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Immunomodulation by Mesenchymal Stem Cells Combats the Foreign Body Response to Cell-Laden Synthetic Hydrogels

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Abstract

The implantation of non-biological materials, including scaffolds for tissue engineering, ubiquitously leads to a foreign body response (FBR). We recently reported that this response negatively impacts fibroblasts encapsulated within a synthetic hydrogel and in turn leads to a more severe FBR, suggesting a cross-talk between encapsulated cells and inflammatory cells. Given the promise of mesenchymal stem cells (MSCs) in tissue engineering and recent evidence of their immunomodulatory properties, we hypothesized that MSCs encapsulated within poly(ethylene glycol) (PEG) hydrogels will attenuate the FBR. *In vitro*, murine MSCs encapsulated within PEG hydrogels attenuated classically activated primary murine macrophages by reducing gene expression and protein secretion of pro-inflammatory cytokines, most notably tumor necrosis factor- α . Using a COX2 inhibitor, prostaglandin E2 (PGE2) was identified as a mediator of MSC immunomodulation of macrophages. *In vivo*, hydrogels laden with MSCs, osteogenically differentiating MSCs, or no cells were implanted subcutaneously into C57BL/6 mice for 28 days to assess the impact of MSCs on the fibrotic response of the FBR. The presence of encapsulated MSCs reduced fibrous capsule thickness compared to acellular hydrogels, but this effect diminished with osteogenic differentiation. The use of MSCs prior to differentiation in tissue engineering may therefore serve as a dynamic approach, through continuous cross-talk between MSCs and the inflammatory cells, to modulate macrophage activation and attenuate the FBR to implanted synthetic scaffolds thus improving the long-term tissue engineering outcome.

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Keywords

poly(ethylene glycol); hydrogel; macrophage; mesenchymal stem cell; foreign body response; immunomodulation

1. Introduction

The foreign body response (FBR) is notorious for causing unwanted degradation of implanted medical devices and scaffolds due to its harsh environment that renders implanted materials non-functional by walling off the device in a dense avascular capsule [1, 2]. It is well known that the FBR occurs ubiquitously to any implanted non-biological material regardless of material chemistry or property [2]. In the context of tissue engineering, synthetic materials are promising as nearly any physical property can be achieved with high fidelity and reproducibility. In addition, *in vivo* placement of cell-laden scaffolds offers many advantages over *in vitro* culture since cells are immersed in a microenvironment containing a milieu of signals unique to that tissue, which may aid in the differentiation or function of the implanted cells. However, synthetically derived scaffolds elicit a FBR [3–7], and we recently reported that the FBR indeed negatively affects cells within a scaffold [8]. Because the FBR subsides once the foreign material is degraded, a strategy that attenuates the FBR in the short-term may improve the *in vivo* outcome of implanted cell-laden synthetic scaffolds.

Mesenchymal stem cells (MSCs) derived from bone marrow are a promising cell source for tissue engineering because of their ability to differentiate into several cell types [9]. MSCs, however, have functions beyond their differentiation potential. Most notably, they have been shown to function as immunomodulators [10, 11]. For example in murine models, MSCs have been shown to reduce inflammation and fibrosis in a lung injury model [12], attenuate sepsis in the lung [13] and reduce inflammation associated with peritonitis [14]. MSCs have also been implicated as a potential therapeutic for treating rheumatic diseases characterized by inflammation [15]. These remarkable capabilities of MSCs are attributed to the trophic factors they secrete and appear to be most effective when MSCs are present at the early stages of inflammation and fibrosis [12, 16]. With these promising findings, questions arise as to whether MSCs are also capable of attenuating the FBR, which is characterized by inflammation and the formation of a fibrous capsule.

The aim of this study was therefore to investigate whether MSCs embedded in a synthetic hydrogel, known to elicit a FBR, are able to attenuate macrophage activation *in vitro* and the FBR *in vivo*, and if so, what are the signaling molecules involved. Additional investigations were performed to determine whether differentiation leads to a loss of this ability. To accomplish these aims, bone marrow derived murine MSCs and bone marrow derived murine macrophages, both isolated from C57BL/6 mice, were employed. Murine MSCs were encapsulated in a poly(ethylene glycol) (PEG) hydrogel, representing a promising platform for tissue engineering, but which we have previously confirmed elicits a FBR [8, 17–19]. A series of experiments were designed to investigate the effects of MSCs on classically activated macrophages that were seeded directly onto a MSC-laden PEG

hydrogel and stimulated with the pro-inflammatory stimulant, lipopolysaccharide. Experiments were also performed using MSC conditioned media to assess the indirect effects of MSCs on macrophage activation and to identify the signaling molecules involved. Finally, MSCs were differentiated down the osteogenic lineage to study the effects of differentiation on MSC modulation of macrophages *in vitro* and the foreign body response *in vivo* when implanted subcutaneously in immunocompetent mice.

