Evaluation of the use of a bioengineered hydrogel containing hyaluronan to reduce inflammation and scarring following spinal cord injury associated with arachnoiditis

by

James W. Austin

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Institute of Medical Science
University of Toronto

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Doctor of Philosophy
Institute of Medical Science
University of Toronto
2012

Abstract

Background: Spinal cord injury (SCI) is heterogeneous in nature and can be complicated by inflammation and scarring in the subarachnoid space (arachnoiditis). The constellation of traumatic injury and arachnoiditis can lead to extensive intraparenchymal cysts or post-traumatic syringomyelia (PTS), due to alterations in fluid flow and pressure dynamics in the subarachnoid space.

Hypothesis: Intrathecal injection of a bioengineered hydrogel containing hyaluronan (HA) will improve functional recovery following severe spinal cord injury associated with arachnoiditis.

Methods: Acute to subacute pathophysiological events were characterized in non-injured sham rats, rats receiving a clip compression/contusion injury (SCI), rats receiving an intrathecal kaolin injection (Arachnoiditis) and in rats receiving SCI plus kaolin injection (PTS). Next, a HA containing hydrogel (HAMC) or artificial cerbralspinal fluid (aCSF) control was injected into the subarachnoid space 24 hours following PTS injury. To assess treatment efficacy, subacute pathophysiology was assessed as was long-term neurobehavioural and neuroanatomical...
recovery. Finally, *in vitro* studies examined the effect of HA on TLR4 activation using lipopolysaccharide in primary rat microglial cultures.

**Results:** PTS animals exhibited a greater parenchymal injury response as compared to the sum of SCI alone or arachnoiditis alone. Injection of HAMC reduced the extent of scarring and inflammation in the subarachnoid space and improved neurobehavioural and neuroanatomical recovery relative to aCSF controls. These improvements were associated with reduced chondroitin sulfate proteoglycan and IL-1α expression and a trend towards and axonal preservation. *In vitro* studies demonstrated that HA is capable of reducing TLR4 mediated inflammation in microglia.

**Conclusions:** Acute arachnoiditis potentiates the intensity of intraparenchymal inflammatory and scarring events following SCI. When HAMC was injected intrathecally following PTS injury, it mitigated some of the pernicious effects of arachnoiditis. Part of the therapeutic action of HAMC can be attributed to the ability of HA to reduce TLR4 mediated inflammation in microglia, possibly through an extracellular mechanism.
Acknowledgments

A big thanks goes out to my committee (Drs. Baker, Eubanks and Shoichet) and Dr. Fehlings for their mentorship and guidance. Also, I’d like to thank the members of the Fehlings lab for support and technical assistance. To all of my collaborators, I appreciate all of the hard work and contributions. I will always value the experience of working with you all. I would also like to thank the funding sources for this work: The Physicians Services Incorporated and the Canadian Syringomyelia Network. Furthermore, I am deeply indebted to Ontario government and the CIHR for financial support through their studentships. Last but not least, I’d like to thank my wonderful partner in life, Katie. I am forever in debt for your patience, kindness and love. Grad school has been a long battle, and I am excited to get on with my life and become a productive member of society.

“When one has become a master in some field one has usually, for that very reason, remained a complete amateur in most other things; but one judges just the other way around…”

Friedrich Nietzsche
## Abbreviations

<table>
<thead>
<tr>
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<th>Definition</th>
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<tbody>
<tr>
<td>aCSF</td>
<td>Artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis inducing factor</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1 (Jun)</td>
</tr>
<tr>
<td>ASIA</td>
<td>American Spinal Injury Association</td>
</tr>
<tr>
<td>B3T</td>
<td>Beta III tubulin</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BBB locomotor rating scale</td>
<td>Basso, Beattie, Breshnahan locomotor rating scale</td>
</tr>
<tr>
<td>BCFB</td>
<td>Blood cerebrospinal fluid barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>BSCB</td>
<td>Blood spinal cord barrier</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>CAM</td>
<td>Cellular adhesion molecule</td>
</tr>
<tr>
<td>Caspase</td>
<td>Cysteine protease, which cleaves at an aspartate residue</td>
</tr>
<tr>
<td>CNP</td>
<td>Central neuropathic pain</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CPA</td>
<td>Canadian Paraplegic Association</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CSPG</td>
<td>Chondroitin sulfate proteoglycan</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglion</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental Autoimmune Encephalitis</td>
</tr>
<tr>
<td>EB</td>
<td>Evan’s Blue</td>
</tr>
<tr>
<td>ECF</td>
<td>Extracellular fluid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbant Assay</td>
</tr>
<tr>
<td>Erk</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FasR</td>
<td>Fas Receptor</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronan or hyaluronic acid</td>
</tr>
<tr>
<td>HMW</td>
<td>High molecular weight</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
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<tr>
<td>IGF</td>
<td>Insulin like growth factor</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>INF-gamma</td>
<td>Interferon gamma</td>
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<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<tr>
<td>IRAK-M</td>
<td>IL-1 receptor associated kinase-M</td>
</tr>
<tr>
<td>IRF3</td>
<td>Interferon regulatory factor 3</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus Kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>K</td>
<td>Potassium</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LMW</td>
<td>Low molecular weight</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAG</td>
<td>Myelin associated glycoprotein</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated kinase-like protein</td>
</tr>
<tr>
<td>MAPKK</td>
<td>Mitogen activated kinase-like protein kinase</td>
</tr>
<tr>
<td>MC</td>
<td>methylcellulose</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>----------</td>
<td>---------------------------------------------------</td>
</tr>
<tr>
<td>MOG</td>
<td>Myelin oligodendrocyte glycoprotein</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>MPSS</td>
<td>Methylprednisolone</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation factor 88</td>
</tr>
<tr>
<td>Na</td>
<td>Sodium</td>
</tr>
<tr>
<td>NASCIS</td>
<td>National Acute Spinal Cord Injury Study</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear factor kappa light polypeptide gene enhancer in B-cells</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NgR</td>
<td>Nerve growth factor receptor</td>
</tr>
<tr>
<td>Nod1</td>
<td>Nucleotide-binding oligomerization domain containing 1</td>
</tr>
<tr>
<td>O</td>
<td>Oxygen</td>
</tr>
<tr>
<td>OEC</td>
<td>Olfactory ensheathing cells</td>
</tr>
<tr>
<td>OMgp</td>
<td>Oligodendrocyte-myelin glycoprotein precursor</td>
</tr>
<tr>
<td>OPC</td>
<td>Oligodendrocyte precursor cell</td>
</tr>
<tr>
<td>p105</td>
<td>NFkB-p105 (NFkB1)</td>
</tr>
<tr>
<td>p38</td>
<td>p38 MAPK</td>
</tr>
<tr>
<td>PAGE</td>
<td>Poly acrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDK</td>
<td>Phosphoinosdtide-dependent protein kinase</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PH</td>
<td>prolyl hydroxylase</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PSGL</td>
<td>P-selectin glycol-protein ligand</td>
</tr>
<tr>
<td>PTS</td>
<td>Post-traumatic syringomyelia</td>
</tr>
<tr>
<td>PVS</td>
<td>Perivascular space</td>
</tr>
<tr>
<td>QA</td>
<td>Quisqualic acid</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RHAMM</td>
<td>Receptor for hyaluronan mediated motility</td>
</tr>
<tr>
<td>RIP</td>
<td>receptor interacting protein</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SAS</td>
<td>Subarachnoid space</td>
</tr>
<tr>
<td>SASc</td>
<td>Subarachnoid scarring</td>
</tr>
<tr>
<td>SCEP</td>
<td>Spinal cord evoked potential</td>
</tr>
<tr>
<td>SCI</td>
<td>Spinal cord injury</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signaling</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal-transducer and activator of transcription</td>
</tr>
<tr>
<td>TBI</td>
<td>Traumatic brain injury</td>
</tr>
<tr>
<td>TIRAP</td>
<td>Toll-interleukin 1 receptor domain containing adaptor protein</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
</tr>
<tr>
<td>Tollip</td>
<td>Toll-interacting protein</td>
</tr>
<tr>
<td>TRAF3</td>
<td>TNF receptor-associated factor 3</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF receptor-associated factor 6</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Deoxynucleotide transferase dUTP nick end-labeling</td>
</tr>
<tr>
<td>Western Blot</td>
<td>Western blot</td>
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</tbody>
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Chapter 1 - General Introduction

1 Spinal Cord Injury

1.1 Epidemiology

People living with a spinal cord injury (SCI) are faced with great emotional and social struggles, financial burden, and impaired quality of life due to loss of motor, sensory and autonomic function [1]. The annual incidence of SCI in the developed world is approximately 15 to 40 cases per million, although the incidence in developing countries is considerably higher [2]. According the Rick Hansen Institute, there are 4,300 new cases of SCI in Canada each year and there are 86,000 Canadians currently living with SCI (www.rickhanseninstitute.org). Interestingly, the Rick Hansen Institute reports that only 42% of new injuries are due to traumatic causes. Similarly, according to the Christopher and Dana Reeve Foundation, there are 1,275,000 people in the United States currently living with paralysis due to SCI (www.christopherreeve.org). Furthermore, the estimated lifetime costs due to SCI range from just under $700,000 up to $3 million for a 25 year old (www.christopherreeve.org). A Canadian Paraplegic Association (CPA) survey of people living with SCI showed that 78% of those surveyed had been injured between the ages of 15 and 34 and that 81% of the newly injured are males. These trends are also seen in other countries around the world [3]. The Christopher and Dana Reeve Foundation reports that 30% of people with a SCI that responded to their survey were between the ages of 40-49, with 24% between ages 50-59, and approximately 10 % in each of the 20-29, 30-39 and 60-69 age brackets. The CPA also reported that between 1983 and 1989, vehicular accidents accounted for approximately 55% of all spinal injuries. Similarly, causational studies from other countries reveal that traffic accidents were responsible for approximately 40-50 % of spinal traumas, while work related injuries, sports and recreation,
falls, and violence made up the other 50-60% [1]. In contrast, the Christopher and Dana Reeve Foundation reports that 24% of traumatic injuries were due to motor vehicle accidents, 28% were due to accidents while working, 16% were due to sports and recreation accidents, 9% were due to falls, 4% due to violence with the rest due to unknown or birth defect causes. In 1999, 53% of new injuries reported to the CPA resulted in paraplegia (lower limbs), while 47% resulted in quadriplegia (upper and lower limbs). Studies suggest that most injuries occur in the cervical region (C1 to C7/T1; 55%) with thoracic (T1 to T11), thoracolumbar (T11-T12 to L1-L2) and lumbosacral (L2 to S5) all having an incidence of 15% each [1].

To summarize, SCI most commonly affects younger males, is mainly due to work related and traffic accidents and occurs most commonly in the cervical region. As SCI is such a debilitating problem psychologically, physically and financially - the need for a cure is great.

### 1.2 Primary and Secondary Injury

The spinal cord is surrounded by the vertebral column, making it vulnerable if physical forces alter the position and structural integrity of the normally protective vertebrae. Traumatic forces such as ones incurred from traffic accidents, diving into shallow water or sports related injuries can cause the types of vertebral column complications associated with SCI and outlined in Table 1. While the majority of SCI is due to blunt injury, penetrating trauma due to knife or gunshot wounds also occurs in a small percentage of cases [4].

The spinal cord is rarely transected during injury, even in cases that result in severe neurological deficits [1]. SCI can involve shear, stretch, laceration and more commonly contusive and compressive forces. In addition, spinal cord laceration due to bone fragments or weapons in violent acts has been observed in a small number of cases. Due to the heterogeneous nature of injuries, numerous animal models have been developed to mimic the human condition.
and shed light on the mechanisms and progression of injury. Animal models include various weight drop devices, spinal cord compression by forceps or modified aneurysm clips, balloon compression, and hemi or full transection injuries in mice, rats, and other small mammals. Studies in rats have demonstrated that neurological impairment increases relative to the force of trauma and the time of compression [5]. Cells, especially neurons and their axons, become permeabilized acutely following injury due to compressive and shear forces [6, 7] leading to cell disability and death. The initial physical trauma also damages local vasculature causing edema and hemorrhaging in the well vascularized gray matter and to a lesser extent in the white matter. Animal studies have also demonstrated that the breach in the blood-spinal cord barrier (BSCB) leads to extravasation of markers from 730 Da in size up to red blood cell size (5-7µm in diameter) 5 minutes after injury to the spinal cord [8]. Damage to the meningeal layers, spinal roots, and bleeding in the subdural and subarachnoid space (SAS) is also common [9].

Acute neuroanatomical outcome includes paralysis to neurons involved in motor, sensory and autonomic functions at the level of injury. Furthermore, at level axonal damage leads to miscommunication in afferent and efferent white matter tracts transmitting signals beyond the injury site. Commonly, there exists a subpial rim of surviving axons transversing the lesion site in varying states of demyelination. SCI has typically been referred to as either complete or incomplete, referring to complete loss of motor and conscious sensory function below the injury site or some quantity of loss, respectively. To circumvent the ambiguity of this definition, the American Spinal Injury Association (ASIA) created a more descriptive measure called the ASIA Impairment Scale [10]. The scale is as follows: A=Complete – no motor or sensory function is preserved below the injury; B=Incomplete – Sensory but not motor function is preserved below the neurological level; C=Incomplete – Motor function is preserved below the neurological level and more than half of key muscles below have a muscle grade less than 3; D = Incomplete -
Motor function is preserved below the neurological level and at least half of key muscles below have a muscle grade of 3 or more; E=Normal – motor and sensory function are normal.

Table 1. Vertebral column injuries associated with SCI

<table>
<thead>
<tr>
<th>Type of Bony Injury</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minor fracture (including compression)</td>
<td>10</td>
</tr>
<tr>
<td>Fracture dislocation</td>
<td>40</td>
</tr>
<tr>
<td>Dislocation only</td>
<td>5</td>
</tr>
<tr>
<td>Burst fracture</td>
<td>30</td>
</tr>
<tr>
<td>SCIWORA</td>
<td>5</td>
</tr>
<tr>
<td>SCIWORET (including cervical spondylosis)</td>
<td>10</td>
</tr>
</tbody>
</table>

SCIWORA = spinal cord injury without obvious radiologic abnormality  
SCIWORET = spinal cord injury without obvious radiologic evidence of trauma

Adapted from [1]

The forces, severity and location of the primary injury dictate the characteristics of secondary events, which together determine the extent of tissue and functional loss, and ultimately patient outcome. The initial trauma triggers a series of systemic, cellular and molecular cascades that expand the lesion from the primary injury site into adjacent white and gray matter, increasing the extent of tissue loss. Concurrently, endogenous beneficial responses occur that limit the spread of the lesion and attempt to regenerate and reconnect damaged signaling pathways. In general, the force of injury determines the ensuing hemorrhage, which in turn dictates the extent of ischemia and secondary damage.

1.3 Anatomy of the Spinal Cord

The spinal cord is comprised of the grey matter and white matter. The former is a well vascularized central region rich in neuronal cell bodies. The less vascularized white matter contains mainly myelinated axons and associated oligodendrocytes. Astrocytes and microglia
are present in both regions, providing support and monitoring the extracellular environment to maintain homeostasis. Surrounding the spinal cord are the meninges - the pia mater is immediately adjacent to the spinal cord parenchyma followed by the SAS and arachnoid layer, with the dura mater on the outside holding everything together. Refer to Figure 1A for a schematic of the gross spinal cord anatomy.

1.3.1 The Meninges

The meninges form the surrounding layers of the spinal cord. The dura is comprised of fibroblasts, collagen, elastic fibers and glycoproteins. The dural border layer with the arachnoid is mainly cellular in nature (fibroblasts). Similarly, the arachnoid barrier cell layer is comprised of closely packed cells and also a basement membrane. Fibroblasts and collagen make up the arachnoid trabeculae that transverse the SAS. The walls of the trabeculae have openings that allow free passage of CSF. Blood vessels also attach to the trabeculae in the SAS. The pia is made up of a single continuous layer of flattened fibroblasts attached to the external glia limitans of the spinal cord parenchyma. The glia limitans are a collection of astrocytic end feet that closely associate with the basal lamina of the parenchyma and form an essential component of the blood-brain barrier. The glia limitans provides a physical barrier into the CNS – controlling molecular trafficking and separating the immune-privileged parenchyma from the non-immune privileged meninges – that provides clear definition of the parenchyma from the vascular and subarachnoid spaces [11, 12]. In Figure 1, the meninges are shown with the dura left out for simplicity.

1.3.2 Blood Flow and Circulation

Blood to the spinal cord is supplied by the left and right radicular arteries that approach the spinal cord along the nerve root. They split into the posterior and anterior radicular arteries. The posterior radicular artery feeds the posterolateral longitudinal spinal artery (one on each
side) and the anterior radicular artery on each side feeds a singular anterior median longitudinal spinal artery. Ventrally (anterior), the intrinsic central arteries originate from the anterior median longitudinal spinal artery and supply the anterior, central and parts of the medial posterior gray matter. Vasocoronaee (pial plexus) from the anterior radicular artery supply the anterior and lateral white matter. Together, this accounts for approximately two thirds of the cross sectional blood supply [13]. Dorsally, vasocoronaee from the posterior radicular artery together with arterioles from the pial plexus and posterolateral longitudinal spinal artery supply the dorsal horns and posterolateral white matter. Similarly, the venous system consists of the posterior spinal vein (medial), posterolateral spinal veins and an anterior spinal vein that feed into an anterior and posterior radicular vein. Refer to Figure 1E for a cross sectional depiction of blood supply to the spinal cord. The gray matter is well vascularized due to the high metabolic activity of neuronal cell bodies (soma). As such, this makes the gray matter vulnerable to ischemic insults following trauma.

### 1.3.3 Cerebrospinal Fluid

Cerebrospinal fluid (CSF) provides cushioning and acts as a pseudo lymphatic system for the CNS. The human body produces approximately 500 mL of CSF daily, mainly via the choroid plexus of the lateral, third and fourth ventricles. CSF flows from the third and fourth ventricles, posteriolaterally down the SAS of the spinal cord and back up towards the brain ventrally. Flow is caused and influenced by arterial pulsations, respiratory function and ependymal cilia [14-16]. In animals (and possibly in humans), there is flow of CSF from the SAS into the spinal cord parenchyma via the perivascular spaces (PVS) to the central canal, allowing for significant mixing of extracellular fluid and CSF and exchange of solutes (electrolytes, proteins etc.) [17]. The central canal itself is not associated with longitudinal (rostral-caudal) flow of CSF as it is not continuous [18]. The choroid plexus filters water and
solutes from the blood and uses various transporters and channels to secrete water and the various electrolytes that comprise the CSF [19]. CSF is reabsorbed into the venous system at the arachnoid granulations (by villi) in the sinuses of the brain (superior sagittal, transverse and straight), the lymphatics across the cribiform plate and the nerve root subarachnoid angles [16].

1.3.4 The Blood -Spinal Cord and -Cerebrospinal Fluid Barriers

Arteries in the SAS form arterioles as they enter the spinal cord. They are surrounded by the pia and glia limitans but they eventually lose the pia as the artery gets smaller and capillaries are formed. With this, the space between the endothelial cells of the blood vessels and pial/glial layers, the PVS or Virchow-Robin space - which is continuous with the SAS - is lost. The blood cerebrospinal fluid barrier (BCFB) is represented as the region at the arteriole level where smooth muscle is no longer present, as in larger arteries, and CSF is in direct contact with endothelial cells via the PVS. This is in addition to the BCFB that exists at the capillary level in the choroid plexus where CSF is made. At the capillary level, only astrocytic and sometimes microglial end feet of the glia limitans and pericytes lie between the vessel walls and parenchyma. This is referred to as the blood-spinal cord barrier (BSCB). It is hypothesized that the PVS is where the flow of CSF and extracellular fluid (ECF) occurs between the central canal, spinal cord parenchyma and SAS [17].

1.4 Neuroanatomy

1.4.1 Motor Function Following Spinal Cord Injury

Motor impairment following SCI is a result of damage to both upper and lower motor neurons. Loss of lower motor neurons in the anterior/ventral horn leads to paralysis of muscles at the level of injury. Additionally, upper motor axons passing through the level of injury are damaged resulting in loss of efferent input to muscles below the level of injury. Tracts involved
with motor control involve pyramidal tracts such as the corticospinal tract (CST; fine voluntary control). Non pyramidal tracts (rubrospinal, reticulospinal, tectospinal, vestibulospinal) control gross motor movement. Since the majority of injuries occur in the cervical level, control of muscles of the upper limbs (in high cervical injuries), trunk, and lower limbs is commonly affected. See Figure 1B, C and E for a schematic of basic motor neuroanatomy. Note that the CST in rats and humans is located in different positions, as represented by the cross-section of the spinal cord. The functional significance of this would be more pronounced in less severe injuries. The CST in rats would be expected to be damaged by most injuries due to its central location and the fact that typically, damaging forces are directed at the center of the spinal cord. In contrast, the lateral CST in humans might be spared following less severe injuries due to their lateral position.

1.4.2 Sensory Function Following Spinal Cord Injury

Sensory input carrying pain and temperature information is relayed from specialized receptors in the periphery to the brain via the spinothalamic tract. These first order neurons enter the CNS, ascend or descend one or two vertebral levels, and synapse on second order sensory neurons in the dorsal/posterior horn, prior to decussating and heading rostrally towards the brain. In contrast, first order neurons of the posterior/dorsal column-medial lemniscus pathway, carrying fine touch and vibration information, enter the spinal cord and travel rostral towards the brain before decussating in the medulla. Damage to first and second order spinothalamic neurons/axons or first order axons of the posterior column pathway disrupt sensory information relayed from dermatomes at and below the level of injury to the brain. Spinocerebellar tracts transmit limb and joint information (proprioception). As such, damage to axons in these tracts can affect locomotion. See Figure 1B, C and E for a schematic of basic spinal cord sensory neuroanatomy.
Figure 1. Spinal cord anatomy

(A) Schematic of meningeal and parenchymal anatomy. The dura mater (outside layer of meninges) was left out for simplicity. (B) Schematic of motor and sensory neurons entering and leaving the spinal cord. Neurons from the motor cortex decussate in the hindbrain and travel down a tract (such as the corticospinal tract) to where they synapse on a lower (secondary) motor neuron in the gray matter – which then exits the anterior horn and innervates a muscle. Sensory information carrying pain and temperature information (spinothalamic) enters the spinal cord via the dorsal roots from a sensory receptor in the periphery in which the neurons synapse on a second order sensory neuron in the dorsal gray matter – which decussates and travels up to sensory cortex via a neuron it synapses on in the thalamus. Note that sensory neurons from the dorsal column pathway enter the spinal cord via the dorsal roots and travel to the medulla before decussating and travelling to the sensory cortex (not shown). (C) Representation of where secondary sensory, autonomic and lower motor neurons originate within the gray matter of the spinal cord. (D) Representation of where ascending and descending sensory and motor axons are located the white matter within a cross section of the spinal cord. Reticulospinal – unskilled and involuntary; Vestibulospinal – extensors of trunk and lower limb and flexors of upper limb; Corticospinal – skilled and voluntary movements; Spinothalamic – simple touch, pain and temperature; Spinocerebellar (ventral) – proprioception lower limbs; Cuneate fasciculus (posterior column)- discriminative touch and proprioception, upper limb; Gracile fasciculus (posterior column)- discriminative touch, lower limb. (E) Illustration of segmental arterial blood supply to the spinal cord.
1.4.3 Autonomic Function Following Spinal Cord Injury

Perhaps even more devastating than the acute motor or sensory loss is the miscommunication between higher centers of the hypothalamus and limbic system and the various effector organs of the autonomic nervous system. Preganglionic cell bodies of the sympathetic system (the final neurons within the CNS that regulate sympathetic output) are found in the intermediolateral horn of the gray matter between T1 and L2 (Figure 1C). Parasympathetic preganglionic cell bodies are located in the brain stem and sacral levels of the spinal cord. The effects of injury on autonomic function depend on the anatomical level. Controls for vasoconstriction, cardiac output, and respiration are found in the T1-T4 regions, whereas input to the gastrointestinal tract and associated organs as well as sexual organs are found below this level from T5-L2. Research suggests that spinal sympathetic interneurons, which are relatively inactive in the uninjured normal spinal cord, become active following injury, resulting in impaired autonomic function commonly referred to as autonomic dysreflexia [20]. Evidence for the existence of these spinal interneurons and their role following SCI is provided by looking at animal transection injury models in which all sympathetic input from the brainstem is lost yet the animals still have sympathetic activity [21]. Parasympathetic branches contributing to respiration, cardiovascular control and digestion generally remain intact due to their cranial nerve location (originating above the cervical spinal cord). In contrast, parasympathetic input to the kidneys, bladder and sexual organs are susceptible in SCI cases due to their sacral location (pelvic nerve).

1.5 Secondary Injury Progression

In mammals such as humans and rats, a cystic cavity (myelomalacia) forms at the injury epicenter and spreads radially and rostrocaudally from the injury site over time, resulting in
extensive functional and morphological alteration. Infiltrating macrophages, lymphocytes, and activated microglia are present within the cavity, along with granular myelin debris and axons in various degrees of demyelination [22, 23]. Typically, a subpial rim of tissue survives the injury and contains axons also in varying states of myelination [24, 25]. Astrocytes proliferate and surround the cavity in an attempt to attenuate the spread of the lesion, forming a glial scar [26, 27]. This astrogliosis also represents a physical and chemical barrier to axonal regeneration. A fibrous scar consisting of collagen and various inhibitory extracellular matrix (ECM) molecules is deposited within and surrounding the lesion. Wallarian degeneration of axons towards their cell bodies and away from the epicenter is a common fate of severed axons [28]. Severed axonal ends distal to the injury site degenerate along with disrupted myelin and are broken down, eventually being phagocytosed by macrophages. A chronic snapshot of the injury demonstrates a cystic cavity containing vascular/glial bundles [29], regenerated nerve roots, collagenous fibers and astrocytes. Refer to Table 3 and Table 4, which summarize the spatio-temporal progression of secondary events.

1.5.1 The Acutely Injured Spinal Cord

Following the primary physical damage and death of neural cells, the acute secondary phase of the injury begins. Typically, the acute phase represents the first 24-48 hours following injury. This phase is characterized by vascular dysfunction including ischemia, energy and ion imbalances, excitotoxicity, and early inflammatory events that lead to necrotic - and to a lesser extent, apoptotic cell death.

The immediate acute injury has often been described as the initial 2 hours post injury [30]. Function below the injury site is immediately lost to a poorly understood phenomenon called spinal shock [31]. During this time, loss of neurons and glia that survived the initial injury but sustained mechanical damage/permeabilization from said injury, undergo necrosis. Within
minutes, edema and hemorrhaging, which correlate to injury severity [32], result in ischemic zones at and adjacent to the injury site causing additional necrotic cell death [33]. Microglia, responding to byproducts of necrosis (DNA, heat shock proteins, ATP, K\(^+\)), become activated and secrete inflammatory cytokines that act to recruit systemic inflammatory cells. In most cases, the gross histology has not been significantly altered and changes may appear normal with MR imaging [34].

1.5.1.1 Changes in Vascular Blood Flow

Vasospasm and impaired autoregulation along with the hemorrhage and loss of microcirculation observed immediately following injury contribute to the overall ischemic pathology. Autoregulation of local spinal cord blood pressure is lost following injury resulting in reduction of blood flow, which is furthered exacerbated by systemic hypotension [35, 36]. Vasospasm is evident after SCI and can be brought by the injury itself or release of vasoactive factors (such as histamine or NO) [37].

1.5.1.2 Energy, ion and glutamate imbalances

Dysfunction in Na\(^+\), K\(^+\), Ca\(^{2+}\), glutamate and metabolism homeostasis is well documented and causes impairment and cell death following SCI. Injury leads to increases in intracellular axonal Na\(^+\) and Ca\(^{2+}\) due to failure of ion pumps, inactivation of ion channels, reverse function of ion exchangers and membrane depolarization [38-43]. Intracellular increases in astrocyte and oligodendroctye Ca\(^{2+}\) through L-type and N-type calcium channels and excess glutamate signaling (via metabotrophic and iontrophic glutamate receptors) [44-48] may also play a role in white matter injury [49]. Impaired glutamate reuptake by astrocytes through dysfunction in glutamate transporters, cell death, and release of glutamate from neurons, axons and glia via reversal of Na(+) dependent glutamate transport, all lead to increased extracellular glutamate.
Increased extracellular concentration of glutamate is observed following injury within 3 hours after injury [51], leading to alterations in glial and axonal function and gray matter neuronal cell death [51, 52]. No direct effects of glutamate on axons have been reported.

Change in acute energy metabolism following spinal cord injury is characterized by depletion of ATP, an initial decrease in glucose and increases in lactate/pyruvate ratios (indicative of hypoxia) [53, 54]. The resultant deficits in energy metabolism are no doubt due to hypoperfusion/ischemia mediated decreases in oxygen and glucose availability to cells and subsequent reperfusion.

1.5.1.3 Intracellular consequences of acute excessive calcium concentration

Excessive intracellular Ca$^{2+}$ results in neuronal cell death and axonal degradation through activation of protein kinases, proteases, and mitochondrial dysfunction. Proteases named calpains are activated acutely following SCI due to increases in intracellular Ca$^{2+}$ and lead to the degradation of cytoskeletal proteins, such as neurofilaments and microtubules, disrupting axonal integrity and function [55-57]. Extreme intracellular calcium levels are detrimental to mitochondria, causing increased reactive oxygen species (ROS) production in neurons and glia.

1.5.1.4 Oxidative stress

Increased production of ROS and reactive nitrogen species (RNS) can be attributed to both intracellular and extracellular sources. These reactive species can be generated intracellularly following SCI due to metabolic imbalances and excess intracellular Ca$^{2+}$ [58-62]. In both cases, mitochondria become dysfunctional and produce increased amounts of ROS, including superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) [63, 64]. If not neutralized, O$_2^-$ can react with nitric oxide (NO) forming peroxinitrite (ONOO$^-$), a RNS that is one of the most reactive and detrimental free radicals known. When generation of ROS increases above the anti-
oxidative capacities of cells, as seen during mitochondrial dysfunction, these reactive molecules can damage proteins and DNA causing necrotic cell death [65, 66]. In some cases, brief oxidative stress can result in apoptosis of oligodendrocytes \textit{in vitro} [67-69] and neurons [70].

In terms of extracellular oxidative stress, myeloperoxidase production by neutrophils has been identified as a detrimental source of ROS in post injured CNS tissue [71]. Myeloperoxidase catalyzes the peroxidization of chloride ions, generating hypochlorous acid [72]. Hypochlorous acid can interact with and disrupt lipid bilayers in surrounding cells, causing increased membrane permeability and eventual cell death. Neutrophils and microglia are also a major source of NO [73-75]. As above, ROS can react with NO to form ONOO\^- [76]. Similar to hypochlorous acid, extracellular ROS/RNS can lead to lipid damage, an increase in membrane permeability and cell death. Experimental evidence suggests that peak ROS production occurs 12 hours following injury, with levels remaining elevated until they return to basal levels within 4-5 weeks [65, 66].

1.5.1.5 Acute inflammation

The inflammatory response following SCI is a complex interaction of local and systemic mediators. Aspects of inflammation as a whole can contribute to the secondary damage and others, such as removal of cellular debris, aid in tissue repair. Similarly, the exact role that each type of immune cell plays has not been fully elucidated – though it is likely each has both beneficial and detrimental aspects. Moreover, a confounding variable in SCI inflammatory research is significant differences in the inflammatory response between species [66] and even between strains [77] of a particular species used to model human SCI. However, with this in mind, the temporal progression of immune events in animal models is similar to humans [78].

Within hours of CNS injury microglia are activated due to vascular compromise, loss of tissue homeostasis and necrotic byproducts such as ATP, DNA, high mobility group box protein
1 (HMGB1), heat shock proteins (HSPs) and high levels of extracellular K⁺. Microglia transition from ramified to amoeboid morphology upon activation and release cytokines, chemokines and NO which act to recruit systemic inflammatory cells, modulate protein expression in neurons and glia, and damage neurons and myelin [71, 77, 79-85]. Microglia are also major cellular sources of matrix metalloproteinases (MMPs) that can increase vascular permeability, enhance leukocyte infiltration, and increase the extent of secondary damage [86].

