TRAUMATIC BRAIN INJURY-ASSOCIATED MICRGOGLIA ADOPT LONGITUDINAL

TRANSCRIPTIONAL CHANGES CONSISTENT WITH LONG-TERM DEPRESSION OF

SYNAPTIC STRENGTH

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1   Traumatic brain injury (TBI) is an under-recognized public health threat. Even mild brain

2   injury, or concussions, may lead to long-term neurologic impairment. Microglia play a

3   fundamental role in the development and progression of this subsequent neurologic

4   impairment. Despite this, a microglia-specific injury signature has yet to be identified. In

5   the current study we hypothesized that TBI-associated microglia would adopt longitudinal

6   changes in their transcriptional profile associated with pathways linked to the

7   development of motor, cognitive, and behavioral disorders. C57BL/6 mice underwent TBI

8   via a controlled cortical impact and were followed longitudinally. FACSorted microglia

9   from TBI mice were subjected to RNA-sequencing at 7, 30, and 90 days post-injury. We

10   identified 4 major patterns of gene expression corresponding to the host defense

11   response, synaptic potentiation, lipid remodeling, and membrane polarization.   In

12   particular, significant upregulation of genes involved in long-term synaptic potentiation

13   including Ptpn5, Shank3, and Sqstm1 were observed offering new insight into a

14   previously unknown role of microglia in the weakening of synaptic efficacy between

15   neurons after brain injury.

16

Introduction

18   Traumatic brain injury is a growing and under recognized public health threat. The CDC

19   estimates nearly 2 million people sustain a traumatic brain injury (TBI) each year in the

20   United States, contributing to over 30% of all injury related deaths [1,2]. In fact, TBI related

21   healthcare expenditures near 80 billion dollars annually with an average cost of 4 million

22   dollars per person surviving a severe TBI [3-5]. The impact of TBI is highlighted not only by

23   its high mortality rate but also by the significant long-term complications suffered by its

24   survivors with the progressive development of motor, cognitive, and behavioral disorders

25   [6-10]. Even subconcussive events, those resulting in subclinical brain dysfunction without

26   the typical symptoms of concussion, may lead to long-term neurologic impairment [11,12].

27   The immune response to TBI plays a fundamental role the development and progression

28   of subsequent neurodegenerative disease and represents a complex interplay between

29   the injured brain and the resident immune cells of the brain — the microglia [13]. The current

30   manuscript is focused on developing a cell-type-specific understanding of the microglial

31   response to injury over time. Here we highlight the major trends in gene expression in

32   response to TBI.

33

34   TBI triggers a robust pro-inflammatory response within the injured brain. The degree of

35   this initial pro-inflammatory response has significant value in predicting more long-term

36   outcomes after TBI [14-16]. Even after the acute inflammatory response has resolved,

37   several studies demonstrated residual long-lasting inflammation within the brain in both

38   animal models as well as in patients [17,18]. One of the main drivers of this continued

39   inflammation is the persistence of activated microglia—characterized by thickening and

40   retraction of their ramified processes, increased IL-1 and IL-6 with concomitant decreases

41   in IL-4 and IL-10, and increased expression of pro-oxidant genes with a reduction of

42   growth and antioxidant genes. A recent study showed an increased inflammatory profile

43   of microglia that persisted for up to 12 months after injury in a murine model of TBI [19].

44   Furthermore, this continued inflammation is associated with lesion volume expansion and

45   loss of neurons in the hippocampus [18-22]. Even once the acute inflammatory process has

46   resolved and infiltrated monocyte-derived macrophages are no longer present within the

47   injured brain, microglia have the potential to remain activated for years after the initial

48   insult [19,23]. A functional consequence of this constitutive activation after brain trauma is

49   an exaggerated neuroinflammatory response to otherwise benign secondary stimuli such

50   a subsequent subclinical head injury [10,24-27]. This may be the mechanism by which

51   patients who have sustained a concussion are more susceptible to subsequent

52   concussions [28-30]. Nonetheless, the molecular mechanisms resulting in the constitutive

53   activation of microglia remain elusive [21]. Therefore, in the current study, we aimed to

54   study the transcriptional dynamics of constitutively activated microglia over the course of

55   brain injury.

