CHARACTERIZING NEUTROPHIL RESPONSE FOLLOWING NATURALLY-OCCURRING SPINAL CORD INJURY

A Dissertation

by

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ABSTRACT

In treating spinal cord injury (SCI), it is important to identify agents that could diminish the inflammatory response that follows primary injury. Human and rodent SCI research shows an increase in neutrophils following injury, yet, little is known about cellular activation post-injury. This study was implemented in dogs with naturally-occurring SCI resulting from intervertebral disk herniation (IVDH) to characterize the post-injury inflammatory response. Canine SCI parallels human SCI with respect to identifiable contusion with sustained compression; treatment modalities; and histopathic, molecular and magnetic resonance lesion phenotype. However, unlike most human SCI, dogs with IVDH-SCI do not have poly-trauma masking inflammatory responses to the injured cord. Cerebrospinal fluid (CSF), blood, and spinal cord tissue were characterized in dogs with SCI and in healthy controls recruited from Texas A&M University. Metabolite concentrations from CSF were measured using enzyme-linked immunosorbent assays and tandem mass spectrometry. Isolated peripheral blood neutrophil activity and expression of L-selectin were evaluated with flow cytometric analysis. Neutrophils were identified in damaged spinal cords of dogs with severe IVDH. The inflammatory response in dogs with SCI is increased in CSF metabolite profiles. Neutrophil activity in circulation is prolonged, and there is evidence to suggest a canine specific expression of neutrophil L-selectin. This is the first observation of neutrophils in the damaged canine spinal cord. This study demonstrates changes in the peripheral immune cells in an animal model that closely resembles human pathogenesis of neuroinflammation after spinal cord injury.

DEDICATION

This work is dedicated to the human and canine patients suffering from spinal cord injury, namely those who were outpatient clients of The Recovery Project in Livonia, Michigan from 2005-2006, inpatient clients of Craig Hospital in Englewood, Colorado, from 2006-2008, and inpatient clients at the Texas A&M Small Animal Hospital Neurology Clinic from 2014-2018.

This work is also dedicated to Anna May Timmons (1925-2017). You will be remembered forever.

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NOMENCLATURE

AA	Arachidonic acid
ASIA	American Spinal Cord Association Impairment Score
BBB	Blood-brain barrier
BBB score	Basso, Bresnahan, Beattie
CD11b	Compliment domain 11b
CD62L	Compliment domain 62L, also L-selectin
CNS	Central nervous system
COX	Cyclooxygenase
CSF	Cerebrospinal fluid
CT	Computed tomography
DAMPs	Damage associated molecular patterns
DHA	Docosahexaenoic acid
DHR 123	Dihydrorhodamine 123
DiHDPE	Dihydroxydocosapentaenoic acid
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EPAs	Eicosapentaenoic acids
FBS	Fetal bovine serum
GCs	Glucocorticoids
HpDHA	Hydroperoxyl docosahexanoic acid
IL-6	Interleukin 6
IL-8	Interleukin 8

IVDH	Intervertebral disc herniation
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
LTs	Leukotrienes
LTC4	Leukotriene C4
MFI	Median fluorescent intensity
MFS	Modified Frankel score
MMPs	Matrix metalloproteinases
MMP-9	Matrix metalloproteinase-9
MPO	Myeloperoxidase
MR	Magnetic resonance imaging
MS/MS	Tandem mass spectrometry
NETs	Neutrophil extracellular traps
NE	Neutrophil elastase
NSAIDs	Non-steroidal anti-inflammatory drugs
OBA	Oxidative burst activity
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PG	Prostaglandins
PGE2	Prostaglandin E2
PLA2	Lipoprotein-associated phospholipase A2
PNS	Peripheral nervous system

ROS	Reactive Oxygen Species
SCI	Spinal cord injury
TNCC	Total nucleated cell count
TNF	Tumor necrosis factor
TLRs	Toll-like receptors
TSCIS	Texas Spinal Cord Injury Score
TXB2	Thromboxane B2
7AAD	7-Aminoactinmycin

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1. INTRODUCTION

Traumatic spinal cord injury (SCI) occurs suddenly and affects the lives of healthy people at a rate of 10,000 new injuries per year across the world [1]. Spinal cord injury results from a traumatic blow to the spine causing contusion, compression, or laceration, that leaves patients partially or completely paralyzed below the level of injured cord. In North America, SCI is primarily caused by a motor vehicular accident [1]. The most visible disability of SCI patients in the eyes of the public is the loss of motor function requiring the use of a wheelchair. However, in addition to a damaged central nervous system (CNS), human patients with SCI often experience a range of other medical problems including but not limited to blood pressure abnormalities, urinary tract infections, and skin infections [2]. Indeed, complications affecting bowel and bladder functions along with impotence are actually more concerning issues for human patients with spinal cord injury, than regaining locomotor ability. Complications from SCI can lead to re-hospitalizations, and in most patients can cost upwards of $300,000 over a lifetime, not including outpatient and long-term care [3]. Besides the additional lifetime costs for the patient, societies as a whole lose valuable members of the workforce due to inability of most SCI patients to return to work. Some patients have a hard time coping and integrating back into their communities they were once part of [4]. There is a higher incidence of depression among SCI patients when compared to the general population [5]. Despite recent advances in stem cell biology and cutting-edge electrical stimulation technologies, there is still no cure for spinal cord injury [6-8].

To understand how a cure for spinal cord injury is imagined and implemented, one needs to first understand the factors that cause and propagate the injury. Injury to the spinal cord is divided into primary and secondary processes. Primary injury is the actual mechanical damage to the spinal cord, and may include compression, contusion, and laceration. Surgical intervention can address the compressive facets of primary injury (contusion and laceration cannot be treated surgically). However, primary injury is not the only complication to human patients suffering from spinal cord damage. After the sudden onset of the primary injury, the body responds by launching secondary injury cascades, including processes such as inflammation, oxidative stress, and excitotoxicity.

There has been a great deal of focus on the inflammatory aspects of secondary injury. A large portion of neuroinflammation research has focused on macrophage activation because activated macrophages can act as either pro-inflammatory agonists, or anti-inflammatory cells promoting growth and repair [9]. Although much is known about the initiation of secondary injury, much less is knowns about when macrophages and other cells switch from a pro-inflammatory to the growth promoting anti-inflammatory state. Therefore, this work aims to answer the central question: when is the appropriate time to administer a therapeutic that will drive the switch of the inflammatory cascade from the damaging pro-inflammatory state, to a growth promoting anti-inflammatory environment? To answer this question, the focus of this work is on a key component of the neuroinflammation cascade: the infiltrating neutrophil, the first cell from circulation to enter into the spinal cord after injury. In order to characterize neutrophil contribution to SCI, a naturally-occurring canine of spinal cord injury was utilized.

1.1 Neuroinflammation

Neuroinflammation is different from peripheral inflammation because an extensive barrier exists between the normal blood flow circulation where cells from the immune system reside, and the brain and spinal cord of the CNS. The blood-brain-barrier is composed of endothelial cells with a series of tight junctions set in place to regulate what goes in and out of the central nervous system during normal homeostasis and also after injury. To solve the “problem” of neuroinflammation, one first as to consider the timing of inflammatory events in CNS damage in order to see at what point neurons are not able to grow or repair. Generally, the sequences of events are known following SCI, however, the timing and overlap between events remains a mystery. There is still debate about whether the immune response is helpful [10] or harmful in model systems of SCI [11]. Furthermore, some immune responses may be helpful in the early stages of neuroinflammation, but the same responses may be harmful at a later time point. Therefore, characterizing the immune response timing in SCI is important in treatments focused on mitigating neuroinflammation in SCI. Questions still remain about why the natural inflammatory response to SCI is insufficient to promote a significant amount of recovery over the lifetime of SCI patients.

1.2 Macrophages as a focus of neuroinflammation research

To date, treatments for mitigating spinal cord damage and secondary inflammation have been focused on controlling macrophage polarization. Activated macrophages infiltrate the CNS after neutrophils and hit peak trafficking around 3 days post-injury, but can remain in the tissue for months or years following spinal cord injury [12]. Macrophages

in SCI and other diseases can exist as either proinflammatory or anti-inflammatory polarized states, or anywhere along a continuum in between. This is a problem because M1 macrophages (proinflammatory) are the dominant cell type following SCI and remain polarized in M1 rather than resolving to an anti-inflammatory M2 state. Besides being unable to drive M1-M2 macrophage polarization, another problem to consider is that macrophages are derived from both microglia and peripheral circulating monocytes. These activated or polarized macrophages are referred to as microglia or macrophages because they are morphologically identical as well as express the same intracellular and extracellular markers [13, 14]. *In vitro*, M2 macrophages stimulate axon regeneration, but *in vivo* environmental factors drive M2 macrophages back into an M1 phenotype [15]. The contents of phagocytosed material can drive the polarization of macrophages [16]. Neutrophils and red blood cells are phagocytosed by macrophages. Therefore, it is possible that neutrophils can be a useful therapeutic target to help drive the macrophage polarization towards axon regrowth. Although, timing of when the immune response should be driven to an anti-inflammatory state and the role of neutrophils in this switch is still poorly understood.

1.3 Neutrophils as first responders to injury and infection

Neutrophils are the most common white blood cells found in peripheral circulation under normal physiological conditions. In humans and in dogs, neutrophil make up 50-70% of the total leukocytes found in circulation. When pathogens or traumatic injury are present in the tissues, neutrophils respond to distress signals, transmigrate through the

endothelium (extravasation), and release their granular contents to break down damaged cells and debris.

Neutrophil generation and function during normal physiological conditions and in response to bacterial infection have been well established [17]. Neutrophils are produced in the bone marrow and are typically found only in blood circulation. If no injury or pathogens are encountered, then neutrophils are transported back to the bone marrow or to the liver to be digested [18, 19]. Neutrophils have constitutive expression of chemokine receptors and produce proteases that are stored in three different kinds of granules: azurophilic, specific, and gelatinase. The most commonly mentioned is myeloperoxidase (MPO), which catabolizes the production of reactive oxygen species (ROS), which will be discussed in the following section.

Neutrophils have the shortest half-life of all leukocytes, but can live longer if activated by certain pathological or injury conditions [20]. When neutrophils encounter distress signals in the blood stream, they activate, extravasate through the endothelium, and ultimately live longer than when they are not activated. Once out of the blood stream, activated neutrophils engulf and breakdown bacteria and also can produce neutrophil extracellular traps (NETs). More specifically, NETs are composed of DNA nucleotide strands peppered with proteases, which can break down bacteria extracellularly.

Neutrophils respond similarly to injury as they do to infection by releasing granular contents to break down cellular debris. This will be discussed in further detail in following sections and throughout this dissertation. However, activated neutrophils use of NETs in response to injury is poorly understood. Furthermore, prostaglandin E2 (PGE2) is elevated soon after traumatic injury [21-23], and is a well-known inhibitor of neutrophil

ROS production [24-26]. More recently, PGE2 has been shown to affect the use of NETs (NETosis) by neutrophils [27, 28] and will also inhibit release of a potent neutrophil chemoattractant, leukotriene B4 (LTB4) [29]. Inhibition of neutrophil activation of disease and injury in which PGE2 is elevated complicates neuroinflammation characterization projects because one would need multiple groups with both inhibited and non-inhibition neutrophil activation to elucidate the role of neutrophil activation.

Questions remain about the role of neutrophils in bacterial infection and in response to injury. Their role in wound healing is shown to be necessary [10]. Depletion of monocytes results in the proliferation of neutrophils [30]. The role of this work is to characterize the role of necessary yet poorly understood neutrophils responding to a specific central nervous system injury, by applying our knowledge of the benefits of neutrophils in their classical function of bacterial infection in peripheral infection or injury.

1.4 Neutrophil granular contents

Neutrophil granule contents contribute to antimicrobial functions, immunomodulation, extracellular matrix remodeling, and cell death (for review of neutrophil granules [31-33]). Neutrophil granules can be contained in one of 3 different types of vesicles: primary, secondary, and tertiary, which are named based on how early in neutrophil development the granules are produced. Primary (azurophilic) granules are produced by neutrophils during the promyelocyte development stage and are composed primarily of MPO and serine proteases, such as neutrophil elastase (NE). Secondary (specific) granules contain lactoferrin and neutrophil gelatinase associated lipocalin and are developed in the myelocyte stage which follows the promyelocyte stage. Granules

that are developed in full matured neutrophils are called tertiary (gelatinase) granules and contain primarily matrix metalloproteinases (MMPs).

A few of the proteases contained in neutrophilic granules have been reported to be involved in SCI inflammation: MPO, neutrophil elastase, and matrix metalloproteases (MMPs) or gelatinase [34]. Neutrophil elastase is a serine proteinase that neutrophils release during transmigration through the blood brain barrier. Inhibition of neutrophil elastase after SCI has shown decreased neutrophil infiltration, vascular leakage and increased endothelial cell preservation [35, 36]. MPO) catalyzes the production of highly reactive and cytotoxic hypochlorous acid from hydrogen peroxide and chloride anions. When neutrophil infiltration is blocked following SCI, decreases in MPO and reaction oxygen species have been reported [37]. There are several isoforms of MMPs. MMP-9 and MMP-3 have been implicated in breakdown of the basal lamina surrounding the endothelium of the BBB and activating macrophages [11, 38, 39]. Neutrophilic granular contents are contained in sealed granules and are not released until the cell has been activated by cytokines or other signals.

1.5 Neutrophils under normal physiological conditions

Under normal physiological conditions, neutrophils can be found in bone marrow, spleen, liver, and lung [40]. Like other granulocytes (basophils and eosinophils), neutrophils develop in the bone marrow by a process called granulopoiesis. It takes 10-14 days to produce mature neutrophils in circulation and this is probably the reason why some studies show a spike in neutrophil infiltration 2 weeks post traumatic injury [41]. Neutrophils exist in four different compartments: in the bone marrow during maturation,

in the bone marrow matured and in storage, in circulation, and migrated to tissues or aggregating on the endothelium surface. Neutrophils have a half-life in circulation of 1.5 hours in mice, and 8 hours in humans [42, 43]. During inflammation, their circulation lifespan increases and they can be primed at sites of injury to persist [40, 44]. Neutrophils in circulation have oxidative burst activity (OBA) profiles in humans following both traumatic brain injury as well as spinal cord injury [45, 46].

Neutrophils, like many cells in the body have receptors on their surface used to recognize foreign bodies like bacteria and even extracellular contents that are typically only found in intact and healthy cells. These receptors are called Toll-like receptors (TLRs), which are transmembrane proteins that are homologs for the drosophila Toll protein that mediates antimicrobial responses in that organism. Their extracellular portion is rich with leucine and cystine motifs that are involved in ligand binding, while their intracellular portion is essential for signaling [47]. Toll-like receptors are constitutively expressed on the surface of neutrophils and depending on what type of TLR, can recognize many pathogens including but not limited to: double stranded RNA seen in viruses, lipopolysaccharide (LPS) a major component of bacterial walls, extracellular DNA, and heat-shock proteins normally secured inside healthy cells. These types of ligands recognized by TLRs are called pathogen molecular patterns and damage molecular patterns (PAMPs and DAMPs, respectively). Molecular patterns expressed by damaged or dying cells will be covered later in this section in regards to neutrophil response to spinal cord injury.

During granulopoesis, the first type of granule formed in the developing neutrophil is the primary or azurophilic granule which has the ability to produce reactive oxygen

species. Oxidative burst is so named because neutrophils utilize oxidative respiration chain reaction to produce the ROS needed to breakdown engulfed bacteria or cell debris [48]. Activated neutrophils are those that are capable of releasing their reactive oxygen species and oxidative enzymes from their granules. Oxidative burst activity in neutrophils is when in response to signals, neutrophils move all their granular contents and reactive oxygen species to the surface of their cells in preparation for tissue and cell degradation [49]. Granular contents, enzymes that are able to safely produce reactive oxygen species are contained in primary or azurophilic granules. This means that ROS production is the very first function neutrophils are able to perform in their mature state. Breakdown of bacteria and cellular debris is covered in following sections.

1.6 Neutrophil extravasation through the blood brain barrier

Despite the extra protections to keep the central nervous system separated from normal circulations, neutrophils can exit the blood vessels and traverse the blood-brain barrier (BBB) to enter the CNS. So far, neutrophil existence in 3 of 4 compartments have been discussed: maturation and storage in the bone marrow, and neutrophils in circulation. The forth compartment discussed in this section will cover migration of neutrophils out of the blood stream.

The BBB is specialized endothelium in the microvessels that form the walls of capillaries in the brain. The endothelial cells of the BBB are very similar to endothelium of other tissues, except that they have more complex tight junctions, which allow for a more restricted regulation of molecules and cells entering the CNS. The BBB functions to regulate ions, neurotransmitters, macromolecules, and neurotoxins from getting into the

brain [50-52]. Under normal physiological conditions, immune cells can enter the brain through the BBB and facilitate neurogenesis in the hippocampus [53].

Some cytokines, like tumor necrosis factor (TNF), Interleukin-6 (IL-6), and IL-1β, can impair BBB function by opening tight junctions which leads to increased neutrophil transmigration [54-56]. Disorganization of VE-cadherin and β-catenin molecules of the tight junctions is part of the neutrophil transmigration process [57]. There is a gate function by T cells to allow for macrophages to enter through the choroid plexus following spinal cord injury in mice [58].

Endothelial cells of the BBB up regulate expression of adhesion molecules in response to astrocytic cytokine secretion of TNF and IL-1 [59-61]. P-selectin and Intracellular adhesion molecule-1 are both transmembrane proteins involved in arresting leukocytes in the blood stream to facilitate transmigration in spinal cord injury in mice [62]. Junctional adhesion molecules on endothelial cells are responsible for the final required step of transmigration of neutrophils through the BBB and into the injured tissue [59, 63, 64]. Also affected by TNF cytokine production is the glycocalyx, a tight meshwork of charged glycosaminoglycans found on the luminal surface of endothelial cells, which is diminished to allow for increased adhesion of neutrophils to the endothelial wall [65].

The neurovascular unit is comprised of the endothelium, astrocytes, pericytes, neurons and the extra cellular matrix surrounding these cells. Pericytes, along with other cells in the neurovascular unit, regulate the recruitment of more neutrophils to the site of injury [66, 67]. Pericytes are cells not dissimilar from astrocytes, with their end feet closely associated with microvasculature in the CNS and both cell types express neuron-glial antigen 2, which makes their cellular boarders difficult to differentiate in

immunohistochemistry [68, 69]. After spinal cord injury, pericytes are thought to control capillary blood flow by squeezing the diameter of the vessel [70], which can affect the tethering and transmigration of neutrophils through the microvasculature. The BBB has close cell-cell interactions with astrocyte end feet and pericytes in the CNS that are thought to drive the more restrictive nature of the endothelium during neuronal development [71].

It is clear that neutrophils have the ability to enter the damaged spinal cord. What remains to be investigated is if neutrophils are entering the damaged cord because there is a physical breakdown of the BBB, or rather, because there is a physical breach in blood vessel walls along with primary injury. The purpose of this work is to contribute to the knowledge of whether or not neutrophils are responding to distress signals sent from the injury site, or if they are just passively arriving in the damaged spinal cord along with hemorrhagic swelling associated with the injury in experimental models of SCI and naturally-occurring injury.

1.7 Role of neutrophils in neuroinflammation following spinal cord injury

Neutrophils are the first responders from peripheral circulation to respond to central nervous system (CNS) injury as an important part of the innate inflammatory response. In the case of SCI, neutrophils respond within hours of initial trauma and are at peak trafficking by 24 hours post injury [34]. Most of the effector molecules contained in neutrophilic granules are proteases and aid in degrading extracellular components, which can be damaging to recovery from SCI [38]. However, neutrophils are also reported to have a beneficial role in wound healing [10, 72], and are necessary for clearance of

debris in peripheral nerve injury [30]. Therefore, blocking transmigration of neutrophils into the injured spinal cord to mitigate neuroinflammation has not been proven to be an effective treatment for spinal cord injury.

Neutrophil function in response to SCI is poorly understood. Cytokines and cell adhesion molecules identified in experimental SCI rodent models as key mediators in neuroinflammation have been corroborated in human studies [73, 74]. Experimental mouse models utilizing specific gene knockouts prior to induced injury in order to characterize neutrophil infiltration are particularly helpful in elucidating neutrophil function [55, 62]. Yet, there are many differences in cell behavior and injury onset between humans with spontaneous injury and laboratory animals with induced SCI.

Initiating the peripheral inflammatory response to SCI is a multi-step process that involves the initial damage alert signals produced by dead or dying cells being released to the extracellular space, followed by signals directly recruiting leukocytes to the CNS. Concurrently, chemokines are produced that alter the tight junctions of the blood brain barrier and increase expression of leukocyte adhesion molecules on the endothelial luminal surface. Once these events have occurred, leukocytes can enter the CNS and the damaged cord. There are several signal cascade events that occur within the damaged cord immediately following spinal cord injury that propagate before signals reach leukocytes in peripheral circulation.