The first systemic immune cells to infiltrate the injured spinal cord are neutrophils. These cells arrive within several hours after injury and are found maximally within 1-2 days [71, 87, 88]. They enter the spinal cord due to direct endothelial cell death (initial hemorrhage) and due to increased expression of selectins, cellular adhesion molecules (CAMs), and integrins on the surface of endothelial cells. Neutrophils express PSGL-1 (P-selectin glycol-protein ligand-1), which interacts with I-type cellular adhesion molecule (I-CAM), vascular cellular adhesion molecule (V-CAM), platelet endothelial cellular adhesion molecule (PE-CAM), and integrins. This interaction allows neutrophils to tether to the endothelial wall, roll, become activated with cytokines and enter the injured tissue (diapedesis or leukocyte extravasation). Once on the scene, they exert their innate immune responses, which include the production of myeloperoxidase and NO – thus inflicting ROS/RNS damage, as discussed earlier. Neutrophils can also potentiate the inflammatory response by producing pro-inflammatory mediators (see section below on cytokines). Additionally, neutrophils secrete MMPs which can degrade collagens, chondroitin sulfate proteoglycans (CSPGs) and endothelial tight junction proteins (see blood spinal cord barrier section below). Whether the presence of neutrophils is beneficial or detrimental following SCI remains undefined as there has been evidence suggesting that neutrophil blockade is detrimental as they can play a role in wound healing [89-92].
1.5.1.6 TLR and CD44 Receptors

Toll-like receptors (TLRs) are a family of ubiquitously expressed receptors involved in innate immune responses to invading pathogens. Activators of these receptors include lipids, proteins, lipoproteins and nucleic acids. Upon activation, signals are transduced to the nucleus where genes responsible for cytokine/chemokine production, production of NO and production of cell surface receptors are activated. TLRs are expressed by cells of the CNS and immune system. Microglia and astrocytes express TLR 1-9, oligodendrocytes express TLR 2 and 3 while neurons express TLR 2, 3, 4, 8 and 9.

TLRs form hetero or homodimers to achieve specificity for a particular ligand. TLR4 and numerous signaling molecules of TLRs are alternatively spliced, producing numerous proteins that can alter the course of the inflammatory response from receptor activation [93]. Signaling in TLRs has been classified as either myeloid differentiation factor 88 (MyD88) dependent or independent. In the MyD88 signaling pathway, MyD88 is recruited to the intracellular surface of TLRs and associates with TIRAP to signal through TRAF6 and on to either; (i) MAPKK -- p38 or JNK -- AP-1, (ii) p105 -- ERK1/2 -- AP-1 or (iii) IKK -- NfκB. In MyD88 independent signaling, TRAM associates at he in intracellular surface of TLRs and associates with TRIF to signal through; (i) RIP-1-- IKK -- NfκB, (ii) TRAF 3 -- IRF3 or (iii) PI3K -- AKT. TLR4 is capable of signaling through either pathway when activated. Additionally, lipopolysaccharide (LPS), a TLR4 agonist, can be internalized within 30 seconds [94] where it can associate with Nod1 and cause NfκB activation [95].

Activation of TLR4 also elicits negative regulatory pathways in macrophage/microglia. IL-1 receptor associated kinase-M (IRAK-M), suppressor of cytokine signaling-1 (SOCS-1), Toll interacting protein (Tollip) and A20 (TNFα-induced protein 3) are elicited during second or continuous exposure to stimuli. IRAK-M prevents dissociation of IRAK and IRAK-4 from
MyD88 and formation of IRAK-TRAF6 complexes [96]. SOCS-1 is thought to regulate the JAK/STAT signal pathway [97]. Tollip, a substrate of IRAK, suppresses IRAK activity [98].

A20 has de-ubiquitinating activity and acts as a negative regulator of TLR signaling by removing ubiquitin moieties from TRAF6 [99]. In contrast, phosphatidylinositol 3-kinase (PI3K) has been implicated in influencing the primary inflammatory stimulus in macrophages through an Akt (PKB) dependent pathway [100]. See Figure 2 for a schematic of TLR4 signaling.

Following trauma to the spinal cord, activation of TLRs is an integral part of the local and systemic immune response to injury. This is mediated in part through endogenous intracellular ligands that become exposed to TLR expressing cells due to necrotic cell death in addition to increased expression of TLRs through; (i) receptor upregulation and proliferation of resident microglia and (ii) recruitment of systemic TLR expressing immune cells. TLRs, normally responding to innate immune challenges from pathogens, seem to produce similar cytokine and chemokine profiles regardless of the source of stimuli [101]. Endogenous intracellular ligands include HSPs and HMBG-1, while extracellular ligands include breakdown products of HA, fibronectin, heparin sulfate proteoglycans and fibrinogen. The nature of their role in the injured spinal cord is varied.
TLR4 Agonist - LPS, HSP, LMW-HA, beta amyloid

TLR4

MD2

CD14

MyD88

TRIF

IRAK

IRAK-M

PI3K

PI2

PI3K

PDK

Akt/PKB

P

IRAK

IRAK

P

TRAF6

MAP3K

MKK3/6

MKK4/7

MKK1/2

p38

JNK

p38

ERK

p38

IKK

AP-1

NFkB

Inflammatory Mediator Production - NO - cytokines - chemokines

Nucleus
Figure 2. TLR4 pathways

TLR4 is capable of signaling through either MyD88 dependent or independent pathways. In the MyD88 signaling pathway, MyD88 is recruited to the intracellular surface of TLRs and associates with TIRAP to signal through TRAF6 and on to either; (i) MAPKK -- p38 or JNK -- AP-1, (ii) p105 -- ERK1/2 -- AP-1 or (iii) IKK -- NFκB. In MyD88 independent signaling, TRAM associates at the intracellular surface of TLRs and associates with TRIF to signal through; (i) RIP-1 -- IKK -- NFκB, (ii) TRAF 3 -- IRF3 or (iii) PI3K -- AKT (not shown). Activation of TLR4 also elicits negative regulatory pathways (red lines). IRAK-M, SOCS-1, Tollip and A20 are elicited during second or continuous exposure to stimuli. IRAK-M prevents dissociation of IRAK and IRAK-4 from MyD88 and formation of IRAK-TRAF6 complexes. SOCS-1 is thought to regulate the JAK/STAT signal pathway. Tollip has been shown to suppress IRAK activity. A20 acts as a negative regulator of TLR signaling by removing ubiquitin moieties from TRAF6. PI3K has been implicated in influencing the primary inflammatory stimulus through an Akt (PKB) dependent pathway.
Animal studies have demonstrated that both TLR2 and TLR4 knockout mice fare worse following injury as compared to their wildtype counterparts [102] while others have pointed to activation of TLR4 to be responsible for neurodegeneration and oligodendrocyte loss [103, 104]. Indeed, TLR signaling following injury is undoubtedly a complicated story involving the temporal activation profile and cell type involved.

CD44 is an extracellular cell-surface glycoprotein receptor that plays a critical role in a variety of physiological processes such as cell proliferation, cell migration, adhesion and inflammation. The multifunctional nature of CD44 is a result of structural heterogeneity obtained through alternative splicing, generating 10 splice variants. The affinity of CD44 for various ligands is dependent on post-translational glycosylation. Receptors can oligomerize/cluster in order to recognize specific glycosaminoglycans (GAGs). The principal GAG ligand for CD44 is HA; however, CD44 also interacts with fibronectin, fibrinogen, collagen IV, CSPG A and C, and osteopontin. Cytokines such as IL-5, IL-3, IL-12, IL-18, and IFN-gamma have been demonstrated to increase CD44 expression. Mechanisms of how these cytokines modulate CD44 expression are complex, however LPS has been shown to activate c-Jun-N-terminal kinase MAPKs leading to increased CD44 expression in macrophages [105].

The involvement of CD44 in inflammation includes regulation of lymphocyte extravasation to sites of inflammation. This has been demonstrated in models of rheumatoid arthritis [106] and EAE [107]. Antibodies against CD44 were demonstrated to reduce entry of T cells to the brain and block secondary influx of leukocytes [107]. CD44 is also involved in inflammatory signaling in immune cells within the CNS. Increased expression of CD44 is seen in microglia following forebrain ischemia at the site of neural damage and also later in infiltrating macrophages [108]. Additionally, CD44-null mice have improved outcome following cerebral ischemia, presumably due to a reduction in IL1-β expression [109]. Following
SCI, Moon et al. suggest that CD44 aids in glial cell attraction to the injury site and tissue repair [110]. They found increased CD44 expression in microglia and astrocytes following injury. They also located CD44 to myelin sheaths, vessels in the core of the lesions and in dorsal horn neurons of the rat spinal cord. Rolls et al. have suggested that CSPGs interact with CD44 on microglia/macrophages creating a beneficial, alternatively activated phenotype [111].

In addition to sharing a common agonist in HA, TLR4 and CD44 have also been shown to upregulate similar gene products and even interact with each other’s signaling pathways. HA fragments (100-150kDa) and not HMW-HA (1900 kDa) were demonstrated to produce IL-6 in cultured peripheral blood mononuclear cells (PBMCs) from wild type animals but to a lesser extent in cells from either TLR knockout and CD44-null mice [112]. Both TLR4 and CD44 neutralizing antibodies produced similar effects. Conversely, HA (ranging in sizes up to 500 kDa) was able to protect mice against LPS-mediated endotoxic shock, a phenomenon not seen in CD44 null mice [113]. This study demonstrated that HA caused an increase in A20 gene expression through CD44 (and TLR4) in peritoneal macrophages in vitro. A20 (TNFα-induced protein 3; TNFAIP3) has de-ubiquitinating activity and acts as a negative regulator of TLR signaling by removing ubiquitin moieties from TRAF6 [99]. Similarly, CD44 has been demonstrated to suppress TLR mediated inflammation through a cytoplasmic association between TLR and CD44, though the exact CD44 agonist was not known [114]. Additionally, the recognition of HA fragments has been reported to utilize a receptor complex involving TLR4, CD44 and MD-2 [115].

1.5.1.7 Early cytokine signaling

Cellular injury leads to activation of microglia, leading to the production of pro-inflammatory cytokines, which in turn causes local cellular responses and recruitment and activation of systemic inflammatory cells [116, 117]. The effectors of this chain reaction are
pro-inflammatory cytokines such as TNF-α, interleukin IL-1β, and IL-6, which are elevated within hours after SCI in humans and rodents [118, 119]. In mice, microglia are the major cellular source of TNF-α, IL-1β and IL-6 within the first 12 hours after SCI [117]. Evidence suggests that astrocytes, other CNS cells and neutrophils produce IL-1β in response to the initial microglial cytokine production, thus acting to potentiate and maintain the inflammatory response [116, 117, 120, 121]. Overall, evidence from humans and animal models suggests that resident cells are the primary producers of pro-inflammatory cytokines with peripheral immune cells playing a supporting role in this respect [116-118].

Resident CNS cells and endothelial cells produce pro-inflammatory cytokines under normal conditions as signaling molecules; however, they increase production above basal levels in response to injury [122, 123]. These cytokines exacerbate the primary damage by causing direct neurodegeneration and preventing axon growth [124, 125]. Additionally, these cytokines continue the recruitment of inflammatory cells by interacting with endothelial cells that line the surrounding vasculature. TNF-α and IL-1β have been shown to increase the expression of vascular cell adhesion molecule-1 (VCAM-1) and E-selectin on the surface of endothelial cells which can help facilitate leukocyte entry into the cord [126, 127]. Further, increased cytokine expression can lead to production of chemokines responsible for the recruitment of systemic inflammatory leukocytes. Increased IL-1β levels in the spinal cord can promote the production of chemokines responsible for recruitment of neutrophils via CXCL1 (GRO/KC or cytokine-induced neutrophil chemoattractant CINC-1) [128] and macrophages via macrophage chemotactic protein-1 (MCP-1) [129]. Additionally, IL-6 has also been shown to play a role in stimulating chemokine production that results in the recruitment of neutrophils and macrophages [125, 130-132].
1.5.1.8 Chemokines

In general, increased cytokine production and signaling induces the production of chemokines, which activate and recruit inflammatory cells to the site of injury or infection. Chemokines are a family of small proteins that can be subdivided into two groups; (i) the α family (C-X-C) and (ii) the β (CC) family [133]. The α subgroup is responsible for recruiting polymorphonuclear leukocytes. CXCL1 and CXCL3 are major factors in the recruitment of neutrophils studied after SCI [134]. The β subgroup recruits macrophages and lymphocytes. CCL2 (MCP-1) has been demonstrated to be a potent monocyte chemoattractant following SCI in mice [129, 133, 134]. Increases in CXCL1 and CCL2 mRNA levels have been detected in mouse and rat SCI models several hours following injury, with CXCL1 peaking between 4-12 hours and CCL2 peaking between 12-24 hours post injury depending on species and method of detection [129, 133, 135]. Astrocytes – and to a lesser extent oligodendrocytes, microglia, endothelial cells and neurons – were shown to produce CCL2 and CXCL1 following SCI in mice [129, 135]. Chemokines such as CXCL1 act on G-protein coupled receptors and induce the production of other chemokines and cytokines in cultured astrocytes [136]. CCL2 has been shown to control immune cell responses that mediate rapid phagocytosis of myelin debris in a model of spinal cord demyelination [137]. There is little of information on chemokines following SCI in humans in addition to few studies on the complete range of known chemokines in animal models of SCI [138].

1.5.1.9 Blood spinal cord barrier compromise

The blood spinal cord barrier (BSCB) remains compromised long after the initial primary mechanical damage to local vasculature, due to the effects of inflammatory mediators on endothelial cells and loss of astrocytes. Tracer studies in rats show that BSCB permeability peaks at 24 hours and remains compromised until approximately 2 weeks following injury [139].
study by Popovich et al. suggests that the BSCB disruption following injury may be biphasic event, as their data show a secondary increase in BSCB permeability at 28 days following injury {Popovich, 1996 #415}. The inflammatory cytokines IL-1beta and TNF-alpha are responsible for the acute increase in vasculature permeability [140]. Increased expression of ROS, NO, histamine, and matrix metalloproteinases all contribute to prolonged permeability [78, 86].

1.5.1.10 Apoptotic versus necrotic cell death

While there are clearly some purely necrotic causes of cell death and purely apoptotic causes, certain studies demonstrate that depending on the intensity of the cell death insult, either necrosis or apoptosis could be the resultant cell death phenotype [141]. There is little evidence to support that neurons undergo apoptosis in human SCI [142, 143] despite evidence of this in animal models [144, 145]. Oligodendrocytes on the other hand undergo apoptosis following SCI [146] and their death leads to axonal demyelination [147]. In general, acute cell death following SCI is necrotic in nature. It should be noted that cell death is most likely on a necrotic-apoptotic continuum. Further, other forms of cell death are possible, including autophagy.

1.5.2 The subacutely injured spinal cord

The subacute period lasts from approximately 2 days to 2 weeks following injury in animal models of SCI. In man, it is likely that the subacute phase lasts from 2 weeks to 6 months. This phase is characterized by massive immune cell infiltration, reactive astrogliosis, remodeling of the ECM, delayed cell death and continuing axonal demyelination/degeneration. Endogenous progenitor cell proliferation, removal of cellular debris, angiogenesis, and astrocyte containment of the injury cavity highlight the beneficial aspects of the body’s response during this period.
1.5.2.1 Inflammation- Continued microglial activation combined with lymphocyte infiltration

Monocytes/macrophages are recruited 3 days following injury and can remain present and activated for several weeks [23, 77, 148, 149]. Once activated, macrophages are morphologically indistinguishable from resident microglia and adopt a similar cytokine expression profile. As with many aspects of inflammation, their beneficial/detrimental role in the injured spinal cord is not clear. [150-154]. Possible discrepancies in these studies could be due to the timeline for prevention of infiltration. Macrophages that are activated and present within the spinal cord during the first week are potentially detrimental whereas after that point they are essential in the recovery process. It appears that the ability of these cells to secrete growth factors and neurotrophins, as well as to phagocytose dead tissue and debris, makes them integral to wound healing and the regenerative process [155-161]. In contrast, peripheral macrophages have been shown to physically induce dystrophic axons to die-back, further inhibiting axonal regeneration [162-164]. Recent studies have also suggested that the microglia/macrophage population is heterogeneous – dependent on spatiotemporal factors [165, 166].

T-lymphocytes enter the spinal cord maximally between 3 and 7 days following injury in response to the cytokine/chemokine signals from activated microglia and macrophages [77, 148, 167]. T-lymphocytes can regulate macrophage/microglial activity, mainly by controlling secretion of both pro- and anti- inflammatory cytokines. Through cytokine signaling, CNS-specific T-cells recruit antigen-independent T-cells to the site of injury, and it is these cells that secrete various trophic factors important for regeneration and growth such as insulin like growth factor (IGF-1) and brain derived neurotrophic factor (BDNF)[168, 169].
1.5.2.2 Subacute cell death and axonal degeneration

There are a number of extra- and/or intracellular events, including removal of trophic factors, increase in inflammatory mediators, death receptor activation, and DNA damage, that can all cause apoptosis in the subacute setting [170]. The specific components involved in the execution of apoptosis differ according to the nature of the initiator and according to cell type.

The occurrence of apoptosis in post traumatic spinal cord tissue was recognized in humans by Emery and coworkers in 1998, through detection of TUNEL and caspase-3 activation [142]. Clinically relevant animal models of SCI have also identified apoptosis as a significant event in the injury pathophysiology, in which neurons and more so oligodendrocytes seem to be vulnerable [145, 171-182]. Activation of caspase-3 and caspase-8 are temporally related to apoptosis after SCI [173, 174, 177]. Caspase-3 activation following experimental SCI has been observed as early as 4 hours and up to 8 days after injury in both neurons and oligodendrocytes at, rostral, and caudal to the injury epicenter [173, 174, 177, 183]. In addition, the presence of cytochrome c in cytoplasmic regions has been measured several hours following SCI in neurons and after several days in oligodendrocytes [173]. Delayed oligodendroglial apoptosis occurs in association with axonal degeneration which might suggest the two phenomena are linked [174, 184].

1.5.2.3 Death receptors

The tumor necrosis factor receptor family are prototypic death receptors. Members that have been implicated in SCI include the tumor necrosis factor receptor (TNFR), FasR, and the TRAIL receptor. Deletion of the tumor necrosis factor receptor (TNFR) has been shown to increase damage and reduce functional recovery in experimental models of SCI. The TRAIL receptor has been associated with various types of experimental spinal cord injuries [185, 186]. Fas has also been associated with both ischemic and traumatic insults in the CNS and has been
shown to be upregulated in cases of ischemia and trauma [84, 174, 187-192]. Additionally, the p75 neurotrophin receptor can induce apoptotic cell death [193-196], a phenomenon that has been associated with oligodendrocytes undergoing apoptosis following experimental SCI [191].

The intrinsic or extrinsic death receptor pathway can be activated following injury, both of which activated caspase-3. Increased intracellular calcium can cause release of cytochrome c from mitochondria. The cleaved form of caspase-3 translocates to the nucleus where it has the ability to further cleave over 40 different proteins. It is recognized that apoptosis may occur in the absence of caspase activation [197, 198], for instance, through mitochondrial release of apoptosis-inducing factor (AIF) [199, 200].

1.5.2.4 The mitochondria and apoptosis

Mitochondria are key regulators of both caspase-dependent and caspase-independent apoptotic signaling. Several proapoptotic proteins in addition to cytochrome c, such as apoptosis inducing factor (AIF), can be released from the mitochondrial intermembrane space under various circumstances. When released, AIF is translocated to the nucleus and induces cell death by triggering chromatin condensation and high molecular weight (50 KDa) fragmentation. Endonuclease activity is not inherent to AIF, thus it does not directly cleave DNA but acts to recruit or activate endonucleases [201]. There is much debate in the literature surrounding how these pro-apoptotic molecules are able to escape from mitochondria, or more specifically, what causes mitochondrial outer membrane permeability (MOMP) allowing their translocation into the cytoplasm [202]. Calcium influx in surviving glial cells occurs almost immediately following injury, a phenomenon that spreads from the injury epicenter over time [203]. Mitochondrial calcium accumulation resulting from increased intracellular calcium concentrations had been demonstrated to cause opening of the MOMP [204].
1.5.2.5 Demyelination

Studies in animals have demonstrated that surviving axons can be found in demyelinated states [205]. This finding has prompted numerous studies looking at stem cell transplantation with the hopes of remyelinating these axons [206], though it is clear that stem cells can have other non-remyelinating effects such as neurotrophic factor secretion [207]. In humans however, studies describing SCI have not validated these findings, suggesting that demyelinated axons are not common to the human pathology [9, 208]. Other studies have detected demyelinated axons in 4 of 7 postmortem human SCI cases [209]. Technical issues or the length of time following injury that the samples were collected could explain these discrepancies.

1.5.2.6 The glial and fibrous scar

Scarring in the injured spinal cord is highly dependent on the severity and type of injury. Transection injury models produce very different patterns of scarring than contusion or compression injuries [210]. Contusion/compression injuries in humans (which are the most common) can involve laceration, breaches in the dura, subarachnoid hemorrhaging, or complete obliteration of the arachnoid layers and will have very different molecular make up and extent of scarring due to greater involvement of meningeal fibroblasts. Schwann cells also have been implicated in production of scar related ECM molecules following human SCI [211].

In rats and humans, astrocytes that survive the primary and acute secondary stages of injury respond by activating/proliferating and surrounding the cystic cavity in an attempt to prevent its spread. This phenomenon is typically referred to as astrogliosis or glial scarring, where astrocytes create a ‘heteromorphic network’ [9]. While partially beneficial due to limiting the spread of the lesion, their physical presence represents a barrier to axonal regeneration whether endogenous or therapeutically initiated. Astrocytes also express and secrete chondroitin sulfate proteoglycans (CSPGs) and other inhibitory molecules which can cause growth cone
collapse and dystrophic end bulb formation in neurons [212-215]. While astrogliosis is prevalent following SCI in rats and mice, it is not as pronounced in human SCI [216] which may have important consequences for the development of SCI therapies that specifically target glial scarring such as ChABC [217, 218].

Inside and around the lesion borders (fibrous scar), the ECM undergoes profound changes as a consequence of injury due to clean up of necrotic and myelin debris, activation of glial and immune cells, and possible infiltration of fibroblasts. The HA component of the normal ECM is degraded by hyaluronidases and ROS [219] leading to astrocyte proliferation following injury [220]. The fibrous scar consists of a collagen IV backbone, which is not inhibitory itself, but is ‘sticky’ and binds other ECM molecules [210]. Collagen IV and laminin expression are upregulated following injury and can be associated with scar formation in rats [221] and humans along with fibronectin [211]. Laminin remains upregulated into chronic time points in rats whereas collagen IV decreases over time, but not to basal levels. Molecules in the fibrous scar responsible for inhibition of neurite outgrowth in animal studies consist of CSPGs (such as NG2) [213, 222-229], tenascin [230-232], myelin associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMgp), brevican, versican, and Nogo A, B & C [214, 233]. In humans, the CSPGs, NG2 and phosphacan were found in scar regions post injury whereas neurocan and versican were not [234].

Astrocytes are one of the cell types responsible for producing CSPGs [223-226][227], which are ECM molecules with membrane and secreted forms, the latter of which typically form a complex with laminin/collagen IV. Following SCI, CSPG expression is up-regulated in reactive astrocytes [213, 222]. The CSPG, NG2 is upregulated 24 hrs after injury and peaks at 7 days [214].
1.5.2.7 Inhibition to regeneration

Myelin associated inhibitors such as MAG, Nogo, and OMgp bind the Nogo receptor (NgR) on neurons and activate the Rho/Rock pathway leading to decreased growth cone mobility and growth cone collapse. Until recently, the receptor for CSPGs was unknown. It was found that PTPsigma, a transmembrane tyrosine phosphatase was expressed on neurons and can act as a receptor for CSPGs [235]. CSPGs also signal through the Rho/Rock pathway.

1.5.2.8 Angiogenesis

Endogenous angiogenesis occurs during the subacute period and is detected at 7 days post injury in the gray matter of adult rats but diminishes as the cystic cavity spreads [221]. At more remote sites from the epicenter there is still significant angiogenesis present that has been associated with regenerating nerve fibers [236].

1.5.2.9 Progenitor cell proliferation

Stem/progenitor cells have been identified in the adult mammalian spinal cord [237-239] and proliferate extensively following SCI [240]. These cells differentiate into glia, as endogenous neurogenesis is generally not seen in the spinal cord. NG2 is a CSPG that is expressed on a subpopulation of progenitor cells and macrophages [214] following injury [214, 241]. It has been suggested that NG2⁺ progenitors can differentiate into astrocytes and oligodendrocytes following trauma, with cues for progenitor differentiation coming from changes in post injury niches [242]. In the spinal cord, cells around the central canal have been identified as a source of progenitors and proliferate following injury, generating mainly astrocytes [243, 244].
1.5.3 The intermediate phase

The intermediate period of SCI occurs from 2 weeks to 6 months post injury. The glial and fibrous scar continue to develop, macrophages remain present and active in the lesion, severed axons continue to degenerate in perilesional areas, axonal sprouting occurs and endogenous remyelination is observed. The severed axon portion distal to the cell body degenerates and myelin breaks down. Macrophages continue to phagocytose debris from degenerating axons/myelin breakdown globules. Regenerative axonal sprouting in corticospinal and reticulospinal tracts has been documented in rats [245]. Peripheral Schwann cells have been found to remyelinate following human spinal cord injury [246]. Oligodendrocyte precursor cells (OPCs) are also responsible for remyelination in the injured spinal cord [247].

1.5.4 The chronically injured spinal cord

The chronic SCI phase is typically considered anywhere after 6 months post injury. Wallarian degeneration of severed axons towards the cell body continues and debilitating neuropathic pain can develop. What is left at the injury site has been described as a multilocular cystic cavity traversed by vascular-glial bundles with regenerated nerve roots [9, 248]. Furthermore, astrocyte and collagenous fibers run through and surround the lesion. Within 1-2 years it is believed that the lesion has ceased to progress and continuing deficits are stabilized.

1.5.4.1 Neuropathic pain

Central neuropathic pain (CNP) describes pain that results from a lesion to the CNS. Pain is experienced in as many as 80% of spinal injuries and poses a significant threat to patient quality of life [249, 250]. CNP experienced by patients can be categorized as either spontaneous or peripherally evoked [251]. Spontaneous pain is described by patients as experiencing stabbing/burning sensations that come and go without any apparent stimuli or trigger. In
contrast, peripherally evoked pain occurs as a result of normally nonnoxious or noxious stimuli. Further classification of pain depends on the anatomical level of sensation relative to the injury site. CNP can be broken down into (i) above level, (ii) at level and (iii) below level pain, corresponding to the affected dermatomes. These regions exhibit different temporal progression and mechanistic pathophysiology of pain. At level pain develops shortly following injury in dermatomes around the lesion whereas below level pain occurs in remote dermatomes distal to the injury site and has a slower onset. Mechanisms of the development and maintenance of at and below level pain can involve decreased inhibitory input, abnormal ion channel and receptor expression, abnormal glutamate transporter expression and function, chronic microglial and astrocyte activation, increased ROS generation and altered cytokine signaling. These generally cause dorsal neuron hyperexcitability through LTP-like mechanisms, resulting in increased pain signaling to the brain (see reference [251] for an excellent review).

In animal studies of CNP after SCI, it is generally accepted that not all injury models (compression, contusion, transection, etc.) will produce neuropathic pain in every animal. Typically, moderate injury models will produce evoked mechanical or thermal allodynia in approximately 50% of animals [252]. This has enabled researchers to study the differential expression of pain related makers in two subsets of animals; ones that exhibit neuropathic pain and ones that do not [252, 253]. These studies have suggested that activation of CREB, ERK and p38 MAPK is increased in animals exhibiting at level mechanical allodynia [252-254]. Animal models also have pointed to chronic astrocyte and microglia activation as a major contributor to the development and maintenance of neuropathic pain below level [255-257].

1.5.4.2  Post-traumatic syringomyelia

Syringomyelia represents a complication of the spinal cord injury where an abnormal fluid filled cyst (syrinx) or multiple cysts (syringes; plural of syrinx) develop (See Figure 3)
Approximately 25% of all syringomyelia cases are associated with SCI, a disorder that has been termed post-traumatic syringomyelia (PTS). The syringes that develop in PTS are called noncommunicating extracanalicular syringes, where there is a clear anatomical distinction between the central canal and the syrinx cavity [259-262]. A syrinx will be found in approximately 28% of SCI patients up to 30 years after injury. However, only 3-5% of cases become symptomatic [263-267] due to the fact that smaller syringes are generally asymptomatic [263]. That being said, 30-50% of PTS patients will have a change in syrinx size in their lifetime [263, 264, 268-270], suggesting the incidence of symptomatic PTS could increase with longer follow up time and that these estimates might be currently underrepresented. Common symptoms include segmental pain due to injury of spinothalamic pathways at or above the level of injury and sensory loss [266, 271, 272], progressive asymmetrical weakness [271, 272] and/or increased spasticity [268]. Through the use of MRI, studies have demonstrated that syringes are often asymmetrical, lobulated and can be multiple separate entities [273-275]. Vannemreddy and coworkers found that PTS was not associated with anatomical level of injury but was associated with complete SCI [276]. They also found that an earlier onset of PTS following injury was associated with increasing age, injuries in the cervical/thoracic levels, and previous spine surgery. Further, they found that the incidence of PTS has increased over the past 15 years and with an earlier onset.

The etiology of PTS is not fully understood. What has been established is that CSF flow is influenced by blockage of the SAS by subarachnoid scarring (SS), causing inflow of CSF into the spinal cord [276-278]. Furthermore, syrinx enlargement can be caused by unbalanced CSF inflow due to increased pressure dynamics from arachnoid lesions or cord compressions [279].
Figure 3. Clinical case of PTS

A T1 weighted MRI of a patient diagnosed with post-traumatic syringomyelia from the Krembil Neuroscience Centre, Toronto Western Hospital, University Health Network (courtesy: Dr. M.G. Fehlings). This patient suffered a SCI and presented with increased pain and functional deficit weeks following the initial injury. Note the subarachnoid scarring and loss of white and gray matter signal differentiation above the injury, indicative of a fluid filled syrinx.

Studies in humans suggest that syringes typically have microglia, macrophages and gliosis in the cyst wall [259-261]. Also, there can be direct communication between a syrinx and the SAS at dorsal nerve root entry zones or ventromedian fissure [259]. Syringes are often found in the vascular watershed regions within the cord [259, 271] and some studies point to the
existence of a “presyrinx state” and that the formation of a syrinx could happen via coalescence of ECF due to edema, which can be imaged prior to syrinx formation [280, 281].

Surgical procedures for treating PTS involve shunting (syringosubarachnoid, syringoperitoneal, and syringopleural) and decompression/arachnolysis (arachnoid and scar dissected away from dura and the pial surface) [277]. Unfortunately, surgical treatment for PTS is prone to failure. Klekamp and coworkers noted in a retrospective study of 107 patients treated for syringomyelia associated with SS that 66% of shunting procedures had a recurrence of symptoms within 2 years, noting a high occurrence of mechanical shunt failure [277]. Further, recurrence rates were 92% and 100% for cases where there was focal or extensive SS, respectively.

Animal models of PTS have attempted to recreate the human condition and determine the disease etiology. Under normal conditions, there is CSF flow from the SAS into the cord parenchyma via the perivascular spaces, exchanging CSF with the central canal. Animal models of PTS suggest that fluid flows from the SAS along the perivascular spaces into the syrinx cavity [282]. Further, animal studies show that even small areas of SS could be associated with significant alterations in fluid flow and pressure dynamics in the SAS [283].

In an animal model of PTS involving intraparenchymal quisqualic acid (QA) injection followed by intrathecal kaolin injection, Brodbelt et al. suggested that the formation of an initial cyst might be a necessary predisposing factor towards the development of a syrinx in the presence of subarachnoid adhesions [284]. The group looked at escalating doses of QA and found that increasing the dose of QA increased the number of animals that developed PTS. Additionally, larger QA doses increased the size and rate of syrinx formation. In another study, CSF fluid flow dynamics was determined in an excitotoxic/arachnoiditis animal model of PTS by using a horseradish peroxidase (HRP) tracer to study the source and root of fluid flow into
syringes [282]. Using the intraparenchymal QA/intrathecal kaolin model, they demonstrated that following injection of HRP into the cisterna magna, HRP was detected in the ventromedian fissure, PVS, central canal, and extracanalicular syrinx. PVS flow was greatest at the level of the syrinx and was mainly from the central penetrating branches of the anterior spinal artery. They also noted that transparenchymal flow was less prominent than PVS flow and that PVS fluid flow moved preferentially into the syrinx and surrounding parenchyma.