56

57   The first step towards any cell-specific transcriptional analysis relies on obtaining

58   sufficient cells of interest with the highest purity. The historical standard for distinguishing

59   between microglia and infiltrating macrophages is immunohistochemistry. Although

60   immunohistochemistry is useful for assessing morphology, proliferation, and sites of

61   activation it has a number of drawbacks limiting its use [31]. Several investigative groups

62   have focused on this problem including column free magnetic separation and CD11b

63   immunomagnetic enrichment combined with the differential expression of CD45 with flow

64   cytometry [31-33]. However, CD45 expression has been reported to vary depending on the

65   pathologic condition; thus, reliable separation of microglia from peripheral myeloid cells

66   is impaired [34,35]. To overcome this issue, fluorescently marked myeloid cells, such as

67   CX3CR1[+/GFP]/CCR2[+/RFP], have been used. However, these mice are on mixed

68   backgrounds, which could greatly affect the immune response. Furthermore, the

69   presence of contaminated nonclassical monocytes could not be excluded [36,37]. Therefore,

70   we use head-shielded bone marrow chimeric mice with CD45.1 cells in the circulation

71   and CD45.2 microglia in the brain allowing definitive and unambiguous differentiation

72   between microglia and infiltrating bone-marrow derived myeloid cells as previously

73   described by our laboratory [38]. To the best of our knowledge, no cell-type-specific study

74   has been conducted to specifically identify transcriptional changes in isolated populations

75   of microglia over the course of TBI. In the current study we demonstrate that TBI-

76   associated microglia adopt longitudinal changes in their transcriptional profile associated

77   with pathways linked to the development of motor, cognitive, and behavioral disorders.

78

Results

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

82   The neuroinflammatory response to TBI is central to both neuroprotection and

83   neurotoxicity after injury, but attempts to broadly target immune activation have been

84   unsuccessful in improving outcomes in TBI patients [39-42]. Because of this, there has been

85   a growing interest in investigating the microglial transcriptome after brain injury. Attempts

86   thus far have been plagued by the use of whole brain homogenates rather than individual

87   cell types as well as the use of microarray analyses restricted to a limited number of genes

88   in the inflammatory response [43,44]. Therefore, we combined our ability to discriminate and

89   sort microglia from infiltrating monocytes and macrophages with unbiased transcriptional

90   profiling (RNA-seq) on FACSorted microglia from 7, 30, and 90 days after TBI. We

91   defined 396 genes that change expression across the time course (see methods). We

92   clustered these differentially expressed genes into 4 main patterns of expression (**Fig. 1**).

93   Clusters 2 represents genes involved in synaptic plasticity and is progressively

94   upregulated over the course of injury. Cluster 4 represents genes involved in the

95   regulation of membrane polarization with gene expression that is upregulated at 30 days

96   post-injury and then downregulated by 90 days post injury. Clusters 1 and 3 represent

97   genes involved in the host response to injury and lipid remodeling and both are

98   progressively downregulated over the course of injury. These data provide new insights

99   into the biology of microglial activation over the course of TBI.

100

101   **Figure 1. Microglia from TBI mice show distinct time-dependent transcriptional**

102   **profiles.** K-means clustering: Four clusters representing 392 genes are shown with

103   distinct time specific expression patterns. Averaged $\log_2$(CPM+1) ≥3 of genes in each

