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Identification of IL-1 β and LPS as Optimal Activators of Monolayer and Alginate-Encapsulated Mesenchymal Stromal Cell Immunomodulation Using Design of Experiments and Statistical Methods

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Abstract

Induction of therapeutic mesenchymal stromal cell (MSC) function is dependent upon activating factors present in diseased or injured tissue microenvironments. These functions include modulation of macrophage phenotype via secreted molecules including prostaglandin E2 (PGE2). Many approaches aim to optimize MSC-based therapies, including preconditioning using soluble factors and cell immobilization in biomaterials. However, optimization of MSC function is usually inefficient as only a few factors are manipulated in parallel. We utilized fractional factorial design of experiments to screen a panel of 6 molecules (lipopolysaccharide [LPS], polyinosinic-polycytidylic acid [poly(I:C)], interleukin [IL]-6, IL-1 β , interferon [IFN]- β , and IFN- γ), individually and in combinations, for the upregulation of MSC PGE2 secretion and attenuation of macrophage secretion of tumor necrosis factor (TNF)- α , a pro-inflammatory molecule, by activated-MSC conditioned medium (CM). We used multivariable linear regression (MLR) and analysis of covariance to determine differences in functions of optimal factors on monolayer MSCs and alginate-encapsulated MSCs (eMSCs). The screen revealed that LPS and IL-1 β potentially activated monolayer MSCs to enhance PGE2 production and attenuate macrophage TNF- α . Activation by LPS and IL-1 β together synergistically increased MSC PGE2, but did not synergistically reduce macrophage TNF- α . MLR and covariate analysis revealed that macrophage TNF- α was strongly dependent on the MSC activation factor, PGE2 level, and macrophage donor but not MSC culture format (monolayer versus encapsulated). The results demonstrate the feasibility and utility of using statistical approaches for higher throughput cell analysis. This approach can be extended to develop activation schemes to maximize MSC and MSC-biomaterial functions prior to transplantation to improve MSC therapies.

Keywords

mesenchymal stromal cells; fractional factorial design; alginate encapsulation; immunomodulation

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Additional Supporting Information may be found in the online version of this article.

Introduction

Mesenchymal stromal cells (MSCs) are a heterogeneous population of multipotent, adult stem-like cells isolatable from many different tissues.¹ These cells exhibit multiple properties that make them attractive as a potential cellular therapeutic, including nonimmunogenicity, migratory potential, and many trophic functions achieved via cytokine secretion that beneficially modulates other cell types, including those involved in innate and adaptive immune responses.^{2,3} We and others have shown that MSCs promote both the attenuation of proinflammatory macrophage behavior, such as elevated tumor necrosis factor (TNF)- α secretion, and the reprogramming of the cells into inflammation-resolving and wound healing macrophages, *in vitro* and *in vivo*,⁴⁻⁶ and that prostaglandin E2 (PGE2) is a particularly potent regulator of macrophage phenotype.^{7,8}

The therapeutic benefit of MSCs may ultimately be limited, however, by several compounding factors. Although MSCs are capable of migrating to damaged and inflamed environments, the efficiency of this homing has been demonstrated to be very low in several *in vivo* systems.⁹⁻¹¹ This is further complicated by relatively rapid clearance of the cells from the body, even when the delivery method is more localized.^{12,13} To address this challenge, we have previously immobilized MSCs in alginate *via* microencapsulation, which maintains cell viability, secretion, and modulation of macrophage phenotype *in vitro* and in a rat model of contusive spinal cord injury.⁴

In addition, most MSC regulatory functions are not constitutive or spontaneous, but require induction by activating signals, such as soluble factors, hypoxia, or other stressors in the microenvironment of damaged tissues.¹⁴ Furthermore, several reports have demonstrated *in vitro* that MSCs, similarly to macrophages and many other cells types, can adopt a spectrum of both pro- and anti-inflammatory phenotypes, dependent upon their exposure to cytokines and toll-like receptor (TLR) ligands at different concentrations, exposure times, and combinations.¹⁵⁻¹⁷ Therefore, from a therapeutic standpoint, it is important to identify potentially exploitable exogenous activation mechanisms that maximize desirable MSC phenotypes. Although the effects of many different activation factors on MSC functions have been investigated, many of these studies limit their investigation to manipulating 1 or 2 factors at a time.^{14,17-21} This approach is time consuming and costly given the scope of potential activators of MSCs. Design of experiments (DoE) and statistical approaches can be used to analyze the effects of several relevant factors on the output of bioprocesses while greatly reducing the number of experiments required, thereby also reducing time and costs.²² This is achieved by using regression analysis to create empirical mathematical models of phenomena based on data collected from the experimental design. From this, the effects of individual soluble factors (main effects) and interactions of these factors can be determined as well the importance of a biomaterial or other environmental cues.²³

To elucidate the effects of components of the *in vivo* inflammatory environment on MSC activation, we utilized a fractional factorial design (FFD) to establish an *in vitro* screening assay to identify factors and/or combinations of factors from a panel of six activating molecules (lipopolysaccharide [LPS], polyinosinic-polycytidylic acid [poly(I:C)], interleukin [IL]-6, IL-1 β , interferon [IFN]- β , and IFN- γ) that concurrently maximize MSC

secretion of anti-inflammatory PGE2 and MSC-mediated attenuation of proinflammatory macrophage TNF- α production. We subsequently used general linear models and statistical methods to compare the functions of alginate encapsulated MSCs to those of monolayer cells in response to selected factors. This knowledge and approach can be extended to investigate and develop activation schemes to maximize specific MSC and MSC-biomaterial functions prior to transplantation in order to better guarantee the action of MSCs *in vivo*.

Methods

Chemicals and reagents

All cell culture reagents and growth factors were purchased from Life Technologies (Carlsbad, CA), unless otherwise stated. Cytokines were purchased from R&D Systems (Minneapolis, MN). LPS and encapsulation reagents (sodium alginate, poly-L-lysine [PLL, 30–70 kDa], 3-(N-morpholino)-propanesulfonic acid [MOPS], calcium chloride, and sodium chloride) were purchased from Sigma Aldrich (Oakville, Ontario, Canada). High molecular weight poly(I:C) was purchased from InvivoGen (San Diego, CA).

