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Evaluating Evolving Leukocyte Populations In Peripheral Blood Circulation Post-Concussion In A Human Longitudinal Analysis Of Female Athletes

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ABSTRACT

Concussions are generating increasing concern due to potential long-term neurological consequences. Currently there is no universally recognized diagnostic approach for concussion. I hypothesize that a signature temporal response of biomarkers of inflammation in systemic circulation will provide an objective diagnosis of concussion and could also be used to track patient recovery. The Western University women's rugby team underwent blood draws at pre-season and post-season as a baseline evaluation, and players determined to have sustained a concussion underwent repeat blood analysis post-concussion. Blood samples were analyzed by flow cytometry to profile immune cell populations alongside accepted concussion assessments, and complete blood count. Immune profiles demonstrated significant changes in total leukocytes and subsets post-concussion compared to baseline. It was demonstrated that we could successfully and feasibly recruit and perform a discovery investigation into potential blood biomarkers of concussion longitudinally. My study provides new insights for future blood biomarker research of concussive injury.

KEYWORDS

Biomarker; Concussion; Flow Cytometry; Inflammation; Lymphocyte; *Lys*-EGFP-*ki* Mouse; Mild Traumatic Brain Injury; Monocyte; Neutrophil.

CO-AUTHORSHIP STATEMENT

The majority of the experimental work presented in this thesis was performed by myself. Dr. Lisa Fischer and Dr. Tatiana Jevremovic oversaw all clinical assessments and dealt with concussion status for participants. Christy Barreira performed all sample collections for the 2012-2013 rugby season, as well as assisted with sample collection in following seasons. Statistical testing by Dr. GY Zou and Artem Uvarov using a Kenward-Roger's F test including a AR(1) first-order autoregression model for covariance structure, testing for fixed effects in a linear mixed model, was performed in sections 3.4.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BBB	Blood Brain Barrier
BD	Becton Dickinson
BS	Beginning of Season
BSA	Bovine Serum Albumin
CBC	Complete Blood Count
CCI	Controlled Cortical Impact
CNS	Central Nervous System
CRP	C-Reactive Protein
CSF	Cerebrospinal Fluid
CT	Computed Tomography
CTE	Chronic Traumatic Encephalopathy
DAI	Diffuse Axonal Injury
DAMPS	Danger Associated Molecular Patterns
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic
EGFP	Enhanced Green Fluorescence Protein
ES	End of Season
FACS	Fluorescence Activated Cell Sorting
FDA	Food and Drug Administration
GCS	Glasgow Coma Scale
G-CSF	Granulocyte Colony Stimulating Factor

GFAP	Glial Fibrillary Acidic Protein
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HBSS	Hank's Balanced Salt Solution
IFN	Interferon
IL	Interleukin
ImPACT	Immediate Post-Concussion Assessment and Cognitive Test
Lys-EGFP-*ki*	Lysozyme M-EGFP-Knock In
MFI	Median Fluorescence Intensity
MRI	Magnetic Resonance Imaging
mTBI	Mild Traumatic Brain Injury
NK	Natural Killer
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PRR	Pathogen Recognition Receptors
RBC	Red Blood Cells
RDW	Red Blood Cell Distribution Width
RPMI	Roswell Park Memorial Institute Medium
SCAT3	Sports Concussion Assessment Tool 3rd Edition
SCI	Spinal Cord Injury
TBI	Traumatic Brain Injury
TNF	Tumour Necrosis Factor

CHAPTER 1 – INTRODUCTION

1.1 OVERVIEW

Traumatic brain injury (TBI) is a massive global healthcare problem currently affecting approximately 10 million people annually.[1] It is reported that on average 39% of individuals sustaining a severe TBI will die of their injury.[2] Those that do survive the initial severe trauma die 3.2 times faster than the general population, and will likely face long-term physical, cognitive, and psychological consequences.[2] This is driven by the fact that central nervous system (CNS) injuries often fail to fully and or properly recover over time, leading to permanent disabilities.[3] Still today there are few therapeutic options for people suffering from brain injuries. New CNS therapeutics take about 18 years to transition from laboratory bench to patient, with 8.1 years on average spent on human testing.[2] This is troubling as TBI has been predicted by the World Health Organization to become the major cause of death and disability worldwide by the year 2020.[1] This has led some to describe TBI as a field with one of the greatest unmet needs in medicine and public health.[4]

Brain trauma exists as a spectrum classically defined into three severity groups; severe, moderate and mild, based on the Glasgow Coma Scale (GCS), which is a clinical evaluation of head trauma. The GCS scores people according to level of consciousness by assessing motor responses, verbalization, and eye movement upon stimulation.[5] Scores range from 3 to 15 with scores <8, 8-12, and >12 representing severe, moderate, and mild injuries respectively.[5] Mild traumatic brain injury (mTBI) afflicts the greatest number of individuals, with 100 to 300 per 100 000 seeking medical attention annually worldwide.[6] Since many individuals sustaining mTBI choose not to seek medical attention, it is estimated that greater than 600 per 100 000 worldwide sustain an mTBI annually, this would be roughly 42 million people.[6,7] This is why the

use of the GCS to categorize brain injury patients has received some criticism as it leads people to dismiss more mild injuries among other issues.[2]

1.2 CONCUSSION

The term concussion has been popularized in sports medicine due to the growing global importance of this injury in the context of sports. A concussion is a biomechanically-induced alteration in brain function, which can result in deficits to memory and orientation, as well as resulting in a potential loss of consciousness.[8] Generally, a concussive injury is considered to exist under the umbrella of mTBI.[9] About 300,000 sports-related concussions occur in the United States annually; however, this number has even been suggested to be as high as 3.8 million when recreation- and sport-related concussions are taken into account due to inadequate reporting.[10] A particularly large group at risk for sports-related concussions are young athletes, especially those playing contact sports. The second leading cause of TBI for individuals of the age 15 to 24 are sports, trailing motor vehicle incidents.[11] Sports-related concussions made up approximately 5.8% of all collegiate sports injures, with contact sports producing the highest numbers.[12]

Unlike more severe forms of TBI, most concussion patients recover quickly, requiring 7 to 10 days of rest for symptoms to dissipate.[10] However, long-term disability, such as seizures, and emotional and behavioural issues, are seen in about 10% of individuals sustaining a concussion.[13] The presence of a constellation of symptoms of concussion beyond the acute period of injury has been described as Post-Concussion Syndrome, and some individuals may experience this for years after insult.[14,15] Within the acute recovery period there is a proposed window of increased neural vulnerability, and if an individual were to sustain a second head trauma in this period the effects can be fatal, this is described as Second Impact Syndrome.[16,17]

While this is a very rare and still a controversial condition, it is especially important to high school aged athletes where the predominant number of fatalities are seen.[18] It is suggested to be the result of loss of auto-regulatory control of intracranial and cerebral perfusion pressure resulting in diffuse cerebral swelling.[18]

Although a single concussion can have important but limited neurological consequences, it is the repetitive nature of these exposures that has largely driven interest in this field. A high school football player, according to a study by Broglio *et al.*, can receive as many as 650 to 900 head impacts over the course of a season.[19] Interestingly, sustaining a concussion makes you more susceptible to future concussions, both acutely as with Second Impact Syndrome and more chronically. In a study of collegiate football players, if a player sustained a concussion they became more likely to sustain another concussion that same season than a player who had not received a concussion.[12] This has led to the effects of concussion to be described of as cumulative, with long-term complications associated with multiple concussions.[20] Supporting this idea, Iverson *et al.* demonstrated that memory impairment was 7.7 times more likely in athletes with multiple concussions than athletes with only a single concussion.[20] At some currently undefined point, concussion stops being a recoverable mild injury, and develops into a chronic neurodegenerative condition.

Chronic Traumatic Encephalopathy (CTE) is a neurodegenerative disease believed to be linked to repetitive concussive as well as sub-concussive trauma.[7] While initially considered to only affect boxers, the at risk population for CTE has grown substantially to include almost every contact sport, and military personal.[21] CTE morbidity often resembles Alzheimer's, Parkinson's, Frontotemporal Dementia, and even Amyotrophic Lateral Sclerosis due to accompanying mood, behaviour, cognitive and/or motor deficits.[7] CTE is characterized in the

brain by the presence of hyperphosphorylated Tau protein as well as aggregated transactive response DNA binding protein 43.[22] The presence of CTE is often not detectable until long after the affected individual is regularly exposed to repeated head impacts, for example after retirement in athletes.[22] Even then, definitive diagnosis is not usually obtainable until a post-mortem examination of the brain.[22] Therefore, while the initial concussion may be hard to prevent, its proper identification and diagnoses can allow for an appropriate treatment and/or a preventative re-injury plan to be implemented.

1.3 CURRENT CONCUSSION DIAGNOSTICS

Across the board, disease identification has become more and more advanced, relying on anatomical, physiological, metabolic, immunological, and/or genetic attributes to provide accurate diagnoses.[2] For TBI however, basic clinical signals makes up the large basis of injury classification.[2] Severe and moderate TBI diagnosis is relatively straightforward, this is not the case for mTBI or concussion. An example of this can be seen using the GCS for concussion, with most individuals scoring 14 or 15 on the 15-point scale.[23] While this may rule out more significant head traumas, it lacks the subtlety to properly assess individuals with concussion. At this point in time, there is no universally established non-subjective "gold standard" concussion diagnostic, nor any prognostic or therapeutic strategies available clinically.[24,25] This leaves individuals at enhanced risk of more severe damage from a subsequent head trauma due to improper recognition and inadequate recovery time.

The most popular sideline assessment of sports related concussion is The Sport Concussion Assessment Tool – Third Edition (SCAT3). This is a pen and paper concussion assessment tool predominantly used to determine any acute effects of concussion.[26] It contains a

self-reported clinical signs and symptoms section using a graded scale, as well as tests informing cognitive dysfunction and physical deficits. While the symptom assessment is effective in that it tracks both presence and severity, these symptoms are not unique to concussion and are considered relatively common in non-concussed individuals with clinical conditions.[26] There are often tremendous pressures on athletes, either from coaches, teammates, parents, or the players themselves, to return to play, and as a result athletes will under report symptoms and create the impression they are recovering faster.[9]

In contrast to the pen and paper approach of the SCAT3, computerized neuropsychological tests have been developed such as the Immediate Post-Concussion Assessment and Cognitive Testing (ImPACT). The ImPACT collects various demographic and personal information about the athlete, followed by any symptoms they may have using the same 7 point grading scale as the SCAT3.[23] Lastly, the athlete is assessed using 6 neuropsychological tests evaluating attention processing, verbal recognition memory, visual working memory, visual processing speed, reaction time, numerical sequencing ability, and learning.[23] The effectiveness of these neuropsychological concussion tests has been challenged. In a study on collegiate football players it was demonstrated that over 25% of ImPACT tests produced invalid results, and this was largely due to players "sandbagging" or intentionally performing poorly.[27] Reliability of the ImPACT is further being evaluated by an ongoing independent investigation by the Federal Interagency Traumatic Brain Injury Research (FITBIR) Informatics System in partnership with the National Institutes of Health.

Due to the lack of gross pathology in concussion, even conventional computed tomography (CT) and anatomical magnetic resonance imaging (MRI) scans cannot detect subtle pathology associated with concussion.[10,21] However, these tests are still valuable in that they can

rule out more severe injury such as hematoma. Overall, the current reliance on neurological examination and neuropsychological evaluation to identify the injury and determine the prognosis is subjective and gives no insight into structural or biochemical alterations at play in the concussed brain.[28] As these tests largely inform return to play decisions it is imperative that they be correct and unbiased. So while these are the best we currently have, they are imperfect. It is necessary that new more objective clinical concussion measures be developed to help bridge this gap.

1.4 BASIC ANATOMY OF THE BRAIN

To discuss TBI and concussion pathology, first a brief introduction to neuroanatomy and function is needed. This information was sourced from the textbook The Human Brain: An Introduction To Its Functional Anatomy, 6th edition by John Nolte, unless otherwise cited.[29] There are two principle categories of cells in the brain: neurons and glial cells. Neurons are information processing and signalling elements. They use electrical signals to pass information within their cellular structure as well as chemical signals, or neurotransmitters, to pass information from neuron to neuron. Neurons contain long cylindrical process called axons, along which information in the form of electrical signals are passed, and while they have universal similarities they occur in a wide variety of shapes and sizes. At times axons are so long they contain 99% of the entire neural cell cytoplasm; they are far too large for simple diffusion to obtain necessary macromolecules, and instead rely on axonal transportation along microtubules to deliver cellular supplies. These long projections are particularly sensitive to damage compared to cell bodies.

The other principle categories of cells tasked with various supporting roles are the glial cells, of which three are highlighted. Oligodendrocytes, whose main role is to provide the insulating protective myelin sheaths that wrap axons and allow for faster transmission of electrical signals. Astrocytes, which provide key structural support within the brain. It is enlarged astrocytic processes that surround the brain vasculature to create the tightly regulated blood brain barrier (BBB). Lastly microglia, which are the principle immune surveillance cells of the CNS, perform constant inspection of the surrounding brain parenchyma in order to maintain homeostasis as well as macrophage-like activities when activated.[30] These long-lived cells are derived from mesenchymal myeloid lineage progenitor cells, which immigrate to the brain early during gestation.[31] These cells are essential components of the neuroinflammatory processes and are the first line of defence post-TBI.[30,32] Upon activation, either by infection or injury, microglia respond promptly to changes in the microenvironment producing inflammatory cytokines and chemokines, as well as secondary messengers and reactive oxygen species.[30,32]

Since the brain contains such a vast network of highly metabolic cells, but no effective way to store oxygen or glucose, it requires a continuous, large supply of oxygenated blood. For perspective, the brain accounts for about 25% of the body's oxygen consumption and uses about 15% of the normal cardiac output, yet it represents only about 2% of our total body weight. Blood vessels are arranged in a dense meshwork within the brain, this is particularly true in the grey matter, which is predominated by neuronal cell bodies. Rows of tight junctions connecting adjacent cerebral endothelial cells form the selective BBB with assistance from the aforementioned astrocytes to separate the nervous system from the rest of the body.

1.5 CONCUSSION MECHANISM AND PATHOPHYSIOLOGY

A concussion is caused by either a direct blow to the head or neck, or by a blow elsewhere on the body producing an impulsive "whiplash" force transmitted to the head. This biomechanical application of acceleration/deceleration and rotational forces induce a non-uniform distribution of pressure and tissue strain driving the pathological alterations seen in concussion.[10,32]

TBI pathophysiology is highly dynamic, evolving over time.[2] Upon application of mechanical force in concussion, axonal projections, which are the links which provide communication for neurons in the brain, can undergo membrane rupture.[10] TBI is classically described as a biphasic injury process; this mechanical damage makes up the primary injury phase, happening immediately at the moment of brain trauma. Concussive injury alone has been shown not to rupture the BBB.[13] The secondary phase of brain trauma, termed secondary injury, is the cascade of events set into motion after the initial insult that can persist for months to years.[33] In concussion, membrane rupture disrupts the ionic gradient by allowing potassium unrestricted trafficking to the extracellular space, this is accompanied by the release of the neurotransmitter glutamate.[10] Excessive glutamate release can drive neurotransmitter excitotoxicity, driving tissue injury and dysfunction after trauma. Simultaneously, calcium rushes into the cell, and it has been suggested that this disrupts calpain proteolytic activity leading to microtubular disorganization, eventually activating pathways causing cell death.[10,32] Disruption of the ionic gradient depolarizes the neuron disturbing electrical signalling and inhibiting neuronal function.[10] By increasing activity of sodium-potassium pumps on the axonal membrane proper ion balance can be returned, however; the energy required to do this can result in an "energy crisis".[10] Effects of this energy crisis can be seen in lactate accumulation and a decrease in cerebral blood flow.[10] Oxygen and glucose delivery will resultantly be decreased and

this further drives neuronal dysfunction due to neuronal depolarization, disruption of ionic gradients, and release of excitatory amino acids.[32] This axonal damage taking place throughout the brain has been referred to as diffuse axonal injury (DAI), and is believed to be the main structural disorder causing brain dysfunction long-term.[14] It has been suggested though that concussive acceleration/deceleration forces do not affect neural cells evenly. Greater susceptibility for damage has been reported to axons that change direction to accommodate a blood vessel, axons at the grey matter white matter interface, or decussate.[20] Damaged axons are not irreversibly injured, and usually complete axonal separation is only seen in severe TBI.[32] It was assumed that 1 to 2 weeks was the required time for axonopathy and metabolic stress reversal by clinicians as that is when symptoms frequently resolve.[34] New evidence though suggests that it takes 30 to 45 days for patients physiological parameters to resolve, well beyond when symptoms have resolved.[34] Current understanding of pathophysiology following concussion is largely dictated by what has been observed from animal studies, so further validation of these mechanism in humans remains to be established.[10]

1.6 NEUROINFLAMMATION

The CNS has classically been considered an immune privileged site, demonstrating restricted immune surveillance largely based on the presence of the BBB which maintains rigorous permeability control for cell entry.[3] However, this theory has been challenged in recent years, with studies demonstrating peripheral immune infiltration to the CNS, making the brain a dynamic immunocompetent organ.[3,32] Even in the absence of pathology in the CNS, research has demonstrated extensive communication between glial cells and the immune system.[3] Neuroinflammation is the name given to inflammatory responses within the brain and spinal

cord, and is a major participant in the secondary injury cascade.[30,32] Due to unique circumstances within the CNS, inflammation becomes much more complicated than in the periphery, which led to early beliefs that immune response could only be detrimental.[3] Neuroinflammation has been revealed to play a role in many CNS diseases, from acute to neurodegenerative to some mental disorders.[35]

An immune response is largely associated with a response to a foreign pathogen, but also plays important roles in response to sterile tissue damage, which is the case in any non-penetrating TBI or closed-head TBI.[3] In the case of sterile TBI, tissue damage causes the release of damage-associated molecular patterns (DAMPS), which can be a number of molecular entities including endogenous proteins, nucleic acids, and metabolites.[36] For concussion, this can be the result of the applied acceleration and rotational forces on the brain producing diffuse axonal tissue injury.[30] These DAMPS are recognized by pattern recognition receptors (PRRs), which upon binding function to both initiate and amplify an immune response.[36] The earliest resulting components of this cascading inflammatory response are cytokines such as Interleukin (IL)-1β tumour necrosis factor (TNF), and IL-6.[3,37] Cytokine release after tissue damage triggers upregulation of adhesion molecules, release of chemokines, and the activation of both local glia, endothelial cells, and circulating inflammatory cells.[37] While microglia are the overarching directors of innate and adaptive immune responses in the CNS, several other CNS resident cells also have innate inflammatory capabilities.[32] Astrocytes can express inflammatory mediators and secrete cytokines.[37] Even neurons have shown some level of immune modulating activity, primarily supressing immune activation and inflammation.[37]

Immune cell trafficking to the brain is dependent upon the upregulation of adhesion molecules on the endothelium such as E- and P-selectin, intercellular adhesion molecule–1, and

vascular cell adhesion molecule, as well as the release of chemotactic chemokines by local inflammatory cells, and also astrocytes and neurons.[32,37] Although the BBB is rigid, it also has shown evidence of having a dynamic quality, allowing trafficking of immune cells through post-capillary venules after manipulation by cytokine and chemokine secretion.[38] There is dispute though that peripheral immune infiltration into the brain occurs mainly through the choroid plexus, which is the special tissue layer that helps separate the blood and cerebrospinal fluid (CSF) in the brain ventricles, rather than at the focal site of injury.[39] The choroid plexus lacks endothelial tight junctions and astrocytic glial limitans, it also constitutively expresses adhesion molecules and chemokines, an observation which has driven this theory.[39]

Inflammation is a normal and essential process, not only providing a defensive mechanism against pathogens and insults, but working to repair tissue damage and restore and maintain homeostasis.[40] This process, irrespective of the trigger or location, involves many cells and protein and peptide mediators in circulation.[40] The brain of an individual suffering a TBI is most often healthy prior to insult, and without vascular or other chronic conditions. Therefore the brain's neuroinflammatory response is believed to be the most universal for biomarker discovery in humans compared to other brain diseases as it lacks potential confounding factors.[35]

1.7 NEUTROPHILS

The most abundant and earliest arriving peripheral immune cell after most injuries are the neutrophils.[3,41] While short lived, surviving about 5 days in humans and 12 hours in mice, these cells are continuously produced in the bone marrow, released into circulation and eventually cleared by macrophages in the bone marrow and liver.[3,42] At the site of injury neutrophils have numerous functions, and while their involvement in pathogen fighting is well documented, their

role in sterile injury response is less well understood.[3] After CNS injury, large pools of neutrophils can be seen to extravasate into the lesion site as early as 4 hours after injury.[3] This is believed to occur due to the TBI-induced release of catecholamines and glucocorticoids into circulation.[41] The catecholamines act to release marginated neutrophils into circulation, while glucocorticoids both encourage neutrophil survival and further neutrophil release from stored pools in bone marrow.[43,44] Neutrophil response peaks in infiltration at about 2 days, and is mostly absent by 3 days.[3,30,32] Upon arrival at the injury site, neutrophils release various effector molecules, which are believed to have deleterious effects on precious neural tissue.[3] An example of some of the effector molecules released include pro-inflammatory cytokines, proteases, and reactive oxygen and nitrogen species.[3,32]

1.8 MONOCYTES/MACROPHAGES

Like neutrophils, monocytes are produced in the bone marrow and are released into circulation, taking up residency there or within the spleen, from which they can traffic to sites of tissue injury.[3,45] Representing 5-10% of circulating immune cells, monocytes are a heterogeneous population.[41] Heterogeneity is largely defined based on the expression of surface markers CD14 and CD16 dividing cells into three subsets; classical ($CD14^{++}$ $CD16^{-}$), intermediate ($CD14^{++}$ $CD16^{+}$), and non-classical ($CD14^{+}$ $CD16^{+}$).[41] These three subsets form an interesting relationship, with each cell type distinguished by a unique function and phenotype. While not formally proven, gene analysis has placed the intermediate and non-classical populations as more closely related compared to the classical group.[46] Proportionally, classical monocytes are the largest population, representing about 85% of total monocytes.[46] They are described as having a superior phagocytic capacity, and gene signature indicating wound healing

and coagulation properties, responsiveness to stimuli, and anti-apoptosis capabilities.[46] Intermediate monocytes represent about 5% of the monocyte population, and are described as having a superior capacity to induce T cell proliferation and activation, likely the result of a gene signature reflective of improved antigen processing ability and MHC class II presentation and processing ability.[46,47] Non-classical monocytes represent about 10% of the monocyte population, and demonstrate a unique patrolling behaviour.[46] An *in vivo* analysis demonstrated non-classical monocytes crawl along the endothelium, suggesting they constantly exhibit a surveillance behaviour for inflammation or damage such that these monocytes can rapidly traffic to the site of interest.[46,48] $CD16^+$ monocytes, more specifically non-classical, have been described as more pro-inflammatory due to their greater capacity to release TNFα and IL-1β, and under inflammatory conditions expansion of the $CD16^+$ subset has been observed.[46] Alterations in responding monocyte subpopulation levels have been identified in conditions such as pre-eclampsia, chronic systolic heart failure, sepsis, Crohn's disease, severe asthma, and stroke.[49–54] Monocytes rely on endothelial activation and chemokine gradients to encourage trafficking into desired sites.[3] Monocyte recruitment is heavily attributed to the release of the chemokine CCL2 after TBI, but other chemokines may be involved, as there is considerable promiscuity in ligand receptor interactions.[55] The importance of the CCL2 chemokine was even demonstrated in a rat model of TBI with its increased secretion encouraging monocyte recruitment.[56]After TBI, studies have shown increases in circulating monocytes at 24 hours after insult.[57] Accumulation of monocytes within neural tissue is reported at 36 to 48 hours after insult, and progressively increase in numbers over the following weeks.[32] Monocytes give rise to tissue resident macrophages or dendritic cells upon infiltration into tissue, but they may also retain their phenotype.[45,58]

Monocyte-derived macrophages are believed to have a biphasic response, upon activation at the injury site they promote inflammation, however over time they are necessary for injury resolution by releasing growth factors and promoting angiogenesis.[3] Overall, they are far less harmful to tissue compared to the earlier arriving neutrophils.[59] Challenging the understanding of the contribution of haematogenous macrophages contribution to neuroinflammation is the fact that they are indistinguishable from activated microglia due to their expression of similar surface markers.[30]

1.9 LYMPHOCYTES

Overall, the adaptive response to CNS trauma is far less prevalent than the innate response, with lymphocyte recruitment and activity in sterile CNS injury still poorly understood. After spinal cord injury (SCI), lymphocyte infiltration appears to be delayed, peaking about 42 days after injury in mice and after several months in humans.[30,32]

After severe TBI, a significant decrease in the number of circulating T cells has been reported.[41] This is true for both CD4 and CD8 T cells, up to 4 days after a severe TBI.[41] It has been suggested that this could be due to increased serum catecholamine levels encouraging lymphocyte retention in lymph nodes.[41]

TBI appears to have little influence on B cells. In one study, no significant alteration in B cell frequency was noted after severe TBI, however only the first 7 days after trauma were investigated.[60] This idea may be challenged though, with one study recently demonstrating an increase in serum anti-S100B autoantibodies as well as serum S100B protein in individuals experiencing repeated subconcussive head insults.[61]

Circulating natural killer (NK) cells have been shown to be reduced for days to weeks following TBI, this is true for mild, moderate, and severe levels of injury.[60,62] It has been suggested this reduction could be the result of glucocorticoid-induced apoptosis of circulating NK cells.[41] It has also been suggested the reduction is due to NK trafficking to the brain where they suppress microglia number and function and thereby controlling amplification of the immune response and any additional damage it may cause.[62]

1.10 BIOMARKERS

A biomarker, as defined by the National Institutes of Health Biomarkers Definitions Working Group, is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention."[63] Biomarkers represent a broad category of medical signs that can be functional, physiological, biochemical, or almost any measurement demonstrating relationship between a hazard and a biological system.[63] Essentially they should provide quantifiable biological information in an objective manner that is in direct relation to the physical insult taking place. Fluid biomarkers of concussion would provide value in the diagnosis of concussion. Currently research has focused mainly on fluid biomarkers sourced from CSF and peripheral blood, although urine and saliva have also been suggested as alternatives for concussion study.[64]

Cerebrospinal fluid is an appealing choice for fluid biomarker analysis for concussion as it freely communicates with the interstitial fluid of the brain that bathes the neurons, and therefore should most accurately reflect CNS injury.[21] Compared to the approximately 4 litres of blood in circulation in a human, there is only about 150 to 200 ml of CSF fluid meaning markers of interest are far less diluted and should be measurable using standard immunoassay

techniques.[21,29] What is problematic about CSF is that it is unclear how quickly there is clearance of brain metabolites into the newly described glymphatic/lymphatic system of the brain and into the blood supply.[21] While the CNS lacks a classical lymphatic drainage system, the glymphatic/lymphatic system made up of astroglial cells forming perivascular channels, lines the dural sinuses and has hallmark lymphatic endothelial characteristics and is capable of carrying fluid and immune cells to deep cervical lymph nodes to generate an immune response.[65,66] CSF is produced at a rate of about ½ L/day, meaning its total brain volume is renewed around 2 to 3 times per day, therefore biomarker levels could fluctuate vastly depending on time of sampling.[29] Additionally, it is unrealistic and ethically unreasonable to perform a highly invasive lumbar puncture for CSF collection on the scale needed to obtain baseline information for non-concussed healthy individuals.

Blood-biomarker use in concussion has already started to be intensely investigated. Currently the most studied TBI biomarker is S100B, which is a calcium binding protein secreted by astrocytes.[67] S100B has been used to rule out minor head injury in emergency room situations in France as part of a prospective study.[68] While S100B is approved for use in certain countries, such as Germany, it is currently not approved by the Food and Drug Administration (FDA) for use in the USA, nor are any other biomarkers for TBI.[16,69] Overall it is an unreliable marker for TBI as it can be released in polytrauma situations where head injury is absent, and this marker does not appear to readily cross the BBB.[67] Alpha-spectrin is another candidate for a blood-biomarker. In a preliminary study of professional hockey players, researchers discovered an increase in the serum concentration of the N-terminal fragment of alpha-spectrin, which is a protein related to DAI, in concussed players compared to preseason levels.[14] The increase of this

protein in blood serum was even shown to correlate with the length of post-concussion symptoms.[14]

Advantages of a blood-biomarker for concussion are numerous. Blood collection is minimally invasive and can be performed on the sideline immediately after injury as well as at the clinic. Blood can be analysed both whole and separated into its components for various analysis options. Disadvantages of blood as a fluid biomarker include the restriction of relevant CNS proteins entering the bloodstream, either at all or in a measurable concentration, due to the presence of the BBB.[13,21] Additionally, markers of interest may undergo degradation or alteration due to proteases in the bloodstream, or clearance by the liver or kidneys, and for this reason the half-life of many investigated protein and serum biomarkers of concussion are unknown.[13,21] There is often no normal range for the concentration of most of the soluble markers evaluated.[40] This is not the case though for blood cell levels themselves, in which there are well-established normal ranges, with clinical use of some of these markers already in place.[40] Therefore, we see potential in tracking the leukocyte response post-concussion as a means to identify concussion.

1.11 INFLAMMATION AS A BIOMARKER

The cascade of immune events that takes place after CNS injury has been distinctly described, starting with cell injury and moving through various molecular and cellular stages.[3] Even the resolution of inflammation is an active process with a signature cascade involving specific cell types and anti-inflammatory cytokines.[40] Using a panel analysis of this cascade of inflammatory factors with different temporal profiles, it is conceivable to track someone through the initial injury, as well as their progression to recovery and safe return to play. Currently, protein and protein fragments have been the focus of fluid biomarker research of CNS injury.[21]

Inflammation itself has been suggested as a powerful biomarker due to its perceived association with the progression of TBI pathologies.[16] Due to the apparent commonality of immune response mechanisms, it would be feasible to apply these biomarkers for both acute and chronic brain injuries.[35] However, during an inflammatory response changes in inflammatory marker concentrations have little specificity.[40] No one marker, or even a small number of makers, can define inflammation as a whole, and that is why it is necessary to consider and evaluate clusters of markers to determine an inflammatory signature.[40] Many markers of inflammation are present at readily detectable levels in healthy individuals; it is the dynamic nature of these levels that may provide a tangible measurement for indicating ongoing inflammation.

A majority of inflammation research investigates the pro-inflammatory cascade as it drives immune amplification and potential tissue damage.[40] Considerably less attention has been paid to the anti-inflammatory resolution cascade, yet it plays a necessary component in preventing chronic long-term inflammation.[40] By capturing both pro- and anti-inflammatory markers as part of an inflammatory marker panel, it could better determine the period of immune propagation and resoloution.[35] A more in-depth analysis of leukocytes, investigating surface expression of relevant markers has been suggest as an additional biomarker component in addition to cell counts and frequencies.[40]

1.12 FLOW CYTOMETRY

Flow cytometry is the process of studying individual cells traveling in suspension through an image plane, and has been called the most powerful tool for probing immune phenotypes.[70] Flow cytometry allows for the rapid, quantitative analysis of multiple parameters on a single cell

in peripheral blood and digested single-cell preparations of tissue.[40,70] This opens up the possibility to detect and phenotype rare cell subsets amongst complicated biological mixtures such as blood, and this could not be performed using bulk assays such as a western blot.[70] This is important as both cell phenotype and function can be demonstrated by the extent of surface marker expression on any given cell.[40] An example of this is CD69, which is a transiently expressed surface marker believed to demonstrate immune cell activation.[70] Flow cytometry has clinical value as a diagnostic tool, and can be used to advance understanding of concussion pathology and etiology due to its ability to rapidly survey biological fluid.[25] For this study flow cytometry will be used to track evolving leukocyte populations.

1.13 MOUSE MODELS OF TBI AND RATIONALE FOR THEIR USE

Currently, concussion research has been largely restricted to humans, while these studies have value they do not allow in-depth mechanistic evaluations.[71] Animal studies are a necessary step in the translational process from laboratory bench to human bedside treatment. Using a mouse model can provide validation for rationale as well as predict an outcome we can compare with later human studies. If clinical outcomes produce results not predicted by an animal model, it can reversely guide modification in future studies of the animal model. Any preparation of future therapeutics for TBI, or more specifically concussion, will require rigorous evaluation in an animal model, making parallel animal model development an important step for future experiments.

