Injury-induced neuroinflammation alters addiction behavior

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Acknowledgement Page

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Dedication Page

This is dedicated to my family and friends, who believed in me every step of the way. You always knew I'd be a doctor, from when I was 2 years old telling anyone who would listen that I wanted to be a doggy doctor!

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Abstract

Traumatic brain injury (TBI) affects 64-74 million people every year worldwide. A history of mild brain injury increases the risk of substance use disorder (SUD) by 2-6 times that of the general population. With the rise of the opiate epidemic, it is imperative to understand the link between TBI and opiate use disorder. Therefore, a model of mild TBI was developed in mice. Mice that received mild TBI had a transient motor deficit at the acute stage of injury and a long-term spatial learning deficit at the chronic stage of injury. The innate immune response in the brain was active at the acute and chronic stage, with macrophage and neutrophil infiltrate peaking at 3 days post injury (DPI), and an increase in the activated macrophage phenotype at both 15 and 30-DPI. Next, to understand how drug seeking behavior is modified, mice with mild TBI or sham injury were subjected to intravenous (IV) self-administration and conditioned place preference (CPP) behavioral assays to evaluate drug seeking behavior. Injured mice had increased consumption of and preference to morphine. The innate immune response was also altered after IV self-administration, with increased macrophage and neutrophil infiltrate on the side ipsilateral to injury. Furthermore, both IV self-administration and CPP with morphine resulted in increased lymphocyte infiltrate on the side ipsilateral to injury. Considering the increased inflammation observed due to morphine, the neuroinflammatory response to a consistent clinical dose morphine was examined, since the majority of people are first exposed to opiates through prescription for pain relief. After TBI or sham injury, mice received morphine twice daily (5mg/kg) for up to 15 days. A biphasic immune response was observed to this dose of morphine with increased macrophage infiltrate and a lymphocyte infiltrate at 15-DPI. This was accompanied by a dynamic, temporally distinct cytokine response, a decrease in the phagocytic activity of macrophages and microglia at 15-DPI and increased leakage of the blood brain barrier in the mid brain. Results from these studies demonstrate a novel increase in the proinflammatory response at the chronic stage of traumatic brain injury when exposed to morphine. The models developed here will provide new insights to help identify new targets to reduce the risk of SUD following TBI.

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List of Abbreviations

α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid ..... AMPA
Arginase 1 ..... ARG1
Blood Brain Barrier ..... BBB
C-C Chemokine Ligand 2 ..... CCL2
C-C Chemokine Receptor 2 ..... CCR2
Central Nervous System ..... CNS
Chronic Traumatic Encephalopathy ..... CTE
Cluster of Differentiation ..... CD
Cognitive Processing Therapy ..... CPT
Computed Tomography ..... CT
Controlled Cortical Impact CCI
C-X-C Motif Chemokine Ligand 10 ..... CXCL10
Diffuse Tensor Imaging ..... DTI
Discrimination Index ..... DI
Evan's Blue ..... EB
Fetal Bovine Serum ..... FBS
Fluid Percussive Injury ..... FPI
Glasgow Coma Scale ..... GCS
High Impact Trauma ..... HIT
Inducible Nitric Oxide Synthase ..... iNOS
Interleukin 1 beta IL1β
Interleukin 4 ..... IL4
Interleukin 6 ..... IL6
Interleukin 10 ..... IL10
Interleukin 15 ..... IL15
Interferon gamma ..... INFɣ
Intravenous IV
Lipopolysaccharide Binding Protein ..... LBP
Migration Inhibiting Factor ..... NIF
NADPH Oxidase ..... NOX
Natural Killer ..... NK
Nicotinamide Adenine Dinucleotide Phosphate ..... NADPH
Nitrous Oxide ..... NO
Novel Object Recognition Test ..... NORT
Open Field Testing ..... OFT
Subcutaneous ..... SC
Substance Use Disorder ..... SUD
T Regulatory ..... Treg
Traumatic Brain Injury ..... TBI
Transforming Growth Factor-Beta ..... TGFβ
Tumor Necrosis Factor α ..... TNFα
Phosphate Buffered Saline ..... PBS
Post Traumatic Headache ..... PTH
Posttraumatic Stress Disorder ..... PTSD
Vascular Access Button ..... VAB

Introduction

Millions of people suffer from traumatic brain injury (TBI) every year, with hundreds hospitalized and tens of thousands dying from their injuries.[1-5] However, these numbers are based on hospital reports and treatment records and may not reflect the extent of people with mild TBI, which is difficult for even the patient to identify. But mild TBI accounts for nearly 75-90% of all TBI.[1,2,6-8] The diagnosis of TBI and classification of severity can be a challenge, while methods to determine which patients will develop long-term consequences remains elusive.[1,2,5,7,9-16] History of TBI increases the risk of many neurological consequences, and in particular increases the risk of substance use disorder by 3-6 times that of the general population.[28,29] Chapter 1 of this project evaluated the existing literature to summarize the known information on the subject and identify gaps in knowledge. The following dissertation designed experiments to elucidate the connection between TBI and substance use disorder (SUD).

To understand the mechanisms behind brain injury and long-term deficits, researchers rely on animal models. Chapter 2 focuses on the neuroinflammatory response to mild TBI and the link to behavioral deficits. While injury has been induced with controlled cortical impactors for many decades, and other laboratories have demonstrated minor motor and cognitive deficits following injury, it is essential to investigate the immune response to injury in the context of behavioral changes from the acute to chronic stage. Following mild TBI, injured mice demonstrated an acute infiltration of neutrophils and macrophages on the ipsilateral side of injury that correlated with minor motor deficits, which resolved at 7 days post injury (DPI). However, at the chronic 15- and 30-DPI time point, macrophages that reside in the brains of injured mice increased the expression of a proinflammatory marker, CD86, on the ipsilateral side of injury. This coincided with a deficit in spatial learning after injury. Changes in the inflammatory milieu was demonstrated by evaluating mRNA of cytokines at the acute and chronic stages of injury. Localization of the response by immunohistochemistry revealed that the inflammatory response spread to areas distal to the injury sites. This consistent and reproducible model of mild TBI with long-term neuroinflammation and cognitive deficits was utilized for all subsequent studies.

Individuals that have had a TBI have 3-6 times the risk of substance use disorder (SUD) than the general population.[132] With the rise of the opiate epidemic, understanding the link between TBI and opiate use disorder is essential.[159,160] Therefore, experiments in Chapter 3 evaluated injured mice for drug seeking behavior in regards to morphine. Intravenous (IV) self-administration is a behavioral assay in which mice can administer morphine to themselves. Injured mice began IV self-administration at 5-DPI and escalated their intake for 10 days higher than sham injured mice. That escalation correlated with increased macrophages, neutrophils and natural killer cells on the ipsilateral side of injury at the conclusion of IV self-administration. Next, sensitivity to morphine was evaluated through conditioned place preference (CPP), which evaluates the time a mouse spends in a drug paired environment vs a saline paired environment. Injured mice showed increased preference to low dose morphine (0.5 and 1mg/kg) when compared to sham injured mice. This again correlated to increased cellular infiltrate on the ipsilateral side of injury, with increases in macrophages, neutrophils, natural killer cells, and lymphocytes at 15 DPI. The behavioral changes here link mild TBI to increased consumption and sensitivity to morphine.

Understanding the temporal changes to the immune response during opiate exposure is essential to identifying targets for treatment and the most effective times for intervention. Therefore, experiments in Chapter 4 utilized a long-term continuous dosing of morphine beginning at the time of injury and followed through 15 DPI. Injured mice exposed to morphine revealed a unique biphasic macrophage response on the ipsilateral side of injury, with an acute infiltrate at 3-DPI that was resolved at 7-DPI, with a subsequent macrophage infiltration at 15-DPI. There was also a lymphocyte infiltration at 15-DPI on the ipsilateral side of injury that was unique to injured mice exposed to morphine. The mRNA of cytokines and chemokines was again evaluated to understand the inflammatory milieu, and there were significant changes in the mRNA of proinflammatory and anti-inflammatory chemokines and cytokines between injured mice exposed to morphine and injured mice exposed to saline. At the chronic 15-DPI timepoint, there was also a decrease in phagocytic activity of macrophages and microglia on the ipsilateral side. Furthermore, the blood brain barrier demonstrated increased leakiness at 15-DPI in the ventral midbrain on both the

ipsilateral and contralateral side of injury. This study demonstrates the unique inflammatory response that occurs in response to morphine exposure after mild TBI.

In the final chapter, the conclusions of the project are examined and discussed. The well-defined mild injury model that was described in Chapter 2 can be further utilized to examine long-term deficits. This model can evaluate risk factors and treatments, enabling the examination of how these factors can modify neuroinflammation and alter behavioral responses. By demonstrating changes in drug seeking behavior in Chapter 3, the connection between mild injury and morphine consumption and sensitivity is evident. This model can further be utilized in the context of modification of the immune response to identify the factors that are critical to behavioral changes. The temporal changes evaluated in Chapter 4 demonstrate the unique changes to the acute and chronic immune response. Further evaluation of this model can identify time sensitive changes that are critical to behavioral alterations. The culmination of the work presented here provides a well described working model to interrogate the mechanisms that link the proinflammatory response to mild TBI and opiate use disorder.

Chapter 1
Review of Literature

Between 64-74 million people sustain a traumatic brain injury (TBI) every year in this world.[1,10,161] Though that number is highly debated, there is clear consensus that brain injuries are under-reported and under-diagnosed.[2-5] While the national average of substance use disorder is 11%, those with a history of TBI have an increased risk of 3-6 times that of the general public.[28,29,132] This confirms the importance of TBI as a key risk factor of substance use disorder, including opiate use disorder. Further investigation into this connection is warranted considering the vast threat to the population.

General epidemiology of TBI

Millions of people in the world suffer from TBI each year,[1] with approximately 5 million people seen for TBI related conditions in the U.S. annually.[2,3] Of those affected, over 200,000 of those patients are hospitalized, and approximately 60,000 people die.[2-5] Though the number of people reported to be affected by TBI is large, it may be highly underestimated, especially since 75-90% of TBI is considered mild, which is the most difficult to identify or diagnose.[1,6] Even patients with mild TBI can fail to identify the injury and thus do not seek medical attention.[7] Furthermore, reports about TBI focus on costs related directly to initial medical care, which underestimates the true impact on patients.[2,8] Approximately 43% of patients can suffer from long-term consequences, and those symptoms can add costs by affecting quality of life, employability, and follow up care.[2,4,5,9]

The majority of civilians in the United States who suffer from TBI are elderly (75+ years old) and the major cause of injury is falls, which seems to be growing more prevalent.[3,17-23] This group of elderly patients also has the highest rate of TBI related death.[3,17-19] Individuals in this group also suffer from more long-term deficits following TBI, especially in terms of dementia, where the earlier injury correlates with earlier onset of dementia.[21-23] Several studies have suggested that younger adults are more resilient to mild injury, thus

there are stronger correlation of mild TBI to long-term deficits at an older age.[162,163] Males and females have approximately equal injury rates among this older age group.[22-24] However, it seems that elderly females have lower scores in quality of life surveys after TBI, suggesting a lowered ability to recover.[19] In fact, age is a major factor in the risk of adverse outcomes following TBI, including more complex and serious injuries.[24,25,164,165]

Another major age group at risk for TBI is adolescents and young adults (15-25 years old), with males representing the majority of injured patients.[22-25] In the younger age group, TBI is more likely to be linked to sporting injuries or motor vehicle accidents.[3,19-23] TBI's caused by motor vehicle accidents also have the highest rate for hospitalizations.[22,23] Impaired driving is a major risk factor for a motor vehicle accident, and with 4x as many males caught driving impaired,[166] young males are considered the major group contributing to this number.[23] Young males are also considered more prone to injury due to risk-related behavior, especially during sporting activities.[15,167]

Soldiers are a major fraction of the young adult population at elevated risk for TBI. This is especially true of those that are deployed to active military zones.[26,27] In the veteran population, even mild TBI is associated with an 56% increased risk of Parkinson's Disease.[43] Within military populations, women are less likely to sustain a TBI or need a craniectomy. However, women are more likely to suffer from post concussive syndrome, and more likely to be treated by standards developed for men since most studies surround the male population.[168] The military population is also at increased risk for polytrauma, where multiple injuries are incurred in at least two areas of the body.[27] Though the incidence of polytrauma exists within the general population as well, approximately 80% of those in the military that have multiple injuries also suffer from TBI.[26] This further complicates the care and outcomes for the military population.

Among the most visible groups at risk for serious consequences of TBI are professional athletes. Movie's such as Concussion the Movie and news reports such as the "League of Denial" have spread awareness about chronic traumatic encephalopathy (CTE) to the general public.[169,170] CTE is a progressive and ultimately fatal brain disease that has been associated with multiple TBI's and devastating sequalae, e.g. dementia.[171-174] While

anyone with repetitive TBI are at risk for CTE, athletes are especially at risk for multiple, and often more serious, brain injuries.[171] While more serious injuries are thought to be important to long-term sequalae, repetitive mild injury can also increase the risk of developing CTE.[163] Though the signs and symptoms are identifiable in some patients (increased aggression, confusion, depression, etc.),[172,174] the diagnosis can only be made post mortem,[173,174] which makes it difficult to discern the incidence of CTE. However, among professional football players there are 3x as many deaths due to neurodegenerative disease, and among boxers, increased bouts and longer careers are linked to higher incidence of CTE.[175-177]

Adolescents and Young Adults with TBI

The leading cause of death and disability for children and adolescents in the United States is TBI.[178,179] The most recent survey of TBI in the US said the adolescents and young adults are the second peak among populations that suffer from TBI.[22-25] The leading cause of injury is motor vehicle accidents and sporting injuries.[3,19-23] Motor vehicle accidents are responsible for the majority of hospitalizations, and can often be linked to impairment of at least one driver involved.[22,23] Young men have been cited 4x more for DWI than women, thus suggesting this behavior contributes to the higher proportion of males injured in this age group.[23,166] Furthermore, young males also participated in high risk behaviors, further exacerbating this number.[15,167]

The second leading cause of concussion in young adults is sports related injury.[3,19-23,25,180,181] In high school students, 15% reported at least one TBI in recreational activity within the year.[18] In fact, among high school students and college athletes, females were more likely to report TBIs than males.[15,182,183] This may be due to gender related expectation, where males subscribe to the masculine norms, with emphasis on risk taking.[15] The societal norms may also affect the behavioral outcomes, such as in one study where young men were more likely to express externalized behaviors (substance abuse and dependence), where females were more likely to internalize behaviors (depression). These results were compounded by a history of adolescent TBI.[184] Young males were also likely

to have less knowledge about TBI, including risk, signs, and symptoms, which could include a lower likelihood of seeking medical aid and subsequent treatment.[15]

Among adolescents, suicide is a leading cause of death.[180,185,186] Recent evidence suggests that having one or more concussive events increases the risk of depression and suicidal ideation.[180,187] Even after controlling for depression and bullying, suicidal thoughts, ideation, and behavior is associated with concussion, mostly attained during sporting activities.[180,185,186,188,189] There is some debate about links to gender effects, but it seems that ideation is more common among girls following concussion,[180,185] while boys are more likely to attempt suicide with more lethal means, higher aggression, and greater lethal intent.[180,186] Recent papers recommend education among parents about concussion symptoms and recognition, including understanding potential risk factors for injury and depression.[180,185,186]

Concussion is also a leading risk factor for substance use disorder (SUD) in adolescents.[132] About 11% of the general population suffer from SUD, but that number jumps to 37-66% with a history of TBI.[28,29,132] When examining adolescents, there is a significant increase in SUD among ages 12 to 18.[190] When examining substance use in adolescents, there is an increase in alcohol use among teens that have a history of TBI as opposed to an orthopedic injury or no reported injuries.[191] Another study illustrated that in teens age 14-16, SUD was significantly more prevalent in individuals that were hospitalized with TBI before 5 years of age.[192] That coincides with findings from a study of inmates in South Carolina, where individuals with a TBI before the age of 13 had higher levels of substance use than those with injury after 13 or no injury at all.[193] The choice of preferred substance use may also be affected by gender, since in one study adolescent males with a history of TBI reported more nicotine use, while females reported increased alcohol use.[194] Though mechanisms behind this association are unknown, and there is still much debate about findings in this adolescent population, it is clear that there is a strong connection between SUD and TBI in childhood and adolescence.

Soldiers and TBI Diagnosis

While soldiers endure many risks while being deployed to war zones, over 450,000 have been diagnosed with TBI since 2000.[2,195] The signature injury from the wars in Iraq and Afghanistan is TBI, though polytrauma, considered injuries to 2 or more organ systems, is more descriptive of the majority of injuries.[30] Of those treated in polytrauma rehabilitation clinics, 80-93% had TBI, 81-96% had pain, and 44 to 52% had a mental health disorder.[26,30,196] In fact, those with polytrauma were diagnosed with 3 or more TBIs, which correlated with pain and posttraumatic stress disorder (PTSD).[30,197,198] These numbers are strikingly consistent between years of reporting.[30]

The vast majority of TBI in soldiers (82%) is considered mild.[8,195] Service-related injuries are described by different criteria than that in many civilian hospitals and is heavily based on time unconscious. When examining soldiers in general, nearly 10% of soldiers were diagnosed with TBI, while a further 29% were suffering from PTSD and 40% were suffering from pain. Acute symptoms can add a variety of cognitive ailments, including vision impairment, anxiety and depression, with headache as the most common.[2,8,30-35,199] A history of TBI is a strong predictor of headache within the first year of care at the Veteran's Association Hospital. Comorbidities can also increase the likelihood of developing headaches after TBI, highlighting not only the importance of TBI diagnosis, but an understanding of the entire patient for accurate prognosis.[33] Soldiers who have been diagnosed with mild TBI from explosion exposure were more likely to suffer long-term side effects. Incidences of mild TBI increased the likelihood of developing more frequent headaches, more severe pain, migraines, PTSD, and sleep impairment.[34] Furthermore, patients with TBI can have issues identifying their own injuries, whether because of their mild injury or issues related to masculinity. Even if soldiers do identify issues, doctors have difficulty diagnosing mild injuries, leading to a low accuracy of diagnosing TBI in the military and skewing of the categorization of severity.[30] The polytrauma triad of TBI is defined as the acute symptoms paired with PTSD and pain, and exists within 6% of the military population.[2,30,36] While the number of veterans has increased, the percentage with TBI, polytrauma triad, and pain conditions remains stable.[30] This also coincides with the

relative increase of pain and PTSD diagnoses, which has benefitted from more targeted awareness campaigns within the military, contributing to more accurate identification by patients and physicians.[30,200-202]

Public attention has increased over recent decades on SUD among the military population, but it remains a significant issue among active military and veteran populations.[203,204] Commonly, alcohol is used for stress relief and socializing, though SUD is also connected to suicide and deaths involving high risk behavior.[203] While there is much information connecting substance use with increased risk of developing TBI, less is known about how the brain injury influences SUD and relapse.[204-207] However, epidemiologic evidence reveals that those with a history of TBI have increased likelihood of dependence or relapse,[205] though that risk may decrease with time.[208] There are very few studies that include whether soldiers with no history of SUD have increased illicit substance use following TBI.[205] More research needs to be done on active military populations to ensure the accuracy of recall about service related injuries, and illicit substance use.[204] One important recent finding has been that mild TBI is highly different from moderate or severe TBI in terms of risk of SUD, thus indicating a need for better diagnosis and prevention strategies follow mild TBI.[208] Even among military population and immediate family, SUD was more likely to be accepted if the soldier reported a history of TBI,[209] thus possibly limiting individuals seeking treatment. That only increases the barriers of care, already greatly hindered by the stigma of seeking treatment.[204]

Sex Differences and TBI

In terms of sheer number, males have a higher rate of diagnosed TBI.[22,210] However, women are underrepresented in the published literate about TBI, and the number of TBIs among women is rising all over the world.[15,161] While the current body of evidence suggests young males are more likely to incur a TBI due to increased risk taking activities, especially in sports related activity, women may have other contributing factors to TBI that are under-investigated, including domestic violence related TBI.[15,167]

Men are more likely to be hospitalized after TBI, thus there may be a skewing of epidemiologic data that relies on hospital records for reporting injuries.[15,211] The number of hospitalizations are decreasing though, reflecting the change in clinical case management.[15,18,212,213] The acute phase of injury seems to have greater adverse symptoms in males,[15] but women may be more likely to die from a head injury, especially if the injury was a result of an assault.[15,214,215,216] Males have a higher likelihood of self-inflicted injury or injury induced by another individual.[15,217] Men are also more likely to leave the hospital against medical advice,[15,217,218] and fewer men follow up with long-term care.[15,218] These observations may be explained by the existence of other health related factors; some studies identify males as having increased incidence of alcohol abuse and assault as the cause of injury, along with other conditions that affect brain and possibly recovery.[15,218]

Men and women's physical differences may affect how TBI is acquired and the severity of injury. For instance, neck strength and physiology differences may alter post injury experience that can affect symptoms and therefore alter reporting and care seeking.[15,219,220] Further differences exist in the cranial bone thickness,[221,222] regional brain volume,[223] connectivity within the brain[224] and symmetry withing the brain.[225] Any of these differences can influence the vulnerability of the patient to TBI, and affect the severity of injury and symptoms.[15] Hormones may also be part of the physiologic differences that affect gender disparities, especially estrogen and progesterone that have been considered neuroprotective within the literature. However, the translation from preclinical models to to clinical evidence and treatment remains inconsistent. [15]

Role expectations seem to be a clear concern that divides the way men and women report and seek care for TBI.[15] Among high school and college athletes, females expressed a greater intention to report future sport-related concussion than males.[15,182,183] This may be further exacerbated by a lower awareness of signs, symptoms and consequences of TBI or by males confirming to the social constructs of masculinity.[15,183] Women are more likely to proactively seek treatment for mild TBI as students at the University level, and later on when mild TBI was sustained in the workplace. [15,182,226]

Following TBI, women report several changes associated with reproductive and sexual health. Although there are changes to menstruation, reproduction, and sexual activity, many women are hesitant to discuss all the subject areas, especially in terms of sexual pleasure. Clinicians also seem to disregard these aspects of recovery and are reticent to even bring up the topic. While the medical community is hesitant to address challenges to women's sexuality after TBI, there is a large set of literature identifying a wide set challenges men face in the area of sexuality post injury.[161]

Further confusion about the impact of sex is compromised by anatomical and physiological differences of humans and animal models used in research. For instance, preclinical differences demonstrated better physiologic outcomes in female animal models in the acute phase. Specifically, animal studies identified sex differences on cerebral hemodynamics, but those same parameters are not different in the clinical presentations.[15,214] These observations highlight the need for more reliable models, which includes understanding which animal model is appropriate to use for the specific question being addressed.

Diagnosis and Classification of Traumatic Brain Injury

The identification and diagnosis of TBI has changed over the years by adapting and updating parameters as more reliable equipment and tests have been developed. However, diagnosis of mild injuries, as well as identifying those patients that will develop long-term consequences remains elusive.[1,5,7,9-15] Even the categories of severity as mild, moderate, and severe are lacking in specificity and description to be of sufficient use,[1,2,16] which has led to more extensive research on diagnosis, clinical care and treatment.

Symptoms of TBI

Symptoms of TBI are wide ranging and vary between patients, but a history of TBI generally causes an increase in long-term mortality and reduces life expectancy.[36] Accuracy of the diagnosis therefore needs to include physical, psychological, and social effects.[2,30] Acute symptoms include headache, vision impairment, insomnia, depression, anxiety, and other cognitive ailments.[2,30-36] When these symptoms pair with pain and

PTSD, it's considered the polytrauma triad.[2,30,36] Care post TBI was originally thought to be an acute concern, but conventional wisdom has moved to understanding this is a chronic condition.[2,36] Many individuals will have symptoms for 6-12 months after an injury of any severity, [2,7,37] with up to a quarter of patients declining over the next 5 years.[2] Even with this evidence, patients continue to not receive information on follow up care after injury.[7]

Most studies focus on moderate and severe TBI, however, mild TBI is overwhelmingly more common at 75-90% of all injuries (as measured by Glasgow Coma Scale) and is connected to the long-term consequences of injury.[1,72,227] Clinical assessment of the vestibular and oculomotor effects after mild TBI may enhance the accuracy of predicting long-term consequences to injury. Furthermore, multiple mild injuries do appear to exacerbate long-term consequences, increasing the amount and severity of symptoms.[1] Mild TBI alone increases the risk of headache development and exacerbation of pre-existing headache conditions. [199] Many patients report new or worsening headaches, but very few are diagnosed with post traumatic headache (PTH). Those with pre-existing headache conditions are even less likely to be diagnosed with PTH, but are more likely to suffer severe cognitive impairments.[45] Patients who are diagnosed with PTH have more severe insomnia symptoms than those who suffer from migraine or healthy patients. The diagnosis is also associated with depression, headache intensity and hyperacusis.[32]

Even decades after injury, patients with a history of TBI are at a higher risk to develop neurological and psychiatric illness, including depression, PTSD, Alzheimer's disease, Parkinson's disease and addiction.[8,30,33,38-43] Depression and PTSD are common diagnoses among individuals with TBI, which may connect with suicide as the leading cause of TBI related death, mirroring the rising level of suicide in the nation.[22] Those that are diagnosed with chronic conditions often need long-term care, with family members often taking on care giver roles, further impacting relationships and quality of life.[2,9,44] The development of long-term disability is heavily affected by socioeconomic status, including location and family related factors, along with access to health care.[2,15] However, insurance status and income level did not have an effect on the amount of individuals seeking follow-up care at 3 months post injury, or even seeking acute trauma care and diagnosis.[7]

Understanding who needs to be referred for long-term care is still unknown and desperately needed.[2] This has led to large scale investigations into neuroimaging, biomarkers, and other technology to improve treatment and understand prognoses.[2,36,44] Improving the accuracy of diagnosis will help all those suffering, since without a diagnosis, patients will not have access to newly developed treatments.[45]

The Glasgow Coma Scale

The Glasgow coma scale (GCS) is a commonly used by medical professionals to diagnose and describe TBI.[16,46] The scale was originally described and published in 1974 by Graham Teasdale and Bryan Jennett,[16,46,47] and is still praised for its reliability and flexibility, though its current form has been updated through multiple studies and revisions.[16,44] It's development began in 1971 to assist with clinical care and prognosis for patients with TBI by providing defined responses to given stimuli.[16,46,228] The scale utilizes three components to analyze areas of responsiveness (eye-opening, motor, and verbal) and come up with an overall score for each patient; the GCS refers to the values from each individual component while the Glasgow coma score refers to the sum of the individual components.[16,46,47,228] The score has also been used to describe and classify the severity of injury, with lower values indicating increased severity. Accuracy in data gathering and reporting of the score can be an issue between clinical care providers.[16,37,47] Many TBIs are tracked through this modality and confirm that 75-90% of injuries are mild in nature.[1,6] The overall scale has a strong relationship to the outcome of acute injury.[77] However that may artificially group patients that have varying risk factors and deficits which more completely inform, thus each patient is best described by the scale, while the overall score is often used to for examining larger data sets and populations.[16,46,47,228] The score's usefulness, as well as the value of each segment has been debated, with some arguing the score is used inappropriately for monitoring, while other experts highlight the motor score as most valuable for patient prognosis.[16,228]

Some groups have adapted the GCS to an extended Glasgow Outcome Scale with several areas of interpretation.[44] The scales that are used describe several patient outcomes, varying from dead and vegetative states to moderate disability and even good recovery prognosis.[44]

Furthermore, they utilize these scales to determine the level of supervision by taking into account the need for care giver assistance, along with determining the cognitive function in terms of functional independence and problem solving/memory retention.[44] However, studies have demonstrated that different patients can response to therapy and treatment in different ways, especially in the acute phase. Thus, medical professionals need to be careful in making early prognoses that take away therapies too early and limit the patient's recovery.[37]

Though appropriate for many situations, the scores can be heavily affected by lifesaving procedures, such as pain control, intoxication, and intubation.[16] Thus, other methods of assessment and monitoring need to be used for many patients, especially those with severe TBI or multiple compounding injuries, to better address the heterogeneity and outcomes of TBI.[1,2,16]

Imaging Modalities

There are several different imaging modalities to diagnose TBI, but the most common by far is computed tomography (CT). This is a rapid imaging technique that identifies patients with urgent neurosurgical intervention needs and is the clear first choice for initial imaging techniques by doctors.[48-51] It provides feedback quickly, and can identify foreign bodies, hemorrhages, herniation and hydrocephalus, while also useful for predicting outcomes.[51-55] However, it does take time, is expensive, and can delay treatment, thus patients need to be evaluated by clinicians for the appropriateness of the test.[53,54] In patients with mild injuries, CT is often normal, so tests should be limited to those with indicating symptoms, such as headache, vomiting, intoxication, and advanced age.[54,55] While useful for initial diagnosis, it offers very little information on follow-up care.[49,229] In follow-up imaging studies, there are very few patients who have any change in care according to new CT scans.[229] Though even among those that have a positive finding on CT scan, only 40% actually had follow up care and were seen by a physician at 3 months post injury,[7] emphasizing the lack of long-term care despite injury severity or diagnosis.

The second most common imaging modality is magnetic resonance imaging (MRI).[48,49,56] MRI is indicated when the CT scan is normal but neurologic symptoms are persistent,[56,57] especially with subtle injury, e.g. mild TBI.[49] MRI can also identify chronic injury pathology, e.g. parenchymal atrophy, white matter injury, and microhemorrhage.[48-50,57] MRI is not indicated for initial diagnosis due to the associated expenses, lack of availability, and lack of sensitivity for fractures.[49] Patients also need to be evaluated for suitability since this modality is incompatible with many medical devices and metal foreign bodies.[49]

CT and typical MRI scans are common but cannot detect all damage to the brain following injury. A common injury in TBI is diffuse axonal injury, which is damage to the neuronal axons during initial force applied to the head.[71,72,230,231] Diffusion tensor imaging (DTI) is an advanced form of MRI that utilizes the directional coherence of water diffusion to evaluate the integrity of white matter microstructures, including axons.[57,71,72,230-233] Utilizing machine learning of multiple indices of DTI can provide a method for identifying more mild cases of TBI by looking at the microstructures of the brain.[230]

Immune Responses

The brain offers a unique and privileged site in which ingress and egress of cells and other physiologically important elements is tightly controlled. While the innate and adaptive systems do respond to inflammatory processes within the brain, the recruitment and management of that response varies, while also interacting with and regulating the cells that are only found within the central nervous system (CNS).

Unique Aspects of the Immune System within the CNS

The brain has long been considered an immunologically privileged sight,[58] and indeed there is experimental evidence demonstrating a lack of rejection of grafted tissue within the brain as opposed to peripheral areas (e.g. skin).[60,234] But that term has fallen out of favor as studies have illustrated strong links and communications between the resident immune cells of the brain and peripheral immune system. Neuroinflammation was long thought to

be only detrimental[235] since many of the disease or degeneration processes feature activated peripheral immune cells in the brain.[236-239] However, new evidence of the last few decades has revealed that infiltrating cells can be neuroprotective or neurotoxic.[240-245] Many normal and recovery processes, including neuronal remodeling and signaling changes, require specific initiation of immune responses.[243,246,247] This has led to the field redefining how the brain interacts with the peripheral immune system, and a wide array of new functions discovered for the main components.

The blood brain barrier (BBB) is responsible for trafficking cells and nutrients into the brain, as well as keeping most cells and substances out.[59,60] The BBB was first demonstrated in 1885, where the Paul Erlich showed that dye injected into other areas of the body could not penetrate the brain. The term was coined in 1900, while the structure was debated into the 1960's.[60] The BBB consists of the cerebrovascular endothelial cells, pericytes and astrocytes.[61,248-250] While this barrier is often thought to confer protection and keep immune cells out, but disruption of the blood brain barrier is not enough for autoimmunity to develop. In one of the original studies, merely disrupting the BBB by grafting foreign tissue in did not induce the proinflammatory T-cell response.[234] Damage in the brain can be exacerbating by hyperosmolar agents or ischemia, yet still the proinflammatory T-cell response was not induced.[242] Even healthy individuals can have areas of increased 'leakiness' and not have increased lymphocyte infiltration or autoimmunity.[60] Therefore, many researchers have redefined the BBB from keeping lymphocytes out, to facilitating the entry of beneficial nutrients and the export of potentially toxic chemicals.[61-63] The control of the blood brain barrier may actually be further under the control of microglia and astrocytes.[64]

The main resident neuroimmune cells are microglia, the tissue resident macrophage and a key innate immune cell of the brain.[65,251,252] Microglia originate from mesenchymal cells that migrate from the yolk sac in early embryonic development.[66,251-253] These cells are extremely sensitive and can respond to signaling throughout the brain,[65-67] including changing homeostatic phenotypes in response to neuronal and astrocyte signaling.[67] Microglia's main interaction with neurons has been eliminating apoptotic cells and non-

functional synapses, thus the cells interact constantly to monitor the neural network and health of the neurons.[67,252] Microglia are highly dynamic in their surveillance even in the resting state, utilizing their motile processes and protrusions to monitor the brain milleu.[254] Oligodendrocytes are also highly affected by microglia, from development and maturation, to later maintaining the progenitor cell population of the oligodendrocytes in the adult brain.[67,255,256] Astrocytes are the largest population of cells in the brain and interact with microglia to maintain homeostasis.[67] Astrocytes actually provide support molecules (CSF1, IL34, TGF-β) to microglia during homeostasis and promote homeostatic morphology and functions.[67,257-259] Astrocytes and microglia can also work together to coordinate synaptic remodeling.[68] While these processes protect the brain, alteration to microglia either by overactivation or by loss of homeostatic processes, is indicated in disease and aging processes.[252,260-262] Ultimately, as previously asserted, the functionality of the microglia along with the context determines the benefit or detriment of their actions.

Astrocytes are the most numerous cells in the brain, but there is still much to discover about their functionality.[67,263] Interestingly, astrocytes are thought to play an important role in the development of consciousness, since humans have a unique increase in the ration of astrocytes to neurons and a distinct phenotype when compared to other mammals.[263,264] Astrocytes initially develop in the neural tube and migrate to other areas of the brain for differentiation.[263,264] It seems that astrocytes actually interact with and alter many processes in the brain. As previously mentioned, astrocytes work in conjunction with microglia for neuronal remodeling[68] and interact with the BBB to control the entry of cells and nutrients into the brain.[64] In fact, the very morphology and phenotype of pericytes is directly tied to the input of astrocytes.[250] However, communication can go both ways, and proinflammatory microglia can activate astrocytes which then promote cell death in neurons and oligodendrocytes, losing their ability to support survival and growth.[69] In stroke, activated astrocytes and active gliosis can help limit secondary damage, but the glial scar formation can inhibit neuronal growth and reconnection.[265] These dueling roles of astrocyte function are typical of many immune cells, and demonstrate the beneficial and detrimental sides of the immune system.

All this has led to a new understanding of the brain and peripheral immune system relationship. Many researchers have determined that though the brain is not as immune privileged as once described, it is still handled differently than any other system in the body.[266,267] One study revealed that interactions with the peripheral immune system may actually take place in dural sinuses, where antigens are trafficked to antigen presenting cells that can communicate with T-cells.[252] Recent studies have demonstrated that there are immune cells found in the dura and meninges of the brain,[268,269] along with a lymphatic system that drains from the dura to local lymph nodes for monitoring.[270,271] In fact, there is a system of channels that exist between the skull and spinal cord bone marrow that supplies the CNS with immune cells.[272-275] This finding demonstrate that the brain is still under regular immune system surveillance.

Inflammatory Responses to TBI

TBI is a result of an external force to the head: direct impact, blast exposure, acceleration/deceleration injury, or even penetration.[70,73] There is a primary stage of injury which consists of the physical injury directly associated with the force applied, e.g. axonal shearing.[70-74] The secondary stage of injury describes the immune system's response and subsequent damage that occurs in the following days to weeks.[70,73,74] A challenging part of understanding TBI and the inflammatory response is the heterogeneity of each injury, from cause and severity to clinical outcomes.[70,93] However, there are some common themes that accompany the response to TBI. This review will focus on the sterile TBI, without puncture or penetration to the brain.

The initial damage following TBI releases cellular contents into the surrounding area and damages the BBB. Though initially described as tools to respond to microbes,[70] toll-like receptors and pathogen-recognition receptors can respond to the release of damage-associated molecular patterns (DAMPs).[75] DAMPs, and other associated alarmins (e.g. S-100 proteins, adenosine triphosphate (ATP), uric acid, DNA or RNA), are released by damaged tissue and sometimes by the effector cells of the immune system to cause a proinflammatory response.[75,76] In one study, the release of ATP caused a neuroprotective inflammatory response after mild TBI,[78] while another demonstrates that dismantling of

the inflammatory response reduces lesion size.[101] The duality of this response is a common theme among all aspects of the inflammatory response to TBI.

Microglia respond to any CNS insult, and are implicated as a main effector cell in any neuroinflammatory response.[70,77,276] Theses cells can rapidly respond to any insult and migrate to areas of damage quickly, which seems to mediated by the release of ATP from damaged cells.[79,277,278] During CNS disease states, it seems that microglia are active in clearing debris through phagocytosis, and can demonstrate a suppressed cellular metabolism at the start of the disease phase.[77-79] In fact, when microglial phagocytosis was inhibited locally, the damage following TBI was more severe.[70,78] Overall, the microglial response seems highly neuroprotective at the acute stage, supporting surviving neurons and astrocytes.[78] The microglial transcriptome actually increases gene expression associated with neuroprotection as individuals age.[279] However, interactions with infiltrating macrophages can activate microglial to a proinflammatory state.[280] Microglia can produce reactive oxygen species (ROS) that are correlated to worsened clinical outcomes.[80-82] Long-term activation of the proinflammatory microglia phenotype may contribute to depressed neuronal signaling, and long-term side effects.[83] Thus while activation of microglia can be beneficial, uncontrolled and chronic proinflammatory reactions can be damaging.

TBI causes a proinflammatory response through assembly of the inflammasome, which recruits the innate immune system.[101] Macrophages are a key feature of that response, though they have similar markers to microglia and can be indistinguishable from microglia on by light microscopy.[77] Macrophages are normally found in different areas of the CNS (e.g. meninges, choroid plexus, and perivascular spaces),[281] and meningeal macrophages are some of the first cells to die after focal injury, possibly initiating the early immune response.[78] Recruitment of peripheral macrophages relies on the engagement of C-C chemokine receptor 2 (CCR2) and C-C chemokine ligand system (CCL2), peaking in the first few days after injury,[282-285] and correlating with increased anxiety-like behaviors and long-term spatial learning deficits after TBI.[282,286] During CNS disease states, infiltrating macrophages are highly phagocytic and proinflammatory, but that action may be

detrimental to the brain since they can be found in areas of demyelination in diseases, as with multiple sclerosis.[77] An important role in antimicrobial inflammatory defense is the release of ROS by macrophages, but this response can also be neurotoxic in the CNS. Following both TBI and spinal cord injury, macrophages, along with microglia, increase their production of ROS, correlating with increased damage to the surrounding tissue.[80-82] Macrophages utilize nicotinamide adenine dinucleotide phosphate (NADPH) production and NADPH oxidase (NOX) to create ROS,[287] and inhibition of NOX improves outcomes after TBI, whether that inhibition is genetic or phamacological.[81,288-292] However, in other cases of CNS damage, e.g. spinal cord injury, unique macrophage phenotypes take on an anti-inflammatory and neuroprotective role.[84] Interestingly, inhibition of NOX actually increases the anti-inflammatory macrophage phenotype.[293] It seems that while macrophage infiltration is essential to the neuroinflammatory response to injury, a switch from the proinflammatory phenotype to an anti-inflammatory response promoting neuroprotection is necessary to improved recovery.

Neutrophils are part of the innate immune systema and some of the first cells to respond to injury within the CNS and throughout the body.[70,294] Following TBI, neutrophils are rapidly recruited into the CNS through meningeal blood vessels and the choroid plexus.[78,283,295,296] While known for being involved in the proinflammatory response, their activities help prepare sites for wound healing, recruit other immune cells, and facilitate acute and adaptive immune responses.[70,294] Within the CNS, they are involved in phagocytosis, surrounding dead cell tissue after injury, and coordinate their response with macrophages.[70,78,294] In fact, reducing the recruitment of neutrophils actually increases damages to the meninges after injury.[70] However, neutrophils can also be highly destructive.[70] After injury, neutrophils can become hyperactive, releasing neurotoxic chemicals that directly damage the BBB,[297] while the same chemicals that damage the BBB can be released and cause neuronal death.[298] Indeed, neutrophils are the most numerous immune cell in circulation after injury and highly activated.[299] In another study, depletion of neutrophils reduces edema and lesion size while also reducing activation of macrophages and microglia, though surprisingly there was no change to the breach in the

blood brain barrier.[300] While it seems neutrophils can cause damage in many ways, it is essential for the clearance of apoptotic and damaged cells after TBI.

While it is very clear that the innate immune system responds to TBI, the role of the adaptive immune response is still under investigation. While autoimmunity to the CNS can be fatally catastrophic, there may be a neuroprotection effect by limiting the secondary damage that spreads from initial injury site.[301,302] In fact, autoimmunity seems to need secondary catalysts to induce disease states, and those autoreactive T cells can actually promote revascularization and healing post-injury.[247] Still, other studies have demonstrated that the production of ROS species can recruit lymphocytes to the injury site and amplify the inflammatory response.[303,304] However, one study that utilized a mouse model deficient in T and B cells found very little difference between deficient and wild type mice in the acute stage of TBI.[305] Thus, the reduction of the infiltrating lymphocytes may offer a novel treatment target,[303,304] but its usefulness may depend on the timeframe of treatment and identification of appropriate patients.

Connections to Neuronal Remodeling

Microglia are highly involved in synaptic plasticity and neuronal remodeling.[252,306] In order to remodel neurons, synaptic pruning needs to occur, and microglia have been concluded to be essential in this process during development.[306,307] In both developmental and disease states, products from the complement cascade are used to identify inactive synapses for elimination, and these products bind receptors on microglia to induce phagocytosis.[308-310] Microglia also use the fractalkine signaling system (chemokine CX3CL1/CX3CR1) in the CNS; neurons mostly express the chemokine ligand CX3CL1 that causes chemotaxis of microglia, which mainly express the associated receptor CX3CR1.[252,311,312] This pathway can modulate synaptic pruning and neurotoxicity,[311,312] but can also protect from over induction of monocyte recruitment by astrocytes.[313] Finally, microglia also utilize lipopolysaccharide binding protein (LBP) to identify synapses for elimination; early life stress reduces the expression of LBP, and leads to increased synaptic density and abnormal dendritic spines in the *hippocampus* which correlates with increased anxiety-like

behavior.[314] Clearly, microglia use several mechanisms to physically manipulate the signaling of neurons within the CNS.

Microglia also release tumor necrosis factor alpha (TNFα), a proinflammatory cytokine that can communicate with a variety of cells.[252] When examining synaptic interactions, TNFα increases the expression of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, increasing synaptic efficiency. The lack of TNFα reduces this effect. Therefore, this proinflammatory cytokine maintains synaptic strength.[315] This effect is solely attributable to TNFα rather than generalized to all the proinflammatory cytokines.[252,316] However, this reaction was seen in hippocampal pyramidal and motor neurons, with the opposite reaction occurring in striatal neurons, demonstrating a region and/or neuron specific response.[315-317] TNFα further alters signaling within other glial cells, specifically altering the AMPA receptors on astrocytes and controlling glutamate release, and thereby controlling astrocyte-dependent synaptic modulation.[318] Thus, this factor can actively manipulate the signaling between neurons through several mechanisms.

Macrophages, like microglia, can have multiple phenotypes that result in different functions in the CNS. Macrophages that are proinflammatory, in concert with microglia, can actually hinder neurogenesis and axonal regrowth,[85-87] but the anti-inflammatory phenotype can increase proliferation in neuron precursor cells, support axonal regrowth, and support integration of new cells into the existing neural circuitry.[84,88-92] While these effects are seen *in vitro*, recapitulation *in vivo* is more varied. Further studies examined how elimination of macrophages infiltration affected recovery through investigation the CCR2 cellular recruitment mechanism.[280,282] In one mouse model, CCR2 negative mice had significantly reduced levels of infiltrating macrophages along with increases in neuronal density in CA1-CA3 regions of the *hippocampus* after injury. These changes were accompanied by improvements in spatial learning memory responses, suggesting alterations associated with infiltrating macrophages is detrimental to recovery.[282] Another study found that infiltrating macrophages *in vivo* are also associated with axonal dieback and retraction from the injury site, and depletion of activated proinflammatory macrophages reduced that effect.[87] However, studies in spinal cord injuries demonstrate

that the detrimental proinflammatory response is only one aspect, and the anti-inflammatory response is essential to recovery and neuronal remodeling.[319] This duality is very similar to the duality of microglia, demonstrating similarities in function and why it is difficult to separate these populations from one another in the CNS.

Lymphocytes can also interact with neurons within the CNS. Autoreactivity seems to be a main concern, however autoreactivity directed at myelin antigens was associated with improved neuronal survival and recovery.[247,301,302] Autoreactive T cells for myelin antigens are found in humans with TBI, and was associated with younger individuals.[320] However, not all of the reactions are so beneficial. In one study, (cluster of differentiation) CD8 T cells invaded the CNS and colocalized with astrocytes overexpressing interleukin 15 (IL15), a cytokine crucial for CD8 T cell development and activation. This resulted in increased neuronal apoptosis that coincided with deficits in neurologic function and memory.[321] Inhibition of neuronal apoptosis was associated with improved neurologic function and the alleviation of brain edema.[322] CD8 T cell depletion, not CD4 T cell depletion, was associated with improved neurologic outcome out to 8 months post injury, with a switch in T cell function to a neuroprotective role.[323] Another proinflammatory cytokine, macrophage migration inhibiting factor (MIF) is also highly expressed, and seems to induce peripheral expansion and activation of lymphocytes, though mitigation did not change the acute parameters of the injury.[324] T regulatory (Treg) cells control the lymphocyte response, and while depletion of these cells does not change the acute TBI parameters, it does increase the amount of T cells within the brain.[325] Treg cells are also implicated in the long-term dysregulation of the inflammatory response after TBI and have a possible connection to inflammatory pain signaling.[326] Coupled with the previous studies demonstrating little connection to acute phase of injury,[305,323] the actual functions of the lymphocyte population may be more pronounced in the chronic stage of injury.

The inflammatory response to TBI is dynamic and diverse, which is of little surprise since the injury itself is completely heterogeneous, even in research-based animal models. But there are common threads that connect each response. Each response, proinflammatory and anti-inflammatory, has areas of benefit and destructiveness. Both systems are essential to

the recovery process, and the immune system within the CNS and the around the periphery have actions associated with that. However, there are gaps in understanding how and when interference with this system can improve recovery and limit the development of chronic conditions. Further studies defining the function of each cell phenotype present after TBI until the chronic stage of injury are needed to identify targets and timeframes for potential treatments.

Modeling TBI in Animals

Animal modeling studies are used to study many aspects of TBI. However, the injury in humans is so variable that exact recapitulation is not possible. Therefore, scientists have developed multiple methods for inducing injury in a variety of species. The utilization of multiple behavioral tests can also allow for analysis of different cognitive and motor functions. Combining this with methods of evaluating neuroinflammation can lead to a better understanding of how injury within the brain can affect clinical outcomes.

Induction of TBI

Modeling TBI is made difficult due to the heterogeneity of the injury. No one animal model can recapitulate the nature of the completely,[93] so multiple models are used to recapitulate different aspects of the injury.[93-96] Here, four common methods for the induction of injury are examined: blast injury, weight drop method, fluid percussive injury (FPI), and controlled cortical impact (CCI).

The blast injury model was developed in reaction to trends in the military injury population; over 50% of injuries received in military combat by soldiers are blast related, and civilian blast related injuries are on the rise as well.[327] Blast injury is a diffuse injury model that results from proximity to an explosive device when it is detonated, and the damage is caused by a non-penetrating supersonic blast-wave impulse.[94,97] There are two common ways to produce this injury: field explosion or the shock tube model.[94] The field explosion model recapitulates the injury by detonating a device near an anesthetized animal and measuring the force of the shock wave through sensors.[94,328-330] While this model is highly

relevant in recapitulating blast injuries in combat scenarios, the presence of an explosive device adds safety concerns and the model itself yields variable injury parameters.[328-330] The shock tube model is generally safer and more contained, providing a more reproducible injury.[94,331,332] The shock tube is a highly complex device, which utilizes a controlled explosion directed down a propagation tube, which has a restraint device for a given animal model, and a catch tank or apparatus to absorb the resulting force.[333-336] As such, this model is expensive to construct and requires constant monitoring and an expertise in engineering to maintain.[333-336] This model can utilize large and small animal models, ranging from rodents, like mice and rats,[97,329,337] to swine,[331,338,339] sheep,[340] and primates.[341] Blast injury seems to result in diffuse axonal injury, brain swelling, and ischemic brain injury.[339,342-345] This model is extremely useful for research in diffuse injury, however, the draw backs, including the dangerous involvement of explosives and the requirement of additional expertise, makes this model unobtainable for many studying TBI.