104   group that had a log fold

105   change of at least 2 was

106   used to generate the heat

107   map. GO processes

108   associated with the genes

109   in each cluster was

110   determined using Gorilla

111   database.
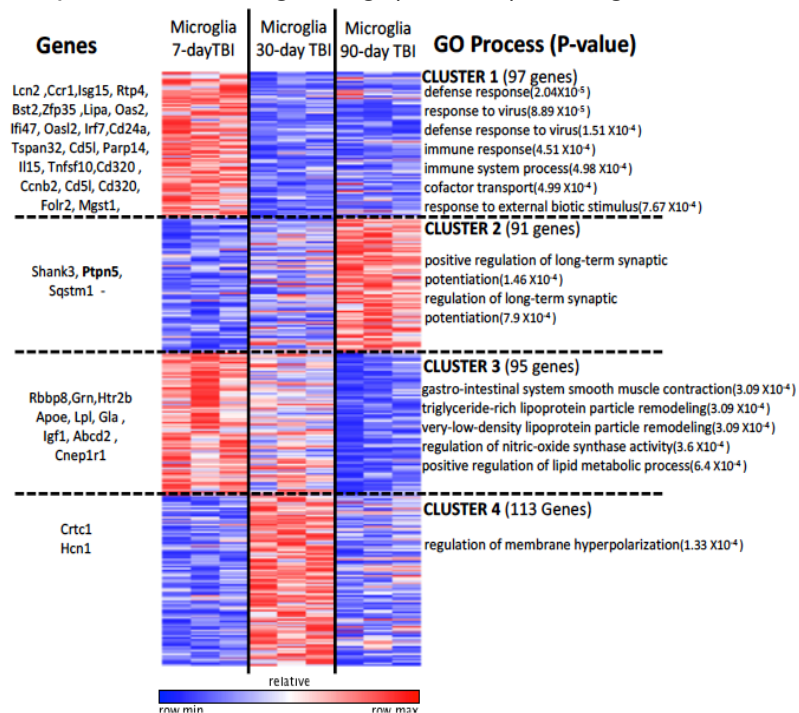
112

113

114   **Pairwise comparison of microglia gene expression between time**

115   **points post-TBI**

116   To further evaluate the gene expression over time in TBI-associated microglia, we

117   investigated differentially expressed genes between microglia across time points (**Fig. 2**).

118   We found 187 genes between days 7 and 30 that are positively differentiated and are

119   likely involved in channel transport activity, as well as defense response. Several genes

120   including genes involved in immune response and tissue repair such as Tnfsf10, Bst2,

121   Igf1, and Ccr1 were upregulated at the earlier time points. However, genes implicated in

122   neurodegenerative diseases such as Ptpn5, Shank3, and Sqstm1 were upregulated by

123   90 days compared to 7 days post TBI. This expression profile is indicative of the chronic

124   inflammatory environment in the TBI brain following injury. To determine whether gene

125   expression trends at 30 days were predictive of 90 days post-TBI, we compared the fold

126   changes between 7 vs. 30 days post-TBI with those between 7 vs. 90 days post-TBI (**Fig.**

127   **3**). We find that these gene expression change were significantly correlated (R=-0.537,

128   p=2.2 X 10^{-16}). This indicates that genes which were upregulated at 90 days post-TBI had

129   already started to increase as early as 30 days post-injury. Conversely, those genes that

130   were downregulated at 30 days post-TBI remained downregulated or continued to fall

131   over time.

132

133   **Figure 2: Differentially**

134   **expressed genes over the**

135   **time course of injury.**

136   Volcano plots of differentially

137   expressed genes in microglia

138   between 7D and 30D post-TBI
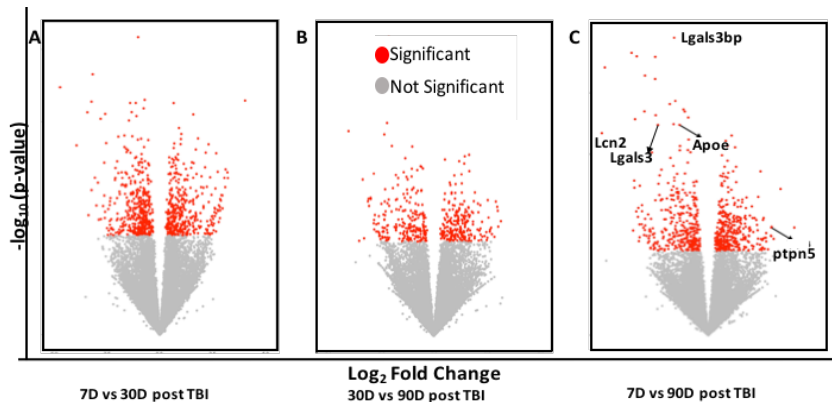
139   (A), 30D and 90D post-TBI (B), and 7D and 90D post-TBI (C). N=3 in each group. Genes

140   with a p- value <0.05 are shown in red.

