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Alginate micro-encapsulation of mesenchymal stromal cells enhances modulation of the neuro-inflammatory response

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Abstract

Background aims—Modulation of inflammation after brain trauma is a key therapeutic goal aimed at limiting the consequences of the subsequent injury cascade. Mesenchymal stromal cells (MSCs) have been demonstrated to dynamically regulate the inflammatory environment in several tissue systems, including the central nervous system. There has been limited success, however, with the use of direct implantation of cells in the brain caused by low viability and engraftment at the injury site. To circumvent this, we encapsulated MSCs in alginate microspheres and evaluated the ability of these encapsulated MSCs to attenuate inflammation in rat organotypic hippocampal slice cultures (OHSC).

Methods—OHSC were administered lipo-polysaccharide to induce inflammation and immediately co-cultured with encapsulated or monolayer human MSCs. After 24 h, culture media was assayed for the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α) produced by OHSC, as well as MSC-produced trophic mediators.

Results—Encapsulated MSCs reduced TNF-α more effectively than did monolayer MSCs. Additionally, there was a strong correlation between increased prostaglandin E_2 (PGE_2) and reduction of TNF-α. 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, from which additional mediators with strong correlations to TNF-α levels were identified.

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Conclusions—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 treating neuro-inflammation.

Keywords

co-culture; inflammation mediators; mesenchymal stromal cells; organ culture; traumatic brain injury

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 [1,2] 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 [3] and traumatic brain injury [4,5]. Rather than serve as a direct cell replacement, in this therapy, MSCs are proposed to act as trophic mediators [6]—responding to several secondary injury mechanisms, including inflammation [7–9].

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 [10,11] as well as migration to other tissues [12,13]. To control long-term effects and localization, we have previously developed and characterized a method to encapsulate MSCs within alginate microspheres [14]. These encapsulated MSCs, but not free MSCs, significantly increased the number of anti-inflammatory macrophages in a spinal cord injury model [15] and prevented tissue degradation of organotypic hippocampal slice cultures (OHSC) [16]. 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 [17,18] 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.

Methods

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 [19]. 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°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.

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 [20]. Briefly, MSCs were cultured in MEM-α medium without ribo- and deoxyribonucleosides (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 μg/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°C in 5% CO_2.

Alginate micro-encapsulation

Alginate poly-L-lysine micro-encapsulation of MSCs was performed as previously described [14]. 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 [15]. 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 μm. 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 re-suspended in 5 mL of MEM-α (Life Technologies) and transferred to a 25-cm^2 tissue culture flask, maintained in an upright position. Encapsulated cells were incubated at 37°C 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 μL of capsules was added to 200 μL 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-μL 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.

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) [21,22] and immediately co-cultured with monolayer or encapsulated MSCs at 2.5×10^4, 5×10^4 or 1×10^5 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.

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.

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.

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).

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). Bio-plex 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 $P \leq 0.05$.

Results

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* [23,24] and *in vivo* [25,26]. 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 [27] and in the clinic [28]. 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 5×10^4 and 1×10^5 cells/well (Figure 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.

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 [17,18,29]. 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 (1×10^5 cells/well) (Figure 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.

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 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.

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 4A). We then compared the effects of exogenous versus MSC-produced PGE_2 on TNF-α reduction. As seen in Figure 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 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 4B, Table I). 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.

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 (1×10^5 cells/well ± 1 μg/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 upregulated or downregulated by encapsulated MSCs (Figure 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 5B).

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 6). These correlations, along with the correlation for PGE_2, are summarized in Table II in order of decreasing correlation. Nine of the 11 analytes demonstrate a significant correlation with TNF-α.

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 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 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.

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 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 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.

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 neuro-protection and promote regeneration after brain trauma [30], several studies have reported low efficiency of engraftment at the injury site and a decrease in cell number at the site over time [10,11]. 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 [13,31]. Our previous efforts have aimed to use alginate micro-encapsulation 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 [15] 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 [15].

