QUANTIFICATION OF GLIAL ACTIVATION THROUGH IMMUNOHISTOCHEMICAL STAINING OF GFAP FOR 3 AND 7 DAYS POST TBI

By

Vaishnavi Malipeddi

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APPROVED

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Dr. Monica J. Carson
Department of Biomedical Sciences, School of Medicine UCR

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Dr. Richard Cardullo, Howard H Hays Jr. Chair and Faculty Director, University Honors
Interim Vice Provost, Undergraduate Education

Abstract

After a traumatic brain injury (TBI), it has been established that inflammation and swelling occurs as part of an immune response. Microglia, the brain's resident macrophage, are activated first in this event and proliferate near the injury site. This process is called microgliosis. After 3 to 5 days, astrocytes also proliferate near the site of injury in a process called astrogliosis. Acute to the time of injury, brain swelling is most prominent; this is detrimental due to the increase in intracranial pressure. Triggering receptor on myeloid cells-2 (TREM2), has been shown to polarize towards an anti-inflammatory phenotype in the brain; therefore, we hypothesized that an absence of TREM2 would lead to a change in lesion size and edema. However, in our previous study, TREM2 knock-out (TREM2KO) mice showed no change in lesion size or edema but did show a decrease in brain swelling. Because TREM2KOs had less swelling, our hypothesis is that TREM2KO mice have less activated astrocytes. In order to investigate this phenomenon, the level of activation of astrocytes was compared between both TREM2KO and wild type mice at 3 and 7 days post-TBI. The activation of astrocytes was quantified using immunohistochemical labeling of glial fibrillary acid protein (GFAP) which is a marker of astrogliosis. Results show that there is less global astrogliosis 7 days post injury for TREM2KOs; therefore, there is less activation of astrocytes in TREM2KO mice.

Acknowledgments

Special acknowledgements go to my mentor, Dr. Monica Carson, my family, my friends, and the especially helpful counselors of Honors. I could not have completed this project without their support. I truly am thankful for the support that was extended to me as I struggled with this project.

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Introduction

Mild to moderate traumatic brain injury (TBI), also known as a concussion, causes brain inflammation, swelling, and may cause long lasting deficits in brain function. Because of this, it's important to understand how the immune system responds to injuries in the brain. The brain is made up of two basic cell types: neurons and glia. Glia form the majority of cells in the brain and perform many supportive functions. One of these functions is protecting neurons by destroying pathogens and removing detritus. A TBI activates certain subtypes of glia with immunological function such as astrocytes and microglia. In response to TBI, astrocytes play many roles including production of neuroprotective growth factors, increased uptake of neurotoxic compounds and regulation of edema. Triggering receptor on myeloid cells-2 (TREM2) is an immunomodulatory receptor expressed by microglia, the resident tissue macrophage of the brain. TREM2 is known for polarizing towards an anti-inflammatory state, and its importance is demonstrated by how an increased risk of loss of function mutations in TREM2 increase the risk for Alzheimer's disease 3-6 fold. In response to TBI, microglial expression of TREM2 increases 10-fold. We recently and surprisingly discovered that TREM2 knockout (KO) mice display decreased brain swelling in response to TBI without any change in size of TBI lesion. Because TREM2KO mice had less TBI triggered brain swelling, we hypothesized that TREM2 deficiency in microglia is sufficient to alter clinically relevant astrocyte responses to TBI. To test this hypothesis, we compared TBI triggered astrocyte activation at the site of impact and rostral to the site of impact (globally) in TREM2KO and wild type (WT) mice at 3 and 7 days post-TBI.

Materials and Methods

The data for the study was generated through 4 overarching steps. The first was the generation of mice (both WT and TREM2KO) who received a TBI. The second step was the preparation of the brains for immunohistochemical staining. The third step was the usage of immunohistochemical staining to identify activated astrocytes in the brain. The fourth step was to quantify the amount of astrogliosis in the samples.

Generation of Traumatic Brain Injury in Mice

All TBIs were caused by controlled cortical impacts (CCI) delivered by an automated piston-driven impactor tip (3mm) for a highly reproducible impact. The CCIs administered at 5 m/s are considered to be a "mild TBI". They did not suffer significant acute brain damage. The mice were treated with care according to documented Animal Use Protocol which was submitted to the Institutional Animal Care and Use Committee (IACUC).

