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ENCAPSULATED MESENCHYMAL STROMAL CELLS FOR SPINAL CORD

INJURY REPAIR

By

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A Dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

The Graduate School of Biomedical Sciences

University of Medicine and Dentistry of New Jersey

In partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Graduate Program in Biomedical Engineering

Written under the direction of

Martin L. Yarmush

and approved by

New Brunswick, New Jersey

October, 2012

Abstract of the Dissertation

ENCAPSULATED MESENCHYMAL STROMAL CELLS FOR SPINAL CORD INJURY REPAIR

Delivery

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Spinal cord injury affects a significant portion of the population and unfortunately, current clinical therapeutic options are limited. The progression of SCI pathology is driven by several cellular and molecular post injury events which culminate in an inhibitory scar. Mesenchymal stromal/stem cells (MSC) robustly produce paracrine factors which successfully attenuate tissue damage and therefore represent a promising cellular SCI therapy. However, current transplantation modalities do not provide control or ensure long term persistence of the cells. To circumvent these limitations, we investigated the efficacy of alginate microencapsulation in developing an implantable vehicle for MSC delivery. We demonstrate that MSCs remain viable after electrostatic encapsulation with alginate and retain paracrine function. Encapsulated MSCs (eMSCs) promote immunomodulatory macrophage action and prevent degradation of hippocampal tissue in an organotypic model of secondary injury. Pre-clinical animal studies demonstrate the feasibility of transplanting eMSC via lumbar puncture (LP). Capsules localize within the intrathecal space for at least 6 weeks after transplantation, without any observable degradation. Free MSC (fMSC) transplants were not detectable 1

week after transplantation, while eMSC persisted for at least 2 weeks after injury. eMSC transplantation led to marked improvements in white matter sparing and locomotor function. Overall, these observations support the inclusion of eMSC for post-SCI therapy.

Syringomyelia after SCI can be attributed, in part, to a non-resolving inflammatory presence at the injury site. The Stromal cell represents a key cellular regulator of immune specific functions. Therefore studies were designed to evaluate the regulatory action of MSCs on macrophages during inflammation. MSC secretion of PGE2 promoted macrophage reprogramming by attenuating pro-inflammatory M1 cytokine secretion and enhancing expression of M2 CD206. PGE2 reprogramming was mediated through the EP4 receptor and CREB signaling indirectly, via GSK3- α inhibition. Lastly, MSCs led to a marked increase in CD206 expressing cells at the injury site 1 week after transplantation. The data here support the role of stromal derived PGE2 in facilitating macrophage reprogramming and establishes GSK3-CREB interactions as a possible regulatory checkpoint in macrophage reprogramming. In conclusion, MSC regulation of immune cell plasticity may be responsible, in part, for their efficacy observed post-SCI transplantation.

Acknowledgments

There are several people that I would like to acknowledge for their support throughout my years at Rutgers. First I would like to thank my advisors Dr. Yarmush and Dr. Schloss for all their guidance over the years. They provided me with the freedom and opportunities which have helped me become the scientist that I am today. I would like to thank my co-advisor Dr. Martin Grumet for his guidance and collaboration. This project was a multidisciplinary endeavor, and without Dr. Grumet the pre-clinical work would not have been feasible. I would also like to thank my other committee members, Drs. David Shreiber and Debabrata Banerjee. Their doors have always been opened for me and over these past years I have received some great advice from both of them.

I have been blessed to have had great lab mates over the years. Nir Nativ, Dr. JP Dolle, Dr. Kevin Nikitczuk, Dr. Lulu Li, Mehdi Ghodbane, Andrea Gray, Serom Lee, David Dong, Dr. Bhaskar Mitra, Dr. Rohit Jundal, Alvin Chen and Dr. Tim Maguire. I enjoyed spending time with you over these last few years. You all have helped make lab a very pleasant place.

My friends outside of lab, Dominick Naczynski, Lavanya Peddada, Jocie Cherry Masa, Shirley Masand, Aaron Carlson and several others. Science can often be a difficult and frustrating process, being able to go through this experience with you has been a pleasure.

My friends outside of Rutgers, Adam Braun, Roman Shikhanovich, Alex Davidovich, Daniel Goldwasser and several others. I have dedicated most of my time these last five years to research, which has diminished the amount of time I have been able to spend with them. However, they continued to support me and I greatly appreciate their understanding and friendships.

I have worked very closely with the Grumet lab the last three years. I would like to thank the efforts of Dr. Kim and Dr. Otsuka, who put in countless hours troubleshooting and performing the animal studies, which represent a significant portion of this dissertation. I would like to thank Joanne Babiarz for her friendship and time. Joanne and I have spent a lot of time discussing the project, designing experiments and life. I appreciate her company and support over these past years. Lastly, I would like to thank Dr. Kurt Gibbs. I looked up to Kurt these last several years, for who he is as a scientist as well as a person. He has taught many things about science and provided perspective on life which I had not entertained until I met him.

I would like to thank my cousins, aunts, uncles, sisters, grandparents and parents. My family has been a source of support and motivation throughout my life. Ben, my brother and closest friend, will argue that he deserves an honorary PhD for spending many nights at lab with me. My Sister Raya, whose love and carrying cannot be over stated. I have cousins who I have had the pleasure of mentoring, Michael and Jeremy, you helped make me a better scientist. I also have cousins who have been mentors to me, Dr. Eric Novik, who made sure I joined a lab early in my undergraduate studies.

My aunts and uncles have been a consistent source support, always motivating me to be the best I can be. I love them very much and appreciate their guidance.

My younger sisters have never really taking me to seriously and have been an outlet for me throughout my graduate studies.

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Finally, I would like to thank my grandparents and parents. They have made great sacrifices in order for me have the opportunities that I have today. From a young age education was made a priority and they ensured that it would remain so throughout my life. It is because of them that I have such an appreciation for knowledge and it is a tremendous privilege to have acquired a degree which has trained me to create knowledge. I love you and thank you.

Dissertation Dedication

I would like to dedicate this dissertation to my grandparents and parents.

"If I have seen further, it is by standing on the shoulders of giants", Sir Isaac Newton.

The accomplishments that I have achieved today are yours as they are mine.

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Chapter 1. Introduction

1.1 Spinal Cord Injury Prevalence

There are approximately 12,000 new cases of SCI annually in the US and currently, ~270,000 people in the US are living with SCI. Of all reported cases, 40.8 % are incomplete tetraplegia, 21.6% complete paraplegia, 21.4 % incomplete paraplegia and 15.8% complete tetraplegia. Less than 1% of SCI patients exhibit complete neurological recovery. Due to medical advancements, life expectancy after SCI has drastically increased and depending upon injury severity, victims 20 years of age at the time of injury can now live for up to 52 years. Unfortunately, only ~11 % of SCI victims are employed after 1 year and after 20 years, only ~35% are employed. As a result, the government has become responsible for supporting SCI victims. The average life-time cost associated with SCI support, ranges from 1.5 million to 4.5 million depending on age and severity of injury. Clinical interventions leading to improvements in neurological outcome would help relieve economic burden and more importantly, decrease patient morbidity.

1.2.1 Spinal cord pathology

SCI is characterized by an initial mechanical or primary injury, which is followed by a cascade of secondary events, leading to progressive destruction of spinal cord tissue. The locations along the spinal column, as well as the force of impact, dictate the degree of neurological impairment. Outcomes correlate with the degree axon damage, loss of neurons and glia, and demyelination. Depending on the nature of the primary injury, the spinal cord initially looses essential parenchymal components. This tissue loss may occur through a multitude of mechanical deformations, which may lead to laceration, solid cord injury, contusion, or massive compression [1, 2]. Solid cord injury (10% of cases), describes a cord whose physical structure has not changed and no cavity formation can be However, histological evaluation reveals significant changes in tissue observed. architecture. Contusion injury, (49% of cases) again, does not disrupt surface anatomy. However, it is associated with a significant degree of hemorrhage and necrosis, which eventually lead to cyst formation. Laceration (21 % of cases) results in a cut to the spinal cord tissue, but generally does not result in complete laceration of the cord [2]. This type of injury does not lead to cavity formation, but is characterized by a deposition of collagenous connective tissue [2]. Massive compression (20% of cases) reduces spinal cord tissue to pulp, which is ultimately replaced by significant fibrous scaring over time [2]. The primary injury is followed by hemodynamic changes at the injury site, but does not result in significant alterations to the tissue architecture [3, 4]. These observations led researchers to posit that most of the deleterious outcomes after SCI result from cascades associated with the secondary injury response[5]. Thus, clinical intervention at the secondary injury phase could potentially alleviate SCI morbidity.

The events associated with secondary injury lead predominantly to the death of neurons and oligodendrocytes [6-8]. The cellular and molecular events associated with secondary injury spread from the site of impact and lead to structural and functional disruption of the spinal cord. The scar which forms during the initial post-injury year is normally larger than the initial primary injury area. Therefore, understanding the temporal cellular and molecular aspects of SCI pathology will be essential in developing successful interventional therapies. In general, the secondary injury mechanisms include: BBB break down, ischemia, oxidative cell stress, glutamatergic excitotoxicity, chronic inflammation, and lipid peroxidation [9, 10]. We now describe the progression of the pathology from days, to weeks and months after the injury.

1.2.1 1-2 Days Post Injury

This first two days post-SCI in humans can be characterized by edema, hemorrhage and inflammation. Breakdown of the BBB results in edema, allowing plasma components to enter the cord [5]. In response to the increased pressure, systemic blood flow to the injury site is reduced, generating an ischemic environment. The increased edema also leads to cytotoxicity due to cellular swelling[11]. The consequences of catastrophic cell death and edema result in elevation of toxic factors in the extracellular space, including glutamate, lactate, K^+ , nitric oxide, arachidonate, reactive oxygen species (ROS) and ammonia [12, 13]. Several of these factors have been shown to cause neurotoxicity [14-17]. Therefore, immediately after trauma the injury milieu becomes extremely unfavorable for cell survival.

The peripheral immune system begins to respond within several hours after the trauma. Neutrophil influx begins within hours after injury, but wanes after 3 days. However, neutrophils secrete potent cytotoxic factors, which contribute to an environment which is already deleterious to cell survival. In addition, immediately after injury, microglia, the resident CNS tissue macrophages, become activated, and assume an

ameboid morphology [18]. Microglia secrete several factors, that can either promote detrimental [19] or beneficial [20] responses. During the initial post-injury, days axonal swelling and demyelination can also be observed [21]. Therefore, many believe that early intervention immediately after trauma, aimed at controlling these events, could provide effective therapeutic options.

1.2.2 Days to Weeks after Injury

The days to weeks after the trauma are marked by tremendous cellular activity. Several days after injury peripheral monocyte influx begins. Most of these monocytes differentiate into macrophages [22]. The macrophage's initial responsibility is phagocytosis of debris. However, in the presence of danger signals released from necrotic cells, microglia/monocytes/macrophages become inflammatory and begin to secrete cytotoxic substances. During normal wound healing responses, diverse and highly controlled functional macrophage subsets are generated. This progression is essential for resolution; however, after spinal cord injury, resolving macrophages are not generated, leading many to believe that chronic macrophage inflammation may directly contribute to SCI pathology. We will discuss the macrophage response to the injury extensively throughout this dissertation. Therefore, more detail will be provided in subsequent chapters.

In addition to the cellular immune response, there are significant phenotypic changes to resident CNS cells. The astrocytes surrounding the lesion undergo hypertrophy several days after injury. These reactive astrocytes are metabolically activated and 2-3 weeks later begin to project processes [23]. Astrocytes are believed to

be the primary contributor to the glial scar, which serves as a barrier to regeneration [24]. However, there is evidence that astrocytes also secrete growth factors which are beneficial for regeneration [5]. Furthermore, astrocyte ablation studies demonstrate pronounced BBB breakdown, which results in a greater infiltration of peripheral immune cells [22]. In general astrocytes are responsible for maintaining the BBB and homeostasis within the CNS. It is likely that astrocytes perform these essential functions after SCI, where there are tremendous imbalances at the lesion sight. However, the consequence of this hypertrophic state results in the deposition of ECM components, which consequently inhibit neuronal outgrowth.

1.2.3 Late Phase (months)

In the months after SCI, the tissue may develop several of the following observable architectural changes: Wallerian degeneration, astroglial scarring, mesenchymal scarring, cysts/ cavities, syrinx and Schwannosis. Wallerian degeneration is a process where severed axons undergo anterograde disintegration. This process occurs within about 1 year [5]. The astroglial scar is composed of several ECM proteins and is produced primarily by astrocytes. A large amount of literature evidence supports the inhibitory effect of ECM proteins on neurite outgrowth [24]. However, others have found that the astroglial scar is necessary for the progression of a normal immune response after injury [25]. In addition, the mesenchymal scar results in the deposition of fibrous scar tissue and also serves as an impediment to neurite outgrowth [5]. Schwannosis is an abnormal proliferation of Schwann cells [5], which occurs in many clinical instances of SCI, but its consequences are poorly understood. Schwann cell

growth seems to interfere with axonal outgrowth, but may also may be a cause of spasticity, pain and other abnormal behaviors associated with chronic spinal cord injury [5].

One year after SCI there is usually some degree of neurological impairment and in all cases, some combination of the events described above can be documented. Therefore, clinical therapies that target these events are being developed with the hope of ameliorating the negative consequences of post-SCI secondary cascades. It is important to understand that while successful therapy may not result in significant motor function improvement, but therapies which enhance quality of life, will be just as beneficial. Many SCI victims experience sexual dysfunction, bladder dysfunction, trunk instability, loss of normal sensation, and neuropathic pain. Improvements in any of these would also provide therapeutic benefits which currently unavailable.

1.3Current Spinal Cord Injury Therapy

Many therapies have been developed to treat SCI. Perhaps the one that has garnered the most attention is methylprednisolone (MP). MP is a glucocorticoid with potent anti-inflammatory function and the only FDA approved treatment for SCI. While MP administration was initially viewed as a tremendous breakthrough in clinical care for SCI, unfortunately, many have recently begun to debate the evidence supporting the safety and efficacy of this approach [26-29]. The treatment is beneficial if administered between 3-8 hours after injury and at the appropriate dose. Any deviation from this administration protocol fails to result in improvements and often leads to side effects [30]. Currently, clinicians are at odds as to whether or not to recommend MP administration after SCI, and the consensus is moving against MP in post-SCI care[28]. Nonetheless, investigation of MP feasibility began a new era in clinically evaluating potential SCI therapies, and indicated that success, while difficult, may be achievable. Several different SCI therapeutic protocols are being developed and some are currently being evaluated in the clinic.

Antibodies against CD11d/CD18 have been developed to mitigate the infiltration of neutrophils/macrophages after injury, in an attempt to reduce the degree of neurotoxicity and scar formation [31]. Other anti-inflammatory treatments such as minocycline, which aims to reduce immune cell production of inflammatory cytokines, free radicals and matrix metalloproteinases, have also been evaluated [32]. In fact, minocycline has been directly compared to MP. Animal model results suggest that minocycline provides superior neuroprotection relative to MP [33], and it is currently being evaluated in clinical trials [34]. In general the therapies described above were designed to target the deleterious consequences of the immune system.

Others have developed molecular therapies aimed at inhibiting neurotoxicity. Na⁺ channels antagonists, HP184 and Riluzole, reduce the extracellular levels of Na+ and subsequently, cytotoxic cell edema and intracellular acidosis [35]. These two drugs are currently being evaluated in clinical trials, but efficacy data has not yet been released. Attempts have also been made to develop and test N-methyl-D-aspartate (NMDA) receptor antagonists [36]. NMDA is a glutamate receptor agonist which, upon binding, stimulates the opening of ion channels. Excess post-SCI levels of extracellular glutamate

over- stimulate the NMDA receptor, leading to neurotoxicity. However, clinical trials have not yielded positive results[30].

Several have taken the neuroregenerative approach, working on the premise that if appropriate factors are delivered, the CNS can regenerate. Within this research realm, growth factors such as brain derived neurotrophic factor (BDNF) [37, 38], basic fibroblast growth factor (bFGF) [39], glial derived neurotrophic factor (GDNF)[40], nerve growth factor (NGF) [41], neurotrophin 3 (NT3)[42], NT4 and NT5[43] have been studied. Unfortunately, systemic delivery of these factors has failed during clinical trials due to side effects and lack of efficacy. However, some have developed biomaterial approaches to localize and target the delivery of these factors, which may reduce non-specific effects and potentially enhance efficacy [37]. These approaches have not yet been evaluated clinically.

Other approaches to induce neuronal regeneration aim to target intracellular signaling of cyclic AMP (cAMP), GTPases and Rho kinase (ROCK). Axonal sprouting is dramatically increased in neurons expressing elevated levels of cAMP [44]. Elevating cAMP levels before SCI resulted in significant improvement [45], however post-SCI cAMP elevation has not provided promising results. Most post-SCI cAMP studies are designed as a combinatorial approach, where the co-cAMP treatment aims to establish a more favorable environment for regeneration [46]. On the other hand, treatments that target GTPase inhibitors of axonal sprouting, such as Rho and Rac, have shown promise *in- vitro* as well as *in vivo* [47]. The bacterial toxin, C3-ADP-ribosyltransferase, which inhibits Rho, promotes axonal sprouting and some functional improvement [48]. The

synthetic Rho inhibitor, Cethrin, delivered via a fibrin conduit, is currently being evaluated in clinical trials [49].

Interestingly, GTPase activity is upregulated when axons interact with particular proteins in the CNS. Some of these proteins are, Nogo-A, myelin associated glycoprotein (MAG), oligodendrocytes myelin glycoprotein (OMG), chondroitin sulphate proteoglycan (CSPG) and ephrin B3. These proteins have been shown to inhibit axonal outgrowth [50, 51]. Therefore, molecular and antibody mediated blocking of these ECM receptor interactions have been designed as treatments to promote axonal outgrowth after SCI. Antibodies have been developed to interfere with Nogo-A binding and have shown promise in SCI animal models [52]. Clinical trials have begun to evaluate a human anti-Small molecule inhibitors of Nogo-A binding have also been Nogo-A antibody. developed and some success has been demonstrated [53]. NEP1-40 is also currently being evaluated in clinical trials.

Another therapeutic approach has been to target the components of the glial scar. CSPGs represent a significant barrier to regeneration. CSPG degradation with chondrotinase ABC (ChABC) promotes axonal sprouting as well as improvement in locomotor function [54]. ChABC treatment is currently in phase II clinical trials.

All of the approaches described above, have promise in development of preventative and some in regenerative therapy for SCI. One caveat is that each approach generally targets only one aspect of the secondary injury cascade and combinatorial approaches will likely provide the best outcomes after SCI [30]. Another limitation with these approaches is that, unless they promote regeneration, they can never replace lost

tissue after the primary injury, which is a predicament the scientific community hopes to address with the implementation of cellular therapy.

The goal of cellular therapy for SCI is to replace CNS tissue or enhance the function of cells remaining after injury. The limiting characteristic of any cellular therapy is the source of cells. Transplantation of embryonic CNS tissue[55], peripheral nerve grafts [56] and Schwann cells [57] have yielded improvements in animal model studies, but the lack of donor material make these approaches impractical in humans. An ideal cell source would be one which is readily available for clinical use. Factors such as ease of clonal expansion as well as autologous transplantation, can often determine the feasibility of cellular therapy. For these reasons stem cell sources have received the most attention during the last decade. Specifically, olfactory nervous system (ONC), embryonic stem (ES) cells and adult stem cells have received the most attention.

ONC comprises a group of stem cells which generate olfactory receptor neurons throughout life. Transplantation after SCI has proven to be efficacious in promoting functional recovery when transplanted 2 days or 2 months after injury [58]. However, transplantation has not consistently resulted in functional improvement [59]. Some clinical trials have been performed, but no apparent improvements in neurological function was demonstrated [30]. Human clinical studies continue to progress as a 1 year follow up since there were no adverse effects associated with autologous treatment [60].

ES cells represent a promising cell source for clinical applications. Theoretically, ES cells can undergo limitless expansion and can differentiate into cells of all lineages. Therefore, if successful, ES cells could be differentiated into several neuronal cell populations and replace cells lost after SCI. ES cell that have been differentiated into oligodendrocytes have shown therapeutic efficacy in animal models of SCI [61]. These studies led to the approval of the first ES cell clinical trial for SCI (or any other application), a milestone in ES cell therapy. However, this trial has since been stopped. In addition, benefit has been documented following undifferentiated ES cell post-SCI transplantation [62]. Overall, ES cells represent a promising cell source for SCI therapy. However, ethical consideration and lack of understanding of their biology, severely limit the progression of clinical research.

Adult stem cell transplantation may represent the compromise between the limitations associated with somatic cell transplantation and the ethical concerns associated with ES cells. Adult stem cells can often be isolated directly from the patient. Stromal (BMSC) and hematopoietic (HSC) cells can be readily isolated from the bone marrow and autologously transplanted. HSC transplantation has proven to promote improvement in function after SCI [63]. BMSC have also been similarly evaluated [64, 65].

Adult neural progenitor cells (NPCs) isolated from the subventricular zone, dentate gyrus and spinal cord, have also been proposed for SCI therapy. NPC transplanted into SCI differentiate into astrocytes and oligodendrocytes [66]. However, the extent of their survival is dependant upon the post-SCI NPC transplantation time Furthermore, clincical NPC transplant would necessarily require allogeneic donors , which may be associated with a number of harmful clinical consequences. Cellular therapy represents the next frontier in clinical SCI care. However, many current studies are unable to adequately control appropriate cellular orientation within the tissue or even the assurance that cells will localize after transplantation. The CNS is comprised of complicated cellular networks and until the scientific community is better able to control cellular microenvironments, cellular replacement therapy may not be able provide substantial benefits for chronic SCI. Nonetheless, there are several potential therapies being evaluated for SCI. As mentioned before, a major limitation with these approaches is that they target one aspect of a multifaceted injury response. Therapies aimed are targeting several different components of the secondary injury response, would theoretically provide the best chance for improvements. Interestingly, the BMSC may represent such a therapy.

1.4 MSC Secreted Factor Therapy

MSC were among several types of stem cells popularized during the last decade or so. The idea was, and partially still is, that MSC and other stem cells sources would provide cellular replacement therapy for various clinical disorders. Limitless expansion, ease of autologous isolation and the potential for trans-differentiation made MSC an enticing cell source for various applications. As a result, many studies focused on characterizing MSC differentiation post or pre-transplantation [67]. However, numerous MSC transplant studies, resulting in significant improvements, occurred without any observable MSC engraftment or differentiation [68, 69]. The contemporary paradigm being popularized is that MSC promote therapeutic benefits via secretion of soluble cues which control immune cell functions and provide trophic support. This idea is supported by *in vitro* co-culture studies as well as *in vivo* transplantation studies. As a result, numerous researchers have begun to regard MSC, not only as traditional differentiating stem cells, but also as cellular drug delivery vehicles [70-72]. MSC secrete bioactive factors which elicit immunomodulatory and trophic action. Studies have also demonstrated that MSC transplantation can reduce trauma-induced apoptosis of parenchyma and stromal cells [73-75], increase tissue vascularization [76] and induce the proliferation of progenitor cells[77]. MSC secrete factors such as SDF-1, VEGF, EGF and IGF-1, which are known to exert protective effects on impaired tissue [73-75]. Furthermore, MSC promote increased axonal sprouting when cultured with neurons [78]. MSC secrete several beneficial post-SCI neurotrophic factors including CNTF, NT-3 and BDNF [78][42]. MSC can also prevent neuronal apoptosis and prolong survival in-vitro [79-81]. In general, MSC express several neuro-regulatory genes [82]. Collectively, these studies corroborate and underscore the tremendous and collective neuroprotective and regenerative MSC functional library, as well as their potential as a post-SCI therapeutic.

In addition to their ability to promote regeneration, MSC can also modulate immune function. The first evidence that MSC can control immunosuppression *in vivo* came from models of GVHD [83]. These studies demonstrated that MSC could reduce allograft rejection [84, 85]. Shortly after, MSC T cell immunosuppression was demonstrated *in vitro* [86] (Figure 1). MSC reduce T cell expansion *in vivo* [87] and have been found to promote differentiation of naïve T cells into T_h2 , providing protection against demyelination and axon loss [88-90].



MSC Figure1: Control Т Differentiation Lymphocyte and Effector **Function**MSC modulate several aspects of T cell behaviors, primarily preventing the differentiation and expansion of pro-inflammatory subsets, TH1 and TH17. Simultaneously, MSC promote T cell phenotypes which possess immunomodulatory behaviors, TH3 and Treg. Additionally MSC can prevent cytotoxic T and natural killer cell functions. Overall, MSC dictate T cell functions which suppress the adaptive immune response, potentiating their application in T cell associated disorders.

