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Encapsulated Mesenchymal Stromal Cells for In-Vivo Transplantation

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Abstract

Immunomodulatory human mesenchymal stromal cells (hMSC) have been incorporated into therapeutic protocols to treat secondary inflammatory responses post-spinal cord injury (SCI) in animal models. However, limitations with direct hMSC implantation approaches may prevent effective translation for therapeutic development of hMSC infusion into post-SCI treatment protocols. To circumvent these limitations, we investigated the efficacy of alginate microencapsulation in developing an implantable vehicle for hMSC delivery. Viability and secretory function were maintained within the encapsulated hMSC population, and hMSC secreted anti-inflammatory cytokines upon induction with the pro-inflammatory factors, TNF- α and IFN- γ . Furthermore, encapsulated hMSC modulated inflammatory macrophage function both *in-vitro* and *in-vivo*, even in the absence of direct hMSC-macrophage cell contact and promoted the alternative M2 macrophage phenotype. *In-vitro*, this was evident by a reduction in macrophage iNOS expression with a concomitant increase in CD206, a marker for M2 macrophages. Finally, Sprague-Dawley rat spinal cords were injured at vertebra T10 via a weight drop model (NYU model) and encapsulated hMSC were administered via lumbar puncture 24 hours post-injury. Encapsulated hMSC localized primarily in the cauda equina of the spinal cord. Histological assessment of spinal cord tissue 7 days post SCI indicated that as few as 5×10^4 encapsulated hMSC yielded increased numbers of CD206-expressing macrophages, consistent with our *in-vitro* studies. The combined findings support the inclusion of immobilized hMSC in post-CNS trauma tissue protective therapy, and suggest that conversion of macrophages to the M2 subset is responsible, at least in part, for tissue protection.

Introduction

Human mesenchymal stromal cells (hMSC) are highly proliferative, tissue culture plastic adherent cells (Friedenstein et al. 1992), which can differentiate into a number of mesodermal cell lineages and serve as a potential cell source for autologous cellular replacement therapies (Dennis et al. 1999; Pittenger et al. 1999), many of which are currently under clinical trial evaluation (NIH 2011). More recently, their cyto-protective role, mediated by secretion of a plethora of cytokines and growth factors (Caplan and Dennis 2006) has also been described (Phinney and Prockop 2007). hMSC secretion profiles have been well characterized *in-vitro* and can be modulated by the local microenvironment (Le Blanc and Ringden 2007). In addition, hMSC therapeutic benefits have been described both *in-vitro* and *in-vivo*, using models of graft versus host disease (Polchert et al. 2008), myocardial infarction (Zhang et al. 2007), fulminant hepatic failure (Parekkadan et al. 2008; Parekkadan et al. 2007; van Poll et al. 2008), central nervous system trauma (Heile et al.

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2009), sepsis (Parekkadan et al. 2011; Yagi et al. 2010a; Yagi et al. 2010b), colitis (Parekkadan et al. 2011), and may occur in the absence of direct cellular replacement or hMSC differentiation (Parr et al. 2007). Many investigators have suggested that hMSC orchestrate biochemical cues that mitigate fibrosis and promote tissue protection, via secretion of soluble factors (Gnecchi et al. 2005; Hardjo et al. 2009; Kunter et al. 2006; Ortiz et al. 2003; Yagi et al. 2010c).

Despite increasing interest in hMSC therapeutic potential, limitations with direct hMSC implantation approaches prevent effective translation of hMSC infusion into the design of safe and controlled therapeutic protocols. Several reports have described wide hMSC distribution to non-targeted tissues after transplantation (Bakshi et al. 2004; Li et al. 2011). Additionally, the hMSC fraction that ultimately reach the targeted destination do not persist long-term (Barker and Widner 2004; Ohtaki et al. 2008), in part because directly transplanted hMSC may be adversely affected by the complex injury environment and may differentiate into undesired end stage cells (Trouche et al. 2010). Resolution of these outstanding issues may be facilitated with the development of an immobilized hMSC delivery approach.