1.5.5 Summary of SCI pathophysiology

The progression of SCI over time results in significant morphological and functional alteration. Inflammation, scarring and continuing axonal degeneration in addition to endogenous attempts at regeneration/remyelination highlight the complex interaction of local and systemic responses. The characterization of SCI pathophysiology has come a long way in recent years however there is still much to be elucidated. A greater understanding of the injured human spinal cord is needed with an increased variety of animal models to better mimic the human condition. Further defining the timeline of inflammatory and glial reactions to injury could better determine which responses should be allowed to proceed and which should be inhibited therapeutically for increased quality of life and functional recovery for patients. Figure 4 provides a schematic of the spatiotemporal dynamics of SCI pathophysiology. Additionally, Table 2 highlights these events.
Table 2. Spatio-temporal secondary pathophysiological events

<table>
<thead>
<tr>
<th>Timeframe</th>
<th>Epicenter</th>
<th>Inflammatory</th>
<th>ECM</th>
<th>Chemical/Biochemical</th>
<th>Other cellular</th>
<th>Perilesional</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immediate (≤2h)</td>
<td>Vascular</td>
<td>Microglial activation</td>
<td>Necrotic products in ECM</td>
<td>Glutamate</td>
<td>Axonal severing</td>
<td>Vascular</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Cyto kinase secretion</td>
<td>- DNA, ATP, K+</td>
<td>- [Ca2+]</td>
<td>- Gray matter necrosis</td>
<td>Inflammatory</td>
</tr>
<tr>
<td>Acute (≤2d)</td>
<td></td>
<td>- Edema (BSCB compromise)</td>
<td>- Degradation of ECM</td>
<td>- Calpain, ROS, MPO, MMP9</td>
<td>- Demyelination from oligodendrocyte loss</td>
<td>ECM</td>
</tr>
<tr>
<td>Subacute (≤2w)</td>
<td></td>
<td>- Hemorrhage (maximal BSCB permeability)</td>
<td>- Increase in HA</td>
<td>- Energy imbalances</td>
<td>- Axonal A-beta accumulation terminal end bulb formation in axons</td>
<td>Biochemical</td>
</tr>
<tr>
<td>Intermediate (≤6m)</td>
<td></td>
<td>- Hypotension</td>
<td>- Microglial activation</td>
<td>- Glutamate excitotoxicity</td>
<td>- Astrocyte proliferation</td>
<td>Other cellular</td>
</tr>
<tr>
<td>Chronic (&gt;6m)</td>
<td></td>
<td>- Ischemia/hypoperfusion</td>
<td>- Angiogenesis</td>
<td>- Wallerian degeneration</td>
<td>- Axonal swelling</td>
<td></td>
</tr>
</tbody>
</table>

With input from [9, 66, 221, 248]. Table adapted from ‘Pathophysiology of Spinal Cord Injury – Chapter 4, by James W. Austin, James W. Rowland and Michael G. Fehlings, in Essentials of
Editors: Michael G. Fehlings, Alexander R. Vaccaro, Maxwell Boakye, Serge Rossignol, John F. Ditunno, Jr., Anthony S. Burns’
Abbreviations: BSCB=blood spinal cord barrier, ECM=extracellular matrix, HA=hyaluronan, MPO=myeloperoxidase, ROS=reactive oxygen species

*The time windows are largely based on preclinical studies in rodent models. It has been estimated that in man, the acute injury lasts up to two weeks, the subacute injury extends from 2 weeks to 6 months and the chronic injury extends beyond the period of 6 months.
A

B

Primary/Immediate Acute

- Axonal swelling
- Gray matter hemorrhage
- Small white matter hemorrhage
- Severe axonal damage

C

Subacute

- Demyelination and axonal dieback
- Hemorrhage and edema
- Activated microglia/macrophages
- Fibroblast infiltration
- Activated astrocytes (glial scar)
- Inhibitory ECM (fibrous scar)

D

Intermediate/Chronic

- OPC myelination
- Vascular-glial bundles
- Astrocite-collagenous fibers
- Wallerian degeneration
- Schwann cell myelination
- Regenerating axon
- Subpial rim of surviving white matter
- Growth cone/inhibitory ECM contact
- Chronic glial activation and neuronal hyperexcitability
Figure 4. Illustration of SCI pathophysiology

Longitudinal and cross sectional representation of the spinal cord at various stages following spinal cord injury. (A) Normal spinal cord. (B) Immediate/acute injury. This phase is characterized by severing of axons at the epicenter and demyelination due to the primary injury. Gray matter hemorrhaging and small white matter hemorrhages are common. Necrosis of gray matter glia and sensory (red), autonomic (green) and motor neurons (blue) occurs along with axonal swelling and axonal transport failure. Microglia become activated due to necrotic byproducts and secrete inflammatory cytokines and NO, further damaging tissue and recruiting systemic inflammatory cells. Necrosis of sympathetic preganglionic neurons (green) causes autonomic dysfunction. (C) Subacute injury. Hemorrhaging and edema continue, resulting in a spread of the hypoperfused/ischemic zone (red area). This continues the necrotic and begins apoptotic cell death. Macrophages (green) infiltrate, contributing to the local damage. At the epicenter, acute necrosis of lower motor neuronal cell bodies results in degradation of the left over axons (blue dashed line). Severing of first order sensory axons causes dieback towards the cell body (DRG). Severing of upper motor neuronal axons at the epicenter results in degradation of the distal end (blue dashed line in caudal cross section). Severing of sensory fibers at the epicenter causes axonal dieback caudal to the injury site (red dashed line in caudal section). As the injury progresses several weeks, hemorrhaging and edema come to an end and microglia/macrophages phagocytose cell and hemorrhagic debris. Oligodendrocytes undergo apoptotic cell death due to inflammation and white matter excitotoxicity, contributing to demyelination. Depending on extent of damage to the meninges, fibroblasts (orange) proliferate and infiltrate the spinal cord, contributing to ECM remodeling. Astrocytes proliferate, acting to seal off the injury, forming a glial scar (black outline of cavity). Macrophages continue to infiltrate and phagocytose debris. At the level of injury, the majority of sensory and motor
neurons are gone. Severed motor, sensory, and autonomic axons moving above and below the injury site have their distal ends (relative to cell body) degraded and proximal ends retract. Angiogenesis also occurs (not shown). (D) Intermediate/chronic. The remaining debris is cleared from the lesion and microglia/macrophages remain active contributing to neuropathic pain. Growth cones of regenerating sensory and motor neurons (dashed lines) meet either a physical barrier in the glial scar or inhibitory chemical signal in the fibrous scar (due to CSPGs and myelin associated proteins). Note that a subpial rim of surviving tissue exists in varying states of demyelination, representing a possible therapeutic target. In the lesion, macrophages, vascular-glial bundles and astrocytes and collagenous fibers can be found. Remyelination is possible via either Schwann cells or OPCs.

*The time windows are largely based on preclinical studies in rodent models. It has been estimated that in man, the acute injury lasts up to two weeks, the subacute injury extends from 2 weeks to 6 months and the chronic injury extends beyond the period of 6 months

1.6 Current State of Treatments for SCI

Currently, researchers and clinicians are moving towards the realization that a multifaceted combinational treatment paradigm will most likely be needed to combat the myriad of pathophysiologic targets that present themselves following SCI. For example, neuroprotective therapies could be combined with anti-inflammatory and cell replacement strategies to maximize patient recovery. However, before this can happen, each individual treatment will most likely have to be tested on its own. The following sections deal with past and present clinical trials in addition to providing a glimpse of what is coming through the pipeline in terms of pre-clinical therapies.

1.6.1 Clinical Studies

Methylprednisolone (MPSS), is currently one of the only pharmacological therapies approved for the treatment of SCI [285-287]. The NASCIS I trial (100 or 1000 mg of MPSS, administered for 10 days), showed no difference. Follow up animal studies suggested that the dose was below the efficacy threshold and thus the NASCIS II was undertaken. This trial looked at a bolus injection plus infusion for the next 23 hours and showed improved neurological recovery with early treatment (less than 8 hours of SCI). As this group represented a subset of the original cohort, the findings were not uniformly accepted. A further MPSS study led by Japanese clinicians showed an improved neurological and sensory recovery if MPSS is given within the first 8 hours following injury using a similar treatment paradigm as the NASCIS I trial. While the mechanism of action remains elusive, the functional improvement seen in animal studies [288, 289] suggests that MPSS reduces TNF-α production, leukocyte infiltration and lipid peroxidation, leading to a reduction in axonal die-back [290, 291]. As MPSS is also associated with the development of infections and wound-related complications, some clinicians recommend against its use [292, 293].
There have been two studies looking at GM-1 ganglioside. Gangliosides are anionic glycosphingolipid molecules with one or more attaching sialic acid residues and are present at high concentration on the outer surface of neuronal cell membranes [294]. The first trial was the Maryland GM-1 trial that looked at a dose of 100mg/day for 18-32 days. This trial showed improved neurological recovery. The Sygen® GM-1 trial looked at a low dose (300 mg loading dose with 100 mg/day for 56 days) and high dose (600 mg loading dose with 200 mg/day for 56 days) treatments given an average of 55 hours post-SCI, both of which were combined with MPSS (same dosing as in NASCIS II). However, this study led to negative primary outcomes and a trend to enhanced secondary outcomes - improved bladder/bowel function and sacral sensation. At the pre-clinical stage, GM-1 was associated with enhanced axonal regeneration [295]. Drug interactions and missing the therapeutic time window for GM-1 were two issues addressed in the discussion of this phase III trial.

The anti-inflammatory compound minocycline has proved to be effective following experimental SCI. Its combined effects from several studies included reduced oligodendrocyte death, reduced apoptosis, less axonal dieback, less tissue loss and improved behavioural outcomes [296-299]. The mechanism of action seems to stem from its ability to attenuate microglial activation and to reduce the production of pro-NGF (nerve growth factor) [300-302]. Despite some pre-clinical findings that suggested there was no benefit from minocycline administration [303, 304], it is currently being studied as a neuroprotective therapy in a pilot Phase I clinical study in Calgary, Alberta.

As mentioned earlier, inhibitory molecules found in the post injury environment can cause Rho activation and neuronal growth cone collapse, leading to failed attempts at regenerating damaged neuronal connections. Further, Rho is associated with apoptosis in neurons, astrocytes and oligodendrocytes [305, 306]. Studies from the McKerracher group
using a bacterially derived C3 trasferase have led to the development of Cethrin®, a Rho antagonist that combines a C3 domain with a transport sequence that enables the protein to cross cell membranes. Bioaxone Therapeutics Inc. is currently testing Cethrin® in a Phase I clinical trials on patients with ASIA-A cervical or thoracic injuries, within two weeks of injury [307].

Aberrant sodium channel functionality is a pathological feature following SCI. Riluzole is a sodium channel blocker that has been shown to preserve tissue and improve functional recovery following SCI in animal models [308]. The fact that it is already approved by the Food and Drug Administration in the U.S. for the treatment of amyotrophic lateral sclerosis makes this a readily available candidate for clinical trials. As such, the Rick Hansen Foundation has funded a clinical trial in Canada for testing the efficacy of Riluzole following SCI in humans (Phase I).

Further to pharmacological treatments, cell replacement clinical trials have also been undertaken. In 2005, an Australian group looked at the transplantation of autologous olfactory ensheathing cells (OECs) in humans with thoracic (T4-T10) SCI [309]. They enrolled 6 patients who had sustained an injury 18-32 months prior and who were classified as ASIA-A (complete loss of sensory and motor function below the site of injury). The OECs were harvested from a nasal biopsy, cultured for 4-10 weeks and cells were transplanted into the damaged cord and into the proximal and distal ends of the intact cord. The 3-year follow up revealed no adverse outcomes (tumor formation, syringomyelia, neuropathic pain, etc.) and greater numbers will be enrolled in the future to determine efficacy.

The Geron® oligodendrocyte progenitor cell (OPC) Phase I clinical trial was commenced in 2010 and is the first to examine stem cells derived from an embryonic source. According to their website (www.geron.com), this multicenter trial using human embryonic stem cell (hESC) derived OPCs (GRNOPC1), will involve ASIA-A patients with thoracic injuries (T3-T10). Cells
will be injected 7-14 days after injury. Unfortunately, in 2011 the trial was halted by the company for strategic/financial reasons.

Procord®, a cell-based therapy currently in a Phase II trial looks at transplantation of ex vivo activated macrophages [310]. The company, Proneuron, has undertaken this study, using macrophages taken from the patient’s own blood. Patients with an ASIA-A grade injury between C5-T11 receive a direct injection of activated macrophages into their spinal cord 14 days after injury. This international, multi-center foray is based on work by Schwartz and colleagues [160, 161, 165, 311].

1.6.2 Pre-clinical Animal Studies

Although cell replacement therapies hold promise, they are in their infancy and thus their efficacy won’t be fully determined for at least a decade. Patients treated with either MPSS or GM-1 ganglioside only show modest neurological improvement following SCI, thus the overall impact of these treatments remains minimal [312-315]. As such, there exists a great need for a broad range of therapeutics to relieve SCI co-morbidities and improve sensory, autonomic and motor outcomes. There are a vast amount of therapies being tested in pre-clinical SCI models. The following represent a broad outline of some of the promising anti-inflammatory and neuroprotective treatments.

The use of immunosuppressants, such as FK506, can reduce the amount of active caspase-3 in oligodendrocytes following injury [316]. Additionally, cyclosporin-A was shown to reduce lipid peroxidation, reduce neuronal cell death, and improve functional outcome in animal models of SCI [317, 318]. Targeting calcium dependent proteases, such as calpains, has also been studied in animal models of SCI, as they have been implicated in the pathophysiology of SCI [319].
CEP-4143 is a calpain inhibitor that has been shown to prevent neurofilament breakdown and improve functional recovery following experimental SCI [57]. Erythropoietin (EPO) is a hormone currently being used in clinical trials for stroke treatment that can protect against ischemic damage by preventing lipid peroxidation, inflammation and apoptosis [320]. Neuroprotection with EPO derivatives has been demonstrated in experimental models of SCI [321].

Glutamate, calcium, and sodium channel blockers have also been used to confer neuroprotection following SCI in experimental models and clinical trials. Non-NMDA receptor antagonists have proven to be safe and show promise. For example, motor function has been improved in experimental models of SCI through application of 1-aminooindan-1,5-dicarboxylic acid or (1)-2-methyl-4-carboxyphenylglycine (LY 367385) [322]. Nimodipine is a calcium channel blocker that has shown improvement in axonal function and increase in blood flow to the spinal cord following injury [323].

Another anti-inflammatory approach to treating spinal cord injury is using an antibody to CD11d in an effort to target the migration of leukocytes to the site of injury [324]. Monoclonal anti-CD11d antibodies were used as a treatment to attenuate neutrophil and macrophage infiltration into the injured spinal cord [325]. These treatments were associated with significant reductions in myeloperoxidase activity (neutrophil associated enzyme), lipid peroxidation, protein nitrosylation, COX-2 expression, mechanical allodynia and autonomic dysreflexia [326-330]. Anti-CD11d treatments were also associated with significant increases in tissue sparing and improvement in hind-limb locomotor function. Unfortunately, confirmatory studies were unable to replicate the initial promising results [331].
Lastly, application of polyethylene glycol (PEG) has proven to be neuroprotective following experimental models of SCI [332, 333]. PEG and similar polymers are fusogens and have also demonstrated the ability to scavenge ROS [334].

Overall, numerous groups worldwide are dedicated to testing a bevy of treatments for SCI. One key obstacle to translation of therapeutics lies in the heterogeneity of SCI. Guidelines for translation of therapeutics have been put forward by a group of SCI basic scientist and clinical experts [335]. They recommend that treatments be tested in a number of different SCI models/species and in numerous independent laboratories before moving into the clinical arena.

1.7 Bioengineering and biocompatible polymers
1.7.1 Drug Delivery Systems and the CNS

Systemic delivery of drugs to the CNS is often associated with severe side effects due to the high doses required to cross the blood-spinal cord barrier (BSCB) [336]. Intrathecal drug delivery is considered an effective way to deliver drugs for CNS conditions, including SCI [337-339], because the BSCB is bypassed, thus decreasing systemic side effects and achieving higher doses than is possible with systemic delivery. Minipump/catheter delivery provides reliable and prolonged administration of therapeutics in diseases such as amyotrophic lateral sclerosis (ALS) [340]. However, this method is associated with problems of blockage, infection and scarring [341, 342]. In certain cases, catheters can cause compression of the spinal cord [343].

Bioengineered drug delivery systems (DDS) provide localized delivery to the injured tissue. A second intervention to remove the DDS from the SAS is unnecessary because it is biodegradable, and the DDS is non-inflammatory so that delivery can be achieved without causing further trauma to the injured spinal cord. A fast-gelling, minimally-invasive system for localized delivery to the spinal cord was pioneered by the Shoichet group and involved a blend
of hyaluronic acid and methyl cellulose (HAMC). In a series of publications, they demonstrated the safety of the methodology [344], efficacy of local release [345] and the mild therapeutic benefit of HAMC alone on early locomotor recovery [346]. HA and MC degrade within 7 days and diffusion of proteins from HAMC is even faster, usually within 24 hours. The introduction of biodegradable/biocompatible polymers such as poly(lactide-co-glycolide), PLGA, used to encapsulate therapeutic agents, including drugs and proteins, for prolonged release of bioactive agents is possible [347]. For example, these agents can be encapsulated in microspheres and then slowly released over time as a function of loading and polymer degradation [348-350], as has been shown with bioactive nerve growth factor [351] and EGF [352]. Additionally, HA and/or MC can be modified (eg. crosslinked) to slow the rate of biodegradation [353].

1.7.2 Hyaluronan

Hyaluronan (HA) is a glycosaminoglycan comprised of repeating chains of the disaccharide D-glucouronic acid β(1-3) N-acetyl-D-glucosamine (Figure 5). It is a major extracellular matrix (ECM) component in most tissues, including the CNS and exists as a high molecular weight polymer of approximately 106 Da. In the CNS, HA is predominantly produced by astrocytes and to a lesser degree neurons, oligodendrocytes and microglia [354-357]. Under normal conditions, HA is localized around myelinated fibers in the white matter [354] and surrounding neuron cell bodies in the gray matter [358, 359]. HA also attracts water by osmosis and resists water flow [360], resulting in changes in interstitial porosity, creating cell free spaces, facilitating the migration of cells in tissues [361]. It also has the capacity to interact with proteins playing a role in growth, development, inflammation, and immune responses. ROS can generate HA fragments [362], suggesting that HA has the capacity to scavenge ROS [363]. HA can also bind cell-bound receptors CD44, RHAMM, TLR 2/4, as well as other proteins found in
the ECM like collagens [364], fibronectin [365], aggrecan [366], versican [367], neurocan [368], and chondroitin sulfates [369].

HA can elicit numerous biological responses depending on its size and cell/protein type it interacts with. RHAMM-HA interactions are thought to be involved in cell locomotion [370] and HA can induce proliferation of endothelial cells [371]. Low molecular weight HA (LMW-HA) has been shown to signal through TLR2 in sterile tissue injury, causing an inflammatory response [372]. However, CD44 is the major HA binding receptor studied and is expressed on most cells including neutrophils, macrophages and lymphocytes [373]. The role of CD44 signaling depends on cell type [374] and the functions associated with HA binding involve a regulated process [375]. CD44 has been postulated to regulate cell adhesion and trafficking [376], homing of T cell to inflamed tissue [377, 378], extravasation of T cells to sites of tissue inflammation [377, 379], antigen-specific T cell responsiveness [380], leukocyte activation [381, 382], expression of other adhesion molecules [383, 384], production of cytokines and chemokines [385, 386], and apoptosis [387]. CD44 on hematopoetic cells appears to be critical to clear LMW-HA from sites of tissue injury [388]. HA-CD44 may play a role in inflammation and T cell recruitment and activation [389]. Also, HA-CD44 interactions are believed to play a role in tissue injury and repair [390]. High molecular weight HA (HMW-HA) can inhibit inflammation [374] and reduce production of pro-inflammatory cytokines [391]. HA inhibits astrocyte proliferation in vitro and its fragmentation causes astrocyte proliferation in vivo [220]. HA becomes degraded following SCI up to a week following injury and then becomes increasingly expressed at 4 weeks following injury [220]. Additionally, application of exogenous HMW-HA was protective against injury, mortality, and epithelial cell apoptosis in a model of lung injury [392].
Figure 5. Chemical structure of HA

The structure of HA (hyaluronic acid) is shown. The polymer consists of repeating units of D-glucuronic acid (left) and N-acetyl-D-glucosamine (right) attached by $\beta(1-3)$ linkages. The linear polymer consists of these disaccharide units attached by $\beta(1-4)$ linkages. Depending on the amount of repeats, the molecular weight can reach as high as 4000 kDa.
Rationale

SCI causes motor, sensory and autonomic impairments that lead to considerable patient suffering and which have substantial economic implications. Subarachnoid inflammation (arachnoiditis) following SCI can lead to the formation of localized SS and the development of post-traumatic syringomyelia (PTS). Damage to meningeal layers is an often overlooked aspect of the primary trauma following SCI. While PTS is a devastating complication of SCI, its relative rarity (occurring symptomatically in about 5% of clinical cases) and lack of fundamental physiological insights have led us to examine an animal model of traumatic SCI with induced arachnoiditis (PTS model). While previous models have been used to study PTS, they do not involve a traumatic insult - they induce parenchymal damage chemically. While they have been useful in generating preliminary pathophysiological insights in terms of syrinx development, a more clinically relevant traumatic model was studied.

Currently, there are few effective pharmacological treatment options to complement surgical and rehabilitation measures taken by physicians and health care practitioners. Moreover, there are no preventive treatment options to reduce arachnoiditis and the onset of SS, which is associated with the development of PTS – a complication of SCI associated with significant neurological impairment and neuropathic pain. Additionally, the likelihood of recurrence following surgical intervention for PTS (arachnolysis/detethering and/or shunting) is high. Due to the debilitating consequences of PTS and lack of effective treatment options available to this subpopulation of injured patients, we set out to test the ability of a bioengineered polymer containing HA to reduce arachnoiditis and SS in an animal model of PTS. A biomaterial containing HA offers the therapeutic advantage of occupying space in the SAS to physically prevent expansion of inflammation and scarring, is a source of HA which is anti-inflammatory and slowly degrades within a week – thus providing sustained delivery of HA. HA has been used
therapeutically to treat inflammatory conditions such as liver and lung injury. Additionally, studies suggest HA can modulate inflammatory reactions and possibly improve functional recovery following SCI.

HA has been used to modulate inflammatory responses in macrophages – a similar cell type to microglia – though the exact mechanism is not clear. Following central nervous system trauma including SCI, microglial Toll-like Receptor 4 (TLR4) signaling is involved in the ensuing inflammatory pathophysiology. Overall, acute microglial activation through TLR4 is most likely seen as a detrimental aspect of SCI that contributes to direct cell toxicity. Due to detrimental microglial TLR4 activation following SCI and the fact that HA has been shown to modulate inflammation following SCI, we sought to determine if HA can modulate TLR4 signaling and if so, elucidate a possible mechanism.

**Overarching Hypothesis**

Intrathecal injection of a bioengineered hydrogel containing HA will improve functional recovery following severe spinal cord injury associated with arachnoiditis.

**Statement of Objectives**

(1) Characterize the acute pathophysiology in a model of SCI associated with arachnoiditis.

(2) Examine the use of a bioengineered hydrogel containing HA to treat severe SCI associated with arachnoiditis

(3) Determine the effect of HA on TLR4 activation in cultured microglia
Publications and Presentations

Publications


Presentations

SpineFest, Department of Surgery, University of Toronto (oral) 2010
Treatment Strategies for Spinal Cord Injury, University of Alberta (poster) 2010
University of Toronto Neuroinflammation Symposium (poster) 2010
Society for Neuroscience (oral) 2009
National Neurotrauma Society Symposium (poster) 2008, 2009
Institute of Medical Science Research Day, University of Toronto (poster) 2009
Toronto Western Research Institute Research Day (poster) 2008
Chapter 2

2 General Materials and Methods

2.1 Animal Models

All animal protocols were approved by the animal ethics board of the University Health Network, Toronto, ON, Canada. Procedures were carried out as previously described [393]. Female Wistar rats approximately 300g in weight were anesthetized with 2% isoflurane with oxygen and NO₂. An incision was made in the skin just below the T2 spinous process, extending approximately 5cm. Next, an incision was made in the subcutaneous fat layer, exposing the underlying muscles. An incision was made in the midline of the muscle and the dorsal aspect of T5-T9 vertebra were exposed and cleared. A 3-level laminectomy was performed from T6-T8, inclusive. For non-injured control animals (sham), no further manipulation was done. For arachnoiditis animals, a 100 µL syringe (Hamilton, Reno NV, USA) with 23g gauge bent needle (Hamilton) was used to inject 5 µL of a 0.5 mg/mL kaolin mixture through a small hole made in the dura with a 35 gauge needle. For SCI animals, a metal hook was used to help pass an open 35g calibrated clip under the spinal cord which was then released to cause the clip to snap on the spinal cord – for 1 minute. For PTS animals, SCI was induced as above followed immediately by a subarachnoid kaolin injection rostral to the injury site as above. Following one of the previous procedures, multilayer tissue closure was performed. See Figure 6 for schematic of model.

Female rats were used due to logistical considerations of animal care following injury. The clip contusion/compression injury is considered a reliable and accurate model of SCI [394]. The rationale for using kaolin to induce arachnoiditis is addressed in the discussion of this chapter.
Figure 6. Schematic of animal models

Model characterization – Chapter 3. Following a 3-level laminectomy from T6-T8 inclusive, animals were assigned to one of 4 groups: (i) non-injured control animals (sham) group, no further manipulation was done, (ii) arachnoiditis group, 5 µL of a 0.5 mg/mL kaolin mixture was injected intrathecally (iii) SCI group, a 35g clip was snapped on the spinal cord and left for 1 minute and (iv) PTS group, SCI was induced followed immediately by a subarachnoid kaolin injection rostral to the injury site. Therapeutic intervention – Chapter 4. 24 hours following PTS injury, either aCSF or HAMC was injected intrathecally.
2.2 HAMC Preparation and Injection

HA (1,700,000 Da) was purchased from Lifecore (Chaska, MN, USA) and MC (13,000 Da) was purchased from Sigma Aldrich (St Louis, MO, USA). HA was sterilized by filtering a 0.1% solution through a 0.2 µm filter and lyophilizing prior to use. MC was sterilized similarly. Following lyophilization, sterile HAMC was produced by mixing polymer solutions in a laminar flow hood. Artificial cerebrospinal fluid (aCSF) was prepared in distilled, deionized water (dH₂O) with 148 mM NaCl, 3 mM KCl, 0.8 mM MgCl₂, 1.4 mM CaCl₂, 1.5 mM Na₂HPO₄, and 0.2 mM NaH₂PO₄. The MC and HA powders were sequentially dissolved in aCSF at 4°C, resulting in a 2% HA and 7% MC solution. Following PTS injury, Animals were randomized and either 10 µL of HAMC or aCSF was injected intrathecally 24 hours following injury below the kaolin injection site.

2.3 Primary Microglia Cultures

Cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA) unless otherwise stated. Routine culturing involved growing cells in Minimum Essential Medium supplemented with 10% heat inactivated FBS (Wisent, St Bruno QC, Canada) and 1% penicillin/streptomycin. Cells were grown in a controlled environment incubator at 37°C and 5% CO₂. All centrifugation steps were done at 300 x g for 5 minutes unless otherwise stated.

Microglia were cultured as previously described [395]. Briefly, 1-3 day old Wistar rat pups were killed by cervical dislocation and their cerebral cortices were removed and minced in ice cold MEM. The resultant minced tissue was passed through a 100-mesh cell sieve (Sigma, St. Louis, MO, USA) with agitation, centrifuged twice, and plated in 75 cm² flasks at a ratio of 1 brain to 5 flasks. The media was changed 2 days after culturing. After 7-10 days in culture, flasks were shaken at 75 RPM in an orbital shaker at 37°C to detach microglia from the surface of the flasks. Media containing microglia was removed from the flasks and centrifuged to pellet
the cells. Isolated microglia were then plated on poly-lysine coated tissue culture plates or multi-chamber slides (Nalge Nunc, Rochester, NY, USA). To coat the wells, a 0.1 % poly-lysine solution in dH$_2$O was incubated in the wells/chambers for at least 2 hours. For 48-well plates used in the nitric oxide (NO) assay, cells were plated at 200,000 cells per well. For 24-well plates used in qPCR assays, cells were plated at 400,000 cells per well. For cell homogenates used in western blotting, cells were plated at 1,000,000 cells per well in 12-well plates. For immunohistochemistry, cells were plated in multi-chamber slides coated with poly lysine as above, at 75,000 cells per well. Experiments were carried out between 12 and 24 hours of final microglial isolation on poly lysine coated dishes/plates.

High molecular weight HA (HMW-HA) with (average molecular weight = 1,700,000 Da) and low molecular weight HA (LMW-HA) (average molecular weight= 230,000 Da) were purchased from Lifecore (Chaska, MN, USA). Lipopolysaccharide (LPS) was purchased from Sigma. Both stock HA and LPS solutions were prepared in serum free media. Vehicle (media) was added as a control to groups not receiving LPS or HA. Each result presented is representative of at least 3 separate experiments. Most experiments involved dosing HA and LPS at the same time. Real-time quantitative polymerase chain reaction (qPCR) was carried out at 6 hours following LPS/HA exposure. IL-6 enzyme linked immunosorbant assay (ELISA) and the NO assay were carried out on media isolated from cells exposed to LPS/HA for 24 hours. The cells from the IL-6 ELISA and NO assays were used to determine DNA content. Cells used for western blot analysis were harvested at 15, 30 and 60 minutes following LPS exposure for acute phosphorylation studies and at 24 hours for A20 expression.
2.4 Immunohistochemistry (IHC)

Animals were fixed by transcardial perfusion with 4% paraformaldehyde (PFA). Prior to PFA fixation, animals were perfused with 120 mL of冰冷 PBS. PFA was prepared by heating distilled deionized water (ddH₂O) to 60 °C and adding the PFA powder and NaOH dropwise until the PFA dissolved. Once cool, the PFA solution was filtered and the pH was adjusted to 7.4. Spinal cords were harvested and postfixed in 4% PFA containing 10% sucrose overnight followed by PBS containing 20% sucrose overnight. Cords were then snap frozen in OCT and sectioned either transversely or longitudinally. Sections were rinsed in PBS for 5 minutes and blocked in blocking solution (0.1% triton-x 100, 1% BSA, 5% non fat milk, and 2.5% normal goat serum in PBS) for 1 hour. Primary antibodies (Table 3) were incubated overnight in blocking solution minus triton-x 100 overnight at 4°C (antibody solution). Sections were rinsed 3 x 10 minutes in PBS and fluorescent secondary antibodies were incubated for 2 hours at room temperature in antibody solution. Sections were rinsed again in PBS (3 x 10min) and coverslipped in a Mowoil mounting medium containing DAPI (Vector Laboratories, Burlington, ON, Canada).

In all cases, sections shown are representative of either the sagittal midline of the spinal cord (longitudinal sections) or are cross sections with stated distances from the epicenter. All longitudinal images are presented in the same orientation with the top of the image representing the dorsal aspect of the cord and the left side of the image representing the rostral side of the epicenter. Cross sections are shown with dorsal aspect of cord at the top of the image.

Immunohistochemistry (IHC) on primary microglia involved cells in multi-well chamber slides being rinsed twice in PBS and fixed with 4% paraformaldehyde in PBS for 30 min. The procedure was carried out as above with no post-fixing.
2.4.1 Lesion Size Analysis

Longitudinal sections were stained with GFAP to delineate the lesion borders. Images were analyzed using ImageJ software (NIH, Bethesda, Maryland, USA). A total of 5 sections were analyzed per animal (4 sections were imaged at every 500 µm on both sides of the epicenter, plus the epicenter section). The lesion borders were traced with ImageJ and an area was summed in each animal. Data are reported as the average area of 8 animals.

