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Prostaglandin E₂ Produced by Alginate-Encapsulated Mesenchymal Stromal Cells Modulates the Astrocyte Inflammatory Response*

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Astroglia are well known for their role in propagating secondary injury following brain trauma. Modulation of this injury cascade, including inflammation, is essential to repair and recovery. Mesenchymal stromal cells (MSCs) have been demonstrated as trophic mediators in several models of secondary CNS injury, however, there has been varied success with the use of direct implantation due to a failure to persist at the injury site. To achieve sustained therapeutic benefit, we have encapsulated MSCs in alginate microspheres and evaluated the ability of these encapsulated MSCs to attenuate neuro-inflammation. In this study, astroglial cultures were administered lipopolysaccharide (LPS) to induce inflammation and immediately co-cultured with encapsulated or monolayer human MSCs. Cultures were assayed for the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) produced by astroglia, MSC-produced prostaglandin E₂, and expression of neurotrophin-associated genes. We found that encapsulated MSCs significantly reduced TNF-α produced by LPS-stimulated astrocytes more effectively than monolayer MSCs, and this enhanced benefit commences earlier than that of monolayer MSCs. Furthermore, in support of previous findings, encapsulated MSCs constitutively produced high levels of PGE₂, while monolayer MSCs required the presence of inflammatory stimuli to induce

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PGE$_2$ production. The early, constitutive presence of PGE$_2$ significantly reduced astrocyte-produced TNF-α, while delayed administration had no effect. Finally, MSC-produced PGE$_2$ was not only capable of modulating inflammation, but appeared to have an additional role in stimulating astrocyte neurotrophin production. Overall, these results support the enhanced benefit of encapsulated MSC treatment, both in modulating the inflammatory response and providing neuroprotection.

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.[1,2] 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.[3,4]

Mesenchymal stromal cells (MSCs) as a therapeutic have been demonstrated as trophic mediators in several models of CNS injury and neuroinflammation, both *in vitro*[5,6] and *in vivo*,[7,8] and in particular, to target astroglial-mediated inflammation.[9,10] 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, as a result of their failure to localize and persist at the injury site,[11,12] and their ability to migrate to other tissues.[13,14] To control long-term effects and localization, we have previously developed and characterized a method to encapsulate MSCs within alginate microspheres,[15] 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,[7] and modulated the inflammatory response in organotypic hippocampal slice culture (OHSC)[16] more effectively than monolayer MSCs. In the latter study, prostaglandin E$_2$ (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 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,[17–19] 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 previous findings 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 we demonstrated the additional role of PGE$_2$ in stimulating astrocyte neurotrophin production. Taken together, these results support the enhanced benefit of encapsulated MSC treatment, both in modulating the inflammatory response and providing neuroprotection.

2. Materials and Methods

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.[20] Briefly, Sprague–Dawley rat pups (Taconic Biosciences Inc., Rensselaer, NY) 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, St. Louis, MO). Cerebral cortices were isolated, cut into small pieces after removal of the meninges, and incubated in Gey's Balanced Salt Solution (GBSS) $+ 0.25\%$ Trypsin–EDTA (Sigma–Aldrich, St. Louis, MO) for 20 min in a 37°C water bath. After 20 min, the tissue suspension was triturated and Dulbecco's Modified Eagle Medium (DMEM) (Sigma–Aldrich, St. Louis, MO) containing 10% Fetal Bovine Serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) was added to stop trypsinization. The cells were pelleted at 1200 rpm for five min, resuspended in DMEM containing 10% FBS, 100 units/ml penicillin and $100\,\mu$g/ml streptomycin (Life Technologies, Carlsbad, CA) ("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 days) and used for experiments at passage one to two. For glial cultures, cells were cultured for 7–10 days, with media exchanged every 2–3 days. To isolate microglia, cultures were shaken at 180 rpm for two hours. The cells in suspension were removed and plated for experiments. Both astrocytes and microglia were plated in 24-well plates (5×10^4 cells/well) two days prior to experiments.

