TRAUMATIC BRAIN INJURY-ASSOCIATED MICROGLIA ADOPT LONGITUDINAL TRANSCRIPTIONAL CHANGES CONSISTENT WITH LONG-TERM DEPRESSION OF SYNAPTIC STRENGTH

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Traumatic brain injury (TBI) is an under-recognized public health threat. Even mild brain injury, or concussions, may lead to long-term neurologic impairment. Microglia play a fundamental role in the development and progression of this subsequent neurologic impairment. Despite this, a microglia-specific injury signature has yet to be identified. In the current study we hypothesized that TBI-associated microglia would adopt longitudinal changes in their transcriptional profile associated with pathways linked to the development of motor, cognitive, and behavioral disorders. C57BL/6 mice underwent TBI via a controlled cortical impact and were followed longitudinally. FACSsorted microglia from TBI mice were subjected to RNA-sequencing at 7, 30, and 90 days post-injury. We identified 4 major patterns of gene expression corresponding to the host defense response, synaptic potentiation, lipid remodeling, and membrane polarization. In particular, significant upregulation of genes involved in long-term synaptic potentiation including Ptpn5, Shank3, and Sqstm1 were observed offering new insight into a previously unknown role of microglia in the weakening of synaptic efficacy between neurons after brain injury.

Introduction

Traumatic brain injury is a growing and under recognized public health threat. The CDC estimates nearly 2 million people sustain a traumatic brain injury (TBI) each year in the United States, contributing to over 30% of all injury related deaths ^{1,2}. In fact, TBI related healthcare expenditures near 80 billion dollars annually with an average cost of 4 million dollars per person surviving a severe TBI ³⁻⁵. The impact of TBI is highlighted not only by its high mortality rate but also by the significant long-term complications suffered by its

survivors with the progressive development of motor, cognitive, and behavioral disorders⁶⁻¹⁰. Even subconcussive events, those resulting in subclinical brain dysfunction without the typical symptoms of concussion, may lead to long-term neurologic impairment^{11,12}. The immune response to TBI plays a fundamental role the development and progression of subsequent neurodegenerative disease and represents a complex interplay between the injured brain and the resident immune cells of the brain — the microglia¹³. The current manuscript is focused on developing a cell-type-specific understanding of the microglial response to injury over time. Here we highlight the major trends in gene expression in response to TBI.

TBI triggers a robust pro-inflammatory response within the injured brain. The degree of this initial pro-inflammatory response has significant value in predicting more long-term outcomes after TBI¹⁴⁻¹⁶. Even after the acute inflammatory response has resolved, several studies demonstrated residual long-lasting inflammation within the brain in both animal models as well as in patients^{17,18}. One of the main drivers of this continued inflammation is the persistence of activated microglia—characterized by thickening and retraction of their ramified processes, increased IL-1 and IL-6 with concomitant decreases in IL-4 and IL-10, and increased expression of pro-oxidant genes with a reduction of growth and antioxidant genes. A recent study showed an increased inflammatory profile of microglia that persisted for up to 12 months after injury in a murine model of TBI¹⁹. Furthermore, this continued inflammation is associated with lesion volume expansion and loss of neurons in the hippocampus¹⁸⁻²². Even once the acute inflammatory process has resolved and infiltrated monocyte-derived macrophages are no longer present within the

injured brain, microglia have the potential to remain activated for years after the initial insult^{19,23}. A functional consequence of this constitutive activation after brain trauma is an exaggerated neuroinflammatory response to otherwise benign secondary stimuli such as a subsequent subclinical head injury^{10,24-27}. This may be the mechanism by which patients who have sustained a concussion are more susceptible to subsequent concussions²⁸⁻³⁰. Nonetheless, the molecular mechanisms resulting in the constitutive activation of microglia remain elusive²¹. Therefore, in the current study, we aimed to study the transcriptional dynamics of constitutively activated microglia over the course of brain injury.

