Targeting chronic and evolving neuroinflammation following traumatic brain injury to improve long-term outcomes: insights from microglial-depletion models

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Microglia, the resident innate immune cells of the central nervous system (CNS), play important roles in brain development, maintenance, and disease. As brain sentinels, microglia adopt a surveillant state in healthy tissue characterized by a ramified scanning morphology that maintains CNS homeostasis and contributes to learning-associated synaptic plasticity. Following acute CNS injury or during chronic disease, microglia undergo dramatic morphological transformations and a phenotypic switch to an activated state that initially plays an important protective role against pathological insult (e.g., clearance of cellular debris by phagocytosis to facilitate effective wound healing responses). However, when microglial activation becomes chronic and dysregulated it can have detrimental effects and lead to neurodegenerative processes. Chronic microglial activation has been reported in patients who suffer moderate-to-severe traumatic brain injury (TBI) and is evident in white matter and distant sites from the primary lesion for many years after the initial brain trauma (Johnson et al., 2013). Microglia are also chronically activated up to 1 year following experimental TBI in rodents, and contribute to chronic neurodegeneration and cognitive impairments (Loane et al., 2014). Thus, chronic non-resolving inflammation with widespread microglial activation is a defining feature of moderate-to-severe TBI, and an important secondary injury mechanism that may be treatable (Simon et al., 2017).

Microglial development and long-term survival is dependent on colony stimulating factor 1 receptor (CSF1R), whereby CSF1R knockout mice are devoid of microglia and die before adulthood. The recent development of CNS-penetrant drugs that inhibit CSF1R to eliminate microglia has enabled the direct investigation of microglial function in the healthy and diseased CNS. When first generation CSF1R inhibitors, PLX3397, were administered to mice for a period of 21 days it led to robust brainwide elimination of microglia (> 95%) (Elmore et al., 2014). Moreover, upon removal of PLX3397 there was a rapid self-renewal and repopulation of microglia in brain, and repopulated cells were capable of mounting an inflammatory response similar to that of non-depleted microglia. A second generation and more selective CSF1R inhibitor, PLX5622, has been developed and has excellent pharmacokinetic properties, including oral bioavailability with a > 20% brain penetrance across multiple species (Spangenberg et al., 2019). PLX5622 rapidly eliminates microglia in brain within 7 days of treatment and can sustain depletion for many months. These new pharmacological tools have propelled much recent preclinical research into microglia depletion strategies as translational therapies for neurological diseases. Accordingly, there is now accumulating evidence that microglial depletion has a broad range of neuroprotective effects by reducing damaging microglial-mediated neuroinflammation (Han et al., 2019). For example, selective removal of microglia in Alzheimer's disease mouse models improves cognitive function, reduces neuronal loss, and partially prevents the progression of Alzheimer's disease pathology, but has no effects on amyloid levels and plaque loads. Furthermore, in autoimmune disorders such as Multiple Sclerosis microglial depletion reduces disease progression in experimental autoimmune encephalomyelitis models, and enhances remyelination and recovery in a cuprizone demyelination mouse model.

In experimental brain injury models, differential effects of microglia depletion have been reported under varying experimental settings. PLX3397-induced depletion of microglia prior to lateral fluid percussion injury in mice promoted neurite outgrowth, preserved dendritic spines, and reduced neuronal apoptosis up to 3 days post-injury (Wang et al., 2020). In another study, depletion of microglia prior to midline fluid percussion injury using PLX5622 decreased astrogliosis and reduced expression of genes associated with complement, cytokines, chemokines, and interferon signaling in the cortex at 7 days post-injury, without any protection against axonal injury (Witcher et al., 2018). In contrast, another study depleted microglia in the pediatric rat brain by intracerebral injection of liposomes containing clodronate immediately following closed head injury, and this led to exacerbated neurodegeneration after closed head injury (Hanlon et al., 2019). In preclinical ischemic stroke models, it has been reported that PLX3397-mediated depletion of microglia prior to middle cerebral artery occlusion in mice resulted in increased infract volume, dysregulated neuronal calcium responses, lack of spreading depressions, calcium overload, and increased neuronal death (Szalay et al., 2016). Notably, in this study, microglial repopulation reduced lesion expansion indicating critical microglia-mediated neuroprotection to excitotoxic injury in stroke. Overall, these conflicting findings suggest that depletion of microglia during acute brain injury may have detrimental effects and further emphasizes the important protective roles of microglia during acute injury. Thus, the concept of depleting microglia prior to or directly following brain injury may be counterintuitive and negate critical immune functions in microglia, and may not be a therapeutically beneficial approach.

