

PROPRANOLOL ELICITS LONG TERM SYSTEMIC EFFECTS AFTER
REPETITIVE MILD TRAUMATIC BRAIN INJURY

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DEDICATION

To my wife, Namratha, for all her encouragement, understanding, humor, and love on all the good days and the bad

To my parents, Thomas and Janet, for their understanding and sacrifices they made so I could have the opportunity to pursue my passions and these degrees.

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PROPRANOLOL ELICITS LONG TERM SYSTEMIC EFFECTS AFTER
REPETITIVE MILD TRAUMATIC BRAIN INJURY

There are almost 2 million new traumatic brain injuries (TBIs) every year in the US. Of these, 80% of these can be classified as mild TBI, also known as concussions, that can lead to pronounced long term symptoms months and years after injury. The presence of post traumatic headaches (PTH) is the most common chronic side effect with prevalence of 47-95% of mTBI patients within a week of injury. Though the mechanisms after TBI leading to these headaches and other post mTBI side effects are poorly understood, recent studies have suggested the role of the immune system after injury plays a causal role in this process. Peripheral immune cells can travel to the brain after mTBI as a result of blood brain barrier dysfunction, sympathetic nervous signaling, and the release of inflammatory mediators. Recent studies have shown sympathetic activation after injury can result in IL-10 dependent systemic immunosuppressive state after mTBI. In this study we sought to limit sympathetic dependent immune alterations after injury by injecting the beta blocker propranolol directly after injury and investigating the immune changes in the blood, brain, spleen, and bone marrow of mTBI animals. Together, these data show mTBI causes immune genetic and pathway level changes at least one month after injury and that propranolol alters genes important for metabolism, cytokine signaling, epigenetic modification, innate, and adaptive immunity. We also find that propranolol reduces the presence of Ly6C+ and increases the presence of Ly6C- monocytes in the blood one month after

injury; however, it leads to increased Ly6C+ monocyte presence in the spleen of mTBI mice. In conclusion, propranolol administration directly after mTBI leads to immune changes that may lead to long-term improvement in post TBI symptomology.

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LIST OF ABBREVIATIONS

AP-1	Activator protein 1
BBB	Blood brain barrier
BM	Bone marrow
CD	Cluster of differentiation
CCL2	Chemokine (C-C motif) ligand 2
CCR2	Chemokine (C-C motif) receptor 2
CNS	Central nervous system
CSF1R	Colony stimulating factor 1 receptor
CX3CL1	C-X3-C motif chemokine ligand 1
CX3CR1	C-X3-C motif chemokine receptor 1
DEG	Differential gene expression
EMT	Epithelial-mesenchymal transition
ENTPD1	Ectonucleoside triphosphate diphosphohydrolase-1
FCGRT	neonatal Fc receptor
HMGB1	High mobility group box 1
JAK1	Janus Kinase 1
Ly6C	lymphocyte antigen 6
IL-1 β	Interleukin 1 beta
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-10	Interleukin 10

IL-18	Interleukin 18
IP	intraperitoneal
ITGAM	Integrin alpha M
MAPK	mitogen-activated protein kinase
MET	Mesenchymal-epithelial transition
MHC	Major histocompatibility complex
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
NFIL3	Nuclear factor, interleukin 3 regulated
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	NLR family pyrin domain containing 3
P13K	Phosphoinositide 3-kinases
PTH	Post traumatic headache
PTGS2	Prostaglandin-endoperoxide synthase 2
RhoROCK	Rho-associated protein kinase
SNS	Sympathetic nervous system
SP	Sham Propranolol
SS	Sham saline
STAT1	Signal transducer and activator of transcription 1
TBI	Traumatic brain injury
TGF β	Transforming growth factor beta
TH1	T helper cell type 1
TLR4	Toll Receptor 4

TNF	Tumor necrosis factor alpha
TNFAIP3	Tumor necrosis factor alpha induced protein 3
TNFAIP6	Tumor necrosis factor alpha induced protein 6
TP	TBI propranolol
TS	TBI saline

Chapter One

General Introduction to Traumatic Brain Injury

Traumatic brain injuries are potential life changing injuries characterized by damage to the central nervous system and surrounding tissue. There are over one million mild TBIs, commonly called concussions, occurring annually leading to substantial economic burden. Concussions are most common in younger individuals with higher occurrence in high-income countries (Lefevre-Dognin., 2021). Common events leading to concussions may be from sport injuries, car accidents, falls, military activity, and physical altercations. Classification of what constitutes a mild TBI utilizes three main criteria: Glasgow Coma Scale (GCS) score, duration of loss of consciousness, and the presence of post-traumatic amnesia (PTA) (Lefevre-Dognin et al., 2021). GCS scores between 13-15, PTA for less than 24 hours, and impaired mental states such as disorientation/confusion at time of injury generally lead to the diagnosis; however, the presence of these defining criteria may last longer than 24 hours.

Issues with diagnosing mild TBIs are complicated by wide variability of clinical symptoms and signs. CT and MRI scans after mTBI often do not show presence of damage. However, human postmortem studies have shown people who have history of mTBI but died of nontraumatic causes show evidence of axonal injury (Blumbergs et al., 1994; Laskowski et al., 2015). Additionally, fMRI studies have shown decreased cerebral blood flow to prefrontal cortex subregions acutely after concussion in athletes that correlated with symptom severity (Chen et al., 2004; Laskowski et al., 2015). Furthermore, in the absence of structural

damage, electroencephalography (EEG) and transcranial magnetic stimulation (TMS) studies have shown both short- and long-term electrophysiological alterations in brain activity in the absence of mTBI symptoms (De Beaumont et al., 2007; Gosselin et al., 2006; Laskowski et al., 2015).

Common symptoms of mild traumatic brain injuries (mTBI) include headache, dizziness, nausea, fatigue, changes in sleep pattern, memory deficits depression, and anxiety although these may vary based on gender (Laskowski et al., 2015). One study reported concussed female athletes reported more drowsiness and noise sensitivity while male athletes reported amnesia and cognitive deficits (Frommer et al., 2011). The most reported side effect is the presence of post traumatic headaches (Long 2018). These usually occur one week after mTBI in 30-90% of patients and resolve; however, they can last months or years (Long 2018). The mechanisms manifesting these post traumatic headaches (PTH) are not well understood.

One avenue gaining traction for PTH and post TBI symptoms is the role of the immune system after injury. After a mild TBI, there may be meningeal inflammation, microglia activation, and perivascular macrophage recruitment to the injured area (Verboon et al., 2021). The primary injury leads to microglia activity characterized by cytokine release and trafficking to the impacted area. These microglia recognize PAMPs (pathogen associated molecular patterns) and DAMPs (Damage associate molecular patterns) through pattern recognition receptors (PRRs), such as TLR4, resulting in rapid response to the stimulus (Verboon et al., 2021; Scheiblich et al., 2020). These cells also lead to the recruitment of peripheral

monocytes, T cells, and neutrophils to aid in injury resolution. Activation of PRRs on microglia lead to inflammasome assembly and production of IL18 and IL1 β . Production of these inflammatory molecules further the inflammatory response by leading to further pro-inflammatory cytokine production. If these mechanisms fail to fully resolve the injury, progression to a more chronic, secondary neuroimmune stage begins with enhanced recruitment from the periphery.

With the wide range of symptoms after a TBI, it becomes apparent that controlling these symptoms is imperative to long-term post concussive management. However, there is no current treatment for concussion. Currently, treatment after concussion is largely supportive and based upon individual patient needs. Physical activity and exercise have shown the most promise for long-term prognosis; however, the benefits for these therapeutic interventions can take months if not years to see benefits (Naugle et al., 2021). Treatment for mTBI largely focuses on symptomatic treatment and interventions. Accordingly, tricyclic antidepressants (TCAs), anticonvulsants, calcium channel blockers, NSAIDs, CGRP antagonists, beta blockers have been emerging as promising molecular treatments to aid in post concussive symptoms (Leung 2020). As for how they work to benefit TBI patients, the mechanisms are not well understood.

There are a couple of models used to model mild traumatic brain injury. As it is tough to model it in humans, rodents are the main vessel to test mild traumatic brain injury. There are many different models used to test brain injury in rodents and each has its own benefit and consequence. The two general classes of modeling brain injury are either open head or close headed injuries. In open head

TBI, the skull is opened/removed, and the brain is exposed to external force. These injuries are akin to a stab or gunshot wound and are severe in nature. Close headed TBI models consist of controlled cortical impact model (CCI), fluid percussion injury (FPI), Blast injury, and weight drop model. These models do not require removal of the skull to induce TBI.

Fluid percussive injury is one type of close headed model used to inflict traumatic brain injuries upon rodents. This model has and focuses on inflicting widespread damage to an area of the brain (Lifshitz et al., 2016). Accordingly, the injuries are widespread and highly variable, another prominent injury model used to model mild traumatic brain injury in rodents is the blast injury. The blast injury is comparable to military IED explosions, and so is very translatable to the military population however, as the civilian population is not well exposed to this type of injury. It is not always the best model to characterize mild TBI (Smith and Garner 2018; Leung 2020). The weight drop model is well received as it is simple to set up; however, due to dropping a weight on the skull, the results are also highly variable as it is difficult to control whether the injury is mild, moderate, or severe (Ma et al., 2019). The model we have decided to use in our studies is the controlled cortical impact model (CCI). This model uses a focal impact concept to drive brain injury and can be used to inflict mild, moderate, and severe TBIs.

In rodents, the typical way to characterize mild, moderate, and severe injuries is not based upon GCS scores, level of consciousness, or post traumatic amnesia as it is in humans. Here, it is based upon the amount of damage to the skull. Mild injuries do not break the skull, and there should not be any noticeable

damage to the underlying brain parenchyma. Moderate TBI may have cracked skulls but no damage to the underlying brain. A severe injury in rodents is usually characterized by skull penetration, the brain itself has been damaged, and tissue may be missing (Siebold et al., 2018).

Headache pain and pain sensitivity post traumatic brain injury

With TBI's becoming more and more common every year, the incidence of post concussive symptoms is also increasing one of the most common complaints after a mild TBI is the presence of chronic persistent post traumatic headaches (PPTH). The presence of these headaches is more common with mild than they are for both moderate and severe injuries. While these post traumatic headaches are more common in the military population compared to the civilian population, these headaches are also the most common pain, side effect of mild traumatic brain injury in the civilian population with a prevalence rate of 47-95% (Leung 2020; Uomoto and Esselman 1993). Sadly, studies have shown that post traumatic headaches may linger and drastically impact quality of life upwards of six months or even five years after a single concussion (Stacey et al., 2017). Many studies have suggested early treatment is critical to avoid this. However, there are not many good treatments for post traumatic headaches with 87% of patients stating dissatisfaction with their post traumatic headache treatment (Stacey et al., 2017).

Post traumatic headaches are defined as a headache that has developed within seven days of a traumatic injury to the head, however, it only becomes persistent if this headache lasts more than three months, was not present prior to the injury (Leung 2020). Along with these headaches, people with this side effect

also report scalp allodynia (Ashina et al., 2020). While the cause for these headaches is not very well understood their cause is thought to have neuropathic or neuroimmune origins. Autonomic nervous system dysfunction is thought to play a role in headache, sensitivity, and onset (Leung 2020).

MTBI related headache pain fits the characteristics of neuropathic pain due to pain after the initial injury with the feeling of burning, numbness, or itching. Sensory or motor dysfunctions may also be present with photo/phono sensitivity, tinnitus, and gait instability. Additionally, recent studies have shown that the presence of chronic inflammation due to the presence of cytokines, deficits in glutamatergic signaling, and neuronal sensitization are leading causes for the development of the persistent headaches after mTBI (Mares et al., 2019). While it is difficult to find any structural abnormality upon neural imaging, diffusion tensor imaging studies have shown that MTBI patients suffer from diffuse axonal injuries to the corpus callosum, anterior corona, radiata, corticospinal, tract and internal capsules (Caeyenberghs et al., 2015).

Treatments for post traumatic headaches are currently an emerging field. TCAs, gabapentin, beta blockers, calcium, channel, blockers, and Calcitonin-gene Related peptide (CGRP) receptor antagonist are emerging tools used to fight off these headaches (Ashina et al., 2020; Ceriani et al., 2019). While it is difficult to study headache, pain and rodents, a recent study showed repetitive mild TBI leads to increases in CGRP levels, increased astrocyte activity, and microglia proliferation in the trigeminal pathway (Tyburksi et al., 2017). This further suggests the role of CGRP onset and potential for management headache management.

However, as CGRP release seems to be a consequence of mild traumatic brain injury, and the levels increase in pain states this suggests that CGRP is more for symptomatic treatment than prevention of mild TBI, pathophysiology, and pain preventions.

Additionally, sympathetic nervous system activity after TBI leads to the production of norepinephrine, epinephrine, and serotonin. The activity of these three monoamine neurotransmitters can lead to sensitized neurons. Therefore, limiting the activity of any of these is thought to modulate descending pain enters after TBI and studies have pursued this post TBI treatment strategy (Sahbaie et al., 2022). Recent mTBI studies showed mechanical sensitization in the periorbital region of the head, forelimbs and hindlimbs is often transient over several days (Sahbaie et al., 2022, Irvine et al., 2019; Ferguson et al., 2020).

Sympathetic nervous system activation with brain injury

In mild traumatic brain injury, the brain may not be structurally damaged, there may be blood brain barrier dysfunction, and neuroimmune activation. The numerous side effects after mTBI underscore the influence of the brain on various processes. The autonomic nervous system is made up of both the parasympathetic and sympathetic nervous systems. The coup-contrecoup force typically found in both human and mouse TBI injuries, can lead to activity in the sympathetic nervous system by way of direct hypothalamic activity through the HPA axis, or indirectly through cerebrovascular signaling by way of damage to the BBB. Vagal nerve activation, hypothalamic-pituitary-adrenal (HPA) axis, blood brain barrier (BBB) dysfunction, and meningeal irritation are amongst many of the

mechanisms leading to the systemic issues common to TBI. From these, HPA axis activation impacts cellular metabolism, immune cell function and production, blood flow, and oxidative stress (Pukrayastha et al., 2019). Activation of the sympathetic nervous system can lead to a catecholamine release from sympathetic nervous terminals, and these terminals can have effects on various target organs. The sympathetic nervous system directly interacts with the bone marrow, spleen, vasculature, heart, G.I. lung, liver, renal, CNS organ systems (Sribnick et al., 2022; Sahbaie et al., 2022). Changes in signaling can have quick systemic effects after TBI.

Neurotrauma, such as a brain injury, can lead to a hyperinflammation state also known as the systemic inflammatory response syndrome (SIRS). This can be simultaneously balanced by the compensated anti-inflammatory response (CARS) leading to immune dysfunction. There are four potential mechanisms of immunosuppression that are thought to be dependent upon sympathetic nervous activation. The first is the release of DAMPs which leads to the CARS response. The second mechanism is the release of MDSCs, which can suppress immune responses. The third mechanism is thought to be sympathetic, nervous terminals throughout the body through the release of Norepinephrine. The fourth and final mechanism thought to lead to post traumatic immunosuppression is due to cortisol release, which has been shown to reduce T cell function, migration, proliferation (Dong et al., 2016).

Post traumatic SNS activation has been linked with the dichotomous release of inflammatory IL6, IL8, and DAMPs lead to release of IL10 and activation

of immunosuppressive myeloid derived suppressor cells (MDSCs) in the first 24 hours after injury. The catecholamines surge can result in production of reactive oxygen species (ROS) followed by IL10 release, which serves to limit inflammation after an injury. This release can be, and typically is over sensitized and leads to an immunosuppressed state after injury. Furthermore, this immunosuppressed state increases susceptibility to infections and ability to respond to pathogens is decreased, thereby increasing the likelihood of further inflammation (Sribnick et al 2022) .

Additionally, sympathetic nervous system activity after TBI leads to the production of norepinephrine, epinephrine, and serotonin. The activity of these three monoamine neurotransmitters can lead to sensitized neurons. Therefore, limiting the activity of any of these is thought to modulate descending pain enters after TBI and studies have pursued this post TBI treatment strategy (Sahbaie et al., 2022).

One study found that two months after neurotrauma referral monocytes still wear anti-inflammatory phenotype (Schwulst et al., 2013). Inflammasome activation after TBI is linked with immunosuppressive state (Bortolotti et al., 2018). Sympathetic nerve synapses in the spleen can lead to immunosuppressive cell phenotypes, forming in this organ due to the presence of beta-2 receptors on T cells (Rosas-Ballina et al., 2008). Increased PD-L1 and functional T cell deficits were reversed with propranolol treatment following TBI (Yang et al., 2019). Sympathetic nerves also innervate the bone marrow. After burn injury, norepinephrine release has been shown to decrease the levels of IL6 and TNF α

production (Cohen et al., 2004). Additionally, catecholamines produced by bone macrophages have been shown to lead to decreased fracture healing and decreased bone volume (Kuhn et al., 2022). With the bone marrow's role in immune cell proliferation and development, injury induced changes here can lead to substantial systemic immunity changes.

Systemic immunity after TBI

Traumatic brain injury results in damage to the neurons and surrounding glial cells. Aiding in the injury response to these cells are peripheral immune cells, such as monocytes, neutrophils, NK cells, dendritic cells, B cells, and T cells, traffic into the CNS to aid in injury resolution. These cells either through the ability to kill, present antigens, make antibodies, phagocytize, or produce cytokines come to the CNS and seek to aid in the TBI inflammation cascade. Through a variety of mechanisms including the hypothalamic-pituitary-adrenal (HPA) axis, microbiome, autonomic nervous system alterations, immune activation in the gut, lung, spleen, thymus, liver, heart and kidney (Faden et al., 2021; McDonald et al., 2020; Royes and Gomez-Pinilla, 2019) immune cells throughout the body may respond to the injury and produce a local inflammatory response or traffic to the CNS. The HPA axis may be most sinister leading to systemic TBI-induced immune changes in vasculature wall thickness, immune cell production and differentiation, stem cell production, inflammatory molecule activity, and metabolism play a substantive role in the systemic effects of TBI.

There have been reported changes in the spleens of TBI animals resulting in increased inflammatory cell production in the spleens of TBI animals. Blood

vessels after TBI have shown increased vessel thickness, increased presence of inflammatory monocytes, and increased risk of atherosclerosis (Chen et al., 2019). The gut and lungs are two organs that are very important for immune system functioning. After TBI, changes in the gut microbiome are very common. The lungs of people after TBI have been shown to change in immune cell composition as well. Given that greater than 70% of immune cells either end up in the lungs or gut, adequate functioning of these two organs is necessary to prevent an immunocompromised state.

Bone marrow is very important to immune cells production. Changes in the bone marrow niche after TBI can then result in changes in the immune system of the impacted individual (Dill et al., 2022;.Feng et al., 2021) The production of stem cells by bone marrow leads to the replenishment of immune cells in various organs (Holmin et al., 1995; Kuhn et al., 2022). Therefore, changes in the bone marrow due to sympathetic nervous activity can have a drastic impact on systemic immunity. The immune cells from the bone marrow travel to various lymph organs and lymph nodes, such as the spleen and thymus, to become educated, properly functioning immune cells (Cano and Lopera, 2013). Furthermore, changes in the environments of these organs can impact immunity by altering the function and basal state of these immune cells. Cells in the thymus and spleen learn to differentiate between self and non-self in these regions; therefore, changes in immunity in these organs can lead to autoimmune conditions and predisposition to sickness (Cano and Lopera, 2013). While its hotly debated to the extent peripheral immune cells replace microglia cells of the CNS, peripheral macrophages do

penetrate the brain parenchyma and stay as perivascular macrophages which help in injury resolution and immune maintenance of the CNS (Ritzel et al., 2015; Theriault et al., 2015).

Beta Blockers and Propranolol

Catecholamines, such as epinephrine and norepinephrine, bind to alpha- and beta-adrenergic receptors and induce various activities. These receptors are throughout the body and can lead to relaxation of the vasculature through cAMP and PKA leading to genomic changes through C/EBP. These receptors are found throughout the CNS, in the heart, spleen, fatty tissue, blood vessels, bone marrow, and on immune cells. Beta blockers are a drug class involving beta-adrenergic receptors. They can either be selective, such as metoprolol, which targets only beta-1 receptors, or non-selective, such as clonidine and propranolol, which both can have activity on beta 1 and beta 2 receptors (Loftus et al., 2019; Lopez et al., 2022). These medicines are typically given in the context of blood pressure management but can also work to lower the heart rate as well (Ding et al., 2021).

Propranolol is one of the best at this due to its activity at both beta receptor types; however, propranolol has side effects including causing headaches, dizziness, and nausea due to its cardiovascular effects (Lewis 1966; Anath and Lin 1986; Siberstein 2015). It has a short half-life of 3-6 hours but can take 15-36 hours to be fully eliminated from the body (Shand 1974; Kalam et al., 2020; Krukemeyer 1990). Propranolol is lipophilic so it can be given peripheral and transition through the blood brain barrier (BBB) to act within the CNS. Many studies have begun to investigate the role of beta blockers and propranolol in the context

of injury. Due to the presence of beta receptors in the bone marrow and on immune cells, the role of beta blockers shows promise in treating inflammation after injury. Propranolol is typically thought to have anti-inflammatory properties by leading to the release of anti-inflammatory cytokines and aiding in injury resolution after injury (Woiciechowsky et al., 1999; Michalovicz et al., 2021). IT has shown much promise in the burn injury field because it not only slows metabolism but also limits inflammation further reducing the energy expenditure (Lam et al., 2022). To get these beneficial effects, propranolol is typically administered multiple times a day clinically (Shand 1974; Kalam et al., 2020).

In the context of traumatic brain injury, beta blockers have shown much clinical promise as they are first line treatments for headaches; however, the mechanism leading to their beneficial effects are not well understood (Leung 2020). One potential mechanism is the relaxing of meningeal blood vessels increasing vascular permeability (Leung 2020; Siberstein 2015; Ha and Gonzalez 2019). However, due to their presence on many cell types, activity on immune cells is another mechanism thought to confer headache relief though decreasing the presence of inflammatory cytokines that are leading to neurogenic inflammation (Lopez et al., 2022; . A growing body of literature has sought to investigate the role of propranolol in mild, moderate, and severe TBI but typically these studies administer the drug multiple times daily for several weeks at a time (Khalili et al., 2020; Ley et al., 2009; Ko et al., 2016; Foley et al., 2008). What we seek to do in this thesis is to understand the impact of a single propranolol dose given at the time of injury and investigate its impact on the immune environment of the blood,

brain, spleen, and bone marrow. Our hypothesis is that giving a beta blocker, such as propranolol, at the time of injury will limit catecholamine dependent sympathetic nervous system activation and result in beneficial long-term changes in these organs. We further hypothesize that a single dose of propranolol will confer long term benefits due to the proximity to injury and control sympathetic dependent immune activation.

Project Outline

The aim of this project is to investigate the systemic effects of mild TBI on the monocyte populations of the blood and spleen while further characterizing the pathway and genetic level changes evident in the bone marrows, spleens, and brains of male C57B6 mice. Additionally, we sought to understand if peri-injury administration of the non-selective beta blocker propranolol would lead to long-term impact on the mild TBI induced immune changes. Our lab and others have shown TBI leads to changes in behavioral sensitivity, peripheral immune changes, and neuroinflammation that can last several weeks and months after injury. In Chapter Two, we investigate how mild TBI influences monocyte populations in the blood and spleen in addition to altering the pathway and genetic expression of immune genes as far as one month after injury. In Chapter Three, we introduce propranolol and characterize how a clinically relevant dose impacts the blood, brain, and spleen immune responses after mild TBI. We found that post mTBI propranolol administration leads to decreased Ly6C⁺ cells but more Ly6C⁻ cells present in the blood one month after injury. In the spleen we found decreased total monocytes and Ly6C⁺ monocyte but no changes in Ly6C⁻ one month after injury.

We found numerous propranolol induced DEG changes in both the brain and spleen leading to changes in several immune pathways. In Chapter Four, we take these immune changes to the next level by investigating the effect of post mTBI propranolol administration on the bone marrow stem cell pathways. Here we found many DEG changes in genes impacting epigenetic, metabolism, and cell signaling up to a month after the injury. We hypothesize that mild TBI induced-SNS activation plays a major role in TBI systemic immune dysfunction and that limiting this activation with beta adrenergic blockade can lead to substantive changes in spleen, blood, bone marrow, and brain that can potentially lead to long-term benefits after mTBI.

Chapter Two

Repeated Mild Traumatic Brain Injury in Mice Elicits Long Term Innate Immune Cell Alterations in Blood, Spleen, and Brain

Introduction

Every year millions of individuals experience mild traumatic brain injuries (mTBI), which often result in post-traumatic headache (PTH). The prognosis of PTH is favorable with most cases resolving within 6 months. (Stovner et al., 2009) Little is known about the pathogenesis of PTH, although primary injury to brain tissues likely results in the initiation of cephalic inflammatory responses which may serve defensive/reparative functions. The importance of the inflammatory response may not be limited to the nervous system as several lines of evidence suggest that peripheral immune cells are affected by the central cellular response activity to injury. (Rowe et al., 2016; Fehily and Fitzgerald, 2017; Jassam et al., 2017; Huber-Lang et al., 2018)

Inflammation, both neuroinflammation and systemic inflammation, triggered by closed head trauma play an essential role in the short and long-term effects injurious events. For example, traumatic head injuries are known to elicit an increase in systemic pro-inflammatory cytokines IL-1b, TNFa, CCL2, HMGB1, which all may serve to sensitize surrounding nervous tissue. (Ji et al., 2018; Domoto et al., 2021) Interestingly, injury-induced early pro-inflammatory cytokine release leads to a strong IL-10 influenced anti-inflammatory wave that seeks to control rampant inflammatory activity and restore homeostasis of the system. (Doganyigit et al., 2022) This natural progression works well in a functional immune

system; however, trauma such as repetitive mTBI, may evoke a condition of continuous immune cell over-activation that interferes with the ability of the innate immune system to adequately respond to challenge. (Kim et al., 2016; Fehily and Fitzgerald, 2017)

Animal studies show an immune challenge after mTBI can lead to deficient cytokine production and release. (Rowe et al., 2016; Fehily and Fitzgerald, 2017; Jassam et al., 2017) This uncoupling of the balance of pro-and anti-inflammatory cytokines results in difficulties in communicating local environment changes and can cause long-term changes in nervous tissue if left unchecked. These changes can include alterations in neuronal firing, changes in gene and protein expression, excessive or defective cytokine presence, and pain symptomology. (Niu et al., 2020). Immunosuppression can also lead to deficient activation and recruitment of peripheral immune cells further leading to incomplete inflammatory resolution to a CNS head injury. Recent findings suggest that repeated mTBI (rmTBI) can impact systemic leukocyte trafficking from the blood and other peripheral lymph organs. (Hiskens et al., 2021; Toutonji et al., 2021) In the present work, we set out to elucidate the influence of rmTBI on populations of circulating and non-circulating innate immune system cells and changes in gene expression changes which may contribute to the possible underlying pathologies of the murine injury model. We chose this highly reproducible animal model as repeated closed head trauma is a major risk factor for active military personnel, contact sports participants and aged population prone to falls.

Methods

Animals

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Indiana University School of Medicine, which are in accordance with National Institutes of Health guidelines for the care and use of laboratory animals. 60 Wild type C57BL/6 mice were obtained from Jackson Laboratory at 8-12 weeks old (~25-30 g) and were randomly assigned to sham and rmTBI for downstream analyses. Mouse groups were further subdivided into one day, one week, and one-month timepoints with a total of n=5 for each group for all immunohistological assessments.