The weight drop method is one of the earliest models developed for injury induction.[74,94,98] The method is relatively simple, utilizing a weight dropped from a given height onto the animals head, and can inflict a focal or diffuse injury.[74,94,98-100] Originally it was developed with a craniotomy placed over the injury site so the damage is directly to the dura; first characterizations found physical damage to the brain including hemorrhage lesions, BBB damage, and cortical contusions which coincided with glial cell activation and immune cell infiltration.[98,346-351] The model can also be used with a closed head paradigm (omitting the craniotomy), creating a more diffuse and varied injury that is more reminiscent of the injuries in humans.[352] Closed TBI allows for investigations of how changes in the skull affect neuroinflammation, while also investigating the movement of substances across barriers and into the meninges.[78] This modeling system allows for the investigation of a variety of injury severities and parameters, and its simplicity has made it a popular choice for decades.

The fluid percussive injury (FPI) is a commonly used model of injury induction utilizing a fluid pulse to induced injury directly on the dura through a craniectomy (open injury model).[94,101-104] The method uses a pendulum to hit a tube filled with fluid that is connected

to the skull creating a closed system. The fluid directly impacts the brain and the associated force can be measured by a fluid pressure detector.[94,96] The type of injury induced is affected by the placement of the craniotomy, with the original midline incisions causing a diffuse injury,[353,354] while a lateral craniotomy produces a more focal injury.[103,104] Further investigation reveals increased variability with midline injury accompanied by increased mortality.[94,355] FPI is used to investigate sterile injury without skull fracture, causing brain edema, tissue shearing, and hemorrhage that is commonly seen in sports activity.[94,96] These injuries can recapitulate some of the symptoms that are seen in human patients, such as motor deficits and impaired cognitive function.[347,356] Rodents are commonly used in this model, but the apparatus can be scaled down for newborn animals or scaled up for use in larger animals, e.g. swine, dogs, and sheep.[104,355-360] This model demonstrates a highly adaptable method of inducing injury, though inherent variability and the lack of ability to perform injury to the skull directly does limit some of its utility.

Controlled Cortical Impact (CCI) is very useful for creating reproducible injury.[95,105] This model was developed originally in ferrets,[361] but was later extended to other species including rodent, swine, and primates.[362-365] The model was originally developed with direct impact to the dura through a craniotomy (open injury model) utilizing a computer controlled rod on a stereotaxic frame to customize the speed and depth of the injury.[94] This creates a discrete and reproducible injury with neuronal and vascular damage generally concentrated on the ipsilateral side of injury.[366] The open CCI model has been used to study diverse outcomes in relation to physical characteristics of the brain, changes to gene expression, and alterations behavioral outcomes.[367-370] While open CCI offers a chance for customization, this was developed to model single impact to the skull and thus closed (without craniotomy) CCI was developed.[94,367,371-374] This model also lends itself to multiple injury studies since the procedure is simplified and less stressful on the animal. Repetitive TBI models have revealed new findings about chronic inflammation following repetitive injury.[371-374] The highly customizable model of TBI lends itself to mechanistic studies, while also making cross study comparisons difficult with individual laboratories varying impact intensity and outcome measures.[74,105,366,375-378]

Animal Models of TBI

Rodents are the primary animal used in TBI research.[106,107,111] This model offers many advantages, including limited associated cost, simple surgical procedures, and the ability to run large cohorts.[106] Since rodent strains have been cultivated in breeding facilities, and generation turn around is relatively quick, there is some homogeneity in the strains that limits the variability within the model.[107,379] In addition, the use of genetic modification is well described in mice, which makes the investigation of specific molecules and pathways more feasible in this model. [106,380] Overall, the mouse immune system is very similar to the human, with corresponding cells, functionality, and signaling processes.[111] However, there are drawbacks to the use of rodents. Mice have lissencephalic brains, as opposed to the gyrencephalic brains of humans and other species, which means they lack cortical sulci and gyri. The lack of these folds greatly diminishes the brains surface area and reduces the subcortical white to gray matter ratio, which would indicate differences in cortical function and development.[107-110] In more severe injury, coma and loss of consciousness are hallmarks of injury and inform severity determination. However, mice and rats are anesthetized for injury, which may be neuroprotective,[381] and do not develop a comatose state after injury.[106,382,383] There are also differences in the rodent immune system, which has been pointed to as a main factor in the lack of success of translational therapies. For instance, there are major differences in the blood composition of neutrophils to lymphocytes (human blood: 50–70% neutrophils, 30–50% lymphocytes; mouse blood: 75–90% lymphocytes, 10–25% neutrophils), which would have major complications in investing the blood neutrophil/lymphocyte ratio (a popular topic in peripheral markers for brain inflammation).[111-116] Another notable immune system difference is that macrophages are a main source of inducible nitrous oxide (NO) in the mouse, but that is not the case for humans, whose macrophages can produce NO but under specific stimuli and in limited quantities.[111,384-388] From the factors discussed here, it is clear that while there are many benefits to this model, the drawbacks have limited the success of this model in translational research.

Swine models demonstrate the potential for more clinically relevant models of TBI.[107,117] The development of the brain in this model is similar to humans, especially in myelin development and water content.[117] Importantly, swine have gyrencephalic brains, with more similar white to gray matter ratios to humans.[107,389] Common models of TBI used in swine involve CCI and rotational acceleration (non-impact rapid rotation of the head on a fixed apparatus).[364,390,391] Importantly, the injuries can be scaled to the size of the brain and animal to model human injuries, and suffer from similar outcomes, such as diffuse axonal injury without the sustained loss of consciousness during sports related injury.[72] While less used, the blast model of injury has been recapitulated in swine.[107] This is especially important since outcomes of the blast injury depend heavily on vascular and tissue structures, along with the structure of the skull.[327] This model is specifically important to model injuries in the military population, and is gaining traction as a significant cause of TBI.[392-395] Also, while the immune system in this model does have clear differences in lymphatic structure and some cellular phenotypes, their macrophages seem limited in production of NO, as are humans, which may lend itself to better modeling of the innate immune system.[388,396] While this model brings ethical concerns, increased cost, complex surgical procedures, and difficulty in performing behavioral assays,[106,107] it demonstrates more similarities to human TBI and may be key in translating preclinical work to success in clinical trials.

Despite the evidence of TBI mechanisms discovered in more traditional species, there are more unconventional models involving bighorn sheep, woodpeckers, whales, and even fruit flies (*Drosophila melanogaster*).[397,398] Here, the focus will be on the role of flies to inform on TBI. Flies offer many advantages as a model of research: their short life span combined with their low cost and easy maintenance make for an ideal species to study.[398,399] 70% of their genes are recognized in human diseases that have an analog, and their internal system has several homologous structures, including a similar diversity of neurons and neurotransmitters.[400-402] There are three main models of TBI in flies: the high-impact trauma (HIT) device, homogenizer model, and close head injury model.[398] In the HIT device, flies are restrained in a plastic tube connected to a spring and a wooden board. The plastic tube and spring is deflected, and hit against Styrofoam, causing variable full

body injury to 50-60 unanesthetized flies.[403] In the homogenizer model, many flies are placed into the Omni Bead Ruptor-24 Homogenizer platform, and shaken at different parameters to cause relatively consistent injuries that result in changes to the immune system and impaired sleep cycles.[404] The closed head injury model delivers individual injuries to the heads of unanesthetized flies, delivering a well-controlled injury.[405] Each of these models demonstrate different types of injury, and have helped researchers to understand the immune response.[398] Importantly, this model demonstrates the importance of utilizing many models of TBI, and that unconventional models can still inform the mechanisms that underlie human disease states.

While the previous work demonstrates the variety of laboratory models that have been developed for TBI, clinical veterinary populations may offer a better understanding of the diversity of injury and serve as an avenue to test potential treatments before human clinical trials. In one study, 25% of canine patients that were seen for blunt force trauma were diagnosed with TBI.[406] SCI is also common; 9% of spinal cord injury in cats is from trauma, while in dogs, 14% of spinal cord injury is from trauma.[407,408] Similar to humans, animals receive CNS injury through a variety of mechanisms, including motor vehicle accidents and falls.[409-411] As in human medicine, understanding the pathophysiology of injury and developing treatments for CNS injury is of critical importance.[412] The clinical model of glioblastoma in dogs has already provided huge gains in understanding and treatments.[413-418] Further use of naturally occurring models in veterinary medicine holds the potential to benefit TBI research.

Behavioral Tests

As previously stated, TBI comes with a variety of short- and long-term consequences.[2,7,30-37] Therefore, in modeling injury, it is important to include behavioral parameters within the study to address the full extent of outcomes in TBI. Rodent models have even linked mild TBI to cognitive and motor deficits, such as increased stress and decreased cooridination.[419,420] This section will highlight how behavioral testing can expose even minor deficits and are necessary to help classify the severity of injury.

Since many cases of TBI have motor deficits, it is a key behavioral response to evaluate in rodent models.[118,119] A useful test in this category is the beam walk test, which measures balance and coordination.[118,421] This model can be used for both mice and rats, as long as the scale of the beams is appropriate to the species, and can be performed at multiple timepoints with the same cohort.[118,421-428] Rodents are trained for 3-5 days before beginning the testing phase, and baseline values can be evaluated before injury.[429] The training stage utilizes a single beam,[421] while testing typically utilizes multiple beams of varied size and shape.[118,421-425] Two to three trials are run on each beam and an average score is gained for the number of foot slips (foot completely misses the beam) and the time to cross the beam. [421,426-428] This test is helpful in sorting through mild to moderate injury and can track the extent of motor deficits overtime. However, if the animal has more severe injury, they may have trouble completing the task, which can skew the results of the test.

Assays evaluating memory after TBI are important since TBI disrupts the retention and processing of both short- and long-term memory aspects. Spontaneous alternation Y maze examines short-term memory by recording how a mouse navigates a maze using spatial working memory.[120,121] In this model, mice are placed within a Y shaped apparatus that has each arm of the maze uniquely labeled for differentiation. The mouse then freely explores the apparatus, with entry into each arm referred to as an alternation; correct alternations require the mouse to consecutively explore each of the three arms of the maze, while returning to a previously visited arm early constitutes an incorrect alternation. [120] This model does require the mouse to be able to move freely and see the reference cues, but its simplicity makes it a useful tool to interrogate for working memory. TBI also causes disruption to memory retention, and the most powerfully retained memory involves fear.[122,430] Therefore, context-based fear conditioning can be used to examine memory retention under varying circumstances.[122,123] In this model, an unconditioned stimulus (foot shocks) is paired with a conditioned stimulus (a context, e.g. a noise or identifiable containment area) during a conditioning program to induce freezing behavior, where a mouse ceases movement temporarily.[122,123,431,432] Subsequently, mice can be placed in the conditioned context to observe freezing behavior without the unconditioned stimulus, and then placed in a non-conditioned context to observe the change in behavior. Memory

retention can be examined in the context of injury by conditioning the stimulus before the TBI.[122,123] This method is time consuming and utilizes expensive equipment to condition stimuli as well as record subsequent freezing behavior, but offers critical insight on how memory retention can be limited after injury, and possibly recover with time.

Spatial learning deficits can be exposed via the Barnes maze assay.[124-126] In this model, mice are placed on a circular apparatus with a spotlight directly over the center. Spatial cues are placed on the walls surrounding the apparatus to orientate the animal. Around the edge of the table are circular holes, with one escape hole that is kept in the same place throughout training. Mice are trained for 4 days to find the escape hole, and on the probe test day, the escape hole is blocked to examine where the mouse explores.[124] Testing groups can demonstrate differences on the amount of time taken to reach the escape hole on testing days, or in the amount of time spent in each zone of the maze on the probe test day.[124-126] This model can be tricky to use since environment can influence the results, and the mouse has to be able to not only see the visual cues and identify the escapes hole, but also crawl into the small box under the escape hole to complete training.[125] However, this paradigm allows insight on a complex learning tasks that requires extensive neuronal network communication, lending itself to questions about how neural networks are altered following mild TBI.

Open field test is a relatively simple behavioral test that evaluates anxiety-like behavior.[124,127-130] In this model, mice or rats are placed in the center of the open field box, which varies in size due to the animal used (e.g. 20 x 20 x 10-inch box for mice), and the animal is allowed to freely explore the apparatus.[124,129] Rodents have a natural inclination to explore new environments and naïve rodents will explore both the inner area and outer area of the apparatus.[433] However, injured or sick rodents spend more time on the outer edges of the open field test, indicating an increase in anxiety-like behavior.[124,130,434] Since increased anxiety has been seen as a long-term consequence of TBI,[184,187,286,337,435] it is important to model this behavior in translational research, and profile any changes with treatment. However, interpretation can be difficult in this test since many conditions including lighting and temperature can influence outcomes.[127,129] Furthermore, a rise in

anxiety-like behavior in rodents is a common comorbid condition with headache,[436] confounding the interpretation of results after a head injury.

Novel object recognition test (NORT) was developed to test the ability to recognize novel objects introduced into a familiar environment.[118,131] This test has three phases: habituation, familiarization, and test stage. The habituation stage is where the rodent is put in the middle of an open field arena with no objects present so the animal can explore the surroundings. The next stage is the familiarization stage, where two identical objects are placed in opposite corners of the arena so the animal can explore them. After a delay, the animal is placed into the arena again, but during the test stage, one of the familiar objects is replaced by a novel one.[118,131,437-441] The placement of the novel object vs. familiar object is counterbalanced to avoid bias.[440] The time with each object can be recorded with computer software, and the data can then be used to determine the discrimination index, which is a calculated value that describes if the animal spends more time with the novel object vs. familiar ($DI = (T_N - T_F)/(T_N + T_F)$).[131] Again, the rodents must be able to move freely and be able to see each object to identify it, but this test can help identify limitations in memory. By adjusting the delay between the familiarization stage, the test can be adjusted to interrogate short- and long-term memory.[131] In further studies, the number of objects, context in which they were presented, and additional familiarization stages demonstrates the versatility of this test.[131,442-446] Thus, it is another powerful tool for assessing cognition.

Drug seeking behavior is an important area of research that has been developed to investigate SUD. Within this realm, IV self-administration is a model of behavior that has been praised for allowing rodents to have a choice over the amount of intake of a specific substance through use of an operant chamber.[133-135] The protocol for IV self-administration of substances begins with training on how to navigate an operant chamber, and rodents learn to associate the pressing of a lever with the delivery of a reward (e.g. sucrose pellets). After that, an IV catheter is placed in the jugular or femoral vein. Following recovery, the subject is placed into the operant chamber and allowed to intake the substance for a given amount of time each day.[133-136,447-452] This model has led to discoveries about molecular and cellular responses that reinforce the effects of various substances at different stages of

use.[136,447-452] Conditioned place preference (CPP) is another model that investigates drug seeking behavior through examining preference to an area paired with administration of a substance, which can induce preference or avoidance depending on the stimuli.[136,137] A two chamber apparatus is utilized that has a removable barrier between two distinct environments. On training days, the subject is administered an unconditioned stimulus and placed into a conditioned environment or administered an inert substance (e.g., saline) and placed in the opposite chamber. On test day, the subject is placed into the apparatus and the barrier between chambers is removed.[453-455] More time spent on the substance paired side indicates the development of place preference, while aversion is the avoidance of the substance paired side.[136,137,456-458] CPP allows for investigation of Pavlovian responses, and offers an avenue for examining the relationship between drug seeking behavior and the surrounding environment.[459] However, many criticize these models as too reductionist, removing the complexity of the condition seen in humans and possibly overlooking key factors of SUD,[111] though changes to the protocol can help to include more complexity. Both models are also highly influenced by the environment around them,[133-137] and the difficulty of maintaining catheters increases the complexity of IV self-administration.[133-135]

Methods for Investigating Neuroinflammation

The neuroinflammatory response to TBI is complex and involves multiple cell types. When examining the response, it is important to understand what cells are involved, whether the response is proinflammatory or anti-inflammatory, where the response is localized to, and what the function of that response is. Highlighted here are a few methods that provide insight into these areas.

Flow cytometry is a powerful tool used for the phenotyping of single cells in solution.[141,142] Samples for flow cytometry are run through a column that allows single cells to pass by a laser light source so they can be analyzed for their size and granularity by forward and side scatter of the laser. The cells can be further analyzed for fluorescent markers that can be expressed by the cell, a dye placed on the cell, or attached to fluorescently conjugated antibodies.[141,142,460-462] The use of fluorescently labeled antibodies is helpful for cellular

identification and analysis of markers both on the surface and intracellularly.[460] When used for evaluation of the inflammatory response, cells can be analyzed for proinflammatory and anti-inflammatory phenotypes, providing much needed insights.[461,462] However, the need to homogenize tissues for single cell analysis destroys the structure, and this method needs to be paired with one that can localize the location of the cell within a given tissue.

Immunohistochemistry (IHC) is a method of tissue analysis that utilizes antigen-antigen binding to localize antigens within a tissue sample.[138-140] The results are mostly qualitative, with fluorescence observed and imaged usually with light microscopy, though the results can be semi-quantitative through examining fluorescent intensities.[139,140,463] This method allows for the convenient use of fixed tissue for analysis.[140] Antibody selection can depend on the sensitivity and specificity necessary for a given antigen, as well as cost concerns. Further complications arise when labeling with more than one antibody, where cross reactivity in the agents and concerns about species the antibody was produced in can complicate analysis.[139] Issues also can arise with labeling if the antigen is interacting with other substances in the tissue, or otherwise inaccessible to the antibody, skewing the staining results and reduced labeling of the target.[139] IHC provides much needed spatial information about the location of responses in tissue, and pairs nicely with more quantitative measures.

Real time reverse transcription polymerase chain reaction (RT-PCR) is a method utilized to measure levels of mRNA in a given sample.[143] During each cycle of a PCR reaction, rt-PCR utilizes a reporter gene to monitor the amplification of products.[143] Benefits include high sensitivity with sequence specific analysis.[464] Normalization of the level of mRNA within samples is key to this method, and can be done by sample size, total RNA or by measuring a house keeping gene, though validation of the chosen method is important for accuracy.[465] Importantly, changes in mRNA levels do not automatically signify translation to protein, so further investigation of the function of the changes observed is necessary. Also, like flow cytometry, these values do not identify an area that the mRNA is located at, so localization is needed. This method offers the potential for high throughput and

analysis of multiple products in a sample,[143,464,465] which can help provide information on the inflammatory milieu.

Functional studies are key to understanding the underlying mechanisms of neuroinflammation following TBI. Of interest is the function of macrophage cells, who are well known for their phagocytic abilities, which is essential to homeostasis and in response to injury or disease.[144] While changes can occur to activation markers of macrophages taken from *in vivo* and cultured *in vitro*, there are studies validating similar phagocytic activities both *in vivo* and *in vitro*.[466,467] Benefits of utilizing the *in vitro* model include isolation of the cells and examination of how phagocytosis is affected by different substances.[468,469] One such model involves the use of streptavidin-coated microspheres, which facilitates the investigation of multiple ligands for phagocytosis,[468,469] rather than focusing on limited targets or receptors.[470,471] Utilizing this method, phagocytosis can be examined in relation to different substrates within different organ systems, allowing a more detailed examination of macrophage function.

While not an exhaustive list of analysis for the neuroimmune response, these methods highlight a several ways of evaluating the response to injury or disease. No one assay can evaluate all the parameters associated with the immune system. However, combining multiple measures can assist in completing a more comprehensive analysis of the factors involved in the response.

Sex differences in Animal Models of TBI

In the human literature, TBI has largely been connected to males since they engage in risk taking behavior and have higher rates of diagnosed TBI.[22,210] However, the rate of TBI in females may be underestimated, since they are prone to injuries are incurred in certain situations under-investigation, e.g. domestic violence.[15,167] The lack of research utilizing female animal models in the study of TBI highlights a glaring gap in understanding how gender differences can affect injury progression, symptom development, and ultimately prognosis and treatment.[15,161] Initial studies utilizing female rodents have demonstrated important differences in the TBI outcomes and treatments.[472,473] Studies in the literature

have already demonstrated differences in neurodegeneration and the response to enriched environments post injury in female rodents.[474-477] Evidence in the literature suggests that females may do better following injury, but more data needs to be gathered to determine how hormonal cycling plays a role and which hormones in particular participate in the response.[478-481] Though the conclusions from each study are highly debated, it is clear that more research is critically needed to close the gap in understanding how sex affects the outcomes of TBI.

TBI induced neuroinflammation increases risk for substance use disorder

A history of TBI raises the risk of development of a SUD by 3-6 times that of the general population.[132] Adolescent substance use disorder was named a top public health concern according to the National Center on Addiction and Substance Use. In fact, the earlier adolescents begin experimentation, the more likely they are to suffer from a long-term and chronic SUD.[482] This makes screening for SUD highly important in primary care, but many doctors lack the proper tools to do so.[483] Substance use disorder is a condition encompassing the use and withdrawal of all substances.[484] The treatment of SUD is often complicated by compounding diagnoses, including major depressive disorder and PTSD, while more consideration also needs to include the violence that often accompanies and contributes to SUD.[485,486] However, many do not seek treatment at all, especially if the substance they are using is legal or semi-legal (e.g. alcohol and marijuana).[487] There is significant hope however in the future with neuroscientists examining the pathways and neurological changes that accompany SUD, and identifying new targets for treatment.[488]

As previously stated, the link between alcohol use and TBI is complex, but animal modeling has allowed us to gain insights into how TBI affects alcohol consumption. In a study observing the acute effects TBI, injured mice actually consumed less alcohol and seemed more sensitive to its effects, which coincides with epidemiologic evidence.[145,146] This correlated with increased astrocyte response at the site of injury and in the *nucleus accumbens*.[146] In other studies where rats were utilized, those rats who consumed more alcohol prior to injury had more drastic increases in alcohol consumption post injury than those who consumed limited amounts.[149,150] While these studies focused on adult rodents,

another study investigated younger animals, revealing that injured female juvenile mice are more apt to increase intake and are sensitive to the rewarding effects of intraperitoneal alcohol, where sham-injured and male mice did not demonstrate these effects.[147] While these studies demonstrate the inherent variability in alcohol consumption models among rodents, they can still offer key insights into this paradigm. In one study, injured adolescent male mice increased their intake of alcohol, but administration of minocycline can return to consumption levels seen in sham-injured animals while also decreasing microglial activation in the *nucleus accumbens*.[148] These models demonstrate a relationship between alcohol intake and TBI, though more research needs to be done to understand the underlying mechanisms.

There are also connections between the inflammatory response to TBI and an increased sensitivity to and consumption of cocaine. In one study, moderate CCI TBI was given to adolescent mice and their preference to cocaine was examined in adulthood utilizing CPP. The injured mice demonstrated increased preference to cocaine with persistent neuroinflammation in the *cortex*, *nucleus accumbens*, and *ventral tegmental area* as evidenced by increased astrocyte and microglial markers and upregulation of inflammatory genes.[151] In female mice, this response was reduced when estrogen and progesterone were at high levels, and that correlated with reduced microglial activation.[152] Another study demonstrated that dexamethasone treatment could attenuate the increase in CPP after TBI to cocaine while also reducing the expression of immune response genes involved in monocyte recruitment. Furthermore, dexamethasone treatment decreased the amount of nonclassical patrolling monocytes peripherally, which the authors suggest reduces the amount available to invade the brain post injury and have a role in chronic inflammation in the reward pathway.[153] Self-administration models demonstrate an increase in cocaine intake following mild and severe TBI with neuroinflammatory proteins correlating with likelihood of drug-taking.[154] Each of these studies demonstrates a clear connection between increased drug seeking behavior in relation to cocaine after TBI that is correlated to alternations in the inflammatory response.

Opiate Use, TBI, and Neuroinflammation

The rise of the opiate epidemic has highlighted the critical need for intervention and treatment. The amount of individuals using opiates as their drug of choice has increased steadily since 2000.[159,160] There has also been a significant rise in SUD among 12-18 year olds.[190] US veterans have shown higher likelihood of using opiates, which is exacerbating when used in conjunction with other substances.[489] Patients seeking treatment are often subjected to opiate detoxification strategies, which can be dangerous due to opiate withdrawals, and thus medication with buprenorphine and methadone is highly recommended.[490] Opiate overdose is common and of extreme concern, and though prescriptions have been increasing for naloxone, there are still missed opportunities to get this life saving drug to people who can implement use during an overdose.[491] The increased risk of developing an opiate use disorder after TBI seems to well supported by epidemiological evidence,[492] but there is a lack of research into understanding the mechanisms behind this risk.

Opiates are immunosuppressive in general and developing an opiate use disorder can increase the likelihood of infection.[155-158] Opiates, like morphine, affect both the innate and adaptive immune system, manipulating the function of cells. In the innate immune system, morphine acts directly on both macrophages and neutrophils. Exposure to morphine increases apoptosis in macrophages and decreases phagocytosis in macrophages and neutrophils.[155,493-495] Recruitment effects seems to be more complex; morphine delays the recruitment of phagocytic cells,[155] but one study in the CNS demonstrated increased recruitment of macrophages at the site of spinal injury.[496] This may be a difference in morphine effects in different tissues,[497] but more research is required. The adaptive immune system is also suppressed by morphine, impairing the functionality of lymphocytes.[155,158] Lymphocyte cytotoxicity can be greatly suppressed by morphine, especially in natural killer (NK) cells, and the expression of opiate receptors on the lymphocyte cell surface can be upregulated.[498-501] While not an exhaustive list, the evidence listed demonstrates the vast consequences of opiate exposure on the immune

system, and highlights the potential of opiates to alter the chronic inflammatory response to TBI.

Conclusion

Millions of people suffer from TBI every year, with adolescents and young adults demonstrating vulnerability with increased incidence of injury and long-term consequences that are associated with risk-taking activities. Soldiers and athletes are at increased risk for TBI, with multiple mild injuries occurring commonly. Sex differences related to injury incidence are more obvious within the young population and ameliorated as patient age increases. Further differences are apparent in injury induction, severity, and recovery, which may be related to lifestyle choices or physiologic and anatomical differences.

TBI is a chronic and lifelong challenge that alters behavioral and physiologic aspects of those affected. Early symptoms can help to inform prognoses; however, it is still impossible to accurately identify those that will benefit from recovery therapies and those that will not develop long-term consequences. Diagnosis depends on multiple indices, from patient reported symptoms to multiple imaging modalities. Objective evidence of injury and accurate classification of injury, especially mild TBI, is still inaccurate and elusive. Diagnosis and management techniques for injury and subsequent conditions is desperately needed, especially since new developments in treatment will not help patients whose diagnoses are not accurate.

The immune response within the brain is unique since ingress and egress of cells and substances is tightly controlled. The interplay between the immune cells of the CNS and peripheral immune systems is altered and cellular infiltrate is increased with the severity of injury. More is understood about the acute aspects of this response, while chronic changes remain elusive. The cellular infiltrate can also interact directly with neurons and alter signaling in neuronal synapses. These interactions can cause wide scale neuronal remodeling, which can alter cognitive processes, motor control of the body, and perception

or stimuli processing. This makes the immune system a prime target for linking behavioral and neuronal changes.

Animal models are used to examine neurologic changes, including the immune response to TBI. There are multiple methods of injury induction that focus the profile of injury; some models demonstrate discrete reproducible injury, while others are aimed at accurately modeling the diffuse and heterogeneous injuries that are seen in clinical populations. Rats and mice are commonly used to study TBI, though everything from flies to swine can be used to recapitulate each aspect of clinical signs. Small animal practice may offer opportunities for clinical studies in veterinary medicine, which could help bridge the gap between pre-clinical research and clinics. Behavioral tests within these species can target specific sites in the brain, determining alterations at the injury site and other areas distal to the physical injury point. Combining these measures with postmortem analysis of neuroinflammation can help reveal the mechanisms that link TBI and long-term deficits. Drug seeking behavioral tests can help identify what changes TBI causes in relation to different aspects of substance exposure. Multiple methods of administration can be used to target specific clinical paradigms, along with helping to understand how different paradigms affect the development of drug seeking behavior.

A history of TBI increases the risk of substance use disorder by up to 6x when compared to the general public. The relationship of immune system to alcohol and cocaine use has already been investigated, while that of opiate use still needs further investigation due to its unique mechanisms of interaction within the brain. The rise of the opiate epidemic has made this investigation particularly relevant, as treatments may help lower the risk of development of SUD. With the immunosuppressive effects of morphine highly documented, the immune system is a prime candidate to connect TBI and opiate use disorder.

All this information considered, the present study was developed to fill the knowledge gaps exposed here. I **hypothesize** that the proinflammatory macrophage response to mild TBI accelerates the remodeling of neuronal synapses in the mesolimbic pathway leading to an increased sensitivity to and consumption of opiates (Fig. 1.1).

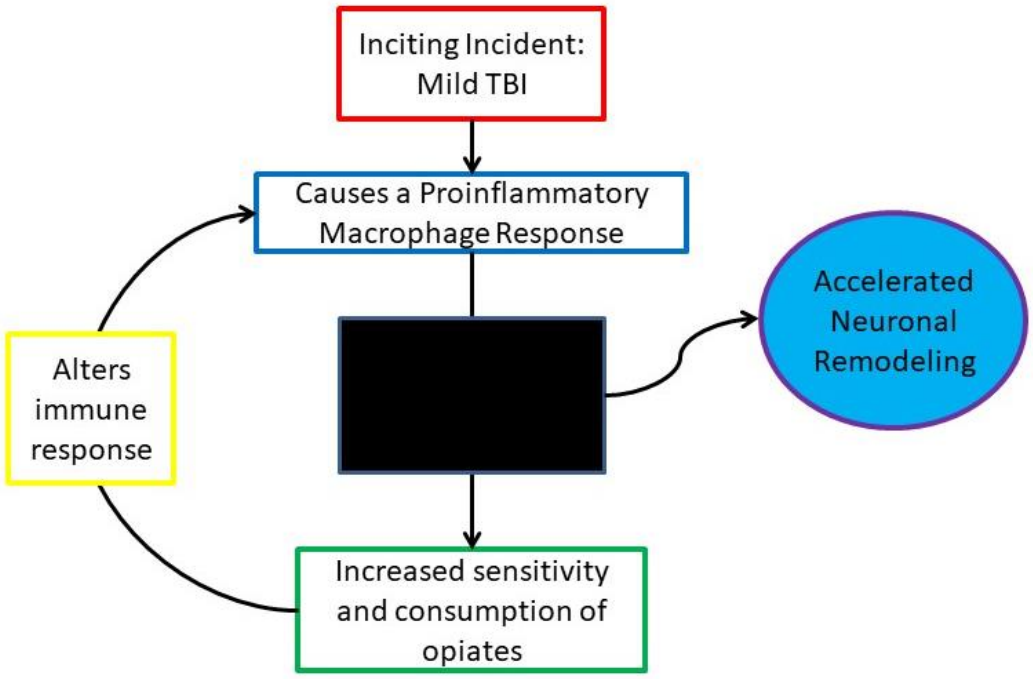

Fig 1.1 Visual representation of the hypothesis. There is an inciting incident that causes a mild traumatic brain injury (TBI) with a proinflammatory macrophage infiltration. After injury, there is an increased sensitivity to and consumption of opiates. That exposure to opiates further alters the immune response, which in turn alters the response to opiate exposure. The mechanism behind this response is unknown, but we hypothesize that the increased proinflammatory response accelerates neuronal remodeling in the reward pathway.

Chapter 2

Neuroinflammatory response to mild traumatic brain injury is associated with long-term behavioral deficits

Abstract

Traumatic brain injury (TBI) affects millions of people every year, and 60-90% of those injuries are considered mild in nature. Though difficult to determine the amount of people affected due to lack of reporting and lack of a definitive test, mild TBI is a serious condition that increases the risk of developing Parkinson's disease, Alzheimer's disease, and depression, potentially through a chronic neuroinflammatory response. Therefore, temporal changes in immune cell phenotypes were characterized after mice were given a mild TBI by controlled cortical impact. Injured mice had infiltrating neutrophils that peaked at 1 day post injury (DPI), and increased macrophages peaking at 3-DPI that were isolated to the ipsilateral side of injury. Though these levels returned to levels seen in sham mice by 7-DPI, proinflammatory markers remained on macrophages that were in the brain on the ipsilateral side of injury. These findings coincided with behavioral deficits. On beam walk, the injured mice had increased foot slips and time to cross the beams at 3-DPI. Though that resolved by 7-DPI, there was a long-term spatial learning deficit observed in injured mice at 30-DPI by Barnes Maze. Further evaluation demonstrated changes in selected cytokines after injury. rtPCR revealed an increase in the level of mRNA associated with TNF-α, TGF-β, INF-ɣ, ARG1 and IL-10 in on the ipsilateral side of the brain of injured mice. At 15-DPI, the level of mRNA for IL1-β and CXCL10 was increased, demonstrating a changing response over time. Immunohistochemistry was also utilized to demonstrate the presence of inflammatory cells at the site of injury the extended into the *hippocampus*. This model can be used to investigate long-term consequences associated with mild TBI. Future studies will investigate the function of inflammatory cells found after injury, and their effect on synaptic activity and function.

Introduction

Traumatic brain injury (TBI) cases have risen by 53% since 2006,[502] with approximately 2.87 million injuries reported in 2014,[17] and a dramatic rise to 61,000 TBI-related deaths in 2019.[18] However, 70-90% of all injuries in the US are mild, and often go unreported or unrecognized by the individual.[11,502,503] Therefore, the number of individuals suffering the effects of TBI may be vastly underestimated.[504] Commonly, these injuries come from falls, motor vehicle accidents, and sports injuries,[3,505] causing disorientation and neurologic deficits.[18,506] Though these injuries are usually classified by severity ranging from mild to severe,[18,506] the injuries themselves are heterogeneous due to the location of impact and brain areas affected.[505,506]

Mild TBI is defined by a transient altered mental state resulting from a low force trauma.[18,506] Common symptoms of mild TBI include headaches, anxiety, and difficulty sleeping,[18,506-508] which can resolve in a few days. However, a subset of 10-50% of patients will develop post-concussive syndrome,[505,508] a chronic condition that can persist months to years after initial injury, with 5-15% developing disabling conditions.[12,503,504,508,509] Mechanisms that link mild TBI and long-term consequences are unknown due to limited evidence of pathology by conventional imaging tools.[510,511] Albeit, epidemiologic evidence illustrates increased risk of long-term neurologic consequences after TBI.

TBI is a biphasic phenomenon; primary physical injury causes structural changes, like tissue deformation and axonal shearing, followed by secondary injury from the immune system exacerbating damage.[132,512,513] Advanced imaging of human patients demonstrate that the effect of the initial trauma can extend beyond the initial injury site to subcortical areas.[14,231,514] Diffuse axonal damage observed in magnetic resonance imaging of the *thalamus* correlates with deficits in executive function and memory.[514] Persistent damage in the *thalamus* and *ventral tegmental area* following mild TBI in patients is associated with cognitive deficits.[231,515] The evidence suggests that mild TBI damages distal areas of the brain, leading to deficits in attention, memory, and apathy.[14,231,514,515] This lends credence to the hypothesis that mild TBI results in long-term deficits through the secondary inflammatory response.

Mild TBI has also been linked to behavioral and physical deficits in rodent models, demonstrating increased stress and decreased cooridination.[419,420] Many types of animal models used in TBI research, including closed head and blast induced injury, which have a high degree of variability in the injury leading to more diffuse damage.[95] This variation has left a gap in understanding how secondary inflammatory damage spreads after primary injury. The controlled cortical impact (CCI) was chosen to induce injury at a focal point to develop a consistent injury profile.[95,105] The majority of studies utilizing CCI models emphasize acute consequences while also varying impact intensity and outcome measures, making comparisons across studies difficult.[105,366,375-378,516] The objective of this study is to develop a thorough understanding of the inflammatory response to a defined mild TBI and assess deficits that follow. In doing so, the severity of the injury is defined in tangible terms to examine the mechanisms underlying the injury and subsequent deficits. I hypothesize that the acute proinflammatory response to mild TBI will extend distally from the site of initial injury to affect deeper brain structures resulting in chronic inflammation associated with long-term behavioral outcomes.

Methods

Animal Use

All procedures and experiments involving animals were conducted in accordance with protocols approved by the University of Minnesota Institutional Animal Care and Use Committee. Female mice at 8-12 weeks of age were purchased from Jackson Labs and housed in environmental controlled micro-isolator cages with Enviro-dry environmental enrichment. The existing literature overwhelmingly utilizes male mice for studies involving TBI; therefore, female mice were utilized to close the gap and provide evidence for the temporally dynamic response to injury.[15,161] Animals were acclimatized to their new environment for at least one week prior to obtaining baseline data for behavioral studies.

Surgical Induction of Traumatic Brain Injury

Mild TBI was surgically induced in mice using an electromagnetic stereotaxic impactor (Impact One Stereotaxic Impactor for CCI, Leica Microsystems Inc). An extended-release

formula of buprenorphine at 2.0 mg/kg was given subcutaneously; animals were anesthetized with isoflurane and placed into a stereotaxic apparatus to secure the head. A midline skin incision was made at the dorsal cranium to expose bregma on the skull. The impact site was positioned over the primary and secondary motor cortices on the right parietal area. A 2.5mm diameter craniotomy was made with a steel drill bit (Dremel, Racine WI) to accommodate a 2mm impact tip. Mild TBI was induced with a velocity of 4m/s, depth of 1mm, and dwell time of 100ms. Sham-injured mice underwent the same procedure with craniotomy but without impact. Incision site was closed, and mice were recovered in a clean home cage on a heating pad. Once fully ambulatory, mice were closely monitored for 3 days post injury (DPI). Mice were placed into groups based on equalizing baseline behavior in fear conditioning due to natural variation.

Behavioral Assays

All behavior assays were performed at the University of Minnesota Mouse Behavior Core facilities. Mice were trained to negotiate the beam walk assay 1 week after arrival. Spontaneous alternation Y maze, context-based fear conditioning, and beam walk baseline assays were obtained 1-3 days prior to TBI, then subsequently at 2-, 6-, 14-, and 29-DPI, while the Barnes maze assay was performed at 25-29-DPI.

Beam Walk

Mice were assessed for motor deficits utilizing the beam walk test as previously described.[421] Briefly, six beams were utilized: a large square beam (25mm square), a large cylindrical beam (25mm diameter), a medium square beam (16mm square), a medium cylindrical beam (19mm diameter), a small square beam (10mm square), and a small cylindrical beam (10mm diameter). Training was performed on the medium square beam for 3 days prior to TBI. Mice were tested for 2 trial runs on each beam. Reported results are the average of trials per beam.

Context-based Fear Conditioning

Med Associates Inc software and chambers were used to assess memory retention in mice with Context-based Fear Conditioning, as previously described.[123] Mice were placed into the fear conditioning apparatus for a 10-minute conditioning program, consisting of an acclimation period followed by 5 shocks of 0.7mA at irregular intervals. This paradigm induces freezing behavior, a fear response where the animal ceases movement momentarily. Baseline context association to electric shocks was examined by utilizing two contexts within the chamber. Freezing time was recorded over 3 min in Context 1, which is the same context they received shocks, with metal siding and flooring with simple green cleaner applied for scent. Memory specificity was examined with exposure to Context 2, which was same chamber, but the scent was changed to vanilla and the metal siding and flooring was replaced with plastic walls and floor.

Spontaneous Alternation Y-Maze

Spontaneous alternation Y-maze has been previously described to examine short-term memory.[120] Briefly, the mouse was placed in the Y-maze for 5 min while the ANY-maze behavioral tracking system (Stoelting Company, Wood Dale, IL) recorded movements. A 'correct' choice in the maze requires the mouse to enter each arm sequentially, and an early return to a previously explored arm without visiting both other arms constitutes an 'incorrect' response.

Barnes Maze

Long-term cognitive deficits were examined with the Barnes maze assay, as described previously.[124] Mice started training 25-DPI on a circular table with circular holes around the periphery of the table. The escape hole was placed at a specific location, which remains constant throughout. Visual cues were provided on the walls as spatial references. Four training days consisted of mice being placed in the middle of the maze with activity recorded with ANY-maze behavioral tracking system (Stoelting Company, Wood Dale, IL). After 3 min of free exploration, the mouse was led to the escape hole if it had not reached it. Each animal was trained with 4 trials per day. On

the 5th day, the mice perform a probe test; the escape hole was removed, and exploration is recorded for a single 90 second trial.

Flow Cytometry

Mice were sacrificed and perfused with cold phosphate buffered saline (PBS; Genesee Scientific). Brains were collected, separated by hemisphere, and homogenized to a single cell suspension in 30% Percoll solution (Sigma-Aldrich) in RPMI media. Gradient was created by gently layering 1.5mL of 70% Percoll solution into the bottom of the 30% Percoll/media solution. This mixture was centrifuged for 30min at 2300 rpm. The cellular interface was then removed, washed with PBS +2% fetal bovine serum (FBS; Sigma-Aldrich), and transferred to a 96-well plate. Cells were incubated with Fc block for 5min, then cell surface antibodies added for a 15min incubation. The cells were then washed with PBS +2% FBS 3 times and fixed with paraformaldehyde (PFA, Millipore Sigma). The cells were again washed with PBS +2% FBS 3 times. Cells were resuspended in FACS buffer with AccuCount beads (Spherotech, Lake Forest, IL, USA) for analysis on a flow cytometer (BD FACSCanto) at University of Minnesota Flow Cytometry Core. Two panels of stains were used to identify immune cells of myeloid and lymphoid lineage (See Table 2.1). Isotype specific antibodies were used for each panel combination to control for nonspecific antibody binding. Immunostained cell populations were analyzed using FlowJo software (TreeStar, Ashland, OR USA). Cell numbers were calculated as the number of cells of interest per one million live cells.

Table 2.1 Flow Cytometry Stains

Macrophage Panel	**Lymphocyte Panel**	**Fluorochrome**	**Manufacturer**
CD45	CD45	PerCP-5.5	eBioscience/Invitrogen
CD11b	CD11b	APC-eFluor 780	eBioscience/Invitrogen
CD86	CD8	FITC	eBioscience/Invitrogen
CD206		Alexa-Fluor647	Biolegend
MHC II	CD19	PE	eBioscience/Invitrogen
Ly6C	CD3	eFluor 450	Biolegend (Ly6C) eBiosciences (CD3)
Ly6G	CD4	PE-Cy7	eBioscience/Invitrogen
	NK 1.1	Brilliant Violet 650	Biolegend

Real time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from brain homogenate using Aurum Total RNA Fatty and fibrous tissue kit (Bio-Rad) per manufacturer's instruction. The purity of RNA was assessed by calculating the ratio of OD260/OD280. RNA was reverse transcribed using high-capacity cDNA reverse transcription kit (Applied Biosystems). The cDNA was amplified by RT-qPCR using Fast SYBR Green master mix (Applied Biosystems) in Quant Studio Real-time PCR system (Applied Biosystems). The specificity of qPCR was assessed by analyzing the melting curves of PCR products. Cycle threshold (Ct), referring to the number of cycles for a sample to go through before reaching a threshold for a positive result, were normalized to ribosomal protein L27 gene, a constitutively expressed gene that has stable expression across tissues,[517] to obtain ΔCt. The difference between the ΔCt values of the TBI sample the sham sample was calculated as ΔΔCt. The fold change was expressed as $2^{-\Delta\Delta Ct}$ as previously described.[518] See Table 2.2 for primer sequences.

Table 2.2 PCR Sequence Information

mRNA Target	**Sequence**
RPL27	Forward - 5' GCA AAG CTG TCA TCG TGA AGA A-3' Reverse - 5' CTT GTG GGC ATT AGG TGA TTG T-3'
CCL2	Forward- 5' TGG CTC AGC CAG ATG CAG T -3' Reverse- 5' TTG GGA TCA TCT TGC TGG TG -3'
CCR2	Forward- 5' TGC AAG TTC AGC TGC CTG CAA A-3' Reverse- 5' TCA TAC GGT GTG GCC CCT T-3'
TNF-α	Forward - 5' CAT CTT CTC AAA ATT CGA GTG ACA A -3' Reverse - 5' TGG GAG TAG ACA AGG TAC AAC CC -3'
IL 1β	Forward - 5' TTG ACG GAC CCC AAA AGA -3' Reverse - 5' AGA AGG TGC TCA TGT CCT CA-3'
CXCL10	Forward- 5' CAG CAC CAT GAA CCC AAG TGC-3' Reverse- 5' GGT CTT CTG AAA GGT GAC CAG C-3'
iNOS	Forward- 5'CCC TTC CGA AGT TTC TGG CAG C -3' Reverse- 5' GGC TGT CAG AGC CTC GTG GCT TTG G -3'
INFɣ	Forward- 5' TCA AGT GGC ATA GAT GTG GAA GAA-3' Reverse- 5' TGG CTC TGC AGG ATT TTC ATG-3'
IL6	Forward- 5' AGA TAA CAA GAA AGA CAA AGC CAG AG-3' Reverse- 5' GCA TTG GAA ATT GGG GTA GGA AG-3'
IL10	Forward- 5' TGC ACT ACC AAA GCC ACA AGG -3' Reverse- 5' TGG GAA GTG GGT GCA GTT ATT G -3'
TGF-β	Forward- 5' TGA CGT CAC TGG AGT TGT ACG G-3' Reverse- 5' GGT TCA TGT CAT GGA TGG TGC-3'
IL4	Forward- 5' ACG AGG TCA CAG GAG AAG GGA-3' Reverse- 5' AGC CCT ACA GAC GAG CTC ACT C-3
Arg1	Forward- 5' ACA AGA CAG GGC TCC TTT CAG G -3' Reverse- 5' GCA GAA GGC GTT TGC TTA GTT CTG-3'

Tissue processing, immunohistochemistry (IHC), and image analysis

Following perfusion by cold PBS (Genesee Scientific) and 4% PFA (Millipore Sigma), brains were removed and submerged in PFA overnight at 4°C for post-fixation. Following 3 rinses in PBS, brains were submerged overnight at 4°C in increasing concentrations of sucrose (10%, 20%, and 30%) dissolved in PBS. When brains equilibrated in 30% sucrose solution, they were rinsed in PBS and embedded in cryosectioning molds with Tissue-Tek OCT (Andwin Scientific, IL and NC) on dry ice. Frozen blocks were then transferred to -80°C until sectioning. Embedded brains were sectioned on a cryostat (-20°C; Leica) in six semi-serial sets in the coronal plane at a thickness of 25 µm (approximately 180 µm between sections) and mounted onto positively charged slides (Superfrost Plus, Fisherbrand). Sections were stained with antibodies for 1) astrocytes (GFAP), neurons (NeuN), and microglia/macrophages (Iba1), and DAPI to visualize nuclei (see Table 2.3 for details). For fluorescent IHC, frozen tissue was mounted onto slides were post-fixed with 4% PFA, rinsed with PBS containing 0.1% Triton X-100 (0.1% PBST), incubated in a 10% blocking solution (10% normal donkey serum in 0.1% PBST) for 60 min at room temperature. Slides were then incubated in a primary antibody solution diluted in with 1% blocking solution overnight at 4°C. Sections were washed in 0.1% PBST, incubated a secondary antibody solution containing DAPI and diluted in 1% blocking solution for 60 min at room temperature. Slides were then rinsed in 0.1% PBST and cover slipped with mounting medium (ProLong Glass Antifade Mountant, Thermo Fisher). Images were captured using Leica DMi8 inverted microscope and Leica LAS software. Representative images were collected at the epicenter of the impact, ipsilateral *hippocampus*, *thalamus*, and ventral *midbrain* area and processed using ImageJ Fiji software (version 1.53q).

Table 2.3 Immunohistochemistry Reagents

Antigen	**1º Antibody**	**Vendor**	**Catalog No.**	**2º Antibody**	**Vendor**	**Catalog No.**
Macrophage/ Microglia	Iba1	Wako	019-19741	Donkey Anti Rabbit (IgG) Alexa Fluor 555	Abcam	ab150074
Reactive Astrocytes	GFAP	Lifespan Biosciences	LS-C7076 GFAP	Donkey Anti Rabbit (IgG) DyLight 488	Novus	NBP1-75278
Neurons	NeuN	Abcam	ab104224	Donkey Anti Mouse (IgG)	Abcam	ab150107

				Alexa Fluor® 647		
Nuclei	DAPI	Thermo Scientific	62248	N/A	N/A	N/A

Statistical analysis

All behavioral, flow cytometry, and RT-PCR assays were analyzed using GraphPad Prism (San Diego, CA). Analysis included two-way ANOVA and post-hoc unpaired t-tests. P-values <0.05 were considered significant.

Results

The inflammatory response to mild TBI and behavioral endpoints were assessed up to 30-DPI (Fig. 2.1A). At 6 and 24h post injury, inflammation was evaluated using flow cytometry and quantitative RT-PCR. Behavioral deficits were assessed beginning at 2-DPI, since changes observed at 6 and 24h could be influenced by surgery and anesthetic effects of isoflurane. Gross characteristics of brains subject to mild TBI were visibly different compared to sham (Fig. 2.1B). Impact on the brain did not result in visible cavitation at any time point tested.

