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Award Number:

W81XWH-08-2-0139

TITLE:

Mission Connect Mild TBI Translational Research Consortium

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REPORT DATE: August 2010

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command, Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

x Approved for public release; distribution unlimited

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| **1. REPORT DATE** *(DD-MM-YYYY)* | **2. REPORT TYPE** | **3. DATES COVERED** *(From - To)* |
|---|---|---|
| 01-08-2010 | Annual | 1 AUG 2009-31 JUL 2010 |

**4. TITLE AND SUBTITLE**
Mission Connect Mild TBI Translational Research Consortium

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-08-2-0139

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**
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**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
University of Texas Medical Branch,
Galveston, TX,
77555-1072

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U. S. Army Medical Research and
and Materiel Command,Fort FortU.S.
Dietrich, MD,21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approval for public release; distribution unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT** Brain injury, particularly mild "blast" injuries due to improvised exploding devices, have long term cognitive and behavioral deficits. Key inflammatory cytokine levels (IL-1 and TNFα) usually increase after traumatic insults to the brain and contribute to the development of long-term deficits via activation of infiltrated monocytes and resident microglia and astrocytes and impaired vascular function; both resulting in persistent inflammation. **Our central hypothesis is that blocking inflammatory cytokine signaling after mild traumatic brain injury (mTBI) will improve outcomes by ameliorating inflammation.** Our goal is to develop, implement and assess interventions with two FDA approved drugs in two rat models of mTBI that will ameliorate the mTBI-induced cognitive and behavioral deficits. We have validated the use of a number of biomarkers that characterize mTBI injury as early as 6h and demonstrated significant behavioral impairment, such as working memory, in our lateral fluid percussion model of mTBI. We have also performed a pilot intervention via ip injection of Kineret, an IL-1 receptor antagonist, at 30 minutes and 6 hours post-mTBI. Preliminary asssessments show improvement in working memory and amelioration of the glial inflammation biomarker GFAP. Improved administration and dosages are underway. While our first year focused on outcomes at 6h, this second year focuses on outcomes at 18days.

**15. SUBJECT TERMS**
Blast head injury; mild head injury; anti-inflammatory therapy; cytokine receptors; IL-1; TNFα

| **16. SECURITY CLASSIFICATION OF:** U | | | **17. LIMITATION OF ABSTRACT** | **18. NUMBER OF PAGES** | **19a. NAME OF RESPONSIBLE PERSON** |
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| **a. REPORT** | **b. ABSTRACT** | **c. THIS PAGE** | | | **19b. TELEPHONE NUMBER** *(include area code)* |
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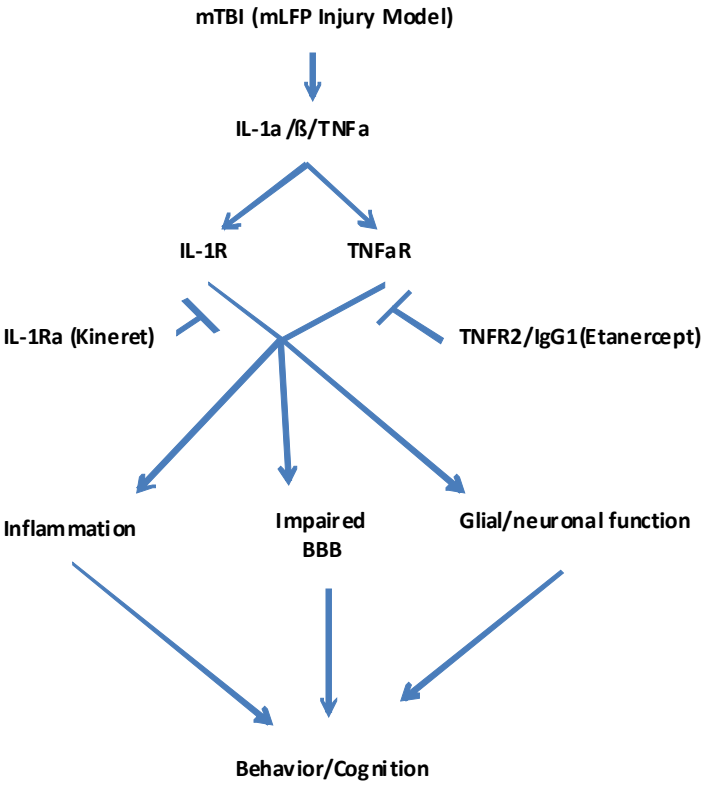
## Introduction

**Figure 1. Specific Aim 3.3 Schematic**




Brain injury, particularly mild "blast" injuries due to improvised exploding devices have long term cognitive and behavioral deficits. The key inflammatory cytokines (IL-1 and TNFα) increase after traumatic insults to the brain and contribute to the development of long-term deficits via activation of infiltrated monocytes and resident microglia and astrocytes, and impaired vascular function, all resulting in persistent inflammation. **Our central hypothesis is that blocking inflammatory cytokine signaling after mild traumatic brain injury (mTBI) will improve outcomes by ameliorating inflammation.** Our goal is to develop, implement and assess interventions with two FDA-approved drugs (Kineret or Interleukin-1 Receptor Antagonist, IL-1Ra and Etanercept or antibody to the Tumor Necrosis Factor Receptor alpha, TNFα) in two rat models of mTBI (mild lateral fluid percussion or mLFP and blast injury) that will ameliorate the mTBI-induced neurological deficits behavioral and cognitive deficits (**Figure 1**). Because of the diversity of clinical outcomes associated with mTBI and the diffuse nature of the injuries classified as mTBI, it is useful to apply diverse biomarker and behavioral assessments to our planned interventions. Furthermore, this approach will also be useful to the other projects and investigators in the development of comprehensive clinical strategies. Brain ischemia, excitotoxicity, oxidative stress, inflammation and edema result from TBI (Coles, 2004; Yi et al., 2006; Israelsson et al., 2008; Donkin et al., 2009).Since human TBI is characterized by a range of features, the use of more than one experimental model is necessary (Laurer et al., 1999).The rat lateral fluid percussion model of TBI (McIntosh et al., 1989) is widely accepted and causes a combination of focal cortical contusion and diffuse injury of subcortical brain areas much like that of human brain neuropathology after TBI (Thompson et al., 2005). The brain is highly susceptible to oxidative stress due to its high content of polyunsaturated fatty acids, high rate of oxidative metabolic actiivty, low antioxidant capacity and high levels of iron, copper and manganese that catalyze the production of ROS and NOS (Evans et al., 1993; Hall et al., 2004; Juurlink and Pataerson, 1998; Leker and Shohami, 2002). During this last year we screened rats suffering mLFP injury (1atm) for cellular biomarkers of inflammation that are known to participate in the various pathways that result in neuropathology and impaired neural/vascular function as well as behavioral assays with a focus on outcomes at 18 days post-injury, indicative and relevant to both the mLFP injury and the military experience. We performed assays for "common biomarkers" for all projects as agreed to by the Modeling and Protection Working Groups (see report from Working Groups); I chair the Protection Working Group. Thus, we validated the use of a number of biomarkers that characterize mTBI injury as early as 6h (see report year one) and as late as 18 days. Also significant in the context of therapy development, we performed a number of behavioral assessments accepted as relevant to trauma outcome in rodents that translate to the human condition. Both biomarker and behavioral screening are being used in our assessments of the planned therapeutic interventions. Finally, we carried out our first intervention with **i.p.** administration of Kineret to block IL-1 inflammatory signaling and observed mild improvement in the working memory and decreased astrocytic activation. after two early injections i.p. of Kineret administered 30min and 6hours after injury. This last finding is important because there was some question by the EAB as to the feasibility of Kineret treatment given it's a large protein in spite of the literature showing penetration of BBB and our demonstration of impaired BBB after mLFP injury – see effects on albumin and SMI-71. Thus, we have proof of concept data that our approach is worth optimizing over the next few months.

**Body of the Report**

**SPECIFIC AIMS**

***Specific Aim #3:*** **To develop new and innovative treatment strategies for MTBI and provide the preclinical and phase 1-2 testing of treatments found to improve outcome.**

***Specific Aim #3.3:*** To study the role of IL-1 and TNF receptor activation in neurological deficits after TBI

***Specific Aim #3.3.1:*** To serially measure brain cytokine levels after MTBI

***Specific Aim #3.3.2:*** To study the role of IL-1 receptor activation in neuronal cell death and in the inflammatory response after MTBI

***Specific aim #3.3.3:*** To study the role of TNF receptor activation in neurological deficits after TBI

**Progress to Date**

Our strategy has been to characterize and validate cytokine expression after mTBI by both immunohistochemistry and a more quantitative array immunoassay and behavioral outcomes relevant to brain trauma. Finally, to determine if Kineret can be administered i.p. to conform with the military setting as discussed with the EAB. From the beginning, another goal of our project has been to have all the mTBI procedures performed by the same individuals that are performing the mTBI in the other Mission Connect Consortium Project at UTMB (Drs. Wu and DeWitt) to allow for crossvalidation and sharing of experiences on a regular basis. Similarly, all immunohistochemistry is validated by performing selected assessments “blind” in the Core managed by Dr. Raymond Grill, at Houston; thus allowing cross validation with all the other Mission Connect Consortium Projects accessing that Core.