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.[21] Briefly, MSCs were cultured in Minimum Essential Medium alpha (MEM-α) without ribo- and deoxyribo-nucleosides (Life Technologies, Carlsbad, CA), supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA), 1 ng/ml basic fibroblast growth factor (Peprotech, Rocky Hill, NJ), 100 units/ml penicillin, and $100\,\mu$g/ml streptomycin (Life Technologies, Carlsbad, CA). Cells were plated at 5,000 cells per cm^2 and allowed to proliferate to 70% confluence (approximately 4–5 days) before passaging. Only MSCs at passages two through five 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 2.5×10^4, 5×10^4, or 1×10^5 cells/well. All cultures were incubated at 37°C in 5% CO$_2$.

2.3. *Alginate microencapsulation*

Alginate poly-L-lysine microencapsulation of MSCs was performed as previously described.[15] A 2.2% (w/v) alginate solution (MW: 100,000–200,000 g/mol, G-content 65–70%, Sigma–Aldrich, St. Louis, MO) was generated with Ca^{2+}-free DMEM (Life Technologies, Carlsbad, CA). Cultured MSCs were dissociated and resuspended in 2.2% alginate to yield a final solution of 4×10^6 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.[7] The cell suspension was transferred to a syringe pump (KD Scientific, Holliston, MA), set at a flow rate of 10 mL/h. Alginate beads were generated using an electrostatic bead generator (Nisco, Zurich, Switzerland) with accelerating electrode at an applied voltage of 6.4 kV. The resulting bead diameter was $500 \pm 50\,\mu$m. The beads were extruded into a bath of CaCl$_2$ (100 mM) (Sigma–Aldrich, St. Louis, MO) containing 145 mM NaCl (Sigma–Aldrich, St. Louis, MO) and 10 mM MOPS (Sigma–Aldrich, St. Louis, MO). Microencapsulated cells were washed once with phosphate buffered saline (PBS) (Sigma–Aldrich, St. Louis, MO) and then treated for two minutes with poly-L-lysine (Sigma–Aldrich, St. Louis, MO, MW: 68,600 g/mol) (0.05% w/v), followed by an additional PBS wash. The microencapsulated cells were resuspended in 5 ml MEM-α (Life Technologies, Carlsbad, CA) 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 one day post-encapsulation. To determine average number of cells per capsule for dosing purposes, $15\,\mu$l of capsules were added to $200\,\mu$l of 1% 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 five minutes to allow lysis of the alginate and release of MSC from capsules. A $10\,\mu$l volume of these cell suspensions was counted on a hemacytometer to determine the 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. Based on the number of capsules necessary to achieve the desired MSC dose, an equivalent number of capsules was chosen for empty capsule controls.

2.4. *LPS injury and co-culture*

Polyester membrane transwell inserts (Corning Inc. Tewksbury, MA, $6.5\,$mm, $0.4\,\mu$m) containing monolayer or encapsulated MSCs (2.5×10^4, 5×10^4, or 1×10^5 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 $100\,\mu$g/ml streptomycin ("low serum media") $\pm 1\,\mu$g/ml LPS (*Escherichia coli* 055:B5, Sigma–Aldrich, St. Louis, MO).[22,23] Nonstimulated and stimulated host cultures without MSC co-culture were used as controls. Cultures were returned to the incubator at 37°C in 5% CO_2 for 6, 12, 24, or 48 h, after which media supernatants were collected and cells were fixed.

2.5. *PGE$_2$ and blocking studies*

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

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 astrocytes or microglia using a rat TNF-α ELISA (Biolegend, San Diego, CA) according to the manufacturer's instructions. Total PGE_2 secretion (rat + human) was evaluated using Prostaglandin E_2 EIA (Cayman Chemical, Ann Arbor, MI), according to the manufacturer's instructions.

