



**UNIVERSITY OF
TECHNOLOGY SYDNEY**

**An Examination of the Cellular and Inflammatory
Response in Rats After Spinal Cord Injury;
the Effects of Age and Survival Time**

A thesis submitted in fulfilment of the requirements of the degree of

Doctor of Philosophy

By

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DECLARATION OF AUTHORSHIP/ORIGINATLITY

I, Theresa Sutherland, declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Life Sciences at the University of Technology Sydney. This thesis is wholly my own work unless otherwise reference or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis. This document has not been submitted for qualifications at any other academic institution. This research is supported by the Australian Government Research Training Program.

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- Mao, Y., Nguyen, T., Sutherland, TC., and Gorrie, CA. (2016) ***Endogenous neural progenitor cells in the repair of the spinal cord.*** *Neural Regeneration Research* 11(7): 1075-1076

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 - Theresa Sutherland, Yilin Mao, Tara Nguyen, Catherine Gorrie: A comparison of the endogenous neural progenitor cell reaction 24 hours after spinal cord injury in adult, juvenile and infant rats. In: New Horizons: 2014; Kolling Institute, Sydney, Australia; 2014.

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ABBREVIATIONS

| | |
|---------------------|---|
| AD..... | Alzheimer's disease |
| ANOVA..... | Analysis of variance (statistic) |
| ASIA..... | American spinal injury association (impairment scale) |
| BBB..... | Blood brain barrier |
| BBB..... | Basso, Beattie and Bresnahan (behavioural score) |
| BDNF..... | Brain derived neurotrophic factor |
| BMDM..... | Bone marrow derived macrophages |
| BMP..... | Bone morphogenic protein |
| BMSC..... | Bone marrow stromal cells |
| BSCB..... | Blood spinal cord barrier |
| CBS..... | Combined behavioural score |
| CNS..... | Central nervous system |
| CNTF..... | Ciliary neurotrophic factor |
| CXCL..... | (C-X-C motif) ligand |
| EAE..... | Experimental autoimmune encephalomyelitis |
| ELISA..... | Enzyme-linked immunosorbent assay |
| eNPC..... | Endogenous neural progenitor cell |
| GDNF..... | Glial derived neurotrophic factor |
| GFAP..... | Glial fibrillary acidic protein |
| hECS..... | Human embryonic stem cells |
| IGF-1..... | Insulin-like growth factor-1 |
| IFN- γ | Interferon-gamma |

IL.....Interleukin-
 INC.....Intermediate nucleus of Cajal
 IRLS.....Infant rat locomotor score
 M1.....Pro-inflammatory macrophage/monocyte
 M2.....Anti-inflammatory macrophage/monocyte
 MBP.....Myelin basic protein
 MDM.....Monocyte-derived macrophages
 MS.....Multiple Sclerosis
 MSC.....Mesenchymal Stem Cells
 MVA.....Motor vehicle accident
 NGF.....Nerve growth factor
 NPC.....Neural progenitor cells
 NSPC.....Neural stem/progenitor cell
 NT-3.....Neurotrophin 3
 NTF.....Neurotrophic Factors
 OEC.....Olfactory ensheathing cells
 OPC.....Oligodendrocyte progenitor cells
 P7.....Post-natal day 7
 P10.....Post-natal day 10
 PD.....Parkinson’s disease
 PNS.....Peripheral nervous system
 RT-PCR.....Reverse transcription polymerase chain reaction
 ROS.....Reactive oxygen species
 RNS.....Reactive nitrogen species
 SCI.....Spinal cord injury
 SCIWORA.....Spinal cord injury without radiographic anomaly

| | |
|---------------------|---|
| SEM..... | Standard error of the mean |
| SEZ..... | Subependymal zone |
| SNI..... | Spinal nerve injury |
| SPC..... | Stem/progenitor cell |
| SVZ..... | Subventricular zone |
| T10..... | 10 th thoracic vertebral level |
| TBI..... | Traumatic brain injury |
| TGF- β | Transforming growth factor-beta |
| TNF- α | Tumour necrosis factor-alpha |
| TNF-R..... | Tumour necrosis factor receptor |
| TLR..... | Toll-like receptor |
| TSCI..... | Traumatic spinal cord injury |

ABSTRACT

Spinal cord injury (SCI) is a complex and devastating condition that has a life-long effect on patients' quality of life, their family, carers and society. Currently there is no cure for SCI, and no proven treatment in the acute phases of SCI. Tissue loss and varying degrees of functional impairment result from a SCI, and only limited repair is exhibited. A great deal of research has focused on reducing the degenerative effects that occur during the secondary injury phase of injury to order to promote tissue repair and regeneration. The immune and inflammatory response is thought to play a significant role in this process, albeit with both beneficial and detrimental responses reported. Most research to date has concentrated on adult SCI, yet it has been suggested that the young show better functional recovery compared to adults both for humans and in a variety of animal models.

The current research project used an animal model of contusive SCI to compare adult (9wk), juvenile (5wk) and infant (P7) Sprague-Dawley rats. One cohort (n=108) was assessed over a 6 week post-injury period for 1) locomotor function using established and newly developed scoring systems, 2) injury progression using histology, and 3) inflammatory cell changes using immunohistochemistry. A second cohort (n=97) was assessed acutely (1h, 24h and 1wk post-injury) for inflammatory mediators using flow cytometry on the injured tissue homogenate and multiplex cytokine ELISA on the tissue supernatant. Finally, an in vitro study was conducted to

explore the possibility of modulating different macrophage populations using conditioned media to create a more anti-inflammatory microenvironment.

The results described in this thesis show that following a SCI of comparative severity there were significant differences between adult and infant injury progression and presentation, inflammatory responses, and behavioural recovery. This research reinforced the inherent difficulties in modelling infant conditions for comparative studies, but it has also highlighted two important avenues of research to be pursued. 1) A better understanding of SCI progression in the young is needed to inform how paediatric SCI is treated and managed, and 2) targeted modulation of the inflammatory response in adult SCI patients may be a promising avenue for better functional recovery.

CHAPTER 1: AN INTRODUCTION TO SPINAL CORD INJURY

1.1 DEFINING SPINAL CORD INJURY

Spinal cord injury (SCI) is a devastating condition that can arise from mechanical trauma to the spinal cord, or from a variety of non-traumatic insults, examples of which include infection, oncogenesis, birth trauma, and even electrocution (Chang and Hou, 2014). Regardless of the cause, SCI will result in either complete or partial loss of motor and sensory function below the lesion site (Barnabé-Heider and Frisé, 2008), as well as some degree of autonomic dysfunction (Karlsson, 2006). SCI will often result in severe loss of tissue, and, after injury, the spinal cord exhibits only limited repair (Schwartz and Yoles, 2006). This can have debilitating effects on the quality of life, and even the life expectancy, of SCI patients (Mathias, 2008). Patients suffering from SCI are also at increased risk of associated pathologies, such as cardiovascular complications, thrombosis, autonomic dysreflexia, and neuropathic pain (Pineau et al., 2010).

There are many different causes and manifestations of SCI. However, they all share a basic pathophysiology (Kwon et al., 2004; Profyris et al., 2004; Rowland et al., 2008), characterised by cell death and inflammation (Kwon et al., 2004; Norenberg et al., 2004), myelopathy, breach of the blood-brain barrier, and damage to the white matter and neural fibre tracts (Ronaghi et al., 2010). This leads to the disruption of nerve tracts and the loss of motor neurons and interneurons, which contributes to the observed loss of function (Ronaghi et al., 2010).

In the adult population, the most common cause of SCI is land transport accidents (42%), including motor vehicle accidents (MVA), motorcyclists and cyclists or

pedestrians (Norton, 2010; Tovell, 2018). This is followed by falls, both low (20%) and high (20%) (Tovell, 2018). In infants and children the common causes of SCI include trauma resulting from MVA and sports injury, infections, neoplasms, congenital malformations, and birth trauma (Lee et al., 2009). The majority of paediatric SCI occurs at the cervical level (Barnabé-Heider and Frisé, 2008), resulting in more severe autonomic dysfunction and a greater loss of function in the body than a similar injury lower in the cord.

There is currently no cure for SCI, and no proven treatment in the acute phases of the injury that will increase the recovery and improve neurological outcomes (Rowland et al., 2008). Immobilisation and surgical interventions to stabilise the vertebral column and decompress the spinal cord are employed with great success in acute spinal column injury, but are not as effective if the spinal cord tissue has been severely compromised. There is extensive research focused on reducing the degenerative secondary injury phase, and promoting tissue repair and regeneration; however, no effective treatment targeting the early secondary injury phase is currently available to SCI patients. To date, patients are treated with therapies that combine pharmaceuticals and intense physiotherapy to alleviate the chronic symptoms (Barnabé-Heider and Frisé, 2008). Recent advances towards a treatment of chronic SCI have provided some promise. For example, the treatment of a 38 year old patient with a thoracic spinal cord transection with autologous olfactory ensheathing cells (OEC) (Tabakow et al., 2014). This treatment resulted in an improvement from a class A injury (complete injury with no sensory or motor function preserved in sacral segments) to a class C (incomplete injury with motor function preserved below the neurologic level; most key muscles below the neurologic level have a muscle grade of less than 3) on the American Spinal Injury Association (ASIA) impairment scale (scoring worksheet and explanation in the Appendix).

1.2 EPIDEMIOLOGY OF SPINAL CORD INJURY

SCI has a high cost to the community, both financially and socially. Although there is a lack of accurate epidemiological data available in many countries (Chang and Hou, 2014), a 2007 estimate of the global incidence of spinal cord injury resulting from trauma (TSCI) was 23 cases per million population each year. The Australian data from 2007 suggests a lower incidence of 15 cases per million (Chang and Hou, 2014). A current estimate in Australia suggests 400-700 people are affected by TSCI per annum and approximately 11,000-19,000 experience persisting SCI (Middleton et al., 2012; Norton, 2010). The medical burden of providing long term care to these patients has been estimated at approximately AU\$500 million per annum (Norton, 2010). This is on top of the loss of productivity, the psychological burden to the patients, and their carers and loved ones, and the indirect financial burden resulting from these. The time spent in hospital for SCI patients averaged between four and five months (Norton, 2010). A study of 443 SCI patients found that 37% suffered from depression and 30% suffered anxiety (Migliorini et al., 2008), which also has a medical and social burden.

These statistics are based on the adult population. Less is known about paediatric SCI. It is rarer, accounting for only 1-13% of all SCI (Apple et al., 1995; Lee et al., 2009; Osenbach and Menezes, 1989; Selvarajah et al., 2014; Tatka et al., 2016); determining an exact figure is difficult as different studies use different age ranges and different parameters to assess the injury, including hospital admissions, ASIA score and associated co-morbidities (Apple Jr et al., 1995; Armstead, 2000; Balasingam et al., 1994; Brown et al., 2001; Chen et al., 2013; DeVivo and Vogel, 2004; Dickman et al., 1989; Osenbach and Menezes, 1989; Parent et al., 2011; Patel et al., 2001; Schottler et al., 2012). In the paediatric SCI population the majority of injuries result from

non-traumatic aetiologies, such as tumours, congenital abnormalities and inflammation, with TSCI being much less common (Lee et al., 2009). One study of 48 cases of paediatric SCI in Seoul found only 37.5% of spinal cord damage in children under four years of age was due to trauma, only 30% in the cohort 4-12 years of age and 50% in adolescents 13-18 years of age (Lee et al., 2009). These relatively uncommon injuries vary substantially with the age of the patient at injury due to multiple factors, including the frequency of the different injury types, the spinal level affected, the severity and the neurological compromise (DeVivo and Vogel, 2004; Hadley et al., 1988). Another complication of SCI in the paediatric population is the presence of spinal cord injury without radiographically anomaly (SCIWORA), an injury that does not present with any evidence of fracture or dislocation due to the inherent elasticity of the young vertebral column (Dickman et al., 1989; Hadley et al., 1988; Kisson et al., 1990). This, however, makes the spinal cord particularly vulnerable to deformation by flexion, hyperextension and longitudinal distraction, and also to ischemic injury (Hadley et al., 1988). In a study of 26 cases of paediatric SCI in 1991, SCIWORA accounted for 32% of SCI in young children and 12% in older children (Dickman et al., 1989).

1.3 THE PATHOPHYSIOLOGY OF SPINAL CORD INJURY

1.3.1 Spinal cord injury is a bi-phasal and progressive insult

It has been long accepted that SCI consists of two distinct phases that contribute to the observed pathology (Figure 1) (Donnelly and Popovich, 2008; Kwon et al., 2004; Ross and Pawlina, 2011; Rowland et al., 2008). The first phase is the initial mechanical trauma that can result in fractures, breaks or damage to the vertebral column, as well as to the spinal cord. This imparts a detrimental force on the spinal cord resulting in the disruption of axons, surrounding glial cells and blood vessels (Profyris et al., 2004). The initial mechanical trauma will directly result in microvascular injuries, haemorrhage and disruption of cellular membranes, releasing cytotoxic molecules (Dumont et al., 2001; Pineau et al., 2010) such as reactive oxygen species and other pro-inflammatory mediators, excess ions and glutamate. This causes damage to the surrounding cells by excitotoxicity (Dumont et al., 2001), oxidative stress (Azbill et al., 1997), lipid peroxidation of membranes (Dubendorf, 1999), disruption of intercellular ion balance and induction of cell death signals. This also initiates a strong pro-inflammatory cascade, characterised by neutrophil and macrophage infiltration, microglial activation and astrogliosis, which can become detrimental if it is unregulated and reaches overwhelming levels (Dumont et al., 2001; Potts et al., 2006; Profyris et al., 2004; Rowland et al., 2008). The secondary phase of SCI is the delayed and continuously developing expansion of the injury, which manifests in a broad spectrum of pathologies that exacerbate the injury (Profyris et al., 2004; Rowland et al., 2008). The lack of regeneration and minimal functional recovery that is associated with SCI is a result of the degeneration of severed axons distally, and the abnormal growth of the proximal section or its failure to regrow (Barnabé-Heider

and Frisé, 2008). To compound this, there are also axonal growth inhibitors in the central nervous system (CNS) after injury, which are associated with reactive astrogliosis, the glial scar and the inflammatory response (Barnabé-Heider and Frisé, 2008; Silver and Miller, 2004; Wang et al., 2011).

The secondary damage phase of SCI is complex and changes over time, making it difficult to identify a simple therapeutic target to alleviate its detrimental effects. The inflammatory response plays a significant role in the profile of the microenvironment of the lesion after SCI, as do the actions of reactive astrocytes and activated endogenous microglia. This basic pathophysiology is common to SCI in both adult and developing cords. The majority of SCI research has been carried out in animal models, with a variety of different mammals used in adult models, including non-human primates. Paediatric models have used pigs (Kuluz et al., 2010), cats (Bregman and Goldberger, 1983a; Bregman and Goldberger, 1983b; Bregman and Goldberger, 1983c) and possums (Lane et al., 2007), as well as the commonly used mice (Hamilton et al., 2009; Kumamaru et al., 2012; Xu et al., 2008) and rats (Bregman et al., 1993; Carrascal et al., 2005; Guest et al., 1997; Horner et al., 2000; Karimi-Abdolrezaee et al., 2006; McDonald et al., 1999; Nakamura and Bregman, 2001; Tzeng, 2002; Yuan et al., 2013). Despite the differences in injury models, time-courses and endpoint analyses, there have been similar findings across all of these models in regard to differences in functional outcomes, cord biomechanics, and cellular and inflammatory responses. This has given a broad view of the similar response in a wide range of mammals. Little has been corroborated in humans; however, as mammals, it is thought that humans will exhibit a similar response to that of the experimental animals used in research (Norenberg et al., 2004).

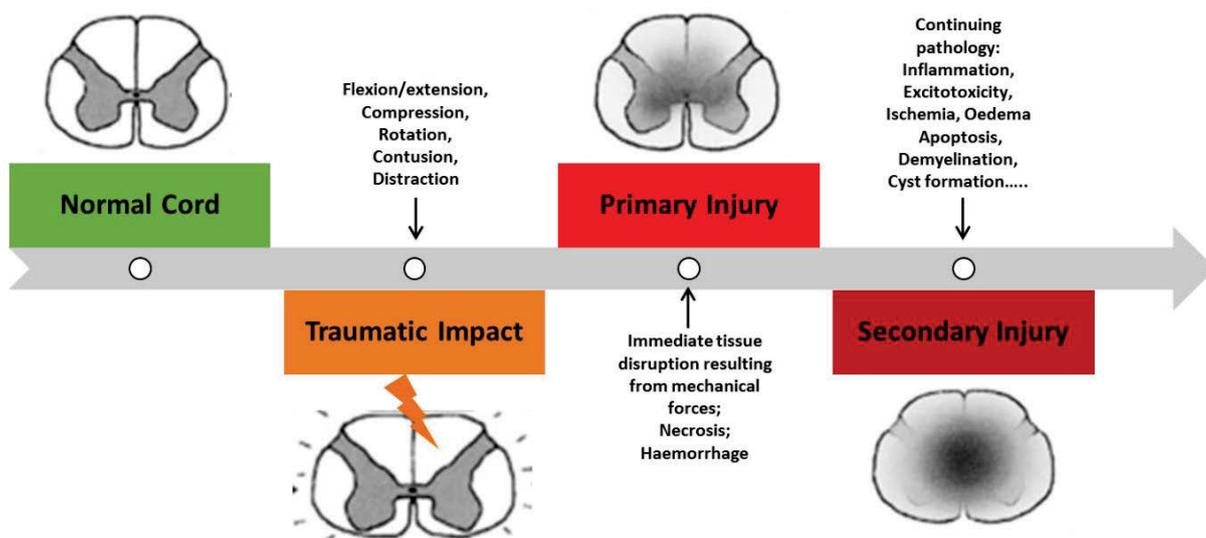


Figure 1: Illustration of the progression of a traumatic spinal cord injury over time, from the normal cord to the chronic secondary injury phase. This is a simplified timeline of a complex injury process.

1.3.2 Reactive astrogliosis and the glial scar

Reactive astrogliosis is the molecular and morphological changes that astrocytes undergo, including the formation of a glial scar (Sofroniew, 2009). Reactive astrocytes are the primary responders to insult in the CNS. After injury, these cells will wall off the damaged areas and seal the lesion to protect the surrounding intact tissue from further damage (Fitch and Silver, 2008). The early reaction to trauma within the spinal cord involves a range of cells, both endogenous and exogenous to the CNS, including endogenous microglia, oligodendrocyte precursors, astrocytes, and stem cells (Fawcett and Asher, 1999), as well as exogenous macrophages and neutrophils (Profyris et al., 2004).

In vitro studies have shown that astrocytes can produce effector molecules, both pro- and anti-inflammatory, that can both help and hinder functional recovery (Silver and Miller, 2004). It is still contentious whether reactive astrogliosis and the glial scar are beneficial or detrimental to functional recovery after SCI (Fawcett and Asher, 1999; Fitch and Silver, 2008; Profyris et al., 2004; Silver and Miller, 2004;

Sofroniew, 2009); much of the literature expounds on both neuroprotective and inhibitory elements of the glial response (Kwon et al., 2004; Sofroniew, 2009). A study by Wang et al. (2011) suggested that reactive astrocytes do not simply physically block the extension of axon across the lesion site, but also contribute to the failure of remyelination by high expression of bone morphogenic protein (BMP) (Wang et al., 2011). Lane et al. (2007) found that the accumulation of glial fibrillary acidic protein (GFAP)-positive astrocytes at the lesion occurred much earlier in animals injured at post-natal day 14 (P14) than those injured at day 7 (P7) (Lane et al., 2007). This delayed timeframe of activation and migration of reactive astrocytes in young subjects may also contribute to the faster and fuller functional recovery of young animals, alleviating some of the negative effects of the glial scar.

Whether reactive astrogliosis is considered to promote or inhibit regeneration, it is the cellular environment in which any therapeutic intervention will have to function. Astrogliosis does not occur in isolation; this process is intertwined with the progression of the inflammatory response and the secondary processes of the injury. Therapies may either augment or attenuate different aspects of this response for greater benefit (Sofroniew, 2009; Woerly et al., 2004).

1.3.3 The neuroinflammatory response to spinal cord injury

In a typical response to CNS injury the first cells to arrive, within hours of the insult, are blood-borne neutrophils, as well as macrophages and endogenous microglia. These are followed by oligodendrocyte progenitor cells (OPC) and macrophages from the surrounding tissue, over the next three to five days (Gensel and Zhang, 2015). The final structure of the glial scar is predominantly composed of reactive astrocytes (Fawcett and Asher, 1999). Fleming et al. (2006) described the

inflammatory response in distinct zones. The first zone is the inflamed and necrotic tissue at the lesion site and the second is the spreading axonal swelling, Wallerian degeneration and further inflamed tissue (Fleming et al., 2006). These areas of damage are surrounded by areas of histologically intact tissue and spared axons, which may be important for therapeutic developments and potential recovery of function (Donnelly and Popovich, 2008; Fleming et al., 2006; Kwon et al., 2004; Rowland et al., 2008).

The immune response plays an important role in the progression (and resolution) of SCI (Chan, 2008); however, it is still contentious whether this cascading immune response (Figure 2) is beneficial or detrimental to recovery (Donnelly and Popovich, 2008; Fleming et al., 2006; Rowland et al., 2008). This cascade begins shortly after the injury with the changes in the spinal cord microenvironment and the release of inflammatory mediators (Donnelly and Popovich, 2008; Hausmann, 2003). The first cellular responders, the neutrophils, infiltrate from the periphery; this is followed by the activation of endogenous microglia and peaks in exogenous macrophage and monocyte activity (Gensel and Zhang, 2015; Schwartz and Yoles, 2006). These inflammatory cells also interact closely with the CNS cells and modulators in the post-injury. Elements of the immune response have both neuroprotective and neurotoxic effects; which of these effects predominates is likely due to the timeframe and scale of expression, and the cells on which they are acting (Kwon et al., 2004). The oxidative and proteolytic enzymes produced by infiltrating neutrophils prepare the area for repair; however, the excessive numbers that are drawn to the lesion can cause further damage to the surrounding tissues (Fleming et al., 2006). It is important to note that this response does not act in a vacuum, but instead is part of a set of complex interactions between the immune and nervous system that take place after injury (Figure 3).

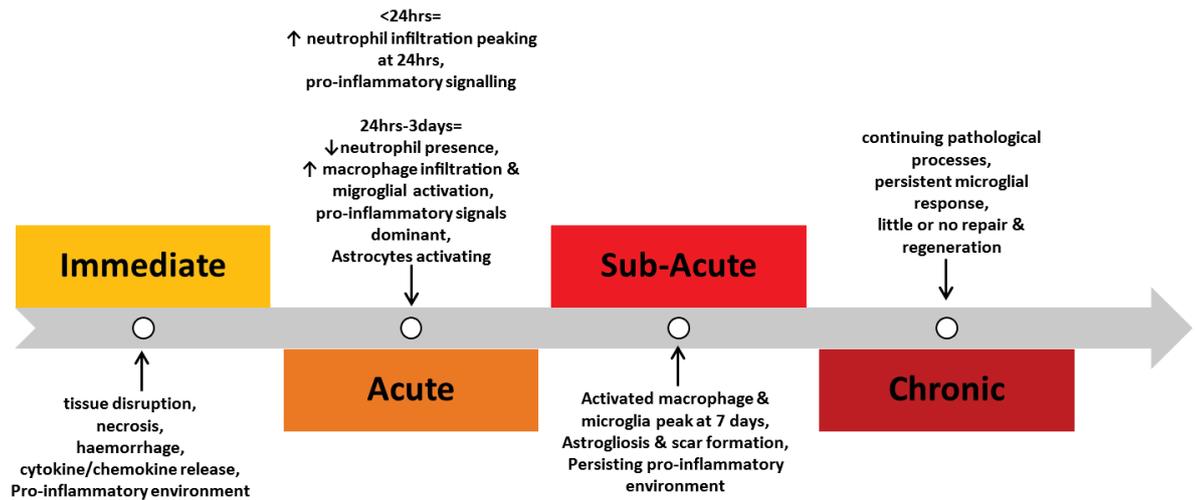


Figure 2: Illustration of the progression of the neuroinflammatory cascade over time after a traumatic spinal cord injury in mature animals, from the immediate biochemical response through to the chronically pro-inflammatory environment. This is a simplified timeline of a complex cascade involving multiple subsets of cytokines, chemokines and cells from both the innate immune system and the nervous system.

The profile of the inflammatory response to SCI differs between young and adult mammals in terms of cellular and molecular response (Kumamaru et al., 2012), as well as timeframe, as shown using animal models (Brown et al., 2005; Lane et al., 2007). Rodent models have shown that microglial cells, the first inflammatory responders, initiate the production and secretion of cytokines into the cellular microenvironment (Kumamaru et al., 2012). The profile of these cytokines, and the expression of inflammatory molecules, differs markedly between adult and infant animals (Kumamaru et al., 2012; Lane et al., 2007). The secretion of pro-inflammatory cytokines interleukin (IL)-6, IL-1 β and tumour necrosis factor (TNF)- α was markedly decreased in young mice after SCI (Kumamaru et al., 2012). This may contribute to the attenuation of the subsequent neutrophil infiltration observed in neonate mice (Kumamaru et al., 2012). Neutrophil infiltration has been found to contribute to the severity of the secondary insult (Donnelly and Popovich, 2008).

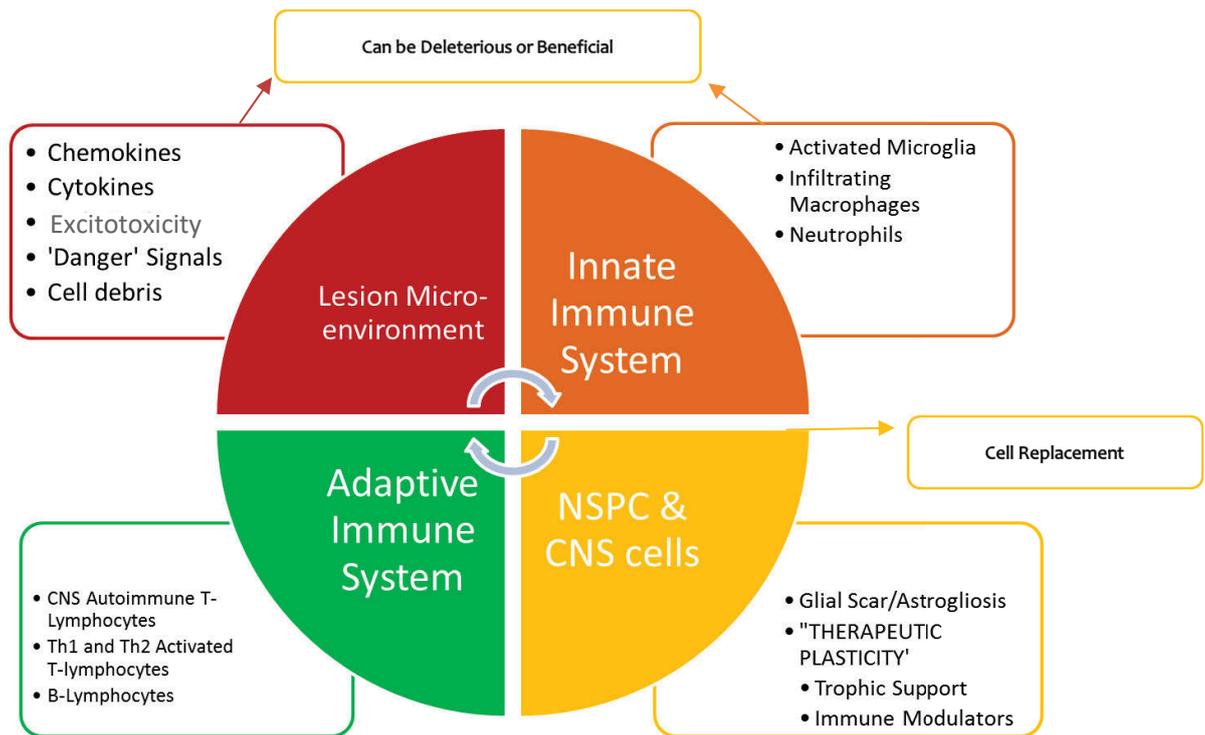


Figure 3: Complex interactions between the immune and nervous systems. This diagram gives a simplified map of the complex interplay between the microenvironment, the neural stem/progenitor cells (NSPC), and the innate and adaptive immune responses.

Macrophages and microglia are capable of producing factors such as brain-derived neurotrophic factor (BDNF) (Dougherty et al., 2000) and macrophage derived protein oncomodulin (Yin et al., 2003; Yin et al., 2006) that will promote axonal growth and regeneration, as well as proteins that are neurotoxic (Fleming et al., 2006). The cells recruited by the glial and immune reaction have the potential to produce molecules that have been demonstrated to inhibit axonal regeneration (Fawcett and Asher, 1999); however, these cells also have a beneficial role to play in the protection of the fragile surrounding tissues and the provision of a cellular support network (Fawcett and Asher, 1999; Profyris et al., 2004).

Microglia are activated as early as 24 hours after SCI, with a markedly less pronounced reaction in young animals compared with the adult counterparts (Vega-Avelaira et al., 2007). One area of interest is the differing effects of classically activated

(M1) or alternatively activated (M2) macrophages (David and Kroner, 2011; Kigerl et al., 2009). M1 macrophages respond to and release pro-inflammatory cytokines and also produce proteases, cytotoxic effectors and reactive oxygen species that contribute to the secondary injury (Stollg and Jander, 1999). In contrast M2, or anti-inflammatory, macrophages help promote a more supportive trophic environment and facilitate neuronal survival and sprouting (Stollg and Jander, 1999).

1.3.4 The neural progenitor cell response in the spinal cord

Stem/progenitor cells (SPC) have received a large amount of attention in relation to a wide variety of disorders, ranging from cancer (Reya et al., 2001) to diabetes (Noguchi, 2010) and cardiovascular disease (Beltrami et al., 2003; Jackson et al., 2001); their use has also been explored for tissue regeneration (Rafii and Lyden, 2003; Zhao et al., 2009). In the CNS different classes of SPC have been explored as a potential therapeutic avenue in a variety of injury models, from neurodegenerative disorders, such as Alzheimer's disease (AD), Multiple Sclerosis (MS) and Parkinson's disease (PD), to traumatic brain injury (TBI) and SCI. Many different sources of these cells have been used, alongside numerous different in vivo and in vitro manipulations and treatment strategies. However, very few of these have progressed beyond animal modelling into the clinical trial phases. The majority of this work has been focused on the transplantation of SPC into afflicted animals or patients; however, the current study will focus on neural progenitor cells (NPC) endogenous to the spinal, rather than the transplantation of different SPC into the injured CNS.

Neural stem/progenitor cells (NSPC) are broadly defined as cells derived from the nervous system that have capacity for some self-renewal and can generate nervous system specific cells (Gage, 2000; Kukekov et al., 1999; McKay, 1997; Temple,

2001). These cells are not confined to the developing nervous system in mammals, but are also present in niches in the fully developed adult CNS. These include the hippocampus, the subventricular zone (SVZ), periventricular subependymal zone (SEZ) (Gage, 2000; Kukekov et al., 1999; McKay, 1997; Temple, 2001) and the ependymal layer of the spinal cord central canal (Beattie et al., 1997; Gage, 2000; Meletis et al., 2008; Weiss et al., 1996). Endogenous neural progenitor cells (eNPC) are partially differentiated cells that are committed to the neural line. These NPC have the self-renewing capacity and multipotency of stem cells, on a limited basis (Gage, 2000). Quiescent in the uninjured CNS, these cells have been shown to activate and proliferate in response to injury (Meletis et al., 2008).

1.3.5 Other cellular responses to spinal cord injury

There are many cellular and pathological processes involved in the progression of SCI, some of which could not be addressed in the scope of this study; however, they are all interconnected. These include disruption of the blood-spinal cord barrier (BSCB), haemorrhage and ischemia, oedema, necrosis and apoptosis, excitotoxicity, demyelination and Wallerian degeneration, as well as axonal swelling, astrogliosis and inflammation.

There is extensive disruption of the spinal cord microvasculature, and the BSCB, involved in SCI. This presents as haemorrhage and ischemia (Dubendorf, 1999; Dumont et al., 2001). Ischemia has long been associated with TBI and SCI due to pathological changes and disruptions in the vasculature at and around the area of direct injury (Dubendorf, 1999). The disruption of blood flow results in an extended area of cell death and the formation of a penumbra of vulnerable cells around the area of damaged cells at the injury epicentre, which is a widely recognised element of TBI

pathophysiology (Greve and Zink, 2009). These vascular effects are coupled with inflammation, excitotoxicity, oxidative stress and oedema, necrosis and apoptosis to perpetrate continued cell death in the secondary injury phase (Arundine and Tymianski, 2004; Dumont et al., 2001).

Demyelination has been associated with the secondary injury pathophysiology of SCI for decades (BLIGHT, 1985), though the exact incidence, extent and chronicity is still under examination (Guest et al., 2005). This process has been observed in acute and chronic SCI and is associated with both axonal damage and the loss of oligodendrocytes (Crowe et al., 1997; Guest et al., 2005). Demyelination of injured axons releases a variety of molecules into the extracellular space, such as myelin basic protein (MBP), that contribute to the pro-inflammatory and detrimental nature of the lesion microenvironment. The myelin debris in the lesion helps promote the recruitment of phagocytes and the activation of microglia (Kopper et al., 2017; Kopper and Gensel, 2018; Lloyd et al., 2017). Elements of the myelin debris, such as IL-34 and MBP, also support the survival of recruited bone-marrow-derived macrophages (Brennan and Popovich, 2018). MBP is a constituent of the myelin sheath in both the peripheral and central nervous systems and has a range of functions in the normal nervous system (Boggs, 2006). MBP-reactive T-lymphocytes, which can cause pathological conditions in naïve rats, are activated after SCI and are thought to have a neuroprotective role (Hauben et al., 2000; Moalem et al., 1999b).

The damage in SCI is not limited to the damaged cells, but spreads into healthy cells (Dumont et al., 2001). Cell death signals and detrimental molecules, such as excess glutamate, can move from damaged and dying cells through normal communication and transport pathways between the cells, such as gap junctions and connexion hemi-channels (Dumont et al., 2001; Mao et al., 2017b; Tonkin et al., 2015).

Excess glutamate and potassium can cause cytotoxic oedema in astrocytes, and transfer via cellular junctions and open hemi-channels (Mao et al., 2017b; Tonkin et al., 2015). Anatomically intact fibre tracts can exhibit a loss of impulse conduction related to shifts in the intracellular ion concentrations, most significantly calcium, which will then have a downstream effect on cell functioning and the release of molecules into the extracellular environment (Dubendorf, 1999). Increased calcium levels in the intracellular environment contribute to the stimulation of protease and lipase activity which subsequently promotes lipid peroxidation of the cell membrane and myelin sheath (Dubendorf, 1999). The molecular changes to the injury microenvironment associated with the cellular responses, necrosis and apoptosis also have downstream effects. Free radicals, fatty acids, glutamate and cellular constituents released from the degrading membrane contribute to further damage, and an extracellular environment that is not permissive of neurological survival and axonal regeneration (Arundine and Tymianski, 2004; David and Aguayo, 1981; Fitch and Silver, 2008; Profyris et al., 2004). The release of excess glutamate and excitatory neurotransmitters from damaged cells into the microenvironment results in accumulation in the extracellular environment and damage to surviving cells (Azbill et al., 1997; Dumont et al., 2001). The generation and accumulation of reactive oxygen species (ROS) and nitrogen species (RNS) after a traumatic injury results in oxidative stress in the surviving cells and further lipid peroxidation (Azbill et al., 1997; Dumont et al., 2001; Fatima et al., 2015; Visavadiya et al., 2016). Mitochondrial activity decreases as early as an hour after injury and remains compromised for up to 24 hours, leading to increased ROS and lipid peroxidation very early in the secondary injury phase (Azbill et al., 1997).

While acknowledging the role of all of these elements in the progression of SCI, the current study is focused on a selection of elements from the innate immune

system and a selection of the endogenous nervous system cells. The area of particular interest to us was the differences in the response and recovery between adult and infant animals, which we had begun to observe previously. In this study we focus on the cellular inflammatory response, and how it correlates to activity of the prominent endogenous responders of the spinal cord, such as astrocytes and eNPCs. The inflammatory response, both cellular and mediators, has come to the fore in previous work from our laboratory in a contusion SCI and in more recent literature (Kumamaru et al., 2012; Lane et al., 2007; Potts et al., 2006; Schultz et al., 2004; Vega-Avelaira et al., 2007). Figure 3 outlines this interplay between the microenvironment, the NSPC and the innate and adaptive immune responses, and provides a simplified map of the complex interactions that are taking place after spinal cord injury.

1.4 THE RESPONSE TO SPINAL CORD INJURY DIFFERS BETWEEN YOUNG AND ADULT ANIMALS

1.4.1 Spinal cord development in young animals

Paediatric SCI accounts for a small percentage of all SCI (Apple et al., 1995; Lee et al., 2009) and therefore is less well characterised. The developing spinal cord is significantly different from the fully developed adult cord in a variety of aspects, from biomechanical (Clarke and Bilston, 2008; Clarke et al., 2009) to molecular (Bregman et al., 1997; Kumamaru et al., 2012; Maisonpierre et al., 1990; Nakamura and Bregman, 2001), cellular and structural (Alizadeh et al., 2018; Firkins et al., 1993; Kuluz et al., 2010; Vega-Avelaira et al., 2007). Biomechanically, using a uniaxial mechanical testing machine on complete spinal cords, similar stress-strain response was seen in neonates and adults; however, neonates had lower modulus and less relaxation, compared to the adults (Clarke et al., 2009). In a rodent model of spinal fracture-dislocation, SCI is produced in the absence of vertebral end-plate fracture in the more flexible immature spine at significantly lower loading than in adults; this may suggest a greater susceptibility to SCI in younger populations (Clarke and Bilston, 2008). The molecular differences that influence the injury microenvironment are complex. In 2001 Nakamura and Bregman found that immature spinal cords exhibit greater anatomical reorganisation after SCI, which could be linked to the differing profile and time-course of neurotrophic factor expression, specifically BDNF, ciliary neurotrophic factor (CNTF), glial-derived neurotrophic factor (GDNF), nerve growth factor (NGF) and neurtrophin-3 (NT-3) (Nakamura and Bregman, 2001). In 2012 Kumamaru et al. found that the magnitude of neutrophil infiltration, and the molecular mechanism behind this, varies greatly with age. Expression of chemoattractants IL-6, TNF- α and (C-X-C motif) ligand

(CXCL)-1 were attenuated in immature cords compared to matched adult counterparts (Kumamaru et al., 2012). The local immune environment is also different in neonatal animals compared to their adult counterparts (Lane et al., 2007). This has led to a heightened interest in SCI in younger subjects, though this is yet to be explored in any depth (Kumamaru et al., 2012). There is a trend in the literature of younger patients or animals having the potential for better functional recovery; several clinical studies seem to support this (Henry et al., 1977; Parent et al., 2011; Wang et al., 2004). A consensus finding was published in 2011 stating that the “potential neurological recovery was higher in children compared to adults”, based on a systematic review of cases in the literature from 1980-2011 (Parent et al., 2011). If this statement is correct, and we can determine why this is occurring, we may be able to identify key differences and use this information to guide a therapeutic approach to SCI in all age groups.

Very little is understood about SCI in young subjects, and less still about the differing responses to comparable injuries between animals of different ages. The mechanisms behind these recovery differences remain a mystery. This study aims to fill in some of the gaps in our knowledge of SCI in younger animals, surrounding the inflammatory response and endogenous cellular responses, and how these differ from their adult counterparts after a comparable injury. Over the course of this study I have attempted to elucidate the response of key cellular players from the innate immune system and the CNS in SCI, to investigate the innate immune cells that are responding to this injury, as well as the key cytokines present in the lesion environment and some adaptive immune elements, and to examine how all of these elements come together to elicit differing responses to SCI in different age groups.

1.5 THIS PROJECT AND THESIS

Based on the literature, and previous work conducted in this lab, the current project was undertaken with the hypothesis that there would be significant differences in the response to SCI in rats of different ages, especially in terms of the innate inflammatory response and functional recovery. This thesis will use a contusion model of SCI in rats of different ages to establish where these differences lie and examine their potential functional consequences. Chapter 2 of this thesis will examine the differences in functional recovery between adult (9 weeks old at the time of injury) and infant (P7) rats over a six week period after a SCI using two different behavioural scoring systems. Chapter 3 will examine the histological post SCI changes in the spinal cord tissue from adult (9wk), juvenile (5wk) and infant (P7) rats with a specific focus on endogenous progenitor cells, astrocytes and innate immune cells. Chapter 4 will examine differences in the post SCI inflammatory response using flow cytometry to explore the macrophage cell phenotypes and cytokines. Chapter 5 will describe a pilot study with in vitro experiments investigating M1, M2 and NPC interactions.

CHAPTER 2: EXPLORING THE RECOVERY DIFFERENCES BETWEEN MATURE AND NEONATAL RATS ON A BEHAVIOURAL LEVEL AFTER A COMPARABLE SPINAL CORD INJURY

2.1 INTRODUCTION

2.1.1 Paediatric spinal cord injury in a clinical setting

Traumatic SCI is most common in young adult males and, as such, this is where the majority of research has focused (Augutis, 2007). As discussed in Chapter 1 (section 1.2), paediatric SCI is much less common and SCI with a traumatic aetiology in the paediatric population even less so (Apple et al., 1995; Lee et al., 2009; Nicholls and Saunders, 1996; Osenbach and Menezes, 1989; Tatka et al., 2016). It has been found in clinical studies and reviews that the incidence of paediatric spinal injury increases with age (Henry et al., 1977). Infants have a higher proportion of non-traumatic than traumatic SCI (Lee et al., 2009). This includes neoplasms, transverse myelitis, congenital abnormalities, and disease (Lee et al., 2009). A retrospective Australian study of 103 patients found that 66% of paediatric patients had non-traumatic spinal cord disease, and of the 34% that had traumatic SCI more than half of these were the result of MVA (Galvin et al., 2013). The cause of injury, frequency of injury type, spinal level at which the injury occurs, and the neurological compromise vary with the age and development of the patient (DeVivo and Vogel, 2004; Hadley et al., 1988).

Paediatric SCI is quite a unique injury and therefore presents unique challenges on a clinical level, as well as ongoing challenges for the patient due to its great effect

on ongoing physical and psycho-social development (Augutis, 2007). In 1988 Hadley et al. found that, of 122 cases examined, 89% with incomplete myelopathy on admission had improved by their last examination and 20% with complete myelopathy had significant functional improvement (Hadley et al., 1988). In a more recent review of 174 hospital admissions Hamilton and Mylks found that, while paediatric patients with complete injuries still showed very little improvement, 74% of patients with incomplete injuries (incomplete damage with some spared motor and sensory function) showed significant improvements and 59% showing complete recovery of neurological function (Hamilton and Mylks, 1992). This has led to the contention that there is a better outcome for paediatric SCI, an outcome that can be quite good in injuries without a physiologically complete cord deficit (Hadley et al., 1988; Hamilton and Mylks, 1992). A 2011 review of the previous paediatric SCI literature concluded that MVA accounted for the largest cause of SCI (52%), cervical spinal injuries were the most common (C1 to C4 in 68% of patients, C5 to C7 in 25%, and both in 7%) and SCIWORA accounted for 43% of injuries (Parent et al., 2011). This review also concluded that neurological recovery in paediatric SCI patients appears to be better than that in adults (Parent et al., 2011).

SCI incurred in early life confers a life-long burden on the patient and their family. An SCI early in life impacts the ongoing growth and development of the patient, and their quality of life with secondary medical complications emerging over a lifetime. A 1998 long-term outcomes study of adults over 25 years that had experienced a paediatric SCI found that in adult life patients experienced pressure ulcers, urinary tract infections, spasticity, pain, dysreflexia, orthopaedic complications, bowel and bladder incontinence, urinary stones, cardiorespiratory complications and hospitalisations (Vogel et al., 1998). Scoliosis is common in children with SCI, more so

when the neurological injury occurs at a younger age (Parent et al., 2011). In a study of 159 individuals a large portion experienced medical complications with 96% developed scoliosis, and 57% hip dysplasia; of those with injuries above T6 level 34% experienced autonomic dysreflexia, 41% suffered from pressure ulcers and 61% experienced spasticity; 82% did not have normal bladder or bowel control (Schottler et al., 2012). Of the 159 patients in this study 131 used a wheelchair, with the median starting age being three years and four months (Schottler et al., 2012), this confers a life-long expense and reduced quality of life for these patients.

There is consensus that significant differences in anatomy and biomechanics exist between adult and infant spinal columns and cords, which lead to unique injury patterns and mechanisms in paediatric SCI (Di Martino et al., 2004; Dickman et al., 1989; Hamilton and Mylks, 1992; Parent et al., 2011; Tatka et al., 2016). Younger patients are more predisposed to suffering cervical spinal injuries, due to a larger head to torso ratio (Di Martino et al., 2004; Stelzner et al., 1979; Tatka et al., 2016). Spinal cord injury without radiographic anomaly (SCIWORA) is also far more common in young infants than adolescents or adults, due to the inherent elasticity of the developing vertebral column and the biomechanics of the cord (Dickman et al., 1989; Kisson et al., 1990; Lee et al., 2009; Osenbach and Menezes, 1989; Schottler et al., 2012). In a 1988 review of 122 paediatric SCI cases Hadley et al. defined four distinct injury patterns; fracture only, fracture with subluxation, subluxation only (a partial dislocation or misalignment of vertebrae), and SCIWORA (Hadley et al., 1988). A 1989 review found that, between 1970 and 1988, 35% of traumatic myelopathy in the paediatric population displayed SCIWORA and this was particularly prevalent in very young children (Osenbach and Menezes, 1989). Since then small single-centre reviews of paediatric admissions and SCI have been conducted with varying parameters as to

what was included, excluded and observed (DeVivo and Vogel, 2004; Hamilton and Mylks, 1992) as well as a few larger reviews of the literature (Dickman et al., 1989; Schottler et al., 2012; Vega-Avelaira et al., 2007).

2.1.2 The response to trauma in developing animals differs from the mature response

A trend of fuller and faster functional recoveries in both infant animals and younger human patients, in contrast to older animals and patients that suffered comparable injuries, has been reported in the literature (Brown et al., 2005; Clarke and Bilston, 2008; Kunkel-Bagden et al., 1992; Parent et al., 2011; Vega-Avelaira et al., 2007) and has yet to be thoroughly investigated. Previous research in the literature has found that spinal repair occurs rapidly and reliably in newborn opossums (Lane et al., 2007; Saunders et al., 1995; Saunders et al., 1998; Treherne et al., 1992) and foetal rats, however this capacity for regeneration decreases as the CNS matures (Nicholls and Saunders, 1996). While the axons regenerate rapidly in the infants they do not necessarily reconnect appropriately or with the specificity needed for functional recovery (Nicholls and Saunders, 1996). The contention that infants exhibit better functional recoveries after SCI has led to a heightened interest in SCI in younger subjects, though this is yet to be explored in any depth (Kumamaru et al., 2012). A 1992 spinal cord hemi-section study by Kunkel-Bagden et al. found that locomotor patterns and motor strategies post-SCI differed between neonate and adult rats, suggesting differences in the underlying mechanisms of the pathology and recovery (Kunkel-Bagden et al., 1992). There are, however, many aspects of the developing cord that are different to the fully mature adult cord worthy of further exploration at a cellular, molecular and biomechanical level.

There have been a range of differences noted in the cellular response to SCI between adult and infant animals. Also, using a thoracic hemi-section model in rats Stelzner et al. found significantly greater density of dorsal root degeneration in the intermediate nucleus of Cajal (INC) on the experimental side in neonatal rats compared to weaning rats (Stelzner et al., 1979). In a T5 transection SCI model the astrogliosis (defined by GFAP immunocytochemistry) was more intense in adult rats and spread rostrally and caudally; in contrast the post-injury gliosis remained confined to the lesion in neonatal rats (Barrett et al., 1984). A 2005 study by Brown et al. found that the spinal cord of infant rats had less myelin, a lower proportion of white to grey matter and increased plasticity (Brown et al., 2005). In a model of spinal nerve injury (SNI) in young rats there is a weak microglial response but a robust astrocytic response that began as early as one day post-injury, followed by a second wave at seven days. This contrasts to the adult response where there is a clear microglial activation in the dorsal horn at five days and astrocytic activation at seven days post-injury (Vega-Avelaira et al., 2007). These studies demonstrate the wide variations in the cellular response to injury in the neonatal CNS and provide a solid basis for further exploration into the mechanisms of SCI in neonatal animal studies and human paediatric trauma.

Not unconnected to the array of cellular differences, there are also differences in the molecular response to SCI and the constituents in the injury microenvironment. There is an argument to be made that the lack of trophic support contributes to the failure of regeneration in mature mammalian spinal cord (Widenfalk et al., 2001). Studies have found differences in expression of neurotrophic factors between developing and mature spinal cords after injury (Bregman et al., 1997; Nakamura and Bregman, 2001; Widenfalk et al., 2001). A 2001 study found relatively limited upregulation of neurotrophic factors in the spinal cord compared with the nerve root

and the peripheral nervous system (PNS) (Widenfalk et al., 2001). This study found upregulation of NGF and GDNF mRNA in meningeal cells adjacent to the lesion, BDNF and p75 in neurons and GDNF in astrocytes close to the lesion in adult rats, and a similar pattern in the newborn rats (Widenfalk et al., 2001). In 2001 Nakamura and Bregman found that there were increased levels of NGF, BDNF, NT-3 and GDNF mRNA expression, and decrease levels of CNTF, in the normal postnatal (P3-P10) spinal cord compared to the normal adult spinal cord. This pattern shifts between P10 and P17 as the expression of CNTF increases and the other factors tested decrease (Nakamura and Bregman, 2001). Findings from this study indicate the contrasting level of BDNF and CNTF in the adult and neonate cord may play a significant role in the improved axonal regrowth after SCI in infants (Nakamura and Bregman, 2001). Inflammation in the CNS is unique to the rest of the body and plays an important role in shaping the injury microenvironment after SCI (Potts et al., 2006). In recent literature a significant difference in the inflammatory response have emerged between infant and adult animals (Kumamaru et al., 2012; Sutherland et al., 2017). A 2012 study by Kumamaru et al. found that young mice (4wk) showed lower pro-inflammatory chemokines and cytokines, such as IL-6, IL-1 β , TNF- α and CXCL1, compared to 10 week old adults in the acute phase of SCI. This study also found that microglia immediately initiate the production of several major pro-inflammatory cytokines and chemokines (IL-6, TNF- α and CXCL1) in adult mice; and the secretion of pro-inflammatory mediators but not anti-inflammatory, such as IL-10 and TGF- β , were significantly lower in young mice (Kumamaru et al., 2012). Cytokines from the local and infiltrating inflammatory cells also assist in modulating reactive astrogliosis (Balasingam et al., 1994). Neonatal SCI produces a more limited astroglial response than comparable adult injuries

(Balasingam et al., 1994; Sutherland et al., 2017) which may coincide with the decreased pro-inflammatory cytokine response in younger animals.

In a 1988 clinical review of paediatric SCI Hadley et al., suggested that the unique anatomical and biomechanical features of the infant spinal cord influence the patterns of injury we see in the human paediatric population (Hadley et al., 1988). Clarke and Bilston have examined the biomechanical differences between mature and developing cords in rodents (Clarke and Bilston, 2008; Clarke et al., 2008; Clarke et al., 2009; Lau et al., 2013). These studies have cumulatively found that age and the amount of spinal motion affect the severity of acute SCI; haemorrhage volume is greater in infants than adults (Lau et al., 2013), normalised axonal density is higher in the infants than the adults (Clarke and Bilston, 2008), and the peak stress and the stress after relaxation are higher for spinal cords from adults than neonates (Clarke et al., 2009). While spinal loading conditions, such as speed and displacement, affect the severity of SCI the relationship between these factors is not fully understood (Lau et al., 2013). There is greater mobility and elasticity in the infant cervical spine, compared to the mature spine (Adamson, 2009; Henrys et al., 1977; Kisson et al., 1990; Tatka et al., 2016). The spinal ligaments in young patients are more elastic than those in adults and increased water content in the annulus helps to increase the elasticity of the vertebral discs in the paediatric spine conferring greater flexibility to the spinal column (Tatka et al., 2016). In a piglet study in 2010 Kuluz et al. concluded that the hypermobility of the infant spinal column and the developing vertebrae can have a protective effect in traumatic SCI as they are less likely to fracture and cause direct damage to the tissue (Kuluz et al., 2010). A 2007 sheep study by Clarke et al. comparing the torque-deflection properties of mature and newborn spinal cords concluded that immature spines are more flexible, and this may be implicated in the

different SCI mechanisms (Clarke et al., 2007). The osteoligamentous structures of the paediatric spine also have greater healing potential that may impact the recovery from SCI in younger patients (Di Martino et al., 2004). The developing spine column undergoes a plethora of changes in the early years of life. The morphology of the vertebrae changes, which increases the stability of the joints with age, and the paraspinal muscles are under-developed in young children, which offers less protection from trauma, (Tatka et al., 2016) potentially leading to the trend of increased initial severity in young patients. Previous unpublished data from this laboratory has found that the spinal cord grows consistently with no difference in the height: width ratio or the proportions between different sections as the cord grows (personal communication, 2014). This indicated the differences are in the mechanical properties and, importantly, the cellular and molecular properties, such as those addressed above.

2.1.3 Concluding remarks

There is clear evidence that the anatomy and biomechanics of the developing spinal column/cord influence the severity of injury. There are also differences described for age-related cellular response to experimental SCI in animal models however, while there are many established behavioural analyses for adult rats and mice and these have been extensively used since their inception, many of these have not been applied to infant models.

There is a trend in our own observations and in the literature, of infants having a different recovery, often better and faster than their adult counterparts. However, this observation hasn't been extensively studied or quantitatively confirmed, due to the technical difficulties in creating comparable scoring systems. In this chapter we use

hind-limb locomotor scoring in the open field to quantify the age-related differences in functional recovery following mild SCI; and also investigate our observations that the infants have a better recovery and cannot be easily distinguished from their respective shams based on their behaviour and hind-limb function.

2.2 HYPOTHESIS AND AIMS

2.2.1 Hypothesis

Distinct differences exist in the functional recovery between adult and infant rats. The infants will exhibit a quantitatively better recovery, compared to their mature counterparts using hind-limb locomotor scoring systems.

2.2.2 Aims

1. To test the applicability of two different scoring systems, the Basso, Beattie and Bresnahan (BBB) scoring system and a simplified infant scoring system, for rats injured at post-natal day 7 (P7).
2. To compare the functional recovery in infants injured at P7 and their adult counterparts using open field behavioural scores over a six week period after injury.
 - a. To determine whether a significant difference can be found between SCI animals and their respective shams, in both adults and infants.
 - b. To explore any differences in functional recovery, as quantified by open field locomotor scoring, between adult and infants injured at P7.

2.3 MATERIALS AND METHODS

2.3.1 Surgery and euthanasia

The surgical procedures, and open field and error ladder behavioural recordings, had been previously undertaken in this laboratory. These procedures were performed under animal ethics approval (UTS ACEC 2013-069/2013-048) on adult/juvenile female Sprague-Dawley rats (ARC, Perth Australia). Infant rats were bred in house, and both sexes were used. These rats were injured at 1wk, 5wk or 9wk of age, and euthanized at 24h, 1wk, 2wk and 6wk post- injury (Table 1). The animals in this table have also been used in the histological analysis detailed in Chapter 3. A small subset of rats were injured at post-natal day 10 (P10), these rats were kept for 6 weeks and associated shams were used to analyse the differences between these rats and the P7 groups.

Rats were anaesthetised using 2% Isoflurane and 1% oxygen. Anaesthetic (0.2ml Bupivacaine, s.c) and iodine were applied to the shaved thoracic region. Prior to the surgery commencing, the rats were given analgesics (Buprenorphine hydrochloride -Temgesic 0.03mg/kg, s.c), antibiotics (Cephazolin sodium 33mg/kg, s.c) and Hartman's replacement solution (Compound sodium lactate 15ml/kg, s.c). An incision was made through the skin at the dorsal midline from the mid to low thoracic region and subsequent layers of tissue parted to expose the spinal column. This was followed by a bilateral laminectomy of the T10 vertebrae to expose the required section of spinal cord. The rats were then moved to the MASCIS weight-drop device, (Basso et al., 1996) stabilised with clamps on the T9 and T11 vertebrae and subjected to a weight-drop contusion injury. The height of the drop and diameter of the impactor head varied between groups to account for different sizes of spinal cords, based on different ages

and stages of growth, in order to produce a comparable injury severity in animals of different sizes as can be seen in Table 2. The surgical incision was sutured closed in layers and the animals were allowed to recover in a warm cage.

Table 1: Table of animal groupings, as distinguished by age and injury status, as well as the numbers of animals in each group.

| Group | Age at Injury | Survival time | Age at Euthanasia | Number of animals |
|------------------------|---------------|---------------|-------------------|-------------------|
| Adult Sham & Normal | 9wk | day 0 | 9wk | 4 |
| | | 6wk | 15wk | 4 |
| Adult Mild SCI | 9wk | 24h | 9wk | 8 |
| | | 1wk | 10wk | 8 |
| | | 2wk | 11wk | 8 |
| | | 6wk | 15wk | 8 |
| Juvenile Sham & Normal | 5wk | 6wk | 11wk | 4 |
| Juvenile Mild SCI | 5wk | 24h | 5wk | 8 |
| | | 1wk | 6wk | 8 |
| | | 2wk | 7wk | 8 |
| | | 6wk | 11wk | 8 |
| Infant Sham | 1wk | 24h | 24h | 4 |
| | | 1wk | 1wk | 4 |
| | | 2wk | 3wk | 4 |
| | | 6wk | 7wk | 4 |
| Infant Mild SCI | 1wk | 24h | 1wk | 8 |
| | | 1wk | 2wk | 8 |
| | | 2wk | 3wk | 8 |
| | | 6wk | 7wk | 8 |
| P10 Infant Sham | 10 days | 6wk | 7wk | 5 |
| P10 Infant SCI | 10 days | 6wk | 7wk | 5 |

Post-surgery the mature animals received regular analgesics (Temgesic 0.03mg/kg, s.c), antibiotics (Cephazolin sodium 33mg/kg, s.c) and fluid replacement (Hartman's Solution (1ml/100g, s.c) and underwent manual bladder expression every 12h until normal voiding was observed. Pups were returned to their dams and monitored closely but were not otherwise disturbed. The animals were humanely

killed at different time points post injury using an overdose of Lethobarb (pentobarbitone, 1ml/100g, i.p) (Virbac Australia). After breathing had ceased the animals were then transcardially perfused with heparinised saline followed by a 4% paraformaldehyde solution to fix the tissue. Finally, the spinal cords were excised and post-fixed for 24h before storage. The excised spinal cords were stored in a phosphate buffered 30% sucrose solution and 0.1% sodium azide in a 4°C fridge labelled with the animals' unique number, the date and the initials of the practitioner. Histological analysis of the spinal cord tissue is outlined in Chapter 3.

Table 2: Table of injury parameters for animal groupings, as distinguished by age and injury status.

| Group | Age at Injury | Weight Drop Height | Impactor Head Diameter |
|----------------------|---------------|--------------------|------------------------|
| Adult Normal/Sham | 9wk | - | - |
| Adult Mild SCI | 9wk | 6.25mm | 2.5mm |
| Juvenile Normal/Sham | 5wk | - | - |
| Juvenile SCI | 5wk | 6.25mm | 2.0mm |
| Infant Sham | 1wk | - | - |
| Infant SCI | 1wk | 3.00mm | 1.5mm |

2.3.2 Behavioural analysis

In the week after their arrival each adult rat was acclimatised to the apparatus used for the behavioural testing. This occurred over a period of four days: on the first day 4 rats at a time were placed in the open field for 10min, on day 2 and day 3, two rats at a time were placed in the field for 10 min, on day 4 1 rat at a time was placed in the field for 5 minutes. Treats were used to encourage the rats to move about. The infants were not acclimatise to the open field before injury as this was impractical for such young animals.

Behavioural testing was conducted on the day prior to surgery to get 'baseline' reading for each animal. Analysis was then conducted on day 1, 3 and 7 post injury and once weekly for 6 weeks post-injury. The baseline scores could then be compared to the post-injury scores to assess the recovery rate and how much of this functioning they can recover. Three different scoring systems were used in this study; the established Basso Beattie and Bresnahan (BBB) Locomotor Rating Score a simple yes/no system to determine injury, and an infant specific scoring system developed in this lab.

2.3.2a Open field test

This behavioural test involved the rat being placed in a flat open field of approximately 90cm diameter, enclosed in 10cm Perspex walls. This allows the rat to move around freely and naturally. The rat was recorded for 2 minutes moving around the field. This digital recording was used to assess the locomotor function of the rats' hind-limbs and its overall stability using the Basso, Beattie and Bresnahan (BBB) scoring system (Bregman and Goldberger, 1982; Goldberger and Murray, 1985).

2.3.2b Scoring the open field test using the Basso, Beattie & Bresnahan hind-limb locomotor scoring system

The BBB Locomotor Rating Score (Figure 4) was used to assess stability and hind-limb motor function after injury. This was conducted blinded to the injury status of the rats using numbered recordings of the rats recorded for 2-3 minutes in the open field. Each adult and infant recording was assessed independently by at least 2 assessors. The juvenile recordings were assessed by one independent assessor.

A small subset of infant rats were injured at P10. These, along with their respective shams, were analysed using the BBB. Further comparisons between the P7 and P10 infants are expanded upon in Chapter 3.

Basso, Beattie, and Bresnahan Locomotor Rating Scale

| | |
|----|--|
| 0 | No observable hindlimb (HL) movement |
| 1 | Slight movement of one or two joints, usually the hip and/or knee |
| 2 | Extensive movement of one joint or extensive movement of one joint <i>and</i> slight movement of one other joint |
| 3 | Extensive movement of two joints |
| 4 | Slight movement of all three joints of the HL |
| 5 | Slight movement of two joints <i>and</i> extensive movement of the third |
| 6 | Extensive movement of two joints <i>and</i> slight movement of the third |
| 7 | Extensive movement of all three joints of the HL |
| 8 | Sweeping with no weight support or plantar placement of the paw with no weight support |
| 9 | Plantar placement of the paw with weight support in stance only (i.e., when stationary) or occasional, frequent, or consistent weight-supported dorsal stepping and no plantar stepping |
| 10 | Occasional weight-supported plantar steps; no FL–HL coordination |
| 11 | Frequent to consistent weight-supported plantar steps <i>and</i> no FL–HL coordination |
| 12 | Frequent to consistent weight-supported plantar steps <i>and</i> occasional FL–HL coordination |
| 13 | Frequent to consistent weight-supported plantar steps <i>and</i> frequent FL–HL coordination |
| 14 | Consistent weight-supported plantar steps, consistent FL–HL coordination, <i>and</i> predominant paw position during locomotion is rotated (internally or externally) when it makes <i>initial contact</i> with the surface as well as just before it is <i>lifted off</i> at the end of stance; or frequent plantar stepping, consistent FL–HL coordination, and occasional dorsal stepping |
| 15 | Consistent plantar stepping and consistent FL–HL coordination <i>and</i> no toe clearance or occasional toe clearance during forward limb advancement; predominant paw position is parallel to the body at initial contact |
| 16 | Consistent plantar stepping and consistent FL–HL coordination during gait <i>and</i> toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off |
| 17 | Consistent plantar stepping and consistent FL–HL coordination during gait <i>and</i> toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact <i>and</i> lift off |
| 18 | Consistent plantar stepping and consistent FL–HL coordination during gait <i>and</i> toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off |
| 19 | Consistent plantar stepping and consistent FL–HL coordination during gait, toe clearance occurs consistently during forward limb advancement, predominant paw position is parallel at initial contact <i>and</i> lift off, and tail is down part or all of the time |
| 20 | Consistent plantar stepping and consistent coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift off, <i>and</i> trunk instability; tail consistently up |
| 21 | Consistent plantar stepping and coordinated gait, consistent toe clearance, predominant paw position is parallel throughout stance, and consistent trunk stability; tail consistently up |

Figure 4: Extract from Basso, Beattie and Bresnahan (1996). Details of the BBB 21 point scoring system used in this study to analyse hind-limb locomotor function in adult and juvenile rats with a T10 contusion spinal cord injury.

2.3.2c Scoring the open field test using a novel infant rat locomotor score

Before conducting infant specific scoring, a test was performed by two independent assessors to see if, from simple observation of the behavioural recordings, it could be determined if the animal was injured or a sham. This was a simple yes/no test, quantified as yes=1 and no=0 and compared to the recorded injury status of the rats to arrive at a percentage correct.

Scoring the infants, especially for the Day 0 (DO) baseline, is challenging as the pups are yet to walk properly at this age (P6-P7). In a 2005 study by Brown et al. uninjured infant rats did not reach 'normal' adult BBB scores of 21 until postnatal day 21 (P21) so using traditional BBB is difficult (Brown et al., 2005). To try and combat this, infants were assessed using not only the traditional BBB but also a simplified, infant-specific scoring system (Infant Rat Locomotor Score, IRLS) developed in this laboratory. This scoring system uses 12 basic motions/behaviours to assess the overall locomotor function based on observations of movement shown in neonatal rats. An example of the scoring sheet used for IRLS assessment when viewing the behavioural recordings can be seen in Figure 5. Each behaviour was scored as 0 (absent), 0.5 (occasional) or 1 (present) in a point system. A small subset of infant rats were injured at P10. These, along with their respective shams, were also analysed using the IRLS to compare to the P7 infants.

| <u>IRLS</u> | Yes = | 1 Point |
|---|---------------------------|------------|
| | Occasionally/Incomplete = | 0.5 Points |
| | No = | 0 Points |
| <u>Behaviour</u> | <u>Score</u> | |
| Head lifted | | |
| Horizontal head movements | | |
| Vertical head movements | | |
| Crawling or walking with trunk free of the ground | | |
| Trunk stability when walking | | |
| Trunk held level from neck to tail, spine straight (vertically and laterally) | | |
| Adducted hind-limbs during walking (feet parallel at initial contact and/or lift-off) | | |
| Fine motor control of toes during lift-off | | |
| Rearing onto hind limbs | | |
| Grooming behaviours | | |
| Symmetry of hind-limb stepping/gait | | |
| Walking in a linear fashion (1) or uneven circular motion (0) | | |
| | Total = | |

Figure 5: Simplified scoring sheet for the Infant Rat Locomotor Score (IRLS) designed to score infant rats' hind-limb locomotor function and stability after an injury, used to score open field recordings.

2.3.2c Statistical analysis

For the BBB scores multiple assessors (2-4) analysed the recordings independently and linear regression was used to compare inter-rater reliability. Linear regression was performed between assessor 1 (TS) and all other assessors, as well as the average of other assessors (3-4). The IRLS was validated by a random sample of 8 recordings being assessed by multiple blinded assessors, and the results compared using regression.

For both age groups the BBB and IRLS scores of all of the cohorts were pooled at each time point after injury as they were consistent and indistinguishable from one another. This pooled data was analysed using two-way analysis of variance (ANOVA) with Bonferroni's post-hoc test, grouped with the injury status against the time post injury in each age group. Multiple t-tests were also performed on the two-way grouped data, before it was pooled.

2.4 RESULTS

2.4.1 BBB scoring of the open field assessment

2.4.1a Adult hind-limb recovery after spinal cord injury

The adult rats started with a baseline of 21, the maximum BBB score for a normal mature rat. This continued through the 6 weeks after surgery in the sham groups. The SCI groups dropped sharply after injury, with day 1 (D1) BBB scores below 10, gradually increasing over the subsequent weeks. The injured adults did not reach a normal score of 21 again in the 6 weeks (W6) after injury, peaking at around 16-18 (Figure 6).

The SCI adults were significantly impaired compared to their shams at all testing points from D1 to W6 after injury (Figure 7). This is very obvious at D1 after the injury and the gap between SCI and sham is slowly closed over time as the BBB scores of the SCI rats increased. The injured adults did not recover normal hind-limb functioning, and still scored significantly lower than their sham counterparts up to 6 weeks after injury (Figure 7). There was a significant variation over time ($P < 0.0001$), and between sham and SCI ($P < 0.0001$), as well as a significant interaction between the two factors using two-way ANOVA.

The BBB scores of all of the assessors, up to 4 for some recordings, were close for the adult rats. The R^2 of the linear regression between assessor 1 (TS) and each other individual assessor was between 0.71 and 0.91. The R^2 of the linear regression between assessor 1 and the average of all of the assessors was 0.93 for the adult groups. This validates the use of the BBB system in these animals as well as the application of this test in this lab, as all of the assessors are consistent in scoring the hind-limb function of these animals.

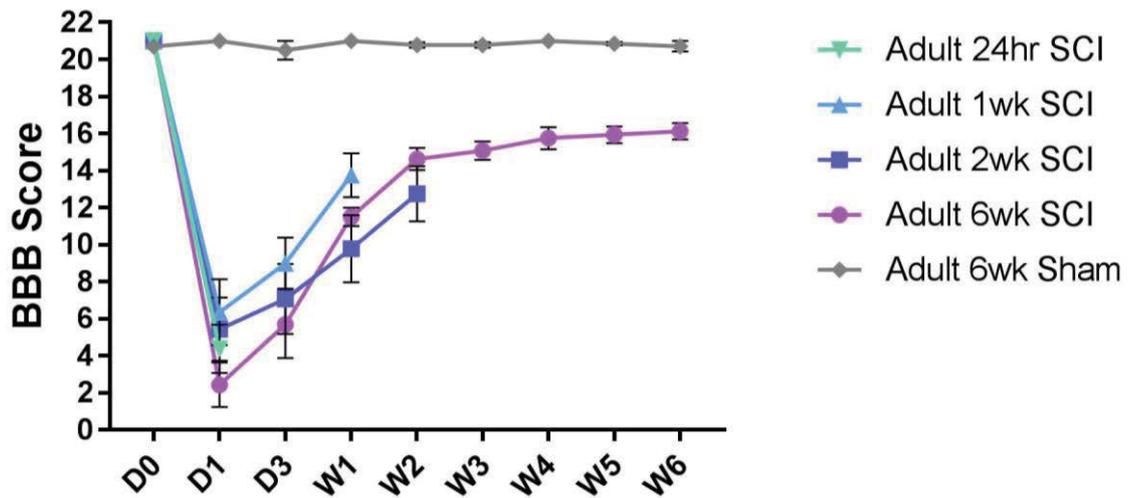


Figure 6: Line plot of the progression of functional recovery in the hind limbs of adult rats as determined by BBB scoring. This graph shows the mean and standard error of the mean for 5 groups of rats (N=8/group), classified by survival time. The maximum score achieved by a normal, uninjured rat is

21.