Surgical recovery time associated with mild TBI was significantly longer in injured than in sham mice.

The differences between injured and sham-injured animals were demonstrated by a significant increase in recovery time (Fig. 2.1D) from surgery, characterized by time to sternal recumbency and return to an alert state. Injured animals took an average of 15.7 (± 0.8) min to recover, while time to recovery was significantly lower in sham-injured mice at 13.4 (± 0.4) min (*paired t-test, $p=0.002$*). The TBI procedure took longer to perform than the sham surgery (Fig. 2.1C). A simple linear regression analysis of surgical time vs. recovery time (Fig. 1E) revealed a positive correlation between the two measures in the sham group (slope= 0.87), while these parameters were poorly related in the mild TBI group (slope=0.16, *$p<0.0001$*). The surgery and injury had no effect on the overall health of the animal, and body weight post injury (Fig. 2.1F).

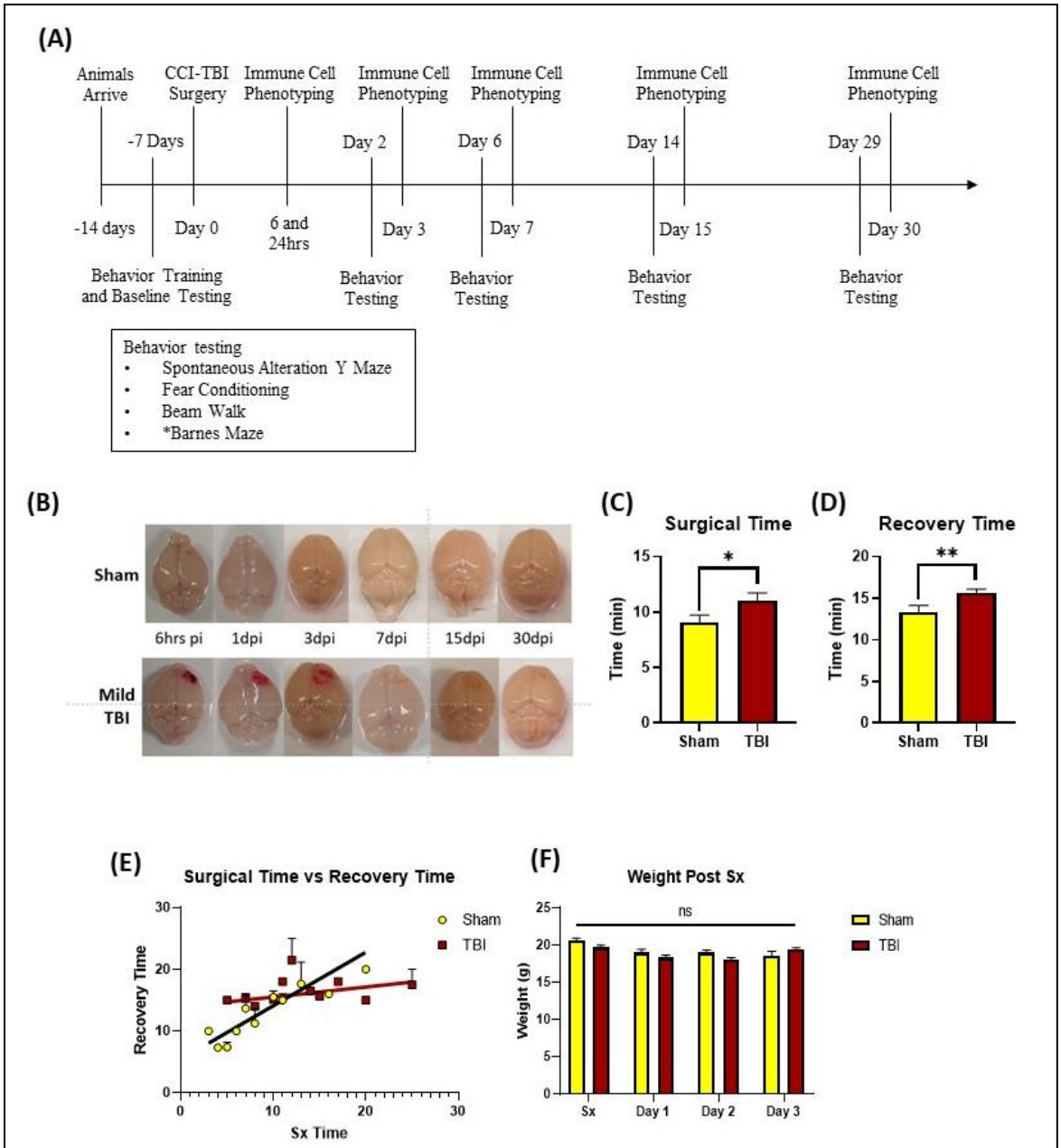

Fig 2.1 Mice receiving mild traumatic brain injury had significantly longer recovery time. (A) Timeline for the experimental paradigm through 30 days post injury (DPI) with (B) examples of sham and injured brains at each sacrifice point. Mice were subjected to controlled cortical impact brain injury under isoflurane. Surgical time (C) was recorded as time from first incision to suture closure and recovery from surgery (D) was the time from removal of anesthesia to the time the mouse could right itself and move around the cage (n=39). Simple linear regression (E) plotted the relationship between surgical time and recovery time. Weight post-surgery (Sx) was measured on the day of surgery and continued through 3-DPI (F). Significance calculated utilizing t-tests and standard linear regression on Prism software.

Following mild TBI, mice had minor motor deficits and memory deficits at two days post injury.

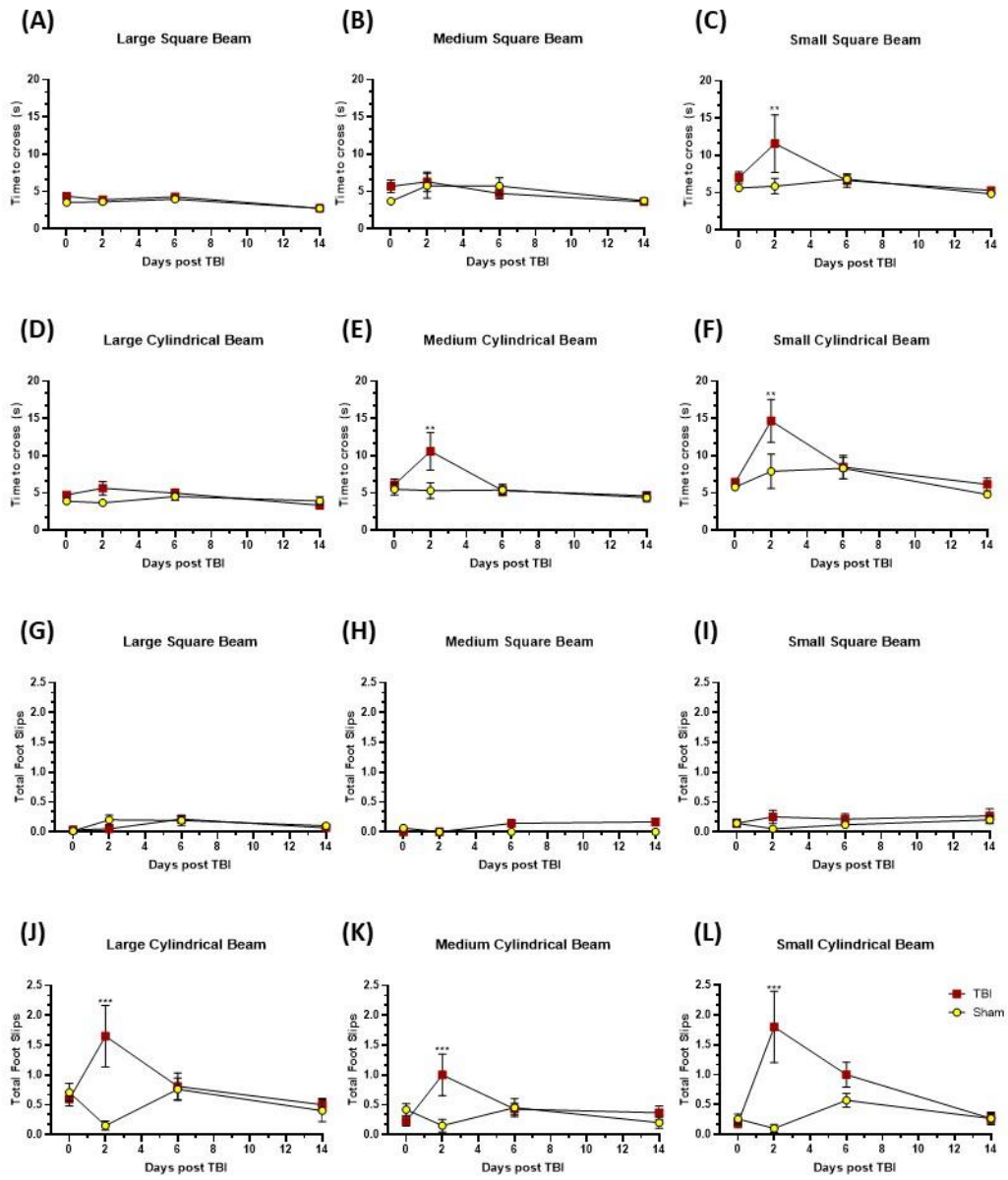

Fig 2.2 Mild traumatic brain injury induces short-term motor deficits. Mice were trained for beam walk 1 week before TBI and were tested for baseline values with 2 trials on each beam tested. After TBI, the mice completed 2 trial runs on each beam at 3-, 6-, 14-, and 29-DPI. Mice were timed crossing the square (A-C) and cylindrical beam (D-F) from time to cross the start line to the finish line. The number of foot slips were also recorded on the square (G-H) and cylindrical beams (J-L). Statistical values presented are the result of multiple unpaired T-tests and were calculated utilizing GraphPad Prism software.

Beam walk was utilized to evaluate fine motor deficits. Injured mice required significantly increased time to cross the beam as the difficulty for crossing increased, either by size (larger vs. smaller) or by shape (square vs. cylindrical) (Fig. 2.2A-F). The mean time to cross significantly increased at 2-DPI in injured mice compared to sham-injured on the medium cylindrical (Fig. 2.2E), small square (Fig 2.2C), and small cylindrical (Fig 2.2F) beams with 10.6 (±2.5) sec in injured mice vs. 5.3 (±1.1) sec in sham mice; 11.6 (±3.9) sec vs. 5.9 (±1.0) sec, and 14.7 (±2.9) sec vs. 5.8 (±2.3) sec respectively (*multiple unpaired t-test, p=0.002, 0.003, 0.003 respectively*). Sham-injured mice had no difference in time to cross from their baseline, while the injured mice doubled their time to cross. This motor deficit was further exhibited as an increased number of foot slips on cylindrical beams (Fig 2.2G-L), which require more precise foot placement. On the large cylindrical beam (Fig 2.2J), average foot slips increased to an average of 1.7 (±0.5) foot slips for injured mice from 0.2 (±0.1) in the sham-injured (*multiple unpaired t-test, p=0.00006*). Similarly on the medium cylindrical beam (Fig 2.2K), foot slips increased to 1.0 (±0.4) in injured mice from 0.2 (±0.1); and on the small beam (Fig 2.2L) to 1.8 (±0.6) from 0.1 (±0.1) (*multiple unpaired t-test, p=0.00006, <0.000001 respectively*). Though the performance of sham and injured mice were significantly different, mice did not fall off the beam walk apparatus in either group during the trials or refuse to complete the task.

Context-based fear conditioning was utilized to determine memory retention of a noxious stimulus given in a unique context, over time. Before injury, all mice were conditioned with the shock stimulus and could distinguish between the two unique environments one with and one without shock conditioning (Fig. 2.3A&B). However, at 2-DPI in the shock-paired side, the injured mice only froze for 12.9 (±3.4) sec, which was not significantly different from freezing in the unpaired side at 6.3 (±4.0) sec (*multiple unpaired t-test, p=0.2311*). Also, at 2-DPI, sham mice froze for a significantly longer time in the fear paired environment at 17.0 (±5.5) sec than in the unpaired side at 1.8 (±0.7) sec (multiple unpaired t-test, p=0.0132). At 6-DPI, the injured mice froze for 11.9 (±2.6) sec in the fear paired environment, which was significantly different from their freezing in the unpaired context at 4.5 (±0.8) sec (*multiple unpaired t-test, p=0.2311*). However, the sham mice froze a similar amount of time in the fear paired environment 6-DPI at 10.8 (±1.9) sec, which was

also significantly increased over the unpaired environment at 6.0 (±1.5) sec (*multiple unpaired t-test, p=0.0384*). Both sham and injured mice had no differences in freezing time after 14-DPI.

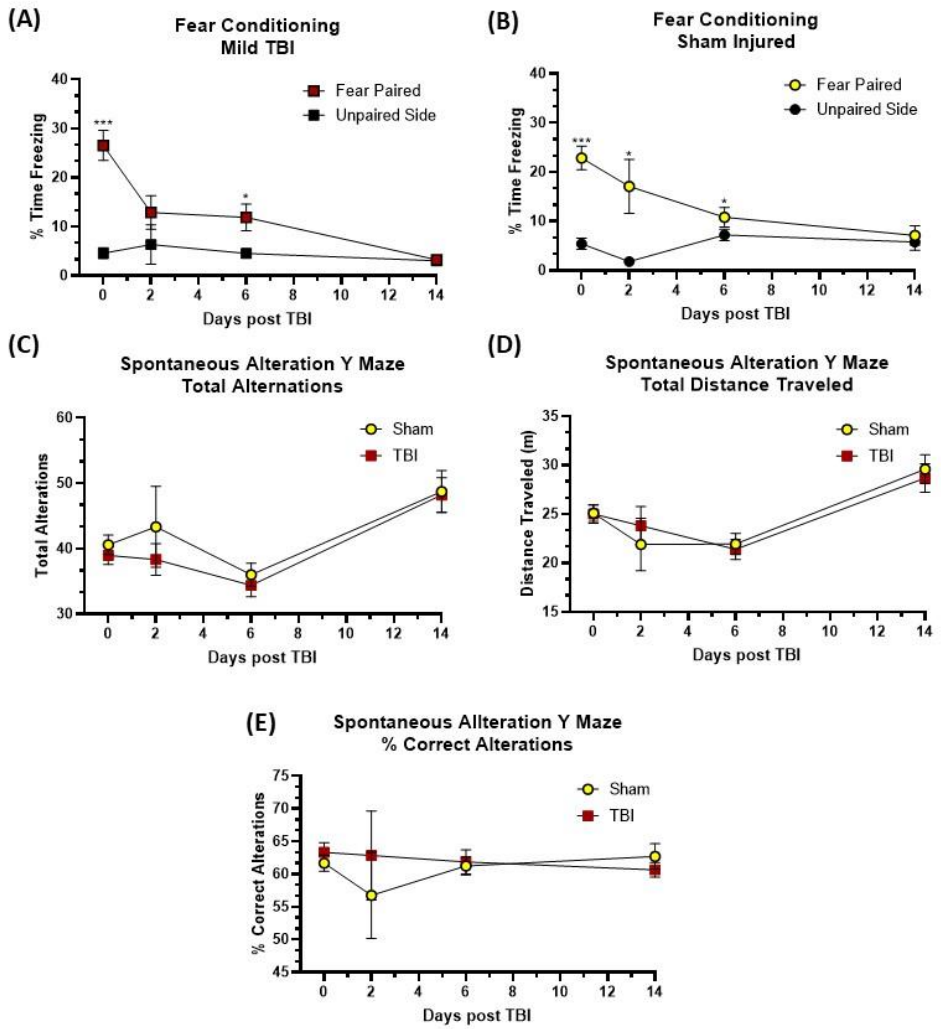

Fig. 2.3 Mice showed minor cognitive deficits as injured mice could not distinguish fear paired or unpaired environments shortly after injury. Context-based fear conditioning utilizes two environments: one in which a mouse has previously been shocked (paired side) or one with a different scent and tactile stimulation (unpaired side). Results are shown as the time spent freezing in paired and unpaired environments by injured mice (A) or sham-injured mice (B). Spontaneous alternation Y maze tracked mice by Any Maze software in a Y shaped maze. Total Alternations (C) shows the total entries into each arm of the maze. Total Distance traveled (D) was tracked and recorded in meters. Correct alternations (E) are defined as consecutively entering each arm of the maze without returning to a previously visited arm. Significance determined by multiple t test with GraphPad Prism (** = $p<0.01$, *** = $p<0.001$).

Mice were also tested in the spontaneous alternation Y maze. As early as 2-DPI, both groups had similar numbers of total alternations (Fig. 2.3C) with injured mice having a mean of 38.3 (±2.4) total entrances into each arm of the maze and sham-injured group having a mean of 43.3 (±6.1) (*multiple unpaired t-test, p=0.492*). In addition, injured mice demonstrated similar mean correct alternations (Fig 2.3E, 62.9±6.8%) as sham (56.8±6.6%; *multiple unpaired t-test, p=0.555*), and distance traveled (Fig 2.3D), with injured at a mean of 23.8 (±2.0) m and sham at a mean of 21.8 (±1.6) m (*multiple unpaired t-test, p=0.581*). This lack of difference was observed throughout the testing period.

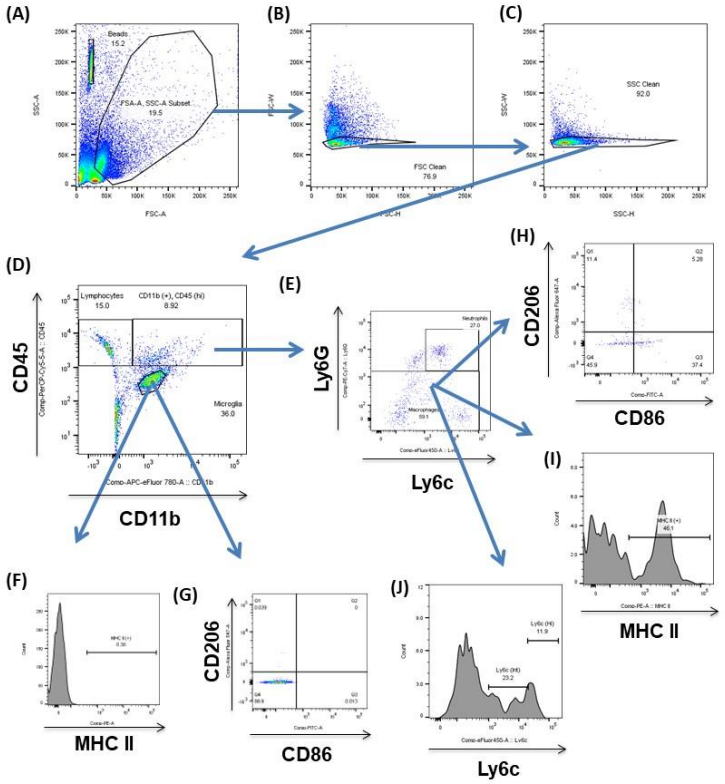

Fig. 2.4 Macrophage gating strategy. Counting beads were isolated with side scatter area on the Y-axis, and forward scatter area on the X-axis (A). Cells are gated on the same dot plot according to previously established parameters, then doublets are eliminated using forward scatter height and width (B), then side scatter height and width (C) to identify single cells. From there, CD45 cells were graphed on the Y-axis and CD11b on the X-axis (D). Cells with intermediate expression for CD45 and positive for CD11b expression were labeled as microglia and evaluated for MHCII (F), CD206 (G), and CD86 (G) expression. Cells with high expression of CD45 and positive for CD11b expression were identified as macrophages ad and evaluated for Ly6G and Ly6c expression (E). Ly6G positive cells were labeled as neutrophils. Ly6G negative cells were labeled as macrophages and evaluated for Ly6c (J), MHCII (I), CD206 (H), and CD86 (H) expression.

Acute inflammatory response to mild TBI is dominated by neutrophil and macrophage infiltration into the brain on the side of injury.

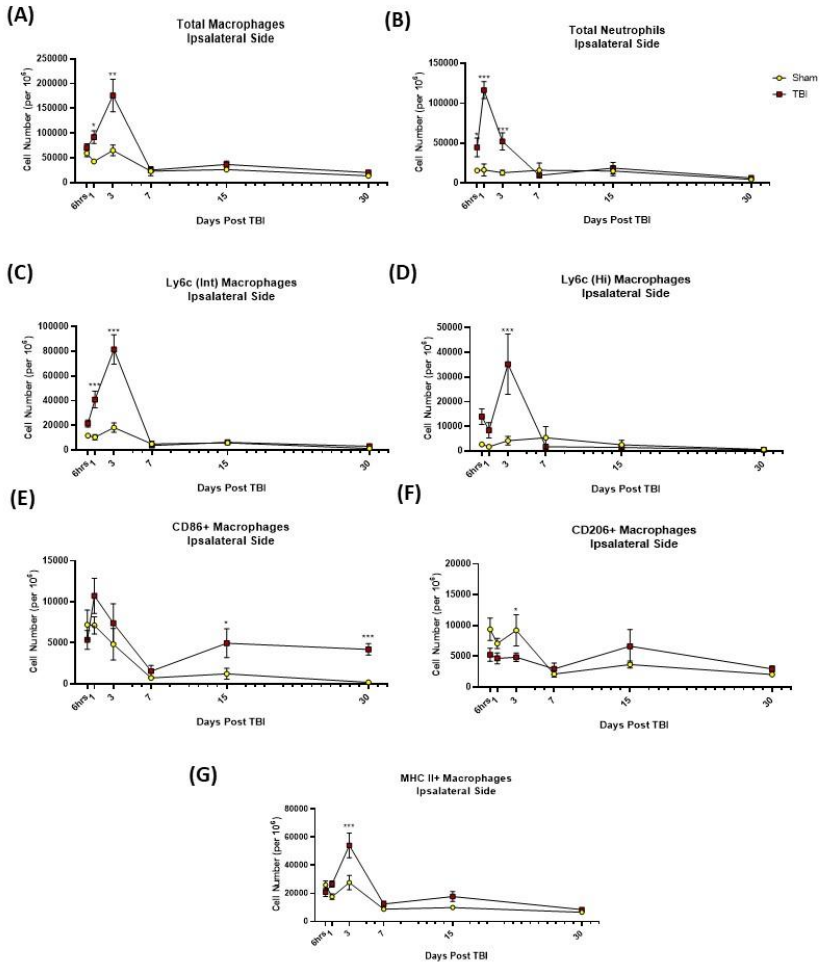

Fig 2.5 The acute phase of inflammation includes an influx of neutrophils and activated macrophage, while long-term CD86+ expression is elevated in resident macrophages weeks after the initial reaction has resolved. Graphs show the number of the cell of interest per 10^6 cells on the Y axis, with the day post injury on the X axis. All graphs shown are from the ipsilateral side of injury. Total macrophages (A) are identified as $CD45^{hi}CD11b^{+}Ly6G^{-}$. Total neutrophils (B) are identified as $CD45^{hi}CD11b^{+}Ly6G^{+}$. The remaining graphs refer to the expression of markers on the total macrophages, with analysis of Ly6c (C&D), CD86 (E), CD206 (F) and MHC II expression (G). Significance calculated utilizing multiple unpaired t-tests on GraphPad Prism software (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$).

Following mild TBI, macrophages (Fig. 2.5A) and neutrophils (Fig. 2.5B) invaded the brain early after injury on the ipsilateral side (See Fig. 2.4 for gating strategy). Neutrophil

numbers peaked early at 1-DPI, with a total of 116,610 (±10,632) neutrophils per 10^6 million cells, compared to 16,518 (±7673) cells in sham-injured brains (*multiple unpaired t-test, p<0.001*). The neutrophil response on the ipsilateral side declined with time, although it was significantly elevated at 3-DPI (39,744±10,631) compared to sham (8,257±3,016; *multiple unpaired t-test, p<0.001*). On the contralateral side, neutrophil response was not significantly changed after injury at all time points tested (injured mice 10,270 (±7,104) neutrophils at 1-DPI compared to sham at 3,035 ±2,682: Fig 2.6B, *multiple unpaired t-test, p=0.633*).

Macrophages migrated to the ipsilateral side at 1-DPI (Fig. 2.5A) and significantly increased in numbers compared to sham-injured mice (91,803±12737 vs. 42,620±3.990; *multiple unpaired t-test, p=0.021*). In contrast to neutrophils, infiltrating macrophages continued to increase and peaked at 3-DPI on the ipsilateral side (175,704±32,765 cells) with 3x greater numbers than in sham-injured brains (64,821±10.732 cells; *multiple unpaired t-test, p<0.001*).

These infiltrating macrophages also expressed increased levels of Ly6C, an activation marker (Fig. 2.5C&D). At 1-DPI, 40,969 (±6,778) infiltrating macrophages expressed intermediate levels of Ly6C after injury (Fig 2.5C), and 8,386 (±3084) expressed Ly6C at high levels (Fig. 2.5D) on the cell surface. These macrophage numbers were higher compared to 10,453 (±2057) $Ly6C^{int}$ macrophages and 1,747 (±229) $Ly6C^{hi}$ cells in sham-injured brain (*multiple unpaired t-test, p<0.001, p=0.369 respectively*). The Ly6C activation marker also peaks on macrophages at 3-DPI, reaching 81,542 (±11,906) $Ly6C^{int}$ macrophages, and 35,197 (±12,213) $Ly6C^{hi}$ cells after injury. In comparison, sham-injured brains had 18,304 (±3746) $Ly6C^{int}$ cells and 4,189 (±1761.2) $Ly6C^{hi}$ macrophages (*multiple unpaired t-test, p<0.001; 0.001 respectively*).

In contrast, CD206 expression on macrophages decreased with injury. While 9211 (±6229) infiltrating macrophages expressed CD206 in sham-injured mice, only 4844 (±1681) expressed CD206 on macrophages (Fig. 2.5F, *multiple unpaired t-test, p=0.02419*).

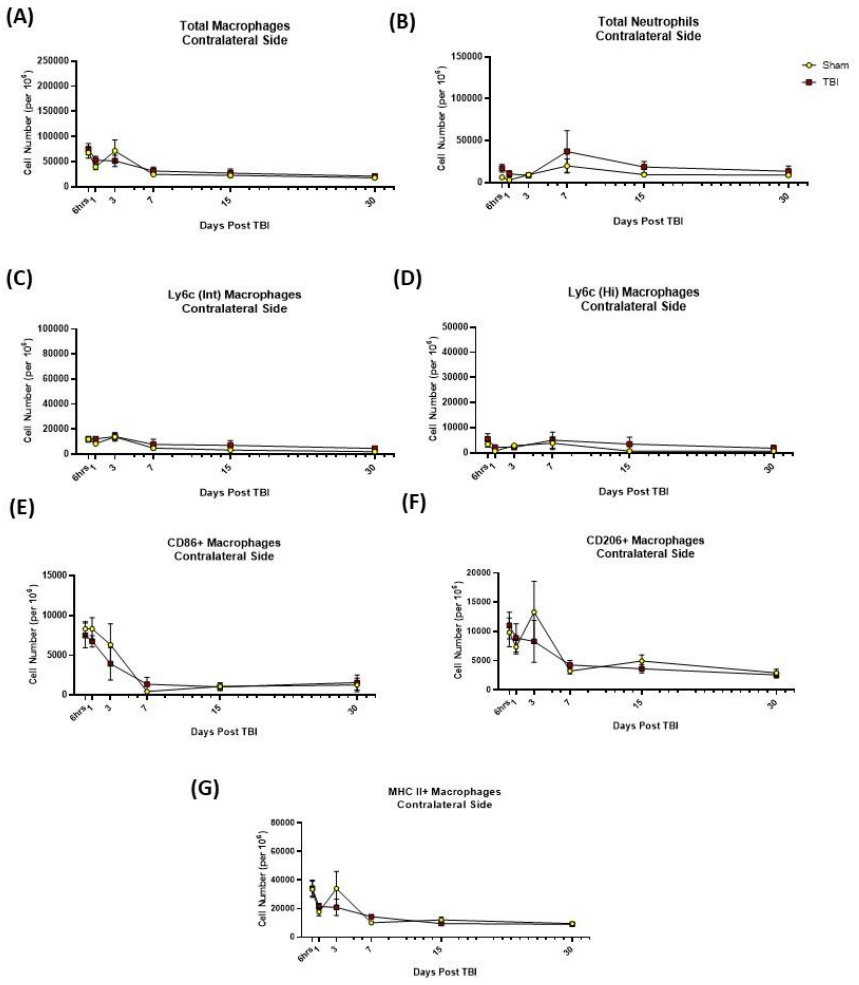

Fig 2.6 Macrophage and neutrophil morphologic evaluation on contralateral side yields no significant differences. Graphs show the number of the cell of interest per 10^6 cells on the Y axis, days post injury (DPI) on the X axis. All graphs shown are from the contralateral side of injury. Total macrophages (A) are identified as $CD45^{hi}CD11b^{+}Ly6G^{-}$. Total neutrophils (B) are identified as $CD45^{hi}CD11b^{+}Ly6G^{+}$. The remaining graphs refer to the expression of markers on the total macrophages, with analysis of Ly6c (C&D), CD86 (E), CD206 (F), and MHC II expression (G). Significance calculated utilizing multiple unpaired t-tests on GraphPad Prism software.

MHC II expression also followed a similar trend to Ly6C with peak expression levels on macrophages at 3-DPI, 54,199 (±8,806) cells expressing MHC II, as opposed to 27,706 (±5,109) infiltrating macrophages expressing MHC II in sham-injured brains (Fig. 2.5G, *multiple unpaired t-test*, $p<0.001$). This infiltrating macrophage response resolved to sham-injured levels at 7-DPI, as there was no longer any significant difference between

groups with injured (12,434±2052) and sham-injured mice (8779±2256) (*multiple unpaired t-test, p=0.901*).

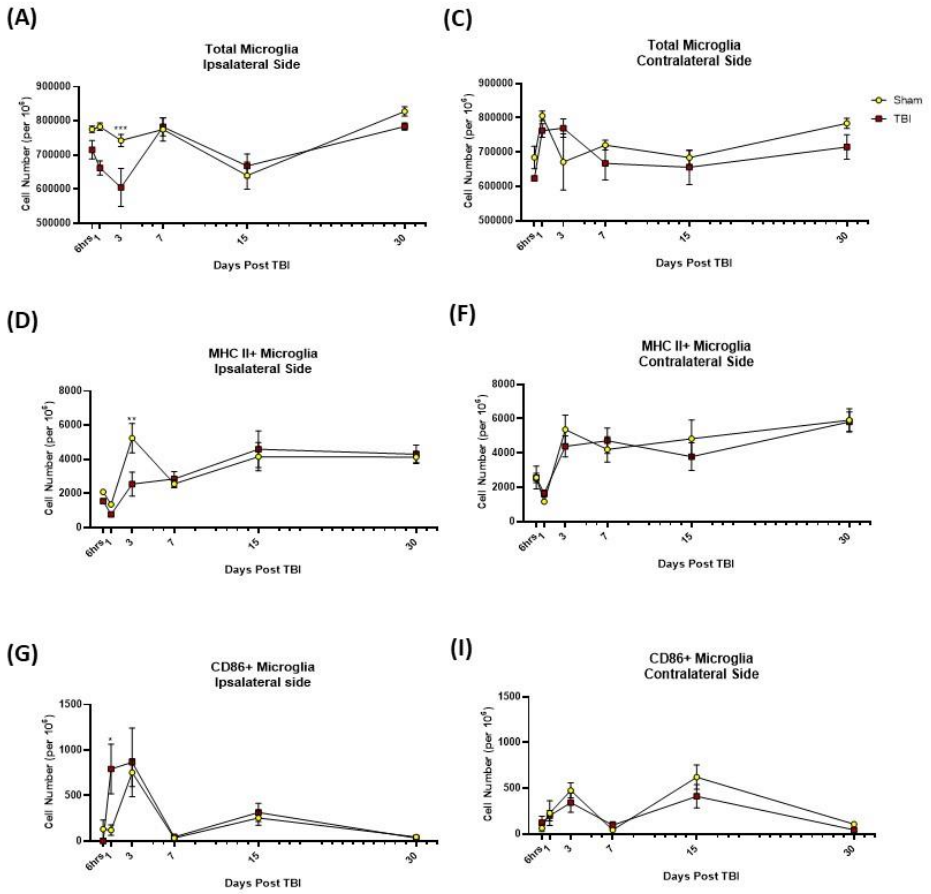

Fig 2.7 Microglial morphological evaluation for the ipsilateral and contralateral side of injury. Graphs show the number of the cell of interest per 10^6 cells on the Y axis, with days post injury on the X axis. Total microglia on the ipsilateral (A) and contralateral (B) side are identified as $CD45^{int}CD11b^{+}$. That population was then examined by for the expression of CD86 on the ipsilateral (C) and contralateral (D) side, and for the expression of MHC II on the ipsilateral (E) and contralateral (F) side. Significance calculated utilizing multiple unpaired t-tests on GraphPad Prism software (**p≤0.01, ***p≤0.005).

Chronic immune response to mild TBI is characterized by increased CD86 macrophages.

While macrophages activation markers, Ly6C and MHCII, peaked at 3-DPI on the ipsilateral side and subsiding thereafter, CD86 expression on macrophages remained elevated at 15 and 30-DPI (Fig 2.5E). The total infiltrating macrophage numbers at 15

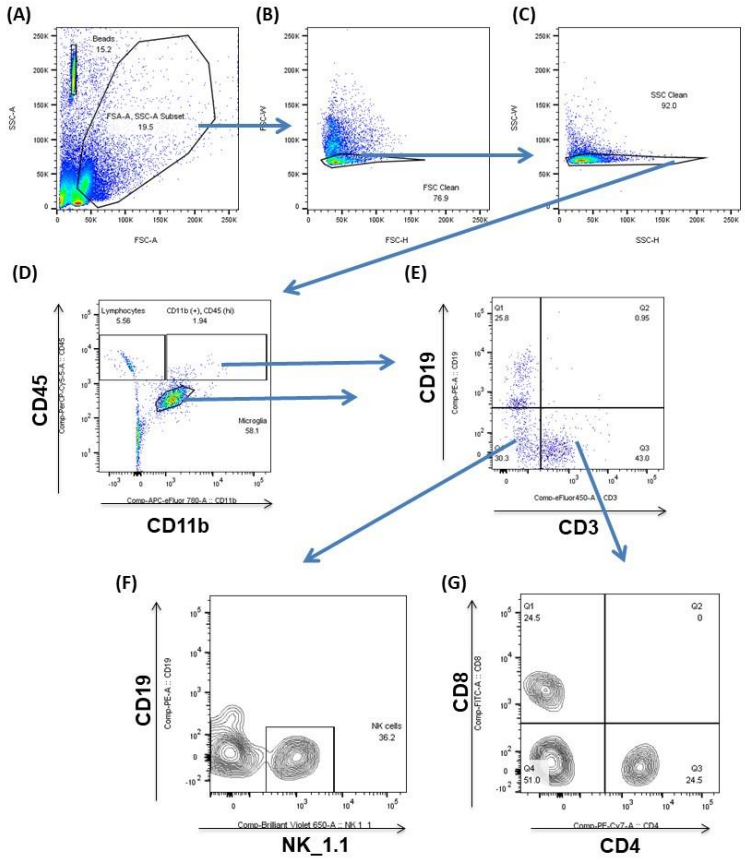

Fig. 2.8. Lymphocyte gating strategy. Counting beads were isolated with side scatter area on the Y-axis, and forward scatter area on the X-axis (A). Cells are gated on the same dot plot according to previously established parameters, then doublets are eliminated using forward scatter height and width (B), then side scatter height and width (C) to analyze single cells. From there, CD45 cells were graphed on the Y-axis and CD11b on the Y-axis (D). Cells that are CD45 positive and CD 11b negative were identified as lymphocytes and further evaluated for CD19 and CD3 expression (E). Those that are CD19 positive were labeled as B cells. Those that are CD3 positive are further evaluated for CD8 and CD4 expression (G). Cells that are CD19 and CD3 negative are evaluated for NK1.1 expression, and positive cells were labeled as natural killer (NK) cells.

and 30-DPI for injured (36,592±6,940 and 20,108±1,647 cells, respectively) and sham-injured (26,203±3296 and 13,659±974 cells, respectively) mice had no significant differences (Fig. 2.5A, *multiple unpaired t-test, p=0.540; 0.703*). At 15-DPI, injured mice had 4,954 (±1749) macrophages expressing CD86 and 1,229 (±670) cells in sham-injured mice (Fig. 2.5E, *multiple unpaired t-test, p=0.002*). This increased CD86+ macrophages were observed through 30-DPI, with 4,202 (±704) macrophages expressing CD86 in injured mice, as opposed to 199 (±53) in sham-injured mice (Fig. 3G, *multiple unpaired t-test, p=0.002*). This observation was exclusive to the ipsilateral side, with the

macrophages on the contralateral side demonstrating no significant difference in CD86 expression at any time point (Fig. 2.6E).

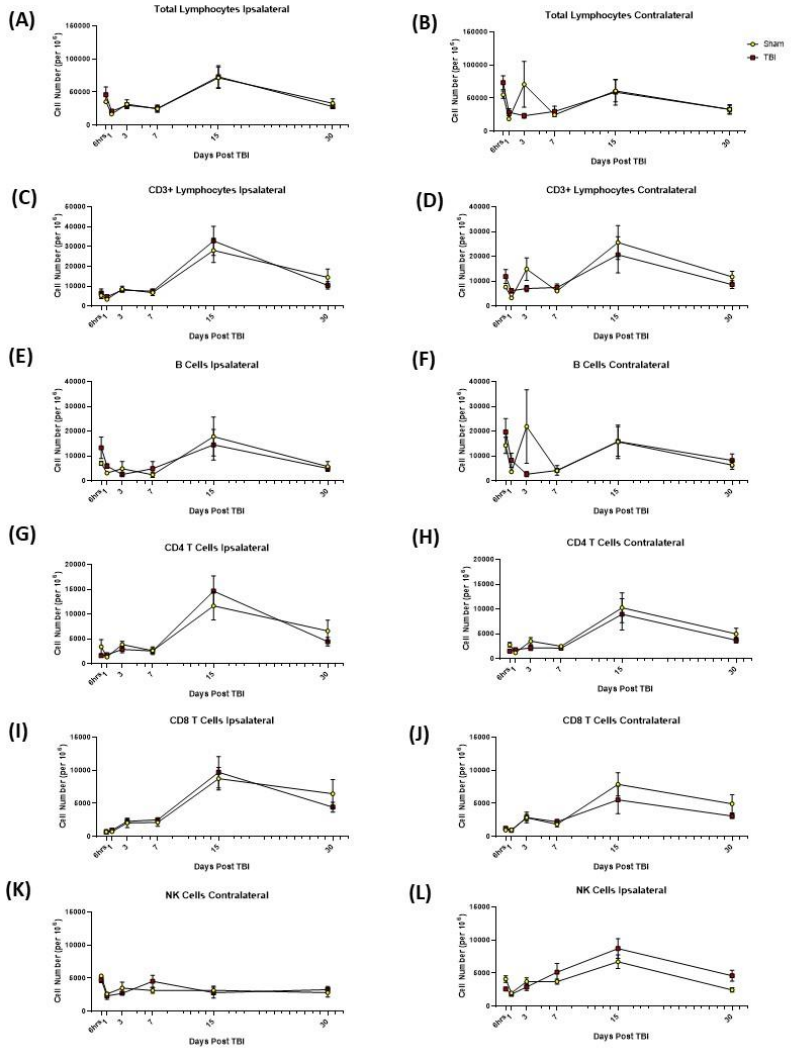

Fig. 2.9 Lymphocyte morphological evaluation for the ipsilateral and contralateral side of injury. Graphs show the number of the cell of interest per 10^6 cells on the Y axis with days post injury on the X axis. Graphs shown are from the ipsilateral and contralateral side of injury. Total lymphocytes (A/B) are identified as $CD45^{hi}CD11b^{-}$. Lymphocytes were then evaluated for CD3 expression (C/D), and those were further evaluated for CD4 (E/F) and CD8 (G/H) expression. Lymphocytes that are $CD3^{-}CD\ 19^{+}$ were labeled as B Cells (I/J). Cells that were $CD19^{-}CD3^{-}$ were evaluated for NK1.1 and positive cells were labeled natural killer (NK) Cells (K/L). Significance calculated utilizing multiple unpaired t-tests on GraphPad Prism software.

The inflammatory macrophage response was localized to the ipsilateral side, while the contralateral side had no difference in total macrophage numbers (at 3-DPI, injured =51,307 (±11,575) and sham =71,203 (±21,905) cells; *multiple unpaired t-test, p=0.142*), or activation markers expression at all time points tested (Fig. 2.6E).

The total numbers and levels of CD86 and MHC II expression were also evaluated on microglia, as well the number of lymphocytes in both the ipsilateral and contralateral side of the brain. Minor differences were observed among these cell populations (Fig 2.7 and 2.9).

The mRNA levels associated with cytokines and chemokines changes from acute to chronic injury response.

PCR analysis of brains for mRNA expression levels of selected cytokines and chemokines involved in cell trafficking and activation at 3 and 15-DPI brains had significant changes compared to the sham groups (Fig. 2.10A-D, *two-way ANOVA, p=0.0372 and 0.0089 respectively*). At 3-DPI on the ipsilateral side (Fig. 2.10A), tumor necrosis factor alpha (TNF-α), transforming growth factor-beta (TGF-β), interferon gamma (INFɣ), arginase 1 (ARG1) and interleukin 10 (IL-10) mRNA expression were elevated over that of sham (3.5±1.1 fold, 2.8±1.1 fold, 3.1±1.1 fold, 2.5±0.5 fold and 1.8±0.6 fold respectively). Additionally, expression of the C-C chemokine receptor type 2 (CCR2) was also elevated (2.1±0.3 fold).

At 15-DPI (Fig. 2.10C), the levels of interleukin 1 beta (IL1-β) and C-X-C motif chemokine ligand 10 (CXCL10) mRNA increased on the ipsilateral side (2.5±0.7, and 4.3±1.1), while previously elevated cytokine levels, including of CCR2, and TNF-α were not significantly increased at 15-DPI, albeit they remained elevated relative to sham-injured animals.

There was no significant difference in overall proinflammatory cytokine response at 3-DPI and 15-DPI on the contralateral side (Fig. 2.10B&D). However, at 15-DPI, interleukin 6 (IL-6) mRNA was significantly decreased in both the ipsilateral and contralateral side (0.1±0.1 fold and 0.2±0.0 fold respectively; *multiple unpaired t-test, p=0.0023 and*

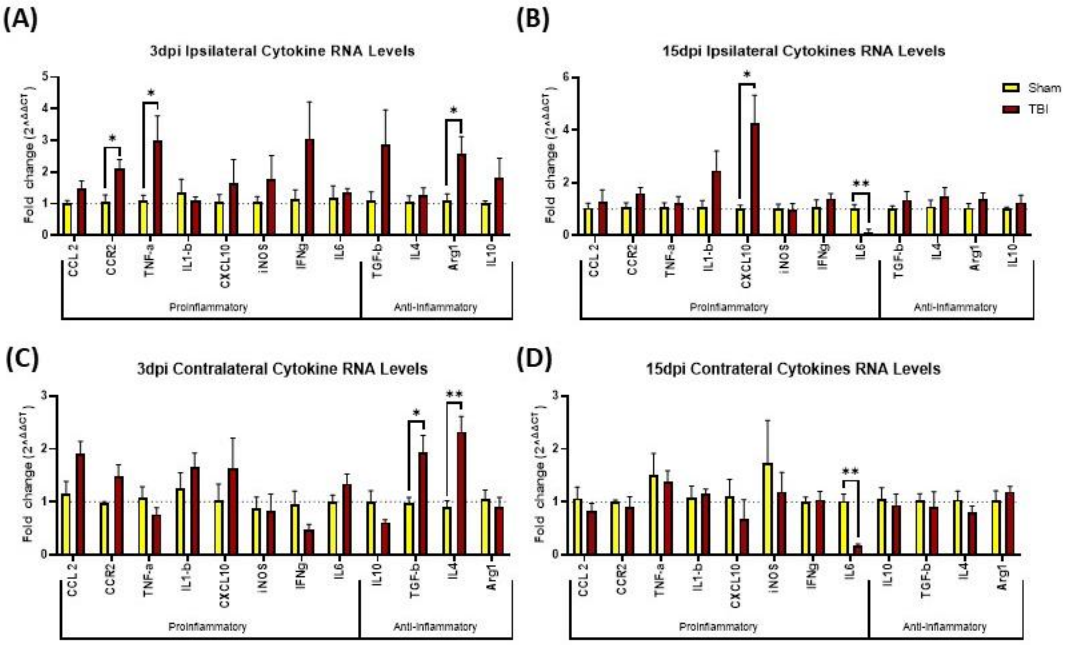

Fig 2.10 The mRNA levels associated with cytokine and chemokine levels changes from acute to chronic injury. The levels of mRNA of several cytokine and chemokines were measure by RT-PCR and normalized to the house keeping gene RPL27. Levels of RNA were measured at the acute 3 days post injury (DPI) timepoint on the ipsilateral (A) and contralateral (B) side of injury, as well as at the chronic stage at 15-DPI on the ipsilateral (C) and contralateral (D) side of injury. Significance calculated utilizing Welch's t-tests on GraphPad Prism software, p values of 0.05 or lower were considered significant (*p≤0.05, **≤0.01, ***≤0.005).

0.0007 respectively). Additionally on the contralateral side, at 3-DPI, the anti-inflammatory cytokine mRNA of IL-4 and TGF-β was significantly increased by 2.3±0.3 fold (*multiple unpaired t-test, p=0.0041*) and TGF-β was significantly increased by 1.9±0.3 fold (*multiple unpaired t-test, p=0.0265*).

Long-term inflammation correlates with spatial learning deficits.

To determine if this long-term inflammatory response was associated with changes in behavior, at 26-30 days post TBI, spatial learning behavior was evaluated on the Barnes maze. Although, initial training days on the Barnes maze demonstrated a trend indicative of a deficit in learning among injured animals (Fig. 2.11A), the differences were not statistically significant. Measurements of distance traveled indicated that both groups traveled the same distance during exploration (Fig 2.11D), with sham-injured mice

traveling 11.1 (±0.8) m and injured mice traveling 10.3 (±1.1) m (*unpaired t-test, p=0.559*). Sham-injured mice also identified and reached the escape hole zone faster (6.5±2.4 sec) than those with mild TBI (11.2±3.6 sec), albeit this difference is not statistically significant.

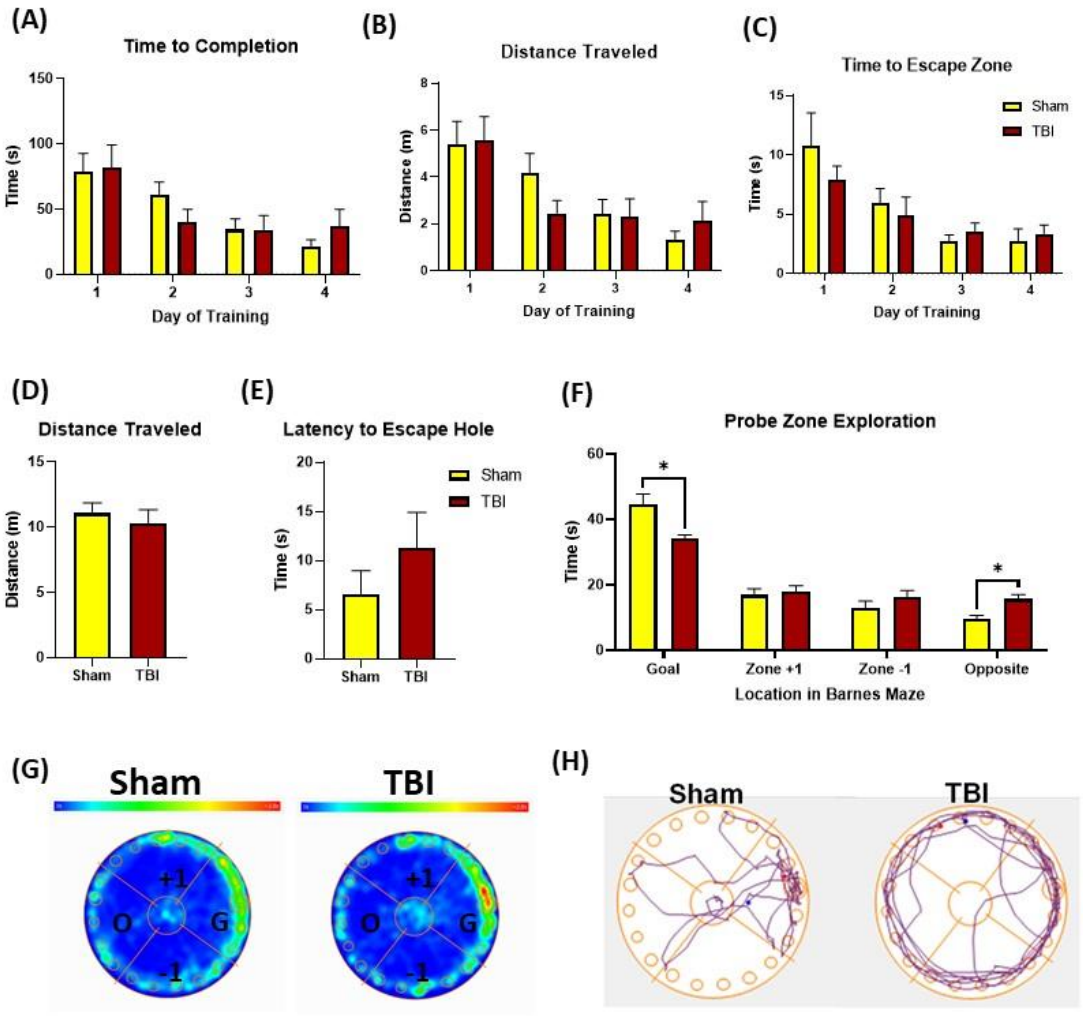

Fig 2.11 Following mild TBI, mice demonstrate a long-term spatial learning deficit. Following TBI, the mice learned to solve the maze by identifying the escape hole. During training, the time to completion (A), distance travelled (B), and time to escape zone were recorded by any maze software. Average distance traveled (D) and (E) latency to escape hole was recorded for sham and injured mice. At the probe trial, the escape hole was removed, and the mice were recorded on the maze for 90 seconds. Probe zone exploration (F) shows the time spent in each quadrant during probe trials. Diagram of the maze is displayed with the heat maps (G) of mice exploring the maze during the probe trial (Blue is approx. 0 seconds and red is approx. 2.3 seconds) and by representative tracing (H) of exploratory behavior. The area marked by the quadrant on the right is the goal zone(G), the top quadrant is zone +1(+1), bottom quadrant is zone -1 (-1), and left quadrant is opposite zone (O). Statistics shown were calculated using multiple unpaired t tests (* = $p \leq 0.05$).