Several studies have demonstrated that cell immobilization can support hMSC survival and functional differentiation into other cell types following differentiation factor supplementation (Xu et al. 2008; Yang et al. 2004). Many investigators have generated cell/material constructs using a variety of natural (e.g. alginate, collagen, chitosan) and synthetic (e.g. cellulose, silicon) materials for improved control over implanted cells. Among these, alginate is a cost effective, non-immunogenic, FDA approved material that has been utilized extensively by many investigators for a variety of stem cell differentiation and cell immobilization protocols (Murua et al. 2008). Previous studies in our laboratory have utilized alginate encapsulation to differentially direct murine embryonic stem cell (ESC) differentiation towards either the hepatocyte or neuronal lineages by varying both the alginate concentration and aggregate formation within the microcapsules (Li et al. 2011; Maguire et al. 2007). The present studies were designed to determine if alginate encapsulation could also be incorporated to preserve hMSC anti-inflammatory function, providing a controlled delivery vehicle that can attenuate inflammation and promote tissue repair *in-vivo*. Our results indicate that alginate encapsulation can sustain hMSC viability and constitutive secretion, and in the presence of pro-inflammatory stimuli, promotes elevated secretion from hMSC of a panel of regulatory cytokines and growth factors. Finally, we demonstrate, via both *in-vitro* and *in-vivo* models of inflammation, that encapsulated hMSC promoted expression of CD206 associated with the alternative activated M2 subtype of macrophages.

Methods

Cell Culture

All cell cultures were incubated in a humidified 37 °C, 5% CO₂ environment. hMSC were purchased from Texas A&M at passage 1 and cultured as previously described (Parekkadan et al. 2007). Briefly, hMSC were cultured in MEM- α (Gibco) medium, containing no deoxy and ribo nucleosides, supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), 1 ng/ml basic fibroblast growth factor (bFGF) (Gibco), 100 units/ml penicillin and 100 μ g/ml streptomycin (Gibco). hMSC were plated at 5000 cells per cm² and allowed to proliferate to 70% confluence (approximately 4 to 5 days). Only hMSC at passages 2 through 5 were used to initiate subsequent experiments. Human acute monocytic leukemia cell line (THP-1) (ATCC, Manassas, VA) was maintained at 8 $\times$ 10⁵/ml in RPMI 1640 (Gibco) medium supplemented with 10% FBS (Gibco), 4mM L-glutamine (Gibco), 100 units/ml penicillin, and 100 μ g/ml streptomycin (Gibco). Medium

was replenished every 3 days and cells were passaged every 6 days. THP-1 cells (3.2×10^5 /ml) were differentiated using 16 nM phorbol-12-myristate 12-acetate (PMA) (Sigma-Aldrich, St. Louis, MO) for 16 hours. The differentiation was further enhanced by removing the PMA-containing medium and incubating the cells for 3 days in culture medium before experiments.

Alginate Microencapsulation

Alginate Poly-L-Lysine microencapsulation of hMSC was performed as previously described (Maguire et al. 2007). The microencapsulated cells were re-suspended in MEM- α (Gibco) and transferred to 25 cm² tissue culture flasks. Medium was changed every 7th day post-encapsulation for a total culture time of 21 days. In all experimental conditions, monolayer culture configurations of hMSC were used as controls for viability, growth kinetics, and functional studies. Microcapsules were synthesized with different concentrations of alginate (1.7%, 2.2% and 2.5%) as well as different initial cell densities (10^6 , 2×10^6 , 4×10^6 and 6×10^6 cells per ml). Based on initial viability post encapsulation, 4×10^6 cells per ml was identified to be optimal for MSC encapsulation and therefore used in all subsequent experiments (data not shown). Capsule diameters ranged from 450 to 550 μ m for all *in-vitro* studies.

Viability/Proliferation

Viability was assessed using calcein (Molecular Probes, Eugene, OR), and ethidium homodimer (Molecular Probes) staining as previously described (Maguire et al. 2007) on days 2, 5, 7, 10, 14, 18, 21, 40, and 60 post encapsulation. Briefly, capsules were washed 3 times with phosphate buffered saline (PBS) (Gibco) and then incubated for 15 minutes with PBS containing calcium and ethidium homodimer, as per vendor's instructions. Capsules were washed 3 times before using an Olympus IX81 spinning disc confocal microscope to acquire 500 μ m Z stacks at 20 μ m intervals for 15 capsules per condition. Digitized images of each cross section for fifteen capsules per condition were analyzed for live cells (green fluorescing) and dead cells (red fluorescing) using Slidebook software.