The first step towards any cell-specific transcriptional analysis relies on obtaining sufficient cells of interest with the highest purity. The historical standard for distinguishing between microglia and infiltrating macrophages is immunohistochemistry. Although immunohistochemistry is useful for assessing morphology, proliferation, and sites of activation it has a number of drawbacks limiting its use³¹. Several investigative groups have focused on this problem including column free magnetic separation and CD11b immunomagnetic enrichment combined with the differential expression of CD45 with flow cytometry³¹⁻³³. However, CD45 expression has been reported to vary depending on the pathologic condition; thus, reliable separation of microglia from peripheral myeloid cells is impaired^{34,35}. To overcome this issue, fluorescently marked myeloid cells, such as CX3CR1^{+/GFP}/CCR2^{+/RFP}, have been used. However, these mice are on mixed backgrounds, which could greatly affect the immune response. Furthermore, the presence of contaminated nonclassical monocytes could not be excluded^{36,37}. Therefore,

we use head-shielded bone marrow chimeric mice with CD45.1 cells in the circulation and CD45.2 microglia in the brain allowing definitive and unambiguous differentiation between microglia and infiltrating bone-marrow derived myeloid cells as previously described by our laboratory³⁸. To the best of our knowledge, no cell-type-specific study has been conducted to specifically identify transcriptional changes in isolated populations of microglia over the course of TBI. In the current study we demonstrate that TBI-associated microglia adopt longitudinal changes in their transcriptional profile associated with pathways linked to the development of motor, cognitive, and behavioral disorders.

Results

Global patterns of gene expression from isolated populations of microglia over the time course of TBI

The neuroinflammatory response to TBI is central to both neuroprotection and neurotoxicity after injury, but attempts to broadly target immune activation have been unsuccessful in improving outcomes in TBI patients³⁹⁻⁴². Because of this, there has been a growing interest in investigating the microglial transcriptome after brain injury. Attempts thus far have been plagued by the use of whole brain homogenates rather than individual cell types as well as the use of microarray analyses restricted to a limited number of genes in the inflammatory response^{43,44}. Therefore, we combined our ability to discriminate and sort microglia from infiltrating monocytes and macrophages with unbiased transcriptional profiling (RNA-seq) on FACSorted microglia from 7, 30, and 90 days after TBI. We defined 396 genes that change expression across the time course (see methods). We clustered these differentially expressed genes into 4 main patterns of expression (**Fig. 1**).

Clusters 2 represents genes involved in synaptic plasticity and is progressively upregulated over the course of injury. Cluster 4 represents genes involved in the regulation of membrane polarization with gene expression that is upregulated at 30 days post-injury and then downregulated by 90 days post injury. Clusters 1 and 3 represent genes involved in the host response to injury and lipid remodeling and both are progressively downregulated over the course of injury. These data provide new insights into the biology of microglial activation over the course of TBI.

Figure 1. Microglia from TBI mice show distinct time-dependent transcriptional profiles. K-means clustering: Four clusters representing 392 genes are shown with distinct time specific expression patterns. Averaged $\log_2(\text{CPM}+1) \geq 3$ of genes in each

group that had a log fold change of at least 2 was used to generate the heatmap. GO processes associated with the genes in each cluster was determined using Gorilla database.

Pairwise comparison of microglia gene expression between time points post-TBI

To further evaluate the gene expression over time in TBI-associated microglia, we investigated differentially expressed genes between microglia across time points (**Fig. 2**). We found 187 genes between days 7 and 30 that are positively differentiated and are likely involved in channel transport activity, as well as defense response. Several genes including genes involved in immune response and tissue repair such as *Tnfsf10*, *Bst2*, *Igf1*, and *Ccr1* were upregulated at the earlier time points. However, genes implicated in neurodegenerative diseases such as *Ptpn5*, *Shank3*, and *Sqstm1* were upregulated by 90 days compared to 7 days post TBI. This expression profile is indicative of the chronic inflammatory environment in the TBI brain following injury. To determine whether gene expression trends at 30 days were predictive of 90 days post-TBI, we compared the fold changes between 7 vs. 30 days post-TBI with those between 7 vs. 90 days post-TBI (**Fig. 3**). We find that these gene expression change were significantly correlated ($R=-0.537$, $p=2.2 \times 10^{-16}$). This indicates that genes which were upregulated at 90 days post-TBI had already started to increase as early as 30 days post-injury. Conversely, those genes that were downregulated at 30 days post-TBI remained downregulated or continued to fall over time.