Damage to cells from SCI causes cellular components normally contained within cells to be suddenly exposed in the extra cellular matrix (ECM). These components, for example, free-floating ATP and non-nuclear DNA are called DAMPs, which activate TLRs on resident CNS cells. Resident cells propagate intracellular signals via activation of their

toll-like receptors, which activates the nuclear factor-κB pathway, and subsequently up-regulates production and release of cytokines and chemokines in the damaged cord. From initial injury, damaged cells release DAMPs that activate astrocytes and microglia to produce cytokines and chemokines.

In the context of acute spinal cord injury, cytokines and chemokines are responsible for alerting immune cells that there is an injury, resulting in the recruitment of proinflammatory cells. There are a vast array of cytokines and chemokines that are elevated in acute SCI and several are directly involved in recruiting immune cells to the site of injury. These cytokines and chemokines can be found in the cerebrospinal fluid (CSF) that bathes the brain and spinal cord. Damage signals are released by resident CNS cells into the CSF. Astrocyte production of CCL-2 (MCP-1) stimulates recruitment of neutrophils and monocytes to the site of injury [75]. Interleukin-8 (CXCL-8), produced by microglia *in vitro* in response to TLR signaling [76], is elevated less than 12 hours after injury in homogenized rat spinal cord [56], and in dog spinal cord following intervertebral disk herniation [77, 78]. CXCL-8 is also elevated in humans and dog CSF 48 hours after injury. Studies utilizing clinical patients, humans and dogs, have longer post-injury tissue sampling times than in experimental SCI models [78, 79], which further separates comparisons made between clinical and experimental injuries.

Other cytokines up-regulated in the first 24 hours after SCI, such as TNF, Interleukin-6 (IL-6), and IL-1β, are not directly related to leukocyte chemoattraction, but rather, can impair BBB by opening tight junctions and therefore contribute to leukocyte recruitment indirectly [54-56]. IL-6 enhances expression of TNF, which reduces the glycocalyx in the blood vessel lumen [80], and expression of IL-1β, which is required for

neutrophil transmigration in peripheral bacterial infection [81]. Disorganization of VE-cadherin and β-catenin molecules of the tight junctions is part of the neutrophil transmigration process [57].

Along with signaling cascades targeting vascular permeabilization, endothelial cells of the BBB also up-regulate expression of adhesion molecules in response to astrocytic cytokine secretion of TNF and IL-1 [59-61]. P-selectin and Intracellular adhesion molecule-1 (ICAM-1) are both transmembrane proteins involved in arresting leukocytes in the blood stream to facilitate transmigration in spinal cord injury in mice [62]. Junctional adhesion molecules on endothelial cells are responsible for the final required step of transmigration of neutrophils through the BBB and into the injured tissue [59, 63, 64].

These actions by cytokines and chemokines to both actively recruit leukocytes to the site of CNS injury, as well as providing an opening of the BBB, allows for cells from the periphery, such as neutrophils and monocyte derived macrophages, to enter the injured cord.

Once in the damaged CNS, neutrophils have always been thought to be harmful for recovery because they release many toxic factors. However, their presence in the injured cord may also help control inflammatory responses once thought to be a passive process. Little is known about what drives the inflammatory response in CNS injury, and targeting neutrophils once in the injured cord may prove to be the key to driving the anti-inflammatory response to promote tissue repair. Activated macrophages are present in the damaged cord of dogs with SCI, but neutrophil infiltration was not documented in these experiments [77, 82].

1.8 Neutrophil communication with macrophages in the injured cord

The recruitment process of neutrophils to the injured spinal cord relies heavily on CNS immune mediators, resident cells that interact with the injured environment and propagate the immune response. The two main CNS immune mediators that interact with neutrophils are astrocytes and microglia/macrophages that produce cytokines to recruit more neutrophils [83, 84].

The neurovascular unit is comprised of the endothelium, astrocytes, pericytes, neurons and the extra cellular matrix surrounding these cells. Pericytes, along with other cells in the neurovascular unit, regulate the recruitment of more neutrophils to the site of injury [66, 67].

Neutrophils interact with macrophages within the CNS by way of phagocytosis after their function of removing and degrading damaged cells. When neutrophils are phagocytosed, they alter the polarized state of the macrophage, shifting it from an M2 back to the proinflammatory M1 state. Whether this happens because neutrophils intentionally drive the environment to their desired state, or because the macrophages are responding to the reactive contents in the neutrophilic granules remains unclear [85, 86].

The contents of phagocytosed material can drive the polarization of macrophages [16]. Following CNS injury, neutrophils and red blood cells are phagocytosed by macrophages. Therefore, it is possible that neutrophils can be a useful therapeutic target to help drive the macrophage polarization towards axon regrowth.

1.9 Cerebrospinal fluid importance in SCI

Along with the vertebral column and the meninges, the CSF functions to protect the brain and spinal cord by cushioning the CNS tissue in fluid. CSF is produced by specialized ependymal cells of the choroid plexus that line the ventricles in the brain. The CSF flows through cavities of the brain, ventricles, and central canal of the spinal cord, as well as around the outside of the brain and spinal in between the pia mater and dura mater of the meninges. The CSF provides nutrients via the blood vasculature [50, 51]. Because the CSF bathes the spinal cord, the CSF can be used as a representative tissue to provide an idea of the condition of the spinal cord during homeostasis, injury, or infection. CSF is also an ideal space for drug delivery, because the continuous flow can be achieved by drug delivery with lumbar puncture or delivery into the cisternal magna which is a routine procedure in both humans and dogs [73, 87, 88]. Analysis of the CSF can be used for biomarker SCI research and used to infuse treatments [52, 89, 90].

During injury, breakdown of the BBB can occur, allowing signals to disperse from the damaged CNS parenchyma out to the blood supply, by way of the cerebrospinal fluid. Therefore, shortly following injury, the CSF is suffused with damage associated molecular patterns (DAMPs) and leukocyte chemoattractant molecules [91-93]. These DAMPs can also travel from the CSF, through the blood-brain barrier, and into the serum [94].

The CSF in normal physiological conditions allows for transport nutrients to CNS tissue from the blood. However, there is little nutrient that is normally part of the CSF. The blood supply to the central nervous system is tightly regulated by way of extra tight junctions between endothelial cells in the BBB, for review see [95]).

Neutrophils can breach the BBB and enter the CSF following injury [96]. Their presence may contribute to the propagation of signaling by DAMPs and other cytokines shown to be elevated in human and dog CSF [79, 97, 98]. Sampling of the CSF in large animals (dogs) and humans can occur without sacrificing the individual [23, 78, 87, 96, 99, 100]. In other smaller species, such as mice and rats, the CSF compartment only contains small volumes and so samples have to be pooled together [101].

The cerebrospinal fluid is an important tissue for identifying damage signals coming from the damaged spinal cord. It is also an important conduit for possible drug delivery or retrieval of neutrophils that have already traversed the blood-brain barrier. The CSF in large animals and in humans can be collected with little to no harm being inflicted on the individual and is often routine practice in neurology clinical settings. Because of this, the CSF was an important part of this work and will be discussed further in this body of work in later sections.

1.10 Arachidonic acid metabolism after spinal cord injury

Arachidonic acid (AA) and docosahexaenoic acid (DHA) are omega-6 and omega-3 polyunsaturated fatty acids, respectively, that are in high concentrations in cell membranes. High concentrations of both AA and DHA are found in adult normal human brain [102], and are important in early human infant development [103, 104]. Leukotrienes (LTs) and prostaglandins (PGs) are eicosanoids derived from AA, while eicosanoids derived from DHA metabolism are eicosapentaenoic acids (EPAs), dihydroxydocosapentaenoic acids (DiHDPEs), hydroperoxyl docosahexanoic acids (HpDHAs), and others. Generally speaking, eicosanoids derived from AA have differing

properties from eicosanoids derived from DHA [105]. For example, prostaglandin E2, derived from AA and regulated by cyclooxygenase enzymes (COX), is known to induce fever and inhibit neutrophil activity. Paradoxically, 17-hydroperoxydocosahexanoic acid (17s-HpDHA) has anti-inflammatory affects in the CNS [106, 107]. Levels of AA and DHA can be influenced by diet, and are in high concentrations in fish oil, which has been used as a dietary supplement in several diseases including spinal cord injury [108-111].

1.11 Neutrophils in damaged spinal cord tissue

Once in the tissue, neutrophils function to breakdown damaged and dying cells by releasing their neutrophilic granular proteases. Cellular morphology and the presence of MPO in primary neutrophilic granules is one way to identify neutrophils in the injured spinal cord [112-114]. Although neutrophils have a short lifespan of less than 10 hours, their longevity can increase during the inflammatory response [40, 44]. Within the tissue, neutrophils can release extracellular traps (NETs). NETs are composed of unraveled neutrophilic DNA strands, antimicrobial histones, and proteases, such as MPO and neutrophil elastase [115]. NETs have been studied in regards to immobilizing and destroying cancer cells or bacteria [116, 117], but have yet to be studied in the context of SCI. It is possible that NET production in spinal cord injury is a transient process difficult to visualize because prolonged NET production leads to neutrophil lysis [116], and neutrophils may be being phagocytosed by macrophages shortly after neutrophils release NETs. Because they are phagocytosed by macrophages, neutrophils help drive the M1/M2 polarization [118]. They may also be inhibited from producing NETs.

Questions remain about neutrophil function following SCI. Perhaps these cells play more of a role in driving the inflammatory response than previous studies suggest, by bringing in their potent granular contents, despite having a short half-life under normal physiological conditions.

1.12 The underappreciated role of neutrophils in SCI

There are two general ways to approach treatments for spinal cord injury. Damage to the spinal cord is marked by an inability of damaged nerve axons to regenerate below the level of injury, resulting in glial scarring and very little, if any recovery of locomotion or sensation. Therefore, one way is to explore the intrinsic mechanisms that control neuron growth or regrowth within the nervous system cells themselves. In this way, scientists can either target specific pathways within the damaged neuron and force it to grow after SCI when naturally it would not, or, replace the damaged cells with neuron stem cells that will develop into new neurons.

A second way to approach treatments for spinal cord injury is to focus on the environment surrounding the damaged neurons. In this approach, targets involved in some way that alters the physical and chemical properties of the extra cellular matrix surrounding the damaged neurons. In theory, if the reason why damaged neurons will not regenerate following spinal cord injury is an intrinsic factor malfunction, then implanting stem cells into the damage cord should work. To date, there has not been a successful treatment with stem cell implantations following SCI [8]. Furthermore, if neurons in the central nervous system were unable to regenerate on their own, then the question arises on how they are able to develop during normal embryological development. Also,

peripheral nerves can regenerate naturally after injury. How the extrinsic factors regulating cells or the extra cellular matrix components surrounding damaged neurons are altered to promote neuron growth or regrowth is important for elucidating what is targeted in SCI therapeutic interventions. If we can better understand the processes involved in neuroinflammation, then we can provide a target window of opportunity and target molecule or cell that regulates the area surrounding damaged nerves.

1.13 Models of spinal cord injury and neutrophils

For more than 30 years of spinal cord injury research, rodents have evolved into the standard injury model for the development of novel therapeutics [119, 120]. Indeed, nearly everything about rodents can be standardized. Housing, light cycles, and diet can be kept consistent throughout an entire experiment. Numbers of animals in each group can be as small or as large as is appropriate for each study. Induced injury, with the help of specialized equipment, can be reliably reproduced in multiple animals and be incomplete or complete, and along different levels of the spinal cord. This general standardization among experiments in rodents allows for optimal circumstances for drug efficacy to be achieved [121, 122]. Much of what we know of regulation of nerve growth, scar formation, and inflammation has come from the use of rodents in SCI [123].

Rats in particular are the primary model for SCI [124, 125]. Both rats and mince qualify as standardized and less costly than larger animal models, but mice and rats differ in regards to neuropathology and recovery from SCI [126, 127]. Complete transection of the spinal cord can be studied in rats, but generally the two main injuries that are used on rats in SCI research are compression or contusion injuries. In humans, often times a

force of impact can cause fractures of the vertebrae that will cause an impact injury to the spinal cord. In an experimental setting, the vertebral laminae are removed to expose the cord for application of induced injury. Compression injures can be induced by clamping the spinal cord in clips or forceps, or by placing a tiny balloon in the vertebral canal and gradually increasing the volume [128, 129]. Contusion injures model well to human impact injuries and are performed using a weight-drop device at different weights and heights [130, 131]. Decompression of the spinal cord can be achieved by removing the force applied by these devices, which improves recovery. Decompression is the only practice included in the standard of care for human SCI [132].

The universal measure for rodents with induced SCI are scored on the Basso, Bresnahan and Beattie (BBB score) [133]. This outcome measurement scale, which ranges from 0-21, was created to include measurements of a broader range of spinal cord damage and expand the distribution of behavioral scores [134]. The walking ability of rats scored with the BBB score is comparable to outcome measurements in the human American Spinal Injury Association impairment score (ASIA) [130]. Rodent locomotor abilities can also be assessed by Catwalk technology, where the animals can walk on a transparent surface and be video recorded from below [135]. Interestingly, small increases in the percent of spared tissue have significant effects on basic locomotor recovery and changes in BBB scores, whereas on the high end of the BBB scale even animals with anywhere from 45-90% tissue sparing are hardly distinguishable behavior wise [133]. This discrepancy between locomotor scoring and recovery at the two ends of the BBB score can be problematic for designing therapeutics or testing pharmacological interventions in rodent models of SCI.

The acute immune response is a complicated multi-pathway process that is still poorly understood in the context of spinal cord injury. Cytokines and cell adhesion molecules identified in experimental SCI models as key mediators in neuroinflammation have been corroborated in human studies [73, 74]. Experimental models that knock out genes prior to induced injury in order to characterize neutrophil infiltration are particularly helpful in elucidating neutrophil function [55, 62]. Yet, there are many differences in cell behavior and injury onset between humans with spontaneous injury and laboratory animals with induced SCI.

The most salient advantage to working with dogs as a translation model of disease is the fact that dogs are clinical patients with spontaneous injuries. Dogs are used in cancer research because they develop tumors spontaneously [136, 137]. Dogs can also suffer spontaneous spinal cord injury, often secondary to intervertebral disk herniation [138-141]. Like humans, there is a notable increases of neutrophils in the CSF of dogs following SCI [96]. Also in the CSF, acute phase proteins including CXCL8 [78], and an arachidonic acid pathway metabolite, prostaglandin E2 [23] have been shown to be elevated in dogs after SCI. In normal circulation, dogs have similar neutrophil percentages to humans [142]. Also, like humans, dog neutrophils express LFA-1 and can develop genetic neutrophil infiltrating abnormalities when the LFA-1 complex is mutated [143]. Activated macrophages are present in dogs with SCI, but neutrophil infiltration was not documented in these experiments [77, 82]. Myelomalacia is a condition occurring in a small population of dogs with severe spinal cord injury and is characterized by hemorrhagic necrosis of spinal cord tissue, and massive infiltration of neutrophils [144]. This progressive oxidative stress is an extreme case of neuroinflammation following

spinal cord injury where the usual defense are inadequate for any kind of recovery, and may be a useful example of secondary injury at the far end of the spectrum [145].

1.14 Aims of the study

The overarching goal of this study was to characterize the inflammatory response, more specifically, neutrophil activity, following naturally occurring spinal cord injury in dogs. The future of testing therapeutic interventions in this large animal model that closely resembles human injury is an important translational step between experimental rodent injury and large-scale human clinical trials. This work will contribute to the field of SCI research by providing key information on the cerebrospinal fluid profiles and behavior of neutrophils in dogs with spinal cord injury

2. DAMAGE SIGNALS PROPOGATED THROUGH CEREBROSPINAL FLUID*

2.1 Background

Several experimental animal models of spinal cord injury (SCI) have been established, including contusion, laceration, clip compression, and crush in a variety of species [126, 146, 147]. These systems generate highly stereotypical injuries and minimize heterogeneity in severity, timing of injury, genetic background, and environmental exposure. While elimination of inter-animal variability likely enhances detection of the effects of putative therapeutic interventions, it does not fully reflect the diverse injury characteristics that complicate naturally-occurring SCI [148].

Canine intervertebral disc herniation (IVDH) causes a naturally-occurring form of SCI that bears critical similarities to human SCI with respect to both injury pathomechanisms and treatment. The resulting SCI occurs spontaneously, consists of varying components of compression and contusion, and is treated with a combination of decompressive surgery and physical rehabilitation [139, 149]. Histologic facets of injury parallel those detected in both humans with SCI and SCI models, including axon destruction demyelination, and centrally-oriented necrosis/cavitation [140]. Spinal cord lesions in affected dogs contain activated microglia [82]; have aberrantly increased expression of IL-6, IL-8 [77], and matrix metalloproteinase-9 (MMP-9) [150]; and contain a population of peripherally-derived leukocytes [82, 151]. Additionally, these

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* Reprinted with permission from Russell RL, Levine JM, Jeffery ND, Young C, Mondragon A, Lee B, Boudreau CE, Welsh CJ, Levine GJ: **Arachidonic acid pathway alterations in cerebrospinal fluid of dogs with naturally occurring spinal cord injury**. *BMC Neuroscience* 2016, **17**(1):1-9.

inflammatory events result in loss of blood-spinal cord barrier integrity and increased oxidative stress [144]. The similarities between human SCI and canine SCI resulting from IVDH have prompted the development of validated outcome measures including ordinal gait scores, kinematics, kinetics, urodynamics, and sensory testing in order to detect subtle improvement associated with experimental therapeutic interventions [152-154]. Furthermore, studies by several independent groups have utilized dogs with IVDH as a second species to evaluate neuroprotective and potential regenerative strategies headed for human clinical trials [139, 153, 155, 156].

The arachidonic acid (AA) pathway is a critical mediator of secondary SCI and an attractive target for pharmacologic interventions. Metabolism of AA is ubiquitous; this polyunsaturated fatty acid is released from cell membranes by phospholipase A2 (PLA2, for review see Schaloske & Dennis, 2006 [157]). In rodent spinal cord contusion models, PLA2 protein expression is induced within minutes of injury, persists for up to 7 days post-injury, and correlates with the development of demyelination and neuronal necrosis [158]. Following its release from the cell membrane, AA is metabolized into leukotrienes (LTs) and prostaglandins (PGs) via 5-lipoxygenase (5-LOX) and cyclooxygenase (COX), respectively [159, 160]. Leukotrienes and PGs function as immune cell chemoattractants, vasodilators, inducers of oxidative stress, and modulators of neurosensory processing. Further, LTs and PGs are increased acutely after experimental SCI and remain aberrantly elevated for months post-trauma [21]. Chronic dysregulation of 5-LOX and COX pathways following experimental spinal cord contusion results in depletion of lipid metabolites, altered amino acid biosynthesis, and pro-inflammatory events. Limited investigation of these pathways has occurred in large animal models of SCI. In one study

that utilized an experimental canine model of compression/contusion, LTs and PGs were increased within the cerebrospinal fluid (CSF) one day following injury and remained elevated for approximately 7 days after the primary event [161].

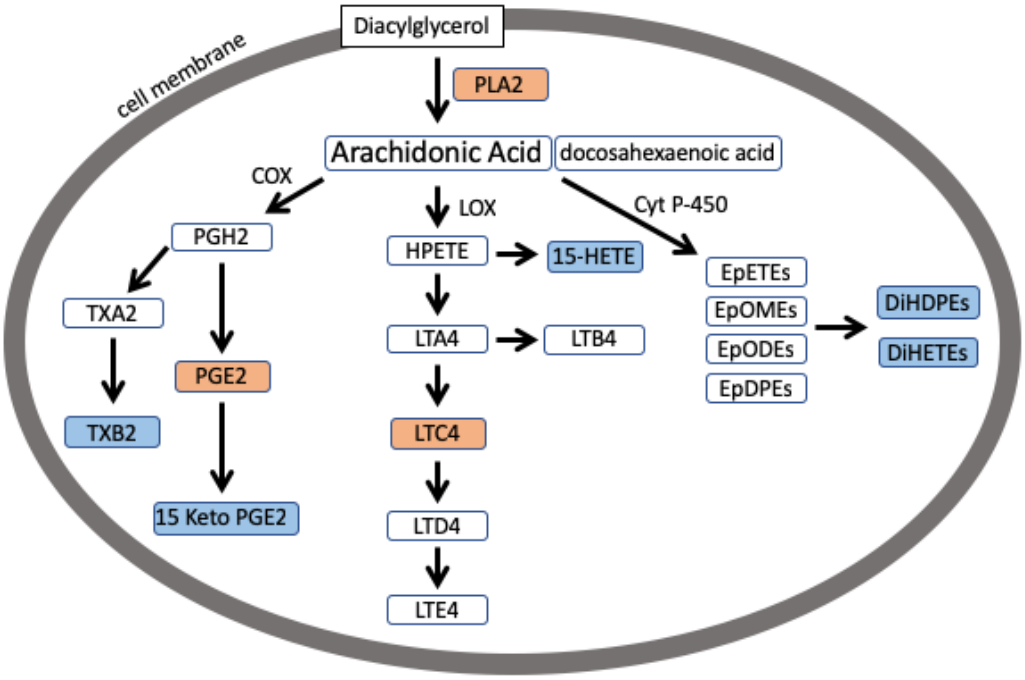

Figure 2.1 Arachidonic acid pathway metabolites and their precursors and products
The fatty acids arachidonic acid (AA) and docosahexaenoic acid (DHA) are precursors for a number of eicosanoid products. These products, or eicosanoids, are catalyzed by 3 main enzymes: cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P-450 (cyt P 450). The main products from the COX pathway are called prostaglandins (PGH2, PGE2, and 15 keto PGE2) and thromoboxanes (TXA2 and TXB2). The lox pathway converts AA to leukotrienes (LTA4, LTB4, LTC4, LTD4, and LTE4), hydroperoxyeicosatetraenoic acid (HPETE) and hydroxyeicosatetraenoic acid (15-HETE). The cytochrome P-450 (cyt P-450) pathway produces epoxide derivatives (EpETEs, EpOMEs, EpODEs, and EpDPEs) which are precursors for dihydroxydocosapentaenoic acid (DiHDPE) and dihydroxy HETEs (DiHETEs). Metabolites from AA breakdown studied in a previous study (Russell et al, 2016) are highlighted in orange. Metabolites from this study are highlighted in blue.