Proliferation was assessed on days 2, 5, 7, 10, 14, 18 and 21 post encapsulation as previously described (Cohen et al. 2010). Briefly, capsules from three samples per condition were counted, subsequently dissociated in 1% EDTA (Sigma Aldrich) for 10 min and centrifuged at 400 g for 5 minutes. The pellet was re-suspended, stained with trypan blue and cells were counted using a hemocytometer. The cell count was then normalized to the number of capsules in the initial sample.

Cytokine Measurement

Evaluation of cytokine secretion was performed on days 2 and 21 post encapsulation. Capsules across different alginate concentrations (1.7%, 2.2% and 2.5%) were placed in 75 μ m inserts (Corning, NY) for a 12 well plate and cultured for 48 hours in hMSC medium supplemented with IL-6 (25 ng/ml) and TNF- α (25ng/ml) /IFN- γ (25ng/ml) (R&D Systems, Minneapolis, MN). Each well had capsules containing a total of 6×10^4 cells per well. hMSC cultured in monolayers served as secretion controls. Supernatants were collected and stored at -20° C. Supernatants were analyzed via multiplex bead analysis (Bio-Rad, Hercules, CA) for 27 different growth factors and cytokines as per vendor's instructions. Data was normalized to cell number and monolayer secretion levels. A 2 way ANOVA was implemented to assess statistical significance within the data set. Post-hoc analysis was performed via the Fisher's least significant difference (LSD) method; p-values < 0.05 and 0.1 were considered significant.

Macrophages

Following the differentiation of THP-1 cells for 4 days, co-cultures were established either with encapsulated hMSC or free hMSC within 8 μ m transwell inserts (Corning). Macrophages were treated with 1 μ g/ml lipopolysaccharide (LPS) (Sigma Aldrich) and hMSC at various cell concentrations (4×10^3 and 4×10^4 cells/ml). The cultures were incubated for 24 hours, after which culture supernatants were collected and macrophages were fixed for immunocytochemistry. Macrophages were immunostained as previously described (Kim and Hematti 2009) with iNOS (Sigma Aldrich, rabbit antihuman, 1:130) and CD206 (Abcam, rabbit anti-human, 1:700). Images were acquired using an Olympus IX81 spinning disc confocal microscope and stereology was performed using Slidebook software. Supernatants were analyzed via ELISA for IL-10, TNF- α (Biolegend, San Diego, CA) and via multiplex bead analysis (Bio-Rad) for IL-1 β , IL-6, IP-10 and MIP-1 α , performed as per vendor's instructions. Encapsulated Chinese hamster ovary (CHO) cells were used as a control.

Spinal Cord Injury and Transplantation

Twenty adult female Sprague-Dawley rats (200-250 g, 77 $\pm$ 2 days old, Taconic, Germantown, NY) were used in this study: SCI + saline (n=5), SCI + capsule (n=5), SCI + hMSC (n=5), and SCI + encapsulated hMSC (n=5). SCI was performed using the MASCIS Impactor as described previously (Hasegawa et al. 2005). Briefly, rats were anesthetized with 2% isoflurane and a 12.5 g-cm contusion was induced at spinal segment T10. hMSC at passage 2 were plated at 5×10^3 cells/cm² 4-5 days before encapsulation or free transplantation. Encapsulated hMSC were prepared with 2.2% alginate at a seeding density of 4×10^6 cells/ml and transplanted 1-3 days post encapsulation. All transplantation of either encapsulated or free hMSC was done with cells at passage 3. One day after SCI, hMSC (5×10^4 cells/70 μ l of saline), encapsulated hMSC (2000 capsules (4.8×10^4 cells)/70 μ l of saline), saline (70 μ l), or hMSC-free capsules (2000 capsules/70 μ l of saline) were injected at lumbar vertebrae L3-L5 through lumbar puncture over a period of 30 seconds; the syringe was left in that place another 60 seconds to prevent leakage (Lepore et al. 2005; Otsuka et al. 2011). All animal experiments were approved by the Animal Care and Use Committee of Rutgers, The State University of New Jersey.

