APPROACHES TO IMPROVING THE THERAPEUTIC POTENTIAL OF MESENCHYMAL STROMAL CELLS

by

ANDREA GRAY

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Martin L. Yarmush, MD, PhD

And approved by

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ABSTRACT OF THE DISSERTATION

Approaches to Improving the Therapeutic Potential of Mesenchymal Stromal Cells

By ANDREA GRAY

Dissertation Director:

Martin L. Yarmush, MD, PhD

Mesenchymal stromal cells (MSCs) hold great potential as a cellular therapy due in part to their tissue protective and regenerative abilities, achieved via the secretion of many bioactive molecules and induced by microenvironmental cues including soluble factors. However, translation of MSCs into the clinic, where variability exists amongst both MSC donors and recipients, has not resulted in dramatic success. Efforts to identify activation inputs that promote specific MSC phenotypes have been inconsistent and lack systematic optimization. The goal of our work is to address these hurdles using a combination of approaches. We aimed to (1) develop a systematic approach for optimizing activation parameters (type and/or combinations of activating molecule, concentration, and duration of exposure) that enhance MSC immunomodulation, (2) characterize MSCs treated with the resulting pre-activation protocol, and (3) test the performance of the pre-activation protocol using multiple MSC donors. In a high throughput *in vitro*

screening assay designed using fractional factorial design of experiments, we identified interleukin (IL)-1β and lipopolysaccharide (LPS), individually and in combination, as optimal activators of MSC attenuation of pro-inflammatory macrophage tumor necrosis factor (TNF)-α production via prostaglandin E2 (PGE2) secretion. Further optimization of dose and duration of pre-activation, however, revealed that brief (1 hour) exposure of to 1 ng/mL IL-1β alone resulted in the highest sustained upregulation of PGE2 secretion post-activation. This pre-activation protocol generated MSCs whose PGE2 secretion was more sensitive to induction by secondary inflammatory molecules than MSCs with no pre-activation. IL-1β pre-activation led to enhanced MSC-mediated attenuation of macrophage TNF-α in a co-culture assay. This protocol was also successful in enhancing these functions for multiple MSC donors, although variability was noted in the absolute secretion levels of PGE2, the level of improvement in macrophage modulation, and the correlation of MSC PGE2 and macrophage TNF-α. Using the previous assays, we found that alginate encapsulated MSCs retained the ability to respond to activation factors. Statistical analysis revealed that macrophage TNF-α level was more significantly a function of PGE2 level, MSC activation factor, and macrophage donor rather than MSC culture format (monolayer versus encapsulated).

DEDICATION

For Dad, my biggest fan.

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Chapter 1: Introduction

1.1 Mesenchymal Stromal Cells

Mesenchymal stromal cells (MSCs) are a heterogeneous population of adult stem-like cells commonly isolated from various tissues including bone marrow, adipose tissue, placenta and umbilical cord [1]. They are identified by their adherence to tissue culture plastic, tri-lineage (osteogenic, adipogenic, chondrogenic) differentiation, and positive expression of cluster of differentiation (CD)73, CD90, and CD105, and the lack of expression of CD11b, CD14, CD19, CD79α, CD34, CD45, and human leukocyte antigen (HLA)-DR [2]. Lack of expression of major histocompatibility complex (MHC) class II led to the prevailing notion that MSCs are non-immunogenic [3], although this has become a hotly contested topic [4]. Nevertheless, MSCs have come to represent a rapidly expandable cell source that is feasibly obtainable and suitable for both autologous and allogeneic use [5]. Initially, interest in using MSCs as a cellular therapeutic focused on exploiting their multipotency to engraft in tissue and replace diseased, damaged, or depleted cell populations [6]. However, *in vivo* studies demonstrating therapeutic effects of MSCs with little to no engraftment in host tissues shifted the paradigm to attribute the therapeutic effects of MSCs to multiple functions [7]. These functions include MSC secretion of a spectrum of bioactive molecules that beneficially modulate other cell types [8]. These paracrine effects of MSCs have been extensively explored *in vitro* and in *in vivo* animal models of human diseases [9-11].

1.2 MSC Immunomodulation

MSCs are being investigated as a treatment for a wide variety of diseases and traumatic injuries, including autoimmune conditions, neurodegenerative disorders and traumas, myocardial infarction, wound healing, and osteoarthritis [12]. Beneficial effects in these applications, including immunomodulation, anti-apoptosis, anti-fibrosis, angiogenesis, chemoattraction, and trophic support of stem and progenitor cell populations for tissue regeneration, are imparted primarily by MSC paracrine secretion of bioactive molecules including cytokines, chemokines, growth factors, and trophic factors [10]. The action of MSC-secreted immunomodulatory factors on target effector cells has been demonstrated to be an integral part of MSC-mediated functional improvements in many autoimmune and inflammatory conditions and tissue trauma [13]. MSCs were first observed to be immunosuppressive in animal models of graft versus host disease (GvHD), in which they reduced allograft rejection in part by suppressing T cell proliferation and activity [14]. Since then, MSCs have been demonstrated *in vitro* and *in vivo* to suppress T cell proliferation and promote generation of regulatory subtypes via secretion of indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), transforming growth factor (TGF)-β1, HLA-G, hepatocyte growth factor (HGF), heme oxygenase (HO)-1, and galactin [15].

MSC modulation of cells of the innate immune system has also been demonstrated [16]. Macrophages are phagocytic innate immune cells that are differentiated from monocytes. They reside in virtually all tissues and are involved in almost all inflammatory and immune processes [17]. In addition to tissue

resident macrophages, monocytes are recruited to areas of tissue damage, at which point they differentiate into macrophages. Consequently, they play a large role in many diseases and conditions, including traumatic injuries, sepsis, chronic obstructive pulmonary disease (COPD), and cancer [17, 18]. It has been established that macrophages are quite plastic, capable of adopting a spectrum of phenotypes dictated by their microenvironments [17]. Two polarized phenotypes within this spectrum are the classical M1 and alternative M2 phenotypes. M1 macrophages have pro-inflammatory functions, while M2 macrophages are anti-inflammatory, promoting resolution and tissue repair [18, 19]. MSCs have been found to promote both the formation of M2 macrophages from monocytes and the reprogramming of M1 macrophages into M2 macrophages, *in vitro* and *in vivo* [16, 20]. Several MSC-secreted molecules have been implicated in promoting the M2 phenotype, including PGE2, interleukin 1 receptor antagonist (IL1-RA), and tumor necrosis factor-stimulated gene 6 (TSG6) [21-24].

1.3 MSC Responsiveness to Microenvironmental Cues

Therapeutically exploitable properties of MSCs are not spontaneous or constitutive, but are induced by cues within their microenvironment [13, 24, 25]. These cues include soluble factors, extracellular matrix components (ECM) and mechanical properties [26], hypoxia [25], and mechanical stimulation [27]. For activation by soluble factors, MSC phenotype is dictated by multiple parameters, including the type of activating molecule, its prevalence/concentration, duration/timing of exposure, and whether it is in combination with another

factor(s) [28]. MSC response to activating factors can be described by measurable descriptors including changes in secretome, surface marker expression, chemotaxis, adherence to ECM, proliferation, and gene expression. These changes in MSC phenotype will presumably result in MSC-mediated changes in target cell characteristics.

There have been several reports in the literature demonstrating changes in MSC response/phenotype induced by exposure to cytokines/growth factors [29-46] and toll like receptor (TLR) ligands [31, 41, 43, 44, 47-51] at different concentrations, exposure times, and combinations (Tables 1.1 and 1.2). Disparate responses can be seen when varying multiples of these determinants at once. For example, Waterman et al. reported that pre-treatment of MSCs with 1 ng/mL lipopolysaccharide (LPS) for 1 hour led to a pro-inflammatory phenotype, characterized by increased secretion of pro-inflammatory cytokines and lack of suppression of T cell activation [50]. Conversely, anti-inflammatory behavior was seen by Opitz et al. for MSCs treated with 5 μg/mL LPS for 24 hours [41]. Differences in MSC function have also been observed for different concentrations of IFN-γ, where low concentrations have induced antigen presenting, immune-stimulating properties while higher concentrations induce classic immunosuppressive functions [30]. These studies reveal the difficulty of comparing data between reports as well as an MSC phenotypic spectrum which includes both immunosuppressive and immune-stimulating arms.

Table 1.1 *In vitro* studies assessing the effect of cytokines and growth factors on MSC phenotypic metrics.

Activating Molecule	Species	Concentration	Duration	Pre-act.?	Effects on MSCs			Ref.
					Secretome	Surface Molecules	Functional	
IL-6	Mouse	0.05 ng/ml	24 hr	yes	↑ VEGF, alone and synergistically with TGF-α			35
	Human	20 ng/ml	24 hr	no			↑ MSC proliferation	29
IL-1α	Mouse	20 ng/ml	24 hr	no		↑ ICAM-1 and VCAM-1 when paired with IFN-γ		42
IL-1β	Mouse	12.5 ng/ml	24 hr	no	↑ VEGF alone and synergistically with TGF-β1			40
	Mouse	25 ng/ml	2-4 hr	no			↑ MSC adhesion to ECM, leukocyte chemotaxis to MSCs	29
	Human	10 ng/ml	24 hr	yes	↑ RANTES, IL-1α, G-CSF, GM-CSF, IL-8			46
IFN-α	Human	1000 U/mL	48 hr	no		↑ TLR3		43
	Mouse	2000 U/mL	18 hr	yes	↑ IL-6 with LPS or Poly(I:C) (48 hr)			
IFN-β	Human	2000 U/ml	6, 24, 72 hr	no	↑ MCP-1, IP-10	↑ CXCR4 and CCR3, alone and synergistically with IFN-γ		32
	Human	500 U/ml	24,48 hr	no			↑ Metabolism of tryptophan (IDO function)	41
IFN-γ	Human	100 U/mL	48 hr	no		↑ TLR3, B7H1		43
		30 ug/mL	18 hr	yes	↑ IL-6, IL-8, CCL5 ↑ TNF-a, IL-12p75 with LPS or Poly(I:C) (48 hr) ↑ IL-6, IL-8, CCL5 with LPS (48 hr)		↑ Chemoattraction of neutrophils with LPS (2 h)	
	Mouse	30 ug/mL	18 hr	yes	↑ IL-6 with LPS or Poly(I:C) (48 hr)			
			18 hr	yes			↑ Immune cell (granulocytes, NK cells) infiltration into matrigel implant with LPS secondary pre-activation (3 hr)	
	Human	n/a	n/a	n/a			Induced MSC suppression of lymphocytes (T, NK, B)	37
	Mouse	20 ng/ml	24 hr	no		↑ ICAM-1 and VCAM-1		42
	Mouse	10, 200 ng/ml	6 hr	no	↑ PGE2, IDO ↑ HGF, alone and synergistically with TNF-α ↓ TGF-β1	↑ PDL-1 alone and synergistically with TNF-α		34
	Human	10, 100 U/ml	4, 8, 12, 16 hr	no		↑MHC-II at low [IFN-γ]		30
	Human	100 U/ml	6, 24, 72 hr	no	↑ MCP-1 ↑CXCL9, IP-10 synergistically with TNF-α	↑ CXCR4 and CCR3		32
	Human	10 ng/ml	24, 48, 72 hrs	no	↑ SOD3 when paired with TNF-α		↑ MSC-mediated neuronal and axonal survival after NO insult (with TNF-α)	36
	Human	1000 U/ml	24,48 hr	no			↑ Metabolism of tryptophan (IDO function)	41
TNF-α	Human	3 ng/mL	48 hr	no		↑ *TLR2, TLR7*		43
	Mouse	20 ng/ml	24 hr	no		↑ ICAM-1 and VCAM-1 when paired with IFN-γ		42
	Mouse	10 ng/ml	6 hr	no	↑PGE2, ↓TGF-β1; ↑HGF, alone and synergistically with IFN-γ			34
	Human	100 U/ml	6, 24, 72 hr	no	↑ MCP-1, IL-8; ↑CXCL9, IP-10 synergistically with IFN-γ	↑ CXCR4 and CCR3 , alone and synergistically with TNF-α		32
	Human	50 ng/ml	24 hr	no	↑ HGF		↑ MSC migration	45
	Human	50 ng/ml	24 hr	no	↑ VEGF, FGF2, IFG1, HGF			31
	Human	10 ng/ml	1 hr	yes	↑ IL-8			44
	Human	10 ng/ml	24, 48, 72 hrs	no	↑ SOD3 when paired with IFN-γ		↑ MSC-mediated neuronal and axonal survival after NO insult (with IFN-γ)	36
	Human	10 ng/mL	48 hr	no	↑ IL-6, IL-8		↑ Endothelial progenitor cell migration to MSC conditioned medium	38
			48 hr	yes			↑ Angiogensis, blood perfusion in mouse ischemic limb	
	Human	0.1, 0.5, 2, 5, 10 ng/mL	6, 12, 24, 48 hr	no	↑ IL-6, IL-8, MCP-1, CXCL6		↑ Monocyte migration to MSC conditioned medium	39
	Human	0.005, 0.05 ng/ml	24 hr	yes	↑ RANTES, IL-1α			46
TGF-β	Mouse	12.5 ng/ml	24 hr	no	↑ VEGF alone and synergistically with IL-1β			40
TGF-α	Human	10 ng/ml	72 hr	yes	↑ VEGF, Ang-2, G-CSF, HGF, IL-6, IL-8, PDGF-BB, leptin, PECAM-I		↑ MCF-7 breast cancer cell chemotaxis to MSCs	33
	Mouse	250 ng/ml	24 hr	yes	↑ VEGF, alone and synergistically with IL-6			35

Table 1.2 *In vitro* studies assessing the effect of TLR ligands on MSC phenotypic metrics.

Activating Molecule	Species	Concentration	Duration	Pre-act.?	Effects on MSCs			Ref.
					Secretome	Surface Molecules	Functional	
LPS	Human	1, 10, 100, 1000 ug/mL	48 hr	no	↑ IL-6, IL-8, CCL5, IL-1β			43
		1 ug/mL	48 hr	no	↑ TNF-a, IL-12p75 with IFN-γ pre-treatment ↑ IL-6, IL-8, CCL5, alone and synergistically with IFN-γ pre-treatment			
	Mouse	1 ug/mL	48 hr	no	↑ IL-6, alone and synergistically with IFN-α or IFN-γ pre-activation			
			3	yes			↑ Immune cell (granulocytes, NK cells) infiltration into matrigel implant, alone and synergistically with IFN-γ primary pre-activation	
	Human	200 ng/ml	24 hr	no	↑ VEGF, FGF2, IFG1, HGF			31
	Mouse	0.01, 0.1, 1, 10 ug/mL	48 hr	no	↑ VEGF		↑ VEGF in rat myocardium ↑ MSC survival and engraftment	51
		n/a	n/a	yes				
	Human	200 ng/ml	4, 8, 24 hr	no	↑ IL-8			44
	Human	10 ng/ml	24, 48 hr	no	↑ IL-6, IL-8 ↓ TNF-α, IL-10, IL-12p70	↓ TLR4	Induced MSC migration	49
	Human	10 ng/ml	1 hr	yes	↑ IL-6, IL-8, IL-4, IL-1RA	↑ JAGGED 1		50
	Human	5 ug/ml	24 hr	no	↑ IL-6		↑ Metabolism of tryptophan (IDO function), alone and synergistically with IFN-γ	41
			24 hr	yes			↑ MSC suppression of T lymphocyte proliferation	
	Mouse	1 ug/ml	12 hr	no			No effect on MSC migration	47
			48 hr	yes			No effect on MSC suppression of T lymphocyte proliferation, or generation of CD4+CD25+Foxp3+ T lymphocytes	
Poly(I:C)	Human	0.02, 0.2, 2, 20 ug/mL	48 hr	no	↑ IL-6, CCL5			43
		20 ug/mL	48 hr	no	↑ TNF-a, IL-12p75 with IFN-γ pre-treatment			
	Mouse	20 ug/mL	48 hr	no	↑ IL-6, alone and synergistically with IFN-α or IFN-γ pre-activation			
	Human	1 μM	24, 48 hr	no	↑ IL-1β, IL-6, IL-8 ↓ TNF-α	↓ TLR3	↑ MSC suppression of T lymphocyte proliferation Induced MSC migration	49
	Human	1 ug/ml	1 hr	yes	↑ IL-8, IL-4, IL-1RA, RANTES, IP-10, PGE2 ↓ TGF-β1, TGF-β3			50
	Human	50 ug/ml	3, 24 hr	no	↑ IL-6, IFN-β			41
			24 hr	no			↑ Metabolism of tryptophan (IDO function), alone and synergistically with IFN-γ	
			24 hr	yes			↑ MSC suppression of T lymphocyte proliferation	
CpG-ODN	Human	1 μM	24, 48 hr	no	↑ IL-1β, IL-6, IL-8, TNF-α, IL-12p70 ↓ IL-10		Induced MSC migration	49
	Human	10 μM	24 hr	no	↑ MMP-13		↑ MSC Invasion into Matrigel	48
Flagellin	Human	5 μM	24, 48 hr	no	↑ IL-6, IL-8 ↓ IL-10, IL-1β			49
Pam3Cys	Mouse	2, 20 ug/ml	12 hr	no			↓ MSC migration	47
		2 ug/ml	48 hr	yes			↓MSC suppression of T lymphocyte proliferation ↓MSC generation of CD4+CD25+Foxp3+ T lymphocytes	

1.4 Clinical Translation

An abundance of promising pre-clinical data has led to the initiation of a multitude of clinical trials to investigate the safety and efficacy of administering MSCs to human patients with a variety of conditions including heart disease, neurodegenerative disorders, and autoimmune conditions [12, 52-54]. Several for-profit companies have MSC products in development [55]. The majority of MSC clinical trials is currently in Phase I, II, or combined I/II, with fewer in Phase III or II/III [53]. In the course of these trials, relatively large doses of single-cell suspensions of either autologous or allogenic MSCs have been demonstrated to be safe in humans, with little to no increased incidence of adverse events after administration [12, 53]. Demonstrating significant efficacy of these cells, however, has been more difficult [6, 12, 56]. This has been illustrated in two early placebo controlled clinical trials carried out by Osiris Therapeutics, Inc. using Prochymal® (allogenic, bone marrow-derived MSCs) [57]. In a Phase III trial investigating the use of MSCs as either a first line treatment or secondary treatment after steroid refractory GvHD, no statistically significant difference was seen between the placebo and MSC treatment groups [58]. Similarly, in a Phase II study of the treatment of chronic COPD with MSCs, no statistically significant improvement in pulmonary function was observed, although systemic inflammation was significantly reduced [58]. Many other studies show variable levels of efficacy, have failed, or have been terminated [12, 53].

The central hypotheses/rationales driving these studies are that (1) MSCs, while heterogeneous, are equipotent, (2) they will home to damaged, diseased,

and inflamed tissues after administration, and (3) the microenvironment will instruct the cells as to what they need to do. It is clear that these driving forces are insufficient to expect efficacy in human patients and that there is a disconnect in the successful translation of MSC therapy from animal models to clinical applications. It is likely that the source of this disconnect is multifactorial, including, but by no means limited to, MSC donor variability, diversity in patient demographic and disease state, cell homing/persistence, and a general lack of thorough mechanistic understanding. These all represent obstacles to successful translation of MSC therapies.

1.4.1 Patient/Disease Diversity and MSC Activation

Although the majority of MSC clinical trials have focused on GvHD, MSC therapies are being investigated and developed for a wide spectrum of other conditions and traumatic injuries [52, 53, 55]. As MSCs have been demonstrated to be capable of a diverse of trophic and homeostatic functions, they are essentially being treated as a magic bullet applicable to virtually all maladies. This paradigm overlooks the fact that different diseases have different and often multifaceted underlying mechanisms and pathologies [55]. Further, within one disease there may be different stages of progress and similarly varying levels of severity in traumatic injuries [55]. The goal of the treatment can also vary, as MSCs are investigated for both preventative purposes as well as reversal or slowing of disease progression [8, 55]. Coupled with this is patient diversity in prior or concurrent treatments, such as steroids, anti-inflammatory drugs, anesthetics, antibiotics, or vasodilators used in first line treatments [59]. All of

these factors can affect the composition of damaged/diseased target microenvironment.

In light of this, the reliance of current protocols on the milieu of the damaged tissue to be sufficient to direct the behavior a “resting” population of “naïve” MSCs is quite tenuous. For instance, in the Prochymal clinical trials discussed earlier, there was a marked difference between MSC performance in patients with gastrointestinal or liver GvHD and patients exhibiting skin GvHD. This concept has also been observed in pre-clinical studies. Polchert et al. reported that MSCs administered at the time of disease induction did not improve survival in a mouse model of GvHD, while injection at day 2 or day 20 significantly improved survival by 40-50% [60]. This suggests that a sufficient accumulation or production of tissue-damage signals must be present in the microenvironment at the time of MSC delivery, which was most likely not the case on the day of disease induction. In a rat model of heart transplantation, Inoue et al. observed that the co-administration of MSCs with the anti-inflammatory drug Cyclosporine A actually led to accelerated allograft rejection rather than graft tolerance, perhaps due the inhibition of interferon (IFN)-γ production by the drug [59].

This body of evidence shows that expectations of how MSCs will respond *in vivo* are generally unclear. Unlike for small molecule drugs, which have a small, limited, and more-or-less known set of mechanisms of action, the response of the patient *to* MSC therapy depends on the response of the MSCs *to* the patient. Therefore it is imperative to further understand the activation of MSCs and how their behavior *in vivo* can be predetermined and manipulated.

1.4.2 MSC Homing/Persistence and Delivery

As mentioned earlier, a tenet of MSC therapy development has been that after administration, which is commonly intravenous injection due its noninvasiveness and convenience, MSCs will preferentially home to sites of tissue damage. MSCs possess the cellular machinery required for chemotaxis, tethering, and diapedesis/extravasation, including chemokine receptors (CXCR and CCR families), adhesion molecules (CD44, integrins), and matrix metalloproteases [42, 61-65], although the expression of these components varies with cell source and exhibits intra-population heterogeneity [66]. There is evidence of endogenous MSC mobilization into circulation in response to skeletal muscle injury [67], hypoxia [68], and myocardial infarction [69]. Observations of preferential homing of MSCs to areas of tissue damage have been reported in animal models or *in vitro* systems using human tissue [55, 66, 70-76].

However, the efficiency of this homing appears to be rather limited. Using various methods of detection (radioactive labeling, fluorescent labeling, reporter genes, probing for species/sex mismatch via specific genes), homing efficiencies reported in literature do not typically exceed 10% [77]. Additionally, the homing efficiency of murine MSCs has been reported to decrease dramatically after culture for as little as 24-48 hours [78], perhaps related to loss of CXCR4 expression [63, 79]. Rather, MSCs injected intravenously have a systemic distribution, with the majority of the cells getting entrapped in the lungs [80] or other filtering organs (e.g. liver, kidneys) [77, 81]. Other routes of delivery have been explored to bypass the pulmonary first pass effect and increase homing,

including intra-arterial and intracardiac infusion [77]. These methods, however, may increase the risk of microvascular occlusion [82]. MSC infusion directly into the target tissue may cause MSC death due to limited oxygen and nutrients [83] and may be too invasive to justify use in the clinic (e.g. the central nervous system).

Despite this, there is some animal data suggesting that MSCs may be capable of modulating tissue damage from a distant tissue/organ or without accumulation in the target tissue [84-87]. However, as the majority of MSC functions are achieved via juxtacrine and paracrine secretion of bioactive molecules it is reasonable to assume that localized MSCs would be more efficacious [66].

Inefficient homing is further complicated by relatively rapid clearance of the cells from the body [12, 88]. These compounding factors result in the use of large doses of MSCs, which poses feasibility challenges for scalable expansion *in vitro* [53-55]. Analogous to pharmaceutical drug considerations, increasing the dose could increase the possibility of adverse side effects such as support of tumor growth [89], or off-target disruption of homeostasis [55, 90].

1.4.3 Donor Variability

An attractive feature of MSCs is their general non-immunogenicity, rendering them a viable cell source for allogenic as well as autologous use [5]. Current screening protocols identify acceptable MSC donors by medical history, viral testing, and satisfaction of the International Society for Cellular Therapy (ISCT) minimal criteria for MSCs (i.e. adherence to tissue culture plastic, surface marker

expression, and tri-lineage differentiation) [53]. This approach is reflective of a dangerous oversight of well-documented donor-to-donor and intra-donor variability [55, 91].

MSCs isolated from children have been reported to proliferate at a faster rate than adult MSCs, which also enter senescence quickly in correlation with telomere length and reactive oxygen species levels [92, 93]. Baxter et al. also showed that *in vitro* culture may age MSCs by increasing the rate of telomere loss [94]. Siddappa et al. described loss of multipotency as well as donor variation related to *in vitro* cell culture [95]. Cell source also contributes to variability in MSC populations [96]. Given the fact that MSCs occur at a relatively low rate in source tissue, massive expansion is needed to generate doses for even a single donor. Culture/passage-induced and cryopreservation-induced changes in the cells are therefore an important factor affecting therapeutic outcome [97, 98]. Additionally, when considering the generation of many doses from only a few doses in the “universal donor” or “off-the-shelf” business models, the potency bias becomes an even greater concern and could potentially make or break the results of a clinical trial [99].

Donor variability has also been observed in functional assessments of MSCs. Bone marrow MSCs isolated from older donors undergoing hip replacement surgery exhibited variable IDO expression and suppression of T cell proliferation after stimulation with IFN-γ, tumor necrosis factor (TNF)-α, or both [100]. Zhukareva et al. described highly variable constitutive secretion of chemokines and cytokines by MSC isolated from healthy adults aged 18 to 45 [46]. Upon

stimulation with pro-inflammatory cytokines or injured rat spinal cord extract, however, secretion among the donors was more comparable.

As MSCs are a heterogeneous cell population, variability is also high within cells isolated from a single donor [91]. Efforts to create a reference material against which to measure the suitability of new MSC preparations for clinical and even pre-clinical use are critically needed [101]. Experts in the field are also pushing to develop potency assays to identify more responsive cells based on immunophenotype [102, 103]. There is currently no consensus on which assay would be the best, but MSC response to IFN-γ has received much of the focus [99, 102, 104].

1.5 Pre-Activation

MSC pre-activation (also referred to as conditioning, pre-conditioning, pre-stimulation, pre-treatment, and priming) has gained momentum as a potential tool to increase the efficacy of MSC therapies [105]. These strategies target several different factors affecting the probability of the success of these cells *in vivo*. To address the issue of cell death upon harvest after cell expansion, cryopreservation, and administration *in vivo*, efforts have been made to increase cell survival and resistance to apoptosis [106]. Approaches to increase migration via increasing the expression of adhesion molecules and chemokine receptors aim to remedy the inefficient homing and engraftment of the cells *in vivo* [66, 71]. Several studies focus on the enhancement of MSC therapeutic mechanisms such as immunomodulation and angiogenesis [106-108]. Much of the

development regarding MSC pre-activation for these purposes has been done in the cardiovascular disease field [108].

Table 1.3 Studies reporting the effects of pre-activated MSCs on therapeutic outcomes in animal models of disease.

Species	Activating Molecule(s)	Concentration	Duration	Disease Model	*In vivo* Results	*In vitro* Pre-Characterization	Ref.
Murine	IFN-γ	5, 50, 500 U/mL	n/a	GvHD (Mouse)	↑ Survival	-none	60
Human, murine	IFN-γ	500 U/mL	6 days	Colitis (Mouse)	↓ Disease score	-IDO, iNOS, surface marker expression -Tri-lineage differentiation Gene expression after 6 days in IFN-γ	118
Human	TNF-α	10 ng/mL	48 hr	Hind limb ischemia (Mouse)	↑ Angiogensis, blood perfusion in mouse ischemic limb	-IL-6, IL-8 secretion - Endothelial progenitor cell migration	38
Human	TNF-α Poly(dA:dT)	20 ng/mL 0.5 µg/mL	24 hr	Lung metastases (Mouse)	↓ Tumor progression synergistically with doxorubicin	-Gene expression (primarily TRAIL) of activated MSCs'-Dose response of poly(dA:dT), ± TNF-α -Cancer cell line cell cycle and apoptosis co-culture assays	122
Human	IL-1β	10 ng/mL	48 hr	DSS-induced Colitis (Mouse)	↓ disease development ↑ COX-2, IL-6, IL-8 ↑ anti-inflammatory macrophages ↑ Migration to inflammation	-Phenotypic surface marker expression, morphology, and viability -CXCR4 expression	119
Murine	IL-1β TGF-β1	12.5 ng/mL 12.5 ng/mL	24 hr	Ex vivo Myocardial I/R (Rat)	↑ post-ischemic myocardial functional recovery	-Dose response measuring VEGF -Signaling pathway studies	40
Murine	IL-6 TGF-α	0.05 ng/ml 250 ng/ml	24 hr	Ex vivo Myocardial I/R (Rat)	↑ post-ischemic myocardial functional recovery	-VEGF secretion	35
Human	LPS	10 ng/mL	1 hr	Epithelial ovarian cancer (Mouse)	↓ Tumor growth	-Gene expression -ECM deposition -T cell activation -IL-6, IL-8, IL-10, PGE2, IDO secretion	120
Human	Poly(I:C)	1 ug/mL	1 hr	Diabetic Neuopathy (Mouse)	↓ Mechanical allodynia ↓ Heat hypoalgesia	-Gene expression -ECM deposition -T cell activation -IL-6, IL-8, IL-10, PGE2, IDO secretion	121

Several strategies are being investigated to achieve these goals, including hypoxic or anoxic culture conditions [109], pharmacological agents and small molecules [110-114], culture substrate mechanical properties [115], shear stress [116], and three dimensional culture [117]. As was described in Tables 1.1 and

Table 1.2, a large portion of studies investigate activation and pre-activation of MSCs with proteins that they are likely to encounter in diseased or injured microenvironment, such as cytokines/growth factors [29-46] and toll like receptor (TLR) ligands [31, 41, 43, 44, 47-51]. These studies serve a dual purpose of enhancing MSC functions and elucidating their mechanisms of therapeutic action.

Recall that MSC phenotype is dictated by multiple parameters, including the type of activating molecule, its prevalence/concentration, duration/timing of exposure, and whether it is in combination with another factor(s) [28]. As discussed in Section 1.3, the studies summarized in Table 1.1 and Table 1.2 demonstrate the spectrum of phenotypes that MSC can adopt, including both immunosuppressive and immune-stimulating, depending on the way they are activated. As summarized in Table 1.3, there have been reports of pre-activation of MSCs with different factors resulting in improved outcomes in animal models of disease [35, 38, 118-122]. For example, Waterman et al. showed that populations of pro-inflammatory MSCs generated by short exposure to a low concentration of LPS and anti-inflammatory MSCs generated by short exposure to a low concentration of poly(I:C) resulted in suppression of tumor growth in a mouse model of epithelial ovarian cancer and attenuation of painful symptoms in a mouse model of diabetic neuropathy, respectively [120, 121]. Therefore the plasticity of MSCs can presumably be exploited by dialing in settings to enhance application-specific phenotypes. This would be equivalent to matching the proper inputs to achieve the desired outputs.

