DYNAMICS OF MESENCHYMAL STROMAL CELL POTENCY DURING *EX VIVO* BIOREACTOR THERAPY

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ABSTRACT OF THE THESIS

Dynamics of Mesenchymal Stromal Cell Potency during *Ex Vivo* Bioreactor Therapy

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Abstract

Mesenchymal stromal cells have become a candidate for cell therapy due to potent immunomodulatory properties exhibited in their secretome. However, they have shown limited clinical success due to issues of dosing and persistence when administered *in vivo*. A scalable hollow fiber bioreactor device has been created to deliver MSC therapeutics in an *ex vivo* manner. The potency and behavior of MSCs in this bioreactor can be explored via a dynamic perfusion system, collecting the MSC secretome in periodic fractions and comparing the MSC behavior at different time points and in response to a variety of factors. Herein, a study was designed to determine the dynamic potency of the MSC secretome using this device and characterize their secreted factors when cocultured with immune cells. Three studies were designed to characterize the dynamic baseline MSC behavior, determine how MSCs behave in a model of an inflamed patient, and understand the effect of prelicensing MSCs on their dynamic potency. Results indicate an ideal dosing time of 24 hours and significant benefits when prelicensing MSCs used in the device. Ultimately, this research can be translated clinically to maximize the potency of the MSC therapy, minimize side effects, and allow greater control for the duration of the *ex vivo* bioreactor therapy.

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List of Abbreviations

MSC	Mesenchymal Stromal Cell
PBMC	Peripheral Blood Mononuclear Cell
LDH	Lactate Dehydrogenase
IFNγ	Interferon Gamma (γ)
TNFα	Tumor Necrosis Factor Alpha (α)
IL-1β	Interleukin-1 Beta (β)
IL-6	Interleukin-6
IL-4	Interleukin-4
IL-10	Interleukin-10
IL-1RA	Interleukin-1 Receptor Antagonist
TGFα	Transforming Growth Factor Alpha (α)
FGF-2	Basic Fibroblast Growth Factor
VEGF	Vascular Endothelial Growth Factor
α-MEM	Minimum Essential Medium Alpha (α)
CFSE	Carboxyfluorescein succinimidyl ester

Chapter I: Introduction

1.1 Mesenchymal Stromal Cell Properties

Mesenchymal stromal cells (MSCs) are considered for cell immunotherapy due to their immunomodulatory potential and properties in regenerative medicine. MSCs can be sourced from a variety of tissues, such as bone marrow, adipose, peripheral blood, umbilical cord, placenta [1]. The International Society for Cellular Therapy proposed minimal criteria for defining MSCs including that they must be plastic-adherent in standard *in vitro* culture, and that they must express CD105, CD73, and CD90 while lacking expression of CD45, CD34, and CD14 amongst others. Finally, they must differentiate into adipocytes, chondrocytes, or osteoblasts when cultured [2]. MSCs display fibroblast-like morphology and can display high potential for self-renewal and differentiation [1]. The International Society for Cellular Therapy further amended the MSC definition to include a bioassay of immunosuppressive properties and functional assays to determine the therapeutic mechanism of action [3].

1.2 Mesenchymal Stromal Cell Mechanism of Action

MSCs have been discovered as a therapeutic for tissue injuries, inflammatory conditions, as well as chronic degenerative diseases. MSC cell therapy assumes the cells' ability to migrate to and reach target tissues [4]. This inherent ability is theoretically controlled by mechanisms including chemoattractant signals. Chemokines and cell adhesion molecules mobilize MSCs to damaged tissue, where they can attach from the blood stream and begin enacting tissue regeneration via secretion of growth, anti-inflammatory, immunomodulatory factors [4, 5].

MSCs' primary mechanism of action is their secretion of a variety of soluble factors, including growth factors, paracrine factors, and pro- and anti-inflammatory cytokines. Secretion of factors and proteins like basic fibroblast growth factor (FGF-2), vascular endothelial growth factor (VEGF), transforming growth factor alpha (TGFα), encourage the increase of cell division and initiate vascularization. Paracrine factors like keratinocyte growth factor and stromal-cell derived factor-1 (SDF-1) can reduce scar tissue at sites of injury [5].

An important feature of MSCs is their immunomodulatory properties. MSCs secrete a variety of cytokines that can modulate suppression or promotion of immune cell proliferation. These cytokines include interleukin-6 (IL-6), transforming growth factor beta (TGFβ), and prostaglandin E2 (PGE2) [6]. This secretion pattern is an example of the complexity of the bulk fluid that can contain a mix of both pro- (IL-6) and anti-inflammatory (TGFβ) cytokines at once. As they also respond dynamically to their environment, MSCs have been found to secrete more anti-inflammatory factors in response to pro-inflammatory cytokines in their microenvironments. Particularly in response to pro-inflammatory tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), and interleukin-1 beta (IL-1β), MSCs further secrete a variety of anti-inflammatory proteins including interleukin-4 (IL-4), IL-10, Indoleamine 2,3-dioxygenase (IDO), IL-1RA, etc. [5, 7].

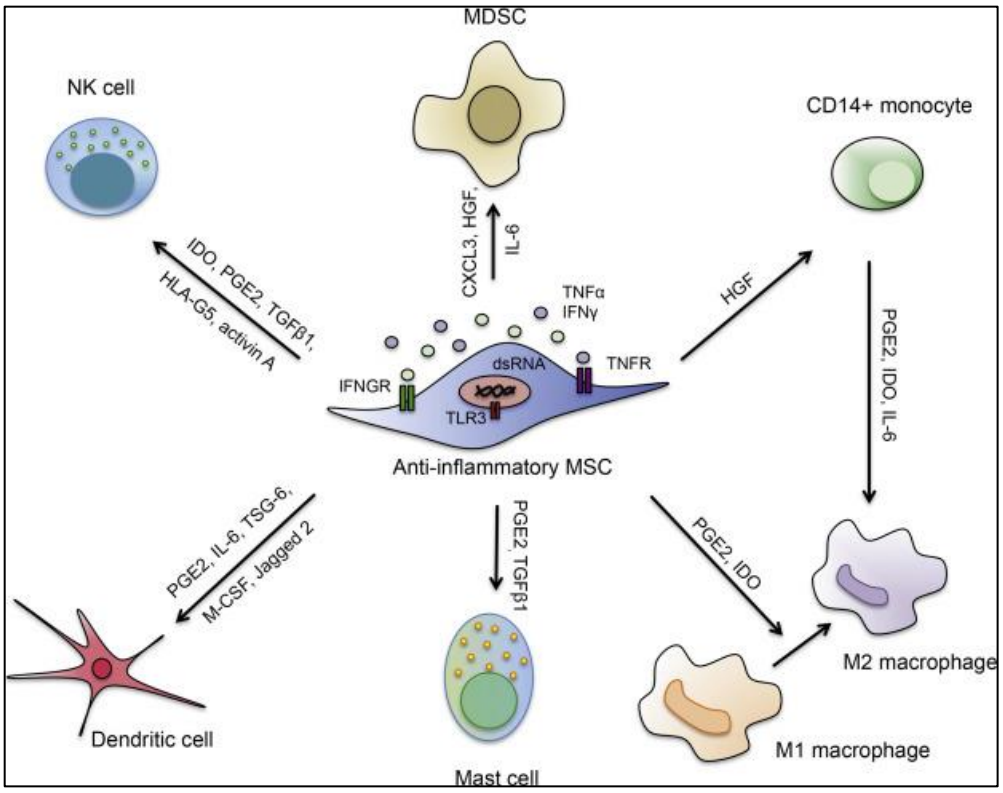


Figure 1*: MSC secretion of immune factors and cytokines.*
In the presence of proinflammatory cytokines, including TNFα and IFNγ, MSCs adopt an anti-inflammatory profile and secrete cytokines that impact a robust profile of immune cells, including NK cells, macrophages, and dendritic cells, among others. Adapted from [8].

However, MSCs are not always immunosuppressive, and their effects are more determined by the conditions of their microenvironment, primarily the presence of the pro-inflammatory cytokines mentioned [6]. It has been found that certain factors can cause a "switch in MSC-mediated immunomodulation" [9]. Without the presence of pro-inflammatory cytokines, MSCs can begin enhancing T cell responses by the secretion of certain "chemokines that recruit lymphocytes to sites of inflammation," like RANTES, CXCL9, etc. [7]. Further, Cuerquis et al. showed that unprimed or activated "MCSs induced a transient increase in IFNγ and IL-2 synthesis by activating T cells before

suppressing T-cell proliferation" [9, 10]. These pathways create a balance and state of equilibrium until prompted to shift.

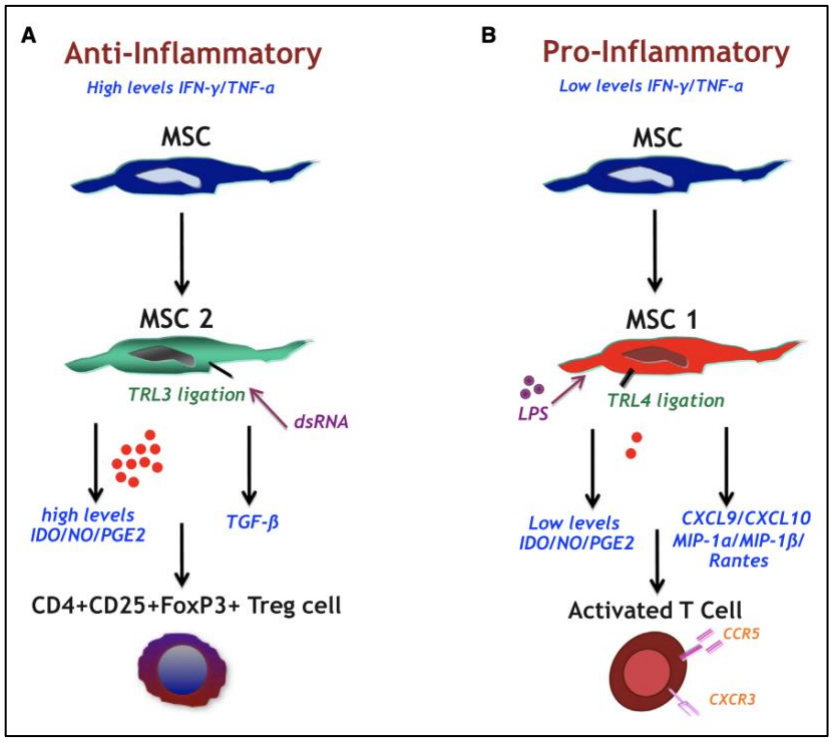


***Figure 2**: MSC pro-inflammatory and anti-inflammatory phenotypes. With an absence of inflammation in the microenvironment, MSCs activate T cells, adopting a pro-inflammatory profile. In the presence of inflammation, MSCs promote the production of regulatory T cells and switch to an anti-inflammatory profile. Adapted from [7]*

Activation, or licensing, of MSCs by exposing them to certain cytokines before they are used therapeutically has been considered to induce MSC-mediated immunosuppression towards a more robust profile of immune cells. This immunosuppressive effect is further enhanced when a combination of pro-inflammatory cytokines, usually TNFα, IFNγ, and IL-1β, are used as opposed to a singular agent [10-13]. MSC secretions of soluble immune factors can be found in **Figure 2**.

1.3 Complications with Clinical Translation of Mesenchymal Stromal Cells

Mesenchymal stromal cells have become a top source of stem cell type for clinical application for a variety of reasons. As previously mentioned, they have strong differentiation potential, are easily expanded in standard *in vitro* culture, can be sourced from a variety of tissue types, and remain viable throughout the cryopreservation and thawing process. They have also been found to display low immunogenicity, making them easily available for allogeneic transplantation without the need for immunosuppression [14]. Many clinical trials evaluating MSC therapy for various diseases have found success in treating various conditions, including "critical-limb ischemia, spinal cord injury, liver cirrhosis," chronic myocardial ischemia, Graft vs. Host Disease, ALS, Type I diabetes, etc. [15-19].

Although there are reported successes with using mesenchymal stromal cells clinically, there have also been complications in clinical findings giving inconsistent results and limited replicability. This has resulted in delayed market therapies and failed clinical translation. One reason is the biological variability in both MSCs and MSC products. This could be due to the differences in tissue source, as well as donor age [3]. Bone marrow-derived MSCs are currently the most frequently used in clinical settings [14]. Additionally, preclinical studies have found long term risks following administration of MSCs, including instigation of malignant tumor growth and immunosuppression [20]. A potential complication could also include the oversuppression of the immune system by MSCs. Patients who received MSC therapy did not developed Graft vs. Host Disease but did experience viral infections. While this may have been expected due to the nature of their

diseases, it could lead to concerns of MSC transplantations affecting the host's immune system against foreign agents [21].

Additionally, a recurring issue has been dosing and persistence of MSCs. Following intravenous infusion in mice, a large majority of the MSCs are no longer found in circulation but become trapped in the lung of the animal [22, 23]. Only a small number of the cells successfully migrate to the damaged tissue. Further, the intravenous infusion of MSCs has been found to display different degrees of procoagulant activity and trigger clot induction *in vivo*, which can compromise the safety of the MSC therapy [24-27]. For these reasons, current MSC research has focused on alternate methods for delivering MSC therapeutics while avoiding the complications with *in vivo* infusion.

