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Prostaglandin E_2 Produced by Alginate-Encapsulated Mesenchymal Stromal Cells Modulates the Astrocyte Inflammatory Response

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Abstract

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 E_2, 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

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levels of PGE_2, while monolayer MSCs required the presence of inflammatory stimuli to induce 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 appears 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.

Keywords

Astroglia; inflammatory mediators; mesenchymal stromal cells; prostaglandin E_2; traumatic brain injury

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 proinflammatory 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 in order to identify the specific cellular targets of MSC anti-inflammatory benefit. We also further investigated the mechanisms of PGE_2-mediated inflammatory modulation. Additionally, because PGE_2 is a pleiotropic molecule

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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 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 μg/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.

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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 μg/ml streptomycin (Life Technologies, Carlsbad, CA). Cells were plated at 5000 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 solution 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 ± 50 μm. The beads were extruded into a bath of $CaCl_2$ (100mM) (Sigma–Aldrich, St. Louis, MO) containing 145mM NaCl (Sigma–Aldrich, St. Louis, MO) and 10mM MOPS (Sigma–Aldrich, St. Louis, MO). Micro encapsulated cells were washed once with phosphate buffered saline (PBS) (Sigma–Aldrich, St. Louis, MO) and then treated for two min 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 5ml 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 μl of capsules were added to 200 μl 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 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 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.

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2.4. LPS injury and co-culture

Polyester membrane transwell inserts (Corning Inc. Tewksbury, MA, 6.5 mm, 0.4 μm) 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 μg/ml streptomycin ("low serum media") ±1 μg/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 incubators 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 ±1 μg/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 μM, or 10 μM. 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 μM, or 10 μM. 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 μM SC-51322, 10 μM PF-04418948, 10 μM L-798,106, or 1 μM 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/mlPGE_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 min, after which trypsinization was neutralized with astrocyte maintenance medium. The cells were harvested and samples pooled per condition, then spun down and resuspended in PBS. Cells were again centrifuged and the PBS supernatants were removed. Pellets were flash frozen on liquid nitrogen, and stored at −80°C. RNA isolation and RT-PCR were performed by Qiagen

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(Frederick, MD), using manufacturer-specific kits and a rat neurotrophin and neurotrophin receptor array (RT^2 Profiler PCR Array, Cat. # PARN 031Z). Fold change/regulation was calculated using the $\Delta\Delta C_T$ method, in which ΔC_T is calculated between gene of interest (GOI) and an average of reference genes (HKG), followed by $\Delta\Delta C_T$ calculations (ΔC_T (Test Group) – ΔC_T (Control Group)). Fold Change was then calculated using $2^{\wedge}(-\Delta\Delta C_T)$ 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 PGE_2 production

Having identified astrocytes as a target of MSC treatment for neuroinflammation, we then further characterized the treatment response over time. Astrocyte cultures were administered 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 PGE_2 were evaluated by ELISA and EIA, respectively. We found that TNF-α production by LPS-

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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] In order to determine the prostaglandin subtypes involved in reducing astrocyte-produced TNF-α, we first used agonists specific for each of the four receptor subtypes—EP1 (iloprost), EP2 (butaprost), EP3 (sulprostone), and EP4 (CAY10598). Astrocyte cultures were administered 1 μg/ml LPS ± 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 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 μM) 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 μg/ml LPS + 20 ng/ml PGE_2 ± 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

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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 μg/ml LPS and treated with monolayer or encapsulated MSCs (1×10^5 cells/transwell) ± 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 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 play 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.

Astrocyte cultures were administered 1 μg/ml LPS and concurrently treated with monolayer or encapsulated MSCs (1×10^5 cells/transwell), or 20 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.

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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 benefit 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

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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 encapsulating 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] as well as having 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 astrocytes are known to express all four receptor subtypes,[64] their contribution to the astrocyte-induced inflammatory response, and attenuation thereof, remains relatively uncharacterized. Our data reveal anti-inflammatory