A limitation of these approaches as they currently stand, however, is that there is not a standardized or systematic method for the optimization of such input/output matching. The lack of such a system has resulted in disparate studies that are difficult to compare. The selection of activation parameter settings in studies utilizing pre-activated MSCs is often arbitrary, with only a portion of relevant factors (activation factor(s), concentration, exposure duration) being optimized inconsistently across studies. Additionally, there is inconsistency in the amount and types of *in vitro* pre-characterization of the pre-activated MSCs prior to animal studies. This is an issue because connections between activation protocols and *in vivo* metrics in relatively homogeneous animal models may not translate or be relevant for diverse human patients with the disease or injury. As discussed in previous sections, the specific MSC functions that would be required to achieve a therapeutic effect depend on the type and state of the disease being treated, which may have multiple underlying mechanisms. Therefore, a pre-activation protocol that determines enhanced efficacy based on only a single metric, such as IDO secretion in response to IFN-γ, may not predict success across the board for conditions potentially treatable by MSCs.

Studies that do perform *in vitro* characterization of the pre-activated MSCs prior to use in animal models often report changes in MSC secretion, migration, or target cell modulation in the presence of an activating factor, but very few investigate how the pre-activated MSCs will respond when introduced into a simulated disease/damaged microenvironment. This has implications for the development of potency assays, where this information would be useful for

correlating *in vitro* metrics with outcomes *in vivo*. These limitations demonstrate the need for a systematic approach to optimize the activation of MSCs for application-specific functions that can be easily adapted to fit any MSC activation/function pair and characterization of the resulting pre-activated cell population.

1.6 Design of Experiments

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 [123]. This is achieved by using regression analysis to create empirical mathematical models of phenomena based on data collected from the experimental design. This approach is extensively used in biotechnological fields for bioprocess development/optimization and is increasingly being utilized for basic and pre-clinical research applications, such as optimization of stem cell differentiation and anti-viral drug studies [124-127].

Two-level factorial experiments allow for analysis of how *n* different factors ($X_{i=1...n}$) affect a response (Y) of a system. In full factorial experiments, all $k=2^n$ possible combinations of factors are tested, resulting in a dataset containing all responses $Y_{j=1...k}$. A regression model (Equation 1.1) of Y as a function of each individual factor $X_{i=1...n}$ and all combinations of factors $X_{i=1...n}X_{j=1...}$ can be fit to this collection of responses, resulting in a calculation of a predicted/fitted response $\hat{Y}$ for each condition, with the residuals defined as the difference between the observed and fitted responses ($Y- \hat{Y}$).

$$\hat{Y}\ X_i = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j + \ldots + \varepsilon \qquad \text{(Equation 1.1)}$$

Here, $\hat{Y}$ is the fitted response, β_0 is a constant, β coefficients represent the main and interacting effects of each factor on the transformed response, and ε is an error term. Transformation of the original data ($Y \rightarrow Y'$) may be needed to make the residuals normal. Main effects of each factor X are defined as the estimated difference in $\hat{Y}$ ($\Delta\hat{Y}$) for all conditions when X_i is present and when X_i is not present. Estimated two-factor interactions are defined as the dependence of the difference in the $\hat{Y}$ at the 2 levels of X_i upon the level of another factor. This determined by subtracting the $\Delta\hat{Y}$ caused by X_1 when X_2 is not present from the $\Delta\hat{Y}$ caused by X_1 when X_2 is present and dividing by 2. The effect estimated for each factor or combination of factors is twice the value of the coefficient for the corresponding term in the linear regression line. In full factorial designs, all main effects and interactions can be analyzed independently. Analysis of variance (ANOVA) can be performed to identify statistically significant effect estimates.

Suppose an investigator wishes to examine the effect of *n*=6 different parameters at 2 levels on a certain process. A full factorial experiment would result in 64 different conditions (2^6). If biological replicates were included or the panel of factors expanded, the resulting experiment would be unreasonably large and economically infeasible to perform. Utilizing a fractional factorial design is an attractive alternative. In half-fraction designs (2^{n-1}), the number of conditions is reduced by aliasing main effects and lower order interactions with high order interactions. In this case of 6 factors, main effects are aliased with fifth-order interactions, two-factor interactions are aliased with fourth-order interactions, and

three-factor interactions are aliased with other three-factor interactions, meaning that they cannot be distinguished mathematically. The results of analyzing main effects and two-factor interactions can be trusted if it is assumed that the higher order interactions they are associated with are negligible, which is commonly the case, classifying this design as resolution VI.

This tool is very attractive for screening purposes and therefore can be useful in developing a systematic approach for optimizing several relevant factors in MSC activation.

1.7 Cell Immobilization

As discussed above, there are several challenges associated with the development of MSCs related to homing and persistence. Although modest therapeutic improvements have been achieved despite the aforementioned limitations, increasing and prolonging the presence of these cells in target tissues or in the body in general may allow more time for the cells to impart their beneficial effects. Additionally, increasing cell number locally could improve efficacy/potency compared to remotely acting MSCs while using fewer total cells can limit side effects and attenuate concerns over tumorigenesis or cell debris accumulation in filtering organs [55, 89, 90].

A commonly investigated approach to improving the “pharmacodynamics” of cellular therapies is to immobilize the cells in a biocompatible matrix fabricated from natural (e.g. alginate, chitosan, dextran, hyaluronic acid, collagen, etc.) or synthetic (e.g. PLGA, PEG, PES, etc.) polymers [128, 129]. These permeable to semi-permeable interfaces allow for the exchange of nutrients/oxygen and

wastes as well as prolonged paracrine communication with the surrounding environment.

Immobilization of cells in semi-permeable biocompatible hydrogels has successfully been performed for many cells types, including primary cells, genetically engineered cells, and stem cells [130]. Alginate encapsulated cells have extensively been investigated for several biomedical applications ranging from stem cell differentiation [131] to delivery of cell-derived bioactive molecules [132, 133] to tissue replacement [134].

1.8 Dissertation Summary

MSCs hold great potential as a cellular therapy due to their tissue protective and regenerative abilities, achieved via the secretion of many bioactive molecules. Translation into the clinic, however, has not resulted in dramatic success. As discussed above, there are several challenges and limitations contributing to this disconnect. The goal of the work done in this dissertation was to develop approaches to address some of these hurdles. Specifically, we aimed to mitigate obstacles regarding MSC activation, donor variability, and inefficient MSC homing and persistence.

The first portion of this dissertation describes the systematic optimization of a pre-activation protocol detailing the type of activating molecule(s), its prevalence/concentration, and duration of exposure that enhance MSC immunomodulatory properties, followed by characterization of the pre-activated cells. In chapter 2, we utilized fractional factorial design of experiments to develop a high throughput *in vitro* screening assay to identify out of panel of 6

candidates soluble factors and/or combinations of factors that enhance MSC immunomodulation. We found that interleukin (IL)-1β and LPS, individually and in combination, upregulated MSC secretion of PGE2 and subsequent attenuation of pro-inflammatory macrophage TNF-α secretion. The effects of IL-1β were much more potent than those of LPS alone.

In chapter 3, we continued the development of the pre-activation protocol by optimizing the dose and duration of exposure for IL-1β with and without LPS to maximize MSC PGE2 secretion. We found that brief exposure to 1 ng/mL IL-1β alone was sufficient to sustain upregulated PGE2 secretion after activation. While LPS provided synergistic increases in PGE2 during activation, this enhancement was transient post-activation. PGE2 secretion in response to secondary inflammatory molecules and modulation of macrophage TNF-α in co-culture after pre-activation with the selected parameters was then characterized. IL-1β pre-activation led to increased sensitivity of the MSCs to secondary stimuli and enhanced attenuation of macrophage TNF-α. In parallel, we pursued interesting, unexpected results observed when MSCs were pre-activated with IFN-γ. While IFN-γ pre-activated MSCs were also more sensitive to secondary stimuli, particularly IL-1β, these MSCs performed the worst in the functional macrophage co-culture assay.

In chapter 4, we tested the performance of the successful IL-1β pre-activation protocol and the less successful IFN-γ pre-activation protocol on MSCs derived from multiple donors and also examined variability amongst the donors. The trends in PGE2 secretion and macrophage TNF-α attenuation were consistent

amongst the donors, demonstrating that differences in the selected metrics induced by pre-activation were detectable with high statistical power. Variability amongst the donors was noted, however, in the absolute levels of secreted PGE2, the degree of PGE2 upregulation compared to no pre-activation, the level of improvement or impairment in macrophage modulation induced by IL-1β or IFN-γ pre-activation, respectively, and the correlation of MSC PGE2 and macrophage TNF-α. Although the age range was narrow, no significant correlations were detected between the metrics and age or sex.

In chapter 5, preliminary work was done to characterize the activation of alginate-encapsulated MSCs (eMSCs) by the factors identified in the high throughput screen in chapter 2 (IL-1β with and without LPS). The eMSCs exhibited increased PGE2 secretion and attenuation of macrophage TNF-α secretion after activation, with apparent superiority to monolayer MSCs. We then utilized statistical methods to determine if these differences were due to alginate encapsulation or other factors. We found that macrophage TNF-α levels were more significantly a function of PGE2 level, MSC activation factor, and macrophage donor rather than MSC culture format (monolayer versus encapsulated).

In chapter 6, we conclude the dissertation with a discussion of key findings, limitations of the approaches taken, implications, and future directions for the work.

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Chapter 2: Identification of IL-1β and LPS as Optimal Activators of Monolayer MSC Immunomodulation Using Design of Experiments

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2.1 Introduction

Mesenchymal stromal cells (MSCs) are a heterogeneous population of multi-potent, 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 many trophic functions achieved via cytokine secretion that beneficially modulate 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 pro-inflammatory macrophage behavior, such as elevated tumor necrosis factor (TNF)-α secretion, and reprogramming of the cells into inflammation-resolving and wound healing macrophages, *in vitro* and *in vivo* [4-6]. Our previous work and that of others have demonstrated 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 the fact that 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 [9]. 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 [10-12]. 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 [9, 12-16]. 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 [17]. 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 factors (main effects) and interactions of these factors can be determined [18].

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-1β, interferon (IFN)-β, and IFN-γ) that concurrently maximize MSC secretion of anti-inflammatory

PGE2 and MSC-mediated attenuation of pro-inflammatory macrophage TNF-α production. This knowledge and approach can be extended to investigate and develop activation schemes to maximize specific MSC functions prior to transplantation in order to better guarantee the action of MSCs *in vivo*.

2.2 Materials and Methods

2.2.1 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 was purchased from Sigma Aldrich (Oakville, Ontario, Canada). High molecular weight poly(I:C) was purchased from InvivoGen (San Diego, CA).

2.2.2 Cell Culture

All cells were maintained in a humidified 37°C incubator with 5% CO_2 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.

2.2.3 MSCs

Human bone marrow-derived MSCs (male, age 28) at passage 1 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 cm^2 flasks at $1.5x10^4$ cells/mL. They were cultured in

MEM-α containing no deoxy- or ribonucleosides, supplemented with 10% FBS (Atlanta Biologics, 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 $2x10^3$ cells/well, and allowed to attach overnight.

2.2.4 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 1X 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 $1x10^7$ cells/175 cm^2 flask in fully supplemented Advanced RPMI 1640. The cells were allowed to attach for 2-6 hours before gently replacing the medium with medium containing 5 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) to induce differentiation into pro-inflammatory macrophages. After 7 days of culture, the differentiated cells were detached with trypsin, seeded into 96-well plates at $1x10^4$ cells/well, and allowed to attach overnight. Different macrophage donors were used in each experimental replication.

Table 2.1 MSC activation factors and levels.

Activation Factor (X_i)		Level (ng/mL)	
		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-1β	0	1
X_5	IFN-β	0	1
X_6	IFN-γ	0	1

2.2.5 Experimental Design

Main and interacting effects of the six activating factors (LPS, poly(I:C), IL-6, IL-1β, IFN-β, and IFN-γ) at two doses (levels, Table 2.1) on MSC secretion and modulation of macrophage phenotype were investigated using a two-level half-fraction (2^{6-1}) factorial design of resolution VI (Table 2.2 and Table 2.3) generated using MiniTAB Statistical Software version 17 (Minitab Inc., State College, PA). This resulted in 32 different conditions (Table 2.4), which were run in triplicate with technical 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 (Table 2.3).

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 2.4 and cultured for 48 hours. Supernatants were collected and frozen at -80°C until analysis. The MSCs were fixed in 4% (w/v) paraformaldehyde (PF) for 20 minutes at room temperature and stored in 1% (w/v) PF at 4°C until further use. To investigate the

Table 2.2 Fractional factorial design (FFD) details. (A...F = X_1…X_6).

Factors:	6
Runs:	192
Blocks:	3
Base Design:	6, 32
Replicates:	6
Fraction:	1/2
Resolution:	VI
Design Generators:	F = ABCDE
Block Generators:	Replicates
Defining Relationship:	I = ABCDEF

Table 2.3 Alias structure for FFD.

Main Effects	Two-Factor Interactions	Three-Factor Interactions
I + ABCDEF	AB + CDEF	ABC + DEF
A + BCDEF	AC + BDEF	ABD + CEF
B + ACDEF	AD + BCEF	ABE + CDF
C + ABDEF	AE + BCDF	ABF + CDE
D + ABCEF	AF + BCDE	ACD + BEF
E + ABCDF	BC + ADEF	ACE + BDF
F + ABCDE	BD + ACEF	ACF + BDE
	BE + ACDF	ADE + BCF
	BF + ACDE	ADF + BCE
	CD + ABEF	AEF + BCD
	CE + ABDF	
	CF + ABDE	
	DE + ABCF	
	DF + ABCE	
	EF + ABCD	

Table 2.4 Two-level, half-fraction factorial design of resolution VI.

	Activation Factor Level					
Run	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
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

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 2.1A). In parallel, medium containing the activating factors according to the experimental conditions described above were similarly cultured for 48 hours 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 hours of culture in CM, the macrophage supernatants were collected and frozen at -80°C until analysis.

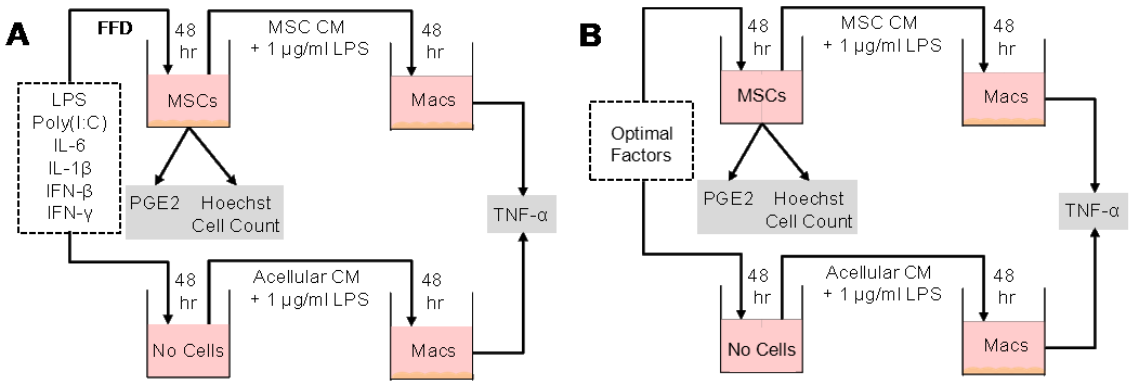

Figure 2.1. Schematics of *in vitro* experiments. (A) To measure the main and interacting effects of the six activating factors on MSC secretion of PGE2 and the modulation of macrophage TNF-α secretion by MSC CM, MSCs or acellular media were incubated for 48 hours (hr) with soluble activating factors (dashed box) in combinations determined by the FFD. MSC or acellular CM was spiked with LPS and used to culture macrophages for 48 hours. Assays were then performed to quantify

metrics of interest (grey shaded boxes). (B) Optimal factors (dashed box) were validated by repeating the aforementioned *in vitro* assay.

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 2.1B). These experiments were run 3 times with 2-6 technical replicates per experiment.

2.2.6 Cell Counting

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

2.2.7 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 (ELISA) 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).

2.2.8 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 (Equation 2.1) to make the residuals random and fit a normal distribution, as evaluated by the Anderson-Darling test for normality in Minitab. A linear regression model was fit to the transformed data in order to determine main effects and two-factor interactions. The general form of this model is seen in Equation 2.2.

$$Y' = \frac{Y^{\lambda}-1}{\lambda} \qquad \text{(Equation 2.1)}$$

$$Y'\ X_i = \beta_0 + \sum_{i=1}^{6} \beta_i X_i + \sum_{i=1}^{5} \sum_{j=i+1}^{6} \beta_{ij} X_i X_j + \varepsilon \qquad \text{(Equation 2.2)}$$

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 nonhomogeneous environmental conditions between experiments. Statistical significance of the estimated effects was determined using analysis of variance (ANOVA), with p-values < 0.05 considered significant.

2.3 Results

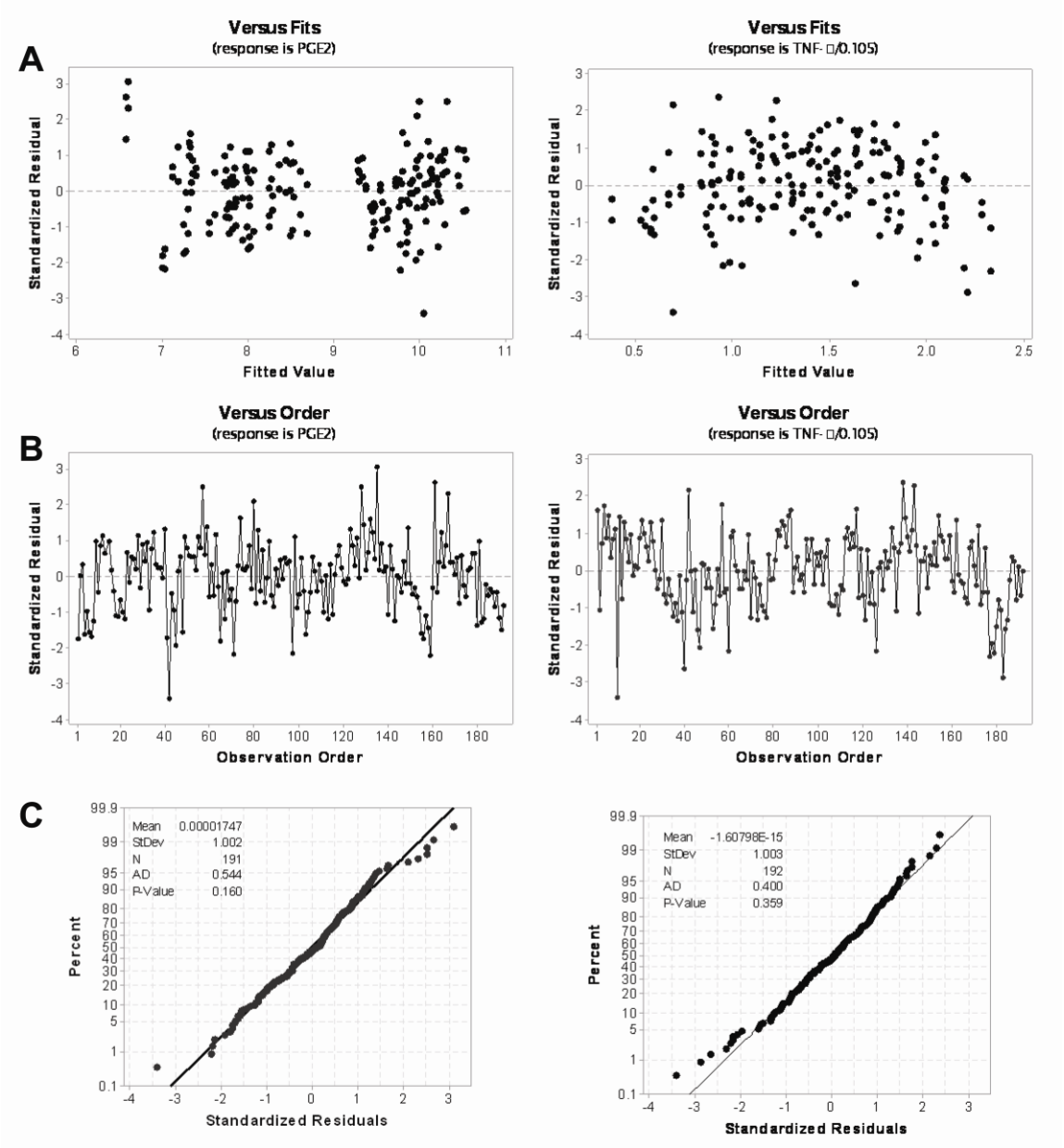

Figure 2.2 Analysis of residuals of linear models fit to PGE2 and TNF-α data. The residuals of the linear models were standardized and plotted versus the (A) fitted values and (B) observation order. (C) Normality of the standardized residuals was determined using Anderson-Darling (AD) test, where the null hypothesis is that the residuals fit a normal distribution.

Table 2.5 Coded (-1,+1) coefficients of terms in linear regression model fit to transformed response variables. Statistical significance ($p<0.05$) are indicated (*).

Regression Term	ln(PGE2)	ln(TNF-α/0.105)
Constant (β_0)	**8.8526***	**1.3998***
LPS	0.2028*	-0.0757*
Poly(I:C)	**0.0061**	**0.0053**
IL-6	0.0258	0.0367*
IL-1β	**1.0589***	**-0.3628***
IFN-β	0.1422*	0.1370*
IFN-γ	**0.0565***	**0.0201**
LPS*Poly(I:C)	-0.0063	0.0167
LPS*IL-6	**0.0092**	**0.0357***
LPS*IL-1β	-0.1177*	0.0562*
LPS*IFN-β	**-0.0341**	**0.0039**
LPS*IFN-γ	0.0080	-0.0074
Poly(I:C)*IL-6	**-0.0288**	**0.0004**
Poly(I:C)*IL-1β	0.0116	0.0013
Poly(I:C)*IFN-β	**0.0015**	**-0.0281**
Poly(I:C)*IFN-γ	-0.0182	0.0107
IL-6*IL-1β	**0.0215**	**0.0148**
IL-6*IFN-β	-0.0081	-0.0056
IL-6*IFN-γ	**0.0075**	**0.0121**
IL-1β*IFN-β	-0.0579	0.0388*
IL-1β*IFN-γ	**-0.0861**	**0.0305**
IFN-1β*IFN-γ	-0.1180	0.0191

Table 2.6 Statistically significant main effects and two-factor effects.

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

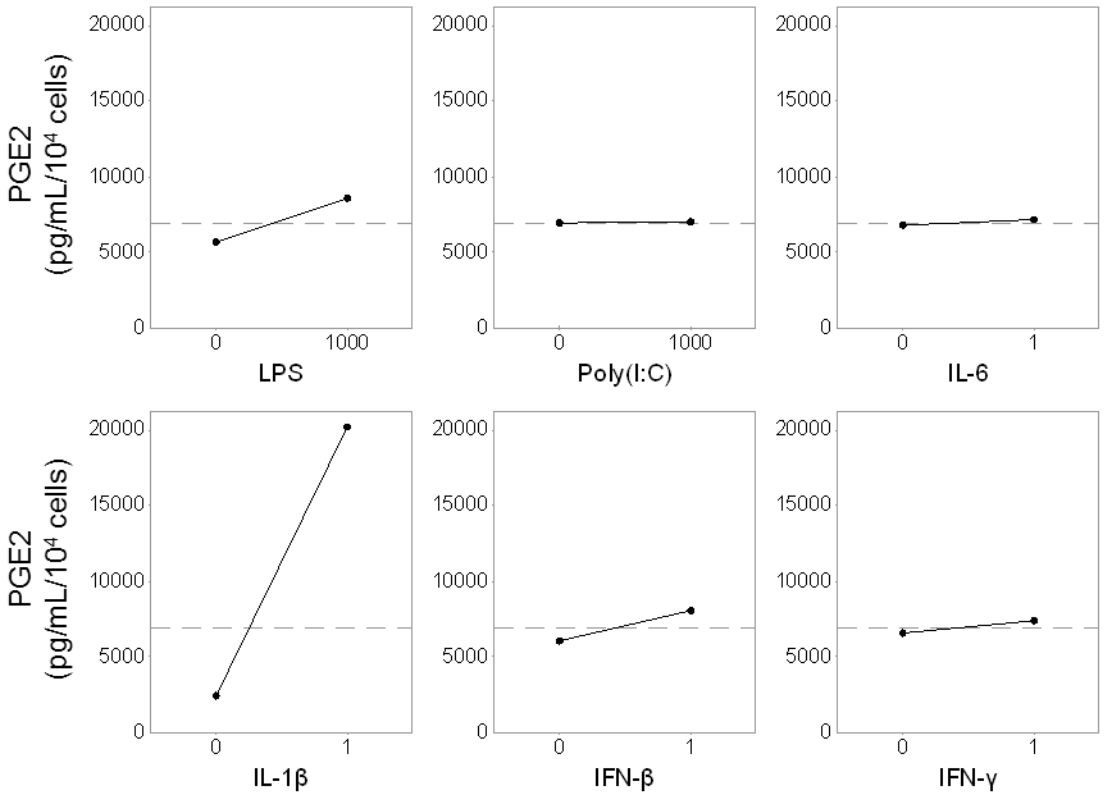

Figure 2.3 Main effects of activating factors on MSC PGE2 secretion. MSCs were incubated for 48 hours with combinations of the six activating factors in the panel. The level of PGE2 in the cell culture supernatants were then quantified and normalized to the number of MSCs present at the end of culture. Plots of main effects 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. Data are average values calculated using the regression model that was fit to 3 individual experiments with 2 technical replicates per experiment.

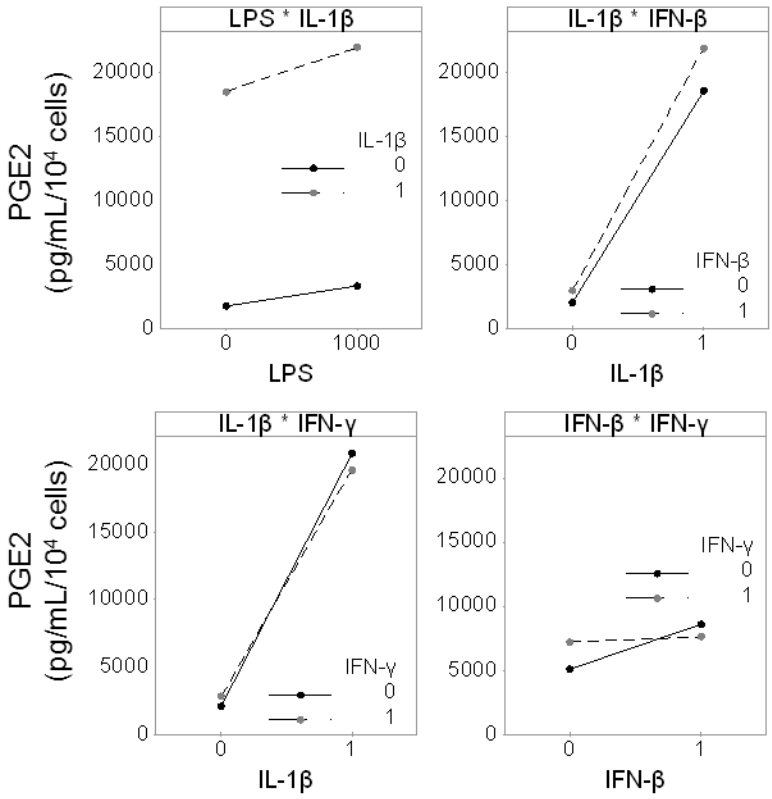

Figure 2.4 Interacting effect of activating factors on MSC PGE2 secretion. MSCs were incubated for 48 hours with combinations of the six activating factors in the panel. The level of PGE2 in the cell culture supernatants were then quantified and normalized to the number of MSCs present at the end of culture. Plots of 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. Data are average values calculated using the regression model that was fit to 3 individual experiments with 2 technical replicates per experiment.

Table 2.7 Interacting effects of activating factors on MSC PGE2 secretion.

Magnitude of the response at indicated level of each factor.
Magnitude and direction of the change (Δ) in the response for the change in the factor level.

LPS*IL-β

Transformed

	LPS -	LPS +	*Δln(PGE2)*
IL-β +	9.8264	9.9958	*0.169*
IL-β -	7.4732	8.1143	*0.641*
Δln(PGE2)	*2.353*	*1.882*	

Untransformed

	LPS -	LPS +	*ΔPGE2*
IL-β +	18515	21952	*3437*
IL-β -	1760	3342	*1582*
ΔPGE2	*16755*	*18610*	

IL-β*IFN-β

Transformed

	IFN-β -	IFN-β +	*Δln(PGE2)*
IL-β +	9.8272	9.9958	*0.169*
IL-β -	7.5937	7.9938	*0.400*
Δln(PGE2)	*2.234*	*2.002*	

Untransformed

	IFN-β -	IFN-β +	*ΔPGE2*
IL-β +	18532	21933	*3401*
IL-β -	1986	2963	*977*
ΔPGE2	*16546*	*18970*	

IL-β*IFN-γ

Transformed

	IFN-γ -	IFN-γ+	*Δln(PGE2)*
IL-β +	9.9410	9.8819	*-0.059*
IL-β -	7.6512	7.9363	*0.285*
Δln(PGE2)	*2.290*	*1.946*	

Untransformed

	IFN-γ -	IFN-γ+	*ΔPGE2*
IL-β +	20765	19573	*-1192*
IL-β -	2103	2797	*694*
ΔPGE2	*18662*	*16776*	

IFN-β*IFN-γ

Transformed

	IFN-γ -	IFN-γ+	*Δln(PGE2)*
IFN-β +	9.0563	8.9333	*-0.123*
IFN-β -	8.5359	8.885	*0.349*
Δln(PGE2)	*0.520*	*0.048*	

Untransformed

	IFN-γ -	IFN-γ+	*ΔPGE2*
IFN-β +	8572	7580	*-992*
IFN-β -	5095	7222	*2127*
ΔPGE2	*3477*	*358*	

2.3.1 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. 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 (Equation 2). 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 λ=0, Equation 2.1) to normalize the residuals (Figure 2.2C) and make them random across fitted values (Figure 2.2A) and order of observation (Figure 2.2B). A regression model (Equation 2.2, Table 2.5) was then fit to this transformed data, with a resulting R^2 of 95.82% (Table 2.6).

Graphs visually present these effects by solving for PGE2 and plotting these untransformed outputs of the regression equation (Figure 2.3, Figure 2.4). Effects and interactions that indicate increases in PGE2 were considered desirable. The main effects of LPS, IL-1β, IFN-β, and IFN-γ indicate that these factors significantly increased PGE2 secretion (Figure 2.3, Table 2.6). Within this group, IL-1β 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-1β 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 2.4). As seen in Table 2.6, the transformed response of PGE2 secretion to the combined presence of LPS*IL-1β, IL-1β*IFN-β, IL-1β*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-1β and IL-1β*IFN-β are additive or synergistic. Furthermore, examination of these interactions using the untransformed fitted values revealed that the interactions of LPS with IL-1β and IL-1β 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 2.7). The interactions of either IL-1β 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.