However, during the probe trial sham-injured mice spent a significantly longer time in the goal zone (44.6±3.2 sec) compared to injured mice (34.2±1.1 sec; Fig. 2.11F, *multiple unpaired t-test, p=0.007*). In addition, injured mice spent significantly more time in the opposite zone (15.6±1.4 sec) than sham-injured (9.7±0.9 sec; *multiple unpaired t-test, p=0.003*). Computer aided heat maps and tracings of maze exploration patterns (Fig 2.11G&H) illustrated that mice with TBI explored largely the outer edges of the entire maze, while the sham-injured mice focused their exploration activity on the target hole.

Inflammation extends beyond the initial site of injury.

Immunohistochemistry (IHC) was utilized to determine where the inflammatory response was located within the brain. At 3-DPI, there was an increase in immunoreactivity for Iba1+ (macrophages) and GFAP+ (astrocyte) cells in injured mice (Fig 2.12A&A') compared to sham-injured mice (Fig 2.12B&B') around the Dentate Gyrus (DG), *Cornu Ammonis* (CA)1, and CA3 regions of the *hippocampus*. This increased immunostaining for GFAP and iba1 was also observed in the same regions at 30-DPI (Fig. 6C-D), indicative of a sustained inflammatory reaction within the *hippocampus*.

In addition, a disorganized pattern of NeuN+ cell staining was observed in the injured mice at 30-DPI, consistent with loss of neuronal bodies (Fig. 2.12C&C'), while the NeuN+ cells localized in an organized pattern in sham-injured mice (Fig. 2.12D&D'). This pattern of decreased NeuN immunostaining was observed to a lesser degree in the CA2 and CA3 areas of the *hippocampus* (Fig. 2.13).

At the site of injury (Fig. 2.14), an increased Iba1+ and GFAP+ labeling was observed at both 3-DPI (Fig 2.14A) and 30-DPI (Fig. 2.14A). This increase in activated astrocytes and macrophages was observed around the injury site with a marked decrease in NeuN immunoreactivity in the area compared to sham.

Immunoreactivity to Iba1 and GFAP were less pronounced in the *thalamus* (Fig. 2.15), ventral *midbrain* (Fig 2.16), and *corpus callosum* (Fig 2.17)) indicating focal inflammatory changes in the brain post mild TBI.

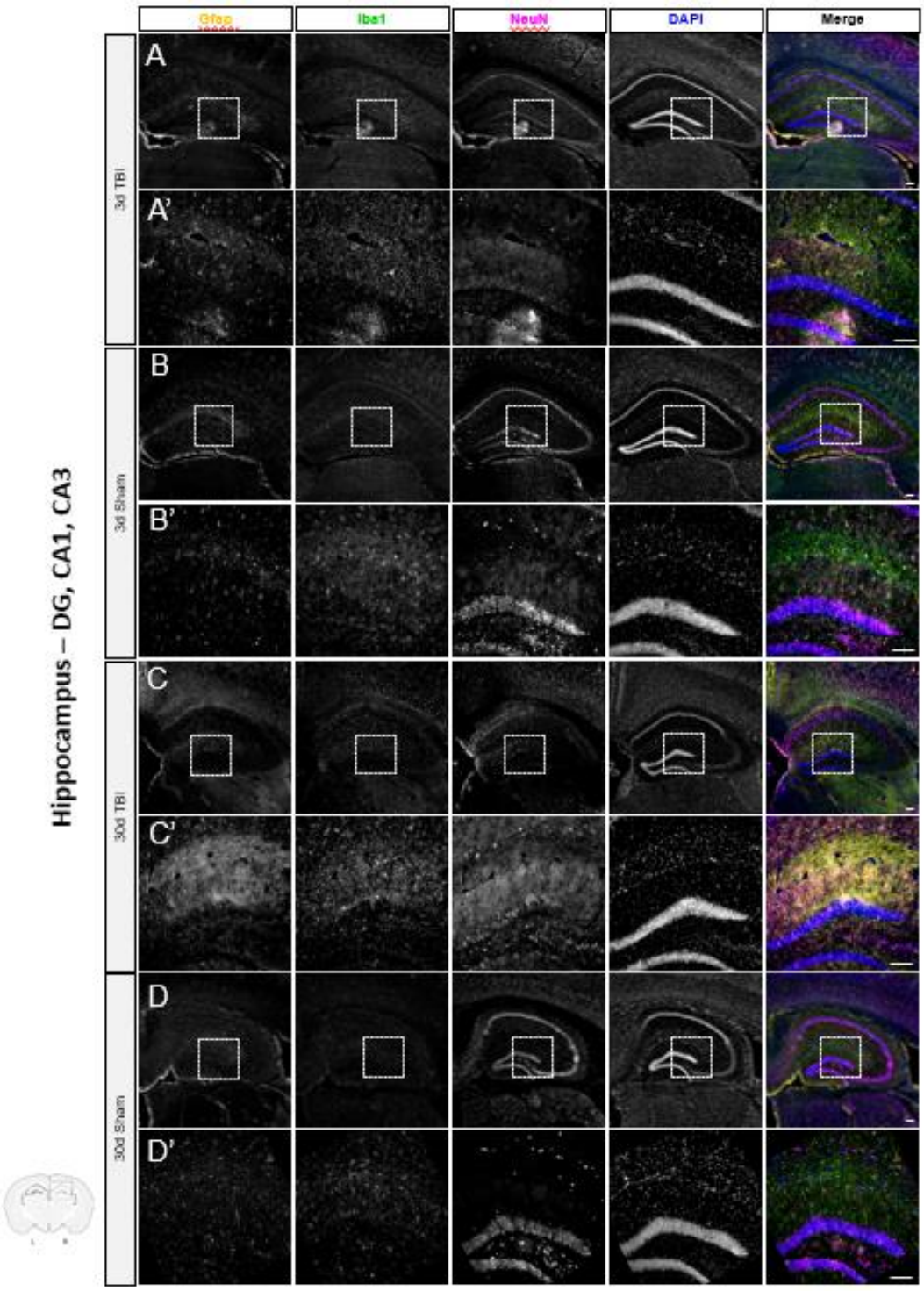

Fig 2.12 Immunohistochemical analysis of inflammation in the *hippocampus* (dentate gyrus, CA1, CA3) after mild TBI. A-D) Gallery-view immunofluorescent images of coronal sections of brains labeled with reactive astrocytes (GFAP), microglia/macrophages (Iba1), and neurons (NeuN), and DAPI at 3-DPI (A, B) and 30-DPI (C, D). (A'-D') Gallery-view immunofluorescent images from insets of A-D. Scale bar: 100µm. Schematic coronal sections created with BioRender.com.

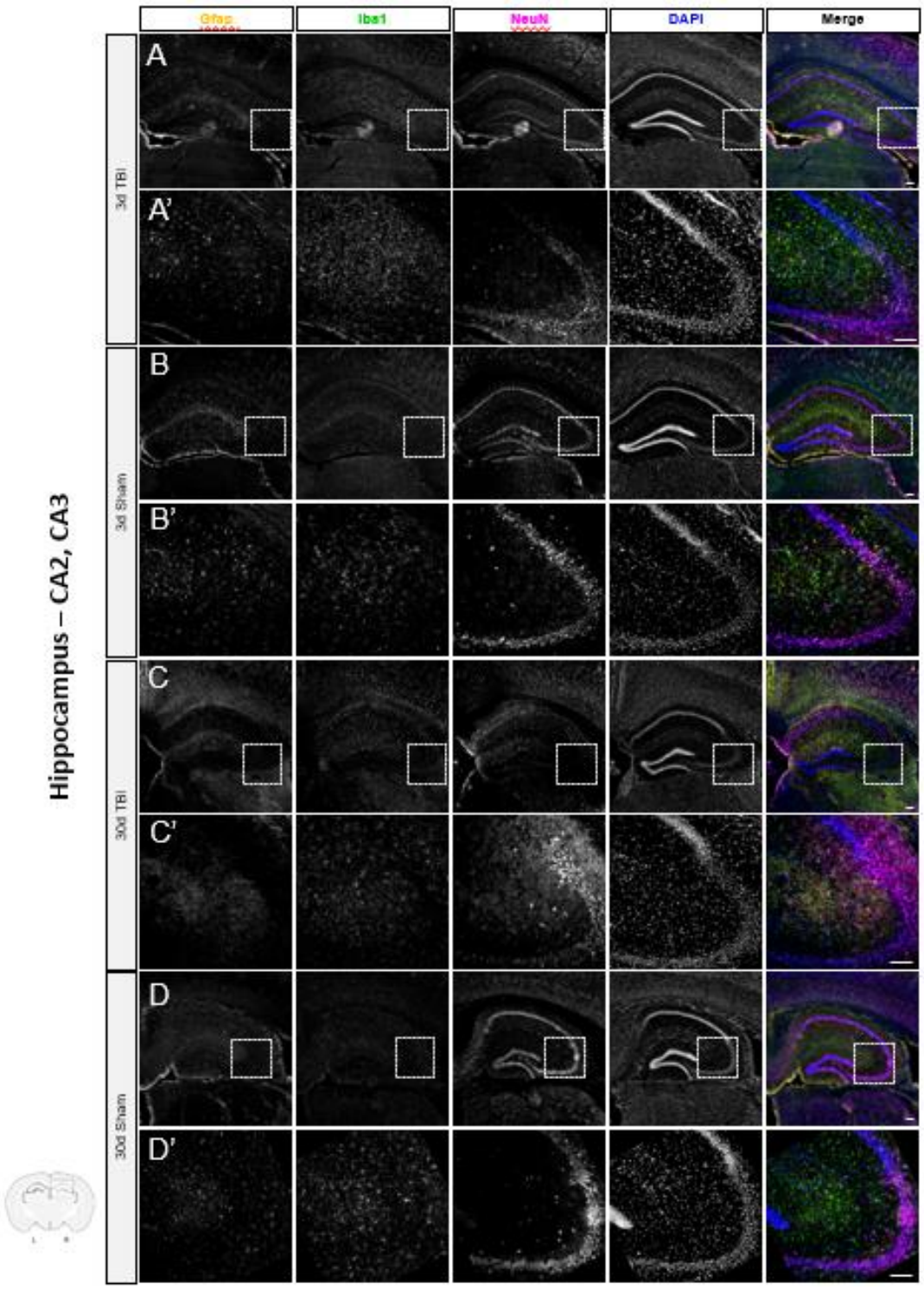

Fig. 2.13 Immunohistochemical analysis of inflammation in the *hippocampus* (CA2, CA3) after mild TBI. A-D) Gallery-view immunofluorescent images of coronal sections of brains labeled with reactive astrocytes (GFAP), microglia/macrophages (Iba1), and neurons (NeuN), and DAPI at 3-DPI (A, B) and 30-DPI (C, D). (A'-D') Gallery-view immunofluorescent images from insets of A-D. Scale bar: 100µm. Schematic coronal sections created with BioRender.com.

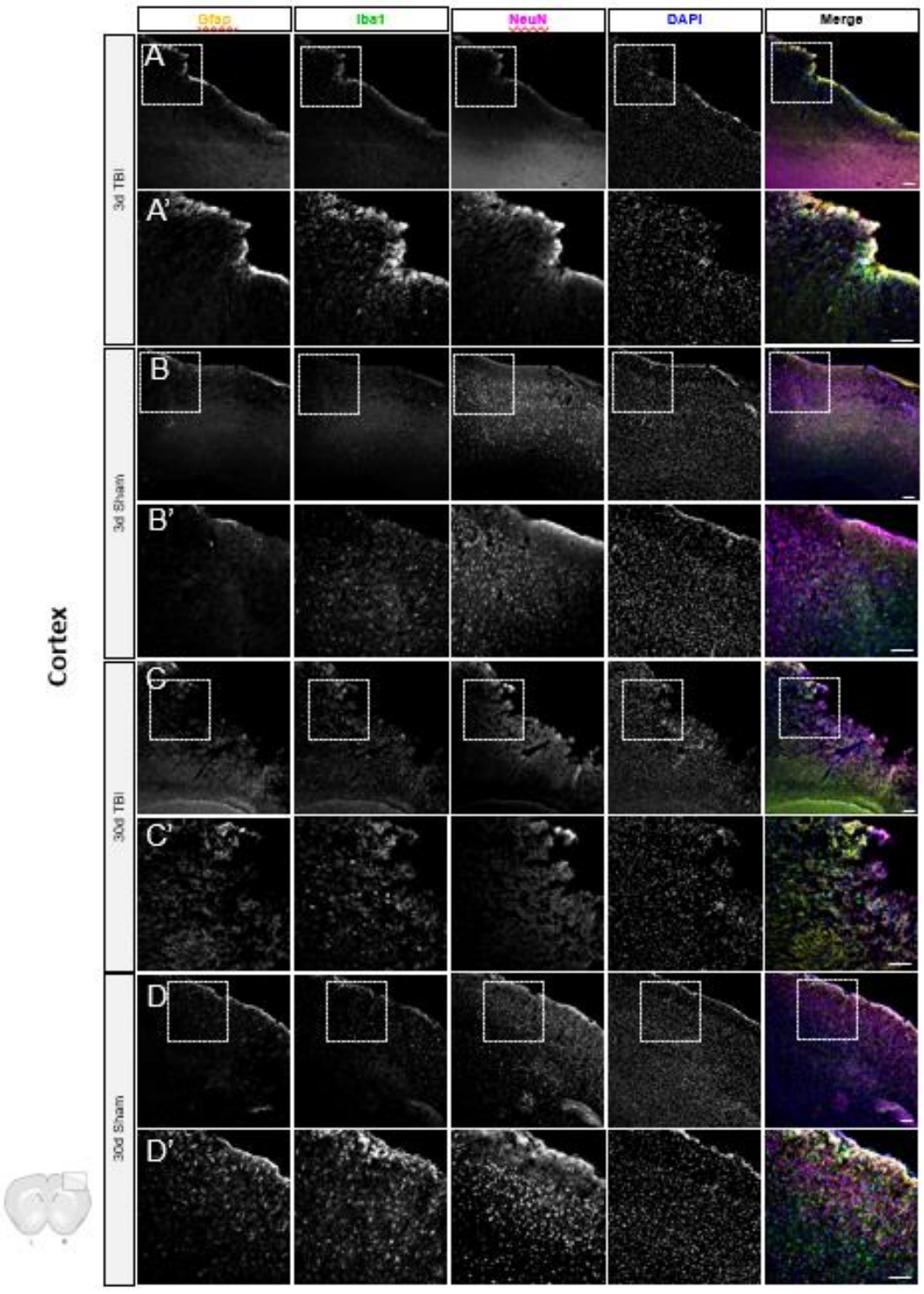

Fig. 2.14 Immunohistochemical analysis of inflammation in the *cortex* after mild TBI. A-D) Gallery-view immunofluorescent images of coronal sections of brains labeled with reactive astrocytes (GFAP), microglia/macrophages (Iba1), and neurons (NeuN), and DAPI at 3-DPI (A, B) and 30-DPI (C, D). (A'-D') Gallery-view immunofluorescent images from insets of A-D. Scale bar: 100μm. Schematic coronal sections created with BioRender.com.

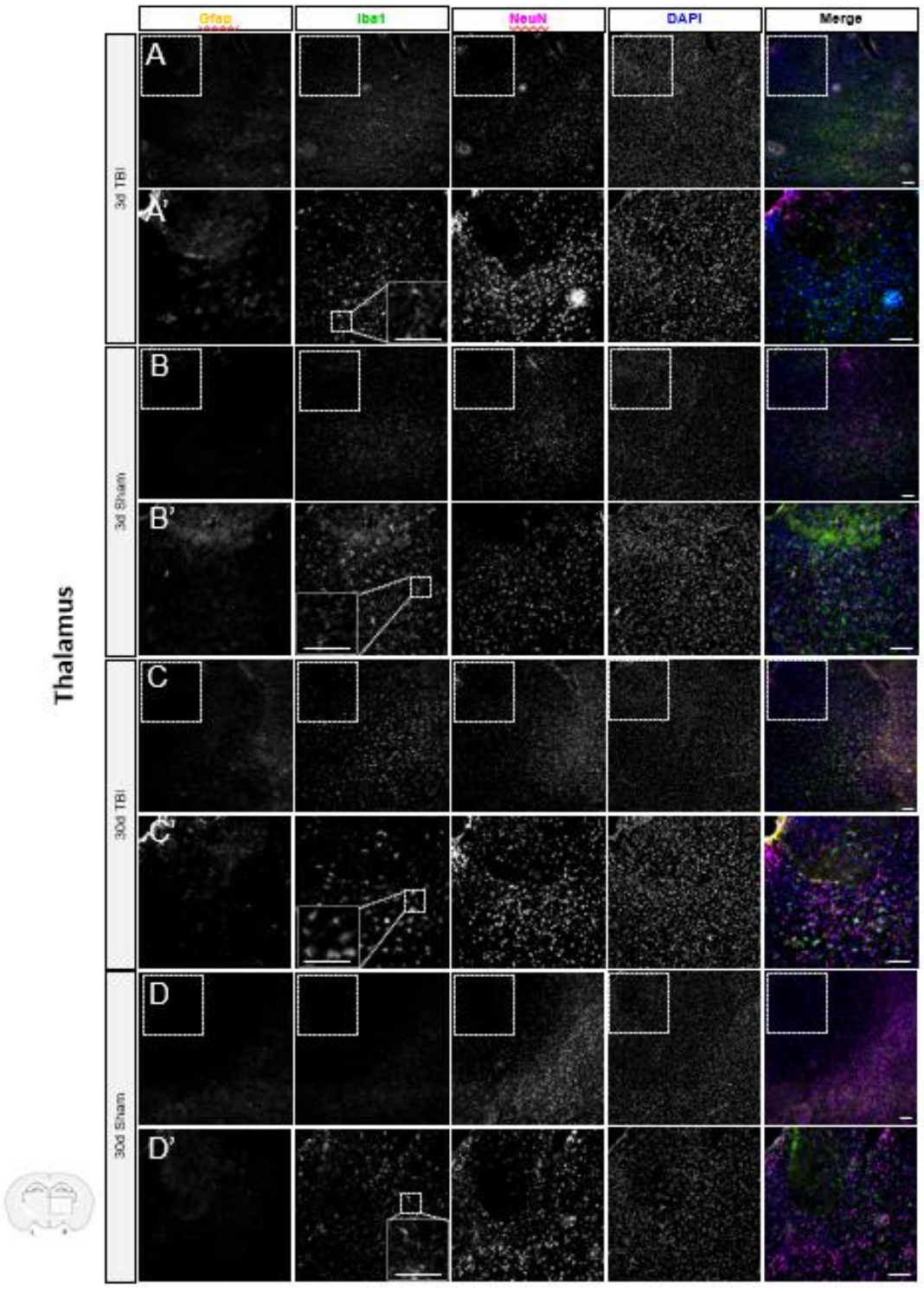

Fig 2.15 Immunohistochemical analysis of inflammation in the *thalamus* after mild TBI. A-D) Gallery-view immunofluorescent images of coronal sections of brains labeled with reactive astrocytes (GFAP), microglia/macrophages (Iba1), and neurons (NeuN), and DAPI at 3-DPI (A, B) and 30-DPI (C, D). (A'-D') Gallery-view immunofluorescent images from insets of A-D. Scale bar: 100µm. Schematic coronal sections created with BioRender.com.

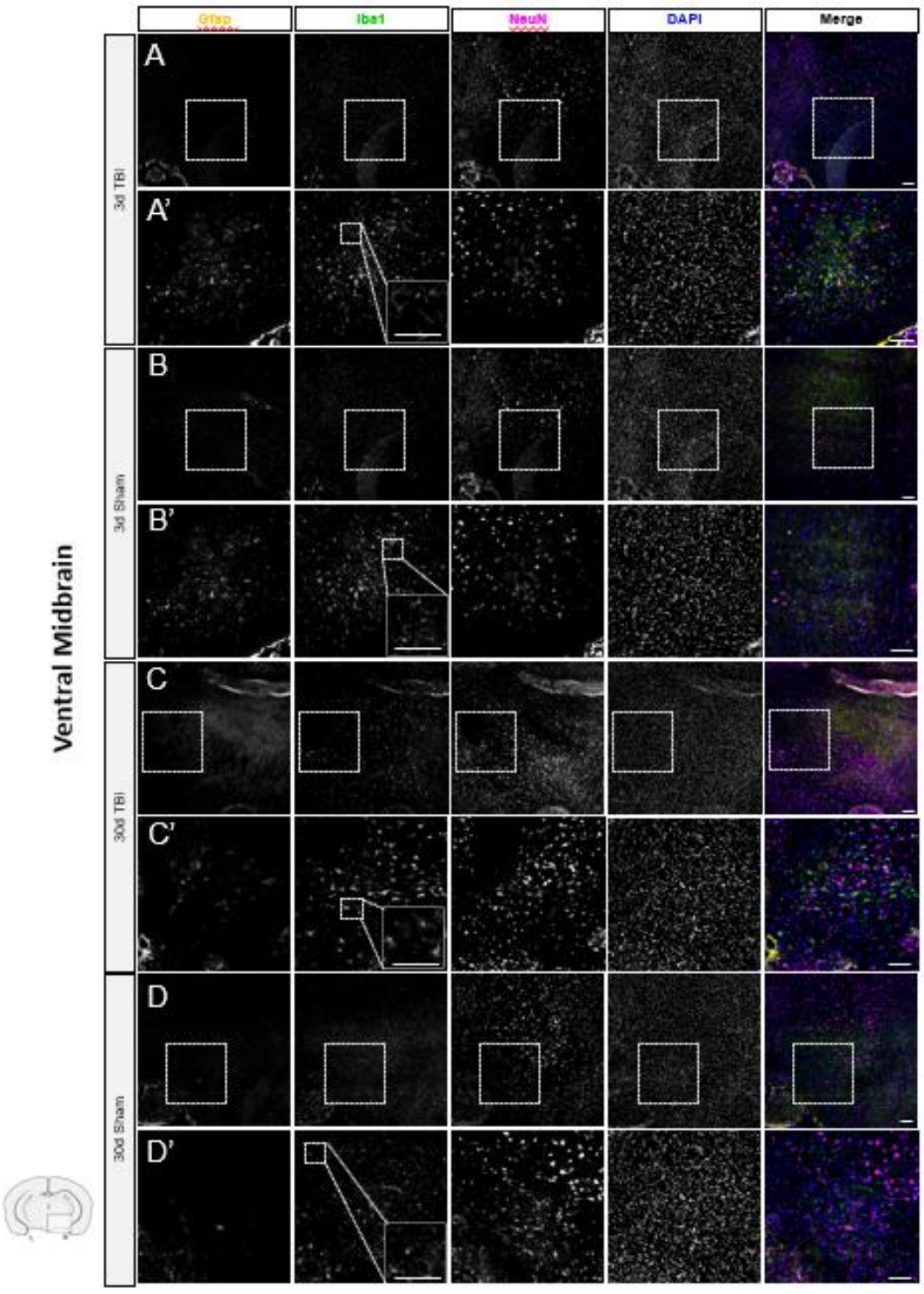

Fig. 2.16 Immunohistochemical analysis of inflammation in the ventral *midbrain* after mild TBI. A-D) Gallery-view immunofluorescent images of coronal sections of brains labeled with reactive astrocytes (GFAP), microglia/macrophages (Iba1), and neurons (NeuN), and DAPI at 3-DPI (A, B) and 30-DPI (C, D). (A'-D') Gallery-view immunofluorescent images from insets of A-D. Scale bar: 100µm. Schematic coronal sections created with BioRender.com.

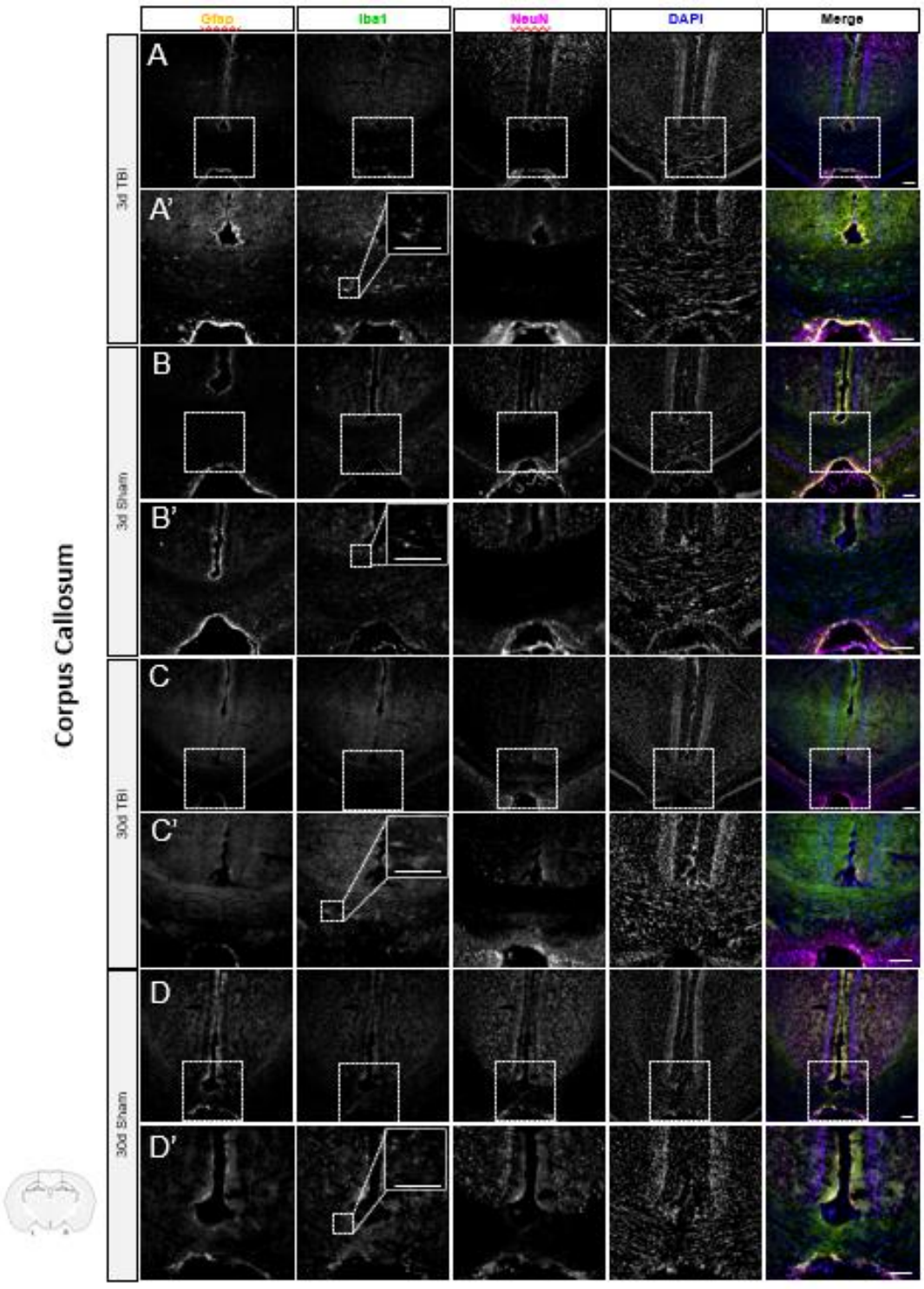

Fig 2.17 Immunohistochemical analysis of inflammation in an inactive area (*corpus callosum*) after mild TBI. A-D) Gallery-view immunofluorescent images of coronal sections of brains labeled with reactive astrocytes (GFAP), microglia/macrophages (Iba1), and neurons (NeuN), and DAPI at 3-DPI (A, B) and 30-DPI (C, D). (A'-D') Gallery-view immunofluorescent images from insets of A-D. Scale bar: 100µm. Schematic coronal sections created with BioRender.com.

Discussion

Mild TBI is defined by a transient alternation in consciousness and quick resolution of acute symptoms. [12,366,377,510,511] but is also well recognized for their ability to lead to long-term neurological effects, including impairment of cognitive function[231]. While there have been many studies[95,366,376,420,519-521] reporting similar acute responses to injury, the long-term response to TBI is not well characterized, especially in mild TBI. The current study describes the characteristics of a murine mild TBI model and demonstrates that after a mild injury, the acute motor deficits and inflammatory responses subside at 7-DPI. However, a chronic inflammatory response mediated by activated macrophages persists in the brain at 15 and 30-DPI. This persistent inflammation was demonstrated by an increase in activation markers on macrophages assessed through flow cytometry, modulation of the cytokine gene responses measured by quantitative real time RT-PCR, and presence of activated macrophages and astrocytes at anatomical locations distal to injury (e.g., the *hippocampus*), as assessed by IHC at 30-DPI. Observed in association with this persistent neuroinflammation is a spatial learning deficit exhibited in injured animals at 30-DPI on a Barnes Maze.

Following mild TBI, mice had a significantly increased recovery time than those with sham injuries. The observation presented is consistent with other published reports that demonstrate increased recovery time after mild injury. [97,522] To rule out exposure to isoflurane as the cause for the increased recovery time,[523] the correlation between recovery and surgical times was examined to determine if a relationship exists between these outcomes. While a positive correlation was seen in the sham group, the recovery time from mild TBI did not correlate with surgical time, thus excluding the effects of surgical intervention or isoflurane anesthesia for the increased time to recovery.

Injured mice took longer to cross the beam on the beam walk test and had larger numbers of foot slips compared to sham-injured mice. These motor deficits were greater when the difficulty to navigate the beams increased (smaller diameter or cylindrical beams). This finding is consistent with the loss of coordination seen shortly after mild TBI.[12,366,509,510] Several studies demonstrate that the magnitude and degree of acute deficits due to brain

injury correlate with the impact force applied or severity of injury.[435,524] In the present study, transient deficits memory retention was confirmed by extinction of the ability of injured animals to differentiate an environment paired with a noxious stimulus (shock) at 2-DPI, with that of an unpaired environment. Both cognitive deficits and motor deficits exhibited on the beam walk test resolved by 6-DPI demonstrating the transient nature of the acute deficits. The minor and transient nature of these acute behavioral deficits corroborate the mild nature of TBI in the model.

Immunophenotyping of the cellular composition in the brain post injury demonstrated a dynamic and temporally distinct inflammatory response post mild TBI. Initially, macrophages ($CD45^{hi}$ $CD11b^{+}$) increased in number from 6 h and peaked at 3-DPI on the ipsilateral side, while neutrophils peaked quickly within 24 hours. This first-line increase in innate response involving neutrophils and proinflammatory macrophages are similar to the initial stages of injury response demonstrated by other studies.[525,526] Both immigrating and resident macrophages when activated, are involved in removing cellular debris and in repairing damage caused by the primary insult.[152,527,528]. In the model of mild TBI, this innate response is isolated to the ipsilateral side of injury and seen only on $CD45^{hi}$ macrophages and not on the microglial ($CD45^{int}CD11b^{+}$) cells. The numbers of infiltrating macrophages return to levels seen in sham mice by 7-DPI, without any elevation of the activation markers assessed.

However, at 15-DPI, macrophages that were still present in the injured brain had increased expression of CD86 and MHC II. This elevation of activation markers was seen through 30-DPI. Presence of CD86 on macrophages is considered an indication of proinflammatory cell activation. CD86 is involved in antigen presentation along with its costimulatory molecule CD80, which induces production of proinflammatory cytokines.[529-533] Presence of CD86 marker indicates activation of macrophages in the brain at chronic time points (15 and 30-DPI) are still in a proinflammatory state and are known to alter neuronal functions either directly by pruning synapses of damaged axons or indirectly through cytokine mediators.[88,152,527,528] Future studies are needed to understand the function of these activated macrophages and how that relates to the behavioral and cognitive deficits.

Since macrophages can alter neural networks and connections through synaptic pruning and cytokine production,[88,152,527,528] the neuroinflammatory milieu was examined using quantitative RT-PCR. Injured brains at 3-DPI, had elevated levels of TNF-α, TGF-β, INF-γ, ARG1 and IL-10 RNA transcripts. TNF-α, TGF-β, and ARG1 are secreted by macrophages,[534-538] while INF-γ is primarily produced by T cells and natural killer cells,[539] and IL-10 is expressed by many immune cells.[540] TNF-α and INF-γ promote inflammatory responses and activate macrophages,[534,535,539] while TGF-β, ARG1, and IL-10 suppress macrophage activation and promote healing.[536-538,540] In the acute phase, activation of the macrophages is required to mobilize response to injury. However, suppression of that response is also necessary to limit the extent of damage.

Chemokines are responsible for trafficking immune cells to sites of injury aiding in the inflammatory response to TBI. The level of mRNA was measured for CCR2, which was also elevated at 3-DPI, coinciding with increasing brain macrophages. CCR2 is the receptor for CCL2 and involved with chemotaxis of monocyte/macrophages. Interestingly, suppression of CCR2 reduces macrophage immigration post TBI and suppresses a proinflammatory type I interferon response in the brain [280]. Interfering with this chemokine axis to reduce monocyte trafficking into the brain has been proven to improve behavioral outcomes [286].

However, most cytokine/chemokine mRNA levels elevated at 3-DPI decreased by 15-DPI. Only, IL1-β and CXCL10 were elevated. IL1-β is a proinflammatory cytokine produced by activated monocytes and macrophages[541], and play an important role in altering behavioral outcomes.[280] CXCL10 is also an activator for macrophages, but is produced by many different cell types, including lymphocytes.[542,543] It is likely that these cytokines are responsible for activating the macrophages into a proinflammatory phenotype at the chronic stage of TBI, and suggests an activated lymphocyte response that was not initially evident in this study. However, these measurements are based on the mRNA levels and do not necessarily reflect translation to protein. The cytokine and chemokine expression data illustrates a change in inflammatory mediators from the acute (3-DPI) to chronic (15-DPI) response after TBI. Further investigation is needed to understand their role in the pathogenesis of TBI.

Long-term spatial learning deficits demonstrated by the Barnes maze test coincided with an activated macrophage response at 30-DPI. The heat maps and trace plots illustrate the difference in exploration patterns between sham and injured mice (Fig 2.11G) and reflects the significantly difference in the time spent in the goal and opposite quadrants (Fig 2.11H).There are a few papers recording long-term side effects in mice following mild TBI,[40,97,337,544,545] and of those that do observe long term effects, more intense and diffuse impacts were used to achieve these effects, or injure the animal multiple time. This study was designed to utilize sensitive behavioral assays to evaluate the minor differences of a single, discrete injury. Many human patients, develop post concussive syndrome,[505,508] which can persist months to years after initial injury. But out of those having long-term deficits, about 5-15% will develop disabling conditions.[12,503,504,508,509]

The IHC analysis revealed the progression of the inflammatory response to areas distal from the initial injury as the chronic stage of the response commences. The most drastic changes occurred in the *hippocampus*, with evidence of increased immunostaining for Iba1 and GFAP in injured mice, suggesting an increased infiltrate of microglia/macrophages, and presence of activated astrocytes. There is also a change in patterns of NeuN staining at 30-DPI in injured mice compared to sham. The inflammatory response resulting from the injury affects areas of the brain distal to injured sites which can occur through the neurochemical dysregulation of neurotransmitter receptor trafficking to synapses in catecholaminergic and cholinergic systems.[88,152,527,528,546,547] Further investigation is needed to determine the functionality of these cells in this region.

IHC analysis also provided evidence for widespread inflammation. At the site of injury, Iba1 staining of microglia/macrophages are increased at 3 and 30-DPI over sham, coinciding with previous findings that these cells infiltrate damaged areas and clear debris.[280,548-552] GFAP+ astrocytes follow the same pattern, which is consistent with astrocytes invading areas of damage to form scars.[551-553] At 30-DPI, there is a large area that has no cells present. Fragility in the tissue, possibly created by glial scaring, during slicing may have contributed to the loss. There is a loss of neurons in the injury site, which persisted and worsened into 30-DPI. In the *thalamus* and ventral midline area, the change in morphology from ramified to bushy of Iba1 staining that persisted at 30-DPI in the TBI

group only may indicate chronic activation of macrophages and microglia.[554-556] Further investigation is needed to confirm the function of the inflammatory cells within these areas.

In the preceding findings, well-defined model of mild TBI was developed that can be used to investigate long-term deficits of brain injury. The study demonstrates an initial proinflammatory response that appears to resolve and re-establish at the chronic stage of recovery. This change in inflammatory response is further validated by the migration of immune cells evidenced in IHC analysis, and the alternation of the cytokine response as evidenced by mRNA levels.

Conclusions

The present study demonstrates that mild TBI leads to a persistent activation state of macrophages. Behavioral deficits start as minor motor deficits which are resolved in 7 days. But injured mice exhibit long-term learning deficits later, which provides a model to test novel treatment paradigms to counteract the long-term cognitive deficits in TBI. These findings demonstrate that the sustained inflammatory response extends from the cortical regions to deeper regions of the brain as the injury progresses from acute (3-DPI) towards a chronic injury phenotype (15-DPI). This sustained inflammation at the *hippocampus* correlates with the spatial learning consequences observed in the mild TBI model. Further investigation into the differences in acute vs. chronic inflammatory milieu may provide the basis for identifying previously unknown mechanisms and targets for treatment.

Chapter 3

Mild traumatic brain injury increases preference to and consumption of opiates in mice and is associated with a chronic proinflammatory neuroimmune response

Abstract

Individuals with a history of TBI have an increased risk of developing substance use disorder (SUD) at 3-6 times that of the general public, and there has been a recent rise in SUD among adolescents. Therefore, the previously described model of mild TBI was used to investigate the effect of mild TBI on morphine's rewarding properties. Controlled cortical impact (CCI) was utilized to induce injury in 8–12-week-old mice and 5 days later, mice were subjected to either intravenous (IV) self-administration or conditioned placed preference behavioral (CPP) protocols. During IV self-administration, injured mice escalated their intake above that of sham-injured mice. CPP was then utilized to examine sensitivity to morphine. Injured mice established place preference at every dose of morphine and spent significantly more time on the morphine paired side at low dose morphine exposure (0.5 and 1 mg/kg) than the sham-injured mice. When examining the inflammatory infiltrate in the brain, there was elevated macrophages, neutrophils, and lymphocytes on the ipsilateral side of injury in injured mice post exposure to morphine compared to sham-injured mice. These findings demonstrate increased proinflammatory infiltrate on the ipsilateral side of injury after exposure to both high and low dose morphine that correlates with an escalation of intake and increased preference to morphine. Future studies will focus on the mechanisms linking the behavioral alterations leading to increased consumption of opiates following TBI and increased neuroinflammation.

Introduction

Individuals who have a history of traumatic brain injury (TBI) have a rate of substance use disorder (SUD) of 37-66%, which is extremely high considering the general public has a rate of 11%.[28,29,132] The second most vulnerable group to receive a TBI is adolescents and young adults, with sports related injury and motor vehicle accidents as the most likely cause.[22-25] There is also a significant rise in SUD among adolescents (age 12-18).[190] These data point to the increased risk for adolescents to have SUD and TBI as comorbidities, and underlines the need for understanding the mechanisms that link these conditions.

In the US, TBI affects approximately 3 million people each year,[17] with approximately 75-90% of the population suffering from mild TBI.[11,502,503] However, many of the symptoms of mild TBI are often unnoticed or not reported, so the true extent of its prevalence may be underestimated.[11,503] A mild TBI is caused by a low force trauma and results in a transient altered mental state with symptoms ranging from headaches and anxiety to sleep disorders.[18,506-508] While most individuals see a resolution in these symptoms within a few days, a small subset can develop chronic conditions that persist for months to years after injury.[12,503-505,508,509] This finding highlights not only the importance of diagnosis and recognition of mild TBI, but also the prevalence that puts millions of people at risk for long-term consequences.

The response to mild TBI is temporally dynamic, with an initial physical injury causing tissue deformation and axonal shearing, followed by the immune system's response that exacerbates damage.[132,512,513] Studies have demonstrated that the trauma can extend from the injury site to subcortical areas.[14,151,153,231,514,557] Studies in humans found damage due to axonal shearing in the *thalamus* and *ventral tegmental area* are linked to deficits in memory and cognition.[231,514,515] Animal studies demonstrate there is chronic inflammation, increased blood brain barrier permeability, and changes in neural networks within the reward pathways of the brain that coincides with increased rewarding effects of cocaine.[151-153] While there are many bidirectional links between TBI and alcohol use, alcohol consumption increases after injury, and this effect can be ameliorated in animal models by modulating the immune response.[207,558-560] These studies indicate that even mild TBI can

cause long lasting damage to distal areas of the brain, and suggests that inflammation within the reward pathways caused by TBI changes the outcome of exposure to illicit substances.

While studies have linked TBI with altered outcomes to alcohol and cocaine exposure,[151-153,207,558-560] the link to opiate use is still not well understood. The previously described model of mild TBI (Chapter 2) using controlled cortical impact (CCI) was used to induce brain injury in a discrete and reproducible manner.[95,105] In order to understand the effects of mild TBI on the rewarding effects of opiates, two drug seeking behavioral assays were employed: intravenous (IV) self-administration and conditioned placed preference (CPP). The IV self-administration paradigm allows the animal to freely dose morphine for a fixed period of time to examine escalation of drug intake post TBI.[133] CPP pairs a controlled substance and dose to a given environment, allowing for investigation of preference for the drug.[453-455] The objective of this present study is to examine how mild TBI affects the escalation and preference to morphine, as well as to determine how exposure to morphine modulates the chronic inflammatory response to injury. I hypothesize that mice with mild TBI will escalate drug intake and increase their preference to morphine.

Methods

Animal Use

All procedures and experiments involving animals were conducted in accordance with protocols approved by the University of Minnesota Institutional Animal Care and Use Committee. Female C57/B6 mice at 8-12 weeks of age were purchased from Jackson Labs and housed in environmental controlled micro-isolator cages with Enviro-dry environmental enrichment. Animals were acclimatized to their new environment for at least one week prior to obtaining baseline data for behavioral studies. For IV self-administration studies, groups consist of injured mice exposed to morphine, injured mice with catheter flushing with saline only, and sham-injured mice exposed to morphine (n=3-5). For CPP assessment, groups consisted of animals exposed to low dose morphine (0.5-5mg/kg) with both sham-injured and injured mice (n=8-16).

Surgical Induction of Traumatic Brain Injury

For full details, please refer to the methods presented in Chapter 2. Briefly, mice were 8-12 weeks old at the time of mild TBI or sham injury induction. However, mice were not given buprenorphine prior to surgery. Buprenorphine is an opiate and as such, the delivery of it can affect the immune response at the induction of injury. Specifically, buprenorphine can affect decrease the migration of CCL2 chemotaxis and acute functions of microglia and astrocytes.[561-564] Thus, mice were anesthetized with isoflurane, placed in a stereotaxis apparatus to secure the head, and topical analgesia was used for pain control. In conjunction with UMN Research Animal Resources (RAR) veterinary consult, lidocaine (4mg/kg) was administered at the incision site for immediate numbing since it has an onset of 1-2 minutes and lasts 1-2 hours. For longer lasting pain control, bupivacaine (1-2mg/kg) was utilized at the incision site; though it's onset of action is 10-15 minutes, it is longer acting than lidocaine (4-8 hours).[565,566] After application of the lidocaine/bupivacaine mixture, an incision was made at the dorsal cranium and a 2.5mm craniotomy was made over the right primary and secondary motor cortices. A 2mm impact tip was used to inflict a CCI at a velocity of 4m/s, depth of 1mm, and dwell time of 100ms. Sham-injured mice had the same procedure without impact. Mice were recovered in a clean home cage with a heating pad and monitored closely for 3 days post TBI.

Jugular Catheterization

Mice that reached acquisition goals on the operant chamber (see IV self-administration protocol and Fig. 3.1 for timeline) were implanted with a jugular catheter at least 5 days before induction of TBI as previously described.[133] Mice were anesthetized with isoflurane and the back and throat area are shaved and swabbed with betadine. Carprofen (5mg/kg subcutaneous (SC)) and gentamycin (5mg/kg SC) were administered to reduce post-operative inflammation and pain. A 1cm incision is made on the dorsal side of the mouse in the mid scapular area. That area was then covered, and the mouse turned over so that a 1cm incision can be made on the neck to expose the right jugular vein. The catheter is a 6cm 3/2 Fr polyurethane catheter tubing (Access Technologies) connected to a 25ga metal tubing of a Vascular Access Button (VAB, Instech). The VAB has a septum needle

connection which allows for quick aseptic connections with a catheterized mouse. The tubing was passed subcutaneously from the dorsal incision to the ventral incision. The jugular vein was exposed through blunt dissection and a small incision was made to pass the catheter into the vein. The catheter was secured with sterile sutures above and below the incision site. The ventral neck incision was then closed, and the mouse was turned over to expose the dorsal incision. The VAB was then secured to the skin with nonabsorbable sutures, and the rest of the incision is closed with a buried suture pattern. The mouse is then recovered in a clean and dry cage on a heating pad. Mice were closely monitored until recovery. For three days following the implantation, mice were injected with Carprofen (5mg/kg SC) to control inflammation and pain. The catheters were flushed with 0.05 ml of heparinized (20 IU/ml) bacteriostatic saline containing gentamicin sulphate (0.33 mg/ml) once every day to maintain patency. For the remainder of the experiment, the mice were weighed daily and monitored for health issues.

Behavioral Assays

All behaviors assays were performed at the University of Minnesota Mouse Behavior Core facilities. All drug seeking paradigms were performed 5 days post injury (DPI).

Short Access IV Self-Administration

The drug IV self-administration paradigm was used to examine escalation of morphine intake by previously described methods.[133] Mice were maintained on a restricted food intake diet of 1.5-2g per day per mouse to facilitate training in operant chambers. Mice were trained to press the right lever to obtain food reinforcement (20uL of Ensure diluted with 25% water), while the left lever does nothing. Training was continued until acquisition criteria are met (20 reinforcements for 3 consecutive days) or for 10 days. Animals that did not meet acquisition criteria were not included in experimental groups were not used for IV morphine infusion but were used in the saline treated injured group that did not utilize the operant chamber and were subjected to only IV flushing each day. Animals then underwent catheter placement (See jugular catheterization

methods) and 5 days later were given mild TBI/sham injury (See Fig 3.1 A for timeline)).

At 5-DPI, mice were placed on a short access IV self-administration protocol derived from published sources (n=7-9).[567] Mice were placed in the operant chamber and connected to 10mL syringe pump through a fluid swivel. They were placed on a single lever-press response on the active lever (FR1) protocol that delivered an intravenous morphine infusion (0.08mg/mL) in 25uL of saline over 1.25sec. A visual cue is paired with the delivery of the morphine, as was done for the food reinforcement, which consisted of a light over the lever. The house light was turned off, and the injection pump sounds with the light cue was followed by an 8 sec time out where lever pressing has no program consequences. Mice are allowed open access to the operant chamber for 2 hours, once daily for 10 days total. The total morphine dose allowed was capped at a 100 infusions per day. No animals approached the capped limit on any test day).