141

142   **Figure 3. Scatter plot of fold change in CPM of microglia over time after TBI.** Scatter

143   plot to examine the relationship between the genes that are up-regulated or down-

144   regulated in the microglia of mice at A) 7 vs 30 days post-TBI compared to 7 vs 90 days

145   post-TBI and at B) 30 vs 90 days post-TBI compared to 7 vs 90 days. A log2 fold change

146   of 1 is equal to a 2-fold change.
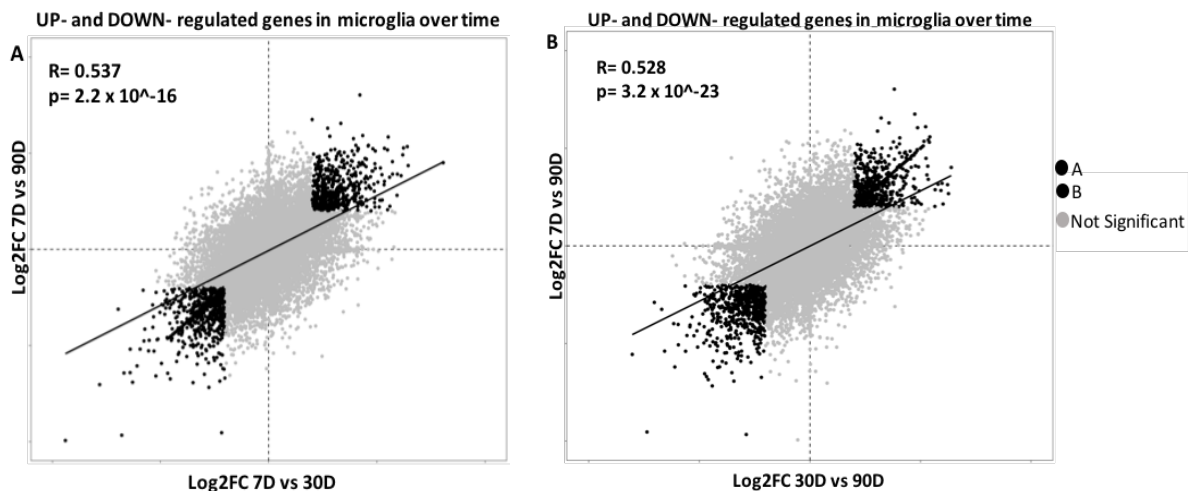
147

148   **Trem2-APOE pathway is not upregulated over the course of traumatic**

149   **brain injury.**

150   Microglia are essential to brain homeostasis but lose this homeostatic function in a

151   number of neurodegenerative disease processes. There has been considerable interest

152   in the Trem2-APOE pathway in the generation of a neurodegenerative microglial

153   phenotype in both Alzheimer's Disease (AD) and multiple sclerosis (MS). In fact, recent

154   data has identified the Trem2-APOE pathway as a pivotal regulator of microglial

155   phenotype in both of these disease processes [45]. Therefore, we aimed to determine if the

156   Trem2-APOE pathway was a major regulator of microglial phenotype after TBI. However,

157   unlike other neurodegenerative processes, our data demonstrates no significant change

158   in TREM2 expression as well as a progressive decrease in APOE expression over the

159   course of TBI (**Fig. 4**). These seemingly contradictory results emphasize the need for

160   microglia-specific transcriptional studies in the setting TBI.

161

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

163   Trem2 did not significantly change

164   over the course of brain injury.

165   APOE   expression   progressively

166   decreased over time following TBI.

167   Ordinary   one   way   ANOVA   with

168   Bonferroni's   multiple   comparison

169   test.