Due to the diffuse nature of the injury associated with mTBI, and the vast diversity of clinical outcomes it produces, it is likely that no single animal model of mTBI will completely reproduce the spectrum of mechanisms that are associated with the corresponding human

injury.[72] While sports-related concussion specific animal injury models are being investigated, there is no universally accepted model.[71] The model of TBI selected for this study is the controlled cortical impact (CCI). This technique uses a computer controlled pneumatic piston to deliver an impact at a predetermined velocity, depth, and duration of tissue deformation.[73] This method is advantageous as it allows for a highly reproducible injury that mirrors many of the attributes seen in human TBI including neurobehavioural and cognitive impairment, BBB disruption, edema, and most importantly inflammation.[73,74]

1.14 THE *lys*-EGFP-*ki* MOUSE

This study utilizes the lysozyme M-EGFP-knock in (*lys*-EGFP-*ki)* transgenic mouse, containing the gene for enhanced green fluorescent protein (EGFP) in place of the lysozyme M coding sequence. The intact *lysozyme M* gene promoter faithfully drives and controls expression of EGFP in the hematopoietic cell types where Lysozyme M protein is normally expressed. This confers EGFP expression to mature hematopoietic granulomyelomonocytic cells, including neutrophils, monocytes, and macrophages.[75] It is important to state that the lysozyme M gene is not essential for mouse viability, and these mice, in addition to being normal in size and fertility, have no defects in the hematopoietic system and contain all cells of myeloid lineage.[76] In previous experiments in the Dekaban laboratory using the *lys*-EGFP-*ki* in a SCI model it was demonstrated that there is no substantial difference between *lys*-EGFP-*ki* and wild-type C57BL/6 mice in leukocyte subset frequencies or leukocyte response to SCI.[75] A comparative in-depth analysis of matching leukocyte frequency patterns, and phenotypic changes in surface markers presence and amount can be performed in the mouse model of TBI to provide context to changes seen in human. This includes mouse monocytes, which are also heterogeneous and can be

compared to the heterogeneous monocyte populations seen in humans. There are at least two distinct phenotypes of circulating mouse monocytes, and these can be separated by the presence of the surface marker Ly6C.[77] $Ly6C^+$ monocytes resemble the human classical monocyte population, and are rapidly attracted and respond to stimuli.[77] $Ly6C^-$ monocytes resemble the human non-classical population, as they exhibit a similar patrolling function of crawling along the endothelium.[77] A third intermediate monocyte population has been described in mice, but it is beyond the scope of my investigation.[45]

1.15 SUMMARY AND RATIONALE

There are a vast number of concussions occurring in sports, and the consequences of each concussion compound upon the next, leading to a potential worsening of clinical outcomes for the affected individual. What is problematic, though, is that current concussion identification methods are imperfect, relying on subjective measures and lacking description with respect to structural or biochemical alterations taking place as a consequence of concussion. Without adequate injury evaluation and assessment knowledge, individuals are at enhanced risk of more severe damage from a subsequent head trauma. The strong desire for a more objective and biologically relevant method of concussion identification and prognostic indictor(s) drives the current interest in evaluating fluid based biomarkers. It is believed that blood is a suitable biofluid for biomarker discovery due to ease of access, processing, and normative data. TBI-induced peripheral inflammation has been suggested as a useful source of blood-biomarkers for concussion identification, as it could provide information on both cause and effect of the secondary injury process.[25] It is anticipated that the evolving changes of leukocyte populations

can act as a biomarker for injury, provide insights into the stage of the ongoing immune response, and help determine patient outcome.

The first stage of this work aims to use a mouse model of TBI as a proof of principle investigation into testing for changes in peripheral immune cell populations. Using flow cytometry, the cellular responses of neutrophils, monocyte subsets, and lymphocyte subsets was evaluated at various points post-TBI in the *lys*-EGFP-*ki* mouse. The second stage of this work was to perform a pilot study as a way to assess the feasibility of evaluating evolving leukocyte populations using basic clinical blood tests as well as by flow cytometry on a cohort of university level female rugby players with and without concussions. It was of immense importance to perform this investigation with the guidance of a baseline analysis of players to provide a framework to objectively determine any changes in players following a concussion. For this reason, a preseason assessment was performed on all the players who consented to the study such that players could act as their own controls. Clinically approved concussion assessments, the SCAT3 and ImPACT, were used alongside our screening panel for inflammation to provide contextual clinical measures.

1.16 HYPOTHESIS AND SPECIFIC GOALS

Hypothesis:

A signature temporal response of biomarkers of neuroinflammation in systemic circulation will provide an objective concussion diagnostic not seen in the pre-concussion baseline assessment. This biomarker panel will also track patient recovery.

Goals:

1) To assess the inflammatory response in systemic circulation and in the spleen post-TBI using the *lys*-EGFP-*ki* mouse.
2) To assess current clinically used concussion diagnostics, the Sports Concussion Assessment Tool 3rd Edition (SCAT3) and Immediate Post-Concussion Assessment and Cognitive Test (ImPACT), for effectiveness and sensitivity on their own and in relation to potential blood biomarkers.
3) To investigate evolving blood leukocyte populations longitudinally after concussion, using both basic clinical blood assessments and a more in depth cell analysis that employed analytical flow cytometry.
4) To determine alterations of inflammatory protein markers and markers of neurotrauma following concussion in peripheral blood plasma.

CHAPTER 2 – MATERIALS AND METHODS

2.1 ETHICS

2.1.1 Animal ethics

All animal experiments in this study were approved by the University of Western Ontario Animal Use Subcommittee (Protocol 2007-104-12 and Protocol 2016-019), and obey to the Canadian Council on Animal Care guidelines. Ethics letters are available in Appendix 1 and 2.

2.1.2 Human ethics

This study was approved by the University of Western Ontario's Health Science Research Ethics Board (protocol 102857). All participants were provided letters of information and signed letters of informed consent prior to participating in the study at the beginning of each season. In keeping with ethical regulations, all players participating in the study have been de-identified and assigned a number for identification. The ethics letter is available in Appendix 3.

2.2 *lys*-EGFP-*ki* MOUSE MODEL OF TBI

2.2.1 *lys*-EGFP-*ki* mice

Heterozygous *lys*-EGFP-*ki* mice were provided by Thomas Graf, from the Albert Einstein College of Medicine (Bronx, NY), and were housed in a barrier at the West Valley Animal Facility at the University of Western Ontario Animal Care and Veterinary Services (London, ON). Homozygous mice were selected for rebreeding to ensure highest EGFP expression, and these mice were transferred to the barrier facility at the Robarts Research Institute (London, ON) before experimentation. Both male and female *lys*-EGFP-*ki* mice were included in experimentation, with a minimum age requirement of 10 weeks at time of surgery; no mice over 17 weeks were used. All mice used in this study are maintained within a barrier facility from

birth till time of experimentally induced brain injury, at which time they were housed in a vented rack in conventional animal room for the duration of their survival.

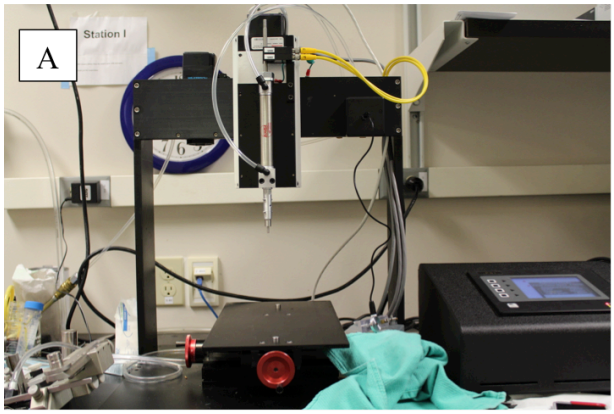
2.2.2 Pre-operative treatment

lys-EGFP-*ki* mice were initially anaesthetized using 3 to 4% Isoflurane (Baxter Corporation, Mississauga, ON) in oxygen, this was reduced and maintained at 1 to 2% Isoflurane during surgery. Mice were placed in a stereotaxic frame with ear bars in place, and the area on the back of the neck was shaved and sterilized with 70 % ethanol then 10% povidone-iodine (Purdue Pharma, Pickering, ON).

2.2.3 Controlled cortical impact injury

TBI was experimentally induced by a computer controlled CCI using the Precision Systems Instruments TBI device (Lintech, Monrovia, CA, USA) (Figure 1A). The mouse scalp was sagittally incised to expose the skull, skin was clipped back, and fascia was cleared from the skull surface using tweezers. A 4 mm diameter hole was trephined into the skull at 2.5 mm right of the sagittal suture, midway between the lambda and bregma suture, and the skullcap was saved. The result of which can be seen in Figure 1B and 1C. A 2 mm metal flat-faced impactor tip was used to apply an impact at a velocity of 3.5 m/s, with a depth of 1 mm, and a dwell time of 500 ms directly to the center of the exposed dura through the cranial window. This was deemed to be a mild TBI in mice.[78] An image of the post-impact result can be seen in Figure 1D. The bone flap was replaced with Vetbond Tissue Adhesive (3M, St. Paul, MN), and the surgical sight was sutured closed with coated vicryl (Johnson & Johnson Medical Ltd, North Ryde, NSW).

Figure 1. Controlled cortical impact TBI mouse model. The Precision Systems Instruments TBI device used to perform the CCI is seen in A. To perform a CCI, a 4mm craniotomy was trephined into the mouse skull to the right of the sagittal suture midway between the lambda and bregma suture (B and C). After CCI any bleeding is stopped and the skull bone flap is replaced and the skin is sutured shut (D). Representative images.


A
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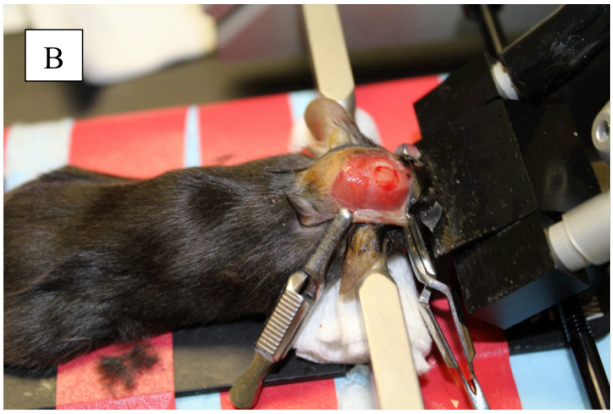
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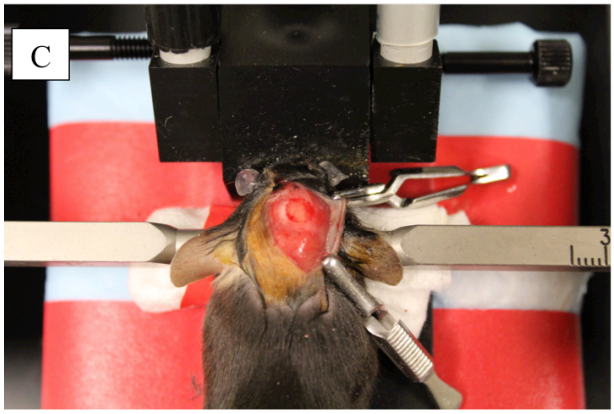

C


D

2.2.4 Post-operative treatment

All TBI mice were administered subcutaneously with Buprenorphine (RB Pharmaceuticals Ltd, Berkshire, UK) (0.05 mg/kg), 0.4 – 0.5 ml saline to prevent dehydration, and Baytril to prevent infection (Bayer, Toronto, ON) using 28g ½ U-100 insulin syringe (Becton Dickinson (BD), Franklin Lakes, NJ), and placed under a heat lamp for recovery and observation after surgery for at least 2 to 3 hours before being placed into conventional vented cages until sacrifice. Buprenorphine, saline, and Baytril was administered twice daily for 3 days post-surgery.

2.2.5 TBI endpoints

lys-EGFP-*ki* were euthanized and evaluated for inflammatory response of circulating blood leukocytes at 2 hours, 8 hours, 12 hours, 24, hours, 72 hours, 7 days, and 14 days post-TBI. Groups of 4 or 5 mice including both males and females made up each group. A pool of 5 uninjured control mice was also included for analysis. Initially one sham injured mouse, receiving a craniotomy but no impact, was included in each time point group, but was subsequently removed from data analysis due to concerns about confounding results from the injury produced by craniotomy.[79] At the appropriate time point, mice were euthanized by an overdose of Isoflurane. Upon euthanization, spleens were individually removed and temporarily placed in 1% Phosphate Buffered Saline (PBS) (Thermo Fischer Scientific, Waltham, MA) on ice for processing. Blood samples were obtained intracardially by first injecting 0.01 mL of 1000 USP units/mL heparin (Sandoz Canada Inc, Boucherville, QC) into the left ventricle, and collected in a 1mL 25g 5/8 needle (BD). Blood was transferred to a lithium heparin coated tube (BD) and kept at room temperature till processing. An incision was made down the middle from

the lower abdomen up to the top of the chest and a perfusion needle attached to tubing and perfusion pump was inserted into the apex of the heart. Intracradial perfusion was performed with tissue Roswell Park Memorial Institute Medium (RPMI) media (Thermo Fisher Scientific) followed by 4% paraformaldehyde (PFA) (Bio-Shop Canada Inc, Burlington, ON). The brains were harvested from each animal. They were cryopreserved in 10% sucrose, 20% sucrose and 30% sucrose solutions for 48 hours each and stored at 4°C until cryosectioning was performed.

2.2.6 Cryosectioning of mouse brain samples

Prior to sectioning, the mouse brain was frozen in optimum cutting temperature compound (Sakura Finetek, Torrance, CA) and sectioned transversely at 20 μm using Leica CM3050S Cryostat (Leica Microsystems, Richmond Hill, ON). Brain tissue was organized in six sets of sections, placed onto positively charged slides and stored at 4°C until further analysis. Hematoxylin and eosin staining was performed at London Health Sciences Center (London, ON) on 20μm brain sections 48 hours post-TBI to examine lymphocyte infiltration to the site of injury.

2.2.7 Confocal microscopy

To obtain representative brain images post-TBI mouse brain slides were selected and stained for analysis by confocal microscopy. Tissue slides were washed with 1x PBS, and then blocked for 3 to 4 hours with 5% normal goat serum (Jackson ImmunoResearch Laboratories Inc., Westgrove, PA) and 0.4% Triton-X (Bio-Shop Canada Inc., Burlington, ON) in PBS. Slides were stained overnight at 4°C with rabbit anti-Iba1 (Clone: EPR16588) (Abcam, Cambridge, United Kingdom) in block solution. Tissue slides were washed with PBS, and then stained with

goat anti-rabbit AF633 (Thermo Fischer Scientific) in PBS. Slides were incubated for 1 hour at room temperature. Slides were washed with PBS, and stained overnight at 4°C with rabbit anti-GFP AF488 (Thermo Fischer Scientific) in block solution. Slides were washed with PBS and cover slipped with hard set DAPI mounting media (Vector Laboratories, Burlingame, CA). Sections were viewed using Leica TCS SP8 microscope (Leica Microsystems, Richmond Hill, ON). Images were analyzed using LAS AF Lite software (Leica).

2.3 HUMAN RUGBY STUDY COHORT AND CLINICAL PROTOCOL

The University of Western Ontario women's varsity rugby team was the selected cohort for this study and players were followed for four consecutive seasons starting August 2012, through to April 2016. Athletes were eligible provided they had not suffered a concussion in the last 10 months and were at least 18 years of age. A total of 58 players participated, with some competing in multiple seasons of the study. Patient cohort characteristics are summarized in Table 1. In the case where a player did complete multiple seasons, each season was counted as a separate event, rather than averaged with a previous season. All assessments were performed by team physicians and trainers at the Fowler Kennedy Sports Medicine Clinic at the University of Western Ontario (London, ON). Players participating in the study underwent a baseline physical and cognitive assessment in August prior to the beginning of the collegiate rugby season. This assessment included a physical performed by a team physician, SCAT3, ImPACT, and blood sample collection. A questionnaire was also completed listing medical history as well as previous concussion information. Participants, who suffered a concussion, as diagnosed by the team physician, were re-examined within 24-72 hours of the concussion-taking place. This re-examination included collecting a blood sample. Blood samples were taken again at as close to 1

week, 1 month, and 3 months post-concussion as could be achieved. Players diagnosed with concussions completed SCAT3 and ImPACT tests at the discretion of the team physician. All participating players in the study underwent an end of season baseline examination in January/February that included a SCAT3, ImPACT, and blood sample. A timeline of the study is outlined in Figure 2. All players also underwent an MRI and MRS scan before and after the season and at time of concussion, at 3 months post-concussion but the analysis the MRI/MRS data will be reported elsewhere.

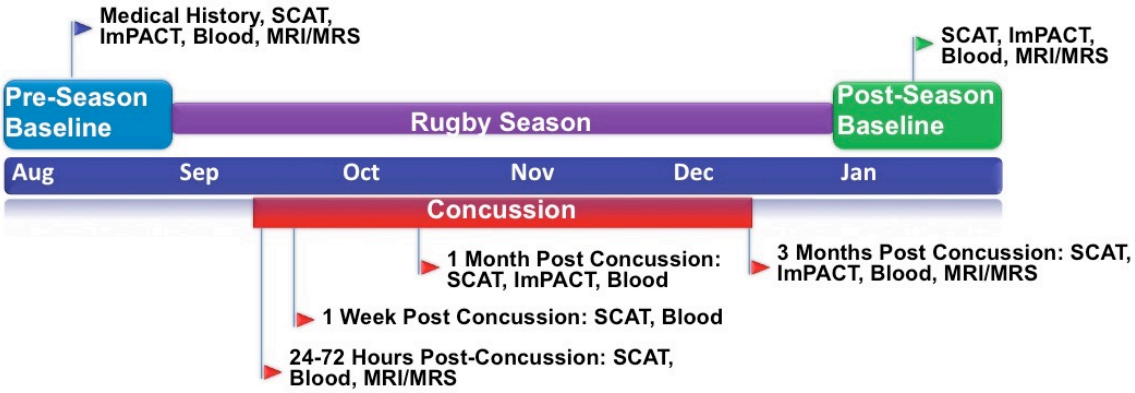
For samples collected from August 2015 to April 2016 a whole blood staining procedure for flow cytometry analysis was included for both baseline and post-concussion assessments. A fifth season (August 2016 – April 2017) was conducted but data was not included due to time constraints.

Table 1. Western Women's Rugby Concussion Study Cohort Characteristics

Cohort characteristics	Combined	2012-2013 Season	2013-2014 Season	2014 – 2015 Season	2015 – 2016 Season
Participants, returning	58	25	26(13)	31(21)	14(4)
Age, mean ± SD (years)	20 ± 1.3	20 ± 1.1	19 ± 1.4	20 ± 1.5	20 ± 1.3
Height, mean ± SD (cm)	168 ± 6.3	167 ± 6.7	166 ± 5.6	168 ± 6.9	171 ± 4.8
Weight, mean ± SD (lbs)	151 ± 17.9	153 ± 21.1	149 ± 17.9	149 ± 17.3	158 ± 12.0
Previous Concussion[1]	31	13	4	9	5
Diagnosed Concussion[2]	20[3]	4	6	5	5

[1] Self reported concussions prior to study entry.
[2] Concussions observed within the time of the study.
[3] 11 athletes had 1 concussion, 3 athletes had 2 concussions, 1 athlete had 3 concussions.

Figure 2. Timeline of the sample collection for the University of Western Ontario Women's Rugby Concussion Study for non-injured baseline participants and concussed participants.


Medical History, SCAT, ImPACT, Blood, MRI/MRS
SCAT, ImPACT, Blood, MRI/MRS
Pre-Season Baseline
Rugby Season
Post-Season Baseline
Aug
Sep
Oct
Nov
Dec
Jan
Concussion
1 Month Post Concussion: SCAT, ImPACT, Blood
3 Months Post Concussion: SCAT, ImPACT, Blood, MRI/MRS
1 Week Post Concussion: SCAT, Blood
24-72 Hours Post-Concussion: SCAT, Blood, MRI/MRS

2.4 BLOOD COLLECTION, PROCESSING, AND FLOW CYTOMETRY

2.4.1 Peripheral blood preperation for flow cytometry from *lys*-EGFP-*ki* mouse model

Anti-mouse CD19, NKp46, CD3ε, CD11c, CD11b, CD45, Ly6G, Ly6C fluorescence conjugated antibodies were purchased from BioLegend Inc. (San Diego, CA). Anti-mouse CD115 fluorescence conjugated antibody was purchased from eBioscience Inc. (San Diego, CA). Anti-mouse F4/80 fluorescence conjugated antibody was purchased from BIO-RAD (Hercules, CA).

From blood samples collected, 100μL aliquots were distributed into fluorescence-activated cell sorting (FACS) tubes (Falcon, BD). Separate staining panels for myeloid and lymphoid lineage cells were performed. For both panels, LIVE/DEAD® Fixable Aqua Dead Cell Stain (Thermo Fischer Scientific) was added to FACS tubes in the dark and left to incubate at room temperature for 20 minutes. Samples were then incubated with one or more of the following fluorescently-labelled antibodies at 4°C for 20 minutes as part of the myeloid panel, PE rat anti-mouse anti-F4/80 (Clone: A3-1), PE/Cy7 rat anti-mouse anti-CD115 (Clone: AFS98), PerCP/Cy5.5 rat anti-mouse anti-CD19 (Clone: 6D5), PerCP/Cy5.5 rat anti-mouse anti-NKp46 (Clone: 29A1.4), PerCP hamster anti-mouse anti-CD3ε (Clone: 145-2C11), PE/Dazzle hamster anti-mouse anti-CD11c (Clone: N418), AF647 rat anti-mouse/human anti-CD11b (Clone: M1/70), AF700 rat anti-mouse anti-CD45 (Clone:30-F11), BV421 rat anti-mouse anti-Ly6G (1A8), BV711 rat anti-mouse anti-Ly6C (Clone: HK1.4). Lymphoid panel cells were incubated with one or more of the following fluorescently-labelled antibodies at 4°C for 20 minutes, PE rat anti-mouse anti-CD19 (Clone: 6D5), APC rat anti-mouse anti-NKp46 (Clone: 29A1.4), AF700 rat anti-mouse anti-CD45 (Clone: 30-F11), and BV421 hamster anti-mouse anti-CD3ε (Clone: 145-2C11). Red blood cells (RBC) were lysed using BD Pharm Lyse lysing buffer (BD) and

samples were washed with cold Hank's Balanced Salt Solution (HBSS) (Thermo Fischer Scientific) + 0.1% (v/v) bovine serum albumin (BSA) (EMD Millipore, Billerica, MA). Samples were spun at 6°C for 5 minutes at 500xg, the supernatant was poured off, another wash with HBSS + 0.1% BSA was performed. Samples were then resuspended in 200µL of HBSS + 0.1% BSA and 100µL of 4% PFA and stored in the dark at 4°. Before analysis by flow cytometry, 50µL of CountBright Absolute Counting Beads (Thermo Fischer Scientific) was added to each all-positive sample. Cells were analyzed by an LSRII analytical flow cytometer (BD). Electronic compensation was performed with UltraComp antibody capture beads (eBioscience Inc.).

2.4.2 *lys*-EGFP-*ki* mouse spleen preperation for flow cytometry

Collected spleens were individually minced in a sterile petri dish. Spleen segments were placed on a sterile 70-micron filter (Falcon, BD) and were pushed through into a 50mL tube with a sterile 10cc plunger. Spleen cells were spun at 6°C for 5 minutes at 500xg and the supernatant was poured off, and cells were resuspended in 4mL incomplete RPMI media. Cells were filtered again using a 40-micron filter (Falcon, BD) and collected in a 15mL tube. Spleen cells were spun at 6°C for 5 minutes at 500xg and the supernatant was poured off, and cells were resuspended in 5mL of Ammonium Chloride Lysis buffer (Stemcell Technologies, Vancouver, BC, Canada), to remove RBCs, and incubated for 7 minutes at room temperature. The volume was adjusted to 15ml with PBS, and tubes were spun at 6°C for 5 minutes at 500xg. The supernatant was discarded, and cell pellets were resuspended in PBS. Cell suspensions were stained using the process listed in 2.4.1 for blood above and analyzed similarly by flow cytometry.

2.4.3 Human blood collection, separation, and processing

Fresh blood was collected via venipuncture into two 10mL tubes and one 5mL tube all containing ethylenediaminetetraacetic (EDTA), and a 7.5mL serum separation tube (all from BD). A small portion of the blood sample collected was sent to the London Health Sciences Centre (London, ON) for a haematology profile including a complete blood count (CBC) with a white blood cell differential. The remaining player's blood sample (12 to 20mL) was overlaid onto LymphoPrep™ (StemCell Technologies Inc.) gradients, which were centrifuged to separate and individually collect the plasma and peripheral blood mononuclear cells (PBMC) from remaining blood fraction. The plasma pool collected was aliquoted and frozen at −80°C. The PBMC layer was collected and resuspended at 10^7 cells/mL in 1:1 (v/v) solution of freezing media (50% Human AB serum (Thermo Fisher Scientific), 30% incomplete RPMI media and 20% dimethyl sulfoxide (DMSO) (Bio-Shop Canada Inc.) to RPMI supplemented with 100 units/ml Penicillin (Thermo Fisher Scientific), 100 µg/ml Streptomycin (Thermo Fisher Scientific), 2 mM L-Glutamine (Thermo Fisher Scientific), 10 mM HEPES (Thermo Fisher Scientific), and 10% Human AB Serum (Thermo Fisher Scientific). This resuspended sample was aliquoted into 2 cryovials (Sarstedt, Newton, MA) and frozen at −80°C in a Mr. Frosty™ Freezing Container (Thermo Fisher Scientific) and eventually transferred to liquid nitrogen for long-term storage. From the serum collection tube, following blood clotting and centrifugation, serum was collected, aliquoted, and frozen at −80°C.

2.4.4 Flow cytometry for collected frozen human samples

Anti-human CD15, and anti-human CD64 fluorescence conjugated antibodies were purchased from Becton Dickinson (BD). Anti-human MDL-1 fluorescence conjugated antibodies were purchased from R&D Systems Inc. (R&D, Minneapolis, MN). Anti-human CD3, CD19,

CD4, CD8, CD69, CD16, and CD56 fluorescence conjugated antibodies were purchased from BioLegend Inc. (San Diego, CA). Anti-human CD14 fluorescence conjugated antibodies were purchased from eBioscience Inc. (San Diego, CA).

Previously frozen PBMCs were removed from liquid nitrogen and thawed quickly in a 37°C water bath. All steps were done at 4°C unless otherwise specified. Samples were washed once with cold HBSS + 0.1% BSA to remove DMSO. Samples were then resuspended in an appropriate amount of cold HBSS + 0.1% BSA, and 100µL of each sample was aliquoted into 5mL polystyrene FACS tubes (BD). Separate staining panels for monocyte and lymphocytes were performed. For both panels, LIVE/DEAD® Fixable Aqua Dead Cell Stain (Thermo Fisher) was added to FACS tubes in the dark and left to incubate at room temperature for 20 minutes. Samples were then incubated with one or more of the following fluorescently-labelled antibodies as part of the lymphocyte panel at 4°C for 20 minutes, PerCP mouse anti-human CD4 (Clone: RPA-T4), PE mouse anti-human CD19 (Clone: HIB19), APC mouse anti-human CD8 (Clone: SK1), APC-Cy7 mouse anti-human CD3 (Clone: SK7), and BV605 mouse anti-human CD56 (Clone: HCD56). Samples for the monocyte panel were incubated with one or more of the following fluorescently-labelled antibodies at 4°C for 20 minutes, FITC mouse anti-human CD15 (Clone: HI98), PE mouse anti-human MDL-1 (Clone: 283834), PE-Dazzle mouse anti-human CD16 (Clone: 3G8), PECy5 mouse anti-human CD69 (Clone: FN50), AF700 mouse anti-human CD64 (Clone: 10.1), APC-Cy7 mouse anti-human CD3 (Clone: SK7), APC-Cy7 mouse anti-human CD19 (Clone: HIB19), eFluor 450 mouse anti-human CD14 (Clone: 61D3). FACS tubes were washed with HBSS and 0.1% BSA. The cells were resuspended in 200µL of HBSS + 0.1% BSA and 100µL of 4% paraformaldehyde (PFA). Lastly, a 50µL aliquot of CountBright Absolute Counting Beads (Thermo Fischer Scientific) was added to obtain absolute cell counts

upon acquisition. Cells were analyzed by an LSRII analytical flow cytometer (BD). Electronic compensation was performed with UltraComp antibody capture beads (eBioscience).

2.4.5 Flow cytometry for collected whole blood samples

For the whole blood staining procedure approximately 1ml of the total blood sample collected, was needed. 100µL aliquots of blood were distributed into FACS tubes. Samples were then incubated with one or more of the following fluorescently labelled antibodies at 4°C for 20 minutes, FITC mouse anti-human CD15 (Clone: HI98), PE-Dazzle mouse anti-human CD16 (Clone: 3G8), PECy5 mouse anti-human CD69 (Clone: FN50), AF700 mouse anti-human CD64 (Clone: 10.1), APC-Cy7 mouse anti-human CD3 (Clone: SK7), APC-Cy7 mouse anti-human CD19 (Clone: HIB19), eFluor 450 mouse anti-human CD14 (Clone: 61D3). To lyse RBCs 2mL of 1x BD Pharm Lyse Lysing Buffer (BD) was added to all whole blood tubes. Tubes were vortexed quickly and then incubated at room temperature for 12 minutes in the dark. Samples were then centrifuged at 500xg for 5 minutes. FACS tubes were washed with HBSS and 0.1% BSA. The cells were resuspended in 200µL of HBSS and 0.1% BSA and 100µL of 4% paraformaldehyde (PFA). Lastly, a 50µL aliquot of CountBright Absolute Counting Beads (Thermo Fischer Scientific) was added. Cells were analyzed by an LSRII analytical flow cytometer as before with frozen samples.

2.5 FLOW CYTOMETRY ANALYSIS

Flow cytometry was run for maximum event collection for all samples. All data was analyzed using FlowJo Software Version 9.7.5 (Tree Star Inc., Ashland, Oregon, USA). Upon analysis a total event gate was set at 200 000 events for whole blood and spleen samples taken

from *lys*-EGFP-*ki* mice, as well as whole blood human samples. A total event gate was set at 100 000 events for frozen PBMC analysis due to reduced cell numbers as resultant from PBMC selection and freeze thaw cycle. The absolute cell count was determined using the following equation for all situations;

Absolute Cell Count = (Gated Cell Event Number/Counting Bead Event Number) x 50 000*

*(Number of beads per 50μL of counting beads added)

This provided the cell numbers presented.

2.6 IMMUNOASSAYS OF HUMAN PLASMA SAMPLES

2.6.1 Human cytokine immunoassay

The Luminex® Human Ultrasensative Cytokine 10-Plex Panel kit was selected and used to determine the concentration of human granulocyte macrophage colony stimulating factor (GM-CSF), interferon (IFN)-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and TNF-α present in the plasma. The methods and materials used were specified by the manufacture's protocol. The Luminex® 100 plate reader and IS 2.3 Software was used to analyze the plates (Luminex Corporation, Austin, TX, USA), this data acquisition and analysis that yielded the standard curve and raw data was performed by the Lawson Health Science Research Institute (London, ON). Samples were analyzed in duplicate and an average was taken.

2.6.2 C-Reactive Protein immunoassay

The Invitrogen Human C-Reactive Protein kit was selected to quantify C-Reactive Protein (CRP) in plasma samples. The methods and materials used were specified by the manufacture's protocol. Plates were read on a Thermo Labsystem Multiskan Ascent Microplate

Photometer, and data collection and analyses were performed by Ascent Software Version 2.6 (Thermo Fischer Scientific). Samples were analyzed in duplicate and an average was taken.

2.6.3 G-CSF immunoassay

The Invitrogen Human granulocyte colony stimulating factor (G-CSF) ELISA kit was selected to quantify G-CSF in plasma samples. The methods and materials used were specified by the manufacturer's protocol. Plates were read on a Thermo Labsystem Multiskan Ascent Microplate Photometer, and data collection and analyses were performed by Ascent Software Version 2.6 (Thermo Fischer Scientific). Samples were analyzed in duplicate and an average was taken.

2.6.4 GFAP immunoassay

The Millipore GFAP ELISA kit was selected to quantify glial fibrillary acidic protein (GFAP) in the plasma (Billerica, MA, USA). The methods and materials used were specified by the manufacturer's protocol. Plates were read on a Thermo Labsystem Multiskan Ascent Microplate Photometer, and data collection and analyses were performed by Ascent Software Version 2.6 (Thermo Fischer Scientific). Samples were analyzed in duplicate and an average was taken.

2.7 STATISTICAL ANALYSIS.

All data, except section 3.4, was analyzed using GraphPad Prism 6 (Graphpad Software, La Jolla, CA) and expressed as means ± standard deviation. Statistical significance was determined using Kruskal–Wallis one-way analysis of variance (ANOVA) followed by a *post-*

hoc Dunn's test. Differences were considered significant if $P < 0.05$. The differences analysis as part of section 3.4 was performed with statistical assistance from Dr. GY Zou and Artem Uvarov using SAS 9.4 (SAS Canada, Toronto, ON) and R package statistics (version 1.0.136). A Kenward-Roger's F test was used including a AR(1) first-order autoregression model for covariance structure, testing for fixed effects in a linear mixed model. Upper and lower 95% confidence intervals were established.