***Specific Aim #3.3.1:*** To serially measure brain cytokine levels after MTBI

In a recent study of 21 post mortem trauma brain samples there was an increase in the expression of inflammatory cytokines at the mRNA and protein levels (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, IFN-γ, and GM-CSF) consistent with earlier reports of cytokines in the cerebrospinal fluid of severe TBI patients (Frugier et al., 2010). Although these were not mild injuries, it showed that human TBI resulted in increased inflammatory cytokine and chemokine protein levels with IL-1β and TNF-α promptly increasing after TBI consistent with a TBI-induced increase in cerebral inflammation.

**Surgical Preparation – Fluid Percussion Injury:** Male Sprague-Dawley rats weighing 350 to 400 g were anesthetized with isoflurane in an anesthetic chamber, intubated, and mechanically ventilated with 1.5-2.0% isoflurane in O2:room air (70:30) using a volume ventilator (EDCO Scientific, Chapel Hill, NC). Rats were prepared for paramedian fluid-percussion TBI as previously described. Briefly, rats were placed in a stereotaxic frame and the scalp was sagittally incised. A 4.0 mm diameter hole was trephined into the skull 2.0 mm to the right of the sagittal suture and midway between lambda and bregma. A modified Luerlok syringe hub was placed over the exposed dura, bonded in place with cyanoacrylic adhesive and covered with dental acrylic. Isoflurane was discontinued; the rats were connected to the trauma device and subjected to mild (1.0 atm) fluid-percussion TBI immediately after the return of a withdrawal reflex to paw pinch. After TBI or sham injury, rats were disconnected from the fluid percussion device and righting reflex was assessed every 60 seconds until a normal righting reflex was observed. Rats were then placed on 2% isoflurane, wound sites were infused with bupivicaine and sutured with prolene. Isolflurane was discontinued and the rats were extubated and allowed to recover in a warm, humidified incubator.

We measured cytokine levels in ipsilateral vs contralateral tissues (hippocampus, cortex, and thalamus) at 3 and 6 hours after 1 atm. mTBI using the Bio-Plex assay kit from Bio Rad, a reliable capture sandwich immunoassay. The Bio- Plex cytokine assay is a multiplex bead-based assay designed to detect up to 100 cytokines in a single well of a 96 well plate. The assay uses up to 100 color–coded bead sets, which are conjugated with different specific reactants. Each reactant is specific for a different target molecule. The

specific reaction is identified based on bead color. Samples in each well are drawn up into the flow based Bio-Plex array reader. The reaction is measured by using fluorescently labeled reporter molecules also specific for each target protein. For our experiment the Bio-Plex rat cytokine assay was designed to detect and quantitate 9 rat cytokines from tissue lysate. The assay is very sensitive detecting cytokines in the pg/g total protein range. In ipsilateral injured hippocampi, we observed a distribution showing significant early increases in IL-1α at 3h and in IL-1β at 6h with IL-1β being the dominant induced cytokine when compared to the contralateral side. Given that both IL-1α and IL-1β bind to the same IL-1R receptor, this result is consistent with our approach to block the IL-1R stimulation of inflammatory cascades with Kineret. There were also increases in IL-10 at 6 h consistent with its demonstrated response in moderate or severe TBI documented in 30 published papers; there are no published results showing an increae in IL-10 in mTBI. While there were significant increases in IL-6 and GM-CSF in hippocampus, their very low levels would suggest their role may not be critical after mTBI.

**Figure 2.** Hippocampal Cytokine/Chemokine Responses to mTBI

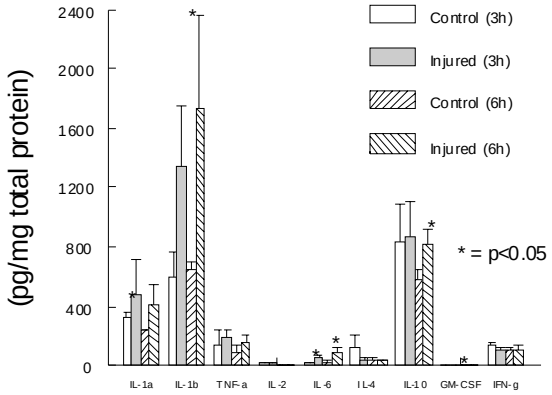



**Figure 3.** Cortical Cytokine/Chemokine Response to mTBI

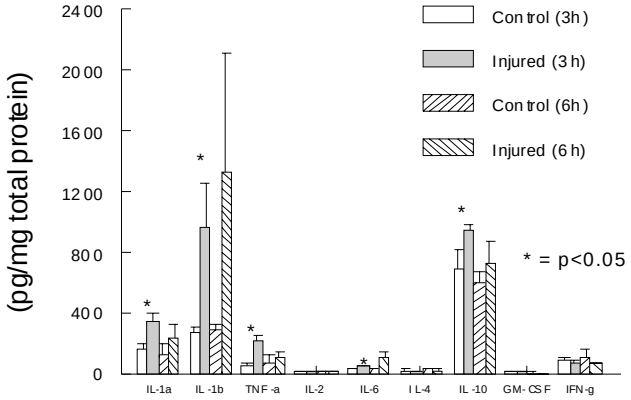
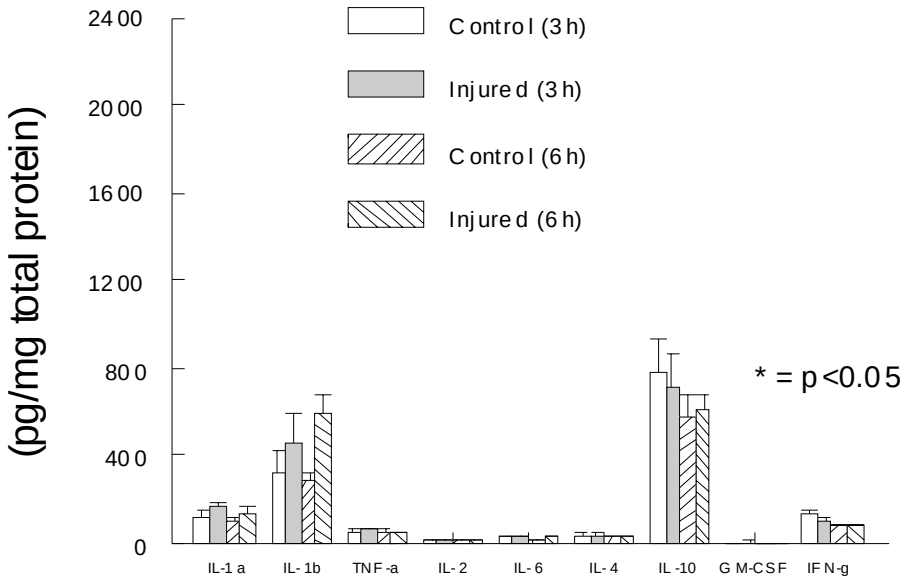


**Figure 4.** Thalamic Cytokine/Chemokine Response to mTBI

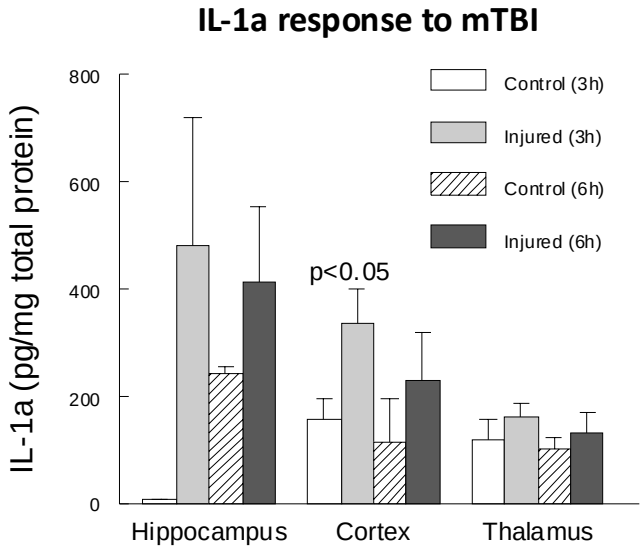



In the injured ipsilateral parietal cortex, in addition to increases similar to those observed in hippocampi (**Figure 2**), we observed significant early increases in TNF-a at 3h when compared to the uninjured contralateral side; again suggesting that blockade of the TNF-a receptor is a good strategy (**Figure 3**).