Tissue Processing for Immunofluorescence

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

Quantitation for Immunofluorescence and Cell counting

Eight ED1 and CD206-immunostained images were taken with a frame size of 334.8 μ m $\times$ 334.8 μ m from coronal sections of injured spinal cord cross-sections 2.5 mm distal to the injury epicenter in each animal. Immunopositive areas were obtained using Zeiss LSM Image Browser and were used to calculate average areas. For cell counting, ED1 and CD206

immunostained images were analyzed by two blinded testers. One-way ANOVA with Tukey's HSD tests were used to analyze statistical significance among the different groups; p -value < 0.05 was considered significant. Data are expressed as the mean $\pm$ standard error of the mean (SEM).

Statistical Analysis

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

Results

Evaluation of Encapsulated hMSC Viability and Proliferation

The ultimate goal of our studies was to determine whether alginate encapsulation could support hMSC immunomodulatory function allowing its utilization as a vehicle for controlled *in-vivo* delivery. However, before function could be evaluated hMSC viability and proliferation in the capsule microenvironment were assessed. Initial experiments incorporated calcein and ethidium homodimer staining to assess hMSC viability within the microcapsules. Encapsulated hMSC remained $> 90\%$ viable for at least 60 days post-encapsulation, indicating that the microenvironment could sustain hMSC survival for long time periods (Figure 1A). Next, we varied the alginate concentration and assessed cell proliferation over time. Our results indicated that hMSC proliferation was dependent upon the alginate concentration since 2.2%, but neither 2.5% or 1.7% alginate, supported hMSC proliferation throughout the 3 week experimental period. By day 21, the final cell concentration per capsule was twice the initial seeding density (Figure 1B), a proliferation rate far lower than monolayer conditions (data not shown). Lower proliferation rates within the capsule microenvironment is consistent with our previous ES cell studies (Maguire et al. 2007).

Encapsulated hMSC Secretion

hMSC secrete numerous factors, many of which have been found to contribute to their immunomodulatory and tissue protective effects (Le Blanc and Ringden 2007). In order to evaluate whether the capsule microenvironment could sustain hMSC secretory function, a 27 factor multiplex assay was employed. Our results demonstrated that, as expected, monolayer-cultured hMSC constitutively secrete a plethora of factors (Table 1). Among these were critical inflammatory cytokines, as well as factors responsible for growth and development. Having established quantitative baseline measurements of monolayer-cultured hMSC, we evaluated whether the capsule microenvironment could support secretory function. The results of our studies indicated that encapsulated hMSC supported comparable constitutive secretion patterns during the first 2 culture days, with the highest overall levels from hMSC in the 1.7% and 2.2% alginate microenvironments (Figure 2A). Subsequent assessment of constitutive secretion at day 21 post-encapsulation revealed diminished secretion from 2.5% alginate encapsulated cells, but constant secretion patterns from 1.7% and 2.2% encapsulated cell conditions (Figure 2B).

A distinct characteristic of hMSC is that upon stimulation with pro-inflammatory cues, secretion levels are increased (Crisostomo et al. 2008; Ren et al. 2008). In the presence of inflammatory cues TNF- α and IFN- γ , monolayer-cultured hMSC could be stimulated to increase secretion of various factors (Figure 2C). When encapsulated hMSC were cultured in the presence of TNF- α and IFN- γ and evaluated 2 days post encapsulation, we observed

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

Assessment of Anti-inflammatory Function with In-vitro Macrophage Co-cultures

hMSC have been suggested to exert their tissue protective benefits, partially, via modulation of immune cell behavior (Le Blanc and Ringden 2007). Therefore, having determined that constitutive and stimulated secretion patterns were sustained for encapsulated hMSC, experiments were designed to determine whether encapsulated hMSC could function to reduce inflammatory macrophage behavior. Inflammatory macrophages have been found to exacerbate pathological events post organ trauma (Kigerl et al. 2009) and hMSC have been shown to attenuate this effector function (Kim and Hematti 2009; Zhang et al. 2010). A THP-1 monocyte co-culture system was employed, where upon LPS stimulation, THP-1 monocytes enter a pro-inflammatory state, referred to as classical activation (M1), represented by elevated TNF- α secretion and iNOS expression (Perez-Perez et al. 1995). The activated macrophages were cultured in the presence of both encapsulated and free hMSC, and modulation of both iNOS and TNF- α expression was assessed. Results of ELISA analysis indicated that free hMSC and encapsulated hMSC, but not encapsulated CHO cells, attenuated inflammatory macrophage TNF- α secretion to a similar degree (Figure 3A). Next, THP-1 cells were analyzed immunocytochemically for expression of the activation marker iNOS. As expected, the activated macrophage population expressed high iNOS levels homogeneously throughout the population (Figure 3B), whereas iNOS expression levels after co-culture with encapsulated hMSC were reduced (Figure 3B).

