Chapter 4: Liposome Study

4.1 Introduction

In the mammalian central nervous system (CNS) axotomised nerves do not regenerate. This is a property characteristic of the CNS and does not apply to injured nerves in the peripheral nervous system (PNS) (Ramón y Cajal, 1928), where axons have long been known to have remarkable regenerative abilities (Guth, 1956). Molecules normally present in the CNS, such as myelin and myelin-associated proteins, have been described to be inhibitory to axonal growth (David and Aguayo, 1981), especially when highly expressed after injury (Caroni and Schwab, 1988a; Schwab, 1990). Consequently, many of the damaged neurons will undergo atrophy and form 'dystrophic end balls' indicative of halted regenerative attempts at axonal extension (Ramón y Cajal, 1928).

In addition, sensitivity changes in injured neurons to myelin decreases the capacity for regrowth, unless myelination can be suppressed (Keirstead et al., 1992). A process, which has been shown to be effective for CNS regeneration in myelin-free spinal cords with the suppression of oligodendrocyte development and myelin formation (Savio and Schwab, 1990). During embryonic development the expression of growth-associated genes such as GAP-43 increases as axonal growth increases, however, as the CNS matures, the presence of these genes decreases (Chong et al., 1992; Fitzgerald et al., 1991). In addition, reduction in the regeneration of CNS neurons is also observed with alterations in the

expression of inhibitory factors on astrocytes and extracellular matrix molecules, also contribute to the limited neurite outgrowth observed after CNS injury (McKeon et al., 1991) by acting as a molecular barrier inhibiting axonal growth of regenerative processes (Pindzola et al., 1993).

Interventions that improve neuronal survival or reduce the exposure of axons to CNS inhibitors has resulted in the successful regeneration of injured CNS neurons and has provided information regarding the intrinsic capacity of adult neurons to regenerate. For instance, as aforementioned evidence for this came from an earlier *in vivo* study by David and Aguayo (1981), where peripheral nerve grafts were used to circumvent the inhibitory properties of the CNS (David and Aguayo, 1981). Their findings indicated that at least some injured CNS axons retained a limited capacity for regrowth, thus overturning previous misconceptions that the CNS was incapable of regenerating after injury (Ramón y Cajal, 1928). This, of course was investigated in earlier studies using peripheral nerves as part of a collective approach to encourage CNS regeneration (Aguayo et al., 1981; Richardson et al., 1982), which also demonstrated the intrinsic ability for CNS neurons to regenerate when provided with a PNS environment.

Furthermore, using a peripheral nerve graft, Richardson and Issa (1984) investigated the regenerative effect of injured CNS afferent neurons after their corresponding peripheral dorsal root ganglion (DRG) branch was also axotomised. This landmark experiment demonstrated a greater amount of

primary sensory neuron regeneration than in any other CNS regenerative *in vivo* model (Richardson and Issa, 1984). Reasons for the robust spinal cord regeneration following a peripheral nerve transection were unclear. Although, it has been proposed that inflammatory cells (e.g. macrophages) as well as satellite cell activation and proliferation near the DRG cell body, as part of the normal PNS response to injury, might be playing a crucial role (Lu and Richardson, 1991). Yet, to this date the precise mechanism regulating this pre-conditioning lesion remains largely unknown.

Generally, in terms of the inflammatory response, previous studies have shown that at the site of SCI, the recruitment number and the phagocytic activity of macrophages can be modulated to improve the clearance of myelin debris via the intraperitoneal administration of inflammatory agents like lipopolysaccharide (LPS) (Vallieres et al., 2006). Similarly, macrophage activation via the intravitreal injections of Zymosan, a potent inflammatory activator, provided important signaling molecules for the successful regeneration of injured axons, which was dependant on the timing of macrophage activation (Yin et al., 2003). Interestingly, the beneficial contribution of macrophage cells in the injured CNS has been reported to being sluggish as compared to what is observed after peripheral nerve injury (Perry et al., 1987). In fact, exposure of macrophages to segments from either the PNS or CNS resulted in the activation of macrophages with different functionality, displaying increased phagocytic activity after PNS exposure as compared to decreased activity after CNS exposure (Zeev-Brann et al., 1998). These peripherally

activated macrophages when transferred to the CNS, resulted in axonal regrowth of injured neurons, which was attributed to the extent of phagocytosis as a determinant of macrophage activation (Lazarov-Spiegler et al., 1996; Rapalino et al., 1998).