2. Materials and Methods

2.1 Preparation of hydrogels

Poly(ethylene glycol) diacrylate (PEG-dA) was synthesized by reacting poly(ethylene glycol) (3000 Da, PEG, Fluka) with acryloyl chloride (Sigma-Aldrich) and triethylamine (TEA) (Sigma-Aldrich) in dry toluene overnight at room temperature as described in [20]. PEG-dA was purified by repeated precipitation in chilled diethyl ether. The degree of acrylation was determined by ^1H NMR to be 95%. Monoacrylated PEG-tyrosine-arginine-glycine-aspartic acid-serine (YRGDS) was synthesized by reacting monoacrylate PEG-N-hydroxysuccinimide (3400 Da, Laysan Bio) with excess of YRGDS (Genscript) at a pH of 8.4 for 2 hours [19]. Monoacrylated PEG-YRGDS was purified by dialysis. The extent of conjugation of the YRGDS oligopeptide to the monoacrylated PEG was determined by ^1H NMR to be 90%. Hydrogel discs (5mm in diameter and ~1 mm in height) were formed by photopolymerization of a sterile filtered solution containing 10% (w/w) PEG-dA and 2.5 mM monoacrylated PEG-YRGDS in PBS with 0.05% photoinitiator (I2959, Irgacure, BASF) under 365 nm ultraviolet light ($5\text{--}10\text{ mW cm}^{-2}$) for 10 minutes. Hydrogels were prepared under sterile conditions and rinsed multiple times in sterile PBS.

2.2 Macrophages

Bone marrow derived murine monocytes were isolated from 6-week-old C57BL/6 mice (Charles River Laboratories) as described previously [21]. Briefly, tibias and femura were extracted from the mice, and the bone marrow flushed using IMDM (Invitrogen). The bone marrow isolates were suspended in IMDM with PSF, layered in Lympholyte M (Accurate Chemicals) and centrifuged per the manufacturer instructions. The portion containing mononuclear cells was collected and resuspended at 10^6 cells/ml in expansion medium, containing IMDM, 20% fetal bovine serum (FBS), 2mM L-glutamine, PSF, 1.5 ng/ml human macrophage colony stimulating factory (R&D systems) and 100 ng/ml huFLT-3 (R&D systems). The cells were plated in non-tissue culture treated polystyrene flasks at 1.7×10^5 cells/cm² and cultured for 10 days. Cells were then seeded onto acellular or MSC-laden hydrogels at 2.5×10^5 macrophages/cm² in MSC culture medium. Hydrogel samples were cultured in the absence or presence of 1 $\mu\text{g/ml}$ lipopolysaccharid (LPS, E. coli, Sigma-Aldrich) for 4, 8 or 24 hours.

2.3 MSC culture and differentiation

Primary murine MSCs from C57BL/6 mice were obtained from Texas A&M University Health Science Center and College of Medicine Institute for Regenerative Medicine. The MSCs were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Mediatech) supplemented with 10% FBS (Atlanta Biologicals), 10% horse serum (Hyclone), penicillin/

streptomycin/amphotericin B (PSF, Invitrogen), and 1% 200 mM L- glutamine (Invitrogen). MSCs were encapsulated into hydrogels at 20×10^6 cells /ml. Hydrogels were cultured in the absence or presence of 1 μ g/ml LPS for 4, 8 or 24 hours.

MSCs were osteogenically differentiated in 2D on tissue culture plastic in the presence of bone differentiation medium (BDM) containing the basic culture medium supplemented with 1 nM dexamethasone (Sigma), 20 mM β -glycerolphosphate (Sigma), 50 μ M L-ascorbic acid 2-phosphate sesquimagnesium salt (Sigma), and 50 ng/mL L-thyroxine sodium pentahydrate (Sigma). MSCs were differentiated for 4, 10, or 21 days prior to encapsulation in hydrogels at 20×10^6 cells /ml.

Osteogenic differentiation was confirmed through alkaline phosphatase activity and mineralization for calcium through the Alizarin Red S stain. Samples for the alkaline phosphatase assay were taken at day 0 (before differentiation) and after 4, 7, 10, 15 and 21 days in BDM. The cells were rinsed in PBS and then lysed in DI water and frozen. The samples were further prepared through freeze-thaw-sonicate cycles to lyse the cells. The alkaline phosphatase activity assay was run according to standard protocol, and normalized to the total DNA content using the Quant-iT PicoGreen dsDNA assay (Invitrogen). For mineralization, cells were seeded in 6-well plates and cultured for 21 days in growth medium or BDM. Cells in wells were washed with PBS and fixed in 10% formalin for 20 minutes. The plates were washed with DI-water and stained with the 1% (w/w) Alizarin Red S (Sigma).