Interestingly, a similar analysis of injured ipsilateral thalamic tissues did not show any signifcant differences when compared to their contralateral cohorts although the pattern of expression was very similar (**Figure 4**). Also, clearly the highest levels were present in the hippocampus and cortex with thalamic levels being about 25% of those in the ipsilateral hippocampi and cortices.25

In **Figures 2-5** we show responses to individual cytokine/chemokines with scales adjusted to show relative changes and grouped according to their responsiveness to mTBI as well as their known roles in inflammatory responses.

**Figure 5a. IL-1a response to mTBI**



# Figure 5b. IL-1b response to mTBI

**Figure 5b.**

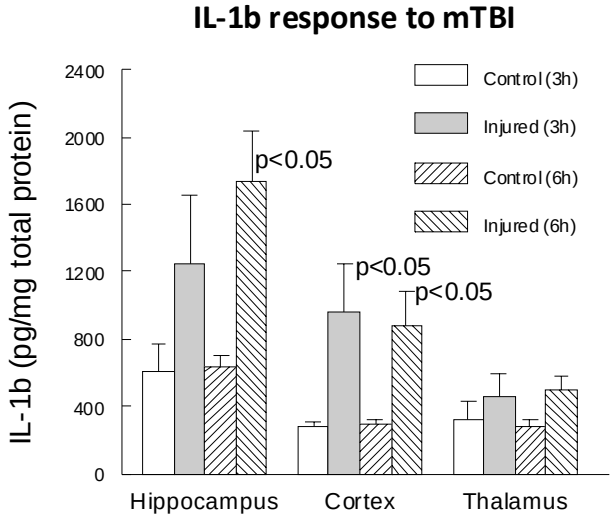



**Figure 6. TNF-a response to mTBI**

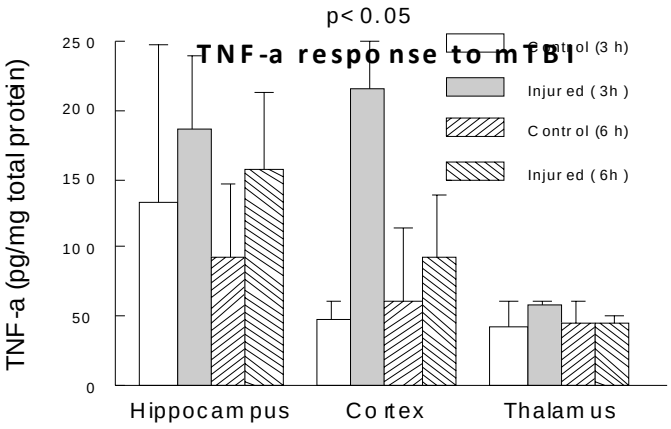


**Figure 7a**

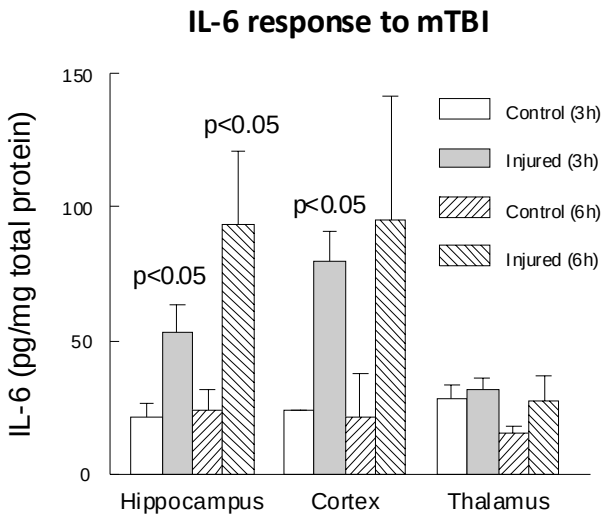



**Figure 7b**

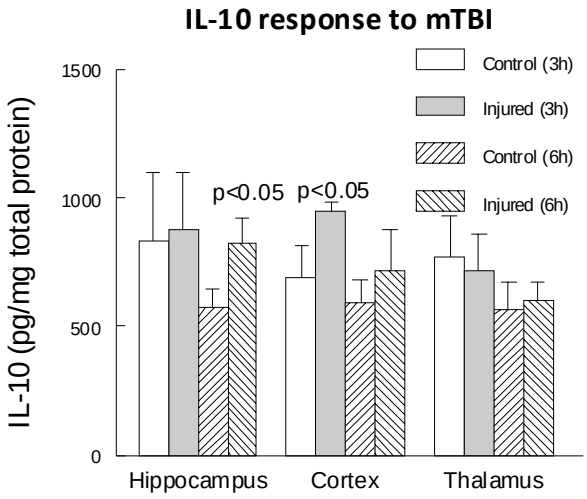


**Figure 8. IL-2, IL-4, IFN-g & GMCSF response to mTBI**

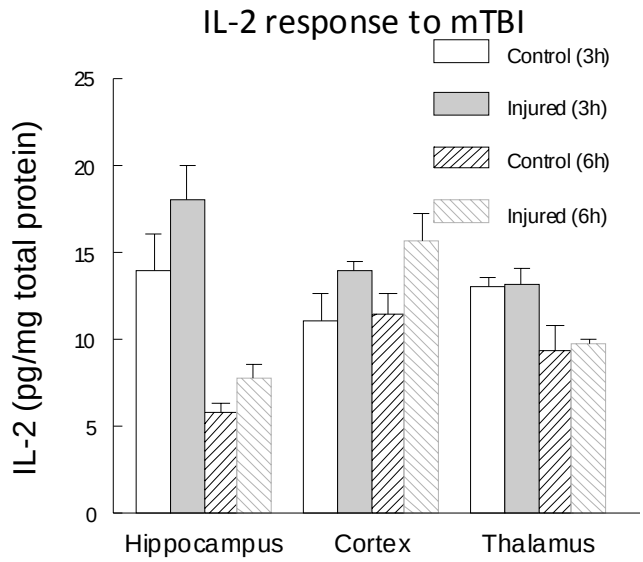



**Figure 8. IL-2, IL-4, IFN-g & GMCSF response to mTBI**

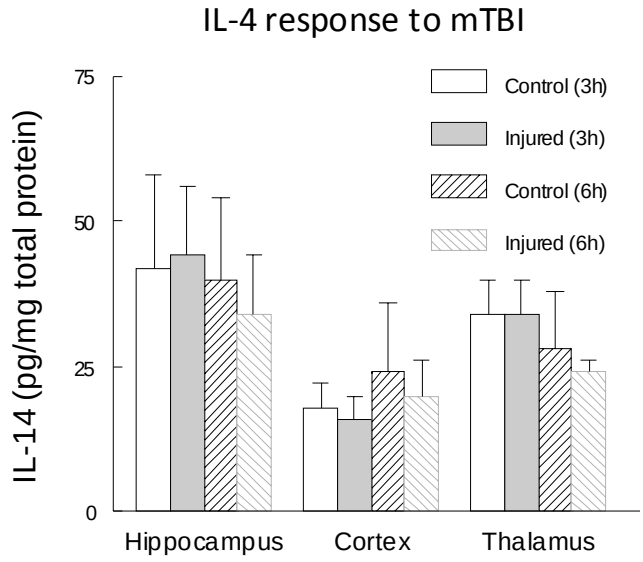


**Figure 8. IL-2, IL-4, IFN-g & GMCSF response to mTBI**

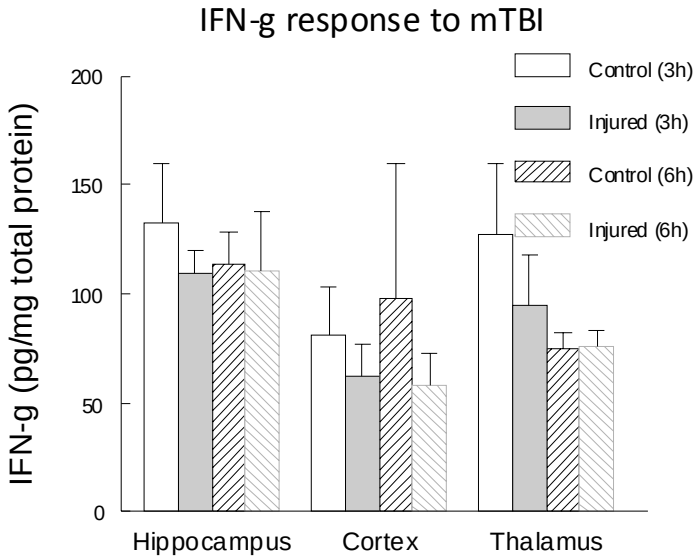
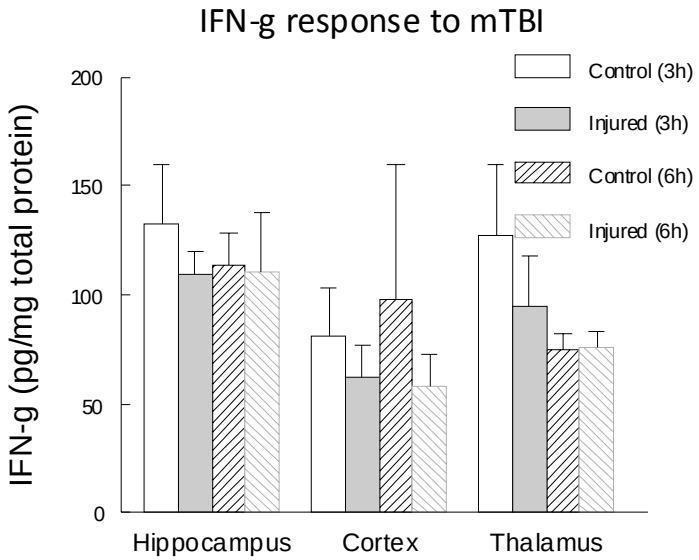