Repeated mild traumatic brain injury (rmTBI) procedure used a technique previously described. (Creed et al., 2011; Han et al., 2020; Nguyen et al., 2021) Briefly, mice were anesthetized with 2–4% isoflurane and heads were stereotactically fixed with heat-pad support below the abdomen. Closed head mTBI was produced using a control cortical impact (CCI) device after shaving, cleaning, and sanitizing the area. After the baseline point of the device was set by lowering the tip to the skull surface and the stage position was set to zero, the impactor was retracted, and the impact depth was set. The approximate center of the impact site was 1 mm posterior to the bregma and 0.5 mm lateral from midline on one cortical hemisphere. The skull was struck with the impactor tip at a speed of 3 m/s to a depth of 1 mm. Sham injuries were shaved, cleaned, sanitized but no impact was performed on the skull. The wounds were sutured, and animal was placed on heating pad until fully recovered. All animals survived the injury without

any skull fracture or hemorrhage. Sham injury or mTBI manipulations were performed once a week for three weeks (total of 3 injuries). Intraocular pressure (IOP) changes were used as a surrogate for injury-induced intracranial pressure changes. These changes were assayed using an ICARE TONOLAB (Vantaa, Finland) tonometer device in both the mouse eyes to confirm skull impact severity and verify the absence of skull fracture. (Wei and Spaeth, 2011; Chen et al., 2019) The IOP measurements were done immediately before and after the injury for up to 30 min (Figure 2.1, n = 5 mice in each group). If the skull bone was fractured by the CCI device, there would be an absence of IOP. Alternatively, if there were no changes in IOP, the delivered impact to the skull bone was deemed to be insufficient and the animal excluded from the study.

Blood Processing

Mice were placed into CO₂ chamber for euthanasia then once breathing stopped, surgical scissors were used to open the chest cavity and 500uL blood was obtained transcardially with an EDTA coated 1mL syringe. Blood was then placed into a 1.5mL microcentrifuge tube for flow cytometry staining.

Spleen processing

Spleen were isolated from mice and placed directly into complete RPMI media (88% RPMI 1640 [ThermoFisher], 10% Fetal Bovine Serum [Sigma-Aldrich], 1% Penicillin Streptomycin [Sigma-Aldrich], and 1% Glutamax [ThermoFisher]). Under sterile conditions and on ice, spleens were minced, cut, and filtered with complete RPMI media through 70 um filter into 50 mL tube,

centrifuged at 1300g x 10 minutes, and the supernatant was discarded. The pellet was then resuspended in 5mL RBC lysis buffer for 10 minutes at RT, centrifuged at 400g x 5 minutes, and resuspended in PBS. The pellet was then once again centrifuged. The Pellet was then resuspended in a 3 mL FACS buffer and 1mL of this resuspended portion was taken for flow cytometry analyses. The remaining 2 mL were frozen down with a combination of DMSO, fetal bovine serum, and complete RPMI media at -80C and then placed in liquid nitrogen for future RNA analyses.

Brain processing

After mice were placed into CO2 chamber and blood was drawn transcardially, mice were decapitated with scissors. The skull was then cut along the midline and the bone surrounding both hemispheres were removed and whole brains were dissected out using sterile techniques and cleaned while in calcium free PBS before placement in liquid nitrogen for future RNA processing.

Flow cytometry

To identify monocyte and monocyte subsets in the blood and spleen, both blood and spleen tissues were stained with CD45-BV711 [Biolegend, San Diego, CA], CD11b-Fitc [BD Biosciences, East Rutherford, NJ], CD115-PE [Biolegend, San Diego, CA], Ly6C-APC-Cy7 [Biolegend, San Diego, CA], and Fc CD16/32 block [BD BioScience, East Rutherford, NJ].

Blood flow staining

Briefly, whole blood was filtered into a 5mL round bottom FACS tube with 35um strainer cap. Next, 10uL of blood from this FACS tube was added to FMO and unstained control tubes. Subsequently, Antibodies were added for 20 minutes, in the dark, at room temperature. Cells were then washed with FACS buffer and centrifuged at 400g for 5 minutes at RT. Supernatant was discarded and the pellet was resuspended in 2mL of RBC lysis buffer for 10 mins at RT, centrifuged, and pellet was resuspended for FACS buffer wash. Lastly, pellet was resuspended in FACS buffer for flow cytometry data acquisition.

Spleen flow staining

Single cell suspended splenocytes were first washed in FACS buffer, centrifuged at 400g x 5 mins, and the supernatant was discarded. Antibodies were added for 30 minutes in the dark at 4C. After 30 minutes, FACS buffer was added, and tubes were centrifuged once again. Supernatant was discarded and cells were resuspended in FACS buffer before data acquisition. For both spleen and blood analyses in our study, fluorescence minus one control were utilized in addition to unstained control samples. Data was acquired on a BD LSR Fortessa X-20 with appropriate voltages, gating, and compensation. Nonclassical monocytes were defined as CD45+CD11b+CD115+Ly6C- and classical monocytes were defined as CD45+CD11b+CD115+Ly6C+. Total monocyte percentages are the summation of CD45+CD11b+CD115+Ly6C- and CD45+CD11b+CD115+Ly6C+ percentages (Figure 2.1).

NanoString RNA processing

100 ng of RNA was isolated from spleen and brain from five separate experiments per group in TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. RNA samples were analyzed by nCounter gene expression analysis and quantified with the nCounter Digital Analyzer (NanoString Technologies). Expressions of 561 genes (including 14 internal reference genes) were analyzed using nCounter Immunology Panel™ (mouse) (NanoString, XT-CSO-MIM1-12). To minimize variability among arrays, densitometry values between arrays were normalized using the Robust Multichip Average function and further transformed to the logarithmic scale (log₂). Gene expression levels in each sample were normalized against the geometric mean of six housekeeping genes including Cltc, Gapdh, Gusb, Hprt1, Pgk1, and Tubb5. A cutoff was introduced at the value of the highest negative control present on the chip. Fold changes were calculated using the average of each group. For each experiment, the fold changes were calculated comparing the rmTBI group to their appropriate sham-injured controls.

Sample detection and analysis were completed on a nCounter® Digital Analyzer. Raw data processing, quality control, and normalization were performed using the nSolver™ 4.0 analysis software. Quality control (QC) and normalization were performed with an imaging QC of >75% field of view registration, binding density QC within 0.1-2.25 range, positive control linearity QC of R² above 0.95, and positive control limit of detection set as 0.5 fM positive control above 2

standard deviations above the mean of the negative controls. Normalization to housekeeping genes, of which genes below 100 were excluded. Pathway scoring, gene set analysis, differential expression analysis were completed using the Advanced Analysis software plugin (version 2.0.115). For gene set analysis, pathway scoring, and differential expression analysis, a p -value of ≤ 0.05 was applied as cutoffs. For all NanoString analyses at 1 day, 1 week, and 1 month after injury, gene expression measurements for each group were normalized to the sham injured ($n=3$) baseline of each timepoint.

Statistics

All flow cytometry statistics were performed using GraphPad Prism 9 (GraphPad Software, La Jolla, California) and values are reported as mean \pm S.E.M. Flow data was analyzed using one-way ANOVA. Data yielding significance was further processed with Tukey's HSD post-hoc analyses were applied for further comparisons between specific groups. NanoString analysis for significant genes used Bonferroni adjusted p -value with a cut off p -value of ≤ 0.05 using sham saline for each timepoint as baseline. Genes were further characterized in Prism with log2 values reported as mean \pm S.E.M. These data were analyzed using two-way ANOVA with Sidak's multiple comparisons post hoc tests.

RESULTS

Repetitive mTBI impact on blood and splenic monocyte populations.

To understand the impact of rmTBI on monocyte populations, PBMCs were isolated from blood and spleen samples at one day, one week and one month

following rmTBI or sham injury (Figure 2.1). In mice, two populations of monocytes exist and can be discriminated by variable expression of lymphocyte antigen 6C (Ly6C). Monocytes expressing high levels of Ly6C (Ly6C⁺ monocytes) have proinflammatory and antimicrobial functions. Monocytes with low expression of Ly6C (Ly6C⁻ monocytes), also known as patrolling monocytes, survey the vasculature by constantly crawling along the lumen of the vasculature and are involved with early responses to inflammation. The rmTBI did not significantly alter total blood monocyte populations at one day or one week after injury ($p=0.0657$ and $p=0.0567$, respectively; Figure 2.2A). However, the repetitive mTBI did produce increases in the total number of monocytes (Figure 2.2A; $p<0.0001$), CD45⁺CD11b⁺CD115⁺Ly6C⁺ ($p<0.0001$), and CD45⁺CD11b⁺CD115⁺Ly6C⁻ ($p<0.001$) compared to sham injured controls one month after injury (Figure 2.2C).

Like the blood monocytes, we did not detect significant changes at the one-day timepoint for any of the splenic monocyte populations (Figure 2.3; *total monocytes*: one day, $p=0.9881$; Ly6C⁺: $p=0.8100$; Ly6C⁻: $p=0.7874$). In contrast to blood at one week post rmTBI spleens derived from rmTBI spleens exhibited significantly decreased splenic monocyte numbers population ($p<0.05$) without significant decreases of splenic Ly6C⁺, ($p=0.9098$) and Ly6C⁻ ($p=0.8908$) monocyte numbers compared to sham injured controls. One month following rmTBI, the spleens exhibited significantly increased numbers of total monocytes ($p<0.0001$), Ly6C⁺ ($p<0.0001$), and Ly6C⁻ ($p<0.001$) numbers. These data mirror the results of the blood monocytes as rmTBI spleens also displayed increased total monocytes, Ly6C⁺ monocytes, and Ly6C⁻ monocytes one month after rmTBI.

Repetitive mTBI (rmTBI) failed to produce immunological gene changes at one day, one week, and one month in the murine spleen.

To further explore transcriptional changes in spleen and brain tissue following rmTBI and sham injury, RNA profiles were analyzed using the NanoString “Mouse immunology CodeSet” which comprises of 561 immunology-related gene transcripts involved in signaling pathways. Murine splenic changes in gene transcript expression using volcano dot plot of NanoString analysis at one day, one week or one month following the rmTBI paradigm generally did not reach statistical significance (Figure 2.4A, C, E). Though overt gene expression was lacking in splenic tissue there were transient decreases in adaptive immunity, apoptosis, B cell receptor signaling, chemokine Signaling, host-pathogen interactions, lymphocyte activation, MHC class I and class II antigen presentation, oxidative stress, T cell receptor signaling, TGF-beta, Th17 differentiation, Th2 differentiation, TLR, TNF signaling pathways. Differential pathway scores were also different between injury and sham conditions for T Cell Receptor Signaling ($p < 0.01$), Th1 Differentiation ($p < 0.01$), and autophagy ($p < 0.01$) at one month (Figure 2.4F).

Murine brain changes in gene expression at one day, one week, or one month were also lacking following the repetitive mTBI paradigm (Figure 2.5A, C, E). Significant differences were observed in signaling pathway at one day following rmTBI including host-pathogen interactions, lymphocyte trafficking, MHC Class II antigen presentation, phagocytosis and degradation, complement system and autophagy. Signaling pathways were also increased in brain tissue at one week for adaptive immunity, cell adhesion, complement system, cytokines, hemostasis,

host-pathogens, innate immunity, lymphocyte activation and trafficking, MHC class I antigen presentation, NF- κ B, phagocytosis and degradation, TGF β , Th2 differentiation, TLR, transcriptional regulation, regulatory T cell differentiation, and Types I and II interferon signaling (Figure 2.5D). One month after rmTBI we still detected significantly increased pathway scores for complement, inflammasomes, MHC class I antigen presentation, phagocytosis, and lymphocyte trafficking (Figure 2.5F). Only signaling pathways associated with phagocytosis, complement system, and MHC class I antigen presentation were significantly increased for all three timepoints in rmTBI brains.

Chemokine and cytokine related differential gene expression (DEG) analysis of *csf1r*, *tnfaip6*, *jak1*, *itgam/cd11b*, *cd3e*, *il10*, *fcgrt*, *nfil3*, and *cd99* at one day, one week, and one month was limited to decreased *cd99* expression one day post injury (Figure 2.6, $p < 0.01$) and increased *jak1* at one month in the spleen (Figure 2.6, $p < 0.01$). Brain tissue DEG exhibited a decrease in *nfil3* mRNA (Figure 2.7H, $p < 0.05$) at one day after rmTBI and increased *csf1r* mRNA at one week and one month ($p < 0.05$). Significant increases of DEG for *tnfaip6* ($p < 0.05$), *itgam/cd11b* ($p < 0.01$), *cd3e* ($p < 0.01$), and *fcgrt* ($p < 0.01$) were limited to one month following rmTBI (Figure 2.7). Additional DEGs for CX3CL1, CX3CR1, CCL2, CCR2, IL1 β , IL-2, IL-4, IL-18, TNFAIP3, PTGS2, ENTPD1, TLR4, CD14, CD19, CD36, CD40 for the brain and spleen are also depicted (Figures 2.8 and 2.9, respectively).

Discussion

The aim of this study was to comprehensively characterize changes in mononuclear cell populations by flow cytometry and immunological transcriptome following rmTBI. Our longitudinal measures showed that, together with statistically significant increases in Ly6C⁺ and Ly6C⁻ monocyte negative cells at one month in both blood and spleen, we noted changes in the expression of genes belonging to pathways associated with the innate immune response in the brain (MHC Class II antigen presentation, phagocytosis and degradation, complement system and autophagy) and spleen (T Cell Receptor Signaling, Th1 Differentiation, and autophagy). Similar to our approach, two previous studies which examined the inflammatory transcriptome following TBI also found long-lasting dysregulation of immune associated genes. (Boone et al., 2019; Toutonji et al., 2021) The study intervals demonstrated that leukocyte mobilization and function is altered in the blood, brain, and spleen after concussive head trauma. These combined findings provide a plausible mechanism by which mild traumatic brain injury may alter cerebral inflammation and modulate systemic leukocyte trafficking.

Being able to predict long-term dysfunction in vivo with clinically relevant blood-based biomarkers offers opportunities for early identification of pathology and progression management of post-traumatic headache. Subsequently the increased prevalence of Ly6C⁺, and Ly6C⁻ monocytes at one month after injury in both the blood and spleen suggests injury-augmented populations of cells remain in both circulation and tissues for prolonged periods of time. Canonical peripheral immune cell trafficking to target tissues such as the CNS post injury typically

involves cells migration through the blood-brain barrier and into the CNS parenchyma and may reflect the increased CSF1R and CD11b signaling present in the brain tissue one month after injury.

The Ly6C⁺ cell population typically expresses higher levels of CCR2 than Ly6C⁻ monocytes, and Ly6C⁻ monocytes canonically express more CX3CR1 to respond to its CX3CL1 ligand. CCL2 can lead to recruitment of both reparative and inflammatory monocytes, however, it predominantly recruits inflammatory monocytes due to the increased expression CCR2 on these cells. (Fenn et al., 2014; Tian et al., 2017) We investigated mRNA levels of CCL2, CCR2, CX3CL1, and its receptor CX3CR1; however, we found no difference in expression levels at any time point for these genes. Although we did not investigate protein levels, it is known that many of these chemokines are robustly upregulated and released by injured or inflamed tissue for extended periods of time. (Semple et al., 2010)

The source of the enhanced populations of Ly6C⁺ inflammatory monocytes may be, in part, due to an enhanced myelopoiesis around one month after injury resulting in the selective accumulation of monocytes, albeit immature monocytes, in the periphery. (Engler et al., 2004; Wohleb et al., 2011; Heidt et al., 2014) These immature monocytes have been identified as Ly6C⁺ and are likely pro-inflammatory in nature. (Geissmann et al., 2003) Previous studies suggest that the release of these immature monocytes from bone marrow is due to prolonged stress resulting in exacerbation of inflammatory conditions and pathology. (Wohleb et al., 2011; Hanke et al., 2012) If chronic stress associated with the mTBI produces the enhanced cell population, it is entirely possible that b-adrenergic

antagonism may reduce the presence of Ly6C⁺ inflammatory monocytes in both the blood and the spleen. (Hanke et al., 2012; Powell et al., 2013; Heidt et al., 2014)

By investigating differential expressions of several interesting genes, we detected only limited changes in the splenic tissue. One gene which was increased, Janus Kinase 1 (JAK1), occurred one month after injury was accompanied by a decrease in autophagy signaling. JAK1 is employed by a variety of cytokine receptors including IL-2, IFN γ , and IFN γ signaling but the underlying molecular mechanisms of JAK1 regulation are still largely elusive. (Zhang et al., 2012; Xu et al., 2022) A similar upregulation of phosphorylated JAK-1 pathway has been observed after moderate TBI. (Gao et al., 2020) Though not tested herein, injury-induced expression of phosphorylated JAK1 pathway and activation of autophagy has been shown to occur when the injury is combined with the use of rapamycin. (Gao et al., 2020)

Most cytokine expression remained largely unchanged across time. There were exceptions which included tumor necrosis factor-inducible gene 6 (TNFAIP6) upregulation in the brain at one month. TNFAIP6 is known to be anti-inflammatory and thought to reduce inflammatory response following postnatal endotoxin exposure in young rats (Bertling et al., 2016) and controlled cortical tissue impact injury in mice. (Mutoji et al., 2021) Another cytokine related to TNFAIP6 is TSG6 which is thought to be important for monocyte differentiation as it helps signaling the change from a pro to anti-inflammatory phenotype. (Mittal et al., 2016) It is

entirely plausible that JAK1 influences IFN γ response and impacts TSG6 expression following brain injury. (Li et al., 2018; Mutoji et al., 2021)

CSF1R/CD115 was found to be significantly differentially expressed at both one week and one month post injury in the brains of male mice. CSF1R is found on a few cell types but most notably microglia, dendritic cells, and monocyte-derived macrophages in the brain with roles in homeostasis, neurogenesis, and neuronal survival in the CNS. (Hume et al., 2020) Its ligand, CSF1, is produced after damage and promotes the chemotaxis and proliferation of CSF1R containing cells. (Hume et al., 2020) The observed increase in CSF1R suggests increased peripheral trafficking to the brain or increased microglia activity although we cannot specify as to which population is most driving the increase. However, the increase in both blood and spleen monocyte numbers coupled with the increased CSF1R mRNA in the brain one month post injury suggest monocytes may be the main contributor to these observations. Concurrent with the increased expression of CSFR1 was enhanced levels of ITGAM/CD11b at one month post injury in the brain. Peripherally, CD11b is typically found on a number of PBMCs, while central expression is limited to microglia (Kim et al., 2016; Jassam et al., 2017; Khan et al., 2018). The degree to which increased expression of CD3e in the brain at one month is representative of modifications of the T cell receptor structure is unknown. Together, these data suggest that there may be ongoing mTBI inflammation at one month which alters adaptive immune signaling pathways, but also modulates the function of innate immune cells of the vasculature, brain, and spleen.

Our study demonstrates that genetic alterations which are functionally associated with innate immune cell types may influence long-term mTBI sequelae and dysfunction. Future studies using spatial resolution techniques may provide insight into these genetic changes which likely occur in both the spleen and brain. Manifest protein level changes due to injury may also provide additional data and understanding of the long-term injury response and potential treatment. Broadening the investigation to include other populations of innate and adaptive immune cells will likely yield a better understanding.

Figures And Tables

Figure 2.1. Experimental setup, flow gating strategy, and mTBI apparatus

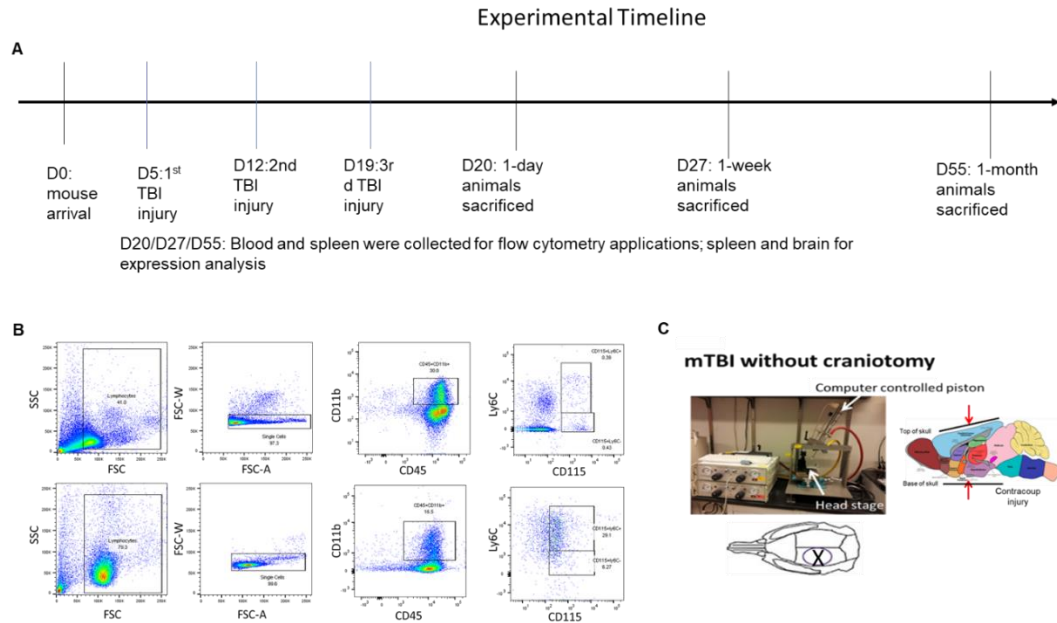


Figure 2.1. (A) depicts the timeline of the experimental setup with injuries and injection schedules. (B) Representative gating strategy used to identify monocytes (CD45+CD11b+CD115+) and monocyte subsets (CD45+CD11b+CD115+Ly6C+/-) from blood and spleen PBMCs. (C) shows our controlled cortical impact TBI setup and location of impact on the mouse skull.

Figure 2.2. Blood total and monocyte subsets vary over time with repeated mTBI.

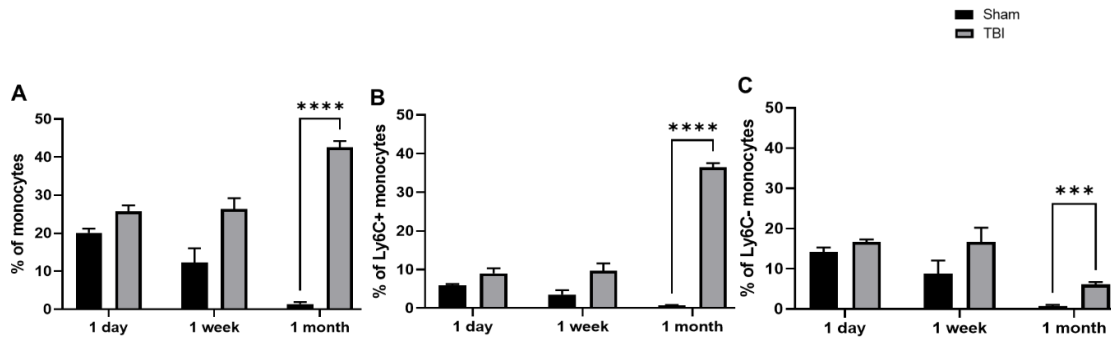


Figure 2.2. Flow cytometric total and subsets blood monocyte percentage varies overtime with rmTBI. **(A)** Total monocyte population percentages at 1 day, 1 week, and 1-month timepoints. **(B)** Displays Ly6C+ monocyte and **(C)** displays Ly6C- monocyte population percentages isolated from PBMCs of the blood from sham injured and rmTBI animals. While there were no changes one day or week after rmTBI, total monocytes, Ly6C+ and Ly6C- monocytes were all increased one month after injury. Data shown as mean +/- SEM, n=5. *P < 0.05, **P < 0.01, ***P < 0.001, ****P<.0001 compared by 2-way ANOVA followed by Šídák's multiple comparisons test.

Figure 2.3. Splenic total and monocyte subsets vary over time after repeated mTBI.

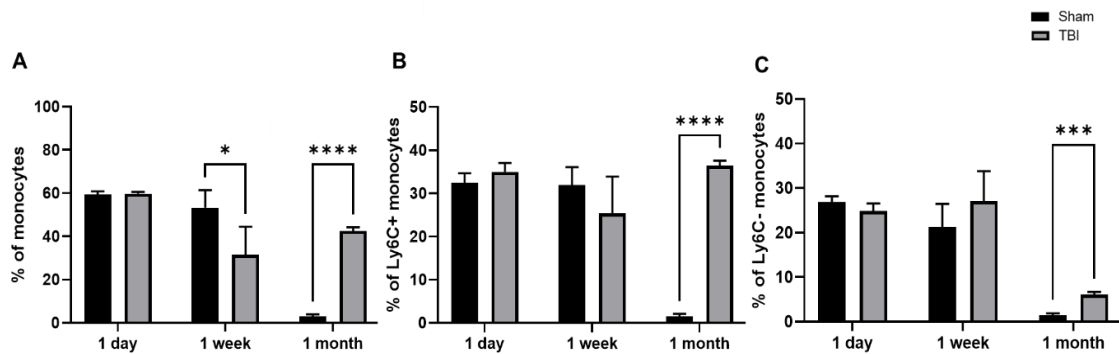


Figure 2.3. Flow cytometric splenic total and monocyte subset populations for rmTBI and sham injured groups over time. **(A)** Total monocyte population percentages at 1 day, 1 week, and 1-month timepoints. **(B)** Displays Ly6C+ monocyte and **(C)** displays Ly6C- monocyte population percentages isolated from the spleens of sham injured and rmTBI. While there were no changes one day or week after rmTBI, total monocytes, Ly6C+ and Ly6C- monocytes were all increased one month after injury. Data shown as mean +/- SEM, n=5. *P < 0.05, **P < 0.01, ***P < 0.001, ****P<0.0001 compared by 2-way ANOVA followed by Šídák's multiple comparisons test.

Figure 2.4. NanoString DEG analysis shows few changes for repeated mTBI spleens.

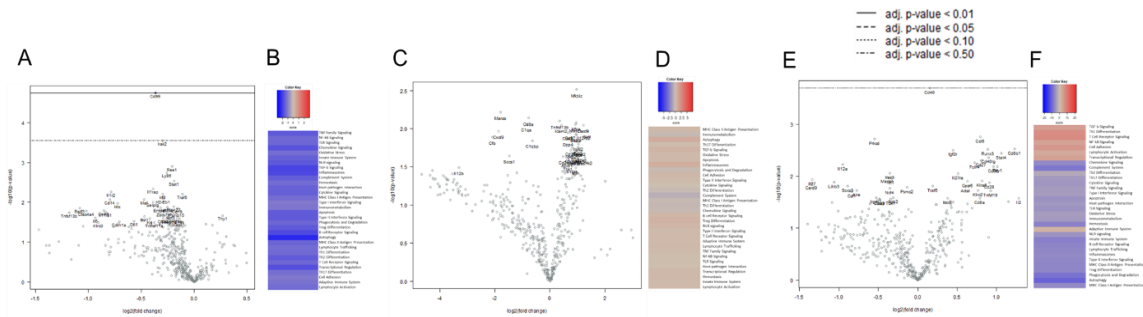


Figure 2.4. Nanostring generated volcano plots (A, C, E) and heatmap showing pathway scores (B, D, F) for sham and mTBI spleens. (A and B) 1 day, (C and D) 1 week, and (E and F) 1 month represent timepoints with comparison to sham as baseline value. Volcano plots are plotted fold change vs. p value with the dotted lines using Bonferroni correction for False Discovery Rate; N=3.

Figure 2.5. NanoString DEG analysis shows changes in genes and pathways in the repeated mTBI brain.

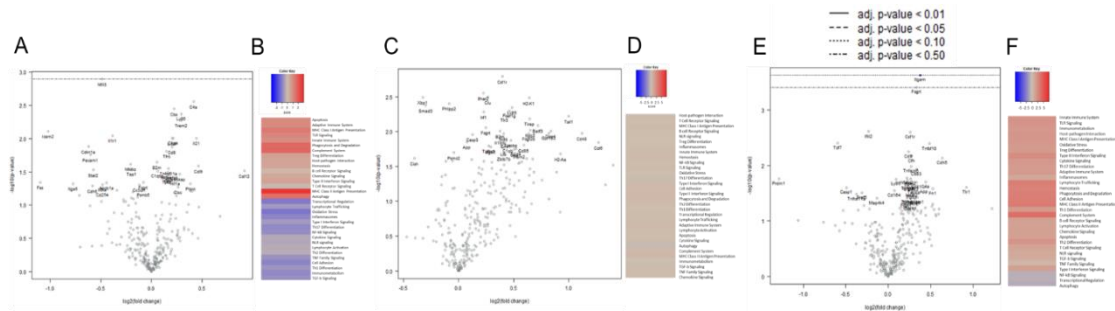


Figure 2.5. NanoString generated volcano plots (A, C, E) and heatmap showing pathway scores (B, D, F) for sham and mTBI brains. (A and B) 1 day, (C and D) 1 week, and (E and F) 1 month represent timepoints with comparison to sham as baseline value. Volcano plots are plotted fold change vs. p value with the dotted lines using Bonferroni correct for False Discovery Rate; N=3.