2.7. *PCR array*

For the analysis of astrocyte neurotrophin and neurotrophin receptor expression after LPS, LPS + monolayer MSC, LPS + encapsulated MSC, and LPS + $20\,$ng/ml PGE_2 treatments, experiments were carried out as described above. After 24 h, medium was collected and assayed for TNF-α as described above. Cells were washed once with PBS, then dissociated with 0.25% Trypsin–EDTA (Sigma–Aldrich, St. Louis, MO) for five minutes, after which trypsin 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°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 the gene of interest (GOI) and an average of reference genes (HKG), followed by $\Delta\Delta C_T$ calculations (ΔC_T (Test Group) $- \Delta C_T$ (Control Group)). Fold Change was then calculated using $2^\wedge(-\Delta\Delta C_T)$ formula. Nonsupervised hierarchical cluster analysis of the entire dataset was generated using the Qiagen data analysis web portal (http://www.qiagen.com/geneglobe).

2.8. *Statistical analysis*

All results are expressed as a mean ± standard error (S.E.). All data presented are averaged from ≥ 3 separate experiments, each with $N = 2$–3 independent replicates. PCR array data are obtained from one experiment, with $n = 6$ cultures per condition, and samples pooled per condition. Kaleida Graph (Synergy Software, Reading, PA) 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. Results

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*[24,25] and *in vivo*.[26,27] To evaluate the ability of MSC treatment to attenuate the astroglial inflammatory response, we stimulated astrocyte or microglial cultures with 1 μg/ml LPS and concurrently treated with monolayer or encapsulated MSCs for 24 h, after which cell culture

media was assayed for the pro-inflammatory cytokine TNF-α produced by the host cultures.

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, [Fig. 1(a)]. Additionally, at 1×10^5 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. In microglia, however, 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 [Fig. 1(b)].

3.2. *Encapsulated MSCs are more effective than monolayer in reducing TNF-α, and exhibit increased PGE₂ 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 1 μg/ml LPS and treated with monolayer or encapsulated MSCs (1×10^5 cells/transwell), and cell culture media was collected at 6, 12, 24, and 48 h. Rat TNF-α and total PGE₂ were evaluated by ELISA and EIA, respectively. We found that TNF-α production by
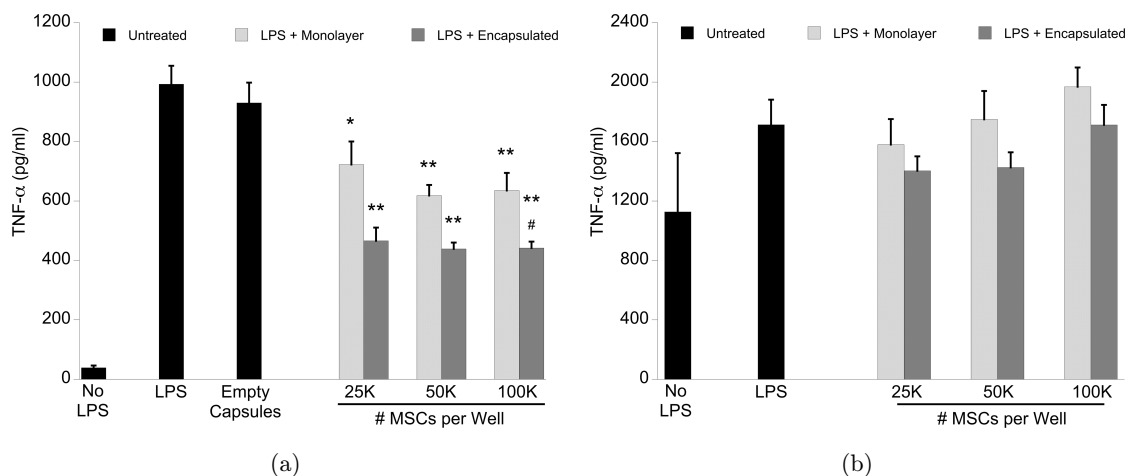

(a)   (b)

Fig. 1. Rat TNF-α ELISA of cell culture media supernatant collected after 24 h of LPS stimulation ±MSC treatment in astrocyte (a) and microglia (b) cultures. Data are represented as mean ±S.E. from three 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 number of monolayer MSC.