MSC immunomodulation is not unique to the T cell; in fact MSC can immunomodulate many immune cells subsets. Gonzales *et al.* co-cultured macrophages with MSC and found that the pro-inflammatory secretion of TNF- α and IL-12 was diminished [91]. Cutler *et al.* suggested that MSC can modulate monocyte functions, which ultimately resulted in the suppression of T cell proliferation [92]. Kim *et al.* observed that macrophages cultured in the presence of MSC



Figure 2: MSC Modulate Macrophage, B and Neutrophil Cell Pro-Inflammatory Functions. MSC direct macrophages towards M2 like phenotypes and simultaneously suppress M1 functions. MSC inhibit B cell differentiation via surface receptors PD-1 which, in turn, reduce immunoglobulin and chemokine production. MSC also provide effector function on neutrophils via HLA-g and IL-6, preventing proinflammatory secretion as well migration.

adopted phenotypes indicative of M2 macrophages (CD206^{high}, IL-10^{high}, IL-12^{low}) [93]. MSC have

been reported to affect the neutrophil recruitment and invasion into tissues in several inflammatory models, including sepsis [94], acute lung injury [95, 96], diabetes [97],



Figure3: MSC Impede DC Maturation and Augment DC Anti-Inflammatory Functions. Dendritic cells are potent APCs possessing a great deal of control over the adaptive immune response. MSC prevent monocyte commitment to DC differentiation and subsequent maturation. Furthermore, MSC favor DC2 phenotypes within mature maDC populations while simultaneously inhibiting DC1 phenotypes. The consequence of these observations is that MSC treated DC will favor type II T cell immune functions *in-vivo*.

and tetrachloride-induced cirrhosis [98] (Figure 2). MSC have also been shown to affect each aspect

of inflammatory DC function via control of differentiation, maturation, and function, in many *in vitro* co-culture systems [99-105].

MSC can regulate mature DCs (maDC) as well endocytic capacity, low immunogenicity, and strong immunoregulatory function [106]. Likewise, Wang *et al.* observed a reduction in maDC expression of maturation markers CD83 and an increase in endocytic activity [107]. These DC orchestrated a shift from pro-inflammatory TH1 to anti-inflammatory Th2 cells, suggesting that MSC can promote DC immunoregulatory phenotypes. Interestingly, DC exhibit type I and II phenotypes, as do most cells of the immune system. These cells have been referred to as DC1 (pro-inflammatory) and DC2 (regulatory) [108]. Studies by Aggarwal *et al.* indicated that MSC enhance DC2 functions (Figure 3), while subduing DC1, within a maDC population [109]. The data suggests that MSC can direct DC to adopt regulatory phenotypes. MSC effects on natural killer [110], cytotoxic T [111], B [112] and TH17 [113] cells have also been described.

Given the wealth of immunomodulatory, neurotrophic and regenerative MSC properties, MSC represent an intriguing option for SCI treatment development. MSC appear to regulate many of the key cellular constituents which orchestrate post-trauma events. MSC could potentially regulate the immune system after SCI, providing cytokine cues, which may regulate and restore a normal resolving course of inflammation. MSC could prevent apoptosis of neuronal cells, as well as potentially provide factors to stimulate their regeneration. Collectively, this functional multiplicity may establish MSC as a potential multimodal SCI therapy, which has to date not been matched (Figure 4).



Figure 4. MSC Multimodal Treatment. MSC secrete several factors which exhibit immuno-modulatory, anti-fibrotic, anti-apoptotic, angiogenic, neurotrophic and regenerative potential.

1.5 MSC Homing

Just as understanding MSC mechanisms of action are important in designing an effective therapy regimen, ensuring that MSC will target the tissue, is equally as important. MSC have been heralded for their ability to specifically home to areas of tissue damage. The ability to non-invasively transplant MSC and then have them specifically home to areas of tissue injury, is an intriguing and controversial concept. Much can be gleaned from leukocyte and hematopoietic stem cell homing, which is a multistage process of (1) chemotaxis, (2) tethering and rolling, (3) firm adhesion and, (4) diapedesis. Most MSC targeting studies have attempted to evaluate potential mechanisms of MSC homing in the context of what is known about leukocyte extravasation. We begin our discussion with molecular cues that initiate MSC migration to areas of tissue trauma.

There are opinion disparities regarding proposed mechanisms of MSC homing. These disparities may be attributed to several factors. MSC expression of homing mediators begin to decline over passage number and is dependent upon culture conditions [114]. Therefore, depending on the isolation techniques and culture conditions, MSC from different laboratories will display varying homing potentials and mechanisms. To complicate the issue further, MSC isolated from different tissues may also express these

homing mediators differently [115]. Some investigators have suggested that MSC homing mechanisms are trumped by their tremendous size [116], as MSC will get trapped in non-specific locations, with reduced chemotaxis independent homing [117]. This may explain why localized delivery of MSC enhances engraftment efficiencies, [118-120] since intravenously injected MSC have been observed to be systemically

delivered to unintended tissues, mainly the lung and liver [118, 121]. Furthermore, MSC have not been found to persist at a tissue site long term and are no longer detectable as early as 1 week post transplantation [122]. To date MSC homing potential as well as the mechanisms which govern homing control continue to be debated.

1.6 MSC as a treatment for SCI

A considerable number of studies have been designed to evaluate the effacy of MSC SCI therapy. Table 1 tabulates, to our knowledge, all BMSC SCI studies which have been reported to date. When evaluating the literature it is important to note that one must consider the type of injury model, number of cells transplanted, time of transplantation, passage and source of MSC, when comparing results. Differences in these parameters may ultimately contribute to study outcomes. In this section we will briefly summarize the current MSC SCI therapy literature.

Chopp et al. reported the first observation that transplanting 2.5x10⁶ directly into the injury epicenter led to improvements in BBB scores 5 weeks post injury [123]. Given that it was thought MSC could transdifferentiate, several publications suggested that improved locomotor function was due to MSC differentiation into neural cells, replacing parenchyma cells, which had died as a result of the injury [64, 124-127]. However, the data became subject to a great deal of criticism and after 2006 only 1 or 2 studies reported MSC trans-differentiation observations. All subsequent literature described the benefits of MSC to be associated with their secretion of trophic factors, which have tremendous effects on tissue health [128].
Table 1. MSC SCI Therapy

	Authors	Journal	year	Ы	Injury	Severity	MSC Species	# MSCs	Model Transplantation	Transplantion Day	Time points	Result
1	Chopp	neuroreport	2000	Rosenblum	Rat Mascis	severe (25 mm)	Rat bone marrow	2.5x10 ⁵	4ul directly into injury epicenter .	7 days post injury	4 & 5 weeks	Improved BBB scoring observed up to five weeks after injury
2	Sasaki	Glia	2001	Kocsis	Focally demylinated	ć	mouse bone marrow	10 ⁴	104 in 1 ul directly into the injury sight	3 days post injury	56 days	remylination of the spinal cord was seen with peripheral pattern of myelination
3												MSC associate with astrocytes and guiding strands.
	Hofetattar	DNAC	000	Drockon	Bat Mascis	severe(25	Rat	5.10 ⁵	Directly into injury sight. 5ul into	Immediately & 7	5 weeks: BBB, GEAD and ED1 7	Delayed transplantation and not immediate
	ווחוזובוובו		7007			mm)	Nat	OTXC	caudal to injury epicenter	days post injury	weeks	promotes improvements in BBB. Present data
												describing MSC neuronal differentiation
4					Focally		ant hou					remylination was seen with both central and
	Akiyama	Glia	2002	Kocsis	demylinated	ć	rat pone marrow	107	10/ IU C. nl through the femoral	3 days post injury	3 weeks	peripheral patterns, conduction velociy of
					lesion							remylinated neurons was improved
5					Focally				-			remylination was seen with both central and
	Akiyama	Glia	2002	Kocsis	demylinated	ć	mouse pone	5x10 ³	5x103 in 1 ul through the femoral	3 days post injury	3 weeks	peripheral patterns, conduction velociy of
					lesion							remylinated neurons was improved
6					0.011000							MSCs migrated towards the injury. Reported that a
	lee	neuropahtology	2003	iwasaki	pneumatic impact (seki	ć	mouse boner	3x10 ³	Directly into injury sight. 1.5 ul into injury and 5 ul 2mm rostral	l day	4 weeks	percetange of MSC expressed astrocytic markers .
					et al.)				נט וווזמו א באוכבוונבו			No BBB assessment
7												MSC nersistance at the initiry reduces over time
	M	Journal of neuroscience research	2003	ide	Rat Mascis	Severe severe (50mm)	Rat bone marrow	1x10 ⁶	20ul directly into injury epicenter	Immediately	4 weeks	MSC transplants resulted in reduced cavity volume
					-							and displayed increased BBB scoring
∞					rat. wieght							MSC formed bridges within the lesion cavity,
	Zurita	Neuroreport	2004	Vaquero	arop zucm height, 25 g	ć	Rat bone marrow	5x10 ⁶	1x10° in 50 ul directly into the injury sight	3 months after injury	4 weeks	expressed neuronal and stroglial markers, and
					rod, 12 mm ²							improved functional recovery
6					Rat ESCID							MSCs filled the lesion cavity, preserved host tissue
	Ankeny	Experimental Neurology	2004	Jakeman	device (Jakeman et	ć	Rat bone marrow	3x10 ³	3x103 in 5 ul directly into the iniury sight	2 days post injury	56 days	and myelin, supported aconal growth, and did not
					al. 1998)				-			improve BBB scores, only air stepping

σ	S						σ		5		>			в	_		s	S		٩	σ	Te	, –	50			17	3	c	
MSC mirgrated to the lesion reduced its size an	improved BBB scores. MSC were seen two week	after transplantation, but were gone after 3 weeks	MSC-derived neurons did not maintain neuronal	differentiationand supported axonal growth to the	same degree as MSCs, but less than that of BDNF	MSCs, however there was no functional recovery	MSCs migrated towards the injury. Reported that	percetange of MSC expressed astrocytic markers	No BBB assessment. Observed cells persisting out fo	4 weeks	Reported improved BBB scores 6 weeks after injur	•	and no neuronal marker expression	MSC migrated towards injury. Improved BB	scoring.reduced cavity formation. Minimal neurons	marker expression	MSC transplanted i.v distributed all over the rat a	seen in gammagraphic images, intralesional MSC	were observed only in the lesion area	MSC differentiated Schwan cells incresed th	numbers of certain nerve fibers, reported improve	MSCs progressivly reduced the lesion cavity an	improved functional recovery over a 1 year period	with virtually all transplanted cells expressin	astroelial markers and improved functional recovery	lantiviral variar mead to kaon track	MSCs more found in the locion antity and immore	ואסכט אבוב וסמומ זוו נווב ובטוסוו רמאויל מוומ וווואוסיסב	functional reevoery but to a much greater extent i	the inter locional manual
_	up to 4 weeks				42 days					,		4 weeks			4 & 5 weeks			10 days			42 days		2 & 6 months and 1	year after MSC	transplantation			_	6 months 1	
	Immediately		mmediately 4				7 dave noet inium	y udys poor injury			7 days post injury			7 days post injury			3 months after iniury	<i>t</i>		Immediately			3 months after	injury			3 months after	injury		
	5x106 in 50 ul through the fourth venticle over 5 min		JSCS, MSC derived neurons an MSCs transduced to over expres tDNF transplanted to the injur- ite				7 ul rostral to injury epicenter.	Animal treated with cyclosporine		3 ul directly into iniury enicenter	ט מו מווכננוץ ווונט וווןמוץ באונכוונכו י		3 ul directly into injury epicent			6x106 in 1 ml intravenously 6x106 100 ul in centromedulla cavity.				ZXLUD IN MALTIGAL PLACED IN A TUDE which they fill the gap with		5x10 ⁶ in 50 ul directly into th					3x106 in 50 µl intralesionally 3x106 .5 ml in intravenously.			
	bone 5x10 ⁶		bone	rrow	NF	nsuction		use			use	1er 3x10 ⁴	rrow	-	bone not-			bone 6x10 ⁶		bone	rrow 2x10 ⁶			bone 5x10 ⁶	rrow			Pone	rrow 3x10 ⁶	
moderate	(12.5mm) & rat severe (25 ma	mm)	rat	ma	f BDI	tra		om vod	un ma		om	2 bor	ma		? Kat			? rat		rat	i? ma	100		2 Rat	ma			rat	2 ma	
	Rat Mascis		Rat transection rat pneumatic impact (seki et al.)					Mouse	compression	at T8 Rat ballo compression at T8-T9			at T8-T9	rat.wieght drop 20cm height, 25 ₆ rod, 12 mm			Rat	transection T 6		rat.wieght drop 20cm height, 25 g rod, 12 mm ² thick. T6-T8				sot windt	drop 20cm	height, 25 g	roa, 12 mm			
	ide		tuszynski			iwaski				Yamazaki				jendelova		vaquero r				Yamazaki		Vaquero					santos			
	2004		2004			f 2005 i				2005			2005 j			2005			u	2005		2006					2006			
	Experimental Neurology			Experimental	Neurology			journal of	neurotrauma			leuroreport			nn N Y Acad Sci			neuroscience etters			journal of neuropathology and expermental		etters			neuroscience etters				
	Ohta											Koda			sykova			de haro			Kamada			Zurita					Vaquero	
10			11				12				13			14			15			16		17					10	2		

Table 1. Current list of pre-clinical SCI studies evaluationg MSC transplant therapy. The table lists 27 studies which evaluate MSC SCI therapy. Question marks in injury severity indicate that we can not determine the severity of injury relative to the method used in this dissertation. There are several different models used to test MSC efficacy. Other variables are different dosing, sources of MSC, passage number and the mode of transplantation. After 2006 several studies focused on demonstrating MSC transdifferentiation after transplantation. Most papers after 2006 describe the trophic and immunomodulatory action MSC impart. There is one group that published several papers on MSC therapy for chronic SCI, using a model which is not utilized by other labs. There has not been an attempt to reproduce these studies. Cumulativley, the data suggests that there are benefits with MSC transplantation for SCI. However, the improvements observed have been modest and several of the later literature can not detect MSC at the injury.

There is evidence that MSC implantation can lead to remylination of injured axons [129, 130] as well as establish microstructures for axon guidance [64]. SCI lesion sites have increased electrophysiological activity [131], white matter content and axonal extension after MSC transplantation [132]. MSC have been shown to reduce the number of macrophages and the degree of reactive astrocytes at the lesion site [133]. Furthermore, there are several reports of reduced cavity formation and increased white matter at the lesion site [65]. These observations are often accompanied by improvements in locomotor functions, as assessed by BBB scoring [65]. However, there have been reports of no improvements in BBB scoring, with MSC treatment [134]. It is difficult to identify the differences between studies which may account for the conflicting results. There is evidence that post MSC transplantation improvements can only be observed in severe models of SCI [133]. Others claim that time of transplantation, as well as the dose of MSC, matter as well [135]. Finally, literature evidence suggests that MSC do not persist long-term after transplantation [74]. Interestingly, many studies do not transplant MSC until 1 week after injury in order to maximize the number of engrafting cells. Those who administer at early time points often do with the administration of antiinflammatory agents, but even with these attempts there is still a considerable amount of evidence that MSC do not persist long term after transplantation [71]. There are, however, those who claim MSC can persist for weeks after transplantation [136], but many of these studies use cellular tracking techniques which may result in the measurement of artifacts associated with transfer of donor labels [137]. The persistence of MSC post-SCI transplantation remains debated but the majority of evidence suggests that they do not persist long-term. Several attempts have been made to study the effect of MSC on chronic SCI in animal models, with some success [125, 127, 131, 138]. However, human clinical trial evidence suggests that chronic applications may not be effective [139]. Furthermore, if in fact, MSC do not persist long-term, the benefits associated with the chronic injury phase, remain unclear. Presumably only cellular replacement could explain chronic injury benefit. Nonetheless, *in-vivo* models suggest that there is some benefit, but these results have not been validated by different laboratories, as all the data on MSC effects of chronic SCI have come from the same group.

A significant body of evidence corroborates the safety of MSC transplantation in both animal studies and human clinical trials [140]. MSC transplantation does not seem to promote increased pain or the formation of cellular growths, side effects observed following transplantation of other groups of stem cells. Although the work cited above provides convincing evidence that MSC transplantation in SCI can provide regenerative effects and contribute to early-partial functional recovery, the long-term therapeutic benefits and the precise mechanisms by which this occurs is completely unknown. For example, the work of Himes et al. (2006) showed very clearly that human MSCs survived less than 11 weeks after transplantation; this lack of long-term survival may explain the extremely weak behavioral improvement [141]. Sheth et al. (2008) were similarly frustrated in their attempts to achieve long-term survival and functional recovery, with only 6 weeks of cell survival and no significant influence on functional recovery [142]. These efforts also failed to discover the precise mechanisms responsible for MSC SCI therapy. The lack of understanding, as well as long-term cell survival has been recently highlighted by Parr et al (2007) where the lack of engraftment, survival and elucidation of protective mechanisms were concluded to have limited the progress of clinical trials and the development of alternative treatments [143, 144].

To resolve these issues we propose a series of studies designed to produce longterm cell survival by cell encapsulation. The objective of the proposed dissertation is to investigate encapsulated MSCs as an effective treatment of spinal cord injury. The encapsulation of MSC is expected to lead to marked cell survival increase, and provide a good environment for sustained cell function. Furthermore, this exploratory platform will address fundamental questions. Among these are, 1) Does prolonged MSC survival enhance MSC SCI therapy? 2) Do MSC need to be at the site of injury or can secreted factors alone promote therapeutic benefit?

In summary, the collective evidence reviewed in this section strongly supports the conclusion that there is a need for adequate cell scaffolds to produce long-term cell survival. Furthermore, in order to develop better therapeutic targets for SCI treatment we must identify the mechanisms by which MSCs can regulate a localized anti-inflammatory effect which, in turn, can prevent apoptosis and enhance neuronal regeneration post -SCI.

Chapter 2 Encapsulated Mesenchymal Stromal Cells for In-Vivo Transplantation

Barminko, J., et al., *Encapsulated mesenchymal stromal cells for in vivo transplantation*. Biotechnol Bioeng, 2011. 108(11): p. 2747-58. 2.1 Abstract

Immunomodulatory human mesenchymal stromal cells (hMSC) have been incorporated into therapeutic protocols to treat secondary inflammatory responses postspinal cord injury (SCI) in animal models. However, limitations with direct hMSC implantation approaches may prevent effective translation for therapeutic development of hMSC infusion into post-SCI treatment protocols. To circumvent these limitations, we investigated the efficacy of alginate microencapsulation in developing an implantable vehicle for hMSC delivery. Viability and secretory function were maintained within the encapsulated hMSC population, and hMSC secreted anti-inflammatory cytokines upon induction with the pro-inflammatory factors, TNF- α and IFN- γ . Furthermore, encapsulated hMSC modulated inflammatory macrophage function both *in-vitro* and *in-vivo*, even in the absence of direct hMSC-macrophage cell contact and promoted the alternative M2 macrophage phenotype. In-vitro, this was evident by a reduction in macrophage iNOS expression with a concomitant increase in CD206, a marker for M2 macrophages. The combined findings support the inclusion of immobilized hMSC for tissue protective therapy, and suggest that conversion of macrophages to the M2 subset is responsible, at least in part, for tissue protection.

2.2 INTRODUCTION

Human mesenchymal stromal cells (hMSC) are highly proliferative, tissue culture plastic adherent cells [145], which can differentiate into a number of mesodermal cell lineages and serve as a potential cell source for autologous cellular replacement

therapies [146, 147], many of which are currently under clinical trial evaluation [148]. More recently, their cyto-protective role, mediated by secretion of a plethora of cytokines and growth factors [70] has also been described [149]. hMSC secretion profiles have been well characterized *in-vitro* and can be modulated by the local microenvironment [150]. In addition, hMSC therapeutic benefits have been described both *in-vitro* and *in-vivo*, using models of graft versus host disease [151], myocardial infarction [152], fulminant hepatic failure [153-155], central nervous system trauma [156], sepsis [157-159], colitis [157], and may occur in the absence of direct cellular replacement or hMSC differentiation [143]. Many investigators have suggested that hMSC orchestrate biochemical cues that mitigate fibrosis and promote tissue protection, via secretion of soluble factors [160-164].

Despite increasing interest in hMSC therapeutic potential, limitations with direct hMSC implantation approaches prevent effective translation of hMSC infusion into the design of safe and controlled therapeutic protocols. Several reports have described wide hMSC distribution to non- targeted tissues after transplantation [119, 165]. Additionally, the hMSC fraction that ultimately reach the targeted destination do not persist long-term [122, 166], in part because directly transplanted hMSC may be adversely affected by the complex injury environment and may differentiate into undesired end stage cells [167]. Resolution of these outstanding issues may be facilitated with the development of an immobilized hMSC delivery approach.

Several studies have demonstrated that cell immobilization can support hMSC survival and functional differentiation into other cell types following differentiation factor supplementation [168, 169]. Many investigators have generated cell/material

constructs using a variety of natural (e.g. alginate, collagen, chitosan) and synthetic (e.g. cellulose, silicon) materials for improved control over implanted cells. Among these, alginate is a cost effective, non-immunogenic, FDA approved material that has been utilized extensively by many investigators for a variety of stem cell differentiation and cell immobilization protocols [170]. Previous studies in our laboratory have utilized alginate encapsulation to differentially direct murine embryonic stem cell (ESC) differentiation towards either the hepatocyte or neuronal lineages by varying both the alginate concentration and aggregate formation within the microcapsules [165, 171]. The present studies were designed to determine if alginate encapsulation could also be incorporated to preserve hMSC anti-inflammatory function, providing a controlled delivery vehicle that can attenuate inflammation and promote tissue repair in-vivo. Our results indicate that alginate encapsulation can sustain hMSC viability and constitutive secretion, and in the presence of pro-inflammatory stimuli, promotes elevated secretion from hMSC of a panel of regulatory cytokines and growth factors. Finally, we demonstrate, via *in-vitro* models of inflammation, that encapsulated hMSC promoted expression of CD206 associated with the alternative activated M2 subtype of macrophages.

2.3 Methods

2.3.1 Cell Culture

All cell cultures were incubated in a humidified $37 \,^{\circ}$ C, $5\% \,^{\circ}$ CO₂ environment. hMSC were purchased from Texas A&M at passage 1 and cultured as previously described [153]. Briefly, hMSC were cultured in MEM- α (Gibco) medium, containing no deoxy and ribo nucleosides, supplemented with 10% fetal bovine serum

(FBS) (Atlanta Biologicals, Lawrenceville, GA), 1 ng/ml basic fibroblast growth factor (bFGF) (Gibco), 100 units/ml penicillin and 100 μ g/ml streptomycin (Gibco). hMSC were plated at 5000 cells per cm² and allowed to proliferate to 70% confluence (approximately 4 to 5 days). Only hMSC at passages 2 through 5 were used to initiate subsequent experiments. Human acute monocytic leukemia cell line (THP-1) (ATCC, Manassas, VA) was maintained at 8x10⁵/ml in RMPI 1640 (Gibco) medium supplemented with 10% FBS (Gibco), 4mM _L-glutamine (Gibco), 100 units/ml penicillin, and 100 μ g/ml streptomycin (Gibco). Medium was replenished every 3 days and cells were passaged every 6 days. THP-1 cells (3.2x10⁵/ml) were differentiated using 16 nM phorbol-12-myristate 12-acetate (PMA) (Sigma-Aldrich, St. Louis, MO) for 16 hours. The differentiation was further enhanced by removing the PMA-containing medium and incubating the cells for 3 days in culture medium before experiments.

2.3.2 Alginate Microencapsulation

Alginate Poly-L-Lysine microencapsulation of hMSC was performed as previously described [171]. The microencapsulated cells were re-suspended in MEM- α (Gibco) and transferred to 25 cm² tissue culture flasks. Medium was changed every 7th day post-encapsulation for a total culture time of 21 days. In all experimental conditions, monolayer culture configurations of hMSC were used as controls for viability, growth kinetics, and functional studies. Microcapsules were synthesized with different concentrations of alginate (1.7%, 2.2% and 2.5%) as well as different initial cell densities (10⁶, 2x10⁶, 4x10⁶ and 6x10⁶ cells per ml). Based on initial viability post encapsulation, 4x10⁶ cells per ml was identified to be optimal for MSC encapsulation and therefore used in all subsequent experiments (data not shown). Capsule diameters ranged from 450 to 550 µm for all *in-vitro* studies.

2.3.3 Viability/Proliferation

Viability was assessed using calcein (Molecular Probes, Eugene, OR), and ethidium homodimer (Molecular Probes) staining as previously described [171] on days 2, 5, 7, 10, 14, 18, 21, 40, and 60 post encapsulation. Briefly, capsules were washed 3 times with phosphate buffered saline (PBS) (Gibco) and then incubated for 15 minutes with PBS containing calcium and ethidium homodimer, as per vendor's instructions. Capsules were washed 3 times before using an Olympus IX81 spinning disc confocal microscope to acquire 500 μ m Z stacks at 20 um intervals for 15 capsules per condition. Digitized images of each cross section for fifteen capsules per condition were analyzed for live cells (green fluorescing) and dead cells (red fluorescing) using Slidebook software.

Proliferation was assessed on days 2, 5, 7, 10, 14, 18 and 21 post encapsulation as previously described [172]. Briefly, capsules from three samples per condition were counted, subsequently dissociated in 1% EDTA (Sigma Aldrich) for 10 min and centrifuged at 400 g for 5 minutes. The pellet was re-suspended, stained with trypan blue and cells were counted using a hemocytometer. The cell count was then normalized to the number of capsules in the initial sample.