Cell culture

All cells were maintained in a humidified 37°C incubator with 5% CO₂ and cultured in the indicated basal medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin, with additional or alternative supplements added as indicated.

MSCs—Human bone marrow-derived MSCs were purchased from the Institute for Regenerative Medicine (Texas A&M College of Medicine, Temple, TX). Differentiation assays and surface molecule expression analysis by flow cytometry to validate the identity of the cells as MSCs were performed by the provider. MSCs at passage 2 were thawed and seeded as a monolayer culture in 175 cm² flasks at 1.5×10^4 cells/mL. They were cultured in MEM- α containing no deoxy- or ribonucleosides, supplemented with 10% FBS (Atlanta Biologicals, Flowery Branch, GA) and 1 ng/mL basic fibroblast growth factor (bFGF). The cells were grown to 70% confluence, detached with trypsin, seeded into 96-well plates at 2×10^3 cells/well, and allowed to attach overnight.

Macrophages—Human peripheral blood from multiple adult donors (The Blood Center of New Jersey, East Orange, NJ) was fractionated on a density gradient (Ficoll-Paque Premium, 1.077 g/mL, GE Healthcare, Piscataway, NJ); the buffy coat was collected and washed twice with $1 \times$ PBS. Antibody-coated microbeads (MACS, Miltenyi Biotec, San Diego, CA) were used according to the manufacturer's instructions to isolate CD14⁺ monocytes, which were seeded at 1×10^7 cells/175 cm² flask in fully supplemented Advanced RPMI 1640. The cells were allowed to attach for 2–6 h before gently replacing the medium with medium containing 5 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) to induce differentiation into proinflammatory macrophages. After 7 days of culture, the differentiated cells were detached with trypsin, seeded into 96-well plates at 1×10^4 cells/well, and allowed to attach overnight. A different macrophage donor was used in each experimental replication.

Alginate microencapsulation

MSCs were thawed and expanded as described above. At 70% confluence, cells at passage 2 were detached with trypsin and seeded into 225 cm² flasks for further expansion. The cells were grown to 70% confluence, detached with trypsin, and encapsulated in alginate as described previously.^{4,24} Briefly, MSCs were suspended in non-supplemented Ca²⁺-free DMEM and subsequently mixed with 2.2% (w/v) alginate in Ca²⁺-free DMEM to achieve a final cell density of 4×10^6 cells/mL and a final alginate concentration of 1.98% (w/v). An electrostatic bead generator (Nisco Engineering AG, Zurich, Germany) was used to generate alginate-MSC microspheres 450–550 µm in diameter.⁴ The capsules were incubated for 10 min in a crosslinking solution containing 100 mM calcium chloride, 145 mM sodium chloride, and 10 mM MOPS in deionized water (pH 7.2). Encapsulated MSCs (eMSCs) were then washed with PBS and coated with PLL by incubation in a 0.05% (w/v) solution. After a final PBS wash, eMSCs were suspended in fully supplemented culture medium and transferred to a 25 cm² flask overnight. Before use, eMSC viability was determined by Hoechst, calcein AM, and ethidium homo-dimer staining, as described previously.⁴ In experiments, eMSCs were cultured in 12-well plates at 2×10^4 cells/well (2×10^4 cells/mL).

Experimental design

Main and interacting effects of the six activating factors (LPS, poly(I:C), IL-6, IL-1β, IFN-β, or IFN-γ) at two doses (levels, Table 1) on MSC secretion and modulation of macrophage phenotype were investigated using a two-level half-fraction (2^{6-1}) factorial design of resolution VI (Tables S1 and S2, Supporting Information) generated using MiniTAB Statistical Software version 17 (Minitab, State College, PA). This resulted in 32 different conditions (Table 2), which were run in triplicate with intraexperimental duplicates. Factor levels were selected based on literature and previous studies. This design allows for the determination of all 6 main effects and all 15 two-factor interactions.

MSC monolayer cultures were washed once with fully supplemented RPMI 1640. Medium containing LPS, poly(I:C), IL-6, IL-1β, IFN-β, and/or IFN-γ at level –1 or 1 (not present or present, respectively) was added to the cells according to the experimental conditions listed in Table 2 and cultured for 48 h. Supernatants were collected and frozen at –80°C until analysis. The MSCs were fixed in 4% (w/v) paraformaldehyde (PF) for 20 min at room temperature and stored in 1% (w/v) PF at 4°C until further use. To investigate the effects of these factors on MSC-mediated modulation of macrophage phenotype, MSC conditioned medium (CM) was generated as described above, collected, spiked with 1 µg/mL LPS, and centrifuged to remove debris before being transferred to macrophages (Figure 1A).

Additionally, medium containing the activating factors according to the experimental conditions described above were similarly cultured for 48 h to generate acellular CM. This acellular CM was also spiked with 1 µg/mL LPS before being transferred to macrophages as controls. Macrophages cultured in fresh medium with and without LPS were included as negative controls. After 48 h of culture in CM, the macrophage supernatants were collected and frozen at –80°C until analysis.

To validate the findings of the fractional factorial screen, the experimental schematic was repeated using only optimal factors present at 2 levels individually and in combination.

MSC, acellular, and macrophage cultures were treated in the same manner as described above, with equivalent controls (Figure 1B). These experiments were run 3 times with 2–6 intraexperimental replicates per experiment. To determine the effects of these factors on encapsulated cells, this experiment was repeated using eMSCs (Figure 1B). These experiments were run 3 times with 3 intraexperimental replicates per experiment.

Cell counting

Fixed monolayer MSCs were brought up to room temperature, washed three times with PBS for 5 min, and incubated with 100 μ L of Hoechst stain (diluted 1:5000 in PBS; Molecular Probes, Eugene, OR) for 30 min in the dark. Cells were then washed twice with PBS and maintained in 200 μ L of PBS for imaging. Stained nuclei were visualized at 4 $\times$ using an inverted fluorescent microscope (IX81, Olympus, Tokyo, Japan) and counted using SlideBook image analysis software version 5.0 (Intelligent Imaging Innovations, Denver, CO).