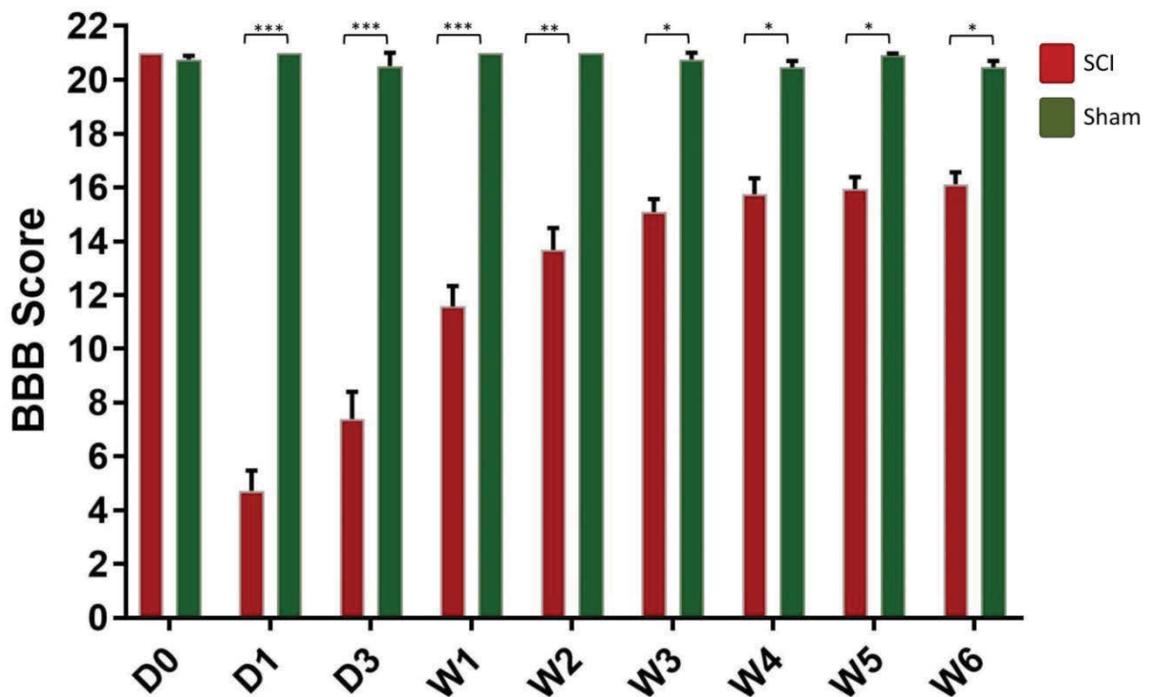


Figure 7: Histogram of the pooled BBB scores of the open field test in adult SCI rats compared with their shams, using means and standard error of the mean (SEM). This represents the combined data generated from four cohorts of rats with different survival times, therefore the N is different for different time points. Day 0 (D0) provides a baseline measure before injury. * (P<0.05), ** (P<0.005), *** (P<0.0001) indicate the statistical significance based on Bonferroni's post-hoc test.

2.4.1b Juvenile hind-limb recovery after spinal cord injury

On D0 the juvenile rats started with a baseline of the maximum BBB score for a normal mature rat. In the 6 weeks after surgery the sham groups maintained a score of 21. The BBB of the SCI groups dropped sharply after injury, with D1 BBB scores of 10 and under, gradually increasing over the subsequent weeks. The juvenile SCI rats did not reach a normal score of 21 again in the 6 weeks after injury, peaking at around 18-19 (Figure 8).

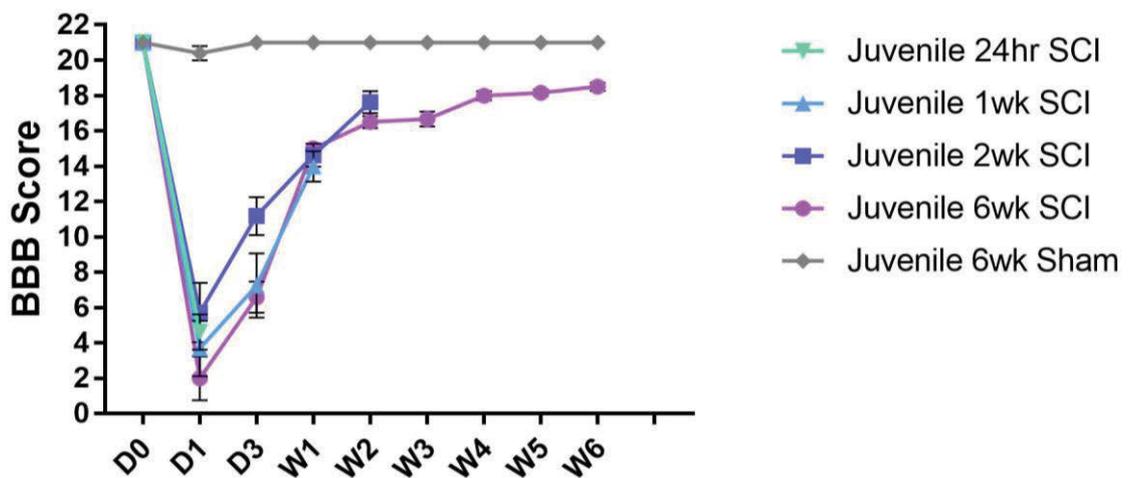


Figure 8: Line plot of the progression of functional recovery in the hind limbs of juvenile rats as determined by BBB scoring. This graph shows the mean and standard error of the mean for 5 groups of rats (N=8/group), classified by survival time. The maximum score for a normal, uninjured rat is 21.

The juvenile SCI were visibly impaired compared to their shams at most testing points from day 1 to week 6 after injury, however this was only statistically significant at D1, D3 and W1 (Figure 8). The gap between SCI and sham is slowly closed over time as the SCI rats increased from very low scores at D1 to plateau around 18, compared to the sham scores of 21 throughout. There was a significant variation over time ($P < 0.0001$), and between sham and SCI ($P < 0.0001$), as well as a significant interaction between the two factors ($P < 0.0001$) using two-way ANOVA (Figure 9).

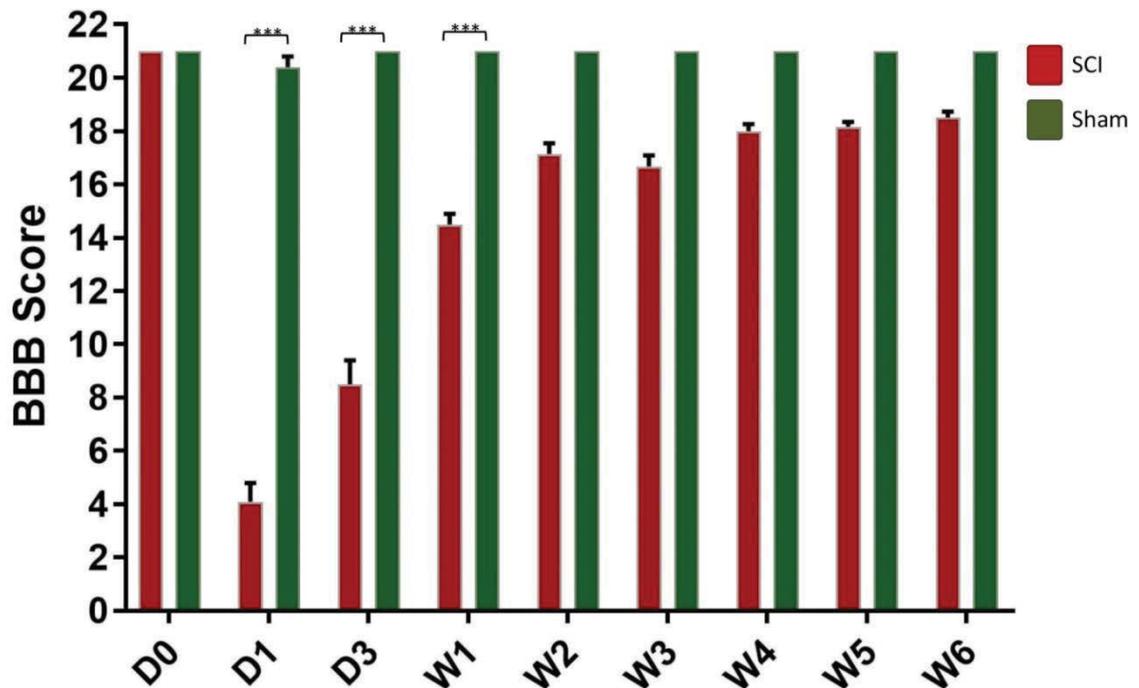


Figure 9: Histogram of the pooled BBB scores of the open field test in juvenile SCI rats compared with their shams, using means and standard error of the mean (SEM). This represents the combined data generated from four cohorts of rats with different survival times, therefore the N is different for different time points. Day 0 (D0) provides a baseline measure before injury. *** ($P < 0.0001$) indicates the statistical significance based on Bonferroni's post-hoc test.

2.4.1c Infant hind-limb recovery after spinal cord injury

The infants started off with a baseline of 8-10 on day 0 and only 12% of those assessed dropped below this on day 1 after the injury. There was no obvious difference in the progression of hind-limb functional development/recovery between the injured and sham animals in the infant groups (Figure 10). It was still not possible to reliably distinguish SCI from sham using the BBB scoring system. There was a significant variation over time ($P < 0.0001$) in the pooled infant data using two-way ANOVA (Figure 11) but not between SCI and sham using Bonferroni's post hoc or t-tests.

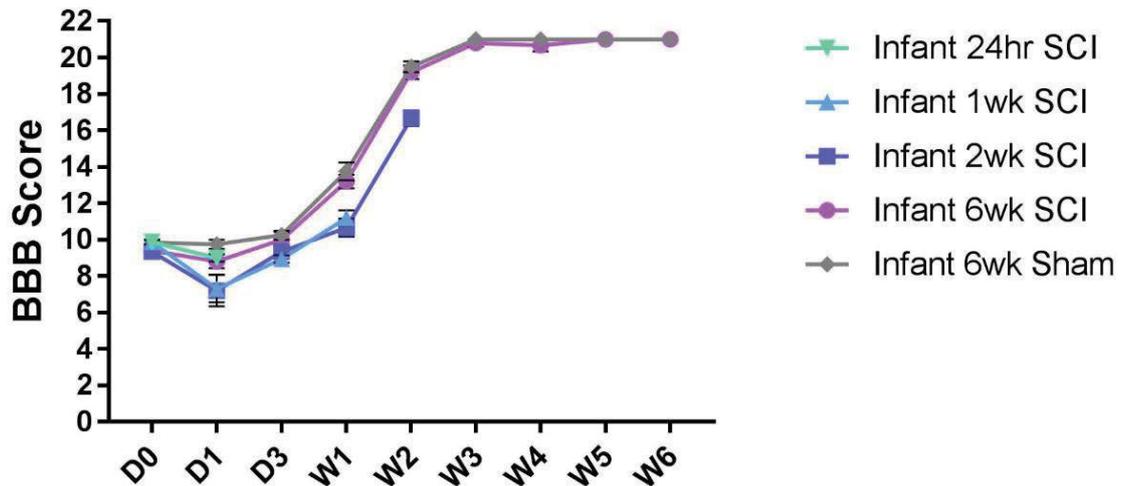


Figure 10: Line plot of the progression of functional recovery in the hind limbs of infant rats as determined by BBB scoring. This graph shows 5 groups of rats (N=8/group), classified by survival time. The maximum score achieved by a mature normal, uninjured rat is 21. These infants have a baseline score around 10 before surgery and do not reach a normal score of 21 until 2-3weeks after surgery, in both sham and injured groups.

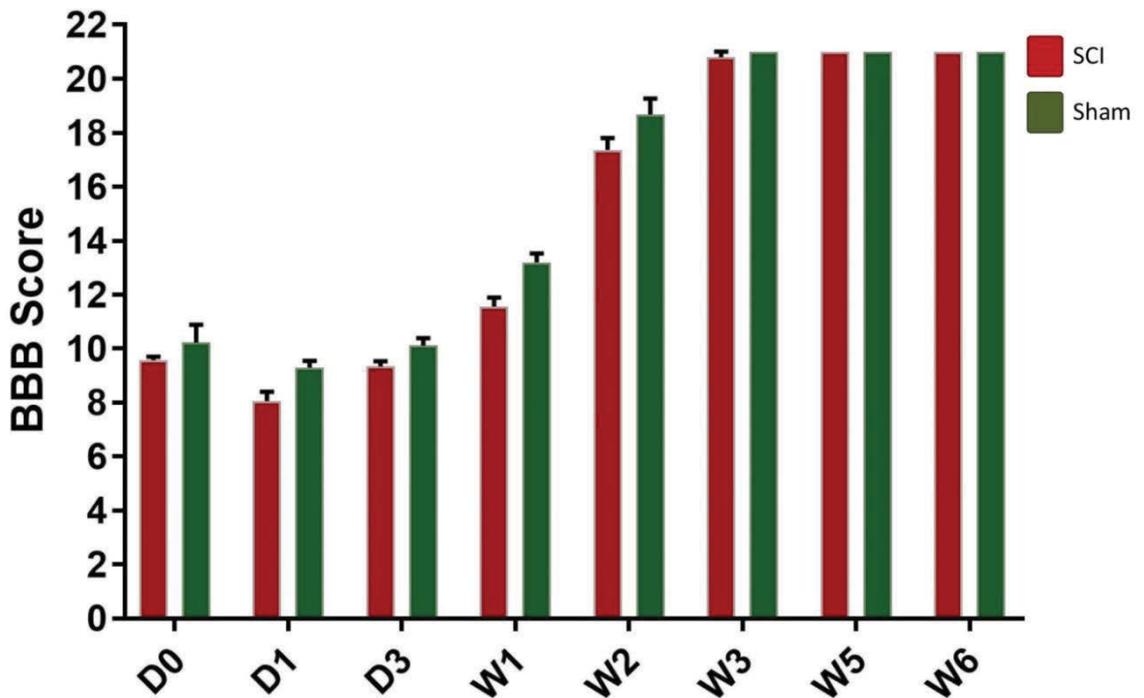


Figure 11: Histogram of the pooled BBB scores of the open field test in infant SCI rats compared with their shams, using means and standard error of the mean (SEM). This represents the combined data generated from four cohorts of rats with different survival times, therefore the N is different for different time points. Day 0 (D0) provides a baseline measure before injury. Due to technical difficulties there was no data generated from the week 4 recordings. There was no statistical significance between SCI and sham based on Bonferroni's post-hoc test.

The BBB scores of both independent assessors were relatively close for the infant rats, with a few outliers. The R^2 of the linear regression between assessor 1 (TS) and the second assessor was 0.82, taking into account all of the scores for the four SCI groups and their corresponding sham groups.

2.4.1d Comparison between adult, juvenile and infant hind-limb recovery

There were obvious differences in the hind-limb motor recovery between adult and P7 infant rats. The adult rats began with normal BBB scores of 21, which dropped significantly after injury and slowly recovered function without fully regaining normal scores (Figure 12). The infants, on the other hand, began with D0 BBBs of around 10 and had BBBs nearly indistinguishable from their shams after the injury. Both the injured and sham infants progressed from a BBB of around 10 to near normal scores by W3 post-injury (Figure 12). The juveniles followed a similar recovery curve to that seen in adults but with numerically better recovery scores. This difference between ages was not statistically significant (Figure 12). There was significant variation over time ($P < 0.0001$) and between the three ages ($P < 0.0001$), as well as interaction between these factors ($P < 0.0001$) using two-way ANOVA.

2.4.1e BBB comparison between P7 and P10 infants after injury

The P7 and P10 infants followed the same developmental curve using BBB scoring. There was very little significant difference between the P7 and P10 cohorts after injury, or in the sham groups. The BBB scoring could not distinguish injured from sham despite gross and histological confirmation of injury. The P10 infants' recovery was slightly slower, with lower post-injury BBB scores than their P7 counterparts at W1, W2 and W3. This was statistically significant at W2 and W3 ($P < 0.05$) based on Bonferroni's post-hoc test (Figure 13). By W5 post injury there was no discernible

difference in locomotor behaviour between infants injured at P7 and P10, or between SCI and sham groups.

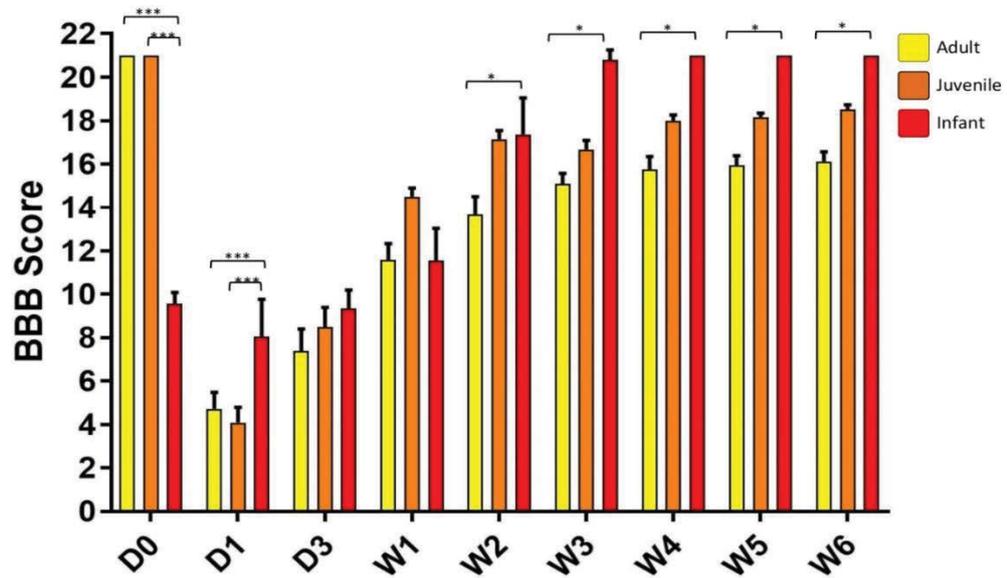


Figure 12: Histogram of the pooled BBB scores of the open field test in adult, juvenile and infant rats after SCI, using means and standard error of the mean (SEM). This represents the combined data generated from four cohorts of rats with different survival times, therefore the N is different for different time points. Day 0 (D0) provides a baseline measure before injury. * (P<0.05) and *** (P<0.0001) indicate the statistical significance based on Bonferroni's post-hoc test.

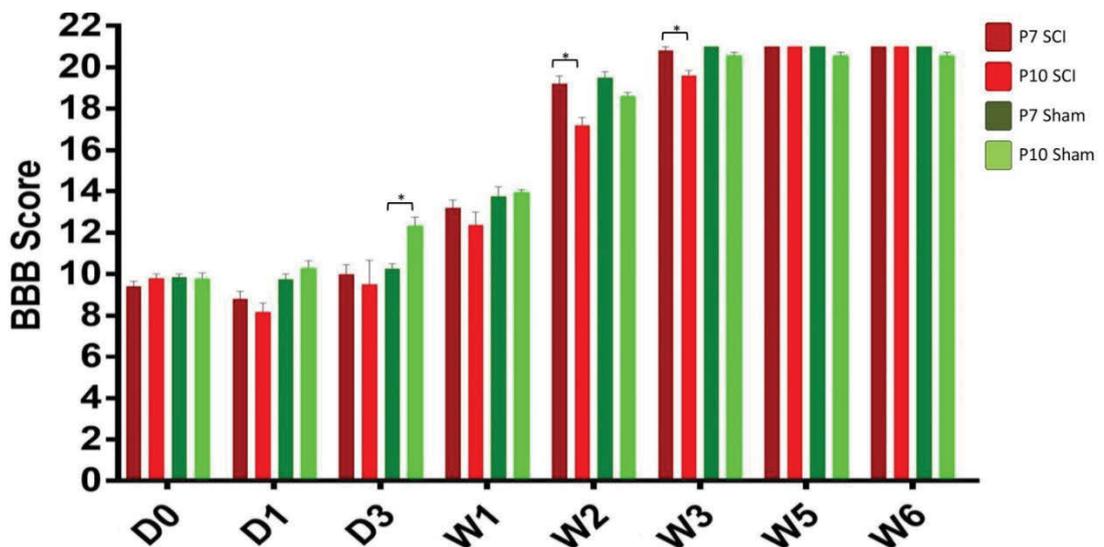


Figure 13: Histogram of the BBB scores of the open field test in both P7 and P10 infant SCI rats compared with their shams, using means and standard error of the mean (SEM). Day 0 (D0) provides a baseline measure before injury. Due to technical difficulties there was no data generated from the week 4 recordings. * (P<0.05) indicates statistical significance based on Bonferroni's post-hoc test.

2.4.2 Blind determination of injury presence in infants

The infants also didn't move around much during the 2-3 minute recordings, so assessing hind-limb motion and stability was often challenging. Before applying the BBB or infant specific scoring system (IRLS) two blinded observers conducted a basic yes/no test to attempt to determine injury status of the infant rats from the open field recordings. At D1 and D3 after injury assessors could determine injury status with an average of over 80% correct. The percentage correct dropped quite drastically to around 55% at 1 and 2wk post-injury. From W3 onwards the percentage correct was 50% or less, which is just even odds for a yes/no test (Figure 14). The times when the determination was incorrect were almost exclusively an injured animal being determined to be a sham, with very few exceptions. The raw data for this can be found in the Appendix.

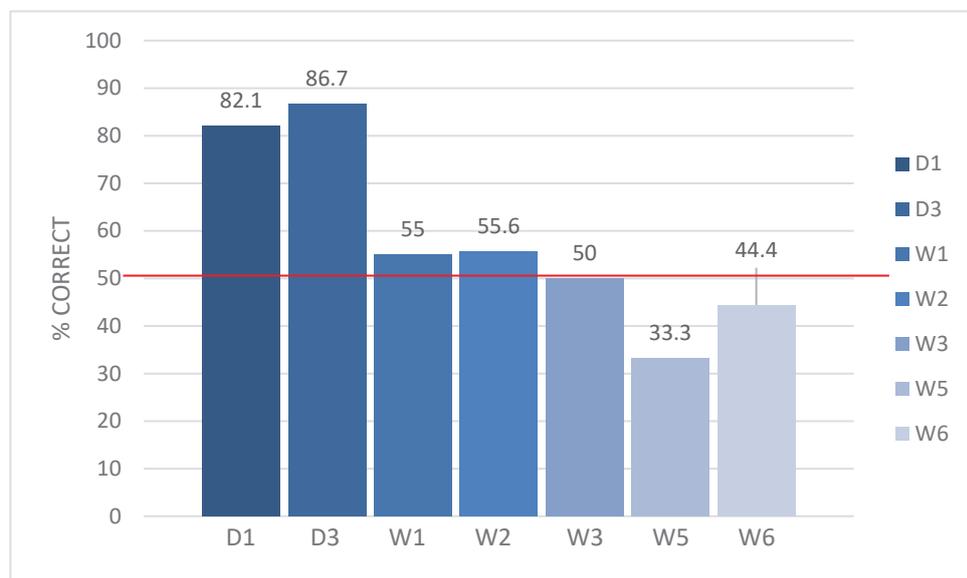


Figure 14: The percentage of infant rats correctly identified as either SCI or sham by two blinded assessors of the open field behavioural recordings.

2.4.3 Infant Rat Locomotor Score (IRLS)

Due to the BBB being unable to discriminate between injured and sham infants a new scoring system that used simpler, and infant-specific, movements was developed. This simpler scoring system specific to infant rats was used alongside the BBB to attempt to discriminate the SCI from sham rats, and chart recovery. Using the 12 point IRLS developed in this laboratory a similar pattern to the infant BBB can be observed. There was a significant variation over time ($P < 0.0001$) and between injury and sham ($P = 0.01$) in the pooled data using two-way ANOVA, but no significant interaction between the two factors ($P = 0.15$). Bonferroni's post-hoc test showed the significance was at day 1 and day 3 only and this was reinforced using t-tests (Figure 15).

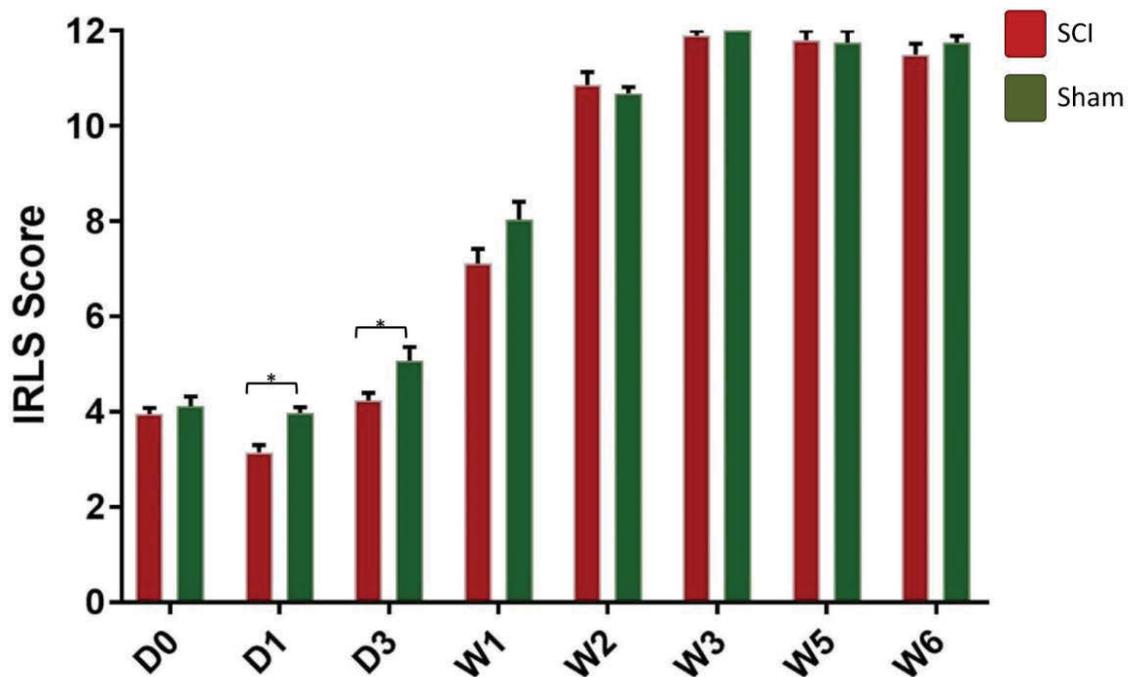


Figure 15: Histogram of the IRLS scores (/12) of the open field test in infant SCI rats compared with their shams. This represents the combined data generated from four cohorts of rats with different survival times, therefore the N is different for different time points. Day 0 (D0) provides a baseline measure before injury. Due to technical difficulties there was no data generated from the week 4 recordings. There was no statistical significance based on Bonferroni's post-hoc test. * ($P < 0.05$) indicate statistical significance based on t-tests.

The IRLS was validated using linear regression on a random sample of eight recordings assessed by multiple blinded assessors. The R-squared value of the linear regression was between 0.81 and 0.94, indicating high similarity between assessors without any training or background (Figure 16).

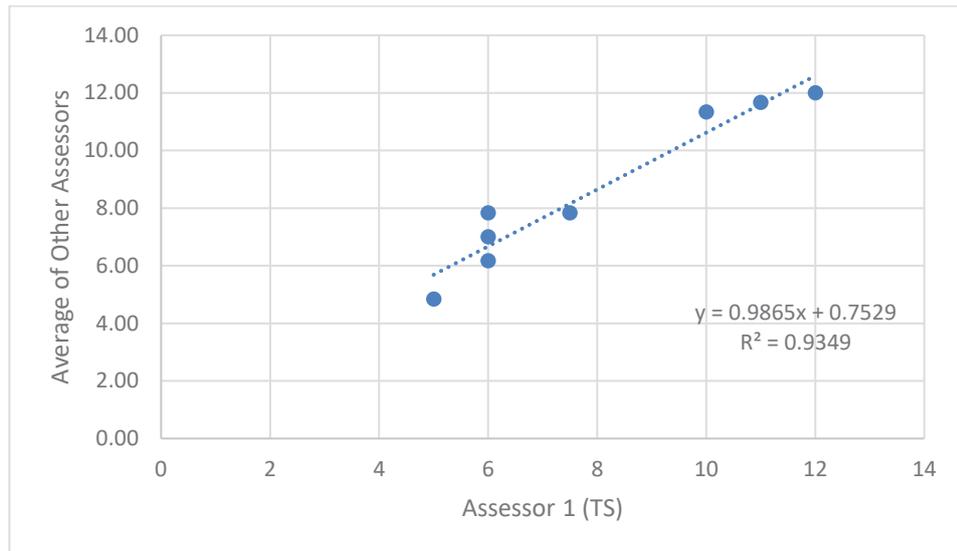


Figure 16: Scatter plot with linear regression comparing my own IRLS scores to the average of all other assessors. The inter-rater reliability is good with an R^2 of 0.94.

There was little visible difference between the infants injured at P7 and P10, and their respective shams, using the IRLS scoring system. The only statistically significant differences were those already noted between P7 SCI and sham groups at day 1 and day 3 post-injury, using Bonferroni's post hoc test (Figures 15 & 17). There was a highly significant difference over time ($P < 0.0001$), some cumulative significance between age and injury groups ($P = 0.0041$) and significant interaction between these factors ($P = 0.048$) using two-way ANOVA.

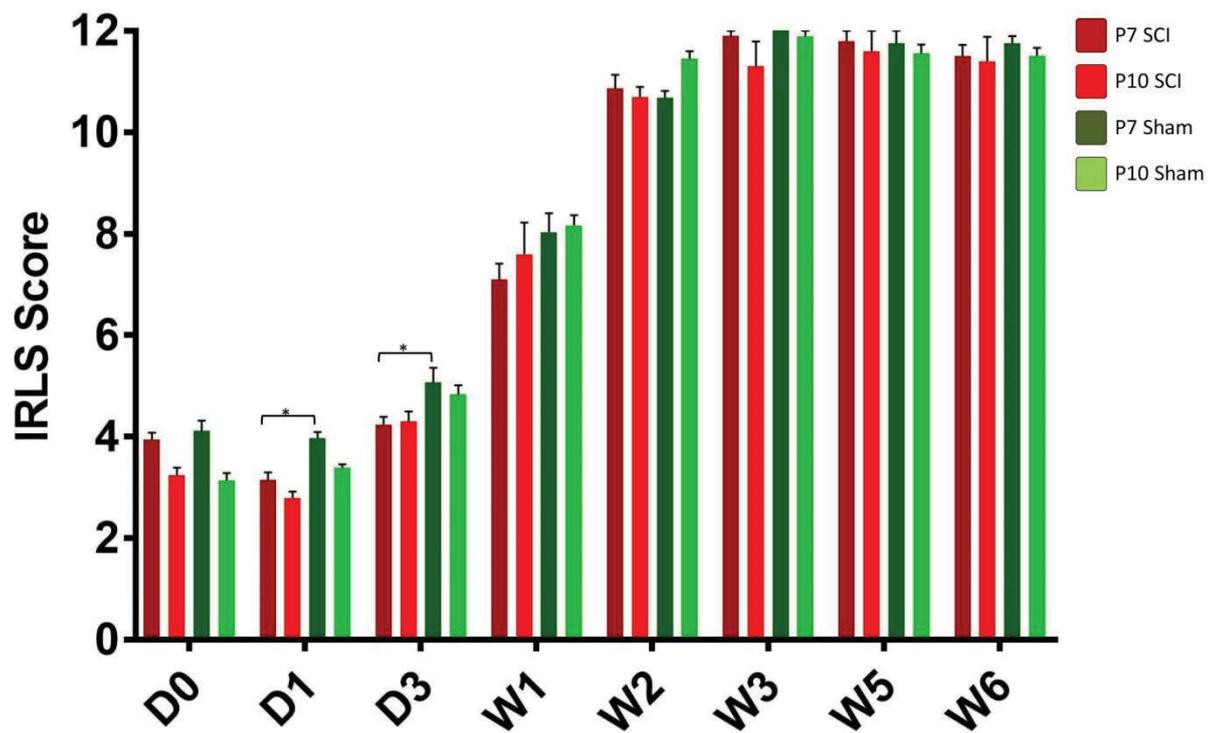


Figure 17: Histogram of the IRLS scores of the open field test in both P7 and P10 infant SCI rats compared with their shams. Day 0 (D0) provides a baseline measure before injury. Due to technical difficulties there was no data generated from the P7 week 4 recordings so this time point has been excluded from the graph. * ($P < 0.05$) indicates statistical significance based on Bonferroni's post-hoc test.

2.5 DISCUSSION

The adult rats in this study showed substantial locomotor deficit following SCI, characterised by no hind-limb stepping or an uneven hind-limb gait, splayed rear feet and loss of weight support and balance. This deficit improved slightly over time, as indicated by the restoration of weight support, some extent of hind-limb stepping and coordination however, the adults never fully recovered back to normal (BBB score of 21). The juveniles followed a similar pattern, namely that they showed a great locomotor deficit with BBBs of around 5 on day 1 after injury and recovered to scores of around 16-18 over 6 weeks; only slightly better outcome than that of the adults. Similarly, injured juveniles never returned to normal functioning. The infants were difficult to score using the established BBB scoring system. Therefore, a new system was developed in our laboratory for the analysis of infant rats. This system differed from the BBB system in its simplicity, by using broader behavioural indicators rather than minutiae of limb and joint movements, in an attempt to take into account the lack of normal movement in infant rats up to P21. Using three different scoring systems for the infant groups (BBB system, injured/uninjured score, and IRLS), a persistent locomotor deficit was unable to be reliably detected when the injury was induced at P7. However, during surgery there was gross confirmation of injury and we can see histological evidence that the injury is present (Chapter 3). A subgroup was injured at P10, and showed complete locomotor recovery over six weeks, as determined using both the BBB system and IRLS.

Observations from our laboratory corroborate previous studies suggesting that younger patients recover faster and more completely from a comparable SCI than their adult counterparts (Bregman and Goldberger, 1983a; Bregman and Goldberger, 1983b;

Bregman and Goldberger, 1983c; Brown et al., 2005; Parent et al., 2011). There are very few studies that directly compare infants to adults in either animal models or human clinical studies (Bregman and Goldberger, 1983b; Kissoon et al., 1990; Kumamaru et al., 2012; Lane et al., 2007; Nakamura and Bregman, 2001; Yuan et al., 2013). Nakamura and Bregman compared the gene expression profile of a variety of neurotrophic factors (NTF) in rats injured at P3, P7, P10 and P17 with that in mature adults, and their normal counterparts, to show a shift in NTF expression in normal developing cords between P10 and P17 that may play an important role in post-injury responses (Nakamura and Bregman, 2001). Lane et al. used an opossum model to compare morphological, cellular and molecular changes after SCI at either P7 or P14 and suggested that the greater ability of younger animals to recover from SCI may be due, at least in part to differing cellular and inflammatory responses (Lane et al., 2007). Kumamaru et al. compared the inflammatory response to SCI in mice injured at 4 weeks and 10 weeks of age and found age-related differences in the pro-inflammatory properties of the endogenous microglia to be potentially an important contributor to the more detrimental inflammatory response seen in adult animals (Kumamaru et al., 2012). Yuan et al. compared locomotor recovery and anatomical regeneration in rats injured at P1, P7, P14 and in maturity, and concluded that the increased functional recovery in neonatal rats was likely independent of spinal nerve regrowth across the transection lesion but rather due to intrinsic adaptations in the spinal circuitry (Yuan et al., 2013). Together these studies show a variety of differences in the response to SCI that may all contribute to the differences in recovery that were also observed. The sparsity of these studies is largely due to the inherent difficulties associated with using infant models of injury, and in creating an injury that is comparable between infant and adult animals. The size and developmental state of the infants engenders

challenges in creating a traumatic injury. This is because younger animals have much smaller cords, which are still growing and developing, as well as a more flexible spinal column (Kuluz et al., 2010) with room around the cord for it to move under the weight-drop. The post-natal age at which the injury is incurred is also of great importance and a source of great variability as the size and developmental state of the rats' spinal cord is rapidly changing.

Finding the optimal time to inflict an injury using a neonate model is problematic. The timeline of postnatal development differs between species, and finding an analogous time between animals and humans is even more challenging (Semple et al., 2013). Using rodent models of CNS injury raises the questions about which ages of rodent best correspond with different human ages and which aspects of development are most significant to match human analogues (Semple et al., 2013). Rodent models of brain injury have commonly utilised rodents of postnatal day 7-10 as analogous to a term human infant, however this is based on the measurement of the post-mortem brain dating to the 1970s (Semple et al., 2013). Spinal cord injury studies have used a wide variety of postnatal time-points from P7 to P21.

The state of overall development of the infants also creates difficulty in finding a comparable behavioural analysis between mature adults and the constantly changing infants. Both male and female infants were used because at the time of injury, and in early recovery, the hormonal cycles were not a confounding factor. As the rats matured this may change. However, for the measurements in this experiment, and the associated histology, we don't believe that this had a biologically significant impact. Further studies are being conducted that will examine this. The widely used BBB score for adult rat hind-limb function can be applied to infants with only limited success. A 2005 study by Brown et al, found that infant rats without an injury did not reach

normal adult behavioural scores using BBB (Basso et al., 1996) and combined behavioural score (CBS) (Kerasidis et al., 1987) until P21 (Brown et al., 2005). This makes scoring infants in SCI experiments, determining the behavioural deficits between SCI and sham infants, and comparing them to their adult counterparts extremely difficult. This is comparable to the results of this study; in which the infants had a BBB of around 9-10 at P7 before injury and both the shams and SCI cohorts reached a score of 21 at week three post-injury.

The young infants are challenging to score using the conventional BBB (with 22 criteria of locomotion) as they are neither very mobile nor do they have normal hind-limb gait and stepping. Therefore, a simpler behavioural scoring system was developed specifically for the infants. This was a 12-point system. However, the newly developed system failed to differentiate the SCI and sham cohorts. This suggests that a more tailored and detailed infant scoring system still needs to be developed for greater insight into the functional recovery of young rats after SCI. This new system should take into account the developmental curve of infant rats that we have seen in both the BBB and our own IRLS and attempt to find a middle ground between the detailed assessment of individual joints and motions that make up the BBB and the broader behaviours examined by the IRLS. One suggestion is to create a compound score from two assessments, a broader behavioural observation in conjunction with a closer anatomical observation. The development of an infant specific scoring system still would not allow for direct comparison to the adult BBBs, however a more detailed, compound scoring system can be made applicable to both infants and adults.

There are two possible interpretations of the results, which need further exploration. The first is that the neonates recovered substantially compared to their adult counterparts, the second is that the injury did not cause significant damage to

the neonatal spinal cord. This second possibility could be due to multiple factors, including the developmental state of the cord at and below the injury site and the plasticity of the developing cord. There has been little done to map the development of connections, the timeline of development and how this effects post-injury recovery (Firkins et al., 1993; Gianino et al., 1999; Joosten et al., 1987). One suggestion is that the recovery of stepping function in younger subjects may be independent of the regrowth of damaged spinal tracts and rely on plasticity of the developing spinal cord and adaptations of the spinal circuitry below the lesion (Yuan et al., 2013).

Previous literature, albeit limited, and the results of this behavioural study, raise the question: is P7 too early to induce the injury in a rodent model? In both the BBB and IRLS scoring systems there was no significant differences between the SCI and sham rats in the functional recovery. Both the SCI and sham followed the same recovery curve which suggest that it is not so much a recovery curve, but a developmental process. One possible explanation is that, while the key motor neuron tracts have reached below the 10th thoracic vertebral level (T10) injury site into the lumbar cord by P7 (Gianino et al., 1999; Joosten et al., 1987), they have not formed fully developed functional connections yet. This may be attributable, in part, to the greater plasticity in the infant spinal cord (Firkins et al., 1993; Pape, 2012; Raineteau and Schwab, 2001). The infant spinal cord has multiple significant differences to the mature cord, on a cellular, developmental and biomechanical level. All of these may come into play in explaining the different injury presentation and recovery in infants. Of especial interest is the plasticity of younger cords and the differences the cellular response to injury.

It is possible that we are seeing greater capacity for recovery from trauma in the infant spinal cord; it is also possible that what we are seeing as functional recovery is instead continuing development of the infant CNS independent of the injury.

2.6 CONCLUSIONS

The BBB scoring system developed for adult rats is not highly applicable or useful in developing animals. Using both the established adult BBB scoring system and a new, simplified infant scoring system developed in this study we were unable to establish a significant quantitative difference between injured and sham animals; with the exception of Day 1 and Day 3 post-injury using the IRLS, which showed a small significance based on t-tests. A more comprehensive system to score and assess hind-limb function and development in infant animals needs to be created. This would encompass a different range of overt behaviours, as well as closer examination of the hind-limb motions in a simplified version of the BBB applicable to the infant locomotor patterns and take into account the developmental aspect. P7 may be too early a time point to injure these rats. Using both the BBB and an infant specific system developed in this lab we could see no significant quantitative difference between SCI and sham infants. Both injury statuses showed the same developmental curve from around 10 at D0 to 21 (BBB) three weeks post-injury; this can be assumed to represent normal hind-limb motor development. This may imply that the normal development of the cord is taking precedence over any injury resolution at this early point in development. The results of this study may also indicate the increased capacity of the developing cord to recover from trauma.

This chapter highlights a very important issue when attempting to design comparable studies in traumatic injury; an issue that we were unable to address using one locomotor scoring system in this study. Further research is required using electrophysiological studies of the functional deficit in the short and long-term after SCI; combined with extensive nerve fibre tracing studies to better validate a neonatal model and timeline. In the following chapters we will investigate the histological and

inflammatory changes that occur in the adult and infant spinal cords after a traumatic injury, despite being unable to establish locomotor deficits in the neonates.

CHAPTER 3: THE PROGRESSION OF THE CELLULAR INFLAMMATORY, REACTIVE ASTROCYTIC AND ENDOGENOUS NEURAL PROGENITOR CELL RESPONSES TO SPINAL CORD INJURY IN DIFFERENT AGES OF RATS

3.1 INTRODUCTION

This chapter focuses on the elucidation of the response of key cellular players from the innate immune system and the CNS in SCI; the neutrophils, and early responding macrophages and microglia (3.1.1), as well as the eNPCs (3.1.3) and resident astrocytes using histological techniques.

3.1.1 The immune response in the central nervous system is unique and significant in the progression of spinal cord injury

Inflammation in the CNS has been receiving increasing research attention as it is being associated with a variety of neuropathology and neurodegenerative states. For a long time the CNS was considered an immune-privileged site. This contention has been rejected, and the brain has recently been implicated in immune-modulation and mediation in the periphery as well as the CNS. The privileged status of the CNS has been discarded with the developing of a deeper understanding of the unique immune responses that occur in the brain and spinal cord under different pathological conditions (Ransohoff and Brown, 2012). During development the relative isolation of the CNS conveys an advantage however in pathological conditions this differing immune response can be detrimental to recovery (Schwartz et al., 1999).

In the case of traumatic SCI or TBI, inflammation has been shown to have both neuroprotective and neurotoxic actions (Das et al., 2012; Ekdahl et al., 2003; Hohlfeld et al., 2007; Kigerl et al., 2009; Lucas et al., 2006; Popovich and Jones, 2003; Schwartz et al., 1999). The outcome of SCI is dependent on a myriad of factors, both intrinsic to the cell types present and extrinsic within the shifting microenvironment of the injury. This duality is the root of the complexity underlying the inflammatory response to traumatic injury and also in targeting elements of this therapeutically (Hohlfeld et al., 2007; Popovich and Jones, 2003).

The inflammatory cascade initiated by SCI follows a stereotypical sequence, necessary to begin the resolution of the injury. In the initial 24 hours post injury neutrophils are recruited from the circulation and the endogenous microglia and astrocytes are activated (Gensel and Zhang, 2015). This is followed by the migration of blood monocytes to the injury site in the next two to three days; these will differentiate into macrophages nearly indistinguishable phenotypically and morphologically from activated microglia (Gensel and Zhang, 2015).

3.1.2 The innate immune response plays an important role in secondary injury after spinal cord injury

The key effectors of the innate immune response in the CNS are the resident tissue phagocytes, the microglia, and the infiltrating neutrophils and activated macrophages (Figure 18) (Nguyen et al., 2002; Ransohoff and Brown, 2012). The early innate response to CNS insult is characterized by infiltration of neutrophils and it is generally accepted that this decreases neuronal survival and regeneration (Schwartz and Yoles, 2006). The next significant responder is the local innate immune cells, the

microglia. These cells, once activated, persist for long periods and the response is often extended beyond the capacity of the CNS to tolerate (Schwartz and Yoles, 2006).

The inflammatory response in SCI differs markedly from that in other tissues (Gensel and Zhang, 2015). In a normal wound healing and repair scenario outside the CNS there are generally considered to be three distinct phases, inflammatory, proliferative and remodelling, although some will add the haemostasis phase to the beginning of this (Adamson, 2009; Koh and DiPietro, 2011; Velnar et al., 2009). The different phases are associated with different macrophage phenotypes, classified as M1, M2a, M2b and M2c (Gensel and Zhang, 2015) as well as different cellular processes. During the haemostasis phase the intrinsic and extrinsic clotting mechanisms are activated and the inflammatory response is initiated. The inflammatory phase sees an influx of polymorphonuclear leukocytes, monocytes and macrophages to clear the wound site of debris and manufacture appropriate growth factors. This is followed by the proliferation phase which consists of processes of fibroplasia, matrix deposition, angiogenesis and re-epithelialization. The final phase is remodelling as the wound contracts and returns to an equilibrium of collagen and a functional homeostasis (Adamson, 2009; Koh and DiPietro, 2011; Mercandetti and Cohen, 2015; Velnar et al., 2009).

In typical somatic tissue injury macrophages and mononuclear phagocytes play an important role in repair and remodelling, not just the initial inflammatory phase (Brown et al., 2014; Mantovani et al., 2013). These cells arrive at the injury site in 24-48 hours and begin to clear the wound bed and initiate the processes that will finally lead to the formation of scar tissue (Brown et al., 2014). The interaction between mesenchymal stem cells (MSC) and mononuclear phagocytes has drawn greater attention in recent years as a two-way interaction has come to light (Cho et al., 2014;

Mantovani et al., 2013). It has been demonstrated that MSC profoundly influence the function and phenotypic expression of macrophages toward an M2 anti-inflammatory phenotype, while M2-like macrophages promote the growth of MSC and their motility towards injured tissue (Cho et al., 2014; Mantovani et al., 2013). Cho et al. (2014) postulated that the immunomodulatory effects of MSC mediated a preferential shift from M1 to M2 phenotypes in a myocardial infarction model (Cho et al., 2014). In the CNS NSPC may have a similar effect, potentially promoting better remodelling events (Cusimano et al., 2012). In non-CNS tissues of the body macrophages undergo dynamic changes in phenotype during the different phases of wound healing (Mantovani et al., 2013; Peiseler and Kubes, 2018). This does not appear to occur in the injured CNS.

During the inflammatory cascade that occurs post injury in the CNS microglia and astrocytes are activated and neutrophils are recruited in the initial 24 hours (Figure 18). Blood monocytes migrate into the injury site over the course of 2-3 days (Gensel and Zhang, 2015). These arrive by specific trafficking through the remote blood-CSF barrier at the ventricular choroid plexus of the brain (Brown et al., 2005) as well as through any disruption in the blood barriers resulting from the injury itself.

Microglia are a unique myloid cell population within the body, with multiple differences to their analogous cells in other tissues (Ransohoff and Brown, 2012; Schwartz et al., 1999). These cells represent 12% of the cell population in the brain, a relatively elevated level, which may have evolved as a compensatory mechanism for the unique immune status that the CNS exhibits (Schwartz et al., 1999). It has been acknowledged that activated microglia and macrophages can induce detrimental effects that are mediated by the pro-inflammatory molecules that they secrete, such as cytokines IL-6, IL-1 α /1 β and TNF- α , and chemokines (Shin et al., 2013). More recently 'alternatively active', or 'M2' macrophages have been seen to modulate

inflammation in the CNS (Shin et al., 2013). It is possible that while the CNS microglia drive the early, and deleteriously extended, pro-inflammatory response it is the infiltrating circulating macrophages that facilitate the M2 restorative phases (Brown et al., 2014). The prolonged pro-inflammatory M1 response induced by the activated microglia has been highlighted as potential driver of the poor remodelling that is observed in CNS trauma (Brown et al., 2014).

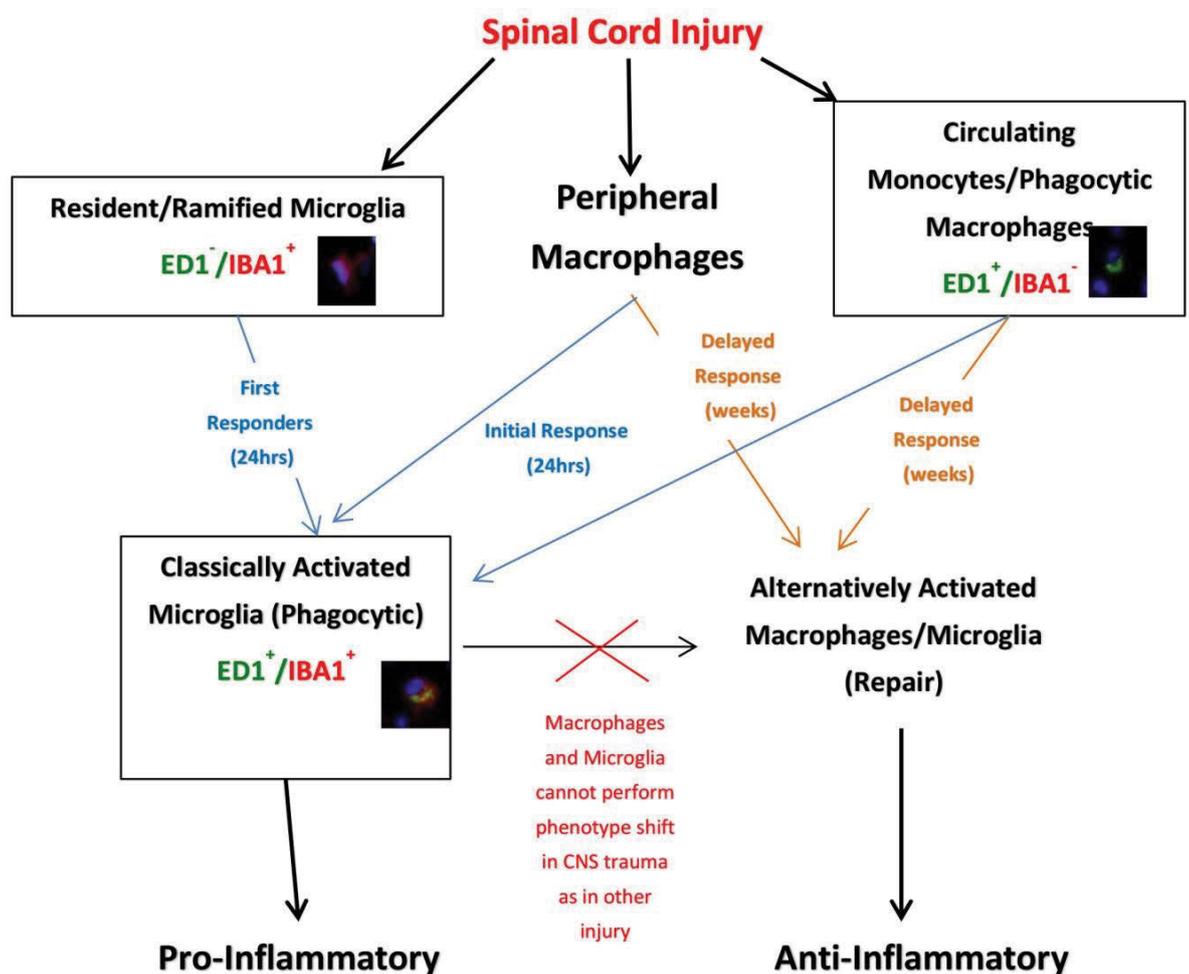


Figure 18: Basic map of macrophage and microglial responders to SCI with proposed identities of the 3 different immunoreactive phenotypes, from our previous data.

In contravention of the misleading and out-dated contention that the CNS is immune-privileged, immune and inflammatory reactions are common and are

modulated by the resident microglia and astrocytes (Ransohoff and Brown, 2012). These responses, however, occur in an isolated manner, irrespective of the normal innate and adaptive interplay, and the innate phagocytes in the CNS do not have a classic role of systematic antigen presentation or long distance signalling (Ransohoff and Brown, 2012). Since it was noted by Ehrlich in 1885 that intravenously injected dyes did not stain the brain (Ehrlich, 1885; Hawkins and Davis, 2005) and the subsequent characterisation of the blood-brain barrier (BBB) the CNS has held a privileged state within the body. The development of an understating of the Blood-CSF barrier (BCSFB) later highlighted the different regulation applied to the entry and exit of signals and molecules to the CNS (Shechter et al., 2009a). The development of a dogma of 'immune-privilege that resulted from these discoveries has since been challenged. The BBB and BCSFB allow for a tightly regulated and restricted interaction between the CNS tissues, tissue resident immune cells and the body's general immune system (Shechter et al., 2009a). There is currently robust evidence showing the presence of inflammatory and immune reactions within the CNS, from both the innate and the adaptive immune systems, which overturns the out-dated dogma of immune-privilege and highlights a robust and unique response.

3.1.3 The potential for stem and progenitor cells in the treatment of spinal cord injury

The use of transplanted cells and cell grafts is a broad, and ever expanding, area of research encompassing a wide variety of cell types as potential therapies (Willerth and Sakiyama-Elbert, 2008). Some, by no means all, of these potential targets include olfactory ensheathing cells (OEC) (Deng et al., 2006; Féron et al., 2005; Gorrie et al., 2010), Schwann cells from the peripheral nervous system (Guest et al., 1997),

oligodendrocyte progenitor cells (OPC) (Keirstead et al., 2005), bone marrow stromal cells (BMSC) (Chopp et al., 2000; Cizkova et al., 2009), and stem cells and progenitor cells derived from a variety of sources (Barnabé-Heider and Frisé, 2008; Cao et al., 2001; Karimi-Abdolrezaee et al., 2006; McDonald et al., 1999; Mothe et al., 2011; Ogawa et al., 2002; Tzeng, 2002). However, the use of these cells is complicated by the need for surgical intervention or an invasive delivery system, as well as issues about what cells to use, how to use them, and when to administer. Since the idea of transplanting various stem or progenitor cells originated the majority of the research was directed toward using these candidates in cell replacement therapies. The use of transplanted cells and cell grafts is a broad area of research encompassing a wide variety of cell types as potential therapies (Willerth and Sakiyama-Elbert, 2008).

Experiments in the early 1990s (Bregman et al., 1993) used the transplantation of foetal spinal cord tissue into the SCI lesion to support axonal growth in the host tissue. A multitude of studies have since isolated neural stem/progenitor cells (NSPC) from different sources for transplantation. These include embryonic, post-natal and adult spinal cords. From these studies it has been observed that transplanted NSPC can survive, migrate and differentiate following transplantation into SCI rats (Mothe and Tator, 2005), however delivery of these cells in human patients raises further complications in the choosing the optimal cell-type, the number of cells to transplant, the transplantation strategy and the timing of intervention (Ohta et al., 2004). Another complication that has been noted across the board in the use of transplanted NSPC in SCI is that these cells will preferentially differentiate along the glial lineage, especially into the astrocytic line, in the absence of any specific intervention. The differentiation of these cells can be directed *in vitro* by exogenous factors (Mothe and Tator, 2005) but there is still much work to be done *in vivo*. In 2001 Cao et al. observed that the

majority of pluripotent human embryonic stem cells (hECS) transplanted differentiated into astrocytes with some oligodendrocytes and undifferentiated cells present as well (Cao et al., 2001). One potential drawback from transplanted embryonic stem cells, that must be addressed is the formation of teratomas from the undifferentiated cells (Ronaghi et al., 2010).

Of the potential cells being explored for cell replacement therapies one class of cells that stands out are autologous olfactory ensheathing cells (OEC). These cells are the glial cells of the olfactory system responsible for ensheathing the axons of the olfactory sensory neurons. OEC are a promising candidate because they have the advantage of stemming from the patient's own body, overcoming the immune rejection issue. In rat models OEC have been found to survive and migrate after transplantation (Deng et al., 2006) and also to improve functional recovery (Gorrie et al., 2010). In a 2005 clinical trial Feron et al. found that these cells promote regeneration and enhance remyelination as well as being feasible and safe 1 year post-implantation (Féron et al., 2005). A three year follow-up also confirmed no adverse effects (Mackay-Sim et al., 2008). Promisingly, results were published in 2014 of a trial of transplantation of OECs with the simultaneous reconstruction of the spinal cord gap with peripheral nerve implants in one 38 year old patient suffering a traumatic transection of the spinal cord at T9 vertebral level. The trial took place 21 months after the injury, when the patient was exhibiting clinically complete SCI (ASIA A), and used OEC and olfactory nerve fibroblasts cultured from one of the patients own olfactory bulbs, resulting in an improvement to an ASIA C class injury. This was exhibited physically by improved trunk stability, partial recovery of the voluntary movements below the injury, and an increase in muscle mass in the left thigh. There was also partial recovery of both superficial and deep sensation (Tamaki et al., 2002).

Bone marrow stromal cells (BMSC) are stem-cell like cells found in the bone marrow that have the potential to differentiate along the neural path into the neuronal or glial lineage (Akiyama et al., 2002). These have also been demonstrated to improve the functional outcomes in rats following SCI (Chopp et al., 2000). In 2000 Chopp et al. transplanted BMSC into the SCI lesion and observed the increased proliferation of ependymal cells, and that some of the transplanted BMSC showed neural protein markers after transplantation (Chopp et al., 2000). Akiyama et al. (2002) used BMSC transplanted by direct microinjection into demyelinated CNS tissue to improve the remyelination of axons. The new myelin showed properties of both central and peripheral myelin under electron microscopy and the conduction velocity showed improvement post-treatment (Akiyama et al., 2002). However, it must be noted that this was a demyelinated plaque with intact neurons to provide a base, not a SCI with destroyed neural structure.

Remyelination has been the focus of a subset of transplantation studies with the theory that transplanted cells could promote remyelination and improve signal conduction in the injured area. Keirstead et al. (2005) experimented with the transplantation of hESC-derived OPCs. These cells were observed to survive and differentiate into oligodendrocytes (Keirstead et al., 2005). Another similar study used adult brain-derived NPCs from transgenic mice, along with a cocktail of pharmaceutical interventions to enhance the survival of these cells (Karimi-Abdolrezaee et al., 2006). In this study approximately 50% of the surviving NPCs formed OPCs or mature oligodendrocytes and were able to assist in myelinating axons (Karimi-Abdolrezaee et al., 2006). In the peripheral nervous system Schwann cells are responsible for myelinating the axon shafts. In 1997 Guest et al. experimented using transplanted human Schwann cells, alongside the administration of the corticosteroid anti-

inflammatory drug methylprednisolone, and found some regenerative growth and improved function (Guest et al., 1997).

Another class of cells that have been extensively studied in this area are cells isolated from foetal and infant spinal cords. Tzeng et al. (2002) isolated NPC from newborn rat spinal cords and found them to differentiate to astroglia and neurons in vitro, to survive and migrate caudal, and rostral, to the lesion when transplanted into the injured rat spinal cord (Tzeng, 2002). Studies have also used foetal stem cells to integrate with the host spinal cord and assist functional improvement (McDonald et al., 1999). In the area of NSPC there are a few cell-lines in various stages of clinical trial. Currently StemCells Inc. have sponsored phase I and II trials for the intermedullary spinal cord transplantation of human CNS derived stem cell (HuCNS-SC) neurospheres in thoracic SCI patients (NCT01321333) (<https://clinicaltrials.gov/ct2/show/NCT01321333>). Recently FDA approval was also given to Neuralstem Inc. to conduct a phase I safety trial for the use of their proprietary NSC, derived from the upper thoracic and cervical regions of an 8 week old human foetus, in treatment of chronic spinal cord injury (NCT01772810) (<https://clinicaltrials.gov/ct2/show/NCT01772810>). To date only one trial has been performed using human embryonic stem cell (ESC) derived neural cells in a clinical setting for CNS injury. This was a phase I safety trial for ESC-derived OPC in neurologically complete SCI at the subacute stage and was stopped by its sponsor Geron Corporation after only a year (NCT01217008) (<https://clinicaltrials.gov/ct2/show/NCT01217008>). As of 2013 Asterias Biotherapeutics Inc. resumed this aborted trial and found no adverse events related to AST-OPC1 administration in a phase 1 trial and received US FDA approval for a phase 1/2a clinical trial in patients with complete cervical injury (SCiSTAR) in 2014 that

reported preliminary efficacy data in 2016 (<https://clinicaltrials.gov/ct2/show/NCT02302157>).

3.1.4 The emerging importance of ‘cross-talk’ between nervous and immune systems

In recent years a high degree of interaction and ‘cross-talk’ has been highlighted between the nervous and immune systems (Cusimano et al., 2012; Kokaia et al., 2012; Ziv et al., 2006). In models for stroke the theory has been raised that MSC release neurotrophic factors (NTF) such as BDNF, that provide trophic support for vulnerable neurons, support oligodendrogenesis, reduce the neural oedema and regulate the anti-inflammatory response (Pluchino and Cossetti, 2013). In the last 10 years multiple studies have also found NSPC capable of attenuating brain inflammation and also decreasing microglial activation, in models of MS and stroke, when injected via various biological fluids into rodents and non-human primates (Pluchino and Cossetti, 2013). There is mounting evidence that glial and stem/progenitor cells of the CNS can switch to what Hauwel et al. referred to as a ‘chaperone’ phenotype to assist in tissue repair via inherent regulatory and protective activities, likely mediated by growth and trophic factors in the microenvironment (Hauwel et al., 2005).

Of particular interest here is the emergence of a significant role for NSPC as mediators and modulators of neuroinflammation and the progression of the immune response within the CNS following trauma (Figure 19). NSPC can exert a beneficial effect post-trauma through immunomodulation and the provision of trophic support for the surviving neurons and glia (Kokaia et al., 2012). Both transplanted and endogenous NSPC can engage in cross-talk with the immune system to help instruct reparative strategies with the immune effector cells (Martino et al., 2011). Martino et al. found that transplanted NSPC in the injured spinal cord secreted a variety of

neurotropic and effector molecules; one theory is that transplanted NSPC can be therapeutically efficacious via bystander, or paracrine, mechanisms. This contention is supported by the behaviour of NSPC after transplantation; the scarce and inappropriate terminal differentiation, propensity for maintaining an undifferentiated phenotype and the co-localisation with immune cells (Martino et al., 2011). This places greater emphasis on the synergy between these progenitors and the cells of the immune system as a therapeutic avenue, rather than the use of NSPCs for cell replacement. Alongside the emergence of a theory of ‘protective auto-immunity’ and the robust innate immune response to SCI the role of NSPC in modulating the immune response is highly significant.

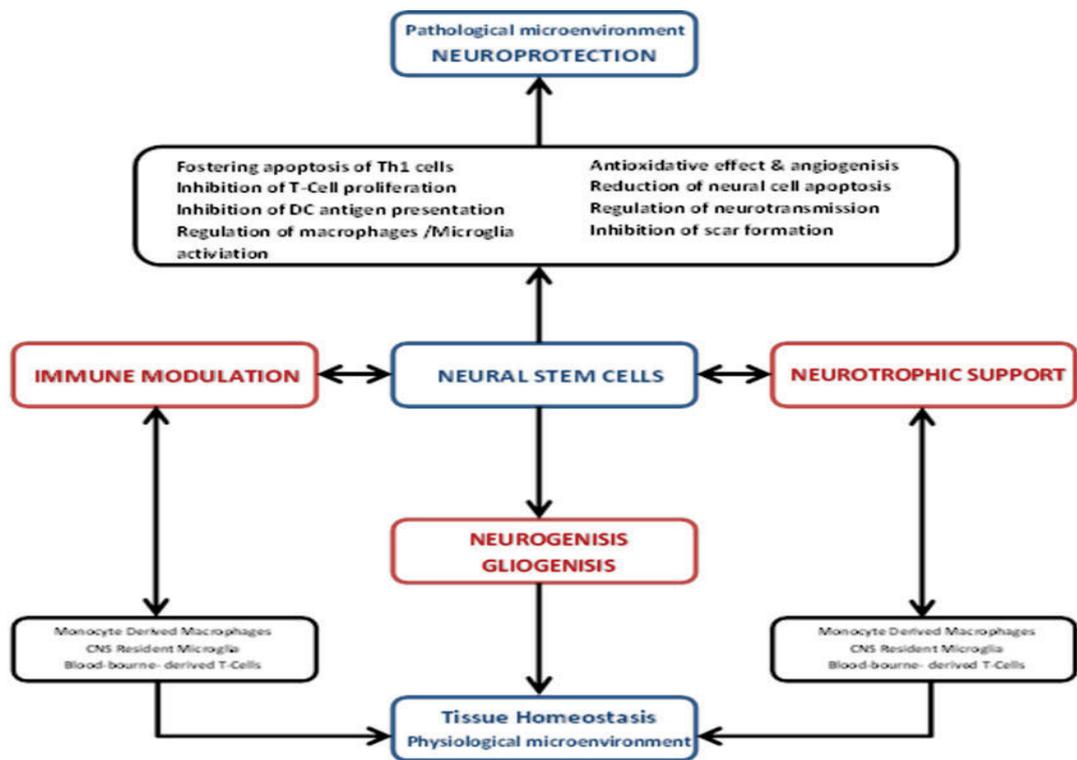


Figure 19: As studies delve further into the area more potential roles for NSPC in the regenerative and reparative phases after injury emerge. This figure highlights the potential interaction of NSPCs with the immune responses as immunomodulators and contributors to shifts in the cellular microenvironment.

(Adapted from (Martino et al., 2011)

One cell type that has received significant attention in recent studies is the mesenchymal stem cell, most commonly the BMSC. To date BMSC have been exploited in experimental autoimmune encephalomyelitis (EAE), the animal model for MS, as well as in TBI and SCI. These have reported an immunosuppressive function as well an ability to attenuate astrocyte reactivity and chronic microglial/macrophage activation (Taoka et al., 1997). A tentative conclusion exists that MSC can modify the inflammatory environment in an acute setting and reduce the inhibitory effects of reactive astrogliosis and the glial scar in a chronic setting (Taoka et al., 1997). In 2014 Urdzikova et al. demonstrated that human MSC transplanted 7 days post injury improved the functional recovery of rats through the modulation of the inflammatory reaction, prevention of apoptosis and remodulation of glial scar formation (Urdzíkova et al., 2014). The results of this study agree with previous work (Cizkova et al., 2009; Mothe et al., 2011). This study also concluded that the triggered changes in the microenvironment may be somewhat transient and may therefore require repeated transplantations (Urdzíkova et al., 2014).

3.1.5 Neural stem and progenitor cells' potential as modulators of the ongoing response to spinal cord injury

While the studies introduced in section 2.1.2 above have classically targeted NSPCs for cell replacement and structural enhancement treatments after SCI, a more significant role in the post-injury environment is emerging, i.e., the role of modulators of the neuroinflammatory response (Cusimano et al., 2012; Kokaia et al., 2012). The term 'therapeutic plasticity' was coined to describe NSPCs emerging abilities to modulate their surrounding environment, as well as respond to its cues, and instruct other innate and immune cells after trauma (Cusimano et al., 2012; Kokaia et al.,

2012). NSPCs can exert a beneficial effects on SCI repair by acting as immunomodulators and providing trophic support (Kokaia et al., 2012). A study by Cusimano et al. in 2012 highlighted the ability of transplanted somatic mouse NPCs to survive undifferentiated in the peri-lesion environment and to modulate the expression of inflammatory gene transcripts. The subacute (7 days) transplantation of these NSPC resulted in a reduction of the M1-like macrophages in the lesion area and the establishment of cellular junction coupling between NSPC and the endogenous phagocytes, with the ultimate result of improved healing and repair (Cusimano et al., 2012).

The study by Chopp et al. (2000) previously mentioned also found that the injured tissue provided a microenvironment conducive to transplantation of stem or progenitor cells because the injured CNS tissue appeared to regress to a developmental state (Chopp et al., 2000). This supports the idea that NSPC have a more complex modulatory role in injury development and resolution than merely replacing lost cell density.

Of particular interest here is the emergence of a significant role for NSPC as mediators and modulators of neuroinflammation and the progression of the immune response within the CNS following trauma. NSPC can exert a beneficial effect post-trauma through immunomodulation and the provision of trophic support for the surviving neurons and glia (Kokaia et al., 2012). Both transplanted and endogenous NSPC can engage in cross-talk with the immune system to help instruct reparative strategies with the immune effector cells (Martino et al., 2011). Martino et al. found that transplanted NSPC in the injured spinal cord secreted a variety of neurotropic and effector molecules; one theory is that transplanted NSPC can be therapeutically efficacious via bystander, or paracrine, mechanisms. This contention is supported by

the behaviour of NSPC after transplantation; the scarce and inappropriate terminal differentiation, propensity for maintaining an undifferentiated phenotype and the co-localisation with immune cells (Martino et al., 2011). This places greater emphasis on the synergy between these progenitors and the cells of the immune system as a therapeutic avenue, rather than the use of NSPCs for cell replacement. Alongside the emergence of a theory of 'protective auto-immunity' and the robust innate immune response to SCI the role of NSPC in modulating the immune response is highly significant.

3.1.6 Central nervous system insult in immature subjects differs from that in adults

As previously mentioned in Chapter 1 (section 1.4) there is a trend of better functional recovery in younger animals contrasted with mature animals with a comparable injury. The reasons for this are not yet understood. The differences between developed adult cords and the developing spinal cord are many and varied, and yet to be adequately explained. During mammalian spinal cord development there are changes in the expression and function of different receptors and transcription factors (Shibata et al., 1997; Yamamoto et al., 2001). Another important aspect of the developing spinal cord is the differences in expression of neurotrophic factors (Bregman et al., 1997; Nakamura and Bregman, 2001). The expression of neurotrophic factors, especially the increased brain-derived neurotrophic factor (BDNF) and decreased ciliary neurotrophic factor (CNTF), differs between the developing and mature spinal cords after injury and this may contribute to increased functional recovery (Nakamura and Bregman, 2001). There is not a great deal supporting this concept in the literature; however, it is an example of the molecular differences

between mature and developing cords and also of the possibility of modulating the cellular niche of the SCI to mirror a younger cord and promote better neural outcomes.

Another significant difference noted in developing cords are the different properties of the spinal cord such as less myelin and a lower proportion of white to grey matter and the increased plasticity of the infant cord (Brown et al., 2005). The developing cord is also protected by developing vertebrae and a hypermobile spinal column which is less likely to fracture and cause direct tissue trauma than the rigid adult vertebrae and spinal cord (Kuluz et al., 2010). In a biomechanical study Clarke et al. (2009) showed similar stress/strain responses between adult and neonate rats however with a higher modulus the strain had greater effect on the adult cords in vitro (Clarke et al., 2009). This study also showed that even with a lower modulus the neonate showed a greater degree of deformation, possibly contributing to the trend of observed initial severity in SCI in young subjects. In another study Clarke and Bilson (2008) observed that the haemorrhage volume did not differ significantly between adults and infants. This study also found that the normalised axonal injury density was higher in neonates however the neonates recovered more rapidly following the contusion (Clarke and Bilston, 2008).