In the CNS, however, the beneficial effects of macrophage activation are not always guaranteed and are often classified as a 'double-edge sword', due to both favourable and detrimental outcomes exerted by these cells (Bethea, 2000; Wyss-Coray and Mucke, 2002). For instance, the inflammatory response to CNS damage such as after SCI, has been described as a contributor to chronic deficits associated with the effects of secondary injury, which is consistent with the robust infiltration of macrophage cells and their level of phagocytic activation (Blight, 1985). Certainly, in the injured CNS, macrophages are the predominant cells observed "in greatest numbers and for the longest duration" (Popovich et al., 1999; Schnell et al., 1999b). Consequently, studies targeting the selective depletion of macrophage cells reported beneficial effect in CNS repair, recovery of locomotor functionality and enhanced regeneration of injured CNS neurons (Blight, 1994; Popovich et al., 1999). Conversely, many studies have argued for the beneficial effects of macrophages after CNS trauma (Batchelor et al., 1999; Bomstein et al., 2003; David et al., 1990; Lazarov-Spiegler et al., 1996; Luk et al., 2003; Ohlsson et al., 2004; Yin et al., 2003). Therefore, it is clear that this dichotomy in macrophage activation has complex characteristics that have proven problematic in the adequate design of regenerative CNS therapies.

4.1.1 Project Rationale

Recent studies have shown that the exogenous application of cAMP (cyclic adenosine monophosphate) to DRGs can mimic the regeneration of the injured CNS afferent branch, normally characteristic of the pre-conditioned lesion (Neumann et al., 2002). This regenerative response in injured CNS axons to cAMP treatment, has mechanistically been attributed to the blocking and to a reduction in sensitivity to myelin inhibitors in the CNS (Qiu et al., 2002). Elevation in cAMP by the priming/exposure of neurons to trophic factors such as BDNF has also resulted in the regrowth of neurons within the presence of inhibitory molecules such as myelin and MAG (Cai et al., 1999). This has provided some useful information regarding the underlying molecular mechanism/s mediating the pre-conditioned lesion model. Given that exposure to trophic factors such as BDNF, has been shown to be beneficial for regeneration (Bouhy et al., 2006; Kobayashi et al., 1997; Mamounas et al., 2000; Xu et al., 1995).

Currently, there is limited information regarding the cellular mechanisms mediating the observed regeneration in pre-conditioned lesion DRG neurons. Although, it has been proposed that the inflammatory response in the DRG following peripheral nerve axotomy might be playing a crucial role in the regeneration of these adult neurons (Lu and Richardson, 1991). Here, to further our understanding into the cellular mechanism/s underlying the regeneration of primary afferent neurons in the CNS after a pre-conditioned peripheral lesion, we will explore the *in vivo* effects of

macrophage depletion and ascertain the relationship between macrophage cells, BDNF, peripheral nerve axotomy and the regeneration of adult DRG neurons. To do this, we will be using a liposome-mediated approach, which was chosen for the following reasons.

4.1.2 Proposed Testing

The role of macrophage cells has been made possible through the selective depletion of these cells via their phagocytic mechanism to examine their functionality *in vivo* (Van Rooijen and Kesteren-Hendrikx, 2003). Over the years, some of these methods have included the use of asbestos (Kagan and Hartmann, 1984), carrageenan (Shek and Lukovich, 1982), antibodies against a macrophage receptor involved in phagocytosis (Bruck and Friede, 1990) and the administration of silica dust (Blight, 1994). However, a more effective approach based on the phagocytic properties of macrophage cells was developed by van Rooijen and van Nieuwmegen in 1984. This involved the intracellular delivery of an artificially constructed phospholipid vesicle (e.g. liposome) encapsulating a particular drug known as clodronate (Van Rooijen, 1989; Van Rooijen and van Nieuwmegen, 1984). Delivery of these liposome-containing drug, resulted in the ingestion and consequent disruption of the phospholipid bilayers of the liposome by cellular phospholipases, thereby releasing clodronate molecules intracellularly (Van Rooijen and Sanders, 1994). In addition, the accumulation of these molecules resulted in intracellular changes causing macrophages to undergo apoptosis (Van Rooijen and Sanders, 1996). More importantly, clodronate molecules released into the

bloodstream from dead macrophages do not re-enter cells, given that these have a short half life and are not able to cross cell wall membranes (Van Rooijen and van Kesteren-Hendrikx, 2002).

In essence, this liposome-mediated approach to macrophage “suicide” has enabled its effective use in a wide range of inflammatory conditions including: rheumatoid arthritis (van Lent et al., 1998), autoantibody-mediated disorders (Alves-Rosa et al., 2000), transplantation (Omer et al., 2003; Slegers et al., 2000), neuropathic pain (Liu et al., 2000), multiple sclerosis (Huitinga et al., 1990), spinal cord injury (Popovich et al., 1999), Wallerian degeneration (Bruck et al., 1996; Kubota and Suzuki, 2000), psoriasis (Stratis et al., 2006) and pancreatis (Shifrin et al., 2005).