170

171   **Microglial STEP expression is progressively upregulated over the**

172   **course of brain injury.**

173   While our data failed to demonstrate a common pathway with Alzheimer's disease and

174   multiple sclerosis through Trem2-APOE, a closer examination of Cluster 2 (**Fig. 1**) revealed

175   a number of upregulated genes associated with long-term synaptic potentiation, including PTPN5

176   also known as STEP (STriatial-Enriched protein tyrosine Phosphatase). STEP is a brain-specific

177   phosphatase and is highly expressed within the cortex, hippocampus, and amygdala [46,47]. Our
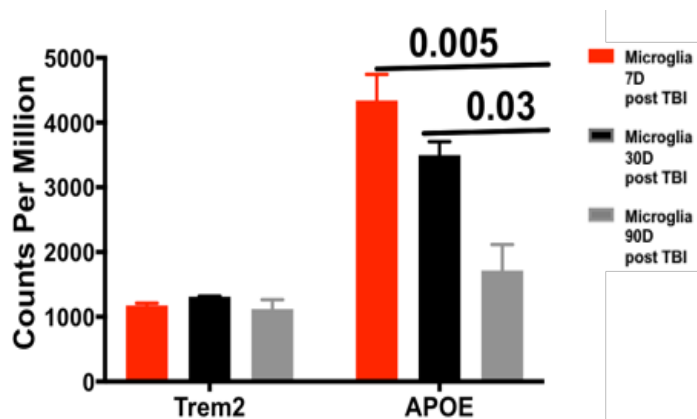
178   data show that expression of STEP is progressively increased in microglia over time following TBI

179   (**Fig. 5**). STEP is critical in the long-term depression, or weakening, of synaptic efficacy between

180   neurons—a process fundamental to learning, memory, and cognition [48,49]. When STEP activity is

181   elevated, several substrates are inactivated resulting in the internalization of NMDA/AMPA

182   glutamate receptors [50]. This disrupts synaptic function and contributes to cognitive deficits [49,51].

183   In fact, elevated STEP is associated with the pathophysiology of Alzheimer's disease,

184   schizophrenia, and ischemic brain injury in both human cortex and mouse models [52-57]. These

185   data suggest that STEP may be one of the common molecular pathways connecting TBI with

186   other known neurodegenerative disorders.

187

188   **Figure 5.   Microglial STEP expression is**

189   **progressively upregulated over the course**

190   **of   brain   injury.** STEP   expression   from

191   FACSorted microglia progressively increased

192   from 7 days post-TBI to 90 days post-TBI;

193   *p*=0.01,   Ordinary   one   way   ANOVA   with

194   Bonferroni's multiple comparison test.

195
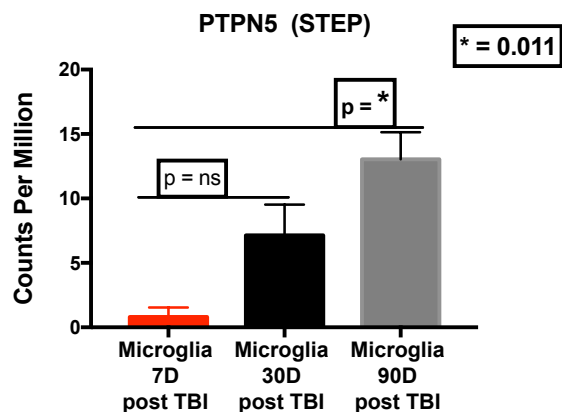
Discussion

197   Microglia are the resident innate immune cells of the CNS. They are ontologically distinct

198   from peripheral bone marrow-derived monocytes and macrophages, arising from the yolk

199   sac as opposed to the developing liver in the embryo [58]. In fact, microglia rely on a

200   distinctive set of transcription factors during development resulting in a lineage of tissue

201   macrophages (microglia) derived from the yolk sac that are genetically distinct from bone

202   marrow-derived macrophages [37,59-62]. Additionally, microglia are self-renewing suggesting

203   that monocyte-derived macrophages do not contribute to the maintenance of the mature

204   microglia pool [63,64]. Distinct developmental origin and renewal mechanisms may suggest

205   that microglia possess discrete functions in pathological processes [58]. Despite this, the

206   cellular mechanisms by which microglia promote or attenuate the progression of injury

207   are largely unknown [65]. Our Data are the first we know of to use unbiased transcriptional

208   profiling of isolated populations of microglia to define the genes/pathways/signatures

209   involved in the generation of TBI-associated microglia over the course of injury.