**Figure 8. IL-2, IL-4, IFN-g & GMCSF response to mTBI**



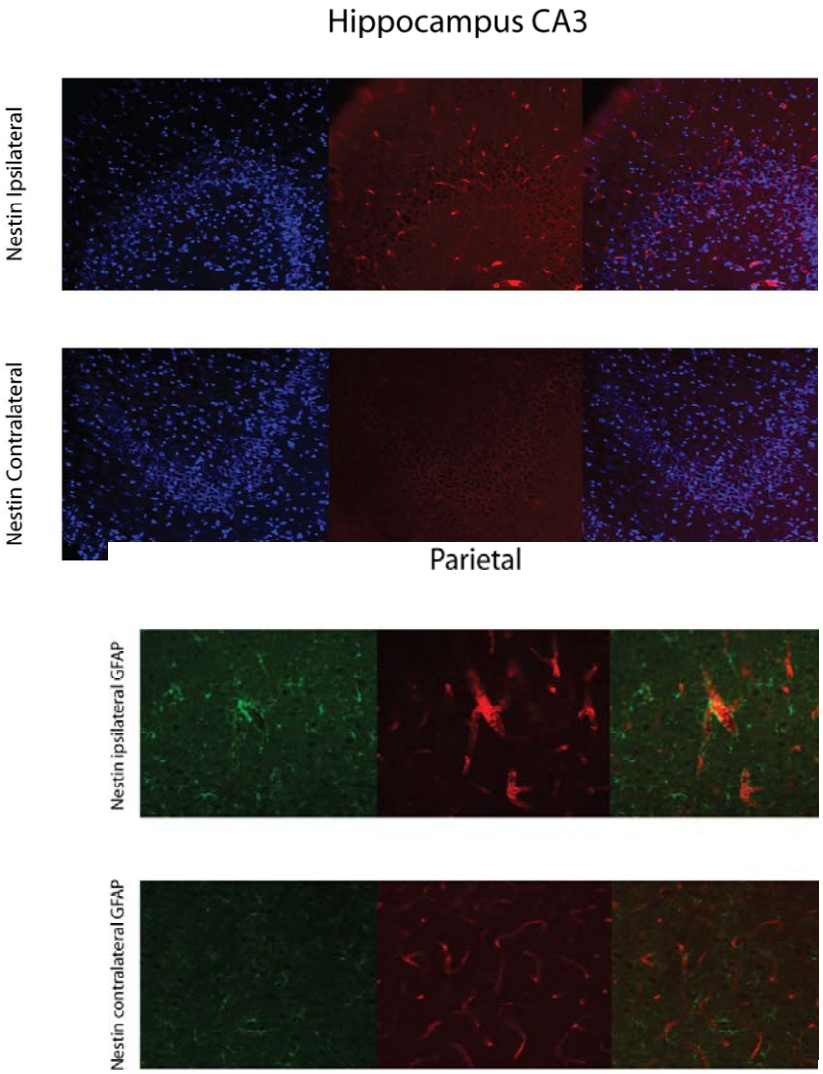
**Biomarker Characterization: Glial activation**

**Figure 9. Demonstration of increased Nestin associated with activated Astrocytes at 6h after 1atm TBI**

**RED is Nestin**

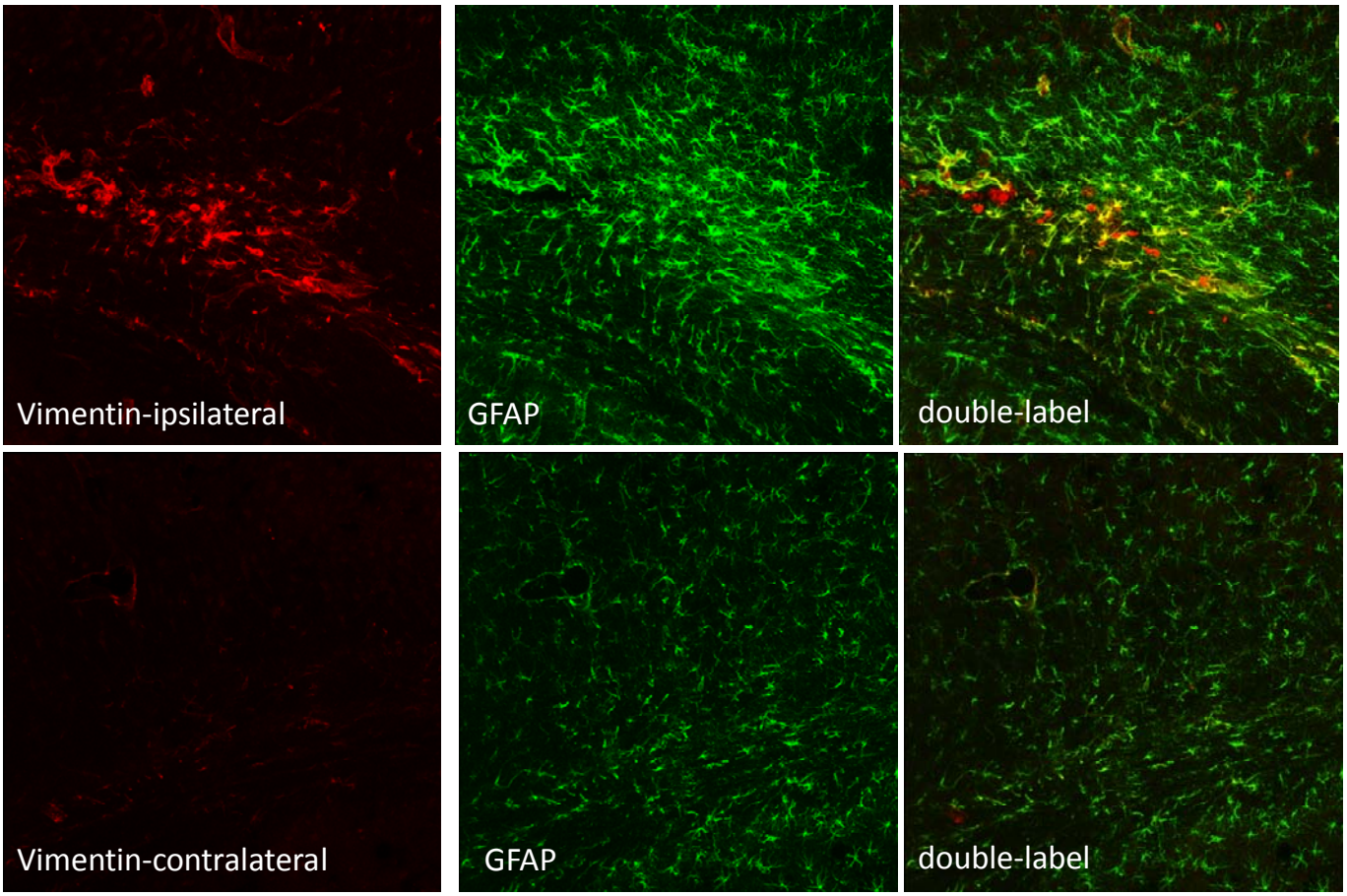
**Blue is nuclear marker**

**Green is GFAP**




We have shown a qualitative increase in astrocytic activation based on the use of a number of markers of astrocytic proliferation and activation indicative of scarring and inflammation such as **nestin** (**Figure 9**), **vimentin** (**Figure 10**) and glial fibrillary acidic protein (**GFAP; Figure 11**). Nestin and vimentin are intermediate filament proteins co-expressed by GFAP-positive astrocytes shown to promote scarring and glial proliferation (Frison et al., 1995; Wei et al., 2002). GFAP is also a common biomarker being screened by all animal mode projects in the Mission Connect Consortium. In order to apply the GFAP marker as a reliable outcome measure, we needed to quantitate *in situ* levels at 18 days post-injury, consistent with the hypothesis of persistent inflammatory outcomes. We performed mTBI, as described in our protocol. In order to model mild TBI and not moderate or severe TBI, we used a 1 atm injury and after six hours and 18 days (18 day animals were also assessed for several behavioral assays) sacrificed animals and removed and prepared parietal cortex (ipsilateral, contralateral) from injured, sham-treated and naïve rats for immunohistochemistry. Slides were prepared at our laboratory or prepared and coded “blind” at Dr. Raymond Grill’s Core and coded slide photographs were then presented to three members of our research team who are not part of this project and scored for number of positive dots in each image. The resulting values were then analyzed individually and as a group by calculating the mean number of dots for each image. This mean value was then considered for each of the images and there was a significant increase in, for example, GFAP positive cells scored for all the ipsilateral (injured) brain slides compared to the contralateral cohort (**Figure 11;** $p<0.05$).