4.1.3 Hypothesis

In adult DRG neurons, the in situ inflammatory response that occurs after peripheral nerve axotomy is believed to be crucial in the regeneration of the CNS afferent branch in the CNS (Lu and Richardson, 1991). It is then conceivable that inflammatory cell depletion prior, during and after axotomy of the peripheral DRG branch (conditioning lesion), could suppress regeneration of the afferent branch in the CNS. A positive finding here would indicate a strong involvement of macrophage cells in the pre-conditioning of the ganglia, responsible for CNS regeneration.

4.1.4 Model of Injury

Here, as aforementioned in Chapter 2, we will be referring back to original model described by Richardson and Issa (1984) where the peripheral DRG branch will be injured (day 0) and this will be followed by injury to the DRG branch extending in the CNS (day 7). Again, the timing of the CNS injury relates to the optimal conditioning of DRG neurons, which occurs at seven days post peripheral nerve axotomy, resulting in the maximum amount of CNS regeneration possible (Richardson and Issa, 1984) (Fig. 27).

4.2 Method and Materials

4.2.1 Animals

Adult female Sprague Dawley (SD) rats (10-12 weeks) were used under the guidelines of the National Health and Medical Research Council of Australia and approved by the Animal Welfare Committee of Flinders University of South Australia.

4.2.1.1 Treatment Groups

Animals were divided into 2 experimental groups, both of which received a sciatic nerve lesion (day 0) followed by a spinal cord injury (SCI, day 7), as per original model described by Richardson and Issa (1984). *Control group* (n=10) received intravenous (i.v.) tail vein injections of sterile saline only (2ml/injection); *Test group* (n=10) received i.v. tail vein injections of

liposome-encapsulated clodronate (2ml/injection). The only difference between these two groups was the use of liposomes in the test group to systemically deplete macrophage cells. Note that animals were randomly allocated to each group and any individual differences found between animals were attributed to the treatment effects.

The main reason for not using liposome-encapsulated saline in our control group as explained by Gray and colleagues (2007), was due to the possibility that liposomes alone may "block macrophage phagocytosis by saturation and may suppress or activate other macrophage functions" (Gray et al., 2007). Consequently, this would have resulted in inconsistent comparisons between our treatment and our control group.

4.2.2 Surgical Procedures

All surgical procedures were conducted under an operating microscope.

4.2.2.1 Sciatic Nerve Injury (SNI)

Briefly, a longitudinal cut was made on the skin adjacent the femur followed by a blunt dissection through the leg muscles. The sciatic nerve was then exposed, ligated just proximal to its trifurcation and cut below the ligation site with fine surgical scissors (refer to chapter 2 section 2.2.3.1 for more information).

4.2.2.2 Spinal Cord Injury (SCI)

Briefly, spinal cords were exposed by a laminectomy between vertebrae T9-T10 followed by a small incision in the dura mater. This type of spinal injury (i.e. dorsal column cut) was performed by positioning the tips of open-blade, fine microsurgical scissors on either side of the exposed spinal cord and subsequently closing them for about 10 seconds. This was carried out at a depth of approximately 1.5-2mm (marked by a black line on the scissors' tip) and resulted in a bilateral cut through the dorsal columns (Qiu et al., 2005). A small piece of gelfoam was temporarily placed over the lesion site to encourage blood clotting and the overlaying muscles were sewn together with a 6/0 surgical suture. The skin was stapled and the animals were returned to their cages.

Note that after each surgery, animals were housed separately and received subcutaneous injections of the analgesic drug buprenorphine (0.03 mg/kg) for a period of up to 5 days to alleviate postoperative pain. For all spinal cord injured animals manual bladder expression was performed twice to three times per day and were treated with antibiotics if required (Brambilla et al., 2005).

4.2.2.2.1 Retrograde Tracer Injection

To investigate the presence of regenerated fibres in the injured dorsal column of the spinal cord (i.e. across the injury epicentre), a somatic retrograde tracer fast blue (FB, 5% in saline, Sigma) was injected into the

proximal stump. As aforementioned, ascending afferent fibres originating from the distal stump, if regenerated, pick up the FB tracer from the proximal stump, retrogradely transport it and consequently label their neuronal cell bodies back to their respective ganglia (Ahmed et al., 2001; Song et al., 2008).

Briefly, immediately after SCI, using a stereotaxic frame and a pulled glass micropipette needle, FB was directly injected into the dorsal column of the proximal stump at 3-4mm rostral from the SCI epicentre (total volume delivered 0.1μl). To absorb any tracer leakage, sterile cotton balls were positioned around the injection site during the procedure. The needle was then carefully removed from the spinal cord, which entailed leaving the needle in situ for an additional 2 minutes post tracer delivery, to avoid fluid drawback. Note that care was taken not to further damage the spinal cord.

4.2.2.2.2 Anterograde Tracer Injection

To further examine the presence of ascending regenerated fibres across the injury epicentre, an anterograde tracer, biotinylated dextran amine (BDA, 10% in saline, 10000mw, Molecular Probes), was injected into the lumbar region of the spinal cord in the ipsilateral column corresponding to the sciatic nerve injury (Feng et al., 2008; Hughes et al., 2003; Novikov, 2001). This type of tracing facilitated labelling of ascending afferent fibres within the dorsal column of the spinal cord.

