ALGINATE-ENCAPSULATED MESENCHYMAL STROMAL CELLS AS A MULTI-POTENT THERAPY FOR TRAUMATIC BRAIN INJURY

By

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ABSTRACT OF THE DISSERTATION

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Primary damage in traumatic brain injury (TBI) occurs instantaneously, and the only method of intervening is prevention. Secondary brain injury, however, involves a complex range of cellular and molecular processes that occur over a period of hours to months after the primary injury, resulting in both immediate cell death and damage, and long-term degenerative changes. Therapies that provide protection against secondary insults and/or restore neural function are critical to survival and functional recovery following TBI. Mesenchymal stromal cells (MSCs) have emerged as a promising therapy, acting as trophic mediators capable of responding to their micro-environment. Current delivery methods, however, limit sustained therapeutic benefit due to lack of long-term persistence, and migration away from the injury site. We have previously developed a method to immobilize MSCs in alginate micro-spheres, enabling greater control and localization.

Herein, we evaluated alginate-encapsulated MSC therapy for the ability to target secondary injury components contributing to progressive damage following TBI. We demonstrated that encapsulated MSCs attenuated the neuro-inflammatory response in

organotypic hippocampal slice culture (OHSC), more effectively than monolayer MSCs, and identified PGE_2 as a key inflammatory mediator produced by MSCs. In contrast to monolayer MSCs, inflammatory signals were not required to stimulate PGE_2 production by encapsulated MSCs. Further encapsulation-stimulated changes were revealed in a multiplex panel analyzing 27 MSC-produced cytokines and growth factors. We also determined that our encapsulated MSC treatment primarily targets astrocyte-mediated inflammation, and that constitutively increased levels of PGE_2 produced by encapsulated MSCs may be a key contributor to their enhanced inflammatory modulation. Furthermore, encapsulated MSC treatment is capable of up-regulating astrocyte expression of several neurotrophic factors. In addition to modulating inflammation, encapsulated MSCs also prevented ischemia-induced cell death and neurite retraction in OHSC and cerebellar granule neuron cultures, and reduced astrocyte activation markers following *in vitro* ischemic injury.

Overall, we have shown that encapsulated MSCs target multiple components of secondary injury following TBI, including inflammation and ischemia, and provide both anti-inflammatory and neuroprotective benefit. These results suggest that alginate encapsulation of MSCs may not only provide an improved delivery vehicle for transplantation but may also enhance MSC therapeutic benefit for TBI recovery.

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DEDICATION

For my family -- my mother and father; my sister, Kim; my favorite little ones, Thomas and Ellie; and for my husband, Rik. Your love, encouragement, and support have carried me throughout.

PRIOR PUBLICATIONS

Several sections of this dissertation have been published elsewhere, or are pending external publication. These publications are acknowledged below:

- Chapter 2 has been published in its entirety and has the following citation: Stucky, EC, Schloss, RS, Yarmush, ML, Shreiber, DI. Alginate micro-encapsulation of mesenchymal stromal cells enhances modulation of the neuro-inflammatory response. Cytotherapy 2015;17:1353-64. doi: 10.1016/j.jcyt.2015.05.002
- Chapter 3 in its entirety is being prepared for publication, estimated submission October 2015.

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CHAPTER 1. INTRODUCTION

1.1. TRAUMATIC BRAIN INJURY (TBI)

Each year in the US, approximately 1.5 million new traumatic brain injuries (TBI) are incurred. Of those new cases, roughly 70,000 – 90,000 will result in long-term disability, which dramatically impacts the quality of life of the patient and his/her family, and may cause economic strain due to the cost of extended healthcare and lost wages. Globally, TBI remains the leading cause of morbidity and mortality in individuals under 45 years of age, with incidence of injury peaking between 15 – 35 years of age [1]. An average of 39% of injuries result in patient death, and 60% result in functional deficits [2]. Because the majority of TBI patients suffer injury early in life, their life expectancy is not significantly altered from the average, non-disabled individual, which further intensifies the drastic and devastating effects of long-term disability and cost of care. It is estimated that in the US alone, the direct and indirect costs of TBI are in excess of $60 billion, representing a substantial economic burden on the healthcare system [3]. Clinical intervention for TBI would relieve this economic burden, improve patient outcome and quality of life, and decrease morbidity due to TBI.

1.1.1. Secondary injury mechanisms following TBI

Traumatic brain injury is characterized by an instantaneous primary injury that is mechanical in nature, followed by a prolonged period of secondary injury, occurring over the days to months after the primary injury is sustained. The mechanisms by which secondary damage is inflicted include: changes in cerebral blood flow and ischemia, excitotoxicity, oxidative stress, free radical production, and chronic inflammation [4].

These secondary insults result in further cell death, neuronal degeneration, increased lesion volume, and formation of an environment that is inhibitory to regeneration. Indeed, the majority of functional deficits experienced after TBI can be directly attributed to the damage caused by delayed injury [5].

Primary injury is a result of immediate mechanical damage, the outcome of which is extensive cellular damage to neurons, axons, astroglia, and blood vessels, due to tearing, stretching, and shearing [6]. The secondary injury cascade begins seconds after the primary mechanical injury, initiating pathological changes on the biochemical, molecular, and cellular levels. The earliest pathological changes are a result of ischemia, energy depletion, and excitotoxicity. Immediate disturbances in cerebral blood flow induce ischemia, and reduced cerebral oxygen tension causes a metabolic switch from aerobic to anaerobic processes, leading to lactose build-up and reduced glucose [7]. This glucose deprivation results in cerebral energy deficits due decreased ATP production, and the subsequent loss of function of ATP-dependent ion channels and proteins [1,8,9]. As these ionic channels fail, neuronal and astroglial cell membranes depolarize, releasing excess glutamate into the extracellular space [10]. Glutamate accumulates even further, as astroglial uptake of excess glutamate, an energy-dependent function, is impeded. Excitotoxic cell death results as elevated levels of glutamate binds NMDA receptors, inducing a massive influx of Ca^{2+} and Na^{+}. This ionic imbalance not only activates several pathways leading to apoptosis and cell death, but also activates pathways leading to free radical generation, membrane degradation, mitochondrial dysfunction, further energy depletion [8,11,12], and release of pro-inflammatory molecules [6].

The intermediate to chronic stages of secondary injury are characterized by inflammation, edema, and glial scar formation. Within minutes to hours post-injury, the early injury mechanisms following TBI activate pathways that result in neuroinflammation and edema, which are primarily mediated by astroglial cells [7]. At the same time, disruption of the blood-brain barrier enables entry of chemokines and peripheral immune cells, further triggering the inflammatory response [13]. These signals induce astroglial cells to produce several pro-inflammatory cytokines, many of which have a positive feedback effect, eliciting further cytokine production [14-16]. The neuroinflammatory response becomes a chronic condition, lasting weeks to years following TBI. In addition to pro-inflammatory molecules, astroglial cells are also stimulated by early injury signals to produce extracellular matrix components that form the glial scar, creating an environment that is inhibitory to neuronal regeneration.

Currently, therapeutic development tends to target individual components of secondary injury. While this approach may be successful in ameliorating TBI symptoms in some patients, damage following TBI is so inherently complex and individually variable, that many show no response whatsoever to treatment [17]. Indeed, more than 30 phase III clinical trials targeting individual secondary injury mechanisms have failed [6,18]. Identification and development of a multi-potent therapy could prove effective in targeting multiple mechanisms of damage, and provide enhanced treatment and recovery following TBI. In addition, a therapeutic strategy that is both multimodal and temporally responsive to the injury environment could further enhance therapeutic benefit, as secondary injury is progressive and the causal agents of damage are continually changing at the lesion site.

1.1.2. Astroglial response to TBI

Astrocytes are multifunctional cells found in abundance in the central nervous system (CNS), performing essential functions in regulating the brain microenvironment including: maintenance of ionic and neurotransmitter homeostasis, free radical scavenging, blood-brain barrier (BBB) structure and development, and synaptic transmission. In response to injury, astrocytes undergo a process known as astrogliosis, leaving their quiescent state and becoming activated [14]. Although the focus of brain injury research and drug development was at one time focused on the role and response of neuronal cells, it has become increasingly clear that reactive astrocytes play a significant role in the pathophysiology of brain trauma and neuron survival [19].

Following astrogliosis, astrocytes cease protective functions such as glutamate uptake, free radical scavenging, and ionic buffering; contributing to excitotoxic and free radical-mediated neuronal cell death [20,21]. Reactive astrocytes are a key contributor to post-injury neuroinflammation, producing increased levels of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β. In addition to activating the inflammatory response and signaling further astroglial activation, these cytokines can also activate the immune response, disrupt the integrity of the BBB, and signal apoptotic pathways [14,22,23]. Glial scar formation is also attributable to reactive astrocytes, by increased production of extracellular matrix molecules in response to injury [14]. Given the key role of astrocytes in the chronic pathology of TBI, it is clear that the study of secondary injury mechanisms and development of TBI therapeutics must include evaluation of both astroglial and neuronal responses.

1.2. CURRENT THERAPEUTIC APPROACHES

As the primary injury cannot be reversed, therapeutic approaches to TBI are aimed at secondary injury mechanisms, with the goal of preventing neurological deficit or restoring function. Early intervention is critical, and current acute stage treatment protocols focus on controlling intracranial pressure, maintaining adequate oxygen supply and blood pressure, and preventing further mechanical injury [24]. While these methods aid in stabilizing the patient, there are currently no approved therapeutics for halting the progression of the secondary injury cascade [25]. The complex pathology and interplay of secondary injury mechanisms, as well as patient and injury variability, make TBI exceedingly difficult to treat.

Several treatment strategies have shown promise in animal models of TBI, but fail to show significant neuroprotective effect in humans [26]. Others have demonstrated inconclusive or variable results, or carry adverse risks that outweigh their benefit. Corticosteroids, such as methylprednisone, have been long thought to reduce edema and consequently, intracranial pressure, following TBI. Methylprednisone, however, was found to be associated with an increased risk of death [24]. As a prophylactic treatment, hyperbaric oxygen has aimed to increase brain tissue oxygenation and halt the progress of ischemic injury. While some trials have demonstrated reduced intracranial pressure and mortality, as well as improved functional outcome; other trials have determined hyperbaric oxygen therapy to be inconclusive, possibly due to variations in administration and trial design [7]. Hypothermia has been evaluated as multifactorial approach to brain injury for decades, in order to slow patient metabolic rate and the progression of the secondary injury cascade [27]. However, despite early patient

benefits, there is no evidence that hypothermia improves long-term functional outcome, and carries the risk of pneumonia and other complications [2,24,28]. Progesterone, a steroid hormone, has been promising as a multi-potent neuroprotective therapeutic, targeting excitotoxicity, apoptotic pathways, edema, and inflammation. It has improved outcomes in TBI patients, but requires further trials [6].

Additional therapies, such as statins, mannitol, excitotoxic amino acid inhibitors, and erythropoietin, have been met with similar challenges or require further investigation and trials. Though single secondary injury mechanisms remain the primary target of TBI therapeutics, multi-potent and cell therapy approaches to development of TBI treatments have become more common, [29] and appear to be more promising in conferring neuroprotection and improving patient outcome. Nonetheless, these approaches target limited time points in the secondary injury cascade, a process that is dynamic and continually progressing. Ideally, therapeutic development for TBI treatment should aim not only to be multifactorial, but also to dynamically respond to the changing injury environment.

1.3. MESENCHYMAL STROMAL CELL THERAPY FOR TBI

Mesenchymal stromal cells (MSCs) have the potential to provide both therapeutic strategies mentioned above – providing multimodal therapeutic benefit, and responding temporally to the injury microenvironment. MSCs are easily isolated from adult bone marrow and propagated on tissue culture plastic, making them a suitable source of cells for autologous cell transplantation [30]. Aside from their ability to differentiate down multiple lineages [31], studies in recent years have shown that MSCs have the ability to

respond to an injury environment, releasing cytokines and trophic factors necessary to modulate the immune response and inflammation [32]. As such, it is possible that MSCs implanted at the site of brain injury would be able to alter their response and secretory profile in reaction to dynamic cellular and molecular changes, progressively targeting specific mechanisms of secondary cell damage. Several studies to date have reported therapeutic benefits of MSCs acting as trophic mediators, using *in vitro* and *in vivo* models of myocardial infarction [33], graft versus host disease [34], wound healing [35], and stroke [36,37], among others [30,38,39].

There have been several reports of functional recovery following administration of MSCs in animal models of both cerebral ischemia and TBI. Intravenous administration of MSCs one week after TBI in a rat model yielded improvements in functional recovery for up to 3 months after treatment, and produced growth factors for this duration as well. Important to note is the therapeutic window – the investigators conducted a previous study administering MSCs one day after TBI, and demonstrated functional recovery with no dose dependence, which became a factor at the one week administration. Even so, the ability of MSCs to improve recovery at more than one time point speaks to their therapeutic efficacy following brain trauma and provides the benefit of an extended therapeutic window [40]. Additional studies evaluating the therapeutic benefit of MSCs in a rat model of transient cerebral ischemia have also demonstrated functional recovery with a therapeutic window extending from one day to one month after injury [41-43].

In regards to the secondary injury cascade following TBI, it has been demonstrated that MSCs are able to act as trophic mediators in response to many of the injury

mechanisms implicated in this process. Treatment of MSCs with pro-inflammatory factors or injured spinal cord extract resulted in an altered cytokine profile, while exposure to anti-inflammatory stimuli had little to no effect [44], implicating the role of MSCs in modulating inflammation and their ability to respond to environmental cues. When co-cultured with cortical neurons exposed to excitotoxic glutamate, MSCs reduced neuronal apoptosis via increased production of the neurotrophic factors NGF and BDNF, as compared to controls [45]. Additionally, transplantation of MSCs into rats after cerebral ischemia improved functional recovery by producing neurotrophic factors to support survival and regeneration of host neurons. MSC treatment increased levels of BDNF, NT-3, and VEGF; and resulted in a reduction of lesion volume and neuronal loss, and promotion of endogenous neuritogenesis [46]. There is also evidence that MSC therapy may have the ability to target several components of the astrocyte-specific response to injury. It has been demonstrated that soluble factors produced by MSCs down-regulated expression of pro-inflammatory cytokines and TLR4, as well as altered expression of metabotropic glutamate receptors, in lipopolysaccharide-stimulated astrocyte cultures [47]. In *in vivo* and *in vitro* models of ischemia, MSCs also reduced production of glial scar components produced by astrocytes [37], as well as astrocytic activation markers [48].

In light of the evidence cited above, it appears that MSC treatment of brain trauma is capable of targeting multiple mechanisms that contribute to functional deficits following TBI. By interacting with the injury environment and the inflammatory response, MSCs are able to provide both neuroprotection (modulation of inflammation, reduction of cell death) and regeneration (secretion of neurotrophic and growth factors,

stimulation of endogenous neuritogenesis), leading to improvements in functional neurological outcomes.

1.4. ALGINATE ENCAPSULATION OF MESENCHYMAL STROMAL CELLS

The ability to provide neuroprotection and promote regeneration after brain trauma make MSCs a promising therapeutic for TBI, however, there has been limited success of clinical translation using direct implantation. Several studies have reported low efficiency of engraftment/localization at the injury site, and a decrease in cell number at the site over time [49,50]. Additionally, several studies have reported that a percentage of intravenously administered MSCs have been detected in the liver, spleen, kidney, lungs, and other tissues, even up to one year after treatment [51,52]. The ability of MSCs to transdifferentiate contributes another potentially adverse outcome to MSC implantation.

In order to control long-term effects, localization, and differentiation, we have encapsulated MSCs within alginate microspheres. Alginate is an FDA approved polymer derived from kelp, and has already been proven to sustain cell viability and function, as well as localization in brain tissue up to 6 months [53]. Encapsulation of MSCs will allow for sustained therapeutic benefit, immobilization of MSCs at the injury site, and protection of cells from exposure to the cytotoxic injury environment. Furthermore, studies have demonstrated that direct cell contact with the injury is not necessary for therapeutic benefit, so there is no concern that the alginate with act as a barrier to cell-cell interactions [54]. We have developed an alginate encapsulation method, and

demonstrated sustained MSC viability and secretion profile within the capsule. The encapsulated MSCs are more responsive to pro-inflammatory stimuli than free MSC, based on secretion of a panel of regulatory cytokines and growth factors. Additionally, we have demonstrated *in vitro* and *in vivo* that encapsulated MSCs can attenuate trauma-induced macrophage-mediated inflammation [55].

The collective evidence reviewed here strongly supports the need to develop a biomaterial platform for delivery of MSCs in order to achieve sustained therapeutic benefit and cell survival; and to evaluate the resultant effects on neuroprotection and regeneration following secondary injury after TBI. Additionally, FDA approval of alginate and current clinical trials evaluating MSC therapy [51], indicate that successful completion of the proposed studies is liable to lead to clinical and translational research endeavors that could improve current treatment and functional outcome of TBI.

1.5. ORGANOTYPIC SLICE CULTURE AS AN INJURY MODEL AND SCREENING TOOL

Dissociated cell culture models of brain injury can provide useful insight into cell-specific responses to stressors and potential treatments, as well as information on mechanisms of action. However, it is known that homogeneous cultures respond differently to experimental injury than cells in co-culture (i.e. neuron-astroglia co-culture), including differences in oxidative metabolism [56], free radical-mediated injury [57,58], and excitotoxic cell death [59,60]. In addition, isolated cell culture models fail to recapitulate the host tissue architecture, which plays an important role in the response to brain trauma, affecting physiologically relevant parameters such as cell morphology

and viability, matrix remodeling, and cytokine production. Animal models of TBI offer the ability to study brain trauma in a system of higher complexity and relevance to human injury, but such studies are costly in resources and time.

Organotypic brain slice cultures have emerged as a "bridge" between dissociated cell culture and animal models of brain injury. Slice cultures offer a distinct advantage over isolated cell culture models in that they retain the cellular heterogeneity and interactions, synaptic function, and cytoarchitecture of the host tissue; but at a reduced cost and higher throughput as compared to animal models [61,62]. We have established an organotypic hippocampal slice culture model, which has been previously used to evaluate neuroprotective compounds. For the purpose of this thesis, we have used organotypic slice culture models to evaluate the ability of encapsulated MSCs to modulate neuroinflammation and attenuate ischemic cell death.

1.6. DISSERTION OVERVIEW AND APPROACH

The broad, long-term objective of this research is to administer alginate-encapsulated MSCs to improve treatment following brain injury. In addition to having a cytoprotective role, MSCs secrete a number of cytokines and growth factors in a pattern that is modulated by the local microenvironment, suggesting that MSCs can be dynamically regulated and used for therapeutic intervention. As such, we believe that encapsulated MSCs have the multi-potent ability to protect against progressive damage caused by multiple secondary injury mechanisms following TBI. Additionally, alginate encapsulation of MSCs allows for control of the capsule microenvironment and MSC behavior, while physically isolating the MSCs from the host tissue. The work presented

herein focuses on MSC modulation of the neuroinflammatory component of the secondary injury cascade, and the ability of encapsulated MSCs to provide neuroprotection following ischemic injury.

In Chapter 2, we evaluated encapsulated MSC treatment in an organotypic hippocampal slice culture (OHSC) model of inflammation, induced by lipopolysaccharide (LPS) administration. We found that encapsulated MSCs markedly reduce levels of the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α) produced by OHSC in response to LPS, more effectively than monolayer MSCs. Our studies confirm that MSC-produced prostaglandin E_2 (PGE_2) is a key inflammatory mediator and further demonstrate that whereas inflammatory signals are required to induce monolayer MSC PGE_2 production, alginate encapsulation of MSCs alone is enough to stimulate PGE_2 production. Similar alginate encapsulation-stimulated changes were also observed across a multiplex panel analyzing 27 MSC-produced cytokines and growth factors, from which additional mediators with strong correlations to TNF-α levels were identified. These results indicate that alginate encapsulation enhances MSC therapeutic benefit for experimental inflammation and induces MSC secretome changes that may be responsible for the improved anti-inflammatory effects of encapsulated MSC treatment.

Given the results in our OHSC model of inflammation, in Chapter 3 we sought to determine the specific cellular target(s) of MSC modulation of the inflammatory response, and to further elucidate the mechanism by which PGE_2 mediates this effect. Using astrocyte, microglia, and neuron cultures to model inflammation *in vitro*, we found astrocytes to be the primary target of our encapsulated MSC treatment. We demonstrated

that MSCs markedly reduced levels of the TNF-α produced by astrocytes in response to LPS, and encapsulated MSCs did so more effectively than monolayer MSCs at higher dose. We again confirmed constitutive production of PGE_2 by encapsulated MSCs regardless of the amount of inflammatory stimuli, and showed that monolayer MSCs only increase production of PGE_2 as the level of TNF-α increases. Because PGE_2 has been described as a pleiotropic molecule, including playing a dual role in the inflammatory response, we then determined EP receptor-specific binding of exogenous PGE_2, as well as the receptor(s) through which MSC-produced PGE_2 is acting to modulation inflammation. Based on these findings, we proposed that MSC-produced PGE_2 might have roles in addition to modulation of the astrocyte inflammatory response. Indeed, a PCR array panel of astrocyte expression revealed that encapsulated MSC and PGE_2 treatments induced an up-regulation of several powerful neurotrophic factors.

In Chapter 4, we evaluate the neuroprotective effects of MSC treatment using *in vitro* models of ischemia, induced by oxygen-glucose deprivation (OGD). Our previous work showing that encapsulated MSC treatment prevented tissue degradation of OHSC cultured on fibronectin [63], along with reports that PGE_2 induces production of neurotrophic factors [64-66], led us to speculate that the elevated levels of PGE_2 produced by encapsulated MSCs, as reported in Chapters 2 and 3, may stimulate astrocytes to produce these molecules. This evidence, as well as reports of neurotrophic factor production by MSCs themselves [67,68], suggests the possibility that encapsulated MSC treatment could provide enhanced neuroprotective benefit, possibly by direct neurotrophin production, or by stimulating host neurotrophin production. Indeed, using an OHSC model, we demonstrated that encapsulated MSC treatment prevented cell

death, while monolayer MSC treatment did not. Additionally, encapsulated MSC treatment reduced neurite retraction in OGD-injured cerebellar granule neuron cultures. Finally, using astrocyte cultures subject to OGD, we found that encapsulated MSC treatment reduced markers of astrocyte activation. These results demonstrate the neuroprotective effects of encapsulated MSC treatment, either directly on neurons or through inhibiting astrocyte activity that leads to neuronal cell death.

Please note, because several chapters of this dissertation have been published, or are being prepared for publication, there may be redundancies in the background, motivation, and methods sections.