Figure 2: Differentially expressed genes over the time course of injury.

Volcano plots of differentially expressed genes in microglia between 7D and 30D post-TBI

(A), 30D and 90D post-TBI (B), and 7D and 90D post-TBI (C). N=3 in each group. Genes with a p-value <0.05 are shown in red.

Figure 3. Scatter plot of fold change in CPM of microglia over time after TBI. Scatter plot to examine the relationship between the genes that are up-regulated or down-regulated in the microglia of mice at A) 7 vs 30 days post-TBI compared to 7 vs 90 days post-TBI and at B) 30 vs 90 days post-TBI compared to 7 vs 90 days. A log2 fold change of 1 is equal to a 2-fold change.

Trem2-APOE pathway is not upregulated over the course of traumatic brain injury.

Microglia are essential to brain homeostasis but lose this homeostatic function in a number of neurodegenerative disease processes. There has been considerable interest in the Trem2-APOE pathway in the generation of a neurodegenerative microglial phenotype in both Alzheimer's Disease (AD) and multiple sclerosis (MS). In fact, recent data has identified the Trem2-APOE pathway as a pivotal regulator of microglial

phenotype in both of these disease processes⁴⁵. Therefore, we aimed to determine if the Trem2-APOE pathway was a major regulator of microglial phenotype after TBI. However, unlike other neurodegenerative processes, our data demonstrates no significant change in TREM2 expression as well as a progressive decrease in APOE expression over the course of TBI (**Fig. 4**). These seemingly contradictory results emphasize the need for microglia-specific transcriptional studies in the setting TBI.

Figure 4. Microglial Trem2 and APOE expression over the course of TBI.

Trem2 did not significantly change over the course of brain injury. APOE expression progressively decreased over time following TBI. Ordinary one way ANOVA with Bonferroni's multiple comparison test.

Microglial STEP expression is progressively upregulated over the course of brain injury.

While our data failed to demonstrate a common pathway with Alzheimer's disease and multiple sclerosis through Trem2-APOE, a closer examination of Cluster 2 (**Fig. 1**) revealed a number of upregulated genes associated with long-term synaptic potentiation, including PTPN5 also known as STEP (STriatal-Enriched protein tyrosine Phosphatase). STEP is a brain-specific phosphatase and is highly expressed within the cortex, hippocampus, and amygdala^{46,47}. Our

data show that expression of STEP is progressively increased in microglia over time following TBI (Fig. 5). STEP is critical in the long-term depression, or weakening, of synaptic efficacy between neurons—a process fundamental to learning, memory, and cognition^{48,49}. When STEP activity is elevated, several substrates are inactivated resulting in the internalization of NMDA/AMPA glutamate receptors⁵⁰. This disrupts synaptic function and contributes to cognitive deficits^{49,51}. In fact, elevated STEP is associated with the pathophysiology of Alzheimer's disease, schizophrenia, and ischemic brain injury in both human cortex and mouse models⁵²⁻⁵⁷. These data suggest that STEP may be one of the common molecular pathways connecting TBI with other known neurodegenerative disorders.

Figure 5. Microglial STEP expression is progressively upregulated over the course of brain injury. STEP expression from FACSsorted microglia progressively increased from 7 days post-TBI to 90 days post-TBI; $p=0.01$, Ordinary one way ANOVA with Bonferroni's multiple comparison test.

Discussion

Microglia are the resident innate immune cells of the CNS. They are ontologically distinct from peripheral bone marrow-derived monocytes and macrophages, arising from the yolk sac as opposed to the developing liver in the embryo⁵⁸. In fact, microglia rely on a distinctive set of transcription factors during development resulting in a lineage of tissue macrophages (microglia) derived from the yolk sac that are genetically distinct from bone

marrow-derived macrophages^{37,59-62}. Additionally, microglia are self-renewing suggesting that monocyte-derived macrophages do not contribute to the maintenance of the mature microglia pool^{63,64}. Distinct developmental origin and renewal mechanisms may suggest that microglia possess discrete functions in pathological processes⁵⁸. Despite this, the cellular mechanisms by which microglia promote or attenuate the progression of injury are largely unknown⁶⁵. Our Data are the first we know of to use unbiased transcriptional profiling of isolated populations of microglia to define the genes/pathways/signatures involved in the generation of TBI-associated microglia over the course of injury.