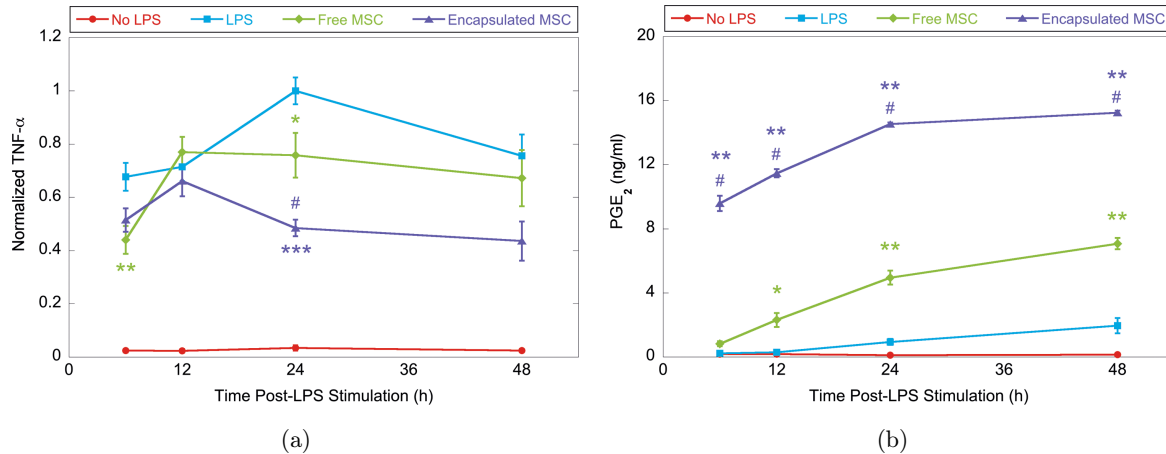Fig. 2. Temporal profile of rat TNF-α and total PGE$_2$ levels in culture media collected after LPS stimulation $\pm$ MSC treatment in astrocytes. TNF-α data are normalized to untreated LPS-stimulated cultures at 24 h. All data are represented as mean $\pm$ S.E. from three experiments, each with $N = 3$ 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 48 h 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 6 h post-stimulation, whereas monolayer MSCs start producing PGE$_2$ at significantly lower levels from 12 h post-stimulation. $*= p < 0.001$, $**= p < 0.0001$ compared to LPS only, $\#= p < 0.0001$ compared to LPS + monolayer MSC.

LPS-stimulated astrocytes reached a maximum at 24 h post-stimulation, and that after 12 h, encapsulated MSC treatment performed better than monolayer MSC treatment, though this effect was only significant at the 24 h time point [Fig. 2(a)]. All data are normalized to untreated, LPS-stimulated astrocytes at 24 h 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[7] and organotypic hippocampal slice cultures.[16] 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 h versus 12 h post-stimulation) [Fig. 2(b)].

