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Real-time Noninvasive Bioluminescence, Ultrasound and Photoacoustic Imaging in NFκB-RE-Luc Transgenic Mice Reveal Glia Maturation Factor-Mediated Immediate and Sustained Spatio-Temporal Activation of NFκB Signaling Post Traumatic Brain Injury in a Gender Specific Manner

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Abstract

Neurotrauma especially traumatic brain injury (TBI) is the leading cause of death and disability worldwide. To improve upon the early diagnosis and develop precision targeted therapies for traumatic brain injury, it is critical to understand the underlying molecular mechanisms and signaling pathways. The transcription factor, nuclear factor kappa B (NFκB) which is ubiquitously expressed plays a crucial role in the normal cell survival, proliferation, differentiation, function as well as in disease states like neuroinflammation and neurodegeneration. Here, we hypothesized that real-time noninvasive bioluminescence molecular imaging allows rapid and precise monitoring of TBI induced immediate and rapid spatio-temporal activation of NFκB

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[i.]**Compliance with Ethical Requirements:** All the experimental procedures performed and described in the studies involving laboratory animals were in accordance with the ethical standards and all applicable institutional and the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals for the care and use of animals were satrictly followed.

[iii.]**Conflict of Interest:** All the authors declare no conflict of interest.

[iv.]**Ethical Approval:** All animal experiments were performed post IACUC approval according to all of the applicable institutional and National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.

[v.]**Informed Consent:** No human subjects were involved in the current study.

signaling pathway in response to Glia maturation factor (GMF) upregulation which in turn leads to neuroinflammation and neurodegeneration post TBI. To test and validate our hypothesis and to gain novel mechanistic insights, we subjected NFκB-RE-Luc transgenic male and female mice to TBI and performed real-time noninvasive bioluminescence imaging (BLI) as well as photoacoustic and ultrasound imaging (PAI). Our BLI data revealed that TBI leads to an immediate and sustained activation of NFκB signaling. Further, our BLI data suggest that especially in male NFκB-RE-Luc transgenic mice subjected to TBI, in addition to brain there is widespread activation of NFκB signaling in multiple organs. However, in the case of the female NFκB-RE-Luc transgenic mice TBI induces a very specific and localized activation of NFκB signaling in the brain. Further, our microRNA data suggest that TBI induces significant upregulation of mir-9-5p, mir-21a-5p, mir-34a-5p, mir-16-3p as well as mir-155-5p within 24 hours and these microRNAs can be successfully used TBI-specific biomarkers. To the best of our knowledge, this is one of the first and unique study of its kind to report immediate and sustained activation of NFκB signaling post TBI in a gender-specific manner by utilizing real-time non invasive BLI and PAI in NFκB-RE-Luc transgenic mice. Our study will prove immensely beneficial to gain novel mechanistic insights underlying TBI, unravel novel therapeutic targets as well as enable us to monitor in real-time the response to TBI-specific gene and stem cell-based precision medicine.

Keywords

Bioluminescence imaging; NFκB; photoacoustic imaging; traumatic brain injury; ultrasound; microRNA

INTRODUCTION

Neurotrauma, especially Traumatic brain injury (TBI) is the leading cause of morbidity, disability as well as mortality worldwide with huge economic burden (Maas et al. 2008; Report to Congress on Traumatic Brain Injury in the United States: Understanding the Public Health Problem among Current and Former Military Personnel 2013; Surveillance Report of Traumatic Brain Injury-related Emergency Department Visits, Hospitalizations, and Deaths 2014; DoD Worldwide Numbers for TBI 2018; Langlois et al. 2006; Nguyen et al. 2016; Roozenbeek et al. 2013). There are a wide variety of causes including combat related, terrorist activities, open field blasts, domestic violence, sports related as well as automobile accidents that induce various degrees of TBI ranging from very subtle mild TBI (mTBI) to very severe fractured and open skull TBI. Currently one of the major challenges is to accurately diagnose mTBI, the most common type of TBI with approximately 2.5 million emergency department visits per year in the US alone (Taylor et al. 2017; Silverberg et al. 2019).

Timely diagnosis of mild TBI can be very crucial to initiate appropriate treatment as soon as possible and prevent or delay any long-term and a wide spectrum of neurological consequences and serious complications including post traumatic stress disorder (Vasterling et al. 2018), sleep-wake disturbances (Ouellet et al. 2015), cognitive impairments (de Freitas Cardoso et al. 2019), dementia (Smith et al. 2013), chronic traumatic encephalopathy (Lucke-Wold et al. 2014), post traumatic epilepsy (Mukherjee et al. 2020) and increased

risk for Alzheimer's disease (AD) (Ramos-Cejudo et al. 2018; Lucke-Wold 2018) and Parkinson's disease (PD) (White et al. 2020). It is in this regard our current research efforts are focused upon developing novel real-time noninvasive imaging modalities including photoacoustic and ultrasound imaging as well as bioluminescence imaging (BLI) for the rapid and accurate diagnosis of invisible mTBI. Multiple earlier studies have demonstrated that TBI leads to an activation of NFκB signaling pathway (Nonaka et al. 1999; Hu et al. 2014; Lipponen et al. 2016; Jassam et al. 2017). However, despite significant progress being made, the spatio-temporal dimension as well as precise molecular and cellular mechanisms underlying NFκB activation post TBI still remain unclear and unexplored. Here, we reasoned and hypothesized that mTBI induced GMF overexpression causes an immediate and rapid localized activation and subsequent wave like progression of NFκB signaling *in vivo* thereby leading to progressive neuroinflammation as well as neurodegeneration. The underlying rationale is that glia maturation factor (GMF) a proinflammatory factor discovered and actively researched upon by our research group has been shown to activate NFκB signaling pathway which is critically involved in neuroinflammation as well as neurodegeneration (Zaheer et al. 2002; Zaheer et al. 2001; Zaheer et al. 2008; Zaheer et al. 2011a; Zaheer et al. 2011b; Zaheer et al. 2012; Kempuraj et al. 2016; Thangavel et al. 2013; Thangavel et al. 2012; Ahmed et al. 2017; Lim et al. 1989; Lim et al. 1981; Lim et al. 1977; Raikwar et al. 2019; Ahmed et al. 2020b). We have very recently demonstrated that neuroinflammation mediated by GMF exacerbates neuronal injury in an *in vitro* model of TBI (Ahmed et al. 2020a). Most recently, utilizing GMF knock out mice we have also successfully demonstrated that lack of GMF protects these mice from axonal injury and motor behavioral impairments post TBI (Selvakumar et al. 2020). These crucial findings lay a solid foundation for our current as well as future studies to decipher novel molecular and cellular mechanisms underlying TBI.

To decipher whether or not mTBI induces NFκB activation we capitalized on a well-established and commercially available murine model BALB/c-Tg(Rela-Luc)31Xen (Model #10499, Taconic Biosciences, Rensselaer, NY). In this particular murine model originally developed by Caliper Life Sciences, a modified luciferase transgene reporter is placed downstream of 6 NFκB-responsive elements (RE) from the CMVα (immediate early promoter) and a basal SV40 promoter. This particular model has been extensively used to study NFκB activation in a wide variety of applications including cancer research, transplantation and immunomodulation (Carlsen et al. 2002; Dohlen et al. 2005; Morlacchi et al. 2011; Roth et al. 2006; Partridge et al. 2007). There are several excellent murine TBI models including weight drop, lateral fluid percussion and controlled cortical impact model to study TBI dynamics as well as molecular and cellular mechanisms underlying TBI pathogenesis (Marklund et al. 2006; Marklund and Hillered 2011; Xiong et al. 2013). Here, we utilized the murine weight drop TBI model to monitor NFκB activation by real-time noninvasive BLI due to simplicity and the fact that this type of TBI is one of the most commonly observed in human patients. We observed that post TBI, there is an immediate and sustained activation of NFκB signaling in both male as well as female mice. In comparison to the female mice which displayed a very localized brain-specific NFκB activation, we observed brain-specific as well as global NFκB activation in the male mice. We also monitored TBI-induced brain injury by means of noninvasive real-time

photoacoustic and ultrasound imaging. Our data suggest that TBI-induces impaired blood flow in the brain region directly impacted by TBI.

Furthermore, we performed microRNA expression profiling to validate our results. Our microRNA data suggest that TBI induces significant upregulation of mir-9, mir-21, mir-34a-5p, mir-16-3p as well as mir-155p within 24 hours. However, the levels of mir-9, mir-21 and mir-155-5p are rapidly downregulated in comparison to mir-34a-5p as well as mir-16-3p. These original findings support our novel hypothesis that TBI-induced GMF overexpression causes an immediate and sustained activation of NFκB signaling cascade which plays a crucial role in neuroinflammation as well as neurodegeneration. Based upon our novel findings, we believe that GMF and NFκB are attractive therapeutic targets for the development of novel TBI-patient-specific precision targeted gene and cell-based regenerative therapies. We believe that our current studies will be pivotal to attain novel mechanistic insights underlying TBI, unravel novel therapeutic targets as well as enable us to monitor in real-time the response to TBI-specific gene and stem cell-based precision medicine.