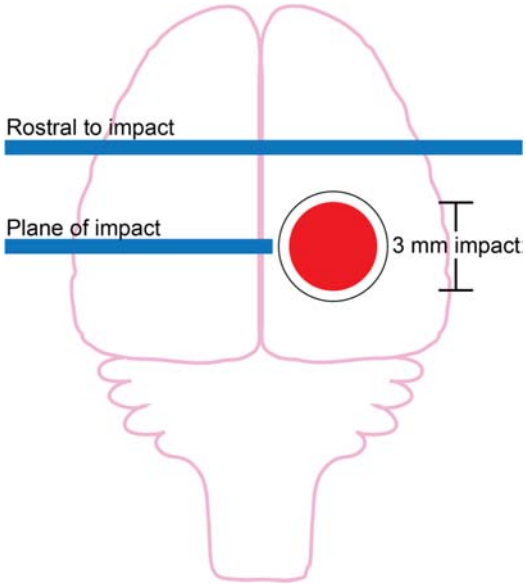

FIGURE 1: THIS FIGURE SHOWS THE PLACEMENT OF THE 3MM IMPACT AS WELL AS THE SLICES PICKED FOR ANALYSIS. ROSTRAL TO IMPACT SHOWS GLOBAL ACTIVATION WHILE THE PLANE OF IMPACT SHOWS REGIONAL ACTIVATION.

Preparation for Immunohistological Analysis

After sacrifice, the animals were perfused with 4% paraformaldehyde (PFA) and the tissues were post-fixed in 30% sucrose in 4% PFA. The brains were then cooled using dry ice and stored in a -80°C freezer. The brain was then taken out of the freezer, and immediately put into a cryostat machine. All the tools used in the cryostat were washed with RNAse-free, water, and ethanol before use to ensure sterility. The brain was then attached to the mount with OCT (optimal cutting temperature) fixative, and mounted onto the

platform. The platform was adjusted to ensure that the angle was correct for the slices we wanted using the help of a mouse brain slice reference book. The brain was then sectioned into 25 μm slices, and these slices were collected into 9 assorted microtubes in cryoprotectant solution and stored in the -80°C freezer

Immunohistochemical Analysis

All the tissues were then stained against glial fibrillary acidic protein (GFAP) which is a marker of astrogliosis. The process for staining the tissues takes two days with the 1st day for binding the primary antibody to GFAP, and the second day for the secondary antibody to bind to the primary antibody.

The tissue samples were thawed out and put into a well plate with a solution of 1x PBS (phosphate-buffered saline). Samples were washed 3 times for 7 minutes each. A blocking buffer solution was made during this step, made of 90% 0.2% Triton in 1x PBS and 10% normal goat serum (S-1000). This blocking buffer was split into 2 aliquots, one to wash the samples with again, and a second to contain the primary antibody dilution. A 1:100 dilution of polyclonal rabbit anti-GFAP antibodies by Dako was added to the 2nd blocking buffer solution. After the samples finished their 1x PBS wash, they were transferred to a blocking buffer wash where they stayed for 1 hour. After this step, they were transferred to wells with primary antibodies in blocking buffer and left in a 4°C fridge overnight on a slow speed shaker.

The procedure for the second day is the same until the 2nd antibody blocking solution. A secondary antibody blocking solution was made with blocking buffer and biotinylated anti-rabbit IgG made by Vector Labs (#BA-100) at a 1:500 dilution. The samples stay in the secondary antibody blocking buffer for an hour and were transferred to a 3-step 7-minute each PBS wash.

The ABC reagent was prepared during this time, which uses 10 ml 1xPBS, 2 drops of reagent A and 2 drops of reagent B for 5 wells(samples). This was prepared using the Vector Labs ABC kit (#PK-4000). After the PBS wash, the samples were transferred to wells with ABC solution and left on the shaker for 1 hour. During this time period, a DAB solution [notable carcinogen, take necessary precautions] was made and distributed into a well plate. The solution consists of 10 ml 1xPBS, 120 µl DAB, and 0.5 µl of 30% hydrogen peroxide. After the ABC solution, the samples were washed in a 3-step 7-minute PBS wash again. Using a timer, each solution was the transferred into DAB solution for exactly 5 minute and 30 seconds. During this time period, the well plates were periodically swirled to ensure consistent exposure to DAB. After 5 minutes and 30 seconds, the samples were immediately transferred to a 2-step 7-minute PBS wash. All materials in contact with DAB were transferred to a tub with bleach solution to be neutralized.