CHAPTER 2. ALGINATE MICRO-ENCAPSULATION OF MESENCHYMAL STROMAL CELLS ENHANCES MODULATION OF THE NEURO-INFLAMMATORY RESPONSE

Note: This chapter is reproduced from the following publication:

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2.1. INTRODUCTION

Neuro-inflammation is a major component of the secondary injury cascade after brain trauma, contributing significantly to tissue damage and the propagation of degenerative mechanisms [1,69] over a period of days to months after the initial trauma. Mesenchymal stromal cells (MSCs) have been reported as a promising therapeutic for such injury through the use of both *in vitro* and *in vivo* models of stroke [70] and traumatic brain injury [40,71]. Rather than serve as a direct cell replacement, in this therapy, MSCs are proposed to act as trophic mediators [30]—responding to several secondary injury mechanisms, including inflammation [47,72,73].

Despite promising evidence for the use of MSCs as a therapeutic for central nervous system (CNS) trauma, there has been varied success with the use of direct implantation of the cells for prolonged treatment of the secondary injury cascade because of diminished localization and survival at the injury site [49,50] as well as migration to other tissues [51,52]. To control long-term effects and localization, we have previously developed and characterized a method to encapsulate MSCs within alginate microspheres

[74]. These encapsulated MSCs, but not free MSCs, significantly increased the number of anti-inflammatory macrophages in a spinal cord injury model [55] and prevented tissue degradation of organotypic hippocampal slice cultures (OHSC) [63]. However, the mechanism by which encapsulated MSCs alleviate CNS inflammation and pathology has not yet been identified. In this study, we used a lipopolysaccharide (LPS)-treated OHSC model to determine whether encapsulated MSCs, compared with monolayer MSCs, could modulate the neuro-inflammatory response. OHSC provides an *in vitro* model with a heterogeneous CNS cell population that maintains cellular interactions and tissue architecture; it is less complex, more tightly controlled and has higher throughput than *in vivo* CNS injury models. With the use of OHSC and transwell co-culture treatment, we studied MSC treatment effects on direct CNS cellular targets and identified MSC-secreted trophic mediators responsible for therapeutic benefit.

We demonstrate that encapsulated MSCs markedly reduce levels of the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α) produced by OHSC in response to LPS and are more effective than monolayer MSCs. Our studies corroborate previous findings that prostaglandin E_2 (PGE_2) is a key inflammatory mediator produced by MSCs [75,76] and further demonstrate that whereas inflammatory signals are required to induce monolayer MSC PGE_2 production, alginate encapsulation of MSCs alone is enough to stimulate PGE_2 production. Similar alginate encapsulation-stimulated changes were also observed across a multiplex panel analyzing 27 MSC-produced cytokines and growth factors, from which additional mediators with strong correlations to TNF-α levels were identified. Together, these results indicate that alginate encapsulation enhances MSC therapeutic benefit for experimental inflammation and induces MSC secretome

changes that may be responsible for the improved anti-inflammatory effects of encapsulated MSC treatment.

2.2. METHODS

2.2.1. Organotypic hippocampal slice culture

All animal procedures were approved by the Rutgers University Institutional Animal Care and Use Committee (Piscataway, NJ, USA), and we carefully adhered to the animal welfare guidelines set out in the Guide for the Care and Use of Laboratory Animals, US Department of Health and Human Services, Publication No. 85-23, 1985. Outbred Sprague-Dawley dams with litters (10 pups/dam) were received and housed together, and approximately two to four rat pups were used per experiment. OHSC were prepared according to established methods [77]. Briefly, Sprague-Dawley rat pups (Taconic Biosciences Inc) at postnatal days 8-10 were decapitated; the hippocampus was rapidly dissected, sliced into 400-μm sections with the use of a McIllwain tissue chopper (Vibratome) and immersed in ice-cold Gey's balanced salt solution (Sigma Aldrich) supplemented with 4.5 mg/mL glucose (Sigma-Aldrich). Slices were separated and plated onto Millicell culture inserts (12 mm, hydrophilic Polytetrafluoroethylene, 0.4 μm, EMD Millipore), one slice per insert, and maintained at 37^{o}C in 5% CO_2 for 14 days. Maintenance medium consisted of 25% heat-inactivated horse serum (Life Technologies), 25% Hank's balanced salt solution (HBSS) (Sigma-Aldrich) and 50% minimum essential medium (MEM) with added Earle's salts (Sigma- Aldrich), supplemented with 1 mmol/L glutamine (Sigma-Aldrich) and 4.5 mg/mL glucose (Sigma- Aldrich). Medium was changed every 3 to 4 days.

2.2.2. Human MSC culture

Human bone marrow MSCs from a single donor (male, 28 years) were purchased from Texas A&M at passage 1 and cultured as previously described [78]. Briefly, MSCs were cultured in MEM-α medium without ribo- and deoxyribo-nucleosides (Life Technologies), supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals), 1 ng/mL basic fibroblast growth factor (Peprotech), 100 units/mL penicillin and 100 mg/mL streptomycin (Life Technologies). Cells were plated at 5000 cells per cm^2 and allowed to proliferate to 70% confluence (approximately 4 to 5 days) before passaging. Only MSCs at passages 2 through 5 were used to initiate subsequent experiments. Monolayer cultures of MSCs, used as controls in all experiments, were seeded 1 day before use in well plates at 2.5×10^4, 5×10^4 or 1×10^5 cells/well. All cultures were incubated at 37^oC in 5% CO_2.

2.2.3. Alginate micro-encapsulation

Alginate poly-L-lysine micro-encapsulation of MSCs was performed as previously described [74]. A 2.2% (wt/vol) alginate solution (molecular weight [MW]: 100,000-200,000 g/mol, G-content: 65% to 70%, Sigma-Aldrich) was generated with Ca^{2+}-free Dulbecco's modified Eagle's medium (DMEM) (Life Technologies). Cultured MSCs were dissociated and re-suspended in 2.2% alginate to yield a final solution of 4×10^6 cells/mL in 2% (wt/vol) alginate (resulting in approximately 150 cells/capsule), which has been previously determined to maintain MSC viability and an undifferentiated state [55]. The cell solution was transferred to a syringe pump (KD Scientific) set at a flow rate of 10 mL/h. Alginate beads were generated with the use of an electrostatic bead

generator (Nisco), at an applied voltage of 6.4 kV. The resulting bead diameter was 500 ± 50 mm. The beads were extruded into a bath of $CaCl_2$ (100 mmol/L) (Sigma-Aldrich) containing 145 mmol/L NaCl (Sigma-Aldrich) and 10 mmol/L 3-(N-morpholino)propanesulfonic acid (MOPS) (Sigma-Aldrich). Micro-encapsulated cells were washed once with phosphate-buffered saline (PBS) (Sigma-Aldrich) and then were treated for 2 min with poly-L-lysine (Sigma-Aldrich, MW: 68,600 g/mol) (0.05% wt/vol), followed by an additional PBS wash. The micro-encapsulated cells were resuspended in 5 mL of MEM-a (Life Technologies) and transferred to a 25-cm^2 tissue culture flask, maintained in an upright position. Encapsulated cells were incubated at 37^oC in 5% CO_2 and used for experiments 1 day after encapsulation. To determine the average number of cells per capsule for dosing purposes, 15 mL of capsules was added to 200 mL of 1% ethylene diamine tetra-acetic acid (EDTA). Capsules were immediately counted in this volume (n = 3), and the average number of capsules/mL was calculated accordingly. The capsule+EDTA solutions were incubated at room temperature for 5 min to allow lysis of the alginate and release of MSC from capsules. A 10-mL volume of these cell suspensions was counted on a hemacytometer to determine average number of cells/mL (n = 3). The average number of cells/capsule was calculated as (cells/mL)/(capsules/mL) and used to determine the number of capsules necessary for experimental treatment. On the basis of the number of capsules necessary to achieve the desired MSC dose, an equivalent number of capsules was chosen for empty-capsule controls.

2.2.4. LPS injury and co-culture

Organotypic slices cultured on membrane inserts were added to 24-well plates containing monolayer or encapsulated MSCs, and maintenance medium was exchanged for serum-free medium (75% MEM with added Earle's salts, 25% HBSS, 1 mmol/L glutamine and 4.5 mg/mL glucose). Slice cultures were randomly placed into treatment and control groups, with each group comprising cultures prepared from at least two different animals. For MSC treatment, cultures were stimulated with 1 μg/mL LPS (Escherichia coli 055:B5, Sigma-Aldrich) [79,80] and immediately co-cultured with monolayer or encapsulated MSCs at 2.5 x 10^4, 5 x 10^4 or 1 x 10^5 cells/well. Non-stimulated and stimulated host cultures without MSC co-culture were used as controls. Cultures were returned to incubators at 37°C in 5% CO_2 for 24 h, after which media supernatants were collected.

2.2.5. PGE_2 treatment

Organotypic slices cultured on membrane inserts were added to 24-well plates containing serum-free medium supplemented with 1 μg/mL LPS ± human PGE_2 (Cayman Chemical) at 2, 4, 6, 8, 10 or 12 ng/mL. Cultures were returned to incubators at 37°C in 5% CO_2 for 24 h, after which media supernatants were collected.

2.2.6. Cytokine measurement

At the end of each treatment, cell culture media supernatants were collected and stored at -20°C. Media supernatants were assayed for TNF-α produced by the organotypic slice culture through the use of a rat-specific TNF-α enzyme-linked

immunosorbent assay (ELISA) (Biolegend) according to the manufacturer's instructions. Total PGE_2 secretion (rat + human) was evaluated through the use of PGE_2 enzyme immunoassay (EIA) (Cayman Chemical), and secretion by MSCs was evaluated with the use of a Bioplex multiplex bead analysis (Bio-Rad Inc) for 27 human-specific growth factors and cytokines, both according to the manufacturer's instructions.

2.2.7. Hierarchical cluster analysis

Bioplex secretome data were normalized to the monolayer MSC condition and analyzed by use of an unsupervised agglomerative clustering algorithm in Matlab (MathWorks).

2.2.8. Statistical analysis

All results are expressed as a mean ± standard error (SE). All data presented, with the exception of Bioplex data, are averaged from ≥3 separate experiments, each with n = 2-3 individual slice cultures per condition (≥6-9 total cultures per condition). Bioplex data are averaged from one experiment, with n = 3 individual slice cultures per condition, assayed in duplicate. KaleidaGraph (Synergy Software) was used for statistical evaluation. Data obtained from individual samples for each condition were pooled, and comparisons between different conditions were performed with the use of one-way analysis of variance followed by post hoc Tukey honestly significant differences test, with statistical significance determined at $P \leq 0.05$. For correlation analyses, data from each sample set were standardized to a normal distribution by calculating the z-score for each sample:

$$z = (x - \mu)/\sigma$$

where x is the sample, μ is the mean and σ is the standard deviation. Pearson's correlation of coefficient, r, was calculated from standard scores and considered significant for values of $P \leq 0.05$.

2.3. RESULTS

2.3.1. Treatment with MSCs inhibits production of pro-inflammatory TNF-α in LPS-stimulated OHSC

The bacterial endotoxin LPS is known to induce experimental inflammation through activation of the immune response and stimulation of cytokine production and has been commonly used to model the neuro-inflammatory component of secondary CNS injury both *in vitro* [81,82] and *in vivo* [83,84]. To evaluate the ability of MSC treatment to mitigate the inflammatory response, we stimulated OHSC with 1 μg/mL LPS and concurrently treated with monolayer or encapsulated MSCs. After 24 h, cell culture media was assayed for TNF-α, a pro-inflammatory cytokine known to be rapidly elevated after brain injury in animal models [85] and in the clinic [86]. With the use of ELISA specific for rat TNF-α, we were able to measure OHSC-produced TNF-α.

In untreated OHSC, LPS caused a significant increase in TNF-α production (13.21 ± 1.44 ng/mL) by the OHSC, and, for the purpose of analysis, we set this condition as maximum TNF-α production (100%). All other conditions are expressed as a relative percentage of this maximum value. Both monolayer and encapsulated MSCs reduced TNF-α production in a dose-dependent manner, which was significant for encapsulated MSCs at all doses (2.5×10^4, 5×10^4, 1×10^5 cells/well) but was only

significant for monolayer MSCs at 5 x 10^4 and 1 x 10^5 cells/well (**Figure 2.1**). Additionally, encapsulated MSCs at all doses had a significantly greater effect on reducing TNF-α as compared with an equivalent dose of monolayer MSCs. Empty-capsule treatment had no significant effect on TNF-α reduction.

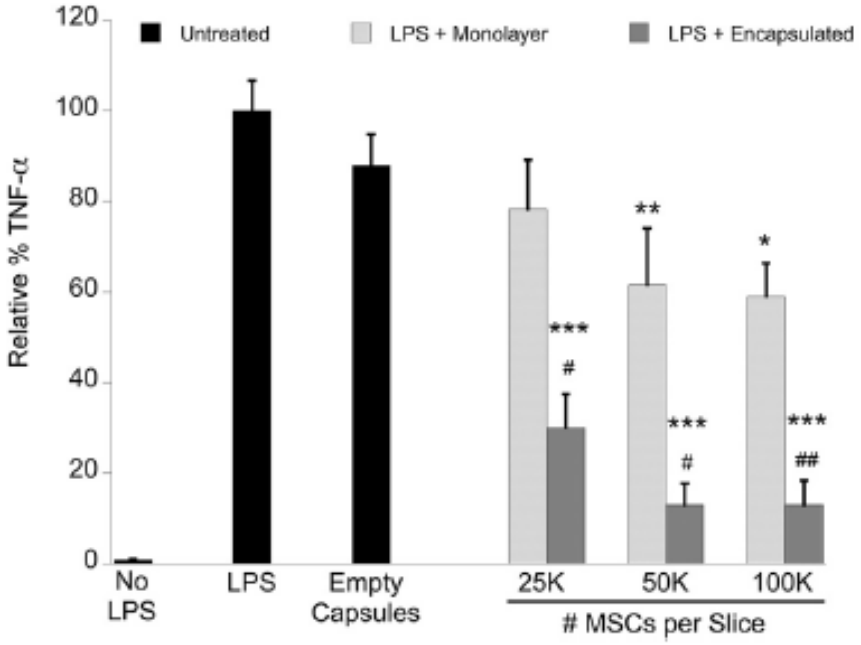

Figure 2.1. Rat TNF-α ELISA of cell culture media supernatant collected after 24 hours of LPS stimulation ± MSC treatment in OHSC. Data is normalized to untreated LPS-stimulated OHSC and represented as mean ± S.E. from 5 experiments, each with N=2-3 cultures per condition. Encapsulated MSC treatment significantly reduced TNF-α levels in a dose dependent manner that was more effective than monolayer MSC treatment. Empty capsule treatment had no significant effect on TNF-α reduction. *=p<0.02, **=p<0.002, ***=p<0.0001 compared to LPS + no treatment, #=p<0.01, ##=p<0.002 compared to treatment with equivalent # of free MSC.

2.3.2. Encapsulated MSCs increase total PGE_2 concentration when co-cultured with LPS-stimulated OHSC

PGE_2 is a potent inflammatory mediator and has been reported to participate in MSC-mediated modulation of the inflammatory and immune responses [14]. We

evaluated total (rat + human) PGE_2 concentration in OHSC media after 24 h with/without stimulation with 1 μg/mL LPS and treatment with monolayer or encapsulated MSC (1 x 10^5 cells/well) (**Figure 2.2**). Both monolayer and encapsulated MSCs increased total PGE_2 production in LPS-stimulated cultures, either through MSC secretion in response to inflammatory factors or by stimulating endogenous PGE_2 production by OHSC. Although the presence of LPS was necessary to induce PGE_2 production in the monolayer MSC condition, encapsulated MSCs produced PGE_2 regardless of LPS stimulation, suggesting that the alginate capsule micro-environment may regulate MSC PGE_2 secretion.

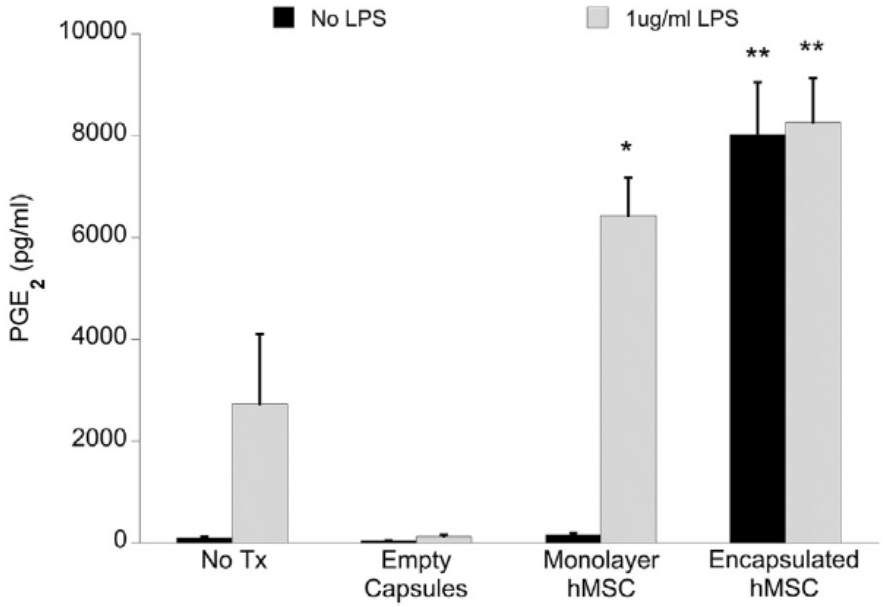

Figure 2.2. Total PGE_2 concentration in cell culture media supernatant collected after 24 hours of culture ± MSC treatment ($1x10^5$ MSCs/well, with or without LPS), as measured by EIA. Data is represented as mean ± S.E. from 3 experiments, each with N=2-3 cultures per condition. Encapsulated MSC conditions resulted in a significant increase of total PGE_2 concentration as compared to untreated LPS-stimulated OHSC, regardless of LPS presence. Only monolayer MSC treatment with LPS stimulation resulted in a significant increase of PGE_2 as compared to untreated LPS-stimulated OHSC. Monolayer MSC treatment without LPS stimulation, as well as treatment with empty capsules, produced negligible amounts of PGE_2. *=p<0.05, **=p<0.0005 compared to OHSC + LPS + no MSC treatment.

2.3.3. Increasing PGE_2 concentration is responsible for TNF-α reduction in OHSC

Given our findings that MSCs (i) reduce TNF-α production and (ii) increase PGE_2 levels in LPS-stimulated slice cultures, we used paired data to determine if a correlation exists between levels of total PGE_2 and rat TNF-α. Data for MSC treatment conditions were first standardized using *z*-score scaling, and Pearson's coefficient of correlation (for linear correlation) was determined from standard scores. We found a strong, significant negative correlation (r =-0.7614, P = 0.0008) between levels of total PGE_2 and rat TNF-α (**Figure 2.3**). Additionally, we observed that treatment conditions cluster together: encapsulated MSC treatment clusters at high PGE_2/ low TNF-α; and monolayer MSC treatment clusters at lower levels of PGE_2 and higher TNF-α.

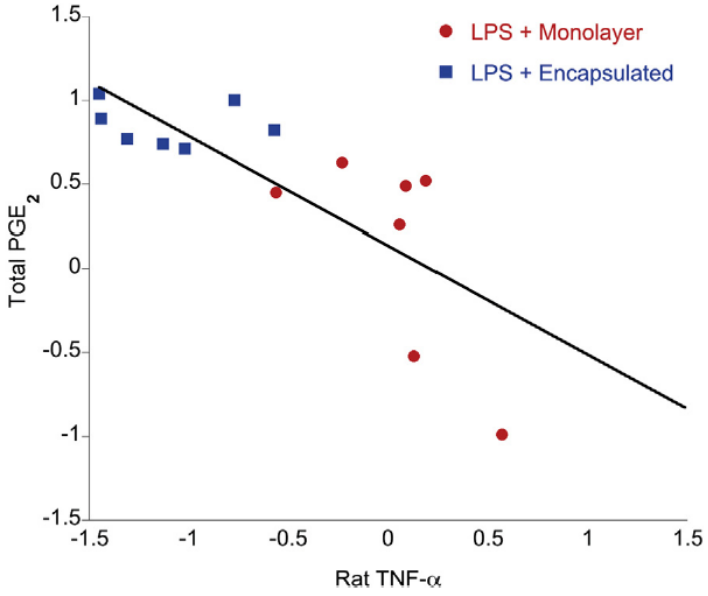

Figure 2.3. Correlation between total PGE_2 and rat TNF-α measured in OHSC culture supernatants for MSC treatment conditions ($1x10^5$ MSCs/well). Linear regression and Pearson's coefficient (r) were derived from z-scores of standardized data. There is a significant (p=0.0008) negative correlation – increasing PGE_2 correlates with decreasing TNF-α – and a clear grouping of treatment conditions: encapsulated MSC treatment clusters at high levels of PGE_2 and low levels of TNF-α, and monolayer MSC treatment clusters at low levels of PGE_2 and higher TNF-α.

To determine if PGE_2 is a direct mediator of TNF-α reduction in our culture model, we added exogenous human PGE_2 to LPS-stimulated slice cultures and evaluated culture media for rat TNF-α secretion after 24 h. There is a clear dose-response effect of increasing human PGE_2 on reducing TNF-α produced by OHSC (**Figure 2.4A**). We then compared the effects of exogenous versus MSC-produced PGE_2 on TNF-α reduction. As seen in **Figure 2.2**, monolayer and encapsulated MSCs produced 6.43 ± 0.74 ng/mL and 8.30 ± 0.87 ng/mL PGE_2, respectively. On the basis of a polynomial curve fit to the exogenous PGE_2 data in **Figure 2.4A**, at these levels of PGE_2 we would expect TNF-α reductions to 32% and 20% of maximum, respectively, if PGE_2 was the primary mediator responsible for TNF-α modulation. Encapsulated MSCs achieved a lower level of TNF-α relative to maximum (13% ± 5.3%) than suggested by this model, but monolayer MSCs did not (59% ± 7.4%) (**Figure 2.4B, Table 2.1**).