MATERIALS AND METHODS

Traumatic Brain Injury Modeling in NFκB-RE-Luc Mice

The NFκB-RE-Luc mice [BALB/c-Tg(Rela-Luc)31Xen (Taconic Catalog #10499-M and 10499-F] and C57BL/6J mice at 8 weeks of age were used for the current study. All animal experiments in NFκB-RE-Luc as well as C57BL/6J mice were performed post IACUC approval according to the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. Eight weeks old NFκB-RE-Luc C57BL/6 mice were subjected to anesthesia by isoflurane inhalation with 2% isoflurane in 100% oxygen at a flow rate of 1 L/min and subsequently maintained in the IVIS system with 1.5% isoflurane with 100% oxygen prior to inducing any TBI or performing any real-time noninvasive bioluminescence (BLI) or PAI. The hair on their scalp was shaved and Betadine was applied on to the scalp and eye ointment was applied to prevent the eyes from drying. In our well-established weight drop TBI model, a fixed weight is released for a free fall according to a well-defined path (Selvakumar et al. 2020). The height and the weight determine the severity of the injury which can range from a mild level of injury to severe brain injury. In the current study, the anesthetized mice were subjected to mild TBI. The weight-drop TBI device as described previously consists of a hollow cylindrical tube (60.96 cm long with an inner diameter of 1.27 mm). The mice were positioned in such a way that they receive the weight drop-induced TBI from the frontal lateral direction either on the top, left or right side of the mouse head equidistant between the eye and the ears. The anesthetized mice were carefully placed on a flat spongy surface that allowed the movement of the head parallel to the injury plane at the time of the weight drop thereby mimicking the most common type of head injury. A metal sphere weighing 48g was dropped from the top of the cylindrical tube to directly strike the head. Immediately post TBI, the mice were carefully examined and subsequently returned back to their original cage for recovery and observation. The mice in the control group received similar treatment except for the weight drop. The mice were injected subcutaneously with Buprenorphine (0.05mg/kg) in 0.5 ml saline solution

and were allowed to return to their home cage once they exhibited normal walking and grooming behavior. We carefully monitored the cognitive and motor function prior to and immediately post TBI. This TBI model very closely mimics the closed head injury with accompanying concussion and contusion, thereby representing one of the most common type of TBI observed in humans. An added advantage is the fact that It is relatively inexpensive, very easy to perform and is capable of producing graded diffused axonal injury.

Real-Time Noninvasive Bioluminescence Imaging (BLI)

All animals were subjected to BLI prior to TBI as well as within 30 minutes post TBI and subsequently at 24 hours, 48 hours and 72 hours post TBI. Briefly, the mice were anesthetized using 2% isoflurane in 100% oxygen at a flow rate of 2L/min and were injected intraperitoneally with D-Luciferin solution in 1XPBS (150mg/kg body weight) and subjected to real-time noninvasive BLI using the IVIS Spectrum (Perkin Elmer, Waltham, MA). During BLI acquisition the mice were maintained in anesthetic state using a constant delivery of 2% isoflurane-oxygen mixture at a rate of 1L/min by means of IVIS anesthesia manifold. The image acquisition parameters were kept constant throughout the study. The acquired BLI images were analyzed using the Living Image Version 4.0 Software (PerkinElmer, Waltham, MA). In order to normalize our BLI data, we applied background correction using the control mice to specific regions of interest to cover the area over the brain and other internal organs. The resulting luminescence signal intensity was quantified as photons/sec/cm^2/steradian. Following the completion of BLI and PAI, all animals were euthanized after 72 hours.

Real-Time Noninvasive Very High-Resolution Photoacoustic-Ultrasound Imaging (PAI)

High-resolution real-time noninvasive PAI was performed 24 hours and 72 hours post TBI using the Vevo LAZR photoacoustic imaging system (Fujifilm, Visualsonics Inc, Canada). The detailed protocols are described in the Vevo LAZR photoacoustic imaging system manual as well as were adapted and modified from another study describing ultrasound and photoacoustic rat deep brain imaging (Giustetto et al. 2015). The mice were anesthetized using 2.5% isoflurane with oxygen level set to 0.5-1L/minute for anesthesia induction and secured to a heating pad to maintain thermoregulation. Paw pinch test was used to confirm the level of anesthesia. Ultrasound gel was copiously applied to provide a clear coupling interface between the imaging area and the ultrasound probe. The PAI system utilizes a pulsed laser light at wavelengths from 680-970 nm to generate acoustic waves that are detected by a linear array transducer. The micro-ultrasound was synchronized with the laser and photoacoustic signals were acquired with a fluence <20 mJ/cm^2 beam formed in the imaging software and were displayed at 5Hz. The LZ400 (center operating frequency of 18-38 MHz, axial resolution 50 μM) probe was used throughout to acquire all the PAI images. The PAI images were acquired in 3D as well as single mode using light at 680, 800 and 850 nm and using the Oxyhemo mode, which enabled us to collect data at 750 and 850 nm and create and display a parametric map of estimated oxygen saturation or total hemoglobin at a rate of 1Hz. The HemoMeaZure tool allowed the measurement of the total hemoglobin content (HbT) within a particular anatomical target area using the PA-Mode. Further, the oxygen saturation (sO_2) of hemoglobin within defined anatomical targets down

to the level of the microenvironment was measured using the OxyZated tool. The acquired images were analyzed using Vevo LAB 3.1.1 software.

MicroRNA Isolation and Analysis

Prior to euthanizing the mice, we collected whole blood from the abdominal aorta of the anesthetized mice. The blood was placed in 1.5 ml Eppendorf tubes and kept on ice for 2 hours to allow clotting while preserving the microRNAs. Serum separation was performed by centrifugation at 3500 rpm for 15 minutes. The collected serum was recentrifuged at 3500 rpm to get rid of any traces of RBCs. The clear serum samples were stored at −80°C until processing for microRNA isolation. The frozen serum samples were thawed on ice and subjected to total RNA isolation and purification using the Plasma/Serum RNA Purification Mini Kit (Norgen Biotek Corp, Thorold, ON, Canada) as per the manufacturer's instructions (Hoey et al. 2019). Total RNA was quantified using Nanodrop 2000 and approximately 10 ng RNA was reverse transcribed and subjected to cDNA synthesis using TaqMan Advanced miRNA cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, MA) as per the instructions provided by the manufacturer. Subsequently, total cDNA was utilized to perform real-time quantitative PCR in MicroAmp Fast Optical 96-Well Reaction Plates (ThermoFisher Scientific, Waltham, MA) using specific TaqMan Advanced miRNA Assays and TaqMan Fast Advanced Master Mix as per the recommendations and guidelines provided by the manufacturer (ThermoFisher Scientific, Waltham, MA). The relative expression of mir-9-5p, mir-16-3p, mir-21a-5p, mir-34a-5p and mir-155-5p was analyzed in triplicates using serum samples collected at different time points. For all the microRNA assays, mir-126-3p was used as the normalizer. All statistical analyses were performed using GraphPad InStat3 and the graphs were generated using GraphPad Prism 8.0 software.

Transcardiac Perfusion and Brain Tissue Processing

Following the completion of the BLI and PAI studies, the mice were anesthetized, transcardially perfused with PBS followed by 4% paraformaldehyde solution and their brains were collected and preserved in 4% paraformaldehyde for 24 hours. Subsequently, these brain specimens were transferred into 30% sucrose solution and stored at 4°C for 48 hours. For the long-term storage, the brains were embedded in OCT compound, allowed to freeze at −20°C for 4-6 hours and then transferred in to −80°C freezer. The cryoprotected brains were allowed to stay in the cryostat for about 2 hours at −22°C prior to sectioning. The whole brain was sectioned with a thickness of 25 μm sections in PBS and were further transferred into the cryoprotectant solution (FD Neurotechnologies, Columbia, MD) and stored at −20°C until necessary for further processing for immunofluorescence analysis. The individual sections were subjected to co-immunostaining with antibodies against GMF and either NeuN, Iba1 or GFAP.