2.4.2 Fluorescence Intensity Analysis

Fluorescence intensity was analyzed using ImageJ software. ImageJ reports the average intensity of each channel in terms of grayscale units (0= black, 255=white). Images taken on a fluorescence microscope were imported into ImageJ. The total area of the spinal cord (minus meninges) was traced and the average intensity was calculated at varying distances from the injury epicenter in each animal. The average intensity at each distance was averaged over 6 animals per group. This grayscale intensity provides a semi-quantitative representation of fluorescene intensity at each distance from the epicenter.

Table 3. Antibodies used in immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host Species</th>
<th>Company</th>
<th>Dilution</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen IV</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>1:500</td>
<td>Overnight (4°C)</td>
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<tr>
<td>GFAP</td>
<td>Rabbit/Mouse</td>
<td>Millipore</td>
<td>1:1000</td>
<td>Overnight (4°C)</td>
</tr>
<tr>
<td>Iba-1</td>
<td>Rabbit</td>
<td>Wako</td>
<td>1:500</td>
<td>Overnight (4°C)</td>
</tr>
<tr>
<td>PMN</td>
<td>Rabbit</td>
<td>Cedarlane Labs</td>
<td>1:1000</td>
<td>Overnight (4°C)</td>
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<tr>
<td>CSPG (CS56)</td>
<td>Mouse</td>
<td>Sigma</td>
<td>1:200</td>
<td>Overnight (4°C)</td>
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<tr>
<td>CD68/ED-1</td>
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<td>Serotec</td>
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<td>2 hrs (RT)</td>
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<tr>
<td>Anti-mouse 546</td>
<td>Rabbit</td>
<td>Sigma</td>
<td>1:200</td>
<td>2hrs (RT)</td>
</tr>
<tr>
<td>Anti-mouse 488</td>
<td>Rabbit</td>
<td>Sigma</td>
<td>1:200</td>
<td>2 hrs (RT)</td>
</tr>
<tr>
<td>Anti-rabbit 488</td>
<td>Mouse</td>
<td>Sigma</td>
<td>1:200</td>
<td>2 hrs (RT)</td>
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<tr>
<td>Anti-rabbit 546</td>
<td>Mouse</td>
<td>Sigma</td>
<td>1:200</td>
<td>2hrs (RT)</td>
</tr>
</tbody>
</table>

2.5 Fresh Frozen Tissue Sectioning

Animals were perfused transcardially with 120 mL of ice cold PBS. Spinal cords were isolated and snap frozen in liquid nitrogen. For in situ zymography (Section 2.7), samples were
embedded in OCT and sectioned at 14 µm using a cryostat. For tissue ELISA (Section 2.11) relating to Chapter 3 and gel zymography (Section 2.7), samples were embedded in OCT and dorsal meningeal isolation was carried out by sectioning off approximately 300 µm of the dorsal surface of 0.5 cm of spinal cord tissue centered at the injury epicenter using a cryostat. This fraction contained the dorsal portion of the meninges exposed to the kaolin and is referred to as the meningeal fraction. The remaining kaolin free portion of the cord is referred to as the parenchymal fraction. Each isolated fraction was homogenized in RIPA buffer (Thermo Scientific, Waltham, MA, USA).

2.6 Immunoblotting

All immunoblot reagents were purchased from Biorad (Hurcules, CA, USA) unless otherwise stated. Animals were anesthetized, perfused with ice cold PBS, and 0.5 cm of spinal cord tissue centered at the epicenter was removed and snap frozen in liquid nitrogen. In some cases, 0.5 cm of spinal cord tissue rostral and caudal to the epicenter portion were also taken. The frozen tissue was crushed with a mortar and pestle in liquid nitrogen and added to a tube of ice-cold RIPA buffer (Thermo Scientific).

Equal protein amounts were determined using the Lowry method. Briefly, 5 µL of each sample was added to 495 µL of ddH2O. Bovine serum albumin (BSA) standards consisted of 2, 20, 40, 60, 80 and 100 µg/mL. Samples and standards were placed on a shaker and 500 µL modified Lowry reagent was added to each tube with shaking. After 10 minutes of incubation, 250 µL of Folin and Ciocalteu’s Phenol Reagent was added with shaking for 10 minutes. Samples were placed in plastic disposable semi-micro cuvettes (Biorad) and the absorption was measured at 750 nm. A standard curve was created using the absorptions and known
concentrations of the BSA standards. The concentration of the samples was determined using this standard curve and a 100 dilution factor (5µL in 500 µL).

Homogenates were dissolved and boiled in sample buffer (for 1 mL of 2x stock solution: 100 µL glycerol, 125 µL 0.5 M Tris-HCl pH 6.8, 50 µL 1% bromophenol blue, 50 µL betamercaptoethanol, 200 µL 10% sodium dodecyl sulfate (SDS), 475 µL ddH₂O) prior to polyacrylamide gel electrophoresis (PAGE) on a 7.5 or 12% gels and transfer to nitrocellulose membranes. The following were mixed together for preparation of 1 gel: 3.75 or 6 mL 30% acrylamide/bis (for 7.5 or 12% gels, respectively), 3.75 mL 1.5 M Tris-HCl pH 8.8, 150 µL SDS, 7.28 or 5.03 mL ddH₂O (for 7.5 or 12% gels, respectively), 7.5 µL tetramethylethylenediamine (TEMED) and 75 µL 10% ammonium persulfate (APS). The stacking gel (for 2 gels) was prepared with 2 mL acrylamide/bis, 3.78 mL 0.5 M Tris-HCl pH 6.8, 150 µL sodium SDS, 9 mL ddH₂O, 15 µL TEMED and 75 µL APS. Approximately 30 minutes was allowed for the separating gel to polymerize prior to the stacking gel being poured. PAGE was carried out at 180 V for approximately 80 minutes. The running buffer (1L of 5x stock) consisted of 5g SDS, 72g glycine and 15 g of Tris base, dissolved in ddH₂O. The transfer to nitrocellulose was carried out at 100 V for 60 minutes. The transfer buffer consisted of 14.4 g of glycine, 3.03 g Tris base and 200 mL of methanol per L, in ddH₂O. Membranes were blocked with 5% nonfat milk in tris buffered saline with 0.05% tween-20 (TTBS) for 1 hour at room temperature followed by the application of primary antibodies in blocking solution overnight at 4°C. Membranes were then rinsed 3 x 10 minutes in TTBS and secondary horseradish peroxidase antibodies were incubated at room temperature for 1 hour in blocking solution. Membranes were rinsed again in TTBS and enhanced chemiluminescence (ECL; Amersham) reagent and x-ray films were used to detect immunoreactivity. Average band densities were measured using a Flouro-S Imaging system and imaging software (Biorad).
For slot blot analysis, 3 µg of protein in RIPA buffer (no sample buffer or boiling) was blotted onto nitrocellulose membranes using a slot blot apparatus from Biorad. Following blotting, membranes were processed as above for Western blot (WB) analysis.

Table 4. Antibodies used in immunoblotting

<table>
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<tr>
<th>Antibody</th>
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<th>Company</th>
<th>Molecular Weight of Target Protein (kDa)</th>
<th>Dilution</th>
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<tbody>
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<td>Millipore</td>
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<td>Iba-1</td>
<td>Rabbit</td>
<td>Wako</td>
<td>15</td>
<td>1:1000 (1 hour)</td>
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<td>NF200</td>
<td>Mouse</td>
<td>Sigma</td>
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<td>(TUJ1)</td>
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<tr>
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</table>

2.7 Zymography

Gel zymography for MMP activity was carried out by first performing PAGE as above with 10% polyacrylamide gels containing 0.1% gelatin B (Sigma Aldrich). Gelatin B is more suited to detect MMP-9 activity, as MMP-9 is a type B gelatinase. Samples in RIPA buffer were processed as in the immunoblotting section and prepared with equal protein concentrations – determined with a Lowry protein assay – in sample buffer. A 2x stock solution of sample buffer consisted of 2.5 mL 0.5 M Tris-HCl pH 6.8, 2 mL glycerol, 4 mL 10% SDS, 0.5 mL 0.1% bromophenol blue, and ddH2O to 10 mL. Importantly, the sample buffer was free of reducing agents (mercaptoethanol) and the samples were not boiled. The running buffer (10x stock) consisted of 29 g Tris base, 144 g glycine, 10 g SDS and ddH2O to 1 L. Following electrophoresis, 2.5 % tritionx-100 (Sigma) in ddH2O was used to renature the gels, with gentle agitation for 30 min. Zymogram developing buffer (10x stock) consisted of 12.1 g Tris base, 63
g Tris-HCl, 117 g NaCl, 7.4 g CaCl$_2$, 0.2% Brij35 and ddH$_2$O to 1 L). The developing buffer was used to equilibrate the gels for 30 min at room temperature with gentle agitation, followed by the addition of fresh developing buffer overnight at 37°C. Gels were then stained with 0.5% Coomassie Blue for 30 min and destained with methanol:acetic acid:water (50:10:40). Gels were imaged with the Flouro-S Imaging system (Biorad).

In situ zymography was performed using DQ gelatin-fluorescein (Invitrogen) as previously published [396]. This conjugate is quenched and will not fluoresce unless cleaved. Thus, MMP activity appears as bright areas with fluorescent microscopy (green channel). Animals were perfused with 120 mL of ice cold PBS. Freshly isolated tissue was snap frozen in liquid nitrogen. Tissue was then sectioned in OCT at a thickness of 14 µm. Tissue was rinsed in PBS and a solution containing 100 µM DQ gelatin-fluorescein in activity buffer (50 mM Tris, 0.2 M NaCl, 5 mM CaCl$_2$, 0.02% Brij35) was applied to slides overnight at 37°C. Slides were rinsed in PBS and cover slipped with Mowoil mounting medium containing DAPI.

2.8 Myeloperoxidase activity

The myeloperoxidase (MPO) activity assay was carried out as per manufacturers instructions (Enzo Life Sciences – Stressgen, Plymouth Meeting, PA, USA). Animals were perfused with 100 mL ice cold PBS and 0.5 cm of spinal cord tissue centered at the epicenter was homogenized in homogenization buffer containing 10 mM NEM and centrifuged at 12000 x g at 4°C. The supernatants were removed, protease inhibitors were added and they were kept for analysis (cytoplasmic/ECM fraction). Solubilization buffer containing 0.5% HTA-Br and 10mM NEM was added to the pellets followed by sonication for 30 seconds and two rounds of freeze thaw cycles before centrifugation as above. The supernatants from this step represent the granular fractions. In the presence of H$_2$O$_2$, MPO contained in the samples oxidized a non-fluorescent detection reagent into a fluorescent analog, which was detected using a fluorescent
plate reader (Perkin Elmer, Waltham MA, USA) at 590-600 nm. Data are reported as U of MPO activity per g wet tissue weight.

2.9 BSCB Permeability

Disruption in the BSCB was measured by extravasation of Evans blue as previously described [397] with few modifications. Evans blue binds to serum albumin (approx. 65 kDa) when injected into the blood, essentially labeling this protein. As serum albumin leaves the vasculature due to barrier permeability, it accumulates in tissue and can be quantified by absorption spectroscopy (620 nm) and visualized in fixed tissue via fluorescence (red channel). Briefly, 2.5 mL/kg of 4% Evans blue in saline was injected intravenously and animals were kept anesthetized for 30 min before sacrifice. Animals were then perfused with 120 mL of saline containing 10 U/mL heparin. Cords were removed – 0.5 cm centered at the epicenter – and weighed prior to homogenization in \(N,N\)-dimethylformamide. Homogenized cords were left at 55°C for 3 days before centrifugation at 20,000 x g for 20 min. Absorbance at 620 nm was measured using a fluorescent plate reader (Perkin Elmer, Waltham MA, USA) in a 96-well glass plate. Data are reported as the amount of Evans blue per tissue weight (µg/g).

For histology, animals were fixed with 4% paraformaldehyde and processed as above (Section 2.4). EB fluorescence was observed in the red channel using a fluorescence microscope.

2.10 qPCR

Crushed frozen spinal cord samples (as in Section 2.6) were added to 1 mL of trizol reagent (Invitrogen). Samples in trizol had 0.2 mL of chloroform added with vigorous shaking and allowed to stand for 2-3 minutes prior to centrifugation at 12,000 x g for 15 min at 4 °C. The colourless aqueous phase was removed and added to a new tube. Isopropyl alcohol (0.5 mL) was
added to each tube and samples were incubated at room temperature for 30 minutes to precipitate the RNA. Samples were then transferred RNA Miniprep columns for purification (Qiagen, Venlo, Netherlands). The precipitated RNA was added to the spin columns and centrifuged at 8000 x g for 15 minutes. Washing and eluting of the RNA was carried out as per manufacturer’s instructions. Following the first series of wash steps, a DNase treatment step was performed. RNA was eluted in molecular grade water provided in the kit and spectrophotometry was used to determine sample purity and RNA concentration by absorbance at 260 nm (Nanodrop Spectrophotometer, Thermo Scientific). Acceptable purity values were a 260/280 ratio of 1.8-2.1 and a 260/230 ratio of 1.8-2.2.

A total of 500 ng of purified RNA was reverse transcribed into cDNA with oligo dT\textsubscript{12-18} primers and Superscript II reverse transcriptase (RT; Invitrogen). Following each step, samples were centrifuged briefly to ensure contents were at the bottom of tube. The first step in this process involved the addition of template RNA, 1 \mu L of oligo dT primers, 1 \mu L of dNTP mix and molecular grade water up to 12 \mu L. This mixture was added to a thermocycler for 5 minutes at 65\degree C. The mixture was cooled on ice and 2 \mu L of DTT and 4 \mu L of 5x first strand buffer were added. The mixture was put back into the thermocycler at 42\degree C for 2 minutes. Finally, 1 \mu L of RT enzyme was added to the tubes with gentle pipetting to mix the reaction mixture. The reverse transcription process was carried out at 42\degree C for 50 minutes. The RT enzyme was inactivated by heating the reaction mixture at 70\degree C for 15 minutes.

For qPCR, a total of 1 ng of cDNA based on mRNA concentration was used per reaction. All reagents including primers were purchased from Applied Biosystems (Carlsbad, CA, USA) and PCR was carried out using a 7900 HT Fast-real time PCR system (Applied Biosystems). In each well, the total volume was 10 \mu L, which included 0.5 \mu L of primers, 4.5 \mu L of template cDNA, and 5 \mu L of Fast-Taqman master mix. The reaction parameters were as follows: UNG
incubation for 2 minutes, polymerase activation for 20 seconds and 40 cycles of [1 second denature and 20 second anneal/extend]. Relative gene expression was determined using the $2^{\Delta\Delta\text{Ct}}$ method [398] using HPRT as the internal control and non-injured control (sham) animals as the calibrator.

Total RNA from primary microglia was isolated and purified using the full procedure of the RNA Miniprep columns (no trizol). Instead of Trizol, buffer RLT was used to homogenize the cells. The procedure was carried out as above with HPRT serving as the internal control and control cells (no treatment or stimulation) serving as the calibrator.

2.11 ELISA

Animals were perfused with 120 mL of ice cold PBS and 0.5 cm of spinal cord tissue centered at the epicenter was isolated. Either sectioned (Chapter 3; tissue processing detailed in Section 2.5) or whole (Chapter 4) spinal cord tissue was homogenized in ice-cold RIPA buffer (Thermo Scientific, Waltham, MA, USA). Samples were processed by Eve Technologies (Calgary, AB, Canada) using Rat Cytokine/Chemokine multiplex ELISA assays available from Millipore (Billerica, MA, USA). Concentrations obtained from the assay were divided by protein concentration determined by the Lowry method and data are expressed as pg/mg protein.

Detection of IL-6 for culture studies (Chapter 5) was carried out using an IL-6 ELISA kit available from Invitrogen which included all buffers and standards. All washing steps were carried out 6 times with 200 µL of wash buffer. All concentrations and incubation times were followed as per manufacturer’s instructions. To begin, 96 well-plates (Nunc) were coated with capture antibody overnight at 4°C. The next day, wells were washed and 100 µL of media samples (and standards in media) were added along with 50 µL of anti IL-6 antibody in assay buffer for 4 hours with shaking. After washing, a secondary HRP-detection antibody was added for 45 minutes. Finally, wells were washed and TMB substrate was added for 30 minutes,
followed by the addition of stop solution. Plates were read on a plate reader (Perkin Elmer, Waltham, MA, USA) at 450 nm.

2.12 NO Assay

To measure NO production in cultures (Chapter 5), a Griess assay kit was used (Promega, Madison WI, USA). The Griess assay detects nonvolatile breakdown products of NO such as nitrite in cell culture media. The reaction was carried out in 96-well plates (Nunc) in duplicate using 50µL of media from the cultures. Standards were also prepared in culture media according to the manufacturer’s instructions. To the media in each well, 50 µL of sulfanilamide was added followed by 10 minutes incubation at room temperature in the dark. Next, 50 µL of NED solution was added, followed by 10 minutes incubation in the dark at room temperature. The resultant Azo compound was detected through absorbance spectrophotometry on a plate reader (Perkin Elmer) at 520 nm.

2.13 DNA Assay

To quantify relative cell numbers for the assays in Sections 2.11 and 2.12, a DNA assay using Hoechst was used. Following removal of media from wells, 200 µL of ddH₂O was added to each well and the plate was incubated at 37 °C for 1 hour. The plate was then frozen at -80 °C and once thawed, 100 µL of each sample was transferred to black 96 well plates. An equal volume of Hoechst dye (5 µg/mL) in TNE buffer (10 mM Tris base, 2M NaCl, 1mM EDTA) was added. The fluorescence intensity was measured using a plate reader (Perkin Elmer). Salmon testis DNA (Sigma) in ddH₂O served as a standard.

2.14 Neurobehavioural Tests

All neurobehavioural tests were carried out with two blinded observers. Hindlimb locomotion was determined weekly for six weeks using the Basso Beattie Bresnahan (BBB)
locomotor rating scale [399]. The movement of the ankle, knee and hip joints were monitored in addition to the characteristics and patterns of hindlimb stepping. Motor function was determined biweekly using the inclined plane test [400]. Animals were placed perpendicularly on an inclined plane of a particular angle and their ability to maintain their position was indicative of a positive result. The highest angle they were able to hold their position was recorded biweekly. Both the right and left side of the animals facing an upward position were tested. The average angle in each group is reported. At level mechanical allodynia was determined biweekly using 2 g and 4 g von Frey monofilaments as previously described [401]. Animals were left in an isolated room for 30 minutes to acclimatize to their environment. The monofilament was applied to the dorsal aspect around the injury site 10 times and animals’ behavioural response to each was recorded. An adverse response to the application of the monofilament (determined in advance of experiments) included vocalization, licking, biting and immediate movement to the other side of the cage. If an animal responded adversely 5 or more times out of 10 applications, it was considered to exhibit mechanical allodynia. The proportion of rats to exhibit allodynia in each group is reported.

2.15 MRI

MR imaging was carried out at the STTARR facility in Toronto, Ontario using a Buker Biospec Scanner and 7T magnet. T2 Turbo RARE (Rapid Acquisition Relaxation Enhanced) - aka Fast Spin Echo (FSE) sequences were run with the following parameters: TE = 8.856ms, Effective TE = 43.28ms, TR = 1500ms, RareFactor = 16, NEX (number of excitations = # of averages) = 3, Scan time = 21m36s. Voxel information collected was as follows: 50x50x16 mm (FOV), 250x150x32 (matrix) and 200x333x500 µm (voxel size/resolution). Respiratory gating was used during imaging. Each slice image was imported into Matlab® and the volume of
hyperintense voxels was determined by a blinded observer. The lesion volume for each animal (in mm$^3$) was calculated by adding up the average volumes from each slice.

2.16 Electrophysiology

*In vivo* recordings of spinal cord evoked potentials (SCEP) were recorded from rats. These electrophysiological outcome measures have been used widely in our laboratory [24, 402]. For spinal cord evoked potentials, the spinal cord at T8-9 was stimulated (2 mA; 0.13 Hz; 0.04 ms) and events were be recorded from the spinal cord at C2-3 (20 sweeps). Amplitude was measured from the first major positive peak to the following negative peak. Response latency was determined by measuring the time between the appearance of the stimulus artifact and the first major positive peak. Conduction velocity was calculated from SCEP recordings by dividing the distance between the stimulating and recording electrodes by the latency. A 3-300 MHz filter was also used.

2.17 Statistics

Statistics were done using StatPlus: Mac Version 2009 software (AnalystSoft Inc., Alexandria, VA, USA). Behavioural data were analyzed by two way analysis of variance (ANOVA) tests with Bonferroni post-hoc tests. Densitometry and SCEPs were analyzed with t-tests. In light of the fact that the neuropathic pain, cytokine and immunohistochemical lesion size analyses exhibited skewed, non-normal distributions, logarithmic transformations were applied prior to statistical analysis. Subsequently, cytokine outcomes were compared between the treatment groups using a two way ANOVA technique to adjust for slight variations in experimental conditions depending on the cohort of animals examined. The number of animals per group is included in the results section and figure legends.
Chapter 3

3  Severe Spinal Cord Injury Associated with Arachnoiditis

3.1  Abstract

Subarachnoid inflammation following spinal cord injury (SCI) can lead to the formation of localized SS and the development of post-traumatic syringomyelia (PTS). While PTS is a devastating complication of SCI, its relative rarity (occurring symptomatically in about 5% of clinical cases) and lack of fundamental physiological insights have led us to examine an animal model of traumatic SCI with induced arachnoiditis. This model has been previously shown to consistently replicate the pathophysiology and clinical symptoms of PTS (Seki and Fehlings, 2008). We hypothesized that arachnoiditis associated with SCI would potentiate early parenchymal pathophysiology. To test this, we examined early spatial pathophysiology in four groups: (i) sham (non-injured control), (ii) arachnoiditis (intrathecal injection of kaolin), (iii) SCI (35g clip contusion/compression injury) and (iv) PTS (intrathecal kaolin + SCI). Overall, there was greater parenchymal inflammation and scarring in the PTS group relative to the SCI group. This was demonstrated with significant increases in cytokine (IL-1α and IL-1β) and chemokine (MCP-1, GRO/KC, MIP-1α) production, MPO activity, blood spinal cord barrier (BSCB) permeability and MMP-9 activity. However, parenchymal inflammation (acute IL-1α and IL-1β production, sub-acute chemokine production, BSCB permeability) and scarring in the PTS group were larger than the sum of the SCI group and arachnoiditis group combined, suggesting that arachnoiditis does indeed potentiate parenchymal pathophysiology. Accordingly, these findings suggest that the development of arachnoiditis associated with SCI can lead to an exacerbation of the parenchymal injury, potentially impacting the outcome of this devastating condition.
3.2 Introduction

Traumatic vertebral fractures and dislocations cause a heterogeneous array of biomechanical forces on the spinal cord, resulting in equally divergent neurological and functional deficits. Meningeal damage and subarachnoid inflammation (arachnoiditis) following spinal cord injury (SCI) occur in patients, although the impact of these events is unclear. The extent and resolution of post SCI arachnoiditis is most likely determined by possible genetic and injury related differences, leaving some patients more prone to the formation of SS or adhesions that disturb CSF flow in the SAS. Although an accurate estimation of the incidence of SS following SCI remains elusive, it is estimated that only 3-5% of cases will become symptomatic with clinical evidence of post-traumatic syringomyelia (PTS), a condition characterized by extensive cysts within the cord parenchyma [264, 268, 270]. Animal studies suggest that CSF blockage by SS results in altered fluid flow and pressure dynamics in the SAS, causing an influx of CSF into the spinal cord parenchyma [282, 283].

Though symptomatic PTS is rare, recent studies suggest that the incidence of PTS is increasing [276]. Currently, PTS receives very little basic science research attention. Moreover, current models of SCI offer little insight into the etiological aspects of PTS due to the lack of significant arachnoiditis. Studies going back almost 80 years have used injections of kaolin into the cisterna magna to cause meningeal inflammation and obstruction of CSF, modeling hydrocephalus and syringomyelia [403]. Furthermore, groups have studied the inflammatory aspects of this model [404]. The use of kaolin has been adapted for use in PTS research, with groups using parenchymal excitotoxic compounds combined with subarachnoid kaolin injections [405, 406]. This model has laid important etiological groundwork, establishing important mechanistic and pathophysiologic details [17, 282, 284]. Importantly, animal models of PTS require a reproducible meningeal inflammatory event that is localized to the site of SCI, as SS
can be seen localized to the injury site clinically [277]. The majority of PTS studies have looked at how the kaolin induced scarring in the subarachnoid space leads to the entry of CSF into the spinal cord [17, 282]. However, looking at the meningeal inflammation that leads to SS has been largely overlooked, particularly in terms of how it can influence the progression of injury.

Recently, our laboratory has modified this classical PTS model [393]. In our variation, animals are subjected to a clip compression/contusion injury followed by an intrathecal injection of kaolin to induce arachnoiditis. While the literature suggests that SS is associated with the formation of a syrinx, we sought to study its root cause – arachnoiditis – and how it relates to early parenchymal injury. We hypothesized that arachnoiditis associated with SCI would potentiate parenchymal pathophysiology. To this end, we studied the early spatial profile of inflammatory and scarring events in four groups of rats: (i) non-injured controls (sham) (ii) arachnoiditis (intrathecal kaolin injection at T7) (iii) SCI (35g clip contusion/compression injury at T7) and (iv) PTS (SCI with induced arachnoiditis at T7). We rationalized that if there was a synergistic relationship between arachnoiditis and parenchymal inflammation induced by SCI, as opposed to an additive relationship, then inflammation and scarring in the PTS group would be greater than the sum of the arachnoiditis group and SCI group combined. Overall, inflammation and scarring were greater in the parenchyma of PTS animals relative to SCI animals. However, acute parenchymal IL-1α and IL-1β production, sub-acute parenchymal chemokine production, BSCB permeability and parenchymal fibrous scarring were much larger in SCI animals with arachnoiditis as compared to the sum of these measures in animals with arachnoiditis alone and SCI alone, suggesting a synergistic relationship. Together, these findings suggest that cases of SCI associated with arachnoiditis (meningeal damage, sub-dural hemorrhaging, etc.) can be expected to experience more severe parenchymal inflammation and scarring.
3.3 Objective

Characterize the acute pathophysiology in a model of SCI associated with arachnoiditis.

3.4 Hypothesis

Arachnoiditis will potentiate parenchymal inflammation and scarring following SCI.

3.5 Specific Aims

(1) Identify cell types involved in kaolin induced arachnoiditis

(2) Establish acute temporal expression of inflammatory mediators

(3) Establish acute spatial expression of inflammatory mediators

(4) Examine sub-acute fibrous scarring

3.6 Methods

There were a total of 114 rats used in this section. Studies were carried out in 4 groups: (i) non-injured sham animals, (ii) arachnoiditis animals (5 µL of kaolin injected into the SAS at T6/T7), (iii) SCI animals (35 g clip compression injury at T6/T7), (iv) PTS animals (clip compression plus kaolin at T6/T7). A total of 6 animals were used for MRI at 6 weeks post injury. For the 2-day IHC and 7-day IHC, 21 animals were used (9 for each time point, 3 animals per group; plus 3 non-injured sham animals). At 2 days, sections were stained for PMN (neutrophils) and Iba-1 (microglia/macrophages). At 7 days post injury, sections were stained for GFAP (astrocytes), Iba-1, CSPGs (fibrous scar) and collagen-IV (fibrous scar). For the cytokine and chemokine experiments at 1 and 3 days post injury, 21 animals were used (9 for each time point, 3 per group; plus 3 non-injured sham animals). For the MPO assay at 1 and 3 days post injury, 21 animals were used (9 for each time point, 3 per group; plus 3 non-injured shams). For PMN staining at 2 days post injury, there were 12 animals used (3 per group, plus 3
non-injured sham animals). For quantitative analysis of Evans blue (EB) extravasation at 1 and 3 days post injury, 21 animals were used (9 for each time point, 3 per group; plus 3 non-injured shams). For qualitative EB extravasation through IHC at 2 days post injury, 12 animals were used (3 per group, plus 3 non-injured sham animals).

3.7 Results

*Visualizing the injury at 6 weeks*

A visualization of the chronic injury using MRI was employed to provide a frame of reference when considering the outcome of the PTS model as compared to a regular injury. Figure 7 shows representative images from SCI and PTS animals taken at 6 weeks following injury. T2 weighted magnetic resonance (MR) images representing a 500 µm thick slice through the center of the spinal cord are shown. The arrow in the PTS image points to a hypointense region corresponding to the kaolin. Note that the PTS injury results in a larger hyperintense injury region compared to the SCI animal. Also note the appearance of multiple lobules of hyperintense regions in PTS animals.

Figure 7. MRI comparison of SCI and PTS animals

Longitudinal T2 weighted MR images from SCI and PTS animals taken at 6 weeks post injury. Images representing a 500 µm thick slice through the center of the spinal cord are shown. The
arrow in the PTS image points to a hypointense region corresponding to the kaolin. Note that the PTS injury results in a larger hyperintense lesion compared to the SCI animal. Also note the appearance of multiple lobules of hyperintense regions in the PTS animal.

*Early Cellular Meningeal Inflammation*

When kaolin was injected into the subarachnoid space, rostral to the site of injury, it remained present and recruited local and systemic inflammatory cells. Figure 8 demonstrates cellular meningeal inflammation in response to SCI and induced arachnoiditis (PTS group). Figure 8A demonstrates a schematic of the region where images were taken. Global representations in Figure 8 are tiled fluorescent microscopic images, whereas higher resolution images are confocal micrographs. At 2 days post injury, there was evidence of both microglia/macrophages (Iba-1, labeled green in right panel) and neutrophils (PMN, labeled green in left panel) in the meningeal layers (dense layer of nuclei) surrounding the kaolin in PTS animals (Figure 8B). By 7 Days post injury, the meningeal layers were more difficult to discern due to the inflammation and scarring in the area, however, some neutrophils (labeled red) remained around the injury epicenter at the dorsal aspect of the spinal cord (Figure 8C) whereas there was little evidence of these cells surrounding the kaolin directly. In contrast, macrophage/microglia (labeled red) completely surrounded the kaolin and were even found in the meninges at very remote sites from the injury epicenter (Figure 8D). GFAP staining for astrocytes (green) was included to delineate the parenchymal boundary. Scale bars in the lower magnification images represent 1mm and in the higher power images they represent 100 µm. In uninjured animals there were very few PMNs and microglia/macrophages in the meningeal layers (data not shown). In contrast, there were many PMNs and few macrophages/microglia found in the meninges of SCI animals (data not shown).
Figure 8. Cellular arachnoiditis in PTS model

(A) Images were taken from sagittal midline spinal cord sections and are oriented with the dorsal aspect of the cord at the top and the rostral end of the epicenter to the left. (B) At 2 days post PTS injury there was evidence of both microglia/macrophage (right panel; Iba-1) and neutrophils (left panel; PMN) in the meningeal layers surrounding the kaolin. (C) At 7 Days post injury some neutrophils (PMN) remained around the injury epicenter whereas there was little evidence of these cells surrounding the kaolin directly. (D) Macrophage/microglia (Iba-1) completely surrounded the kaolin and were even found in the meninges at very remote sites from the injury epicenter (right panel). Scale bars represent 1 mm in lower power images and 100 μm in higher resolution images.
Meningeal Cytokine and Chemokine Expression

To determine spatial information regarding the expression of inflammatory mediators, kaolin exposed meningeal fractions were isolated and analyzed separately from the remaining cord tissue (see Figure 9 for a schematic of sectioning). Multiplex ELISA was used to assay cytokines (IL-1α, IL-1β and IL-6) and chemokines (MCP-1, MIP-1α and GRO/KC) in these fractions. The results are shown in Figure 9 and are reported as pg/mg protein (n=3 per group). Non-injured sham animals are represented by hatched lines on the graphs. As seen in Figure 9A, induced arachnoiditis significantly increased all cytokines and chemokines in meningeal fractions relative to sham animals at 1 and 3 days post injury (ANOVA, p<0.05 for each day and each molecule; p<0.05 Bonferroni post-hoc test) with the exception of IL-1β at 3 days (p=0.25). In contrast, SCI alone resulted in significant increases in meningeal IL-6, MCP-1, and GRO/KC at 1 day (p<0.05) with no significant differences found at 3 days relative to sham animals. Additionally, relative to sham animals, the combination of SCI and induced arachnoiditis (PTS group) resulted in significant increases in all of the meningeal cytokines and chemokines at 1 day post injury (p<0.05) and only IL-1β and IL-6 at 3 days post injury (p<0.05). Relative to SCI alone, PTS animals had a significant increase in all inflammatory mediators reported at 1 day post injury (p<0.05) with the exception of IL-6 (p=0.18) and a significant increase in IL-1β and IL-6 at 3 days post injury (p<0.05). Relative to arachnoiditis alone, PTS animals had a significant increase in only MCP-1 at 1 day post injury (p<0.05).

