Functional phenotyping of microglia highlights the dark relationship between chronic traumatic brain injury and normal age-related pathology

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Traumatic brain injury (TBI) is a major cause of death and disability worldwide. Age-related TBI differences demonstrate the third peak of prevalence and incidence of TBI within the elderly population. This is due to the elderly being at a higher risk of sustaining falls, which have been identified as the main cause (40–50%) of TBI. With advances in healthcare and technology, millions of TBI survivors live for decades after the initial injury; however, these individuals suffer from varying degrees of neurological impairment, including long-term cognitive deficits. Epidemiological studies show that the occurrence of TBI significantly increases the risk for the development of Alzheimer’s disease (AD) or non-AD forms of dementia, with the latter appearing to be most prevalent. Although aging is considered a key risk factor for AD/AD-related dementias (ADRD), age-associated neuropathology and neurobehavioral abnormalities can be potentiated both during aging after TBI and in patients sustaining TBI at an older age. Thus, there is an emerging confluence of TBI and AD/ADRD in the older adult population, as well as an increased risk of ADRD in patients aging after TBI, both of which reflect significant unmet healthcare challenges.

In humans, outcomes associated with TBI have been found to differ between young and older adults, all of which worsen with advanced age, emphasizing the need to elucidate not only how younger individuals age with TBI, but also how TBI and old age affects the elderly. To date, there have been limited studies in aged animal TBI models investigating the cellular and molecular mechanisms that lead to worse outcomes. While the pathophysiology of TBI is very complex, the physical impact initiates molecular and biochemical changes that cause prolonged neurotoxic microglial activation, which may serve as a mechanistic link between TBI and subsequent development of chronic neurodegeneration. Numerous experimental observations (Witcher et al., 2015), including ours (Ritzel et al., 2020), indicate that TBI induces widespread alterations in central and peripheral immune responses. As the major cellular component of the innate immune system in the central nervous system (CNS), microglia play a critical role in neuroinflammation following TBI. However, the disease-associated microglia (DAM) signatures in the aged brain following TBI have not yet been studied. Despite considerable research, there is still no established effective treatment to improve recovery in the elderly population following TBI. In part, this reflects an incomplete understanding of the complex secondary pathobiological mechanisms involved.

In a recent study (Ritzel et al., 2022), we investigated whether old age impacts long-term recovery after TBI in a controlled-cortical impact injury model in mice. The clinical rationale of our study was premised on the prevailing view that aggressive management and rehabilitation of older TBI patients is futile given their longer recovery times and higher rates of disability, secondary complications, and mortality (Gardner et al., 2018). This notion is also supported by the increased rate of pre-existing conditions and normal age-related functional decline in individuals chronologically older than 65 years of age. The stereotypic view that all elderly people have confounding comorbidities and similar rates or degrees of decline across neurological measures may justify the exclusion of older patients from clinical trials, but ultimately limits the generalizability of findings to younger age groups. Undoubtedly, we too found that nearly 30% of aged male mice did not survive to 4 months after moderate-to-severe TBI using the controlled cortical impact model, while the mortality rate was negligible (~3%) in the young group. Within the mortality group, all mice died on or after the 9-week mark, further underscoring the importance of the chronic and evolving consequences of TBI. Surprisingly, however, we observed significant recovery gains in motor function of surviving mice after repeated testing as far as 4 months post-injury, the murine equivalent of roughly a decade in human years. Considering the mice were already aged at the time of injury, and that normal age-related decline accelerates with faster increasing age, these gains were rather remarkable, even if the restored level of function remained significantly lower than for younger TBI mice. These data suggest that for a subset of aged animals, presumably the healthier subjects with fewer comorbidities, there is a strong potential for motor recovery. Identifying this resilient (or conversely, at-risk) population will require rigorous investigation, including a comprehensive analysis of pre- versus post-TBI measures. For example, recent findings suggest that individuals with higher frailty index scores have more unfavorable TBI outcomes, regardless of age (Galamberti et al., 2022). It is important to note that our data were less promising as it pertained to cognitive outcomes, which were generally worsened over time in older TBI mice as in humans (Gardner et al., 2018). However, given the negative influence that disability has on mental health and cognition, we believe this may be an important aspect of the neurological recovery process.