In a review of 122 cases of spinal cord and vertebral injuries in America Hadley et al. (1988) defined four distinct injury patterns; fracture only, fracture and sublexation, sublexation only and spinal cord injury without radiographical abnormalities (SCIWORA). This review agreed that there are several anatomical and biomechanical features of the paediatric spinal cord that distinguish it from the adult and influence the injury patterns (Hadley et al., 1988). SCIWORA is far more common in paediatric subjects than in adults with the innate elasticity of the developing spinal cord rendering it especially vulnerable to deformation (Pang and Wilberger Jr, 1982). The damage in

SCIWORA arises from flexation, hyperextension, longitudinal distraction and ischemia (Pang and Wilberger Jr, 1982).

The immature brain has a distinctive inflammatory response and also different vasculature to the mature brain which conveys a unique vulnerability to TBI (Potts et al., 2006). The inflammatory response has been observed to differ in developing cords as well (Kumamaru et al., 2012) resulting in a widely observed faster and fuller functional recovery in younger subjects. In a model of neuropathic pain in the dorsal root ganglion Vega-Aelaira, Geranton and Fitzgerald found that different sets of genes were up-regulated in infant and adult rats after nerve injury. These genes were principally related to immune regulation and manifested in differences in the macrophage response between the age groups. Specifically, adults exhibited increased numbers clustering around sensory neurons in the DRG whereas the infants resembled the controls with macrophages spread evenly through the parenchyma (Garraway et al., 2005).

3.2 HYPOTHESIS AND AIMS

3.2.1 Hypothesis

There will be a significant decrease in the innate immune response between the infant groups and the two mature groups, and the infants will exhibit a less pro-inflammatory environment. Infants will show a lower magnitude response, less innate immune cells at the injury site and a higher proportion of “M2-like” cells than the adults. There is expected to be less histological signs of injury, less damaged axons and subsequently less astrogliosis (GFAP).

3.2.2 Aims

1. To investigate the innate immune and endogenous stem cell response in the rat spinal cord 24h, 1wk, 2wks and 6wks following a mild contusion injury.
 - a. To quantify the neutrophil infiltration post injury using haematoxylin and eosin staining.
 - b. To quantitate the proportions of ramified microglia, activated macrophages/monocytes and activated microglia present after injury using immunohistochemistry.
 - c. To measure the endogenous progenitor cell and astrocyte response post injury using immunohistochemistry.
2. To determine the age-related changes in injury progression and immune cell responses by comparing the spinal cord tissue from infant, juvenile and adult rats.

3.3 MATERIALS AND METHODS

3.3.1 Animal numbers and groups

This study used a total of 140 outside-sourced Sprague-Dawley rats (ARC, Perth Aus) injured at different ages (1 week, 5 weeks and 9 weeks) and euthanized at 24 hours, 1 week, 2 weeks and 6 weeks post induction of contusion SCI. These are separated into distinct groups, with injured (n=8) and control (n=4) groups at all three ages and all survival times (Table 2, Section 2.3.1). During experimentation, all animals were numbered and randomised as a blinding technique. However, it was easy to distinguish infant from adult spinal cords and injured from non-injured spinal cords based on size and appearance, this could not be prevented.

For the adult and juvenile rats, controls consisted of age matched sham surgeries that underwent the laminectomy but not the SCI portion of the surgery, and also age matched normal non-injured rats at day 0 and 6 weeks. This was to reduce animal numbers as there is no evidence that there will be any changes in the uninjured cord. For the infant rats controls consisted of age matched rats that underwent sham surgery, to see if the surgery procedures themselves had any effect on the infants.

A small cohort of infants were injured at P10, as opposed to the large P7 cohort, and euthanised 6 weeks post injury, with age matched controls. This was because the cortico-spinal neurons that have passed T10 by P7 have made it into the lumbar cord and begun to form functional connections in the thoracic region at 10 days postnatal (Joosten et al., 1987).

3.3.2 Surgery and euthanasia

The surgical procedures and removal of spinal cord tissue had been previously undertaken in this laboratory and stored spinal cord tissue was used in the current study. These procedures were performed under animal ethics approval (UTS ACEC 2013-069/ 2013-048) and followed closely with those outlined in a previous paper from this laboratory (Gorrie et al., 2010). This is outlined previously in Section 2.3.1.

3.3.3. Sectioning

The excised spinal cords of the animals, both injured and controls were sectioned as frozen sections using a Leica cryostat. A 1cm section of the thoracic spinal cord, approximately 5mm on each side of the epicentre of the injury at T10, was set in OCT TissueTek and frozen. This was then mounted dorsal to the top and caudal forward in the cryostat and cut into transverse sections of 15 micron thick at -15°C, every third section of which was collected. The sections were mounted in series of 10 (A to J) gelatinised slides using a small amount of phosphate buffer to flatten them and left overnight to air dry. Slide A) of the series was used for Haematoxylin and Eosin staining, B) for ED1/IBA1 double labelling, and C) for nestin and D) GFAP immunohistochemistry. Slides E through J were wrapped and stored in a -80°C freezer for use as negative controls or as spares. Each slide had approximately 18 sections, at 450µm intervals; giving a cross-section of the injury of just over 5mm (2.5mm each side of the epicentre).

3.3.4 Staining and data analysis

For each of the three age groups the transverse spinal cord was examined at six different anatomical locations (Figure 20) in three different spinal cord levels (Figure

21). Different combinations of these six different locations; namely DWM, DGM, VWM, VGM, LWM and the central canal, were used for the different analyses. This was conducted for both SCI animals and age matched controls.

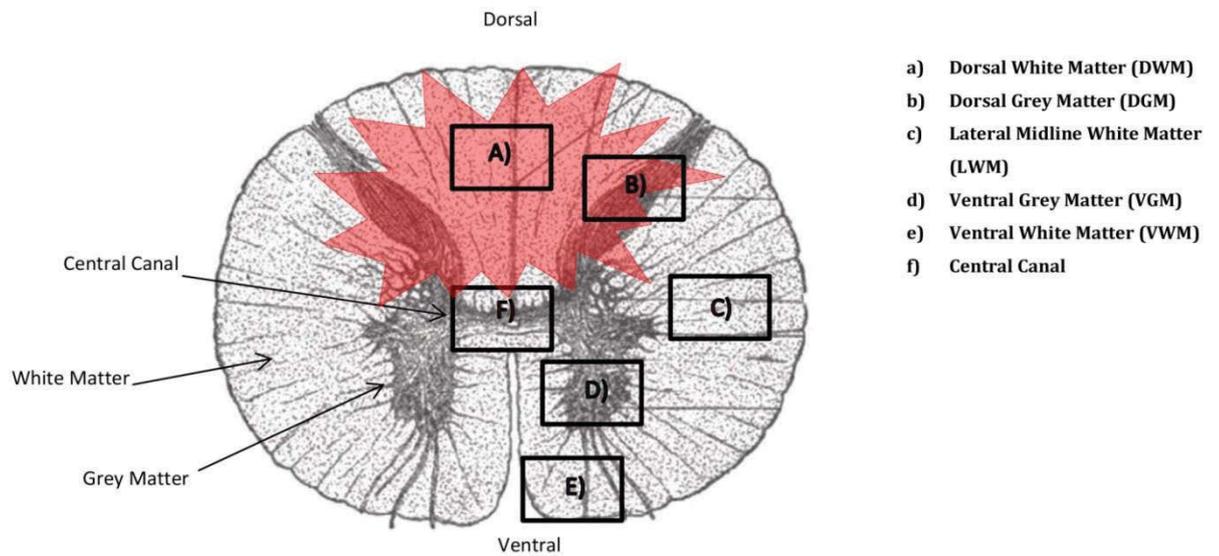


Figure 20: Diagram depicting the anatomical locations used for swollen axons in H&E sections and immunohistochemistry images. A) dorsal white matter, B) dorsal grey matter, C) midline lateral white matter, D) ventral grey matter and E) ventral white matter.

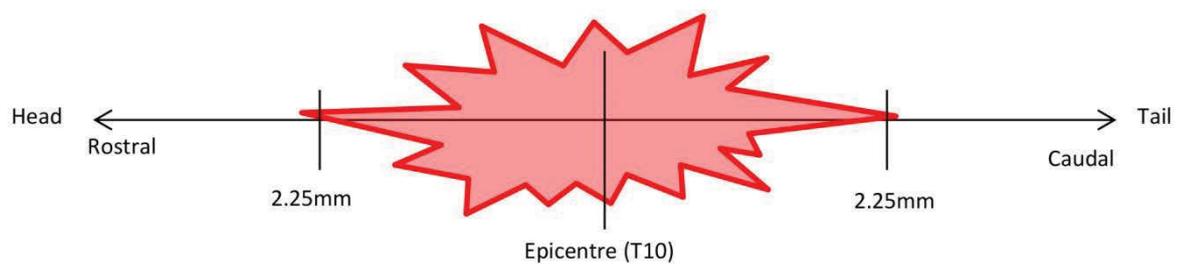


Figure 21: Diagram depicting locations of the sections on each slide used to get a cross section of the injury.

3.3.4a Haematoxylin and eosin staining

Slide A of the series was stained using Mayer's Haematoxylin and Eosin method. The slides were bought through changes of xylene, absolute, 95% and 70%

alcohol to water, despite being frozen sections rather than paraffin, as this resulted in better staining. The slides were then placed in Mayer's Haematoxylin solution for 3 minutes, rinsed in water, Scott's Bluing solution for 1 minute, rinsed further, and finally placed in alcoholic Eosin for 3 minutes before being brought back through absolute alcohol to xylene and coverslipped using DPX. This slide was used to observe the area of the lesion, the extent of the haemorrhage, the distribution of the swollen axons and magnitude of neutrophil infiltration.

3.3.4b Quantification of lesion

Lesion area as percentage of total area

Images were taken of each section using a PixeLINK camera attached to an Olympus BH-2 light microscope on the 2x and 4x objectives. Using the pixel analysis program Image J the total transverse area of each section and the area of the evident tissue damage that constituted the lesion was measured in millimetres. From this the lesion area was calculated as a percentage of the total area. For adults and juveniles the boundary of the lesion area was defined by areas of paler apoptosed cells with few small or no viable nuclei and obvious tissue disruption or the cystic cavity, including any necrotic zones on the edge. Picnotic nuclei at the edges were not routinely included unless they showed a significant patch of dying cells or areas of clearly defined haemorrhage. In cases where the lesion boundaries were not abundantly clear, including haemorrhage masking the disrupted tissue that constituted the lesion and diffuse patches laterally and ventrally to the lesion, conservative estimates were used.

For the infants difficulties were encountered when assessing the lesion in the infant groups after 24hours survival time (1wk, 2wk and 6wk groups). This was due

different manifestations of the injury. In these groups no cystic cavity formed and there was no distinct lesion. Instead the injury manifested in distorted development resulting in one half of the spinal cord smaller than the other. This was measured as a percentage difference between the two halves, using the ventral fissure to determine the midline dorsal to ventral.

Swollen axon counts

Sections were examined for the presence of swollen axons, identified as having a rounded appearance, staining homogenously pale and eosinophilic, and be located in a lacunae. This is specifically to distinguish swollen axons from the large motor neurons especially in the grey matter which are dark staining and usually more irregular in shape. The morphology described above, and illustrated in Figure 22, was consistent in infants, juveniles and adults.

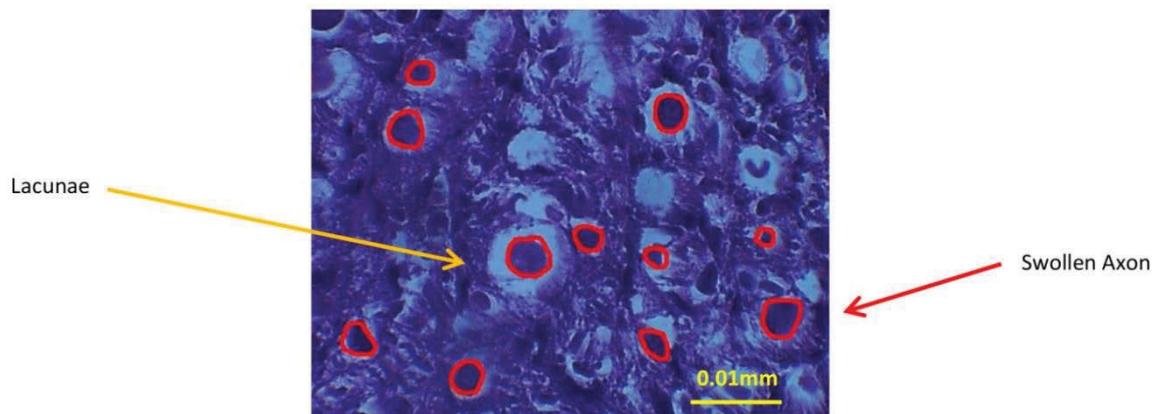


Figure 22: Representative sections of swollen axons (circled in red) in the ventral white matter of an injured adult rat, using the 40x objective, depicting the morphology and size criteria.

Counts per field of view were used to quantify the number of swollen axons/100 μm^2 (0.10 mm^2) in 5 different locations within the sections using the

Olympus light microscope on the 40x objective on 3 of the sections on slide A, the lesion epicentre, and approximately 2.25mm caudal and 2.25mm rostral of the epicentre. These positions were the dorsal grey matter (where intact) and white matter, the lateral white matter, and the ventral mid-line white matter and grey matter. Results are only reported for white matter as this is where the axons are located and where the changes occurred.

Neutrophil quantitation

To explore the differences in the basic immune response the number of neutrophils/ 0.1mm^2 was calculated. This was done using cell counts, based on morphology in the H&E sections, of three different 400x fields of view (0.04mm^2) at the epicentre of the injured spinal cord and the equivalent section of the normal/sham cord. These locations were the lesion centre, lesion edge and ventral to the lesion. The mean of these three counts was taken and then converted to neutrophils/ 0.1mm^2 for the transverse section.

3.3.4c Immunohistochemical staining

General immunohistochemical protocol

One slide from each series of 10 was used for nestin, one for GFAP, and another for ED1/IBA1 double-immunohistochemical staining. This was done to highlight the endogenous neural progenitor cells, which express nestin only, astrocytes expressing GFAP and macrophages/microglia with ED1/IBA1 (Frisén et al., 1995; Pekny, 2003; Wei et al., 2002). The central canal was examined for nestin staining in the ependymal layer, the proposed location of an eNPC niche in the spinal cord.

In each staining run a negative and a positive control was included. The negative control was an adjacent slide using the same protocol, but with the omission

of the primary antibodies. The positive was a slide from an injured animal that was known to be positive, as it has previously been observed in this laboratory. These were put through the complete protocol including the primary and secondary antibodies and counterstain.

As with the H&E staining the slides were first brought through two changes of xylene and decreasing grades of alcohol to water. To begin the staining process the slides were immersed in pH7.4 PBST (phosphate buffered sodium and Triton-X) and incubated for 10 minutes. Following the PBST buffer the slides were incubated in 5% NGS (normal goat serum) as a blocking agent for 30 minutes. The primary antibodies were diluted in PBG (phosphate buffered goat serum) as specified in Table 3 and applied to all slides except the negative control. PBG alone was applied to this control. This was incubated overnight at 4° in a humid chamber.

| Primary Antibody | Secondary Antibody | Tissue Element |
|-------------------------------------|---|--|
| Mouse anti-Nestin (Abcam, 1:200) | AlexaFlour 568 anti-Mouse (Invitrogen, 1:1000) | Neural Progenitor Cells (Dahlstrand et al., 1995; Xu et al., 2008) |
| Rabbit anti-GFAP (Dako, 1:1000) | AlexaFlour 488 anti-Rabbit (Invitrogen, 1:200) | Astrocytes (Eng, 1985) |
| Mouse anti-ED1 (Serotec, 1:500) | AlexaFlour 488 Anti-Mouse (Invitrogen, 1:200) | Activated Macrophages and Microglia (Damoiseaux et al., 1994) |
| Rabbit anti-IBA1 (Abcam, 1:500) | AlexaFlour 568 anti-Rabbit (Invitrogen, 1:200) | Ramified and Activated Microglia (Le Blon et al., 2014; O'Carroll et al., 2013) |

Table 3: Primary and secondary antibodies used for immunohistochemical staining

The slides were washed in 3 changes of PBST. The secondary AlexaFlour antibodies were diluted in PBG as specified above (Table 3), applied to all slides and

left to incubate for 2 hours at room temperature. This was followed by a further 3 washes of PBST before counterstaining. Slides were placed in a Hoechst counterstain solution (Invitrogen, 1:5000) for 10 minutes to highlight the nuclei in blue under UV light. Finally, all of the slides were washed again with PBST and coverslipped using Dako fluorescent mounting medium. The slides were left to air dry in a dark environment before being stored in a dark box in the 4°C refrigerator. A small subset (2 animals from each 1 week age group) was double-stained with nestin (Abcam, 1:200) and RECA1 (Serotec, 1:500) to see if nestin was localised to blood vessels in the parenchyma.

Imaging protocols

The immunohistochemistry fluorescent labels were visualised using an Olympus BX51 Upright Fluorescence Microscope with Olympus U-RFL-T fluorescent burner and images were captured using an Olympus DP70 camera with *Olympus CellSens* software.

The AF568 anti-mouse secondary antibody used for nestin staining showed red using the UMWIG3 filter and the AF488 anti-rabbit secondary antibody for GFAP and ED1 fluoresce green through the UMWIB3 filter. This was combined with the Hoechst nuclear staining that presented as blue through the UMNUA2 filter. Composite images were created using *CellSens* to combine the different fluorescent images. Images were taken on the 40x objective lens (400x magnification) with the black balance adjusted as a built-in function of the *Olympus CellSens* software. The exposure time was kept constant for each stain. Images were taken at 400x magnification of different anatomical locations within the transverse section of the spinal cord as shown in Figure (20) and at three caudal-rostral levels (rostral 2.25mm, epicentre of the injury and caudal 2.25mm) as shown in Figure (21).

Immunohistochemistry image analysis protocols

Due to the thickness of the frozen spinal cord sections, containing multiple layers of cells, it was not possible to count individual cells stained for nestin and GFAP. As an alternative the Mean Greyscale Value (MGV) was used as a measure of the fluorescence in each section. The black balance was set for all of the images using a built-in function of the *Olympus CellSens* software to normalise the intensities. Using ImageJ software the MGV was calculated for the central canal, selected using the freehand tool, as well as areas of approximately $150\mu\text{m}^2$ (0.15 mm^2) in the different anatomical locations within the transverse section. In the GFAP sections the background was corrected for, to account for any auto-fluorescence within the sections, using the MGV of a dorsal root taken from the same slide. For the nestin sections the VWM was used. To normalise the background to 0 this value was subtracted from all of the values. The MGVs were compared between animals of different ages, between locations within the animals and between positions on the spinal cord.

The images that were taken of the ED1/IBA1 stained sections were used to establish absolute counts of three subsections of macrophages/microglia at the epicentre and distal 2.25mm caudal. These were ED1⁺/IBA1⁺ (classically activated microglia, M1-like), ED1⁺/IBA1⁻ (infiltrating phagocytic macrophages/monocytes) (Ajami et al.; Chiu et al., 2013), and ED1⁻/IBA1⁺ (ramified microglia). From these counts the percentage of total macrophages was calculated. Counts were taken in a field of view at 400x (using 40x objective lens) of the white matter, grey matter, lesion edge, lesion centre and the central canal on all slides. For the sham and control animals the dorsal white and grey matter were used while on the injured sections counts were taken in the lesion centre and at the lesion edge. The ED1 positive cells in the dura

mater were not counted as the dura was not present on all sections and had a relatively high population when it was intact. Henceforth cells stained ED1⁺/IBA1⁺ are referred to as activated microglia/macrophages, ED1⁺/IBA1⁻ as infiltrating macrophages, and ED1⁻/IBA1⁺ as ramified microglia.

Randomised data collection

The cords in this study were sectioned in a random order blind, as much as possible in this situation, to the animal groups. Complete blinding to the condition of the cords was not possible as observation of the cords showed obvious injury and the size variations between the groups.

The collection of all the data was done chronologically as cords were sectioned and stained using only the randomised animal numbers. In the case of the swollen axons the data was collected in age-based groups, randomised for injury status, to keep the judgement of size consistent for all the animals as there were some small differences between the age groups. The data for haemorrhage and the swollen axons was collected in large blocks to reduce any differences in judgement of the criteria, reduce human error and increase consistency.

3.3.5 Statistical analysis

The data collected from these methods; namely the lesion area percentage, the swollen axon counts and the immunohistochemistry, were continuous and assumed to be of a normal distribution and were therefore analysed statistically using one-way analysis of variance (ANOVA) with the Bonferonni post-hoc test. Student's t-tests were used to compare specific data sets. This was done using GraphPad Prism software version 6. This software was also used to produce graphs of the data for visualisation

of means and standard deviations. For all analyses a P-value of <0.05 was taken as indicator of statistical significance.

Analyses were first conducted within each age group, between injured and sham, and between time points, shown in results 3.4.1 (adults), 3.4.2 (juveniles) and 3.4.3 (infants); then between age groups in results section 3.4.5. For adult and juveniles the control groups (Day 0 and 6 week normal and 6 week shams) were compared and as there was no significant differences these were pooled. In the infants each SCI time point was compared to its age matched sham. For each measurement multiple factors were compared, however only the findings with the most significant differences have been presented in the results section.

3.4 RESULTS

3.4.1 Injury progression

3.4.1a Adult

The adult rats showed a progression from a necrotic, disrupted lesion to an expanding cystic cavity that has stabilised by 6 weeks post injury. This lesion or cavity tapers out rostral and caudal to the epicentre, eventually disappearing out of the transverse sections, at around 2.25mm generally although there is a high degree of variation between animals (Figure 23). Significant differences in lesion size were found between the epicentre and the caudal and rostral sections at all four time points post injury (with the exception of the rostral sections at 1 week post injury) using one-way ANOVA and Bonferroni's post hoc test ($P < 0.001$) (Figure 24A). Swollen axons are a common marker of trauma in the central nervous system. There was a peak of swollen axons at 24h that reduced in the other four time points. There was a statistically significant increase in number of swollen axons in the ventral and lateral white matter at 24 hours compared to the same areas of the rostral, caudal and epicentre sections at 1 week, 2 weeks and 6 weeks using ANOVA and Bonferroni's post hoc ($P < 0.0001$) (Figure 24B). There was a significant difference in the number of swollen axons between the epicentre and the two distal locations at 24 hours post injury, however not at any of the other survival times.

The progression of the neutrophil infiltration at the epicentre of the injured adult groups reflected what was expected, with a large peak at 24 hours post injury subsiding significantly in the 1 week, 2 week and 6 week groups. A significant decrease was found between the 24 hour SCI group and the control groups as well as the other

three time points using ANOVA and Bonferroni's ($P < 0.0001$). The sections 2.25mm rostral and caudal of the injury centre showed negligible neutrophil infiltration.

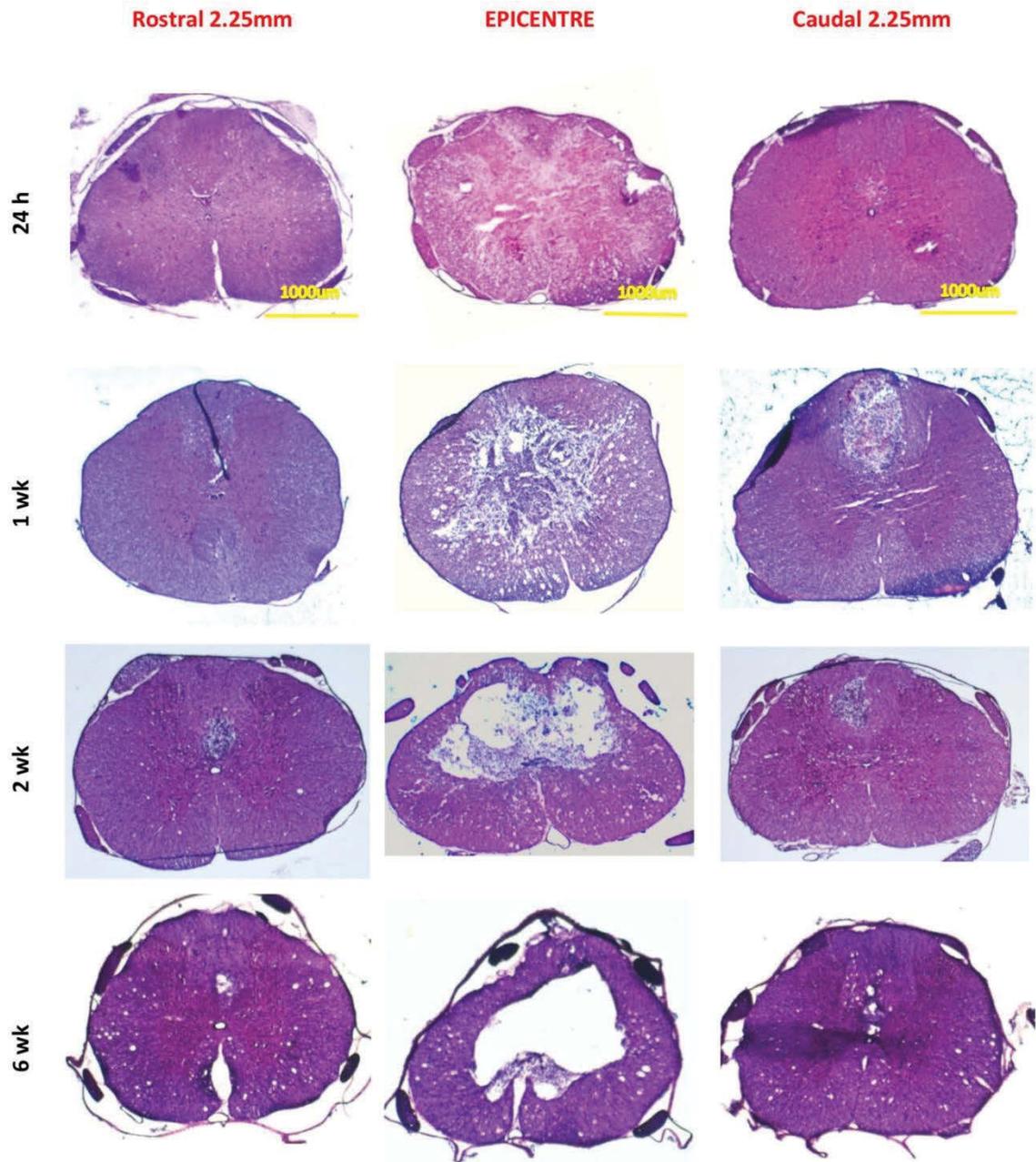


Figure 23: A visual representation of the injury progression and lesion development at the epicentre and 2.25mm on either side at four time points post-injury (H&E staining) in the adult rat.

Exogenous macrophages/monocytes and activated endogenous microglia respond in the early stages of SCI and can persist for some time, with an expected peak

at around a week post-injury. In adult rats there was a peak in the proportion of ED1⁺/IBA1⁻ (phagocytically activated macrophages/ monocytes) over 1 and 2 weeks post injury. Using ANOVA and Bonferroni's post hoc test there was only a statistically significant difference in the ED1⁺/IBA1⁻ percentage between 24 hour and 2 weeks post-injury at the epicentre. At all time points phagocytically activated macrophages/ monocytes were significantly increased from the shams both at the epicentre and 2.25mm distal (P<0.001) (Figure 24C). The proportion of ED1⁺/IBA1⁺ (activated macrophages/microglia) was highest at 24 hours post-injury and decreased with time post-injury (P<0.001) (Figure 24D). At all survival times activated macrophages/microglia were significantly increased from the shams at both the epicentre and distally. Conversely, the proportion of ED1⁻/IBA⁺ (ramified) microglia in all of the injured groups, both at the epicentre and distally, was significantly decreased from the shams (Figure 24E).

The astrocytic response in adult rats begins as early as 24 hours post injury at very low levels and increases as the injury progresses. At 24 hours post injury there is a small visible increase in GFAP MGV from the sham levels around the lesion but this was not found to be statistically significant. This increase around the lesion edge is more prevalent at 1 and 6 weeks; both at the epicentre of the lesion and 2.25mm distal. There was a small dip in the GFAP MGV around the lesion edge at 2 weeks, the increase from sham levels dropping to below a level of statistical significance, before escalating once again at 6 weeks (P<0.0001) (Figure 24F). The GFAP MGV was highest in the white matter of the normal and sham animals and this relatively high level was maintained in the injured animals. There was no significant difference between the injured and control animals at any of the time points tested and also no difference found between the time points (Figure 24F).

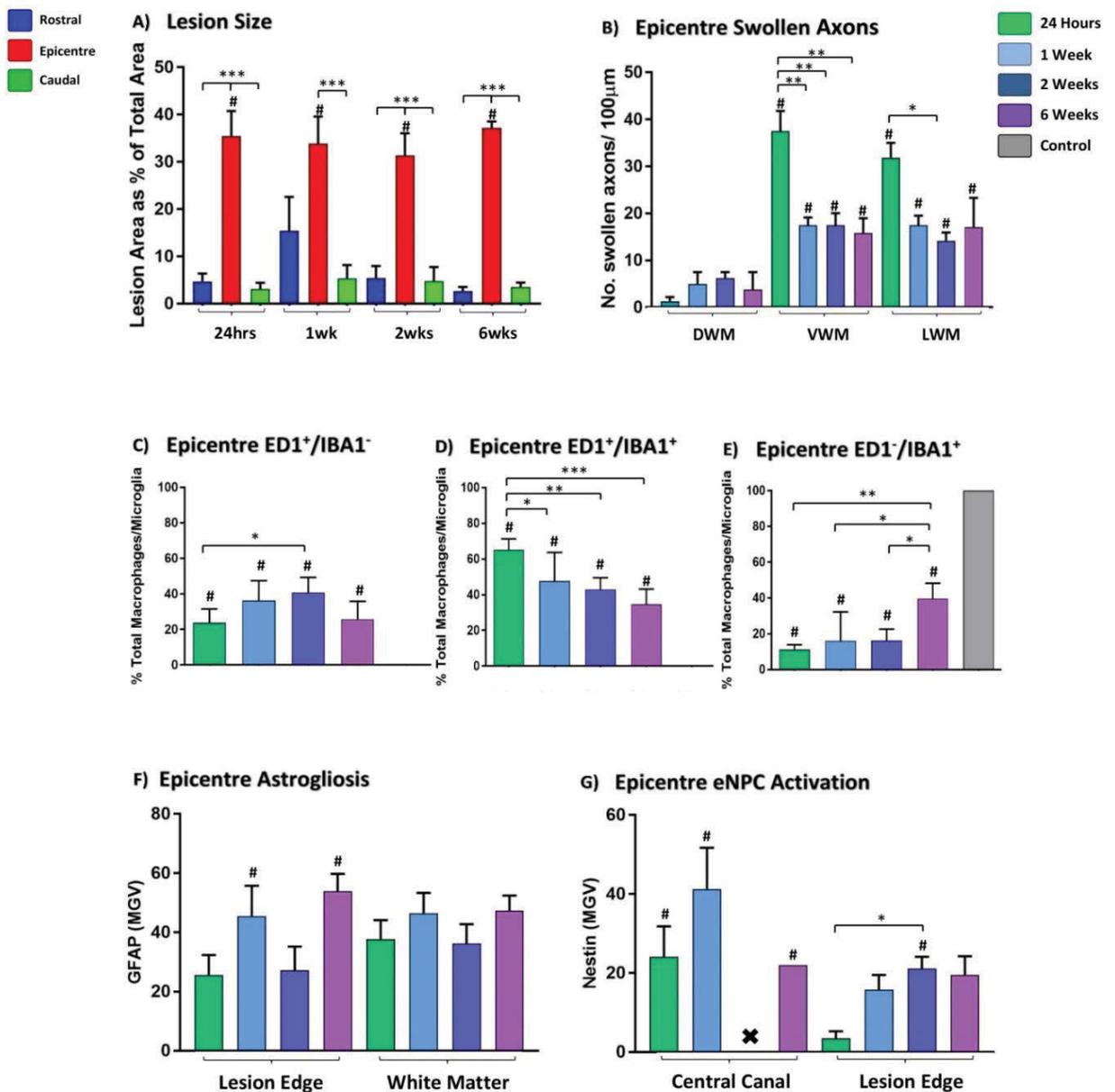


Figure 24: Histograms representing a progression of the injury in adult rats as A) the lesion area as a percentage of the total transverse area at the lesion epicentre and 2.25mm rostral and caudal of the epicentre; B) the number of swollen axons per 100µm in the white matter of adult rats at the epicentre of the spinal cord lesion; the macrophage and microglial populations present as a percentage of the total macrophage/microglia including C) the infiltrating activated macrophages (ED1⁺/IBA1⁻), D) activated macrophages/microglia (ED1⁺/IBA1⁺), and E) resting microglia (ED1⁻/IBA1⁺); F) astrogliosis at the lesion edge and white matter at the epicentre using the GFAP fluorescent intensity (MGV); and G) the activation of endogenous neural progenitor cells at the central canal and lesion edge of the lesion epicentre using the nestin fluorescent intensity (MGV). Controls are not shown here where they were not at detectable levels. * (P<0.05), ** (P<0.005) and * (P<0.001) indicate the significant differences between groups based on Bonferroni's post hoc test and # indicates a significant difference from the respective control (P<0.05). X indicates where the central canal was destroyed at the epicentre.**

Endogenous neural progenitor cells are present in the ependymal layer of the spinal cord central canal and are known to respond to injury (Cizkova et al., 2009; Gage, 2000; Horky et al., 2006; Ohta et al., 2004). The intensity of the nestin staining was visibly increased at all of the time points (24h, 1wk, 2wk and 6wk) post injury at the two locations 2.25mm distal from the epicentre and also at the epicentre, when the central canal was present and intact. This staining was dominant around the ependymal layer and showed long basal processes extending into the parenchyma, the expected morphology of activated eNPCs (Figure 25). There was also some nestin scattered in the parenchyma; this was associated with blood vessels, as shown by nestin/RECA-1 staining. There was a significant increase in nestin MGV at all three locations (epicentre and 2.25mm rostral and caudal) at 24 hours and 1 week post injury, with the MGV peaking at 1 week. The increase in staining intensity was only significant rostrally at 2 weeks and at the epicentre at 6 weeks post injury ($P < 0.001$) (Figure 24G). Previous studies have also shown that the eNPC migrate towards the injury site. This was borne out by the increase in nestin MGV at the lesion edge at 1 week, 2 weeks and 6 weeks post injury. However, with the exception of 2 weeks post injury, this increase was not shown to be significant using ANOVA and Bonferroni's post hoc test due to that variations between animals that resulted in large standard deviations (Figure 24G).

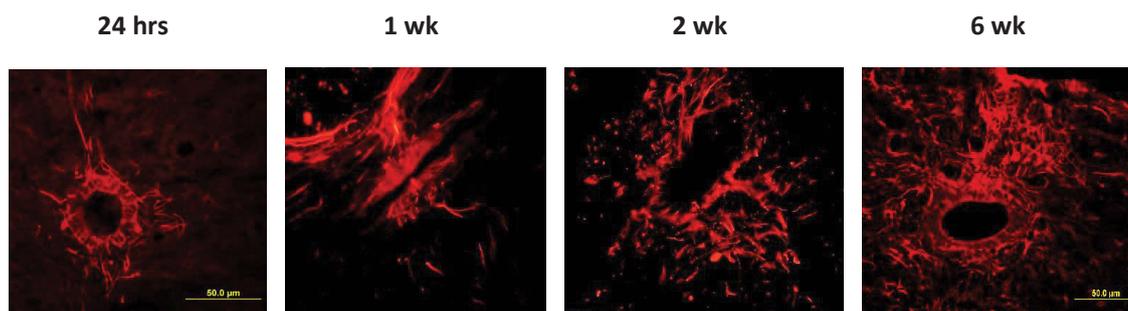


Figure 25: Fluorescent microscopy images of the central canal taken at 400x, 2.25mm distal to the epicentre of the injury, stained with nestin as a marker for endogenous neural progenitor cells in adult rats. The ependymal layer of the central canal and long processes extending into the parenchyma can be seen.

3.4.1b Juvenile

The juvenile rats showed a very similar lesion progression to that shown in adults; that is a necrotic, disrupted lesion to a stabilising cystic cavity at 6 weeks post injury. The lesion or cavity tapers out rostral and caudal to the epicentre, eventually disappearing out of the transverse sections (Figure 26). Significant differences were found between the epicentre and the caudal and rostral sections at all four time points post injury using one-way ANOVA and Bonferroni's post hoc test (with the exception of the rostral sections at 2 week post injury). The shams had no lesion and were therefore significantly different to the lesion epicentre at all four time points ($P < 0.001$) (Figure 27A). The number of swollen axons peaked at 24hrs then reduced in the other four time points; this is shown most prominently at the epicentre (Figure 27B). Statistically there was no significance between the time points at any of the three locations. The only significance was found in the 24 hour survival group. This was between the epicentre and the caudal section in the lateral and ventral white matter ($P < 0.05$) (Figure 27B). Due to the large standard deviations there was very little significance between the injured and controls however the epicentre at 24 hours was highly

significantly increased ($P < 0.0001$). Although there was an increase from the controls at all other time points and locations this was not often statistically significant.

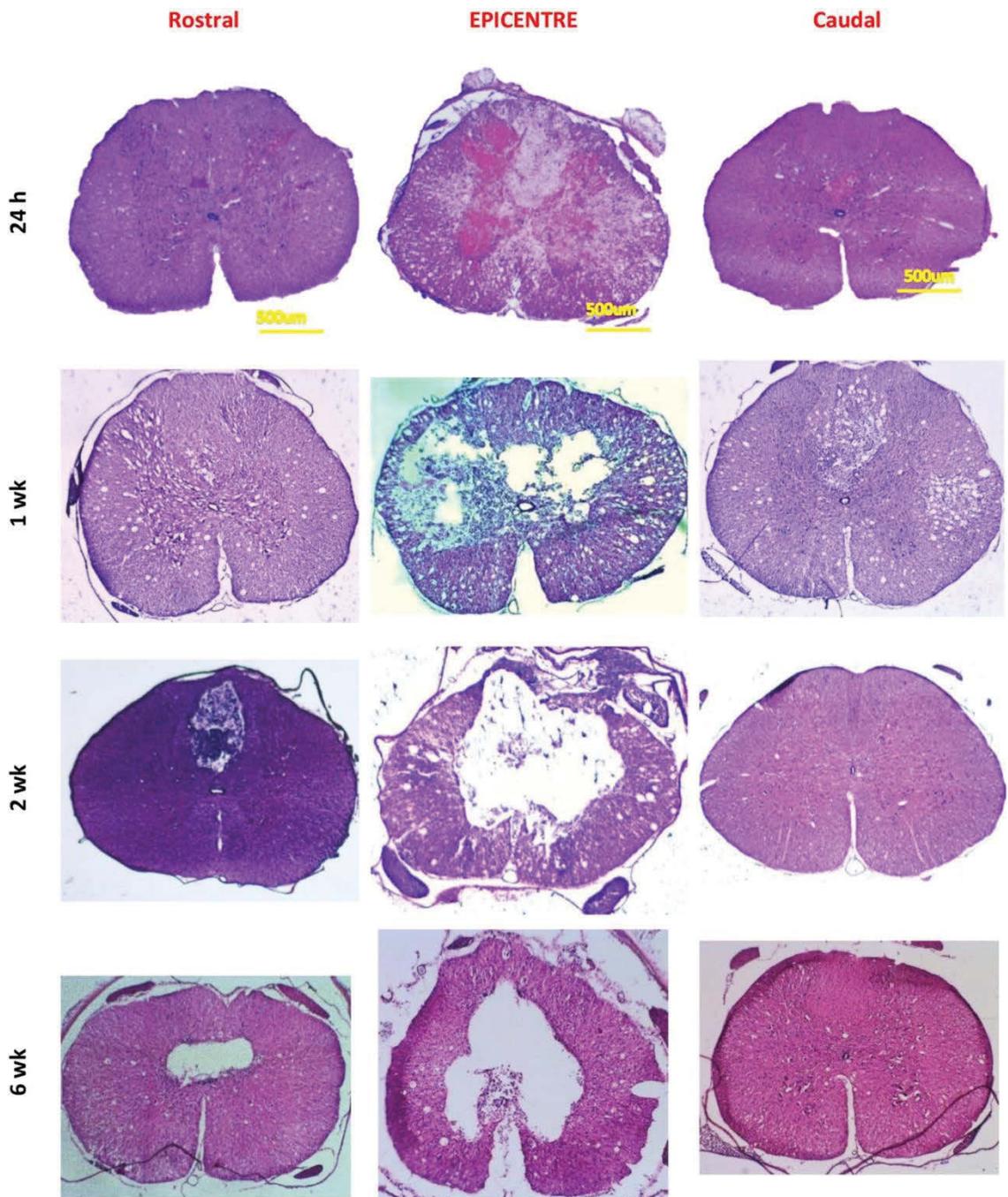


Figure 26: A visual representation of the injury progression and lesion development at the epicentre and 2.25mm on either side, at four time points post-injury (H&E staining) in the Juvenile rat.

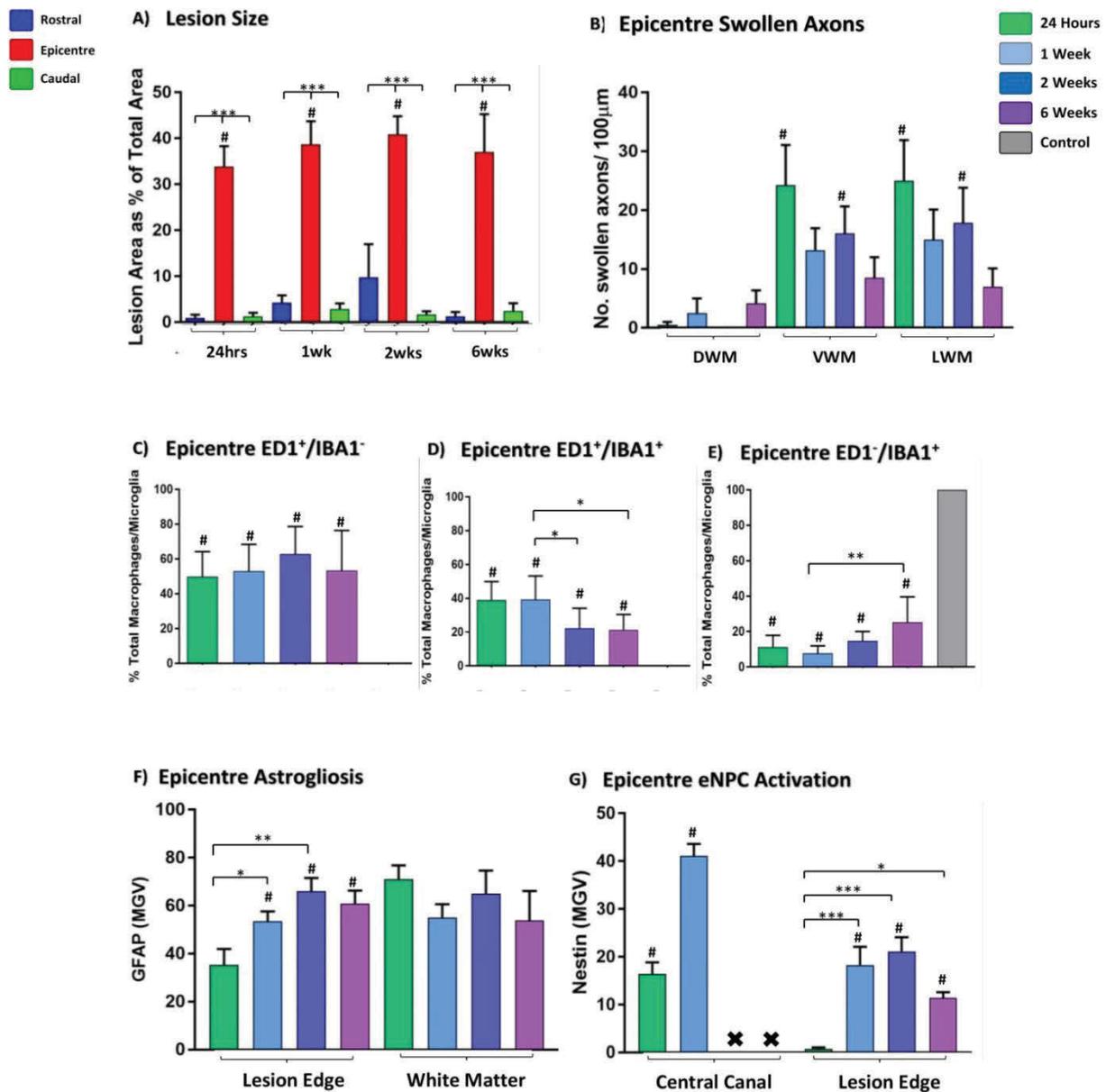


Figure 27: Histograms representing a progression of the injury in juvenile rats as A) the lesion area as a percentage of the total transverse area at the lesion epicentre and 2.25mm rostral and caudal of the epicentre; B) the number of swollen axons per 100µm in the white matter of adult rats at the epicentre of the spinal cord lesion; the macrophage and microglial populations present as a percentage of the total macrophage/microglia including C) the infiltrating activated macrophages (ED1⁺/IBA1⁻), D) activated macrophages/microglia (ED1⁺/IBA1⁺), and E) resting microglia (ED1⁻/IBA1⁺); F) astrogliosis at the lesion edge and white matter at the epicentre using the GFAP florescent intensity (MGV); and G) the activation of endogenous neural progenitor cells at the central canal and lesion edge of the lesion epicentre using the nestin florescent intensity (MGV). Controls are not shown here where they were not at detectable levels. * (P<0.05), ** (P<0.005) and * (P<0.001) indicate the significant differences between groups based on Bonferroni's post hoc test and # indicates a significant difference from the respective control (P<0.05). X indicates where the central canal was destroyed at the epicentre.**

The progression of the neutrophil infiltration at the epicentre of the injured juvenile groups reflected the pattern seen in the adult groups, as was expected. This pattern consists of a large peak at 24 hours post injury subsiding significantly in the longer time points. A significant decrease was found between the 24 hour SCI group and the control groups as well as the other three time points using ANOVA ($P < 0.0001$). As with the adult injured groups the results reported here are only for the epicentre as there is a highly localised response from infiltrating neutrophils.

The proportion of phagocytically activated macrophages/ monocytes ($ED1^+/IBA1^-$) was significantly increased from the shams at all four survival times, both at the epicentre and 2.25mm distal, using ANOVA and Bonferroni's (Figure 27C). There was no significant differences between the time points ($P < 0.001$). Similarly, the proportion of endogenous activated macrophages/microglia ($ED1^+/IBA1^+$) was also significantly increased from the shams at all points and survival times, except 2.25mm distal at 24h post-injury. Activated macrophages/microglia were elevated at 24h and at 1 week, both distally and at the epicentre, before dropping in numbers. This was only statistically significant at the epicentre between 1 week and 2 weeks, and 1 week and 6 weeks; and distally between 1 week and 2 weeks ($P < 0.001$) (Figure 27D). The proportion of ramified microglia ($ED1^-/IBA^+$) in all of the injured groups, both at the epicentre and distally, was significantly decreased from the shams. At the epicentre ramified microglia were increased at 6 week post-injury compared to the earlier time points, however there was only statistical significance between 6 weeks and 1 week ($P < 0.005$) (Figure 27E). At 2.25mm distal the highest proportion was seen at 24 hours post-injury, this was only significant compared to 1 week ($P < 0.001$).

The juvenile animals showed increasing GFAP MGV around the lesion edge as survival time increased. The GFAP intensity at the lesion edge at 24 hours showed

some increase from the sham but this was not significant. At 1 week post injury a further increase can be seen, statistically significant at the epicentre and 2.25mm caudal. Another small increase from the 1 week levels can be seen at 2 weeks post injury, however the increase from sham levels was only statistically significant at the epicentre ($P < 0.0001$). Both 1 and 2 weeks post injury the GFAP MGV was significantly increased from the levels seen at 24 hours. By 6 weeks post SCI the GFAP intensity at the lesion edge was significantly increased from the sham at all three levels ($P < 0.0001$) (Figure 27F). The juveniles showed no significant increase from their sham GFAP levels in the white matter at any of the injury survival times (Figure 27F).

The intensity of the nestin staining, indicating endogenous neural progenitor cells, was visibly increased at all of the time points post injury 2.25mm distal from the epicentre and also at the epicentre, when the central canal was present and intact. This staining was visible around the ependymal layer and showed long basal processes extending into the parenchyma, the expected morphology of activated eNPCs (Figure 28). At both 2 and 6 weeks post SCI there were very few intact central canals at the epicentre in the juveniles. The nestin staining shows eNPC are activated at 24 hours post injury and peaked at 1 week both distally and at the epicentre. There was less variation between animals than that seen in the adults, resulting in smaller standard deviations, allowing for more significant differences to be visible using ANOVA and Bonferroni's post hoc (Figure 27G). The most significant differences were found in the central canal 2.25mm rostral to the epicentre ($P < 0.001$). There was an increase in nestin MGV at the lesion edge at 1 week, 2 weeks and 6 weeks post injury ($P < 0.001$), this was statistically significant both rostrally and at the epicentre (Figure 27G). At 1 and 2 weeks post injury this was also significantly higher than at 24 hours ($P < 0.001$).

There was also some scattered nestin in the parenchyma associated with blood vessels. This was confirmed by nestin/RECA-1 double staining.

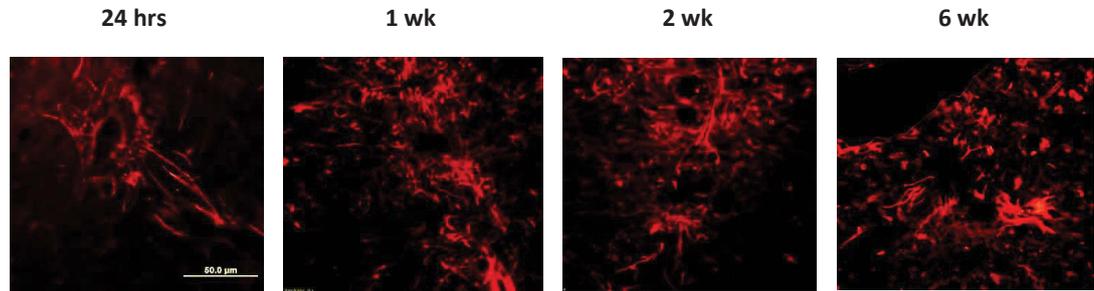


Figure 28: Fluorescent microscopy images of the central canal taken at 400x, 2.25mm distal to the epicentre of the injury, stained with nestin as a marker for endogenous neural progenitor cells in juvenile rats. The ependymal layer of the central canal and long processes extending into the parenchyma can be seen.

3.4.1c Infant

The infant rats showed a vastly different injury progression to that observed in the adult and juvenile groups. This consisted of a necrotic, disrupted lesion at 24 hours similar to that seen in the mature groups progressing to a unilateral asymmetry and only a small indent rather than a cystic cavity (Figure 29)¹. Lesion size was greater at the epicentre compare to the distal sections, this was statistically significant ($P < 0.05$), except at 6 weeks post injury. This reflects a common pattern seen in both adult and juvenile injuries, though the manifestation of the injury differs. The infants showed a less developed corticospinal tract in the shams, shown in Figure 30. None of the sham surgeries resulted in any visible injury or asymmetry therefore all SCI lesions were significantly different from the controls (Figure 31A).

¹ At 24 hours the lesion size was measured as a percentage of the total transverse area, for the later time points this is expressed as a percentage difference between the two sides of the midline on the transverse sections (see Methods 2.3.4).

In all locations and time points in the infant groups the number of swollen axons was negligible. There was no difference between the injured and sham groups, or between locations and time points within the injured groups (Figure 31B).

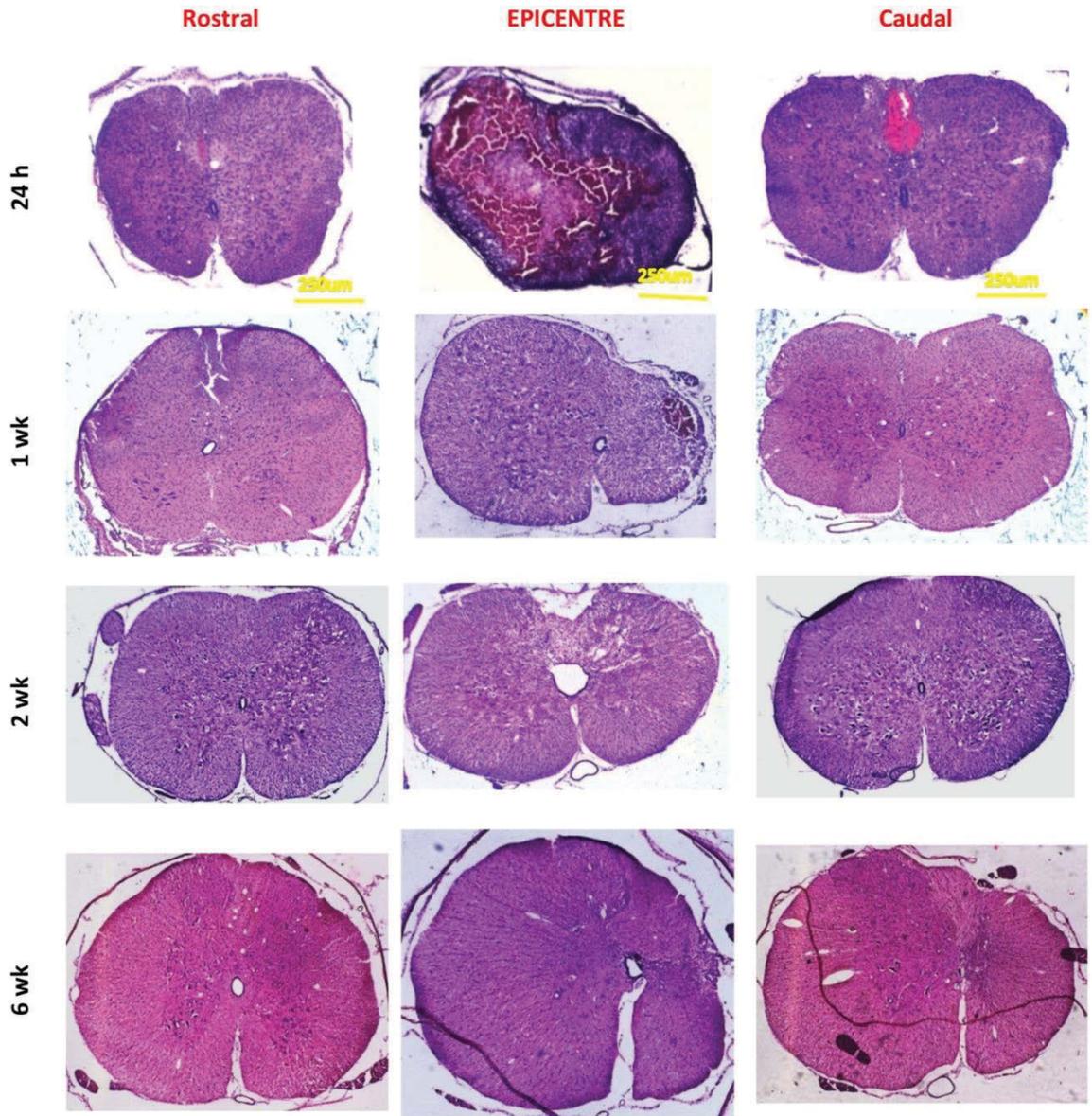


Figure 29: A visual representation of the injury progression and lesion development at the epicentre and 2.25mm on either side, at four time points post-injury (H&E staining) in the infant rat.

The neutrophil peak at 24 hours that was seen in the adults and juveniles was also visible in the infant groups as well, however on a decreased scale. A significant

decrease was found between the 24 hour SCI group and the control groups as well as the other three time points using ANOVA ($P < 0.0001$). The results reported here are only for the epicentre as examination of the rostral and caudal sections showed almost no neutrophil infiltration.

The infants showed a vastly different pattern of ED1/IBA1 immunohistochemical staining, compared to both the adult and juvenile groups. The pattern was reversed from that seen in the mature groups. The proportion of phagocytically activated macrophages (ED1⁺/IBA1⁻) was not significantly increased from the shams at any of the time points, either distally or at the epicentre (Figure 31C). The only significant difference seen was between ED1⁺/IBA1⁻ staining at the epicentre at 2 weeks and 6 weeks post-injury ($P < 0.001$). Similarly, the proportion of activated macrophages/microglia (ED1⁺/IBA1⁺) was much lower in the infants than in the mature groups. At the epicentre, it was significantly increased from the shams at 24 hours, 1 week and 2 weeks post-injury; peaking at 1 week and decreasing to close to sham levels at 6 weeks (Figure 31D). The only significant difference observed between time point was between 1 week and 6 weeks post-injury at the epicentre ($P < 0.001$). As with the ED1⁺/IBA1⁻ macrophages, there were no significant differences found 2.25mm distal. The proportion of ramified microglia (ED1⁻/IBA1⁺) was significantly decreased from the shams at the epicentre at 24 hours, 1 week and 2 weeks post injury, returning to close to the sham levels at 6 weeks (Figure 31E). There was only significant difference found between 24 hours and 1 week post injury at the epicentre ($P > 0.001$). There were no significant differences seen in the distal sections.

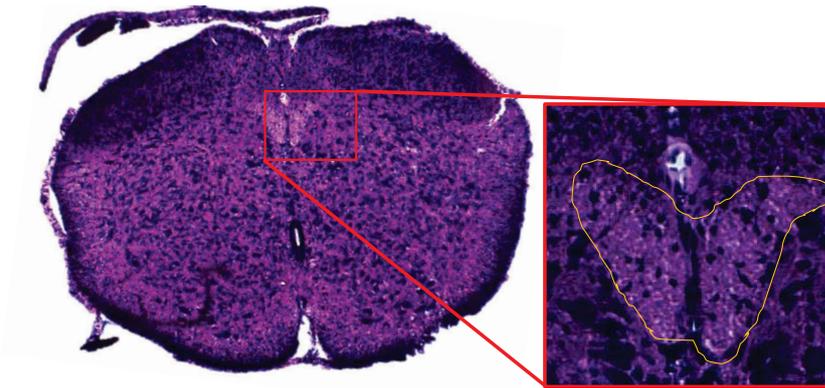


Figure 30: Infant sham with 24 hour survival time showing the pale staining corticospinal tract in the dorsal white matter (indicated by the red box), also shown at 400x magnification in the inset.

GFAP staining in the infants showed a relatively consistent pattern compared to the sham controls. There was no definitive cavity or lesion edge and this was reflected in the GFAP staining intensity. The GFAP MGV is highest at 1 week post injury, dropping at 2 weeks and then increasing again at 6 weeks but there is no statistically significant differences from sham levels or between groups (Figure 31F). The white matter showed the largest changes with an early (24 hours) increase from sham levels ($P < 0.005$) dropping back at later time points. This peak was significantly higher than the sham and 2 week levels at all three locations, the 1 week level at the epicentre and 2.25mm rostral, and the 6 week level 2.25mm rostral and caudal (Figure 31F).

Nestin staining was visible around the central canal ependymal layer and showed long basal processes extending into the parenchyma on the 24 hour and 1 week sections. Nestin is also apparent staining the epithelial cells of blood vessels at all time points. This blood vessel staining in the parenchyma was confirmed by nestin/RECA-1 staining. There was quite a high degree of variation between animals, the strongest staining at each time point shown in Figure 32. Nestin immunoreactivity

was highest early after the injury at both the central canal and 'lesion edge'² with a small, but visible, increase in intensity at 24 hours and 1 week after injury that dropped off substantially at 2 weeks. This was most visible at the epicentre. There was no visible lesion in the infants after 24 hours however there was still an increase in nestin MGV at the 'lesion edge' at the epicentre at 1 week, 2 weeks and 6 weeks post injury, and distally at 1 week. This was only statistically significant at the epicentre at 1 week post SCI ($P < 0.0001$) (Figure 31G).

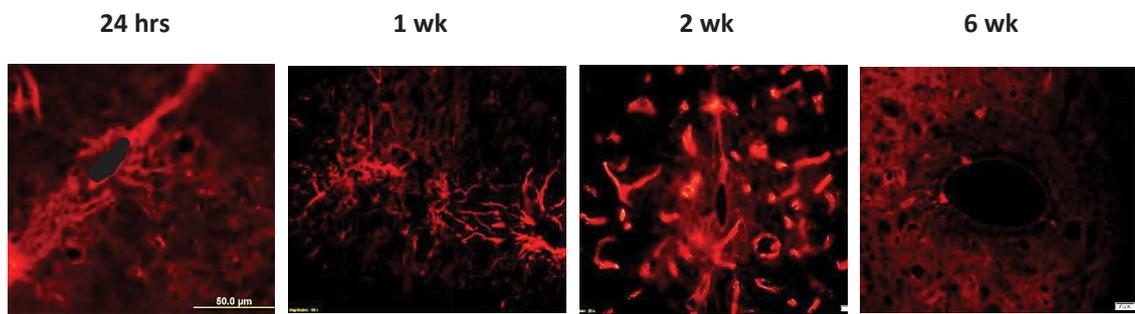


Figure 31: Fluorescent microscopy images of the central canal taken at 400x, 2.25mm distal to the epicentre of the injury, stained with nestin as a marker for endogenous neural progenitor cells in infant rats. The ependymal layer of the central canal and long processes extending into the parenchyma can be seen. Numerous nestin positive blood vessels are also visible.

² Measurements made of the nestin clustered in the lateral edge of the smallest side of the asymmetrical cord are described as the 'lesion edge' to be consistent with the adults and juveniles

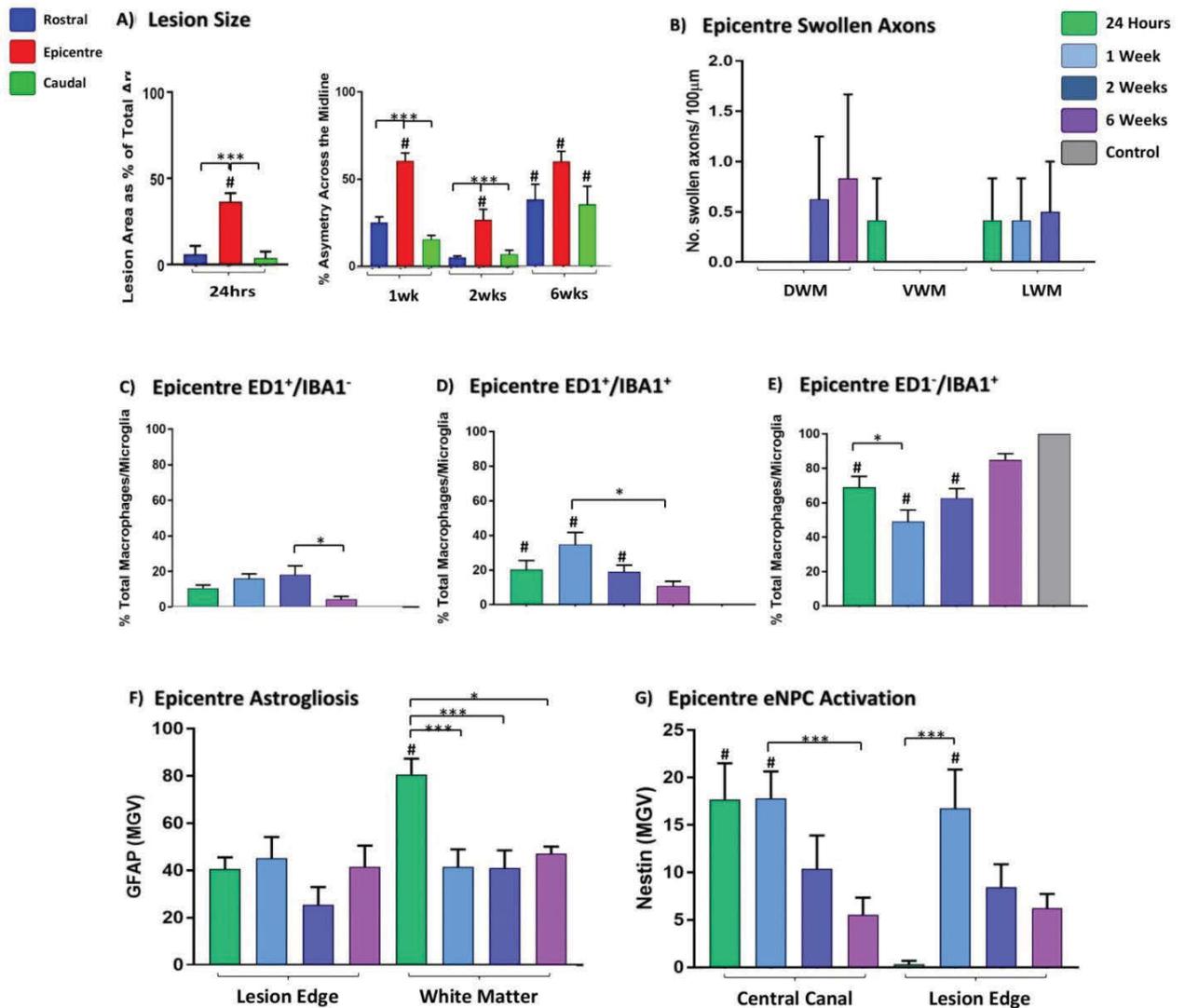


Figure 32: Histograms representing a progression of the injury in infant rats as A) the lesion area as a percentage of the total transverse area at the lesion epicentre and 2.25mm rostral and caudal of the epicentre; B) the number of swollen axons per 100µm in the white matter of adult rats at the epicentre of the spinal cord lesion; the macrophage and microglial populations present as a percentage of the total macrophage/microglia including C) the infiltrating activated macrophages (ED1⁺/IBA1⁻), D) activated macrophages/microglia (ED1⁺/IBA1⁺), and E) resting microglia (ED1⁻/IBA1⁺); F) astrogliosis at the lesion edge and white matter at the epicentre using the GFAP florescent intensity (MGV); and G) the activation of endogenous neural progenitor cells at the central canal and lesion edge of the lesion epicentre using the nestin florescent intensity (MGV). Controls are not shown here where they were not at detectable levels. * (P<0.05), ** (P<0.005) and *** (P<0.001) indicate the significant differences between groups based on Bonferroni's post hoc test and # indicates a significant difference from the respective control (P<0.05).

3.4.2 Comparisons between age groups

3.4.2a Lesion size and location

The SCI presented differently in infants compared to adults and juveniles. At the later time points; 1 week, 2 week and 6 weeks, there is a stark difference in appearance of the injured cords. In both the juvenile and adult groups the area of necrosis and disrupted tissue that comprised the lesion at 24 hours has become a cystic cavity, presenting as a large hole in the cross-section of the tissue, tapering out caudal and rostral. In contrast the P7 infant groups do not present with a cavity (with a few exceptions) but instead show a distinct asymmetry across the midline of the transverse spinal cord. This is shown in Figure 34.

There was a significant difference between the epicentre and the caudal and rostral sections in all three age groups using one-way ANOVA and Bonferroni's post hoc ($P < 0.0001$). At 24 hours post-injury, where the lesion morphology was similar, the lesion size was measured as a percentage of the total transverse area of the cord in all age groups. The pattern of injury is very close in all three age groups at this acute point in injury development (Figure 33).

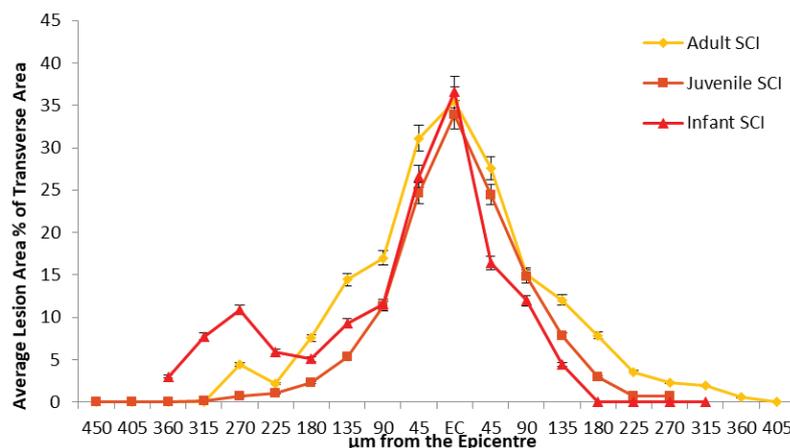


Figure 33: Comparison of the extent of the lesion along the spinal cord length in all three age groups showing a similar pattern of injury confirming a comparable injury. Sham and control groups are not shown as they did not display any lesion.

One week post-injury the adult and juvenile groups show increased lesion size at the epicentre compared to the caudal and rostral sections, the lesion developing into a cystic cavity and exhibiting tissue loss. A significant difference was found between the epicentre and the caudal sections in both the adults and juveniles, and between the epicentre and rostral section in the juveniles using one-way ANOVA and Bonferroni's post hoc test ($P < 0.0001$). The infants showed a similar pattern however it presented as a disparity between the two sides of the midline on the transverse sections. There was a statistically significant difference between the epicentre and both the rostral and caudal sections in the infant ($P < 0.001$). All three age groups had a significant difference between the epicentre and the controls. At 2 weeks post-injury significant differences between the epicentre and the caudal and rostral sections continued in all three age groups ($P < 0.0001$, $P = 0.0004$). As with the previous time points there was a significant difference between the epicentre and the respective controls in all age groups.

At 6 weeks post-injury the epicentre of the lesion shows significantly greater injury than the caudal and rostral sections, as well as being significantly different to the controls ($P < 0.001$). Interestingly the infants showed no statistically significant difference between any of the three locations within the injury, although all three locations were significantly different to the shams ($P < 0.0001$).

3.4.2b Swollen axons

The number of swollen axons was quantified in the dorsal grey (DGM) and white matter (DWM), the ventral grey (VGM) and white matter (VWM), and the lateral white matter (LWM) at three spinal levels in each age group. There was no significance between groups in the DGM ($P = 0.6729$) and VGM ($P = 0.0246$); the DWM was severely

decimated by the initial injury in both adults and juveniles, so no significance was found here. In the VWM and LWM there were significant increases in both the adults and juveniles at the three spinal cord locations, especially the epicentre ($P = <0.0001$). There was also a significant difference between the injured and control groups in the adults and juveniles in the VWM and LWM. This was not the case for the infants.

The adult and juvenile groups had significantly higher numbers of swollen axons than the infant groups in the VWM and LWM ($P < 0.0001$) at 24 hours post-injury (Figure 35A), 1 week (Figure 35B), 2 weeks (Figure 35C), and 6 weeks (Figure 35D). There was also a significant difference between the injured and control groups in the adults and juveniles in the VWM and LWM at 24 hours, 1 week and 2 weeks post-injury. At 6 weeks post-injury there was a high degree of variation within groups that resulted in large standard deviations. The highest concentration of swollen axons is in the LWM and VWM. There were observable differences between the three spinal cord levels in both the juvenile and adult groups, with the epicentre being higher than the distal locations (data not shown). The adult and juvenile groups retained significantly higher numbers of swollen axons than the infant groups at 6 weeks although it was only statistically significant at the epicentre ($P < 0.0001$).