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 [32,33]. 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 [17]. 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 [34,35], 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 [36]. In experimental models of cerebral ischemia, PGE_2 signaling through the EP_2 receptor was found to have neuro-protective effects [37,38], and induction of PGE_2 synthesis was demonstrated to reduce inflammation in experimental pleuritis [39,40]. 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 [17,29,41], 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 [18]. 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 micro-environment enhances MSC secretion patterns as compared with monolayer MSCs, both in the presence and absence of inflammatory cues [15]. Though alginate has been widely used and studied as a biomaterial for immobilization and delivery of cell therapies [42,43] and is generally accepted as a biocompatible material for implantation and long-term efficacy [44–46], some studies have reported activation of host immune and inflammatory responses to alginate [47] that may be dependent on the purity and composition of alginate [48–50]. 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 [51–53]. 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 [54], and, in cross-linked methacrylated hyaluronic acid hydrogels, MSC secretion of cytokines and angiogenic factors was found to be dependent on hydrogel stiffness [55]. In addition, MSCs encapsulated in alginate hydrogels were reported to upregulate secreted growth factor expression when subject to compression forces, suggesting the ability of MSCs to modulate gene expression in response to their mechanical environment [56]. Finally, there is recent evidence that spheroid aggregate culture of MSCs enhances anti-inflammatory properties [57,58], 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*.

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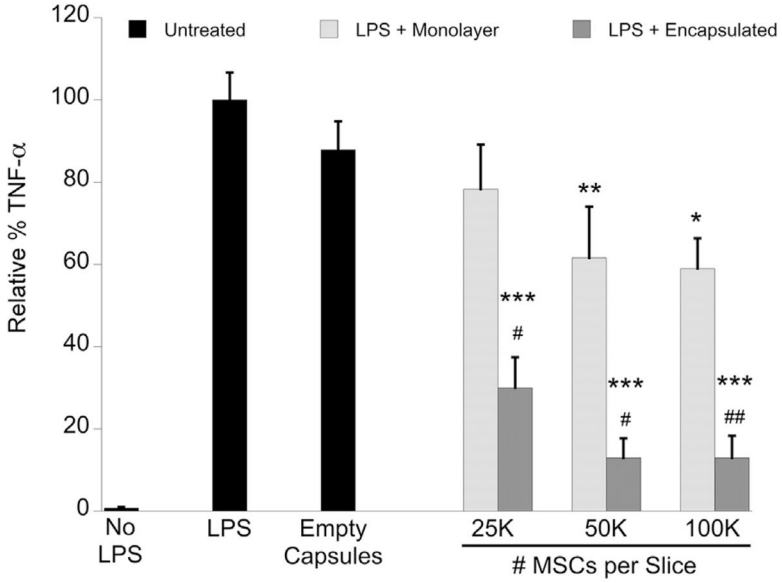

Figure 1.

Rat TNF-α ELISA of cell culture media supernatant collected after 24 h of LPS stimulation ± MSC treatment in OHSC. Data are normalized to untreated LPS-stimulated OHSC and represented as mean ± SE from five 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 with LPS + no treatment; $^{\#}P < 0.01$, $^{\#\#}P < 0.002$ compared with treatment with equivalent number of free MSCs.

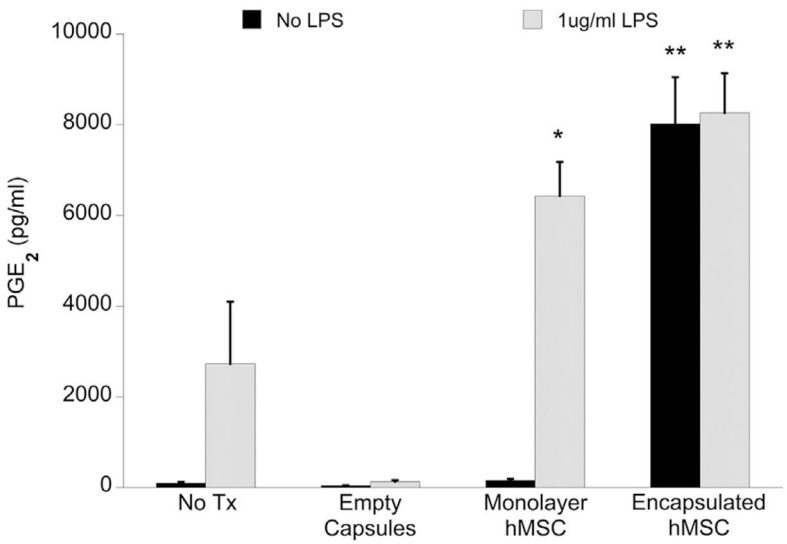

Figure 2.

Total PGE_2 concentration in cell culture media supernatant collected after 24 h of culture ± MSC treatment (1×10^5 MSCs/well, with or without LPS), as measured by EIA. Data are represented as mean ± SE from three experiments, each with n = 2–3 cultures per condition. Encapsulated MSC conditions resulted in a significant increase of total PGE_2 concentration as compared with 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 with 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 with OHSC + LPS + no MSC treatment.