2.3.4 Cytokine Measurement

Evaluation of cytokine secretion was performed on days 2 and 21 post encapsulation. Capsules across different alginate concentrations (1.7%, 2.2% and 2.5%) were placed in 75 um inserts (Corning, NY) for a 12 well plate and cultured for 48 hours in hMSC medium supplemented with IL-6 (25 ng/ml) and TNF- α (25ng/ml) /IFN- γ (25ng/ml) (R&D Systems, Minneapolis, MN). Each well had capsules containing a total of 6x10⁴ cells per well. hMSC cultured in monolayers served as secretion controls. Supernatants were collected and stored at -20° C. Supernatants were analyzed via multiplex bead analysis (Bio-Rad, Hercules, CA) for 27 different growth factors and cytokines as per vendor's instructions. Data was normalized to cell number and monolayer secretion levels. A 2 way ANOVA was implemented to assess statistical significance within the data set. Post-hoc analysis was performed via the Fisher's least significant difference (LSD) method; p-values < 0.05 and 0.1 were considered significant.

2.3.5 Macrophages

Following the differentiation of THP-1 cells for 4 days, co-cultures were established either with encapsulated hMSC or free hMSC within 8 μ m transwell inserts (Corning). Macrophages were treated with 1 μ g/ml lipopolysaccharide (LPS) (Sigma Aldrich) and hMSC at various cell concentrations (4x10³ and 4x10⁴ cells /ml). The cultures were incubated for 24 hours, after which culture supernatants were collected and macrophages were fixed for immunocytochemistry. Macrophages were immunostained as previously described [173] with iNOS (Sigma Aldrich, rabbit antihuman, 1:130) and CD206 (Abcam, rabbit anti-human, 1:700). Images were acquired using an Olympus IX81 spinning disc confocal microscope and stereology was performed using Slidebook software. Supernatants were analyzed via ELISA for IL-10, TNF- α (Biolegend, San Diego, CA) and via multiplex bead analysis (Bio-Rad) for IL-1 β , IL-6, IP-10 and MIP-1 α , performed as per vendor's instructions. Encapsulated Chinese hamster ovary (CHO) cells were used as a control.

2.3.6 Statistical Analysis

Each data point represents the mean of three or more experiments (each with biological triplicates), and the error bars represent the standard deviation from the mean, unless otherwise specified. Statistical significance was determined using the student t-test for unpaired data. Differences were considered significant if the p-value was less than or equal to 0.05, unless otherwise stated.

2.4 Results

2.4.1 Evaluation of Encapsulated hMSC Viability and Proliferation

The ultimate goal of our studies was to determine whether alginate encapsulation could support hMSC immunomodulatory function allowing its utilization as a vehicle for controlled *in-vivo* delivery. However, before function could be evaluated hMSC viability and proliferation in the capsule microenvironment were assessed. Initial experiments incorporated calcein and ethidium homodimer staining to assess hMSC viability within the microcapsules. Encapsulated hMSC remained > 90% viable for at least 60 days post-encapsulation, indicating that the microenvironment could sustain hMSC survival for long time periods (Figure 1A). Next, we varied the alginate concentration and assessed cell proliferation over time. Our results indicated that hMSC proliferation was dependent upon the alginate concentration since 2.2%, but neither 2.5% or 1.7% alginate, supported hMSC proliferation throughout the 3 week experimental period. By day 21, the final cell concentration per capsule was twice the initial seeding density (Figure 1B), a proliferation rate far lower than monolayer conditions (data not shown). Lower

proliferation rates within the capsule microenvironment is consistent with our previous ES cell studies [171].



Figure 2.1. Viability and proliferation within various alginate micro-environments. A) hMSC viability up to 2 months post 2.2% alginate encapsulation, with each data point representing mean of sample size for three experiments. Viability was >90% for hMSC encapsulated in 1.7% and 2.5% alginate as well (data not shown), B) Evaluation of hMSC proliferation within the capsule microenvironment over 21 days of culture. Each time point represents cell number means normalized to initial cells per capsule for a given experiment. Typical initial seeding densities ranged from 80 to 100 cells per capsules.

2.4.2 Encapsulated hMSC Secretion

hMSC secrete numerous factors, many of which have been found to contribute to their immunomodulatory and tissue protective effects [150]. In order to evaluate whether the capsule microenvironment could sustain hMSC secretory function, a 27 factor multiplex assay was employed. Our results demonstrated that, as expected, monolayer-cultured hMSC constitutively secrete a plethora of factors (Table1). Among these were critical inflammatory cytokines, as well as factors responsible for growth and development. Having established quantitative baseline measurements of monolayer-cultured hMSC, we evaluated whether the capsule microenvironment could support secretory function. The results of our studies indicated that encapsulated hMSC supported comparable constitutive secretion patterns during the first 2 culture days, with the highest overall levels from hMSC in the 1.7% and 2.2% alginate microenvironments (Figure 2A). Subsequent assessment of constitutive secretion at day 21 post-encapsulation revealed diminished secretion from 2.5% alginate encapsulated cells, but constant secretion patterns from 1.7% and 2.2% encapsulated cell conditions (Figure 2B).

A distinct characteristic of hMSC is that upon stimulation with pro-inflammatory cues, secretion levels are increased [174, 175]. In the presence of inflammatory cues TNF- α and IFN- γ , monolayer-cultured hMSC could be stimulated to increase secretion of various factors (Figure 2C). When encapsulated hMSC were cultured in the presence of

TNF- α and IFN- γ and evaluated 2 days post encapsulation, we observed elevated secretion patterns relative to un-stimulated monolayer cultures (Figure 2C) and elevated levels comparable to those found for TNF- α and IFN- γ stimulated monolayer cultures. Furthermore, this induction was observed over time and found to be sustained for the 21 day culture period (Figure 2D). Overall, our data indicate that 1.7% and 2.2% alginate microenvironments augment constitutive secretion relative to monolayer hMSC cultures and amplify secretion post hMSC activation by inflammatory cues.

Concentration (pg/ml)	Basal Medium	TNF-α/IFN-γ
2-10	IL2,IL1b,IL15 IL4	
10-30	IL13	IL4,IL13,IL2,,IL1b
30-60	IL9, MIP1 α , MIP1 β , PDGFbb, RANTES, FGFbasic	IL9
60-135	IL1Rα, IL-12, Eotaxin, GCSF,IL10,IP10, GMCSF	IL10,II12,FGFbasic,GCSF, PDGFbb
135-180		MIP1α,MIP1β,IL15,Eotaxin
200-400		GMCSF, IL1Rα
1000-3000	IL6, IL8, MCP-1	RANTES, MCP-1, IL6
10000>	VEGF	IL8,IP10, VEGF

Table 2.1. General levels of hMSC secretion from 6×10^5 cells in basal and activated monolayer cultures.



Figure 2.2. Multiplex analysis of protein secretion from encapsualted hMSC. Secretion from encapsulated hMSC cultures normalized to baseline levels of monolayer hMSC secretion A) day2 B) day21 post-encapsulation. Secretion from TNF- α and IFN- γ stimulated encapsulated hMSC normalized to baseline levels of monolayer hMSC secretion C) 2 days D) 21 days post encapsulation. Statistical significance was established by 2 way ANOVA with confidence value of .05. Post hoc analysis to determine inidivdual differences within the population was determined via Fisher LSD. For general ranges of protein secretion reference Table 1.

2.4.3 Assessment of Anti-inflammatory Function with In-vitro Macrophage Cocultures

hMSC have been suggested to exert their tissue protective benefits, partially, via modulation of immune cell behavior [150]. Therefore, having determined that constitutive and stimulated secretion patterns were sustained for encapsulated hMSC, experiments were designed to determine whether encapsulated hMSC could function to reduce inflammatory macrophage behavior. Inflammatory macrophages have been found to exacerbate pathological events post organ trauma [176] and hMSC have been shown to attenuate this effector function [173, 177]. A THP-1 monocyte co-culture system was employed, where upon LPS stimulation, THP-1 monocytes enter a proinflammatory state, referred to as classical activation (M1), represented by elevated TNF- α secretion and iNOS expression [178]. The activated macrophages were cultured in the presence of both encapsulated and free hMSC, and modulation of both iNOS and TNF- α expression was assessed. Results of ELISA analysis indicated that free hMSC and encapsulated hMSC, but not encapsulated CHO cells, attenuated inflammatory macrophage TNF- α secretion to a similar degree (Figure 3A). Next, THP-1 cells were analyzed immunocytochemically for expression of the activation marker iNOS. As expected, the activated macrophage population expressed high iNOS levels homogenously throughout the population (Figure 3B), whereas iNOS expression levels after co-culture with encapsulated hMSC were reduced (Figure 3B).



Figure 2.3. Encapsulated hMSC attenuate macrophage activation *in-vitro***.** A) TNF-a secretion from macrophages activated with $1\mu g/ml$ LPS over 24 hours treated with at $1x10^3$ and $1x10^4$ free or encapsulated hMSC. Data is represented as mean TNF-levels normalized to activated macrophage conditions from three experiments. Asterisks (*) designate statistical significance (p<.05) compared to activated conditions. B) Representative immunocytochemical staining for iNOS depicting encapsulated hMSC mitigation of macrophage nitric oxide levels compared to activated macrophage cultures.

2.4.4 Encapsulated hMSC promotion of an anti-inflammatory macrophage phenotype

Macrophages have been identified to exhibit a great degree of phenotypic plasticity [179] and it has been suggested that hMSC could control this plasticity, by promoting anti- rather than pro- inflammatory function [173, 177]. Therefore, experiments were designed to determine if this function was maintained by encapsulated hMSC. LPS activated macrophage protein secretion was evaluated via multiplex protein analysis to determine whether hMSC co-cultured macrophages merely reverted to a quiescent state or if they assumed an alternative phenotype. Activated macrophages characteristically secreted pro-inflammatory factors (IL-1b, IP-10, and MIP1a) at elevated levels compared to quiescent macrophages (Figure 4A-C). In the presence of

MSC, macrophage secretion of inflammatory factors was attenuated (Figure 4A-C). In contrast, secretion of IL-6 was elevated with respect to both quiescent and activated macrophage cultures Figure 4D), a phenotype which has been found to be associated with anti-inflammatory M2 macrophage alternative activation [173]. In an effort to gain further insight into the mechanism of macrophage inflammation attenuation by hMSC, we quantified expression of surface CD206 and secretion of the anti-inflammatory mediator IL-10, both of which are characteristic of a M2 macrophage phenotype. We observed that co-culture of encapsulated hMSC with activated THP-1 resulted in elevated levels of CD206 expression (Figure 5A). Furthermore, CD206 expression was regulated in a dose dependent manner with only the 10^3 encapsulated hMSC resulting in elevated CD206 expression (Figure 5A). In addition, IL-10 secretion levels were elevated when encapsulated hMSC were present during activation (Figure 5C). Therefore, both attenuation of M1 and promotion of M2 phenotypes may both be induced by co-culture with encapsulated hMSC. However, precise control over time and dose with hMSC treatment may be required to balance this transition.



Figure 2.4. Evaluation stimulated macrophage protein secretion in the presence of encapsulated hMSC. A) Non-activated macrophages baseline secretion. B) Macrophage secretion levels after activation with 1µg/ml LPS. C) LPS treated macrophage secretion in the presence of encapsulated 1×10^4 hMSC. Asterisks (*) designate statistical significance (p<.05) compared to quiescent macrophages. Double Asterisks (**) represent statistical significance (p<.05) compared to activated macrophages.



Figure 2.5. Evaluating alternatively activated macrophage phenotypes in THP-1 -encapsulated hMSC co-cultures. A) Hoechst and representative CD206 staining of activated macrophages versus macrophages treated with 1000 hMSC. B) Quantitation of CD206 expression in activated macrophages and macrophages treated with 1×10^3 to 1×10^4 encapsulated hMSC. Data points represent sample means from four different experiments. Data was normalized to activated macrophage CD206 expression. C) IL-10 secretion from encapsulated hMSC treated macrophages. Asterisk (*) designates statistical significance (p<.05) compared to activated macrophages.

2.5 Discussion

The development of engineered hMSC delivery systems is vital for clinically relevant therapeutic protocol translation. However, current hMSC infusion strategies can neither regulate unwanted cell migration nor ensure hMSC persistence at the injury site. In fact, recent findings suggest that transplanted hMSC no longer persist at injury sites as early as 7 days post transplantation [137]. Cell immobilization systems have long been proposed as a vehicle for delivering controlled release of therapeutic agents. However, to date no biological vehicle has been described that can maintain the secretion of the wealth of therapeutic factors hMSC provide, as well as circumvent fibrosis post encapsulation for extended periods [180].

Alginate has been found to avoid biodegradation within the CNS up to 6 months post transplantation [181]. Our studies have shown that hMSC remain viable within the alginate micro-environment for at least 2 months and, depending on alginate concentration, support proliferation, although not at the high rates found in monolayer cultures. This finding is consistent with previous reports where mouse embryonic stem cell proliferation within alginate microcapsules was only supported at 2.2% alginate concentration [171, 182] . In addition, hMSC have been found to differentiate into several mesodermal cell lineages depending on the cell source, passage number and culture conditions. Studies have indicated that the alginate microenvironment may be designed to control hMSC differentiation [167], a feature which may be important in controlling anti-inflammatory function as well. In fact, recent studies in our laboratory support this observation (data not shown).

In many models of trauma, hMSC treatment benefits have been attributed to their unique ability to control inflammatory responses. hMSC have been found to mediate inflammation and promote tissue repair through the secretion of a variety of soluble mediators [150]. Here, the capsule microenvironment not only sustained but also enhanced the secretion of these soluble mediators. While the mechanism(s) of hMSC inflammation control is unclear, it has been suggested that in the presence of inflammatory factors, such as TNF- α and IFN- γ , the hMSC anti-inflammatory phenotype is promoted [175]. We have replicated this response with our encapsulated hMSC populations. Overall, our analyses indicate that the capsule microenvironment enhances hMSC secretion, a finding corroborated by recent findings that 3 dimensional culture of hMSC enhances anti-inflammatory therapeutic potential [183]. However, within 2.5% alginate capsules, the secretion rates were diminished compared to the other concentrations. Cumulatively based on viability, secretion profile and our previous alginate encapsulation studies [171, 184, 185] the 2.2% alginate encapsulation condition was chosen for further functional evaluation. However, hMSC in a 1.7% alginate microenvironment also demonstrated sustained secretion patterns and may also have been a suitable choice.

Several publications have attributed organ pathology expansion to M1 macrophage secretion of pro-inflammatory cytokines and tissue degrading enzymes [176, 186]. The ability of hMSC to attenuate macrophage activation *in-vitro* underscores the tremendous potential of these cells in preventing tissue degradation [187]. Our studies indicate that encapsulated hMSC co-cultures can attenuate M1 secretion of TNF- α as well as the percentage of iNOS expressing macrophages in LPS stimulated cultures.

Furthermore, multiplex analyses revealed that encapsulated hMSC attenuate secretion of several pro-inflammatory factors (MIP-1 α , RANTES and IL-1 β), which have been found to be elevated early in tissue pathologies and are associated with facilitating tissue fibrosis [188].

Interestingly, depending on the time and hMSC to macrophage ratio, CD206, a marker for the M2 phenotype, was elevated. The time and ratio dependency of M2 phenotypic acquisition may be due to accumulation of additional factors in static culture which drive the adoption of other macrophage phenotypes. Overall, this finding is consistent with previous reports that have highlighted a novel type of macrophage referred to as the hMSC educated macrophage (M2m) [173]. However, to date, this phenomenon has not been explored in an immobilized platform. M2m macrophages exhibited levels of CD206 ^{high}, IL-10 ^{high}, IL-6 ^{high} and TNF- α ^{low}, a profile analogous to the one measured here, suggesting that this may be the macrophage function may also occur via a combination of M2 phenotype promotion as well as M1 macrophage attenuation.

Overall our studies support the incorporation of encapsulated hMSC as an immunomodulatory delivery vehicle *in-vivo*. Alginate parameters were identified to maximize hMSC survival and protein secretion. We also demonstrated that encapsulated hMSC can attenuate macrophage activation *in-vitro*. Additionally, the encapsulated hMSC were able to modulate macrophage function to a state which, *in-vivo*, could promote tissue regeneration. This hypothesis was corroborated with an *in-vivo* model of SCI where encapsulated hMSC were able to promote pro-inflammatory macrophage

attenuation at the site of injury. These macrophages expressed higher levels of alternatively activated phenotypic markers. The immobilization system developed here should circumvent many of the drawbacks in current hMSC administration platforms and at the same time may serve to augment hMSC tissue protective behavior.

Acknowledgments

These studies were supported by NIH 5R21NS070175 and the graduate fellowship programs IGERT on stem cell research NSF DGE 0801620, Rutgers-UMDNJ Biotechnology Training Program 2T32GM008339-21 and the New Jersey commission on spinal cord injury 10-2947-SCR-R-0. We thank Purvee Patel and Kamal Siugh for technical assistance.

Chapter 3 Reversal of Fibronectin Induced Hippocampal Degeneration with Encapsulated Mesenchymal Stromal Cells

Manuscript to be Submitted to The Journal of Experimental Neurology

3.1 Abstract

Chapter 2 conveyed the feasibility of MSC alginate encapsulation. We show that the MSC remian viable after encapsulation. eMSC secretion was not affected in the microenvironment and immuno-modulatory function sustained. Before, moving to preclinical animal studies, we wanted to further validate eMSC efficacy in-vitro. To do so we turned to organotypic model of CNS injury. The nice thing about these systems is that complex microstructure of the tissue is maintained in culture. The complex cellular interactions between the paryenchma and glia is intact, therefore, one can evluate the ability of a therapy to mitigate the secondary injury cascade on the lose of neuronal tissue. Here we plate hippocampel slices on fibronectin to induce an injury over 2 weeks of culture. This observation is not intergral to this dissertation, but may represent an interesting aspect of the secodary injury cascade. What is more important is that we show that eMSC can reverse the degradation observed with this injury model.

3.2 Introduction

Central nervous system (CNS) associated pathologies are difficult to resolve, in part because they are induced by a wide range of biological processes, and therefore represent a significant challenge to our healthcare system. While the underlying cause of CNS pathologies may be different, there are aspects of the observed pathologies that are similar. Therefore, the identification of conserved mechanisms among similar pathological outcomes may potentially provide an avenue for the development of standardized treatment regimens. Interestingly, a common consequence of all CNS diseases and traumas is the extravasation of blood plasma constituents through the normally selective blood brain barrier (BBB). Disease states such as Alzheimer's (AD), multiple sclerosis (MS), meningitis, neoplasms and others, all occur in concert with increased blood brain barrier (BBB) permeability [189] which leads to the entry of blood plasma constituents into CNS tissue [190]. CNS traumas such as spinal cord injury (SCI) and traumatic brain injury (TBI) result in a significant amount of tissue hemorrhage leading to the extravasation of blood constituents into healthy and damaged CNS tissue [191]. In addition to immune and red blood cells, blood plasma contains dissolved proteins, glucose, clotting factors, mineral ions, hormones and carbon dioxide. Recently, evidence suggests that particular plasma proteins may have deleterious effects after experimental SCI [192]. In particular, fibronectin interaction with immune components has been implicated in organ transplant failure [193] and has been shown to directly activate microglia [194-196]. Fibronectin, a large dimeric glycoprotein (400-500 kDa) composed of independent domains FN I, II and III, is present in plasma protein at approximately 300 µg/ml [197] and has been shown to permeate the CNS following an *in* vivo controlled cortical impact injury [198], mouse autoimmune encephalomyelitis (EAE) [194], neoplasm [199] and AD [200] models. Fibronectin has been shown to remain elevated and is found in regions with high concentrations of activated microglia/macrophages [198]. Despite these unfavorable observations, fibronectin has

been implemented as a CNS regenerative material [201-203]. However, the interaction of fibronectin with immunoregulatory cells may lead to immuno-pathological destruction of CNS tissue and can overall lead to poor morbidity rates in the clinic.

The clearly established role of immune-inflammatory networks in the progression of many CNS diseases underscores the importance of maintaining clinical immunological homeostasis after disease onset. MSC secrete a plethora of immunomodulatory factors [204] in addition to facilitating tissue regeneration and preservation [205]. However, general transplantation modalities do not ensure long term MSC persistence and would require repeated injections to sustain benefits for patients with chronic CNS diseases. Therefore, engineered polymer based systems have been developed to encapsulate MSC (eMSC) and deliver them to CNS intrathecal spaces [206], where within the cerebral spinal fluid (CSF) the eMSC could respond to injury cues by providing immunomodulatory and trophic support. The studies described here utilized organotypic hippocampal slice cultures (OHC) as an in vitro model to evaluate the potential pathological effect of fibronectin on CNS tissue and the ability of eMSC to prevent CNS degeneration. The results suggest that fibronectin is not a permissive CNS substrate and induces significant tissue degeneration overtime. eMSC were able to reverse fibronectin induced degeneration and sustain overall OHC health.

3.3 Methods

3.3.1 Human MSC Culture

All cell cultures were incubated in a humidified $37 \,^{\circ}$ C, $5\% \,^{\circ}$ CO₂ environment. Human MSCs (hMSC) were purchased from Texas A&M at passage 1 and

cultured as previously described [206]. Briefly, hMSCs were cultured in MEM- α (Gibco) medium, containing no deoxy and ribo nucleosides, supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), 1 ng/ml basic fibroblast growth factor (bFGF) (Gibco), 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco). hMSC were plated at 5000 cells per cm² and allowed to proliferate to 70% confluence (approximately 4 to 5 days). Only hMSCs at passages 2 through 5 were used to initiate subsequent experiments.

3.3.2 Alginate Microencapsulation

Alginate Poly-L-Lysine microencapsulation of hMSCs was performed as previously described [171] however with some modifications. A 2.2% (w/v) alginate (Sigma-Aldrich, MW: 100,000 -200,000 g/mol, G-content 65%-70%) solution was generated with Ca²⁺ free DMEM (GIBCO). The solution was filtered using a 25-micron syringe filter (Fisher Brand, Pittsburg, PA). A 70% confluent monolayer culture of MSC was removed with trypsin (Gibco) and counted. The cells were spun down at 400 g's and re-suspended at a concentration of $4x10^7$ cells / mL with Ca²⁺ free DMEM. The cell solution was then diluted 10x with 2.2% alginate yielding a final cell concentration of $4x10^6$ cells/ml and a 2% (w/v) alginate solution. The solution was transferred to a syringe pump (KD Scientific, Holliston, MA). Alginate beads were generated using an electrostatic bead generator (Nisco, Zürich, Switzerland) with an accelerating electrode at a flow rate of 2 mL/h and an applied voltage of 6.4 kV. The resulting bead diameter was 225 µm ± 25. The beads were extruded into and 100 mL bath of CaCl₂ (100 mM) (Sigma Aldrich) containing 145 mM NaCl (Sigma-Aldrich) and 10 mM MOPS (Sigma-Aldrich). Microencapsulated cells were washed once with PBS and then treated for 2 minutes with poly-L-Lysine (PLL) (sigma –Aldrich, Mw: 68,600 g/mol) (0.05% w/v). The capsules were washed one more time and then re-suspended in MEM- α (Gibco) and transferred to 25 mm² tissue culture flasks.

3.3.3 Surgical Procedures

The surgical procedures were all performed in accordance with protocols approved by the NIH guidelines for the care and use of laboratory animals. A total of 20 (male/female) postnatal day 4-6 Sprague-Dawley pups (Taconic, NY) were used. First the brains of the pups were removed and placed in ice cold Gey's Balanced Salt Solution (Sigma-Aldrich Corp, MO) supplemented with 10mM D-glucose (Sigma-Aldrich Corp, MO) and 3µM Kynurenic Acid (Sigma-Aldrich Corp, MO). The hippocampi were separated from the surrounding cortex and sliced into 400µm thick slices using a McIlwain Tissue Chopper (Vibratome, IL). The slices were then carefully placed onto fabricated strips of polydimethylsiloxane (PDMS) (Sylgard 184, Fischer Scientific, MA). The petri dish was filled with 750µl of serum containing media (1:1:2 of heat inactivated horse serum, Hanks Balanced Salt Solution, Basal Medium Eagle, supplemented with 0.5mM L-Glutamine, 30µg/ml gentamycin and 10mM HEPES, all from Invitrogen, CA). After 24hours the media was changed to a serum free media (Neurobasal A, 1X B27, 0.5mM L-glutamine, 30µg/ml gentamycin and 10mM HEPES, all from Invitrogen CA). Thereafter, half of the media was changed every 48hours.

3.3.4 Condition Preparation and Tissue Processing

Prefabricated strips of PDMS (150µm thickness) were sterilized with 70% ethanol, and then placed on top of a round, sterile, microscope cover slip. The coverslips with PDMS were then placed within individual 35mm petri dishes (Becton, Dickinson, NJ). Oxygen plasma treatment sterilized the entire assembly and irreversibly bonded the PDMS to the glass coverslip. After plasma treatment the petri dish assemblies were treated with 1.5mL of sodium hydroxide for 1hour, followed by two 1mL washes with deionized water. Fibronectin (Sigma-Aldrich Corp, MO) was applied at a concentration of 25ug/mL for 3 hours to some conditions while poly-D-lysine (PDL, Sigma-Aldrich Corp, MO) and laminin (Sigma-Aldrich Corp, MO) were applied, in that order, to control culture conditions at a concentration of 80ug/mL and 1mg/mL respectively. The PDL was applied first overnight, while the laminin was applied for 2hours the following day. Finally, each petri dish was filled with 750ul of serum free media after the initial coatings for 24hours before removal and organotypic hippocampal slice addition. Hippocampus slice explants of 450 µm thickness were placed on top of the treated PDMS in the assembly to complete each organotypic hippocampus slice culture (OHC).