In this study, we assessed alterations of AA metabolism after SCI in dogs with IVDH by using enzyme-linked immunosorbent assays (ELISA) to measure CSF

concentrations of PLA2, leukotriene C4 (LTC4) and prostaglandin E2 (PGE2) (Figure 2.1). We selected these mediators because they represent critical nodes in the AA pathway that are altered in experimental SCI. Our primary objective in this exploratory study was to determine if there were higher concentrations of AA metabolites in the CSF of dogs with SCI than in healthy control dog CSF. Our secondary objective was to determine if, in dogs with SCI, CSF AA pathway metabolites were correlated with functional deficits at the time of sampling and 42-day post-injury recovery as measured by a validated ordinal score. Because so little is known about the factors that drive the inflammatory response following SCI coupled with the ease of CSF sampling in humans and in dogs, there is value in large metabolomic discovery-based studies in dogs with naturally-occurring spinal cord injury. Therefore, our third objective was to determine differentially expressed arachidonic acid pathway metabolites in the CSF of dogs with SCI by using unbiased tandem mass spectrometry discovery-based metabolomics approach (Figure 2.1).

2.2 Methods

2.2.1 Sample size determination ELISA

Sample size was determined *a priori* and was based on previous studies that examined inflammatory mediators (e.g., IL-8, C-reactive protein, MMP-9) in the CSF of dogs with IVDH-associated SCIs compared to CSF of healthy control dogs[78, 162]. In those studies, samples from 8-21 healthy controls and 35-47 dogs with IVDH were used to identify significant inter-group differences. Based on these data, we elected to utilize all control (n=21) and SCI samples (n=44) available within our biobank.

2.2.2 Sample size determination MS/MS

A second set of samples were sent to the West Coast Metabolomics center for metabolomics analysis. Similarly, CSF was collected cranial to the injury from the cerebellomedullary cistern in dogs with naturally-occurring SCI (N=21) and purpose-bred dogs (N=21) with owner consent and/or approval from the Texas A&M University Animal Care and Use Committee. All animal procedures consisted of standard medical and surgical care, and cerebrospinal fluid samples were collected between 2009 and 2012 and then stored at -80°C until being shipped on dry ice to the West Coast Metabolomics Center in 2018. Only CSF samples from SCI animals given no glucocorticoids or non-steroidal anti-inflammatories prior to collection, with red blood cell counts less than 50 cells/μL, and an admit motor score of 3 (non-ambulatory) or less, were sent for further analysis. Healthy dogs had to have a normal physical and neurological examination; normal complete blood count, serum biochemistry, and urinalysis; and CSF red blood cell counts less than 50 cells/μL.

2.2.3 Inclusion and exclusion criteria

A repository of CSF aliquots collected from the cerebellomedullary cistern, stored at -80°C, and housed at Texas A&M University since December 2009 was screened in February 2014 for samples from dogs with IVDH-associated SCI that met the following inclusion criteria: 1) lesion between T3 and L5 vertebrae; 2) neurologic impairment of <7 days duration; 3) surgical decompression of IVDH with post-operative rehabilitation; and 4) complete medical records including neurologic score at admission and follow-up

scoring at day 42 post-surgery. Dogs that were part of on-going clinical trials or had a myelogram performed as part of pre-operative diagnostics were excluded from this study.

Healthy control CSF samples were collected from purpose-bred dogs with normal physical and neurologic exams, normal complete blood counts, and normal serum biochemical analysis. All CSF samples from healthy control dogs were collected and stored in the same manner as described for dogs with IVDH-associated SCI and were required to have a normal total nucleated cell count (TNCC) (<5 cells/μL) and total protein concentration (<35 mg/dL).

2.2.4 Sample collection and therapeutic procedures

Procedures in dogs with naturally-occurring SCI were performed with owner consent and consisted of standard medical and surgical care. Purpose-bred dogs were obtained and used with approval from the Texas A&M University Animal Care and Use Committee (AUP 2007-115; AUP 2011-145). All studies adhered to the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Dogs with SCI underwent complete physical examination, neurologic examination, complete blood count, and serum biochemistry prior to anesthesia. Data including age, gender, duration of SCI, and recent delivery of non-steroidal anti-inflammatory drugs (NSAIDs) or glucocorticoids (GCs) were collected (Table 2.1, reprinted with permission from Russell et. al., 2016). Dogs were considered treated with these drugs if NSAIDs or GCs were administered within the 7-day period prior to CSF collection. Dogs were then pre-medicated with glycopyrrolate (Robinul-V, West-Ward, Eatontown, NJ, USA) and oxymorphone (Numorphan, Endo Pharmaceuticals, Chadds Ford, PA, USA) or

hydromorphone (West-Ward, Eatontown, NJ, USA). Following pre-medication, dogs were induced with propofol (Rapinovet, Abbott Labs, Chicago, IL, USA) and intubated, and anesthesia was maintained with sevoflurane (SevoFlo, Abbott Labs, Chicago, IL, USA). Thoracolumbar vertebral column imaging was performed either via magnetic resonance imaging (MR) or computed tomography (CT). Cerebrospinal fluid was then collected via needle puncture of the cerebellomedullary cistern with an aliquot saved and stored at -80° C for further analysis. Following CSF collection and diagnostic imaging, a hemilaminectomy was performed to remove herniated, compressive intervertebral disc material and associated hemorrhage from the epidural space. Either gross appearance of the disc material or histopathology was used to confirm the diagnosis of IVDH.

Following surgery, dogs were recovered and provided intravenous fentanyl citrate (Hospira Inc., Lake Forest, IL, USA) analgesia and bladder evacuation if unable to voluntarily void. Twenty-four hours later, physical rehabilitation consisting of supported overland walking, passive range of motion, and standing strength exercises were initiated. Dogs were released to their owner's care after pain control was achieved via oral analgesics (tramadol hydrochloride, Amneal Pharaceuticals, Hauppauge, NY, USA) and urine could be voluntarily voided or the bladder manually expressed. The owners continued physical rehabilitation exercises for 6 weeks post-operatively.

Variable		SCI dogs (n=44)	Healthy control dogs (n=21)
Dogs			
	Median age	5.75 yrs	3 yrs
	MFS at admission	2.5	N/A
	Injury to anesthesia time	36.5 hrs	N/A
Sex characteristics	Female intact	4(9%)	0(0%)
	Female spayed	18(41%)	3(14%)
	Male intact	8(18%)	5(24%)
	Male neutered	14(32%)	13(62%)
Breeds			
	Dachshund	34(76%)	0(0%)
	Labrador Retriever	0(0%)	7(33%)
	Mixed breed	4(9%)	5(24%)
Injuries			
	T12-13	6(14%)	N/A
	T13-L1	13(30%)	N/A
	L1-L2	5(11%)	N/A
	L2-L3	8(18%)	N/A
	Other thoracic	8(18%)	N/A
	Other lumbar	4(9%)	N/A
Treatments			
	Non-steroidal anti-inflammatory drugs	17(39%)	0(0%)
	Glucocorticoids	14(32%)	0(0%)
	Both	3(<1%)	0(0%)

Table 2.1 Population characteristics for dogs with spinal cord injury (SCI) and healthy control dogs used in ELISA experiments, Reprinted with permission from (Russell et. al., 2016).

2.2.5 Neurological scoring

Two separate ordinal SCI scores were used in this study, and were applied at initial evaluation and at a 42-day post-SCI re-evaluation. The modified Frankel score (MFS), and the Texas Spinal Cord Injury Score (TSCIS) have both been validated previously in dogs with IVDH-associated SCI and have been shown to have excellent inter-rater agreement, correlate well with MRI-based measures of SCI, and predict 42-day post-SCI motor outcome[163]. For both assessment tools, dogs were considered ambulatory if they could rise unassisted and take 10 or more steps without falling. Dogs that were non-

ambulatory had pelvic limb movements evaluated using tail support. Postural reaction scores were determined by supporting the dog in a standing position and placing the dorsum of the paw in contact with the ground. Conscious perception of mild and severe stimuli was evaluated by pinching the interdigital webbing and clamping the nail bed with hemostats, respectively. Pain sensation was considered intact based on demonstration of a behavioral (e.g., orienting to the stimulus, vocalization) or physiological (e.g., tachycardia, tachypnea) response to stimulation.

Variable		SCI dogs (n=21)	Healthy control dogs (n=21)
Dogs			
	Median age	5 yrs	3 yrs
	MFS at admission	2	N/A
	Injury to anesthesia time	21 hrs	N/A
Sex characteristics	Female intact	0(0%)	0(0%)
	Female spayed	0(0%)	3(14%)
	Male intact	4(19%)	5(24%)
	Male neutered	17(81%)	13(62%)
Breeds			
	Dachshund	15(71%)	0(0%)
	Shih Tzu	1(5%)	0(0%)
	Poodle	1(5%)	0(0%)
	Coton De Tulear	1(5%)	0(0%)
	Labrador Retriever	0(0%)	7(33%)
	Mixed breed	3(14%)	5(24%)
Injuries			
	T12-13	2(9%)	N/A
	T13-L1	6(29%)	N/A
	L1-L2	1(5%)	N/A
	L2-L3	3(14%)	N/A
	Other thoracic	6(29%)	N/A
	Other lumbar	3(14%)	N/A
Treatments			
	Non-steroidal anti-inflammatory drugs*	0(0%)	0(0%)
	Glucocorticoids	0(0%)	0(0%)
	Both	0(0%)	0(0%)

Table 2.2 Population characteristics for dogs with spinal cord injury (SCI) and healthy control dogs used for MS/MS

* Non-steroidal anti-inflammatory drugs (NSAIDs) directly affect actions of PGE2 and other eicosanoid products from arachidonic acid metabolism. Therefore, samples from dogs treated with NSAIDs were removed from analysis.

Variable	Median	Range
Microprotein (mg/dL)	24	<10 - 63
Red blood cells (/µL) *	2	0-50
White blood cells (/µL)	2	0-30

Table 2.3 Cerebrospinal fluid analysis for injured dogs in MS/MS
* samples with red blood cell counts higher than 50 were not analyzed.

The MFS was used as a cordinal system to stratify injured dogs into groups that parallel those in the American Spinal Cord Injury Association Impairment Scale (ASIA). The MFS consists of 6 strata where 0 = paraplegic with absent pain sensation from the hindquarters; 1 = paraplegic with pain sensation intact to severe stimuli; 2 = parapaplegic with intact sensation for mild stimuli; 3 = non-ambulatory paraparetic; 4 = ambulatory paraparetic; and 5 = signs consistent with spinal pain only.

The TSCIS was developed as a more refined system than the MFS and was used for all analyses that did not require stratification into broad functional categories. With this system, individual limbs are assessed independently and given a score based on sensation, gait, and proprioceptive placing. Sensation was scored as 0 = absent, 1 = sensation present to severe stimuli, but absent for mild stimuli, and 2 = sensation intact. Proprioceptive placing was scored as 0 when absent, 1 when delayed (correction to normal posture taking > 2 seconds), and 2 when considered normal. For gait assessment, scores ranged from 0-6 for each limb as follows: 0 = no voluntary movement present when supported; 1 = intact limb protraction with no ground clearance; 2 = intact limb protraction with inconsistent ground clearance; 3 = intact limb protraction with consistent ground clearance; 4 = ambulatory with moderate paresis/ataxia (will fall

occasionally); 5 = ambulatory with mild paresis/ataxia (does not fall even on slick surfaces); and 6 = normal gait.

2.2.6 Measurement of AA pathway metabolite concentrations

Cerebrospinal fluid concentrations of PLA2, LTC4, and PGE2 were measured using commercially available ELISA (MyBioSource, San Diego, CA). The PLA2 ELISA (for the lipoprotein-associated isoform), catalog # MBS015390, was performed following the manufacturer's protocol, using a standard curve ranging from 800-25 ng/mL. The LTC4 and PGE2 ELISAs (catalog #MBS013956 and MBS705363, respectively) were also performed following the manufacturer's protocol, and the standard curves used were 31.2 -1000 pg/mL for LTC4 and 31.25-2000 pg/mL for PGE2. All control CSF samples were run as technical duplicates. A single run was performed on injured dog samples, because volume available was limited.

2.2.7 Mass spectrometry

CSF samples from dogs with SCI (N=21) and healthy dogs (N=21) were sent to the West Coast Metabolomics center for solid phase extraction-liquid chromatography-electrospray ionization tandem mass spectrometry (MS/MS). Briefly, samples were thawed on ice and then mixed by vortex. 50 μL of CSF sample was mixed with 200 μL of surrogate standards and incubated at -20°C for 30 minutes. Samples were centrifuged at 15,000 rcf at 6°C for 5 minutes. Filtered supernatant was transferred into a PVDC filter plate and stored at -20°C until further analysis.

2.2.8 Statistics

The primary objective of this study was to determine whether the concentrations of the AA pathway metabolites in CSF are associated with functional recovery status at 42 days. These relationships were explored first by using univariable linear regression with TSCIS at 42 days as the outcome (dependent variable). Multivariable linear regression was then used to examine the effects of the various possible interactions including initial injury severity, time delay between injury and sampling, NSAID and GC administration, through their inclusion as covariates. All analyses were conducted using commercially available software (Stata 11, StataCorp, College Station, TX).

Secondary objectives of this study were to explore relationships between injury and AA metabolite concentrations. We compared the CSF metabolite concentrations between control and SCI dogs using Mann-Whitney tests. Association between AA metabolite concentration and cell count in the CSF and with SCI severity at presentation were analyzed using linear regression. Figures were generated using GraphPad Prism, version 6.0 (GraphPad Software, San Diego, CA).

Descriptive statistics were calculated for dog demographics including age, sex, breed, injury to CSF collection interval, and injury severity (Table 2.2). Concentrations of 71 metabolites were collected for each of the 21 injured dog CSF samples and 21 healthy control samples. For the specific MS/MS equipment used here, there is a limit of detection (LOD) and a limit of quantification (LOQ) for each metabolite analyzed. Because MS/MS is optimized for use with urine and blood samples, most of the sample metabolite concentration from the CSF samples were below the LOQ values. Therefore, in order to utilize more of the information from this data set, a “limit of use” was set for each

metabolite half-way between the LOD and the LOQ values. Anything below this half-way value was deemed below the limit to which we could reliably trust the data and changed to a value of 0.00. From this analysis, out of 2,982 metabolite concentrations for 71 metabolites in 41 animals, 2,092 concentrations were changed to 0.00, leaving 890 metabolite concentrations that were non-zero values. Differences in abundance of CSF metabolites between dogs with SCI and healthy purpose bred controls were analyzed for biomarker analysis and pathway analysis in the publicly available MetaboAnalyst software [164]. Data was normalized using pareto scaling (mean-centered and divided by the square root of the standard deviation of each variable). The heatmap of the top 25 metabolites, the fold change graph, and the volcano plot were all generated on MetaboAnalyst. Volcano plot statistical analysis was also generated in MetaboAnalyst. Dot plots were generated using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA).

2.3 Results

2.3.1 Population characteristics ELISA

There were 44 dogs in the SCI group (Table 2.1). The median age was 5.75 years (range, 1-12 years). The 3 most common breeds were dachshunds (n=34; 76%), mixed breeds (n=4; 9%), and shih tzus (n=3; 7%). There were 4 intact females (9%), 18 spayed females (41%), 8 intact males (18%), and 14 neutered males (32%). The median duration between the time of initial injury and CSF collection was 36 hours (range, 3-182 hours). The median MFS before CSF acquisition was 2.5 (a score indicating non-ambulatory paraparesis; scores ranged from 0-5). The median TSCIS sub-scores at presentation

were as follows: nociception 4 (range, 0-4), proprioceptive placing 0 (range, 0-3), and motor 2 (range, 0-10). The most common vertebral levels at which compressive/contusive lesions were located based on MR and CT imaging included: T12-T13 (N = 6; 14%), T13-L1 (N = 13; 30%), L1-L2 (N = 5; 11%), and L2-L3 (N = 8; 18%); there were 8 dogs with thoracic injuries at other levels (18%) and 4 with lumbar injuries at other levels (9%). There were 17 dogs (39%) that received NSAIDs, 14 that received glucocorticoids (32%), and 3 dogs (<1%) that received both NSAIDs and glucocorticoids.

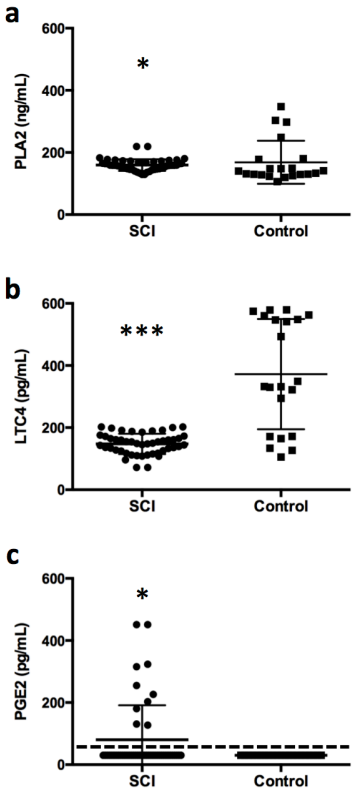

Figure 2.2 Scatter plots and line and whisker plots
CSF concentrations of AA metabolites from 44 dogs with SCI and 21 control dogs. There was a significantly higher CSF PLA2 concentration in dogs with SCI compared to control dogs (asterisks, p= 0.0370) (Panel a). The concentration of LCT4 in the CSF of SCI dogs was significantly lower than that in control dogs (asterisks, p<0.0001) (Panel b). The concentration of PGE2 in the CSF of SCI dogs was significantly higher (Panel C, asterisks, p = 0.0273) compared to that in control dogs (<31pg/mL, dotted line). Reprinted with permission from Russell et. al., 2016.

The 21 control dogs had a median age of 3 years (range, 1-4). The 2 most common breeds were Labrador retriever and beagle (33% and 24%, respectively, Table 2.1). There were 0 intact females, 3 spayed females (14%), 5 intact males (24%), and 13 neutered males (62%). No control dogs received glucocorticoids or NSAIDs.

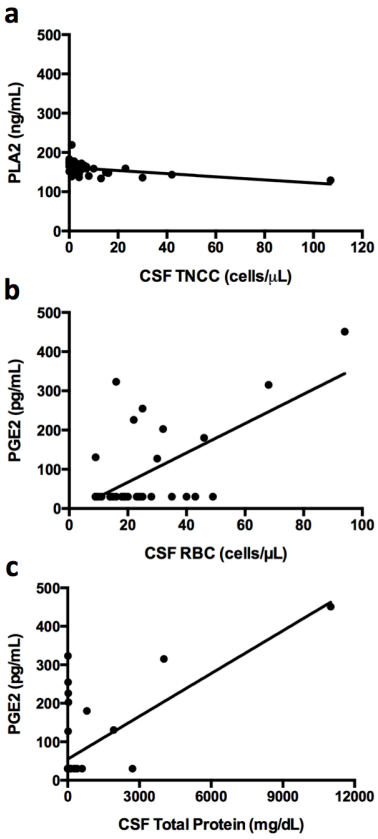

Figure 2.3 Linear regressions amongst CSF AA pathway metabolite concentrations
Compared to total nucleated cell counts (TNCC, cells/μL), CSF total protein (mg/dL), and CSF red blood cell count (cells/μL) in 44 SCI dogs. PLA2 concentration was negatively associated with TNCC in dogs with SCI (r^2= 0.178, slope = -0.400; p= 0.004) (Panel a). CSF PGE2 concentration correlated positively with CSF total protein concentration (Panel b, r^2= 0.422, slope= 3.75; p<0,0001, and CSF RBC (Panel c, R squared= 0.451, slope= 0.370; p<0,0001). Reprinted with permission from Russell et. al., 2016.

2.3.2 Population characteristics MS/MS

There were 21injured dogs that received decompression surgery and 21 healthy dogs that were used as controls (Table 2.2). The median age was 5 years (range 3-12 years). The most common breed was dachshunds (n=15, 71%) and mixed breed (n=3, 14%) while the remaining dogs were of various breeds. There were 17 neutered males (81%), 4 intact males (19%). Females were excluded in this study. The median duration from injury to sampling was 20.5 hours. The range of injury duration to time of sampling was from 7- 44.5 hours. The median MFS at the time of collection was 2 (range 0-3), indicating non-ambulatory paraparesis. Dogs did not receive GCs or NSAIDs within 30 days of being enrolled in the study nor did they receive any during the duration of the study as per standard animal care procedures.