Briefly, using a stereotaxic frame and a pulled glass micropipette needle, BDA was directly injected into the dorsal column of the spinal cord, ipsilateral to the sciatic nerve lesion at a depth of 1-1.2mm (total volume delivered 0.1μl). As previously mentioned, the possibility of tracer leakage was minimised by placing sterile cotton balls around the injection site.

4.2.3 Clodronate-Encapsulated Liposome

Liposomes are artificially prepared spheres consisting of concentric phospholipids bilayers (Fig. 28). These layers form when phospholipids (e.g. phosphatidylcholine) are dispersed in water by conforming the hydrophobic fatty acid chain part of the inner side of the bilayer and allowing the hydrophilic head groups form the outer parts of the bilayer (Van Rooijen and Sanders, 1994). In addition, part of the aqueous solution together with hydrophilic molecules dissolved in it (e.g. clodronate) are encapsulated during liposomal formation (Van Rooijen and van Kesteren-Hendrikx, 2002) (Fig. 28).

4.2.3.1 Liposome Preparation

The following method was prepared according to previous studies using this particular technique (Buiting and Van Rooijen, 1994; Popovich et al., 1999; Van Rooijen and Kesteren-Hendrikx, 2003; Van Rooijen and Sanders, 1994) and through personal communications with liposome pioneering author Dr Nico van Rooijen (Netherlands).

Briefly, 86mg of egg phosphatidylcholine and 8mg of cholesterol were dissolved in 5ml of chloroform in a round-bottom flask. The chloroform was removed by using a low-vacuum rotary evaporator at 37°C to form a thin lipid film around the flask. The lipid was then dispersed with 10ml sterile phosphate buffered saline (PBS, 0.1M, pH 7.4) containing 2.5gm of clodronate (dichloromethylene-diphosphonate-DMDP, Sigma) and incubated on a gentle stirrer at room temperature (RT) for 2 hours.

After incubation, the suspension was sonicated for 3 minutes (50 Hz) at RT and incubated again for 2 hours at RT with no stirring to allow for liposome formation. Liposomes were centrifuged for 15 minutes at 10,000g RT to remove any free clodronate. The remaining pellet was washed twice in sterile PBS at 20,000g for 30 minutes RT and finally resuspended in 4ml sterile PBS to be used immediately or stored under nitrogen at 4^0C to use within 7 days (Buiting and Van Rooijen, 1994; Claassen and Van Rooijen, 1986; Van Rooijen and Sanders, 1994).

4.2.3.2 Liposome Administration

In the pre-conditioned lesion, for the greatest amount of afferent CNS regeneration to occur, the SCI must be performed 7 days post peripheral nerve lesion (Richardson and Issa, 1984). This is believed to be due to the normal inflammatory response in the ipsilateral DRG post peripheral injury (Lu and Richardson, 1991).

To verify this, cytotoxic liposomes targeting inflammatory cell depletion were delivered intravenously at specific time intervals to temporarily suppress the inflammatory response that occurs post peripheral nerve lesion. For all test animals, liposomal delivery was administered at: a) three days prior to; b) immediately after; and c) four days post peripheral conditioning lesion (Fig. 29). All control animals received injections of sterile saline only, delivered in the same timely fashion as per test group intervals. The specific timing of liposome injections ensured macrophage depletion commencing 3 days prior to peripheral nerve lesion and ending 3 days post SCI. It is known that macrophage cells are depleted within 24 hours post liposomal treatment and begin to slowly repopulate approximately 5-7 days post treatment completion (personal communication with Dr Nico van Rooijen). This allowed us to modify our methods according to those described by Popovich and colleagues (1999).

Note that there were no adverse side effects associated with this particular treatment in any of the experimental animals used in this study.

4.2.4 Perfusion

All animals were injected i.p. with 5% chloral hydrate in distilled water and perfused transcardially with 1% sodium nitrite ($NaNO_2$) in phosphate buffer (PB, 0.1 M, pH 7.4) followed by a 4% paraformaldehyde (PFA) fixative flush in PB (0.1 M, pH 7.4). Perfusions were performed at 4 weeks post CNS lesion with all dissected tissues post-fixed in 4% PFA and

cryoprotected in 30% sucrose solution in PB (0.1 M, pH 7.4), each for 48 hours at 4^0Celsius (refer to chapter 2 section 2.2.4 for more details).