While acute microglia activation likely confers neuroprotection following TBI, there is now a substantial body of evidence that chronic and evolving microglial activation is associated with neurodegenerative processes and long-term impairments in functional recovery (Simon et al., 2017). Importantly, experimental studies implicate NADPH oxidase (NOX2) as a common and necessary mechanism of microglia-mediated neurotoxicity during chronic neurodegeneration. Previous work from our laboratory demonstrated that severe TBI in mice induces chronic microglial activation up to 1 year post-injury and contributes to progressive lesion expansion, hippocampal neurodegeneration, and white matter damage (Loane et al., 2014); inhibiting NOX2 activity suppresses microglial neurotoxicity, reduces chronic tissue loss and improves long-term motor function recovery after TBI. We subsequently demonstrated that inhibition of chronic microglial activation beginning 1 month after TBI using either mGluR5 agonists or delayed aerobic exercise, reduced neurodegeneration and improved long-term neurological recovery. Notably, both interventions significantly reduced NOX2 expression in chronically activated microglia.

In our most recent preclinical study (Henry et al., 2020), we hypothesized that selective removal of chronically activated neurotoxic microglia by short-term administration of the CSF1R inhibitor, PLX5622, at a highly delayed timepoint of 1 month post-injury followed by microglial repopulation would reduce chronic neurodegeneration and long-term neurological impairments in injured animals. To test this hypothesis, adult male C57Bl/6J mice underwent moderate-level controlled cortical impact or sham surgery and were orally administered PLX5622 or vehicle in rodent chow for 1 week starting at 4 weeks post-injury. All mice were returned to normal chow at 5 weeks post-injury and comprehensive motor and cognitive testing was performed from 8–12 weeks post-injury, prior to collection of brain tissue for histological assessments of TBI neuropathology (**Figure 1A**).

We found that the delayed depletion of chronically activated microglia followed by repopulation resulted in significantly reduced numbers of pro-inflammatory microglia in the injured cortex. Further, the repopulated microglia had an altered phenotype that was markedly less inflammatory, including reduced expression of NOX2. Stereological assessments demonstrated that repopulated microglia in the injured cortex displayed more ramified scanning morphologies, similar to that of uninjured sham controls. In contrast, vehicle-treated TBI mice had microglia with typical chronic post-traumatic hypertrophic/bushy morphologies. Furthermore, delayed short-term microglial depletion followed by repopulation significantly improved neurological recovery through 3 months post-injury, as demonstrated by a variety of complementary motor (beam walk and rotarod) and cognitive (Y maze, novel object recognition, and Morris water maze) function tests. Improved functional outcomes in PLX5622-treated TBI mice were associated with decreased cortical lesion volume and neuronal cell loss in the hippocampus. Thus, short-term depletion of microglia chronically after TBI largely eliminates the destructive neurotoxic microglial phenotype that appears to contribute to chronic post-traumatic neurodegeneration and associated neurological dysfunction (**Figure 1B**).

Molecular and cellular analysis in a second cohort of animals revealed that the lesion microenvironment in PLX5622-treated animals was markedly less inflammatory and there was reduced expression of microglial-related genes for complement, pro-inflammatory cytokine and chemokines, and oxidative stress. Moreover, cellular studies showed that repopulated microglia had decreased NOX2, NOD-like receptor family pyrin domain-containing 3 (NLRP3), caspase-1 activation, and interleukin-1β expression. Of note, the NLRP3 inflammasome is regulated by NOX2 such that NOX2 inhibition markedly attenuates its activity following TBI. Thus, PLX5622 treatment confers neuroprotection after TBI, in part, by removing chronic NOX2-NLRP3 inflammasome signaling in microglia.

Overall, our preclinical studies strongly support the concept that chronic and evolving microglial activation contributes to post-traumatic neurodegeneration and related neurological dysfunction after TBI (Henry et al., 2020). The neuroprotective effects of short-term depletion of chronically activated microglia late after TBI followed by repopulation likely reflect multifactorial mechanisms including less NOX2-mediated neuroinflammation associated with altered oxidative stress responses, and reduced