The healthy control dogs had a median age of 3 years (range 1-4). The 2 most common breeds were Labrador Retriever (33%) and beagle (24%). There were 0 intact females, 3 spayed females (14%), 5 intact males (24%), and 13 neutered males (62%). None of the healthy controls received GCs or NSAIDs.

2.3.3 CSF analysis ELISA

In the SCI group, the median TNCC was 2 cells/μL (range, 0 - 107 cells/μL), the median red blood cell count (RBC) was 10 cells/μL (range, 0 - 11005 cells/μL) and the median total protein concentration was 18 mg/dL (range, 9-94 mg/dL). Twelve dogs had pleocytosis (TNCC > 5 cells/μL); of these the median percentage of neutrophils was 49% (range, 0-85%), monocytes 24.5% (range, 0-100%), lymphocytes 12% (range, 0-63%), and eosinophils was 0% (range, 0-3%). No pleocytosis was detected in control CSF samples. In the control group, the median TNCC was 0 cells/μL (range, 0 - 2 cells/μL),

the median RBC was 3 cells/μL (range, 0 - 730 cells/μL) and the median total protein was 26 mg/dL (range 10-35 mg/dL).

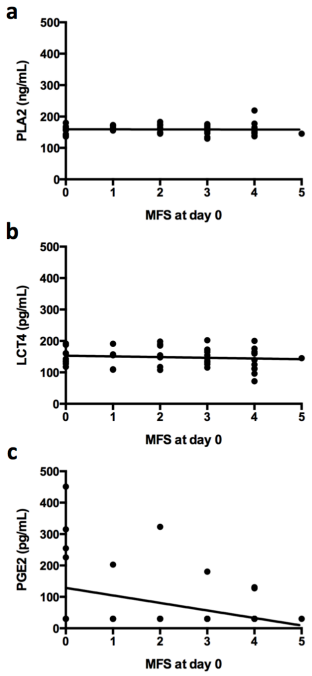

Figure 2.4 Linear regressions of CSF AA pathway metabolites
Modified Frankel scores (MFS) at day of hospital admission (day 0). PLA2 and LTC4 were not significantly correlated to MFS at day 0 (r^2= 0.0004, slope = -0.221; p= 0.894, and r^2= 0.012, slope = 0.-2.23; p= 0.4756, respectively) (Panels a and b). PGE2 was higher in SCI dogs with lower MFS at day 0 (r^2 = 0.137, slope = -23.9; p= 0.013) (Panel c). Reprinted with permission from Russell et. al., 2016.

2.3.4 CSF analysis MS/MS

Dogs in the injured group (N=21) had CSF collected and cell counts were totaled (Table 2.3). CBC analysis was performed before anesthesia and before decompression

surgery. The RBC counts were used to determine if CSF samples were of high enough quality to use by only using samples that had RBC counts of less than 50 cells/μL.

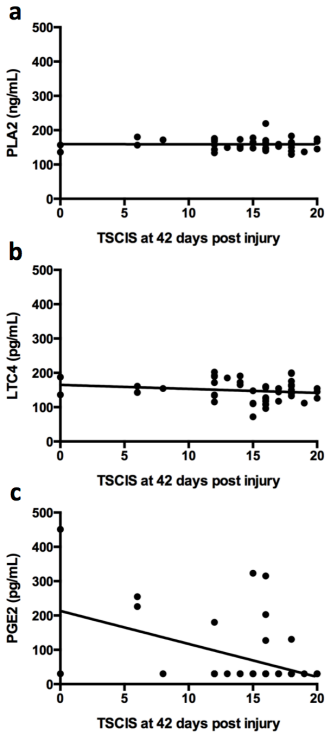

Figure 2.5 Linear regressions of AA pathway metabolites and Texas Spinal Cord Injury Scores (TSCIS) at day 42 post-injury
PLA2 and LTC4 were not significantly correlated to TSCIS at day 42 (r^2= 0.00003, slope = -0.021; p= 0.970, and r^2= 0.030, slope = 0.-1.166; p= 0.262, respectively) (Panels a and b). PGE2 was significantly correlated to lower TSCIS at day 42 in dogs with SCI (r^2= 0.199, slope = -9.62; p= 0.002) (Panel c). Reprinted with permission from Russell et. al., 2016.

2.3.5 AA pathway mediators are dysregulated in the CSF of SCI dogs

There was significantly higher CSF PLA2 concentration (p= 0.0370) in dogs with SCI (median=158.65 ng/mL, range, 129.47-219.45 ng/mL) compared to control dogs (Figure 2.2a, median=140.08 ng/mL, range, 106.04-347.93 ng/mL, reprinted with

permission from Russell et. al., 2016). The concentration of LCT4 in the CSF of SCI dogs (median=148.69 pg/mL, range, 71.88 - 202.37 pg/mL) was significantly lower ($p<0.0001$) than that in control dogs (median=332.27 pg/mL, range, 105.49 - 579.09 pg/mL) (Figure 2.2b). The concentration of PGE2 in the CSF of SCI dogs (median<31pg/mL, range <31 – 451.07 pg/mL) was significantly ($p = 0.0273$) greater compared to control dogs (Figure 2.2c; all control dogs had CSF containing concentrations that were below the limit of detection for this kit <31pg/mL).

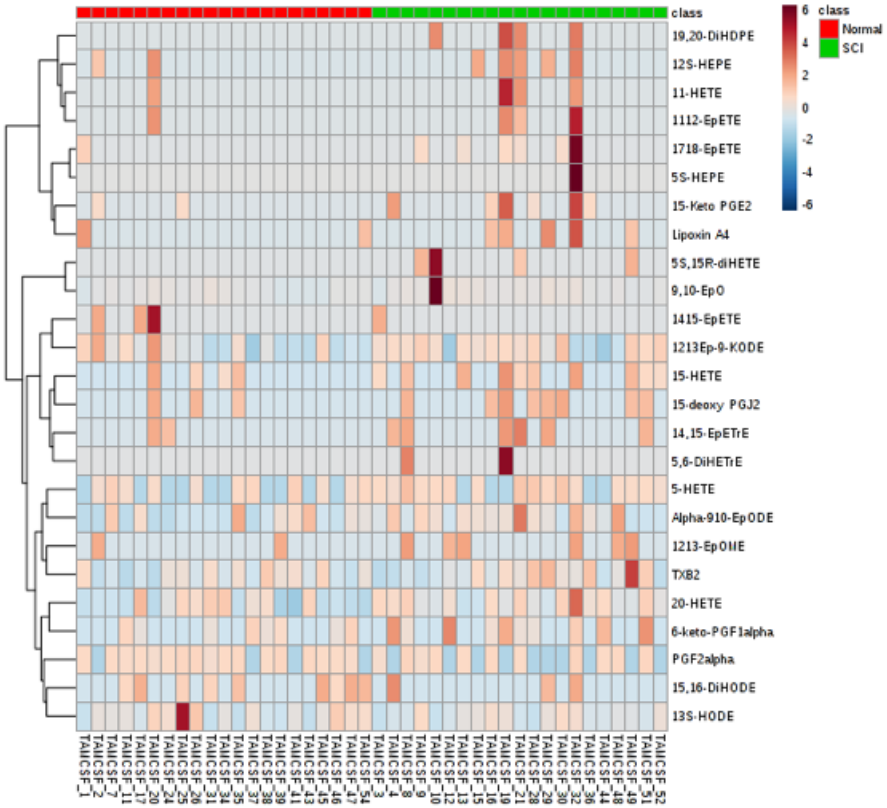

Figure 2.6 Hierarchical clustering heatmap of the top 25 metabolites tested for all SCI and normal healthy dogs analyzed.
Each individual dog is in a column, with all normal on the left and SCI dogs on the right. Each metabolite is a single row. The lines on the left represent hierarchical clustering. The Scale bar is in the top right corner.

Metabolite	Log fold change
15- Keto PGE2	1.84
17,18-EpETE	1.82
5S-HEPE	1.64
5S, 15R- diHETE	1.63
19,20 DiHDPE	1.53
17,18-DiHETE	1.51
9,10-EpO	1.49
9,10-EpOME	1.47
12,13-EpOME	1.41
15,16-EpODE	1.40
17-HDoHE	1.37
Lipoxin A4	1.12
14,15-EpETrE	1.07
15-HETE	1.05
19,20-EpDPE	-1.21
14,15-EpETE	-1.47
13-HOTE	-1.51
4-HDoHE	-1.89

Table 2.4 Cerebrospinal fluid log fold changes (FC) for metabolites FC > 1.0

We explored associations between CSF analytes including TNCC, RBC, total protein concentration, and percentage of leukocytes and AA pathway metabolite concentrations in the CSF of SCI dogs. The only significant associations were between PLA2 and TNCC (Figure 2.3a, r^2= 0.178, slope= -0.400; p=0.004, reprinted with permission from Russell et. al., 2016), PGE2 and CSF total protein (Figure 2.3b, r^2= 0.422, slope= 3.75; p<0.0001), and PGE2 and CSF RBCs (Figure 2.3c, r^2= 0.451, slope= 0.370; p<0.0001).

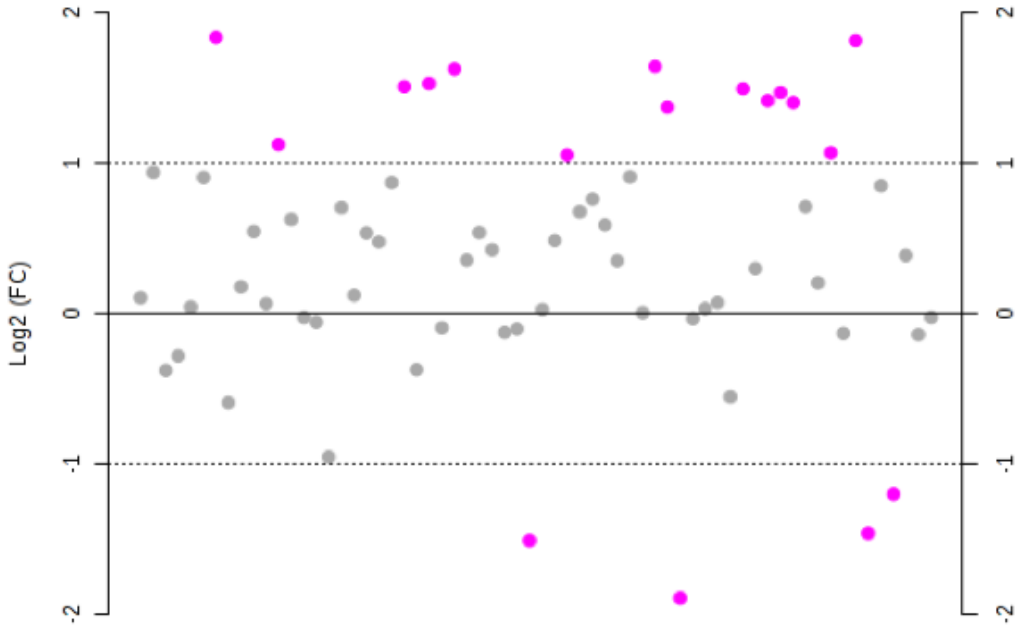

Figure 2.7 Fold changes of metabolites from cerebrospinal fluid (CSF) from spinal cord injured (SCI) and healthy dogs
Dotted lines are at +/- 1 log fold-change. There are 14 metabolites elevated in SCI dog CSF compared to healthy dogs (pink dots > 1) and 4 metabolites that are decreased in SCI dog CSF (pink dots < -1).

2.3.6 CSF PGE2 is correlated with SCI severity and 42-day outcome

Prostaglandin E2 concentration in the CSF was significantly and positively associated with increasing severity of SCI at the time of sampling, as measured by the MFS in univariate and multivariate models (Figure 2.4c, p=0.029 and p=0.041, respectively). No other AA mediators were associated with SCI severity at the time of sample acquisition (Figure 2.4a-b, reprinted with permission from Russell et. al., 2016). The CSF concentrations of PLA2 and LTC4 were not significantly associated with 42 day post-SCI TSCIS (Figure 2.5 a-b, p=0.970 and 0.262, respectively, reprinted with permission from Russell et. al., 2016). Prostaglandin E2 concentration was significantly

and negatively associated with 42-day post-SCI recovery as measured by the TSCIS in univariate and multivariate models (Figure 2.5c, r^2= 0.199, slope= -9.61, p=0.003 and p=0.006, respectively). Because of the low number of SCI dogs in which PGE2 reached detectable concentrations we examined the sensitivity of this result to the numerous null values by repeating the test but including only the dogs with detectable values; both univariable (r^2=0.39, slope= -10.47, p=0.073) and multivariable (r^2=0.51, slope= -1.77, p=0.137) analysis revealed a non-significant association, although this may also result from the much-reduced power of these tests.

2.3.7 AA metabolites heat map and fold changes determined by MS/MS

The hierarchical clustering and heatmap of the top 25 metabolites can be seen in Figure 2.6. Dogs with SCI had 12 metabolites elevated higher in SCI dogs compared to healthy controls. There were 4 metabolites downregulated more than 1.0-fold change difference (Figure 2.7). The log fold change values are shown in Table 2.4.

2.3.8 Volcano plot of AA pathway metabolites determined by MS/MS

A volcano plot, which is a scatter plot that compares the fold change difference (threshold FC > 1.5) to the p-values from t test ($p < 0.05$, Figure 2.8). The only metabolite to be significantly different on the volcano plot ($p<0.05$, FC=1.53) was 19,20 dihydroxydocosapentaenoic acid (DiHDPE). Along with 19,20 DiHDPE, the next four highest metabolites scatter plots are shown in Figure 2.9a-e. Thromboxane B2 (TBX2) concentrations were not different between groups, but all measurements were above the limit of detection for this analysis (Figure 2.9f).

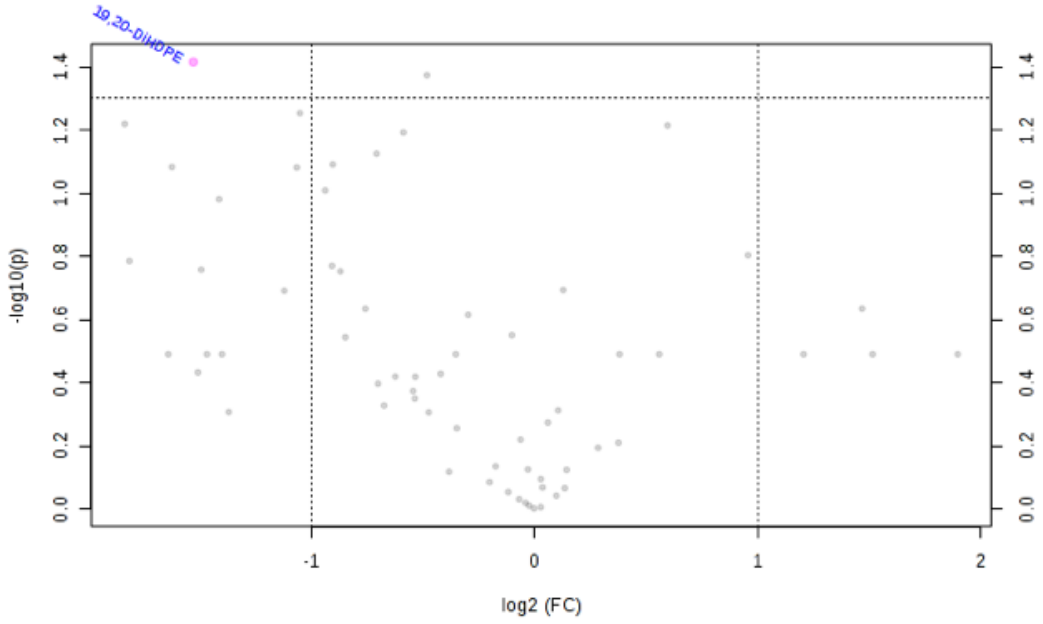

Figure 2.8 Volcano plot analysis of differentially expressed metabolite concentrations for all cerebrospinal fluid samples from spinal cord injured and healthy dogs.
The further the metabolite position from 0,0, the more significant the difference from the entire group. The pink dot represents the metabolite above thresholds for fold change (FC, x-axis) and t-test p-value (p, y-axis). 19,20 HiHDPE is above the 1.5-fold change cutoff with a p-value > 0.05 (horizonal dotted line).

2.4 Discussion

This study broadly compared measures of AA metabolism in dogs after naturally-occurring SCI with that in healthy control dogs. The concentration of PLA2, which frees AA from phospholipid membranes, was significantly higher in SCI versus control dogs, and had a weak negative association with the total nucleated cell count in the CSF. The CSF concentration of LTC4, a pro-inflammatory leukotriene, was significantly lower in dogs with naturally-occurring SCI compared to control dogs. The CSF concentration of PGE2 was significantly higher in SCI dogs compared to control dogs, and significant

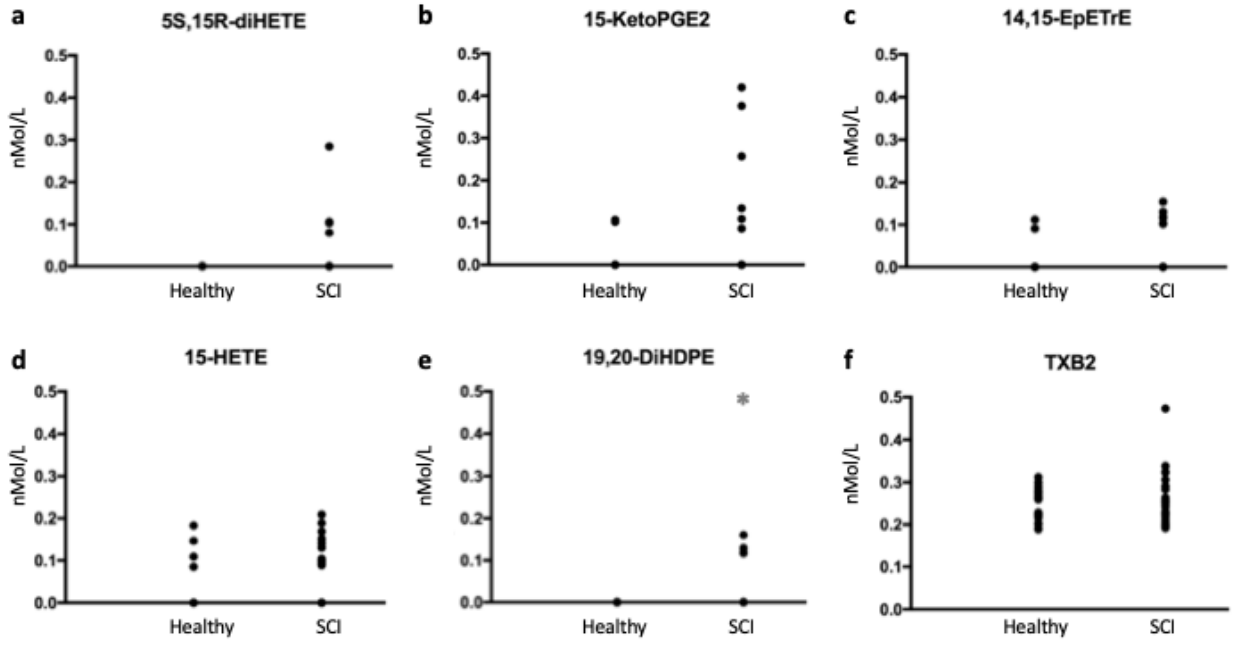

Figure 2.9 Dot plots for metabolite concentrations of 6 metabolites from cerebrospinal fluid (CSF) of healthy and spinal cord injured dogs.
There were 4 metabolites that trend towards elevated concentrations in CSF of dogs with SCI (a-d). Metabolite 19,20- DiHDPE was significantly elevated in CSF of injured dogs with volcano plot analysis (e, gray asterisk, $p < 0.05$, $FC > 1.5$). Thromboxane B2 (TXB2) has measurable amounts for every individual CSF sample analyzed (f). TXB2 levels were not significantly different between the two groups ($p > 0.05$).

relationships existed between CSF PGE2 concentration, initial SCI severity, as well as 42-day post-SCI recovery.

Here, we found CSF concentration of lipoprotein-associated PLA2 was significantly higher in SCI dogs compared to control dogs. In studies performed on rodent spinal cord homogenates post-SCI, the secretory isoform of PLA2 increases within hours of injury, is over-expressed for days following SCI, and is negatively correlated with recovery of locomotion [159]. Critically, there are 27 isoforms of PLA2, of which only 7 have been clearly demonstrated to be dysregulated in SCI [159]. We chose to measure lipoprotein-associated PLA2 because it has not been previously evaluated in the context of SCI, up-

regulation has been established in neuro-inflammatory diseases such as Alzheimer's [165], it is secreted from inflammatory cells known to be present within injured cords, and validated methodologies existed to measure it in dogs. While findings here suggest lipoprotein-associated PLA2 is released following injury, there was no association between CSF PLA2 concentration and injury severity at the time of sampling or 42-day post-SCI outcome. Additionally, CSF PLA2 concentration was weakly, but negatively, associated with CSF TNCC. The complex and overlapping role of PLA2 isoforms, treatment of dogs with immune-modulating drugs, and sample size may explain our inability to detect associations between CSF lipoprotein-associated PLA2 concentrations and certain facets of injury.