Figure 2.6. NanoString derived mRNA immunological changes in the spleen were only for *jak1* and *cd99*.

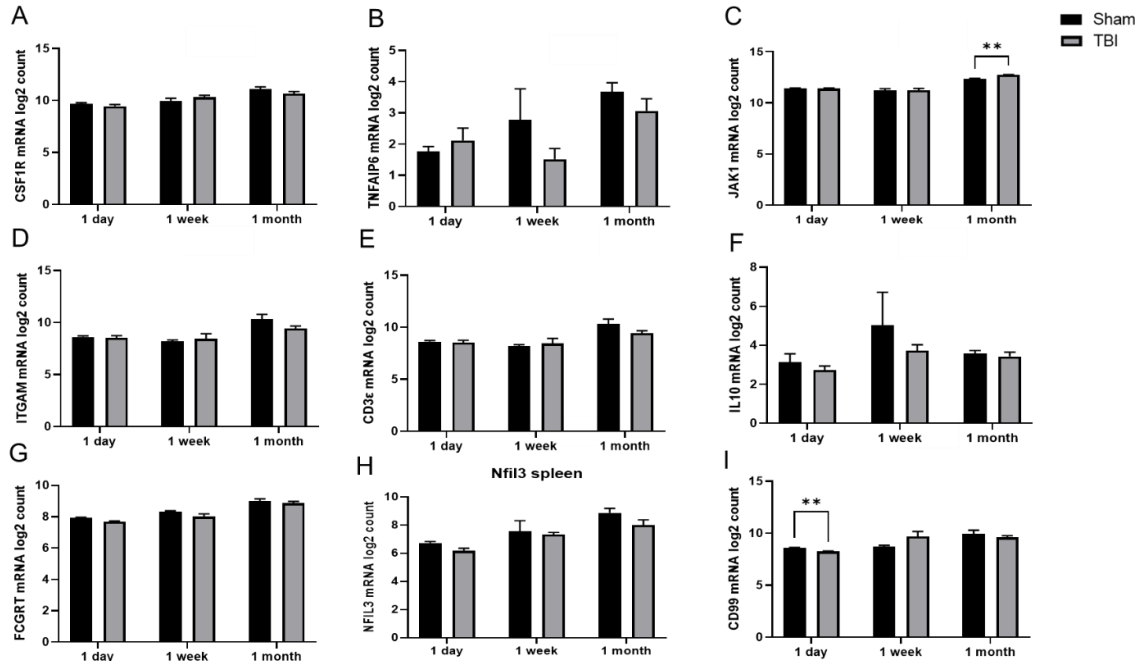


Figure 2.6. NanoString derived immunology mRNA in the mouse spleen for (A) CSF1R, (B) TNFAIP6/TSG, (C) JAK1, (D) ITGAM, (E) CD3e, (F) IL-10, (G) FCGRT, (H) NFIL3, (I) CD99 for rmTBI samples using sham as baseline value. Splenic mRNA shows no changes in except for CD99 (decreased, $p < 0.01$) at one day, and JAK1 (increased, $p < 0.01$) at one month Data is expressed as log₂ count 2-way ANOVA followed by Bonferroni post hoc test $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***; N=3.

Figure 2.7. NanoString DEG analysis shows changes in *csf1r*, *tnfaip6*, *itgam*, *cd3ε*, *fcgrt*, and *nfil3* immune mRNA in the repeated mTBI brain

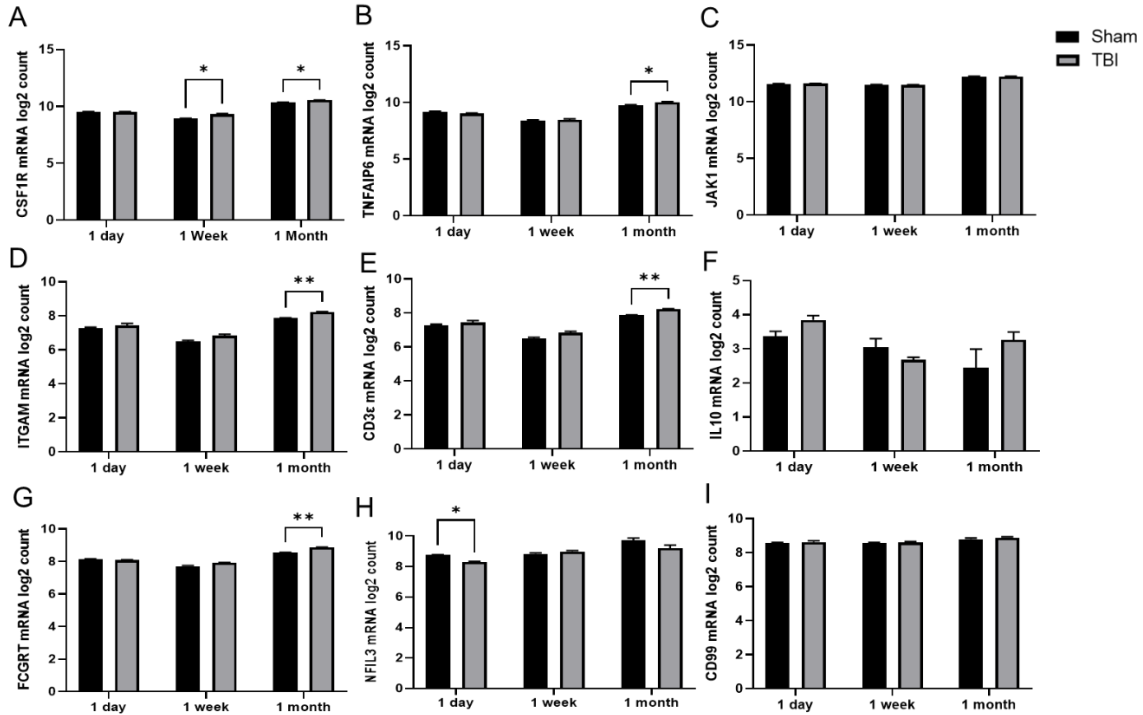


Figure 2.7. NanoString derived immunology mRNA in the mouse brain for (A) CSF1R, (B) TNFAIP6/TSG, (C) JAK1, (D) ITGAM/CD11b, (E) CD3ε, (F) IL-10, (G) FCGRT, (H) NFIL3, (I) CD99 for rmTBI samples using sham as baseline value. Here we found changes one day after injury for NFIL3 (decreased, $p < 0.05$). One week timepoint yielded only significant increases in CSF1R ($p < 0.05$). One month after rmTBI displayed the most changes as we found increases in CSF1R ($p < 0.05$), TNFAIP6 ($p < 0.01$), ITGAM ($p < 0.01$), CD3ε, ($p < 0.01$), and FCGRT ($p < 0.01$) Data is expressed as log2 count statistic analysis by 2-way ANOVA followed by Bonferroni post hoc test $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***; $N = 3$.

Figure 2.8. NanoString analysis showed no changes in many cytokine and chemokine genes in the brain

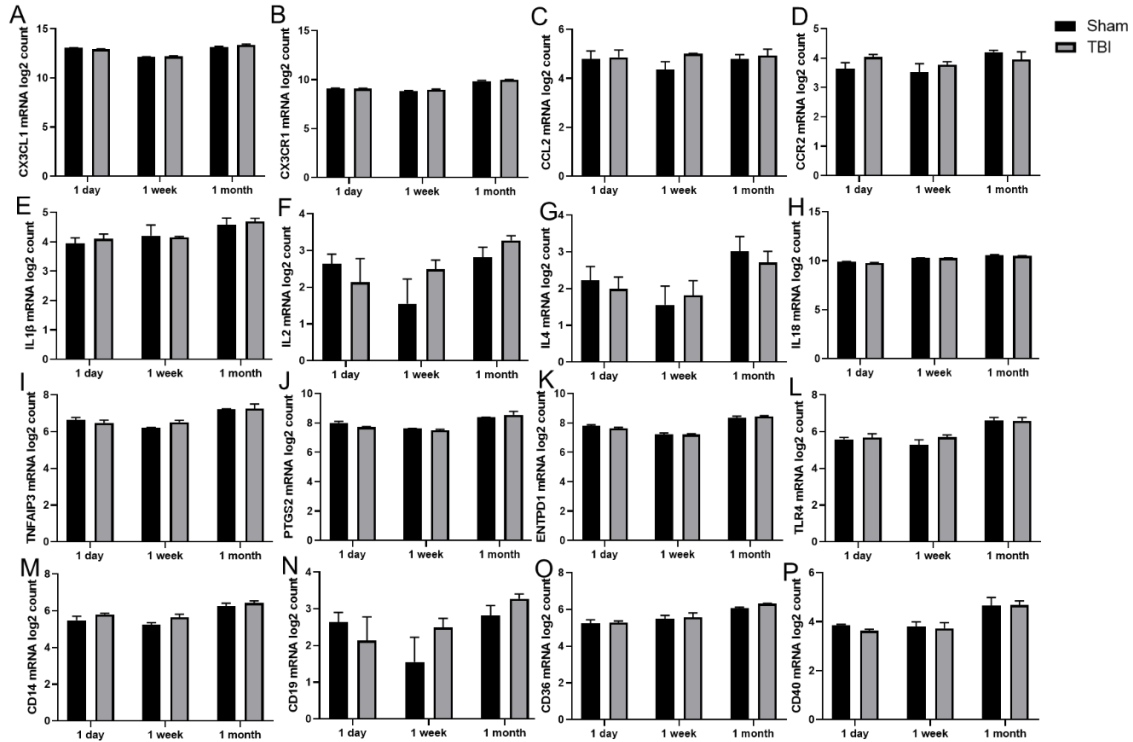


Figure 2.8. NanoString derived immunology mRNA in the mouse brain for (A) CX3CL1, (B) CX3CR1, (C) CCL2, (D) CCR2, (E) IL1beta, (F) IL-2, (G) IL-4, (H) IL-18, (I) TNFAIP3, (J) PTGS2, (K) ENTPD1, (L) TLR4, (M) CD14, (N) CD19, (O) CD36, (P) CD40 using sham as baseline value. Data is expressed as log2 count with $p < .05$ *, $p < .01$ **, $p < .001$ ***; $N = 3$.

Figure 2.9 NanoString analysis showed no changes in many cytokine and chemokine genes in the repeated mTBI spleen.

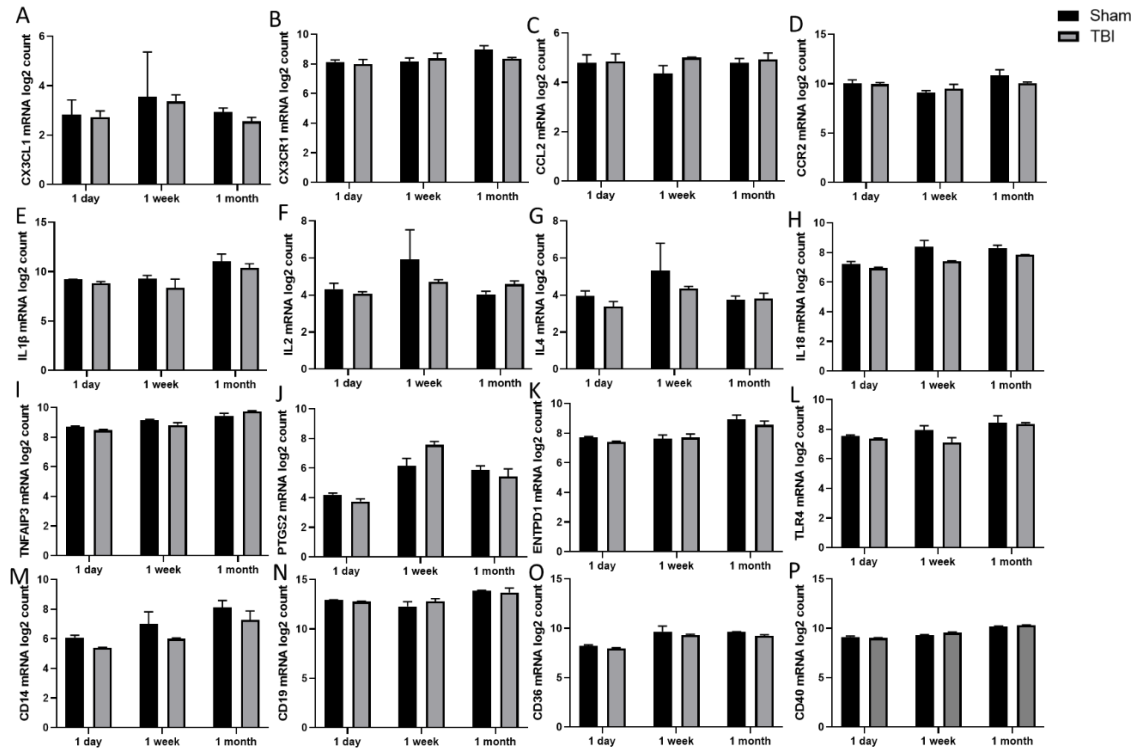


Figure 2.9. NanoString derived immunology mRNA in the mouse spleen for (A) CX3CL1, (B) CX3CR1, (C) CCL2, (D) CCR2, (E) IL1beta, (F) IL-2, (G) IL-4, (H) IL-18, (I) TNFAIP3, (J) PTGS2, (K) ENTPD1, (L) TLR4, (M) CD14, (N) CD19, (O) CD36, (P) CD40 using sham as baseline value. Data is expressed as log2 count with $p < .05$ *, $p < .01$ **, $p < .001$ ***; $N = 3$.

Table 2.1. NanoString derived pathway scores show widespread immune changes over the course of one month in the brain.

Brain	1 day			1 week			1 month		
	Sham mean	rmTBI mean	p value	Sham mean	rmTBI mean	p value	Sham mean	rmTBI mean	p value
Adaptive Immune System	-0.706	0.706	0.066	-1.166	1.166	0.032	0.011	0.245	0.245
Apoptosis	-0.389	0.389	0.128	-0.479	0.479	0.113	0.038	0.676	0.676
Autophagy	-0.034	0.034	0.748	-0.165	0.165	0.529	0.176	0.382	0.382
B cell Receptor Signaling	-0.192	0.192	0.479	-0.469	0.469	0.054	0.018	0.616	0.616
Cell Adhesion	-0.663	0.663	0.006	-0.831	0.831	0.022	0.007	0.163	0.163
Chemokine Signaling	-0.506	0.506	0.057	-0.907	0.907	0.062	0.021	0.325	0.325
Complement System	-0.508	0.508	0.007	-0.744	0.744	0.030	0.010	0.042	0.042
Cytokine Signaling	-0.908	0.908	0.067	-1.589	1.589	0.027	0.009	0.091	0.091
Hemostasis	-0.050	0.050	0.896	-0.947	0.947	0.021	0.007	0.076	0.076
Host-pathogen Interaction	-1.134	1.134	0.003	-1.647	1.647	0.028	0.009	0.086	0.086
Immunometabolism	0.305	-0.305	0.108	-0.235	0.235	0.330	0.110	0.076	0.076
Inflammasomes	0.089	-0.089	0.602	-0.207	0.207	0.065	0.022	0.008	0.008
Innate Immune System	-0.886	0.886	0.053	-1.316	1.316	0.030	0.010	0.806	0.806
Lymphocyte Activation	0.546	-0.546	0.378	-1.372	1.372	0.032	0.011	0.024	0.024
Lymphocyte Trafficking	-0.558	0.558	0.003	-0.507	0.507	0.033	0.011	0.581	0.581
MHC Class I Antigen Presentation	-0.495	0.495	0.015	-0.734	0.734	0.015	0.005	0.037	0.037
MHC Class II Antigen Presentation	-0.200	0.200	0.373	-0.660	0.660	0.064	0.021	0.097	0.097
NF- κ B Signaling	0.036	-0.036	0.930	-0.711	0.711	0.028	0.009	0.532	0.532
NLR signaling	-0.605	0.605	0.010	-0.654	0.654	0.107	0.036	0.342	0.342
Oxidative Stress	-0.512	0.512	0.019	-0.543	0.543	0.151	0.050	0.130	0.130
Phagocytosis and Degradation	-0.553	0.553	0.029	-0.933	0.933	0.031	0.010	0.045	0.045
T Cell Receptor Signaling	0.047	-0.047	0.867	-0.601	0.601	0.072	0.024	0.252	0.252
TGF- β Signaling	-0.003	0.003	0.983	-0.272	0.272	0.017	0.006	0.104	0.104
Th1 Differentiation	0.121	-0.121	0.569	-0.053	0.053	0.717	0.239	0.145	0.145
Th17 Differentiation	-0.343	0.343	0.099	-0.190	0.190	0.397	0.132	0.086	0.086
Th2 Differentiation	-0.027	0.027	0.873	-0.368	0.368	0.030	0.010	0.103	0.103
TLR Signaling	-0.274	0.274	0.461	-0.966	0.966	0.041	0.014	0.994	0.994
TNF Family Signaling	-0.427	0.427	0.101	-0.548	0.548	0.098	0.033	0.519	0.519
Transcriptional Regulation	0.452	-0.452	0.339	-0.737	0.737	0.017	0.006	0.069	0.069
Treg Differentiation	0.080	-0.080	0.579	-0.357	0.357	0.010	0.003	0.182	0.182
Type I Interferon Signaling	-0.368	0.368	0.080	-0.578	0.578	0.018	0.006	0.104	0.104
Type II Interferon Signaling	-0.017	0.017	0.941	-0.832	0.832	0.038	0.013	0.197	0.197

Table 2.1. NANOSTRING-derived pathway scores in the mouse brain at the one day, one week, and one month timepoints for various immune pathways. One day post mTBI displays significant increases in cell adhesion, complement system, host-pathogen interaction, lymphocyte trafficking, MHC Class I antigen presentation, NLR signaling, and Phagocytosis and degradation. One week timepoint is characterized by significant increases in pathways such as adaptive immunity, cell adhesion, complement system, cytokine signaling, hemostasis, host-pathogen interaction, innate immunity, lymphocyte activation and trafficking, MHC Class I antigen presentation, oxidative stress, TGF β signaling, Th2 differentiation, TLR signaling, transcriptional regulation, regulatory T cell differentiation, and Types I and II Interferon signaling. Lastly, one month post mTBI brains display significant increases in complement, innate immunity, MHC class I antigen presentation, and phagocytosis; yet, shows a decrease in inflammasome activation. Data is expressed as mean Z score with two-tailed t test significance set at $p < .05$; $n = 3$

Table 2.2. NanoString derived pathway scores show widespread immune changes over the course of one month in the spleen.

Spleen	1 day			1 week			1 month		
	Sham mean	rmTBI mean	p value	Sham mean	rmTBI mean	p value	Sham mean	rmTBI mean	p value
Adaptive Immune System	0.965	-0.965	0.043	1.525	-1.525	0.488	1.400	-1.400	0.242
Apoptosis	0.612	-0.612	0.016	0.880	-0.880	0.464	0.362	-0.362	0.560
Autophagy	0.137	-0.137	0.255	-0.655	0.655	0.072	0.337	-0.337	0.031
B cell Receptor Signaling	0.451	-0.451	0.020	-0.456	0.456	0.574	0.593	-0.593	0.219
Cell Adhesion	0.611	-0.611	0.133	1.247	-1.247	0.389	0.883	-0.883	0.247
Chemokine Signaling	1.023	-1.023	0.036	1.362	-1.362	0.430	0.942	-0.942	0.238
Complement System	0.448	-0.448	0.411	1.226	-1.226	0.292	0.729	-0.729	0.261
Cytokine Signaling	1.839	-1.839	0.010	2.197	-2.197	0.502	1.865	-1.865	0.284
Hemostasis	0.473	-0.473	0.329	1.218	-1.218	0.464	0.853	-0.853	0.365
Host-pathogen Interaction	1.579	-1.579	0.026	2.225	-2.225	0.457	1.819	-1.819	0.286
Immunometabolism	0.436	-0.436	0.096	0.729	-0.729	0.539	0.598	-0.598	0.383
Inflammasomes	0.160	-0.160	0.234	-0.597	0.597	0.057	0.179	-0.179	0.516
Innate Immune System	0.543	-0.543	0.479	2.284	-2.284	0.334	1.727	-1.727	0.330
Lymphocyte Activation	1.510	-1.510	0.018	1.830	-1.830	0.530	1.674	-1.674	0.244
Lymphocyte Trafficking	0.403	-0.403	0.185	-0.539	0.539	0.490	0.246	-0.246	0.622
MHC Class I Antigen Presentation	0.575	-0.575	0.016	1.161	-1.161	0.277	0.710	-0.710	0.330
MHC Class II Antigen Presentation	0.179	-0.179	0.015	-0.028	0.028	0.962	0.122	-0.122	0.723
NF- κ B Signaling	0.867	-0.867	0.007	1.262	-1.262	0.418	0.408	-0.408	0.643
NLR signaling	0.595	-0.595	0.115	1.257	-1.257	0.309	0.742	-0.742	0.386
Oxidative Stress	0.529	-0.529	0.038	0.846	-0.846	0.456	0.518	-0.518	0.472
Phagocytosis and Degradation	0.454	-0.454	0.281	1.037	-1.037	0.421	0.958	-0.958	0.368
T Cell Receptor Signaling	0.715	-0.715	0.009	0.824	-0.824	0.554	-1.159	1.159	0.005
TGF- β Signaling	0.284	-0.284	0.037	0.402	-0.402	0.605	-0.464	0.464	0.098
Th1 Differentiation	0.334	-0.334	0.187	0.791	-0.791	0.390	-0.815	0.815	0.015
Th17 Differentiation	0.598	-0.598	0.010	0.834	-0.834	0.480	0.340	-0.340	0.568
Th2 Differentiation	0.421	-0.421	0.029	0.241	-0.241	0.786	0.161	-0.161	0.606
TLR Signaling	0.819	-0.819	0.070	1.374	-1.374	0.388	1.032	-1.032	0.330
TNF Family Signaling	0.768	-0.768	0.029	1.165	-1.165	0.337	0.882	-0.882	0.258
Transcriptional Regulation	0.926	-0.926	0.006	0.700	-0.700	0.642	0.681	-0.681	0.321
Treg Differentiation	0.289	-0.289	0.066	0.711	-0.711	0.256	-0.279	0.279	0.341
Type I Interferon Signaling	0.354	-0.354	0.083	0.681	-0.681	0.531	0.538	-0.538	0.286
Type II Interferon Signaling	0.495	-0.495	0.084	0.919	-0.919	0.380	0.520	-0.520	0.456

Table 2.2. NANOSTRING-derived pathway scores in the mouse spleen at the one day, one week, and one month timepoints for various immune pathways. Here we denote rmTBI display decreased activity in adaptive immunity, apoptosis, cell adhesion, chemokine signaling, cytokine signaling, host-pathogen interaction, lymphocyte activation, MHC class I and II antigen presentation, oxidative stress, T cell receptor signaling, Th17 differentiation, Th2 differentiation, TNF family signaling, and transcriptional regulation. While there were no significant differences at the one week timepoint, one most post rmTBI the spleens showed significantly decrease autophagy but increases in T cell receptor signaling and Th1

differentiation. Data is expressed as mean Z score with two-tailed t test
significance set at $p < .05$; $n = 3$

Chapter Three

Effect of the Nonselective Beta Blocker, Propranolol, on Murine Monocytes Populations and Immune Dysfunction Post Repeated Mild Traumatic Brain Injury

Introduction

There are almost 2 million individual instances of traumatic brain injuries (TBIs) every year in the US. Of these, 75% of these injuries are classified as mild TBI, or concussions, but even the side effects of these mild injuries may last several months, if not, years (Leung 2020; Faul and Coronado 2015). While there are many potential mechanisms likely resulting in mTBI side effects, such as post traumatic headaches, prevention of these side effects is currently challenging. Furthermore, prevention and treatment of the side effects likely do not address the underlying TBI-driven physiological changes.

Mild TBI elicits an inflammatory response in the brain that typically requires the help of peripheral immune cells to aid in resolving the injury (Verboon et al., 2021; Laskowski et al., 2015). With the brain important in controlling and organizing many pathways, many organ systems are involved in sending cells to the brain after TBI. The bone marrow, blood, and spleen are all reservoirs of immune cells that are immediately active after injury and possess the ability to traffic into the post TBI brain (Xu et al., 2017; Shi et al., 2021; Otto et al., 2020). Although mTBI acutely has substantial inflammation, the inability to resolve the inflammation quickly leads to a sympathetic nervous system influenced immunosuppressive state that further complicates the injury resolution process (Sribnick et al., 2022) . High levels of damage associated molecular patterns

(DAMPs), IL6, TNF α , and IL-1 β lead to a sympathetic driven catecholamine surge most characterized by even higher levels of the anti-inflammatory cytokine, IL10 (Sribnick et al., 2022). In attempting to restore the body back to per-injury homeostasis, this IL10 surge prolongs injury resolution and increases the risk for sickness and infection. Additionally, incomplete injury resolution leads to enhanced inflammation in the CNS which may lead to further long-term consequences.

One treatment used to inhibit this immunosuppressive state and simultaneously treat the presence of TBI-related headaches is beta blockers (Leung 2020). Beta blockers inhibit adrenergic receptor signaling of the beta subtype of these receptors. One in particular, propranolol, is a non-selective β -blocker that can block both β 1 and β 2 receptor types (Lopez et al., 2022). With the ubiquitous presence of these receptors on many cell types, including immune cells and neurons, they have provided an interesting avenue for post traumatic headache treatment. In high enough concentrations, this lipophilic drug can diffuse through the blood brain barrier (BBB) and block sympathetic nervous activity peripherally and in the CNS (Pardridge et al., 1984; Ammar and Hussein 2018). In doing so, propranolol has the potential to prevent sympathetic activity induced immunosuppression after mild traumatic brain injury.

In these studies, we set out to elucidate the role of propranolol on immune cells following head trauma. Male mice were subjected to a repetitive model of concussive TBI and subsequently injected with 10mg/kg of propranolol following each injury. We performed flow cytometry, pain behavior, and NanoString based mRNA differential expression analysis for brain and spleen genes as far out as

one month after the last mild traumatic brain injury. We show that the combination of head trauma alone and in combination with propranolol reveals important differences in blood, brain, and spleen that add to the interpretation of the underlying post TBI pathology.

Methods

Repeated mild traumatic brain injury procedure.

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Indiana University School of Medicine, which are in accordance with National Institutes of Health guidelines for the care and use of laboratory animals. 60 Wild type C57BL/6 mice were obtained from Jackson Laboratory at 8-12 weeks old (~25-30 g) and were randomly divided into sham saline (SS), mTBI saline (TS), and mTBI propranolol (TP) and sham propranolol (SP) for downstream analyses. Mice were further subdivided into 1 day, 1 week, and 1-month timepoints with a total of n=5 for each group at each timepoint. Wild type C57BL/6 mice were used for immunohistological assessments.

To produce rmTBI, we used a technique previously described [12-14]. Briefly, mice were anesthetized with 2–4% isoflurane and heads were stereotactically fixed with heat-pad support below the abdomen. Closed head mTBI was produced using a control cortical impact (CCI) device after shaving, cleaning, sanitizing, and surgical exposure of the skull bone in animals. After the baseline point of the device was set by lowering the tip to the skull surface and the stage position was set to zero, the impactor was retracted, and the impact depth was set. The approximate center of the impact site was 1 mm posterior to the

bregma and 0.5 mm lateral from midline on one cortical hemisphere. The skull was struck with the impactor tip at a speed of 3 m/s to a depth of 1 mm. Sham injury animals were shaved, cleaned, sanitized, and subjected to skull exposure surgery but no impact was performed on the skull. Wounds were sutured and animals were placed on heating pad until fully recovered. All animals survived the injury without any skull fracture or hemorrhage. Sham injury or mTBI manipulations were performed once a week for three weeks (total of 3 injuries). Intraocular pressure (IOP) changes were used as a surrogate for injury-induced intracranial pressure changes. These changes were assayed using an ICARE TONOLAB (Vantaa, Finland) tonometer device in both the mouse eyes to confirm skull impact severity and verify the absence of skull fracture [15-16]. The IOP measurements were done immediately before and after the injury for up to 30 min (Figure 3.1, n = 5 mice in each group). If the skull bone was fractured by the CCI device, there would be an absence of IOP. Alternatively, if there were no changes in IOP, the delivered impact to the skull bone was deemed to be insufficient and the animal excluded from the study.