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$_2$ they produce from early time points post-LPS stimulation, as well as previous data correlating increased PGE$_2$ with decreased TNF-α,[16] 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 h after and evaluated culture media for rat TNF-α secretion 24 h post-LPS stimulation. There is a dose-response effect of increasing human PGE$_2$ on reducing TNF-α when immediately administered (0 h), but no significant reduction of TNF-α by any PGE$_2$ dose when administered 6 h post-stimulation (Fig. 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,[28,29] 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.[30] 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 $1\,\mu g/ml$ LPS $\pm$ receptor agonists, and cell culture media was collected at 24 h. Using ELISA for rat TNF-α, we found that the EP2 and EP4 receptors are highly involved in reducing TNF-α, and the
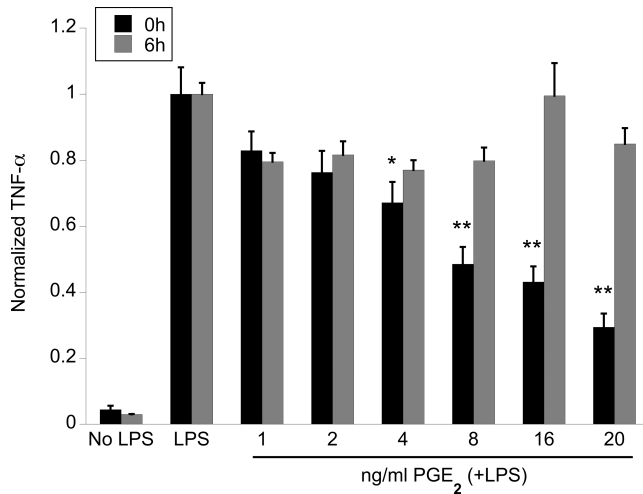
Fig. 3. Rat TNF-α ELISA of cell culture media collected from astrocyte cultures after 24 h of LPS stimulation $\pm$human PGE$_2$. Data is normalized to untreated LPS-stimulated astrocytes and represented as mean $\pm$ S.E. from three experiments, each with $N = 3$ cultures per condition. Addition of exogenous human PGE$_2$ significantly reduced TNF-α levels in a dose dependent manner when immediately administered, but had no effect when administered 6 h after LPS. *= $p < 0.01$, **= $p < 0.0001$ compared to LPS only.

EP1 receptor to a lesser, but significant, extent [Fig. 4(a)], 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 $(10\,\mu\text{M})$ 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 $1\,\mu\text{g/ml}$ LPS + $20\,\text{ng/ml}$ PGE$_2$ $\pm$ receptor antagonists for 24 h, 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 [Fig. 4(b)]. 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.

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 modulating TNF-α production. Astrocyte cultures were administered $1\,\mu\text{g/ml}$ LPS and treated with monolayer or encapsulated MSCs $(1 \times 10^5$ cells/transwell) $\pm$ receptor antagonists, and cell culture media was collected after 24 h for evaluation by TNF-α ELISA. Significant blocking of the MSC-mediated TNF-α reduction is achieved with
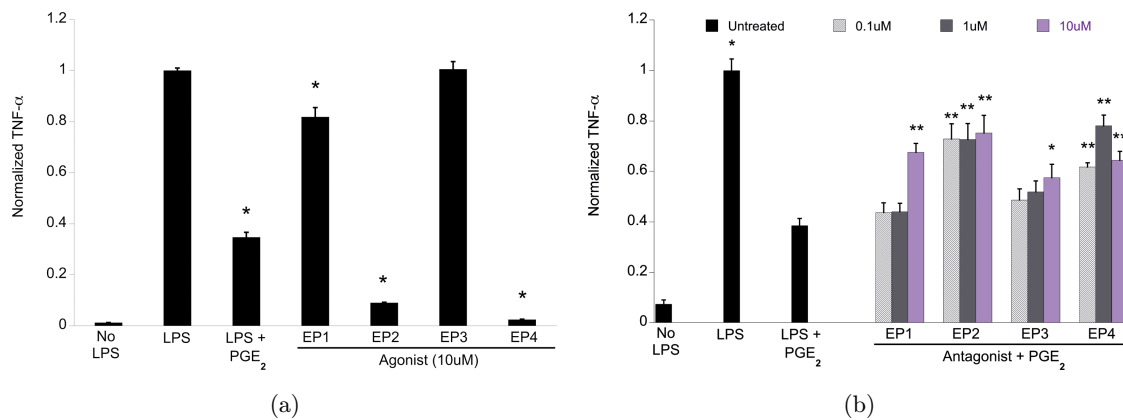

(a)

(b)