Conditioned Place Preference

Conditioned placed preference (CPP) was carried out through the UMN Behavior Core as previously described.[453-455] CPP is a behavioral test that allows for the evaluation of the rewarding or adverse effects of a given substance, in this case, morphine. A 2-chamber apparatus was used to evaluate the response to morphine exposure. The apparatus is separated by an opaque wall with a guillotine style door. The two resulting chambers had a grid or mesh style floor to assist in providing conditioning cues for the mice. One floor was paired with morphine exposure and the other with saline. The pairing of drug or saline was randomly assigned and counterbalanced to eliminate the flooring style as a factor in preference. During training, the mice were exposed to either morphine or saline each day and confined to the paired chamber for 15 minutes. On testing days, mice were not exposed to any substance, and the guillotine style door was open, allowing for free exploration for 20 minutes. All data was recorded by ANY-maze software (Stoelting Co., Wood Dale, IL).

Mice were initially trained for 1 week on subcutaneous injections of 0.1mL saline once daily to lessen the stress associated with injection and restraint. After injection training, mice received mild TBI or sham surgery. Baseline preference between chambers was then evaluated at 4-5-DPI. Preference was calculated by subtracting the time on saline paired side from the time on the drug paired side, and mice were divided in groups so that baseline preference for the drug side was close to zero. Rewarding effects are demonstrated by positive differences, where aversion is indicated by negative differences. There were 4 training sessions per week, 1 per day, with saline or morphine administration for a total of 8 days. At 5-DPI and 15-DPI, the guillotine style doors were removed, and the mouse freely traveled between the two environments to determine the establishment of preference after training.

Flow Cytometry

Mice were sacrificed after behavioral protocols at 15-DPI as described in Chapter 2 methods. Briefly, mice were anesthetized with isoflurane and perfused with cold PBS, brains collected and divided by hemisphere for homogenization into single cell suspension in a Percoll gradient. Cells were removed and washed with PBS+2% FBS and transferred to a 96-well plate. Cells were incubated with Fc block for 5min, followed by adding cell surface antigens. Cells were washed, fixed with PFA, and again washed before resuspension in FACS buffer with AccuCount beads (Spherotech, Lake Forest, IL, USA) for analysis on a flow cytometer (BD FACSCanto) at UMN Flow Cytometry Core. See table 2.1 for details on the staining panels. Isotype specific antibodies were again used to control for nonspecific antibody binding. Immunostained cell populations were again analyzed using FlowJo software (BD Biosciences, Ashland OR). See Chapter 2: Fig. 2.4 for macrophage panel gating strategy and Fig 2.8 for lymphocyte panel gating strategy.

Statistical analysis

All assays were analyzed using Graph Pad PRISM with one and two-way ANOVA, Welch's t-test and multiple unpaired t-tests. p-values < 0.05 were considered as significant differences.

Results

Brain injured mice increase morphine intake during short access IV self-administration protocol

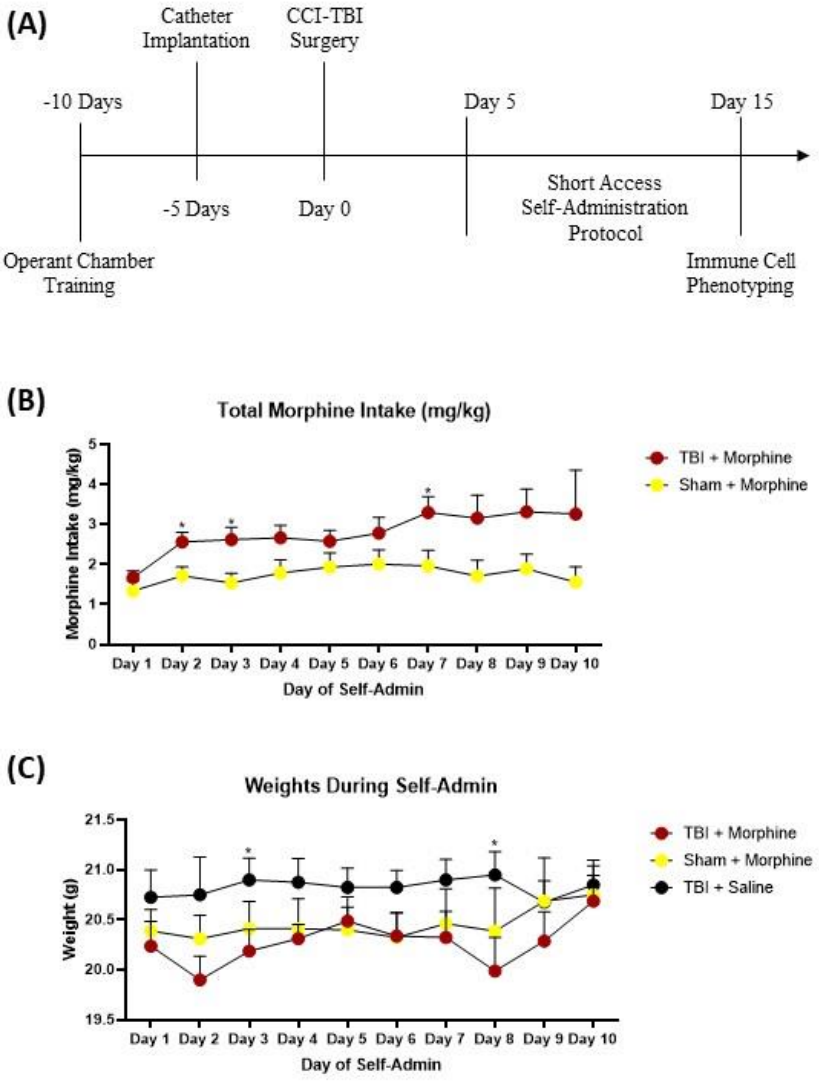

Fig 3.1 Injured mice increased opiate intake during self-administration. The timeline (A) of the experiment is presented through 15-DPI. Total opiate intake (B) was calculated as the total opiate infused during the 2hr short access self-administration session divided by the weight of the mouse taken that day (mg/kg). Body weights throughout the experiment (C) are presented for injured and sham-injured mice, with the weights of the third experimental group who had mild TBI received saline. Significance calculated utilizing multiple unpaired t-tests on GraphPad Prism software, p values of 0.05 or lower were considered significant (*p≤0.05, **p≤0.01, ***p≤0.005).

Five days after injury, mice implanted with a jugular catheter were put on a short access IV self-administration protocol (Fig. 3.1A). Injured mice escalated their intake of morphine (Fig. 3.1B) above that of sham-injured mice, with significant increases on day 2, with injured mice consuming 2.6±0.7 mg/kg morphine and sham at 1.7±0.6 mg/kg morphine. At day 3, injured mice consumed 2.6±0.9 mg/kg morphine and sham at 1.5±0.7 mg/kg morphine; and at day 8, injured mice consumed 3.3±1.1 mg/kg morphine and sham at 0.34±0.02 mg/kg morphine (*multiple unpaired t-tests, p=0.0209, 0.0158, 0.0315 respectively*). Two-way ANOVA reveals that the TBI group had significantly elevated consumption of morphine in comparison to the non-TBI group (*p=0.0221*). However, there was no group x time interaction. When examining body weight (Fig. 3.1C), injured mice exposed to opiates had lower weights than the injured mice that were given saline. This decreased body weight was evident by significant differences at 3-DPI, where injured mice exposed to morphine weighed 20.2±0.2 g and saline treated injured mice weigh 20.9±0.2 g; and day 8, where injured mice exposed to morphine weigh 20.0±0.3 g and saline treated injured weigh 21.0±0.2 g (*p=0.0464, 0.0405 respectively*).

Brain injured mice exposed to morphine have increased macrophage and neutrophil infiltration on the ipsilateral side of the injury

Total macrophage ($CD45^{hi}CD11b^{+}Ly6G^{-}$) and total neutrophil ($CD45^{hi}CD11b^{+}Ly6G^{+}$) infiltrate were increased in the injured mice exposed to morphine on the ipsilateral side of injury (Fig 3.2). Total macrophage infiltrate (Fig. 3.2A) was significantly increased in injured mice exposed to morphine (84,579±6,018 cells) over saline treated injured mice (45,084±11,344 cells) and morphine treated sham-injured mice (53,903±9381 cells; *Welches t-test, p=0.0296, 0.0200; one-way ANOVA, p=0.0147*). Total neutrophil infiltrate (Fig. 3.2B) in morphine exposed injured mice (24,848±4,054 cells) was elevated significantly over saline treated injured mice (8,940±4,870 cells; *Welches t-test, p=0.0407*). There was a trend towards increased expression of CD206 (Fig, 3.2D) and Ly6c (Fig. 3.2E&F) on macrophages in injured mice exposed to morphine, but it did not rise to the level of significance.

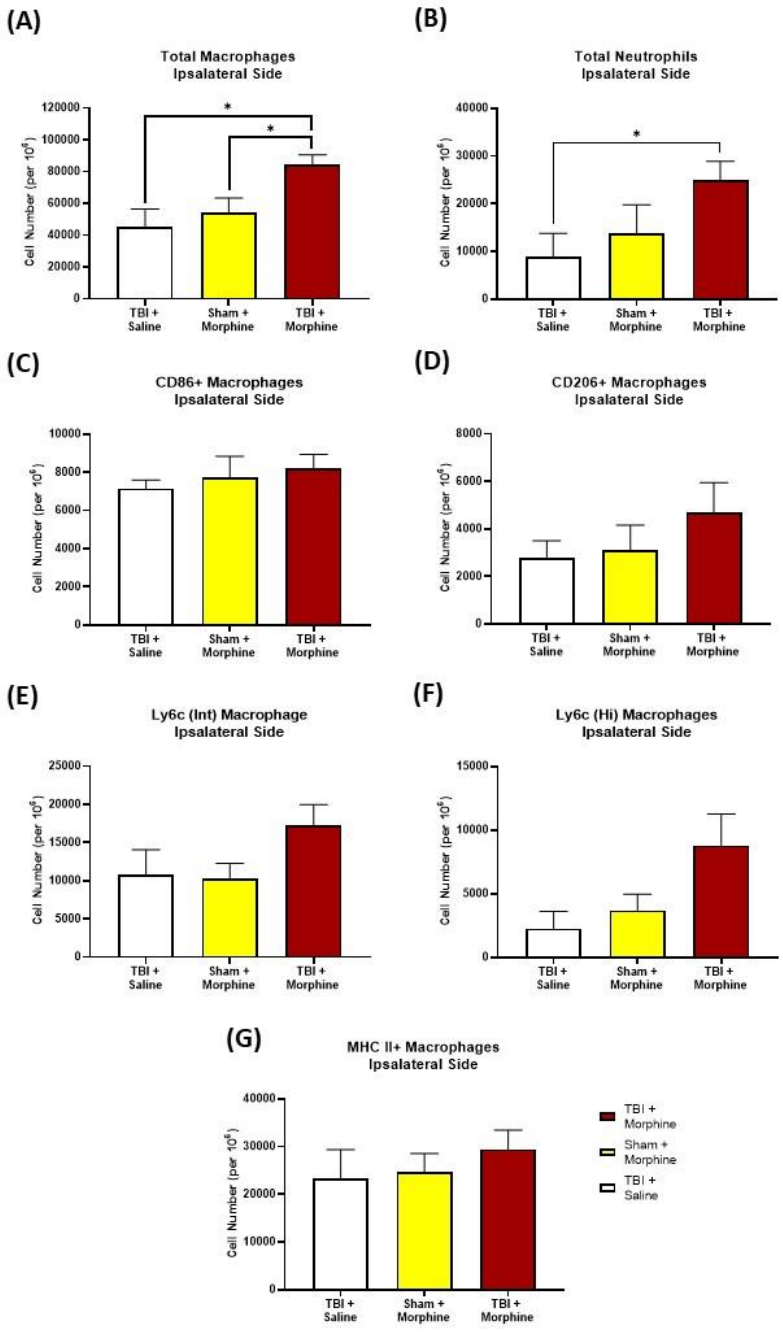

Fig. 3.2 Injured mice have increased macrophage and neutrophil infiltration in the ipsilateral side of the brain. Graphs show the number of the cell of interest per 10^6 cells on the Y axis, with group label on the X axis. All graphs shown are from the ipsilateral side of injury. Total macrophages (A) are identified as $CD45^{hi}CD11b^{+}Ly6G^{-}$. Total neutrophils (B) are identified as $CD45^{hi}CD11b^{+}Ly6G^{+}$. The remaining graphs refer to the expression of markers on the total macrophages, with analysis of CD86 (C), CD206 (D), Ly6c (E&F), and MHC II expression (G). Significance calculated utilizing Welch's unpaired t-tests on GraphPad Prism software, p values of 0.05 or lower were considered significant (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$).

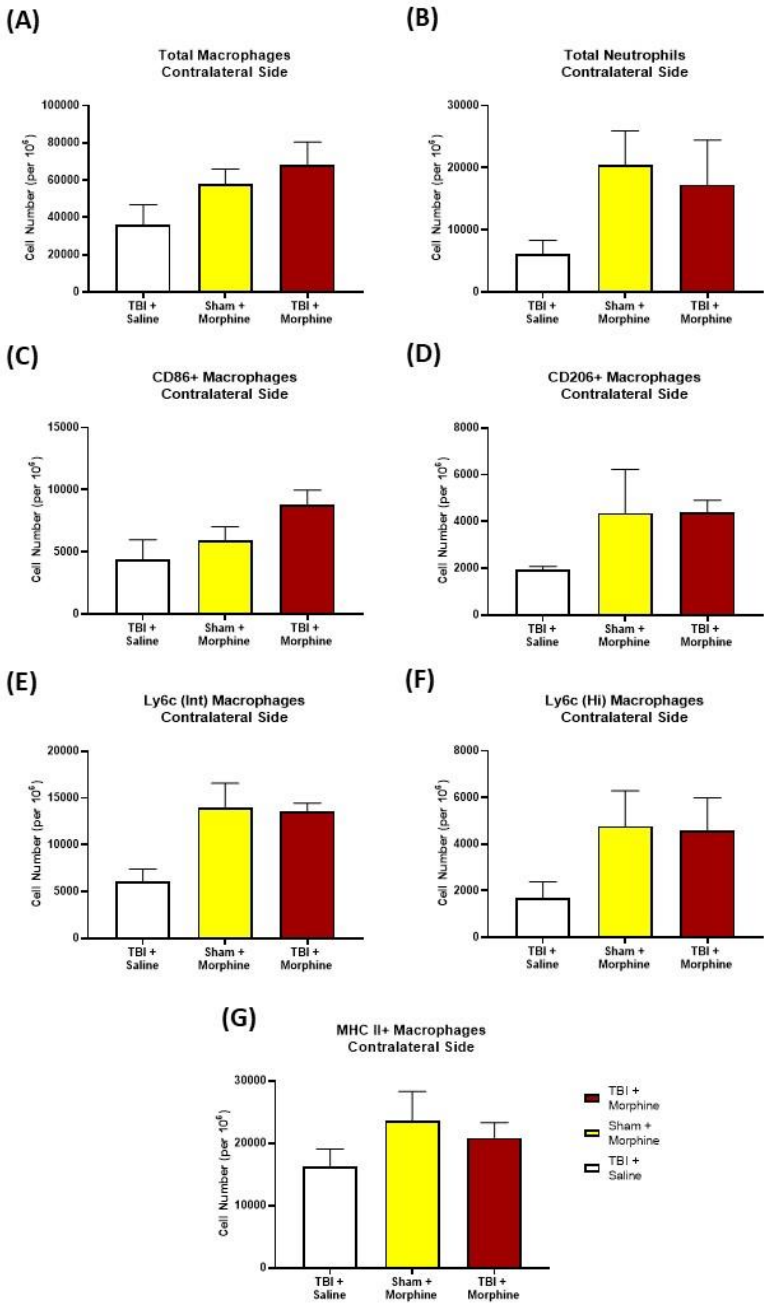

Fig. 3.3 There are no significant changes to the macrophage and neutrophil infiltrate on the contralateral side. Graphs show the number of the cell of interest per 10^6 cells on the Y axis, with the group label on the X axis. All graphs shown are from the contralateral side of injury. Total macrophages (A) are identified as $CD45^{hi}CD11b^{+}Ly6G^{-}$. Total neutrophils (B) are identified as $CD45^{hi}CD11b^{+}Ly6G^{+}$. The remaining graphs refer to the expression of markers on the total macrophages, with analysis of CD86 (C), CD206 (D), Ly6c (E&F), and MHC II expression (G). Significance calculated utilizing Welch's unpaired t-tests on GraphPad Prism software, p values of 0.05 or lower were considered significant.

The increased infiltrate is isolated to the ipsilateral side of injury. There are increases in different cellular populations on the contralateral side to injury (Fig, 3.3) in groups exposed to morphine, but none of the trends rise to the level of significance. The same pattern is true for the microglia population (Fig. 3.4) on both sides of the brain.

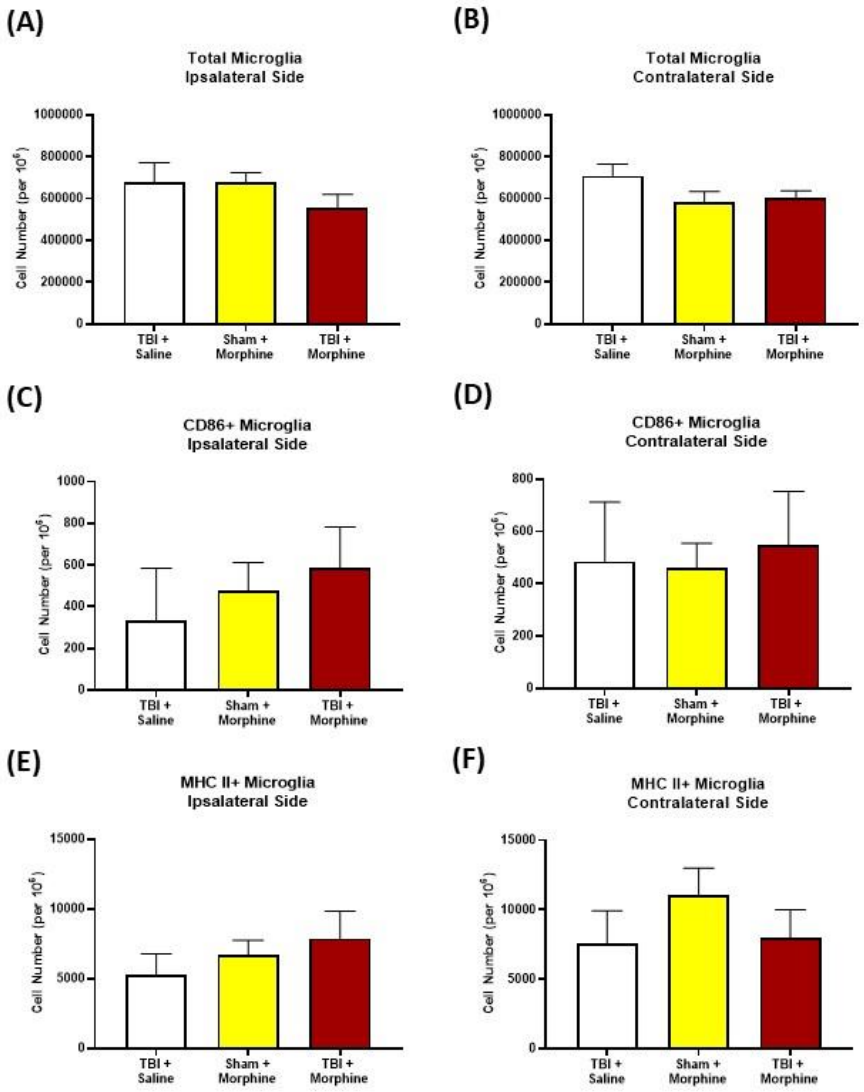

Fig. 3.4 There are no significant changes to the microglia population. Graphs show the number of the cell of interest per 10^6 cells on the Y axis, with the group label on the X axis. Total microglia on the ipsilateral (A) and contralateral (B) side are identified as $CD45^{int}CD11b^{+}$. That population was then examined by for the expression of CD86 on the ipsilateral (C) and contralateral (D) side, and for the expression of MHC II on the ipsilateral (E) and contralateral (F) side. Significance calculated utilizing multiple unpaired t-tests on GraphPad Prism software, p values of 0.05 or lower were considered significant.

Injured mice exposed to morphine have increased lymphocyte infiltrate on the ipsilateral side of injury

Injured mice that are exposed to morphine have increased lymphocyte infiltrates into the ipsilateral side of the brain. The total lymphocyte ($CD45^{hi}CD11b^{-}$) number (Fig. 3.5A) was

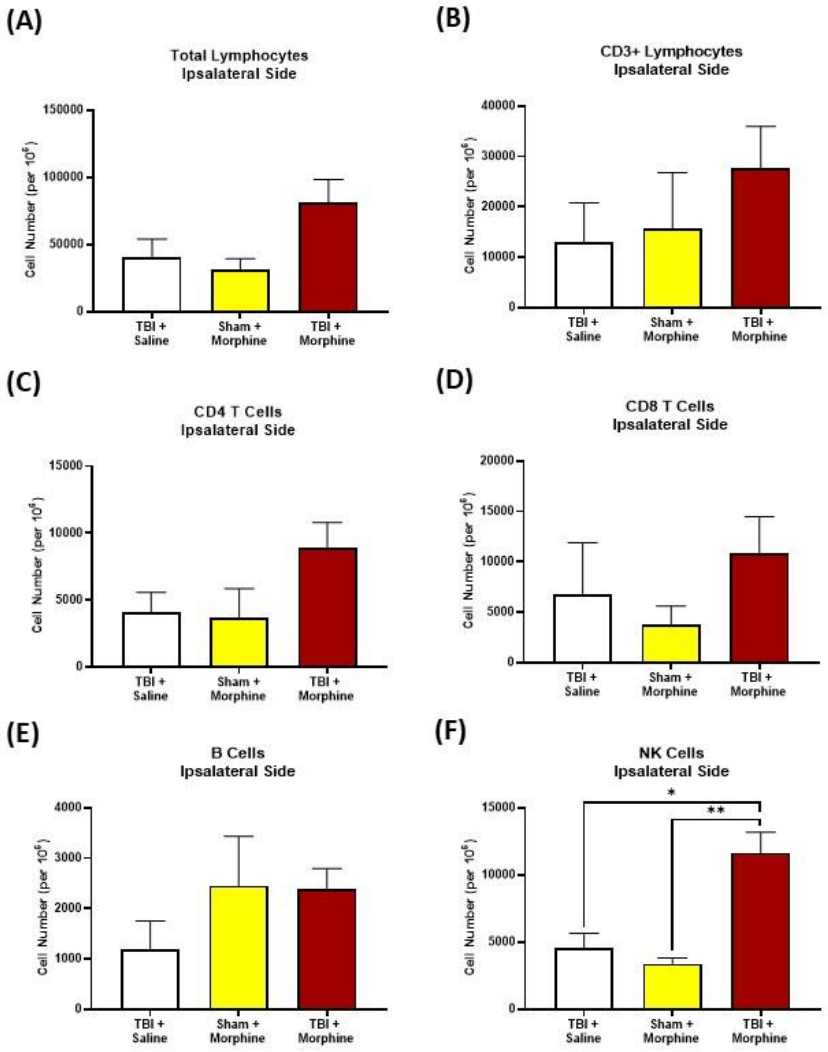

Fig. 3.5 Injured mice have an increase in lymphocyte infiltrate on the ipsilateral side of injury. Graphs show the number of the cell of interest per 10^6 cells on the Y axis, with the group label on the X axis. All graphs shown are from the ipsilateral side of injury. Total lymphocytes (A) are identified as $CD45^{hi}CD11b^{-}$. Lymphocytes were then evaluated for CD3 expression (B), and those that were $CD3^{+}$ were further evaluated for CD4 (C) and CD8 (D) expression. Lymphocytes that are $CD3^{-}$CD 19^{+} were labeled as B Cells (E). Cells that are $CD3^{-}CD19^{-}$ were evaluated for NK 1.1 expression, and positive cells were labeled natural killer (NK) cells. Significance calculated utilizing Welch's t-tests on GraphPad Prism software, p values of 0.05 or lower were considered significant (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$).

significantly different among groups; injured mice exposed to morphine had 81,514±16,953 cells, saline treated injured mice had 40,520±13,576 cells, and sham-injured mice exposed to morphine had 31,635±7,649 cells (*one-way ANOVA, F=-4.638 p=0.0409*). Total NK cells ($CD3^-CD19^-NK1.1^+$, Fig. 3.5F) are also significantly different from one another, with morphine-exposed injured mice had 11,642±1,539, and saline treated injured mice had 4,590±1,072 cells, and exposed sham- injured mice had

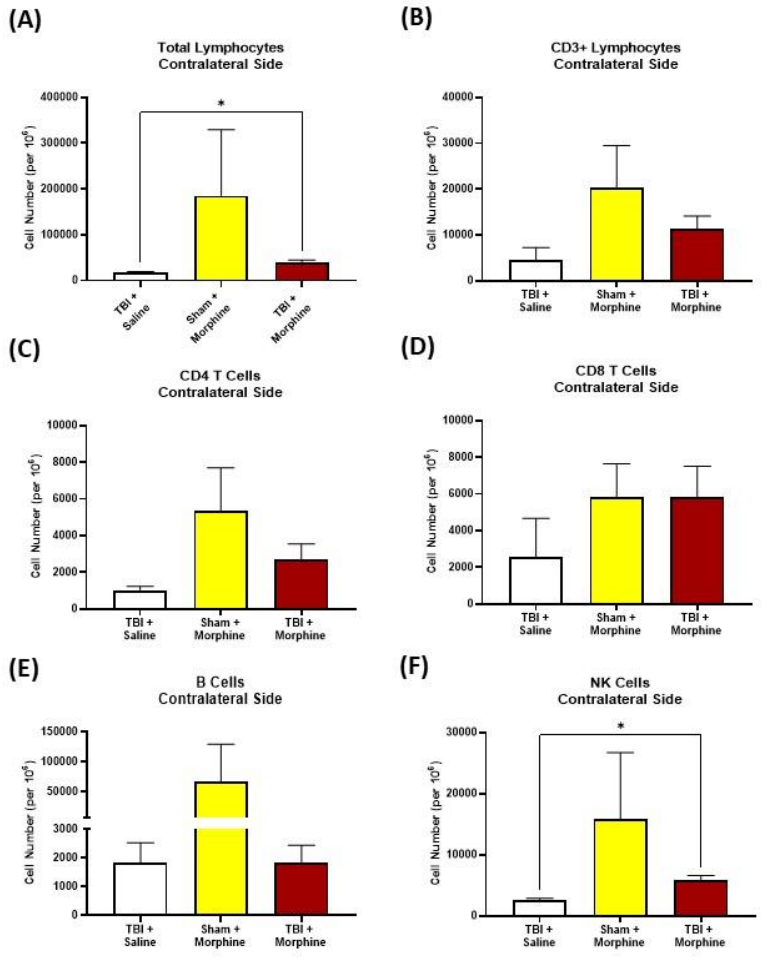

Fig. 3.6 Injured mice have a decreased in lymphocyte infiltrate on the contralateral side of injury. Graphs show the number of the cell of interest per 10^6 cells on the Y axis, with the group label on the X axis. All graphs shown are from the contralateral side of injury. Total lymphocytes (A) are identified as $CD45^{hi}CD11b^-$. Lymphocytes were then evaluated for CD3 expression (B), and those that were $CD3^+$ were further evaluated for CD4 (C) and CD8 (D) expression. Lymphocytes that are $CD3^-CD\ 19^+$ were labeled as B Cells (E). Cells that are $CD3^-CD19^-$ were evaluated for NK 1.1 expression, and positive cells were labeled natural killer (NK) cells. Significance calculated utilizing Welch's t-tests on GraphPad Prism software, p values of 0.05 or lower were considered significant (*$p \leq 0.05$).

3,379±442 (*Welch's t- test, p=0.0137, 0.0096; one-way ANOVA, p=0.0006*). The $CD3^+$ (Fig. 3.5B), CD4+ (Fig. 3.5C), and CD8+ (Fig. 3.5D) T cell populations were all elevated in the morphine exposed injured mice, but those trends did not rise to the level of significance.

On the contralateral side of injury, there were less lymphocytes in injured mice than in the sham-injured mice regardless of morphine exposure, though these trends did not rise to the level of significance. In the total lymphocyte population (Fig. 3.6A), morphine exposed injured mice (39,221±5,141 cells) were significantly elevated over saline treated injured mice (18,295±1,038 cells; *Welch's t-test, p=0.244*), but both were less than morphine exposed sham-injured mice (185,232±144,270 cells). NK cells (Fig. 3.6F) were also elevated in morphine exposed injured mice (5,781±816 cells) over saline treated injured mice (2,611±296 cells; *Welch's t-test, p=0.0244*), but again sham-injured mice (15,868±10,827 cells) had higher average numbers and not significantly different from injured morphine exposed animals.

Brain injured mice establish conditioned placed preference (CPP) to low dose morphine

Mice tested for CPP were first given mild TBI or sham injury. (Fig. 3.7A). The surgical time (Fig. 3.7B) to complete the mild TBI (7.9±0.3min) took significantly longer than the time for sham (4.7±0.1 min; *unpaired t-test, p<0.0001*). The recovery time from surgery (Fig. 3.7C) also took significantly longer for injured mice (13.3±0.3min) than for sham-injured mice (8.3±0.3min; *unpaired t-test, p<0.0001*). A simple linear regression (Fig. 3.7D) was utilized to examine the relationship between surgery time and recovery time. For sham-injured mice, there was a positive correlation with a slope of 0.4668, while injured mice had a negative correlation of -0.4339. When examining the two groups, there is a 0.4117% chance of randomly generating data with slopes this different, thus confirming this finding as significantly different. This data is consistent with that seen in Chapter 2.

At 4-5-DPI, baseline preference data was obtained and were used to divide mice into dosage groups, so that had no overall preference within groups for either side. Mice were given morphine sulfate SC at 0.5-5mg/kg dose and tested for the establishment of place preference at day 5 and 10 of CPP (10 and 15-DPI). By the week 2 preference test,

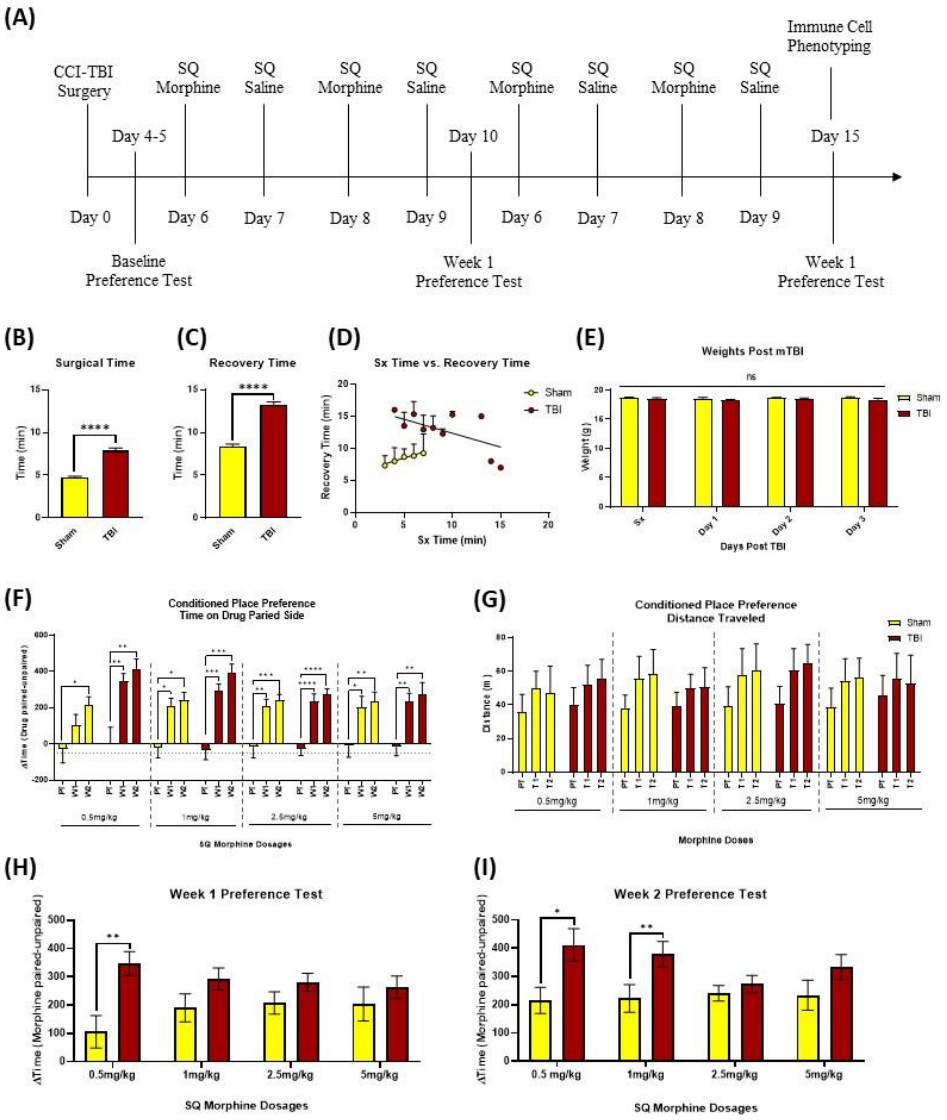

FIG 3.7 Mice receiving mild traumatic brain injury establish preference to low dose morphine. (A) Timeline for the experimental paradigm through 15 days post injury. Mice were subjected to controlled cortical impact brain injury under isoflurane. Surgical time (B) was recorded as time from first incision to suture closure and recovery from surgery (C) was the time from removal of anesthesia to the time the mouse could right itself and move around the cage (n=39). Simple linear regression (D) plotting the relationship between surgical time and recovery time. Weight post-surgery (Sx) was measured on the day of surgery and continued to 3-DPI (E). During conditioned placed preference, the change in time on drug paired (F) was calculated by subtracting the time on the nonpaired side from the time on the drug paired side. Distance travels during preference tests was also tracked by Any Maze software. Change in time between drug paired and saline paired side was compared between injured and sham injured mice at week 1 (H) and week 2 (I) preference trials. Significance calculated utilizing multiple unpaired t-tests on GraphPad Prism software, p values of 0.05 or lower were considered significant (*p≤0.05, **p≤0.01, ***p≤0.005).

all groups had established place preference (Fig 3.7F) as evident from a significant difference from baseline behavior, showing a positive change with increased time spent on the drug paired side. In the group given 0.5mg/kg morphine, injured mice demonstrated a pronounced difference in time spent on the drug paired side, with baseline change time at 6.6±86.4 sec to week 1 preference test at 346.8±42.3 sec (*unpaired t-test, p=0.0080*). The sham-injured mice had no significant difference, with baseline change time at -27.6±78.3 sec to week 1 preference test at 105.4±57.3 sec (*unpaired t-test, p=0.1919*). There were slight differences in the distance traveled (Fig. 3.7G) between the baseline, week 1, and week 2 preference test, but none rose to the level of significance.

At week 1 and week 2 preference trials, the time spent on the drug paired side was compared between injured and sham-injured mice. At the week 1 preference trial (Fig. 3.7H), injured mice exposed to 0.5 mg/kg morphine spent significantly more time (346.8±42.3 sec) on the drug paired side than the sham-injured mice (105.4±57.3 sec; *multiple unpaired t-tests, p=0.0079*). At the week 2 preference trial (Fig 3.7I), injured mice spent more time on the drug paired side when exposed to 0.5 mg/kg (411.4±153.4 sec) and 1 mg/kg (379.6±168.2 sec) than sham-injured mice (215.1±131.0 sec, 222.2±169.8 sec; *multiple unpaired t-tests, p=0.0101, 0.0068 respectively*).

Brain injured mice exposed to low dose morphine have increased macrophage and neutrophil infiltration on the ipsilateral side of injury

After completion of the CPP protocol, brains were collected and divided by hemisphere for analysis of immune cells by flow cytometry. The total number of macrophage infiltrate (Fig. 3.8A) was significantly increased in injured mice with 0.5mg/kg morphine. When administered 0.5mg/kg morphine 4 times over 10 days, injured mice had 44,000±3,729 macrophages ($CD45^{hi}CD11b^{+}Ly6G^{-}$, *multiple unpaired t-tests, p=0.0061*). The neutrophil infiltration ($CD45^{hi}CD11b^{+}Ly6G^{+}$, Fig. 3.8B) was also significantly increased in injured mice at both 1- and 2.5mg/kg doses. When administered 1mg/kg morphine, injured mice had 5,120±1366 infiltrating neutrophils, while sham-injured mice have only 1,776±392 neutrophils (*multiple unpaired t-tests, p=0.0024*). Injured mice receiving 2.5mg/kg

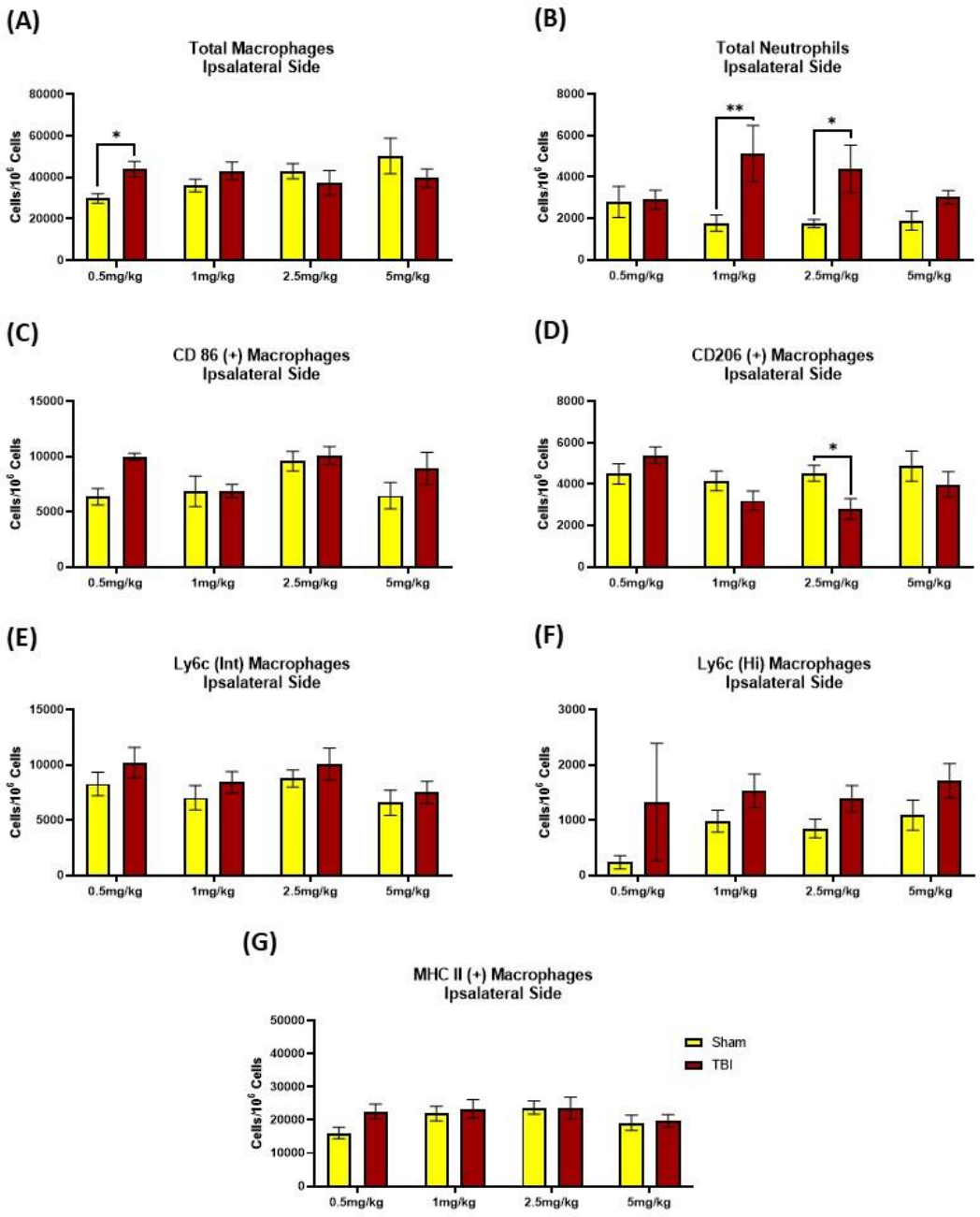

Fig. 3.8 Injured mice have increased macrophage and neutrophil infiltration in the ipsilateral side of the brain. Graphs show the number of the cell of interest per 10^6 cells on the Y axis, with the dose of morphine given subcutaneously during conditioned placed preference on the X axis. All graphs shown are from the ipsilateral side of injury. Total macrophages (A) are identified as $CD45^{hi}CD11b^{+}Ly6G^{-}$. Total neutrophils (B) are identified as $CD45^{hi}CD11b^{+}Ly6G^{+}$. The remaining graphs refer to the expression of markers on the total macrophages, with analysis of CD86 (C), CD206 (D), Ly6c (E&F), and MHC II expression (G). Significance calculated utilizing multiple unpaired t-tests on GraphPad Prism software, p values of 0.05 or lower were considered significant (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$).

morphine had 4,387±1,146 infiltrating neutrophils, while sham-injured had only 1,752±194 neutrophils (*multiple unpaired t-tests, p=0.0156*). When examining the two-way ANOVA, there is not a significant interaction between morphine dosage and injury, but there is a significant difference between mice with mild TBI and sham-injured mice regarding neutrophil infiltrate (*p=0.6041, p=0.0025 respectively*).

When examining the expression of markers on infiltrating macrophages, significant differences began to emerge. $CD86^+$ expression on infiltrating macrophages (Fig 3.8C) was significantly increased in injured mice receiving 0.5mg/kg morphine, with injured mice having 9,984±326 $CD86^+$ macrophages while sham have 6,354±734 $CD86^+$ macrophages (*multiple unpaired t-tests, p=0.0330*). When examined by two-way ANOVA, there is a significant difference between $CD86^+$ macrophage response between dosages, and between groups, but there was no significant interaction (*p=0.0297, p=0.0399, p=0.3012 respectively*). The $CD206^+$ macrophage infiltrate (Fig. 3.8D) decreased significantly in injured mice at the 2.5mg/kg, with sham-injured mice at 4,524±380 CD206+ macrophages and injured mice at 2,798±496 $CD206^+$ macrophages (*multiple unpaired t-tests, p=0.0114*). Two-way ANOVA demonstrated that there is a dose effect regardless of injury on this cellular population (*p=0.0274*). In the $Ly6c^{hi}$ macrophage population (Fig 3.8F), two-way ANOVA demonstrates an effect of injury on this cell population that does not interact with morphine dose (*p=0.0099*), demonstrated as a non-significant increase in this population with injured mice at every dose investigated. No other significant results were observed among the $Ly6c^{int}$ or MHC II^+ macrophage populations.

Injured mice had few significant changes in the contralateral side of the brain. There were no significant changes in the total population of the macrophage (Fig. 3.9A) or neutrophil infiltrate (Fig 3.9B). The $CD206^+$ macrophage infiltrate (Fig. 3.9D) was reduced in injured mice at 2.5mg/kg morphine dosage, with sham-injured mice at 6,766±712 $CD206^+$ macrophages while injured mice had 3,908±541 $CD206^+$ macrophages (*multiple unpaired t-tests, p=0.0081*). Two-way ANOVA revealed that there is a significant effect of morphine dosage on this population of cells that interacts with injury state, though alone the injury does not have a significant effect (*p=0.0471, p=0.0044, 0.4396*). The MHC II^+ population (Fig 3.9G) follows the same pattern, with a significant effect of morphine on cells that

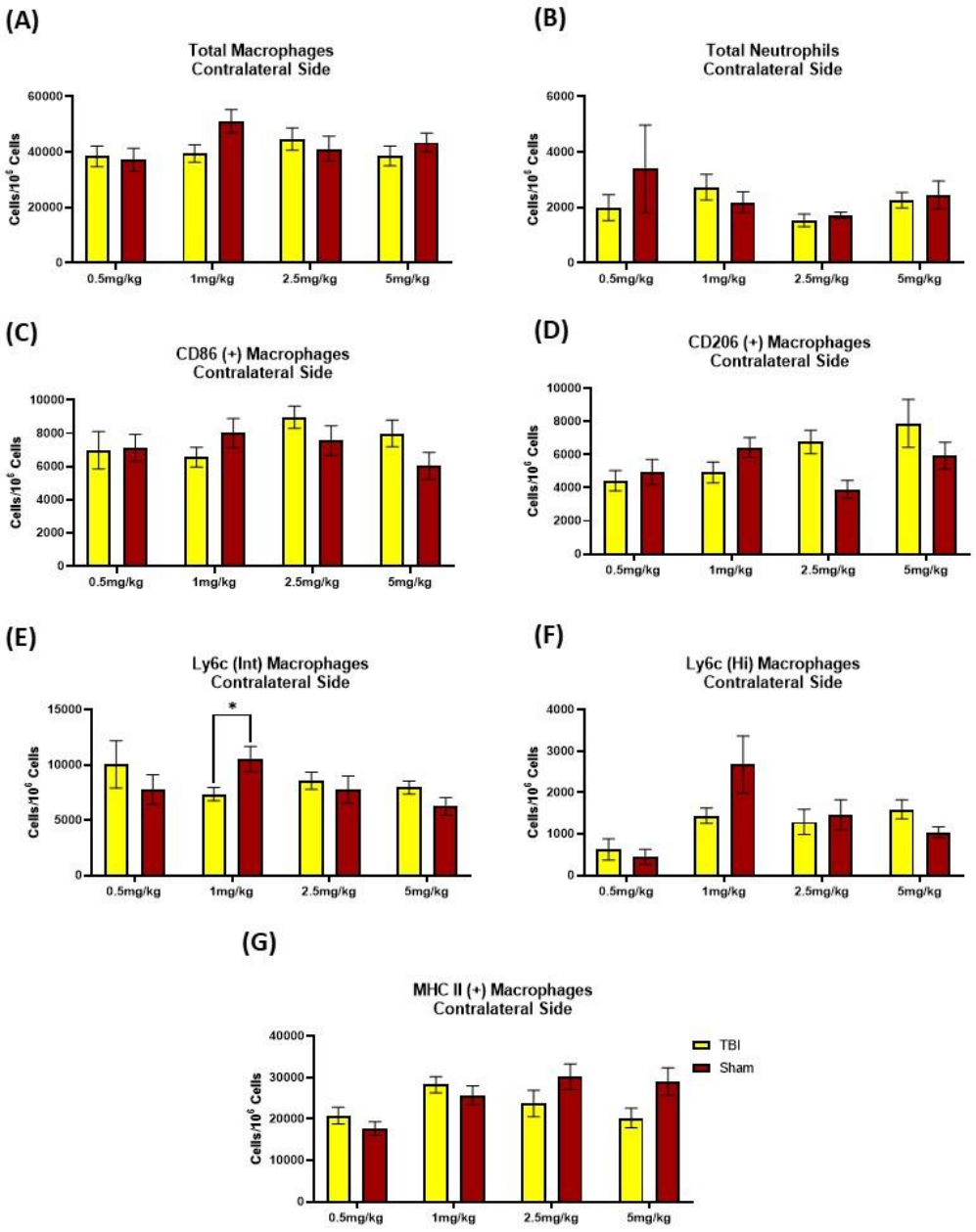

Fig. 3.9 Injured mice have few changes in macrophage or neutrophil populations on the contralateral side of the brain. Total macrophages (A) are identified as $CD45^{hi}CD11b^{+}Ly6G^{-}$. Total neutrophils (B) are identified as $CD45^{hi}CD11b^{+}Ly6G^{+}$. The remaining graphs refer to the expression of markers on the total macrophages, with analysis of CD86 (C), CD206 (D), Ly6c (E&F), and MHC II expression (G). Significance calculated utilizing multiple unpaired t-tests on GraphPad Prism software, p values of 0.05 or lower were considered significant (*$p \leq 0.05$).

interacts with injury status, without injury as a significant factor (*p=0.0489, 0.0128, 0.4123*). Indeed, both populations demonstrate injured animals had a non-significant increase in cells at 1mg/kg, and deceases at 2.5 and 5mg/kg dosages. In the $Ly6c^{+}$

macrophage population, there is a significant increase in the intermediate expression (Fig. 3.9D) of Ly6c in injured mice (10,516±1,149 cells) as opposed to sham mice (7,355±603 cells) at 1mg/kg morphine dosage (*multiple unpaired t-tests, p=0.0234*). Two-way ANOVA reveals that morphine dosage has a significant impact on the Ly6c^{hi} macrophage population, though there is no impact of injury and no interaction between the variables (*p=0.0081, 0.5210, 0.0857*).