Immunofluorescence Staining and Confocal Microscopy

Immunofluorescence staining was performed as previously described (Ahmed et al. 2017; Raikwar et al. 2019). Briefly, free-floating mouse brain sections were placed in 24 well plates and washed with PBS. Sections were subjected to antigen retrieval using sodium citrate buffer containing 0.1% Triton X-100 and blocked with 3% BSA in PBS/0.1% Triton

X-100 for 1h at room temperature. The primary antibodies anti-GMF (1:250; Proteintech and Santa Cruz Biotechnology), anti-GFAP (1:250; PA5-85109, Invitrogen) anti-IBA1 (1:250, WAKO Chemicals, USA) and anti-NeuN (1:250, EMD Millipore) were diluted in blocking buffer solution. Primary antibody solutions (500μl) were added to each well and the sections were incubated at 4°C overnight. The next day, primary antibodies were removed and sections were washed with 1X PBS for 3 times at 5 minutes intervals. The corresponding fluorescent secondary antibodies (Alexa Fluor-488 (green) or Alexa Fluor -568 (red) were diluted in blocking solution (1:500) and 500 ul of secondary antibodies were incubated with sections for 1h at room temperature. The secondary antibodies were removed and sections were washed with 1X PBS three times at 5 minutes intervals. After the final wash with 1X PBS, sections were mounted on Fisher Super Frost Plus slides and kept for air drying to remove excessive moisture for 1-2 minutes. The sections were treated with Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (H-200; Vector Laboratories). The slides were subjected to confocal microscopy using Leica TCP SP8 confocal microscope equipped with a 405-nm diode laser and a tunable supercontinuum white light laser. We used the 405/420-480nm (DAPI), 495/505-550 nm (Alexa Fluor 488) and 570/580-630 nm (Alexa Fluor 568) excitation/emission band-pass wavelengths to acquire confocal images which were subsequently processed using the Leica Application Suite X software.

Statistical Analysis

To achieve reproducibility as well as statistical significance, all the TBI experiments were performed in triplicates. The counting of GMF, NeuN, IBA-1 and GFAP positive cells was performed using images captured at 63X magnification. The results were analyzed using GraphPad InStat 3 software and are represented as arithmetic mean±SD. Further, the comparison between different groups was performed by one-way analysis of variance (ANOVA) and post hoc Tukey-Kramer multiple comparison analysis was used to determine statistical significance. The p value $p<0.05$ was considered to be statistically significant.

RESULTS

TBI Induces Rapid and Sustained Activation of NFκB

Acute neurotrauma induces a series of signaling events which in turn are responsible for progressive neuroinflammation as well as neurodegeneration (Jassam et al. 2017). We were interested in determining what is the earliest time point at which we are able to monitor the activation of acute neurotrauma-induced signaling events. Here, based upon our prior research, we decided to investigate the activation of NFκB *in vivo* in a weight drop TBI model. We subjected NFκB-RE-Luc mice to real time noninvasive BLI prior to and post TBI. We observed that there was minimal to no BLI baseline signal prior to inducing TBI in both males as well as female mice (Fig 1). The baseline BLI signal prior to TBI was mainly localized in the fore as well as hind limbs with a relatively small BLI signal over the nostrils in one male as well as one female and also over the ears in one female. These results suggest that there is very minimal activation of NFκB in mice prior to inducing TBI. However, real-time noninvasive BLI performed within 30 minutes of TBI induction revealed a significant and specific increase in the BLI signal intensity specifically in the brain region

(Fig. 2). Additionally, in the case of male mice, we observed strong BLI signals in the abdominal area representing kidneys. However, in the case of the female mice, we observed very specific BLI signals only in the brain region and heart region. These results confirm and validate *in vivo* activation of the NFκB signaling pathway immediately post TBI.

Next, we wanted to address the issue whether or not TBI-induced NFκB activation is transient in nature. We addressed this crucial issue by subjecting the mice that have undergone TBI to real-time noninvasive BLI at 24, 48 and 72 hours (Fig. 3-5). We observed that real-time noninvasive BLI at 24, 48 and 72 hours post TBI led to an increase in the BLI signal intensity in the brain region as compared to the non TBI mice. In the case of all the male mice, there were specific BLI signals not only in the brain but also in the abdominal areas indicative of kidneys at 24 hours post TBI (Fig. 3). Interestingly, in one of the male mice the BLI signal was very diffuse throughout the body. Interestingly, in the case of female mice, we observed a significant increase in the BLI signal intensity in the brain as well as heart regions with very small and relatively faint renal BLI signal at 24 hours post TBI. Subsequent BLI at 48 hours post TBI revealed a significant increase in BLI in one of the male mice with predominant BLI signal in the area representing the liver and the kidneys (Fig. 4). In the case of the female mice, BLI signals were very focused in the brain and heart regions. We observed similar results at 72 hours in both male and female mice (Fig. 5). Overall, these interesting results suggest that TBI induces an immediate and sustained activation of the NFκB signaling cascade not only in the brain but also heart and kidneys thereby suggesting a global activation due to TBI.

TBI Induces Alterations in Cerebrovascular Function

Here, we were interested to investigate, whether or not TBI induces any alterations in the cerebrovascular function. To address this critical question, the mice were subjected to real-time noninvasive photoacoustic and ultrasound imaging prior to and post TBI at 24, 48 and 72 hours. As is evident at 0 hours the cerebrovascular blood flow is clearly visible with color Doppler overlay on the B mode images indicating flow direction within the posterior and middle cerebral arteries wherein the red color indicates blood flow towards the transducer and the blue color represents blood flow away from the transducer (Fig. 6). However, at 24 hours post TBI, we observed noteworthy changes in the cerebrovascular blood flow (Fig. 7). In the mice M1 and M3 there were slight changes but in comparison the mouse M2 displayed very very slight to almost negligible blood flow that could be readily monitored. A closer look at the blood perfusion within the brain parenchyma at 24 hours revealed that certain areas exhibited enhanced blood flow while some areas they were relatively poorly vascularized. At 48 hours post TBI the blood vessels appeared to be less dense while the perfusion of the blood within the brain parenchyma appeared to be significantly increased in the mice M1 and M3 while it was greatly diminished in the mouse M2 (Fig. 8). In fact, in the mouse M2 there was marked hypoxia and very poor blood flow which is indicative of a significant injury to the brain. Interestingly, the mouse M3 exhibited an aberrant cardiovascular function in comparison to the mice M1 and M2. Surprisingly, at 72 hours post TBI, the vascularization of brain parenchyma was suboptimal in all of the three mice as compared to 24 and 48 hours post TBI (Fig. 9). Very similar results were

observed in the case of the female mice. However, in comparison to the male mice the female mice had a minimal effect on the cardiovascular function.

TBI-Induced NFκB Activation Perturbs microRNA Expression

Emerging evidence suggest that microRNAs play a crucial role in TBI pathogenesis and could potentially be used as TBI biomarkers. Here, we were interested to investigate whether or not our BLI and PAI results correlate with differential microRNA expression. The underlying rationale being able to reliably establish and validate our BLI and PAI results in concert with novel microRNA biomarkers for the accurate diagnosis of TBI. As a consequence, our current studies using the weight drop TBI model include serum microRNA profiling in male mice (Fig. 10). We observed a greater than 20-fold expression of mir-9-5p in the serum 24 hours post TBI which subsequently declined to less than 5-fold by 72 hours post TBI. These findings indicate the crucial role of mir-9 in TBI pathogenesis and suggest that mir-9-5p might have a potential utility as an early TBI-specific biomarker. Analysis of mir-16-3p revealed approximately 6-fold upregulation 24 hours post TBI and approximately 4-fold upregulation 72 hours post TBI as compared to healthy control mice. Although, mir-16-3p is widely used as an endogenous microRNA normalizer, in our studies we found significant upregulation of mir-16-3p post TBI and therefore there is a need to evaluate other microRNAs that might be more stable (eg. mir-331, mir-223, mir-423, mir-191-5p, mir-484). Further, we analyzed the expression of mir-21a-5p in the control mice as well as mice that were subjected to weight drop TBI. Our current research findings reveal that as compared to the healthy control mice, mir-21a-5p expression is upregulated more than 14-fold 24 hours post TBI and decreased by more than 5-fold 72 hours post TBI. Our microRNA profiling data revealed a four-fold upregulation of mir-34a-5p 24 hours post TBI which was also almost stable at 72 hours post TBI. Since mir-34a-5p remains upregulated even at 72 hours, it might serve as a robust early and late TBI specific biomarker. Furthermore, We observed a greater than 2-fold upregulation of neuroinflammation associated mir-155-5p at 24 hours post TBI as compared to healthy control mice. By 72 hours the mir-155-5p levels returned to the baseline values. Therefore, mir-155-5p can serve as an early TBI specific biomarker. Overall our microRNA profiling data suggest that mir-9, mir-16-3p, mir-21 and mir-34a-5p can serve as robust TBI biomarkers in conjunction with BLI and PAI for an accurate and robust diagnosis of TBI.

TBI Induces GMF Expression, Neuronal Damage and Glial Activation

Having established the BLI and PAI modalities for the real-time noninvasive diagnosis of TBI, we were particularly interested to investigate whether our BLI and PAI results correlate with TBI-induced neuropathology. In order to conclusively prove the validity of BLI and PAI results, we performed immunofluorescence analysis on TBI brain specimens using classical markers NeuN, Iba1 and GFAP as well as TBI relevant neuroinflammation marker GMF. In comparison to the control mice, we observed significant neuropathological differences at 72 hours post TBI. Especially, we observed a significant upregulation of GMF expression.