Chapter II: Ex Vivo MSC Therapeutic Delivery

2.1 Alternate Mesenchymal Stromal Cell Therapeutic Delivery

2.1.1 Alternate Mesenchymal Stromal Cell Therapeutics

Due to the complications found with translating MSC properties to *in vivo* models, research has been done on alternate ways of increasing their bioavailability to human body and delivering the therapeutic benefits of MSCs without the complications with dosing and persistence. Further research into MSC-secreted exosomes function has found that the extracellular vesicles derived from MSCs are the primary source of MSC therapeutic function, mitigating T cell-proliferation at a dose-dependent level [28]. Both *in vitro* and *in vivo* results have been corroborated immune cell suppression, decreases in pro-inflammatory cytokines, and increases in anti-inflammatory cytokines from MSC-EV injection [29].

Additionally, a study has shown that MSC apoptosis is required for their immunosuppressive effects, and deletion of apoptotic favors attenuated these effects in *in vivo* disease models [30]. Though there has been conflicting research in this area, studies are being conducted researching the function of apoptotic MSCs.

Modification of MSCs is also being studied to increase site-specific delivery. Genetic modification via viral and nonviral vectors and nongenetic modification have become candidates for causing MSCs to express certain cytokines to slow tumor growth and exhibit anticancer activity [31]. These methods also have their complications. Certain groups have investigated "nanoparticle-encapsulated forms of drugs" to increase MSC drug-carrying capacity, allowing delivery of the drugs to the tumors for a longer duration [31].

2.1.2 Indirect Contact Mesenchymal Stromal Cell Therapeutic Delivery

Because MSCs primary therapeutic mechanism of action seems to be through their secretome, the secreted factors from the MSCs and resulting effect on immune cells are paramount to understand. Many current coculture methods use Transwell™ inserts to study a peripheral blood mononuclear (PBMC) and MSC coculture without direct cell contact as a model of human therapeutic conditions. Using this Transwell model, MSC secreted factors are able enact an inhibitory effect on the PBMC proliferation through the indirect contact with the cells [32]. Additionally, various studies have been conducted on the conditioned medium of the MSCs, or the medium the MSCs were cultured in. MSC-conditioned medium was found to improve hindlimb movement in spinal cord injured rats *in vivo* [33]. This further supports MSC's therapeutic effects being delivered via paracrine function.

These studies provide a solid argument for approaches to control the MSC secretome as a potent therapeutic to induce immunomodulation, while avoiding the complications that occur with *in vivo* administration.

2.2 Novel Engineered Approach for Delivering *Ex Vivo* MSC Therapeutics

The Parekkadan Lab has developed a method for administered MSC-derived molecules and secreted factors in an *ex vivo* manner via a hollow fiber bioreactor. In a scaled down model, there are nine hollow fibers per bioreactor that are fabricated from polyethersulfone (PES) with a lumen diameter of 500 μm and a pore size of 0.2 μm [34, 35]. The bioreactors have an inner port and an outer port. The outer port connects to the extraluminal space, where the cells seeded in the hollow fibers remain throughout the experiment. Media flows through hollow fiber-adhered cells via the inner port.

The bioreactor intra- and extracapillary spaces are first primed with sterile PBS. MSCs were thawed and suspended in culture medium. The cell suspension was syringe injected into the extraluminal space of the bioreactor. Culture media and cells flow through the bioreactor. The media flows out, but due to the pore size of the fibers, the MSCs are unable to be flushed out the other port, and therefore remain in the bioreactor. Once cells are seeded to the bioreactor, they incubated for 2 hours to allow for the cells to adhere to the fibers. They are then ready to be used. During the perfusion, culture media is flowed through the bioreactor. As it passes through cells, MSCs secrete various factors and cytokines into this media, which is then collected. Images of the cell-seeded bioreactors can be seen in **Figure 3.**

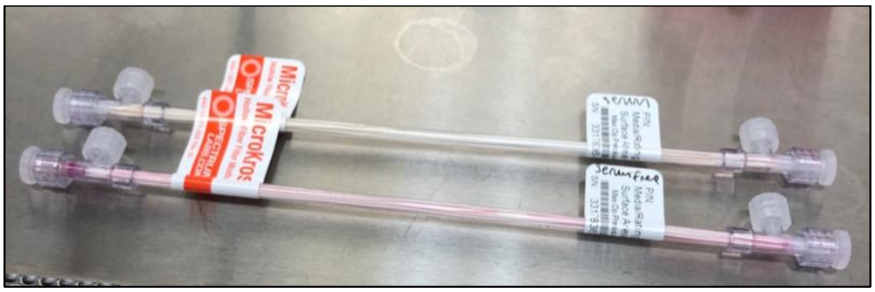


***Figure 3**: Hollow fiber bioreactors used in perfusion system. MSCs are seeded to the PES fibers, allowing culture media to flow through the bioreactor and collect secretions from the MSCs.*

Various studies have been conducted with the hollow fiber bioreactors to characterize and deliver the secreted factors from the MSCs. Li et al. found that bone marrow-MSCs adhere to the surface of the fibers and are viable in the bioreactor under flow conditions. Additionally, it was shown that cytokine and growth factor secretion was maintained in the bioreactor system via IL-6 and VEGF analysis at 24h and 48 timepoints. Finally, it was found that the secretions are able to retain potency when used in a potency assay with human PBMCs [34]. Further studies explored the use of these bioreactors for clinical therapeutics potential. Patients with severe acute kidney injury had their blood filtered to MSC-seeded bioreactors to explore the immunoregulatory mechanism of the cells. As a result, treated patients experienced greater IL-10 and less TNFα and IFNγ in their blood profile compared to control patients. Additionally, there were decreases in monocyte populations in treated patients [36]. This study corroborates that this *ex vivo* delivery system can successfully deliver MSC secreted factors to patients and successfully induce an immunotherapeutic response from the MSCs.

With this system, we are able to determine the profile of the MSC secretome in each fraction, giving us a deeper understanding of MSC behavior in the dynamic perfusion system. Once we understand the baseline MSC behavior, we can further use the system to

test how sensitive MSCs are to changes in the system via an introduction of inflammatory cytokines.

2.3 Objectives

2.3.1 Introduction of Dynamic Perfusion System

Previous research in the Parekkadan Lab has tested the scaled down hollow fiber bioreactor system in a long-term, closed-loop, continuous perfusion platform. In this system, they were able to show that "MSCs were viable, able to sense inflammatory signals and to dynamically respond with differential secreted factors" [35]. Additionally, after perfusion of T cells through the circuit, it was found that "increased perfusion time did not lead to more immunomodulation… the shorter time frame of MSC exposure (24 hours) led to the most significant anti-inflammatory effects" [35]. Samples are collected at "0, 0.5, 4, 17, 24, 48, 72, 96, 144, and 196 hours of perfusion" and at "0.5 and 20 hours post-stimulation" in perfusion.

This previous research has provided substantial background and understanding of the MSCs behavior and secretome in perfusion with the hollow fiber bioreactors. However, there is still room to explore the MSC behavior further. Primarily, the dynamic potency of the MSCs can be explored to understand MSC behavior over time. Additionally, being able to understand the potency and profile of the MSC secretome at more frequent intervals within a perfusion would be vital to understanding their behavior. To begin answering these questions, we used a dynamic one-way perfusion system engineered by the Parekkadan Lab. In this system, culture media is flowed through the MSC-seeded bioreactors at a constant flow rate via a syringe pump. Upon exiting the bioreactor, the media flows to a fraction collector to collect the media, with the MSC secretions, in fractions. This will

occur for the entirety of the perfusion. A diagram of the system can be viewed in **Figure 4**. Another image of the setup with an additional inlet stream can be viewed in **Figure 5**.

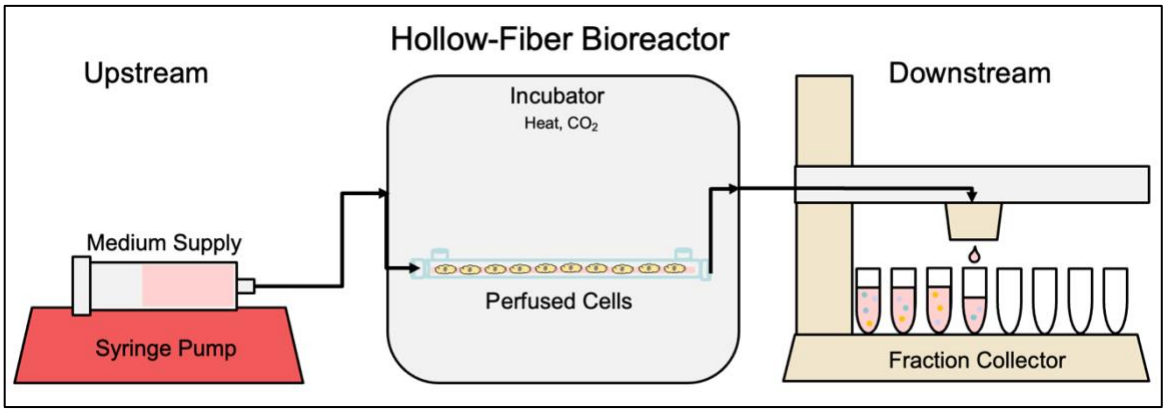


***Figure 4**: Dynamic perfusion system using hollow fiber bioreactors. Culture media is pumped via a syringe pump through tubing. Media flows through MSC-seeded bioreactors, collecting the secreted factors. Media is perfused into a fraction collector, where secretions are collected in various fractions to quantify the secretome profile in a time-bearing manner. Adapted from [37]*

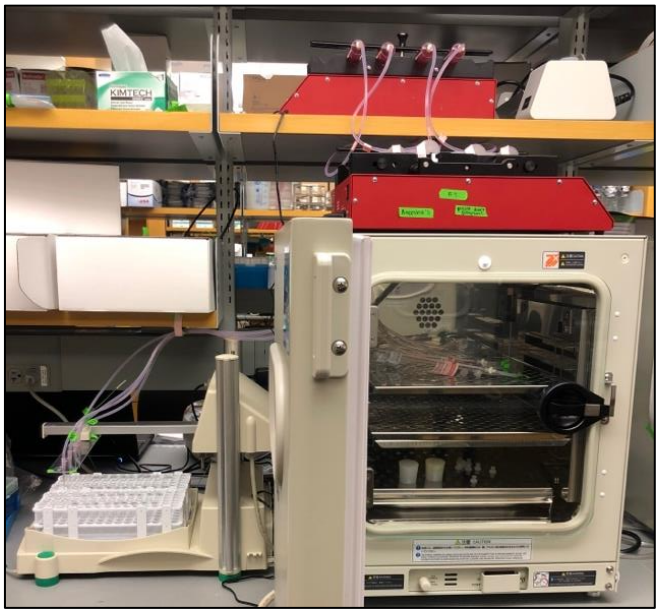


***Figure 5**: Two stream perfusion setup for modeling inflammation in patient. One pump contains untreated culture media and second pump contains cytokine-treated culture media. After 24 hours, step change is performed by changing input of one media to another to introduce it to the system without interrupting perfusion.*

This dynamic perfusion system using hollow fiber bioreactor enables greater visibility and control to the questions of persistence and dosing that have prevented clinical translation of mesenchymal stromal cells. There are three primary objectives we hoped to answer with this research: (1) to understand the baseline behavior and potency of MSCs in this system, (2) determine how the MSCs respond to inflammatory cytokines introduced via the system, and (3) use the system to determine how behavior of MSCs changes in response to being prelicensed, or activated, by exposure to inflammatory cytokines before being used in the system. Ultimately, further understanding into this system and the dynamic potency of MSCs will enable us to maximize their therapeutic potency, minimize side effects, and ultimately increase control over the therapeutic effects for the duration of ex vivo bioreactor treatment. We also could expand the use of the system to understand the dynamic behavior of other biological products.

2.3.2 Hypothesis

Based on the previous research by Allen et al., we propose a hypothesis that there is a time bearing dynamic to MSC potency. We further propose a second hypothesis that, MSCs would respond to proinflammatory cytokines stimulation by switching behavior and secreting greater anti-inflammatory cytokines, increasing their potency.

Chapter III: Baseline MSC Potency and Behavior

3.1 Experimental Conditions

The first study was conducted to understand the baseline behavior of mesenchymal stromal cells in the bioreactor perfusion system. To understand their behavior over a longer period, the perfusion was conducted for 72 hours. Culture media used was an exosome-depleted Alpha Minimum Essential Medium (α-MEM). Exosomes were removed from the Fetal Bovine Serum (FBS) to make the α-MEM via a recommended protocol of ultracentrifugation for 18 hours at 100,000g's [38]. Previous studies conducted with this system confirmed that MSCs were viable and function normally in the hollow fiber bioreactors after perfusion with exosome depleted α-MEM.

Bioreactors were seeded with $1x10^6$ and $3x10^6$ MSCs per device (n=2) and then perfused at 0.5ml/hour. Fractions of perfused media with the MSC supernatant were collected every 2 hours after perfusion began. Every 24 hours after perfusion began, completed fractions were collected and stored at 4°C until assayed.