The samples were then mounted onto glass slides. After being mounted onto slides, all the slides were immersed into a solution of hematoxylin for 5 minutes in order to co-stain the samples. After drying, the samples went through a series of dehydration baths which included: 70% ethanol, 95% ethanol, 100% ethanol, 1:1 chloroform:ethanol, 100% ethanol, and toluene solution. Each sample is dipped in each step but stays in toluene solution for 1 hour. Afterwards, coverslips were attached to the slides and stored for a later date. The slides were then imaged using a light microscope, and then three representative sections from each plane were imaged for each animal, respectively.

Quantification of Astrocyte Activation

Astrocyte activation (astrogliosis) was measured by quantifying GFAP immunoreactivity. Activated astrocytes were marked using pixel intensity and particle size thresholds. In order to remove bias from our histological analysis, we developed an automated quantification protocol

(macro) using the NIH software, ImageJ (Appendix 1). A 0.5376 mm2 area was cropped from each 10x objective cortical image, and analyzed with the macros for GFAP (Figure 2). The data was analyzed using a one-way ANOVA with a Tukey's post test, where: * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

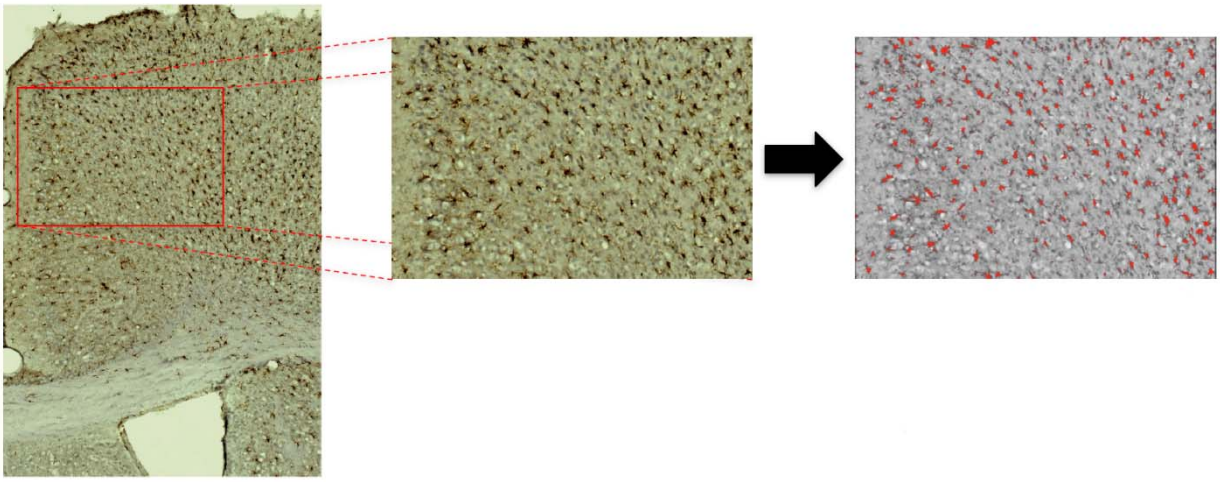

FIGURE 2: COMPARABLE CORTICAL AREAS WERE DEFINED AND CROPPED FROM EACH IMAGE (2ND IMAGE). A REPRESENTATIVE IMAGE OF THE IMAGEJ QUANTIFICATION OVERLAY DEMONSTRATES THE ACCURACY IN THRESHOLDING IMAGES BASED ON PIXEL INTENSITY AND PARTICLE SIZE (3RD IMAGE).

Results

3 days post-TBI

Site of Impact (Figure 3)

WT and TREM2KO mice have the same level of activation in the impacted hemisphere. The impacted hemisphere for both the WT and TREM2KO is significantly more activated than non-impacted contralateral. TREM2KO