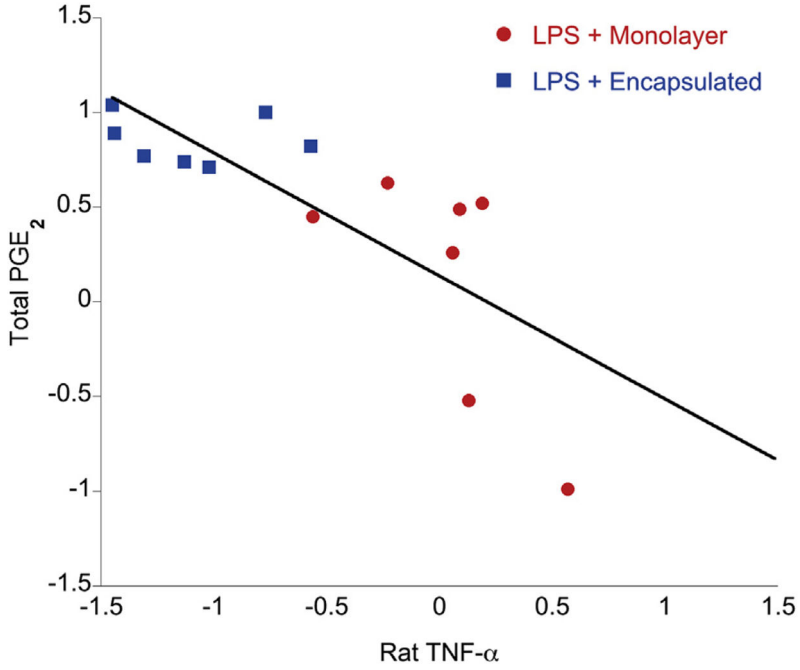

Figure 3.

Correlation between total PGE_2 and rat TNF-α measured in OHSC culture supernatants for MSC treatment conditions (1×10^5 MSCs/well). Linear regression and Pearson's co-efficient (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.

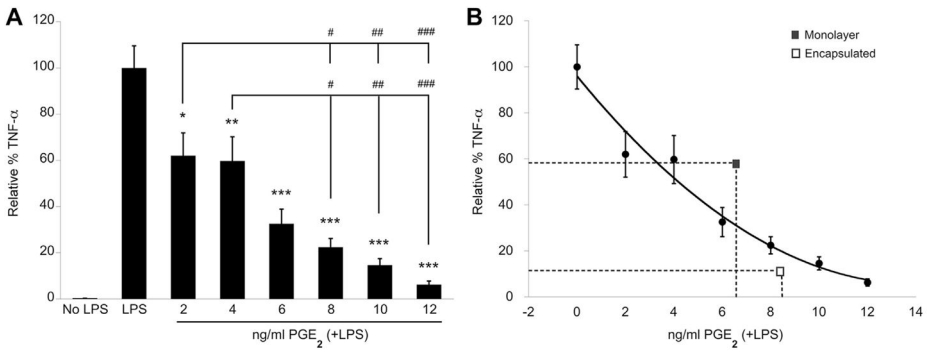

Figure 4.

(A) Rat TNF-α ELISA of cell culture media supernatant collected from organotypic cultures after 24 h of LPS stimulation ± human PGE_2. Data are normalized to untreated LPS-stimulated OHSC and represented as mean ± SE from three 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 with LPS + no treatment. $^{\#}P < 0.01$, $^{\#\#}P < 0.001$, $^{\#\#\#}P < 0.0001$ between dose groups. (B) Polynomial curve fit of data presented in A, overlaid with corresponding mean levels of PGE_2 production and TNF-α reduction by monolayer and encapsulated MSCs.

