Running Title: TREM2 deficiency alters acute macrophage distribution and improves recovery after TBI

Authors:
Maha Saber[1,2], Olga Kokiko-Cochran[4], Shweta S. Puntambekar[4], Justin D. Lathia[3] , Bruce T. Lamb[1,4*]

Affiliations:

[1]Dept. of Neurosciences, Cleveland Clinic, Cleveland, OH, USA.

[2]Dept. of Molecular Medicine, Case Western Reserve University, Cleveland, OH, USA.

[3]Dept. of Cellular Molecular Medicine, Cleveland Clinic, Cleveland, OH, USA.

[4] Stark Neurosciences Research Institute, Indianapolis, IN, USA

*Stark Neurosciences Research Institute, Indiana University School of Medicine, Neuroscience Building Suite 414, 320 W. 15th Street, NB Building Indianapolis IN 46202-2266, Phone: 317-278-5811, Fax: 317-231-0203, btlamb@iu.edu

AUTHOR CONTACT INFORMATION

Maha Saber
Department of Neurosciences
Cleveland Clinic Lerner Research Institute
9500 Euclid Avenue
Cleveland, Ohio 44195
Phone:(216) 444-1142
Fax:(216) 444-7927
saberm@ccf.org

Olga Kokiko- Cochran, PhD
Stark Neurosciences Research Institute
Indiana University School of Medicine
Neuroscience Building, Suite 414
320 W. 15th Street, NB Building
Indianapolis, IN 46202-2266
Phone: 317-278-5811
Fax: 317-231-0203
olkokiko@iu.edu

Shweta Puntambekar, PhD
Stark Neurosciences Research Institute
Indiana University School of Medicine
Neuroscience Building, Suite 414
320 W. 15th Street, NB Building
Indianapolis, IN 46202-2266
Phone: 317-278-5811
Fax: 317-231-0203
sspuntam@iu.edu

Justin Lathia, PhD
Department of Cellular and Molecular Medicine (NC10)
Cleveland Clinic Lerner Research Institute
9500 Euclid Avenue
Cleveland, Ohio 44195
Phone: (216) 444-9340
Fax:(216) 444-9404
lathiaj@ccf.org

Bruce Lamb, PhD
Stark Neurosciences Research Institute
Indiana University School of Medicine
Neuroscience Building, Suite 414
320 W. 15th Street, NB Building
Indianapolis, IN 46202-2266
Phone: 317-278-5811
Fax: 317-231-0203
btlamb@iu.edu

ABSTRACT

Traumatic brain injury (TBI) affects 1.7 million people annually in the USA (Center of Disease Control). There is increasing evidence that individuals exposed to TBI have increased risk of developing multiple neurodegenerative conditions, including Alzheimer Disease (AD). TBI triggers a strong neuroinflammatory response characterized by astrogliosis, activation of microglia, and infiltration of peripheral monocytes. Recent evidence suggests that alterations in innate immunity promote neurodegeneration. This includes genetic studies demonstrating that mutations in *Triggering Receptor Expressed on Myeloid cells 2 (TREM2)* is associated with a higher risk for not only AD but multiple neurodegenerative diseases. To examine whether TREM2 deficiency affects pathological outcomes of TBI, *Trem2* knockout (*Trem2*$^{-/-}$) and C57BL/6J (B6) mice were given a lateral fluid percussion injury (FPI) and sacrificed at 3 and 120 Days Post Injury (DPI) to look at both acute and chronic consequences of TREM2 deficiency. Notably, at 3 DPI, B6 mice exposed to TBI exhibited increased expression of TREM2 in the brain. Furthermore, *Trem2*$^{-/-}$ mice exposed to TBI exhibited enhanced macrophage activation near the lesion, but significantly less macrophage activation away from the lesion when compared to B6 mice exposed to TBI. In addition, at 120 DPI, *Trem2*$^{-/-}$ mice exposed to TBI demonstrated reduced hippocampal atrophy, and rescue of TBI-induced behavioral changes when compared to B6 mice exposed to TBI. Taken together, this study suggests that TREM2 deficiency influences both acute and chronic responses to TBI, leading to an altered macrophage response at early time points, and improved pathological and functional outcomes at later time points.

Key Words: Inflammation, Neurodegenerative Disorders, Behavior, Traumatic Brain Injury, Immunohistochemistry

INTRODUCTION

Increasing evidence suggests that traumatic brain injury (TBI) represents a significant risk factor for multiple neurodegenerative diseases including Chronic Traumatic Encephalopathy (CTE) and Alzheimer's disease (AD) [1-6]. Several studies have documented an increase in both the development and onset of AD-related neuropathology following TBI, including the formation and accumulation of intracellular aggregates of the microtubule-associated protein tau and extracellular aggregates of the β-amyloid (Aβ) peptide[1, 5, 7-10]. Notably, one phenomenon observed in both TBI and neurodegenerative diseases includes the activation of the innate immune system within the brain[11-13]. This includes activation of resident innate immune cells (microglia), infiltration and activation of peripheral innate immune cells (monocytes) into the brain and the release of pro- inflammatory cytokines[12, 14-16]. While the chronic activation of the innate immune system within the brain is generally thought to contribute to neurodegeneration, the exact role of specific innate immune pathways has remained somewhat elusive.

TBI induces a vast array of pathological consequences including neuronal cell death, altered cytokine/chemokine production, tissue loss, oxidative stress, cognitive deficits, behavioral changes, and brain atrophy[1, 15, 17-20]. Initially, the mechanical stress from the injury induces primary damage including neuronal death, shearing of blood vessels, blood- brain barrier damage, etc[21-23]. However, following the initial injury, a wide variety of secondary injury cascades contribute to later pathological and functional impairments, including the leakage, recruitment and activation of monocytes, as well as activation of microglia[23, 24]. Considerable evidence suggests that macrophages (derived from both monocytes and microglia), may contribute to the phagocytosis of neuronal debris at early time points following injury[16, 25]. However, continued and chronic macrophage activation and release of accompanying

cytokines/chemokines is thought to contribute to pathological and functional outcomes at later time points[26]. While normally macrophage activation and secretion of cytokines/chemokines regress over time, the inflammatory cascade can become chronic in subsets of patients[7, 27, 28]. While chronic macrophage activation is observed both in TBI and numerous neurodegenerative diseases, the exact contribution of specific macrophage activation pathways in neurodegenerative disease pathologies and outcomes following TBI have remained elusive and proven difficult to discern from cause and effect.

Recent genetic studies have provided evidence for a direct link between immune pathways and neurodegeneration, including genome-wide association studies of AD, FTD, and other diseases that have revealed single nucleotide polymorphisms associated with increased risk for neurodegenerative diseases in a variety of genes associated with immunity, including CD33, progranulin, etc[29, 30]. Furthermore, recent compelling evidence linking innate immune pathways and neurodegeneration has been provided by studies of the *Triggering Receptor Expressed on Myeloid cells 2 (TREM2)* gene[31-34]. Notably, TREM2 is a receptor exclusively found on myeloid-derived immune cells, including dendritic cells, monocytes, and microglia[35, 36]. Homozygous, loss of function mutations in *TREM2* are associated with Nasu-Hakola disease, in which patients develop bone cysts and age-related dementia[34, 37]. Finally, in late 2012, rare heterozygous coding mutations in TREM2 were identified that are associated with substantially increased risk for late-onset AD[38-41]. Additional genetic studies have provided evidence that TREM2 variants are also associated with FTD, PD, and ALS[33, 42]. While these studies have provided compelling evidence linking innate immunity and TREM2, in particular, to neurodegeneration, the exact contribution of TREM2 in both TBI and neurodegeneration remains to be determined.

Several *in vitro* studies have provided evidence that suggests that TREM2 is anti-inflammatory and potentially linked to phagocytosis, including studies demonstrating that reduction of TREM2 within microglia leads to increased pro-inflammatory cytokine release while overexpression of TREM2 leads to decreased cytokine production[43, 44]. It has been suggested that TREM2 negatively regulates the toll-like receptor (TLR) pathway, a class of proteins that plays a key role in the innate immune system and inflammation[45-47]. In contrast to what have been observed *in vitro*, TREM2 appears to play divergent roles in various *in vivo* models of injury and damage both within the CNS and in the periphery. For example, in a model of stroke, *Trem2*$^{-/-}$ mice showed less inflammatory cytokines in the brain compared to wild-type mice[48]. However, many of these studies have provided evidence that TREM2 alters the macrophage response, either by blocking the appearance and proliferation or increasing the death of macrophages in both neurodegenerative and infectious models[49, 50]. Given the unique macrophage response following TBI and epidemiological studies linking TBI and neurodegenerative disease, the current studies sought to examine the acute and chronic roles of TREM2 in macrophage activation, neurodegeneration and behavior following experimental TBI.

MATERIALS AND METHODS

Mice

The *Trem2*$^{-/-}$ (Trem2$^{tm1(KOMP)Vlcg}$) mouse model used in this study contains a lacZ reporter cassette knocked into the endogenous *Trem2* locus replacing exons 2 and 3 and the majority of exon 4, making a nonfunctional protein and is maintained on the (C57BL/6J) B6 background[50]. This mouse was originally generated by the trans-NIH Knockout Project (KOMP). Wildtype B6 mice were used as controls and originally purchased from Jax laboratory and breed in house for experimentation. Animals were housed in the Cleveland

Clinic Biological Resources Unit (BRU) on a 14:10 hour light/dark cycle with free access to food and water. For all studies, age and sex matched animals were exposed to sham or fluid percussion injury (FPI) between 8 to 12 weeks of age at an average of 10 weeks. Approximately half female and half male cohorts were used for all experiments. All procedures were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic.

Surgical Preparation and Injury

Standard procedures were used to administer lateral fluid percussion or sham injury to mice[51, 52]. Briefly, 2-month-old mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), weighed, and attached to a stereotaxic frame. Bupivacaine (0.25%, 50µl) was administered subcutaneously for topical pain relief. Following a midline incision, a 3.0 mm craniotomy was performed midway between bregma and lambda using a trephine on the skull. A modified Leur-Loc needle hub (3.0 mm inside diameter) was placed over the exposed intact dura and surrounded by dental acrylic. Sterile PBS was added to the hub and then sealed with a modified syringe tip filled with cotton to limit fibrosis. Mice were then returned to their home cage to recover. Twenty-four hours later, animals in the injured groups were re-anesthetized, had their seals removed, and received a moderate (1.0 atm of pressure) level TBI using the Amscien instrument FPI device. Animals in the sham group were re-anesthetized and connected to the injury device; however, no fluid pulse was delivered. Following FPI or sham injury, the hub was removed. Mice were then sutured and returned to their home cage for observation and analysis as described.

Primary Antibodies

The following antibodies against inflammatory markers were utilized: sheep polyclonal anti-TREM2 (R&D Systems, catalog number AF1729); rat monoclonal anti-CD45 (Serotec, catalog number MCA1388, clone IBL-3/16); mouse monoclonal anti-F4/80 (Serotec, catalog number MCA497, clone CI: A3-1); rabbit polyclonal anti-glial fibrillary acidic protein (GFAP, Sigma, catalog number G4546).