Parenchymal Cytokine and Chemokine Expression

Spinal cord tissue free from areas of kaolin/arachnoiditis was also analyzed for cytokine and chemokine expression (Figure 9B). Parenchymal fractions from arachnoiditis animals contained significantly more MIP-1α, and GRO/KC at 1 day post injury as compared with sham
animals (p<0.05) with no significant differences seen at 3 days post injury. SCI alone resulted in a significant increase in IL-6, MCP-1, MIP-1α and GRO/KC at 1 day post injury (p<0.05) and IL-1α, MCP-1, MIP-1α and GRO/KC at 3 days post injury (p<0.05) compared to sham animals. Relative to sham animals, PTS animals exhibited significant increases in all of the parenchymal cytokines and chemokines at 1 day and 3 days post injury (p<0.05). Relative to SCI alone, PTS animals had a significant increase in IL-1α and IL-1β at 1 day post injury (p<0.05) and a significant increase each chemokine at 3 days post injury (p<0.05). Relative to arachnoiditis alone, PTS animals had a significant increase in all inflammatory mediators reported, at 1 and 3 days post injury (p<0.05).
Figure 9. Spatiotemporal cytokine expression

Dorsal meninges were removed from the parenchyma and each fraction was analyzed for cytokine/chemokine expression with multiplex ELISA. A schematic of the sectioning is presented. Non-injured sham animals are represented by hatched lines on the graphs. (A) Meningeal cytokines/chemokines. Arachnoiditis with kaolin significantly increased all cytokines and chemokines relative to sham animals at 1 and 3 days post injury with the exception of IL-1β at 3 days (p=0.25). SCI significantly increased IL-6, MCP-1, and GRO/KC at 1 day post injury. PTS significantly increased all of the cytokines and chemokines at 1 day post injury and only IL-1β and IL-6 at 3 days post injury compared to sham animals. Relative to SCI alone, PTS significantly increased IL-1α, IL-1β and all chemokines reported and significantly increased IL-1β and IL-6 at 3 days post injury. Relative to arachnoiditis alone, PTS significantly increased MCP-1 at 1 day post injury. (B) Parenchymal cytokines/chemokines. Fractions from arachnoiditis animals contained significantly more MIP-1α, and GRO/KC at 1 day post injury as compared with sham animals. SCI significantly increased IL-6, MCP-1, MIP-1α and GRO/KC at 1 day post injury and IL-1α, MCP-1, MIP-1α and GRO/KC at 3 days post injury compared to sham animals. Relative to sham animals, PTS significantly increased all of the meningeal cytokines and chemokines at 1 day and 3 days post injury. Relative to SCI alone, PTS significantly increased IL-1α and IL-1β at 1 day post injury and significantly increased each chemokine at 3 days post injury. Relative to arachnoiditis alone, PTS animals had a significant increase in all inflammatory mediators reported, at 1 and 3 days post injury. n=3 per group. ANOVA, p<0.05 for each cytokine/chemokine for each day; *p<0.05 Bonferroni post-hoc test.
Neutrophil Infiltration and MPO Activity

The relative extent of neutrophil extravasation in animals was determined using a myeloperoxidase activity assay and qualitative immunohistochemistry. Figure 10A demonstrates results from a myeloperoxidase assay that analyses the enzyme activity in granular fractions, measured in U/g of whole cord tissue (n=5 per group). Non-injured sham animals are represented by the black-hatched line. Arachnoiditis animals did not contain significantly more MPO activity compared with sham animals at 1 and 3 days post injury (ANOVA, p<0.05 for each day; Bonferroni post-hoc test p<0.05). SCI alone and PTS alone resulted in a significant increase in MPO activity relative to sham animals at 1 and 3 days post injury (p<0.05 for each). Relative to SCI alone, PTS animals did not have a significant increase in MPO activity at 1 day post injury (p=1.0) but did exhibit an increase at 3 days (p<0.05). Relative to arachnoiditis alone, PTS animals had a significant increase in MPO activity at 1 and 3 days post injury (p<0.05).

As neutrophils are the main source of post-SCI MPO, we qualitatively assessed neutrophil accumulation 1 day following injury using immunohistochemistry. Representative images were taken from the sagittal midline of spinal cords and are shown in Figure 10B. Note the significant increase in PMN (green) immunoreactivity in the parenchyma of SCI and PTS cords compared to animals with induced arachnoiditis. Further, the presence of parenchymal neutrophils in PTS animals compared to SCI animals is increased, suggesting that increases in MPO activity correlate with an increase in neutrophil influx.
Figure 10. Acute neutrophil extravasation

(A) Neutrophil extravasation was determined by assessing MPO activity in granular fractions at 1 and 3 days post injury (measured in U/g tissue). There was a significant increase in MPO activity in SCI and PTS animals compared to sham animals and arachnoiditis animals at 1 and 3 days post injury. At 3 days following injury, there was a significant increase in MPO activity in PTS animals compared to SCI animals. n= 3 per group. ANOVA, p<0.05; * Bonferroni post-hoc test p<0.05. (B) Longitudinal immunohistochemical images taken at 2 days post injury show neutrophils stained with a PMN antibody (green) in arachnoiditis, SCI and PTS animals. Images were taken from sagittal midline spinal cord sections and are oriented with the dorsal aspect of the cord at the top and the rostral side of the epicenter to the left. Note the increased amount of labeled cells in PTS animals. Scale bars represent 1mm in lower power images and 100 µm in higher resolution images.
Blood Spinal Cord Barrier Permeability

In order to determine BSCB permeability, EB was injected intravenously into injured animals and its extravasation into the spinal cord was imaged visually in fixed frozen tissue via fluorescence and measured in homogenized tissue by absorbance spectrophotometry. Figure 11A shows representative EB fluorescence (red) images from arachnoiditis, SCI and PTS animals counterstained with DAPI (blue) and taken 2 days following injury (with equal exposure and intensity settings). These images demonstrate that there is evidence of parenchymal EB extravasation in arachnoiditis animals in addition to EB fluorescence in the meninges. Also, note the area of EB fluorescence in the parenchyma is larger in PTS animals as compared to SCI animals. Further, there is a strong fluorescence signal in the meninges in PTS animals, which includes areas remote to the injury epicenter and on the ventral side of the spinal cord.

Figure 11B represents the level of EB detected in whole tissue homogenates at 1 and 3 days post injury. Animals with induced arachnoiditis alone had significantly more EB extravasation as compared with sham animals at 1 but not 3 days post injury (ANOVA, p<0.05; Bonferroni post-hoc test, p<0.05). SCI alone resulted in a significant increase in EB extravasation compared to sham animals at 1 day following injury (p<0.05) but not at 3 days (p=1.0). In contrast, PTS animals exhibited significant increases EB extravasation at 1 and 3 days post injury compared to sham animals (p<0.05 for each day). Relative to SCI alone, PTS animals did not exhibit increases in EB extravasation at 1 day post injury (p=0.18) but did show significantly more at 3 days (p<0.05). Additionally, PTS animals exhibited significantly more EB extravasation compared to arachnoiditis animals at 1 and 3 days post injury (p<0.05 for each).

As MMP-9 has been implicated in promoting acute blood vessel permeability associated with injury, we assessed its spatial activity at 2 days post injury. Figure 11C demonstrates a
representative gel zymogram of MMP-9 activity in meningeal and parenchymal fractions. MMP-9 activity was not detected in sham animals and was only very slightly increased in arachnoiditis animals in both fractions. Similarly, there was slight MMP-9 activity in the meninges in SCI animals with the parenchymal activity more pronounced. When induced arachnoiditis was associated with SCI (PTS group), there was an increase in MMP-9 activity in both meningeal and parenchymal fractions compared to SCI and arachnoiditis animals. Additional spatial information is demonstrated in Figure 11D, which demonstrates results from in situ zymography. Fresh tissue was incubated with a fluorescent gelatin conjugate 2 days post injury. When cleaved, the previously quenched substrate becomes fluorescent, demonstrating areas of gelatinase (MMP) activity. Arachnoiditis animals exhibited gelatinase activity in the meninges and slight activity in the parenchyma. In contrast, SCI animals exhibited increased gelatinase activity in the parenchyma with little in the meninges. Also, note the increase in fluorescence in the parenchyma and meninges in PTS as compared to SCI animals.
Figure 11. Blood spinal cord barrier permeability

(A) Evans blue (EB) extravasation into the spinal cord was determined in injured animals. Representative EB (red) fluorescence images from arachnoiditis, SCI and PTS animals 2 days following injury show a larger area of extravasation in PTS animals. Note the intense fluorescence in the meninges of PTS animals. Slides were counterstained with DAPI (blue). (B) EB extravasation was measured in homogenized tissue by absorbance spectrophotometry. There was a significant increase in the amount of EB in SCI and PTS animals compared to sham and arachnoiditis animals at 1 day post injury. PTS animals contained significantly more EB relative to SCI animals at 3 days post injury. N=3 per group. * Bonferroni post hoc test p<0.05. (C) PTS animals exhibited an increase in MMP-9 activity in both parenchymal and meningeal fractions at 2 days post injury. (D) In situ zymography was used to determine spatial MMP activity at 2 days following injury. Note the increase in fluorescence in parenchyma and meninges in PTS sections compared to SCI and arachnoiditis animals. Images were taken from sagittal midline spinal cord sections and are oriented with the dorsal aspect of the cord at the top and the rostral side of the epicenter to the left. Scale bar represents 1 mm.
**Fibrous scarring**

Next we set out to determine the impact of arachnoiditis on sub-acute fibrous scarring. Assessment of fibrous scarring was carried out immunohistologically on midline sagittal sections using collagen IV (red) and CSPGs (green) at 7 days following injury. Collagen IV has been described as providing the adhesive matrix that binds inhibitory molecules such as CSPGs [210]. Figure 12A demonstrates the extensive scarring in the meninges caudal to and surrounding the kaolin in arachnoiditis and PTS animals. While there was little parenchymal collagen IV and CSPG immunoreactivity in arachnoiditis animals, SCI and PTS animals contained extensive parenchymal fibrous scarring. Note the increase in parenchymal fibrous scarring in PTS animals compared to SCI animals. Figure 12B is a LFB/H&E image taken to show the extent of scarring in the meninges of PTS animals to support the previous immunohistological image.
Figure 12. Fibrous scarring

(A) Longitudinal fluorescent immunohistochemical images from SCI and PTS animals at 7 days post injury labeled for collagen IV (red) and CSPGs (green). Note the extensive scarring in the meninges caudal to and surrounding the kaolin. Additionally, this image shows an increase in parenchymal CSPG and collagen IV immunoreactivity. (B) A longitudinal LFB/H&E image taken to show the extent of scarring in the meninges of PTS animals. Scale bars represent 1 mm.
3.8 Discussion

This study has demonstrated that when we induced arachnoiditis following SCI, the intramedullary pathology was altered. This was associated with the evidence of increased inflammation, increased BSCB permeability and fibrous scarring. To our knowledge, this is the first study to separately examine meningeal and parenchymal inflammation and study the contribution of arachnoiditis to the early pathophysiology of SCI. Animals subjected to SCI alone experienced some dorsal meningeal inflammation, including increased IL-6 and MCP-1 expression, MMP-9 activity and permeability of blood vessels. When this meningeal inflammation was synthetically increased by the introduction of kaolin (PTS group), animals exhibited greater parenchymal cytokine/chemokine expression, neutrophil extravasation, MMP-9 activity, BSCB permeability and fibrous scarring compared to SCI alone. In some cases, these increases were not merely additive, suggesting that arachnoiditis potentiates parenchymal inflammation synergistically. Based on our data, we propose that if sufficient arachnoiditis were to exist following SCI, parenchymal inflammation would be increased, patients would have a poorer prognosis and possibly be more susceptible to the development of syringes.

Relevance of the Model

Though PTS is considered a chronic condition, several studies have described cases developing within several weeks to months following SCI [276, 407, 408]. While the presentation of PTS occurs in a delayed fashion following injury, it is quite plausible that the inflammatory events leading to the development of SS progress as part of the acute/sub-acute response to the pathophysiology of SCI. As such, we are studying these early inflammatory interactions.
Studies going back almost 80 years have used injections of kaolin into the cisterna magna to cause meningeal inflammation and obstruction of CSF, modelling hydrocephalus and syringomyelia [403]. In addition to using kaolin to obstruct CSF flow, groups have studied the inflammatory aspects of kaolin in the CNS [404]. PTS researchers have developed models by injecting excitotoxic compounds into the parenchyma combined with subarachnoid kaolin injections [405]. The majority of PTS studies have looked at how the kaolin induced scarring in the SAS leads to the entry of CSF into the spinal cord [17, 282]. However, looking at the early meningeal inflammation that leads to eventual SS has been largely overlooked, particularly in terms of how it can influence the progression of injury. The model developed by our group is a modification of the excitotoxic/arachnoiditis model [405] and results in increased neuropathic pain, a larger lesion/cyst and increased functional deficits – mimicking some of the pathophysiological features of PTS seen in humans [393].

As no clinical pathophysiological data exist that can offer insights into the events and magnitude of post-SCI arachnoiditis, and due to the likelihood that these events are variable between patients, our model relies on three main assumptions: (1) post-SCI arachnoiditis is localized at the injury epicenter, (2) post-SCI arachnoiditis involves infiltration of systemic blood cells to the meninges and (3) post-SCI arachnoiditis involves increased meningeal inflammatory mediator production. As PTS related SS is localized [277, 409], it is highly likely the causative arachnoiditis occurs at the injury epicenter. Damage to the meninges following SCI can include direct cell death and hemorrhaging. Based on what is known about inflammation, these two events result in the recruitment of systemic blood cells and increased production of inflammatory mediators – making it highly likely that this is seen in SCI patients who develop SS. As we demonstrated, the kaolin used in our model remained localized, recruited inflammatory cells and increased production of meningeal inflammatory mediators. The obvious
question is how closely kaolin replicates the inflammation seen in humans that experience post SCI-arachnoiditis. Overall, it is likely that the presence of kaolin induces a more robust inflammatory response than seen in humans - involving different cellular activation pathways.

Evidence suggests that kaolin induces inflammatory events that could possibly be seen in the meninges of humans following SCI. Kaolin has been found to cause endothelial death *in vitro* [410]. This suggests that kaolin can induce endothelial cell death and increase vascular permeability in meningeal blood vessels. Studies that have looked at cellular responses to kaolin in the SAS, report recruitment of macrophage/microglia [406, 411], which is consistent with our data. We also showed increased neutrophil infiltration. Furthermore, results from a 23-plex ELISA demonstrated that kaolin did not increase the expression of any inflammatory mediator that was not already increased by SCI alone – it just increased the magnitude (Figure 9 and additional data not shown).

Inflammatory activation pathways are undoubtedly different in cells exposed to kaolin compared to clinical events post-SCI. Evidence on the activation of inflammatory cells by kaolin – which is comprised of silica and aluminum – comes from research on alveolar macrophages in models of silica inhalation. While SCI can cause activation of resident and immune cells via TLR-2 and TLR-4 [102], evidence has shown that AMs can bind and phagocytose silica particles through scavenger receptors, such as scavenger receptor (SR) AI, SR-AII and MARCO [412]. Upon phagocytosis of silica, AMs are thought to cause tissue damage through production of ROS, NO, cytokines, chemokines and also through possible necrotic and apoptotic biproducts from silica exposure [413]. Though produced via an alternate mechanism, these inflammatory molecules are similar to what macrophage/microglia produce in response to SCI and other inflammatory insults - as described in detail in the introduction.
Overall, we believe that our model can offer insights into acute PTS etiology as it involves a localized meningeal inflammatory reaction that causes recruitment of inflammatory cells and the production of inflammatory mediators – similar to those caused by injury alone, although more severe. Furthermore, induced arachnoiditis was combined with a validated, clinically relevant clip compression/contusion injury model [394].

*Arachnoiditis, Inflammation and SCI*

At the basic science level, little has been studied regarding meningeal inflammation following SCI. Some studies of relevance demonstrate that the presence of blood in the CSF following injury can stimulate cells in the arachnoid layers to act as antigen presenting cells and to initiate an inflammatory response [414]. Additionally, when inflammatory mediators are introduced in the SAS, they cause a breakdown in the BCFB and potentiate inflammation [415]. Following human SCI, inflammatory mediators have been detected in the CSF [416], though the source of these molecules is likely from a combination of parenchymal and meningeal cells. Importantly, we acknowledge that it was likely some dorsal parenchyma was included in meningeal fractions due to the sectioning procedure utilized in our study. Further, we acknowledge that meningeal inflammatory mediators produced in response to the kaolin were able to diffuse via the CSF and influence meninges that were included in the ‘parenchymal’ fractions. As such, the ‘parenchymal’ fractions contained lateral and ventral meninges that contributed to the various metrics of inflammation.

To our knowledge, no study has specifically isolated the meninges and looked at the expression of inflammatory mediators. As such, data from the literature are typically representative of a combination of products from parenchymal and meningeal cells. Inflammatory mRNA (TNF-α, IL-1β and IL-6) is elevated within minutes after SCI, peaks after several hours and returns to basal levels after 3 days [116-120]. In light of this, our assay is most
likely assessing slightly post-peak production of cytokines. Additionally, maximal expression of chemokines is achieved between 12 and 24 hours post SCI [129, 133, 135], suggesting that we are looking at peak chemokine production at our 1 day post injury time point.

Maximal BSCB permeability reported in the literature is reached 1 day following injury with some permeability still evident at 1 week [139, 417]. Our results are consistent with this literature (Figure 11). However, in PTS animals there was no decrease in EB extravasation from 1 to 3 days, as was the case in the SCI group, suggesting that there is prolonged BSCB permeability in these animals. This increase was likely due to a combination of meningeal and parenchymal blood vessel permeability. It should be noted that we did not look at other vasoactive substances that could have influenced BSCB, such as reactive oxygen species, kinins, histamines, nitric oxide and elastases [78, 86].

Upon the association of arachnoiditis with SCI (PTS animals), some parenchymal inflammatory mediators detected were merely additive, ie the inflammation seen was equal to that of arachnoiditis alone plus SCI alone. However, others were greater than that each combined, suggesting a synergistic increase. These include IL-1α and IL-1β at 1 day post injury (Figure 9), the inflammatory chemokines reported at 3 days post injury (Figure 9), BSCB permeability at 3 days (Figure 11) and the extent of parenchymal scarring (Figure 12).

**Insights into human SCI**

There was some modest dorsal meningeal inflammation induced by a moderate clip compression/contusion injury in our study (SCI group). From this observation, we postulate that not all SCI patients develop PTS because; (i) arachnoiditis is either cleared in a timely fashion following injury or (ii) it is not severe enough to lead to alterations in parenchymal pathophysiology or cause SS.
Our data demonstrated that induction of arachnoiditis following SCI can have a significant impact on parenchymal pathophysiology. This increase could make the spinal cord more susceptible to the development of syringes, as it has been demonstrated that the severity of parenchymal lesion increases the incidence of syrinx formation [284]. Furthermore, studies suggest that complete – presumably more severe – injuries are more prone to syrinx development in humans [276].

There is most likely a reciprocal inflammatory relationship when arachnoiditis is associated with SCI. Inflammatory cells from the parenchyma can migrate to sources of inflammation in the meninges, and vice versa. Similarly, local inflammatory mediators can flow via extracellular fluid/CSF to alter inflammation in areas remote to the site of inflammatory stimulus. It is believed that approximately 30% of CSF is derived from ECF (see [418] for a review of CSF pathways), allowing for movement of such macromolecules from the parenchyma to the SAS and vice versa. We did not look at meningeal fibroblasts in this study, though they are expected to play a role in cytokine and chemokine production as well as deposition of the fibrous scar in the parenchyma. Indeed, others have demonstrated that following SCI, proliferative meningeal cells can enter the spinal cord parenchyma [210, 419].

As we begin to recognize the heterogeneity in SCI, models like these are needed to properly mimic the permutations seen in certain subpopulations of patients. Previous studies from our lab demonstrated that our PTS model significantly decreased locomotor recovery and increased neuropathic pain [393]. This finding, combined with results from the present study, suggests that arachnoiditis could have a substantial impact on patient quality of life.
3.9 Conclusions

Our model of PTS used a synthetic means to induce a more robust and local meningeal inflammatory response following SCI – an event that is thought to contribute to SS and eventual parenchymal influx of CSF. The induction of arachnoiditis potentiated parenchymal inflammation and scarring following injury, suggesting that if sufficient arachnoiditis were to exist following SCI, patients might experience a more severe injury and might be more susceptible to the development of syringes. Specific models, like this one, are needed to address the biological permutations seen in certain populations of patients in order to eventually develop targeted treatment options.
Chapter 4

4 Evaluation of the use of a bioengineered hydrogel to treat spinal cord injury associated with arachnoiditis

4.1 Abstract

SCI comprises a heterogeneous condition caused by a complex array of mechanical forces that damage the spinal cord – making each case somewhat unique. In addition to parenchymal injury, a subset of patients experience severe inflammation in the SAS or arachnoiditis, which can lead to the development of fluid filled cavities/syringes, a condition called post-traumatic syringomyelia (PTS). Currently, there are no therapeutic means to address this devastating complication in patients and furthermore once PTS is diagnosed, treatment is often prone to failure. We hypothesized that reducing subarachnoid inflammation using a novel bioengineered strategy would improve outcome in a rodent model of PTS. A hydrogel containing HA (HAMC) was injected into the SAS 24 hours post PTS injury in rats. Intrathecal injection of HAMC reduced the extent of fibrosis and inflammation in the SAS. Furthermore, HAMC promoted improved neurobehavioural recovery, enhanced axonal conduction and reduced the extent of the lesion as assessed by MRI and histomorphometric assessment. These findings were additionally associated with a reduction in the post-traumatic parenchymal fibrous scar formation as evidenced by reduced CSPG deposition and reduced IL-1α cytokine levels. Together, our data suggest that HAMC is capable of modulating inflammation and scarring events, leading to improved functional recovery following severe SCI associated with arachnoiditis.
4.2 Introduction

Traumatic SCI can lead to motor, sensory and autonomic impairments that cause considerable suffering and economic hardship. Currently, there are few effective pharmacological treatment options to complement surgical and rehabilitation measures undertaken by physicians and health care practitioners. Damage to meningeal layers is an often overlooked aspect of the primary trauma following SCI. Acute arachnoiditis can not only potentiate parenchymal inflammation and scarring but also lead to the formation of chronic SS or adhesions, a phenomenon associated with the development of parenchymal fluid-filled cavities – or what is known as post-traumatic syringomyelia (PTS) [277]. It has been estimated that up to 5% of injuries will develop symptomatic PTS from weeks to years following injury [263, 264, 268-270]. Syrinx development is thought to occur due to SS mediated alterations in CSF flow dynamics, resulting in increased inflow of CSF [276-279]. Importantly, PTS represents a complication of SCI that is responsible for increased neuropathic pain and decreased motor function [409, 420].

Our PTS model increases neuropathic pain, increases lesion/cyst volume, and causes increased functional deficits [393] – mimicking some of the disease pathologies seen in PTS patients. Although arachnoiditis is artificially induced in this model, the kaolin injection produces more severe arachnoiditis/scarring that is seen clinically in PTS patients and also increases parenchymal inflammation, gliosis and decreases functional recovery relative to SCI alone [393]. This suggests that targeting early arachnoiditis could have a significant impact on SCI pathology during this time period and improve long-term functional recovery. Currently, there are no preventive treatment options to reduce SS. Further, the likelihood of recurrence following surgical intervention for PTS (arachnolysis/detethering) can be as high as 83% in cases of extensive scarring [277].
Hydrogels, such as fibrin [421], polyethylene glycol [422, 423], chitosan [424] and HA-based biomaterials [425, 426] have been studied in a variety of spinal cord injury repair strategies. Hyaluronan (HA) is particularly compelling, as high molecular weight HA plays a role in inflammation and tissue repair by interacting with inflammatory cells and ECM proteins (see [374] for a review). When HAMC was injected in the intrathecal space (the fluid-filled cavity that surrounds the spinal cord) it attenuated the inflammatory response after spinal cord injury [346], and degraded/dissolved after 4-7 days therein [427]. We hypothesized that intrathecal injection of HAMC [346] would reduce arachnoiditis and improve functional recovery in our rat model of PTS [393]. In the present chapter, to test this hypothesis, HAMC was injected into the intrathecal space 24 hours after severe SCI with arachnoiditis was induced in a rat animal model. When HAMC was injected into the SAS, it reduced arachnoiditis and SS, promoted neurobehavioural recovery, enhanced axonal conduction, reduced lesion size and reduced the extent of fibrous scar formation. Furthermore, there was a reduction in IL-1α expression in HAMC treated animals. We conclude that HAMC represents a promising treatment for arachnoiditis associated with SCI and modestly attenuates the pathological and clinical complications of PTS. Moving forward, we propose that HAMC could also be used for a myriad of conditions involving meningeal damage and/or arachnoiditis.

4.3 Objective

Determine the efficacy of a bioengineered hydrogel (HAMC) in treating SCI associated with arachnoiditis (PTS).

4.4 Hypothesis

HAMC will reduce inflammation and scarring, leading to improved functional recovery.
4.5 Specific Aims

(1) Evaluate neurobehavioural recovery in aCSF (control) and HAMC treated animals.

(2) Determine neuroanatomical/histological outcome in aCSF and HAMC treated animals.

(3) Assess acute inflammation and scarring in aCSF and HAMC treated animals.

4.6 Methods

There were a total of 132 rats used in this section. PTS animals (35g clip compression injury at T6/T7 immediately followed by subarachnoid kaolin injection rostral to the injury site) were randomly divided into 2 groups: (i) intrathecal aCSF injection at 24 hours after injury and (ii) intrathecal HAMC injection at 24 hours after injury. A total of 6 animals were used for IHC at 7 days post injury (3 per group). Longitudinal sections were stained for fibrous scarring (CSPG, collagen-IV), macrophages (Iba-1) and astrocytes (GFAP). For the 6-week behavioural study (BBB, inclined plane, neuropathic pain) 24 animals were used (12 per group). For MRI and electrophysiology at 6 weeks, 24 animals were used (12 per group). For the labeled HA study at 2 days post injury, 6 animals were used (3 per group). For Iba-1 gene expression and western blotting at 2 and 7 days post injury, 24 animals were used (6 per group, per time point). For cross sectional IHC (Iba-1, GFAP and CSPGs) at 7 days, 12 animals were used (6 animals per group). For MPO, MMP-9 and EB extravasation at 2 days post injury, 12 animals were used (6 per group). For cytokine and chemokine analysis, 24 animals were used (12 per group).

4.7 Results

HAMC reduces the spread of scarring and inflammation in the SAS

Since kaolin remains static in the SAS after its injection immediately following injury we sought to qualitatively look at the caudal spread of the scarring and inflammation its presence
caused. To accomplish this we perfused aCSF and HAMC treated animals one week following injury and processed the spinal cord tissue immunohistochemically. Sagittal sections were stained for fibrous scarring and macrophage/microglia markers. Representative images from aCSF and HAMC treated animals are presented in Figure 13A and B, for collagen-IV/CSPGs and Iba-1/GFAP respectively. Note that the presence of kaolin in the SAS of aCSF and HAMC treated animals is similar, as expected (arrows) and has elicited intense inflammation and scarring due to its presence. However, with HAMC treatment, the spread of collagen IV (red in part A) and macrophage/microglia (green in part B) from the site of kaolin injection below the injury site (boxed area) is reduced.
Figure 13. HAMC reduces meningeal scarring and inflammation

(A) Representative sagittal sections stained for collagen-IV (red) and CSPGs (green) in aCSF and HAMC treated animals, 7 days following injury. In HAMC treated animals, the scarring from the site of kaolin injection that has spread below the injury site (boxed area) is reduced. (B) Iba-1 (green) staining demonstrates that HAMC also reduced the caudal presence of macrophage/microglia in the meninges at 7 days post injury. GFAP is shown in red. Arrows are pointing to the kaolin. Scale bar represents 1mm.
HAMC improves hind limb locomotion and reduces the incidence of at level mechanical allodynia

HAMC or aCSF control was injected intrathecally (caudal to the injury site/area of scarring) 24 hours following injury and animals were monitored for hind limb locomotor activity according to the BBB locomotor rating scale. Figure 14A shows that there was an overall treatment effect with HAMC injection (two-way ANOVA p=0.04). Upon post-hoc analysis, the only week that was significant was 6 weeks post injury - where aCSF animals had an average score of 6.1 ± 0.9 versus HAMC animals which had a score of 7.8 ± 0.8, corresponding to a difference of 1.7 (Bonferroni post hoc, p=0.049). For reference, a score of 7 represents extensive movement of ankle, hip and knee joints. Above a score of 7, animals start to gain co-ordinated movement (8) and stepping (9). Below 7, animals have variations of slight to extenstive movement of the three joints. In addition to weekly locomotor assessment, motor function was monitored biweekly using the inclined plane apparatus. Figure 14B demonstrates that HAMC did not improve motor function on the inclined plane (two-way ANOVA, p=0.88). Control aCSF animals were able to achieve an average maximal angle of 23 ± 2, 33 ± 4 and 35 ± 3 degrees at weeks 2, 4 and 6, respectively. HAMC animals achieved an average angle of 27 ± 2, 34 ± 4 and 36 ± 3 degrees at each time point. There were 12 animals studied in each group and error bars represent SEM.

Treated and control animals were also monitored for the incidence of at level mechanical allodynia, a measure of neuropathic pain. Application of von Frey monofilaments (2 g or 4 g) was carried out on the dorsal aspect of the rats around the level of injury for a total of 10 times at 4 and 6 weeks following injury. The average number of adverse reactions in aCSF and HAMC treated animals is shown Figure 14C (n=12 animals per group). There was an overall treatment effect with HAMC using the 2 g monofilament (ANOVA, p<0.05). However, group
comparisons revealed non-significant decreases at 4 weeks and 6 weeks (Bonferroni post-hoc test; $p=0.23$ and $p=0.1$, for 4 and 6 weeks, respectively). There was no difference between aCSF and HAMC treated animals when using the 4 g monofilament (two way ANOVA, $p=0.24$).
Figure 14. HAMC improves neurobehavioural outcomes following injury

HAMC or aCSF control was injected intrathecally caudal to the injury site 24 hours following injury and animals were monitored for neurobehavioural recovery. (A) HAMC injection significantly improved the locomotor recovery compared to aCSF controls according to the BBB locomotor rating scale (two way ANOVA p=0.04). (B) Motor function was monitored using the inclined plane apparatus, biweekly. HAMC did not improve motor function on the inclined plane (Two way ANOVA, p= 0.88). (C) The incidence of at level mechanical allodynia, a measure of neuropathic pain, was monitored with von Frey monofilaments (2 g or 4 g). Data are expressed as the average number of adverse reactions out of 10 applications of the monofilament. There was an overall treatment effect with HAMC using the 2 g monofilament (ANOVA, p<0.05). However, group comparisons revealed non-significant decreases at 4 weeks and 6 weeks (Bonferroni post-hoc test; p=0.23 and p=0.1, for 4 and 6 weeks, respectively). There was no difference between aCSF and HAMC treated animals when using the 4 g monofilament (two way ANOVA, p=0.24). * Bonferroni post hoc p<0.05. Error bars represent SEM. n=12 per group.
**HAMC improves axonal conduction**

*In vivo* spinal cord evoked potentials (SCEP) were recorded from rats 6 weeks following injury (n=4 per group). A schematic of the stimulating and recording paradigm is shown in Figure 15A. The average conduction velocity is shown in Figure 15B. Control aCSF injected animals had a conduction velocity of approximately 2.3 ± 0.3 m/s compared to 3.3 ± 0.2 m/s in HAMC treated animals, representing a significant increase (t-test, p<0.05). The average amplitude is shown in Figure 15C. HAMC animals exhibited a significant increase in the average amplitude compared to aCSF controls. aCSF animals had an average amplitude of 34.1 ± 27.3 µV compared to 74.6 ± 13.7 for HAMC treated animals (t-test, p<0.05). Representative SCEP traces are shown below the graphs.
Figure 15. HAMC improves axonal conduction

*In vivo* spinal cord evoked potentials (SCEPs) were recorded from rats 6 weeks following injury (n=4 per group). The average conduction velocities (B) and amplitudes (C) are shown. HAMC treated animals exhibited a significant increase in average conduction velocity compared to aCSF controls. Similarly, HAMC animals exhibited a significant increase in the average amplitude compared to aCSF controls. Representative traces are shown below the graphs. * t-test, p<0.05. Error bars represent SEM.
HAMC reduces lesion size

Following the 6-week behavioural analysis, animals were imaged with a MR scanner to determine spinal cord lesion sizes. Longitudinal T2-weighted images were produced using the parameters described in the Methods section, with each image corresponding to an approximately 500 µm thick sagittal section. Figure 16A shows a representative slice through the midline of the spinal cord in an aCSF control and HAMC treated animal. Hyperintense voxels in each image slice were traced using Matlab® software and summed, generating a lesion volume for each animal. The average lesion volume from each group is reported in Figure 16B (n=12 per group). HAMC reduced the lesion volume compared to aCSF controls, however this was not found to be statistically significant. The average lesion volume of aCSF animals was 7.6 ± 0.8 mm³ compared to 5.8 ± 0.5 mm³ for HAMC treated animals (t-test, p=0.11).