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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 PGE_2–mediated changes. However, expression of a number of genes differed following the three treatments. For example, exogenous PGE_2 and encapsulated MSC treatment, but not monolayer MSCs, up-regulated astrocyte expression of the neurotrophic factors BDNF and NT-3. Additionally, cluster analysis of the entire dataset showed expression patterns to be most similar between PGE_2 and encapsulated MSC treatment conditions, suggesting that encapsulated MSC-induced changes in expression may be largely due to increased PGE_2 production. Dissimilarities between these conditions also exist, where encapsulated MSC-induced gene regulation more closely matches that of monolayer MSCs than exogenous PGE_2. The changes previously observed in the MSC secretome in response to OHSC inflammatory signals, [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 PGE_2, and the necessity of early PGE_2 administration to reduce inflammation. Additionally, we determined EP receptor subtypes through which exogenous and MSC-produced PGE_2 are acting to modulate inflammation, and demonstrated additional therapeutic benefit of encapsulated MSCs through induction of astrocyte neurotrophin expression. These results suggest that alginate encapsulation may be a novel and effective method to deliver MSCs for TBI treatment, and may provide sustained, multi-potent benefit by modulating inflammation and providing neuroprotection through induction of neurotrophin expression. 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

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encapsulated MSC following SCI demonstrated efficacy in mitigating inflammation when the treatment was applied 24 h after the initial trauma.

Supplementary Material

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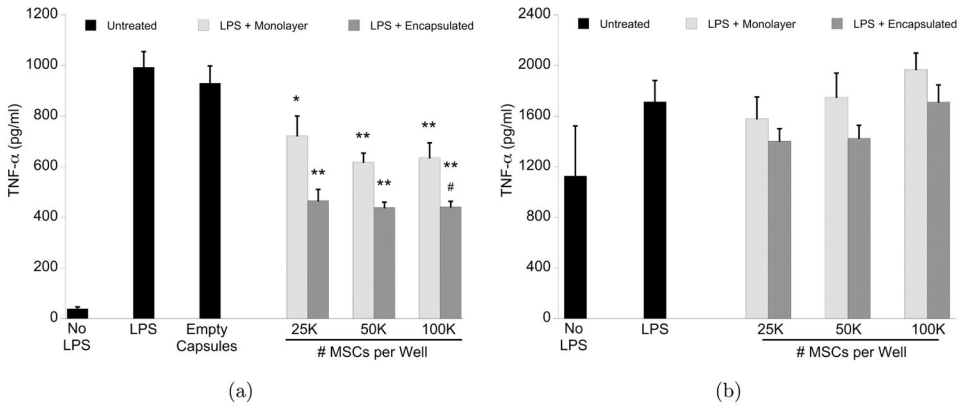

Fig. 1.

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.

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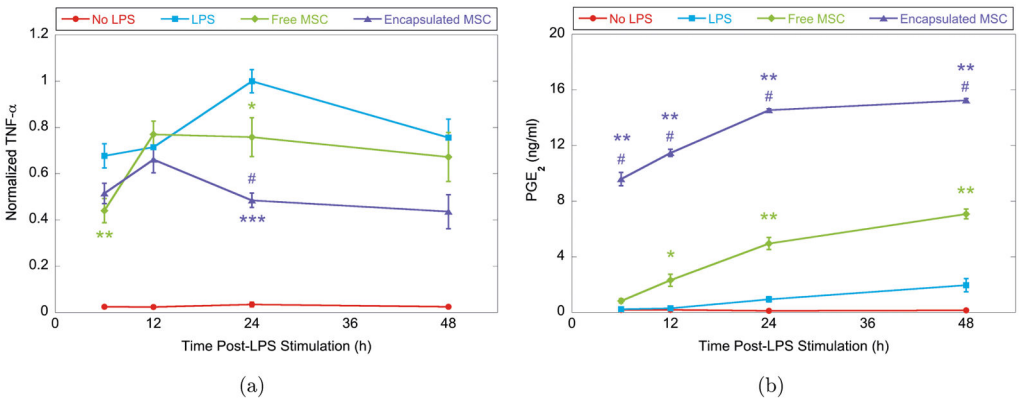

Fig. 2.

Temporal profile of rat TNF-α and total PGE_2 levels in culture media collected after LPS stimulation ± MSC treatment in astrocytes. TNF-α data are normalized to untreated LPS-stimulated cultures at 24 h. All data are represented as mean ± 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.