3.2 Baseline MSC Potency and Behavior Evaluation

Three assays were chosen for the analysis to determine a robust and thorough understand of the potency of the MSC secretions and how it changes over time. The conditions for the assays and results are presented in the following subsections.

3.2.1 Cytotoxicity Assay of MSCs in Perfusion

Lactate Dehydrogenase (LDH) is an enzyme released by cells upon tissue damage after loss of cytoplasm [39]. Levels remain in the serum and can be measured to assess cell and tissue health in a system. To evaluate the dynamics of cell health and metabolism in the perfusion system, we performed a cytotoxicity assay by measuring the amount of LDH

secreted from cells in the bioreactor system. The fractions were measured via Cedex Bio Analyzer, and concentrations were plotted in **Figure 6**.

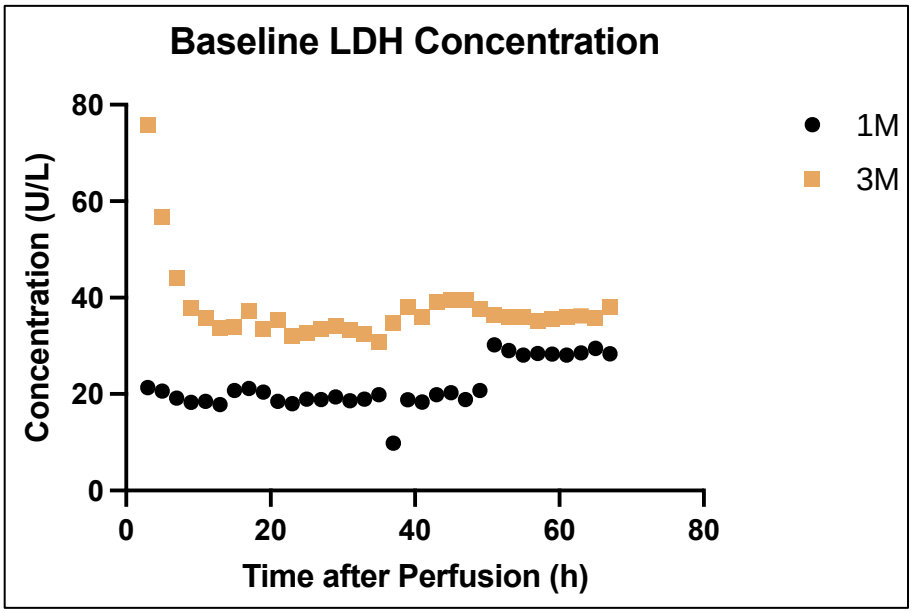


***Figure 6**: Cytotoxicity assay of MSCs in baseline perfusion. LDH (U/L) quantified directly from fractions after perfusion via Cedex Bio Analyzer. Concentrations from 1M cell group are plotted in black (●) and concentrations from 3M cell group are plotted in yellow (▪). LDH concentrations peak initially and stabilize for the remainder of perfusion.*

We can see results from the cytotoxicity assay of the baseline perfusion in **Figure 6**. From the 3M cell group, there is a high initial concentration of LDH secreted from the cells, at 74.75 U/L at 3 hours, with a sharp decline to 33.79 U/L at 10 hours. It remains around these values for the remainder of the perfusion, with a slight uptick to 39.66 U/L at 45 hours, where it remains consistent. The 1M cell group remains consistent from the initial concentration of 21.37 U/L at 3 hours to an increase to 30.24 U/L at 51 hours, where it remains consistent until the end of the perfusion.

3.2.2 Dynamic MSC Potency

To evaluate dynamic MSC potency, we conducted a potency assay using the fractions with MSC secretions on immune cells, specifically, peripheral blood

mononuclear cells (PBMCs) isolated from blood. In a high throughput manner, MSC secreted factors from fractions were plated with CFSE-stained and CD28- and CD3-stimulated PBMCs at a seeding density of 1 million cells/1ml medium. Conditioned medium from fractions were added after 24 hours of PBMC stimulation to emulate the inflammation that is likely in patients who would receive this *ex vivo* MSC therapy. PBMCs were cultured in 75ul of the conditioned fraction medium and 75ul of RPMI medium for 4 days before being evaluated via flow cytometry to determine percent proliferation. Using FlowJo software, the T cell population was selected from the PBMC population. Percent proliferation via percentage of CFSE+ cells of each cell group was compared to a control group which was PBMCs cultured in 75ul of RPMI and 75ul of unconditioned exosome-depleted α-MEM.

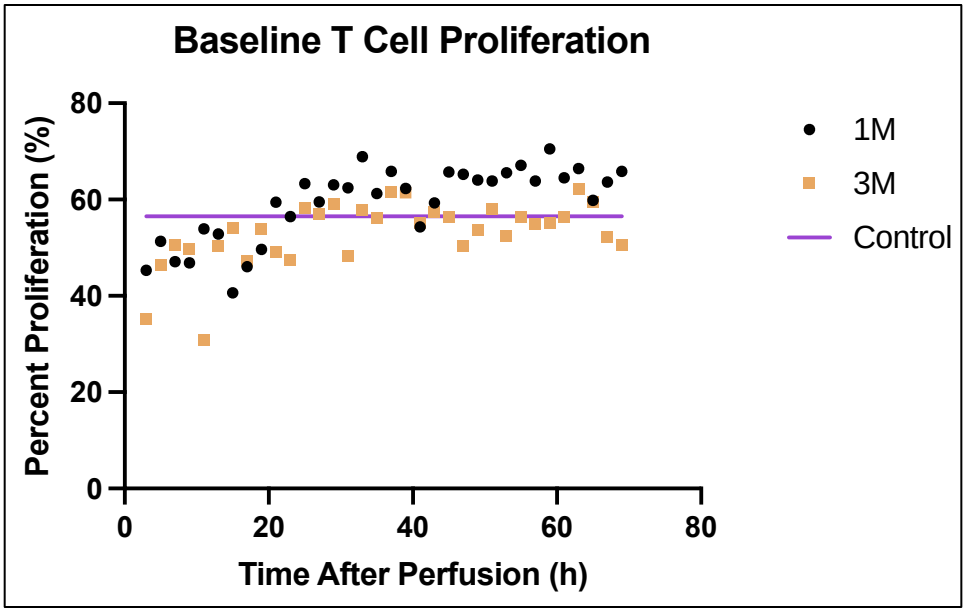


***Figure 7**: Percent proliferation of T cells after coculture with baseline perfusion fractions.*
PBMCs stained with CFSE, stimulated (CD3/CD28), and cocultured with baseline perfusion fractions for 3 days. T cell proliferation was evaluated and quantified via flow cytometry and FloJo. Fractions from first 24 hours of perfusion are more potent, and fractions from both groups lose potency after.

Proliferations from 1M cell group are plotted in black (•) and concentrations from 3M cell group are plotted in yellow (▪).

Figure 7 shows the percent proliferation of the T cells after coculture with the conditioned media from each fraction. The control group experienced a percent proliferation of 56.5%. From the figure, it is evident that the conditioned media of the fractions from the first 24 hours cause the highest T cell suppression. Percent proliferation is the least in the earlier fractions, with the lowest percent proliferation from 3M cells at 35.3% and from 1M cells at 45.3%. Percent proliferation of both groups increases after the first 24 hours. Later fractions from the 3M cell group oscillate close to the control value of 56.5%, not causing significant suppression or proliferation. Fractions from the 1M group after 24h appear to promote T cell proliferation, with the conditioned media at 57h causing 70.5% proliferation.

A one-way ANOVA was performed between the three groups: control, 1M cells, and 3M cells. From here, Tukey's Multiple Comparisons Test was performed between these groups to determine the differences between the means at three different time periods: 0 -24 hours, 25 – 48 hours, and 49 – 72 hours. The results can be viewed in the **Figure 8** below.

	0 – 24h	24 – 48h	49 – 72h
1M vs. 3M	ns	**	***
1M vs. Control	*	***	****
3M vs. Control	**	ns	ns

***Figure 8**: Tukey's Multiple Comparisons Test between three experimental groups. 3M vs. Control shows significance for first 24 hour fractions. 1M vs Control shows increasing significance in difference as fraction time increases. 1M vs. 3M shows significance in difference after 24 hour fractions.*

Within the first 24 hours, there is no significant difference between the 1M cell group and the 3M cell group, but there is greater significance between the 3M and control compared to the 1M and control, as these 3M fractions appear to be more potent than the 1M. For 24 – 72 hours, there is no significant difference between the 3M group and the control, as the later fractions are not as potent. There is a significant difference between the 1M and 3M groups and the 1M and control group, as the 1M cell group begins promoting proliferation after 25 hours until the end of perfusion. For the final 49 – 72h period, the significance of the 1M group compared to both groups increases compared to the 25 – 48 hours, indicating that these fractions become more proliferative in the final stage of the perfusion.

3.2.3 Secreted Factors from PBMCs and MSCs

After 4 days of culture, supernatants from the PBMC and conditioned media coculture were collected and frozen at -80°C until assayed using the Milliplex® Human Cytokine/Chemokine Magnetic Bead Panel with Luminex xMAP technology. Analytes selected included IFNγ, TNFα, IL-1β, IL-4, IL-6, IL-10, IL-1RA, TGFα, FGF-2, VEGF. Analytes were selected based on previous research that had found presence of these cytokines from the MSC secretome as well as PBMC secretions [32, 35]. IL-6 is predominantly secreted from MSCs. IFNγ, TNFα, IL-4, and IL-10, are predominantly secreted by the PBMCs. IL-1β and IL-1RA are secreted both by MSCs and PBMCs. Additionally, research into the relationship between IL-6 and IL-10 has found a positive correlation between the two cytokines and patients with an inflammatory condition [40]. Results can be seen in **Figure 9**.

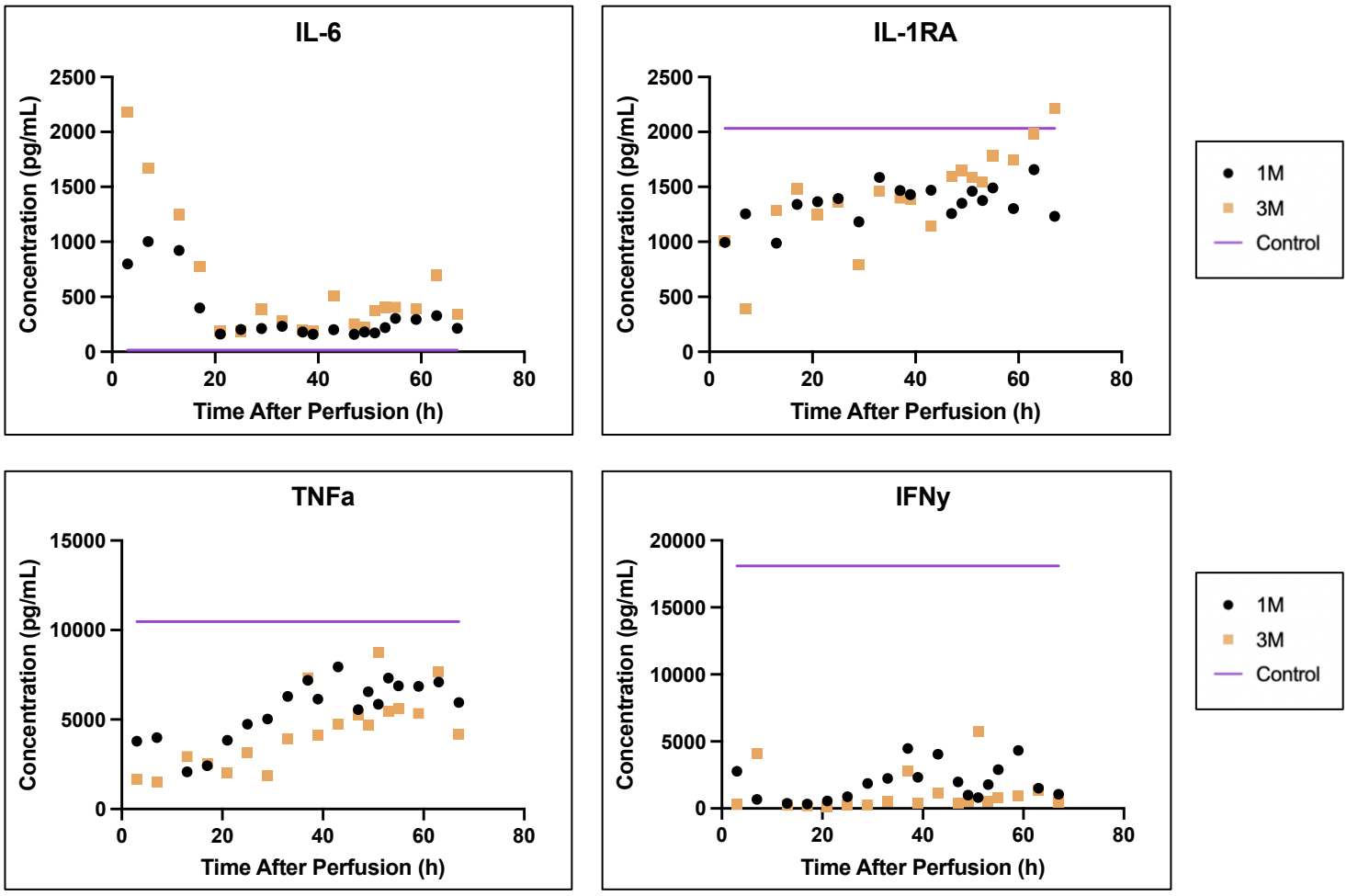


***Figure 9**: Secreted factors from coculture of stimulated PBMCs and MSC conditioned media from baseline perfusion.*
IL-6, IL-1RA, TNFα, and IFNγ concentrations are quantified (pg/mL). Concentrations from 1M cell group are plotted in black (●), concentrations from 3M cell group are plotted in yellow (▪), and control values are plotted in purple.