Immunohistochemistry

Mice were deeply anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and perfused with sodium based 0.1M Phosphate Buffer (PB) (pH 7.4). Brains were collected and drop-fixed in 4% paraformaldehyde in PB. After brains were left in fixation for 24hrs, brains were cryoprotected in 30% sucrose. Brains were embedded in optimal cutting temperature (OCT) compound, and free-floating 30 µm coronal sections were collected and stored at 4°C in PBS with .05% sodium azide to prevent microbial contamination. 3,3′-diaminobenzidine (DAB; vector solutions) staining was performed first by boiling samples at 90^{o}C in 10mM sodium citrate. Endogenous peroxidases were then quenched by incubating sections in 1% H_2O_2 in phosphate buffered saline (PBS) for 30 min. Sections were blocked in 5% normal goat serum (NGS)/0.3% Triton X-100 in 1× PBS at room temperature for 1 h. Sections were then incubated with primary with the following dilutions: TREM2: 1:100; CD45 1:500; F4/80 1:500; GFAP 1:500; all incubated at 4°C overnight. Sections were washed and then incubated in secondary antibodies conjugated to biotin (Vector Laboratories; 1:1000) for 1 hr at room temperature. Sections were incubated with Avidin/Biotinylated enzyme complex (ABC reagent, Vector Laboratories; for immunohistochemistry) reagent for 1 hour at room temperature followed by DAB until a brown reaction was observed. Sections were then mounted with Permount (Thermo Fisher Scientific). Immunoreactivity was measured using threshold difference through Image J. The percent of the area that was covered with DAB

immunoreactivity was measured and analyzed using prism® where the percent area covered with immunoreactivity was compared between all groups. An investigator blinded to the conditions of the experiment scored all images. n=6 mice per group for all IHC experiments.

Lesion Analysis

Sections traversing the lesion cavity from -0.20 to -3.50 mm from bregma were stained with .1% cresyl violet for 1-2 minutes. Slides were then washed with PBS (2x 5 min) and dehydrated as done for IHC. Slides were then scanned into a computer using Quickscan via Pathscan Enabler IV (Meyer instruments) and stacks of the sections were made using Image J (NIH). The stacks were imported to Reconstruct® software (SynapseWeb) for volumetric analysis and 3D reconstruction[53]. First, the entire coronal section was outlined. Then the lesion cavity defined as any cavitation, or cellular disruption (abnormal cellular spacing, discoloring of cells within that region compared to healthy tissue, etc) found in the right hemisphere in the parietal associated cortex was outlined and a percentage ratio to the entire coronal section was calculated and compared across groups. Experimenters were blinded to the conditions and genotypes of all samples. The ipsilateral and contralateral hippocampus from -0.20 to -3.50 mm from bregma was also outlined and a percentage ratio to the remaining tissue was calculated and compared across groups (10 sections, 330 μm apart). n= 4 per group for all groups at 3 DPI. N=6 for B6 and *Trem2*$^{-/-}$ sham groups and n=5 for B6 and *Trem2*$^{-/-}$ mice exposed to TBI groups at 120 DPI time points. One B6 mouse exposed to TBI was excluded from lesion analysis for a lesion that was more than 2 standard deviations away from the mean for a final n=4 for the B6 mice exposed to TBI group.

qPCR

For analysis of TREM2, IL-1β, TNFα, and Il-6 mRNA levels following TBI, dissected brains were flash frozen using liquid nitrogen and the entire ipsilateral cortex homogenized in 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and1:100 protease inhibitor cocktail in PBS. RNA was isolated using chloroform extraction, purified using Purelink RNA Mini kit (Life Technologies), and genomic DNA removed via treatment with DNase Purelink (Life Technologies). cDNA was prepared from 1.5 µg total RNA using a QuantiTect Reverse Transcription kit (QIAGEN) and real-time PCR performed for 40 cycles with the StepOne Plus Real Time PCR system (Life Technologies) using *Trem2, il1β, tnfα, and il6* TaqMan probes (Life Technologies). Relative changes in gene expression were determined using the ΔΔCT method. N=3 per group was used for *Trem2* time course study in B6 mice. N=5 per group for all other experiments.

Flow cytometry

Mice were perfused, brains removed, chopped and digested using the Macs Neural tissue Dissociation kit (Miltenyl Biotec) at 3 DPI and subsequent percoll gradients (30% percoll [GE healthcare], 10% FBS) was use to purify myeloid cells and incubated to antibodies against Cd11b and CD45. Cells were gated on Cd11b+ and Cd45+. $CD45^{high}$Cd11b+ cells were defined as macrophages and $CD45^{low}$CD11b+ as resident microglia. n=4 for B6 and $Trem2^{-/-}$ sham mice, n= 6 for B6 exposed to TBI, and n= 5 for $Trem2^{-/-}$ mice exposed to TBI.

Behavioral Analysis

Rotarod

To assess motor coordination and locomotion, the accelerating rotarod (Rotarmex-5, Columbus Instruments, Columbus, OH) was utilized[54]. Each mouse received baseline training before surgical preparation and was assessed at 1, 3, 6, 30, 60, and 90 DPI. Rod rotations increased from 4 to 30 rotations per minute (RPM) during each five-minute trial. An average

latency to fall over three trials was calculated for each testing day and compared across all groups. n= 12 for all groups.

Y-Maze

The Y-maze is a standard behavioral test used to assess spatial working memory. Each mouse was placed in the Y-maze and manually scored for spontaneous alternations (the sequential entry of all arms before entering another arm) and arm entries[55, 56]. Each mouse was tested at 7, 31, 61, and 91 DPI. At the beginning of each trial, the mouse was placed in the center of the Y-maze and allowed to freely explore for 5 minutes. n= 12 for all groups. One mouse was excluded for analysis at 91 DPI in the *Trem2*$^{-/-}$ exposed to TBI for not entering more than 3 arms, a requirement to determine one spontaneous alternation.

Elevated Plus Maze

Alterations in inhibition and exploratory behavior were examined via elevated plus maze at 31 DPI[57, 58]. Mice were placed in the center of the elevated plus maze and the latency to enter all arms as well as time spent in all arms were measured using EthoVision XT (Noldus) video tracking software where a mouse was considered in an arm if its entire body (from nose to the beginning of the tail) was in the arm[57]. All trials were 10 minutes long. n= 12 for all groups.

Morris Water Maze

Finally, to examine spatial learning and reference memory, the visual and hidden platform versions of the Morris water maze were utilized at 110 DPI[4, 59]. Procedural learning was assessed through visible platform training, which required mice to complete four trials a day over three testing days. During visible platform training, the circular pool was filled with water to a depth approximately 0.5 cm below the top of the goal platform. A visual cue

(plastic syringe [no needle] colored black with a red piece of tape at the top) was placed on the platform to attract the animal's attention[59]. Mice were placed in the pool from one of four start locations and given 60 seconds to locate the platform. The location of the platform and the start location changed between every trial for every mouse. Mice were left on the platform for 15 seconds before being removed from the pool by the investigator. Mice unable to find the platform in 60 seconds were guided to the platform where they remained for 15 seconds before being removed from the pool.

Next, in the hidden platform version of the test, the circular pool was filled with water to a depth approximately 2 cm above the top of the goal platform. The visual cue was removed from the platform and animals were required to use constant spatial cues around to pool to locate the submerged goal platform, which remained in the same position throughout hidden platform testing. This design required mice to remember the position of the platform between trials and testing days to successfully complete the task in the shortest period of time. Mice were placed in the pool from one of four start locations and given 60 seconds to locate the platform. The start location changed between every trial for every mouse. Mice were left on the platform for 15 seconds before being removed from the pool. Mice unable to find the platform in 60 seconds were guided to the platform by the investigator where they remained for 15 seconds before being removed from the pool. The latency to find the platform was measured at all days and compared between days and between groups. n= 12 for all groups.

Statistics

For experiments with only two groups particularly the experiments looking at TREM2 expression in wild- type mice, unpaired, two-tailed T-tests were performed to compare significant differences. For experiments with multiple groups (groups of 4) 2-way analysis of variances (ANOVAs) were performed to measure significance using Prism (GraphPad). Post

hoc test correcting for multiple comparisons was used to compare individual groups to each other using QuickCals (GraphPad) http://graphpad.com/quickcalcs/posttest1.cfm. *p <0.05, **p<0.01, ***p<0.001. Bonferroni post hoc test was used to correct for repeated measures in rotarod, y-maze and, water maze. Comparisons and significance are shown between mice exposed to TBI groups compared to their respective shams and between both genotypes exposed to TBI.

RESULTS

TREM2 Expression Increases Acutely after TBI

Given that TREM2 is implicated in neurodegenerative diseases with increased expression in both human AD and mouse models of AD, we predicted that TREM2 expression would increase after TBI. We previously reported that TREM2 expression (as detected by immunohistochemistry) increased following a moderate experimental TBI at 3DPI[53]. Previously published work also confirms that macrophage infiltration and activation begins to peak at 3DPI[60]. Therefore, we selected 3 DPI as the optimal time point to characterize the expression of TREM2 on central and infiltrating cells. In the current study, animals were exposed to lateral FPI and examined for altered expression of TREM2 by IHC for TREM2 protein and RT-PCR for TREM2 mRNA. A time course was done to look at the acute expression of TREM2 after TBI using RT-QPCR. There was no increase of TREM2 at 1DPI, however TREM2 peaked at 3DPI showing a significant difference between mice exposed to TBI and sham injured mice and stayed elevated at 7DPI (Figure 1a-c). To follow this result, immunohistochemical staining was performed on 30 μm thick coronal slices isolated from B6 mice at 3 days following TBI using antibodies against TREM2. Notably, TREM2 staining was elevated in the TBI group and quantification revealed that there was significantly more TREM2 immunoreactivity in TBI mice compared to sham mice (Figure 1f and g). By

contrast to the findings at 3 DPI, TREM2 expression levels were greatly reduced at 120 DPI both using IHC and RT- PCR (Figure 1d, h, i). Taken together, this data suggests that TREM2 expression increases acutely after TBI but returns to near baseline levels at chronic post-injury time points.