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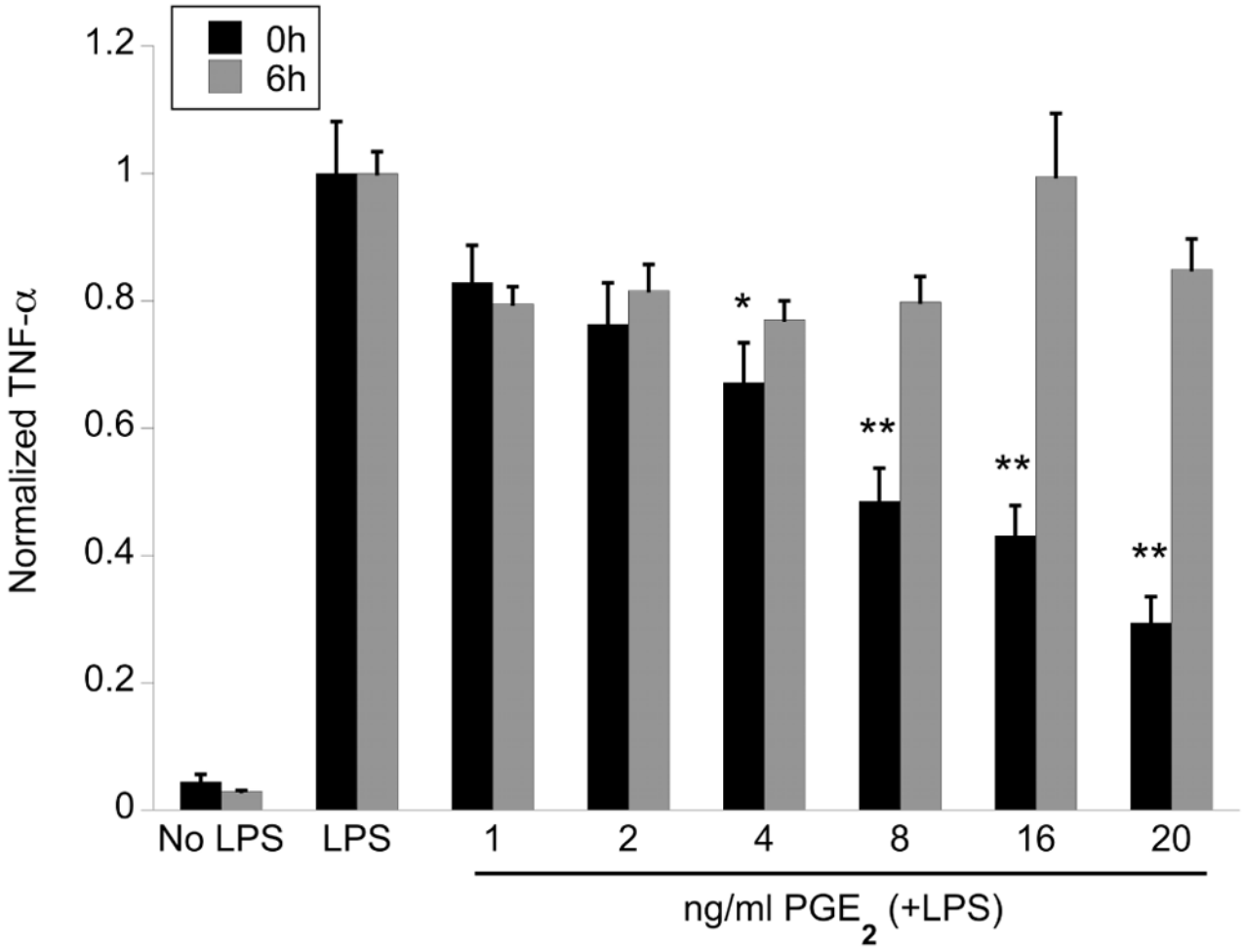

Fig. 3.

Rat TNF-α ELISA of cell culture media collected from astrocyte cultures after 24 h of LPS stimulation ±human PGE_2. Data is normalized to untreated LPS-stimulated astrocytes and represented as mean ± 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.