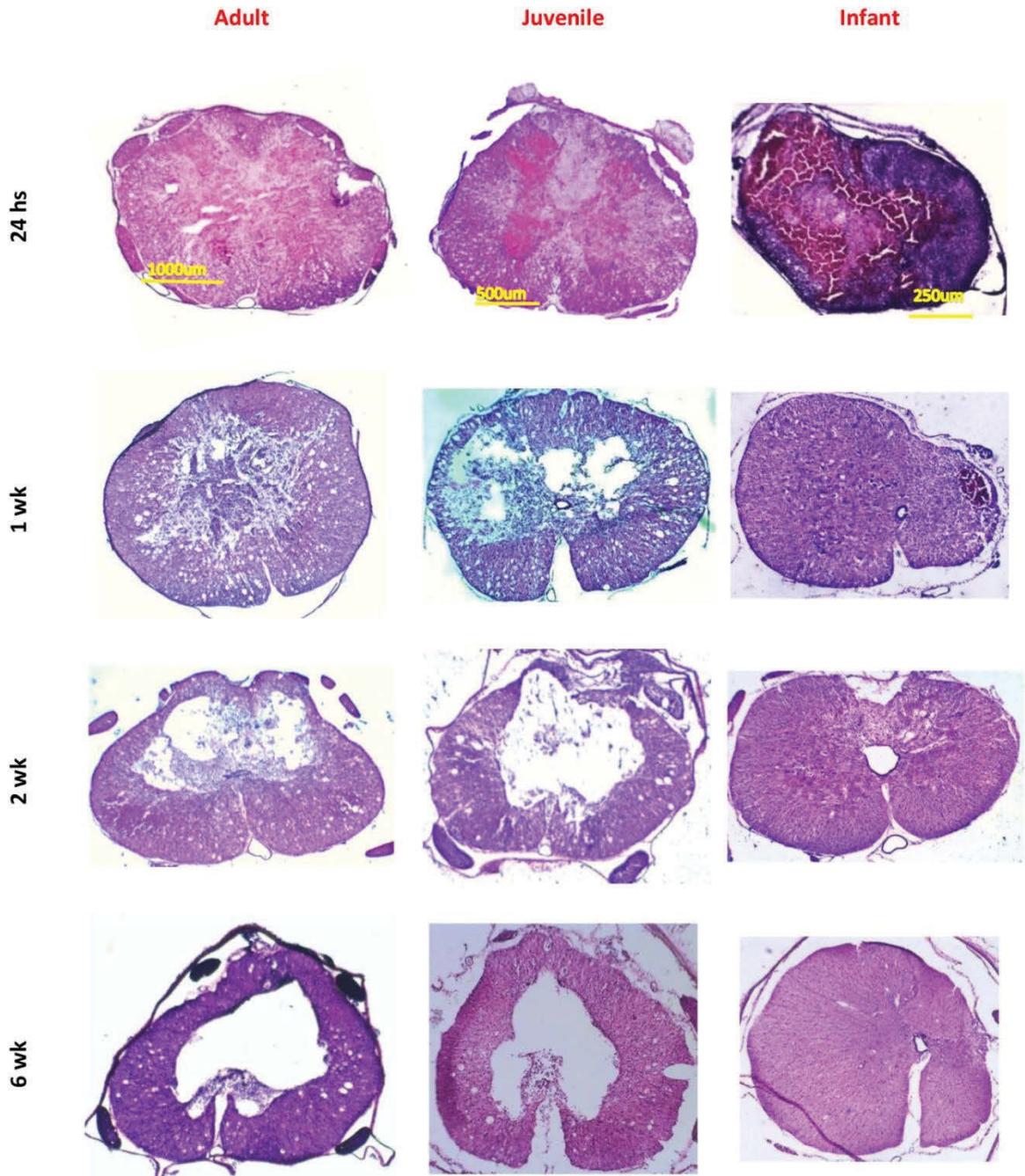


Figure 34: A visual comparison, using H&E staining, to highlight the differences in lesion presentation at the epicentre between adult, juvenile and infant rats at the four time points post-injury. All images have been normalised in size to allow for direct comparison.

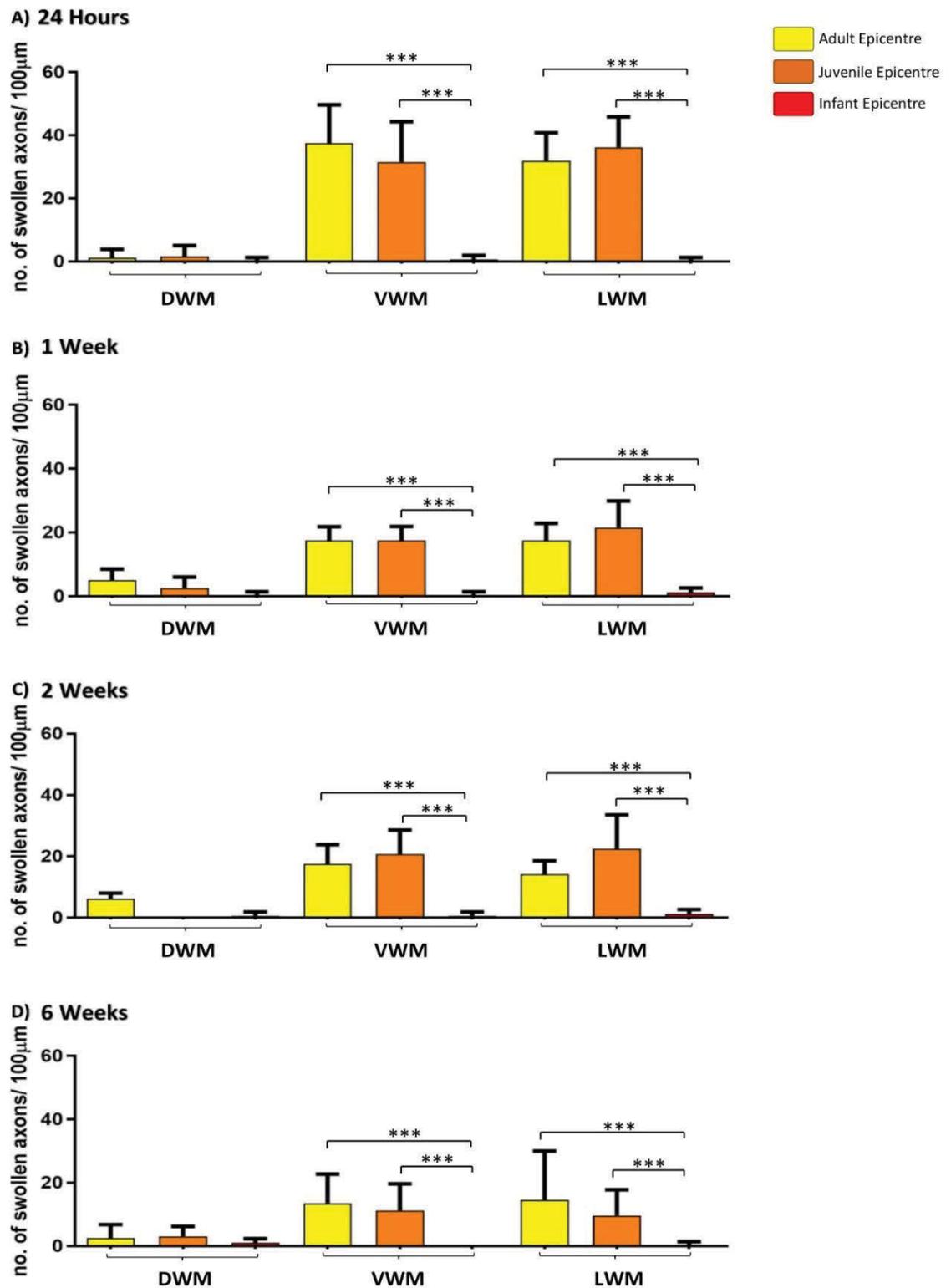


Figure 35: Graph of the number of swollen axons per 100µm at the epicentre by age groups at A) 24h, B) 1wk, C) 2wks and D) 6wks post-injury in the dorsal (DWM), ventral (VWM) and lateral white matter (LWM). Adults are shown in yellow, juveniles in orange and infants in red. *** indicates a statistically significant increase ($P < 0.0005$) based on Bonferroni's post hoc test. Controls are not shown as they are 0 with little variance.

3.4.2c Neutrophil infiltration

The neutrophils observed at 24 hours post-injury show a concentration in the epicentre for all age groups therefore the neutrophils/0.1mm² were calculated at the epicentre for all three age groups and their relative controls. As expected there were no neutrophils infiltrating into the parenchyma of the cord in the normal and sham controls. There was a greater infiltration of neutrophils in the SCI lesion of the adult and juvenile animals in comparison to the infant SCI group as well as a significant increase in the injured groups from their respective controls ($P < 0.0001$) (Figure 36A). At 1 week the neutrophil infiltration is concentrated at the epicentre of the injury in all age groups. This infiltration is greatly decreased from that at 24 hours. There was no statistically significant difference between the infant injured epicentre and the infant shams. A significant difference was seen in neutrophil numbers at the epicentre between the adult and infant cords, as well as between the adult and juvenile SCI and their respective controls ($P < 0.0001$) (Figure 36B).

The neutrophil infiltration evident at 2 weeks post-injury follows the same pattern as that observed at both 24 hours and 1 week, and there was also no significant difference between the injured and control groups in the infant age group ($P < 0.0001$). A significant difference was found in neutrophil numbers between the infant injured group and both the adult and juvenile groups ($P < 0.001$) (Figure 36C). At 6 weeks post-injury the neutrophil infiltration continues in the same pattern. There is a statistically significant increase neutrophil numbers in both the adult and juvenile SCI groups compared to the infant SCI group, as well as a significant difference between the injured and control groups in the adults and juveniles ($P < 0.0001$) (Figure 36D). As with the 1 week and 2 week data there is no significant difference between the infant injured and infant sham groups.

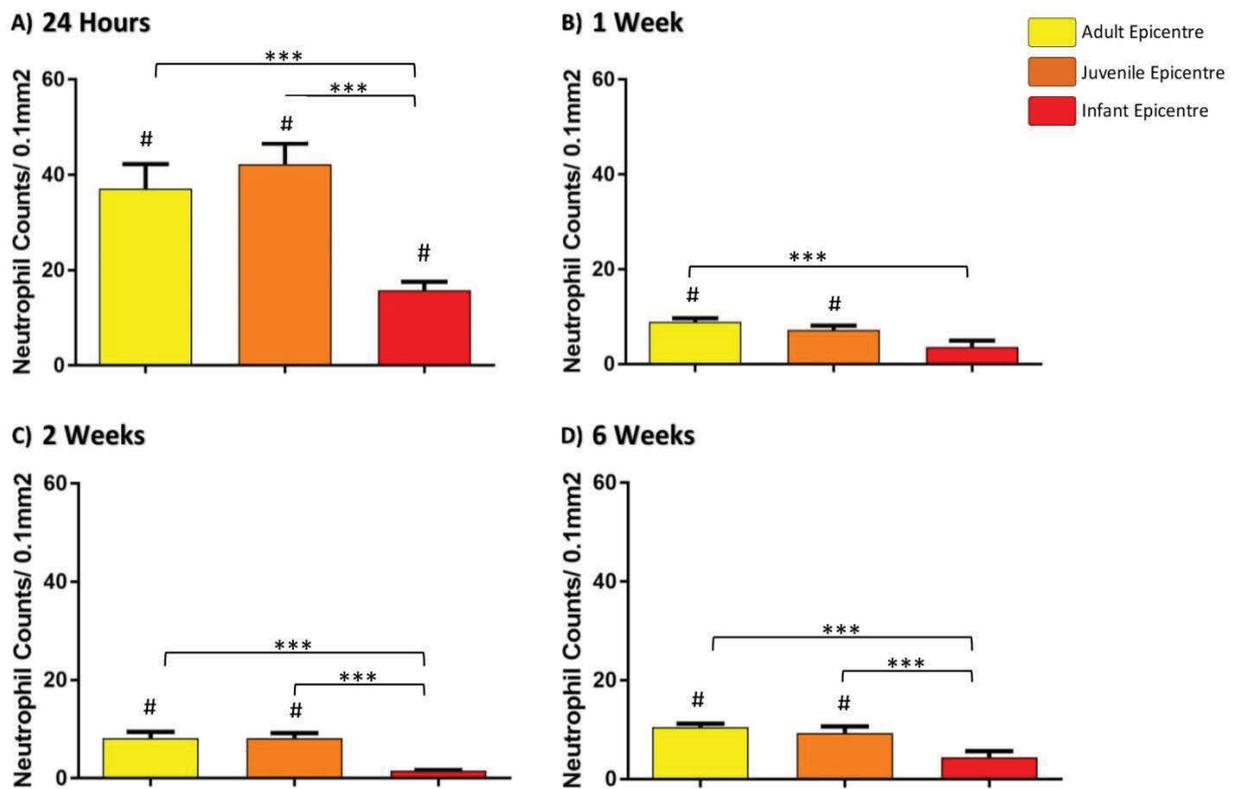


Figure 36: Histogram of the Neutrophils/0.1mm² at the epicentre of the SCI lesion at A) 24 hours, B) 1 week, C) 2 week and D) 6 weeks. * (P<0.005) indicate the significant differences between groups based on Bonferroni's post hoc test. # (P<0.05) indicates significance from the respective control. Controls showed no infiltration and are therefore not shown.**

3.4.2c Macrophages and microglia

24 hours post-injury the pattern of staining in the adult and juvenile groups showed higher levels of both infiltrating macrophages (ED1⁺/IBA1⁻) and activated macrophages/microglia (ED1⁺/IBA1⁺) compared to the ramified microglia (ED1⁻/IBA1⁺) at the epicentre. In the infant group this pattern was reversed, with a significantly higher level of ramified microglia than both of the ED1 positive subsets. At the epicentre there was a statistically significant increase in the proportion of activated macrophages/microglia in the adults and juveniles compared to the infants (P<0.001) and in the proportion of infiltrating macrophages between juveniles and infants (P<0.005). The proportion of activated macrophages/microglia was increased in adults

compared to infants however this was not statistically significant. The infants showed a significantly increased proportion of ramified microglia compared to the two mature groups ($P<0.001$) (Figure 37A).

The pattern of staining seen at 24 hours post spinal cord injury, both at the epicentre and distally, remains fairly consistent for all three age groups at all time points post-injury (Figure 37B-D). There was statistically significant increase in the proportion of activated macrophages/microglia in the adults and juveniles compared to the infants ($P<0.001$) at the epicentre. The proportion of infiltrating macrophages showed no significant differences between ages at the epicentre while the infants showed a significantly increased proportion of ramified microglia compared to the two mature groups ($P<0.001$) (Figure 37B).

Two weeks after SCI there was a statistically significant difference in the proportions of infiltrating macrophages between the adult and juvenile groups, adult and infant groups, and juvenile and infant groups ($P<0.001$) at the epicentre. The ramified microglia were significantly increased at the epicentre in the infant group compared to both of the mature groups ($P<0.001$). Mirroring this the activated macrophages/microglia were significantly decreased in the infant group compared to both the adult and juvenile groups ($P<0.05$) (Figure 37C).

Finally, at 6 weeks post injury, the proportion of infiltrating macrophages was significantly different between the adult and juvenile groups, and the juvenile and infant groups at the epicentre ($P<0.001$). The ramified microglia were significantly decreased at the epicentre in the mature groups compared to the infants ($P<0.001$). At the epicentre the activated macrophages/microglia were significantly increased in the adult and juvenile groups compared to the infant group ($P<0.05$) (Figure 37D).

Distally, the proportions of all three subsets in the adult and juvenile groups reduce to sham levels while the pattern remains consistent in the infant group at 24 hours. At 24 hours the proportion of infiltrating macrophages in both the adult and juvenile groups are still significantly increased from the infants ($P < 0.001$) though only the adult group shows a significant increase in activated macrophages/microglia compared to the infants ($P < 0.05$). The infants still maintained a significantly increased proportion of ramified microglia compared to the adults and juveniles ($P < 0.001$). At 1 week the infants still maintain a significantly increased proportion of ramified microglia compared to the adults and juveniles ($P < 0.001$) and the proportion of infiltrating macrophages in the juvenile groups is still significantly increased from the infants ($P < 0.005$); although the adult group appears increased as well this was not statistically significant. Both the adult and juvenile groups showed significantly higher proportions of activated macrophages/microglia compared to the infants ($P < 0.005$). At 2 weeks there was a statistically significant difference in the proportions of infiltrating macrophages between the adult and juvenile groups, and juvenile and infant groups ($p < 0.001$). Following the pattern demonstrated throughout the ramified microglia were significantly decreased distally in the mature groups compared to the infants ($P < 0.001$). As at the epicentre the activated macrophages/microglia were significantly increased in the adult and juvenile groups compared to the infant group ($P < 0.005$). By 6 weeks there was a statistically significant difference in the proportions of infiltrating macrophages between the adult and juvenile groups, and juvenile and infant groups distal to the epicentre ($P < 0.001$). The ramified microglia were significantly increased 2.25mm distally in the infant group compared to both of the mature groups ($P < 0.001$). Mirroring this the activated macrophages/microglia were significantly decreased in the infant group compared to both the adult and juvenile groups ($P < 0.05$).

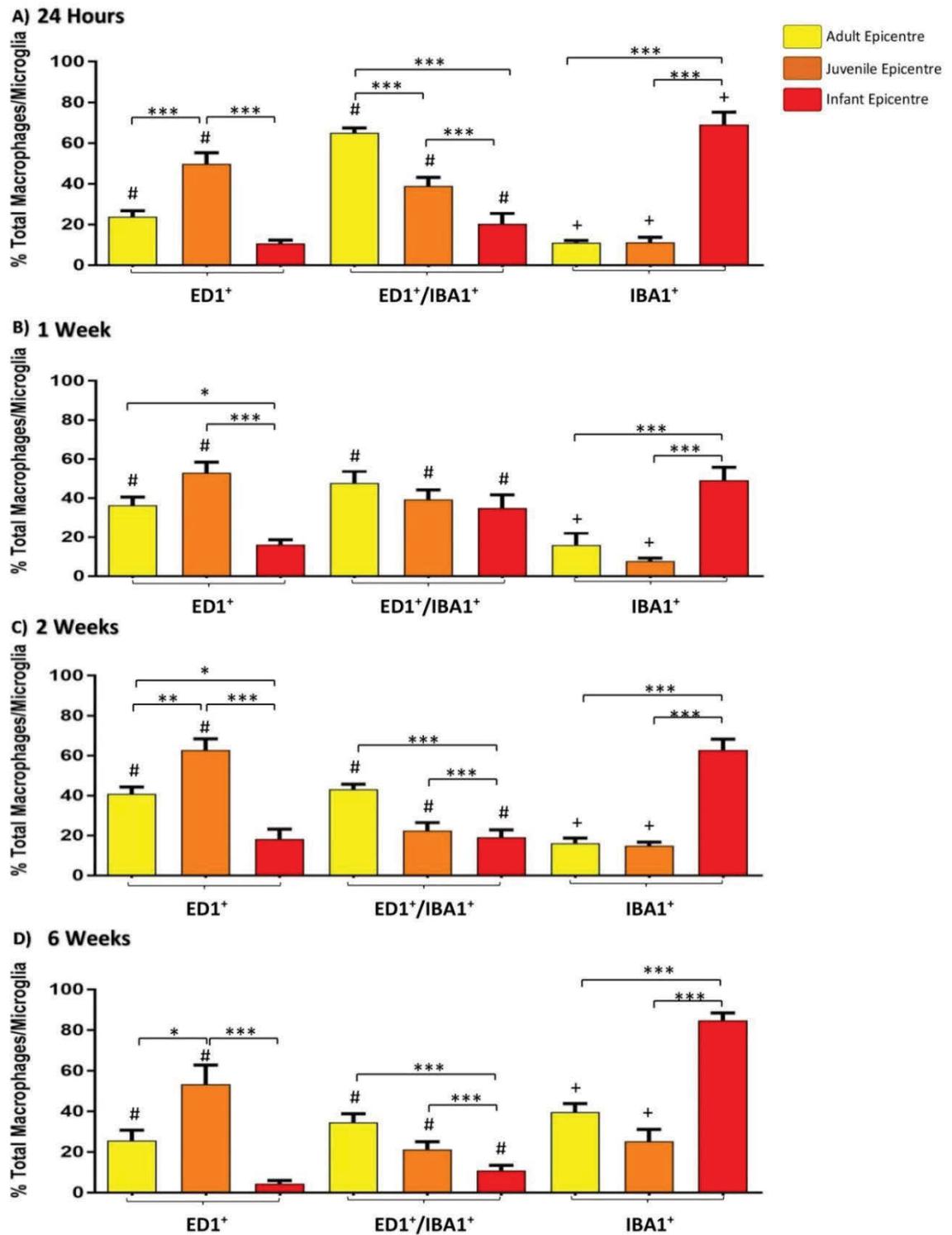


Figure 37: Histograms of the proportion of the total macrophage/microglial population staining ED1+ (Infiltrating macrophages), ED1+/IBA1+ (activated macrophages/microglia) and IBA1+ (resting microglia) at the injury epicentre A) 24hours, B) 1 week, C) 2 weeks and D) 6 weeks post-injury. * (P<0.05), ** (P<0.005) and *** (P<0.001) indicate the significant differences between groups based on Bonferroni's post hoc test and # (P<0.05) indicates a significant increase from sham and + a significant decrease.

3.4.2e Astrocytes

The pattern of distribution of the astrocytes in the normal and sham rats of all three age groups followed the same general pattern, as can be seen in the adult example in Figure 38, with the highest GFAP staining seen in the white matter. This distribution of GFAP is maintained in the injured animals (Figure 39), with an increase overall as well as around the lesion edge, and is consistent between age groups.

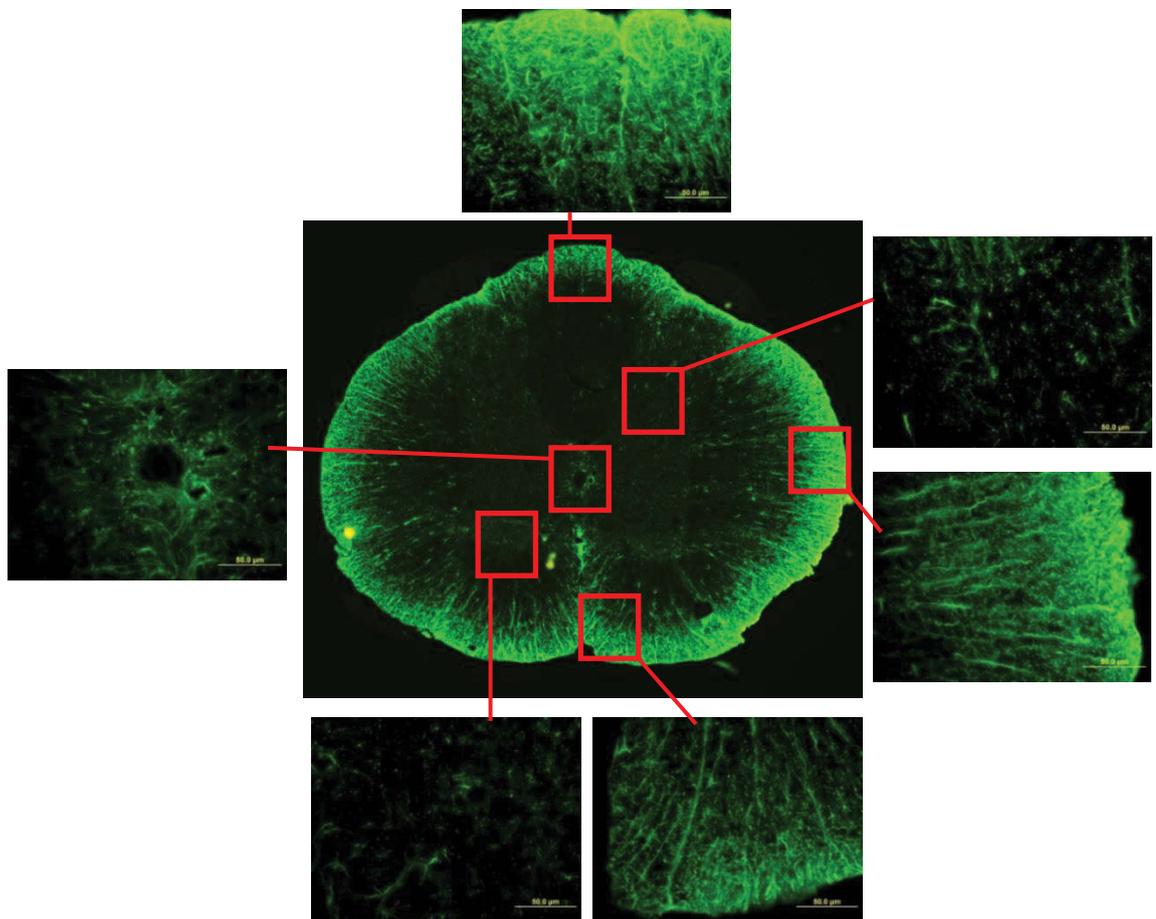


Figure 38: A map of the distribution of GFAP in the normal spinal cord of an adult rat, mirrored in juveniles and infants, and maintained to a high degree in the lesioned cord.

At 24 hours after the induced SCI there is a small increase in the GFAP MGV around the lesion edge, however this was not significant (Figure 40A). 1 week after SCI the GFAP MGV is still increasing at the lesion edge as the glial scar begins to form. This

is fairly consistent between the age groups with no significant differences found in the GFAP intensity around the lesion between adult, juvenile and infant rats (Figure 40B). 2 weeks post injury the GFAP MGV around the lesion edge is still increased in the mature groups. The juvenile rats are the only group to show significant increases from the sham level at all three locations on the cord, however there is still a visible increase in the adults as well. The infants have returned to sham levels (Figure 40C). By 6 weeks post injury the adult and juvenile rats have significantly increased GFAP intensity around the lesion edge at all three locations on the spinal cord tested, compared to their respective shams, consistent with the formation of the glial scar (Figure 40D).

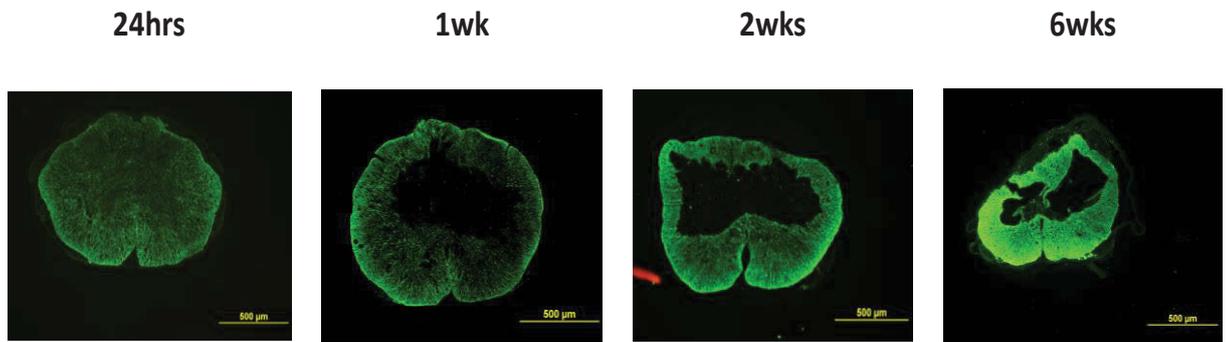


Figure 39: Fluorescent microscopy examples of the distribution of GFAP in injured adult cords, a pattern that is fairly consistent between age groups.

The GFAP staining is most prominent in the white matter. 24 hours post injury there is a visible increase in the GFAP MGV in the juvenile and infant groups; however, this is not statistically significant. The only statistically significant difference is between the infant and adult GFAP MGV in the ventral white matter, with the infants having significantly higher GFAP intensity (Figure 40A). There was a small increase in GFAP intensity visible in the injured white matter compared to the respective shams in all three age groups, but this was not found to be statistically significant. There were also no significant differences in MGV between ages found in the white matter (Figure

40B). Essentially all three age groups have dropped back to the levels of their respective shams in the white matter by 2 weeks post-injury. There were also no significant differences in MGV between ages found in the white matter (Figure 40C).

The infants had greater GFAP immunoreactivity in the spinal cord than the adults and juveniles; greater differences are seen when the results are presented as a fold change from the baseline immunoreactivity. The fold increase of GFAP from sham levels is obviously decreased in the white matter and at the lesion edge compared to the adult and juvenile groups after 24 hours (Figure 41A). From 1 week post-injury onward the infants increase in GFAP intensity is consistently less than half that seen in the two mature groups (Figure 41B-D). In the white matter the fold increase in GFAP was highest in the adults compared to the infants at all time points, although the fold change was not as high in the white matter as at the lesion edge (Figure 41A-D). GFAP MGV remained at a constant level in the infants while in the mature groups it increased at 1, 2 and 6 weeks. This results in a fold increase in the infants after 24 hours that is, once again, less than half that seen in the adults and juveniles.

3.4.2f Endogenous neural progenitor cells

The endogenous neural progenitor cells in the ependymal layer of the central canal are activated as early as 24 hours post SCI in all three age groups tested (P7, 5wks and 9wks). The fluorescence images in the first row of Figure 42 show the typical morphology of activated eNPCs with long basal processes extending into the parenchyma. The central canal was not always present or intact at the epicentre of the injury, because of this one section 2.25mm distal of the epicentre was also examined in detail. Nestin was also seen in the blood vessels of both the injured and sham spinal cords.

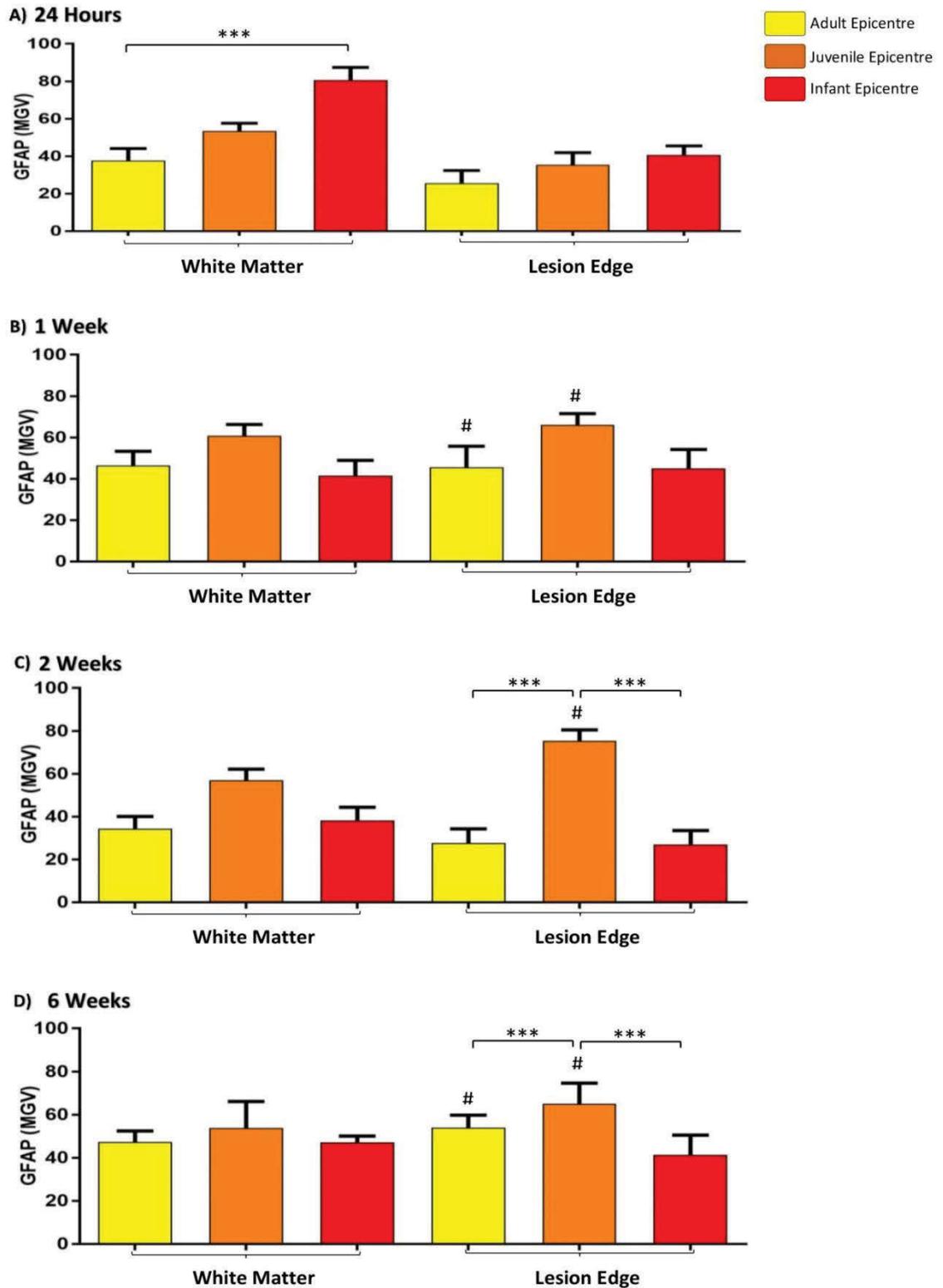


Figure 40: Histograms of the staining intensity, using the mean greyscale value (MGV), of GFAP at the lesion edge (LE) and in the remaining white matter in the epicentre of the lesion A) 24hours, B) 1 week, C) 2 weeks and D) 6 weeks post-injury. *** ($P < 0.001$) indicates the significant differences between groups based on Bonferroni's post hoc test and # ($P < 0.05$) indicates a significant difference from the respective sham.

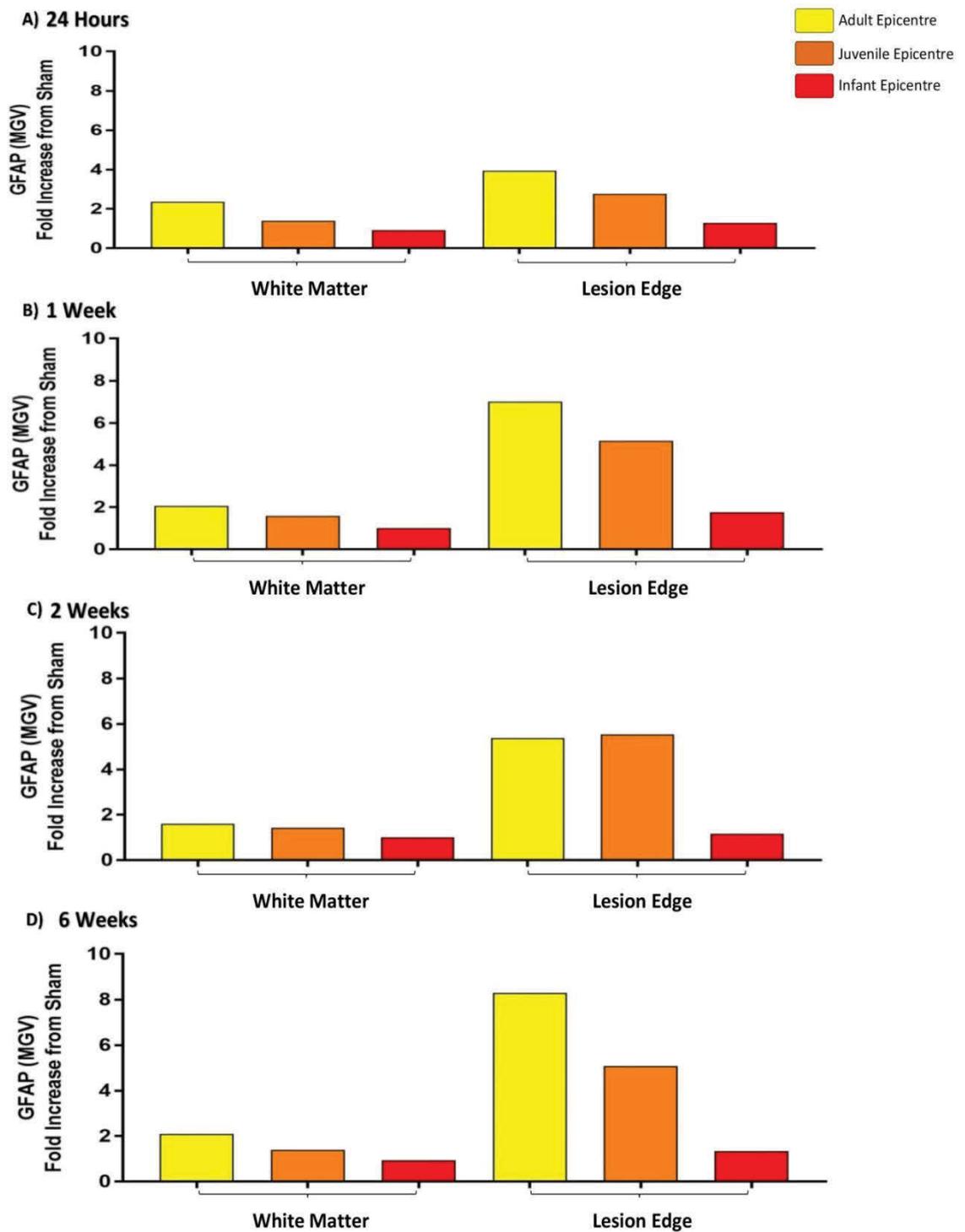


Figure 41: Histograms of the fold increase in staining intensity of GFAP (MGV) in the SCI groups from their respective shams, using the mean greyscale value at the lesion edge and in the remaining white matter in the epicentre of the lesion A) 24hours, B) 1 week, C) 2 weeks and D) 6 weeks post-injury.

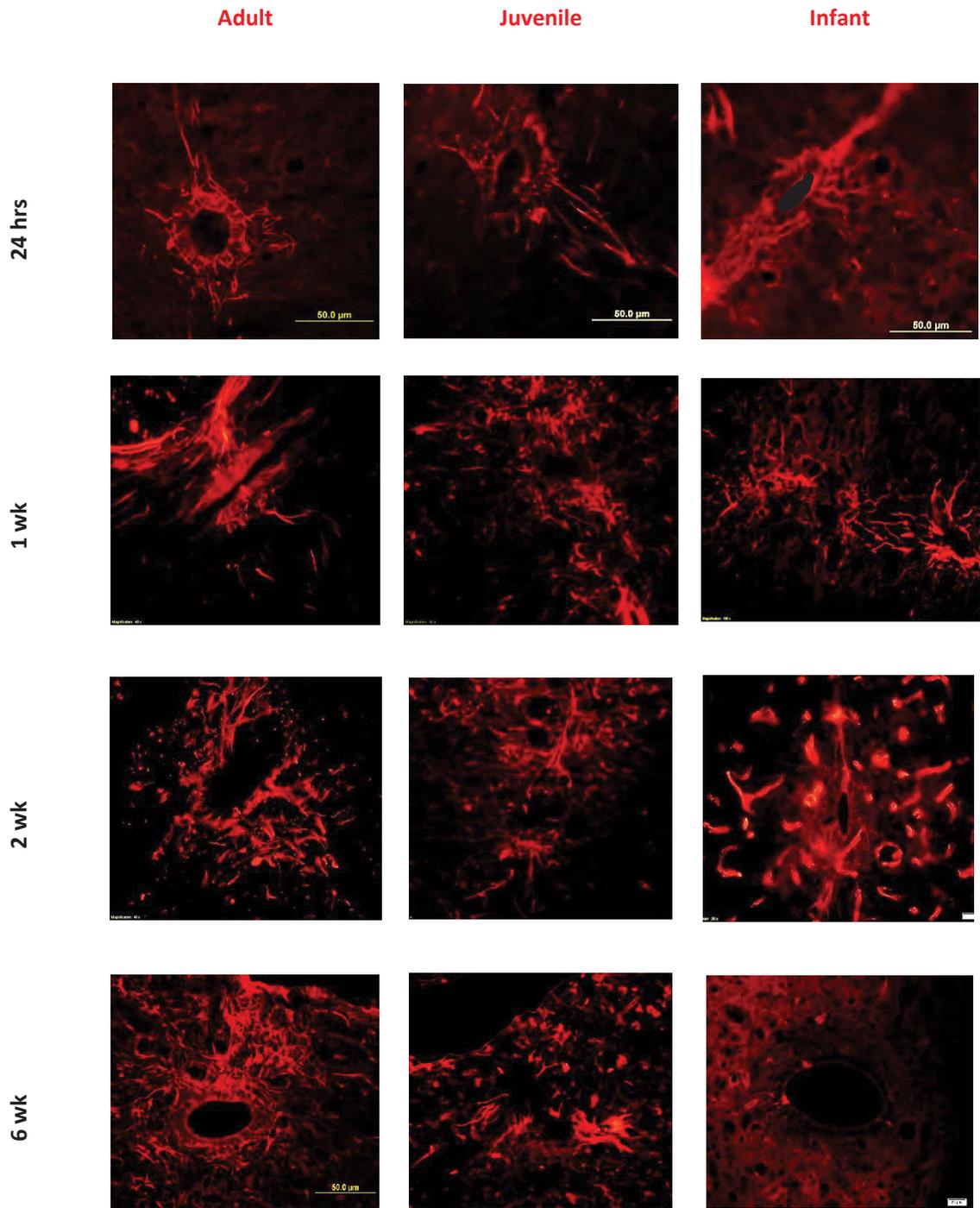


Figure 42: Fluorescent microscopy images of the central canal taken at 400x, 2.25mm distal to the epicentre of the injury, stained with nestin as a marker for endogenous neural progenitor cells. The ependymal layer of the central canal and long processes extending into the parenchyma can be seen, some blood vessels are also visible as strongly nestin positive. The staining intensity in the infants is visibly lower than the adults and juveniles at the 2wk and 6wk time points.

At the epicentre at 24 hours all three age groups showed significantly higher nestin MGV at the epicentre central canal (Figure 44A). 1 week post injury exhibited higher nestin MGV around the central canal in the adult and juvenile groups than the infants ($P < 0.0001$) (Figure 44B). The infant central canal only showed a statistically significant increase from the shams at the epicentre ($P < 0.0001$) (Figure 44B). At both the epicentre and 2.25mm distal the nestin MGV was significantly higher at the central canal in the adults compared to the infants ($P < 0.0001$).

Distally, the nestin staining intensity at the central canal showed the greatest differences between age groups at 2.25mm rostral to the epicentre at 2 weeks post SCI. Both the adult and juvenile groups showed significant increases, from their respective shams, as well as compared to the infants ($P < 0.0001$). The increased nestin levels in the central canal that were seen at 24 hours and 1 week post SCI are being maintained, to some degree, in the adults and juveniles but have dropped off considerably in the infants. The only significance 2.25mm caudal ($P < 0.0001$) (Figure 45C) and at the epicentre ($P < 0.0001$) (Figure 44C) was between the adult and its respective sham. The lack of significance at the epicentre may be, in part, due to the lack of a central canal in the juvenile rats (Figure 44C). At 6 weeks post injury the nestin levels at the ependymal layer of the central canal have dropped off considerably in all three age groups, especially in the infant rats. The only significance between age groups can be seen between the juvenile and infant groups 2.25mm distal of the epicentre ($P < 0.0001$) (Figure 45D).

The nestin MGV around the lesion edge shows no increase from the respective shams at 24 hours (Figure 44A). At 1 week post-injury the edge of the lesion site showed a visible increase in nestin staining, from the appropriate sham, in all three age

groups both at the epicentre (Figure 44B) and distally (Figure 45B). This was only statistically significant for all three ages at the epicentre ($P < 0.0001$) (Figure 44B). At 2 weeks post injury there is a peak in nestin MGV at the lesion edge in the adult and juvenile rats but very little nestin staining present in the infants (Figure 45C). Nestin staining in the adults were significantly higher distally as well as the epicentre, while the juveniles were significantly higher only rostrally and at the epicentre compared to the shams ($P < 0.0001$). At the epicentre both the adult and juvenile groups had significantly higher nestin MGV at the lesion edge compared to the infants ($P < 0.0001$). This is to be expected as there was no true lesion in the infants after the 24 hour time point. Visually, there was substantial GFAP and nestin staining individually around the lesion edge in adults and juveniles, and also a high level of dual staining (Figure 43). This was not quantified.

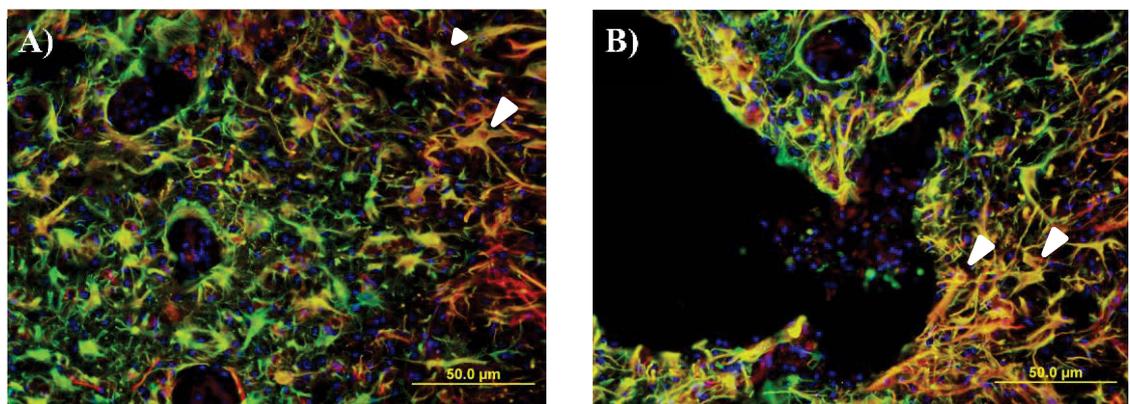


Figure 43: Fluorescent microscopy images of adult grey matter (A) and lesion edge (B) taken at 400x, 2.25mm distal to the epicentre of the injury, stained with nestin as a marker for endogenous neural progenitor cells (red) and GFAP for astrocytes (green), with blue indicating the cell nuclei. This shows a number of dual stained cells and processes in an orange colour, many of which have an astrocytic appearance (white arrows).

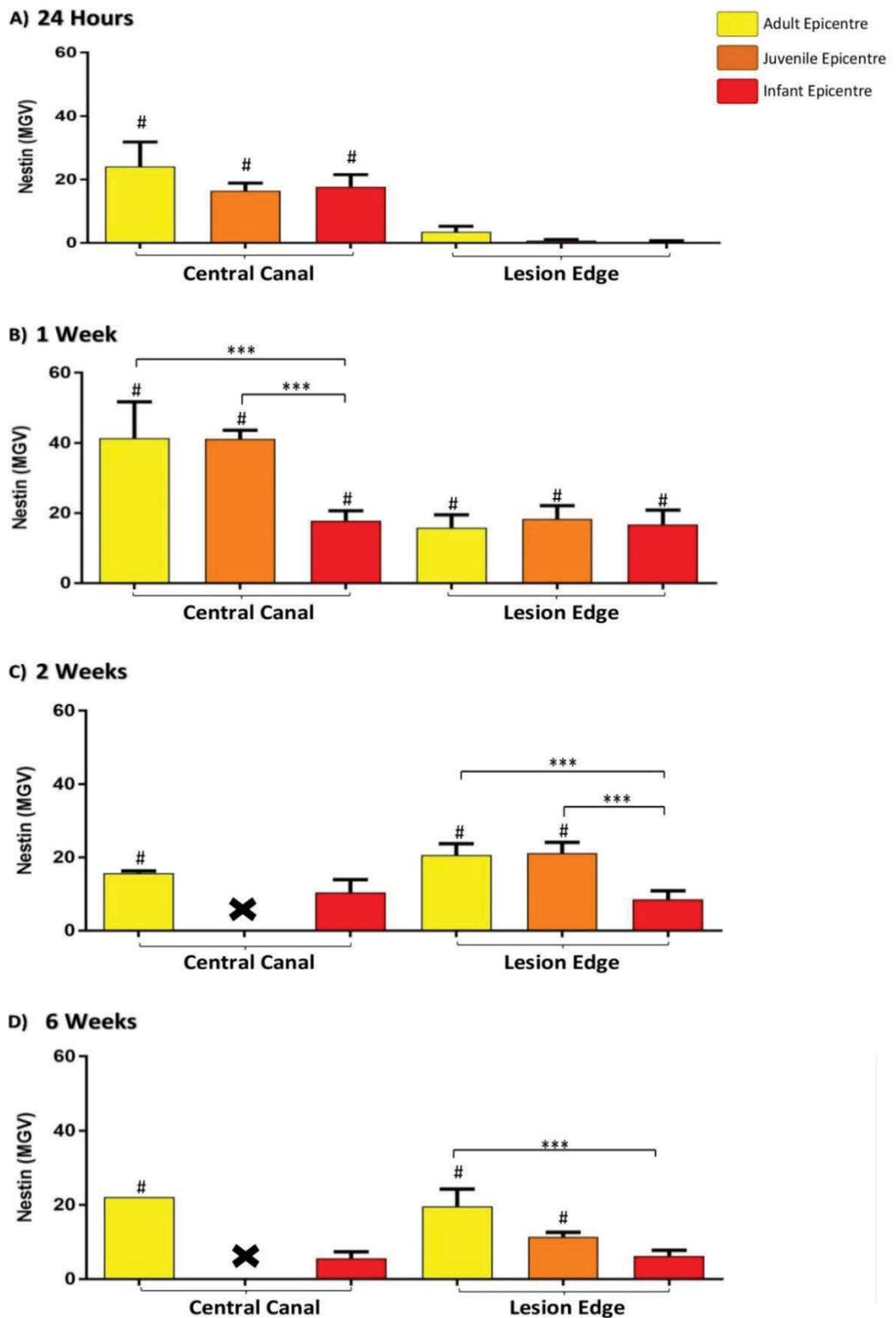


Figure 44: Histograms of the staining intensity, using the mean greyscale value (MGV), of nestin at the epicentre in the ependymal layer of the central canal (CC) and the lesion edge (LE) A) 24hours, B) 1 week, C) 2 weeks and D) 6 weeks post-injury. *** (P<0.001) indicates a significant difference between groups based on Bonferroni's post hoc test and # (P<0.05) indicates a significant difference from the respective sham. X indicates where the central canal was completely destroyed.

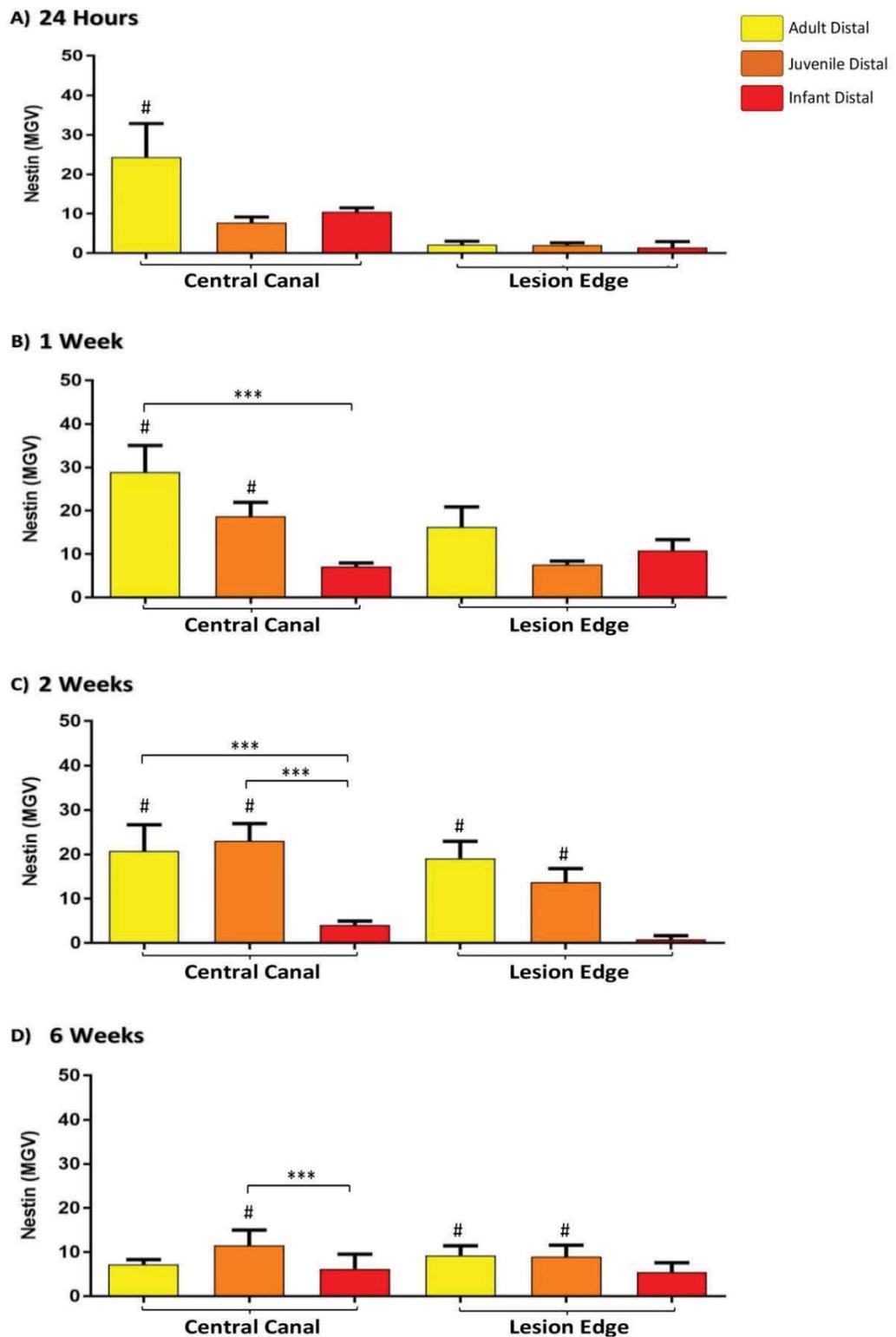


Figure 45: Histograms of the staining intensity, using the mean greyscale value (MGV), of nestin 2.25mm distal to the epicentre in the ependymal layer of the central canal (CC) and lesion edge (LE) A) 24hours, B) 1 week, C) 2 weeks and D) 6 weeks post-injury. *** ($P < 0.001$) indicates a significant difference between groups based on Bonferroni's post hoc test and # ($P < 0.05$) indicates a significant difference from the respective sham.

3.4.3 There is no significant difference between seven day and 10 day old infants

3.4.3a Lesion Size and Presentation

It can be observed through H&E staining that the SCI induced presents differently in infants compared to adults and juveniles. A small group of animals were injured at P10 and allowed to recover for 6 weeks before culling, to compare to the P7 response. As with the P7 group these P10 animals showed a disparity in size of the left and right sides (Figure 46). In addition to this two of these P10 animals had a fairly significant lesion cavity (#1398 #1389) (Figure 47) while the rest of the group presented with a cavity resembling a small bite out of the smallest side.

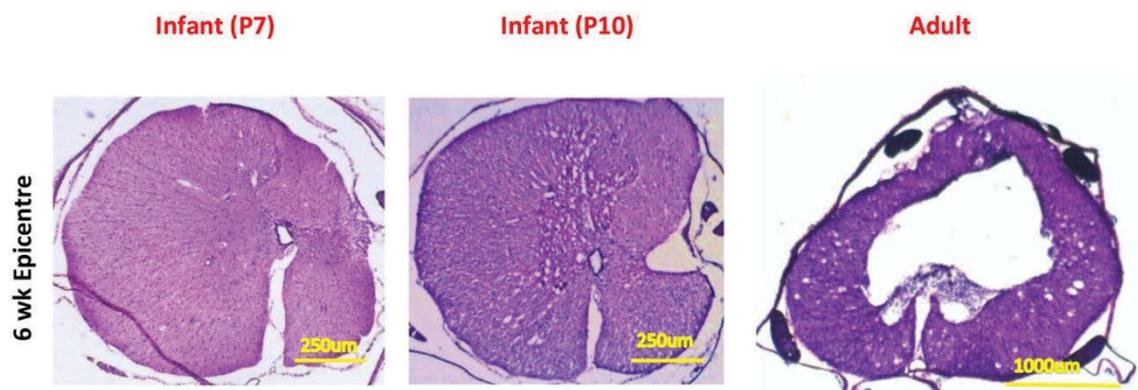


Figure 46: A visual comparison, using H&E staining, to highlight the differences in lesion presentation at the epicentre between adult, P7 and P10 infant rats at 6 weeks post-injury. These images are not to scale but have been matched in size.

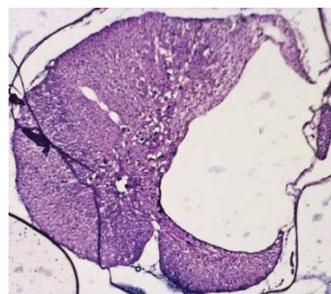


Figure 47: Caudal to the epicentre of P10 animal 1389, showing a much more pronounced cavity than the rest of the P10 group.

There were no significant differences found between the P7 and the P10 groups at any of the three points in the injury with 6 weeks survival time and also no significance between the locations within the P10 injured group (Figure 48). Details on the P7 lesion can be found in section 3.4.1c.

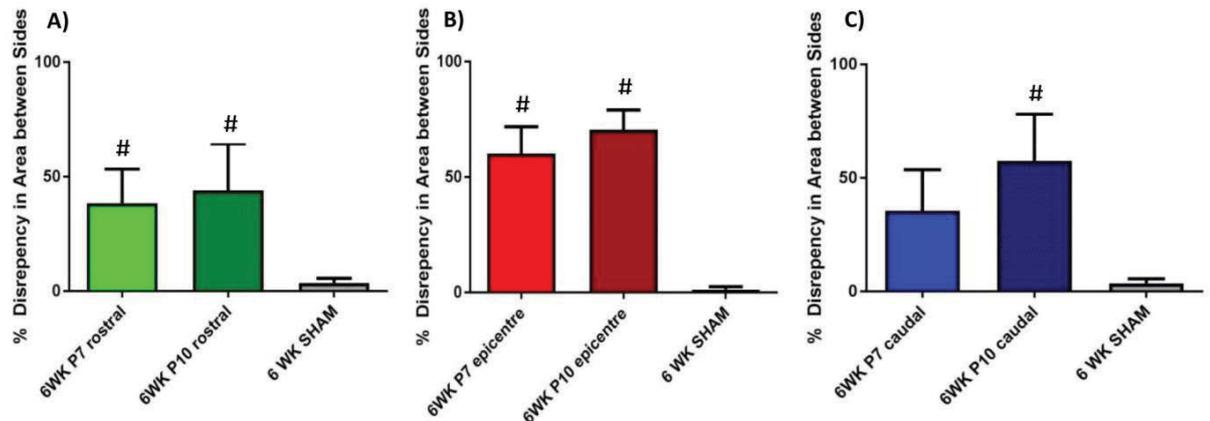


Figure 48: Histogram depicting the lesion area in infant rats (P7 and P10) at three levels of the spinal cord at 6wks post-injury. A) is 2.25mm rostral, B) is the epicentre and C) is 2.25mm caudal. # ($P < 0.05$) indicates difference from the respective sham based on Bonferroni's post hoc test.

3.4.3b Swollen Axons

There was no difference in the number of swollen axons was found between the P7 ($0.5 \pm 1.3/100\mu\text{m}^2$) and P10 ($2.5 \pm 1.8/100\mu\text{m}^2$) injured groups at 6 weeks post-injury.

3.4.3c Neutrophils

There were few neutrophils observed at the epicentre of the injury in either the P7 or P10 groups 6 weeks post-injury. There was no statistically significant difference in neutrophil infiltration between the P7 and P10 infant groups. There was a statistically significant increase in the number of neutrophils following SCI ($P < 0.05$) and it was similar for both P7 and P10 (Figure 49).

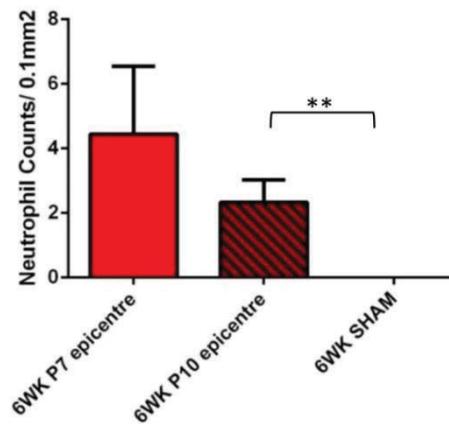


Figure 49: Histogram of the Neutrophils/0.1mm² at the epicentre of the SCI lesion at 6 weeks in 7 day old (P7) and 10 day old (P10) infants, with associated controls. ** (<0.005) indicate the significant differences between groups based on Bonferroni's post hoc test.

3.4.3d Macrophages and Microglia

Both the P7 and P10 groups followed the same pattern observed previously for ED1/IBA1 staining in the infant groups (section 3.4.1c), i.e., greatly decreased numbers of the two ED1⁺ (active) subsets compared to the ED1⁻/IBA1⁺ (ramified) subset. However, there were more IBA1⁺ cells in the P10 infant group both at the epicentre ($P < 0.05$) and 2.25mm distal ($P < 0.001$), with the P7 being higher. It can be seen that the majority of difference is within the percentage of ED1⁻/IBA1⁺ ramified cells; however there are few differences between the P7 and P10 groups in the ED1⁺/IBA1⁺ and one difference in the ED1⁺/IBA1⁻ (Figure 50).

3.4.3e Astrocytes

At 6 weeks post-injury the P10 SCI group exhibited significantly higher GFAP MGV at the lesion edge, compared to the P7 infants. However only the P10 epicentre was significantly increased from the 6 week sham level. There was no significant difference between the GFAP in P7 and P10 groups in the white matter (Figure 51).

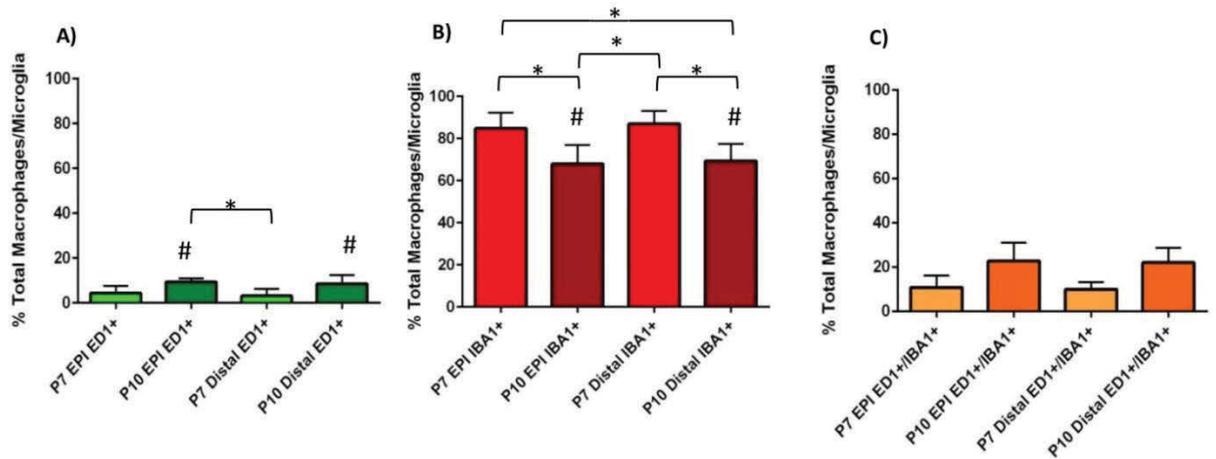


Figure 50: Histogram of the proportion of the total macrophage/microglial population staining A) ED1+/IBA1-, B) ED1-/IBA1+ and C) ED1+/IBA1+ at 6 weeks in 7-day old (P7) and 10-day old (P10) infants.

This represents both the epicentre of the SCI lesion and 2.25mm distal. * (P<0.05) indicate the significant differences between groups based on Bonferroni's post hoc test and # (P<0.05) indicates a significant difference from the sham.

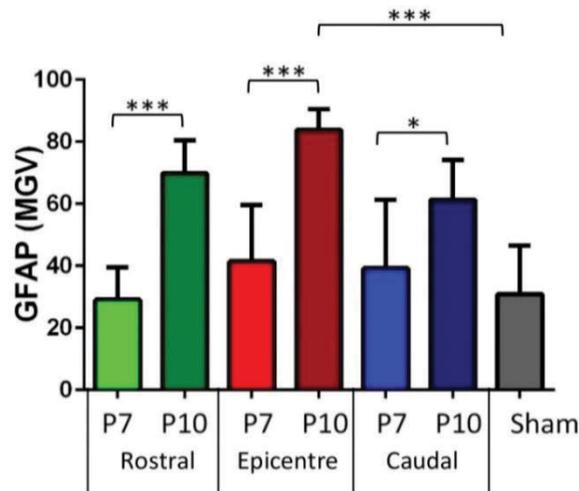


Figure 51: Histograms of the staining intensity, using the mean greyscale value (MGV), of GFAP at the lesion edge in infant rats, at the ages of P7 and P10. * (P<0.05) and * (P<0.001) indicate the significant differences between groups based on Bonferroni's post hoc test.**

3.4.3f Endogenous Neural Progenitor Cells

As can be seen earlier in this chapter, these cells are activated quite substantially after SCI in adult, juvenile and infant rats, though the increased staining intensity is sustained for a shorter period of time in the infants. This again raises the

question of the influence of age. Both the P7 and P10 groups showed some visible increase in nestin staining at 6 weeks post-injury compared to the sham (Figure 52).

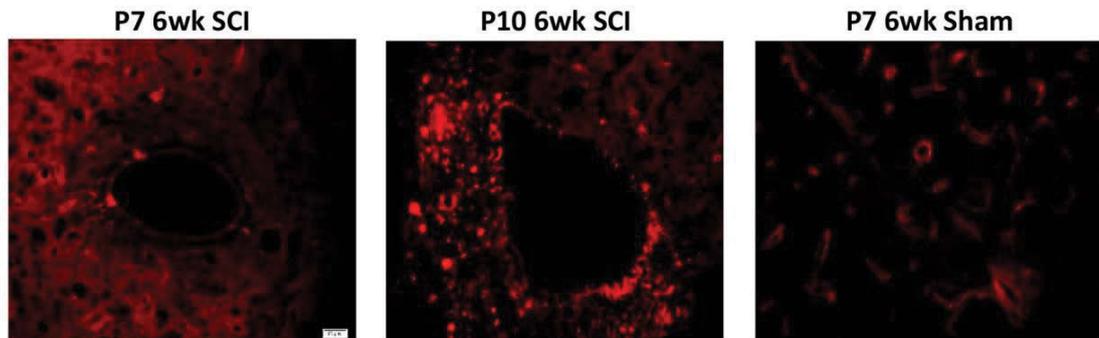


Figure 52: Fluorescent microscopy images of the central canal taken at 400x, 2.25mm distal to the epicentre of the injury, stained with nestin as a marker for endogenous neural progenitor cells. The ependymal layer of the central canal and few processes extending into the parenchyma can be seen in the injured animals.

The nestin staining was very similar in both P7 and P10 infant age groups. The values for both ages, at all three locations on the spinal cord, showed an increase from the sham values however this was not statistically significant with the exception of the lesion edge at 1 week post-injury in the P10 group.

3.4.4 Results summary

The lesion initially presented as a necrotic and haemorrhagic area in the spinal cord, extending rostrally and caudally of the impact epicentre. This then developed into a cystic cavity in the older animals but presented as asymmetry across the midline in the infant animals. There were significantly more swollen axons and neutrophils at the earliest time point in the older animals, than the infants and their later time points.

There was also significant difference in the proportions of the different populations of macrophages and microglia between the age groups with the infants

favouring the ramified microglia, while the older animals had greater numbers of active and phagocytic cells. The astrocytic response was of a much greater magnitude in the older animals compared to the infants, and increased sharply at 1 week post injury. The activation pattern of eNPC was similar in all three ages.

There were no significant difference in the chronic response to SCI between P7 and P10 infants, except in the Macrophage/Microglial response which was increased in the P10 rats.

Table 4: Summary of the results from Chapter 2. + and ↓ indicate trends of increases or decreases respectively relative to the appropriate sham, averaged across the injury length. The - indicates no significant difference from the sham. + indicates a trend toward increase, ++ denotes an increase with statistical significance (P<0.05) and +++ an increase with significance (P<0.005).

| | ADULT (9wks) | | | | JUVENILE (5wks) | | | | INFANT (P7) | | | |
|---|--|-----|------|------|--|-----|------|------|--|-----|------|------|
| | 24h | 1wk | 2wks | 6wks | 24h | 1wk | 2wks | 6wks | 24h | 1wk | 2wks | 6wks |
| Lesion Progression | Begins as large necrotic lesion and progresses to cystic cavity covering around 50% of the total transverse area | | | | Begins as large necrotic lesion and progresses to cystic cavity covering around 50% of the total transverse area | | | | Begins as a large necrotic lesion (24hs) and progresses as simply a disparity in size between the sides across the midline, no obvious tissue disruption | | | |
| Swollen axons | ++ | - | - | - | ++ | - | - | - | - | - | - | - |
| Neutrophil infiltration | +++ | - | - | - | +++ | - | - | - | +++ | - | - | - |
| Resting microglia (Iba1⁺/ED1⁻) | ↓↓↓ | ↓↓↓ | ↓↓↓ | ↓↓↓ | ↓↓↓ | ↓↓↓ | ↓↓↓ | ↓↓ | ↓↓ | ↓↓ | ↓↓ | - |
| Activated phagocytic cells (ED1⁺) | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | - | + | + | - |
| Reactive astrogliosis in the white matter | - | - | - | - | - | - | - | - | ++ | - | - | - |
| Astrocytic glial scar | - | +++ | + | ++ | + | +++ | +++ | +++ | - | - | - | - |
| Activation of eNPC in the central canal | ++ | +++ | ++ | - | + | +++ | +++ | - | ++ | +++ | + | - |
| Migration of activated eNPC to lesion site | - | + | ++ | + | - | ++ | ++ | + | - | + | + | - |

3.5 DISCUSSION

3.5.1 Differences in spinal cord injury response between ages of rats

3.5.1a The injury progression shows histological differences between mature and infant rats that may have a significant impact on recovery

In SCI the first cells to arrive, within hours of the insult, are neutrophils. These are closely followed by exogenous macrophages/monocytes and the activation of endogenous microglia (Zhang and Gensel, 2014). The injury microenvironment progresses through a largely pro-inflammatory response (Beck et al., 2010b; Stammers et al., 2012) that persists for some time until reactive astrocytes are also recruited to form a glial scar (Sofroniew, 2009). In 2006 Fleming et al. described the typical tissue response to SCI in three distinct zones; the inflammation and necrosis at the lesion site, the spreading axonal swelling, Wallerian degeneration, and further inflamed tissue (Fleming et al., 2006; Sofroniew, 2009). These areas of damage are surrounded by areas of histologically intact tissue and spared axons, of significance therapeutically and in the recovery of function (Donnelly and Popovich, 2008; Fawcett and Asher, 1999; Hamilton et al., 2009; Sofroniew, 2009).