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

Due to 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, 19]. Macrophage conversion from pro-inflammatory to anti-inflammatory phenotypes is characterized in part by decreased TNF-α secretion [20]. 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 λ=0, Equation 1) to make residuals normal (Figure 2.2C) and random across fitted values (Figure 2.2A) and order of observation (Figure 2.2B). 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 (Equation 2.2, Table 2.5) fit to the transformed TNF-α response had an R^2 of 83.86% (Table 2.6).

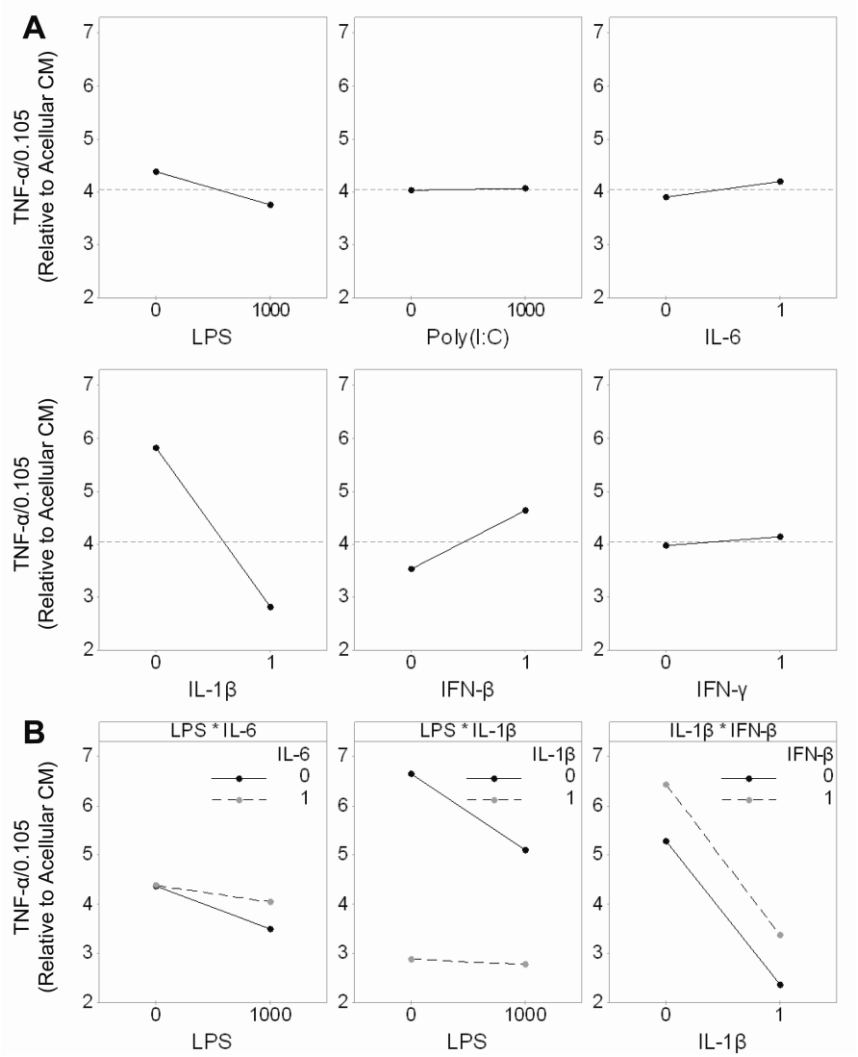

Figure 2.5 Effects of activating factors on MSC modulation of macrophage TNF-α secretion. Macrophages were incubated for 48 hours in CM collected from MSCs activated with combinations of the six factors in the panel. The

level 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 MSC activation cocktail. Data are average values calculated using the regression model that was fit to 3 individual experiments with 2 technical replicates per experiment.

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-1β (Figure 2.5A), with the effect of IL-1β being ~5 times that of LPS (Table 2.6). 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 2.6). A subset of the combinations of these factors also had significant interactions (Table 2.6). Activation of MSCs with IL-6 decreased the effects of LPS on MSC-mediated reduction of macrophage TNF-α (Figure 2.5B). Similarly, the effects of IL-1β 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-1β, 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 2.8). 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-1β is present (Figure 2.5B).

Table 2.8 Interacting effects of factors on MSC modulation of macrophage TNF-α secretion.

Magnitude of the response at indicated level of each factor.
Magnitude and direction of the change (Δ) in the response for the change in the factor level.

LPS*IL-β

Transformed

	LPS -	LPS +	*Δln(TNF-α/0.015)*
IL-β +	1.057	1.018	*-0.039*
IL-β -	1.895	1.631	*-0.264*
Δln(TNF-α/0.015)	*-0.839*	*-0.613*	

Untransformed

	LPS -	LPS +	*Δ(TNF-α/0.015)*
IL-β +	2.876	2.767	*-0.110*
IL-β -	6.649	5.108	*-1.541*
ΔTNF-α	*-3.773*	*-2.341*	

LPS*IL-6

Transformed

	LPS -	LPS +	*Δln(TNF-α/0.015)*
IL-6 +	1.477	1.397	*-0.080*
IL-6 -	1.475	1.252	*-0.223*
Δln(TNF-α/0.015)	*0.002*	*0.145*	

Untransformed

	LPS -	LPS +	*Δ(TNF-α/0.015)*
IL-6 +	4.369	4.042	*-0.327*
IL-6 -	4.377	4.042	*-0.336*
ΔTNF-α	*-0.008*	*0.000*	

IL-β*IFN-β

Transformed

	IFN-β -	IFN-β +	*Δln(TNF-α/0.015)*
IL-β +	0.861	1.213	*0.352*
IL-β -	1.664	1.861	*0.197*
Δln(TNF-α/0.015)	*-0.803*	*-0.648*	

Untransformed

	IFN-β -	IFN-β +	*Δ(TNF-α/0.015)*
IL-β +	2.366	3.363	*0.997*
IL-β -	5.282	6.429	*1.147*
ΔTNF-α	*-2.916*	*-3.066*	

IL-β*IFN-γ

Transformed

	IFN-γ -	IFN-γ+	*Δln(TNF-α/0.015)*
IL-β +	0.9864	1.0878	*0.101*
IL-β -	1.773	1.7522	*-0.021*
Δln(TNF-α/0.015)	*-0.787*	*-0.664*	

Untransformed

	IFN-γ -	IFN-γ+	*Δ(TNF-α/0.015)*
IL-β +	0.2816	0.3116	*0.030*
IL-β -	0.6183	0.6056	*-0.013*
ΔTNF-α	*-0.337*	*-0.294*	

Examination and comparison of the TNF-α levels at each level individually in both the transformed and untransformed output (Table 2.8), however, indicates that the macrophage TNF-α level when both LPS and IL-1β are used to activate MSCs is lower than that of activation with LPS or IL-1β alone. Therefore the effect of combined MSC activation by IL-1β and LPS is beneficial for mitigating the pro-inflammatory macrophage phenotype, although the effects are not mathematically additive or synergistic.

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

We next aimed to validate the results of the FFD by using LPS and IL-1β, alone and in combination without the presence of the non-optimal factors, in our monolayer MSC and macrophage *in vitro* assays. As determined in the high throughput screening assay, IL-1β exhibited a much higher potency than LPS. LPS and IL-1β increased MSC PGE2 secretion ~2- and ~7-fold on a per cell basis, respectively (Figure 2.6A). 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 2.6B), this reduction was greater when MSCs were activated with LPS. MSC activation with IL-1β more potently reduced macrophage TNF-α secretion compared to LPS activation of MSCs. In contrast to the effects of these two factors on MSC PGE2 secretion, the combined use of LPS and IL-1β to activate MSCs did not result in a synergistically enhanced attenuation of macrophage TNF-α secretion.

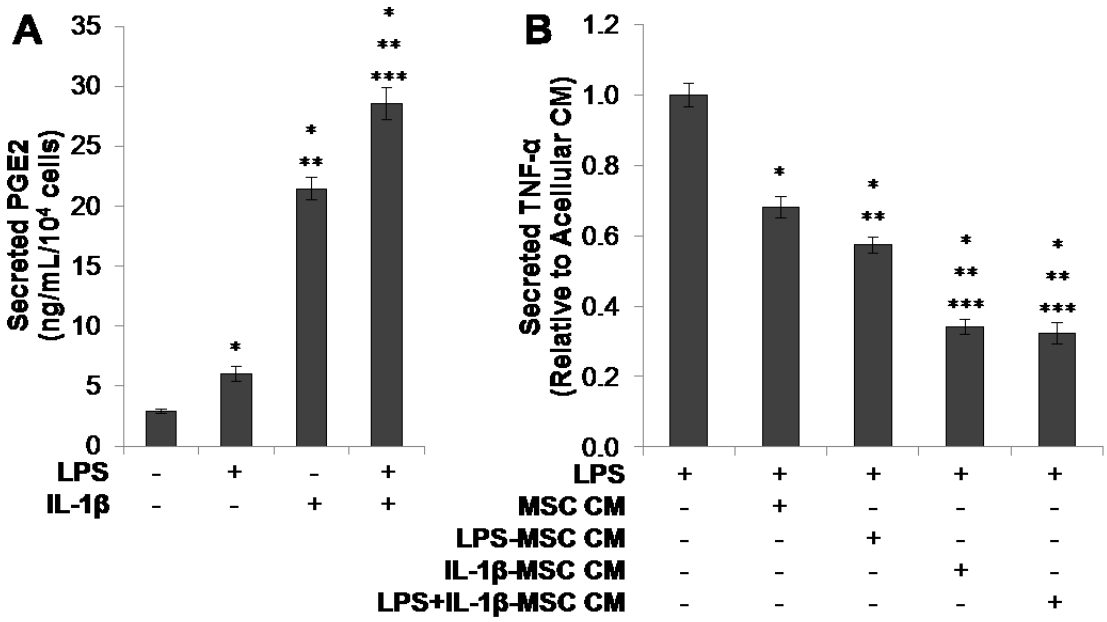

Figure 2.6 Validation of effects of LPS and IL-1β on MSCs. MSCs were incubated for 48 hours with LPS or IL-1β, individually and in combination. (A) The level of PGE2 in the cell culture supernatants were then quantified and normalized to the number of MSCs present at the end of culture. Data are means ± SEM of 3 independent experiments with 3-6 replicates per experiment ($p<0.05$ compared to LPS^- $IL\text{-}1\beta^-$ (*), LPS^+ $IL\text{-}1\beta^-$ (**), or LPS^- $IL\text{-}1\beta^+$ (***) by ANOVA with Fisher's LSD *post hoc* test. (B) Macrophages were incubated for 48 hours in CM collected from activated MSCs. The level of TNF-α in the macrophage cell culture supernatants were then quantified and normalized to that of corresponding acellular CM. Data are means ± SEM of 3 independent experiments with 2-6 technical replicates per experiment ($p<0.05$ compared to LPS (*), MSC CM (**), or LPS-MSC CM (***) by ANOVA with Fisher's LSD *post hoc* test).

2.4 Discussion

Many current approaches are being developed to optimize MSC-based cellular therapies, including pre-conditioning using soluble activating factors [21-23]. 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 DoE methodology to screen a panel of soluble molecules for improved MSC immunomodulatory function.

Using a $2^{(6-1)}$ 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 accurate estimation of the effects of all individual factors and all two-factor interactions, assuming that higher order interactions (≤ 4^{th} order) are negligible. DoE methodology is extensively used in biotechnological fields for bioprocess development/optimization and is increasingly being utilized for basic and pre-clinical research applications, such as optimization of stem cell differentiation and anti-viral drug studies [24-27]. We have previously utilized FFD to screen the effects of 13 MSC-secreted factors on the conversion of macrophages from a pro-inflammatory 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 [9, 12-16], knowledge of their effects on MSC modulation of macrophages has been limited. IL-1β has been reported to induce migration,

chemoattraction and secretion of several cytokines and growth factors from MSCs [13, 28, 29]. 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 [10, 12, 30-33]. 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-1β and LPS activate myeloid differentiation primary response gene 88 (MyD88)-dependent pathways leading to nuclear factor (NF)-κB and activator protein (AP)-1 transcriptional activity, which increases expression of cyclooxygenase-2 (COX-2) and subsequent enzymatic production of PGE2 [34]. The activity of the NF-κB 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 pro-inflammatory macrophages into IL-10-secreting cells *in vivo* [7]. LPS and IL-1β stimulate different receptors with similar intracellular signaling machinery (TLR4 and IL1R1, respectively) [35]; 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 [10, 12, 32, 36, 37]. 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 [11, 12]. 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-1β. 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-1β-activated MSC CM, despite the synergistic interaction of IFN-β with IL-1β to increase PGE2 secretion. Upregulation of NF-κB activity and COX-2 transcription by IFN-β has been reported in Huh7 cells [38]

and microglia [39] and IFN-γ has previously been reported to increase COX-2 expression and secretion of PGE2 from MSCs [40, 41]. 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-1β activity [42], 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-κB pathways. STAT3 activity has been reported to inhibit NF-κB activity, potentially via competitive binding to DNA promoter regions [43, 44]. 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 [45]. The divergence of downstream effects of type I and type II interferon 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-κB pathway. Supplementation of MSC culture medium with exogenous IL-6 has

been shown to increase MSC proliferation [13] and vascular endothelial growth factor (VEGF) secretion in combination with transforming growth factor (TGF)-α [15]. Additionally, PGE2 secretion from murine MSCs has been reported to be dependent on MSC *IL6* expression, suggesting an autocrine IL-6 function [46]. 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. Due to 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.

There are limitations of the *in vitro* platform and approach described here. FFDs operate on the assumption that higher order interactions (≥ 4^{th} order) are negligible 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 2 levels and does not include center points, which only allows for linear relationships to be described. Although non-linear 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 3-level or mixed level designs, for which analysis is also well defined [17]. 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 [29]. Therefore, future studies will also include analysis across multiple MSC donors.

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 [47], 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 co-culture 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, co-administration of inflammatory molecules with MSCs would not be desirable. Rather, MSCs would be pre-exposed to activation factors and washed prior to injection. There have been several reports of pre-activation and pre-conditioning generating different MSC functional phenotypes and having enhanced therapeutic behavior *in vitro* and *in vivo* [10, 22, 28, 48, 49].

The approach described here can easily be adapted to facilitate *in vitro* investigations of pre-activation. 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.

In conclusion, we have identified IL-1β from a screen of a panel of activating molecules as an optimally potent enhancer of MSC immunomodulatory function, with LPS increasing the potency of IL-1β's effect on some aspects of this function. We demonstrated the feasibility and utility of using statistical approaches to facilitate these types of investigations with higher throughput. These studies will be used to further elucidate MSC therapeutic mechanisms and harness control of these functions by developing pre-activation schemes for improved MSC function.

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Chapter 3: Optimization and Characterization of MSC Pre-Activation

3.1 Introduction

Pre-activation of mesenchymal stromal cells (MSCs) by cytokines/growth factors [1-18] and toll like receptor (TLR) ligands [3, 13, 15, 16, 19-23] is currently being explored as a strategy to enhance the therapeutic functions of MSCs. There have been reports of pre-activation of MSCs with different factors resulting in improved outcomes in animal models of disease [7, 10, 24-28].

A limitation these approaches as they currently stand, however, is that there is not a standardized or systematic method for the optimization of inputs to achieve specific MSC outputs. As discussed in chapter 1, elicitation of MSC functions depends on several factors including the type of activating molecule, its prevalence/concentration, duration/timing of exposure, and whether it is in combination with another factor(s) [29]. The selection of activation parameter settings in studies utilizing pre-activated MSCs is often arbitrary, with only a portion of relevant factors being optimized inconsistently across studies. This limitation is in part responsible for disparate results and difficulty comparing approaches.

There are also inconsistencies in the amount of characterization performed on pre-activated MSCs prior to testing in an animal model. Of the studies that perform *in vitro* characterization, very few investigate how the pre-activated MSCs will respond when introduced into a simulated disease/damaged microenvironment. This is important because connections between activation protocols and *in vivo* metrics in relatively homogeneous animal models may not

translate or be relevant for diverse human patients with the disease or injury [30, 31]. Therefore, a pre-activation protocol that determines enhanced efficacy based on only a single metric may not predict success across the board conditions potentially treatable by MSCs.

This demonstrates a need for a systematic approach to optimizing MSC pre-activation conditions to induce desired phenotypes and characterization of pre-activated MSCs by multiple relevant functional metrics. In chapter 2, we identified interleukin (IL)-1β and lipopolysaccharide (LPS) as optimal activators of MSC prostaglandin E2 (PGE2) secretion and subsequent attenuation of macrophage tumor necrosis factor (TNF)-α secretion using a flexible high throughput design of experiments approach. In this chapter, we continue the development of an MSC pre-activation for the specific purpose of enhancing MSC-mediated macrophage phenotype by optimizing IL-1β and LPS dose and duration of pre-exposure. We then characterize the secretion of PGE2 from pre-activated MSCs in response to secondary pro-inflammatory stimuli and the modulation of macrophage TNF-α secretion in a co-culture assay. Our results deliver a pre-activation protocol for enhancing MSC immunomodulation and demonstrate the need for multi-metric characterization of pre-activated MSCs.

3.2 Materials and Methods

3.2.1 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 was purchased from InvivoGen (San Diego, CA).

3.2.2 Cell Culture

All cells were maintained in a humidified 37°C incubator with 5% CO_2 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.

3.2.3 MSCs

Human bone marrow-derived MSCs (male, age 28) at passage 1 were purchased from the Institute for Regenerative Medicine (Texas A&M College of Medicine, Temple, TX, USA) and cultured as previously described [32]. The cells were grown to 70% confluence, detached with trypsin, seeded into 96-well plates at $2x10^3$/well, 48-well plates at $3x10^3$ cells/well or transwell inserts, as required for specific experiments, and allowed to attach overnight. Experiments were set up using MSCs at passage 3 – 4 and fully supplemented RPMI 1640 medium.

3.2.4 Macrophages

$CD14^+$ monocytes were isolated from human peripheral blood from healthy adult donors (The Blood Center of New Jersey, East Orange, NJ; New York Blood Center, Long Island City, NY), differentiated to generate macrophages as described previously [32], and cryopreserved. Macrophages at passage 1 were thawed, seeded into well plates as required for specific experiments, and allowed to attach overnight.

3.2.5 Cell Counting

After cell culture supernatants were collected and stored at -80°C, MSCs were fixed in 4% (w/v) paraformaldehyde (PF) for 20 minutes at room temperature and stored in 1% (w/v) PF at 4°C until further use. The nuclei of fixed monolayer MSCs stained with Hoechst and counted via microscopic imaging, as previously described [32].

Alternatively, cell culture supernatants were collected, stored at -80°C, and replaced with fully supplemented RPMI 1640 medium containing Alamar blue (Molecular Probes, Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. In parallel, a standard curve was created by seeding MSCs into a 48-well plate at 3 $x10^3$, 6 $x10^3$, 1.2 $x10^4$, or 2.4$x10^4$ cells/well and allowing the cells to attach overnight. MSC cell culture medium was replaced with fully supplemented RPMI 1640 medium containing Alamar blue. Fluorescent Alamar blue readings were recorded after 4 hours of incubation using a microplate reader (DTX 880 Multimode Detector, Beckman Coulter, Fullerton, CA), and the standard curve was used to calculate cell number.

3.2.6 MSC/Macrophage Co-Culture

Macrophages at passage 1 were thawed, washed in fully supplemented Advanced RPMI 1640 medium, seeded into 24-well plates at 5$x10^4$ cells/well, and allowed to attach overnight. MSCs were seeded into 24-well transwell inserts (0.4 μm pore size polyester membrane, Corning, Lowell, MA) at 5$x10^4$ cells/transwell and allowed to attach overnight. MSC cell culture medium was replaced with fully supplemented RPMI 1640 medium with activation factors for 1

hour. After thoroughly washing with fresh medium 3 times, fully supplemented RPMI 1640 medium with LPS (1 μg/mL) was added to the transwell apical chamber. Macrophage cell culture medium was replaced with fully supplemented RPMI 1640 medium with or without LPS (1 μg/mL) and MSC transwell inserts were added to LPS conditions. Basolateral supernatants were collected after 48 hours and frozen at -80°C.

3.2.7 Cytokine Measurement

Cell culture supernatants were thawed and analyzed using an enzymatic immunoassay for PGE2 (Cayman Chemical, Ann Arbor, MI), enzyme-linked immunosorbent assays (ELISAs) for TNF-α, IL-1β, and interferon (IFN)-γ (Biolegend, San Diego, CA), and a bead-based 27 multiplex immunoassay (Bio-Plex Pro Human Cytokine Group I, Bio-Rad Laboratories Inc., Hercules, CA), according to the manufacturer's instructions. Absorbances were recorded using microplate readers and data was analyzed in Matlab software version R2013b (MathWorks, Natick, MA) or Microsoft Excel (Microsoft, Redmond, WA).

3.2.8 Statistical Analysis

Data points represent the mean ± standard error of the mean (SEM) for the indicated number of independent observations (n). Statistical differences between the conditions were determined using Student's *t* test or analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) *post hoc* analysis in KaleidaGraph software version 4.1 (Synergy Software, Reading, PA, USA). P values <0.05 were considered significant.

3.3 Results

3.3.1 Dose Response

We previously identified IL-1β and LPS, individually and in combination, as optimal inducers of MSC PGE2 secretion and subsequent attenuation of macrophage TNF-α secretion [32]. We next performed dose response studies to optimize the concentration of each of these activating factors for efficient and maximal secretion of PGE2 from MSCs. The cell culture medium of MSCs in 96-well plates was replaced by medium without or with increasing doses of IL-1β (0.1, 1, or 10 ng/mL) and/or LPS (10, 100, 1000 ng/mL) (Figure 3.1A). Cell culture supernatants were collected after 6, 24, and 48 hours and analyzed for PGE2. As was previously observed [32], IL-1β more potently induced PGE2 secretion compared to LPS (Figure 3.1B,C). After just 6 hours of activation, dose dependent effects of IL-1β and LPS were observed. IL-1β at 1 ng/mL (Figure 3.1B) and LPS at 100 ng/mL (Figure 3.1C) increased PGE2 secretion to a greater degree than their respective lower dose. PGE2 levels were higher after 24 hours of activation, but plateaued at 48 hours. Further increasing the concentration of either factor did not further increase PGE2. In LPS activated MSCs, using a concentration of 100 ng/mL induced more PGE2 secretion in 6 hours than did 10 ng/mL in 48 hours, demonstrating more efficient induction.

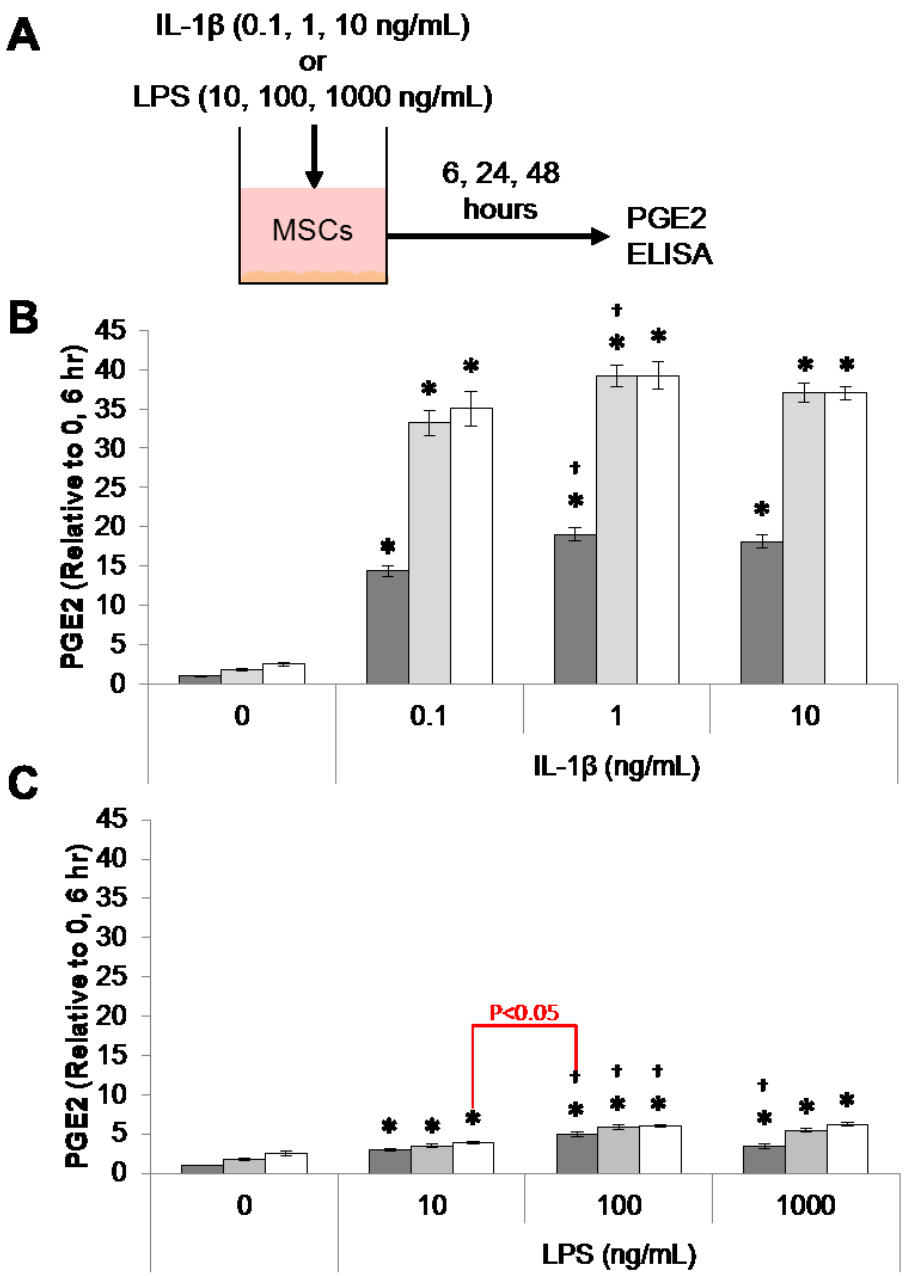

Figure 3.1 Dose response of MSC PGE2 secretion to IL-1β or LPS. (A) MSC culture medium was replaced with fully supplemented RPMI 1640 medium without or with increasing doses of (B) IL-1β or (C) LPS for 6 (dark grey bars), 24 (light grey bars), or 48 hours (white bars) and then analyzed for PGE2. Data are the mean ± SEM for PGE2 normalized to cell number and

medium control (0 ng/mL) at 6 hours for n = 6 replicates. *p<0.05 compared to 0 ng/mL, †p<0.05 compared to previous dose by ANOVA and Fisher's LSD. Other statistical comparisons were performed using Student's *t* test.

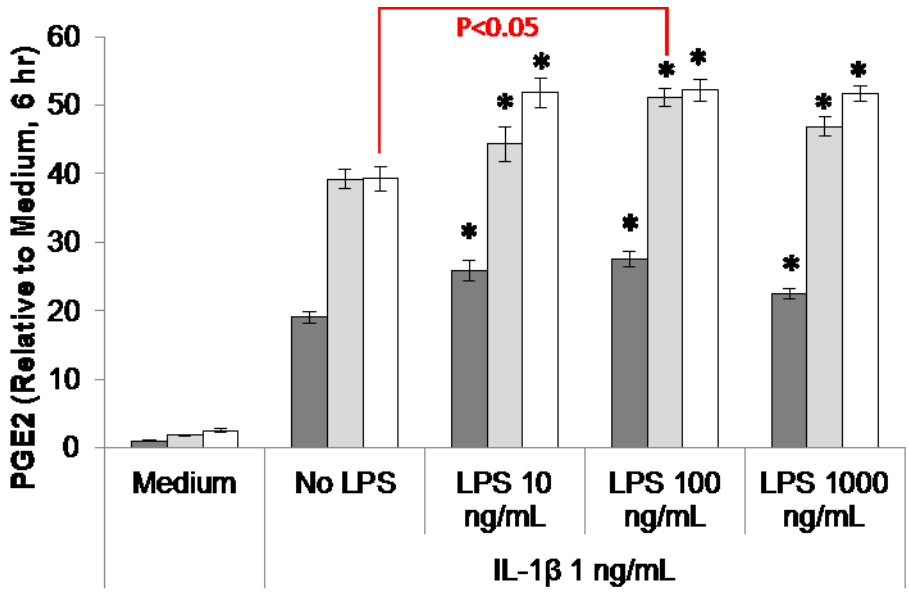

Figure 3.2 Dose response of MSC PGE2 secretion to LPS and IL-1β. MSC culture medium was replaced with fully supplemented RPMI 1640 medium without or with IL-1β and an increasing dose of LPS for 6 (dark grey bars), 24 (light grey bars), or 48 hours (white bars) and then analyzed for PGE2. Data are the mean ± SEM for PGE2 normalized to cell number and medium control at 6 hours for n = 6 replicates. *p<0.05 compared to No LPS by ANOVA and Fisher's LSD. Other statistical comparisons were performed using Student's *t* test.

We next determined which doses of LPS most synergistically upregulate MSC PGE2 secretion with 1 ng/mL IL-1β, which was selected as optimal, based on the previously described results. Supplementation of the activation medium with LPS in addition to IL-1β significantly increased MSC PGE2 secretion for all

time points and LPS concentrations (Figure 3.2). LPS at 100 ng/mL, which was the best performing condition in the experiments using LPS alone, induced more PGE2 secretion in 24 hours than IL-1β alone did in 48 hours.