TREM2 Deficiency Increases Macrophage Accumulation Near the Lesion at 3 DPI

Given that TREM2 is implicated in neuroinflammation and our results demonstrating that TREM2 expression levels increase at early time points following experimental TBI, we next examined if TREM2 deficiency would affect the inflammatory profile of mice exposed to TBI. IHC was utilized to assess the accumulation of macrophages and astrocytes in the brain in both *Trem2*$^{-/-}$ and B6 mice at 3 DPI. Antibodies against CD45, a common marker for leukocytes that is expressed at high levels on peripherally derived macrophages and expressed lowly on brain resident microglia, F4/80, a marker specifically for macrophages, and GFAP, a marker for reactive astrocytes were used. There was a significant increase in CD45 positive staining in the *Trem2*$^{-/-}$ compared to their wild type counterparts lateral to the lesion including the lesion (Figure 2b and e). *Trem2*$^{-/-}$ mice showed significantly more positive F4/80 staining in this same brain region at 3DPI compared to their wild-type counterparts (Figure 2c and f). Regardless of genotype, brain injured animals demonstrated increased CD45 and F4/80 staining near the injury site compared to sham animals (Figure 2b, c, e, f). The GFAP staining revealed enhanced astrogliosis in both TBI groups compared to their respective shams in the same brain region lateral to the lesion (Figure 2d and g). However, there were no significant differences in GFAP staining between the *Trem2*$^{-/-}$ and B6 mice exposed to TBI groups at 3 DPI. These IHC experiments demonstrate that there is increased staining for macrophage markers near the lesion of *Trem2*$^{-/-}$ mice exposed to TBI compared to wild type counterparts independent of brain-injury induced astrogliosis.

TREM2 Deficiency Reduces Macrophage Accumulation Away From the Lesion at 3 DPI

Previous studies indicate that macrophage survival or migration may be compromised in *Trem2*$^{-/-}$ mice[49, 50], which prompted us to examine sites distal to the epicenter of primary cortical damage. Notably, IHC with antibodies against F4/80 revealed that there was significantly more F4/80 staining in B6 mice exposed to TBI compared to *Trem2*$^{-/-}$ mice exposed to TBI in a location inferiolateral to the lesion within the ipsilateral cortex (Figure 3b and c). In fact, *Trem2*$^{-/-}$ mice exposed to TBI did not exhibit statistically significant increases in F4/80 staining when compared to shams. Taken together, this data provide evidence for an altered macrophage response in the *Trem2*$^{-/-}$ mice based upon the distance to the injury that could be due to alterations in macrophage migration or survival.

TREM2 Deficiency Reduces Macrophage Activation and Accumulation Throughout the Brain at 3 DPI

To further examine the effects of TREM2 deficiency on specific macrophage populations following TBI, flow cytometry was utilized. As IHC staining with specific antibodies is notoriously unable to reliably differentiate between macrophages derived from microglia versus monocytes, flow cytometry allows a more accurate method to discriminate between these cells. Although there is no universally accepted marker to definitively distinguish peripherally derived macrophages from microglia, differences in levels of CD45 expression by flow cytometry have been used in multiple studies to distinguish these two macrophage populations within the brain, namely CD45^{low}Cd11b+ cells derived from microglia and CD45^{high}Cd11b+ cells derived from monocytes[61-63]. Notably, flow cytometry at 3 DPI, revealed that the percentage of CD45^{high}Cd11b+ macrophages in the *Trem2*$^{-/-}$ mice exposed to TBI was significantly less than their wild type counterparts suggesting that there are

significantly less peripherally derived macrophages then those resident-derived (Figure 3d and e). qpCr was also performed to look at inflammation and activation of immune cells in the brain. There was significantly less *tnfα* production in *Trem2*$^{-/-}$ mice exposed to TBI was compared to their wild type counterparts (Figure 3f). These levels were not significantly different then either B6 sham mice or *Trem2*$^{-/-}$ sham mice groups. There was also no significant increase in *il6* or *il1β* in *Trem2*$^{-/-}$ mice exposed to TBI compared to *Trem2*$^{-/-}$ sham mice while there was a significant difference in B6 mice exposed to TBI compared to their respective shams (Figure g and h). This data suggests that TREM2 is important in the peripheral macrophage response after TBI since there are comparatively less peripheral macrophages in *Trem2*$^{-/-}$ mice brains exposed to TBI and comparatively less inflammatory cytokine production.

Macrophage Activation Resolves in Both B6 and TREM2 Deficient Mice at 120 DPI

Given that we observed an altered macrophage response at acute time points (3 DPI) following TBI in TREM2 deficient mice and that chronic activations of the innate immune systems is a feature observed in a wide variety of neurodegenerative diseases, we next examined the effects of TREM2 deficiency at chronic time points (120 DPI) following TBI. Notably, and unlike what was observed at 3 DPI, no significant differences were observed in CD45 IHC staining between any of the groups at 120 DPI lateral to the lesion including the lesion, regardless of genotype or treatment (Figure 4b and e). F4/80 IHC revealed a similar pattern in staining where there were no differences between any of the groups at 120 DPI (Figure 4c and f). Finally, GFAP staining revealed reactive astrocytes in both *Trem2*$^{-/-}$ and B6 mice exposed to TBI when compared to sham controls in the region near the lesion (Figure 4d and g). These results suggest that macrophage activation patterns observed at 3 DPI, largely have resolved at 120 DPI, particular in *Trem2*$^{-/-}$ mice exposed to TBI.

TREM2 Deficiency Reduces Hippocampal Atrophy at 120 DPI

Given the unique pattern of macrophage activation following TBI in TREM2 deficient mice that appeared to change between 3 DPI and 120 DPI, we next sought to examine whether there were alterations in brain structure at these acute and chronic time points. Cresyl violet staining of the brain was utilized to examine the volume of specific brain structures via three-dimensional reconstructions. All volumetric measurements were expressed relative to the entire brain volume in order to correct for inter-individual brain differences. Hippocampal volume was assessed on 10, representative 30-micron brain sections. At 3 DPI, reconstruction analysis revealed there were no significant alterations in ipsilateral and contralateral hippocampal volumes or lesion volume between the genotypes (B6 versus *Trem2*$^{-/-}$) or experimental groups (TBI versus sham; Figure 5 c, e, g). By contrast, at 120 DPI, while B6 mice exposed to TBI exhibited a significant decrease in contralateral and ipsilateral hippocampal volume relative to B6 shams, no significant alterations were observed in *Trem2*$^{-/-}$ mice exposed to TBI when compared to either B6 or *Trem2*$^{-/-}$ shams (Figure 5f and h). Though both groups exposed to TBI had a significant increase of lesion volume loss compared to their respective shams, there was no genotype difference (Figure 5d). In summary, TREM2 deficiency had no detectable effects on relative brain volumes at an acute time point, but resulted in reduced hippocampal volume loss at a chronic time point.

TREM2 Deficiency Ameliorates Behavioral Alterations Associated with TBI

TBI and sham mice aging to the chronic post-injury time point completed a variety of behavioral assessments, which provided functional outcomes measures to correlate with injury-induced histological changes (see Fig. 6a). First, to examine spatial working memory, the Y-maze was performed at 7, 30, 60, 90 DPI. Both the spontaneous alternation ratio

(animals entering the three separate arms consecutively over the total number of arm entries) and total number of arms entered were measured. Notably, TREM2 deficient mice exposed to TBI exhibited a reduced number of arm entries at all post-injury time points compared to B6 mice exposed to TBI (Figure 6f-i). B6 brain injured mice demonstrated a significant reduction in the spontaneous alternation ratio at 90 DPI compared to B6 shams, which was not observed between *Trem2*$^{-/-}$ mice exposed to TBI and sham injured mice (Figure 6e). Together, these data suggest that *Trem2*$^{-/-}$ mice were protected from brain injury induced spatial working memory deficits; however, performance may have been compromised by a unique motor or anxiety phenotype.

To examine whether alterations in arm entries observed in the Y-maze could be due to motor deficits, the rotarod (a test of an animal's ability to maintain itself on an accelerating rod) was utilized. Importantly, no significant differences were observed in rotarod performance between groups at any time point (Fig 6j). To identify potential anxiety-related deficits following TBI, the elevated plus maze was completed to characterize exploratory behavior and/or inhibition. Previous studies report that increased exploration of open arms reflects a lack of inhibition or disinhibition while increased time in closed arms reflects a lack of exploratory behavior. Disinhibition is a common occurrence after TBI and in AD in both humans and mice where subjects will take more risks or be impulsive [58, 64, 65]. Though rodents will typically spend more time exploring the elevated plus maze, naïve B6 mice have been shown to spend a majority of time in the closed arm[57]. We observed that *Trem2*$^{-/-}$ mice exposed to TBI spent significantly more time in the closed arm compared to B6 mice exposed to TBI and acted similarly to both sham groups (Figure 6k). This suggests that TREM2 deficiency protects against TBI induced disinhibition.

Finally, to examine spatial reference memory, mice were tested in the visible and hidden platform version of the Morris water maze beginning at 110 DPI. In the visual platform version of the task, mice were trained to swim to a goal platform identified by a visual cue over 4 training trials per day for three days[59]. Notably, both B6 and *Trem2*$^{-/-}$ mice exposed to either sham or TBI did not exhibit any significant differences in the latency to find the visible platform over the three days of testing. Next, to examine spatial reference memory, the cue identifying the platform was removed and the mice were trained to find the submerged platform on the basis of spatial cues located on the walls around the swimming pool. As expected, in the hidden platform version of the task, there was significant impairment in the latency to find the platform between the B6 mice exposed to TBI and sham controls (Figure 6l). By contrast, *Trem2*$^{-/-}$ mice exposed to TBI did not exhibit any significant differences in latency to find the platform when compared to either B6 or *Trem2*$^{-/-}$ sham controls (Figure 6l). Taken together, this data suggest that TREM2 deficiency protects form the chronic effects of TBI on spatial reference memory as detected in the Morris water maze.

DISCUSSION

TBI represents a significant risk factor for multiple neurodegenerative diseases[1-6]. Both acute and chronic outcomes must be studied to determine the exact role TBI induced neuroinflammation may play in the development of neurodegenerative pathologies. In this study, acutely after TBI a) TREM2 expression increased in the brain of B6 mice exposed to TBI b) TREM2 deficiency altered the macrophage response in a spatially dependent manner, with increased activation near the lesion site and decreased activation at a distal location c) TREM2 deficiency resulted in a reduced proportion of peripherally derived macrophages recruited to the brain following TBI and decreased the production of inflammatory cytokines. Chronically after TBI, a) macrophage activation substantially declined in brain injured mice

regardless of genotype b) *Trem2*$^{-/-}$ mice had less hippocampal volume loss after TBI compared to their wild type counterparts c) *Trem2*$^{-/-}$ mice displayed rescue of TBI-induced disinhibition and deficits in spatial memory. These data suggest that TREM2 plays a role in the macrophage response acutely after TBI and promotes volume loss of sensitive regions of the brain and behavioral deficits after TBI (see Figure 7).