The CSF concentration of LTC4 was significantly higher in healthy control dogs compared to those with SCI. This finding is in contrast to data from a guinea pig spinal cord contusion model, which showed increased parenchymal LTC4 10 minutes after SCI that persisted 60 minutes post-SCI. Data from dogs with experimental spinal cord contusion likewise showed abrupt, early increases in LTC4 concentration, measured within the CSF [161]. Our results may have differed from these previous studies for a variety of reasons. First, the median time between SCI and CSF sampling in our population was 36 hours; thus, we may not have captured many dogs with post-injury elevations in LTC4. Secondly, a proportion of the naturally injured dogs studied here received either NSAIDs or glucocorticoids, both of which could reduce LT production. Additionally, post-SCI increases in CSF or parenchymal LTC4 may be species- specific, or model- specific. In a study of cats with experimental compressive SCI, LTC4 concentration was not significantly different between sham and SCI animals [166].

Finally, we believe post-SCI shunting within the LT pathway could be possible and might explain the higher CSF LTC4 concentration in control dogs compared to those with injury in this study. For example, macrophages (the predominant inflammatory cell that releases LTC4) that are exposed to a pro-inflammatory environment *in vitro* have reduced LTC4 synthase mRNA expression [167].

A prominent finding from this study is the 1.84 log fold change difference of 15-Keto PGE2 in CSF of injured dogs over healthy control dogs. Although this finding was not mathematically significant, our group has previously seen a similar pattern of significantly increased concentrations of PGE2 in CSF of dogs with SCI [23]. Prostaglandin E2 is ubiquitous throughout the body and is most known for its deleterious effects as a vasodilator and fever inducer [168]. However, PGE2 is also a well-known inhibitor of neutrophil reactive oxygen species (ROS) release [24, 25, 169]. The more reactive form is PGE2, while the downstream product 15-Keto PGE2 is the less active and more stable form of PGE2 [170]. It is unclear whether cells within the CSF of dogs with spinal cord injury are actively converting the more reactive PGE2 isoform into the less reactive 15-Keto PGE2, or if the conversion is a product of unchecked inflammation. More studies are needed to explore PGE2 and its isoforms.

The finding of elevated 19,20 dihydroxydocosapentaenoic acid (DiHDPE) in the CSF of SCI dogs is notable because of its association with fish oil as a dietary supplement. This diol is a downstream product from docosahexaenoic acid (DHA), which is an omega-3 poly unsaturated fatty acid which is a main component of fish oil. Increases in ingested DHA causes increases in serum levels of 19,20 DiHDPE [171, 172]. Interestingly, administration of DHA improves neurological outcomes in rats with SCI

[173, 174]. In this study, the log fold change difference between dogs with SCI and healthy controls was 1.53. It is not clear whether elevated levels of 19,20 DiHDPE in the CSF of injured dogs is a pathway that is beneficial for SCI and should be promoted, or if increased levels of the diol in the CSF is an indication that lipid and protein oxidation are detrimental processes that may be potential targets for therapy. More research is needed to investigate the specific role of 19,20 DiHDPE in naturally-occurring SCI.

Interestingly, the eicosanoid thromboxane B2 was one of the few metabolites from this study that had detectable concentrations in every CSF sample. However, there were no differences between dogs with SCI and healthy controls ($p > 0.05$). In rodent studies, TXB2 concentration in the injured spinal cord are highest 1-hour post injury and then reduced back down to normal levels as soon as 8 hours post injury [175-177]. Our findings from the current study are consistent with these results. The duration of injury before sample collection ranged from 7 hours to up to 44.5 hours post-injury. One advantage to working with experimental models of SCI is the time of CSF sampling is less variable. However, in a clinical environment with both humans and dog, collecting CSF samples less than 12 hours post injury is not feasible [79]. Likewise, targeting mediators of inflammation that are elevated minutes to a few hours after injury are probably not feasible targets for SCI therapeutics that can be tested in large scale clinical trials.

Cerebrospinal PGE2 concentration was significantly increased in dogs with SCI compared to healthy control dogs. Additionally, CSF PGE2 concentration was significantly and positively associated with CSF protein concentration and RBC in injured dogs; both these CSF analytes are increased as a result of intrathecal bleeding and blood-spinal cord barrier disruption [178]. Finally, CSF PGE2 concentration was significantly

and positively associated with more severe injury at the time of sampling and also 42-day post-SCI recovery as measured by an ordinal scoring system. The relationship between CSF PGE2 and 42-day post-SCI recovery was assessed using multivariate logistic regression. The strength of this approach is that it takes account of other contributory factors such as immunomodulatory drug administration, initial injury severity, and timing of injury. When we assessed associations between CSF PGE2 and 42-day outcome only using dogs with SCI that had detectable CSF PGE2, the relationships were non-significant. While examining relationships in this manner does eliminate bias from null values, it substantially reduces statistical power (9 dogs assessed). Biologic facets of SCI and recovery are typically investigated in rodent contusion models under a series of highly controlled conditions. Here, we assessed AA pathway metabolites in a naturally-occurring, large animal model of SCI that recapitulates many features of human injury. There are limitations inherent to utilizing samples from dogs with naturally occurring SCI, one of which is the administration of immune response-modulating drugs prior to sample collection. Multivariate logistic regression was used to mitigate influence of administration of these drugs when assessing relationships between CSF AA metabolite concentration and 42-day outcome. We did not, however, directly examine the impact of prior NSAID or GC delivery on CSF AA metabolite concentration in injured dogs. This study could not be adequately powered to investigate the influence of these drugs because of the great variety of interactions between time of injury, time of drug administration, and time of sample collection. Certainly, it is possible that interactions between these drugs and targets in the AA pathway impacted data reported here. Additionally, heterogeneity in injury severity, timing of injury, and vertebral level of compression that are inherent to

clinical studies can affect the ability to detect significant inter-group differences. Despite these limitations, this study suggests that lipoprotein associated PLA2, LTC4, and PGE2 are all associated with SCI and may provide information relevant to recovery of function. These data, combined with those from other model systems, provide further evidence that AA metabolites are a viable target for pre-clinical SCI trials.

2.5 Future directions

Future directions of this study include tests for the notable AA pathway contributors, such as up and downstream products of PGs using ELISAs rather than large scale metabolomics platforms with less specificity. It would be interesting to know the levels of these metabolites in the plasma of dogs with SCI and compare them to humans with SCI. One of the limitations of this study was low and sometimes undetectable levels of metabolites in the watery CSF, especially in healthy controls where arachidonic acid levels are low to nonexistent. Because CSF collection in dogs is routine clinical practice, samples can be pooled from dogs of similar age, breed and sex. These future studies might help the field characterize the inflammatory response following SCI and could contribute to the background knowledge needed to find an appropriate therapeutic for the cure for SCI.

3. NEUTROPHIL ACTIVATION IN CIRCULATION

3.1 Background

A problem in treating spinal cord injury is mitigating secondary injury and inflammation. One of the key components of inflammation following spinal cord injury is leukocyte infiltration into the damaged spinal cord [10, 179-182]. The first cells to infiltrate from circulation into the damaged cord are neutrophils. Traditionally, neutrophils have been studied mostly in the context of bacterial infection and their effective role in breaking down infected cells and dismantling bacteria is recognized as their principal role in the inflammatory response. However, neutrophils recently have garnered more attention in their role following SCI [183, 184]. In rodents, controlling neutrophil and monocyte recruitment and infiltration into the injured spinal cord have led to improved neurological outcomes and reduced lesion size [185-188]. Although, other studies in rodents have shown the benefits of neutrophils in SCI and peripheral nerve crush injury [10, 30]. It is probable that infiltrating neutrophils are both beneficial and detrimental to regrowth and repair of the damaged spinal cord, and therefore, special attention should be given to the spatial and temporal changes in neutrophil activity following SCI. In addition to inconsistent results in rodent studies, questions remain about neutrophil activation and infiltration in humans and large animal models of SCI.

Neutrophils studied in the context of acute spinal cord injury are known to respond to damage-associated molecular patterns (DAMPs), activate granules and produce reactive oxygen species (ROSs), and express L-selectin in order to leave the blood vessel and enter the damaged spinal cord. Neutrophils are transient cells under physiological conditions and have a half-life of several hours in humans [42, 43]. However, when

activated, neutrophils can live for multiple days [20, 189]. Neutrophils are granulocytes produced in the bone marrow that function primarily to break down pathogens and cellular debris using ROSs and proteases. After responding to DAMPs and producing ROS, neutrophils can express varying amounts of L-selectin on their surface. L-selectin is a firm adhesion marker that neutrophils in circulation express constitutive amounts of, but can also upregulate L-selectin. Once the glycocalyx is reduced, ligands for L-selectin and other adhesion molecules are exposed on the endothelium surface. Neutrophils then shed L-selectin, release more of their granular

contents to get through the basement membrane, and leave the blood stream [190] (Figure 3.1). Lymphocytes and monocytes also express L-selectin on their surface, but enter the damaged cord after neutrophils [191]. Although much is known about initial neutrophil recruitment and activation in acute SCI, much less is known about how long activated and infiltrating neutrophils exist in SCI [34, 59], especially in humans and large animal models of SCI.

Neutrophils respond to DAMPs non-specifically, meaning that they can be activated by broken bones or soft tissue damage (such as lacerations or incisions) at the same time responding to spinal cord injury. Therefore, neutrophils after SCI are best studied under conditions without polytrauma. Secondly, because neutrophils respond to skin incisions and soft tissue damage, models of naturally-occurring injury are also ideal for the study of neutrophils. Therefore, experiments measuring neutrophil activity were carried out in dogs with intervertebral disk herniation (IVDH) with spontaneous SCI, a model that closely resembles human injury but without polytrauma. IVDH is common in

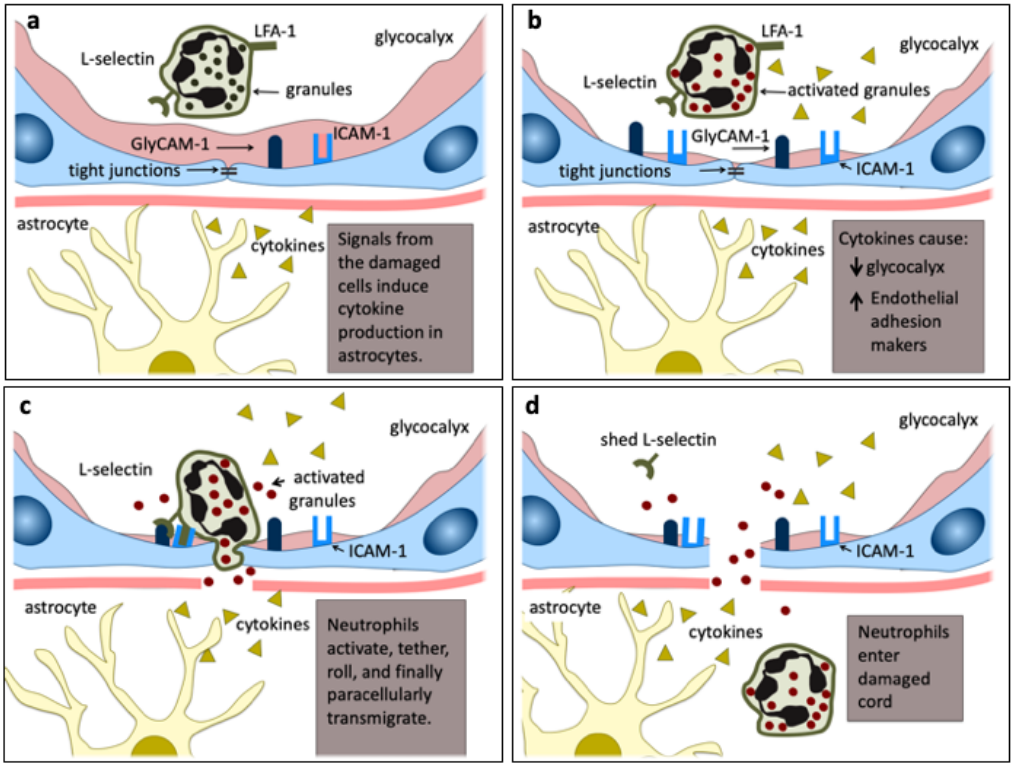

Figure 3.1 Neutrophils in circulation
Neutrophils extravasate from circulation by releasing activated granular contents and expressing adhesion molecules on the cell surface. Neutrophils express constitutive amounts L-selectin and LFA-1 and have non-activated granules in normal circulation before damage signals enter the blood stream (a). When damage signals reach the blood stream, the glycocalyx is reduced, exposing ligands on the endothelial surface. Damage signals cause neutrophil granule activation (b). Neutrophils release activated granules and adhere to the endothelial membrane using the firm adhesion molecule, L-selectin (c). After passing through the endothelium, neutrophils can shed L-selectin and are now activated and in the damaged spinal cord (d).

dachshunds, and is diagnosed with CT or MRI and treated with decompression injury [138-141], very similar to humans. In addition to diagnosis and treatment similarities, dogs suffering from IVDH and secondary SCI have a similar window for treatment modalities. Often times, rodent studies are “proof of principal” studies using genetically modified animals, or with treatment modalities administered within hours of injury. With clinical

patients, dogs or humans, proof of principal studies are out of the scope of what is possible for characterization of inflammation events following SCI.

This work has been done to characterize the response of neutrophils to SCI in dogs for several days and weeks following spinal cord injury. To our knowledge, this is the first time peripheral neutrophil activity in dog SCI has been monitored beyond 0-3 days post injury.

Subject Group	Case #	Breed	Age (years)	Sex	Spinal Level(s)	Injury to anesthesia time	Modified Frankel Score				
SCI Surgical							Day 0	Day 3	Day 7	Day 30	Day 90
	TAMU # 17	Dachshund	4	MC	T11-12, T12-13	10	2	2	3	4	4
	TAMU # 19	Dachshund-LH	7	FS	T12-13	48	3	2	3	4	5
	TAMU # 20	Dachshund-LH	3.5	MC	L1-L2	15	2	2	3	4	4
	TAMU # 21	Dachshund-M	4	FS	L1-2; L2-3	22	2	2	NA	NA	NA
	TAMU # 22	Dachshund	5	MC	L3-4; L4-5	12	2	3	3	NA	NA
	TAMU # 23	Chihuahua	3	MC	T11-13; 12-13	18	0	0	0	0	0
	TAMU # 24	Havanease	9	MC	T13-L1; L1-L2	23	3	3	3	3	4
	TAMU # 27	Chiweenie	2	F	L1-2; L2-3	45	3	3	4	4	4
	TAMU # 28	Dachshund-M	9	SF	T13-L1; L1-L2	13	3	NA	NA	4	4
Uninjured							Single Day Neurology Evaluation				
	TAMU # 5	Beagle	7	M					Normal		
	TAMU # 16	Beagle	5	FS					Normal		
	TAMU # 18	Dachshund-M	3	MC					Normal		
	TAMU # 25	Dachshund	3.5	MC					Normal		
	TAMU # 26	Dachshund	9.5	FS					Normal		
	TAMU # 29	Shih Tzu	9	MC					Normal		
	TAMU # 30	Chihuahua	3	MC					Normal		
	TAMU # 31	Chiweenie	2	FS					Normal		
	TAMU # 33	Cocker Spaniel	8	FS					Normal		

Table 3.1 Population characteristics for subjects
-M = miniature -LH= long-haired, MC= male castrated, FS= female spayed.

3.2 Methods

3.2.1 Dogs

All animal procedures were conducted with approval from the Texas A&M University Animal Care and Use Committee (AUP# 2015-0129). Procedures in dogs with naturally-occurring SCI were performed with owner consent and consisted of standard medical and surgical care. Enrollment in this study was from July 2015 to June 2018. Dogs with SCI underwent physical and neurologic examination. Dogs were included in the study if they met the following criteria: 1) non-ambulatory for less than 48 hours from time of hospital admission; 2) no history of back pain; 3) no delivery of non-steroidal anti-inflammatory drugs (NSAIDs) or glucocorticoids (GCs) within the last 30 days; 4) no vaccine administration within the last 14 days; 5) and SCI between the spinal levels of T3-L3. Dogs were excluded from further participation if they had additional neurologic disease unrelated to SCI, illness or disease in which the immune system was functioning abnormally (e.g. cancer or inflammatory bowel disease), inflammation unrelated to SCI (e.g. clinically significant skin infection), uncooperative, pregnant, or weighed less than 4 kg (9lbs). If all inclusion criteria were met, then owners would give consent for the dogs to participate in 5 blood draws on the day of hospital admission (day 0) and days 3,7,30, and 90 post-surgery. The inclusion criteria of non-ambulatory status was assigned using the modified Frankel score [192]. Only dogs with a grade of 0 defined as paraplegia with no deep nociception, grade 1 defined as paraplegia with no superficial nociception, grade 2 defined as paraplegic with nociception, or grade 3 defined as non-ambulatory paraparesis (weight bearing and non-weight bearing), were enrolled in this study.

Healthy dogs were recruited from Texas A&M University employees and 6 out of the 10 were matched for breed, sex, and age within 20% of the injured match. After matching 6 healthy dogs to the injured dogs, the AUP was amended to include an increased number of reference population dogs, and 4 of the 10 control animals were enrolled as reference controls that were chondrodystrophic breeds [193]. Healthy matched and reference control dogs (N=9) met the same inclusion and exclusion criteria as the injured dogs, with the exclusion of spinal cord injury.

3.2.2 Neutrophil isolation

Blood draws of 10 mLs were collected from the jugular vein using a 22 G needle and syringe and placed in 2 EDTA coated plastic 6 mL tubes. Blood in the EDTA tubes was transported from the clinic to the laboratory on ice. The following experiments were conducted at 4°C or using ice cold reagents. Whole blood was transferred to a 15 mL conical tube and centrifuged at 4°C for 10 minutes at 1800 rpm using a swinging bucket rotor. The supernatant was collected as plasma and was aliquoted and stored at -80°C. The remaining blood was mixed 1:1 with ice cold Phosphate-Buffered Saline, pH 7.4 (PBS) and was carefully layered onto room temperature Histopaque®-1077 (Millipore-Sigma, Darmstadt, Germany, cat# 10771) on top of Histopaque®-1119 (cat# 11191) and centrifuged at room temperature for 30 minutes at 1600 rpm with the break off. The top layer of cells was collected as peripheral blood mononuclear cells, and aliquoted and stored at -80°C in RPMI 160 Medium (ThermoFisher, Waltham, MA, cat# 11875093) with 10% Fetal Bovine Serum (cat# 10437-036, FBS) and 20% Dimethyl Sulfoxide (Millipore-Sigma, cat# D8418, DMSO). The bottom layer was collected as neutrophils into a fresh

15 mL conical tube and washed twice with ice cold PBS and centrifuged at 4°C at 1050 rpm for 4 minutes. Red blood cells were lysed by first freeing the cells from the bottom of the tube by gently pipette mixing with 80 μL of ice-cold PBS. Then, 2-3 mLs of ice-cold H_2O was applied to the loosened cell pellet for promptly 30 seconds. Isotonicity was restored by adding a 1:1 volume of 2X PBS. Cells were washed twice with PBS and centrifuged at 4°C at 1050 rpm for 4 minutes. Cells were counted using 0.2% trypan blue exclusion dye and a Cellometer Auto 1000 (Nexcelom Bioscience LLC, Lawrence, MA). Neutrophils were brought to a concentration of $1x10^6$ cells/mL with RPMI.

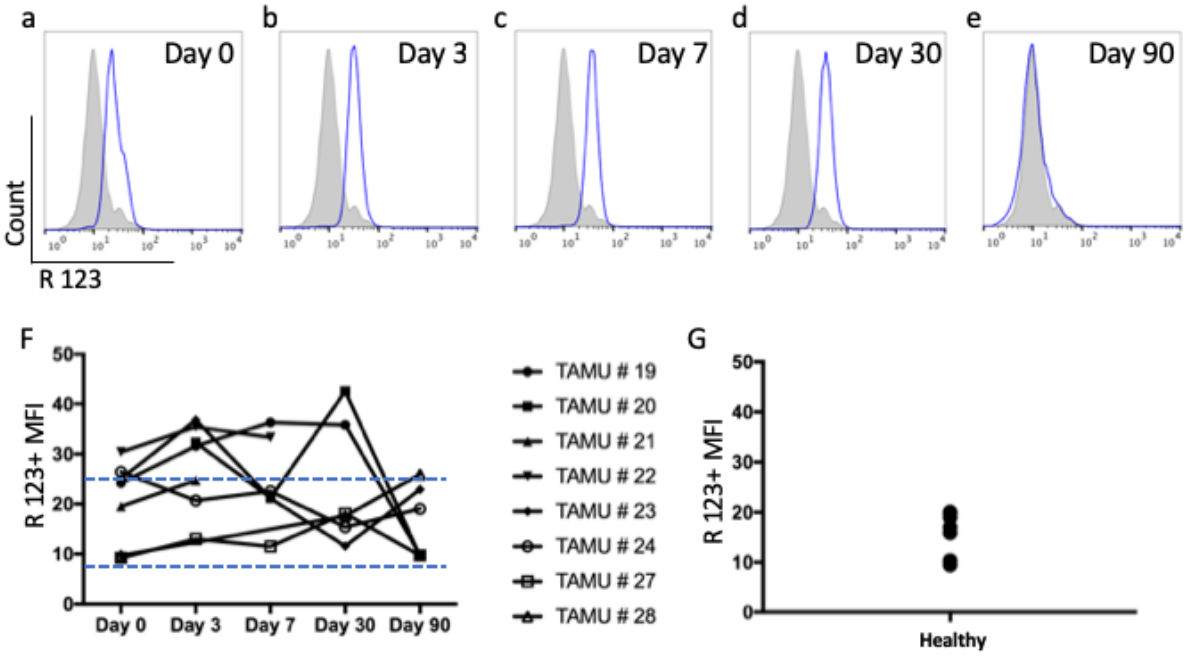

Figure 3.2 Neutrophils oxidative burst activity
Oxidative burst activity in healthy and injured dogs as a measurement of median fluorescent intensity (MFI) of dihydrorhodamine 123 conversion to rhodamine 123 (R 123). A representative sample of a healthy dog (gray shaded) and the injured matched control on days 0,3,7, 30 and 90 (a-e). The summary of all 8 injured dogs on a Bland-Altman plot (f). The healthy dogs OBA were measured from a single blood draw (g).