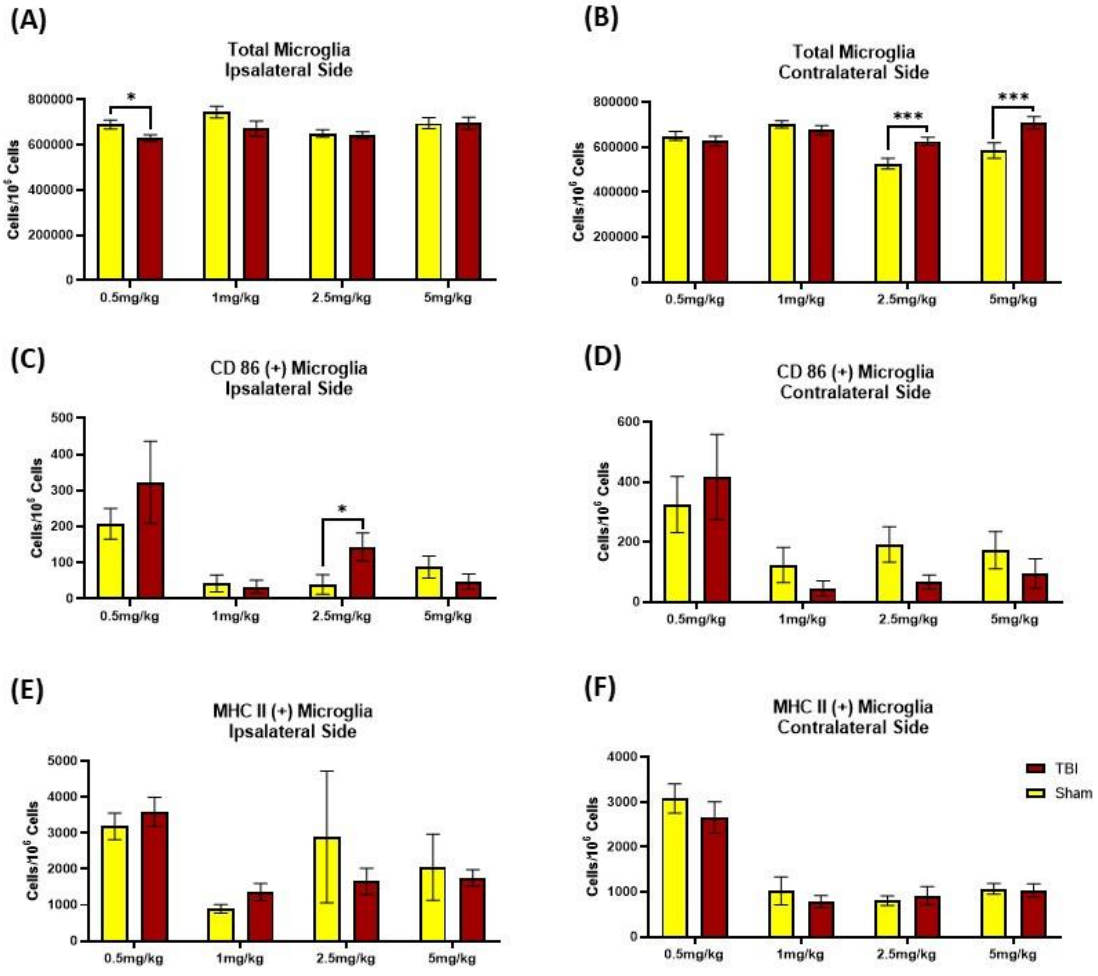

Fig. 3.10 Minor changes occur in the microglia population on the ipsilateral and contralateral sides of the brain. Graphs show the number of the cell of interest per 10^6 cells on the Y axis, with the dose of morphine given subcutaneously during conditioned placed preference on the X axis. Total microglia on the ipsilateral (A) and contralateral (B) side were identified as CD45^{int}CD11b^{+}. That population was then examined by for the expression of CD86 on the ipsilateral (C) and contralateral (D) side, and for the expression of MHC II on the ipsilateral (E) and contralateral (F) side. Significance calculated utilizing multiple unpaired t-tests on GraphPad Prism software, p values of 0.05 or lower were considered significant (*p≤0.05, **p≤0.01, ***p≤0.005).

The microglia population ($CD45^{int}CD11b^{+}$) also demonstrates some changes in the ipsilateral and contralateral sides of the brain. On the ipsilateral side of the brain, the total number of microglia (Fig. 3.10A) is significantly reduced in injured mice exposed to 0.5mg/kg morphine (629,963±15,290 cells) as opposed to sham-injured mice (745,530±26013 cells; *multiple unpaired t-tests, p=0.0301*). Two-way ANOVA revealed a morphine dose effect on the population, but that did not interact with injury status (*p=0.0465, p=0.2930*). On the contralateral side of injury (Fig. 3.10B), there is a significant increase in total microglia in injured mice exposed to 2.5 and 5 mg/kg morphine (TBI: 626,364±18117; 708,792±26874 cells; Sham: 527,114±23,813, 585,379±34,550; *multiple unpaired t-tests, p=0.0018, 0.0004 respectively*). Two-way ANOVA also revealed a significant effect of morphine dose on the cell population that interacts with injury status, though there is no effect of injury on its own (*p=<0.0001, 0.0003, 0.0540*). When examining the expression of CD86 on microglia, two-way ANOVA reveals a significant effect of morphine dose on both the ipsilateral (Fig. 3.10C) and contralateral (Fig. 3.10D) sides of the brain (*p=0.0005, 0.0029 respectively*). On the ipsilateral side, there was a significant increase in $CD86^{+}$ microglia in injured mice exposed to 2.5mg/kg morphine (143±38 cells) as opposed to sham-injured mice (39±27cells, *multiple unpaired t-tests, p=0.0458*). In examining the MHC II expression (Fig. 3.10 E&F) in macrophages, two-way ANOVA revealed a significant effect of morphine dose on the contralateral side (*p<0.0001*). However, expression of CD86 or MHC II on microglia is less than 1% of the total population of microglia.

Brain injured mice have increased lymphocytes centralized on the ipsilateral side of the brain

Many cells of lymphocytic origin are elevated on the ipsilateral side of injury in the brain. Total lymphocytes (Fig. 3.11A) in the brain are significantly increased in injured mice at each morphine dose delivered. At 0.5mg/kg morphine, injured mice had 66,511±7,138 cells while sham had 46,221±6,084 cells; at 1mg/kg morphine, injured mice had 44,367±5,083 cells while sham had 20,669±2,847 cells; at 2.5mg/kg morphine, injured mice had 38,879±4,160 cells while sham had 26,192±1,822 cells; at 5mg/kg morphine, injured mice had 43,518±4,476 cells while sham has 24,516±2,436 cells (*multiple paired*

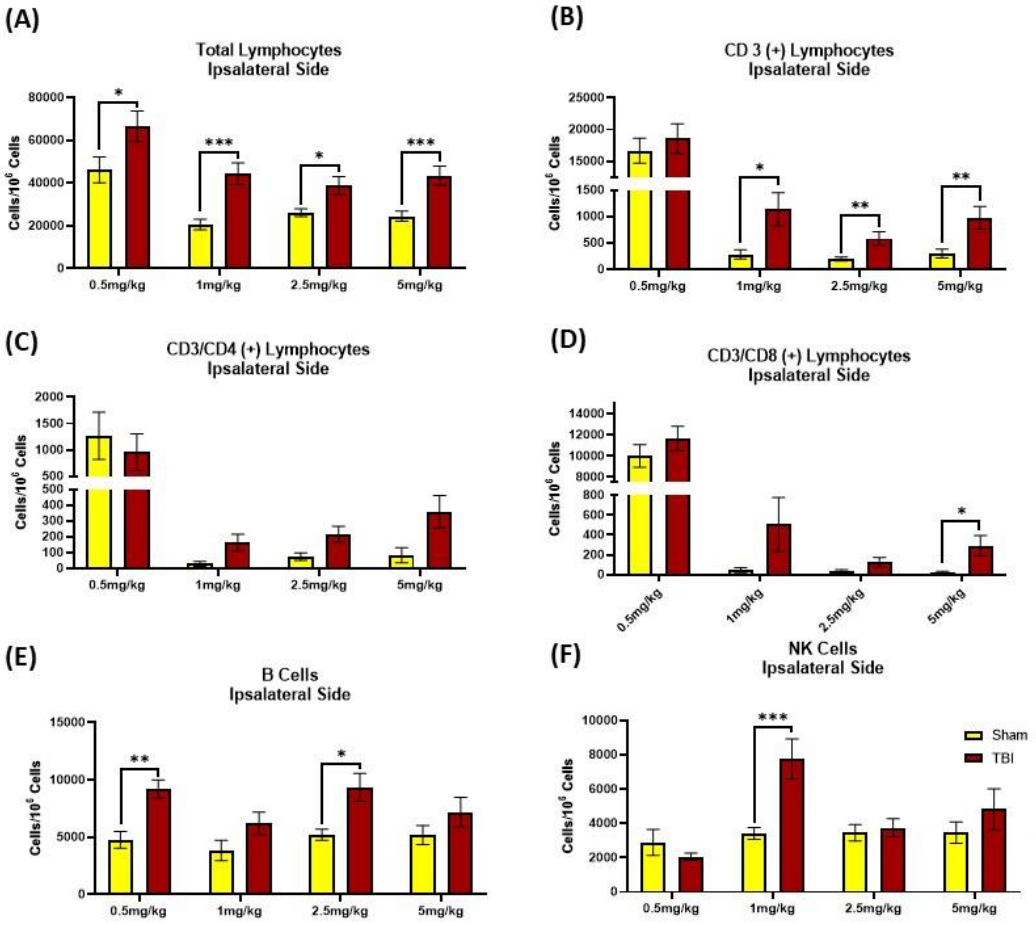

Fig. 3.11 Injured mice have an increase in lymphocyte infiltrate on the ipsilateral side of injury. Graphs show the number of the cell of interest per 10^6 cells on the Y axis, with the dose of morphine given subcutaneously during conditioned placed preference on the X axis. All graphs shown are from the ipsilateral side of injury. Total lymphocytes (A) are identified as $CD45^{hi}CD11b^{-}$. Lymphocytes were then evaluated for CD3 expression (B), and those that were positive were further evaluated for CD4 (C) and CD8 (D) expression. Lymphocytes that are $CD3^{-}CD19^{+}$ were labeled as B Cells (E). Cells that are $CD3^{-}CD19^{-}$ were evaluated for NK1.1, and those that were positive were labeled natural killer (NK) cells (F). Significance calculated utilizing multiple unpaired t-tests on GraphPad Prism software, p values of 0.05 or lower were considered significant (*p≤0.05, **p≤0.01, ***p≤0.005).

t-tests, p=0.0483, 0.0003, 0.0106, 0.0015 respectively). According to two-way ANOVA, there is a significant effect of both morphine dosage and injury status, though no interaction between the two (*p=<0.0001, <0.0001, 0.5716 respectively*). This elevation continued in the $CD3^{+}$ population (Fig. 3.11B), with significant increases at all doses from 1-5mg/kg. At 1mg/kg morphine, injured mice had 1,136±310 cells while sham had 283±86 cells; at 2.5mg/kg morphine, injured mice had 585±127 cells while sham had 197±38 cells; at

5mg/kg morphine, injured mice had 973±213 cells while sham has 300±82 cells (*multiple paired t-tests, p=0.0147, 0.0780, 0.0087 respectively*). Two-way ANOVA analysis revealed an effect of morphine dose on the cell population (*p<0.0001*). The $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphocyte (Fig. 3.11C) populations were affected by morphine dose (*Two-way ANOVA, p=<0.0001, <0.0001 respectively*). The $CD3^+CD4^+$ T cell population was elevated in injured mice across three morphine doses (1-5mg/kg), with injured mice at 163±15 cells at 1mg/kg dose, 216±52 cells at 2.5mg/kg, and 357±102 cells at 5mg/kg as opposed to sham-injured mice at 29±15 cells at 1mg/kg, 74±23 cells 2.5mg/kg, and 83±47 cells at 5mg/kg (*multiple unpaired t-tests, p=0.0296, 0.0216, 0.0300 respectively*). The $CD3^+CD8^+$ T cell population (Fig. 3.11D) was also significantly elevated in injured mice at 5mg/kg morphine dosage; injured mice had 292±104 cells while sham had 16±16 cells (*multiple unpaired t-test, p=0.0170*). B cells (Fig. 3.11E) were affected by injury status (*two-way ANOVA, p<0.0001*), and was significantly elevated at 0.5 and 2.5mg/kg morphine dosages (injured mice at 9216±778 and 9358±1276 cells; sham-injured mice at 4772±722 and 5209±485 cells; *multiple unpaired t-test, p=0.0009, 0.0043 respectively*). The natural killer (NK) cell population ($CD45^{hi}CD11b^-CD3^-CD19^-NK1.1^+$, Fig. 3.11F) was affected by both morphine dose and injury status, and those factors interacted with each other (*two-way ANOVA, p=0.0020, 0.0459, 0.0039 respectively*). There is also elevation in the NK cells in injured mice exposed to 1mg/kg morphine, with injured mice at 7760±1170 cells while sham was at 3413±338 cells (*multiple unpaired t-tests, p=0.0001*).

Total lymphocytes ($CD45^{hi}CD11b^-$) on the contralateral side (Fig. 3.12A) were affected by the dose of morphine, and that interacted with injury status (*two-way ANOVA, p=<0.0001, 0.0305 respectively*). At the 1mg/kg morphine dosage, injured mice had 39,325±6,370 lymphocytes while sham mice had 24,935±2,021 lymphocytes (*multiple unpaired t-test, p=0.0099*). All $CD3^+$ lymphocytes (Fig. 3.12B), including CD4 and CD8 positive cells (Fig. 3.12C&D), were affected by morphine dosage (*two-way ANOVA, p=<0.0001, 0.0003 <0.0001 respectively*). In the B cell population ($CD45^{hi}CD11b^-CD19^+CD3^-$, Fig. 3.12E), there was an interaction between morphine dosage and injury status, but no effect of either factor alone (*two-way ANOVA, p=0.0101, 0.2957, 0.2486*). There is also a significant

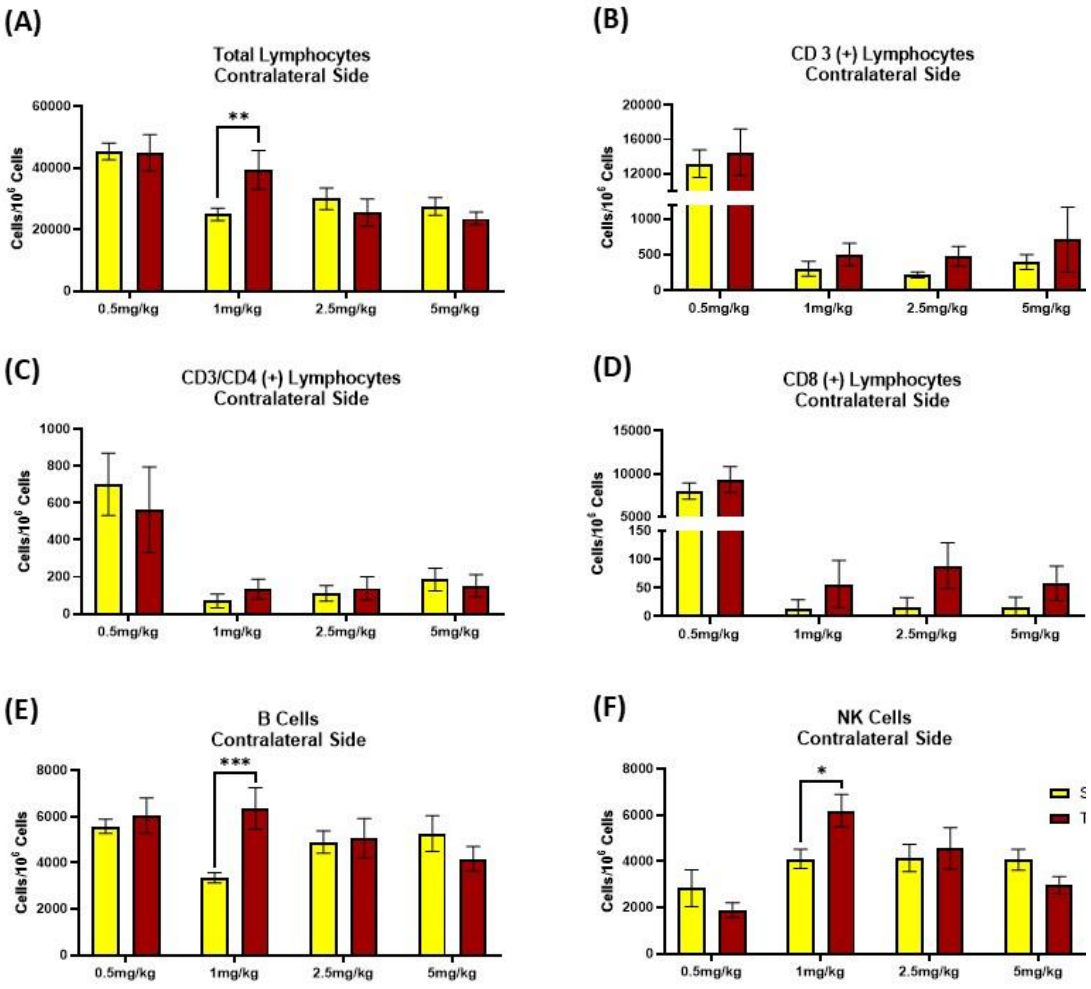

Fig. 3.12 Injured mice have minor elevations in the lymphocyte population on the contralateral side of injury. Graphs show the number of the cell of interest per 10^6 cells on the Y axis, with the dose of morphine given subcutaneously during conditioned placed preference on the X axis. All graphs shown are from the contralateral side of injury. Total lymphocytes (A) are identified as $CD45^{hi}CD11b^{-}$. Lymphocytes were then evaluated for CD3 expression (B), and those that were positive were further evaluated for CD4 (C) and CD8 (D) expression. Lymphocytes that are $CD3^{-}CD19^{+}$ were labeled as B Cells (E). Cells that are $CD3^{-}CD19^{-}$ were evaluated for NK1.1, and those that were positive were labeled natural killer (NK) cells (F). Significance calculated utilizing multiple unpaired t-tests on GraphPad Prism software, p values of 0.05 or lower were considered significant (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$).

elevation in the B cell population at the 1mg/kg morphine dose in injured mice (6,354±898 cells) than in sham-injured mice (3352±224 cells; *multiple paired t-test, p=0.0029*). The NK population ($CD45^{hi}CD11b^{-}CD3^{-}CD19^{-}NK1.1^{+}$, Fig. 3.12F) was affected by the morphine dose, and that interacted with injury status (*two-way ANOVA, p=0.0016, 0.0472*). At a morphine dose of 1 mg/kg, there is significant elevation in NK cells injured

mice (6,195±710 cells) than in sham-injured mice (4113±413 cells; *multiple paired t-test, p=0.0158*).

Discussion

Mild TBI and substance use disorder have been linked through epidemiological data; individuals with a history of TBI have an increased risk of addiction of 3-6 times than that of the general population.[28,29,132] With adolescents as one of the groups most vulnerable to injury,[22-25] and a significant rise in reported adolescent SUD,[190] there is a critical need to understand the mechanisms connecting these observations. Imaging studies have demonstrated in both humans and animal models that the initial damage caused by injury can extend to the reward pathways.[14,151,153,231,514,557] Animal studies have linked chronic inflammation and changes to the blood brain barrier with increased rewarding effects of alcohol and cocaine.[148,151-154,558,568] However, with the rise of the opiate epidemic, the need to understand the mechanisms linking opiate SUD and TBI is critical.

The IV self-administration protocol was used in examine the early escalation of morphine intake. Injured mice escalated their opiate intake throughout the 10 days of short access IV self-administration, with significant differences noted at days two, three, and seven. This coincides with previous studies demonstrating increased ethanol intake following TBI; even mild injury induced an escalation of intake of ethanol in IV self-administration protocol.[148,149,568] The same escalation of intake was seen with cocaine IV self-administration after TBI.[154] Studies with repetitive blast injury rats demonstrated an increased consumption of ethanol and oxycodone, though that model causes a different and more diffuse pattern of damage than CCI or other induced injuries.[569,570] This study demonstrated an increase in morphine administration after a single mild TBI, thereby recapitulating some of the epidemiological data presented earlier.

Following mild TBI and IV self-administration, mice have an increase in macrophages and neutrophils on the ipsilateral side of injury at 15-DPI. Chapter 2 previously demonstrated an increase in numbers of macrophages peaking at 3-DPI and in neutrophils peaking at 1-DPI in injured mice, but a return to levels seen in sham-injured mice by 7-DPI that persisted through 30-DPI. Evidence has concluded that macrophages can have a proinflammatory

response after injury. Modulation of that response by converting macrophages to an anti-inflammatory phenotype or impairing macrophage infiltration can preserve neurofunction and limit secondary damage.[70,282,571] Morphine also acts directly on macrophages, and has been observed to increase macrophage apoptosis and depress phagocytic activity,[155,493-495] as well as causing a decrease in phagocytosis of neutrophils.[155] Recruitment of neutrophils is also delayed with morphine exposure, increasing susceptibility to infection.[155] Overall, morphine causes a general immunosuppression and impairment of function for macrophages and netrophils.[155-158] However, there is evidence from one study that morphine increases the amount of macrophages at the lesion site in spinal cord injury.[496] Thus, there may be diversity in the effect of morphine based on context of the injury and tissue involved.[497] Further research is needed to understand the function and the mechanisms of recruitment of macrophage and neutrophil populations at 15 DPI with morphine exposure.

The adaptive immune system is also affected in injured mice exposed to morphine after IV self-administration. The overall number of lymphocytes in morphine exposed injured mice is similar to sham and injured mice in Chapter 2. However, total lymphocytes on the contralateral side are also significantly elevated, and the number of NK cells is significantly elevated on both the ipsilateral and contralateral side. Though this bilateral response was not seen in the mild TBI model described previously, lymphocytes can infiltrate the brain following TBI with increased ROS species elevating the number of cells that infiltrate.[303,572-574] T-cell recruitment can also be correlated with worse and chronic clinical outcomes.[574] Morphine is generally immunosuppressive and can impair functionality.[155,158] In terms of lymphocytes, and particularly NK cells, morphine can reduce cytotoxicity and increase the number of opioid receptors on the cell surface.[498,499,501,575] However, small changes seen in this experiment are difficult to interpret without performing appropriate functional assays.

To examine how mild TBI would affect the drug preference to several doses of morphine (0.5-5mg/kg). Injured mice established place preference to every dose at the week 1 test day and spent significantly more time on the morphine paired side than sham-injured mice at 0.5 mg/kg (weeks 1 and 2 test) and 1 mg/kg (week 2 test) morphine doses. This

corresponds with an increase in preference for ethanol after injury in female mice.[147,576] Preference for cocaine was also increased after injury, with increased effects in animals that were injured earlier in life.[151-153,557] This study demonstrates that preference to low dose morphine is enhanced after mild TBI.

On the ipsilateral side of injury, there were increases in the macrophage population and neutrophil populations at different doses. Though increases within this population are consistent with the results following IV self-administration, the increases are not consistent in dose (macrophages are elevated at 0.5mg/kg dose, neutrophils at 1 and 2.5 mg/kg doses). There is also some increase in microglia on the contralateral side, with differences in activation markers. However, less than 1% of the microglia express activation markers. Though information has been presented about the effect of TBI and morphine on this cell population, the present study is limited to description, so more experiments need to be done to determine the functionality of the changes presented. This observation may be explained in part due to the dose dependent effects of morphine that can be both proinflammatory as well as immunosuppression effects on immune cells.[156,158]

There was an increase in total lymphocytes observed across all morphine doses on the ipsilateral side in injured mice compared to sham-injured mice. Further analysis demonstrated that this is mainly due to an increase in $CD3^+$ lymphocytes in groups given 1 to 5mg/kg morphine doses. As previously stated, lymphocytes can enter the brain after injury and can correlate with worse clinical outcomes and the possibility of chronic issues.[303,572-574] Morphine is generally immunosuppressive, hindering the functionality and cytotoxicity of lymphocytes.[155,158,498,499,501,575] Thus, further research needs to be done to understand the function of the cells.

The data presented here suggests that the response to mild TBI is altered by exposure to morphine, which is in line with the evidence connecting increases in alcohol and cocaine drug seeking behavior to TBI. After TBI, one study demonstrated that mice had increased sensitivity to the effects of alcohol and increased placed preference when compared to sham-injured mice, which correlated with increased astrocyte activation in the *nucleus accumbens*.[145,146] Young mice have increased alcohol consumption and increased preference to alcohol after TBI,[147,148] relating well with epidemiological findings that

adolescents are at increased risk of SUD after injury. Rats also increased their consumption of alcohol post injury.[149,150] However, treatment with minocycline decreased the increased consumption seen in injured mice and decreased microglial activation in the *nucleus accumbens*.[148] Research utilizing cocaine exposure post injury reveals increased neuroinflammation in the reward pathway. Mice that received TBI in adolescence had increased preference to cocaine as an adult, again reflecting epidemiologic evidence in humans, which correlated with increased microglia / astrocyte activation in the *nucleus accumbens* and *ventral tegmental area*.[151] Consumption of cocaine was also increased after both mild and severe TBI which correlated neuroinflammatory proteins with likelihood of increased drug intake.[154] In another study, dexamethasone treatment reduce preference to cocaine and attenuated the increase in inflammatory genes after TBI.[153] These studies demonstrate that after injury, exposure to addictive substances results in an increase in neuroinflammatory mediators and proinflammatory activation markers that correlates with enhancement of sensitivity to and consumption of the given substance. Treatment that reduces proinflammatory responses ameliorates the increased reaction to addictive substances, suggesting a key role for the immune system.

For the findings described in this chapter, the previously defined model of mild TBI (Chapter 2) was used to evaluate the effect of injury on morphine exposure. This model demonstrates an increase in consumption and sensitivity to morphine. The inflammatory response is enhanced, though inconsistently, after exposure to morphine. This model can be further used to evaluate the response to morphine exposure at more chronic timepoints, as well as examine temporal and functional changes within the immune response.

Conclusions

This study demonstrates that mild TBI alters the rewarding effects of morphine. Injured mice escalated their intake of morphine during IV self-administration over that of sham-injured mice and demonstrated increased sensitivity to low dose morphine by spending more time on the morphine paired side at 0.5 and 1mg/kg of morphine compared to sham-injured mice. Furthermore, inflammatory responses were altered by morphine

administration, with increased macrophage, neutrophil, and lymphocyte infiltrate on the ipsilateral side of injury. Further studies are needed to determine the change in functionality of the immune cells with morphine treatment. This model will be used for further interrogation of drug seeking behavior, as well as investigating how modulation of the inflammatory system can ameliorate the behavioral changes.

Chapter 4

Chronic dose morphine increases the proinflammatory response after mild traumatic brain injury

Abstract

The rise of the opiate epidemic has brought public attention to opiate use disorder, and individuals with history of traumatic brain injury (TBI) are at a 2-6 times increased risk of developing a substance use disorder (SUD). There is a clear connection between TBI and alcohol or cocaine use disorder that implicates inflammatory responses in the enhancement of the rewarding effects of these substances. However, the connection between inflammation and opiate use disorder is still not well understood. Utilizing the previously developed model of mild TBI, morphine was chronically administered to mice at a clinically relevant dose for pain relief for up to 15 days post injury (DPI). The inflammatory response was assessed with flow cytometry. Injured animals exposed to morphine had a biphasic macrophage response on the ipsilateral side of injury, with significant increases at 3- and 15-DPI. There was also an increased lymphocyte infiltrate at 15-DPI on the ipsilateral side of injury which was predominantly made up of $CD8^+$ cytotoxic T-cells. These infiltrates correlated with increased mRNA expression of proinflammatory and anti-inflammatory cytokines, though the composition of the inflammatory milieu changes from the acute to chronic phase of the response. In examining the function of macrophages at the chronic (15-DPI) timepoint, phagocytosis was reduced on the ipsilateral side of injury by macrophages and microglia. This study demonstrated that the immune response to injury is drastically changed in the brain when exposed to morphine and provides a new model to identify targets in reducing the risk of SUD.

Introduction

Traumatic brain injury (TBI) affects approximately 70 million people worldwide.[1,10,161] However, brain injuries are under reported and under diagnosed,[2-5] especially considering that 75-90% are mild in nature, which are the hardest to recognize.[11,502,503] TBI is caused by an external force applied to the head, which commonly occurs in sports, car accidents, and falls.[3,18,70,73,505] While the initial injury causes physical damage with tissue deformation and axonal shearing,[70-73] the immune response extends the damage in the following days to weeks.[70,73] TBI induces a proinflammatory response mediated by infiltrating macrophages and activated microglia, which remove cellular debris and repair damage.[152,528,549] These injuries are usually classified by severity ranging from mild to severe,[18,506] but the injuries themselves are heterogeneous due to the location of impact and brain areas affected.[505,506]

Mild TBI is the most common type of injury seen, which usually results in minor symptoms that resolve in a few days, such as headaches, anxiety, and difficulty sleeping,[18,506-508] However, some patients will develop a chronic condition following injury that can last months to years.[12,503-505,508,509] Understanding the links of mild TBI to long-term consequences is essential, but there is limited evidence of pathology using conventional imaging tools.[510,511] Animal models are commonly used to evaluate the consequences of injury, and recent studies demonstrate increased stress and decreased cooridination.[419,420] Advanced imaging techniques unavailable to most physicians and patients demonstrated long-term chronic inflammation in multiple subcortical areas distal to the original injury site, e.g. the mesolimbic system, which was also observed in animal models.[14,151,153,231,514,557] Changes in these areas coincided with increased rewarding effects with exposure to alcohol and cocaine in rats and mice, which were recapitulated in chapter 2 in regards to morphine.[151-153,207,558-560] This evidence seems to connect mild TBI and chronic inflammation to increased rewarding effects of addictive substances. However, temporal changes to the inflammatory response during exposure and the mechanisms connecting mild TBI and opiate use disorder are still not fully understood.

Many people are prescribed opiate pain relievers following a painful injury or disease condition, and this is often their first exposures to opiates. However, among the general public, approximately 11% develop a substance use disorder (SUD),[28,29,132] and many misuse their prescriptions by using a higher dose or in a way other than prescribed, using another person's prescription, and using their prescribed opiate to become intoxicated.[577-579] Since this is such a common introduction to opiates, and a common first step before a SUD develops, the clinical pain relief dose (5mg/kg subcutaneously (SC)) for a mouse was administered every 12 hours for up to 15 days post injury (DPI), to model the effects of morphine misuse.[580-583] The proinflammatory response post TBI is enhanced after exposure to alcohol or cocaine, and modulation of the proinflammatory response with minocycline and dexamethasone coincides with reduction in drug seeking behavior.[152,153,558,560] Utilizing this information, experiments were designed to examine how morphine administration affects both the acute and chronic immune response to injury. The previously described controlled cortical impact (CCI) model was used to investigate the inflammatory response at multiple timepoints (1-, 3-, 7-, and 15-DPI) since data from Chapter 2 demonstrates a distinct acute and chronic proinflammatory response at 3- and 15-DPI. The objective of the present study is to assess changes in immune responses from acute to chronic stages of injury with morphine exposure. I hypothesized that there will be an increase in macrophage and neutrophil recruitment in the acute response to injury in mice that receive a mild TBI with chronic morphine exposure.

Methods

Animal Use

All procedures and experiments involving animals were conducted in accordance with protocols approved by the University of Minnesota Institutional Animal Care and Use Committee. Female C57/BL6 mice at 8-12 weeks of age were purchased from Jackson Labs and housed in environmental controlled micro-isolator cages with Enviro-dry environmental enrichment. Animals were acclimatized to their new environment for at least one week prior to obtaining baseline data for behavioral studies.

Surgical Induction of Traumatic Brain Injury

For full details, please refer to the data presented in Chapter 2 and 3. Briefly, mice were 8-12 weeks old at the time of mild TBI or sham injury induction. Mice were anesthetized with isoflurane, placed in a stereotaxis apparatus to secure the head, and a mixture of lidocaine (4mg/kg) and bupivacaine (1-2mg/kg) delivered at the incision site. The incision was made on the dorsal cranium and a 2.5mm craniotomy was made over the right primary and secondary motor cortices. A 2mm impact tip was used to inflict a mild CCI TBI at a velocity of 4m/s, depth of 1mm, and dwell time of 100ms. Sham-injured mice had the same procedure without impact. Mice were recovered in a clean home cage with a heating pad and monitored closely for 3 days post TBI.

Morphine Subcutaneous Injections

Mice were trained for 1 week prior to injury with 0.1mL subcutaneous (SC) saline injection to reduce handling stress. Immediately following mild TBI or sham injury surgery, mice were injected with morphine sulfate (5mg/kg, 1mg/mL) SC or saline depending on experimental group, and that continued throughout the experimental timeline twice daily. All mice were closely examined for health issues before injection and weighed twice daily for accurate morphine dosing.

Behavioral Assays

All behavior testing was done sequentially 2-, 6-, and 14-DPI. Mice were acclimated to the testing room for at least 15 mins and were given SC morphine or saline treatment 20 mins before testing. Mice were first tested on spontaneous alternation Y maze, followed by open field testing (OFT) and novel object recognition test (NORT).

Open Field Testing

The open field test (OFT) was performed as previously described.[124] Mice were placed in the center of a 20 x 20 x 10-inch box for 30 mins of free exploration. The outer ~3.5-

inch perimeter area of is the outside area of the apparatus, and the remaining ~11 x 11-inch area was considered the center area. Mouse time in each area, distance travelled, and center entries were recorded with ANY-maze behavioral tracking system (Stoelting Company, Wood Dale, IL).

Novel Object Recognition Test

Novel object recognition test (NORT) was performed as previously described.[118] The same apparatus was used for NORT as in OFT, so the acclimation stage of NORT was actually the OFT. After OFT, two identical objects were placed in opposite corners of the apparatus in a 2 x 2-inch square area. Mice were placed in the center of the apparatus and allowed free exploration for 10 mins for the familiarization stage. ANY-maze behavioral tracking system (Stoelting Company, Wood Dale, IL) was utilized to record nose pokes into the area of each object, along with time spent in each area and distance traveled. After 1 hr, mice were returned to the apparatus for the novel object testing stage. In this stage, one of the familiar objects was replaced with a novel object, alternating between placement in either object area to avoid site bias. After testing, the ability to discriminate between familiar and novel objects was analyzed utilizing the discrimination index (DI): $DI = (T_N - T_F)/(T_N + T_F)$; T_N= Time/nose pokes with novel object and T_F= Time/nose pokes with familiar object.

Spontaneous Alternation Y Maze

Spontaneous alternation Y-maze has been previously described to examine short-term memory,[120] and is described in Chapter 2. Briefly, the mouse was placed in the Y-maze for 5 min while the ANY-maze behavioral tracking system (Stoelting Company, Wood Dale, IL) recorded movements. A ‘correct’ choice in the maze requires the mouse to enter each arm sequentially, and an early return to a previously explored arm was an ‘incorrect’ response.

Flow Cytometry

Mice were sacrificed after behavioral protocols at 1, 3, 7, and 15-DPI as described in Chapter 2 methods. Briefly, mice were anesthetized and perfused by cold PBS. Each hemisphere was homogenization into single cell suspension. Cells were then washed with PBS+2% FBS and transferred to a 96-well plate. Cells were incubated with Fc block for 5min, followed by adding cell surface antigens. Cells were washed, preserved with PFA, and again washed before resuspension in FACS buffer with AccuCount beads (Spherotech, Lake Forest, IL, USA) for analysis on a flow cytometer (BD FACSCanto) at UMN Flow Core. See table 2.1 for details on the staining panels. Isotype specific antibodies were used to control for nonspecific antibody binding. Immunostained cell populations were again analyzed using FlowJo software. See Fig 4.5 for macrophage panel gating strategy and Fig 2.8 for lymphocyte panel gating strategy.

Real time reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR was performed as described in chapter 2. Briefly, the brain was separated into hemispheres for homogenization and total RNA was extracted using Aurum Total RNA Fatty and fibrous tissue kit (Bio-Rad). Purity was assessed by calculating the ratio of OD260/OD280. RNA was reverse transcribed using high-capacity cDNA reverse transcription kit (Applied Biosystems). RT-qPCR was used to amplify cDNA with Fast SYBR Green master mix (Applied Biosystems) in Quant Studio Real-time PCR system (Applied Biosystems). The specificity of qPCR was assessed by analyzing the melting curves and CT values were used to determine a positive result and all values were normalized to ribosomal protein L27 gene to obtain ΔCt. Difference between the ΔCt values of the TBI sample the sham sample was calculated as ΔΔCt. The fold change was expressed as $2^{-\Delta\Delta Ct}$ as previously described.[518] See Table 2.2 for primer sequences.

Evaluation of the Blood Brain Barrier with Evan's Blue dye

Evaluation of the blood brain barrier (BBB) with Evan's blue (EB) dye was done as previously described.[584] Mice were administered with 2% EB dye in PBS utilizing a tail

vein restraint device for intravenous (IV) injection (Braintree Scientific) 1 hour prior to perfusion. Mice were then anesthetized with isoflurane and perfused intracardially with cold PBS. The brains were then collected, isolated by hemisphere, and the *cortex*, *midbrain*, and *hippocampus* were separated for analysis. Each sample was weighed with an analytical balance and manually homogenized in 50% trichloroacetic acid. The homogenate was then centrifuged for 20 mins at 10,000 x g and the supernatant removed. The supernatant was then diluted with ethanol 4-fold and transferred to a 96-well plate for analysis. The Clariostar microplate reader (BGM Labtech) was then used to measure fluorescent intensity. Along with samples, a concentration curve was created for EB with 100, 250, 500, 750, and 1000 ng/ml, to assess the concentration of EB dye in each sample. The concentration extrapolated from the standard curve was divided by sample weight, to express EB concentration as ng/mg brain tissue.

Macrophage Phagocytosis Ex-Vivo Study

At 15-DPI, mice were anesthetized and perfused by cold PBS. Each hemisphere was homogenized into a single cell suspension. Cells were then washed with RPMI media (Thermo Fischer Scientific) +2% FBS (Sigma-Aldrich) and transferred to cell culture dishes. During that time, Streptavidin Coated Microspheres (Bangs Laboratory Inc) were incubated with Cy5 oligo biotin probe (/5Cy5/TCAGTTCAGGACCTCGGCT/3Bio/; Integrated DNA Technologies) for 30 min in PBS + 2% FBS to label the beads. The isolated cells were then counted to determine the total number of beads needed for each sample, and Cy5 oligo biotin probe coated streptavidin coated microspheres were added to each sample at a 10:1 ration. The cells were then placed into an incubator for 5 hours at 37°C and 5% CO_2. After incubation, the cells were treated with and Oligo-Quencher for 20min at 20°C (AGCCGAGGGTCCTGAACTGA/3IAbRQSp; Integrated DNA technologies). After, the cells were isolated and processed for flow cytometry as previously described (see Flow cytometry methods). See table 4.1 for details on staining antibodies used.

Table 4.1 Flow Cytometry Stains

Staining Panel	Fluorochrome	Manufacturer
CD45	PerCP-5.5	eBioscience/Invitrogen
CD11b	APC-eFluor 780	eBioscience/Invitrogen
CD19	PE	eBioscience/Invitrogen
CD3	eFluor 450	eBiosciences
Ly6G	PE-Cy7	eBioscience/Invitrogen
NK 1.1	Brilliant Violet 650	Biolegend

Statistical analysis

All assays were analyzed using Graph Pad PRISM with one and two-way ANOVA, Welch's t-test and multiple unpaired t-tests. P-values < 0.05 were considered as significant differences.

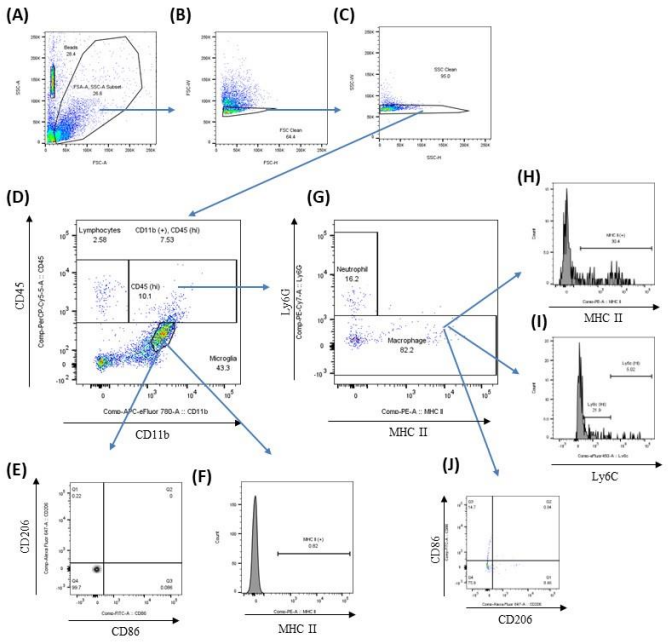

Fig. 4.1 Macrophage gating strategy. Counting beads are isolated with side scatter area on the Y-axis, and Forward scatter area on the X-axis (A). Cells are gated on the same dot plot according to previously established parameters, then doublets are eliminated using forward scatter height and width (B), then side scatter height and width (C) to analyze single cells. From there, CD45+ cells were graphed on the Y axis and CD11b on the X axis (D). Cells with intermediate expression of CD45 and positive for CD11b positive were labeled as microglia and evaluated for CD206 (E), CD86 (E), and MHC (F) expression. Cells that are CD45 positive and CD11b positive were identified as macrophages and evaluated for Ly6G and MHC II expression (G). Ly6G positive cells are labeled as neutrophils. Ly6G negative cells were labeled as macrophages and evaluated for MHCII (H), Ly6c (I), CD206 (J), and CD86 (J) expression.

Results

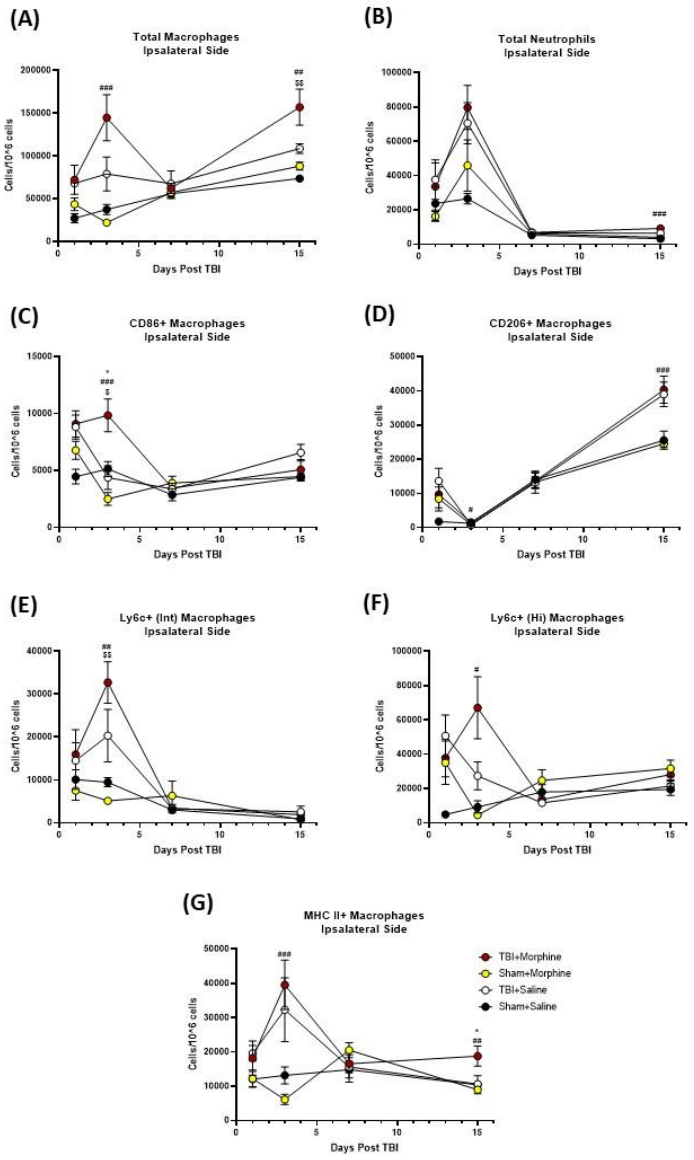

Fig 4.2 Changes in the macrophage population on the ipsilateral side of injury illustrate differences due to morphine exposure, injury status, or an interaction of both. Graphs show the number of the cell of interest per 10^6 cells on the Y axis, with the days post injury on the X axis. All graphs shown are from the ipsilateral side of injury. Total macrophages (A) are identified as $CD45^{hi}CD11b^{+}Ly6G^{-}$. Total neutrophils (B) are identified as $CD45^{hi}CD11b^{+}Ly6G^{+}$. The remaining graphs refer to the expression of markers on the total macrophages, with analysis of CD86 (C), CD206 (D), Ly6c (E&F), and MHC II expression (G). Significance calculated utilizing Welch's unpaired t-tests on GraphPad Prism software (* refers to significant differences between morphine exposed and saline treated injured mice, # refers to significant differences between morphine exposed injured and sham-injured mice, $ refers to differences between morphine-exposed and unexposed sham injures mice; */#/$p≤0.05, **/##/$$≤0.01, ***/###/$$$≤0.005).

Chronic morphine exposure causes a biphasic macrophage response

Macrophages are significantly elevated at 3- and 15-DPI in morphine exposed injured mice on the ipsilateral side (see Fig. 4.1 for gating strategy). In morphine exposed injured mice, total macrophages ($CD45^{hi}CD11b^{+}Ly6G^{-}$, Fig. 4.2A) are elevated at 3-DPI (144,671±26,884 cells) and 15-DPI (156,822±21,161 cells) over that of morphine exposed sham-injured mice (3-DPI: 22,214±2,780 cells, 15-DPI: 88,079±4,699 cells; *multiple t-tests, p=<0.0040, 0.0100 respectively*). Morphine exposed sham-injured mice had significantly elevated macrophages over saline treated sham-injured mice (73,690±4,314 cells) at 15-DPI (*multiple unpaired t-tests, p=0.0477*). Morphine exposed injured mice also had more neutrophils ($CD45^{hi}CD11b^{+}Ly6G^{+}$, Fig. 4.2B; 9,180±1,130 cells) than morphine exposed sham mice (6,465±676 cells) at 15-DPI (*multiple unpaired t-tests, p=0.0016*).

