

Influence of Peripheral immune-derived EphA4 on Microglial dynamics following
Traumatic Brain Injury

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ABSTRACT

Traumatic brain injury (TBI) elicits an immediate neuroinflammatory response that involves resident glia and infiltrating peripheral immune cells that coordinate tissue damage and functional deficits. The activation of resident microglia has been associated with a change in their morphology from a branched-like ramified cell to an amoeboid state. This activation is thought to initiate a pro-inflammatory response leading to the release of neurotoxic, immune chemoattractant, and antigen-presenting signals. Subsequently, peripheral-derived immune cells (PICs), such as neutrophils and monocytes, travel to the site of injury and help coordinate this response. However, little is known regarding whether PICs influence the progressive activation state of microglia in the acute and chronic phases of injury. Overactivation of microglia can lead to neuroinflammation-mediated tissue damage and death or dysfunction of healthy neurons. Therefore, understanding how microenvironmental cues may regulate the microglial response may aid in strategies to retool their activation state in the brain. EphA4 receptor tyrosine kinase has been identified as a potential cell-to-cell contact protein on PICs that could be involved in the inflammatory changes following TBI. While microglial activation changes have been described in TBI models, the mechanistic role of infiltrating peripheral-derived immune cell (PIC) recruitment on microglial fate and function is not well understood. The purpose of my project is to gain a better understanding of the temporospatial influence that EphA4-expressing PICs, specifically monocyte/macrophages, have on microglial proliferation, survival, activation phenotype, and debris clean-up using bone marrow GFP chimeric mice and the cortical contusion injury TBI model.

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General Abstract

Traumatic brain injury (TBI) triggers an immediate response from the brain's immune system, involving both local glial cells and immune cells from outside the brain.

These cells work together to mediate the initial injury but, in some cases, cause development of a secondary injury. Microglia, the brain's resident immune cell, change their shape and behavior when activated by a TBI, becoming more aggressive and releasing inflammatory proteins. At the same time, immune cells from the bloodstream, like neutrophils and monocytes, rush to the injury site to assist. Yet, it is unclear how

these immune cells affect microglia over time during the injury's acute and chronic phases. If microglia become too active, they can cause further damage to brain tissue and harm healthy neurons. Therefore, understanding the signals that control microglial activity could help us develop therapies to manage brain inflammation. One protein of interest in this process is the EphA4 receptor found on immune cells, which might play a

crucial role in inflammation following TBI. While we know that microglia change post-TBI, we do not fully understand how the recruitment of immune cells from outside the brain affects them. My research aims to clarify how EphA4-expressing immune cells, especially monocytes/macrophages, influence microglia in terms of growth, behavior,

and their ability to mediate a TBI.

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CHAPTER ONE

The following Literature Review has been published in the International MDPI Journal: Cells

Crosstalk and Subset Control of Microglia and Associated Myeloid Cells in Neurological Disorders

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Abstract

Neurological disorders are highly prevalent and often lead to chronic debilitating disease. Neuroinflammation is a major driver across the spectrum of disorders, and microglia are key mediators of this response, gaining wide acceptance as a druggable cell target. Moreover, clinical providers have limited ability to objectively quantify patient-specific changes in microglia status, which can be a predictor of illness and recovery. This necessitates the development of diagnostic biomarkers and imaging techniques to monitor microglia-mediated neuroinflammation in coordination with neurological outcomes. New insights into the polarization status of microglia have shed light on the regulation of disease progression and helped identify a modifiable target for therapeutics. Thus, the detection and monitoring of microglia activation through the inclusion of diagnostic biomarkers and imaging techniques will provide clinical tools to aid our understanding of the neurologic sequelae and improve long-term clinical care for patients. Recent achievements demonstrated by pre-clinical studies, using novel depletion and cell-targeted approaches as well as single-cell RNAseq, underscore the mechanistic players that coordinate microglial activation status and offer a future avenue for therapeutic intervention.

Distinct role for microglia and other myeloid cells in the neurological disorders

- a. Overview of the roles of CNS myeloid cells in the pathogenesis of neurological diseases

Myeloid cells of the central nervous system (CNS) have distinct ontogeny and function in health and disease [1]. Microglia, the parenchymal mononuclear phagocytic cells of the CNS, are detected in the brain during the early stages of embryonic development following emigration from the yolk sac [2] and remain self-maintained through-out adulthood [3-6]. While microglia

are the most abundant myeloid cells within the brain, an immunologically diverse population of non-parenchymal macrophages occupy the border regions of the immune-privileged CNS [7-9]. In response to neuroinflammation, circulating mononuclear myeloid cells, such as monocytes and dendritic cells, can infiltrate the brain and influence recovery. Unlike microglia, circulating myeloid cells are routinely re-placed by hematopoietic stem cells from the bone marrow (BM) throughout life. [10-12].

CNS resident microglia

Following birth, microglia play numerous roles essential to healthy brain development and maintenance; such as buffering of excessive and apoptotic cells, pruning of inefficient synapses, and regulating neuronal activity at the synaptic level ultimately fostering circuit plasticity [13]. Microglia primarily accomplish this via phagocytosis, a process through which cells recognize, engulf, and digest degraded cellular particles and debris. Following acquired brain injury, microglia are capable of acting as antigen-presenting cells to initiate activation of neighboring T-cells and orchestrate an inflammatory response that can impair proper neurological function [14-19]. In addition, microglia are activated following brain injury and undergo significant morphological changes to execute different tasks. Within the healthy brain, a predominant trait of homeostatic microglia is a ramified morphology which enables continuous monitoring of local environments and fine-tuning of neuronal circuits. De-ramification of microglia occurs upon activation; microglia retract their processes to acquire a bushy-like, amoeboid shape.

The contribution of microglia to the neuroinflammatory response is context-dependent and temporally regulated [2, 5, 12, 14-16, 18-38]. In the neuroinflammatory milieu, different mediators activate microglia polarization to pro-inflammatory (e.g. TNF α , INF γ producing) or anti-inflammatory (e.g. IL-4, IL-13, IL-10) phenotypes [39]. Pro-inflammatory microglia secrete abundant amounts of inflammatory cytokines and chemokines (e.g. TNF α , IL-6, IL-1 β) that not only initiate inflammation in the brain but aid in the recruitment of peripheral-derived immune cells. Together with peripheral-derived macrophages, microglia contribute to inflammation-mediated cytotoxicity of neurons, glia, and endothelium [2, 15, 17, 21, 33, 40-43].

Peripheral-derived monocytes/macrophages

The inflammatory response initiated by CNS glia is responsible for the recruitment of peripheral-derived myeloid cells including monocytes [16, 44-46]. Monocyte subset polarization has been demonstrated to drive early progressive tissue loss and dysfunction - serving as a good predictor of injury outcome following brain trauma [47, 48]. Traditional stratification of monocytes includes classical (CD14^{hi}CD16⁻ human; Ly6ChiCD43⁻ mouse), non-classical (CD14^{dim}CD16⁺⁺ human; Ly6CloCD43⁺ mouse) and intermediate (CD14⁺CD16⁺; Ly6C^{int}CD43⁺) subsets [10, 49, 50]. It is well established that these monocyte subsets infiltrate the brain following traumatic injury in mice and that the CCR2-CCL2 axis is essential for the recruitment of the Ly6Chi subset [51]. Classical monocytes are involved in phagocytosis, pro-inflammatory responses, intermediate cells exclusively express CCR5 and mediates antigen presentation, while non-classical cells are involved in complement and Fc gamma-mediated phagocytosis and adhesion [52-54]. A subset of non-classical monocytes express CD9 and SLAN which suggests increased efferocytosis and migration functions compared to the SLAN-negative population [53, 55]. Subsets of macrophages have been identified in the injured brain [48].

Monocyte depletion studies demonstrated that peripheral-derived monocytes contribute to the pathophysiology of several neurological disorders including but not limited to Alzheimer's disease (AD), intracerebral hemorrhage, traumatic brain injury and multiple sclerosis [51, 52, 56-65]. Mice receiving intravenous injection of clodronate had reduced numbers of CCR2⁺Ly6chi monocytes infiltrating the brain following injury suggesting subset control modulates tissue damage [52]. Importantly, the use of CCR2^{-/-} mice revealed that altering monocyte influx directly affects the production of type1 interferon genes in resident microglial subsets [47]. Loss of Ccr2 also accelerates early disease progression, cognitive impairments, A β plaque burden and alters microglia accumulation and phenotype in murine models of Alzheimer's disease [62, 63]. These studies demonstrate the likely influence of peripheral-derived monocytes on microglia activation in the diseased brain. Bone marrow-derived macrophages (BMDMs) play crucial roles in the pathophysiology of numerous neurological conditions and their modulation, through bone

marrow transplantation or gene therapy, represents a key therapeutic strategy to treatment, as reviewed elsewhere [66].

CNS border-associated macrophages

Single-cell RNA sequencing (scRNA-seq) of the immune compartments within and bordering the CNS identified microglia as the long-lived, yolk sac-derived myeloid cells within the CNS parenchyma, while border-associated macrophages (BAMs) were the primary myeloid cells within the peri-vascular (pvM ϕ), choroid plexus (cpM ϕ), and meninges (mM ϕ) [67-69]. The central role for BAMs in normal brain physiology and the pathogenesis of disease is reviewed elsewhere [1, 9]. Briefly, pvBAMs may contribute to vascular inflammation, blood brain barrier (BBB) permeability, nutrient exchange and metabolism, as well as HPA axis regulation [70, 71]. Given that the choroid plexus interfaces between the periphery and CNS, immune cell activation in this compartment may aid in T cell trafficking and antigen presentation in the meninges [67, 68, 72], along with neutrophil invasion [69, 73]. With an opportune location, cpM ϕ are crucial in the development of neurological sequelae of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE), and TBI. In meninges, the fenestrated endothelium grants passage of microorganisms to prime the brain against future potential harm from circulating microorganisms in the context of injury; this concept has been termed the “gut-meningeal immune axis” [74]. mM ϕ are both physically and transcriptionally distinct from microglia [33] as demonstrated in mild TBI patients where meningeal vascular damage was resolved owing to local and infiltrating CD206+ myeloid cells that scavenged dead cells and promoted angiogenesis [75]. As the immune gatekeepers of the CNS, BAMs control over immune cell entry, CSF/blood ex-change, and debris clean-up makes them key contributors to neuroinflammation in acquired neurological disease.

b. New insights into disease-associated CNS myeloid subsets

CNS resident microglial subsets

There is a transcriptional distinction between homeostatic resident microglia and their disease-associated counter-parts [76, 77]. The main classifications of microglia have been extensively reviewed [18, 39, 78]. Briefly, mature microglia display a homeostatic, surveilling phenotype, express key TFs Jun, Fos, Mef2a, and Mafk, and the characteristic markers TMEM119, purinergic and chemokine receptors P2ry12, Csf1R and Cx3cr1. Csf1R, for example, is critical for microglial survival and its null mutation removes 99.7% of the microglial cell population, while a few morpho-logically-distinctive microglia remain near the piriform cortex, hippocampus, thalamus and dentate gyrus [79]. Zhan L, et.al. used CX3CR1-CreERT2/Rosa26-stop-DsRed mice to show that repopulation after administration of the Csf1R inhibitor, PLX5622 (PLX), [80, 81] required a PLX-resistant, nestin-positive microglial progenitor that displays upregulation of Galectin-3 or Mac2 (a ligand for Trem2 [82]) and other immature microglial genes and downregulation of mature markers Tmem119, Mafk, Cx3cr1, and Csf1r [83]. Others showed that the Itgax (CD11c+) microglia sub-set is highly proliferative and prominent in the developing CNS in areas of myelination that express Igf1, Spp1, and Gpnmb [37, 84].

While recent scRNAseq findings show several distinct homeostatic subtypes based on cluster analysis in the human and mouse brain [76, 85], putative phenotypes with unique specifications, such as axonal interaction, supporting myelination and neurogenesis, and synaptic pruning have been described in different brain regions [36]. However, following insult, it is well-established that microglia diversify their phenotypic identity and function, resulting in a continuum of states that include pro-inflammatory and/or anti-inflammatory signatures in response to environmental stimuli [18, 21, 30, 42, 86]. In general, their expression of pro-inflammatory cytokines, such as TNF, IL1 β , IL12 and reactive oxygen and nitrogen species (ROS and RNS, respectively) can mediate the recruitment of innate immune cells and induce neurotoxic effects [14]. Microglia expression of nitric oxide synthase (iNOS) also contributes to the neuroinflammatory environment and neuronal cell death [25]. Review of traditional classifications of phenotypic states identified in brain injury has been discussed previously [29, 42, 87]. More recently, however, lineage tracing and scRNA-seq studies [3, 8, 76, 85, 88] suggest that more distinct subsets exist, highlighting a complex landscape in the context of neurological disease, where microglia may adopt both anti- [22] and pro-inflammatory features

[48] with transcriptional states that vary based on brain region, age, and disease pathology [7, 37, 85, 89]. Therefore, the traditional classifications [29, 43, 48], have been reconsidered given the heterogeneity now observed across health and disease.

Subsets of microglia in the human brain

scRNAseq and time-of-flight mass cytometry performed by Sankowski et al. revealed a spectrum of transcriptional states in human microglia during homeostasis, aging, and disease [35]. Nine clusters (C1-C9) were identified: C2-C3 were homeostatic clusters with high core gene expression CX3CR1, CSF1R, P2RY12, P2RY13 and TMEM119; C6-C7 clusters showed low CX3CR1, high MHC-II, and metabolism genes (such as ApoE and LPL); C1, 5, 8, 9 were characterized by high transcript expression of chemokines and cytokines. Importantly, cluster enrichment was region-specific with MHC-IIhigh clusters residing in white matter and MHC-IIlow residing in gray matter microglia as well as age-specific with >50 years having enriched C6 and C7 with higher expression of SPP1 (osteopontin), a pro-inflammatory cytokine. Similarly, following analysis of cortical microglia isolated from healthy human brains (with no evidence of CNS pathology), Masuda et al., identified 4 clusters of microglia (C1-C4). C1 and C2 had higher expression of CST3 and P2RY13 than C3 and C4. In addition, C4 had high levels of chemokine genes CCL2 and CCL4 and transcription factors EGR2 and EGR3 indicating that homeostatic human microglia include subsets with distinct gene expression patterns [89].

Esaulova E, et. al, surprisingly showed a discrete population of microglia having homeostatic genes *cx3cr1*, *csf1r*, *slc2a5*, *marcks* and *P2ry13* in the CSF and blood of patients with MS. When applying the classifier tool from Sankowski et al., the cells were identified as microglia cluster 6 and 7 with gene ontology described as antigen processing [88]. Microglia heterogeneity was further validated in the brains of patients with temporal lobe epilepsy (TLE), mild cognitive impairment (MCI), and Alzheimer's disease (AD) showing 9 distinct clusters that expressed microglia-enriched genes. Clusters 1 and 2 were the most common across all patients and were considered typical homeostatic states. C1-3 comprised cells with closely related signatures, as did C5 and C6, but the remaining micro-glial clusters showed more distinct signatures. Further, cluster inter-relatedness suggested that differentiation of each cluster

emerged radially from a common cell fate. The temporal neocortex of TLE patients showed an increased frequency of three microglia clusters: C5 (CD83-positive cells enriched with transcription factors CREB and ATF and anti-inflammatory genes IL4, IL10, and IL13), C6 (CD83-positive cells enriched with anti-inflammatory genes), and C7 (CD74hi cells enriched with antigen presenting genes). However, the dorsolateral prefrontal cortex of AD patients showed reduced frequency of C7 microglia [90]. In addition, brains of healthy humans and patients with MS showed 7 myeloid clusters expressing microglia core genes [89]. C5, C6, and C7 microglia are entirely from healthy brain showing highest expression of core genes. C4 clusters contained microglia from both healthy and MS brains showing reduced core gene expression and elevated CCL2, CCL4, EGR2, and other cytokine and chemokine genes. C2, C3, and C8 microglia were enriched in brains of MS patients showing downregulation or absence of core genes including TMEM119 and upregulation of APOE and MAFF. C3 microglia showed increased expression of MHCII-related molecules indicating an immunomodulatory role of this microglial subset. A detailed summary of the alterations in gene expression between microglial clusters in healthy and diseased human brains can be found in Table 1.

Subsets of microglia in the murine brain

Evaluations of microglia in the murine brain have also revealed a more complex cell-state using scRNA-seq and clustering analysis, which demonstrated distinct microglial clusters across age and injury [85, 89]. Hammond, et.al., showed nine clusters (C1-C9) that exist across age and injury with differential gene expression observed to be cluster-specific, except canonical genes C1qa, Fcrls, P2ry12, Cx3cr1, Trem2 which were expressed in most clusters and C1qa, Fcrls, Trem2 expressed regardless of age or perturbation. Likewise, F13a1, H2Aa, Ccr2, Lyve1 and Mgl2 were identified as monocyte/macrophage specific [85]. Embryonic brains were enriched with C6 microglia expressing MS4a7 and sharing a similar transcriptional profile with mature microglia, peripheral macrophages, and BAM suggesting an intermediate state that possibly downregulates macrophage-related genes upon entry into the brain. Interestingly, no specific clusters appeared with aging; instead, an expansion of microglia expressing inflammatory and interferon-responsive signals was seen. Similarly, Masuda et.al., revealed ten clusters (C1-C10) with variable distribution, both spatially and temporally, across embryonic and post-natal

development [89]. Greater heterogeneity in subtypes occurred during embryonic development (C1-C6) compared to the postnatal period (C7-C10), whose post-natal expression includes homeostatic genes *Tmem119*, *Selplg* and *Slc2a5*. In this study, embryonic clusters showed high expression of lysosomal genes (e.g. *ctsb*, *ctsd*, *lamp1*) and the amyloid cell activation marker (*ApoE*). These distinct populations disappeared in postnatal brains. Additional clusters emerged after facial nerve axotomy (C11) and cuprizone treatment (C12-C13) in a time-dependent fashion. Disease-specific signatures revealed upregulation of demyelination, remyelination, and MHCII genes along with downregulation of *TMEM119*. The variable distribution of clusters across brain regions suggests that the local microenvironment drives subtype needs fostering expeditious expansion, enhanced synaptic pruning, cell motility or immunological needs of the brain [23, 91-93].