750uL of serum containing media was added to the cultures, a volume chosen to optimize the media-oxygen interface, and placed in a humidified 5% CO₂ incubator at 37° C. 24 hours later, the serum containing media was exchanged for 750µl of serum-free media. In later experiments, alginate capsules containing 50,000 hMSCs were distributed

evenly into petri dishes of both the control and injury conditions in order to assess the models' response to the cellular therapeutic.

Half the media was changed every 48 hours until day 14, the end of each experimental period. Exchanging half the media served to replenish essential nutrients without entirely removing secreted growth factors or cytokines, preserving each culture's micro-environmental condition. Following each media change, the extracted solutions were frozen and stored for future analysis. Phase contrast, images were taken of each condition to qualitatively assess slice degradation, axonal outgrowth, and overall slice health using an Olympus CKX41 microscope (Olympus, PA).

3.3.5 Assessing Cell Viability

The organotypic slices were stained on days 3, 7, and 14 using 5ug/mL propidium iodide (PI), a DNA intercalating stain, diluted in 37°C serum free media. The stain was allowed to permeate through the slice for 30 minutes by returning the cultures to the incubator and was subsequently removed before imaging. After washing the slices with 37°C serum free media, they were imaged using the TRIT-C channel on an Olympus IX81 spinning disk confocal microscope (Olympus, PA) using Slidebook software (Intelligent Imaging Innovations; Denver, CO). The cultures were then fixed by applying 4% paraformaldehyde for 20 minutes. The paraformaldehyde was removed and replaced with Tris Buffered Saline solution (TBS - 0.5M Tris Base, 9% NaCl, pH 7.4) to prevent dehydration. Each organotypic slice acted as its own PI control. After being fixed, each slice was again introduced to PI. 850uL of a 0.1% Triton-X, TBS solution was applied to the slices for approximately 45 minutes. The solution was removed; the cultures were

washed and then exposed to PI for 48 hours at 4°C. After a final wash, the slices were ready to be imaged.

3.3.6 Histology and Immunohistochemistry

Slices were fixed on days 3, 7 and 14 in 4% paraformaldehyde to study temporal expressions. After being fixed, slices were blocked using a 10% goat serum, 0.1% Triton-X 100, 1% bovine serum albumin, TBS solution for 45 minutes at room temperature. Slices were stained with either a rabbit primary antibody (1:800) to IBA-1 (IgG) (Abcam, MA), or a rabbit primary antibody (1:400) to Tau (IgG) (Bectin, Dickinson, NJ) overnight at 4°C. IBA-1 staining was followed by an Alexa Fluor 488 goat anti-rabbit secondary (1:450) (Invitrogen, CA) for 2 hours at room temperature, and Tau staining this was followed by an Alexa Fluor 647 goat anti-rabbit secondary (1:500) (Invitrogen, CA) for 2 hours at room temperature. Slices were imaged on either the GFP channel (IBA-1) or Cy-5 channel (Tau) at all three time points. An isotype control was used to account for background staining using rabbit IgG (Zymed, CA) at the same concentration as the rabbit primary antibody also overnight at 4°C. All sections were washed in room temperature Phosphate Buffered Saline (PBS) after each antibody addition and before imaging.

3.3.7 Image Analysis

Bright field and fluorescent images were taken of each condition to qualitatively assess slice degradation, axonal outgrowth, and overall slice health during tissue processing. To establish quantitative results concerning slice surface area, images were analyzed using ImageJ software (NIH). Regions of interest were drawn along the boundary of each organotypic slice at the beginning of each culture and superimposed on the slice at each subsequent time point to calculate changes in slice area. PI stained images were analyzed using ImageJ software, and specific tissue regions were scrutinized including the dentate gyrus, CA1, and CA3 hippocampal regions. IBA-1images were captured using the same microscope and GFP channel. These images were also analyzed using ImageJ software following a similar protocol to the PI stain results.

3.3.8 Statistical Analysis

A one-sided student t-test for unpaired data was used to compare the fluorescent signal of each of the stains for 5 experiments with between 1-3 cultures per condition in each individual experiment. The areas analyzed were the dentate gyrus, CA1, and CA3 region of the hippocampus. Statistical significance was considered for differences with a P-value less than or equal to 0.05, unless stated otherwise.

3.4 Results

3.4.1 Using Fibronectin to Induce Injury-like Conditions in Organotypic Hippocampal Slice Cultures

Given the influx of fibronectin post CNS injury, an in vitro hippocampal slice culture system was established to investigate the effect of plasma fibronectin on health and survival of CNS tissue. Hippocampal slices were obtained from 4-6 day old rat pups, sectioned to 400 μ m, and then plated on fibronectin or poly-d-lysine (PDL) and laminin (referred to as PDL+Laminin), a known permissive substrate. Slices were cultured for fourteen days and phase contrast images were acquired every other day to observe temporal changes in slice morphology. Day 3 slices on fibronectin and PDL+Laminin exhibited sustained hippocampal superstructure with visible CA1-CA3 and dentate gyrus regions (Figure 1A). By day 13 there was considerable tissue degradation with slices plated on fibronectin in which hippocampal superstructures were essentially unidentifiable (Figure 1A). At the same time point slices plated on PDL+Laminin exhibited less dramatic decreases in tissue area and hippocampal superstructures were still identifiable, although the slice had shrunk without the degree of degradation seen in the fibronectin condition (Figure 1A). To quantify the extent of tissue degradation, slice areas were measured every other day from day 3 until day 13. Areas were normalized to the initial slice area at each time point. On day 5 there was statistically significant degradation on fibronectin coated surfaces compared to PDL+Laminin conditions (Figure 1B). By day 13 there was a 45% decrease in slice area compared to 20% on PDL+Laminin surfaces (Figure 1B). The decrease in tissue area on PDL+Laminin was mainly due to cellular outgrowth. However, on fibronectin there was drastic alteration in cellular morphology suggestive of necrosis. Overall, fibronectin is deleterious to hippocampal in-vitro tissue survival.


Figure 3.1 Fibronectin to Induced Injury-like Conditions in Organotypic Hippocampal Slice Cultures. A) Representative images of an OHC placed on fibronectin or PDL+Laminin coated surface from days 3 and 14. OHC degenerate significantly by day 13 when cultured on a fibronectin-coated surface, however, Slices cultured on a surface coated with PDL+Laminin did not. B) The surface areas of the OHCs were quantified revealing approximately a 45% loss in tissue when slices were cultured on fibronectin. Asterisks represent statistical significance (p < 0.01) and error pars represent standard error, where N=7.

3.4.2 Cell Death and Damaged Hippocampal Regions

To better evaluate the degree of cell death in the hippocampal cultures, with respect to the different surface coatings, slices were stained with propidium iodide (PI) exclusion dye. PI will only be taken up by cells with damaged nuclear membranes and is used extensively to assess cell death in hippocampal slice cultures (Macklis and Madison, 1990). OHCs were cultured on fibronectin or PDL+Laminin for 14 days and PI staining was performed immediately after isolation and then at days 3, 7, and 14. Immediately after isolation there is a considerable amount of PI staining. This baseline level of cell death remains 3 days after culture (Figure 2A). By day 14 PI staining has diminished significantly on PDL+Laminin conditions, but on fibronectin PI staining is considerably

elevated (Figure 2A). The staining was localized to the CA1-CA3 and DG regions of the hippocampal slice where the majority of neuronal cell bodies are found (Figure 2A). In order to quantify the degree of PI staining, image thresholds were adjusted to levels acquired from images of paraformaldehyde fixed slices. Average fluorescent intensities were calculated from regions of interest created around the CA1-CA3 and DG regions. The data depicts that initially there are elevated PI levels on both coated surfaces, which proceeds to subside by day 7 (Figure 2B). By day14 there is a significant increase in PI levels in fibronectin coated slice cultures, which remained diminished for PDL+Laminin cultured surfaces (Figure 2B). These results suggest that PDL+Laminin is a favorable culture surface to maintain OHC survival. There is no increase in PI staining after the initial elevation due to the isolation procedure. However, fibronectin coated surfaces result in an additional spike in PI staining at day 14, suggesting that fibronectin has deleterious effects on OHC survival.



Figure 3.2 Cell Death in Damaged Hippocampal Regions. A) Representative images of PI stained OHCs placed on fibronectin or PDL+Laminin from days 3 and 14. On day 3 there is initial cell death due to the isolation of the hippocampal slice. By day 14 OHC PI levels decrease on PDL+Laminin conditions, however, they remain elevated when coated on fibronectin. B) The staining in the OHC was quantified by creating ROIs around the DG, CA1 and CA3 regions and then calculating the average PI fluorescent intensity. All images were thresholded to PI stained paraformaldehyde fixed OHCs before quantification. The intensity values diminished by day 14 in healthy control conditions; however it was drastically higher in the fibronectin conditions. Asterisks represent statistical significance (p < 0.01) and error bars represent standard error, where N=5.

3.4.3 Fibronectin mediated activation of Microglia

In order to gain insight to the mechanism of fibronectin mediated OHC degradation, immune-staining of key cellular components (microglia, astrocytes, and neurons) were performed. Microglia/macrophages in the CNS have been implicated in driving the pathological consequences of TBI [208]. Therefore, OHC cultures were probed for activated microglia/macrophages at days 3, 7, and 14 of culture with IBA-1. IBA-1 is a calcium-binding protein that is specific to macrophages and is elevated following macrophage activation. Initially there is a baseline level of IBA-1, once again due to the nature of the isolation procedure, which was greatest at day 3, when OHCs were cultured on fibronectin (Figure 3A). By day 7 IBA-1 levels dropped significantly on PDL+Laminin conditions (Figure 3A). OHC fibronectin cultures also displayed a

drop in IBA-1 levels by day 7 (Figure 3A), however, they remained significantly elevated relative to PDL+Laminin conditions. IBA-1 levels remained significantly elevated compared to PDL+Laminin conditions on fibronectin cultures on day 14 with the localization of the staining showing an even distribution throughout the three regions of CA1, CA3 and DG (Figure 3B).



Figure 3.3 Fibronectin Mediated Activation of Microglia and Hippocampal Slice Health assessed through Tau Staining. A) Pictures depicting IBA-1 staining of OHCs cultured on fibronectin or PDL+Laminin. Initially, there is a considerable amount of intensely fluorescing IBA-1 regions. By day 14 on PDL+Laminin this intensity significantly diminishes; however on fibronectin there is still considerable amount of intensely fluorescing microglia. B) Average fluorescent intensity of IBA-1 was quantified over the entire slice area. The average fluorescent intensity of the IBA-1 stain in the slice was always higher in slices placed on fibronectin surfaces. This is evident throughout the fourteen day culture period. C) Representative images of OHC's stained with Tau. i) OHC on Fibronectin on Day 3, iii) OHC on Fibronectin on Day 14, iv) Day 14 OHC on PDL+Laminin MSCs cultures, v) Enlarged section of iv) Scale bar = 1mm. Asterisks represent statistical significance (p < 0.05) and error bars represent standard error, N=3.

3.4.4 Hippocampal Slice Health assessed through Tau Staining

In addition to assessing slice health through changes in hippocampal slice area and cell death, slices were stained for Tau and glial fibrillary acidic protein (GFAP). Staining for Tau allows for changes in neurons and axonal extension to be observed. Tau staining for axons did not reveal any observable changes in axon morphology at the micron level. By Day 14 we observe good maintenance of the distinct Hippocampal regions of PDL+Laminin conditions as compared to Fibronectin (Figure 3C iii, iv). This demonstrates that the PDL+Laminin surface generates a more favorable environment for neuronal health whereas fibronectin conditions result in a complete breakdown in neuronal hippocampal structure. Not only does PDL+Laminin create a favorable environment for slice health but facilitates axonal extension from the periphery of the slice, as seen by the extensive axonal extension network that originates from the periphery of the CA1 region (Figure 3C v - enlarged section of 3C iv). Glial fibrillary acidic protein (GFAP) staining for reactive astrocytes was also performed at the same time points. GFAP levels were no different between conditions at any of the time points (data not shown).



Figure 3.4 Encapsulated Mesenchymal Stromal Cells Preserve Tissue in OHCs on Fibronectin. Encapsulated MSC were cultured with OHC plated on either fibronectin or PDL+Laminin coated surfaces for 14 days. Consistently, fibronectin coated surfaces resulted in significant tissue degradation over time relative to PDL+Laminin conditions. When Co-cultured with eMSC OHC fibronectin cultures did not have significant degradation relative to PDL+Laminin. Asterisk represent statistical significance (p < 0.05) and error bars represent standard error, N=3.

3.4.5 Encapsulated Mesenchymal Stromal Cells Preserve Tissue in OHCs on Fibronectin

OHC culture on fibronectin led to significant tissue degradation and substantial cell death over time, events which are hallmarks of CNS pathology. MSC secreted factors result in cyto-protection and have been found to be efficacious in ameliorating CNS pathologies (Kim et al., 2010). Alginate encapsulation of MSC's has been previously evaluated as an effective means of sustaining MSC tissue protective properties

in an attempt to provide a transplantation modality that can improve MSC persistence at a site distant from the injury (Barminko et al., 2011). Therefore, experiments were designed to determine if MSCs could mitigate the deleterious effects of fibronectin on OHCs. Slices were co-cultured with 5x10⁵ eMSC on fibronectin or PDL+Laminin for 14 days. Phase contrast images were acquired every other day to observe and quantify slice Fibronectin coated cultures treated with eMSCs did not demonstrate degradation. substantial OHC degradation and the superstructure remained intact. The necrotic areas observed with fibronectin cultured conditions were not present when MSCs were introduced into the environment. Next, we quantified the tissue degradation. There was no difference between the PDL+Laminin conditions treated with or without eMSC (Figure 4). However, as early as day 5 there were significant tissue degradation differences, between cultures placed on fibronectin alone and those treated with eMSCs (Figure 4). In fact, in the presence of eMSC, fibronectin did not demonstrate significant differences in slice area at any time point when compared to PDL+Laminin cultures (Figure 4).



Figure3.5. Encapsulated Mesenchymal Stromal Cells Impart Cytoprotective Action. PI Staining of the OHC was quantified by defining a ROI around the DG, CA1 and CA3 regions and then calculating the average fluorescent intensity. The fluorescent intensities were considerably higher in the fibronectin conditions on day 14, the presence of eMSC's significantly mitigated cell death. This trend was also observed at days 3 and 7. Asterisk represent statistical significance (p< 0.05) and error bars represent standard error, N=3.

3.4.6 Encapsulated Mesenchymal Stromal Cells Impart Cytoprotective Action

In order to qualify the cytoprotective action of eMSCs on OHC cultures, PI staining for dead cells was performed. OHC fibronectin and PDL+Laminin cultures were incubated in the presence of eMSCs for two weeks and PI staining performed immediately after isolation and at days 3, 7 and 14. Qualitative observations suggest that the initial cell death observed at day 3, due to the isolation procedure, was significantly decreased in eMSC conditions. This was evident in both fibronectin and PDL+Laminin conditions. By day 7 PI intensity diminished in all conditions, however the eMSC treated conditions displayed the greatest drop in intensity (Figure 5). By day 14 untreated OHCs cultured on fibronectin displayed a considerable increase in PI staining, consistent with our previous observations (Figure 5). However, eMSC treated slices did not display

increased PI staining, suggesting that the negative effects of fibronectin on OHC culture from Day 7 to 14 are either diminished or prevented due to eMSC secreted factors. PI intensities remained diminished in PDL+Laminin conditions whether or not they were treated with eMSCs (Figure 5). We next assessed whether eMSCs have an effect on the elevated IBA-1 microglia expression induced by fibronectin. eMSC's attenuated microglia IBA1-expression on day 7 as well as day 14 to levels comparable to PDL+Laminin conditions (Figure 6). Lastly, in the presence of eMSC, even as early as Day 3, we observe higher expression levels of Tau in both PDL+Laminin and Fibronectin conditions as compared to without eMSC (Figure 6B i, iv). By Day 14, not only do we see a remarkable improvement of neuronal structure in fibronectin conditions with the addition of eMSC (Figure 6B ii, v), but we also observe axonal extension from the periphery of the slice as observed in PDL+Laminin conditions (Figure 6B vi). The extension observed in eMSC added PDL+Laminin conditions appears to be different to those observed without eMSC, where axonal extensions occur sporadically along the periphery with the most extensive occurring in the subiculum area (Figure 6B iii)



Figure 3.6 Encapsulated Mesenchymal Stromal Cells Attenuate Microglial Activation and enhance Tau expression. EMSC's were cultured with OHCs plated on fibronectin and PDL+Laminin for 14 days. At days 3, 7 and 14 slices were immunostained for IBA-1, an activated microglia marker. Average fluorescent intensity of the entire slice area was calculated at each time point. IBA-1 levels on day 3 in eMSC treated fibronectin conditions were reduced to levels comparable to that seen in PDL+Laminin conditions. Consistent with earlier observations OHC IBA-1 levels remained elevated at days 7 and 14 of culture on Fibronectin conditions, however, eMSC's diminished the levels significantly at both time points. B) Representative images of OHC's stained with Tau. i) OHC on Fibronectin in the presence of MSC's on Day 3, iv) OHC on PDL+Laminin in the presence of MSC's on Day 3, ii) OHC on Fibronectin in the presence of MSC's on Day 14, v) OHC on PDL+Laminin in the presence of MSC's on Day 14, iii) Enlarged section of ii vi), enlarged section of v). Scale bar = 1mm. Asterisk represent statistical significance (p < 0.05) and error bars represent standard error, N=3.

3.5 DISCUSSION

Organotypic slice cultures are powerful in-vitro platforms to identify novel and efficacious therapeutic interventions for CNS pathology. To this end, several in-vitro manipulations to OHC cultures have been implemented to initiate pathological associated events in an attempt to make physiologically relevant in-vitro systems. Some of these approaches include: induction of excitotoxicity [210, 211], inflammation [212] and mechanical damage [213] all which have been reviewed previously [214]. However, a major component of secondary pathological processes associated with CNS pathology is the interaction with systemic components. In particular, there is an abundant amount of plasma protein, which extravasate into the injured tissue after BBB breakdown [215, Fibronectin represents a considerable fraction of these infiltrating proteins. 216]. Fibronectin is detectable in the CNS during development [217], but essentially absent in the mature CNS [218, 219]. After maturation the only way the CNS will be exposed to fibronectin is if there is breakdown of the BBB, which only occurs under pathological circumstances. Fibronectin has been observed to be persistent at the tissue adjacent to brain injury sites for up to fourteen days post controlled cortical injury [198] and colocalizes with activated microglia/macrophages. The findings here, suggest that fibronectin is an unfavorable substrate for maintaining OHC culture health. By 2 weeks, marked tissue degradation was observed in fibronectin cultured surfaces. Culture on a favorable PDL+Laminin surface coating did not result in significant tissue loss. Necrotic tissue evident at the injury core was observed with OHC fibronectin cultures, however not on PDL+Laminin cultures. This was corroborated with increased PI levels in fibronectin cultures compared to PDL+Laminin cultures. These results imply that fibronectin is a non-permissive CNS substrate. Interestingly, fibrinogen has been implicated as an inhibitor to SCI regeneration [192], but has previously been shown invitro to support neuronal growth [220]. Fibronectin has displayed similar capabilities [221]. However, most *in-vitro* systems do not support the complex interaction between all CNS cellular components. Furthermore, fibronectin in-vivo studies [197, 201-203] do not

report motor or cognitive function assessment which will be the ultimate benchmark to determine positive vs. negative fibronectin effects on CNS tissue.

There is an abundant body of literature claiming that Fibronectin enhances microglia inflammatory activity. It has been demonstrated that microglia cultured on fibronectin exhibit increased secretion of pro-inflammatory cytokines and enhancement of phagocytic activity [195]. In-vivo models of EAE identify fibronectin adhesion to integrin subunits $\alpha_5 \beta_1$ and $\alpha_{\nu} \beta_5$ on microglia engendering deleterious activation [194]. Others have reported adhesion to fibronectin induces microglial expression of interleukin-1 β [222], which is a potent mediator of the pro-inflammatory response post injury. Fibronectin also stimulates elevation in signal transduction pathways associated with pro-inflammatory microglial action [223]. These studies observed elevation in the microglial/macrophage activation marker IBA-1 when OHCs were cultured on fibronectin. Initially, IBA-1 was elevated in all conditions; however, on control conditions PDL+Laminin microglia/macrophages reverted to a quiescent state. Fibronectin OHCs displayed elevated IBA-1 expression at 14 days of culture, suggesting that either directly or in-directly, fibronectin promotes microglia/macrophage activation. Activated microglia/macrophages produce harmful free radicals and superoxide factors. Furthermore, increased IBA-1 levels correlate with enhanced phagocytic action. Therefore, fibronectin induced stimulation of microglia/macrophages may be responsible for the considerable amounts of cell death and tissue degradation seen here. These events resemble the progression CNS pathology post-trauma where healthy tissue is damaged. The microglia elevation here corresponds with tremendous tissue degradation, which may

therefore serve as a platform to evaluate potential treatments of chronic pathological events post TBI.

MSCs represent a unique stem cell therapeutic platform in that their main benefits in CNS disease models are attributed to secreted factors and not differentiation [206]. Considering this mode of effector function, previous work has led to an encapsulation platform designed to immobilize MSCs locally at sites of injury [206]. The efficacy of the platform was evaluated to determine if the tissue degradation associated with fibronectin culture would be reverted in the presence of eMSCs. EMSCs reversed the tissue degradation associated with OHC fibronectin culture to levels similar to PDL+Laminin cultures. Similar observations have been made *in-vivo* where MSCs reversed pathological events associated with experimental TBI [17]. The amount of cell death 14 days in fibronectin OHC cultures was also reduced significantly with eMSC treatment, consistent with MSC benefits in glucose-deprivation models as well as neuroinflammatory models [212, 224]. Additionally, eMSCs attenuated IBA-1 levels in fibronectin cultures to levels similar to PDL+Laminin. There is evidence that MSCs mitigate microglia activation *in-vitro* where lower microglia levels of iNOS and secreted TNF- α were observed in the presence of MSCs [225]. Similarly, *in-vivo* models of ischemia reveal that MSCs modulate microglia function to simultaneously mitigate proinflammatory and promote anti-inflammatory functions [122]. Recently, in a neuroinflammatory OHC model it was established that MSC PGE2 secretion is responsible for their tissue protective action. Specifically, MSC PGE2 secretion modulated microglia inflammatory behaviors. Therefore, eMSC benefits may be attributed, in part, to secretion of PGE2. However, this does not exclude the plethora of other factors MSCs secrete, ranging from neuroprotective [226] to regenerative [67], which may also be contributing to the benefits observed in these systems.

MSCs have been reported to be efficacious in several instances of CNS pathology [209, 227]. However, it has been found that MSCs injected directly into CNS tissue do not survive 7 days post transplantation [122]. Others have observed that .0005% of intravenously transplanted MSC reach the cerebral parenchyma and were not detected 2 weeks post infusion [121]. An encapsulated platform would ensure MSC persistence at the site of injury and potentially augment the benefits achieved with MSC transplantation. The results here suggest that encapsulated MSCs maintain their tissue protective behaviors and could be used as a transplantation modality. Future studies will be performed to determine the efficacy of such an approach relative to free MSC transplants in experimental models of CNS pathology.

In summary, the system described here may provide an additional injury modality, which could be implemented to model the secondary injury tissue degeneration driven by plasma protein extravasation. The use of PDMS as the coating substrate allows for mechanical manipulation. One can envision incorporating several injury modalities (mechanical, toxicity, inflammatory and systemic) in an attempt to create more physiologically relevant models encompassing several mechanisms of CNS tissue degeneration. The system is amenable to high-through-put configuration platforms which permit the simultaneous evaluation of several drug candidates. Finally, the data

implies that the negative effects of fibronectin on CNS tissue should be considered before it is implemented as a suitable ECM for CNS transplantation.

3.6Acknowledgements

Funding for this work was partially funded by the New Jersey Commission for Brain Injury Research (BIR-1.08.016), NIH Biotechnology Training Grant (2T32 GM008339-21), and graduate fellowship programs IGERT on stem cell research NSF DGE 08016 Chapter 4 Encapsulated Macrophages Promote Recovery after Spinal Cord Injury Sections in this chapter are taken from a published manuscript with citation:

Barminko, J., et al., *Encapsulated mesenchymal stromal cells for in vivo transplantation*. Biotechnol Bioeng, 2011. 108(11): p. 2747-58.

4.1 Abstract

Having demonstrated in chapter 2 and 3 that the capsule platform can sustain MSC function and improve, *in-vitro*, secondary CNS degeneration, here we begin preclinical animal studies. In these studies we aim to show feasibility of eMSC transplantation as well as to demonstrate prolonged eMSC presence in the spinal cord. We also evaluate the effects of MSC on immune function, specifically looking at M1 vs M2 macrophage subset distribution after SCI. Finally, we perform long term studies to evaluate enhanced tissue sparing and improvements in locomotor function after eMSC transplantation. It is important to note that some of the data in this chapter is preliminary and this will be pointed out when the data is presented. However, the data suggests that the eMSC do have a positive effect in animal models of SCI. Also, I would like to acknowledge Dr. Jae Hwan Kim for his contributions in generating a significant portion of the data described in this chapter. Dr. Kim performed all of the animal injuries and MSC transplantations. He completed the histological staining for M2 macrophages and white matter sparing. He also, in conjunction with others at the KECK Center, performed the BBB locomotor assessments. Joanne Babiarz must also be thanked for the human actin data presented here.