TBI has been shown to accelerate the rate of normal age-related brain atrophy (Cole et al., 2015), which would imply that older individuals with TBI exhibit an increased risk, onset, or worsening of neurodegenerative disease. Indeed, older patients with mild TBI are more likely to have a positive CT scan for acute traumatic lesions and an increase in preexisting lesions (Isokoru et al., 2018), whereas those with more severe TBI are shown to have larger lesion volumes and smaller grey matter volumes in neo-cortical brain regions compared to their younger counterparts (Schonberger et al., 2009). The findings from our experimental study in mice neatly align with these clinical data. However, there is a paucity of information relating to the neuropathological features associated with older TBI subjects. Likely because of differences in the mechanism of TBI, heterogeneity issues, and difficulty in teasing out prior history of TBI from normal age-related pathology and other underlying conditions or neurodegenerative disease, much of our current understanding of the age-dependent features of TBI neuropathology is derived from imaging studies. Thus, preclinical models of TBI in older mice are essential for laying the groundwork for future work in humans. Our study noted a significant age-related decrease in hippocampal neuron densities of older TBI mice that was consistent with more pronounced cognitive deterioration. Interestingly, we also found a reduction in white matter myelin intensity. The balance between demyelination and remyelination requires further investigation as our transcriptomic data revealed a large number of differentially regulated genes in older TBI mice associated with oligodendrocyte function in the cortex, but not hippocampus. A recent clinical study suggests that age at injury contributes to an age-related decline in the callosal genu, highlighting the main finding that large commissural and intra-hemispheric structures are at high risk for post-traumatic degradation (Robles et al., 2022). We independently reported significant demyelination in the medial corpus callosum of older mice at four months post-TBI, consistent with brain injury patients’ age-dependent deficits in information processing speed and interhemispheric communication. These findings suggest that neuropathology is generally more severe in older TBI populations, but future studies will be needed to determine the relative contribution of primary versus secondary injury processes and whether there are any unique identifying cellular characteristics or cytoarchitectural changes associated with head trauma in old age. As tissue-resident macrophages that primarily function to maintain neuronal homeostasis in the CNS, microglia are exquisite sensors of their environment. Microglial interactions with neurons are mediated by soluble factors as well as contact-dependent mechanisms. Receptor-ligand interactions can mediate the engulfment of dead and dying neurons, pruning of synapses, and clearance of myelin debris. Post-mortem human studies have reported persistent inflammatory pathology and ongoing white matter degeneration for many years after a single TBI; however, it is not clear whether microglia are promoting degradation or reacting to myelin debris (Johnson et al., 2013). Recent work in animals suggests that forced turnover of microglia during the chronic stages of TBI can ameliorate neuroinflammation.
and cognitive impairment. These findings imply that microglia adopt a neurotoxic phenotype after TBI. However, it is important to note that these studies utilized young mice, in which microglia have a significantly different inflammatory profile, response sensitivity, and tissue environment. The elevated basal activation state of microglia in older mice may have an additive effect on the injury response or aging in an aberrant inflammatory reaction. The secondary mechanisms that govern microglial activation in relation to inflammatory neurodegeneration have remained elusive, particularly in older TBI subjects, where microglial burden, white matter loss, and lesion volumes are generally more severe.