Our immunofluoresecnce analysis (Fig. 11A) revealed an increased GFAP expression 72 hours post TBI as compared with the control brain thereby suggesting initiation and

progression of astrogliosis. GMF analysis indicated a significant increase in the TBI mouse brain as compared with the control. Surprisingly, there was only a minimal colocalization of GMF and GFAP in an isolated region which could possibly be a very small blood vessel. Next, we investigated the morphological alterations and changes in the staining intensity of microglia by performing Iba1 and GMF co-immunostaining of TBI brain sections and compared them with the control brain sections. In comparison to the control mouse brain there was a significant increase in both Iba1 as well as GMF expression thereby representing an increase in the number of microglia as well as increase in the staining intensity (Fig. 11B). Additionally, we observed increased microglial activation as reflected by increase in the number of microglial projections as well as greater thickness of the microglial projections. Furthermore, we observed that in the in the case of the control mice brain the microglia morphology was highly ramified with multiple branched processes which are typical characteristic of the resting microglia. In comparison to the control mice brains, the TBI brain displayed hypertrophic microglia with primary branches. Our results suggest that TBI induces neuronal damage and induces activation of microglia in a time dependent manner. These observations point towards acute neuroinflammatory responses mediated by proinflammatory factors including cytokines and chemokines as well as immune cells. Our NeuN immunofluorescence analysis (Fig. 11C) revealed a slight reduction in the number of neurons post TBI when compared with the control mouse brain. These results are expected and are indicative of TBI-induced neuronal loss. Further, we observed neuronal degeneration and as expected we also observed a significant increase in GMF expression as well as colocalization of GMF and NeuN in the TBI brain. These results suggest that TBI-induced GMF overexpression leads to NFκB activation which in turn is responsible for the observed neuronal loss as well as TBI-specific neuropathology.

DISCUSSION

Acute neurotrauma, especially TBI is a major cause of morbidity and mortality worldwide. In the absence of accurate diagnosis and imprecise medical treatment TBI prognosis can become grave rapidly. Hence, there is an urgent and unmet need to develop novel diagnostic modalities for an accurate and rapid TBI diagnosis. Towards, achieving this goal, we have utilized a robust weight drop murine TBI model due to its relative simplicity, reproducibility and due to the fact that it very closely mimics human TBI. Real-time noninvasive BLI has been successfully used to study neuropathology, stem cell transplantation as well as neurogenesis in the brain (Aelvoet et al. 2014; Akimoto et al. 2009; Aswendt et al. 2019; Fricke et al. 2017; Fukuchi et al. 2017; Hochgrafe and Mandelkow 2013; Lopez de Heredia et al. 2011; Rogall et al. 2018; Tennstaedt et al. 2013). We performed real-time noninvasive BLI to monitor the spatiotemporal regulation of NFκB signaling post TBI. Further by utilizing the high-resolution multimodal *in vivo* ultrasound and photoacoustic Vevo LAZR imaging we monitored the 3D anatomical, functional and molecular brain imaging including cerebral blood flow, perfusion, concentration and oxygen saturation of hemoglobin in the NFκB-RE-Luc mice at 24, 48 and 72 hours post TBI.

Our current results suggest that TBI induces an immediate and sustained activation of NFκB signaling. Further we observed that as compared to the female mice the male NFκB-RE-Luc mice exhibited a robust global NFκB activation as reflected by strong BLI signal intensity in

the regions that are indicative of heart, liver and kidneys. Whether or not male and female sex hormones play a crucial role in TBI-induced NFκB activation remains controversial and yet to be determined (Roof and Hall 2000; Hall et al. 2005; Clevenger et al. 2018; Villapol et al. 2017; Acaz-Fonseca et al. 2015; Bruce-Keller et al. 2007). However, recent studies have demonstrated that the sexually dimorphic responses in acute neuroinflammation post experimental TBI are mediated by infiltrating myeloid cells and are more robust in the males as compared to the females (Doran et al. 2019). We also believe that in addition to NFκB-RE-Luc mice, it would be interesting to utilize other transgenic reporter models such as GFAP-Luc mice to study and validate GFAP expression as well as gliosis post TBI induced neuroinflammation in real-time (Cordeau and Kriz 2012; Cordeau et al. 2008; Luo et al. 2014). In the recent past, BLI has been successfully used to monitor LPS-induced sterile neuroinflammation following intracranial administration of NFκB reporter lentiviral vector (Buckley et al. 2015). Most recently, development of a synthetic luciferin analogue, AkaLumine-HCL has been shown to significantly enhance the sensitivity (100-1000 fold) of *in vivo* BLI for deep tissue imaging as well as single cell imaging in mice as well as non-human primate marmoset (Iwano et al. 2018; Kuchimaru et al. 2016). This could potentially also be due to the fact that in comparison to D-Luciferin which has limited and relatively low tissue permeability and therefore heterogeneous biodistribution, AkaLumine-HCL produces near-infrared emission peaking at 677 nm which can easily penetrate most animal tissues (Kuchimaru et al. 2016). Therefore, our future studies will utilize AkaLumine-HCL for BLI of TBI in NFκB-RE-Luc and GFAP-Luc mice. Further improvements in BLI would incorporate InVivoPLOT which unifies the *in vivo* quantification and automated analysis of bioluminescence reporters in animals across entire animal cohorts (Klose and Paragas 2018).

Recent studies have indicated that microRNAs play a crucial role in TBI-induced neuropathology (Toffolo et al. 2019; Taheri et al. 2016; Sharma et al. 2014; Pan et al. 2017; Hicks et al. 2018; Di Pietro et al. 2018; Di Pietro et al. 2017; Atif and Hicks 2019). We have analyzed a defined set of neuroinflammation and TBI-specific microRNAs to validate our results. We found increased expression of mir-9-5p, mir-21a-5p, mir-34a-5p, mir-16-3p as well as mir-155-5p. Increased mir-9 expression causes neuronal apoptosis and decreased neurogenesis. Since GDNF 3'UTR has mir-9 binding site, it is expected that elevated mir-9 expression causes degradation of GDNF transcripts thereby causing reduced GDNF expression. Recent studies have shown that upregulation of mir-9 is associated with microcephaly and zika virus infection in mice. Further, mir-9 targets Hes1 and Notch2 to attenuate Notch signaling and promote neuronal differentiation. Additionally, mir-9 has been shown to directly target the transcription factors TLX and ONECUT to regulate VEGF-A expression. Most recently, upregulation of mir-9-5p has been shown to promote angiogenesis and cortex neuron survival post TBI by inhibiting Pitch1 (Wu et al. 2020). Thus, it would be interesting to monitor subtle changes in the gene expression of mir-9 targets to potentially discover novel TBI biomarkers.

mir-16 regulates crosstalk in NFκB tolerogenic inflammatory signaling by binding to IKKβ 3'UTR and its other targets include BCL2, CDK6, CDC27, CARD10, PRDM4, MAP7, CDS2, SESN1, MEK1 and TWIST1. Whether or not these multiple mir-16 target genes play a crucial role in TBI pathogenesis remains to be determined yet but is part of our ongoing studies to discover novel TBI biomarkers. Our data are consistent with the human

TBI studies wherein mir-16 has been shown to be upregulated at 0-10 hours in mild TBI (Redell et al. 2010).

We believe that TBI induced neuroinflammation causes GMF induced NFκB activation which leads to increased expression of mir-21. Prior studies have shown that overexpression of mir-21 causes dysregulation/inhibition of the multiple target genes including Angiopoietin, Tie2, PDCD4, PTEN, STAT-3, IL-12, PPARα, RECK, FASLG and TIMP3. Upregulation of mir-21 by the neurons post TBI appears to be neuroprotective as it downregulates NFκB signaling. Upregulation of mir-21 has been shown to reduce brain edema induced by blood brain barrier disruption and dysfunction (Ge et al. 2015). Thus mir-21 appears to play a crucial role in TBI pathogenesis (Harrison et al. 2016). Previous studies have revealed that exosomal mir-21 is responsible for microglia M1 polarization leading to aggravated release of neuroinflammatory factors (Yin et al. 2020). Most recently, administration of mir-21 rich exosomes derived from hypoxia preconditioned mesenchymal stromal cells were shown to significantly improve learning and memory capabilities in APP/PS1 mice with a concomitant reduction in the levels of GFAP, Iba1, TNF-α, IL-1β and decreased activation of STAT3 and NFκB signaling (Cui et al. 2018). Our current mir-21 expression profile matches with the human mir-21 upregulation post TBI as well as early mir-21 upregulation in rats and mice TBI models (Di Pietro et al. 2017; Hu et al. 2015; Sandhir et al. 2014; Lei et al. 2009; Ge et al. 2014).

mir-34a has been shown to regulate cell survival, neural stem/progenitor cell differentiation, migration and tissue remodeling via modulation of downstream signaling pathways. Further, upregulation of mir-34a is involved in auditory fear conditioning in the basolateral amygdala through Notch signaling and causes a decrease in hippocampal levels of BDNF as well as suppression of multiple target genes including Caspase 2, p53, BCL2, SIRT1, SIRT6, Stx1A, Syt1 and TREM2. Elevated mir-34a is a significant risk factor for behavioral and psychiatric disorders including AD, PD, bipolar disorder as well as age related hearing loss. Therefore, it would be very interesting to analyze various mir-34a target genes in conjunction with neurocognitive function.