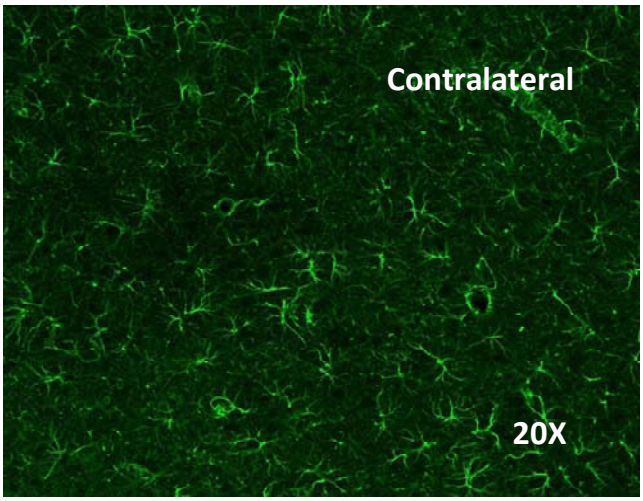
**Figure 10**. Vimentin, like GFAP, appears upregulated in astrocytes as early as 6 hours post-FPI

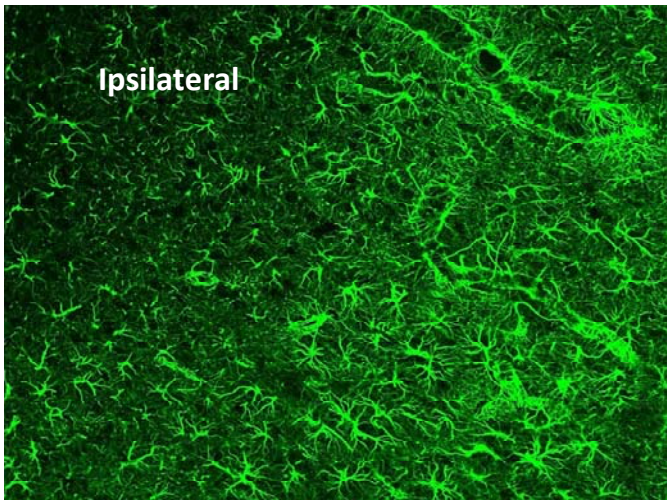



Profiles of (nuclei, cell soma, etc.) were counted in specific anatomic regions, ex. hippocampus CA1, dendate gyrus, etc. on histolgical sections that were consistently 14.5 to 15.5 um in thickness from at least three sections per animal for at least three different animals, averaged and calculated as profile counts within that specific region/per animal/per side and considered as one data point per side. Thus, the final number is a mean number of profiles counted within a three dimensional region (area of anatomical region x thickness of section). Since each region compared is similar, and the thickness of the sections were not statistically significant between the ipsilateral and the contralateral side (being from the same section) we report our counts as number of (nuclei, or cells) with the understanding that the counts are stereological estimates based on profile counts. All counts were repeatedly done blind by three different individuals to eliminate bias. Thus, we averaged for each individual brain the average of counts as determined by the three individuals counting each slice and then determined the mean for the slice +/- SD. The resulting values were then analyzed individually and as a group by calculating the mean number of dots for each image. This mean value was then considered for each of the images and there was a significant increase in GFAP positive cells scored for all the ipsilateral brain slides compared to the contralateral cohort (**Figure 11;** $p<0.05$).

While there were differences in resulting counts for any slice for the three different individuals counting, the relative differences between samples were consistent and there was a 10% or less variation for any sample count across individual counts from the three individuals performing counts. Our results confirm the qualitative results that are consistent with the persistent activation of astrocytes and serve as a measure of chronic inflammation. Thus, there was a significant increase in ipsilateral vs contralateral levels of GFAP in the injured animals 18 days after mTBI (**Figure 11**) but not in naïve or sham animals (data not shown). This is an important finding from the perspective of being able to introduce therapeutic proteins in the injured brain.

Also consistent with the concept of impaired function associated with increased chronic inflammation we saw increased IBA 1 and ATF3 with decreased BDNF.



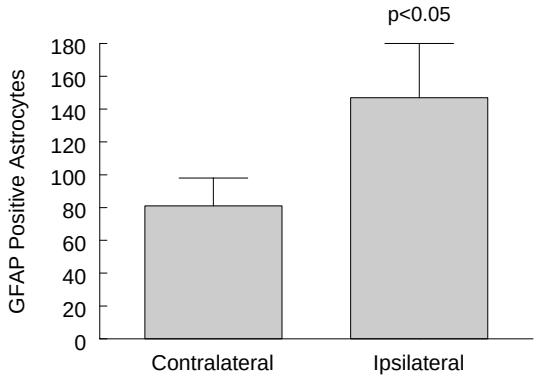
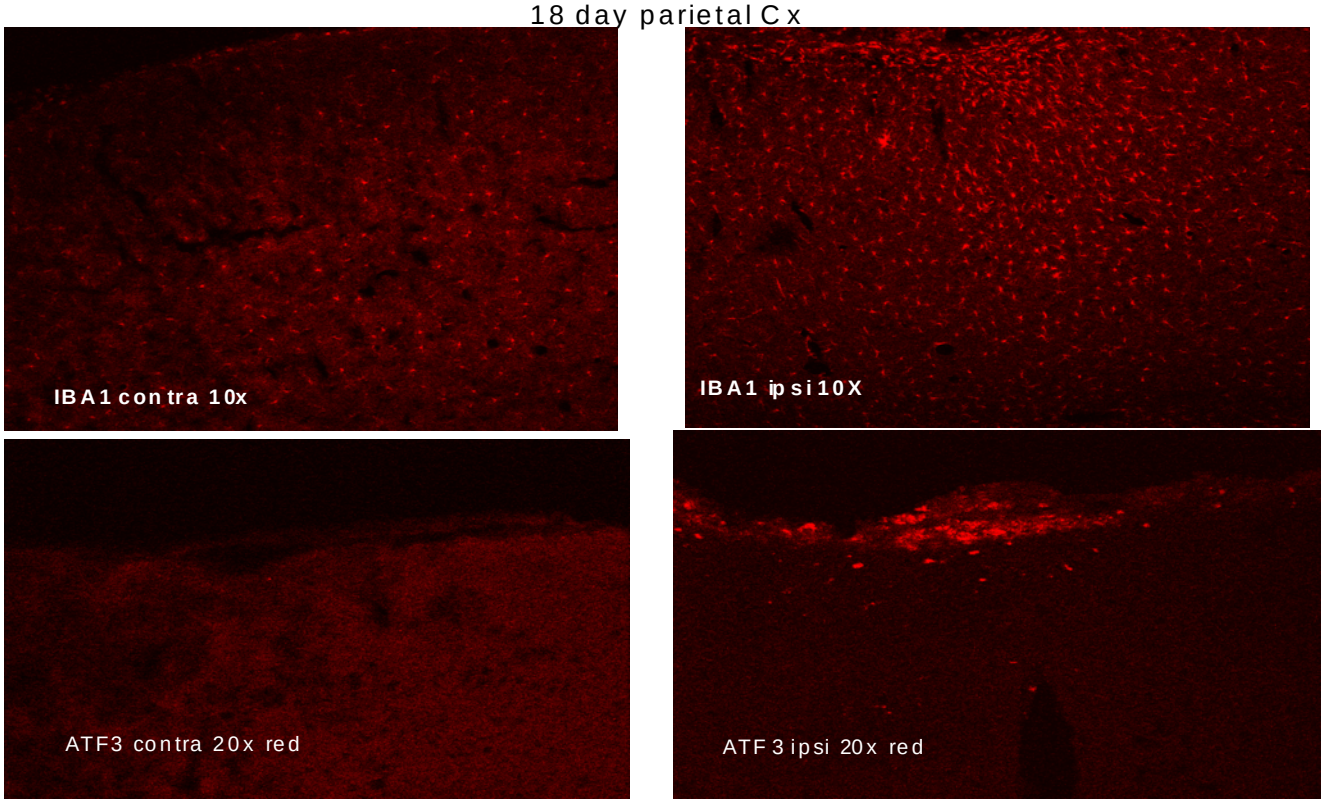
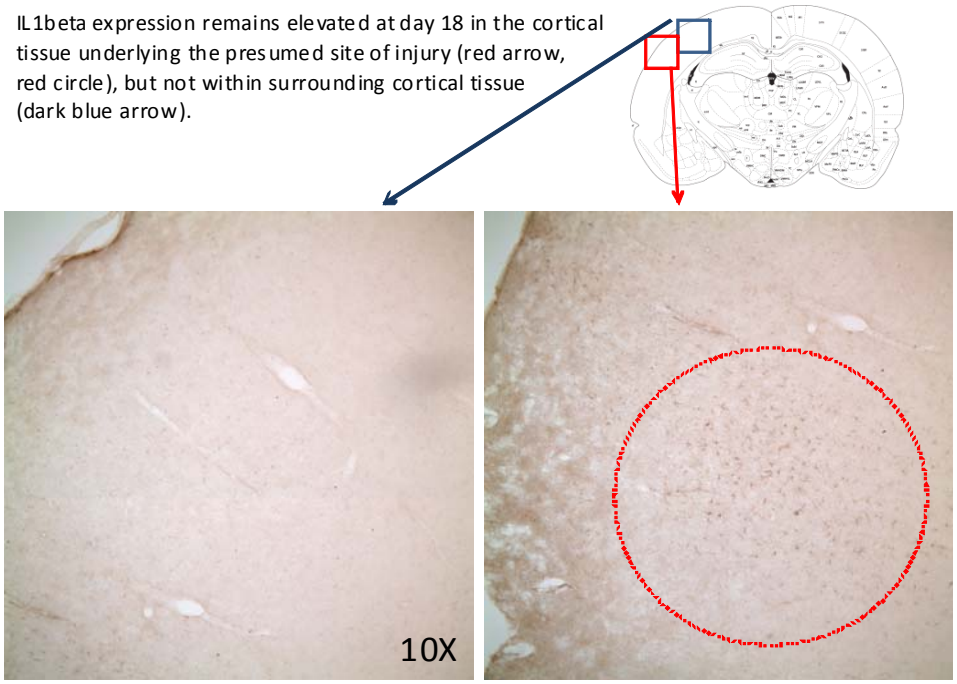