4.2 Introduction

The development of effective treatments for SCI has been elusive. Unfortunately, effective SCI treatments are not currently available. Lack of success may be attributed to the complexity of the secondary injury cascade which ensues after primary SCI [5]. Ultimately, an effective treatment for SCI would be one which simultaneously targets the different components of the secondary injury cascade. The mesenchymal stromal cell (MSC) has particularly been an enticing stem cell option, as it operates via secretion of paracrine factors [72] and can potentially simultaneously target many secondary cascade effectors. The ease of MSC isolation and transdifferentiation potential initially made MSC a popular option for SCI replacement therapy [70]. There is some evidence that MSCs can differentiate into neuronal like cells, however, this area of research has become controversial and most agree that the major MSC benefit is achieved following secretion of paracrine factors [70-72]. MSC secrete angiogenic factors [76], growth factors to promote regeneration [78], cytokines which modulate immune cell phenotypes [93], as well as factors which prevent apoptosis [79-81] and facilitate differentiation [77]. MSC paracrine factor SCI therapy has recently been corroborated [128]. Others have observed reduced macrophage inflammatory and reactive astrocyte function following MSC transplants [133]. All of these observations occur in the absence of neuronal differentiation, which suggests that MSC effects are mediated either through the secretion of paracrine factors or through cell to cell contact. Therefore, MSC may represent a multimodal cellular therapy with properties that SCI treatments to date, have not yet matched.

What remains highly debated in the field is the persistence of MSC posttransplantation. There are reports that MSC remain as long as 5 weeks posttransplantation [136] however, other reports indicate that they are no longer able to detect MSC as early as 1 week post-transplantation [122]. There is no doubt that the degree of MSC persistence will depend on autologous vs allogeneic and xenograft transplantation. However, the SCI milieu is an unfavorable environment for cell survival and may lead to MSC death after transplantation independent of graft source. There is now a significant amount of data claiming that MSC do not persist long term after transplantation [70-72]. Additionally, due to the lack of control over MSC persistence, experiments cannot be designed to determine the optimal dosing regimen to maximize MSC therapy or whether long term therapy would provide enhanced benefits. To circumvent these issues, an MSC encapsulation (eMSC) platform was designed to ensure persistence of MSC posttransplantation [206]. The eMSC platform maintained MSC secretory and immunomodulatory function as well as prevented *in-vitro* secondary CNS degradation. The purpose of the current study was to use the encapsulation platform to assess long term effects of free MSC (fMSC) and encapsulated MSC (eMSC) transplantation. The studies aimed at evaluating the long-term persistence of eMSC and to evaluate their respective effects on locomotor outcomes in combination with immune function. The results demonstrate that while fMSC do not persist at 7 days post –ransplantation, eMSC persist for at least 2 weeks post-transplantation. MSC transplantation results in macrophage expression of M2 markers and a reduction in pro-inflammatory cytokine levels at the injury site. Only eMSC resulted in significantly increased white matter sparing and BBB locomotor scoring. Overall the data suggests that prolonged MSC persistence after injury may provide enhanced MSC benefits after SCI.

4.3 Methods

4.3.1 Alginate Microencapsulation

Alginate Poly-L-Lysine microencapsulation of hMSCs was performed as previously described [171] however with some modifications. A 2.2% (w/v) alginate (Sigma-Aldrich, MW: 100,000 -200,000 g/mol, G-content 65%-70%) solution was generated with Ca^{2+} free DMEM (GIBCO). The solution was filtered using a 25-micron syringe filter (Fisher Brand, Pittsburg, PA). A 70% confluent monolayer culture of MSC was removed with trypsin (Gibco) and counted. The cells were spun down at 400 g's and re-suspended at a concentration of 4×10^7 cells / mL with Ca²⁺ free DMEM. The cell solution was then diluted 10x with 2.2% alginate yielding a final cell concentration of 4×10^{6} cells/ml and a 2% (w/v) alginate solution. The solution was transferred to a syringe pump (KD Scientific, Holliston, MA). Alginate beads were generated using an electrostatic bead generator (Nisco, Zürich, Switzerland) with accelerating electrode at a flow rate of 2 mL/h and an applied voltage of 6.4 kV. The resulting bead diameter was 225 μ m ± 25. The bead were extruded into and 100 mL bath of CaCl₂ (100 mM) (Sigma Aldrich) containing 145 mM NaCl (Sigma-Aldrich) and 10 mM MOPS (Sigma-Aldrich). Microencapsulated cells were washed once with PBS and then treated for 2 minutes with poly-L-Lysine (PLL) (sigma –Aldrich, Mw: 68,600 g/mol) (0.05% w/v). The capsules were washed one more time and the re-suspended in MEM- α (Gibco) and transferred to 25 mm^2 tissue culture flasks.

4.3.2 Qiazol Extraction of RNA from Alginate encapsulated hMSCs

Flash frozen tissues samples with encapsulated hMSCs or capsules alone were homogenized in Qiazol for 30 sec using a Polytron 1200. Samples were spun at 12,000 x g for 10 minutes at 4°C to pellet out debris. Supernatants were transferred to fresh tubes and chloroform was added. After centrifugation at 12,000 xg for 15 mins. at 4°C, the clear aqueous phase was transferred to a fresh tube. RNA was precipitated with isopropanol for 10mins. at RT. and centrifuged at 12,000 x g for 10 mins. at 4°C. The RNA pellet was then washed with 75% ethanol and centrifuged at 7,500 x g for 5 min. The pellet was allowed to air dry for 5-10 minutes and was then redissolved in 35 ul of nuclease free H₂O. All RNA concentrations were read on a NanoDrop ND-1000 spectrophotometer. cDNA was made using random primers with the Applied Biosystems High Capacity Reverse Transcription Kit #4368814. The RT- PCR primers for the human beta actin were specifically designed for the non-homologous 3' UTR of human beta actin. The RT-PCR primers for human beta actin were; sense 5'-CACAGGGGAGGTGATAGCAT and anti-sense 5'-CACGAAGGCTCATCATTCAA, and for GAPDH sense 5'-AACTCCCTCAAGATTGTCAG and anti-sense 5'-GGCTAAGCAGTTGGTGGTGC. QRT-PCR was done using AB Power Sybr Green Master Mix and the AB 7500 Fast Real Time PCR System. The pcr data are reported as the relative expression compared to GAPDH.

4.3.3 Spinal Cord Injury and Transplantation

Twenty adult female Sprague-Dawley rats (200-250 g, 77 ± 2 days old, Taconic, Germantown, NY) were used in this study: SCI + saline (n=5), SCI + capsule (n=5), SCI

+ hMSC (n=5), and SCI + encapsulated hMSC (n=5). SCI was performed using the MASCIS Impactor as described previously [228]. Briefly, rats were anesthetized with 2% isoflurane and a 12.5 g·cm contusion was induced at spinal segment T10. hMSC at passage 2 were plated at $5x10^3$ cells/cm² 4-5 days before encapsulation or free transplantation. Encapsulated hMSC were prepared with 2.2% alginate at a seeding density of $4x10^6$ cells/ml and transplanted 1-3 days post encapsulation. All transplantations of either encapsulated or free hMSC was done with cells at passage 3. One day after SCI, hMSC (5×10^4 cells /70 µl of saline), encapsulated hMSC (2000 capsules (4.8×10^4 cells) /70 µl of saline), saline (70 µl), or hMSC-free capsules (2000 capsules/70 µl of saline) were injected at lumbar vertebrae L3-L5 through lumbar puncture over a period of 30 seconds; the syringe was left in that place another 60 seconds to prevent leakage [229, 230]. All animal experiments were approved by the Animal Care and Use Committee of Rutgers, The State University of New Jersey.

4.3.4 Tissue Processing for Immunofluoresence

Animals were sacrificed, perfused with cold PBS, and fixed with 4% paraformaldehyde 8 days after SCI. Spinal cords were removed and additionally fixed at 4°C overnight. For cryo-sections, spinal cords were equilibrated in 25% sucrose for 72 hours at 4°C, embedded in OCT compound (Fisher Scientific, Pittsburgh, PA) and cut into 20-µm coronal sections. For immunofluorescence, sections were blocked with 10% normal goat serum at room temperature for 2 hours and incubated with the primary antibodies against ED1 (AbD Serotec, mouse anti-rat, 1:800) and CD206 (Abcam, rabbit anti-rat, 1:400) at 4°C overnight. Sections then were washed with PBS and incubated with appropriate secondary antibodies (Molecular Probes, goat anti-mouse conjugated with Alexa 568; goat anti-rabbit conjugated with Alexa 647, 1:1000) at room temperature for 2 hours. After washing, sections were counterstained with Hoechst 33342 (Sigma Aldrich, 1:2000). Image analysis was performed using Zeiss 510 confocal laser scanning microscope.

4.3.5 Quantitation for Immunofluorescence and Cell counting

Eight ED1 and CD206-immunostained images were taken with a frame size of 334.8 μ m × 334.8 μ m from coronal sections of injured spinal cord cross-sections 2.5 mm distal to the injury epicenter in each animal. Immunopositive areas were obtained using Zeiss LSM Image Browser and were used to calculate average areas. For cell counting, ED1 and CD206 immunostained images were analyzed by two blinded testers. One-way ANOVA with Tukey's HSD tests were used to analyze statistical significance among the different groups; p-value < 0.05 was considered significant. Data are expressed as the mean ± standard error of the mean (SEM).

4.3.6 White Matter Sparing

Tissue sections from each condition, at 1 mm distances rostral and caudal from the injury epicenter as far out as 3 mm away from the epicenter were stained for myelin as previously described [231]. Briefly, sections were dehydrated at room temperature in a series of graded ethanols for 5 min each and cleared in hemoDe for 5 min. The sections were then rehydrated in a reverse-graded ethanol series. Myelinated fibers were stained for 10 min with a solution of 0.16% Eriochrome cyanine-R, 0.4% sulfuric acid, 0.4% iron chloride, and 0.12% HCL. The sections were then rinsed under running tap water for 10 min. Tissue sections were imaged using Zeiss microscope. Images were thresholded and total positive area stained represented the percentage of myelinated tissue.

Motor Function Evaluation

Locomotor recovery was assessed weekly using the 21 point BBB score [232, 233] by BBB scoring teams that were unaware of experimental conditions.

4.4 Results



Figure 4.1 Free MSC transplants after SCI do not persist long term. One day after spinal cord injury $2x10^6$ MSC were transplanted via LP. 5 mm spinal cord segments were extracted around the epicenter and two distal and proximal segments to the epicenter at 2, 4 and 7 days after injury. mRNA was extracted from these segments and PCR was performed using primers to human actin. Figure 1 depicts the human beta actin signal level detected from all the segments combined at each time point. Over the course of a week the beta actin signal diminished to undetectable levels.

4.4.1 Free MSC Do Not Persist Long Term after Transplantation

In order to determine MSC persistence, $2x10^6$ cells were transplanted, tissue segments were removed and mRNA was extracted. QPCR was performed on these samples at 2, 4, and 7 days post injury, with primers specific to human actin. The signal from the five 5 mm segments, injury site and two distal and proximal to the injury site, were summed for each time point The data suggests that a significant hMSC-derived actin signal, is detectaable2 days after injury. However by day 4, ~80% of the signal has decreased and by day 7, there is no detectable signal. The rapid loss of fMSC after transplantation post-transplantation motivated the implementation of encapsulated MSCs (eMSCs) to prolong hMSC presence after transplantation. Our previous work has shown that encapsulation does not inhibit MSC inflammatory function [206]. Furthermore, we have demonstrated that eMSC can be used in an organotypic model of secondary injury to prevent neuro-degeneration (chapter 3). Initial experiments were performed to determine the feasibility of transplanting alginate capsule using the LP approach. Transplanted capsules, coated with PLL conjugated to FITC, were imaged 1 week after injury using an upright dissecting microscope. We observed the presence of capsules at the lumbar enlargement, which localized around the injection point and reached as close as 25 mm to the injury site (Figure 2). In order to better assess capsule persistance posttransplantation magnetic nano-particles were co-encapsulated within alginate and T2 weighted MRI imaging was used to non-invasively image the capsules over several weeks. Capsules were imaged for as long as 6 weeks after transplantation and no apparent signs of degradation were apparent. The localization of the capsules did not vary overtime (Figure 3).

We next wanted to determine eMSC survival compared to fMSC. Therefore, $5x10^4$ eMSC were transplanted 1 day after SCI and after 2 weeks 5 mm tissue segments were collected beginning at the LP point and extending up to the injury site. mRNA was isolated from these segments using a QIAzol extraction method and QPCR was performed with primers to human beta actin. A beta actin signal was detectable from

eMSC 2 weeks after transplantation (Figure 4). The strongest human actin signal was located at lumbar vertebrae 4 and 5 (Figure 4), consistent with the eMSC localization observed with MRI and microscopy. The signal begins to decline within segments closer to the injury site.



Figure 4.2 FITC-conjugated PLL-coated alginate capsules localizing within the subarachnoid spaces of the lumbar region of the spinal cord. Empty capsules coated with PLL conjugated to FITC were transplanted via LP in non-injured animals. 1 day later, the animal was sacrificed and a full laminectomy was performed, exposing the entire cord. The entire cord was imaged using an upright fluorescent microscope. The capsules were observed to localize distal and proximal to the injection point.



Figure 4.3 Intrathecal MRI imaging of transplanted capsules. Capsules were prepared by encapsulating 100 nm chitosen coated magnetite nano-particles (6mg/ml) (ChMNP) in 1.98 % (w/v) alginate. A) Approximately $4x10^3$ capsules containing 6 mg/ml ChMNP were implanted into the CSF in the lumbar cistern of an adult female Sprague-Dawley rat via lumbar puncture. Under anesthesia, MRI was performed on the rat at 1T using a T1-weighted gradient echo sequence with TR 25 ms, TE 3 ms using the M2TM Compact High-Performance MRI at the Rutgers Molecular Imaging Center. The imaging was performed at 1 day, 1 week, and 6 weeks after implantation in order to track the presence of the capsules in the lumbar cistern over time. This signal was present throughout the course of the study, indicating that the capsules persist *in vivo*.



Figure 4.4 eMSC display prolonged persistence after transplantation compared to fMSC transplants. A) data from figure 1. B) eMSC were transplanted 1 day after SCI at doses of 10^4 and $5x10^4$. Two weeks later spinal cord segments were isolated from the LP point to the injury site at 5 mm segments. mRNA was obtained from each segment using a QIAzol extraction method. Human actin signal was detected at multiple segments around the LP point with the $5x10^4$ condition. Very weak signals were detected for the 10^4 eMSC transplant. Moving up the cord closer to the injury site, the $5x10^4$ signal disappears. This suggests that the capsules are persisting at least 2 weeks after transplantation and longer then fMSC.

4.4.2 MSC SCI Immunomodulation

Previously, we demonstrated anti-inflammatory encapsulated hMSC function *in-vitro [206]*. We next assessed immunomodulation in our *in-vivo* model of spinal cord injury, since an overly aggressive M1 inflammatory response post-SCI has been associated with decreased regeneration [234]. Contusions to the spinal cord at vertebra T10 via the NYU impactor [235] were performed and 24 hours post-contusion, approximately 5x10⁴ encapsulated or free hMSC were administered via lumbar puncture (LP) at vertebra L4-L5 [230]. Animals were sacrificed 8 days post contusion, a prime time point to evaluate immunotherapy, since macrophage infiltration is known to be robust at this time, and since macrophage activation is known to promote and exacerbate tissue damage post-SCI

[236]. Spinal cord coronal sections were prepared and macrophages immunostained at the site of injury (Figure 5A-D). Qualitative and quantitative evaluation of the macrophage population was performed, via ED1 (Figure 5E-H) and CD206 (Figure 5E'-H') expression, at the post-contusion injury site. These experiments indicated that the number of ED1⁺ cells at the injury site was not significantly different among the experimental conditions 1 week post-hMSC infusion, (Figure 5I, J). However, a greater percentage of the macrophages was positive for CD206 (Figure 5I, J) compared to control conditions Therefore, encapsulated hMSC converted macrophages to a M2 phenotype *invivo*. At the same time point, protein was extracted from the injury site and multiplexed for a panel of pro-inflammatory cytokines. The data suggests that MSC are able to attenuate the levels of inflammatory cytokines at the injury site one week after transplantation (Figure 6). There was no significant difference in the level of attenuation between fMSC and eMSC. Nevertheless, these observations suggest that MSC can promote immunomodulatory action after SCI.



Figure 4.5 Effect of Encapsulated hMSC on macrophage phenotype in an *in-vivo* model of spinal cord trauma. Transplantation of encapsulated hMSC increases the number of CD206 positive cells 8 days after SCI. Many activated macrophages were immunostained with ED1 antibody (pseudo-red) in injured spinal cords from all groups injected with saline (A, E), empty capsules (B, F), free hMSC (C, G), or encapsulated hMSC (D, H). Immunostaining for the M2 macrophage marker CD206 (pseudo-green) was higher in hMSC transplanted groups (free hMSC and encapsulated hMSC) compared to saline and capsule control group (E' – H'). Overlays of ED1 and CD206 staining are shown in E'' – H''. Ratios of the CD206 positive areas and the ratio of CD206⁺ to ED1⁺ cells were significantly increased in the encapsulated hMSC transplanted group compared to saline and capsule controls (I and J, respectively). Note that free hMSC increased CD206⁺ areas significantly while the increases in cell number were not significant. Scale bar is 50 μ m. (*, P < 0.05; **, P < 0.01 in ED1⁺; ##, P < 0.01; ###, P < 0.001 in CD206⁺; ++, P < 0.01 in ED1 and CD206 double-positive/ ED1⁺, one-way ANOVA with Tukey's HSD test). Data represent mean ± standard error with 4-5 rats per group.



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Figure 4.6 MSC attenuate the levels of pro-inflammatory cytokines at the injury site after spinal cord injury. 1 day after SCI 5x10⁴ fMSC and eMSC were transplanted via LP. 1 week after injury the animals was perfused with PBS and the injury site explanted. Protein was extracted from the injury site and then multiplexed for several pro-inflammatory cytokines. Cytokine levels were normalized to the total protein extracted for each sample and then level detected in non-injured SC tissue. The data demonstrates that MSC transplantation attenuates the levels of several pro-inflammatory cytokines 1 week after injury.

4.4.3 MSC Improve White Matter Sparing and Locomotor Function

We next performed long term studies with eMSC vs fMSC to determine if prolonged presence of MSC after SCI improves outcomes. One day after injury eMSC, fMSC and saline were transplanted via LP and animals were allowed to recover for 8 weeks. To analyze the effect of MSC transplantation on the white matter preservation, animals were perfused and fixed after eight weeks of recovery. Coronal sections were prepared and stained for white matter. Spinal cords exhibited minimal white matter staining at the epicenter of the injury, which gradually increased in rostral and caudal regions. However, white matter increased to a greater degree in caudal regions relative to the rostral regions. Quantification of the percent white matter spared at 1-mm intervals rostral and caudal from the injury epicenter yielded higher values for eMSC treatments in the rostral regions and 1 mm caudal as well (Figure 7). FMSC did not promote significant improvement in white matter sparing above saline controls (Figure 7). These data suggest that eMSC treatment results in reduced white matter loss after SCI. Next, we assessed improvements in locomotor function over the course of the 8 week recovery period. BBB locomotor evaluation was performed every week by a team of evaluators blinded to the animal conditions. BBB scores of eMSC transplanted animals were approximately two points above saline controls throughout the 8 week recovery period. FMSC transplanted animals had a 2 point higher BBB score relative to saline controls at 3 weeks, but remained within a point above saline controls for the remainder of the assessment period. At the 4th week, only eMSC transplanted animals exhibited significant improvement in locomotor recovery.



Figure 4.7 eMSC therapy increases the percentage of spared white matter 8 weeks after injury. After an 8 week recovery period, animals were perfusion fixed and coronal tissue sections from the epicenter at 1 mm intervals rostrally and caudally were prepared. ECR staining for white matter was performed on each section. Section images were thresholded and percent area positive for staining was calculated. A) Representative images of staining across conditions and sections B) Percent white matter for each conditions demonstrating that eMSC result in statistically significant increases in white matter sparing relative to sham and fMSC treated conditions. This increase was observed from segments 1mm caudal to 3mm rostral.





(*, P < 0.05 vs Saline by One-Way ANOVA with Tukey HSD test)

Figure 4.8 eMSC therapy promotes significant locomotor recovery. Every week throughout the course of an 8 week recovery period animals were assessed for locomotor improvements by the BBB open field locomotor scale. Measurements were acquired in a teamed double blind fashion. EMSC and fMSC conditions display significant improvements as early as 3 weeks after transplantation. However, week 4 fMSC and eMSC benefits diverge and only the eMSC condition remains significantly above the sham condition.

4.5 Discussion

MSCs represent a cellular drug delivery vehicle, which is currently unmatched. MSC secrete factors which prevent apoptosis, promote regeneration, modulate immune function, promote angiogenesis and mitigate scar formation. Thus, MSC represent a truly multimodal therapy for SCI. MSC derived factors can potentially act on several secondary injury cascades, which left unchecked, ultimately lead to morbidity. However, lack of fMSC transplantation control as well as survival uncertainty has led to inconsistent results in the literature. Therefore, a controllable eMSC platform was implemented to evaluate MSC therapy relative to fMSC transplantation. We showed that fMSC transplants are not detectable at 7 days post injury while, eMSC transplants can survive at least 2 weeks after injury. Alginate capsules localize around the LP injection point up to 6 weeks and reach as far as 25 mm from the injury site. Both the free and encapsulated MSC transplants lead to an attenuation of pro-inflammatory cytokine levels, as well as the promotion of M2 CD206 expression at the injury site. After an 8 week recovery period, increases in white matter sparing were observed in eMSC and not fMSC treated animals. At 3 weeks into the recovery period, eMSC animals displayed significant improvement in locomotor function and this continued for duration of the 8 week recovery period. FMSC exhibited a modest but insignificant increase in locomotor function.

Previous studies have indicated that following SCI, the M1 phenotype dominates post-trauma and is linked to tissue pathology progression [176]. HMSC transplantation post-SCI has been attempted by several investigators, yielding inconsistent results with regards to cell survival as well as homing to the injury site, as reviewed by Parr et al

[143]. In our studies, immobilized hMSC were administered 24 hours post-contusion via lumbar puncture. We observed that fluorescent capsules localized along the spinal cord in the subarachnoid spaces, primarily at the cauda equina of the lumbar region 1 hour and 1 week after transplantation. The utility of the capsule platform is that the capsule microenvironment can accommodate MSC co-encapsulated with magnetic particles and MRI imaging can be used to track the position and presence of the capsule throughout a course of treatment. Here we show the feasibility of such an approach, demonstrating that capsules containing magnetic nano-particles can be imaged via MRI up to at least 6 weeks after transplantation. Additionally, these experiments also confirm that the capsule can remain intact for at least 6 weeks after transplantation and that the capsule does not move significantly from the initial implantation site.

Recent reports have described promising hMSC therapeutic benefits in animal models of SCI, but the mechanism of action is still unclear. Several of these reports hypothesized that the *in-vivo* hMSC benefits are attributed to trans-differentiation. However, experimental evidence to support this concept is questionable [237]. The proposed paradigm in the current study is that hMSC modulate the inflammatory environment dynamically via the secretion of soluble factors, since direct cell to cell contact of hMSC with the tissue is not possible when cells are encapsulated within alginate beads. Previous animal studies have not been unable to de-couple the contributions of hMSC secreted factors from direct cellular interactions with the trauma area. Our results indicate that within alginate capsules, hMSC promoted the M2 phenotype at the injury site. This was supported by the observation of elevated levels of CD206 in animals treated with encapsulated hMSC. Interestingly, while the number of

ED1⁺ cells did not differ significantly among the experimental conditions, the ED1⁺ population expressed elevated levels and increased co- localization with CD206, suggesting that macrophages which would predominantly adopt M1 phenotypes are instead induced into an M2 phenotype that is favorable for tissue recovery. A similar phenomenon has recently been observed in animal models of wound healing, where it was reported that hMSC treated wounds displayed increases in CD206 positive macrophages [177]. Consistent with our observations, a recent SCI study demonstrated that macrophages at the injury site display M2 properties after fMSC transplantation [136]. However, considering the fact that fMSC promoted the same response, but did not result in improved BBB score and white matter sparing, this suggests that other mechanisms are also involved in MSC SCI therapy as well. However, our results suggest that endogenous macrophages are diverting to a more favorable phenotype, which could potentially provide a means of harnessing the inflammatory response to yield therapeutic benefits.

To date, no one has reported therapeutic benefits in animal injury models using cell densities as low as were used here. On average, $\sim 5x10^4$ encapsulated hMSC were administered within the subarachnoid space in our study. Previous reports of lumbar puncture delivery of hMSC achieved maximal therapeutic benefits after 3 transplantations of $2x10^6$ hMSC, beginning 1 week post contusion [135]. The immunomodulatory benefits observed in our studies were also detected following fMSC transplants. However 8 week outcome measurements did not indicate significant fMSC improvement in BBB and white matter outcomes. Interestingly, there have been studies which did not report improvements when MSC ($3x10^5$) were transplanted in moderate
injury models [133], like the one used here. However, several studies using several SCI models, have reported improvements in BBB scores following MSC transplantation [65, 123, 133, 238]. These findings suggest that MSC mediated improvements may be further amplified using severe, relative to moderate, injury models. Furthermore, eMSC, in more severe models, may display an even greater improvement in recovery compared to saline and fMSC conditions.