Propranolol administration

Propranolol chloride (a nonselective beta blocker) was purchased from Tocris and dissolved in saline (3mg/ml). The drug was immediately injected intraperitoneally into each mouse in the SP and TP group following mTBI at a dosage of 10mg/kg as previously stated by other studies. A total of 3 propranolol injections were performed for each mouse in the SP and TP groups throughout the study.

Blood Processing

Mice were placed into CO₂ chamber for euthanasia then once breathing stopped, surgical scissors were used to open the chest cavity and 500uL blood was obtained transcardially with an EDTA coated 1mL syringe. Blood was then placed into a 1.5mL microcentrifuge tube for flow cytometry staining.

Spleen processing

Spleen were isolated from mice and placed directly into complete RPMI media (88% RPMI 1640 [ThermoFisher], 10% Fetal Bovine Serum [Sigma-Aldrich], 1% Penicillin Streptomycin [Sigma-Aldrich], and 1% Glutamax [ThermoFisher]). Under sterile conditions and on ice, spleens were minced, cut, and filtered with complete RPMI media through 70 um filter into 50mL tube, centrifuged at 1300g x 10 minutes, and the supernatant was discarded. The pellet was then resuspended in 5mL RBC lysis buffer for 10 minutes at RT, centrifuged at 400g x 5 minutes, and resuspended in PBS. The pellet was then once again centrifuged. The pellet was then resuspended in 3mL FACS buffer and 1mL of this resuspended portion was taken for flow cytometry analyses. The remaining 2mL were frozen down with a combination of DMSO, fetal bovine serum, and complete RPMI media at -80C and then placed in liquid nitrogen for future RNA analyses.

Brain processing

After mice were placed into CO₂ chamber and blood was drawn transcardially, mice were decapitated with scissors. The skull was then cut along the midline and the bone surrounding both hemispheres were removed and whole

brains were dissected out using sterile techniques and cleaned while in calcium free PBS before placement in liquid nitrogen for future RNA processing.

Flow cytometry

To identify monocyte and monocyte subsets in the blood and spleen, both blood and spleen tissues were stained with CD45-BV711 [Biolegend], CD11b-Fitc [BD Bioscience], CD115-PE [Biolegend], Ly6C-APC-Cy7 [Biolegend], Fc CD16/32 block (BD BioScience).

Blood flow staining

Briefly, 500uL of whole blood was filtered via a 35 µm strainer cap into a 5mL round bottom FACS tube. Next, 10 µL of blood from this FACS tube was added to FMO and unstained control tubes. Subsequently, antibodies were added, and cells were incubated for 20 minutes in the dark at room temperature. Cells were then washed with FACS buffer and centrifuged at 400 g for 5 minutes at room temperature. Supernatant was discarded, and the pellet was resuspended in 2mL of RBC lysis buffer, and then incubated for 10 mins at room temperature, and centrifuged. Cell pellet was resuspended and washed with the FACS buffer. Lastly, pellet was resuspended in FACS buffer for flow cytometry data acquisition.

Spleen flow staining

Single cell splenocytes were first resuspended and washed with the FACS buffer, centrifuged at 400 g for 5 mins, and the supernatant was discarded. Cell pellet was resuspended in the FACS buffer, antibodies were added, and cells were incubated for 30 minutes in the dark at 4° C. After 30 minutes, cells were washed

with the FACS buffer, and tubes were centrifuged once again. Supernatant was discarded, and cells were resuspended in the FACS buffer for data acquisition.

Flow data acquisition

For both spleen and blood analyses in our study, fluorescence minus one controls were utilized in addition to unstained control samples for gating. Data was acquired on a BD LSR Fortessa X-20 with appropriate voltages, gating, and compensation. FCS files were analyzed using FlowJo 10 (Treestar, OR). A minimum number of 25,000 gated events from forward scatter versus side scatter gating were collected using the BD FACSDiva software. Cells were then gated for singlets, and CD45+CD11b+ cells and downstream monocyte cell subset populations were defined following the representative gating strategies depicted in Figure 3.1. Comparison of cell populations was performed in GraphPad Prism using two-way ANOVA followed by Šídák's multiple comparisons test with an alpha of 0.05 to determine significance. Again, nonclassical monocytes were defined as CD45+CD11b+CD115+Ly6C-, and classical monocytes were defined as CD45+CD11b+CD115+Ly6C+. Total monocyte percentages were determined as the summation of CD45+CD11b+CD115+Ly6C- and CD45+CD11b+CD115+Ly6C+ percentages (Figure 3.1).

NanoString nCounter gene expression panels

We analyzed the expression of 561 genes (including 14 internal reference genes) related to the immune response to repeated mTBI using the nCounter Immunology Panel™ (mouse) (NanoString, XT-CSO-MIM1-12). 100 ng of total RNA was used as input and sample hybridization was performed according to the

manufacturer's instructions. Sample detection and analysis were completed on a nCounter® Digital Analyzer. Raw data processing, quality control, and normalization were performed using the nSolver™ 4.0 analysis software. Quality control (QC) and normalization were performed with an imaging QC of >75% field of view registration, binding density QC within 0.1-2.25 range, positive control linearity QC of R^2 above 0.95, and positive control limit of detection set as 0.5 fM positive control above 2 standard deviations above the mean of the negative controls. Normalization to housekeeping genes, of which genes below 100 were excluded, and pathway scoring, gene set analysis, differential expression analysis were completed using the Advanced Analysis software plugin (version 2.0.115). For gene set analysis and differential expression analysis, a p -value of ≤ 0.05 was applied as cutoffs. For all NanoString analyses at 1 day, 1 week, and 1 month after injury, gene expression measurements for each group were normalized to the sham saline (n=3) baseline of each timepoint.

Differential Expression

Differentially expressed (DE) genes were calculated using fold change by comparing the transcript levels between the combined samples within a group of SP, TS, or TP bone marrow and sham saline bone marrow baseline. The results of these multiple t test analyses are summarized in volcano plots (Figures 4.2-4.4) Data was graphed $-\log_{10}(p\text{-value})$ vs. fold change with Bonferroni false discovery rate (FDR) adjusted p value dotted lines shown to depict varying levels of significance at $p < 0.50$, $p < 0.10$, $p < 0.05$, and $p < 0.01$ with the top 40 differentially expressed genes labeled in each volcano plot. 12 of the most significantly changed

genes across the experimental groups and timepoints were chosen for further characterization. (Table 4.1 and Figure 4.5). These data were generated by NanoString advanced analysis and selected due to high differential expression. Log₂ fold changes for these mRNAs were further assessed across the experimental timepoints to determine group dependent changes for these transcripts.

Pathway Scoring

Panel derived transcripts from each sample were grouped into pathways by NanoString Advanced Analysis software and Scores are displayed on the same scale via a Z-transformation. Mean z score for each group was compared at each timepoint to the sham saline baseline control to understand how pathway scores cluster together and which samples exhibit similar pathway score profiles at each timepoint (Figures 3.3-3.5).

Pain behavior

Briefly, mice were allowed to acclimate for 30-45 minutes in the cage before trial onset. Both left and right hindpaws were initially applied with Von-Frey type filaments in ascending order with forces of 0.5 mN, 1.0 mN, 2.0 mN, and 4.0 mN on the six surfaces of the hindpaw three times for each surface. These surfaces were the plantar, dorsal, distal plantar, distal dorsal, proximal plantar, and proximal dorsal hindpaw. Paw withdrawal, indicative of pain response, was denoted as positive if paw was removed upon filament application in all trials on that surface.

The mouse was placed on a metal mesh floor and covered with a transparent plastic dome. Typically, the animals rest quietly in this situation after

an initial exploration period. Animals were habituated to this testing apparatus for 30-45 minutes a day, two days prior to the behavioral assays. Following acclimation, each filament was applied to the six locations spaced across the nerve distribution of hind paw glabrous skin. The filaments were tested in order of ascending force, with each filament delivered in sequence from the 1st to the 6th location alternating from one hind paw to the other. The duration of each stimulus was 1 second and the interstimulus interval was 10–15 second. Each location was assayed three times. In each behavioral testing sequence, the operator was blinded to the animal treatment condition and the blinding codes were not revealed until the completion of the data collection (Wilson et al., 2016; Bhangoo et al., 2007).

The incidence of foot withdrawal was expressed as a percentage of the six applications of each filament as a function of force. A Hill equation was fitted to the function (OriginPro 2022, Microcal Software, Northampton MA) relating the percentage of indentations eliciting a withdrawal to the force of indentation. From this equation, the paw withdrawal threshold (PWT) force was obtained and defined as the force corresponding to a 50% withdrawal (Wilson et al., 2016; Bhangoo et al., 2007). Animal pain behavior was determined by establishing baseline tactile pain paw withdrawal threshold one day prior to injury and comparing each timepoint to sham saline control animals at each timepoint.

Statistics

All flow cytometry statistics were performed using GraphPad Prism 9 (GraphPad Software, La Jolla, California) and values are reported as mean \pm S.E.M. Flow data

was analyzed using one-way ANOVA. Data yielding significance was further processed with Tukey's HSD post-hoc analyses were applied for further comparisons between specific groups. NanoString analysis for significant genes used t-test followed by Bonferroni false discovery rate (FDR) adjusted p-value with a cut off p-value of ≤ 0.05 using sham saline for each timepoint as baseline. Genes further characterized in Prism with log₂ fold changes were reported as mean \pm S.E.M using 2-way ANOVA (treatment vs. time) test (GraphPad Prism 9, La Jolla, California). These data were further analyzed Sidak's multiple comparisons post hoc test. Pathway score significance was calculated using unpaired t test with significance set as $p < 0.05$ with mean (n=3/group) established by averaging pathway Z scores of individual samples within a group at one timepoint. For all NanoString derived data analyses, our sample size was n=3.

RESULTS

Propranolol impacts total and subset populations of monocyte populations at 1 day, 1 week and 1 month time points after repeated mTBI.

To understand the impact of propranolol on monocyte populations, PBMCs were isolated from blood and spleen samples at 1 day, 1 week and 1 month following repeated mTBI or sham injury in combination with either propranolol or saline treatment (Figure 3.1). In mice, two populations of monocytes exist and can be discriminated by variable expression of lymphocyte antigen 6C (Ly6C). Monocytes expressing high levels of Ly6C (Ly6C⁺ monocytes) have proinflammatory and antimicrobial functions. Monocytes with low expression of Ly6C (Ly6C⁻ monocytes), also known as patrolling monocytes, survey the

vasculature by constantly crawling along the lumen of the vasculature and are involved with early responses to inflammation. Total monocyte numbers were unchanged 1 day after injury for all groups; However, we detected substantially increased monocyte percentages in both the TS and TP groups ($p < 0.01$ and $p < 0.0001$, respectively, 2-way ANOVA). SP derived monocytes were most increased one week after injury. 30 days after injury we find that both TS and TP total monocytes are still increased compared to both SS and SP controls. There was no difference between TS and TP total monocytes at this timepoint.

Ly6C+ monocytes were unchanged 1 day after injury for all groups (Figure 3.2B). We denote a trend increase in SP derived Ly6C+ cells here along with a significant increase in Ly6C+ cells from TP animals compared to TS derived Ly6C+ cells ($p < 0.05$). Interestingly, we found that TS blood Ly6C+ cells are present more 30 days after injury than Ly6C+ cells from TP animals ($p < 0.01$).

Ly6C- monocytes percentages too did not change 1 day after repeated mTBI (Figure 3.3C, $p > 0.05$, 2-way ANOVA). We found significant increases in Ly6C- cells in the blood for both the SP and TP groups ($p < 0.0001$) but not for the TS group 7 days after injury. There was no difference between TP and TS Ly6C- monocyte percentages at this timepoint. However, we found TP derived Ly6C- monocytes were significantly increased compared to both TS ($p < 0.01$) and SP ($p < 0.001$) 30 days after injury.

Propranolol treatment alters splenic monocytes populations in mTBI animals at 1 month after injury.

We investigated whether the repeated mTBI paradigm in the presence or absence of propranolol induces changes monocyte subsets within the spleen (Figure 3.3). Like the blood monocytes, we did not detect significant changes at the 1-day timepoint for total monocyte numbers. 7 days after injury, we found TS and TP specific total monocyte decreases (Figure 3.3A, $p < 0.05$ and $p < 0.0001$, respectively) with the most pronounced deficits in the TP group. TP group was also significantly decreased compared to TS splenic monocyte numbers ($p < 0.05$) 7 days after injury. At the 30-day post injury timepoint, splenic monocytes are most increased in both TS and TP groups compared to SS splenic monocytes (Figure 3.3A, $p < 0.0001$) with the TP monocytes exhibiting even higher percentage than TS splenic monocytes ($p < 0.01$).

Interestingly, Ly6C⁺ monocytes were decreased in the SP spleen 1 day after injury compared to SS spleen (Figure 3.3B, $p < 0.05$). Here too, we found significantly decreased monocyte percentages in the TP group as TP spleen displays low presence of Ly6C⁺ monocytes 7 days after injury across all groups. The Ly6C⁺ splenic monocyte levels rebound for this group by the 30-day timepoint as the TP group exhibits the highest percentage of Ly6C⁺ cells amongst the groups (Figure 3.3B, $p < 0.0001$). This is noteworthy as TP Ly6C⁺ monocytes were higher than TS Ly6C⁺ cells ($p < 0.0001$) which also displayed high presence of these cells compared to SS splenic tissue.

Surprisingly, Ly6C- monocytes were unchanged 1 day after injury across all groups (Figure 3.3C, $p > 0.05$). These cells also showed low percentages in the TP spleen compared across the groups ($p < 0.0001$) 7 days after injury. Unlike the Ly6C- changes we saw in the blood 30 days after injury, we found no changes across the groups 30 days after injury for Ly6C- monocytes.

Propranolol leads to pronounced, substantive changes in gene expression 1 week post mTBI in the mouse spleen.

Differential expression analysis was performed for each group by comparing a gene in one group against its level of expression in the sham saline spleen at the timepoint followed by correction for multiple t test using Bonferroni false discovery rate (FDR) adjustment (Figure 3.4A). Significant differential expression changes in the mouse spleen 24 hours after concussive traumatic brain injury was limited to drug-treated sham control animals. Here we see propranolol alone led to a only one gene, *cd8b1*, reaching our significance cut off of $p < 0.05$. Neither the TS nor TP spleens have any genes reaching significance 1 day after injury. Of note, five of the transcripts with the most non-significant changes for the TP group were *nfkbi2*, *tfaip3*, *trem1*, *casp2*, and *ccl3*.

DEG analysis for 7-day post injury spleen shows no significant genes in the volcano plots for TS and SP groups (Figure 3.4B). The TP spleen at this timepoint had 213 significant changes here with all but 13 of these transcripts displaying decreased expression compared to sham saline expression levels. The top 5 TP mRNA changes here were *tmem173*, *cd274*, *h2-ab1*, *cd48*, and *nfkbia*.

30-day post injury, DEG analysis once again shows no significant genetic changes for the TS and SP groups (Figure 3.4C). At this timepoint, we only find one TP spleen gene significantly altered. *Ifna1* was the most significantly changed gene in this group and it increased its expression more than 8-fold compared to SS *ifna1* levels. Although not significant, the most pronounced TP spleen transcript differences also included *ccl19*, *c1s*, *cd40lg*, and *cxcl13*. NanoString pathway analysis suggested that the difference in the top 5 genes at the 1 day, 7 days, and 30-day timepoints were associated with MHC class II, phagocytosis, cell adhesion, lymphocyte trafficking, innate immunity, and transcriptional regulation pathways.

Additionally, we investigated chemokine and cytokine differential gene expression analysis for IL10, IL1 β , IL18, CCL2, CCR2, CX3CL1, and CX3CR1 (Figure 3.8) at one day, one week, and one-month after the third injury which revealed dynamic changes in cytokine gene expression. We further characterized these transcripts due to their role in impacting various immune processes. IL10 displays decreases 24 hours post injury in the TBI saline group with propranolol treated spleens displaying sham saline expression levels; however, 1 week after injury, propranolol treated spleens are characterized by decreased IL10 expression. IL-1 β displays no differential expression changes 24 hours after injury but propranolol treated repeated TBI spleens displayed decreased IL-1 β at this time. Interestingly, IL18 decreases in untreated TBI spleens 24 hours after injury. IL18 is still decreased 1 week after injury before returning to sham saline levels at the one-month timepoint. We did not detect changes in splenic CCL2 expression at any of our timepoints, however, we did denote changes in its

receptor. TBI propranolol spleens displayed decreased expression of the CCR2 compared to all other groups 1 week after injury. There were no further CCR2 changes 1 month after injury. While CX3CR1 did not portray any changes in our study, its ligand, CX3CL1 did display an interesting expression pattern.

Pathway scores

In the spleen, we find several significant pathway score changes 1 day after rmTBI. TS spleens show decreased pathway scores for chemokine signaling, cytokine signaling, immunometabolism, innate immune activation, lymphocyte activation, NK- κ B, oxidative stress, TGF β , Th17 differentiation, Th2 differentiation, TLR signaling, TNF family signaling, transcriptional regulation, T reg differentiation, and Type 2 interferon signaling 1 day after injury (Figure 3.5, $p < 0.05$, unpaired t-test). Interestingly, TP spleens do not show any significant pathway score differences unlike SP spleens which shows increased activity in many of the pathways decreased in the TS group such as cytokine signaling, innate immune activity, NF- κ B, oxidative stress, Th17 differentiation, and Th2 differentiation 1 day after injury.

Surprisingly, TS spleens do not display any significant pathway changes 7 days post rmTBI while TP spleens display decreases in every pathway assayed on the NanoString Immunology panel compared to sham saline spleens ($p < 0.05$, unpaired t test). This decrease was not significant when compared to TS spleen.

30 days after injury we find that the TS spleen has decreased pathway scores for T cell receptor signaling but increases in TGF β and Th1 differentiation pathways while the TP spleen shows decreases in B cell receptor signaling and

lymphocyte trafficking. 30 days after injury the TP spleens also show increases in Th2 differentiation (Figure 3.5C). When we directly compare TP spleen pathway scores to TS pathway scores at this timepoint we find that TP spleens have decreased B cell receptor signaling and lymphocyte trafficking while showing enhancements in Th2 differentiation and transcriptional regulation.

Repeated mTBI induces few yet interesting changes in the mouse brain.

DEG analysis in the brain depicts gene expression changes across all the groups 1 day after injury reaching significance. TP genes in the brain 1 day after injury show that the top 5 genes changing non significantly were *nfil3*, *ctss*, *c1qa*, *tnfrsf11a*, and *tlr1*. Genes at this timepoint for the TP group seem to generally be increased in fold change compared to sham saline brain levels (Figure 3.6). Interestingly, the TS brain also shows the *nfil3*, *ctss*, and *c1qa* as three of the top 5 genetic changes at this timepoint although, these did not change significantly compared to sham saline baseline.

7 days after injury, we did not find any DEG changes for the TS or TP group; however, we found 264 DEG changes in the SP brain at this timepoint. 80% of these DEG changes here display increased fold change (Figure 3.6B, $p < 0.05$). Interestingly, of the DEG showing decreased expression, *cx3cl1*, *frmpd4*, *tnfaip6*, *tgfb2*, and *ncf4* were 5 of the most significantly decreased amongst all the DEG changes at this timepoint.

At the 30-day post injury timepoint, we see DEG changes from all groups. We found 17 DEG changes for the SP brain with increased expression, most notably for *csf1r*, *tnfaip6*, *cd164*, *cfh*, and *itgam* (Figure 3.6C). The TS brain at this

timepoint displayed the least amount of significant DEG changes as the only significant change here was for increased *itgam* expression compared to sham saline. TP brain displays 10 significantly changed DEGs here as we find increased expression of *tnfaip6*, *cd9*, *itgam*, *csf1r*, *ly86*, *ptgs2*, *fcgrt*, *cd81*, and *vtn*. The only significant DEG decrease here was for *mx1*.

Further investigation of cytokine changes across the experiment shows changes in *il1 β* , *il10*, *ccr2*, *cx3cl1*, *cx3cr1* at different timepoints (Figure 3.9). While we did not identify many changes in cytokine expression 1 day after injury, we found increased *ccr2* ($p < 0.05$, one way ANOVA) and *cx3cr1* ($p < 0.01$, one way ANOVA) in the TP brain 1 day after injury when compared to SP brains. 7 days later our data shows significant increases in *il1 β* ($p < 0.01$, one way ANOVA), *il10* ($p < 0.05$, one way ANOVA), *ccr2* ($p < 0.0001$, one way ANOVA), and *cx3cr1* ($p < 0.001$, one way ANOVA) for the SP group compared to SS baseline 7 days after injury. Interestingly, we found decreased *cx3cl1* ($p < 0.01$, one way ANOVA) in the SP brain at this timepoint as well. While there were not many changes 30 days after injury, we did find that both SP and TP brains expressed increased expression of *cx3cl1* ($p < 0.01$, one way ANOVA).

Pathway Scores

The DEG changes we found across the groups at 1 day, 7 days, and 30 days after injury led to interesting pathway score changes. 1 day after injury SP brains show increased pathway scores for MHC class I antigen presentation, phagocytosis, and Toll receptor signaling. The 1-day post injury TS brain shows increased pathway scores for adaptive immunity, complement activation, cytokine

signaling, host pathogen interaction, innate immunity activation, MHC class I antigen presentation, Nod-like receptor (NLR) signaling, oxidative stress, and TNF family signaling while also displaying decreases in lymphocyte trafficking and cell adhesion. The TP brain portrays increased adaptive immunity, B cell receptor signaling, cytokine signaling, MHC Class I antigen presentation, NLR signaling, oxidative stress, TNF family signaling, and Type I interferon signaling pathways (Figure 3.7, $p < 0.05$, unpaired t test). Furthermore, the only pathway displaying significant changes between TS and TP brains 1 day after injury was increased regulatory T cell differentiation pathways in the TP brain ($p < 0.05$, unpaired t test).

The 7-day timepoint displays widespread changes amongst the groups when compared to sham saline brains (Figure 3.7). The SP brain displays increased pathway scores for all pathways available on the NanoString Immunology panel ($p < 0.05$, unpaired t test). The TS brain also displays increases in all pathways with the exceptions of apoptosis, autophagy, T cell differentiation and signaling, MHC class II antigen presentation, and inflammasome activation. TP brains only show increases in complement activation ($p < 0.05$, unpaired t test) compared to sham saline baseline. However, TP brains have decreased pathway scores for genes in the Th2 differentiation pathway ($p < 0.05$, unpaired t test) when compared to TS brains at this timepoint. Furthermore, TP brains here show significant decreased pathway scores for all pathways compared to SP brains (Figure 3.7, $p < 0.05$, unpaired t test).

30 days after injury we find that the SP group still displays the most increases across all pathways with 24/32 pathways displaying increases (Figure

3.7, $p < 0.05$, unpaired t test). We find that TS brains at this time show increases in chemokine signaling, inflammasome activation, lymphocyte activation, MHC Class I antigen presentation, and phagocytosis vs sham saline brains ($p < 0.05$, unpaired t test). Additionally, TP brains display increased pathway scores for adaptive immunity, apoptosis, cell adhesion, chemokine signaling, complement activation, cytokine signaling, hemostasis, immune metabolism, inflammasome activation, MHC class I and II antigen presentation, oxidative stress, phagocytosis, and Type 1 interferon signaling while also showing decreased scores for TLR signaling ($p < 0.05$, unpaired t test). When directly compared, TP brains 30 days after injury show increased innate immune activation, immunometabolism, and type 1 interferon response compared to TS brains at this timepoint.

Pain behavior displays inconclusive results across the various groups over the course of the experiment.

Von Frey filaments were used to test the mechanical pain threshold for male mice once a week, after each injury throughout the timepoints of the experiment. Briefly, mice hind left and right paws were prodded 6 times each with 0.5mN, 1mN, 2 mN, and 4 mN probes in succession to determine the paw withdrawal response. A response was considered positive if paw was raised or withdrawn upon stimulation. If withdrawal response was 6/6 on a probe prior to 4mN probe stimulation, no further probing was performed on that paw or animal. Our data shows mice begin to experience sensitivity as soon as 1 day after the first injury and this response remains low throughout the duration of the experiment. These data are difficult to evaluate as external factors such as building construction

began after study onset and disrupted our post baseline timepoints. By the end of the 1-month post injury timepoint, sham saline mice displayed the most significant paw withdrawal response amongst all groups resulting in no baseline for these data (Figure 10).

Discussion

In the present study, propranolol treatment in a validated murine model of repeated concussive injury to the brain which was utilized to validate changes in populations of mononuclear. The study intervals demonstrated that leukocyte mobilization and function is altered in the blood, brain, and spleen by propranolol administration in combination with concussive head trauma. These combined findings provide a plausible mechanism by which non-selective beta-blockade may alter cerebral inflammation and leukocyte trafficking.

Evidence indicates that circulating peripheral immune cells and factors may permeate brain tissue and contribute to secondary inflammation (Sulhan et al., 2020; Chiu et al., 2016; Feng et al., 2021; Verboon et al., 2021). In this study, we found that repetitive closed head concussive injury in combination with propranolol treatment increases the number of circulating monocyte subpopulations but blunts the injury-induced genetic changes in splenic monocyte subpopulations 7 days after injury. At one month post injury, we observed both increases in anti-inflammatory monocytes and decreases in Ly6C⁺ blood monocytes compared to monocytes derived from injured saline-treated control mice. In contrast, concurrent decreases in total and Ly6C⁺, and Ly6C⁻ splenic monocytes were evident 7 days after injury; however, 1 month after injury the combination of repetitive TBI and

propranolol resulted in the increased presence of splenic monocytes and splenic inflammatory monocytes. We also found DEG changes were most evident for propranolol treated TBI splenic genes impacting immune activation and cellular responses to injury. For the brain, we found that propranolol treatment after TBI can lead to enhanced expression of genes important for adaptive immune activation, antigen presentation, and cytokine signaling.detail spleen brain gene changes

The innovation in our study lies in the treatment paradigm of only a single propranolol injection given along with each injury and the lasting impact this has on blood, spleen, and brain, cell types and pathways. mTBI results in activation of the sympathetic nervous system (SNS) very soon after initial injury and this activation can impact various organs such as the heart, bone marrow, spleen, liver, lungs, blood, and GI systems. Additionally, many immune cell types in these organs have alpha and beta adrenoreceptors that can directly respond to the catecholamines released due to SNS activity (Zuo et al., 2016). Our goal is early administration of propranolol immediately after injury to limit the sympathetic nervous system dependent immunosuppressive activities on the blood, brain, and spleen. It is worthwhile pointing out that propranolol alone in both the brain and spleen one week after injury resulted in high differential expression in pathways impacting immunometabolism, adaptive immune system, lymphocyte activation, TGF beta signaling, and cytokine signaling pathways. Propranolol impacts all these pathways regardless of a mild traumatic brain injury. Therefore, mild traumatic brain injury treated with propranolol seemingly reverts or inhibits many

of these pathways from changing as they do in TBI conditions untreated with propranolol. Collectively, these data display the wide-ranging impact of propranolol on various organ systems.