There are several changes that occur in the expression of pro- and anti-inflammatory markers on the macrophage population. Morphine exposed injured mice have increased $CD86^{+}$ macrophages (Fig. 4.2C; 9,847±1,445 cells) over both saline treated injured mice (4,379±1,041 cells) and morphine exposed sham-injured mice (2,508±562 cells.) at 3-DPI (*multiple unpaired t-tests, p=0.0219, 0.0032*). Morphine exposed sham-injured mice also had lower levels of $CD86^{+}$ macrophages than the saline treated sham-injured mice (5,156±656 cells) at 3-DPI (*multiple unpaired t-tests, p=0.0225*). When examining $CD206^{+}$ macrophages (Fig. 4.2D), morphine exposed injured mice had elevated levels at both 3-DPI (1,528±253 cells) and 15-DPI (40,299±3,969 cells) over morphine exposed sham-injured mice (3-DPI: 846±112 cells, 15-DPI: 24,501±1,105 cells; *multiple unpaired t-tests, p=0.0489, 0.0032*). Morphine exposed injured mice have increased levels of $Ly6c^{int}$ macrophages (Fig. 4.2E) and $Ly6c^{hi}$ macrophages (Fig. 4.2F) (67,019±18,055 cells) when compared to morphine exposed sham-injured mice (5,123±186 cells; 4,554±1,950 cells respectively) at 3-DPI (*multiple unpaired t-tests, p=0.0013, 0.0138 respectively*). Saline treated sham-injured mice also have more $Ly6c^{int}$ macrophages (9,464±1,047 cells) than morphine exposed sham-injured mice at 3-DPI (*multiple t-tests, p=0.0065*). When examining MHC II expression (Fig. 4.2G), exposed injured mice have increased MHC II^{+} macrophages (18,771±2,895 cells) over saline treated injured mice (10,634±888 cells) at 15-DPI (*multiple unpaired t-tests, p=0.0228*). Morphine exposed injured mice also had

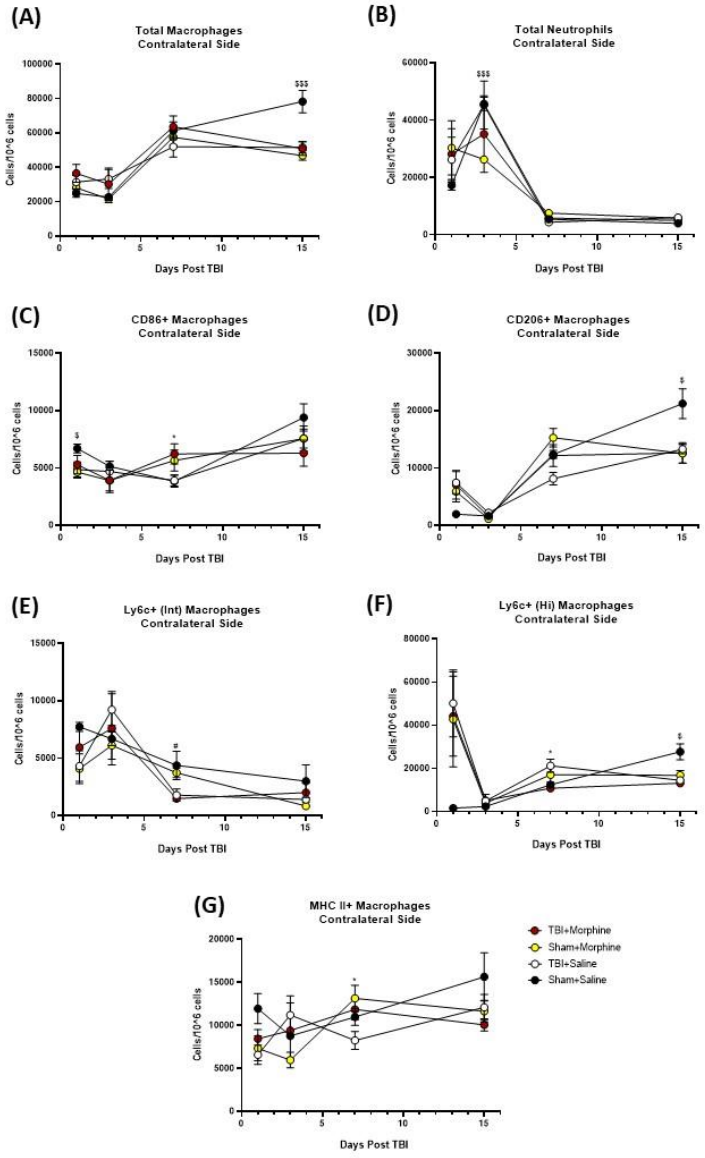

Fig 4.3 Changes in the macrophage population on the contralateral side of injury illustrate differences due to morphine exposure, injury status, or an interaction of both. Graphs show the total number of the cell of interest per 10^6 cells, on the Y axis, and days post injury on the X axis. All data shown are from the contralateral side of injury. Total macrophages (A) are identified as $CD45^{hi}CD11b^{+}Ly6G^{-}$. Total neutrophils (B) are identified as $CD45^{hi}CD11b^{+}Ly6G^{+}MHC\ II^{-}$. The remaining graphs refer to the expression of markers on the total macrophages, with analysis of CD86 (C), CD206 (D), Ly6c intermediate (E), Ly6c high (F), and MHC II expression (G). Significance calculated utilizing Welch's unpaired t-tests on GraphPad Prism software (* refers to significant differences between morphine exposed and unexposed injured mice, # refers to significant differences between morphine exposed injured and sham-injured mice, $ refers to differences between morphine-exposed and unexposed sham injures mice; */#/$p≤0.05, **/##/$$≤0.01, ***/###/$$$≤0.005).

elevated levels of MHC II+ macrophages at 3-DPI (39,548±7,187 cells) and 15-DPI (18,771±2,895 cells) over exposed sham-injured mice (3-DPI: 6.141±1,488 cells; 15-DPI: 9,014±512; *multiple unpaired t-tests, p=0.0039, 0.0078*).

The contralateral side had much fewer changes than the ipsilateral side (Fig. 4.3). There are significantly more macrophages (Fig. 4.3A) in saline treated sham-injured mice (78,272±6,552 cells) than in morphine exposed sham-injured mice (46,823±2,707 cells) at 15-DPI (*multiple unpaired t-tests, p=0.0013*). There were also significantly more neutrophils (Fig. 4.3B) in the saline treated sham-injured mice (45,695±2,355 cells) than in the morphine exposed sham-injured mice (26,285±998 cells) at 3-DPI (*multiple unpaired t-tests, p=0.0013*).

Smaller changes are present in the expression of markers on macrophages on the contralateral side to injury. As early as 1-DPI, morphine exposed sham-injured mice had significantly less $CD86^+$ macrophages (4,648±518 cells) than saline treated sham-injured mice (6,704±392 cells) at 1-DPI (*multiple unpaired t-tests, p=0.0237*). Morphine exposed injured mice have significantly more $CD86^+$ macrophages (Fig. 4.3C) and significantly less $Ly6c^+$ macrophages (Fig 4.3E&F) at 7-DPI. Morphine exposed injured mice have 6,238±891 $CD86^+$ macrophages while saline treated injured mice have only 3,915±481 cells $CD86^+$ macrophages (*multiple unpaired t-tests, p=0.0446*). In terms of $Ly6c^+$ macrophages at 7-DPI, morphine exposed injured mice exhibited significant decrease in $Ly6c^{int}$ macrophages (1,489±308 cells) when compared to morphine exposed sham-injured mice (3,729±428 cells; *multiple unpaired t-tests, p=0.0017*), and a significant decrease in $Ly6c^{hi}$ macrophages where morphine exposed injured mice had 10,834±1,282 $Ly6c^{hi}$ macrophages while saline treated injured mice had 21,228±3,024 $Ly6c^{hi}$ macrophages (*multiple unpaired t-tests, p=0.0101*). At 15-DPI, morphine exposed sham-injured mice had significantly less $CD206^+$ macrophages (Fig. 4.3D; 12,549±1,700 cells) than saline treated sham-injured mice (21,223±2,590 cells) at 15-DPI (*multiple unpaired t-tests, p=0.0188*). Morphine exposed sham-injured mice also have significantly less $Ly6^{hi}$ macrophages (16,817±2,177 cells) than saline treated sham-injured mice (27,734±3,692 cells) at 15-DPI (*multiple unpaired t-tests, p=0.0290*). Morphine exposed injured mice

have significantly more MHC II^+ macrophages (Fig. 4.3G; 11,855±1,193 cells) than saline treated injured mice (8,252±1,042 cells) at 7-DPI (*multiple unpaired t-tests, p=0.0462*).

The microglia ($CD45^{int}CD11b^+$) population had few changes on the ipsilateral or contralateral side (Fig. 4.4). The morphine exposed sham-injured mice have significantly increased total microglia (Fig. 4.4A) at 3-DPI (680,881±33,862 cells), 7-DPI (488,371±13,689 cells), and 15-DPI (620,035±8.272 cells) over morphine exposed injured mice (480,096±40,556, 335,463±18,482, and 502,249±8,745 cells respectively) on the ipsilateral side (*multiple unpaired t-tests, p=0.0090, 0.0001, <0.0001*). Morphine exposed

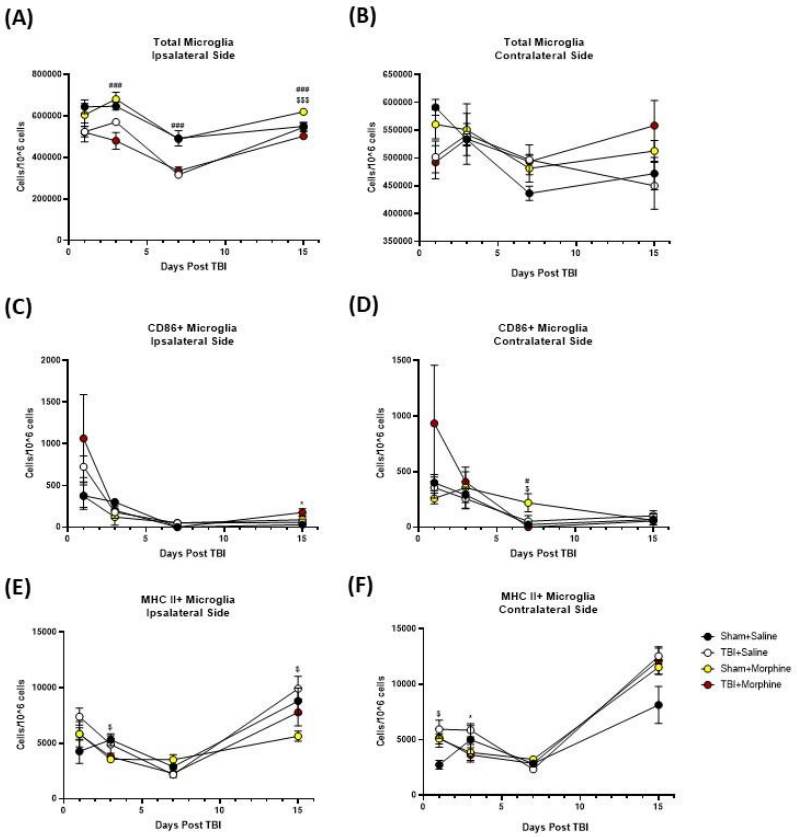

Fig. 4.4 Minor changes occur in the microglia population. Graphs show the number of the cell of interest per 10^6 cells on the Y axis, with days post injury on the X-axis. Total microglia on the ipsilateral (A) and contralateral (B) side are identified as $CD45^{int}CD11b^+$. That population was then examined by for the expression of CD86 on the ipsilateral (C) and contralateral (D) side, and for the expression of MHC II on the ipsilateral (E) and contralateral (F) side (* refers to significant differences between morphine exposed and unexposed injured mice, # refers to significant differences between morphine exposed injured and sham-injured mice, $ refers to differences between morphine-exposed and unexposed sham injures mice; */#/$p≤0.05, **/##/$$≤0.01, ***/###/$$$<0.005).

sham-injured mice also have elevated microglia levels (620,035±8,272 cells) on the ipsilateral side of injury over saline treated sham-injured mice (549,621±19,161 cells) at 15-DPI (*multiple unpaired t-tests, p=0.0070*). There are no significant changes on the contralateral side to the microglia populations (Fig. 4.4B). On the ipsilateral side, there is a significant increase in $CD86^+$ macrophages (Fig. 4.4C) in morphine exposed injured mice (176±46 cells) over saline treated injured mice (58±26 cells) at 15-DPI (*multiple unpaired t-tests, p=0.0499*). On the contralateral side, morphine exposed sham-injured mice have elevated $CD86^+$ microglia (Fig. 4.4D; 219±81 cells) over morphine exposed injured mice (0 cells) and saline treated sham-injured mice (24±24 cells) at 7-DPI (*multiple unpaired t-tests, p=0.0221, 0.0430 respectively*). There was a significant increase in MHC II^+ microglia on the ipsilateral side (Fig 4.4E) in saline treated sham-injured mice at 3-DPI (5,316±530 cells) and 15-DPI (8,804±1,178 cells) over morphine exposed sham-injured mice (3-DPI: 3,551±294 cells, 15-DPI: 5,635±456 cells; *multiple unpaired t-tests, p=0.0270, p=0.0309 respectively*). On the contralateral side, there is a significant decrease in MHC II^+ microglia (Fig 4.4D) in exposed injured mice (3,638±650 cells) when compared to saline treated injured mice (5,863±594) at 3-DPI (*multiple unpaired t-tests, p=0.0447*). There is also a significant increase in morphine exposed sham-injured mice levels of MHC II^+ microglia (5,078±467 cells) over saline treated sham-injured mice (2,741±401 cells) at 1-DPI (*multiple unpaired t-tests, p=0.0084*). However, less than 1% of microglia have expressed any marker across all groups.

Morphine-exposed injured mice have increased CD3/$CD8^+$ lymphocytes on the ipsilateral side of injury at 15-DPI

On the ipsilateral side of injury, majority of significant changes in cell infiltrate occur at 15-DPI. When examining the total lymphocytes ($CD45^{hi}CD11b^-$) (Fig 4.5A), there is an increase in morphine exposed injured mice (46,287±10,116 cells) over morphine exposed sham-injured mice (14,778±2,117) at 15 DPI (*multiple unpaired t-tests, p=0.0123*). Saline treated sham-injured mice also have significantly more total lymphocytes (26,530±3,087 cells) than morphine exposed sham-injured mice (*multiple unpaired t-tests, p=0.0105*). Morphine exposed injured mice also had elevated $CD3^+$ lymphocytes (Fig. 4.5B;

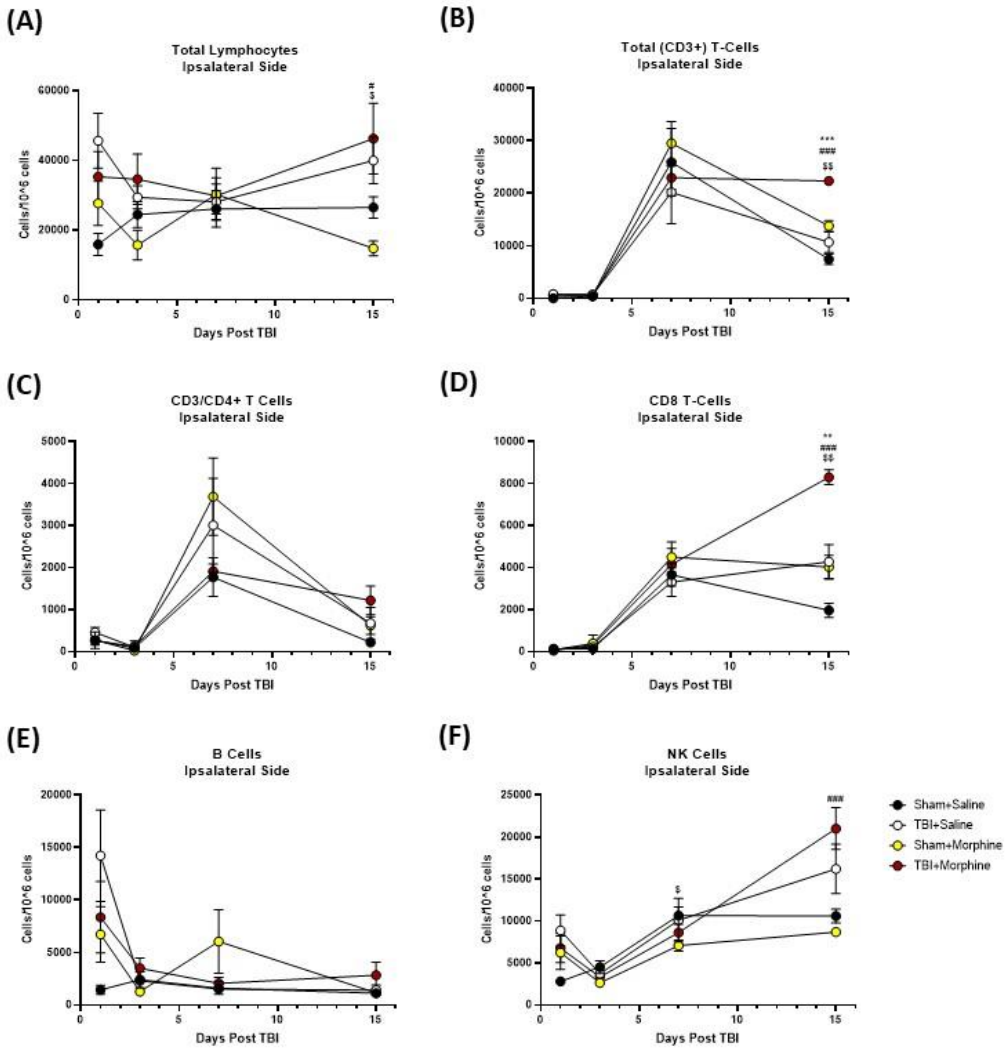

Fig. 4.5 Morphine-exposed injured mice have increased $CD3^+/CD8^+$ lymphocytes on the ipsilateral side of injury at 15-DPI. Graphs show the number of the cell of interest per 10^6 cells on the Y axis, with days post injury on the X axis. All graphs shown are from the contralateral side of injury. Total lymphocytes (A) are identified as $CD45^{hi}CD11b^-$. Lymphocytes were then evaluated for CD3 expression (B), and those that were positive were further evaluated for CD4 (C) and CD8 (D) expression. Lymphocytes that are $CD3^-CD19^+$ positive were labeled as B Cells (E). Cells that were $CD3^-CD19^-$ were evaluated for NK1.1 (F) and cells that were positive were labeled natural killer (NK) cells. Significance calculated utilizing t-tests on GraphPad Prism software (* refers to significant differences between morphine exposed and unexposed injured mice, # refers to significant differences between morphine exposed injured and sham-injured mice, $ refers to differences between morphine-exposed and unexposed sham injures mice; */#/$p≤0.05, **/##/$$≤0.01, ***/###/$$$≤0.005).

22,353±523 cells) over both saline treated injured mice (10,681±1,922 cells) and morphine exposed sham-injured mice (13,795±1,014 cells) at 15-DPI (*multiple unpaired t-tests*,

p=0.0002, <0.0001 respectively). Morphine exposed sham-injured mice also have significantly more $CD3^+$ lymphocytes than saline treated sham-injured mice (7,447±990 cells) at 15-DPI (*multiple unpaired t-tests, p=0.0012*). When examining the $CD3^+$ population, there are no significant changes in the $CD3^+/CD4^+$ lymphocyte population (Fig. 4.5C). However, the $CD3^+/CD8^+$ lymphocytes are elevated significantly in morphine exposed injured mice (8,303±357 cells) over saline treated injured mice (4,279±823 cells) and morphine exposed sham-injured mice (4,302±550 cells) at 15-DPI (*multiple unpaired t-tests, p=0.0012, 0.0001*). Additionally, $CD3^+/CD8^+$ lymphocytes are also elevated in morphine exposed sham-injured mice over saline treated sham-injured mice (1,965±338 cells) at 15-DPI (*multiple unpaired t-tests, p=0.0095*). There are no significant changes to the B cell population (Fig. 4.5E). But in the natural killer (NK) cell population ($CD45^{hi}CD11b^-CD3^-CD19^-NK1.1^+$), there is a significant increase in exposed injured mice (20,997±2,492 cells) over exposed sham-injured mice (8,693±523 cells) at 15-DPI; at 7-DPI, there is a significant decrease in morphine exposed sham-injured mice (7,083±653 cells) over saline treated sham-injured mice (10,656±1,034 cells; *multiple unpaired t-tests, p=0.0007, 0.0152*).

There were no significant changes to the total lymphocytes on the contralateral side (Fig. 4.6A). There was significantly more $CD3^+$ lymphocytes in the morphine exposed sham-injured mice (20,369±2,312 cells) over the morphine exposed injured mice (12,803±1,199 cells) and the saline treated sham-injured mice (17,504±1,840 cells) at 7-DPI (*multiple unpaired t-tests, p=0.0157, 0.0007 respectively*). There were also more $CD3^+/CD8^+$ lymphocytes (Fig. 4.6C) in morphine exposed sham-injured mice (1,161±245 cells) over the morphine exposed injured mice (479±126 cells) at 15-DPI (*multiple unpaired t-tests, p=0.0328*), albeit the numbers are small. Exposed injured animals have significantly more $CD3^+/CD8^+$ lymphocytes (Fig. 4.6D, 221±77 cells) than saline treated injured mice (42±22 cells) and morphine exposed sham-injured mice (0 cells) at 1-DPI (*multiple unpaired t-tests, p=0.0261, 0.0147*). At 7-DPI, there were significantly more $CD3^+/CD8^+$ lymphocytes in morphine exposed sham-injured mice (4,931±680 cells) than in saline treated sham-injured mice (3,317±319; *multiple unpaired t-tests, p=0.0497*). At 7-DPI, morphine exposed sham-injured mice have elevated B cells (Fig 4.6E, 3,779±819 cells)

over morphine exposed injured mice (1,240±401) and saline treated sham-injured mice

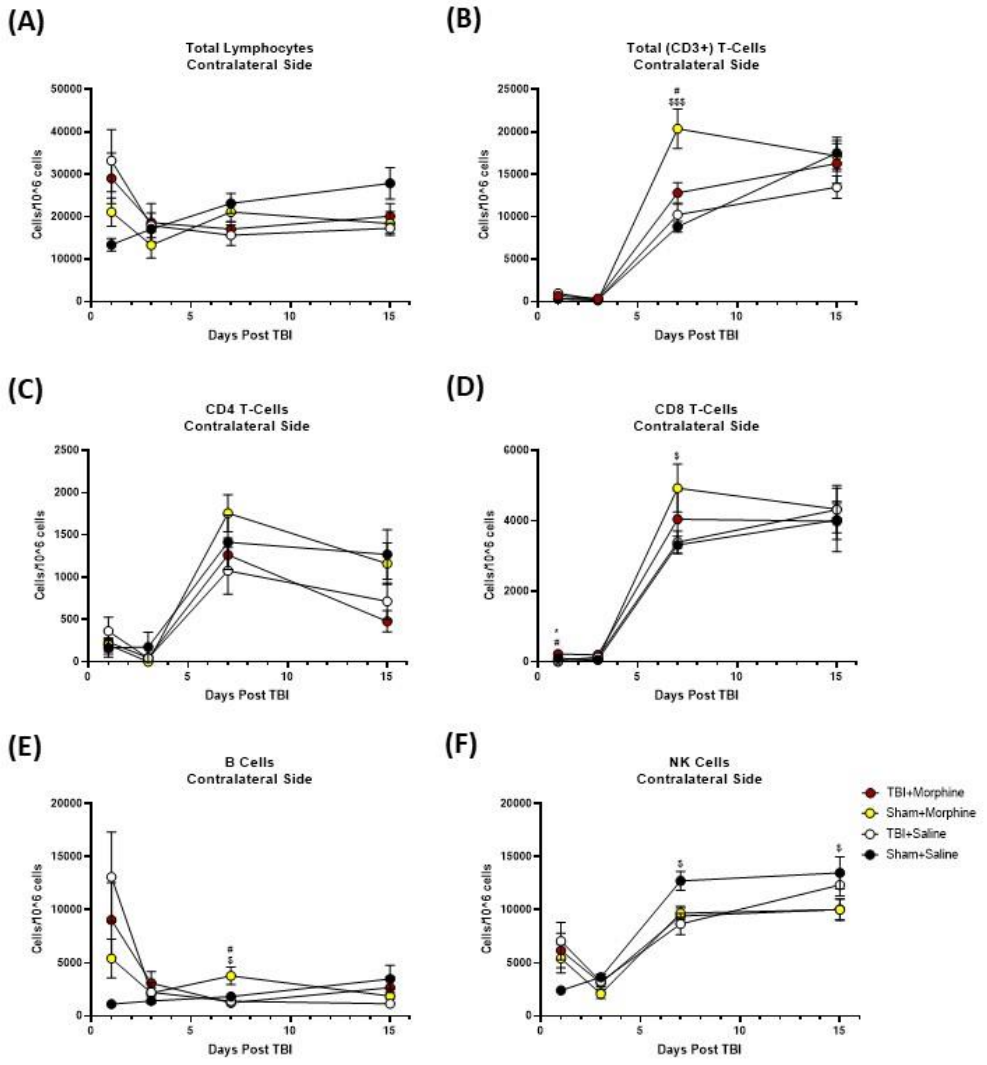

Fig. 4.6 Minor changes in the lymphocyte population on the contralateral side of injury. Graphs show the number of the cell of interest per 10^6 cells on the Y axis, with the days post injury on the X axis. All graphs shown are from the contralateral side of injury. Total lymphocytes (A) are identified as $CD45^{hi}CD11b^{-}$. Lymphocytes were then evaluated for CD3 expression (B), and those that were positive were further evaluated for CD4 (C) and CD8 (D) expression. Lymphocytes that are $CD3^{-}CD19^{+}$ positive were labeled as B Cells (E). Cells that were $CD3^{-}CD19^{-}$ were evaluated for NK1.1 (F) and cells that were positive were labeled natural killer (NK) cells. Significance calculated utilizing t-tests on GraphPad Prism software (* refers to significant differences between morphine exposed and unexposed injured mice, # refers to significant differences between morphine exposed injured and sham-injured mice, $ refers to differences between morphine-exposed and unexposed sham injures mice; */#/$p≤0.05, **/##/$$≤0.01, ***/###/$$$≤0.005).

(1,828±241 cells; *multiple unpaired t-tests, p=0.0194, 0.0456 respectively*). When examining the NK cell population, there is significantly less cells in morphine exposed

sham-injured mice at 3-DPI (2,084±445 cells) and 7-DPI (9,681±645 cells) when compared to saline treated sham-injured mice (3-DPI: 3,652±130 cells; 7-DPI: 12,732±896 cells; *multiple unpaired t-tests, p=0.0334, 0.0200*).

The mRNA levels of cytokines and chemokines changes in the brain with morphine exposure

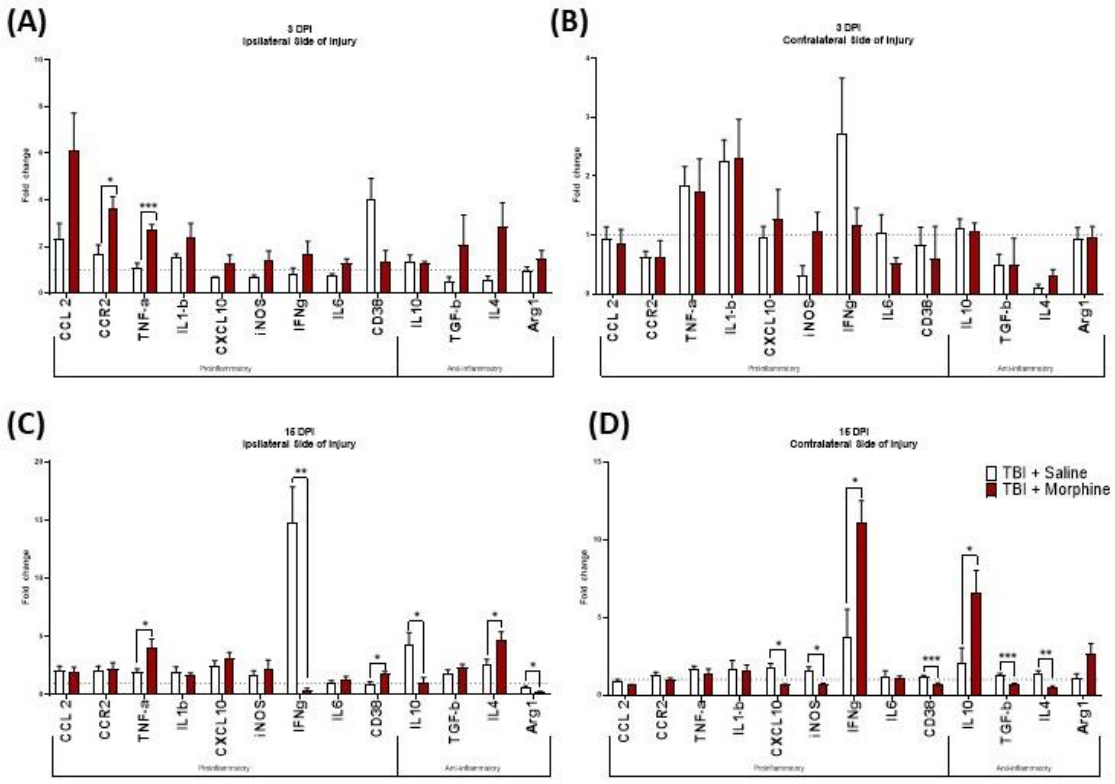

Fig 4.7 The RNA levels associated with pro- and anti- inflammatory chemokines and cytokines after injury changes based on exposure to morphine. Levels of RNA were measured at the acute 3 days post injury (DPI) timepoint on the ipsilateral (A) and contralateral (B) side of injury, as well as at the chronic stage at 15-DPI on the ipsilateral (C) and contralateral (C) side of injury. Significance calculated utilizing Welch's t-tests on GraphPad Prism software, p values of 0.05 or lower were considered significant (*p≤0.05, **≤0.01, ***≤0.005).

The mRNA levels alter according to morphine exposure, days post injury, and side of the brain relative to injury. All values were normalized to saline treated sham-injured controls on the respective sides of the brain, as illustrated by the black dotted line on each graph

(Fig 4.71). At 3-DPI on the ipsilateral side of injury (4.7A), two-way ANOVA reveals individual effects of injury and mRNA that interact with one another (*p=<0.0001, 0.0218, 0.0005*). The level of C-C chemokine receptor 2 (CCR2) mRNA was significantly elevated in morphine exposed injured (3.6±0.9 fold) mice as compared to saline treated injured mice (1.7±0.8 fold; *multiple unpaired t-test, p=0.0420*). At the same time, the level of tumor necrosis factor-alpha (TNFα) was increased in morphine exposed injured mice (2.7±0.4 fold) over saline treated injured mice (1.1±0.4 fold; *multiple unpaired t-tests, p=0.0018*). There were no significant changes at 3-DPI on the contralateral side of injury (4.7B).

The 15 DPI response represents the chronic stage of injury. On the ipsilateral side (Fig. 4.7C, two-way ANOVA reveals individual effects of morphine exposure and cytokine/chemokine mRNA expression, which interact with one another (*p=0.0488, 0.0023, <0.001*). There is a significant increase in levels of TNFα in morphine exposed injured mice (4.1±1.4 fold) over saline-treated injured mice (2.0±0.6 fold; *multiple unpaired t-tests, p=0.0477*). Interferon gamma (INF-ɣ) is significantly decreased in morphine exposed injured mice (0.4±0.3-fold vs 14.8±6.9 fold), as is interleukin 10 (IL10) and Arginase 1 (Arg1) as compared to saline treated controls (IL10: 1.0±1.037-fold vs 4.3±2.3 fold; Arg1: 0.2±0.1 fold vs. 0.6±0.3 fold; *multiple paired t-tests, p=0.0096, 0.0257, 0.0125 respectively*). Interleukin 4 (IL4) and CD38 were both significantly increased in morphine exposed injure mice (4.7±1.3 fold, 1.8±0.4 fold) than in saline treated injured mice (2.6±1.1 fold, 0.9±0.3 fold; *multiple unpaired t-tests, p=0.0419, 0.0128*). On the contralateral side, there is no overall effect of morphine exposure (Fig. 4.7D; *two-way ANOVA, p=0.0778*). There were significant increases in INF-ɣ and IL10 in morphine exposed injured mice (11.1±2.8 fold; 6.6±2.8 fold respectively) over saline treated injured mice (3.7±4.4 fold; 2.1±2.3 fold; *multiple unpaired t-tests, p=0.0120, 0.0385 respectively*). There were also significant decreases in C-X-C motif chemokine ligand 10 (CXCL10), inducible nitric oxide synthase (iNOS), transforming growth factor (TGF-β), interleukin 4 (IL4), and CD38 in exposed injured mice (0.7±0.1 fold; 0.7±0.1 fold; 0.6±0.2 fold; 0.5±0.1 fold; 0.7±0.2 fold) as opposed to saline treated injured mice (1.8±0.6 fold; 1.6±0.6 fold; 1.3±0.3 fold; 1.3±0.6 fold; 1.2±0.2 fold; *multiple unpaired t-tests, p=0.0191, 0.0120, 0.0015, 0.0169, 0.0113 respectively*).

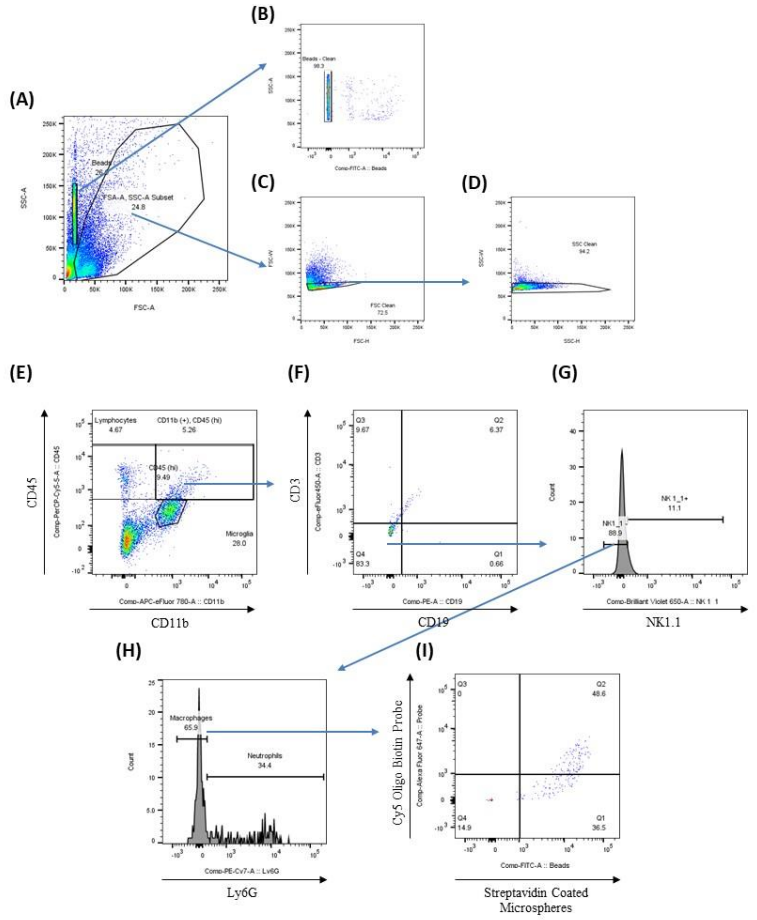

Fig. 4.8 Macrophage phagocytosis gating strategy. Beads were isolated with side scatter area on the Y-axis, and forward scatter area on the X-axis (A). Counting beads are similar size to the streptavidin coated microspheres, beads were analyzed for FITC expression, and beads negative for FITC were labeled as the counting beads (B). Cells are gated on the same dot plot as the beads (A) according to previously established parameters, then doublets are eliminated using forward scatter height and width (B), then side scatter height and width (C) to analyze single cells. From there, CD45+ cells were graphed on the Y-axis and CD11b on the X-axis (D). Cells with high expression of CD45 and positive for CD11b expression and cells with intermediate expression of CD45 and positive for CD11b were evaluated for CD3(F), CD19 (F), NK1.1 (G), and Ly6G (H) expression. $CD45^{hi}CD11b^{+}$ cells that are negative for CD3, CD19, NK1.1, and Ly6G expression were labeled as macrophages, and $CD45^{int}CD11b^{+}$ cells that are negative for CD3, CD19, NK1.1, and Ly6G expression were labeled as microglia. Macrophage and microglia cells were then evaluated for streptavidin coated microspheres and the Cy5 oligo biotin probe (I). Cells that were double positive for the streptavidin coated microspheres and the Cy5 oligo biotin probe were considered to have phagocytosed beads. Cells positive for only the microspheres were considered to be coated in beads, since the oligo-quencher would quench the signal of the Cy5 biotin probe if it was not inside a cell.

At 15-DPI, brains were isolated from saline treated and morphine exposed mice, separated into the ipsilateral and contralateral side of injury, and homogenized into single cell solution. White blood cells were separated out utilizing a percoll gradient (see flow cytometry protocol) and washed. Cells were then incubated with streptavidin coated FITC conjugated microspheres that were further labeled with a Cy5 oligo biotin probe. After incubation for 5hr, Cy5 fluorescence in non-phagocytosed microspheres were quenched with an oligo-quencher and cells evaluated by flow cytometry (see Fig. 4.8 for gating strategy) to identify macrophages/ microglia that were double positive for the microspheres (FITC) and oligo probe (Cy5), thus indicating the cell had phagocytosed the beads (see methods for details).

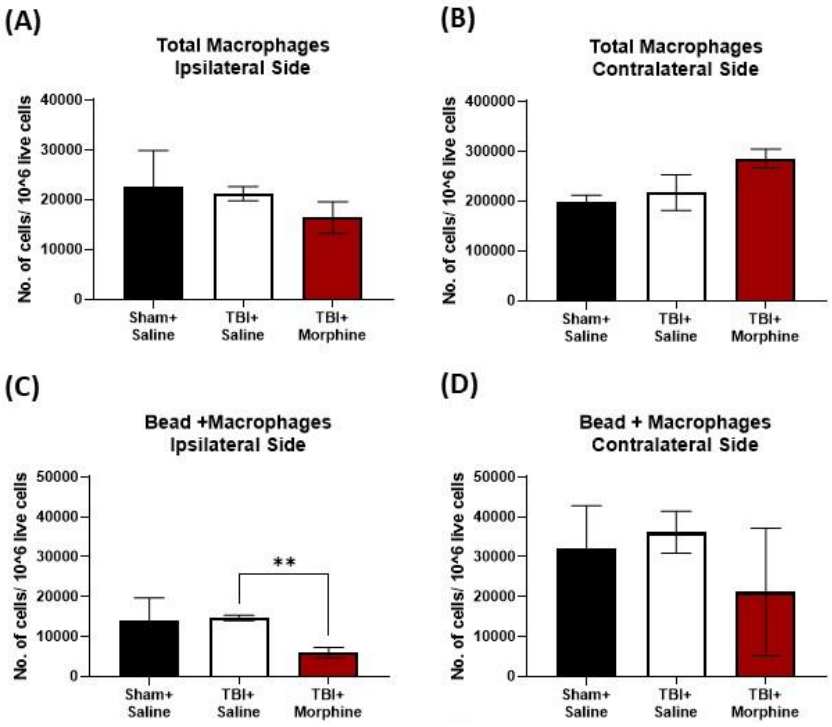

Fig. 4.9 Macrophage phagocytosis is reduced on the ipsilateral side to injury. Graphs show the number of the cell of interest per 10^6 cells on the Y axis (A-D), and group on y axis. Total macrophages are displayed on the ipsilateral (A) and contralateral side (B). Bead + macrophages were double positive for streptavidin coated microspheres and the Cy5 oligo biotin probe on the ipsilateral (C) and contralateral (D) side. (**$p \leq 0.01$).

When examining the macrophage population, there were similar amounts of macrophages in each group on the ipsilateral (Fig. 4.9A) and contralateral sides (Fig 4.9B). There was a

decrease in the number of beads+ macrophages on the ipsilateral side of injury (Fig. 4.9C) in morphine exposed injured mice (5,951±1,285 cells) in comparison to saline treated injured mice (14,651±704 cells, *Welch's t-test, p=0.0087*). There were no differences in bead+ macrophages on the contralateral side of injury (Fig. 4.9D)

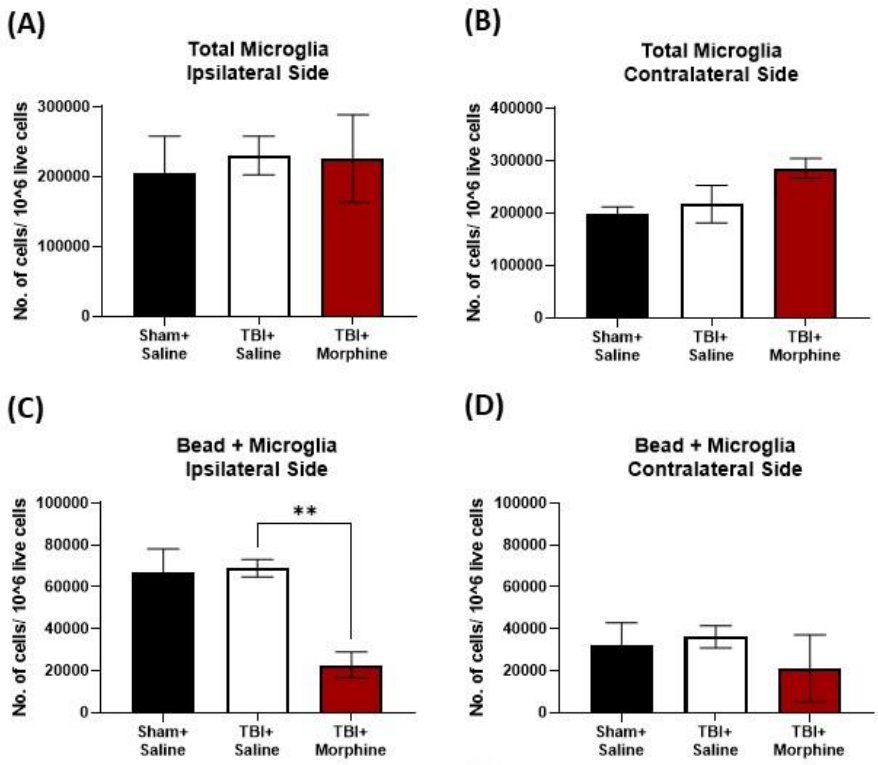

Fig. 4.10 Microglia phagocytosis is reduced on the ipsilateral side to injury. Graphs show the number of the cell of interest per 10^6 cells on the Y axis (A-D), and group on y axis. Total microglia are displayed on the ipsilateral (A) and contralateral side (B). Bead + microglia were double positive for streptavidin coated microspheres and the Cy5 oligo biotin probe on the ipsilateral (C) and contralateral (D) side. Significance calculated utilizing Welch's t-tests on GraphPad Prism software, p values of 0.05 or lower were considered significant (**p≤0.01).

There were also similar amounts of microglia in each group on the ipsilateral (Fig. 4.10A) and the contralateral side (Fig. 4.10B). Again, there was a 3-fold decrease in the bead+ microglia on the ipsilateral side of injury (Fig. 4.10C) in morphine exposed injured mice (22,717±6,194 cells) when compared to saline treated injured mice (68,982±4,146 cells; *Welch's t-test, p=0.0053*). There was no significant difference in phagocytosis on the contralateral side of the injury (Fig 4.10D).

Blood brain barrier leakage was detected in the midbrain of morphine-exposed injured mice

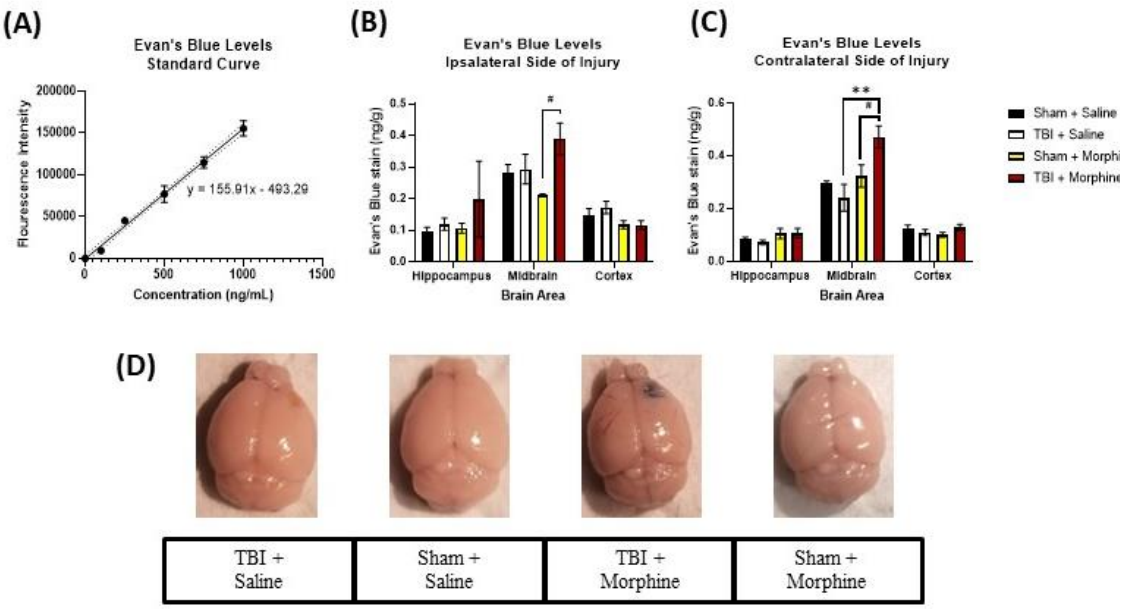

Fig. 4.11 Evan's blue dye leaks into the *midbrain* of morphine-exposed injured mice. The standard curve (A) of Evan's blue (ng/mL) on the x-axis vs the fluorescence intensity on the y axis. The equation displayed was used to calculate the ng of EB in each sample normalized to weight of the brain sample giving ng EB/g of brain tissue for the ipsilateral (B) and contralateral side of injury (C). Representative images of brains from each group (D). Significance calculated utilizing Welch's t-tests on GraphPad Prism software, p values of 0.05 or lower were considered significant (*p≤0.05, **≤0.01).

A standard concentration curve (Fig. 4.11A) was used to quantify (ng/mL) EB in each brain sample and normalized to the total weight of the brain sample to give the quantity of Evan's blue (ng/g) in the brain region. On the ipsilateral side to injury 15 DPI (Fig. 4.11B), morphine exposed injured mice had significantly more EB (0.390±0.050 ng dye/g brain tissue) in the *midbrain* than morphine exposed sham-injured mice (0.210±0.005 ng dye/g, *multiple unpaired t-tests, p=0.0360*). Similarly, on the contralateral side of injury (Fig. 4.11C), there was more EB dye in the morphine exposed injured mice (0.471±0.042 ng dye/g brain tissue) than in the saline treated injured mice (0.241±0.050 ng dye/g brain tissue) or in the exposed sham-injured mice (0.323±0.042 ng dye/g brain tissue; *multiple unpaired t-tests, p=0.0130, 0.0461 respectively*). A gross anatomical examination of select

brains from each group are also displayed presence of EB in the brains of morphine exposed and injured animals (Fig. 4.11D).

Morphine exposed injured mice have difficulty maintaining and gaining weight post-injury.

The timelines for the training protocol and experimental endpoints are shown in Fig. 4.12A. Surgical times were similar for saline treated mice and morphine exposed injured mice (Fig. 4.12B). Surgical time for inducing injury took significantly longer (5.5±0.2 mins) than for sham-injured animals (3.4±0.02 mins) to complete in the saline treated group (*multiple unpaired t-test; p<0.0001*). Morphine exposed sham surgery took 3.4±0.3 mins to complete while morphine exposed injury took significantly longer at 5.5±0.2 mins to complete (*multiple unpaired t-test; p<0.0001*); though no significant differences existed between morphine exposed and saline treated groups. The recovery time was also significantly increased by injury, though not significantly increased by morphine exposure (Fig. 4.12C). Saline-treated sham-injured mice took 6.4±0.5 mins while saline treated injured mice took 10.7±0.6 mins to recover. Morphine exposed sham-injured mice took 7.0±0.5 mins while morphine exposed injured mice took 11.4±0.7 mins to recover (*multiple unpaired t-test; p<0.0001, 0.0001 respectively*). A simple linear regression curve (Fig. 4.12D) revealed that the sham-injured mice had a significantly different elevation in their slopes than injured mice (*p=0.0004*).

Body weight changes were seen throughout the experiment. Morphine exposed injured mice had weights below baseline (Fig. 4.12E), while saline treated injured mice gain weight, maintaining a weight above baseline from 2 DPI throughout the experimental timeline. When analyzing with a two-way ANOVA, there is a significant effect of days post injury, as well as exposure, and these variables interact with one another (*p=<0.0001, 0.0080, 0.0433*). When examining the sham injury group (Fig. 4.12F), there is an effect of days post injury, but not of exposure status, thought these variables do interact (*p=<0.0001, 0.9185, <0.0001*).

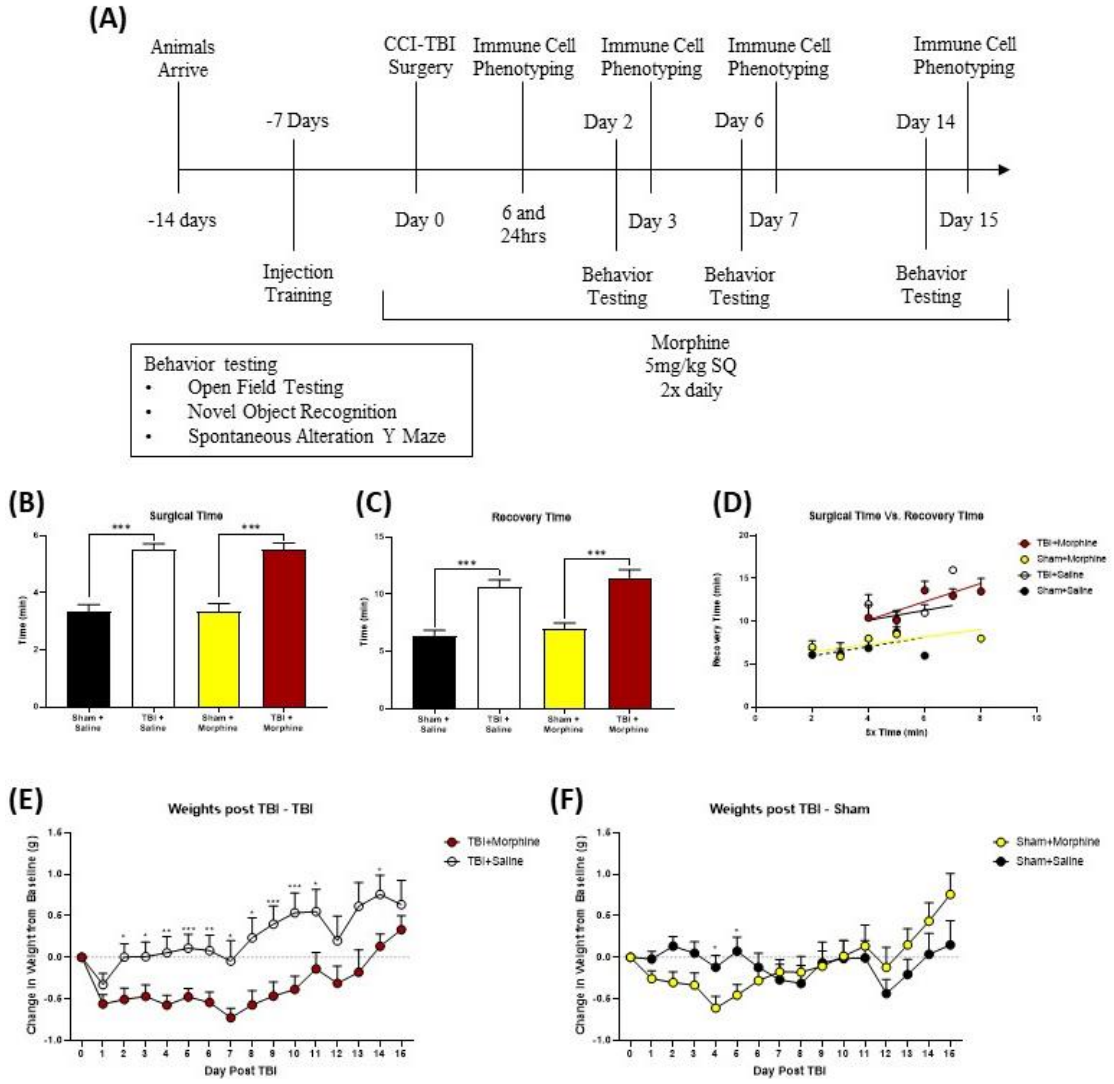

Fig. 4.12 Morphine exposed injured mice have difficulty maintaining and gaining weight post-injury. Timeline (A) is shown from mice arrival to 15 days post injury (DPI). The time of surgery (B) was time from first incision to incision closure. Mice were given SC 5mg/kg morphine or saline immediately following incision closure. Time for recovery (C) was time from the mouse taken off anesthesia to sternal recumbency. Simple linear progression was used to examine the relationship of surgery to recovery time; the red line represents morphine-exposed injured mice, the black line represents unexposed injured mice, the yellow line represents the morphine-exposed sham-injured mice, and the black dotted line represents saline treated sham-injured mice. Surgical vs recovery time (D) is given as time of surgery in min on x axis, and time of recovery in min on y axis. Weights post-surgery of injured mice (E) and sham-injured mice (F) with both morphine-exposed and unexposed mice shown with change in weight from base line on the Y-axis and DPI on the X-axis. Significance calculated utilizing Welch's t-tests and multiple unpaired t-tests on GraphPad Prism software, p values of 0.05 or lower were considered significant (*p≤0.05, **p≤0.01, ***p≤0.005).