CHAPTER 3 – RESULTS

The results below are presented as follows: Firstly, peripheral leukocytes are compared at various time points post-TBI using the *lys*-EGFP-*ki* mouse model. Secondly, collected SCAT3 and ImPACT concussion assessments were analyzed both pre- and post-concussion in a cohort of female rugby players. Thirdly, inflammatory measurements of evolving leukocyte populations by basic clinical blood tests as well as by analytical flow cytometry were performed on the same cohort. Lastly, alterations of inflammatory protein markers and markers of neurotrauma following concussion in peripheral blood plasma were evaluated.

3.1 EXAMINATION OF PERIPHERAL GFP$^+$ IMMUNE RESPONSE POST-TBI IN *lys*-EGFP-*ki* MICE

3.1.1 Frequency of circulating blood neutrophils and monocytes

Using the *lys*-EGFP-*ki* mouse model mature cells of hematogenous myelomonocytic origin (predominantly neutrophils, monocytes, and macrophages) can be distinguished by the presence of EGFP. Further cell discrimination was performed by presence or absence of established cell surface markers. Neutrophils have been identified as Ly6G$^+$ F4/80$^-$ (Figure 3A), while monocytes have been identified as F4/80$^+$ CD115$^+$ Ly6G$^-$ (Figure 4A).[80] Using the surface marker Ly6C, classical and non-classical monocytes can be separated as Ly6C$^+$ and Ly6C$^-$ respectively.[80] A full outline of the gating strategy used for cell selection, including singlet discrimination, viability assessment, and negative and positive selection can be seen in Appendix 4.

Using the strategy outlined above, the absolute count of blood neutrophils and monocytes was compared between uninjured control and post-TBI mice. Neutrophils increased acutely post-TBI, with a 7.0, 5.9, and 9.0 fold increase compared to control at 2, 8, and 24 hours respectively

post-TBI, all these increases were significant (Figure 3B). At 1 and 2 weeks post-TBI the neutrophil count had returned to levels comparable to the uninjured control. Relative surface expression of the neutrophil marker Ly6G was evaluated by capturing its median fluorescence intensity (MFI). MFI was highest for Ly6G at 24 hours post-TBI, where it was 2.7 times higher than control (Figure 3C). This increase was not significant though ($p=0.0595$), and all other time points were not substantially changed from control. Evaluation of monocytes demonstrated an initial dip in counts for both classical and non-classical monocytes compared to control post-TBI. The initial dip in classical monocyte number had recovered by 8 hours post-TBI and was at their highest concentration at 72 hours post-TBI with 1.8 times the cell count compared to control (Figure 4B). The initial dip in non-classical monocyte numbers lasted much longer, with about 1/2, 1/4, and 1/3 of the cell counts present at 2, 8, and 24 hours post-TBI compared to control cell counts (Figure 4C). However, non-classical monocyte counts recovered 72 hours post-TBI where it was at its highest average cell count, and then returned to levels more similar to control at 1 and 2 weeks post-TBI.

To confirm $EGFP^+$ cell infiltration into the brain, representative images were taken of mouse brain tissue at the CCI site as well as on the contralateral side of the cortex. A hematoxylin and eosin stained section was included for anatomical reference, and a 4x tiled image was taken. A clear $EGFP^+$ cell presence was seen at 20x and 63x magnification however; no $EGFP^+$ cell presence was seen on the uninjured contralateral side (Figure 5). An Iba1 stain (red) was included, which would stain for monocytes/macrophages as well as tissue resident microglia, however very little red stain is present in the images taken.[81] No quantification was performed here, and only one mouse was examined to date.

Figure 3. Blood neutrophil counts increased acutely post-TBI in *lys*-EGFP-*ki* mouse model. Using flow cytometry neutrophils were identified as $Ly6G^{+}$ $F4/80^{-}$ cells and were then confirmed as $EGFP^{+}$ (A). Representative dot plots are shown. Neutrophil blood counts were significantly elevated compared to control at 2, 8, and 24 hours post-TBI (B). Ly6G surface expression on neutrophils was evaluated by median fluorescence intensity (MFI), and although it increased at 24 hours post-TBI this was not significant (C). Both counts and MFI were similar to control levels at 72 hours and onward post-TBI. Kruskal–Wallis ANOVA followed by Dunn's test, * = $P<0.05$. n=4-5 per time point (no replicates).

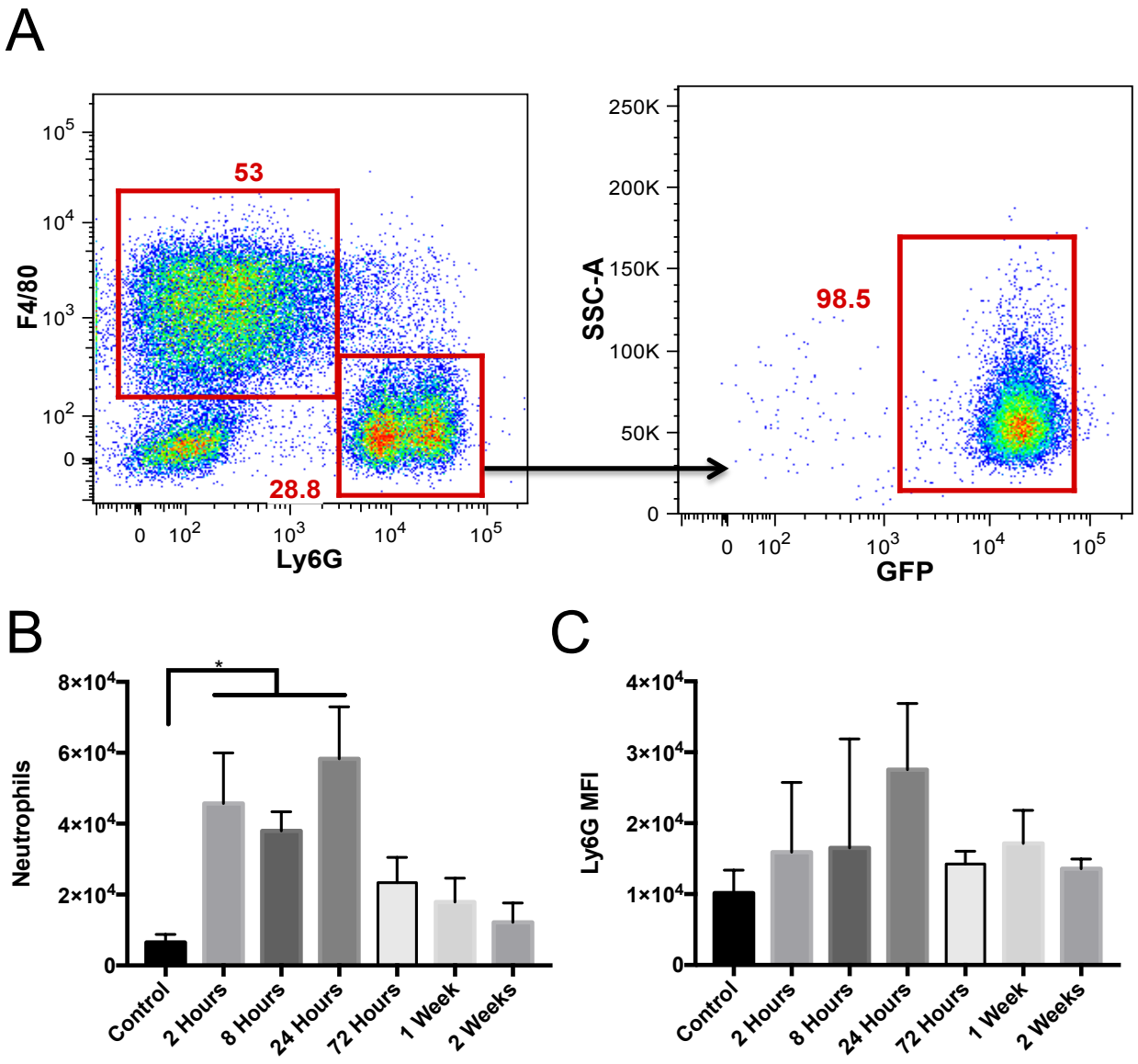
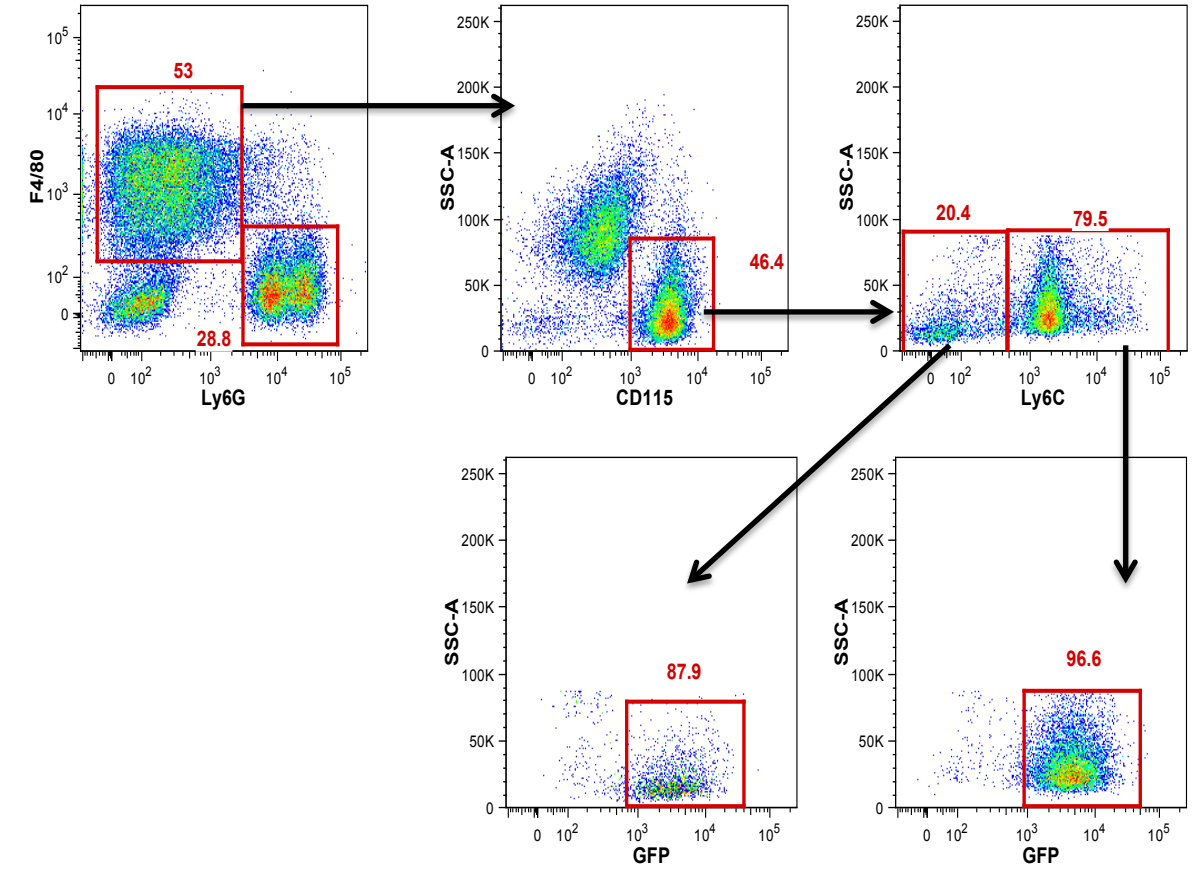
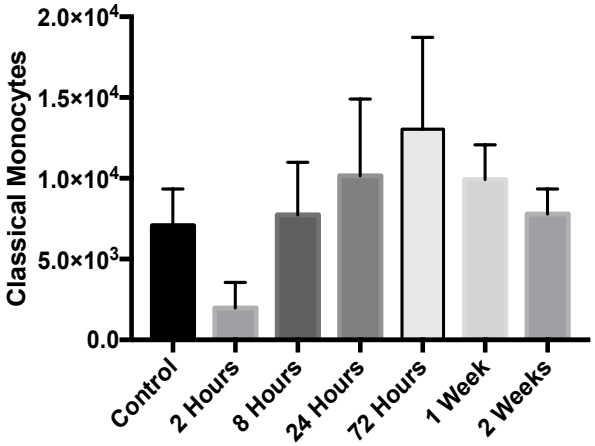

A
53
28.8
F4/80
Ly6G
SSC-A
GFP
98.5
B
Neutrophils
*
Control
2 Hours
8 Hours
24 Hours
72 Hours
1 Week
2 Weeks
C
Ly6G MFI

Figure 4. Dynamic blood monocyte response to TBI in *lys*-EGFP-*ki* mouse model. Using flow cytometry monocytes were identified as $F4/80^{+}$ $Ly6G^{-}$ $CD115^{+}$ cells, and subdivided into classical and non-classical populations were then confirmed as $Ly6C^{+}$ and $Ly6C^{-}$ respectively. Lastly cells were confirmed for the presence of EGFP (A). Representative dot plots are shown. Both classical (B) and non-classical (C) population counts acutely decreased before peaking at 72 hours post-TBI eventually returning to levels closer to control. None of these changes were significant according to Kruskal–Wallis ANOVA followed by Dunn's test. n=4-5 per time point (no replicates).

A


F4/80
Ly6G
53
28.8
SSC-A
CD115
46.4
SSC-A
Ly6C
20.4
79.5
SSC-A
GFP
87.9
SSC-A
GFP
96.6

B


Classical Monocytes
2.0×10^4
1.5×10^4
1.0×10^4
5.0×10^3
0.0
Control
2 Hours
8 Hours
24 Hours
72 Hours
1 Week
2 Weeks

C

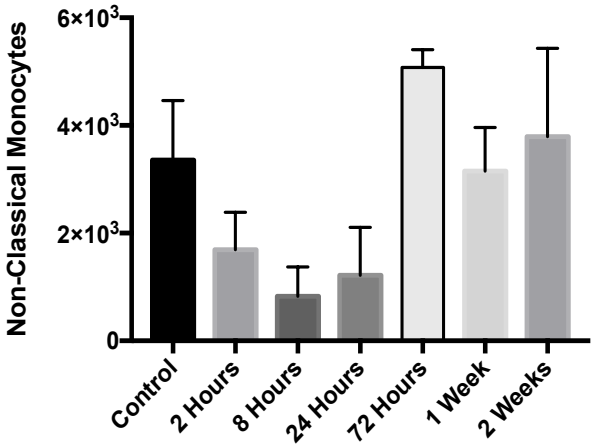
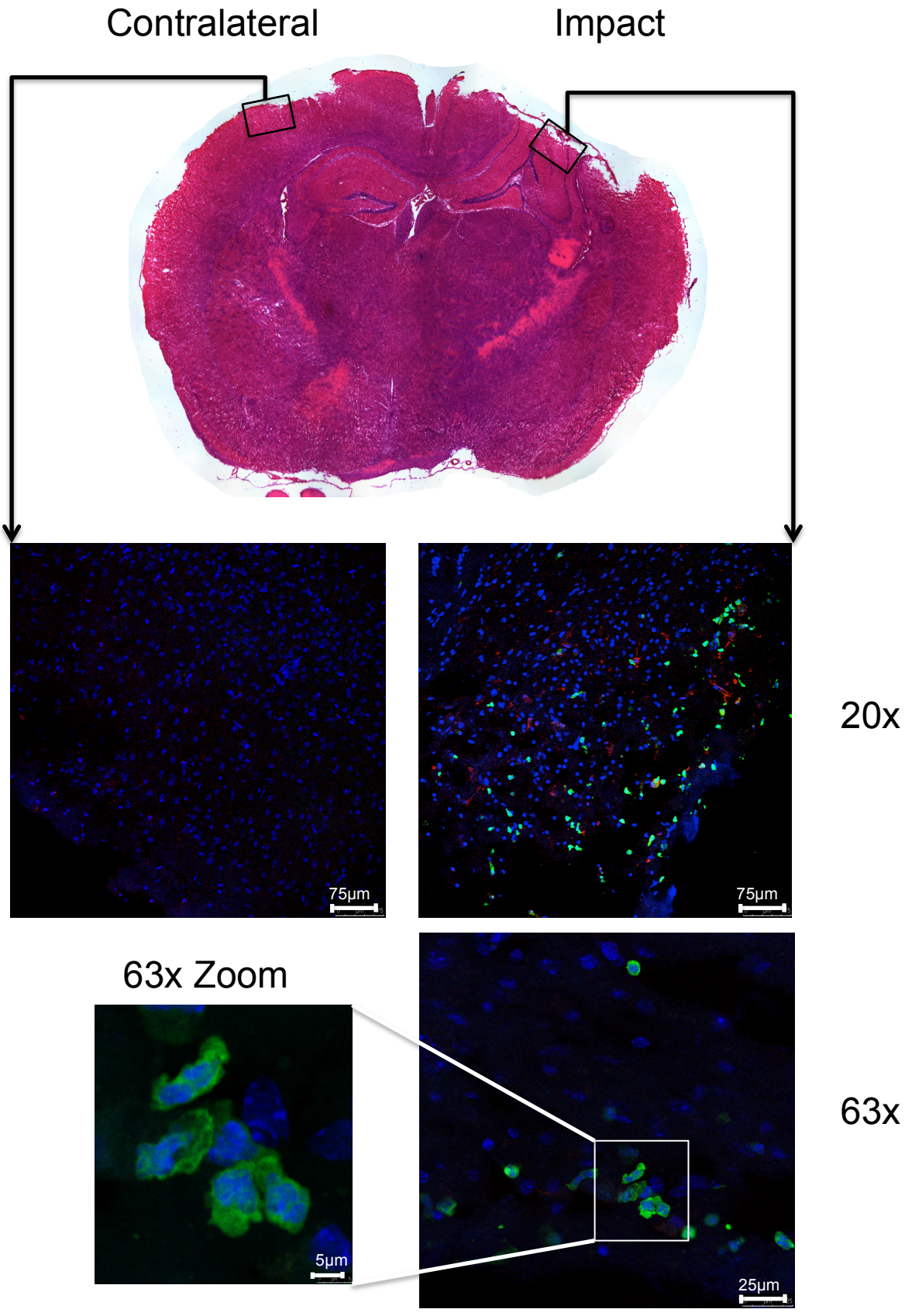

Non-Classical Monocytes
6×10^3
4×10^3
2×10^3
0
Control
2 Hours
8 Hours
24 Hours
72 Hours
1 Week
2 Weeks

Figure 5. EGFP infiltration into brain post-TBI. Mouse brains post sacrifice were collected and processed for hematoxylin and eosin stain and immunohistochemistry, and representative images were taken at the 48 hour post-TBI time point. Hematoxylin and eosin stained section was included for anatomical reference, and a 4x tiled image was taken. Tissue sections were stained for nuclei (DAPI, blue), hematogenous $EGFP^+$ neutrophils and monocyte/macrophages (green), and Iba1 (red). EGFP positive cells, identifying either blood borne monocytes/macrophages or neutrophils were clearly present at the impact site post-TBI at 20x, and 63x magnification, however there was no infiltration into the contralateral side.

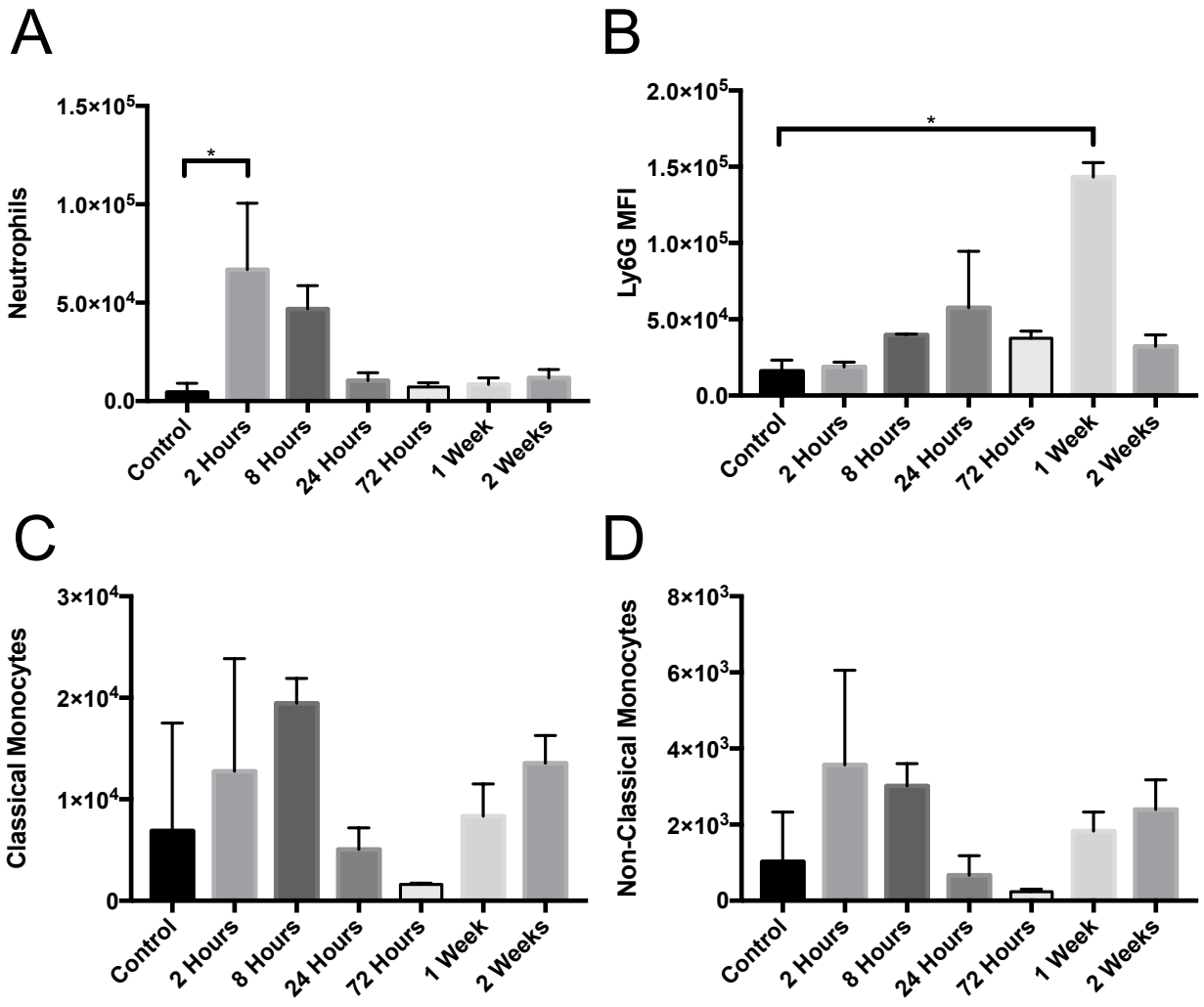

Contralateral
Impact
75µm
75µm
20x
63x Zoom
5µm
25µm
63x

3.1.2 Frequency of splenic neutrophils and monocytes

The spleen provides an important monocyte reservoir, and the leukocyte population present within it can inform the state of the systemic inflammatory response to TBI, and therefore was considered a valuable organ for analysis.[45,82] The neutrophil and monocyte counts in the spleen in response to TBI were compared for the *lys*-EGFP-*ki* mouse model. The same gating strategy and selection markers were used as in the blood analysis above. The splenic neutrophil count increased significantly immediately post-TBI compared to control with a 15-time increase in neutrophils 2 hours post-TBI (Figure 6A). Neutrophils counts were still high at 8 hours post-TBI, but returned to control levels at 24 hours post-TBI and onward. It is important to mention that for the splenic flow cytometry analysis, the 8 hour post-TBI time point has an n=2 due to spleens lost in processing. Ly6G MFI was evaluated for splenic neutrophils and was significantly elevated at 1 week post-TBI compared to control with all other time points showing non-significant change (Figure 6B). The splenic monocyte response to TBI was highly dynamic. Both classical and non-classical monocyte/macrophages showed similar trends with initial increases in cell counts at 2 and 8 hours post-TBI compared to control (Figure 6C and 6D). Cell counts dropped below uninjured control for both classical and non-classical at 72 hours post-TBI, 24% and 23% of control cell counts respectively. At 1 and 2 weeks both classical and non-classical had returned to values similar to control.

Between blood and spleen, there was a clear trend for the neutrophils, with both showing acute cell count peaks before returning to control like levels for the later time points. The monocyte subsets showed a reciprocal relationship between blood and spleen counts. Increases in blood counts for classical and non-classical monocytes in the blood demonstrated no increase or even a decrease in that subset in the spleen. This is true for the reverse situation.

Figure 6. Comparison of splenic neutrophil and monocyte counts post-TBI compared to control in *lys*-EGFP-*ki* mouse model. Splenic leukocytes were evaluated via flow cytometry and monocytes and neutrophils were gated using the same strategy as for blood. Splenic neutrophil counts significantly increased post-TBI and remained elevated at 8 hours post-TBI before returning for control-like levels for remaining time points (A). The MFI for Ly6G was significantly elevated at 1 week post-TBI, but all other time points were closer to control levels (B). Both classical (C) and non-classical (D) monocyte/macrophage counts increased acutely post-TBI before decreasing below control levels at 72 hours, both cell counts then recovered to control like levels. Kruskal–Wallis ANOVA followed by Dunn's test, * = $P<0.05$. n=4-5 (except for the 8 hour time point which is n=2) per time point (no replicates).


A
Neutrophils
1.5×10^5
1.0×10^5
5.0×10^4
0.0
*
Control
2 Hours
8 Hours
24 Hours
72 Hours
1 Week
2 Weeks
B
Ly6G MFI
2.0×10^5
1.5×10^5
1.0×10^5
5.0×10^4
0.0
*
Control
2 Hours
8 Hours
24 Hours
72 Hours
1 Week
2 Weeks
C
Classical Monocytes
3×10^4
2×10^4
1×10^4
0
Control
2 Hours
8 Hours
24 Hours
72 Hours
1 Week
2 Weeks
D
Non-Classical Monocytes
8×10^3
6×10^3
4×10^3
2×10^3
0
Control
2 Hours
8 Hours
24 Hours
72 Hours
1 Week
2 Weeks

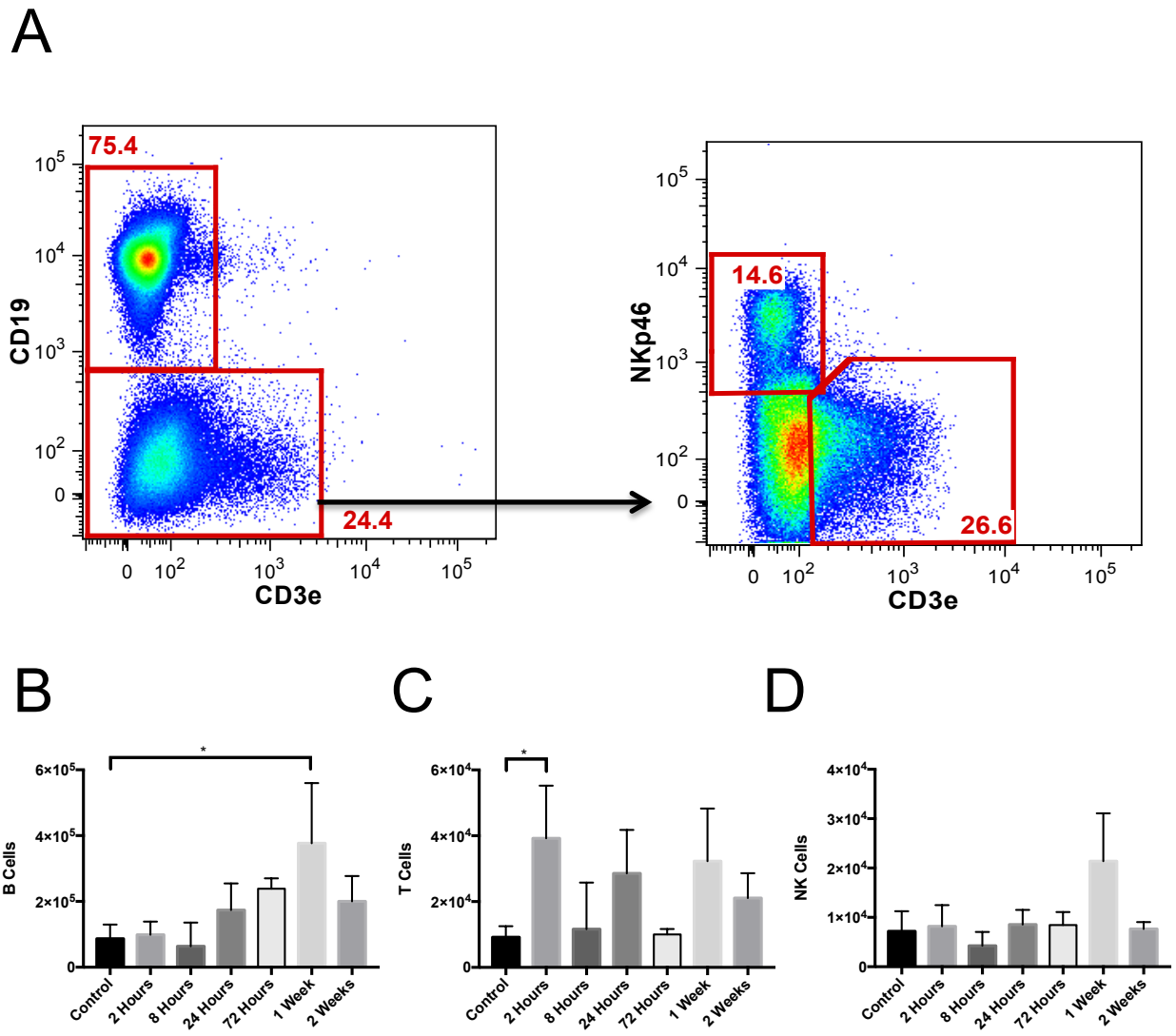
3.2 EXAMINATION OF PERIPHERAL EGFP$^-$ IMMUNE RESPONSE POST-TBI IN *lys*-EGFP-*ki* MICE

3.2.1 The circulating lymphocyte response

Lymphocytes of the *lys*-EGFP-*ki* mouse model do not endogenously express EGFP and must be selected for based on specific surface marker presence. The pan surface marker CD19 identifies B cells. CD3ε is the pan surface marker used to identify T cells. Lastly, NKp46 is the marker used for the recognition of NK cells (Figure 7A). A full outline of the gating strategy used for cell selection, including singlet discrimination, viability assessment, and negative and positive selection can be seen in Appendix 5.

Lymphocyte response to TBI is described to be a delayed process occurring long after the initial insult.[32] This appeared to be true for both B cells and NK cells with cell count peaking at 1 week post-TBI (Figure 7B and 7D). For B cells, counts increased at 24 and 72 hours post-TBI and peaked at 1 week, this significant increase was 4.4 times larger than control population, but counts returned to control like levels at 2 weeks. NK cell count increase was almost 3 times greater than control, but was not significant ($p=0.0914$), with all other time points closely resembling control. Opposite to the B and NK cells, T cells demonstrated a cyclic response, with a significant rise in count at 2 hours post-TBI (Figure 7C). T cells counts dropped and then rose over the remaining time points, although not reaching significance.

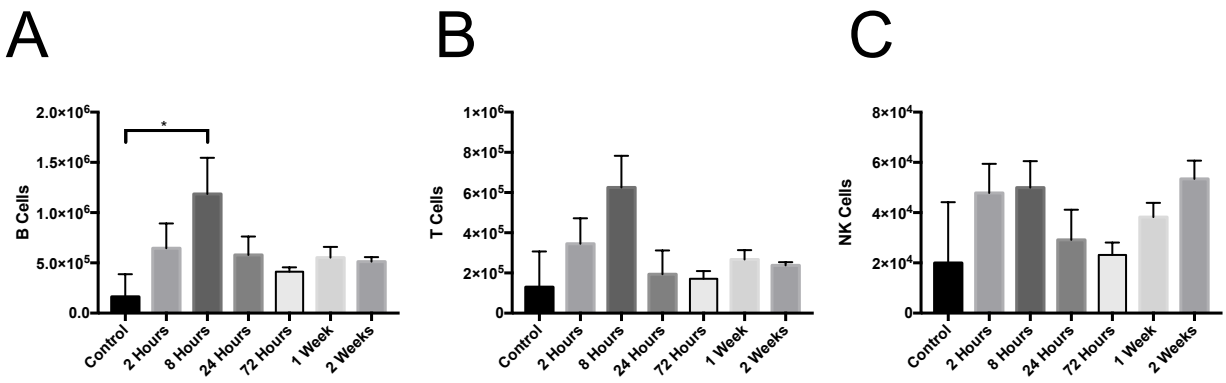
Figure 7. Examination of circulating blood lymphocyte subsets post-TBI in *lys*-EGFP-*ki* mouse model. Using flow cytometry lymphocyte subsets were identified, this included B cells as $CD19^+$, T cells as $CD3\varepsilon^+$ and lastly NK cells as $NKp46^+$ (A). Representative dot plots are shown. Circulating B cell counts began increasing 24 hours post-TBI peaking at 1 week compared to control before counts decreased at 2 weeks post-TBI to control like levels (B). T cell counts increased significantly post-TBI immediately after insult (C). T cell counts cycled up and down for the remainder of the time points evaluated. Blood NK cells were largely unchanged until they increased at 1 week post-TBI, then returning to control like counts at 2 weeks post-TBI. Kruskal–Wallis ANOVA followed by Dunn's test, * = $P<0.05$. n=4-5 per time point (no replicates).