Following MR imaging, animals were perfused with PFA and 20µm thick frozen sagittal sections were generated. Sections were processed for GFAP immunoreactivity to delineate the lesion boundaries. Representative sagittal GFAP images for aCSF and HAMC animals are shown in Figure 16C. The lesion area was calculated in 5 sections per animal, spaced 500µm apart using ImageJ® software and summed. Average lesion area for each group is demonstrated in Figure 16D (n=8 per group). HAMC treatment reduced the lesion area from 9.9 ± 1.5 mm² in aCSF controls to 6.7 ± 1.1 mm², representing a reduction of 32% (t-test, p<0.05).
Figure 16. HAMC reduces lesion size

(A) At 6-weeks following injury longitudinal T2-weighted MR images were taken in aCSF and HAMC treated rats. Representative slices through the midline of the spinal cord in an aCSF control and HAMC treated animal are shown. (B) The volume of hyperintense voxels in each image slice was determined and the average lesion volume from each group is reported (n=12 per group). HAMC did not significantly reduce the lesion volume compared to aCSF controls. (t-test, p=0.11). (C) Following MR imaging, animals were perfused, cords were sectioned and stained with GFAP to delineate the lesion borders. Representative sagittal GFAP images for aCSF and HAMC animals are shown. (D) The average lesion area from aCSF and HAMC groups is shown (n=8 per group). HAMC treatment reduced the lesion area approximately 32% (* t-test p<0.05) Error bars represent SEM.
HA enters the injured spinal cord parenchyma as HAMC biodegrades

As previous reports suggest that the HAMC blend biodegrades in the SAS within 3-5 days, we sought to determine if HA enters the spinal cord parenchyma as it is liberated from the gel. To accomplish this, we labeled HA with Alexa-fluor 568-hydrazide (HA-568) using carbodiimide chemistry. Labeled HA was used to generate a HAMC blend that was injected into the SAS 24 hours following injury. One day later (2 days post injury), animals were perfused with PFA and frozen cords were sagittally sectioned to observe HA-568 fluorescence. Unlabeled HAMC served as a control. Figure 17 demonstrates fluorescence images of HAMC-568 injected and control (non-labeled) HAMC animals counterstained with DAPI. Below each colour image is a grayscale representation of the red channel for clarity.
Figure 17. HA enters the spinal cord parenchyma as HAMC biodegrades

HAMC was injected into the SAS and 24 hours later, spinal cords were fixed and sectioned sagittally. The left panel represents unlabelled HAMC. A merged image of DAPI (blue) and red channel fluorescence is shown with a grayscale representation below. The right panel shows results from labeled HA (Alexa-568) used in the HAMC formulation. A DAPI and Red channel fluorescence image is shown with a grayscale representation of the red channel below.
**HAMC does not reduce the influx/activation of macrophage/microglia**

We examined if HAMC injection was associated with a decrease in acute to sub-acute macrophage/microglia activation and accumulation in the injured spinal cord. Through qPCR and immunoblotting, we looked at Iba-1 mRNA and protein expression at 2 and 7 days post injury as measure of macrophage and microglia (Figure 18). In both cases, results are presented relative to an uninjured control (sham) animal. Overall, there were no differences in Iba-1 gene expression at 2 and 7 days post injury (Figure 18A). At 2 days post injury, aCSF animals had a relative Iba-1 gene expression level of $1.3 \pm 0.1$ compared to $1.4 \pm 0.1$ in HAMC treated animals ($t$-test, $p=0.46$). Similarly, there were no differences in Iba-1 gene expression at 7 days in any fractions sampled (ANOVA, $p=0.65$). The epicenter region in aCSF animals had a relative Iba-1 expression level of $2.3 \pm 0.1$ compared to $2.6 \pm 0.3$ in HAMC treated animals. In rostral regions, the expression was $2.3 \pm 0.2$ compared to $2.2 \pm 0.3$, respectively. In caudal regions, the expression was $2.9 \pm 0.4$ versus $2.7 \pm 0.3$, respectively.

In terms of Iba-1 protein expression, Figure 18B shows Western blot densitometry results at 2 days following injury. There was no difference between aCSF ($2.15 \pm 0.32$) and HAMC ($2.7 \pm 0.2$) treated animals ($t$-test, $p=0.2$). A representative Western blot is shown beside the relative expression graph. Similarly, at 7 days, regions immunoblotted for Iba-1 revealed no differences between aCSF and HAMC treated animals (two way ANOVA, $p=0.9$). The 7 day results are shown in Figure 18C (ANOVA, $p=0.89$). Epicenter Iba-1 protein expression was $3.3 \pm 0.2$ in aCSF animals compared to $3.3 \pm 0.1$ for HAMC treated animals. In rostral regions, the numbers were $2.4 \pm 0.3$ and $2.3 \pm 0.5$, respectively. In caudal regions, the numbers were $2.8 \pm 0.5$ and $2.9 \pm 0.5$, respectively (Bonferroni post hoc, $p=0.77$). In each case there was 6 animals studied per group, per time point for both mRNA and protein studies. Error bars represent SEM.
We also looked at Iba-1 immunohistochemical staining to gain additional spatial information on the presence of macrophage/microglia in the injured spinal cord at 7 days post injury (Figure 19). Fixed frozen cross sections, 20 \( \mu \)m thick, were stained for Iba-1 immunoreactivity at 1000 and 2000 \( \mu \)m rostral and caudal to the injury epicenter. Representative sections are shown in Figure 19A. Mean fluorescence intensities were recorded for the entire section and the averages for aCSF and HAMC animals are presented in Figure 19B. This semi-quantitative immunohistochemical analysis demonstrated that there were spatial differences in the Iba-1 staining between aCSF and HAMC animals (two way ANOVA, \( p=0.00031 \)). At 1000 \( \mu \)m rostral, the average Iba-1 intensity for aCSF animals was 28.1 ± 9.6 versus 9.4 ± 5.6 for HAMC animals (Bonferroni post hoc, \( p=0.0003 \)). At 1000 \( \mu \)m caudal, the average Iba-1 intensity was 34.9 ± 11.6 for aCSF animals versus 12.4 ± 7.1 in HAMC treated animals (Bonferroni post hoc \( p<0.001 \)). There were no statistical differences at 2000 \( \mu \)m rostral (17.9 ± 17.5 versus 8.3 ± 2.8, for aCSF and HAMC; Bonferroni post hoc, \( p=0.19 \)), and 2000 \( \mu \)m caudal (11.4 ± 3.4 versus 16.9 ± 6.5, for aCSF and HAMC; Bonferroni post hoc, \( p=0.36 \)).
Figure 18. HAMC does not reduce overall presence of macrophage/microglia following injury
(A) qPCR at 2 and 7 days following injury revealed no significant changes in Iba-1 mRNA levels between aCSF and HAMC treated animals [t-test (2d) p=0.46 and ANOVA (7day), p=0.9]. (B) At 2 days post injury, immunoblotting for Iba-1 revealed no significant differences between aCSF and HAMC treated animals (t-test, p=0.2). (C) At 7 days post injury Western blot analysis revealed no significant changes in Iba-1 expression at the epicenter (0.5 cm centered at the injury site) or adjacent (0.5 cm rostral and caudal) regions in aCSF and HAMC treated animals (ANOVA, p=0.9). Error bars represent SEM.
A

Rostral 2000μm 1000μm 1000μm 2000μm

HAMC

Rostral

aCSF

2000μm 1000μm 1000μm 2000μm

Caudal

Lba-1

DAPI

B

Average Iba-1 Intensity

Rostral 2000 1000 1000 Caudal 2000

Distance from Epicenter (μm)

aCSF

HAMC

*
Figure 19. HAMC reduces macrophage/microglia at the injury epicenter

HAMC treatment shows a spatial decrease in Iba-1 immunoreactivity relative to aCSF controls. (A) Representative Iba-1 immunohistochemical staining at 7 days is shown. (B) Analysis of Iba-1 fluorescence intensities from entire sections (minus meninges) at various distances from the injury epicenter are shown (two way ANOVA p=0.0003). There were decreases in the Iba-1 staining between aCSF and HAMC animals at both 1000 µm rostral and caudal from the injury epicenter. n=6 per group. * Bonferroni post hoc p<0.05. Error bars represent SEM.
**HAMC does not reduce neutrophil extravasation or BSCB permeability**

Neutrophils are the first systemic immune cell recruited to the injury site following SCI, with maximal accumulation at 24 hours following injury. We assessed granular and cytoplasmic/ECM fractions for MPO activity in aCSF and HAMC treated animals (n=6 per group) at 2 days following injury (1 day following HAMC injection) to see if HAMC had any effect on recruitment of these cells (Figure 20A). In granular fractions, MPO activity was 28.8 ± 8.7 in aCSF animals compared to 28.7 ± 14.5 in HAMC animals (t-test, p=0.919). In cytoplasmic/ECM fractions MPO activity was 5.3 ± 1.8 in aCSF animals compared to 4.3 ± 0.6 in HAMC animals (t-test, p=0.18). Neutrophils are a source of MMP-9, which acts to degrade ECM components and tight junction proteins of the BSCB. Gel zymography was used to determine MMP-9 activity and it was found that there was no difference between aCSF and HAMC groups (Figure 20B). BSCB permeability is a hallmark of SCI, to which MMP-9 can contribute. Animals in aCSF and HAMC treated groups were injected with EB and its extravasation was measured in spinal cord tissue through absorbance spectrophotometry. Figure 20C shows the results from the quantification of EB in spinal cord homogenates. Animals injected with an aCSF had 440.8 ± 47.6 compared to that of 461.0 ± 74.1 µg/g tissue (t-test, p=0.84). Error bars represent SEM (n=6 per group).
Figure 20. HAMC does not reduce MPO activity or BSCB permeability

(A) Granular and cytoplasmic/ECM fractions were assessed for MPO activity in aCSF and HAMC treated animals. HAMC did not alter MPO activity in either fraction (t-test, p=0.6, p=0.23, respectively). (B) Gel zymography revealed no difference in MMP-9 activity between HAMC and aCSF animals. (C) BSCB permeability was assessed using Evans blue (EB) extravasation. There was no difference in aCSF and HAMC treated animals (t-test, p=0.84). n=6 per group. Error bars represent SEM.
Acute cytokine and chemokine expression

Next, we looked at cytokine and chemokine expression 2 days following injury (1 day after HAMC injection) with the expectation that if HAMC can reduce scarring and inflammation in the SAS and HA is present in the spinal cord, then overall (meningeal and parenchymal) production of inflammatory mediators would be reduced. Figure 21A shows average cytokine levels and Figure 21B shows average chemokine levels from 0.5 cm of spinal cord tissue, centered at the epicenter. Data are presented as pg/mg protein (n=12 per group). HAMC significantly reduced IL-1α, from 47.3 ± 11.2 to 30.6 ± 7.5 in aCSF treated animals, representing a 40% decrease (p=0.04). HAMC did not effect the expression of IL-1β (p=0.28), IL-6 (p=0.12) or TNF-α (p=0.74). In terms of chemokine expression, HAMC did not significantly reduce MCP-1 (p=0.33), GRO/KC (p=0.55) or MIP-1α (p=0.11).
Figure 21. HAMC reduces IL-1α cytokine expression

Cytokine and chemokine expression was determined by multiplex ELISA, 2 days following injury from 0.5 cm of spinal cord tissue centered at the epicenter (n=12 per group). (A) Average cytokine levels are shown and were calculated by normalizing analyte concentration to total protein concentration of each sample. HAMC significantly reduced IL-1α (p=0.04) but did not significantly alter the expression of IL-1β (p=0.28), IL-6 (p=0.12) or TNF-α (p=0.74). (B) Average chemokine levels are shown. HAMC did not significantly reduce MCP-1 (p=0.33), GRO/KC (p=0.55) or MIP-1α (p=0.11) protein levels relative to aCSF controls. Error bars represent SEM.
HAMC does not reduce the astrocytic scar but does reduce fibrous scarring

Due to the link between inflammation and scarring, we examined the immunoreactivity and gene expression of GFAP as a measure of glial scarring. We looked at spatial GFAP expression using IHC. Figure 22A demonstrates representative GFAP (green) IHC images taken from 1000 and 2000 µm rostral and caudal from the injury epicenter at 7 days following injury. Figure 22B demonstrates quantification of fluorescence intensity and shows HAMC did not alter GFAP expression at these distances relative to aCSF controls (two way ANOVA, p=0.78). The numbers for aCSF versus HAMC animals were: 9.0 ± 2.4 and 9.5 ± 2.1 at 2000 µm rostral; 1.6 ± 0.4 versus 1.7 ± 0.4 at 1000 µm rostral; 1.4 ± 0.5 versus 0.7 ± 0.2 at 1000 µm caudal; and 12.1 ± 3.1 versus 7.7 ± 1.1 at 2000 µm caudal, respectively. Figure 22C shows that there was no difference in GFAP immunoreactivity in aCSF and HAMC treated animals at 2 and 7 days post injury using immunoblotting techniques (ANOVA, p=0.43). The average GFAP immunoreactivity from slot blot analysis in aCSF and HAMC treated animals and representative bands from each group are shown (n=6 per group). At 2 days post injury, aCSF animals had a relative GFAP expression of 1.3 ± 0.1 versus 1.2 ± 0.1 in HAMC treated animals. At 7 days, these numbers were 1.4 ± 0.1 and 1.2 ± 0.1, respectively.

In addition to glial scarring, we looked at fibrous scarring in terms of the deposition of CSPGs. Figure 22A also shows representative CSPG (red) IHC images taken from 1000 and 2000 µm rostral and caudal from the injury epicenter at 7 days following injury (minus meninges). Fluorescence intensity quantification in part B demonstrates that there were decreases in CSPG immunoreactivity in HAMC animals relative to aCSF controls (two way ANOVA, p=0.03) A significant difference seen was at 2000 µm caudal, where aCSF animals had an average intensity of 4.5 ± 0.9 versus 1.8 ± 0.7 for HAMC animals (Bonferroni post hoc, p=0.047). For aCSF and HAMC animals, the numbers were: 3.7 ± 1.0 versus 3.3 ± 2.0 at 2000
µm rostral; 2.3 ± 0.6 versus 0.68 ± 0.1 at 1000 µm rostral; and 4.2 ± 0.8 versus 3.8 ± 0.8 at 1000 µm caudal, respectively. Additionally, we looked at CSPG protein expression thorough slot blot analysis (Figure 23D). HAMC significantly decreased CSPG expression (two way ANOVA, p=0.002). At 2 days post injury, aCSF animals had an average CSPG expression of 7.8 ± 0.3 compared to 10.1 ± 0.9 in HAMC treated animals (Bonferroni post hoc, p=0.08). At 7 days, aCSF animals had an average CSPG expression of 9.6 ± 0.6 versus 5.6 ± 0.8 for HAMC animals, representing a significant decrease in CSPG expression (Bonferroni post hoc, p=0.007). The average relative CSPG protein expression for aCSF and HAMC treated animals in addition to representative CSPG slot blots are shown (n=6 per group). Error bars represent SEM.
Figure 22. HAMC reduces CSPG fibrous scarring following injury

(A) Representative GFAP (green) and CSPG (red) IHC images taken from 1000 and 2000 µm rostral and caudal from the injury epicenter at 7 days following injury. (B) Fluorescence intensity analysis revealed HAMC did not alter GFAP expression at these distances relative to aCSF controls (two way ANOVA p=0.9), but did decrease CSPG immunoreactivity (two-way ANOVA p<0.05). A significant difference between HAMC animals relative to aCSF controls was seen at 2000 µm caudal. (C) Average GFAP immunoreactivity from slot blot analysis in aCSF and HAMC treated animals is shown. There was no difference in band densities between aCSF and HAMC treated animals at 2 or 7 days post injury (ANOVA, p=0.8). Representative bands from each group are shown. (D) Slot blot analysis of CSPG immunoreactivity demonstrated that HAMC reduced the deposition of CSPGs relative to aCSF controls at 7 days post injury. Representative CSPG blots are shown. Error bars represent SEM. n=6 per group * Bonferroni post hoc p<0.05
**Axonal/neuronal protection following injury**

Since the acute inflammation and cytokine neurotoxicity has been established in the literature, we set out to determine if the modest reduction in cytokine expression translated acute cellular and axonal preservation. Figure 23A shows a representative Western blot and quantification of relative MAP-2 expression at 2 days post injury. Control aCSF animals had a relative MAP-2 expression of $0.6 \pm 0.1$ compared to $0.5 \pm 0.1$ in HAMC treated animals (t-test, $p=0.42$). Samples were also immunoblotted for NF200 at 2 days following injury as a measure of the presence of axons in the tissue (Figure 23B). Relative NF200 expression was $0.4 \pm 0.2$ in aCSF animals compared to $0.6 \pm 0.2$ in HAMC treated animals (t-test, $p=0.063$). Figure 23 C shows results from Beta III tubulin (B3T) immunoblotting at 2 and 7 days post injury. HAMC treatment did not alter B3T expression relative to aCSF controls. At 2 days, aCSF animals had a relative B3T expression of $0.33 \pm 0.08$ compared to $0.5 \pm 0.1$ in HAMC treated animals (t-test, $p=0.35$). At 7 days, aCSF animals had a relative B3T expression of $0.2 \pm 0.03$ compared to $0.3 \pm 0.1$ in HAMC treated animals (t-test, $p=0.36$). Note that MAP-2 was only faintly detected at 7 days post injury. Error bars represent SEM.
Figure 23. HAMC treatment does not lead to significant axonal and neuronal preservation.

Western blot results for MAP-2 (A), NF200 (B), and B3T (C) are shown. HAMC did not alter the expression of MAP-2 (t-test, p=0.42), B3T (t-test, p=0.35 and 0.36, for 2 and 7 days respectively) or NF200 (t-test, p=0.063). n=12 per group. Error bars represent SEM.
4.8 Discussion

We have demonstrated that injection of a hydrogel containing HA improved neurobehavioural recovery and histological outcome following SCI associated with SS. The physical blend of HAMC reduced the extent of scarring and inflammation in the SAS. While there was no overall reduction in macrophage/microglia or neutrophils following injury, HAMC showed a significant reduction in both IL-1α and CSPG expression following injury.

While others have used urokinase to reduce fibrosis in models of arachnoiditis (not associated with SCI) [428], to our knowledge this is the first study to look directly at reducing SS following traumatic injury to the spinal cord. Some groups have suggested that HA can modulate inflammatory reactions [346], fibrous scarring and improve functional recovery following SCI [425, 426]. With HAMC, HA is likely the putative therapeutic molecule based on in vitro studies in microglia (see Chapter 5).

Endogenous and exogenous HA in the spinal cord

Following SCI, endogenous HA (10^6 Da) is degraded [220] – possibly by endogenous hyaluronidases (see [429] for a review) and reactive oxygen species [362]. While the anti-inflammatory benefits of exogenous HMW-HA (above 1,000 kDa) have been demonstrated [430, 431], there is evidence that HA of lower molecular weights (LMW-HA) can be pro-inflammatory [115]. However, studies have also shown that LMW-HA can also be anti-inflammatory [114] and pro-angiogenic [432]. Moreover, delivery of HA oligomers (from 2 to 12 saccharides; corresponding to 372 to 2233 g/mol) to the injured spinal cord showed improved functional recovery in a weight drop model of SCI in rats [425]. Together, it is expected that the molecular state of exogenous HA in regards to the pathophysiology of SCI is complex and could influence inflammation, fibrous scarring and angiogenesis at various stages of biodegradation.
HA turnover has been observed following SCI in animal models. Most notably, Struve and colleagues observed substantially reduced HA expression up to a week, followed by an increase in expression at 4 weeks post clip compression SCI in rats [220]. They postulated that HMW-HA plays a role in regulating gliosis and that HA degradation contributes to the observed astrocyte proliferation following SCI. In our studies, we did not see any HAMC mediated alterations in GFAP expression through immunoblot and IHC analyses at 2 and 7 days post SCI (Figure 22).

The presence of exogenous HA (from HAMC) appears to be in the spinal cord at a time when previous reports suggest that endogenous HA has been degraded or is in the process of degradation (Figure 17). This time (1 to 2 days) corresponds to a time when microglia, neutrophils and astrocytes are responding to injury and coordinating a systemic immune response, hence exogenous HA in this setting is present to influence inflammatory signaling in these cells.

**Mechanism of action of HAMC**

HAMC showed a modest improvement in hindlimb locomotion following SCI compared to aCSF controls (Figure 14A). Though not statistically significant, there was a possible trend in axonal preservation as demonstrated with NF200 immunoblotting at 2 days following injury (Figure 23C). Even though a relatively large number of animals was used (12 per group), the post-hoc power analysis gave a value of 53% (G*Power, Institute for Experimental Psychology, Heinrich Heine University, Dusseldorf, Germany) thus the analysis was under powered (based on the convention of 80% being an appropriate power level). That being said, axonal preservation/functionality was supported by improved SCEP axonal conduction (Figure 15), though this measured afferent cord conduction. In contrast, motor function was not improved as measured by the inclined plane test (Figure 14B). This discrepancy could be explained by the
fact that the inclined plane test was developed using a cervical injury (C7-T1) [400, 433] whereas the BBB was developed using thoracic injuries [399]. Thus, the inclined plane test, while still useful, is likely not as sensitive for thoracic injuries as the BBB in terms of measuring small changes in functional improvement.

We also observed that there was an overall treatment effect on at-level mechanical allodynia (Figure 14C). However, this treatment effect was not found to be significant at 4 and 6 weeks post injury when post-hoc tests were performed. Interestingly, only half of the untreated animals developed mechanical allodynia in our model, which is similar to what others have observed in different models of neuropathic pain [434], yet which also limited our power to determine a treatment effect. Similar to our PTS model, not all cases of human SCI develop neuropathic pain [249, 250]. It should be noted that survival of dorsal horn neurons caudal to the injury site (data not shown) and SCEP recordings (Figure 15) suggest there was not a bias in pain relaying infrastructure that could account for any differences in pain response between HAMC and aCSF animals.

HAMC treatment did not significantly change the overall lesion volume as determined with MRI. Upon post-hoc analysis, this comparison was found to be under powered (55%; G*Power). Additionally, T2 weighted MRI analysis is somewhat subjective due to the fact that both gliosis and the lesion appear white (hyperintense). As tracing the lesion volume in each slice was based on hyperintensity of voxels, the lesion volume calculation is subject to experimenter error. Another source of error was likely the resolution of the MR images. In contrast, when IHC was used to measure lesion area, there was found to be a significant decrease with HAMC treatment. IHC is prone to fixation issues and subject to some morphological changes during the cutting and slide mounting procedure. This is expected to introduce some error when calculating the lesion size. An additional weakness with the IHC analysis was that
the lesion area was calculated at every 500 µm in longitudinal slices – and the total area was summed in each animal. A greater resolution (less distance in between slices analyzed) could have provided a better spatial analysis and accurate result. Additionally, rather than summing the total area in each animal, the area at each distance from the injury epicenter could have been averaged to provide spatial information. Despite these differences, as both analyses demonstrated a decrease in lesion size (one significant and one not – but underpowered) it is likely HAMC is having an effect on lesion size.

Representative IHC lesion images in Figure 16 were taken from the center of the spinal cord. With these sections, differences in lesion size are reflected in the lesion length. In lateral sections (not shown), there are differences in lesion width – from dorsal to ventral margins. The increased non-lesional tissue in lateral sections likely represents sparing in axonal tracts (such as the rubrissipinal tract) that can explain the increased behavioural recovery (BBB) and improved SCEPs. This relationship is only a correlation and was not validated. The use of fluorogold retrograde labeling could help confirm preservation of motor tracts.

Through immunohistochemical analysis, previous studies have suggested that HA reduces microglia/macrophage activation and CSPG expression following SCI [425, 426]. We also replicated this finding in our study for both Iba-1 and CSPG immunoreactivity (Figure 19). However, using Western blotting and qPCR, we did not detect a reduction in microglia/macrophage activation through Iba-1 immunoreactivity and gene expression, respectively (Figure 18). It should be noted that the source and size of HA, delivery methods and injury models were different in our study and those published previously. While immunohistochemical fluorescence intensity analysis can offer spatial and cellular information to an extent, is not seen as a robust indicator of protein expression due to variability in staining between sections – even though exact protocols were followed in each case. That being said, it is
possible that small spatial decreases in macrophage/microglia, that are detectable with immunohistochemistry, are lost when homogenized tissue is analyzed. The reduction in Iba-1 staining was seen closer to the injury epicenter and as such, perhaps HAMC had more influence in the lesion as opposed to the prenumbra. It should be noted that our study also looked at the gene expression of two other macrophage/microglia markers -OX42 (CD11b) and ED-1 (CD68) - using qPCR and found no treatment differences (data not shown).

Our study also showed a reduction in CSPG expression (Figure 22), which is consistent with previous studies [425, 426]. Importantly, through immunohistochemistry we demonstrated that CSPGs were reduced in the parenchyma of HAMC treated animals, suggesting that the reduction seen in CSPG expression from homogenate samples in Figure 22 was not solely due to less CSPGs in the meninges as shown in Figure 13. Through IHC, the CSPG reduction was seen most prominently caudal to the injury epicenter. This could be associated with the presence of HAMC caudal to the injury site. As HAMC was found to influence fibrous scarring, it might have been able to promote endogenous regeneration due to decreased CSPG expression. Indeed, studies that have reduced CSPG expression following injury through enzymatic degradation have led to enhanced endogenous regeneration and plasticity [217, 218]; however, we recognize that some of the effects in these studies could be attributable to other mechanisms [435].

We were able to detect a significant decrease in IL-1α expression in HAMC treated animals compared to aCSF controls. Previous studies have demonstrated that IL-1R antagonism prevents apoptosis [436] and improves the severity of SCI [437, 438]. Additionally, we saw non-significant decreases in IL-6 and MIP-1α. Upon performing a post-hoc power analysis, the values were 28% and 32%, respectively (G*Power Software). Both tests for these molecules were underpowered and as such, there is a possibility that greater numbers could determine whether there is an actual reduction in IL-6 and MIP-1α expression. Overall, there is a
possibility of a very modest reduction of inflammatory cytokine/chemokine expression with HAMC treatment. The link with inflammatory cytokines/chemokines and neurotoxicity in addition to preventing axonal growth is well established in the literature [439, 440], thus the decreases, though not all statistically significant, may have had a biological effect on injury pathophysiology. Related to this, we observed a possible trend in axonal preservation (Figure 23) that could be due to less cytokine/chemokine production. Due to the lack of statistical significance and direct evidence, this proposed relationship is only suggestive. In summary, HAMC significantly increased functional recovery as demonstrated by increases in axonal conduction (Figure 15) and neurobehavioural recovery (Figure 14). The mechanism could be attributed to HAMC’s influence on both the meninges and parenchyma. The injection of HAMC into the SAS put it into contact with inflammatory cells and local meningeal fibroblasts activated from SCI and induced arachnoiditis. The physical presence of HAMC and/or chemical interactions of free HA released into the CSF upon biodegradation may have acted in tandem to influence the number of inflammatory cells recruited to the meninges (as in Figure 13) and the inflammatory mediators produced by inflammatory cells and local meningeal fibroblasts. As there is significant exchange of extracellular fluid and CSF (see [418] for a review of CSF pathways), reduced meningeal inflammation is expected to influence the severity of the parenchymal injury as we have demonstrated with reduced lesion size (Figure 16), increased axonal conduction (Figure 15), and reduced CSPG expression (Figure 22). Alternatively, we observed that HA entered the parenchyma (Figure 17) – suggesting that HA could interact with resident parenchymal cells and recruited inflammatory cells directly.

**Clinical applications of HAMC**

It is interesting to think about whether we could translate this strategy to the clinic. On the one hand, we have demonstrated that administration of HAMC is beneficial following SCI
associated with arachnoiditis based on acute application of the therapeutic. While PTS is considered a chronic complication of SCI, studies have described cases developing within several weeks to months following SCI [276, 407, 408]. Furthermore, it is possible that acute to sub-acute arachnoiditis – which we are targeting in this study – is the cause of the chronic SS associated with PTS.

This study provides implications not only to SCI but also other CNS conditions. Any time the dura is breached, the risk of causing local inflammation and SS exist. These include subdural surgical procedures such as tumor removal, stem cell injections, surgery for a subdural/subarachnoid hemorrhage and decompressive/arachnolysis treatment for PTS. In particular, the surgical procedures of shunting and arachnolysis for chronic PTS are prone to failure, including a high recurrence of meningeal scarring/fibrosis [277, 441] and return of the syrinx. Overall, we feel that an anti-inflammatory hydrogel like HAMC would be suitable as an adjuvant therapy to subdural surgical procedures. The physical and chemical properties of HAMC could offer a prolonged effect until the inflammatory response has subsided.

4.9 Conclusion

In summary, HAMC dampened induced arachnoiditis associated with SCI and improved functional recovery. In addition to improving neurobehavioural and neuroanatomical outcomes, HAMC exhibited reduced IL-1α cytokine expression and CSPG expression. To our knowledge this is the first study to therapeutically target arachnoiditis/meningeal inflammation following SCI and show that reducing arachnoiditis can have an effect on parenchymal pathophysiology. These findings should be of interest to the PTS community, who currently are without effective treatment options. Furthermore, HAMC represents a possible strategy for surgeons to mitigate arachnoid inflammation and scarring related to subdural surgical procedures. Though its effects
were modest in our model, future studies elucidating the efficacy of HAMC are certainly warranted. Specifically, HAMC could be tested in different models of SCI associated with arachnoiditis, different formulations of HAMC could be generated by altering the size of HA and the rate of biodegradation and also anti-inflammatory or neuroprotective drugs could be dissolved in HAMC to enhance recovery.
Chapter 5

5  Hyaluronan Mediated Reduction of TLR4 Activation in Microglia

5.1  Abstract

Toll-like Receptor 4 (TLR4) signaling has been implicated in microglial activation and propagation of the secondary inflammatory response following spinal cord injury (SCI). As such, modulating microglial activation through TLR4 represents an attractive therapeutic approach to treat SCI. High molecular weight HA (HMW-HA), a polymer with multiple therapeutic uses, has been previously shown to modulate TLR4 activation in macrophages and has shown early promise as a therapeutic agent in SCI. However, the mechanism associated with HMW-HA has not been fully elucidated or tested in microglia, a similar cell type. In the current study, we sought to determine the effects of HMW-HA on TLR4 activation in microglia in order to gain insights into the mechanism of action of this promising therapy for CNS injuries.

Primary microglia in culture were exposed to LPS to simulate an inflammatory trigger. HMW-HA or vehicle control were applied at the same time. Measures of inflammation included acute ERK 1/2 activation along with inflammatory gene and protein production. Analysis of TLR4 negative regulation included examination of Akt phosphorylation and the induction of A20 expression.

HMW-HA decreased LPS-mediated IL-1β, IL-6 and TNF-α gene expression and IL-6 and nitric oxide production. This decrease was associated with a reduction in ERK 1/2 phosphorylation seen as early as 15 minutes post stimulation and was not seen with HA of a lower molecular weight. The continued presence of HA was necessary for a therapeutic effect and HMW-HA reduced the LPS-mediated activation of Akt and A20 protein expression.
Together, our results show that HMW-HA can reduce LPS-mediated inflammatory signaling in microglia. Since the continued presence of HA was necessary to obtain a therapeutic effect and considering that HA did not stimulate negative regulatory pathways, we conclude that HA possibly mediates its effects by blocking the induction of inflammatory signaling through an extracellular mechanism. HMW-HA represents an anti-inflammatory molecule that can be used to modulate microglial TLR4 inflammatory signaling, and hence could show promise as a therapeutic strategy for CNS injuries including SCI.