The injury in our experimental model presented very similarly in all groups at 24 hours survival time but appeared vastly different at the three later time points (1 week, 2 weeks and 6 weeks). In both the juvenile and adult groups the area of necrosis and disrupted tissue that comprised the lesion at 24 hours progressed to become a cystic cavity by 6 weeks, presenting as a large hole in the cross-section of the tissue, tapering out caudally and rostrally. In contrast to this, after the initial haemorrhagic lesion at 24 hours, the P7 infant groups showed an asymmetry between the left and right sides of the spinal cord and did not present with a cavity or visible tissue

disruption. There are a few potential explanations for this phenomena; based on the increased plasticity of the infant cords (Bregman and Goldberger, 1982; Goldberger and Murray, 1985; Pape, 2012) as well as the developmental state of the cord at post-natal day 7. It was established in the 1980s that the young and still developing spinal cord has greater neural plasticity, this may allow for greater cellular survival and neural re-wiring compensating for the injury (Bregman and Goldberger, 1982; Bregman and Goldberger, 1983a; Pape, 2012); however, this is still poorly understood and has not been studied to a great extent. Embryonic spinal cord transplants have previously been shown to promote greater CST sprouting in the lesioned adult spinal cord, though the factors behind this are unknown (Schnell and Schwab, 1993). It is also possible that the developmental state of the cord allowed for axons that had not yet developed or reached the T10 region of the cord to grow through or around the injury site (Joosten et al., 1987). Why this appeared in one side of the cord only is unknown at this stage. The impact was centred on the midline and was supposed to be equally distributed across both sides. Certainly the lesion appearance at 24 hours indicated no overall left or right bias in tissue disruption. We can speculate that there was in fact slight asymmetry in the impact and that the less injured side atrophied at the expense of the regenerating/protected side. Left and right asymmetries were seen in equal degrees so there did not appear to be a systemic methodological issue, but it cannot be ruled out that one side was simply more damaged than the other. This model of neonatal spinal cord injury has not been used previously so we are unable to verify if this is the normal pattern of injury. Even so, the development of the lesion over time was very different to that seen in both the juvenile and adult spinal cords, and most importantly, there was no central cavity observed in any of the infant spinal cords over time.

These stark differences in the injury beg the question as to the mechanisms underlying. To this question we still have no answer. There are a few possible explanations, behind the injury differences involving both the simple mechanics of the injury itself and the complex biomechanics and functional plasticity of the cord itself. One possibility is simply that the gross mechanics of the infant cord make it difficult to induce a central injury therefore the impact causes uneven cell death and results in one side exhibiting more apoptosis than the other; this may also result in some compensation from the other side of the cord. This difficulty in producing a central, bilateral injury has been encountered previously by other infant studies, such as the one by Brown et al. in 2005 using P14-15 rats (Brown et al., 2005). Studies using a vertebral displacement model in rats highlight the biomechanical influences on injury severity (Clarke and Bilston, 2008; Jones et al., 2012; Lau et al., 2013). Other possibilities involve more complex mechanisms within the developing cord. In P14-15 pups Brown et al. found that while the initial injury and behavioural deficits were similar to previous adult studies there were also striking differences. Specifically, differences in the timing of neuronal loss and the speed of functional recovery (Brown et al., 2005). This study found little obvious correlation between the neuropathy and the rapid behavioural recovery in the infant rats or any obvious asymmetry in locomotor function, leaving the underlying mechanisms of the functional recovery differences in question. The different results from different studies may be strongly influenced by the different injury mechanisms (Jones et al., 2012), the different ages at injury and the different animal models used (Karimi-Abdolrezaee et al., 2010), be it rodent (Brown et al., 2005; Kumamaru et al., 2012; Yuan et al., 2013), feline (Bregman and Goldberger, 1982; Bregman and Goldberger, 1983a; Bregman and Goldberger, 1983b; Bregman and

Goldberger, 1983c) or marsupial (Fry and Saunders, 2000; Knott et al., 1999; Lane et al., 2007; Saunders et al., 1995; Saunders et al., 1998; Treherne et al., 1992).

The lesion area, or in the case of the later infant groups the percentage difference in size between the right and left sides of the midline, followed a strong pattern of greater disruption at epicentre tapering off distally. There was no statistically significant difference between adult and juvenile groups at all four time points and the infants followed the same pattern, although they could not be directly compared after 24 hours. It is very difficult to design a model of paediatric SCI that creates a comparable injury in infants that matches their adult counterparts. There are obvious differences in animal size and weight, and also in the maturity of the CNS, although the specifics of this are not well documented in very young rats. It can be concluded from the comparison between the lesion area in the three age groups at 24 hours post injury that the surgically induced SCI was consistent in terms of severity and initial histological presentation and therefore we are confident that this model has created a comparable severity of spinal cord injury in infants, juveniles and adults. This is of critical importance to the entire study as it allows for the interpretation of all the other data and allows for a valid comparison between the three age groups.

Swollen axons were quantified as markers of injury. There was a trend toward higher numbers at the epicentre compared to distal in adults and juveniles; however, these regional differences were only statistically significant at 24 hours. The infants showed less swollen axons than the adults and juveniles at all four time points, though this did not reach statistical significance at 6 weeks due to large standard deviations. Differences in the number of swollen axons adds another dimension to the injury presentation for infants and older animals. The significant decrease in the numbers of swollen axons observed in the infants is potentially linked to the developmental state

of the spinal cord. By P7 the majority of axonal tracts, such as the CST, should be past the T10 level of the spinal cord according to previous retrograde and anterograde tracing studies (Gianino et al., 1999; Joosten et al., 1987). The developmental plasticity of these axons may account for the stark differences in the injury presentation in the infant groups post 24 hours in this study. In the 1980s Bregman and Goldberger ran a series of studies on spinal cord sparing, recovery and plasticity in young cats (Bregman and Goldberger, 1982; Bregman and Goldberger, 1983a; Bregman and Goldberger, 1983b; Bregman and Goldberger, 1983c). These studies concluded that there was some sparing of function in neonatal injury, though the response never fully matured, that was likely dependant on the contralateral sensorimotor cortex (Bregman and Goldberger, 1983a) and there was still deficits and differences in motor patterns (Bregman and Goldberger, 1983b; Bregman and Goldberger, 1983c). This plasticity may contribute to the observed lower incidence of swollen axons in the infants, as well as the developmental state of the cord.

The changes in lesion size, distribution and damage to axons over time indicate differences in injury progression in the neonates (P7) compared to the adults and juveniles. This suggests that the younger animals' spinal cord tissue is either underdeveloped, more protected or has a better regenerative capacity. These factors may have great influence on the infants' recovery. However, they are not alone; our results also suggest that the innate immune system plays an important role.

For the adult and juvenile age groups only female rats were used. This laboratory has used female rats for many years due to the lower chance of complications we have experienced, and therefore has a good baseline of what to expect. There is some evidence in the literature of females having better neurological recovery than males in both human clinical data (Sipski et al., 2004) and animal studies

(Farooque et al., 2006; Hauben et al., 2002; Roof and Hall, 2000). The estrous cycle was not monitored for in this experiment as it was thought to have limited biological effect on the outcomes measured, though further studies are being conducted that will examine this in detail. The infants were too young at the time of injury and in early recovery for hormonal cycles to be a factor, although in later stages of post-injury recovery this may emerge as a confounding factor that needs further investigation.

3.5.1b The innate immune response to spinal cord injury is significantly different in magnitude and nature between infants and mature animals and may play an important role in recovery differences

The long-established privileged status of the CNS has been called into question in recent years with the developing of a deeper understanding of the unique immune responses that occur in the brain and spinal cord under different pathological conditions (Ransohoff and Brown, 2012). In the early stages of development the semi-privileged status of the CNS conveys an advantage in controlling the environment, however in pathological conditions this differing immune response can hinder normal recovery (Schwartz et al., 1999). In the case of traumatic SCI, inflammation has been shown to have both neuroprotective and neurotoxic actions (Das et al., 2012; Ekdahl et al., 2003; Hohlfeld et al., 2007; Kigerl et al., 2009; Lucas et al., 2006; Popovich and Jones, 2003; Schwartz et al., 1999). Which of these prevails in the injury environment is influenced by a myriad of factors, both intrinsic to the cell types present and extrinsic within the shifting microenvironment. This duality is the root of the complexity underlying the inflammatory response to traumatic injury and also in targeting elements of this therapeutically (Hohlfeld et al., 2007; Popovich and Jones, 2003).

In a typical response to CNS injury the innate immune response begins very quickly with neutrophils arriving within hours of the injury (Taoka et al., 1997). In terms of the inflammatory response to SCI, neutrophils play an important role. The character of this role is contentious and the magnitude of neutrophil infiltration may depend on the severity of the trauma (Taoka et al., 1997; Trivedi et al., 2006; Xu et al., 1990). Neutrophils are capable of producing reactive oxygen species, and other neurotoxic factors, within the lesion environment that can further contribute to lipid peroxidation and lesion spread in the local area (Carlson et al., 1998; Sadik et al., 2011; Taoka et al., 1997; Trivedi et al., 2006). However they are also the first responding cells that begin the inflammatory process in earnest and secrete chemo-attractants and activators for other important cells types (Trivedi et al., 2006). Neutrophils are one of the first extrinsic responders to spinal cord injury and are expected to peak within 2 days post injury in rats (Hausmann et al., 1999; Zhang and Gensel, 2014), persisting for more than 4 weeks (Hausmann et al., 1999). In humans this happens within hours (Hausmann et al., 1999). At all age groups in the current study there were significantly higher numbers of neutrophils infiltrating into the injury site at 24 hours compared to the other three time points (1 week, 2 weeks, and 6 weeks), which coincides with the expected peak (Carlson et al., 1998). The early innate immune response, that is characterized by invasion of neutrophils, is widely contended to be unfavourable for neuronal survival and regeneration (Schwartz and Yoles, 2006). The potential of neutrophils for both beneficial and detrimental effects on the development of the secondary injury is dependent on a number of factors, especially the magnitude and length of the response (Donnelly and Popovich, 2008). The oxidative and proteolytic enzymes produced by infiltrating neutrophils prepare the area for repair, however the overwhelming numbers that are drawn to the lesion can cause further damage to the

surrounding tissues (Fleming et al., 2006). Interestingly there were significant differences between the adult and infant groups at all four time points, as well between juvenile and infant at all time points except 1 week. The numbers of neutrophils in the infant spinal cords were lower than the adult and juvenile groups, even when this was not statistically significant. This could be of increased benefit for the infant cohort as there are still neutrophils present to play their essential role however the numbers are not high enough to have a significant detrimental effect. This could also be indicative of the lower numbers of neutrophils in the P7 rats at this stage of development. As early as the 1980s research found differences in neutrophil pool sizes and kinetics between neonates and adults (Basha et al., 2014; Carr, 2000; Christensen, 1989; Erdman et al., 1982; Kumar and Bhat, 2016; Levy et al., 1999). It was found that newborn rats' neutrophils cell mass per gram of body weight was only one quarter that of adult rats and reaches adult levels after about 4 weeks (Carr, 2000; Erdman et al., 1982). Christensen et al. observed that newborn rats have lower numbers of granulocyte-monocyte colony-forming units (GM-CFU) per gram of body weight than their adult counterparts who have large pools of quiescent GM-CFU able to respond to infection (Christensen and Rothstein, 1984; Christensen, 1988; Christensen, 1989). Depletion of neutrophils has also been shown to decrease the brain swelling in a neonatal rat model of hypoxic-ischemic brain injury (Hudome et al., 1997). This trend is also thought to flow over into humans. It has previously been found that neonates have a decreased ability to mount a neutrophil response or extravasate neutrophils (Anderson et al., 1981; Anderson et al., 1990).

Extrinsic macrophages and intrinsic microglia are also inflammatory responders belonging to the innate immune system. Microglia are a unique myloid cell population that are the innate phagocytes of the CNS, quite apart from analogous cells in other

tissues (Ransohoff and Brown, 2012; Schwartz et al., 1999). These cells likely evolved as a compensatory mechanism for the unique immune status that the CNS exhibits (Schwartz et al., 1999). Extrinsic monocytes and macrophages only have access to the CNS in cases of pathology, trauma and disruption to the BBB or BSCB. Immediately following SCI and intensive inflammatory response is initiated local to the lesion, involving the activation of microglia and additional populations of monocyte derived macrophages from the bloodstream (Shechter et al., 2009a). The action and distribution of endogenous microglia and infiltrating monocyte-derived macrophages has been shown to differ after SCI (Hausmann, 2003; Klusman and Schwab, 1997; Shechter et al., 2009a). Both of these subsets play essential roles in the progression of injury, however the spontaneous levels of M2-like activated macrophages infiltrating after injury may not be sufficient for efficient tissue repair (Shechter et al., 2009a). It must be noted that the majority of this work has been conducted in adult models with very few comparing the mature and developing inflammatory response (Kumamaru et al., 2012; Vega-Avelaira et al., 2007; Yuan et al., 2013).

The macrophages and microglia showed an opposing profile of ED1/IBA1 staining in the infants compared to the adults and juveniles at all four survival times. The adult and juvenile groups showed increases in the proportions of ED1+/IBA1- (phagocytic macrophages/monocytes) and ED1+/IBA1+ (activated microglia) and decreases in the proportion of ED1-/IBA1+ (ramified microglia) compared to the shams. The infants, on the other hand, presented with only small increases in the proportion of the two ED1+ subsets and a small decrease in the proportion of ED1-/IBA1+ cells from the sham levels. The different subsets of macrophage and microglia are potentially having a great impact on the development of the lesion through their phagocytic role as well as the cytokines that they are secreting. Previous studies have

found that profile of cytokines, and the expression of inflammatory molecules, differs markedly between adult and infant mice, and opossums, after injury (Kumamaru et al., 2012; Lane et al., 2007). The secretion of pro-inflammatory cytokines, in contrast to the anti-inflammatory, was markedly decreased in young mice (Kumamaru et al., 2012). This may contribute to the attenuation of the subsequent neutrophil infiltration observed in neonate mice (Kumamaru et al., 2012). However, the current study is more focussed on the differences between activated pro-inflammatory (M1) and anti-inflammatory (M2) phenotypes, which will be further explored in Chapter 4 of this study.

The age groups, especially with the proportions of ED1+/IBA1- and ED1-/IBA1+ cells, were different at all four time points. This is potentially an important factor contributing to the differences in recovery between the age groups; and the different injury progression observed between infants and adults. The higher proportion and numbers of classically activated macrophages and microglia in the adults is likely contributing to a more robust pro-inflammatory lesion environment and the detrimental propagation of the secondary injury. This pro-inflammatory cascade is initially essential in SCI, as it is in all tissues of the body, to clear debris and begin the process of inflammation by the activation of other subsets of cells, and finally repair (Donnelly and Popovich, 2008). However, if this process is sustained for too long without progressing to the tissue repair stage dominated by alternatively activated inflammatory cells, as it does in adult SCI, it becomes a detriment to tissue repair and promotes poor injury resolution (Martinez and Gordon, 2014). The presence of these phagocytically activated cells at lower levels in the infants may be a sign of a less robust pro-inflammatory response. It has been suggested that the manipulation of the inflammatory cascade towards the alternatively activated phenotype would be

beneficial, and that blood-derived macrophages play a vital role in the resolution of SCI through their M2-like capabilities (Hausmann, 2003; Klusman and Schwab, 1997; Shechter et al., 2009a). The elucidation of the activation phenotype of these inflammatory cells is essential to shed yet more light on this important distinction and guide toward a therapeutic application. This will be addressed in detail in Chapter 4 of this thesis.

It is well established that the immature brain has a distinctive inflammatory response, compared to the adult brain, and also different vasculature (Potts et al., 2006). The inflammatory response has been observed to differ in developing mouse spinal cords as well (Kumamaru et al., 2012) which has contributed to a trend of observed faster and fuller functional recovery in younger subjects. These differences are highlighted in the current study through the examination of the neutrophil, macrophage and microglial response over a six week time course post TSCI. The results of this study strongly suggest that the differences in the innate inflammatory response cannot be overlooked when searching for the mechanisms behind the observed trend of a better outcome in infant animals and younger patients. Further study is definitely warranted to explore the age-related changes in inflammatory response after SCI. The next experimental chapter of this thesis will use flow cytometry to examine the macrophage/microglial phenotypes that are responding in the acute stages of SCI in adult and infant rats, as well use ELISA to investigate differences in cytokine profiles in different aged rats.

3.5.1c Differences in reactive astrogliosis between mature and infant animals is directly linked to the differences in histological injury progression

Reactive astrogliosis refers to the molecular and morphological changes that astrocytes undergo, culminating in the formation of a glial scar to seal of the injured area (Sofroniew, 2009). This process is well established, widely studied and contentious. The debate continues as to whether this is ultimately beneficial or detrimental after SCI (Faulkner et al., 2004; Fawcett and Asher, 1999; Fitch and Silver, 2008; Hu et al., 2010; Karimi-Abdolrezaee and Billakanti, 2012; Silver and Miller, 2004) with much of the literature expounding on both neuroprotective and inhibitory elements of the glial response (Faulkner et al., 2004; Karimi-Abdolrezaee and Billakanti, 2012; Pineau et al., 2010; Sofroniew, 2009). It has been suggested that reactive astrocytes do not simply physically block the extension of axon across the lesion site but may also contribute to the failure of re-myelination due their high expression of inhibitory bone morphogenic protein (BMP) (Wang et al., 2011). The principal role of reactive astrocytes is to form the glial scar that walls off the damaged tissue to help protect the intact surrounding tissue (Fitch and Silver, 2008) however, they are not simply inert structural elements. Previous in vitro studies have shown that reactive astrocytes produce and secrete a variety of different effector molecules into the complex and shifting lesion microenvironment (Aloisi et al., 1992; Balasingam et al., 1994; Dougherty et al., 2000; Fitch and Silver, 2008; Karimi-Abdolrezaee and Billakanti, 2012; Pineau et al., 2010). These may be both pro- and anti-inflammatory, that can both help and hinder functional recovery (Silver and Miller, 2004).

In the current study, regional differences in GFAP intensity were noted within the transverse sections that were statistically significant in all age groups, however the areas of greatest interest were the white matter and lesion edge. This is due to higher

densities of astrocytic projections in the white matter and lower densities in the grey matter (Baldwin et al., 1998; Yang et al., 1993). It follows then that the most prominent changes in GFAP staining intensity were noted in the white matter as well as around the lesion edge as the glial scar forms. The developmental state of the infant cords led to the expectation of a higher initial astrocytic density (Vega-Avelaira et al., 2007; Yang et al., 1993). This may be due, in part, to the higher levels of radial glia and radial precursor cells expressing GFAP (Sancho-Tello et al., 1995). This expectation was borne out by the higher GFAP immunoreactivity in the infants compared with their adult and juvenile counterparts; however, this was only considered significant between the infants and adults at 24 hours post injury. This experiment resulted in a peak in GFAP MGV in the P7 infants' white matter at 24 hours post injury, that receded to sham levels by 1 week. This would suggest that the resident astrocytes respond promptly to injury even without the development of a classic lesion cavity. The acute response returns to normal levels by 1 week. In contrast, the adult response remained elevated at the lesion edge for an extended timeframe and remained unaltered in the uninjured tissue.

24 hours is a fairly acute time point in the injury development and while reactive astrogliosis is in its early stages, indicated by observed slight increase in astrocytic density, the glial scar has not yet begun to form. This is to be expected as astrocytes typically react to CNS injury over the course of days to weeks, so at this acute time point in the development of SCI the astrocytic response will be only in its preliminary stages (Fitch and Silver, 2008; Silver and Miller, 2004). There were significant increases in GFAP in the adults and juveniles at the lesion edge at 1 week post injury; this represents the beginnings of the glial scar structure and a peak in fluorescent intensity as the reactive astrocytes are proliferating and in a highly active

state. 2 weeks post-injury the adults GFAP levels were beginning to drop, and the infants had returned to their sham level. Only the juveniles continued to be significantly higher than their shams. This indicates a stabilization of the glial response and the beginning of the glial scar. These results highlight further differences between the infant and mature rats in the response to SCI which are likely intrinsically connected to the stark differences in the histological progression of the injury. As observed earlier in this chapter the infant rats do not exhibit a defined lesion or cavity in the spinal cord tissue after 24 hours post injury, so there is less need for a glial scar to wall off the affected area. Reactive astrocytes do still play a role in the post injury environment outside the formation of the scar tissue (Karimi-Abdolrezaee and Billakanti, 2012); this is indicated through the increase in GFAP still visible where the lesion edge would be without the lesion.

According to a study by Lane et al. (2007) using opossums the accumulation of GFAP-positive astrocytes at the site of the lesion will occur earlier in animals injured at P14 compared with those injured at P7 (Lane et al., 2007). A consequence of this is the theory that the delayed timeframe of reactive astrocyte activation and migration in the younger animals may be a contributor to the better recovery observed by alleviating some of the negative effects of the process. This especially highlights the importance of timing, both in the body's own response and in any potential therapeutic intervention. The results found in the current experiment agree with Lane et al.'s findings somewhat as the levels of GFAP were starkly and significantly increased at 24 hours, compared with the mature animals. However, our results differ in that the GFAP at the lesion edge at the later time points remained unchanged, this could be a result of the different mode of injury (contusion rather than complete transection), the

different animal species used and the use of transverse rather than longitudinal sections.

3.5.1d The endogenous neural progenitor cell response is present after spinal cord injury in rats of all ages, providing a potentially promising target for manipulation

Endogenous neural progenitor cells have been the subject of substantial research in the last decade as a potential candidate for tissue replacement after SCI, with the majority of research conducted in rodent models (Barnabé-Heider et al., 2010; Cao et al., 2001; Hamilton et al., 2009; Horky et al., 2006; Horner et al., 2000; Marichal et al., 2009; Mothe and Tator, 2005; Mothe et al., 2011; Tzeng, 2002). Many studies have demonstrated a proliferative burst that occurs in progenitor cells after SCI. One such paper by Horkey et al. (2006) showed a surge in proliferation of CNS-derived progenitor cells as early as 24 hours after injury, with evidence of oligodendrocyte and astroglial differentiation but no evidence of neural differentiation (Horky et al., 2006). This observation is supported by the results of the current study. The adults, juveniles and infants all showed activation of eNPCs in the ependymal layer of the central canal 24 hours after a SCI, demonstrated by significant increases in nestin intensity. Horkey's study showed these cells to persist in the parenchyma for up to 9 weeks post injury (Horky et al., 2006). This is somewhat supported in this study in the adults and juveniles by the persistent nestin staining in the central canal and lesion edge that was seen up to 6 weeks post injury in this study. The persistence of the nestin staining in the infants was substantially lower, especially in the later stages of the injury progression, as the central canal levels dropped back towards normal by 2 weeks post injury. In 2005 Zai & Wrathall postulated that the eNPCs are stimulated to proliferate in the first week after SCI, develop mature phenotypes and assist in

restoration of cell density but not necessarily functionality in the post injury environment (Zai and Wrathall, 2005).

As many studies have shown activated NPCs proliferate and migrate towards the injury site in a post injury environment. This is visible in this study at the 1, 2 and 6 week survival times by the increasing nestin MGV around the lesion. This was significant in all three ages at 1 week post injury and peaks at 2 weeks post injury in both the adult and juvenile rats. This differs from the infant rats that peaked at 1 week but drop off substantially by 2 and 6 weeks. A 2008 study by Meletis found that the ependymal cells of the spinal cord central canal can act as NSC in vitro, and also confirmed that the proliferation and migration of ependymal cells will also contribute to the formation of the glial scar in vivo (Meletis et al., 2008). Recently Mothe et al. (2011) released a study confirming this idea and supporting the model that the eNPCs from the central canal differentiate largely down the astrocytic pathway and will assist in the formation of the glial scar tissue (Mothe et al., 2011). In the current study this process was confirmed using GFAP and nestin double staining on a cohort of the animals. There was substantial GFAP and nestin staining individually around the lesion edge and when these two stains are used together (Figure 43) a high level of co-localisation can be observed. Many of the dual stained cells had an astrocyte-like phenotype, suggesting the potential fate of these cells along the astrocytic lineage. This contention is supported in the literature as, similarly to many of the transplanted stem and progenitor cells studied, most studies of eNPCs have found them to generate a majority of cells with an astrocytic phenotype and some in the oligodendrocyte lineage (Cao et al., 2001; Meletis et al., 2008).

Based on the trend of differences between mature and infant cords, which has been touched on by numerous authors throughout the literature as well as in this

thesis, it was expected that the response of eNPC in the infants would be significantly greater than that evident in the rats injured at 5 and 9 weeks of age. As early as the 1980s Bregman and Goldberger described the 'infant lesion effect' (Bregman and Goldberger, 1983a; Bregman and Goldberger, 1983b; Bregman and Goldberger, 1983c) and in 1997 Beattie et al. observed greater regeneration in younger animals after a contusion SCI (Carrascal et al., 2005). The greater cellular plasticity of the infant cord (Bregman and Goldberger, 1982; Carrascal et al., 2005) and the physiological hypermobility and malleability (Kuluz et al., 2010; Tatka et al., 2016) are all factors that may contribute to this observed trend, however we do not yet have adequate knowledge to determine the exact cause. More pertinent to this current study is the continued developmental state of the neonatal cord and the levels of progenitor cell populations present in the infants CNS (Carrascal et al., 2005). This led to an expectation that there would be a greater nestin response visible in the infant SCI compared to that seen in the mature groups.

At 24 hours post injury the intensity of the nestin staining at the central canal was moderately consistent between all three age groups; each of the groups showed an increase in MGVI from control levels, taking into account the high degree of variation between animals. However, this significant increase was not sustained in infants as the injury progressed. Nestin staining in the infants was still increased from control levels at 1 week after the injury, however it wasn't statistically significant. In the adults and juveniles, the nestin intensity was increased from control levels at the same time. Chronically, all three age groups had begun to decrease by 2 weeks post injury, but this was most significant in the infants which are almost back to sham levels. Potentially, this may be a result of the eNPC differentiating into mature

neurons; which may be another mechanism to explain the functional recovery differences.

This has strong implications in terms of developing potential therapies based on eNPC as it shows that there is a population of cells in the ependymal layer of the central canal that is present at all ages and also responds to SCI at all ages, even though to a different degree. The response of these cells has been documented to consist of proliferation, differentiation and migration (Martino and Pluchino, 2006; Mothe and Tator, 2005; Ziv et al., 2006) providing multiple avenues by which these cells can possibly be manipulated to improve the recovery of SCI patients. The uniformity of the response at the acute time point of 24 hours illustrates that this is a therapeutic target that is applicable to all patients regardless of age, if administered in a timely manner. Further highlighting the emerging potential of ependymal cells as a therapeutic target, these cells have previously been shown not to apoptose in response to minimal SCI (Mothe and Tator, 2005); and injury adjacent to the ependyma induces a response (Mothe and Tator, 2005). This is supported by the results of this study that show the persistence of nestin positive cells at the central canal after a mild contusion SCI and the migration of nestin positive cells and processes to the lesion edge. The infant rats also showed a persistence of nestin positive cells in the central canal early post injury. This demonstrates the potential of these cells as a target for a therapeutic intervention in the early stages of the injury progression in all ages.

3.5.1e The potential significance of these injury differences

This chapter has shown that differences exist in the progression of the SCI, and the responses of the CNS endogenous and exogenous cells to the injury. It is a complex

process that still eludes complete characterisation; however this study sheds some light on the different cellular processes occurring. The injury progression is starkly different between adult and infant rats; visible on multiple levels from the ability of the infants to 'bounce-back' post-injury to the gross and histological appearance of the injury. The potential reasons behind this become clearer on a cellular level where there are differences in astrocytic and inflammatory responses. This could provide a target to a therapeutic pathway, manipulating these responses to be more beneficial to recovery and even regeneration.

This study aligns with the current literature suggesting that completely suppressing the inflammatory response, using therapies such as Methylprednisolone, has little long term benefits (Hurlbert, 2000; Short et al., 2000); however the manipulation of the character and magnitude of the response may hold great therapeutic potential (Chan, 2008). Ideally, a therapeutic intervention could act to boost the positive role of immune cells early after injury and then moderate the response to less damaging levels and more beneficial phenotypes as the injury progresses, similar to the response seen in non-CNS tissues; however, dissociating the beneficial from the detrimental may highly challenging (Popovich and Jones, 2003). The introduction of secreted factors such as cytokines, and the use of NPC as immune-modulators are just potential strategies to achieve this; however more study is needed before this is possible. This is just one part of any potential therapy as SCI is a complex injury, any therapy would have to address more than a single aspect of the response to be more effective. This study has highlighted the importance of the inflammatory response and, also, the interaction between immune cells and endogenous nervous system cells.

3.5.2 'Cross-talk' between nervous and immune systems

These cellular elements of the post injury environment all play a role in the resolution, or lack thereof, of the SCI; however, they do not act alone. The post-injury microenvironment is a complex milieu of cells, molecules and signals that interact with each other on multiple levels. This is brought to attention by the synergy in the response of the astrocytes, phagocytes and eNPCs in the mature groups in this study. In recent years it has become apparent that there is a high degree of interaction between the nervous system and the immune system, especially in the emerging role of progenitor cells as immunomodulators (Cusimano et al., 2012; Hohlfeld et al., 2007; Kokaia et al., 2012; Ziv et al., 2006).

In the adult rats the nestin MGV peaked at the central canal 1 week after injury and at the lesion edge 2 weeks after, suggesting a migration of these cells towards the injury site. The GFAP MGV peaked at the lesion edge at 1 week post injury and in the white matter over 1 and 2 weeks. Meanwhile, in terms of the cellular inflammatory response there was the expected acute peak in neutrophils at the lesion site at 24 hours after injury which coincided with a peak in ED1/IBA1 dual positive phagocytes, likely activated microglia, this was followed by a peak in ED1+/IBA1- activated macrophages or monocytes at 1 and 2 weeks post injury. Together these point to the neutrophils having a significant role in the initiation of the responses that follow and a progression from the prevalence of endogenous to infiltrating phagocytes. The juvenile rats showed very similar results but with the GFAP peaking over 1 and 2 weeks post injury.

Compared to the mature animals the infants had a much less significant rise in neutrophils at 24 hours post injury followed by much smaller peaks in ED1/IBA1 positive phagocytes at 1 week, and ED1+/IBA1- macrophages/monocytes at 2 weeks

post SCI. This data suggests that the initial attenuation of the neutrophil response may have implications for the continued injury resolution and decreased cellular responses; less activation of endogenous phagocytes is seen as well as a decreased number of infiltrating cells. There was also only a slight increase in GFAP MGV around the lesion at 1 week post injury, which dropped back quite quickly as there was no true lesion around which to form a glial scar; there was however a large peak in GFAP in the white matter at 24 hours post injury, indicating some other function of the astrocytes was required to further resolve the injury. Similar to the mature groups there was a peak in nestin MGV around the ependymal layer of the central canal at 1 week however this dropped off substantially by 2 and 6 weeks survival time, with a small increase in nestin being visible at the lesion edge at 1 and 2 weeks post injury. This is potentially a symptom of the quicker and fuller injury resolution leaving less functional need for these cells to be active, and no need for them to migrate towards the lesion edge to contribute to the glial scar.

Traditionally stem and progenitor cells have been considered to have potential as cell replacement and structural restoration treatments after SCI, however in recent years a high degree of interaction and 'cross-talk' has been highlighted between the nervous and immune systems (Cusimano et al., 2012; Kokaia et al., 2012; Ziv et al., 2006). Due to this research another, more significant, role in the post-injury environment is emerging. That is the role of stem and progenitor cells, especially NSPCs and MSCs, as modulators of the neuroinflammatory response (Beck et al., 2010b; Clarke et al., 2008; Martino and Pluchino, 2006; Nakajima et al., 2012; Stammers et al., 2012). The results of the current study also indicate some interplay between the immune, progenitor and glial responses on a cellular level; however this is based on observation and timing, more molecular and signalling data is needed to

explore this. The term 'therapeutic plasticity' was coined to describe the emerging role of NSPCs to modulate their surrounding environment, as well as respond to its cues, and instruct other innate and immune cells in the post-injury environment (Martino and Pluchino, 2006; Martino et al., 2011). NSPCs can exert beneficial effects on SCI repair by acting as immunomodulators and providing trophic support (Martino and Pluchino, 2006; Martino et al., 2011).

3.5.3 Spinal cord injury in immature subjects

3.5.3a A comparison between P7 and P10 neonates

The initial promising results from the infant groups, in terms of the lack of an injury cavity and the decreased pro-inflammatory response, raised the possibility that perhaps 7 day old neonates (P7) were too young at the time of the induced injury and the positive results may have stemmed from the fact that the spinal cord was still developing. Certainly, the histology demonstrated an underdeveloped cortico-spinal tract (CST) in the 24 hour groups. This is shown by the pale-staining section of the dorsal white matter in Figure 30. An anterograde tracer study from 1987 suggested that cortico-spinal axons of rats have progressed past the T10 level in P7 rats (Joosten et al., 1987) and study in 1998 using retrograde tracing placed the CST at the 13th thoracic segment at P7 in mice (Gianino et al., 1999). Studies like these suggest that CST axons should be present in the P7 neonatal rats, however they may not have made their full mature connections.

To examine this potential a small group of 10 day old neonates (P10) were compared to P7s. There were some obvious visual differences in the lesion presentation in the H&E staining. The 1 week, 2 week and 6 week juvenile and adult groups presented with large cystic cavities in the centre of the injured cord, while the

P7 groups for these survival times instead showed a disparity in size between either side of the midline. The P10 injured animals' injury presented differently to the adult, juvenile and P7 groups. There was not a large central cavity, as there was with the adults; however, there was some varying amount of tissue loss to the edge of the smaller side, unlike the P7 infants. Statistically there was no difference between the P7 and P10 groups in terms of lesion size. The swollen axons showed a trend of higher numbers in the P10 group compared to the P7 however, due to high variations between animals resulting in large standard deviations.

The developing spinal cord exhibits significant difference to the fully developed adult cord in a variety of aspects, from biomechanical (Clarke and Bilston, 2008; Clarke et al., 2009), cellular and structural (Kuluz et al., 2010) to molecular (Bregman et al., 1997; Kumamaru et al., 2012; Nakamura and Bregman, 2001). The developmental state of the cord may have an impact on the response of the animal to spinal cord injury, this is supported by the differences in the injury presentation between the infant and adult groups, as observed in the H&E results. In a 2005 study of T8 contusion SCI in P14-15 rats Brown, Wolfe and Wrathall saw a different injury progression to that observed in this study's adult or P7 infants. The P14 infants showed the development of a cavity and significant tissue loss over a month after injury, with weight-drops from both 2.5 and 5cm, though these animals still showed a better behavioural recovery than their adult counterparts (Brown et al., 2005). This greatly emphasises the difference the age at injury makes in our rat model and brings up questions about the mechanism behind this. Is it based on the developmental plasticity of the cord, or more dependent on the biomechanics of the cord and the injury itself? It highlights the possibility that in our current study the injury in the P7 infants is not

entirely centred on the cord and is resulting in uneven cell death and compensation by the other side of the cord.

The innate immune response was similar in the P7 and P10. There was no significant difference between the neutrophil infiltration at the epicentre between these groups, with both groups being lower than the mature response. This very similar response demonstrates that the neutrophil response has not developed further in the few days between P7 and P10, so in this case the promising P7 results may be considered a fair representation of the infant response to SCI. There was a statistically significant increase between the proportions of ED1-/IBA1+ ramified microglia in the P7, compared to the P10 group. Conversely, there was a statistically significant decrease in the proportions of ED1+/IBA1+ activated microglia in the P7 group compared to the P10s. This suggests a developmental difference in the infiltration of innate immune phagocytes and the response of endogenous microglia. At P10 this response has shifted towards a more mature response, however it still has a much closer resemblance to that of a P7 infant than that of a juvenile or adult, suggesting some development but only in small increments.

The astrocytic response in P7 infants was significantly lower than that of the juvenile and adult groups. There was no difference in the GFAP levels in the white matter between the 7 day and 10 day old infants. However, the presence of some cavity in the P10 group resulted in the GFAP at the lesion edge being significantly increased from the P7s in the P10s at 6 weeks post injury. These results suggest that the astrocytic response is very similar in both P7 and P10 infants, the differences occurring due to the histological progression of the injury itself. The increases in the P10 group were due to the need to respond to a forming cavity that is not present in the P7 group. This is further linked to the differences between adults and infants

stemming directly from the injury progression. These types of observations may indicate that, as reported in the marsupial spinal cord (Knott et al., 1999; Lane et al., 2007; Saunders et al., 1998), there may be very discrete and exact critical developmental time periods in which injury progression and functional outcomes may intrinsically differ.

3.6 CONCLUSIONS

There are still many grey areas concerning SCI in young subjects, not least of these comparing the responses to the same injury between animals of different ages. The basis of the research in this chapter is the established trend of better recoveries in young animals, as well as in instances of human SCI. The mechanisms behind the observed recovery differences is still yet to be elucidated. This study aims to start to develop an understanding of the interactions between cells and systems that governs the progression of SCI, and by filling in some of the gaps in our knowledge of SCI in younger animals to potentially develop how the better recovery observed in these infants can be used therapeutically in mature SCI. In order to achieve this the response of key cellular players from the innate immune system and the CNS in SCI were examined. These cells were the first responders of the innate immune response, neutrophils and early responding microglia, as well as the infiltrating macrophages, reactive astrocytes and eNPCs. The results suggested significant differences between mature animals and infants in all of the aspects examined, from the histological progression of the injury to the cellular response with the most significant differences observed in the cellular inflammatory response. This suggests that the inflammatory response is a key player in the observed better functional recovery in younger subjects. The results of this study also hint at connections and correlations between all of these separate cellular aspects and processes. More work is needed to solidify this; however, it does suggest that none of these elements operate in a vacuum, they are interconnected and operate as parts of a complete response to the injury. This is a complicated landscape however, the synergy between the responses that is hinted at in this study bears much greater research and suggests that a therapeutic intervention to assist in favourable injury resolution need to focus on more than a single aspect.

CHAPTER 4: CHARACTERISTICS OF THE CELLULAR INNATE IMMUNE AND T-LYMPHOCYTE RESPONSE TO SPINAL CORD INJURY IN ADULTS AND INFANTS

4.1 INTRODUCTION

4.1.1 Macrophages play an important role in the spinal cord injury secondary cascade

The activation states of macrophages fall in a spectrum with overlapping states between the two phenotypes, M1 and M2 (Gensel and Zhang, 2015). In normal wound repair this spectrum can be represented as a dynamic transition from M1 to M2a, M2b and M2c (Gensel and Zhang, 2015); each associated with different activators, expressing a variety of receptors and secreting different factors (Martinez et al., 2008). M1 are considered to be 'classically' activated pro-inflammatory macrophages, as compared to the M2 'alternatively' activated anti-inflammatory phenotype (Kigerl et al., 2009). This dichotomy is a simplification and is being reassessed constantly; a more accurate paradigm needs to take into account more complex and mixed phenotypes and levels of response (Martinez and Gordon, 2014). The traditional paradigm is associated with the Th1/Th17 vs Th2/T-reg (regulatory T-lymphocytes) response. The predominance of M1 or M2 helps determine the nature of the T-lymphocyte response and these two adaptive immune responses are mutually antagonistic. M1 macrophages are attributed the characteristics of efficient producers of potentially neurotoxic effector molecules and pro-inflammatory cytokines, while the M2 phenotype is involved in dampening the inflammatory response and promoting tissue remodelling (Mantovani et al., 2013). These phenotypes are associated with different cytokine and chemokine profiles; significantly, M1 is more associated with pro-

inflammatory cytokines such as interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), IL-6 and IL-1 while M2 is associated with anti-inflammatory cytokines IL-10, IL-4, IL-13 and transforming growth factor beta (TGF- β) (Bastien and Lacroix, 2014; Bethea et al., 1999; Guerrero et al., 2012; Huang et al., 2014; Klusman and Schwab, 1997; Schwartz, 2010). The heterogeneity of the macrophage response and phenotypes is demonstrated in Figure 53 (Gordon, 2003).

The three phases of normal wound healing in non-CNS tissues of the body; the inflammatory, proliferative and remodelling (Gensel and Zhang, 2015), involve dynamic changes in macrophage phenotype during wound healing (Mantovani et al., 2013). In the initial inflammatory phase macrophages with predominantly a pro-inflammatory M1 phenotype, as well as some M2a, attract neutrophils and help to boost the response. This phase occurs in CNS trauma as well however it is prolonged and dysregulated, with a prolonged inflammatory phase propagated by pro-inflammatory macrophages (Gensel and Zhang, 2015). In normal wound repair following the inflammatory phase there is shift towards the M2b phenotype for the proliferative phase (Mantovani et al., 2013). In SCI this phase is typically incomplete and the M2b macrophages improperly or inadequately activated, resulting in poor wound resolution (Gensel and Zhang, 2015). In normal wound healing the third phase, the remodelling phase, will be marked by a prevalence of the M2c phenotype to assist in tissue remodelling, however little is known about the role of macrophages in the remodelling phase of SCI. Endogenous remodelling event in CNS trauma do not lead to successful resolution and this phase is not properly executed (Gensel and Zhang, 2015). The environment of the SCI lesion also affects the function of phagocytes and the polarisation of macrophages between M1 and M2 (Brennan and Popovich, 2018). Myelin debris in the injured spinal cord can switch infiltrating macrophages and

monocytes towards an M1 phenotype, helping to propagate the pro-inflammatory response (Brennan and Popovich, 2018; Guo et al., 2016). The role of M1 and M2 macrophages as well as the M1-M2 transition is less understood in the context of the CNS (Brown et al., 2014; Chen et al., 2015; Shechter et al., 2009b).

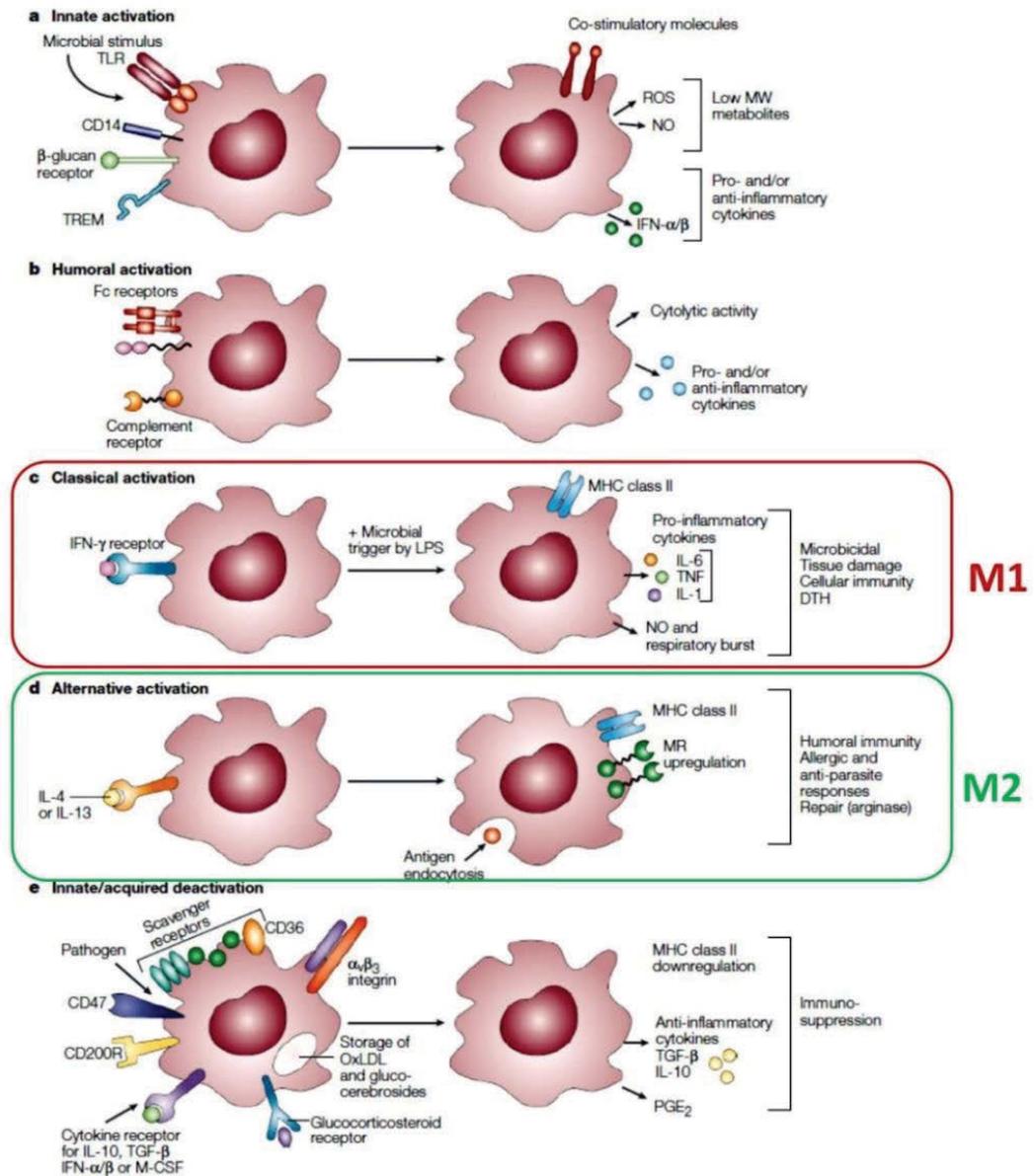


Figure 53: The multiple paths and phenotypes of innate and acquired immune activation of macrophages showing how simplified the M1/M2 dichotomy is compared to the complexity of the responses (adapted from (Gordon, 2003)). This shows the complexity and potential heterogeneity of the of the macrophage response depending on the stimulus and the nature of the microenvironment.

Cytokines play a significant role in this response.

The temporal distribution and the magnitude of the M1-like and M2-like response after SCI may have a significant role in determining the efficacy of any injury resolution and degree of functional recovery (Chen et al., 2015; Gordon, 2003; Klusman and Schwab, 1997; Martinez et al., 2008; Schwartz, 2010). It is generally accepted that the classically activated, or M1-like, macrophages are prominent in the initial inflammatory response to SCI and persist at relatively increased levels for many weeks post injury (Gordon, 2003). The injury microenvironment also pushes the resident microglial activation towards a pro-inflammatory M1-like phenotype as well (Turtzo et al., 2014). A population of infiltrating blood-borne macrophages is thought to be responsible for the presence of M2-like macrophages in the injury milieu (Klusman and Schwab, 1997; Shechter et al., 2009a); these cells are less prominent than the M1-like populations and are only transiently present (Gordon, 2003). The transient nature of the anti-inflammatory macrophage response, and its lower magnitude, contribute to the pro-inflammatory response continuing unabated and the development of a neurotoxic inflammatory state (Hausmann, 2003; Ren and Young, 2013). This all translates into poor neural tissue regeneration and wound resolution, therefore poorer functional recovery. Infants have recently demonstrated a more M2-like shift in spinal cord microglia, compared to the adults strong M1 response, after nerve injury (Gong et al., 2017). This may be highly beneficial to a more permissible post-injury microenvironment.

Many studies have shown the necessity of populations of macrophages and microglia after SCI. Recently it has emerged that the activated resident microglia and the infiltrating blood-derived monocytes and macrophages do not play redundant roles, and that both are necessary for a beneficial post-injury inflammatory response (Hausmann, 2003; Klusman and Schwab, 1997; Shechter et al., 2009a). Similarly, the

pro- and anti-inflammatory phenotypes of macrophage are required, however the response needs to be regulated and the magnitude and timing closely controlled. It is this balance and regulation that is currently missing in the inflammatory response to CNS injury.

The role of blood-borne macrophages in wound healing and resolution in other tissues has been quite extensively studied and continues to be characterised in more detail. This M1-M2 spectrum of phenotypes and their temporal response to trauma is important for the resolution of injury; however, in the central nervous system this response is dysregulated and skewed towards a persistent pro-inflammatory M1 response that causes further tissue damage. Understanding this response, and subsequently manipulating it, may assist in the attenuation of the secondary injury and the treatment of SCI.

4.1.2 The microglial response to spinal cord injury is important and yet to be fully elucidated

Microglia are the population of tissue phagocytes in the CNS and are unique from analogous cells in other tissues, and blood-borne monocytes and macrophages (Klusman and Schwab, 1997; Ransohoff and Brown, 2012; Schwartz et al., 1999). As one of the support cells in the CNS matrix microglia are the monitors of the CNS, sensitive to changes in the brain and spinal cord microenvironment (Barron, 1995; Streit et al., 1988). These glial cells are implicated in a range of processes within the CNS and are attributed with a wide variety of functions involved in CNS development, maintaining health and homeostasis, and in pathological responses (Boche et al., 2013). Differentiating activated amoeboid microglia from activated blood-borne macrophages in injured CNS tissue is difficult using most common visualisation or

quantification techniques as these cells have many similar markers and features. These two subsets of mononuclear phagocytes involved in the inflammatory response post-SCI have unique functions and both are necessary for a complete response (Hausmann, 2003; Klusman and Schwab, 1997; Shechter et al., 2009a).

Usually in a quiescent state, microglia are known to exhibit both hypertrophy and hyperplasia after an injury to the CNS and up-regulate the expression of a variety of surface molecules in their activated state (Barron, 1995; Dougherty et al., 2000; Eric Thomas, 1992; Gehrman et al., 1995; Kreutzberg, 1996). These molecules include the production of NTF and their receptors (Dougherty et al., 2000), inflammatory cytokines and their receptors (Gehrman et al., 1995) and activation markers such as ED1 or CD68 (Damoiseaux et al., 1994). In pathological states these cells can also function as macrophages and antigen presenting cells, contributing to the innate immune and inflammatory response as well as recruiting T-lymphocytes (Streit et al., 1988). As with peripheral macrophages in vitro studies of microglia have suggested different activation states along the M1/M2 paradigm; however the mechanisms that regulate microglial phenotype are comparatively poorly understood (Boche et al., 2013). Similarly to blood-borne macrophages, the activation state of microglia can have a significant effect on the nature of the injury response; these cells can be both beneficial and detrimental to tissue repair and functional recovery (Boche et al., 2013; Schwartz et al., 2006).

The role of microglia after a traumatic SCI, and how it compliments and differs from that of blood-borne macrophages, is still an area that requires greater study. These cells are endogenous to the spinal cord and have a significant role in the post-injury environment, responding to local signals, activating and perpetuating the

inflammatory response. The characteristics of these cells after activation may play a significant role in the ongoing nature of the inflammatory response.

4.1.3 The role of cytokine signalling in the central nervous system after a spinal cord injury is complex and links many different cell types

Cytokines are immune signals that assist in determining the nature of the immune response and the perpetuation or dampening of inflammation. The profile of these is different for different pathologies and also changes temporally as pathologies progress (Nakamura et al., 2003). It is becoming clear that inflammation post-SCI becomes detrimental due to the magnitude and persistence of the pro-inflammatory response. In most other body tissues this pro-inflammatory response is balanced and dampened by an appropriate anti-inflammatory response; this does not occur after SCI. After SCI the pro-inflammatory modulators and phenotypes of certain cells prevail. Chief amongst the mediators of this response are the 'Pro-inflammatory', or Th1, cytokines. The most prominent of these studied include TNF α , IL-6, IL-1 α /1 β and IFN- γ (Bastien and Lacroix, 2014), all of which are generally accepted to be detrimental to functional recovery when overexpressed or persistent. Cytokines are not necessarily directly toxic but may be indirectly toxic through their interactions with cells (Bethea et al., 1999; Toulmond et al., 1996). For example, TNF- α and IL-1 are not neurotoxic in a pure neuronal culture but become so in a mixed cell culture (Toulmond et al., 1996). Classically activated M1 macrophages are generally a product of stimulus such as Lipopolysaccharide (LPS) and are associated with Th1 cytokines such as TNF- α and IFN- γ . In the CNS these cells can perpetuate adverse effect on the damaged area in both humans and rodents (Guerrero et al., 2012). IL-1 α is a prominent danger signal after SCI that acts through the NF κ -B or MapK signalling pathways to promote inflammation,

and a knockout of IL-1 α is accompanied by increased oligodendrocyte survival after SCI (Bastien et al., 2015). IL-6 has been shown to also enhance the expression of other pro-inflammatory cytokines and play a significant role in perpetuating the pro-inflammatory response to a detrimental end; however this cytokine is still necessary for the beneficial inflammatory response as its complete blockade has detrimental effects on recovery (Guerrero et al., 2012). A temporary blockade of IL-6 activity was shown to have positive effects on the injury recovery in mice; decreasing Th1 cytokine expression and M1 dominance while increasing Th2 cytokine expression and the M2 response (Guerrero et al., 2012). HMG CoA reductase inhibitor drugs that reduce low density lipoproteins (called statins) such as Atorvastatin and Lovastatin have been shown to attenuate the pro-inflammatory response in rodents in conditions such as SCI (Pannu et al., 2005) and EAE (Greenwood et al., 2003; Stanislaus et al., 2001), respectively. A potential mechanism proposed for the efficacy of Atorvastatin in SCI is mediated by the attenuation of the expression of pro-inflammatory cytokines TNF- α and IL-1 β which correlated with a reduction in ED1 positive macrophages and reduced reactive astrogliosis (Pannu et al., 2005). This speaks to the important role these cytokines play in the post-injury response and how manipulating their expression and action can have significant effects on the injury outcome.

Alongside this pro-inflammatory response is the 'anti-inflammatory' response involving Th2 cytokines; the most common examples of which include TGF- β , IL-10, IL-4, and IL-13 (Bastien and Lacroix, 2014). These cytokines are associated with a wound healing and injury resolution response and alternatively activated macrophages, which are prominently induced by IL-4, IL-10 and IL-13 (Guerrero et al., 2012). IL-10 has received a lot of attention as a mediator of the pro-inflammatory response, capable of dampening the expression of pro-inflammatory mediators and switching to an anti-

inflammatory response. Previous studies have reported neuroprotective effects of IL-10 after SCI (Brewer et al., 1999; Plunkett et al., 2001). This cytokine has been shown to suppress most macrophage/monocyte pro-inflammatory responses in the peripheral immune system and is well known to deactivate macrophages (Bethea et al., 1999; Bogdan et al., 1991) and inhibit Nitrogen oxide production from pro-inflammatory activated macrophages (Gazzinelli et al., 1992). IL-10 can be produced by cells of both myeloid and lymphoid lineages and is a significant immune suppressor, however it can also have immune stimulatory effects in some specific circumstances (Soleymanejadian et al., 2012). A study by Bethea et al. found that a dose of IL-10 in the acute phase of SCI reduced the injury-induced synthesis of pro-inflammatory TNF- α and improved tissue sparing and hind-limb functional recovery (Bethea et al., 1999). As well as suppressing expression of pro-inflammatory cytokines, such as TNF- α , IFN- γ and IL-1, IL-10 can also suppress the MHC-II dependant proliferation of T-lymphocytes (Toulmond et al., 1996) and recruit regulatory T-lymphocytes (T-regs) (Soleymanejadian et al., 2012). TGF- β can have a significant impact on immune cells to induce apoptosis and suppress growth (Soleymanejadian et al., 2012), as well as suppressing the production of pro-inflammatory cytokines by lymphocytes and macrophages (Bogdan and Nathan, 1993). IL-4 and IL-13 are intrinsically linked to the alternative M2 activation of macrophages, although this is less well understood than the classical activation dependant on IFN- γ (Gordon, 2003; Gordon and Martinez, 2010). Different subsets of M2 macrophages are associated with IL-4 and IL-13 (M2a), immune complexes, IL-1 β or LPS (M2b) and TGF- β , IL-10 or glucocorticoids (Gensel and Zhang, 2015; Martinez et al., 2008). These pathways are complicated and there are many more, less well characterised factors that contribute to these responses and activation pathways (HAYASHI et al., 2000).

Cytokines, and their receptors, are expressed by and on a variety of cells not confined to cells of the immune system. In the CNS there are different cytokines are secreted from different glial cells, and receptors to a range of cytokines are expressed on the surface of microglia, astrocytes, neuron and progenitor cells, both constitutively and in pathological states (Lee et al., 2002; Rothwell et al., 1996; Sawada et al., 1993; Szelényi, 2001). Intact CNS cells can secrete macrophage colony stimulating factor (M-CSF) which influences the injury microenvironment and polarises microglia towards an M1-like phenotype (Mao et al., 2017a). Microglia, as the resident immune cell of the CNS and monitor of the extracellular environment, express a large range of cytokine and chemokine receptors across the spectrum of inflammatory signalling (Lee et al., 2002). Astrocytes appear to have a more active involvement in the inflammatory than originally assumed, expressing receptors to a variety of signal molecules such as IL-6, IL-7, granulocyte macrophage colony stimulating factor (GM-CSF) and M-CSF (Sawada et al., 1993). This means that the astrocytic response is not just scar-forming but is also contributing to the inflammatory microenvironment. Depending on the expression of these signal molecules the reactive astrocytes may be increasing the push towards a detrimental pro-inflammatory response. Oligodendrocytes express some cytokine receptors as well, differing from the expression profiles of astrocytes and microglia, although there is some overlap (Sawada et al., 1993). Even mature neurons express some cytokine receptors, though at a significantly lower level (Sawada et al., 1993).

In recent years a significant role as modulators of neuro-inflammation has emerged for NSC and NPC (Cusimano et al., 2012; Kokaia et al., 2012; Martino et al., 2011; Ziv et al., 2006). These cells express a range of receptors for cytokines and growth factors, including insulin-like growth factor 1 (IGF-1), BDNF, CDNF, GDNF, IL-15, TNF- α , IL-10, IL-6 (Gazzinelli et al., 1992; Karamita et al., 2017; Ziv et al., 2006) and can

secrete and respond to cytokines, such as IL-6, IL-11, M-CSF and stem cell factor (SCF) (Bogdan et al., 1991). Recently some cytokines, such as IL-15 and TNF- α , have been implicated in the normal proliferation and self-renewal of NSPCs (Gómez-Nicola et al., 2011; Iosif et al., 2006). The cytokine profile in the cellular microenvironment, and the phenotype of the macrophages/microglia, may also have an impact on the differentiation of NSPCs (Barkho et al., 2006; Butovsky et al., 2006; Johansson et al., 2008; Ling et al., 1998; Nakanishi et al., 2007). This further highlights the extensive cross-talk between the immune and nervous systems that has a great impact on the pathological response. IL-1 β , IL-4, IL-6, IFN- γ and TNF- α have all been proposed to have modulatory effects on the neurogenic niches in inflammatory conditions (Gómez-Nicola et al., 2011). In 2011 Gomez-Nicola et al. signalled out pleiotropic pro-inflammatory cytokine IL-15 as having a role in maintaining the self-renewal of NSC and promoting neurogenesis (Gómez-Nicola et al., 2011). TNF- α has a duality in its interaction with NSC and its effects on neurogenesis in the brain, likely based largely on which receptor it is acting through (Iosif et al., 2006). Tumour necrosis factor receptor 1 (TNF-R1) and R2 are both expressed on hippocampal progenitor cells and the deletion of one or the other has different effects. In the adult brain TNF- α signalling through the TNF-R1 is thought to suppress NSPC proliferation and the resulting neurogenesis. In this study the deletion of TNF-R1 elevated the number of new hippocampal neurons and increase SGZ cell proliferation (Iosif et al., 2006).

The role of cytokine and chemokine signalling after SCI is manifold and complex, linking cells from the nervous, innate and adaptive immune systems through multiple pathways. These molecules permeate the injury microenvironment, secreted from a variety of cells and effecting even more, to guide the nature of the cellular response. The manipulation of the molecules could potentially have significant effect

on the nature of the inflammatory response and the phenotypes of the cellular response to resolve the damage after a traumatic SCI.

4.1.4 There exists extensive molecular linking of the nervous and immune systems after spinal cord injury

There is extensive 'cross-talk between elements of the central nervous system and both the innate and adaptive immune systems (section 2.1.3) (Cusimano et al., 2012; Kokaia et al., 2012; Ziv et al., 2006). This is seen prominently in the multifaceted roles of the inflammatory cytokines and widespread expression of their receptors. This is further emphasised by the sharing of multiple signals and pathways between the two systems (Hohlfeld et al., 2007). One prime example of this is Brain-derived Neurotrophic Factor (BDNF). BDNF is a member of the nerve growth factor (NGF) neurotrophin family involved in the regulation of survival and differentiation of neurons in normal development and the growth of axons post-trauma (Bregman et al., 1997; Hohlfeld et al., 2007; Nakamura and Bregman, 2001). This NGF is produced in large amounts by neurons and also produced by activated T-lymphocytes, B-lymphocytes and monocytes (Hohlfeld et al., 2007) and has the potential to support axonal growth and regeneration in the injured cord (Nakamura and Bregman, 2001; Thoenen and Sendtner, 2002).

NSC and MSC constitutively express chemokine receptors and release chemokines; this expression is up-regulated by exposure to TNF- α and IFN- γ (Pluchino and Cossetti, 2013). It has recently been found that transplanted NSC in a multiple sclerosis model (experimental autoimmune encephalomyelitis) demonstrate decreased demyelination, decreased production of myelin-specific antibodies and also decreased T- and B-cell infiltration (Pluchino and Cossetti, 2013). These transplanted

cells also functioned to prevent the differentiation and maturation of monocytes into dendritic cells (DC) *in vivo* and promote macrophage activation down the alternative pathway (Nakajima et al., 2012; Pluchino and Cossetti, 2013). Martino et al. (2011) found that transplanted NSPC in the injured spinal cord secreted BDNF, glial-derived neurotrophic factor (GDNF), Neurotrophin-3 (NT-3) and nerve growth factor (NGF). This raised the idea that transplanted NSPC can be therapeutically efficacious via bystander, or paracrine, mechanisms which is supported by the observation of a propensity for maintaining an undifferentiated phenotype and the co-localisation with immune cells observed in transplanted NSPCs (Martino et al., 2011). These studies suggest that, with greater understanding of the mechanisms underlying this cross-talk, a therapy that uses these cells can also manipulate the inflammatory response for greater attenuation of the secondary injury.

The nervous and immune systems are extensively linked through the sharing of signalling molecules and receptors that allow them to have significant effect on each other after a SCI. This extends beyond the traditional cytokines to neurotrophins and growth factors that affect both endogenous CNS cells and immune cells. This molecular interlinking highlights the complexity of the secondary injury response and the milieu of cells and signals that are interlinked to perpetrate the progression of this response.

4.1.5 The T-lymphocyte response plays a role in the response to central nervous system injury that is still largely unknown

The adaptive immune response has long been thought to be divorced from the CNS, however this idea has been abandoned as greater understanding of the unique CNS response develops. In recent years the potential role of adaptive immune responses in relation to CNS insults has come to light. T-lymphocytes specific to CNS

self-antigen can be found in the blood of healthy individuals (Moalem et al., 1999a) and are present in small numbers in the healthy CNS (Martino et al., 2011). In recent years it has become apparent that these cells play a protective role in injury states, although the mechanisms underlying this are still unknown (Martino et al., 2011; Moalem et al., 1999a; Schwartz et al., 1999; Schwartz et al., 2009). T-lymphocytes specific for myelin components have been shown to help protect neurons from the effects of spreading secondary damage (Moalem et al., 1999a; Schwartz et al., 1999). The BBB is normally impenetrable to resting T-lymphocytes but selectively permeable to activated subsets, however the disruption of the BBB generally associated with traumatic injury allows for more ready access by these cells (Schwartz et al., 1999).

In controlled and regulated responses auto-reactive T-lymphocytes that respond to elements of the CNS can have a positive, neuro-protective effect. This has been coined as “protective autoimmunity” (Cusimano et al., 2012; Hohlfeld et al., 2007; Kokaia et al., 2012; Martino et al., 2011). The perception that the mere occurrence of an immune or inflammatory response in the CNS was detrimental and the cause of a failure to regenerate has been refuted (Martino et al., 2011) as evidence for the benefits of immune-nervous system interactions mounts. In recent years a number of studies have focused on the role of the adaptive immune system in normal CNS development (Martino et al., 2011) and in CNS pathologies (Cusimano et al., 2012; Hohlfeld et al., 2007; Kokaia et al., 2012). In 2007 Hohlfeld, Meinl & Kerschensteiner discussed the potential of T-lymphocytes specific to CNS self- antigens to protect neurons from secondary damage processes and the ability of MBP reactive T-lymphocytes to produce growth factors and NTF, such as BDNF, GDNF and NT-3 (Hohlfeld et al., 2007). This has since been supported by Kokaia et al. (2012) who describe the potential beneficial and detrimental effects of T-lymphocytes in brain

repair (Kokaia et al., 2012). Self-reactive T-lymphocytes have the potential to facilitate the recruitment of monocytes and macrophages to the injury CNS tissue and also of supporting CNS cell renewal through Toll-like receptor (TLR) mediated production of effector molecules (Kokaia et al., 2012). It is through these mechanisms that auto-reactive T-lymphocytes are said to produce a protective autoimmunity after CNS insult. Another key point raised in this paper is the influences that innate and adaptive immunity have on the phenotypic and functional characteristics of NSPC, further extending the idea of cross-talk and linking these systems.

In 2012 Cusamino et al. used a severe contusion SCI model in mice to test the effect of transplanted NSPC administered in two treatment schedules, subacute (7 days) and chronic (21 days). This study found that the NSPC survived undifferentiated in the peri-lesion environment and used connexin43-mediated cellular-junctional coupling to establish connections with the endogenous phagocytes and helped to modulate inflammatory transcript expression (Cusimano et al., 2012). A consequence of this was a reduction in the proportion of M1-like macrophages and an increase in the levels of T-regs. This may have a ripple effect on the further shifting of the inflammatory environment as activated T-lymphocytes can facilitate the recruitment of monocytes and support CNS repair processes (Kokaia et al., 2012). One hypothesis behind this observed effect is that NSPC increase the availability of diffusible tissue trophic factors and modulate the inflammatory environment (Cusimano et al., 2012).

Historically the mere presence of activated T-lymphocytes in the CNS was thought to be indicative of a sustained pathology. This has been challenged as emerging evidence suggests a neuroprotective role for these cells (Moalem et al., 1999b). Activated T-lymphocytes are capable of synthesising a variety of cytokines as well as various neurotrophic factors that have the potential to exert a beneficial effect

in the aftermath of a TBI or SCI (Kokaia et al., 2012). Auto-reactive T-lymphocytes reactive to CNS antigens can enter the healthy CNS via the choroid plexus and support the functioning of the brain, unless overwhelming or out of control (Martino et al., 2011). These self-reactive T-lymphocytes also facilitate the recruitment of monocytes and macrophages and interact with NSPC through bystander or paracrine mechanisms (Martino et al., 2011). As seen in Figure 54, this interaction with other cells of the CNS and the innate immune system may facilitate the T-lymphocytes neuroprotective roles.

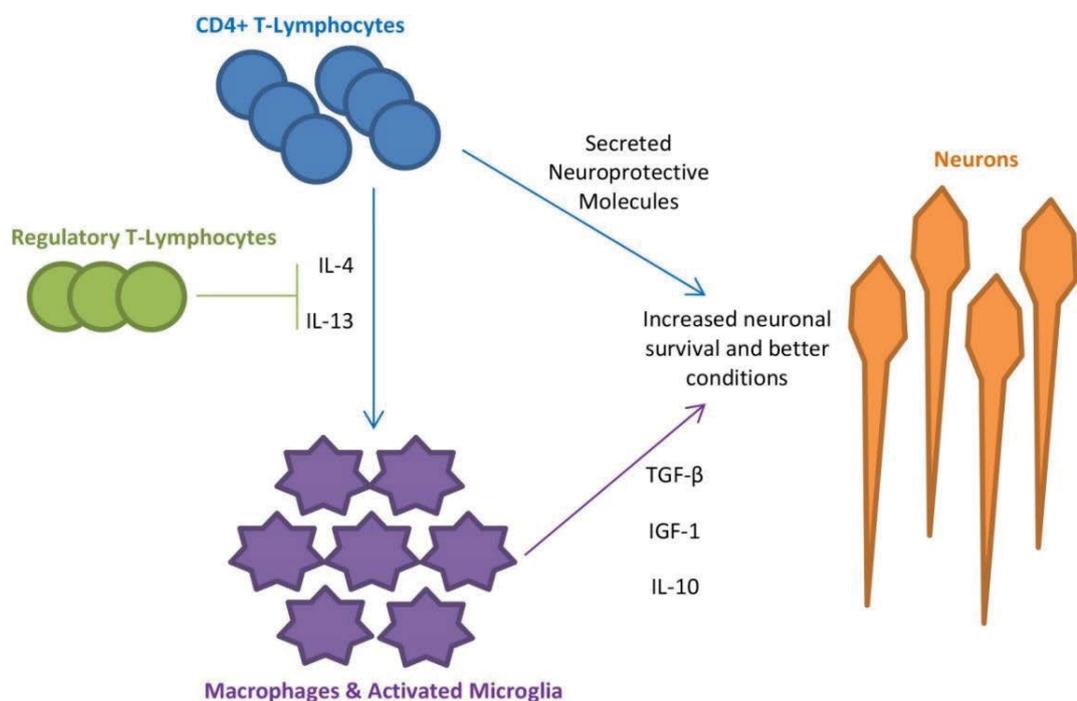


Figure 54 : A mechanism proposed by Walsh, Watson and Kipnis for the neuroprotective effects of CD4+ effector T and regulatory T-lymphocytes within the injured CNS (Walsh et al., 2014). This is based on the secretion of cytokines, chemokines and neuroprotective molecules into the injury microenvironment as well as the effects on other innate immune cells such as macrophages and microglia to create a more permissive microenvironment for neuronal survival.

One class of T-lymphocytes that has been singled out in recent studies is the CD25⁺ T-reg. These are an endogenous dampener of the adaptive immune response and have the capacity to suppress autoimmune reactions (Walsh et al., 2014). T-reg

could have different roles at different stages of SCI. That is, the deactivation of these cells during the initial phase of injury could allow for other T-lymphocytes to mediate their beneficial effects, the later restoration of Treg numbers could then control and dampen the pro-inflammatory adaptive immune response (Walsh et al., 2014). T-lymphocytes can recruit monocytes in a CCR2 dependant manner and induce them to display an anti-inflammatory phenotype, characterised by the expression of the anti-inflammatory cytokine IL-10. These M2-like phagocytes can then produce tissue building and trophic molecules such as IGF-1 and TGF- β (Walsh et al., 2014). This proposed beneficial role of T-lymphocytes is summarised in Figure 54. Another cell that activated T-lymphocytes interact with are the astrocytes. T-lymphocytes can signal astrocytes to increase glutamate buffering and to up-regulate their production of protective thiol compounds, resulting in a more permissible microenvironment (Walsh et al., 2014).

The role that the adaptive immune system, specifically the different classes of T-lymphocytes, plays in the bodies' response to a traumatic injury to the CNS is still to be discovered. The ability of T-regs to manipulate the macrophage or monocyte response, and visa versa, to react to auto-antigens within the CNS and act as an endogenous dampener of the adaptive response may be an important aspect of the post-injury inflammatory response.

4.1.6 Concluding remarks

The post-injury immune and inflammatory response is complicated and multifaceted. It plays an important role in the injury progression and the post-injury environment, making it an exciting target for therapeutical manipulation. Complete suppression of the response is largely not beneficial in the long term. It has become

clear that nonspecific inflammatory therapies are ineffective and may in contribute to worse outcomes (Brennan and Popovich, 2018), meaning that we need specific and directed modulation of the inflammatory response. The inflammatory response has both beneficial and detrimental effects that are intrinsically linked, which makes figuring out how to manipulate the response towards its more beneficial ends.