Increasing evidence implicates the macrophage response as a key mediator in long-term outcome following TBI. Clearly distinguishing between resident derived and peripherally derived macrophages has been a challenge in the field. One method using flow cytometry as discussed before is CD45^{low}Cd11b+ cells derived from microglia and CD45^{high}Cd11b+ cells derived from monocytes[61-63]. However, depleting a certain set of macrophages to look at pathological outcomes using CD45^{low}Cd11b+ versus CD45^{high}Cd11b+ is inefficient and challenging. One method of overcoming this problem is by distinguishing resident macrophages using CX3CR1, a receptor involved in adhesion and most commonly found on resident microglia, and peripherally derived macrophages using CCR2, a receptor needed for the chemotaxis or migration of peripheral macrophages/monocytes. CCR2 has been shown to be particularly important in TBI pathology. For example, CCR2+ macrophages were found to form around the hippocampus after TBI[66]. Another study showed that impairing macrophage infiltration using CCR2 deficient mice is beneficial for TBI recovery and cognitive outcome after TBI[67]. Though TREM2 is expressed in both microglial and monocytic derived macrophages, TREM2 deficiency significantly decreased the presence of peripherally derived macrophages in the brain of an AD mouse model[50]. In this study *Trem2*$^{-/-}$ mice exhibited a decrease in peripheral macrophages after TBI throughout the brain, and chronically less hippocampal volume loss. If other studies show that peripheral macrophages tend to target sensitive regions such as the hippocampus and that blocking infiltrating monocytes is beneficial for long term outcomes after TBI, this study helps support the idea

that blocking a particular set of infiltrating macrophages may be beneficial for TBI recovery in preventing chronic pathology.

Brain volume loss is a common consequence of both chronic and severe single occurrence TBI[20, 68]. Even a mild to moderate single occurrence injury lead to brain volume loss particularly in more sensitive regions like the hippocampus, a phenomenon repeated in wild type B6 mice in this study as well[69]. Hippocampal volume loss is also a major pathology of neurodegenerative diseases such as AD[70, 71]. Chronic hippocampal volume loss is correlated to behavioral changes and cognitive deficits in both rodents and humans[20, 72, 73]. This data suggests that TREM2 deficiency may be neuroprotective in reducing hippocampal volume loss after TBI.

TREM2 has been thought to be anti-inflammatory and potentially beneficial and preventing inflammatory related pathologies[43, 74]. However many *in vivo* studies have obtained differing results. In an AD mouse model, TREM2 deficient mice had dramatically reduced numbers of peripherally derived macrophages associated with Aβ deposits and overall reduced Aβ pathology[50]. Another study found that TREM2 activation promotes the development of chronic lung disease after a viral infection in mice[49]. This study also found that TREM2 deficiency lead to reduced numbers of infiltrating macrophages, specifically showing that TREM2 promoted macrophage survival. Notably, in the current study, TREM2 deficiency lead to enhanced macrophage activation around the lesion, but overall reduced macrophage activation and production of inflammatory cytokines throughout the brain at early time points following injury. A model similar to TBI where there is ischemic damage, dead tissue, etc is the stroke model [75, 76]. Though, the role of TREM2 in stroke is controversial, one study focusing on TREM2-deficient mice also showed attenuated inflammation and better functional outcomes[48, 77]. These data suggest that TREM2 deficiency may be beneficial particularly by modulating the inflammatory state of the brain.

Another aspect that is linked to both neurodegeneration and TBI is changes in cognitive behavior. In this study, we see a rescue of inhibitory behavior and cognition through y maze, elevated plus maze, and water maze in TREM2 deficient mice exposed to TBI compared to wild type controls. Though elevated plus maze is typically used as a measure of anxiety, naive B6 mice spend more time in the closed arms of elevated plus maze[57]. TREM2 deficient mice exhibited similar behavior to B6 sham mice regardless of treatment. However, B6 mice exposed to TBI showed an increase of disinhibited behavior, a common consequence of TBI[58, 64, 65]. This suggests that TREM2 deficiency protects against TBI- induced disinhibition specifically seen on the B6 background. Though the fact that *Trem2*$^{-/-}$ mice spend more time in the closed arms in the elevated plus maze may suggest that these mice are more anxious, it is important to note that they exhibit behaviors similar to wild type controls and that it is the B6 mice exposed to TBI that are different from all other groups. With water maze testing it is common to see deficits in reference memory after TBI[78-80]. In this study, we were also able to show that wild type B6 mice exposed to TBI take significant longer to reach a submerged goal platform compared to B6 sham mice. However TREM2 deficient mice exposed to TBI performed just as well on this task as either sham group, suggesting a rescue of TBI-induced cognitive dysfunction. There were largely no statistically significant phenotypic differences between sexes, specifically for the *Trem2*$^{-/-}$ mice. Together these data suggests that TREM2 deficiency reduces TBI- induced behavioral changes.

Briefly, TREM2 deficiency attenuates both hippocampal atrophy and TBI induced behavioral changes. This neuroprotection may be due to the altered macrophage response seen acutely after TBI where there was a decrease of peripheral macrophages found throughout the brain, but significantly found near the lesion site. Given the important role of TREM2 in neurodegenerative disease and brain injury, additional studies must be completed to fully characterize the expression profile of microglia and monocytes at both acute and chronic time

points. Additional studies are needed to specifically identify which macrophage populations express TREM2 after TBI, brain-resident microglia, infiltrating peripheral monocytes, or both. Subsequent studies may then be designed to potentially develop therapeutic targets that would only affect this particular subset of macrophages, instead of targeting all peripheral macrophages that could be detrimental by promoting neurodegenerative pathologies[81]. In conclusion, this study gives support to exploring TREM2 as a potential target for therapeutic treatment for the long-term recovery of TBI patients in reducing neurodegenerative pathologies.

ACKNOWLEDGEMENTS

We thank Guixang Xu for genotyping all mice and assisting with lab maintenance. We thank the Najm lab for allowing us to use their FPI device for our studies. We thank

Grahame Kidd for assistance with volumetric analysis and 3D reconstruction. We thank Josephine Dermawan for assistance with technical support for figure development. We also thank the Rodent Behavioral Core and the Flow Cytometry Core for technical support. **Funding:** This work was supported by the Department of Defense (W81XWH-14-1-0265, Bruce Lamb). **Author Contributions:** M.S. designed, performed, and analyzed all experiments and wrote the manuscript. O.K-C. analyzed behavioral experiments, trained MS in performing and analyzing all experiments, and co-edited the manuscript; S.S.P. helped with Flow analysis; J.D.L advised with the writing of the manuscript and co-edited the manuscript. B.T.L. provided funding and expertise and co-edited the manuscript. **Author Disclosure Statement:** No competing financial interests exist.

REFERENCES

1. Breunig, J.J., Guillot-Sestier, M.V. and Town, T. (2013). Brain injury, neuroinflammation and Alzheimer's disease. Front Aging Neurosci 5, 26.

2. Draper, K. and Ponsford, J. (2008). Cognitive functioning ten years following traumatic brain injury and rehabilitation. Neuropsychology 22, 618-625.

3. Goldstein, L.E., Fisher, A.M., Tagge, C.A., Zhang, X.L., Velisek, L., Sullivan, J.A., Upreti, C., Kracht, J.M., Ericsson, M., Wojnarowicz, M.W., Goletiani, C.J., Maglakelidze, G.M., Casey, N., Moncaster, J.A., Minaeva, O., Moir, R.D., Nowinski, C.J., Stern, R.A., Cantu, R.C., Geiling, J., Blusztajn, J.K., Wolozin, B.L., Ikezu, T., Stein, T.D., Budson, A.E., Kowall, N.W., Chargin, D., Sharon, A., Saman, S., Hall, G.F., Moss, W.C., Cleveland, R.O., Tanzi, R.E., Stanton, P.K. and McKee, A.C. (2012). Chronic traumatic encephalopathy in blast-exposed military veterans and a blast neurotrauma mouse model. Sci Transl Med 4, 134ra160.

4. Hamm, R.J., Dixon, C.E., Gbadebo, D.M., Singha, A.K., Jenkins, L.W., Lyeth, B.G. and Hayes, R.L. (1992). Cognitive deficits following traumatic brain injury produced by controlled cortical impact. J Neurotrauma 9, 11-20.

5. Sivanandam, T.M. and Thakur, M.K. (2012). Traumatic brain injury: a risk factor for Alzheimer's disease. Neurosci Biobehav Rev 36, 1376-1381.

6. Van Den Heuvel, C., Thornton, E. and Vink, R. (2007). Traumatic brain injury and Alzheimer's disease: a review. Prog Brain Res 161, 303-316.

7. Johnson, V.E., Stewart, W. and Smith, D.H. (2012). Widespread tau and amyloid-Beta pathology many years after a single traumatic brain injury in humans. Brain Pathol 22, 142-149.

8. Tran, H.T., LaFerla, F.M., Holtzman, D.M. and Brody, D.L. (2011). Controlled cortical impact traumatic brain injury in 3xTg-AD mice causes acute intra-axonal amyloid-beta accumulation and independently accelerates the development of tau abnormalities. J Neurosci 31, 9513-9525.

9. Ikonomovic, M.D., Uryu, K., Abrahamson, E.E., Ciallella, J.R., Trojanowski, J.Q., Lee, V.M., Clark, R.S., Marion, D.W., Wisniewski, S.R. and DeKosky, S.T. (2004). Alzheimer's pathology in human temporal cortex surgically excised after severe brain injury. Exp Neurol 190, 192-203.

10. Emmerling, M.R., Morganti-Kossmann, M.C., Kossmann, T., Stahel, P.F., Watson, M.D., Evans, L.M., Mehta, P.D., Spiegel, K., Kuo, Y.M., Roher, A.E. and Raby, C.A. (2000). Traumatic brain injury elevates the Alzheimer's amyloid peptide A beta 42 in human CSF. A possible role for nerve cell injury. Ann N Y Acad Sci 903, 118-122.

11. Cameron, B. and Landreth, G.E. (2010). Inflammation, microglia, and Alzheimer's disease. Neurobiol Dis 37, 503-509.

12. Dardiotis, E., Karanikas, V., Paterakis, K., Fountas, K. and Hadjigeorgiou, G.M. (2012). Traumatic Brain Injury and Inflammation: Emerging Role of Innate and Adaptive Immunity. In: *Brain Injury – Pathogenesis, Monitoring, Recovery and Management*. Agarwal, A. (ed). InTech: Rijeka, Croatia, pps. 23-38.

13. Schmidt, O.I., Leinhase, I., Hasenboehler, E., Morgan, S.J. and Stahel, P.F. (2007). [The relevance of the inflammatory response in the injured brain]. Orthopade 36, 248, 250-248.

14. Lloyd, E., Somera-Molina, K., Van Eldik, L.J., Watterson, D.M. and Wainwright, M.S. (2008). Suppression of acute proinflammatory cytokine and chemokine upregulation by post-injury administration of a novel small molecule improves long-term neurologic outcome in a mouse model of traumatic brain injury. J Neuroinflammation 5, 28.

15. Acosta, S.A., Tajiri, N., Shinozuka, K., Ishikawa, H., Grimmig, B., Diamond, D., Sanberg, P.R., Bickford, P.C., Kaneko, Y. and Borlongan, C.V. (2013). Long-term upregulation of inflammation and suppression of cell proliferation in the brain of adult

rats exposed to traumatic brain injury using the controlled cortical impact model. PLoS One 8, e53376.