Post TBI, mir-155 is induced within macrophages and microglia in response to TNFα-mediated stimulation and NFκB dependent TLR signaling and induces IL-6 expression and is responsible for neurogenic deficits including gliogenesis as well as abnormalities in proliferation, differentiation and migration of neural stem cells. Mir-155 targets include SOCS1, SHIP1, C/EBP-β, IL-13Rα1, c-Maf, HDAC4, Smad2, Smad5, AID, IKKε, TAB2, MyD88, CTLA-4, Rufy2, Nova1, Nav1, Thoc1 and Sumo3. Our mir-155 results are in sync with those reported earlier in a rat TBI model (Wang et al. 2015). We believe that mir-155 plays a crucial role in TBI pathogenesis and could represent a novel TBI biomarker as well as a potential therapeutic target.

Although we did not investigate the levels of mir-873a-5p, recent studies have shown that mir-873a-5p inhibits LPS-activated NFκB signaling pathway and inflammatory response by promoting microglial M2 polarization post TBI. Also, it would be very interesting to investigate spatio-temporal and gender specific dimorphic alterations in microRNA profiles post TBI especially because female mice have been shown to be acutely more

resistant to moderate-to-severe TBI as compared with male mice primarily due to reduced neuroinflammation (Villapol et al. 2017). Hence, our future efforts will be directed to decipher novel molecular mechanisms underlying spatio-temporal and gender specific dimorphic differences in various TBI models.

Our prior as well as very recent studies have shown that GMF plays a crucial role in neuroinflammation as well as neurodegeneration in AD, PD as well as TBI (Ahmed et al. 2017; Raikwar et al. 2019; Thangavel et al. 2018; Thangavel et al. 2013; Thangavel et al. 2017; Thangavel et al. 2012; Zaheer et al. 2002; Zaheer et al. 2007; Zaheer et al. 2008; Ahmed et al. 2020a). In our current studies we observed TBI-induced neuropathological changes including significant upregulation of GFAP and Iba1 expression as well as reduction in NeuN expression with a corresponding enhanced GMF co-expression and colocalization thereby suggesting significant glial activation post TBI. Multiple prior studies have also reported similar results with respect to GFAP (Susarla et al. 2014; Villapol et al. 2014) and NeuN (Sato et al. 2001; Gao and Chen 2011; Fluiter et al. 2014).

Microglia play a crucial role in normal healthy brain by continuously performing surveillance and contributing to synaptic maturation and function as well as participate in the phagocytic removal of cellular debris (Nimmerjahn et al. 2005; Paolicelli et al. 2011; Schafer et al. 2012). Microglia which also play a pivotal role in neuroinflammation, are highly responsive to stimulus and can rapidly become activated thereby causing altered microglia morphology especially during TBI (Donat et al. 2017). We have also most recently demonstrated that absence of GMF is neuroprotective, reverses TBI-induced neuropathology and improves motor and cognitive functions post TBI (Selvakumar et al. 2020). These crucial findings highlight the significant role of GMF in TBI-induced neuroinflammation, neurodegeneration as well as cognitive dysfunction.

As such there are no major limitations to either BLI or photoacoustic and ultrasound imaging. However, as far as BLI is concerned, it is somewhat difficult to pinpoint the exact depth and the precise location of the BLI signal within an organ at depths more than 1 cm below the surface. This issue can be significantly mitigated by using a highly *in vivo* stable and BBB permeable analog AkaLunmine which has a BLI signal strength >40 fold as compared to D-Luciferin (Kuchimaru et al. 2016; Iwano et al. 2018). BLI data analysis currently relies on the assumption that the measured BLI intensity at the body surface of the subject being examined has a linear correlation with its unknown luminescence source inside the tissue. However, the luminescence is strongly attenuated by the tissue and the measured intensities are in fact nonlinearly dependent on multiple factors including the heterogeneous optical tissue properties, spatial location of bioluminescence source, the imaging view, size, shape and relative position of the subject to the optical CCCD.

Similarly, ultrasound and photoacoustic imaging suffer from the caveat that bony structures do not allow deep penetration and as a result suffer from poor resolution at greater depths. Furthermore, limited bandwidth, and the finite size of the transducer also contribute to the loss of resolution. Some of these limitations can potentially be overcome by using second near-infrared (NIR) window (NIR-II) which offers higher spatial resolution, deeper penetration depth, reduced optical absorption, reduced tissue scattering, minimal

autofluorescence, maximum oxyhemoglobin absorption thereby allowing the measurement of tissue oxygenation levels deep inside the tissue (Upputuri and Pramanik 2019). Furthermore, there are much more sophisticated molecular imaging modalities like microCT, microPET, single photon emission tomography computed tomography (SPECT), single-impulse panaromic photoacoustic computed tomography (SIP-PACT), and MRI that are capable of very high resolution structural and functional imaging but they are very expensive and do require the use of radiotracers or contrast agents and highly trained research personnel. Our future studies will therefore incorporate some of these sophisticated molecular imaging modalities for the long term longitudinal monitoring of TBI dynamics as well as response to TBI-specific therapies.

Overall, our results, suggest that real-time noninvasive BLI, as well as high-resolution multimodal *in vivo* ultrasound, PAI and neuropathological analyses provides an accurate validation of TBI pathology. Further, we believe that real-time noninvasive PAI alone or in combination with other neuroimaging modalities can be successfully utilized as a robust diagnostic tool for rapid TBI diagnosis, rapid monitoring of TBI dynamics as well as for long-term longitudinal preclinical monitoring of precision targeted TBI therapies. Our current as well as future studies will be directed towards developing novel GMF targeted patient-specific precison and regenerative gene and stem cell based therapies not only for acute neurotrauma in military and civilian patients especially TBI, stroke and sports-related head injuries but also for AD and PD.

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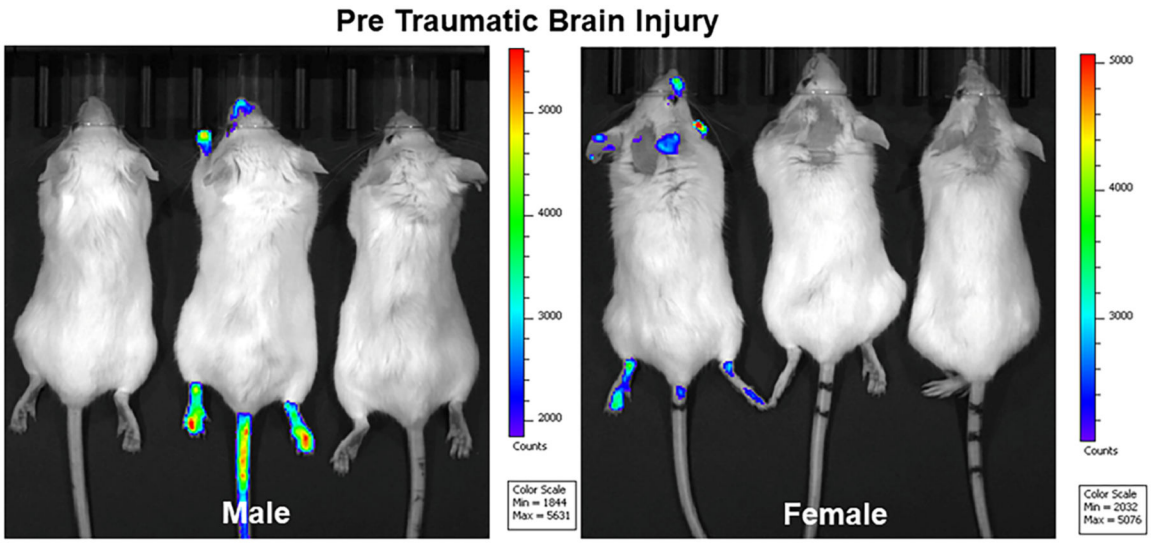

Figure 1: Lack of NFκB Activation in Non TBI Mice:

Real-time noninvasive BLI performed on NFκB-RE-Luc mice immediately prior to TBI indicates that there is lack of NFκB Activation in non TBI mice especially in the head region. Since these are global transgenic mice, certain regions like extremities as well as nose tip reveals minimal NFκB activation. As such there is no observable difference between the male and female mice.