In conclusion, these initial experiments support the feasibility of implementing the eMSC approach as a SCI therapy. Prolonged encapsulated MSC in vivo persitance leads to significant improvements in white matter content and locomotor function relative to saline controls. We also demonstrated MSC can drive immunomodulation of macrophages to express M2 phenotypes. Future work will focus on determining the optimal dose of eMSC as well as the optimal delivery time point.

4.6 Future Work

Long term eMSC persistence studies must be completed to determine how long the cells remain viable within the spinal canal. If we see that eMSC begin to die this may suggest that the CSF is not an ideal environment for MSC survival and modification of the capsule microenvironment may be able to provide survival factors that are not available within the CSF. It will also be important to determine the degree of MSC presence that is necessary to achieve improvement. Even though we measured a human actin signal at 2 weeks after eMSC transplantation we do not know how many cells are represented by that signal. Therefore, future studies must be accompanied by a baseline control, which should include the sacrifice of be animals immediately after transplantation, along with a measure of human actin levels. All subsequent time points can be normalized to the initial time point to determine the relative decrease in eMSC over time.

Experiments should also be designed to determine if MSC are activated after transplantation. The PCR method to measure actin can be modified to measure additional human proteins, which can provide insights into MSC function after transplantation. There are several transcription factors and cytokines that are elevated after MSC activation. These factors can be probed to determine if MSC are activated.

In-vitro studies should be performed which combine several intracellular factors or regulators of stimulated MSC secretion. This approach should identify a factor, or combination of factors, that are elevated after MSC activation. eMSC can then be transplanted into injured and non-injured animals and sacrificed at various time points within the first week, to isolate mRNA. Human actin can be used as an internal control for f MSC number and then relative increases in the factors chosen to represent MSC activated, increases in mRNA transcripts should be detected with this technique. FMSC at the injury site can serve as control. I believe this is an important control, because I hypothesize that the MSC, in the absence of an injury environment are not significantly activated within the capsule. This hypothesis is supported by the observation that we have not measured significant levels of cytokines within the CSF, which would be necessary to induce cell activation.

The modest improvement in observed BBB scores was perhaps due to a very low dose of eMSC. In other laboratories, most of the doses which have led to the degree of

improvement observed here are achieved with much higher fMSC doses. Therefore, in order to determine the true potential of this approach, higher doses of MSC need to be tested. Right now this should be done with multiple transplantations of capsules or by increasing the number of cells per capsule. This latter method would need some validation as minimal work has been done thus far in our lab, increasing the amount of cells per capsule. Initial observations suggest that this approach would be feasible, but effort would be needed to optimize the approach. An additional note on the capsule synthesis, the system implemented here is robust and effective. However there are limitations when capsule synthesis below 300 µm is desired. The increase in shear stress that occurs in conjunction with the decrease in needle diameter needed to obtain smaller capsules, results in cell death during encapsulation. As one attempts to increase the number of cells/capsule the degree of cell death will ultimately increase. To overcome this limitation, an alternative encapsulation system may be needed. An aerodynamic unit, developed by NISCO, may overcome this limitation. They report that the final capsule size is a fraction of the needle diameter. Therefore to obtain 200 um capsules the diameter of the needle would be larger, rather than smaller, which would overcome the shear stress problem. However, I digress; increasing the number of transplanted cells could potentially enhance the difference between the fMSC and eMSC transplantations. I believe this study will be essential in determining the true efficacy of the approach and MSC therapy in general.

The MSC effect on macrophage function after SCI is intriguing, but currently, it is out of context. What I mean is: how do CD206 expression values in our studies, correlate with the normal course of CD206 expression in the peripheral nervous system or other tissue? What is the true phenotype of these macrophages and how does their phenotype change over time? When is the transition to CD206 positive macrophages needed? Experiments that attempt to address the ineffective macrophage response after SCI will help to understand the effect of MSC on this inflammatory cell type. To do this, a flow cytometry approach, which can be implemented to isolate the immune cells at and around the injury site, would be optimal. As a first attempt to begin these studies, we developed a method described previously[239]. The protocol was followed identically for injured spinal cords at 1 and 4 days post injury. The data shows that from day 1 to day 2,



there is a significant increase in the number of ED1 positive cells at the injury site. This is consistent with the course of macrophage infiltration after SCI [176]. A small percentage of the population on day 4 expressed CD206, however, the population was predominantly CD206 negative, which is also consistent with histological macrophage data [176]. Some optimization must still be completed to maximize the isolation of cells from the tissue, as

well as to determine the optimal staining procedures for these populations. The method will be a powerful tool in studying immune cell phenotype distribution after SCI.

Chapter 5: Stromal Cell Regulation of Macrophage Plasticity: The PGE2/EP4 Axis Promotes Reprogramming Through CREB Signaling

5.1 Macrophage function after SCI

The inflammatory response post SCI was thought to represent an obstacle to regeneration for several years. Many believed that pro-inflammatory immune-cell the function directly and indirectly promoted neurotoxicity as well as instigated the development of a glial scar, which prevents regeneration [240]. This dogma prevailed for a considerable amount of time. Therefore, SCI therapies were designed to mitigate the initiation and/or progression of the inflammatory cascade after injury. Drugs were developed to attenuate the M1 macrophage response were reported to show improvements [241]. Therapies were designed to reduce the degree of immune cell infiltration after spinal cord injury [242]. It was even shown that the depletion of peripheral macrophages infiltrates led to hind limb recovery [243]. The most successful attempt was the FDA approved antiinflammatory drug, MP, for SCI therapy. MP is a potent glucocorticoid, which displays remarkable anti-inflammatory function. MP administered 3-8 hours after SCI mitigates the inflammatory response and subsequently, benefit [244]. However, towards the end of the 20th century confidence in MP clinical utility for SCI was fading.

It is clear that the spinal cord does not regenerate well after trauma, but other organs do. For instance, the peripheral nervous system seems to have an impressive ability to regenerate itself after experiencing trauma [245]. Furthermore, recovery of nerve function can be achieved after complete peripheral nerve laceration, when the size of the gap is not to large. Intriguingly, the healing response post peripheral nerve injury has a macrophage presence which seems to be essential for regeneration [246]. In 1998 a

study published in nature medicine reported motor function improvements when macrophages cultured with peripheral nerve explants are transplanted after experimental SCI [247]. This result represented a turning point in the role the macrophage and the immune system plays in SCI pathology.

What we understand now is that the innate immune system is essential for wound healing. Macrophage depletion in skin wound healing models prevents wound closure [248]. There is now evidence that extensive or complete macrophage depletion after SCI worsen outcomes [25], suggesting that the macrophages are essential for healing. It turns out that the macrophage exhibits an extraordinary degree of phenotypic plasticity. These phenotypes are broadly characterized under pro- and anti- inflammatory M1 and M2 macrophages, respectively. Skin wound healing macrophages exhibit a gradual transition from M1 to M2 phenotypes over the course of healing [249]. The initial recruitment of monocytes, which differentiate into M1 macrophages are responsible for inflammatory/debriding function [250]. The macrophage will then transition into an M2 macrophage which will promote regeneration of parenchyma as well angiogenesis [251]. Studies treating SCI with macrophages obtained from skin wounds result in improved outcomes[252]. This same course of macrophage function is observed in the healing response after peripheral nerve injury [246]. However, for some reason after spinal cord injury this does not happen and a predominating M1 population persists long term after trauma [176]. The absence of an M1 to M2 transition is believed to be responsible for the lack of inflammatory resolution after SCI. Overall, an appropriate innate immune response is necessary for efficient wound healing. Therefore, immunotherapies aimed at

restoring this transition post SCI may potentially provide a novel approach for dealing with SCI secondary injury.

5.2 MSC Macrophage Modulation

Macrophages are phagocytic cells of the myeloid lineage, differentiated from monocytes and present in essentially all tissues. They play major roles in adaptive and innate immunity and are able to perform pathogen clearance in the absence of phagocytic labels for pathogen ingestion/destruction (opsinzation) and act as antigen presenting cells (APC). Considering their abundance throughout the body, the macrophage is an essential player in tissue damage as well as the overall immune response. Macrophage behaviors have been implicated in pathology after organ trauma [176], allograft organ rejection [253], as well as atherosclerosis [254]. Over the past several years, the complexity of the macrophage response has been documented as well as the role of phenotypic plasticity in macrophage responses. The major implication of these observations have been the distinction of classically (M1) and alternatively (M2) activated macrophages [251]. M1 macrophages represent the pro-inflammatory arm of the macrophage response while M2 is the anti-inflammatory arm. Intriguingly, MSC secrete several of the factors found to promote M2 phenotypes either constitutively or in the presence of certain soluble cues. Furthermore, considering the tremendous amount of data supporting the ability of MSC to modulate immune responses, it is no surprise that MSC have been found to promote M2 macrophage phenotypes in the presence of stimuli which normally lead to M1 phenotypes. Kim et al. were the first to observe that macrophages cultured in the presence of MSC adopted phenotypes indicative of M2 macrophages (CD206^{high}, IL-10^{high}, IL-12^{low}) after 48 hours of culture [93]. These studies were performed in the

absence of cell-cell contact, suggesting that soluble factors were responsible for the phenomenon. Gonzales et al. co-cultured colitis derived macrophages with MSC and found that the pro-inflammatory secretion of TNF- α and IL-12 was diminished [91]. Anti-inflammatory IL-10 secretion was found to be elevated and when PGE2 blocking antibodies were introduced, inflammatory functions were partially reverted. Reports by Cutler *et al.* suggested that MSC can modulate monocyte functions, which ultimately resulted in the suppression of T cell proliferation [92]. They suggested that this response was dictated by MSC secretion of PGE2. Similarly, Maggini et al. observed that thioglycolated peritoneal macrophages cultured with MSC adopted a regulatory phenotype [255]. These macrophages exhibited reduced secretion of pro-inflammatory mediators and enhanced secretion of anti-inflammatory mediators [255]. Furthermore, LPS dependent up-regulation of major histocompatibility complex (MHC) class II and co-stimulatory CD86, factors which are responsible for macrophage antigen presentation, were mitigated [255]. They claimed that MSC secretion of PGE2 was responsible for these changes. Zhang et al. also observed that macrophages assumed M2 phenotypes in the presence of MSC. Macrophages expressed mannose receptors (CD206) and secreted IL-10, hallmarks of M2 macrophage phenotypes. This was observed with a concomitant reduction in M1 secretion of TNF- α as well as the ability to stimulate T_h17 expansion [177]. It was suggested that MSC drive macrophage phenotype through the synergistic interaction between granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-6, which when blocked, reduced macrophage expression of CD206 [177]. The studies of Barminko et al. support these findings as well. They observed that THP-1 proinflammatory secretion of IL-1 β , TNF- α , IP-10 and MIP1- α was reduced in the presence of MSC. These macrophages exhibited elevated CD206 expression as well as IL-10 secretion [256]. Collectively, these observations strongly suggest that MSC can dictate macrophage plasticity towards regulatory M2 behavior. MSC promote similar phenomena's in vivo. In an animal model of sepsis, Nemeth et al. found that MSC reprogrammed macrophages to secrete IL-10 and this was dependant on macrophage secretion of PGE2 [257]. Gonzalez et al. reported that MSC reduced TH1 driven histopathology as well stimulated systemic levels of IL-10 in an experimental model of colitis [91]. They claimed that MSC act directly on activated macrophages to partially facilitate these benefits. In skin injury models, MSC transplantation accelerated wound healing by increasing the number of macrophages infiltrating the wound site [168]. Zhang et al. observed that subcutaneous administration of MSC increased M2 macrophages and enhanced wound healing [177]. Ohtaki et al. explored the effects of MSC on inflammation in an animal model of stroke and found that microglia exhibited M2 phenotypes. The implication of these findings is that if appropriately implemented in vivo, MSC could be utilized as a means of driving endogenous macrophage expression of M2 phenotypes. This could potentially provide an approach to enhance resolution of chronic inflammation. Therefore, the next studies aimed to understand MSC macrophage interaction. We specifically focused on the factors MSC secrete to mediate macrophage modulation and in what intracellular manner they impart change. The hope is to gain more insight into the MSC macrophage interaction so that the capsule microenvironment could be tailored to maximize eMSC benefits.

5.3 Abstract

Understanding the regulatory networks which govern the presentation and control of specific macrophage phenotypes, will be essential for the identification of novel targets for treating conditions associated with improper macrophage function. The Stromal cell represents a key cellular regulator of immune specific functions, therefore studies were designed to evaluate the regulatory action stromal cells impart on macrophages during inflammation. Human bone marrow stromal cells were cultured with human blood derived M1 macrophages and stimulated with LPS. Stromal cells were observed to attenuate pro-inflammatory M1 cytokine secretion and enhance expression of M2 CD206. Objective evaluation of several stromal secreted mediators revealed that PGE2 was able to facilitate macrophage reprogramming, while IL4 only provided partial reprogramming. Inhibiting stromal PGE2 secretion with a COX2 inhibitor, indomethacin, reversed the macrophage reprogramming effect. PGE2 reprogramming was mediated through CREB signaling indirectly, via GSK3-α inhibition. GSK3 specific Inhibitors administered after LPS stimulation, promoted M1 macrophage CD206 expression. This reprogramming pathway functioned independent of the one which led to M1 suppression, as CREB or GSK3 inhibition did not reverse PGE2 TNF- α attenuation. Lastly, we show that PGE2 reprogramming effects are mediated through the EP4 receptor. In conclusion, the data here supports the role stromal derived PGE2 has in facilitating macrophage reprogramming and establishes GSK3-CREB interactions as a possible regulatory checkpoint in macrophage plasticity.

5.4 Introduction

Macrophages exhibit tremendous phenotypic plasticity which is regulated by the physiological or pathological environments to which they are recruited. Wound healing in particular, is characterized by an exorbitant degree of recruited macrophages undergoing a gradual transition from inflammatory/debriding to wound healing phenotypes. These phenotypic states can broadly be categorized as pro-inflammatory M1 and antiinflammatory M2. When the transitions between these phenotypic states are not regulated appropriately, chronic inflammation ensues and tremendous tissue damage occurs. Deleterious macrophage function has been observed in models of sepsis (1), wound healing (2), spinal cord injury (3) and myocardial infarction (4). In particular, observations suggest that the absence of an M1 transition to M2 phenotypes impairs healing (3). Others observed that the predominating macrophage population in chronic wounds expresses both M1 M2 characteristics (2). Mycordial infarcts are notorious for the persistence of non-resolving macrophages (5). Conversely, the tumor associated macrophages (TAM) display M2 properties and positively contribute to cancer malignancy (6). Collectively, these observations suggest that the macrophage has an integral role in neoplasim and pathology progression. Therefore, understanding the regulation of macrophage plasticity may be essential for the identification of novel targets for therapy.

Stromal cells have been traditionally regarded as the tissue cell responsible for the maintenance and modification of the connective tissue supporting parenchyma function. However, there is a burgeoning of evidence supporting the fact that stromal cells regulate a myriad of cellular interactions within several different organs. Stromal cells display selective control over immune cell extravasations and dictate the development of immune cell phenotypes in both primary and secondary lymphoid organs (7). Fibroblasts have been observed to promote inflammatory Th1 reprogramming to regulatory T cell phenotypes (8). They can also control antigen presentation in lymphoid organs and display IFN- γ induced immunosuppressive T cell and monocyte function (9-11). There is also an abundance of data supporting the regulatory potential of mesenchymal stromal cells (MSCs). MSC promote T cell anergy (12, 13), reprogramming (8) and apoptosis (14). MSC prevent dendritic cell differentiation (15, 16) as well as pro-inflammatory cytokine secretion (13). There are now numerous pre-clinical studies which demonstrate MSC immune cell regulation (1, 17, 18) as well as specific evidence that stromal cells regulate macrophage inflammatory functions *in-vivo* (1, 19). Thus, understanding stromal/macrophage interactions may provide novel insights into the cellular and molecular interaction which govern macrophage reprogramming events.

There have been a plethora of studies exploring the effects of macrophages on stromal cells, but few exploring the reverse interaction. Some *in-vitro* studies demonstrate monocyte differentiation and tissue macrophage conversion to M2 phenotypes when co-cultured with MSC (18, 20). However, the M1 macrophage stage of the wound healing process seems to be an essential component of normal healing (21) and macrophage depletion exacerbates inflammatory responses (1). Since the M1 presence precedes the M2 macrophage in the course of inflammation and that most observed macrophage pathologies manifest in an inappropriate transition between the two phenotypes, we thought it appropriate to study the regulation of the M1 macrophage. Therefore, experiments were designed to evaluate stromal cell regulation of mature M1

function. We demonstrate that MSC have the ability to engender reprogramming of M1 macrophages. The paradigm was determined to be driven by MSC secretion of PGE2 which potentiates CREB transcriptional regulation indirectly through GSK-3 α inhibition. Finally, we demonstrate the PGE2/EP4 axis facilitates stromal macrophage reprogramming.

5.5 Methods

5.5.1 Reagents

IL4, MCSF, IL6, HGF, IL-10, IL-13, TNF- α R were purchased from Peprotech (Rocky Hill, NJ), GMCSF, TGFb-1, IDO were purchased from R&D (Minneapolis, MN) and IGF-1 from Lonza (Basel,Switzerland). GSK3 inhibitors, Lithium chloride and SB415286, CREB inhibitors, pamoic acid and RO- 31–8220, COX2 inhibitor Indomethacin and LPS were purchased from Sigma-Aldrich (St. Louis, MO). EP receptor antagonists, L161982 and Ah6809, and PGE2 were purchased from cayman chemical (Ann Arbor, MI). Rabbit polyclonal Abs to CD206 and α-Tubulin were purchased from Abcam (Cambridge, MA). Rabbit monoclonal Abs to phosphorylated CREB (ser-133), GSK- α (ser-21), GSK3- β (ser-9), and IL-6 (tyr-641) as well as to CREB, GSK3- α , GSK3- β , and IL-6 were purchased from Cell Signaling (Boston, MA).

5.5.2 Mesenchymal stem/stromal cells: All cell cultures were incubated in a humidified 37 °C and 5% CO₂ environment. Human MSC (hMSC) were purchased from Texas A&M at passage 1 and cultured as previously described (23). Briefly, hMSC were cultured in MEM- α (Gibco) medium, containing no deoxy and ribo nucleosides, supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), 1 ng/ml basic fibroblast growth factor (bFGF) (Gibco), 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco). hMSC were plated at 5000 cells per cm² and allowed to

proliferate to 70% confluence (approximately 4 to 5 days) before passaging. Only hMSC at passages 2 through 5 were used to initiate subsequent experiments.

5.5.3 Macrophage/MSC Co-cultures: Peripheral blood mononuclear cells were collected from blood of healthy donors (Blood Center of New Jersey) by density gradient centrifugation using ficoll at a density of 1.077 (GE Healthcare). Monocytes were isolated to high purity (>90%) by magnetic cell sorting using anti-CD14 coated beads (per manufacturer recommendation (Milteny Biotec, Auburn, CA). 10⁷ monocytes were cultured on 175 cm² flasks (BD) in RPMI (GIBCO). RPMI was supplemented with 10% fetal bovine serum (FBS) (GIBCO), 100 units/ml Penicillin (GIBCO), 100ug/ml streptomycin (GIBCO) and 400 mM L-glutamine (GIBCO). Monocytes were cultured for 2 hours and then washed 3 times with PBS to remove non-adherent cell. Monocytes were cultured for seven days in RPMI supplemented with 5 ng/ml GMCSF. On the 7th day of culture macrophages were washed once with PBS and detached with trypsin-EDTA (GIBCO) for 30 minutes at room temperature. Cells were re-suspended in RPMI, counted and re-plated at 1×10^5 cells/ ml in a 24 well plate (Corning) and allowed to attach overnight. The following day cells were used for experimentation. Macrophage/MSC cocultures were established with 8 µm transwell inserts (Corning, Corning, NY). Macrophages were treated with 1 µg/ml LPS from Escherichia Coli (serotype 055:B5, Sigma-Aldrich, St. Louis, MO) and MSC at various cell concentrations $(2x10^3, 2x10^4 \text{ and }$ 10⁵ cells /ml) within transwell inserts . COX-2 blocking was performed under the identical culture conditions, but in the presence of Indomethacin (10µM) or 1MT (1 mM) (negative Control). The cultures were incubated for 2 or 5 days, after which culture supernatants were collected and macrophages fixed with 4% paraformaldehyde (PF).

5.5.4 Fractional factorial design

MSC immunomodulatory candidates were chosen based on previous publications. Concentrations corresponding to MSC secreted levels for each factor were determined experimentally or from literature. A two level 13 factor fractional factorial experiment was performed. The various conditions were developed based on montgomery's suggestions (1994). Two levels for each factor were evaluated, 0 which corresponds to the absence of the factor and 1 which corresponds to the presence of the factor at a designated concentration. A 1/64 fraction experiment was designed with a resolution of four. With this design, all main effects and two factor interactions are determinable. Each cocktail condition was created within 96 well plates and then spiked with LPS. The cocktail array was then transferred to M1 macrophages seeded in 96 well plates at a density of 10^5 cells/ml. The macrophages were cultured for 48 hrs then supernatants collected for TNF- α ELISA detection and cells PF fixed for CD206 immunostaining. The data from the factorial experiment was analyzed using statistical analysis software 9.2 (SAS Institue Inc., Cary, NC).

5.5.5 Macrophage PGE2 and Blocking Studies: Cytotoxic thresholds for Lithium chloride, SB415286, pamoic acid and RO-31–8220 were determined by titration experiments for each inhibitor. Macrophages were pre-treated with 20 µm SB415286 (1hr), 40 mM Lithium Chloride (1hr), 100 µm Leflunomide (1hr) and 1µm RO-31-8220 (10 min). Macrophages were treated with cocktails containing various combinations of LPS, PGE2 and IL4 in the presence of the different inhibitors for 48 hours before supernatant collection for ELISA and cell fixation for CD206 immunostaining.

Conditions for macrophage protein immunoblotting studies were established identically except protein extraction was performed after .25, 1, 4 and 24 hours of culture.

5.5.6 Immunostaining and Cytokine Measurement: Macrophages were immunostained as previously described (20) with Rabbit pAb to CD206 (1 μ g/ml). Images were acquired using an Olympus IX81 spinning disc confocal microscope and stereology was performed using Slidebook software (Olympus, Center Valley, PA). To analyze CD206 positive cells within the M1 population, images were thresholded based on levels determined from isotype controls wells. There was slight basal expression of CD206 throughout the M1 populations, however after induction with either PGE2 or IL4 a fraction of the population expressed high levels of CD206. The threshold for CD206 high expressing cells was set by taking the average CD206 expression in the medium populations and adding one standard deviation. All CD206 percentages represent the percentage of the population above basal levels. Supernatants were analyzed via ELISA for IL-10, TNF-α, IL-12 (Biolegend, San Diego, CA) and PGE2 (Ann Arbor, MI)

5.5.7 Immunoblotting: Whole macrophage extracts were obtained and protein levels quantitated by BCA protein Assay kit (Thermo Scientific Waltham, MA). 40 μ g of cell lysates were fractioned on 10% polyacrylamide gels (Biorad, Hercules, CA) by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Nitrocellulose membranes were blocked for 90 min with 5% BSA (phosphorylated protein) or 5% milk (total protein). Membranes were then incubated with specific antibodies overnight and the following day enhanced chemiluminescence was used for detection. Membranes were stripped for 15 minutes with a solution of 1.5% (w/v) glycine, .1% (w/v) SDS, 1% tween and brought to a pH of 2.2. Membranes were washed

twice with PBS, twice with TBST and then incubated with primary antibody to α -Tubulin overnight and the next day enhanced chemiluminescence was used for detection.

5.5.8 Statistical analysis: Each data point represents the mean of three or more experiments (each with biological triplicates), and the error bars represent the standard deviation from the mean, unless otherwise specified. Statistical significance was determined using one or two way ANOVA analysis with 95% confidence intervals.

5.6 RESULTS

5.6.1 MSC facilitate M1 Macrophage reprogramming to M2 phenotypes

To investigate the regulatory effects MSC have on macrophage function, monocyte derived M1 macrophages were obtained from human blood donors by CD14+ monocyte isolation and GMCSF (5 ng/ml) differentiation for 7 days. This macrophage subset has been shown to express a repertoire of M1 macrophage phenotypes, which has been validated through epigenetic (24), genetic (25, 26) and protein (27, 28) characterization. M1 macrophages were plated at 10⁵ cells/ml and within transwell inserts cultured with MSC at ratios of 1:1, 5:1, and 50:1 in the presence of LPS stimulation. After 48 hours of culture TNF- α level was assessed via ELISA. At ratios of 50:1 and 5:1 MSC mitigated M1 macrophage TNF- α secretion ~45% and ~60% at a 1:1 ratio (Fig. 1A). We next looked at M1 macrophage IL-12 secretion 2 and 5 days after LPS stimulation, focusing on the 1:1 ratio, which promoted the highest TNF- α attentuation. Following LPS stimulation IL-12 macrophage secretion was maintained at steady levels for at least 5 days, as opposed to TNF- α , which was not detectable 5 days after stimulation MSC were able to attenuate IL-12 secretion $\sim 60\%$ on day 2 and $\sim 80\%$ by day 5 (Fig. 1B).