Partially contributing to the difficulty in treating concussions is that the primary head injury causes initial dysfunctions that manifest in secondary neuroinflammatory mechanisms as the CNS seeks to heal itself (Leung 2020; Sulhan et al., 2020). While necessary for general homeostatic functioning, these secondary mechanisms can lead to further propagation of the immune response, heightened immune sensitivity, and a lack of resolution of the injury (Sulhan et al., 2020). These secondary neuroinflammatory mechanisms can require the recruitment and activity of peripheral immune cells, such as monocytes, from distant sources trafficking to the brain due to increased blood brain barrier leakage, release of inflammatory molecules from the brain parenchyma, and the altered ability of the brain to control both CNS and systemic immunity (Xu et al., 2017; Chiu et al., 2016; Hanscom et al., 2021). While we were unable to detect the infiltration of peripheral immune cells to the CNS in this study, lymphocyte trafficking pathway scores were decreased in the saline treated mTBI brain one day after injury; yet, increased by the 7-day timepoint. Interestingly, our data shows propranolol treated TBI brains do not show differences in lymphocyte trafficking over the course of a month post injury.

The release of these catecholamines, epinephrine and norepinephrine (NE), results in increased immune activity but also induces immune suppression due to divergent effects on various cytokine pathways. SNS activity can increase

cortisol levels leading to an increase in NF-kb, IL-1B, TNFa, CCL2 (Zuo et al., 2016; Sribnick et al., 2022). NE promotes inflammation through NLRP3 inflammasome assembly and cAMP-ERK dependent IL-1beta production; however, it can also work through beta adrenoreceptors to block downstream NF-kb activity induced inflammatory cytokine production (Zuo et al., 2016; Johnson et al., 2019). Moreover, catecholamines promote the translation of anti-inflammatory regulators IL-10, IL-1RA, and type 2 IL-1 decoy receptors (Salazar et al., 2017; . Trauma-induced immune suppression is common after infection, burn injury, surgery, and is heavily reliant on the anti-inflammatory environment post IL-10 release (Salazar et al., 2017; Elenkov et al., 2000). IL-10 release into serum begins 6-12 hours post injury but usually peaks after 24 hours. Additionally, IL-10 mRNA is upregulated following catecholamine release (Elenkov et al., 2000). Our data did not show IL10 mRNA changes one day after injury in the brain; however, we did see that IL10 mRNA is reduced in the spleens of propranolol treated TBI mice 1 week after injury.

When we correlate the changes in the brain and spleen at the 1-day timepoint we do not see many changes in the spleen one day after injury for any of the groups . This is in support of our flow cytometry data where we did not detect any monocytic population changes one day after injury for any of our groups. Moreso, we did not denote any changes in the blood at this timepoint either. Interestingly we do see a mTBI effect one day after injury in the brains. DEG Changes in pathways such as cytokine signaling, adaptive immunity, MHC class 1 antigen presentation, NLR signaling, oxidative stress, TNF family signaling

pathways are equitable one day after injury in the brain for TBI saline and TBI propranolol groups. Altogether, the one day post injury timepoint did not yield much statistically significant data.

Interestingly, one week after injury in the brains and spleens the TBI propranolol group portray low differential expression in for all pathways assayed. This is in stark contrast once again to the TBI saline group which shows opposing trends in the brain but mirrors the mTBI propranolol group in the spleen. Due to the widespread presence of beta receptors, the blocking effect of propranolol is not clear-cut; however, these data suggest its effect here displays the ability to prevent immunosuppression as the TBI propranolol group in the spleen mirrors the sham saline group at the 1-week time point.

The innate immune system typically gives way to the adaptive arm of the immune system over the course of the 1st week after injury. Directly comparing the spleen and brains we begin to understand how propranolol opposes the TBI saline pathophysiology at the one-week time point. These data show that innate and adaptive immunity genes are lowly differentially expressed in the TBI propranolol group along with various cytokine pathways at the 1-week time point. Moreover, the splenic monocytes population changes identified by flow cytometry experiments also showed the same data trends. We found in flow cytometry that the TBI propranolol group resulted in the fewest total, Ly6C+, and Ly6C- monocytes percentages present at this time point. We theorize based on these data that TBI propranolol conditions result in a decreased demand for monocytes and the immune system to be present and active.

Furthermore, when we look one month after injury the changes that seem to happen in the brain and spleen at the 1-month timepoint seemingly are most attributed to propranolol administration as both the TBI propranolol and sham propranolol groups mirror similar differential expression pathway levels. Typically, the adaptive immune system is more active than its innate counterpart one month after an injury for canonical injury resolution. Here we do note that both the innate immune system and adaptive immune system (especially T cell dependent pathways) are very active in TBI propranolol conditions. However, this is also occurring in the sham injured propranolol treated conditions which suggest propranolol impacts these pathways especially. 1 month post mTBI flow cytometric data in the spleens showed that TBI propranolol treated spleens result in higher monocyte numbers compared to TBI saline and that these monocytes were predominantly of the inflammatory nature.

TBI increases the prevalence of Ly6C⁺ cells in the spleens after injury and stress (Wohleb et al., 2015; Saber et al., 2020). Activation of the SNS after middle cerebral artery occlusion (MCAO) in rodents has correlated with splenomegaly that can be reversed with propranolol treatment (Yan and Zhang 2014). Our data supports this literature and shows propranolol can reduce the presence of these inflammatory cells one week after injury. These data suggest less inflammation in the spleen of TP animals at this timepoint and that the increased Ly6C⁺, Ly6C⁻, and total monocyte presence in the blood is the mobilization of these cells to go where they are more needed one week after injury. Ly6C⁺ cells typically express higher levels of CCR2 than Ly6C⁻ monocytes, and Ly6C⁻ monocytes canonically

express more CX3CR1 to respond to its CX3CL1 ligand (Lee et al., 2016). CCL2 can lead to recruitment of both reparative and inflammatory monocytes, however, it predominantly recruits inflammatory monocytes due to the increased expression CCR2 on these cells (Wohleb et al., 2015; Sribnick et al., 2022). Although we did not investigate CCL2 and its sources in this study, injured inflamed tissue upregulates and releases CCL2, CX3CL1, and other chemokines (Lee et al., 2016).

Taken together this suggests propranolol can result in a more inflamed phenotype. This is potentially beneficial because immunosuppression post mild traumatic brain injury seems to be the driving factor behind long-term mild traumatic brain injury consequences. While the brains of TBI saline animals do exhibit more differential expression in pathways compared to sham saline baseline, we see that sham propranolol has even higher differential expression at both the one week and one month time points in the brain thereby suggesting propranolol leads to high differential expression in immunologic genes in the mouse brain. We did not see this to the same levels in the TBI saline group. TBI propranolol at the one-month time point in the brain most resembles the sham propranolol state where there was high differential gene expression in many pathways important in immune functioning such as TGF beta, adaptive immunity, immunometabolism, MHC class I antigen presentation, and MHC class II antigen presentation pathways.

We take these data to further suggest that the high differential expression in both propranolol treatment groups suggest decreased immunosuppression as

evident by increased monocytic presence in the spleen and blood at later timepoints. The presence of these cells can indicate an ongoing inflammatory response and decreased immune resolution. Mild TBI Typically results in high IL-1 β , TNF α , and CCL2 cytokine response soon after injury (Sribnick et al., 2022; Foley et al., 2008, Woiciechowsky et al., 1999). However, days and weeks after mild traumatic brain injury the brain, spleen, heart, and GI can show signs of inflammation due to insufficient resolution. Collectively, we take these data to suggest propranolol increases the ability of the body and immune cells to respond accordingly to inflammation post repeated mTBI by limiting the initial sympathetic induced IL10-dependent immunosuppressive state and the downstream effects on the brain, blood, and spleen.

Further highlighting the notion of an acute-to-chronic crossover, 1 month post rmTBI, both TBI conditions displayed increases in Ly6C⁺ inflammatory monocytes, but not the reparative Ly6C⁻ monocytes, with the spleens of propranolol treated TBI animals displaying significantly more Ly6C⁺ cells. While both TBI conditions display increased Ly6C⁺ and total monocyte numbers, the reasons for these increases can be very different. The increases for propranolol treated spleens may occur due to decreased immunosuppression. Therefore, this increase may be a reasonable compensation threshold indicating adequate monocyte functioning and decreased immunosuppression. Typically, one month after an injury is characterized by the activity of B and T cells of the adaptive arm of the immune system (Mauri and Bosma, 2012; Khan and Ghazanfar 2018). While we did not directly investigate their roles in this study, both B and T cells can

influence the behavior and activity of monocytes/macrophages due to the release of antibodies, molecules such as IL12, or directly binding to the dendritic cell/monocyte/macrophage through CD40 (Diaz et al., 2021). While highly probable that propranolol has direct and indirect effects on B and T cells as well, the feedback mechanism of B and T cells on monocytes may best explain the changes we detected at 1 month post injury in both the blood and spleen as the short monocyte lifespan would suggest the monocytes present at this time were not present around the time of injury.

Our study, though interesting, is limited in that the functional phenotype of these monocytic changes in the spleen and blood is not correlated to a behavioral outcome. Propranolol's use in migraine treatment suggests these animals would experience less post mTBI short- and long-term side effects although we cannot say with certainty. Although using a stress model, one group suggests that 24 days after experiencing stress paradigms, the spleens of mice predominate with inflamed monocytes (Reader et al., 2014; Wohleb et al., 2015). Our data suggests our 1-month spleen TBI propranolol treated spleen are potentially more inflamed than untreated TBI spleen. However, we do not think this to be the case in our study as the propranolol treatment limits sympathetic nervous system-induced immune suppression and the resultant Ly6C+ increase at 1 month could be the new homeostatic normal for these animals. Another plausible explanation is that the increase in splenic Ly6C+ monocytes at one month is a mobilizable reservoir for cells to be quickly recruited to resolve the next injury.

Conclusions

Future directions of this study will direct us to better understand how propranolol impacts repeated mTBI animals on a behavioral level. Pain is one of the main long-term consequences of mild traumatic brain injuries so understanding the etiology of the pain in accordance with immunological changes will provide much needed information as to continuing to understand the ever-changing mTBI pathophysiology. Also, we plan to functionally understand how monocyte subsets are directly influenced by propranolol to change their cytokine production at various timepoints post rmTBI. In summary, our study shows propranolol can cause shifts in mice monocyte subset populations after repeated mild traumatic brain injury in both the blood and spleen. We also can conclude propranolol causes differential gene expression changes impacting many pathways in the brain and spleen over the course of a month. Our data further suggests that propranolol can mobilize Ly6C⁺ monocytes better than untreated rmTBI animals from the spleens into the blood. 1 month after injury, the blood of rmTBI animals treated with propranolol depict increased Ly6C⁻ monocytes. We conclude this to suggest that the rmTBI animals treated with propranolol are less immunosuppressed over time as propranolol limited immediate sympathetic nervous system activation directly after injury. We hope that the results of this study will support repurposing of this drug for immediate use after concussions and mild brain injuries will lead to enhanced behavioral outcomes for the millions of people who are impacted by the long-term consequences of concussions every year.

Figures and Tables

Figure 3.1. Experimental Timeline

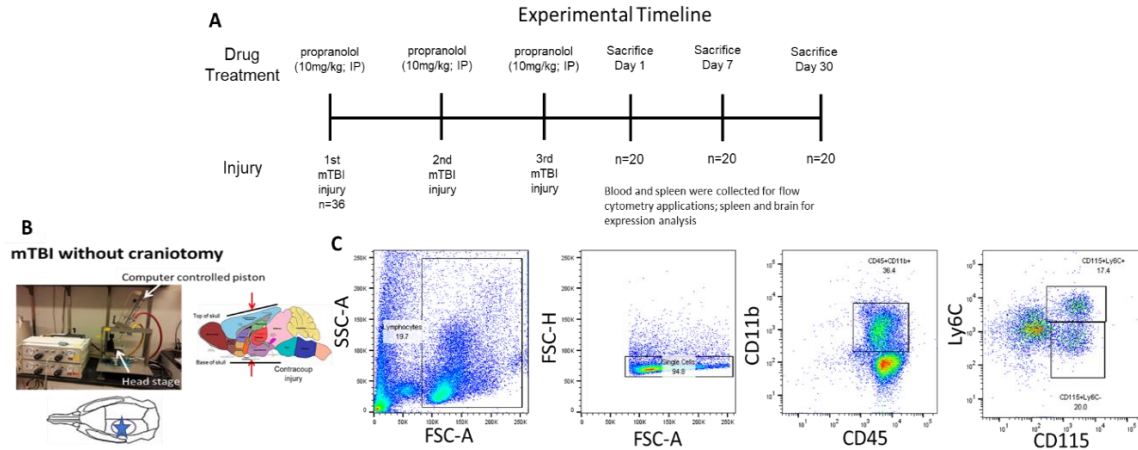


Figure 3.1. (A) depicts the timeline of the experimental setup with injuries and injection schedules. **(B)** Displays our close headed TBI set up and where we injury the cortex. **(C)** Representative gating strategy used to identify monocytes (CD45+CD11b+CD115+) and monocyte subsets (CD45+CD11b+CD115+Ly6C+/-) from blood and spleen PBMCs.

Figure 3.2. Blood monocyte percentages show no changes at 1 day but by day 30 shows population shifts from Ly6C+ to increased Ly6C- cells in propranolol treated TBI tissue.

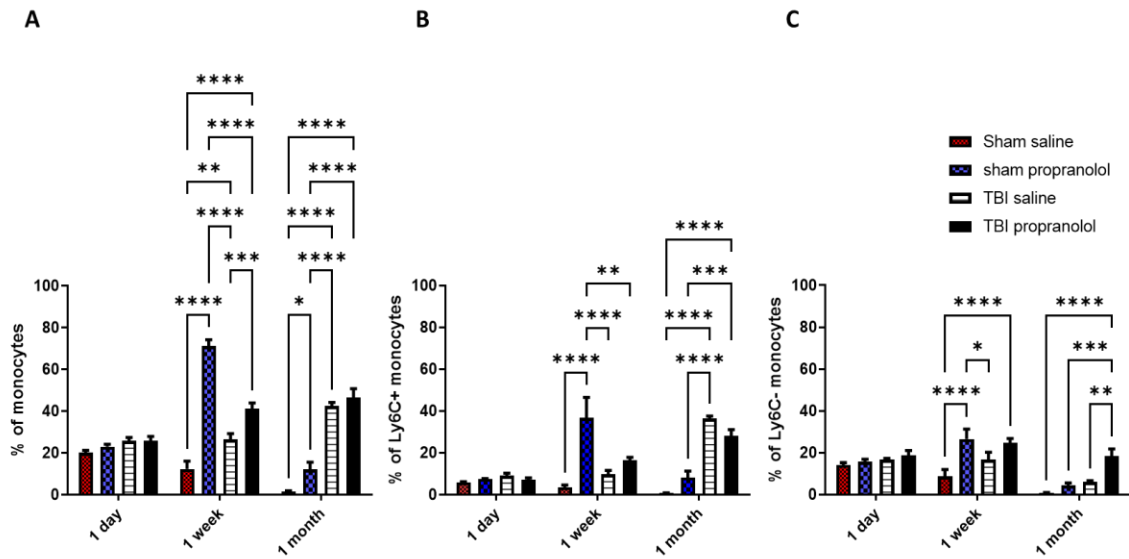


Figure 3.2. Total (A), Ly6C+ (B), and Ly6C- (C) blood monocyte percentage varies overtime with TBI and/or propranolol treatment. (A) While there does not seem to be any population changes 24 hours after mTBI. Total monocyte numbers increase significantly for all groups compared to sham saline treated animals 1 week post injury with sham injured propranolol animals displaying the most significant increases. However, by 1 month, the monocyte increases seem to be more TBI based as both TBI saline and TBI propranolol display significant increases compared to sham injured animals. (B). Interestingly, Ly6C+ blood monocytes were significantly increased in TBI treated animals compared to untreated TBI animals at 1 week, however, this completely reverses by the 1-month timepoint. (C) While there were no significant population differences between saline treated sham and TBI isolated Ly6C- monocytes at the 1 week timepoint, sham injured with

propranolol displays increases in Ly6C- monocytes over both groups with the TBI propranolol only displaying significant increases in this population over uninjured saline treated controls. This continues at the 1-month timepoint as the TBI animals treated with propranolol have significant Ly6C- monocyte increases over the other groups. Data shown as mean +/- SEM, n=5. *P < 0.05, **P < 0.01, ***P < 0.001, ****P<.0001 compared by 2-way ANOVA followed by Bonferroni post hoc test.

Figure 3.3. TBI spleens from propranolol treated mice show decreased monocyte percentages 7 days after injury but increased monocyte numbers 30 days after repeated mTBI.

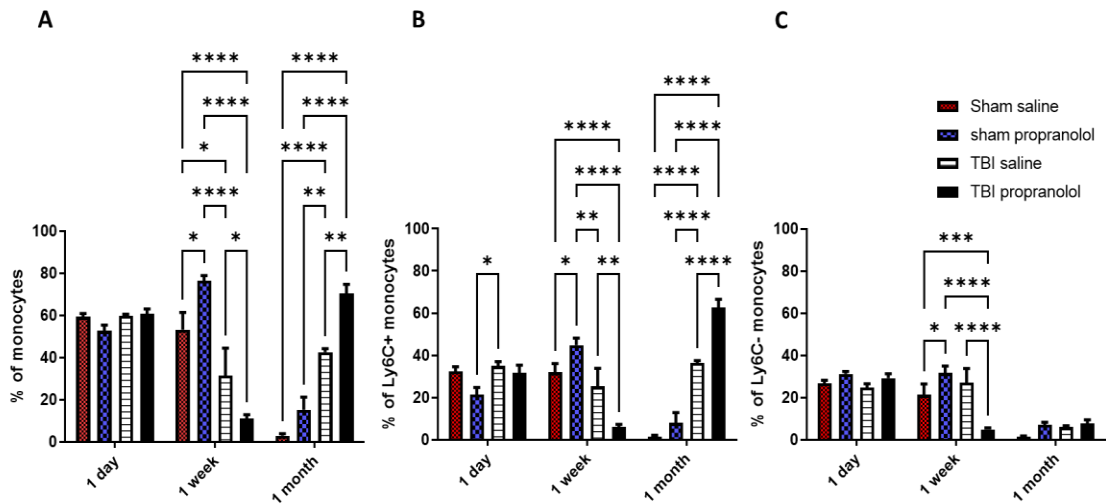


Figure 3.3. Spleen total and monocyte subset populations across TBI and propranolol groups over time. (A) Total monocyte population percentages at 1 day, 1 week, and 1-month timepoints. Total monocyte numbers remained unchanged in the spleen 24 hours after injury; however, 1 week after injury TBI animals display significantly decreased monocyte numbers compared to injured controls with the TBI propranolol treated animals exhibiting the most pronounced decreases. Ironically, this effect reverses by the 1-month timepoint with the TBI animals, especially those treated with propranolol, now displaying increases in total monocyte numbers. (B) Displays Ly6C+ monocyte and (C) displays Ly6C- monocyte population percentages isolated from the spleens of sham injured and TBI mice injected with propranolol or saline. Data shown as mean +/- SEM, n=5.

*P < 0.05, **P < 0.01, ***P < 0.001, ****P<.0001 compared by 2-way ANOVA followed by Bonferroni post hoc test.

Figure 3.4. DEG expression after repeated mTBI shows various splenic DEG changes at 1 day, 7 days, and 30 days after injury.

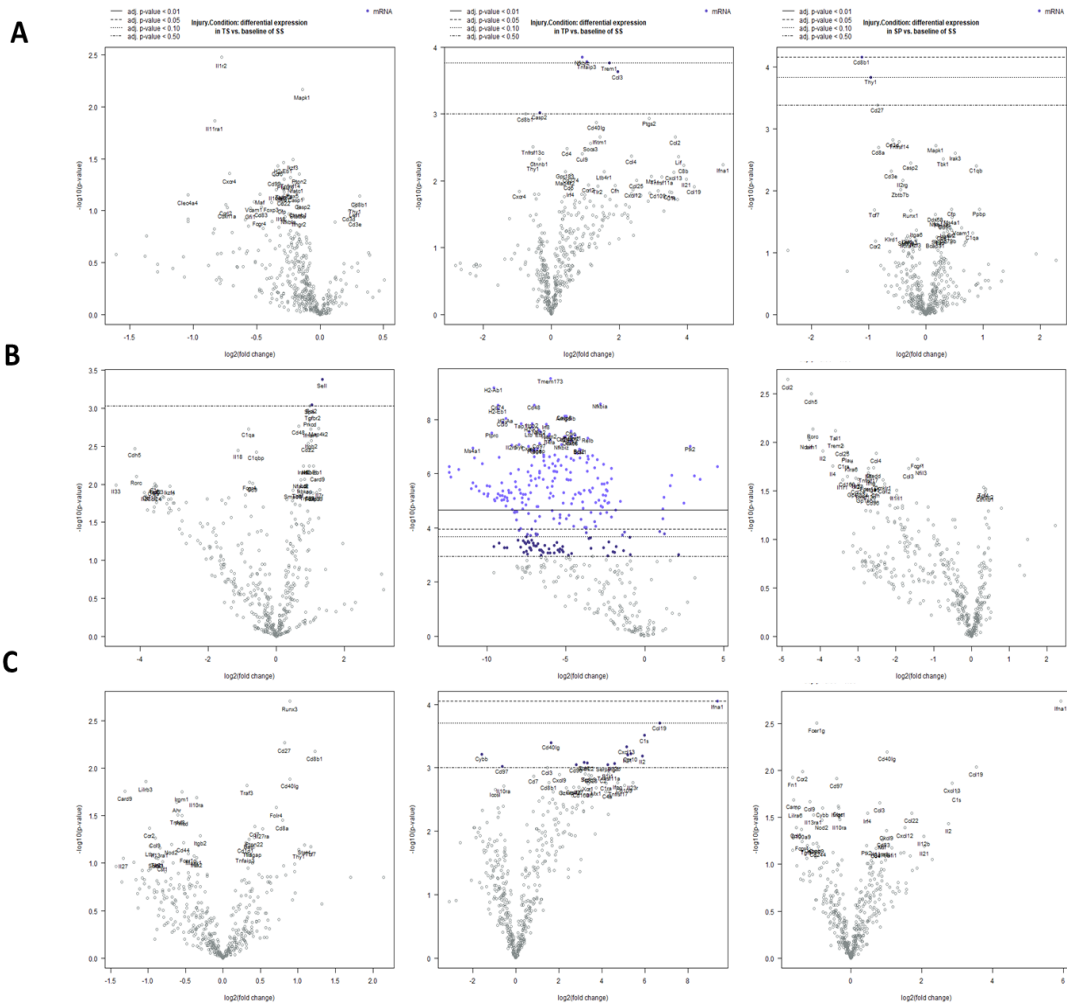


Figure 3.4. NanoString generated volcano plots of mRNA fold changes in the mouse spleen. (A) 1 day, (B) 1 week, and (C) 1 month represent timepoints with comparison to sham saline as baseline value. TP= TBI + propranolol, TS= TBI + saline, SP= Sham + propranolol. Data is expressed log2 fold change vs. p value; N=3.

Figure 3.6. DEG expression after repeated mTBI shows various brain DEG changes at 1 day, 7 days, and 30 days after injury.

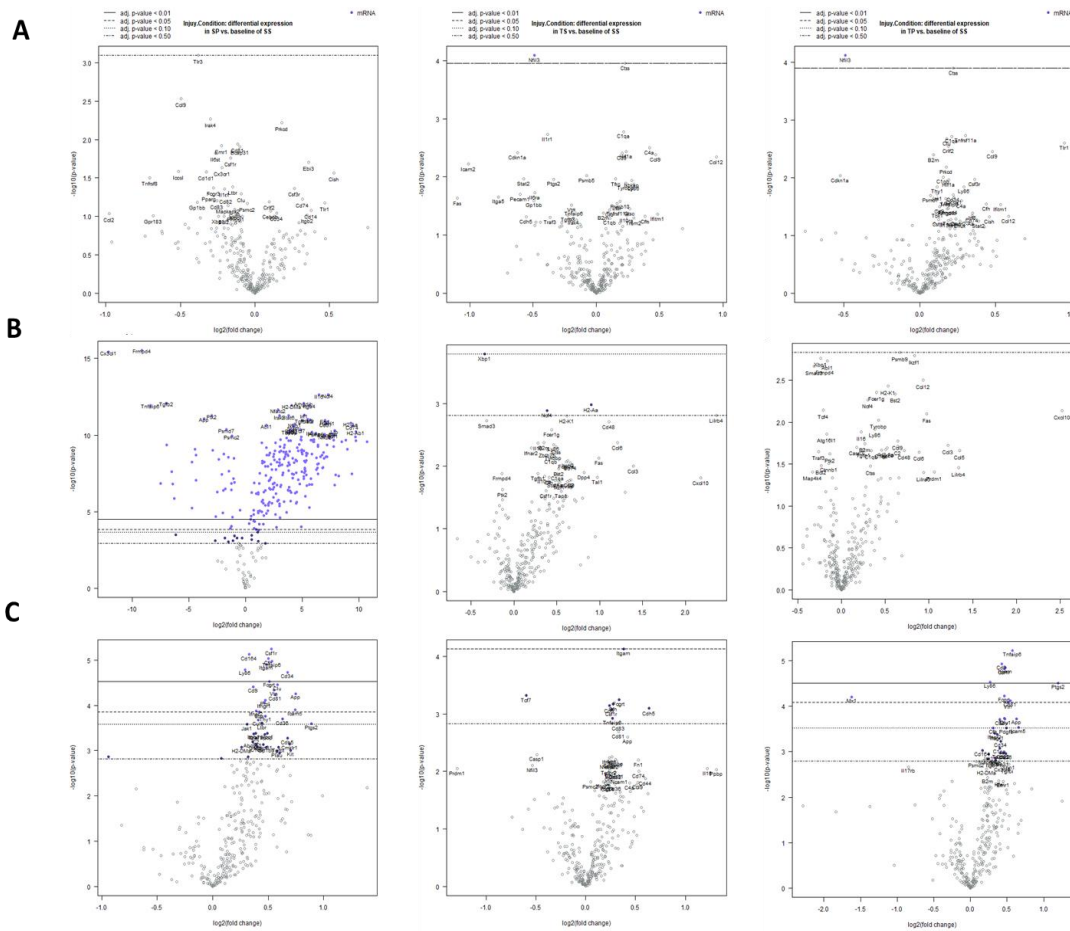


Figure 3.6. NanoString generated Volcano plot of mRNA fold changes in the mouse brain. (A) 1 day, (B) 1 week, and (C) 1 month represent timepoints with comparison to sham saline as baseline value. TP= TBI + propranolol, TS= TBI + saline, SP= Sham + propranolol. Data is expressed log2 fold change vs. p value; N=3.

Figure 3.7 Pathway scores for the brain after propranolol at 1 day, 7 days, and 30 days after mTBI and propranolol injection.

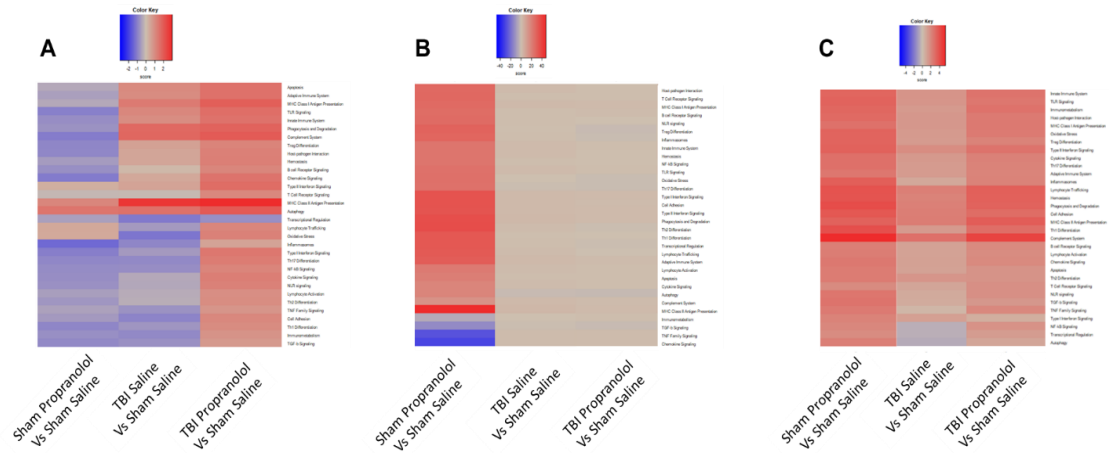


Figure 3.7. Heatmap displays for each treatment condition as exhibited by global significance scores. Directed global significance statistics measure the extent to which a pathways genes are up-or down-regulated. Red denotes gene sets whose genes exhibit extensive overexpression while blue denotes gene sets with extensive under expression compared to the sham saline baseline at A) 1 day B) 7 days, and C) 30 days after repeated mTBI. Data are expressed as Z scores for genes from brains; n=3.