Similar studies showed that TBI resulted in 4 distinct clusters over the course of progressive injury each (C1-C4) related to host defense response (C1), synaptic potentiation (C2), lipid remodeling (C3), and membrane polarization (C4) [31]. In EAE, four disease-associated microglial clusters (DAMg 3-6) were observed showing downregulation of canonical genes and upregulation of genes related to immune regulation, cell activation, proliferation, and chemokine/cytokine production [76]. Flow cytometric analysis can aid in the identification of pro- and anti-inflammatory DAMs in AD models leading to studies on the response to various drug therapeutics. For example, use of the Kv1.3 channel blocker, ShK223, was found to reduce expression of pro-inflammatory while also influencing production of anti-inflammatory DAM genes, phagocytic uptake, and clearance of AB [94]. The discovery of DAMs has created an opportunity to develop more targeted therapy aimed at modulating this inflammatory subset to quell neuronal injury in diseases of the nervous system. Collectively, these studies have advanced our understanding of the multi-faceted nature of microglial subpopulations which offer potentially innovative approaches designed to target defined subsets for therapeutic intervention. A summary of genes altered in murine microglia clusters within healthy and diseased brains is shown in Table 1.

CNS border-associated and peripheral-derived macrophage subsets

Each region-specific BAM population is endowed with distinct transcriptomic clustering, which have been shown to mediate immune responses at the brain boundaries and whose generation requires the transcription factor (TF) PU.1, unlike short-lived peripheral-derived myeloid cells such as monocytes and dendritic cells [7, 76, 95, 96]. High-dimensional single-cell proteome mass cytometry shows that surface marker expression of CD38 and MHC-II are distinguishing features of BAMs at steady state and their 4 subsets include Cd38+/MHCII-/Ccr2-, Cd38+/MHCII+/Ccr2-, Cd38-/MHCII+/Ccr2-, Cd38-/MHCII+/Ccr2+ [8]. Only Ccr2+ BAMs are enriched in the choroid plexus and are proposed to be replaced routinely by BM-derived monocytes [7, 8]; these homeostatic phenotypes were also identified by scRNA-seq [76].

Multiparametric single-cell analyses have revealed subsets that represent either distinct stages of linear differentiation or functionally distinct subsets that emerge under emergency conditions, such as neutrophil-like monocytes [97] to initiate inflammation or promote healing [50]. Recent studies using Ccr2-CreER-based fate-mapping showed that monocytes infiltrating the brain after hypoxic-ischemic (HI) injury were maintained as TNF/MHC-II macro-phages while others were Tmem119/Sall1/P2ry12 ramified microglial-like cells that persisted for months [17]. In addition, monocyte-derived macrophages were analyzed by scRNA-seq profiling in TBI and revealed a mixture of polarized subsets [98]. However, this may reflect the early stage (1-day post-injury) where the initial wave of immune cell entry may be from one subpopulation. These and other findings discussed later, highlight the ability of peripheral-derived monocytes to generate several subsets including those with microglial-like phenotypes and functions in the brain.

c. Peripheral-derived monocyte engraftment and cross-talk with microglia & BAMs

Under defined experimental conditions, peripheral-derived macrophages have demonstrated a unique ability to replace the long-lived BAMs and microglia during neuroinflammation [99-101]. It has been demonstrated, using transcriptomic and proteomic analysis, that these populations have distinct regional ontogeny with divergent transcriptional and immunological signatures both in development and diseases of the brain [102]. A number of previously identified microglial-specific markers, such as TMEM119, show reduced

immunoreactivity in disease [103, 104] and are expressed by peripheral-derived cells that enter the brain under pathological conditions such as ischemic stroke, EAE, MS, 6-OHDA [105-107], which we further demonstrated in our own work following focal traumatic cortical impact injury [108]. Furthermore, peripheral-derived macrophages have been shown to express transcripts for common microglia-specific markers early during development, including TMEM119 as well as Fcrls, P2ry12 and Trem2 [109]. Fate mapping experiments with reporter-labeling models have been instrumental in identifying cell origins and cross-talk between populations [15, 95]; these include GFP bone marrow chimeras, and Cx3cr1GFP/Ccr2RFP, Cx3cr1CreER/R26tdTomato/stopRFP mice (Figure 1).

Blood brain barrier integrity and peripheral-derived immune cell invasion

In non-pathologic states, the brain parenchyma is a highly immune-privileged tissue separated from the blood via the BBB, a non-fenestrated endothelial barrier with tight junctions composed of transmembrane proteins such as claudins, occludins and junctional adhesion molecules [110]. Yet in the absence of neuroinflammation, perivascular BAMS and microglia interact with peripheral-derived T cells via antigen presentation to facilitate immune surveillance [111]. Activated T cells then cross the BBB endothelium via paracellular (tight junction-dependent) or trans-cellular (endothelial cell receptor and charge-dependent) transport mechanisms, while preserving the integrity of the BBB [112, 113]. As neuroinflammation progresses, chemo attractants (e.g., chemokine-like factor-1) mediate neutrophil migration, receptors (e.g., CCR2) recruit monocytes, and endothelial cell receptor expression (P-selectin, ICAM-1) promotes paracellular permeability, causing blood-borne immune cell infiltration into a disrupted BBB [114].

External factors influencing CNS engraftment of peripheral-derived monocytes

Peripheral-derived monocytes that infiltrate the CNS can acquire a microglial-like phenotype and function that is driven by environmental signals [101, 115, 116]. Under defined conditions, peripheral-derived monocytes/macrophages give rise to microglia that are phenotypically indistinguishable [101, 115, 116]. The micro-glia-vacant niche is replaced, in

part, by peripheral-derived monocytes following lethal whole-body γ irradiation, in response to microglia depletion using the pharmacological agent PLX5622, genetic modification using CD11b-HSVTKI, and in tamoxifen-treated Cx3cr1CreER^{+/+}/R26DTA^{+/+} or Cx3cr1CreER^{+/+}/Csf1rf/f mice (Figure 2) [81, 117]. The BBB is not disrupted, and microglia replacement requires the sustained engraftment of the Ly6chi/Ccr2⁺ subpopulation that gives rise to F4/80hi macrophages [99, 115, 117]. Long-term engraftment did not occur when the brain was shielded from irradiation or in control Cx3cr1CreER^{+/+} mice. Increased cytokine production is postulated as a key attractant of Ly6chi monocytes [51]. While these cells upregulate microglial-specific genes (TMEM119, P2ry12, Siglech, etc.) they remain transcriptionally independent from naïve microglia, lacking Sall1 transcription factor but notably, upregulating transendothelial migration genes (Itga4, Vcam1, Itgal), which correlated with vascular activation suggesting that they gain entry through transcytosis mechanisms and have distinct morphology and function such as higher motility in response to stimuli [117]. This may be due to their unique epigenetic programming from the origin of birth. It should be noted that the source of irradiation, gamma vs x-ray, and shielding from irradiation differs across studies. Microglia replacement has not been demonstrated using x-ray irradiation [7, 52, 118] but shows extensive repopulation in gamma irradiated conditions [101, 115]. Due to the recent discovery of a skull bone marrow and myeloid cell reservoir, it is plausible that engraftment may also occur as a result of this population [11, 119-121]. However, parabiotic experiments [115] and head shielded chimeras using CD45.1 and CD45.2 cells [117] suggest that the CNS-engrafted cells are primarily from circulation.

The loss of Cx3cr1hiCcr2lo-neg meningeal macrophages after mild TBI can also result in the invasion of Cx3cr1loCcr2hi monocytes, however this response is transient and temporally regulated [75]. Studies have shown that BAMs are readily, and almost completely, exchanged after artificial whole-body irradiation and bone marrow transplantation [122, 123]. Meningeal macrophages (mM ϕ) serve as sentinels for foreign invaders and are gatekeepers of the neuroimmune response. Peripheral-derived monocytes can also repopulate an open niche created by meningeal macrophage loss, for example by viruses, however they fail to fully mimic their predecessors [116, 124]. Lineage tracing via Cx3cr1CreER/Stopfl/fl TdTomato mice showed that viral destruction of mM ϕ resulted in their repopulation by circulating monocytes whose gene

profile was enriched for IFN- γ -related genes paired with a lower frequency of MHC-II. This event appears to be context-dependent as additional KitMerCreMer/R26YFP fate-mapping analysis [125, 126] has demonstrated that microglia and F4/80hiCD206+ CNS border-associated macrophages were stable during the progression of AD pathology whose turnover rate by circulating monocytes was less than 10% (Figure 2) [126].

Maintenance of distinct identities following cell replacement

Using fate tracing methods to discriminate BAMs, microglia, and PDMs whole genome sequencing (WGS) has identified several specific signatures. Cronk, J. et. al., identified 52 microglia genes that were not present in brain engrafted peripheral-derived macrophages [115]. Grassivaro F, et. al., identified 65 unique transcripts that were distinct throughout the life of microglia that were not expressed by peripheral macrophages [109]. When comparing these lists, we observed that *sparc*, *capn3*, *rtn4r11*, *sall1*, and *sall3* were unique to microglia regardless of myeloid comparison. The use of *Sall1*GFP reporter mice confirmed that this transcription factor is restricted to microglia and not BAM or peripheral-derived macrophages [8]. The Spalt-like transcription factors *sall1* and *sall3* were also noted to be microglia-specific when comparing the competitive repopulation after microglia depletion [117] and using gene expression profiling [127-129]. *Sall1*-deficient mice display proinflammatory, amoeboid microglia [129], altered neurogenesis, and tissue homeostasis [127]. *Sall1* is also downregulated in microglia following intracerebral hemorrhage (ICH) at 1 day but is restored at later time points suggesting that suppressing its expression mediates the pro-inflammatory state in the acute phase of disease. Among the core microglial genes, the *Sparc* transcript has been shown to be stable during EAE pathology [76] but is rapidly downregulated at the protein level in ischemic and excitotoxic lesions, mediating microglia proliferation [130]. These studies demonstrate that while peripheral-derived macrophages can act as a cell source for microglial replacement (due to damage or senescence), microglia remain transcriptionally distinct from other myeloid cell subsets.

d. Divergent neuroinflammatory functions of CNS macrophages in diseases of the brain

Peripheral-derived myeloid cells gain access to the brain in the presence of an intact BBB, due to the local inflammatory environment created by experimental elimination of microglia following gamma irradiation or depletion. Their recruitment and coordination with resident microglia in mediating neuroinflammation as a consequence of acquired injury such as stroke, TBI, AD, and cancer (glioblastoma, gliomas) has been extensively evaluated [5, 12, 14, 21, 40, 63, 90, 131-137].

Early clonal expansion of resident microglia occurs alongside infiltrating monocytes/macrophages which transiently integrate into the parenchyma following EAE [76]. Using single-cell profiling in this same model, distinct myeloid fates were identified in the neuroinflammatory milieu, where microglia generate defined subsets while their role in antigen presentation is co-opted by dendritic and monocyte-derived cells with prolonged T-cell interactions, which are presumed to be the major players in disease progression [88]. This comports with additional findings that show higher expression of MHCII (H2aa, H2ab1 and Cd74), as well as more efficient antigen presentation and T-cell proliferation, in peripheral-derived macrophages compared to microglia in the hemorrhagic brain [102]. Transcriptional profiles of resident microglia and CNS-infiltrating macrophages showed enriched gene sets that remained distinct over time post-injury, with microglia displaying genes necessary for immune migration, complement (C1qb, C1qa, Trem2 for synaptic pruning), and inflammation [3, 8, 76, 89]. Subsets of disease-specific myeloid cells also can be identified in the brain and pia following ischemic stroke. Single cell transcriptomic analysis described 4 microglia clusters with Tmem119, Hexb, Cx3cr1, Sparc, P2ry12, Cst3 as transcript signatures and five macrophage clusters with CAM, SAMC, MARCO, Lyz2 and Apoe transcripts, one of which showing BAM expression of Pf4 and Lyve1 [138]. These studies suggest that while resident and CNS-infiltrating macrophages co-exist in the diseased brain, they play complementary roles that are not mutually exclusive in neuroinflammation.

Clinical assessment of microglial activation and CNS-infiltrating macrophages

Infiltration of peripheral-derived monocyte/macrophages along with activation of resident and CNS-associated macrophages play a significant role in the progressive pathology of brain

disorders such as acquired injury and de-generative conditions. A number of clinical approaches have emerged to evaluate these changes using CT, PET, and MRI imaging. To improve clinical management across a variety of brain disorders, monitoring of microglial activation in vivo will aid in the selection and implementation of novel therapeutics. Several studies have demonstrated that new imaging techniques offer direct visualization of neuroinflammatory sequelae. When these imaging techniques are combined with microglial-specific biomarkers and clinical assessments, their value in predicting disease outcome may be greater than alone. As disease-associated microglia and CNS or peripheral macrophages orchestrate the progression of the CNS inflammatory response, advanced imaging techniques in the clinical setting to monitor these myeloid changes may aid in developing prognostic biomarkers for neurological disorders.

a. Imaging techniques: Positron emission tomography (PET), diffuse-weighted MRI, and Magnetic Resonance Imaging (MRI)

To date, several microglia-related PET radiotracers and MRI contrast agents have been administered to patients with AD, IS, and TBI to assess the extent of microglia-mediated neuroinflammation. Overall, findings utilizing these new modalities suggest that cell-specific neuroinflammation can be visualized with whole-brain imaging. Translocator protein 18 kDa (TSPO), a benzodiazepine receptor expressed in the mitochondrial outer layer of microglia, is a target in PET imaging (Yao et al., 2020). One TSPO-targeting radioligand, 1-(2-chlorophenyl)-N-[¹¹C] methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide ([¹¹C]PK11195), is lipophilic and exhibits non-specific binding but its R--enantiomer binds TSPO with 2x greater affinity [139] and demonstrates comparable levels in amyloid load in the brains of AD patients [140]. In TBI [¹¹C] DAA1106 is highly selective for TSPO and localizes to the region of injury for a longer time than the [¹¹C]PK11195 R--enantiomer [141]. TSPO is also expressed in blood-borne infiltrating monocytes/macrophages, suggesting that this radioligand target may be reflective of broad myeloid activation [142]. To mitigate this limitation of PET imaging, a recent preclinical study investigated the utility of diffusion-weighted MRI to map microglia activation [143]. This method is specific to micro-glia through the combination of three MRI parameters: the stick fraction, the stick dispersion, and the small sphere size. Lastly, MRI imaging of blood-borne macrophage invasion across several disease states is possible when using ultra-small particles of

iron-oxide (USPIO) [144]. The combination of TSPO-targeting PET (local microglia and blood-borne macrophages) and iron-oxide-enhanced MRI (blood-borne macrophages) may elucidate the extent of microglial vs peripheral-derived mediated changes.

b. CSF and blood biomarkers to detect microglia activation

While MRI and PET may detect microglia activation *in vivo*, limitations such as cell specificity and radioligand quality require inclusion of additional assessments such as soluble biomarkers present in blood or CSF. There is a growing need to develop biomarkers to detect microglia subsets in the clinical setting. To date, several micro-glia-related biomarkers have been measured in CSF and serum of patients with neuroinflammation. These are summarized in Table 2.

These markers include triggering receptor (TREM2) expressed on myeloid cells [145, 146] whose cleaved, soluble form (sTREM2) is detectable in CSF and may reflect the microglial response, especially in AD, sports-related repetitive TBI, ischemic stroke, and MS [135, 145-154]. NLRP3 inflammasome [155-158] is increased in CSF and associated with worse functional outcome in pediatric TBI patients [159]. Adiponectin, an adipocyte-secreted protein signal [160] is also increased under a prolonged state of neuroinflammation in AD [161], is used as a diagnostic tool in severe TBI [162] and associated with disease severity in MS [163]. Finally, high mobility group box protein 1 (HMGB1) is highly expressed in phagocytic microglia [164] which may reflect AD progression [165, 166] as well as ischemic stroke, TBI and MS severity [167-170]. Galectin-3 drives microglial activation [171-174] and is associated with a worse AD [175, 176], TBI, and stroke [173, 177, 178]. Fractalkine/CX3 chemokine ligand 1 (CX3CL1) is higher in plasma of patients with lower stroke severity [179]. Progranulin (PGRN) may attenuate aberrant microglia/macrophage-mediated neuronal damage [180-182] and its diagnostic utility in AD is sex- and age-dependent [147, 183]. Higher serum levels of GPRN are associated with a high risk of adverse functional outcome in stroke and differentiate radiologically isolated MS from healthy controls [184, 185]. Finally, the transmembrane Protein 119 (TMEM119) could be a biomarker of AD progression [186].

A combinatory approach that includes advanced imaging and blood/CSF biomarkers reflecting microglial changes, along with clinical assessments may have greater predictive value in monitoring disease progression. Recently it was shown that combining (non-microglial) MRI, PET, CSF biomarkers, and clinical assessment better predicted a patient's conversion to AD than a clinical assessment alone [187]. Thus, we propose that the development of a suit-able pipeline that includes microglia-specific imaging, selected biomarkers, and clinical assessment may more accurately predict disease outcome than each of these approaches alone.

Summary

Neuroinflammation caused by disease, or through experimental strategies to deplete long-lived resident microglia, can allow entry of peripheral-derived monocytes/macrophages (PDMs) into the brain. This may act as a cell source for microglia replacement or as a partner in mediating the neuroinflammatory milieu. Recently it was shown that regardless of how many PDMs entered the brain, their polarization state was most important in dictating out-come from brain injury. It remains unresolved which transcriptionally distinct PDM subsets influence early vs chronic stages of disease and whether they work in concert with resident microglia to restore homeostatic balance. Importantly, future studies may address sex differences and how PDM subsets interact with ontologically distinct microglia to regulate their phenotypic state and whether this cross-talk can be recalibrated to control expression of pro- and anti-inflammatory responses. Overall, resident microglia are key players in the progression and resolution of injury to the CNS. Microglia production of inflammatory cues aid in the recruitment of peripheral-derived immune cells, including monocyte/macrophages, whose coordination may be central to regulating the neuroinflammatory milieu across numerous neurological disorders. The use of single-cell RNA sequencing has helped detail the complex transcriptomic characteristics of newly identified myeloid derivatives, as well as enhanced our understanding of their origins and local responsibilities across the lifespan.