To adequately capture the heterogeneity and complexity of microglia at different stages of injury, a comprehensive, genome-wide sampling of individual cell types is required^{37,66}. A cell-specific delineation of innate immune function based on transcriptional profiling in TBI has yet to be undertaken. Therefore, we combined our ability to discriminate and sort microglia from infiltrating monocytes and monocyte-derived macrophages with unbiased transcriptional profiling (RNA-seq) on FACSorted microglia. Our analysis identified 4 sequentially upregulated or downregulated gene clusters involved in processes such as the host defense response, synaptic potentiation, lipid remodeling, and membrane polarization each providing new insights into the biology of microglial activation over the course of TBI (**Fig. 1**). While there has recently been considerable recent interest in the Trem2-APOE pathway in the generation of a neurodegenerative microglial phenotype in Alzheimer's Disease and multiple sclerosis⁴⁵, our data demonstrates no significant change in Trem2 expression as well as a progressive decrease in APOE expression over the course of TBI (**Fig. 4**). However, an examination of cluster II revealed a number of

genes associated with long-term synaptic potentiation, including PTPN5 also known as STEP (STriatal-Enriched protein tyrosine Phosphatase). STEP is a brain-specific phosphatase that is highly expressed within the striatum, cortex, hippocampus, and amygdala^{46,47}. STEP is critical in the long-term depression, or weakening, of synaptic efficacy between neurons—a process fundamental to learning, memory, and cognition^{48,49}. Elevated STEP is associated with the pathophysiology of Alzheimer’s disease (AD), schizophrenia, and ischemic brain injury in both human cortex and mouse models⁵²⁻⁵⁷. In fact, genetic knockout of STEP reverses many of the cognitive and behavioral deficits in AD models^{53,67}. To the best of our knowledge, STEP expression has never been studied within the context of TBI. Our RNA-seq analysis demonstrates a 13-fold increase in microglial expression of STEP over the course of injury (**Fig. 5**). Previous studies have shown that STEP affects neuronal communication by opposing synaptic strengthening. High levels of STEP are believed to disrupt synaptic function and to contribute to learning deficits in neurodegenerative disease^{52,68}. When STEP activity is elevated, several substrates are inactivated resulting in the internalization of NMDA/AMPA glutamate receptors⁵⁰. This disrupts synaptic function and contributes to cognitive deficits^{49,51}. In other words, STEP activation modulates learning and memory by removing glutamate receptors from synaptic membranes. This important discovery suggests that TBI may share a common molecular pathway with several other cognitive disorders previously regarded as distinct. These data are remarkable in demonstrating the power of longitudinal transcriptional profiling, which provides important biologic insights into the state of microglial processes even in the complex and dynamic model of traumatic brain

injury. Furthermore, these data strongly implicate longitudinal changes in microglial gene expression in the development of long-term neurocognitive changes.

In conclusion, our data demonstrate that TBI-associated microglia adopt longitudinal transcriptional changes consistent with long-term depression of synaptic strength. The contribution of altered microglial gene expression to the pathogenesis of TBI has not been previously investigated. Our data suggest that TBI-associated microglia may play a previously unknown role in the weakening of synaptic efficacy between neurons after brain injury. As a result, learning, memory, and cognitive performance may all be affected leading to the resultant long-term neurocognitive impairments seen after TBI. Moving forward it will be important to study larger cohorts of brain-injured mice during both the acute and chronic phase of TBI. Furthermore, it has been shown that microglia display different transcriptional identities depending on the brain region in which they reside as well as their age⁶⁹. This will necessitate side-by-side comparison with age-matched naïve control mice to account for transcriptional changes associated with aging. Additionally, a single-cell RNA-seq approach may be required to account for inherent microglial heterogeneity at the site of injury. This could allow for the identification of novel microglial subpopulations within and surrounding the site of injury. Regardless of the techniques used, once the molecular mechanisms underlying the transcriptional changes in microglia after injury are further delineated, targeting the microglial response after TBI may soon represent a target for future therapeutic intervention.