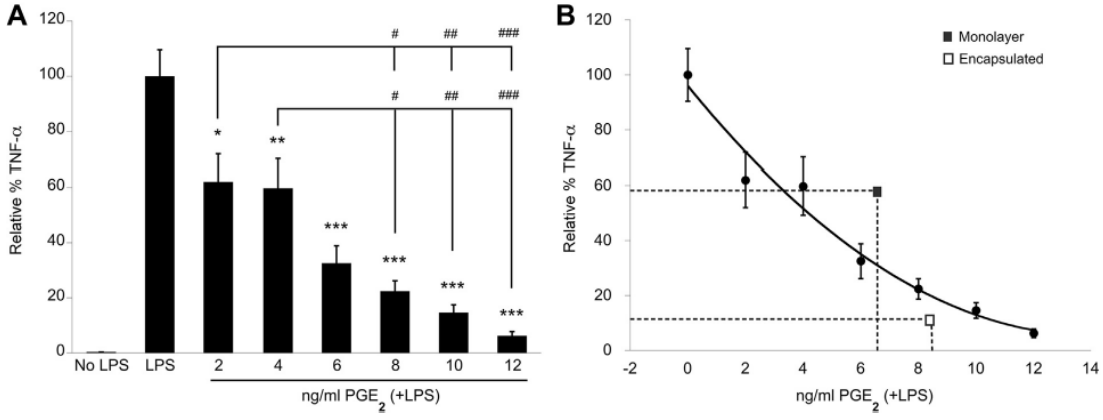

Figure 2.4. (A) Rat TNF-α ELISA of cell culture media collected from OHSC 24 hours after LPS stimulation ± human PGE_2. Data is normalized to untreated LPS-stimulated OHSC and represented as mean ± S.E. from 3 experiments, each with N=3 cultures per condition. Addition of exogenous human PGE_2 significantly reduced TNF-α levels in a dose dependent manner. *=p<0.01, **=p<0.005, ***=p<0.0001 compared to LPS + no treatment. #=p<0.01, ##=p<0.001, ###=p<0.0001 between dose groups. (B) Polynomial curve fit of data presented in **Figure 2.4A**, overlaid with corresponding mean levels of PGE_2 production and TNF-α reduction by monolayer and encapsulated MSCs.

Table I. Experimental versus PGE_2-estimated TNF-α reduction by hMSCs.

	Monolayer	Encapsulated
PGE_2 (ng/mL)	6.43 ± 0.74	8.30 ± 0.87
TNF-α (% maximum, experimental)	59 ± 7.4	13 ± 5.3
TNF-α (% maximum, estimated)	32	20

Table 2.1. Experimental vs. estimated values of rat TNF-α (% of maximum production) after treatment with monolayer or encapsulated MSCs ($1x10^5$ cells/well). Estimated TNF-α values were calculated from a polynomial curve fit of TNF-α levels following exogenous PGE_2 administration (**Figure 2.4B**), using the concentration of PGE_2 detected in media collected from LPS-stimulated OHSC treated with MSCs (**Figure 2.2**).

These deviations suggest that inflammatory mediation is regulated differently by encapsulated MSCs compared with monolayer MSCs and may be explained by differences in encapsulated versus monolayer MSC secretion of other factors that enhance or limit the effect of MSC-produced PGE_2.

2.3.4. Alginate-encapsulated MSCs exhibit secretome changes

To further understand the mechanisms by which encapsulated MSCs differentially modulate the inflammatory response, we used a multiplex assay to screen for 27 human growth factors and cytokines that may be differentially regulated in monolayer versus encapsulated MSCs. Using cell culture media from monolayer and encapsulated MSCs (1 x 10^5 cells/well ± 1μg/mL LPS ± OHSC), we first examined secretion by encapsulated MSCs compared with monolayer MSC secretion, for conditions in which MSCs were co-cultured with LPS-stimulated OHSC. With the use of heat-map representation of the 17 analytes detectable by multiplex assay, encapsulated MSC secretion was normalized relative to monolayer MSC secretion, and we identified distinct panels of cytokines either up-regulated or down-regulated by encapsulated MSCs (**Figure 2.5A**). We then

compared quantitative levels of secretion and determined that 10 of the 17 analytes detected in the multiplex assay exhibited significantly different levels of secretion by encapsulated MSCs as compared with monolayer MSCs, after co-culture with LPS-stimulated OHSC (**Figure 2.5B**).

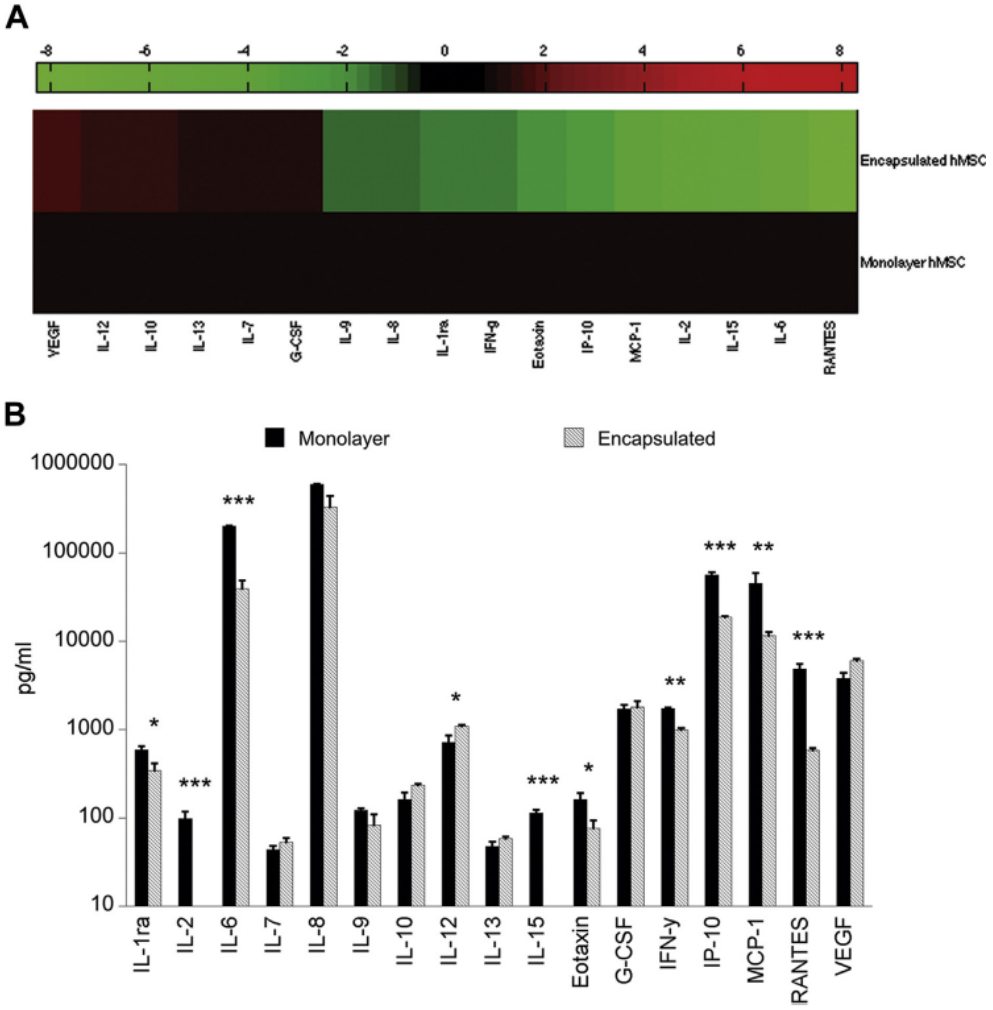

Figure 2.5. (A) Heat map representation of multiplex (human) secretome data. Secretion by encapsulated MSCs ($1x10^5$ cells/well) co-cultured with LPS-stimulated OHSC is normalized to secretion by monolayer MSCs ($1x10^5$ cells/well) co-cultured with LPS-stimulated OHSC. Increased levels of secretion are represented in shades of red, and decreased levels in shades of green. (B) Multiplex analysis of cell culture media collected after 24 hours of MSC co-culture with LPS-stimulated hippocampal slices. Data is represented as mean ± S.E. from 1 experiment, with N=3 cultures per condition. Of 17 detectable analytes, 10 were identified as exhibiting significantly different levels of secretion by encapsulated MSCs as compared to monolayer MSCs. *=$p<0.05$, **=$p<0.005$, ***=$p<0.0001$.

From this subset of 10 analytes, we sought to identify potential candidates responsible for the improved benefit of encapsulated MSCs by using paired data to correlate levels of MSC-secreted factors to rat TNF-α. Data for MSC treatment conditions was first standardized through *z*-score scaling, and Pearson's coefficient of correlation (for linear correlation) was determined from standard scores. We found a strong, significant correlation for several MSC-secreted mediators (**Figure 2.6**). These correlations, along with the correlation for PGE_2, are summarized in **Table 2.2** in order of decreasing correlation. Nine of the 11 analytes demonstrate a significant correlation with TNF-α.

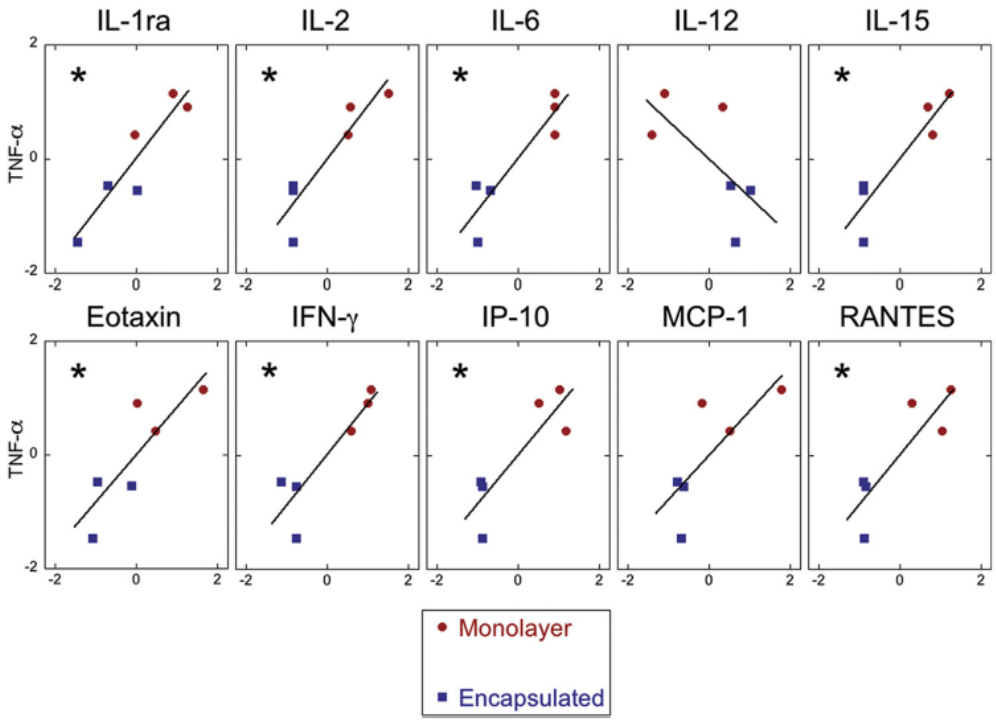

Figure 2.6. Correlation between rat TNF-α and MSC-secreted factors measured by multiplex bead assay, for monolayer and encapsulated MSC treatment conditions. Only analytes that exhibited significant differences between treatment groups (monolayer vs. encapsulated MSC) are depicted. Linear regression and Pearson's coefficient (*r*) were derived from z-scores of standardized data. Coefficients of correlation (*r*) and significance for each analyte can be found in **Table 2.2**. *=$p<0.005$.

Table II. Correlations between levels of hMSC-secreted factors and OHSC-produced TNF-α detected in culture media.

Analyte	*r* (Pearson)	*P* value	TNF-α ↓
PGE_2[a]	−0.7614	0.0008	↑
IL-1ra	0.9230	0.0087	↓
IL-15	0.9170	0.0100	↓
IL-6	0.9158	0.0103	↓
IL-2	0.9138	0.0108	↓
IFN-g	0.8994	0.0147	↓
RANTES	0.8622	0.0257	↓
IP-10	0.8590	0.0284	↓
Eotaxin	0.8402	0.0363	↓
MCP-1	0.7687	0.0741	↓
IL-12	−0.6570	0.1563	↑

Table 2.2. Correlation between MSC-secreted factors found to be significantly different between treatment groups (monolayer v. encapsulation MSC), and rat TNF-α measured in OHSC culture supernatants. Pearson's coefficient of correlation (*r*) and significance of correlation, calculated from z-scores of standardized data, are listed for each detectable analyte and ranked in order of increasing significance. Of 11 MSC-secreted factors significantly different between treatment groups, 9 demonstrate a significant ($p<0.05$) strong correlation ($r>0.75$) with rat TNF-α. Whether the factor is detected as increased or decreased when TNF-α is reduced, is denoted as '↑' or '↓'. N=14 for PGE_2, N=6 for all others.

2.3.5. Alginate is an effector of MSC secretion

It is clear that alginate encapsulation enhances MSC modulation of the inflammatory response through TNF-α reduction and induces changes in the MSC secretome. To better understand the influence of alginate encapsulation on MSC behavior in isolation, we compared the effects of inflammatory stimuli (LPS and/or co-culture) on the secretion profile of monolayer and encapsulated MSCs. We first evaluated PGE_2 concentration in media collected from MSCs cultured alone (1×10^5 cells/well) after 24 h ± stimulation with 1 μg/mL LPS. We have already reported in **Figure 2.2** that, when co-cultured with OHSC, encapsulated MSCs are stimulated to produce PGE_2 even in the absence of LPS-stimulation. Correspondingly, encapsulated MSCs cultured alone

produced PGE_2 regardless of LPS presence, whereas neither condition induced PGE_2 production by monolayer MSCs (**Figure 2.7**). These data suggest that alginate encapsulation alone is capable of inducing MSC PGE_2 production, regardless of inflammatory stimuli, and that monolayer MSC culture requires the presence of both slice co-culture and LPS to stimulate PGE_2 production.

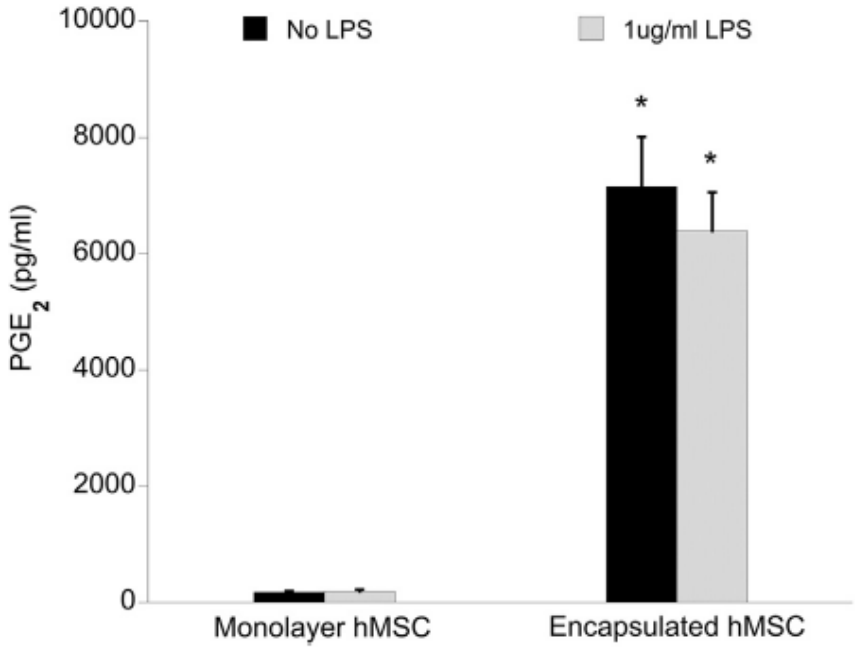

Figure 2.7. Total PGE_2 concentration in MSC cell culture media supernatant ($1x10^5$ cells/well) collected after 24 hours ± 1μg/ml LPS, as measured by EIA. Data is represented as mean ± S.E. from 3 experiments, each with N=2-3 cultures per condition. Encapsulated MSC produced a significantly greater amount PGE_2 compared to monolayer MSC, regardless of LPS presence. *=p<0.0001 compared to monolayer MSC counterpart.

Through the use of hierarchical cluster analysis (HCA) of our multiplex data, we identified similar patterns governing changes in the MSC secretome when examining the effects of (i) OHSC ± LPS or (ii) ± LPS (MSCs cultured alone). In the first instance, we normalized all MSC "treatment" conditions (co-culture with OHSC ± 1 μg/mL LPS) to

"baseline" secretion: monolayer MSC cultured alone (no LPS) (**Figure 2.8A**). The first node in the dendrogram clusters "No LPS + Monolayer" nearest to the "Monolayer Only (Basal)" condition, indicating similar secretion patterns and little-to-no change above baseline secretion. Farthest away from the baseline node is the secretion pattern of the "LPS + Monolayer" condition, demonstrating a larger change relative to baseline secretion. The "No LPS + Encapsulated" and "LPS + Encapsulated" conditions cluster together and branch further away from the baseline condition. These patterns mirror those found in our PGE_2 data: the presence of both LPS and slice co-culture were necessary to stimulate secretome changes by monolayer MSCs, and the presence of LPS had little direct effect on encapsulated MSC secretion.

HCA conducted for secretion by MSCs cultured alone reveals similar trends (**Figure 2.8B**). The addition of LPS to monolayer MSC culture induced very little change above baseline secretion. Alginate encapsulation stimulated greater secretome changes, seemingly regardless of LPS stimulation. These data combined suggest that the presence of alginate and the capsule micro-environment exert dominant effects on MSC secretion.

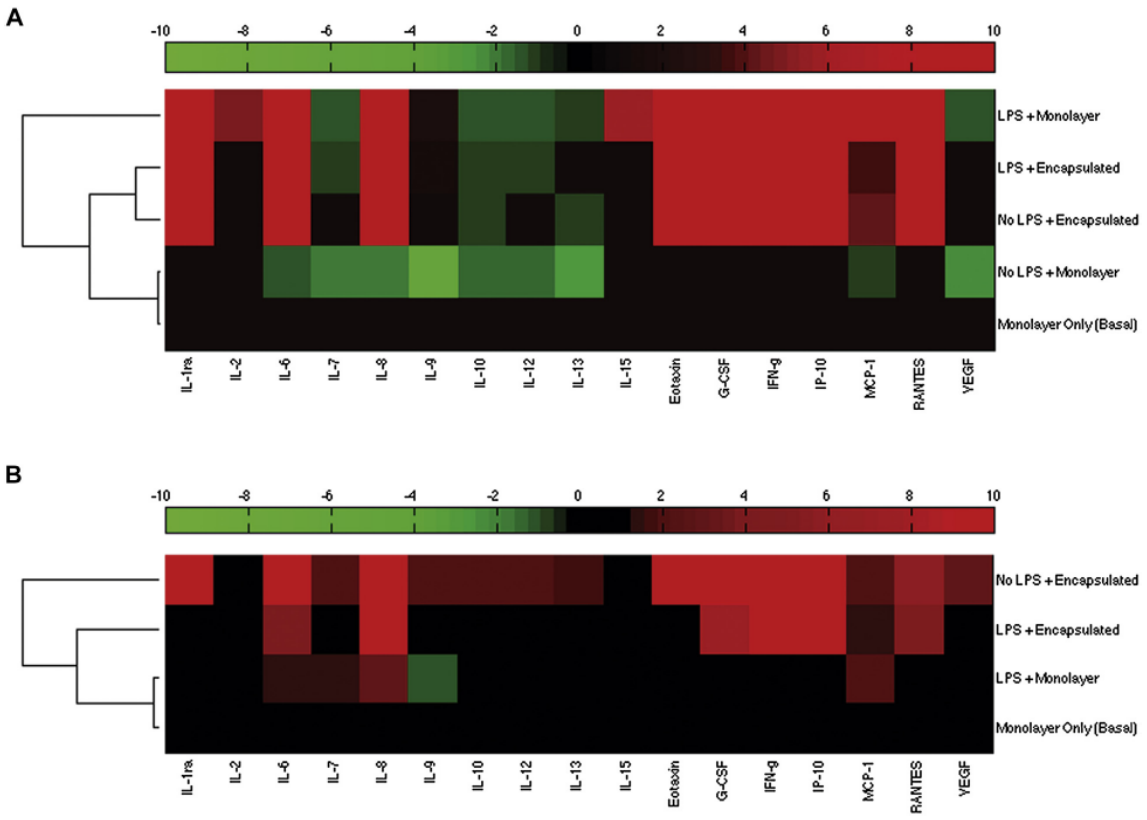

Figure 2.8. Heat map representation of hierarchical cluster analysis on secretome data of (A) MSC treatment conditions (+OHSC, ± LPS) normalized to baseline monolayer MSC only condition (no LPS, no co-culture), and (B) monolayer (+LPS) and encapsulated MSC (± LPS) culture alone normalized to baseline monolayer MSC only condition (no LPS). For all conditions, $1x10^5$ MSCs/well were used. Increased levels of secretion are represented in shades of red, and decreased levels in shades of green.

2.4. DISCUSSION

There is a clear need to develop a delivery platform to facilitate clinical translation of MSC therapeutics. Although MSCs have been shown to provide neuroprotection and promote regeneration after brain trauma [24], several studies have reported low efficiency of engraftment at the injury site and a decrease in cell number at the site over time [49,50]. Additionally, several studies have reported that a percentage of intravenously administered MSCs have been detected in the liver, spleen, kidney, lungs and other tissues even up to 1 year after treatment [52,87]. Our previous efforts have

aimed to use alginate microencapsulation of MSCs to deliver cells after spinal cord injury (SCI). The MSC encapsulation process has been optimized to maintain cell viability up to 2 months, support proliferation up to 3 weeks within the alginate capsule environment [55] and maintain MSCs in an undifferentiated state. With the use of these encapsulated MSCs, we demonstrated, using both *in vitro* macrophage culture and an *in vivo* model of SCI, that encapsulated MSCs promote the anti-inflammatory M2 macrophage phenotype, even in the absence of direct cell contact. Furthermore, encapsulated MSCs co-cultured with LPS-stimulated macrophages reduced levels of pro-inflammatory TNF-α and the activation marker inducible nitric oxide synthase [55].

In this study, we sought to evaluate the ability of encapsulated MSCs to specifically attenuate the neuro-inflammatory component of CNS injury with the use of LPS-stimulated OHSCs, in which astrocytes and glial cells are the primary mediators of the inflammatory response [14,88]. Our results demonstrate that MSCs are capable of attenuating TNF-α produced by OHSC, in a dose-dependent manner. The findings in monolayer MSC treatment are consistent with previous studies [75]. However, our studies demonstrated that encapsulated MSC treatment results in a significantly greater reduction of TNF-α compared with equivalent doses of monolayer MSC treatment.

To determine a mechanism for the improved action of encapsulated MSCs, we evaluated PGE_2 as a potential inflammatory mediator. PGE_2 is a critical component of the early inflammatory response, and, although it has been previously recognized for its pro-inflammatory actions [89,90], recent studies provide evidence that PGE_2 acts as an anti-inflammatory mediator dependent on receptor subtype binding and affinity, as well as local PGE_2 concentration [91]. In experimental models of cerebral ischemia, PGE_2

signaling through the EP_2 receptor was found to have neuro-protective effects [92,93], and induction of PGE_2 synthesis was demonstrated to reduce inflammation in experimental pleuritis [94,95]. Moreover, MSC PGE_2 production has been identified as a primary mediator responsible for the anti-inflammatory and immunomodulatory effects of MSC treatment in several *in vitro* models [75,96,97], and our previous studies evaluating MSC treatment in LPS-stimulated macrophage culture have demonstrated that MSC-secreted PGE_2 facilitates macrophage reprogramming by attenuating the pro-inflammatory M1 phenotype and promoting the anti-inflammatory M2 phenotype [76]. Our results, consistent with such previous reports, demonstrate the role of PGE_2 as an important mediator of LPS-induced inflammation and that increased levels of MSC-secreted PGE_2 are correlated with decreased production of TNF-α by OHSC. Interestingly, although not statistically significant, our observations suggest that encapsulated MSCs may be capable of producing, or stimulating host production of, more PGE_2 in response to inflammatory stimuli than their monolayer MSC counterpart.