Encapsulated hMSC promotion of an anti-inflammatory macrophage phenotype

Macrophages have been identified to exhibit a great degree of phenotypic plasticity (Porchery et al. 2005) and it has been suggested that hMSC could control this plasticity, by promoting anti-rather than pro-inflammatory function (Kim and Hematti 2009; Zhang et al. 2010). Therefore, experiments were designed to determine if this function was maintained by encapsulated hMSC. LPS activated macrophage protein secretion was evaluated via multiplex protein analysis to determine whether hMSC co-cultured macrophages merely reverted to a quiescent state or if they assumed an alternative phenotype. Activated macrophages characteristically secreted pro-inflammatory factors (IL-1b, IP-10, and MIP1a) at elevated levels compared to quiescent macrophages (Figure 4A-C). In the presence of hMSC, macrophage secretion of inflammatory factors was attenuated (Figure 4A-C). In contrast, secretion of IL-6 was elevated with respect to both quiescent and activated macrophage cultures (Figure 4D), a phenotype which has been found to be associated with anti-inflammatory M2 macrophage alternative activation (Kim and Hematti 2009). In an effort to gain further insight into the mechanism of macrophage inflammation attenuation by hMSC, we quantified expression of surface CD206 and secretion of the anti-inflammatory mediator IL-10, both of which are characteristic of a M2 macrophage phenotype. We observed that co-culture of encapsulated hMSC with activated THP-1 resulted in elevated levels of CD206 expression (Figure 5A). Furthermore, CD206 expression was regulated in a dose dependent manner with only the 10^3 encapsulated hMSC resulting in elevated CD206 expression (Figure 5A). In addition, IL-10 secretion levels were elevated when encapsulated hMSC were present during activation (Figure 5C). Therefore, both attenuation of M1 and promotion of M2 phenotypes may both be induced by co-culture with encapsulated hMSC. However, precise control over time and dose with hMSC treatment may be required to balance this transition.

In-vivo Model of spinal cord trauma

Having demonstrated anti-inflammatory encapsulated hMSC function *in-vitro*, we next assessed immunomodulation using an *in-vivo* model of spinal cord injury, since an overly aggressive M1 inflammatory response post-SCI has been associated with decreased regeneration (Jones et al. 2005). Contusions to the spinal cord at vertebra T10 via the NYU model (Basso et al. 1996) were performed and 24 hours post-contusion, approximately 5×10^4 encapsulated or free hMSC were administered via lumbar puncture (LP) at vertebra L4-L5 (Otsuka et al. 2011). Animals were sacrificed 8 days post contusion, a prime time point to evaluate immunotherapy since macrophage infiltration is known to be robust and since macrophage activation is known to promote and exacerbate tissue damage post-SCI (Donnelly and Popovich 2008). Capsules coated with FITC- conjugated PLL were visualized within the lumbar cistern of the subarachnoid spaces at 1 week post transplantation (Figure 6). Next, we prepared coronal sections of the spinal cords and immunostained the macrophages at the site of injury (Figure 7A-D). Qualitative and quantitative evaluation of the macrophage population was performed, via ED1 (Figure 7E-H) and CD206 (Figure 7E'-H') expression, at the post-contusion injury site. These experiments indicated that the number of ED1⁺ cells at the injury site was not significantly different among the experimental conditions 1 week post-hMSC infusion, (Figure 7I, J). However, a greater percentage of the macrophages was positive for CD206 (Figure 7I, J) compared to control conditions. Therefore, encapsulated hMSC attenuated and/or converted macrophages to a M2 phenotype *in-vivo*.