Cytokine measurement

The cell culture supernatants collected from MSCs were thawed and analyzed using an enzymatic immunoassay for PGE2 (Cayman Chemical, Ann Arbor, MI), according to the manufacturer's instructions. Macrophage supernatants were thawed and analyzed using an enzyme-linked immunosorbent assay (ELISAs) for TNF- α (Biolegend, San Diego, CA), according to the manufacturer's instructions. Absorbances were recorded using a microplate reader (DTX 880 Multimode Detector, Beckman Coulter, Fullerton, CA).

Data analysis and statistics

The dataset for each cytokine measured in the supernatants from the screening assay was tested for outliers using Grubb's Test in Minitab and "anchored" to 1.00 (normalized to the lowest values in the dataset), if necessary. This data was then further transformed (i.e., manipulated using mathematical operations such as square root, natural log, etc.) as necessary using a Box-Cox power (λ) transformation to make the residuals random and fit a normal distribution, as evaluated by the Anderson-Darling test for normality using Minitab. A linear regression model (Eq. (1)) was fit to the transformed data in order to determine main effects and two-factor interactions. The general form of this model is:

$$Y(X_i) = \beta_0 + \sum_{i=1}^6 \beta_i X_i + \sum_{i=1}^5 \sum_{j=i+1}^6 \beta_{ij} X_i X_j + \varepsilon. \quad (1)$$

Here, Y is the transformed response, β_0 is a constant, X 's are the individual factors, XX 's are two-factor interactions, and ε is an error term. The coefficients of this equation (β 's) represent the main and interacting effects of each factor on the transformed response. Each replicate of the experiment was isolated into separate blocks within the analysis, over which the regression was averaged, to account for nonhomogenous environmental conditions between experiments. Statistical significance of the estimated effects was determined using analysis of variance (ANOVA), with P -values < 0.05 considered significant. To determine correlation between PGE2 and TNF- α in the smaller scale experiments, Spearman's

correlation coefficients (ρ) were calculated separately for MSC and eMSC conditions and scatter plots were generated. Total secreted PGE2 levels and relative TNF- α levels were anchored to 1.00 and transformed by taking the natural log. Linear regression was then performed in Microsoft Excel (Microsoft, Redmond, WA). To further compare the effect of the different MSC culture formats (monolayer vs. encapsulated), multivariable linear regression and covariate analysis were performed in Minitab to adjust the effects on natural log transformed TNF- α ($\ln[\text{TNF-}\alpha]$) to account for the activating factor used, the culture format, and differences in macrophage donor (nested within the culture format), with the anchored, natural log transformed total PGE2 ($\ln[\text{PGE2}]$) level as a covariate.

Results

Main effects and two-factor interactions for MSC PGE2 secretion

MSC-secreted PGE2 is an important factor in converting macrophages from pro-inflammatory to anti-inflammatory cells. To evaluate individual and interacting effects of the panel of factors on MSC PGE2 secretion, a regression model was fit to the results of the FFD (Eq. (1)). The data was first normalized to cell number to account for the effects of these factors on proliferation and then transformed by taking the natural log (Box-Cox transformation, $\lambda = 0$) to normalize the residuals (Figure S1C, Supporting Information) and make them random across fitted values (Figure S1A, Supporting Information) and the order of observation (Figure S1B, Supporting Information). A regression model (Eq. (1), Table S3, Supporting Information) was then fit to this transformed data, with a resulting R^2 of 95.79% (Table 3).

Graphs visually present these effects by solving for PGE2 and plotting these untransformed outputs of the regression equation (Figure 2). Effects and interactions that indicate increases in PGE2 were considered desirable. The main effects of LPS, IL-1 β , IFN- β , and IFN- γ indicate that these factors significantly increased PGE2 secretion (Figure 2A, Table 3). Within this group, IL-1 β had the largest effect, followed by LPS, whose effect was ~5 times less and accounted for only ~3% of the variability. Although, statistically significant, the estimated effects of IFN- β and IFN- γ were ~7 and ~19 times less than that of IL-1 β and accounted for only a combined 1.74% of the variation in the transformed data.

Subsets of these factors had significant interactions with each other in their effects on MSC PGE2 secretion (Figure 2B). As seen in Table 3, the transformed response of PGE2 secretion to the combined presence of LPS*IL-1 β , IL-1 β *IFN- β , IL-1 β *IFN- γ , or IFN- β *IFN- γ were of a similar negative magnitude, indicating that the magnitude of the change in the natural log of PGE2 caused by one factor was smaller in the presence of the other factor. However, in the graphical representations of these interactions, which present the calculated PGE2 level for each condition, it is apparent that the interactions of LPS*IL-1 β and IL-1 β *IFN- β are additive or synergistic. Furthermore, examination of these interactions using the untransformed fitted values revealed that the interactions of LPS with IL-1 β and IL-1 β with IFN- β are synergistic: the sum of the changes in PGE2 for the individual factors is less than the change in PGE2 caused when both factors are present (Table S4, Supporting Information). The interactions of either IL-1 β or IFN- β with IFN- γ , however, are indeed

mildly antagonistic. Poly(I:C) and IL-6 did not significantly affect PGE2 secretion, individually or in combination with other factors.

Main effects and two-factor interactions for macrophage TNF- α secretion

Because of the prevalence of resident and recruited macrophages and monocytes in most tissues and their involvement in almost all inflammatory and immune processes, these cells play a large role in many diseases and conditions.^{6,25} Macrophage conversion from proinflammatory to anti-inflammatory phenotypes is characterized in part by decreased TNF- α secretion.²⁶ Therefore we next explored the effect of this panel of factors on influencing MSC-mediated modulation of macrophage TNF- α secretion. The FFD was repeated in order to generate CM to culture LPS-activated macrophages. To determine the background effects of residual activating factors that may be in the MSC CM, LPS-activated macrophages were also cultured in acellular CM. The levels of macrophage TNF- α secretion in response to MSC CM were normalized to that of acellular CM (background) and anchored to 1.00 by dividing by the lowest value in the dataset (0.105) prior to analysis. This response was then transformed by taking the natural log (Box-Cox transformation, $\lambda = 0$) to make residuals normal (Figure S1C, Supporting Information) and random across fitted values (Figure S1A, Supporting Information) and the order of observation (Figure S1B, Supporting Information). Anchoring the dataset to 1.00 prior to the natural log transformation results in a dataset with positive values, which eases interpretation of the results. The regression model (Eq. (1), Table S3, Supporting Information) fit to the transformed TNF- α response had an R^2 of 83.86% (Table 3).