16. Gentleman, S.M., Leclercq, P.D., Moyes, L., Graham, D.I., Smith, C., Griffin, W.S. and Nicoll, J.A. (2004). Long-term intracerebral inflammatory response after traumatic brain injury. Forensic Sci Int 146, 97-104.

17. Franzblau, M., Gonzales-Portillo, C., Gonzales-Portillo, G.S., Diamandis, T., Borlongan, M.C., Tajiri, N. and Borlongan, C.V. (2013). Vascular damage: a persisting pathology common to Alzheimer's disease and traumatic brain injury. Med Hypotheses 81, 842-845.

18. Bamberger, M.E. and Landreth, G.E. (2002). Inflammation, apoptosis, and Alzheimer's disease. Neuroscientist 8, 276-283.

19. Pierce, J.E., Smith, D.H., Trojanowski, J.Q. and McIntosh, T.K. (1998). Enduring cognitive, neurobehavioral and histopathological changes persist for up to one year following severe experimental brain injury in rats. Neuroscience 87, 359-369.

20. Smith, D.H., Chen, X.H., Pierce, J.E., Wolf, J.A., Trojanowski, J.Q., Graham, D.I. and McIntosh, T.K. (1997). Progressive atrophy and neuron death for one year following brain trauma in the rat. J Neurotrauma 14, 715-727.

21. Shlosberg, D., Benifla, M., Kaufer, D. and Friedman, A. (2010). Blood-brain barrier breakdown as a therapeutic target in traumatic brain injury. Nat Rev Neurol 6, 393-403.

22. Williams, A.J., Wei, H.H., Dave, J.R. and Tortella, F.C. (2007). Acute and delayed neuroinflammatory response following experimental penetrating ballistic brain injury in the rat. J Neuroinflammation 4, 17.

23. Ray, S.K., Dixon, C.E. and Banik, N.L. (2002). Molecular mechanisms in the pathogenesis of traumatic brain injury. Histol Histopathol 17, 1137-1152.

24. Morganti-Kossmann, M.C., Satgunaseelan, L., Bye, N. and Kossmann, T. (2007). Modulation of immune response by head injury. Injury 38, 1392-1400.

25. Neumann, H., Kotter, M.R. and Franklin, R.J. (2009). Debris clearance by microglia: an essential link between degeneration and regeneration. Brain 132, 288-295.

26. Ziebell, J.M. and Morganti-Kossmann, M.C. (2010). Involvement of pro- and anti-inflammatory cytokines and chemokines in the pathophysiology of traumatic brain injury. Neurotherapeutics 7, 22-30.

27. Roberts, G.W., Gentleman, S.M., Lynch, A., Murray, L., Landon, M. and Graham, D.I. (1994). Beta amyloid protein deposition in the brain after severe head injury: implications for the pathogenesis of Alzheimer's disease. J Neurol Neurosurg Psychiatry 57, 419-425.

28. Driscoll, I., Troncoso, J.C., Rudow, G., Sojkova, J., Pletnikova, O., Zhou, Y., Kraut, M.A., Ferrucci, L., Mathis, C.A., Klunk, W.E., O'Brien, R.J., Davatzikos, C., Wong, D.F. and Resnick, S.M. (2012). Correspondence between in vivo (11)C-PiB-PET amyloid imaging and postmortem, region-matched assessment of plaques. Acta Neuropathol 124, 823-831.

29. Malik, M., Simpson, J.F., Parikh, I., Wilfred, B.R., Fardo, D.W., Nelson, P.T. and Estus, S. (2013). CD33 Alzheimer's risk-altering polymorphism, CD33 expression, and exon 2 splicing. J Neurosci 33, 13320-13325.

30. Llado, A., Sanchez-Valle, R., Rene, R., Ezquerra, M., Rey, M.J., Tolosa, E., Ferrer, I. and Molinuevo, J.L. (2007). Late-onset frontotemporal dementia associated with a novel PGRN mutation. J Neural Transm (Vienna) 114, 1051-1054.

31. Guerreiro, R., Wojtas, A., Bras, J., Carrasquillo, M., Rogaeva, E., Majounie, E., Cruchaga, C., Sassi, C., Kauwe, J.S., Younkin, S., Hazrati, L., Collinge, J., Pocock, J., Lashley, T., Williams, J., Lambert, J.C., Amouyel, P., Goate, A., Rademakers, R.,

Morgan, K., Powell, J., St George-Hyslop, P., Singleton, A. and Hardy, J. (2013). TREM2 variants in Alzheimer's disease. N Engl J Med 368, 117-127.

32. Borroni, B., Ferrari, F., Galimberti, D., Nacmias, B., Barone, C., Bagnoli, S., Fenoglio, C., Piaceri, I., Archetti, S., Bonvicini, C., Gennarelli, M., Turla, M., Scarpini, E., Sorbi, S. and Padovani, A. (2013). Heterozygous TREM2 mutations in frontotemporal dementia. Neurobiol Aging.

33. Rayaprolu, S., Mullen, B., Baker, M., Lynch, T., Finger, E., Seeley, W.W., Hatanpaa, K.J., Lomen-Hoerth, C., Kertesz, A., Bigio, E.H., Lippa, C., Josephs, K.A., Knopman, D.S., White, C.L., 3rd, Caselli, R., Mackenzie, I.R., Miller, B.L., Boczarska-Jedynak, M., Opala, G., Krygowska-Wajs, A., Barcikowska, M., Younkin, S.G., Petersen, R.C., Ertekin-Taner, N., Uitti, R.J., Meschia, J.F., Boylan, K.B., Boeve, B.F., Graff-Radford, N.R., Wszolek, Z.K., Dickson, D.W., Rademakers, R. and Ross, O.A. (2013). TREM2 in neurodegeneration: evidence for association of the p.R47H variant with frontotemporal dementia and Parkinson's disease. Mol Neurodegener 8, 19.

34. Soragna, D., Papi, L., Ratti, M.T., Sestini, R., Tupler, R. and Montalbetti, L. (2003). An Italian family affected by Nasu-Hakola disease with a novel genetic mutation in the TREM2 gene. J Neurol Neurosurg Psychiatry 74, 825-826.

35. Thrash, J.C., Torbett, B.E. and Carson, M.J. (2009). Developmental regulation of TREM2 and DAP12 expression in the murine CNS: implications for Nasu-Hakola disease. Neurochem Res 34, 38-45.

36. Paloneva, J., Mandelin, J., Kiialainen, A., Bohling, T., Prudlo, J., Hakola, P., Haltia, M., Konttinen, Y.T. and Peltonen, L. (2003). DAP12/TREM2 deficiency results in impaired osteoclast differentiation and osteoporotic features. J Exp Med 198, 669-675.

37. Numasawa, Y., Yamaura, C., Ishihara, S., Shintani, S., Yamazaki, M., Tabunoki, H. and Satoh, J.I. (2011). Nasu-Hakola disease with a splicing mutation of TREM2 in a Japanese family. Eur J Neurol 18, 1179-1183.

38. Jonsson, T., Stefansson, H., Steinberg, S., Jonsdottir, I., Jonsson, P.V., Snaedal, J., Bjornsson, S., Huttenlocher, J., Levey, A.I., Lah, J.J., Rujescu, D., Hampel, H., Giegling, I., Andreassen, O.A., Engedal, K., Ulstein, I., Djurovic, S., Ibrahim-Verbaas, C., Hofman, A., Ikram, M.A., van Duijn, C.M., Thorsteinsdottir, U., Kong, A. and Stefansson, K. (2013). Variant of TREM2 associated with the risk of Alzheimer's disease. N Engl J Med 368, 107-116.

39. Pottier, C., Wallon, D., Rousseau, S., Rovelet-Lecrux, A., Richard, A.C., Rollin-Sillaire, A., Frebourg, T., Campion, D. and Hannequin, D. (2013). TREM2 R47H variant as a risk factor for early-onset Alzheimer's disease. J Alzheimers Dis 35, 45-49.

40. Singaraja, R.R. (2013). TREM2: a new risk factor for Alzheimer's disease. Clin Genet 83, 525-526.

41. Guerreiro, R., Wojtas, A., Bras, J., Carrasquillo, M., Rogaeva, E., Majounie, E., Cruchaga, C., Sassi, C., Kauwe, J.S., Younkin, S., Hazrati, L., Collinge, J., Pocock, J., Lashley, T., Williams, J., Lambert, J.C., Amouyel, P., Goate, A., Rademakers, R., Morgan, K., Powell, J., St George-Hyslop, P., Singleton, A., Hardy, J. and Alzheimer Genetic Analysis, G. (2013). TREM2 variants in Alzheimer's disease. N Engl J Med 368, 117-127.

42. Sessa, G., Podini, P., Mariani, M., Meroni, A., Spreafico, R., Sinigaglia, F., Colonna, M., Panina, P. and Meldolesi, J. (2004). Distribution and signaling of TREM2/DAP12, the receptor system mutated in human polycystic lipomembraneous osteodysplasia with sclerosing leukoencephalopathy dementia. Eur J Neurosci 20, 2617-2628.

43. Takahashi, K., Rochford, C.D. and Neumann, H. (2005). Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. J Exp Med 201, 647-657.

44. Hsieh, C.L., Koike, M., Spusta, S.C., Niemi, E.C., Yenari, M., Nakamura, M.C. and Seaman, W.E. (2009). A role for TREM2 ligands in the phagocytosis of apoptotic neuronal cells by microglia. J Neurochem 109, 1144-1156.

45. Gao, X., Dong, Y., Liu, Z. and Niu, B. (2013). Silencing of triggering receptor expressed on myeloid cells-2 enhances the inflammatory responses of alveolar macrophages to lipopolysaccharide. Mol Med Rep 7, 921-926.

46. Ito, H. and Hamerman, J.A. (2012). TREM-2, triggering receptor expressed on myeloid cell-2, negatively regulates TLR responses in dendritic cells. Eur J Immunol 42, 176-185.

47. Ford, J.W. and McVicar, D.W. (2009). TREM and TREM-like receptors in inflammation and disease. Curr Opin Immunol 21, 38-46.

48. Sieber, M.W., Jaenisch, N., Brehm, M., Guenther, M., Linnartz-Gerlach, B., Neumann, H., Witte, O.W. and Frahm, C. (2013). Attenuated inflammatory response in triggering receptor expressed on myeloid cells 2 (TREM2) knock-out mice following stroke. PLoS One 8, e52982.

49. Wu, K., Byers, D.E., Jin, X., Agapov, E., Alexander-Brett, J., Patel, A.C., Cella, M., Gilfilan, S., Colonna, M., Kober, D.L., Brett, T.J. and Holtzman, M.J. (2015). TREM-2 promotes macrophage survival and lung disease after respiratory viral infection. J Exp Med 212, 681-697.