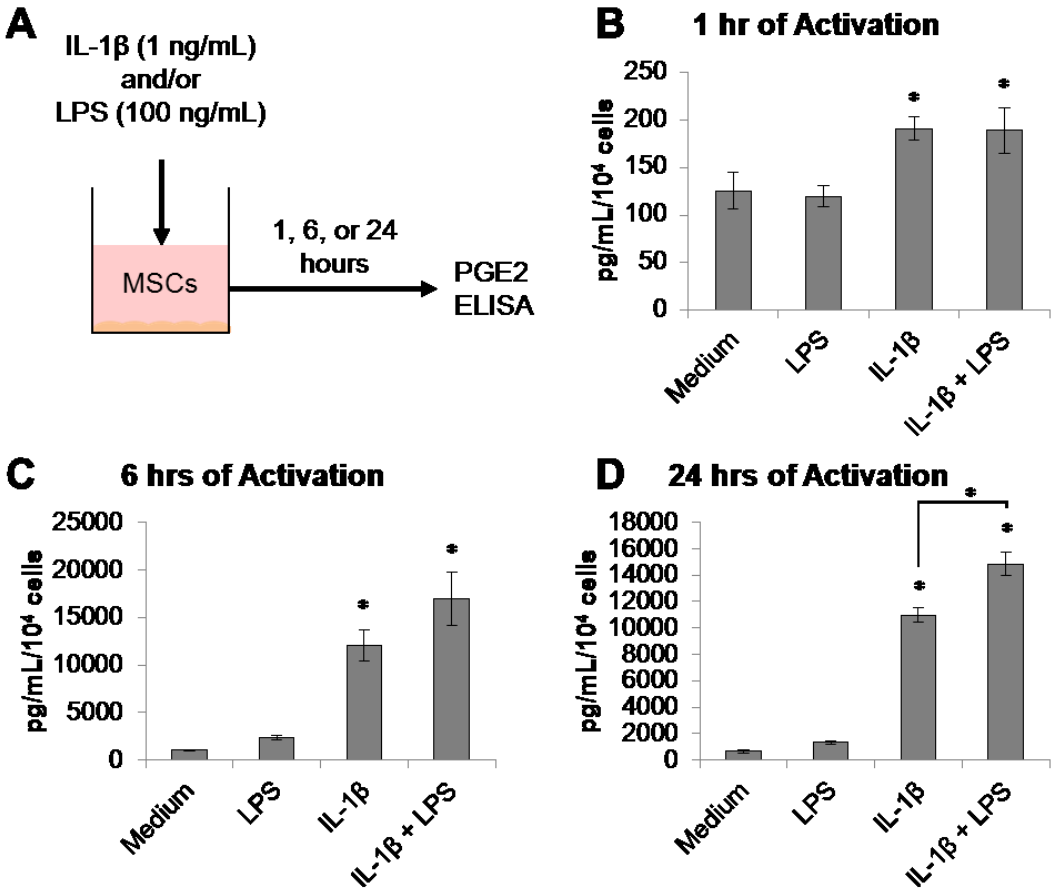

Figure 3.3 MSC PGE2 secretion at the end of the activation period. (A) MSC culture medium was replaced by medium without or with IL-1β, LPS or both for (A) 1, (B) 6, or (C) 24 hours (hrs). These supernatants were collected and analyzed for PGE2. Data are the mean ± SEM for PGE2 normalized to cell number for n = 6 replicates. *p<0.05 compared to medium by Student's *t* test.

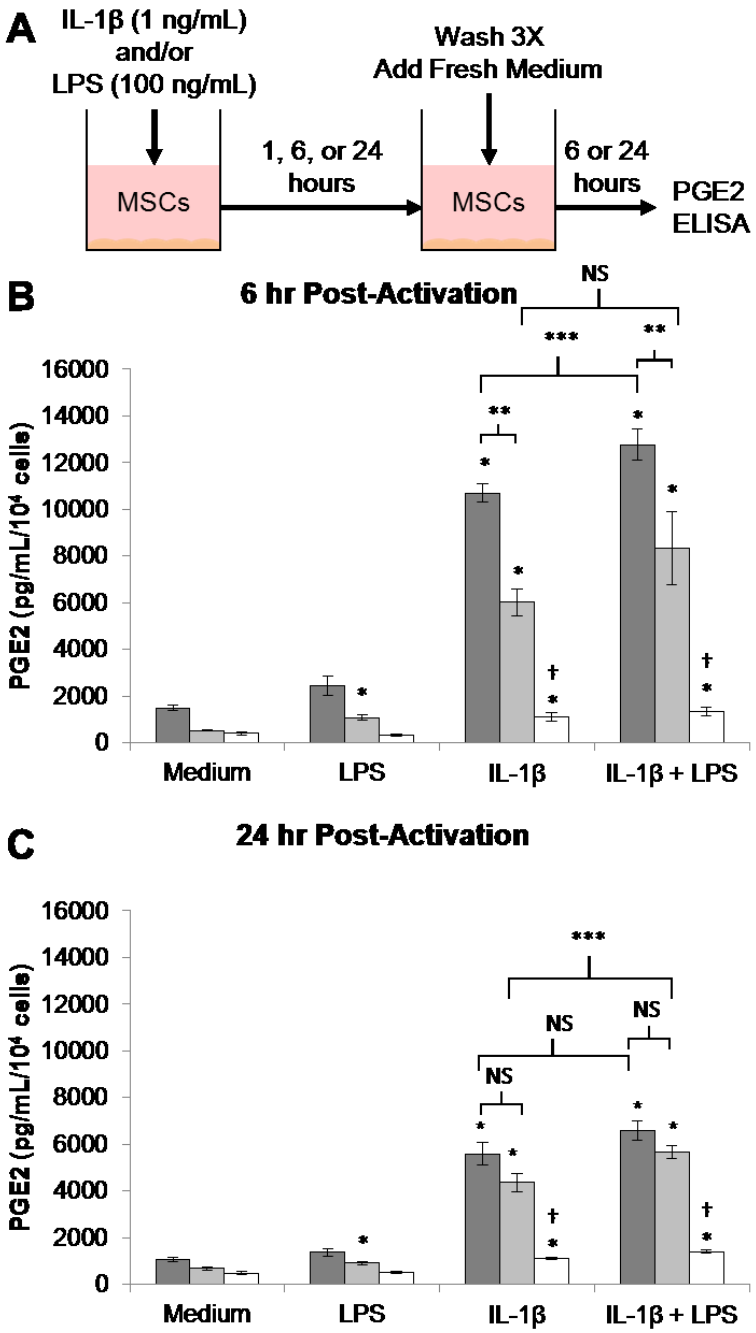

Figure 3.4 MSC PGE2 secretion after pre-activation. (A) MSC culture medium was replaced by medium without or with IL-1β, LPS or both for 1 (dark grey bars), 6 (light grey bars), or 24 (white bars) hours (hr). Pre-activated cell culture supernatants were replaced by fresh medium for (B) 6 or (C) 24 hours. Data are the mean ± SEM for PGE2 normalized to cell

number for n = 6 replicates. *p<0.05 compared to no pre-activation (medium) by Student's *t* test; **p<0.05 by Student's *t* test; ***p<0.05 by ANOVA and Fisher's LSD; †p<0.05 compared to 1 or 6 hours of pre-activation by ANOVA and Fisher's LSD.

3.3.2 Optimization of Duration of Pre-Activation

Based on the results of the dose response studies, 1 ng/mL IL-1β with and without 100 ng/mL LPS were used to pre-activate MSCs. The cell culture medium of MSCs in 48-well plates was replaced by medium without or with IL-1β, LPS or both for 1, 6, or 24 hours (Figure 3.3A). In as little as 1 hour of exposure, MSCs upregulated their release of PGE2 in response to IL-1β (Figure 3.3B). Further increases in PGE2 secretion with the addition of LPS became apparent starting at 6 hours of exposure (Figure 3.3C) and became significant and synergistic after 24 hours of exposure (Figure 3.3D).

To determine if pre-activation leads to sustained upregulated PGE2 levels and how the pre-activation duration and choice of activator(s) affects this, activated MSC culture media were replaced by fresh medium for 6 or 24 hours (Figure 3.4A). To confirm that the pre-activation factor was completely removed from the culture, the discard of the third wash was collected and analyzed for IL-1β, which was not detected (data not shown). LPS pre-activation of MSCs did not have a large effect on PGE2 secretion at 6 hours (Figure 3.4B) or 24 hours (Figure 3.4C) post-activation; significant increases in PGE2 from LPS pre-activated MSCs compared to MSCs without pre-activation were only seen when the cells were pre-activated for 6 hours. Pre-activation by IL-1β with or without

LPS resulted in sustained upregulated levels of PGE2 secretion compared to MSCs without pre-activation at both 6 and 24 hours post-activation. As cells proliferated from 6 to 24 hours post-activation, levels of PGE2 per cell decreased while still remaining upregulated compared to MSCs without pre-activation. Pre-activation for just 1 hour was sufficient to achieve the highest level of sustained upregulated PGE2 post-activation. Extending the duration of pre-activation to 6 and 24 hours resulted in significantly lower levels of PGE2 post-activation. At 6 hours post-activation, MSCs pre-activated with both IL-1β and LPS for 1 hour resulted in slightly higher levels of PGE2 compared to pre-activation with IL-1β alone, but this effect did not persist after 24 hours.

3.3.3 MSC Secretome After Optimal Pre-Activation

After selecting 1 hour of pre-activation with IL-1β as optimal based on PGE2 secretion, we next examined a larger panel of MSC secreted factors to further characterize activation induced secretome changes more thoroughly. To do this, a bead-based multiplex immunoassay was used to quantify 27 analytes in pre-activated MSC supernatants collected at the end of the pre-activation period (Figure 3.5A) and 6 or 24 hours post-activation (Figure 3.6A). Out of the 27 probed secreted factors, 15 were detectable in MSC supernatants at the end of the 1 hour activation period (Figure 3.5B). These factors included chemokines, pro-inflammatory, anti-inflammatory/trophic, and pleiotropic molecules. MSCs that were exposed to IL-1β for 1 hour had significant release of IL-8, monocyte Chemoattractant Protein (MCP)-1, TNF-α, IL-1RA, fibroblast growth factor (FGF)-basic, vascular endothelial growth factor (VEGF), granulocyte-colony stimulating

factor (G-CSF), and IL-6 at the end of the activation period compared to MSCs without pre-activation.

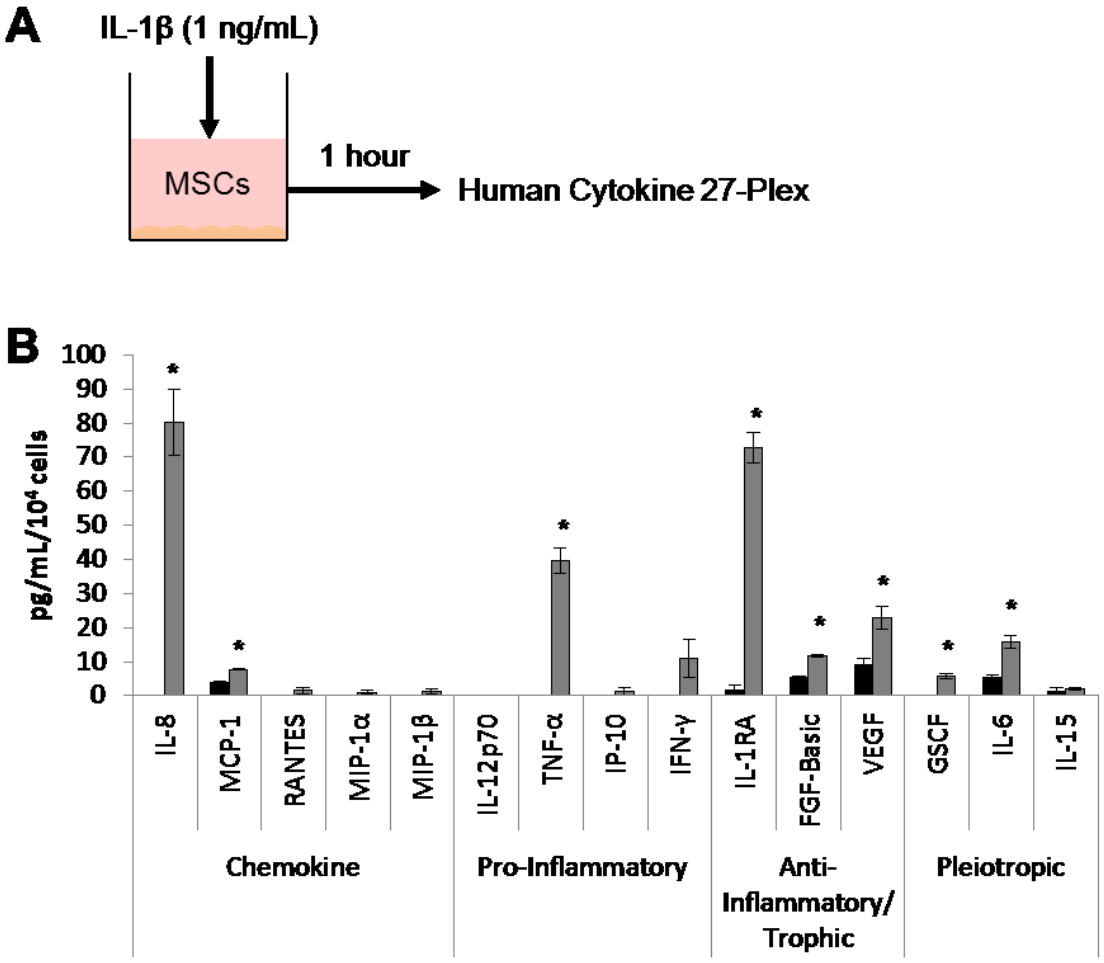

Figure 3.5 MSC secretome changes at the end of the activation period. (A) MSC culture medium was replaced by medium without (black bars) or with IL-1β (grey bars) for 1 hour. (B) Out of 27 secreted factors quantified, 15 were detected in supernatants using a bead-based multiplex immunoassay. Data are the mean ± SEM for secreted level normalized to cell number for n = 3 replicates. *p<0.05 compared to medium by Student's *t* test.

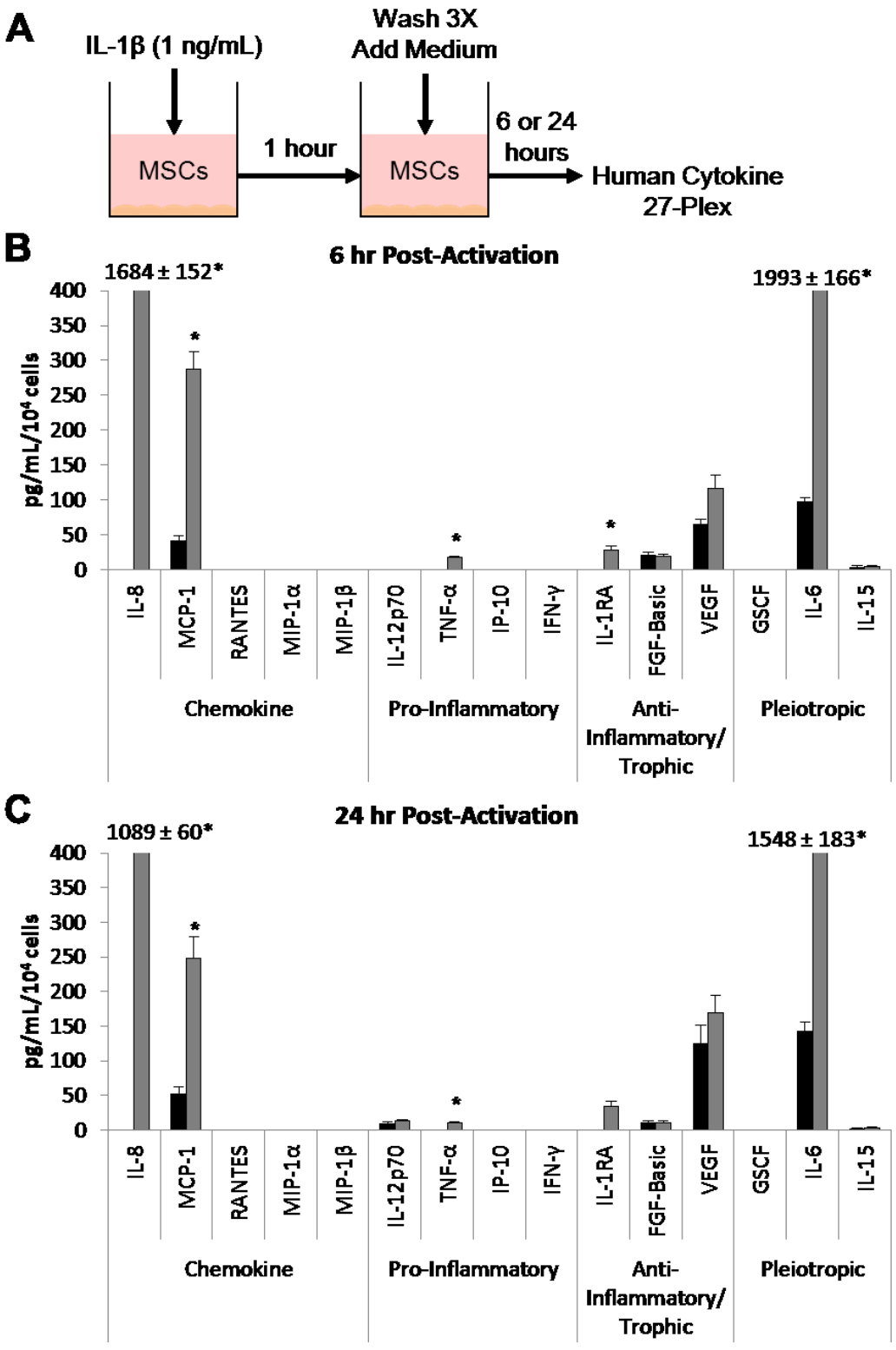

Figure 3.6 MSC secretome changes post-activation. (A) MSC culture medium was replaced by medium without (black bars) or with IL-1β (grey

bars) for 1 hour. Pre-activated cell culture supernatants were replaced by fresh medium for (B) 6 or (C) 24 hours. Secreted factors in supernatants were quantified using a bead-based multiplex immunoassay. Data are the mean ± SEM for secreted level normalized to cell number for n = 3 replicates. *p<0.05 compared to medium by Student's *t* test.

We next examined the levels of these factors 6 hours (Figure 3.6B) and 24 hours (Figure 3.6C) post-activation. At both 6 and 24 hours, secretion of IL-8 and IL-6 remained highly upregulated from IL-1β pre-activated MSCs. TNF-α was also detectable at these time-points but only at very low levels. Secretion of IL-1RA remained significantly upregulated from IL-1β pre-activated MSCs at 6 hours post-activation.

3.3.4 Secondary Stimulation of Pre-Activated MSCs

After demonstrating that MSCs sustain upregulated secretion of PGE2 and other factors after brief pre-activation with IL-1β, we next determined whether this pre-activated population of MSCs is more sensitive to secondary stimuli that they are likely to encounter upon reintroduction into an inflammatory environment involving macrophages. To do this, MSCs were pre-activated with IL-1β for 1 hour and then supernatants were replaced with fresh medium without or with IL-1β, TNF-α, IFN-γ, or LPS for 24 hours (Figure 3.7A). Consistent with previous results, IL-1β pre-activated MSCs exhibited upregulated PGE2 secretion when exposed to fresh medium (Figure 3.7B). The upregulation was further enhanced when IL-1β pre-activated MSCs were then stimulated with TNF-α, IFN-γ, or LPS compared to MSCs stimulated with these secondary factors

without pre-activation. Interestingly, PGE2 secretion from MSCs pre-activated with IL-1β and secondarily exposed to IL-1β again was not different than the response of MSCs with no pre-activation.

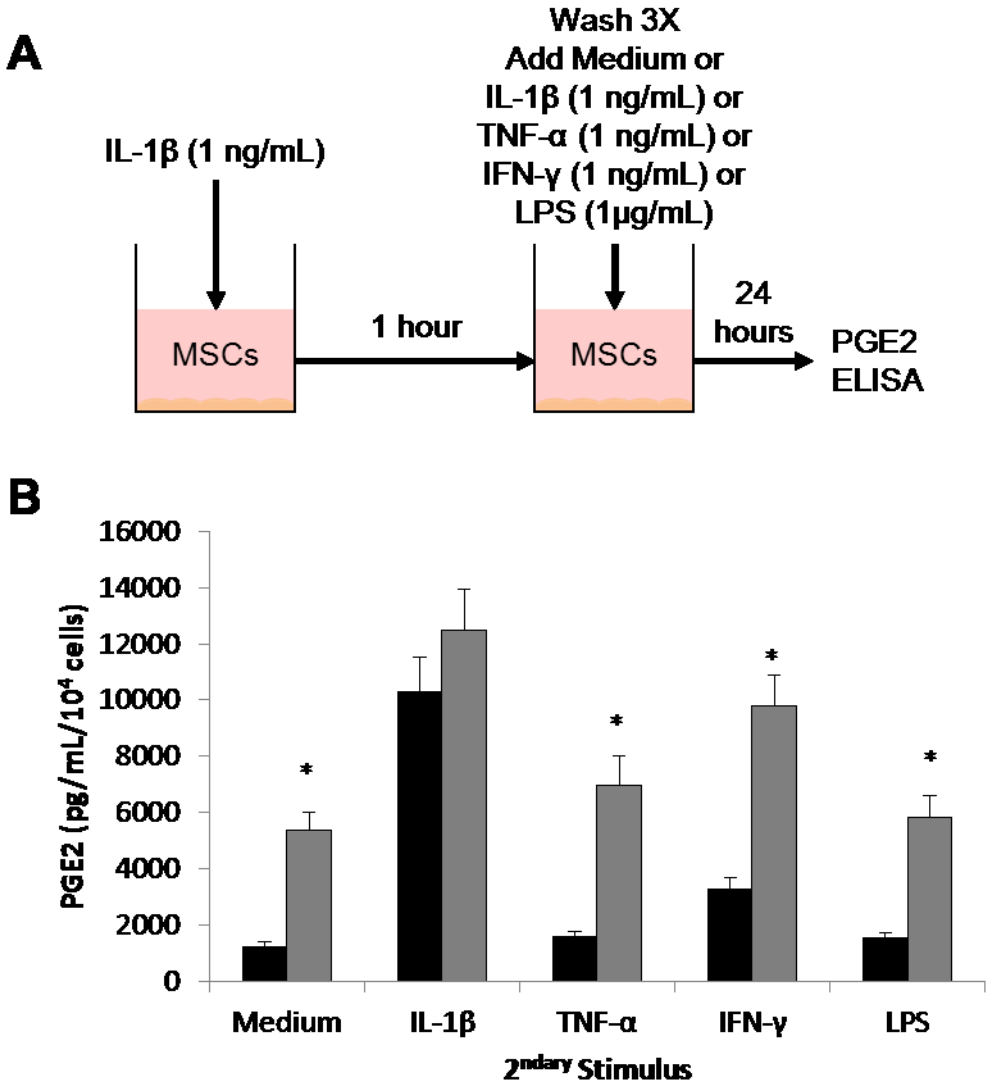

Figure 3.7 Sensitivity of IL-1β pre-activated MSCs to pro-inflammatory secondary stimuli. (A) MSC culture medium was replaced by medium without (black bars) or with (grey bars) IL-1β for 1 hour. Pre-activated cell culture supernatants were replaced by fresh medium without or with IL-1β, TNF-α, IFN-γ, or LPS for 24 hours. (B) PGE2 was quantified in the resulting

supernatants. Data are the mean ± SEM for secreted PGE2 level normalized to cell number for n = 9 replicates. *p<0.05 compared to MSCs without pre-activation by Student's *t* test.

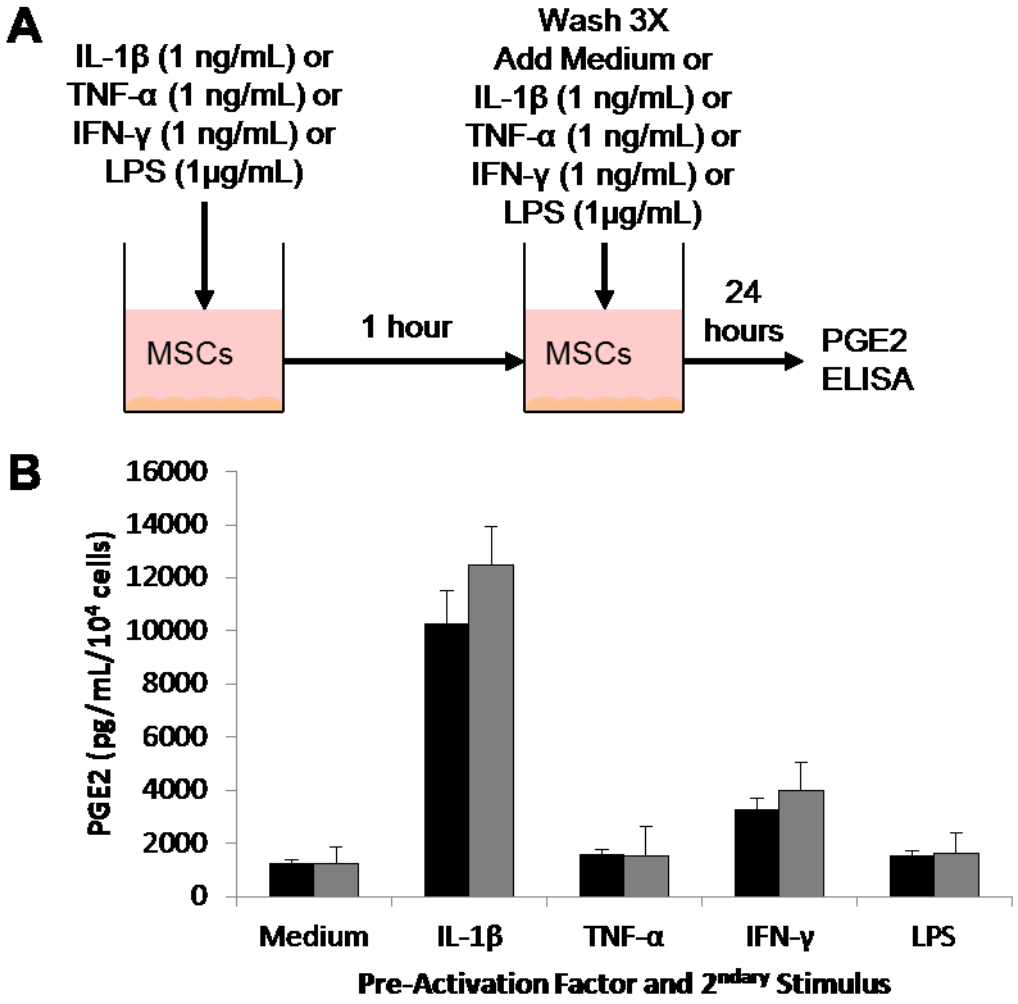

Figure 3.8 Response of pre-activated MSCs to secondary exposure to the pre-activation factor. (A) MSC culture medium was replaced by medium without (black bars) or with (grey bars) IL-1β, TNF-α, IFN-γ, or LPS for 1 hour. Pre-activated cell culture supernatants were replaced by fresh medium without or with the same factor. (B) PGE2 was quantified in the resulting supernatants. Data are the mean ± SEM for secreted PGE2 level

normalized to cell number for n = 9 replicates. *p<0.05 compared to MSCs without pre-activation by Student's *t* test.

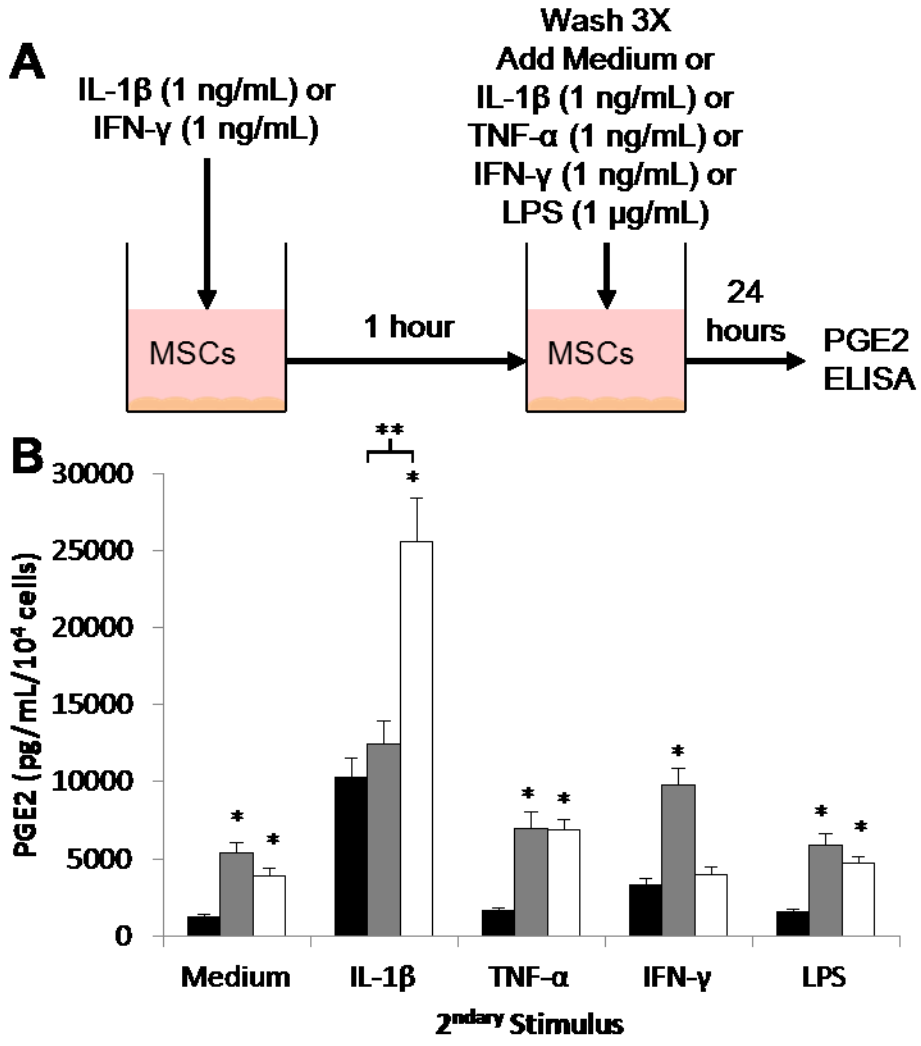

Figure 3.9 Response of pre-activated MSCs to secondary exposure to a panel of pro-inflammatory secondary stimuli. (A) MSC culture medium was replaced by medium without (black bars) or with IL-1β (grey bars) or IFN-γ (white bars) for 1 hour. (B) PGE2 was quantified 24 hours after pre-activated cell culture supernatants were replaced by fresh medium without or with IL-1β, TNF-α, IFN-γ, or LPS. Data are the mean ± SEM for secreted

PGE2 level normalized to cell number for n = 9 replicates. *p<0.05 compared to MSCs without pre-activation by Student's *t* test.

To determine if the lack of enhanced PGE2 secretion from IL-1β pre-activated MSCs in response to a second dose of IL-1β is specific to this cytokine, the experiment was repeated using TNF-α, IFN-γ, or LPS as pre-activators as well as secondary stimuli (Figure 3.8A). No enhancement in PGE2 secretion was observed for MSCs pre-activated and secondarily stimulated with the same factor (Figure 3.8B). The secretion of PGE2 from TNF-α or LPS pre-activated MSCs exposed to the rest of the panel of pro-inflammatory secondary stimuli was not different from MSCs that were not pre-activated (data not shown). IFN-γ pre-activated MSCs, however, did exhibit enhanced PGE2 secretion in response to IL-1β, TNF-α, and LPS (Figure 3.9). Further, the secretion of PGE2 in response to secondary exposure to IL-1β was dramatically enhanced.

3.3.5 Pre-Activated MSC Modulation of Macrophage TNF-α

After determining that the population of IL-1β pre-activated was more sensitive to secondary inflammatory stimuli, we next tested the MSC pre-activation protocol in a functional assay. MSCs in transwell inserts were pre-activated with IL-1β for 1 hour and then co-cultured with macrophages in the presence of LPS for 48 hours (Figure 3.10A). IFN-γ pre-activated MSCs were also used due to the unexpected observation of the enhanced PGE2 secretion, particularly after secondary IL-1β stimulation.