Since our aim is to activate MSCs for increased attenuation of the macrophage pro-inflammatory phenotype, decreases in macrophage TNF- α were considered desirable. The MSC activating factors with the largest main effects on macrophage TNF- α in the desirable direction were LPS and IL-1 β (Figure 3A), with the effect of IL-1 β being ~5 times that of LPS (Table 3). Conversely, IL-6 and IFN- β activation of MSCs increased macrophage TNF- α . These four main effects contributed to over half of the variation in the transformed data (Table 3). A subset of the combinations of these factors also had significant interactions (Table 3). Activation of MSCs with IL-6 decreased the effect of LPS on MSC-mediated reduction of macrophage TNF- α (Figure 3B). Similarly, the effects of IL-1 β activation on MSC-mediated reduction of macrophage TNF- α were lessened in the presence of IFN- β . The strongest interaction was seen between LPS and IL-1 β , where the magnitude of the effect of one factor on MSC modulation of macrophage TNF- α was mitigated in the presence of the other. The interpretation of these interactions was maintained when examining the relationships using the untransformed fitted values (Table S5, Supporting Information). This is also evident in the interaction plot depicting the untransformed fitted data, where the slope of the decrease in TNF- α is smaller in magnitude for the effect of LPS MSC activation when IL-1 β is present (Figure 3B). Examination and comparison of the TNF- α levels at each level individually in both the transformed and untransformed output (Table S5, Supporting Information), however, indicates that the macrophage TNF- α level when both LPS and IL-1 β are used to activate MSCs is lower than that of activation with LPS or IL-1 β alone. Therefore, the effect of combined MSC activation by IL-1 β and LPS is

beneficial for mitigating the pro-inflammatory macrophage phenotype, although the effects are not mathematically additive or synergistic.

Validation of the effects of LPS and IL-1 β on monolayer MSCs

We next aimed to validate the results of the FFD by using LPS and IL-1 β , alone and in combination without the presence of the nonoptimal factors, in our monolayer MSC and macrophage *in vitro* assays. As determined in the high throughput screening assay, IL-1 β exhibited a much higher potency than LPS. LPS and IL-1 β increased MSC PGE2 secretion ~2- and ~7-fold on a per cell basis, respectively (Figure 4A). Secretion of MSC PGE2 was higher and synergistic when both activating factors were used in combination.

Although unstimulated MSC CM attenuated TNF- α secretion from LPS-stimulated macrophages (Figure 4C), this reduction was greater when MSCs were activated with LPS. MSC activation with IL-1 β more potently reduced macrophage TNF- α secretion as compared LPS activation of MSCs. In contrast to the effects of these two factors on MSC PGE2 secretion, the combined use of LPS and IL-1 β to activate MSCs did not result in a synergistically enhanced attenuation of macrophage TNF- α secretion.

Effects of LPS and IL-1 β on eMSCs

After confirming the effects of LPS and IL-1 β using the monolayer MSC *in vitro* system, we investigated the effect of alginate encapsulation on this function. The alginate encapsulated MSCs exhibited a round morphology with small amounts of clustering, as opposed to the elongated spindle-shaped MSCs grown on tissue culture plastic (Figure S2, Supporting Information). PGE2 secretion from nonactivated eMSCs was lower than monolayer MSCs (1.08 vs. 2.88 ng/mL/10⁴ cells). Activation with IL-1 β alone resulted in a ~7.5-fold increase in PGE2 secretion from eMSCs per cell. LPS alone did not significantly induce PGE2 secretion (Figure 4B). Similarly to monolayer MSCs, the presence of both LPS and IL-1 β further increased eMSC PGE2 secretion compared to IL- β alone.

CM generated using non-activated eMSCs reduced macrophage secretion of TNF- α (Figure 4D). When eMSCs were stimulated with LPS, macrophage TNF- α secretion was further attenuated. Stimulation of MSCs with IL-1 β reduced macrophage TNF- α secretion to a greater degree as compared to no stimulation or LPS-activation, but this effect was not further enhanced when IL-1 β was used in combination with LPS.

Relation of MSC PGE2 secretion to macrophage TNF- α secretion

We next examined whether the general trends seen for MSCs and eMSCs reflected the known effects of MSC-secreted PGE2 on the modulation of macrophage function by calculating Spearman's correlation coefficient and plotting the relative levels of macrophage secreted TNF- α against total MSC-secreted PGE2 (i.e., not normalized to cell number). As seen in Figure 5A, the relationships between macrophage TNF- α secretion and PGE2 secreted from MSCs or eMSCs are nonlinear in nature and have statistically significant negative correlations. Data points representing eMSC conditions are shifted down, indicating higher total PGE2 levels and lower resulting macrophage TNF- α levels. To more analytically determine differences between the effects of the two MSC culture formats on

PGE2 secretion and macrophage modulation, the datasets were first manipulated to make the data more linear and comparative prior to plotting them against each other. Both metrics were normalized to their lowest values before natural log transformation. The differences between MSCs and eMSCs seen in Figure 5A were observed quantitatively in the slopes of the linear regression lines through the transformed data (Figure 5B). The slope of the linear relationship between macrophage TNF- α and eMSC-secreted PGE2 is significantly more negative than that of MSCs. This trend communicates that relative TNF- α levels extrapolated for higher values of PGE2 secreted from monolayer MSCs would not be reduced to the same level as a similar amount of eMSC-secreted PGE2.

Analytical comparison of MSC and eMSC function

The analysis above describes MSC and eMSC immunomodulation but does not account for the effects of activation factors, differences in macrophage donor (Figure S3, Supporting Information), or the variance of total secreted PGE2 levels across these conditions. To further investigate these apparent differences, multivariable linear regression was used to fit a general linearized model to the natural log transformed macrophage TNF- α secretion versus MSC culture format (MSC or eMSC), activation factor (none, LPS, IL-1 β , LPS +IL-1 β), and macrophage donor nested within MSC culture format (donors 1–3 for MSCs; donors 4–6 for eMSCs), with the natural log transformed, anchored total PGE2 level as a covariate. Analysis of covariance was then used to determine the significance of each of these variables. The PGE2 level, activation factor, and macrophage donor significantly affected the macrophage TNF- α response, indicated by their low *P*-values (Table 4). After adjusting for the effects of these other variables, the difference observed between MSCs and eMSCs became insignificant. This indicates that the encapsulation of MSCs in alginate can provide the benefits of immobilization without altering the activation and subsequent immunomodulation of MSCs by LPS and IL-1 β .