4.2.5 Cryosectioning

All dissected tissues were post-fixed and cryoprotected accordingly prior to cryosectioning (refer to section 4.2.4 for more information). Cryosectioning was performed on a cryostat microtome at -20^0 Celsius and due to the morphological variation of dissected tissues, these were cryosectioned at different thicknesses. Spleens were cut at 20μm (cross-sections), DRGs were cut at 20μm (coronal sections) and spinal cords were cut at 40μm (longitudinal sections). All specimens were mounted on 2% gelatine-coated glass slides.

4.2.6 Immunohistochemistry

All immunohistochemistry (IHC) procedures were done as per specified protocols using single or double labelling immunoreactivity where appropriate (Li et al., 2002; Song et al., 2008).

4.2.6.1 Fluorescence Staining

The primary antibodies used in this study for single and/or double labelling were: mouse-anti-rat cluster differentiation 68 (CD68, macrophage, 1:400, Serotec), rabbit-anti-glial fibrillary acidic protein (GFAP, astrocyte, 1:500, Dako).

A combination of the following secondary antibodies for single and/or double labelling were: sheep-anti-mouse-cy3-IgG (Jackson, 1:500), donkey-anti-mouse-cy2-IgG (Jackson, 1:500), donkey-anti-rabbit-488-IgG (Jackson, 1:500), sheep-anti-rabbit-cy3-IgG (Jackson, 1:500).

Note that a similar general IHC protocol was followed throughout this thesis (refer to chapter 2 section 2.2.6 for more information).

4.2.6.2 Immunoperoxidase Staining

BDA injected tissue was treated in 3% hydrogen peroxide (H_2O_2) in 100% methanol at RT for 10 minutes, rehydration in phosphate buffer solution (PBS, 0.1M, pH 7.4) and thorough washing in PBS containing 0.1% detergent Tween-20 (PBST). This was followed by incubation with streptavidin-HRP conjugated antibody (1:2000, Vector Laboratories) in PBST at RT for 60 minutes. After extensive washing with PBST, sections were developed in a solution containing 0.05% 3'3-diaminobenzidine tetrahydrochloride (DAB, Sigma), 0.06% nickel sulfate ($NiSO_4$) and 0.005% glucose oxidase (Novikov, 2001; Shu et al., 1988).

4.2.7 Relative DRG Somatic Count

The relative number of retrograde labelled FB^+ DRG neurons was obtained by serially counting every fourth section of the ipsilateral and contralateral L4 DRG (total of 5 sections/animal, n=5). This method of counting allowed for the relative estimation of FB labelled cell bodies and

to avoid the possibility of double counting, only those neurons where the nucleus was visible were counted (Li et al., 2002; Zhou et al., 1991). Note that in all instances, FB^+ neurons were considered regenerated neurons (Song et al., 2008).

4.2.8 Cell Quantification Method

Briefly, using ImageJ (image processing program, NIH version 1.37) 20x magnification images stained for the aforementioned antibodies were converted to binary contrast images (black and white). This provided a threshold by subtracting background levels from the immunoreactive stained areas and allowed the determination of the percentage area fraction per image to be collected, tabulated and statistically analysed (Gray et al., 2007; Hu et al., 2007; Maruyama et al., 2005; van Wijngaarden et al., 2007) (refer to chapter 2 section 2.2.9 for more details).

4.2.8.1 Statistics

In all graphs, columns represent an averaged mean (n=5 as specified per figure) and error bars indicate standard error of mean (+/- S.E.). Comparisons between groups were made by ANOVA and/or independent samples t-test where appropriate. Results were considered significant if $P<0.05$.

4.2.9 Sandwich BDNF ELISA Test

BDNF expression is known for its favourable action on regeneration (Mamounas et al., 2000; Plunet et al., 2002), its neuroprotective effects (Carson, 2002; Donelly and Popovich, 2008) and its secretion from macrophage cells (Bouhy et al., 2006; Moalem et al., 2000; Schulte-Herbruggen et al., 2005) after peripheral and/or CNS injury. Therefore, this was also tested here to examine BDNF concentrations in relation to each of the treatments provided to each animal.

Briefly, at the end of the experimental period serum samples were collected from all animals. These samples were tested using a commercially available ELISA kit pre-coated with BDNF (Millipore, USA, www.millipore.com). This provided an estimate of the BDNF serum concentration level that was measured and analysed in all animals (refer to chapter 2 section 2.2.10 for protocol information) (refer to Appendix A Fig. A4 for standard BDNF curve).

4.3 Results

4.3.1 Effectiveness of Macrophage Depletion

IHC analysis of spleen sections immunostained for macrophage cells ($CD68^+$) were used to determine the efficacy of macrophage depletion using clodronate liposomes (Gray et al., 2007). Our results revealed that during liposome treatment there were no macrophage cells present in the spleens of liposome-treated animals (Fig. 30A-B). The effectiveness of

liposome use in macrophage depletion was confirmed in organs with an open vascular supply, such as in the spleen (Popovich et al., 1999). Spleens tested at 2-weeks post liposome treatment completion showed early signs of macrophage repopulation evident by the amount of $CD68^+$ immunoreactivity in the tissue (Fig. 30C-D). Qualitatively, at the end of the experimental period (day 35) macrophage numbers in the spleens of liposome-treated animals had returned to normal levels, as compared to untreated spleens (Fig. 30E-H).