2.4 Conditioned media experiments

MSCs encapsulated in hydrogels (as described above) were allowed to condition media for 24 hours in the presence of 1 μ g/ml LPS without or with 1 μ M cyclooxygenase 2 (COX2) inhibitor (NS-398, Sigma). Macrophages were seeded on acellular hydrogels for 24 hours. After 24 hours media from MSCs were collected and centrifuged. Medium was completely removed from the samples containing macrophages and replaced with medium that consisted of 0, 25, 50, or 75% MSC conditioned medium and the remainder consisting of fresh medium with a final concentration of 1 μ g/ml LPS. In order to examine the effect of prostaglandin E2 (PGE2) on macrophage cytokine secretion, exogenous PGE2 (Cayman Chemical) from 1–1000 nM was delivered to macrophages seeded on top of acellular hydrogels in the presence of 1 μ g/ml LPS for 24 hours. Additionally, secretion of PGE2 from encapsulated MSCs was measured in the presence of 0.01 to 10 μ M of NS-398, the COX2 inhibitor.

2.5 Gene Expression

Hydrogels were removed from culture at predetermined time points and placed in TRK lysis buffer (Omega), frozen in liquid nitrogen and stored at -80°C . RNA was isolated from the two cell populations as described previously [8], with an initial 10s immersion in TRK lysis buffer, which minimizes cross contamination. RNA was isolated using E.Z.N.A. microelute kit (Omega) per the manufacturer instructions. The amount of pure RNA was quantified using a Nanodrop instrument (ND-1000, Thermo Scientific) with A260/280 greater than 1.90. Purified RNA was reverse transcribed into cDNA using a High Capacity cDNA

Reverse Transcription Kit (Applied Biosystems) per the manufacturer instructions. Samples were immediately used for qPCR with Fast SYBR Green Master Mix (Applied Biosystems) on a 7500 Fast system (Applied Biosystems). Custom primers were designed using Primer Express 3.0 software (Applied Biosystems) and were evaluated for efficiency. Primer sequences, efficiencies, and accession numbers can be found in Supplemental Table 1. The data are presented as relative expression (RE) to the housekeeping gene L32: Relative

expression $(RE) = \frac{E_{HKG}^{C_{t(HKG)}}}{E_{GOI}^{C_{t(GOI)}}}$ where E is the primer efficiency, HKG is the house-keeping gene, GOI is the gene of interest, Ct is the cycle number where the sample crosses the threshold.

2.6 Protein Secretion

Supernatant was collected from samples at prescribed time points, snap frozen and stored at -80°C until further processing. Protein concentration was determined by commercially available ELISA kits for tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) (eBioscience) and EIA kits for PGE2 (Cayman Chemical) following manufacturers' instructions.

2.7 Immunocytochemistry

Macrophages were seeded on hydrogels in the presence of 1 $\mu\text{g/ml}$ LPS alone or with either 1000 nM PGE2 or 10 μM glycogen synthase kinase (GSK)-3 β inhibitor (SB216763, Sigma) for 30 minutes. Cells were fixed in 10% formalin. Hydrogels were washed and blocked prior to incubating with rabbit anti-murine β -catenin (ab16051, Abcam) antibody at 1 $\mu\text{g/ml}$ followed by a goat anti-rabbit AlexaFluor $^{\text{®}}$ 488 (1:200, Molecular Probes, Life Technology) secondary antibody. Hydrogels were stained with 4',6-diamidino-2-phenylindole (DAPI, Life Technology) to visualize the nuclei. Samples were imaged by laser scanning confocal microscopy (Zeiss LSM5 Pascal).

2.8 Subcutaneous implantation

Hydrogel discs (5mm in diameter and ~ 1 mm in height) containing syngeneic MSCs and differentiating MSCs were implanted into dorsal subcutaneous pockets on six- to eight-week old, male C57BL/6 mice. Acellular hydrogels were confirmed to be endotoxin-free using an E-TOXATE $^{\text{TM}}$ Kit (Sigma) per manufacturer's instructions. One incision was made along the spine, and a pocket was bluntly dissected on either side, above the shoulder blade. One hydrogel was inserted on either side of the spine in the described pocket. A single animal contained samples of an identical condition. The incision was then closed with a single surgical staple. Animals were monitored daily for wound abnormalities. After 28 days, the mice were sacrificed by CO_2 asphyxiation and cervical dislocation. Implants were excised with surrounding tissue and processed for immunohistological analysis (n=4).

2.9 (Immuno)histological analysis

Explants with attached dorsal skin were pinned in a dissection dish and immediately fixed in 4% paraformaldehyde at room temperature for 24 hours. The samples were removed from the paraformaldehyde and stored in a 15% sucrose solution at 4°C until dehydration.