A
75.4
24.4
CD19
CD3e
14.6
26.6
NKp46
CD3e
B
B Cells
C
T Cells
D
NK Cells
Control
2 Hours
8 Hours
24 Hours
72 Hours
1 Week
2 Weeks

3.2.2 The splenic lymphocyte response

Next, I compared the count of lymphocytes in the spleen in response to TBI. This was done using the same gating strategy and selection markers used as in the blood analysis above. In contrast to the blood analysis, B cells in the spleen increased at 2 hours and peaked at 8 hours post-TBI with a significant increase 7.3 times greater than control counts. B cell counts remained elevated, but were much closer to control level at 24 hours post-TBI onward (Figure 8A). It is important to mention that for the splenic flow cytometry analysis, the 8 hour post-TBI time point has an n=2 due to spleens lost in processing. The T cell response directly mirrored the B cell response, increasing at 2 hours, peaking at 8 hours post-TBI, and returning to control-like levels for the remaining time points (Figure 8B). Lastly, the NK cell count initially increased post-TBI at 2 and 8 hours post-TBI, briefly dipped to control-like levels and elevated again at 2 weeks post-TBI (Figure 8C). The only trend that was seen between the blood and spleen analysis is that cell counts either increased or stayed relatively constant compared to control. There didn't appear to be a consistent trend between blood and spleen counts for each subset.

Figure 8. Splenic lymphocyte subsets counts were evaluated post-TBI in *lys*-EGFP-*ki* mouse model. Using flow cytometry lymphocyte subsets were identified using the same strategy employed in the blood. Splenic B cell counts increased 2 hours post-TBI peaking at 8 hours compared to control before counts decreased to control like levels (A). T cell (B) and NK cell (C) counts were largely unchanged although they did both show increases post-TBI compared to control. Kruskal–Wallis ANOVA followed by Dunn's test, * = $P<0.05$. n=4-5 (except for the 8 hour time point which is n=2) per time point (no replicates).


A
B
C
B Cells
T Cells
NK Cells
2.0×10^6
1.5×10^6
1.0×10^6
5.0×10^5
0.0
1×10^6
8×10^5
6×10^5
4×10^5
2×10^5
0
8×10^4
6×10^4
4×10^4
2×10^4
0
*
Control
2 Hours
8 Hours
24 Hours
72 Hours
1 Week
2 Weeks

3.3 EXAMINATION OF APPROVED CLINICAL CONCUSSION ASSESSMENTS

3.3.1 Self-reported symptoms increase acutely after concussion from SCAT3

Over 4 seasons, 230 SCAT3 assessments were collected from participants in the study. It is important to note that SCAT3 forms were not always returned complete, and some data was lost because of how the test may have been performed at the clinic. There was a significant increase in score for the mental concentration (counting Digits Backwards) assessment from beginning of season (BS) in the non-concussed group to end of season (ES) in both the non-concussed and concussion group (Table 2). It must be stated that a score increase on this assessment is an indication of a better test performance. Included in the SCAT3 is a 22-symptom self-evaluation chart, where symptoms can be graded from 0 to 6. At 24-72 hours post-concussion, the number of self-reported symptoms was significantly higher compared to both BS and ES for the non-concussed group (Figure 9A). The mean number of reported symptoms for the non-concussed group at BS and ES were 1.8 and 1.5 respectively, this increased to 10.6 symptoms out of the possible 22 at 24-72 hours post-concussion. At ~1 week post-concussion, symptoms were significantly elevated from the ES non-concussed group. There were no significant changes in symptom scores post-concussion at ~1 month and onward compared to the non-concussed group, with the mean number of symptoms dropping to 2.3, 0.8, and 1.0 for the ~1 month, ~3 months, and ES time points in the concussed group. A list of the symptoms evaluated by the SCAT3 protocol and the amount they were reported can be seen in Table 3. This same pattern was seen for the symptom severity assessment, which totals the 1 to 6 score from reported symptoms, in Figure 9B. The mean reported symptom severity for the non-concussed group at BS and ES was 2.8 and 2.2, and this increased about 10 fold to 20.4 at 24-72 hours post-concussion. Again, the symptom severity score fell to levels similar to the non-

concussed group at ~1 month, ~3 months, and ES post-concussion with respective scores of 2.9, 0.9, and 1.1. A breakdown of the average scores for the remaining SCAT3 assessments containing both the non-concussed group as well as post-concussion group can be seen in Table 2.

Table 2. Mean scores of SCAT3 assessments

Test	Non-concussed Players		Concussion Players					
	BS	ES	BS	~24-72hrs	~1 Week	~1 Month	~3 Months	ES
Cognitive Orientation (n)	4.97 ± 0.16 (78)	4.97 ± 0.17 (66)	5 ± 0 (10)	4.94 ± 0.25 (16)	5 ± 0 (12)	4.92 ± 0.29 (12)	4.93 ± 0.27 (14)	5 ± 0 (9)
Immediate Memory (n)	14.17 ± 1.94 (78)	14.78 ± 0.49 (67)	14.8 ± 0.42 (10)	14.76 ± 0.56 (17)	14.92 ± 0.29 (12)	14.83 ± 0.39 (12)	14.93 ± 0.27 (14)	14.78 ± 0.44 (9)
Concentration (DB)[1] (n)	2.94 ± 0.93 (78)	**3.43 ± 0.80** (67)	3.5 ± 0.71 (10)	3.29 ± 0.77 (17)	3.58 ± 0.67 (12)	3.67 ± 0.65 (12)	3.64 ± 0.63 (14)	**3.89 ± 0.33** (9)
Concentration (MR) (n)	0.96 ± 0.20 (70)	0.99 ± 0.12 (67)	1 ± 0 (10)	0.94 ± 0.24 (17)	1 ± 0 (12)	1 ± 0 (12)	0.91 ± 0.30 (11)	1 ± 0 (9)
Balance (n)	2.33 ± 2.25 (78)	2.21 ± 2.16 (67)	1.56 ± 1.24 (9)	2.13 ± 1.96 (15)	1.73 ± 1.62 (11)	2.23 ± 2.35 (13)	1.85 ± 1.77 (13)	1.33 ± 1.41 (9)
Coordination (n)	1 ± 0 (73)	0.97 ± 0.17 (67)	1 ± 0 (9)	1 ± 0 (14)	1 ± 0 (10)	1 ± 0 (12)	1 ± 0 (13)	0.89 ± 0.33 (9)
Delayed Recall (n)	3.86 ± 1.08 (74)	4 ± 1.04 (66)	3.8 ± 1.03 (10)	3.4 ± 1.30 (15)	4.2 ± 1.23 (10)	4.33 ± 0.98 (12)	4.46 ± 0.88 (13)	4.56 ± 0.73 (9)

[1] Bold indicates significantly increased from underlined values.
Digits Backwards (DB), Months Reversed (MR).
Values mean ± SD

Figure 9. Participants diagnosed with a concussion reported significantly higher symptoms in the acute stages following injury compared to their own non-injured baseline values and the baseline values of non-concussed participants. SCAT3 tests were collected and analyzed for all participants in the study. The Symptom Score (A), which is the number of symptoms out of a list of 22 the individual reported, and Symptom Severity Score (B), which is how severe they reported those symptoms to be, assessments showed significant changes between pre-concussion baseline and post-concussion scores. The acute post-concussion scores returned back to non-concussed baseline levels for participants over the remainder of the study period. Beginning of Season (BS) End of season (ES). Kruskal–Wallis ANOVA followed by Dunn's test, * = $P<0.05$. Non-Concussed Group: BS (n=79) ES (n=66), Concussed Group: BS (n=10), 24-72 Hours (n=18), ~1 Weeks (n=18), ~1 Month (n=16), ~3 Months (n=14), ES (n=9).

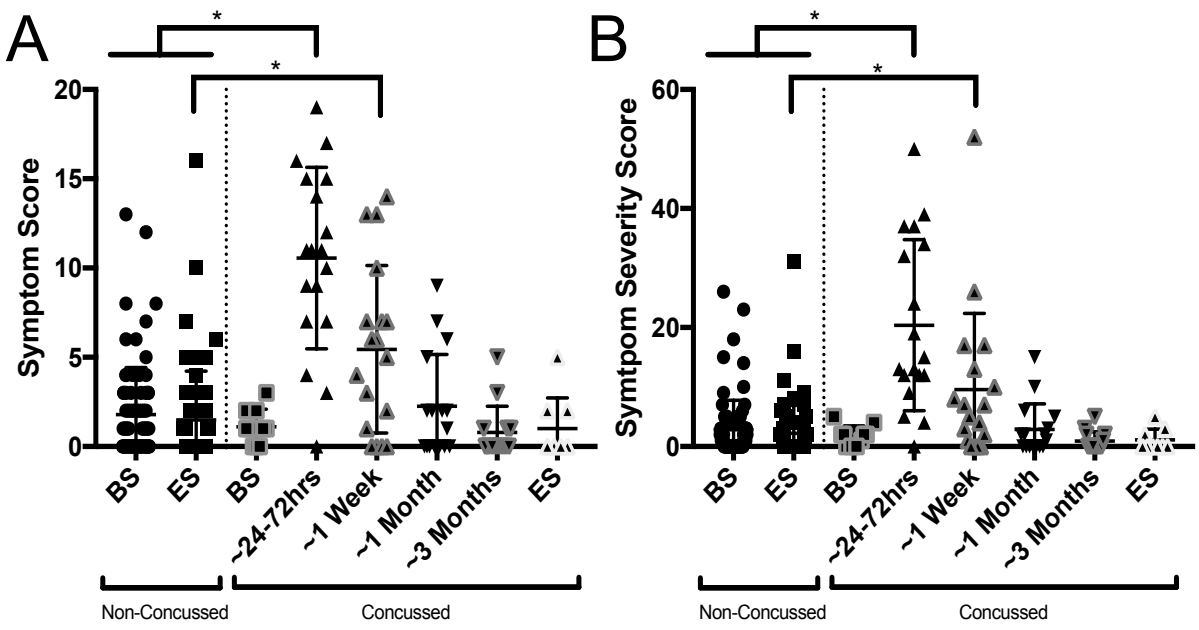

A
*
*
Symptom Score
20
15
10
5
0
BS
ES
BS
~24-72hrs
~1 Week
~1 Month
~3 Months
ES
Non-Concussed
Concussed
B
*
*
Symtpom Severity Score
60
40
20
0
BS
ES
BS
~24-72hrs
~1 Week
~1 Month
~3 Months
ES
Non-Concussed
Concussed

Table 3. Frequency of reported symptoms according to SCAT3

Symptom	Baseline-non-concussed players		Concussion					
	BS (n=79)	ES (n=67)	BS (n=9)	~24-72hrs (n=17)	~1 Week (n=19)	~1 Month (n=16)	~3 Months (n=15)	ES (n=10)
Headache	8	7	0	14	14	3	3	1
Pressure in Head	5	4	0	14	12	3	1	0
Neck Pain	19	6	5	12	7	4	0	0
Nausea	1	0	0	3	1	0	0	1
Dizziness	4	2	0	5	1	1	0	0
Blurred Vision	4	2	0	2	1	0	1	0
Balance Problems	4	6	0	5	2	0	0	0
Sensitivity to Light	8	1	0	11	7	0	1	0
Sensitivity to Noise	2	1	0	10	7	2	0	0
Feeling Slowed Down	4	6	0	10	7	2	0	0
Feeling like "in a fog"	3	5	0	10	5	2	0	0
"Don't feel right"	8	2	0	14	9	4	0	1
Difficulty Concentrating	5	6	0	10	7	3	1	0
Difficulty Remembering	4	2	0	6	6	2	0	0
Fatigue or low energy	17	18	1	11	8	7	2	2
Confusion	1	0	0	2	3	0	0	0
Drowsiness	6	6	1	10	3	2	2	1
Trouble Falling Asleep	15	11	2	4	2	2	2	2
More Emotional	8	3	1	7	7	2	0	0
Irritability	7	4	0	6	8	1	0	1
Sadness	5	2	0	6	4	0	0	1
Nervous or Anxious	14	4	2	8	1	1	0	0

3.3.2 ImPACT assessments lack detectability

There were 205 ImPACT assessments collected over 4 seasons, and much like the SCAT3 there are instances where data is missing due to how the test may have been conducted in clinic. From the ImPACT assessments collected, there was a significant increase in the visual motor speed composite assessment between BS for the baseline group and ~3 months post-concussion (Table 4). This increase in score is an indication of better test performance. There were no other significant alterations between the non-concussed group and any of the post-concussion time points. A breakdown of the average scores for the ImPACT assessment can be seen in Table 4.

Table 4. Mean scores of ImPACT assessments

Test	Non-Concussed Players		Concussion Players					
	BS (n=74)	ES (n=67)	BS (n=8)	~24-72hrs (n=8)	~1 Week (n=10)	~1 Month (n=16)	~3 Months (n=12)	ES (n=10)
Memory Composite (Verbal)	89.84 ± 8.41	93.37 ± 6.71	89.88 ± 10.37	92.38 ±5.45	91 ± 8.33	93 ± 6.99	92.5 ± 5.92	91.2 ± 6.94
Memory Composite (Visual)	76.03 ± 13.13	81.18 ± 12	78.63 ± 12.40	77.25 ± 11.40	76 ± 16.41	78.88 ± 14.34	80.17 ± 15.83	82.5 ± 9.29
Visual Motor Speed Composite[1]	42.77 ± 5.91	44.74 ± 5.19	43.44 ± 6.98	42.68 ± 6.97	41.04 ± 6.28	44.92 ± 5.77	**48.36 ± 5.35**	45.5 ± 5.45
Reaction Time Composite	0.55 ± 0.06	0.53 ± 0.05	0.57 ± 0.07	0.55 ± 0.04	0.55 ± 0.06	0.53 ± 0.05	0.5 ± 0.05	0.52 ± 0.06
Impulse Control Composite	6.53 ± 3.54	6.13 ± 3.63	4.38 ± 4.60	5 ± 2.27	6.5 ± 4.25	7.13 ± 4.57	6.25 ± 5.33	6.6 ± 5.76
Cognitive Efficiency Index	0.43 ± 0.12	0.46 ± 0.11	0.41 ± 0.14	0.47 ± 0.06	0.44 ± 0.12	0.52 ± 0.08	0.48 ± 0.12	0.48 ± 0.12
Total Symptom Score	4.82 ± 7.14	4.42 ± 6.86	7.75 ± 6.45	9.63 ± 12.13	4.4 ± 6.64	4.31 ± 5.38	1.42 ± 2.39	2.7 ± 3.68

[1] Bold indicates significantly increased from underlined
Values mean ± SD

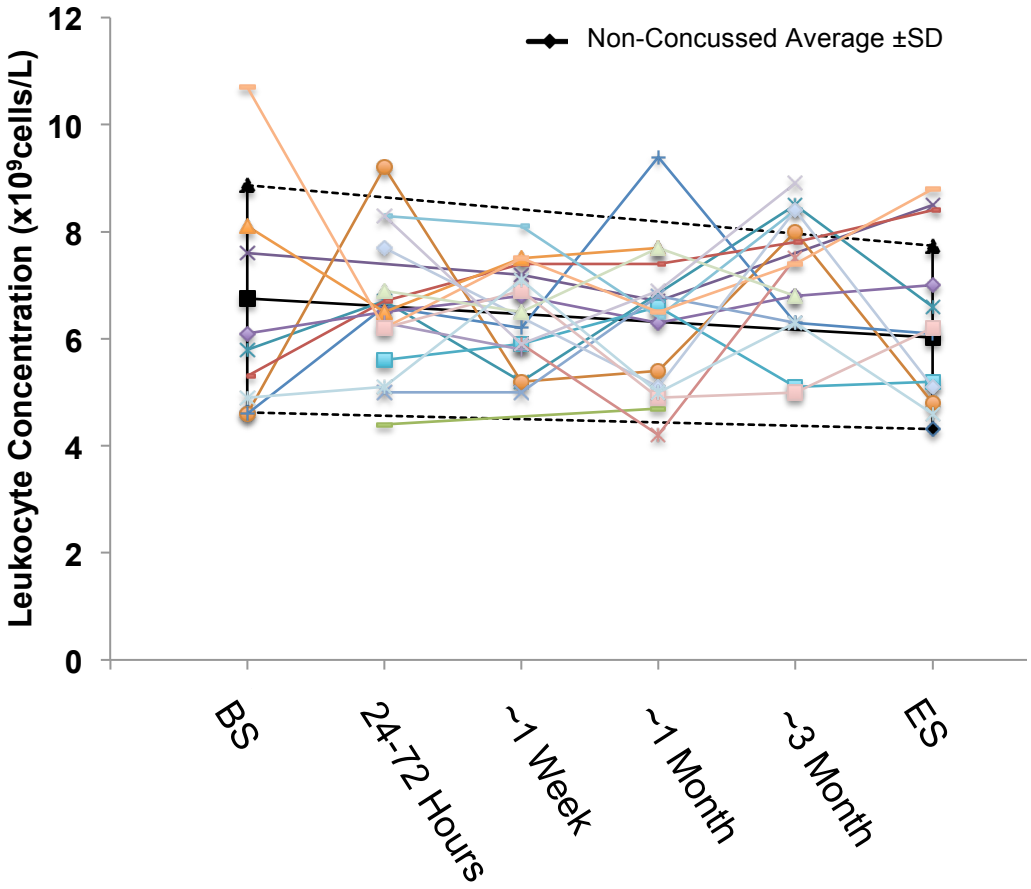
3.4 DIFFERENCES EVALUATION OF IMMUNE RESPONSE IN BLOOD SAMPLES

Based off the results of my investigation of peripheral inflammatory cell response post-TBI using the *lys*-EGFP-*ki* mouse model it was determined that objective changes in cell counts can be determined via flow cytometry. This provided the basis to go ahead with a pilot investigation tracking immune cell populations longitudinally in human blood as a biomarker of concussion.

The results in sections 3.4.1 and 3.4.2 were performed with statistical assistance from Dr. GY Zou and Artem Uvarov. It was of immense importance to perform a baseline analysis of players, as this allows for a personal comparison to determine objective changes in players following a concussion. Natural inter-human variability could hide important individual changes in the concussed group due to the wide range of values seen. As an example, changes to total leukocyte concentration post-concussion, while increasing or decreasing from a players personal baseline value level, were often still within the wide range of reported values seen in the non-concussed baseline population (Figure 10). To attempt to correct for this high degree of variation amongst individual players a differences analysis was undertaken, using a player's BS evaluation results, before any injury has occurred, as a set 0 mark to compare against. For the non-concussed group this is demonstrated as a player's ES value minus their own individual BS value (ES – BS) for each marker tested. For the concussed group, each player's time point, 24-72 hours, ~1 week, ~1 month, ~3 months, and ES post-concussion values, was subtracted by that individual player's BS values for each marker tested. In the circumstance where a player did not have a baseline before their concussion that season, a previous non-concussed baseline for that player from an earlier season was substituted if available. It is anticipated that the individual increases or decreases for each marker analyzed could be measured and pooled for each time

point and compared to the set 0 of their baseline. Time plots were created for each marker, and changes, denoted by Δ then the marker measured, resulting in a 95% confidence interval not spanning the 0 mark demonstrates significant alteration from the set 0 baseline (represented by the red dotted line at 0).

Figure 10. Variability between humans hides important individual alterations. Total blood leukocyte concentrations calculated by CBC, and non-concussed player values were averaged for beginning (BS) and end of season (ES), represented by the respective black dots at each time point, and the standard deviation is the dotted black line above and below. Each colour line represents a different concussed player and their completed time points over the course of the study. Although changes are occurring for each player, they are hidden within the large natural variability seen within players in the non-concussed group.


Non-Concussed Average ±SD
Leukocyte Concentration (x10⁹cells/L)
12
10
8
6
4
2
0
BS
24-72 Hours
~1 Week
~1 Month
~3 Month
ES

3.4.1 Complete blood count

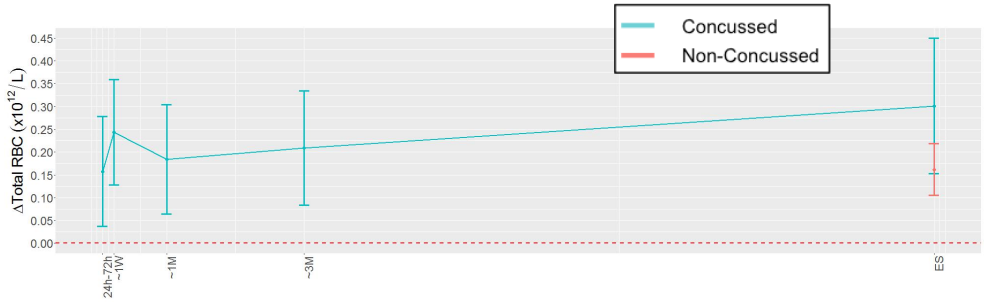
In total, 238 haematology profiles containing a CBC with a white blood cell differential were collected over 4 seasons, from this 66 non-concussed differences (ES – BS) evaluations were performed, and 14, 15, 14, 13 and 9, concussion differences evaluations were performed at 24-72 hours, ~1 week, ~1 month, ~3 months, and ES post-concussion respectively. Basic clinical blood tests were performed and provided a collection of leukocyte cell concentrations, as well as important information about RBCs and their characteristics. The total RBC concentration was significantly elevated from 0 at all time points post-concussion (Figure 11A). However, the non-concussed group ES was also significantly elevated from 0. The same pattern was seen for haemoglobin (Figure 11B) and hematocrit (Figure 11C), which is the ratio of the total volume of RBCs to the total volume of blood in the sample analysed.[83] All three of these factors show peaks at ~1 week post-concussion, followed by a decrease and then rise in levels to ES, which is slightly higher than the ES difference for the non-concussed group. The variable red blood cell distribution width (RDW), which is a numerical measure of the size variability of circulating erythrocytes, was initially elevated post-concussion, but dropped below the 0 mark on average by ES post-concussion, this is opposite to the non-concussed group's ES difference which was significantly higher than 0 (Figure 11D).[84] Thrombocytes were significantly elevated from 0 at 24-72 hours post-concussion, but returned to elevated but not significant levels at ~1 week to ~3 months post-concussion (Figure 11E). Thrombocytes were significantly elevated again compared to 0 for ES for both the concussed and non-concussed group.

From the leukocyte factors included in the white blood cell differential as part of the CBC, a significant elevation in both total leukocytes and neutrophil concentration from 0 was seen at ~3 months post-concussion (Figure 12A and B). Although no other post-concussion time

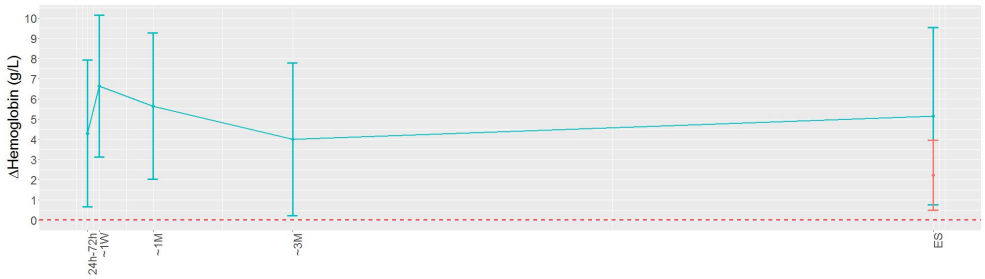
points were significantly altered from 0, the non-concussed groups ES was actually significantly decreased from 0 for both these factors. The only other significant alterations from 0 were seen at ES in the non-concussed group, which was increased at ES for lymphocyte concentration (Figure 12C), but decreased at ES for monocytes (Figure 12D). While the concussed group did also follow these trends at ES for lymphocytes and monocytes respectively, it did not reach significance. All remaining time plots can be seen in Appendix 6. Interestingly, RBC factors were consistently above 0 at ES, while leukocyte factors, excluding lymphocytes, were consistently below 0 at ES for the non-concussed group.

Figure 11. Red blood cells and red blood cell associated factors increase post-concussion. From collected CBC, differences evaluations were undertaken to allow more individualistic changes to be represented for values. All players collected marker values were subtracted from their own baseline vale and a comparison to the set baseline (0) was made. RBCs (A), haemoglobin (B), and hematocrit (C), were all significantly elevated from 0 at every time point post-concussion with no overlap of the 95% confidence interval with the 0 line. This was also true for ES in the non-concussed group. RDW (D) had a significant elevation from 0 for the non-concussed group at ES, although there were no post-concussion alterations. Thrombocytes showed a significant elevation from 0 at 24-72 hours post-concussion for the concussed group (E). Thrombocytes were also significantly elevated from 0 at ES for both the concussed and non-concussed group. Non-Concussed group differences (n=66), Concussion group differences; 24-72 hours (n=14), ~1 week (n=15), ~1 month (n=14), ~3 months (n=13), and ES (n=9).

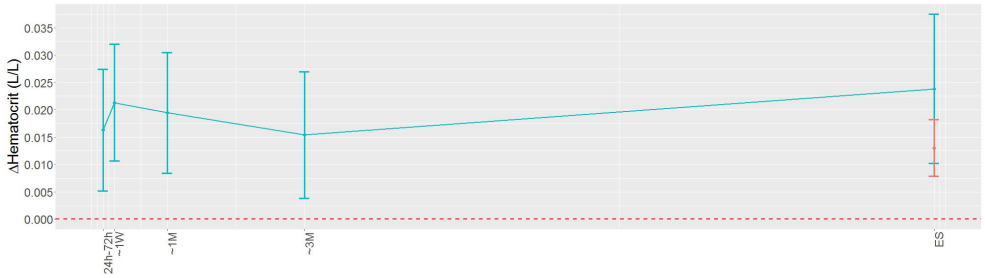
A


Concussed
Non-Concussed
ΔTotal RBC (x10^12/L)
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
24h-72h ~1W
~1M
~3M
ES

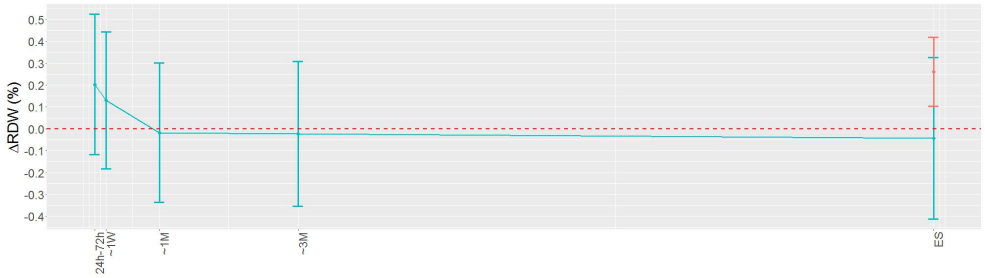
B


ΔHemoglobin (g/L)
10
9
8
7
6
5
4
3
2
1
0
24h-72h ~1W
~1M
~3M
ES

C


ΔHematocrit (L/L)
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
24h-72h ~1W
~1M
~3M
ES

D


ΔRDW (%)
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
24h-72h ~1W
~1M
~3M
ES

E

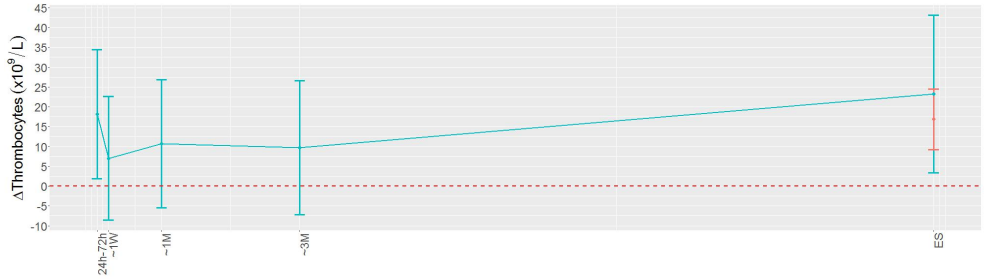
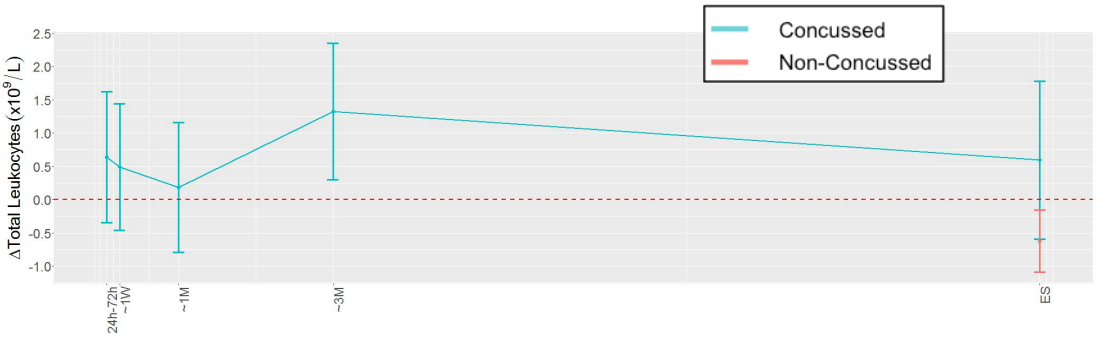
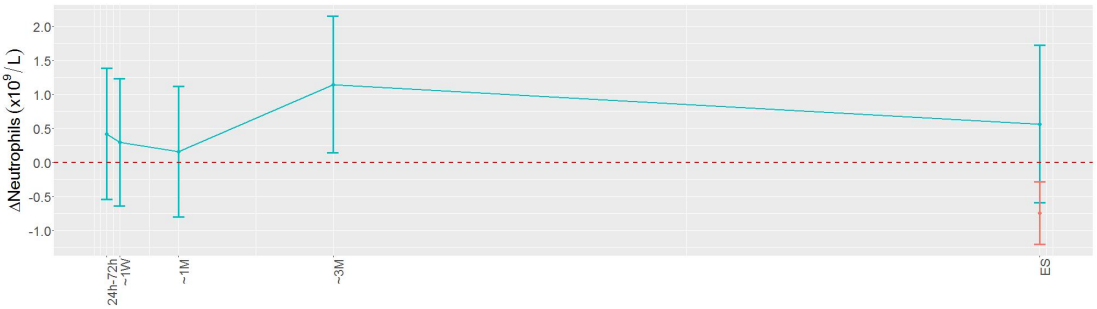

ΔThrombocytes (x10^9/L)
45
40
35
30
25
20
15
10
5
0
-5
-10
24h-72h ~1W
~1M
~3M
ES

Figure 12. Dynamic response of blood leukocyte population post-concussion. Using our established differences analysis for the leukocyte factors included in the CBC a significant elevation in both total leukocytes (A) and neutrophil (B) concentration from 0 was seen at ~3 months post-concussion. Although no other post-concussion time points were significantly altered from 0, the non-concussed groups ES was actually significantly decreased from 0 for both these factors. The only other significant alterations from 0 were seen at ES in the non-concussed group, which was increased at ES for lymphocyte concentration (C), but decreased at ES for monocytes (D). Non-Concussed group differences (n=66), Concussion group differences; 24-72 hours (n=14), ~1 week (n=15), ~1 month (n=14), ~3 months (n=13), and ES (n=9).