Lastly, there is a need to develop new combinatory diagnostic tools (blood-based and/or imaging biomarkers) in the clinical setting to track microglial/PDM activation states in the brain. This advancement would improve prognostic indicators of disease outcome and enable the

successful monitoring of new therapeutic strategies aimed at quelling a progressive pro-inflammatory response. Using murine models, we understand that PDMs can enter the brain in the absence of BBB disruption through upregulation of genes required for transcytosis. The identification of pro-resolving PDM subsets that are long-lived in the brain under disease conditions may be an attractive cell therapy to combat chronic neuroinflammation.

Figures & Tables

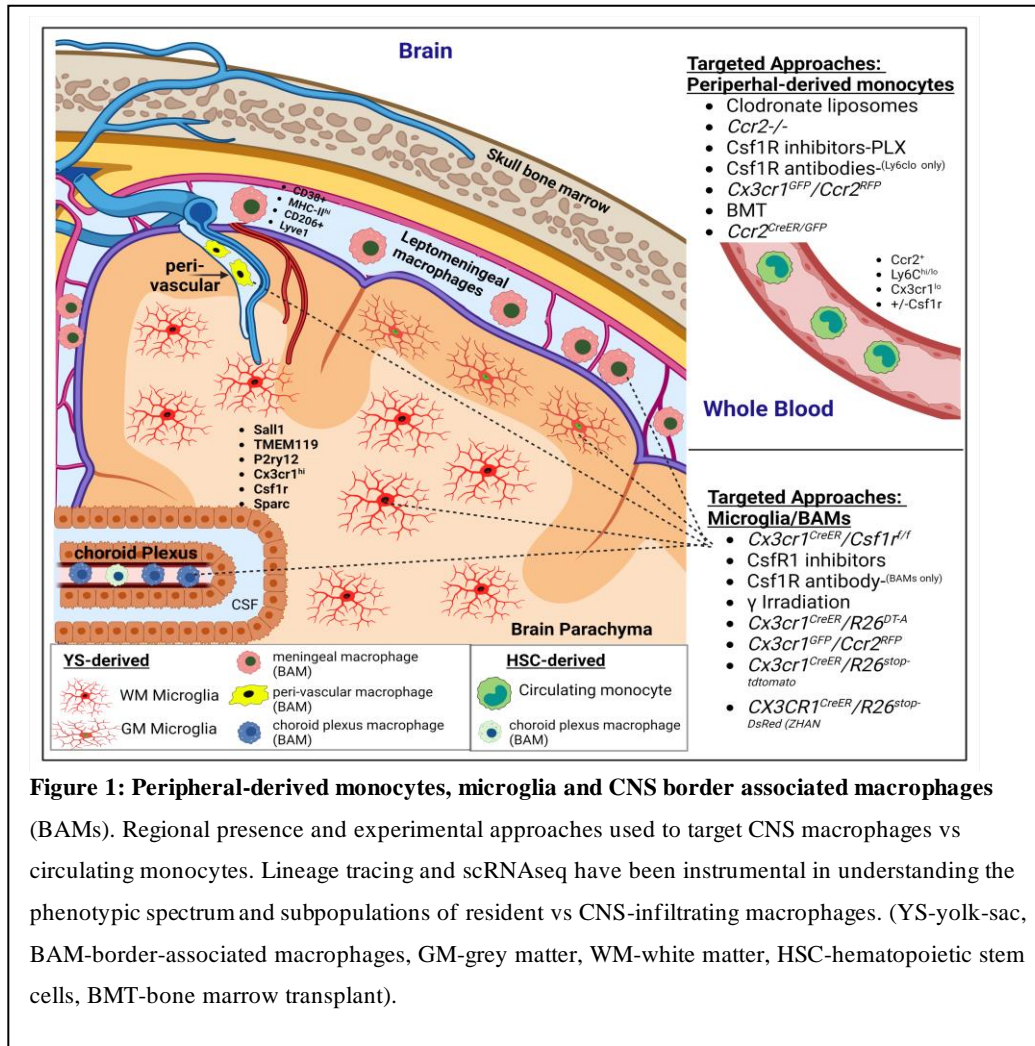


Figure 1: Peripheral-derived monocytes, microglia and CNS border associated macrophages (BAMs). Regional presence and experimental approaches used to target CNS macrophages vs circulating monocytes. Lineage tracing and scRNAseq have been instrumental in understanding the phenotypic spectrum and subpopulations of resident vs CNS-infiltrating macrophages. (YS-yolk-sac, BAM-border-associated macrophages, GM-grey matter, WM-white matter, HSC-hematopoietic stem cells, BMT-bone marrow transplant).

Table 1. Human gene signatures of microglia subsets in healthy and diseased brain. Microglia clusters identified by the expression of core genes.

Study	Human Tissue	Techniques	Subsets/Cluster:	Enriched genes	Function
Sankowski et al.,	Healthy tissue	scRNAseq	9 clusters (C1-C9)		
	Temporal lobe (Grey & white matter)	Time-of-flight mass cytometry	C1	<i>CCL2, IL1B</i>	Chemokine and cytokine inflammatory genes
			C2 (WM)	<i>MHC-II, HLA-DRA, CD7, IFI44L</i>	Chemokine and cytokine inflammatory genes
		C3 (GM)	<i>CX3CR1, TMEM119</i>	Homeostasis c	
		C5 (WM)	<i>MHC-II, CCL2, IL1B</i>	Homeostasis	
		C8 (GM)	<i>CCL2, IL1B</i>	Chemokine and cytokine inflammatory genes	
		C6-C7 (WM)	<i>MHC-II, SPP1, APOE (>50 y) and LPL</i>	Integrin-receptor-binding protein and metabolism genes	
Masuda et al.,	Healthy tissue	scRNA -seq (Cel-Seq2 protocol)	4 clusters (C1-C4)		
	MS tissue		C1-C2	<i>CST3, P2RY13</i>	Microglia activation and homeostasis
			C4	<i>CCL4, CCL2, EGR2, EGR3</i>	Cytokine inflammatory genes and zinc finger transcription factors
		7 clusters (C2-C8)			
		C2	<i>APOE, MAFB, CTSD, APOC1, GPNMB, ANXA2, LGALS1</i>	Microglia activation	
		C3	<i>APOE, MAFB, CD74, HLA-DRA, HLA-DRB1, HLA-DPB1</i>	Microglia Activation & Immunoregulation	
		C4	<i>CCL2, CCL4, EGR4</i>	Cytokines and zinc finger TF	
		C5-C7	<i>TMEM119, P2RY12</i>	Homeostasis	
	C8	<i>APOE, MAFB, SPP1, PADI2, LPL</i>	Microglial Activation & Demyelination		

Olah et al.,	TLE, MCI, AD	scRNA-seq	9 clusters (C1-C9)
			C4 (MS, AD) <i>IRF1, IRF8, IFITM3, ISG15+</i> IFN-response
			C5 (TLE) <i>CREB, ATF, IL10, IL4, IL13, CD83</i> TF & anti-inflammatory
			C6 (TLE) <i>IL10, IL4, IL13, CD83+</i> Anti-inflammatory
			C7 (TLE, MCI; reduced in AD) <i>APOE, TREM2, CD74^{hi}</i> Microglia activation and Antigen presentation
			C9 <i>E2F1, CBFB, NRF1</i> Cell cycle

* WM=white matter; GM=grey matter; TLE=temporal lobe epilepsy; MCI=malignant cerebral infarction; scRNA-seq=single cell RNA sequencing.

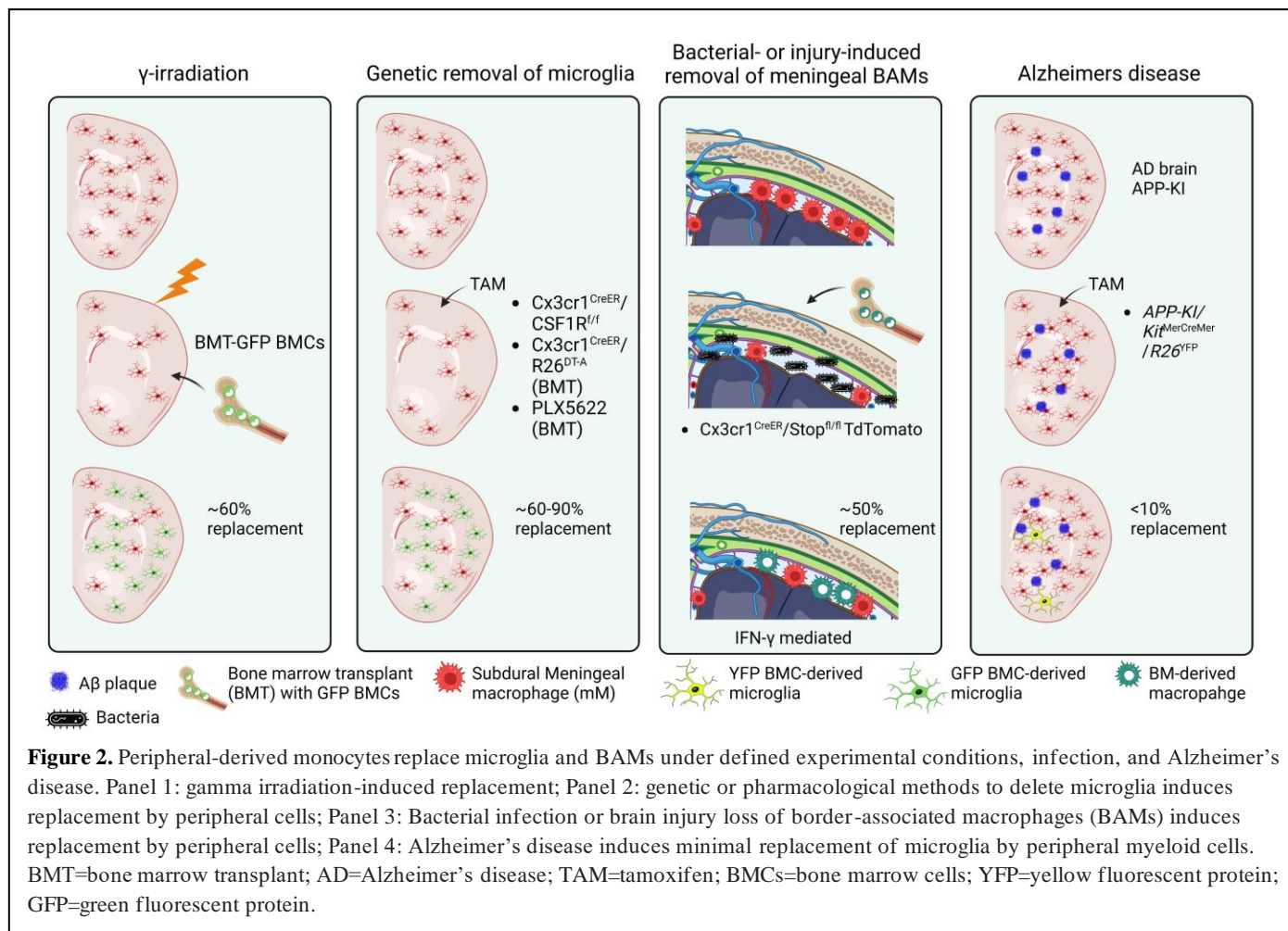


Table 2: Blood/CSF biomarkers targeting microglia in AD, IS, TBI, and MS

Biomarker name	Abbreviation	Microglia connection	Disease state	Source	Direction	Sources
Soluble triggering receptor expressed on myeloid cells 2	sTREM2	Expressed in microglia, increases inflammatory cytokines	AD	CSF	Up	Bekris et al., 2018; Gratuze et al., 2018; Suárez-Calvet et al., 2019
			IS	Blood	Up	Rauchmann et al., 2019; Kwon et al., 2020; Lu et al., 2022; Zhu et al., 2022
			TBI	CSF	Up	Alosco et al., 2018
			MS	CSF	Up	Dong et al., 2022; Ionnides et al., 2021; Öhrfelt et al., 2016
Nod-leucine rich repeat and pyrin containing protein 3	NLRP3 inflammasome	Activated by microglial cytokines IL-1 β and IL-18	AD	CSF	Up	Feng et al., 2020; Hu et al., 2019
			IS	Brain tissue	Up	Gao et al., 2017
			TBI	CSF	Up	Wallisch et al., 2017
			MS	PBMC	Up	Malhotra et al., 2020
Adiponectin	Adiponectin	Modulates microglia through PPAR- γ signaling	AD	CSF / Blood	Down / Up	Waragai et al., 2016
			IS	Blood	Up	Tu et al., 2020; Z. Wang et al., 2019
			TBI	Blood	Up	L.-J. Shen et al., 2014
			MS	Blood	Up	Signoriello et al., 2018
High mobility group box protein 1	HMGB1	DAMP, expressed in phagocytic microglia	AD	Blood	Up	Festoff et al., 2016; Jeong et al., 2022
			IS	Blood	Up	Le et al., 2018; J. Wang et al., 2020
			TBI	Blood	Up	K.-Y. Wang et al., 2012
			MS	Blood	Up	Bucova et al., 2020
Galectin-3	Gal-3	TLR-4 ligand, promotes microglial activation	AD	Blood	Up	X. Wang et al., 2015; Yazar et al., 2021
			IS	Blood	Up	Q. Wang et al., 2021; Zhuang et al., 2021

			TBI	Blood	Up	Y.-F. Shen et al., 2016
			MS	Blood/AA	Up	Nishihara et al., 2017
Fractalkine	CX3CL1	Expressed in neurons, facilitates neuron-microglia communication	AD	CSF / Blood	Down / -	Perea et al., 2018
			IS	Blood	Down	Grosse et al., 2014
			TBI	Blood / CMD	Mixed	Begum et al., 2020; Dyhrfort et al., 2019; Thompson et al., 2020
			MS	PBMC	Up	Stojković et al., 2020
Progranulin	PGRN	Expressed in microglia, attenuates neural damage	AD	CSF	Up, sex/age-bias	Piscopio et al., 2013; Suárez-Calvet et al., 2019
			IS	Blood	Up	Xie et al., 2016
			TBI	Serum	Mixed	Menzel et al., 2017; Olczak et al., 2021
			MS	CSF	Up	Pawlitzki et al., 2018

*AD=Alzheimer's disease; IS=Ischemic stroke; TBI=Traumatic brain injury; MS=Multiple sclerosis; CSF=Cerebral spinal fluid; PBMC=Peripheral blood monocytes; AA=Autoantibodies; CMD=Cerebral microdialysis; DAMP=damage associated protein.

CHAPTER TWO

Acute vs. Chronic Investigation

Background

Upon injury to the central nervous system (CNS), several reactions ensue in order to assist in mediating the problem to achieve homeostasis. Microglial cells are known as “resident macrophages” of the CNS and are constantly surveilling for signs of damage or of foreign intruders with the use of their long dendritic arms [188]. These cells are able to detect their surroundings, perform phagocytosis, and act as antigen-presenting cells to initiate activation of neighboring T-cells. Once microglial cells encounter a foreign substance, this is when they are converted from a resting to an activated state. Upon activation of the microglial cell, the cell changes morphology from its resting “ramified” state to an ameboid phenotype. During this activated state, microglial cells can release inflammatory cytokines that not only cause inflammation within the CNS but initiate the recruitment of peripheral derived monocytes to the site of injury. These steps following CNS disruption are all activated in the hopes of restoring homeostasis². Following activation of the cell, microglia are able to diversify their phenotypic functionality, resulting in what are considered pro-inflammatory and anti-inflammatory phenotypes³. Pro-inflammatory phenotype microglial cells can not only release inflammatory cytokines but also produce reactive oxygen species (ROS) and reactive nitrogen species (RNS). A major enzyme involved in inflammatory microglia is the inducible nitric oxide synthase (iNOS). Although there have been some key markers associated with this inflammatory phenotype of microglia, there is still much need for investigation into the differences associated with microglial phenotypes and their effects in the CNS⁴. Homeostasis post-TBI is not always accomplished due to the inflammatory environment caused by microglial cells thus producing what is referred to as a “secondary inflammation” resulting in a secondary injury, i.e., Dementia or Alzheimer’s disease (AD) [189].

Communication between glial cells within the CNS is crucial to the development, function, and regulation of the nervous system. Erythropoietin-producing human hepatocellular (Eph) receptors and their Ephrin ligands have been found to be not only the largest known receptor tyrosine family but also crucial to the communication between neuronal immune cells. Eph receptors and Ephrin ligands are unique in their ability to conduct both forward and reverse

signaling resulting in bidirectional crosstalk between neuronal immune cells⁶. Eph receptors are able to bind to ephrin ligands to influence cell changes affecting proliferation, migration, motility, and shape [190]. Eph receptors can initiate their signaling similar to other receptor tyrosine kinases (RTKs) by way of autophosphorylation following the attachment of their corresponding ligand, this results in “forward signaling” by the Eph receptor. Unlike the average RTK, the ephrin ligand is able to produce a “reverse signal” thus creating bidirectional cell-to-cell communication [191]. Majority of the Eph receptors and ephrin ligands are expressed within the mammalian hippocampus [192].

Due to Eph-Ephrin's roles in both neuronal development and immune regulation, it is possible that disorders within the CNS can arise due to malfunction in Eph-ephrin signaling. Alterations of Eph-ephrin's provide further evidence that these membrane-bound signaling proteins play a functional role in the immune response following CNS damage. In the case of TBI Eph/ephrin signaling has been found to be involved in the neuroinflammatory response following the injury. The upregulation of the Eph receptor, EphA4, on Cx3Cr1 microglial cells has been found as early as 2hrs. following a TBI. Findings also included that genetic deletion of EphA4 globally showed a significant reduction in the cortical size of the lesion (as shown in Figure 1), implying that the EphA4 receptor may have a role in the inflammatory phenotype of immune cells in response to TB I193]. Recent reviews have concluded that the amount of PDIs entering the brain made no difference but instead the polarization state was found to dictate the outcome of the brain injury [194]. Further proving that investigation of the role EphA4 on peripheral immune cells plays in the hindrance of the repair process post-TBI is needed as a possible therapeutic pathway.