Dehydration and paraffin embedding were performed according to standard protocols. Sections, 10 μm in thickness, were stained with Masson's Trichrome, Hematoxylin & Eosin, and von Kossa with nuclear red counter stain, according to standard protocols. Sections were also stained with α -smooth muscle actin (α -SMA) antibody (1:1000) overnight at 4°C and a secondary antibody conjugated to horseradish peroxidase and developed with 3, 3'-diaminobenzidine and counterstained with nuclear red. Tissue sections were imaged using light microscopy (Zeiss, Axioskop 40) with either a 10 $\times$, 20 $\times$ or 40 $\times$ objective using a digital camera (Diagnostic Instruments, MN 14.2 Color Mosaic) with SPOT software v. 4.6. Fibrous capsule thickness and inflammatory cells were evaluated semi-quantitatively using NIH ImageJ software.

2.10 Statistical analysis

One-way analysis of variance was performed using KaleidaGraph software for relative gene expression. Pair-wise comparisons were performed using Tukey's post-hoc test with $\alpha=0.05$. A p value of 0.05 was considered statistically significant. All data are presented as mean with standard deviation as error bars.

2.11 IACUC approval

NIH guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) were observed in use of all animals. The University of Colorado at Boulder Institutional Animal Care and Use Committee approved protocols for animal use.

3. Results

3.1 Effects of encapsulated MSCs on macrophage activation *in vitro*

High MSC viability *in vitro* was confirmed immediately after encapsulation with ~97% viable cells and after 48 hours with ~93% viable cells based on a membrane integrity assay. To capture macrophage interrogation of a cell-laden hydrogel in an inflammatory environment similar to that seen during the FBR *in vivo*, macrophages were seeded on top of the PEG hydrogel and cultured in the presence of LPS. The hydrogel contained tethered RGD peptides to enable encapsulated MSCs to interact with the hydrogel. The experimental conditions are shown in Fig. 1a. We first confirmed that pro-inflammatory cytokines, interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α), were up-regulated at the gene and protein levels in macrophages cultured on hydrogels and with LPS over the course of 24 hours (Fig. 1b). Encapsulated MSCs also responded to LPS with increased secretion of IL-6 protein, which was ~400% higher than macrophages, but no TNF- α protein was detected. For macrophages, the LPS-induced up-regulation in gene expression for IL-6 and TNF- α was partially or completely abrogated when MSCs were present in the hydrogel at 24 hours. With LPS, TNF- α secretion in this co-culture system was significantly lower compared to the monoculture of macrophages on PEG hydrogels. IL-6 protein was also secreted in the co-culture system with LPS. However, the levels of each cytokine in the co-culture system were lower than the combined levels secreted by macrophages and encapsulated MSCs in monocultures. Macrophage gene expression of arginase, a marker of wound healing, was elevated in the presence of MSCs at 24 hours with LPS (Fig. 1c).

3.2 Identification of paracrine signaling molecules that mediate MSC-modulation of macrophages

The primary mechanism by which MSCs signal macrophages is through paracrine signaling, since macrophages are adhered to the outer surface of the hydrogel and MSCs are entrapped within the hydrogel. To this end, we investigated the signaling molecule, PGE2. PGE2 secretion was first measured in each of the different experimental conditions (Fig. 2a). Encapsulated MSCs secreted high levels of PGE2 on their own and even higher levels in the presence of LPS. Macrophages also secreted PGE2, which increased with LPS presence, but the levels were much lower than MSCs. With LPS, PGE2 levels in the co-culture were lower than the combined levels secreted by macrophages and encapsulated MSCs in monocultures.

To determine whether PGE2 mediated the MSC immunoregulation of LPS-stimulated macrophages on hydrogels, a series of experiments were performed. Specifically, we investigated the effects of exogenous PGE2 delivered to macrophages seeded on top of the hydrogels in the presence of LPS on cytokine secretion (Fig. 2b). The amount of TNF- α decreased in a dose dependent manner ($p < 0.0001$). However, PGE2 did not significantly affect IL-6 protein levels. Macrophages were subsequently treated with MSC conditioned medium (CM), i.e., medium conditioned by encapsulated MSCs for 24 hours in the presence of LPS, referred to as normal CM. MSCs were also cultured in medium supplemented with a small molecule inhibitor for COX2 to knockdown PGE2 synthesis by encapsulated MSCs, which was confirmed in Fig. 2c. Treatment of MSCs with a COX2 inhibitor reduced MSC secretion of IL-6, but did not result in any detectable secretion of TNF- α (Fig. 2d). With increasing concentrations of normal MSC CM, a dose-dependent decrease ($p < 0.0001$) in macrophage secreted TNF- α was observed. On the other hand, IL-6 levels increased with increasing amounts of normal MSC CM. In the COX2 inhibited MSC CM, there was partial recovery of LPS-induced macrophage secreted TNF- α (Fig. 2e). IL-6 increased with increasing MSC CM, but to a lesser degree compared to the normal MSC CM (Fig. 2e). We confirmed that the COX2 inhibitor had no impact on macrophage cytokine secretion of TNF- α or IL-6 in the presence of LPS (Supplemental Fig. 1). Taken together, these experiments confirm that under LPS stimulation, PGE2 secreted by MSCs attenuates LPS-induced TNF- α secretion by macrophages.