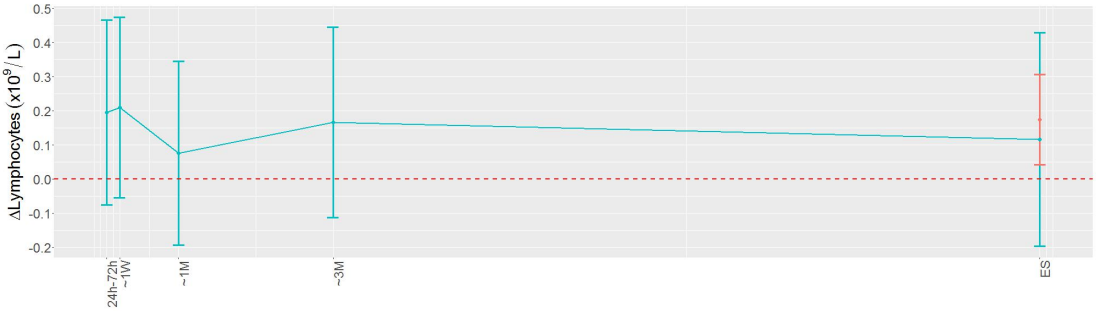
A


Concussed
Non-Concussed
ΔTotal Leukocytes (x10^9/L)
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
24h-72h
~1W
~1M
~3M
ES

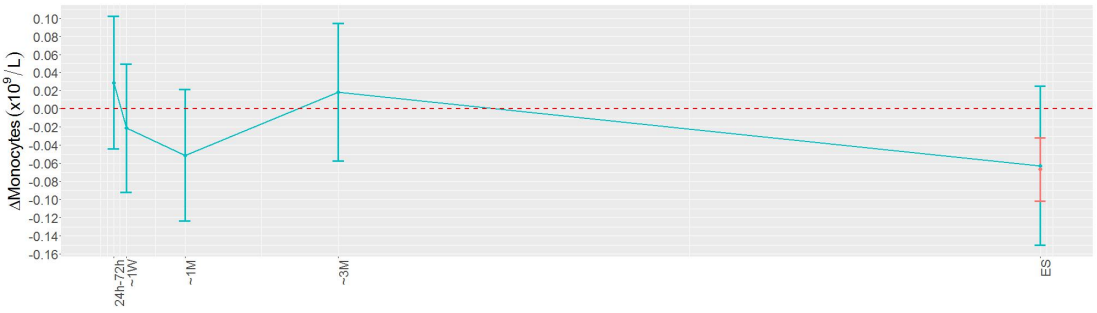
B


ΔNeutrophils (x10^9/L)
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
24h-72h
~1W
~1M
~3M
ES

C


ΔLymphocytes (x10^9/L)
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
24h-72h
~1W
~1M
~3M
ES

D


ΔMonocytes (x10^9/L)
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
-0.06
-0.08
-0.10
-0.12
-0.14
-0.16
24h-72h
~1W
~1M
~3M
ES

3.4.2 Frozen PBMC evaluation by flow cytometry

Over 4 seasons, 146 collected frozen PBMC samples were collected and analyzed, including 40 non-concussed differences (ES – BS). For the concussed group; 11, 11, 9, 8 and 7, concussion differences evaluations were performed at 24-72 hours, ~1 week, ~1 month, ~3 months, and ES post-concussion respectively. Two separate analyses were done to investigate monocyte and lymphocyte populations. Monocytes were distinguished by the presence of the pan surface marker CD14. Subdivision of the monocytes was done using the surface markers CD64 and CD16, $CD64^+$ $CD16^-$, $CD64^+$ $CD16^+$, and $CD64^-$ $CD16^+$, representing classical, intermediate, and non-classical subsets respectively (Figure 13C). The surface marker CD69 was included as a transient marker of cell activation (Figure 14A). A gating strategy for the monocyte analysis can be seen in Appendix 7. With the assistance of counting beads, absolute cell counts were obtained and differences were calculated from those counts.

On analysis of total monocytes (Figure 13A), there was a significant alteration from 0 for the concussed group at ES dropping below the 0 mark (Figure 13B). Although the initial differences showed increases above 0 at 24-72 hours, ~1 week, ~1 month, ~3 months post-concussion, the decrease at ES matches the decrease seen for the monocyte count derived from the CBC. When comparing monocyte subtypes, this drop appears to be driven by the intermediate (Figure 13 E) and non-classical populations (Figure 13F). Both populations showed significant decreases from 0 at ES post-concussion, with decreases seen at all time points except for ~1 week post-concussion where both had means greater than 0. Corresponding significant decreases were seen in the number of $CD69^+$ cells for both intermediate (Figure 14D) and non-classical monocytes (Figure 14F) at ES in the concussed group with the overall trend mirroring the respective subset count. The classical count was also decreased at ES in the concussed group,

however not significantly, but it was elevated above 0 for all prior time points post-concussion (Figure 13D). The non-concussed group was consistently just below 0 for each of the populations, which matches the decrease seen in this group for the total monocyte count from the CBC differential.

For the lymphocyte evaluation, a separate flow cytometry staining panel was used to discriminate subtypes and obtain cell counts. B cells were selected for using the pan surface marker CD19, while the pan surface marker CD3 was used to determine T cells (Figure 15A). T cells were further separated as either $CD4^+$ or $CD8^+$ to classify those respective subtypes.[70] Lastly, NK cells were distinguished as $CD56^+$ (Figure 15A).[70] A full gating strategy is available in Appendix 8. From the previous analysis looking at total lymphocyte concentration in the CBC, there was an increase from 0 at all time points post-concussion and at ES for the non-concussed group. This same pattern was seen for the total lymphocyte differences analysis from frozen PBMCs, except the concussed groups ES mean was below 0 (Figure 15B). There was a significant increase from 0 for the CD4 T cells in the non-concussed group, which contrast with a non-significant decrease from 0 for the ES difference in the post-concussion group (Figure 15D). At ~1 week post-concussion there was a significant increase from 0 for the concussed group in CD8 T cells (Figure 15E). NK cells were also significantly increased at ~1 week post-concussion (Figure 15F). This was a sharp peak in count compared to the time points before and after which both averaged differences below 0.

Figure 13. Blood monocyte population decreases at end of season from frozen PBMC flow analysis. Frozen PBMC samples were analyzed by flow cytometry to obtain monocyte population information. The monocyte fraction was gated on to determine total monocytes counts and then evaluated using our differences strategy (A). Representative dot plots are shown. Although total monocyte increases were seen immediately post-concussion, there was a significant decline from 0 at ES for the concussed group. Upon subdivision of the monocytes into $CD64^{+}$ $CD16^{-}$, $CD64^{+}$ $CD16^{+}$, and $CD64^{-}$ $CD16^{+}$ subsets, representing classical, intermediate, and non-classical subsets respectively counts were obtained and evaluated using our differences strategy (C). While classical (D) populations on average were above 0 till ES, average intermediate (E) and non-classical (F) counts were below 0, except for at ~1 week post-concussion. All populations counts averaged below 0 for both concussion and non-concussion group with both the intermediate and non-classical significantly below 0. Non-Concussed group differences (n=40), Concussion group differences; 24-72 hours (n=11), ~1 week (n=11), ~1 month (n=9), ~3 months (n=8), and ES (n=7).

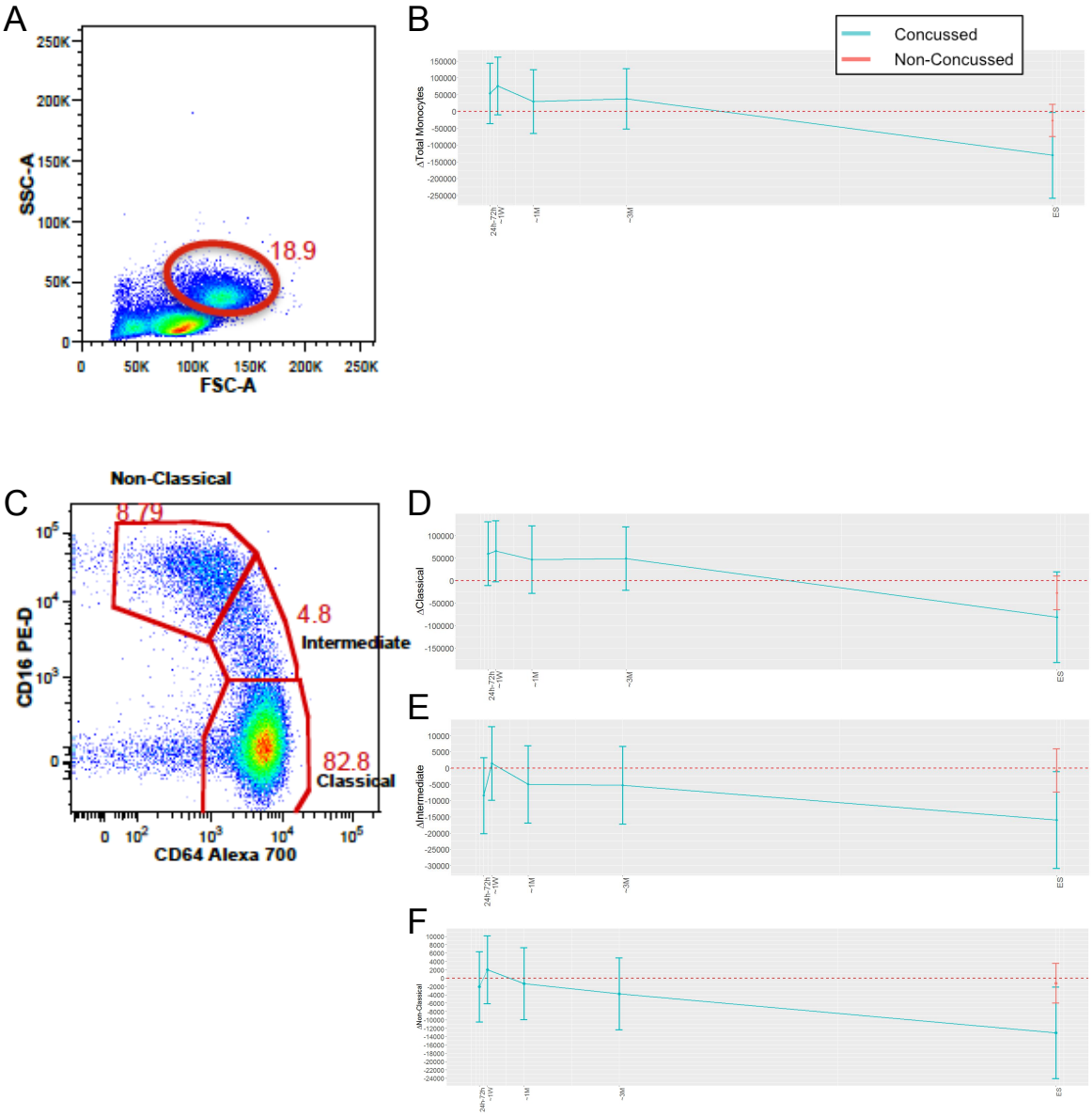

A
SSC-A
FSC-A
18.9
B
Concussed
Non-Concussed
ΔTotal Monocytes
C
Non-Classical
8.79
4.8
Intermediate
82.8
Classical
CD16 PE-D
CD64 Alexa 700
D
ΔClassical
E
ΔIntermediate
F
ΔNon-Classical

Figure 14. Monocyte cell counts positive for CD69 surface marker decrease at ES. Transient surface marker of cell activation, CD69, was evaluated on monocyte subsets from frozen PBMC samples analyzed by flow cytometry for both count and MFI and evaluated using our differences strategy (A). Representative histograms are shown. Classical cells positive for CD69 (B) on average were above 0 till ES, average intermediate (D) and non-classical (F) counts were below 0, except for at ~1 week post-concussion. All population counts averaged below 0 for both the concussion and non-concussion group with both the intermediate and non-classical significantly below 0 in the concussed group. Non-Concussed group differences (n=40), Concussion group differences; 24-72 hours (n=11), ~1 week (n=11), ~1 month (n=9), ~3 months (n=8), and ES (n=7).

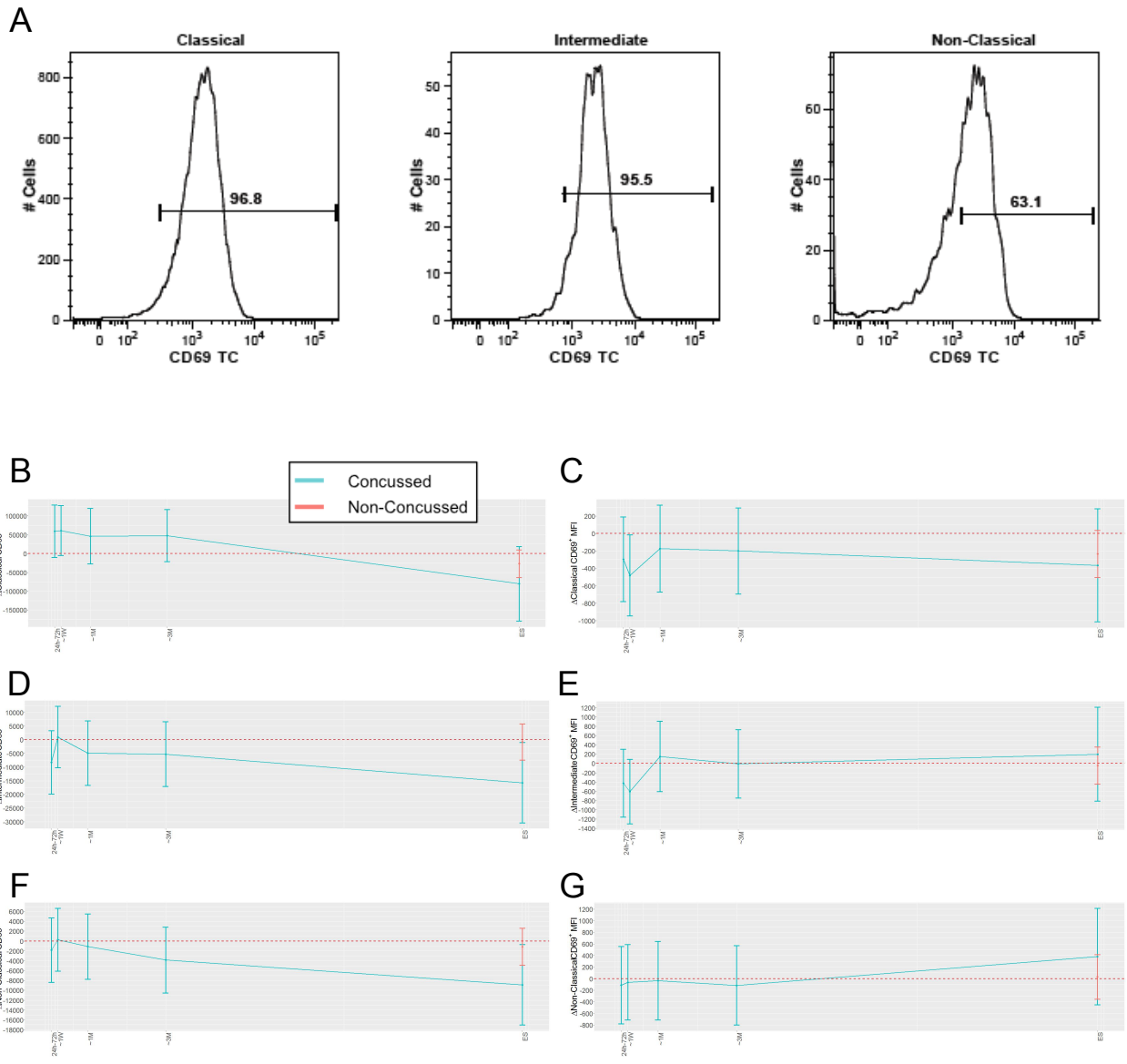
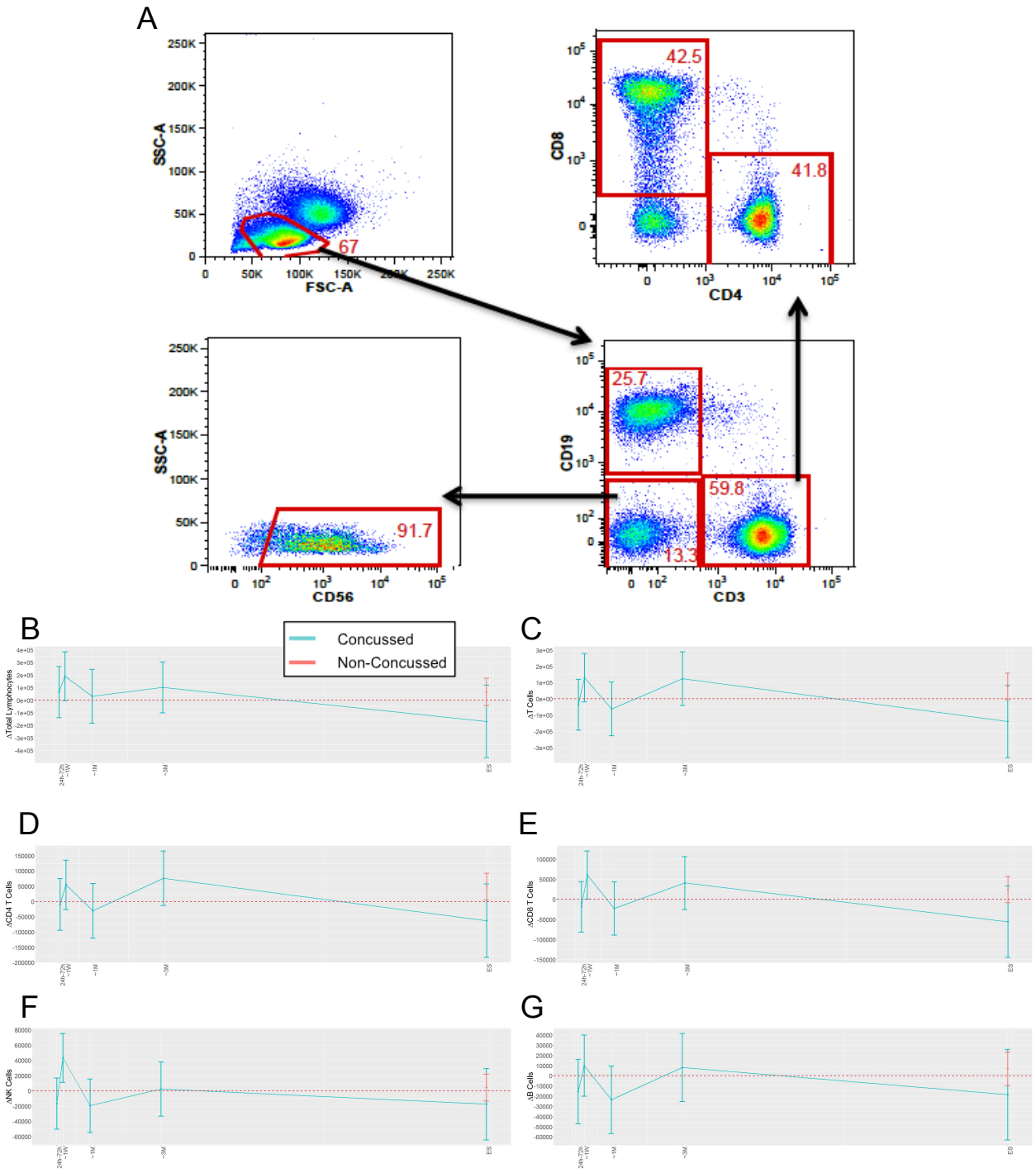

A
Classical
Intermediate
Non-Classical
Cells
CD69 TC
96.8
95.5
63.1
B
C
D
E
F
G
Concussed
Non-Concussed

Figure 15. Identification of blood lymphocyte subset counts post-concussion. Frozen PBMC samples were analyzed by flow cytometry to obtain lymphocyte subset information. The lymphocyte fraction was gated on first to determine total counts and then evaluated using our differences strategy (A). Representative dot plots shown. Although total lymphocyte increases were seen immediately post-concussion, there was a decline by ES, which was opposite to the non-concussed group, which increased average counts above 0 (B). CD4 T cell counts significantly increased from 0 for the non-concussed group, which contrast with a non-significant decrease from 0 for the ES difference in the post-concussion group (D). CD8 T cells were significantly increased from 0 at ~1 week post-concussion from 0 (E). NK cells were significantly increased at ~1 week post-concussion (F). Non-Concussed group differences (n=40), Concussion group differences; 24-72 hours (n=11), ~1 week (n=11), ~1 month (n=9), ~3 months (n=8), and ES (n=7).


A
SSC-A
FSC-A
67
CD8
CD4
42.5
41.8
CD19
CD3
25.7
59.8
13.3
CD56
91.7
B
Concussed
Non-Concussed
ΔTotal Lymphocytes
C
ΔT Cells
D
ΔCD4 T Cells
E
ΔCD8 T Cells
F
ΔNK Cells
G
ΔB Cells

3.5 EVALUATON OF WHOLE BLOOD FLOW CYTOMETRY IN HUMAN BLOOD SAMPLES

3.5.1 Circulating neutrophil response to concussion

For the fourth season of the human concussion study a whole blood evaluation by flow cytometry was included in addition to the frozen PBMC evaluation done previously. An advantage of whole blood flow analysis is neutrophils, which were lost during the PBMC purification and storage process, can now be captured and evaluated by flow cytometry. The disadvantage of this technique though was it was no longer possible to capture as many players at BS since the costs of antibody requirements for flow cytometry would be beyond what was available. For this reason, only 10-12 players were selected for baseline analysis and new players to the study were a majority of those selected. A whole blood flow baseline will therefore only be available if one of the players captured in these reduced selection groups also received a concussion in season. This made doing the differences analysis carried out in the human blood evaluations above unrealistic.

Neutrophils were identified with their characteristic high forward and side scatter and presence of the surface marker CD16 using flow cytometry (Figure 16A). Using the expression of surface marker CD15 neutrophils were subdivided into three groups; low ($CD15^-$), middle ($CD15^+$), and high ($CD15^{++}$) (Figure 16A). A full outline of the gating strategy used for neutrophil selection can be seen in Appendix 9. There was no significant alteration in number of $CD16^+$ neutrophils post-concussion compared to non-concussed baseline, although there was about a 2 times increase in cells at ~3 months post-concussion compared to BS (Figure 16C). This aligns with the neutrophil differences analysis from the CBC, which had a significant increase in neutrophils from 0 at ~3 months post-concussion. Interestingly, there was an almost 1.6 times increase in mean cell count from BS to ES for the non-concussed group, which also

matched the increase seen at ES in the non-concussed group from the previous haematology profile with CBC blood difference analysis. On comparison of the CD15 subsets for the non-concussed group there was a 4.3, 2.4, and 1.27 fold increase, for the low, middle, and high group respectively for counts between BS and ES, demonstrating this increase is a combined effect (Figure 17A-C). A significant decrease in the CD15 low count was observed between ES in the non-concussed group and ~1 week post-concussion, however this effect may be driven by the presence of a couple outliers. There were no significant alterations post-concussion compared to the non-concussed group for CD15 MFI for each of the CD15 neutrophil subsets (Figure 17G-I). Upon looking at the CD15 subsets positive for the surface marker CD69 (Figure 16B), a significant decrease in CD15 high $CD69^{+}$ cell count was seen between BS and ~1 week post-concussion. For CD69 MFI, there was a significant increase at ~1 week post-concussion for both the middle and high CD15 subgroups, but for the middle this was to the ES in the non-concussed group, and for the high group it was against the BS of the non-concussed group (Figure 17K and L). This effect too may have been driven by potential outliers and will need to be investigated further.

Figure 16. Neutrophil analysis from whole blood samples for concussed and non-concussed participants. Using flow cytometry neutrophils were selected as $CD16^{+}$ cells and then were further subdivided into three groups using the expression of surface marker CD15; low ($CD15^{-}$), middle ($CD15^{+}$), and high ($CD15^{++}$)(A), lastly surface marker CD69 was used to assess cell activation upon each subset (B). Representative dot plots and histograms are shown. Total neutrophil counts were relatively unchanged at 24-72 hours, ~1 week, and ~1 month post-concussion, but increased at ~3 months post-concussion compared to BS (B). There was an increase in mean cell counts from BS to ES for the non-concussed group as well. Beginning of Season (BS) End of season (ES). None of these differences were significant according to the Kruskal–Wallis ANOVA followed by Dunn's test. Data only for the fourth season, Non-Concussed Group: BS (n=11) ES (n=9), Concussed Group: 24-72 Hours (n=3), ~1 Weeks (n=5), ~1 Month (n=3), ~3 Months (n=3), ES (n=1).

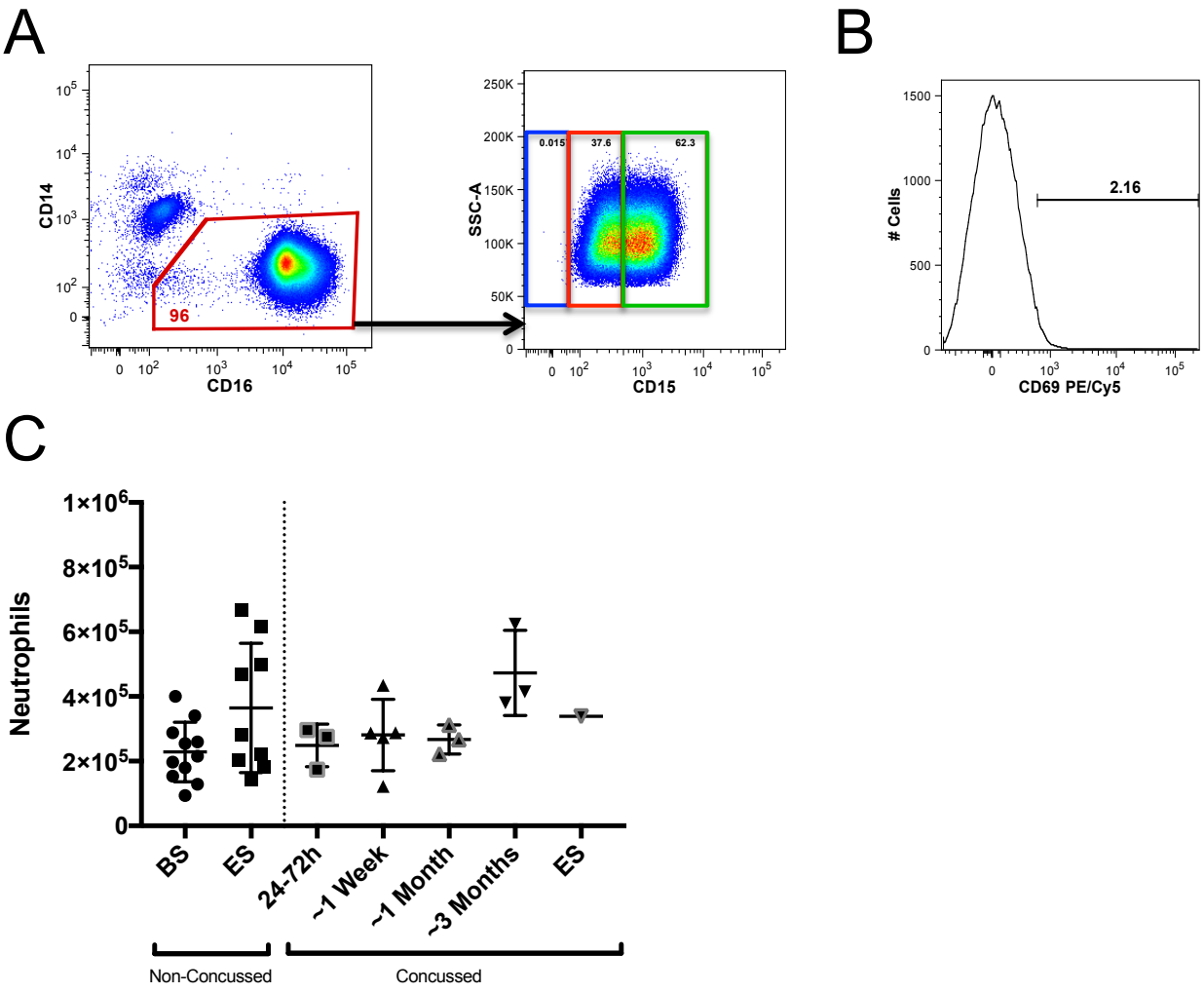
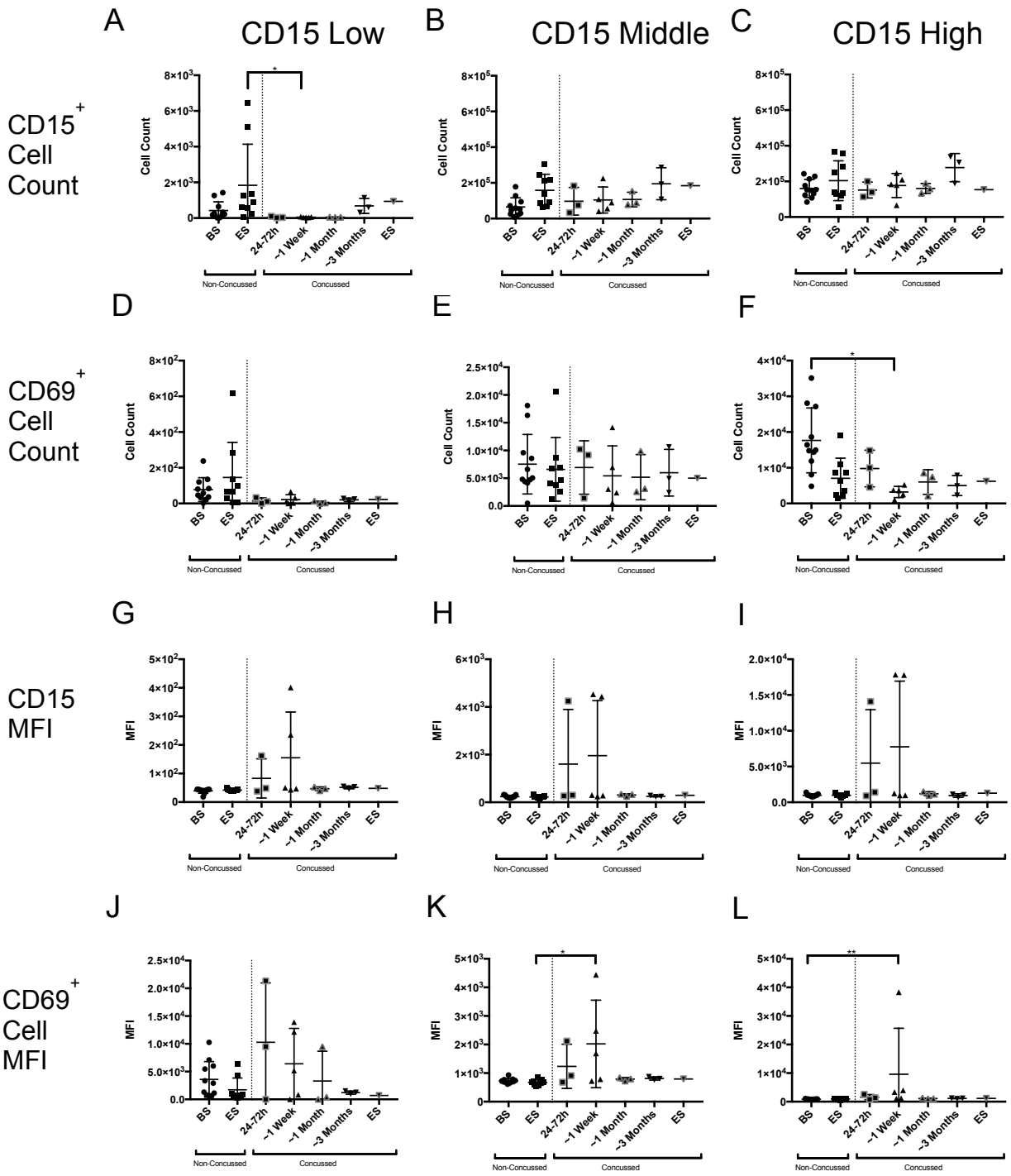

A
CD14
CD16
96
SSC-A
CD15
0.015
37.6
62.3
B
Cells
CD69 PE/Cy5
2.16
C
Neutrophils
1×10^6
8×10^5
6×10^5
4×10^5
2×10^5
0
BS
ES
24-72h
~1 Week
~1 Month
~3 Months
ES
Non-Concussed
Concussed

Figure 17. Neutrophil subset evaluation post-concussion via whole blood flow cytometry. The CD15 subsets for the non-concussed group were increased for the low (A), middle (B), and high (C) group respectively for counts between BS and ES. A significant decrease in the CD15 low count was observed between ES in the non-concussed group and ~1 week post-concussion (A). A significant decrease in CD15 high $CD69^{+}$ cell count was seen between BS and ~1 week post-concussion (F). For CD69 MFI, there was a significant increase at ~1 week post-concussion for the CD15 middle subgroup compared to ES of the non-concussed group (K). The CD15 high subgroup was significantly increased at ~1 week post-concussion for CD69 MFI compared to BS of the non-concussed group (L). Beginning of Season (BS) End of season (ES). Kruskal–Wallis ANOVA followed by Dunn's test, * = $p \leq 0.05$. Data only for the fourth season, Non-Concussed Group: BS (n=11) ES (n=9), Concussed Group: 24-72 Hours (n=3), ~1 Weeks (n=5), ~1 Month (n=3), ~3 Months (n=3), ES (n=1).