Material & Methods

All animal studies are be carried out under the NIH and the Virginia Tech's IACUC guidelines. *Adoptive Transfer (AT)*: Male CD1 mice purchased from Charles River laboratories were irradiated and injected with mTmG harvested GFP+ bone marrow cells either having WT or KO EphA4. Following the adoptive transfer, recipient mice are given a period to allow the donor cells to engraft, proliferate, and differentiate into functional immune cells. Hematopoietic

cells derived from the bone marrow are able to fill vacate bone marrow niches' left behind as a result of irradiation. The differentiation of these cells leads to the generation of various immune cell types, including monocytes and other myeloid cells, that can populate the peripheral tissues and central nervous system. To support this critical engraftment and differentiation phase, the recipient mice were provided with Gentamycin water for 2 weeks post-irradiation. This antibiotic treatment helps prevent infections, which the mice are particularly susceptible to during the initial stages of immune reconstitution, ensuring the survival and proper function of the newly engrafted cells.

Controlled Cortical Impact (CCI): At-WT and AT-KO mice were injected via subcutaneously with ketamine (100 mg/kg) and xylazine (10 mg/kg) in order to anesthetize before surgery. Temperature of the mice was monitored using a rectal probe and a controlled heating pad set to 37°C. Following anesthetization, the mice were positioned in a stereotaxic frame and a 4mm diameter craniotomy was made over the right parietal-temporal cortex with the use of a dremel. The injury is caused by a program-controlled cortical impactor ($\Phi = 3$ -mm beveled tip) connected to an eCCI-6.3 device (Custom Design & Fabrication, LLC) at a velocity of 5.0 m/s, depth of 2.0 mm, and 100ms impact duration. Following injury, the incision was closed using Vetbond tissue adhesive (3M, St. Paul, MN, USA) and sutured. The animals are then placed into a heated cage and monitored every 20 min until fully recovered from anesthesia. Cardiac perfusion with 4% PFA, was performed at 4hr, 1d, 3d, and 60dpi.

Immunohistochemistry: Staining/imaging of Iba1 (Invitrogen), Click-iT Plus TUNEL assay (Invitrogen), ephrinA4 (Invitrogen), and phospho-Histone H3(pH3) (Cell signaling) were performed on 30 μ m-thick cryostat coronal cut serial sections of perfused brains. These samples were blocked using 2% fish gel (Sigma, Inc.) /0.2% triton before being stained overnight with the respective primary antibody. TUNEL staining will be conducted following the provided protocol published by the company. Images of the injured hippocampus were captured using FITC/TEXR/Cy5/DAPI filters on a fluorescent confocal microscope and analyzed using non-biased stereology or Fiji ImageJ.

Non-biased Stereology: With the use of a Stereo Investigator (MBF Biosciences) and an upright Olympus BX51TRF motorized microscope, quantification of both PICs and resident microglia were analyzed by the Optical Fractionator probe from MBF's Stereo Investigator

software version 2017.03 to provide an unbiased estimate. Imaging was captured utilizing a Nikon ECLIPSE Ti2 inverted confocal microscope equipped with a motorized stage and a Nikon C2 laser system. A contour of the injury is produced and sites of the contoured tissue were randomly selected by the software for counting. Contours for ipsilateral cortex were created, and the optical fractionator's grid size was set to 400×400 mm with a 200×150 mm counting frame.

ImageJ: At the 4hr, 1dpi, and 3dpi timepoints, mice were euthanized, brain cryo-sectioned, and stained with IBA1 and TUNEL as detailed previously. Confocal images were taken using a C1 Nikon confocal device at 40x magnification. These images are uploaded to the Fiji ImageJ software in which the Cell Counter plugin was utilized to identify both the number of TUNEL+ microglia, peripheral immune cells, and peripheral derived macrophages within the injured cortex.

IMARIS: At the 1dpi, 3dpi, and 60dpi timepoints, mice will be euthanized, brain cryo-sectioned, and stained with IBA1 as detailed previously. Confocal images will be taken using a C1 Nikon confocal device at 40x magnification. These images will then be uploaded into the IMARIS software to be used for Sphericity, Oblate, Prolate, and Sholls analytical quantification of the microglia at the various timepoints to compare morphology at the acute and chronic phases of TBI.

Novel Object Recognition (NOR): Mice were habituated to the monitoring box for 5 minutes each with no objects in the box. At -2dpi, a baseline assessment was conducted in which each mouse is exposed to two identical objects for 5 minutes and 1 hour later exposed to one of the identical objects and 1 novel object replacing the space of the previous identical object. These tests were conducted between the hours of 7am to 9am to best accommodate the nocturnal sleeping patterns of the mice. CCI was performed at day 0 and NOR testing will resume 1d, 3d, 7d, 30d, and 60dpi. Following the 60dpi testing, the mice were euthanized for tissue collection.

T-Maze: The T-maze was ordered from Maze Engineers (Conduct Science) and assembled in the lab. It features clear acrylic walls and doors, and a smoked acrylic floor. The design is a loading arm (stem) and two goal arms, forming a T shape. The T-maze is utilized to assess spatial working memory through spontaneous alternation. It is based on the natural tendency of rodents to prefer exploring a novel arm over a familiar one, which causes them to alternate their choice of arm over repeated trials. The T-maze was consistently positioned within

a quiet and dimly lit room, maintaining the same layout for all trials and mice. Prior to testing, the functionality of the timer was checked to ensure accurate measurements. A sample trial (T0) was conducted, during which the mouse was placed at the start of the maze and allowed to explore freely. Timing commenced when the mouse turned toward the central zone, and upon entering a goal arm, the door was closed behind it, and the chosen arm along with the latency to choose were noted. The mouse was then confined in the chosen arm for 30 seconds before being returned to the start. This procedure was repeated for five test trials (T1-T5) and a final trial (T6). After completing the trials, the mouse was returned to its home cage. Between trials, the maze was thoroughly cleaned to remove any visual and odor cues that could influence subsequent trials. This process was repeated for all experimental mice.

RNA Isolation: Neuronal dissociation was conducted on AT-WT and AT-KO mice at 90dpi in order to collect resident microglial cells of the injured cortex and hippocampus for transcriptomic analysis. Hippocampal samples were sent out to MedGenome for scRNAseq.

Statistical Analysis: Data was quantified and graphed with the use of GraphPad (GraphPad Software, Inc., San Diego, CA). Mean values were reported together with the standard error of mean (SEM) and group comparisons will be identified as significant if P is less than 0.05.

Cell Death

Secondary damage following the initial neuronal injury is due to a complex cascade of events contributing to the neurotoxic milieu that includes blood brain barrier breakdown and influx of peripheral blood-derived components and immune cell recruitment, reactive astrogliosis and microgliosis. Microglia, the first line innate immune response in the brain, play a pivotal role in regulating neuroinflammation in traumatic brain injury (TBI) [194]. The role of activated microglia after TBI was once believed to be either protective or detrimental but that narrative has since evolved. New findings have proven that microglial cells, although small in size, play a larger role in the mediation of neuronal injuries. In order to better understand what role these cells have, we must gain a greater understanding of the temporospatial changes that can occur for these cells post-CCI. The relationship with their surroundings seems to have a high influence in

the characteristics of resident microglial cells. Recent findings have illustrated that conditional deletion of EphA4 on microglial cells fails to increase neuroprotection post-TBI unlike previous data utilizing a global knock-out of EphA4 [195]. In order to further characterize the role of microglial cells post-TBI, quantification of the cellular survival of microglial cells within the AT-WT mouse model has been demonstrated at the acute timepoints using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining method. With the use of TUNEL staining, investigation of dead resident microglia (IBA1+), peripheral derived macrophages (PDMs) having both GFP and IBA1 expression, as well as peripheral immune cells (PICs) from GFP tagged mTmG bone marrow cells at the site of injury is shown in Figure 1.

Figure 1A demonstrates the production of the chimeric Adoptive transfer mouse model with WT expression of EphA4 on invading myeloid cells from the body post-TBI. Beginning with irradiation of the CD1 mice, donor bone marrow cells from mTmG mice are injected via the tail vein followed by a cellular turnover period of two weeks before the initial CCI injury is initiated. Neuronal tissue was later perfused and sectioned in preparation for TUNEL IHC staining at 4hr, 1d, and 3dpi. Confocal images of the immune-stained tissue are represented in Figure 1B indicated that at 3dpi, GFP+ infiltrating immune cells are highly expressed at the site of injury, specifically the perilesional area. Confocal images of the injured cortex were used to investigate fluorescent expression of IBA1, GFP, and TUNEL utilizing the application of the Cell counter Plugin within Fiji ImageJ. Cellular quantification displayed in Figure 1C indicate that as the injury increases in time, so does the amount of IBA1+/GFP+ peripheral derived macrophages within the injury. In relation to the significant decrease of resident microglia at 1dpi, there is also a slight increase in the number of TUNEL+/IBA1+ microglial cells at 1dpi. This can be further analyzed in Figure 1D where it illustrates resident microglia having 3% increase in the number of dead microglia at 1dpi with a plateau at 3dpi. The highest percentage of cell death at the acute phase of TBI can be found in PDMs as early as 4hrs post-injury. This shows that as the injury progresses in time so does the number of immune cells passing the damaged blood brain barrier (BBB) thus infiltrating toward the damaged cortex leading to engulfment by microglia. As the number of PDMs increases, its percentage of cellular death also decreases, this may be due to increased proliferation of microglia at 3dpi, depicted in Figure 2. These results may also give reason to why microglia cell death decreases also at 3dpi.

In terms of the overall cell death, for all three cell types, there is little significance between the timepoints. Indicating no significant linear correlation between total number of cell death caused by a TBI and the time progression of said injury. Cell death for TUNEL+/GFP+ cells show a large increase in number at the 1dpi timepoint (Figure 1C) followed by a decrease in the number of dead immune cells at 3dpi. The percentage of TUNEL+ cells within the cortical TBI region were calculated to illustrate that although insignificant, evidence of both microglia and PIC cell death can be found as early as 4hr post-TBI (Figure 1D). Microglia have the highest percentage of cell death compared to PICs and PDMs, roughly 2% at 4hr, 5% at 1dpi, followed by a decrease to about 2% at 3dpi. Immunohistochemistry analysis was conducted in order to quantify the cell death of both IBA1+ and GFP+ cell types. Confocal images of these IHC stained tissues at 4hr, 1d, and 3dpi neuronal tissue sections were acquired for evaluation of cellular death within the ipsilateral (Figure 1 E-J).

Proliferation of Microglia

Additional analysis of the temporospatial changes occurring within the cortex involved measurement of the proliferation rates of resident microglia and GFP+/IBA1+ PDMs at 4hr, 1d, and 3dpi in both AT-WT and AT-KO mice (Figure 2). Proliferation analysis was quantified with the use of the Stereo Investigator optical fractionator application following phospho-histone3 (pH3) IHC staining of neuronal tissue sections collected at the experimental timepoints. The results are in line with the preliminary hypothesis of proliferating resident microglia increasing in number within the AT-WT model as time progresses acutely. The increased number of proliferating microglial cells mimics the increased number of total microglia at 3d found in the previous TUNEL data in Figure 1. For both the 4hr. and 1dpi timepoints, there is little proliferation of resident microglia, with no significance between the WT and KO adoptive transfer mouse models (Figure 2A). At 3dpi, microglia proliferation increases in number in the WT mouse model compared to the 1dpi timepoint. In comparison to the WT model, there is a significant decrease in proliferation within the AT-KO mice indicating an influence from the EphA4 receptor to increase proliferation in the WT setting (Figure 2A). The percentage of proliferating microglia was also calculated illustrating significance again at the WT 3dpi

timepoint compared to both 1d and 4hr. The highest percentage of dividing microglia can be found within the AT-WT model at 3dpi. Both the AT-WT and AT-KO mouse models demonstrate increased proliferation within the acute progression of TBI at the 3dpi timepoint. The estimated total density of resident microglia was compared to the estimated total density of the infiltrating GFP labeled immune cells within the ipsilateral region (Figure 2C). Cellular analysis of immune-stained neuronal tissue revealed a sustained progression of infiltrating GFP+ immune cells in both the AT-WT and AT-KO mouse models at acute timepoints. Compared to the increasing amount of GFP PICs, the amount of cellular division amongst the resident microglia also increases as the injury progresses (Figure 2C). Confocal imaging of phosphor-Histone-H3 immuno-stained cells is represented in (Figure 2 D-H) illustrating the identification of proliferating microglia and GFP labeled PICs. It is also notable that the morphology of the H3+ resident microglia at 3dpi are more amoeboid in shape

Proliferation of microglia during the chronic phase of TBI, investigated at 60dpi, was also investigated to compare the changes occurring acutely versus chronically post-TBI (Figure 2I). The estimated number of microglial proliferation rates within the AT-WT mouse model at 60dpi is roughly halved in comparison to the acute 3dpi timepoint. Although, the proliferation of resident microglia in the KO is higher than WT, it is not significant at 60dpi. Notably, in contrast to acute analysis of proliferation at 3dpi, the AT-KO 60dpi model displays a higher expression of pH3+ microglial cells within the ipsilateral (Figure 2I). Previous data illustrated in Figure 2A suggested the opposite with WT having significantly higher number of proliferating microglia. At 60 dpi, no discernible significance between the AT-WT and AT-KO mouse models was observed, suggesting subtle yet impactful cellular changes throughout chronic TBI progression. This evidence underscores the necessity for further mechanistic exploration to elucidate the underlying rationale behind post-TBI cellular alterations and the modulatory role of peripherally derived EphA4 receptor infiltration in this context.

Proliferation of Peripheral Derived Macrophages

To address the infiltration of PICs to the site of injury and their involvement with resident microglial cells, proliferation marker phospho-Histone H3 (pH3), was used to identify GFP+/IBA1+/pH3+ proliferating PDM cells within the cortex of AT-WT and AT-KO mice. First, the estimated number of pH3+ macrophages expressing both GFP and IBA1 indicated little to no expression of proliferation within the acute timepoints of 4hr and 1dpi. Proliferation is increased for the AT-WT mouse model at 3dpi with a significance difference compared to the low number of KO PDM proliferation (Figure 3A). Upon evaluation of the percentage of proliferating PDMs, again there is only evidence of this cell type dividing during 3dpi WT analysis (Figure 3B).

Proliferation of PDMs at the chronic time point of 60dpi shows no significant difference between WT and KO experimental mouse models (Figure 3C). Evidence shows the estimated number of microglia proliferating during chronic progression of TBI are substantially lower than those of dividing microglia at 3dpi. The AT-WT PDMs at 60dpi are significantly lower compared to previous findings at 3dpi, indicating that in WT mice, EphA4 expression from PICs leads to a decrease in PDM proliferation during the chronic phase of TBI repair. Visual representation of a proliferating resident microglial cell are illustrated in Figure 3D-H, showing phospho-Histone H3 in red (E), GFP PICs in green (F), DAPI in blue (G), and Iba1 in pink (H).

Morphology of Microglia

The spatiotemporal activation of microglia after injury is a determining factor of their function. Previous studies have reported that the two activation phenotypes ‘pro-inflammatory’ and ‘anti-inflammatory’ contribute to the pathophysiology of TBI and their function varies according to their distribution in the brain and the time after injury [196]. Recent findings indicate that PDIs responding to neuronal injury also play a role on the resident microglial cells’ morphology. The majority of activated or amoeboid shaped microglia could be found at the injuries core compared to the more ramified or branch-like shaped microglia closer to the perilesional and contralateral areas. The results of microglial morphology within the ipsilateral cortex were analyzed using 3D reconstructed confocal images with IMARIS software (Figure 4). With the use of confocal Z-stack imaging, microglial cells expressing IBA1 in TexasRed

fluorescence and with a complete cell nucleus in DAPI, demonstrated by DAPI fluorescence, were selected for 3D-reconstruction. The cell bodies were constructed using the IMARIS function “Surfaces” to identify the cells sphericity, oblate, and prolate index (Figure 4A-C). Sphericity at the acute timepoints post-TBI, resident microglia illustrate greater spheric morphology at 1dpi contrasted to 3dpi in the WT mouse model. Visually, the morphology of the 3d WT microglial cells can be seen visibly more ramified than at 1dpi where the cells have less processes stretching outward from the soma, as well as, a rounder cell body (Figure 4x).

Sphericity index at 1dpi showed no significance between the WT vs. KO mouse models but did have significance in comparison to the 3dpi WT (Figure 4B). From the 1d to 3dpi acute timepoints there is evidence of a significant decrease in the cells WT sphericity, indicating the cells increase their number of processes and mimic more closely to the ramified state versus at 1dpi. Sphericity of the KO 3dpi microglial cells is more similar to those at 1d and have a significantly higher sphericity index in contrast to the WT 3dpi analysis (Figure 4B). Oblate index for 1d WT and KO mice showed a slight significance with the KO decreasing in value (Figure 4D). The 3dpi microglial oblate index showed no significant difference between the two experimental models but did show changes when statistically compared to 1dpi (Figure 4D). The ipsilateral oblate values of 1dpi WT and KO are significantly lower than both the WT and KO values at 3dpi, showing an increase within the acute progression of TBI. The contralateral values of these morphological analysis show little significance between them, Sphericity contra showed a decrease at 3dpi within the KO model (Figure 4A), while for Oblate, the index decreases at 3dpi WT and increases within its KO countermodel (Figure 4C). Representative images captured from the IMARIS software for the 3d WT and KO mouse models have been illustrated to show the contrasting morphological changes occurring between the microglia of each experimental environment (Figure 4E-K).