**Figure 11. Effect of 1 atm mTBI of astrocytic activaton (GFAP positive cells) after 18 days.**

**Figure 12. Demonstration of increased ipsilateral IBA1 and ATF3 after 1atm LFP mTBI.**



IBA1 is a marker for activated microglia and a potent indicator of inflammation and was seen throughout tissue with strongest concentrations seen just below injury area occipital /parietal cortex with an increase in the ipsilateral (injured) over contralateral (control) side. ATF3, an important inflammatory transcription factor, was found to be sparse and sporadic in tissue with high concentrations below the injury area in the ipsilateral vs contralateral side (**Figure 12**). Consistent with these images of inflammation at 18 days post-injury, we also see a similar aggregation on the ipsilateral injured cortex of the IL-1b inflmmatory cytokine (**Figure 13).**




**Biomarker Characterization: BBB permeability and Blood Bourne Protein Levels Post-mTBI**

In order to confirm the published literature on increased BBB permeability after mTBI (the bulk of that literature addresses moderate TBI and/or stroke) we also assessed the presence of blood borne proteins in brain at 18 days post-trauma. When we stained for albumin and IgG and compared results between naïve's and injured, there was a qualitatively very definitive presence of blood borne proteins in the injured brains at 18 days post injury (**Figure 13),** clearly documenting BBB impairment up to 18 days post-injury. This is important from the perspective of being able to introduce therapeutic proteins in the injured brain. A more precise and better characterized "window of opportunity is being determined.

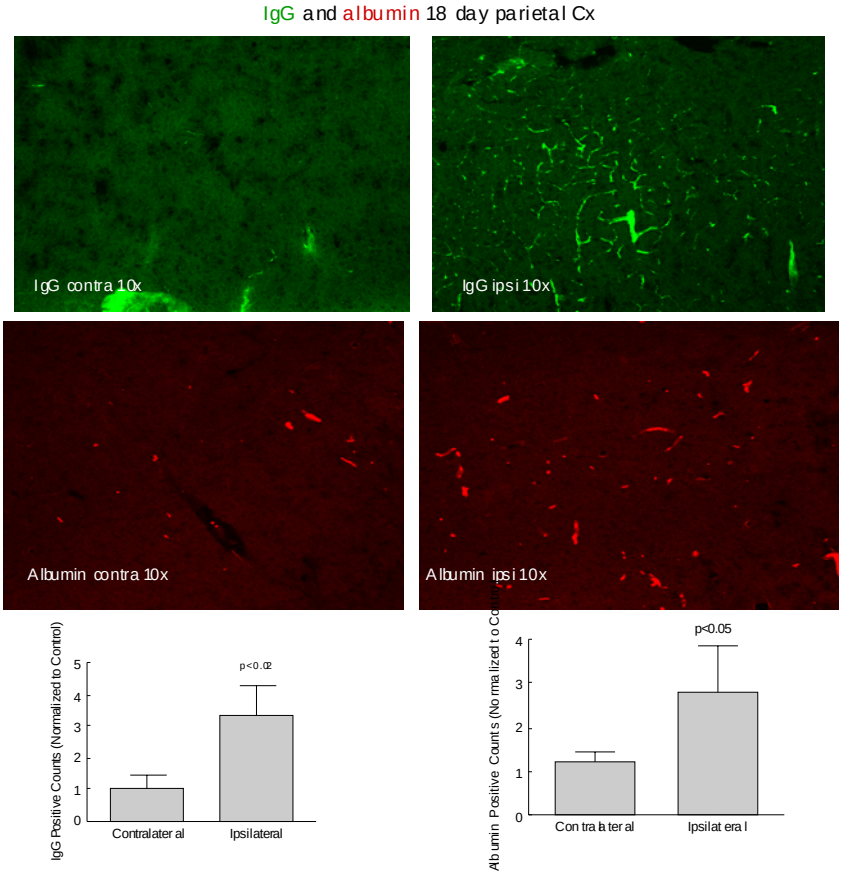
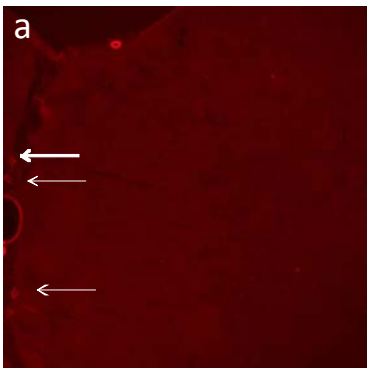



**Figure 14. Demonstration of significant presence of inflammatory proteins in parietal cortex 18 days after injury normalized to control (contra-lateral).**



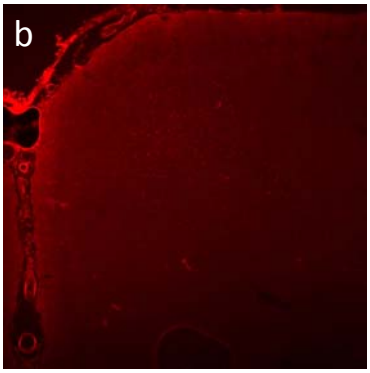
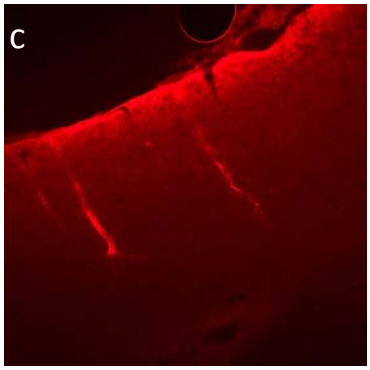




**Figure 15. Evans Blue/albumin deposition in the co 72 hours after 2 atm fluid percussion injury**

a) Cortex from animal receiving IV evans blue 1 hr prior to sacrifice. EB labeling can be detected around meningeal vessels, but not in parenchyma
b) Cortex 72 hours post-2atm FPI, 1 hr-post EB delivery Note the parenchymal labeling
c) EB distribution around the cavitation site at 72 hours. At low mag, can detect large, penetrating blood vessels as well as halo around injury site.