4.3.2 Retrograde Tracing in DRG

Quantification of retrograde labelled FB^+ neuronal cell bodies in the ipsilateral L4 DRG revealed some interesting variations between experimental groups. As with previous observations by Richardson and Issa (1984), our results were consistent with their findings where saline-treated control animals displayed extensive positive retrograde labelling in the ipsilateral DRG (white arrows, Fig. 31A-B) (Richardson and Issa, 1984). Intriguingly, however no FB^+ labelled cell bodies were found in the ipsi- and contra-lateral DRGs of liposome-treated animals (red arrows, Fig. 31C-D). Statistically, the presence of FB^+ labelled neurons in our control group as compared to unlabelled neurons in our liposome-treated group indicated a lack of retrograde labelling in the latter group ($P<0.001$; Fig. 31E). This could have resulted from poor attempts at axonal regeneration, thus far attributed to the absence of macrophage cells. The possibility that regeneration could still be present in the spinal cord of liposome-treated

animals was further explored using anterograde tracing (refer to section 4.3.3).

It is worthwhile to note that although it is possible that tracer leakage could have affected retrograde labelling in the liposome-treated group, this seems unlikely given that tracer leakage out of the injection site and into the SCI epicentre, would have provided similar numbers of positively labelled cell bodies in both ipsilateral and contralateral DRG, however this was not found (Fig. 31E). Note that in all instances, the contralateral DRG was used as the control (uninjured) side.

4.3.3 Anterograde Tracing in Spinal Cord

Normally in the pre-conditioned lesion model, regenerated ascending afferent fibres can be seen extending through the dorsal column and across the SCI site (Richardson and Issa, 1984). Here, we assessed this by using an anterograde BDA tracer previously injected into the lumbar region of the injured spinal cord. The results from our control group corroborated aforementioned findings by Richardson and Issa (1984), displaying the presence of ascending afferent fibres extending from the distal stump, across the SCI and into the proximal stump, in a rostral direction (black arrows, Fig. 33A-G) (Richardson and Issa, 1984).

Conversely, in our liposome-treated group no regenerated fibres were found across the SCI epicentre (Fig. 32A-G). Higher magnification images of BDA labelled fibres in the distal stump, revealed fibre retraction and

collapse near the SCI site, with no BDA labelled fibres observed rostrally in the proximal stump (black arrows, Fig. 32B). The lack of regenerated fibres across the SCI epicentre was consistent with the absence of retrograde labelled FB^+ DRG neurons in this liposome-treated group (refer to section 4.3.2). This finding confirmed previous assumptions relating to abortive attempts at axonal regeneration and offers tangible explanations for the role of macrophage cells in the pre-conditioned model of injury.

4.3.4 Macrophage and Astrocyte Quantification

Macrophage quantification at 1-2mm both rostral and caudal from the SCI epicentre revealed greater macrophage numbers ($CD68^+$) in the saline-treated group, as compared to the liposome-treated group (Fig. 34A, $P<0.05$ & $P<0.01$, respectively). No statistically significant difference between experimental groups was found at the SCI epicentre with macrophage numbers remaining constant in both experimental groups. As expected, the highest number of macrophage cells was observed caudally to the SCI as opposed to rostrally. This variation, although not examined in detail, most likely reflects Wallerian degeneration effects resulting from the mechanical injury and/or extent of myelin clearance by macrophage cells (Vargas and Barres, 2007). Hypothetically, this difference in macrophage numbers between these experimental groups might be directly responsible for the pro-regenerative response observed in the control group, as opposed to the abortive one in the liposome-treated group. Several reasons supporting these observations are addressed in the discussion section.

On the other hand, astrocyte quantification ($GFAP^+$) 3mm caudal from the SCI epicentre reached similar levels in both experimental groups with no statistical differences found (Fig. 34B). Conversely, 3mm rostral from SCI, there was a significantly higher astrocyte expression ($GFAP^+$) in the liposome-treated group as compared to the control group ($P<0.05$). Furthermore, this constant increase in astrocyte expression located at both extremes of the SCI epicentre, was only observed in the liposome-treated group and not in the saline-treated group. In the latter group, there was a noted significant reduction in astrocyte expression in the proximal stump as compared to the distal stump (Fig. 34B, $P<0.05$). Again, this was not examined further, but this difference could be associated to the effects of Wallerian degeneration. Hypothetically, this difference in GFAP expression in the proximal stump might be a cellular representation for the amount of scar formation in and around the SCI epicentre, consequently inhibiting axonal regeneration (Faulkner et al., 2004; Sofroniew et al., 2001).