From **Figure 9**, we can begin to understand the dynamics of how the factors in the conditioned media affect the secret ion of factors from PBMCs. Looking at pro-inflammatory IL-6, we can see a high presence of IL-6 from the conditioned media in the earliest fraction, decreasing until about 20 hours before stabilizing, with a slight uptick at 59 hours. The 3M cell group produced the highest concentration at 3 hours of 2183.44 pg/mL. The 1M cell group produced a high of 1002.28 pg/mL at 3 hours before decreasing as well in later fractions. These values were all greater than the amount secreted from the

control group 15.38 pg/mL. For IL-1RA, there was an oscillatory increase in concentration of the anti-inflammatory cytokine, based on fraction, from a low of 395.36 pg/mL at the 7 hour fraction to a high of 2218.22 pg/mL in the 67 hour fraction while the 1M cell group remained more stable in the range of 995.76 to 1657.60 pg/mL. All but one of these values is under the concentration from the control group, 2032.66 pg/mL.

Looking at the TNFα, there is an increase in concentration based on the fraction number. Earlier fractions before 17 hours have lower concentrations, while fractions after this number have steadily increasing amounts. The 3M cell group has overall a lower concentration of the proinflammatory cytokine secreted by the PBMCs, with the lowest value of 1513.04 pg/mL to the highest of 8726.68 pg/mL from the 51 hour fraction, ending with 4161.7 pg/mL at the final 67 hour fraction. The 1M cell group experienced higher concentrations, from a low of 2083.42 pg/mL at 13 hours, rising to a high of 7939.00 pg/mL at the 48 hour fraction, finishing with 5950.62 pg/mL at the 67 hour fraction. Both groups remain under the concentration of TNFα secreted by the control well, 10474.28 pg/mL. Overall, we have established a baseline state of dynamic factors secreted by the MSCs at different times throughout the perfusion cultured with stimulated PBMCs.

3.3 Discussion

This study has enabled us to establish a baseline for dynamic MSC potency and secreted factors in the perfusion bioreactor system. From the cytotoxicity assay, we could see that the LDH concentration in both groups was initially high, perhaps as a response to the seeding procedure but eventually stabilizes with a slight uptick towards the end of perfusion, giving confidence that the cells are viable and metabolically active throughout the experiment.

From the potency assay, we could see how the percent proliferation of the T cells changed based on the fraction. Cells cultured with fractions from before 24 hours for both groups experienced the greatest degrees of suppression. After 24 hours, the 1M cell group appears to switch behavior to a proliferative state, promoting T cell proliferation over the control group. This is expected, as it has been reported that MSCs can switch behavior depending on their microenvironments [7]. As these cells were not in an inflammatory microenvironment, it is likely that they began to promote T cell proliferation after an initial suppression period.

From the Tukey's Multiple Comparison Test conducted, preliminary conclusions suggest that MSC therapies are dose dependent, as the 3M cell group had a greater immunosuppressive effect and concentrations of a greater magnitude compared to the 1M cell group. The differences between the control and 1M group differ after 25 hours of perfusion, as these fractions become proliferative, while the control and 3M group do not have statistically significant differences.

This is further corroborated by an increase in TNFα secreted by the PBMCs in response to factors in the MSCs, as the concentration rises throughout the duration of the perfusion. Additionally, the IL-6 concentration is initially high but decreases after 24 hours, likely in an inverse response to the concentration of TNFα or in response to the IL-10 [41]. The IL-1RA also increases after about 48 hours towards the end of perfusion.

From establishing the baseline state of MSC function in the system, we have determined that there are indeed time-varying changes in MSC secreted factor potency. Further changes can be made in the upstream conditions, like adding inflammatory cytokines to our culture media, and the MSC behavior can be quantified. We can use our

system to model various states of inflammation in patients and determine how the MSC response in potency and secreted factors change in response.

Chapter IV: MSC Behavior in Response to Inflammatory Signals Introduced in Perfusion

4.1 Experimental Conditions

With an understanding of the baseline MSC potency and behavior in our dynamic perfusion system, we began to consider how to evaluate its therapeutic benefits. The purpose of this study was to model an inflamed patient and measure how the cells respond to this upstream change. To do so, exosome-depleted α-MEM was treated with a cytokine cocktail: 10 ng/mL of IFNγ, 10ng/ml of TNFα, and 10 ng/mL of IL-1β [10-12].

Bioreactors were seeded with $3x10^6$ MSCs per device (n=4) and then perfused at 0.5ml/hour. For the first 24 hours of perfusion, bioreactors with 3M cells (n=4) were perfused with untreated exosome-depleted α-MEM. At 24 hours, we conducted a step change and began perfusing the treated cytokine media throughout the system. Fractions of perfused media with the MSC supernatant were collected every 2 hours after perfusion began. Every 24 hours after perfusion began, completed fractions were collected and stored at 4°C until assayed.

4.2 MSC Potency and Behavior Evaluation

Based on previous Residence Time Distribution experiments, it is estimated that it took the cytokine media about 5 hours to flow from the syringe pump the beginning of the bioreactor. Therefore, we can assume that our signal reached the bioreactors around 29 hours of perfusion and began reacting with the MSCs at this time.

We conducted the same three assays as the baseline perfusion to compare the outcomes and behaviors of the MSCs to the baseline state. The conditions for the assays and results are presented in the following paragraphs.

4.2.1 Cytotoxicity Assay of MSCs in Perfusion in Inflamed Model

To evaluate the dynamics of cell health and metabolism in the perfusion system and in response to the inflammatory cytokines, we performed an identical cytotoxicity assay by measuring the amount of LDH secreted from cells in the bioreactor system. The fractions were measured via Cedex Bio Analyzer. Results can be seen in **Figure 10**.

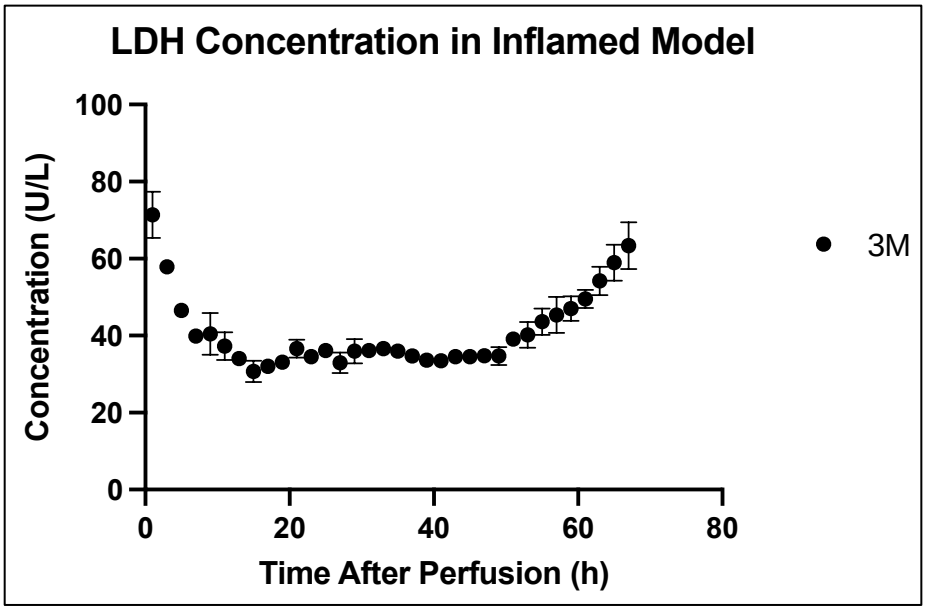


***Figure 10**: Cytotoxicity assay of MSCs in inflamed model. LDH (U/L) quantified directly from fractions after perfusion via Cedex Bio Analyzer. Concentrations from 3M cell group are plotted in black (●). LDH concentrations peak initially and stabilize before rising again towards the end of perfusion.*

From **Figure 10**, there is a similar high initial concentration of LDH secreted from the cells as the previous baseline study. In the first fraction, the concentration was 71.37 U/L. This number declines steadily to 30.75 U/L at 15 hours, where it remains between 31 U/L and 35 U/L until 51 hours. At 51 hours the LDH concentration begins to increase steadily again, from 39.11 U/L to 63.34 U/L at 67 hours of perfusion.

4.2.2 Dynamic MSC Potency

To evaluate the potency of this conditioned media, we conducted an identical potency assay using the fractions with MSC secretions on PBMCs as in 3.2.1 CFSE-stained and CD28- and CD3-stimulated PBMCs were cultured with the conditioned media for 4 days and evaluated using flow cytometry to determine percent proliferation. Results from the assay can be seen in **Figure 11**.

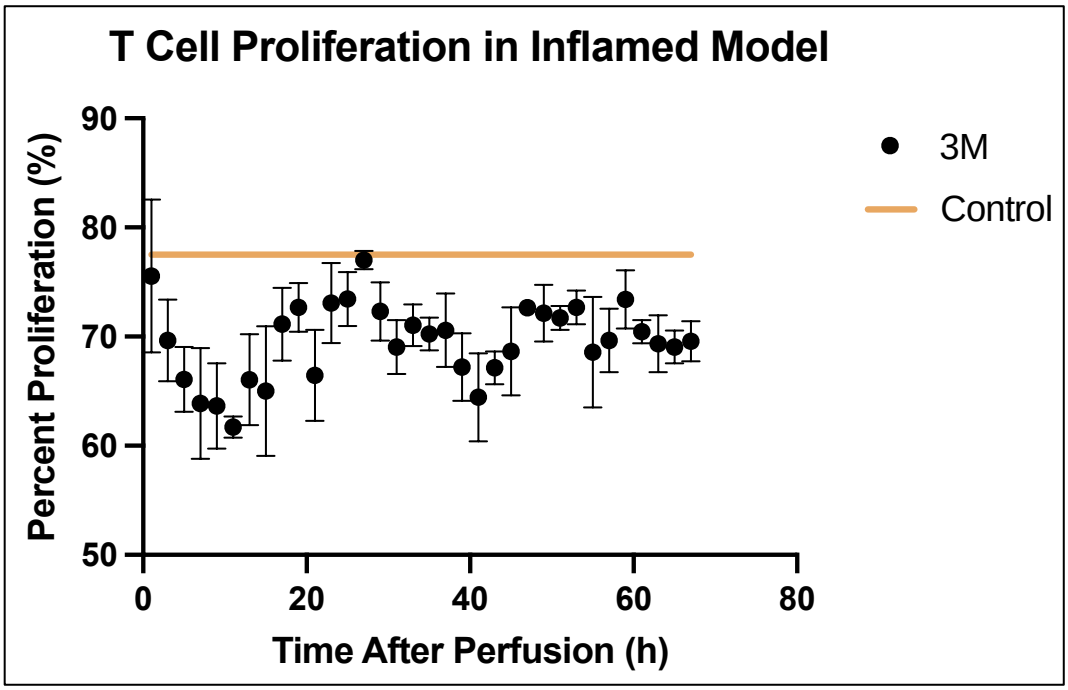


***Figure 11**: Percent proliferation of T cells after coculture with fractions from inflamed model.*
PBMCs stained with CFSE, stimulated (CD3/CD28), and cocultured with baseline perfusion fractions for 3 days. T cell proliferation was evaluated and quantified via flow cytometry and FloJo. Proliferations from 3M cell group are plotted in black (•) and control value plotted in yellow. MSC fractions are potent during first 24 hours, similar to baseline. After introduction of cytokine cocktail (10 ng/mL IFNγ, 10ng/ml TNFα, and 10 ng/mL IL-1β) at 24h, MSC experiences behavior switch. Potency is reactivated and continues throughout the later fractions.

In **Figure 11**, the percent proliferation of all the T cells cultured with the conditioned media from each well is less than the control proliferation of 77.5%. The greatest proliferation is 77% at 27 hours and the lowest is 61.7% at 11 hours. Percent

proliferation decreases from 75.5% at the 2 hour fraction to 61.7% at the 11 hour fraction. It then begins to rise again in the fractions from 13 - 27 hours up to 77%. After this point, the percent proliferation begins to decrease again until 64.45% for the 41 hour fraction before rising again and remaining in the range of 67.15% - 73.4% for the remainder of the perfusion.