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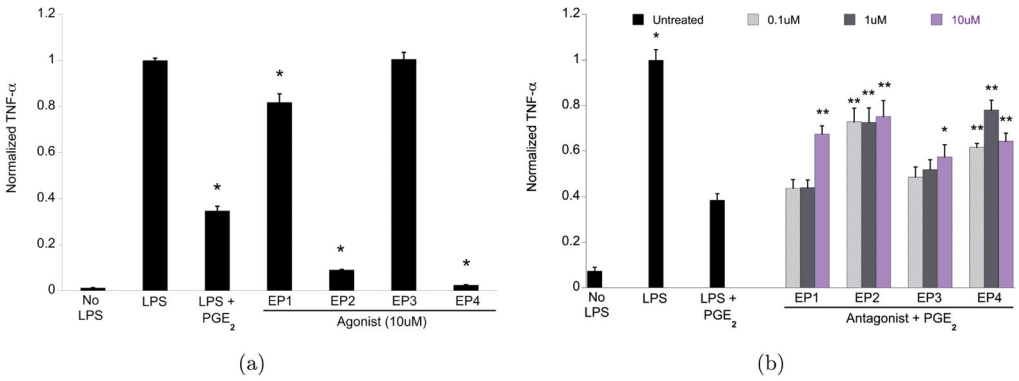

Fig. 4.

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 ± 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 ± 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 astrocytes after 24 h of LPS stimulation + 20 ng/ml PGE_2 ± EP receptor antagonist SC-51322 (EP1), PF-04418948 (EP2), L-798,106 (EP3), or L-161,982 (EP4). Significant antagonist blocking is observed for all EP receptor subtypes. *= $p < 0.05$, **= $p < 0.0005$ compared to LPS + PGE_2.

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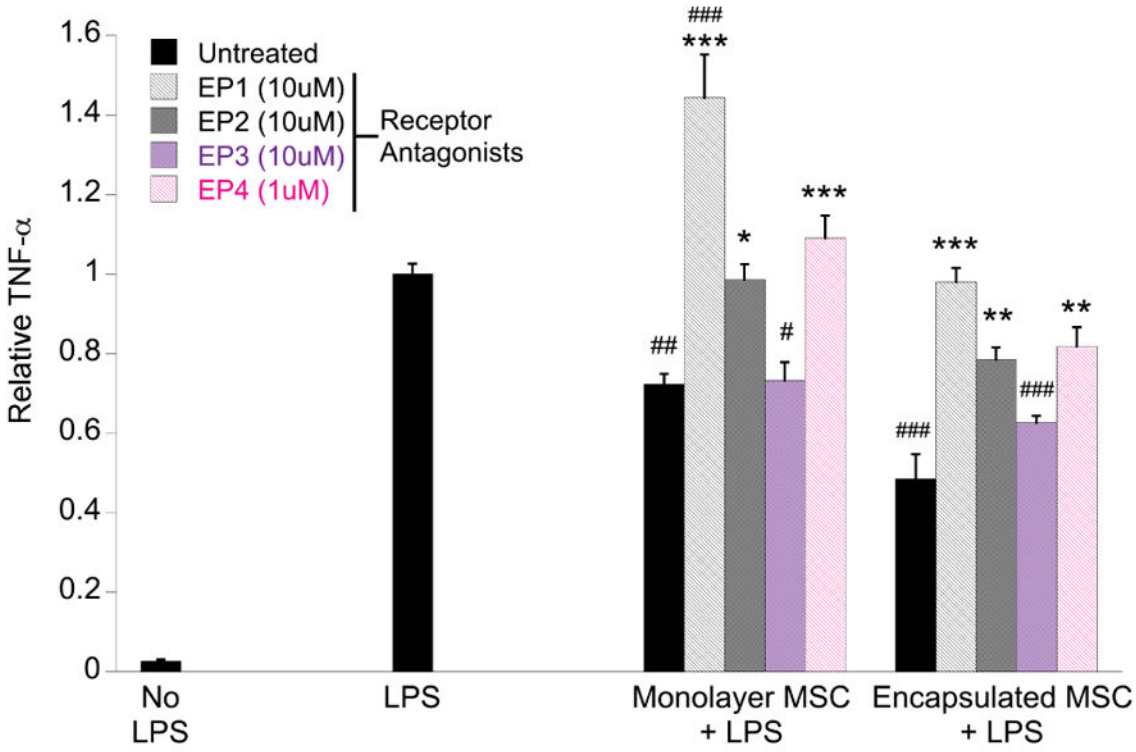

Fig. 5.

PGE_2 receptor antagonist blocking of MSC treatment. Rat TNF-α produced by astrocytes after 24 h of LPS stimulation + MSC (monolayer or encapsulated) ± 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.