Starting with surface analysis of the contralateral microglial cells at 3d (Figure 4E), followed by comparison to the WT surface structure of microglia at 3dpi within the perilesional region of the TBI (Figure 4F & G) and its KO counterpart which illustrates microglia have a more ameboid morphology (Figure 4H & I). The following figures demonstrates the varying morphological changes that occur for microglial cells within the ipsilateral post-TBI (Figure 4K). Sholls analysis was also performed in order to further characterize the morphological changes occurring progressively post TBI between the AT-WT and AT-KO mouse models (Figure 4L).

Sholl analysis was performed using the same confocal images as used for IMARIS surface analyses and are similar in terms of the spatial resolution and imaging parameters, ensuring consistency in the quantitative assessment of neuronal morphology. The data presented for Sholl analysis is comparable to that of the Sphericity index for both 1d and 3d mouse models. Sholl analysis of microglia at 1 dpi for both WT and KO mice shows a similar pattern, with the number of intersections peaking around 5 and consistently decreasing with increasing distance from the soma. Values for 3dpi KO mice are similar to those at 1dpi, but there is evidence of increased ramification in the 3dpi WT mouse model, with a greater number of intersections as the distance from the soma increases (Figure 4L). These findings are consistent with the previously presented Sphericity index data. Representative images of the Sholl analysis for 3d WT and KO ipsilateral and contralateral sides are provided to illustrate the process of Sholl analysis, as well as the increased intersections observed not only in the contralateral images but also in the WT 3dpi microglia (Figure 4L).

Morphological analysis was also conducted, using the same methods, for the chronic timepoint of the TBI at 60dpi. IMARIS analysis was conducted in order to calculate the Sphericity and Ellipticity values shown previously. The Sphericity index at 60 dpi indicated no significant difference between WT and KO experimental groups; however, both were significantly higher than their contralateral comparisons, which aligns with expectations due to the resting, ramified state of microglia unaffected by injury (Figure 5A). Similarly, the Oblate index at 60 dpi was consistent between WT and KO experiments, with both showing significant differences compared to their contralateral values (Figure 5B). Ellipticity value, Prolate, also showed no significance between the WT and KO experimental groups but both did have small significance in comparison to the WT contralateral index (Figure 5C). Sholl's analysis was also conducted to investigate the influence of peripheral derived EphA4 signaling on the morphological changes chronically post-TBI (Figure 5D). Both the AT-WT and AT-KO analysis for 60dpi have a peak number of intersections at around 5 with the steady decline as the distance from soma increases, similar to the statistics previously presented for 1dpi WT and KO. Representative images of both the ipsi- and contralateral cell morphology of WT and KO resident microglial cells at 60dpi (Figure 5E).

Neurological Behavioral Analysis

For the purpose of investigating the effects of TBI on short-term memory, *Novel Object Recognition* (NOR), was tested in mice at 1d, 3d, 7d, and 30d post-injury (Figure 6). NOR is a commonly used open field test to investigate the short-term memory of experimental mice. Illustration of the experimental protocol shows how mice are introduced to two identical objects for 5 minutes of testing, recorded by EthoVision software analysis, and reintroduced to one familiar object from the previous test and one novel object to score preference index (Figure 6A). Previous studies utilizing this tool has provided evidence of neuroprotection in the murine EphA4 KO mouse model upon investigation. Although the decline of short-term memory for both the AT-WT and AT-KO mice were both significant in comparison to the baseline analysis at 1dpi, the WT group is significantly lower in preference to the KO group, showing higher cognition retainment of memory upon the KO of EphA4 (Figure 6B). For the following timepoints (3d, 7d, and 30dpi) there are no significant differences between the two experimental groups although there is significance when comparing each groups chronic investigation to the acute. The AT-KO experimental group show evidence of recovery by the chronic progression timepoint of 30dpi.

T-maze experimental testing was used to evaluate spatial working memory by observing spontaneous alternation (Figure 6C-D). It capitalizes on the rodents' innate preference for exploring new arms over familiar ones, driven by the need to find food or detect threats. This behavior results in the rodents alternating their choice of arms across trials, relying on spatial memory to remember previously visited arms. The arm least recently visited becomes the more novel choice after both arms have been explored. This assessment requires no prior training and solely relies on the novelty response. The results illustrated in Figure 6 show evidence of a significant decrease in spatial function of the AT-WT mouse model at 60dpi in comparison to the KO experimental group. As the injury progresses, the KO group shows a decrease in working memory and loses its significant difference in comparison to the WT group. There is also evidence of the control group increasing their average time to selection. Future directions for these findings will include including to number of experimental mice for each group to gain a greater insight of the true progression of these murine behavioral deficits and the role EphA4 signaling plays in the remedy of these deficits.

Ephrin Ligand A4

In relation to EphA4 signaling, the receptor is able to bind to multiple ephrin ligands, including ephrinA4. We investigated the expression of this particular ligand within the ipsilateral cortex of AT-WT mice at 3dpi. Our findings showed that the ephrinA4 ligand is highly expressed not only within the injured cortex but expansively throughout the coronal serial section on both the ipsi- and contralateral (Figure 7). EphrinA4 signaling was imaged using confocal microscopy, proving that it is indeed expressed within the cortex post-CCI (Figure 7A). Upon further investigation, there was evidence that expression of the ephrinA4 ligand was mainly found to be expressed on resident microglial cells (Figure 7E). There was little to no evidence of GFP labeled infiltrating PICs expressing the ephrinA4 ligand (Figure 7C). Lastly, confocal imaging of the contralateral exhibited fluorescent signaling of the ephrinA4 ligand and resident microglial cells (Figure 7F-I). This work will be a guide for future directions to investigate the relationship between infiltrating expression of the receptor EphA4 from peripheral derived PICs and the ephrinA4 ligand expressed on resident microglial cells within the cortex of an injured brain. Evidence of ephrinA4 expression on resident microglial cells provided us with useful information to better understand the relation between the two cell types in relation to mediating TBI.

Summary

This chapter provides an in-depth analysis of the influence of peripheral immune-derived EphA4 on microglial dynamics during acute and chronic timepoints following TBI, focusing on microglial cell death, proliferation, and morphology. The primary aim was to investigate the influence of peripheral immune-derived EphA4, which not only includes cellular and temporospatial changes of the cells themselves but also the behavioral changes in vivo that could provide further explanation of the neurodegenerative effects of the TBI. The experimental investigation began with understanding that there is little cell death during the acute timepoint of microglia and peripherally derived immune cells. The greatest percentage of cell death was found for microglial cells at 1dpi having an average of 5% cell death within the ipsilateral.

Proliferation of microglial cells of AT-WT mice showed a significant increase from 1d to 3dpi, having an average of 600 estimated pH3+ microglial cells with another decrease during the 60dpi chronic timepoint. This data concludes that the proliferation of microglial cells with the AT-WT mouse model peaks at the 3dpi acute timepoint. For the AT-KO experimental model, microglial cells showed little to no proliferation during the early acute timepoints with a small increase at 3dpi, still significantly lower than that shown for WT. Chronic investigation of AT-KO proliferation indicated an increase to about 500 estimated pH3+ microglial cells within the ipsi region, similar to that of 3d WT findings. Peripherally derived macrophages, PDMs, had the highest number of proliferation microglia with an estimated number of about 1500 pH3+ microglial cells and was significantly higher than its KO countermodel. In comparison to the acute proliferation of PDMs, AT-WT PDMs at 60dpi are substantially lower than at 3dpi and little difference between the KO analysis of the two timepoints. Morphological analysis of the resident microglial cells post-TBI indicated that both AT-WT and AT-KO at the 1dpi timepoint are more ameboid in shape than at the 3dpi timepoint, based on the Sphericity index of the two experiments. The chronic investigation of 60dpi microglial sphericity also showed that although cells lowered in sphericity at 3dpi, they again increase toward a more ameboid morphology. This finding highlights the adaptive responses of microglia as they transition from acute to chronic injury states. Additionally, Sholl analysis revealed similar data, corroborating the observed trends in microglial sphericity.

In reference to behavioral analysis, NOR Results indicated a significant decline in short-term memory for both AT-WT and AT-KO mice at 1dpi compared to baseline. However, the AT-KO group exhibited better memory retention than the AT-WT group. No significant differences were observed between the groups at later time points, 3, 7, 30, and 60dpi, though both groups showed recovery by 60 days post-injury. The OLT results showed no significant differences between the AT-WT and AT-KO groups, suggesting that factors such as experimental objects and the animals' recognition abilities might influence the outcomes. Lastly, T-maze results revealed a significant decrease in spatial function in the AT-WT group at 60 days post-injury compared to the Flox control group, while the AT-KO experimental group maintained performance levels similar to the control. Overall, these findings demonstrate differential impacts of TBI on short-term and spatial memory in WT and KO mouse models, highlighting the role of EphA4 in cognitive recovery following injury.

With the use of this study, we explored the expression of the ephrinA4 ligand in the cortex of mice following TBI. We found high expression of ephrinA4 not only in the injured cortex but also throughout the coronal serial section on both sides of the brain. Confocal microscopy confirmed ephrinA4 expression in the cortex post-TBI, mainly on resident microglial cells rather than infiltrating peripheral immune cells. This finding suggests a potential role for microglial cells in mediating TBI through hinderance of ephrinA4 signaling by EphA4 binding and provides insight for future investigations into the relationship between infiltrating immune cells expressing EphA4 and resident microglial cells expressing ephrinA4 in the injured brain.

In conclusion, our study provides novel insights into the complex interplay between peripheral immune-derived EphA4 and microglial dynamics following traumatic brain injury. By elucidating the temporal and cellular responses in WT and KO mouse models, we highlight the potential therapeutic implications of targeting EphA4 in mitigating cognitive deficits post-injury. These findings contribute to a deeper understanding of neuroinflammatory processes and underscore the importance of tailored therapeutic interventions for improved outcomes in TBI patients.

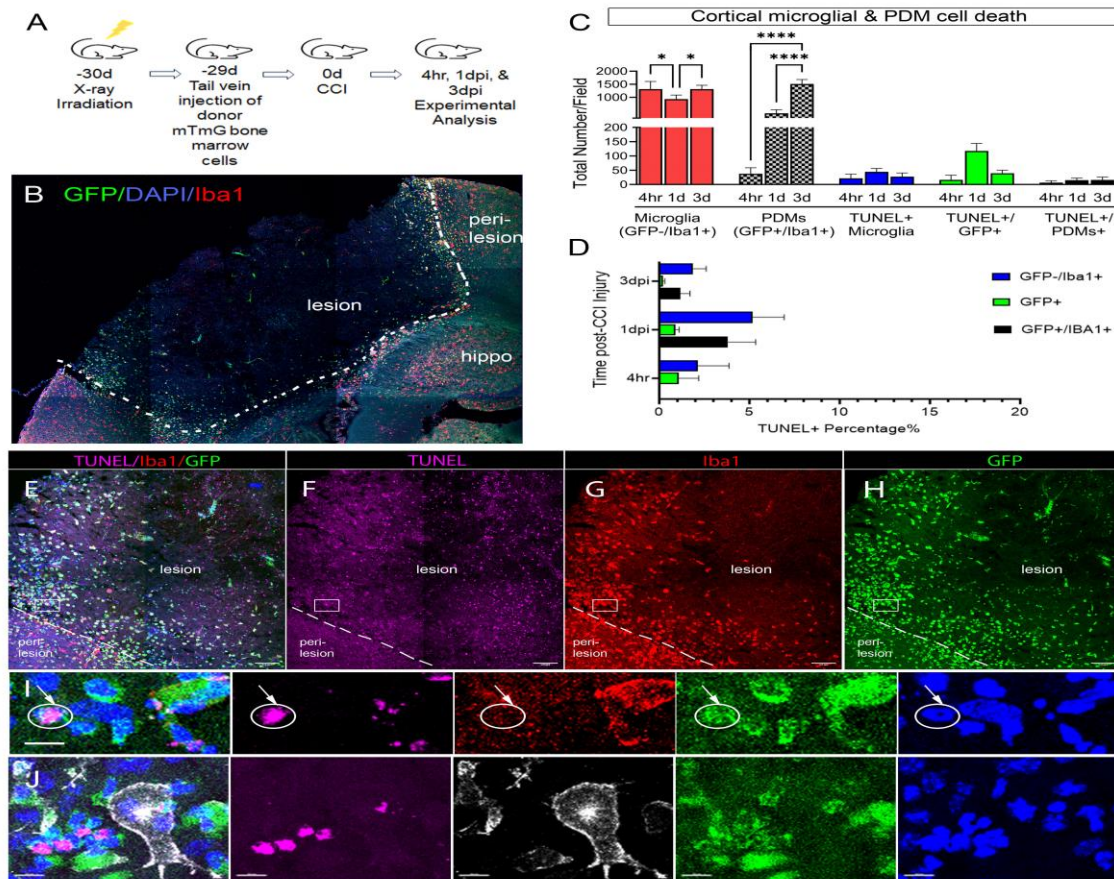


Figure 1. Development of Adoptive Transfer (AT) chimeric mouse model and TUNEL Immunohistochemistry. (A) Experimental timeline of AT chimeric mouse model illustrated. (B) Confocal image of AT-WT 3dpi cortex expressing GFP, DAPI, and IBA1. (C) Total Number of live and dead cells at 4hr, 1d, and 3dpi in the AT-WT model using Fiji ImageJ. (D) Percentage of TUNEL positive microglia, peripheral immune cells (PICs), and peripheral derived macrophages (PDMs). (E) Confocal image illustrating IHC fluorescence of TUNEL, IBA1, GFP labeled immune cells, and DAPI. (F) Confocal image of TUNEL expression within the cortex of AT-WT 3dpi mouse model. (G) Confocal image of IBA1 expression within the cortex of AT-WT 3dpi mouse model. (H) Confocal image of GFP labeled immune cell expression within the cortex of AT-WT 3dpi mouse model. (I) Representative confocal image of a GFP+ immune cell expressing TUNEL. (J) Representative confocal image of the relationship between GFP+ immune cells and resident microglia expressing TUNEL. * $P < 0.05$; $n = 5$ per group; represented as mean \pm SEM.

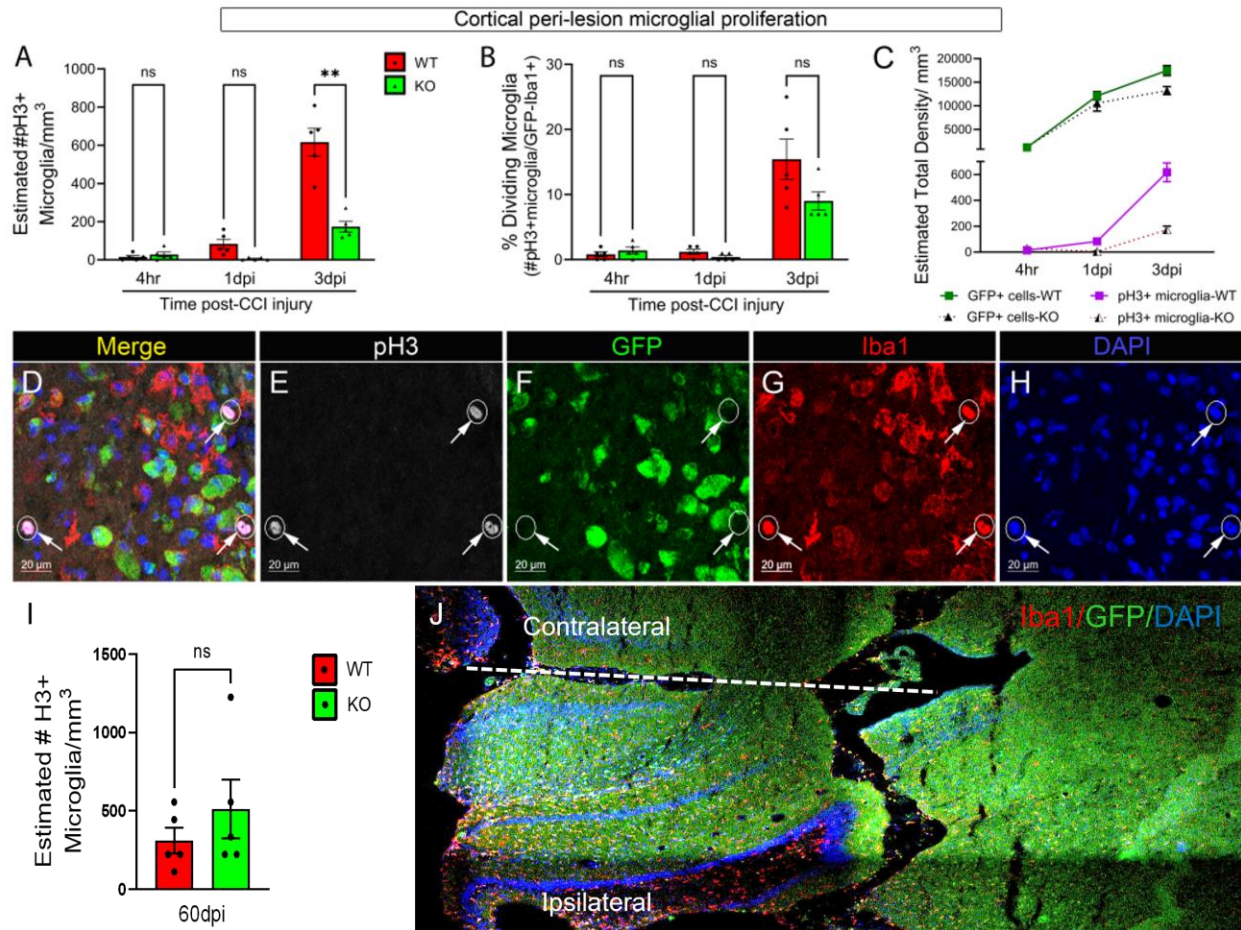


Figure 2. Proliferation of resident microglial cells within the cortex of murine AT model.