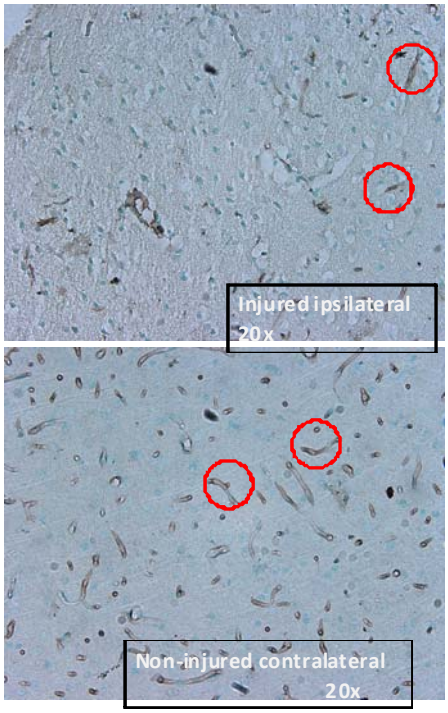
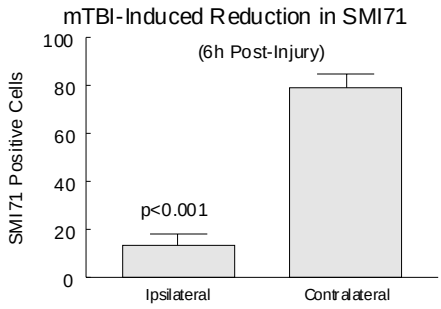



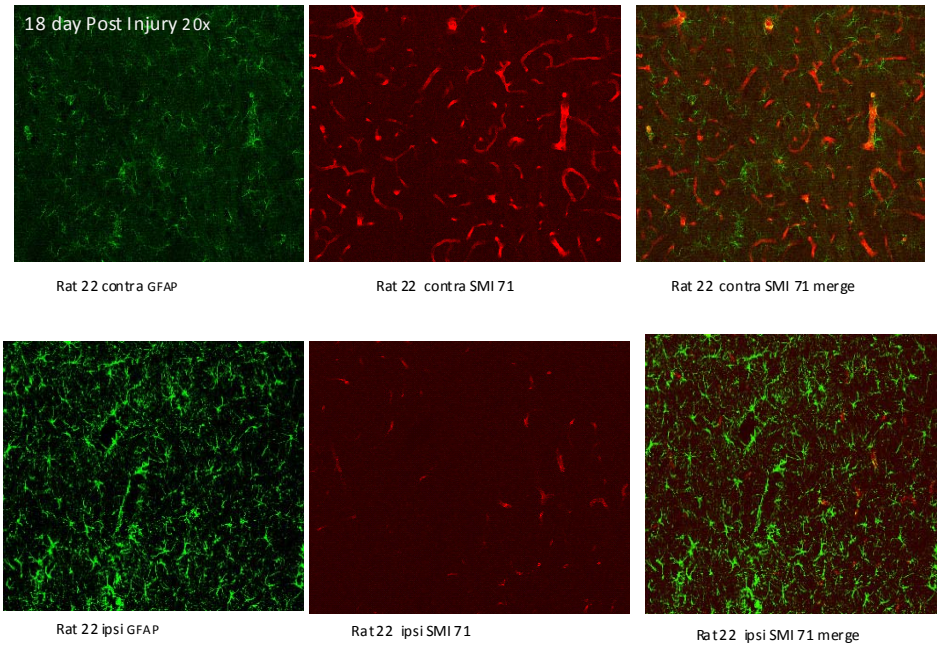

**Figure 16. Effect of mTBI on cortical levels of SMI71**




In order to quantitate the presence of blood bourne proteins in the brains exposed to 1atm TBI (mild TBI, no cell death but chronic inflammation with mild behavioral impairments) we used a stereological approach. Profiles of (nuclei, cell soma, etc.) were counted in specific anatomic regions, ex. hippocampus CA1, dendate gyrus, etc. on histolgical sections that were consistently 14.5 to 15.5 um in thickness. Profile counts were taken from at least three sections per animal for at least three different animals, averaged and calculated as profile counts within that specific region/per animal/per side and considered as one data point per side. Thus, the final number is a mean number of profiles counted within a three dimensional region (area of anatomical region x thickness of section). Since each region compared is similar, and the thickness of the sections were not statistically significant between the ipsilateral and the contralateral side (being from the same section) we report our counts as number of (nuclei, or cells) with the understanding that the counts are stereological estimates based on profile counts. All counts were done blind by three different individuals to eliminate bias. Our results confirm the qualitative results that are consistent with the persistent presence of blood bourne proteins indicative of persisitent inflammation. IgG and albumin were sparsely scattered throughout tissue concentrating in parietal cortex with an increase in ipsilateral over contralateral.This is an important finding from the perspective of being able to introduce therapeutic proteins in the injured brain.

In agreement with these findings, we also found Evans Blue (**EB; Figure 15**), which binds to circulating albumin and is normally excluded from the CNS parenchyma by the BBB, present after TBI. EB fluoresces in the red-far red range and can be used to assess areas of ACTIVE permeability/leakage in the brain/spinal cord. Whereas albumin immuno-histochem-istry will indicate the presence of albumin at different time points, it will not determine whether that leakage is active or if it occurred at an earlier time point. EB passage into CNS tissue indicates an actively leaky barrier. Finally, we also showed decreased appearance of SMI-71, a marker for BBB integrity (**Figure 16-17**). The resulting mTBI-induced decrase in SMI-71 expression is indicative of BBB dysfunction; a conclusion based on the known association of decreased SMI-71 with CNS injury resulting

**Figure 17. Effect of mTBI on thalamic levels of SMI71**




in BBB impairment and the induction of BBB impairment following treatment with antibodies to SMI-71 (Lu et al., 2010; Skold et al., 2005).

In summary, we have an array of biomarkers relevant to our anti-inflammatory intervention and the common histopathological markers used in common with other Mission Connect Consortium Projects and in each case are validating our results via the Core run by Dr. Ray Grill.

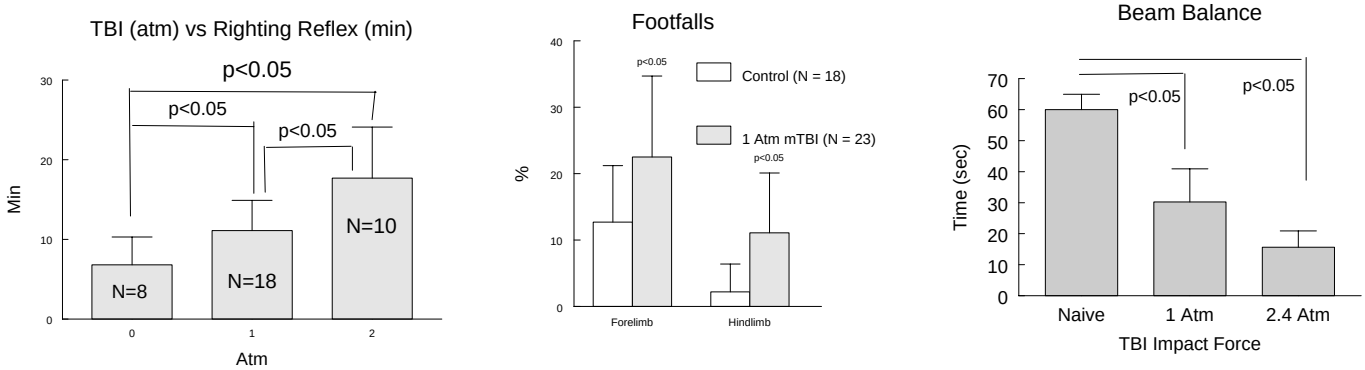
**Behavioral and Cognitive Testing:**

We also began to evaluate a number of behavioral outcomes using the same bimodal strategy we have applied throughout. Thus, we assessed early recovery processes such as righting reflex time, beam balance and foot faults, all known reliable assays that provide an accepted and fairly global index of behavioral recovery based on coordinated locomotor function.

**Beam Balance.**
The balance beam apparatus consists of a beam 91.5cm L x 1.7cm W and elevated 30cm off the surface below and secured to a platform on either end. The rat is placed on the center of the beam and released (start time) and is allowed to walk to either end. The animal is returned to the center of the platform if he walks onto one of the end platforms. This is continued for 2 minutes or until the rat falls off (stop time).

**Foot Fault.**
A wire mesh 69.5 cm W x 45 cm L with 3 cm gaps is stretched out over a wooded frame. The number of times out of 10 that the forelimb or hindlimb falls through the gaps is counted and is recorder as % foot faults.

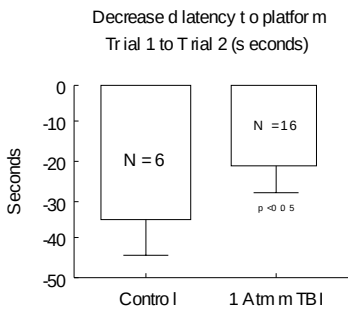
**Figure 18. mTBI-Induced effects on behavior**



**Morris Water Maze.**
The animals are assessed on days 11 through 15 post-injury. Four trials are run each day. The water maze is a 6' diameter tank, filled to 2 cm above the invisible platform that is 4 inches diameter. The water temperature is held at 22-24 degrees. The platform is stationary through out the experiment. The tank is divided into four quadrants and stationary cues are marked on the wall in each quadrant. Before the first trial, the rat is placed on the platform for 30 seconds. The animals' starting point is randomly chosen each day based on these quadrants, one trial is started from each quadrant. The SMART computer system is used to track and monitor the animal during the trials. After placing the animal in the water facing the wall of the tank, the handler leaves the room. The animal is allowed two minutes to find and climb the platform and escape the water (latency to platform); he must remain on the platform for 30 seconds. If the animal does not find the platform, the handler places him on it for 30 seconds before removing him from the maze. The animals are given a four-minute rest period in a warming chamber between each trial.