We next assessed macrophage expression of CD206, an M2 marker, to determine if MSC can facilitate macrophage reprogramming events in addition to pro-inflammatory cytokine attenuation. Initially, ~ 10% of the M1 populations, in basal or activated conditions, were positive for CD206. When MSC were introduced into the environment, a steady increase in CD206 positive cells within the population was observed (Fig. 2A), correlating with greater ratios of MSC (Fig. 2A). IL-4, a cytokine which promotes M2 macrophage phenotypes (29), was used as a positive control for macrophage CD206 expression. IL-4 (10 ng/ml) treatment led ~50% of the population to express CD206 (Fig. 2A, B). Similarly, MSC at 1:1 ratio resulted in ~50% of the M1 population expressing CD206 (Fig. 2A, B). Increases in MSC to macrophage ratios did not lead to increases in the percentage of CD206 positive cells (data not shown). Consistently, with either MSC or IL4 treatment, ~ 40% of the population could not be induced to express CD206.



Figure 5.1. MSC attenuate macrophage pro-inflammatory cytokine secretion. (A) At day 2 M1 macrophage TNF- α secretion was diminished with MSC co-culture. There was a dose dependent reduction in TNF- α , maximum attenuation (55% reduction) was achieved at an M1:MSC ratio of 1:1.* represent statistical significance relative to LPS and ** to M1:MSC ratios of 1:50 and 1:5. (B) MSCs attenuate M1 macrophage IL-12 (p40) secretion at days 2 and 5 after LPS stimulation. * represent statistical significance relative to LPS and ** to M1:MSC day 2 cultures.



Figure 5.2 MSC promote LPS stimulated M1 macrophage CD206 expression. (A) At day 2 MSC co-culture resulted in a significant increase in the percentage of macrophages expressing M2 associated CD206 relative to LPS and medium conditions. There was a dose dependent increase in the percentage of CD206 expressing cells which plateaus at ~50 %. IL4 (10 ng/ml) promotes CD206 expression to approximately the same levels as PGE2. * and ** represent statistical significance relative to LPS. (B) Images depicting the elevated levels of CD206 observed when M1 macrophages are treated with MSC and IL4.

5.6.2 Fractional factorial design determination of MSC mediator(s) of M1 Reprogramming

To objectively identify the specific MSC secreted factors responsible for the M1 macrophage inflammatory cytokine attenuation and increase in CD206 expression a fractional factorial experiment was performed. A panel of potential MSC secreted immune mediators was identified from the literature (Fig. 3A). Two levels for each factor were examined: 1) representing the absence of the factor and 2) representing the approximate level MSC secrete the factor in-vitro, which was determined based on ELISA and/or literature. Statistical analytical system (SAS) software was employed to design an experiment with the minimum resolution needed to identify the combinations of factors which maximize the main effects, CD206 and TNF- α . Macrophages were seeded on 96 well plates and the different cocktails of factors, spiked with LPS, were added to each well. After 48 hours, supernatants were collected for TNF- α ELISA analysis and cells fixed for CD206 immunostaining. The results indicate that PGE2 was the factor which maximally promoted CD206 expression (Fig. 3B). While other factors also promoted CD206 expression, when in combination with PGE2, no combinatorial elevation in CD206 expression was observed. We next performed an ELISA for TNF- α and PGE2 was again, the main factor responsible for macrophage TNF- α attenuation (Fig. 2C). Interestingly, IL-4 (100 pg/ml), while able to promote CD206 expression, did not mitigate TNF- α levels (Fig. 2C). Overall, the results suggest that within the panel evaluated here, PGE2 alone was able to promote both M1 macrophage immunosuppression and CD206 expression.



Figure 5.3 Main factor effects on macrophage reprogramming. 128 combinations of each factor (supplemental Figure 1) were designed to determine the main factor and two factor effects on macrophage reprogramming. LPS was spiked into each condition and transferred to M1 macrophage 96 well cultures. After 48 hours of cells were immunostained for CD206 and supernatant TNF- α levels were measured via ELISA. A) CD206 B) TNF- α represent main factor effects for each potential cytokine. -1 and 1 represents the levels of the response in the absence and presence of the factor respectively. The slope of the line represents that magnitude of the change. Degree of changes were considered statistical significance based on a confidence interval of 95% (p<.05). PGE2 and IL4 demonstrated the ability to significantly promote CD206. However, only PGE2 was able to significantly attenuate TNF- α secretion.

5.6.3 MSC PGE2 Secretion Mediates Macrophage Inflammatory Action and Reprogramming

Next we wanted to test the dose response of PGE2 in attenuating M1 secretion as well as promoting CD206 expression. Therefore, M1 macrophages were stimulated with LPS in the presence of several different concentrations of PGE2. TNF- α ELISA measurement revealed that there was a strong dose dependent attenuation of TNF- α secretion with increasing PGE2 concentrations. However, at and above a concentration of 2.5 ng/ml, PGE2 attenuation was ~ 75%, or about 15% greater than post-MSC co-culture (Fig. 4A). The percent of macrophages within the population that was positive for CD206 correlated with PGE2 dose as well. However, the CD206 expression plateaued at 10 ng/ml in contrast to the 2.5 ng/ml for maximal TNF- α attenuation (Fig. 4B).

We next designed experiments to confirm the role of MSC PGE2 secretion in macrophage immune-modulation. PGE2 levels in supernatants collected from MSC/machrophage co-cultures were measured via ELISA. Macrophages in both basal and stimulated conditions did not secrete PGE2 (Fig 5A). Conversely, MSC/macrophage co-cultures secreted PGE2 at a concentration of ~7.5 ng/ml (Fig 5A). Implementation of a nonsteroidal anti-inflammatory drug (NSAID), indomethacin, to block COX-2 activity prevented MSC secretion of PGE2 (Fig 5A).

We next assessed MSC ability to attenuate macrophage TNF- α and promote CD206 elevation in the presence of indomethacin. Our results indicated that both TNF- α (Fig. 5B) attenuation and CD206 promotion (Fig. 5C) were completely reversed in the presence of a COX2 inhibitor. As a control, an IDO inhibitor was added to evaluate MSC macrophage modulation specificity. Unlike indomethacin inhibition of PGE2, IDO

inhibition with 1MT did not diminish the ability of MSC to attenuate TNF- α and promote CD206 (Fig. 5B, C).



Figure 5.4 PGE2 attenuates M1 TNF- α secretion and promotes reprogramming. M1 macrophages were stimulated with LPS and immediately treated with increasing concentrations of PGE2. After 2 days macrophage A) TNF- α secretion and B) CD206 expression were quantified. Increasing concentration of PGE2 attenuated M1 macrophage TNF- α secretion, which plateaued at 2.5 ng /ml at a reduction of ~75%. The percentage of CD206 expressing macrophages displayed the reverse relationship to increasing PGE2 concentration, however displayed a more linear response, and reached a maximum value of ~50 %.



Figure 5.5 MSC secretion of PGE2 promotes macrophage reprogramming. (A) Supernatants from MSC: macrophage co-cultures treated with LPS (1 μ g/ml) were assessed for their levels of PGE2. M1 macrophages in basal and stimulated conditions do not produce PGE2, however in the presence of MSC ~6.5 ng/ml was detected. When the COX2 inhibitor, indomethacin (10 μ m), is administered to the co-culture MSC PGE2 secretion is eliminated. * represent statistical significance (p<.01) relative to LPS, medium and indomethacin. (B) CD206 expression and (C) TNF- α secretion were assessed with indomethacin present in the MSC:macrophage co-culture. The inhibition of MSC PGE2 secretion completely prevented the increase in percent positive CD206 expressing cells as well as the attenuation of TNF- α previously observed with MSC co-culture. * represent statistical significance (p<.01) between MSC:macrophage conditions in the presence and absence of indomethacin.

5.6.4 PGE2 Induces Macrophage reprogramming Through CREB Signaling

The transcription of M2 macrophage associated genes has been identified to be mediated by STAT6 transcription factor binding. Therefore, we assessed p-STAT6 expression and found that, M1 macrophage p-STAT6 was only detected with IL-4 treatment (Fig. 6A). Therefore, a different signaling mechanism was explored to explain PGE2 regulation of macrophage plasticity. Recently, cAMP response element-binding (CREB) has been implicated in macrophage reprogramming (30) and PGE2 mediated cAMP elevation has been shown to activated CREB through phosphorylation of ser-133 (31). Therefore, experiments were designed to assess whether CREB is involved in PGE2 macrophage regulation. Two CREB inhibitors were identified, Pamoic Acid and RO-31-8220. Pamoic acid (PA) is known to prevent p-CREB/CREB binding protein (CBP) binding, which is imperative for target gene induction (32). RO-31-8220 is a protein kinase inhibitor, which prevents phosphorylation of CREB at ser-133 (30). PA and RO-31-8220 both prevented PGE2 promotion of CD206 (Fig. 6B), but did not alter PGE2 attenuation of TNF- α (Fig. 6c).

Next, immunoblotting for p-CREB was performed to determine if PGE2 activated CREB by enhancing phosphorylation at ser-133. Macrophages stimulated with LPS exhibited enhanced pCREB expression above basal levels, which was not enhanced by PGE2 after 15 min of treatment (Fig. 6d). This suggests that TLR-4 signal transduction leads to CREB phosphorylation at ser-133 and that PGE2/CREB mediated reprogramming occurs through a mechanism independent of direct CREB phosphorylation. Phosphorylation of Ser-129, in addition to the ser-133, by glycogen synthase kinase 3 (GSK3) inhibits CREB binding affinity to CRE promoter regions (33). GSK3 activity is negatively regulated by several factors, two of which PGE2 has been shown to induce, PI3K and cAMP pathways (34, 35). It was therefore posited that overtime, PGE2 treatment would phosphorylate GSK3, preventing its inhibition of CREB. M1 macrophages stimulated with LPS and treated with PGE2, were cultured over a 24 hour period. Protein was extracted at 1, 4 and 24 hours for immunoblotting. PGE2 co-culture resulted in prolonged and enhanced phosphorylation of GSK3- α at serine 21 over 24 hours compared to all other conditions (Fig. 7A). GSK3- β did not display differences in phosphorylation at ser-9 with PGE2 treatment (Fig. 7A). CREB dephosphorylation was slightly diminished overtime with PGE2 treatment (Fig. 7A). At no time point was phosphorylation of STAT6 observed in any condition except IL-4, which was present at 1 hour, but undetectable by 4 hours (data not shown).

To substantiate GSK3 involvement in CREB signaling and subsequent macrophage CD206 expression, inhibitors, lithium and SB415286, to GSK3 were administered to LPS stimulated M1 macrophages. Inhibition of GSK3 led to the elevation of CD206 after 48 hours (Fig. 7B). However, GSK3 inhibition did not result in TNF- α attenuation (Fig. 7C). Experiments were then designed to determine what signaling network may regulate PGE2 M1 TNF- α attenuation. The PI3K pathway has

been found to regulate pro-inflammatory cytokine secretion (36). M1 macrophages were stimulated with LPS, treated with PGE2 in the presence and absence of wortmanin, a specific PI3K inhibitor. Wortmanin reversed PGE2 TNF- α attenuation (Fig. 7D), but had no affect on CD206 promotion (data not shown).







p-CREB	-		-	-
α-Tubulin	-	-	-	-
LPS	-	+	+	+
PGE2		-	+	+
RO- 31-8220	-	-	-	+

Figure 5.6. PGE2 promotes increased M1 macrophage CD206 expression through CREB signaling. (A) M1 macrophages were stimulated with LPS (1 μ g/ml) and treated with IL-4 or PGE2 for 15 minutes before protein extraction. Immunoblotting for phosphorylated STAT6, at tyr-641, shows that PGE2, unlike IL4, does not promote STAT6 activation. (B) M1 macrophages were pre-incubated with RO- 31–8220 for 10 minutes, before stimulation and treatment with PGE2. Pamoic acid was administered at the time of LPS stimulation and PGE2 treatment. Inhibition of CREB signaling with Pamoic acid or RO- 31–8220 prevented MSC promotion of M1 CD206 expression, but had no effect on (C) TNF- α secretion. (D) Immunoblotting for phosphorylated CREB, at ser-133 15 minutes after LPS stimulation and treatment with PGE2 did not show increased expression of pCREB. LPS stimulation alone was able to significantly increase p-CREB expression. Immunoblotting confirmed RO- 31–8220 inhibition of CREB activation.



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increase p-CREB expression. Immunoblotting confirmed RO- 31-8220 inhibition of CREB activation.

5.6.5 PGE2 EP4 Axis Regulates Macrophage Inflammatory Phenotype

To investigate which PGE2 receptor mediates M1 macrophage reprogramming, inhibitors to EP receptors were employed. Traditionally, EP2 and EP4 binding results in cAMP elevation which activates PKA and subsequently activates several mediators which have been implicated in PGE2 immunosuppression [287, 288]. Recently, it has been identified that PGE2 binding to EP4 results in the downstream activation of the PI3k pathway [286], which can inhibit GSK3 activity [289]. Therefore, we anticipated that PGE2-EP4 interactions would specifically be responsible for M1 macrophage reprogramming. Specific inhibitors (L161982) to EP4 were used to convey its binding specificity to CD206 elevation. Macrophages were incubated for 48 hours in the presence of LPS with IL4 or PGE2 as well as with and without inhibitors to EP receptors. The data reveals that EP4 blocking results in significant reduction in the amount of CD206 positive cells (~30%) (Fig. 8A, C), however blockade of the other receptors did not inhibit PGE2's ability to promote CD206 (Fig. 8A). Likewise, inhibition with EP4 blocker inhibits PGE2 attenuation of TNF- α (Fig. 8B). Consistently, IL4 promoted CD206, but did not significantly attenuate TNF- α (Fig. 8A, B), again suggesting that the pathways for M1 attenuation and reprogramming are independent. However, PGE2 is able to regulate both pathways exclusively though the EP4 axis.



Figure 5.8 PGE2 mediates M1 macrophage reprogramming through the EP4 receptor. LPS stimulated M1 macrophages were treated with IL4, PGE2, PGE2 with an EP4 inhibitor (L161982) or a general inhibitor to the other EP receptors (AH6809). (A) The increase in the percentage of CD206 positive cells previously observed with PGE2 treatment was diminished with an EP4 antagonist. Antagonists to the other EP receptors did not interfere with PGE2 promotion of CD206. (B) PGE2 attenuation of TNF- α was also reversed with an EP4 antagonist. Blocking the other EP receptors did not reverse PGE2 TNF- α attenuation. (C) Representative images of CD206 depicting the reduction of percent positive cells when the EP4 receptors is blocked during PGE2 treatment.

5.7 Discussion

The ability of macrophages to exhibit plastic behaviors is emerging as an essential characteristic of inflammation in a normal wound healing response. There is now evidence supporting the notion that macrophages can express a combination of different phenotypes defining a spectrum, which, on one end has the pro-inflammatory M1 and on the other anti-inflammatory M2 macrophage phenotypes [251]. The immune system exploits this repertoire of phenotypic states to enact effective immune responses and when these phenotypes are regulated inappropriately, immunopathology may ensue [258]. The contemporary resolution paradigm is that inflammation is an active, as opposed to passive, process involving several biochemical cues leading to the control and eventual cessation of inflammation [290]. Interestingly, regulation of macrophage inflammatory function in numerous disease models [257, 259, 272, 281, 291-293] has been attributed to MSC. Here we show that MSC have the ability to promote reprogramming of human monocyte derived M1 macrophages [278]. MSC co-culture attenuated M1 macrophage pro-inflammatory cytokine levels and promoted expression of M2 markers. Through objective evaluation of several potential immunomodulatory mediators PGE2 was identified as the predominant factor that initiates M1 macrophage reprogramming. PGE2 binding to the macrophage EP4 receptor potentiates CREB transcriptional regulation indirectly, via the inhibition of GSK-3a. This mode of PGE2 action differs from IL-4 in that IL-4 was not able to attenuate M1 macrophage secretions and mediates expression of M2 associated transcripts via STAT6 regulatory networks.

There is now evidence, which suggests that normal wound healing occurs in the absence of IL-4 and IL-13 regulation [250]. In the course of a normal wound healing response no detectible levels IL-4, IL-13 or pSTAT6 were measurable [249]. Furthermore, IL-4 and IL-13 knockout mice did not exhibit reduced expression of M2 macrophages overtime [249]. Therefore, alternative factors may regulate the innate immune response throughout a wound healing response.

PGE2 represents one of the initial biochemical cues in an inflammatory cascade. Initially, PGE2 was considered as a pro-inflammatory mediator; however, there is accumulating support for anti-inflammatory PGE2 function. COX-2 inhibition in experimental models of colitis yields exacerbated inflammatory damage [294]. Knockout of COX-2 results in defective resolution events in models of peritonitis [295-297] and PGE2 has been demonstrated to facilitate Th2 polarization [298]. The data here suggests that through PGE2, MSC facilitate M1 macrophage reprogramming to M2 like cells. MSC transplantation in sepsis models have been shown to facilitate macrophage reprogramming through PGE2 [257]. Furthermore, macrophage ablation eradicated MSC benefits and led to exacerbated inflammation [257], which suggests that macrophage reprogramming may be an essential mechanism of MSC therapy. Similar MSC macrophage reprogramming effects have been observed in several pre-clinical models of chronic immune pathology [206, 259, 272, 292]. There is now evidence that dysregulation of fibroblast PGE2 production leads to lung fibrosis [299]. Interestingly, fibroblasts exhibit similar inflammatory control in T cell co-culture systems to MSC [263]. Therefore, stromal/epithelial cell PGE2 regulation of macrophage function may represent a regulatory checkpoint in an innate immune response. Additionally, PGE2

may be the factor responsible for promoting the M2 phenotype during a wound healing response.

Our observations are consistent with previous reports [173, 255, 272] which have suggested that MSC can promote M2 macrophage phenotypes. However, there are some discrepancies in the factors identified to be responsible for MSC promotion of M2 phenotypes. The identification of PGE2 as the mediator here is consistent with *Maggini* et al. observations using thioglycolate elicited peritoneal macrophages. Zhan et al. identified IL-6 and GMCSF to be responsible for MSC promotion of M2 phenotypes. IL-6 and GMCSF did not facilitate macrophage reprogramming in our system which may be attributed to macrophage maturity, where Zhang et al. used monocytes in their MSC studies. This may imply that different factors facilitate macrophage differentiation and reprogramming to specific phenotypes. Maggini et al. report elevation in macrophage IL-10 secretion after treatment, which was not observed here. This may be explained by possible differences in murine and human MSC functions [263] . Alternatively, peritoneal macrophages as tissue macrophages, may exhibit particular phenotypic plasticity that may not be an attribute of circulating monocytes after differentiation. There may also be subsequent cues post PGE2 regulations which control the temporal release of macrophage anti-inflammatory factors. Interestingly, IL4 receptor binding did not result in the attenuation of TNF- α secretion, but maintained the ability to promote CD206. Others have consistently observed the inability of IL-4 to mitigate TNF- α after 7 days of monocyte differentiation [300]. In contrast, IL-4 can prevent monocyte secretion of TNF- α [300]. The studies here suggest that PGE2 can elicit effects on differentiated macrophages, suggesting that PGE2 is able to promote macrophage reprogramming.

These observations suggest that the roles of these cytokines have environmental and temporal dependent actions, which would need to be leveraged to obtain a particular effect on a macrophage response. Furthermore, MSC in particular disease applications may impart different effects depending on the microenvironment.

There are several intracellular signaling pathways which define various macrophage states. STAT-6 is an intracellular mediator of macrophage alternative activated phenotypes [276]. However, in our studies STAT6 phosphorylation was not observed upon PGE2 treatment, suggesting that a different regulatory network is responsible for reprogramming. CREB is a transcription factor that binds Cre promoter regions, which are present in $\sim 49\%$ of all genes, but binding accessibility is under complex epigenetic regulation [301]. Accumulating evidence supports CREB's involvement in immune cell functions, specifically, as a transcription factor responsible for mediating antiinflammatory function [302]. Several M2 transcripts, like IL-10, contain Cre promoter regions, and several CREB transcripts stimulate M2 associated protein expression [282]. Additionally, CREB is necessary for macrophage secretion of IL-10, which was shown to be diminished by IFN- γ mediated activation of GSK CREB inhibition [303]. Here, inhibition of CREB/CBP interactions or phosphorylation of CREB at ser-133, prevented PGE2 mediated macrophage reprogramming. However, PGE2 did not enhance M1 pCREB expression relative to the increase observed with LPS. CREB can be phosphorylated by mitogen associated protein kinase (MAPK), PKA, PKC, $Ca^{2+}/calmodulin$ as well as others [304]. In particular, MAPK is activated downstream of the myD88 dependent pathway upon TLR4 binding [305], which may explain the immediate elevation in pCREB observed subsequent to LPS stimulation. However,

CREB is regulated by several intracellular factors; one in particular is GSK3, which phosphorylates CREB at ser-129, thereby reducing its affinity for Cre promoter regions [283, 285]. PGE2 has been shown to inhibit GSK3 activity [306], consistent with the GSK3 inhibition observed here. Specific inhibitors to GSK3 activity, Lithium chloride and SB415286, were able to promote CD206 expression in LPS stimulated M1 macrophages. This suggests that GSK-3 alone was preventing CREB activity. Lithium Chloride has been shown to enhance CREB binding affinity by preventing GSK3 inhibition of CREB-CRE interaction [285]. Furthermore, GSK3 imparts kinase activity to protein phosphatase (PP) [307], a phosphatase which has been identified to dephosphorylate activated CREB [308]. This may explain our observed reduction of CREB de-phosphorylation with PGE2 treatment. In general, there is a considerable amount of evidence that GSK3 plays a significant role in the regulation of innate and adaptive immune responses [309]. GSK3 activity negatively regulates the production of antiinflammatory mediators such as IL-10 and IFN- β [303, 310, 311]. The administration of GSK3 inhibitors in an animal model of endotoxic shock promoted the secretion of antiinflammatory mediators and increased animal survival by 70% [312]. Others have observed a reduction in the damage associated with collagen induced arthritis after GSK3 inhibition which specifically promoted macrophage anti-inflammatory function [303]. In our system, PGE2 only regulated the GSK3- α isoform, which may be explained by the fact that GSK3 isoforms can be differentially regulated [307, 313]. Interestingly, GSK3- α has been shown to exhibit enhanced CREB kinase function compared to GSK3- β [313]. However, it will be important to elucidate the regulatory mechanisms which govern the functions of the different isoforms and which ultimately lead to CREB
inhibition. Overall, GSK3 regulation of CREB may be an important regulatory checkpoint in macrophage reprogramming which could be exploited for therapy.



Figure 5.9 PGE2 signal transduction leading to CREB transcriptional regulation. LPS binding to the TLR-4 receptor stimulates a MYD88 dependent pathway which eventually leads to NF-KB translocation to the nucleus and subsequently proinflammatory cytokine secretion. PGE2 is able to attenuate this pathway through PI3k signalling. PGE2 binding to the EP4 receptor also activates cAMP signaling, which in

exhibits kinase function to GSK3- α . This will prevent GSK3- α from phosphorylated CREB at ser-129, which reduces CREB affinity to its Cre promoter regions.

Interestingly, the effect of PGE2 on cellular function is dependent on the expression of cell surface EP receptors. EP4 has specifically been found to activate PI3K/ERK in addition to the anti-inflammatory cAMP/PKA pathways [286]. Antagonists to the EP4 receptor were used to corroborate this hypothesis. Antagonists to the EP4 receptor attenuated macrophage CD206 expression. Recent evidence has shown cAMP to be essential for regulating macrophage transition into resolving macrophages [314]. Consistent with our observation, others have found EP4 dependent attenuation of pro-inflammatory human macrophage secretion with PGE2 treatment [315]. This report also did not detect the expression of the EP2 receptor on human macrophages, which may also explain the lack of M1 attenuation with EP2 antagonists observed here.

Interestingly, the M1 macrophages here, in either stimulated or basal conditions did not secrete PGE2, indicating that they do not have either COX1 or COX2 function at this stage of the inflammatory cascade. However, PGE2 secretion from macrophages has been shown to be part of a feedback loop and it is possible that COX2 could be present at a later time point. Since, IL-4 did not decrease pro-inflammatory secretion but did increase CD206, this would suggest that cAMP may be essential for M1 attenuation and a dichotomy exists in the signal transduction pathways which regulate M1/M2 phenotypes.

The data here suggests that through the PGE2/EP4 axis, MSC impart macrophage reprogramming function via intracellular CREB transcriptional regulation. The findings here implicate GSK3-α as a regulatory checkpoint in macrophage reprogramming during innate immune responses (Fig. 9). Therefore, *in-vivo*, an essential benefit from MSC transplantation in many disease models may be the restoration of the normal course of macrophage inflammatory function. These in-vitro observations may be highlighting a stromal/epithelial biological responsibility, which when disturbed leads to chronic immune pathology. Thus, PGE2, and other lipid mediators may have a distinct role in defining the initial phase of the resolution process. As such, failure in the appropriate course of COX2 prostaglandin production will result in improper macrophage cell regulation and subsequently, chronic immunopathology.