(A) Estimated number of proliferating microglial cells at acute timepoint post-TBI using Stereo Investigation. (B) Percentage of proliferating microglia within the peri-lesion. (C) Estimated total density comparing infiltrating GFP+ immune cells and proliferating resident microglial cells acutely post-TBI. (D) Representative confocal images of pH3 expressing microglia at 3dpi. (E) Confocal image of phospho-Histone H3 IHC. (F) Confocal image of GFP+ infiltrating immune cells. (G) Confocal image of Iba1 expressing microglial cells. (H) Confocal image of DAPI IHC. (I) Estimated number of proliferating microglial cells at the chronic timepoint post-TBI using Stereo Investigation. (J) Representative confocal image portraying immunohistochemical analysis of the cortex at 60dpi expressing Iba1, GFP, and DAPI. *P<0.05; n=5 per group; represented as mean ± SEM.

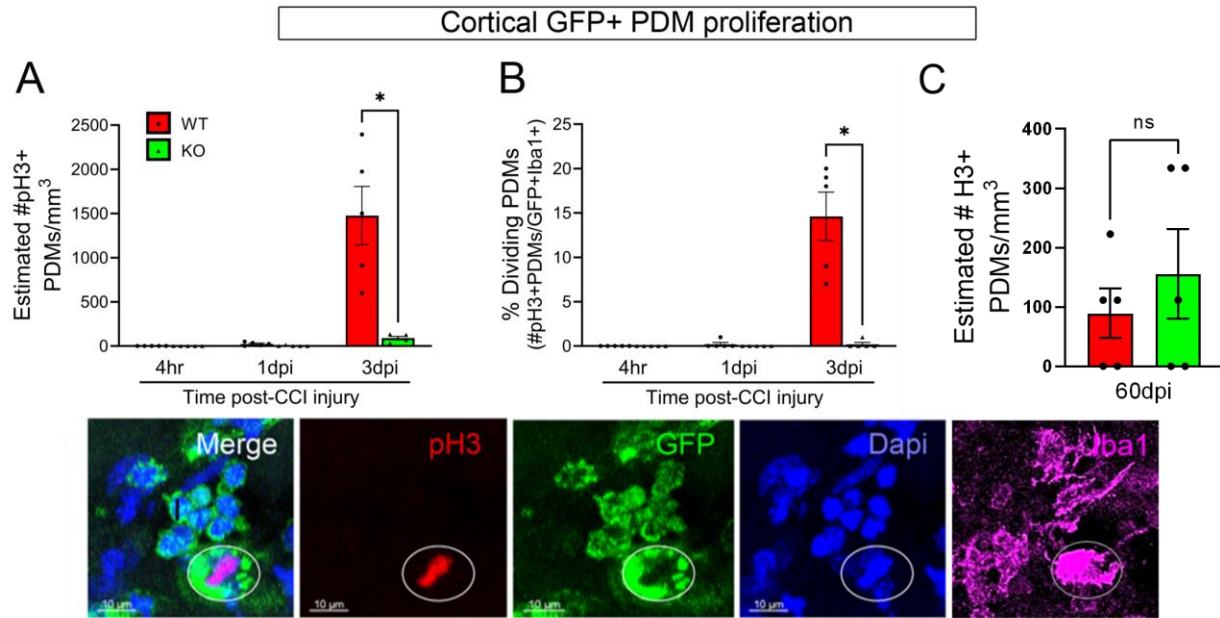


Figure 3. **Proliferation of peripheral derived macrophages within the cortex of murine AT model.** (A) Estimated number of proliferating macrophage cells at the acute timepoint post-TBI expressing GFP and Iba1 using Stereo Investigation. (B) Percentage of proliferating macrophage cells. (C) Estimated number of proliferating macrophage cells at the chronic timepoint 60d following CCI. (D) Representative confocal images of the relationship of proliferating H3+ (red), GFP+ immune cells (green), DAPI (blue), and Iba1+ (pink) macrophages at 3dpi. * $P < 0.05$; $n = 5$ per group; represented as mean \pm SEM.

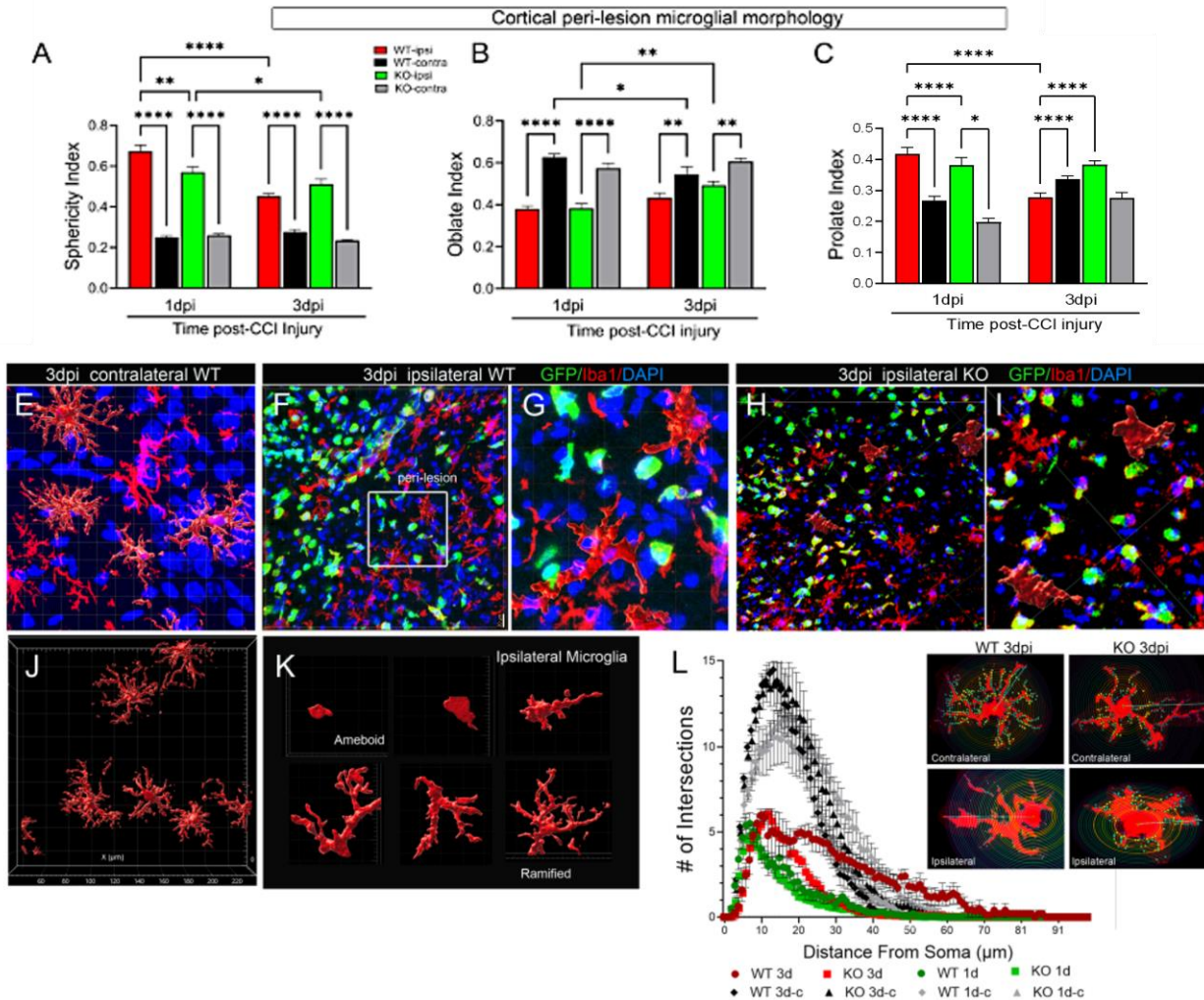


Figure 4. Morphological analysis of resident microglia during acute progression of TBI.

(A) Sphericity index of the within the peri-lesion at 1dpi and 3dpi analyzed using IMARIS. (B-C) Ellipticity values, Oblate and Prolate, analyzed within the peri-lesion 1d and 3dpi following CCI using IMARIS surface analysis. (E) Representative image of 3D generated surface analysis by IMARIS of a 3dpi contralateral with overlapping IHC of Iba1 and DAPI. (F-G) IMARIS 3D generated imaging of an AT-WT 3dpi peri-lesion focusing in on surface analysis of microglia. (H-I) IMARIS 3D generated imaging of an AT-KO 3dpi peri-lesion focusing in on surface analysis of microglia. (J) Representative confocal image of 3dpi WT contralateral region with surface analyzed microglia generated by IMARIS. (K) 3D graphical representation of the various morphological changes that can occur within microglial cells following injury. (L) Sholl analysis comparing the ipsilateral and contralateral morphology of microglia at 1 and 3dpi along with representative images from ImageJ at 3dpi for WT and KO mouse models.

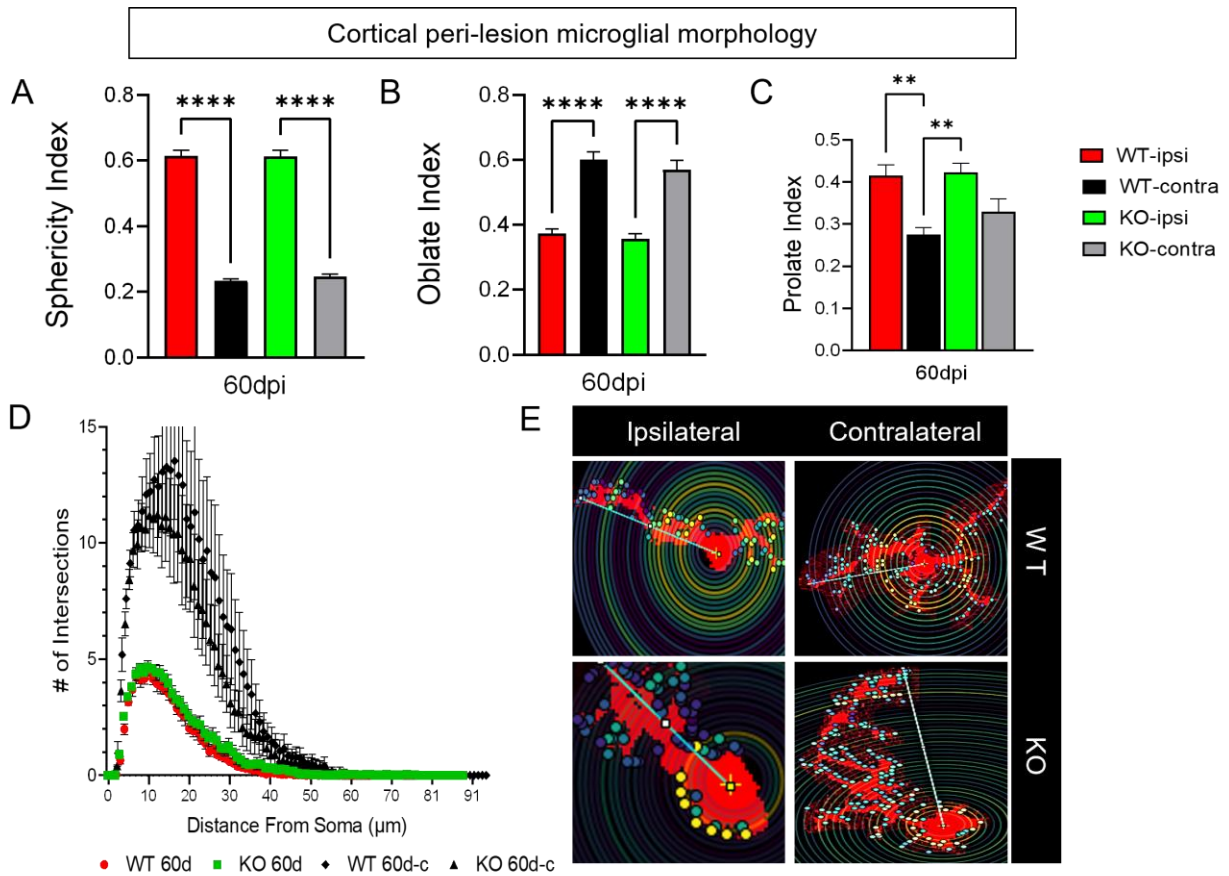


Figure 5. **Morphological analysis of resident microglia during chronic progression of TBI.** (A) Sphericity index of the within the peri-lesion at 60dpi analyzed using IMARIS. (B-C) Ellipticity values, Oblate and Prolate, analyzed within the peri-lesion 60dpi following CCI using IMARIS surface analysis. (D) Sholls analysis comparing the ipsilateral and contralateral morphology of microglia at 60dpi. (E) Representative images from ImageJ for WT and KO ipsi/contralateral microglial cells.

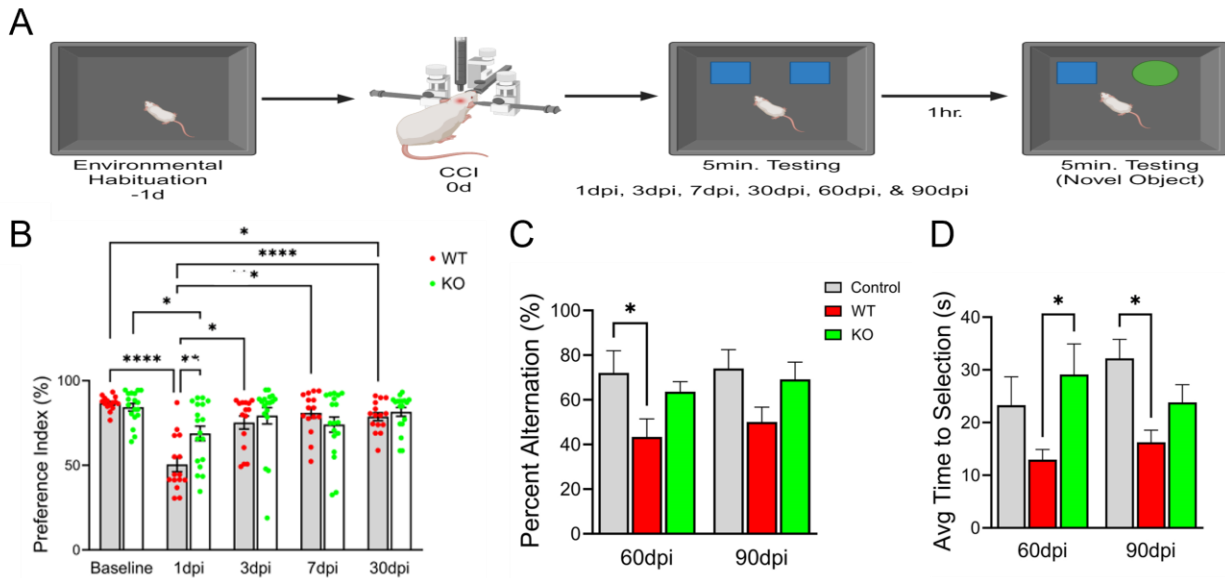


Figure 6. Behavioral analysis post-TBI of AT mouse models.

(A) Illustration of Novel Object Recognition (NOR) testing experimental timeline. (B) NOR analysis of both AT-WT and AT-KO mice show significant decrease in memory. (C-D) T-Maze behavioral analysis.

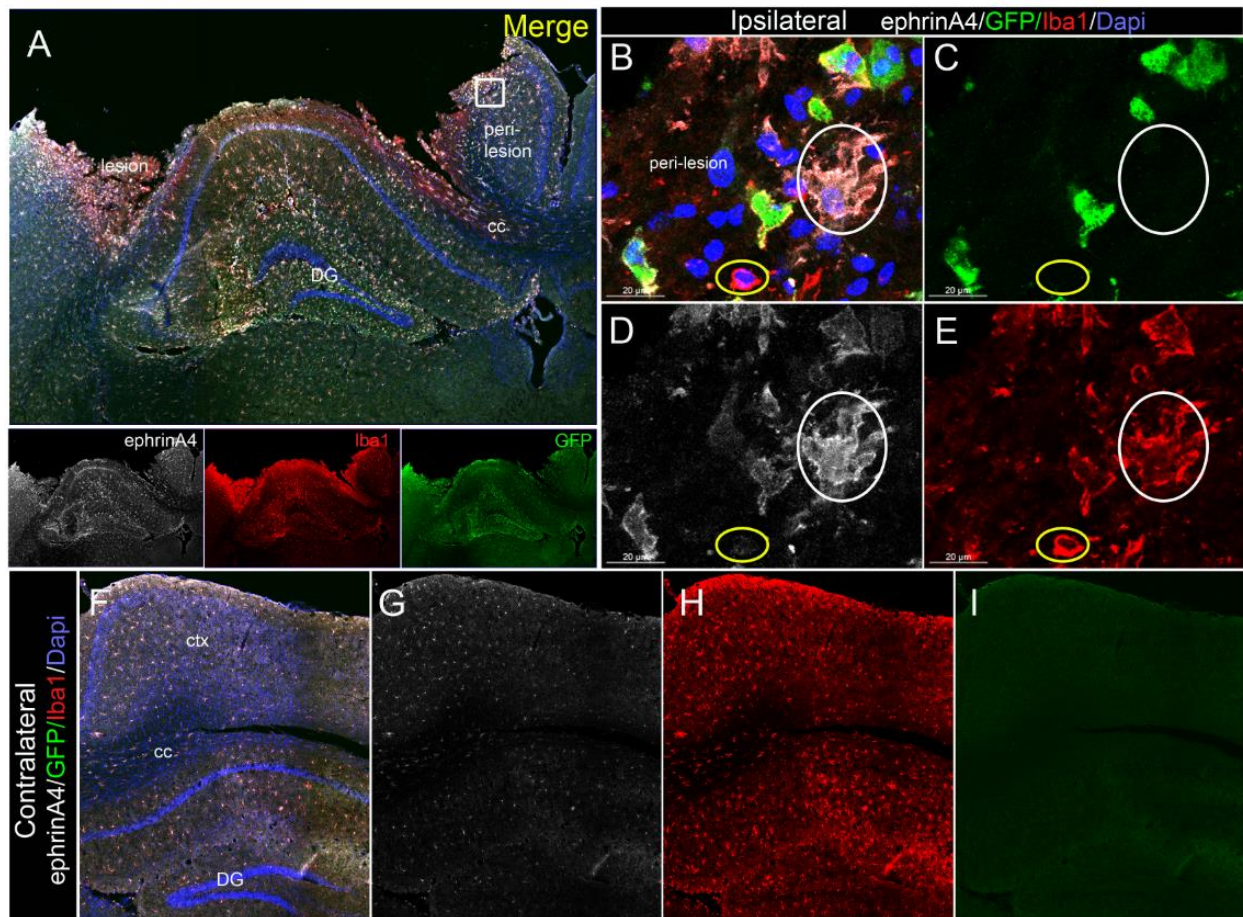


Figure 7. **Investigation of EphrinA4 signaling within the ipsilateral of AT-WT mice at 3dpi.** (A) Representative confocal image of WT 3dpi cortex expressing ephrinA4 (white), Iba1 (red), GFP+ immune cells (green), and DAPI overlapping in blue. (B-E) Resident microglial cell expressing ephrinA4 within the ipsilateral region at 3dpi (white) and resident microglial cell overlapping with DAPI only (yellow). (F-I) Representative confocal image of a 3dpi contralateral showing evidence of only DAPI (blue), ephrinA4 (white), Iba1 (red), and GFP+ infiltrating immune cells (green).