Discussion

The development of engineered hMSC delivery systems is vital for clinically relevant therapeutic protocol translation. However, current hMSC infusion strategies can neither regulate unwanted cell migration nor ensure hMSC persistence at the injury site. In fact, recent findings suggest that transplanted hMSC no longer persist at injury sites as early as 7 days post transplantation (Coyne et al. 2006). Cell immobilization systems have long been proposed as a vehicle for delivering controlled release of therapeutic agents. However, to date no biological vehicle has been described that can maintain the secretion of the wealth of therapeutic factors hMSC provide, as well as circumvent fibrosis post encapsulation for extended periods (Goren et al. 2010).

Alginate has been found to avoid biodegradation within the CNS up to 6 months post transplantation (Nunamaker et al. 2007). Our studies have shown that hMSC remain viable within the alginate micro-environment for at least 2 months and, depending on alginate concentration, support proliferation, although not at the high rates found in monolayer cultures. This finding is consistent with previous reports where mouse embryonic stem cell proliferation within alginate microcapsules was only supported at 2.2% alginate concentration (Li et al. 2011; Maguire et al. 2007). In addition, hMSC have been found to differentiate into several mesodermal cell lineages depending on the cell source, passage number and culture conditions. Studies have indicated that the alginate microenvironment may be designed to control hMSC differentiation (Trouche et al. 2010), a feature which may be important in controlling anti-inflammatory function as well. In fact, recent studies in our laboratory support this observation (data not shown).

In many models of trauma, hMSC treatment benefits have been attributed to their unique ability to control inflammatory responses. hMSC have been found to mediate inflammation and promote tissue repair through the secretion of a variety of soluble mediators (Le Blanc and Ringden 2007). Here, the capsule microenvironment not only sustained but also enhanced the secretion of these soluble mediators. While the mechanism(s) of hMSC inflammation control is unclear, it has been suggested that in the presence of inflammatory

factors, such as TNF- α and IFN- γ , the hMSC anti-inflammatory phenotype is promoted (Ren et al. 2008). We have replicated this response with our encapsulated hMSC populations. Overall, our analyses indicate that the capsule microenvironment enhances hMSC secretion, a finding corroborated by recent findings that 3 dimensional culture of hMSC enhances anti-inflammatory therapeutic potential (Bartosh et al. 2010). However, within 2.5% alginate capsules, the secretion rates were diminished compared to the other concentrations. Cumulatively based on viability, secretion profile and our previous alginate encapsulation studies (Li et al. 2008; Maguire et al. 2007; Maguire et al. 2006) the 2.2% alginate encapsulation condition was chosen for further functional evaluation. However, hMSC in a 1.7% alginate microenvironment also demonstrated sustained secretion patterns and may also have been a suitable choice.

Several publications have attributed organ pathology expansion to M1 macrophage secretion of pro-inflammatory cytokines and tissue degrading enzymes (Kigerl et al. 2009; Ricardo et al. 2008). The ability of hMSC to attenuate macrophage activation *in-vitro* underscores the tremendous potential of these cells in preventing tissue degradation (Kim et al. 2009). Our studies indicate that encapsulated hMSC co-cultures can attenuate M1 secretion of TNF- α as well as the percentage of iNOS expressing macrophages in LPS stimulated cultures.

Furthermore, multiplex analyses revealed that encapsulated hMSC attenuate secretion of several pro-inflammatory factors (MIP-1 α , RANTES and IL-1 β), which have been found to be elevated early in tissue pathologies and are associated with facilitating tissue fibrosis (Tsai et al. 2008). Interestingly, depending on the time and hMSC to macrophage ratio, CD206, a marker for the M2 phenotype, was elevated. The time and ratio dependency of M2 phenotypic acquisition may be due to accumulation of additional factors in static culture which drive the adoption of other macrophage phenotypes. Overall, this finding is consistent with previous reports that have highlighted a novel type of macrophage referred to as the hMSC educated macrophage (M2m) (Kim and Hematti 2009). However, to date, this phenomenon has not been explored in an immobilized platform. M2m macrophages exhibited levels of CD206^{high}, IL-10^{high}, IL-6^{high} and TNF- α ^{low}, a profile analogous to the one measured here, suggesting that this may be the macrophage phenotype hMSC are promoting. However, hMSC regulation of inflammatory macrophage function may also occur via a combination of M2 phenotype promotion as well as M1 macrophage attenuation.