5.8 Perspective and Future Work

The results just described suggest stromal cells possess macrophage regulatory function. However, this leaves one with several questions: Why does this interaction not oc5ur in the spinal cord? Does the CNS contain MSCs? If so, why don't they help? Most literature describing the cellular and molecular events which lead to the development of a fibrotic scar implicates astrocyte functions [206]. However, clinicians suggest that the progression of fibrosis after SCI is not solely dependent on a glial response [5]. In fact, 30 years ago a stromal contributor to the SCI scar was described [5, 316]. This should be of little surprise, considering that scar formation in all organs represent remarkable similarities [317] and in all instances of scarring stromal cells play an integral role in the progression of fibrosis [318]. Therefore, what and where are the stromal components of the fibrotic response after SCI? Recent evidence identified a

pericyte presence after SCI [319]. The pericyte is a perivascular cell responsible for sustaining normal BBB function. The pericyte resides at the neurovascular unit of CNS tissue and is responsible for controlling endothelial cell function through the secretion of paracrine factors. There is evidence substantiating the pericyte's fibrotic functions in chronic kidney diseases [320] and now in SCI [319]. Goritz et al. reports the presence of a specific subset of percives at the epicenter of the injury sight after SCI [319]. There was a temporal accumulation of pericytes at the injury site, and a subsequent reduction until a constant level was reached, 7 months after a hemisection injury. These pericytes were secreting collagen and fibronectin as well as interacting with endothelial cells. They report that normally 10 times the amount of astrocytes to pericytes are present in uninjured spinal cord tissue, but 2 weeks after injury there are twice as many pericytes as there are asotroytes [319]. They implemented a Cre-lox recombinase technique to delete ras genes from the pericyte population, which reduced the numbers of pericytes after injury. Without a pericyte population, the lesion site remained open and no observable scarring was detected. This was the first evidence that stromal cells play an integral role in scar formation after SCI. Interestingly, there is currently a substantial amount of evidence supporting the similarities between pericytes and MSC [321]. MSC and pericytes display identical cell surface marker expression [317] and there is evidence the pericyte can exhibit all the clonal and differentiation potential of an MSC [322]. So, if there is a cell with MSC like properties present at the site of injury why is there a suboptimal healing response after SCI?

Earlier in this thesis we described an area of therapy being evaluated for SCI which focused on inhibiting an intracellular family of Rho GTPases. These GTPases,

such as Rho/ROCK prevent axonal regeneration by promoting growth cone collapse [323]. Intriguingly, there is now evidence that some of these GTPases can directly inhibit COX-2 PGE2 production [299]. Liu et al. reported that with increased matrix stiffening, similar to what occurs during fibrosis, there is reduced PGE2 synthase gene expression. They show that an increase in Rho/ROCK expression directly suppresses the levels of COX2 PGE2 synthesis, which subsequently leads to the excessive deposition of ECM [299]. Inhibitors, to Rho/ROCK restored PGE2 levels as well normal fibroblast functions. It has been demonstrated that PGE2 can directly prevent fibroblast proliferation and deposition of ECM through cAMP signaling [324]. Comparably, fibroblast derived COX2/PGE2 regulation of fibrosis has been observed in liver and wound healing models [325, 326]. Overall, a balance between COX2/PGE2 and Rho/Rock levels are necessary to maintain tissue homeostasis.

The injury site post SCI is comprised of several myelin inhibiting proteins. The most prominent ones are Nogo, myelin-associated glycoprotein (Mag) and oligodendrocyte myelin glycoprotein (Omgp) [327]. A receptor to Nogo (NGR) was cloned by Strittmatter and colleagues [323] and there is now evidence that Mag and Omgp can also bind NGR [327]. The NGR does not have a transmembrane domain and therefore cannot directly affect intracellular signal transduction, but rather functions with a co-factor, p75 neurotrophin receptor, to do so [328]. The p75 neurotrophin receptor directly interacts with Rho GTPase. Therefore, cells entering the lesion site after SCI, which express an NGR-p75 complex, will bind inhibitory myelin protein and result in GTPase activation. Furthermore, there are several glial derived factors which after binding also transduce signals which lead to GTPase activation [47]. There is evidence that non-neuronal cells,

specifically stromal cells, can express the nogo receptors [329] as well CSPG receptors [330]. Therefore, pericyte interactions with the myelin inhibitory proteins within the scar may activate intracellular GTPases and subsequently prevent sufficient production of PGE2. The reduced PGE2 levels at the injury may terminate the resolution cascade. GTPases have been found to be imported regulators of LPS induced pro-inflammatory cytokine secretion [331]. In-vivo Rho kinase inhibitors reduce renal failure in animal models of LPS challenge [332]. Infiltrating inflammatory macrophages and T cells in autoimmune models express elevated levels of RhoA [333]. We have performed experiments with LPA, a RhoA activator, and have seen elevated M1 macrophage secretion of TNF- α above the observed baseline level with LPS stimulation (Figure 10A). We have also shown that IL-10 secretion from LPS stimulated M2 macrophage is significantly diminished with RhoA activation (Figure 10B). In the same experiment LPS stimulated M2 macrophages were give C3 transferase, a non-specific Rho inhibitor, and an increase in IL-10 was observed (Figure 10B). These experiments suggest that Rho activation can inhibit M2 macrophage IL-10 secretion.



Figure 5.10 Effect RhoA activation has on M1 and M2 functions. A) M1 macrophage were simulated with LPS in the presence of LPA (Rho activator). The data demonstrated that Rho activation enhances M1 TNF- α secretion. B) M2 macrophages were stimulated with LPS together with LPA or C3 (Rho inhibitor). LPA significantly reduced the levels of IL-10 secretion from M2 macrophage and inhibiting Rho promoted secretion.

The work of hu et al. indentified CREB as an essential positive regulator of IL-10 production from M2 macrophages [303]. Interestingly, RHO activation results in activation of GSK3, which was described earlier to inhibit CREB activity [334]. Therefore, Rho activation may inhibit the secretion of IL-10 through GSK3 inhibition of CREB. Furthermore, considering the role CREB was shown to play in macrophage reprogramming Rho activation after SCI would represent another barrier for the M1 transition into M2 macrophages.

Treatments such as Cethrin, a RhoA inhibitor, may be indirectly promoting improvements after SCI by restoring pericyte regulation of macrophage function, promoting IL-10 secretion and removing an obstacle to inflammatory resolution. Therefore, RhoA may represent a target for multimodal treatment. Cethrin, has presented promising results in phase 1 clinical trials. Intriguingly, their best outcomes were observed with patients that received the drug 3 days after injury, which is the time point monocytes begin to enter the wound. (This data was presented in a seminar at the KECK center and should be published in the near future. The presenter was Lisa McKerracher CEO of bioaxone and her lab was one of the first to isolate NGR.) Rho inhibits several of the signaling cascades which lead to the macrophage reprogramming and the secretion of anti-inflammatory factors. One will argue that increases in axonal sprouting will be of limited benefit if they will not be able to transverse the lesion site. Therefore transplanting Cethrin when immune cells have entered the injury site may mitigate fibrosis while simultaneously promoting axonal sprouting.

Lithium chloride may also have indirect effects on SCI recovery. Lithium chloride is a potent inhibitor of GSK3, preventing CREB inhibition. There is a considerable amount data implicating CREB transcriptional regulation in the promotion of neural plasticity. Therefore, lithium chloride has been evaluated as a potential SCI therapy which can promote axon regeneration. The results here suggest that lithium chloride may promote macrophage plasticity after injury and may therefore represent an alternative benefit from lithium chloride care. However, lithium chloride did not mitigate the M1 TNF- α secretion therefore may have to be administered with a co-therapy which would mitigate pro-inflammatory secretion. None the less, it will be interesting to evaluate the dual effect

these therapies exhibit. Insights, into these mechanisms will provide information which will potentially enhance treatment regiments.

The results from the Cethrin trial suggest that it will be essential to determine the correct time points to administer a therapy. Currently, fMSC transplantation is performed mostly 1 week after injury. Some of the more recent reports have evaluated earlier time points. However, none of these studies evaluated the effect the dosing time points have on the inflammatory response. Furthermore, transplanting MSC directly into the injury site may be a suboptimal way to obtain MSC SCI therapy. The same interactions which may prevent pericyte function may affect MSC functions as well. MSC would be exposed to the same myelin inhibiting proteins and may lead to a reduced secretion of PGE2. This may provide an explanation for why the improvements with MSC transplantation for SCI have not been very impressive to date, considering MSC act at the injury site. Therefore, remote MSC function within an alginate capsule, will prevent MSC interactions with factors in the SCI milieu which, may not be favorable for wound healing. This may represent an additional benefit to the localization and persistence benefits, the MSC capsules platform provides.

Stromal cell secretions illicit action on several of the injury cascades post SCI. However, PGE2 alone may be able to provide benefits at multiple levels independent of the other factors MSC secrete. PGE2 binding to EP2 and EP4 receptors increases intracellular cAMP [283] which will induce axonal plasticity via the same mechanism which lithium chloride does [44]. cAMP has also been shown to inhibit the activity of Rho kinase [335], which suggest that PGE2 can potentially provide the same benefits as

Chapter 6 Methlyprednisolone Exhibits None Specific Immunosuppressive Function

6.1 Abstract

The previous studies identified that the macrophage is a target of MSC therapy. However, immunotherapy has been attempted for SCI. MP was approved as an antiinflammatory drug to treat SCI, but it does seem to reducing morbidity. What will differentiate MSC immunosuppression from the previous anti-inflammatory therapies, which have unfortunately been unsuccessful? Here we looked at the effects different GC have on M1 and M2 macrophages after LPS stimulation. The goal was identify differences in GC vs MSC therapy which would differentiate the immune action these two therapies impart. What we observed is that GC work differently depending on their potency and that MP specifically exhibits non-specific immunosuppressive action affecting both M1 and M2 functions. MSC on the other hand did not inhibit M2 functions, which suggests that they promote M1 conversion to M2 without interfering with M2 function.

Introduction

The current FDA approved treatment for SCI is MP, a drug which has tremendous antiinflammatory behavior. However, MP SCI care has fallen under tremendous scrutiny and may soon be considered an unacceptable option for treatment [26-29]. Interestingly, MP efficacy is observed when given to patients 3-8 hours after injury. Any deviation from this protocol does not lead to positive outcomes and often results in side effects. Yet, one should not ignore the efficacy observed with early administration of MP. Understanding the mechanism by which early transplantation leads to improvements may provide valuable knowledge. MP inhibits COX2 expression [336], which is a responsible for prostaglandin secretion and hence will affect vascular permeability. MP administration within the first 8 hours after SCI would potentially reduce the amount of infiltrating immune cells. The neutrophil would be particular affected by this initial dose, considering its presence after SCI is observed within 24 hours [5]. However, MP is administered for 48 hours after injury which can potentially reduce the infiltration of immune cells, which enter the lesion site days after injury. Although, in light of the observations made in chapter five of this dissertation, administration of MP after monocyte infiltration may lead to inflammatory dysregulation. Inhibition of COX2 expression would decrease PGE2 levels and subsequently lead to arrest in the M1 phase of immune progression. Furthermore, considering the current understanding of the dichotomy in macrophage phenotypes, it is important to consider what effect MP has both M1 and M2 macrophages. Therefore, experiments were designed to determine the effect MP and different glucocorticoids have on M1 and M2 functions. The results suggest that MP at all concentrations imparts nonspecific immunosuppression of M1 and M2 macrophages. On the other hand cortisol and prednisolone displayed dose dependent inhibition of M2 IL-10 secretion and a concentration of dexamethasone could be optimized to maximize M1 TNF- α inhibition without effecting M2 IL-10 secretion [337]. MSC did not inhibit M2 IL-10 secretion and thus inhibit M1 functions not at the expense of M2 functions.

6.3 Methods

6.3.1 Macrophage/MSC Co-cultures

Human blood (Blood Center of New Jersey) was used to derive Monocytes. Peripheral blood mononuclear cells were collected from blood of healthy donors by density gradient separation using ficol at a density of 1.077(GE Healthcare). Monocytes were isolated to high purity (>90%) by magnetic cell sorting using anti-CD14-coated beads (per manufacturer recommendation (Milteny Biotec, Auburn, CA). 10⁷ monocytes were cultured on 175 cm² flasks (BD) in RPMI-1640(GIBCO). RPMI was supplemented with 10% fetal bovine serum (FBS)(GIBCO), 100 units/ml Penicillin(GIBCO), 100ug/ml streptomycin (GIBCO) and 400 mM L-glutamine(GIBCO). Monocytes were allowed to adhere for 2 hours and then washed 3 times with PBS to remove non-adherent cell. Monocytes were cultured for seven days in RPMI supplemented with 5 ng/ml GMCSF (M1) or 50 ng/ml MCSF (M2) (R&D). On the 7th day of culture macrophages were washed once with PBS and then detached with trypsin-EDTA (GIBCO) for 30 minutes at room temperature. Cells were re-suspended in RPMI, counted, re-plated at 1×10^5 cells/ ml in a 24 well plate (Corning) and allowed to attach overnight. The following day cells were used for experimentation. Cortisone, prednisolone, MP and dexamethasone at various concentrations (1, .5, .1 and .01 ug/ml) were administered to M1 or M2 macrophage after LPS stimulation. Co-cultures were established with MSC within 8 µm transwell inserts (Corning, Corning, NY). M2 Macrophages were treated with 1 µg/ml LPS from Escherichia Coli (serotype 055:B5, Sigma-Aldrich, St. Louis, MO) and MSC at a cell concentrations of 10^5 cells /ml. COX-2 blocking was performed under the identical culture conditions, but in the presence of Indomethacin (10µM) or 1MT (1 mM). The

cultures were incubated for 48 hours or 5 days, after which culture supernatants were collected and macrophages fixed for immunocytochemistry as described earlier in the dissertation.



Figure 6.1 GC promote M1 macrophage CD206 expression. GCs at all concentrations were able to promote M1 macrophage expression of CD206.



Figure 6.2 MP impart non-specific immunosuppressive action. A) GCs exhibited a dose dependent reduction of M1 macrophage TNF- α . The level attenuation correlated with increasing levels of GC potency were cortisol the weakest and dexamethasone being the strongest. B) MP at all concentrations completely inhibited M2 macrophage IL-10 secretion. The two weaker GCs promoted II-10 at lower concentration at higher ones had no effect. Lower concentration of dexamethasone did not affect IL-10 secretion.



Figure 6.3 MSC do not inhibit M2 IL-10 secretion. MP consistently attenuated M2 macrophage IL-10 secretion. However, MSC co-culture did not affect secretion.

6.4 Results

In order to ascertain the differential effects different GCs have on M1 and M2 function, different doses of cortisone, prednisolone, MP and dexamethasone were administered to MCSF derived M2 and GMCSF derived M1 macrophage in the presence of LPS stimulation. The cells were cultured for 48 hours at which point supernatant was collected and cells fixed for immunostaining. M1 macrophage cultures were immunostained for M2 marker CD206 and the percent of cells expressing CD206 above one standard deviation of untreated M1 macrophages were quantified. Every GC produced and increase in CD206 expression above untreated controls (Figure 1). Dexamethasone at a dose of 100 ng/ml promotes ~60% of the M1 population to express CD206 (Figure 1). The others, depending on the dose, yielded CD206 percentages between 40 and 50% (Figure 1).

The ability of GCs to promote M2 macrophage protein expression has been documented previously, and these observations substantiate those results [338]. However, the effects different GCs have on the different macrophage subsets have not been studied. We therefore analyzed IL-10 secretion from M2 macrophage cultured with varying doses of GCs. The results suggest that the natural occurring GCs, cortisone and prednisolone, enhance IL-10 secretion from M2 macrophages (Figure 2B). Conversely, the synthetic GCs, MP and dexamethasone, significantly attenuated IL-10 secretion from M2 macrophages (Figure 2B). Dexamethasone at 10 ng /ml dose did not significantly attenuate IL-10 secretion (Figure 2B). We next looked at the attenuation of M1 TNF- α secretion with these GCs. There was a dose dependent reduction in TNF- α secretion with all GCs (Figure 2A). Dexamethasone was the most potent attenuator of TNF- α secretion.

However, at higher concentration MP and dexamethasone attenuated TNF- α equivalently (Figure 2B). At those doses both synthetic GCs attenuate IL-10 secretion from macrophages.

We next set out to assess whether MSC treatment of M2 macrophage would affect IL-10 secretion. Chapter 5 of this thesis identified that MSC secretion of PGE2 promotes M1 macrophage reprogramming. If this phenomenon occurs concomitantly with inhibited M2 macrophage function, MSC treatment may not be as effective. Therefore, MSC M2 macrophage co-cultures studies were established to determine MSC effects on M2 IL-10 secretion. The cultures were established in the presence of LPS and MP was used as a control. The data suggests that MSC do not interfere with M2 macrophage IL-10 secretion (Figure 3). MP significantly attenuated IL-10 secretion from M2 macrophages. This suggests that PGE2 does not negatively affect M2 macrophage function. In fact, in a later study PGE2 was administered to M2 macrophages at doses corresponding to the ones implemented in the GC studies. The data did not reveal any reduction in IL-10 secretion, but rather at some of the higher doses, enhanced IL-10 secretion was observed (data not shown).

6.5 Discussion

GCs are utilized clinically for a myriad of different applications aimed at controlling inflammatory responses. In several instances of auto-immune, chronic, pain and others GC administration proves to be effective. GCs, MP in particular, became the subject of pioneering work for the treatment of SCI, leading to its FDA approval for acute SCI care. Since this occurrence, now over 20 years ago, the literature does not completely accept MP as an effective SCI treatment. Our observations may help partially explain some of the more negative consequences of MP treatment. These experiments suggest that MP acts as a nonspecific attenuator of immune function. Therefore, while it is more potent then natural GCs in its ability to attenuate pro-inflammatory secretion, the fact that MP attenuates the beneficial aspects of M2 immune function may be a deleterious consequence. The immune system is able to present a versatile repertoire of phenotypic states, which are utilized to impart effective immune function. If all of these states are inhibited then inappropriate immune function would ensue. Intriguingly, the natural occurring GCs did not diminish IL-10 secretion. Some of these observations may be explained by the different affinities GCs have to mineralocorticoid (MR) and glucocorticoids (GR) receptors. Interestingly, MR and GR have opposing effects on inflammatory function, where MR has pro-inflammatory properties [338] and GR is antiinflammatory [339]. In general most GCs have a greater binding affinity to MR over GR [340]. Cotrisol exhibits equal binding affinity to the two receptors. Prednisolone has 6.25 and MP 15 times greater affinity for MR. Dexamethasone does not bind MR and has low potency for GR. It would be interesting to quantify the degree of MR to GR expression on M1 vs. M2 macrophages. The differences in GC affinity coupled with the varying expression levels of MR and GR may shed light on the physiological hormonal regulation of immune responses to stress.

There is evidence that GCs can be detrimental to the CNS through mechanisms independent of immune cells. In general, MR and GR binding results in the intracellular activation of different regulatory networks [341]. Furthermore, it is known that in tissues lacking 11 β HSD2, like the brain, GC MR binding trumps GR activity [338]. GCs binding to MR has been shown to engender impaired hippocampal function [342].

Therefore, it is possible that the neurotoxicity may account for the harmful observations made after MP administration for SCI care. Others feel that prolong exposure to GC result in unresponsiveness which leads to harmful alternative function [343].

MSC did not attenuate M2 macrophage IL-10 secretion, which supports their role as a facilitator of regulatory immune cell phenotypes [160]. Considering, PGE2 was identified to be the mediator of macrophage immune modulation; it would be safe to say that PGE2 does not interfere with M2 functions as well. In fact, at certain concentrations we have found that PGE2 may actually enhance IL-10 secretion. Identifying, that PGE2 function through CREB, in chapter 5 of this thesis, would support this observations, considering that CREB has been implicated in IL-10 secretion from MCSF derived M2 macrophages [303]. MSC through PGE2 secretion can facilitate M1 conversion of M2 macrophage, and potentially enhance the function of M2 macrophages already in the system. In conclusion, these studies highlight the unique immunomodulatory action MSC impart compared to previous SCI immunotherapy.

Chapter 7 Conclusions, Future Directions and Perspective

In conclusion, this dissertation established the feasibility of implementing an encapsulation based bioengineering approach to enhance MSC treatment for SCI. We show that the capsule microenvironment can be designed to cultivate MSC paracrine functions. The eMSC was able to exhibit MSC immune modulation in response to inflammatory signals, similar to the signals which would be present in the CSF after SCI. More impressive support for the platforms therapeutic potential was obtained from an organotypic model of CNS secondary injury. EMSC were able to completely reverse degradation as well promote axonal extensions. The capsule microenvironment presents an avenue for multifunctional modification. Current MSC therapy relies on cues endemic to the transplantation milieu to dictate MSC activation. The philosophy being, MSCs will respond to these factors and act accordingly, a wishful thinking ideology in retro respect. I hope that after reading this dissertation, one will gain an appreciation for the complexity of stromal regulation. I believe that it is no longer appropriate to think that MSC uniquely



possess the ability to provide trophic support, this is an attribute of several stromal cell types. Therefore, future work with the capsule platform should aim at modifying the capsule microenvironment to manipulate a desired MSC response. For instance, it known that different MSC activation can be achieved with different factors. Some of our own preliminary

work demonstrates that depending on the factor one can induce MSC to secrete neuro-

trophic factors. The graph below shows that $TNF-\alpha$ administered to MSC promotes BDNF secretion. BDNF is a neurotrophic factor which can facilitate axonal regeneration. Agonists to the TNF- α R can be incorporated into the alginate microenvironment to continuously stimulate the secretion of neurotrophic factors. This could theoretically be done to maximize a desired MSC response. A similar approach can be taken to maximize MSC secreted factors which would prevent apoptosis. These MSC activators can be incorporated into the system through various modalities. MSC stimulants can be conjugated to the alginate or potentially via co-encapsulation of MSCs with liposomes containing these factors. The capsule microenvironment could be designed to tailor specific MSC functions. One can then envision creating separate batches of capsules biasing MSCs to a particular response and then pooling those capsules before transplantation. Of course, it will be important to identify the right factors to activate MSC for a particular response. The fractional factorial approach implemented in chapter 5 can provide a powerful technique for identifying these factors. For instance, if an optimal activation cocktail was needed to stimulate MSC to promote axon extension one could identify a panel of potential agonists to receptors which transduce signals to activate the transcription of neurotrophic factors. This panel can then be evaluated with a fractional factorial designed experiment to stimulate the MSC, transfer the condition medium to axons and determine which combination promoted the greatest axonal extension. This approach can be taken to maximize MSC effects on any desired response.

The capsule multi-functionality can be extended to make the therapy more robust. We already described multifunctionalization with contrast agents to provide a non-invasive imaging modality. Other modification can be made to induce degradation of the capsule and or apoptosis of the MSC after a desired course of therapy is achieved. This may be important in the future if the treatment does advance to clinical trials. After the MSCs have provided a desired response they would not be needed in the intrathecal space. The capsules we are using now would degrade over time, but we currently do not know how long this would take.

Pre-clinical preliminary data demonstrated the potential the eMSC platform possesses at several levels of application. The obvious one being that eMSC exhibited an ability to promote improved outcomes after SCI. Additionally, we were able to show that prolonged presence of the MSC enhances their therapeutic action. This result substantiates the fact that fMSC transplantation may not result in prolonged persistence. This may also explain the variable and modest improvements observed to date from other studies. A different spin on the benefit of this approach is that it may represent a novel exploratory frame work to answer fundamental questions about MSC therapy. It essentially provides an in-vivo transwell system, where benefits from the capsules can only be achieved through secretion. This may provide conclusive evidence that MSC do not elicit benefits in SCI through transdifferentiation. While, there is more to be studied here, this is one of the more exciting aspects of this approach. It is a bio-engineering application which provides a way of studying the MSC response from a novel perspective. I am excited to see what future results can be obtained from this system.

We show that stromal cells act as a regulator of macrophage specific functions. We highlight that one of the benefits MSC impart from in inflammation is the attenuation of pro-inflammatory M1 and promotion of reprogramming to an anti-inflammatory like cell. In chapter 5 we study this interaction in depth and identify that it is mediated by PGE2 and intracellular CREB signaling. PGE2 potentiates CREB activity by inhibiting GSK3- α , thus establishing GSK3- α as a potential checkpoint in the macrophage response in wound healing. One of the more interesting observations from these studies was the fact that only a fraction of the population was able to undergo reprogramming. It would be interesting to identify the phenotypes which differentiate the two populations (it could be more than two). Monocytes are a heterogeneous population of cells and it may be that certain cells in the population can undergo this plastic behavior. I believe these types of studies would lead to potentially important contribution to the innate immunity field. Another study which would be interesting is to inhibit some of these signaling pathways in a wound healing model and observe if healing is impaired. I believe this would be essential to establish these pathways in facilitating an appropriate course of macrophage function for a normal wound healing response. Another study would be looking at RhoA activation in the pericyte population after SCI and determine if PGE2 synthase function is reduced. This may provide an explanation for the lack of M1 to M2 transition after spinal cord injury. In these same experiments looking at RhoA in the macrophage population and determining if GSK3 is activated in these cells would also provide interesting insights into the interactions which prevent resolution. These experiments would all implicate the myelin inhibiting and glia scar proteins as a driving force for the immune pathology. While the fibronectin results in chapter 2 are not integral to this thesis, it would be interesting to observe its interaction with the macrophage after injury to determine if fibronectin induces signals which will lead to RhoA activation.