50. Jay, T.R., Miller, C.M., Cheng, P.J., Graham, L.C., Bemiller, S., Broihier, M.L., Xu, G., Margevicius, D., Karlo, J.C., Sousa, G.L., Cotleur, A.C., Butovsky, O., Bekris, L., Staugaitis, S.M., Leverenz, J.B., Pimplikar, S.W., Landreth, G.E., Howell, G.R.,

Ransohoff, R.M. and Lamb, B.T. (2015). TREM2 deficiency eliminates TREM2+ inflammatory macrophages and ameliorates pathology in Alzheimer's disease mouse models. J Exp Med 212, 287-295.

51. Dixon, C.E., Lyeth, B.G., Povlishock, J.T., Findling, R.L., Hamm, R.J., Marmarou, A., Young, H.F. and Hayes, R.L. (1987). A fluid percussion model of experimental brain injury in the rat. J Neurosurg 67, 110-119.

52. McIntosh, T.K., Vink, R., Noble, L., Yamakami, I., Fernyak, S., Soares, H. and Faden, A.L. (1989). Traumatic brain injury in the rat: characterization of a lateral fluid-percussion model. Neuroscience 28, 233-244.

53. Kokiko-Cochran, O., Ransohoff, L., Veenstra, M., Lee, S., Saber, M., Sikora, M., Teknipp, R., Xu, G., Bemiller, S., Wilson, G., Crish, S., Bhaskar, K., Lee, Y.S., Ransohoff, R.M. and Lamb, B.T. (2015). Altered Neuroinflammation and Behavior after Traumatic Brain Injury in a Mouse Model of Alzheimer's Disease. J Neurotrauma.

54. Hamm, R.J., Pike, B.R., O'Dell, D.M., Lyeth, B.G. and Jenkins, L.W. (1994). The rotarod test: an evaluation of its effectiveness in assessing motor deficits following traumatic brain injury. J Neurotrauma 11, 187-196.

55. Hamm, R.J. (2001). Neurobehavioral assessment of outcome following traumatic brain injury in rats: an evaluation of selected measures. J Neurotrauma 18, 1207-1216.

56. Hamm, R.J., Temple, M.D., Pike, B.R. and Ellis, E.F. (1996). The effect of postinjury administration of polyethylene glycol-conjugated superoxide dismutase (pegorgotein, Dismutec) or lidocaine on behavioral function following fluid-percussion brain injury in rats. J Neurotrauma 13, 325-332.

57. Komada, M., Takao, K. and Miyakawa, T. (2008). Elevated plus maze for mice. J Vis Exp.

58. Shultz, S.R., Tan, X.L., Wright, D.K., Liu, S.J., Semple, B.D., Johnston, L., Jones, N.C., Cook, A.D., Hamilton, J.A. and O'Brien, T.J. (2014). Granulocyte-macrophage colony-stimulating factor is neuroprotective in experimental traumatic brain injury. J Neurotrauma 31, 976-983.

59. Morris, R. (1984). Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 11, 47-60.

60. Turtzo, L.C., Lescher, J., Janes, L., Dean, D.D., Budde, M.D. and Frank, J.A. (2014). Macrophagic and microglial responses after focal traumatic brain injury in the female rat. J Neuroinflammation 11, 82.

61. Ford, A.L., Goodsall, A.L., Hickey, W.F. and Sedgwick, J.D. (1995). Normal adult ramified microglia separated from other central nervous system macrophages by flow cytometric sorting. Phenotypic differences defined and direct ex vivo antigen presentation to myelin basic protein-reactive CD4+ T cells compared. J Immunol 154, 4309-4321.

62. Becher, B. and Antel, J.P. (1996). Comparison of phenotypic and functional properties of immediately ex vivo and cultured human adult microglia. Glia 18, 1-10.

63. Hickman, S.E., Kingery, N.D., Ohsumi, T.K., Borowsky, M.L., Wang, L.C., Means, T.K. and El Khoury, J. (2013). The microglial sensome revealed by direct RNA sequencing. Nat Neurosci 16, 1896-1905.

64. Kim, E. (2002). Agitation, aggression, and disinhibition syndromes after traumatic brain injury. NeuroRehabilitation 17, 297-310.

65. Pang, F.C., Chow, T.W., Cummings, J.L., Leung, V.P., Chiu, H.F., Lam, L.C., Chen, Q.L., Tai, C.T., Chen, L.W., Wang, S.J. and Fuh, J.L. (2002). Effect of neuropsychiatric symptoms of Alzheimer's disease on Chinese and American caregivers. Int J Geriatr Psychiatry 17, 29-34.

66. Morganti, J.M., Jopson, T.D., Liu, S., Riparip, L.K., Guandique, C.K., Gupta, N., Ferguson, A.R. and Rosi, S. (2015). CCR2 antagonism alters brain macrophage polarization and ameliorates cognitive dysfunction induced by traumatic brain injury. J Neurosci 35, 748-760.

67. Hsieh, C.L., Niemi, E.C., Wang, S.H., Lee, C.C., Bingham, D., Zhang, J., Cozen, M.L., Charo, I., Huang, E.J., Liu, J. and Nakamura, M.C. (2014). CCR2 deficiency impairs macrophage infiltration and improves cognitive function after traumatic brain injury. J Neurotrauma 31, 1677-1688.

68. Povlishock, J.T. and Katz, D.I. (2005). Update of neuropathology and neurological recovery after traumatic brain injury. J Head Trauma Rehabil 20, 76-94.

69. MacKenzie, J.D., Siddiqi, F., Babb, J.S., Bagley, L.J., Mannon, L.J., Sinson, G.P. and Grossman, R.I. (2002). Brain atrophy in mild or moderate traumatic brain injury: a longitudinal quantitative analysis. AJNR Am J Neuroradiol 23, 1509-1515.

70. Basiratnia, R., Amini, E., Sharbafchi, M.R., Maracy, M. and Barekatain, M. (2015). Hippocampal volume and hippocampal angle (a more practical marker) in mild cognitive impairment: A case-control magnetic resonance imaging study. Adv Biomed Res 4, 192.

71. Chincarini, A., Sensi, F., Rei, L., Gemme, G., Squarcia, S., Longo, R., Brun, F., Tangaro, S., Bellotti, R., Amoroso, N., Bocchetta, M., Redolfi, A., Bosco, P., Boccardi, M., Frisoni, G.B., Nobili, F. and Alzheimer's Disease Neuroimaging, I. (2016). Integrating longitudinal information in hippocampal volume measurements for the early detection of Alzheimer's disease. Neuroimage 125, 834-847.

72. Yoshiyama, Y., Higuchi, M., Zhang, B., Huang, S.M., Iwata, N., Saido, T.C., Maeda, J., Suhara, T., Trojanowski, J.Q. and Lee, V.M. (2007). Synapse loss and microglial activation precede tangles in a P301S tauopathy mouse model. Neuron 53, 337-351.

73. Guderian, S., Dzieciol, A.M., Gadian, D.G., Jentschke, S., Doeller, C.F., Burgess, N., Mishkin, M. and Vargha-Khadem, F. (2015). Hippocampal Volume Reduction in Humans Predicts Impaired Allocentric Spatial Memory in Virtual-Reality Navigation. J Neurosci 35, 14123-14131.

74. Takahashi, K., Prinz, M., Stagi, M., Chechneva, O. and Neumann, H. (2007). TREM2-transduced myeloid precursors mediate nervous tissue debris clearance and facilitate recovery in an animal model of multiple sclerosis. PLoS Med 4, e124.

75. Bramlett, H.M. and Dietrich, W.D. (2004). Pathophysiology of cerebral ischemia and brain trauma: similarities and differences. J Cereb Blood Flow Metab 24, 133-150.

76. Ost, M., Nylen, K., Csajbok, L., Ohrfelt, A.O., Tullberg, M., Wikkelso, C., Nellgard, P., Rosengren, L., Blennow, K. and Nellgard, B. (2006). Initial CSF total tau correlates with 1-year outcome in patients with traumatic brain injury. Neurology 67, 1600-1604.

77. Kawabori, M., Kacimi, R., Kauppinen, T., Calosing, C., Kim, J.Y., Hsieh, C.L., Nakamura, M.C. and Yenari, M.A. (2015). Triggering Receptor Expressed on Myeloid Cells 2 (TREM2) deficiency attenuates phagocytic activities of microglia and exacerbates ischemic damage in experimental stroke. J Neurosci 35, 3384-3396.

78. Hamm, R.J., White-Gbadebo, D.M., Lyeth, B.G., Jenkins, L.W. and Hayes, R.L. (1992). The effect of age on motor and cognitive deficits after traumatic brain injury in rats. Neurosurgery 31, 1072-1077; discussion 1078.

79. Hamm, R.J., Lyeth, B.G., Jenkins, L.W., O'Dell, D.M. and Pike, B.R. (1993). Selective cognitive impairment following traumatic brain injury in rats. Behav Brain Res 59, 169-173.

80. White-Gbadebo, D. and Hamm, R.J. (1993). Chronic corticosterone treatment potentiates deficits following traumatic brain injury in rats: implications for aging. J Neurotrauma 10, 297-306.

81. El Khoury, J., Toft, M., Hickman, S.E., Means, T.K., Terada, K., Geula, C. and Luster, A.D. (2007). Ccr2 deficiency impairs microglial accumulation and accelerates progression of Alzheimer-like disease. Nat Med 13, 432-438.

FIGURE LEGENDS

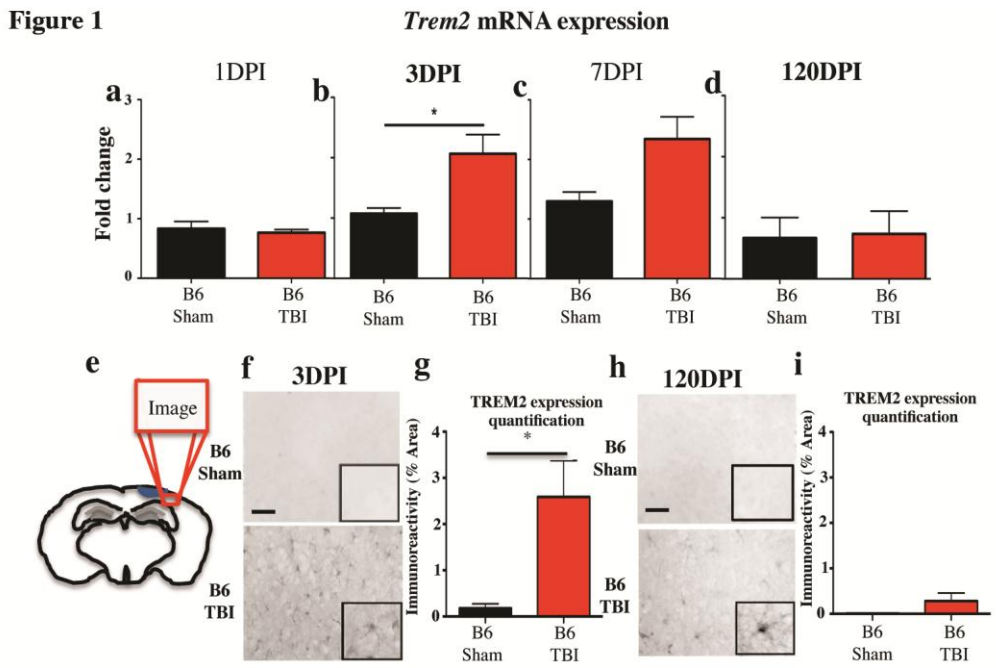

Figure 1: TREM2 expression increases in B6 mice acutely after TBI starting at 3DPI. RT-pcr was completed to look at the time course of *Trem2* expression acutely after TBI in the ipsilateral cortex. **a)** 1DPI **b)** 3DPI **c)** 7DPI **d)** 120DPI**. e)** Diagram showing where images were taken. **f)** DAB staining was performed on 30-micron thick coronal slices of B6 mouse brains after 3DPI. TREM2 expression increased after a mild TBI in wild type b6 mice 3DPI compared to shams. **g)** Percentage of area that showed immunoreactivity was quantified using Image J. **h)** DAB staining was performed after 120 DPI to look at chronic TREM2 expression. **i)** Immunoreactivity was quantified using Image J. Scale bar indicates 20µm and *p<0.05.