Discussion

Many current approaches are being developed to optimize MSC-based cellular therapies, including preconditioning using soluble activating factors and immobilization of the cells in biomaterial scaffolds.^{11,27,28} However, most studies are limited to manipulating 1 or 2 factors at a time, increasing resource consumption and cost while limiting the investigation throughput. Here we used design of experiments (DoE) methodology to screen a panel of soluble molecules for improved MSC immunomodulatory function and statistical tools to compare functions of optimal factors on monolayer MSCs and MSCs encapsulated in alginate.

Using a 2⁶⁻¹ fractional factorial experimental design (FFD), we developed a screening assay to identify soluble molecules that activate MSCs to upregulate PGE2 secretion and subsequently attenuate macrophage TNF- α secretion. This design allows for accurate estimation of the effects of all individual factors and all two-factor interactions, assuming that higher order interactions (4th order) are negligible. DoE methodology is extensively used in biotechnological fields for bioprocess development/optimization and is increasingly being utilized for basic and preclinical research applications, such as optimization of stem

cell differentiation and antiviral drug studies.^{29–32} We have previously utilized FFD to screen the effects of 13 MSC-secreted factors on the conversion of macrophages from a proinflammatory to an anti-inflammatory phenotype.⁸ This study identified PGE2 as the primary MSC-secreted molecule involved in this macrophage conversion.

While many factors have been previously reported to effect MSC secretome and function,^{14,17–21} knowledge of their effects on MSC modulation of macrophages is limited. IL-1 β has been reported to induce migration, chemoattraction, and secretion of several cytokines and growth factors from MSCs.^{18,33,34} Several studies have investigated the effects of TLR ligands, such as LPS, on MSC secretion and function, with conflicting results as both pro- and anti-inflammatory functions have been reported.^{15,17,35–38} We observed in our screening assay that these two factors optimally activated MSCs to upregulate PGE2 secretion and attenuate macrophage TNF- α secretion. When used together, there was a synergistic increase in PGE2 secretion, although this same synergistic effect was not seen for the reduction of macrophage TNF- α . This may indicate a lower limit in macrophage TNF- α attenuation in our system, where further increases in PGE2 will not further reduce TNF- α . Both IL-1 β and LPS activate myeloid differentiation primary response gene 88 (MyD88)-dependent pathways leading to nuclear factor (NF)- κ B and activator protein (AP)-1 transcriptional activity, which increases expression of cyclooxygenase-2 (COX-2) and subsequent enzymatic production of PGE2.³⁹ The activity of the NF- κ B pathway in MSC modulation of macrophages was shown to be critical in a mouse model of sepsis, where LPS and TNF- α stimulation of MSCs led to a PGE2-dependent reprogramming of proinflammatory macrophages into IL-10-secreting cells *in vivo*.⁷ LPS and IL-1 β stimulate different receptors with similar intracellular signaling machinery (TLR4 and IL1R1, respectively)⁴⁰ therefore the simultaneous presence of these two molecules may additively increase the activity of their overlapping pathways. While TNF- α may not be further reduced, other molecules may be modulated to further resolve macrophage function.

Poly(I:C), a synthetic analog of double stranded RNA, is a TLR3 ligand that has been reported to stimulate the secretion of many cytokines, including interleukins, type I interferons, and PGE2, from MSCs and has also been observed to enhance MSC migration and lymphocyte suppression.^{15,17,37,41,42} Interestingly, poly(I:C) did not have significant main effects or interacting effects in our system. This may be due to differences in cell source, molecular weight, concentration, and exposure duration between previous studies and our studies.

The effects of interferons on MSC modulation of lymphocyte phenotype and proliferation have been extensively explored. Both IFN- β (a type I interferon) and IFN- γ (a type II interferon) stimulation of MSCs have been reported to increase the production of indoleamine-2,3-dioxygenase-1 (IDO-1) and suppression of T-cell proliferation.^{16,17} To our knowledge, their explicit effect on MSC-macrophage interactions, however, has not been studied. In this study, the effects we observed for interferon activation of MSCs were small in magnitude and accounted for very little of the variation in the regression models, but were nonetheless statistically significant. Increases in MSC PGE2 production were observed upon IFN- β or IFN- γ activation, albeit far less than when activated with IL-1 β . However, despite this increase, combinatorial effects of these factors were disparate. IFN- β activation of

MSCs led to increases in macrophage TNF- α and antagonized its attenuation by IL-1 β -activated MSC CM, despite the synergistic interaction of IFN- β with IL-1 β to increase PGE2 secretion. Upregulation of NF- κ B activity and COX-2 transcription by IFN- β has been reported in Huh7 cells⁴³ and microglia⁴⁴ and IFN- γ has previously been reported to increase COX-2 expression and secretion of PGE2 from MSCs.^{45,46} However, discrepancies in cell type and source as well as higher concentrations of IFN- γ make comparisons with the current study difficult. It has been also suggested that IFN- γ can inhibit c-Jun, a subunit of AP1, binding to COX-2 promoter regions, thereby potentially antagonizing the effects of LPS and IL-1 β activity,⁴⁷ although this function may be dependent on concentration and cell type. Type I and type II interferons signal primarily through signal transducer and activator of transcription factor (STAT) proteins in heterodimeric or homodimeric combinations, respectively. There is ample evidence in the literature of a complicated crosstalk between the janus kinase (JAK)/STAT and NF- κ B pathways. STAT3 activity has been reported to inhibit NF- κ B activity, potentially via competitive binding to DNA promoter regions.^{48,49} Alternatively, STAT1 signaling induces pro-inflammatory cellular stress, which is essential for the induction of MSC therapeutic properties, and may also be inhibited by STAT3 activity.⁵⁰ The divergence of downstream effects of type I and type II inter-feron signaling could be explained by their differing ratios of STAT1 and STAT3 activity. Further studies are needed to clearly elucidate the interactions of these signaling pathways in human MSCs and the downstream effects on secretion and immunomodulation.