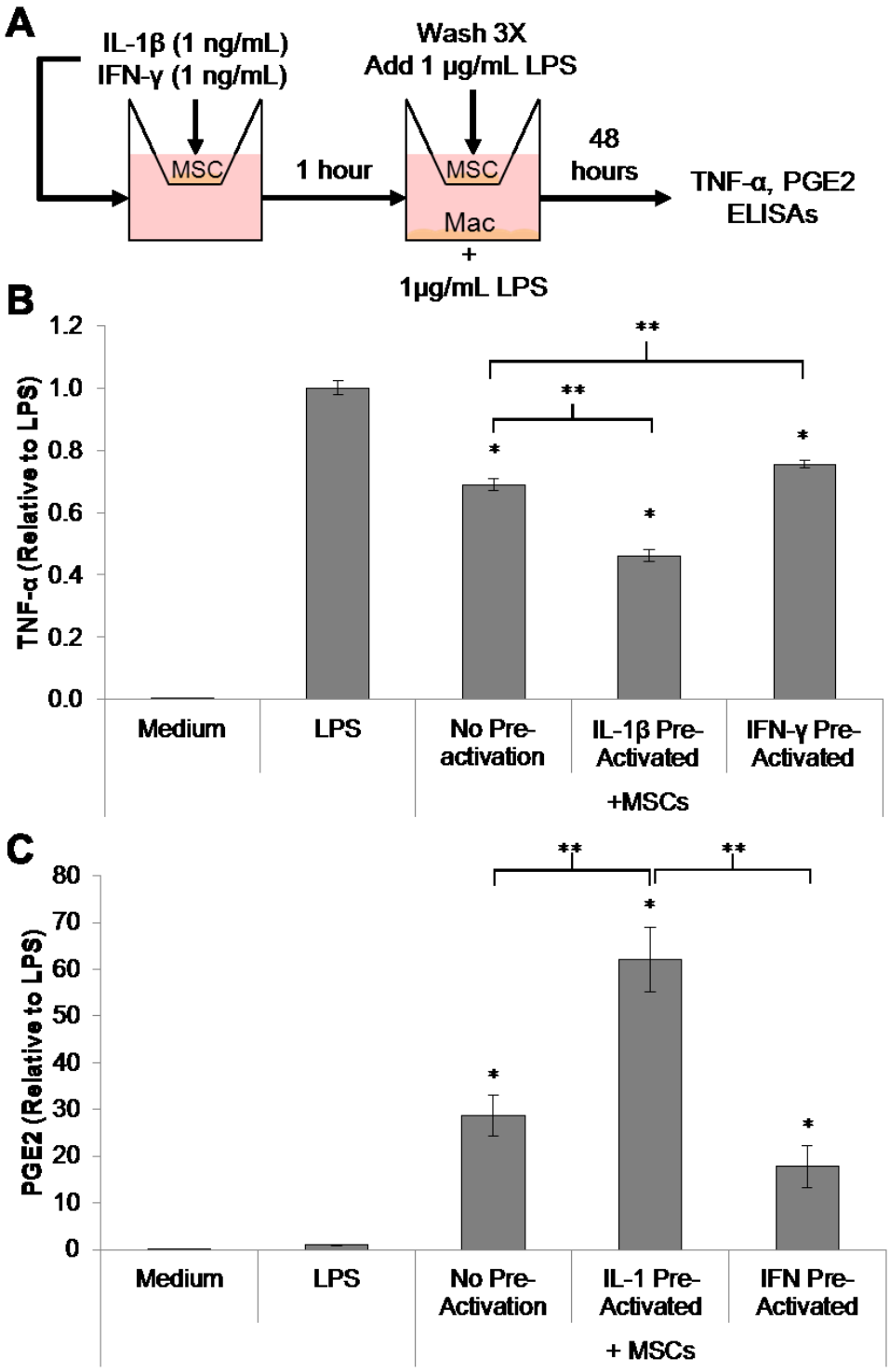

Figure 3.10 Pre-Activated MSC modulation of macrophages. (A) MSC transwell culture medium was replaced by medium without or with IL-1β or IFN-γ for 1 hour, and then transwells were washed and transferred to

macrophage cultures with LPS for 48 hours. Co-culture supernatants were analyzed for (B) TNF-α and (C) PGE2. Data are the mean ± SEM for secreted level normalized to the LPS control for n = 9 – 19 replicates. *p<0.05 compared to LPS by Student's *t* test. **p<0.05 determined by Student's *t* test.

Without pre-activation, MSCs attenuated macrophage TNF-α secretion by ~31% (Figure 3.10B). Pre-activation of the MSCs with IL-1β resulted in enhanced macrophage modulation, attenuating TNF-α secretion by ~54%. Surprisingly, IFN-γ pre-activation of MSCs not only failed to enhance MSC-mediated attenuation of macrophage TNF-α, but these cells performed slightly worse than MSCs without pre-activation (~24% attenuation of TNF-α secretion). PGE2 levels in the co-culture supernatants (Figure 3.10C) complemented the trends in TNF-α levels, with IL-1β pre-activated MSC conditions containing the highest amount of PGE2, followed by MSCs without pre-activation and then IFN-γ pre-activated MSCs.

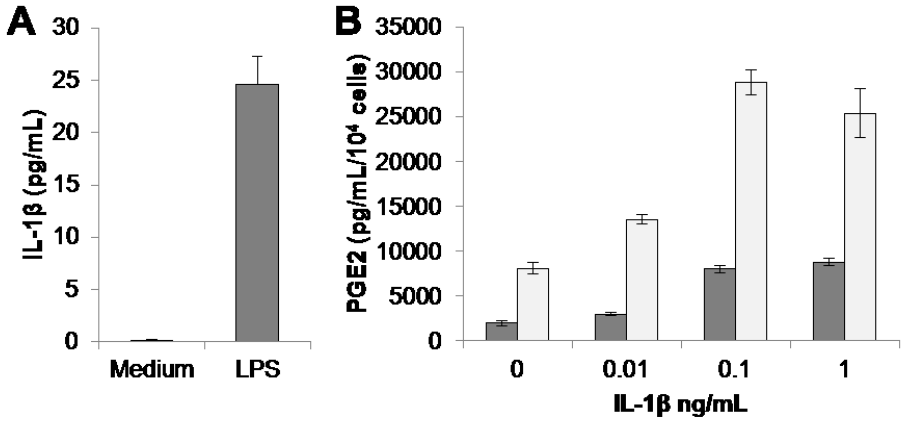

Figure 3.11 Effect of IL-1β concentration on IFN-γ pre-activated MSC PGE2 secretion. (A) The level of IL-1β present in LPS-activated macrophage

supernatants at 48 hours was quantified. (B) PGE2 secretion from MSCs without (dark grey bars) and with (light grey bars) IFN-γ pre-activation in response to an increasing secondary dose of IL-1β was measured. Data are the mean ± SEM for (A) IL-1β level or (B) PGE2 level normalized to cell number for (A) n = 27 or (B) n = 3 replicates.

To begin to investigate why the enhanced anti-inflammatory properties of IFN-γ pre-activated MSCs in isolated culture in response to IL-1β did not translate into the co-culture system, the level of IL-1β secreted from LPS-activated macrophages was first quantified (Figure 3.11A). LPS-activated macrophages secreted IL-1β at levels on the order of 10^1 pg/mL, whereas the dose of secondary IL-1β used to secondarily stimulate IFN-γ pre-activated MSCs in isolated culture was on the order of 10^3 pg/mL (Figure 3.9B). Therefore to determine if enhanced PGE2 secretion from IFN-γ pre-activated MSCs is dose dependent, these MSCs in isolated culture were secondarily exposed to increasing doses of IL-1β (Figure 3.11B). Even at 10^1 pg/mL IL-1β, the secretion of PGE2 from IFN-γ pre-activated MSCs was still higher than that of MSCs without pre-activation.

IFN-γ has been reported to enhance TNF-α production from LPS-stimulated macrophages [33] and was also observed in our system in a dose-dependent manner (Figure 3.12A). It was additionally observed that ability of high levels of PGE2 may be impaired in the presence of high levels of IFN-γ (Figure 3.12B). The MSC/macrophage co-culture supernatants were then probed for

IFN-γ (Figure 3.12C). IFN-γ levels on the order of 10^1 pg/mL were only detected in IFN-γ pre-activated MSC conditions.

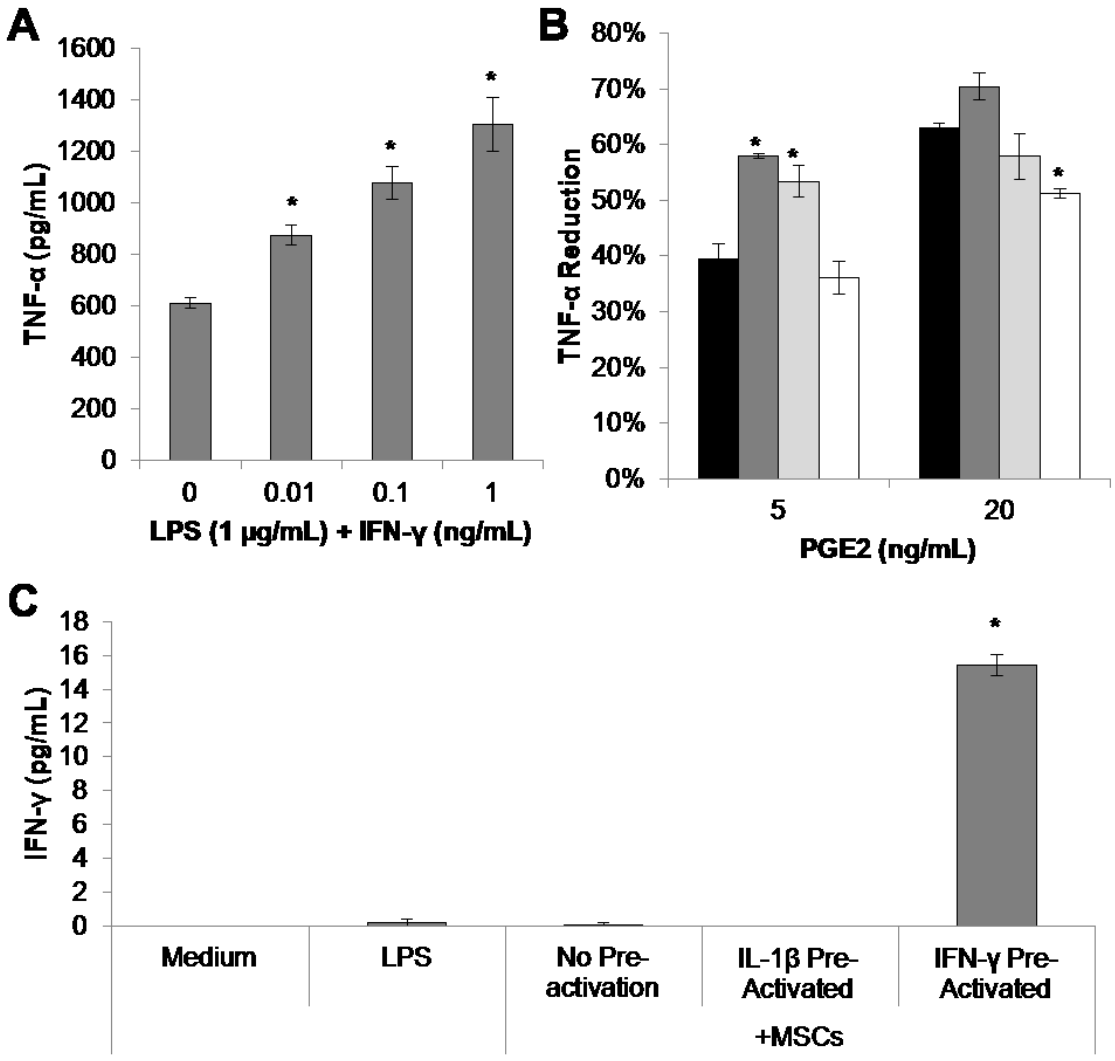

Figure 3.12 Effect of IFN-γ on macrophage TNF-α secretion and PGE2 action. (A) TNF-α was quantified in supernatants collected from macrophages stimulated with LPS and increasing concentrations of IFN-γ for 48 hours. (B) TNF-α was quantified in supernatants from macrophages cultured with LPS without (black bars) and with 0.01 (dark grey bars), 0.1 (light grey bars), or 1 (white bars) ng/mL IFN-γ, and PGE2 at 5 or 20 ng/mL.

(C) IFN-γ was quantified in 48 hour MSC/macrophage co-culture supernatants. Data are the mean ± SEM for (A) TNF-α level, (B) percent reduction in TNF-α level compared to corresponding baseline (0 ng/mL PGE2) levels, and (C) IFN-γ level for (A,B) n = 3 – 8 or (C) n = 9 replicates. *p<0.05 compared to LPS by (A, B) ANOVA and Fisher's LSD or (C) Student's *t* test.

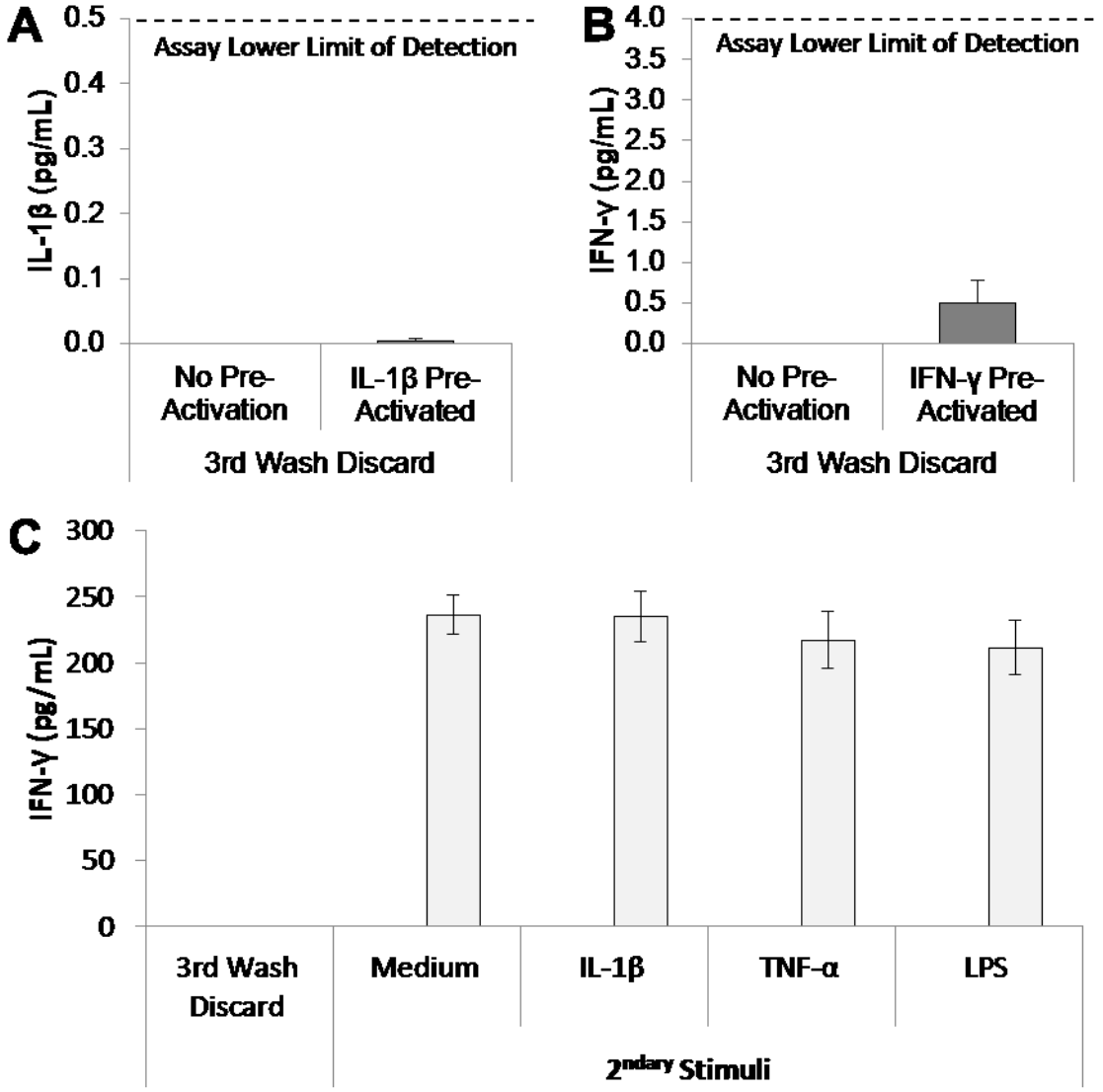

Figure 3.13 MSC secretion of IFN-γ. The discarded media from the third wash of the transwells containing (A) IL-1β or (B) IFN-γ pre-activated MSCs

were probed for residual activation factors. (C) IFN-γ was quantified in 24 hour isolated culture supernatants from MSCs without pre-activation (dark grey bars) or IFN-γ pre-activated MSCs (light grey bars) and the discarded media from the third wash after pre-activation. Data are the mean ± SEM for secreted levels for (A) n = 12, (B) n = 14, or (C) n = 9 replicates.

To determine if this was residual IFN-γ from the MSC pre-activation, the discarded third washes of the transwells were also probed for IFN-γ. IFN-γ was not detected at levels above the ELISA's lower limit of detection (Figure 3.13B), nor was IL-1β in the corresponding discarded third washes from IL-1β pre-activated MSCs (Figure 3.13A). To determine if the MSCs could be the source of the IFN-γ in the co-culture supernatants, IFN-γ was measured from IFN-γ pre-activated MSCs secondarily stimulated with medium, IL-1β, TNF-α, or LPS (as in Figure 3.9A) as well as the discarded media from the third wash after pre-activation (Figure 3.13C). While IFN-γ was not detected in the third wash discard or in supernatants from MSCs without pre-activation, significant levels were observed in supernatants from IFN-γ pre-activated MSCs. Secondary stimulation with another pro-inflammatory factor did not alter the levels of IFN-γ detected. When the corresponding supernatants from IL-1β pre-activated MSCs and discarded third washes were probed for IL-1β, none was detected (data not shown).

3.4 Discussion

In this chapter, we developed a pre-activation protocol for the enhancement of MSC-mediated modulation of macrophages by PGE2. We further

characterized the pre-activated MSCs after reintroduction in pro-inflammatory environments in isolated culture and in co-cultures with macrophages. We found that brief (1 hour) exposure of MSCs to IL-1β (1 ng/mL) induced PGE2 secretion that remained upregulated after pre-activation and was enhanced upon exposure to pro-inflammatory stimuli. IL-1β pre-activation enhanced MSC-mediated attenuation of macrophage TNF-α secretion in co-culture, simultaneous with increases in PGE2. Tests in animal models will be needed to determine if this enhancement will translate into therapeutic benefit.

The studies in chapter 2 and the dose response studies in the current chapter demonstrated that activating MSCs with LPS in combination with IL-1β results in synergistically enhanced PGE2 secretion. In the current pre-activation studies, using LPS together with IL-1β enhanced upregulation of PGE2 secretion post-activation, but this effect was only seen at the acute (6 hour) time point, and did not persist as time went on. Additionally, the use of LPS in a therapy developed for clinical use may not be ideal, as it is an endotoxin, difficult to remove, and may compromise the safety of the therapy even in trace amounts.

In addition to PGE2, we also observed that other elements of the MSC secretome, specifically IL-6, IL-8, and MCP-1, exhibited sustained upregulation after pre-activation with IL-1β. IL-6 is constitutively produced by MSCs and is also highly inducible by factors activating the nuclear factor (NF)-κB pathway. The role of IL-6 is pleiotropic, exhibiting both pro-inflammatory and anti-inflammatory functions [34-36]. MSC secreted IL-6 has been reported to prevent the maturation of dendritic cells [37] but also to prevent apoptosis of lymphocytes

and neutrophils [38]. Similarly, IL-8, which is not constitutively expressed but is also highly inducible, is a chemokine which primarily attracts neutrophils [39]. MCP-1 is also a chemokine, attracting monocytes and macrophages [40]. These potentially pro-inflammatory functions speak to the possibility of off-target side effects described in chapter 1. However, macrophages are first responders directing the action of other immune and inflammatory cells, therefore increased recruitment of monocytes and immune cells may be offset by MSC-mediated modulation of macrophages to promote anti-inflammatory behavior by factors such as PGE2 and IL-1RA, which was also induced and remained upregulated 6 hours post-activation. This was evident in a mouse model of sepsis, where prophylactic delivery of MSCs enhanced the ability of the immune system to clear the bacterial pathogens and then resolve inflammatory processes that would otherwise become exacerbated [41].

The IL-1β pre-activation protocol produced a population of MSCs that was more sensitive to secondary inflammatory stimuli with respect to enhanced PGE2 secretion. IL-1β activates several intermediate intracellular signaling molecules including mitogen-activated kinases (MAPKs) which lead to activation of transcription factors activator protein 1 (AP-1) and NF-κB [42]. Transcriptional activity of these factors increases expression of cyclooxygenase-2 (COX-2) and subsequent enzymatic production of PGE2 [43]. The secondary stimuli TNF-α and LPS also activate some of the same pathways as IL-1β via different receptors, thereby perhaps additively increasing the activity of their overlapping pathways [44, 45]. PGE2 secretion from IL-1β activated MSCs was also

enhanced by secondary exposure to IFN-γ, a type II interferon, which signals primarily through signal transducer and activator of transcription 1 (STAT1). IFN-γ has also previously been reported to increase COX-2 expression and secretion of PGE2 from MSCs [6, 46]. The promotor region for *COX2* contains binding sites for both STAT1 and NF-κB [47]. Conflicting data has been reported regarding the interaction of STAT1 with NF-κB and COX-2 [48] and the effect of IFN-γ on COX-2 activity and PGE2 secretion [49] in different cell types. In chapter 2 we observed that the presence of IFN-γ with IL-1β for 48 hours antagonized IL-1β-induced PGE2 secretion. However, several studies have shown that IFN-γ activated STAT1 can synergize with NF-κB activated by molecules including LPS, TNF-α, and IL-1 [50, 51]. This could explain the enhanced secretion of PGE2 that was also seen from IFN-γ pre-activated MSCs after secondary exposure to IL-1β, TNF-α, or LPS. Held et al. reported that IFN-γ priming of RAW 264.7 cells for 30 minutes resulted in increased NF-κB binding to DNA when secondarily stimulated with IL-1 or TNF-α [52]. The dramatic increase observed for secondary exposure to IL-1β in our results could be due to “head-start” elicitation of STAT1 combined with the already high potency of IL-1β in inducing PGE2. Apparent conflict with our previous data may suggest a time- or sequence of exposure-dependent relationship.

Pre-activation with IL-1β or IFN-γ and then secondary stimulation with the same cytokine did not enhance PGE2 secretion compared to the response of MSCs with no pre-activation to those factors. The same was true for pre-activation with LPS or TNF-α. This suggests autocrine regulatory mechanisms

that dampen the activity of stimuli over time. IL-1β has several known regulatory mechanisms. Attenuation of the response to IL-1β could be the result of a combination of decreased of expression of the signaling receptor (IL-1R1) or its accessory protein (IL-1RAcP), increased expression of the non-signaling receptor (IL-1R2), and induced expression of IL-1RA [53]. We observed that IL-1RA was induced by IL-1β during the 1 hour activation period and 6 hours post-activation. IFN-γ can negatively regulate its activity decreasing recycling and surface expression of its receptor (IFNGR1), dephosphorylation of intracellular signaling intermediates, and induction of suppressor of cytokine signaling 1 (SOCS-1), which inhibits signaling by interfering with janus kinase 1/2 (JAK1/2) [50]. These mechanisms could also hold the key to why 1 hour of pre-activation was optimal. In addition to dilution of PGE2 induction by cell proliferation, extension of the pre-activation duration to 6 and 24 hours may allow time for autoregulatory mechanisms to reach their peak effect. Outside of the scope of the main goal of developing an optimal pre-activation protocol, it would be interesting to explore this hypothesis by testing how these autoregulated cells respond to secondary stimuli. This type of characterization sheds light not only on what phenotypic changes the pre-activation factor induces, but also how the cells might react after reintroduction into an inflammatory microenvironment, where they will be imparting their therapeutic benefits. Current pre-activation studies in the literature often stop at characterizing the cells only at the end of the pre-activation period and therefore may miss the opportunity to detect unexpected phenotypic changes.

The results discussed above were observed in isolated cultures of MSCs manipulated with a single molecule at a time in order to tease out specific effects. When IL-1β pre-activated MSCs were tested in the more complex, dynamic macrophage co-culture assay, we observed that they enhanced MSC-mediated attenuation of macrophage TNF-α secretion, as predicted based on the PGE2 results in isolated culture. The supernatants from these co-cultures also contained more PGE2. In tangentially continuing to explore the unexpected results of IFN-γ pre-activation, we saw that the promising observation of dramatically enhanced PGE2 secretion after IL-1β stimulation of IFN-γ pre-activated MSCs did not translate into enhanced performance in the co-culture assay. IFN-γ pre-activation of the MSCs resulted in higher TNF-α levels and lower PGE2 content compared to MSCs with no pre-activation. Interestingly, low levels of IFN-γ were detected only in co-cultures containing IFN-γ pre-activated MSCs. As discussed above and seen in our results, IFN-γ enhances macrophage TNF-α secretion in response to LPS [50]. Therefore the increased TNF-α in macrophage co-cultures with IFN-γ pre-activated MSCs may be explained by the presence of IFN-γ in those conditions.

The MSCs could potentially be the source of the IFN-γ, as IFN-γ was not detected in LPS-activated macrophage supernatants while secretion of IFN-γ was observed from isolated cultures of IFN-γ pre-activated MSCs. It is possible that, although IFN-γ was not detected in the third wash discards, the IFN-γ detected in supernatants from IFN-γ pre-activated MSCs is residual from the activation process. The probability of this is weakened by the lack of detection of

IL-1β in both third wash discards and supernatants from in IL-1β pre-activated MSCs.

The presence of IFN-γ in the macrophage supernatants does not, however, explain why the PGE2 levels in those conditions were lower than expected, since PGE2 secretion was still enhanced from IFN-γ pre-activated MSCs even at low concentrations of secondary IL-1β. This may tie back to the results from chapter 2 mentioned above, where we observed that the presence of IFN-γ with IL-1β for 48 hours slightly antagonized IL-1β-induced PGE2 secretion, although the concentrations of those factors were much higher there. This is also complicated by the bolus versus dynamic reintroduction of MSCs to pro-inflammatory mediators. Analysis of the macrophage secretome and the secretome of isolated cultures of MSCs with secondary stimulation by cocktails of macrophage secreted factors or macrophage conditioned medium may shed additional light on the response of IFN-γ pre-activated MSCs to more complex inflammatory environments. If the observed phenomena are true, this IFN-γ MSC pre-activation protocol, while not optimal for reducing macrophage mediated inflammation, could be useful in treating cancer, as it has been reported that MSCs genetically engineered to secrete IFN-γ exerted anti-tumor effects in a nude mouse model of lung carcinoma by persistent activation of TNF-related apoptosis-inducing ligand (TRAIL) [54].

In conclusion, working off the foundation laid in chapter 2, we further optimized an IL-1β MSC pre-activation protocol to enhance immunomodulatory properties. In addition to this deliverable, our parallel exploration of IFN-γ pre-

activation demonstrated that using a single metric in isolated cultures may be insufficient to predict performance in a more complex, dynamic microenvironment. This has implications for suggestions in the field to use IFN-γ induction of indoleamine 2,3-dioxygenase (IDO) as a single metric to characterize MSC potency. While this may be predictive in treating diseases dominated by T cell responses, our approach to link activation mechanisms with functional outputs can be tailored to develop disease-specific potency assays.

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Chapter 4: MSC Donor Variability

Note: We would like to acknowledge and thank Alec Lee and Extem Bioscience Corporation for collaboration and donation of cells.

4.1 Introduction

Although contested [1], MSCs have generally been viewed as non-immunogenic and therefore are being developed as both autologous and allogenic cell therapies [2]. This paradigm has resulted in the harvest and use of MSCs from many donors, providing they meet the International Society for Cellular Therapy (ISCT) minimal criteria for MSCs (i.e. adherence to tissue culture plastic, surface marker expression, and tri-lineage differentiation) [3, 4]. Additional criteria for screening potential donors include lack of adventitious agents and satisfactory medical history. These criteria, however, have no bearing on the functionality of the cells. Additionally, the current approaches to donor selection communicate an assumption that all MSCs are equipotent. This is a serious oversight of well-documented donor-to-donor and intra-donor variability [5, 6].

MSC proliferation, multipotency, and viability have been shown to vary amongst donors based on age and cell source (e.g. bone marrow, adipose tissue, placenta, umbilical cord, etc.) [7-10]. MSCs from different donors have also been reported to vary in their response to different *in vitro* culture conditions [11], which has important consequences for the development of MSC therapies due to low prevalence in source tissue and subsequent requirement for massive *ex vivo* expansion and cryopreservation [12, 13].

Variability has also been observed in MSC secretion, response to inflammatory stimuli, and immunomodulation, which are the basis of several proposed therapeutic actions of MSCs [14, 15]. Therefore generation of many doses from only a few donors in the “universal donor” or “off-the-shelf” models results in a potency bias that could potentially make or break the results of a clinical trial [16].

It is clear that focus needs to be put on determining the suitability of MSCs from different donors for use in pre-clinical and clinical evaluations. Towards this goal, there are efforts to create an MSC reference material and to develop potency assays to identify more responsive cells based on immunophenotype [16-19]. In this chapter, we characterize the performance of the MSC pre-activation protocol developed in chapters 1 and 2 for 6 MSC donors using key metrics identified through that work. Specifically, we examine variability in the effect of pre-activation on MSC PGE2 secretion and modulation of macrophage TNF-α secretion in a co-culture assay. Our results demonstrate the need for both isolated culture and functional assays in determining the potency of MSCs from different donors. They additionally show the utility of our protocol as a means of enhancing MSC functions and present a potential potency assay to screen and select suitable MSC donors.

4.2 Materials and Methods

4.2.1 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 was purchased from InvivoGen (San Diego, CA).

4.2.2 Cell Culture

All cells were maintained in a humidified 37°C incubator with 5% CO_2 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.

Table 4.1 MSC donor information provided by the supplier.