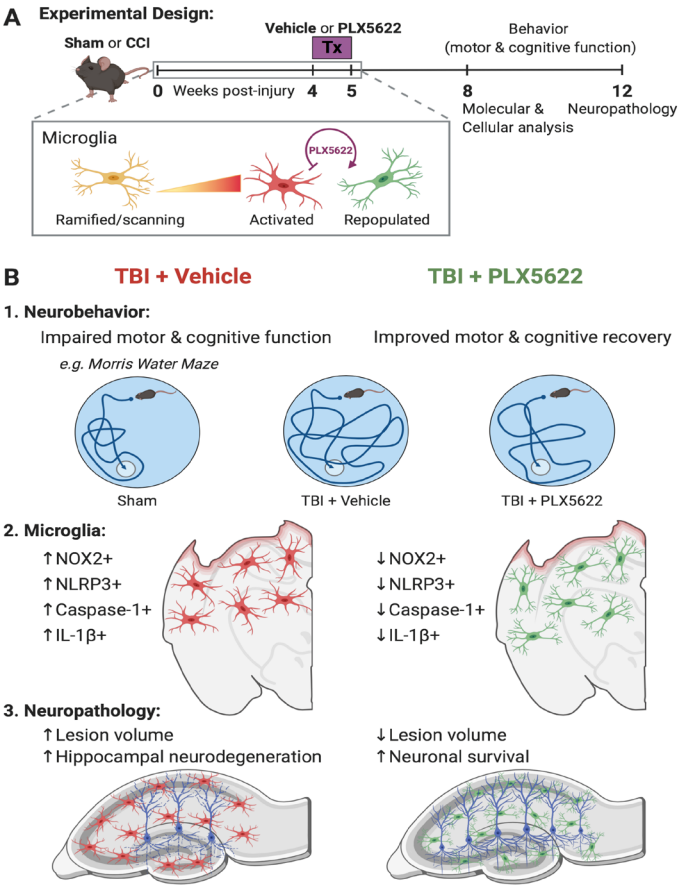

Figure 1 | Microglial depletion with CSF1R inhibitor during chronic phase of experimental TBI reduces neurodegeneration and neurological deficits.
(A) Experimental design and hypothesis for delayed microglial depletion/repopulation TBI model (Henry et al., 2020). Adult male C57Bl/6 mice underwent CCI or sham surgery, and at 4 weeks post-injury mice were placed on CSF1R inhibitor, PLX5622 (1200 ppm), or normal chow (Vehicle) for 1 week, and were then returned to normal chow for the remainder of the study. One cohort of mice were sacrificed at 8 weeks post-injury and samples were collected for molecular (nanostring) and cellular (flow cytometry) analysis. A separate cohort of mice underwent a battery of neurobehavioral tasks for motor and cognitive function through 12 weeks post-injury, and samples were collected for histological analysis of TBI neuropathology. Inset – predicted changes in microglial morphology and activation status in the model. Prior to TBI, microglia are in a surveillant state with ramified/scanning morphologies. Following moderate-level CCI, microglia transform to a highly activated state with hypertrophic/bushy morphologies. At 4 weeks post-injury, activated microglia are depleted by PLX5622 for 1 week and upon removal of CSF1R inhibitor, microglia repopulate the brain and have a less inflammatory activation profile and display ramified/scanning morphologies. (B) Summary of major findings from Henry et al. (2020), in TBI + vehicle-treated (left side) and TBI + PLX5622-treated (right side) mice. 1. Neurobehavior: TBI + Vehicle-treated mice had impaired motor and cognitive function through 12 weeks post-injury. Representative swim patterns from mice during the Morris Water Maze test for spatial learning and memory are shown. During the final day of acquisition learning, sham-injured mice quickly located the hidden submerged escape platform. In contrast, TBI + vehicle-treated mice spent significantly more time to find the escape platform. Notably, PLX5622-treated TBI mice spent less time to find the escape platform than TBI + vehicle-treated mice, indicating improved cognitive function recovery after TBI. 2. Microglia: Molecular and cellular analysis demonstrated that TBI + vehicle-treated mice had reactive microglia in the injured cortex at 8 weeks post-injury, with upregulated expression of pro-inflammatory and neurotoxic proteins, NOX2, NLPR3, cleaved caspase-1, and IL-1β. In contrast, microglia from TBI + PLX5622-treated mice were less inflammatory, and had significantly reduced expression of all proteins. 3. Neuropathology: Stereological assessment of TBI neuropathology at 12 weeks post-injury demonstrated that TBI + vehicle-treated mice had a large cortical lesion and extensive neuronal loss in the hippocampus, and this was associated with presence of highly activated microglia with hypertrophic and bushy phenotypes. In contrast, analysis of TBI + PLX5622-treated mice revealed that microglial depletion/repopulation reduced the cortical lesion and increased neuronal survival in the hippocampus following TBI. PLX-5622-treated TBI mice also had reduced numbers of hypertrophic and bushy microglia and increased numbers of ramified/scanning microglia. CCI: Controlled cortical impact; CSF1R: colony stimulating factor 1 receptor; IL: interleukin; NLRP3: NOD-like receptor family pyrin domain-containing 3; NOX2: NADPH oxidase; TBI: traumatic brain injury.

expression of the NLRP3 inflammasome in microglia.

Finally, a recent study by Willis et al. (2020) demonstrated that short-term depletion and subsequent repopulation of microglia in the injured brain attenuated learning deficits and stimulated hippocampal neurogenesis in a interleukin-6-dependent manner. This preclinical study used genetic and pharmacological (PLX5622) models to show that microglial repopulation was required to reset microglial phenotype to a neuroprotective and pro-regenerative phenotype after TBI that supported functional neurogenesis and cognitive recovery in injured animals. Such detailed studies are essential to gain a deeper understanding of the underlying mechanisms by which microglial depletion/repopulation confers neuroprotection following TBI. Thus, further research is needed before targeted microglial depletion strategies based on CSF1R signaling are translated to the clinic for chronic neurological diseases such as TBI.

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