210

211   To adequately capture the heterogeneity and complexity of microglia at different stages

212   of injury, a comprehensive, genome-wide sampling of individual cell types is required [37,66].

213   A cell-specific delineation of innate immune function based on transcriptional profiling in

214   TBI has yet to be undertaken. Therefore, we combined our ability to discriminate and sort

215   microglia from infiltrating monocytes and monocyte-derived macrophages with unbiased

216   transcriptional profiling (RNA-seq) on FACSorted microglia. Our analysis identified 4

217   sequentially upregulated or downregulated gene clusters involved in processes such as

218   the host defense response, synaptic potentiation, lipid remodeling, and membrane

219   polarization each providing new insights into the biology of microglial activation over the

220   course of TBI (**Fig. 1**). While there has recently been considerable recent interest in the

221   Trem2-APOE pathway in the generation of a neurodegenerative microglial phenotype in

222   Alzheimer's Disease and multiple sclerosis [45], our data demonstrates no significant

223   change in Trem2 expression as well as a progressive decrease in APOE expression over

224   the course of TBI (**Fig. 4**). However, an examination of cluster II revealed a number of

225   genes associated with long-term synaptic potentiation, including PTPN5 also known as

226   STEP (STriatial-Enriched protein tyrosine Phosphatase).   STEP is a brain-specific

227   phosphatase that is highly expressed within the striatum, cortex, hippocampus, and

228   amygdala [46,47]. STEP is critical in the long-term depression, or weakening, of synaptic

229   efficacy between neurons—a process fundamental to learning, memory, and cognition

230   [48,49]. Elevated STEP is associated with the pathophysiology of Alzheimer's disease (AD),

231   schizophrenia, and ischemic brain injury in both human cortex and mouse models [52-57].

232   In fact, genetic knockout of STEP reverses many of the cognitive and behavioral deficits

233   in AD models [53,67]. To the best of our knowledge, STEP expression has never been

234   studied within the context of TBI. Our RNA-seq analysis demonstrates a 13-fold increase

235   in microglial expression of STEP over the course of injury (**Fig. 5**). Previous studies have

236   shown that STEP affects neuronal communication by opposing synaptic strengthening.

237   High levels of STEP are believed to disrupt synaptic function and to contribute to learning

238   deficits in neurodegenerative disease [52,68]. When STEP activity is elevated, several

239   substrates are inactivated resulting in the internalization of NMDA/AMPA glutamate

240   receptors [50]. This disrupts synaptic function and contributes to cognitive deficits [49,51]. In

241   other words, STEP activation modulates learning and memory by removing glutamate

242   receptors from synaptic membranes. This important discovery suggests that TBI may

243   share a common molecular pathway with several other cognitive disorders previously

244   regarded as distinct. These data are remarkable in demonstrating the power of

245   longitudinal transcriptional profiling, which provides important biologic insights into the

246   state of microglial processes even in the complex and dynamic model of traumatic brain

247   injury. Furthermore, these data strongly implicate longitudinal changes in microglial gene

248   expression in the development of long-term neurocognitive changes.