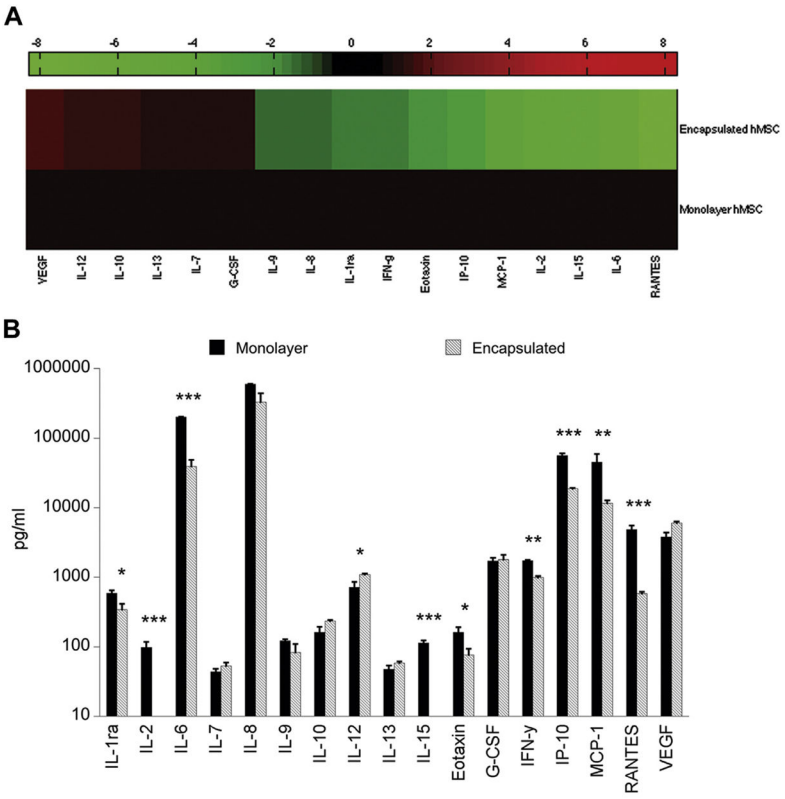

Figure 5.

(A) Heat map representation of multiplex (human) secretome data. Secretion by encapsulated MSCs (1×10^5 cells/well) co-cultured with LPS-stimulated OHSC is normalized to secretion by monolayer MSCs (1×10^5 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 h of MSC co-culture with LPS-stimulated hippocampal slices. Data are represented as mean ± SE from one 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 with monolayer MSCs. $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0001$.

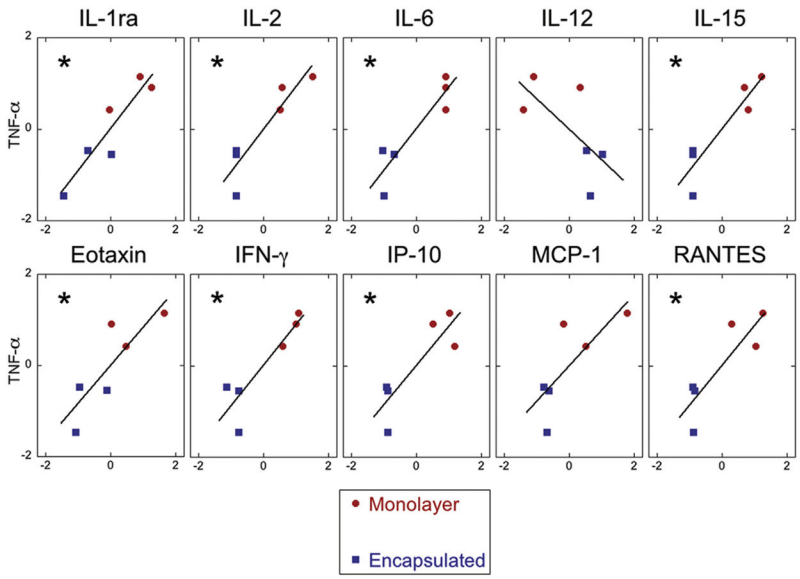

Figure 6.

Correlation between rat TNF-α and MSC-secreted factors measured by means of multiplex bead assay for monolayer and encapsulated MSC treatment conditions. Only analytes that exhibited significant differences between treatment groups (monolayer versus encapsulated MSC) are depicted. Linear regression and Pearson's coefficient (r) were derived from z-scores of standardized data. Co-efficients of correlation (r) and significance for each analyte can be found in Table II. $*P < 0.05$.