IL-6, also a stimulator of JAK/STAT pathways, is secreted constitutively from MSCs at high levels and is highly inducible by factors activating the NF- κ B pathway. Supplementation of MSC culture medium with exogenous IL-6 has been shown to increase MSC proliferation¹⁸ and vascular endothelial growth factor (VEGF) secretion in combination with transforming growth factor (TGF)- α .²⁰ Additionally, PGE2 secretion from murine MSCs has been reported to be dependent on MSC *IL6* expression, suggesting an autocrine IL-6 function.⁵¹ In our system, IL-6 activation of MSCs did not affect PGE2 secretion and antagonized the LPS-activated MSC attenuation of macrophage TNF- α secretion. Similar to the effects observed for interferon stimulation, these effects were small in magnitude and accounted for very little of the variation in the regression model, but were nonetheless statistically significant. Because of the potential saturation of MSC IL-6 receptors by the abundance of IL-6 by constitutive secretion and induction by other cytokines, additional exogenous IL-6 may not be effective in eliciting a desirable response.

Immobilization of cells in semi-permeable biocompatible hydrogels, such as alginate, can address inefficient homing and rapid clearance *in vivo* and has successfully been performed for many cell types, including primary cells, genetically engineered cells, and stem cells.²⁷ This approach allows for exchange of nutrients and therapeutic molecules from the entrapped cells. Alginate encapsulated cells have extensively been investigated for several biomedical applications ranging from stem cell differentiation²⁴ to delivery of cell-derived bioactive molecules^{52,53} to tissue replacement.⁵⁴ We encapsulated the cells in alginate microspheres as we have previously described for the use of intrathecally delivered eMSCs to treat inflammation associated with acute spinal cord injury.^{4,55} While the primary purpose of the hydrogel is to immobilize the cells, the mannuronate subunits of alginate have been reported to have biological activity similar to that of TLR2 and 4 ligands, primarily on cell

line derived and primary monocytes and macrophages.^{56–61} Additionally, three-dimensional culture has been demonstrated to enhance certain properties of MSCs as compared to two-dimensionally cultured cells, including PGE2 secretion.^{62–64} We have previously observed that the MSC secretome was enhanced when encapsulated in alginate, constitutively and after activation with TNF- α and IFN- γ .⁴ Therefore, after validating the results of the optimal factors LPS and IL-1 β identified in the FFD screen using monolayer cells, we next determined if the effects of these factors are altered in alginate-encapsulated MSCs. We found that although constitutive levels of PGE2 were lower for eMSC, trends in PGE2 secretion and macrophage TNF- α secretion were similar to those observed for IL-1 β activated monolayer cells. LPS activation did not significantly increase eMSC secretion, although it did synergize with IL-1 β to further increase PGE2 production.

The apparent reduced activity of LPS for eMSCs when used alone may possibly be attributed to the structural and chemical aspects of LPS, which is an 10 kDa amphiphilic molecule that forms micelles in aqueous solutions.⁶⁵ These aggregates have been reported to contain 10¹ to 10³ molecules per micelle, resulting in a macromolecule with a molecular weight of 10² to 10⁴ kDa.^{65,66} The portion of LPS that is responsible for the elicitation of biological responses is hydrophobic and requires LPS binding protein (LBP) and soluble CD14 (sCD14), which are present in serum.^{67,68} Other serum molecules such as albumin also bind to LPS.⁶⁹ These molecules have molecular weights <100 kDa, thereby also increasing the size of the LPS complex. At this point, effects of the molecular weight cut-off (MWCO) of the semipermeable alginate hydrogel are more likely to become a concern. The MWCO for diffusion into alginate capsules depends on several factors including viscosity of the alginate and the degree of coating by PLL, which can tune the MWCO between values on the order of 10¹ to 10³ kDa.^{66,70–73} Therefore, it is possible that the MWCO of the capsules used in these studies was below that of LPS aggregates and serum complexes, resulting in suppressed action of LPS on the eMSCs. We also speculate that interaction of these complexes with the PLL coating of the capsules or the alginate itself and interactions of mannuronate residues with TLR4 receptors may present physical and chemical barriers of LPS action on the entrapped cells. The enhancement of MSC PGE2 secretion when LPS and IL-1 β were used together compared to IL-1 β alone, however, suggests that the interaction of these two molecules on MSCs may extend past additive stimulation of redundant pathways. Attenuation of macrophage TNF- α secretion was observed for non-activated eMSC CM, with enhanced attenuation seen for LPS-activated eMSC CM. Further attenuation was observed for IL-1 β activated eMSC CM, with no further benefit seen by adding LPS coactivation. Studies examining the biophysical interactions of LPS and MSCs in this alginate capsule format will be needed to determine the mechanism underlying these observations.

The function of PGE2 as a modulator of macrophage function has been extensively established in literature.^{7,8,74–76} While IL-1 β and LPS synergistically increased PGE2 secretion from both monolayer and encapsulated cells, their activation of MSCs and eMSCs did not further reduce macrophage TNF- α secretion as compared to IL-1 β MSC activation alone. This implies that there may be a limit to TNF- α depression achievable. However, increased PGE2 has been shown to affect other macrophage phenotypic changes such as increased IL-10 secretion as well as the functions of other immune and inflammatory cells.

^{5,77} When comparing the relationship of PGE2 secretion and macrophage TNF- α for MSC and eMSC CM, we initially observed that the linear relationship between macrophage TNF- α and eMSC-secreted PGE2 was more negative than that of MSCs. This analysis suggested a difference between the modulation of macrophage secretion by CM generated by eMSC and MSCs, but does not account for the other variables embedded within the dataset, including differences in total PGE2 secretion due to variance in cell concentration and selection of activation molecule or differences in macrophages derived from different donors. ANOVA is case of the application a general linear model to data containing a continuous dependent variable and a categorical independent variable. Analysis of covariance is an extension of this using a continuous covariate(s) as an independent variable in addition to a categorical independent variables in order to adjust the output of the linear relationships for the effects of that covariate. The inclusion of multiple categorical predictors expands the method to multivariable linear regression. This approach is often used to elucidate the results of clinical/epidemiological studies and market research, where there are many uncontrolled but potentially important covariables, such as gender, age, preferences, and lifestyle parameters.^{78–81} Statistical analysis using multivariable linear regression revealed that the variables of activation factor, macrophage donor differences (nested within the MSC culture format), and the covariance of total PGE2 level more significantly explained differences in macrophage TNF- α secretion than did the MSC culture format, which was not significant. These results regarding the effect of macrophage donor further highlight the importance of patient variability in determining the effectiveness of MSC functions. The field may benefit from similar *in vitro* studies emphasizing human epidemiological effects on MSC therapeutic benefit, as these types of challenges are not a factor in controlled animal models.