5.2 Introduction

Following CNS trauma including SCI, TLR4 signaling is involved in the ensuing inflammatory pathophysiology. Cell death and extracellular matrix breakdown expose local and systemic immune cells to TLR4 agonists, leading to the production of cytokine/chemokines, increased cell surface receptor expression and cell motility [93]. Such TLR4 ligands include heat shock protein 60 [442], high mobility group protein B1 [443], HA fragments [444], fibronectin [445], fibrinogen [446], gangliosides [447] and fibrillar A beta protein [448]. Microglia respond quickly to such cues and contribute to immediate responses as well as influence subsequent sub-acute phase inflammation [449, 450]. While some studies have shown that activation of TLR4 is responsible for neurodegeneration and oligodendrocyte loss [103, 104], others studies have demonstrated that TLR4 knockout mice fair worse after injury compared to their wild type counterparts [102]. Undoubtedly, there are cell specific and temporal aspects of receptor expression and activation to consider [451]. Overall, acute microglial activation through TLR4 is most likely seen as a detrimental aspect of SCI that contributes to direct cell toxicity [83].

High molecular weight HA (HMW-HA) has been used to modulate inflammatory responses in macrophages [113, 430, 431, 452] – a similar cell type to microglia – though the exact mechanism is not clear. Additionally, groups have examined the therapeutic use of HA to
treat inflammatory conditions such as liver and lung injury [392, 453]. In terms of SCI research, HMW-HA has been used in bioengineered drug delivery vehicles [346] and recent studies suggest HA can modulate inflammatory reactions and possibly improve functional recovery following injury [425, 426].

Mechanistic studies involving HA and TLR4 activation are complicated due to the promiscuity of HA for several cell surface receptors, co-receptors and associated proteins. HA can bind the receptor for hyaluronan-mediated motility (RHAMM), TLR2, TLR4, CD44 and intercellular adhesion molecule-1 (ICAM-1) [374]. Extracellular recognition of HA has been reported to utilize a receptor complex involving CD44, TLR4 and MD-2 [115]. Similarly, inflammatory TLR4 agonists bind comparable receptor complexes. For example, LPS binds a receptor complex consisting of TLR4, MD-2 and CD14 [454], whereas fibrillar A-beta and gangliosides can use TLR4 and CD14 [447, 448].

The effects of HA on TLR4 signaling have been previously examined in macrophages, although the effects on CNS-resident microglia are unknown. HMW-HA has been shown to reduce LPS-mediated inflammation in the U937 macrophage cell line through interaction with ICAM-1 and CD44 [430, 431]. Similarly, it has been demonstrated that HA inhibits LPS-mediated inflammation in mouse peritoneal macrophages via interaction with CD44 by increasing A20 expression [113]. A20 (TNFα-induced protein 3; TNFAIP3) has de-ubiquitinating activity and acts as a negative regulator of TLR signaling by removing ubiquitin moieties from TRAF6 [99]. However, other studies suggest that HA can inhibit LPS-mediated inflammation in CD44 knockout and wild type bone marrow mouse macrophages, suggesting that CD44 might not be necessary [114]. HA and TLR4 activation has also been examined in mouse chondrocytes [455]. Similar to macrophages, chondrocytes exposed to HMW-HA exhibited reduced LPS-mediated inflammation, however the authors suggested that interaction
with TLR4 was necessary. Together, the inhibitory mechanism of HA on LPS activation is not fully elucidated, could involve multiple receptors and could vary depending on cell type.

In the present study, when added at the same time as LPS, HMW-HA (and not LMW-HA) was able to reduce LPS-mediated IL-1β, IL-6 and TNF-α gene expression and production of NO and IL-6. This decrease was associated with an early reduction in LPS-mediated Erk 1/2 phosphorylation. The continual presence of HA was necessary for a therapeutic effect and HMW-HA reduced LPS-mediated Akt activation and A20 induction – two negative regulators of TLR4 activation [99, 100].

To our knowledge, this is the first study to show that HMW-HA can reduce inflammatory responses in LPS stimulated microglia, albeit a similar cell type to macrophages. The fact that the continual presence of HA was necessary and that HA decreased LPS-mediated activation of Akt and A20 negative regulatory pathways suggests that HA might be blocking LPS extracellularly as opposed to modulating intracellular signaling. HA mediated receptor masking has been previously described [455, 456] and could be at play in this setting. HMW-HA represents an anti-inflammatory molecule that can be used to modulate microglial TLR4 inflammatory signaling. We suggest that the anti-inflammatory effects of HMW-HA shown following SCI by several groups might be due to the modulation of TLR4 inflammatory signaling in microglia.

5.3 Objective
Determine if HA reduces LPS-mediated inflammation in microglia and elucidate a mechanism.

5.4 Hypothesis
HA will reduce TLR4 mediated inflammation through negative regulation of TLR4.
5.5 Specific Aims

(1) Characterize LPS-mediated inflammation in primary microglia

(2) Measure the effect of HA on LPS stimulated microglia

(3) Determine mechanism by which HA reduces inflammatory reactions in LPS stimulated microglia

5.6 Methods

Primary microglia isolated from 2-day old rat pups were exposed to LPS alone or LPS and HA at the same time, except in Figure 31. Gene expression was examined at 6 hours post exposure with qPCR. NO and IL-6 levels were detected in the media at 24 hours after exposure with a nitrite Griess assay and ELISA, respectively. Acute phosphorylation of Akt and ERK 1/2 was analyzed at 15, 30 and 60 minutes after exposure. A20 expression was analyzed at 24 hours after exposure.

5.7 Results

Primary microglia were cultured from 2-day old rat pups. Their isolation was based on differential adhesion [395], and the purity of the cultures was monitored by immunohistochemistry. Figure 24 shows confocal images taken from cultures stained for Iba-1 in green (A) and CD68 in red (B). DAPI was used as a nuclear marker (blue). Iba-1 (ionized calcium binding adapter molecule 1) is an intracellular protein that binds filamentous actin, localizing to membrane ruffles and phagocytic cups in macrophage/microglia. CD68 (ED-1) is a member of the lysosomal/endosomal-associated membrane glycoprotein family and is a scavenger receptor responsible for clearing cellular debris and recruiting and activating macrophages. The protein is primarily found in lysosomes and endosomes and to a lesser extent, the cell membrane. Cellular localization of these proteins shown in Figure 24 is consistent with
the known localization. The purity of the cultures was approximately 95%. Contaminating cells present were astrocytes (GFAP) and what are believed to be fibroblasts (data not shown).

Figure 24. Microglial immunohistochemistry

Microglia were fixed and stained for Iba-1 (A; green) and CD68 (B; red). DAPI was used as a nuclear marker (blue). Cultures were 95% pure based on cell counting. Scale bar is 100 µm.
Inflammation in LPS stimulated microglia

Microglia were stimulated with 1, 10 and 100 ng/mL of LPS (a specific TLR4 agonist), and several lines of inflammatory evidence were monitored. LPS exposure caused microglia to adopt an ameboid-like morphology (data not shown). Inflammatory gene expression (IL-1β IL-6, iNOS and TNFα) was monitored with qPCR on mRNA isolated from cells exposed to LPS for 6 hours (Figure 25A). Additionally, nitrite in the media, a measure of NO production, was monitored at 24 hours following LPS stimulation (Figure 25B). Finally, IL-6 protein production was detected in the media from cells exposed to LPS for 24 hours using ELISA (Figure 25C). For NO determination and the IL-6 ELISA, DNA content was measured with a fluorometric assay using Hoechst DNA dye and salmon testis DNA as a standard. Both the nitrite concentration and IL-6 amounts were normalized to DNA content of each well. As seen in Figure 25, inflammatory gene signaling and the production of inflammatory mediators plateaued with above the 10 ng/mL dose, with the 100 ng/mL dose eliciting less of a response. For IL-1β gene expression, the escalating doses elicited relative gene expressions of 16.5 ± 4.2, 39.0 ± 1.1 and 17.2 ± 0.7 for 1, 10 and 100 ng/mL respectively (ANOVA, p<0.0001). Similarly, the numbers for IL-6, iNOS and TNF-α were: 33.3 ± 10.6, 241.4 ± 25.5 and 101.2 ± 9.1; 72.2 ± 16.9, 602.2 ± 14.8, and 421.6 ± 33.7; and 8.5 ± 1.9, 32.8 ± 4.8 and 15.9 ± 2.1, respectively. In terms of IL-6 protein production, the levels were 55.7 ± 28.3, 305.6 ± 26.9 and 170.7 ± 56.2 for 1, 10, and 100 ng/mL, respectively (ANOVA, p<0.0001). For nitrite in the media, the numbers were 2.8 ± 0.3, 9.9 ± 1.2 and 8.3 ± 0.8 respectively (ANOVA, p<0.0001). Each dose was significantly greater than the control (media vehicle) (Bonferroni post hoc, p<0.05), except for IL-6 protein production at the 1 ng/mL dose (Bonferroni post hoc, p=0.57).
Figure 25. LPS dose response

Microglia were stimulated with 1, 10 or 100 ng/mL of LPS, and inflammatory responses were measured. (A) IL-6, IL-1β and TNFα gene expression were monitored with qPCR at 6 hours following the addition of LPS. (B) Nitrite in the media (a measure of NO production) was detected in the media from cells exposed to LPS for 24 hours using the Griess reagent assay. (C) IL-6 levels in the media at 24 hours of LPS exposure were quantified using ELISA. (n=3 per group; ANOVA, p<0.0001; Bonferroni post-hoc, p<0.05 for each dose relative to control for A, B and C) except for IL-6 protein at 1 ng/mL (Bonferroni post-hoc, p=0.57). Error bars represent SD.
HA modulates inflammatory gene expression in LPS stimulated microglia

Microglia cultures were treated with LPS at the same time as HA was added to cells. PCR, a Griess assay for NO and ELISA were employed at 6 or 24 hours following the application of HA/LPS (10 ng/mL) to measure inflammatory reactions. Figure 26A shows a representative qPCR gene expression analysis of the cytokines IL-1β, IL-6, and TNF-α at 6 hours following HA/LPS application. Treatment with HA reduced the expression on all 3 inflammatory cytokines (ANOVA; $p=0.0008$, $p=0.001$, $p=0.0001$, respectively). In terms of IL-1β, the highest dose of HA (0.5 μM) reduced gene expression from 334 ± 70 in LPS samples to 183 ± 11 (Bonferroni post hoc, $p=0.03$). In terms of IL-6, 0.5 μM HA reduced gene expression from 1079 ± 365 in LPS samples to 319 ± 9 (Bonferroni post hoc, $p=0.019$). In terms of TNF-α, HA reduced gene expression from 108 ± 15 in LPS samples to 59 ± 9 (Bonferroni post hoc, $p=0.07$). Note that methyl cellulose (MC) had no effect on inflammatory gene expression (Bonferroni post-hoc test; $p=0.7$, $p=1.0$ and $p=1.0$, respectively). Figure 26B shows representative results from an IL-6 ELISA at 24 hours following stimulation which verifies the qPCR finding in part A (ANOVA, $p<0.0001$; Bonferroni post-hoc for 0.5 μM HA versus LPS, $p<0.0001$). Additionally, Figure 26B demonstrates results from a Griess assay for NO, 24 hours following stimulation. HA significantly reduced the production of NO in LPS stimulated microglia (ANOVA, $p<0.0001$; Bonferroni post-hoc for 0.5 μM HA versus LPS, $p=0.0004$). Note that MC had no effect on any measure of inflammation (Bonferroni post-hoc test $p=1.0$ and $p=0.11$ for IL-6 and NO respectively). In part 26C, 0.5 μM HA was dosed at the same time as either 1 ng/mL or 10 ng/mL LPS and IL-6 and NO were measured in the media. IL-6 was significantly reduced by HA (two way ANOVA $p=0.0001$). There was a non-significant decrease at a dose of 1ng/ml LPS (Bonferroni post hoc, $p=0.19$) and significant decrease at 10
ng/mL (Bonferroni \( p < 0.00001 \)). For NO, the same trend was seen (Bonferroni post hoc, \( p = 0.63 \) and \( p = 0.0004 \) for 1 and 10 ng/mL respectively).

**HMW-HA is required for inflammatory modulation**

Studies that use macrophage cultures suggest that only HMW-HA is anti-inflammatory. To support this, we exposed our primary microglia to either 0.5 \( \mu M \) HMW-HA (1,700 kDa) or 0.5 \( \mu M \) LMW-HA (230 kDa) together with 10 ng/mL LPS and measured the inflammatory response. Figure 27 demonstrates the results from NO and IL-6 detection, 24 hours following LPS stimulation. Whereas HMW-HA was able to reduce NO and IL-6 production, LMW-HA was not. Part A demonstrates representative results from an IL-6 ELISA demonstrating that there was no difference in IL-6 levels between “LMW-HA+LPS” group and the LPS group (259 ± 43 versus 226 ± 26; Bonferroni post hoc, \( p = 1.0 \)). Similarly, part B shows results from a Griess assay demonstrating that “LMW-HA+LPS” NO levels were no different than LPS levels (9.4 ± 1.7 versus 8.48 ± 1.9; Bonferroni post hoc, \( p = 1.0 \)).
Figure 26. HA modulates LPS-mediated inflammation

(A) qPCR analysis from cultures 6 hours following the addition of LPS and HA is shown. Treatment with the highest dose of HA reduced the expression of all three inflammatory cytokines relative to LPS alone (ANOVA, p<0.05 with Bonferroni post hoc, IL-1β p=0.03, TNF-α p=0.07; IL-6 p=0.019). (B) Representative IL-6 ELISA results are shown using media following 24 hours of stimulation. HA significantly reduced IL-6 protein production (ANOVA, p=0.02) compared to LPS alone. A Griess assay was used to detect nitrite in culture media (a measure of NO production) 24 hours following stimulation (right panel). HA significantly reduced the production of NO in LPS stimulated microglia (ANOVA, p=0.03). (C) 0.5 µM HA was dosed at the same time as 1 ng/mL or 10 ng/mL LPS and IL-6 and NO were measured in the media. IL-6 was significantly reduced by HA (two way ANOVA p=0.0001). There was a non-significant decrease at a dose of 1 ng/ml LPS (Bonferroni post hoc, p=0.19) and significant decrease at 10 ng/mL (Bonferroni post hoc, p<0.0001). For NO, the same trend was seen (Bonferroni post hoc, p=0.63 and p=0.0004 for 1 and 10 ng/mL respectively). Error bars represent SD (n=3 per group).
Figure 27. High but not low molecular weight HA modulates LPS-stimulated microglia.

LMW-HA was unable to reduce LPS-mediated inflammation in microglia. (A) Results from a representative IL-6 ELISA (ANOVA p=0.0014) demonstrate that there was no difference in IL-6 levels between “LMW-HA+LPS” group and the LPS group (Bonferroni post hoc, p=1.0). (B) Results from a representative Griess assay demonstrate that “LMW-HA+LPS” NO levels were no different than LPS levels (Bonferroni post hoc, p=1.0). HMW-HA significantly reduced IL-6 and NO production relative to LPS alone (Bonferroni post hoc, *p< 0.05 ** p<0.01). Error bars represent SD (n=3 per group).
**HA reduces ERK 1/2 Phosphorylation**

Phosphorylation of ERK 1/2 is part of the TLR4 signal transduction pathway that leads to the activation of transcription factors and production of inflammatory cytokines. A representative Western blot demonstrating the temporal phosphorylation of ERK 1/2 is shown in Figure 28A. Densitometric quantification is shown below and is expressed as relative ERK 1/2 phosphorylation (ANOVA p=0.0083). Phospho-ERK 1/2 was detected as early as 15 minutes following addition of 10 ng/mL LPS (Bonferroni post hoc, p=0.004). The phosphorylation increased at 30 and 60 minutes following addition of LPS (p<0.0001 for both relative to control). In part B, HMW-HA was added at the same time as LPS and significantly reduced ERK 1/2 phosphorylation at 15, 30 and 60 minutes following LPS exposure (t-test, p=0.038, p=0.05 and p<0.0001, respectively). Representative Western blots are shown in addition to densitometric quantification expressed as relative density. In both parts, non-phosphorylated ERK 1/2 served as a loading control (n=3 per group).
Figure 28. HA reduces acute phosphorylation of ERK 1/2

(A) LPS stimulation results in ERK-1/2 phosphorylation (ANOVA p=0.0083). A representative Western blot demonstrating the temporal phosphorylation of ERK 1/2 is shown with densitometric quantification. Phospho-ERK 1/2 was detected as early as 15 minutes following addition of 10 ng/mL LPS (Bonferroni post hoc, p=0.004). The phosphorylation increased at 30 and 60 minutes following addition of LPS (p<0.0001 for both relative to control).

(B) HMW-HA reduces LPS-mediated ERK 1/2 phosphorylation at 15, 30 and 60 minutes following LPS exposure (t-test, p=0.038, p=0.05 and p<0.0001, respectively). Representative Western blots are shown in addition to densitometric quantification. (n=3 per group). Error bars represent SD.
Continual exposure with LPS is essential for maximal inflammatory response

Next, we set out to determine how crucial the early stages of LPS stimulation was to the overall inflammatory response. More specifically, we wanted to see what the effect of removing the initial LPS stimulus would have on overall inflammatory mediator production. We incubated microglia with 10 ng/mL LPS for 1, 3 or 6 hours and replaced the LPS media with fresh media (no LPS) for the balance of time, up to 24 hours at which point we assayed NO and IL-6 levels. LPS exposure for 24 hours served as a control for maximal mediator production. As shown in Figure 29, LPS exposure for 6 hours or less resulted in under one third of the total amount of NO or IL-6 produced compared to LPS exposure for the full 24 hours (ANOVA, p=0.003 and p=0.002, respectively).

The addition of HA following LPS exposure still reduces LPS-mediated inflammation

Since microglial activation depends on both the initial presence of agonists (LPS) and prolonged exposure, we wanted to determine if we could delay the addition of HA to block only prolonged exposure to LPS. Figure 30 shows results from experiments where HA was added 1 hour following the addition of LPS. Relative IL-1β gene expression was reduced from 147 ± 3 to 102 ± 7 (t-test, p=0.0018). IL-6 was reduced from 2997 ± 232 to 1649 ± 149 (t-test, p=0.0064). TNF-α was reduced from 101 ± 3 to 63 ± 3 (t-test, p=0.0019). In terms of NO production, post-treatment with HA reduced nitrite in the media from 14 ± 1 to 10 ± 1 (t-test, p=0.004). For IL-6, post-treatment with HA reduced its production from 368 ± 114 to 87 ± 74 (t-test, p=0.02).
Microglia were incubated with 10 ng/mL LPS for 1, 3 or 6 hours and replaced the LPS media with fresh media (no LPS) for the balance of time (24 hours) at which point NO and IL-6 levels were assayed. LPS exposure for 24 hours served as a control for maximal mediator production. (A) LPS exposure for 6 hours or less resulted in significantly less NO production compared to continual exposure for 24 hours (ANOVA, p=0.003). (B) Similarly, LPS exposure for 6 hours or less resulted in significantly less IL-6 production compared to LPS exposure for the full 24 hours (ANOVA, p=0.002). Error bars represent SD.
Figure 30. Post treatment with HA lowers LPS-mediated inflammation

HA was added 1 hour following the addition of LPS. (A) LPS-mediated IL-1β, IL-6 and TNF-α gene expression were reduced by delayed HA treatment. (B) In terms of NO production, post-treatment with HA reduced nitrite in the media. Similarly, post-treatment with HA reduced IL-6 production. Error bars represent SD, n=3 per group. (t-test, *p<0.05).
The continual presence of HA is required for effect on LPS inflammatory response

If there was any intracellular negative regulation associated with HA, removing it following a pre-incubation period should still have reduced LPS-mediated microglial activation. On the other hand, if there was no reduction, it would suggest that there might not be an internal pathway elicited and would point to an extracellular mechanism. Experimental groups consisted of a control group exposed to media for 2 hours, at which point the media was removed and replaced with media for the balance of time (Media-Media). The next group was exposed to media for 2 hours, which was then replaced with media containing 10 ng/mL LPS for the balance of time (Media-LPS). The third group was exposed to 0.5 μM HA in media for 2 hours, which was replaced with media containing 10 ng/mL LPS (HA-LPS). The final group was exposed to media for 2 hours, then replaced with media containing 0.5 μM HA and 10 ng/mL LPS (Media-HA+LPS). Upon each exchange, cells were rinsed once with media. Levels of IL-6 and NO were measured in each group at 24 hours following the initial 2 hour exposure. Results from the NO assay are shown in Figure 31A (ANOVA, p<0.0001). The Media-LPS group produced 7.8 ± 0.5 μM of nitrite/μg DNA following 24 hours LPS exposure. Pre-incubation with HA prior to LPS exposure (with removal of HA before LPS exposure; HA-LPS) did not reduce NO production (8.1 ± 0.1; Bonferroni post hoc, p=1.0). However, if HA was present with LPS following the initial 2 hours, there was a significant reduction in NO produced (3.0 ± 0.5; Bonferroni post hoc, p<0.0001). Additionally, there was a significant difference between the HA-LPS and Media-HA+LPS groups (Bonferroni post hoc, p<0.0001). Results from IL-6 production are shown in Figure 31B (ANOVA, p<0.0001). Similar to NO, there was no difference in the IL-6 levels of the Media-LPS group (339 ± 54) compared to the HA-LPS group (294 ± 17; Bonferroni post hoc, p=0.63). The presence of HA with LPS reduced the production of IL-6 to 223 ± 21 pg/μg DNA (Bonferroni post hoc, p=0.009). There was slight, non-
significant difference seen between the HA-LPS and Media-LPS+HA groups (Bonferroni post hoc, p=0.1). Error bars represent SD.

HA reduces LPS-mediated increases in CD44 gene expression but not TLR4

Next, we considered that HA might have an effect on the expression of TLR4 and CD44, receptors implicated in LPS and HA signaling. We employed qPCR to determine receptor gene expression at 6 hours post stimulation. Figure 32 demonstrates results from these experiments. Gene expression for TLR4 and CD44 after microglia were exposed to increasing concentrations of LPS (1, 10 and 100 ng/mL) is shown in Figure 32A. CD44 gene expression (ANOVA, p<0.0001) and TLR4 gene expression (ANOVA, p=0.02) were significantly increased upon LPS exposure. CD44 expression was increased by all three doses (Bonferroni post-hoc test, p<0.05). In contrast, TLR4 expression was only increased by the 10 ng/mL dose (p<0.05). Figure 33B shows that when using a dose of 10 ng/mL LPS, 0.5 µM HA was able to significantly reduce the expression of CD44 (t-test, p=0.03) but not TLR4 (t-test, p=0.59). CD44 receptor expression was reduced from $5.2 \pm 0.1$ to $4.6 \pm 0.3$ upon the addition of HA, while TLR4 receptor expression was $1.5 \pm 0.2$ and $1.4 \pm 0.2$, respectively.
Figure 31. Continual presence of HA is needed to reduce LPS-mediated response

Microglia were exposed to media for 2 hours followed by full media replacement (Media-Media); media for 2 hours, replaced with media containing 10 ng/mL LPS (Media-LPS); 0.5 μM HA in media for 2 hours, replaced with media containing 10 ng/mL LPS (HA-LPS); or media for 2 hours, replaced with 0.5 μM HA and 10 ng/mL LPS (Media-HA+LPS). (A) Pre-incubation with HA with removal prior to LPS exposure (HA-LPS) did not reduce NO production relative to Media-LPS (ANOVA p<0.0001; Bonferroni post hoc, p=1.0). When HA was present with LPS following the initial 2 hours, there was a significant reduction in NO. (B) Similarly, pre-incubation with HA with removal prior to LPS exposure (HA-LPS) did not reduce IL-6 production relative to Media-LPS (ANOVA p<0.0001; Bonferroni post hoc, p=0.63). When HA was present with LPS following the initial 2 hours, there was a significant reduction in IL-6 produced. * Bonferroni post hoc, p<0.001. n=3 per group. Error bars represent SD.
Figure 32. HA alters CD44 but not TLR4 gene expression

(A) Gene expression for CD44 and TLR4 exposed to increasing concentrations of LPS (1, 10 and 100 ng/mL) for 6 hours is shown. CD44 and TLR4 gene expression was significantly increased (ANOVA, p<0.0001 and p=0.02, respectively). (B) When microglia were stimulated with a dose of 10 ng/mL LPS, 0.5 µM HA was able to significantly reduce the expression of CD44 (t-test, p=0.03) but not TLR4 (t-test, p=0.59). Error bars represent SD (n=3 per group).
**HA does not increase A20 expression**

The increase in A20 expression has been linked to negatively regulating TLR4 signaling. We tested whether HA influenced A20 expression in primary microglia. Figure 33A demonstrates that A20 is upregulated by LPS in a dose dependent manner at 24 hours following stimulation (ANOVA, p<0.001). Average A20 density is shown, as is a representative Western blot (n=3 per group). Actin was used as a loading control. Part B demonstrates that at 24 hours, 0.5 µM HA did not cause upregulation of A20 relative to control cells exposed to media alone. Figure 34C demonstrates that 0.5 µM HA showed did not alter A20 expression elicited from a dose of 10 ng/mL LPS (t-test, p=0.07). The average A20 densities are shown in addition to a representative Western blot. The average A20 density in LPS treated microglia was 0.22 ± 0.08 compared to 0.09 ± 0.03 in LPS+HA treated microglia. (n=3 per group).

**HA reduces Akt phosphorylation**

Of all the known negative regulatory pathways that can influence TLR4 signaling, the PI3K-Akt pathway is activated early enough to influence primary – first exposure – LPS-mediated signal transduction of which ERK 1/2 phosphorylation is involved. Figure 34A demonstrates that exposure to 10 ng/mL LPS results in the phosphorylation of Akt. Representative Western blots shown in Figure 34A demonstrate that maximal phospho-Akt (ser473) was detected at 30 minutes post LPS exposure. Semi-quantitative densitometry is also shown (ANOVA, p<0.05; * Bonferroni post-hoc test for each time relative to controls, p<0.05). Additionally, HMW-HA on its own had no influence on Akt phosphorylation at 30 minutes compared to media alone for 30 minutes (Figure 34A, right panel). When HMW-HA (0.5 µM) was dosed at the same time as LPS (10 ng/mL), it reduced the LPS-mediated phosphorylation of
Akt at 30 and 60 minutes (t-test, p<0.05, for both times). Figure 34B shows representative Western blots and semi-quantitative densitometry (n=3 per group). Error bars represent SD.

Figure 33. HA reduces A20 protein expression

(A) A20 is upregulated by LPS in a dose dependent manner at 24 hours following stimulation (ANOVA, p<0.001). (n=3 per group). Actin was used as a loading control. (B) At 24 hours, 0.5 µM HA did not cause upregulation of A20 relative to control cells exposed to media alone. (C) 0.5 µM HA showed a trend in reducing A20 expression elicited from a dose of 10 ng/mL LPS (t-test, p=0.07; n=3 per group). Error bars represent SD.
Figure 34. HA reduces LPS induced phosphorylation of Akt

(A) Exposure to 10 ng/mL LPS results in the phosphorylation of Akt. Maximal Akt phosphorylation at serine 473 was detected at 30 minutes post LPS exposure (ANOVA, p<0.05; * Bonferroni post-hoc test, p<0.05 compared to control). At 30 minutes, 0.5 μM HMW-HA on its own had no influence on Akt phosphorylation minutes compared to media alone (right panel).

(B) HMW-HA (0.5 μM) reduces the LPS-mediated phosphorylation of Akt at 30 and 60 minutes
(t-test, p<0.05, for both times). Non-phosphorylated Akt is also shown in both parts. n=3 per group. Error bars represent SD.

5.8 Discussion

To our knowledge, this is the first study to look at HMW-HA modulating TLR4 activation in microglia. HMW-HA significantly decreased LPS-mediated inflammatory gene expression and IL-6 and NO production. The decrease in inflammation was associated with a reduction in ERK 1/2 phosphorylation seen as early as 15 minutes post stimulation and was not seen with HA of a lower molecular weight. The continual presence of HA was necessary for a therapeutic effect and HMW-HA reduced the LPS-mediated activation of Akt – a negative regulator of TLR4 activation. Together, our results show that HMW-HA can reduce LPS-mediated inflammatory signaling in microglia. The fact that the continual presence of HA was necessary and that HA did not stimulate negative regulatory pathways suggests that HA might be blocking LPS extracellularly. HMW-HA represents an anti-inflammatory molecule that can be used to modulate microglial TLR4 inflammatory signaling following neurotrauma such as SCI.

Microglial activation with LPS

The dose response achieved in this study is consistent with the literature [457]. The plateau/decrease in inflammatory mediators with the 100 ng/mL LPS dose did not alter DNA content relative to the lower doses, suggesting cells were still alive at 24 hours (data not shown). However, the decrease could have been a product of decreased cell viability or by increased negative regulation of TLR4 as described previously [96-98, 458].

TLR4 gene expression was upregulated by the 10 ng/mL dose of LPS compared to control cells (Figure 32). This finding is not consistent with the literature, as studies suggest that TLR4 expression in macrophages is not changed upon LPS exposure [459]. In some cases TLR4
is even downregulated upon LPS activation [460]. It is uncertain if the LPS stimulus upregulated the expression of other co-receptors or signaling molecules; however, it has been suggested that LPS does upregulate proteins such as CD14 and MyD88 in macrophages [460, 461]. Figure 32 demonstrated that HA was not able to reduce TLR4 gene expression but did significantly reduce CD44 gene expression. It is unknown if HMW-HA was able to influence expression of other molecules involved in TLR4 signaling or the protein expression of TLR4 and CD44.

Anti-inflammatory mechanism of HMW-HA in LPS stimulated microglia

Our data are supported by studies in macrophage cultures [113, 114, 430, 431]. In the present study, we did not test the involvement of various receptors in HMW-HA mediated reduction of LPS activation. Based on the literature, CD44 [113, 430], TLR4 [455] and ICAM-1 [431] might all play a role in anti-inflammatory HA/TLR4 interactions. Furthermore, due to the fact that there are several putative receptors, it is possible that there are several mechanisms at play. Combined with signaling complex overlap between anti-inflammatory HA (CD44-TLR4-MD2 [115]) and inflammatory TLR4 agonists (TLR4-MD2-CD14 [454]), involvement of a particular receptor mediated mechanism is a complex interaction to elucidate.

There are several lines of evidence that lead us to suspect HA is altering extracellular receptor activation as previously described for several receptor types [455, 456]. The first is that fact that HA was found to reduce the acute (15 minutes post exposure) LPS-mediated phosphorylation of ERK 1/2 (Figure 28). In order to reduce such an acute event, HMW-HA would have to either block LPS extracellularly or activate an alternate signal cascade that would negatively regulate the inflammatory signal of ERK 1/2 activation. Additionally, as Figure 34 demonstrates, HMW-HA was found to significantly reduce LPS-mediated Akt phosphorylation. This finding is supported in the literature using macrophage cultures [452], however, our interpretation of this event differs. The previously mentioned study suggested that the PI3K/Akt
pathway was part of a signaling pathway that activated NF-κB and inflammatory mediator production. However, evidence from the literature suggests that a PI3K-Akt negative regulatory pathway is activated upon the primary exposure to LPS [100, 462]. As such, we felt that Akt-phosphorylation was an event involved in negative regulation and thus we wanted to see if HA could possibly potentiate this. It is also worth mentioning that Akt was not found to be phosphorylated until 30 minutes following the addition to LPS (also supported by the literature [100]), which is 15 minutes after we saw HMW-HA reduce ERK 1/2 phosphorylation. To our knowledge, there is no other negative regulatory pathway that is capable of intersecting inflammatory TLR4 signaling at such an early time point.

Though A20 is not expected to reduce acute ERK 1/2 phosphorylation, we looked at its expression to see if another HA mediated mechanism could be influencing TLR4 inflammation due to prolonged LPS exposure. A previous study pointed to A20 playing a role in HA mediated TLR4 regulation and demonstrated an increase in A20 gene expression after several hours of exposure to HA [113]. Our results showed that HMW-HA did not increase A20 expression at 24 hours, thus it is unlikely that A20 is playing a role in the inflammatory reductions seen. In fact, we demonstrated that there was a slight decrease in A20 expression when HA was present with LPS, though this was not found to be significant. A power analysis yielded a 30% chance of detecting significance at p<0.05 (G*Power). Increasing the n to n=6 would have increased the chance to of finding significance to 91%.