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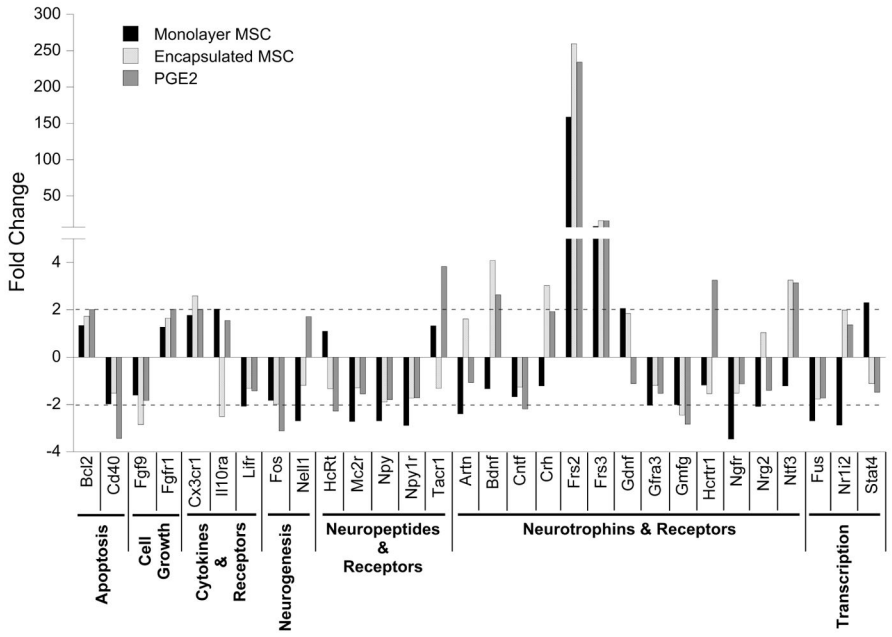

Fig. 6.

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.

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Table 1

A list of the neurotrophin and neurotrophin-receptor genes assessed with the Qiagen PCR array. Fold changes are in comparison to the untreated, un-stimulated control. More than two-fold up-regulated genes are marked in bold; more than two-fold down-regulated genes are marked in bold italics.

			Fold change relative to no treatment			
Gene symbol	**Gene name**	**Accession Number**	**LPS only**	**Monolayer MSC**	**Encapsulated MSC**	**PGE_2 (20 ng/ml)**
Apoptosis						
Bax	Bcl2-associated X protein	NM_017059	1.56	1.37	1.84	1.66
Bcl2	B-cell CLL/lymphoma 2	NM_016993	−1.99	−1.48	−1.15	1.01
Cd40	CD40 molecule, TNF receptor superfamily member 5	NM_134360	**12.73**	**6.45**	**8.46**	**3.71**
Fas	Fas (TNF receptor superfamily, member 6)	NM_139194	**3.27**	**3.51**	**4.00**	**3.39**
Hspb1	Heat shock protein 1	NM_031970	−1.59	1.04	−1.19	1.13
Cell Growth and Differentiation						
Fgf2	Fibroblast growth factor 2	NM_019305	1.88	1.83	1.39	**2.08**
Fgf9	Fibroblast growth factor 9	NM_012952	1.91	1.19	−1.49	1.04
Fgfr1	Fibroblast growth factor receptor 1	NM_024146	−1.87	−1.46	−1.13	1.09
Nf1	Neurofibromin 1	NM_012609	−1.12	−1.33	1.17	1.27
Tgfa	Transforming growth factor alpha	NM_012671	1.64	1.43	1.31	1.01
Tgfb1	Transforming growth factor, beta 1	NM_021578	1.09	−1.31	1.29	1.43
Tgfb1i1	Transforming growth factor beta 1 induced transcript 1	NM_001191840	**2.41**	**2.64**	**3.29**	**3.61**
Tp53	Tumor protein p53	NM_030989	1.79	1.32	1.60	1.77
Cytokines and Receptors						
Cx3cr1	Chemokine (C-X3-C motif) receptor 1	NM_133534	***−202.25***	***−114.56***	***−77.71***	***−99.73***
Il10	Interleukin 10	NM_012854	**104.69**	**56.49**	**58.08**	**128.89**
Il10ra	Interleukin 10 receptor, alpha	NM_057193	−1.21	1.69	***−3.01***	1.28
Il1b	Interleukin 1 beta	NM_031512	**43.41**	**30.7**	**36.5**	**37.01**
Il1r1	Interleukin 1 receptor, type I	NM_013123	**3.48**	**2.91**	**5.39**	**5.94**
Il6	Interleukin 6	NM_012589	**45.25**	**35.26**	**29.24**	**60.97**
Il6r	Interleukin 6 receptor	NM_017020	**6.63**	**5.50**	**6.77**	**7.26**
Il6st	Interleukin 6 signal transducer	NM_001008725	**2.45**	1.69	**2.39**	**2.35**
Lif	Leukemia inhibitory factor	NM_022196	**5.62**	**3.32**	**4.38**	**4.11**
Lifr	Leukemia inhibitory factor receptor alpha	NM_031048	**2.71**	1.31	**2.06**	1.91