To test the therapeutic potential of encapsulated hMSC, alginate encapsulated cells were implanted into the lumbar region of the spinal cord and evaluated in a rat trauma model. Previous studies have indicated that following SCI, the M1 phenotype dominates post trauma and is linked to the overall tissue pathology (Kigerl et al. 2009). hMSC transplantation post-SCI has been attempted by several investigators, yielding inconsistent results with regards to cell survival as well as homing to the injury site, as reviewed by Parr et al (Parr et al. 2007). In our studies, immobilized hMSC were administered 24 hours post-contusion via lumbar puncture. We observed fluorescent capsules localized along the spinal cord in the subarachnoid spaces, primarily at the cauda equina of the lumbar region 1 hour and 1 week post- transplantation. Recent reports have described promising hMSC therapeutic benefits in animal models of SCI; the mechanism of action, however, is still unclear. Several of these reports hypothesized that the *in-vivo* hMSC benefits can be attributed to trans-differentiation. However, experimental evidence to support this concept is questionable (Lu and Tuszynski 2005). The proposed paradigm in the current study is that hMSC modulate the inflammatory environment dynamically via the secretion of soluble factors, since direct cell to cell contact of hMSC with tissue is not possible within the alginate beads. Previous animal studies have been unable to dissect contributions of hMSC secreted factors from those due to direct cellular interactions with the trauma area. Our results indicate that within an immobilization platform, hMSC promoted the M2 phenotype

in the injury site. This was supported by the observation of elevated levels of CD206 in animals treated with encapsulated hMSC. Interestingly, while the number of ED1⁺ cells was not significantly different across conditions, the ED1⁺ population expressed elevated levels and increased co-localization with CD206, suggesting that macrophages which would predominantly adopt M1 phenotypes are induced into an M2 phenotype, favorable for tissue recovery. A similar phenomenon has recently been observed in animal models of wound healing, where it was reported that hMSC treated wounds displayed increases in CD206 positive macrophages (Zhang et al. 2010). Our studies suggest that endogenous macrophages are diverting to a more favorable phenotype, which could potentially provide a means of harnessing the inflammatory response to yield therapeutic benefits.

Furthermore, to date no one has reported therapeutic benefits in animal models at cell densities as low as were used here. On average, $\sim 5 \times 10^4$ encapsulated hMSC were administered within the subarachnoid space. Previous reports of lumbar puncture delivery of hMSC achieved maximal therapeutic benefits after 3 transplantations of 2×10^6 hMSC starting 1 week post contusion (Bakshi et al. 2006). The benefits observed in our studies were also provided by the free hMSC populations. Future studies will determine whether this response can be sustained longer than 1 week, as free hMSC may not persist long term at the site of injury (Coyne et al. 2006).

Overall our studies support the incorporation of encapsulated hMSC as an immunomodulatory delivery vehicle *in-vivo*. Alginate parameters were identified to maximize hMSC survival and protein secretion. We also demonstrated that encapsulated hMSC can attenuate macrophage activation *in-vitro*. Additionally, the encapsulated hMSC were able to modulate macrophage function to a state which, *in-vivo*, could promote tissue regeneration. This hypothesis was corroborated with an *in-vivo* model of SCI where encapsulated hMSC were able to promote pro-inflammatory macrophage attenuation at the site of injury. These macrophages expressed higher levels of alternatively activated phenotypic markers. The immobilization system developed here should circumvent many of the drawbacks in current hMSC administration platforms and at the same time may serve to augment hMSC tissue protective behavior.

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Figure 1. Viability and proliferation within various alginate micro-environments

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

Figure 2. Multiplex analysis of protein secretion from encapsulated hMSC

Secretion from encapsulated hMSC cultures normalized to baseline levels of monolayer hMSC secretion A) day 2 B) day 21 post-encapsulation. Secretion from TNF- α and IFN- γ stimulated encapsulated hMSC normalized to baseline levels of monolayer hMSC secretion C) 2 days D) 21 days post encapsulation. Statistical significance was established by 2 way ANOVA with confidence value of 0.05. Post hoc analysis to determine individual differences within the population was determined via Fisher LSD. For general ranges of protein secretion, reference Table 1.