Fig. 4. Effect of PGE$_2$ receptor subtype-specific agonists and antagonists on TNF-α reduction. Data are normalized to untreated LPS-stimulated astrocytes and represented as mean $\pm$ S.E. from three experiments, each with $N = 3$ cultures per condition. (a) Rat TNF-α produced by astrocyte cultures after 24 h of LPS stimulation $\pm$ EP receptor agonist iloprost (EP1), butaprost (EP2), sulprostone (EP3), or CAY1058 (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 astrocyte cultures after 24 h of LPS stimulation + $20\,\text{ng/ml}$ PGE$_2$ $\pm$ 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 + PGE$_2$.
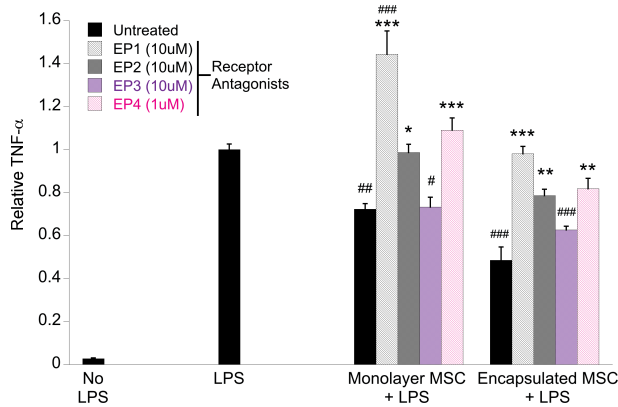
Fig. 5. PGE$_2$ receptor antagonist blocking of MSC treatment. Rat TNF-α produced by astrocytes after 24 h of LPS stimulation + MSC (monolayer or encapsulated) $\pm$ EP receptor antagonist SC-51322 (EP1), PF-04418948 (EP2), L-798,106 (EP3), or L-161,982 (EP4). Significant blocking of MSC-mediated TNF-α 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.

EP1, EP2, and EP4 receptor antagonists, but as with the agonist study, no effect is seen when targeting the EP3 receptor (Fig. 5). Hence, it appears

MSC-produced PGE$_2$ is anti-inflammatory via binding to the EP1, EP2, and EP4 receptors.

3.5. *Comparative responses of LPS-stimulated astrocytes to MSC and PGE$_2$*

MSC appeared to reduce the inflammatory response via the secretion of PGE$_2$. Of course, PGE$_2$, is just one of many molecules secreted by MSC, and MSC and PGE$_2$ affect other aspects of cell behavior. For example, although PGE$_2$ is best known for its role in the inflammatory response, several studies have demonstrated additional downstream effects in stimulating expression and/or production of neurotrophic factors[17–19] as well as neuroprotective effects.[31–33] Given the potential for broader responses to these two therapies, as well as differences between free and encapsulated MSC, we preliminarily screened gene expression by astrocytes of a number of factors that may contribute to the neuroprotective and/or regenerative environment following TBI. We were particularly interested in identifying similarities and differences in the expression profiles of astrocytes induced to an inflammatory state with LPS that were treated with PGE$_2$ or with MSC.
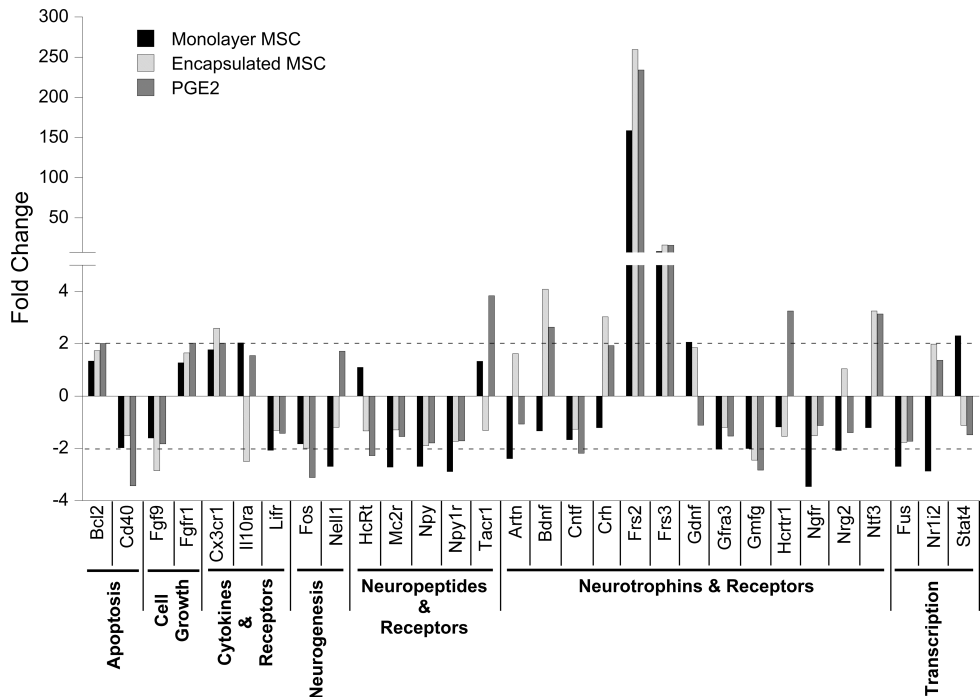