One of the cellular pathways known to be affected during both normal brain aging and neurodegenerative disease is autophagy. The autophagy-lysosomal pathway plays an essential role in cellular homeostasis as well as a protective function against a variety of pathological conditions including neurodegeneration and inflammation. After neurotrauma, inhibition of autophagy occurs in several cell types including microglia/macrophages (Wu and Lipinski, 2019). Emerging data, including ours (Wu and Lipinski, 2019), also suggest that the restoration and/or augmentation of proper autophagy function, may be a potential therapeutic strategy for CNS injuries in aging, and may mitigate a decline in autophagic efficiency including both an age-dependent decline in the expression of autophagy genes and a decrease in lysosomal function. Although autophagic mechanisms have been found to decrease with age in many experimental models, whether they do so in the aging brain with TBI is unclear. It is also widely accepted that microglial autophagy is dysregulated in both brain aging and injury. Recent data from our group suggests there is an interaction between old age and TBI that exacerbates the effect of injury in aging animals (Ritzel et al., 2022). This was evident in the cortical and hippocampal gene signatures showing both age- and TBI-dependent changes in transcriptional networks involved in autophagy and innate immune activation. Given that microglia become chronically activated and contribute to inflammatory neurodegeneration after TBI, we hypothesized that these cells may be the primary driver of this disease signature. Interestingly, the chronic TBI gene expression profile resembled that described for DAM shown to be upregulated in other neurodegenerative diseases (Keren-Shaul et al., 2017). Among the DAM genes were those involved in lysosomal, phagocytic, and lipid metabolism pathways, including several genes associated with increased risk for AD, such as apolipoprotein E and triggering receptor expressed on myeloid cells. In addition to identifying perturbations in autophagic and lysosomal function, we also confirmed the presence of a hyperphagocytic phenotype, which we have previously reported as far out as eight months after TBI in mice (Ritzel et al., 2020). The validation of the DAM gene signature, or more accurately, integrated gene networks, at the level of both protein and function in microglia during the chronic phase of TBI provides further evidence that targeting innate immune activation at early or delayed stages of injury may prevent or slow the progression of neurodegenerative disease and lower the risk of dementia. Moreover, the age-related increases in DAM genes found in sham and chronically injured old mice support studies indicating higher risk/rates of AD in elderly TBI patients. Autophagy may therefore serve as a dual-purpose therapeutic target to attenuate age-related pathology, injury progression, and risk of dementia later in life.

The precise timing of TBI-induced autophagic dysregulation is still up for debate. Activation of autophagy is a normal response to cellular stress and can be observed in many experimental injury models. Surprisingly, the reduction in lipofuscin-associated autofluorescent protein- and lipofuscin- degradation seen in microglia from aged mice during the acute stage (i.e., 48 hours) of TBI implies that autophagy may be activated adaptively. However, the reestablishment of these pathological hallmarks suggests the block in flux may be a more gradual or delayed process. Thus, the requirement for continuous administration of autophagy enhancers to achieve therapeutic benefit needs to be investigated further, as it may be possible short duration or single-dose treatments at a dose sufficient to enhance autophagic flux and confer protection in older mice.

We also shed light on potential mechanisms driving the conversion of healthy microglia to a chronically activated state characterized by autophagic impairment. The persistent changes in microglial morphology, transcriptional activation, and functional state strongly imply epigenetic regulation. Both global protein acetylation and histone acetylation marks were significantly reduced in microglia as a function of age and TBI, suggesting a deleterious increase in histone deacetylation (HDAC) activity. This post-translational epigenetic modification of microglia was captured early after TBI, sustained for months, and attenuated with trehalose treatment, indicating that TBI reprograms microglia towards a more aberrant, inflammatory state. However, it is still unclear whether defects in autophagic flux are cause, consequence, or coincidence, as both changes occurred in parallel, and the effects of enhancing autophagy may be secondary. While numerous studies have demonstrated that HDAC inhibition can improve outcomes after brain injury, the relationship with autophagy is still unresolved. A more granular time course of these early events and interrogation of deacetylase activity may help further elucidate the upstream drivers of epigenetic-mediated disease progression.

Our data highlight the value and potential of functional phenotyping of microglia using flow cytometry. Immunophenotypic identification of cells to determine compositional or numerical changes in brain leukocyte populations is an important indicator of neuroinflammation, but does not provide any detail regarding what the cells are doing on a functional level. By multiplexing a combination of surface and intracellular antibodies indicative of various functional processes, activity-based fluorescent dyes, and phagocytosis engulfment assays, we were able to better understand how microglia respond to aging and TBI, including the similarities and differences between disease settings. While removing cells from the intact brain may cause subtle data artifacts, cells remain functional in the initial hours following isolation and are more likely to exhibit preserved activation states reflective of their native tissue environment compared to in vitro primary cell culture conditions. Furthermore, compared to traditional immunohistochemistry in which cells are fixed in situ, microglia may be acutely stimulated ex vivo to evaluate secondary responses. Flow cytometry allows for quantitative measurements of these changes. While individual cell surface proteins may be simple, useful biomarkers of microglia activation, the concerted activation of the proteome provides a more comprehensive investigation of the differential biological response as injury and other stimuli are undoubtedly a complex process. Activity-based assays are more accurate measures of these dynamic effector functions. Multiplexing provides the ability to understand how cellular functions coincide and are coordinated across time in particular cell types. Using this strategy, we were able to validate the interaction between older age and TBI on the expression of the DAM gene signature seen at the tissue level. Although pharmaceutical treatments known to enhance levels of autophagy have been reported to improve TBI outcomes, all the drugs tested have multiple targets of action, making it unclear as to whether or not their effects on TBI were mediated via autophagy. An mTOR-independent inducer of autophagy, the disaccharide trehalose, has been shown to improve outcomes in rodent models of neurodegenerative diseases and a rabbit model of spinal cord ischemia (Khaliﬁeh et al., 2021).