A
CD15 Low
B
CD15 Middle
C
CD15 High
CD15+ Cell Count
D
E
F
CD69+ Cell Count
G
H
I
CD15 MFI
J
K
L
CD69+ Cell MFI
Cell Count
MFI
BS
ES
24-72h
~1 Week
~1 Month
~3 Months
ES
Non-Concussed
Concussed

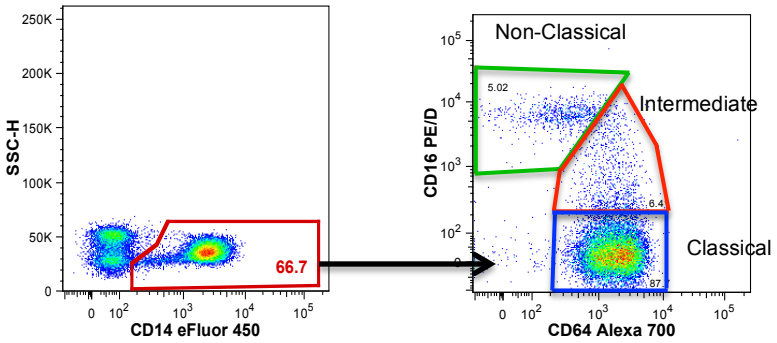
3.5.2 Circulating monocyte response to concussion

For the assessment of the monocyte subset populations by flow cytometry from fresh blood samples, the same gating strategy previously optimized from my monocyte analysis using frozen PBMC samples was used (Figure 18A and B). Gating on total monocyte population, there was an increase in all post-concussion time points average counts compared to BS of the non-concussed group, the ES of the non-concussed group was also similarly elevated though (Figure 18C). However, none of these increases reached significance. Previous monocyte analysis from the CBC, the opposite effect was seen, with monocyte concentration differences decreasing at ES for both the concussed and non-concussed group. On analysis of the classical monocyte subset, a steady increase can be seen from an average of 20 377, 24 400, 28 543, and 35 050 cells, at 24-72 hours, ~1 week, ~1 month, and ~3 months post-concussion time points respectively compared to non-concussed BS which averaged 11 965 cells (Figure 19A). This increase from BS was significant at ~3 months post-concussion. This same trend was seen for $CD69^+$ classical monocyte count, although none of the changes were significant (Figure 19D). The total monocyte count for ES of the non-concussed group was also elevated compared to BS. The highest mean cell counts came post-concussion at 24-72 hours and ~1 week for the intermediate population, before moving to counts slightly below both BS and ES of the non-concussed group (Figure 19B). Again, this trend was seen for the $CD69^+$ intermediate monocytes count (Figure 19E). The non-classical subset demonstrated little change between the non-concussed group and the post-concussion time points (Figure 19C). Although there are no significant alterations between the non-concussed and post-concussion group for the CD69 MFI, there was a significant decrease in CD69 MFI from BS to ES in the non-concussed group for both the classical (Figure 19G) and intermediate subsets (Figure 19H). Monocyte subpopulation counts in comparison to

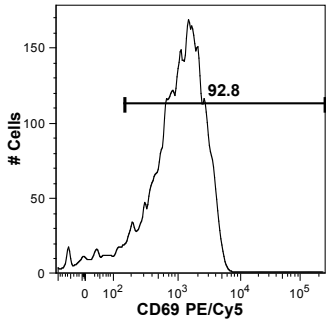
one another can be seen in Figure 20 (these proportions are produced from subset values seen in Figure 19A-C). While subtypes remained fairly constant both for the concussed and non-concussed groups, there does appear to be an expansion of the intermediate monocyte population at 24-72 hours post-concussion that has returned to a more normal proportion by ~1 week post-concussion.

Figure 18. Circulating blood monocyte population from whole blood flow analysis. Whole blood samples were analyzed by flow cytometry to obtain monocyte population information. The previously optimized gating strategy for monocytes from my frozen PBMC analysis was used to identify monocytes. Subdivision of the monocytes into $CD64^{+}$ $CD16^{-}$ (classical), $CD64^{+}$ $CD16^{+}$ (intermediate), and $CD64^{-}$ $CD16^{+}$ (non-classical) subsets was performed as before (A). Surface marker CD69 was used to assess cell activation upon each subset (B). Representative dot plots and a histogram are shown. Slight total monocyte count increases were seen post-concussion; this was matched by similar increases at ES in the non-concussed group (C). Kruskal–Wallis ANOVA followed by Dunn's test, * = $p \leq 0.05$. Data only for the fourth season, Non-Concussed Group: BS (n=11) ES (n=9), Concussed Group: 24-72 Hours (n=3), ~1 Weeks (n=5), ~1 Month (n=3), ~3 Months (n=3), ES (n=1).

A


SSC-H
CD14 eFluor 450
66.7
CD16 PE/D
CD64 Alexa 700
Non-Classical
Intermediate
Classical
5.02
6.4

B


Cells
CD69 PE/Cy5
92.8

C

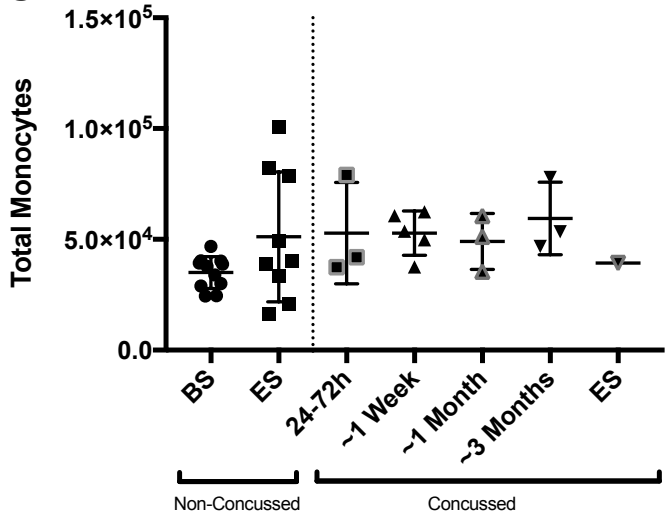

Total Monocytes
1.5×10^5
1.0×10^5
5.0×10^4
0.0
BS
ES
24-72h
~1 Week
~1 Month
~3 Months
ES
Non-Concussed
Concussed

Figure 19. Classical monocytes increase chronically post-concussion. Monocyte subset counts were obtained and analyzed for whole blood samples via flow cytometry, CD69 surface expression was also evaluated by count and MFI. Classical monocytes increased steadily post-concussion and were significantly greater than counts at BS in the non-concussed group. There was a significant decrease in CD69 MFI from BS to ES in the non-concussed group for both the classical (G) and intermediate subsets (H). Kruskal–Wallis ANOVA followed by Dunn's test, * = $p \leq 0.05$. Data only for the fourth season, Non-Concussed Group: BS (n=11) ES (n=9), Concussed Group: 24-72 Hours (n=3), ~1 Weeks (n=5), ~1 Month (n=3), ~3 Months (n=3), ES (n=1).

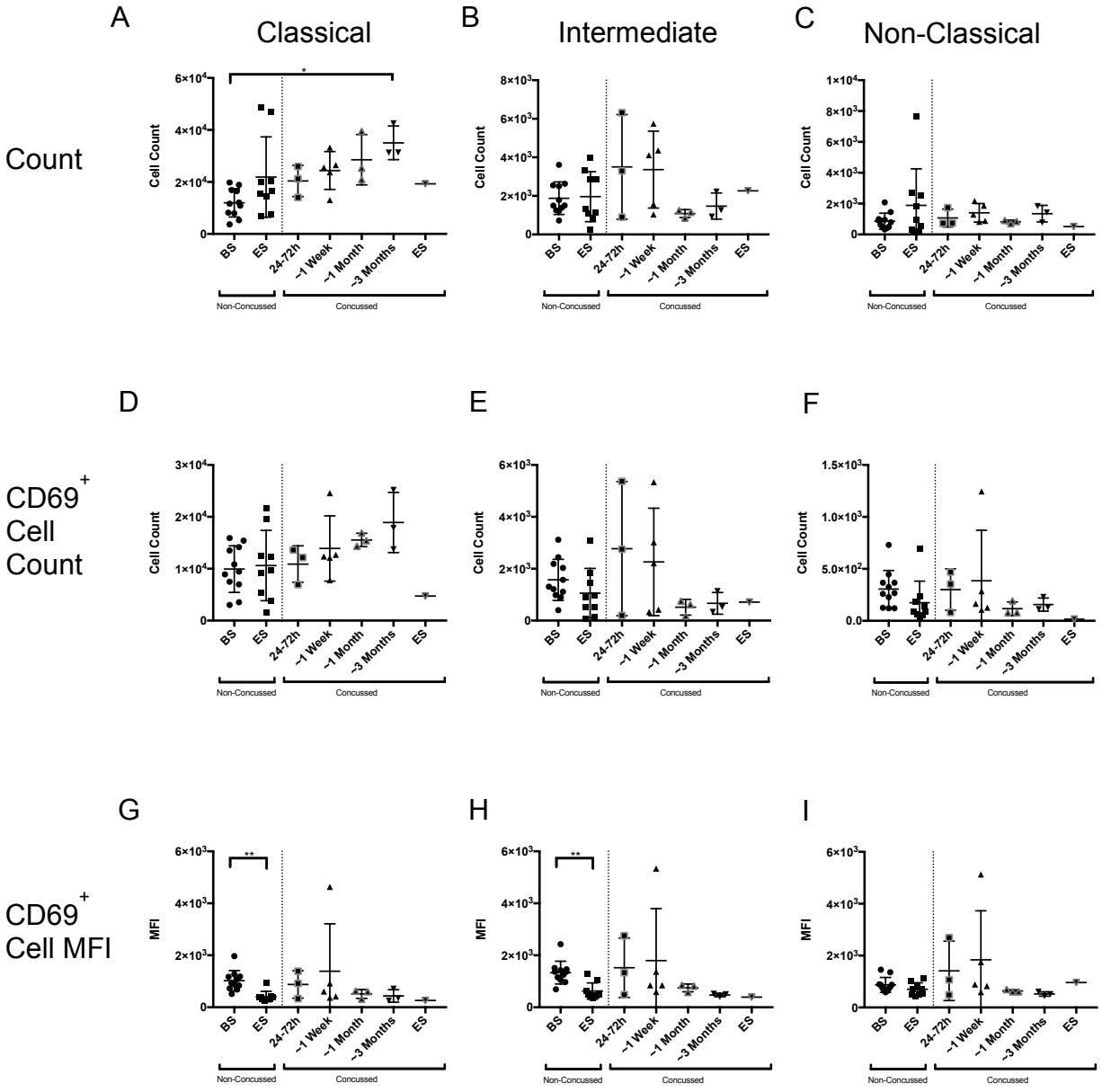
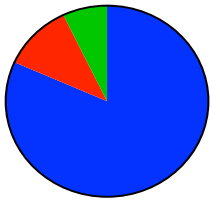

A
Classical
B
Intermediate
C
Non-Classical
Count
D
E
F
CD69+ Cell Count
G
H
I
CD69+ Cell MFI
Cell Count
MFI
BS
ES
24-72h
~1 Week
~1 Month
~3 Months
ES
Non-Concussed
Concussed
*
**

Figure 20. Monocyte subset proportional comparison in concussed and non-concussed participants. From monocyte subset counts, proportional representation was evaluated. The intermediate monocyte subset showed expansion at 24-72 hours post-concussion compared to other time points, and quickly reduced to previous levels. No statistical calculation was performed. Data only for the fourth season, Non-Concussed Group: BS (n=11) ES (n=9), Concussed Group: 24-72 Hours (n=3), ~1 Weeks (n=5), ~1 Month (n=3), ~3 Months (n=3), ES (n=1).

Non-Concussed

BS



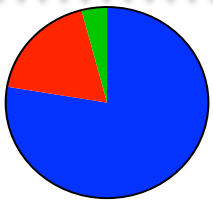
81.68% Classical
11.22% Intermediate
7.10% Non-Classical

ES

88.46% Classical
6.14% Intermediate
5.40% Non-Classical

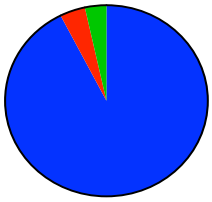
Concussed

24-72 Hours



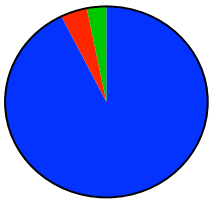
77.65% Classical
18.29% Intermediate
4.06% Non-Classical

~1 Weeks



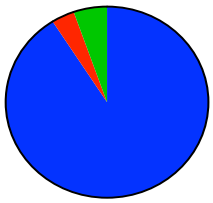
92.53% Classical
4.05% Intermediate
3.42% Non-Classical

~1 Month



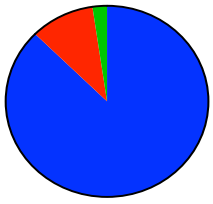
92.67% Classical
4.21% Intermediate
3.11% Non-Classical

~3 Months



90.99% Classical
3.68% Intermediate
5.34% Non-Classical

ES

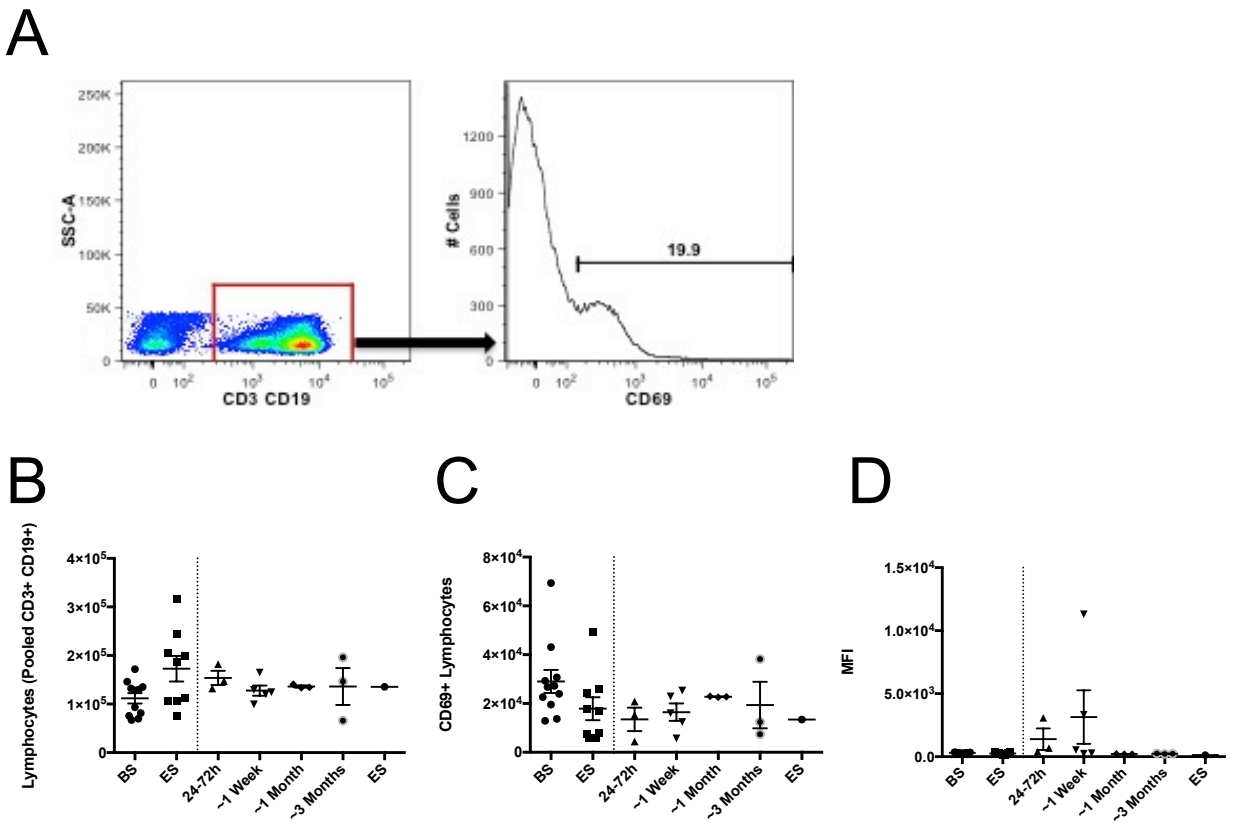


87.45% Classical
10.25% Intermediate
2.31% Non-Classical

3.5.3 Pooled lymphocytes response post-concussion

Although there was no lymphocyte specific panel included in my whole blood analysis, some lymphocyte information can be obtained. A pooled collection of CD3 and CD19 surface expressing cells were selected for from the lymphocyte fraction and analyzed for absolute cell count and surface expression of CD69 (Figure 21A). This allows for a rough assessment of B and T cells. The full gating strategy for this analysis can be seen in Appendix 10. From the CD3-CD19 pooled count, there was a small increase in counts post-concussion compared to BS, however the largest increase from BS was the ES for the non-concussed group (Figure 21B). Again, this increase at ES in the non-concussed group matches changes seen from the CBC and frozen PBMC evaluation. Additionally, when analyzing the number of $CD69^+$ cells, the BS has a greater count than ES for the non-concussed group, it was also greater than all the average counts for the post-concussion time points (Figure 21C). The CD69 MFI showed no significant alterations (Figure 21D).

Figure 21. No signature changes were detected in the lymphocyte population analyzed from whole blood following concussion. Whole blood was analyzed by flow cytometry and a gating strategy was developed to pool $CD3^+$ and $CD19^+$ lymphocyte cells, and cell activation using CD69 (A). A representative dat plot and histogram are shown. There were no significant changes between BS or ES compared to post-concussion for total cell populations (B), the $CD69^+$ count (C), or the CD69 MFI (D). Kruskal–Wallis ANOVA followed by Dunn's test. Data only for the fourth season, Non-Concussed Group: BS (n=11) ES (n=9), Concussed Group: 24-72 Hours (n=3), ~1 Weeks (n=5), ~1 Month (n=3), ~3 Months (n=3), ES (n=1).


A
SSC-A
CD3 CD19
Cells
19.9
CD69
B
Lymphocytes (Pooled CD3+ CD19+)
C
CD69+ Lymphocytes
D
MFI
BS
ES
24-72h
~1 Week
~1 Month
~3 Months
ES

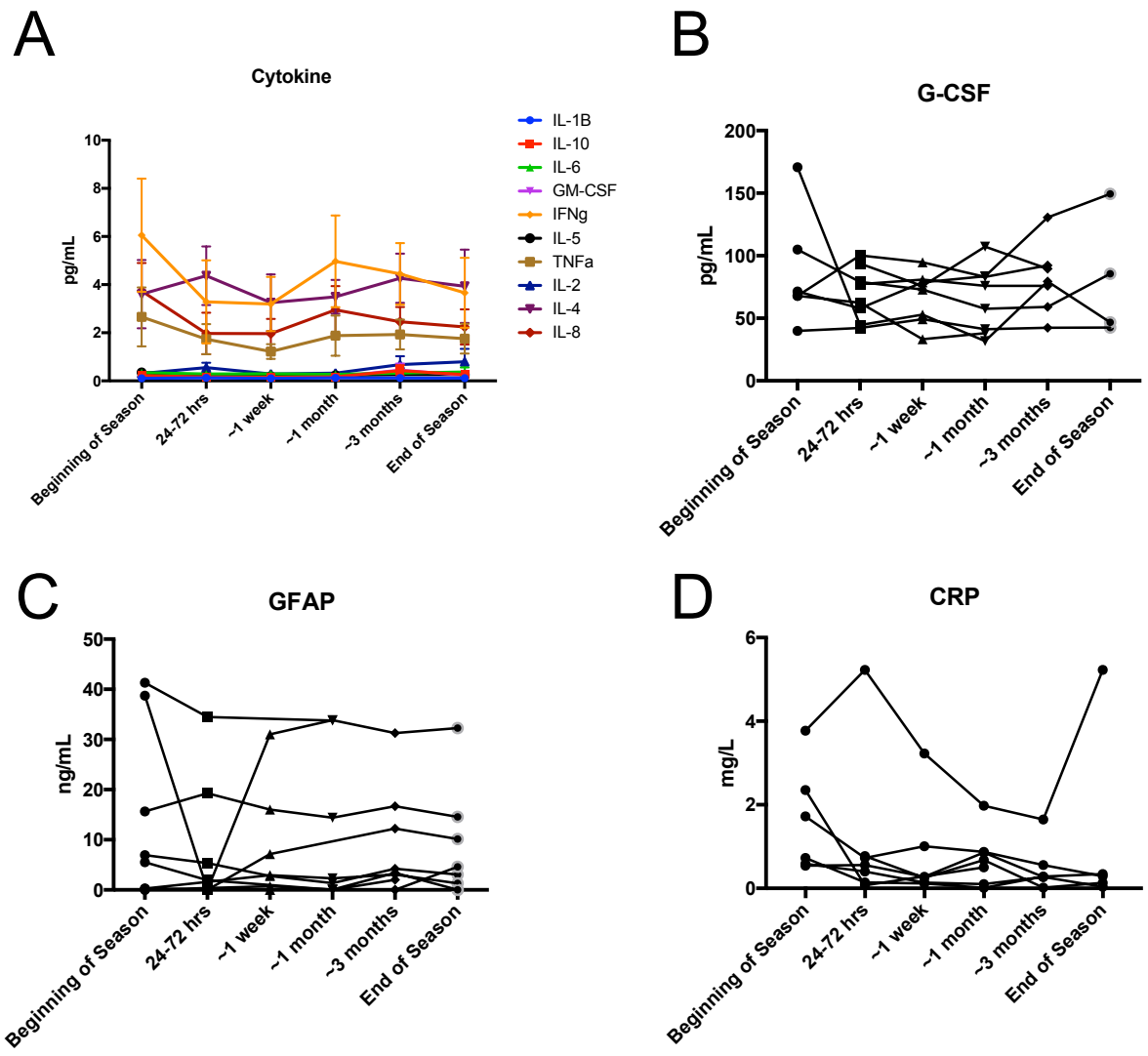
3.6 EXAMINATION OF ALTERATIONS OF INFLAMMATORY PROTEIN MARKERS AND MARKERS OF NEUROTRAUMA FOLLOWING CONCUSSION IN PERIPHERAL BLOOD PLASMA

Protein and protein fragments make up the majority of targets for concussion blood biomarkers studied to date.[21] Using plasma samples collected immunoassays were performed for proteins of interest. Due to the number of samples collected and the limited space available on immunoassay plates only a subset of players receiving a concussion and their collected time points were analyzed. A multiplex immunoassay for GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and TNF-α revealed no significant alterations for the concussion participants at any time point post-concussion compared to their uninjured baseline (n=7)(Figure 22A). Several cytokines included in the assay registered concentrations of around 0 pg/mL. A separate ELISA immunoassay was performed for the cytokine G-CSF, which also demonstrated no significant alterations between any post-concussion time points compared to pre-injury baseline (n=8) (Figure 22B).

GFAP is an intermediate filament protein expressed almost exclusively in astroglia, and has demonstrated signs of upregulation during glial injury and astrogliosis process of brain injury.[85] GFAP has been considered a valuable marker of study as it is neurotrauma-specific and elevated levels have been detected in the serum of patients with TBI.[34] My plasma GFAP immunoassay did not demonstrate any significant alterations from pre-injury baseline though (n=9) (Figure 22C). CRP is a general biomarker of systemic inflammation, increasing in instances of infection, trauma, surgery, burns, tissue infarction, advanced cancer, and chronic inflammatory conditions.[86] CRP has been suggested as a blood biomarker of concussion, and may provide additional insights to ongoing inflammatory responses.[86]An ELISA immunoassay

for CRP was performed, however no significant alterations were seen between post-concussion measurements and pre injury baseline (n=7) (Figure 22D).

Figure 22. No signature changes were detected in any of the plasma biomarkers analyzed for the concussed participants compared to their uninjured baseline test. Collected plasma samples were assayed for proteins of interest. A luminex immunoassay for cytokines (A) showed no significant differences for mTBI injured participants compared to their uninjured baseline (n=7), this was also the case for an ELISA immunoassay for G-CSF (B) (n=8). An ELISA immunoassay was performed for neurotrauma specific protein GFAP (C) (n=9) and acute phase protein CRP (D) (n=7) showed similar inconclusive results with no significant differences between concussed participants uninjured baseline and post-concussion results. Kruskal–Wallis ANOVA followed by Dunn's test.


A
Cytokine
pg/mL
IL-1B
IL-10
IL-6
GM-CSF
IFNg
IL-5
TNFa
IL-2
IL-4
IL-8
Beginning of Season
24-72 hrs
~1 week
~1 month
~3 months
End of Season
B
G-CSF
pg/mL
C
GFAP
ng/mL
D
CRP
mg/L

CHAPTER 4 – DISCUSSION

In this study, I have shown via a proof of principle mouse study that alterations in immune cell populations in peripheral circulation can be detected post-mTBI using flow cytometry. Expanding from this result, it was demonstrated that it was feasible to successfully recruit and perform an evaluation of both approved clinical concussion assessments and a discovery evaluation into potential inflammatory blood biomarkers of concussion longitudinally in a female athletic cohort. Clinically approved concussion assessments provided valuable symptom information post-concussion, but lacked diagnostic effectiveness in other areas. While fluid biomarker discovery for CNS injury has focused on protein and protein fragments thus far, my protein investigations into plasma based markers of inflammation and neurotrauma were inconclusive.[21] A cursory analysis of blood profiles from basic clinical haematology profile identified interesting changes post-concussion compared to a set baseline, as well as alterations in a non-concussed group. A more in-depth immune cell analysis via flow cytometry on frozen PBMC and whole blood leukocytes were performed which evaluated immune cell counts as well as functional information. This was the first study to my knowledge to use flow cytometry to quantify and track leukocyte subset frequencies post-concussion, and provided information for further evaluation.

4.1 THE INFLAMMATORY RESPONSE POST-TBI IN *lys*-EGFP-*ki* MOUSE MODEL

I have shown that mTBI in a mouse model initiates an inflammatory response producing significant alterations to circulating leukocyte populations. Post-TBI circulating blood neutrophil counts were immediately elevated, and remained so for 24 hours post-TBI. As neutrophils are the most acute responding cell this fits exactly with what is anticipated in experimental TBI.[87]

Splenic neutrophils peaked 2 hours after TBI and then returned to normal levels. In a similar CCI mouse TBI study, splenic neutrophil levels remained unchanged from control at 1 day post-TBI and onwards, which aligns exactly with what I have seen, but no comparison can be made to the response observed before 24 hours post-TBI as this was their earliest time point.[82] Neutrophil reservoirs exist in bone marrow, spleen, liver, and lungs, providing a pool of cells to flood into circulation when needed in an inflammatory response, this explains the immediate spike in neutrophil numbers seen post-TBI.[88]

The monocyte response was not as dramatic as anticipated post-TBI, however it still showed a dynamic quality. Both $Ly6C^+$ classical and $Ly6C^-$ non-classical population counts dipped immediately post-TBI, recovering by 8 hours for classical and by 72 hours for non-classical. Unlike in humans, where monocytes make up 10% of total blood leukocytes, monocytes only comprise 4% of total blood leukocytes in mice.[89] This considerably smaller proportion in mice could result in smaller population shifts in circulation to TBI. In a weight drop TBI mouse model using C57BL/6 mice, Schwulst *et al.* demonstrated a reduction in blood monocytes at 1 day post-TBI followed by an increase at 3 days post-TBI, although this increase was still below control levels.[90] This was true for both $Ly6C^+$ classical and $Ly6C^-$ non-classical population in their study. While this is directly comparable with the results I observed, the authors saw consistent monocyte level depression chronically, which I did not.[90] Non-classical monocytes, which have a unique patrolling phenotype involving crawling along blood vessel walls, have been shown to rapidly infiltrate infected tissue within an hour of infection.[89] Potential population dips acutely seen in my study post-TBI may be due to extravasation of non-classical cells into the injury site in the brain. However, blood monocyte count decreases for both

populations were matched with splenic count increases, which may also explain the movement of circulating cells.

In mice, Ly6C^{+} classical monocytes are found in circulation and at steady state in the spleen as a reservoir, however, Ly6C^{-} non-classical monocytes remain predominantly in the blood, rolling along the vasculature.[58] This was true for my uninjured control mice, as there were some non-classical monocytes in the spleen, but it was only about 15% of the amount of classical monocytes present. Unlike neutrophils, which have multiple marginated pools of cells, monocytes rely only on the spleen for storage; this may explain why we saw inversely fluctuating counts of cells between spleen and blood.[88,91]

There is little research to compare against for the circulating lymphocyte response post-TBI in mouse models. B cell infiltration into the brain was seen at 4 to 6 days post-TBI in a rat contusion model, while I saw a significant increase in circulating B cells at 7 days post-TBI in my study.[92] Without further immunohistochemistry of the injured brain tissue though, no confirmation can be made as to whether B cell increases were due to or a result of infiltration into the brain. T cell levels fluctuated up and down at 1, 3, 7, and 14 days post-TBI in a mouse CCI model, while I saw increased counts at 2 and 8 hours post-TBI, there was little change at 1 day and beyond.[82]

No animal model ideally replicates human disease, and this is especially true of TBI. While neuroprotective treatment of TBI has been successfully performed in animal models, none of these have been successfully translated to humans.[71] Few currently established mouse models of TBI reach appropriate criteria for properly modeling sports-related concussion.[71] While the fact that blood leukocyte populations are changing is encouraging for progression into a human

blood evaluation of circulating leukocytes post-concussion, direct relation between mouse and human studies may be challenging and may require methodological revision.

4.2 EVALUATING CURRENT CLINICAL ASSESSMENTS THE SCAT3 AND ImPACT

A majority of concussion studies in humans have focused on male participants, leaving the growing global population of females in sport largely understudied; yet, females have been reported to be at a higher risk for sports-related concussions than males.[10,93] While this difference may be attributed to hormonal and biomechanical differences between men and women, it is also suggested that women tend to be more honest about reporting symptoms and seeking medical attention.[93] This factor, if true, makes women an incredibly valuable and relevant cohort for evaluation in concussion research, as current methods for concussion identification and eventual return to work or play rely heavily on self-reporting. Over 4 seasons, there were 20 diagnosed concussions in my study population. The symptom evaluation as part of the SCAT3, demonstrated the women's rugby team cohort was honest about reporting symptoms and demonstrating that they have a definitive injury and it has a traceable, although subjective, recovery path post-concussion.

Participants with concussion reported significantly higher symptom number and severity at 24-72 hours and at ~1 week post-concussion compared to the non-concussed group, but this had returned to baseline levels by ~1 month post-concussion and remained there. This matches expectations reported in the literature, with 80-90% of individuals with a concussion requiring 7 to 10 days for symptom resolution.[94] It is important to state that this symptom checklist is not specific to concussion, resulting in many non-concussed individuals reporting some symptom level at baseline.[26] Neck pain, at a self-reported severity score from 1 to 6, was the highest

reported symptom at pre-season baseline in my study, reported 19 times in participants, followed by “fatigue or low energy” and “trouble falling asleep”, with 17 and 15 players reporting, respectively. It is conceivable that many of the symptoms listed could easily be driven by daily stresses and fatigue of university life with the addition of regular hard physical exertion associated with daily team practices and weekly games, which would be anticipated in this cohort. Studies attempting to predict which mTBI patients will go on to develop post-concussion syndrome using clinical symptoms have shown ineffectiveness, and currently do not influence clinical decisions.[95] Additionally, there were no deficits observed in any of the other SCAT3 assessments used between post-concussion and baseline. Both the cognitive portion, and the balance evaluation of the SCAT3 have demonstrated poor sensitivity and specificity for detecting mTBI in other studies.[96] Improved sensitivity and specificity can be seen in the cognitive portion of the SCAT3 as long as the test is performed within 12 hours post-concussion, but players did not receive their first assessment until 24-72 hours post-concussion, which hinders the effectiveness of this section.

Assessment of the ImPACT computerized concussion assessment was only able to demonstrate the improvement in score over time of repeated tests in one of its assessments, but no actual deficits in score due to a concussion. ImPACT Applications, Inc, the creators of the ImPACT test, suggest that the test be used within 24-72 hours post-concussion to begin an initial evaluation of a players concussion status.[97] ImPACT assessments in this study were typically only performed at 2 weeks or longer post-concussion at the direction of the team physician to prevent further exacerbation of player symptoms in the initial period following a concussion. This could explain why most test scores did not show significant differences between concussed and non-concussed participants as the concussed players were given enough time to recover

before their initial post-concussion ImPACT evaluation. The unreliable nature of these tests supports the need for better objective, biologically relevant, concussion assessment techniques.

4.3 INVESTIGATION OF BLOOD LEUKOCYTE RESPONSE POST-CONCUSSION

The blood analysis began simply with a routine haematology profile to obtain a CBC and differential, which provided a variety of measures for both red and white blood cell components. It is important to say that all but two tests showed blood factors within normal human ranges. Two tests as part of the beginning of season baseline had elevated leukocyte and neutrophil concentrations in the same player. The differences analysis performed in this study was limited by the availability and timing of concussion in my cohort. On several occasions, players were either unable to make a post-concussion follow up, or dropped out of the study before all time points could be collected. Additionally, some players had acquired a concussion just prior to their arrival for their initial BS baseline assessment during pre-season training. These players had to be handled differently as they did not have a BS baseline to make a judgment against. When studying a unique population such as a group of collegiate athletes, it can be challenging to establish population reference intervals that are appropriate for biomarker interpretation. Inter-individual variation can be considerable for the number of circulating leukocytes as well as soluble protein markers.[40] Currently, it was felt that it was most valuable to evaluate players against their own set baseline for each variable collected for this reason. It is anticipated that upon collection of enough data relevant baseline levels can be determined to help analyze players in the situation of not having full pre-injury baseline evaluation going forward.