4.2.3 Secreted Factors from PBMCs and MSCs

After 4 days of culture, supernatants from the PBMC and conditioned media coculture were collected and frozen at -80°C until assayed using the Milliplex® Human Cytokine/Chemokine Magnetic Bead Panel with Luminex xMAP technology. Analytes selected included IFNγ, TNFα, IL-1β, IL-4, IL-6, IL-10, IL-1RA, TGFα, FGF-2, VEGF. Results can be seen in **Figure 12.**

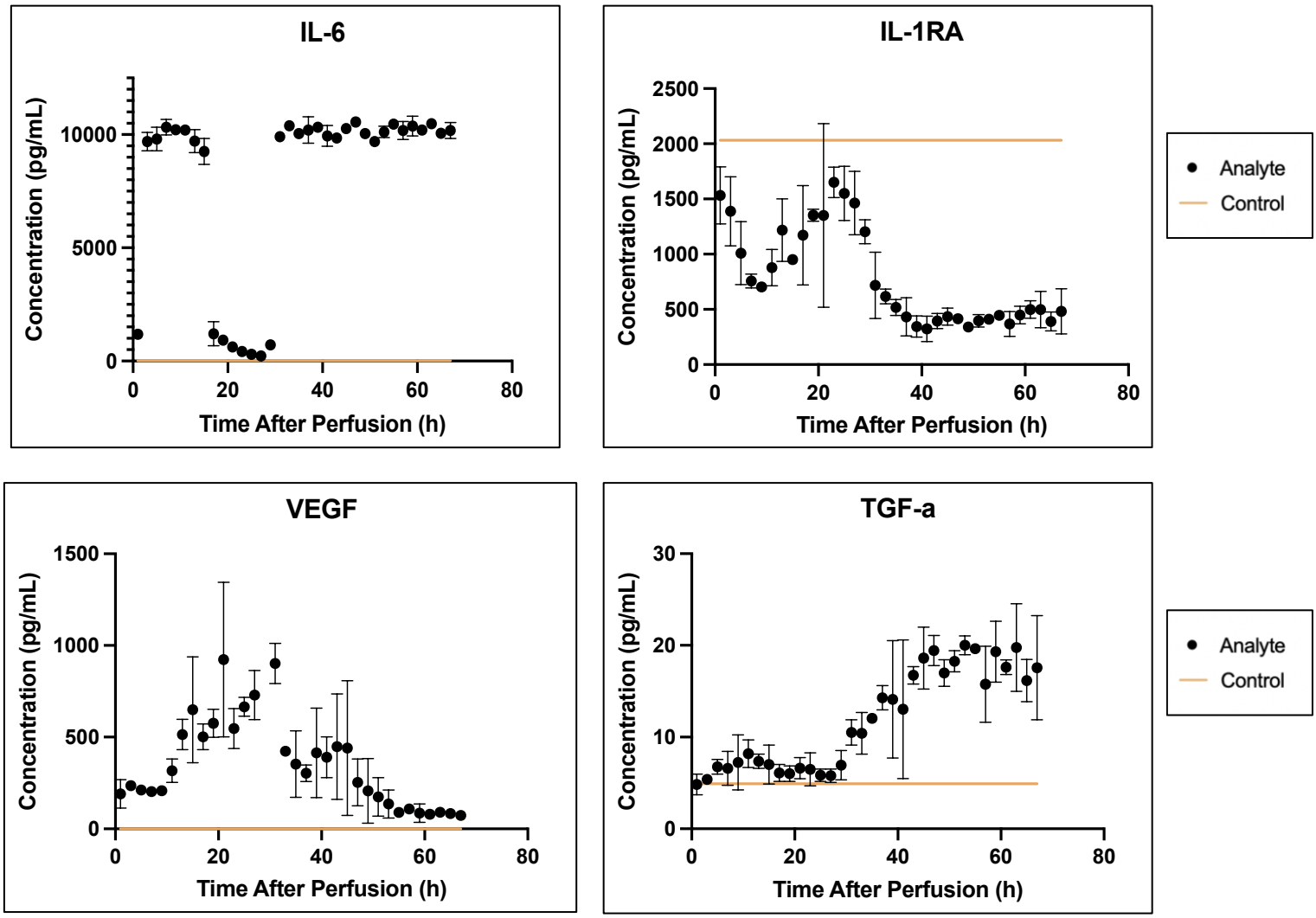


***Figure 12**: Secreted factors from coculture of stimulated PBMCs and MSC conditioned media from inflamed model.*
IL-6, IL-1RA, VEGF, and TGF-α concentrations are quantified (pg/mL). Concentrations from 3M cell group are plotted in black (●) and control values are plotted in yellow. Changes in concentrations of cytokines correlates with introduction of cytokine cocktail into perfusion system.

After analysis of the secreted factors from the PBMC and MSC conditioned media coculture, we can compare the dynamics of these factors to the baseline secretions in **Figure 12.** For IL-6, there is a similar increase in IL-6 concentrations at a similar time range as the previous perfusion. IL-6 concentrations begin at 1183.09 at the 1 hour fraction, range from 9259.25 pg/mL - 10324.8 pg/mL from the 3 hour – 15 hours, decreasing back

between 1576.33 at 17 hours and dropping to 231.79 pg/mL at 27 hours, then rising to 630.80 pg/mL at 29 hours and increasing back around 10000 pg/mL for the remainder of the fractions.

Looking at the anti-inflammatory cytokines in the analysis, there appears to be a correlation between the IL-1RA secretion and the concentration of pro-inflammatory cytokines. All wells with PBMCs and conditioned media contained less IL-1RA than the control value of 2032.66 pg/mL. The concentration begins at 1716.29 pg/mL at 1 hours, decreasing to 702.29 pg/mL at 7 hours. It then increases to 101.1651.38 pg/mL at 23 hours. From here, there is a decrease to 324.21 pg/mL at 41 hours, where it slightly increases for the remainder of the perfusion, remaining under 500 pg/mL.

For TGFα, the concentration from the 1 hour – 29 hour fractions remains between 5.38 – 8.175 pg/mL. At the 31 hour fraction, the concentration increases to 10.525 pg/mL and continues to rise for the remainder of the perfusion, reaching a maximum of 20.025 pg/mL at the 55 hour fraction.

Finally, for VEGF, there is a steadily increasing concentration up to the 31 hour fractions, with the 1 hour fraction having a concentration of 191.1 pg/mL and the 31 hour fraction having a concentration of 901.70 pg/mL. After this point, the concentration drops down to 423.14 pg/mL at the 33 hour fraction and continues to steadily drop for the remainder of the perfusion to 73.07 pg/mL at the last fraction.

4.3 Discussion

This study has allowed us to understand how the baseline behavior of MSCs in the dynamic perfusion system change in response to inflammatory cytokines IFNγ, IL-1β, and TNFα. These cytokines are commonly found in patients with inflammation, and this

response of MSCs could shed light on how MSCs in the bioreactor would behave when used on patients with a large amount of these cytokines in their system. Based on the potency assay and secreted factors, we can see that the inflammatory cytokines reach the bioreactors and MSCs around 29 hours of perfusion, as this is likely the time it took for the media to reach the fraction collector from the syringe pump. Many of the behavior changes or response to these cytokines happens around the 29 hour fractions.

After analyzing the LDH concentration, the results for the first 49 hours are consistent with the baseline perfusion. There is an initial high concentration, perhaps from the seeding procedure, which quickly stabilizes to a similar concentration as the baseline. However, the increase in concentration for the last 24 hours of perfusion could indicate the cells' metabolism was damaged in response to the increased inflammation. However, this does not affect the potency of these fractions

From the potency assay we could see that the PBMCs cultured with the conditioned media experienced less proliferation than the control, for every reaction. At 29 hours, it appears that instead of behavior switching back into a pro-inflammatory state, the MSCs become activated and began to take on an anti-inflammatory response and producing factors to suppress PBMCs [7, 9]. Therefore, after the 29 hours there is another decrease in PBMC proliferation, which remains the most potent up to 41 hours of perfusion. The potency was still lowest within the first 24 hours but experienced a close low point at 41 hours. In response to inflammation, MSCs secrete factors that suppress immune cells at a high potency.

From the secreted factors, we can see changes in cytokine concentration correlated with the introduction of the cytokine media. There is an increase in proinflammatory IL-6

in response to the inflammation in the media. For the anti-inflammatory cytokine IL-1RA, there is an apparent response to the inflammation. After the 29 hour fraction, there is a sharp decrease in the concentration of IL-1RA, likely in response to the increase in IL-1β concentration, as it is unable to significant block the activity [42]. For TGFα, there is a steady increase in concentration after the 29 hour fractions in response to the cocktail media. Finally for VEGF, there is a steady increase concentration before the 31 hour fraction, and then sharp decrease in concentration from the 31 hour fraction until the end of perfusion. Overall, we can see that many cytokines secreted by MSCs and PBMCs change in response to inflammatory cytokines introduced in the system. Changes in the MSC secretions promoted TGFα and IL-6 concentrations to increase while simultaneously prompting IL-1RA and VEGF concentrations to decrease.

Overall, this system has enabled us to understand that the baseline anti-inflammatory properties of MSCs can be extended in an inflammatory environment, as the fractions were all potent to cause suppression below the control in the potency assay. Additionally, in response to inflammation, MSCs do experience a behavior switch to express anti-inflammatory properties. This system can be used for patients in an inflamed state to deliver greater anti-inflammatory therapeutic effects from the MSCs while maximizing control over the dose and duration of the delivered therapeutic.

Chapter V: MSC Behavior after Prelicensing with Inflammatory Signals

5.1 Experimental Conditions

For the final study of this thesis, we wanted to determine the effects of prelicensing or activating the MSCs before they were inserted into the bioreactor system. As previous research has found, and as our last study showed, exposing MSCs to inflammatory cytokines changes their behavior to an anti-inflammatory state, promoting further suppression of immune cells [5]. Additionally, it has been found that prelicensing the cells, or exposing them to inflammatory cytokines before using them for a cell therapy purpose promotes an anti-inflammatory profile that is then maintained in further potency assays [11, 43, 44]. This study was designed to understand whether MSC prelicensing could enhance their potency, and if so, to determine how long this potency could be maintained for.

To conduct this study, exosome-depleted α-MEM was treated with a cytokine cocktail containing 10 ng/mL of IFNγ, 10ng/ml of TNFα, and 10 ng/mL of IL-1β [10-12]. MSCs were plated and cultured for 24 hours in this cocktail media. After 24 hours, MSCs were trypsinized and seeded to the bioreactors (n=4), with 3M cells per bioreactor. Bioreactors were connected to the dynamic perfusion system and perfused for 72 hours with untreated exosome-depleted α-MEM at 0.5 ml/hour. Fractions of perfused media with the MSC supernatant were collected every 2 hours after perfusion began. Every 24 hours after perfusion began, completed fractions were collected and stored at 4°C until assayed.

5.2 Prelicensed MSC Potency and Behavior Evaluation

We conducted the same three assays as the baseline perfusion to compare the outcomes and behaviors of the MSCs to the baseline state. The conditions for the assays and results are presented in the following paragraphs.

5.2.1 Cytotoxicity Assay of Prelicensed MSCs

To evaluate the dynamics of cell health and metabolism in the perfusion system after prelicensing the cells, we performed an identical cytotoxicity assay by measuring the amount of LDH secreted from cells in the bioreactor system. The fractions were measured via Cedex Bio Analyzer.

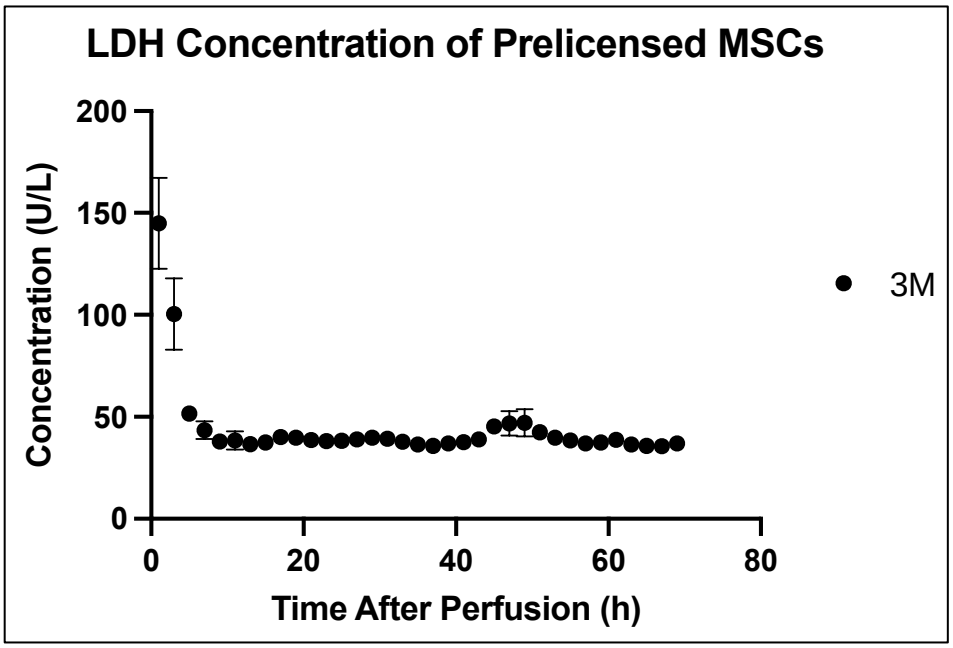


***Figure 13**: Cytotoxicity assay of prelicensed MSCs during perfusion. LDH (U/L) quantified directly from fractions after perfusion via Cedex Bio Analyzer. Concentrations from 3M cell group are plotted in black (●). LDH concentrations peak initially and stabilize throughout perfusion.*

As shown in **Figure 13**, the LDH begins around 150 U/L at 1 hours of perfusion and quickly decreases to 39.91 U/L by 7 hours of perfusion. Similar to the previous two cytotoxicity assays, the level begins high and quickly drops to a stable level. For the

remainder of the perfusion the level remains stable within a range of 35.83 – 47.05 U/L until the end of the perfusion.