**Working Memory.**

**Figure 19. mTBI Effects on Working Memory**




One suggestion of the EAB was to develop and use a cognitive assay that is more relevant to the clinical outcomes observed in the field. That is, the goal is to have a reliable and sensitive assessment of "executive function". There are three ways to measure higher order cognitive function or executive function: working memory, reversal learning and recognition memory (Hamm, 2001). Of these the most sensitive is working memory. To that end we developed a working memory assessment that is now operational. Our initial validation has been carried out with moderate TBI and we are presently extending this to mild TBI. As shown in **Figure 19,** the assay yielded significant differences between shams and TBI exposed rats.

The procedures for using the MWM to assess working memory are described in detail by Hamm, et al. (1996). Briefly, the MWM is a black tank (180 cm diameter, 28 cm depth) filled with water and containing a clear plastic platform beneath the surface of the water. Each animal received four pairs of trials per day. For the first trial of each pair, the animal was randomly assigned a start point (north, south, east, west) and a goal platform position (1, 2, 3, 4). Rats were placed in the maze facing the wall of the pool and allowed a maximum of 120 sec to find the platform. If the rats failed to locate the platform within 120 sec, they were placed on the platform for 10 seconds. Rats that found the platform during the testing interval were allowed to remain on the platform for 10 seconds. After 10 seconds, the rats were placed back in the original starting position for the second trial. On this second trial of each pair, the starting position and platform position were the same as in the first trial of the pair. Rats were allowed 120 seconds to find the hidden platform and were required to stay on it for 10 sec. After the second trial, the rats were placed in a warmed chamber for 4 minutes. The second, third and fourth pairs of trials were performed identically to the first pair except that the start and platform locations were changed for each subsequent pair of trials. The rats were monitored continuously while they were in the water and any unable to swim were removed from the water immediately. The water maze performance assessments are done 7-14 days after TBI to allow time for any TBI-induced motor deficits to resolve (Hamm et al., J Neurotrauma 1996).

Our initial validation had been carried out with 2.4 atm moderate TBI and here we have extended our evaluations to a 1 atm. mTBI. Rats were anesthetized with isoflurane and subjected to a 1.0 atm mild parasagittal fluid percussion TBI (n = 16) and compared to controls (n = 6). Working memory was tested as described above on post-injury days seven through 11. As shown in **Figure 19,** there was a significant longer latency to find the hidden platform in the injured rats when compared to controls. These results indicate that significant impairment in working memory after mTBI can be measured. Working memory was tested as described above on post-injury days seven through 11.

In summary, we now have an array of behavioral assays: righting reflex, foot fault, beam balance (documented in third quarterly report, final report and report to EAB) as well as Morris water maze and working memory assessment. Interestingly, the Morris water maze appears to detect early deficits after TBI while the working memory assessment detects later deficits, suggesting the latter may be more clinically relevant to the clinical situation.

**IL-1Ra (Kineret) treatment.**

Treatment with Kineret (7.5 µg/µl, 75 ng/µl, and 750 ng/µl; injected i.p at 30min and 6h) resulted in an improvement in working memory (**Figure 20**) and a reduction in the presence of GFAP positive activated astrocytes (**Figure 20**). These proof of concept results demonstrate improved functional outcomes as well as a decreased expression of a biomarker that is associated with glial inflammation.

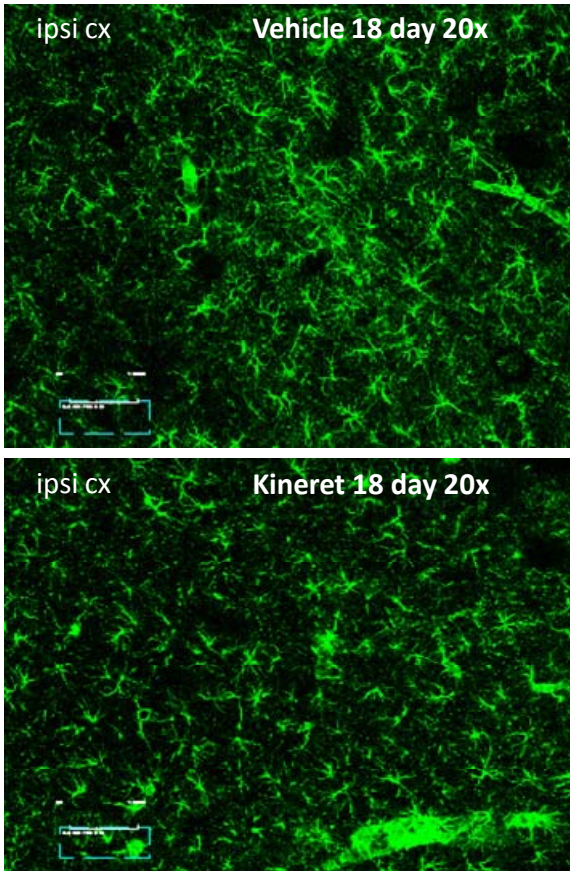
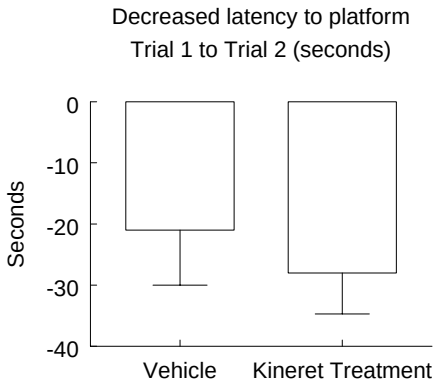



**Figure 20.Effect of Kineret Treatment of mTBI on Astrocytic Activation (GFAP) and Working Memory**




**Key Research Accomplishements**

- Determined mTBI-induced changes in a spectrum of cytokines and chemokines for three brain regions
- Developed a panel of reliable biomarkers to assess mTBI-induced perturbations in:
    - Inflammation
    - Increased BBB permeability
- Developed and applied behavioral assessments for evaluation of mTBI functional outcomes
- Performed proof of concept intervention with i.p. Kineret treatment with successful outcomes

**Reportable Outcomes**

• Presented Posters at following meetings:

Perez-Polo, J, DeWitt, DS, Rea, HC, Dash PK, Grill RJ, Parsley MA, Unabia G, Hulsebosch CE. Anti-inflammatory treatment of traumatic brain injury (TBI). Society for Neuroscience, 2009.

Hulsebosch, CD, DeWitt DS, Dash PK, Grill RJ, Parsley MA, Unabia, BC, Rea HC, & Perez-Polo, JR Role of IL-1 and TNF receptor activation in neurological deficits at TBI. Military health Research Forum, 2009.

Hulsebosch, CD, DeWitt DS, Dash PK, Grill RJ, Parsley MA, Unabia, BC, Rea HC, & Perez-Polo, JR Role of IL-1 and TNF receptor activation in neurological deficits at TBI. Natl. Neurotrauma Soc., 2009.

Dewitt, DS, Dash PK, Grill R, Parsley MA, Unabia GC, Rea C, Perez-Polo JR & Hulsebosch CE. Measures of neurological deficits after mild traumatic brain injury. Soc. Neurosci., 2009.

Rea, HC, DeWitt DS, Dash PK, Grill RJ, Parsley M, Unabia G, Hulsebosch CE, Perez-Polo, JR. Inflammatory mediators after TBI. 2010 Transaction of the American Society for Neurochemistry p 145 PTW07-01.

Rea, HC, DeWitt DS, Dash PK, Grill RJ, Parsley M, Unabia G, Johnson, KM, White, MM, Hulsebosch CE, Perez-Polo, JR. The Role of Specific Inflammatory mediators in Neurological Deficits after TBI. Neurotrauma Meeting, 2010.

Perez-Polo, JR, Rea HC, White MM, Johnson KM, Gril R, DeWitt D, Hulsebosch CE. Outcomes of mild traumatic brain injury in a rodent model. Soc. Neurosci., 2010.

**Conclusion**

We have a fully developed and validated treatment and evaluation protocol that is relevant to the military setting. We are now optimizing the treatment protocol by treating animals with a 2x dose at 30 min. and 6h. and a 1x dose every 24h for 11 days thereafter. We are also increasing the number of animals to 10 per trial component. Our goal being to proceed to a second treatment (Aim 3.33) as originally planned with Etanercept in early 2011 and to apply both treatments to blast injury during the last two years of the project. We will optimize our treatments to provide a realistic approach to treating soldiers in the field by a medic to administer an injection and after evacuation to a hospital setting there will be follow up injections, which will diminish the extent of long term behavioral and cognitive impairments. In the present proposal, we rely on two approaches to two targets of inflammation that are appropriate in a time frame of hours to days. We also believe there may remain targets beyond this time frame to be further explored once the approach here assessed has run its course in a clinical/military context.

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**Appendices None**