Despite the strong evidence indicating a direct effect of PGE_2 on reduced TNF-α production, our data comparing the effects of exogenous versus MSC-produced PGE_2 suggests that other MSC-secreted factors might play a role in enhancing or limiting their therapeutic benefit. As such, we identified several additional MSC-secreted mediators with strong correlations to TNF-α levels. Several pro-inflammatory factors demonstrated strong positive correlations with TNF-α and were produced in higher quantities by monolayer MSCs compared with encapsulated MSCs. The increased production of known inflammatory mediators by monolayer MSCs may explain the limited effect of monolayer MSC-produced PGE_2 on TNF-α reduction. Although administration of the

equivalent amount of exogenous PGE_2 predicts greater inflammatory modulation, monolayer MSC-produced PGE_2 may not be sufficient to overcome the concurrent effects of MSC-produced pro-inflammatory mediators.

Of particular interest is our observation that, although production of inflammatory mediators and/ or changes in secretion patterns by monolayer MSCs are dependent on inflammatory stimuli (co-culture with LPS-stimulated OHSC), encapsulated MSCs exhibit these changes regardless of LPS stimulation or the presence of stimuli produced by OHSC co-culture. These results indicate that the alginate material or the 3D culture environment within the micro-capsule effects changes in the MSC secretome. The data are corroborated by our previously reported findings that the alginate capsule microenvironment enhances MSC secretion patterns as compared with monolayer MSCs, both in the presence and absence of inflammatory cues [55]. Though alginate has been widely used and studied as a biomaterial for immobilization and delivery of cell therapies [98,99] and is generally accepted as a biocompatible material for implantation and long-term efficacy [100-102], some studies have reported activation of host immune and inflammatory responses to alginate [103] that may be dependent on the purity and composition of alginate [104-106]. It is possible that encapsulated MSC secretion is changing in response to such cues, and, consequently, MSCs are becoming primed to modulate the host inflammatory response. Alternatively, alginate-encapsulated MSCs may be responding to the capsule micro-environment. It has been well documented that cells respond to the mechanical properties of the substrate on which they are cultured [107-109]. MSCs encapsulated in a gellan gum hydrogel modified with extracellular matrix-like peptides demonstrated enhanced proliferation and secretion of neurotrophic

factors when compared with MSCs in unmodified capsules [110], and, in cross-linked methacrylated hyaluronic acid hydrogels, MSC secretion of cytokines and angiogenic factors was found to be dependent on hydrogel stiffness [111]. In addition, MSCs encapsulated in alginate hydrogels were reported to up-regulate secreted growth factor expression when subject to compression forces, suggesting the ability of MSCs to modulate gene expression in response to their mechanical environment [112]. Finally, there is recent evidence that spheroid aggregate culture of MSCs enhances anti-inflammatory properties [113,114], suggesting that the 3-D conformation of MSC culture plays a role in improving therapeutic benefit.

In summary, our results demonstrate that alginate encapsulation of MSCs enhances their ability to modulate experimental inflammation, through reduction of the pro-inflammatory cytokine TNF-α. Our results suggest that the enhanced benefit conferred by alginate encapsulation is due to changes in encapsulated MSC secretion patterns relative to monolayer MSC. Alginate encapsulation appears to be an effector of changes in MSC secretion regardless of external stimuli, indicating that the capsule material or environment may induce functional changes in MSCs that enhance their therapeutic properties, perhaps through priming MSCs to elevate beneficial factors. Overall, our results suggest that alginate encapsulation of MSCs may not only provide an improved delivery vehicle for transplantation and extended treatment but may also provide for enhanced MSC therapeutic benefit for CNS trauma. Future studies aim to investigate delivery modes, feasibility and long-term effects *in vivo*.

2.5. ACKNOWLEDGMENTS

We would like to acknowledge Serom Lee, PhD, and Andrea Gray for their assistance and contribution to the statistical analyses, and Mehdi Ghodbane, PhD, for assistance with multiplex assay and analysis. This research was supported by the New Jersey Commission on Brain Injury Research (SNJ-DHSSCBIR- CBIE12IPG019 and 10-3215-BIR-E-0), National Institute of Health Grant P41EB002503, Rutgers-UMDNJ Biotechnology Training Fellowship T32GM00008339e21 and NSF Stem Cell IGERT Fellowship 0801620. These funding agencies were not involved in the conduct of the research or preparation of the article.

CHAPTER 3. PROSTAGLANDIN E_2 PRODUCED BY ALGINATE-ENCAPSULATED MESENCHYMAL STROMAL CELLS MODULATES THE ASTROCYTE INFLAMMATORY RESPONSE AND PROMOTES NEUROTROPHIN EXPRESSION

3.1. INTRODUCTION

Astrocytes and microglia are well known for their role in the secondary injury cascade following traumatic brain injury (TBI). In the uninjured central nervous system (CNS), these cells are responsible for homeostasis, as well as carrying out protective and developmental functions. In response to injury or stimuli, however, astrocytes and microglia take on a "reactive" phenotype. Though this phenotypic switch is initially aimed at neuroprotection and creation of a barrier between the injury and surrounding tissue, chronic cell reactivity propagates further damage, and creates an environment inhibitory to neuron survival and regeneration [14,115]. Neuroinflammation, one of the most damaging chronic injury mechanisms following TBI, is primarily mediated by these reactive astrocytes and microglia, through increased secretion of pro-inflammatory cytokines that propagate further reactivity and activate the inflammatory and immune responses [116,117].

Mesenchymal stromal cells (MSCs) as a therapeutic have been demonstrated as trophic mediators in several models of CNS injury and neuroinflammation, both *in vitro* [44,75] and *in vivo* [55,118], and in particular, to target astroglial-mediated inflammation [47,119]. Despite these promising results, there has been varied success with the use of direct implantation of cells for treatment of chronic and prolonged injury mechanisms,

due to diminished localization and survival at the injury site [49,50] as well as migration to other tissues [51,52]. To control long-term effects and localization, we have previously developed and characterized a method to encapsulate MSCs within alginate microspheres [74], in order to achieve sustained therapeutic benefit by immobilizing MSCs at the injury site and limiting their exposure to the cytotoxic injury environment.

These encapsulated MSCs significantly increased the number of anti-inflammatory macrophages in a spinal cord injury model [55], and modulated the inflammatory response in organotypic hippocampal slice culture (OHSC) [120], more effectively than monolayer MSCs. In the latter study (presented in Chapter 2), PGE_2 was identified as a key mediator of MSC-mediated inflammatory modulation. Here, we have expanded on that particular study, isolating the cellular components of OHSC in order to identify the specific cellular targets of MSC anti-inflammatory benefit. We also further investigated the mechanisms of PGE_2-mediated inflammatory modulation. Additionally, because PGE_2 is a pleiotropic molecule that has also been demonstrated to stimulate neurotrophin production [64-66], we sought to determine if MSC and/or PGE_2 treatment might have neuroprotective, as well as anti-inflammatory, effects.

In this study, we demonstrated that encapsulated MSCs significantly reduced TNF-α produced by lipopolysaccharide (LPS)-stimulated astrocytes, more effectively than monolayer MSCs. However, LPS and MSC treatment had no significant effect on microglia. We further characterized the response of LPS-stimulated astrocytes to MSC treatment and found that the enhanced benefit of encapsulated MSCs begins early and is maintained over time. Additionally, we confirmed our previous finding that encapsulated MSCs constitutively produce high levels of PGE_2, and that monolayer MSCs require the

presence of inflammatory stimuli to induce PGE_2 production. We have also shown that while the early presence of PGE_2 significantly reduces astrocyte-produced TNF-α, delayed administration has no effect. Finally, we determined the receptor subtype binding through which exogenous and MSC-produced PGE_2 are modulating inflammation, and demonstrated the additional role of PGE_2 in stimulating astrocyte neurotrophin production. Taken together, these results support the enhanced benefit of encapsulated MSCs treatment, both in modulating the inflammatory response and providing neuroprotection.

3.2. MATERIALS AND METHODS:

3.2.1. Primary cell culture

All animal procedures were approved by the Rutgers University Institutional Animal Care and Use Committee (Piscataway, NJ). Primary rat cortical astrocyte cultures were prepared according to established methods [121]. Briefly, Sprague-Dawley rat pups (Taconic Biosciences Inc.) at postnatal day 2-3 were decapitated, the brain rapidly removed, and placed in a dish of ice cold Hank's Balanced Salt Solution (HBSS) (Sigma-Aldrich). Cerebral cortices were isolated, cut into small pieces after removal of the meninges, and incubated in GBSS + 0.25% Trypsin-EDTA (Sigma-Aldrich) for 20 minutes in a 37°C water bath. After 20 minutes, the tissue suspension was triturated and Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS (Atlanta Biologicals) was added to stop trypsinization. The cells were pelleted at 1200 rpm for 5 minutes, resuspended in DMEM containing 10% FBS, 100 units/ml penicillin and 100μg/ml

streptomycin (Life Technologies) ("maintenance medium"), and filtered through a cell strainer. The final suspension was cultured in 75-cm^2 flasks (one flask per cortex), and incubated at 37°C in 5% CO_2. For astrocyte culture, cells were passaged at confluency (5-7 day), and used for experiments at passage 1 to 2. For glial cultures, cells were cultured for 7-10 days, with media exchanged every 2-3 days. To isolate microglia, cultures were shaken at 180rpm for 2 hours. The cells in suspension were removed and plated for experiments. Both astrocytes and microglia were plated in 24-well plates ($5x10^4$ cells/well) 2 days prior to experiments.

3.2.2. Human MSC culture

Human bone-marrow mesenchymal stromal cells from a single donor (male, 28 years) were purchased from Texas A&M at passage one and cultured as previously described [78]. Briefly, MSCs were cultured in MEM-α medium without ribo- and deoxyribo-nucleosides (Life Technologies), supplemented with 10% FBS (Atlanta Biologicals), 1ng/ml basic fibroblast growth factor (Peprotech), 100 units/ml penicillin and 100μg/ml streptomycin (Life Technologies). Cells were plated at 5000 cells per cm^2 and allowed to proliferate to 70% confluence (approximately 4 to 5 days) before passaging. Only MSCs at passages 2 through 5 were used to initiate subsequent experiments. Monolayer cultures of MSCs, used as controls in all experiments, were seeded one day prior to use in transwells at $2.5x10^4$, $5x10^4$, or $1x10^5$ cells/well. All cultures were incubated at 37°C in 5% CO_2.

3.2.3. Alginate microencapsulation

Alginate poly-L-lysine microencapsulation of MSCs was performed as previously described [74]. A 2.2% (w/v) alginate solution (MW: 100,000-200,000 g/mol, G-content 65%-70%, Sigma-Aldrich) was generated with Ca^{2+}-free Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies). Cultured MSCs were dissociated and resuspended in 2.2% alginate to yield a final solution of $4x10^6$ cells/ml in 2% (w/v) alginate (resulting in approximately 150 cells/capsule), that has been previously determined to maintain MSC viability and an undifferentiated state [55]. The cell solution was transferred to a syringe pump (KD Scientific), set at a flow rate of 10mL/h. Alginate beads were generated using an electrostatic bead generator (Nisco), with accelerating electrode at an applied voltage of 6.4kV. The resulting bead diameter was 500 ± 50μm. The beads were extruded into a bath of $CaCl_2$ (100mM) (Sigma-Aldrich) containing 145mM NaCl (Sigma-Aldrich) and 10mM MOPS (Sigma-Aldrich). Encapsulated cells were washed once with phosphate buffered saline (PBS) (Sigma-Aldrich) and then treated for 2 minutes with poly-L-lysine (Sigma-Aldrich, MW: 68,600 g/mol) (0.05% w/v), followed by an additional PBS wash. The microencapsulated cells were resuspended in 5ml MEM-α (Life Technologies) and transferred to a $25cm^2$ tissue culture flask, maintained in an upright position. Encapsulated cells were incubated at 37°C in 5% CO_2 and used for experiments one day post-encapsulation.

3.2.4. LPS injury and co-culture

Transwells containing monolayer or encapsulated MSCs ($2.5x10^4$, $5x10^4$, or $1x10^5$ cells/transwell) were added to host cultures in 24-well plates, and maintenance

medium was exchanged for DMEM + 1% FBS, supplemented with 100 units/ml penicillin and 100μg/ml streptomycin ("low serum media") ± 1μg/ml LPS (*Escherichia coli* 055:B5, Sigma-Aldrich) [79,80]. Non-stimulated and stimulated host cultures without MSC co-culture were used as controls. Cultures were returned to incubators at 37°C in 5% CO_2 for 6, 12, 24, or 48 hours, after which media supernatants were collected and cells were fixed.

3.2.5. PGE_2 and blocking studies

Before all experiments, astrocyte medium was exchanged for low serum media ± 1 μg/mL LPS. For exogenous PGE_2 treatment, human PGE_2 (Cayman Chemical) at 1, 2, 4, 8, 16 or 20ng/mL was added immediately, or 6 hours after LPS. For agonist studies, iloprost (EP1, Cayman Chemical), butaprost (EP2, Cayman Chemical), sulprostone (EP3, Cayman Chemical), or CAY10598 (EP4, Cayman Chemical) was added at 10nM, 100nM, 1μM, or 10μM. For antagonist studies, 20ng/mL PGE_2 was added along with SC-51322 (EP1, Cayman Chemical), PF-04418948 (EP2, Cayman Chemical), L-798,106 (EP3, Sigma-Aldrich), or L161,982 (EP4, Cayman Chemical) at 10nM, 100nM, 1μM, or 10μM. For antagonist blocking studies, monolayer or encapsulated MSCs were co-cultured with astrocytes and antagonists were added concurrently at doses determined by antagonist studies (10μM SC-51322, 10μM PF-04418948, 10μM L-798,106, or 1μM L-161,982). All cultures were returned to incubators at 37°C in 5% CO_2, and media supernatants were collected 24 hours post-LPS stimulation.

3.2.6. Cytokine measurement

At the end of each treatment, cell culture media supernatants were collected and stored at -20^{o}C. Media supernatants were assayed for TNF-α produced by astrocytes or microglia using a rat TNF-α ELISA (Biolegend) according to the manufacturer's instructions. Total PGE_2 secretion (rat + human) was evaluated using Prostaglandin E_2 EIA (Cayman Chemical), according to the manufacturer's instructions.

3.2.7. PCR array

For the analysis of astrocyte neurotrophin and neurotrophin receptor expression after LPS, LPS + monolayer MSC, LPS + encapsulated MSC, and LPS + 20ng/ml PGE_2 treatments, experiments were carried out as described above. After 24 hours, medium was collected, cells were washed once with PBS, then dissociated with 0.25% Trypsin-EDTA (Sigma-Aldrich) for 5 minutes, after which trypsinization was neutralized with astrocyte maintenance medium. The cells were harvested and samples pooled per condition, then spun down and resuspended in PBS. Cells were again centrifuged and the PBS supernatants were removed. Pellets were flash frozen on liquid nitrogen, and stored at -80^{o}C. RNA isolation and RT-PCR were performed by Qiagen (Frederick, MD), using manufacturer-specific kits and a rat neurotrophin and neurotrophin receptor array (RT^2 Profiler PCR Array, Cat. # PARN_031Z). Fold change/regulation was calculated using the $\Delta\Delta C_T$ method, in which ΔC_T is calculated between gene of interest (GOI) and an average of reference genes (HKG), followed by $\Delta\Delta C_T$ calculations (ΔC_T (Test Group) - ΔC_T (Control Group)). Fold Change was then calculated using 2^ ($-\Delta\Delta C_T$) formula.

Non-supervised hierarchical cluster analysis of the entire dataset was generated using the Qiagen data analysis web portal (http://www.qiagen.com/geneglobe).

3.2.8. Statistical analysis

All results are expressed as a mean ± standard error (S.E.). All data presented is averaged from ≥ 3 separate experiments, each with N=2-3 independent replicates. PCR array data is obtained from one experiment, with n = 6 cultures per condition, and samples pooled per condition. KaleidaGraph (Synergy Software) was used for statistical evaluation. Comparisons between different conditions were performed using one-way ANOVA followed by post-hoc Tukey-HSD test, with statistical significance determined at $p \leq 0.05$.

3.3. RESULTS

3.3.1. MSCs attenuate production of pro-inflammatory TNF-α in LPS-stimulated astrocytes

The bacterial endotoxin lipopolysaccharide (LPS) is known to induce inflammation through activation of the immune response and stimulation of cytokine production, and has been commonly used to model the neuroinflammatory component of secondary CNS injury both *in vitro* [81,82] and *in vivo* [83,84]. To evaluate the ability of MSC treatment to attenuate the astroglial inflammatory response, we stimulated astrocyte or microglial cultures with 1μg/ml LPS and concurrently treated with monolayer or encapsulated MSCs for 24 hours, after which cell culture media was assayed for the pro-inflammatory cytokine TNF-α produced by the host cultures.

In microglia, LPS did not cause a significant increase in TNF-α production over control cultures, and neither monolayer nor encapsulated MSC treatment resulted in significant changes in TNF-α (**Figure 3.1A**). However, in astrocyte culture, LPS induced a significant increase in TNF-α and both monolayer and encapsulated MSCs significantly reduced TNF-α production at all doses, (**Figure 3.1B**). Additionally, at $1x10^5$ cells/well, encapsulated MSCs had a significantly greater effect on reducing TNF-α as compared to the same dose of monolayer MSCs. Empty capsule treatment had no significant effect on TNF-α reduction in astrocytes.

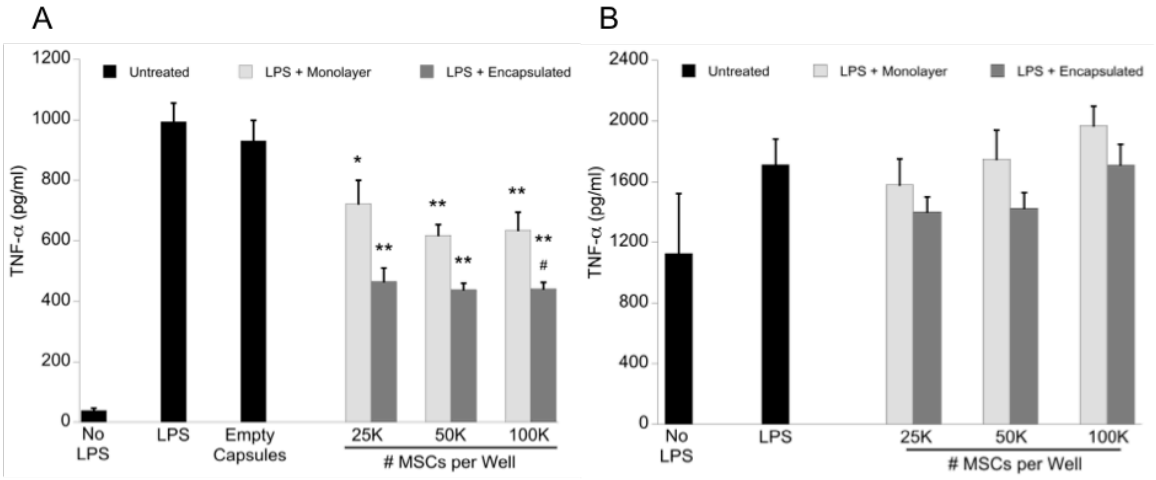

Figure 3.1. Rat TNF-α ELISA of cell culture media supernatant collected after 24 hours of LPS stimulation ± MSC treatment in astrocyte (A) and microglia (B) cultures. Data is represented as mean ± S.E. from 3 experiments, each with N=2-3 cultures per condition. In astrocyte culture, encapsulated MSC treatment significantly reduced TNF-α levels, and was more effective than monolayer MSC treatment at the highest dose evaluated. Empty capsule treatment had no significant effect on TNF-α reduction. MSC treatment had no effect in microglia cultures. *=p<0.02, **=p<0.002, ***=p<0.0001 compared to LPS + no treatment, #=p<0.01, ##=p<0.002 compared to treatment with equivalent # of monolayer MSC.

3.3.2. Encapsulated MSCs are more effective than monolayer in reducing TNF-α, and exhibit increased PGE_2 production

Having identified astrocytes as a target of MSC treatment for neuroinflammation, we then further characterized the treatment response over time. Astrocyte cultures were administered 1μg/ml LPS and treated with monolayer or encapsulated MSCs ($1x10^5$ cells/transwell) and cell culture media was collected at 6, 12, 24, and 48 hours. Rat TNF-α and total PGE_2 were evaluated by ELISA and EIA, respectively. We found that TNF-α production by LPS-stimulated astrocytes reached a maximum at 24 hours post-stimulation, and that after 12 hours, encapsulated MSC treatment performed better than monolayer MSC treatment, though this effect was only significant at the 24-hour time point (**Figure 3.2A**). All data is normalized to untreated, LPS-stimulated astrocytes at 24 hours post-stimulation.

PGE_2 is a critical component of the early inflammatory response, and we have previously identified PGE_2 as a key mediator of MSC-mediated inflammatory modulation in macrophage [55] and organotypic hippocampal slice cultures [120]. Here, we have shown that while both monolayer and encapsulated MSCs produce increased PGE_2 in response to inflammatory stimuli, encapsulated MSCs produce significantly higher levels at all time points, and begin production earlier than monolayer MSCs (6 hours vs. 12 hours post-stimulation) (**Figure 3.2B**).