Figure 3.8. Repeated mTBI spleens treated with propranolol show alterations in cytokine mRNA expression 1 week after injury.

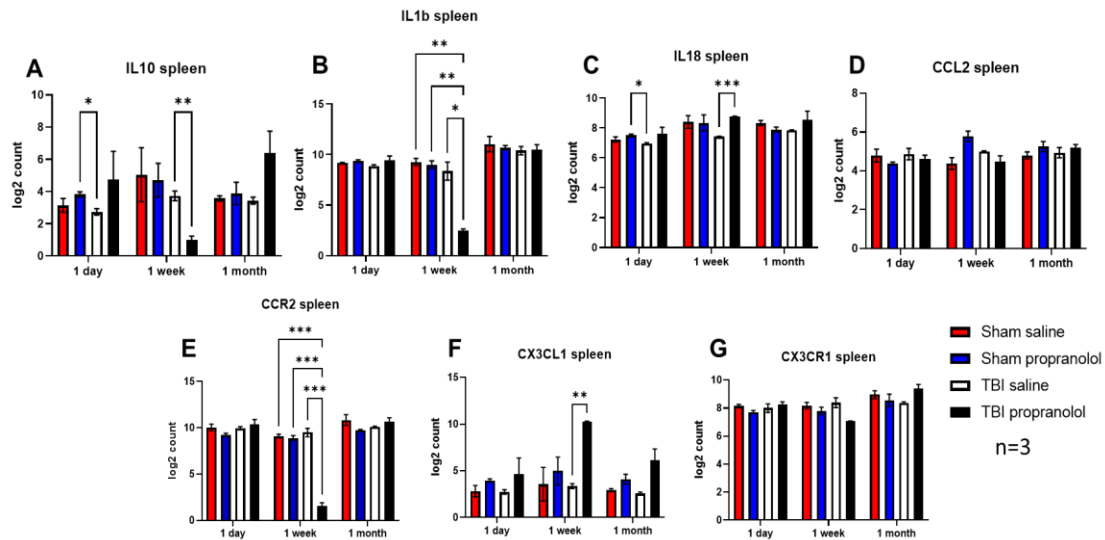


Figure 3.8. Effect of injury alone or in combination with propranolol on genes in the spleen. Fold change in mRNA expression between sham injury plus saline tissue (red), sham injury plus propranolol (blue), rmTBI plus saline (white) and rmTBI plus propranolol (black) at one day, seven days and one month following injury. (A) IL10, (B) IL1 β , (C) IL18, (D) CCL2, (E) CCR2, (F) CX3CL1, (G) CX3CR1 represent mean log₂ count \pm SEM. Data was analyzed 2-way ANOVA followed by Sidak's multiple comparison test (p<0.05) *, (p<0.01) **, (p<0.001) ***, (p<0.0001) ****.

****.

Figure 3.9. Propranolol alone drives cytokine expression changes in the brain 1 week after injury.

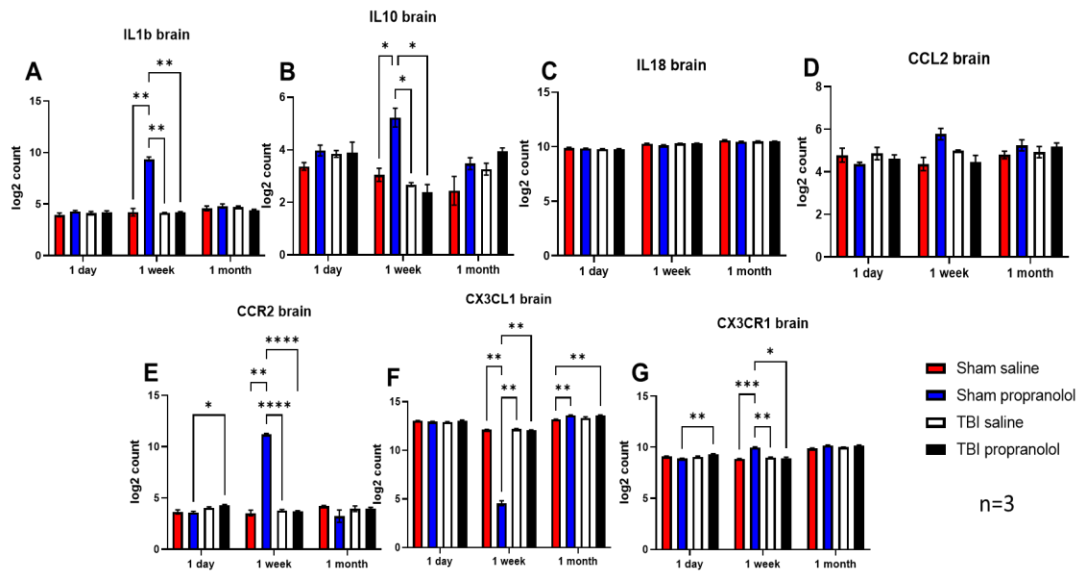


Figure 3.9. Effect of injury alone or in combination with propranolol on genes in the brain. Fold change in mRNA expression between sham injury plus saline tissue (red), sham injury plus propranolol (blue), rmTBI plus saline (white) and rmTBI plus propranolol (black) at one day, seven days and one month following injury. (A) IL10, (B) IL1 β , (C) IL18, (D) CCL2, (E) CCR2, (F) CX3CL1, (G) CX3CR1 represent mean log₂ count \pm SEM. Data was analyzed 2-way ANOVA followed by Sidak's multiple comparison test ($p < 0.05$) *, ($p < 0.01$) **, ($p < 0.001$) ***, ($p < 0.0001$) ****.

Figure 3.10. Left and right hindpaw withdrawal testing yields inconclusive results due to external factors.

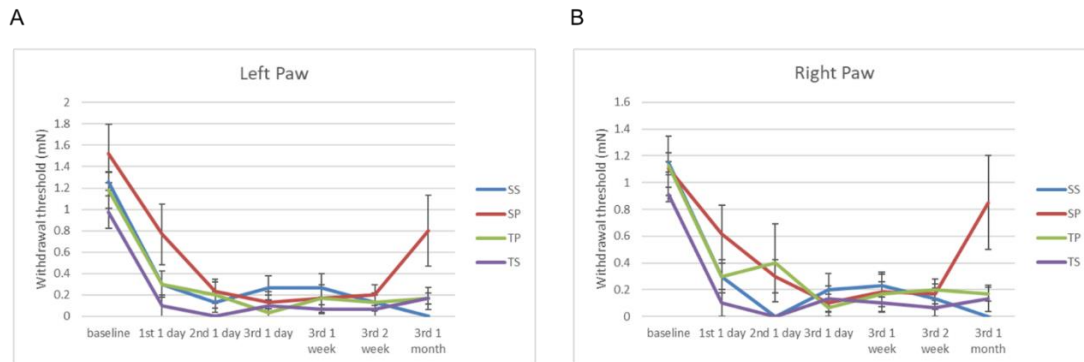


Figure 3.10. Mouse hindpaw withdrawal tests tactile pain sensitivity to mechanical stimuli at 0.5 mN, 1.0 mN, 2.0 mN, and 4.0 mN. Our data is shown with withdrawal thresholds at various timepoints after repeated mTBI. Due to external forces, we were unable to establish an adequate baseline for which to compare TBI changes as sham saline control displays substantial pain response throughout the trials. Data is shown mean \pm SEM.

Table 3.1. Number of differentially expressed genes in each tissue.

Differentially Expressed Genes										
Tissue	TBI saline			TBI propranolol			sham propranolol			
	1D	1W	1M	1D	1W	1M	1D	1W	1M	
Brain		2	0	1	2	0	10	0	264	17
Spleen		0	0	0	0	213	1	1	0	0

Table 3.1. This table displays the number of differentially expressed genes (DEGs) in each tissue compared to sham saline controls. Data was NanoString generated using multiple t-tests for each gene in a group compared to its sham saline counterpart. DEG were considered significant if Bonferroni FDR adjusted $p < 0.05$.

Chapter Four

Propranolol Treatment During Repetitive Mild Traumatic Brain Injuries Induces Pronounced Transcriptomic Changes in the Bone Marrow of Mice

Introduction

Every year, millions of individuals suffer from mild traumatic brain injuries, commonly known as concussions, that result in potentially debilitating long-term complications. Healthcare burden of treatment for concussion patients is estimated to be up to \$1.35 billion annually (McGinity et al., 2018; Pavlov et al., 2019). Due to limited understanding of the underlying mechanisms, treatments for concussion-associated conditions, such as headache, are mostly supportive and do not effectively address underlying pathophysiology.

Concussion can trigger a multitude of molecular processes and mechanisms that are generally ignored in favor of symptom management. These processes may eventually lead to downstream disease or deleterious injury responses. Factors influencing these responses may include tissue specific DNA methylation and histone modification and alterations in patterns of microRNA expression. These changes affect gene activation or repression and in turn determine cellular responses to a broad spectrum of environmental signals by altering protein expression.

Studies of disease genetics have used drug effects to identify putative genes contributing to variation in therapeutic drug responses and adverse drug reactions (Giacomini et al., 2017; Castonguay et al., 2022). Though most drugs have not been effective in treating headache symptoms associated with mTBI,

some pharmacological options are available and are thought to be relatively free of serious or long term off-target effects. One such class of drugs is the β -adrenergic receptor antagonist, or beta-blockers. As the concussive effect of mTBI is largely thought to be associated with a hyperadrenergic state, a disrupted blood brain barrier, and high local norepinephrine levels, treatment with a beta-blocker, such as propranolol, offers a potentially beneficial approach to blunting the cascade of post-mTBI sympathetic activation (Alali et al., 2017; Chen et al., 2017; Ding et al., 2021; Florez-Perdomo et al., 2021).

In this study, we compared the effects of propranolol on transcriptome in the bone marrow of mice subjected either to mTBI or to the Sham surgery procedures. Additionally, we performed the pathway enrichment analysis to identify the common cellular pathways affected by propranolol alone, mTBI alone, or the combination of propranolol with head injury, focusing on those pathways that are related to fatty acid, glucose, and glutamine metabolism, stem cell epigenetic modifications, and inflammatory signaling. Our study reveals new disease-related genes and informs on the molecular and cellular basis for post-concussion sequela across time.

Methods

Animals

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Indiana University School of Medicine, in accordance with National Institutes of Health guidelines for care and use of laboratory animals. 36 Wild type male C57BL/6 mice were obtained from Jackson Laboratory at 8-12

weeks old (~25-30 g) and were randomly divided into sham saline (SS), mTBI saline (TS), mTBI propranolol (TP), and sham propranolol (SP) for downstream gene expression analyses. Mice were further subdivided into day 1, day 7, and day 30 timepoints with a total of n=3 for each group at each timepoint.

Repeated mild traumatic brain injury procedure.

mTBI was inflicted using a technique described recently (Nguyen et al., 2021). Briefly, mice were anesthetized with 2–4% isoflurane and heads were stereotactically fixed with heat-pad support below the abdomen. Closed head mTBI was produced using a control cortical impact (CCI) device after shaving, cleaning, and sanitizing the area, and making a skin incision to expose the skull. After the baseline point of the device was set by lowering the tip to the exposed skull surface and the stage position was set to zero, the impactor was retracted, the impact depth was set, and the impact was made. The approximate center of the impact site was 1 mm posterior to the bregma and 0.5 mm lateral from midline on one cortical hemisphere. The skull was struck with the impactor tip at a speed of 3 m/s to a depth of 1 mm. Wounds were sutured and the animal placed on heating pad until fully recovered. Sham injury animals were shaved, cleaned, sanitized, an incision was made to expose the skull, but no impact was performed on the skull, followed by suturing. All animals survived the process without any skull fracture or hemorrhage. The injury treatment paradigm was performed once a week for three weeks for a total of 3 injuries (Figure 4.1).

Intraocular pressure (IOP) changes in mouse eyes were used to both confirm the severity of the skull impact and verify the absence of skull fracture, as

a surrogate of changes in brain pressure (Chen et al., 2019). An ICARE TONOLAB (Vantaa, Finland) tonometer was used to measure IOP. IOP measurements were done immediately before and after the injury for up to 30 min (data not shown). If the calvarium had been fractured there would have been an absence of IOP. Alternatively, if there were no changes in IOP, injury to the calvaria was deemed insufficient, and the animal excluded from the study. No animals were subsequently removed from this study.

Propranolol administration.

Propranolol chloride (Tocris Bioscience, Bristol, United Kingdom) was dissolved in saline (3mg/ml). Based on the treatment group designation, the drug (10 mg/kg) or saline vehicle was injected intraperitoneally into each mouse immediately following mTBI or sham surgery as previously stated by other studies (Haffner-Luntzer et al., 2019; Loftus et al., 2019). Each mouse was subjected to either a total of three propranolol or saline injections.

Bone marrow tissue processing.

We followed previous bone marrow protocols to extract bone marrow tissue from the femurs of mice. (Marim et al., 2010) Briefly, scissors were used to cut the femur from the pelvis and knee joint. The femur was then placed into ice cold PBMC culture media composed of complete RPMI media (88% RPMI 1640; ThermoFisher), 10% Fetal Bovine Serum (Sigma-Aldrich), 1% Penicillin Streptomycin (Sigma-Aldrich), and 1% Glutamax (ThermoFisher). Next, a 23G needle attached to a 3 mL syringe filled with ice cold complete RPMI was inserted into the medullary cavity of the femur and the bone marrow was flushed out into a

15 mL tube. The procedure was repeated 2-3 times until the femur diaphysis was white. The resulting tissue was centrifuged at 400G for 10 mins at 4 °C, and the supernatant was discarded. Remaining tissue was then resuspended in RBC lysis buffer for 10 minutes at room temperature, recentrifuged, and resuspended in calcium free PBS. The suspension was again centrifuged at 400G for 10 minutes at room temperature, resuspended in complete RPMI media, and then added to an equal volume of FBS supplemented with 10% DMSO for freezing. The frozen bone marrow was stored at -196°C until future analyses.

NanoString nCounter gene expression panels.

mRNA was isolated from each bone marrow sample using the TRIzol reagent (Invitrogen) as described in the manufacturer's protocol. RNA samples were evaluated by nCounter gene expression analysis technology (NanoString Technologies) and quantified with nCounter Digital Analyzer (NanoString Technologies). The expression of 770 genes (including 14 internal reference genes) was determined using the nCounter Stem Cell Characterization Panel™ (mouse, NanoString, XT-CSO-MSCC-12). To minimize variability among arrays, densitometry values between arrays were normalized using the Robust Multichip Average function and further transformed to the logarithmic scale (log₂). Gene expression levels in each sample were normalized against the geometric mean of six housekeeping genes, specifically *Cltc*, *Gapdh*, *Gusb*, *Hprt1*, *Pgk1*, and *Tubb5*. A cutoff was introduced at the value of the highest negative control present on the chip. 100 ng of total mRNA was used as input and sample hybridization was performed according to the manufacturer's instructions.

Raw data processing, quality control, and normalization were performed using nSolver™ 4.0 analysis software. Quality control (QC) and normalization were performed with an imaging QC of >75% field of view registration, binding density QC within 0.1-2.25 range, positive control linearity QC of R2 above 0.95, and positive control limit of detection set as 0.5 fM positive control above 2 standard deviations above the mean of the negative controls. Normalization to housekeeping genes, of which genes below 100 were excluded, and pathway scoring, gene set analysis, differential expression analysis, and cell type profiling were completed using the Advanced Analysis software plugin (version 2.0.115). Fold changes were calculated using the average of each group compared to appropriate sham-injured saline treated control at each timepoint. For gene set analysis and differential expression analysis, a *p*-value of ≤ 0.05 was applied as cutoffs. For all NanoString analyses at 1 day, 1 week, and 1 month after injury, gene expression measurements for each group were normalized to the sham saline (n=3) baseline of each timepoint.

Statistics.

All pathway score data values (Tables 4.2-4.4) are reported as mean Z-score values (n=3) for each group and corresponding *p* value post unpaired t test using sham saline at each timepoint for comparison. NanoString analysis for significantly differentially expressed genes shows the 40 most statistically significant increased or decreased genes on volcano plots (Figures 4.2-4.4) graphed fold change for the mRNA of interest in the experimental group versus the sham saline mRNA amount plotted against the Bonferroni false discovery rate

adjusted *p*-value of ≤ 0.05 using the sham saline for each timepoint as baseline. Normalized log₂ values from the 12 top mRNA changes (Figure 4.5) amongst the SP, TS, and TP groups were further characterized across the 1 day, 7 days, and 30-day timepoints in GraphPad Prism 9 (GraphPad Prism 9, GraphPad Software, La Jolla, California) with log₂ values reported as mean \pm S.E.M with significance set at $p < 0.05$. These data were analyzed using two-way ANOVA (treatment vs. timepoint) with Sidak's multiple comparisons post hoc tests.

RESULTS

A high-resolution transcriptional profile of rmTBI in mice subjected to propranolol or saline treatment.

A sample total of three mice per treatment group were used for each post-injury/drug treatment time point in this study (Figure 3.1). This gives detailed coverage of acute, subacute, and chronic responses of bone marrow tissue to rmTBI in the absence or presence of propranolol treatment.

Effect of propranolol combined with repeated mTBI at 1-day post-injury.

Mice sacrificed on 1-day following completion of the 3x injury/drug treatment paradigm showed no significant changes of gene expression in the SP treatment group (Figure 4.2A), whereas the TS treatment group exhibited increased gene expression in branched chain amino acid transaminase (*Bcat2*), valosin-containing protein (*Vcp*) and neurolysin (*Nln*) (Figure 4.2B). The TP treatment group exhibited the greatest change at 1-day as there were 66 genes which displayed decreased expression including *Bcat2* (Figure 4.2C).

Complex biological processes such as those present in bone marrow tissue involve the coordinated regulation of multiple intracellular signaling pathways controlling gene expression. The signaling pathway changes found in the SP treatment group were limited to an increase in Hedgehog signaling (Table 4.2). In contrast, the decreases in gene expression observed in both TS and TP bone marrow tissue were accompanied by decreases in signaling pathway scores on the 1-day time point following injury paradigm for all assayed pathways (Table 4.2).

Effect of propranolol combined with repeated mTBI at 7-days post-injury.

Seven days post cessation of the 3x injury/drug treatment paradigm, the SP group exhibited 57 genes which were significantly different when compared with the SS group (Figure 4.3A). Of this group, 48 genes exhibited decreased expression and 9 genes showed increased expression (Figure 4.3A). A significant decreased expression for 21 genes in the TS group was evident (Figure 4.3B). The SP and TS groups at 7-days exhibited similar decreases in *Usp7*, *tfdp2*, *add1*, *pp2r5b1*, *cir1*, and *cul1*. Unlike the 1-day post injury paradigm, TP bone marrow failed to exhibit either increases or decreases in gene expression at 7-days (Figure 4.3C).

Signaling pathway changes in SP bone marrow tissue exhibited the greatest number of pathway changes (26 of 32) at 7-days post-injury (Table 4.3; Figure 4.6). The SP bone marrow tissue displayed decreases in amino acid metabolism, apoptosis, autophagy, cell cycle, cytoskeletal reorganization, epigenetic modifications, glutamine metabolism, hedgehog signaling, notch signaling, senescence and quiescence, and TGF β signaling (Table 4.3; Figure 4.6).

Increases in signaling pathway were also apparent. The significant changes were found in AP1 signaling, ectodermal lineages, endodermal lineage, fatty acid metabolism, glucose metabolism, hippo signaling, hypoxia response, MAPK signaling, mesodermal lineages, mTOR signaling, Na.QZ.ve state, oxidative stress, partially reprogrammed, pluripotency markers, and RhoROCK signaling (Table 4.3; Figure 4.6).

The TS bone marrow tissue exhibited numerous changes in 15 of 32 signaling pathways (Table 4.3; Figure 4.6). These changes were largely decreases in signaling pathways such as, autophagy, cell cycle, cytoskeletal reorganization, epigenetic modifications, glutamine metabolism, hedgehog signaling, HOX gene activation, integrin signaling, JAK-STAT signaling, notch signaling, primed state activation, and senescence and quiescence. Significant increases were found only in three signaling pathways such as amino acid metabolism, AP-1 signaling, and partially reprogrammed cells (Table 4.3; Figure 4.6).

Despite a lack of gene expression changes in the TP animal group, we observed significant increases in signaling pathway scores for glutamine metabolism, hedgehog signaling, and JAK-STAT signaling. Increases in signaling pathway were limited to only glutamine metabolism in TP bone marrow when compared to TS bone marrow (Table 4.3; Figure 4.6).

Effect of propranolol combined with repeated mTBI at 30-days post-injury.

We observed significant differential expression of 40 genes in the SP group and 52 genes in TP group but no changes in gene expression in TS group at 30-days after completion of the injury/drug treatment paradigm. Among the genes

which exhibited decreased expression in both treatment conditions of SP and TP were *Cul1*, *Itgb1*, *Sorbs*, *Tsc22d1*, *E2f4*, *Brd7*, *Mark3*, *Cir1*, and *Amd1/2* (Figure 4.4). This differential gene expression profiling clearly demonstrates the long-lasting impact of propranolol on bone marrow tissue in both SP and TP animal groups. None of the genes associated with TS met the established criterion for significant change in expression levels.

Characterization of signaling pathway changes following the injury/drug treatment paradigm demonstrated significant signaling pathway changes present in SP, TS, and TP bone marrow tissue 30 days after injury. SP bone marrow tissue exhibited significantly decreased signaling in 25/32 pathways when compared to the SS bone marrow tissue. The pathway score changes for the various groups are summarized in (Table 4.4 and Figure 4.6). The TS bone marrow was found to have increased signaling in amino acid metabolism, apoptosis, cell cycle signaling, cytoskeletal reorganization, glucose metabolism, hedgehog signaling, and oxidative stress pathways. Additional signaling changes were observed in the TP bone marrow tissue 30 days following injury as displayed by significant decreases in 24/32 pathways compared to baseline SS tissue. Here, we again observe a high crossover of decreased signaling changes common between SP and TP bone marrow tissue. Signaling pathway changes which were different between SP and TP were limited to an increase in AP-1 Signaling. Compared to TS bone marrow tissue, mTBI bone marrow from TP mice exhibit significant decreases in 24/32 signaling pathways.

Long term bone marrow tissue mRNA changes impact several genes important for numerous signaling pathways.

Expression of numerous genes were affected by the drug/injury paradigm across the three timepoints (Figure 4.5). The SP group changes at one day were most evident by decreased gene expression of *hmgb1*, *rock1*, *hdac6*, *vegfa*, *tfdp2*, *sorbs1*, *bcat2*, *camk1*, and *cul1* when compared to SS treated animal mRNA transcripts. The only gene expression which increased at 1-day was *usp7*. This change was restricted to a comparison between sham saline (SS) bone marrow and not to rmTBI (TS) bone marrow.

Seven days after injury TP mRNA transcripts were increased for *hmgb1*, *usp7*, *tfdp2*, *itgb1*, *sorbs1*, and *cul1* when compared to TS derived bone marrow mRNA expression levels. *Jak2* was also increased in TP bone marrow when compared to SP bone marrow tissue. Two gene transcripts, *hdac6* and *vegfa*, were decreased in TP bone marrow but only when compared to SP bone marrow. Interestingly, TP genes did not differ from SS derived gene transcripts for the genes we investigated at this timepoint.

The 30-day timepoint displayed genetic changes for several gene transcripts derived from TP animals. We observed increased *hmgb1*, *rock1*, *hdac6*, *usp7*, *vegfa*, *jak2*, *tfdp2*, *itgb1*, *sorbs1*, *bcat2*, and *cul1* compared to TS-derived bone marrow tissue. It is worth noting that some gene expression increases in the TP group were considerably less when the data were compared to SS-derived bone marrow tissue, specifically for *itgb1* and *cul1* genes.

Discussion

Deciphering transcriptomic changes elicited by rmTBI is important for understanding the cellular and molecular mechanisms underlying the post-concussion complications. Beta-blockers are widely used in TBI patients, and it is important to determine how such treatment modulates TBI-associated changes in gene expression and whether it affects any cellular signaling pathways. In this study we investigated the effects of propranolol treatment on bone marrow tissue derived from mice subjected to repetitive mild trauma to the calvarium. TBI is known to cause systemic and neuro-inflammation, whereas bone marrow tissue is a powerhouse for production of immune cells and is critical for body responses to infections and injuries. Our data revealed that propranolol treatment variably modulated TBI-induced changes in gene expression and signaling pathways within bone marrow tissue.

Bone marrow dysfunction is common in severely injured trauma patients as the tissue is known to be influenced by factors including elevated levels of circulating catecholamines and inflammatory mediators (Livingston et al., 2003; Robinson et al., 2006; Loftus et al., 2018). The early use of propranolol following severe traumatic head injury appears to be beneficial as it blunts aspects of Cushing reflex, which include early tachycardia, reduce hematopoietic progenitor cell mobilization, and results in faster return to baseline of the inflammatory granulocyte colony-stimulating factor peak often seen with head injury. (Fonseca et al., 2004; Cook et al., 2013; Bible et al., 2014). However, the degree to which propranolol benefits the mTBI patient is relatively unknown but observational data

suggests that early usage of the drug, post-injury, likely controls hemodynamics and blood sugar with decreased catecholamine levels (Alali et al., 2017). Subsequently, the non-selective beta-blocker, propranolol, limits activation of these pathways in many injury paradigms; however, its role in bone marrow tissue function after mTBI requires further investigation. Overall, our study suggests propranolol may impact mTBI bone marrow tissue by altering metabolic pathways, and potentially limiting inflammation and epigenetic modifications.

Our data suggests that epigenetic changes occurring after mTBI can be modulated by short term dosing of propranolol. Four genes important for epigenetic modifications that we further investigated based on significant expression changes across the three time points were *hdac6*, *camk1*, *vegfa*, and *usp7*. Collectively, these genes' expression were all decreased one day after injury yet returned to sham saline levels 30 days later in the drug-treated bone marrow tissue compared to non-treated bone marrow. While these genes have important roles in other pathways such as oxidative stress, metabolism, autophagy, and intracellular signaling, the changes in the mRNA expression here reflect potential widespread bone marrow tissue dysfunction after mTBI. Further direct measurement of changes in epigenetic markers accompanying changes in gene levels will further elucidate effects.

One mRNA transcript that impacts many of the possible signaling pathway changes is branched chain amino transferase 2 (*bcat2*). This enzyme appears to be important for mitochondrial protein and energy metabolism. Branch chain amino acids (leucine, isoleucine, and valine) serve as precursors to many proteins,

particularly neurotransmitters (Holecek, 2018). Bcat2 enzyme is pivotal for BCAA catabolism and helps in the generation of glutamic acid, glutamate, and glutamine (Dimou et al., 2022). BCAA levels decrease following a TBI event and supplementation of BCAAs improved post TBI cognitive recovery in humans. (Aquilani et al., 2008; Sharma et al., 2018; Dickerman et al., 2022). The fact that we observed increased *bcat2* and mTOR pathway scores one day after injury but decreased levels 30 days later in TS bone marrow tissue may indicate that *bcat2* expression in TP bone marrow could prove to have a restorative function. Bcat2, glutamate, and glutamate activity can also lead to PI-3K/mTOR pathway activation. Accordingly, we found mTOR signaling at 30 days to be decreased in the drug-treated injury paradigm while injury alone tissue displayed increases in this signaling pathway. Therefore, it is entirely possible that blocking beta receptors with propranolol helps reduce bone marrow metabolic processing of inflammatory mediators following traumatic injury.