3.3 Identification of intracellular signaling molecules involved in PGE2 attenuation of LPS-stimulated macrophages

To determine whether GSK-3 β and β -catenin, shown through the pathway summarized in Fig. 3a, are involved in the PGE2-mediated modulation of LPS-stimulated macrophages, we stained for β -catenin after a 30-minute stimulation with LPS (Fig. 3b). There was no detectable β -catenin in LPS-stimulated macrophages. However, in the presence of either PGE2 or an inhibitor for GSK-3 β , activation of intracellular β -catenin was observed in macrophages (Fig. 3b). We also investigated c-myc gene expression and confirmed elevated levels in macrophages when cultured on MSC-laden hydrogels regardless of LPS (Fig. 3c).

3.4 Effects of encapsulated differentiating MSCs on macrophage activation in vitro

To investigate whether the degree of differentiation affected MSC immunomodulation of macrophages, MSCs were differentiated down the osteogenic lineage for 4, 10, and 21 days

on tissue culture polystyrene (TCPS) and then encapsulated into hydrogels (Fig. 4b). Differentiation was confirmed by alkaline phosphatase activity and Alizarin Red S staining for mineral deposits (Fig. 4a). Similar to the previous experiments, macrophages were seeded on top of the cell-laden hydrogels and cultured with LPS. After 24 hours, LPS-induced expression of genes for TNF- α and IL-6 were all significantly down regulated in macrophages when MSCs and osteogenically differentiated MSCs were present (Fig. 4c), regardless of the degree of differentiation. Arginase expression in LPS activated macrophages was elevated when MSCs and MSCs that were differentiated for 4 and 10 days were present when compared to the 21 day differentiated MSC and PEG samples (Fig. 4d).

3.5 Effects of differentiating MSCs encapsulated in a hydrogel on the FBR in vivo

Given that the *in vivo* response is more complex, hydrogels containing syngeneic MSCs or differentiating MSCs were implanted subcutaneously in C57BL/6 male mice for 28 days. Representative histological micrographs of sections stained with Masson's Trichrome to assess collagen deposition and formation of a fibrous capsule and Hematoxylin & Eosin to visualize cell presence around the implant are shown. Additionally, a semi-quantitative assessment of capsule thickness and number of cells at the implant surface is shown in Fig. 5. Sections were also stained using von Kossa for mineral and for α -SMA using immunohistochemistry as a marker of stemness (Supplemental Fig. 2). In the hydrogels with encapsulated MSCs, the MSCs displayed a characteristic round morphology. A thin layer of inflammatory cells approximately 1–2 cells thick surrounded the hydrogels with no evidence of cell infiltration. Beyond the inflammatory cell layer, a loose collagenous capsule is seen. The encapsulated cells have some pericellular staining for collagen within the hydrogel. There was minimal staining for mineral deposition but positive staining for α -SMA. With differentiation of the MSCs, the number of inflammatory cells at the hydrogel surface increased, as did the thickness of the collagenous capsule, but there was no observable α -SMA staining in the MSCs. Mineral deposition was present in the hydrogels with MSCs that were differentiated for 4 or 21 days. Interestingly, there was minimal mineral deposition in the hydrogels with MSCs that were differentiated for 10 days, which corresponds to an elevated FBR. In the acellular hydrogel, the fibrous capsule was visibly denser. The layer of inflammatory cells at the acellular hydrogel surface was greater than the MSCs, but similar to or less than differentiated MSCs.

4. Discussion

Although PEG hydrogels are highly hydrophilic, we have recently shown that they elicit a FBR when implanted into immunocompetent mice [18, 19], and that cells within the hydrogel are not only negatively affected by the FBR but also effect the severity of the FBR [8]. These previous findings suggested that a cross-talk occurs between inflammatory cells and the cells within the hydrogel, which ultimately impacts both cell types. Based on these observations, we hypothesized that MSCs, given the recent and growing evidence of their ability to attenuate inflammation and fibrosis, may be able to also combat the FBR through cross-talk with macrophages, the primary orchestrators of the FBR [2].