There are limitations of the *in vitro* platform and approach described here. FFDs operate on the assumption that higher order interactions (4th order) are negligible as compared to main effects and two-factor interactions. This is a reasonable assumption for generalized screening purposes. However in complex biological organisms, where much remains unknown, this assumption must be considered carefully in conjunction with prior knowledge of the system. Additionally, the design used here utilizes the factors of interest at two levels and does not include center points, which only allows for linear relationships to be described. Although nonlinear relationships and interactions that may be present will not be detected, this design is useful for initial screens with fewer conditions focusing on general phenomena. The power of subsequent experimental designs can be increased by using three-level or mixed level designs, for which analysis is also well defined.²² Regardless of the design used, however, it must keep kept in mind that while the results are very informative, further cognitive analysis is sometimes needed uncover the true meaning of the output of the design, as was seen in the interpretation of interactions in the current study.

In the current study, MSC from a single donor were used, which is appropriate for such a proof-of-concept investigation. However, MSC donor variability has also been shown to be an important factor in determining MSC behavior.³⁴ Therefore, future studies will also include analysis across multiple MSC donors, particularly in light of our findings regarding the importance of macrophage donor variability.

Some of the factors in our panel are either secreted constitutively or upon stimulation with other factors used, adding a degree of convolution to the system. This is less important in screening experiments, in which the mechanism of action for the observed results is contained in a black box where inputs and outputs of interest are the focus. Mechanistic questions become more appropriate in subsequent studies, which further investigate a smaller number of expanded variables identified in the screen. TNF- α has been shown to be a potent activator of MSC functions,⁷⁷ but was not included in the current screen due its use as a metric of macrophage phenotype in the assay. Related to this, the use of CM rather than coculture in the present study allows for information to be gained more explicitly about the effect of the activating factors on the MSCs themselves rather than the dynamics of MSC-macrophage cross-talk. However, residual amounts of these activating factors may be present in the CM and may have an effect on the macrophages in subsequent culture. The control of acellular CM used in our study represents the maximum possible level of residual factors in the CM. Additionally, the CM is representative of constant exposure of MSCs to an activating factor. In a clinical setting, coadministration of inflammatory molecules with MSCs would not be desirable. Rather, MSCs or eMSCs would be pre-exposed to activation factors and washed prior to injection. There have been several reports of preactivation and preconditioning generating different MSC functional phenotypes and having enhanced therapeutic behavior *in vitro* and *in vivo*.^{15,28,33,82,83} The approach described here can easily be adapted to facilitate *in vitro* investigations of preactivation. Additionally, the screening assay can be adjusted to study the main and interacting effects of other factors on additional MSC therapeutic functions, such as angiogenesis and neuroprotection, by simply modifying the inputs and output metrics.

Conclusion

We have identified IL-1 β from a screen of a panel of activating molecules as an optimally potent enhancer of MSC and eMSC immunomodulatory function, with LPS increasing the potency of IL-1 β 's effect in some aspects of this function. Furthermore, macrophage donor variability was found to play a significant role in the assessing the potency of these responses. The results underscore the need to systematically investigate the effects and interactions of multiple relevant factors, including biomaterials, soluble factors and patient variability, on MSC therapeutic characteristics. We demonstrated the feasibility and utility of using statistical and computational approaches to facilitate these types of investigations in a higher throughput and in depth manner. These studies will be used to further elucidate MSC therapeutic mechanisms and harness control of these functions by developing pre-activation and delivery schemes for improved MSC function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematics of *in vitro* experiments. (A) To measure the main and interacting effects of the six activating factors on MSC secretion of PGE2 and the modulation of macrophage TNF- α secretion by MSC CM, MSCs, or acellular wells were incubated for 48 h (hr) with soluble activating factors (dashed box) in combinations determined by the FFD. MSC or acellular CM was spiked with LPS and used to culture macrophages (Macs) for 48 h. Assays were then performed to quantify metrics of interest (gray shaded boxes). (B) Optimal factors (dashed box) were validated by repeating the aforementioned *in vitro* assay for monolayer MSCs and comparatively tested using eMSCs.

Figure 2.

Effects of activating factors on MSC PGE2 secretion. MSCs were incubated for 48 h with combinations of the six activating factors in the panel. The level of PGE2 in the cell culture supernatants were then quantified and normalized to the number of MSCs present at the end of culture. Plots of (A) main effects and (B) statistically significant two-factor interactions of the six MSC activating factors were then generated. The absence of the factor is indicated by “0” while “1” or “1000” indicates its presence and concentration (ng/mL) in the activation cocktail. The largest increases in MSC PGE2 secretion were seen for IL-1 β and LPS. The combination of IL-1 β with either LPS or IFN- β synergistically increased MSC PGE2 secretion. The presence of IFN- γ antagonized the desirable effects of IFN- β and IL-1 β . Data are average values calculated using the regression model that was fit to three individual experiments with two replicates per experiment.

Figure 3.

Effects of activating factors on MSC modulation of macrophage TNF- α secretion. Macrophages were incubated for 48 h in CM collected from MSCs activated with combinations of the six factors in the panel. The levels of TNF- α in the macrophage cell culture supernatants were then quantified and normalized to that of corresponding acellular CM. Plots of (A) main effects and (B) statistically significant two-factor interactions of the six activating factors as calculated using the regression model were then generated. The absence of the factor is indicated by “0” while “1” or “1000” indicates its presence and concentration (ng/mL) in the activation cocktail. LPS- and IL-1 β -activated MSC CM reduced macrophage TNF- α secretion. The magnitude of this reduction caused by LPS activation was lessened in the presence of IL-1 β , but the macrophage TNF- α level was ultimately lower when both factors were used to activate MSCs. The presence of IFN- β antagonized the desirable effects of IL-1 β , as did IL-6 for LPS. Data are average values calculated using the regression model that was fit to three individual experiments with two replicates per experiment.