Though understudied, rmTBI appears to increase metabolism of amino acids, fatty acids, glucose, and glutamine one day after injury, but propranolol administration resulted in lower expression of genes in these pathways. Importantly, TP bone marrow showed no changes from SS bone marrow 7 days after injury; however, TS bone marrow showed decreases in amino acid and glutamate metabolism. Interestingly, though both TS and TP bone marrow showed decreased pathway scores 30 days after injury, propranolol treated rmTBI tissue displayed substantially less expression, further displaying decreased bone marrow metabolism. This may result in a less inflamed bone marrow environment for stem

cells and bone marrow derived immune cells to develop and mature. Collectively, TP bone marrow may respond more appropriately to future challenges.

The rmTBI paradigm also appears to disrupt signaling pathways for integrin signaling, JAK-STAT, oxidative stress, and TGF β , as all these pathways were decreased with propranolol treatment when compared to untreated bone marrow tissue at both one day and 30 days after injury. Activity in these pathways can lead to increased white blood cell modulation and inflammatory cytokine production. (Kalra et al., 2022). For example, TGF β production and activity leads to activation of immunosuppressive T cells that may further increase inflammation by limiting injury resolution. (Sanjabi et al., 2017; Simon et al., 2017). While we did not measure TGF β protein, our data supports this premise, as we detected increased TGF β signaling in saline treated TBI animals. Interestingly, TGF β signaling was reduced in the TP group at 1 month. This may be a compensation response as there was no difference in TGF β signaling in propranolol treated bone marrow tissue at the one-week timepoint.

Our observations are limited in that we did not measure what bone marrow cell types exhibited the mRNA transcript changes or directly measure changes in protein expression. Subsequently it is difficult to attribute a direct mechanism for drug action in the bone marrow tissue after rmTBI. Another uncontrolled variable was the systemic administration of propranolol. Parallel direct injection into bone marrow tissue would allow additional insight regarding direct or indirect effects of the drug on bone marrow tissue. Nevertheless, the fact that propranolol appears to produce long-term effects on the tissue suggests impactful changes in several

signaling pathways including cellular metabolism, TGF β signaling, integrin signaling, and JAK-STAT signaling pathways. An additional element which could not be controlled in this study was the use of a sham injury as a baseline gene expression control. The type of sham operation commonly utilized in mTBI rodent models is objectively damaging to the rodent scalp. Evidence of changes in gene expression due to the traditional sham operation as a control confers proinflammatory and morphological damage, which confounds some interpretation of conventional experimental brain injury models. (Cole et al., 2011)

In summary, our study concludes that short term propranolol exposure causes differential gene expression changes impacting many bone marrow tissue pathways over the course of the post-injury month. Collectively, this could be an important step towards a better understanding of mechanisms which may be central to the effects of mTBI on the individual.

Figures and Tables

Figure 4.1. Experimental Timeline.

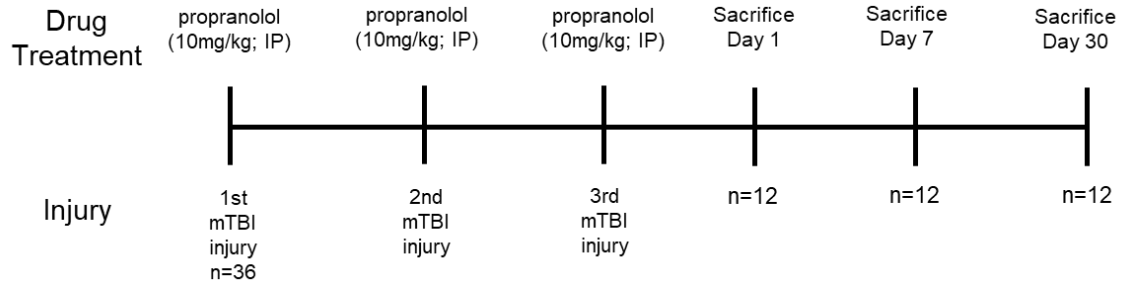


Figure 4.1. The experimental timeline and injury schedule is displayed along with animal numbers per each timepoint. 10mg/kg Propranolol dissolved in 0.9% Saline was injected intraperitoneally (i.p) immediately following each TBI to each mouse that was to receive propranolol.

Figure 4.2. Volcano plots showing differential gene expression in bone marrow 1 day after repeated mTBI.

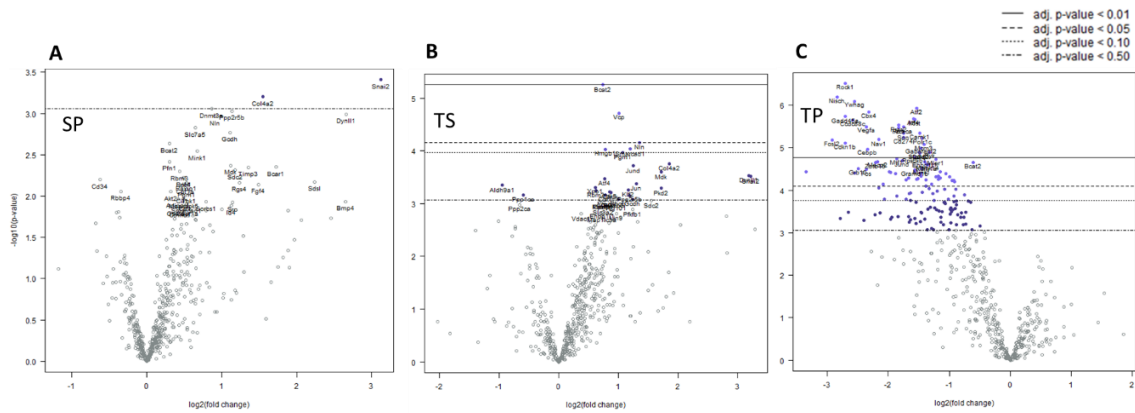


Figure 4.2. Volcano plot showing differential gene expression in bone marrow 1 day post (A) sham injury plus propranolol (SP), (B) rmTBI plus saline (TS), and (C) rmTBI plus propranolol (SP). Sham plus saline is utilized as a baseline comparator value. Data is expressed log₂ fold change vs. p value and graphed with Bonferroni false discovery rate adjusted p value lines of 0.01, .05, 0.10, and 0.50 to denote levels of significance; n=3.

Figure 4.3. Volcano plots showing differential gene expression in bone marrow 1 week after repeated mTBI.

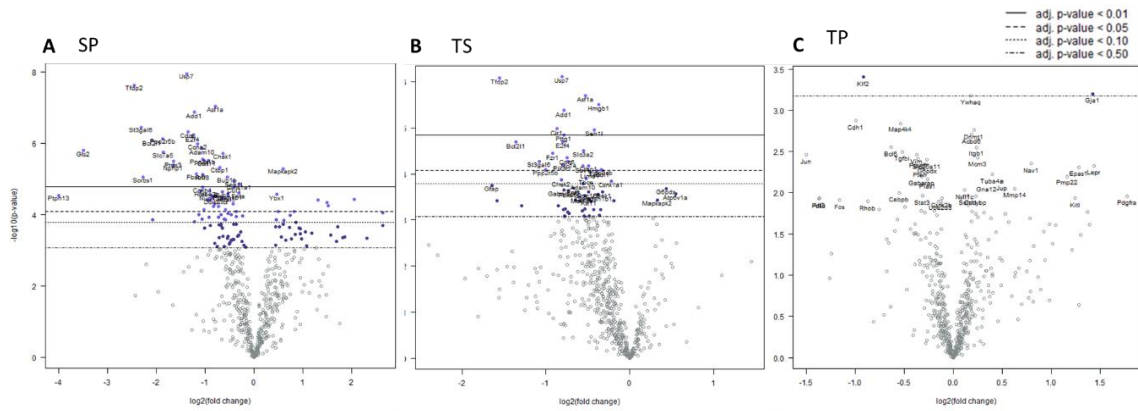


Figure 4.3. Volcano plot showing differential gene expression in bone marrow 7 days post (A) sham injury plus propranolol (SP), (B) rmTBI plus saline (TS), and (C) rmTBI plus propranolol (SP). Sham plus saline is utilized as a baseline comparator value. Data is expressed log₂ fold change vs. p value and graphed with Bonferroni false discovery rate adjusted p value lines of 0.01, .05, 0.10, and 0.50 to denote levels of significance; n=3.

Figure 4.4. Volcano plots showing differential gene expression in bone marrow 1 month after repeated mTBI.

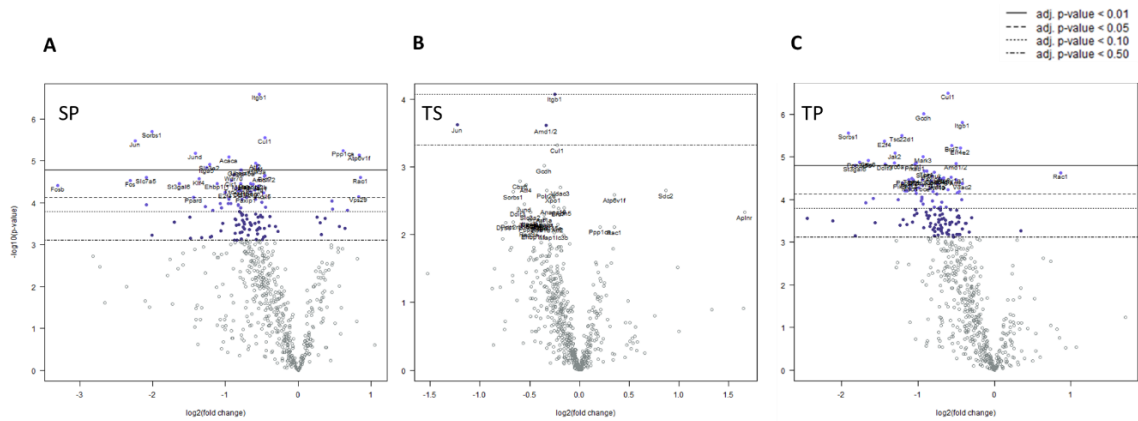


Figure 4.4. Volcano plot showing differential gene expression in bone marrow 30 days post (A) sham injury plus propranolol (SP), (B) rmTBI plus saline (TS), and (C) rmTBI plus propranolol (TP). Sham plus saline is utilized as a baseline comparator value. Data is expressed log₂ fold change vs. p value and graphed with Bonferroni false discovery rate adjusted p value lines of 0.01, .05, 0.10, and 0.50 to denote levels of significance; n=3.

Figure 4.5. mRNA changes in the bone marrow were most evident for propranolol treated TBI bone marrow one day and one month after injury.

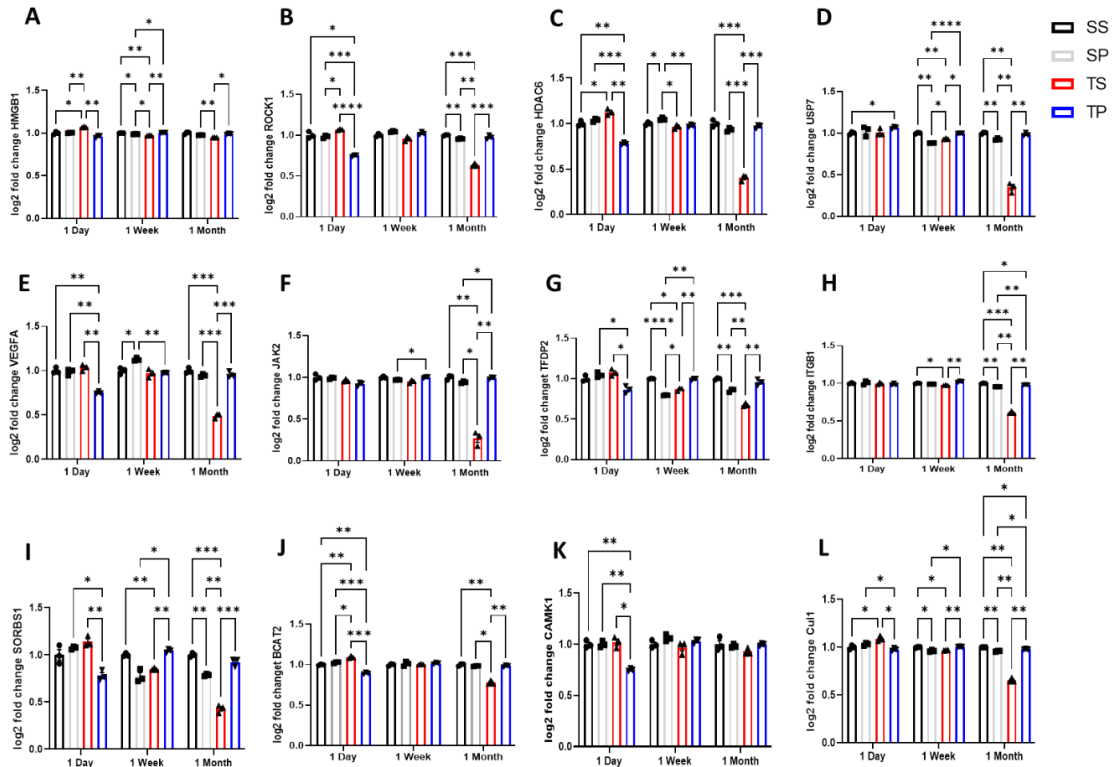


Figure 4.5. Effect of injury alone or in combination with propranolol on genes in bone marrow. Fold change in mRNA expression between sham injury plus saline tissue (black bar), sham injury plus propranolol (light gray bar), rmTBI plus saline (blue) and rmTBI plus propranolol (red bar) at one day, seven days and one month following injury. (A) HMGB1, (B) ROCK1, (C) HDAC6, (D) USP7, (E) VEGFA, (F) JAK2, (G) TFDP2, (H) ITGB1, (I) SORBS1, (J) BCAT2, (K) CAMK1, AND (L) CUL1 values represent mean \pm SEM. Data is expressed as log₂ fold change 2-way ANOVA followed by Sidak's multiple comparison test ($p < 0.05$) *, ($p < 0.01$) **, ($p < 0.001$) ***, ($p < 0.0001$) ****.

Figure 4.6. Bone marrow pathway scores show dysregulation in many pathways important for metabolism, epigenetics, and cell signaling after repeated mTBI. Propranolol reverts these pathway changes 1 day after TBI.

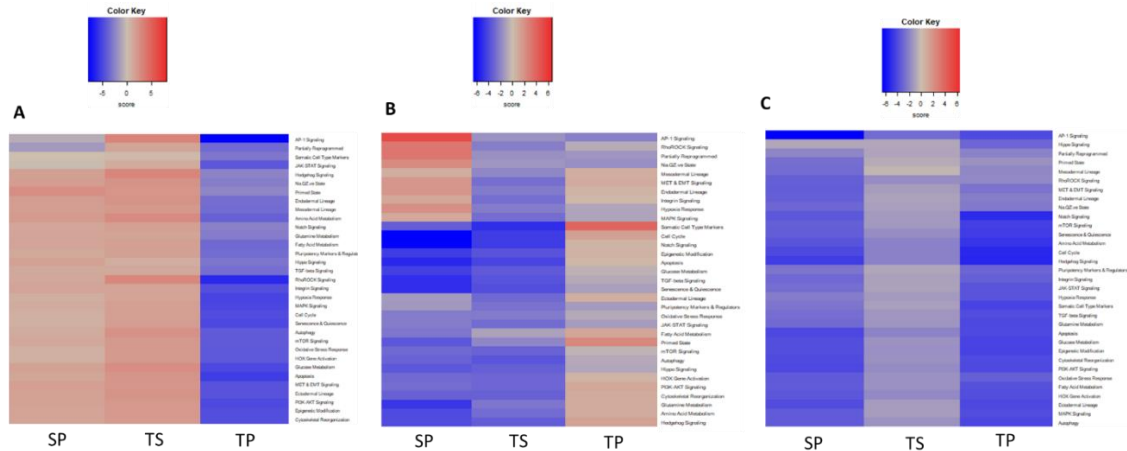


Figure 4.6. Heatmap displays for each treatment condition as exhibited by global significance scores. Directed global significance statistics measure the extent to which a gene set's genes are up-or-down-regulated. Red denotes gene sets whose genes exhibit extensive overexpression while blue denotes gene sets with extensive under expression compared to sham saline baseline at A) 1 day, B) 7 days, C) 30 days after injury. Data are expressed as Z scores for genes from brains; n=3.

Table 4.1. The top mRNA changes across all groups and timepoints.

Gene	Name	Bone marrow pathways involved
HMGB1	High mobility group box protein 1, amphoterin	Apoptosis, Autophagy, Glucose Metabolism, MET & EMT Signaling
ROCK1	Rho-associated, coiled-coil-containing protein kinase 1	Apoptosis, Cytoskeletal Reorganization, MET & EMT Signaling, RhoROCK Signaling, TGF-beta Signaling
HDAC6	Histone deacetylase 6	Autophagy, Ectodermal Lineage, Epigenetic Modification, Notch Signaling, Oxidative Stress Response
USP7	Ubiquitin-specific protease 7	Epigenetic Modification, Glucose Metabolism
VEGFA	Vascular endothelial growth factor A	Epigenetic Modification, Hypoxia Response, MAPK Signaling, MET & EMT Signaling, PI3K-AKT Signaling
JAK2	Janus kinase 2	JAK-STAT Signaling
TFDP2	Transcription factor Dp2	Apoptosis, Cell Cycle, Notch Signaling, Senescence & Quiescence, TGF-beta Signaling
ITGB1	Integrin beta subunit 1	Cytoskeletal Reorganization, Integrin Signaling, MET & EMT Signaling, PI3K-AKT Signaling
SORBS1	Sorbin and SH3 domain containing 1	Glucose Metabolism
BCAT2	Branched chain amino acid transaminase 2	Amino Acid Metabolism
CAMK1	Calcium/calmodulin-dependent protein kinase 1	Epigenetic Modification
Cul1	Cullin 1	Cell Cycle, Hedgehog Signaling, Notch Signaling, TGF-beta Signaling

Table 4.1. Table depicts the top 12 mRNA changes that varied in all 4 treatment groups and lists pathways their gene products are involved in.

Table 4.2. 1-day post mTBI bone marrow pathway scores.

Bone marrow Pathway	SS mean	SEM	1 Day								
			SP mean	SEM	SP p value	TS mean	SEM	TS p value	TP mean	SEM	TP p value
Amino Acid Metabolism	0.494	0.333	1.162	0.293	0.20629	1.878	0.258	0.0303	-3.534	0.410	0.0016
AP-1 Signaling	1.118	0.121	0.525	0.550	0.35117	2.447	0.422	0.0389	-4.090	0.050	0.0000
Apoptosis	0.899	0.374	1.296	0.512	0.56511	2.874	0.316	0.0156	-5.070	0.155	0.0001
Autophagy	0.606	0.293	1.091	0.273	0.29171	2.955	0.263	0.0040	-4.652	0.220	0.0001
Cell Cycle	0.717	0.344	0.896	0.402	0.75270	2.485	0.452	0.0358	-4.098	0.120	0.0002
Cytoskeletal Reorganization	0.921	0.441	1.802	0.354	0.19424	3.339	0.263	0.0092	-6.062	0.574	0.0006
Ectodermal Lineage	0.824	0.258	1.281	0.525	0.47791	2.876	0.220	0.0038	-4.981	0.382	0.0002
Endodermal Lineage	0.522	0.245	0.940	0.572	0.53827	2.204	0.324	0.0144	-3.666	0.437	0.0011
Epigenetic Modification	0.804	0.199	1.636	0.624	0.27328	3.354	0.289	0.0019	-5.794	0.462	0.0002
Fatty Acid Metabolism	0.582	0.103	0.688	0.286	0.74390	2.057	0.307	0.0104	-3.328	0.324	0.0003
Glucose Metabolism	0.746	0.380	1.039	0.431	0.63805	3.037	0.268	0.0079	-4.822	0.266	0.0003
Glutamine Metabolism	0.119	0.161	0.473	0.109	0.14231	0.408	0.133	0.2388	-1.000	0.030	0.0024
Hedgehog Signaling	0.154	0.142	0.644	0.078	0.03926	1.480	0.071	0.0011	-2.278	0.131	0.0002
Hippo Signaling	0.261	0.296	1.010	0.433	0.22650	2.072	0.312	0.0136	-3.343	0.210	0.0006
HOX Gene Activation	0.453	0.078	0.560	0.251	0.70726	1.377	0.294	0.0385	-2.390	0.144	0.0001
Hypoxia Response	0.850	0.188	0.829	0.386	0.96368	1.837	0.244	0.0329	-3.516	0.308	0.0003
Integrin Signaling	0.549	0.164	1.280	0.213	0.05299	1.698	0.235	0.0160	-3.526	0.572	0.0024
JAK-STAT Signaling	1.073	0.374	0.623	0.454	0.48632	2.241	0.328	0.0786	-3.937	0.310	0.0005
MAPK Signaling	1.194	0.316	1.162	0.659	0.96660	3.409	0.382	0.0111	-5.765	0.429	0.0002
Mesodermal Lineage	0.473	0.322	0.909	0.631	0.57182	2.691	0.286	0.0068	-4.073	0.450	0.0012
MET & EMT Signaling	1.012	0.746	1.830	0.825	0.50319	3.651	0.163	0.0259	-6.493	0.798	0.0023
mTOR Signaling	0.618	0.338	0.957	0.447	0.57761	2.465	0.308	0.0156	-4.040	0.135	0.0002
Na,Q,Z ve State	0.380	0.022	0.515	0.431	0.77001	1.587	0.189	0.0031	-2.482	0.121	0.0000
Notch Signaling	0.250	0.180	0.793	0.372	0.25907	1.772	0.265	0.0090	-2.815	0.208	0.0004
Oxidative Stress Response	1.114	0.436	0.998	0.566	0.87942	3.290	0.370	0.0191	-5.402	0.356	0.0003
Partially Reprogrammed	0.327	0.118	0.115	0.204	0.41774	1.057	0.293	0.0820	-1.499	0.152	0.0007
PI3K-AKT Signaling	0.993	0.540	1.727	0.604	0.41632	3.587	0.259	0.0123	-6.307	0.674	0.0011
Pluripotency Markers & Regulators	0.904	0.364	1.209	0.580	0.67895	2.958	0.391	0.0184	-5.072	0.393	0.0004
Primed State	-0.018	0.093	0.452	0.296	0.20485	0.840	0.094	0.0029	-1.274	0.176	0.0032
RhoROCK Signaling	0.426	0.188	0.345	0.181	0.77015	1.263	0.192	0.0358	-2.034	0.155	0.0005
Senescence & Quiescence	1.172	0.346	1.140	0.519	0.96223	3.100	0.428	0.0248	-5.412	0.223	0.0001
TGF-beta Signaling	0.337	0.234	1.103	0.270	0.09898	2.368	0.206	0.0029	-3.809	0.189	0.0002

Table 4.2. Pathway scores from each timepoint of the experiment as shown with means for each group 1 day after injury with the resultant p value. Significant pathway scores were denoted as p<0.05 by T test with sham saline at each timepoint as comparison.

Table 4.3. 1 week post mTBI bone marrow pathway scores.

Bone marrow Pathway	SS mean	SEM	SP mean	SEM	7 Days		TS p value	TP mean	SEM	TP p value	
					SP p value	TS mean					
Amino Acid Metabolism	0.778	0.136	-2.098	0.232	0.0004	0.041	0.156	0.0236	1.280	0.181	0.0907
AP-1 Signaling	-0.765	0.579	2.946	0.052	0.0031	0.050	0.591	0.3803	-2.231	0.191	0.0741
Apoptosis	1.013	0.383	-2.906	0.037	0.0005	0.011	0.436	0.1591	1.882	0.188	0.1113
Autophagy	1.122	0.025	-1.442	0.136	0.0000	-1.310	0.225	0.0004	1.629	0.207	0.0715
Cell Cycle	1.392	0.164	-2.399	0.075	0.0000	-0.687	0.235	0.0019	1.693	0.140	0.2370
Cytoskeletal Reorganization	0.485	0.641	-0.084	0.473	0.5143	-2.636	0.503	0.0186	2.235	0.724	0.1446
Ectodermal Lineage	-0.421	0.415	2.194	0.589	0.0222	-1.983	0.731	0.1366	0.210	0.188	0.2380
Endodermal Lineage	-0.584	0.522	2.009	0.596	0.0306	-1.727	0.691	0.2572	0.302	0.301	0.2151
Epigenetic Modification	1.109	0.291	-2.608	0.118	0.0003	-0.274	0.249	0.0225	1.772	0.062	0.0896
Fatty Acid Metabolism	-0.678	0.297	1.655	0.185	0.0026	-0.300	0.349	0.4557	-0.677	0.086	0.9972
Glucose Metabolism	-1.006	0.342	2.675	0.194	0.0007	-0.609	0.481	0.5379	-1.060	0.142	0.8904
Glutamine Metabolism	0.297	0.080	-0.915	0.247	0.0096	-0.146	0.068	0.0134	0.764	0.075	0.0130
Hedgehog Signaling	0.303	0.108	-0.405	0.180	0.0280	-0.832	0.090	0.0013	0.934	0.148	0.0263
Hippo Signaling	-0.680	0.152	2.032	0.142	0.0002	-0.160	0.204	0.1100	-1.192	0.107	0.0508
HOX Gene Activation	0.039	0.176	0.792	0.246	0.0675	-1.031	0.317	0.0419	0.201	0.083	0.4519
Hypoxia Response	-0.117	0.289	1.372	0.288	0.0217	-1.565	0.612	0.0993	0.309	0.201	0.2927
Integrin Signaling	0.234	0.506	0.440	0.375	0.7600	-1.881	0.544	0.0466	1.208	0.668	0.3097
JAK-STAT Signaling	-0.061	0.218	1.053	0.588	0.1505	-2.000	0.620	0.0419	1.008	0.316	0.0495
MAPK Signaling	-0.343	0.485	2.376	0.445	0.0145	-2.099	0.814	0.1376	0.066	0.193	0.4772
Mesodermal Lineage	-0.911	0.573	2.102	0.523	0.0178	-1.322	0.443	0.6003	0.131	0.400	0.2101
MET & EMT Signaling	-0.635	0.904	2.396	0.745	0.0609	-3.448	1.071	0.1151	1.687	0.819	0.1297
mTOR Signaling	-0.915	0.222	1.851	0.190	0.0007	-0.060	0.320	0.0933	-0.876	0.190	0.9003
Na.QZve State	-0.370	0.245	1.511	0.376	0.0138	-0.358	0.342	0.9796	-0.783	0.072	0.1806
Notch Signaling	1.194	0.163	-2.124	0.030	0.0000	-0.263	0.159	0.0031	1.194	0.084	0.9993
Oxidative Stress Response	-0.835	0.553	3.047	0.227	0.0029	-0.962	0.837	0.9051	-1.251	0.269	0.5359
Partially Reprogrammed	-0.300	0.141	0.603	0.229	0.0283	0.341	0.101	0.0208	-0.644	0.156	0.1763
PI3K-AKT Signaling	-0.213	0.689	1.626	0.791	0.1544	-2.942	1.013	0.0899	1.530	0.690	0.1483
Pluripotency Markers & Regulators	-0.372	0.550	2.631	0.654	0.0245	-1.848	0.614	0.1477	-0.411	0.290	0.9530
Primed State	0.343	0.046	-0.466	0.316	0.0645	-0.461	0.123	0.0036	0.584	0.108	0.1104
RhoROCK Signaling	-0.187	0.138	1.253	0.168	0.0002	-0.600	0.399	0.3838	-0.465	0.046	0.1283
Senescence & Quiescence	1.306	0.309	-2.999	0.019	0.0002	-0.411	0.304	0.0167	2.103	0.113	0.0726
TGF-beta Signaling	0.994	0.279	-2.379	0.148	0.0004	0.213	0.294	0.1266	1.171	0.069	0.5709

Table 4.3. Pathway scores from each timepoint of the experiment as shown with means for each group 7 days after injury with the resultant p value. Significant pathway scores were denoted as p<0.05 by T test with sham saline at each timepoint as comparison.

Table 4.4. 1-month post mTBI bone marrow pathway scores.