5.2.2 Dynamic Potency of Prelicensed MSCs

To evaluate the potency of the fractions from this study, we conducted an identical potency assay using the fractions with MSC secretions on PBMCs as in 3.2.1 CFSE-stained and CD28- and CD3-stimulated PBMC were cultured with the conditioned media for 4 days and evaluated using flow cytometry to determine percent proliferation. Results from the assay can be seen in **Figure 14.**

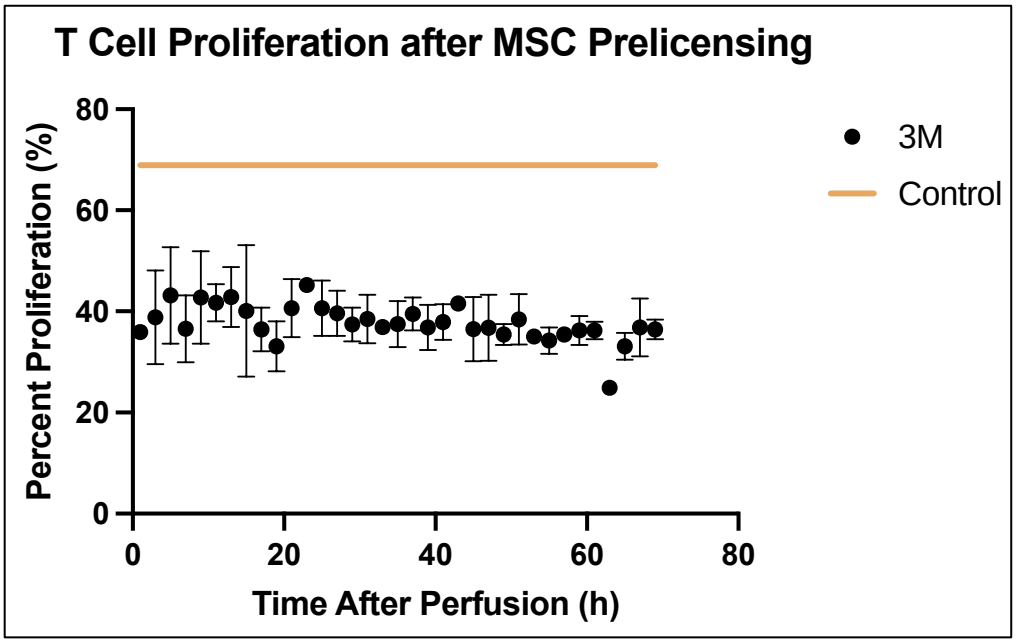


Figure 14*: Percent proliferation of T cells after coculture with fractions from prelicensed MSCs.*
PBMCs stained with CFSE, stimulated (CD3/CD28), and cocultured with baseline perfusion fractions for 3 days. T cell proliferation was evaluated and quantified via flow cytometry and FloJo. Proliferations from 3M cell group are plotted in black (•) and control value plotted in yellow. MSC fractions are most potent after prelicensing with cytokine cocktail (10 ng/mL IFNγ, 10ng/ml TNFα, and 10 ng/mL IL-1β). Potency is greatest in this model and maintained throughout the perfusion.

From **Figure 14**, we can see that the percent proliferation of all the fractions is greatly under the control percent proliferation of 68.9%, compared to the previous potency assays. The percent proliferations from each of the fractions appear more stable do not vary greatly from each other, as they are all within the range of 24.9% - 45.2% for the entirety of the perfusion.

5.2.3 Secreted Factors from PBMCs and Prelicensed MSCs

After 4 days of culture, supernatants from the PBMC and conditioned media coculture were collected and frozen at -80°C until assayed using the Milliplex® Human Cytokine/Chemokine Magnetic Bead Panel with Luminex xMAP technology. Analytes selected included IFNγ, TNFα, IL-1β, IL-4, IL-6, IL-10, IL-1RA, TGF-a, FGF-2, VEGF. Results can be seen in **Figure 15.**

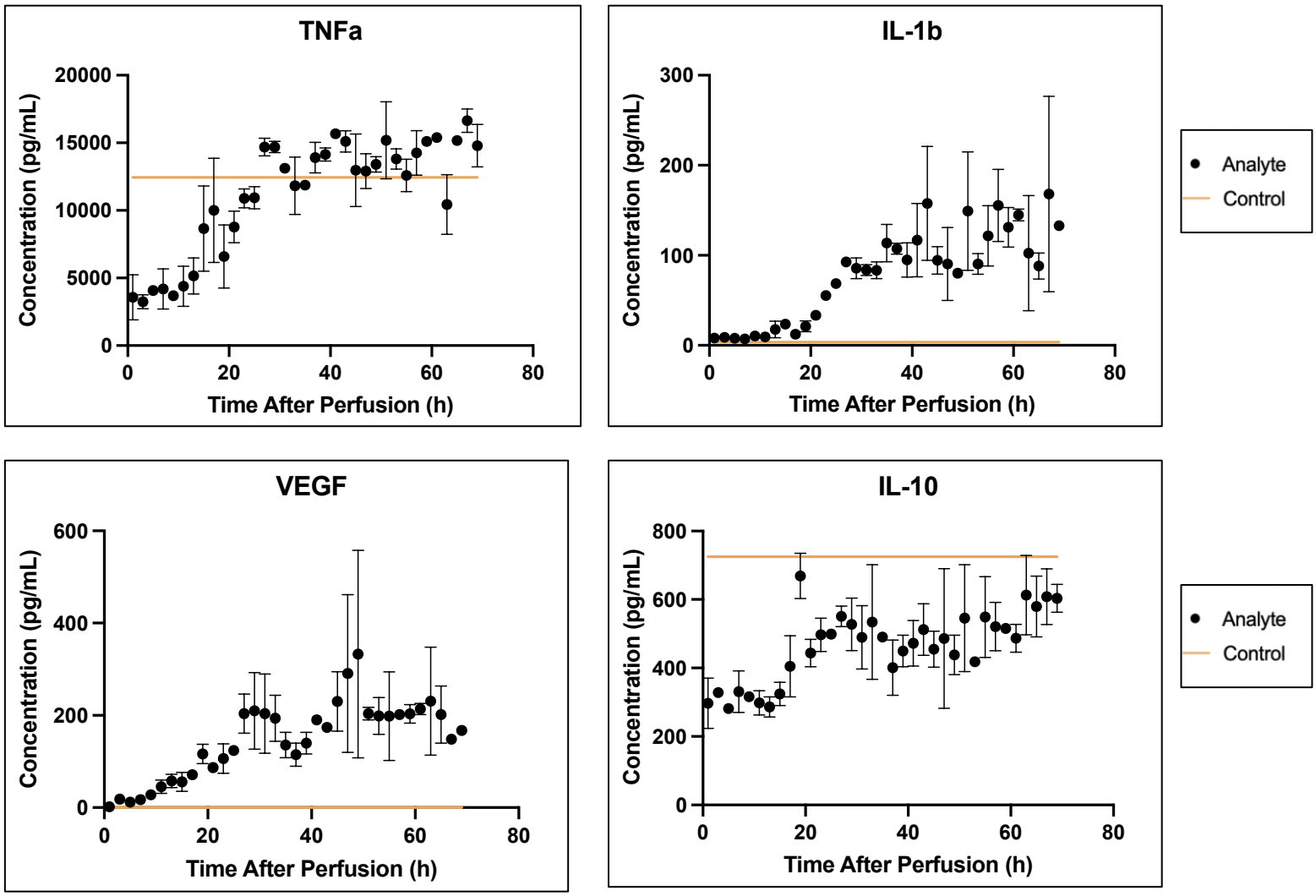


***Figure 15**: Secreted factors from coculture of stimulated PBMCs and prelicensed MSC conditioned media.*
TNFα, IL-1β, VEGF, and IL-10 concentrations are quantified (pg/mL). Concentrations from 3M cell group are plotted in black (●) and control values are plotted in yellow. Increases in cytokine concentrations increase after the 25 hour fractions.

From **Figure 15**, we can see the factors secreted from the prelicensed MSCs cultured with PBMCs. Looking at the proinflammatory cytokines, both TNFα and IL-1β increase as fraction number (time after perfusion) increases. Later fractions have greater amounts of the proinflammatory cytokines. The lowest concentration of TNFα occurs in the 3 hour fraction at 3241.51 pg/mL. The concentration steadily increases, passing the control concentration of 12438.2 pg/mL after the 25 hour fraction. Fractions after this point

are generally higher than the control, with the greatest concentration of the cytokine occurring at 16646.6 pg/mL at 67h. Similarly, concentrations of IL-1β are significantly lower in the earlier fractions and begin to rise after the 19 hour fraction. The number continues to consistently rise and then begins to oscillate between 83.41 pg/mL and 168.19 pg/mL from the 33 hour fraction to the 67 hour fraction. For IL-6, the concentration remains relatively consistent around 10,000 pg/mL except for the 9-21 hour fractions, where the concentrations range from 5671.54 pg/mL – 8340.72 pg/mL.

We can see increases in anti-inflammatory Il-10 in later fractions as well. The concentration is relatively stable and low for the first 1 – 15 hour fractions, and after the 16 hour fractions, the concentration begins to rise. For IL-10, the 17 – 24 hour fractions rise in concentration to 550.75 pg/mL at 27 hours, then begin to decrease. At the 37 hour fraction, the concentration is 400.99 pg/mL. The concentration begins to slightly rise again remaining under 612.90 pg/mL for the remainder of the perfusion.

Additionally, VEGF concentrations remain low for first 17 hour fractions, from a concentration of 1.91 pg/mL at the 1 hour fraction to 71.36 pg/mL at the 17 hour fraction. From here, the concentration begins to rise more sharply, reaching 332.56 pg/mL at the 49 hour fraction. From here it decreases slightly, remaining around 200 pg/mL for the remainder of the perfusion.

5.3 Discussion

This study was designed to explore the dynamic potency and secreted factors of prelicensed MSCs in the bioreactor perfusion system. Prelicensing, or activating, MSCs switches their behavior to an anti-inflammatory state before they are used in a cell therapy product, enabling them to be in the most potent state from the start of the therapy.

From the cytotoxicity assay, we can see that there is an initial high concentration of LDH, but that quickly stabilizes for the remainder of perfusion to a value like the first cytotoxicity study. It is notable that the concentration of LDH is slightly higher with the prelicensed cells compared to the unstimulated cells. The initial peak is closer to 150U/L as opposed to 80 U/L, and the stable concentration is closer to 50 U/L as opposed to 39 U/L. However, this does not appear to reduce their potency, and it could just be a side effect of the prelicensing. This indicates the health of the cells is maintained once they are acclimated to the bioreactor system after the prelicensing procedure.

The potency assay suggests that this form of activating the MSCs has the greatest effect on suppression of T cells when cultured when MSC secretions. Percent proliferation was lowest in this assay compared to the previous two potency assays, and potency was maintained for longer than 24 hours. Additionally, the differences in potency between each fraction appeared to be more stable and less dynamic than the previous assays, indicating that prelicensing promotes a more stable MSC potency.

From the secreted factors, many changes in cytokine concentration occurred in fractions from 19 hours and later. Both pro-inflammatory TNFα and IL-1β concentrations increased in these later fractions. Anti-inflammatory IL-10 concentrations rose in response as well. Additionally, VEGF concentrations began increasing sharply around the 19 hour fraction until around 49 hours of perfusion. Though the percent proliferation was lowest in this study, and the value was relatively stable in all the fractions, due to the lower concentration of pro-inflammatory cytokines, a dose duration of 24 hours still appears to be best for patients, enabling a potent therapeutic response without the risk of producing other cytokines. MSC behavior appears to change after 19-24 hours of being in the

perfusion system, primarily in terms of the secreted factors produced from the coculture with PBMCs. This could be because the MSCs secretions are responding to the lack of inflammation in the microenvironment in the later fractions. However, we can see that this does not affect the overall potency of the cells in the system. Overall, there is a time bearing dynamic of certain cytokines in response to prelicensed MSCs, and most of the fractions increase in concentration after around 19h of perfusion.

Overall, prelicensing MSCs enables the most stable potency and action of the cells when they are used in the *ex vivo* bioreactor system. The potency of the cells is the least dynamic and highest when cells are prelicensed and activated before being used in the therapy. When secreted factors are cultured with PBMCs, the lower cytokine concentrations in the earlier fractions support the 24 hour duration of therapy to obtain the most therapeutic benefit of the system.