Figure 4.

Validation of effects of LPS and IL-1 β on MSCs and eMSCs. MSCs or eMSCs were incubated for 48 h with LPS or IL-1 β , individually and in combination. The level of PGE2 in the cell culture supernatants were then quantified and normalized to the number of (A) MSCs or (B) eMSCs present at the end of culture. MSC PGE2 secretion was increased in the presence of LPS and IL-1 β , individually and synergistically in combination, after 48 h of culture. eMSC PGE2 secretion was not significantly increased in the presence LPS but was increased in the presence IL-1 β and synergistically in combination with LPS. Data are means $\pm$ SEM of three independent experiments with 3–6 replicates per experiment ($P < 0.05$ compared to LPS⁻ IL-1 β ⁻ (*), LPS⁺ IL-1 β ⁻ (**), or LPS⁻ IL-1 β ⁺ (***) by ANOVA with Fisher's LSD *post hoc* test). Macrophages were incubated for 48 hours in CM collected from activated (C) MSCs or (D) eMSCs. The level of TNF- α in the macrophage cell culture supernatants were then quantified and normalized to that of corresponding acellular CM. TNF- α secretion was reduced from LPS-activated macrophages cultured with MSC or eMSC CM. CM from either LPS- and IL-1 β -activated MSC or eMSC further decreased macrophage TNF- α production; coactivation of MSCs or eMSCs with both LPS and IL-1 β did not result in further reduction in TNF- α as compared to IL-1 β -activated MSC or eMSC CM alone. Reductions in TNF- α were larger in magnitude for eMSC CM conditions. Data are means $\pm$ SEM of three independent experiments with 3–6 replicates per experiment ($P < 0.05$ as compared to LPS (*) , MSC CM (**), or LPS-MSC CM (***) by ANOVA with Fisher's LSD *post hoc* test).

Figure 5.

Statistical analysis of relationship between MSC and eMSC secreted PGE2 and macrophage TNF- α . (A) Total PGE2 and coresponding reative TNF- α levels from experiments testing the effects of LPS and IL-1 β on MSCs (open circles) and eMSCs (black circles) were plotted against each other. Spearman's correlation coefficients (ρ) and their statistical significance were calculated. The relationship was non-linear with a significant negative correlation for both MSC and eMSC datasets. (B) PGE2 and TNF- α levels were normalized to their lowest value, transformed by taking the natural log, and plotted against each other. Linear regression was then performed. The negative slope for eMSC (dashed line) was significantly steeper than that of MSCs (solid line), as determined by Student's t -test with a 95% confidence interval. Data points represent the individual replicates in each experiment (MSCs: $n = 43$; eMSCs: $n = 36$).

Table 1

MSC activation factors and levels

		Level (ng/mL)	
Activation Factor (X_i)		Low (-1)	High (+1)
X_1	LPS	0	1000
X_2	Poly(I:C)	0	1000
X_3	IL-6	0	1
X_4	IL-1 β	0	1
X_5	IFN- β	0	1
X_6	IFN- γ	0	1

Table 2

Two-level, half-fraction (2^{6-1}) fractional factorial design of resolution VI

Run	Activation Factor Level					
	X_1	X_2	X_3	X_4	X_5	X_6
1	-1	-1	-1	-1	-1	-1
2	1	-1	-1	-1	-1	1
3	-1	1	-1	-1	-1	1
4	1	1	-1	-1	-1	-1
5	-1	-1	1	-1	-1	1
6	1	-1	1	-1	-1	-1
7	-1	1	1	-1	-1	-1
8	1	1	1	-1	-1	1
9	-1	-1	-1	1	-1	1
10	1	-1	-1	1	-1	-1
11	-1	1	-1	1	-1	-1
12	1	1	-1	1	-1	1
13	-1	-1	1	1	-1	-1
14	1	-1	1	1	-1	1
15	-1	1	1	1	-1	1
16	1	1	1	1	-1	-1
17	-1	-1	-1	-1	1	1
18	1	-1	-1	-1	1	-1
19	-1	1	-1	-1	1	-1
20	1	1	-1	-1	1	1
21	-1	-1	1	-1	1	-1
22	1	-1	1	-1	1	1
23	-1	1	1	-1	1	1
24	1	1	1	-1	1	-1
25	-1	-1	-1	1	1	-1
26	1	-1	-1	1	1	1
27	-1	1	-1	1	1	1

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Activation Factor Level						
Run	X ₁	X ₂	X ₃	X ₄	X ₅	X ₆
28	1	1	-1	1	1	-1
29	-1	-1	1	1	1	1
30	1	-1	1	1	1	-1
31	-1	1	1	1	1	-1
32	1	1	1	1	1	1

Table 3

Statistically significant main effects and two-factor effects

Response (Transformed)	Regression Term	Transformed Response Estimated Effect	Contribution (%)	P-Value
<i>ln</i> (PGE2) ($R^2 = 95.79\%$)				
	LPS	0.4057	2.86	0.000
	IL-1 β	2.1177	82.66	0.000
	IFN- β	0.2843	1.51	0.000
	IFN- γ	0.113	0.23	0.003
	LPS*IL-1 β	-0.2354	1.04	0.000
	IL-1 β *IFN- β	-0.1158	0.25	0.002
	IL-1 β *IFN- γ	-0.1721	0.55	0.000
	IFN- β *IFN- γ	-0.2360	1.03	0.000
<i>ln</i> (TNF- α /0.105) ($R^2 = 83.86\%$)				
	LPS	-0.1513	2.20	0.000
	IL-6	0.0734	0.52	0.021
	IL-1 β	-0.7255	50.65	0.000
	IFN- β	0.274	7.23	0.000
	LPS*IL-6	0.0715	0.49	0.025
	LPS*IL-1 β	0.1124	1.22	0.000
	IL-1 β *IFN- β	0.0775	0.58	0.015

Table 4

Summary of general linear model with covariate and nested factors

Source	df	Adj SS	F-Statistic	P-Value
PGE2 Level (Covariate)	1	5.916	111.35	0.000
MSC format	1	0.154	2.89	0.094
Activation factor	3	1.541	9.67	0.000
Macrophage donor (nested in MSC Format)	4	14.181	66.73	0.000
Error	69	3.666		
Total	78			