Fig. 6. Fold changes in astrocyte neurotrophin-associated gene expression after MSC or PGE$_2$ treatment for 30 genes (of 84 assayed) that exhibited at least two-fold up- or down-regulation (dashed line) in one or more treatment conditions evaluated, relative to untreated, LPS-stimulated astrocytes.

Astrocyte cultures were administered $1\,\mu\text{g/ml}$ LPS and concurrently treated with monolayer or encapsulated MSCs (1×10^5 cells/transwell), or $20\,\text{ng/ml}$ PGE$_2$ for 24 h. Separate astrocyte cultures were not stimulated with LPS and were left untreated. After 24 h, astrocytes were harvested for RNA isolation and analysis by PCR array for expression of 84 neurotrophin, neurotrophin receptor, and neurotrophin-associated genes. Separate TNF-α levels in these cultures were consistent with results shown in Figs. 1 and 2, indicating that the cultures were representative of the typical response. Fold changes in expression relative to unstimulated, untreated astrocytes are shown in Supplemental Table 1. To compare the responses in the therapeutic conditions, the data for LPS-stimulated astrocytes treated with free MSC, encapsulated MSC, and PGE$_2$ were normalized to the response of untreated, LPS-stimulated astrocytes (Fig. 6). For 30 genes, a greater than two-fold change in expression was observed for at least one condition, and for six of these 30 genes, fold changes induced by encapsulated MSC treatments paralleled those observed with PGE$_2$ treatment. These preliminary but encouraging trends suggest that increased PGE$_2$ production by encapsulated MSCs may confer an enhanced neuroprotective effect over monolayer MSCs.

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,[23,34] excitotoxicity,[35] and free radical-mediated injury.[36,37] 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,[38] 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.[39,40]

Several studies have demonstrated the therapeutic potential of MSCs to target multiple components of the secondary injury cascade following TBI, including neuroinflammation[9,41,42] — specifically, through modulation of the tissue and cellular environment.[43] Direct delivery of cells, however, presents limitations to long-term benefits and clinical translation due to lack of persistence at the injury site and a decrease in cell number at the site over time.[11,44,45] Additionally, it has been demonstrated that intravenously administered MSCs migrate to nontarget tissues, including the liver, spleen, kidney, and lungs, even up to one year after treatment.[14,46] To overcome these limitations, we have immobilized MSCs in alginate microspheres, which have been shown to persist in the brain up to six months.[47] Our previous studies have used alginate microencapsulation of MSCs to deliver cells after spinal cord injury (SCI). These encapsulated MSCs promoted the anti-inflammatory M2 macrophage phenotype, in both *in vitro* macrophage culture and an *in vivo* model of SCI, and reduced levels of pro-inflammatory TNF-α and the activation marker inducible nitric oxide synthase (iNOS) when co-cultured with LPS-stimulated macrophages.[7] The treatments were delivered 24 h after spinal cord injury, which suggests a long therapeutic window.