Indeed we found that MSCs encapsulated in a synthetic hydrogel were able to attenuate classical activation (i.e., M1 phenotype) of macrophages seeded on top of the hydrogels and stimulated with LPS. The *in vitro* experimental set-up enabled direct probing into the mechanisms by which MSCs influence macrophages. It is well established that LPS binds to toll like receptor 4 on macrophages and leads to an up-regulation in NF- κ B-mediated gene transcription of pro-inflammatory cytokines (e.g., TNF- α and IL-6) [22]. This in turn amplifies the inflammatory response since binding of TNF- α up-regulates inflammatory gene expression via an NF- κ B dependent pathway. Macrophages are able to self-limit the inflammatory response [23] by simultaneously secreting inflammatory regulators, which we observed in this study by an increased secretion of PGE2 from macrophages stimulated with LPS. However, these molecules are insufficient at 24 hours to attenuate the inflammatory response. On the other hand, MSCs constitutively express inflammatory regulators including PGE2 at relatively high levels in a non-inflammatory environment, which was observed in this study and reported by others [24]. PGE2 has been implicated in MSC immunomodulation in sepsis and acute lung injury [13, 25, 26]. MSCs responded to LPS by secreting pro-inflammatory cytokines (specifically IL-6), but this was accompanied by even higher PGE2 synthesis. Interestingly, no TNF- α secretion was detected by MSCs, which may be attributed to high PGE2 levels that have been shown to inhibit transcription of TNF- α [27]. When MSCs were present and encapsulated in the hydrogel, macrophage expression of the pro-inflammatory cytokines, TNF- α and IL-6, was significantly down-regulated at the gene level. Concomitant with the decreases in macrophage gene expression of pro-inflammatory cytokines by encapsulated MSCs, we observed an increase in arginase expression. While classically activated macrophages typically convert arginine into nitric oxide, wound healing macrophages alternatively convert arginine into ornithine, a precursor to collagen synthesis, via the enzyme arginase [28, 29]. Therefore, increased arginase expression accompanied by decreased pro-inflammatory cytokine expression is characteristic of a shift from a classically activated macrophage to a wound healing macrophage. This finding is also consistent with previous work, which found that 3D MSCs spheroids pushed LPS stimulated macrophages from a classically activated (M1) to an alternatively activated (M2) phenotype [30]. Work by others has also shown that MSCs are able to promote a regulatory phenotype in macrophages through the secretion of PGE2 [31].

These findings pointed towards MSC-secreted PGE2 as being a potent inflammatory regulator of LPS-stimulated macrophages. Through the MSC conditioned medium experiment, we confirmed that MSC-secreted PGE2 is likely one of the key paracrine signaling molecules that attenuates LPS-activation of macrophages. Because TNF- α was not produced by MSCs (at least in any detectable amounts), TNF- α provided an indicator of macrophage activation. Our data showed that with increasing amounts of MSC conditioned medium, TNF- α secretion decreased; a finding that correlated well with decreased TNF- α secretion observed in the co-culture system. When PGE2 synthesis was blocked in the MSC conditioned medium with a COX2 inhibitor, TNF- α secretion by macrophages was partially recovered. Our results are consistent with previous work showing that when macrophages were cultured in the presence of 3D MSC spheroids and LPS, pro-inflammatory cytokine secretion was inhibited and that this effect was abolished through treatment with a COX2

inhibitor [30, 32], but show for the first time in the context of tissue engineering with MSCs encapsulated within a synthetic scaffold.

It is well known that LPS initiates an inflammatory cascade, which leads to increased gene expression of pro-inflammatory cytokines (e.g., TNF- α and IL-6) through a NF- κ B dependent pathway [33]. PGE2 binds to specific prostaglandin receptors on the surface of cells, which have been implicated in regulating the inflammatory response in macrophages [13]. Upon binding of PGE2, several intracellular signaling pathways are activated one of which involves inactivation of GSK-3 β [34]. Active GSK-3 β leads to degradation of β -catenin, while inactive GSK-3 β does not [35]. β -catenin is able to translocate to the nucleus and interact with transcription factor 4, which induces gene expression of anti-inflammatory genes (e.g., c-myc) [36]. β -catenin in the nucleus is also able to interact with NF- κ B by down-regulating expression of pro-inflammatory cytokines [37]. Staining for β -catenin in Fig. 3 supports our proposed mechanism that in the presence of LPS alone β -catenin is degraded, but accumulates in the presence of either PGE2 or a small molecule inhibitor for GSK-3 β . The elevated levels of c-myc expression in macrophages when cultured in the presence of MSCs further supports the proposed mechanism, as it is known that β -catenin activation is required for c-myc expression [38, 39]. While the exact role of c-myc in the FBR is not known, studies have shown that c-myc is important to cell growth and angiogenesis [40], but abnormally high or low expression of c-myc has been linked to chronic inflammation [41].