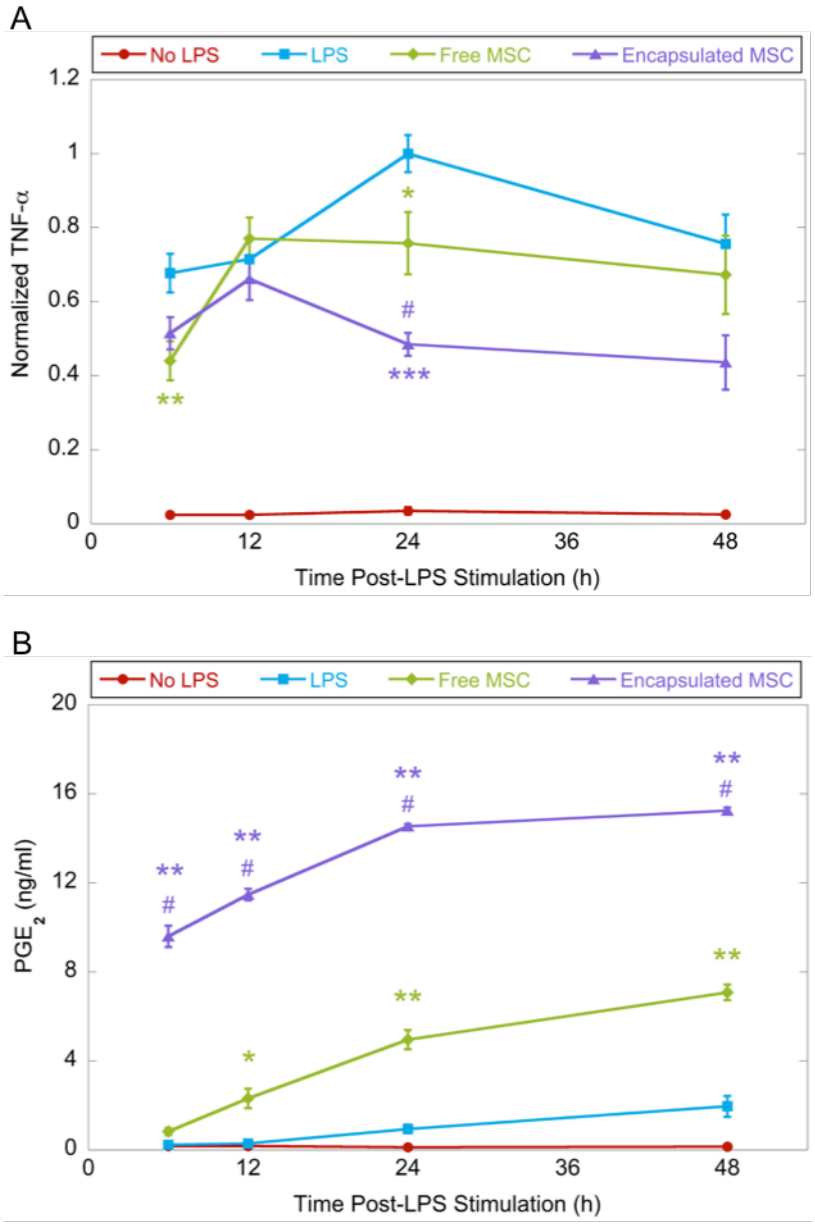

Figure 3.2. Temporal profile of rat TNF-α and total PGE_2 levels in cell culture media collected after LPS stimulation ± MSC treatment in astrocyte cultures. TNF-α data is normalized to untreated LPS-stimulated cultures at 24 hours. All data is represented as mean ± S.E. from 3 experiments, each with N=3 cultures per condition. (A) Encapsulated MSC treatment shows an early trend in reducing TNF-α more effectively than monolayer MSCs, which is maintained to the 48h endpoint. *=$p<0.05$, **=$p<0.01$, ***=$p<0.0001$ compared to LPS only, #=$p<0.05$ compared to LPS + monolayer MSC. (B) High levels of PGE_2 are produced by encapsulated MSCs from 6h post-stimulation, whereas monolayer MSCs start producing PGE_2 at significantly lower levels from 12h post-stimulation. *=$p<0.001$, **=$p<0.0001$ compared to LPS only, #=$p<0.0001$ compared to LPS + monolayer MSC.

3.3.3. Early presence of PGE_2 benefits inflammatory modulation

Given the enhanced anti-inflammatory benefit of encapsulated MSCs, and the high levels of PGE_2 produced by encapsulated MSCs from early time points post-LPS stimulation, as well as previous data correlating increased PGE_2 with decreased TNF-α [120], we sought to determine if the early PGE_2 presence, as seen with encapsulated MSC treatment, benefits inflammatory modulation. To achieve this, we added exogenous human PGE_2 to LPS-stimulated astrocyte cultures at the time of LPS administration or 6 hours after, and evaluated culture media for rat TNF-α secretion 24 hours post-LPS stimulation. There is a clear dose-response effect of increasing human PGE_2 on reducing TNF-α when immediately administered (0h), but no significant reduction of TNF-α by any PGE_2 dose when administered 6 hours post-stimulation (**Figure 3.3**). This data corroborates findings that PGE_2 is a key inflammatory mediator, and supports the enhanced benefit of encapsulated MSC treatment.

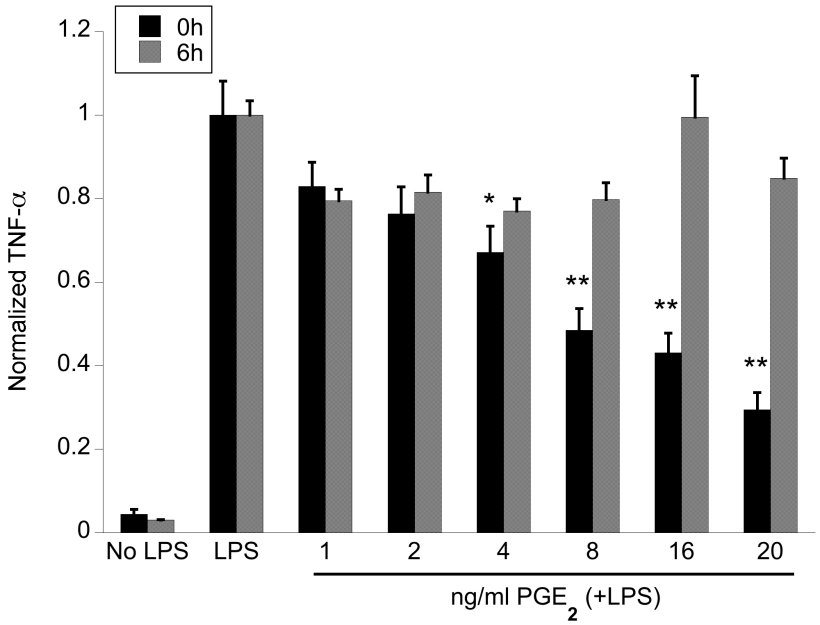

Figure 3.3. Rat TNF-α ELISA of cell culture media collected from astrocyte cultures after 24 hours of LPS stimulation ± human PGE_2. Data is normalized to untreated LPS-stimulated astrocytes and represented as mean ± S.E. from 3 experiments, each with N=3 cultures per condition. Addition of exogenous human PGE_2 significantly reduced TNF-α levels in a dose dependent manner when immediately administered, but had no effect when administered 6h after LPS. *=$p<0.01$, **=$p<0.0001$ compared to LPS only.

3.3.4. PGE_2 reduces TNF-α through specific prostaglandin receptor subtypes

Although PGE_2 has been previously recognized for its pro-inflammatory actions [89,90], recent studies provide evidence that PGE_2 acts as an anti-inflammatory mediator dependent on receptor subtype binding and affinity, as well as local PGE_2 concentration [91]. In order to determine the prostaglandin subtypes involved in reducing astrocyte-produced TNF-α, we first used agonists specific for each of the four receptor subtypes – EP1 (iloprost), EP2 (butaprost), EP3 (sulprostone), and EP4 (CAY10598). Astrocyte cultures were administered 1μg/ml LPS ± receptor agonists, and cell culture media was collected at 24 hours. Using ELISA for rat TNF-α, we found that the EP2 and EP4 receptors are highly involved in reducing TNF-α, and the EP1 receptor to a lesser, but significant, extent (**Figure 3.4A**), though this may be an effect of relative receptor subtype expression by astrocytes. The EP3 receptor is not involved in reducing TNF-α in our culture model. Again, this may due to lack of EP3 expression by astrocytes, which was not evaluated. A range of doses was evaluated, but only the most effective dose (10μM) is represented in the figure.

To confirm these findings, we then evaluated antagonist blocking of PGE_2 inflammatory mediation for each receptor subtype – EP1 (SC-51322), EP2 (PF-04418948), EP3 (L-798,106), and EP4 (L-161,982). Astrocyte cultures were administered 1μg/ml LPS + 20ng/ml PGE_2 ± receptor antagonists for 24 hours, after

which cell culture media was assayed by TNF-α ELISA. Again, we found the EP1, EP2, and EP4 to be significant targets of antagonist blocking (**Figure 3.4B**). In contrast to the agonist study, EP3 appears to be a target of antagonist blocking at the highest dose evaluated, but this could potentially be due to non-specific binding to other receptor subtypes.

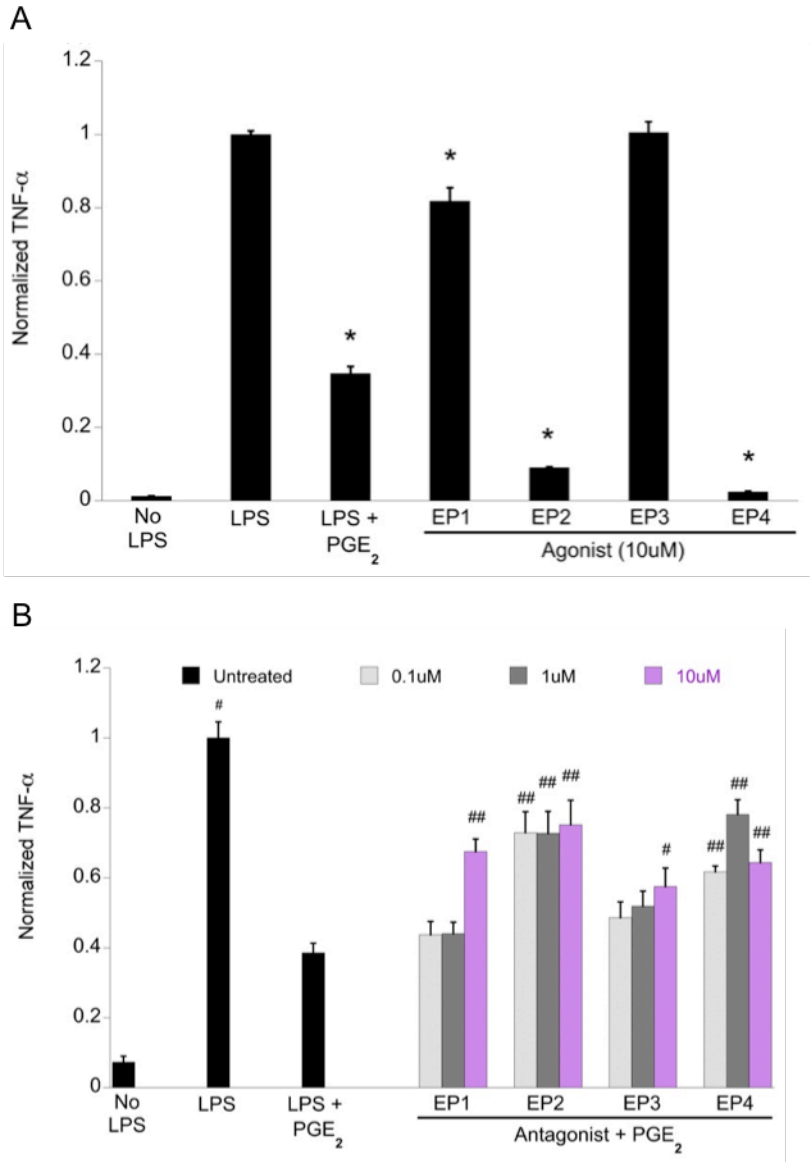

Figure 3.4. Effect of PGE_2 receptor subtype-specific agonists and antagonists on TNF-α reduction. Data is normalized to untreated LPS-stimulated astrocytes and represented as mean ± S.E. from 3 experiments, each with N=3 cultures per condition. (A) Rat TNF-α produced by astrocyte cultures after 24 hours of LPS stimulation ± EP receptor agonist iloprost (EP1), butaprost (EP2), sulprostone (EP3), or CA1058 (EP4). A significant, strong agonist effect is observed for the EP2 and EP4 receptors, and a milder, but significant effect for the EP1 receptor. No effect is seen on the EP3 receptor. *=$p<0.0001$ compared to LPS only. (B) Rat TNF-α produced by astrocytes after 24 hours of LPS stimulation + 20ng/ml PGE_2 ± EP receptor antagonist SC-51322 (EP1), PF-04418948 (EP2), L-798,106 (EP3), or L-161,982 (EP4). Significant antagonist blocking is observed for all EP receptor subtypes. #=$p<0.05$, ##=$p<0.0005$ compared to LPS only.

Having determined effective doses for antagonist blocking of PGE_2-mediated inflammatory modulation, and the receptor subtype targets, we then carried out EP receptor antagonist blocking of MSC treatment, to determine through which receptor subtype(s) MSC-produced PGE_2 is modulation TNF-α production. Astrocyte cultures were administered 1μg/ml LPS and treated with monolayer or encapsulated MSCs ($1x10^5$ cells/transwell) ± receptor antagonists, and cell culture media was collected after 24 hours for evaluation by TNF-α ELISA. Significant blocking of the MSC-mediated TNF-α reduction is achieved with EP1, EP2, and EP4 receptor antagonists, but as with the agonist study, no effect is seen when targeting the EP3 receptor (**Figure 3.5**). Hence, it appears MSC-produced PGE_2 is anti-inflammatory via binding to the EP1, EP2, and EP4 receptors.

3.3.5 Neurotrophin production is stimulated by encapsulated MSCs

PGE_2 is best known for its role in the inflammatory response, but several studies have demonstrated additional downstream effects in stimulating expression or production of neurotrophic factors [64-66], as well as the neuroprotective effects of PGE_2 [92,93,122].

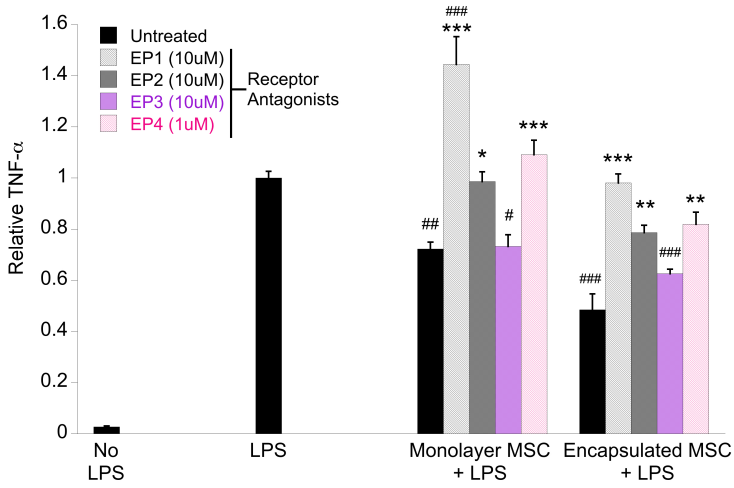

Figure 3.5. PGE_2 receptor antagonist blocking of MSC treatment. Rat TNF-α produced by astrocytes after 24 hours of LPS stimulation + MSC (monolayer or encapsulated) ± EP receptor antagonist SC-51322 (EP1), PF-04418948 (EP2), L-798,106 (EP3), or L-161,982 (EP4). Significant blocking of MSC-mediated TNF-α reduction was observed with antagonists specific for the EP1, EP2, and EP4 receptors. No effect was seen using the EP3 receptor-specific antagonist. *=p<0.05, **=p<0.005, ***=p<0.0005 compared to MSC only counterpart, #=p<0.05, ##=p<0.005, ###=p<0.0001 compared to LPS only.

As we have demonstrated that encapsulated MSCs produce significantly higher levels of PGE_2 (as compared to monolayer MSCs) early after LPS-stimulation in astrocytes, and have previously shown constitutive production of PGE_2 by encapsulated MSCs regardless of inflammatory stimuli [120], we speculated that encapsulated MSCs may not only demonstrate inflammatory modulation, but may also provide neuroprotection by stimulating endogenous neurotrophin production.

Astrocyte cultures were administered 1μg/ml LPS and concurrently treated with monolayer or encapsulated MSCs ($1x10^5$ cells/transwell), or 20ng/ml PGE_2 for 24 hours, after which cells were harvested for RNA isolation and analysis by PCR array for astrocyte expression of 84 neurotrophin, neurotrophin receptor, and neurotrophin-associated genes. Fold changes in expression were calculated for LPS-stimulated astrocytes treated with monolayer MSCs, encapsulated MSCs, or PGE_2, relative to

expression by untreated, LPS-stimulated astrocytes. For 30 genes, a greater than two-fold change in expression was observed for at least one condition (**Figure 3.6, Table 3.1**), and for 6 of these 30 genes, fold changes induced by encapsulated MSC paralleled those observed with PGE_2 treatment.

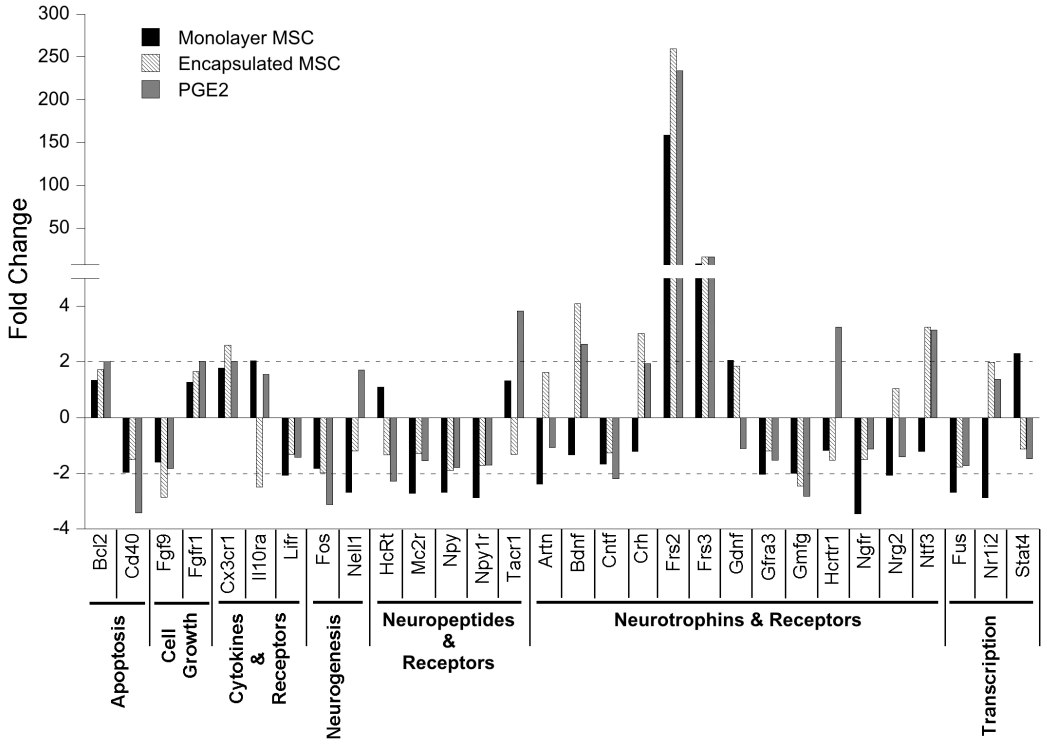

Figure 3.6. Fold changes in astrocyte neurotrophin-associated gene expression after MSC or PGE_2 treatment, for 30 genes (of 84 assayed) that exhibited at least two-fold up- or down-regulation (dashed line) in one or more treatment conditions evaluated, relative to untreated, LPS-stimulated astrocytes.

Additionally, hierarchical cluster analysis on magnitude of expression for all 84 genes shows that the expression patterns induced by encapsulated MSCs and PGE_2 are more similar to each other than to any other condition (**Figure 3.7**) These trends suggest that increased PGE_2 production by encapsulated MSC treatment may confer an enhanced neuroprotective effect over monolayer MSCs, through increased expression of potent neurotrophic factors, including BDNF and NTF3.