In previous studies, 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.[16] This is consistent with report that MSC-secreted PGE$_2$ is an important mediator of inflammation,[6] and that 3D aggregates of MSCs (spheroid culture) display enhanced PGE$_2$ production and anti-inflammatory properties.[48,49] Following these results, we herein 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 h) and is maintained to at least 48 h post-treatment. Previous characterization of viability and proliferation of MSCs within the alginate microcapsule reveals a far lower proliferation rate than that of monolayer MSCs,[7] which is consistent with our previous findings with encapsulated embryonic stems cells in an alginate microenvironment.[50] Though not explored in this study, these data render it unlikely that enhanced reduction of TNF-α is a result of differences in cell number over the culture period.

Having previously identified PGE$_2$ as a key MSC-produced inflammatory mediator in macrophage[51] and OHSC[16] 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-[29,52] and anti-inflammatory,[53,54] and has roles in pain,[55–57] cancer,[58,59] neuroprotection,[31–33] and wound repair,[60,61] among others.[62] This diversity of functions is largely attributed to the ability of PGE$_2$ to bind four receptor subtypes — EP1, EP2, EP3, and EP4[30] — that mediate PGE$_2$ actions through distinct downstream signaling pathways.[63] In neurological pathology alone, PGE$_2$ displays signaling versatility dependent on receptor binding, affinity, and expression levels — often with opposing actions.[64] The EP1 and EP3 receptors have been implicated in excitotoxic cell death and exacerbation of injury in models of cerebral ischemia,[65–67] while the EP2 and EP4 receptors have demonstrated

neuroprotection against excitotoxic insult[68,69] and cerebral ischemia.[32,33,70] In contrast to the neuroprotective effects in models of excitoxicity, EP2 elicits an opposing, neurotoxic response in models of neurodegeneration[71,72] and has demonstrated conflicting roles in neuroinflammation. Activation of the EP2 receptor induced neurotoxicity in LPS-stimulated OHSC[73] and microglia-neuron co-cultures,[74] but was also shown to reduce IL-1β production[75] and iNOS expression[76] by LPS-stimulated microglia. Signaling through the EP4 receptor attenuated neuroinflammation in LPS-stimulated microglial[77] and macrophage[51] cultures, and deletion of microglial EP4 in a mouse Alzheimer's model increased inflammation and Aβ deposition.[78]

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 all four receptor subtypes are known to be expressed by astrocytes,[64] 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.[75,76] 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.[79] The role of EP1 in neuroinflammation, specifically, has not been thoroughly explored, but EP1 activation has been shown to propagate inflammatory pain.[56,80] To the best of our knowledge, this is the first study to demonstrate a role of the EP1 receptor in modulating astrocyte-mediated inflammation.

PGE$_2$ and MSC treatments may affect a number of cellular responses in addition to their anti-inflammatory effects, including neuroprotection[32,78] and neurotrophic factor production,[19,81] both of which may further enhance recovery for TBI. To preliminarily evaluate similarities and differences between PGE$_2$- and MSC-treatments following induction of inflammation in astrocyte cultures, and to identify additional potential benefits of MSC

encapsulation, we screened an array of the genes using PCR. The PCR array panel revealed many genes that were affected by all three treatments, which is consistent with PGE₂–mediated changes. However, expression of a number of genes differed following the three treatments. For example, exogenous PGE₂ 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 PGE₂ and encapsulated MSC treatment conditions, suggesting that encapsulated MSC-induced changes in expression may be largely due to increased PGE₂ production. Dissimilarities between these conditions also exist, where encapsulated MSC-induced gene regulation more closely matches that of monolayer MSCs than exogenous PGE₂. The changes previously observed in the MSC secretome in response to OHSC inflammatory signals,[16] 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 PGE₂, and the necessity of early PGE₂ administration to reduce inflammation. Additionally, we determined EP receptor subtypes through which exogenous and MSC-produced PGE₂ 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. The goals of the current study were to examine the mechanisms of MSC-mediated regulation of the inflammatory response of astrocytes, and a number of studies must be completed before clinically translating the therapy, such as identifying a therapeutic window for administering the treatment. However, previous work with encapsulated MSC following SCI demonstrated efficacy in mitigating inflammation when the treatment was applied 24 h after the initial trauma.

Acknowledgments

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