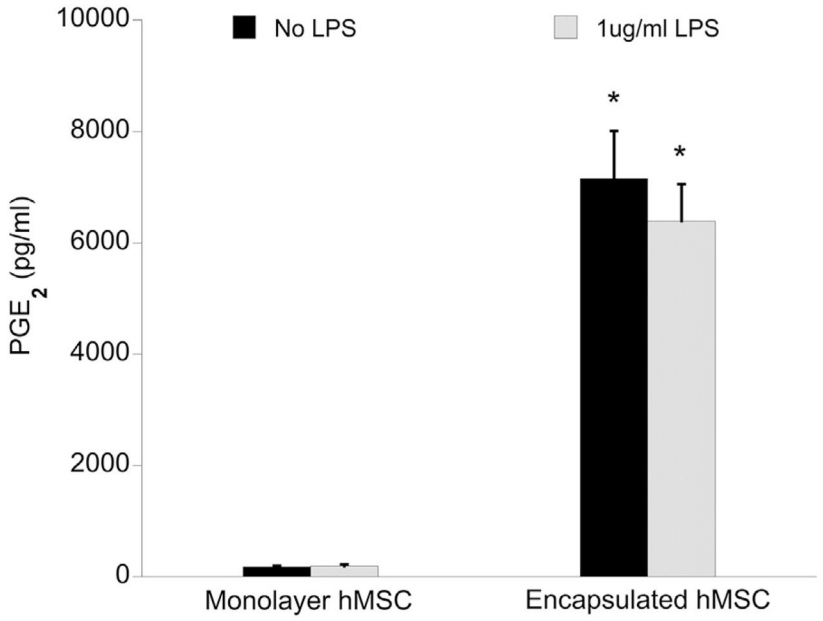

Figure 7.

Total PGE_2 concentration in MSC cell culture media supernatant (1×10^5 cells/well) collected after 24 h ± 1 μg/mL LPS, as measured by EIA. Data are represented as mean ± SE from three experiments, each with $n = 2–3$ cultures per condition. Encapsulated MSCs produced a significantly greater amount of PGE_2 compared with monolayer MSC, regardless of LPS presence. $*P < 0.0001$ compared with monolayer MSC counterpart.

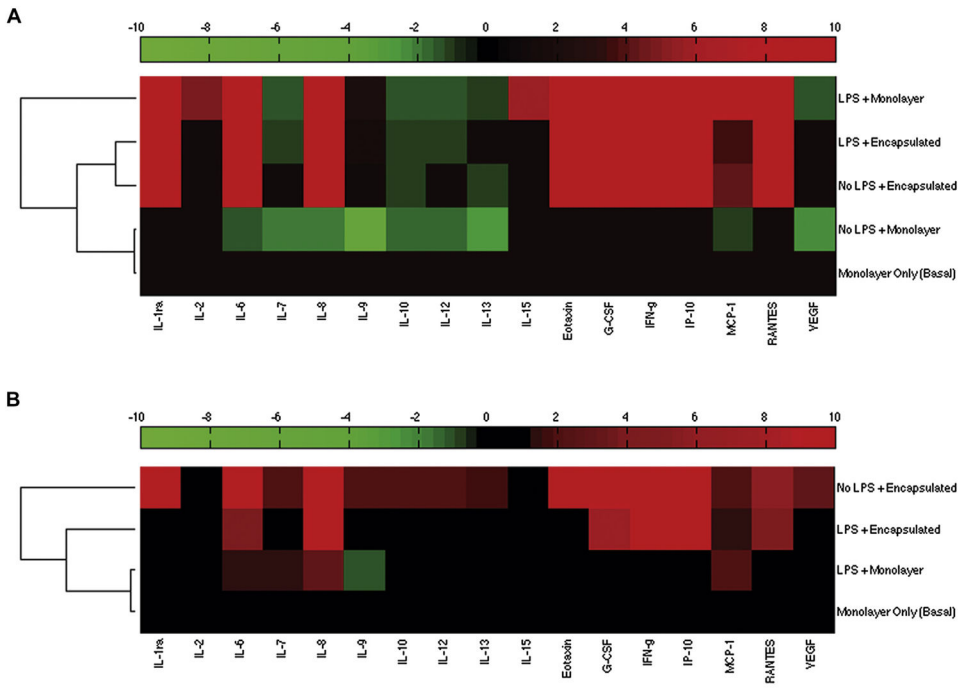

Figure 8.

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, 1×10^5 MSCs/well were used. Increased levels of secretion are represented in shades of red and decreased levels in shades of green.

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

Experimental versus estimated values of rat TNF-α (percentage of maximum production) after treatment with monolayer or encapsulated hMSCs (1×10^5 cells/well). Estimated TNF-α values were calculated from a polynomial curve fit of TNF-α levels after exogenous PGE_2 administration (Figure 4B), with use of the concentration of PGE_2 detected in media collected from LPS-stimulated OHSC treated with hMSCs (Figure 2).

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	↑

Correlation between hMSC-secreted factors found to be significantly different between treatment groups (monolayer versus encapsulation hMSC) 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 hMSC-secreted factors significantly different between treatment groups, 9 demonstrate a significantly ($P < 0.05$) 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. $n = 14$ for PGE_2, $n = 6$ for all others.

[a]Total PGE_2 in supernatant measured, all other analytes measured are human-specific.