4.3.5 BDNF ELISA Serum Levels

Calculated serum levels from both experimental groups revealed a significantly higher BDNF concentration level in saline-treated animals, as compared to liposome-treated animals (Fig. 35, $P<0.05$). The fact that immune cells, such as macrophage cells are able to support neurite outgrowth by secreting neurotrophic factors such as BDNF (Batchelor et al., 1999; Bouhy et al., 2006; Deng et al., 2000), offers a plausible

explanation as to why regenerated fibres appeared absent in liposome treated animals.

4.4 Limitations

Successful macrophage depletion by the administration of liposomal clodronate has been well documented in several studies (Bruck et al., 1996; Huitinga et al., 1990; Liu et al., 2000; Shifrin et al., 2005; Stratis et al., 2006) and here our results have also confirmed the effectiveness of this technique in macrophage depletion. However, this phagocytic suicide technique, has been suggested to result in impaired spleen functionality due to platelet displacement from the blood to the spleen (Shibazaki et al., 1998), as well as causing neutrophil depletion and toxicity following liposomal administration (Duffield et al., 2005). Undoubtedly, these factors could have played a role in the *in vivo* model tested here, however, these were not examined during the study.

Neutrophils, according to a recent review, are often neglected even though these cells are capable of mediating immune responses by either promoting or liming the extent of inflammation and also by providing instructions to white blood cells such as macrophages (Nathan, 2006). Therefore, their depletion and/or cytotoxicity could have potentially altered the immunological repertoire of restored macrophage cells within the liposome-treated group. Nevertheless, regardless of this, our results still offer a plausible explanation for the role of inflammatory cells in the pre-conditioned lesion model. Methodologically, this validates the

administration of saline alone in our control group without incorporating it into a phospholipid vesicle.

4.5 Discussion

Intravenously injected liposomes containing clodronate molecules phagocytosed by macrophages causing a rapid, temporary and selective depletion of these cells from the circulation within 24-48 hours post treatment administration (Van Rooijen et al., 1990; Van Rooijen and Sanders, 1994). In our study, this phagocytic suicide approach resulted in successful macrophage cell depletion and subsequent abrogation of the inflammatory response associated with the pre-conditioning peripheral lesion. Furthermore, the timely delivery of these liposomes ensured the depletion of macrophages prior to, during and after the 'critical 7 day period' post peripheral axotomy; determined as the conditioning time of DRG neurons responsible for the greatest amount of CNS regeneration (Richardson and Issa, 1984).

4.5.1 Effects of Macrophage Depletion

Interestingly, our results revealed that while normally the pre-conditioned peripheral lesion supports afferent CNS regeneration of adult DRG neurons (Richardson and Issa, 1984), depletion of inflammatory cells completely abolished this pro-regenerative effect. Comparisons between the control and liposome group indicated a considerable lack of regenerated fibres in the latter group as confirmed by retrograde tracing.

Whereby, in macrophage depleted animals no FB^+ neurons were found in either the ipsilateral or contralateral DRG, as compared to a significantly greater number of FB^+ labelled neurons found in the DRG of control animals. This absence in retrograde labelled DRG neurons was further investigated by anterograde tracing of ascending afferent fibres.

Concomitantly, evidence from the anterograde tracing study revealed no BDA labelled fibres found across the SCI epicentre in macrophage depleted animals. Closer examination of ascending BDA labelled fibres in liposome-treated animals displayed extensive axonal collapse and retraction neighbouring the SCI epicentre, represented by the presence of bulb-like structures at the end of axons (Ramón y Cajal, 1928), as opposed to lengthy BDA labelled regenerated afferent fibres found both rostral and caudal to the SCI epicentre in saline-treated animals. In fact, astrocyte quantification in macrophage-depleted animals indicated higher astrocytic expression levels both rostral and caudal to the lesion. Comparatively, in control animals this level of astrocyte expression although similar caudally, was significantly reduced rostral to the lesion, as observed in liposome-treated animals. This level of astrocyte expression is likely to represent the amount of glial scar formation, thus creating an inhibitory barrier against neurite growth (Faulkner et al., 2004; Silver and Miller, 2004; Sofroniew et al., 2001).

On the other hand, macrophage quantification at the end of the experimental period in both groups revealed greater macrophage numbers

in saline-treated animals, as compared to liposome-treated animals. Specifically, in the former group, this difference in macrophage numbers was significantly higher in regions rostral and caudal to the lesion, as compared to liposome test animals. Note that, we do not attribute variations in macrophage numbers between groups solely to the depletion of these cells given that spleens of liposome-treated animals showed cellular repopulation to normal levels. Instead, we propose a reduced activation and infiltration of macrophage cells into the CNS parenchyma due to their absence during the acute conditioning phase following peripheral axotomy, which we believe is critical for the early immune cell infiltration to the CNS (Zhang et al., 2007).