4.2 HYPOTHESIS AND AIMS

4.2.1 Hypothesis

There will be significant differences in the acute immune response between adult and infant rats. There will be higher levels of M1-like macrophages, compared to M2-like, and a higher level of neutrophil infiltration in the adults. The cytokine profile of the adults will reflect a much more robust more pro-inflammatory environment than that in the infants.

4.2.2 Aims

1. To determine the key differences in the cellular inflammatory response to SCI between adult and infant rats.
 - a. Use flow cytometry to quantify the presence of neutrophils, T-lymphocytes and the proportions of activated macrophages expressing an M1-like or M2-like phenotype in both adults and infants 1h, 24h, and 1wk post SCI.
 - b. To compare the response of these cell and how it differs between adult and infant animals.
2. To compare the inflammatory associated cytokines and factors in the injured and non-injured spinal cord between adult and infant rats.
 - a. To determine which are present, and in what proportions, at 1h, 24h and 1wk after injury in both adults and infants using multiplex cytokine analysis.
 - b. To identify where the key differences in cytokines and inflammatory factors lie between adult and infant animals in the acute period of SCI.

4.3 MATERIALS AND METHODS

4.3.1 Animal numbers and groups

This study used a total of 97 Sprague-Dawley rats, 30 adult females and 67 infants, both males and females (sourced from ARC, Perth; UTS ACEC 2015-462). These rats underwent surgery at 1 week of age (infant) and 9-11 weeks of age (adult) and were euthanized at 1h, 24h, and 1wk post-surgery. These were separated into injured and sham control groups at both ages and all survival times (as seen in Table 5). The adults (N= 5) were used singly and the infants were pooled two animals per sample. Due to the numbers of pups per litter there was an N of 4-6 in the infant groups. During experimentation all animals were numbered and randomised as a blinding technique.

Table 5: Table of animal groupings, as distinguished by age and injury status, as well as the numbers of animals in each group.

| Group | Age at Injury | Survival time | Number of samples |
|-----------------|----------------------|----------------------|--------------------------|
| Adult Sham | 9-11wks | 1h | 5 |
| | | 24h | 5 |
| | | 1wk | 5 |
| Adult Mild SCI | 9-11wks | 1h | 5 |
| | | 24h | 5 |
| | | 1wk | 5 |
| Infant Sham | 1wk | 1h | 4 |
| | | 24h | 5 |
| | | 1wk | 5 |
| Infant Mild SCI | 1wk | 1h | 4 |
| | | 24h | 6 |
| | | 1wk | 6 |

4.3.2 Surgery and euthanasia

The surgical procedures for this experiment were performed under animal ethics approval (UTS ACEC 2015-462). Surgery to induce a mild contusion SCI was performed as previously described (section 2.3.2). At the end of the experimental survival time rats were euthanised by Lethobarb (Virbac Australia) overdose, the rat was then cardially perfused with heparinised saline for one minute and approximately 1.5cm of the fresh spinal cord tissue, surrounding the T10 lesion, was extracted and placed in HBSS buffer (ThermoFisher Scientific) on ice and transported to be homogenised for flow cytometry. The same physical length of cord was collected for both adults and juveniles as the histology (Chapter 3) suggests that the lesion spans the same length of cord in both ages.

4.3.3. Flow cytometry

4.3.3a Tissue homogenisation

The extracted spinal cords were initially sliced up using sterile scissors whilst in the HBSS buffer, directly after extraction. Following this the tissue was homogenised using the GentleMACS Dissociator desktop system (Miltenyi Biotec) preset brain protocol. Adult samples were processed singly while infants were pooled from this point onward, two animals to a sample, to ensure adequate numbers for cell population separation. This involved a short dissociation step in HBSS, followed by the addition of trypsin 1:250 (USB)(1mg/ml) and collagenase (Sigma-Aldrich) (2mg/ml) in HBSS incubated at room temperature (RT) for 15min before another brief dissociation, the addition of 10% foetal bovine serum in DMEM (FBS-DMEM) (ThermoFisher Scientific) to counteract the trypsin and collagenase and a final dissociation. The

homogenate was then spun down briefly and 150µl of supernatant was removed and stored at -80°C for later analysis. The pellet was then resuspended and filtered through a 70µm strainer using 10ml of FBS-DMEM and centrifuged at 300xg for 10min. The supernatant was removed, and the pellet resuspended in 1ml of red blood cell lysis buffer and left for 5min RT. Finally, this was centrifuged for 10min again, the supernatant removed, and the pellet resuspended in 6ml of HBSS.

4.3.3b Removing debris using an Optiprep gradient

The Optiprep density gradient was performed as previously described (Beck et al., 2010a). Stock Optiprep solution was diluted using a 1:1 ratio with MOPS buffer (0.15M NaCl and 10mM MOPS pH 7.4) to create the diluted Optiprep solutions for use in the gradient. The density gradient was made up of four solutions of different concentrations of diluted Optiprep in HBSS buffer, to a final volume of 1 ml for each gradient tube.

These four solutions were layered carefully into a 15ml conical centrifuge tube with solution 1 at the bottom followed by solution 2, 3 then 4 in order. This places the least dilute at the bottom of the tube and the most dilute at the top. Approximately 1.5ml of homogenised spinal cord in HBSS buffer was carefully layered on top of the gradient solutions in the centrifuge tube and this was centrifuged for 15min at 1900rpm on an Eppendorf centrifuge. This separated the gradient and cell solution into layers and separated the myelin and debris from the cells. As can be seen in Figure 55 the top of the tube contained the debris which was discarded. Beneath this were layers of large neuronal cells, which were also discarded, leaving inflammatory cells, glia, and red blood cells in the pellet. Some of the solution was left above the pellet as some larger macrophages could be located here (Beck et al., 2010a).

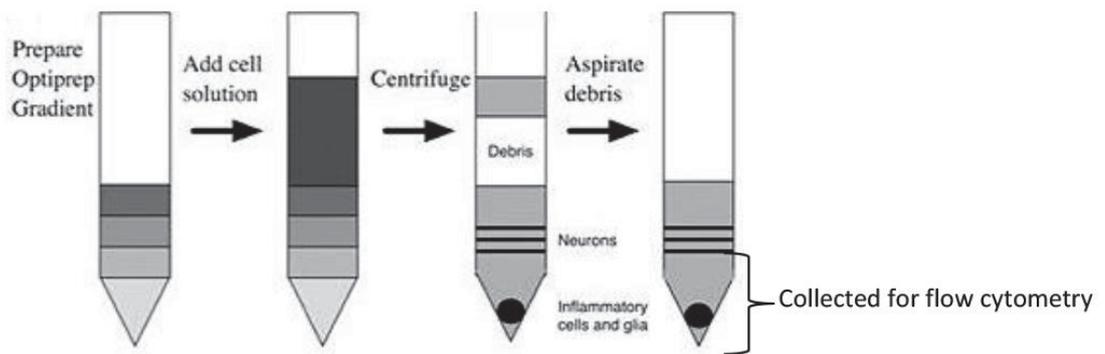


Figure 55: Optiprep gradient of different densities to separate the majority of debris from the spinal cord and inflammatory cells of interest (Beck et al., 2010a).

4.3.3c Flow cytometry

Using Trypan Blue manual cell counts were performed on 20µl of the isolated solution. From the results of these cell counts the isolate was separated into a number of 1.5ml Eppendorf tubes, indicated in Table 6. Sufficient isolate was placed in the stained and isotype control tubes for 1×10^6 cells and sufficient for 1×10^5 cells in the unstained control tubes. Compensation Beads (CB) were used for the single colour controls (BD CompBeads #552843, BD Biosciences). For the single colour controls 4 tubes of positive and negative control beads were prepared to the manufacturers' specifications and 1µl APcCy7, 2µl AF488, 5µl PE, and 2µl AF647 were added to separate tubes.

The separated isolate was centrifuged for 2-3 6 second pulses to pellet out the cells. Once the supernatant has been aspirated, 20µl of 5% v/v normal mouse serum (NMS) in faccine wash (FSW) (PBS, FBS, BSA and Sodium Azide) was added to each tube as a blocking agent, and the cell pellet was resuspended and incubated on ice for 30 min. The two different antibody mixes for the macrophage (1) and neutrophil (2) panels, as well as the isotype control panel, were made up using the antibodies in Table 7 (quantities can be seen in Appendix Table 1-4). 80µl of the antibody mixes

were placed on top of the blocking solution in the appropriate stained tubes and 50µl of the isotype mix in the isotype control tubes. This was incubated on ice for 30min, after which the cells were washed twice in FSW then resuspended in 350µl of NMS in FSW. Finally, this was strained into 5ml round-bottom tubes and topped up with 300µl FSW.

Table 6: Minimum number of tubes for one flow cytometry run using two animals, a sham and an injured.

| Macrophage Panel | Neutrophil + T-Cell Panel | Controls |
|----------------------|---------------------------|----------------------------|
| Sham stained | Sham stained | ApcCy7 colour control (CB) |
| Sham Isotype control | SCI stained | AF488 colour control (CB) |
| SCI stained | | PE colour control (CB) |
| SCI Isotype control | | AF647 colour control (CB) |
| | | Unstained control |

Table 7: Conjugated antibodies used for fluorescent cells staining for flow cytometry

| Primary Antibody | Conjugate | Supplier (Dilution) | Isotype Control |
|----------------------|-----------|-----------------------|-------------------------------------|
| Mouse Anti-Rat CD45 | APC-Cy7 | BD Biosciences (1:20) | APC-Cy7 Mouse IgG1 (BD Biosciences) |
| Mouse Anti-Rat CD68 | FITC | BioRad (1:10) | FITC Mouse IgG2a (BD Biosciences) |
| Mouse Anti-Rat CD86 | PE | BD Biosciences (1:20) | PE Mouse IgG1 (BD Biosciences) |
| Mouse Anti-Rat CD163 | AF647 | BioRad (1:10) | AF647 Mouse IgG1 (BD Biosciences) |
| Mouse Anti-Rat CD3 | PE | BD Biosciences (1:20) | PE Mouse IgG1 (BD Biosciences) |
| Mouse Anti-Rat HIS48 | FITC | BD Biosciences (1:10) | FITC Mouse IgM (BD Biosciences) |

Flow cytometry was performed on the samples using an LSRII Flow Cytometer (BD Biosciences) and using Sytox Blue as a cell viability stain. The relative numbers of activated macrophages, M1-like and M2-like macrophages was found. Leukocytes were gated as CD45⁺ and then specific populations of macrophages were discriminated

using the CD markers 68, 86 and 163, T-lymphocytes using CD3, and neutrophils using HIS-48 (Table 7). Activated macrophages were indicated by staining with CD45⁺/CD68⁺, the M1-like phenotype was typified by CD68⁺/CD86⁺ staining while the M2-like phenotype was typified by CD68⁺/CD163⁺ staining (Hadley et al., 1988; Turtzo et al., 2014). Neutrophils were indicated using an anti-granulocyte marker (HIS48) and T-lymphocytes using the pan-T-Lymphocyte marker CD3.

4.3.3d Flow cytometry gating strategies

The gating strategies used to select the sub-populations of cells were hierarchical and based on forward- (FSC) and side-scatter (SSC) as well as fluorescent staining of specific markers. The same initial strategy was used for both the macrophage (1) panel and the neutrophil/T-lymphocyte (2) panel (Figure 56). This involved an initial gate based on forward and side scatter properties of the cells of interest (P1) followed by a height against area gate to restrict to single cells events (P2). Finally, this was further gated using Pacific Blue fluorescence from the Sytox Blue cell viability marker to remove dead cells and debris from analysis as much as possible (P3).

Populations were gated hierarchically from the total events using scatter properties and fluorescent staining to define populations of single, viable cells expressing specific fluorescent markers. In the macrophage panel leukocytes were broadly gated as positive in the APC-Cy7 (CD45⁺) channel with a subpopulation of APC-Cy7 (CD45) and AF488 (CD68) channel positive. Within the CD45⁺ gated population M1-like cells were quantified as AF488 channel (CD68) and PE channel (CD86) positive, while M2-like were AF488 channel (CD68) and APC channel (CD163) positive (Figure 57).

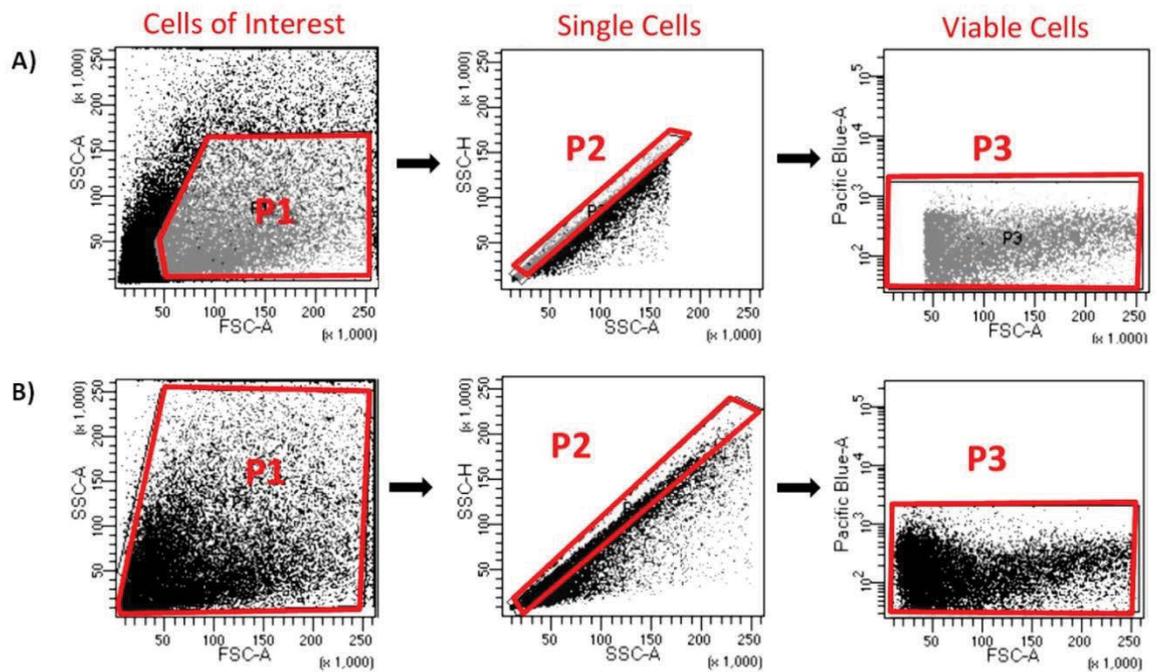


Figure 56: Initial hierarchical gating strategies used to identify viable single cell events in both A) the macrophage panel and B) the neutrophil and T-lymphocyte panel. Gating was based on forward and side scatter properties of the cells (P1), SSC height and area to restrict to single cell events (P2) and Sytox Blue viability staining (P3).

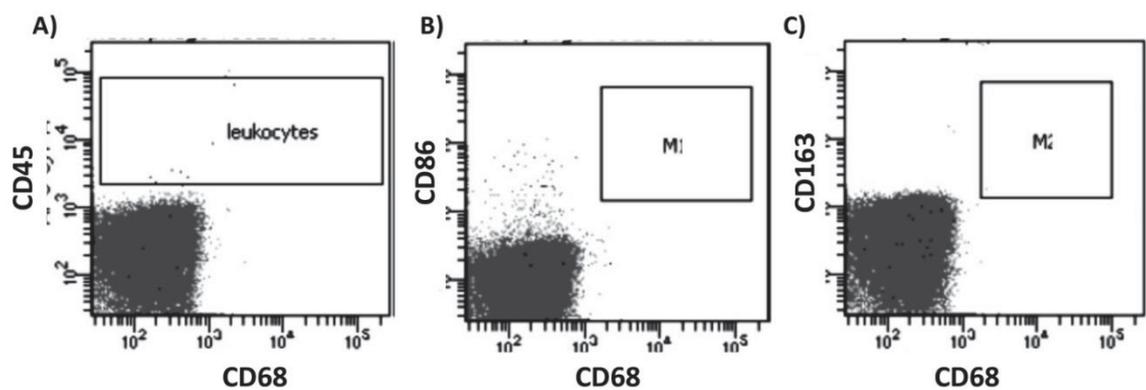


Figure 57: Example flow cytometry scatter-plots from Adult shams to demonstrate the gating strategy used for A) CD45+ leukocytes, B) CD68+/CD86+ M1-like cells and C) CD68+/CD163+ M2-like cells.

In the second flow cytometry panel neutrophils were gated using APC-Cy7 (CD45) channel and PE (CD3) channel positivity. T-lymphocytes were gated out both as PE (CD3) channel positive and as both PE (CD3) and APC-Cy7 (CD45) positive (Figure

58). The neutrophil and T-lymphocyte panel was plagued by issues of inconsistent non-specific staining and subsequent gating difficulties. In both panels the forward- and side-scatter of the gated populations were checked to ensure these events fell into the broader FSC and SSC properties expected of the cells of interest.

The infants demonstrated a consistent auto-fluorescence in the APC/APC-Cy7 channel that had to be gated around. This was addressed by using unstained controls, unstained with Sytox Blue viability stain and isotype controls as references.

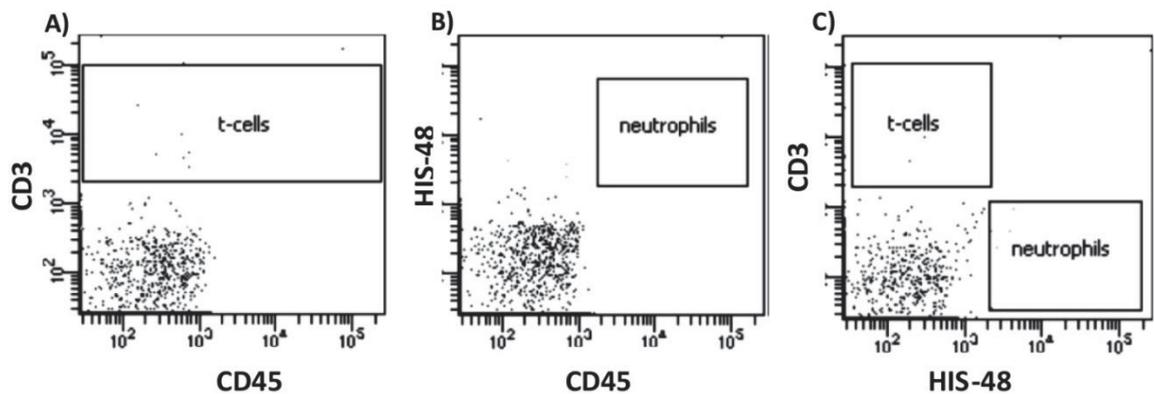


Figure 58: Example flow cytometry scatter-plots from Adult shams to demonstrate the gating strategy used for A) CD3+ T-lymphocytes, B) CD45+/HIS48+ neutrophils and C) combined CD3+ T-lymphocytes and HIS48+ neutrophils hierarchically gated out of CD45+ events.

4.3.4 Multiplex cytokine analysis

Cytokine analysis was performed on the supernatant from the spinal cord homogenate using the Bio-Rad Pro Rat TH1/Th2 12-Plex panel for the Bio-Rad MagPix. The 96 well plate was prepared to the manufacturers' specifications, using the 20x coupled beads and respective antibodies, with the supernatant diluted 1:2 with FSW and the standards prepared as 1:4 serial dilutions. The standards and sample dilutions were prepared the previous day and stored at 4°C and -20°C overnight respectively.

The plate was analysed on the Bio-Rad MagPix using the standard settings recommended by the manufacturer.

Table 8: BioRad Bio-Plex Pro Rat Cytokine 12plex Th1/Th2 panel

| Cytokine | Th1 Associated (M1) | Th2 Associated (M2) |
|-----------------|----------------------------|----------------------------|
| GM-CSF | | * |
| IFN- γ | * | |
| IL-1 α | * | |
| IL-1 β | * | |
| IL-2 | * | |
| IL-4 | | * |
| IL-5 | | * |
| IL-6 | * | |
| IL-10 | | * |
| IL-12 (p70) | * | |
| IL-13 | | * |
| TNF- α | * | |

For data analysis, where the values recorded for the shams were below the range of detection for the assay the lowest recorded value was used to calculate the fold increase from sham levels in the injured animals. This only occurred in a few cases and where it has occurred is indicated on the histograms by #.

4.3.5 Statistical analysis

Two-way ANOVA with Bonferroni's post hoc test was used to analyse the CD68⁺/CD86⁺ and CD68⁺/CD163⁺ population data, as both a percentage of the viable cells (gate P3, Figure 56) and as a percentage of the CD45⁺ population. This method was also used to analyse the CD45⁺, CD45⁺/CD3⁺ and CD45⁺/HIS48⁺ populations as percentages of the viable cells (P3). The main effects analysed were age against post-injury interval (survival time) and injury status against survival time. These are reported as the impact and significance of each factor alone (ie/age, injury status or

survival time), and any significant interaction between the two factors being tested. Bonferroni's post hoc test was used to find the specific differences between groups.

To test the specific population differences within the cohort one-way ANOVA with Bonferroni's post hoc test and unpaired T-tests were performed. This was between SCI groups to test age groups at specific post-injury intervals; and between different cell populations (CD68+/CD86+ versus CD68+/CD163+) within each age group. The following graphs represent group means with the standard error of the mean (SEM).

Multiplex data was analysed using the raw observed concentration (pg/ml) data as well as the SCI observed concentration as a fold increase from their respective shams. This data was analysed in GraphPad Prism using two-way ANOVA with Bonferroni's post hoc test. In some instances, there was not enough data points to run ANOVA due to the levels of cytokines being below the range registered by this test.

4.4 RESULTS

4.4.1 Progression of the cellular inflammatory response after injury

4.4.1a Progression of the cellular inflammatory response after injury in the adults

Leukocyte (CD45⁺) cell number increased over time in the SCI animals but not in the shams (Figure 59). There was no macrophage/microglial response at 1h post-injury, increasing at 24h and peaking at 1wk (Figure 60A). This followed the pattern expected from the previous results. The increase in the CD45⁺ cell percentage of viable cells was statistically significant between both 1h (0.06%) and 24h (2.9%), and between 24h (2.9%) and 1wk (4.2%) using two-way ANOVA. The percentage of CD45⁺ cells at 1wk was significantly higher than both the shams (0.6%) and at 1h post-injury using ANOVA and Bonferroni's post-hoc test ($P < 0.0001$).

The progression of the M1-like (CD68⁺/CD86⁺) response follows this increase over time in the SCI animals as well, with an increase in the percentage of CD45⁺ from 1h to 1wk (Figure 61). The CD68⁺/CD86⁺ cells as a percentage of the viable cells showed a significant increase between both 1h (0.7%) and 24h (0.6%), and between 24h and 1wk (0.7%) (ANOVA $P < 0.001$). The CD68⁺/CD86⁺ cells as a percentage of viable cells were significantly higher at 24h and at 1wk compared to the shams using ANOVA and Bonferroni's post-hoc test ($P < 0.001$) (Figure 60B). The 1wk peak percentage was significantly higher than the 1h and 24h post-injury groups ($P < 0.001$) (Figure 60B).

The M2-like (CD68⁺/CD163⁺) response also increased over time in the SCI animals, with the highest percentage of total leukocytes at 1wk post injury (26.8%) (Figure 62). The CD68⁺/CD163⁺ cells response showed no significance in the increase in percentage of viable cells between 1h (0%) and 24h post-injury (0.6%) or between 24h and 1wk (1.0%). CD68⁺/CD163⁺ cells as a percentage of the viable cells showed a

significantly higher percentage at 1wk compared to 1h and the sham ($P < 0.001$) with no other statistical significance present using ANOVA and Bonferroni's post-hoc test. This is shown in Figure 60C. There was a significant effect for both injury status ($P < 0.001$) and survival time ($P < 0.001$) as well as a significant interaction between these factors ($P < 0.001$) for $CD45^+$, $CD68^+/CD86^+$ and $CD68^+/CD163^+$ cells using two-way ANOVA.

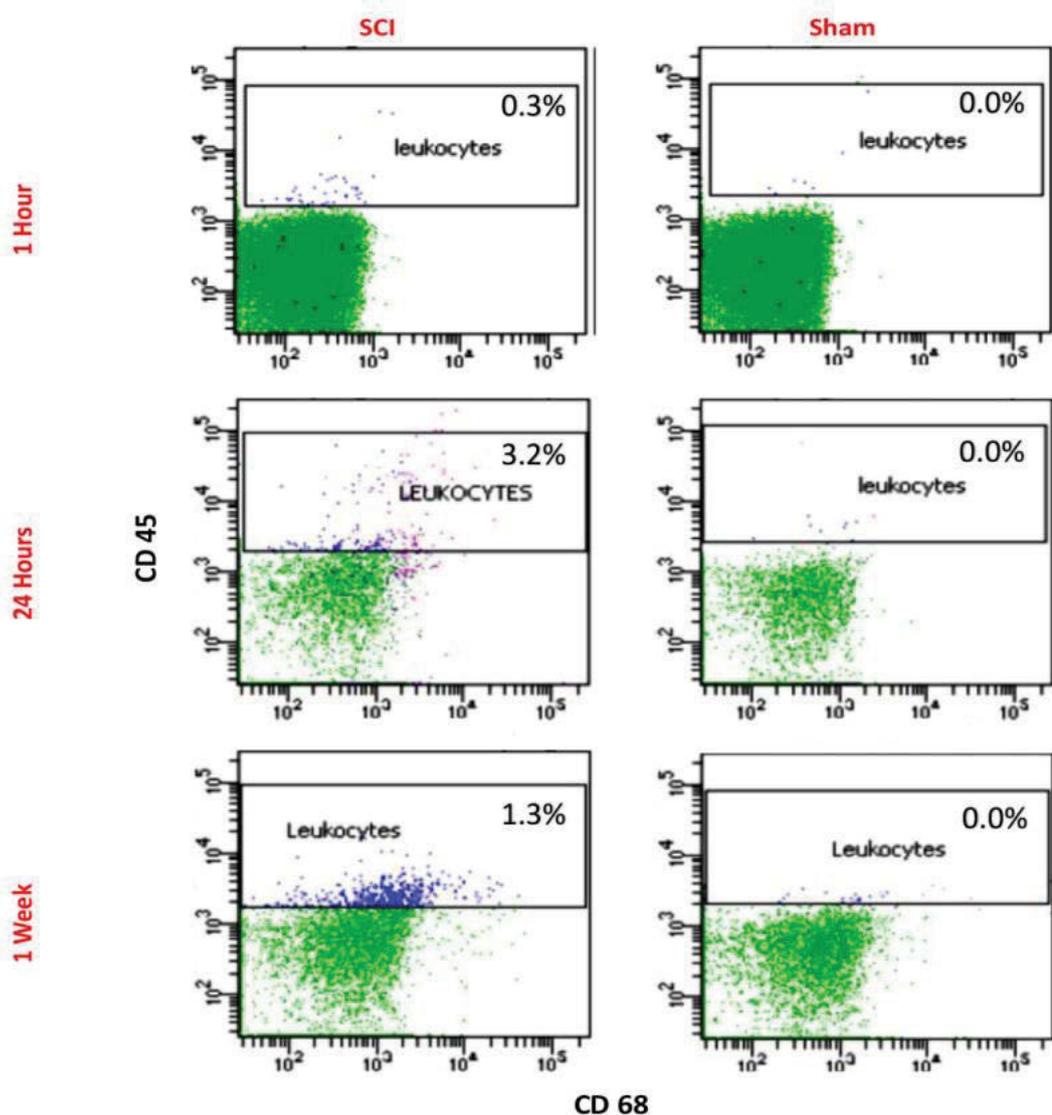


Figure 59: Representative flow cytometry scatter-plots for the total leukocyte population ($CD45^+$) in Adults over the three time-points. These show a pattern of increasing numbers of leukocytes over time, peaking at 1wk post-SCI. Percentages represent percentages of the total viable cell population.

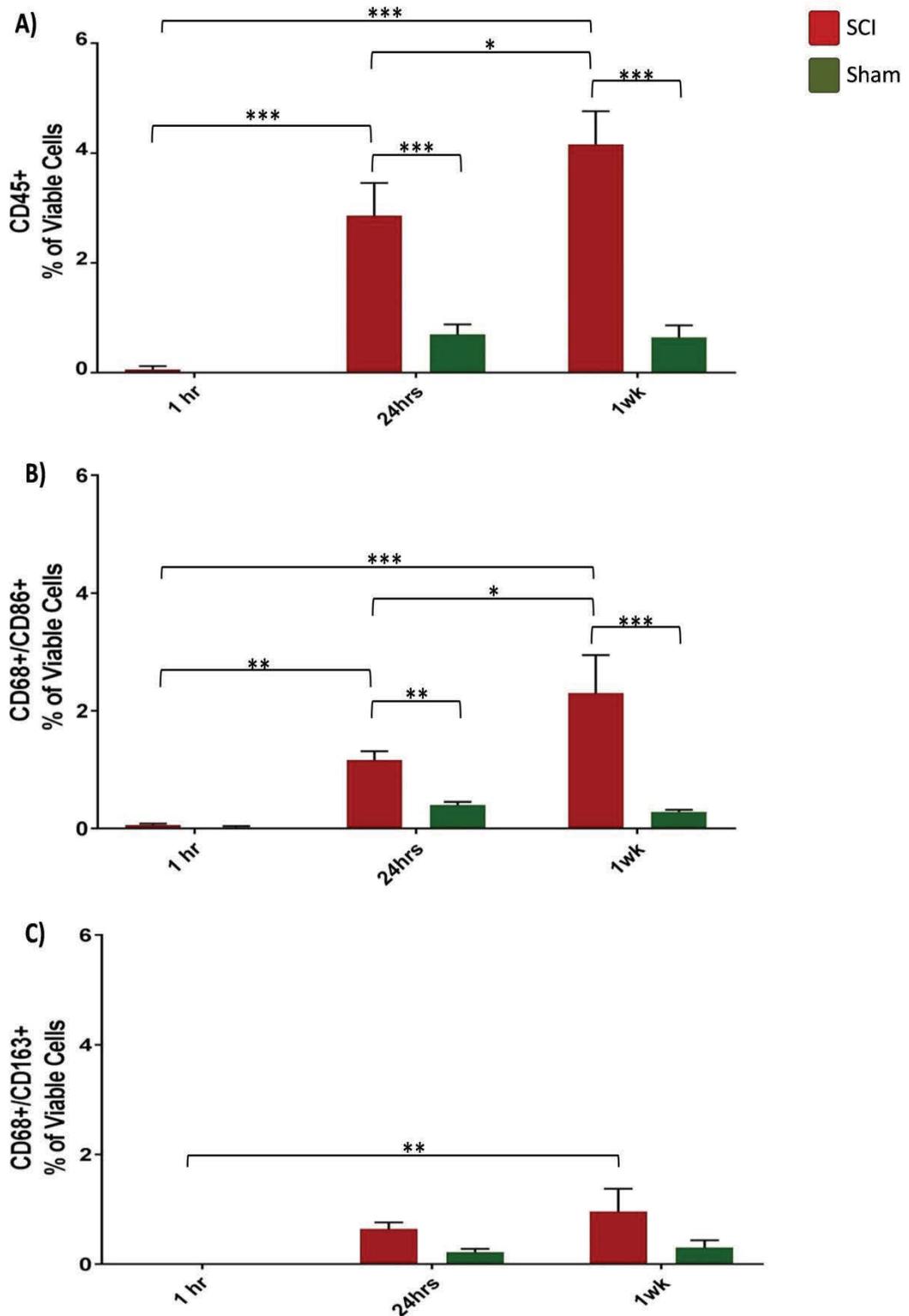


Figure 60: Histograms of the A) CD45+ leukocytes, B) CD68+/CD86+ M1-like cells and C) CD68+/CD163+ M2-like cells as a percentage of the total viable cells in both sham and SCI adults 1h, 24h and 1wk post injury. * (P<0.05), ** (P<0.005) and *** (P<0.001) indicate the statistical significance based on Bonferroni's post-hoc test.

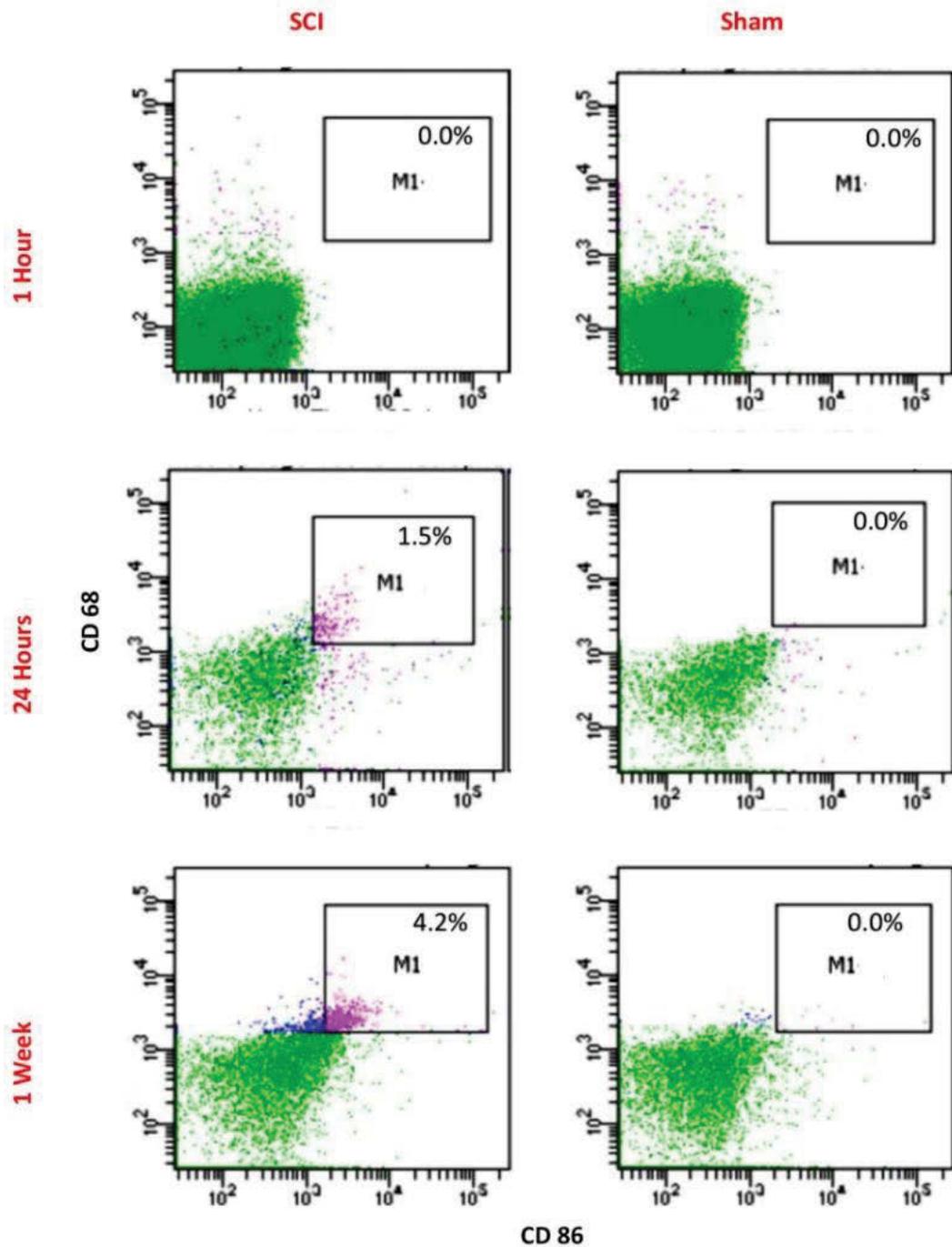


Figure 61: Representative flow cytometry scatter-plots for the M1-like population (CD45⁺/CD86⁺) in adult spinal cord tissue over the three time points for spinal cord injured (SCI) (left column) and sham rats (right column). Percentages represent percentages of the total viable cell population.

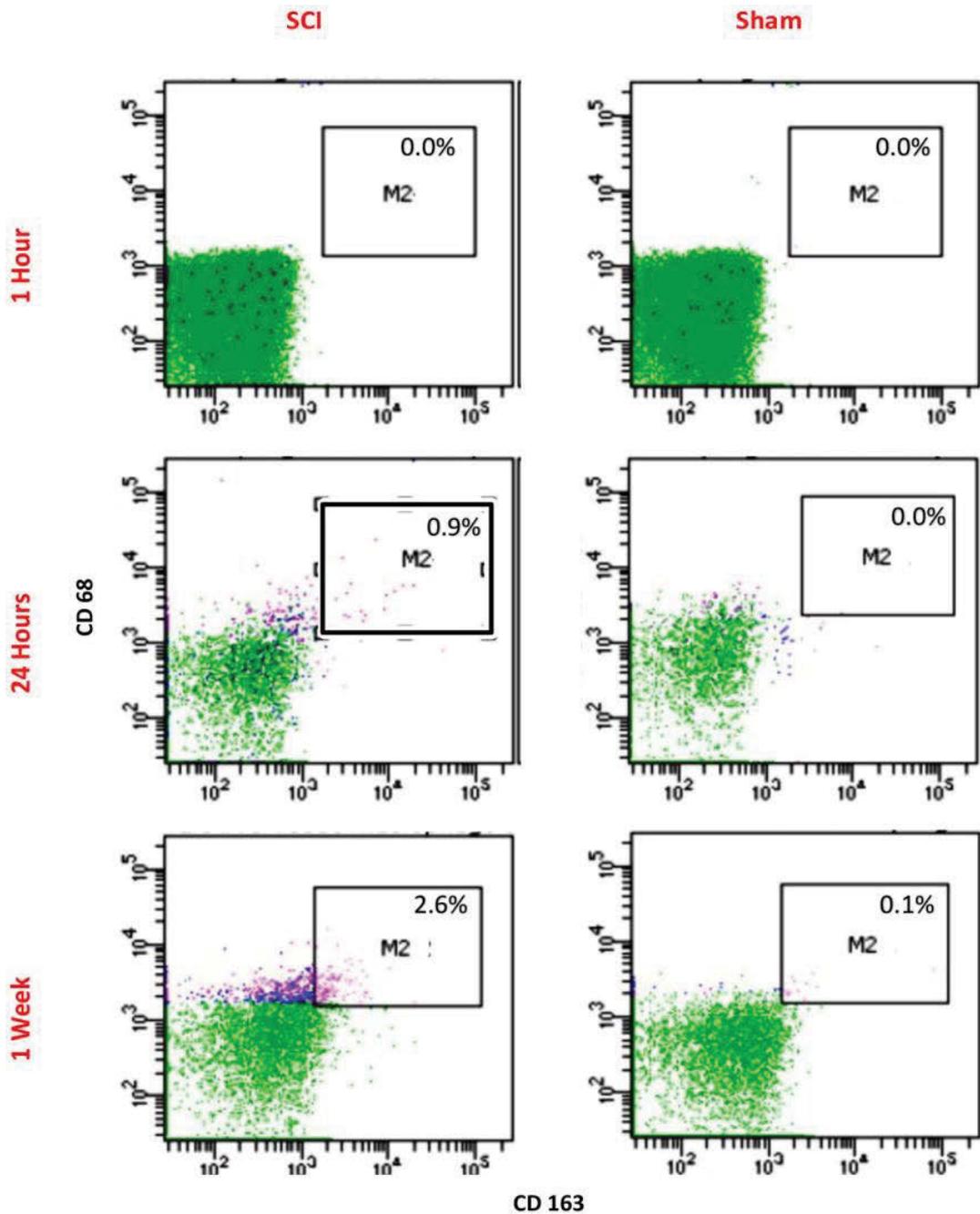


Figure 62: Representative flow cytometry scatter-plots for the M2-like population (CD68⁺/CD163⁺) in adult spinal cord tissue over the three time points for spinal cord injured (SCI) (left column) and sham rats (right column). Percentages represent percentages of the total viable cell population.

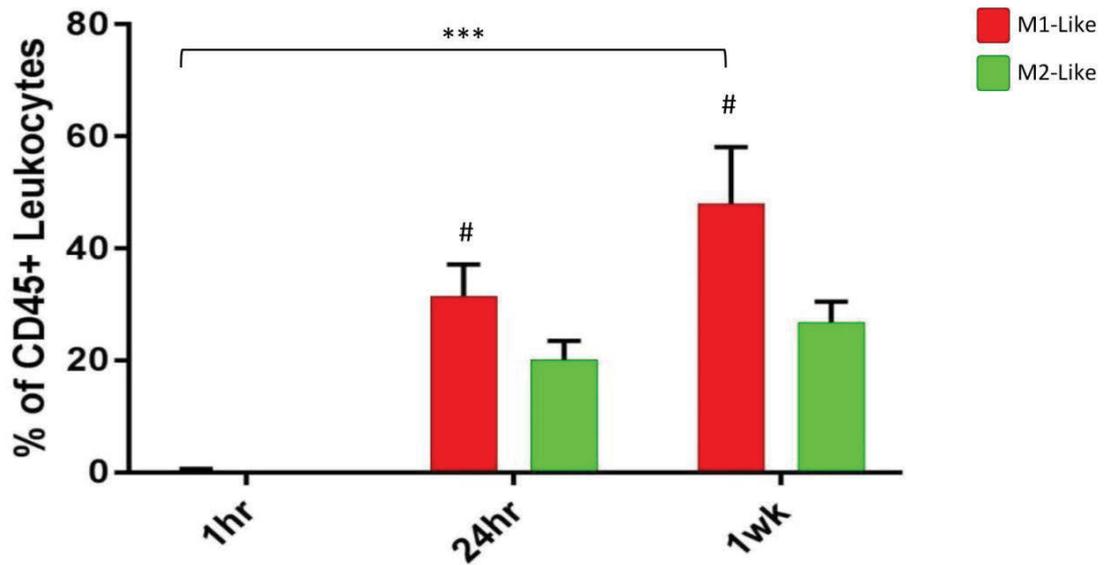


Figure 63: Histogram of the proportion of M1-like and M2-like cells as a percentage of total leukocytes in adult rat spinal cord tissue, progressing from 1h, 24h and 1wk post-spinal cord injury. # indicates a significant increase from sham levels. *** (P<0.001) indicates the statistical significance based on Bonferroni's post-hoc test.

As seen in Figure 63, the CD68⁺/CD86⁺ (M1) cells represented a higher percentage of the total CD45⁺/CD68⁺ population than the CD68⁺/CD163⁺ (M2) cells at both 24h (31.4%, 20.1%) and 1wk (48%, 26.8%) post injury in the adults, however this was not statistically significant using ANOVA and Bonferroni's post-hoc test (ANOVA P=0.8). There was an increase in the CD68⁺/CD86⁺ cells as percentage of CD45⁺/CD68⁺ cells over time; however, this was only statistically significant between 1h (0.3%) and 1wk (48%) post-injury (P<0.0001).

Neutrophil (CD45⁺/HIS48⁺) numbers peaked at 24h post injury. The CD45⁺/HIS48⁺ cells as a percentage of viable cells at 24h (4.3%) showed a significant increase from 1h post-injury (0.08%) and was also significantly higher than its sham (0.5%) using ANOVA and Bonferroni's post-hoc test (Figure 64). There was a significant effect for both injury status (P<0.05) and survival time (P=0.005) as well as a significant interaction between these factors (P<0.05) using two-way ANOVA. Neutrophil data for 1wk post injury was unavailable for comparison.

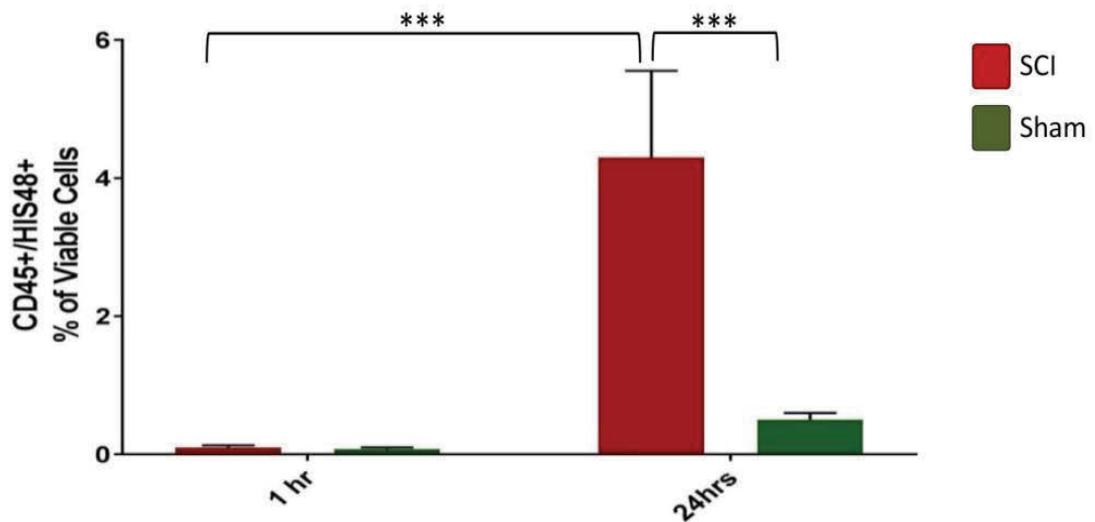


Figure 64: Histograms of the CD45+/HIS48+ neutrophils as a percentage of the total viable cells in both sham and injured (SCI) adult rat spinal cord tissue 1h and 24h post injury. *** (P<0.001) indicates the statistical significance based on Bonferroni's.

T-lymphocytes (CD45⁺/CD3⁺) as a percentage of viable cells increased over time in the SCI animals and also in the shams, with no significance between the different injury status'. At 24h (0.6%) there was significant increase from 1h (0.1%) post injury using ANOVA and Bonferroni's post-hoc test (ANOVA P=0.0005) (Figure 65). These percentages were much lower than the other inflammatory cells examined however, not reaching 1% of the viable cells present. There was a significant effect only for survival time (P=0.0005) using two-way ANOVA.

4.4.1b Progression of the cellular inflammatory response after injury in the infants

In infants there is a small peak in total macrophage percentage of CD68⁺/CD86⁺ cells at one week although no statistical significance was found using ANOVA and Bonferroni's post-hoc test (P>0.4) (Figure 67A). The percentage of viable cells expressing CD68⁺/CD163⁺ was higher at 1h (0.25%) than 24h (0.07%), as was the total macrophage percentage (0.65%, 0.55%), however these were not at all significant

(ANOVA $P=0.5$). The percentage of viable cells expressing $CD68^+/CD86^+$ remains fairly consistent. There was a population of auto-fluorescent cells high in the APCy7 channel that was not included in the leukocyte gate as it appeared on all SCI, sham, isotype controls and unstained controls (indicated by the red box in the representative scatterplots) (Figure 66).

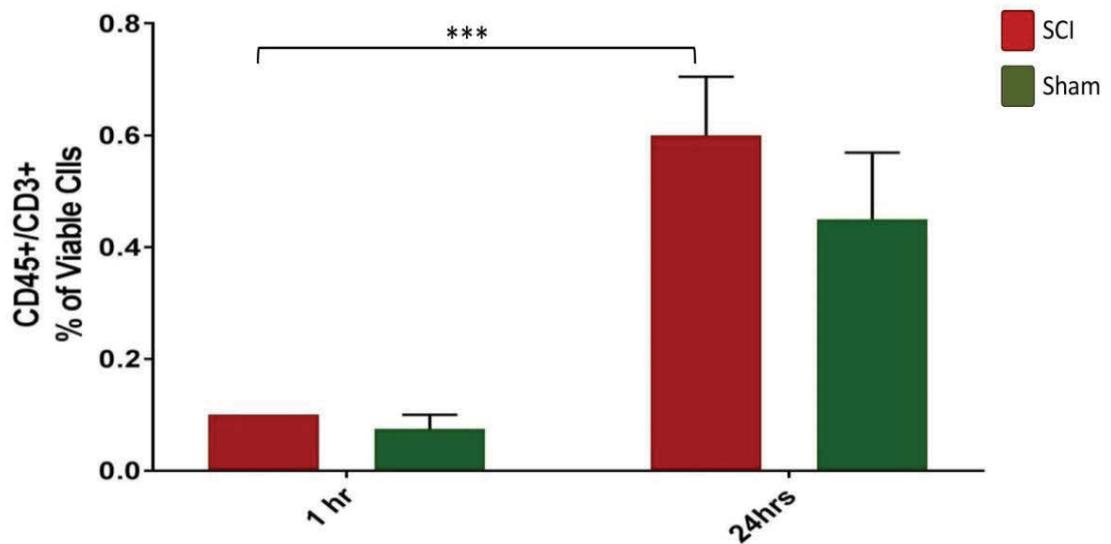


Figure 65: Histogram of the $CD45^+/CD3^+$ T-lymphocytes as a percentage of the total viable cells in both sham and injured (SCI) adult spinal cord tissue 1h and 24h post injury. * ($P<0.001$) indicates the statistical significance based on Bonferroni's post-hoc test.**

The number of the M1-like ($CD68^+/CD86^+$) as a percentage of viable cells increased with time (Figure 68). There was no statistical significance using one-way or two-way ANOVA (Figure 67B). The progression of the M2-like ($CD68^+/CD163^+$) as a percentage of viable cells increased with time, similar to the $CD68^+/CD86^+$ cell response (Figure 69). The $CD68^+/CD163^+$ cells peaked at 1wk (0.36%) post-injury and was higher than the corresponding $CD68^+/CD86^+$ percentage at 1h and 1wk post-injury. This was not statistically significant (ANOVA $P=0.236$) (Figure 67C).

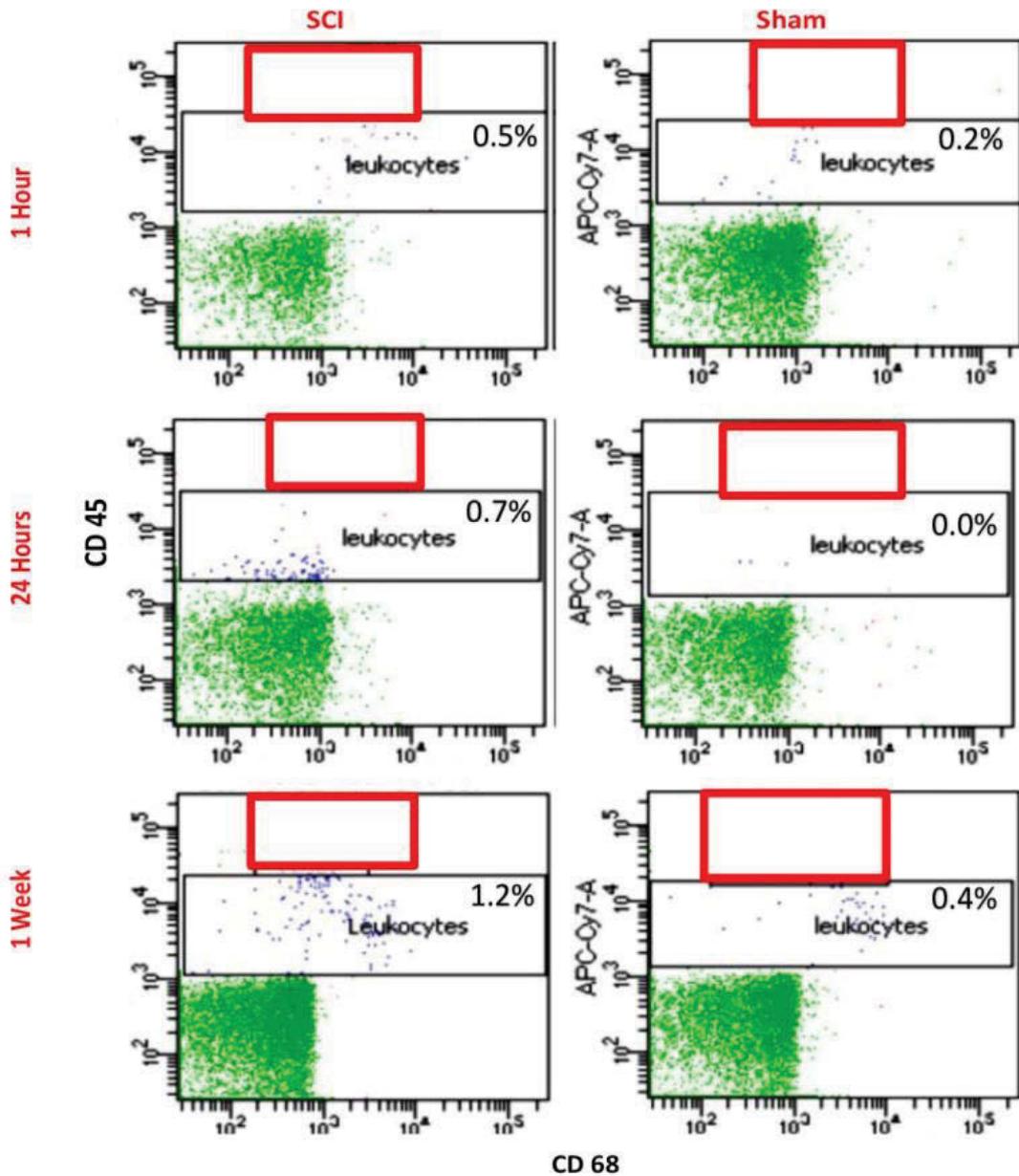


Figure 66: Representative flow cytometry scatter-plots for the total leukocyte (CD45⁺) population in Infants over the three time points. Percentages represent percentages of the total viable cell population. The red boxes indicated the location of a population of auto-fluorescent cells high in the APCy7 channel that appeared in all infant samples.

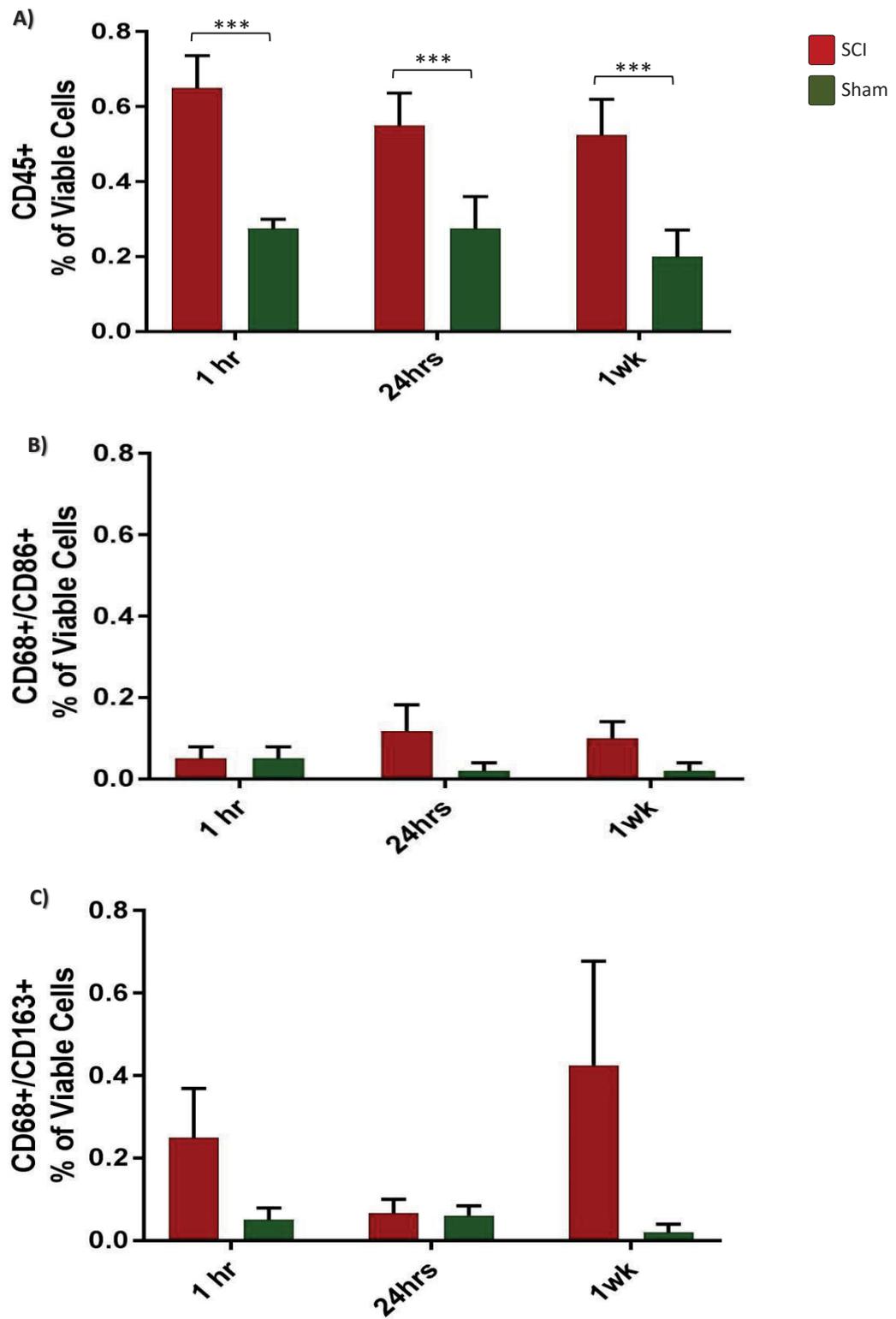


Figure 67: Histograms of the A) CD45+ leukocytes, B) CD68+/CD86+ M1-like cells and C) CD68+/CD163+ M2-like cells as a percentage of the total viable cells in both sham and injured (SCI) infant rat spinal cord tissue at 1h, 24h and 1wk post injury. *** (P<0.001) indicates the statistical significance based on Bonferroni's post-hoc test.

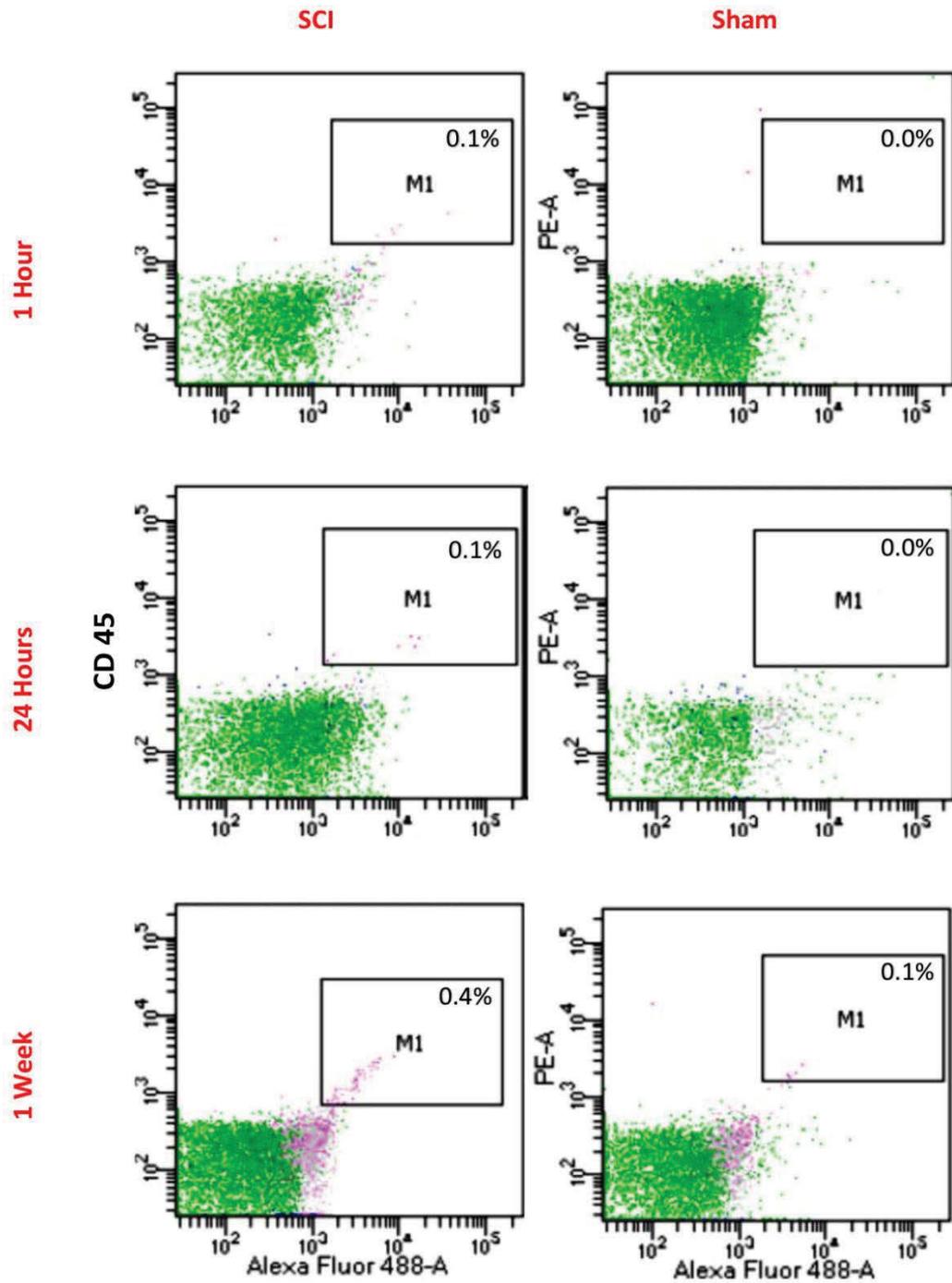


Figure 68: Representative flow cytometry scatter-plots for the M1-like population (CD45+/CD86+) in infant spinal cord tissue over the three time points for spinal cord injured (SCI) (left column) and sham rats (right column). Percentages represent percentages of the total viable cell population.

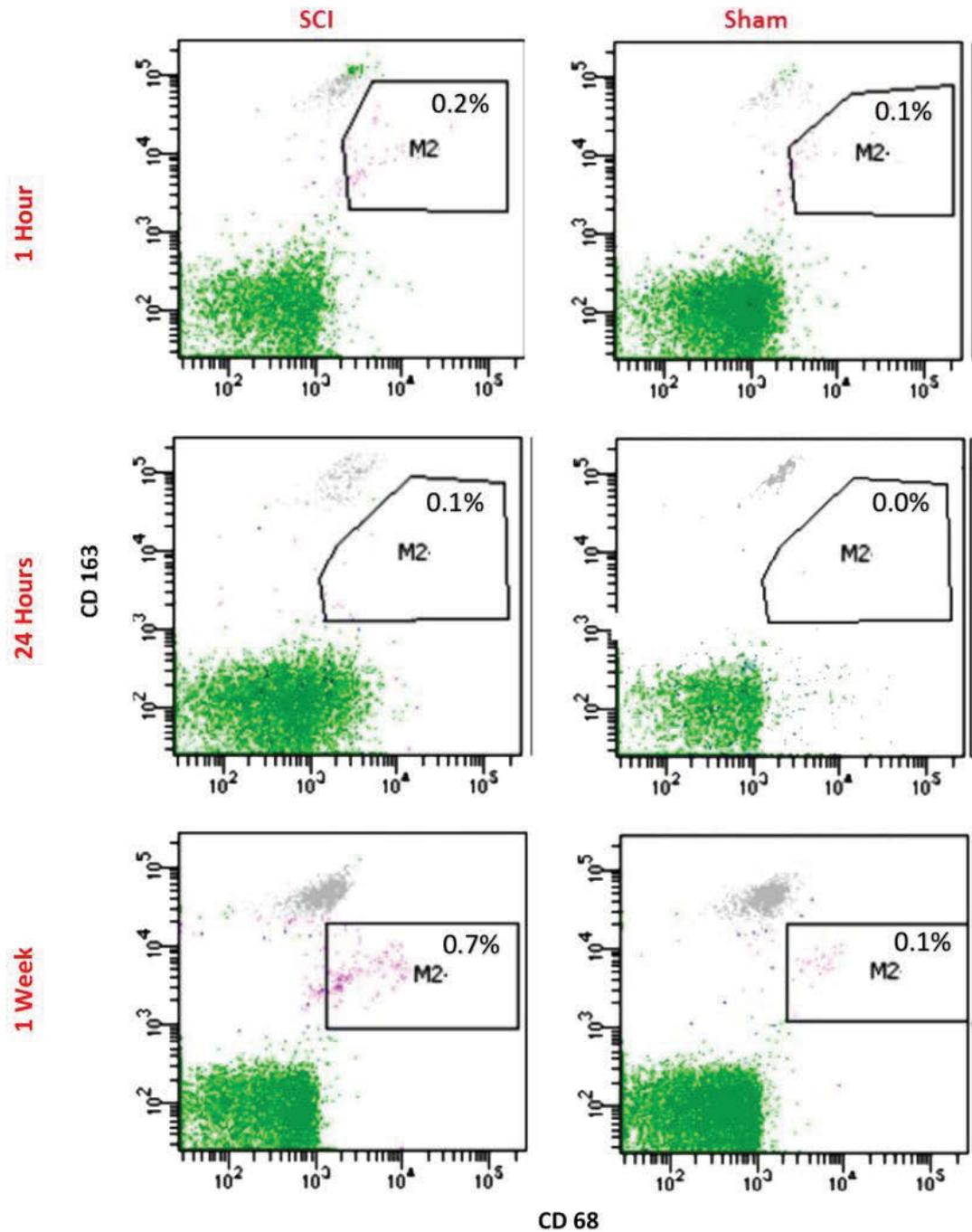


Figure 69: Representative flow cytometry scatter-plots for the M2-like population (CD68⁺/CD163⁺) in Infant spinal cord tissue over the three time points for spinal cord injured (SCI) (left column) and sham rats (right column). Percentages represent percentages of the total viable cell population.

Eight additional rats were used to determine what the normal basal levels of these populations in the spinal cord and to check the auto-fluorescence that was observed in the sham and SCI groups. The normal infant spinal cord showed only very

low background levels of fluorescence for the populations of interest. The levels of fluorescence for the CD68⁺/CD86⁺ and CD68⁺/CD163⁺ cells in the normal spinal cord were not significantly different from the time-matched shams at any point. There was a small increase in the shams compared to the normal samples at 1h post injury in the CD45⁺ population using a T-Test (P=0.014).

At all three post-injury time points the percentage of CD68⁺/CD86⁺ leukocytes is greater than the percentage of CD68⁺/CD163⁺ leukocytes. Due to high variation between animals creating a high standard deviation there was no statistical significance (ANOVA P=0.236) between the CD68⁺/CD86⁺ and CD68⁺/CD163⁺ percentages even though the trend is clearly visible (Figure 70). Overall, the percentage of CD68⁺/CD163⁺ cells was higher than that of CD68⁺/CD86⁺ cells with a peak in both at 1wk post-injury.

Neutrophil (CD45⁺/HIS48⁺) numbers peaked at 24h post injury in the infants. The CD45⁺/HIS48⁺ cells as a percentage of viable cells at 24h (0.97%) showed a significant increase from 1h (0.19%) and 1wk (0.14%) post-injury and was also significantly higher than its sham using ANOVA and Bonferroni's post-hoc test (ANOVA P=0.007) (Figure 71). There was a significant effect for both injury status (P<0.005) and survival time (P=0.005) as well as a significant interaction between these factors (P<0.05) using two-way ANOVA.

T-lymphocytes (CD45⁺/CD3⁺) as a percentage of viable cells decreased over time in the SCI animals and also in the shams, with no significance between the different injury statuses. The CD45⁺/CD3⁺ cells as a percentage of viable cells is visibly much higher at 1h (0.35%) post injury but due to variability between animals this was only significant between 1h and 1wk (0.02%) SCI using ANOVA and Bonferroni's post-hoc test (ANOVA P=0.015) (Figure 72). These percentages were much lower than the

other inflammatory cells examined however, not reaching 1% of the viable cells present. There was a significant effect only for survival time ($P=0.05$) using two-way ANOVA.

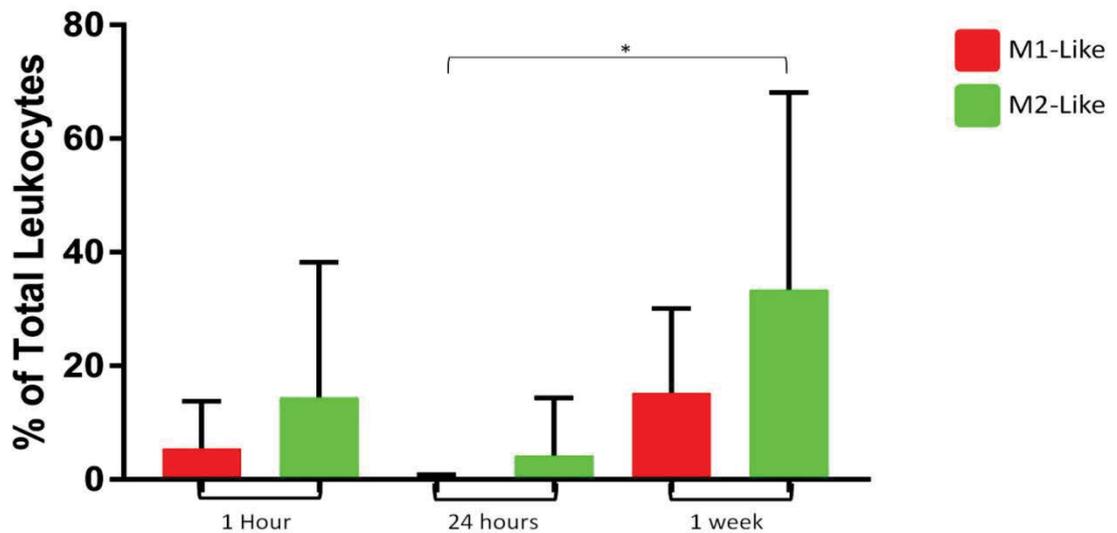


Figure 70: Histogram of the percentage of total leukocytes in infant rat spinal cords expressing CD68+/CD86+ (M1-like) and CD68+/CD163+ (M2-like) progressing from 1h, 24h and 1wk post-injury.

* ($P<0.05$) indicates the statistical significance based on Bonferroni's post-hoc test.

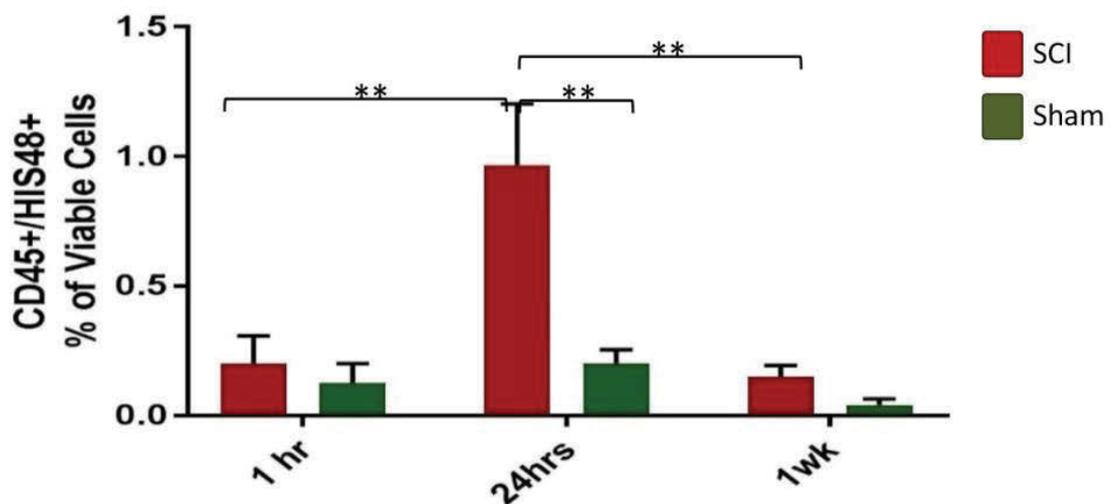


Figure 71: Histogram of the CD45+/HIS48+ neutrophils as a percentage of the total viable cells in both sham and injured (SCI) infant rat spinal cords 1h and 24h post injury. ** ($P<0.005$) indicates the statistical significance based on Bonferroni's post-hoc.