Methods

Mice

All procedures were approved by the Northwestern University Institutional Animal Care and Use committee and all experiments were carried out in accordance with the ARRIVE guidelines on the reporting of in vivo experiments. Two mouse strains were used, they are C57BL/6 and B6.SJL-*Ptprca*^a *Pepcb*^b/BoyJ (CD45.1). All mice were purchased from the Jackson Laboratory and housed at a barrier facility at the Center for Comparative Medicine at Northwestern University (Chicago, IL, USA). Sixteen-week-old mice were used for all experiments.

Shielded Bone Marrow Chimeras

Bone marrow was aseptically harvested from tibias and femurs, from 8-week-old B6.CD45.1 donor mice, erythrocytes were lysed and the cells were counted using a Countess automated cell counter. 8 weeks old B6.CD45.2 mice received a single 1000-cGy γ -irradiation dose using a Cs-137-based Gammacell 40 irradiator. The mice heads were shielded with a lead bar so as to deliver the irradiation to the body only. 6 hours after shielded irradiation, busulfan (30 mg/kg) was administered to completely ablate the bone marrow of the recipient mice. Donor bone marrow (CD45.1) was transplanted 12 hours after busulfan ablation. Shielded bone marrow chimeras were maintained on antibiotics trimethoprim/sulfamethoxazole (40 mg/5 mg, respectively). Eight weeks after irradiation, 95% of the circulating monocytes were of donor origin (**Fig. 6**)³⁸.

Figure 6. Microglia from head-shielded bone marrow chimeric mice are host origin.

Brains isolated from chimeric mice post TBI were analyzed by flow cytometry. CD45.1^{neg}

'B, T, NK cells, Eosinophils' acted as the gating control for CD45.2^{hi}, showing that resident

microglia (CD45^{lo})

can be

unambiguously

differentiated from

infiltrating monocyte-

derived macrophages

(CD45.1⁺). Arrows

indicated the

directionality of gating.

Controlled cortical impact

Controlled cortical impact was induced as previously described by our laboratory³⁸. In

brief, mice were anesthetized with 100 mg/kg Ketamine and 10 mg/kg Xylazine via

intraperitoneal injection. A 1cm scalp incision was performed and a 5mm craniectomy

was performed 2 mm left of the sagittal suture and 2 mm rostral to the coronal suture.

The dura was left intact. Mice were then placed in a stereotaxic operating frame and the

impactor (Leica Biosystems Inc., Buffalo Grove, IL) was maneuvered into position. A

controlled cortical impact was then applied with a 3mm impacting tip at a velocity of 2.5m/s

and an impacting depth of 2mm with the dwell time set at 0.1s. Immediately following

injury all animals had their scalps sealed with VetBond (3M). All animals received post

injury analgesia with Buprenorphine SR via subcutaneous injection and were allowed to recover in separate cages over a warming pad. Mice were euthanized at 7, 30, and 90 days post TBI and brains were harvested.

Tissue preparation and fluorescence activated cell sorting

Immediately following euthanasia, mice were transcardially perfused first with ice cold PBS. The brains were then excised and place in ice cold HBSS until time to process. The brains were weighed, cut into pieces and placed into C-tubes containing digestion buffer (2.5 mg/mL Liberase TL (Roche, Basel, Switzerland), and 1 mg/mL of DNase I in HBSS). The C-tubes were placed on a MACS dissociator (Miltenyi Biotec) and run on the M_Brain_3 protocol, after which they were placed in an incubator for 30 minutes at 37°C with shaking at 200 rpm. After incubation, the C-tubes were placed back on the MACS dissociator and run on the same protocol as before. The cells released were then passed through a 40 µm nylon mesh and washed with 100ml of wash buffer (1% BSA in HBSS) per brain sample. Microglia and infiltrating cells were isolated using a 30/70 percoll gradient (Percoll Plus, GE Healthcare). The cells collected from the interphase of the gradient were washed with HBSS and counted using Countess automated cell counter (Invitrogen); dead cells were discriminated using trypan blue. Cells were stained with live/dead Aqua (Invitrogen) viability dye, incubated with Fc-Block (BD Bioscience) and stained with fluorochrome-conjugated antibodies (**Table 1**). Data were acquired on a BD FACS Aria cell sorter (BD Biosciences, San Jose, CA), and microglia were sorted for further analyses. “Fluorescence minus one” controls were used when necessary to set up gates. Pelleted sorted cells were immediately lysed in extraction buffer from a PicoPure RNA isolation kit (Arcturus Bioscience), and

lysates were stored at -80°C until RNA was extracted. Analysis of the flow cytometric data was performed using Flowjo software (TreeStar, Ashland, OR).