Chapter VI: Conclusion

As the development of MSC therapy continues, there is increasing evidence supporting the use of an *ex vivo* MSC products to utilize the immunomodulatory benefits of their secreted factors while avoiding complications from *in vivo* delivery. An *ex vivo* bioreactor perfusion system allows a controlled delivery of MSC secreted factors into a patient to optimize their potency and therapeutic benefit. These studies were conducted to understand the MSC behavior in the bioreactor delivery system and optimize the benefits of the therapy, enhancing MSC potency and giving more control over the duration of the therapy.

Based on previous research, a hypothesis was proposed there is a time bearing dynamic to MSC potency. This hypothesis was supported by the potency assay of the baseline perfusion, as the baseline MSCs were potent and exhibited PBMC suppression amongst the first 24 hours. However, based on cell dosage, after 24 hours this potency was reduced and the cells had either no effect on the proliferation or experienced a behavior switching to begin promoting proliferation, as previously suggested [6, 7, 9]. Both pro- and anti-inflammatory cytokine secretion was lowest in the baseline unstimulated model compared to the other two studies.

A second hypothesis was proposed that MSC potency would be change after exposure to proinflammatory cytokines, both when introduced in the bioreactor system and when MSCs were prelicensed with these cytokines. This was most evident in the two potency assays conducted where percent proliferation from all the fractions were under the control proliferation, and potency was enhanced and strongest after MSC prelicensing. With cytokines introduced within the system, there was a greater concentration of

responding cytokines secreted by both the MSCs in the bioreactor system as well as the PBMCs during the coculture.

To translate these conclusions to clinical applications, from the studies conducted, it is evident that to obtain the most potent and therapeutic effect of the MSCs, prelicensing the MSCs with inflammatory cytokines for 24 hours before introducing them to the patient via bioreactor would activate them, ensuring greatest suppression of PBMCs and secretion of anti-inflammatory cytokines. Additionally, though potency is maintained for the duration of the 72 hour perfusion, dosing the patients for a duration of 24 hours with this therapy would ensure maximum suppression of PBMCs and a minimum production of proinflammatory cytokines secreted.

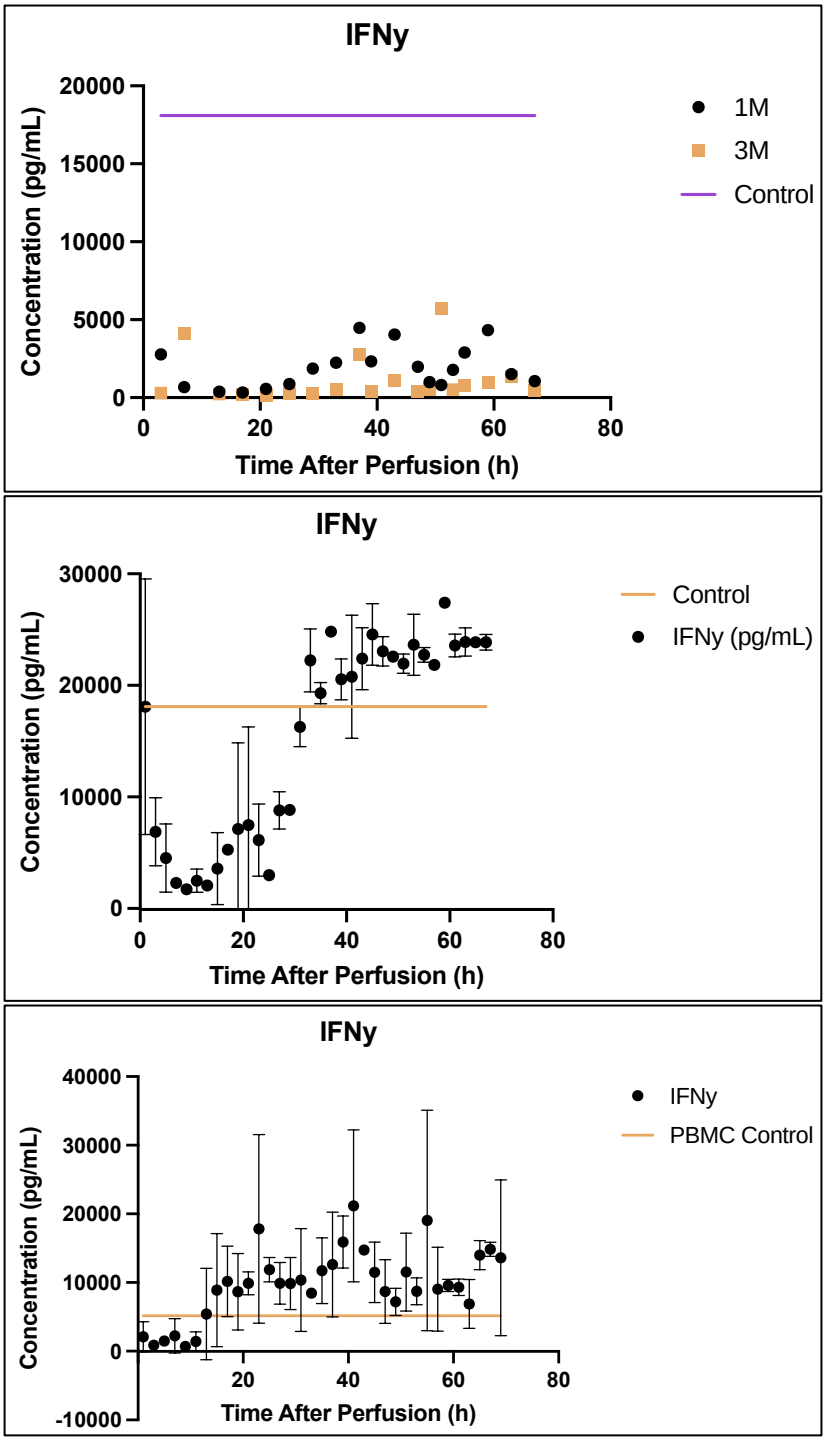
Further understanding of the dynamic behavior of MSCs in this bioreactor system can allow for a more controlled and effective therapy when the device is used clinically. With a deeper understanding of the ideal duration for delivering this therapy, we are furthering the use of mesenchymal stromal cells as a powerful source for an *ex vivo* cell therapy product for autoimmune disease and inflammation.

Supplementary Figures

Baseline Perfusion

Inflamed Model

Prelicensed Model

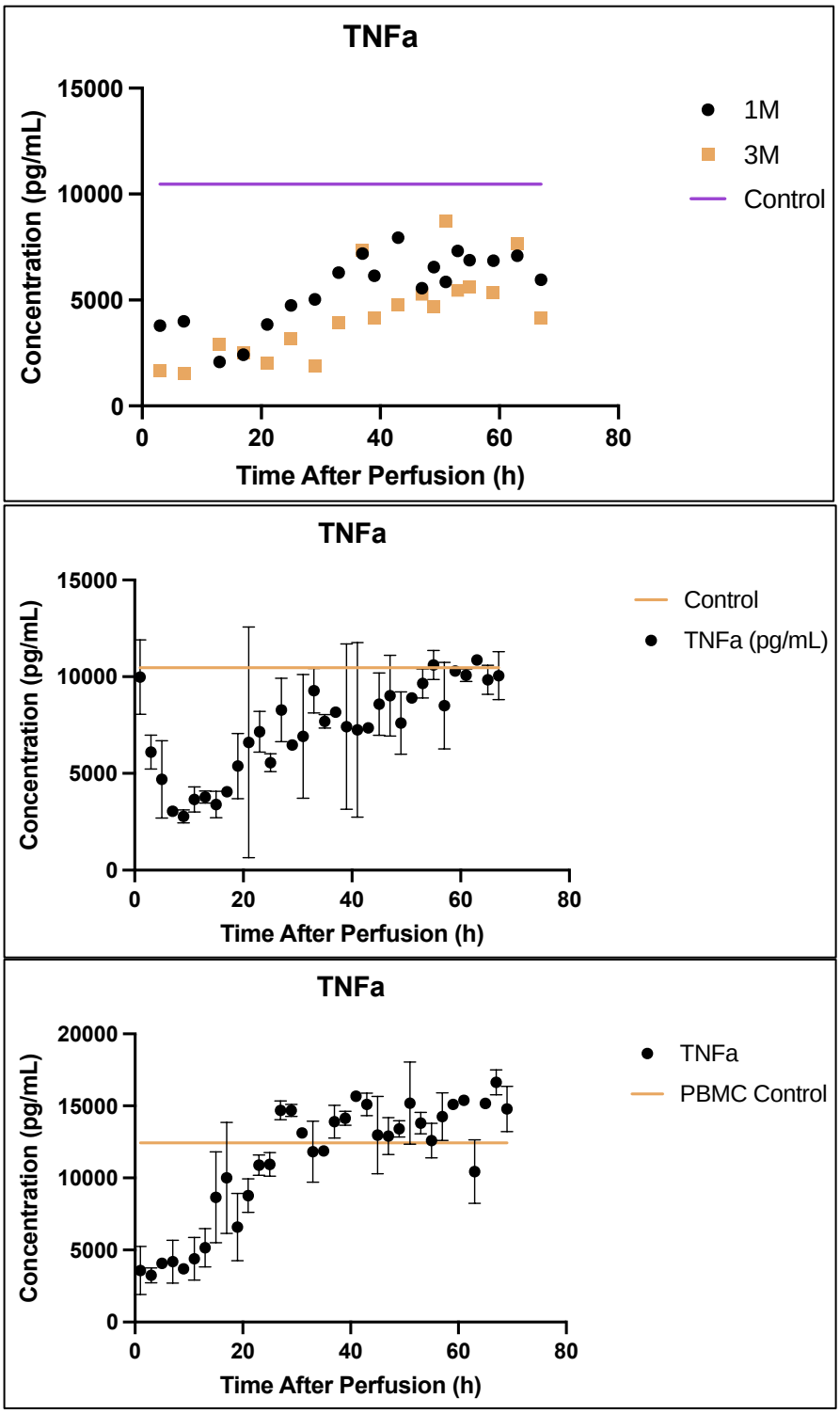


Supplemental Figure 1*: Secreted IFNγ from PBMCs and MSC conditioned media in three models: baseline perfusion, inflamed model, and prelicensed MSCs.*

Baseline Perfusion

Inflamed Model

Prelicensed Model

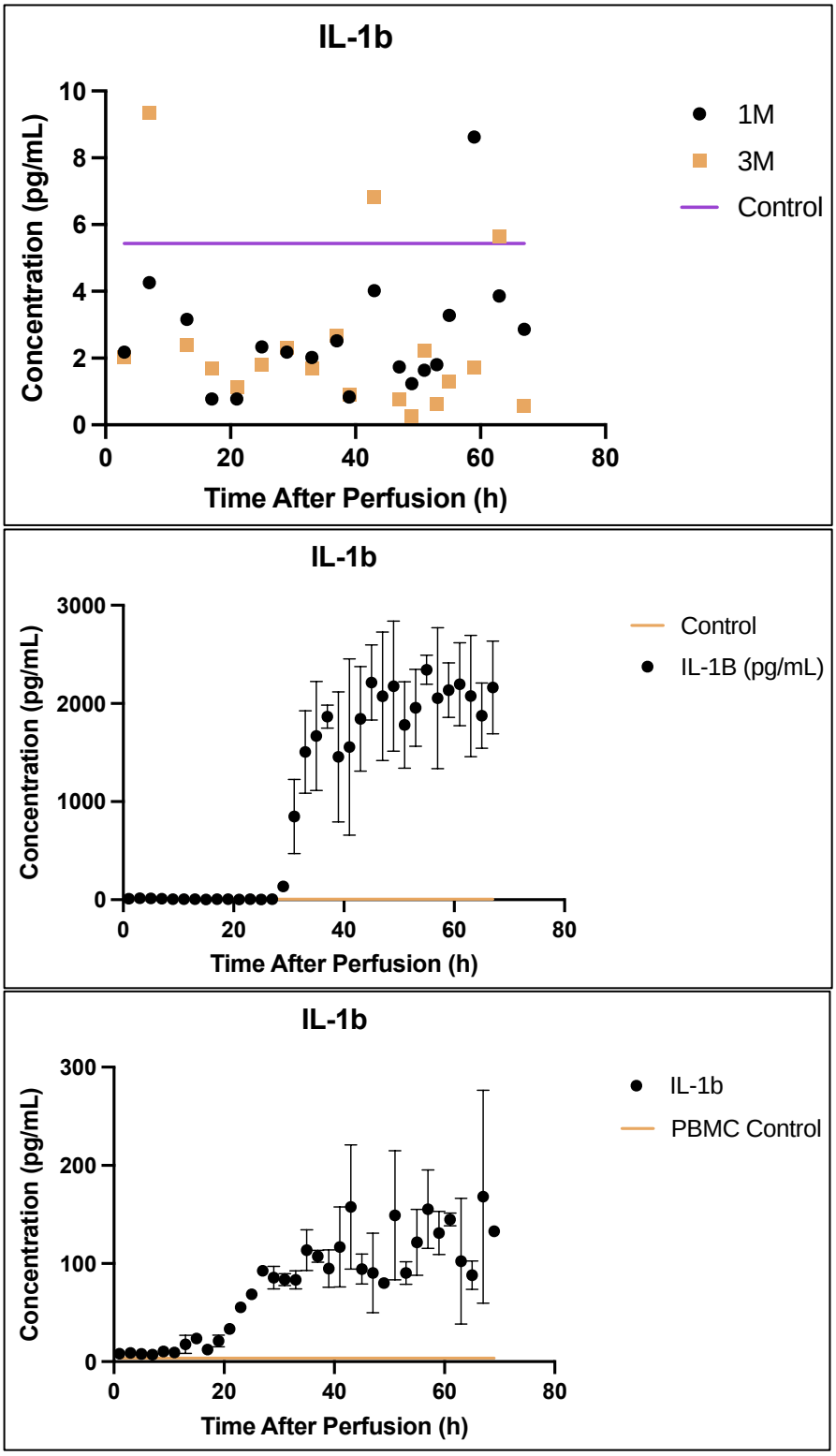


Supplemental Figure 2*: Secreted TNF α from PBMCs and MSC conditioned media in three models: baseline perfusion, inflamed model, and prelicensed MSCs.*