Bone marrow Pathway	SS mean	SEM	SP mean	SEM	30 Days		TS p value	TP mean	SEM	TP p value	
					SP p value	TS mean					
Amino Acid Metabolism	1.596	0.191	-1.011	0.206	0.0008	0.655	0.184	0.0239	-1.240	0.394	0.0029
AP-1 Signaling	1.838	0.486	-2.219	0.137	0.0013	0.247	0.455	0.0750	0.133	0.368	0.0488
Apoptosis	2.257	0.215	-1.737	0.240	0.0002	0.771	0.309	0.0169	-1.292	0.088	0.0001
Autophagy	1.949	0.261	-0.960	0.179	0.0008	0.844	0.330	0.0584	-1.832	0.285	0.0006
Cell Cycle	2.183	0.307	-0.902	0.133	0.0008	0.797	0.372	0.0453	-2.078	0.637	0.0038
Cytoskeletal Reorganization	2.604	0.365	-1.679	0.188	0.0005	1.026	0.233	0.0220	-1.952	0.166	0.0003
Ectodermal Lineage	1.665	0.421	-1.457	0.250	0.0031	0.993	0.317	0.2711	-1.201	0.338	0.0061
Endodermal Lineage	0.970	0.942	-1.545	0.490	0.0768	0.879	0.337	0.9320	-0.303	0.360	0.2754
Epigenetic Modification	2.347	0.533	-1.529	0.284	0.0030	0.857	0.502	0.1115	-1.675	0.105	0.0018
Fatty Acid Metabolism	0.339	0.162	0.675	0.099	0.1524	0.383	0.322	0.9087	-1.397	0.522	0.0336
Glucose Metabolism	2.031	0.237	-1.771	0.111	0.0001	0.873	0.225	0.0239	-1.133	0.086	0.0002
Glutamine Metabolism	0.577	0.197	-0.481	0.072	0.0073	0.124	0.055	0.0915	-0.220	0.088	0.0209
Hedgehog Signaling	1.044	0.102	-0.776	0.153	0.0006	0.395	0.200	0.0447	-0.663	0.037	0.0001
Hippo Signaling	1.284	0.308	-0.998	0.349	0.0080	0.811	0.029	0.2008	-1.096	0.355	0.0072
HOX Gene Activation	0.925	0.309	-0.545	0.111	0.0110	0.314	0.276	0.2143	-0.694	0.076	0.0070
Hypoxia Response	0.914	0.458	-1.029	0.157	0.0160	0.303	0.363	0.3551	-0.187	0.171	0.0877
Integrin Signaling	1.050	0.579	-1.098	0.531	0.0523	0.265	0.272	0.2875	-0.217	0.380	0.1416
JAK-STAT Signaling	1.575	0.354	-0.860	0.227	0.0044	0.963	0.261	0.2374	-1.678	0.333	0.0026
MAPK Signaling	1.956	0.673	-1.700	0.362	0.0088	1.353	0.499	0.5118	-1.609	0.325	0.0089
Mesodermal Lineage	0.392	0.994	-1.724	0.306	0.1117	0.739	0.590	0.7796	0.593	0.584	0.8705
MET & EMT Signaling	1.448	1.724	-2.783	1.006	0.1014	1.564	1.028	0.9565	-0.229	0.868	0.4338
mTOR Signaling	1.706	0.283	-1.118	0.128	0.0008	0.672	0.257	0.0537	-1.220	0.161	0.0008
Na,QZve State	0.599	0.224	0.151	0.082	0.1337	0.416	0.079	0.4820	-1.166	0.064	0.0016
Notch Signaling	1.689	0.215	-0.912	0.144	0.0006	0.967	0.280	0.1103	-1.744	0.420	0.0019
Oxidative Stress Response	2.214	0.381	-2.372	0.276	0.0006	0.712	0.351	0.0440	-0.554	0.243	0.0036
Partially Reprogrammed	0.771	0.289	-0.715	0.146	0.0101	0.393	0.126	0.2969	-0.449	0.276	0.0379
PI3K-AKT Signaling	2.323	0.928	-2.401	0.645	0.0139	1.307	0.499	0.3896	-1.229	0.575	0.0313
Pluripotency Markers & Regulators	1.335	0.826	-1.609	0.393	0.0323	1.105	0.226	0.8017	-0.831	0.524	0.0912
Primed State	0.409	0.140	-0.508	0.120	0.0076	0.169	0.081	0.2114	-0.070	0.146	0.0773
RhoROCK Signaling	0.812	0.293	-0.720	0.198	0.0123	0.094	0.361	0.1971	-0.186	0.363	0.0992
Senescence & Quiescence	2.314	0.420	-1.019	0.199	0.0020	0.967	0.404	0.0818	-2.261	0.460	0.0018
TGF-beta Signaling	1.663	0.270	-1.182	0.134	0.0007	0.864	0.155	0.0626	-1.344	0.225	0.0010

Table 4.4. Pathway scores from each timepoint of the experiment as shown with means for each group 30 days after injury with the resultant p value. Significant pathway scores were denoted as $p < 0.05$ by T test with sham saline at each timepoint as comparison.

Chapter Five

Concluding remarks

Mild traumatic brain injuries, or concussions, continue to lead to debilitating long-term side effects severely impacting the quality of life of impacted individuals. While it is known mTBI side effects have a neuroimmune component, the way by which mTBI impacts various organs and alters immune gene expression outside the brain is not as well characterized. In this thesis we explored how mTBI causes immune changes in the brain, spleen, blood, and brain while investigating whether a peri-injury injection of the non-selective beta blocker propranolol could reverse or prevent mTBI induced alterations that occur over the course of one month after injury.

Impact of mTBI on sympathetic activation of the bone marrow, brain, spleen, and vasculature

Mild TBI is hypothesized to influence sympathetic nervous system activation and output through several mechanisms. These are due to disruptions in the hypothalamic-pituitary axis, increased release of neurotransmitters, activation of inflammatory pathways, neuronal damage, and changes in cerebral blood flow (McEwen 2007; Bao et al., 2021; Carney et al., 2017). While we did not investigate neurotransmitter release, directly assay neuronal damage, nor investigate changes in cerebral blood flow in this project, we sought to limit beta-adrenergic receptor dependent sympathetic activation to reduce systemic immune dysfunction. After mTBI, the brain releases corticotropin releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus. This hormone stimulates

the anterior pituitary gland to release adrenocorticotrophic hormone (ACTH) into the vasculature (Herman et al., 2016). Once this is released, this hormone will stimulate the adrenal glands to release cortisol. Cortisol is known to have many various functions upon the immune system by directly and indirectly influencing metabolism and immune cell suppression (McEwen 2007; Weil et al., 2022).

The sympathetic nervous system, which is part of the autonomic nervous system, is made up of both preganglionic and postganglionic fibers. Ganglia are clusters of neuronal cell bodies in the peripheral nervous system. Preganglionic fibers originate in the intermediolateral cell column of the thoracic and lumbar spinal cord regions, and synapse on a postganglionic neuron (LeBouef et al., 2023). The postganglionic neuron is stimulated by preganglionic release of acetylcholine due to the presence of nicotinic acetylcholine receptors on postganglionic neurons. These postganglionic fibers release norepinephrine from post ganglionic nerve fibers into various target organs such as the vasculature, bone marrow, and spleen. (LeBouef et al., 2023). Sympathetic postganglionic neurons are clustered into ganglia near the spinal cord, far from their target organs. The celiac ganglia contain the cell bodies of the postganglionic neurons that will send sympathetic fibers to the spleen, while the femur bone marrow is supplied by both celiac and superior mesenteric ganglionic fibers (LeBouef et al., 2023). The release of norepinephrine from these postganglionic fibers can stimulate alpha and beta receptors in these organs after TBI.

Recently, a TBI study found catecholamine levels were highly correlated with endotheliopathy and coagulopathy within 24 hours after TBI and that these

biomarkers of endotheliopathy and coagulopathy are further associated with poor outcomes after TBI (Di Battista et al., 2016). This group also had previously shown that inflammatory cytokine profiles in patients were dependent upon the hyperadrenergic state following mild TBI. A positive association between serum norepinephrine with IL1 β , IL10, TNF α , IL-8, IP-10, and MCP-1 levels were identified post hospital admission after TBI (Di Battista et al., 2016b). Interestingly, blockage of alpha-1 receptors after TBI can lead to edema in the striatum and hippocampus to suggest alpha-1 receptors may act to stabilize the peri-injury BBB after TBI injury (Dunn-Meynell et al., 1998).

While the impact of TBI on various organ systems requires further investigation, a recent study found that TBI lead to decreased splenic monocyte presence 7 days after injury (Saber et al., 2020). While they did not further characterize the monocytes to inflammatory or anti-inflammatory subsets, our data supports these findings as we too found decreased monocyte presence one week after mTBI. As previously stated, alpha and beta receptors are also located on immune cells within these organs. Therefore, the release of these two neurotransmitters can greatly impact systemic immune function after a concussion. Beta receptor activation of immune cells is characterized by increased immune cell proliferation, cytokine production, and cell signaling (Loftus et al., 2018). Moreso, blocking the beta receptors could have the opposing effect. Our data suggests that the blocking of these receptors with propranolol has led to widespread immune changes and activation in many pathways after mTBI. Interestingly, we found that many pathways were showing decreased activity 1 day

after TBI in the bone marrow. This suggests the administered propranolol is acting peripherally and potentially centrally to inhibit these immune cell pathways. Furthermore, the immune changes we found after TBI and propranolol were most apparent 1 month after injury. These data collectively reveal peri-injury propranolol dosing can lead to long-term immune changes in the blood, brain, spleen, and bone marrow after mild TBI. These data collectively reveal peri-injury propranolol dosing can lead to long-term beta adrenergic-dependent immune changes in the blood, brain, spleen, and bone marrow after mild TBI.

Further studies and experiments are needed to characterize the behavioral outcome of propranolol administration after TBI; however, preliminary preclinical and clinical studies suggest promising results. Propranolol is currently administered for headache pain and post traumatic headaches are one of the most common side effects after TBI. One study has even shown peri-injury propranolol administration is associated with decreased BBB permeability, improved cognitive/memory function, and decreased microglia activation 120 days after TBI (Kota et al., 2016). Furthermore, post injury propranolol has been reported to reduce leukocyte mobilization and microvascular permeability into the brain (Lopez et al., 2022a; Lopez et al., 2022b).

Summary

This project explores the ways in which repeated mTBI impacts systemic immunity and how peri-injury propranolol injection may alter mTBI induced immune changes. First, we found that TBI leads to increased total, Ly6C+, and Ly6C- monocytes in both the blood and spleen 1 month after injury. While there were not

many DEG changes over the course of 1 month in this study, we did find mTBI changes in genes such as CD99 and JAK1 in the spleen. In the brain, we found CSF1R, TNFAIP6, CD3 ϵ , ITGAM increase 1 month after injury, but NFIL3 and CSF1R show increases in expression at 1 day and 1 week after injury, respectively. Lastly, we found TBI changes gene expression in many immune pathways in the brain one day after injury such as cell adhesion, lymphocyte trafficking, antigen presentation, and phagocytosis. In the spleen we found TBI lead to changes in adaptive immunity, apoptosis, MHC antigen presentation 1 day after injury, but no pathway changes at the 1-week timepoint. 1 month after injury, TBI spleens had increased complement, inflammasome, lymphocyte trafficking, antigen presentation, and phagocytosis.

Secondly, we found that the addition of propranolol significantly altered the monocyte populations in the blood and spleen across 1 month after injury. TBI propranolol blood derived total and Ly6C⁺ monocytes were more prevalent 1 week after injury compared to monocytes derived from saline treated mTBI mice. Although there were not changes in total monocyte numbers by 1 month, there were subset differences as the TBI propranolol group had less Ly6C⁺ cells, but more Ly6C⁻ cells. In the spleen we saw the TBI propranolol group had significantly less total, Ly6C⁺, and Ly6C⁻ monocytes than the TBI saline group 1 week after injury. By 1 month, we found the TBI propranolol spleen had more total and Ly6C⁺ monocytes, but no change in Ly6C⁻ monocytes percentages. We found numerous propranolol influenced DEG changes at the 1 week and 1-month timepoints, but not at 1 day for both the spleen and brain. These DEG changes were most

concentrated for pathways involving cytokine signaling, innate immunity, adaptive/T cell receptor signaling, phagocytosis, and immunometabolism. When we further characterized the cytokine response, we found propranolol induced mTBI changes at 1 week for IL1 β , IL10, CX3CL1, and CCR2 in the spleen. Interestingly, the brain did not show TBI induced propranolol changes, but rather propranolol alone led to increased IL1, IL10, CCR2, and CX3CR1 but decreased CX3CL1. Due to external issues, we were unable to obtain reliable behavior data to determine the impact of propranolol on mTBI induced pain states.

Lastly, we investigated the mTBI effect on bone marrow gene expression. Our data showed TBI increased *bcat2*, *vcp*, and *nln* expression 1 day after injury. However, bone marrow from propranolol treated TBI mice shows numerous DEGs that significantly decreased in expression at this timepoint. TBI groups showed significant pathway changes for nearly every stem cell pathway on the NanoString panel although the propranolol treated group shows decreases in those pathways while saline treated TBI bone marrow shows increases 1 day after injury. Moreover, TBI propranolol bone marrow does not show any DEG changes compared to sham saline bone marrow 1 week after injury. TBI saline bone marrow shows 21 DEG changes with these concentrated in pathways impacting metabolism, cell priming, senescence, and epigenetic modifications. 30 days after injury we found over 50 genes that had significantly decreased in expression in TBI propranolol bone marrow; however, there were not any significant DEGs in the TBI saline group. The differences between these two groups at this timepoint were for genes in apoptotic, epigenetic, metabolism, and stress signaling pathways.

Taken together, these data depict the wide-ranging impact of both mTBI and propranolol across time and on various organ systems. Our aim was to determine the role of propranolol on immune cells and identify whether a dose given with each injury will yield long-term benefits suggesting improvements in mTBI immune functioning. Mild traumatic brain injuries occur commonly and there is no reliable treatment to alleviate the effects. In conclusion, a dose of propranolol given along with each injury prevented many pathway DEG changes in the bone marrow, brain, and spleen at various timepoints after mTBI. Propranolol also caused a shift in the blood inflammatory to anti-inflammatory monocyte balance one month after injury. While we cannot conclude whether there were long-term phenotypic benefits due to the lack of behavior results, the changes we see between propranolol treated and untreated tissue immune and stem cell pathways suggests propranolol leads to altered immune functioning that may suggest better long-term outcomes. With propranolol already used to treat headaches, understanding its systemic impact after TBI can lead to further utilization of this treatment.

Implications and Limitations

The work presented in this thesis gives many novel insights into the immune mechanisms underlying mTBI and further characterizes the role of propranolol in modulating these immune mechanisms. Previous studies have shown that mTBI leads to immune cell population changes in the brain, serum, and spleen. We found increased total, inflammatory, and anti-inflammatory monocytes in the blood and spleen as far out as 1 month after mTBI. We also found a peri-injury

propranolol injection can lead to changes in this population resulting in more anti-inflammatory cells and less inflammatory cells present weeks after injury.

The changes we see in the blood and spleen, monocyte populations. Suggest that propranolol can alter the monocyte presence in these two regions. What this suggests is that propranolol has shifted these populations to a more anti-inflammatory phenotype. This is significant because mild traumatic brain injury is usually characterized by the occurrence of increased inflammation; therefore, this change in population due to the presence of propranolol further highlights, the anti-inflammatory nature of propranolol administration after MTBI.

Furthermore, we found that there were no differences for any pathway between one day propranolol treated and untreated TBI brains. This suggests the changes that we see for propranolol do not occur within one day of injury. One caveat to this conclusion is our mTBI model is for repeat injuries. Therefore, the one-day timepoint is 22 days after the initial injury. At the one-week timepoint, the only pathway change we found between propranolol treated and untreated TBI brains was that propranolol treated brains lead to increases in Th2 differentiation. This is in accordance with studies that show propranolol leads to Th2 differentiation (Seyedi et al., 2012). Interestingly, we found that many TP group pathways at this timepoint remained unchanged compared to sham saline controls. This suggests that the one-month TBI brain with propranolol may be somewhat comparable to the sham injured brain at this time point. To this point, we did not find any DEG changes in the TBI propranolol brain at this time point interestingly, though the changes we do see in this group all occurred in the spleen where there were 213

DEG changes one week after injury. This is interesting because we did not see any pathway changes in the TP group spleens one day after injury, nor any DEG changes as well. This further suggests either of the changes that we see in the spleen in this group are delayed, not present, or that we could not find them based on the methods of investigation we utilized.

The changes we see at one week in the spleen for the TP group further suggest that propranolol has substantial impact upon the functioning of the spleen after TBI. 80% of the DEG changes we see here are decreases in expression, and we found decreases in every NanoString immunology pathway on the panel. When we compare these one-week changes that we see in the blood, brain, and spleen in the TP group to the bone marrow at the same time point we find that the TP bone marrow also did not show any differential expression changes at this time point. Furthermore, there were very few pathway level changes at this time point. TP bone marrow shows increases in glutamine, metabolism, and Jak-STAT signaling yet decreases in hedgehog signaling. This is in stark contrast to the changes we see in the untreated TBI bone marrow at this time point where we found generalized decreases in stem cell pathway activity. This implies that the bone marrow and the brain of TP treated animals are genetically and epigenetic similar to sham saline brains and bone marrow.

The spleen not only filters the blood but also stores white blood cells for development, maturation, and future immune responses. Splenic monocytes are clustered in red pulp in the spleen separate from macrophages and dendritic cells. Splenic monocytes exit the spleen and accumulate in injured tissues, such as the

brain, to participate in wound healing (Swirski et al., 2009). One study finds that Ly6C⁺ splenic monocytes are critically important for repair after injury. Splenectomy in this study lead to decreased numbers of Ly6C⁺ cells penetrating injured tissues (Rizzo et al., 2020). The changes we see for splenic monocytes over the course of one month after injury suggest propranolol is leading to more inflammatory monocytes present after mTBI than in saline treated TBI spleens. While we do not know the level of function of these cells, their increased presence suggests increased ability to combat inflammation. We also found changes in innate immune cell pathways such as MHC I and II signaling, inflammasome activation, and innate immune signaling in propranolol treated TBI animal spleens at the one week and one-month timepoints.

On the cellular level, one week after injury, we found increases in Ly6C⁺ and total monocytes but not Ly6C⁻ monocytes at this time point. Taken together, we can see that the one-week time point perhaps restores the brains and bone marrow to SS levels. However, perhaps accomplished by modulation of the spleen immunological niche. One limitation to drawing this conclusion is that we did not measure the protein expression for any of the genetic changes we have identified. Therefore, we do not know if there were post-transcriptional modifications at play where the protein levels for these genes may be unchanged.

One month after the injury, we once again see an interesting pattern of expression for genes in the brain, spleen, and bone marrow. The TP bone marrow, one month after injury shows, decreases in many pathway scores. Moreover, compared to TBI saline derived bone marrow, TBI, propranolol, derived bone

marrow shows, increases in pathways associated with epigenetics, cell cycle, apoptosis, stem cell reprogramming, and oxidative stress compared to untreated TBI bone marrow. These increases are corroborated by our data in the blood. They showed decreased Ly6C + monocytes, but increased Ly6C -monocytes at this time point in the blood. The spleen at this point depicts increased total monocytes and increased Ly6C+ monocytes but no change in Ly6C- monocytes between TBI saline and TBI propranolol groups, one month after injury. Interestingly, the changes we found for the splenic monocytes do not translate for the pathway and DEG changes. The TP spleen shows decreases in B-cell receptor signaling and lymphocyte trafficking; however, there were increases in TH2 differentiation. It should be noted, though, that there were not many pathway changes for either group in the bone marrow at this time point. TP bone marrow at this time point shows 53 differentially expressed gene changes and these genes were all increased in expression compared to sham saline controls.

The brain, one month after injury and propranolol treatment, shows increases in 16 out of 32 pathways. Moreover, only three of these pathways were significant deviations from the TBI saline pathway scores. TP brain shows increased, immunometabolism, innate, immune, signaling, and increase type one interferon signaling. Taking these data together, we see that the changes in the blood, bone marrow, spleen, and brain do not necessarily all correlate to each other. The bone marrow and spleen show decreases in pathways scores and DEG expression, while the brain and blood suggest increases in immune pathways. This

highlights the dichotomous nature of propranolol, as it seems to have different impacts on different organs when compared to untreated TBI tissue.

Though we used two different NanoString panels to identify the changes in the spleen, brain and bone marrow, there were five pathways that were similar to both panels. Metabolism, oxidative, stress, TGF, beta, autophagy, and apoptosis. What are the five pathways that we can compare between all three organs. Comparing TBI propranolol to TBI untreated tissue at similar time points we were able to better understand how propranolol impacts TBI tissue. We find that propranolol reduces metabolism, oxidative, stress, TGF, beta, signaling autophagy and apoptosis one day after injury in the bone marrow but there is no change between the two groups in the brain and spleen of TBI animals. At the one-week time point we see no change between the TBI conditions for the five different pathways in the three organs, apart from increased metabolism and oxidative stress in the TBI propranolol brain. This may suggest increased inflammation in the TBI propranolol brain. One month after injury, we see decreases in metabolism, oxidative, stress, TGF, beta, signaling autophagy and apoptosis in the bone marrow once again, but no change in these pathways for the brains and spleens of TBI animals.

While we do not have behavioral data to correlate with these findings, previous studies have shown that administration of propranolol after TBI can lead to cognitive and behavioral benefits subacutely (Kota et al., 2016; Zeeshan et al., 2019). Therefore, the increased metabolism and oxidative stress that we see in the TBI propranolol brain, but not the TBI saline brain one month after injury could

be a new homeostatic basis for long-term improvement in these animals. Further limitations for this study lie in our lack of proteomic changes and data to correlate with the genetic findings in the TBI tissues we investigated and characterized. Another limitation is that we did not directly block the sympathetic nervous system by cutting axons to identify its contributions to stem and immune function of the tissues we investigated.

In conclusion, propranolol treatment post mTBI has shown benefits, clinically, and in rodent models of TBI to alleviate cognitive and behavioral deficits. The immunological and stem cell niche changes we have characterized after propranolol administration post TBI highlights the role of catecholamines and sympathetic signaling in controlling stem cell and immune pathways in the body. Re-purposing propranolol for mild traumatic brain injury treatment may lead to long-term benefits by altering mild traumatic brain injury pathophysiology and symptomology for concussed individuals based on these and other data.

Future directions

Future studies are needed to further understand—

1. Role of T cells and other adaptive immune cells in these processes and how propranolol administration functionally impacts them after mTBI.
2. Proteomic changes after mTBI by measuring cytokine/chemokine measurements with and without propranolol administration .
3. Pain behavior after propranolol administration
4. Image propranolol treated organs to understand potential histological changes.
5. Repeat study in female mice to understand whether the changes we have identified are sex dependent.

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Curriculum Vitae

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Education

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 - University honors
 - Completed April 2015
- Doctor of Philosophy (PhD) in Medical Neuroscience
 - Indiana University-Purdue University Indianapolis
 - Completed July 2023
- Doctor of Medicine (MD)
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 - 2016-present

Research experience

- Medical Scientist Training Program; 2016-present
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 - Mentors: Benjamin Gaston, MD and Brittney-Shea Herbert, PhD
- Graduate Student; 2018-2023
 - Indiana University-Purdue University Indianapolis
 - Mentor: Fletcher White, PhD
- IPREP Research Fellow; 2015-2016
 - Indiana University-Purdue University Indianapolis
 - Mentor: Fletcher White, PhD
- Undergraduate Research Assistant; 2012-2015
 - University of Alabama at Birmingham
 - Mentor: Rita Cowell, PhD
- Undergraduate Research Assistant; 2014-2015
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 - Mentor: James Patterson, PhD

- Undergraduate Research Assistant; May 2015-July 2015
 - The Ohio State University
 - Mentor: Jonathan Godbout

Conference presentations

- **Smith, JA.**, Allette Y., Randolph, A., Kim., Y., Ripsch M., White., FA. “Targeting neuronal TLR4 signaling by TLR4 Toll/IL-1 receptor domain-derived decoy peptides therapeutically disrupts nociceptive signaling”. [Poster] Veterans Administration Research Day, Indianapolis, IN. April 2016
- **Smith, JA**, Hayes J, Lindsay A., Randolph, A., Ripsch, M., White, FA. “Oxaliplatin-induced Chemotherapy Peripheral Neuropathy can be Reversed by Carbamazepine in the Rodent”. [Poster] Indiana University Research Day, Indianapolis, IN. May 2016
- **Smith, JA.**, Ripsch M., and White, FA. “Validation of a Rodent Model of Chemotherapy-Induced Peripheral Neuropathy Using Oxaliplatin”. [Poster]. Annual Biomedical Research Conference for Minority Students, Seattle, WA. November 2015.
- **Smith JA**, Xie J, Kim Y, Carey C, Kang H, Saxe J, Iyengar S, Naugle KM, White FA. “Inflammatory biomarkers and anxiety traits in the development of persistent post-traumatic headaches following mild traumatic brain injury in a patient cohort: A pilot study.” [poster] Indiana Society for Neuroscience, Indianapolis, IN. September 2019
- **Smith JA**, Xie J, Malicky P, Carey C, Saxe J, Dorsey S, Naugle KM, White FA. “The influence of mild traumatic brain injury on the immune response and development of post traumatic headache in healthy adults.” [poster] Society for Neuroscience, Chicago, IL. November 2019
- **Smith JA**, Xie J, Malicky P, Carey C, Saxe J, Dorsey S, Naugle KM, White FA. “The influence of mild traumatic brain injury on the immune response and development of post traumatic headache in healthy adults.” [virtual poster] Society for Neuroscience, Chicago, IL. November 2021
- **Smith JA**, Nguyen T, Davis B, Juboori M, White FA. “The effect of the nonselective beta blocker propranolol on mice splenic and blood monocyte populations post repeated mild traumatic brain injury.” [poster] Clinical and Translational Science Institute Annual Meeting, Indianapolis, IN. September 2022
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Manuscript Publications

- Salazar TE, Richardson MR, Beli E, Ripsch MS, George J, Kim Y, Duan Y, Moldovan L, Yan Y, Bhatwadekar A, Jadhav V, **Smith JA**, McGorray S, Bertone AL, Traktuev DO, March KL, Colon-Perez LM, Avin KG, Sims E, Mund JA, Case J, Deng X, Kim MS, McDavitt B, Boulton ME, Thinschmidt J, Li Calzi S, Fitz SD, Fuchs RK, Warden SJ, McKinley T, Shekhar A, Febo M, Johnson PL, Chang LJ, Gao Z, Kolonin MG, Lai S, Ma J, Dong X, White FA, Xie H, Yoder MC, Grant MB. Electroacupuncture Promotes Central Nervous System-Dependent Release of Mesenchymal Stem Cells. *Stem Cells*. 2017 May;35(5):1303-1315.
- Allette YM, Kim Y, Randolph AL, **Smith JA**, Ripsch MS, White FA. Decoy peptide targeted to Toll-IL-1R domain inhibits LPS and TLR4-active metabolite morphine-3 glucuronide sensitization of sensory neurons. *Sci Rep*. 2017 Jun 16;7(1):3741.
- Naugle KM, Corrona S, **Smith JA**, Nguyen T, Saxe J, White FA. Physical activity behavior in the first month after mild traumatic brain injury is associated with physiological and psychological risk factors for chronic pain. *Pain Rep*. 2021 Oct 29;6(4):e969.
- Naugle KM, Nguyen T, **Smith JA**, Saxe J, White FA. Racial Differences in Head Pain and Other Pain-Related Outcomes after Mild Traumatic Brain Injury. *J Neurotrauma*. 2023 Feb 24.
- **Smith JA**, Nguyen T, Karnik S, Davis B, Juboori M, Kacena MA, Obukhov AG, White FA. Repeated Mild Traumatic Brain Injury in Mice Elicits Long Term Innate Immune Cell Alterations. *J Neuroimmunology*. 2023 May 16;380:578106.
- **Smith JA**, Nguyen T, Davis B, Obukhov AG, White FA. Propranolol Treatment During Repetitive Mild Traumatic Brain Injuries Induces Pronounced Transcriptomic Changes in the Bone Marrow of Mice. *Scientific Reports*. (in review)