3.2.3 Oxidative burst activity assays

Neutrophils were isolated from dogs with spinal cord injury on the day of hospital admission (day 0) and days 3,7,30 and 90 post-surgery, and from healthy controls on a single day. A count of 400,000 cells were incubated in a 24-well culture plate in RPMI at 37°C with 5.0% CO_2 for 1.5 hours before the addition of 50 μM Dihydrorhodamine 123 (DHR 123, Millipore-Sigma, cat# 109244-58-8). After 20 minutes incubation with DHR 123, cells were transferred to round bottom tubes, centrifuged at 1050 rpm for 4 minutes at 4°C, and the supernatant was removed. Cells were resuspended in ice cold PBS with 6% FBS (FACS Buffer) and underwent flow cytometry on a BD FACS Calibur (BD Biosciences, San Jose, CA) to measure the conversion of non-fluorescent DHR 123 to Rhodamine 123 in the FITC channel.

Subject Group	Case #	OBA, Median Fluorescent Intensity (MFI)				
SCI Surgical		Day 0	Day 3	Day 7	Day 30	Day 90
	TAMU # 17	NA	NA	NA	NA	NA
	TAMU # 19	24.2	31.6	36.3	35.8	9.8
	TAMU # 20	NA	32.3	21.2	42.5	9.6
	TAMU # 21	19.5	24.7	NA	NA	NA
	TAMU # 22	30.4	35.4	33.3	NA	NA
	TAMU # 23	25.0	36.8	21.1	11.6	22.9
	TAMU # 24	26.4	20.7	22.5	15.4	19
	TAMU # 27	9.24	13	11.5	18.1	9.73
	TAMU # 28	9.73	NA	NA	17.6	25.9
Uninjured		Single Day Blood Draw				
	TAMU # 5	17				
	TAMU # 16	8.1				
	TAMU # 18	NA				
	TAMU # 25	9.5				
	TAMU # 26	10.3				
	TAMU # 29	15.9				
	TAMU # 30	19.9				
	TAMU # 31	20				
	TAMU # 33	19.9				

Table 3.2 Individual changes in oxidative burst activity as measured by median fluorescent intensity changes for all dogs

3.2.4 Statistical analysis

Flow cytometry standard files were exported and analyzed using FlowJo version 10.4.2 (FlowJo, LLC, Ashland, Oregon). The ellipse tool was used to gate neutrophils based on forward and side scatter values, and MFI values were obtained. GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA) was used to create scatter and box plots. Data from injured animals were plotted on a Bland-Altman plot to detect differences among samples that were collected on different days [194].

3.2.5 L-selectin expression

A count of 300,000 neutrophils was incubated with 10 ng/mL Lipopolysaccharide from *E. coli* (Millipore-Sigma, cat# L2630) in RPMI at room temperature for 10 minutes. The remaining neutrophils were aliquoted and centrifuged 4°C at 1050 rpm for 4 minutes and resuspended in ice cold FACS Buffer and Human BD Fc Block (BD Biosciences, San Jose, CA, cat# 564219) following the manufacturer's protocol. After blocking, cells were brought to a volume of 350 μL with FACS buffer and incubated with the following antibodies following the manufacturer's protocols (1:80 dilution) and incubating on ice in the dark for 30 minutes: rat anti-mouse CD11b:FITC, Clone M1/70 (Biolegend, San Diego, CA, cat# 101206); mouse anti-human CD62L:RPE, Clone FMC46 (Bio-Rad, Hercules, CA, cat# MCA1076PET); and 7 Aminoactinomycin D staining solution for cell viability (7AAD, TONBO biosciences, San Diego, CA, cat# 10140-986). Neutrophils were centrifuged 4°C at 1050 rpm for 4 minutes and resuspended in 350 μL of fresh FACS buffer. Anti-Rat and Anti-Mouse BD CompBeads were incubated with 1 μL of antibody as per the manufacturer-s protocol and run on the BD FACS Calibur prior to all neutrophil

samples and used for compensation. Neutrophil suspensions with and without antibodies (unstained control) fluorescent intensities were measured on the FACS Calibur (BD Biosciences). Flow cytometery standard files were exported and analyzed using FlowJo version 10.4.2 (FlowJo, LLC, Ashland, Oregon). In FlowJo, the ellipse tool was used to gate neutrophils based on forward and side scatter values. Next, dead cells were excluded using a rectangle gate on cells that were 7AAD negative. Then, viable neutrophils were gated using the rectangle tool to isolate only cells that were CD 11b+. Finally, a rectangle gate was used to demarcate $CD62L^{High}$ and $CD62L^{Low}$ cells based on the average of the matched control animals mean fluorescent intensity scores (Figure 3.3).

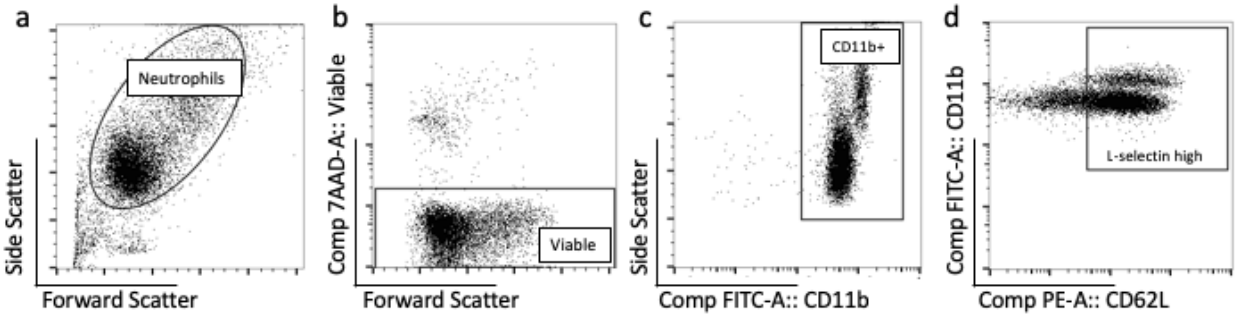

Figure 3.3 Gating strategy for neutrophils expressing high amounts of L-selectin
Neutrophils are isolated from blood and then the ellipse gate is applied to the side and forward scatter plot (a). Next, cells are gaited for negative 7-Aminoactinmycin (7AAD), a fluorescent dye that has a strong affinity for nuclear DNA and therefore will only bind to cells with dead cells (b). All neutrophils express constitutive amounts of CD11b, an adhesion marker that is functional for cell adhesion when in complement with CD18. Only neutrophils that were CD11b+ were gated for further analysis (c). Finally, L-selectin high cells were gated using a rectangle gate applied to the healthy control neutrophil scatter plots (not pictured). All L-selectin high gates were identical for all scatter plots obtained and percentage of L-selectin high neutrophils were calculated (d).

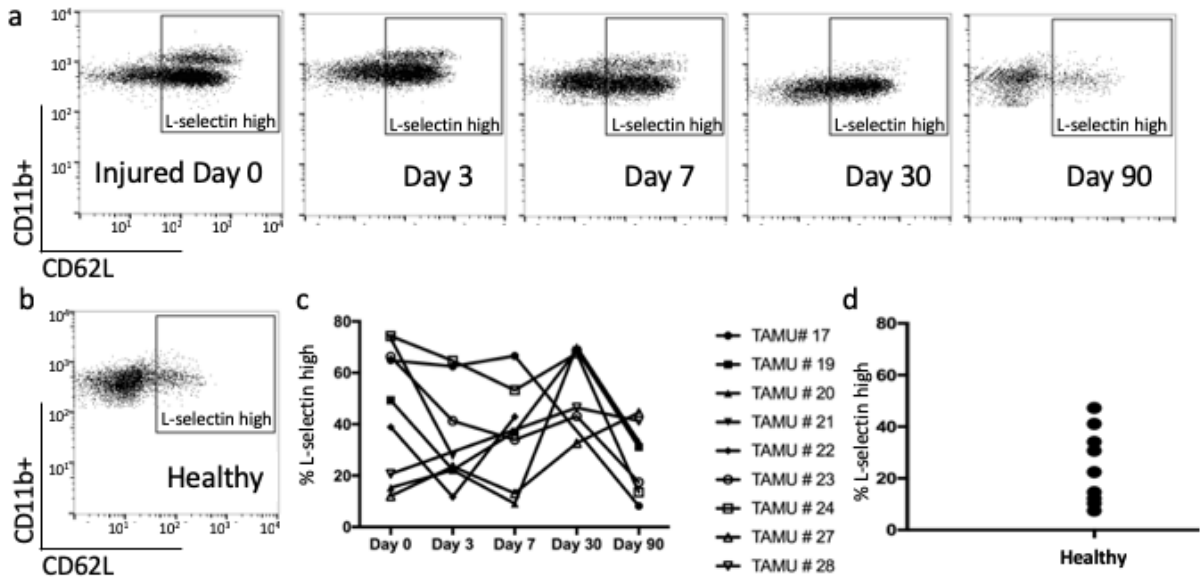

Figure 3.4 Percentage of cells with L-selectin high expression
A representative of an injured animal with all 5 time-points, where there are more cells with L-selectin high expression 0-30 days post injury and very few at 90 days post injury (a). The healthy matched control dog has fewer cells that are L-selectin high expressing (b). The percentage of neutrophils expressing high amounts of L-selectin among all injured animals (c). Healthy animal expression of high L-selectin (d).

3.3 Results

3.3.1 Population characteristics

There were 9 injured dogs that received decompression surgery and 9 healthy dogs that were used as age, breed, and sex matched controls or reference controls (Table 3.1). For the 9 injured dogs, the median age was 4 years (range 2-9 years). The most common breed in the injured group were dachshunds (n=6, 67%), while the remaining dogs were of various breeds (n=3, 33%). There were 5 neutered males (56%), 3 spayed females (33%), and 1 intact female (11%). The median duration between first reported time of injury and blood collection on day 0 was 18 hours (range 10-48 hours). The median

MFS at the time of the blood draw on day 0 was 2 (range 0-3), indicating non-ambulatory paraplegia.

There were 6 healthy control dogs that had the same age, breed, and sex characteristics as the injured animals, with the exception of all the dogs in the control group were either spayed or neutered, respectively (Table 3.1). After collecting 6 matched from chondrodystrophic breeds to be used as reference controls [195], the AUP was amended to allow for more healthy control blood collections.

3.3.2 Oxidative burst activity in peripheral circulating neutrophils

To measure OBA in circulating neutrophils, the conversion of non-fluorescent DHR 123 to fluorescent R123 was measured in SCI and healthy dogs (Table 3.2). Higher MFIs indicate higher levels of OBA by isolated neutrophils. One individual injured dog and a second individual matched-control dog is shown in Figure 3.2. Of the 9 injured dogs enrolled in the study, 7 of them were measured for OBA. On day 0, the range of MFIs was between 9.73-30.4 for 6 dogs that had blood drawn on day 0. The range of neutrophil activity on day 3 was 20.7-36.8 for 6 dogs. Day 7 MFIs ranged from 21.1-32.8 for 5 dogs. For the 5 dog neutrophils measured on day 30, the MFI range is the largest, from 11.6-35.8. Measurements of MFI for all injured animals were plotted on a Bland-Altman plot to detect differences in measurements outside the range of normal values or +/- 2 standard deviations away from the mean (Figure 3.2F, dotted lines).

Subject Group	Case #	L-selectin (% cells)				
SCI Surgical		Day 0	Day 3	Day 7	Day 30	Day 90
	TAMU # 17	64.9	62.5	66.6	NA	8.2
	TAMU # 19	49.3	22.2	37	68.8	31.1
	TAMU # 20	15.3	22.1	9.1	70.1	33.2
	TAMU # 21	73.4	27.5	NA	NA	NA
	TAMU # 22	38.9	11.7	42.9	NA	NA
	TAMU # 23	66.2	41.3	33.9	43.1	17.4
	TAMU # 24	74.3	64.7	53.2	67.6	13.5
	TAMU # 27	12	23.5	13.1	32.6	44.5
	TAMU # 28	20.6	NA	NA	46.4	41.3
Uninjured		Single Day Blood Draw				
	TAMU # 5	7.5				
	TAMU # 16	NA				
	TAMU # 18	41.1				
	TAMU # 25	47.2				
	TAMU # 26	12.2				
	TAMU # 29	22.4				
	TAMU # 30	30.7				
	TAMU # 31	34.1				
	TAMU # 33	10.3				

Table 3.3 Individual changes in L-selectin ^{high} as measured by rectangle gate inclusion

3.3.3 L-selectin expression in peripheral circulating neutrophils

L-selectin expression was measured using an antibody to L-selectin. The gating strategy for identifying L-selectin expression is shown in Figure 3.3A-D. The percentage of cells that were expressing high levels of L-selectin were measured using a rectangle gate, and an identical gate was applied to all scatter plots. A representative sample of one injured animal (Figure 3.4A) and the matched healthy control (Figure 3.4B) are shown. Expression of L-selectin^{high} neutrophils for all injured animals are shown in Figure 3.4C and individual dog L-selectin^{high} neutrophils are shown in Table 3.3. The percentage of cells that were expressing high amounts of L-selectin for the healthy controls is shown in Figure 3.4D.

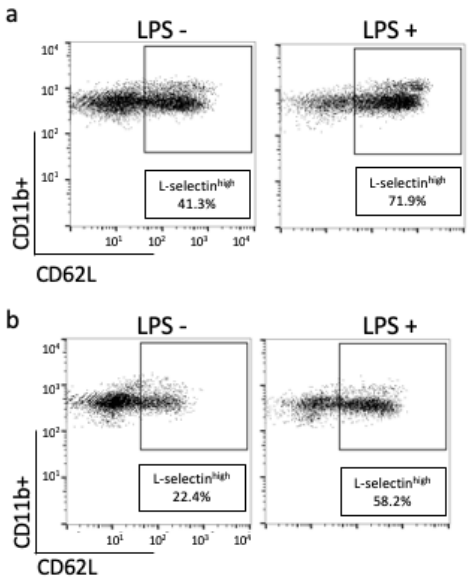

Figure 3.5 Changes in L-selectin ^{high} neutrophils after stimulation with LPS
Lipopolysaccharide (LPS) measured by rectangle gate inclusion and then subtracting the LPS+ L-selectin ^{high} from the LPS- sample. All neutrophils stimulated with LPS expressed higher amounts of L-selectin on the cell surface. Representative samples of LPS- and LPS+ from single subject from the injured group (TAMU # 28, A). Representative samples of LPS- and LPS+ from single subject from the control group(TAMU # 29, B).

Neutrophils were incubated with LPS to artificially regulate L-selectin expression on the cell surface (Figure 3.5). In all cases, LPS induced L-selectin^{high} expression. Differences between the amount of L-selectin^{high} expression were calculated by subtracting L-selectin^{high} values from the LPS- sample from the L-selectin^{high} LPS+ values (Table 3.4). The highest values indicate a higher capacity for expressing higher amounts of L-selectin. The largest differences between LPS+ and LPS- values were from day 90 samples and the healthy controls, although these results were not significant ($p<0.05$).

3.3.4 Trends from day 0 and day 3 from OBA and L-selectin

OBA values on day 0 all increased on day 3, with the exception of one subject (Figure 3.6A). Inversely, L-selectin expression decreased from all animals with the exception of 1 individual (Figure 3.6B).

3.4 Discussion

These experiments were conducted to characterize the neutrophil response in peripheral circulation following a naturally-occurring spinal cord injury in canine clinical patients. Even with the abundance of successful pre-clinical trial data in experimental rodent studies, there is still a high failure rate for human large-scale clinical SCI studies [73, 196]. Development of highly reproduceable and standardized injuries that show statistical significance between groups have lead researchers to rely on rodents for pre-clinical trials [119]. Recent attention has been given to the importance of large animal SCI research to bridge the gap between human and experimental rodent SCI [121, 197]. Likewise, the role of neutrophils in inflammation following SCI has been given more credence than in years past [184]. More studies characterizing the immune response in large animal models are needed to help identify areas where preclinical work can be improved and large animal models can be used when preclinical work fails to reach full potential in human clinical trials.

This study in dogs with SCI addresses some of the concerns with experimental rodent SCI since the individuals in this study were suffering from spontaneous injury, clinically relevant patient enrollment (i.e. non-ambulatory inclusion criteria), sufficient

blood volumes for repeat sampling, no polytrauma, and no prior administration of GCs or NSAIDs.

SCI Surgical	Day 0	Day 3	Day 7	Day 30	Day 90
TAMU # 17	NA	NA	9.7	NA	NA
TAMU # 19	11.1	15	32.2	5.1	24
TAMU # 20	NA	9.8	15.8	NA	28.3
TAMU # 21	8.3	0.2	NA	NA	NA
TAMU # 22	35.8	51.4	25.1	NA	NA
TAMU # 23	4.1	23.6	45.6	33.6	NA
TAMU # 24	1.7	20.7	33	NA	32.1
TAMU # 27	NA	NA	20.9	10.2	NA
TAMU # 28	NA	NA	NA	11.2	30.6
Uninjured	**Single Day Blood Draw**				
TAMU # 5	12.58				
TAMU # 18	38.5				
TAMU # 25	34.9				
TAMU # 26	27.1				
TAMU # 29	35.8				
TAMU # 30	17.8				
TAMU # 31	34.2				
TAMU # 32	NA				
TAMU # 33	14.6				

Table 3.4 Individual changes in L-selectin ^{high} neutrophils after stimulation with LPS
Values were obtained by subtracting the LPS+ value from the LPS- value for each sample on each day.

Patients were not given GCs or NSAIDs prior to enrollment or during the course of the study. This is imperative to mention because anti-inflammatories have been shown to directly influence the amount of ROS production and L-selectin expression in circulating neutrophils. In humans, the NSAID diclofenac induces shedding of L-selectin [198]. This mechanism is believed to be achieved by the induction of ROS production to break thiol bonds in L-selectin [199]. Therefore, as ROS production inside neutrophils increases, then L-selectin expression is reduced.

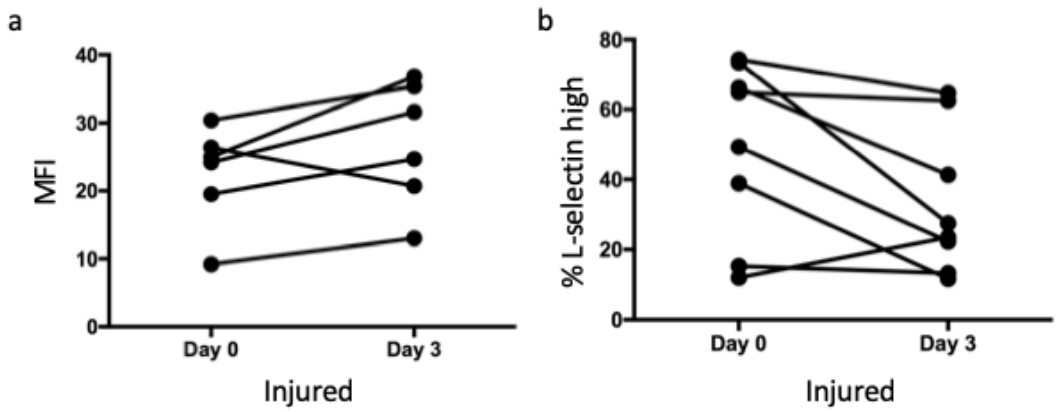

Figure 3.6 Opposing patterns of OBA of L-selectin expression on days 0 and 3 in dogs with spinal cord injury.
In OBA of dogs injured dogs, all activity increases from day 0 to day 3 with one exception (TAMU # 24, panel A). In the same dogs, with the addition of 2 additional dogs, all cells have fewer L-selectin high expressing cells on day 3 compared to day 0 (exception is TAMU # 27, panel B).

The inclusion of a day 0 time-point in this study was significant. Dogs enrolled in this study were clinical patients and the sustained injury for all individuals was naturally-occurring. All blood draws for this time-point were taken prior to surgery, laminectomy, and skin and muscle incisions. This means that neutrophil activity on day 0 was in response to SCI and not any extraneous damage to the skin, muscle, or bone, as there would be in experimental SCI.