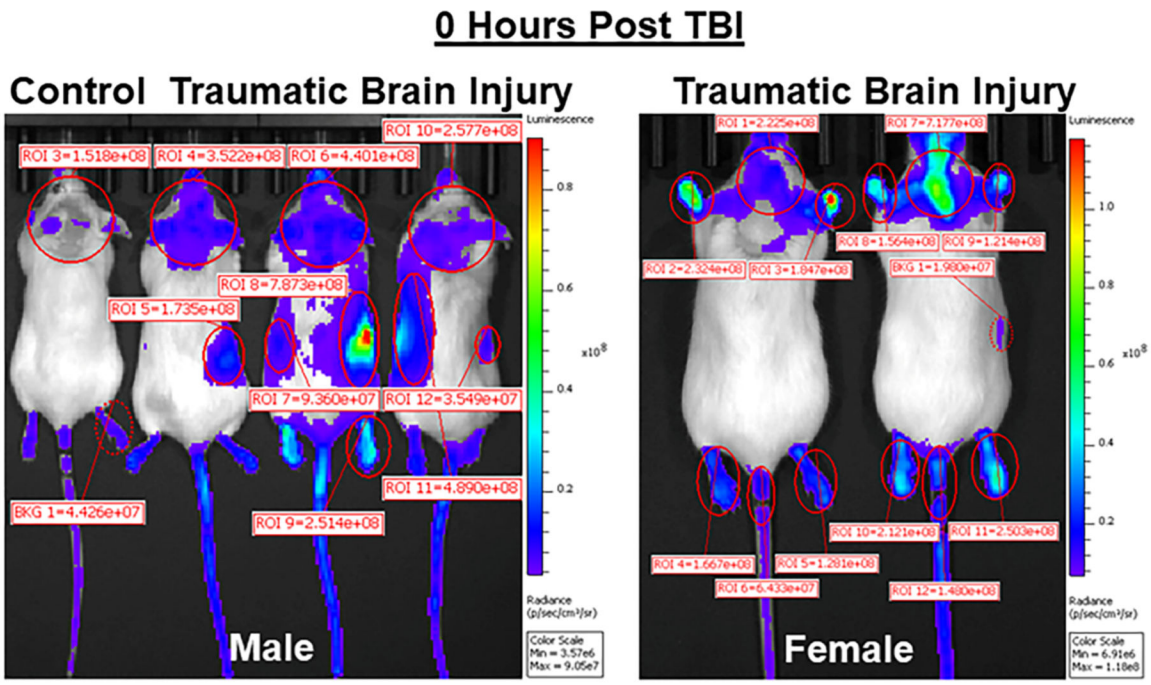

Figure 2: TBI Induces Rapid and Sustained Activation of NFκB:

Real-time noninvasive BLI performed on NFκB-RE-Luc mice within 30 minutes post TBI revealed a rapid activation of NFκB signaling cascade which was consistently sustained at 24, 48 and 72 hours post TBI. In the case of male NFκB-RE-Luc mice in addition to the brain region, BLI signals could be detected in the region of the heart, liver as well as kidneys. However, in the case of the female NFκB-RE-Luc mice the BLI signals remained very localized in the brain region throughout the duration of the study.

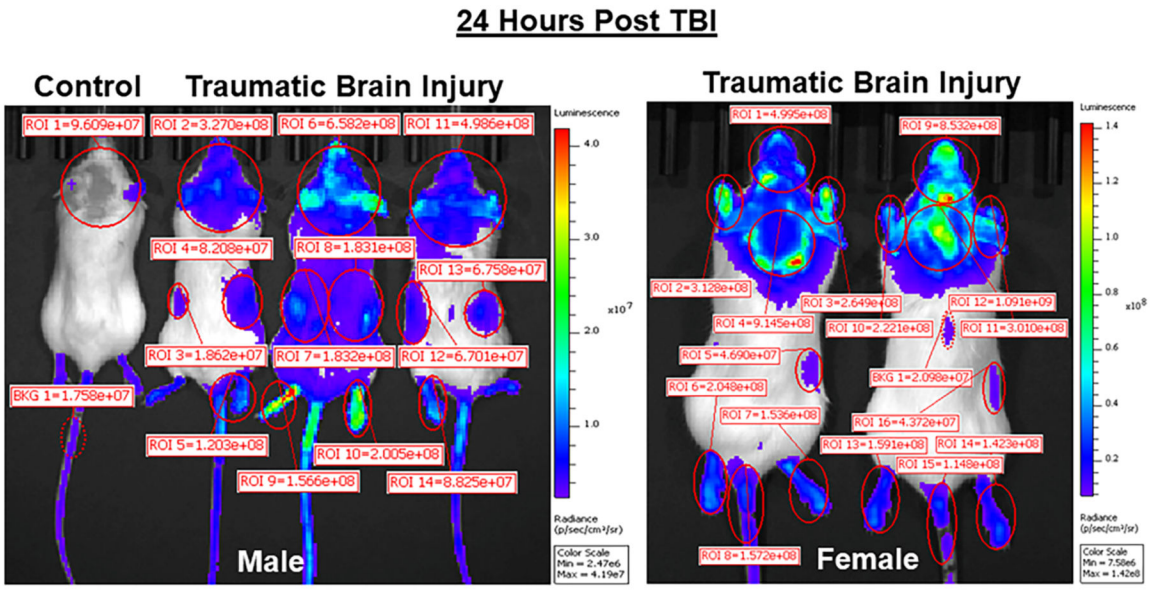

Figure 3: TBI Induces Sustained Activation of NFκB:

Real-time noninvasive BLI performed on NFκB-RE-Luc mice 24 hours post TBI revealed an enhanced and sustained activation of NFκB signaling cascade. In the case of male NFκB-RE-Luc mice in addition to the brain region, BLI signals could be detected in the region of the heart, liver as well as kidneys. In the case of the female NFκB-RE-Luc mice the BLI signals remained very localized in the brain and heart region throughout the duration of the study.

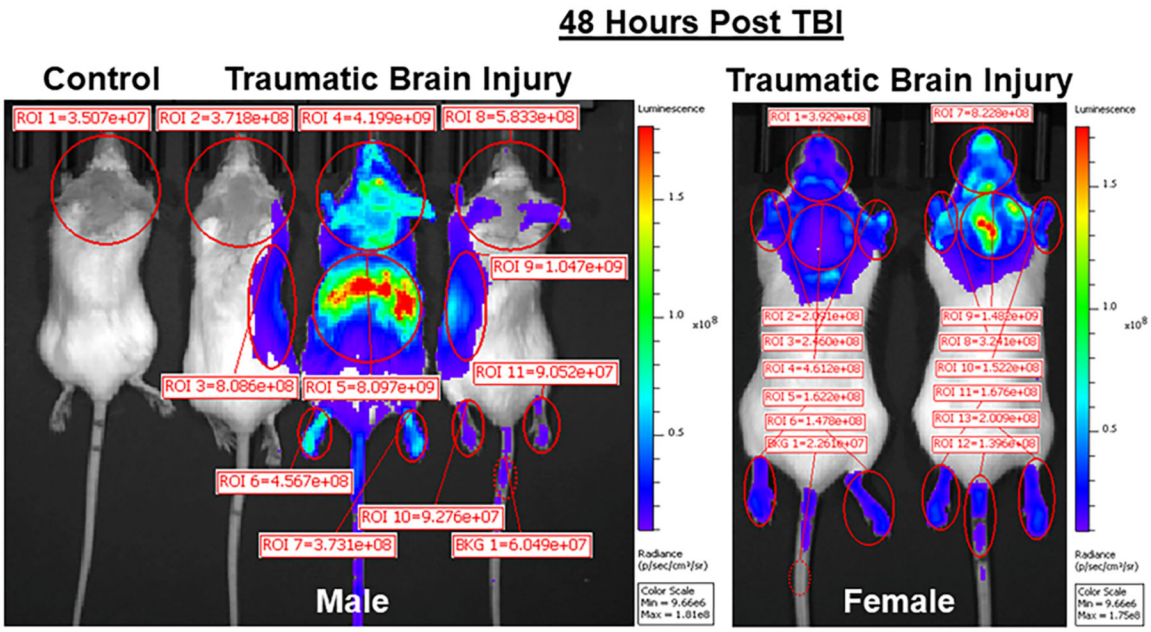

Figure 4: TBI Induces Sustained Activation of NFκB:

Real-time noninvasive BLI performed on NFκB-RE-Luc mice 48 hours post TBI revealed an enhanced and sustained activation of NFκB signaling cascade in one of the male mouse. In the case of male NFκB-RE-Luc mice in addition to the brain region, BLI signals could be detected in the region of the heart, liver as well as kidneys. In the case of the female NFκB-RE-Luc mice the BLI signals remained very localized in the brain and the heart region throughout the duration of the study.