Our final observation that led us to suspect an extracellular mechanism was the fact that the continual presence of HA was necessary for a reduction in LPS inflammation (Figure 31). When HA was removed and cells were rinsed, there was no reduction in subsequent LPS inflammation as was seen when HA and LPS were together in the media. If HMW-HA was
activating a negative regulatory pathway, its removal would have no effect and there would still have been a reduction in LPS-mediated inflammation in its absence.

When we suggest that the external presence of HWM-HA is responsible for its anti-inflammatory action, there are two possible ways for HA to do so; competitive inhibition and/or receptor masking. Since HA and LPS can bind the same receptor, competitive inhibition is possible. However, LPS and HA bind different receptor complexes, complicating the kinetics and interactions. Studies suggest that LPS has a greater affinity for its receptor complex compared to HA [454, 463], however it is likely HMW-HA was present at 10 to 100 fold greater concentrations. Without further experiments, it would be hard to speculate on such competitive kinetics, particularly because LPS is quite heterogeneous in terms of molecular weight. It is worth noting that a solution of LMW-HA of the same molarity as HMW-HA was unable to reduce LPS-mediated inflammation (Figure 27). If a competitive inhibition mechanism was responsible, we would have expected to see a similar decrease with the LMW as the HMW-HA. Furthermore, our findings suggest that HA is not binding LPS directly and preventing interaction with TLR4. If this were the case, we would expect both HMW-HA and LMW-HA to bind LPS and reduce inflammation in a similar fashion, as their chemical structure is identical. Additionally, when HMW-HA was added in a delayed fashion (1 hour after the addition of LPS) it still elicited a significant anti-inflammatory effect, suggesting that HMW-HA was likely not adsorbing LPS or preventing its diffusion in solution (Figure 30). Overall, we suggest that both a competitive inhibitory mechanism and the possibility of HA adsorbing LPS are unlikely.

Receptor masking has been previously described in the literature. One study suggested that CD44, an ErbB2 interacting protein, acted as an anchor for an “obstructive meshwork” of HA, which was able to block or mask antibody binding to ErbB2 [456]. Another study suggested that HMW-HA binds to TLR4 and masks/blocks other agonists from receptor
engagement [455]. Evidence suggests that HA solutions experience intramolecular associations such as loops, hairpin like structures and even coiled structures [464]. Furthermore, HMW-HA has been demonstrated to experience interchain associations/interactions [465, 466]. We suggest such a meshwork of HA could bind extracellular receptors, such as CD44, that are in close proximity to TLR4 (as part of a signaling complex) or even TLR4 itself, thus hindering TLR4 agonists from binding. Although evidence suggests that LMW-HA intermolecular associations are not as stable [467], based on the literature, the LMW-HA we used in this study could have still formed aggregates [468] and could have been inhibitory. We used the same molar concentration of LMW-HA and HMW-HA (0.5 µM), however, the corresponding concentration of LMW-HA by weight was lower that that of the HMW-HA (0.12 mg/mL and 0.85 mg/mL, respectively). This could explain why LMW-HA did not produce the same anti-inflammatory effects as seen in Figure 22 and 25. However, studies using the same concentrations of HMW and LMW-HA by weight and which used similar or higher molecular weights of LMW-HA than used in this study demonstrated that LMW-HA did not have an anti-inflammatory effect [431, 455].

It should be noted that the terms LMW and HMW were used to differentiate between the two sizes of HA used. Based on the literature, it is apparent that the cutoff that classifies HA as LMW or HMW is somewhat arbitrary. While there are groups that have classified sizes of HA in the 200 kDa range (200-280 kDa range) as LMW [469, 470] or HMW as 950 kDa [114] we acknowledge there is ambiguity when using such notation.

In summary, HMW-HA reduces acute ERK 1/2 activation, does not elicit Akt or A20 negative regulatory pathways and its continual presence was required to reduce LPS activation in microglia. Together, HA seems to be blocking both the primary inflammatory signal of LPS and associated negative regulatory pathways – or in effect, reducing all LPS induced signaling. These
results are suggestive of an extracellular inhibitory mechanism. While our study does not definitively support a mechanism of receptor masking, future studies will hopefully uncover the validity of our inferences.

*Application to neurotrauma*

Our *in vitro* studies have relevance to neurotrauma associated with inflammation through activation of TLR4, such as SCI. At a high enough concentration and if able to persist long enough, HMW-HA is expected to block TLR4 activation and reduce cytotoxic effects of inflammatory mediators such as cytokines and NO. Though we used LPS as a TLR4 agonist, endogenous activators of TLR4 expressed following neurotrauma/neuroinflammation use similar receptor complexes as LPS. For example, fibrillar A-beta and gangliosides use TLR4 and CD14 in activating microglia [447, 448].

5.8.1 Conclusions

HA was found to reduce the extent of TLR4 activation by LPS possibly through an extracellular mechanism. To our knowledge, this is the first study to look at HA/TLR4 interactions in microglia. Considering that microglial activation through TLR4 is a key aspect of neuroinflammation, the use of HA as an immune modulatory compound *in vivo* warrants consideration. Additionally, these results could partly explain the therapeutic benefits of HAMC seen in Chapter 4.
Chapter 6

6 General Discussion and Future Directions

We have demonstrated that when arachnoiditis was synthetically induced with kaolin following SCI, it significantly increased inflammation and increased parenchymal scarring. Our attempt to mitigate the effects of induced arachnoiditis using a bioengineered hydrogel containing HA modestly reduced cytokine production and parenchymal fibrous scarring. This was associated with a greater neurobehavioural improvement, a slight decrease in lesion size and greater axonal functionality. In microglial cultures, HA was able to reduce the inflammatory reactions in primary microglial cultures exposed to LPS, a TLR4 agonist, possibly through an extracellular mechanism that perturbed LPS binding its receptor complex. Together, this suggests that: (i) managing clinical arachnoiditis therapeutically could possibly promote histological and functional improvement in patients following SCI, (ii) a HA based biomaterial might have the potential to achieve this aim and (iii) HA could have the potential to modulate inflammatory responses in microglia following neurotrauma.

6.1 SCI associated with arachnoiditis

Arachnoiditis can have a substantial impact on parenchymal pathophysiology in the spinal cord. Using the results from Chapter 3 in this study in combination with the literature, we have put together a schematic for the development of post-traumatic syringomyelia (Figure 35). During SCI, damaged vertebrae become displaced and impose traumatic forces on the spinal cord. Inflammatory events are elicited due to physical damage to the parenchyma. In some cases of SCI, meningeal inflammation results from necrotic cell death and hemorrhaging in the meninges (Figure 35A). Chemical (eg. cytokine and chemokine) signals are carried with ECF/CSF from the area of meningeal inflammation to the parenchymal lesion and vice versa.
Activated meningeal fibroblasts invade the parenchyma as neutrophils and microglia/macrophages respond to inflammatory cues in the meninges (Figure 35B). Over time, inflammation contributes to SS in addition to glial and fibrous scarring in the parenchyma. The arachnoiditis either subsides or is not significant enough to alter CSF flow and pressure dynamics. If severe enough, SS and adhesions block CSF fluid flow in the SAS, causing increased fluid flow into the parenchyma, forming a syrinx. Additionally, there is potentially a larger primary lesion formed and more fibrous and glial scarring due to the acute arachnoiditis.

6.1.1 Future studies

The contribution of fibroblasts to SCI and SCI associated with arachnoiditis would be a potential future area of interest as little is known. Following injury, meningeal fibroblasts are activated and can enter the spinal cord parenchyma – contributing to the inflammatory response, although the exact details remain unclear. They can produce inflammatory cytokines and also produce collagen IV, which can act as a sticky ECM substrate that binds CSPGs – contributing to impaired regeneration [210]. SCI causes increases in cytokines and chemokines in the CSF [416] which activate and recruit meningeal fibroblasts to the site of injury. Meningeal damage or hemorrhaging introduces additional inflammatory reactions that are expected to increase the activation of fibroblasts. As such, we expect that arachnoiditis would increase meningeal activation and proliferation of fibroblasts as well as increase the number of fibroblasts that migrated to the parenchymal injury site. We attempted to assess fibroblasts following injury by looking at fibroblast activation protein expression using qPCR, WB and IHC analyses. Unfortunately, using several commercially available reagents, we were unable to generate any convincing labeling of fibroblasts. Additionally, we assessed the expression of prolyl hydroxylase (PH), an enzyme involved in the collagen biosynthesis pathway. We did find an increase in PH expression in PTS animals compared to SCI animals (data not shown), however
the source of PH is not limited to fibroblasts, as astrocytes are also expected to contribute to its expression levels.

To solve the problems with properly identifying meningeal fibroblasts, other markers could be used. For example, class 3 semaphorins are exclusively expressed by meningeal fibroblasts [471]. Additionally, meningeal fibroblasts express EphB2 [472]. If problems exist with the antibodies, creating a genetic mouse strain that expresses a fluorescent protein under control of the promoters for either of these molecules would be a next step – albeit quite a large undertaking.
A

Vertebral damage on cord:
- acute compression
- impact
- missile
- distraction
- laceration
- shear

Possible necrosis and hemorrhaging in meninges due to primary trauma

Axonal damage, hemorrhaging, necrosis in parenchyma due to primary trauma

B

Microglia and neutrophils migrate to inflammatory signals in meninges

Meningeal fibroblasts migrate into parenchyma in response to the tissue damage

Cytokines and chemokines diffuse with ECF/CSF flow between parenchyma and subarachnoid space

Persistent arachnoiditis
- SAS
- more severe parenchymal injury

Insignificant arachnoiditis or resolved
- no SAS
- no parenchymal effects

PTS - Chronic

Parenchymal fibrous and glial scarring

SCI - Chronic

SAS

Perivascular space

Inflow of CSF

Syrinx (separate or a within initial lesion)
Figure 35. Schematic of the contribution of arachnoiditis to SCI

Presented is a schematic of PTS development based on results from this study and the literature [409]. (A) During SCI, damaged vertebrae become displaced and impose traumatic forces on the spinal cord. Inflammatory events are elicited due to physical damage to the parenchyma. In some cases of SCI, meningeal inflammation results from necrotic cell death and hemorrhaging in the meninges. (B) Chemical (eg. cytokine and chemokine) signals are carried with ECF/CSF from the area of meningeal inflammation to the parenchymal lesion and vice versa. Activated meningeal fibroblasts invade the parenchyma as neutrophils and microglia/macrophages respond to inflammatory cues in the meninges. Arachnoiditis can be resolved or is not significant enough to alter CSF flow and pressure dynamics – in which case, patients will not develop PTS. If severe enough, SS and adhesions block CSF fluid flow in the SAS, causing increased fluid flow into the parenchyma, forming a syrinx. Additionally, if arachnoiditis is present early, it can cause increased parenchymal inflammation and fibrous scarring (as has been demonstrated by this study).
6.2 The use of HAMC to treat SCI associated with arachnoiditis

We demonstrated that acute administration of HAMC was beneficial following SCI associated with arachnoiditis. The therapeutic effect of HAMC is most likely due to preventing inflammation and scarring in the SAS, which is likely temporally linked to the time of injury and application of the polymer. Based on the model characterization in Chapter 3, which outlined the significance of meningeal inflammation to the parenchymal pathophysiology, it is likely that the meningeal effects of HAMC lessened the impact on the parenchyma. Figure 36 provides a schematic of the proposed effects of HAMC on arachnoiditis and how this leads to improving parenchymal outcomes.

The average CSF in a 300g rat is approximately 580 µL [473]. The amount of HA in 10 µL of HAMC is 0.2 mg, making the maximal concentration of HA in the CSF of animals 0.345 mg/mL or 0.2 µM. This concentration is within the range of HA used in our *in vitro* studies in Chapter 5 (effective range between 0.05- 0.5 µM). It has been suggested that there is a large exchange in CSF and ECF of the parenchyma, with approximately 30% of CSF coming from ECF (see [418] for an excellent review). This suggests that molecules in the CSF are exchanged with the parenchyma. Such a large molecule like HA is able to enter the spinal cord through exchange of CSF/ECF due to the absence of astrocytic end feet and glia limitans at the injury epicenter. At the injury epicenter, there is a large reduction in the GFAP (astrocyte) immunoreactivity (Figure 22). Additionally, we observed blood vessels in the injury epicenter at 1- week post injury without GFAP positive astrocytes surrounding them (data not shown).
Early SCI associated with arachnoiditis can form scarring in the SAS. Intrathecal HAMC treatment can lessen arachnoiditis and subarachnoid scarring. This can reduce the blockage of CSF and the disruption of fluid dynamics in the SAS which has been implicated in the development of syringes [17]. Further, HAMC treatment could lessen the severity of the parenchymal injury – possibly reducing the susceptibility of the parenchyma to syrinx development [284].

Figure 36. Mechanism of HAMC on SCI associated with arachnoiditis
Together, this suggests that blood vessels from the meninges are entering the spinal cord with no protective glia limitans to prevent molecules in the PVS from entering the cord.

Though syringomyelia can take years to develop, there are clinical cases where PTS develops months following injury [276, 407, 408, 474]. Though little is known regarding meningeal inflammatory reactions following SCI, it is likely that they are due to the initial trauma following SCI and cause SS associated with PTS. It is uncertain if a neurosurgeon would opt to open the dura acutely to administer HAMC, unless it was already breached by the injury – especially in a patient that has a small chance of developing arachnoid adhesions/scarring and PTS. However, the literature does suggest that certain patient populations have a greater risk of developing PTS, including those with complete injuries [276]. With advances in medical imaging, patient follow up and the understanding of PTS etiology, it is expected that a more detailed description of predisposing factors will better identify patients at risk of developing PTS. It should be noted that kaolin was injected rostral to the injury epicenter to allow for the spread of scarring and inflammation to develop at and below the injury site. Our logic was that a therapeutic intervention would be undable to reduce the scarring over the injury site if the kaolin had occupied that space.

Where our HAMC study benefits the scientific community most significantly is providing a proof of principle study demonstrating the effects of a targeted meningeal anti-inflammatory treatment – to our knowledge this is the first of its kind. This is of particular significance to a range of conditions affecting the spinal cord including chronic PTS patients, patients with subdural hemorrhaging, patients with spinal or meningeal tumors and patients receiving direct stem cell transplantation to the spinal cord. HAMC could be delivered at the conclusion of the surgical procedure. Each of these procedures is invasive and can cause
inflammation in the meninges, a consequence that could be minimized with the use of HAMC. Animal studies would also have to be used to confirm any efficacy in these situations.

6.2.1 Limitations and further interpretation of experimental results

*Western blotting for neuronal/axonal proteins*

NF200 is an axonal protein that exists in phosphorylated and dephosphorylated forms. It has been shown that SCI causes degradation of NF200 following injury [319]. The fate of NF200 following nerve injury – particularly the phosphorylation status – is complicated as NF200 accumulates in dystrophic neuronal end bulbs and plays a role in axonal regeneration [475, 476]. This could explain why Western blots at 7 days demonstrated that injured animals contained more NF200 than noninjured control animals (data not shown). We therefore assessed total NF200 (phospho and dephospho) only at 2 days following injury as a semi-quantitative measure of axonal number. To support this result, we also immunoblotted for beta III tubulin, but found only small non-significant increases in this neuronal specific protein at both 2 and 7 days. Additionally, MAP-2 was not significantly altered by HAMC treatment over aCSF controls (Figure 23A). It should be noted that MAP-2 expression at 7 days was almost undetectable by Western blot analysis. As such, data suggesting there is neuronal/axonal preservation following HAMC treatment are not robust. In addition to neuronal markers, it was demonstrated that HAMC did not preserve oligodendrocytes as determined through CNPase western blotting at 2 and 7 days post injury (data not shown).

*Neuropathic pain data*

Survival of axons in ascending pathways is necessary to transmit pain information to the brain [477]. As mentioned earlier, SCEP recordings showed that HAMC treated animals had better axonal conductance in ascending pathways (Figure 15), suggesting these animals could
have had increased survival of axons in these pathways and a greater chance of transmitting
neuropathic pain information. This would indicate that the decrease in neuropathic pain in
HAMC treated animals was most likely not due to loss of ascending spinothalamic pathways.
Additionally, neurons in the gray matter that receive information from the surrounding
dermatomes are required for relaying neuropathic pain information. Cell counts for the neuronal
cell body marker NeuN revealed there was no difference in dorsal horn neurons between aCSF
and HAMC treated animals at 1000 and 2000 µm caudal from the injury epicenter (data not
shown). Together, this suggests the reduction in neuropathic pain attributed to HAMC treatment
was not likely due to any treatment differences in the pain infrastructure but rather likely due to
effects at the neuronal-microglial level involving putative neuropathic pain molecules.

Microglial activation has been identified as a major player in neuropathic pain following
SCI [256, 257]. HAMC’s ability to reduce the microglia/macrophage caudal spread in the SAS
(Figure 13) could have reduced the sensitivity of dorsal horn sensory neurons and their
subsequent neuropathic pain response. Further, previous reports using a clip compression injury
in rats suggest that a more severe injury results in increased at-level mechanical allodynia [401].
This suggests that a therapeutic intervention that reduces injury severity, such as HAMC, could
be expected to reduce the incidence of neuropathic pain. It is unknown whether HAMC altered
axons involved in negative modulation of pain pathways via descending pathways.

Cellular inflammation

Currently, there are no immunohistochemical methods to distinguish microglia from
systemic monocytes/macrophages. However, we can use the published temporal profile of
macrophage influx to the injured spinal cord as a way to distinguish early microglial proliferation
from the sub-acute combination of microglial/macrophage. By looking at Iba-1 expression 2
days following injury as in Figure 18, we are more than likely looking at a predominantly
microglial response as it has been suggested that macrophage don’t enter the spinal cord until 3 days post injury [66]. Iba-1 expression measured at 7 days post injury in Figure 18 and 19 therefore represents a combined microglial and macrophage response. In either case, HAMC did not reduce the overall proliferation or influx of microglia/macrophage following injury (qPCR and Western blotting). In contrast, our fluorescence intensity analysis of parenchymal Iba-1 revealed some spatial differences (Figure 19). This could suggest that there are some spatial differences closer to the injury epicenter in the cellular accumulation of microglia/macrophages that qPCR and western blot was unable to detect. It is worth noting that the use of protein as an indirect measure of a cell population/cell numbers is subject to some error. For example cells can increase expression of a protein in response to a stimulus while the proliferation remains static.

If taken alone, our immunohistochemical data fit with earlier publications from Khaing and Wakao [425, 426]. However, it is hard to ignore the more solid qPCR and western blotting data. While we used Iba-1 to look at the presence of macrophage microglia, we also looked at gene expression of both OX42 (CD11b) and ED-1 (CD68) and did not find a significant difference between HAMC and aCSF controls (data not shown). This is of particular importance as Wakao and colleagues demonstrated that there was a significant difference in ED-1 immunoreactivity at 1 week following injury but not Iba-1 [425]. In contrast to our study, Khaing et al. used ED-1 cell counts to determine the efficacy of HA in their study [426].

The timing of maximal neutrophil influx following SCI is approximately 24 hours [71]. Since our treatment was applied at 24 hours, we examined the notion that HAMC might curb this influx and possibly rid the spinal cord of neutrophils in a more timely fashion. As demonstrated in Figure 20, HAMC did not reduce MPO activity compared to aCSF treated animals, indicating that the presence of neutrophils were unaffected. MPO is almost solely produced by neutrophils
and therefore provides an indirect measure of their numbers [72]. Although no supporting cell counts or molecular evidence was provided, the extent of MPO roughly corresponded with the presence of neutrophils in the spinal cord, as demonstrated qualitatively in Figure 10 from the previous chapter and from previous unpublished cell counting studies from our lab.

Reduction of CSPG deposition by HAMC

We demonstrated that HAMC was able to significantly reduce CSPG deposition following injury (Figure 22). It is possible that HAMC reduces the parenchymal production of collagen IV which acts as a meshwork to trap CSPGs as part of the fibrous scar [210], however we did not analyze levels of collagen IV post injury. It is also possible that HAMC reduced the presence of CSPGs following injury as a consequence of or in combination with the modest reduction in cytokine production by influencing cellular production. When HAMC biodegrades, it was shown that HA was able to enter the spinal cord. This put it in direct contact with inflammatory and resident cells that are responsible for production of CSPGs. As such, HA could have interacted directly with parenchymal cells to reduce CSPGs or reduced meningeal cytokines that would have promoted the production of parenchymal CSPGs.

HAMC and the clinical presentation of PTS

While we did not quantify the incidence of fluid filled syringes that accompanied the lesion, upon qualitative analysis we did not detect a decrease in syrinx occurrence between the HAMC and control groups. This finding makes sense, considering that the presence of kaolin remains chronically in both groups and thus can influence the CSF flow and formation of syringes regardless of treatment. In other words, we were unable to determine if HAMC reduced the incidence of PTS (presence of a syrinx) based on the limitations of our model. What we were able to determine that is of relevance to the PTS community was that the effects of HAMC
possibly help disease progression in two ways; (i) reducing inflammation and scarring in the SAS – reducing blockage of CSF and disruption of fluid dynamics in the SAS which has been implicated in the development of syringes and (ii) lessening the severity of the parenchymal injury – possibly reducing the susceptibility of the parenchyma to syrinx development. As an aside, we did notice that in some cases, the syringes were located within the lesion and in other circumstances they were completely separate entities.

Another limitation of our study is that our model speeds up a meningeal inflammatory process that most likely takes longer to develop in humans. While the kinetics of post-injury arachnoid scarring and adhesion development is not fully understood, clearly the injection of kaolin represents an accelerated inflammatory event.

### 6.2.2 Future studies

Examining the timing of treatment with HAMC represents a subject for future experiments. Specifically, studies could look at extending the treatment time to 3 days and 1 week following injury. Furthermore, the treatment of chronic PTS could represent the focus of future experiments. Animals could be anesthetized 6 weeks following injury and the scarring could be released and kaolin excised with microscissors. The dura would then be sealed with a synthetic patch secured with fibrin glue. Prior to sealing the dura, 10 µL of HAMC or aCSF control would be injected. Each animal would have the lesion volume quantified and presence of syringes confirmed by MR imaging at week 6, prior to surgery. Animals will then be randomized into 3 treatment groups; (i) surgical decompression plus aCSF control, (ii) surgical decompression plus HAMC injection and (iii) no treatment. Efficacy of treatment could be monitored with neurobehavioural, lesion/syrinx size and electrophysiological assessments for 6 weeks following surgical/HAMC intervention. Animals would then be used for immunoblotting/qPCR and immunohistochemistry following the 6-week assessment.
Future research for HAMC would be to determine the effect of HAMC on angiogenesis and endogenous regeneration. HA fragments have been described as being pro-angiogenic [432]. It is likely that HA is somewhat degraded once HAMC is injected following SCI and is therefore capable of generating pro-angiogenic byproducts. Experiments could look at counting RECA-1 positive blood vessels following injury. Systemic injection of a fluorescent lectin (eg. FITC-LEA) and double labeling with RECA-1 could determine if the vessels are functional, while staining for Ki67 and RECA-1 could indentify proliferating, or angiogenic, endothelial cells.

In terms of endogenous regeneration, experiments could analyze regenerating sensory axons through the injection of tracer dyes. For example, Texas Red–Dextran conjugates could be injected into the freshly crushed sciatic nerve as has been reported previously [478]. After waiting several days, Texas red fluorescence could be detected in the spinal cord in and around the lesion site. Counting neurons that were able to regenerate into or through the lesion could provide evidence of a less inhibitory environment (less CSPGs).

Another avenue of research would be to investigate HAMC as an anti-inflammatory compound to help mitigate the effects of stem cell injections. Invariably, when stem cells are injected directly into the spinal cord, the invasive procedure causes damage that impairs functional recovery associated with the cell therapy for several weeks post injection [205]. HAMC could be applied immediately following injection to help dampen the inflammation caused in the parenchyma and meninges in addition to helping reduce leakage of CSF through the hole created in the dura. Histological and behavioural studies could determine the effect of HAMC in this situation.
6.3 Mechanism of HA mediated reduction in TLR4 signaling

We employed HMW-HA to reduce the effect of TLR4 mediated activation of microglia. Based on the literature, evidence suggests that HA can either block access to TLR4 (receptor masking) or induce either A20 expression or Akt phosphorylation – negative regulators of TLR4 signaling. HMW-HA reduced acute LPS signal transduction and LPS induced inflammatory mediator production. The continual presence of HA was a requisite for reduction of LPS-mediated activation and HA did not alter A20 expression or increase Akt phosphorylation. Based on these findings, we postulated that HA participates in an extracellular mechanism. The literature suggests that a HA mediated receptor masking mechanism is in play for ErbB2 [456] (using CD44 as a co-receptor) just as CD44 uses TLR4 as a co-receptor [115]. This could also be the case in our study, however without further investigation, this is only speculative. Figure 37 provides a mechanistic visualization of our findings. We feel that given the current knowledge, this is the most plausible explanation; however, there could be an unknown pathway that HA-CD44 interaction activated to negatively regulate TLR4 activation. While our study definitively demonstrated that HMW-HA reduced LPS induced activation of microglia, and that an extracellular pathway was likely – the suggestion of a specific receptor masking mechanism was not tested or validated.

6.3.1 Limitations and further interpretation of experimental results

A note about microglial activation with LPS

Maximal activation of microglia required the continual presence of LPS. Figure 29 demonstrates that if the initial 10 ng/ml dose of LPS is removed at 1, 3 or 6 hours after stimulation, inflammatory mediators detected at 24 hours were significantly reduced. According to the literature, maximal cytokine gene expression is achieved around 2 to 5 hours following
LPS exposure [479], thus the removing LPS prior to this might have prevented reaching the maximum expression - explaining the decrease in IL-6 and NO levels at 24 hours. Another explanation could be that in addition to removing the LPS, we also removed cytokines or other mediators (already produced due to LPS exposure) capable of potentiating the initial LPS stimulus via increased mediator or receptor expression. Indeed, cytokines can promote production of more cytokines and can increase expression of various pro-inflammatory receptors [480, 481]. Studies suggest that microglia produce cytokines as early as 3 hours after LPS exposure with maximal production occurring at 24 hours [457]. This same study showed that cytokines produced in the first 6 hours account for less than 50% of the maximal amount. Together, this would suggest that we could have removed some of the initial cytokines produced when removing the media/LPS in the early stages (under 6 hours). Alternatively, the decrease could be that microglia are able to detect a decrease in external stimulus and adjust their response accordingly. There are three main molecules elicited by TLR4 to ensure the inflammatory response in macrophages is measured over repeated/prolonged exposure; IRAK-M, Tollip and SOCS-1. IRAK-M prevents dissociation of IRAK and IRAK-4 from MyD88 and formation of IRAK-TRAF6 complexes [96]. Tollip, a substrate of IRAK, suppresses IRAK activity [98]. SOCS-1 is thought to regulate the JAK/STAT signal pathway [97].

**Difficulties with reagents**

We experienced problems with the specificity of our reagents employed to aid in mechanistic insights. Mainly, we used a hyaluronic acid binding protein (HABP) and anti-CD44 antibody reagents that produced unexplainable results. We expected that if we pre-incubated HA with HABP, it would occupy epitopes required for HA binding cell surface receptors, thus nullifying the effect of HA and telling us whether a HA binding event was necessary. However, the HABP on its own blocked LPS induced microglial activation thus we were not able to
determine if HA-receptor binding was a necessity. In regards to the anti-CD44 antibody, both the control antibody and the anti-CD44 antibody blocked LPS induced inflammation in microglia. It is possible that the control IgG antibodies came from animals that experienced a previous bacterial infection – and thus LPS specific antibodies were generated as part of their host defense system and present in the pooled IgG product. If the anti-CD44 receptor antibody was specific, it would have blocked HA from binding CD44 and given us information regarding the role of CD44 receptor in this mechanism. For example, if the inflammatory action of HA was reduced, we could have concluded at the very least that CD44 binding has something to do with the observed effect of HA. Together, the use of an anti-CD44 antibody and HABP would not rule out one mechanism over the other but would have pointed us down the road to future experiments.
TLR4 Activation of Microglia

Inflammatory Mediator Production
- NO
- cytokines
- chemokines

High molecular weight HA present

### Negative TLR4 regulation
- Reduced IL-6
- Reduced NO
- no A20 upregulation
- no increase in Akt phosphorylation

### Receptor Masking
- Reduced A20
- Reduced Akt-P
- early acute blockade of signal transduction
- continual presence of HA necessary
- reduced LPS mediated negative regulation (A20, Akt-P)
- no decrease in TLR4 expression

Overall: - most likely extracellular mediated mechanism
LPS binds a TLR4 receptor complex that involves TLR4/MD2/CD14. MyD88 or TRIF can transmit the signal to the nucleus where inflammatory genes are upregulated via NFκB and/or AP-1. Based on the literature, HA can negatively regulate TLR4 through upregulation of A20 or phosphorylation of Akt. However, in our studies we did not find that HA increased A20 expression or increased phosphorylation of Akt in microglia. Based on the fact that the continual presence of HA was necessary for a therapeutic effect and that HA reduced early ERK 1/2 phosphorylation, we concluded that HA was blocking the LPS signal extracellularly. When we consulted the literature, we discovered a mechanism where HA was able to mask ErbB2 activation by binding CD44 (part of the receptor complex with ErbB2) [456]. The fact that HA binds CD44 in a complex with TLR4 suggests that HA could be masking TLR4 (as HA masked CD44 in the previously mentioned study) from potential agonists such as LPS.
6.3.2 Future studies

Although we were unable to find a rat specific anti-CD44 antibody that was capable of blocking the binding of HA it might be worth while conducting studies in microglia isolated from mice, as such commercially antibodies are abundant. Further, if studies were moved to mice, the power of genetic manipulation would be gained. Such knockout candidates would include CD44. While the CD44 antibody blocking experiment might have yielded interesting results, the literature already points to the importance of CD44 with HA and TLR4 activation. Specifically, Yasuda used a 2700 kDa HA (highest dose 1 mg/mL) which was pre-incubated for 1 hr prior to LPS insult in macrophages – showing that HA reduced LPS-induced inflammation [430]. When an anti-CD44 antibody was used, it negated some of the HA effects on stimulation, suggesting CD44 is required for HA to influence TLR4 signaling. Similarly, Muto et al. demonstrated the necessity of CD44 by showing that a 500 kDa HA (25 µg/mL) had no effect on LPS-mediated stimulation in macrophages isolated from CD44 knockout mice [113].

In order to strengthen our findings and go deeper into the mechanism, we would propose the following studies. The first would be utilizing a siRNA for A20. Though we showed that there was not an increase in A20 expression, we are unaware of whether basal A20 activity was influenced by HA. As mentioned earlier, A20 removes ubiquitin molecules from TRAF6 and it is possible that the expression of A20 could remain at basal levels while its activity is increased. By reducing basal A20 we could determine more convincingly if A20 is required for HA mediated TLR4 modulation. Additional studies could look at longer pre-incubations with HA. In the experiment where we pre-incubated microglia with HA for 2 hours then rinsed it away (Figure 32), there is a possibility that cells needed longer than 2 hours of exposure to HA to sufficiently upregulate A20. Other studies could look at the using siRNA for Akt, which is part
of the PI3K pathway, to solidify that Akt is indeed a prominent negative regulator in microglia as it was shown to be in macrophages.

Further to continuing studies in microglia, it would be of interest to study the effects of HA on cultured fibroblasts, endothelial cells and neutrophils as these cells possess HA receptors and play a role in inflammatory pathophysiology following SCI.

6.4 Final Conclusions

These studies have made important advances in understanding how early arachnoiditis contributes to the progression of SCI, began the initial validation of the use of a therapeutic polymer to reduce scarring and inflammation in the SAS and also provided some insight into the mechanism by which HA reduces TLR4 mediated inflammation in microglia. To our knowledge this is the first study to look at early arachnoiditis following SCI and also the first to treat it with a therapeutic polymer. Further, this is the first study to look at how HA modulates TLR4 activation in microglia. These results form the basis of what could lead to the development of a potential treatment for the myriad of pathologies/procedures that involve subarachnoid inflammation and scarring.
References