Oxidative burst activity of circulating neutrophils in dogs with SCI was highest 3 and 7days post-injury. This is remarkable considering that neutrophils are only expected to be entering the damaged cord 1-3 days post-injury. Although neutrophil activation in circulation does not directly indicate their end goal is to enter the injured cord, increased activity in the periphery does leave room for speculation the reasons why this is happening 7 days after spinal cord injury but not in healthy animals. Measurements of

OBA were decreased back to normal levels by day 90, compared to matched and reference healthy control dogs.

All measurements of OBA from injured dogs were plotted on a Bland-Altman plot to detect differences between two groups. Traditionally, a Bland-Altman plot is used to identify difference in measurements between two different tests or protocols. However, this method has been used to characterize neutrophil surface molecule expression in other disease models [194]. In this experiment, 4 dogs of the 7 measured had elevated levels of oxidative burst activity on day 3 when compared to the healthy control dogs. This result is consistent with studies from humans where neutrophil activity is high days after spinal cord injury [200].

The percent of neutrophils with L-selectin^{high} expression was the most variable on day 0, and surprisingly highest on day 30 in injured dogs compared to healthy dogs. The variability on day 0 in understandable because the inclusion criteria for this study included dogs that sustained injury anywhere from 0- 48 hours prior to hospital admission. The large variability of measurements on day 0 includes a large gap between 3 animals that had smaller percentages of cells producing high amounts of L-selectin (range 12.0-20.6) and 6 animals with higher percentages of L-selectin^{high} expression (range 38.9 – 73.4). One may conclude that the 3 lowest measurements on day 0 are from dogs that sustained less severe injuries when compared to the other injured animals. However, the 3 lowest measurements on day 0 were collected from dogs 13,15, and 45 hours after injury. Similarly, the MFS for these animals (grades 2,3,3) were similar to the median for the group. Because L-selectin has a cyclical rhythm throughout the day [201], the time of blood collection from day zero was analyzed. The blood draws on day 0 ranged from 8:00

am – 4:30 pm. The healthy control dog blood draws were all collected at 8:00 am. On days 3 and 7, blood draws were performed from 8:00-10:00am. On days 30 and 90, the blood draws occurred from 9:00 am-11:00 am. The finding that most consistently high percentage of L-selectin^{high} expression on neutrophils on day 30 is of note because neutrophil activity is thought to be insignificant at 30 days post-injury. Although, these cells are collected from peripheral circulation and could therefore be responding to other systems other than the injured CNS, for example, the incisions from surgery or the laminectomy that all these injured animals were treated with.

Neutrophils were incubated with LPS, the main component in bacterial cell walls. LPS exposure causes activation of neutrophil granules in a calcium dependent manner. Because the blood samples were collected in EDTA coated tubes that chelates all available Ca^{2+}, induced neutrophil activation could not be determined in these experiments. However, LPS does interact with surface expression of L-selectin in a Ca^{2+} independent manner. In these experiments, neutrophils incubated with 10ng/mL LPS all expressed more L-selectin on their cell surface. This result is inconsistent with both rodents and human neutrophils. When exposed to LPS, human and rodent neutrophils will robustly shed L-selectin [202, 203]. It is possible that induced expression of L-selectin by dog neutrophils when exposed to LPS is a species-specific condition.

The two most limiting factors for this study were patient enrollment due to exclusion criteria, and variability of enrolled patients demographics (age, sex, and breed, etc.). Often times, dogs with IVDH do not show obvious signs of acute non-ambulation, but rather, mild symptoms progress over time. Dogs enrolled in this study had a clear event in which the owner noticed their dog “went down” at a specific time, or small window of

time, for example, when they were at work and arrived home. Many cases seen at the Texas A&M small animal hospital during the enrollment period were put on observation for more than 48 hours before receiving surgical intervention, and were therefore not eligible to participate. Another related complication to the 48-hour injury onset inclusion is the administration of GCs or NSAIDs prior to enrollment. Often time these animals are in pain and so primary care physicians will administer pain medication as well as recommending Texas A&M for consulting. GCs and NSAIDs directly affect both OBA and L-selectin expression on leukocytes in circulation, and therefore these dogs were excluded from the study. Another limitation of this study was the amount of variability seen across the population of dogs suffering from IVDH treated at Texas A&M Small Animal Hospital. A power calculation was performed using human data to detect a 1.5-fold change difference between groups. This result yielded 19 animals per group, however, due to patient availability and the stringent inclusion criteria, the enrollment period was closed after only 9 animals were enrolled.

In conclusion, this study is the first to characterize neutrophil function in a longitudinal analysis following naturally-occurring spinal cord injury. Like in humans, dog neutrophils are producing reactive oxygen species for a prolonged period post-injury. Like rodents, dog neutrophils have reduced expression of L-selectin when ROS production is high. However, repeated studies with larger samples sizes should be performed to confirm the results seen here.

3.5 Future directions

The AUP for this project from Texas A&M University included amendments for the addition of two groups: SCI injured non-surgical dogs, and dogs with long bone fractures.

With a longer recruitment period and additional research coordinating staff, these groups of dogs could be included in future studies of neutrophil activation characterization in naturally-occurring spinal cord injury. It would also be interesting to include another group of dogs suffering from bacterial meningitis to have both an infection control and a trauma control (long bone fractures). The addition of the non-surgical SCI cases would be to further elucidate the action of neutrophils after major surgery. These experiments should be repeated in rodent models of SCI where injuries are induced after incisions through muscle and skin and laminectomy are performed.

4. NEUTROPHILS IN DAMAGED SPINAL CORD TISSUE

4.1 Background

In humans, spinal cord injury (SCI) most often occurs secondary to car accidents, and there are thousands of new injuries each day in the United States and around the world [1]. Damage to the spinal cord occurs after a mechanical injury to the spine, which causes damage to blood vessels, tissue swelling, cord laceration or transection, cell death, and eventually glial scarring. Human injury can be classified based on pathological features into 4 different types of lesions: 1) contusion/cyst, 2) cord maceration from massive compression, 3) cord laceration due to open injuries, or 4) solid cord injury (injury affects mostly white matter with gray matter sparing) [204, 205]. In human injury, there is often damage to the ventral part of the cord due to vertebral luxation or disk herniation.

In experimental SCI, rats are subjected to compression or contusion injuries by first anesthetizing the animal and performing a laminectomy to expose the spinal cord and then inducing compression or contusion injury with clips or weight-drop devices, respectively [128, 130, 131]. Contusion injuries with a weight-drop device induce injury to the dorsal side of the cord, although sufficient weight can cause complete paralysis in most cases[130, 133]. Histopathological features of rat experimental injury are comparable to human injury in regards to MR signal enhancement, gross lesion size, and amount of intact or destroyed tissue as seen in histological staining patterns [130]. In both human and rodent SCI, there is an initial influx of neutrophils that peaks 1-3 days post injury [35, 206-208], followed by a secondary infiltration of monocyte-derived macrophages that can last for several months or years [118, 209, 210].

Case #	Breed	Age (years)	Sex	Spinal Level(s)	Injury to tissue collection (days)
1	Maltese	9	MN	Lumbar	2
2	Chihuahua	3	FS	T12-13	4
3	Yorkshire Terrier	4	MN	T13-L1-Myelomalacia	4

Table 4.1 Case summaries for 3 dogs with spinal cord injury
Spinal levels are the areas of diagnosed injury from necropsy reports.

Although experimental SCI is analogous to structural changes seen in human injury, rodent models of induced injury lack heterogeneity inherent to clinical injured patients. To this end, pet dogs with naturally-occurring SCI can be viewed as translational clinical trial model of SCI. Injured dog spinal cords bear many of the same lesion patterns, early myelin abnormalities and axon damage as seen in humans [140]. Like humans but unlike rodents, dogs can sustain naturally-occurring spinal cord injuries. Commonly, this transpires secondary to intervertebral disk herniation (IVDH) [151].

Like both humans and rodents, dogs have an inflammatory response following SCI [77, 82, 211]. In small number of IVDH cases, dogs can sustain a myelomalacia that is accompanied by hemorrhage and extensive neutrophil and macrophage infiltration [144, 145, 212]. This progressive oxidative stress is an extreme type of neuroinflammation following spinal cord injury where the usual mechanisms are inadequate for any kind of recovery, and may be a useful example of secondary injury at the far end of the spectrum [145]. Although there is mention of neutrophil infiltration in these cases, there has yet to be confirmed presence of neutrophil infiltration in dog spinal cord following injury. In this study, neutrophil infiltration in the injured spinal cords of 3 dogs will be described.

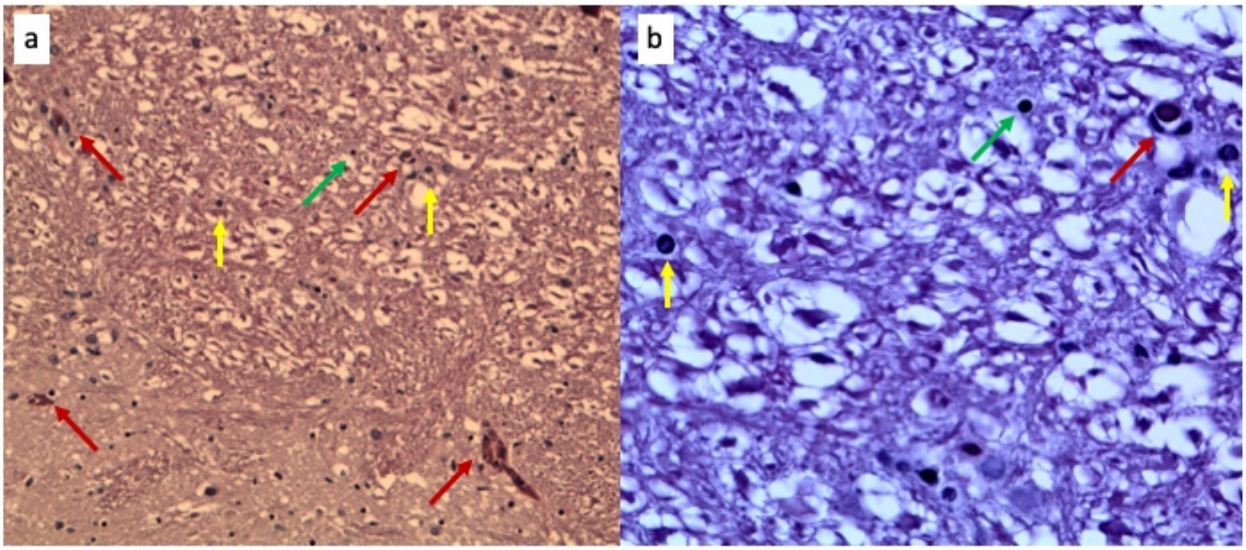

Figure 4.1 Case 1 neutrophils in the spinal cord at the level of injury
20x magnification (a) and 60x magnification (b). Red arrows are pointing to intact blood vessels. Yellow and green arrows indicate neutrophils and lymphocytes, respectively.

4.2 Neutrophils in the injured spinal cords of dogs

Three cases of dogs with IVDH and SCI from patients seen at Texas A&M Small Animal Hospital were included in this description (Table 4.1). Cases were identified from necropsy cases completed by the pathology department in 2017 and 2018 in collaboration with Dr. Brian Porter. The inclusion criteria for these cases were, 1) dogs suffered from acute spinal cord injury, 2) dogs were euthanized 0-7 days post-injury (range for these cases: 2-4 days), 3) dogs were not given glucocorticoids (GCs) or non-steroidal anti-inflammatory drugs (NSAIDs) one week prior to euthanasia, and 4) histology was included as part of necropsy procedures.

Case 1 was a 9-year-old, neutered male Maltese who was first discovered to have discomfort in his hindlimbs while attempting to lie down. The injury progressed until the

patient was non-ambulatory and deep pain negative. Euthanasia was elected by the owner 48 hours after the initial onset of discomfort. Case 2 was a 3-year-old spayed female long-haired Chihuahua who had a history of urinary tract infections two months prior to the onset of IVDH. The patient was non-ambulatory and deep pain negative for 4 days prior to euthanasia. This patient was given unknown pain medication from a primary care veterinarian prior to admission at Texas A&M Small Animal Hospital. Case 3 was a 4-year-old neutered male Yorkshire Terrier who was first ataxic and in pain when held. The situation progress until the third day when the patient became non-ambulatory and deep pain negative. On the third day, the patient was given carprofen, a common NSAID prescribed for pain in veterinary medicine. On the fourth day the patient was taken to Texas A&M. An MRI was performed and the patient was shown to have mildly compressive extradural intervertebral disc extrusion at L1-2 with severe SCI including intraparenchymal hemorrhage. Also, on physical examination on the fourth day, the patient had hyperesthesia, and euthanasia was elected that same day.

In all cases, neutrophils were seen in the injured spinal cords. In case 1, there was no intervertebral disk space collapse observed grossly, but histopathology revealed a subdural hemorrhage with extruded disk material in the lumbar spinal cord. There were multiple examples of neutrophils in the tissue, near but outside of intact blood vessels (Figure 4.1). Case 2 gross pathological findings included acute, extradural hemorrhage extending from T11 to the sacrum. There were some, but not many neutrophils in the tissue of that were outside intact blood vessels (Figure 4.2). Case 3 gross diagnosis was consistent with the clinical diagnosis of T13-L1 IVDH with myelomalacia. Histopathological analysis confirmed extensive myelomalacia and hemorrhage. There

were multiple neutrophils present in the lumbar spinal cord (Figure 4.3). There was obvious extensive hemorrhage in the tissue, along with some intact blood vessels.

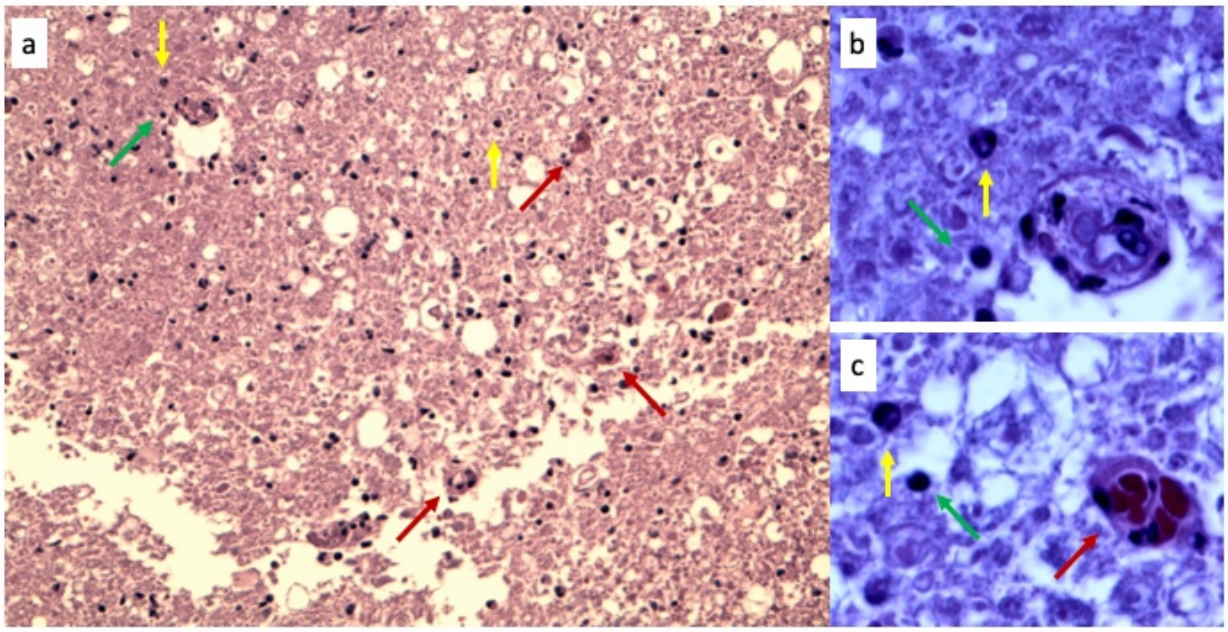

Figure 4.2 Case 2 neutrophils in the spinal cord at the level of injury
20x magnification (a) and 60x magnification (b,c). Red arrows are pointing to intact blood vessels. Yellow and green arrows indicate neutrophils and lymphocytes, respectively.

4.3 Discussion

As with human injury, findings varied between cases. However, one consistent finding between all 3 cases was that neutrophils were present in the injured spinal cords of all three cases. There were more neutrophils observed in the spinal cord of case 3 compared to cases 1 and 2. This is likely due to the fact that the amount of hemorrhage was greater in case 3 compared to the others. In both case 2 and case 3, dogs had received pain medication prior to tissue collection.

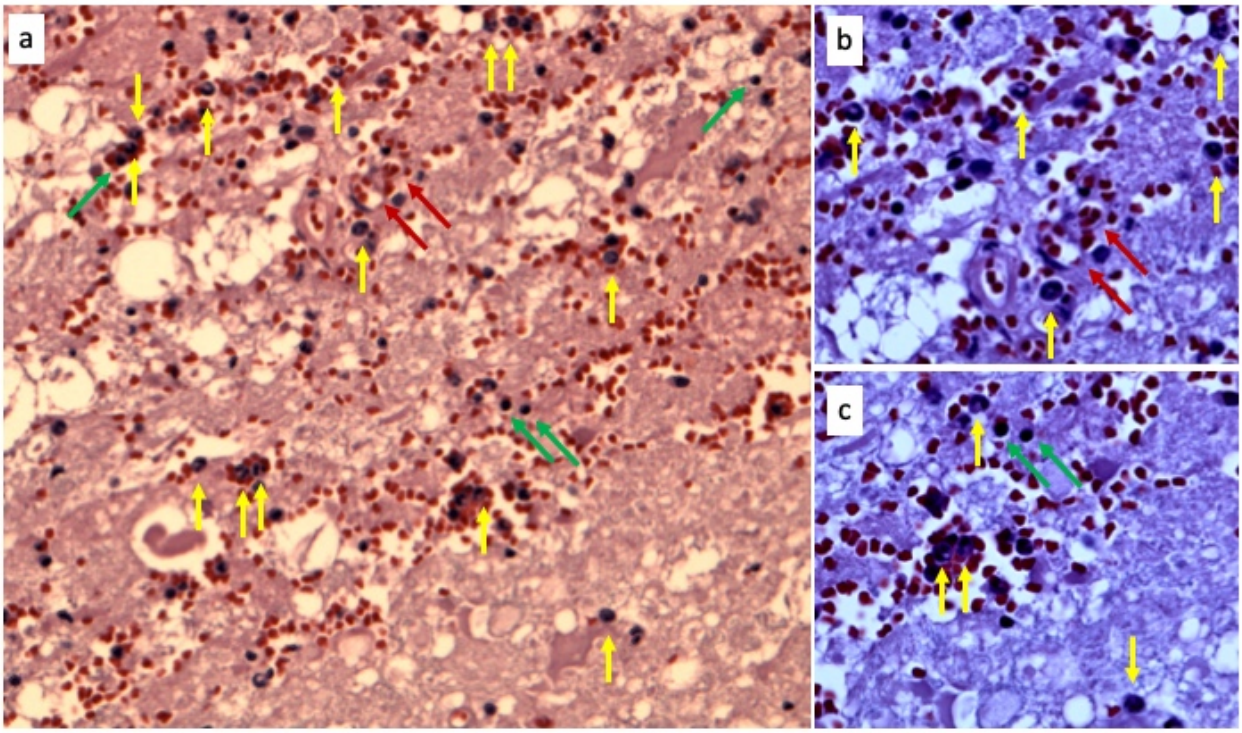

Figure 4.3 Case 3 neutrophils in the spinal cord at the level of injury
20x magnification (A) and 60x magnification (B-C). Red arrows are pointing to intact blood vessels. Yellow and green arrows indicate neutrophils and lymphocytes, respectively.

In summary, neutrophils were identified in the spinal cords of 3 cases of canine IVDH and secondary SCI. This result is consistent with what is seen in both spontaneous human injuries, as well as in rodents with experimental injury. In addition, these results are also consistent with the high variability seen in human clinical settings. This characterization of neutrophil infiltration is a valuable contribution to the effort to make dog spinal cord injury clinical trials more prolific in order to advance more successful treatments into large-scale human clinical trials that might otherwise fail by going straight from rodent to humans.

5. DISCUSSION

The current studies were designed to take advantage of a naturally-occurring clinical model of spinal cord injury and allowed us to characterize the activity of neutrophils over time following spinal cord injury in dogs. Because neutrophils are transient cells under normal physiological conditions, and experiments in rodents identify macrophages, not neutrophils, as the primary driver of post-injury inflammation, one might expect to see low levels of neutrophil activity 1-3 days after injury. However, data reported here indicates that neutrophils in circulation and in the damaged cord have prolonged activation and increased presence, respectively. In addition, there is evidence reported here to support the hypothesis that dogs, like humans and also rodents, have a higher incidence of arachidonic acid metabolism in the cerebrospinal fluid following SCI than is seen in homeostatic conditions.