Figure 2

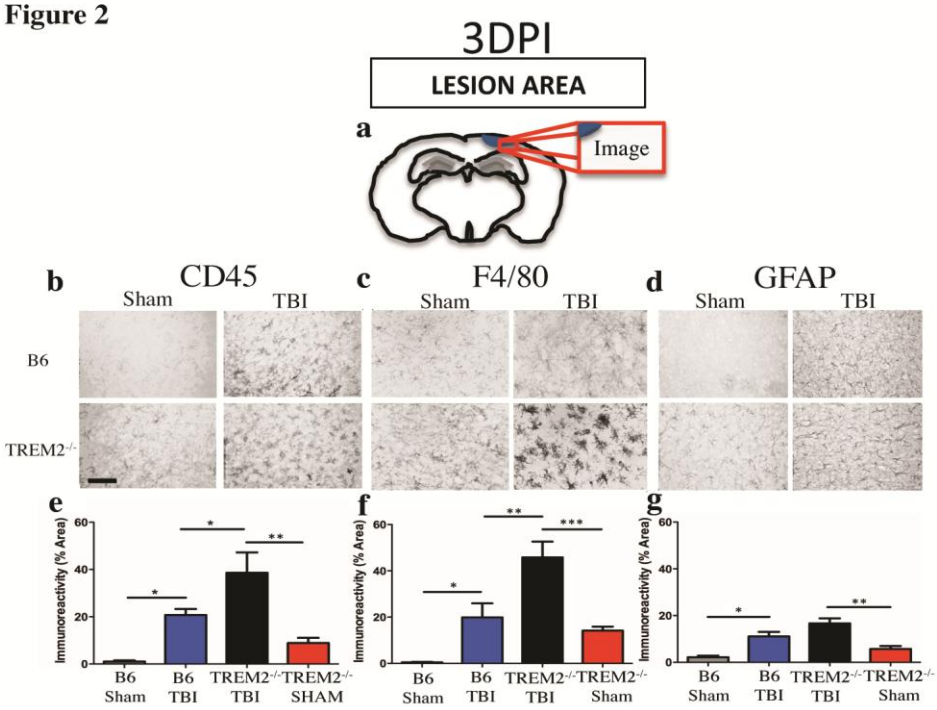

Figure 2: Inflammatory cell activation increases acutely after experimental TBI in TREM2 deficient mice compared to B6 mice near lesion. DAB staining was performed on 30-micron thick coronal slices of B6 and TREM2$^{-/-}$ mouse brains after 3DPI. **a)** A diagram showing where images were taken from. **b)** CD45 staining was performed and images were taken ipsilateral to the lesion. The same was done for **c)** F4/80 **d)** GFAP. Percentage of area that showed immunoreactivity was quantified using Image J for **e)** CD45 **f)** F4/80 and **g)** GFAP.

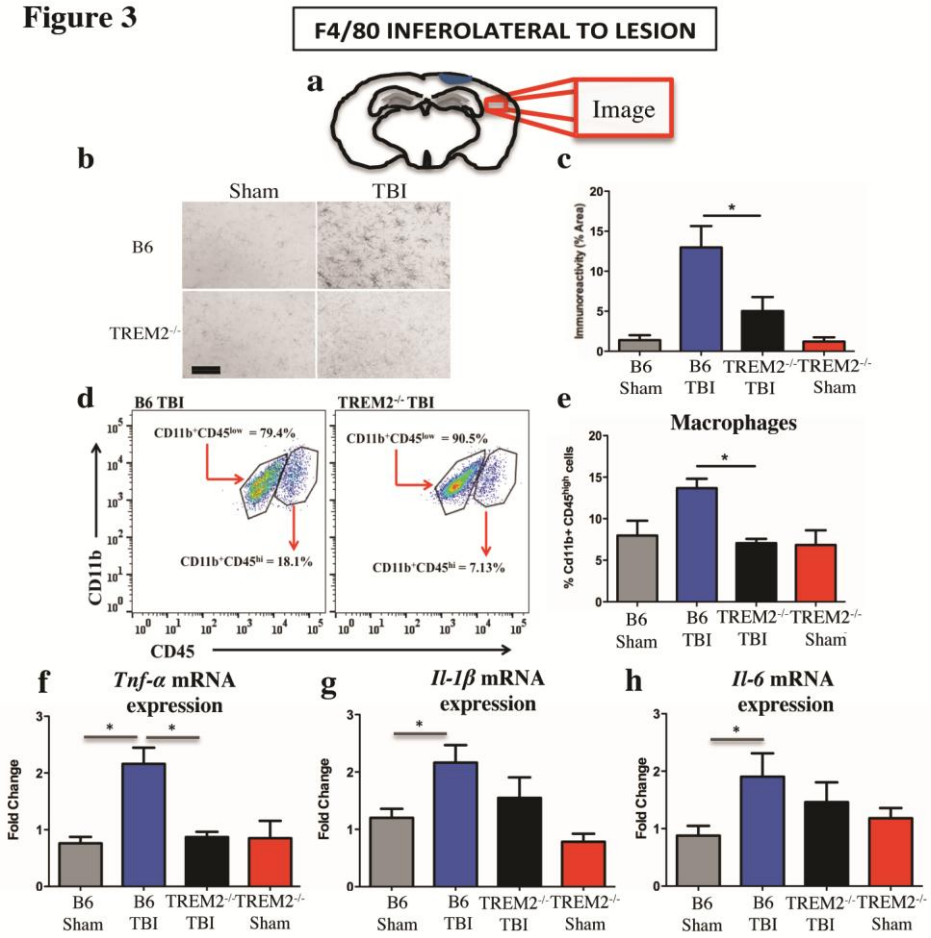

Figure 3: Decrease of inflammation and peripheral macrophage activation throughout the cortex in *Trem2*-/- mice compared to B6 mice after experimental TBI. a) A diagram showing where images were taken for inferiolateral images. **b)** Images were taken in the cortex away from lesion (Inferiorlaterally). **c)** Quantifications of the inferolateral images were taken. **d)** Flow cytometry was performed to look at macrophage percentage. **e)** The percentage of macrophages was quantified. qPCR was performed to examine mRNA production for the

pro-inflammatory cytokines **f)** *tnfα* **g)** *il-1β* **h)** *il6*. Scale bar indicates 20μm and *p<0.05, **p<0.01, ***p<0.001.

Figure 4

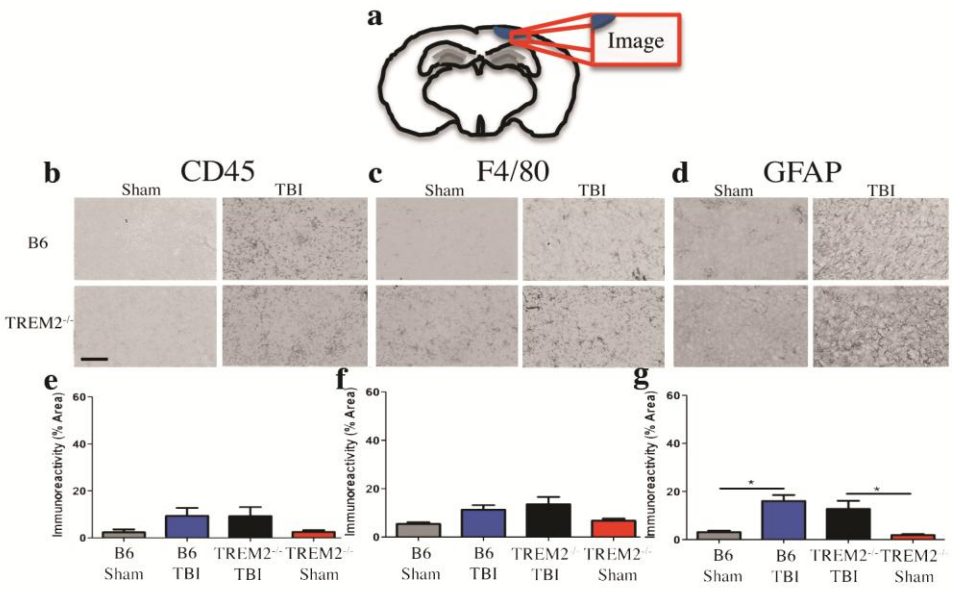

Figure 4: Neuroinflammation resolves in both B6 and TREM2 deficient mice. DAB staining was performed on 30-micron thick coronal slices of B6 and TREM2$^{-/-}$ mouse brains after 120DPI. **a)** Diagram showing where images were taken **b)** CD45 staining was performed and images were taken ipsilateral to the lesion. The same was done for **c)** F4/80 **d)** GFAP. Percentage of area that showed immunoreactivity was quantified using Image J for **e)** CD45 **f)** F4/80 and **g)** GFAP. Scale bar indicates 20μm and $p<0.05$.