CHAPTER THREE

Transcriptomic Analysis

Background

In the pursuit of a deeper comprehension of cellular responses and the impact of immune cells within the brain following traumatic brain injury (TBI), transcriptomic investigations in both model organisms and humans have taken center stage. Nevertheless, these studies typically rely on aggregated RNA from either mixed cell populations, which may exhibit age-related variations, or from cell populations isolated using established markers, which themselves may undergo age-related changes. Consequently, while significant progress has been made in identifying key aging-related genes and pathways, prior transcriptomic analyses have not distinguished between aging-related alterations common to all brain cells and those specific to certain cell types. Thus, there is a pressing need to uncover how aging affects individual cell types and to ascertain whether the aging process follows a uniform pattern across all cell types or if certain cells undergo distinct transcriptional changes. This insight is crucial for determining whether aging at the tissue level is a universal phenomenon, resulting from specific alterations in particular cell populations leading to functional decline and deterioration, or a combination of both factors. Such understanding could inform the development of targeted aging-related therapies, tailored to affect specific cell types or broader interventions impacting all cells.

Eph receptors, short for erythropoietin-producing human hepatocellular receptors, are crucial for axon growth and guidance during early brain development, playing a vital role in embryonic and early postnatal stages [197]. Their function involves bidirectional signaling, which significantly impacts Eph/ephrin biology [198]. This family of receptor tyrosine kinases, divided into classes A and B and expressed throughout the body, holds particular importance in brain injury response, including blood-brain barrier regulation [199, 200]. For instance, EphA6 expression increases post-TBI, correlating with reduced neurite density and impaired motor function [201]. Additionally, ephrinB2/EphB4 signaling is involved in arteriovenous specification [202]. Notably, EphA4 loss of function in the bone marrow immune compartment mitigates neuroinflammation and promotes neuroprotection after TBI [203], while EphA2 activation following ischemic stroke exacerbates injury outcome by increasing BBB permeability [204]. Thus, Eph receptors emerge as potential targets in the secondary injury response following

brain injury. The present study aims to investigate the cell-autonomous role of EphA4 in modulating functional deficits and its impact on resident microglia in the context of post-TBI repair processes.

Material & Methods

All animal studies are carried out under the NIH and the Virginia Tech's IACUC guidelines. *Adoptive Transfer (AT)*: Male CD1 mice purchased from Charles River laboratories were irradiated, injected with mTmG harvested GFP+ bone marrow cells either having WT or KO EphA4 and provided with Gentamycin water for 2 weeks following irradiation as detailed in the previous chapter.

Controlled Cortical Impact (CCI): At-WT and AT-KO mice were injected via subcutaneously with ketamine (100 mg/kg) and xylazine (10 mg/kg) in order to anesthetize before surgery. Temperature of the mice was monitored using a rectal probe and a controlled heating pad set to 37°C. Following anesthetization, the mice are positioned in a stereotaxic frame and a 4mm diameter craniotomy is made over the right parietal-temporal cortex with the use of a dremel. The injury is caused by a program-controlled cortical impactor ($\Phi = 3$ -mm beveled tip) connected to an eCCI-6.3 device (Custom Design & Fabrication, LLC) at a velocity of 5.0 m/s, depth of 2.0 mm, and 100ms impact duration. Following injury, the incision was closed using Vetbond tissue adhesive (3M, St. Paul, MN, USA) and sutured. The animals are then placed into a heated cage and monitored every 20 min until fully recovered from anesthesia.

Tissue Procession for scRNAseq: AT-KO and AT-WT murine mouse models were anesthetized using isoflurane exposure and euthanized at 90dpi. Both WT and KO ipsilateral regions of the brain were separated in preparation for neuronal dissociation of the hippocampus of both experimental regions. RNA isolation of the microglia was performed using the Miltenyi Biotec neural tissue dissociation kit (p) followed by its preservation in CryoStor CS10. RNA sequencing was conducted by MedGenome. The 10x genomics cellranger (version 7.1.0) mkfastq analysis pipeline was used for demultiplexes raw base call (BCL) files generated by Illumina sequencers into FASTQ files. Seurat (package version 4.1.0) was used for QC analysis and exploration of single-cell RNA-seq data. Doublets were identified using only gene expression data with the use of DoubletFinder package (v2.0.3). Global-scaling normalization

method “LogNormalize” was used to normalize the data after removing unwanted cells. Wilcoxon Rank Sum test was used to define gene markers and DESeq2 method was also used to detect populations greater than log₂ FC. GO enrichment was performed by Enrichr Ontologies, GO Biological Processes. Transcription factor analysis was performed with Enrichr Submissions TF-Gene Cooccurrence on Enrichr.

Hippocampal scRNAseq

To gain more insight into the effects of EphA4 signaling on resident microglia, the use of unbiased high-throughput single-cell RNA sequencing (scRNA-seq) was implemented to examine the transcriptional profiles of resident microglia post-TBI in AT-WT and KO mouse models (Figure 1). Mouse models underwent a CCI procedure and were euthanized at 90dpi for neuronal dissociation of the ipsilateral hippocampal region. The initial analysis led to the identification of several cell types with distinct expression profiles including, astrocytes, endothelial cells, immune cells, microglia 1, microglia 2, neural progenitor cells, neuroblasts, oligodendrocytes, OPCs, pericytes, smooth muscle cells, and one unidentifiable group (Figure 1A). These cell types were then separated by expression within the AT-WT versus AT-KO mouse models, with the KO group showing a visibly higher expression of microglia cell type 2 than in WT (Figure 1B). GFP features were measured from GFP-tagged mTmG injected immune cell, previously explained, and identified cell type microglia1 as the highest percentage of GFP expression within the hippocampus (Figure 1C). Immune cells have also show expression of GFP in the hippocampal region 90dpi indicating the residence of peripheral derived immune cell infiltration occurring during chronic progression of TBI. As a phagocytic cell type, microglia are able to engulf these GFP tagged immune cells and present immunofluorescence of both the microglia and GFP+ immune cell, this may give reason as to why microglia1 have expression of GFP features. Confirmation of these microglial cell types were tested utilizing various well known cell type specific marker genes for microglia including, *Iba1*, *Cx3cr1*, *Mertk*, and *CD45* (Figure 1D).

Further investigation of cell type microglial1 is illustrated by heatmap of the top 10 upregulated genes for the WT and KO hippocampal samples 90dpi (Figure 1E). The top gene expressed in WT samples following TBI appears to be predominantly involved in meeting the energy demands of microglia post-TBI. Although specific data on *FGGY* expression in microglia are limited, it is well-documented that metabolic genes play crucial roles in the activation and function of immune cells [205]. Microglial activation, which occurs in response to CNS injury or disease, can lead to metabolic shifts within the cells. Upregulation or changes in *FGGY* expression might be indicative of altered metabolic states associated with different activation states of microglia. The *SLC5A2* gene encodes the sodium/glucose cotransporter 2 (*SGLT2*), which is primarily known for its role in glucose reabsorption in the kidneys. The expression and function of *SLC5A2* in microglia are not as well-characterized as in other tissues [206-212]. However, considering general principles of cellular biology and glucose metabolism, some potential implications of *SLC5A2* expression in microglia can be explored involving its active role in transport of glucose into cells, coupled with sodium ions. In the context of microglia, the expression of *SLC5A2* could facilitate glucose uptake from the extracellular environment, supporting the metabolic needs of these cells. Microglia require energy for their various functions, including surveillance, phagocytosis, and response to injury or infection. Efficient glucose uptake would be crucial for meeting the energy demands of mediating a TBI. The *1700126G02Rik* gene, also known as *P2RY12*, encodes the *P2Y12* receptor, which is a G-protein-coupled receptor involved in purinergic signaling. *P2Y12* is known to be highly expressed in microglia and has important functions related to their physiology and activity in the CNS [213-214]. The *Etl4* gene, also known as *EPM2AIP1* (Epilepsy, Progressive Myoclonus Type 2A, Interacting Protein 1), has been studied in various cellular contexts, but its specific role and implications in microglia are not well-documented. Given *Etl4*'s known association with glycogen metabolism, it would suggest that the gene could be involved in regulating the energy needs of these cells [215-217]. Since the metabolic processes in microglial function and activation are crucial to mediating TBI, *Etl4* expression might influence how microglia respond to CNS injuries and diseases. In summary, while the specific roles of these genes in microglia are not fully understood, their differential expression post-TBI highlights their potential importance in microglial energy metabolism and activation. The observed changes in gene expression also underscore the impact of the loss of EphA4-binded signaling on microglial function post-injury.

Analysis of the genes most upregulated in the KO mouse model for cell type microglial were also investigated to further identify the role of EphA4 signaling in the cellular activation phenotype of microglia post TBI (Figure 1E). Majority of the genes show evidence of cellular regulation of not only metabolism but shape as well as influence in cell signaling. The *TPM3-RS7* gene is not widely recognized in the current scientific literature, however, TPM3 refers to the gene encoding tropomyosin 3, a protein involved in the regulation of the actin cytoskeleton, which is crucial for various cellular functions including movement, stability, and shape [218-220]. While *TPM3* is primarily known for its role in actin cytoskeleton regulation, its expression in microglia could imply involvement in cytoskeletal dynamics necessary for microglial functions such as migration, phagocytosis, and morphological changes [221]. Understanding the specific role of TPM3 in microglia would require further research, including verification of its expression and functional studies to elucidate its impact on microglial behavior in the CNS. The *PPFIA4* gene encodes liprin- α 4, a member of the liprin family of proteins that are involved in the organization of synaptic junctions and intracellular signaling pathways [222]. While the specific role of *PPFIA4* in microglia is not extensively documented, we can infer potential implications based on the general functions of liprin- α proteins and their known roles in other cell types. In microglia, *PPFIA4* might be involved in cell signaling pathways that regulate microglial activation, motility, and phagocytosis. By organizing signaling complexes, liprin- α 4 could influence how microglia respond to extracellular cues and mediate their interactions with other CNS cells. Overall, while the specific role of *PPFIA4* (liprin- α 4) gene expression in microglia is not well-documented, it is likely involved in intracellular signaling, cytoskeletal regulation, and interactions with synapses. The *CXCL1* gene encodes the chemokine (C-X-C motif) ligand 1, also known as growth-regulated alpha protein (GRO- α). CXCL1 is a member of the CXC chemokine family and plays a crucial role in inflammation and immune responses [223]. In the context of microglia, *CXCL1* gene expression has several important implications including but not limited to microglial activation due to injury or infection, cell-to-cell communication, and neuroinflammatory regulation. Elevated *CXCL1* levels in microglia have been associated with various CNS diseases, indicating its potential as a target for therapeutic interventions to modulate inflammation and immune responses in the brain [224-227]. The *Trnaulap* gene, also known as *TRNA-UI* (adenosine(58)-N(1))-methyltransferase interacting protein 1 (*TRNAUIAP*), encodes a protein that interacts with tRNA methyltransferase [228]. This

interaction is important for the proper modification and maturation of transfer RNAs (tRNAs), which are essential for protein synthesis. *Trnauiap* gene expression in microglia likely plays a role in supporting the protein synthesis machinery through tRNA modification. This function is essential for maintaining microglial health and enabling their responses to activation and neuroinflammatory conditions. While specific studies on *TRNAUIAP* in microglia are limited, understanding its role can provide valuable insights into the molecular mechanisms underlying microglial function and their involvement in CNS diseases. Further research is needed to fully elucidate the implications of *TRNAUIAP* expression in microglial biology [229-235]. Overall, the expressed genes of microglia1 within the KO model show no direct implication of being solely pro- or anti-resolving of TBI.

scRNAseq analysis of upregulated genes were investigated for the cell type microglia2 group as well, illustrated by heatmap (Figure 1F). In comparison to the genes expressed in the WT model for microglia1, WT microglia2 genes are also heavily involved in the cellular function of the cell. With top regulated genes including, *Gdpd3*, also known as glycerophosphodiester phosphodiesterase domain-containing protein 3 (*GDPD3*), an enzyme involved in lipid metabolism. *GDPD3* is part of the glycerophosphodiester phosphodiesterase (*GDPD*) family, which plays a role in hydrolyzing glycerophosphodiesters to produce glycerol-3-phosphate and alcohol. By generating signaling molecules such as glycerol-3-phosphate, *GDPD3* can influence various signaling pathways that control cell proliferation, differentiation, and response to external stimuli [236-238]. *GDPD3* gene expression in microglia is likely related to its roles in lipid metabolism, signal transduction, and membrane remodeling. These functions are essential for microglial activation, inflammatory responses, and phagocytic activity. The *LARS2* gene encodes the enzyme leucyl-tRNA synthetase 2 (*LARS2*), which is located in the mitochondria. *LARS2* is responsible for the aminoacylation of tRNA with leucine, an essential step in mitochondrial protein synthesis. This process is crucial for the proper function of mitochondria, which are the powerhouses of the cell, providing energy through oxidative phosphorylation. *Lars2* gene expression in microglia is likely essential for maintaining mitochondrial function and energy metabolism. This role supports various microglial activities, especially during activation and in response to CNS challenges. Proper *LARS2* function is crucial for preventing mitochondrial dysfunction and oxidative stress, thereby maintaining the neuroprotective roles of microglia [239-245]. The *mt-Nd6* gene, which stands for mitochondrial

NADH dehydrogenase subunit 6, is a component of the mitochondrial genome. It encodes a subunit of *NADH* oxidoreductase, commonly known as Complex I, which is the first enzyme in the mitochondrial electron transport chain. Complex I is crucial for cellular respiration and ATP production through oxidative phosphorylation [246-248]. Efficient mitochondrial function in microglia is also essential for their role in neuroprotection. Adequate *mt-Nd6* expression helps maintain mitochondrial integrity and function, supporting microglia in their protective roles against neurodegeneration [248-249]. Lastly, the *KIF5A* gene encodes kinesin family member 5A, a motor protein involved in the intracellular transport of organelles, vesicles, and proteins along microtubules. *KIF5A* is a crucial component of the cellular machinery that ensures proper distribution and localization of various cellular components, which is vital for cell function and survival [250-255]. In summary, these genes regulating the function of resident microglial cells represented in the microglia2 cell type seem to play a crucial role in the structural integrity and efficient operation of microglia following TBI.

Interestingly, when the top upregulated genes of microglia2 within the KO hippocampus were examined, the genes showed evidence of both anti- and pro-resolving protein expressions. The *NFKBIA* gene which encodes *IκBα* (inhibitor of nuclear factor kappa-B kinase subunit alpha), is a critical regulator of the nuclear factor-kappa B (NF-κB) signaling pathway. *NF-κB* is a transcription factor that plays a central role in the immune response and inflammation by regulating the expression of genes involved in immune responses, cell survival, and inflammation [256]. Microglia express *NFKBIA* to tightly regulate *NF-κB* activation. Upon activation by CNS injury, infection, or neurodegenerative stimuli, microglia upregulate *NFKBIA* as part of the negative feedback mechanism to control the inflammatory response [257]. In contrast of *NFKBIA*, the *CXCL2* gene encodes a chemokine called C-X-C motif ligand 2, also known as macrophage inflammatory protein-2 alpha (*MIP-2α*). *CXCL2* acts as a pro-inflammatory mediator by inducing the activation of microglia and promoting the production of inflammatory cytokines and chemokines. Elevated *CXCL2* expression in microglia often correlates with their activation state. It can influence microglial morphology, phagocytic activity, and secretion of inflammatory molecules, thereby shaping the local immune environment in the CNS [258-267]. It is unclear at this time whether the microglia1 or microglia2 can be considered specifically helpful or harmful to the progression of a TBI but it is understood that these cells are working hard to mediate the injury.

Lastly, our findings lead us to investigate further into the microglia² cell type illustrated in the Umap of Figure 2A. These genomic analysis of microglia² was then split between the AT-WT and AT-KO hippocampal tissue samples and identified an outlier group of proteins being expressed within the KO model and is further identified by cluster frequency (Figure 2B-C). The top five upregulated genes for cluster 4 of the KO group are identified as *CYRR1*, *ST6GALNAC3*, *ADGRF5*, *PREX2*, and *ECE1* as illustrated by heatmap (Figure 2D). The genes *CYRR1*, *ST6GALNAC3*, *ADGRF5*, *PREX2*, and *ECE1* in relation to microglia seem to share common themes of involvement in cell signaling and communication, immune and inflammatory responses, glycosylation and cell surface interactions, development and neuroplasticity, and responses to environmental changes and stress. *ADGRF5* encodes a member of the adhesion *GPCR* family, which is involved in cell-cell communication, signaling, and possibly migration and adhesion of microglia in response to CNS changes [268-274]. *PREX2* is involved in signaling pathways that regulate cell migration, adhesion, and the cytoskeleton, all of which are crucial for microglial responses to injury and inflammation [275]. *CYRR1*, while its specific role in microglia is less well-defined, its involvement in cysteine and tyrosine-rich domains often play roles in redox regulation and signaling, which are important in the context of immune responses and microglial activation [276-278]. To further substantiate these findings and elucidate the potential influence of this cell type, Gene Ontology (GO) biological process analyses were conducted. The results demonstrated a significant involvement in microglial cell activation, regulation of cell migration, and gene expression (Figure 1F). Transcription factor (TF) data for cluster 4 KO genes presented evidence of microglia² cell type being neuroprotective at 90dpi (Figure 2G). *ZFP36*, the top expressed transcription factor expressed, is known to bind to adenine-uridine (AU)-rich elements in the 3' untranslated regions of target mRNAs, leading to their destabilization and degradation. Many of these target mRNAs encode pro-inflammatory cytokines such as *TNF- α* , *IL-6*, and *IL-1 β* . By promoting the degradation of these cytokines' mRNAs, *ZFP36* helps to regulate and potentially reduce the inflammatory response in microglia. By controlling the levels of inflammatory cytokines, *ZFP36* contributes to neuroprotection [279-281]. Within the TF's identified, *ZFP36* is one of many genes found to play this role in microglia², *JUNB*, *JUND*, *CEBPB*, *ID2*, and *ZFP36L2* all play a role in regulating not only inflammation but cell survival and proliferation through regulation for gene expression of inflammatory factors [282-283].