Table 3.1. PCR array of astrocyte neurotrophin and neurotrophin receptor expression

Gene Symbol	Gene Name	Accession Number	Fold Change Relative to No Treatment: Monolayer MSC	Encapsulated MSC	PGE2 (20ng/ml)
Apoptosis					
Bax	Bcl2-associated X protein	NM_017059	-1.14	1.18	1.06
Bcl2	B-cell CLL/lymphoma 2	NM_016993	1.34	1.73	**2.01**
Cd40	CD40 molecule, TNF receptor superfamily member 5	NM_134360	-1.97	-1.51	***-3.43***
Fas	Fas (TNF receptor superfamily, member 6)	NM_139194	1.07	1.22	1.04
Hspb1	Heat shock protein 1	NM_031970	1.65	1.34	1.79
Cell Growth & Differentiation					
Fgf2	Fibroblast growth factor 2	NM_019305	-1.03	-1.35	1.11
Fgf9	Fibroblast growth factor 9	NM_012952	-1.6	***-2.85***	-1.83
Fgfr1	Fibroblast growth factor receptor 1	NM_024146	1.27	1.65	**2.03**
Nf1	Neurofibromin 1	NM_012609	-1.19	1.31	1.42
Tgfa	Transforming growth factor alpha	NM_012671	-1.14	-1.25	-1.61
Tgfb1	Transforming growth factor, beta 1	NM_021578	-1.42	1.19	1.32
Tgfb1i1	Transforming growth factor beta 1 induced transcript 1	NM_001191840	1.09	1.37	1.49
Tp53	Tumor protein p53	NM_030989	-1.36	-1.12	-1.01
Cytokines & Receptors					
Cx3cr1	Chemokine (C-X3-C motif) receptor 1	NM_133534	1.77	**2.6**	**2.03**
Il10	Interleukin 10	NM_012854	-1.85	-1.8	1.23
Il10ra	Interleukin 10 receptor, alpha	NM_057193	**2.04**	***-2.5***	1.55
Il1b	Interleukin 1 beta	NM_031512	-1.41	-1.19	-1.17
Il1r1	Interleukin 1 receptor, type I	NM_013123	-1.2	1.55	1.71
Il6	Interleukin 6	NM_012589	-1.28	-1.55	1.35
Il6r	Interleukin 6 receptor	NM_017020	-1.21	1.02	1.09
Il6st	Interleukin 6 signal transducer	NM_001008725	-1.44	-1.02	-1.04
Lif	Leukemia inhibitory factor	NM_022196	-1.69	-1.28	-1.37
Lifr	Leukemia inhibitory factor receptor alpha	NM_031048	***-2.07***	-1.32	-1.42
Neurogenesis					
Cbln1	Cerebellin 1 precursor	NM_001109127	-1.73	-1.88	-1.2
Cxcr4	Chemokine (C-X-C motif) receptor 4	NM_022205	1.36	1.69	1.61
Fos	FBJ osteosarcoma oncogene	NM_022197	-1.83	-1.99	***-3.12***
Nell1	NEL-like 1 (chicken)	NM_031069	***-2.69***	-1.19	1.72
Neuropeptides & Receptors					
Cckar	Cholecystokinin A receptor	NM_012688	-1.21	-1.32	-1.48
Galr1	Galanin receptor 1	NM_012958	-1.21	-1.32	-1.48
Galr2	Galanin receptor 2	NM_019172	-1.52	-1.19	-1.19
Grpr	Gastrin releasing peptide receptor	NM_012706	1.95	-1.32	-1.48
HcRt	Hypocretin	NM_013179	1.1	-1.33	***-2.28***
Mc2r	Melanocortin 2 receptor	NM_001100491	***-2.71***	-1.29	-1.55
Npffr2	Neuropeptide FF receptor 2	NM_023980	-1.21	-1.06	-1.4
Npy	Neuropeptide Y	NM_012614	***-2.69***	-1.89	-1.79
Npy1r	Neuropeptide Y receptor Y1	NM_001113357	***-2.89***	-1.73	-1.71
Npy2r	Neuropeptide Y receptor Y2	NM_023968	-1.21	-1.32	-1.48
Ntsr1	Neurotensin receptor 1	NM_001108967	-1.21	-1.32	1.65
Npy4r	Pancreatic polypeptide receptor 1	NM_031581	-1.21	-1.32	-1.07
Tacr1	Tachykinin receptor 1	NM_012667	1.33	-1.32	**3.84**

Gene Symbol	Gene Name	Accession Number	Fold Change Relative to No Treatment: Monolayer MSC	Encapsulated MSC	PGE2 (20ng/ml)
Neurotrophins & Receptors					
Adcyap1r1	Adenylate cyclase activating polypeptide 1 receptor 1	NM_133511	1	1.79	1.71
Artn	Artemin	NM_053397	***-2.39***	1.62	-1.07
Bdnf	Brain-derived neurotrophic factor	NM_012513	-1.33	**4.08**	**2.64**
Cntf	Ciliary neurotrophic factor	NM_013166	-1.67	-1.27	***-2.19***
Cntfr	Ciliary neurotrophic factor receptor	NM_001003929	-1.88	-1.51	-1.46
Crh	Corticotropin releasing hormone	NM_031019	-1.21	**3.03**	1.93
Crhbp	Corticotropin releasing hormone binding protein	NM_139183	1.25	-1.32	-1.47
Crhr1	Corticotropin releasing hormone receptor 1	NM_030999	-1.33	1.36	1.22
Crhr2	Corticotropin releasing hormone receptor 2	NM_022714	-1.54	-1.16	-1.72
Frs2	Fibroblast growth factor receptor substrate 2	NM_001108097	**158.68**	**259.57**	**233.94**
Frs3	Fibroblast growth factor receptor substrate 3	NM_001017382	**8.22**	**16.11**	**15.89**
Gdnf	Glial cell derived neurotrophic factor	NM_019139	**2.06**	1.85	-1.11
Gfra1	GDNF family receptor alpha 1	NM_012959	1.16	1.09	1.13
Gfra2	GDNF family receptor alpha 2	NM_012750	-1.03	1.69	1.88
Gfra3	GDNF family receptor alpha 3	NM_053398	***-2.03***	-1.19	-1.53
Gmfb	Glia maturation factor, beta	NM_031032	-1.88	-1.33	-1.21
Gmfg	Glia maturation factor, gamma	NM_181091	***-2.01***	***-2.45***	***-2.83***
Hcrtr1	Hypocretin (orexin) receptor 1	NM_013064	-1.18	-1.54	**3.25**
Hcrtr2	Hypocretin (orexin) receptor 2	NM_013074	-1.21	1.1	-1.48
Mt3	Metallothionein 3	NM_053968	-1.15	-1.03	1.25
Ngf	Nerve growth factor (beta polypeptide)	NM_001277055	-1	-1.06	-1.04
Ngfr	Nerve growth factor receptor (TNFR superfamily, member 16)	NM_012610	***-3.46***	-1.51	-1.13
Ngfrap1	Nerve growth factor receptor (TNFRSF16) associated protein 1	NM_053401	-1.23	-1.1	1.09
Nrg1	Neuregulin 1	NM_031588	-1.2	1.51	1.09
Nrg2	Neuregulin 2	NM_001136151	***-2.08***	1.04	-1.4
Ntf3	Neurotrophin 3	NM_031073	-1.21	**3.25**	**3.14**
Ntf4	Neurotrophin 4	NM_013184	-1.21	1.37	1.14
Ntrk1	Neurotrophic tyrosine kinase, receptor, type 1	NM_021589	-1.21	-1.32	-1.48
Ntrk2	Neurotrophic tyrosine kinase, receptor, type 2	NM_012731	-1.14	1.36	1.25
Pspn	Persephin	NM_013014	-1.38	1.09	-1.62
Ptger2	Prostaglandin E receptor 2 (subtype EP2)	NM_031088	-1.54	-1.33	-1.1
Tfg	Trk-fused gene	NM_001012144	-1.34	-1.02	1.01
Ucn	Urocortin	NM_019150	-1.64	1.49	-1.77
Vgf	VGF nerve growth factor inducible	NM_030997	-1.39	-1.08	-1.28
Transcription					
Fus	Fusion (involved in t(12;16) in malignant liposarcoma) (human)	NM_001012137	***-2.69***	-1.77	-1.73
Maged1	Melanoma antigen, family D, 1	NM_053409	-1.58	-1.71	-1.3
Myc	Myelocytomatosis oncogene	NM_012603	-1.27	-1.01	-1.03
Nr1i2	Nuclear receptor subfamily 1, group I, member 2	NM_052980	***-2.87***	1.99	1.37
Stat1	Signal transducer and activator of transcription 1	NM_032612	-1.91	-1.14	-1.36
Stat2	Signal transducer and activator of transcription 2	NM_001011905	-1.82	-1.16	-1.22
Stat3	Signal transducer and activator of transcription 3	NM_012747	-1.22	-1.06	-1.17
Stat4	Signal transducer and activator of transcription 4	NM_001012226	**2.31**	-1.12	-1.48
Zfp110	Zinc finger protein 110	NM_001024775	-1.34	-1.01	-1.6
Zfp91	Zinc finger protein 91	NM_001169120	-1.35	1.04	-1.14

Table 3.1. A list of the neurotrophin and neurotrophin-receptor genes assessed with the Qiagen PCR array. Fold changes are in comparison to the untreated, LPS-stimulated control. More than two-fold up-regulated genes are marked in bold; more than two-fold down-regulated genes are marked in bold italics. Genes that are similarly regulated in encapsulated MSC and PGE_2 conditions are outlined.

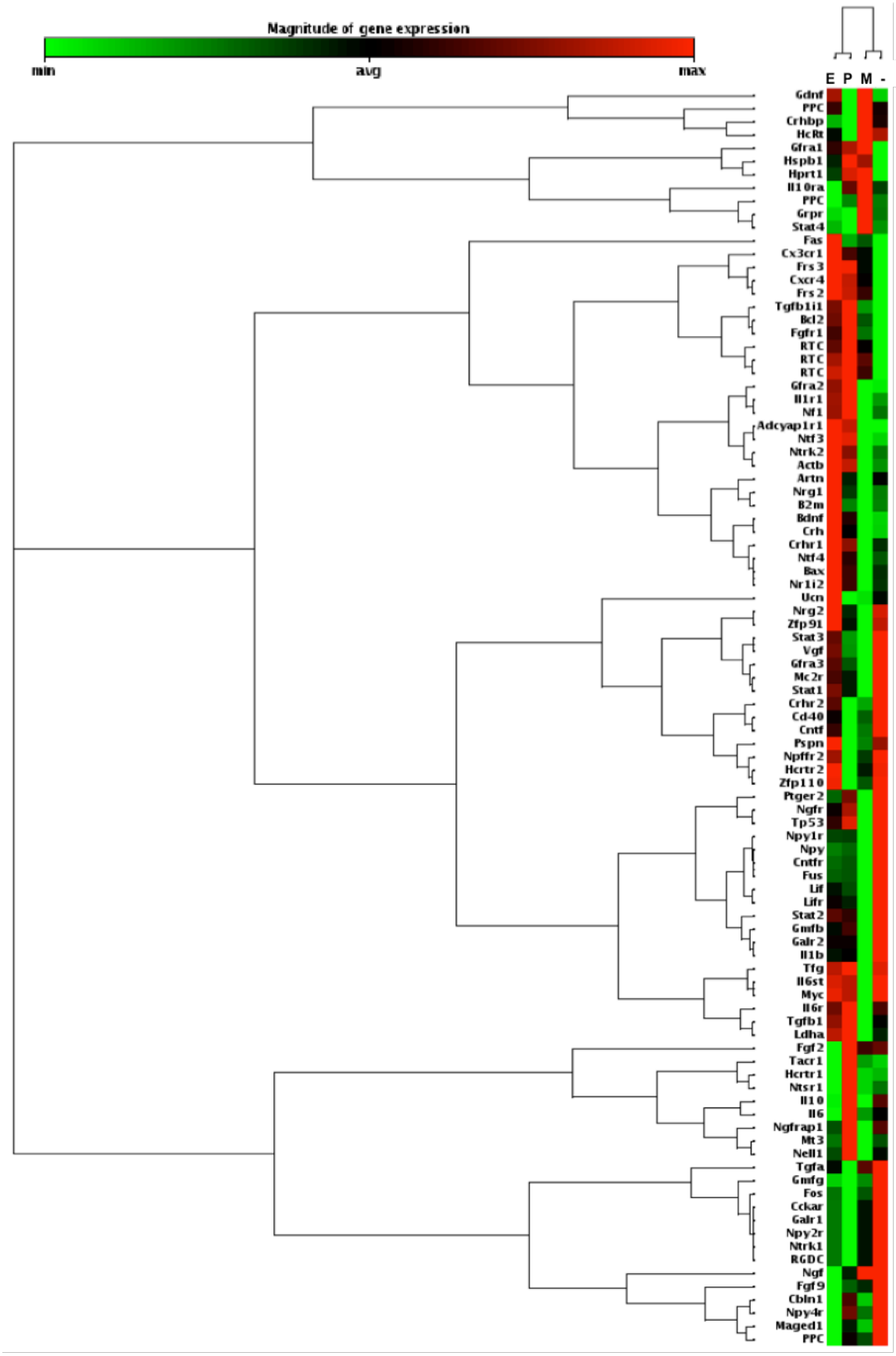
Magnitude of gene expression
min
avg
max
E P M -
Gdnf
PPC
Crhbp
HcRt
Gfra1
Hspb1
Hprt1
Il10ra
PPC
Grpr
Stat4
Fas
Cx3cr1
Frs3
Cxcr4
Frs2
Tgfb1i1
Bcl2
Fgfr1
RTC
RTC
RTC
Gfra2
Il1r1
Nf1
Adcyap1r1
Ntf3
Ntrk2
Actb
Artn
Nrg1
B2m
Bdnf
Crh
Crhr1
Ntf4
Bax
Nr1i2
Ucn
Nrg2
Zfp91
Stat3
Vgf
Gfra3
Mc2r
Stat1
Crhr2
Cd40
Cntf
Pspn
Npffr2
Hcrtr2
Zfp110
Ptger2
Ngfr
Tp53
Npy1r
Npy
Cntfr
Fus
Lif
Lifr
Stat2
Gmfb
Galr2
Il1b
Tfg
Il6st
Myc
Il6r
Tgfb1
Ldha
Fgf2
Tacr1
Hcrtr1
Ntsr1
Il10
Il6
Ngfrap1
Mt3
Nell1
Tgfa
Gmfg
Fos
Cckar
Galr1
Npy2r
Ntrk1
RGDC
Ngf
Fgf9
Cbln1
Npy4r
Maged1
PPC

Figure 3.7. Heat map representation of hierarchical cluster analysis on magnitude of astrocyte gene expression, as induced by LPS stimulation only ('-'), and LPS + monolayer MSCs ('M'), encapsulated MSCs ('E'), or PGE_2 ('P'). For LPS + MSC conditions, $1x10^5$ cells/transwell were used, and for LPS + PGE_2 condition, 20ng/ml human PGE_2 was used. Maximal levels of expression are represented in shades of red, and minimal levels in shades of green.

3.4. DISCUSSION

The traditional "neurocentric" approach to developing therapies for TBI has focused on regenerating neurons and repairing synapses at the injury site. However, it is important to consider all cell types present that contribute to the ongoing cell death, degeneration, and inhibition of regeneration. Astrocytes exhibit distinct responses to brain injury, and are a key player in several components of secondary injury including inflammation [80,123], excitotoxicity [124], and free radical-mediated injury [125,126]. Here, we focus on the role of these cells in mediating the neuroinflammatory component of secondary injury. Rapidly after insult, astrocytes release several pro-inflammatory cytokines – including TNF-α, IL-6, IFN-γ and IL-1β. These cytokines are responsible for signaling infiltration of other inflammatory mediators to the injury site and stimulating production of additional cytokines [23], thus continually amplifying the inflammatory response. This chronic perpetuation of neuroinflammation by astrocytes, as well as their reaction to other TBI-related insults, significantly contributes to the prolonged cascade of injury, and is linked to neuronal cell death and degradation [127,128].

Several studies have demonstrated the therapeutic potential of MSCs to target multiple components of the secondary injury cascade following TBI, including

neuroinflammation [37,45,47] – specifically, through modulation of the tissue and cellular environment [30]. Direct delivery of cells, however, presents limitations to long-term benefit and clinical translation due to lack of persistence at the injury site and a decrease in cell number at the site over time [36,49,129]. Additionally, it has been reported that a percentage of administered MSCs have been detected in the liver, spleen, kidney, lungs and other tissues even up to 1 year after treatment [52,130]. To overcome these limitations, we have immobilized MSCs in alginate microspheres. Our previous studies have used alginate microencapsulation of MSCs to deliver cells after spinal cord injury (SCI). We demonstrated, using both *in vitro* macrophage culture and an *in vivo* model of SCI, that encapsulated MSCs promote the anti-inflammatory M2 macrophage phenotype, even in the absence of direct cell contact. Furthermore, encapsulated MSCs co-cultured with LPS-stimulated macrophages reduced levels of pro-inflammatory TNF-α and the activation marker inducible nitric oxide synthase (iNOS) [55].

In Chapter 2, we further explored the mechanism by which encapsulated MSCs alleviate CNS inflammation and pathology, using an OHSC model of inflammation. We found that encapsulated MSCs conferred enhanced inflammatory modulation, compared to monolayer MSCs, and identified PGE_2 as a primary mediator in attenuating the inflammatory response [120]. Following these results, in this Chapter we aimed to identify and distinguish cell-specific responses to inflammation and MSC therapy – specifically, the role of astroglial cells -- and to further elucidate the mechanism(s) underlying the improved efficacy of encapsulated MSCs. Our results highlight the contribution of astrocytes to the neuroinflammatory component of TBI, and demonstrate that astrocytes, but not microglia, are highly responsive to our encapsulated MSC

treatment. As with OHSC, our findings show that encapsulated MSC treatment results in a significantly greater reduction of TNF-α compared with an equivalent dose of monolayer MSC treatment. This improved reduction of TNF-α commences early after treatment (12 hours) and is maintained to at least 48 hours post-treatment.

Having previously identified PGE_2 as a key MSC-produced inflammatory mediator in macrophage [76] and OHSC [120] culture models, we continued to evaluate and characterize the role of this molecule in contributing to the enhanced benefit of encapsulated MSC treatment. In LPS-stimulated astrocyte culture, we found that encapsulated MSCs constitutively produce higher levels of PGE_2 than monolayer MSCs, and begin doing so at earlier time points. Together with our data demonstrating that early presence of PGE_2 significantly reduces astrocyte-produced TNF-α, while delayed administration has no effect, these results further support the importance of PGE_2 in modulating inflammation and the advantage of encapsulating MSCs for treatment.

Though we have shown it to have a strong anti-inflammatory effect in our culture models, PGE_2 is a highly pleiotropic molecule known to be both pro- [90,131] and anti-inflammatory [95,132], as well as having roles in pain [133-135], cancer [136,137], neuroprotection [92,93,122], and wound repair [138,139], among others [140]. This diversity of functions is largely attributed to the ability of PGE_2 to bind four receptor subtypes –EP1, EP2, EP3, and EP4 [91] – that mediate PGE_2 actions through distinct downstream signaling pathways [141]. In neurological pathology alone, PGE_2 displays signaling versatility dependent on receptor binding, affinity, and expression levels – often with opposing actions [142]. The EP1 and EP3 receptors have been implicated in excitotoxic cell death and exacerbation of injury in models of cerebral ischemia [143-

145], while the EP2 and EP4 receptors have demonstrated neuroprotection against excitotoxic insult [146,147] and cerebral ischemia [92,93,148]. In contrast to the neuroprotective effects in models of excitotoxicity, EP2 elicits an opposing, neurotoxic response in models of neurodegeneration [149,150] and has demonstrated conflicting roles in neuroinflammation. Activation of the EP2 receptor induced neurotoxicity in LPS-stimulated OHSC [151] and microglia-neuron co-cultures [152], but was also shown to reduce IL-1β production [153] and iNOS expression [154] by LPS-stimulated microglia. Signaling through the EP4 receptor attenuated neuroinflammation in LPS-stimulated microglial [155] and macrophage [76] cultures, and deletion of microglial EP4 in a mouse Alzheimer's model increased inflammation and Aβ deposition [156].

Given the multitude of actions PGE_2 exhibits in CNS pathology, we sought to determine which EP receptors subtypes were involved in our observed PGE_2- and MSC-mediated inflammatory modulation. Though astrocytes are known to express all four receptor subtypes [142], their contribution to the astrocyte-induced inflammatory response, and attenuation thereof, remains relatively uncharacterized. Our data reveal anti-inflammatory actions of exogenous and MSC-produced PGE_2 through the EP1, EP2, and EP4 receptors, corroborating previous studies describing EP2 and EP4 as anti-inflammatory in microglial cultures [153,154]. Not surprisingly, PGE_2 binding to EP2 and EP4 is known to activate similar downstream pathways via increased intracellular cAMP. The dichotomous roles of EP2 in the inflammatory response, however, may be due to evidence that EP2-induced cAMP is capable of binding two separate effectors – PKA and Epac – whose signaling pathways mediate different effects [157]. The role of EP1 in neuroinflammation, specifically, has not been thoroughly explored, but EP1

activation has been shown to propagate inflammatory pain [134,158]. To the best of our knowledge, this is the first study to demonstrate a role of the EP1 receptor in modulating astrocyte-mediated inflammation.

Because PGE_2 signaling through the EP2 and EP4 receptors has been shown to provide neuroprotection [93,156] and stimulate neurotrophic factor production [66,159], we surmised that the high levels of PGE_2 produced by our encapsulated MSCs might confer treatment benefits additional to inflammatory modulation. A PCR array panel revealed that both exogenous PGE_2 and encapsulated MSC treatment, but not monolayer MSCs, up-regulated astrocyte expression of the neurotrophic factors BDNF and NT-3. Additionally, cluster analysis of the entire dataset showed expression patterns to be most similar between PGE_2 and encapsulated MSC treatment conditions, suggesting that encapsulated MSC-induced changes in expression may be largely due to increased PGE_2 production. Dissimilarities between these conditions also exist, where encapsulated MSC-induced gene regulation more closely matches that of monolayer MSCs than exogenous PGE_2. The changes previously observed in the MSC secretome in response to OHSC inflammatory signals [120] (presented in Chapter 2), could point to other MSC-produced mediators responsible for astrocyte gene regulation.

In summary, our results further confirm that alginate encapsulation of MSCs enhances their ability to modulate inflammation through reduction of the pro-inflammatory cytokine TNF-α, and identify astrocytes as the primary target of this treatment. We show that the improved anti-inflammatory benefit of encapsulated MSCs may be due to early, constitutive production of high levels of PGE_2, and the necessity of early PGE_2 administration to reduce inflammation. Additionally, we determined EP

receptor subtypes through which exogenous and MSC-produced PGE_2 are acting to modulate inflammation, and demonstrated additional therapeutic benefit of encapsulated MSCs through induction of astrocyte neurotrophin expression. These results suggest that alginate encapsulation may be a novel and effective method to deliver MSCs for TBI treatment, and may provide sustained, multi-potent benefit by modulating inflammation and providing neuroprotection through induction of neurotrophin expression.

3.5. ACKNOWLEDGMENTS

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CHAPTER 4. ALGINATE-ENCAPSULATED MESENCHYMAL STROMAL CELLS ARE NEUROPROTECTIVE IN AN ORGANOTYPIC MODEL OF CEREBRAL ISCHEMIA

4.1. INTRODUCTION

Secondary injury following traumatic brain injury (TBI) is progressive; occurring over days to months after the primary injury is sustained. It is characterized by cell death, neuronal degeneration, increased lesion volume, and formation of an environment that is inhibitory to regeneration. The majority of functional deficits experienced after TBI are a direct result of damage due to secondary injury [4]. Ischemia is one of the initial secondary injury events, initiating a wave of additional insults including glutamatergic excitotoxicity, oxidative stress, free radical production, and chronic inflammation. Clinically, it is one of the most important predictors of morbidity and mortality following TBI. Signs of ischemic trauma are seen in 90% of non-surviving TBI patients and is clinically associated with poor functional recovery in those that do survive injury [1,160,161].

Mesenchymal stromal cells (MSCs) as a therapy have been reported to promote functional recovery in animal models of both cerebral ischemia and TBI3. Intravenous administration of MSCs after TBI in a rat model yielded improvements in functional recovery for up to 3 months after treatment [40], and studies evaluating MSCs in a rat model of transient cerebral ischemia have demonstrated functional recovery and reduced thickness of the ischemic boundary zone, which would provide a more permissive

environment for neurite outgrowth and regeneration. Engraftment at the injury site was detected in these studies as well, but as only a small percentage of injected cells [41-43].

Despite evidence demonstrating the neuroprotective and regenerative benefits of MSC treatment, there are limitations using current delivery methods due to lack of long-term persistence at the injury site. In the studies cited above, there was low efficiency of engraftment and a decrease in cell number at the implantation site over time. Additionally, several studies have reported that a percentage of intravenously administered MSCs have been detected in the liver, spleen, kidney and lungs, and other tissues, even up to one year after treatment [41,51,162]. To control long-term effects and localization, we have encapsulated MSCs within alginate microspheres, in order to achieve sustained therapeutic benefit, immobilize MSCs at the injury site, and protect cells from exposure to the cytotoxic injury environment. Additionally, it has been demonstrated that direct cell contact with the injury is not necessary for therapeutic benefit [54].

Our previous work using organotypic hippocampal slice culture (OHSC) and astrocyte models of inflammation has demonstrated the anti-inflammatory action of encapsulated MSCs. This effect was strongly mediated by increased levels of MSC-produced PGE_2 [120], a molecule that has also been reported to induce production of neurotrophic factors [64-66]. In Chapter 3, we demonstrated that both encapsulated MSCs and PGE_2 up-regulate neurotrophin expression by astrocytes. We have also shown that encapsulated MSC treatment prevented tissue degradation of OHSC cultured on fibronectin [63]. This evidence, along with reports of neurotrophic factor production by MSCs themselves [67,68], suggests that encapsulated MSC treatment could provide

enhanced neuroprotective benefit, possibly by direct neurotrophin production, or by stimulating host neurotrophin production.