n = 3; WT n = 3; $p < 0.0001$

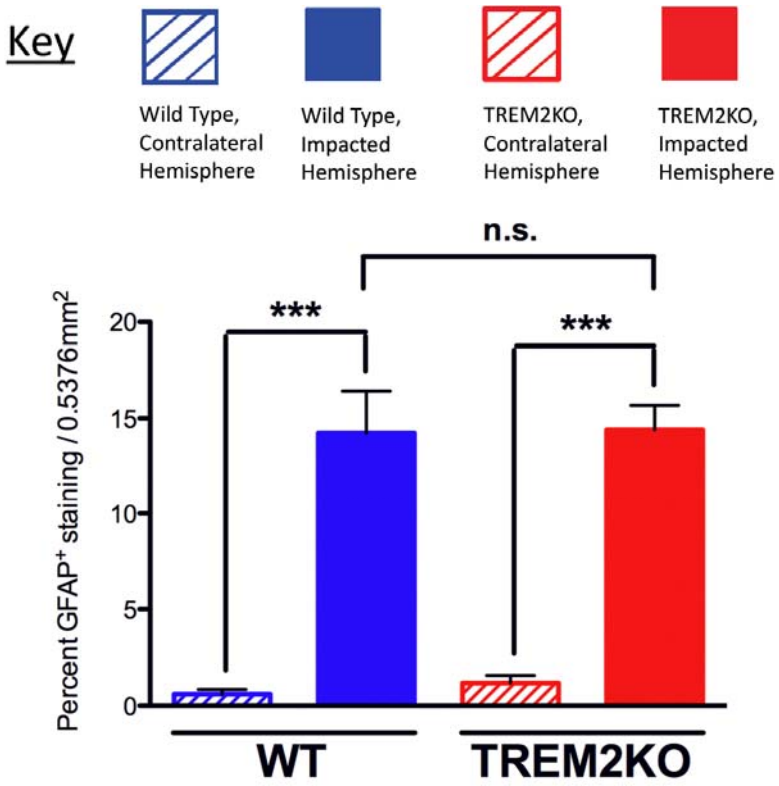

FIGURE 3: BY 3 DAYS POST-TBI, ROBUST ASTROGLIOSIS OCCURS AT IMPACT SITE BUT NOT CONTRALATERAL TO INJURY IN BOTH WT AND TREM2KO MICE

Rostral to Impact (Figure 4)

There are non-significant differences between both WT and TREM2KO; activation is the same across the board. Also, activation is reduced compared to the plane of impact.

TREM2KO n = 3; WT n = 3; $p = 0.2296$

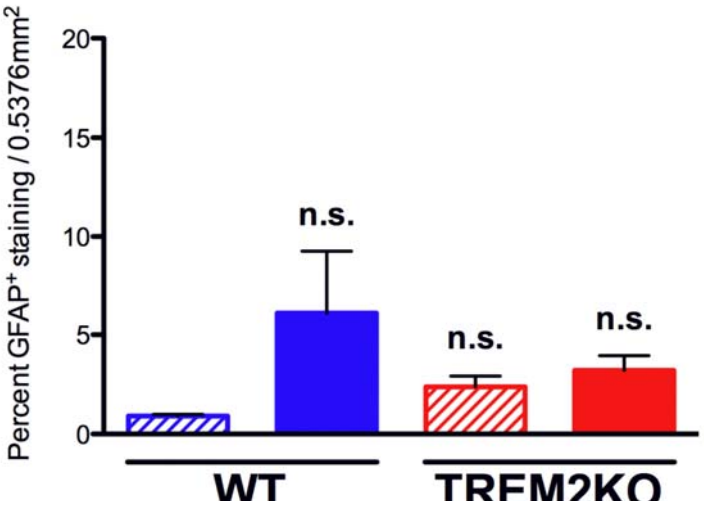

FIGURE 4: BY 3 DAYS POST TBI, NO SIGNIFICANT DIFFERENCE IN ASTROGLIOSIS BETWEEN IMPACTED AND CONTRALATERAL SIDE IN BOTH WT AND TREM2KO MICE

7 days post-TBI

Site of Impact

WT and TREM2KO mice have the same level of activation, but the difference between TREM2KO impacted and contralateral hemispheres is non-significant while the difference between the WT impacted and contralateral is significant.

TREM2KO n = 4;

WT n= 3; p = 0.0062

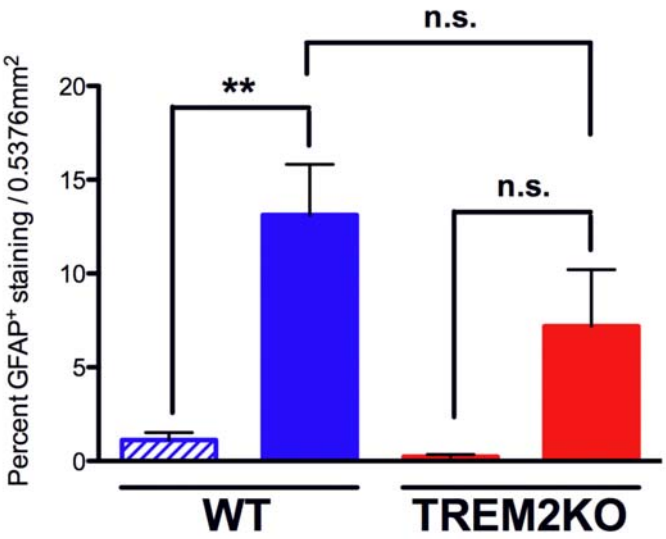

FIGURE 5: BY DAY 7, WT BUT NOT TREM2KO MICE DISPLAY SIGNIFICANTLY HIGHER LEVELS OF ASTROGLIOSIS AT IMPACTED BUT NOT CONTRALATERAL SITE

Rostral to Impact

The WT impacted hemisphere is significantly more activated than TREM2KO impacted side globally. TREM2KO has no significant differences between impacted and contralateral, so no significant changes in activation were detected globally.