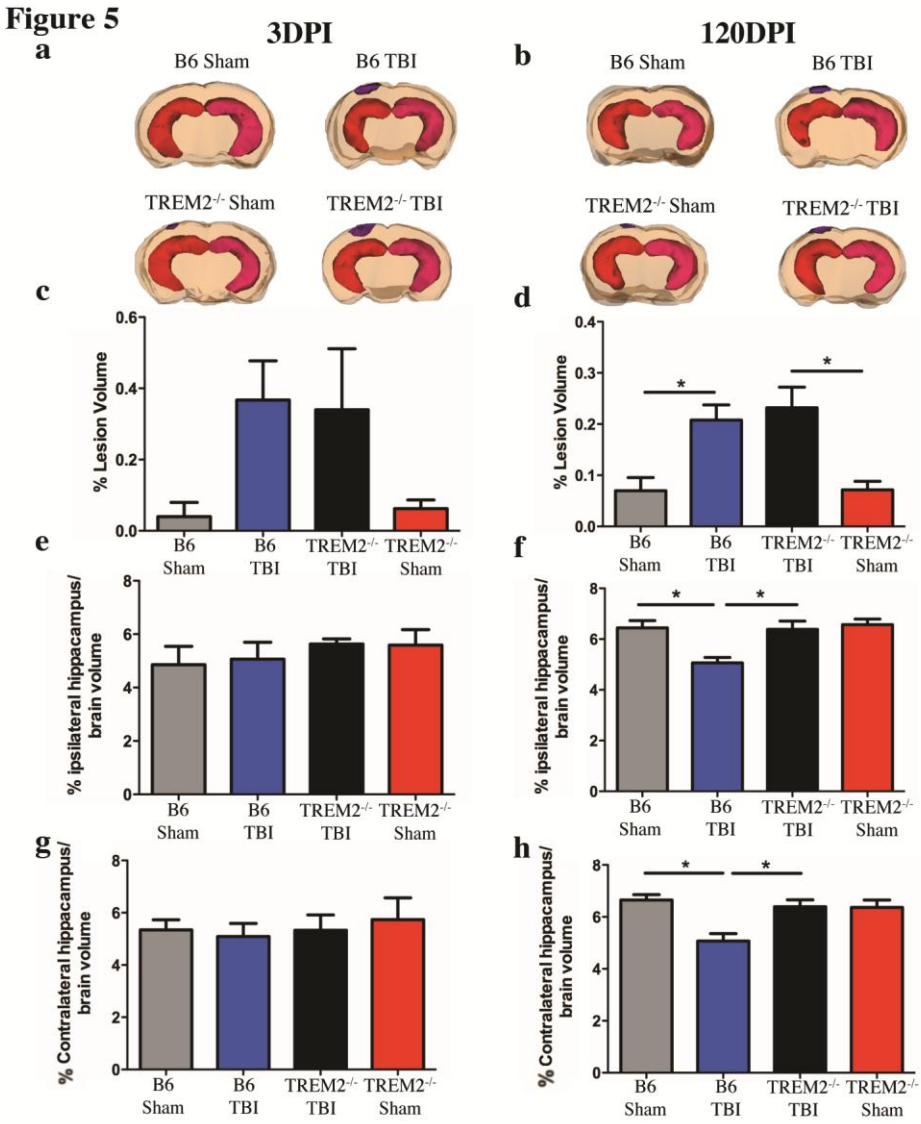

Figure 5: TREM2 deficiency is neuroprotective against TBI induced chronic hippocampal atrophy. Cresyl violet staining was performed on 30- micron thick sections and the program Reconstruct® was used to make 3D reconstructions of brains using 10 serial representative brain sections around the lesion cavity. **a)** representative reconstructions are shown in both b6 and Trem2$^{-/-}$ at 3DPI and **b)** 120DPI. Abnormal cresyl violet staining was used to measure lesion size at **c)** 3DPI and **d)** 120DPI. Percentage of ipsilateral hippocampus over entire brain volume were measured at **e)** 3DPI and **f)** 120DPI. Percentage of contralateral hippocampus over entire brain volume were taken at **g)** 3DPI and **h)** 120DPI. *p<0.05

Journal of Neurotrauma
TREM2 deficiency alters acute macrophage distribution and improves recovery after TBI (doi: 10.1089/neu.2016.4401)

Figure 6

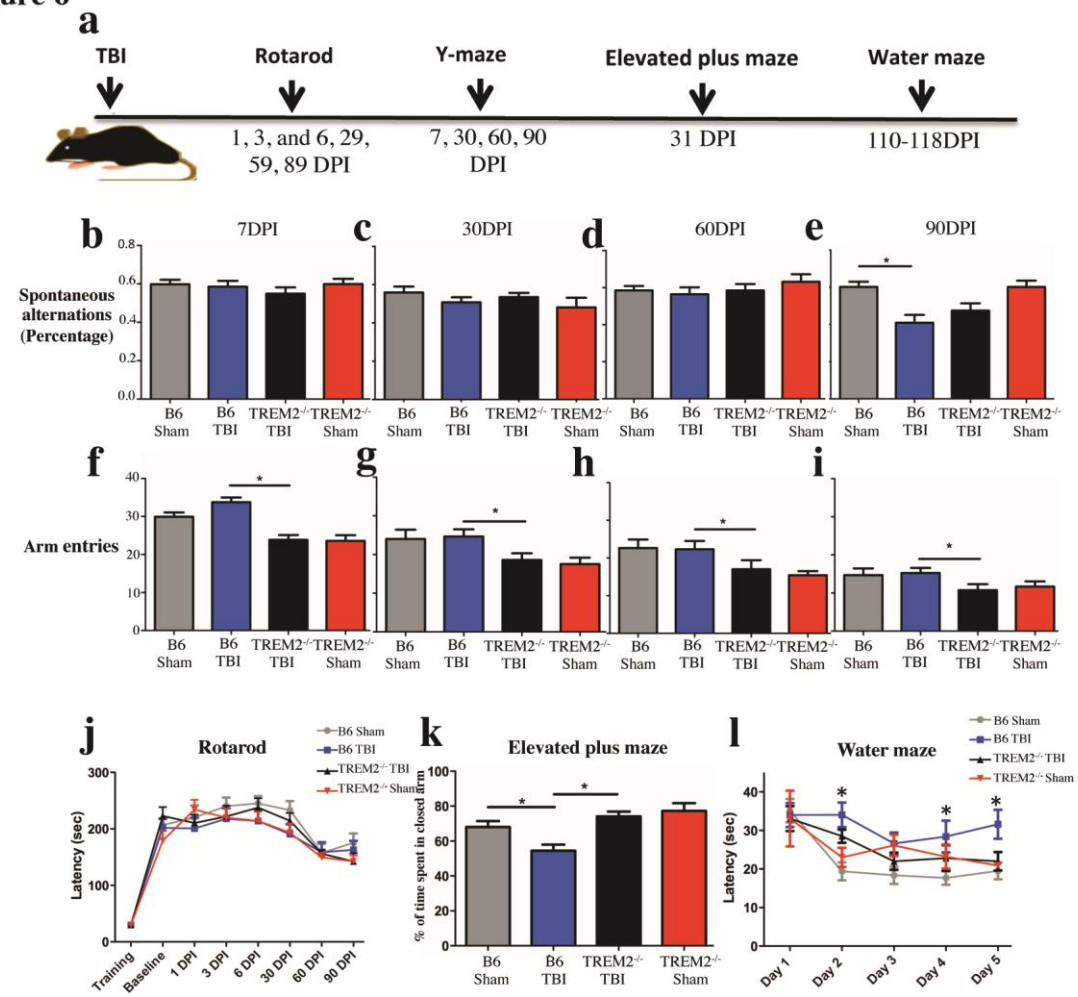

Figure 6: TREM2 deficient mice show less cognitive deficits after TBI and are less exploratory. a) A schemetic is shown to describe behavioral tests done and timing. Y-maze testing was performed at 7,30, 60, 90DPI. Spontaneous alternations at **b)** 7DPI **c)** 30DPI, **d)** 60DPI **e)** 90DPI Differences in arm entries are shown at **f)** 7DPI **g)** 30DPI **h)** 60DPI **i)** 90DPI. **j)** Mice were tested on rotarod 3 trials a day before injury and at 1DPI, 3DPI, 6DPI, 30DPI, 60DPI, 90DPI. The average latency to fall of the rod was measured. **k)** Elevated plus maze was performed for 10 minutes 31 DPI. **l)** mice were trained in visual platform 3 days prior to memory testing. Mice were then subjected to 5 days of memory testing were latency to reach the platform was measured. *p<0.05

Journal of Neurotrauma
TREM2 deficiency alters acute macrophage distribution and improves recovery after TBI (doi: 10.1089/neu.2016.4401)

Figure 7

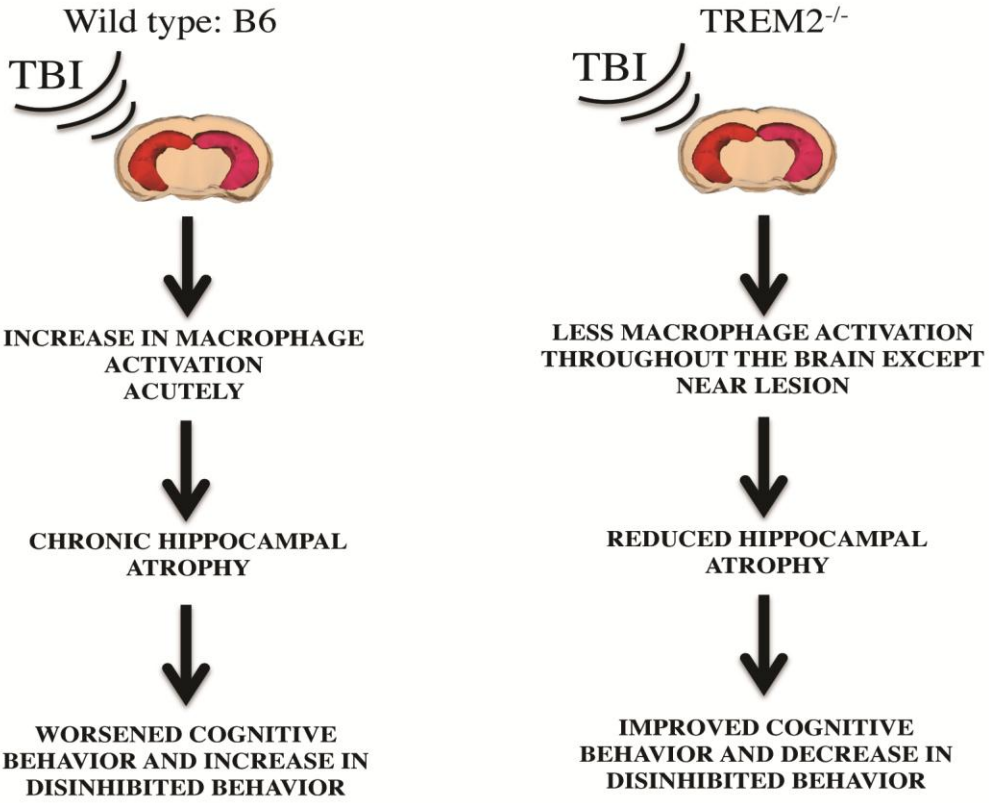

Figure 7: The effect of TREM2 deficiency may be neuroprotective compared to B6 mice after a mild TBI. A schematic summarizing the major findings of this project. In wild type B6 mice there is an increase of macrophage activation and infiltration acutely throughout the brain while in TREM2 deficient mice this increase is only found near the lesion. Chronically, there is an attenuation of TBI- induced hippocampal atrophy and a rescue of TBI -induced behavioral changes.