In this study, we evaluated the neuroprotective ability of encapsulated MSCs using *in vitro* models of ischemia induced by oxygen-glucose deprivation (OGD). We demonstrate that encapsulated MSC treatment significantly reduced cell death in OHSC after OGD, whereas monolayer MSC did not. In cerebellar granule neuron culture, encapsulated MSC treatment prevented neurite retraction induced by OGD. Additionally, encapsulated MSCs reduced astrocyte production of glial-fibrillary acidic protein (GFAP), and the glial scar component, neurocan, after OGD. These results indicate the enhanced neuroprotective benefit of encapsulated MSC treatment, both through direct targeting of neuronal populations, as well as by modulating astrocyte activity that results in neurodegeneration and creation of an inhibitory environment for neuronal growth (the glial scar).

4.2. METHODS

4.2.1. Organotypic hippocampal slice culture

All animal procedures were approved by the Rutgers University Institutional Animal Care and Use Committee (Piscataway, NJ, USA), and we carefully adhered to the animal welfare guidelines set out in the Guide for the Care and Use of Laboratory Animals, US Department of Health and Human Services, Publication No. 85-23, 1985. Outbred Sprague-Dawley dams with litters (10 pups/dam) were received and housed together, and approximately two to four rat pups were used per experiment. OHSC were prepared according to established methods [77]. Briefly, Sprague-Dawley rat pups

(Taconic Biosciences Inc) at postnatal days 8-10 were decapitated; the hippocampus was rapidly dissected, sliced into 400-μm sections with the use of a McIllwain tissue chopper (Vibratome) and immersed in ice-cold Gey's balanced salt solution (Sigma Aldrich) supplemented with 4.5 mg/mL glucose (Sigma-Aldrich). Slices were separated and plated onto Millicell culture inserts (30 mm, hydrophilic Polytetrafluoroethylene, 0.4 μm, EMD Millipore), 4 slices per insert, and maintained at 37°C in 5% CO_2 for 14 days. Maintenance medium consisted of 25% heat-inactivated horse serum (Life Technologies), 25% Hank's balanced salt solution (HBSS) (Sigma-Aldrich) and 50% minimum essential medium (MEM) with added Earle's salts (Sigma-Aldrich), supplemented with 1 mmol/L glutamine (Sigma-Aldrich) and 4.5 mg/mL glucose (Sigma-Aldrich). Medium was changed every 3 to 4 days.

4.2.2. Primary astrocyte culture

All animal procedures were approved by the Rutgers University Institutional Animal Care and Use Committee (Piscataway, NJ, USA). Primary rat cortical astrocyte cultures were prepared according to established methods [121]. Briefly, Sprague-Dawley rat pups (Taconic Biosciences Inc.) at postnatal day 2-3 were decapitated, the brain rapidly removed, and placed in a dish of ice cold Hank's Balanced Salt Solution (HBSS) (Sigma-Aldrich). Cerebral cortices were isolated, cut into small pieces after removal of the meninges, and incubated in HBSS + 0.25% Trypsin-EDTA (Sigma-Aldrich) for 20 minutes in a 37°C water bath. After 20 minutes, the tissue suspension was triturated and Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS (Atlanta Biologicals) was added to stop trypsinization. The cells were pelleted at 1200 rpm for 5 minutes,

resuspended in DMEM (Sigma-Aldrich) containing 10% FBS (Atlanta Biologicals, Lawrenceville, GA), 100 units/ml penicillin and 100μg/ml streptomycin (Life Technologies), and filtered through a cell strainer. The final suspension was cultured in 75-cm^2 flasks (one flask per cortex), and incubated at 37°C in 5% CO_2. Cells were passaged at confluency (5-7 day), and used for experiments at passage 1 to 2.

4.2.3. Primary cerebellar granule neuron culture

All animal procedures were approved by the Rutgers University Institutional Animal Care and Use Committee (Piscataway, NJ, USA). Primary rat CGN cultures were prepared according to established methods [163]. Briefly, Sprague-Dawley rat pups (Taconic Biosciences Inc.) at postnatal day 8-9 were decapitated, the brain rapidly removed, and immersed in ice-cold Gey's balanced salt solution (GBSS) (Sigma Aldrich) supplemented with 4.5 mg/mL glucose (Sigma-Aldrich). The cerebellum was isolated, cut into small pieces, and incubated in GBSS + 0.25% Trypsin-EDTA (Sigma-Aldrich) in a 37°C water bath. After 15 minutes, the trypsinization reaction was stopped with trypsin inhibitor/DNase (Sigma-Aldrich), and the tissue was triturated. An equal volume of Neurobasal medium (Life Technologies) supplemented with B27 (Life Technologies) and 25mM KCl (Sigma-Aldrich) ('neuron medium') was added to the tissue suspension. The cells were pelleted at 1000rpm for 5 minutes, resuspended in neuron medium, and filtered through a 40μm nylon mesh. The cells were plated in 24-well plates (5 x 10^4 cells/well), and used for experiments 1-2 days after plating.

4.2.4. Human MSC culture

Human bone marrow MSCs from a single donor (male, 28 years) were purchased from Texas A&M at passage 1 and cultured as previously described [78]. Briefly, MSCs were cultured in MEM-α medium without ribo- and deoxyribo-nucleosides (Life Technologies), supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals), 1 ng/mL basic fibroblast growth factor (Peprotech), 100 units/mL penicillin and 100 mg/mL streptomycin (Life Technologies). Cells were plated at 5000 cells per cm^2 and allowed to proliferate to 70% confluence (approximately 4 to 5 days) before passaging. Only MSCs at passages 2 - 5 were used to initiate subsequent experiments. Monolayer cultures of MSCs, used as controls in OHSC experiments, were seeded 1 day before use in well plates at 1 x 10^5 cells/well. All cultures were incubated at 37^{o}C in 5% CO_2.

4.2.5. Alginate micro-encapsulation

Alginate poly-L-lysine micro-encapsulation of MSCs was performed as previously described [74]. A 2.2% (wt/vol) alginate solution (molecular weight [MW]: 100,000-200,000 g/mol, G-content: 65% to 70%, Sigma-Aldrich) was generated with Ca^{2+}-free DMEM (Life Technologies). Cultured MSCs were dissociated and re-suspended in 2.2% alginate to yield a final solution of 4 x 10^6 cells/mL in 2% (wt/vol) alginate (resulting in approximately 150 cells/capsule), which has been previously determined to maintain MSC viability and an undifferentiated state [55]. The cell solution was transferred to a syringe pump (KD Scientific) set at a flow rate of 10 mL/h. Alginate beads were generated with the use of an electrostatic bead generator (Nisco), at an applied voltage of 6.4 kV. The resulting bead diameter was 500 ± 50 mm. The beads

were extruded into a bath of $CaCl_2$ (100 mmol/L) (Sigma-Aldrich) containing 145 mmol/L NaCl (Sigma-Aldrich) and 10 mmol/L 3-(N-morpholino)propanesulfonic acid (MOPS) (Sigma-Aldrich). Micro-encapsulated cells were washed once with phosphate-buffered saline (PBS) (Sigma-Aldrich) and then were treated for 2 min with poly-L-lysine (Sigma-Aldrich, MW: 68,600 g/mol) (0.05% wt/vol), followed by an additional PBS wash. The micro-encapsulated cells were resuspended in 5 mL of MEM-a (Life Technologies) and transferred to a 25-cm^2 tissue culture flask, maintained in an upright position. Encapsulated cells were incubated at 37^oC in 5% CO_2 and used for experiments 1 day after encapsulation. To determine the average number of cells per capsule for dosing purposes, 15 mL of capsules was added to 200 mL of 1% ethylene diamine tetra-acetic acid (EDTA). Capsules were immediately counted in this volume (n = 3), and the average number of capsules/mL was calculated accordingly. The capsule+EDTA solutions were incubated at room temperature for 5 min to allow lysis of the alginate and release of MSC from capsules. A 10-mL volume of these cell suspensions was counted on a hemacytometer to determine average number of cells/mL (n = 3). The average number of cells/capsule was calculated as (cells/mL)/(capsules/mL) and used to determine the number of capsules necessary for experimental treatment. On the basis of the number of capsules necessary to achieve the desired MSC dose, an equivalent number of capsules was chosen for empty-capsule controls.

4.2.6. Oxygen-glucose deprivation (OGD) and MSC co-culture

Prior to OGD, cultures were transferred to serum-free medium (SFM) – DMEM supplemented with 100 units/mL penicillin and 100 mg/mL streptomycin (Life

Technologies) - containing 5μg/ml propidium iodide (PI) (Life Technologies) and allowed to equilibrate for 30 minutes before imaging. Cultures exhibiting PI fluorescence at this stage were excluded from further study. To induce ischemia, cultures were transferred to serum- and glucose- free medium, then sealed into an airtight chamber and gassed with 1% O_2, 5% CO_2, 94% N_2 for 10 minutes before being placed at 37^{o}C for an additional 50 minutes (OHSC), 5 hours (astrocytes), or 80 minutes (CGN). After ischemic exposure, cultures were returned to normoxic SFM + 5μg/ml PI (OHSC), or normoxic SFM (astrocytes, CGN). Cultures selected for treatments were co-cultured with encapsulated or monolayer MSCs (1 x 10^5 cells/well for OHSC; 1 x 10^4, 5 x 10^4, or 1 x 10^5 cells/well for astrocytes and CGN) (**Figure 4.1)**. Treatment with 30μM MK-801 (Sigma-Aldrich) was used as a control. All cultures were returned to the incubator (37^{o}C, 5% CO_2) for 24 hours.

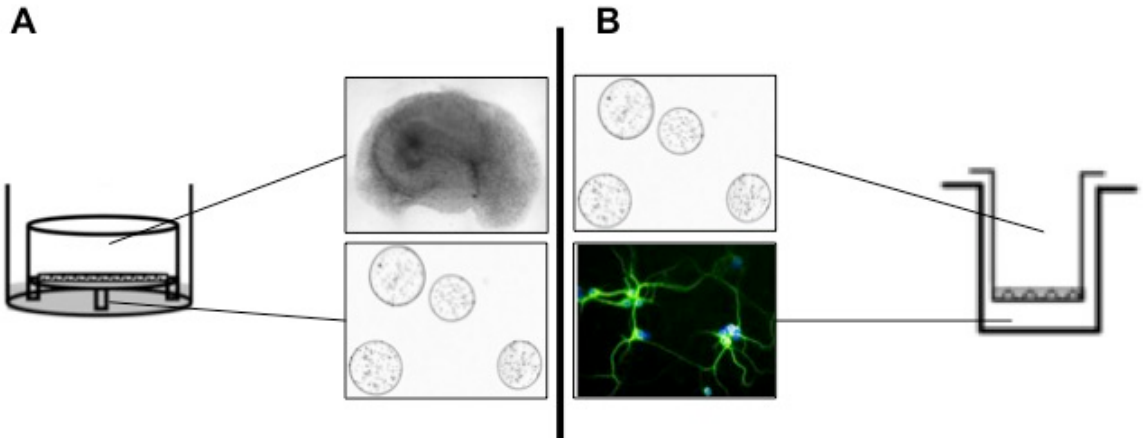


Figure 4.1. MSC co-culture configuration for (A) organotypic hippocampal slice cultures, and (B) astrocyte and cerebellar granule neuron cultures.

4.2.7. Imaging and quantification of slice cultures

Slice cultures were imaged with PI pre-injury and at 24 hours post-injury. Cell death was calculated as the percent area of staining above a threshold in a given

anatomical region, determined from a bright field image of the same slice prior to injury (**Figure 4.2)**. Values were averaged across experiments and expressed as the mean ± standard error (SE). Statistical comparison of treatment groups was performed using ANOVA followed by post-hoc Student-Newman-Keuls test, with significance set at $P \leq 0.05$.

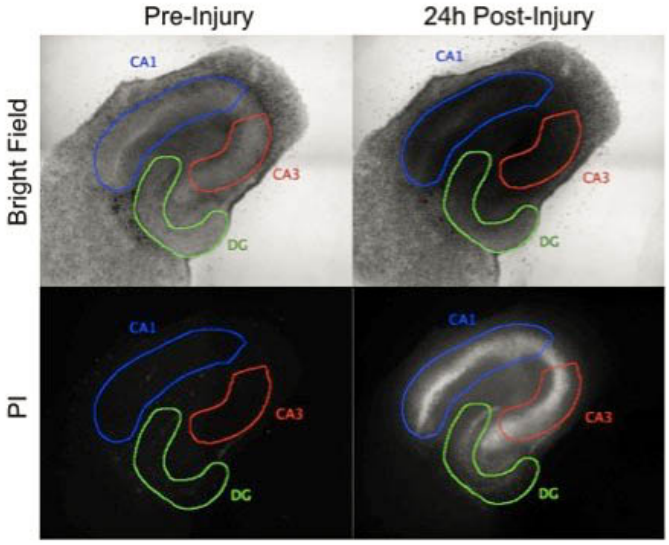


Figure 4.2. Region determination for quantification of cell death in OHSC

4.2.8. Immunocytochemistry

After treatment period, astrocyte and CGN cultures were washed once with PBS, fixed with 4% paraformaldehyde (Sigma-Aldrich) for 30 minutes at room temperature, and then washed 3 times with immunobuffer - PBS supplemented with 1% bovine serum albumin (BSA) (Sigma-Aldrich) and 0.5% Triton-X (Sigma-Aldrich). Cultures were blocked with 10% goat serum (Sigma-Aldrich) for one hour, then incubated in primary antibody solution of mouse α-neurocan (1:50) (EMD Millipore), rabbit α-glial fibrillary acid protein (GFAP) (1:500) (Dako) or rabbit α-microtubule-associated protein 2 (MAP2) (1:500) (EMD Millipore) overnight at 4^{o}C. Cultures were washed with 3 times

with immunobuffer, for 10 minutes each, and then incubated with secondary antibody solution of goat α-rabbit Alexafluor-488 (1:500) (Life Technologies) and/or goat α-mouse Alexafluor-568 (1:500) (Life Technologies), plus 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Life Technologies). Fluorescence was examined and photographed with the use of an Olympus fluorescence microscope. Fluorescence was quantified per cell, dividing the fluorescence area by the number of DAPI-stained nuclei. For each experiment (N=1 for CGN or astrocytes), 3 cultures were used per condition, and 3 images were captured per culture for quantification. Statistical comparison of treatment groups was performed using ANOVA followed by post-hoc Tukey-HSD test, with significance set at $P \leq 0.05$.

4.3. RESULTS

4.3.1. Alginate-encapsulated MSCs inhibit OGD-induced cell death

We evaluated the neuroprotective ability of MSCs with the use of hippocampal slice cultures subject to oxygen-glucose deprivation (OGD). Cultures were transferred to glucose-free media, and subject to OGD for 60 minutes. After completion of OGD, the cultures were returned to normoxia and glucose-containing media and immediately co-cultured with encapsulated or monolayer MSCs (1×10^5 cells/well). After 24 hours, the slice cultures were stained with propidium iodide (PI) to assess for cell death, which was calculated as the percent area of PI staining above a threshold in a given anatomical region (CA3, CA1, DG), determined from a bright field image of the same slice obtained prior to injury.

In slice cultures, oxygen-glucose deprivation for 60 minutes produced significant injury in each hippocampal region. Treatment with encapsulated MSCs significantly reduced cell death in the CA1 region. No significant reduction in cell death was observed in any region of slices treated with monolayer MSCs or the neuro-protectant MK-801 (**Figure 4.3**). Negligible cell death was observed in uninjured slices co-cultured with either monolayer or encapsulated MSC.

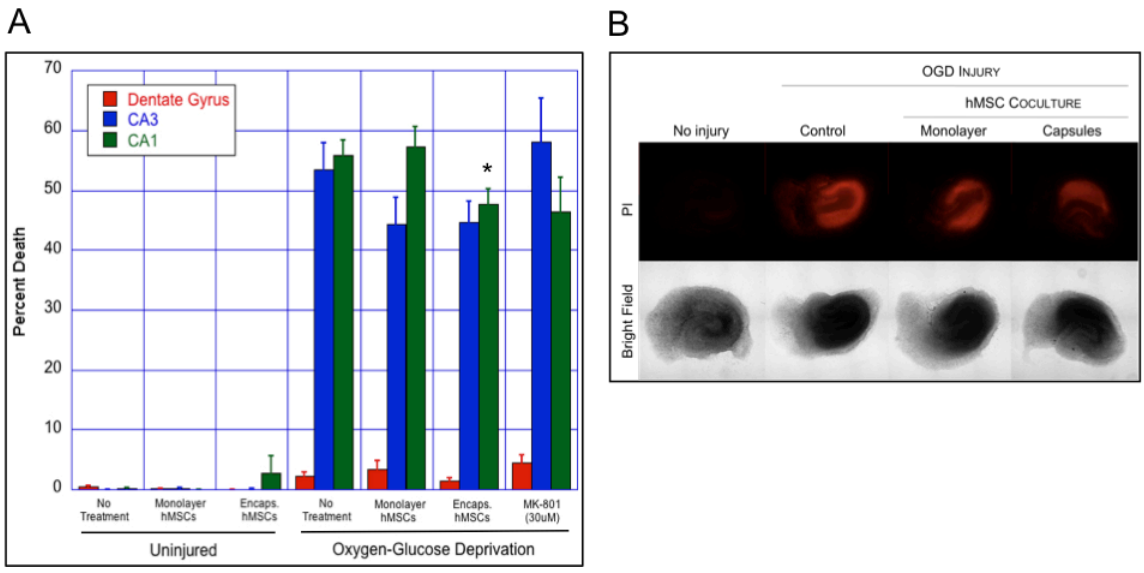


Figure 4.3. Alginate-encapsulated MSCs are neuroprotective against oxygen-glucose deprivation (OGD). Slices were exposed to 60 minutes OGD, immediately co-cultured with MSCs (1 x 10^5 cells/well), and assessed for cell death by propidium iodide (PI) staining 24 hours later. Data is represented as mean ± S.E. from 4 experiments, each with $N \geq 4$ cultures per condition. (A) Encapsulated MSCs significantly reduced cell death in the CA1 region of the hippocampus, exhibiting an enhanced ability for neuroprotection as compared to monolayer MSCs. * = $P \leq 0.05$ compared to OGD only. (B) Representative images of hippocampal slices 24 hours after OGD +/- MSC treatment.

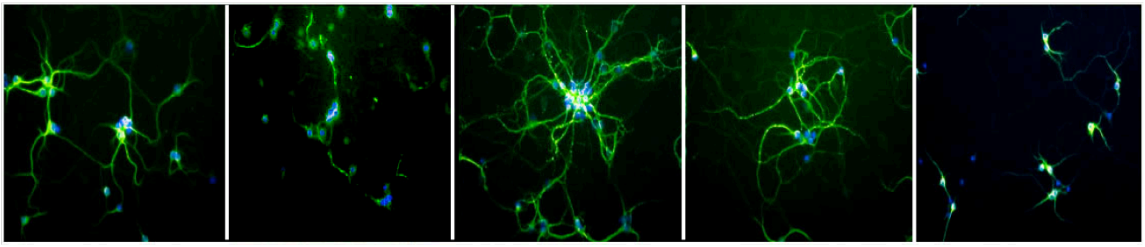
4.3.2. Alginate-encapsulated MSCs prevent neuronal degradation after OGD

Given the neuroprotective benefits of encapsulated MSCs on OGD-injured hippocampal slices, we then investigated the specific cellular targets and possible mechanisms by which MSC therapeutic benefits are exerted. First, we evaluated the

effects of encapsulated MSC treatment in cerebellar granule neuron (CGN) culture subject. CGN were subject to 90 minutes OGD, followed by immediate treatment with encapsulated MSCs (1×10^4, 5×10^4, or 1×10^5 cells/well). After 24 hours, CGN were fixed and labeled with anti-MAP2, to visualize neuronal processes.

After 90 minutes of OGD and 24 hours of recovery, neuronal processes are markedly retracted. All doses of encapsulated MSC treatment appear to prevent process degradation (**Figure 4.4A)**. To quantify neuronal degradation, MAP2 was quantified as an area of fluorescence per number of cells. We found that OGD results in a marked decrease of MAP2 per cell, as compared to the uninjured control, indicating degradation and/or neurite retraction. Encapsulated MSC treatment at all doses results in increased MAP2 per cell, as compared to OGD injury without treatment (**Figure 4.4B)**

A



B

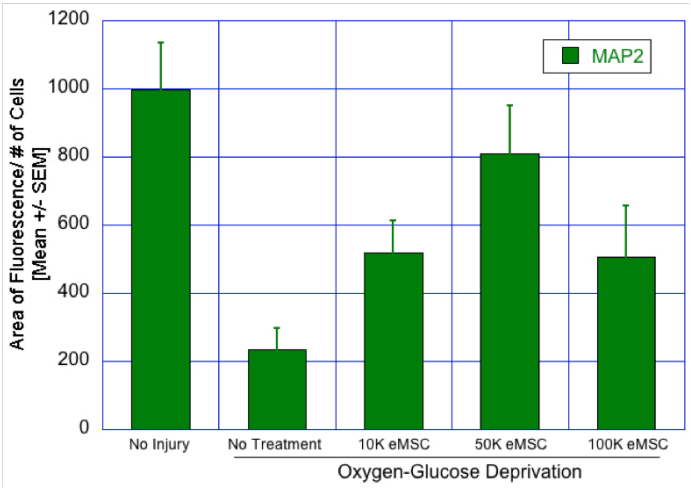


Figure 4.4. (A) MAP2 labeling of cerebellar granule neurons (representative images). MAP2 (green) was visualized using immunohistochemistry, and cell nuclei were counter-stained with DAPI (blue). OGD induces retraction/degradation of neuronal processes, which is prevented with all doses of encapsulated MSC treatment. (B) MAP2 fluorescence per cell, expressed as mean ± S.E. OGD markedly reduces MAP2 labeling, which is prevented at all doses of encapsulated MSC treatment.

4.3.3. Alginate-encapsulated MSCs modulate astrocyte activation after OGD

We have demonstrated the direct neuroprotective effects of encapsulated MSC treatment in hippocampal slice cultures, as well neuronal cultures subject to OGD injury, identifying neurons as a target of MSC treatment. Astroglial cells in the brain also respond to injury - undergoing a process known as astrogliosis, leaving their quiescent state and becoming activated [14]. Activated astrocytes cease neuroprotective functions [20,21], induce inflammation and the immune response [14,22], and increase production of extracellular matrix (ECM) molecules that contribute to the glial scar [14].