249

250   In conclusion, our data demonstrate that TBI-associated microglia adopt longitudinal

251   transcriptional changes consistent with long-term depression of synaptic strength. The

252   contribution of altered microglial gene expression to the pathogenesis of TBI has not been

253   previously investigated. Our data suggest that TBI-associated microglia may play a

254   previously unknown role in the weakening of synaptic efficacy between neurons after

255   brain injury. As a result, learning, memory, and cognitive performance may all be affected

256   leading to the resultant long-term neurocognitive impairments seen after TBI. Moving

257   forward it will be important to study larger cohorts of brain-injured mice during both the

258   acute and chronic phase of TBI. Furthermore, it has been shown that microglia display

259   different transcriptional identities depending on the brain region in which they reside as

260   well as their age [69]. This will necessitate side-by-side comparison with age-matched naïve

261   control mice to account for transcriptional changes associated with aging. Additionally, a

262   single-cell RNA-seq approach may be required to account for inherent microglial

263   heterogeneity at the site of injury. This could allow for the identification of novel microglial

264   subpopulations within and surrounding the site of injury. Regardless of the techniques

265   used, once the molecular mechanisms underlying the transcriptional changes in microglia

266   after injury are further delineated, targeting the microglial response after TBI may soon

267   represent a target for future therapeutic intervention.

268

269   **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-*Ptprc^a Pepc^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].

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

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

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

293 microglia (CD45^{lo})

294 can be

295 unambiguously

296 differentiated from

297 infiltrating monocyte-

298 derived macrophages

299 (CD45.1^{+}). Arrows

300 indicated the

301 directionality of gating.

302

303 ## Controlled cortical impact

304 Controlled cortical impact was induced as previously described by our laboratory [38]. In

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

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

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

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

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

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

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

312 injury all animals had their scalps sealed with VetBond (3M). All animals received post
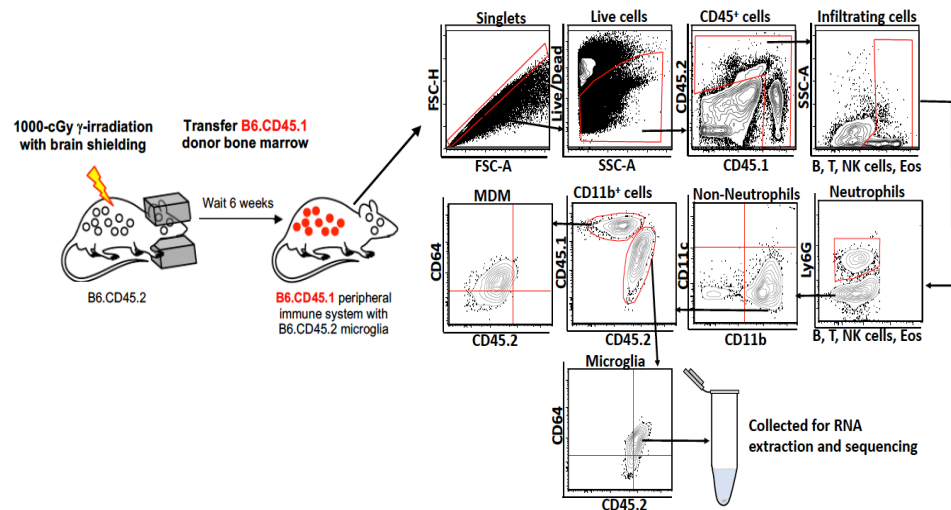
313   injury analgesia with Buprenorphine SR via subcutaneous injection and were allowed to

314   recover in separate cages over a warming pad. Mice were euthanized at 7, 30, and 90

315   days post TBI and brains were harvested.

316

317   **Tissue preparation and fluorescence activated cell sorting**

318   Immediately following euthanasia, mice were transcardially perfused first with ice cold PBS.

319   The brains were then excised and place in ice cold HBSS until time to process. The brains

320   were weighed, cut into pieces and placed into C-tubes containing digestion buffer (2.5

321   mg/mL Liberase TL (Roche, Basel, Switzerland), and 1 mg/mL of DNase I in HBSS). The

322   C-tubes were placed on a MACS dissociator (Miltenyi Biotec) and run on the M_Brain_3

323   protocol, after which they were placed in an incubator for 30 minutes at 37°C with shaking

324   at 200 rpm. After incubation, the C-tubes were placed back on the MACS dissociator and

325   run on the same protocol as before. The cells released were then passed through a 40 µm

326   nylon mesh and washed with 100ml of wash buffer (1% BSA in HBSS) per brain sample.