Our data point towards the importance of the cross-talk between macrophages and encapsulated MSCs. Most notably, encapsulated MSCs secreted high levels of PGE2 in the presence of LPS, which is thought to depend on toll-like receptor ligands that trigger an immunosuppressive response in MSCs [42]. However, in the co-culture system, the levels of PGE2 were 50 times lower. Because macrophages and MSCs are able to communicate with each other at the onset of LPS stimulation, our data suggest a continuous cross-talk between the two cell types reduces the amplification of the inflammatory response. We, therefore, hypothesize that MSC-secreted PGE2 signals to the macrophages immediately and is able to dampen the effect of LPS and the up-regulation of pro-inflammatory cytokines. In turn, MSCs sense an overall reduced inflammatory environment and accordingly produce less PGE2. Indeed others have suggested that the anti-inflammatory capabilities of MSCs are possible only if MSCs are present at the beginning of the inflammatory or fibrotic response [12, 16]. However, we recognize that there may be other factors not yet known, which may be secreted by macrophages and may influence the MSCs leading to the observed lower levels of PGE2.

While we demonstrate that MSCs are able to attenuate LPS activation of macrophages and the severity of the FBR *in vivo*, our previous work suggested that a model differentiated cell type, fibroblasts, had the opposite effect, enhancing LPS activation and inducing a more severe FBR [8]. Therefore to determine whether stemness of MSCs was critical to MSC-driven attenuation of LPS-activated macrophages, MSCs were differentiated down the osteogenic lineage for varying times, up to 21 days, which is typical for when osteogenic markers, e.g. mineralization, are evident. Under *in vitro* conditions that simulate an acute inflammatory response, osteogenically differentiated MSCs regardless of their extent of

differentiation were able to attenuate LPS-activation of macrophages after 24 hours of co-culture. MSCs have been shown recently to modulate inflammation [43], and previous work from Ylostalo *et al.* [30] found that MSCs cultured in 3D spheroids attenuated LPS induced pro-inflammatory cytokine secretion from macrophages.

In vivo, however, the FBR begins with acute inflammation followed by chronic inflammation and eventually transitions to a resolution phase accompanied with low levels of chronic inflammation. The resolution phase is an altered wound healing response that is characterized by fibrous encapsulation and macrophages that reside at the implant surface for its lifetime. The cell-laden hydrogels with MSCs at varying stages of osteogenic differentiation had a significant effect on the FBR at the resolution phase. Interestingly, the number of inflammatory cells at the implant surface was higher for day 10 and day 21 osteogenically differentiated MSC when compared to the acellular gels. This finding is consistent with previous reports that differentiated cells may signal to the inflammatory cells elevating the response, even though this did not lead to a thicker capsule [8]. Most notably, the fibrous capsule was visibly less dense around hydrogels containing MSCs and differentiating MSCs when compared to the acellular hydrogel. However, the fibrous capsule thickness, measured in terms of the number of cell layers, increased with MSCs differentiated for longer amounts of time. Several studies have reported that MSCs and pre-differentiated MSCs are capable of osteogenic differentiation and ectopic bone formation when subcutaneously implanted within a biomaterial in immunocompromised mice [44, 45]. In this study, we observed many cells that appeared to maintain their stemness as evident by positive staining for α -SMA and minimal staining for mineral deposits. The protein, α -SMA, has been associated with MSCs [46, 47] and used as a marker for mesenchymal progenitors that have self-renewing and multipotency capabilities [46, 48–50]. On the contrary, there was no obvious staining for α -SMA in any of the pre-differentiated MSCs. There was evidence of mineral deposits in the day 4 osteogenically differentiated MSCs suggesting that these cells were able to continue to differentiate *in vivo*, which is consistent with other reports [44, 45]. The lack of positive staining for mineral deposits in the day 10 osteogenically differentiated MSCs may indicate a negative effect of the FBR on MSC osteogenic differentiation, which is consistent with an elevated FBR. Because the day 21 osteogenically MSCs had mineral deposits prior to implantation, it is difficult to determine if there was a negative effect on the encapsulated cells. Nonetheless, these findings suggest that *in vivo* the presence of MSCs, including differentiating MSCs (to a certain degree) are able to attenuate the fibrotic response in the FBR; however, this effect diminishes as osteogenic differentiation progresses. Additional studies are needed to better understand the effect of the FBR and the *in vivo* environment on the differentiation fate of encapsulated MSCs.