Moreover, analysed blood samples from both experimental groups, revealed higher BDNF serum concentration levels in saline-treated animals. This difference in endogenous BDNF levels, at least for injured peripheral nerves has been demonstrated to support axonal regeneration and myelination following trauma (Zhang et al., 2000) and this is most likely also contributing to the regeneration of ascending afferent fibres in spinal injured animals (Song et al., 2008). Certainly, axotomy of the peripheral DRG branch could potentially be stimulating macrophage cells with a phenotype beneficial for CNS regeneration, given that their activation with peripheral nerve fragments *ex vivo* resulted in an enhanced phagocytic ability and capacity to secrete trophic factors like BDNF (Lazarov-Spiegler et al., 1996; Zeev-Brann et al., 1998). Which provides a

probable mechanistic cause for the successful regenerative capability of mature neurons in the pre-conditioned lesion.

4.5.2 Possible Macrophage Contribution

In fact, it appears that the timely liposomal depletion of macrophage cells completely abolished the pre-conditioning regenerative effects of the peripheral nerve injury. Thus, transforming this regenerative model into a normal CNS lesion, where mature neurons do not regenerate. Undoubtedly, several contributing factors could have affected this pro-regenerative mechanism and from our point of view, hereafter we will briefly attempt to address two of these main factors: myelin clearance and CNS milieu priming.

4.5.2.1 Poor Myelin Clearance

One explanation why the pre-conditioned lesion model is consistently able to trigger CNS regeneration of the afferent DRG branch, might be due to the larger numbers of peripheral macrophage cells entering the CNS and aiding myelin removal (Zeev-Brann et al., 1998). Indeed, myelin clearance during Wallerian degeneration by circulating macrophage cells has been previously described as one of the major differences between the PNS and the CNS (Bruck et al., 1996; Vargas and Barres, 2007). Furthermore, the poor clearance of myelin debris has been reported to impede the necessary repair process in inflammatory conditions, such as multiple sclerosis (MS) (van Rossum et al., 2008). Finally, indirect evidence from

our earlier study investigating the effects of sciatic nerve vaccination revealed that, proportionally, animals with the greatest amount of macrophage cells experienced more CNS regeneration. Which is most likely associated with a rapid myelin clearance from the CNS environment, consequently creating a growth-supporting matrix for injured CNS neurons to regenerate.

4.5.2.2 CNS Milieu Priming

In a study Zhang and colleagues (2007), authors demonstrated that peripheral nerve injury induced circulating macrophage cell infiltration into the spinal cord, where these cells proliferated and differentiated into microglia cells (Zhang et al., 2007). Similarly, in our previous chapter investigating the effects of SNI and ONC, we demonstrated an increased influx of macrophage cells into the CNS, found throughout the spinal cord and optic nerve tissue, post peripheral nerve axotomy. This observation as aforementioned, suggests a plausible explanation for this cell infiltration into CNS milieu, consequently increasing immune cell surveillance, which could be regarded as a form of compartmental priming not well understood (Kleine and Benes, 2006). Nonetheless, immune surveillance into the uninjured CNS environment introduces new functions in “bi-directional communication between the CNS and immune system” (Quan and Banks, 2007). Therefore, here we believe we have provided further evidence regarding the neuroimmune interaction in CNS regeneration, presumably by supporting a complex communication process between the peripheral

and central nervous system, despite of the immune privilege status of the CNS (David and Ousman, 2002; Pachter et al., 2003).

4.6 Conclusion

Here, we have highlighted the importance of the normal inflammatory cascade following peripheral nerve injury and its contribution to afferent CNS regeneration of pre-conditioned adult DRG neurons. In particular, we ascribe a beneficial role in CNS regeneration to inflammatory cells, since *in vivo* macrophage depletion led to astrocyte up-regulation, reduced macrophage infiltration into the CNS and a down-regulation in endogenous BDNF serum concentration. Collectively, we believe these factors suppressed the beneficial role characteristic of the pre-conditioned lesion model, possibly by inhibiting trophic support to injured neurons, delaying environmental CNS priming, interruption of cell-cell interactions and/or poor myelin clearance.

Conclusively, in the pre-conditioned lesion model, inflammatory cells are able to modify the matured, normally inhibitory CNS environment into a growth-supporting milieu for axonal regeneration. This renders macrophage cells as highly attractive for promoting repair provided that pro-regenerative processes, such as phagocytosis, are not coupled to undesired inflammation and harmful T cell activation. It is then clear that further research into the functionality of these cells is needed to decipher their role during PNS/CNS injury and their involvement in CNS repair and in regeneration.

4.7 Future Studies

These studies should investigate the extent and mechanistic properties of the neuro-immune interaction following peripheral nerve axotomy, as a means to encourage CNS repair, which appears to be dependant on a specific macrophage phenotype.

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