TREM2KO n = 4; WT n = 3; p = 0.0004

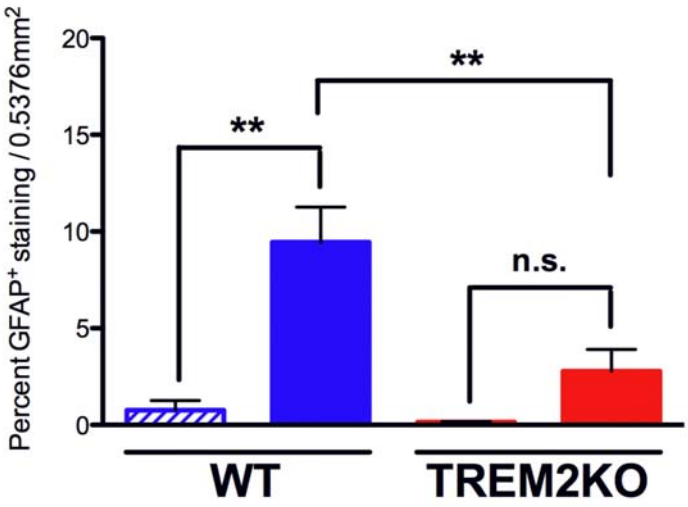

FIGURE 6: BY 7 DAYS POST-TBI, WT BUT NOT TREM2KO DISPLAY HIGHER ASTROGLIOSIS ROSTRAL TO IMPACT SITE AS COMPARED TO EQUIVALENT CONTRALATERAL AREA. WT ASTROGLIOSIS IS ALSO HIGHER THAN THAT IN TREM2KO MICE.

Discussion

Early after TBI, astrogliosis in wild-type and TREM2KO is equivalent both acute to the site of the injury (high level of gliosis as expected) and globally in the brain (low level of gliosis as expected). However, by 7 days post-TBI, astrogliosis has extended to other regions of the brain in wild-type but not TREM2KO mice; in addition to this, astrogliosis appears to be in the process of decreasing at the site of impact in TREM2KO mice. Within the brain, TREM2 is only expressed in microglia and is not expressed in astrocytes. Therefore, the fact that TREM2 deficiency altered the level of astrogliosis 7 days post-TBI shows that TREM2 plays an important role in microglia-astrocyte communication following brain injury. This has significant implications, as previous experiments have shown that TREM2 expression increases 10-fold in the event of a TBI. Although TREM2 is well-known as an anti-inflammatory immunomodulator and that its loss increases risk of Alzheimers. However, lack of TREM2 could be beneficial in the short term by preventing unnecessary immune activation globally in the brain, as well as decreased intracranial pressure due to swelling.

Appendix

Appendix 1: Image J Quantification Process

- Open the Image J application. Navigate to the below menu options in the order written:
- Edit → Options → Conversions
  - Scale when converting → switch to OK
  - No weighted RGB conversions (OK)
- Image → Type → HSB Stock (Hue, Saturation, Brightness)
  - Only 8 bit conversions
  - Choose "saturation" image (middle image)
- Image → Duplicate
- Select Copy of image
  - Image → Adjust → Threshold →
    - Set threshold lower limit: 165
    - Click apply (will get a binary image)
- Select binary image → duplicate
  - Analyze → Set Measurements → OK
    - Select desired measurements:
      - Area, StdDev, Min, Max, Grey values, Shape description, Integrated Density, Mean Gray Value, Modal Gray Value, Perimeter
  - **must** select "limit to threshold" and "display label"
  - **must** redirect to original grey scale image (If not selected, it will quantify the binary image)
- Select new copy

- Analyze → Analyze particles
  - Size: 0.0004 – 0.02 (do NOT click fixed units)
  - Check Summarize
  - Check display results
  - **Must** show Overlay Masks

- All of these commands can be compiled into a macro using imageJ's macro tool. We suggest using a macro as there will be a lot of images to analyze using this method.