Morphine-exposed injured mice demonstrate increased anxiety during open field testing.

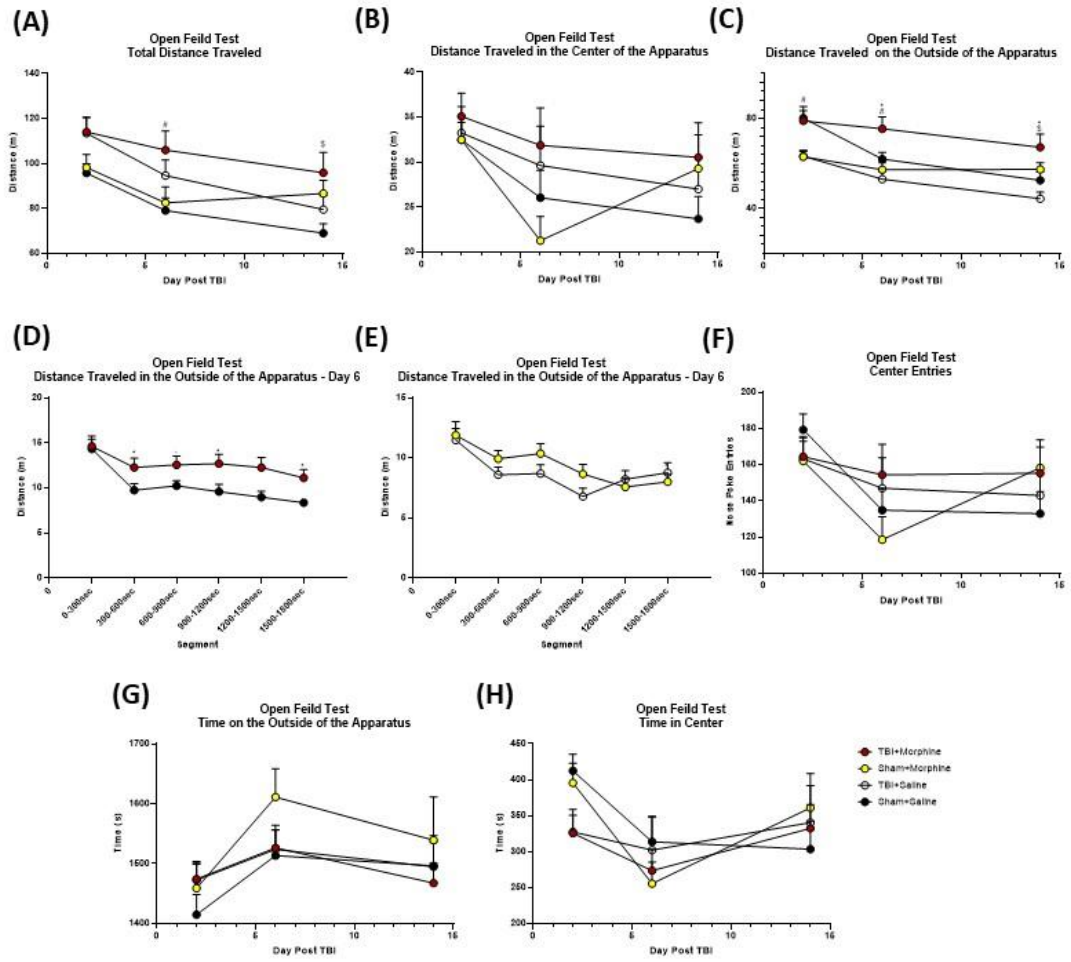

Fig 4.13 Morphine-exposed injured mice demonstrate increased anxiety during open field testing. Total distance traveled (A) over all time points displayed as distance in meters on the Y-axis, and days post injury on the X-axis. Distance traveled is broken down into distance traveled in the center (B) and outside (C) of the apparatus. Distance traveled in the outside of the apparatus at 6-DPI is presented for injured (D) and sham-injured (E) per segment of testing on the X-axis (Data recorded in 300 sec intervals). The number of center entries (Y axis) is displayed over days post injury (F). The time spent in seconds (Y axis) is displayed for the outside (G) and center area (H). Significance determined by multiple t-test with GraphPad Prism (* refers to significant differences between morphine exposed and unexposed injured mice, # refers to significant differences between morphine exposed injured and sham-injured mice; */#/$p≤0.05).

Mice were tracked in the OFT for time and distance traveled within the apparatus. When looking at the total distance traveled over the experimental timeline (Fig. 4.13), at 6-DPI,

morphine exposed injured mice traveled 106.0±8.4 m while morphine exposed sham-injured mice traveled significantly less at 82.6±7.1 m (*multiple t-tests, p=0.0471*). At 14-DPI, morphine exposed sham-injured mice traveled 86.7±5.8 m while saline treated sham-injured mice traveled significantly less at 69.1±4.2 (*multiple t-tests, p=0.0175*). There were no significant differences examining the distance traveled in the center of the apparatus Fig 4.13B). However, there are differences between the groups when examining the distance traveled on the outside of the apparatus (Fig. 4.13C). Morphine exposed injured mice traveled significantly further than saline treated injured mice on 6-DPI (75.4±5.3 m vs 61.9±3.0 m respectively) and 14-DPI (67.3±5.9 m vs 52.5±3.7 m; *multiple unpaired t-test, p=0.0331, 0.0396 respectively*). Morphine exposed injured mice also traveled significantly farther than morphine exposed sham-injured mice at 2-DPI (79.0±4.6 m vs 63.0±2.4 m) and 6-DPI (75.4±5.3 m vs 57.3±3.1 m; *multiple unpaired t-test, p=0.0042, 0.0088 respectively*). Morphine exposed sham-injured mice (57.4±2.9 m) traveled significantly further than saline treated sham-injured mice (44.4±3.0 m) at 14-DPI (*multiple unpaired t-tests, p=0.0050*). There are no differences in center entries (Fig. 4.13F) or time spent in each area (Fig. 4.13G&H) for any group.

Upon further examination, the most dramatic differences center at 6-DPI. The recording of data is broken down into 300 second segments (Fig. 4.13D&E). All groups start the first segment at a higher rate of travel, but the morphine exposed injured mice maintain higher activity (Fig. 4.13D) as evidenced by significantly higher values of meters traveled at segment 300-600s (12.3±4.7 m vs 9.7±3.3 m), 900-1200s (12.7±4.9 m vs 9.6±3.7 m), 1200-1500s (12.3±5.3 m vs 9.0±3.0 m), and 1500-1800s (11.1±4.4 m vs 8.4±1.8 m) over saline treated injured mice (*multiple unpaired t-tests, p=0.0495, 0.0249, 0,0165, 0.0111 respectively*). There are no significant differences between morphine exposed and saline treated sham-injured mice in the 6-DPI segment data (Fig. 4.13E).

NORT demonstrates an increase in distance traveled in the exposed injured mice. At 6-DPI, injured mice in the familiarization stage (Fig. 4.14A) traveled (35.2±2.1 m) significantly further than exposed sham-injured mice (27.7±1.9 m; *multiple paired t-test, p=0.0131*). At the test stage (Fig 4.14B) at 6-DPI, injured mice again traveled (29.7±1.8

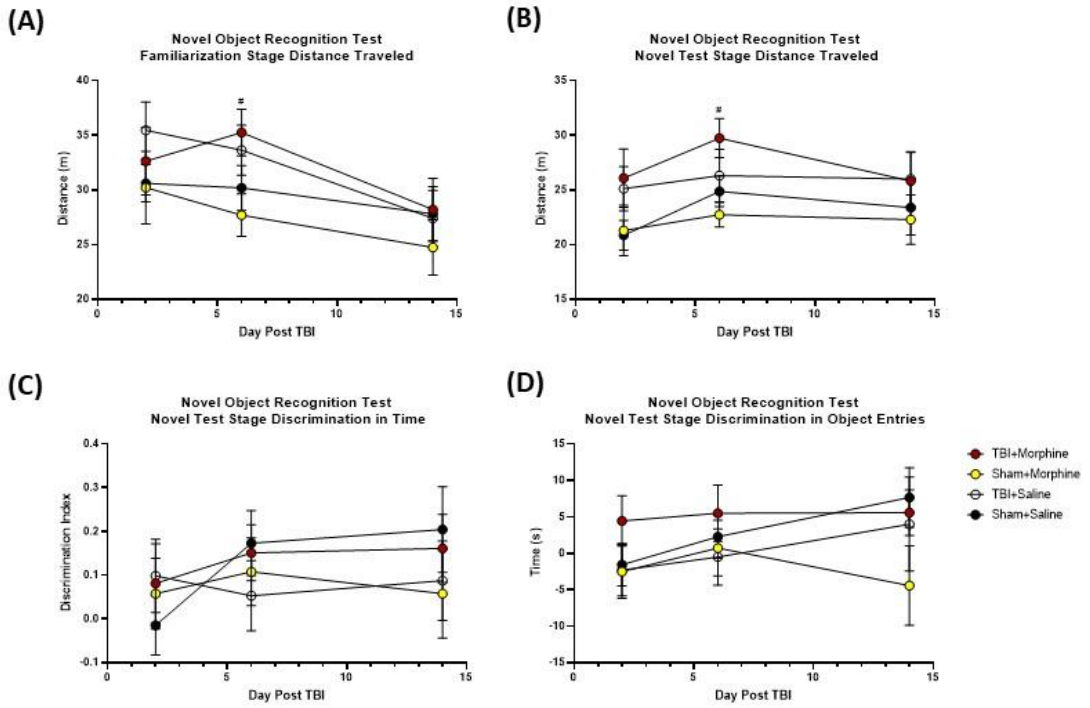

Fig. 4.14 Novel object recognition test (NORT) reveals no deficits in short-term memory following injury or morphine exposure. Total distance traveled is displayed as distance during the familiarization (A) or novel test (B) stage of NORT. The ability to discriminate between novel and familiar objects is displayed by evaluating time spent with each object (C), or entries into the object areas (D). Significance determined by multiple t test with GraphPad Prism (# refers to significant differences between morphine exposed injured and sham-injured mice; #p≤0.05).

m) significantly further than saline treated sham-injured mice (22.7±1.1 m; *multiple paired t-test, p=0.0020*). There was no difference between any groups on novel object discrimination (Fig. 4.14C&D).

Spontaneous alternation Y maze also demonstrated group differences. At 6-DPI, morphine exposed injured mice traveled 47.0±6.0 m (Fig. 4.15A) and made 86±11 alternations (Fig. 4.15B) which is significantly higher than saline treated injured mice that traveled 29.5±1.6 m and made 57±3 alternations (*multiple paired t-tests, p=0.0191, 0.0078 respectively*). At the same timepoint, morphine exposed sham-injured mice traveled farther (41.8±5.2 m) and had more alternations (77±10) than saline treated sham-injured mice (28.1±1.1 m, 52±2 alternations; *multiple paired t-tests, p=0.0154, 0.0180*). At 14-DPI, morphine exposed injured mice again traveled significantly farther (58.5±9.2 m) and made significantly more alternations (112±18) than saline treated injured mice (21.6±1.8 m;

42±4 alternations; *multiple paired t-tests, p=0.0008, 0.0007*). Again, exposed sham-injured mice traveled farther (49.2±9.0 m) and had more alternations (86±17) than saline treated sham-injured mice (24.8±2.3 m, 46±4 alternations) at 14-DPI (*multiple paired t-tests, p=0.0158, 0.0320*). There were no differences in the % correct alternations (Fig. 4.15C) between any groups.

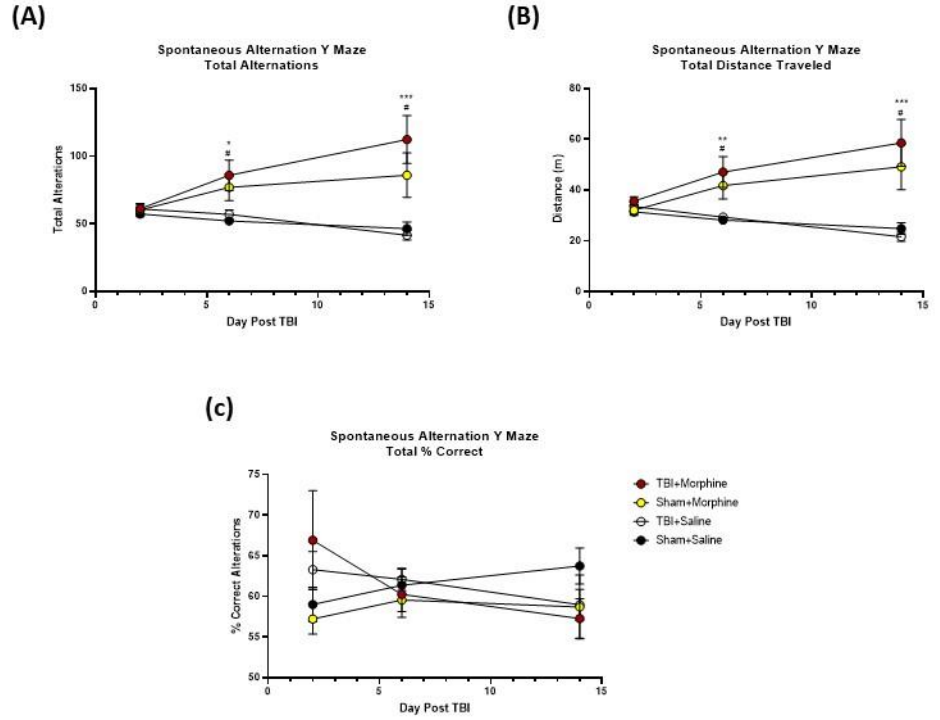

Fig. 4.15 Morphine-exposed mice have increased distance traveled and total alternations regardless of injury status. Mouse movement in spontaneous alternation Y maze was tracked with Any Maze software. Total Alternations (C) shows the total entries into each arm of the maze. Total Distance traveled (D) was tracked and recorded in meters. Correct alternations (E) are defined as consecutively entering each arm of the maze without returning to a previously visited arm. Significance determined by multiple t test with GraphPad Prism (* refers to significant differences between morphine exposed and unexposed injured mice, # refers to significant differences between morphine exposed injured and sham-injured mice, $ refers to differences between morphine-exposed and unexposed sham injures mice; */#/$p≤0.05, **/##/$$≤0.01, ***/###/$$$≤0.005).

Discussion

A distinct and temporally dynamic inflammatory response was observed in mice that were injured and exposed to chronic morphine as revealed through immunophenotyping of the

cellular composition of the brain. Injured mice exposed to morphine had increased number of infiltrating macrophages on the ipsilateral side of injury at 3 and 15-DPI, though they returned to levels seen in sham-injured mice at 7-DPI. At 3-DPI, macrophages had increased CD86, CD206, Ly6c and MHC II expression. This acute stage increase in macrophage infiltration is consistent with other studies,[525,526] and the model of mild TBI described in Chapter 2. As previously stated, activated immigrating and resident macrophages remove cellular debris and repair the damage involved with mild TBI.[152,527,528] CD86, Ly6c, and MHCII are all proinflammatory markers, giving some clues to the possible functions of the recruited macrophages.[319,529-533,585-587] CD86 is involved with antigen presenting, as is MHCII, which can both communicate with the adaptive immune response.[319,529-533,585] CD86 has also been implicated in the pruning synapses of damaged axons or indirectly through cytokine mediators.[88,152,527,528] Ly6c has been implicated in maintaining inflammatory responses, and possible in maintaining chronic inflammation.[586,587] CD206 is considered an anti-inflammatory marker and has been indicated in repair and remodeling stage of injury.[319] CD206 was also the only marker elevated at 15-DPI on macrophages. While this marker naturally occurs in injury models, it may also be connected to administration of morphine. While commonly associated with macrophage apoptosis,[155,493-495] CD206 is generally immune suppressive and has a role in neuronal remodeling.[155-158] There is also evidence that morphine increases the amount of macrophages when the CNS is damaged which could point to different effects based on tissue site.[496,497] However, the response is isolated to macrophages, with no change in activation markers on microglia.

When examining the adaptive immune system, there is an increase in lymphocyte infiltration on the ipsilateral side of injury in morphine exposed injured mice. This response was dominated by $CD3^+/CD8^+$ cytotoxic T-cells. While lymphocyte numbers can increase after a TBI and in response to increased ROS levels, it was not previously seen in the model of mild TBI (see Chapter 2).[303,572-574] However, when T-cells do invade, it is correlated with worsened prognosis and more chronic outcomes.[574] Morphine's immunosuppressive effects extend to the adaptive immune system and can reduced the cytotoxic effects of NK cells and T cells, along with increasing the number of opiate receptors on the cell surface.

[155,158,498,499,501,575] Take together, it seems that an increase in lymphocyte infiltration is to be a novel finding. Further studies need to be done to determine the function associated with these cells.

Next, the inflammatory milieu was examined through the analysis of mRNA expression of proinflammatory and anti-inflammatory cytokines and chemokines by rtPCR. At the acute 3-DPI timepoint, changes in mRNA levels were isolated to the ipsilateral side of injury with significant increases in CCR2 and TNFα level in morphine exposed injured mice. CCR2 is associated with the chemotaxis of monocytes/macrophages in response to its ligand, CCL2, both which were elevated in the morphine exposed brain, though not statistically significant.[280] Increases in monocyte trafficking into the brain is linked to worsened behavioral outcomes,[286] thus may contribute to the alteration of the rewarding effects of morphine demonstrated in Chapter 3, and the increased anxiety-like behavior during the open field test. Once macrophages have invaded the brain, they can remodel neural networks though synaptic pruning and cytokine production.[88,152,527,528] TNFα is secreted by macrophages and promotes proinflammatory responses and macrophage activation.[534,535] This response suggests that the acute phase of injury recruits and activates macrophages in the brain on the ipsilateral side of injury. However, tests are needed to see if the mRNA changes seen here are translated to changes in protein levels.

At 15-DPI, there is a shift in chemokine/cytokine levels of mRNA on the ipsilateral side. INF-γ, IL10 and Arg 1 are all significantly decreased; IL4 and CD38 were both significantly increased. INF-γ is secreted by T cells, promotes proinflammatory responses and can activate macrophages.[539] Therefore reduction in this cytokine could be reflective of the immunosuppression caused by morphine.[155,158] Arginase 1, produced by macrophages, and IL-10, produced by many cells, suppress macrophage activation and promote healing,[537,538,540] so reduction in these signals seems counterintuitive, but the repair mechanisms may be governed through alternate signaling cascades.[70,588,589] IL4 is primarily expressed by Th2 cells, mast cells, eosinophils and basophils, and can support T cell growth and cytolytic activity (in conjunction with IL2).[590-592] This increase in INF-γ may be responsible for the support of the infiltrating $CD3^+/CD8^+$ lymphocytes. CD38 is expressed in the brain on a variety of cells (e.g., neurons, astrocytes, microglia) and has

been linked to autoimmunity and neuroinflammation.[593,594] Thus, CD38 could be supporting proinflammatory and cellular infiltration seen at this timepoint.

On the contralateral side at 15-DPI, there are several changes in the levels of mRNA associated with the inflammatory response. This demonstrates a change at the chronic timepoint where the inflammatory response has expanded to sites that are distal to the initial injury. In morphine exposed injured mice, there were significant increases in INF-γ and IL10, and significant decreases in CXCL10, iNOS, TGF-β, IL4, and CD38. Again, INF-γ promotes proinflammatory responses,[539] and may be the main driver of any proinflammatory response. There were significant decreases in CXCL10, iNOS, IL4, and CD38, all of which can support proinflammatory responses, cell recruitment and growth, and thus may be decreased in response to morphine immunosuppression.[155,158,534,535,542,543,590-595] IL10 and TGF-β are both involved in suppressing macrophage activation and promote healing.[536,540] The increase in IL10, coupled with the decrease in TGF-β, suggests that IL10 is more involved with immunosuppression, and thus may work in concert with morphine. Again, without evidence that the changes in mRNA are reflected in protein levels leaves most of these conclusions as speculation. Further investigation is needed to determine how these signaling molecules interact with the inflammatory response in the context of morphine exposure.

To understand the function of inflammatory cells at the chronic timepoint, the phagocytic activity of macrophages and microglia was evaluated at 15-DPI. This is of particular interest since after invading the brain, macrophages can remodel neural networks and its function though synaptic pruning and cytokine production.[88,152,527,528] A marked decrease in phagocytic activity of both macrophages and microglia was observed on the ipsilateral side of injury in morphine exposed injured mice, which is surprising considering there were no changes in the activation phenotype of microglia by flow cytometry. However, this is in line with the effects of morphine, which reduces phagocytic activity.[155,493-495] This reduced phagocytosis may be isolated to activated macrophages on the injured side.

An increased leakage of Evan's blue dye was observed within the *midbrain* of morphine exposed injured mice. This would suggest that there is alteration of the BBB. The BBB has long thought to be the main barrier that keeps the brain an immunologically privileged site.[59] However, disruption to the BBB is not enough to induce a proinflammatory infiltrate, even when exacerbating by hyperosmolar agents or ischemia.[234,242] Healthy individuals can also have variations in 'leakiness' within the BBB without increases in cellular infiltrate or autoimmunity.[60] Thus, the more compelling evidence states that disruption of the BBB causes deregulation of the influx of nutrients and efflux of toxins.[61-63] Furthermore, many aspects of the BBB are altered by signaling through microglia and astrocytes.[64] Therefore, more investigation is needed into the functional changes that have occurred, the buildup of potentially damaging substances, and into the inflammatory milieu present in each area.

Mice are also affected in physical and behavioral aspects after mild TBI and morphine exposure. The administration of morphine as a pain reliever for surgery can delay recovery, impeding functional recovery and increasing nociception.[596] Morphine has an extended analgesic effect and extended sedation in the dog by 30 minutes.[597,598] Interestingly, these effects were not seen with morphine administration in mice that received sham injury or TBI. However, injured mice exposed to morphine lost a significant amount of weight and took longer the saline treated mice to return to baseline levels. While morphine has been implicated in reducing food intake 24 hours following surgery,[599] this effect seems enhanced in injured mice and reflects the prevalence of adverse health-related consequences to the dosing of opiates.[600-603] Morphine exposed injured mice also spent increased time in the outside of the open field apparatus, indicating an increase in anxiety-like behavior.[124,130,434] Increased anxiety is a long-term consequence of TBI that has been profiled in animal models as well,[184,187,286,337,435] but that response seems to be elongated when injured mice are exposed to morphine. Opiate use can cause increased anxiety with chronic administration, and that can be exacerbating with withdrawal.[604-606] It seems that this effect is additive, with the increased anxiety response to mild TBI being maintained at a higher level through 15-DPI with administration of chronic dose morphine.

The findings of the study point to a unique paradigm where the inflammatory response to mild TBI is altered with chronic morphine exposure. This chronic dosing model offers a unique opportunity to evaluate the impact of morphine on immune response to TBI over time, given that the temporal response to injury is markedly dynamic. Future studies will focus on the functional aspects of the inflammatory cells that were increased post injury and with morphine exposure. This offers the chance to identify new mechanisms of action underlying the relationship between TBI and SUD that are targets for development novel treatment modalities, where only limited treatment is currently available for both TBI and SUD.[45,490]

Conclusions

The evidence presented here indicates that the immune response to mild TBI is uniquely altered with the addition of chronic morphine administration. Macrophage infiltration peaked at 3 and 15-DPI in morphine exposed injured mice, demonstrating a biphasic response that is not present in injury alone. At 15-DPI, $CD3^+$/$CD8^+$ lymphocytes were also elevated in morphine exposed injured mice exposing a novel change in recruitment of the lymphocyte population after mild TBI. These changes were accompanied by dynamic changes in the mRNA of cytokine/chemokines associated with proinflammatory and anti-inflammatory responses. At the 15-DPI chronic timepoint, macrophage and microglia phagocytosis were decreased in morphine exposed injured mice as well, demonstrating a functional change in the immune system. These novel findings allow for further investigation of the mechanism linking TBI and SUD, along with an opportunity to identify new targets for treatment in reducing the risk of SUD after mild TBI.

Chapter 5
Summary and Future Directions

The preceding chapters have demonstrated a gap in understanding of how the proinflammatory response to mild traumatic injury (TBI) increases the risk of opiate use disorder. To address this gap, a refined model of mild TBI in mice was used to evaluate temporal changes in the neuroinflammatory response to controlled cortical impact (CCI). This mouse model exhibits acute motor deficits and long-term spatial learning deficits. Next, sensitivity to and consumption of morphine was examined using this mild TBI model. Mice that received mild TBI increased their consumption and preference for morphine. This coincided with an increase in the proinflammatory response in the brain, both in cellular infiltration and activation level. The investigation proceeded to evaluate how this neuroinflammatory response is affected over time with chronic morphine administration. The inflammatory response to mild TBI was altered by chronic morphine administration in that macrophages exhibited a biphasic proinflammatory reaction, invading at 3- and 15-days post injury (DPI), and lymphocytes invading at 15-DPI. Expression of inflammatory mediators was altered with morphine exposure, as was the functionality of macrophages. Results presented here suggest that the inflammatory response is implicated in the connection between TBI and opiate use disorder. This model provides the opportunity to study the mechanisms underlying the connection between inflammation and behavioral outcomes of TBI.

Mild TBI Model

Mild TBI is difficult to recognize and diagnose but is extremely common and constitutes 75-90% of all TBI.[1,6,72] With millions suffering each year, and connections to long-term deficits overwhelmingly demonstrated in the literature, it is important to investigate how these long-term conditions develop.[1-5,8,30,33,38-43] Decades after TBI, patients are still at higher risk of developing PTSD, Alzheimer's disease, Parkinson's disease and substance use disorder.[8,30,33,38-43] Inherent variability within the injuries among humans and in the

parameters used in the model has necessitated an in-depth profiling of the behavioral outcomes and immune response to injury.[95]

To describe the mild TBI model and define the severity of injury, several behavioral responses were examined post injury. Injured mice had significantly longer recovery time than sham-injured mice, which was observed in other models of mild injury.[97,522] Furthermore, there was a transient motor deficit and minor deficit in memory shortly after injury, indicating a lapse in coordination acutely that correlates with the severity of injury, in this case mild injury.[12,366,435,509,510,524] Furthermore, this mild TBI demonstrates deficits in spatial learning at the chronic stage of injury. Most injury models utilize repetitive injury or diffuse injury to cause long-term deficits, but the Barnes maze assay is sensitive enough to detect this change.[40,97,337,544,545] In modeling these changes from the acute to chronic stage, this animal model can help investigate issues related to post concussive syndrome which can persist for years after TBI and can be disabling.[12,503-505,508,509]

To understand which cells are crucial to the neuroimmune response to TBI, the phenotype of immune cells in the brain was evaluated by flow cytometry. At the acute timepoint, both neutrophils and activated macrophages infiltrate into the brain, which is similar to the initial response to other injuries.[525,526] Activated infiltrating and resident macrophages remove cellular debris and repair the damage caused by physical trauma.[152,527,528] This response was isolated to the ipsilateral side of injury. In the chronic stage of injury, resident macrophages had elevated CD86 expression, which is a proinflammatory activation marker that is involved in the production of proinflammatory cytokines.[529-533] These proinflammatory macrophages can alter neuronal function through cytokine mediators and synaptic pruning,[88,152,527,528] but more studies are needed to understand the functional role of these cells in recovery form TBI.

To gain evidence of cytokine involvement in the inflammatory response to mild TBI, the neuroinflammatory milieu was evaluated utilizing quantitative Real time reverse transcription polymerase chain reaction (RT-PCR). There was ample evidence of increases in the level of mRNA associated with pro- and anti-inflammatory cytokines on the ipsilateral side of injury at 3-DPI. These cytokines are produced by both innate and

adaptive immune cells,[534-540] suggesting roles for immune cells that did not change their overall population size in the brain after injury. While many promote and support the proinflammatory macrophage response, e.g. tumor necrosis factor-alpha (TNF-α) and interferon gamma (INF-γ),[534,535,539] others suppress macrophage activation and promote healing, e.g. transforming growth factor-beta (TGF-β), arginase 1 (ARG1), and interleukin 10 (IL-10).[536-538,540] These two opposing functions are necessary for control of the immune response, since excess proinflammatory response needs to be limited to decrease the spread of damage.

Cytokines are also involved in cell trafficking to the brain. An important mechanism for macrophage recruitment is the C-C chemokine receptor 2 (CCR2)- C-C chemokine ligand (CCL2) system.[280,286] CCR2 mRNA levels were increased at 3-DPI, correlating with increased macrophage infiltration. Post injury, reduction in the activity of macrophage recruitment coincides with improved behavioral outcomes,[286] providing more evidence for the essential role of macrophages in linking post TBI inflammation to behavioral deficits. At the chronic stage of brain injury, the tissue inflammatory milieu has drastically changed. All the elevations in mRNA seen at 3-DPI are decreased except for interleukin (IL1-β) and C-X-C motif chemokine ligand 10 (CXCL10). IL1-β is a proinflammatory cytokine that has been implicated in altered behavioral outcomes,[280,541] and CXCL10 is an important cytokine for macrophage activation.[542,543] Both of these cytokines can play a role in inducing the proinflammatory macrophage phenotype observed at the chronic stage of injury, however, more investigation is needed to determine if the changes in mRNA are reflected at protein level.

While thus far the inflammatory response has been isolated to the ipsilateral side of injury, immunohistochemistry was used to further localize the acute and chronic response to mild TBI. At both the acute and chronic timepoints, there were increases in staining for markers on microglia/macrophages and astrocytes at local areas, coinciding with evidence that microglia/macrophages migrate to areas of damage to clear debris and participate in astrocytic scar formation.[280,548-553] Furthermore, there was a loss of neuronal marker staining at 30-DPI. Changes within the *hippocampus* were the most apparent, with increased staining for markers on microglia/macrophages and activated astrocytes at the

acute stage of injury. There is also a change in the pattern of NeuN staining at 30-DPI, suggesting alterations to neurons at these sites. Sites distal to the injury can be effected through the dysregulation of neurotransmitter trafficking.[88,152,527,528,546,547] There was also evidence of modified morphology of microglia and macrophages at 30-DPI, possibly demonstrating chronic activation.[554-556] Further research needs to be done to determine the functional role of these cells in TBI.

The limitations of this study can affect the interpretation of the results. Importantly, a mild TBI was induced utilizing a controlled cortical impact model which produces a focal and consistent injury. However, clinical data emphasizes variations exists within injured patients. This model is a simplified injury with one impact that was chosen for its lack of variability that would be beneficial in evaluating mechanisms of pathogenesis but does not reflect the clinical presentation accurately. Furthermore, the present study is entirely descriptive. There were no functional assays performed to distinguish how the immune cell infiltrate is interacting with the brain, or how its manipulation would affect behavior. Therefore, changes in the immune cellar population demonstrated may not be causative to the behavioral deficits observed. Attention also needs to be drawn to the animal model utilized. Though the mouse model was chosen for its versatility (in regard to assays available) to observe immune system changes and behavioral deficits, it is different from the human condition, e.g., the increased complexity of behavioral paradigms and cognition in the human. All observations made in the murine model need to be carefully compared to the human injury state to specify their importance for translational purposes.

The data presented here demonstrates the development of a consistent model of mild TBI in mice. The transient acute effects on motor and memory assays demonstrates the initial deficits seen after mild injury, while the long-term spatial learning deficit links this model to long-term consequences. In profiling the immune response at the acute and chronic stages, the temporally dynamic response to injury can be examined to identify new targets for TBI treatment. Further investigation needs to be done on immune functions at each stage of recovery.

Enhancement of Drug Seeking Behavior

Epidemiological evidence links mild TBI and the development of substance use disorder (SUD), identifying that even mild TBI can increase the risk of SUD by 3-6 times.[28,29,132] Imaging studies in both humans and animals link TBI to changes in the reward pathway.[14,151,153,231,514,557] Following injury, changes associated with the blood brain barrier (BBB) integrity and chronic inflammation are linked to increased rewarding effects of alcohol and cocaine.[148,151-154,558,568] Millions suffer from TBI every year, and the epidemic rise in SUD cases makes it essential to understand the mechanisms linking injury and drug seeking behavior essential.[22-25,190]

To understand how mild TBI influences morphine consumption, the intravenous (IV) self-administration model was utilized. Mice were given mild TBI and trained to administer morphine through an implanted catheter. Injured mice increased their intake of morphine over the 10 days of short access IV self-administration. This finding was consistent with models demonstrating increased alcohol and cocaine intake after a single injury.[148,149,154,568] Other diffuse injury models involving multiple injuries demonstrated increased consumption of ethanol and oxycodone.[569,570] This model demonstrates that a single mild injury is sufficient to observe this drug seeking behavior response, which is supported by epidemiologic evidence.

The immune response to mild TBI is also altered following IV self-administration. Injured mice have increased macrophage and neutrophils on the ipsilateral side of injury. Increase in the macrophage population has been associated with proinflammatory responses, and modulation of that response can limit the spread of damage and preserve neuronal function.[70,282,571] Morphine exposure alone affects macrophages directly, increasing apoptosis and depressing phagocytosis.[155,493-495] Generally, morphine is immunosuppressive and can depress immune cell recruitment,[155-158] which seems to be in contrast to the observations in this model. However, evidence exists that the effect of morphine changes in different tissue types.[497] In one study, increased number of macrophages were recruited to the site of spinal cord injury.[496] Injured mice also had elevations in lymphocytes on both sides of the brain. Post injury, lymphocytes can infiltrate

the brain when there are increases in the production of reactive oxygen species, and their presence correlates with worse clinical outcomes.[303,572-574] But again, morphine is typically immunosuppressive, altering cellular functionality (e.g. reduced cytotoxicity).[155,158,498,499,501,575] The apparent effect of morphine on the immune system is complex, and contradictory based on some of the information in the literature. Therefore, more investigation needs to be done to understand the functional modulation of the immune response to TBI after morphine administration.

To examine if mild TBI affects sensitivity to opiates, conditioned place preference (CPP) was used to investigate preference behavior to increasing doses of morphine (0.5-5mg/kg). Injured mice had increased preference to lower dose morphine. When examining other models of drug seeking behavior after injury, preference for cocaine and alcohol was enhanced after TBI.[147,151-153,557,576] In fact, mice that were exposed to ethanol were more sensitive to its effect following injury.[145,146] This alteration may offer some key insights to the connection between TBI and drug seeking behavior, and may be a factor in the escalation of morphine consumption in TBI patients.

When examining the immune response post mild TBI after the CPP test, there were changes observed across the innate and adaptive immune systems. However, the response is not consistent across the low dose groups and makes interpretation difficult. There are some responses that are consistent with the inflammation seen after IV self-administration of morphine. On the ipsilateral side of injury, there were increases in the neutrophil and macrophage populations at several doses. As previously stated, modulation of this response that limits macrophage infiltrate and promotes the anti-inflammatory response preserves neuronal function.[70,282,571] This finding may be further substantiate that morphine effects are dependent on the context of injury and tissue involved, or simply a reflection of dose dependent effects.[496,497] Total lymphocytes were also increased post injury and low dose morphine exposure, which are correlated with chronic worsened clinical outcomes.[303,572-574] Once again, further research into the functions associated with the cells that have increased presence within the brain is needed to understand the possible links to behavior.

The study provides new insights into the relationship between the proinflammatory response to mild TBI and drug seeking behavior but is not without its limitations. Importantly, drug seeking behavior is not equivalent to substance use disorder in humans and cannot recapitulate all aspects of the condition. The assays here focus on the escalation phase of drug seeking behavior, or initial experiences with morphine. However, this phase is highly dependent on the rewarding effects of morphine. The development of substance use disorder in humans takes a long period to develop and is influenced by the avoidance of withdrawal symptoms rather than seeking a pleasurable experience. Also, the changes to the immune responses were varied and only evaluated at a single timepoint. The immune response is dynamic in any injury, and it is possible to over conclude the importance of some cellular populations by only examining one snapshot in time. A stronger connection could be established through manipulation of the immune response and examining the resulting changes in drug seeking behavior, which is an important future direction for this work.

Mild TBI alters drug seeking behavior; the data presented in this dissertation demonstrates that following mild TBI, mice have increased preference for and consumption of morphine. Concurrently, injured mice have increased levels of immune cells within their brains, implicating the role of the immune system. Some insight into these connections can be gleaned from research utilizing other addictive substances. In regards to alcohol studies, injured mice demonstrated increased sensitivity to alcohol, which coincided with increased astrocyte activation in the *nucleus accumbens*, a key area in the mesolimbic reward pathway.[145,146] Mice also increase their consumption of alcohol post TBI, and that effect can be reversed by minocycline, a drug that inhibits macrophage activation.[148] When examining the response to cocaine exposure, injured mice again increased preference for the drug and had increased neuroinflammation in the *nucleus accumbens* and *ventral tegmental area*.[151] Treatment with dexamethasone can reverse the increased preference while decreasing neuroinflammation.[153] Further studies are needed to test the hypothesis that modulating the inflammatory response post mild TBI can attenuate increased drug seeking behavior to opiates in a similar manner that it did for alcohol and cocaine.

Profiling the immune response to mild TBI in the context of chronic morphine exposure

Injured mice exposed to chronic dose morphine have a distinct and temporally dynamic inflammatory response. Flow cytometry was used for immunophenotyping of the cellular composition of the brain and revealed a biphasic macrophage infiltrate first at 3 and then at 15-DPI. Acute infiltration at 3-DPI demonstrated a highly activated macrophage phenotype, which is consistent with studies reported in the literature.[525,526] Activated macrophages can be involved with removal of cellular debris, communication with the adaptive immune system, and neuronal remodeling.[152,319,527-533,585-587] This could also be influenced by morphine considering morphine enhances recruitment of macrophages to the site of injury in the spinal cord.[496,497] During the chronic stage of TBI, macrophages have reduced activation markers. This could be reflective of the immunosuppressive properties of morphine, and as a result promoting an anti-inflammatory phenotype that supports healing and neuronal remodeling.[155-158,493-495] There is also an increase in $CD3^+CD8^+$ lymphocytes at the chronic stage, which is associated with worsened clinical outcomes post TBI.[574] More studies need to be done to determine the function of these cells and how that changes from the acute to chronic stage.

To gain insight into the potential mechanisms behind the unique changes observed in the immune response to mild TBI during chronic morphine administration, RT-PCR was used to analyze expression levels of mRNA in the inflammatory milieu. At 3-DPI, there were significant increases in CCR2 in injured and morphine exposed mice, which suggests increased activity of the macrophage recruitment CCR2-CCL2 pathway.[280] This CCR2 increase coincides with the acute increase in macrophages in the ipsilateral brain. mRNA levels of TNFα were also elevated at 3-DPI, which denotes a proinflammatory response and macrophage activation.[534,535]

At 15-DPI, there were several changes in the inflammatory milieu. Importantly, there was a reduction in the expression of proinflammatory mediators in injured and morphine exposed mice, which may demonstrate the immunosuppression induced by morphine.[155,158] There was also a rise in the mRNA levels of cytokines involved in suppressing macrophage

activation and promoting healing,[537,538,540] which may signal a change in the function of the immune response. Further, elevation in mRNA levels of IL4, which supports T cell growth and cytolytic activity,[590-592] may play a role in the increase lymphocyte infiltrate at 15-DPI. While further investigation is needed to determine if changes in the level of mRNA are reflected in protein levels. This data suggests functional alterations in the immune response to mild TBI from the acute to chronic stage when morphine is chronically administered.

To investigate the functional changes in macrophages post injury, the phagocytic activity of microglia and macrophages was evaluated in an *ex vivo* experiment at 15-DPI. Phagocytosis is a particularly important outcome measure since activated microglia and macrophages can remodel neural networks through synaptic pruning and cytokine production.[88,152,527,528] Surprisingly, phagocytosis was markedly decreased in both microglia and macrophages on the ipsilateral side of injury even though there was no notable change in the markers used when phenotyping microglia. This effect seems to be due to morphine exposure since morphine reduces phagocytic activity.[155,493-495] However, this effect was seen only on the side of injury, suggesting that it is somehow enhanced by injury. More phagocytosis studies are needed to understand this phenomenon, as well as determine if the reduction in phagocytosis is generalized, or changes with the substrate involved

The chronic morphine dosing model introduces a new paradigm of exposing mice immediately post injury, to model a continuous morphine dose that eliminates fluctuations related to withdrawal periods seen in drug seeking assays. However, it still presents a number of important limitations. Importantly, individuals with mild TBI would not be prescribed opiates clinically unless other painful injuries were present. Much of the data that supports the link of SUD and TBI stresses that injury can happen well before substance exposure. An important question remains whether the changes observed here are present when the time between injury and exposure to morphine is prolonged. The functional implications of the changes in the immune response also needs to be evaluated. Importantly, only one functional assay was utilized that targeted a single cellular population. These observations need to be more fully interrogated, along with additional

functional assays for other populations, to understand the mechanism behind the change. Furthermore, changes to the blood brain barrier in the midbrain were observed, but evaluation of how that affects movement of nutrients into the brain and toxins out will be important to fully understand its role in the development of SUD. While these limitations must be acknowledged, they do not detract from the unique findings throughout the study when injured mice are exposed to morphine.

This model reveals that there is a unique and dynamic response following mild TBI and chronic morphine exposure. By following the response from the acute to chronic stage, a biphasic proinflammatory response in macrophages was identified at 3 and 15-DPI, accompanied by an increase in lymphocyte infiltration at 15-DPI. The inflammatory milieu reveals hints in the possible functional pathways that are involved in the acute and chronic inflammatory response. Investigation into phagocytosis revealed a depression in activity after mild TBI and morphine exposure. This model can be used to evaluate the connection between mild TBI and drug seeking behavior in relation to morphine. Furthermore, it can provide insights into treatment targets to reduce the increased risk of SUD following TBI.

Future Directions

The work presented in this dissertation has brought new insights into the relationship between mild TBI and opiate use disorder, but the work is far from done. Like most research, each discovery inspires more questions for investigation, and a few are highlighted here.

All of these studies have identified a key role of the innate immune system in linking mild TBI and drug seeking behavior, but there is still much not known about their function. An important component of the inflammatory response is localization of the involved cells. The UMN imaging core offers services to use the PEGASOS method of tissue clearing[607] that can be used to localize macrophages to specific areas of the brain in situ and examine their proximity to synapses. Also important is the expression of inflammatory mediators and genes associated with the response. Single-cell RNA sequencing is also a method of investigation that has revealed more comprehensive information about gene expression in cells which led to the identification of new cellular populations and new relationships in

gene interactions.[608,609] Using this method could help to identify differentially expressed genes with morphine treatment while also identifying unique macrophage populations within the brain. This could also help guide investigations into understanding changes in protein levels and future functional assays. Analysis of protein levels is needed to determine if changes in RNA levels are reflected in protein levels, so ELISA or western blot can be used.[610] These analyses can help to better understand the roles of macrophages in mild TBI and its relation to drug seeking behavior, and reveal how the macrophage phenotype changes over time.

Modulation of the inflammatory response *in vivo* can also help determine the role of the immune system in mild TBI and chronic morphine expression. Modulation of the inflammatory response to TBI previously demonstrated an amelioration of the increased preference and sensitivity alcohol and cocaine.[148,153] Clodronate-encapsulated liposome (CPL) administration can deplete peripheral macrophages when the cells phagocytose the CPLs and induce apoptosis. Alternatively, it can be administered in the CNS to deplete macrophages and microglia.[611] This model can be used at multiple time points to reduce these macrophage populations and observe the changes to the immune response and behavioral consequences. While this CPL model offers benefits of being a reductionist approach to study the immune response, cell-based therapies have a more conservative modulatory effect on immune responses and are thus have much promise for TBI therapies.[612] It has been previously demonstrated that UCBSC treatment of ischemic stroke reduced macrophage and T cell infiltration into the brain, along with reducing microglia cell numbers to baseline levels.[613] This method can modulate the innate and adaptive immune response, and is a prime candidate for reducing the increase in opiate seeking behavior following mild TBI.

These further methods of investigation will help to understand how the immune system is involved in the response to mild TBI, and how exposure to chronic dose morphine alters that response. Further investigation of the immune response and characterization of the functional characteristics of the cells can help provide insight on how morphine exposure alters the proinflammatory response to mild TBI. This leads to the identification of targets for treatment. Developing clinical treatments to decrease the risk of SUD after TBI is

desperately needed. Modulation of the response in animals can help demonstrate connections between the immune system and drug seeking behavior. By targeting multiple populations at different time points, the most beneficial time frames for each new treatment can be identified. The work presented here has provided a model that can be used to further understand of the link between TBI and opiate use disorder,

Conclusion

The model of mild TBI presented here has many advantages for research. This model demonstrates that mild TBI induces a persistent proinflammatory activation of macrophages. This is accompanied acutely by transient minor memory and motor deficits, and a long-term spatial learning deficit. The extent of damage within the brain also changes from acute to chronic injury, spreading to the deep cortical regions of the brain. This model provides a method to study the mechanisms that link mild TBI to long-term consequences and may help identify new targets for treatment.

Evaluation of drug seeking behavior post injury provided evidence that the inflammatory response to mild TBI is implicated in the increased rewarding effects of morphine. Injured mice had increased preference to and consumption of morphine, with increased inflammatory infiltrate post injury and morphine exposure. Increased macrophage, neutrophil and lymphocyte levels were observed on the ipsilateral side of injury, demonstrating an enhanced response. This model offers an opportunity to further investigate the functions of these cells post injury and can help identify new treatments to reduce drug seeking enhancement.

Modeling chronic morphine administration post mild TBI provided the opportunity to profile the immune response out to the chronic stage. Following injury, chronic morphine exposure causes a biphasic proinflammatory response in macrophages at 3 and 15-DPI. At 15-DPI, there was also an increase in lymphocytes entering the brain. These results were accompanied by a primarily proinflammatory milieu at 3-DPI, with a more anti-inflammatory milieu at 15-DPI. Concurrently, macrophages and microglia have decreased phagocytosis activity on the ipsilateral side of injury, demonstrating morphine's effect on activated macrophages. These novel findings require further investigation to determine the functional changes that occur in the immune response from acute to chronic injury.

The research presented here demonstrates the development of a model of mild TBI that can be utilized to study long-term deficits. The consistency of the model allows for the evaluation of more complex studies, e.g., the evaluation of drug seeking behavior. The IV

self-administration and conditioned place preference behavioral paradigms allow for the analysis of how the consumption of and sensitivity to morphine is altered after mild TBI. Further investigation utilizing chronic dose morphine allowed for the profiling of changes in the immune response from acute to chronic injury, identifying distinct and dynamic response post TBI and morphine exposure. More research needs to be done to understand the functional aspects of this response and determine the efficacy of treatments in reducing neuroinflammation and drug seeking behavior. However, this work provides a comprehensive description of the behavioral and immune system changes that occur post mild TBI and morphine exposure.

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