From the RBC factors collected as part of the CBC several alterations were observed. Total RBC concentrations, based on my differences evaluation, were significantly increased

from baseline (set arbitrarily to 0) post-concussion at all time points. This was also true for haemoglobin and hematocrit, with the highest mean difference at ~1 week post-concussion. Linking this change to head trauma is quite challenging though. In individuals with severe TBI, anemia brought on by low haemoglobin levels can lead to greater complications.[98] While still conflicting, blood transfusions have shown to improve TBI outcome by increasing oxygen delivery and brain tissue oxygenation. It is conceivable that increased haemoglobin post-concussion could be part of a compensatory mechanism, with the addition of functional MRI analysis, which relies on the magnetic properties of haemoglobin to make a measurement, this could be better evaluated in my study subjects.[99] It seems more likely that it is not so much concussion causing an increase in RBC post-injury, but rather player circumstances causing a decrease in RBC at BS baseline, since the non-concussed groups ES difference is also elevated from baseline.

Thrombocyte levels increased significantly from the set baseline (0) at 24-72 hours post-concussion, but by ~1 week post-concussion this level had dropped. This acute increase and drop may be explained by endothelial damage to brain vasculature.[100] In a study by Schwarzmaier *et al.* vascular diameter was seen to increase post-TBI in a CCI mouse model; however, cerebral blood flow was decreased in the brain.[101] On inspection, a microthrombi had formed on the cerebrovascular endothelium containing leukocytes and platelets, and this reduced blood flow. While it is possible that circulating thrombocytes may be playing a role in microthrombi formation causing a resulting upregulation of thrombocyte formation, this would be very hard to prove going forward in a concussion model. In this study, the authors did not measure circulating thrombocytes, and no measurements were performed more than 2 hours after insult.[101] There were also significant increases in thrombocytes from baseline (0) at ES for both the concussed

and non-concussed group in my study, which further complicates this understanding. One study reported a drop in mean platelet volume after mild head trauma; however, I was unable to see a similar result from my blood analysis after concussion.[102]

Total leukocyte concentration, as part of the differences analysis from values collected in the CBC, showed a significant increase from baseline (0) at ~3 months post-concussion. This was also true for neutrophil concentrations, which is logical as they are the most abundant leukocyte and any increase in its population will largely drive up total leukocyte count. In a study of white blood cell counts from mild, moderate, and severe TBI patients, Rovlias and Kotsou noticed a significant increase in white blood cell counts severe TBI compared to mild and moderate TBI, but there was no comparison to an uninjured control so no increase in the mTBI population could be determined.[103]

Although neutrophils could not be analyzed as part of my frozen PBMC evaluation by flow cytometry, they were included in my whole blood flow cytometry experiments as part of the fourth season of study. Due to its ability to rapidly evaluate immune cells for phenotype and function, flow cytometry has been suggested as a valuable tool in advancing the understanding of concussion pathology and etiology.[25] Whole blood flow cytometry analysis also demonstrated the highest neutrophil counts at ~3 months post-concussion, this was only slightly higher than the non-concussed groups ES count. This delayed response to concussion is unexpected though as neutrophils are one of the most acute responding cells in an inflammatory response. What exactly caused this consistently observed delayed response will require much further investigation.

Using neutrophil levels calculated via a haematology profile with CBC of human blood, Petrone *et al.* observed higher than normal neutrophil percentages at 0 to 6 and 24 hours post-

mTBI, with neutrophils returning to normal ranges by 48 hours post-mTBI.[104] From histopathological analysis of post-mortem brains, it appears that neutrophils infiltrate only at the earliest stages after injury in small numbers.[105] My mouse evaluation even showed greatest blood neutrophil counts at 2, 8, and 24 hours post-TBI, returning to normal by 72 hours. It is possible that I didn't see acute rises in neutrophil counts post-concussion because the set initial human blood draw was too late to properly detect an earlier response. Twenty four to 72 hours was the selected initial blood draw as it allowed participants more time to report to clinicians, especially if players were traveling back from away games, or had delayed symptom onset after concussion and reported late. Future study data may be improved by a more stringent time line of early blood collection, sampling both within the first 8 to 24 hours, which should hopefully better identify neutrophil responses, as well as 72 hours post-concussion, which may improve monocyte response details. This would require a very engaged and responsive cohort that may be hard to achieve with university athletes, rather requiring a close evaluation of a professional team where players are more closely managed.

Based on the success in a concurrent concussion study in paediatric boys hockey players, $CD16^{+}$ neutrophils were subdivided based on CD15 expression into low ($CD15^{-}$), middle ($CD15^{+}$), and high ($CD15^{++}$) populations. CD15, also known as Lewis X, is a mature human neutrophil surface marker, and is believed to play a role in key neutrophil functions such as cell-cell interactions, phagocytosis, stimulation of degranulation, and respiratory burst, although more research is needed.[106] An upregualtion of CD15 has been connected to alterations to immune function due to interactions with dendritic cells.[107] While an increased shift in CD15 surface expression was seen 24 to 72 hours post-concession in the paediatric boys' hockey players (G.A. Dekaban, manuscript in preparation), I was unable to detect the same shift on analysis from the

rugby cohort. This could be a timing of collection issue, but it also could be a demonstration of how results cannot be generalized across very different participant demographics. At this point, my sample size for the whole blood flow evaluation is very small, and interpretation may not become clear until greater numbers are recruited.

While monocyte concentrations obtained from haematology profile with a CBC and differential did show a change, it was only between beginning and end of season in the baseline population. Limited understanding can be gained from the monocyte count as part of the CBC as it does not allow separate analysis of monocyte subpopulations as can be performed using flow cytometry. From the total monocyte analysis acquired by flow cytometry from my frozen PBMC samples, I saw consistency with the haematology profiles collected in that there were population decreases from BS to ES in the non-concussed group and a significant decrease in total monocytes for the concussed group. From analyzing the monocyte subsets there were significant decreases in intermediate, and non-classical monocytes at ES post-concussion compared to baseline (0). The absolute monocyte counts did increase post-concussion compared to baseline (0), with the highest counts coming for classical and non-classical monocytes at 24-72 hours and ~1 week post-concussion, while it was the ~1 week and ~1 month time point for intermediate monocytes. However, there was no significant change over this time period or the activation of those monocytes based on CD69 expression from my frozen PBMC analysis. Analysis of frozen PBMC samples was made challenging due to the variable viability of cells after the thawing and staining process, this was especially true for monocytes.

The introduction of a whole blood analysis by flow cytometry removed the viability issue in samples. It also allowed for a more instantaneous analysis of samples as opposed to before when all frozen PBMC samples from a player were analyzed together after all time points had

been collected and frozen down. Although I could not use the differences analysis that had been used before due to the reduced number of baselines and therefore reduced odds of a player having a baseline post-concussion, I was still able to demonstrate leukocyte population changes post-concussion. There was a steady increase in classical monocytes reaching significance at ~3 months post-concussion. Like the increase in neutrophils at ~3 months post-concussion, this late increase is an unexpected result. Unlike with the CBC or frozen PBMC monocyte analysis, the ES counts for whole blood monocytes were higher than at BS baseline, but both the classical and intermediate subsets had significantly reduced CD69 MFI, suggesting a reduced state of activation of those cells, despite being at an increased count. Upon comparison of the subset proportions for monocytes there was an expansion of the intermediate subset at 24-72 hours post-concussion, before returning to levels similar with baseline.

Circulating monocytes have been reported to increase post-severe TBI, specifically classical and intermediate populations, within the first 48 hours.[57,108] While aspects of these findings do match my results, the magnitude of the injury in this study, being an mTBI, was much smaller which may explain the smaller or lack of cell population changes. Currently, there is little research on monocyte subsets, and how these individual subsets are involved in either a pathogenic or protective role in inflammation in general.[46] The expansion of intermediate and non-classical monocyte populations has been seen in various inflammatory conditions, often of a chronic nature.[46,109] In a mouse model of TBI, Ly6C$^-$ non-classical monocytes only became the predominant blood monocyte population at 60 days post-TBI.[90] While purely speculative, changes in proportions of monocyte subset populations may only become visible if an individual progresses towards a neurodegenerative state much later in life, and not within the time frame of this short study in young women. There is scarce research currently evaluating monocyte subset

population changes post-TBI, and my concussion analysis is a first in providing such detailed peripheral blood cell lineage analysis.[41] The combined analysis of these three monocyte populations is encouraging for future biomarker panel consideration.

The lymphocyte response to TBI is the much more poorly understood in comparison to the myeloid cells. I observed a significant increase from baseline (0) in total lymphocytes at ES for my non-concussed group, with increases in concentration at all time points-post concussion. As the mean increases were fairly similar across all time points it appears as if the BS, which was compared against, may have been abnormally low. Moving to my frozen PBMC evaluation, a significant increase was seen from baseline (0) for the CD4 T cells at ES for the non-concussed group, matched with increases at ES in all subsets in the non-concussed group explaining the total lymphocyte count seen. The exact opposite effect was seen for the non-concussed group with mean differences all below 0, none of which were significant though. The pooled $CD3^{+}CD19^{+}$ lymphocyte as part of the whole blood flow cytometry analysis also demonstrated increased counts at ES for the non-concussed group, but the post-concussion time point counts were unaltered. In a study by Petrone *et al.* looking at lymphocyte percentages, as determined from CBC, were below the normal range at 0 to 6 and 24 hours post-mTBI, but had recovered to normal levels by 48 hours post-mTBI.[104]

NK cells had a significant increase at ~1 week post-concussion, which was a steep peak compared to the relatively unchanging counts at other post-concussion time points and at ES for the non-concussed group. In a study by Kong *et al.* circulating NK cell % was diminished at 1 and 3 days post-mTBI, but at 7 days had risen above control levels, matching patterns seen in my study.[62] When split into $CD56^{DIM}$ and $CD56^{BRIGHT}$ there was a specific depletion seen in the

$CD56^{DIM}$ population after TBI, providing an interesting further evaluation for collected NK cells in the future of this study.[60]

To this point in data evaluation, I have relied on individual statistical assessment of separate biomarkers. A more robust machine learning methodology to identify more elaborate patterns and changes in inflammatory biomarker will likely provide more fruitful interpretation of the data collected.[104] This is a direction that will be taken going forward with both the current data and any future data collected.

A potential limitation of this blood analysis is that a natural increase in peripheral leukocyte concentration, specifically monocytes and neutrophils, but not lymphocytes, has been reported up to 4 hours after exercise in females compared to their pre-exercise values.[110] Exercise additionally can affect various RBC related factors measured by the CBC. Exercise can induce increased erythrocyte destruction, which would in turn result in decreased levels for erythrocytes, haemoglobin, and hematocrit as is seen at beginning of season.[111] This exercise effect in the strenuous tryout period during beginning of season baseline testing may have driven some of the results I have seen by encouraging lower RBC concentrations and increased leukocyte concentrations which all comparisons were then made against. This is an important discovery though, as influence of continuous strenuous exercise will be an important element in identifying and developing blood-biomarkers of concussion moving forward. An example of this can be seen in the measurement of serum levels of S100B, which has been shown to be predicative of mild TBI when increased, but can also be elevated strictly due to physical activity without head trauma.[69,112] Beyond the acute effects of exercise on the immune system, research has demonstrated that regular physical activity, reducing visceral fat, stimulates an anti-inflammatory environment.[113,114] In a study on circulating leukocytes it was demonstrated that

regular exercises lowered the number of circulating leukocytes and this effect was majorly driven by a reduction in neutrophils.[115]

Sex differences may also influence blood-biomarker discovery in athletes. Fluctuation of circulating leukocyte populations is induced at the menstrual phase and secretary phases of the menstrual cycle, with reported increases in total leukocyte count, which was mainly attributable to neutrophil percentage.[116] This was not controlled for in the current study, and will also be an unavoidable factor in blood-biomarker development for concussion identification in female athletes. Overall, adult females are reported to mount stronger innate and adaptive immune responses compared to males.[117] This would suggest that any concussion driven inflammatory changes would be more visible in a female cohort compared to males. Further evaluation of similar markers of inflammation post-concussion in male and females will be required to establish if this holds true. Animal experiments have consistently demonstrated improved survival and cognitive function in females post-TBI compared to males, suggesting gonadal steroids progesterone and oestrogen may have neuroprotective functions.[118] Human studies consistently suggest the opposite, with females showing worse outcomes post-TBI compared to males, driving a belief that experimentally induced TBI in animal models does not disrupt the gonadal hormone stimulating anterior pituitary gland in the CNS, but this is disrupted in human TBI leaving females in a state of withdrawal of oestrogen and/or progesterone.[118] More work will need to be performed in this area.

It is unclear if using players with a past history of concussion or just general exposure to head trauma from collision sports may influence blood biomarkers. Plasma tau levels were increased in males who have played a contact sport compared to non-collision sport athletes.[119] Female athletes with a history of past concussions had elevated plasma levels of monocyte

chemoattractant protein-1 compared to a healthy control.[119] However, recruiting university athletes with no history of concussion is also incredibly challenging especially at the university level where players have likely been exposed to head impacts throughout their playing career from a younger age. Selecting only for individuals with absolutely no history of head trauma though would likely reduce participants for enrolment. It could also limit the potential understanding of biomarkers evaluated as they are no longer representative of the true population they clinically will be applied to.[120] Additionally, drug and alcohol abuse and the use of steroids that often go unreported can influence study results while also contributing long-term to health problems as well as CNS disorders.[71] Even supplement use such as branched chain amino acids have been demonstrated to influence outcomes post-TBI in a mouse model.[121] The effect of co-existing factors such as these are what make animal models so valuable as these can be controlled for.[71]

4.4 INCONCLUSIVE PROTEIN ALTERATION POST-CONCUSSION

As the evaluation of protein biomarkers make up the majority of blood biomarker research in concussion, and plasma samples were easily attainable within the framework of this study, it was a valuable opportunity to assess important inflammatory and neurotrauma protein markers used in the literature. However, I was unable to demonstrate clear changes between players' pre-concussion and post-concussion protein levels. It should be stated though that I only investigated a small subset of the total population via protein assay.

Cytokine evaluation, both by a multiplex immunoassay for various cytokines, and for G-CSF as a separate ELISA showed no obvious differences between post-concussion samples and pre-injury baseline in the subset of players analyzed. Changes in cytokine levels have been

successfully seen in both mouse models of mTBI and human models of severe TBI. Using a similar multiplex immunoassay for cytokines, Yang *et al.* showed significant elevation of serum IL-6 among other cytokines in a mTBI plus hypoxia mouse model.[122] Analyzing the hippocampus and cortex after a mild fluid percussion TBI in BALB/c mice, Fenn *et al.* saw transient increases in IL-1β, TNFα, within 4 hours after injury.[123] Il-1β has been shown to rapidly increase hours after injury in both human and rat TBI studies.[33] TNFα levels also have shown acute increases in human TBI, peaking between 3 and 8 hours, but are reported to return to normal at 24 hours after injury.[33] However, little success has been seen in human concussion studies. Comparing plasma cytokine levels post-TBI between severe and moderate/mild brain injury patients, severely injured patients had significantly higher IL-6 and TNFα from 6 hours to 2 weeks after injury.[57] Looking at a plasma samples from a cohort of athletes with a previous history of concussion, although currently healthy, no differences were seen in inflammatory markers such as IL-1β, IL-6 and IL-10, between contact and non-contact athletes, and many markers measured did not reach the level of detection.[119] Improved results may be obtained by acquiring more acute blood draws, or through development of a more sensitive assay technique. More work will need to be conducted in this area.

The acute phase protein CRP has been a widely investigated biomarker of general inflammation, therefore it seemed like a relevant marker to investigate after concussion in the women's rugby cohort.[124] I was unable to determine any changes though between post-concussion and pre-concussion values. Increased circulating CRP has been reported to be a result of IL-6 release into circulation.[40] As I saw no alterations in IL-6 in plasma samples this could explain this. Regular exercise has the effect of decreasing circulating IL-6 concentrations; this results in subsequently lower CRP levels, which could play a factor in my cohort.[125]

Sources of inflammatory markers, as well as targets of trafficking leukocytes are extremely hard to distinguish as blood comes into contact with all tissues and organs and represents such a general process in humans.[64] Evaluating cranial and non-cranial sources of biomarkers may be incredibly challenging and will require the assistance of additional neural specific markers. GFAP has been a highly researched potential blood biomarker for TBI as it was supposedly neural specific, and immunoreactivity to GFAP has been evaluated as an indicator of brain injury in experimental mTBI models.[64,126] My assay for GFAP produced no noticeable differences in protein levels before and after concussion. Currently, only a few studies have focused on GFAP specifically for mTBI. In one study, where plasma GFAP levels were assessed, it out performed S100B in predicting the outcome in mTBI. However, the authors still considered GFAP to be a weak indicator.[64,127] This may be due to the fact that GFAP has been found detectable in non-CNS cells such as Schwann cells, chondrocytes, and lymphocytes.[128] This may explain why measurable GFAP levels were detected even before the concussion had taken place in some players. In a recent study comparing serum GFAP levels, higher concentrations were seen in individuals with orthopaedic injury compared to individuals with mTBI.[128] Further evaluation of this marker will need to be performed going forward.

Blood plasma was used for all immunoassays performed; however, serum is the predominantly used biofluid for marker evaluation.[64] During the clotting process to obtain serum, coagulating cells may release proteins into solution not previously there, while fibrinogen and other proteins can be removed from solution. [64] This is important as pro-inflammatory cytokines may be released by platelets during clotting, confounding measurements seen in blood before clotting.[129] It is currently unclear if one is preferable to the other for biomarker detection,

although it is clear that uniformity may not be seen in individual markers between the two fluids.[64]

It is conceivable that it is not a knowledge gap that needs to be bridged to develop a better blood-biomarker for concussion, but a technological one. The current limits of detection for most standard ELISAs and ELISA-like technologies are around 10 to 100 pg/ml.[21] Due to the amount of dilution of brain specific proteins in peripheral blood circulation, more sensitive detection methods will be required. New ultrasensitive techniques, such as Erenna and Simoa, should help bridge this gap by providing femtomolar range detection capabilities.[21,130] Additional advances in point of care and rapid detection have allowed for sideline readouts of blood-biomarker data. A recently developed smartphone-enabled optofluidic platform demonstrated ability to rapidly detect exosomes containing the concussion biomarker $GluR2^{+}$ in a murine model of concussion.[13]

4.5 SIGNIFICANCE AND CONCLUSIONS

In conclusion, my concurrent investigation using a mouse model of mTBI has been established and provided the groundwork for determining changes in leukocyte subsets post-TBI and provides a framework for contextualizing future blood changes in human studies. Future work will be taken to characterize leukocyte infiltration into the brain after injury in this mouse model. My human study evaluated a novel method to identify and track concussive injury by assessing leukocyte response via flow cytometry. This is the first study to my knowledge that has used this approach and included evaluations such as monocyte subsets. The evaluation of responding leukocytes was far more successful for identifying changes post-concussion than the more commonly used protein immunoassay method I used to investigate inflammatory protein

markers as well as GFAP, which came back fully inconclusive. From approved clinical concussion assessment, the SCAT3 and ImPACT, valid symptom assessments provided important information, but the lack of other useful tests highlights the need for better blood-biomarker development.

While a clear signature constellation of biomarkers will still require further work, I have made important steps forward to obtaining this goal. The main focus of a pilot investigation is not statistical significance, but rather feasibility, and it was demonstrated we could successfully recruit and perform blood analysis longitudinally for a large portion of a highly competitive athletic team.[131] It is likely this study was underpowered due to limitations in recruitment and concussion samples obtained. It is important to state that "no evidence of effect" is not "evidence of no effect". Based on the lessons learned throughout this project vast improvement in sample collection and procedural techniques were achieved, and new ideas for an improved methodology can be implemented to future investigations.

Advances are being made on multiple fronts for the identification of a marker that more accurately identifies concussion. Using head impact telemetry systems, the application of forces, both translational and rotational, can be established and have been in various studies in various sports as a way to help recognize concussion.[71] While the output force values for head impacts can be instantaneously recorded and reported, it currently has not been able to predict concussion.[71] More advanced clinical imaging systems are currently being developed including diffusion tensor MRI, which is capable of identifying damage to white matter tracts.[64] Yet, despite these advances and improvements in MRI techniques, no clinical guidelines currently exist for the diagnosis of concussion using MRI.[64] Using metabolomics profiling, a signature collection of markers were obtained accurately separating concussed from non-concussed

samples in a study using adolescent male hockey players, although this was a relatively small sample size and may not prove generalizable to a larger population.[132] This clearly demonstrates that all approaches have limitations, and no one technique is a clear solution for this problem. However, in combination my data will help to provide a more comprehensive understanding of concussion along with these other metrics that can contribute to the field as a whole.

The evaluation process for biomarkers is an ever-developing topic, and any selected inflammatory markers from this study would be subjected to this process. The Institutes of Medicine, now The National Academy of Medicine (Washington, DC), recently laid out some general concepts for the evaluation process, providing three steps. The first is analytical validation; referring to the limits of detection, reference ranges, and reproducibility of the assay used.[40,133] Sensitivity and specificity are the quantitative values necessary to determine the diagnostic value of a biomarker.[17] Sensitivity denotes the number of tests predicted to be positive that are actually present, or true positives. Specificity denotes the number of tests predicted to be false that are correctly false, or true negatives.[17] Second is qualification; referring to the relationship of the biomarker and disease state based on available evidence, with data demonstrating effects of intervention on biomarker and disease outcome.[133] Third is utilization; referring to the sufficient support from the last two steps on the specific use designed for this biomarker.[133]

It is important to not generalize the results of inflammatory response for all CNS injuries, as research has shown the inflammatory response to SCI is far greater than it is in TBI.[134] Increased infiltration of macrophages, neutrophils, T cells, and B cells, along with higher activation of astrocytes and microglia has been observed post-SCI compared to TBI.[134] This could potentially be explained by the fact that the brain is 30-40x larger in mass than the spinal

cord, and despite standardizing for injury severity, the amount of damage to intact tissue will be greater in the spinal cord.[134] Increased permeability of the blood spinal cord barrier compared to BBB may also influence this.[134]

The growing acknowledgment of concussion as a serious health threat has already started to result in noticeable changes in athletes and sport. In a study on professional boxers in the United Kingdom and Australia, when analyzed from 1930 to 2003, it was discovered that professional careers dropped on average from 19 years to 5 years, and the number of professional fights from 336 bouts to 13 bouts.[135] Medical oversight has increased dramatically in recent years in American football, in addition to rule changes to make the game safer.[7] The adoption of a concussion protocol is currently a must in almost every sporting profession. The idea that a "ding" or "seeing stars" is something to be toughed out with an immediate return to play is all but expected to become an out-dated notion. From 1997 to 2008, there was an increase of about 16% a year in the number of reported concussions in high school athletes in one prospective study.[136] It is unlikely this is due purely to increase in the number of concussions, but rather an increased attention to the injury encouraging players and coaches to properly report this injury. As reporting increases, this puts more emphasis on the development of a better diagnostic for concussion, which an objective blood-based marker would ideally be able to meet.

Using a panel of markers that identifies temporally different responding elements within the cascade of post-concussion events, a better return to play/work guideline could be put in place.[64] This should encourage a better understanding of both a player's concussion history and recovery paths, and how it changes over time so as to properly inform clinicians as to how to handle concussions on an individual basis. An ability to detect at the earliest stages an athlete progressing towards CTE would allow for immediate intervention to be made into that athlete's

life to prevent subsequent exposure as well as provide an opportunity to test new therapeutics and therapies.[21] It is conceivable that blood biomarkers can develop to a point that this would become a possibility. The progressively evolving cascade of the secondary injury may be preventable or at least controllable, such that only beneficial elements are permitted. This has been described as the therapeutic window.[32] A successful biomarker candidate may have value in determining the effectiveness of any future concussion therapeutic or treatment.[9] A better understanding of the peripheral response to concussion may be able to guide future therapeutic research.

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Appendix

Appendix 1. Animal ethics approval 2007-104-12 letter.

Western

2007-104-12::5:

AUP Number: 2007-104-12
AUP Title: Experimental Spinal Cord Injury

Yearly Renewal Date: 06/01/2013

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2007-104-12 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee

The University *of* Western Ontario
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, ● London, Ontario ● CANADA – N6A 5C1
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Appendix 2. Animal ethics approval 2016-019 letter.

Western

AUP Number: 2016-019
PI Name: Dekaban, Gregory A
AUP Title: Experimental Cns Injury

Approval Date: 10/28/2016

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Experimental Cns Injury

" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2016-019::1

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care

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PH: 519-661-2111 ext. 86768 ● FL 519-661-2028
Email: auspc@uwo.ca ● http://www.uwo.ca/animal/website/

Appendix 3. Human ethics approval HSREB 102857 letter.

Western Research

Research Ethics

Western University Health Science Research Ethics Board
HSREB Annual Continuing Ethics Approval Notice

Date: July 18, 2016
Principal Investigator: Dr. Arthur Brown
Department & Institution: Schulich School of Medicine and Dentistry\Anatomy & Cell Biology,Robarts Research Institute

Review Type: Delegated
HSREB File Number: 102857
Study Title: London Brain Injury Consortium: A Longitudinal Patient Centered Program to Uncover the Cellular and Molecular Determinants of Neurological Outcomes after Brain Injury.

HSREB Renewal Due Date & HSREB Expiry Date:
Renewal Due -2017/07/31
Expiry Date -2017/08/10

The Western University Health Science Research Ethics Board (HSREB) has reviewed the Continuing Ethics Review (CER) Form and is re-issuing approval for the above noted study.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice (ICH E6 R1), the Ontario Freedom of Information and Protection of Privacy Act (FIPPA, 1990), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

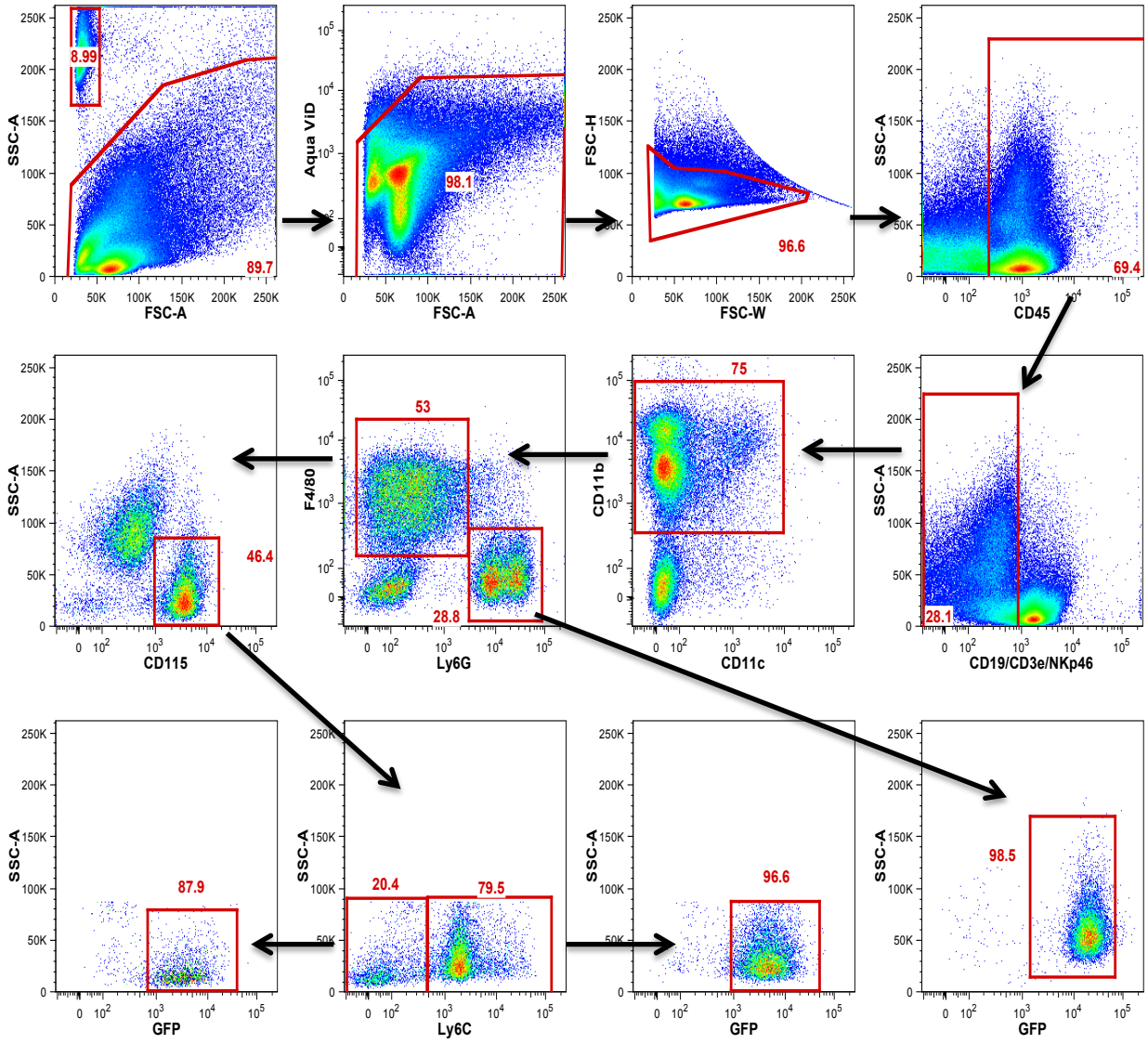
Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

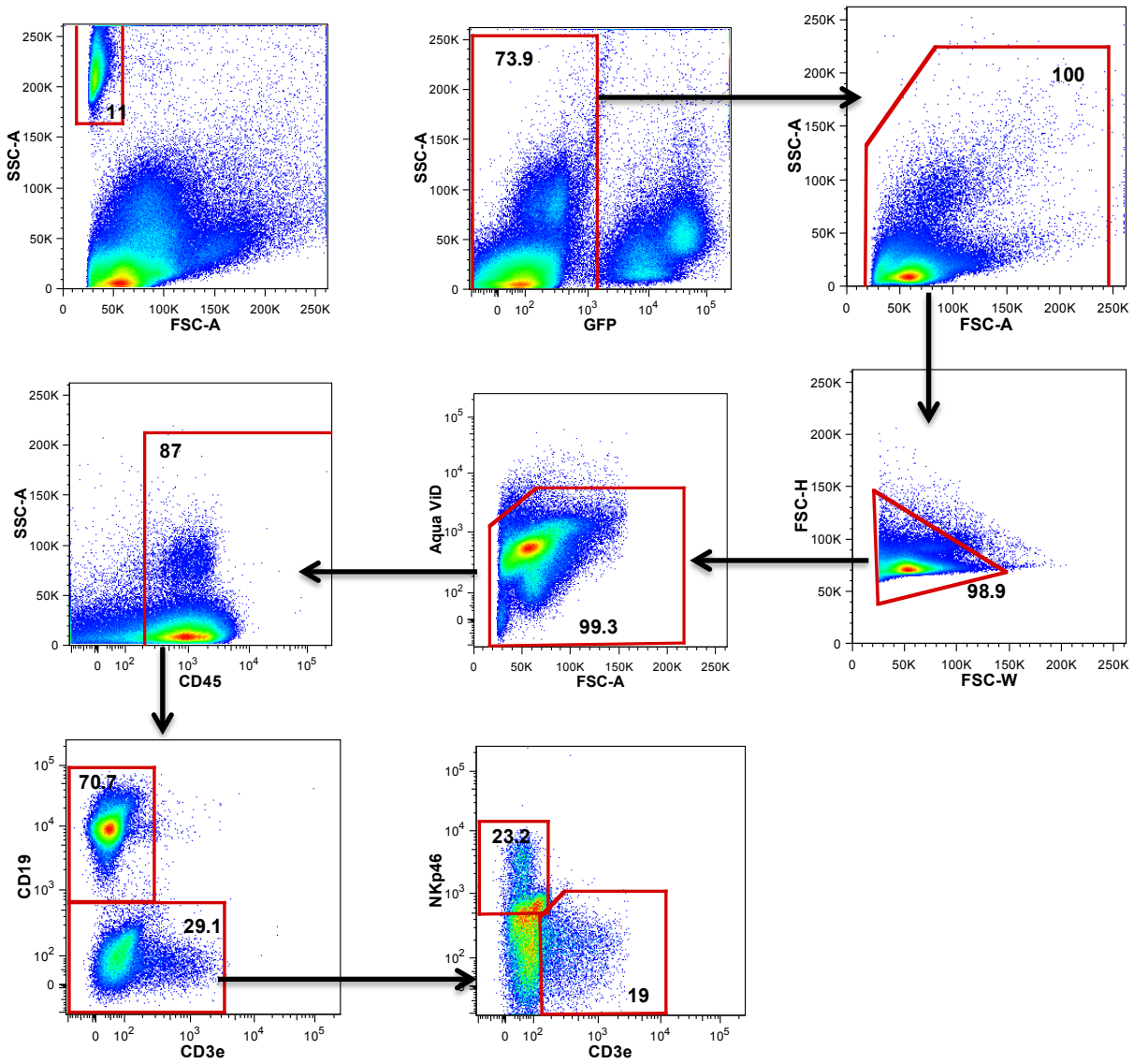
Ethics Officer, on behalf of Dr. Joseph Gilbert, HSREB Chair

Ethics Officer: Erika Basile ___ Katelyn Harris___ Nicole Kaniki___ Grace Kelly ✓ Vikki Tran ___ Karen Gopaul ___

Appendix 4. Optimized gating strategy used to identify myeloid lineage cells in *lys*-EGFP-*ki* mouse model by flow cytometry. Blood and spleen samples collected were analyzed by flow cytometry, first separating the counting beads from the cell scatter, from which viability, singlets were selected. Myeloid cells were selected as $CD45^{+}CD11b^{+}$, and a dump channel containing CD19 CD3ε and NKp46 was used to remove any cells with those surface markers. From there neutrophils were selected as $Ly6G^{+}EGFP^{+}$, classical monocytes as $CD115^{+}Ly6C^{+}EGFP^{+}$, and non-classical monocytes as $CD115^{+}Ly6C^{-}EGFP^{+}$. Representative dot plots are shown.