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			Fold change relative to no treatment			
Gene symbol	**Gene name**	**Accession Number**	**LPS only**	**Monolayer MSC**	**Encapsulated MSC**	**PGE_2 (20 ng/ml)**
Neurogenesis						
Cbln1	Cerebellin 1 precursor	NM_001109127	−1.01	−1.74	−1.89	−1.21
Cxcr4	Chemokine (C-X-C motif) receptor 4	NM_022205	***−4.56***	***−3.36***	***−2.69***	***−2.83***
Fos	FBJ osteosarcoma oncogene	NM_022197	**2.89**	1.58	1.45	−1.08
Nell1	NEL-like 1 (chicken)	NM_031069	***−6.45***	***−17.39***	***−7.67***	***−3.76***
Neuropeptides and Receptors						
Cckar	Cholecystokinin A receptor	NM_012688	***−3.86***	***−4.69***	***−5.10***	***−5.74***
Galr1	Galanin receptor 1	NM_012958	−1.06	−1.28	−1.39	−1.57
Galr2	Galanin receptor 2	NM_019172	1.30	−1.16	1.09	1.09
Grpr	Gastrin releasing peptide receptor	NM_012706	−1.84	1.06	***−2.43***	***−2.73***
HcRt	Hypocretin	NM_013179	***−3.43***	***−3.12***	***−4.56***	***−7.84***
Mc2r	Melanocortin 2 receptor	NM_001100491	**5.70**	**2.10**	**4.41**	**3.68**
Npffr2	Neuropeptide FF receptor 2	NM_023980	1.44	1.19	1.37	1.03
Npy	Neuropeptide Y	NM_012614	**2.10**	−1.28	1.11	1.17
Npy1r	Neuropeptide Y receptor Y1	NM_001113357	**5.54**	1.92	**3.20**	**3.25**
Npy2r	Neuropeptide Y receptor Y2	NM_023968	1.44	1.19	1.09	−1.03
Ntsr1	Neurotensin receptor 1	NM_001108967	1.04	−1.17	−1.27	1.71
Npy4r	Pancreatic polypeptide receptor 1	NM_031581	1.44	1.19	1.09	1.35
Tacr1	Tachykinin receptor 1	NM_012667	***−3.39***	***−2.55***	***−4.47***	1.13
Neurotrophins and Receptors						
Adcyap1r1	Adenylate cyclase activating polypeptide 1 receptor 1	NM_133511	−1.99	−1.99	−1.11	−1.16
Artn	Artemin	NM_053397	**5.1**	**2.13**	**8.28**	**4.76**
Bdnf	Brain-derived neurotrophic factor	NM_012513	−1.62	***−2.16***	**2.51**	1.62
Cntf	Ciliary neurotrophic factor	NM_013166	**2.91**	1.74	**2.3**	1.33
Cntfr	Ciliary neurotrophic factor receptor	NM_001003929	1.09	−1.72	−1.38	−1.34
Crh	Corticotropin releasing hormone	NM_031019	1.22	1.01	**3.71**	**2.36**
Crhbp	Corticotropin releasing hormone binding protein	NM_139183	***−7.73***	***−6.19***	***−10.2***	***−11.39***
Crhr1	Corticotropin releasing hormone receptor 1	NM_030999	**2.79**	**2.1**	**3.78**	**3.41**
Crhr2	Corticotropin releasing hormone receptor 2	NM_022714	1.83	1.19	1.58	1.06
Frs2	Fibroblast growth factor receptor substrate 2	NM_001108097	***−229.13***	−1.44	1.13	1.02