					Surface Marker (% Positive)								
Donor ID	Supplier	Sex	Age	Tri-lin. Diff.	CD44	CD73	CD90	CD105	CD166	CD14	CD34	CD45	HLA-DR
1	Texas A&M	M	28	✓	99.20%	99.13%	99.27%	99.27%	99.39%	0.06%	0.14%	0.14%	3.18%
2	Texas A&M	F	29	✓	93.97%	97.56%	96%	99.04%	97.97%	0.05%	0.13%	0.18%	1.48%
3	Texas A&M	M	22	✓	99.51%	99.99%	100%	100%	100%	0.28%	0.04%	0.67%	1.51%
4	Texas A&M	F	23	✓	99.28%	99.99%	99.97%	99.99%	99.97%	0.01%	0.03%	0.31%	1.18%
5	Extem, Inc.	M	28	✓	97%	100%	100%	100%	100%	<1%	<1%	<1%	<1%
6	Extem, Inc.	F	27	✓	99%	100%	100%	100%	98%	<1%	<1%	<1%	<1%

4.2.3 MSCs

Human bone marrow-derived MSCs were purchased at passage 1 from the Institute for Regenerative Medicine (donors 1 – 4, Texas A&M College of Medicine, Temple, TX) or donated at passage 2 from Extem, Inc. (donors 5 – 6, San Francisco, CA). Donor information is summarized in Table 4.1. 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 – 3 were thawed and seeded as a monolayer culture in 175 cm^2 flasks at $1.5x10^4$ cells/mL. They were cultured in MEM-α

containing no deoxy- or ribonucleosides, supplemented with 10% FBS (Atlanta Biologics, Flowery Branch, GA) and 1 ng/mL basic fibroblast growth factor (bFGF). The cells were grown to 70% confluence, detached with trypsin, seeded into 48-well plates at $3x10^3$ cells/well or transwell inserts, as required for specific experiments, and allowed to attach overnight. Experiments were set up using MSCs at passage 3 – 5 and fully supplemented RPMI 1640 medium.

4.2.4 Macrophages

$CD14^+$ monocytes were isolated from human peripheral blood from a healthy adult female donor (New York Blood Center, Long Island City, NY), differentiated to generate macrophages, as described previously [20], and cryopreserved. Macrophages at passage 1 were thawed, seeded into 24-well plates and allowed to attach overnight.

4.2.5 Cell Counting

Cell culture supernatants were collected, stored at -80°C, and replaced by fully supplemented RPMI 1640 medium containing Alamar blue (Molecular Probes, Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. In parallel, a standard curve was created by seeding MSCs into 48-well plate at 3 $x10^3$, 6 $x10^3$, 1.2 $x10^4$, or $2.4x10^4$ cells/well, allowed to attach overnight, and then cell culture medium was replaced with fully supplemented RPMI 1640 medium containing Alamar blue. Fluorescent Alamar blue readings were recorded after 4 hours of incubation using a microplate reader (DTX 880 Multimode Detector, Beckman Coulter, Fullerton, CA, USA), and the standard curve was used to calculate cell number.

4.2.6 MSC/Macrophage Co-Culture

Macrophages at passage 1 were thawed, washed in fully supplemented Advanced RPMI 1640 medium, seeded into 24-well plates at $5x10^4$ cells/well, and allowed to attach overnight. MSCs were seeded into 24-well transwell inserts (0.4 μm pore size polyester membrane, Corning, Lowell, MA) at $5x10^4$ cells/transwell and allowed to attach overnight. MSC cell culture medium was replaced with fully supplemented RPMI 1640 medium with activation factors for 1 hour. After thoroughly washing with fresh medium 3 times, fully supplemented RPMI 1640 medium with LPS (1 μg/mL) was added to the transwell apical chamber. Macrophage cell culture medium was replaced with fully supplemented RPMI 1640 medium with or without LPS (1 μg/mL) and MSC transwell inserts were added to LPS conditions. Basolateral supernatants were collected after 48 hours and frozen at -80°C.

4.2.7 Cytokine Measurement

Cell culture supernatants were thawed and analyzed using an enzymatic immunoassay for PGE2 (Cayman Chemical, Ann Arbor, MI, USA) and enzyme-linked immunosorbent assays (ELISAs) for TNF-α, IL-1β, and IFN-γ (Biolegend, San Diego, CA), according to the manufacturer's instructions. Absorbances were recorded using microplate readers and data was analyzed in Matlab software version R2013b (MathWorks, Natick, MA) or Microsoft Excel (Microsoft, Redmond, WA).

4.2.8 Statistical Analysis

Data points represent the mean ± standard error of the mean (SEM) for the indicated number of independent observations (n). Statistical differences between the conditions were determined using analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) *post hoc* analysis in KaleidaGraph software version 4.1 (Synergy Software, Reading, PA, USA). P values <0.05 were considered significant. To determine correlation between PGE2 and TNF-α, the data was first linearized using the Box Cox power transformation (λ). Pearson's correlation coefficients (r) were then calculated and scatter plots were generated using MiniTAB Statistical Software version 17 (Minitab Inc., State College, PA). Correlations between age and PGE2 or TNF-α were calculated and scatter plots were generated in Microsoft Excel. Two-tailed *t* tests-based *post hoc* power analysis with α = 0.05 was performed on the metrics for the set of donors using the difference between 2 dependent means for matched pairs in G*Power software version 3.1.9.2 (University of Kiel, Kiel, Germany [21]).

4.3 Results

4.3.1 Response to Secondary Pro-inflammatory Stimuli

The secretory response, upon reintroduction into a simulated pro-inflammatory environment after pre-activation, was assessed for MSCs derived from 6 healthy adult donors. MSCs were pre-activated with IL-1β for 1 hour and then supernatants were replaced with fresh medium without or with IL-1β, TNF-α, IFN-γ, or LPS for 24 hours (Figure 4.1A). IFN-γ pre-activated MSCs were also

tested due to the interesting observation of the enhanced PGE2 secretion, particularly after secondary IL-1β stimulation, as described in chapter 3.

The major trends observed for donor 1, as described in chapter 3, were similar for the other donors (Figure 4.1B). Specifically, all donors exhibited upregulated PGE2 secretion after pre-activation with IL-1β or IFN-γ. Secretion of PGE2 was enhanced to variable degrees for IL-1β pre-activated MSCs in response to IFN-γ, TNF-α, or LPS and for IFN-γ pre-activated MSCs in response to IL-1β TNF-α, and LPS. Upregulation of PGE2 for IFN-γ pre-activated MSCs in response to IL-1β was most dramatic for donors 1 and 2, followed by donors 4 and 5. This phenomenon was not as pronounced for donor 3. Generally, IL-1β or IFN-γ pre-activated MSCs were not much more sensitive to the same secondary stimuli compared to MSCs without pre-activation.

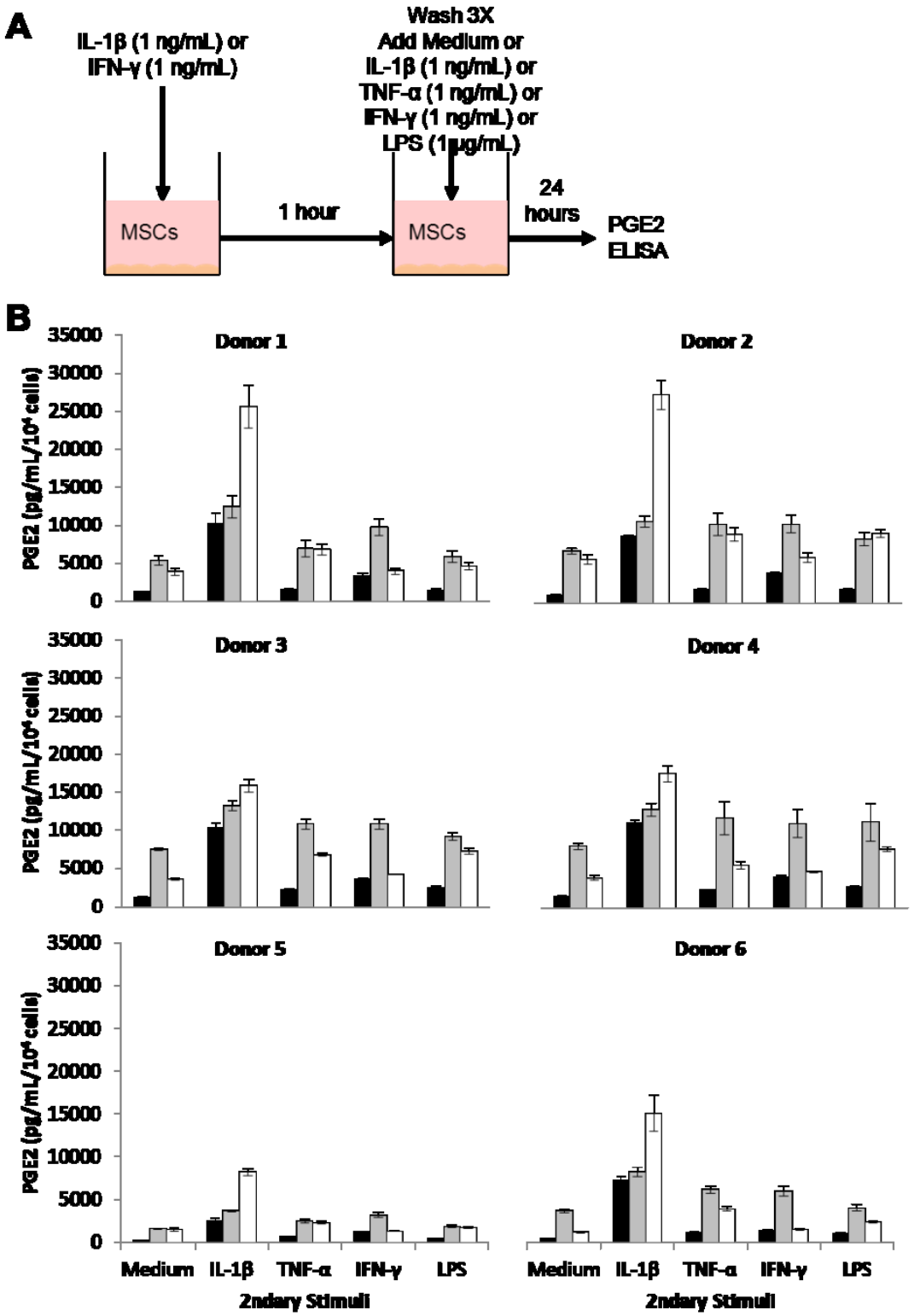

Figure 4.1 Response of pre-activated MSCs from multiple donors to secondary pro-inflammatory stimuli. (A) MSC culture medium was replaced

by medium without (black bars) or with IL-1β (grey bars) or IFN-γ (white bars) for 1 hour. (B) PGE2 was quantified 24 hours after pre-activated cell culture supernatants were replaced by fresh medium without or with IL-1β, TNF-α, IFN-γ, or LPS. Data are the mean ± SEM for PGE2 normalized to cell number for n = 3 (Donors 2 – 6) or n = 9 (Donor 1) replicates.

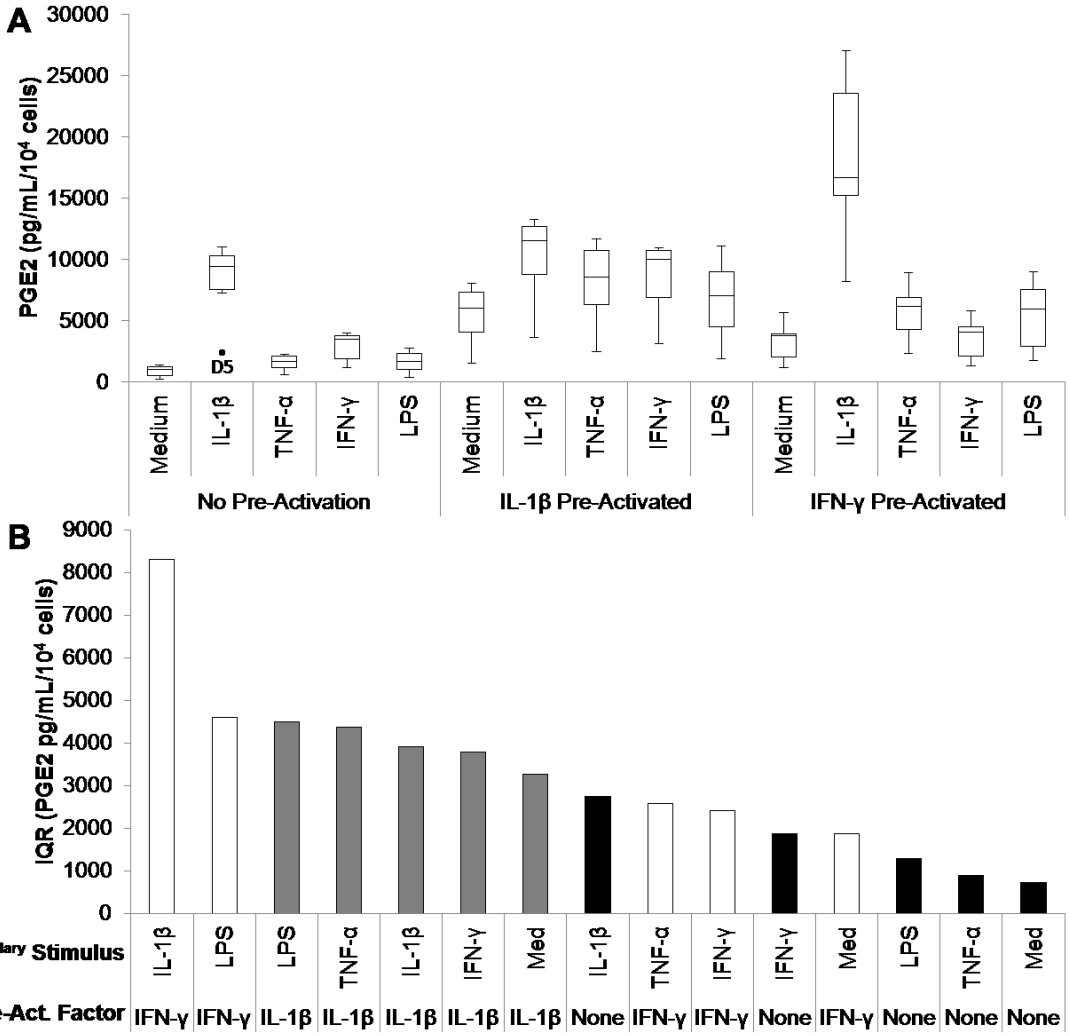

Figure 4.2 Donor variability in pre-activated MSC response to secondary pro-inflammatory stimuli. (A) Box and whisker plots display the minimum, first quartile, median (second quartile), third quartile, maximum, and outliers in the normalized PGE2 level secreted by the donors for each

activation condition. (B) The interquartile range (IQR) is the difference between the first and third quartile and is plotted for each activation condition in the order of highest to lowest (black bars are no pre-activation; grey bars are IL-β pre-activated; white bars are IFN-γ pre-activated).

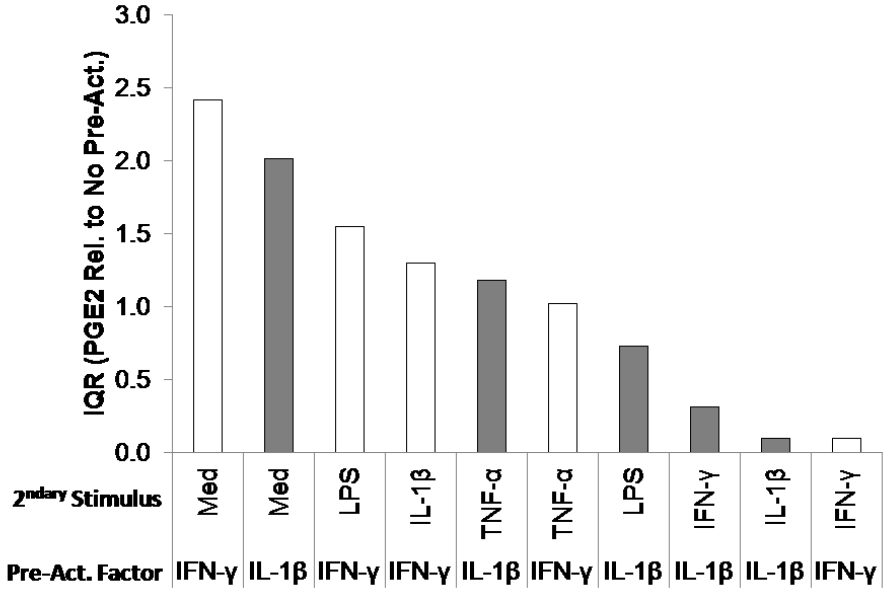

Figure 4.3 Donor variability in fold change of PGE2 secretion from pre-activated MSCs in response to secondary pro-inflammatory stimuli. The interquartile range (IQR) is the difference between the first and third quartile and is plotted for the fold change in PGE2 compared to no pre-activation for each activation condition in the order of highest to lowest (grey bars are IL-β pre-activated; white bars are IFN-γ pre-activated).

Although trends were fairly consistent, the quantitative levels of secreted PGE2 varied noticeably amongst the donors. This is particularly apparent when comparing donor 5 to donors 1 or 2. To obtain a condensed view of the donor variability in these results, a box and whisker plot was generated for the donor pool (Figure 4.2A). MSCs without pre-activation had the least amount of

variability in PGE2 levels across the donors constitutively and in response to pro-inflammatory stimuli, despite the extreme low value observed for donor 5 in response to IL-1β. After pre-activation, the variability in PGE2 secretion amongst the donors increased. To more quantitatively describe the variability, the interquartile range (IQR) was calculated for each condition and then plotted in order from highest to lowest (Figure 4.2B). Larger IQRs indicate higher variability. The largest IQR was observed for PGE2 from IFN-γ pre-activated MSCs in response to IL-1β. IL-1β pre-activated conditions had similar IQRs, which clustered together towards the higher end. As was seen in the box and whisker plots, the three lowest IQRs were observed for MSCs without pre-activation.

The fold changes in PGE2 compared to no pre-activation were also calculated to determine if the relative upregulation of PGE2 was similar amongst the donors, simply at different quantitative levels. The IQRs for this metric varied greatly amongst the activation conditions, with both the highest and lowest IQRs being observed for IFN-γ pre-activated MSCs (Figure 4.3). This indicates that the degree of upregulation in addition to the quantitative PGE2 levels varied amongst donors in an activation condition-dependent manner.

4.3.2 Pre-Activated MSC Modulation of Macrophage TNF-α

The performance of the IL-1β pre-activation protocol as well as IFN-γ pre-activation across the set of donors were next evaluated in the MSC/macrophage co-culture assay described in chapter 3 (Figure 4.4A). All donors were tested using a single macrophage donor, to prevent convolution of the results by macrophage donor variability.

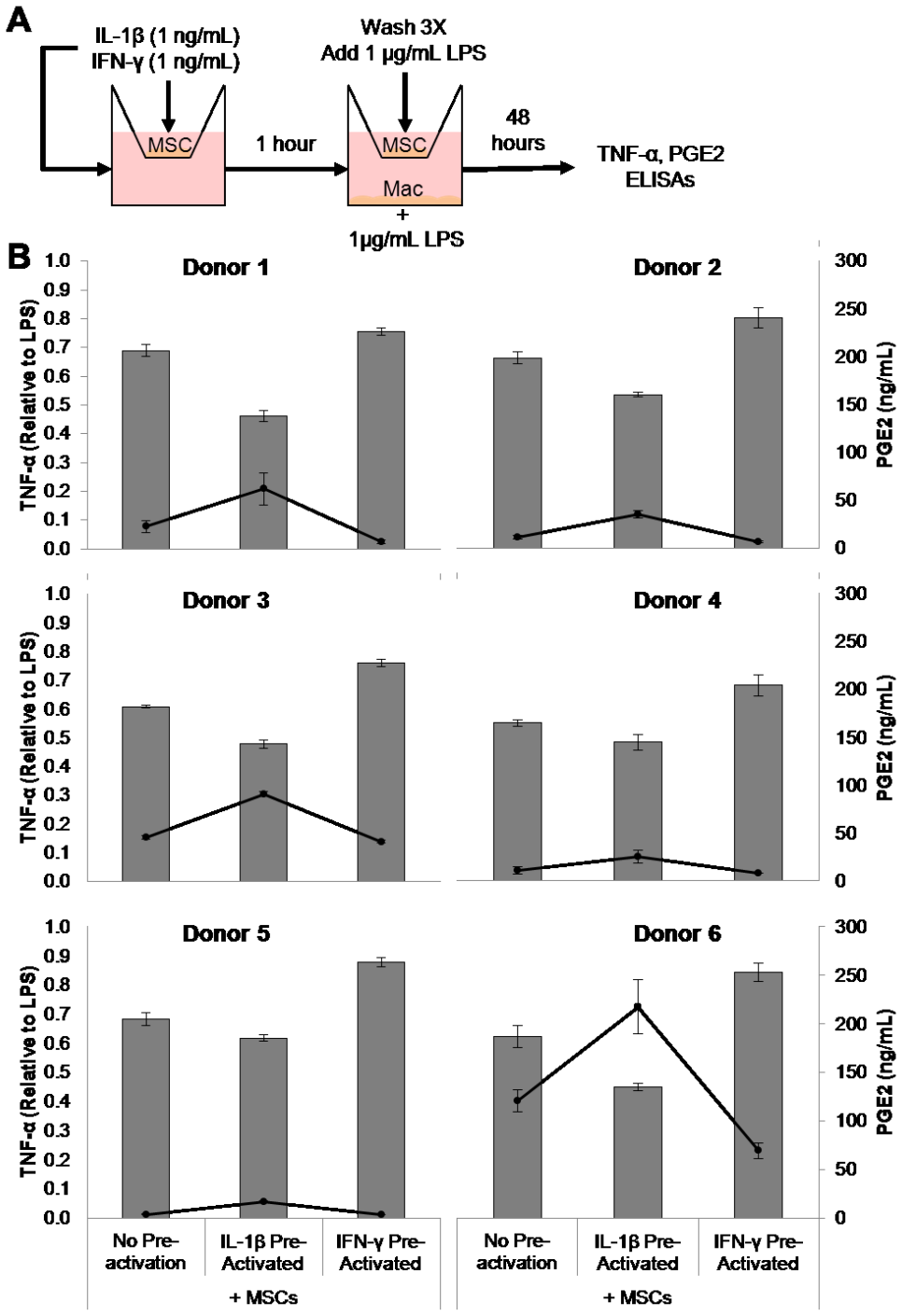

Figure 4.4 Pre-activated MSC modulation of macrophages. (A) MSC transwell culture medium was replaced by medium without or with IL-1β or IFN-γ for 1 hour, and then transwells were washed and transferred to macrophage cultures with LPS for 48 hours. (B) Co-culture supernatants

were analyzed for TNF-α (grey bars) and PGE2 (black circles/line). Data are the mean ± SEM for secreted level the LPS control for n = 3 (Donors 2 – 5) or n = 9-19 (Donor 1) replicates.

MSCs from all 6 donors were able to significantly reduce TNF-α without pre-activation, with MSCs from donor 4 achieving the greatest baseline attenuation (Figure 4.4B), and similarly trended towards further reduction with IL-1β pre-activation and diminished attenuation with IFN-γ pre-activation. PGE2 levels present in the co-culture supernatants varied greatly amongst the donors, but all trended toward higher PGE2 in IL-1β pre-activated conditions and lower PGE2 in IFN-γ pre-activated conditions, compared to no pre-activation. This variability was again visually condensed using box and whisker plots. The variability in relative PGE2 levels was relatively large, with donor 6 exhibiting extreme highs (Figure 4.5B). Despite this, variability in the relative TNF-α level was lower (Figure 4.5A).

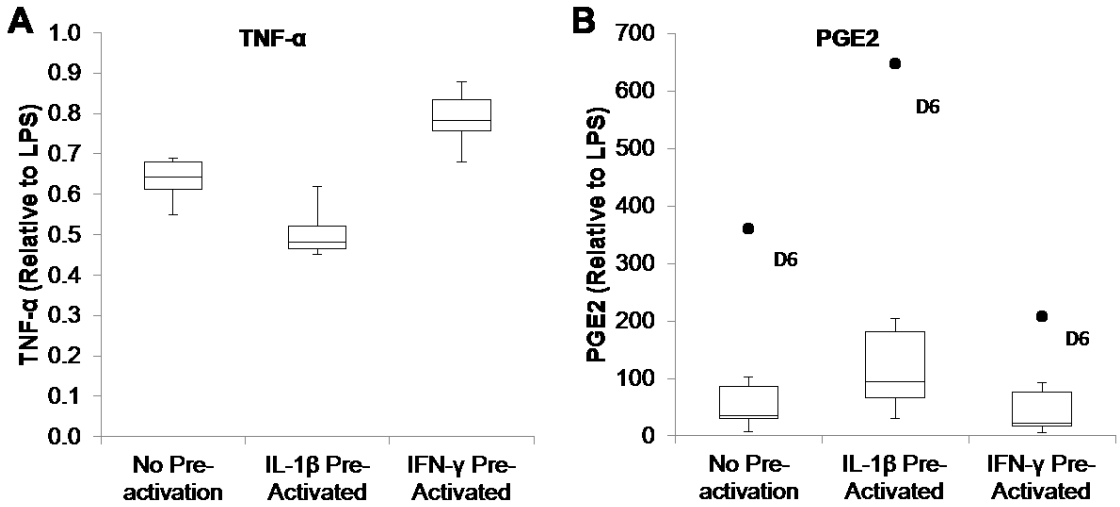

Figure 4.5 Donor variability in pre-activated MSC modulation of macrophages. Box and whisker plots display the minimum, first quartile,

median (second quartile), third quartile, maximum, and outliers in the (A) TNF-α level (normalized to LPS) and (B) PGE2 (normalized to LPS) present in the co-cultures for each activation condition.

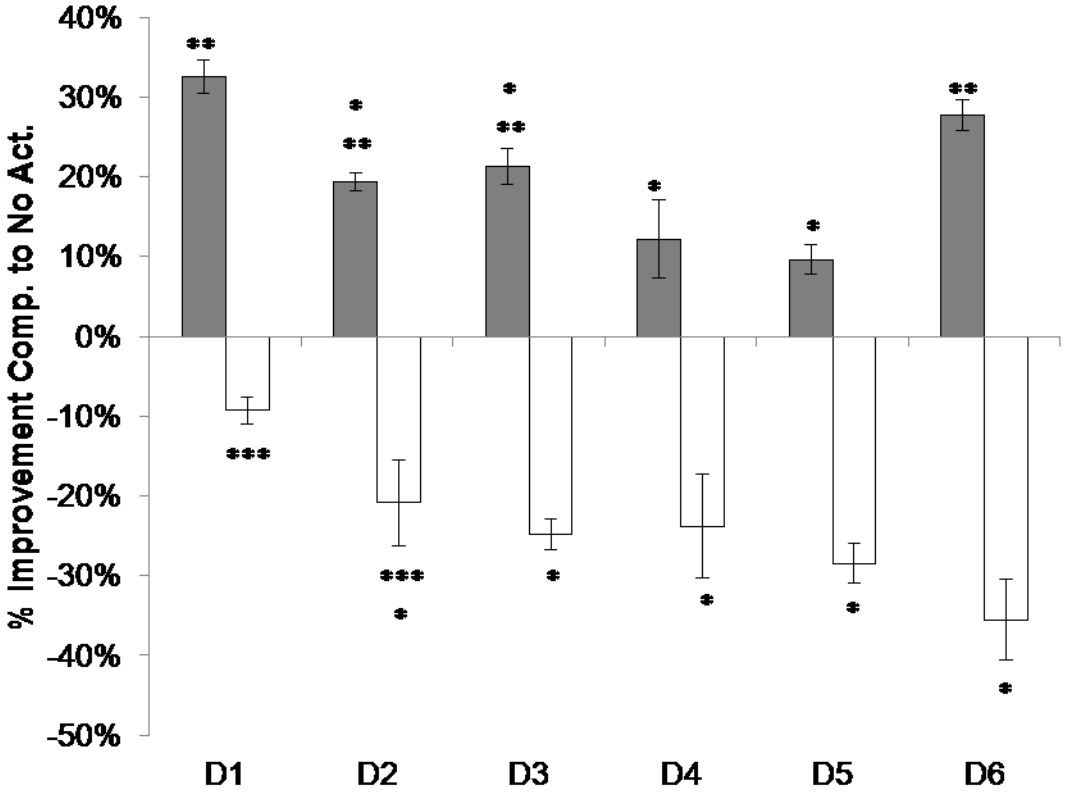

Figure 4.6 Comparison of percent improvement in TNF-α reduction imparted by pre-activation. Improvement in attenuation of TNF-α was determined using the percent change in relative TNF-α level for IL-1β pre-activated (grey bars) and IFN-γ pre-activated (white bars) MSC conditions compared to no pre-activation for each donor. Negative values indicate impaired attenuation of TNF-α. *p<0.05 compared to donor 1, **p<0.05 compared to donor 5, ***p<0.05 compared to donor 6 by ANOVA with Fisher's LSD.

To further tease out the performance of each donor, the improvement in macrophage modulation was determined by calculating the percent change in relative TNF-α level for IL-1β pre-activated and IFN-γ pre-activated MSC conditions compared to no pre-activation (Figure 4.6). MSCs from donors 1 and 6 had similar levels of improvement with IL-1β pre-activation which were the highest in the group. MSCs from donor 5, and similarly donor 4, exhibited the least amount of improvement after pre-activation with IL-1β. Donor 1 MSCs also exhibited the least amount of diminished macrophage modulation after pre-activation with IFN-γ. MSCs from donors 3 – 5 and particularly donor 6 showed the largest impairment of macrophage modulation after pre-activation with IFN-γ.

4.3.3 Correlation of TNF-α and PGE2

PGE2 is known to be a potent modulator of macrophage TNF-α secretion [22]. We have previously shown that PGE2 reduces macrophage TNF-α secretion in a dose dependent manner [23]. In the macrophage co-culture assay, similar levels of TNF-α reduction were observed despite large variations in the PGE2 levels. To detect differences amongst the donors in the relationship between PGE2 and TNF-α, we calculated the linear correlation of these two metrics after mathematical transformation of the data (Figure 4.7). The relationship between TNF-α and PGE2 was negative for all donors and statistically significant, with the exception of donor 4. The strength of these relationships, however, indicated by the absolute value of the correlation coefficients, varied amongst the 6 donors. MSCs from donor 6 had the steepest negative correlation between TNF-α and PGE2 while donor 1 had the lowest.