327   Microglia and infiltrating cells were isolated using a 30/70 percoll gradient (Percoll Plus, GE

328   Healthcare). The cells collected from the interphase of the gradient were washed with

329   HBSS and counted using Countess automated cell counter (Invitrogen); dead cells were

330   discriminated using trypan blue. Cells were stained with live/dead Aqua (Invitrogen) viability

331   dye, incubated with Fc-Block (BD Bioscience) and stained with fluorochrome-conjugated

332   antibodies (**Table 1**). Data were acquired on a BD FACSAria cell sorter (BD Biosciences,

333   San Jose, CA), and microglia were sorted for further analyses. "Fluorescence minus one"

334   controls were used when necessary to set up gates. Pelleted sorted cells were immediately

335   lysed in extraction buffer from a PicoPure RNA isolation kit (Arcturus Bioscience), and

336 lysates were stored at -80°C until RNA was extracted. Analysis of the flow cytometric data

337 was performed using Flowjo software (TreeStar, Ashland, OR).

338

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

339 **Table 1.** List of antibody conjugated fluorochromes used to differentiate microglia from

340 infiltrating leukocytes.

341

342 **RNA sequencing**

343 RNA from the FACSorted microglia of brain-injured mice were extracted using a PicoPure

344 RNA isolation kit according to manufacturer's instructions. Sample quality control,

345 processing, and library preparation were performed by the Northwestern University Next

346 Generation Sequencing Core (NUSeq). RNA quality and quantity were measured using

347 Agilent High Sensitivity RNA ScreenTape System (Agilent Technologies). RNA

348 sequencing (RNA-seq) libraries were prepared from 3ng of total RNA using the QuantSeq

349 3' biased mRNA-seq Library Prep Kit for Illumina (Lexogen). DNA libraries were

350   sequenced on an Illumina NextSeq 500 instrument with a target read depth of ~20 million

351   reads per sample.

352

353   **RNA-seq analysis**

354   Raw sequencing files were first de-multiplexed using bcl2fastq. The resulting fastq files

355   were trimmed of low-quality reads and bases, polyA tails, and adaptors using bbduk

356   (http://jgi.doe.gov/data-and-tools/bb-tools/). The trimmed fastq files were aligned to the

357   mouse reference genome (mm10, Genome Reference Consortium GRCm38) using the

358   STAR (Spliced Transcripts Alignment to a Reference) algorithm [70]. HTSeq was run on

359   the resulting BAM files to provide raw gene counts. Raw gene counts for each sample

360   were merged into a single gene expression table and normalized for read depth using

361   counts per million (CPM). The three highest quality samples, based on RNA quality and

362   library quality from each experimental group were included for subsequent analyses. For

363   the RNA-seq analysis, we focused on the expressed genes which were defined as average

364   log CPM ($\log_2$(CPM+1)) expression > 4 in each experimental group. For visualization, GENE-E

365   (https://software.broadinstitute.org/GENE-E/) was used to perform K-means clustering

366   (K=4) on differentially expressed genes across all time points as defined by ANOVA test

367   (p<0.05) across any two groups shown in the heatmap. Gene Ontology associations and

368   the related p-values were determined by GO analysis (by GOrilla— [Gene Ontology

369   enRIchment *anaLysis* and visuaLizAtion tool]).

370   [71]. Pairwise differential genes between time points were determined using DEseq2.

371   Volcano plots were generated using the log2 fold change of normalized gene counts

372   between microglia at different time points on the x-axis and corresponding p-values (-

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

374   R Studio software.

375

Data Availability

377   The data that support the findings of this study are available from the corresponding

378   author on request. RNA sequencing data is available through the NCBI Sequence

379   Read Archive (SRA accession number: SRP160379).

380

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Author Contributions

S.S and H.M wrote the main manuscript text and prepared all figures. H.M, T.J

and S.S carried out the experiments. H.M and D.W performed the computational analysis

of the sequencing data.