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			Fold change relative to no treatment			
Gene symbol	**Gene name**	**Accession Number**	**LPS only**	**Monolayer MSC**	**Encapsulated MSC**	**PGE_2 (20 ng/ml)**
Frs3	Fibroblast growth factor receptor substrate 3	NM_001017382	***−8.06***	1.02	**2**	1.97
Gdnf	Glial cell derived neurotrophic factor	NM_019139	**3.51**	**7.21**	**6.5**	**3.16**
Gfra1	GDNF family receptor alpha 1	NM_012959	−1.46	−1.27	−1.34	−1.29
Gfra2	GDNF family receptor alpha 2	NM_012750	−1.19	−1.22	1.42	1.58
Gfra3	GDNF family receptor alpha 3	NM_053398	**6.77**	**3.34**	**5.7**	**4.47**
Gmfb	Glia maturation factor, beta	NM_031032	1.28	−1.46	−1.04	1.06
Gmfg	Glia maturation factor, gamma	NM_181091	**2.36**	1.17	−1.04	−1.2
Hcrtr1	Hypocretin (orexin) receptor 1	NM_013064	1.83	1.55	1.19	**5.94**
Hcrtr2	Hypocretin (orexin) receptor 2	NM_013074	1.44	1.19	1.45	−1.03
Mt3	Metallothionein 3	NM_053968	***−2.58***	***−2.97***	***−2.66***	***−2.07***
Ngf	Nerve growth factor (beta polypeptide)	NM_001277055	**12.64**	**12.64**	**11.88**	**12.21**
Ngfr	Nerve growth factor receptor	NM_012610	**2.35**	−1.47	1.56	**2.07**
Ngfrap1	Nerve growth factor receptor associated protein 1	NM_053401	1.09	−1.13	−1.01	1.19
Nrg1	Neuregulin 1	NM_031588	−1.06	−1.27	1.41	1.03
Nrg2	Neuregulin 2	NM_001136151	**5.78**	**2.77**	**6.02**	**4.11**
Ntf3	Neurotrophin 3	NM_031073	***−4.41***	***−5.35***	−1.36	−1.4
Ntf4	Neurotrophin 4	NM_013184	−1.67	***−2.03***	−1.22	−1.46
Ntrk1	Neurotrophic tyrosine kinase, receptor, type 1	NM_021589	1.44	1.19	1.09	−1.03
Ntrk2	Neurotrophic tyrosine kinase, receptor, type 2	NM_012731	***−2.04***	***−2.33***	−1.51	−1.64
Pspn	Persephin	NM_013014	1.31	−1.05	1.43	−1.24
Ptger2	Prostaglandin E receptor 2 (subtype EP2)	NM_031088	**6.63**	**4.32**	**4.99**	**6.02**
Tfg	Trk-fused gene	NM_001012144	1.49	1.12	1.46	1.52
Ucn	Urocortin	NM_019150	1.64	−1	**2.45**	−1.08
Vgf	VGF nerve growth factor inducible	NM_030997	**47.5**	**34.3**	**44.02**	**37.01**
Transcription						
Fus	Fusion (human)	NM_001012137	1.51	−1.79	−1.17	−1.15
Maged1	Melanoma antigen, family D, 1	NM_053409	1.52	−1.04	−1.13	1.16
Myc	Myelocytomatosis oncogene	NM_012603	1.33	1.04	1.32	1.29
Nr1i2	Nuclear receptor subfamily 1, group I, member 2	NM_052980	−1.57	***−4.5***	1.27	−1.15
Stat1	Signal transducer and activator of transcription 1	NM_032612	**5.24**	**2.75**	**4.59**	**3.86**

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		Fold change relative to no treatment				
Gene symbol	**Gene name**	**Accession Number**	**LPS only**	**Monolayer MSC**	**Encapsulated MSC**	**PGE_2 (20 ng/ml)**
Stat2	Signal transducer and activator of transcription 2	NM_001011905	**6.59**	**3.63**	**5.66**	**5.39**
Stat3	Signal transducer and activator of transcription 3	NM_012747	**4.23**	**3.46**	**4**	**3.61**
Stat4	Signal transducer and activator of transcription 4	NM_001012226	1.44	**3.34**	1.29	−1.03
Zfp110	Zinc finger protein 110	NM_001024775	1.91	1.42	1.89	1.19
Zfp91	Zinc finger protein 91	NM_001169120	1.24	−1.09	1.28	1.09

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