Antibody	Fluorochrome	Company and Clone
CD45.1	FITC	A-20 / BD Bioscience
CD45.2	BV421	104 / Biolegend
CD64	APC	X54-5/7.1/ BD Bioscience
CD11b	APC- Cy7	M1/70 / BD Biosciences
CD11c	PE-Cy7	HL3 / BD Bioscience
Ly6G	Alexa Flour 700	1A8 / BD Bioscience
MHC II	Percp- Cy 5.5	M5/114.15.2 / Biolegend
Siglec H	PE	551 / Biolegend
B220	PECF594	RA3-6B2 / BD Bioscience
Siglec F	PECF594	E50-2440 / BD Bioscience
CD4	PECF594	RM4-5/ BD Bioscience
CD8	PECF594	53-6.7 / BD Bioscience
NK1.1	PECF594	PK136/ BD Bioscience
Viability	e-bioscience Fixable Viability Dye eFluor 506	Invitrogen

Table 1. List of antibody conjugated fluorochromes used to differentiate microglia from infiltrating leukocytes.

RNA sequencing

RNA from the FACSorted microglia of brain-injured mice were extracted using a PicoPure RNA isolation kit according to manufacturer's instructions. Sample quality control, processing, and library preparation were performed by the Northwestern University Next Generation Sequencing Core (NUSeq). RNA quality and quantity were measured using Agilent High Sensitivity RNA ScreenTape System (Agilent Technologies). RNA sequencing (RNA-seq) libraries were prepared from 3ng of total RNA using the QuantSeq 3' biased mRNA-seq Library Prep Kit for Illumina (Lexogen). DNA libraries were

sequenced on an Illumina NextSeq 500 instrument with a target read depth of ~20 million reads per sample.

RNA-seq analysis

Raw sequencing files were first de-multiplexed using bcl2fastq. The resulting fastq files were trimmed of low-quality reads and bases, polyA tails, and adaptors using bbdut (<http://jgi.doe.gov/data-and-tools/bb-tools/>). The trimmed fastq files were aligned to the mouse reference genome (mm10, Genome Reference Consortium GRCm38) using the STAR (Spliced Transcripts Alignment to a Reference) algorithm⁷⁰. HTSeq was run on the resulting BAM files to provide raw gene counts. Raw gene counts for each sample were merged into a single gene expression table and normalized for read depth using counts per million (CPM). The three highest quality samples, based on RNA quality and library quality from each experimental group were included for subsequent analyses. For the RNA-seq analysis, we focused on the expressed genes which were defined as average log CPM ($\log_2(\text{CPM}+1)$) expression > 4 in each experimental group. For visualization, GENE-E (<https://software.broadinstitute.org/GENE-E/>) was used to perform K-means clustering (K=4) on differentially expressed genes across all time points as defined by ANOVA test ($p < 0.05$) across any two groups shown in the heatmap. Gene Ontology associations and the related p-values were determined by GO analysis (by GOrilla— [Gene Ontology enrichment *anaLysis* and visualiZation tool]).

⁷¹. Pairwise differential genes between time points were determined using DEseq2. Volcano plots were generated using the log2 fold change of normalized gene counts between microglia at different time points on the x-axis and corresponding p-values (-

log10) from DEseq2 on the y-axis. Plots were generated using the ggplot2 package from R Studio software.

Data Availability

The data that support the findings of this study are available from the corresponding author on request. RNA sequencing data is available through the NCBI Sequence Read Archive (SRA accession number: SRP160379).

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594 **Author Contributions**

595 S.S and H.M wrote the main manuscript text and prepared all figures. H.M, T.J
596 and S.S carried out the experiments. H.M and D.W performed the computational analysis
597 of the sequencing data.

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