Conclusion

In summary, our comprehensive analysis of microglial gene expression post-TBI in AT-WT and KO mouse models reveals significant insights into the roles of various genes in microglial activation and function. The differential expression patterns of key metabolic, signaling, and structural genes underscore the complex regulatory mechanisms at play in microglial response to CNS injury. Genes such as *FGGY*, *SLC5A2*, and *P2RY12* highlight the importance of metabolic and signaling pathways in microglial activation, while *TPM3-RS7*, *PPFIA4*, and *CXCL1* emphasize the roles of cytoskeletal regulation and immune response. The identification of genes involved in lipid metabolism, mitochondrial function, and immune regulation in the KO model further illustrates the intricate balance between pro- and anti-inflammatory responses in microglia. Furthermore, our understanding of the microglia2 cell type indicates that loss of EphA4 expression for PICs show evidence of neuroprotection through the alteration of the cells' genomic expression. It is also important to note that the microglia2 cell type, unlike microglia1, showed little to no expression of GFP features for in both WT and KO environmental settings. This provides additional evidence of the negative effects post-CCI that PICs play in mediating TBI even during the chronic progression of an injury. Based on the scRNAseq results, we can confirm that the microglial cell type, although complex in expression, leads to a more pro-inflammatory environment given its genomic expression and their roles in microglial activation. Microglia2 was proven to be more anti-inflammatory when analyzing its transcription factors found in cluster 4 of its KO group. This cluster was not found in the WT group and leads us to confirm loss of EphA4 binding as a main factor in the cell's progression toward a pro-resolving phenotype. These findings not only enhance our understanding of microglial biology but also suggest potential therapeutic targets for modulating microglial activity and improving outcomes in CNS injuries and diseases. Future studies will be essential to elucidate the specific functional roles of these genes in microglial physiology and their implications in neuroinflammation and neuroprotection.

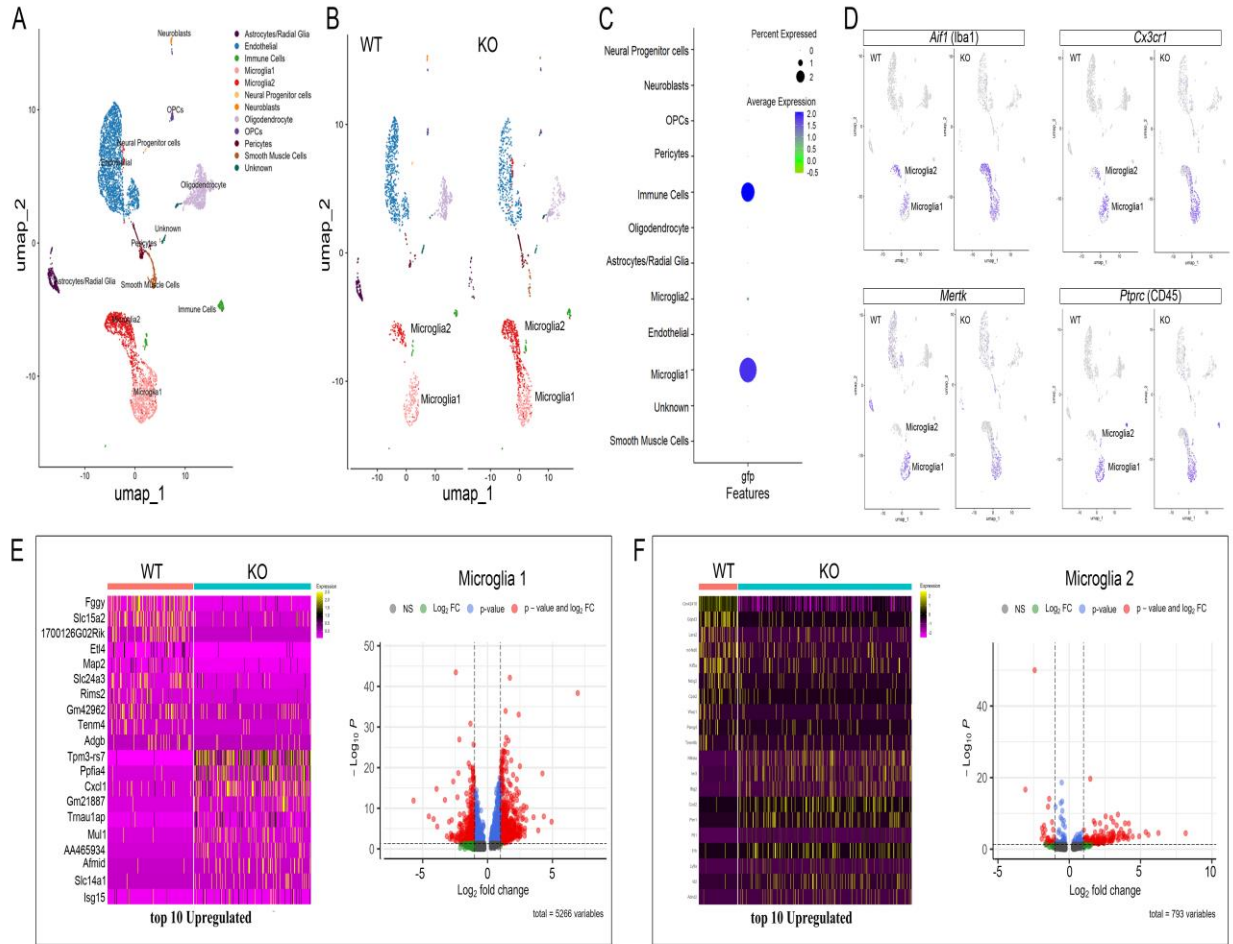


Figure 1. Gene ontology of microglial cells from the hippocampus of the ipsilateral at 90dpi. (A) Ipsilateral cells of the hippocampus are clustered based on RNA gene expression in a Uniform Manifold Approximation and Projection (UMAP) plot. (B) Microglial 1 group cells were further classified into AT-WT and AT-KO subclusters for UMAP analysis. (C) Gene Ontology (GO) analysis of GFP features. (D) Genomic markers to identify resident microglial cells, IBA1, Cx3cr1, Mertk, and CD45. (E) Heatmap of top DEG's (Y axis) per cluster (X axis) for AT-WT and AT-KO showing distinctive gene expression profiles between Microglia type1. Yellow and purple represent higher and lower gene expression, respectively. Volcano plot showing significant genes vs. log2Fold change. (F) Heatmap of top DEG's (Y axis) per cluster (X axis) for AT-WT and AT-KO showing distinctive gene expression profiles between Microglia type2. Yellow and purple represent higher and lower gene expression, respectively. Volcano plot showing significant genes vs. log2Fold change.

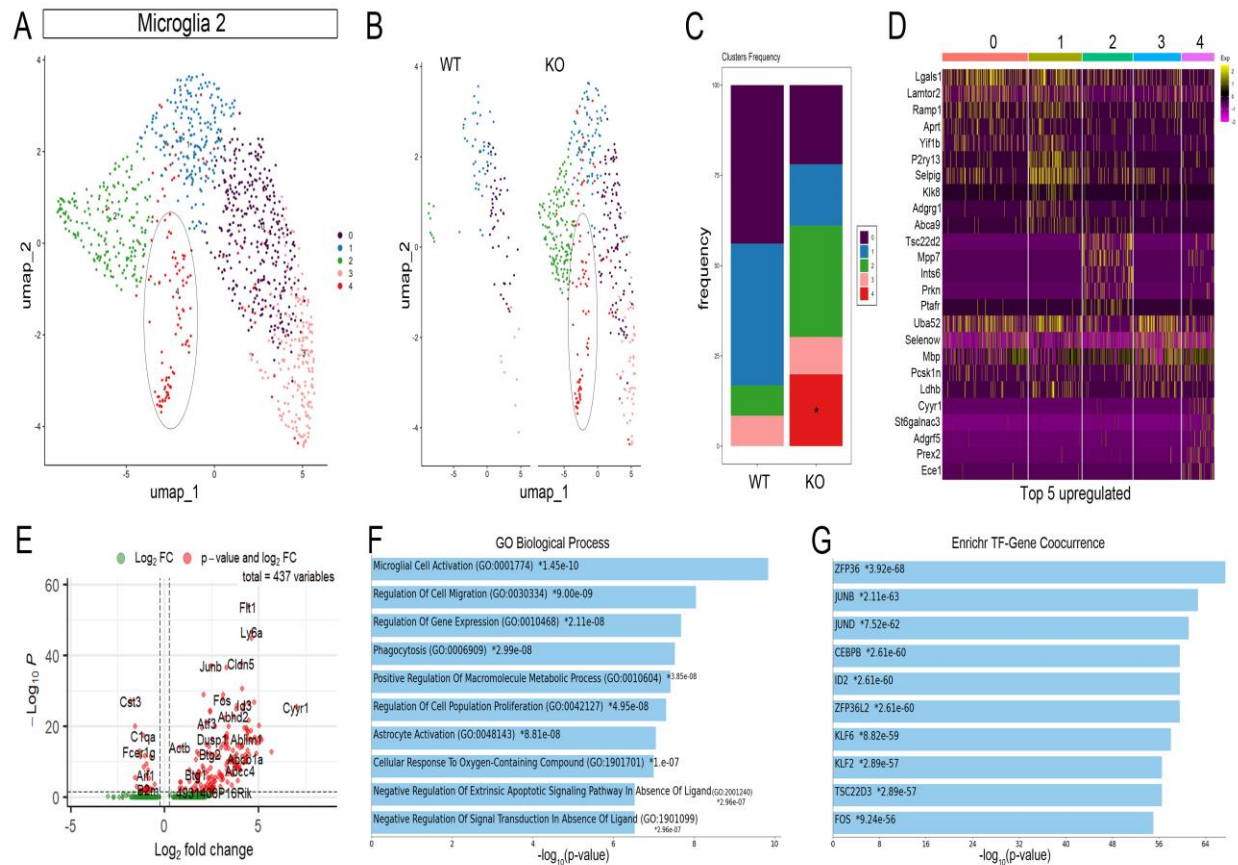


Figure 2. scRNAseq transcriptomic signatures of ipsi-hippocampal resident microglia 2 following CCI Injury chronically post-TBI at 90dpi.

(A) Cell type Microglia2 cells of the hippocampus are clustered based on RNA gene expression in a Uniform Manifold Approximation and Projection (UMAP) plot. (B) Microglial type 2 cells were further classified into AT-WT and AT-KO subclusters for UMAP analysis. (C) Frequency chart detailing WT and KO microglia 2 subclusters. (D) Heatmap of top DEG's (Y axis) per cluster (X axis) for AT-WT and AT-KO showing distinctive gene expression profiles for cell type Microglia2. Yellow and purple represent higher and lower gene expression, respectively. (E) Volcano plot showing significant genes vs. \log_2 Fold change. (F) Gene Ontology (GO) of the biological processes based on the identified top upregulated genes. (G) Transcription factor analysis performed with Enrichr Submissions for microglia2 KO group 4 cluster.

CHAPTER FOUR

Future Directions

Various experimental approaches have been employed to investigate the interaction between peripherally derived EphA4 binding and resident microglial cells, exploring its impact on cell activation and neuroinflammation mediation following traumatic brain injury (TBI). These investigations have encompassed immunohistochemical analysis, single-cell RNA sequencing, murine behavioral testing, and other detailed methodologies outlined in the preceding sections. Initial findings suggest a potential shift towards a more neuroprotective phenotype in microglia following TBI when EphA4 signaling from peripheral-derived immune cells, likely monocyte/macrophages, is absent, although this area of study remains in its early stages. Drawing from my background and recent research experience in this field, several future research directions can be proposed to enhance our understanding of these complex resident immune cells, microglia, and advance this line of inquiry.

Single-cell RNA sequencing (scRNA-seq) has emerged as a crucial tool for gaining detailed insights into the genomic expression profiles of various cell types. Given the complexity and diversity of microglial cells, especially upon activation during CNS injury, a thorough examination is essential. In this dissertation, scRNA-seq was employed to characterize the transcriptomic progression of microglial cells within the hippocampal region in the chronic phase of TBI when cognitive deficits emerge. Evidence showed two significant groups of microglial cells identified as Microglia1 and Microglia2. Microglia1 showed an array of genomic features involving cellular function and metabolism within the AT-WT mouse model and a stronger influence in cell signaling within the AT-KO group. The featured genes of microglia1 were involved more closely to neuroinflammatory production in comparison to those of microglia2 that had expression of both pro- and anti-inflammatory influencing genes. TF's of microglia2 further characterized the cell types influence in the KO model as neuroprotective when further analysis of the KO cluster 4 was conducted. Future studies should focus on investigating microglia specifically at the injured cortex, with a more precise division between the core and perilesional regions which show visual evidence of significantly differing level of GFP expressed PICs. It would also be beneficial to investigate the variety of cellular expression of PICs infiltrating in the WT vs. KO experimental settings to further understand their variation in expression post-TBI. These results may show evidence of one cell type being more neuroprotective in the

mediation of neuroinflammation or supporting of the resident microglia. The myeloid cells involved in infiltration post-TBI could also differ in expression when comparing acute and chronic progression of the injury, further illustrating the cellular influence of PICs involved in neuroinflammation.

Complementing these genomic analyses with immunohistochemical (IHC) staining of microglia would provide a visual illustration of the cellular processes occurring at chronic time points post-TBI. Detailed analysis of cell morphology using IHC, categorized by core, peri-lesion, and boundary areas, could confirm the influence of proximity to the initial impact on cellular phenotype and morphology. Spatial transcriptomics of the microglia within these identifiable regions of the injury can also provide greater context of the tissue's architecture as well as insight into microglia's phenotype based on their perspective microenvironments. Other parameters that could be used to characterize microglia's phenotype include the use of oxidative burst assays to identify reactive oxygen species showing evidence of microglial activation, multiplex assays can be implemented to investigate cytokine expression, and even patch-clamp recordings to measure the ionic currents of microglia in both acute and chronic progression to show changes in microglial electrical properties.

Additionally, examining infiltrating immune cells, particularly monocytes' ability to adopting a microglial morphology during prolonged cellular interaction with microglia would enhance our understanding of cell's morphological changes post-TBI. In terms of the cellular interaction between these cell types, further investigation into the expression of ephrin ligands, specifically ephrinA4, is necessary to elucidate the preferred ligands of microglial cells and their binding to EphA4-expressing peripheral immune cells (PICs). Studies using microglia-specific ephrinA4-KO mice may help support the role of ephrin reverse ligand signaling on acute and chronic microglial changes in TBI. This multi-faceted approach will provide a comprehensive understanding of microglial activation and function in response to CNS injury and their contribution to the inflammatory environment during the progression of chronic neurological symptom onset.

Given the results of the two microglial cell types identified within the hippocampus, it appears that microglial cell subset alteration could be a direct target for quelling the neuroinflammatory effects of TBI. Their roles extend beyond neurodevelopment to encompass the mediation of CNS injury, involving both pro- and anti-resolving mechanisms, and the current

work supports to the role of peripheral immune cells regulating microglia activities. Although microglia can recruit peripheral immune cells to manage neuroinflammatory responses, the influence of these external cells is just being elucidated. Our findings here demonstrate that by eliminating the influence of peripheral derived EphA4 signaling from infiltrating immune cells, microglial cells can be manipulated into a more pro-resolving phenotype. Upon knocking out the EphA4 receptor specifically on PICs, we have found evidence of decreased proliferation of both resident microglia and PDMs during acute progression of TBI, increased behavioral function during chronic progression, and genomic alteration of microglia toward a more pro-resolving phenotype. Although further research is essential to expand our understanding of peripheral derived EphA4 signaling, this research provides a plausible route for therapies targeting neuroinflammation resulting from TBI.

While this study provides valuable insights into the role of peripheral immune-derived EphA4 on microglial dynamics following TBI, several limitations need to be acknowledged. Firstly, tissue degradation at the site of injury within both the acute and chronic timepoints of progression can be hindering to the replication of this study. Tissue loss is also evidence of cellular loss and can become a hinderance when performing cellular quantifications based on immunofluorescent staining. Our studies have shown evidence of tissue degradation due to the neuroinflammatory progression following TBI. Secondly, the study's focus on acute and chronic timepoints (1 dpi, 3dpi, and 60dpi) provides a snapshot of microglial responses, but continuous monitoring over the entire injury and recovery period could yield a more comprehensive understanding of the temporal dynamics involved. Additionally, the use of single-cell RNA sequencing, while powerful, is inherently limited by its resolution and the potential for missing subtle but important cellular changes. Furthermore, the study primarily investigates microglial cells and peripherally derived macrophages, potentially overlooking the contributions of other cell types, such as astrocytes and neurons, which also play critical roles in the TBI response. Lastly, while the study highlights the role of EphA4 in microglial activation and behavior, it does not fully explore the downstream signaling pathways and molecular mechanisms involved, which are crucial for developing targeted therapeutic interventions. Addressing these limitations in future research will be essential for translating these findings into effective treatments for TBI in humans.

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