Here, we have used primary astrocyte cultures to determine whether encapsulated MSCs target multiple CNS cell populations, exerting potential neuroprotective benefits indirectly via modulation of astrocyte activation. Astrocyte cultures were subject to 5.5 hours of OGD, followed by immediate treatment with encapsulated MSCs (1×10^4, 5×10^4, or 1×10^5 cells/well). After 24 hours, astrocytes were fixed and labeled with anti-GFAP, a common marker of astrocyte activation; and anti-neurocan, an ECM component of the glial scar. We found that astrocyte production of GFAP and neurocan is significantly increased after OGD, as compared to uninjured cultures. All doses of encapsulated MSC treatment significantly reduce both molecules (**Figure 4.5**), indicating an ability of encapsulated MSCs to modulate astrocyte activation and/or inhibit glial scar formation.

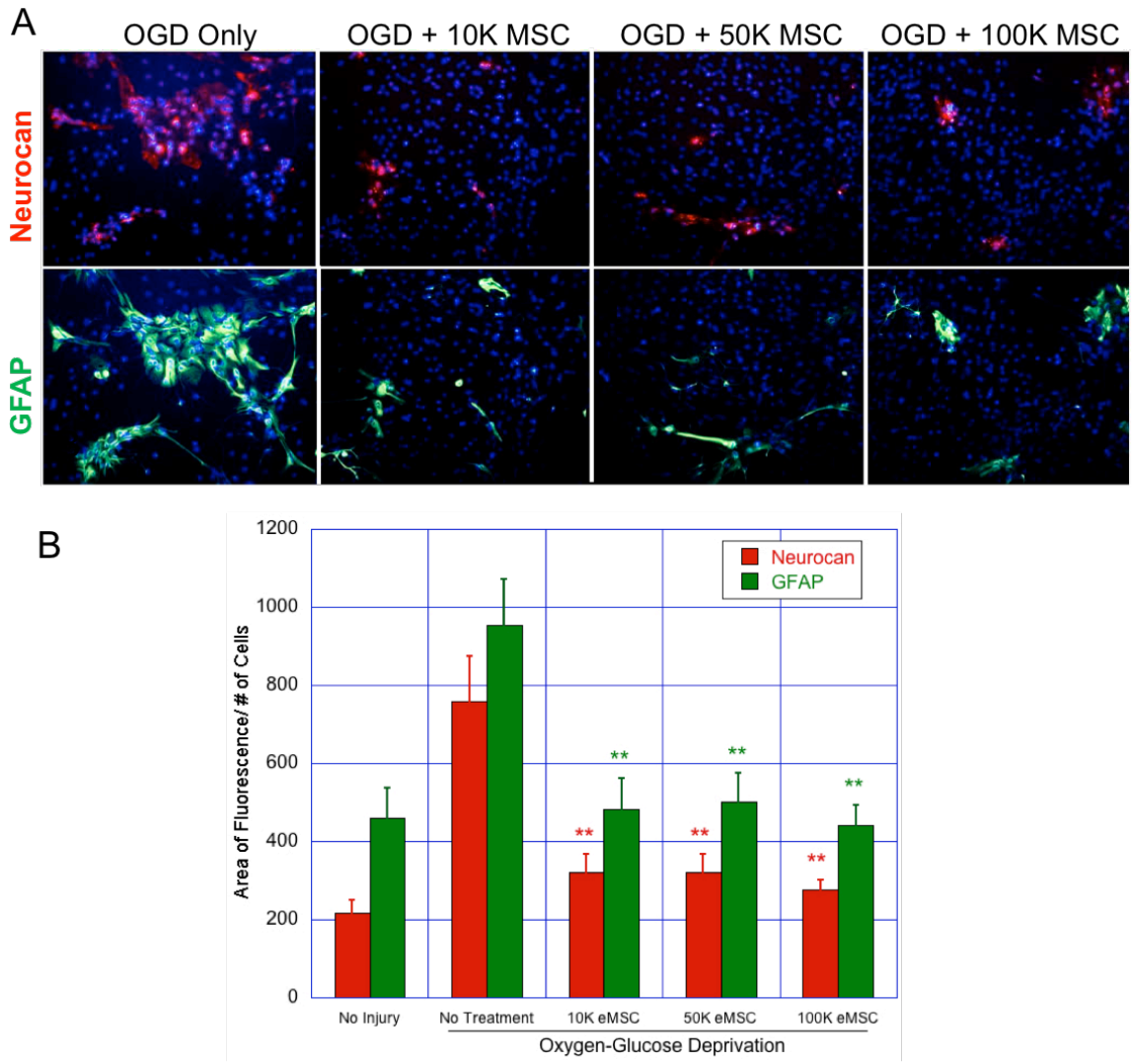


Figure 4.5. (A) GFAP and neurocan labeling of astrocytes (representative images). GFAP and neurocan were visualized using immunohistochemistry, and cell nuclei were counter-stained with DAPI (blue). Encapsulated MSC treatment at all doses reduces astrocyte production of both GFAP and neurocan. (B) GFAP and neurocan fluorescence per cell, expressed as mean ± S.E. OGD markedly increases production of both molecules, which is reduced at all doses of encapsulated MSC treatment. ** = $P \leq 0.01$ compared to OGD + no treatment.

4.4. DISCUSSION

Therapies that provide protection against secondary insults and/or restore neural function are critical to survival and functional recovery following TBI. Ischemia, in particular, is reported to be one of two initial insults in secondary injury and may be the most significant cause of progressive damage, evoking a cascade of changes that lead to additional injury processes [164,165] such as excitotoxicity, free radical generation, and inflammation [166]. In a study evaluating perfusate from the ischemic hippocampi of rats, extracellular glutamate and aspartate levels were found to elevated [167], and increased reactive oxygen species (ROS) were detected in organotypic hippocampal slices subject to OGD [168]. Additionally, in rat models of ischemia, investigators found an increase in several pro-inflammatory chemokines and cytokines [169], as well as immune cell recruitment at the lesion site [170]. Combined, these events are linked to neuronal death and damage, as well as initiation of a chronic inflammatory response.

Mesenchymal stromal cell (MSC) therapy has been shown to provide both neuroprotective and regenerative benefits in models of ischemia and TBI [40,130,171], most likely as a result of paracrine signaling and MSC secretion of growth factors and anti-inflammatory cytokines [30]. The ability to control long-term survival and localization of the implanted cells remains questionable, however, and limits the potential for treatment efficacy of MSCs in brain trauma. We circumvent these limitations by immobilizing MSCs within alginate capsules for enhanced delivery and prolonged treatment of TBI. We have previously shown these alginate-encapsulated MSCs to be anti-inflammatory in macrophage [55], OHSC, and astrocyte [120] cultures, and to exert neuroprotective benefit in a model of fibronectin-induced hippocampal slice degradation

[63]. We have also found encapsulated MSCs to up-regulate host expression of several neurotrophic factors [120] (presented in Chapter 3).

In this study, we evaluated the therapeutic potential of alginate-encapsulated MSCs to provide neuroprotection and support regeneration following *in vitro* ischemia. Our results reveal that encapsulated, but not monolayer, MSCs protect against ischemic cell death in OHSC within 24 hours of injury. Treatment effects are significant in the CA1 region, but a trend of reduced cell death after MSC treatment is also observed in the CA3 region. Additionally, in cerebellar granule neuron (CGN) culture subject to OGD, encapsulated MSCs reduced the amount of injury-induced neurite retraction. Though the mechanism behind encapsulated MSC neuroprotection was not evaluated in this study, our previous results demonstrating MSC-induced up-regulation of the neurotrophins brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and glial cell line-derived neurotrophic factor (GDNF) may indicate a possible means for their improved therapeutic benefit.

Neurotrophic factors function to promote and support neuron survival, and are involved in neuron development, as well as synaptic function and plasticity [172]. Dysregulation of these factors is a contributing element to the pathogenesis of brain injury. In a study of TBI patients, lower levels of circulating BDNF on day-of-injury correlated with increased injury severity and higher odds of incomplete recovery [173], and up-regulation of BDNF has been associated with induction of neurogenesis and functional recovery in rat models of TBI [174,175]. Increased NT-3 has been shown to decrease lesion size and promote motor function [176], and reduce cell death [177] following *in vivo* TBI, and has been extensively studied as a promising therapeutic for

spinal cord injury (SCI) [178-180]. Finally, TBI-injured rats administered GDNF showed reduced lesion size [181] and neurodegeneration, increased neuron survival, and more rapid functional recovery [182]. In addition to therapeutic benefits demonstrated with exogenous delivery or induction of host production of neurotrophic factors, MSCs are known to produce several neurotrophins that support neuronal cell survival and regeneration [67,183]. Taken together, this evidence suggests that the neuroprotective action of encapsulated MSCs observed in our studies may be a result of stimulation of host neurotrophin production, or neurotrophin production by the MSCs themselves.

In addition to demonstrating the neuroprotective actions of encapsulated MSC treatment following *in vitro* ischemia to neuron and slice culture preparations, we also evaluated the effect of encapsulated MSCs on astrocyte cultures subject to OGD. Astrocyte activation following TBI injury is responsible for initiating several mechanisms of secondary damage, resulting in neuronal death and inhibition of regeneration [128]. One function of reactive astrocytes is to isolate the lesion site in response to trauma. Astrocytic processes induced by activation become tightly interwoven to form a network "mesh" of cells, and extracellular matrix (ECM) molecules, such as proteoglycans, are deposited by astrocytes. This cellular and structural reorganization forms a dense barrier along the border of necrotic tissue, known as the glial scar. While the glial scar is thought to be initially aimed at sparing healthy brain tissue from the cytotoxic effects of the injury environment, long term it can be an obstruction to axon regrowth and regeneration [20,128,184]. As such, the glial scar has become an important target of therapeutic development for TBI. Presented here, our results show that encapsulated MSC treatment is capable of targeting the glial scar component of secondary injury, by

reducing levels of OGD-induced GFAP and neurocan – a marker of astrocyte activation and a proteoglycan scar component, respectively.

The ability to exert greater control over delivery and long-term potential of MSC therapy is necessary in order to maximize and prolong therapeutic efficacy, particularly in cases of injury characterized by persistent cell death and degeneration. Herein, we have evaluated the neuroprotective and regenerative potential of encapsulated MSCs. Our results demonstrate that encapsulated MSCs have multi-potent benefit in protecting against ischemic cell death, maintaining neurite morphology and integrity, and reducing astrocyte activation and secretion of glial scar components. Though we have only investigated acute time points in this study, our results form the basis for future studies, which aim to investigate the long-term therapeutic effect of alginate-encapsulated MSCs for TBI treatment.

4.5. ACKNOWLEDGEMENTS

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CHAPTER 5. DISCUSSION AND FUTURE DIRECTIONS

5.1. DISCUSSION

The ability to successfully ameliorate secondary injury following TBI is necessary to facilitate regeneration and functional recovery. Achievement of this outcome requires a multi-potent, dynamic therapy capable of (i) targeting multiple components of the secondary injury cascade and (ii) responding to the continually changing injury environment. Mesenchymal stem cell (MSC) therapy has achieved a degree of success in several models of TBI and CNS injury, but the ability of MSCs to provided sustained therapeutic benefit is impeded by lack of long-term persistence and localization of MSCs at the injury site. Cell encapsulation technology provides a potential method to overcome these obstacles, by allowing for sustained treatment and immobilization, as well as reduced immunogenicity, while still maintaining cellular viability and secretory functions [98].

In these studies, we have evaluated alginate-encapsulated MSCs for their ability to provide multi-potent benefit using *in vitro* models of inflammation and ischemia – two critical components contributing to secondary damage following TBI. Alginate is an FDA approved polymer derived from kelp, and has already been proven to sustain cell viability and function [55], as well as localization in brain tissue up to 6 months [53]. Encapsulation of MSCs in alginate microspheres will allow for sustained therapeutic benefit, immobilization of MSCs at the injury site, and protection of cells from exposure to the cytotoxic injury environment. Furthermore, studies have demonstrated that direct cell contact with the injury is not necessary for therapeutic benefit, so there is no concern

that the alginate with act as a barrier to cell-cell interactions [185]. In previous studies, we demonstrated that our alginate-encapsulated MSCs are more responsive to pro-inflammatory stimuli than monolayer MSCs, based on secretion of a panel of regulatory cytokines and growth factors. Additionally, we have demonstrated *in vitro* and *in vivo* that encapsulated MSCs can attenuate trauma-induced macrophage-mediated inflammation [55] and prevent tissue degradation of organotypic hippocampal slices cultured on the inhibitory substrate, fibronectin [63]. Building on that evidence, this dissertation further investigated the anti-inflammatory and neuroprotective properties of encapsulated MSCs, and the mechanism(s) by which encapsulated MSCs alleviate CNS inflammation and pathology.

We first evaluated the ability of encapsulated MSCs to attenuate LPS-induced inflammation in an organotypic hippocampal slice culture (OHSC) model. Our results corroborated previous studies describing the anti-inflammatory benefit of MSCs for LPS-induced inflammation [47,75,119], but demonstrated the enhanced action of encapsulated MSC therapy. Following treatment with encapsulated MSCs, TNF-α produced by LPS-stimulated OHSC was reduced significantly and more effectively than treatment with monolayer MSCs. To investigate the possible mechanism behind the improved inflammatory modulation of encapsulated MSC treatment, we initially assayed for PGE_2 produced by MSCs. PGE_2 is a well-known inflammatory mediator, and several studies have shown that inflammatory stimuli can induce MSC production of PGE_2 [97,186-188]. Indeed, we demonstrated that both monolayer and encapsulated MSCs produced increased levels of PGE_2 in response to stimuli, however, encapsulated MSCs exhibit this increase regardless of external inflammatory stimuli, suggesting that constitutive PGE_2

production is encapsulation-regulated. Furthermore, we found a direct correlation between increased PGE_2 and reduction of TNF-α, indicating PGE_2 as a key inflammatory mediator produced by MSCs.

In addition to the constitutive increase of PGE_2 production, a Bioplex screen for 27 MSC-produced cytokines and growth factors revealed additional encapsulation-regulated changes. Again, changes in monolayer MSC secretion patterns required stimulation by LPS and co-culture factors, but encapsulation alone was capable of influencing the MSC secretome. Moreover, we found several additional cytokines produced in significantly different quantities between monolayer and encapsulated MSCs, and with strong correlations to levels of TNF-α. These results highlight the enhanced therapeutic action of alginate-encapsulated MSCs for neuro-inflammation, which is likely conferred by altered secretome regulation compared to monolayer MSCs. It is yet unknown how alginate-encapsulation induces alteration of MSC secretion, but it is possible that the effect is mediated by the encapsulation material or 3-D culture environment. Recent evidence has shown alginate to act as an inflammatory stimulus depending on purity and composition [106], and that modification of the 3-D cellular environment by culturing MSCs as spheroids enhances their anti-inflammatory properties [113] and activates PGE_2 production [114]. It is possible that, in response to such cues, encapsulated MSCs are becoming primed to modulate the host inflammatory response.

Having demonstrated inflammatory modulation by encapsulated MSCs in LPS-stimulated OHSC, we then sought to further characterize the host response to MSC treatment. Using isolated cultures of astrocytes and microglia -- the primary cellular mediators responsible for the neuro-inflammatory response -- we found that astrocytes

are the primary target of our encapsulated MSC treatment and confirmed, in astrocyte culture, that encapsulated MSCs are more effective than monolayer MSCs in reducing LPS-stimulated TNF-α. We also demonstrated that this enhanced effect is maintained over time and is temporally responsive to the co-culture environment, supporting our hypothesis that encapsulated MSC therapy is capable of responding to a dynamic injury environment. Additionally, in characterizing MSC treatment over an extended evaluation period, we confirmed that monolayer MSCs require the presence of increasing inflammatory stimuli in order to produce PGE_2. Encapsulated MSCs are also responsive to increasing levels of TNF-α, however, they exhibit significantly higher levels of PGE_2 from an earlier time point, and maintain this increase over monolayer MSC production throughout the evaluation period. Our data showing that early administration of exogenous PGE_2 significantly reduced TNF-α produced by LPS-stimulated astrocytes, while delayed administration of PGE_2 had no effect, further supports the advantage of alginate-encapsulation of MSCs for therapeutic delivery, and the critical role of PGE_2 as an inflammatory mediator.

PGE_2 is a multi-functional molecule, capable of a multitude of (sometimes opposing) actions depending on receptor subtype binding (EP1-4) and the activation of distinct downstream signaling pathways. Using receptor subtype specific agonists and antagonists, we found that both exogenous and MSC-produced PGE_2 exert their anti-inflammatory effect through astrocyte EP1, EP2, and EP4 receptors. The extent to which this effect is mediated through each subtype though, appears to differ depending on PGE_2 source (exogenous or MSC). Recalling data presented earlier, showing MSC secretome changes induced by inflammatory stimuli, this difference could be due to other MSC-

produced factors altering EP receptor expression or the expression of other interacting factors in the PGE_2 signaling pathways, in a manner that exogenous PGE_2 is not capable of. Nevertheless, our results support the anti-inflammatory role of EP4 in CNS pathology [76,155] and corroborate select reports that EP2 is capable of neuro-inflammatory modulation [153]. This is the first report, to the best of our knowledge, demonstrating the ability of the EP1 receptor to attenuate neuro-inflammation.

In addition to reducing astrocyte-mediated inflammation, we also found that encapsulated MSC and PGE_2 treatments were capable of regulating astrocyte neurotrophin expression, inducing increased expression of potent neurotrophins such as BDNF, NT-3, and GDNF. Both quantitative and hierarchical cluster analysis revealed that expression patterns regulated by encapsulated MSC and PGE_2 treatments were most closely related, indicating that increased PGE_2 production by encapsulated MSCs may be largely responsible for astrocyte neurotrophin induction. However, a subset of neurotrophin expression patterns, such as that for GDNF, displayed similar trends between monolayer and encapsulated MSC treatment groups that contrasted those observed with PGE_2 treatment. Again, because of additional inflammation-induced changes in the MSC secretome, other MSC-produced regulatory factors could be enacting astrocyte expression changes. Overall, these data reveal that alginate-encapsulated MSCs are not only anti-inflammatory, but are capable of neuroprotective benefit as well, through regulation of host neurotrophic factor expression.

Finally, using a model of *in vitro* ischemia, induced by oxygen-glucose deprivation (OGD), we confirmed the ability of encapsulated MSCs to provide neuroprotection following injury. In OHSC, encapsulated MSCs mitigated ischemia-

induced cell death, while monolayer MSC treatment had no effect. Additionally, using cerebellar granule neuron (CGN) culture subject to OGD, we saw that ischemia caused neurite retraction/degradation, which was prevented by encapsulated MSC treatment. In addition to the direct neuroprotective effect of encapsulated MSCs observed in both mixed CNS and isolated neuronal cell preparations, we also demonstrated indirect neuroprotective actions using astrocyte culture. Encapsulated MSCs reduced OGD-induced production of GFAP, an astrocyte activation marker, and neurocan, a glial scar component produced by reactive astrocytes. Reducing activation mitigates several components of the secondary injury cascade that are initiated by reactive astrocytes, preventing neuronal cell death caused by these injury mechanisms. The glial scar, in particular, is detrimental to neuronal growth and regeneration across the lesion site. As such, decreased GFAP and neurocan production following encapsulated MSC treatment suggests downstream neuroprotective and regenerative benefit.

5.2. FUTURE DIRECTIONS

In our studies, we demonstrated the enhanced anti-inflammatory and neuroprotective benefit of encapsulated MSC treatment. Mechanistically, we identified PGE_2 as a key MSC-produced inflammatory mediator, determined that alginate-encapsulation alone is an effector of MSC secretome changes, and found that encapsulated MSC treatment induced up-regulation of host neurotrophic factors. Though we have elucidated some of the underlying mechanisms for the improved action of encapsulated MSCs, future work aims to better characterize the means by which they

confer multi-potent benefit. We also aim to evaluate the long-term effects of encapsulated MSC treatment, as well as therapeutic optimization and translation.

First, given that alginate encapsulation alone is capable of inducing secretome changes, regardless of inflammatory stimuli, it is possible that the capsule material and/or environment can be optimized to further activate MSC therapeutic mediators. We have seen the dramatic increase in PGE_2 production upon encapsulation, however, our Bioplex screen revealed significant changes in additional mediators. The effect of these mediators on inflammatory modulation must be further explored in order to identify therapeutic factors that may work in addition to or synergistically with PGE_2. Additionally, we must determine the causal mechanisms responsible for activation of encapsulated MSCs -- whether related to material, micro-environment, or 3-D culture conformation -- in order to facilitate the optimization process.

Secondly, while we have demonstrated both the induction of neurotrophin expression and the neuroprotective ability of encapsulated MSCs, a direct causal relationship has yet to be shown in our model. Evidence exists linking these neurotrophic factors to neuron survival and regeneration, so it is reasonable to surmise that MSC-regulated expression changes may be responsible for the neuroprotective benefit of encapsulated MSC treatment. It would be beneficial, however, to determine actual production of these neurotrophic factors by astrocytes, and their direct effect on neurons using mixed or co-culture preparations. Given that MSCs have been shown to produce neurotrophic factors themselves, it would be interesting to determine the contribution of MSC-produced neurotrophins to their neuroprotective actions. Co-culture preparations could also be used to determine the direct effect of MSC-induced down-regulation of

astrocyte GFAP and neurocan, and whether encapsulated MSC treatment does indeed result in a more permissive environment/substrate for neuron growth and regeneration.

Finally, the therapeutic benefit of encapsulated MSCs must be demonstrated *in vivo*, to determine whether the multi-potent effects demonstrated *in vitro* translate to prolonged treatment and improved functional outcome following *in vivo* TBI. Our encapsulated MSC treatment has previously been evaluated using an *in vivo* model of spinal cord injury (SCI), with promising results [55]. In these studies, the encapsulated MSCs were delivered to the lumbar space, a relatively un-invasive procedure. Delivery of encapsulated MSCs in an animal model of TBI proves more cumbersome, however, and the least invasive route of delivery must be established for the success of future clinical translation. In addition to delivery method, the optimal dose, time of administration, and duration of treatment must also be determined, as well as the metrics by which functional outcome will be measured. The long-term stability (>6 months) of encapsulated MSCs, and their terminal fate following treatment, should also be evaluated.

5.3. CONCLUSIONS

The long-term objective of this research is to administer alginate-encapsulated MSCs to improve treatment following brain injury. Collectively, the work presented in this dissertation demonstrates that encapsulated MSCs achieve our goal of developing a therapeutic strategy that is both multi-potent and temporally responsive to the injury environment. We have shown that encapsulated MSC treatment targets distinct components of secondary injury, including inflammation and ischemia, and responds temporally to injured tissue. Our findings revealing the anti-inflammatory,

neuroprotective, and regenerative potential of encapsulated MSC treatment could pave the way for clinical evaluation of encapsulated MSC therapy in large animal and human models of TBI, as well as point to key components of its action for development of potential alternate or complementary therapies. We believe that alginate-encapsulation of MSCs has the potential for therapeutic translation to TBI and other CNS injuries, in which prolonged activity in the injury environment is essential to recovery.

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