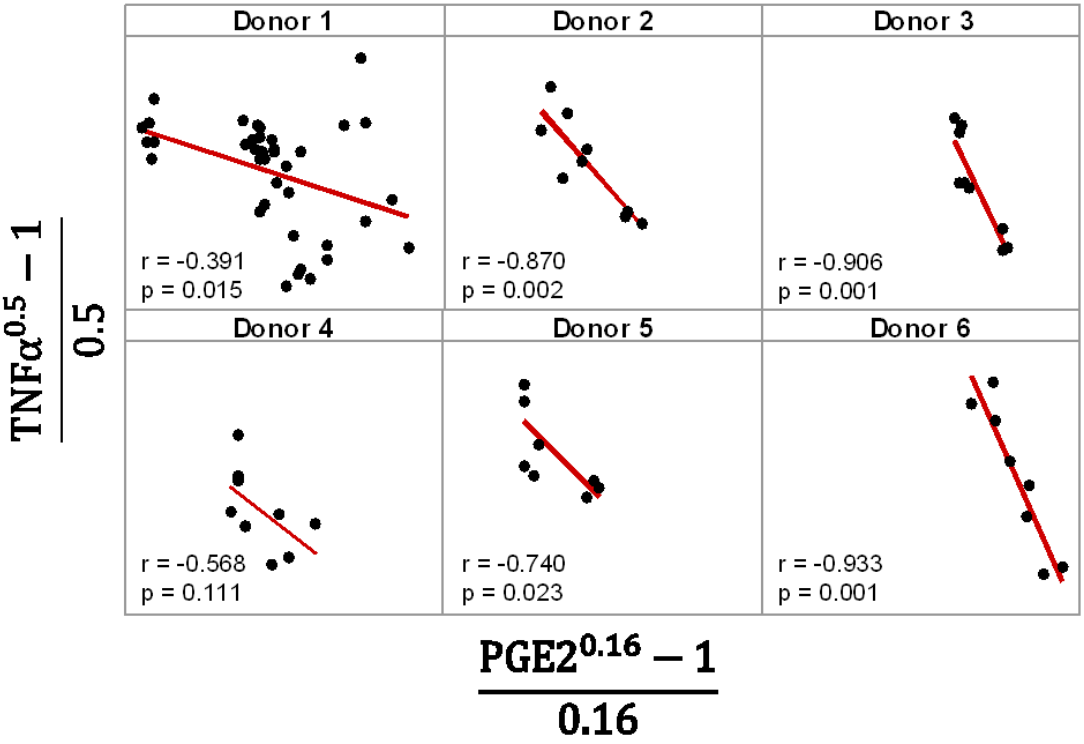

Figure 4.7 Correlation of PGE2 and TNFα for different MSC donors. PGE2 and TNF-α data for no pre-activation, IL-1β pre-activation, and IFN-γ pre-activation conditions (black circles) were linearized using the Box Cox power transformation and plotted against each other with a linear regression line (red line). Pearson's correlation coefficients (r) were calculated and their statistical significances (p) were determined using Student's *t* test. Each data point represents an experimental replicate.

4.1.1 Effect of Age and Sex on MSC Pre-Activation

We next determined if differences in age or sex correlated with any of the data. No significant correlations were observed for PGE2 secretion in response to pro-inflammatory stimuli after pre-activation (Figure 4.8) or relative TNF-α levels in MSC/macrophage co-culture supernatants (Figure 4.9). Similarly, no

significant differences were detected in these metrics between male and female donors (Figure 4.10 and Figure 4.11).

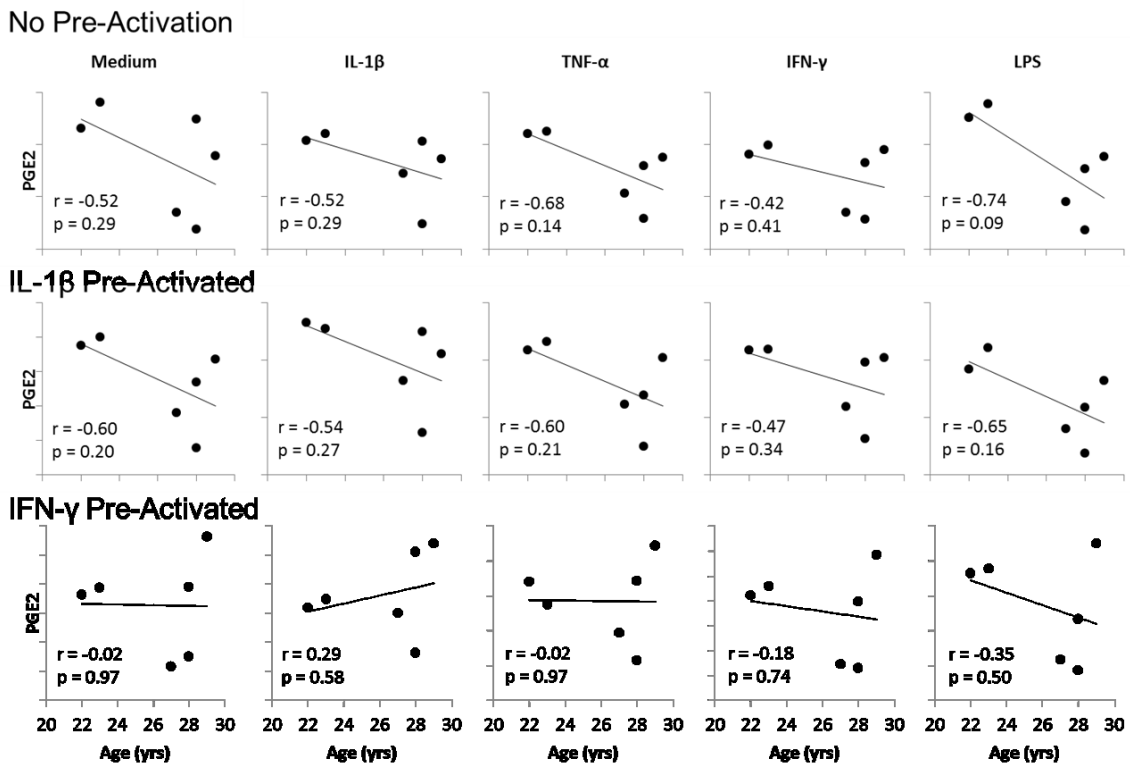

Figure 4.8 Correlation of donor age and PGE2 secretion in response to pro-inflammatory stimuli after pre-activation. PGE2 levels (black circles) were plotted against the age of the corresponding donor with a linear regression line (black line). Pearson's correlation coefficients (r) were calculated and their statistical significances (p) were determined using Student's *t* test. Each data point represents the average PGE2 secretion for a single donor.

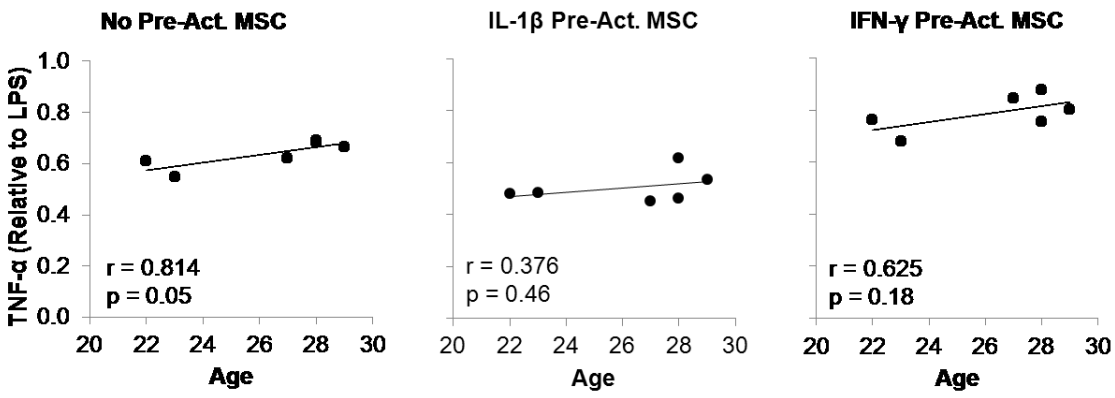

Figure 4.9 Correlation of donor age and relative TNF-α level in MSC/macrophage co-cultures. Relative TNF-α levels (black circles) were plotted against the age of the corresponding donor with a linear regression line (black line). Pearson's correlation coefficients (r) were calculated and their statistical significances (p) were determined using Student's *t* test. Each data point represents the average TNF-α level for a single donor.

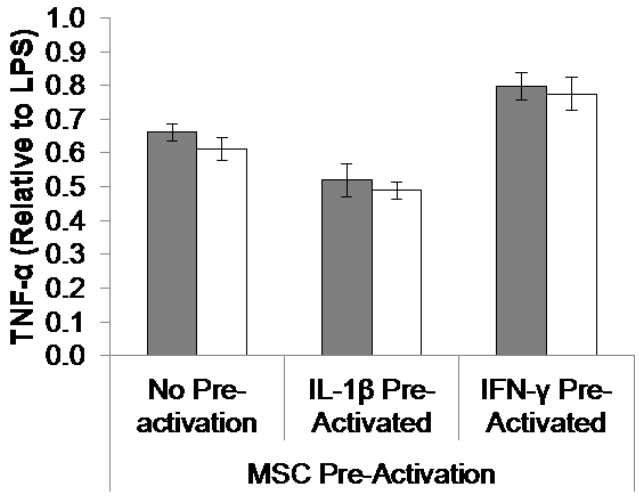

Figure 4.10 Comparison of relative TNF-α level in MSC/macrophage co-cultures between male and female MSC donors (grey bars and white bars, respectively). Data are the mean ± SEM for relative TNF-α level for equal numbers of male and female biological replicates (n = 3 each).

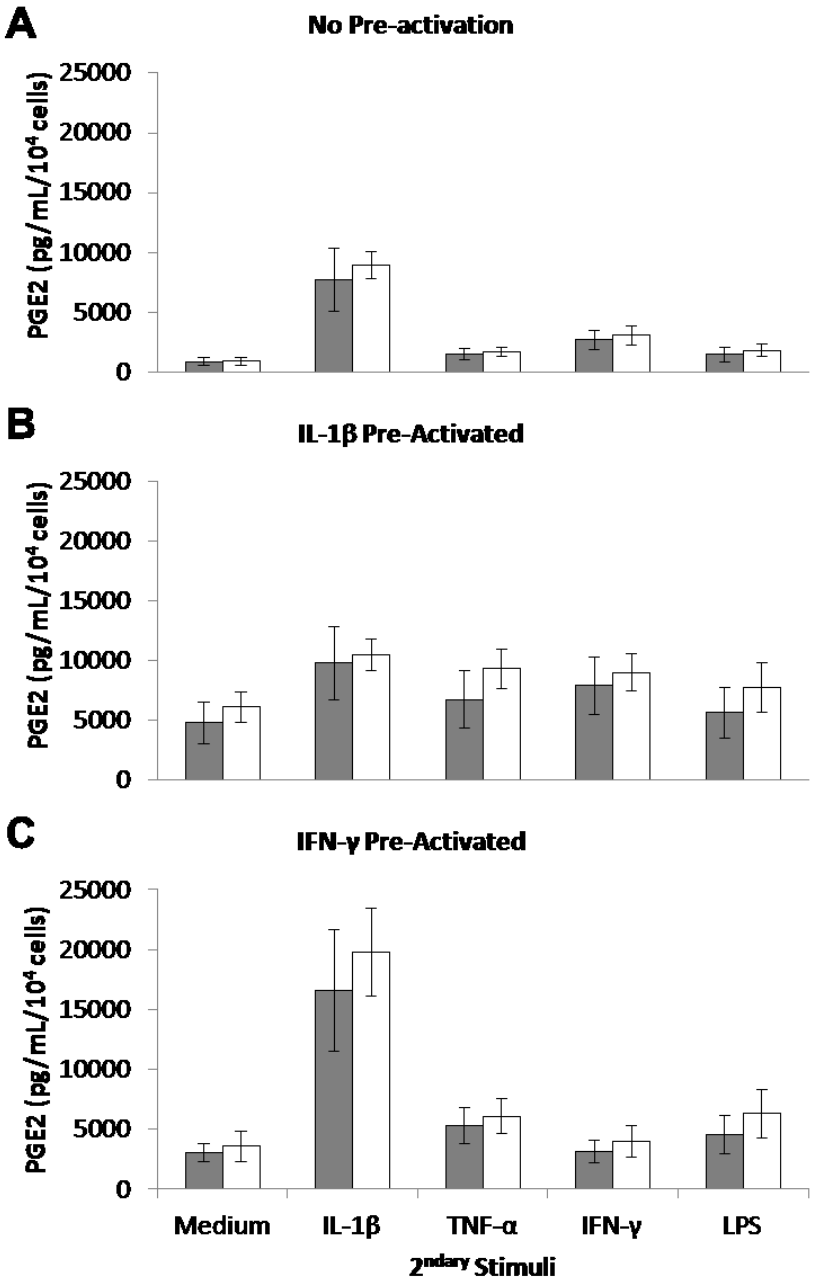

Figure 4.11 Comparison of PGE2 secretion in response to pro-inflammatory stimuli between male and female MSC donors (grey bars and white bars, respectively) after (A) no pre-activation, (B) IL-1β pre-activation, or (C) IFN-γ pre-activation. Data are the mean ± SEM for PGE2 normalized to cell

number for equal numbers of male and female biological replicates (n = 3 each).

4.3.4 Statistical Power

To determine the statistical power of the metrics used to test the performance of the successful IL-1β pre-activation protocol, power analyses were performed using the difference in means for pre-activation and no pre-activation averaged across the 6 donors used. The power of measuring the enhanced response of IL-1β pre-activated MSCs in response to secondary stimulation with TNF-α, IFN-γ, and LPS was calculated to be very high (Table 4.2), as was the power of detecting enhanced attenuation of macrophage TNF-α secretion (Table 4.3). Enhanced PGE2 content in the MSC/macrophage co-cultures had less statistical power (Table 4.4).

Table 4.2 Power analysis for PGE2 secretion in response to secondary stimuli after pre-activation with IL-1β for 6 biological replicates (MSC donors).

Effect	Mean of Difference	St. Dev. Of Difference	Effect Size	Power (1-β)
TNF-α-induced PGE2 levels	6471	2880	2.247	0.9903
IFN-γ-induced PGE2 levels	5615	2003	2.803	0.9996
LPS-induced PGE2 levels	5071	2546	1.992	0.9695

Table 4.3 Power analysis for the relative TNF-α level in macrophage co-cultures with MSCs for 6 biological replicates (MSC donors).

Effect	Mean 1	Mean 2	St. Dev. 1	St. Dev. 2	Correlation	Effect size	Power (1-β)
No Pre-Activation vs. IL-1β pre-activation	0.636	0.504	0.054	0.063	0.435	2.107	0.9814

Table 4.4 Power analysis for the PGE2 level in macrophage co-cultures with MSCs for 6 biological replicates (MSC donors).

Effect	Mean of Difference	St. Dev. Of Difference	Effect Size	Power (1-β)
No Pre-Activation vs. IL-1β pre-activation	38780	28440	1.364	0.7606

4.4 Discussion

In this chapter, we tested the IL-1β pre-activation protocol developed in chapters 2 and 3 on MSCs derived from 6 human donors. These donors consisted of equal numbers of healthy males and females between 20 and 30 years of age. The cells all met the ISCT minimum criteria for MSCs, but this does not guarantee equipotency. This was demonstrated in the variability observed amongst the donor set for the key metrics identified in chapter 3 (response to secondary stimuli after pre-activation and modulation of macrophage TNF-α secretion in co-culture). Due to the interesting results described in chapter 3, IFN-γ pre-activation was also tested in parallel.

In examining PGE2 secretion from the MSCs in response to secondary stimuli after pre-activation, we observed activation condition-dependent degrees of variability. The least amount of variability was seen when the MSCs were stimulated with inflammatory molecules without pre-activation. After pre-activation with either IL-1β of IFN-γ and exposure to these molecules, variability in the secreted PGE2 level and in the degree of PGE2 upregulation compared to no pre-activation was increased, with the highest variability being seen in IFN-γ pre-activated conditions. Differences between donors were also observed in the baseline (no pre-activation) levels of macrophage TNF-α attenuation and the

degree of enhancement or impairment of this function imparted by IL-1β or IFN-γ pre-activation, respectively. Performance in the isolated MSC culture assays did not consistently correlate with performance in the co-culture assay. MSCs from donor 5 and 6 had the lowest PGE2 secretion in the isolated culture assays. While MSCs from donor 5 also had the least improvement in macrophage TNF-α modulation after IL-1β secretion and the lowest co-culture PGE2 levels, MSCs from donor 6 exhibited one of the highest improvements and highest co-culture PGE2 levels. This again speaks to the need discussed in chapter 3 for both isolated culture and more complex assays in identifying suitable MSCs donors.

Due to the known effects of PGE2 on modulating macrophage TNF-α secretion [23], we examined the correlation between these two metrics in the co-culture assay for each donor. All relationships exhibited a negative correlation as expected, but the magnitude of these correlations varied amongst the MSC donors. This could indicate the presence of other factors in the co-culture affecting the secretion of TNF-α or the action of PGE2 on the macrophages. Other MSC-secreted factors have been shown to modulate macrophage phenotype, including interleukin 1 receptor antagonist (IL1RA), transforming growth factor (TGF)-β, and tumor necrosis factor stimulated gene 6 (TSG6) [24-27]. Therefore the active components with the co-cultures should be teased out with further experimentation.

We did not observe any correlation between our results and the age or sex of the donor. Observations of donor variability and lack of correlation between metric and sex are consistent with results reported in the literature [5, 28]. Age,

however, has been consistently reported to affect MSC proliferation and function [7-9]. The age ranges in those studies are much larger than the very narrow range of the donors used in our studies. Therefore future studies should include a wider range of donor ages.

While the donor pool in this study was relatively small, power analyses showed that the sample size was large enough to accurately detect changes in response to secondary stimuli and macrophage modulation after pre-activation with high statistical power. This analysis is affected, however, by several factors including the effect size [29]. The effect sizes in our metrics were large, meaning they could be noted in the data without extensive searching. Therefore while sufficient for this analysis, this effect size consideration also increases the motivation to expand the donor pool.

Additionally, the MSCs used in these studies were all derived from donors characterized as “healthy” by the supplier. There is conflicting evidence as to whether MSCs derived from disease or injured donors exhibit impaired functions compared to MSCs from healthy donors [30-39]. As discussed in chapter 1, variability also exists amongst potential recipients of the MSCs in terms of disease type and state as well as concurrent or previous treatments [6]. These points together have implications for both autologous and allogenic uses of MSCs and motivate future investigations to evaluate the performance our MSC pre-activation protocols across several macrophage donors as well as MSC donors.

In conclusion, donor variability is an important factor in the field of developing MSC therapies and cell therapies in general. We have demonstrated that, although variability is present, our IL-1β pre-activation protocol for the purpose of enhancing MSC-mediated macrophage modulation is successful for MSCs derived from multiple donors.

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Chapter 5: Evaluation of MSC Activation in a Biomaterial Scaffold

Note: This chapter is reproduced in part from the following publication:

Gray A, Maguire T, Schloss R, Yarmush ML. “Identification of IL-1β and LPS as Optimal Activators of Monolayer and Alginate-Encapsulated Mesenchymal Stromal Cell Immunomodulation Using Design of Experiments and Statistical Methods.” *Biotechnology Progress*, 31 (2015) 1058 – 1070.

5.1 Introduction

MSCs have the ability to migrate and home to areas of tissue damage and inflammation *in vitro* and *in vivo* [1-9]. This efficiency of this homing, however, has been shown to be very low *in vivo*, with most cells becoming trapped in capillary beds particularly in the lungs after intravenous injection [4, 10]. This is further complicated by short persistence of the cells [11, 12]. Although therapeutic benefit has still been observed [13-16], these challenges generate the need for very large doses of MSCs, which has implications on the probability of potential side effects and cell expansion to meet clinical needs [3, 17-20]. Therefore approaches which localize MSCs and increase their persistence in the target tissue could allow the entire dose of MSCs more time to impart their therapeutic benefits and therefore also reduce the number of cells needed to achieve a therapeutic effect [2].

Immobilization in semipermeable biomaterials has been commonly investigated as a means to localize many types of cells while maintaining their viability, exchange of nutrients, oxygen, and waste, and sometimes directing their behavior through microenvironmental cues [21, 22]. Alginate encapsulated cells

have extensively been investigated for several biomedical applications ranging from stem cell differentiation [23] to delivery of cell-derived bioactive molecules [22, 24] to tissue replacement [21]. We have previously investigated the encapsulation of MSCs in alginate hydrogel capsules and have demonstrated their ability to localize and increase the persistence of these cells while maintaining their ability to secrete bioactive molecules and modulate macrophage phenotype *in vitro* and in a rat model of contusive spinal cord injury [25-27].

In chapter 2, we identified IL-1β and LPS as optimal activators, individually and in combination, of MSC PGE2 secretion and subsequent modulation of macrophage TNF-α secretion [28]. Combining activation and cell immobilization approaches holds promise to address multiple challenges in MSC therapies simultaneously. However, interaction with biomaterials [29-36] and different culture configurations [37-39] can direct MSC behavior, including PGE2 secretion. Therefore in this chapter, we assess the function of IL-1β and LPS on alginate-encapsulated MSC (eMSC) secretion of PGE2 and macrophage modulation.

5.2 Materials and Methods

5.2.1 Chemicals and Reagents

All cell culture reagents and growth factors were purchased from Life Technologies (Carlsbad, CA, USA), unless otherwise stated. IL-1β was purchased from R&D Systems (Minneapolis, MN, USA). LPS and encapsulation reagents (sodium alginate, poly-L-lysine (PLL), 3-(N-morpholino)propanesulfonic

acid (MOPS), calcium chloride, and sodium chloride) were purchased from Sigma Aldrich (Oakville, Ontario, Canada).

5.2.2 Cell Culture

All cells were maintained in a humidified 37°C incubator with 5% CO_2 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.

5.2.3 MSCs

Human bone marrow-derived MSCs were purchased from the Institute for Regenerative Medicine (Texas A&M College of Medicine, Temple, TX, USA). 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 cm^2 flasks at $1.5x10^4$ cells/mL. They were cultured in MEM-α containing no deoxy- or ribonucleosides, supplemented with 10% FBS (Atlanta Biologics, Flowery Branch, GA, USA) 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 $2x10^3$ cells/well, and allowed to attach overnight.

5.2.4 Macrophages

$CD14^+$ monocytes were isolated from human peripheral blood from multiple healthy adult donors (The Blood Center of New Jersey, East Orange, NJ) and differentiated to generate macrophages, as described previously [28]. The differentiated cells were detached with trypsin, seeded into 96-well plates at

$1x10^4$ cells/well, and allowed to attach overnight. A different macrophage donor was used in each experimental replication.

5.2.5 Alginate Encapsulation

MSCs were thawed and expanded as described above. At 70% confluence, cells at passage 2 were detached with trypsin and seeded into 225 cm^2 flasks for further expansion. The cells were grown to 70% confluence, detached with trypsin, and encapsulated in alginate as described previously [23, 25]. Briefly, MSCs were suspended in non-supplemented Ca^{2+}-free DMEM and subsequently mixed with 2.2% (w/v) alginate in Ca^{2+}-free DMEM to achieve a final cell density of $4x10^6$ 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 [25]. The capsules were incubated for 10 minutes 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 cm^2 flask overnight. Before use, eMSC viability was determined by Hoechst (nucleus), calcein acetomethoxy (AM) (live), and ethidium homodimer (dead) staining, as described previously [25]. In experiments, eMSCs were cultured in 12-well plates at $2x10^4$ cells/well ($2x10^4$ cells/mL).

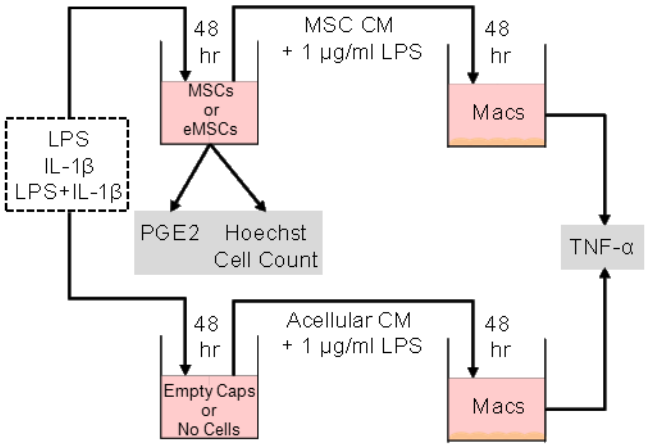

Figure 5.1 Schematic of the *in vitro* assay. (A) MSCs, eMSCs, or acellular media were incubated for 48 hours (hr) with soluble activating factors (dashed box). MSC, eMSC, or acellular CM was spiked with LPS and used to culture macrophages for 48 hours. Assays were then performed to quantify metrics of interest (grey shaded boxes).

5.2.6 Experimental Design

MSC monolayer cultures were washed once with fully supplemented RPMI 1640. Medium containing IL-1β, LPS, or both was added to the cells cultured for 48 hours. Supernatants were collected and frozen at -80°C until analysis. The MSCs were fixed in 4% (w/v) paraformaldehyde (PF) for 20 minutes 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 5.1). Additionally, medium containing IL-1β, LPS, or both were similarly cultured for 48 hours 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 hours of culture in CM, the macrophage supernatants were collected and frozen at -80°C until analysis. These experiments were run 3 times with 2-6 technical replicates per experiment. In order to determine the effects of these factors on encapsulated cells, this experiment was repeated using eMSCs (Figure 5.1). These eMSC experiments were run 3 times with 3 technical replicates per experiment.

5.2.7 Cytokine Measurement

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

5.2.8 Data Analysis and Statistics

To determine correlation between PGE2 and TNF-α, 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 (normalized to the lowest values in the dataset) 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 versus encapsulated), multivariable linear regression and covariate analysis was performed using MiniTAB Statistical Software version 17 (Minitab Inc., State College, PA) to adjust the effects on natural log transformed TNF-α (ln(TNF-α)) to account for the MSC activating factor used, the MSC culture format, and differences in macrophage donor (nested within the culture format), with the anchored, natural log transformed total PGE2 (ln(PGE2)) level as a covariate.

5.3 Results

5.3.1 Effects of LPS and IL-1β on MSCs and eMSCs

IL-1β exhibited a much higher potency than LPS. LPS and IL-1β increased MSC PGE2 secretion ~2- and ~7-fold on a per cell basis, respectively (Figure 5.2A). 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 5.2C), this reduction was greater when MSCs were activated with LPS. MSC activation with IL-1β more potently reduced macrophage TNF-α secretion compared to LPS activation of MSCs. In contrast to the effects of these two factors on MSC PGE2 secretion, the combined use of LPS and IL-1β to activate MSCs did not result in a synergistically enhanced attenuation of macrophage TNF-α secretion.

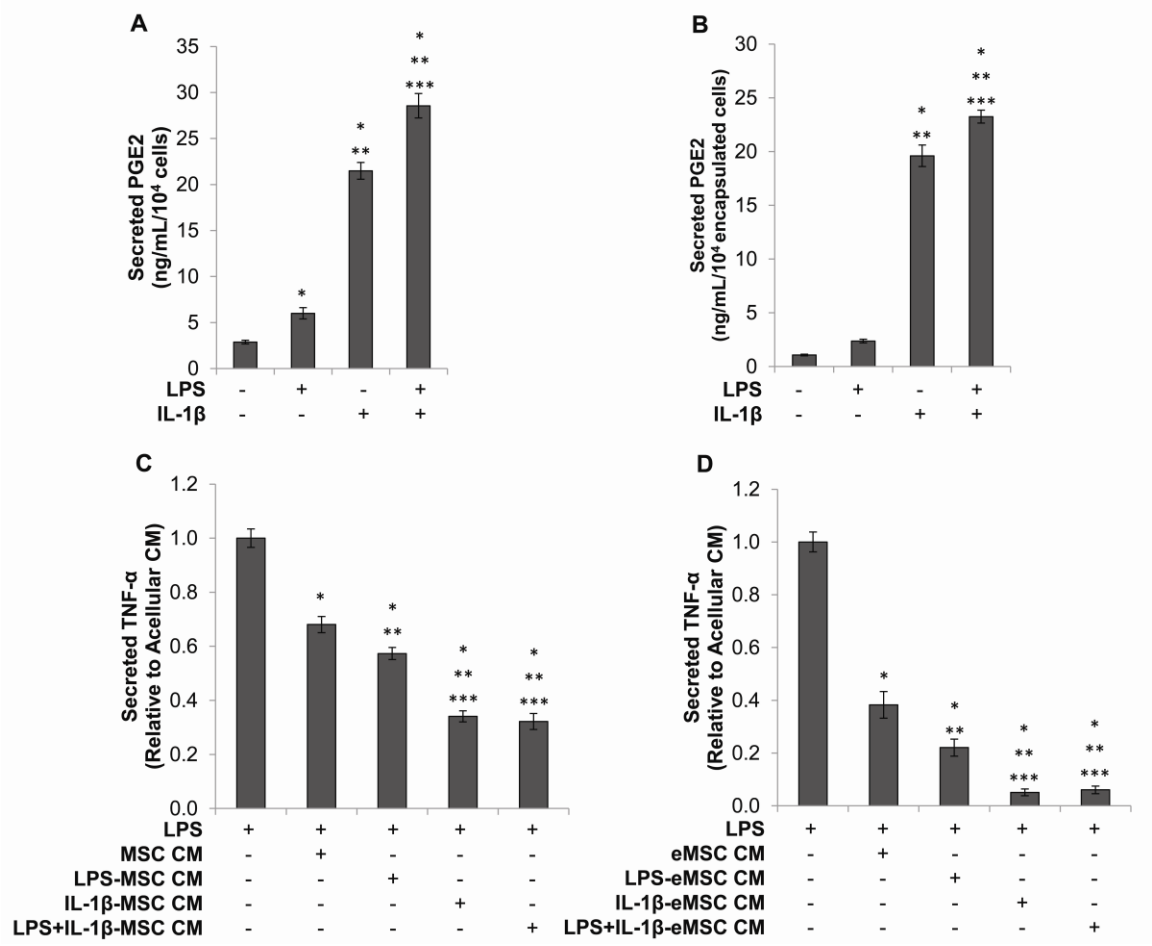

Figure 5.2 Effects of LPS and IL-1β on MSCs and eMSCs. (A) MSCs or (B) eMSCs were incubated for 48 hours with LPS or IL-1β, individually and in combination. The level of PGE2 in the cell culture supernatants were then quantified and normalized to the number of MSCs or eMSCs present at the end of culture. Data are means ± SEM of 3 independent experiments with 3-6 technical replicates per experiment ($p<0.05$ compared to LPS^- $IL\text{-}1\beta^-$ (*), LPS^+ $IL\text{-}1\beta^-$ (**), or LPS^- $IL\text{-}1\beta^+$ (***) 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. Data are means ± SEM of 3 independent experiments with 3-6 technical replicates per experiment ($p<0.05$ compared to LPS (*), MSC CM (**), or LPS-MSC CM (***) by ANOVA with Fisher's LSD *post hoc* test).

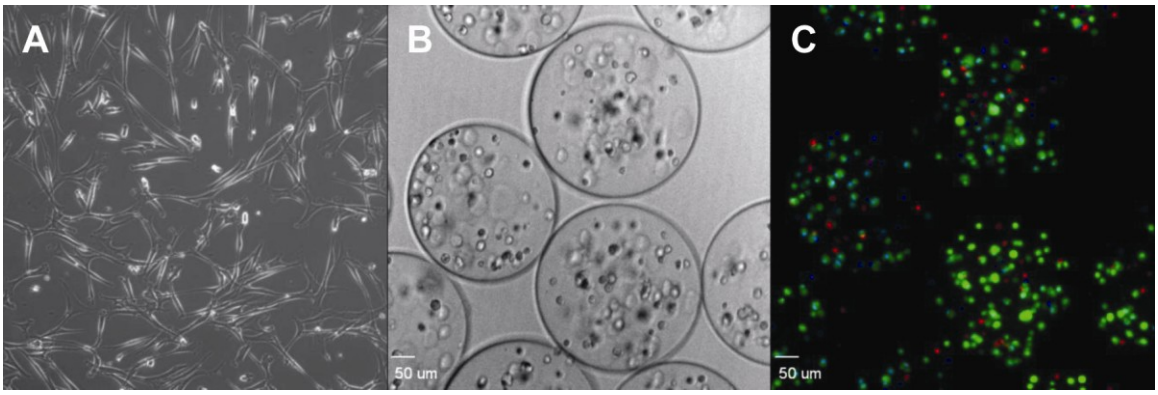

Figure 5.3 Morphology and viability of eMSCs. (A) Monolayer MSCs grown on tissue culture plastic (10X magnification). (B) MSCs encapsualted in alginate and their (C) viability (10X magnification; Hoescht staining of nuclei in blue; calcein AM positive live cells in green; ethidium homodimer positive dead cells in red). Scale bar = 50 µm.