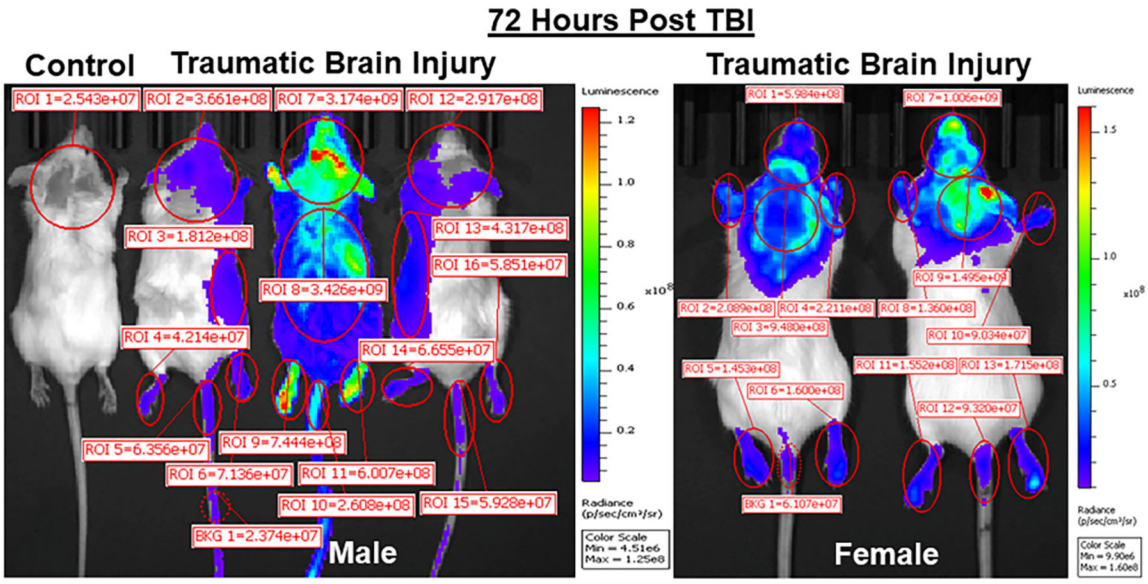

Figure 5: TBI Induces Sustained Activation of NFκB:

Real-time noninvasive BLI performed on NFκB-RE-Luc mice 72 hours post TBI revealed an enhanced and sustained activation of NFκB signaling cascade in one of the male mouse. In the case of male NFκB-RE-Luc mice in addition to the brain region, BLI signals could be detected in the region of the heart, liver as well as kidneys. In the case of the female NFκB-RE-Luc mice the BLI signals remained very localized in the brain and the heart region throughout the duration of the study.

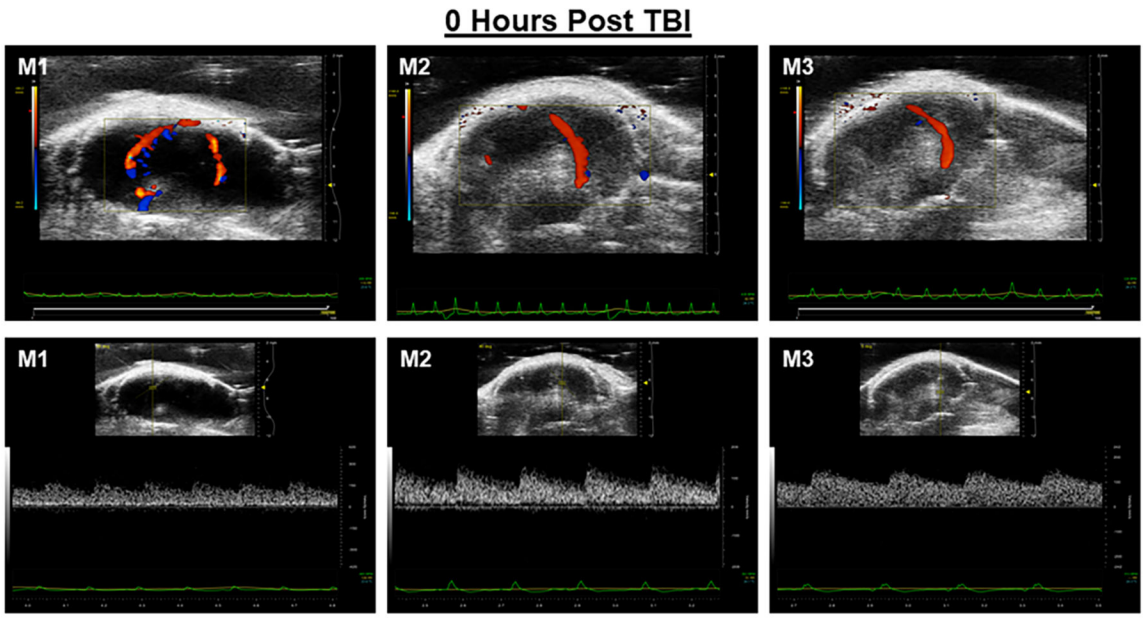

Figure 6: TBI Induces Alterations in Cerebrovascular Function:
Real-time noninvasive PAI revealed minimal alterations in the cerebrovascular function immediately post TBI. Especially, the blood flow appeared to be consistently normal in all the areas post TBI. In M1 mouse the Circle of Willis was relatively more clear.

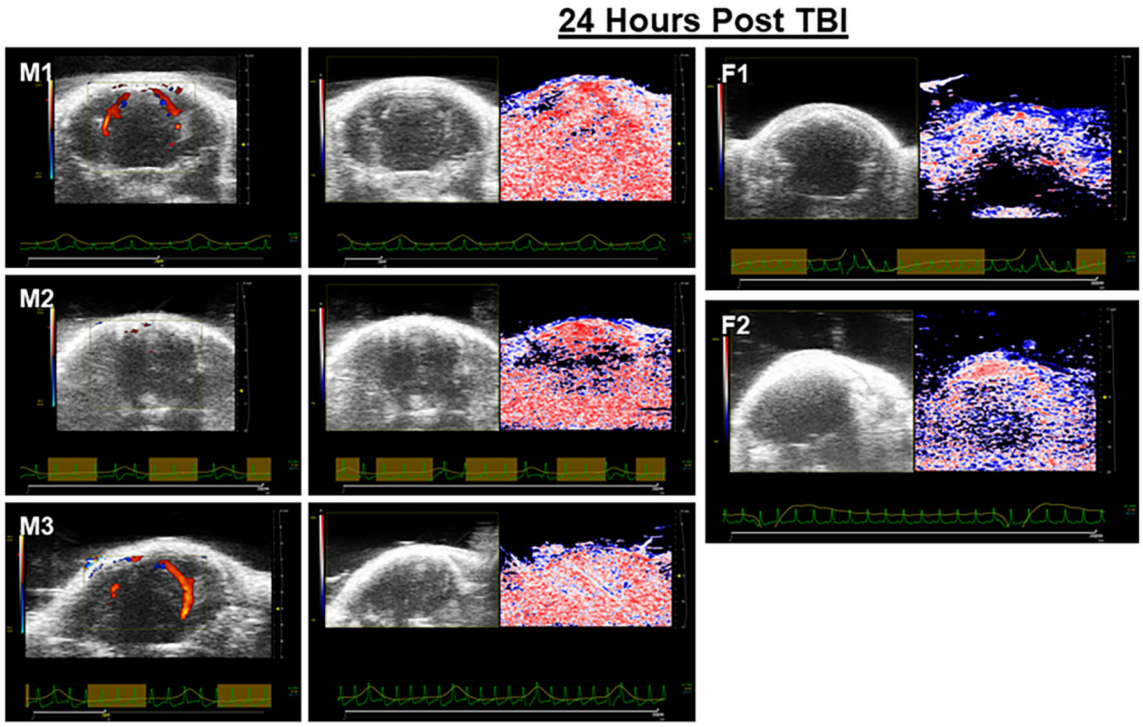

Figure 7: TBI Induces Alterations in Cerebrovascular Function:

Real-time noninvasive PAI 24 hours post TBI revealed alterations in the cerebrovascular function in TBI mice as compared to the TBI mice at 0 hours post TBI. Especially, the blood blow appeared to be consistently reduced in the area representing TBI in a time dependent manner. In the brain there was enhanced blood flow in the TBI region in the male as well as female mice.

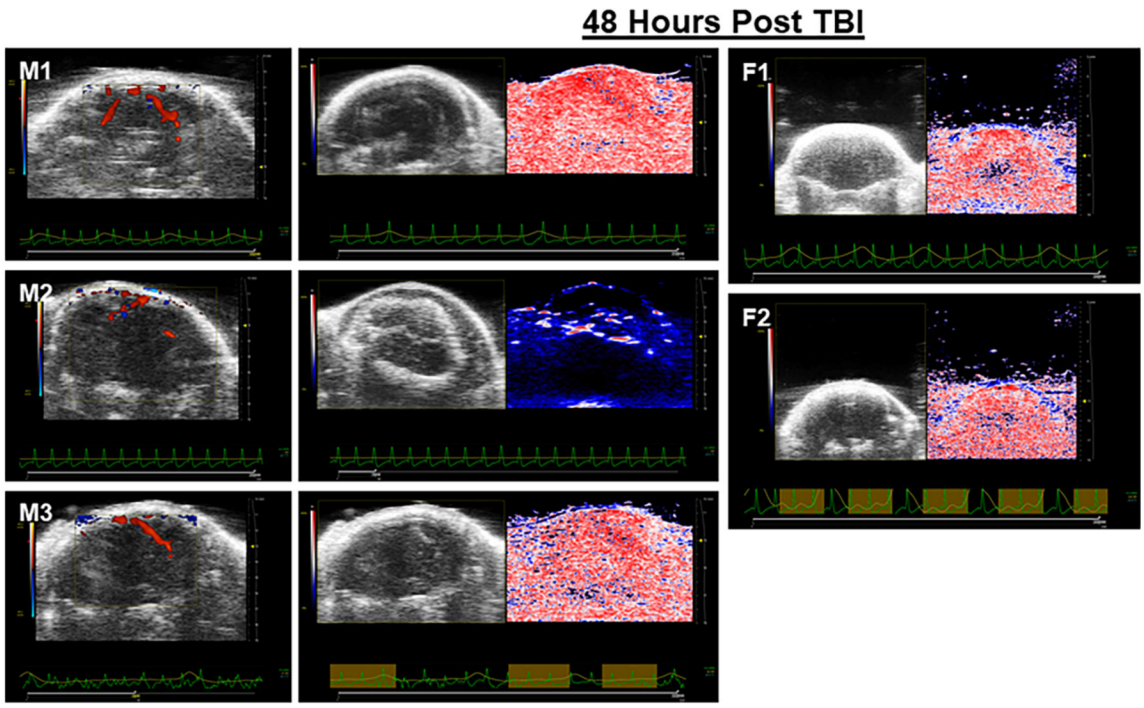

Figure 8: TBI Induces Alterations in Cerebrovascular Function:

Real-time noninvasive PAI 48 hours post TBI revealed alterations in the cerebrovascular function in TBI mice as compared to the TBI mice at 0 and 24 hours post TBI. Especially, the blood blow appeared to be consistently reduced in the area representing TBI in a time dependent manner especially in the M2 mouse brain. In the brain there was enhanced blood flow in the TBI region in the two males as well as two female mice. However, there was a significant reduction in the blood flow in one of the female mice.

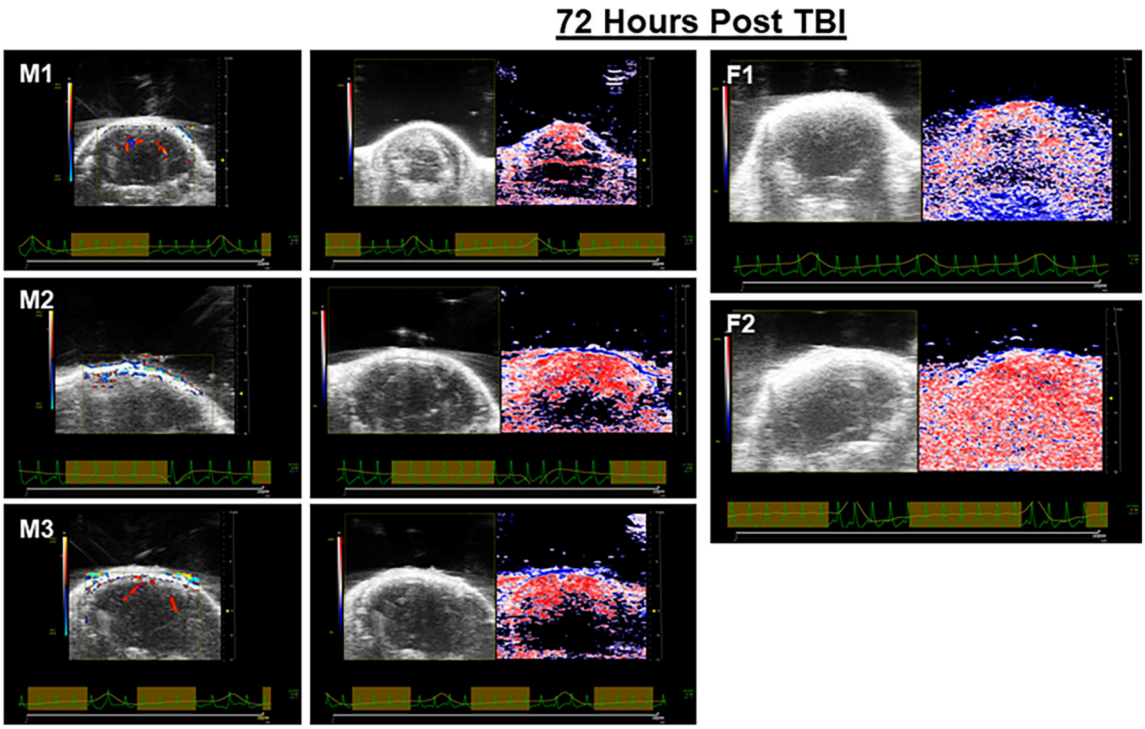

Figure 9: TBI Induces Alterations in Cerebrovascular Function:

Real-time noninvasive PAI 72 hours post TBI revealed alterations in the cerebrovascular function in TBI mice as compared to the TBI mice at 0, 24 and 48 hours post TBI. Especially, the blood blow appeared to be consistently reduced in the area representing TBI in a time dependent manner in all the three males and one female mice.

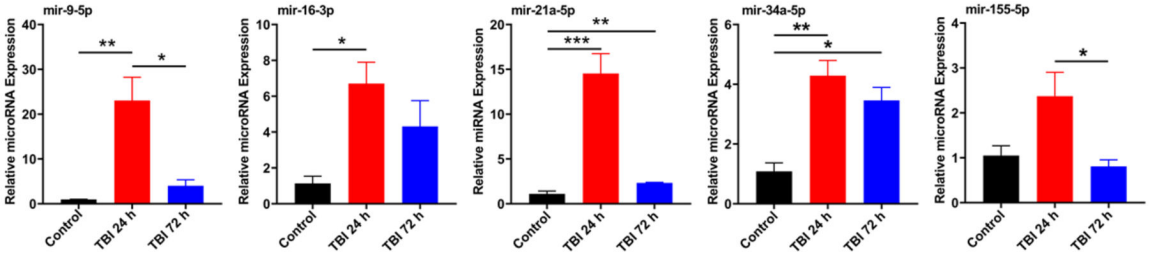

Figure 10: TBI-Induced NFκB Activation Perturbs microRNA Expression:

Changes in the levels of microRNAs were determined by TaqMan advanced miRNAs assays. Quantitative data were analyzed using the $2^{-\Delta\Delta Ct}$ method. A comparative analysis of microRNAs in the control and TBI serum revealed a significant upregulation of mir-9-5p, mir-16-3p, mir-21a-5p, mir-34a-5p as well as mir-155-5p within 24 hours post TBI. However, the most of the microRNA levels except mir-16-3p and mir-34a-5p declined to near baseline levels within 72 hours post TBI. Comparisons between different groups were performed by one-way analyses of variance (ANOVA) with Tukey's multiple comparisons test was used to determine statistical significance between different groups. (***P0.0001, **P<0.005, *P<0.01 versus the control group, values represent mean+SD, n=3 mice/group).

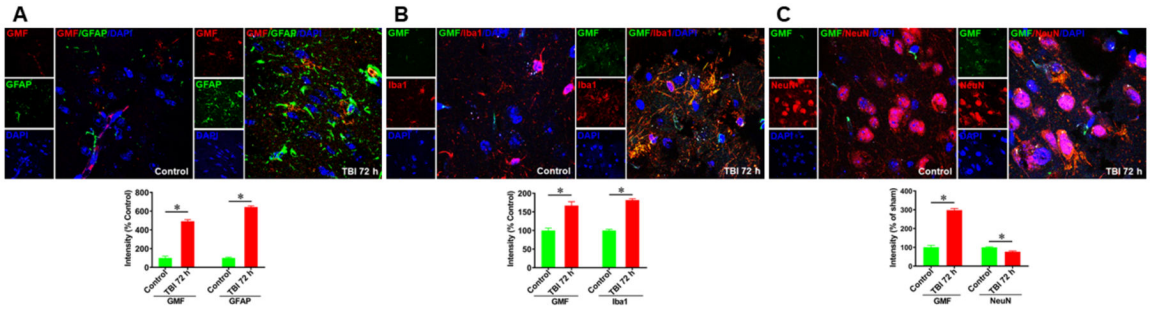

Figure 11: Immunofluorescence analysis reveals TBI induced neuropathology:

(A) The representative brain sections from the control and TBI mice subjected to GFAP immunostaining reveal TBI-induced neuropathology. As compared to the control brain, TBI brain exhibit significant upregulation of GFAP as well as GMF expression 72 hours post TBI. Enhanced GFAP expression indicates glial activation post TBI. $p<0.01$ control vs 72 hours post TBI. **(B)** Microglia in Iba1 stained representative brain sections in the TBI brain reveal alterations in numbers, staining intensity as well as morphology. *$p<0.05$ control vs 72 hours post TBI. **(C)** In comparison to the control mouse brain, representative NeuN staining exhibits neuronal damage as well as neuronal loss post TBI. In comparison to the control, TBI brain exhibits upregulation of GMF expression post TBI. *$p<0.05$ control vs 72 hours post TBI. Furthermore, TBI induces significant overexpression of GMF which is colocalized with GFAP, Iba1 and NeuN and is responsible for TBI associated neurpoinflammation and neuropathology. Results are representative analyses of 4-5 brain sections from each group (n=3 mice per group). Statistical analyses were conducted by performing Student's t test. *$P< 0.05$ versus control group. Scale bar = 50 μm.