest that a single, closed-head mild TBI can produce persistent changes to the neuroimmune response.

However, further experimentation, such as gene expression changes and antibody labeling for acute and chronic neuroinflammatory cytokines, is needed to provide more contextual detail to microglial morphological changes and provide knowledge of their role in neuronal health and synaptic function post TBI. Absent these additional studies, interpretation of these phenotypic microglia changes is limited. Morphological changes to microglia suggest a change in neuroimmune homeostasis, which may be driven by numerous extracellular signals, such as disrupted BBB or damaged neurons. While we have not observed overt neuronal loss in this model, microglia may be monitoring injured neurons and stabilizing their synaptic function [104]. Based on these observed chronic microglial phenotype changes in our validated model of injury, we propose that preclinical models of mild TBI also be validated on neuroinflammatory criteria, in addition to clinical, biomechanical, and neuropathological (Fig. 4).



Biomechanical Etiology - closed-head rotational acceleration, scaled to levels experienced by humans during TBI (91-94); brain anatomy/architecture sharing key similarities to humans, e.g. gyrencephalic, white:gray matter ratio (57,87)

Clinical Presentation - transient confusion/memory dysfunction, loss of consciousness lasting less than 30 minutes, Glasgow Coma Scale greater than 13-15, normal CT or MRI
Animal Subjects - delayed neurological recovery (94)

Neuropathology - diffuse axonal injury (56-58), neuronal hypertrophy (104), synaptic alterations (104), mild edema and astrogliosis (102)

Neuroinflammation - microglia phenotype changes (58,59,67), microglia density changes (104)

Fig. 4 Mild TBI preclinical modeling criteria. After evaluation of numerous preclinical studies, as well as the clinical observations of mild TBI, we propose that preclinical models of mild TBI be validated through biomechanical, clinical, neuropathological, and neuroinflammatory criteria

Large animal models do have distinct disadvantages, however. Large animal subjects themselves cost much more than rodents, as does the necessary specialized housing. Veterinary staff needs to be trained for larger animal handling and care, which may impact feasibility. In addition, post-TBI behavioral and cognitive tests are not thoroughly validated in a porcine model and many commercially available antibodies do not cross-react with porcine tissue, thus restricting some experimental measures. Finally, genetic and mechanistic modulations are much more difficult in porcine models compared to rodent models. As such, rodent and large animal models may have complementary roles as the field seeks to examine neuroinflammation after TBI and better understand the chronic consequences of brain injury.

Prospects for therapy

Post-traumatic neuroinflammation provides vital physiological functions as we have outlined in previous sections. However, this response can be pushed beyond

homeostatic parameters or may continue beyond the acute injury period, potentially contributing to a lifetime of disability and neurodegenerative disease. Unfortunately, targeting and treating inflammation after TBI has proven challenging as many clinical trial have failed that directly or indirectly influenced inflammatory processes, including corticosteroids, hypothermia, and hypertonic saline infusion [105–108]. One anti-inflammatory clinical trial had mixed results; minocycline administration after TBI in 15 patients reduced chronic microglial activation but increased neurodegeneration [109].

As clinical trials continue to optimize the neuroimmune response following TBI, a new intervention framework has been proposed by Simon et al. [8]: limit the acute proinflammatory response to essential debris clearance and danger signals, promote anti-inflammatory and pro-regenerative immune cells, and prevent chronic inflammation. This framework suggests that specific components of neuroinflammation should be modulated in a time dependent manner. For instance, due to the role of the complement system in enhancing phagocytic activity and inflammation after TBI, blocking this cascade has become an attractive therapeutic strategy. Blocking different steps in the complement cascade has mitigated neuronal loss and chronic inflammation in rodent models of TBI [110, 111].

Another method that allows finer control over microglia involves inhibiting the microglia colony stimulating factor 1 receptor (CSF1R), a receptor expressed by microglia, macrophages, and osteoclasts, which can deplete the brain's microglia population when the gene is eliminated [112, 113]. One study of note administered mice a CSF1R inhibitor for 1 week at 28-days post-moderate TBI, which improved motor and cognitive function, decreased lesion volume, and attenuated cortical and dentate gyrus lesion loss [62]. Even though these data are promising, many other factors will need to be assessed to determine the true effectiveness of therapeutics for post-TBI inflammation. Primarily, all immune cell types should be carefully assessed as one study suggests that CSF1R inhibitors also affect peripheral immune cells and inhibit circulating tissue macrophages [114]. In addition, candidate therapeutics should carefully consider various factors that may affect the neuroimmune response to TBI, such as age, sex, mechanism and degree of injury, and secondary insults, with the overall goal of promoting regeneration and mitigating degeneration following TBI.

Conclusions

Microglia are necessary immune cells in the CNS that may contribute to both neuroprotection and neurodegeneration after TBI. Microglia can be driven by a variety of extracellular signals and microglial activation is often sustained out to chronic timepoints. Preclinical modeling of TBI-induced neuroinflammation is a challenge as injury severity, species, and biological sex are among the many factors than can influence the inflammatory response. However, the most translatable preclinical research will strive to mirror human TBI according to neuroinflammatory responses, in addition to the three classic benchmarks of biomechanical etiology, clinical presentation, and neuropathological sequelae. Further research is needed to elucidate the potential benefits and/or neurotoxic effects of microglia after TBI. Only a multipronged approach of genomic analysis, pharmacological intervention, neuroimaging, and behavioral assays will improve our understanding of the chronic neuroinflammatory sequela to TBI and advance treatment options for patients that suffer from deleterious post-TBI effects.

Abbreviations

TBI	Traumatic brain injury
CNS	Central nervous system
LTP	Long-term potentiation
TNF	Tumor necrosis factor
DAMPs	Damaged-associated molecular patterns
HMGB1	High mobility group protein B1
ATP	Adenosine 5′-triphosphate
TLR	Toll-like receptors
NLR	NOD-like receptors
NMDA	*N*-Methyl-D-aspartate
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
PET	Positron emission tomography
DPI	Days post-injury
CSF1R	Colony stimulating factor 1 receptor

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Author details
[1]Center for Neurotrauma, Neurodegeneration & Restoration, Corporal Michael J. Crescenz VA Medical Center, Philadelphia, PA, USA. [2]Department of Neurosurgery, Center for Brain Injury & Repair, University of Pennsylvania,

105E Hayden Hall/3320 Smith Walk, Philadelphia, PA 19104, USA. [3]School of Biomedical Engineering, Science and Health Systems, Drexel University, Philadelphia, PA, USA. [4]Department of Neurobiology and Anatomy, Drexel University College of Medicine, Philadelphia, PA, USA. [5]School of Biochemistry and Immunology, Trinity College Dublin, Dublin, Ireland. [6]Department of Anesthesiology and Shock, Trauma, and Anesthesiology Research (STAR) Center, University of Maryland School of Medicine, Baltimore, MD, USA. [7]Department of Bioengineering, School of Engineering and Applied Science, University of Pennsylvania, Philadelphia, PA, USA.

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