Although trehalose is known to act as a chemical chaperone and may improve recovery independently of autophagy (Khaliﬁeh et al., 2021), therapeutically targeting early components of the autophagy-lysosomal pathway represents a promising avenue for clinical intervention following TBI in the aging population. Our ﬁndings revealed that continuous administration of trehalose supplemented in drinking water enhanced long-term functional recovery after TBI, attenuated microgliosis, and reduced synaptic engulfment. Furthermore, trehalose treatment prevented TBI-mediated reductions in autophagy-related 7 (ATG7) protein and increased lysosomal enzyme activity in microglia. The dosing regimen was selected to ensure that acute-, subacute-, and/or late-onset of autophagic dysfunction would be blunted. These results are proof-of-principle that continuous trehalose treatment is a viable neurotherapeutic approach for older TBI populations, but the exact mechanistic target of action needs to be elucidated. Moreover, the response to dose termination requires further investigation to better understand the persistence of these therapeutic benefits.

In summary, although TBI primarily affects younger individuals, there is a third peak incidence in persons 65 years of age and older. TBI older patients have higher mortality, worsened long-term outlook, and are ~40% more likely to develop a neurodegenerative disorder than younger individuals. This often contributes to the assumption that aggressive management of geriatric TBI is futile. Indeed, there is no cure for TBI, and our pathophysiological and mechanistic understanding of age-related TBI outcomes is limited because of the exclusion of older animals in pre-clinical studies. The present study (Ritzel et al., 2022) showed that long-term mortality was associated with older age at injury, but found that surviving mice exhibit a surprising level of endogenous functional recovery and therapeutic responsiveness. Molecular and cellular analyses identiﬁed age- and injury-related changes in innate immune and autophagy pathways late after TBI (Figure 1).
Continued oral treatment with a well-tolerated activator of autophagy, trehalose, was found to enhance long-term functional recovery and ameliorate microglial-mediated inflammation after TBI. This is one of the first experimental studies to demonstrate therapeutic efficacy in older mice late after TBI.

We would like to thank Miss Kavitha Brunner (University of Maryland School of Medicine) for her assistance with the illustration and proofreading of the manuscript.

This work was supported by the National Institutes of Health Grants K99 NS116032 to RMR and R01 AG077541, RF1 NS110637, 2RF1 NS094527, R01 NS110825, and R01 NS110635 to JW.

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Date of submission: May 21, 2022
Date of decision: July 4, 2022
Date of acceptance: July 20, 2022
Date of web publication: September 16, 2022

https://doi.org/10.4103/1673-5374.353487

How to cite this article: Ritzel RM, Wu J (2023) Functional phenotyping of microglia highlights the dark relationship between chronic traumatic brain injury and normal age-related pathology. Neural Regen Res 18(4):811-813.

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C-Editors: Zhao M, Liu WJ, Wang Lu; T-Editor: Jia Y

Perspective

Figure 1 | Relationship between microglial functions in normal aging and chronic TBI.

The diagram depicts various cellular and molecular changes in microglial function across the normal lifespan and chronically following traumatic brain injury (TBI). The resemblance in the functional phenotype of microglia from young chronic TBI (< 12 months old) and normal aged (> 18 months old) mice, and progressive worsening with increased age at the time of injury is consistent with the notion of injury-induced accelerated aging (created with Biorender.com).