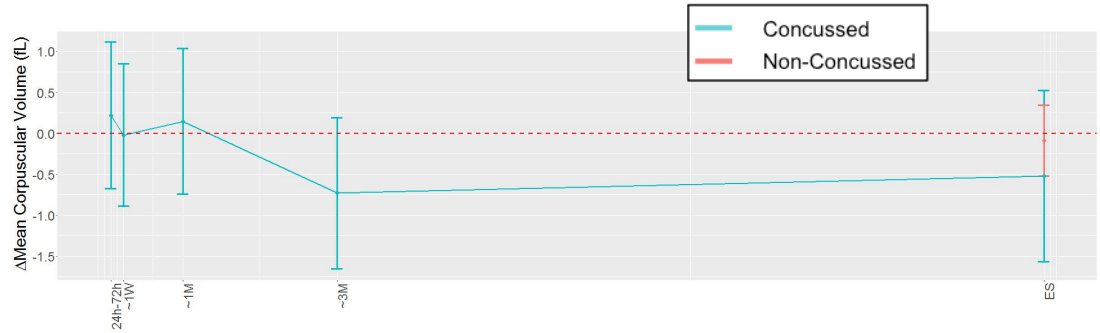

SSC-A
FSC-A
8.99
89.7
Aqua ViD
98.1
FSC-H
FSC-W
96.6
CD45
69.4
CD19/CD3e/NKp46
28.1
CD11b
CD11c
75
F4/80
Ly6G
53
28.8
CD115
46.4
GFP
87.9
Ly6C
20.4
79.5
96.6
98.5

Appendix 5. Optimized gating strategy used to identify lymphoid lineage cells in *lys*-EGFP-*ki* mouse model by flow cytometry. Blood and spleen samples collected were analyzed by flow cytometry, first separating the counting beads from the cell scatter, then gating on $EGFP^-$ cells as the lymphocytes will form this group. Singlets, viability, and then $CD45^+$ cells were selected. CD19 CD3ε and NKp46 was used to remove any cells with those surface markers. From there B cells were selected as $CD19^+$, T cells $CD3\varepsilon^+$, and NK cells as $NKp46^+$. Representative dot plots are shown.

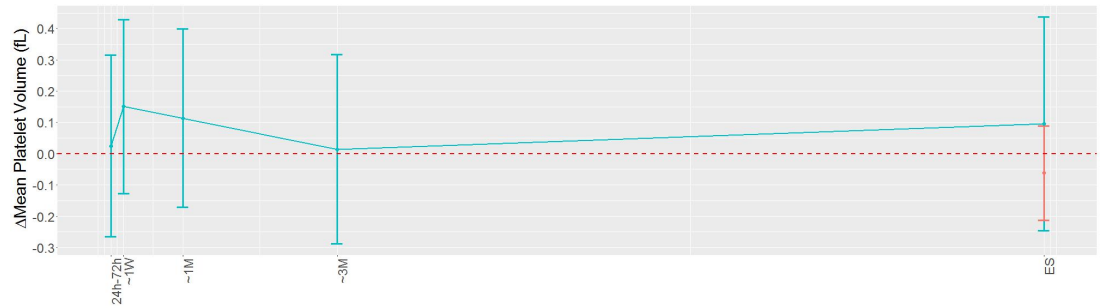

11
SSC-A
FSC-A
73.9
GFP
100
98.9
FSC-H
FSC-W
99.3
Aqua ViD
87
CD45
70.7
29.1
CD19
CD3e
23.2
19
NKp46

Appendix 6. Time plots for CBC differences analysis. From collected CBC, differences evaluations were undertaken to allow more individualistic changes to be represented for values. All players collected marker values were subtracted from their own baseline vale and a comparison to the set baseline (0) was made. The plots above are as follows; mean corpuscular volume (A), mean platelet volume (B), eosinophils (C), and basophils (D). Non-Concussed differences (n=66) Concussion differences; 24-72 hours (n=14), ~1 week (n=15), ~1 month (n=14), ~3 months (n=13), and ES (n=9).

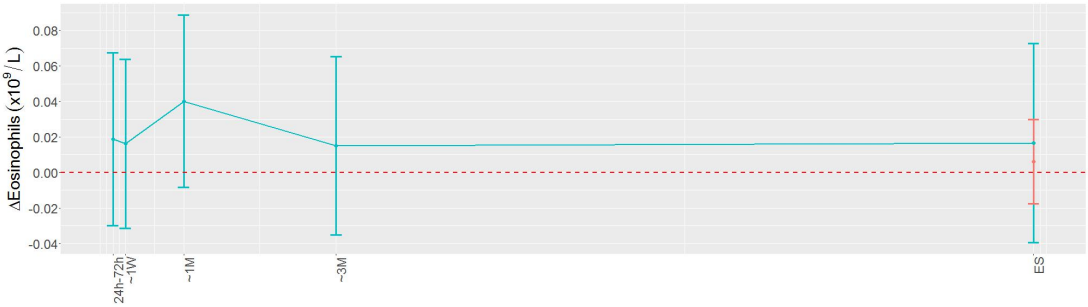
A


Concussed
Non-Concussed
ΔMean Corpuscular Volume (fL)
1.0
0.5
0.0
-0.5
-1.0
-1.5
24h-72h
~1W
~1M
~3M
ES

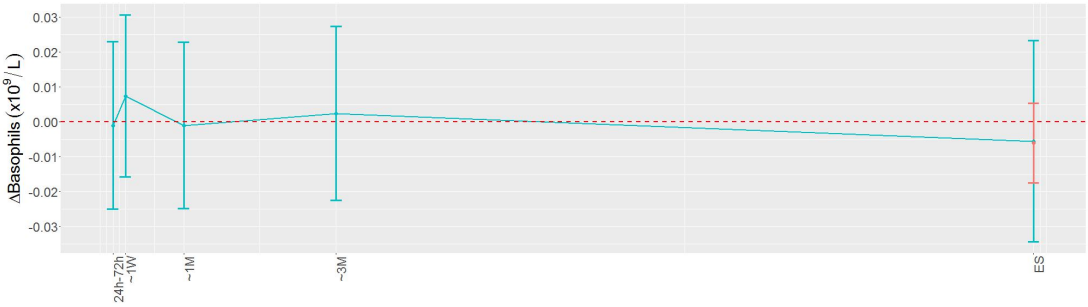
B


ΔMean Platelet Volume (fL)
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
24h-72h
~1W
~1M
~3M
ES

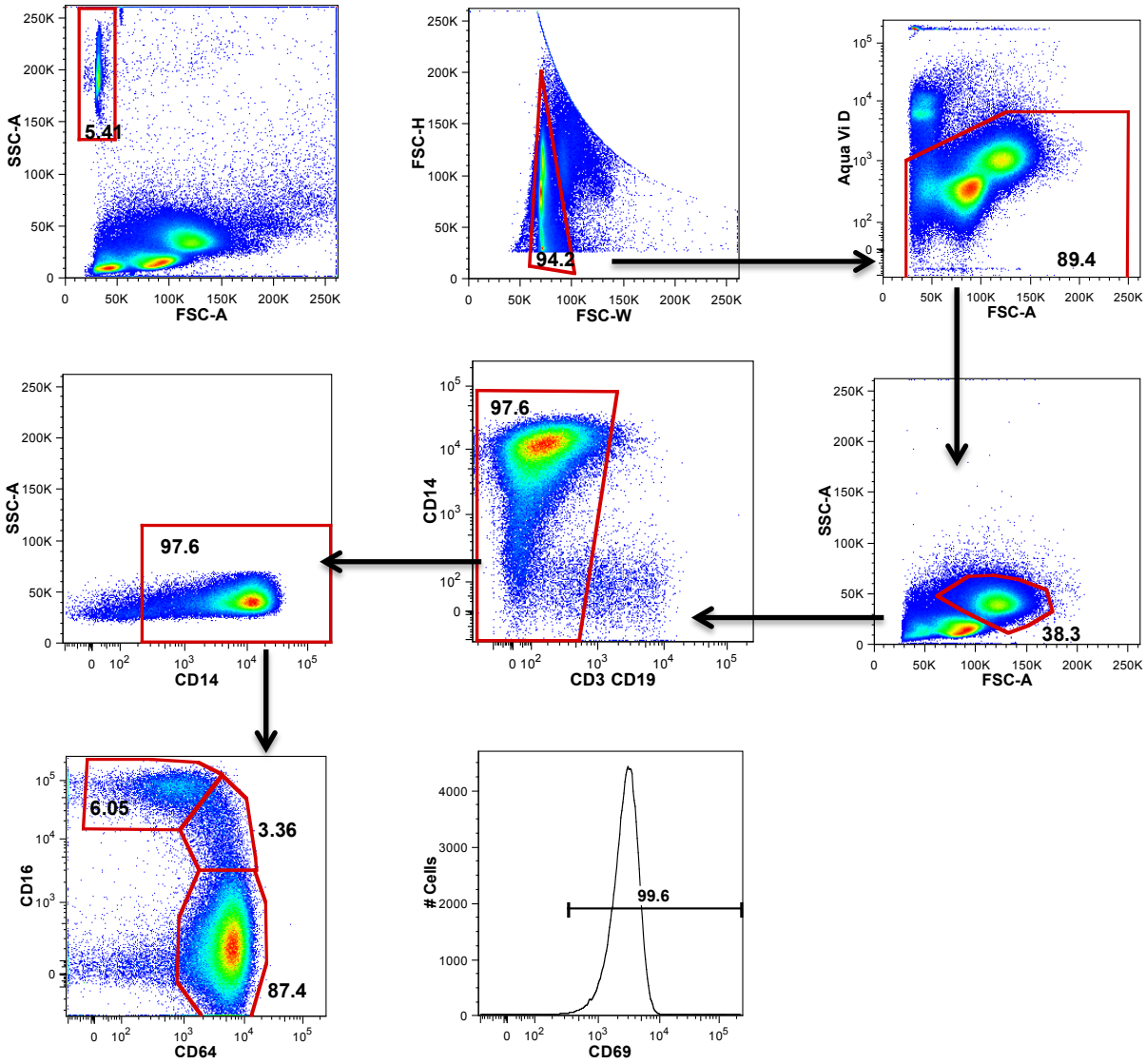
C


ΔEosinophils (x10⁹/L)
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
24h-72h
~1W
~1M
~3M
ES

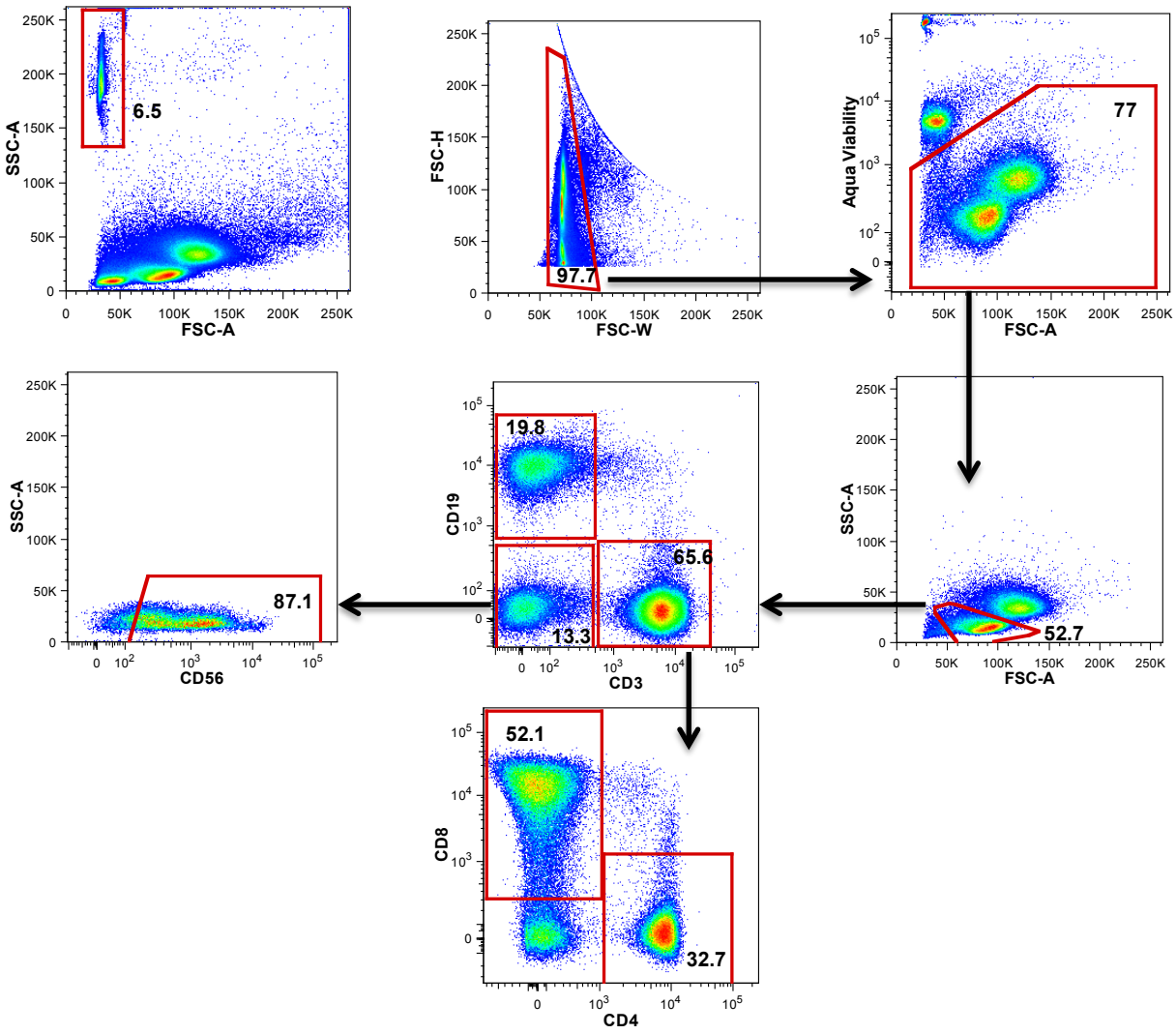
D


ΔBasophils (x10⁹/L)
0.03
0.02
0.01
0.00
-0.01
-0.02
-0.03
24h-72h
~1W
~1M
~3M
ES

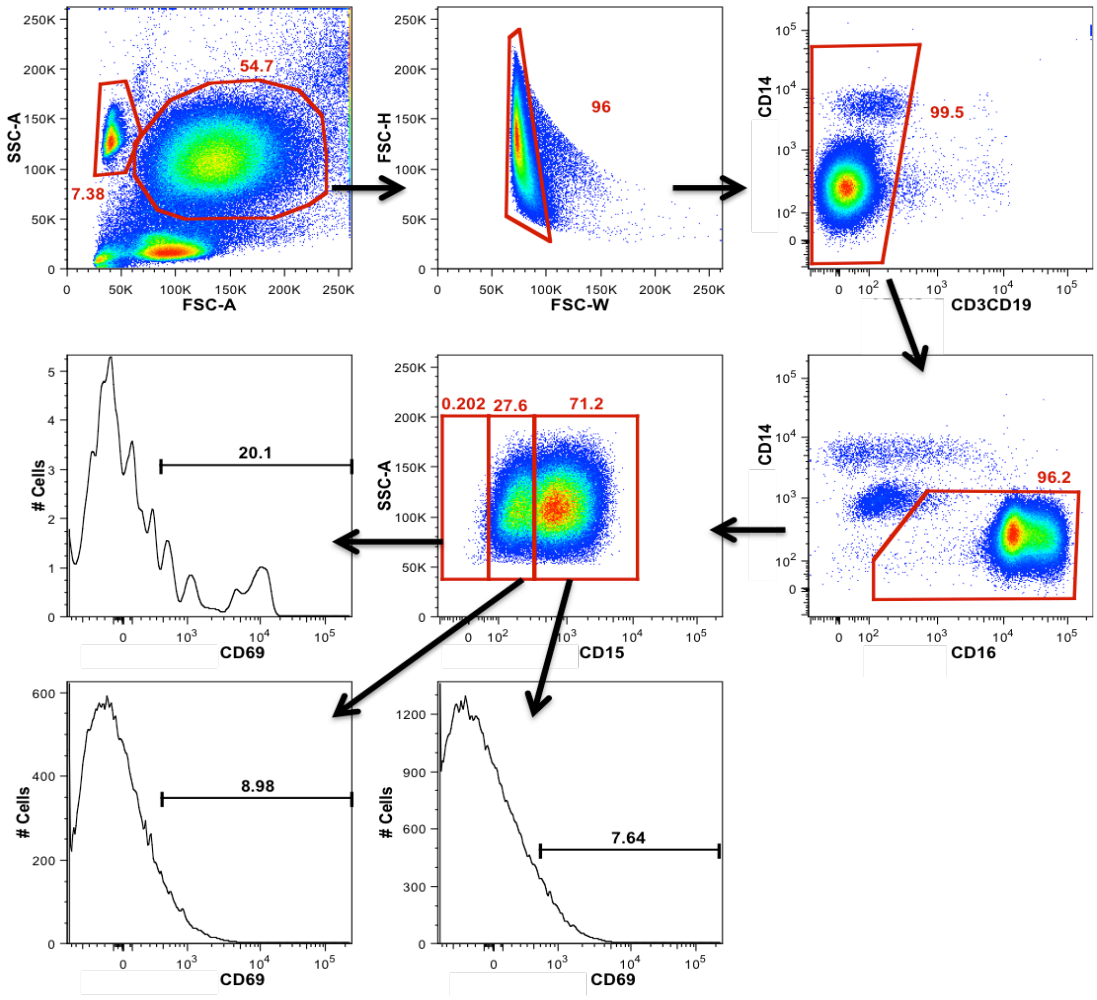
Appendix 7. Optimized gating strategy used to select the monocyte subset populations, as well as determine cell activation using cell surface marker CD69. Frozen PBMC samples collected were analyzed by flow cytometry, first separating the counting beads from the cell scatter, from which singlets, and viable cells were selected. The monocyte fraction was selected and a dump channel containing CD19 CD3 was used to remove any cells with those surface markers. From there monocytes were selected as $CD14^{+}$ then subdivided into $CD64^{+}$ $CD16^{-}$ (classical), $CD64^{+}$ $CD16^{+}$(intermediate), and $CD64^{-}$ $CD16^{+}$ (non-classical). Lastly CD69 expression was obtained for each subset. Representative dot plots and a histogram are shown.


SSC-A
FSC-A
5.41
FSC-H
FSC-W
94.2
Aqua Vi D
FSC-A
89.4
SSC-A
FSC-A
38.3
CD14
CD3 CD19
97.6
SSC-A
CD14
97.6
CD16
CD64
6.05
3.36
87.4
Cells
CD69
99.6

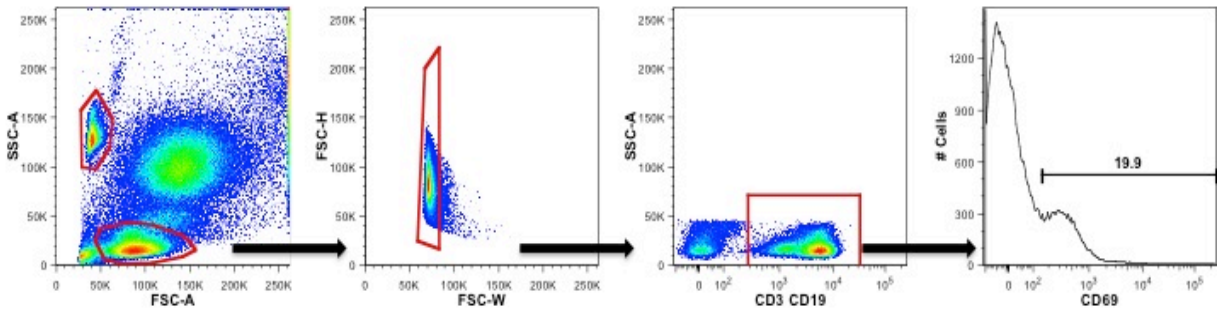
Appendix 8. Optimized gating strategy used to select the lymphocyte subset populations. Frozen PBMC samples collected were analyzed by flow cytometry, first separating the counting beads from the cell scatter, from which singlets, and viabile cells were selected. The lymphocyte fraction was selected, from there B cells were selected as $CD19^+$, T cells as $CD3^+$ and further subdivided into $CD4^+$ and $CD8^+$ T cells. Lastly, NK cells were selected as $CD56^+$. Representative dot plots are shown.


SSC-A
FSC-A
6.5
FSC-H
FSC-W
97.7
Aqua Viability
FSC-A
77
SSC-A
CD56
87.1
CD19
CD3
19.8
65.6
13.3
SSC-A
FSC-A
52.7
CD8
CD4
52.1
32.7

Appendix 9. Optimized gating strategy used to identify neutrophils, subdivided into $CD15^{lo}$, $CD15^{mid}$, and $CD15^{hi}$, as well as assess cell activation using CD69 in whole blood samples. Whole blood samples collected were analyzed by flow cytometry. First the counting beads were selected from the cell scatter, as well as the neutrophil fraction. A dump channel containing CD19 CD3 was used to remove any cells with those surface markers. From there neutrophils were selected as $CD16^{+}$ then subdivided into $CD15^{lo}$, $CD15^{mid}$, and $CD15^{hi}$. Lastly CD69 expression was obtained for each subset. Representative dot plots and histograms are shown.


SSC-A
FSC-A
54.7
7.38
FSC-H
FSC-W
96
CD14
CD3CD19
99.5
CD14
CD16
96.2
SSC-A
CD15
0.202
27.6
71.2
Cells
CD69
20.1
Cells
CD69
8.98
Cells
CD69
7.64

Appendix 10. Whole blood was analyzed by flow cytometry and a gating strategy was developed to pool $CD3^+$ and $CD19^+$ lymphocyte cells, and cell activation using CD69. Whole blood samples collected were analyzed by flow cytometry. First the counting beads were selected from the cell scatter, as well as the lymphocyte fraction. Pooled $CD19^+$ $CD3^+$ cells were selected. Lastly CD69 expression was obtained for each subset. Representative dot plots and a histogram are shown.


SSC-A
FSC-A
FSC-H
FSC-W
SSC-A
CD3 CD19
Cells
19.9
CD69

Curriculum vitae

Kevin Blackney

Education

Master of Science Candidate — September 2014 – Present
Microbiology and Immunology
University of Western Ontario, London, Ontario

Bachelor of Medical Science — 2010 – 2014
Honours Specialization in Microbiology and Immunology
University of Western Ontario, London, Ontario

Research Experience

Master's Student in Dr. Gregory Dekaban's Laboratory — September 2014 – Present
University of Western Ontario and Robarts Research Institute, London, Ontario

- Undertook two intensive projects as part of my graduate research investigating inflammation following traumatic brain injury, with a focus on systemic and brain-specific myeloid cell responses.
- Conducted extensive literature evaluations of inflammation and brain injury to guide analysis and interpretation of both research direction and data.
- Trained to prepare and run multi-colour panels on BD LSR II flow cytometer and used an appropriate gating strategy to determine distinct leukocyte populations in human and mouse samples.
- Recorded, processed, and analyzed data using software such as Excel and Graphpad Prism to develop graphs and determine significance of results.
- Arranged figures and slides on my research for public presentations as well as for collaborative researchers to present at international conferences.
- Project 1: Investigating Blood Biomarkers of Concussion in Female Rugby Players
    - Helped organize and conduct a longitudinal human study requiring patient consenting and sample collection for analysis.
    - Worked alongside clinicians and patients at the Fowler Kennedy Sports Medicine Clinic performing balance assessments, and interpreting approved concussion assessments, the Sports Concussion Assessment Tool 3rd Edition and the Immediate Post-Concussion Assessment and Cognitive Test, to better understand how concussions are currently diagnosed.
    - Processed blood samples to separate the peripheral blood mononuclear cells, plasma, and serum for storage as well as analysis by flow cytometry.
    - Selected and performed immunoassays for proteins of interest to determine changes post-concussion in plasma and serum samples.
    - Performed both independent and collaborative data analysis with research partners to obtain a multifactorial evaluation of concussion.

- Project 2: Differentiating haematogenous myelomonocytic and microglial populations after traumatic brain injury using the lys-EGFP-ki mouse model
    - Received advanced animal training according to the Canadian Council on Animal Care guidelines in handling, injections, surgery, and sacrifice of mice. Applied these techniques appropriately in my own mouse experiments in a controlled cortical impact traumatic brain injury model using genetically engineered Lys-EGFP-KI mouse strain.
    - Carefully collected blood and fixed mice via cardiac puncture with perfusion. Upon fixation harvested organs for cryosectioning on a Leica CM3050 S Research Cryostat.
    - Processed collected spleen and blood samples for flow cytometry with individual myeloid and lymphoid cell analysis panels.
    - Trained in confocal microscopy on a Leica TCS SP5 II microscope for the immunohistochemical analysis of immune cell infiltration and activity in mouse brain and other organs.

Undergraduate Thesis Student in Dr. Gregory Dekaban's Laboratory 2013 – 2014
University of Western Ontario and Robarts Research Institute, London, Ontario

- Assisted in a spinal cord injury study requiring fluorescence microscopy of mouse lung sections to quantify leukocytes.
- Investigated mild traumatic brain injury and the resulting biomarker production as part of a fourth year thesis research project. Was encouraged by my supervising professor to continue this project as part of a Masters degree.
- Collected and processed human blood samples to separate out plasma for immunoassays and leukocytes for flow cytometry and analyzed the resulting data.
- Developed a thesis report from my research, and presented this and took questions from peers as well as professors.

Microbiology and Immunology Laboratory Student 2012 – 2013
University of Western Ontario, London, Ontario

- Carefully followed instructions and made insightful decisions with experiments to obtain meaningful results that can be reported in scientific papers.
- Administered immunizations, performed blood collection, and dissected mice following ethical standards to obtain samples for further investigation.
- Work efficiently with fellow lab members to achieve common goals and help problem solve difficulties in the experiment methodology or results.

Abstracts/Posters

Society for Neuroscience Annual Meeting November 2016
San Diego, California, United States of America
"Evidence for neuroreparitive mechanisms after a season of concussion-naïve play in female varsity rugby players."
Manning K, Blackney K, Brown A, Bartha A, Dekaban G, Barreira C, Doherty T, Fischer L, Shaw S, Fraser D, Menon R.

Organization of Human Brain Mapping June 2016
Geneva, Switzerland
"Diffusion, connectivity and hematology changes in female rugby players after a single season"
Manning K, Blackney K, Brown A, Bartha A, Dekaban G, Barreira C, Doherty T, Fischer L, Shaw S, Fraser D, Menon R.

ISMRM 24th Annual Meeting May 2016
Singapore,
"Diffusion tensor imaging changes in rugby players without diagnosed concussion"
Manning K, Blackney K, Brown A, Bartha A, Dekaban G, Barreira C, Doherty T, Fischer L, Shaw S, Fraser D, Menon R.

ISMRM 24th Annual Meeting May 2016
Singapore,
"Single-subject diffusion tensor imaging after concussion"
Manning K, Blackney K, Brown A, Bartha A, Dekaban G, Barreira C, Doherty T, Fischer L, Shaw S, Fraser D, Menon R.

London Health Research Day April 2016
London Convention Centre, London, Ontario
"Investigating evolving blood leukocyte populations longitudinally after concussion in adult female athletes"
Blackney K, Fischer L, Doherty T, Menon R, Bartha R, Fraser D, Brown A, Dekaban G

Infection and Immunity Research Forum November 2015
London Convention Centre, London, Ontario
"Identification of signature biomarkers in adult female athletes following mild traumatic brain injury"
Blackney K, Fischer L, Doherty T, Menon R, Bartha R, Fraser D, Brown A, Dekaban G

Society for Neuroscience Annual Meeting October 2015
Chicago, Illinois, United States of America
"Identification of signature biomarkers in adult female athletes following mild traumatic brain injury"
Blackney K, Manning K, Fischer L, Doherty T, Menon R, Bartha R, Fraser D, Brown A, Dekaban G

Infection and Immunity Research Forum November 2014
University of Western Ontario, London, Ontario
"Identification of signature biomarkers in adult female athletes following mild traumatic brain injury"
Blackney K, Fischer L, Doherty T, Menon R, Bartha R, Fraser D, Brown A, Dekaban G

Presentations

Molecular Medicine Data Club April 2016

Robarts Research Institute, London, Ontario
"Investigating Inflammatory Biomarkers for Mild Traumatic Brain Injury"
Blackney K, Dekaban G

London Health Research Day Feature Platform Presentation April 2015
London Convention Centre, London, Ontario
"Identification of signature biomarkers in adult female athletes following mild traumatic brain injury"
Blackney K, Dekaban G

Awards

Dr. Frederick W. Luney Graduate Travel Award in Microbiology & Immunology October 2015
Value $2000

Ontario Graduate Scholarship September 2015 – August 2016
Value $15000

Western Graduate Research Scholarship September 2014 – August 2016
Value $9000

Academic All-Canadian Fall/Winter 2010 – 2015
80% or higher grade average as a member of a varsity team

Dean's Honour List Fall/Winter 2010 – 2014
80% or higher grade average

The Western Scholarship of Distinction September 2010
Value $1000

Volunteer Experience

American Society for Virology Annual Conference Volunteer July 2015
University of Western Ontario, London, Ontario

Robarts Molecular Medicine Student Leader September 2014 – Present
Robarts Research Institute, London, Ontario

Leadership Experience

Robarts Research Day Conference Organizer June 2014/June 2015
London, Ontario

- Worked with a team of graduate students to help organize and run a 1 day conference featuring poster presentations, oral presentations, and an international keynote speaker.
- Worked with Robarts Administration and Principle Investigators to decide budget, venue, and schedule.

- Collected, organized, and selected abstracts for poster and oral presentations by research theme.

Varsity Cross Country and Track and Field Athlete 2010 – 2015
University of Western Ontario, London, Ontario

- Part of two highly competitive varsity teams requiring a large commitment of time and effort on top of a full time undergraduate and graduate school schedule.
- Selected as captain of the varsity cross-country team in my fourth year and first year of my MSc as a result of my dedication and commitment to both my personal training and development of the team.
- Acquired essential time management skills to be both a high achieving student and athlete.
- Constantly working towards achieving personal and team goals as well as setting new ones.

Employment Experience

University of Toronto Youth Summer Program Counsellor May 2013 – August 2013
University Of Toronto, Toronto, Ontario

- Acted as a university mentor and guide to Canadian and international high school students participating in both the medical or law modules of this high achieving summer camp.
- Answered complicated scientific questions in an understandable manner for high school students who have not had advanced scientific courses.
- Acted as a residence don to campers to enforce rules and maintain a safe environment.

Health and Safety Department Employee May 2012 – August 2012
SNC Lavalin Engineering and Construction Firm, Toronto, Ontario

- Collected and categorized data for all Toronto employees and mapped them into new organizational system.
- Devised new health and safety strategies when challenged with existing problems on and off construction sites.
- Researched and read health and safety acts and laws for various construction projects to devise new company forms.
- Received excellent experience working in a business environment within a large company.