5. Conclusion

Findings from this study show for the first time that in the context of tissue engineering, encapsulated MSCs are able to reduce macrophage activation *in vitro*, simulating acute inflammation, and the FBR *in vivo*, at the resolution (and fibrotic) phase. Our *in vitro* studies indicate that PGE2 is a central mediator of MSC-driven attenuation of macrophage activation. Our *in vivo* findings indicate that as MSCs differentiate (at least down an

osteogenic lineage) they lose some of their immunomodulatory properties and the ability to mitigate the FBR. The results from this study support the working hypothesis that MSCs modulate the immune response when encapsulated in a synthetic hydrogel, specifically a PEG hydrogel with immobilized RGD, through continuous cross-talk with interrogating inflammatory cells that involves PGE2 and partially prevents the activation and/or amplification of pro-inflammatory cytokines, specifically TNF- α . Our findings demonstrate that MSCs and MSCs at the early stages of differentiation, when incorporated into synthetic scaffolds, are able to regulate the FBR and may ultimately improve the tissue engineering outcome for synthetic scaffolds.

Supplementary Material

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Figure. 1. Effect of MSCs on macrophage (MΦ) activation. a) Experimental design for *in vitro* studies. b) Relative gene expression and cytokine secretion of pro-inflammatory cytokines, TNF- α and IL-6, in macrophage and MSCs monocultures and co-culture in the absence and presence of the inflammatory stimulant, LPS. c) Relative gene expression of arginase in macrophage and MSC monocultures and co-culture in the absence and presence of LPS. Gene expression is relative to the housekeeping gene, L32. Data are presented as mean with standard deviation as error bars (n=4). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Figure. 2.

Role of PGE2 in MSC immunomodulation of LPS-activated macrophages (Mφ). a) Secretion of PGE2 from macrophages and MSC monocultures and co-culture in the absence and presence of LPS. b) Macrophage cytokine secretion of pro-inflammatory cytokines, TNF-α and IL-6, with increasing concentrations of PGE2. c) MSC secretion of PGE2 in the presence of increasing amounts of a cyclooxygenase 2 (COX2) inhibitor. d) MSC secretion of pro-inflammatory cytokines, TNF-α and IL-6, in the absence (gray) and presence (green) of a COX2 inhibitor. e) Macrophage secretion of pro-inflammatory cytokines, TNF-α and IL-6, with increasing amounts of normal MSC conditioned medium (gray) and MSC conditioned medium with a COX2 inhibitor (green). Data are presented as mean with standard deviation as error bars (n=4).). * p<0.05, ** p<0.01, and *** p<0.001.

Figure 3.

Proposed mechanism of MSC-driven attenuation of LPS-activated macrophages ($M\phi$). a) Proposed mechanism in macrophages where red lines indicate signaling by LPS, an inflammatory stimulant and black lines indicating signaling by PGE2 signaling. b) Immunocytochemistry analysis of macrophages seeded onto acellular hydrogels. Macrophages were stained for β -catenin (green) in the presence of LPS alone, or LPS with PGE2 or a GSK-3 β inhibitor. Nuclei were stained with DAPI (blue). Scale bar is 20 μ m. c) c-myc gene expression in macrophages on PEG hydrogels in the absence and presence of LPS and with and without encapsulated MSCs at 4 hours. Gene expression is relative to the

housekeeping gene, L32. Data are presented as mean with standard deviation as error bars (n=4). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

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Figure 4.

Effect of MSC differentiation on macrophage ($M\phi$) activation. a) Confirmation of osteogenic differentiation of murine MSCs by alkaline phosphatase (ALP) activity over the 21 days differentiation period normalized to total DNA content and by Alizarin Red S staining for mineral deposits. b) Experimental design for the differentiation and encapsulation of MSCs. c) Relative gene expression in macrophages cultured on PEG hydrogels laden with MSCs and osteogenically differentiating MSCs in the presence of LPS for pro-inflammatory cytokines, TNF- α and IL-6, and d) arginase. Gene expression is relative to the housekeeping gene, L32. Data are presented as mean with standard deviation as error bars ($n=4$). * $p<0.05$, ** $p<0.01$, and *** $p<0.001$. Scale bar equals 100 μ m.

Figure. 5.

In vivo response to PEG hydrogels laden with MSCs and osteogenically differentiating MSCs. Hydrogels were implanted subcutaneously for 28 days in immunocompetent C57bl/6 mice. Masson's Trichrome staining and H&E staining for PEG hydrogels containing: MSCs, 4 day differentiated MSCs, 10 day differentiated MSCs, 21 day differentiated MSCs, and no cells (referred to as PEG). Arrows in Masson's Trichrome stained sections indicate the fibrous capsule, while arrows in the H&E stained sections indicate the layer of inflammatory cells at the material / host interface. Fibrous capsule and inflammatory cell layer thicknesses are given as the number of cells layers present. Data are presented as mean with standard deviation as error bars (n=4). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Scale bar is 100 μm .