Figure 3. Encapsulated hMSC attenuate macrophage activation *in-vitro*

A) TNF- α secretion from macrophages activated with 1 μ g/ml LPS over 24 hours treated with 10³ and 10⁴ free or encapsulated hMSC. Data is represented as mean TNF-levels normalized to activated macrophage conditions from three experiments. Asterisks (*) designate statistical significance ($p < 0.05$) compared to activated conditions. Gammas (γ) represent statistical significance ($p < 0.05$) compared to 10³ hMSC conditions. B) Representative immunocytochemical staining for iNOS depicting encapsulated hMSC mitigation of macrophage nitric oxide levels compared to activated macrophage cultures.

Figure 4. Evaluation of stimulated macrophage protein secretion in the presence of encapsulated hMSC

A) Non-activated macrophages baseline secretion. B) Macrophage secretion levels after activation with 1 μ g/ml LPS. C) LPS treated macrophage secretion in the presence of encapsulated 10⁴ hMSC. Asterisks (*) designate statistical significance (p < 0.05) compared to quiescent macrophages. Double Asterisks (**) represent statistical significance (p < 0.05) compared to activated macrophages.

Figure 5. Evaluating alternatively activated macrophage phenotypes in THP-1 -encapsulated hMSC co-cultures

A) Hoechst and representative CD206 staining of activated macrophages versus macrophages treated with 10³ hMSC. B) Quantitation of CD206 expression in activated macrophages and macrophages treated with 10³ to 10⁴ encapsulated hMSC. Data points represent sample means from four different experiments. Data was normalized to activated macrophage CD206 expression. C) IL-10 secretion from encapsulated hMSC treated macrophages. Asterisks (*) designates statistical significance ($p < 0.05$) compared to activated macrophages.

Figure 6. FITC-conjugated PLL coated alginate capsules localizing within the subarachnoid spaces of the lumbar region of the spinal cord

The capsules volumetrically measured $\sim 30 \mu\text{l}$. An equivalent volume of empty capsules, as well as saline, were administered as controls. Animals were observed for several weeks without any adverse treatment affects.

Figure 7. Effect of encapsulated hMSC on macrophage phenotype in an *in-vivo* model of spinal cord trauma

Transplantation of encapsulated hMSC increases the number of CD206 positive cells 8 days after SCI. Many activated macrophages were immunostained with ED1 antibody (pseudo-red) in injured spinal cords from all groups injected with saline (A, E), empty capsules (B, F), free hMSC (C, G), or encapsulated hMSC (D, H). Immunostaining for the M2 macrophage marker CD206 (pseudo-green) was higher in hMSC transplanted groups (free hMSC and encapsulated hMSC) compared to saline and capsule control group (E' – H'). Overlays of ED1 and CD206 staining are shown in E'' – H''. Ratios of the CD206 positive areas and the ratio of CD206⁺ to ED1⁺ cells were significantly increased in the encapsulated hMSC transplanted group compared to saline and capsule controls (I and J, respectively). Note that free hMSC increased CD206⁺ areas significantly while the increases in cell

number were not significant. Scale bar is 50 μm . (*, $P < 0.05$; **, $P < 0.01$ in ED1⁺; ##, $P < 0.01$; ###, $P < 0.001$ in CD206⁺; ++, $P < 0.01$ in ED1 and CD206 double-positive/ED1⁺, one-way ANOVA with Tukey's HSD test). Data represent mean $\pm$ standard error with 4-5 rats per group.

Table 1

General levels of hMSC secretion from 6×10^4 cells in basal and activated monolayer cultures.

Concentration (pg/ml)	Basal Medium	TNF- α /IFN- γ
2-10	IL2,IL1b,IL15,IL4	
10-30	IL13	IL4,IL13,IL2,IL1b
30-60	IL9, MIP1 α ,MIP1 β , PDGFBb, RANTES, FGFbasic	IL9
60-135	IL1R α , IL-12, Eotaxin, GCSF,IL10,IP10, GMCSF	IL10,IL12,FGFBbasic,GCSF,PDGFBb
135-180		MIP1 α ,MIP1 β ,IL15,Eotaxin
200-400		GMCSF, IL1R α
1000-3000	IL6, IL8, MCP-1	RANTES, MCP-1, IL6
10000>	VEGF	IL8,IP10, VEGF