CSF was collected in a single sampling from dogs with spinal cord injury and from purpose bred healthy control dogs. Increased concentrations of AA pathway metabolites were detected in the CSF of several canine patients who suffered from spinal cord injury, compared to purpose bred healthy controls. Because neutrophils are found in increased numbers in the CSF of dogs with SCI [96], and neutrophils are main cells induced to metabolize arachidonic acid [27, 159, 213], increased AA metabolism in CSF of dogs with spinal cord injuries supports this result. In our studies here as well as previously reported findings indicate that levels of some AA metabolites are steady state (TXB2) or decreased levels (LTC4 [23]) in dogs with spinal cord injury seem to contradict results from studies

conducted in rabbits and rats [214-216]. However, timing of sampling as well as collection techniques differed between dog clinical patients and experimental laboratory animals.

Neutrophils were isolated from peripheral blood of dogs with spinal cord injuries on the day of hospital admission (day 0) and then at 3,7,30 and 90 days post-injury, to evaluate the degree and length of neutrophil activity following spinal cord injury and decompression surgery. Despite the central dogma that neutrophils contribute only negligible amounts to inflammatory cascades after 1-3 days following spinal cord injury, we present evidence to suggest that neutrophils are activated in circulation longer than the 1-3 days, and that neutrophils are present in the injured spinal cords of dogs. Peripheral blood neutrophil production of ROS was elevated in dogs with spinal cord injury compared to healthy control dogs. This result is consistent with peripheral blood neutrophil activity in human SCI [200]. Peripheral blood neutrophil expression of L-selectin was variable across all measurements of injured animals at 0,3,7,30 and 90 days, but was particularly variable on the day of hospital admission. This variability is inherent in clinical studies and is particularly notable because L-selectin expression is cyclical under normal physiological conditions [201], and the dog enrolled on day 0 occurred at the time of hospital admission which varied for each animal. This evidence supports the hypothesis that neutrophils may play a key role in inflammation along with macrophages following spinal cord injury.

In recent past, focus has been on macrophage polarization as the main driver of neuroinflammatory cascades, while the role of neutrophils has been largely ignored [191]. Indeed, a study from Hannover, Germany has yielded important information on the activation of macrophages in SCI in dogs, but the same study also suggested that

neutrophils are not present in the injured dog cord [77]. In these experiments, we report the presence of neutrophils in the injured cord of 3 dogs with spinal cord injuries. In all 3 cases, neutrophils were found to be in the parenchyma and outside of intact blood vessels in the area and in areas lacking large areas of hemorrhage. This result is inconsistent with other studies of dog SCI, but consistent with models of rodent and human SCI.

The following sections are dedicated to the discussion of these results in addition to the unexpected findings, limitations of clinical studies in dogs, and the implications of study for future research of large animal naturally-occurring spinal cord injury.

5.1 Neutrophil activity following SCI

We addressed the question of whether or not neutrophil activity was prolonged in dogs with SCI for longer than 1-3 days post injury. The reason for exploring this area is due to the fact that the neutrophil contribution to inflammatory events after CNS injury have been largely ignored. This makes sense when considering the naturally short half-life of neutrophils in circulation and tissues under normal physiological conditions. After injury neutrophils can survive for longer than a few hours and contribute to inducing and prolonging inflammatory cascades [20]. However, macrophages can survive in the injured cord for several months and even years [12]. Thus, research has rightfully focused on factors driving macrophage polarization. And yet, phagocytosis of neutrophils by stimulated macrophages leads to a shift in polarization to an anti-inflammatory state [16], an increase in release of IL-10 and TGFβ [217], increased regulation of arginase-1, and decreased regulation of nitric oxide synthesis [218]. Because of this ability by neutrophils to drive the polarization state of macrophages in spinal cord injured tissue, more work is

needed to further elucidate the important role played by neutrophils in neuroinflammation. Additionally, because neutrophils are reactive to soft tissue damage and skin incisions, neutrophil activity needs to studied in a naturally occurring model of neuroinflammation as seen in clinical human and dog patients.

5.1.1 Neuroinflammation in dogs with SCI

These experiments were conducted in dogs with spinal cord injuries because of the spontaneous nature of the injury. On the day of hospital admission, day 0, blood draws were conducted before the dogs underwent decompression surgery. This is notable because this means that all results from day 0 are a direct result of neutrophil responses to the herniated disc and SCI. Dogs as clinical patients with spontaneous injury are used in other models of CNS disease. Canine tumors have been increasingly more common in cancer research settings because they develop spontaneously [136, 137]. Similar to spinal cord injury in dogs and in humans, gliomas in dogs are highly variable and cover a broad range of tumor subtypes and have varying genetic backgrounds [219, 220].

These experiments were also carried out in dogs because dogs have a more similar immune system to humans compared to rodents. In normal circulation, dogs have similar neutrophil percentages of total white blood cells to humans [142]. Also, like humans, dog neutrophils constitutively express LFA-1 (CD 11/CD 18 in complex). Dogs have been reported to have mutations in the LFA-1 complex [143]. Activated macrophages are present in the damaged cord of dogs with SCI, but neutrophil infiltration was not documented in these experiments [77, 82]. Like humans, there is a notable increases of neutrophils in the CSF of dogs following SCI [96].

5.1.2 Cerebrospinal fluid arachidonic acid pathway metabolism from dogs

The hypothesis was that AA metabolism would have an overall increase in pathway regulation in the cerebrospinal fluid of dogs with SCI when compared to healthy dogs. CSF samples from both injured and healthy dogs were sent for analysis on a solid phase extraction-liquid chromatography-electrospray ionization tandem mass spectrometry (MS/MS). This technique was optimized for blood and urine samples [221-223]. CSF samples have also been analyzed with MS/MS [224]. Metabolites in the gut microbiome have utilized MS/MS [225, 226], but to our knowledge, this is the first report of CSF metabolite analysis in dogs with spinal cord injury.

Out of the over 70 metabolites measured in dog CSF with MS/MS, only 19,20 19,20 DiHDPE was significantly overexpressed in dogs with SCI when compared to healthy dogs. Although this result is consistent with elevated levels of free-floating AA and DHA after SCI, elevation of 19,20 DiHDPE in the serum of humans and rats is associated with increased ingestion of fish oil as a dietary supplement 19,20 DiHDPE [171, 172]. It is unclear from this discovery-based sampling of CSF in spinal cord injured dogs if an increased amount of 19,20 DiHDPE is an attempt to mitigate pro-inflammatory events by shifting AA metabolism to more anti-inflammatory pathways and this pathway should be targeted for promotion. On the other hand, increased 19,20 DiHDPE could simply just be a biomarker for AA metabolism in CSF of animals with CNS injury. Follow up experiments on the levels of DiHDPE need to be explored before any speculation on the meaning of 19,20 DiHDPE levels in CSF of dogs with SCI.

The studies presented here, as well as previously reported studies, have detected steady levels of TXB2 and decreased levels of LTC4 in the CSF of dogs with SCI [23]. These results seem to contradict results from small mammal studies where TXB2 and LTC4 levels were high in the CSF of rabbits [227], and in the CSF and spinal cord of rats [214-216]. In these experimental SCI studies, sampling of CSF, blood, and spinal cord tissue was done minutes to hours after injury, whereas in our study the average duration from injury onset to sample collection was 23 hours.

5.1.3 Neutrophil activity is prolonged in circulation following SCI in dogs

We set out to characterize neutrophil activity in circulation from dogs with SCI by isolating neutrophils from peripheral blood and measuring the amount of oxidative burst activity and the amount of L-selectin expressed on the cell surface. Additionally, neutrophils were also cultured with LPS, the main component of bacterial cell membranes, to induce L-selectin expression. In these experiments, OBA was increased above normal levels in some individual dogs with SCI. Expression of L-selectin was variable in all dogs with SCI and healthy individuals, and was dependent on the time of day the sample was collected. Inducing L-selectin expression increased L-selectin expression in all cases. The characterization experiments discussed here are important to report for future experiments where treatments for humans are going to mitigate neuroinflammatory events.

Oxidative burst activity is a measure of the level of reactive oxygen species production by neutrophils. In this study, neutrophil OBA was increased to above basal levels in some individuals at the time of hospital admission, and also 3- and 7-days post

injury. In two individuals, OBA was increased 30 days after injury. The high variability of these clinical samples is consistent with what is seen in human patient neutrophil OBA [228]. These results are also consistent with what is seen in human neutrophils at 1-7 days post injury [200], but to the best of our knowledge, 30 and 90 days post injury is the longest neutrophil activity has been measured in clinical patients. These results are inconsistent with data from rodents that find neutrophil activity is reduced as soon as 1-3 days post injury [113, 207, 229]. However, in experimental rodents, neutrophil activity has not been measured up to 30- and 90-days post injury. It is possible that rodent neutrophils in circulation respond differently to SCI those in dogs and humans, or due to life-span differences between species that the temporal axis of neutrophil activation is different between humans and large animals versus rodents.

L-selectin expression was found to be highly variable in dogs with SCI compared to healthy animals. This result is consistent with both human and rodent expression of L-selectin, as the adhesion molecule is constitutively expressed and regulated on a cyclical pattern throughout the day. Although consistent with human data, it is of note that the results reported here were from a low number of individuals (N=9) and with high variability within groups of measurements from each day.

Because induction of neutrophil ROS production is Ca^{2+} dependent, in these experiments induced OBA activity could not be measured. Blood samples were collected in EDTA tubes which chelated all excess Ca^{2+}. However, LPS to L-selectin binding is a calcium independent process [230]. Isolated neutrophils from healthy and injured dogs were incubated with LPS prior to flow cytometric analysis. In LPS treated neutrophils, the amount of increased varied with each animal and the amounts were highest on days 90

and in controls, although these increases were not significant. In all samples measured, LPS induced expression of L-selectin. This result is contrary to other studies where LPS induced the shedding of L-selectin in both rats [230-233] and humans [234-236]. Inability of dog neutrophils to react to fMLP, a peptide released by and unique to bacteria, has been well documented [142, 237, 238]. To the best of our knowledge, this appears to be the first documented result of dog neutrophil response to LPS. The unique ability of canine neutrophils to respond to LPS stimulation by inducing L-selectin expression appears to be species specific. Furthermore, this could explain the result of highly variable L-selectin expression on neutrophils from dogs with SCI, in which neutrophil extravasation is regulated by another selectin or integrin complex other than L-selectin.

5.1.4 Neutrophils are present in the injured spinal cords of dogs

The identification of neutrophils in the spinal cords of dogs with SCI reported here is the first documented instance of neutrophil infiltration in this model system. This finding is consistent with observations in human and rodent injury [35, 206-208]. Infiltrating neutrophils have been mentioned as a key characteristic in a small population of severe SCI in dogs called myelomalacia [144, 145, 212]. And yet, neutrophil identification was not confirmed in these studies. Most studies of neuroinflammation following SCI do not focus on neutrophil contribution, but rather the more robust monocyte derived macrophage that lasts in the damaged tissue for months or years after injury. Research in dogs with SCI is no exception to this rule, as focus in recent studies has been on macrophage polarization in the injured cord [77]. Although the number of cases was

minimal, the first observation of neutrophils in the damaged cords of dogs in a valuable contribution to the field of neuroinflammation research in a large animal model of SCI.

5.2 Canine clinical research models

Using dogs in translational medicine as pre-human clinical studies is increasing in popularity in regards to characterizing and treating neurological deficits prior to studies in human medicine [197]. Indeed, clinical trials with pet dogs are already carried out very similar to experimental design in human medicine [121]. Dogs have similar environmental variations when comparted to humans, such as genetic variability between breeds, age, and sex. Furthermore, dogs live in the exact same environments as humans. In fact, humans and dogs have the similar gut microbiomes [239]. Many drugs used in human medicine are also safe for use in dogs. However, using clinical canine patients in pre-human clinical trials is not without difficulties. There are challenges in translating any study from one species to another [240]. Dogs in clinical trials have human owners, and thus have the same limitations as human studies including response bias and missing data. Like in human studies, there are difficulties in collecting consistent and high-quality samples post-mortem. Some limitations of using pet dogs in research studies are outlined in this section.

5.2.1 Use of non-steroidal anti-inflammatory drugs

Dogs were excluded from enrollment in this study if they received glucocorticoids or non-steroidal anti-inflammatory drugs within the previous 30 days. This enrollment requirement was included for two reasons. First, administration of NSAIDs causes

adverse effects on the kidney in dogs [241-243]. Second, NSAIDs and GCs directly affect neutrophil activation [243-245]. Despite the acute adverse effects of NSAIDs on kidney function, some NSAIDs are still used in veterinary medicine for short-term use for pain management [246, 247]. In the context of spinal cord injury, GCs do not benefit inured dogs [248]. However, NSAIDs have been associated with success of recovery after SCI injury in dogs and dogs administered NSAIDs were less likely to experience injury recurrence [248, 249]. Although NSAIDs do have a positive effect on SCI outcomes, it should be noted that the effects of NSAIDs are only associated with minor improvements in outcome and recovery. The use of NSAIDs alone have not been proven to be an effective treatment for spinal cord injury in dogs or in humans [250].

5.2.2 Repeated measures in clinical studies

The ability to serially measure neutrophils in the circulation of clinical patient dogs with SCI was one of the most compelling aspects of this work. Attempting to analyze serial measures of 9 dogs with SCI and multiple missing timepoints was the most challenging barriers to overcome. Clinical veterinary patient dogs with spinal cord injury enrolled in this study were subjected to 5 blood draw repeated measurements. In this study, there were multiple missing measurements throughout the study. Missing values are inherent in human clinical trials with repeated measures [251, 252]. Missing values can be categorized in 3 different ways as either completely random, missing at random, or non-random [253].This study was no exception from human clinical studies with repeated measurements as we had all 3 types of missing values. The most common days for missing values were at the end of the study. This makes sense as owners enrolled their

dogs and then dropped out for various reason such as too far travel distance from the clinic, missed or forgotten appointments, poor outcome, or even good outcome. On days 30 and 90 animals were mostly recovered from the injury and surgery, so their neutrophil counts were lower than days just after surgery. On a couple of occasions, the dogs recovered earlier than 7 days post-surgery. On those cases when the owner was traveling from rural Texas hundreds of miles away, it did not make sense to keep the animal enrolled in the study for a single blood draw. In these cases, blood draws could occur on earlier days, but in some cases the animals were discharged before communication with the laboratory occurred.

Statistical analysis of the repeated measurements in circulating neutrophil activity was a challenge in this study. There are methods for interpolating missing data in repeated measure studies [253]. However, because data were collected days and weeks between measurements, and the variability of dog breeds, ages, and sex was so high, interpolating missing data was deemed inappropriate for this study.

5.2.3 Collecting tissue from clinical patients with SCI

A challenge of relying on clinical patients for research work is the mercurial collection of tissue for histological analysis. In this study, pathological cases were collected over a 2-year timeframe and only a limited number of cases that underwent necropsy had accompanying histology performed. Some cases had vertebral luxation, in which the spinal cord was severed. Histology need not be performed on these animals with an obvious mechanism of injury. This is unfortunate, as acute spinal cord transection would be an interesting subpopulation to include in future studies.

Another limitation of collecting tissue from clinical patients is the quality of preserved tissues. In experimental SCI with purpose bred animals, rodents can be perfused with fixative that arrests biochemical reactions and allows for clearer tissue that is not riddled with artifact. However, in larger patients such as dogs and humans, perfusion can be a challenge across larger tissue surface areas and volumes. Handling larger volumes of fixative is both more dangerous and also costly.

Some of these limitations can be overcome with proper experimental design and facility approval of animal use protocols. In future research of SCI in dogs, care should be taken to collect high quality samples following standardized protocols.

5.3 Future of dog SCI research

As of right now, translating rodent studies into dogs prior to human clinical trials is viewed as too risky. However, spending years and millions of dollars on a rodent preclinical study that does not translate to human medicine is also a risk for society and particularly to the percentage of the population suffering from SCI. This is particularly of note for treatments involving differential regulation of the immune system. Before starting a human clinical trial, safety and drug efficacy information based on rodent studies is sufficient for approval from the United State Food and Drug Administration (FDA) [6, 254, 255]. The risk is that a successful treatment in rodents could be not proven to be successful in dogs but might still work for human injury. If a canine patient dies during the course of a clinical trial, that death must be explained before research can continue. Sometimes the death of the animal is unrelated to the treatment, or the treatment dosage was not correct, but a different dose would be successful. There is value in studying and

improving policies through the FDA and other regulatory bodies across the world. For now, studies providing characterization of dogs with spinal cord injuries, with particular focus on the immune response, are valuable contributions to the field of SCI research.

5.3.1 Short comings of translating rodent models of SCI directly to human medicine

Although many issues and concerns to producing a standardized and reproducible model for SCI in rodents have been addressed in the introduction, there are still limitations to using rodents as models for human injury in SCI. First, there is a large size difference between rats and mice and humans. This could be significant for validation of human SCI therapies made for humans but tested in rodents because the ability of regenerating neurons to bridge a smaller gap in an injured rat spinal cord is easier than in the larger human cord [256, 257]. Furthermore, testing drug efficacy in smaller animals may not translate well to humans if the drug delivery system is in normal circulation, i.e. it might take longer for the drug to arrive at the injury site than the drug is effective and scaling up the dosage would not necessarily help with this problem [121]. Axonal tract organization between rats and humans differs in location of the corticospinal fibers and other tracts [258]. Spinal cord organization is even different between subspecies of different rats [259]. Besides differences in morphological features of injury and spinal cord organization between rodents and humans, there are also significant differences in inflammatory responses between the two, which will be discussed in greater detail in future sections.

These limitations in using rodents as the only model for SCI research can be costly in both dollar amounts spent on treatments that are not translatable to human medicine, and time it will take to discover a successful therapy. To date, there is no cure for spinal

cord injury, and very few treatments from rodent research have been successful in large-scale human clinical trials. The most tangible example of failed treatment for SCI that had promising results in preclinical trails was administration of methylprednisolone. For decades, this treatment was trialed in humans and several other species before the attempt as a treatment was eventually abandoned in the mid 2000s [6, 260, 261]. For these reasons, there is value in including large animal or non-human primate models of SCI instead of just going from rodents to humans which has not worked now for multiple decades of research [197].

Neutrophils of mice and rats responding to injury and infection are different in many ways than humans who have endured spontaneous traumatic injury such as SCI. Both rats and mice have low blood and CSF volumes, meaning that samples often have to be pooled [101]. Neutrophils in humans make up 50-70% of the total white blood cells in circulation, while in mice they are around 10-25% [262]. Reduction of the glycocalyx begins within minutes of spinal cord injury in rats and mice, and is repaired 3 days post-injury in rats [65], and 5-7 days post-injury in mice [80]. The glycocalyx recovery time has not been studied in humans following SCI, but the differences in neutrophil percentages of white blood cells counts coupled with the differences in recovery time between mice and rats leaves room for speculation on whether neutrophil trafficking is mimicked in rodent models of SCI. Furthermore, mouse leukocytes express Ly6 family surface markers, which are standard use for identification of neutrophils in peripheral circulation as well as within the tissues in SCI research [10, 58, 187]. Other species including humans, dogs, cats, and rats do not express Ly6 gene products on their leukocytes [263]. Defensins are proteins rich with cysteine residues that are used primarily in antimicrobial

host defense, but are also reported to function as monocyte recruitment tools by neutrophils in humans, however, mouse neutrophils do not produce defensins [264]. In rats, there is a notable lymphocytic (T cells and B cells) inflammatory response in experimental SCI models, but in humans, there is a larger innate inflammatory response by microglia and neutrophils [265]. Another key difference between rodent models of SCI and human injury is the fact that humans undergo spontaneous, naturally-occurring injury often accompanied by polytrauma (example, broken bones). In rodent models, the injury to the spinal cord occurs after the animal is anesthetized and after significant skin incisions and laminectomy have been performed.

5.3.2 Availability of dog reactive antibodies and assays

One of the most limiting factors for research conducted in dogs is the lack of commercially available reagents that are reactive in dogs. Commercial availability is an issue for every canine study [266, 267], whereas in rodent research availability is almost never an issue when determining feasibility of a study. Often in canine research, a validation study precedes any hypothesis driven research [268]. It might even be necessary to produce novel antibodies specific for work in dogs [269]. However, as dogs are recognized more and more as valuable models with clinical patients, commercial availability and validation of cross-species reactivity is increasing. This is especially apparent in glioma research, where dogs are now recognized as a naturally-occurring, translationally relevant animal model for human brain cancer [270].

6. CONCLUSIONS

Similar to cancer research, the hope is to make naturally-occurring canine SCI a successful pre-human clinical trial model in order to improve drug efficacy and safety for use in both human and veterinary medicine. This study in a large animal model of contributes to the field of SCI research by providing dog CSF profiles that are similar to humans in regards to timing of both sample collection and a possible window in time and location for therapeutic intervention. This is the first report of a species-specific lipopolysaccharide-induced increase of surface L-selectin expression on canine neutrophils. This is also the first documented instance of neutrophils in the injured spinal cord of dogs following spinal cord injury. I hope that this work contributes to advancing the knowledge for the betterment of human and dog clinical trials. I hope that these trials can become standardized for use in all areas of SCI research and characterization of neuroinflammation. I hope that these treatments lead to a cure for spinal cord injury in both human and veterinary medicine.

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