We next 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 5.3). PGE2 secretion from non-activated eMSCs was lower than monolayer MSCs (1.08 versus 2.88 ng/mL/10^4 cells). Activation with IL-1β alone resulted in a ~7.5-fold increase in PGE2 secretion from eMSCs per cell. LPS alone did not significantly induce PGE2 secretion (Figure 5.2B). Similarly to monolayer MSCs, the presence of both LPS and IL-1β further increased eMSC PGE2 secretion compared to IL-β alone.

CM generated using non-activated eMSCs reduced macrophage secretion of TNF-α (Figure 5.2D). When eMSCs were stimulated with LPS, macrophage TNF-α secretion was further attenuated. Stimulation of eMSCs with IL-1β reduced macrophage TNF-α secretion to a greater degree compared to no stimulation or LPS-activation, but this effect was not further enhanced when IL-1β was used in combination with LPS.

5.3.2 Relationship between MSC PGE2 secretion and 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 5.4A, the relationships between macrophage TNF-α secretion and PGE2 secreted from MSCs or eMSCs are non-linear 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 5.4A were observed quantitatively in the slopes of the linear regression lines through the transformed data (Figure 5.4B). 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.

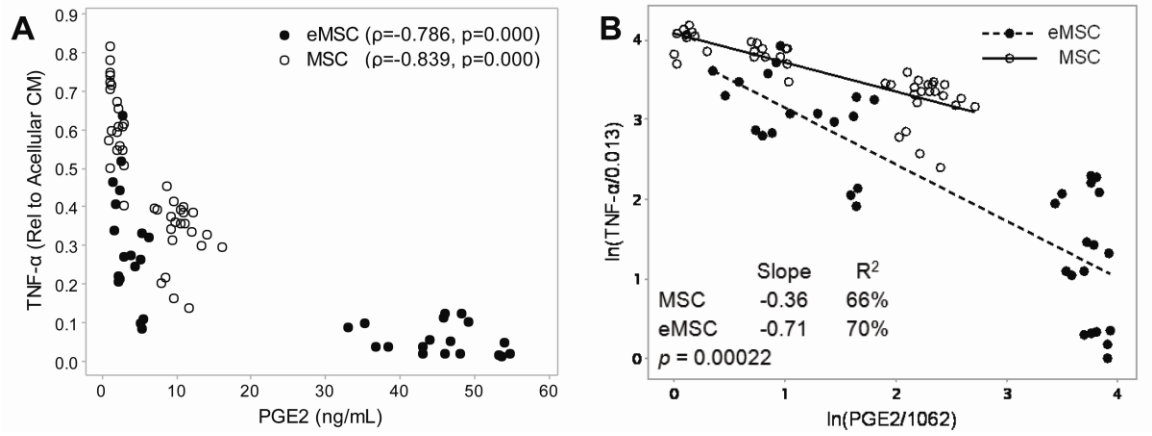

Figure 5.4 Statistical analysis of relationship between MSC and eMSC secreted PGE2 and macrophage TNF-α. (A) Total PGE2 and cooresponding realtive TNF-α levels from experiments testing the effects of LPS and IL-1β on MSCs (○) and eMSCs (●) were plotted against each other, Spearman's correlation coefficients (ρ), and their statistical signficance was calculated. (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. Statistical significance of the slope for eMSC (----) and MSCs (—) was 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).

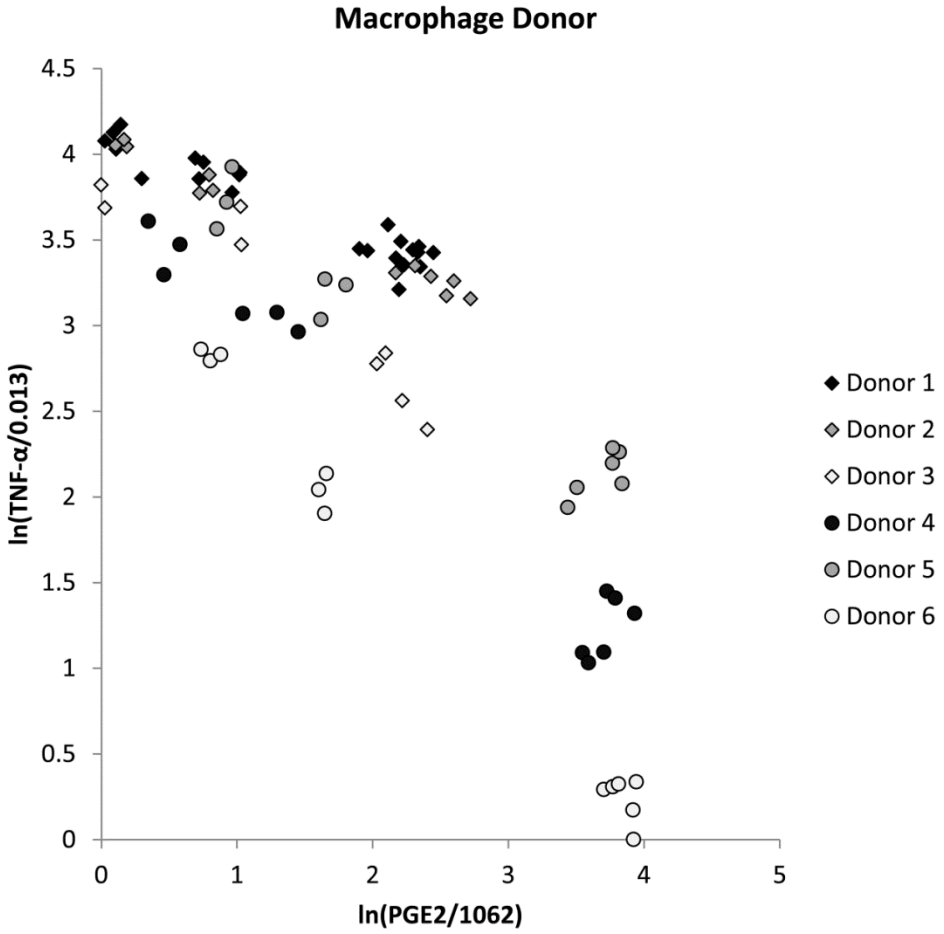

Figure 5.5 Relationship of transformed TNF-α and PGE2 per macrophage donor. PGE2 and TNF-α levels for MSC (diamonds) and eMSC (circles) conditions were normalized to their lowest value, transformed by taking the natural log, and plotted against each other for each macrophage donor.

5.3.3 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 5.5), 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-1β, LPS+IL-1β), 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 5.1). 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-1β.

Table 5.1 Summary of general linear model with a covariate and a nested factor.

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			

5.4 Discussion

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 cells types, including primary cells, genetically engineered cells, and stem cells [40]. We previously encapsulated MSCs in alginate microspheres for intrathecal delivery to treat inflammation associated with acute spinal cord injury [25, 41]. Although alginate is considered relatively, inert there is potential for interaction with the encapsulated cells. The mannuronate subunits of alginate have been reported to have biological activity similar to that of toll like receptor 2 (TLR2) and TLR4 ligands, primarily on cell line derived and primary monocytes and macrophages [29, 32-36]. Additionally, three-dimensional culture has been demonstrated to enhance certain properties of MSCs as compared to two-dimensionally cultured cells, including PGE2 secretion [37-39]. We have previously observed that the MSC secretome was enhanced when encapsulated in alginate, constitutively and after activation with TNF-α and IFN-γ [25]. Therefore, after observing the effects of LPS and IL-1β on monolayer MSCs in chapter 2, we next determined if the effects of these factors are altered in alginate-encapsulated MSCs. We found that although constitutive levels of PGE2 secretion were lower for eMSC, trends in PGE2 secretion and macrophage TNF-α secretion were similar to those observed for IL-1β activated monolayer cells. LPS activation did not significantly increase eMSC secretion, although it did synergize with IL-1β 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 a 10 kDa amphiphilic molecule that forms micelles in aqueous solutions [42]. These aggregates have been reported to contain 10^1 to 10^3 molecules per micelle, resulting in a macromolecule with a molecular weight of 10^2 to 10^4 kDa [42, 43]. 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 [44, 45]. Other serum molecules such as albumin also bind to LPS [46]. 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 10^1 to 10^3 kDa [43, 47-50]. 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 TLRs may present physical and chemical barriers of LPS action on the entrapped cells. The enhancement of MSC PGE2 secretion when LPS and IL-1β were used together compared to IL-1β 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-1β activated eMSC CM, with no further benefit seen by adding LPS co-activation. 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 [26, 51-54]. While IL-1β 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 compared to IL-1β 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 [55, 56]. 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 from eMSCs 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 MSC 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 variable 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 [57-60]. Statistical analysis using multivariable linear regression revealed that the variables of MSC 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 seen here combined with the results for different MSC donors described in chapter 4 further highlight the importance of patient and donor 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.

The limitations of these studies discussed in chapter 2 carry over to the current studies. To reiterate, only one MSC donor was used to compare activation of MSCs and eMSCs. As discussed in chapter 4, MSC functions can

vary between multiple donors [61-65]. Therefore, future studies should include analysis across multiple MSC donors, particularly in light of our findings regarding the importance of macrophage donor variability. Additionally, the use of CM in this study does not provide insight into how pre-activated eMSCs would perform after re-introduction into an inflammatory environment with dynamic crosstalk with immune cells. Therefore the studies of chapters 3 should be extended to included assessment of the pre-activation protocol on eMSCs as well. The immobilization of MSCs in alginate also introduces other variables, including cell morphology as was shown in this chapter; the effect of these biomaterial-specific factors on MSC activation should also be investigated.

We have demonstrated that IL-1β, identified from a screen of a panel of activating molecules in chapter 2 as an optimally potent activator, enhanced MSC and eMSC immunomodulatory function, with LPS increasing the potency of IL-1β's effect on some aspects of this function. Furthermore, macrophage donor variability was found to play a significant role in 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.

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Chapter 6: Dissertation Conclusion

6.1 Key Findings

The goal of the work done in this dissertation was to develop approaches to address challenges in successfully translating the promise of MSCs into the clinic pertaining to MSC activation, donor variability, and inefficient MSC homing and persistence. We developed an approach to identify activation parameters that induce specific MSC functions. This flexible approach yielded an MSC pre-activation protocol which enhanced MSC immunomodulatory functions. We further demonstrated the performance of this protocol for multiple MSCs donors. Finally, we show that encapsulation of MSCs in alginate microcapsules can offer the localization and persistence benefits of immobilization while maintaining the effect of soluble factors on MSC immunomodulatory functions. In addition to these deliverables, our results underscore the need for continued improvement and innovation in the field to overcome obstacles to success.

6.1.1 IL-1β Pre-activation Protocol

The field of MSC therapy currently lacks systematic approaches to manipulating MSCs to enhance their functions in an effort to improve therapeutic efficacy. To develop an MSC pre-activation protocol, we utilized a systematic approach to selecting activation parameters related to the type of activating molecule, combinations of molecules, concentration, and duration of pre-activation. Our specific MSC function of interest was modulation of macrophage TNF-α secretion via PGE2 secretion. Fractional factorial design of experiments was used to increase the throughput of screening potential activation factors and

combinations of factors for the upregulation of specific MSC functions. We found that IL-1β and LPS, individually and in combination, upregulated MSC secretion of PGE2 and subsequent modulation of TNF-α secretion, with the effects of IL-1β being more potent than those of LPS alone. We next performed dose response studies to select concentrations of these factors which maximize MSC secretion of PGE2. These studies indicated 1 ng/mL of IL-1β with and without 100 ng/mL LPS led to higher PGE2 levels earlier than other concentrations. Temporal studies were the performed to select the optimal duration of pre-activation which sustains and maximizes PGE2 secretion after the activating factors are removed. We identified that IL-1β alone at 1 ng/mL for just 1 hour activated MSCs to secrete PGE2 at high levels in subsequent fresh medium.

6.1.2 IL-1β Pre-Activation Enhances MSC Immunomodulation

To characterize the sensitivity of the pre-activated MSCs upon reintroduction into inflammatory microenvironments, we measured PGE2 secretion from IL-1β pre-activated MSCs exposed to IL-1β, TNF-α, IFN-γ, or LPS individually for 24 hours. We additionally co-cultured IL-1β pre-activated MSCs with LPS-activated macrophages for 48 hours and measured TNF-α and PGE2 in the co-culture supernatants. Pre-activated MSC secretion of PGE2 was upregulated in response to TNF-α, IFN-γ, or LPS, compared to MSCs without pre-activation. PGE2 secretion by pre-activated MSCs after secondary exposure to IL-1β, however, was not different than that of no pre-activation. This unchanged sensitivity to the same factor was also observed if the MSCs were pre-activated with any of the other factors in the secondary stimuli panel;

therefore this was not specific to IL-1β. In co-culture, IL-1β pre-activated MSCs attenuated macrophage TNF-α to a greater degree than MSCs without pre-activation. PGE2 levels were also further increased in these conditions.

6.1.3 Duality in MSC Functions

MSCs have been demonstrated to adopt immune-stimulating as well as immunosuppressive phenotypes [1]. In our studies, we observed both anti-inflammatory behavior and suggestions of pro-inflammatory behavior. A 27-cytokine multiplex immunoassay was used to examine the secretome of MSCs pre-activated for 1 hour with IL-1β and then cultured in fresh medium for 6 or 24 hours. Secretion of chemokines, anti-inflammatory molecules, pleiotropic factors, and some pro-inflammatory factors was upregulated for IL-1β pre-activated MSCs. The pro-inflammatory factors were secreted only at very low levels post-activation, but pleiotropic factors and chemokines remain highly upregulated. Due to the dual roles these factors in inflammation, it is difficult to classify them as indicating a pro- or anti-inflammatory phenotype.

In parallel to IL-1β pre-activated MSCs, the sensitivity to secondary stimuli studies were also performed for MSCs pre-activated with the rest of secondary stimuli panel (TNF-α, IFN-γ, or LPS), as described above. Like IL-1β pre-activation, IFN-γ pre-activation resulted in upregulated PGE2 secretion in response to IL-1β, TNF-α, or LPS compared to MSCs without pre-activation. The upregulation in response to IL-1β was particularly dramatic. However when IFN-γ pre-activated MSCs were co-cultured with LPS-activated macrophages, the attenuation of TNF-α was actually impaired compared to that of MSCs without

pre-activation. PGE2 levels in these co-cultures were slightly lower than that of MSCs with no pre-activation. Preliminary data suggests that IFN-γ secreted from IFN-γ pre-activated MSCs increased TNF-α secretion from macrophages synergistically with LPS. Future work will include investigations into the cause of the lower PGE2 levels in the co-cultures. This work demonstrates that relying on a single isolated culture metric to characterize MSC potency or predict efficacy is not sufficient.

6.1.4 Performance of the Pre-Activation Protocols Varied Amongst Different MSC Donors

To determine the effect of donor variability on the performance of the IL-1β pre-activation protocol and IFN-γ pre-activation, the secondary stimuli and macrophage co-culture assays were repeated using MSCs derived from 6 donors. The effect of IL-1β pre-activation on TNF-α-, IFN-γ-, and LPS-induced PGE2 secretion and macrophage TNF-α attenuation was detectable with high statistical power. Although the major trends in the data were consistent among the 6 donors, variability was observed in the absolute levels and degree of upregulation of secreted PGE2, level of improvement or impairment in macrophage modulation induced by IL-1β or IFN-γ pre-activation, respectively, and the correlation of MSC PGE2 and macrophage TNF-α. Correlations of these metrics with age were not significant and differences between males and females were not detected.

6.1.5 Alginate Encapsulation Sustains MSC Activation

To address the challenges in MSC therapy related to inefficient cell homing and transient persistence, we encapsulated MSCs in semipermeable alginate hydrogel microcapsules, as we have previously demonstrated maintains MSC viability and localization *in vitro* and *in vivo* [2, 3]. To determine if alginate encapsulation affects the activation of MSCs by the optimal factors identified in the high throughput screen, we exposed monolayer or encapsulated MSCs (eMSCs) to IL-1β and LPS, individually and in combination, for 48 hours and then measured PGE2 secretion. Conditioned medium (CM) generated this way was also used to culture LPS-activated macrophages from multiple donors, after which TNF-α secretion was quantified. Similar to monolayer MSCs, IL-1β increased eMSC PGE2 secretion potently alone and synergistically with LPS. LPS alone did not significantly induce PGE2. Attenuation of macrophage TNF-α was increased when LPS was used to activate MSCs and further when IL-1β was used. No further attenuation was observed when LPS and IL-1β were used together. Decreases in TNF-α were correlated with increases in PGE2 secretion for both monolayer MSCs and eMSCs. Using general linear models and analysis of covariance, we determined that the TNF-α level was significantly affected by PGE2 level, MSC activation factor, and macrophage donor, but not MSC culture format. Therefore we concluded that alginate encapsulation can provide the benefits of cell immobilization while maintaining MSC response to activating factors.

6.2 Limitations

6.2.1 Conditioned Medium

To increase the feasibility of executing the high throughput screening assay, we measured the modulation macrophage TNF-α secretion by culturing them in activated MSC conditioned medium (CM) spiked with LPS rather than with the activated MSCs themselves, which are adherent and require enzymatic detachment. While the use of CM facilitated exploitation of the benefits provided by fractional factorial design, it introduces some limitations into the assay. In our assay, MSCs were exposed to combinations of activating factors for 48 hours and then this CM was used to culture the macrophages. Although the macrophage-modulating factor of interest present in the CM is PGE2, residual activation factors in the CM, which we tried to control for using acellular CM, can have an effect on the macrophages. Additionally, 48 hours of culturing the MSCs changes the amount of nutrients available for the macrophages as well as the levels of cellular waste products in the CM. The CM in our study was centrifuged to remove cellular debris and any MSCs detached by pipetting. However, it is still possible that trace numbers of MSCs were inadvertently added to the subsequent macrophage cultures. All of these factors have the potential convolute the results of the assay.

Using CM can also alter the question being asked in the experiment. Instead of investigating the dynamic crosstalk between the two cell types, our assay asked questions regarding the effect of MSC response to different inputs on the action of the MSC secretome on macrophage behavior. However our

ultimate goal was to create a pre-activation protocol where the activated MSCs themselves are destined to dynamically interact with pro-inflammatory macrophages. Due to the flexibility of this assay, it can easily be adapted to investigate MSC pre-activation. Therefore in moving forward to optimize activation for other MSC phenotypes/functions or for other MSC tissue sources and to ask mechanistic questions, an intermediate culture of the MSCs in fresh medium after activation with factors of interest can be introduced.

6.2.2 Fractional Factorial Design of Experiments and Statistical Approaches

Design of experiments (DoE) can be a powerful tool in studying factors affecting the output of a process [4, 5]. In fractional factorial designs (FFDs), reduction in the number of experimental conditions is achieved by mathematically aliasing main effects and lower order interactions with higher order interactions, based on the assumption that these higher order interactions are negligible. This assumption is often safe in examining most processes including bioprocesses [5-8], given that the investigator has some prior knowledge of the system being studied. As the mechanisms of action of MSCs continue to be elucidated, their complexity increasingly comes to light as well. Therefore the validity of this assumption must continue to be carefully considered based on the specific aspects of the MSC system being investigated.

Within the DoE approach, there are many opportunities to improve the quality of the design, some of which were not utilized in our studies [9]. We used a 2-level fractional factorial design, where activating factors of interest were

either not present or present at a single concentration. This approach is limited in that, in analyzing the results, only linear relationships will be described whereas some factors may have a non-linear effect on the output metric. This can be addressed by using a 3-level experiment for which the DoE approach is also well established [9]. Using this approach, non-linear relationships can be detected and minimization/maximization of the equation modeling the input/output relationship can be used to aid in optimization [10]. Regardless, as was demonstrated in chapters 2 and 5, the results of the mathematical analysis cannot be taken as face value, but must rather be considered carefully to elucidate the true message in the data.

Randomization of the order in which the experimental conditions are tested and blocking of groups of conditions to be run at a certain time can reduce systematic error introduced by uncontrollable environmental factors, such as room temperature and lot-to-lot differences in reagents [9]. This is a useful tool when it is not practical or feasible to test all conditions at once or in parallel, which is common in optimization of manufacturing and biotechnological processes. Randomization was not a large issue in our studies as we were feasibly able to run all the conditions at once and in duplicate using 96 well plates and multichannel liquid handling. Also, as is particularly necessary in measuring biological samples with good statistical confidence, we performed 3 experimental replicates in addition to duplicate technical replicates in each experiment. These experimental replicates served as the blocking factor for analysis. In running so many conditions at once, however, there is an increased probability of systematic

error in the process of making the activation cocktails in the same order each time. Therefore introducing randomization in the preparation of the experimental conditions could increase the power of the data analysis.

Finally, in several parts of this thesis work, general linear models and correlation between metrics are used to analyze results. This again is a useful engineering tool to aid in biological investigations. As in the FFD approach, however, these analyses must be performed in the context of prior knowledge of the system, as correlation does not guarantee causation.

6.2.3 Donor Studies

Our evaluation of the pre-activation protocols on MSC from several donors was motivated by well-documented donor-to-donor variability [11]. Variability was observed in PGE2 secretion and modulation of macrophage TNF-α after pre-activation. We used MSCs isolated from the bone marrow of equal numbers of males (3) and females (3), all aged between 20 and 30. Power analysis determined that this was a sufficient number of subjects to detect differences in our metrics with high statistical confidence. However, there are several limitations to this study.

The high statistical power calculated in the power analysis may be inflated by the large effect size in our metrics [12]. Therefore, a larger size donor pool is needed to detect more subtle differences. Additionally, these studies were performed using a single macrophage donor in order to make the data from each donor more comparable. We demonstrated in chapter 5, however, that

macrophage donor variability can significantly affect the interaction between MSCs and macrophages.

Variability in MSC donor demographics can be based on many different factors, including ethnicity, method of isolation, medical history, and cell culture methods [11]. Due to the limited amount of information provided by suppliers, we could not account for these other factors that could affect the results. Additionally, two of the donors were received cryopreserved at passage 2 while the other 4 donors were supplied at passage 1. There is evidence that passage and cell culture methods can affect MSC functions [13]. While gender has not been reported to affect MSC properties, as we also observed in this work, age has been documented to play a significant role [14, 15]. The age range used in these studies was very narrow. Therefore conclusions based on our results showing lack of significant correlation of our metrics with age are limited in scope. The diversity of the age of the donors should be increased to make stronger conclusions about the effect of age on our metrics.

6.2.4 Encapsulated MSCs

The combination of cells, including MSCs, and biomaterials is commonly studied for many purposes related to controlling the fate of the cells *in vivo* [16]. These include direction of differentiation for tissue replacement purposes and simple immobilization for localization and increased cell persistence. Alginate is an attractive material for this purpose as it can be easily be cross-linked with divalent cations and creates semipermeable hydrogels that can maintain cell viability and allow for the exchange of nutrients, oxygen, soluble factors, and

waste products [17]. We have previously successfully used alginate encapsulation for the differentiation of embryonic stem cells into hepatocytes and immobilization of MSCs for modulating inflammatory cascades in acute contusive spinal cord injury [2, 18]. While providing potential benefits, alginate encapsulation comes with several challenges of its own. These issues are related to alginate purity, composition, biocompatibility, routes of delivery, tracking *in vivo*, consistent preparations, and scale up.

As a naturally derived product, alginate is inherently variable in its composition. The ratios of its constitutive polymeric components, guluronic (G) and mannuronic (M) acids, have implications on the mechanical properties of the alginate, with higher G ratios resulting in stiffer hydrogels [19]. This heterogeneity can make comparisons between studies in the literature difficult. Also, due to its natural source, contamination of alginate by endotoxins, purification chemicals, and proteins make sterility/purity a concern [20, 21]. Relatedly, alginate is generally thought to be biocompatible and has been approved for use in several clinical applications [22]. However, mannuronic acid residues have been reported to elicit pro-inflammatory reactions from immune cells [23-26]. Alginate microcapsules are also commonly coated with polycations to improve material stability, such as the poly-L-lysine (PLL) as used in our studies [27]. However, PLL has been shown to induce immune reactions and fibrosis *in vivo* [28, 29]. The composition and coating of the alginate can also affect the molecular weight cutoff for permeability of the capsules [22, 27].

Another looming issue is the generation of uniformly sized, consistent preparations of encapsulated cells, which has significant implications for the scale up of production [30]. Evidence suggests that very small diameter capsules may compromise biocompatibility, emphasizing the importance of this issue [31]. Uncrosslinked alginate is generally viscous, depending on the composition, and has presented challenges in our previous work in generating sufficient quantities of eMSCs for animal studies as has operator-to-operator variability [3].

Finally, issues also arise when contemplating the administration of encapsulated cells to patients. The most common method of delivering single cell suspensions of MSCs is intravenous injection. While convenient, it is limited by inevitable lung entrapment, systemic distribution, inefficient homing, and transient persistence [32]. While alginate encapsulation can address these issues, it also poses problems for selecting feasible and safe transplantation sites [33]. Theoretically, eMSCs should remain viable and functional in places in the body that provides oxygen and nutrients. Transplantation sites include fluid filled cavities (lumbar cistern, ventricles, peritoneum, etc.), subcutaneous spaces, the portal vein in the liver, under the kidney capsule, and intraocular spaces [17]. While some of these routes are quite invasive even for single cell suspensions, we have had some previous success with intrathecal delivery of eMSCs into the lumbar cistern [2, 3]. Passing concentrated boluses of capsules through a syringe/needle compatible with rat intrathecal delivery, however, presented many feasibility issues. Intraperitoneal transplantation seems to be the most feasible, but several groups have reported better stability, viability, and function using

subcutaneous transplantation and injection under the kidney capsule [33-35]. Once situated *in vivo*, tracking the fate of the capsules also presents a challenge. We have demonstrated stable detection of alginate containing superparamagnetic iron oxide nanoparticles in the rat lumbar cistern for 8 weeks by magnetic resonance imaging (MRI), but the effect of these particles on eMSC function needs to be further investigated [3].

Based on these points, development of alginate encapsulation technology for regenerative medicine including MSC therapies remains promising. The initiation of clinical trials investigating the safety and efficacy of using alginate encapsulated pancreatic islets and choroid plexus cells in type I diabetes and Parkinson's disease is encouraging [36]. Much work is still needed, however, to successfully integrate it into clinical use.

6.3 Future Directions

Despite the limitations discussed above, this body of work raises many interesting questions in addition to delivering an effective pre-activation protocol and a systematic approach to enhancing MSC functions. One of the metrics used to optimize the pre-activation protocol was the MSC-mediated modulation of macrophage TNF-α secretion. However, MSCs have been demonstrated to modulate several other macrophage characteristics [37]. We and others have previously demonstrated the ability of MSCs to promote the adoption of an anti-inflammatory phenotype by macrophages in addition to suppressing pro-inflammatory behavior [2, 38, 39]. Therefore more macrophage metrics can be utilized to supplement our screening assays, including secretion of anti-

inflammatory and trophic factors (e.g. IL-10, TGF-β), surface expression of pro-inflammatory (e.g. CD86) and anti-inflammatory (e.g. CD206) markers, and phagocytic activity. Future work should additionally include adaptation of the approach to optimizing activation parameters for other MSC functions, such as promoting angiogenesis. The most widely studied function of MSCs has been suppression of lymphocyte proliferation and it is widely known and accepted that IFN-γ stimulation of MSC indoleamine 2,3-dioxygenase (IDO) secretion is essential for this [40]. Therefore successful identification of IFN-γ from a panel of factors using secretion of IDO and modulation of lymphocyte proliferation as metrics could further validate the approach.

We observed that eMSCs retain their ability to respond to exogenous activation. However we did not test the performance of the pre-activation protocols using eMSCs. Therefore we plan to repeat the assays described in this dissertation using alginate encapsulated MSCs. Furthermore, we plan to test the efficacy of pre-activated MSCs and eMSCs versus MSCs or eMSCs without pre-activation in animal models of conditions in which macrophages play a known role, such as sepsis and wound healing. As discussed above, some results using IL-1β or IFN-γ pre-activation indicated some potential pro-inflammatory characteristics of the MSCs. Using the pre-activated MSCs in these animal models may allow us to detect off target side effects caused by these unintended characteristics.

We made several interesting and unexpected observations in the course of this work. We observed that extending the duration of pre-activation with IL-1β to

6 or 24 hours did not further enhance sustained upregulated PGE2 secretion post-activation but actually impaired it. Additionally, we observed that IL-1β pre-activated MSCs did not have an enhanced response to secondary exposure to IL-1β. This may indicate the involvement of autoregulatory mechanisms in dampening MSC responses. Bellehumeur et al. reported that endometrial cells regulate their response to IL-1β by altering the expression of signaling and non-signaling receptors and secretion of IL-1RA [41]. In the multiplex analysis of the MSC secretome, we observed that IL-1β pre-activation induced significant IL-1RA secretion. Therefore we suspect similar mechanisms of autoregulation are utilized by MSCs. We plan to test this hypothesis by further tracking IL-1RA secretion and IL-1 receptor expression, and by blocking the activity to these factors to detect loss or gain of MSC PGE2 secretion.

We also observed that the promising dramatic enhancement of PGE2 secretion from IFN-γ pre-activated MSCs in response to secondary IL-1β did not translate into enhanced modulation of pro-inflammatory macrophages in co-culture. While increased TNF-α levels in the co-culture supernatants were most likely due to the presence of IFN-γ, possibly secreted by the IFN-γ pre-activated MSCs, it remains unclear why the PGE2 levels in these conditions were unexpectedly low. Further characterization of the dynamics of the co-culture microenvironment may help to elucidate this.

Both MSC donor and macrophage “recipient” variability were observed to have an effect on the metrics in our studies. This is highly reflective of concerns regarding screening MSC donors and issues pertaining to variability in patient

disease state and underlying pathologies. To increase the scope of our studies, we plan to increase the diversity of our MSC donor set to include a wider age range and pathologies. To gain insight into the importance of matching MSC donors with appropriate MSC recipients, we plan to perform our assays using multiple MSCs donors in parallel with multiple macrophage donors. Differences detected in these studies and in animal studies using high and low performing MSCs could further support the use of our protocol as a predictive potency assay.

6.4 References

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