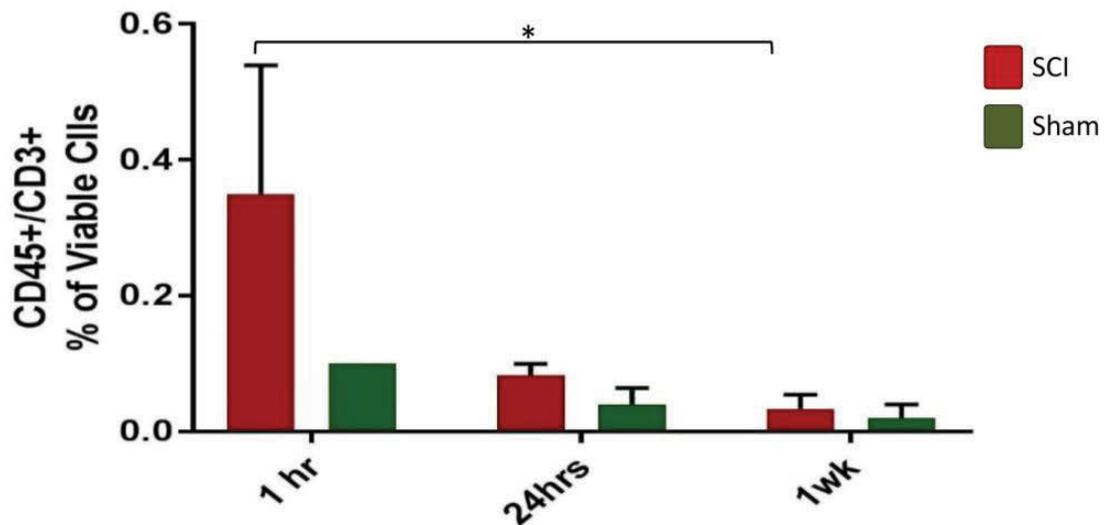


Figure 72: Histogram of the CD45+/CD3+ T-lymphocytes as a percentage of the total viable cells in both sham and Injured (SCI) infant rat spinal cords 1h and 24h post injury. * (P<0.05) indicates the statistical significance based on Bonferroni's post-hoc test.

4.4.1c Differences in cellular inflammation between adults and infants

There was significant difference in the cellular inflammatory response between adults and infants, especially in CD68⁺/CD86⁺ and CD68⁺/CD163⁺ cells. Expressing the increase in macrophages as a fold increase from the sham levels shows a significant difference in the phenotype of the macrophage response between adults and infants (Figure 73). At 1h post-injury the infants had a greater increase from sham levels in CD45⁺/CD68⁺ cells than the adults. This evened out at 24h and then the adults had a greater increase from sham levels at 1wk post-injury. The differences in M1 (CD68⁺/CD86⁺) and M2-like (CD68⁺/CD163⁺) responses are more easily seen in Figure 73B & C, with the adults showing greater increases in CD68⁺/CD86⁺ cells at 24h and 1wk post injury. The infants were opposite to this, showing greater increases in CD68⁺/CD163⁺ cells than CD68⁺/CD86⁺ cells at 1h and 1wk post injury, with a greater increase in CD68⁺/CD86⁺ cells visible at 24h.

The CD68⁺/CD86⁺ and CD68⁺/CD163⁺ percentages of the total CD45⁺ cells showed no statistical significance between CD68⁺/CD86⁺ and CD68⁺/CD163⁺ cells at any time point in either the adults or infants using ANOVA or T-Tests. There are, however, visible differences and trends in the data. The adults' percentage of CD68⁺/CD86⁺ cells increased from 1h through to 1wk as the total leukocyte numbers increased. The CD68⁺/CD163⁺ percentages also increased from 1h to 1wk but were consistently lower than the CD68⁺/CD163⁺ percentages at each time point. The infants showed a higher percentage of both CD68⁺/CD86⁺ and CD68⁺/CD163⁺ cells at 1h compared to 24h post-injury and a peak in both CD68⁺/CD86⁺ and CD68⁺/CD163⁺ cell percentages at 1wk post-injury. The key difference between the infants and adults is that at this 1wk peak there was a higher percentage of CD68⁺/CD163⁺ cells than there was CD68⁺/CD86⁺. The high levels of variability, especially in the infant groups, lead to low statistical significance found in using ANOVA and Bonferroni's (Figure 73 & 74).

Both age groups showed a peak in CD45⁺/CD68⁺ cells at 1wk post-injury, however the infants were on a much smaller scale, as can be seen in Figure 74A. 1h post injury the infants showed greater CD45⁺/CD68⁺ percentage of the viable cells, compared to their adult counterparts who had negligible macrophage presence, with a greater number of these being CD68⁺/CD163⁺ cells (Figure 74C). At 24h after the injury the percentage of CD45⁺/CD68⁺ cells were the same in both age groups, however the phenotype distribution was slightly different (Figure 74A). The adults showed almost equal percentages of CD68⁺/CD86⁺ and CD68⁺/CD163⁺ cells, while the infants were beginning to show greater percentages of CD68⁺/CD163⁺ compared to CD68⁺/CD86⁺ cells (Figure 74B & 74C). At the 1wk peak in macrophage response the adults showed the highest percentage of viable cells expressing CD45⁺/CD68⁺, with higher percentage of CD68⁺/CD86⁺ cells compared to CD68⁺/CD163⁺ cells (Figure 74). The infants also

showed their highest CD45⁺/CD68⁺ percentage of viable cells; however, this was approximately 8 times lower than the adults (Figure 74A). The infants also displayed higher percentages of CD68⁺/CD163⁺ cells than CD68⁺/CD86⁺ (Figure 74B & 74C).

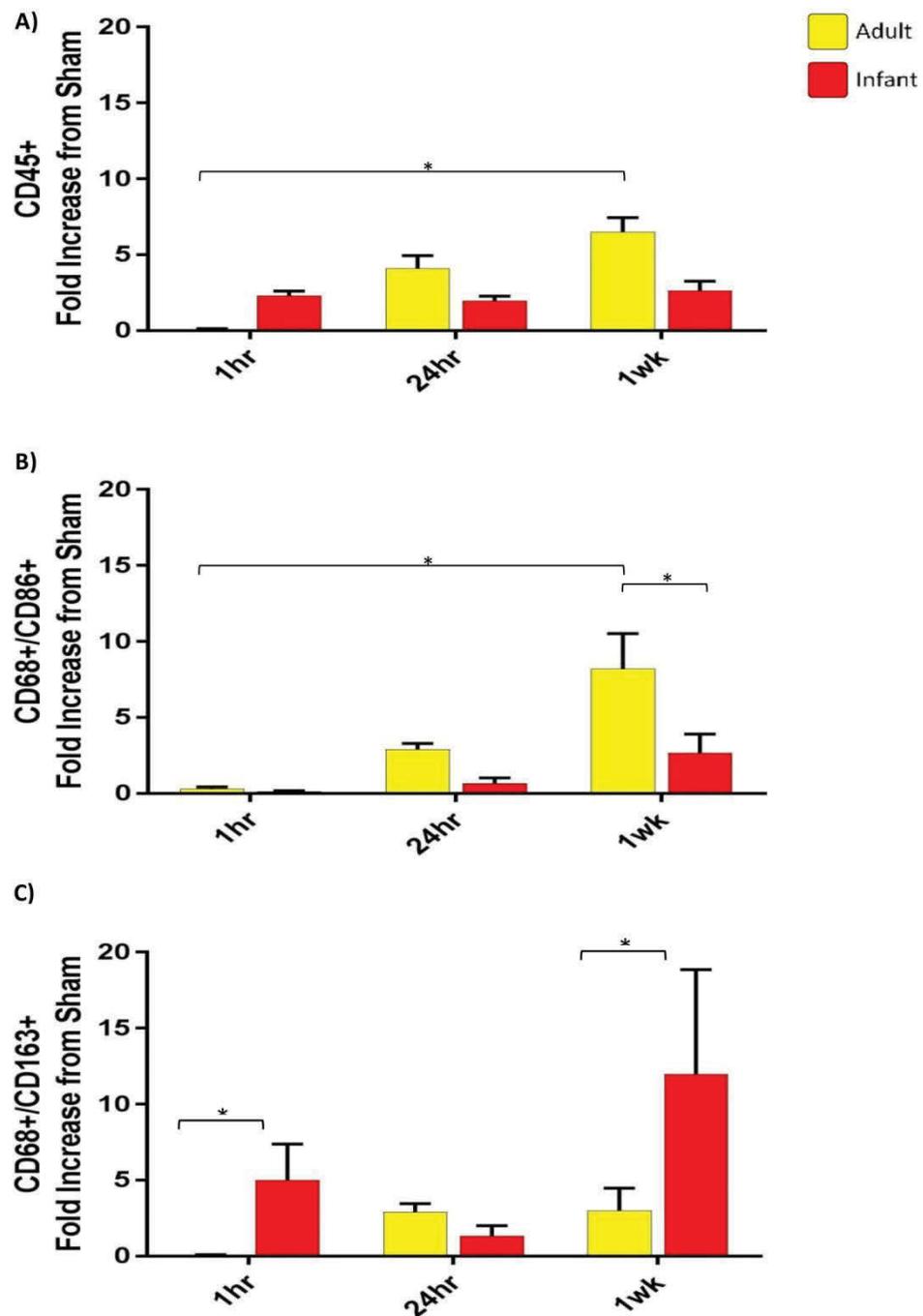


Figure 73: Histograms representing the fold increase from sham levels A) CD45⁺ leukocytes, B) CD68⁺/CD86⁺ M1-like cells and C) CD68⁺/CD163⁺ M2-like cells as a percentage of the total viable cells in the Adult and Infant SCI groups. *(P<0.05) indicates the statistical significance between groups.

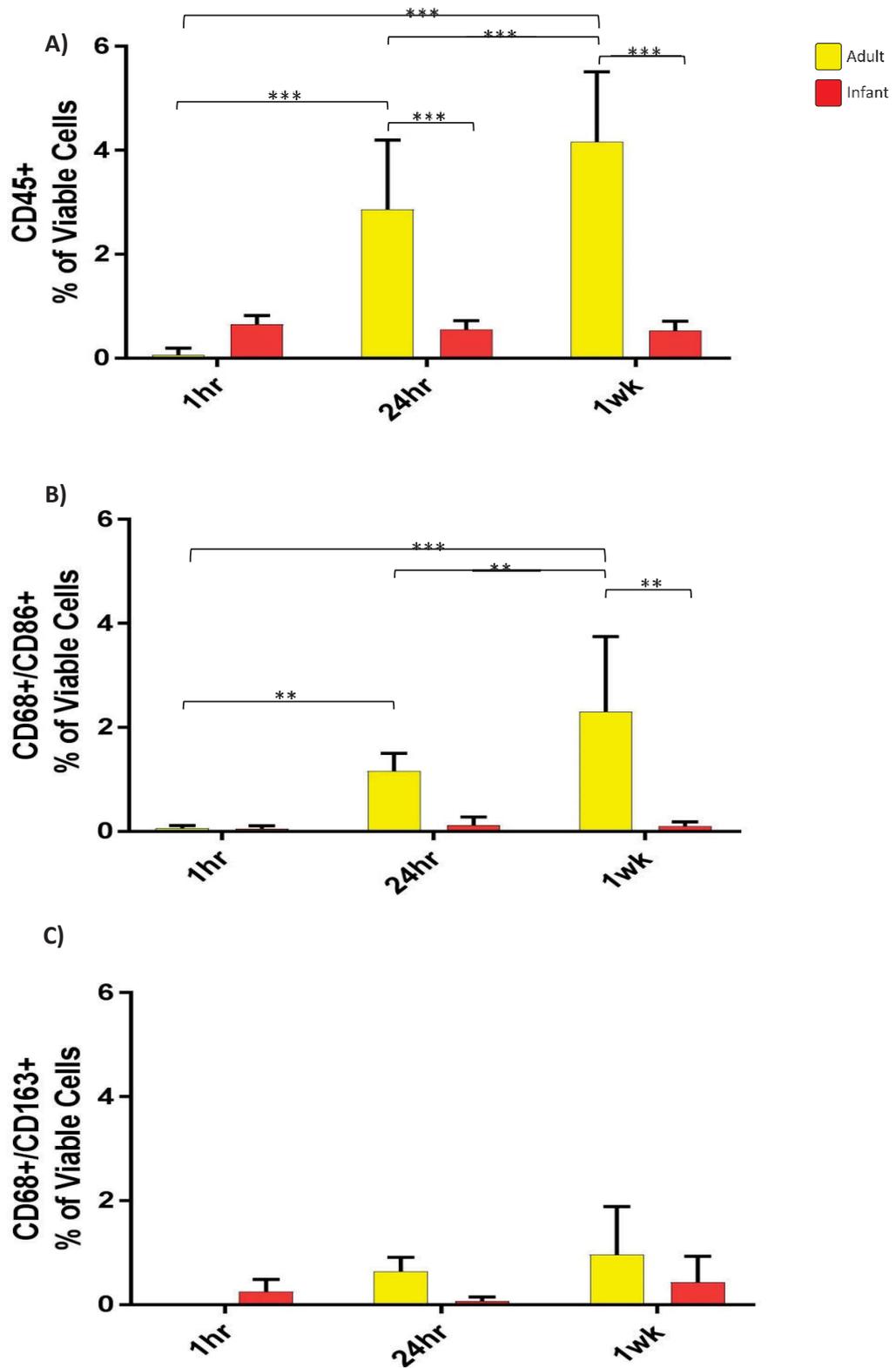


Figure 74: Histograms of the A) CD45+ leukocytes, B) CD68+/CD86+ M1-like cells and C) CD68+/CD163+ M2-like cells as a percentage of the total viable cells in both adult and infant SCI animals 1h, 24h and 1wk post injury. ** (P<0.005) and *** (P<0.001) indicate the statistical significance based on Bonferroni's post-hoc test.

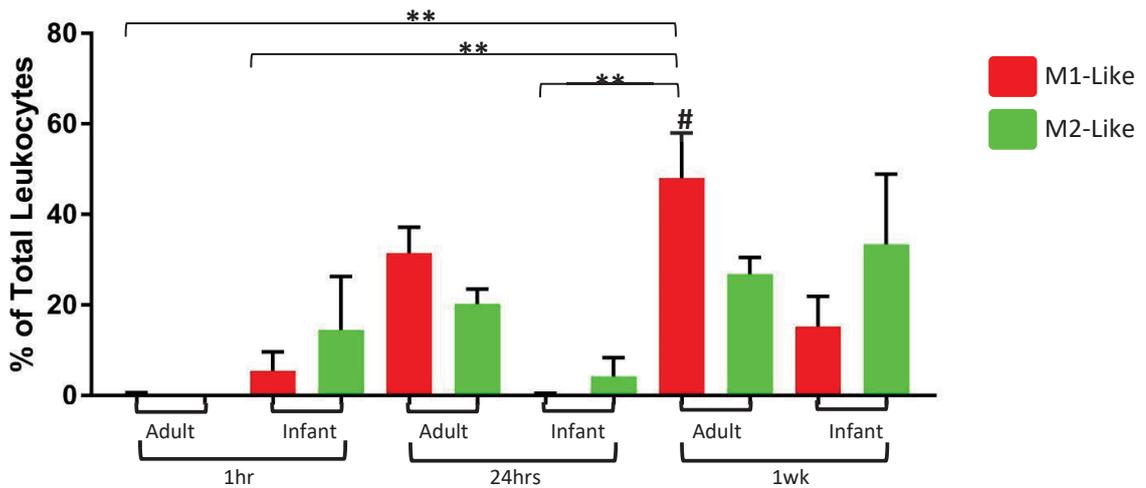


Figure 75: Histogram comparing the CD68+/CD86+ (M1) and CD68+/CD163+ (M2) cells as percentages of the CD45+ (leukocyte) population in adults and infants at 1h, 24h and 1wk post-injury. # indicates a significant increase from sham levels. ** (P<0.005) indicates the statistical significance based on Bonferroni's post-hoc test.

The CD45⁺/HIS48⁺ (neutrophil) cell response was consistent between the adults and infants; however the magnitude was decreased in the infants. This was only statistically significant at the 24h peak using ANOVA and Bonferroni's post-hoc test (P<0.0001). The CD45⁺/CD3⁺ (T-lymphocyte) response in the adults increases from 1h to 24h post-injury, it appears that the response is increasing over time however without the 1wk dataset it is difficult to comment. The infants, conversely, had the highest percentage at 1h post-injury and decreased over time until 1wk.

4.4.2 Cytokine expression in the rat spinal cord post-spinal cord injury

4.4.2a Cytokine signalling in the adult spinal cord

The cytokine response to SCI in adult rats was largely pro-inflammatory and persisted as such. There was significant variability between individual animals that resulted in large standard deviation and less statistical significance in the data. The highest concentration was found in IL-1 β at 24h post injury with an average

concentration of 137pg/ml across five animals. This represented a peak in IL-1 β , significantly higher than its corresponding sham and the SCI concentration at 1h and 1wk post injury (ANOVA $P < 0.001$) (Figure 76A). This 24h peak, significantly increased from its sham and visibility increased from the other SCI time points, was mirrored in the IL-6 concentration though much lower with an average of 45pg/ml across 5 rats (ANOVA $P < 0.001$) (Figure 76B). IL-1 α showed steady concentrations at both 1h and 24h post injury (15pg/ml) before dropping down sharply at 1wk (7pg/ml) (ANOVA $P < 0.001$) (Figure 76C). TNF- α peaked at 1h post injury in adult rats (7pg/ml) and dropped sharply by 24h and back to below detectable levels by 1wk. This was not statistically significant (ANOVA $P = 0.5$) (Figure 76D). IL-12 (p70) demonstrated steady, low concentration levels at 1h and 24h with an increase at 1wk post injury, though this was not significantly higher than the corresponding sham (ANOVA $P = 0.006$) (Figure 76E). Finally, IFN- γ had the lowest concentration levels; below detectable at 1h and then steadily increasing up to 2pg/ml at 1wk post injury (Figure 76F). This increase was not significant at all (ANOVA $P = 0.8$).

In the adult rats the classically anti-inflammatory cytokines were generally expressed at lower concentrations than their pro-inflammatory counterparts. The highest concentration of anti-inflammatory cytokine was in IL-10, which increased sharply at 24h with an average concentration of 67pg/ml and remained elevated at 1wk post injury (58pg/ml) (ANOVA $P = 0.009$) (Figure 77A). IL-5 showed fairly steady concentration, at sham levels, at 1h and 24h post injury, peaking at 1wk (18pg/ml) where it was higher than the sham and previous time points (ANOVA $P = 0.003$) (Figure 77B). The two cytokines generally linked to M2-like activation, IL-4 and -13, increased from 1h to 1wk post injury, however were present at very low concentrations (< 1 pg/ml). IL-4 was at sham levels at 1h post injury and then increased steadily to 1wk

where it peaked at 0.5 pg/ml, with no statistical significance (ANOVA P=0.2) (Figure 77C). IL-13 was present at even smaller concentrations, peaking at 0.3pg/ml 1wk post injury (Figure 77D). This peak was increased from its respective sham, though not statistically significant (ANOVA P=0.4).

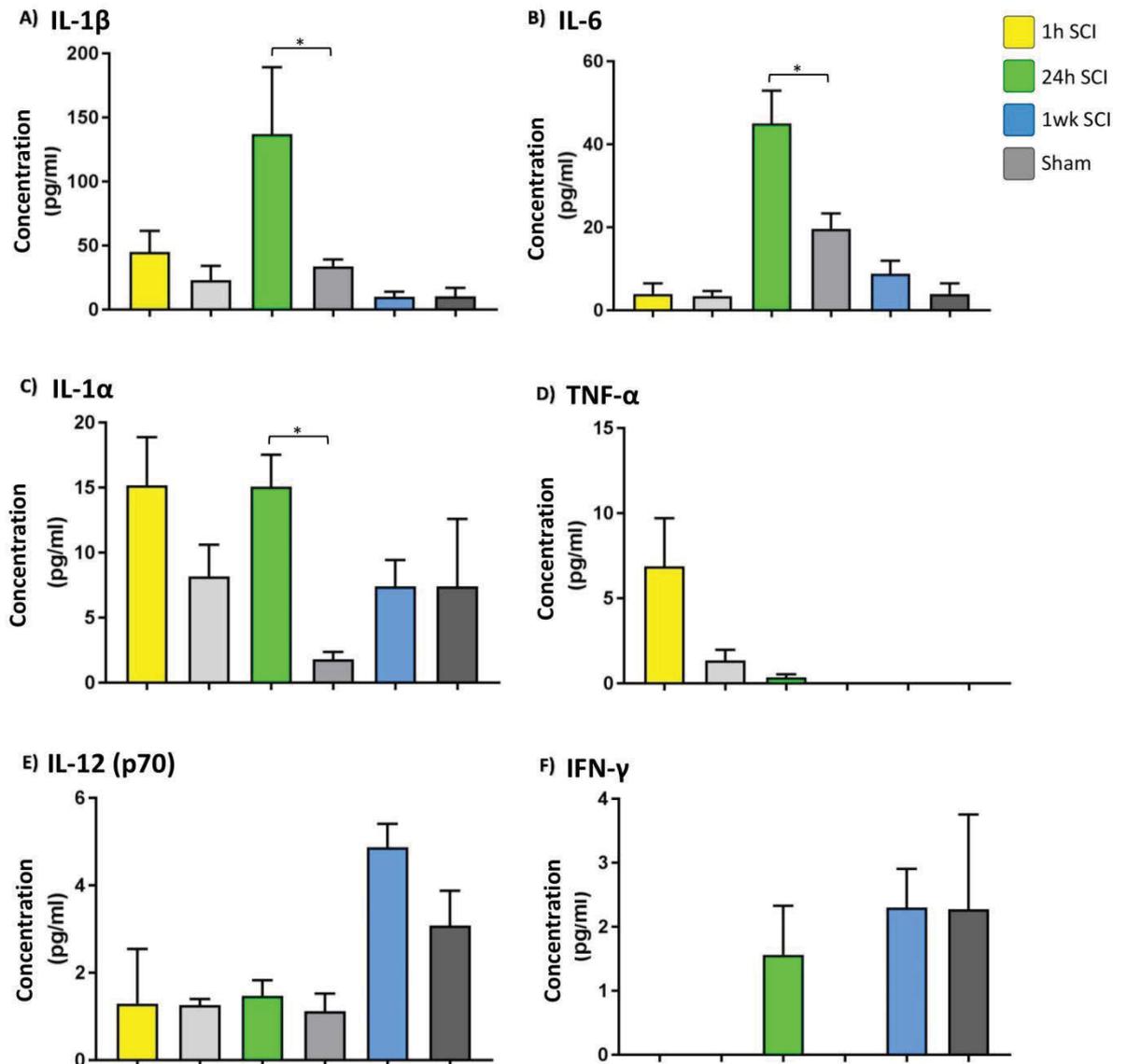


Figure 76: Histograms representing the observed concentration levels (pg/ml) of pro-inflammatory cytokines A) Interleukin-1 β (IL-1 β), B) IL-6, C) IL-1 α , D) Tumour necrosis factor- α (TNF- α), E) IL-12 (p70) and F) Interferon- γ (IFN- γ) in the adult SCI and sham groups from highest to lowest expression (A-F).

*($P < 0.05$) indicates the statistical significance between groups based on Bonferroni's post hoc test. The axes have not been normalised as there was a huge variation in concentration ranges between cytokines.

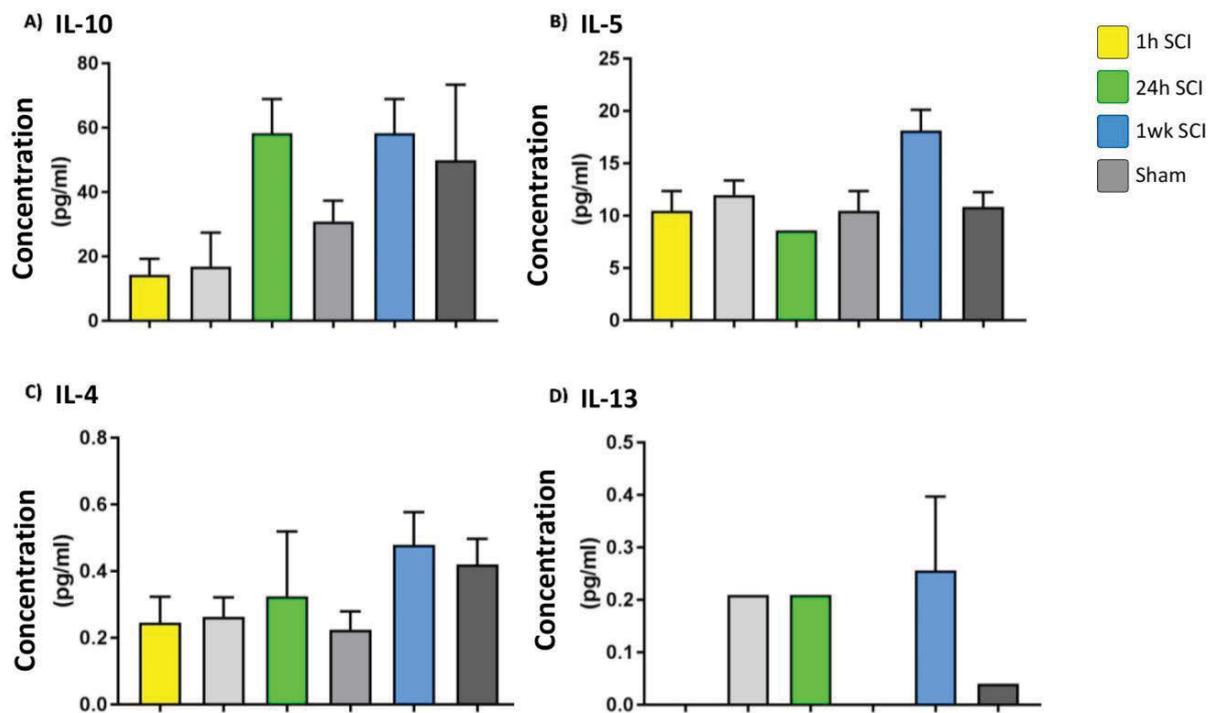


Figure 77: Histograms representing the observed concentration levels (pg/ml) of anti-inflammatory cytokines Interleukins (IL) A) IL-10, B) IL-5, C) IL-4, and D) IL-13 in the adult SCI and sham groups. The axes have not been normalised as there was a huge variation in concentration ranges between cytokines.

4.4.2b Cytokine signalling in the infant spinal cord

The cytokine expression after SCI in infant rats showed no significant increase from sham levels and a more balanced response. There was significant variability between individual animals that resulted in large standard deviation and no statistical significance. IL-1 β was highest in the injured spinal cord and peaked at 24h post injury (21pg/ml) (Figure 78A). The IL-6 concentration was relatively steady at 1h and 24h (9-11pg/ml) and decreasing at 1wk post injury (ANOVA $P < 0.001$) (Figure 78B). IFN- γ concentrations increased by small, steady amounts from 1h (2pg/ml) to 1wk (5pg/ml) post injury (ANOVA $P = 0.8$) (Figure 78C). IL-1 α concentration peaked at 1h after injury (8pg/ml) and dropped steadily to 1wk post-injury (3pg/ml), visibly higher than the respective shams (ANOVA $P < 0.001$) (Figure 78D). Conversely IL-12 (p70) was at its

lowest concentration at 1h, with the SCI levels significantly lower than the sham levels, and then increased up to 1wk post-injury, this was not statistically significant (ANOVA $P=0.006$) (Figure 78E). Finally, $TNF-\alpha$ increase steadily from negligible levels ($<1\text{pg/ml}$) at 1h post-injury to 3pg/ml at 1wk. This increase was not significant at all, nor were the increase from sham. (ANOVA $P=0.5$) (Figure 78F).

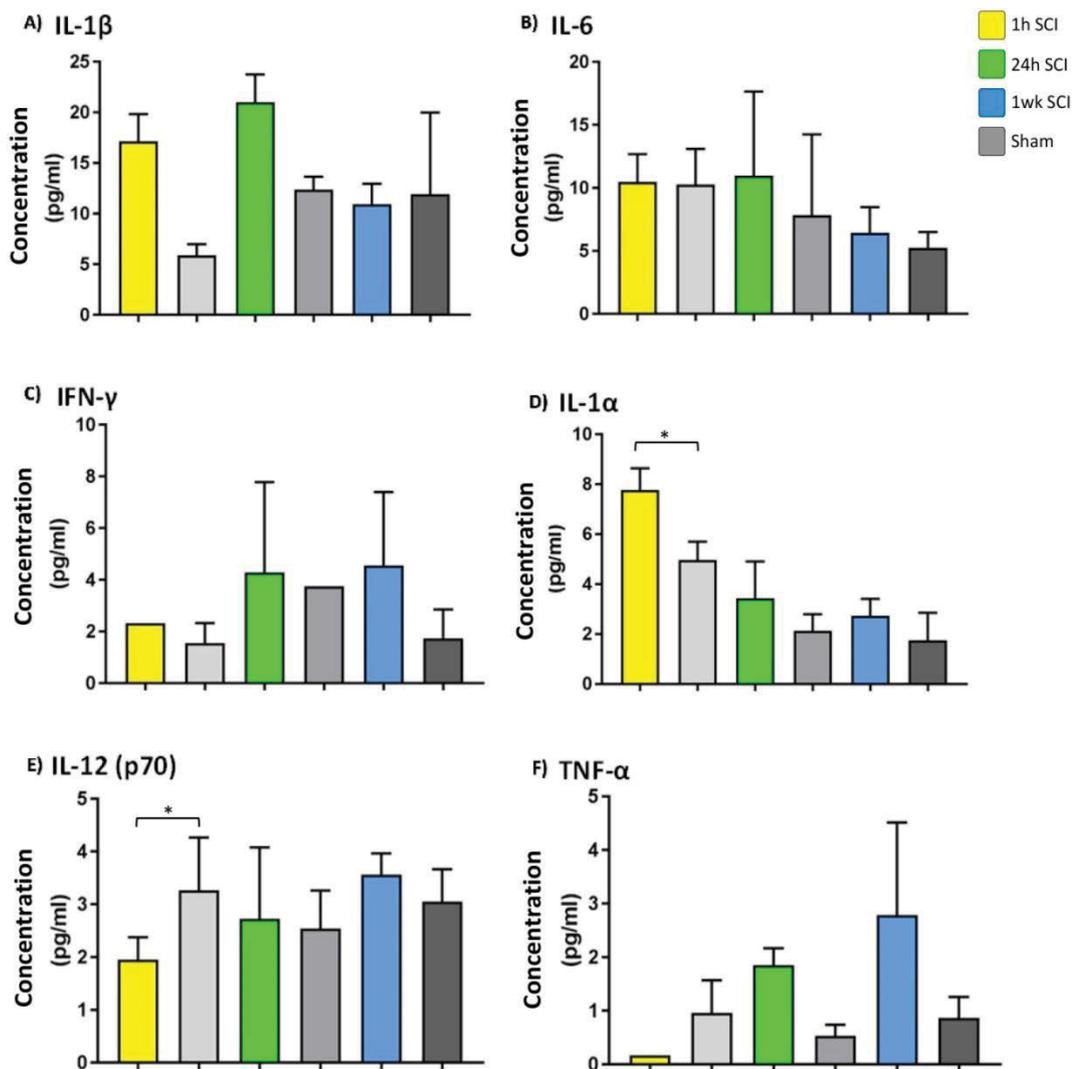


Figure 78: Histograms representing the observed concentration levels (pg/ml) of typically pro-inflammatory cytokines A) Interleukin-1 β (IL-1 β), B) IL-6, C) Interferon- γ (IFN- γ), D) IL-1 α , E) IL-12 (p70) and F) Tumour necrosis factor- α (TNF- α) in the infant SCI and sham groups from highest to lowest expression (A-F). The axes have not been normalised due to variation in concentration ranges between cytokines. * ($P < 0.05$) indicates statistical significance between groups based on Bonferroni's post hoc test.

The typically anti-inflammatory cytokines all peaked at 24h and were visibly lower than their respective shams at 1h post-injury in the infant rats, though this was not statistically significant. The highest concentration of anti-inflammatory cytokine was in IL-10, which increased at 24h with an average concentration of 47pg/ml and dropped again at 1wk post injury (ANOVA $P=0.009$) (Figure 79A). IL-5 also showed a peak 24h (26pg/ml) post injury and dropped again at 1wk (ANOVA $P=0.003$) (Figure 79B). IL-4 and -13, both peaked at 24h post injury, however were present at much lower concentrations (<2 pg/ml), however there was no statistical significance (ANOVA $P=0.2$, $P=0.4$) (Figure 79C & 79D).

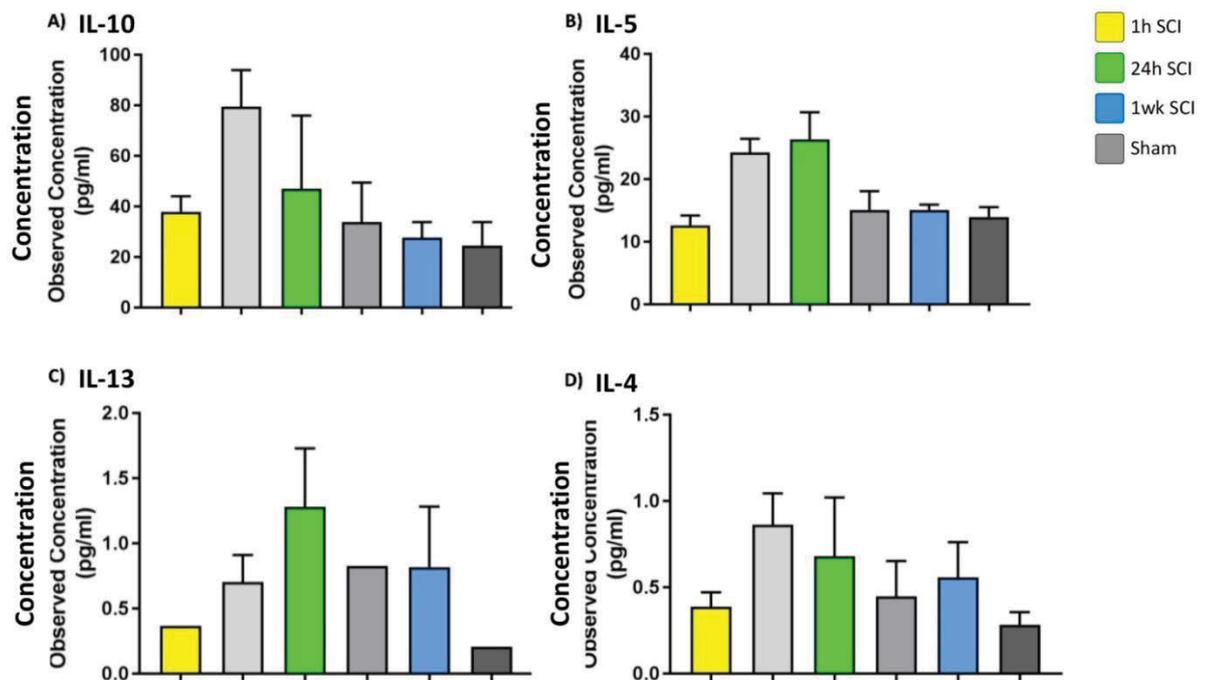


Figure 79: Histograms representing the observed concentration levels (pg/ml) of typically anti-inflammatory cytokines Interleukins (IL) A) IL-10, B) IL-5, C) IL-13, and D) IL-4 in the infant SCI and sham groups. The axes have not been normalised as there was a huge variation in concentration ranges between cytokines.

4.4.2c Differences in the inflammatory environment between adult and infant rats

Overall the adults and infant cytokine profiles differed in nature. They exhibit a different balance between the classically pro- and anti-inflammatory cytokines. The trends and differences are more readily visible by expressing the observed concentration in the SCI cohort as a fold increase from the corresponding sham level. In general, the adults had greater increases in pro-inflammatory cytokines than their infant counterparts and higher peaks in expression. The magnitude of anti-inflammatory cytokine expression was similar in the adults and infants; however, the infants showed a response more balanced between Th1 and Th2 cytokines and a steady increase in M2-associated cytokines (IL-4 and IL-13) over time.

Pro-inflammatory Cytokine Expression is more Pronounced in Adult Rats

The expression of 6 of the 7 cytokines classically considered to be pro-inflammatory were generally higher in the adults than the infants. IL-2 levels were too low to measure using this assay. The highest fold increases were seen in the adults IL-1 α and TNF- α . The expression of IL-1 α peaked in the adults at 24h post-injury and exhibited a significantly higher fold increase than the corresponding infants (Figure 80A). TNF- α level showed the greatest fold increase at 1h post-injury in the adults, while the infants showed a lower peak at 24h that remained increased at 1wk (Figure 80B). IL-1 β peaked at 24h in the adults and decreased sharply by 1wk; the infants showed a smaller peak at 1h and then steadily decreased up to 1wk post-injury (Figure 80C). The fold increase in IL-6 in the adults increased sharply at 24h and was higher than that in the infants at both 24h and 1wk post-injury. The IL-6 levels were more consistent across the 3 time points in the infants (Figure 80D). IL-12 showed a fairly consistent fold change in the adult SCI, and in the infants after a larger increase

between 1h and 24h (Figure 80E). There were a lot of values below the detectable range for IFN- γ so it is difficult to visualise the trends in expression (Figure 80E). From the observed concentration data both the adults and infants appear to have the highest expression at 1wk post-injury, with the infants being more consistent in the recorded concentrations over the 3 time points.

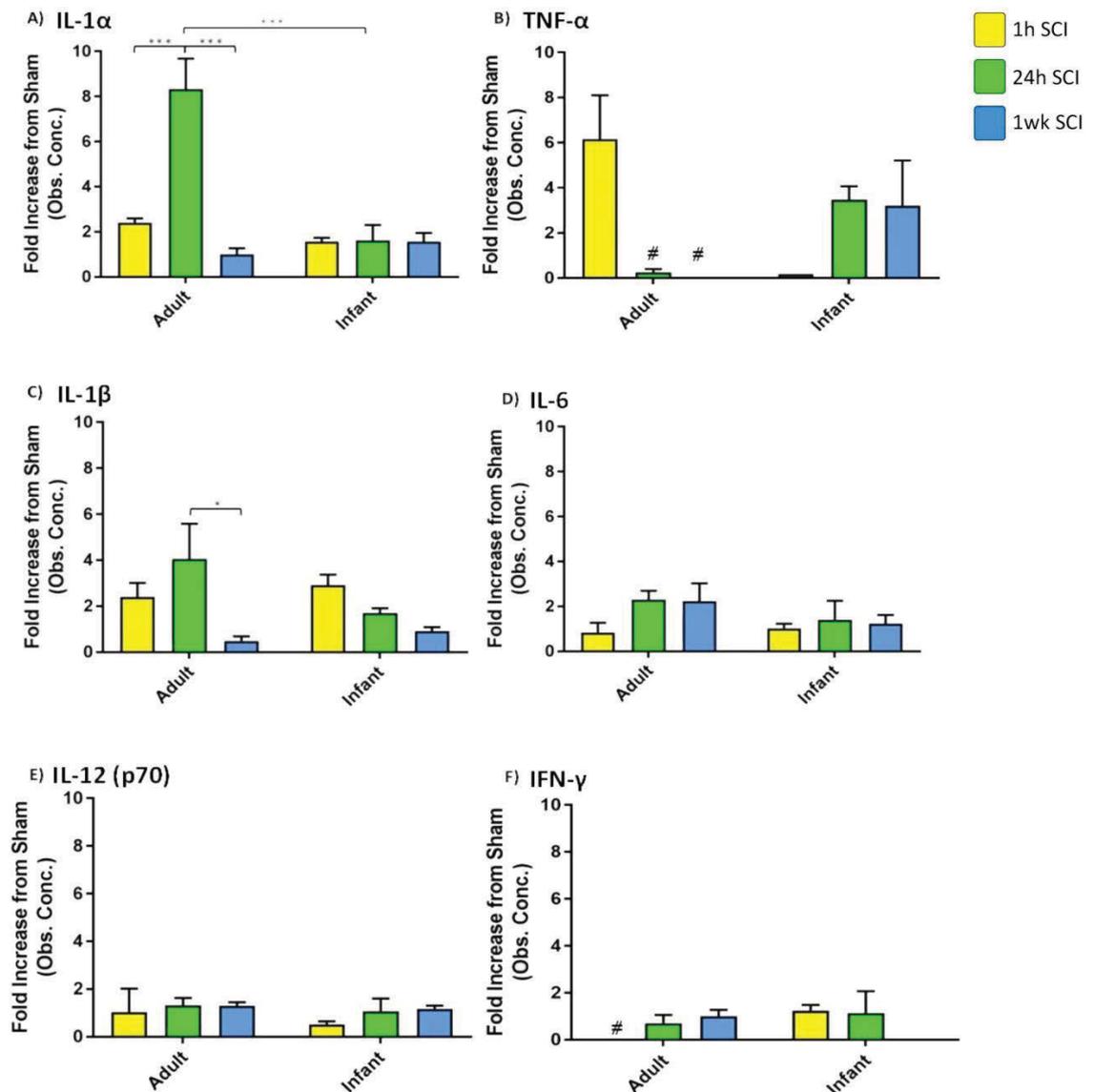


Figure 80: Histograms representing the fold increase from sham levels of classically pro-inflammatory cytokines A) Interleukin-1 α (IL-1 α), B) Tumour necrosis factor- α (TNF- α), C) IL-1 β , D) IL-6, E) IL-12 (p70) and F) Interferon- γ (IFN- γ) in the Adult and Infant SCI groups. * (P<0.05) and * (P<0.0001) indicate the statistical significance between groups based on Bonferroni's post hoc test. Where levels were below detectable levels for the assay the lowest value was used (indicated by #).**

Table 9: Summary table of the timing and magnitude of the pro-inflammatory cytokine peaks in adult and infant rats.

| Cytokine | Adult | | Infant | |
|----------------|--------|-------------------------|--------|-------------------------|
| | Peak | Fold Increase from Sham | Peak | Fold Increase from Sham |
| IL- 1 α | 24h | 8 | Steady | 2 |
| IL- 1 β | 24h | 4 | 1h | 4 |
| IL- 6 | 1wk | 3 | 24h | <2 |
| TNF- α | 1h | 6 | 24h | 4 |
| IL- 12 | Steady | <2 | 1wk | <2 |
| IFN- γ | Steady | <2 | 1h | <2 |

Anti-inflammatory Cytokine Expression is more Balanced in Infant Rats

Of the 5 typically anti-inflammatory cytokines measured in this assay the fold increases were only able to be calculated for 4; the levels of GM-CSF were mostly below the detectable range. Generally the fold increases from sham in these cytokines was lower than in the pro-inflammatory subset. The adults exhibited a steady expression of IL-4 across the 3 time points while the infants showed a steady and continuing increase from 1h to 1wk post-injury (Figure 81A). The most significance was, interestingly, in the fold increases of IL-5. The adults showed a significant peak at 1wk post-injury and a greater increase than their corresponding infants; where the infants peaked at 24h and where significantly higher than the adults at this time point (Figure 81B). IL-10 expression peaked at 24h in both ages but then decreased sharply in the adults at 1wk while remaining fairly steady in the infants (Figure 81C). The fold increase in IL-13 concentrations in adults was highest at 24h and this level maintained up to 1wk. The infants showed higher concentrations and also, in the fold increases, demonstrate a steady increase from 1h to 1wk (Figure 81D).

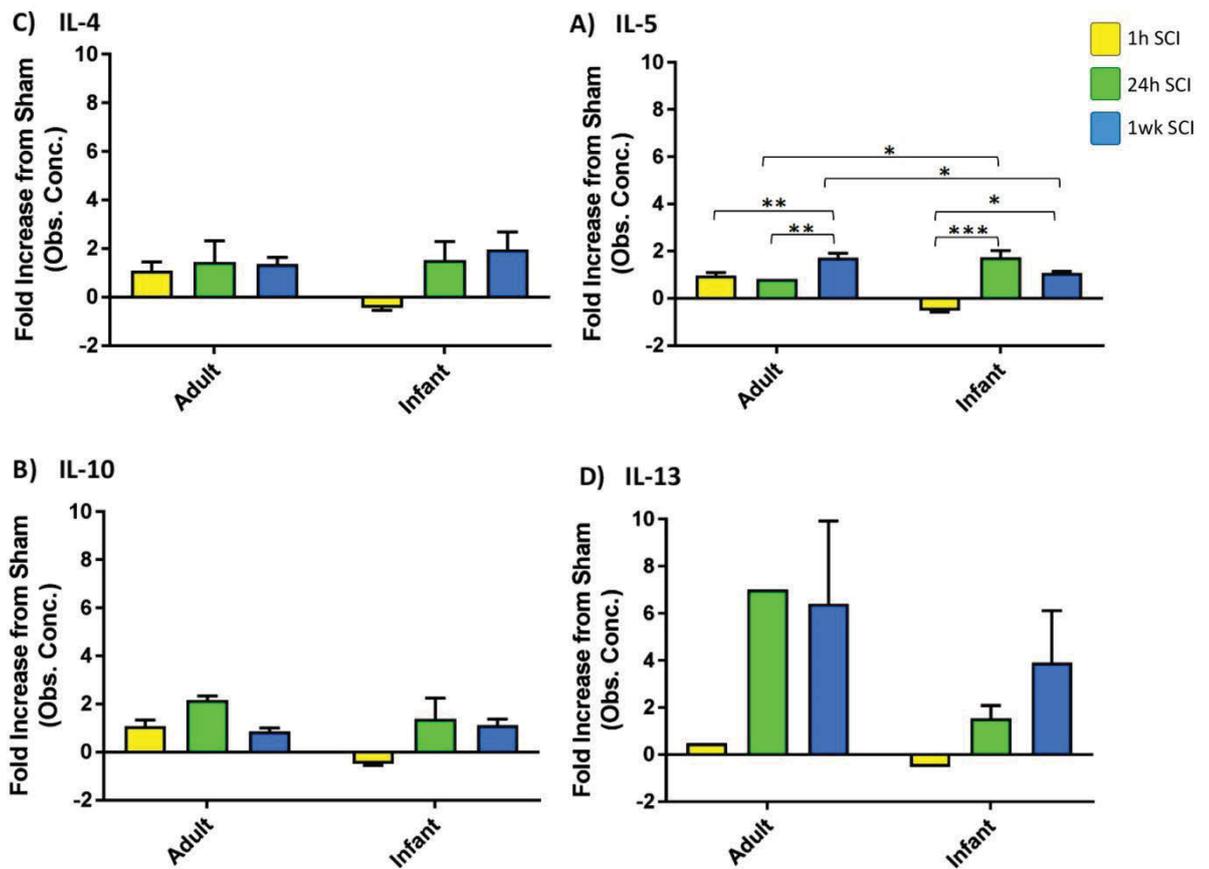


Figure 81: Histograms representing the fold increase from sham levels of classically anti-inflammatory cytokines, Interleukins (IL) A) IL-4, B) IL-5, C) IL-10, and D) IL-13 in the Adult and Infant SCI groups at 1h, 24h and 1wk post spinal cord injury. * (P<0.05), ** (P<0.005) and * (P<0.0001) indicate the statistical significance between groups based on Bonferroni's post hoc test. Where the concentrations of either SCI or shams were < the detectable range of the assay the lowest value was used (#).**

Table 10: Summary table of the timing and magnitude of the anti-inflammatory cytokine peaks in adult and infant rats

| Cytokine | Adult | | Infant | | |
|----------|--------|-------------------------|--------|-------------------------|-----------------------------------|
| | Peak | Fold Increase from Sham | Peak | Fold Increase from Sham | |
| IL-4 | Steady | <2 | 1wk | 3 | Alternative macrophage activation |
| IL-13 | 24h | 7 | 1wk | 5 | |
| IL-10 | 24h | 2 | 24h | 2 | Pro-inflammatory suppression |
| IL-5 | 1wk | 2 | 24h | 2 | |

4.5 DISCUSSION

4.5.1 Differences in the cellular and molecular inflammatory response to spinal cord injury between infant and adult rats

The cellular response to SCI has been extensively examined in Chapter 3 of this thesis, this chapter took a closer look at the macrophage/microglial phenotypes, specifically, and the nature of the inflammatory response. Macrophages and microglia play a vitally important role in the SCI secondary injury cascade that can be both beneficial and detrimental (David and Kroner, 2011; Gensel and Zhang, 2015; Hohlfeld et al., 2007; Schwartz, 2003). For a long time these have been considered in the simplified M1/M2 dichotomy to somewhat match the Th1/Th2 inflammatory responses; however, this concept is currently undergoing considerable review (Mantovani et al., 2013; Martinez and Gordon, 2014) as it has been acknowledged that activation states of macrophages fall on a spectrum between 'M1' and 'M2' (Gensel and Zhang, 2015) each characterised by a variety of receptors and secretions (Martinez et al., 2008). In the current study, the simplified "M1-like"/"M2-like" terminology will be used to report on the differences in the inflammatory environment between adults and infants. The other cellular candidate re-examined in this chapter is the neutrophils, first responders of the immune system and significant players in the early stages of SCI (Chapter 3, Section 3.42c, 3.5.1b)(Schwartz and Yoles, 2006). Together these cells help create the inflammatory environment of the spinal cord after injury and have a great effect on the injury progression. This environment may be affected, to a lesser degree by the T-lymphocytes of the adaptive immune system (Hohlfeld et al., 2007; Kokaia et al., 2012; Martino et al., 2011), though this is a relatively new contention and has not

been extensively studied. T-lymphocyte numbers were assessed here to see if there was any difference between adults and infants.

Cytokines are important immune/inflammatory effectors after trauma and play an important role in determining the nature and perpetuation of the response. These effectors are released from and responded to by a wide range of cells involved in the secondary injury phase of SCI, from infiltrating neutrophils and macrophages to endogenous microglia (David and Kroner, 2011; Pineau and Lacroix, 2007), and astrocytes (Watkins et al., 2001). Cytokines, much like macrophages, are often grouped into Th1 'Pro-inflammatory' and Th2 'anti-inflammatory' responses. Both Th1 and Th2 cytokines are necessary in a normal inflammatory response, however, the response to SCI is dysregulated and the excess of pro-inflammatory responders becomes detrimental. The most prominently studied examples of the Th1 cytokines include TNF α , IL-6, IL-1 α /1 β and IFN- γ (Bastien and Lacroix, 2014), all of which are not necessarily directly neurotoxic or damaging but may be indirectly toxic through their interactions with cells and build up after SCI (Popovich and Jones, 2003; Toulmond et al., 1996). Commonly studied examples of Th2 cytokines associated with wound healing, injury resolution and 'alternatively activated' macrophages include TGF- β , IL-10, IL-4, and IL-13 (Bastien and Lacroix, 2014). These are involved in the modulation of the inflammatory response after the initial pro-inflammatory cascade in normal wound healing, however they are also dysregulated after SCI.

4.5.1a The acute neutrophil infiltration and the nature of the macrophage and microglial response differed significantly between adults and infants

The first responders expected to peak at 24h in the adults were the neutrophils (Chapter 3, Section 3.4.2c). This was borne out by the flow cytometry results that

showed no CD45⁺/HIS48⁺ cells at 1h after injury and a sharp increase in CD45⁺/HIS48⁺ cells as a percentage of the total viable cell population at 24h. This high peak in neutrophils in the acute stages of the injury is, in part, responsible for the later inflammatory response as these cells secrete effectors to attract macrophages and activate microglia (McTigue et al., 1998; Oyinbo, 2011; Sadik et al., 2011). In general, it is thought that this initial response is highly pro-inflammatory and so promotes the recruitment of more pro-inflammatory effectors (Carlson et al., 1998; Chan, 2008; Hausmann, 2003; Hohlfeld et al., 2007; Lucas et al., 2006). The CD45⁺ macrophage/microglial population that followed this neutrophil infiltration at 24h and 1wk was predominately 'M1-like', with only a small subset of 'M2-like' cells present. This macrophage infiltration and microglial activation peaked at 1wk, as expected (Chapter 3, Section 3.4.2b).

The neutrophil response in the infant was negligible at 1h and 1wk, exhibiting a sharp peak at 24h that tracked with what we have previously observed (Chapter 3, Section 3.4.2c). Once again, this peak was of a fairly low magnitude, compared to the adults. This may be a result of the lower numbers of circulating neutrophils and the lower numbers of GM-CFU per gram of body weight at this stage of development (Basha et al., 2014; Carr, 2000; Kumar and Bhat, 2016; Levy et al., 1999). The lower magnitude neutrophil response may have beneficial results downstream and be less damaging to the tissue than the response seen in the adults (Chapter 3, Section 3.5.1b). The infants exhibited a macrophage/microglial response that was similar in timing, though it began as early as 1h after injury, to that seen in the adults but was of a much lower magnitude. This response was more prominently 'M2-like' than 'M1-like' at 1h, 24h and 1wk post-injury in infant rats.

The key differences between the adults and the infants in terms of the cellular inflammatory response lies in the decreased magnitude and shifted phenotypes observed in the infants. This decreased magnitude is a continuation of that which was observed using histology and immunohistochemistry in Chapter 3. After SCI, inflammation has been shown to have both neuroprotective and neurotoxic effects (Das et al., 2012; Ekdahl et al., 2003; Hohlfeld et al., 2007; Kigerl et al., 2009; Lucas et al., 2006; Popovich and Jones, 2003; Schwartz et al., 1999). This duality is the root of the complexity underlying the inflammatory response (Hohlfeld et al., 2007; Popovich and Jones, 2003) and may also be a key in the recovery differences between infants and adults. A simplified view of this is that the inflammatory response is necessary and has vital functions to perform, however when it continues to build and persist in a highly pro-inflammatory nature it becomes detrimental to injury resolution and potentially harmful to the surviving tissue.

The immune response within the CNS has been shown to be very different than that seen in other somatic tissue trauma (Gensel and Zhang, 2015). The response to trauma outside the CNS exhibits a progression from an inflammatory phase characterised by M1 macrophages through to a remodelling phase characterised by subsets of M2 macrophages (Adamson, 2009; Gensel and Zhang, 2015; Koh and DiPietro, 2011; Velnar et al., 2009). In a typical adult SCI, the response is overwhelmingly and persistently pro-inflammatory, characterised by high numbers of infiltrating neutrophils early in the progression and then M1-like macrophages and pro-inflammatory activated microglia. The infant response to SCI more closely resembles the normal wound healing paradigm than the adult response, with a larger proportion of M2-like macrophages present. This more balanced response could have important implications in the differences in the histological presentation of the injury

in infants seen in Chapter 3 and previously published (Sutherland et al., 2017). With a more balanced inflammatory response the lesion microenvironment becomes less damaging to the surrounding cells, and more permissible to healing of the surviving tissues and regeneration of the neural tissue/tracts.

The decreased magnitude of the inflammatory response may have been an unintended consequence of the size differences. This is possibly due to size differences; in the infant sample, the vertebral span of the spinal cord section is much larger than the adult. This begs the question as to whether using the same physical distance or vertebral span provides a better comparison. For this experiment it was decided to use the same physical distance as the histology indicated that the lesion spanned the same length of the spinal cord in each age group at the beginning of the post-injury progression (Chapter 3.4.2, Figure 33). Using the same physical length of sample ensured that the edges of the lesion, and any associated immune cells, were not missed.

4.5.1b The response of T-lymphocytes to spinal cord injury

CD3⁺ T-lymphocytes were present in small numbers in the shams and at 1h post-injury, increasing significantly at 24h in adult rats. These lymphocytes in the shams and early injury could be the small population of T-lymphocytes specific to CNS self-antigen that can be found in small numbers in the CNS of healthy individuals (Martino et al., 2011; Moalem et al., 1999a). The increase at 24h may be indicative of T-lymphocytes entering through the damaged BBB or a small adaptive immune response being mounted, greater exploration into the nature of these lymphocytes would be needed to be sure.

The CD3⁺ Lymphocytes were present in the infants in very low numbers in the shams at all time points and peaked significantly at 1h after injury. This low number of cells in the shams may be a small population of T-lymphocytes specific to CNS self-antigen, as seen in the adults (Martino et al., 2011; Moalem et al., 1999a). Infants are not “immunodeficient” in their adaptive immune response, as was long thought, but instead show great variability and a Th2 bias in rodent studies (Adkins, 2000; Adkins et al., 2004; Basha et al., 2014; Levy, 2007; Zhang et al., 2017). In mice, the infant adaptive immune response is still developing after birth and can show reduced ability compared to adults in some situations, it can also mount an adult-like response in others (Adkins et al., 2004; Kumar and Bhat, 2016). In this SCI study the T-lymphocytes peaked at 1h after injury, although the cell percentage was still very low, but did not persist. This could be indicative of a rush of T-lymphocytes from the circulation, through the damaged BBB, that do not maintain a response in the tissue.

The role that the adaptive immune system plays in the post-injury milieu is still poorly understood and has received very little research attention compared to the innate immune response. As with the innate immune response, the adaptive immune response could be both beneficial and detrimental depending on a number of variables (Schwartz et al., 1999). Post-injury T-lymphocytes reactive to CNS self-antigens may have a neuroprotective role rather than a detrimental autoimmune function (Moalem et al., 1999a). As with the innate immune cells, the T-lymphocyte response in infants was of a decreased magnitude than that seen in adults, it also had a very early peak and a very transient response. The reasons for this, and the biological implications are still unclear and require further exploration.

4.5.1c The cytokine signalling in response to spinal cord injury appeared more balanced in infant rats compared to adults

The cytokine response to SCI in adult rats observed in this study was largely pro-inflammatory and persisted as such in a less balanced fashion than that observed in the infants. There were elevated levels of IL-1 α and TNF- α as early as 1h after injury and peaks of IL-1 α , IL-1 β and IL-6 at 24h. These did not persist out to 1wk after injury; however, IL-12 (p70) peaked 1wk post-injury. The typical Th2 cytokines were generally expressed at much lower levels than their Th1 counterparts in the adults. IL-10 was elevated at 24h, persisting to 1wk, while IL-4 and IL-13 were present in very low concentration and increased from 1h out to 1wk. There was no obvious pattern in the expression of the Th1 or Th2 cytokines and the concentration levels showed a large variation from a peak of 137pg/ml of IL-1 β to a peak of just 0.3pg/ml of IL-13; which is suggestive of a fairly unbalanced response. IL-10 can be produced by cells from both the myeloid and lymphoid lineages and has received a lot of attention as a mediator of the inflammatory response and a potent suppressant of the pro-inflammatory response (Popovich and Jones, 2003; Soleymaninejadian et al., 2012). IL-4 and IL-13 have been linked to the alternate activation of macrophages towards an injury resolution phenotype (Gordon, 2003). The emerging finding that these Th2 cytokines of increased in concentration over time may be indicative of an attempt to modulate the response away from the overwhelmingly pro-inflammatory; however, the concentrations of the cytokines are too low to effectively switch the response.

The cytokine expression in the infants appeared a little more balanced. The levels of all the Th1 cytokines was generally lower than that seen in the adults. IL-1 α peaked at 1h post-injury, IL-1 β was highest at 24h, IL-6 was steady from 1h to 24h, and IFN- γ , TNF- α and IL-12(p70) increased by small but steady amounts from 1h to 1week.

The Th2 cytokines were more significantly expressed in the infants with IL-10 increasing sharply at 24h post-injury peaking at 1wk, IL-5 peaked at 34 hrs, and IL-4 and IL-13 both peaked at 24hrs, though still at quite low concentrations. This generally lower Th1 response and stronger Th2 response suggest a more balanced inflammatory response in the infants that can only be more beneficial. The Th1 response is not overwhelming and is lowered faster than that observed in the adults and the Th2 response is earlier and of higher concentration possibly in an attempt to switch the cellular response. While the Th2 response may still not be sufficient to switch the environment towards an injury resolution phenotype it may be enough to balance the early pro-inflammatory response.

The cytokine responses observed in this study were quite highly variable not just between but also within groups and ages, exhibited by the high standard deviation seen on the histograms in section 4.4.2. This makes interpreting the data quite challenging. An overall observation was that the response in the infants appeared to be more balanced between the Th1 and Th2 cytokines than that seen in the adults. It is this generally lower Th1 response and stronger Th2 response in infant, that we have observed in the cellular response as well, that may be much more beneficial to injury resolution. In the infants it appears that the Th1 response is not overwhelming, as it may be in the adults, and is lowered faster, and the Th2 response appears earlier and of higher concentration in an attempt to switch the cascading response through the spectrum of macrophage phenotype, such as in other tissues. The differences seen in the cytokine response may be closely associated with those observed in the cellular response; however, we cannot discount the effect that the development of the immune system may have. Previous studies in humans have shown TLR-mediated cytokine production by blood mononuclear cells to be different between neonates,

toddlers and adults (Corbett et al., 2010; PrabhuDas et al., 2011). The infant blood monocytes exhibited decreased IFN- α , IFN- γ and IL-12 (P70); and a greater capacity to produce immune-modulating IL-10 and helper T-lymphocyte associated IL-17 (PrabhuDas et al., 2011). The cytokine response in infant rats after SCI bears greater scrutiny, alongside the cellular immune response, to determine the exact mechanisms involved and to pinpoint the key effectors for therapeutic manipulation.

4.5.2 Future directions and significance of this work

The results of this chapter, taken alongside those of Chapter 3, suggest great differences in the inflammatory response to SCI in infant rats. The infants' response is of a lower magnitude, appears to be more balanced and have a stronger Th2 response compared to the adult counterparts. There is still a lot that we do not know about the infants' response to SCI in this model. Chapter 3 raised questions about the causes of the vastly different injury presentation; is it due to the developmental state of the cord, to increased plasticity, to neuronal sparing and compensation, or simply the different mechanics of the cord? This chapter raises further questions as to the complex nature of the post-injury immune response and how we can separate the good from the bad.

The M1/M2 macrophage phenotype distinction used in this study is commonly utilised, however, the activation states of macrophages and microglia are more accurately represented as a spectrum with different cells falling along a continuum with M1 at one end and M2 at the other (David and Kroner, 2011; Gensel and Zhang, 2015; Kigerl et al., 2009; Mantovani et al., 2013). This is based on the expression of a wide variety of receptor and antigens, as well as a range of secretory products. Therefore, deeper analysis is needed to fully classify the macrophage and microglia cell

populations after a SCI. In Chapter 5 a pilot study will be undertaken to isolate macrophages from spinal cords from rats with and without spinal cord injury to further investigate M1-like and M2-like macrophages and the effect of their secreted soluble factors, including cytokines, in influencing macrophage phenotype.

4.6 CONCLUSIONS

Following on from Chapter 3, this chapter has further shown significant differences in the inflammatory response between adults and infants. We have previously shown that the infants' neutrophil and macrophage responses were of a lower magnitude, with less cells responding in the cord (Chapter 3). These findings are supported by the results in this chapter. The cellular response, however, differs not just in magnitude but also in the nature and phenotype of the responders. The numbers of infiltrating neutrophils is much lower in the infants than the adults. The basic macrophage/microglial populations examined using immunohistochemistry in Chapter 3 have been investigated further here using five markers to demonstrate a simplified M1-like/M2-like distinction. This simplified distinction gave great insight into the differences between the infants' and adults' responses. The adults were overwhelmingly M1-like and in far greater numbers than their infant counterparts. The infants on the other hand showed lower numbers of both phenotypes but also a profoundly increased M2-like cell response. This was mirrored to some degree in the cytokine expression in the spinal cord supernatant. The response showed high variation between animals and also between cytokine expression levels. In general, the adult rat spinal cords showed greater increases in expression of the Th1 (pro-inflammatory) cytokines after injury and lower levels of Th2 (anti-inflammatory) cytokine in the than the infant rat spinal cords. The infants' response appeared more balanced between the Th1 and Th2 cytokines, but with such high variability there was little statistical significance. Taken together with the cellular analysis it appears that there is a more balanced, and likely more beneficial, inflammatory response in the infants compared with their adult counterparts. The challenge will now be to create an inflammatory environment in the injured adult spinal cord that mimics that found in the infant condition, and determine whether this improves cellular, and ultimately functional, outcomes.

CHAPTER 5: MODULATION OF SPINAL CORD DERIVED MACROPHAGES *IN VITRO* POST-SPINAL CORD INJURY- A PILOT STUDY

5.1 INTRODUCTION

5.1.1 M1-like versus M2-like cells after a spinal cord injury

Macrophages are highly plastic cells and can change their phenotype and function to respond to external cues in the microenvironment (Mosser and Edwards, 2008b), as well as influence the microenvironment by secretion of mediators, such as cytokines, and modulation of receptor expression (notably MHCII and its costimulatory molecules). Mosser and Edwards (2008) classified macrophage phenotypes into three broad categories each exhibiting specific and different homeostatic functions; host defence, wound healing and immune regulation (Mosser and Edwards, 2008b). The M1/M2 paradigm uses a dichotomy between classically activated M1 cells and alternatively activated M2 cells, with subsets in between and has been used for decades (Gensel and Zhang, 2015). The M1/M2 dichotomy is overly simplified but allows for a simple model to use in studying macrophage populations. The M1/M2 distinction has come under review in recent years, as the complexity of the macrophage spectrum of responses has become clearer (Martinez et al., 2008; Martinez and Gordon, 2014; Mosser and Edwards, 2008b; Murray and Wynn, 2011a). It is now accepted that the activation states of macrophages fall along a spectrum, dependent upon their expression of receptors as well as secretion of immune mediators. A simplified characterisation of this spectrum is the progression from M1 to M2a, M2b and M2c in a normal wound healing paradigm (Gensel and Zhang, 2015).

M1 cells are stimulated by INF- γ , LPS and TNF- α , are defined by high levels of reactive oxygen species (ROS) and IL-12 production and low IL-10 expression, and are associated with IL-1 β , TNF- α , IL-6, CD16, CD32, CD86, and iNOS markers. M2 subset are stimulated by different cytokines and receptors; M2a is stimulated by IL4/IL13, M2b is stimulated by toll-like receptors (TLRs) and immune complexes and M2c are stimulated by IL-10. All three subsets of M2s are associated with decreased ROS and M2b cells are associated with high IL-10 production (Gensel and Zhang, 2015). However, the phenotypes of macrophages can fall anywhere on the spectrum from highly pro-inflammatory phagocytes, characterised by expression of higher levels of pro-inflammatory mediators such as IL1 β , IL-6 and TNF- α , to anti-inflammatory and injury resolution mediators characterised by higher expression of mediators such as IL-10, TGF- β and VEGF (Mantovani et al., 2007; Martinez and Gordon, 2014; Mosser and Edwards, 2008a; Xue et al., 2014).

The plasticity of these macrophages allows them to alter their functional phenotype in response to cues in the injury environment, such as cytokines, chemokines and growth factors (Heusinkveld and van der Burg, 2011; Wang et al., 2014). Once a macrophage has responded to tissue trauma and expressed a specific functional phenotype, characterised by surface markers and soluble factor secretion, a macrophage may not remain inflexibly set in a particular phenotype but instead has the ability to shift its function in response to altered environmental cues (Ricardo et al., 2008; Stout et al., 2005; Wang et al., 2014). Generally, in a wound healing paradigm there is a progression of macrophage phenotypes that assist in different phases of wound healing and repair (Gensel and Zhang, 2015; Mantovani et al., 2013; Murray and Wynn, 2011a). In a typical somatic injury circumstances macrophages play an important role not just in the inflammatory phase of the injury but also in repair and

remodelling (Brown et al., 2014; Mantovani et al., 2013). Macrophages arrive at the site of trauma within the first days after injury to clear debris and initiate an inflammatory cascade characterised by high expression of pro-inflammatory mediators (Brown et al., 2014). Outside the CNS there are generally considered to be 3 distinct phases, inflammatory, proliferative and remodelling. These are associated with different macrophage phenotypes, classified as M1, M2a, M2b and M2c (Chapter 4, section 4.1.1) (Gensel and Zhang, 2015).

The inflammatory response in SCI differs markedly from the progression that occurs in other tissues (Gensel and Zhang, 2015) and this is thought to be to the detriment of repair and regeneration. In traumatic CNS injury inflammation has both neuroprotective and neurotoxic effects (Das et al., 2012; Ekdahl et al., 2003; Hohlfeld et al., 2007; Kigerl et al., 2009; Lucas et al., 2006; Popovich and Jones, 2003; Schwartz et al., 1999). The duality of this response is the root of the complexity underlying the inflammatory response to SCI and targeting elements of this therapeutically (Hohlfeld et al., 2007; Popovich and Jones, 2003). Previous work in this study has shown that the inflammatory response after SCI tends towards a strong pro-inflammatory response characterised by high levels of acute neutrophil infiltration, activated microglia, CD68⁺/CD86⁺ pro-inflammatory macrophages and pro-inflammatory cytokines (Chapter 3 and Chapter 4), which agrees with the current literature. As opposed to the multi-phased response in normal somatic tissue injury (Adamson, 2009; Gensel and Zhang, 2015; Koh and DiPietro, 2011; Velnar et al., 2009) the inflammatory response after SCI is more dysregulated, and persists as strongly pro-inflammatory, less permissive towards injury resolution (Gensel and Zhang, 2015). In a normal injury the responding macrophages participate in the repair and remodelling phases, not just the initial inflammatory phase (Brown et al., 2014; Mantovani et al., 2013), however in SCI

the lingering macrophage population tends towards the pro-inflammatory and show little resolution.

Modulation of macrophage phenotypes has been explored in the autoimmune degenerative disease Multiple Sclerosis (MS). In the animal model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), M1 macrophages participate in the induction and development of disease releasing pro-inflammatory mediators and causing further damage to the CNS tissue (Jiang et al., 2014). M1 and M2 phenotypes co-exist at all stages of the disease however the predominant phenotype shifts as the disease progresses, in the later phase of the disease the macrophages and microglia in the CNS are less activated and have a more M2-like phenotype (Jiang et al., 2014). Understanding the balance between these phenotypes and how it affects the disease progression can allow for the modulation of these cells to achieve a better recovery outcome. There is some evidence that the alteration of the macrophage response towards a more M2-like phenotype can be beneficial to neurologic function (Denney et al., 2012). A 2011 study of EAE in rats demonstrated that an equilibrium between M1 and M2-like cells favours a mild disease while the predominance of the M1-like phenotype is associated with relapsing EAE. Furthermore, the administration of M2 activated cells had the effect of suppressing the severe ongoing EAE (Mikita et al., 2011). In a rat model of white matter demyelinating lesions the switch from a dominant M1 to M2 macrophage phenotype is associated with enhanced oligodendrocyte differentiation *in vitro* and proved to be important for efficient remyelination *in vivo* (Miron et al., 2013). MS is another example of a CNS disease where the therapeutic modulation of the macrophage/microglial response would be beneficial, specifically the modulation towards an M2-like phenotype predominance.

Modulating the inflammatory response, especially the macrophage response, towards a progression from pro-inflammatory phenotypes to wound healing and repair phenotypes could drastically change the nature of the secondary injury environment and help to alleviate degeneration. A current phase 1 clinical trial in Australia aims to test the viability of intravenous immunoglobulin (IVIg) as an immunomodulatory therapy in the acute phase of SCI to improve neurological outcomes (<http://www.anzctr.org.au/ACTRN12616001385437>). If the macrophages can be modulated towards the M2 end of the spectrum this will also change the nature of the extracellular environment and help push the other cellular responders towards injury resolution and potentially allow for greater neuronal survival and regeneration of nervous tracts.

5.1.2 Can activated macrophages that respond to central nervous system injury be modulated?

Macrophage categorisation has long been based on gene expression and secretion of immune mediators induced by cytokines and pathogen-derived stimuli (Murray and Wynn, 2011b). M1 macrophages are commonly associated with LPS and IFN- γ activation and are characterised by the production of type 1 interferons, IL-6 and IL-1 β , and proteolytic enzymes (Murray and Wynn, 2011b); whereas M2 macrophages are associated with TH2 responses, cytokines such as IL-4 and IL-13, and produce growth factors such as TGF- β 1 (Murray and Wynn, 2011a). This has allowed for the modulation of the immature cells of macrophage lineages towards specific phenotypes. One complicating factor in studying the M1/M2 dichotomy *in vitro* is that the techniques used to grow, maintain and characterise the phenotypes of macrophages can influence the expression of these phenotypes. For example, the use

of MCSF or GM-CSF can result in polarisation of macrophages towards M2 or M1-like, respectively, and is often used in procedures to achieve just such a polarisation (Hamilton et al., 2014; Lescoat et al., 2018; Rey-Giraud et al., 2012). In the current pilot, the macrophages extracted from the rats' spinal cords were terminally differentiated and sorted according to their specific phenotype; extraneous growth factors to the media may skew the phenotype of the macrophages and influence the results, and so were not used in this experiment.

There are many studies that use and modulate primary macrophages *in situ* and *in vitro*, associated with many different conditions (Heusinkveld and van der Burg, 2011). Some of the diseases commonly associated with the use of macrophages *in vitro* are cancer, autoimmune diseases, chronic and parasitic infections. Helminth parasites have been demonstrated to trigger a polarised immune response skewed towards a T-helper (Th2), M2-like phenotype which has led to a focus on the macrophage response to these worms (Kreider et al., 2007). In renal disease there has been some success in modulating macrophage phenotype to reduce renal injury in mice models (Ricardo et al., 2008); the focus of which has been a switch to a more balanced M2-like response (Wang et al., 2007).

Tumour-associated macrophages (TAMs) are involved in a wide range of processes involved in the spread of the tumour and cancer associated inflammation, and are often studied for their M2-like phenotype (Biswas and Mantovani, 2010; Mantovani et al., 2002; Mantovani and Sica, 2010; Qian and Pollard, 2010). In cancer scenarios macrophages have long been known to play a role in tumour development and high numbers of TAMs associate with poorer prognosis; however this varies depending on the type and aetiology of the cancer (Heusinkveld and van der Burg, 2011). This has led to the contention that the functional phenotype of the TAMs is an

important factor, and a high M1/M2 ratio may actually be beneficial for patients survival (Heusinkveld and van der Burg, 2011). Previously, Stout et al. (2005) used *in vitro* modulation with sequential treatments of cytokines to induce functional phenotype switching of primary macrophages from aged and tumour-bearing mice (Stout et al., 2005). This approach holds promise for multiple diseases, not just cancer, and could potentially be applied to MS, SCI and other immune based conditions.

Immature macrophages and monocytes can easily be modulated into different phenotypes and functions, mature macrophages can also be manipulated but are less plastic than MDMs and BMDMs in culture. The primary macrophages extracted from injured spinal cords in this pilot study were fully mature and activated cells so will be more challenging to maintain and manipulate in culture, as they are passed their dividing phase.

5.1.3 The emerging role for neural progenitor cells as mediators of inflammation

In Chapter 3 (Chapter 3, Section 3.1.4) the concept of ‘cross-talk’ between the nervous system was addressed, especially between the neural progenitor cells and the immune system (Cusimano et al., 2012; Kokaia et al., 2012; Ziv et al., 2006). This is an idea that holds great interest and promise in the development of a therapeutic strategy for SCI, but still requires more exploration. Over the past decade there have been multiple studies that have found various NSPC capable of influencing inflammation in the brain in models of MS and stroke in rodents and non-human primates (Pluchino and Cossetti, 2013). Glial cells and NSPC can play a role in tissue repair after injury that is most likely mediated by extrinsic growth and trophic factors in the microenvironment (Hauwel et al., 2005). Mesenchymal stem cells have been shown to influence the phenotypical expression of macrophages toward an M2

phenotype, while concurrently M2-like macrophages promote the growth of MSC and their motility towards injured tissue (Cho et al., 2014; Mantovani et al., 2013).

The post-injury environment is modulated by expressed growth factors, such as BDNF, GDNF and CNTF, cytokines, both Th1 and TH2, and chemokines from immune cells as well as endogenous cells of the CNS, as well as cell signals from the injured tissue. The 'cross-talk' between the immune and nervous system is based on the expression and response to NTF such as BDNF (Pluchino and Cossetti, 2013), cytokines and chemokines (Hauwel et al., 2005; Martino et al., 2011). Stem cells are sources of a variety of cytokines, chemokines, growth factors, micro- RNAs and exosomes, and can have strong regulatory effects on immune cells (Lin and Du, 2018). The exact mechanisms as to how NSPC can manipulate and modulate the injury microenvironment are still yet to be elucidated. However, the question then becomes, how can we reproduce and use these effects therapeutically? If we can understand the mechanisms behind NSPC modulation of the immune response we can use these to prompt a beneficial response in patients using either the application of the appropriate factors to activate endogenous progenitor cells, or the introduction of exogenous stem cells, and switch the immune response towards a wound healing paradigm.

5.1.4 Concluding remarks

Before potential modulations can be tested *in vivo*, their potential efficacy is examined *in vitro* on cultured cells. In this case there is some difficulty inherent in this approach. To test the effects of one cell type on another requires the precise separation of not only the eNPC of the spinal cord, but also the M1-like from M2-like macrophages, and the maintenance of these mature and activated primary cells in culture. However, if we can find a combination of factors to help manipulate the injury microenvironment and the phenotype of the macrophages/microglia we can use this therapeutically to push towards a more beneficial wound healing and repair paradigm.

5.2 HYPOTHESIS AND AIMs

5.2.1 Hypothesis

M1 and M2 macrophages and NPC secrete soluble factors (cytokines) that can modulate the phenotype of macrophages isolated following SCI.

5.2.2 Aims

1. To isolate primary NPCs, 'M1-like' and 'M2-like' macrophages/microglia from fresh rat spinal cord, and to culture these cells.
2. To collect the conditioned media from each cell type and measure pro-inflammatory and anti-inflammatory cytokine levels using ELISA.
3. To treat M1 like cells and M2 like cells with conditioned media from each cell type and then examine these cells for a switch of phenotype using flow cytometry.

5.3 MATERIALS AND METHODS

5.3.1 Animal numbers and groups

For this study 6 adult female Sprague-Dawley rats were used (ARC, Perth). All procedures were performed according to previously applied methodology and approved by the UTS animal ethics committee (UTS ACEC 2016-1027). These rats were either left as uninjured normal and not given surgery or were given a surgically induced contusion SCI at 10 weeks of age (adult), as previously described in Chapter 2. These animals were euthanized 1 week post-surgery (n=3), with a corresponding normal rat (n=3).

5.3.2 Surgery and euthanasia

Surgery to induce a mild contusion SCI was performed as previously described (section 2.3.2)(Gorrie et al., 2010). The rat was then cardially perfused with heparinised saline for one minute and approximately 2cm of the fresh spinal cord tissue, surrounding the T10 lesion, was extracted and placed in sterile HBSS buffer (ThermoFisher Scientific) on ice. This was then transported to the tissue culture facility for, homogenisation, disassociation and sorting.

5.3.3 Spinal cord homogenation, dissacociation and cell sorting

Substantial optimisation and protocol testing was required to achieve a viable protocol from dissociation to cell sorting and primary cell culture. This protocol was tested using just MACS columns, just fluorescent sorting without MACS columns and a combination of both. Below is the finalised protocol utilising both MACS columns and fluorescent sorting.

5.3.3a Spinal cord disassociation using GentleMACS

Spinal cord dissociation was performed using a Neural Tissue Dissociation Kit (T) (130-093-231 Miltenyi Biotec Australia) in conjunction with the GentleMACS system (Miltenyi Biotec Australia). The recommended procedures for these products were followed and the existing pre-set brain program on the GentleMACS machine was used.

5.3.3b MACS cell sorting columns for macrophages/microglia and neural progenitor cells

The homogenised tissue was then strained through a 70µm SmartStrainer (Miltenyi Biotec Australia) and put through two MACS MS columns with different microbeads to positively select for macrophages/microglia (CD11b/c microbeads, 130-105-634, Miltenyi Biotec Australia) and NPCs (Anti-PSA-NCAM microbeads, 130-092-966, Miltenyi Biotec Australia). The MS column with the CD11b/c beads was used first and the positively selected macrophages/microglia taken to be stained for fluorescent cell sorting. The column with Anti-PSA-NCAM bead was then used and the positively selected NPCs taken straight to culture. This was performed as per the manufacturer's recommended procedures.

5.3.3c Fluorescent activated cell sorting (FACS) of macrophages/microglia

The positively selected macrophages/microglia plunged from the MACS column were centrifuged (16000rpm for 10min) and the supernatant removed. The cells were resuspended in 10µl of 5% NMS in FSW and incubated on ice for 30min. 40µl of the fluorescently conjugated antibody mix (CD45, CD68, CD86 and CD163), as used previously in section 4.3.3c, was added on top of this and gently mixed. This was

incubated for a further 30min on ice. After washing in 700µl of FSW with EDTA the stained cells were resuspended in 500µl of FSW with EDTA ready for sorting.

These cells were sorted into 'M1-like' and 'M2-like' populations using either a BD Influx or BD Aria cell sorter. The gating strategy used to sort the two populations of macrophages/microglia was hierarchical and based on forward- (FSC) and side-scatter (SSC) as well as fluorescent staining of specific markers. These populations were gated as follows: firstly, the single cells were gated from a larger total population, within this gate the CD45 positive cells were selected, then within the CD45 positive gate 'M1-like' cells were gated as both CD68 and CD86 positive and this population was collected, the 'M2-like' cells were gated within the CD45 positive gate as being CD68 and CD163 positive and excluding any CD86 crossover (Figure 82). This resulted in two populations of macrophages/microglia for each sample. These populations were sorted into complete basic media (DMEM + 10% FBS + Penicillin/Streptomycin) and transported on ice to be cultured separately. There were three successful sorting runs of one normal and one SCI cord. One on the BD Influx cell sorter at UTS and two on the BD Aria cell sorter at the Garvan Institute.

5.3.4 Tissue culture

The sorted 'M1-like' and 'M2-like' cells were spun down (10min at 1600rpm) and the collected media from the sorting was aspirated. These cells were then resuspended in warm basic media (DMEM+ 10% v/v FBS + penicillin/streptomycin) to seed 0.5ml per well on a 24 well plate. These cells were cultured two animals per 24 well plate, as seen in Figure 83, at 37°C and 5% CO². They were left undisturbed the next day. On day two the conditioned media from these cells was collected and then used to treat the plate as shown in Figure 83. The cells were fed 350µl of conditioned

media and an additional 150µl of warm, fresh base media per well. The remaining conditioned media was kept at -80°C for further analysis.

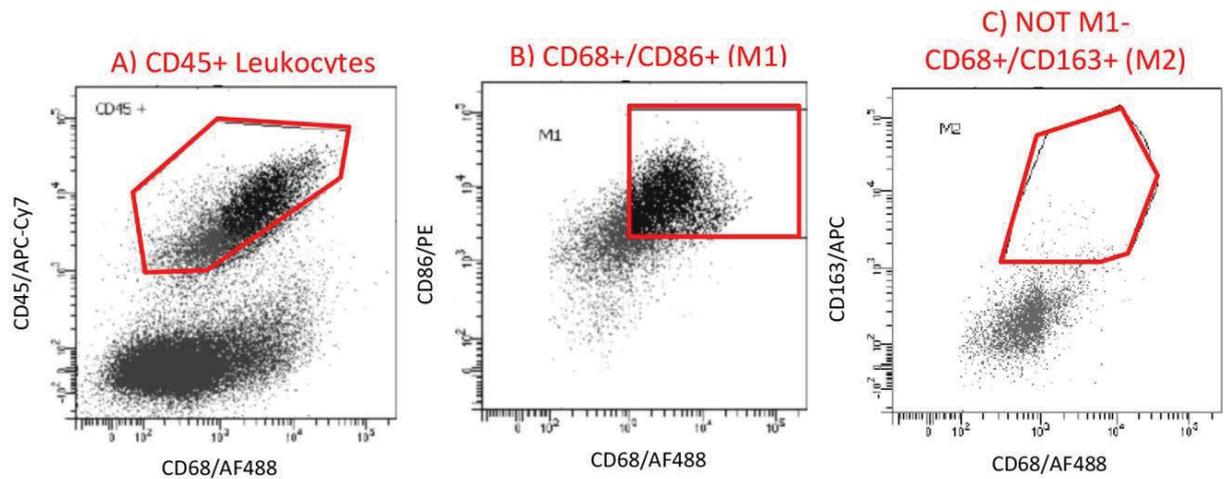


Figure 82: Representative scatter-plots of SCI-derived cells to illustrate flow cytometry gating strategy for M1/M2 FACS cell sorting. After selecting single cells and live cells using forward and side scatter the leukocytes were selected using CD45 (A). CD45+ leukocytes were sorted into an M1 population using CD68⁺ and CD86⁺ (B), and M2 (not M1) population using CD68⁺/CD163⁺ and CD86⁻ (C).

The NPCs were sorted from the tissue homogenate using anti-PSA-NCAM microbeads (Miltenyi Biotech) in an MS column, according to the manufacturer's suggested protocol. After separation these cells were seeded into poly-lysine coated T25 tissue culture flasks, 1 per animal, with 4ml of basic media and kept in 37°C, 5% CO₂. These cells had difficulty adhering to the cell culture surface and so were left as undisturbed as possible for the first week after seeding. After a week 2ml of supernatant was removed, spun down to remove the non-adherent cells, and frozen at -80°C for later use. This 2ml was replaced with fresh media. These cells were maintained for use in a related project for a few weeks, with regular feeding and as little disruption as possible, but did not settle or adhere well.

| | | M1 | | | M2 | | | |
|----------|--|--|---|--|---|---|---|--------------------------------|
| (Normal) | | M1 cells + M1 media = Flow Cyt. | M1 cells + M2 media = Flow Cyt. | M1 cells + NPC media = Flow Cyt. | M2 cells + M2 media = Flow Cyt. | M2 cells + M1 media = Flow Cyt. | M2 cells + NPC media = Flow Cyt. | Day 0 Day 2 Day5 |
| | | M1 cells + M1 media = RNA | M1 cells + M2 media = RNA | M1 cells + NPC media = RNA | M2 cells + M2 media = RNA | M2 cells + M1 media = RNA | M2 cells + NPC media = RNA | Day 0 Day 2 Day5 |
| | | M1 cells + M1 media = Flow Cyt. | M1 cells + M2 media = Flow Cyt. | M1 cells + NPC media = Flow Cyt. | M2 cells + M2 media = Flow Cyt. | M2 cells + M1 media = Flow Cyt. | M2 cells + NPC media = Flow Cyt. | Day 0 Day 2 Day5 |
| | | M1 cells + M1 media = RNA | M1 cells + M2 media = RNA | M1 cells + NPC media = RNA | M2 cells + M2 media = RNA | M2 cells + M1 media = RNA | M2 cells + NPC media = RNA | Day 0 Day 2 Day5 |

Figure 83: 24 well tissue culture plate plan, with treatment over 5 days.

5.3.5 Flow cytometry for M1-like/M2-like phenotypes

On day 5, once the manipulation of the cells *in vitro* has been completed, the wells were checked under the microscope for cell density and condition. Then cells in one row of normal wells and one row of SCI wells were harvested using room temperature TrypLE (Gibco) at 1ml/10cm² of plate. The TrypLE was inactivated by dilution using 0.5ml of media per well, and then transferred to 1.5ml Eppendorf tubes, centrifuged (1600rpm for 10min), the supernatant aspirated and replaced with 5% normal mouse serum blocking solution. The staining for flow cytometry followed the protocol used for macrophages/microglia in Chapter 4 (Chapter 4 section 4.3.3c & d).

Compensation beads (BD Biosciences) were again used (Chapter 4 section 4.3.3c). The gating strategy is demonstrated in Figure 84. The percentages of CD45⁺ leukocytes exhibiting M1 and M2-like phenotypes were compared using two-way ANOVA, with phenotype and treatment as the variables, and Bonferroni's post hoc test. Individual t-tests were used to compare phenotypes between specific treatments.

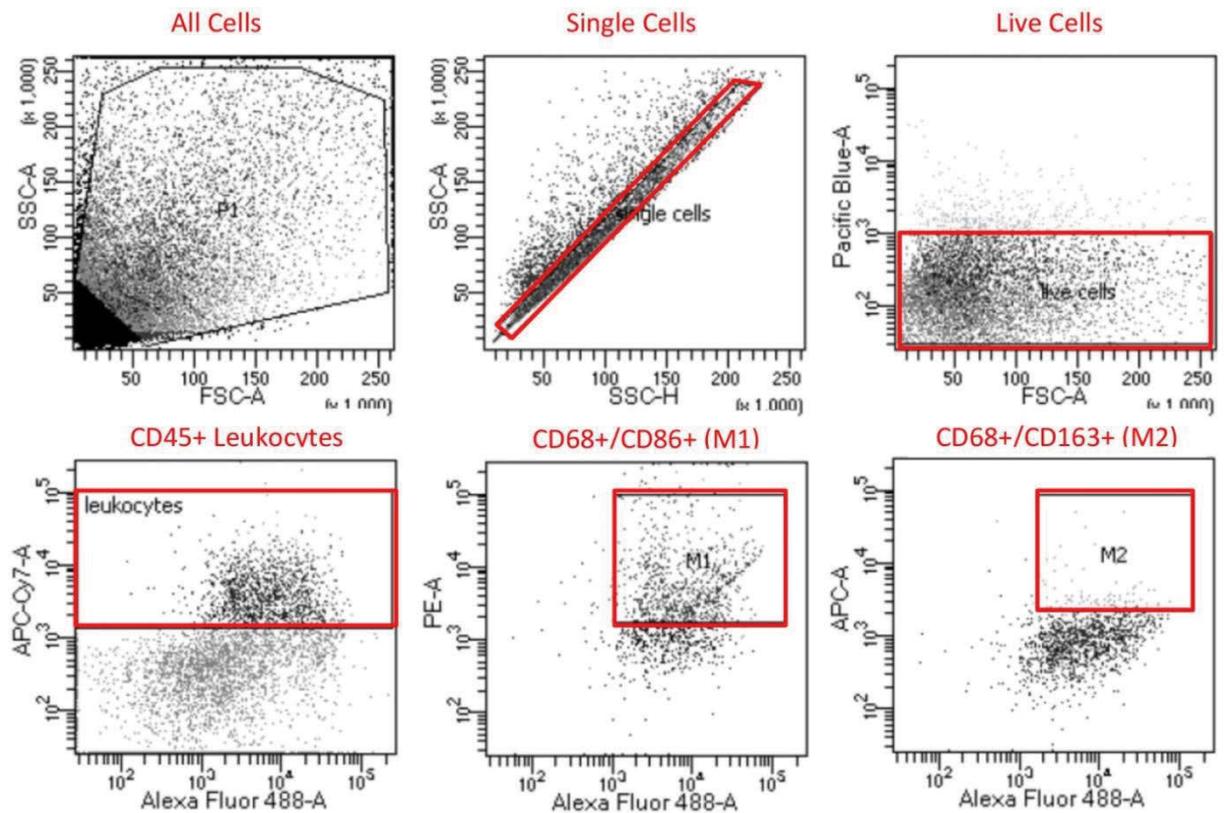


Figure 84: Representative scatter-plots of SCI-derived cells to illustrate flow cytometry gating strategy for M1/M2 distinction after modulation.

5.3.6 IL-1 β , IL-6, IL-4 and IL-13 ELISA

The media was collected from the cells at day 2, representing the un-manipulated M1, M2 and NPC supernatant; and at day 5, after manipulation. This resulted in conditioned media from M1 cells, M1+ M1 supernatant, M1+ M2 supernatant, M1+ NPC supernatant, M2 cells, M2+ M2 supernatant, M2+ M1

supernatant and M2+ NPC supernatant. These were stored at -80°C until analysis.

Specific cytokine ELISAs were conducted for classic pro-inflammatory cytokines IL-6 (Sigma-Aldrich, #RAB0311) and IL-1 β (Invitrogen, #BMS630); as well as IL-4 (Invitrogen, #BMS628) and IL-13 (Sigma-Aldrich, #RAB0258), cytokines associated with M2 phenotype. Technical repeats of all of the injured animals were conducted and a pooled repeat of the normal animals (Figure 85). IL-6 and IL-13 ELISAs were conducted according to the Sigma-Aldrich suggested sandwich ELISA protocol (see Appendix). IL-1 β and IL-4 ELISAs were conducted according to the suggested protocol for Affymetrix ELISA kits (Invitrogen). The supernatant samples for each ELISA were diluted 1:2 with the provided sample diluent, according to the Affymetrix recommendation. This data was analysis using two-way ANOVA, with the injury status (normal or SCI) and well treatments as variables, and Bonferonni’s post hoc test.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|--------|--------|----------------|----------------|----------------|--------------------------|----------------|----------------|----------------|----------------|----------------|----------------|
| A | STD. 1 | STD. 1 | 1707 M1 | 1709 M1 | 1711 M1 | Pooled Norm M1 | 1708 M1 | 1708 M1 | 1710 M1 | 1710 M1 | 1712 M1 | 1712 M1 |
| B | STD. 2 | STD. 2 | 1707 M1+M1 | 1709 M1+M1 | 1711 M1+M1 | Pooled Norm M1+M1 | 1708 M1+M1 | 1708 M1+M1 | 1710 M1+M1 | 1710 M1+M1 | 1712 M1+M1 | 1712 M1+M1 |
| C | STD. 3 | STD. 3 | 1707 M1+M2 | 1709 M1+M2 | 1711 M1+M2 | Pooled Norm M1+M2 | 1708 M1+M2 | 1708 M1+M2 | 1710 M1+M2 | 1710 M1+M2 | 1712 M1+M2 | 1712 M1+M2 |
| D | STD. 4 | STD. 4 | 1707 M1+NPC | 1709 M1+NPC | 1711 M1+NPC | Pooled Norm M1+NPC | 1708 M1+NPC | 1708 M1+NPC | 1710 M1+NPC | 1710 M1+NPC | 1712 M1+NPC | 1712 M1+NPC |
| E | STD. 5 | STD. 5 | 1707 M2 | 1709 M2 | 1711 M2 | Pooled Norm M2 | 1708 M2 | 1708 M2 | 1710 M2 | 1710 M2 | 1712 M2 | 1712 M2 |
| F | STD. 6 | STD. 6 | 1707 M2+M2 | 1709 M2+M2 | 1711 M2+M2 | Pooled Norm M2+M2 | 1708 M2+M2 | 1708 M2+M2 | 1710 M2+M2 | 1710 M2+M2 | 1712 M2+M2 | 1712 M2+M2 |
| G | STD. 7 | STD. 7 | 1707 M2+M1 | 1709 M2+M1 | 1711 M2+M1 | Pooled Norm M2+M1 | 1708 M2+M1 | 1708 M2+M1 | 1710 M2+M1 | 1710 M2+M1 | 1712 M2+M1 | 1712 M2+M1 |
| H | BLANK | BLANK | 1707 M2+NPC | 1709 M2+NPC | 1711 M2+NPC | Pooled Norm M2+NPC | 1708 M2+NPC | 1708 M2+NPC | 1710 M2+NPC | 1710 M2+NPC | 1712 M2+NPC | 1712 M2+NPC |

Figure 85: 96 well plate plan for Cytokine ELISA for IL-1 β , IL-6, IL-4 and IL-13, with technical repeats.

5.4 RESULTS

5.3.1 FACs sorting of macrophages

The sorting was based on the CD45⁺ population with the M1-like cells sorted as those expressing CD68 and CD86 within this population and the M2-like cells as those positive for CD68 and CD163 that were not within the M1-like gate. The initial sorts resulted in an average of 8014 M1-like cells and 8282 M2-like cells in the normal spinal cords, 255,505 M1-like cells and 9463 M2-like cells in the injured cords (Figure 86A) (t-test $P=0.05$). This allowed for seeding an average of 1335 M1-like cells and 1380 M2-like cells in the respective normal wells, 42,584 M1-like cells and 1577 M2-like cells in the respective injured wells of the 24 well tissue culture plate. The SCI derived macrophages showed significantly higher proportions of M1-like cells than M2-like ($P<0.005$) and the normal leukocytes also resulted in higher proportions of M1-like than M2-like, however the percentages were much closer and there was no statistical significance (Figure 86B).

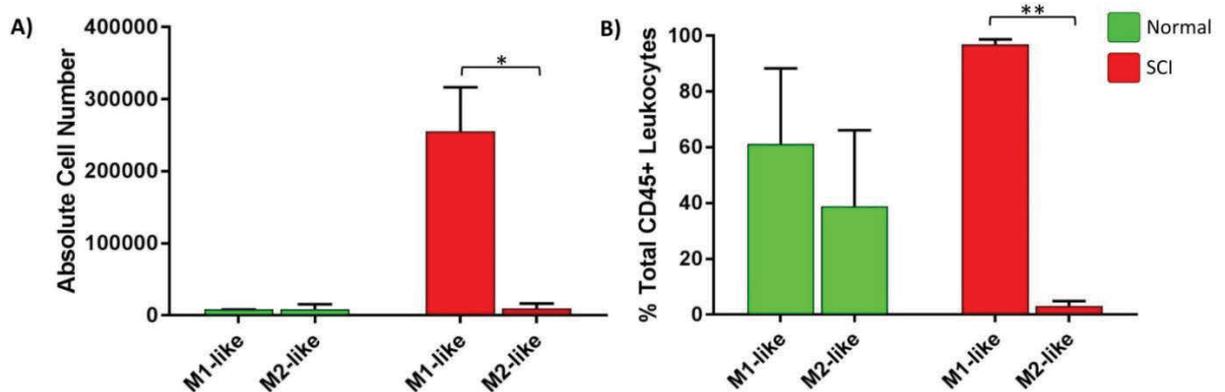


Figure 86: Histograms comparing M1-like to M2-like cells resulting from the initial fluorescent cell sort in both normal and SCI spinal cords using A) the numbers of M1-like and M2-like cells and B) the numbers of M1-like and M2-like cells as a percentage of the total leukocyte (CD45⁺) population.

* ($P=0.05$) and ** ($P<0.005$) and indicate statistical significance.

5.3.2 Characterisation of cytokines in the extracellular supernatant using ELISA

The cytokine ELISAs showed very little pro-inflammatory cytokines in the cultured cell media. There was almost no IL-1 β detected within the standard curve of the Affymetrix ELISA, with the exception of the M2 wells from one SCI animal and one normal well (Figure 87A). There was also very little IL-6 detected within the standard curve of the Sigma Aldrich ELISA kit. There were a few scattered wells with low concentrations of IL-6, interestingly these were in the normal wells, largely in the M2-like cell wells (Figure 87B).

There was a much greater anti-inflammatory cytokine detection. Levels of IL-4 were detected in all wells, using the Affymetrix ELISA kit, and IL-13 in the majority of wells, using a Sigma Aldrich kit. This could be indicative of the higher sensitivity of these kits, specifically the IL-4 kit. IL-4 concentration was higher in the M2 cell wells than the M1 cell wells in the SCI cells and generally in the normal wells, and highest in the M2 + NPC conditioned media. The SCI and normal levels of IL-4 were similar in the M1 wells, however the levels appeared increased in the SCI M2 wells compared to their normal counterparts (Figure 87C). However, due to large standard deviations, there was no statistical significance to be found using two-way ANOVA and Bonferonni's post hoc test. The levels of IL-13 showed a random pattern with no significant trends. The concentration of IL-13 was generally higher in the normal M1 wells, dropping to a comparatively lower level in the SCI M1 wells. Levels were relatively high in the normal M2 untreated well and at fairly consistently low levels in all of the subsequent M2 wells treated with conditioned media (Figure 87D). Due to large standard deviations, there was no statistical significance to be found using two-way ANOVA and Bonferonni's post hoc test.

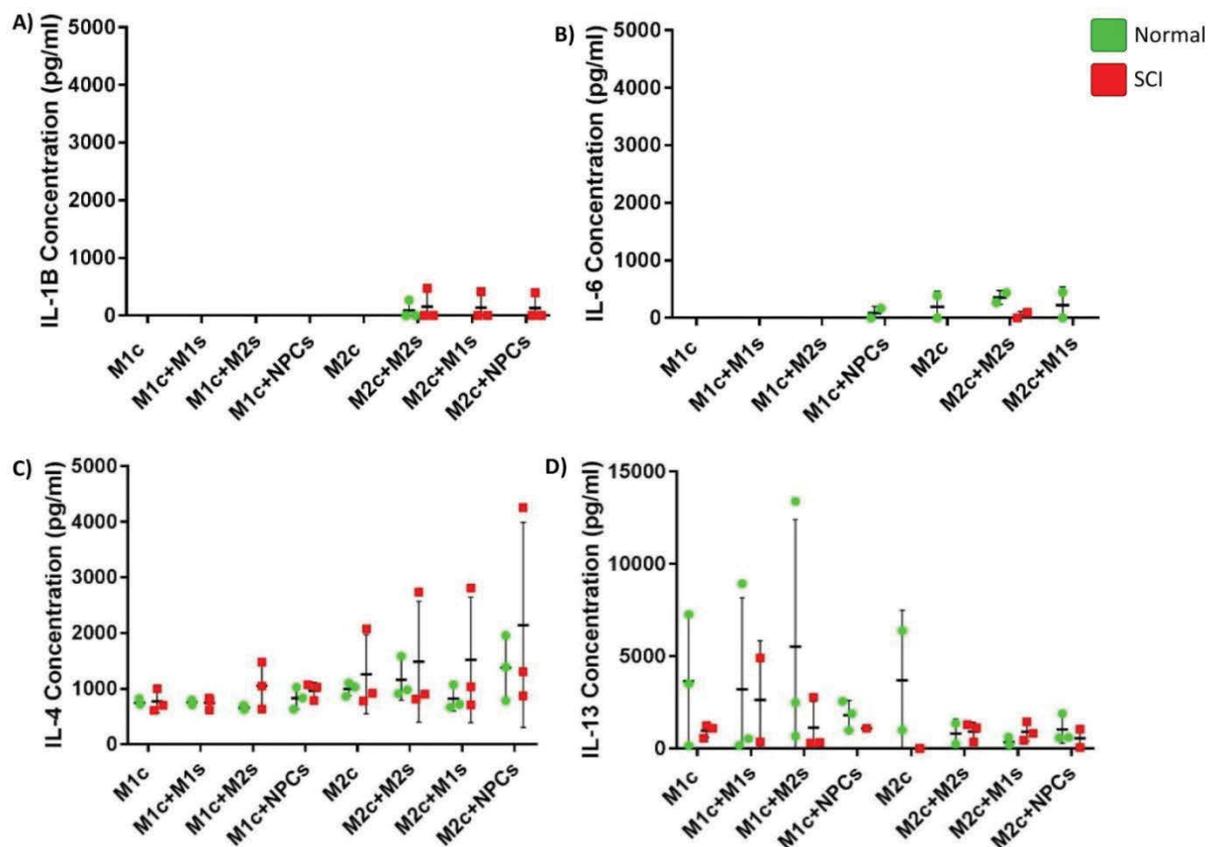


Figure 87: Individual value scatter graphs of the sparse pro-inflammatory cytokines, A) IL-1 β and B) IL-6, and the anti-inflammatory cytokines associated with alternative (M2) macrophage activation, C) IL-4 and D) IL-13, and, found in the tissue culture supernatant before and after treatments with different conditioned media using ELISA. This shows stark differences in the magnitude of the pro-inflammatory and anti-inflammatory responses. On the x-axis the subscript c (cells) indicates the initial cells in the well while s (supernatant) indicates the conditioned media applied. Each point represents the average of 2 technical repeats in 1 animal. There was no statistical significance found in this data.

5.3.3 Characterisation of cells by flow cytometry

There were very few significant differences found between wells, and M1-like and M2-like cells, using flow cytometry. The cells derived from the normal rats showed a greater percentage of the total CD45⁺ leukocytes with an M1-like (CD68⁺/CD86⁺) phenotype rather than an M2-like (CD68⁺/CD163⁺) in the initial sort (Figure 86B) and in all the subsequent wells (Figure 88A), regardless of treatment with conditioned media. The conditioned media showed no effects in the normal wells, and the only statistical

significance was a decrease in M1-like cells from the initial sorting percentage in all subsequent wells ($P < 0.001$). This could simply be due to cell death over the 5-day culture period.

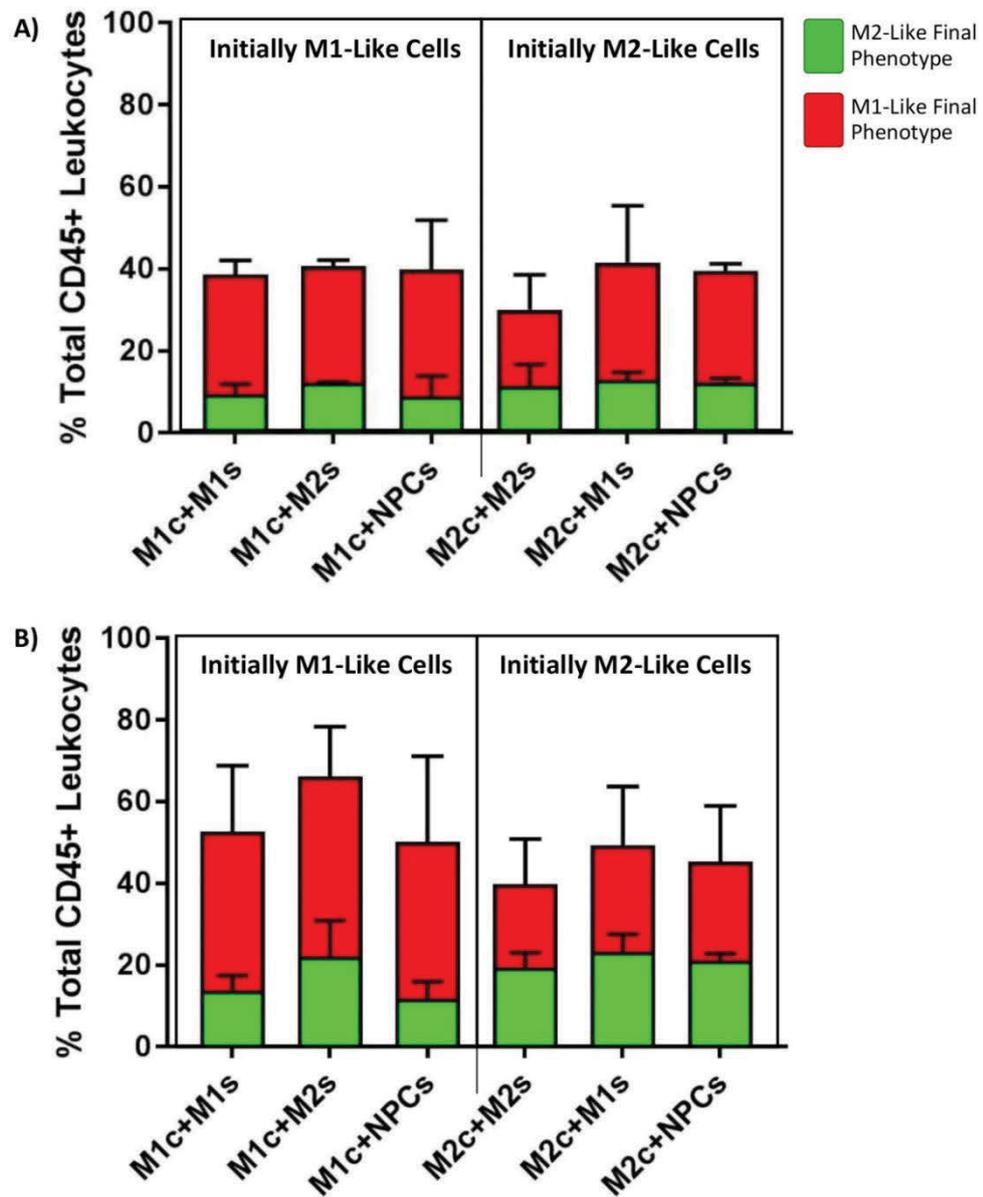


Figure 88: Histograms comparing the percentage of the CD45⁺ leukocyte population expressing M1-like (CD68⁺/CD86⁺) and M2-like (CD68⁺/CD163⁺) markers, in A) the cells derived from normal animals and B) the cells derived from SCI animals, after modulation with conditioned media. On the x-axis the subscript c (cells) indicates the initial cells in the well while s (supernatant) indicates the conditioned media applied to modulate these cells. The graphs are divided in 2, the cells that were initially M1 and those that were initially M2. There were no significant differences between the modulated wells.

The SCI derived cells showed a significantly higher percentage of M1-like than M2-like cells at the initial sort (Figure 86B). All of the M1 cell wells showed higher percentages of M1-like cells, with some M2-like cells. The addition of M2 conditioned media to M1 cells (M1c + M2s) showed a small increase in the percentage of M2-like cells. The conditioned media treatment in the M2 cell wells had no observable effect and all of the initially M2 cells showed similar percentages of M2-like and M1-like cells despite the conditioned media applied (Figure 88B). None of these observed trends in the raw data were at all statistically significant using either ANOVA or t-tests.

5.5 DISCUSSION

The data from the flow cytometry showed little change and suggested that the conditioned media was not robust enough to induce a change in phenotype. This could be due to many factors; the time period before conditioned media was collected resulting in too low levels of cytokine secretions, the fully mature and differentiated macrophages being unresponsive to the new media, and attrition of the cells due to apoptosis in culture. The low cell numbers in this pilot were a severe limitation and may not have been enough to produce and sustain levels of cytokines high enough to have a biologic effect on the cell phenotype. There were very small trends and changes in the raw data but these were far too subtle to translate into anything practically or statistically significant. These trends suggested that the M2 conditioned media may be able to shift the M1-like cells present in high levels after injury towards an M2-like functional phenotype. This could be of great therapeutic significance as modulating the post-injury macrophage response towards an M2-like phenotype would be more neuroprotective, decrease the detrimental secondary injury inflammation, and allow for some injury resolution. The presence of these trends in this pilot study would suggest that more animals are needed, but also that the timing and set up of the culture experiment needs to be revisited for greater efficacy. Previous studies have shown that primary macrophages, usually BMDM and MDM, can be manipulated in culture (Heusinkveld and van der Burg, 2011; Ying et al., 2013) so further optimisation needs to be pursued for this technique.

The high proportion of M1-like cells in all of the cultured wells, whether they were initially M1 or M2 cells, may be in response to other factors in the conditioned media. The cells were never confluent in the wells of the 24 well plate, so a smaller

plate would allow for better visualisation of these populations. In observing the cultures under a microscope there was an attrition of cells over the 5 days of the experiment that suggests a level of cell death that was not quantified in this pilot. This cell death will influence the extracellular environment in way that we did not consider or quantify. The release of cell constituents and cell death signals are likely to be an inducement towards a pro-inflammatory, phagocytic phenotype in the remaining cells and make the environment less conducive to the survival of M2 macrophages.

The goal of this experiment was to use the M2 supernatant to switch the M1-like cells to an M2-like phenotype. It was expected that the M1 supernatant would be rich in the pro-inflammatory IL-6 and IL-1 β as these are commonly associated with this functional macrophage phenotype and have often been used as markers of inflammation. The M2 supernatant was expected to show more expression of IL-4 and IL-13. IL-4 and IL-13 are anti-inflammatory cytokines that are associated with the switch to an M2-like phenotype, and have been shown to stimulate M2a macrophages (Gensel and Zhang, 2015). The anti-inflammatory cytokines in the supernatant from the M2 cells were hypothesised to be able to shift the M1-like cell phenotype to an M2-like phenotype *in vitro*.

The cytokine ELISAs showed almost no detectable pro-inflammatory cytokines (IL-6 and IL-1 β) which is unexpected in the injured animals as previous evidence suggests that the response to SCI is highly pro-inflammatory. There was a higher proportion of M1-like cells than M2-like however the cell numbers were relatively low and may not have been sufficient for high levels of secretion of these cytokines. There are also many different pathways and overlapping functions for different cytokines, so it is likely that the four cytokines assessed in this pilot was too narrow a subset. It is possible that the M1-like cells are expressing and responding to higher levels of

another cytokine such as TNF- α , rather than IL-1 β , which is prompting the low expression of this particular pro-inflammatory cytokine in the conditioned media. Levels of IL-4 were generally higher in the SCI animals than their normal counterparts, and this was particularly obvious in the initially M2 cells and the M1 cells subjected to M2 conditioned media. This is promising as IL-4 and IL-13 are associated with alternative (M2) macrophage activation. M2 macrophages are considered to be anti-inflammatory macrophages that help to suppress the pro-inflammatory response and facilitate wound healing outside the CNS. IL-13, however, was strangely present in higher concentrations in all of the normal M1 wells, as well as the normal M2 original well. It was expected that the IL-1 β and IL-6 levels would be high in the M1 cells and increase in the wells treated with M1 conditioned media. It was also expected that IL-4 and IL-13 levels would be higher in the M2 cells and increase in the wells treated with M2 conditioned media. These cytokine results do not exhibit the expected trends, aside from some IL-4 increases in the SCI M2 SCI wells. There is little that can be inferred from this data and it further confirms that the conditioned media was not robust enough to induce any phenotype or functional changes in the culture cells.

There was great variability between animals, as with all animal models, so greater numbers are needed for statistical power. There was also some variation between the duplicate wells, which suggests that the thawed supernatant samples needed to be mixed more thoroughly before analysis. To validate this experiment, further animals, triplicate wells and repeat analysis is necessary. More sensitive tests for IL-6 and IL-1 β are necessary, as well as the analysis of further pro-inflammatory cytokines such as IL-1 α , TNF- α and IFN- γ . The examination of IL-10, associated with pro-inflammatory suppression and alternative macrophage responses is also necessary to characterise the potential phenotype switch. This study used ELISA to assess the

expression of cytokines at a protein level, the assessment of these factors at an RNA level would also be informative and can be multiplexed to get a cross-section of the response. RNA extraction was performed on one well of each condition for each animal in this current pilot however the yield was too low to perform RT-qPCR. This was due to the high levels of cells death and low remaining cell numbers. In future optimisation pooling of spinal cords or specific culturing of BMDM as M1 or M2 cells would boost cells numbers and allow for effective RNA analysis.

The final results of this pilot did not support the initial hypothesis, however, there are some trends that suggest that with further work, and optimisation, the use of conditioned media could successfully manipulate these cells. There are easier ways to modulate the cells in culture if we can isolate specific factors and cytokines to introduce to the cells. Ideally the use of a multiplex approach to highlight the key cytokines secreted, and treatment with selected cytokines. This is a stepping stone towards modulating the nature and phenotype of the inflammatory response in vivo, a very promising therapeutic avenue. I believe that the results obtained in this experiment have been influenced more by experimental design factors than by biological factors, and as such should be considered inconclusive rather than negative. Also, further expansion of this experiment to include more cell types that are responding to SCI would also be useful. Co-culture or transwell experiments to mirror the complexities of the response to these factors in the spinal cord, using combinations of macrophages, microglia, neurons and astrocytes, would be an important step forward from this experiment.

Using cultured cell lines allow for an intricate knowledge of the properties of the original cultured cells and a prediction of their response to manipulations. Bone marrow derived macrophages (BMDM) and monocyte-derived macrophages (MDMs)

are often used to study macrophage polarisation as they can be specifically manipulated to polarise towards M1 or M2 phenotypes (Ying et al., 2013). The fully mature, differentiated primary macrophages used in the current pilot are a more challenging prospect. These cells are in the final stage of maturation and have a defined phenotype and functionality, this make them harder to maintain in culture for any extended period of time and manipulate towards specific polarisation. A repetition of this experiment using BMDM to produce M1 and M2 specific populations would allow for greater control over the initial phenotype and also for greater cell numbers to utilise in the subsequent modulation.

This experiment was a pilot study and still requires extensive optimisation before any substantial results can be obtained. The flow cytometry results suggested that the conditioned media was not robust enough to induce a change in phenotype. There were very small trends and changes in the cytometry raw data and some statistically significant changes in the concentration in the extracellular cytokines. These trends would suggest that, with optimisation, this approach would hold promise. Manipulating the phenotype of responding macrophages and the inflammatory nature of the lesion microenvironment is a promising therapeutic avenue and should be given further investigation.

CHAPTER 6: CONCLUSIONS

6.1 SUMMARY AND CONCLUSIONS

This study used a contusion model of SCI in rats of different ages to explore the differences between the mature and developing spinal cord in response to SCI, especially in terms of the innate inflammatory response and functional recovery. The overall goal of this study was to develop our understanding of the interactions between cells and systems that govern the progression of SCI. By filling in some of the gaps in our knowledge of paediatric SCI in animals, it may be possible to develop therapies for mature SCI that mimic the superior recovery observed in these infants. There have previously been studies that compared some aspects of the post injury response in adults and young animals, but these are few and far between. This is one of very few studies to directly compare different ages of rats over an extended post-injury period, behaviourally, histologically and molecularly.

Chapter 2 aimed to analyse rats with a spinal cord injury at P7 and compare the hind-limb locomotor function over 6 weeks to the adult and juvenile rats using the BBB scoring system. The BBB scoring system is a well validated scoring system used worldwide that can be successfully applied to adult rats; however, it is much less applicable to infants as they have not developed the fine motor traits and locomotion used in the BBB scoring criteria. As a result, a new infant rat locomotor scoring (IRLS) system was developed for the current study. Significant differences were found in the behavioural responses between mature and developing rats post-SCI. However, hind-limb locomotor function in the infants was indistinguishable between the spinal cord injury cohort and the respective shams using either scoring system. This may be an indication

of greater capacity for recovery from trauma in the infant spinal cord or it may be simply indicative of developmental differences in the infant spinal cord that continue independently to an injury at P7.

Chapter 3 investigated the innate immune and endogenous cell response to SCI in the rat spinal cord using histology to determine if there are differences between adult, juvenile and infant rats, with specific focus on endogenous neural progenitor cells, astrocytes, neutrophils, macrophages and microglia. The results of this chapter showed significant differences between mature animals and infants in all of the aspects examined, from the histological progression of the injury to the cellular response. The most significant differences were observed in the cellular inflammatory response. This highlights the importance of the prominent inflammatory response in the ongoing injury progression, and suggests it is a viable target for therapeutic exploration.

Chapter 4 aimed to determine the key differences in the cellular inflammatory response to SCI between adult and infant rats and compare the associated cytokine response. The results of Chapter 4, alongside those from Chapter 3, show great differences in the inflammatory response to SCI in infant rats, when compared to adults and juveniles. The infant's inflammatory response is of a lower magnitude and exhibits a different, and potentially much more beneficial functional phenotype, which helps to create a lesion microenvironment more permissible to injury resolution axon sprouting. This may contribute significantly to the differences in injury progression and recovery. This is a significant finding as it points towards the modulation of the inflammatory response as an important therapeutic avenue to explore.

Chapter 5 presented an *in vitro* study exploring the viability of modulating different primary macrophage populations using conditioned media from primary 'M1-

like' and 'M2-like' cells, and eNPCs. This was a pilot study and yielded inconclusive results. However, the premise behind this *in vitro* pilot remains a promising avenue to be pursued in future studies; with pooling of spinal cords for higher cell populations, further consideration of timing, and a broader focus on the active cytokines this could prove an important step towards the modulation of the inflammatory response in SCI. The understanding of which inflammatory effectors are having a major role in the post injury environment and which could be used to modulate the nature of the response could lead to the application of these to reduce the detrimental effects of the post-injury inflammatory response.

Taken together, this study provides evidence of substantial differences between paediatric and adult SCI. These differences merit greater investigation and could help to inform the way that this condition is treated and managed. By developing and using an animal model to better understand the mechanisms of paediatric SCI we can begin to develop therapeutic strategies more tailored to the intricacies of the injury in the developing spinal cord and apply these in the clinical setting. Understanding the infant response may also help us to develop therapeutic targets that can be applied to adult SCI to help reduce its severity.

6.2 FUTURE DIRECTIONS

This study raised numerous questions and highlighted how little we actually know about paediatric spinal cord injury, from basic science to a clinical level. The first, and most fundamental, question raised is whether the contention that infant recovery from SCI is better than adult recovery is an artefact or whether it has clinical significance that can be utilised therapeutically. Much of the evidence supporting this statement is anecdotal, with very little quantitative data to support it. This thesis has highlighted some areas where there are clear differences between infant and adult spinal cord injury responses and some areas that require further investigation. As a result, there are several questions that could be addressed with more detailed experimentation.

First, how does the lack of cavity formation in the infants affect the differences in infant and adult responses to SCI? In Chapter 3 we observed that the large cystic cavity that formed in the mature SCI was not present in the infants. This could have significant flow on effects for the rest of the injury response. Without the formation of this cavity there is no need for reactive astrocytes to form the glial scar and less debris for the immune cells to respond to. Second, what is the impact of neurological and CNS development on the infant response? This question is linked to the possibility that the injury in our model occurred before functional connections had been formed, making it difficult to differentiate between development and recovery. The behavioural results in Chapter 2 suggest that what we are seeing could be interpreted as development, recovery or a combination of both. Third, what impact does the developmental stage of the systemic immune system have on the differences observed in the inflammatory response to SCI? To answer these questions a larger experiment to

characterise this animal model of paediatric SCI is required, tracing these factors through rats injured at multiple different developmental ages as well as over different survival times.

There are many difficulties inherent in experimentally exploring SCI in the paediatric population. The complexities of creating an infant model of injury have resulted in the use of exclusively traumatic animal models, despite the prevalence of non-traumatic SCI in the paediatric population compared to TSCI. This is because traumatic models are logistically easier, more readily reproducible and comparable to similar models in adults.

Adding to the complexity of studying paediatric SCI is the fact that we have a limited understanding of the analogous ages between the model animals and human development, as well as the developmental timing of the model animals. The landmark developmental stages – significant events in the CNS development – are also poorly understood in our model animals. There has been little work done to map the development of the spinal cord as a whole in neonatal animals with tracing studies focused on the extension of neurons and progression of the main axonal tracts down the cord. This creates challenges in aligning animal models with the same landmarks in human development, which is necessary to account for the impact that the development of the spinal cord, the CNS and exogenous systems has on the response to SCI incurred at younger ages. To further validate these models and allow for greater utility in studying the pathophysiology of SCI and developing potential therapies, a deeper understanding of the model animals is essential.

Over the course of this study it became quite clear why there are so few studies that directly compare spinal cord injury between mature and developing animals. The creation of an analogous injury in this study proved quite challenging due to the

variable size and vastly different biomechanics of the infant spinal cord, and the more flexible nature of the developing spinal column. We were able to achieve a histologically comparable injury paradigm in the early stages of this study that was used throughout.

Another significant hurdle in studying developing animals, and comparing them to older counterparts, is the behavioural analysis of the infants. The 'normal' behaviour of infant and neonatal animals is inherently different to that of fully developed adults, and changes with different stages of development. This study showed that in a paediatric model of SCI it is hard to accurately ascertain where development ends and recovery begins. Very little is known about the impact of the developmental state and the plasticity of young spinal cords on injury recovery. Another complicating factor in paediatric models of injury is the presence of central pattern generation in the spinal cord. Central pattern generation allows for the development of reflex movements without significant input from descending pathways; it is common in infant animals. This can complicate the assessment of locomotor function in these animals after injury.

The stark differences seen in the immune response to SCI in this study are exciting and potentially important. To fully understand their significance, we must develop a better understanding of neonatal SCI. Injury presentation of paediatric SCI in animal models is different to that in mature adults on a basic pathophysiological, developmental and behavioural level, and a greater understanding of the mechanisms behind paediatric SCI is needed. The aetiology, injury presentation and progression of paediatric SCI are also vastly different in humans. The development of clinically relevant animal models is challenging and still requires substantial exploration. While current traumatic SCI models have found promising avenues for research and

discovered a trend of better recovery in younger animals, the developmental and behavioural complexities inherent in paediatric models of SCI need to be addressed. The results of paediatric SCI experiments also help to guide potential therapeutic approaches in adult SCI, and so cannot be overlooked.

SCI in the paediatric population may be rarer, however it is an injury that incurs 'life-long' ramifications. Unfortunately, we still understand very little about how the developing spinal cord responds to injury, or how the state of development affects this response. A great deal of work is required to answer some of the questions raised in this study. A proposed study to begin filling in the gaps surrounding paediatric SCI would involve rats injured at different post-natal ages, subjected to a range of immunohistochemistry, ELISA and flow cytometry similar to that employed in this study, as well as retrograde and anterograde tract tracing, to elucidate significant developmental events and time-points. Also, investigation to develop models to understand the progression of non-traumatic injuries, as well as the post-injury sensory and autonomic impacts, is another important step in both paediatrics and adult SCI research. This presents a whole new set of challenges.

The *in vitro* pilot study in Chapter 5 raised promising possibilities but will require considerable additional experimentation to produce robust and conclusive data. Resourcing for the necessary extensive research was outside the scope of the current study. These experiments need to be optimised and repeated with more animals, the pooling of spinal cords to increase the viable cell numbers as well as the exploration of a broader range of inflammatory mediators using both ELISA and RNA analysis. This initial pilot only used ELISA to assess the expression of cytokines at a protein level as the RNA yield from the extraction performed was too low to perform RT-qPCR. This was due to the high levels of cell death and low remaining cell numbers.

The assessment of these factors at an RNA level can be multiplexed to get a cross-section of the Th1 and Th2 cytokines involved in the response. An *in vitro* experiment using Bone marrow derived macrophages (BMDM) to produce cultures of specific M1 and M2 macrophages with more defined phenotypes and more robust cell numbers could also be used to investigate the soluble factor these cells produce and the effect they have when applied to other each other and other cultured immune and CNS cells. Co-culture or transwell experiments can be designed to begin to mirror the complexities of the response to these factors in the spinal cord, using combinations of macrophages, microglia, neurons and astrocytes. This may lead to a promising therapeutic path by modulating the immune response post-SCI in both adults and infants; and allowing the adult response to reflect the more beneficial infant response.

Overall, this thesis has reported significant differences between adult and infant injury progression and presentation, cellular response, inflammatory response, and behavioural recovery. The findings in this thesis may be used to guide two important avenues of research

1. By better understanding the unique nature and response to injury in immature spinal cords this can better inform the way that paediatric SCI is treated and managed. SCI incurred at a young age may be rarer than in adults, but it has a significant and life-long impact on the patient, their family and the healthcare system and is deserving of special attention.
2. If there are clear differences identified in the cellular (inflammatory) response in paediatric spinal cord injury that are deemed to be beneficial to functional recovery, then there is potential for these to be harnessed for the treatment of adult spinal cord injury. The targeted modulation of the inflammatory response after SCI could have great therapeutic benefit and understanding the paediatric response will also assist in informing the final goal of inflammatory modulation.

Muscle Function Grading

- 0 = total paralysis
- 1 = palpable or visible contraction
- 2 = active movement, full range of motion (ROM) with gravity eliminated
- 3 = active movement, full ROM against gravity
- 4 = active movement, full ROM against gravity and moderate resistance in a muscle specific position
- 5 = (normal) active movement, full ROM against gravity and full resistance in a functional muscle position expected from an otherwise unimpaired person
- 5+ = (normal) active movement, full ROM against gravity and sufficient resistance to be considered normal if identified inhibiting factors (i.e. pain, disuse) were not present
- NT = not testable (i.e. due to immobilization, severe pain such that the patient cannot be graded, amputation of limb or contracture of > 50% of the normal ROM)

Sensory Grading

- 0 = Absent
- 1 = Altered, either decreased/impaired sensation or hypersensitivity
- 2 = Normal
- NT = Not testable

When to Test Non-Key Muscles:

In a patient with an apparent AIS B classification, non-key muscle functions more than 3 levels below the motor level on each side should be tested to most accurately classify the injury (differentiate between AIS B and C).

| Movement | Root level |
|---|------------|
| Shoulder: Flexion, extension, abduction, adduction, internal and external rotation | C5 |
| Elbow: Supination | |
| Elbow: Pronation | C6 |
| Wrist: Flexion | |
| Finger: Flexion at proximal joint, extension. | C7 |
| Thumb: Flexion, extension and abduction in plane of thumb | |
| Finger: Flexion at MCP joint | |
| Thumb: Opposition, adduction and abduction perpendicular to palm | C8 |
| Finger: Abduction of the index finger | T1 |
| Hip: Adduction | L2 |
| Hip: External rotation | L3 |
| Hip: Extension, abduction, internal rotation | L4 |
| Knee: Flexion | |
| Ankle: Inversion and eversion | |
| Toe: MP and IP extension | |
| Hallux and Toe: DP and PP flexion and abduction | L5 |
| Hallux: Adduction | S1 |

ASIA Impairment Scale (AIS)

- A = Complete.** No sensory or motor function is preserved in the sacral segments S4-5.
 - B = Sensory incomplete.** Sensory but not motor function is preserved below the neurological level and includes the sacral segments S4-5 (light touch or pin prick at S4-5 or deep anal pressure) AND no motor function is preserved more than three levels below the motor level on either side of the body.
 - C = Motor incomplete.** Motor function is preserved at the most caudal sacral segments for voluntary anal contraction (VAC) OR the patient meets the criteria for sensory incomplete status (sensory function preserved at the most caudal sacral segments (S4-S5) by LT, PP or DAP), and has some sparing of motor function more than three levels below the ipsilateral motor level on either side of the body.
(This includes key or non-key muscle functions to determine motor incomplete status.) For AIS C – less than half of key muscle functions below the single NLL have a muscle grade ≥ 3 .
 - D = Motor incomplete.** Motor incomplete status as defined above, with at least half (half or more) of key muscle functions below the single NLL having a muscle grade ≥ 3 .
 - E = Normal.** If sensation and motor function as tested with the ISNCSCI are graded as normal in all segments, and the patient had prior deficits, then the AIS grade is E. Someone without an initial SCI does not receive an AIS grade
- Using ND:** To document the sensory, motor and NLL levels, the ASIA Impairment Scale grade, and/or the zone of partial preservation (ZPP) when they are unable to be determined based on the examination results.

Steps in Classification

The following order is recommended for determining the classification of individuals with SCI.

1. **Determine sensory levels for right and left sides.**
The sensory level is the most caudal, intact dermatome for both pin prick and light touch sensation.
2. **Determine motor levels for right and left sides.**
Defined by the lowest key muscle function that has a grade of at least 3 (on same testing), providing the key muscle functions represented by segments above that level are judged to be intact (graded as a 5).
Note: In regions where there is no myotome to test, the motor level is presumed to be the same as the sensory level, if testable motor function above that level is also normal.

3. **Determine the neurological level of injury (NLI)**
This refers to the most caudal segment of the cord with intact sensation and anal sphincter (3 or more) muscle function strength, provided that there is normal (intact) sensory and motor function rostrally respectively.
The NLI is the most cephalad of the sensory and motor levels determined in steps 1 and 2.

4. **Determine whether the injury is Complete or Incomplete.**
(i.e. absence or presence of sacral sparing)
If voluntary anal contraction = No AND all S4-5 sensory scores = 0 AND deep anal pressure = No, then injury is Complete.
Otherwise, injury is Incomplete.

5. **Determine ASIA Impairment Scale (AIS) Grade:**
Is injury Complete? If YES, AIS=A and can record ZPP (lowest dermatome or myotome on each side with some preservation)

- Is injury Motor Complete? If YES, AIS=B
- NO ↓ (No=voluntary anal contraction OR motor function more than three levels below the motor level on a given side, if the patient has sensory incomplete classification)

Are at least half (half or more) of the key muscles below the neurological level of injury graded 3 or better?

- NO ↓ AIS=C
- YES ↓ AIS=D

If sensation and motor function is normal in all segments, AIS=E

Note: AIS E is used in follow-up testing when an individual with a documented SCI has recovered normal function. If at initial testing no deficits are found, the individual is neurologically intact, the ASIA Impairment Scale does not apply.



Figure 2: Explanation of the scoring worksheet for the ASIA spinal cord injury scale from the International Standards for Neurological Classification of Spinal Cord Injury (pg 2).

CHAPTER 2:

| | | Injured Y/N? | | | Correct (=1) Incorrect (=0) | | | % Correct | | |
|-------|----|--------------|------------|--------|-----------------------------|------------|---------|------------|------------|---------|
| | | Assessor 1 | Assessor 2 | Actual | Assessor 1 | Assessor 2 | Average | Assessor 1 | Assessor 2 | Average |
| 13108 | D1 | n | n | y | 0 | 0 | 0 | 71.4 | 92.9 | 82.1 |
| 13109 | | n | n | n | 1 | 1 | 1 | | | |
| 13110 | | y | y | y | 1 | 1 | 1 | | | |
| 13111 | | n | n | n | 1 | 1 | 1 | | | |
| 13112 | | y | y | y | 1 | 1 | 1 | | | |
| 13113 | | n | n | n | 1 | 1 | 1 | | | |
| 13114 | | y | y | y | 1 | 1 | 1 | | | |
| 13115 | | y | n | n | 0 | 1 | 0.5 | | | |
| 13116 | | y | y | y | 1 | 1 | 1 | | | |
| 13117 | | y | n | n | 0 | 1 | 0.5 | | | |
| 13118 | | y | y | y | 1 | 1 | 1 | | | |
| 13119 | | n | n | n | 1 | 1 | 1 | | | |
| 13120 | | n | y | y | 0 | 1 | 0.5 | | | |
| 13121 | n | n | n | 1 | 1 | 1 | | | | |
| 1375 | D3 | n | n | n | 1 | 1 | 1 | 86.7 | 86.7 | 86.7 |
| 1382 | | y | y | y | 1 | 1 | 1 | | | |
| 1393 | | n | n | n | 1 | 1 | 1 | | | |
| 1392 | | y | y | y | 1 | 1 | 1 | | | |
| 1367 | | y | y | y | 1 | 1 | 1 | | | |
| 1379 | | y | n | n | 0 | 1 | 0.5 | | | |
| 1388 | | y | y | y | 1 | 1 | 1 | | | |
| 1385 | | n | y | n | 1 | 0 | 0.5 | | | |
| 1365 | | n | n | n | 1 | 1 | 1 | | | |
| 1380 | | y | y | y | 1 | 1 | 1 | | | |
| 1377 | | n | n | y | 0 | 0 | 0 | | | |
| 1379 | | y | y | y | 1 | 1 | 1 | | | |
| 1391 | | y | y | y | 1 | 1 | 1 | | | |
| 1370 | | y | y | y | 1 | 1 | 1 | | | |
| 1362 | | y | y | y | 1 | 1 | 1 | | | |
| 1337 | W1 | n | y | y | 0 | 1 | 0.5 | 60 | 50 | 55 |
| 1317 | | n | n | y | 0 | 0 | 0 | | | |
| 1338 | | y | y | y | 1 | 1 | 1 | | | |
| 1306 | | n | y | n | 1 | 0 | 0.5 | | | |
| 1323 | | n | n | y | 0 | 0 | 0 | | | |
| 1308 | | n | n | y | 0 | 0 | 0 | | | |
| 1331 | | n | n | n | 1 | 1 | 1 | | | |
| 1332 | | n | n | n | 1 | 1 | 1 | | | |
| 1324 | | n | y | n | 1 | 0 | 0.5 | | | |
| 1350 | | y | y | y | 1 | 1 | 1 | | | |
| 1357 | W2 | n | n | n | 1 | 1 | 1 | 55.6 | 55.6 | 55.6 |
| 1361 | | y | y | y | 1 | 1 | 1 | | | |
| 1366 | | n | n | y | 0 | 0 | 0 | | | |
| 1373 | | y | y | n | 0 | 0 | 0 | | | |
| 1359 | | n | n | y | 0 | 0 | 0 | | | |
| 1368 | | n | n | y | 0 | 0 | 0 | | | |
| 1396 | | n | n | n | 1 | 1 | 1 | | | |
| 1363 | | n | n | n | 1 | 1 | 1 | | | |
| 1390 | y | y | y | 1 | 1 | 1 | | | | |
| 1357 | W3 | n | n | n | 1 | 1 | 1 | 55.6 | 44.4 | 50.0 |
| 1361 | | y | n | y | 1 | 0 | 0.5 | | | |
| 1366 | | n | n | y | 0 | 0 | 0 | | | |
| 1373 | | n | n | n | 1 | 1 | 1 | | | |
| 1359 | | n | n | y | 0 | 0 | 0 | | | |
| 1368 | | n | n | y | 0 | 0 | 0 | | | |
| 1396 | | n | n | n | 1 | 1 | 1 | | | |
| 1363 | | n | n | n | 1 | 1 | 1 | | | |
| 1390 | n | n | y | 0 | 0 | 0 | | | | |
| 1357 | W5 | y | n | n | 0 | 1 | 0.5 | 33.3 | 33.3 | 33.3 |
| 1361 | | n | n | y | 0 | 0 | 0 | | | |
| 1366 | | n | n | y | 0 | 0 | 0 | | | |
| 1373 | | n | n | n | 1 | 1 | 1 | | | |
| 1359 | | y | n | y | 1 | 0 | 0.5 | | | |
| 1368 | | n | n | y | 0 | 0 | 0 | | | |
| 1396 | | n | n | n | 1 | 1 | 1 | | | |
| 1363 | | y | y | n | 0 | 0 | 0 | | | |
| 1390 | n | n | y | 0 | 0 | 0 | | | | |
| 1357 | W6 | n | n | n | 1 | 1 | 1 | 44.4 | 44.4 | 44.4 |
| 1361 | | n | n | y | 0 | 0 | 0 | | | |
| 1366 | | n | n | y | 0 | 0 | 0 | | | |
| 1373 | | n | n | n | 1 | 1 | 1 | | | |
| 1359 | | n | n | y | 0 | 0 | 0 | | | |
| 1368 | | n | n | y | 0 | 0 | 0 | | | |
| 1396 | | n | n | n | 1 | 1 | 1 | | | |
| 1363 | | n | n | n | 1 | 1 | 1 | | | |
| 1390 | n | n | y | 0 | 0 | 0 | | | | |

Figure 3: Raw data for blinded determination of injured or sham infants by two independent assessors.

CHAPTER 4:

Table 1: Mixture of conjugated antibodies for the M1/M2 macrophage panel

| Macrophage Panel: | |
|--------------------------|--------------------|
| Antibody (1) | 1 tube (μl) |
| CD45 (BD Biosciences) | 5 |
| CD68 (BioRad) | 10 |
| CD86 (BD Biosciences) | 5 |
| CD163 (BioRad) | 10 |
| + 5% NMS in FSW | 50 |

Table 2: Mixture of antibodies for the isotype controls equivalent to the M1/M2 macrophage panel

| Macrophage Isotype Control: | |
|------------------------------------|--------------------|
| Isotype control (1) | 1 tube (μl) |
| ApcCy7 (CD45) (BD Biosciences) | 5 |
| AF488 (CD68) (BD Biosciences) | 1 |
| PE (CD86) (BD Biosciences) | 5 |
| AF647 (CD163) (BD Biosciences) | 5 |
| + 5% NMS in FSW | 34 |

Table 3: Mixture of conjugated antibodies for the neutrophil and T-Cell panel

| Neutrophil and T-Cell Panel: | |
|-------------------------------------|--------------------|
| Antibody (2) | 1 tube (μl) |
| CD45 (BD Biosciences) | 5 |
| CD3 (BD Biosciences) | 5 |
| HIS-48 (BD Biosciences) | 10 |
| + 5% NMS in FSW | 60 |

Table 4: Mixture of antibodies for the isotype controls equivalent to the neutrophil and T-Cell panel

| Neutrophil and T-Cell Isotype Control (2) | |
|--|--------------------|
| Control (2) | 1 tube (μl) |
| ApcCy7 (CD45) (BD Biosciences) | 5 |
| PE (CD3) (BD Biosciences) | 1 |
| AF488 (HIS-48) (BD Biosciences) | 2 |
| + 5% NMS in FSW | 42 |

CHAPTER 5:

SIGMA-ALDRICH

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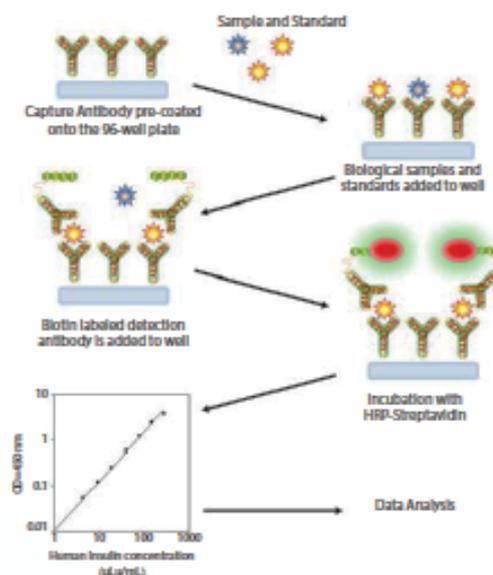
Product Information

Sigma's Sandwich ELISA kits are *in vitro* enzyme-linked immunosorbent assays for the quantitative measurement of soluble proteins in a variety of species. Our extensive ELISA selection includes cytokines, growth factors, proteases, soluble receptors, apoptosis effectors, and many other soluble proteins.

Sandwich Assay Procedure

1. Bring all reagents and samples to room temperature (18–25 °C) before use. It is recommended that all standards and samples be run at least in duplicate.
2. Add 100 μ L of each standard and sample into appropriate wells. Cover wells and incubate for 2.5 hours at room temperature or overnight at 4 °C with gentle shaking.
3. Discard the solution and wash 4 times with 1x Wash Solution. Wash by filling each well with Wash Buffer (300 μ L) using a multi-channel Pipette or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
4. Add 100 μ L of 1x prepared Detection Antibody to each well. Cover wells and incubate for 1 hour at room temperature with gentle shaking.
5. Discard the solution. Repeat the wash procedure as in step 3.
6. Add 100 μ L of prepared Streptavidin solution to each well. Cover wells and incubate for 45 minutes at room temperature with gentle shaking.
7. Discard the solution. Repeat the wash as in step 3.
8. Add 100 μ L of TMB One-Step Substrate Reagent (Item H) to each well. Cover wells and incubate for 30 minutes at room temperature in the dark with gentle shaking.
9. Add 50 μ L of Stop Solution (Item I) to each well. Read at 450 nm immediately.

How It Works



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Figure 4: Sigma-Aldrich recommended sandwich ELISA protocol.

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