Baseline Perfusion

Inflamed Model

Prelicensed Model

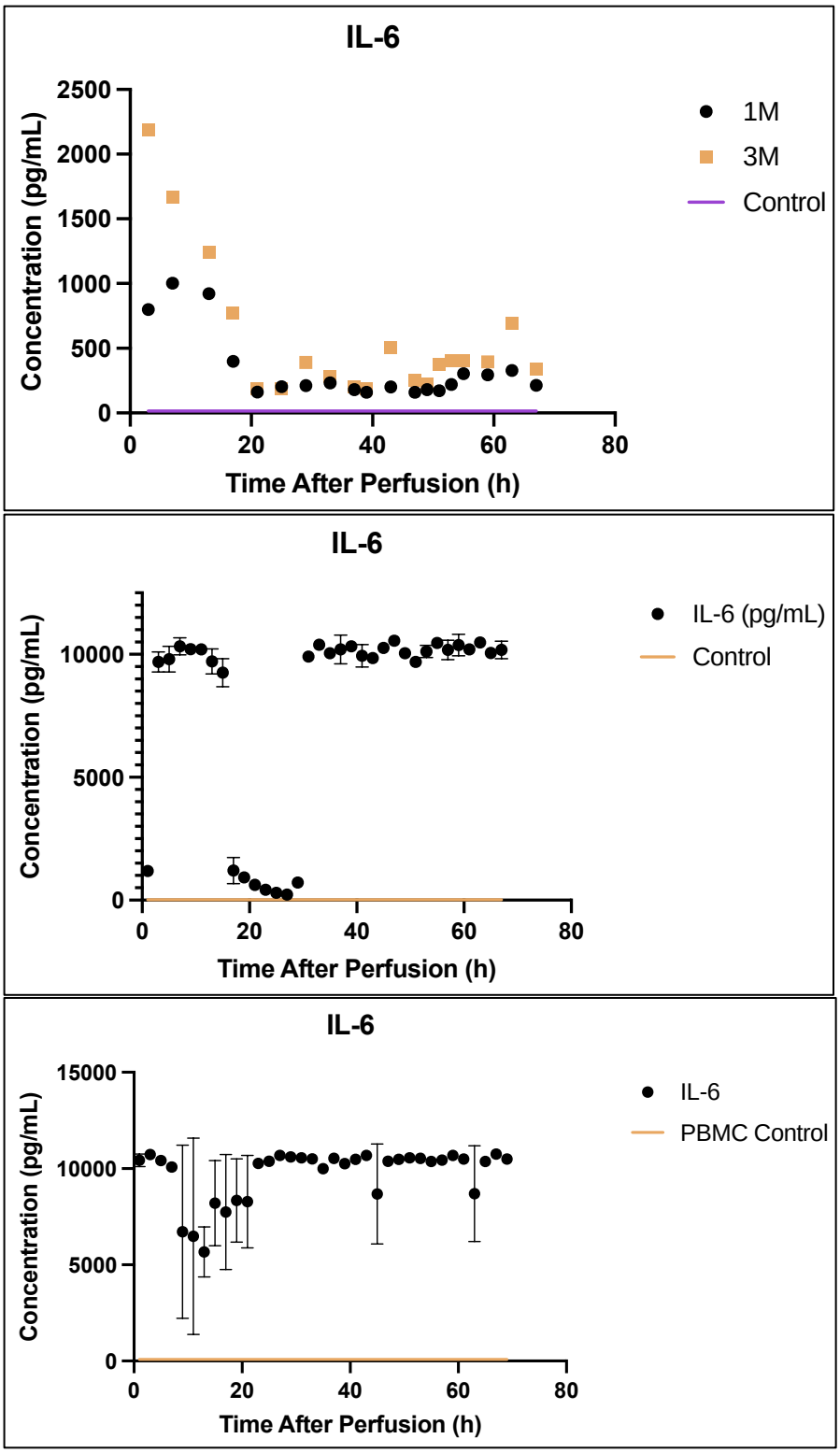


Supplemental Figure 3*: Secreted IL-1β from PBMCs and MSC conditioned media in three models: baseline perfusion, inflamed model, and prelicensed MSCs.*

Baseline Perfusion

Inflamed Model

Prelicensed Model

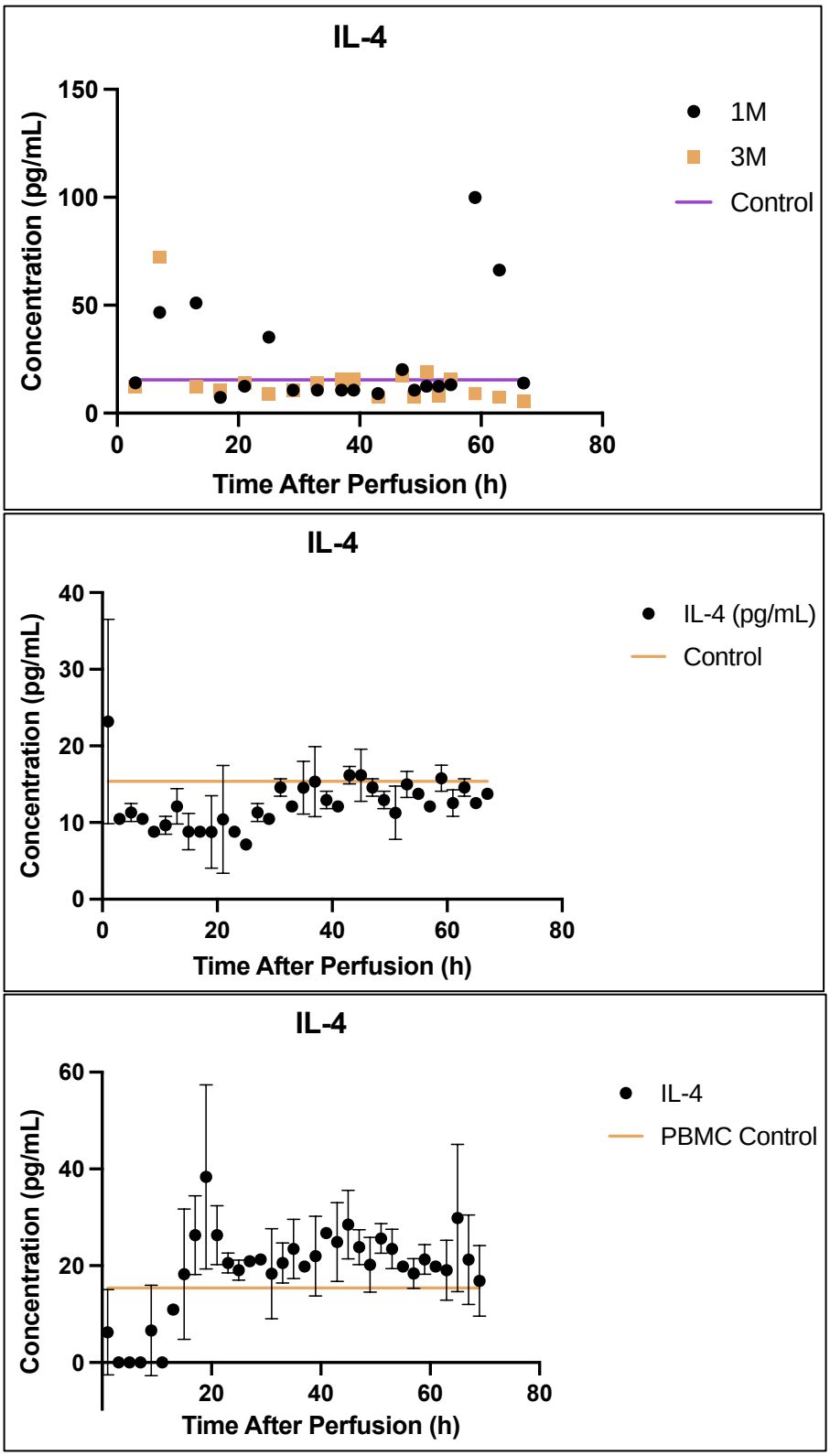


Supplemental Figure 4*: Secreted IL-6 from PBMCs and MSC conditioned media in three models: baseline perfusion, inflamed model, and prelicensed MSCs.*

Baseline Perfusion

Inflamed Model

Prelicensed Model

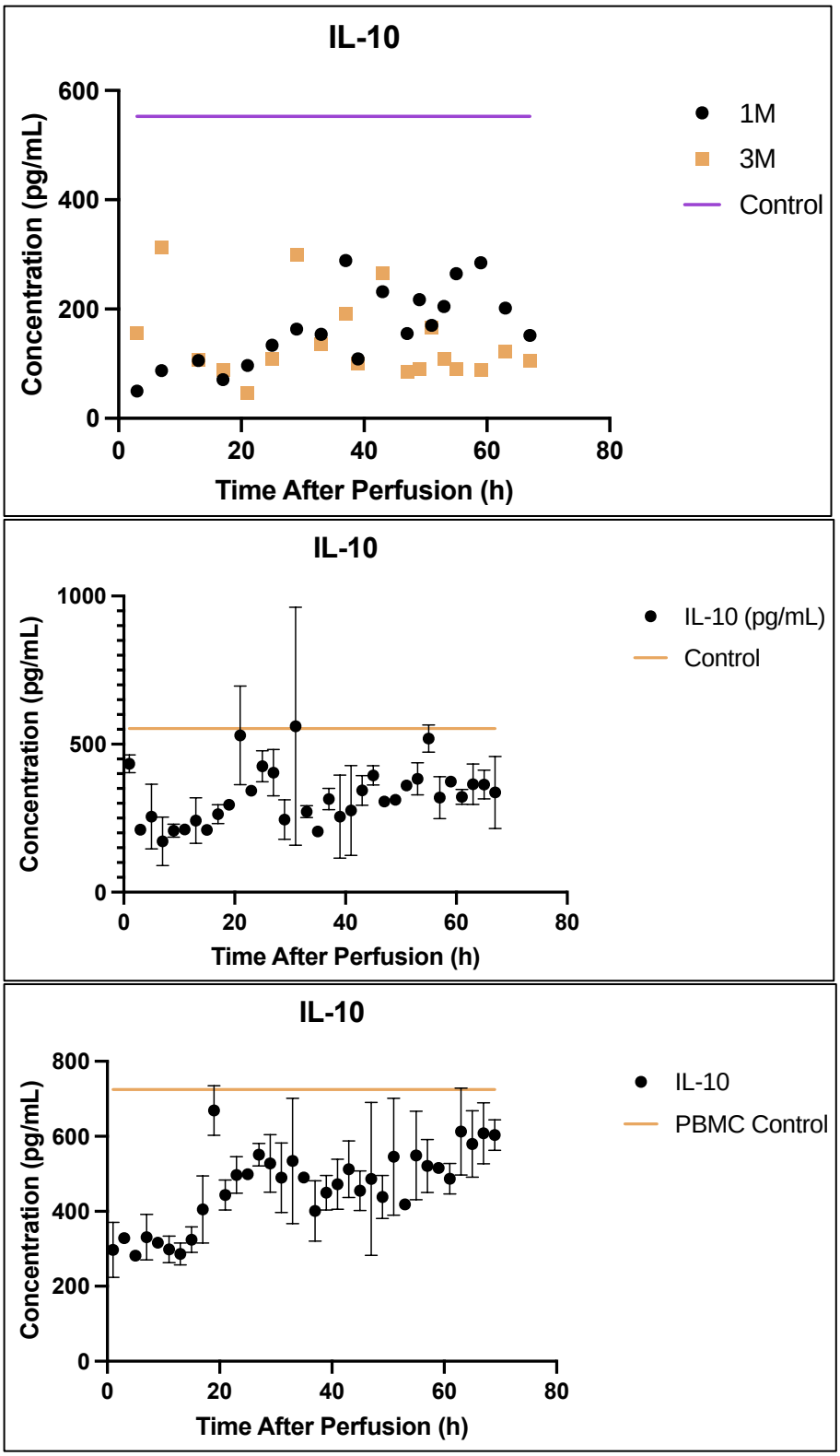


Supplemental Figure 5*: Secreted IL-4 from PBMCs and MSC conditioned media in three models: baseline perfusion, inflamed model, and prelicensed MSCs.*

Baseline Perfusion

Inflamed Model

Prelicensed Model

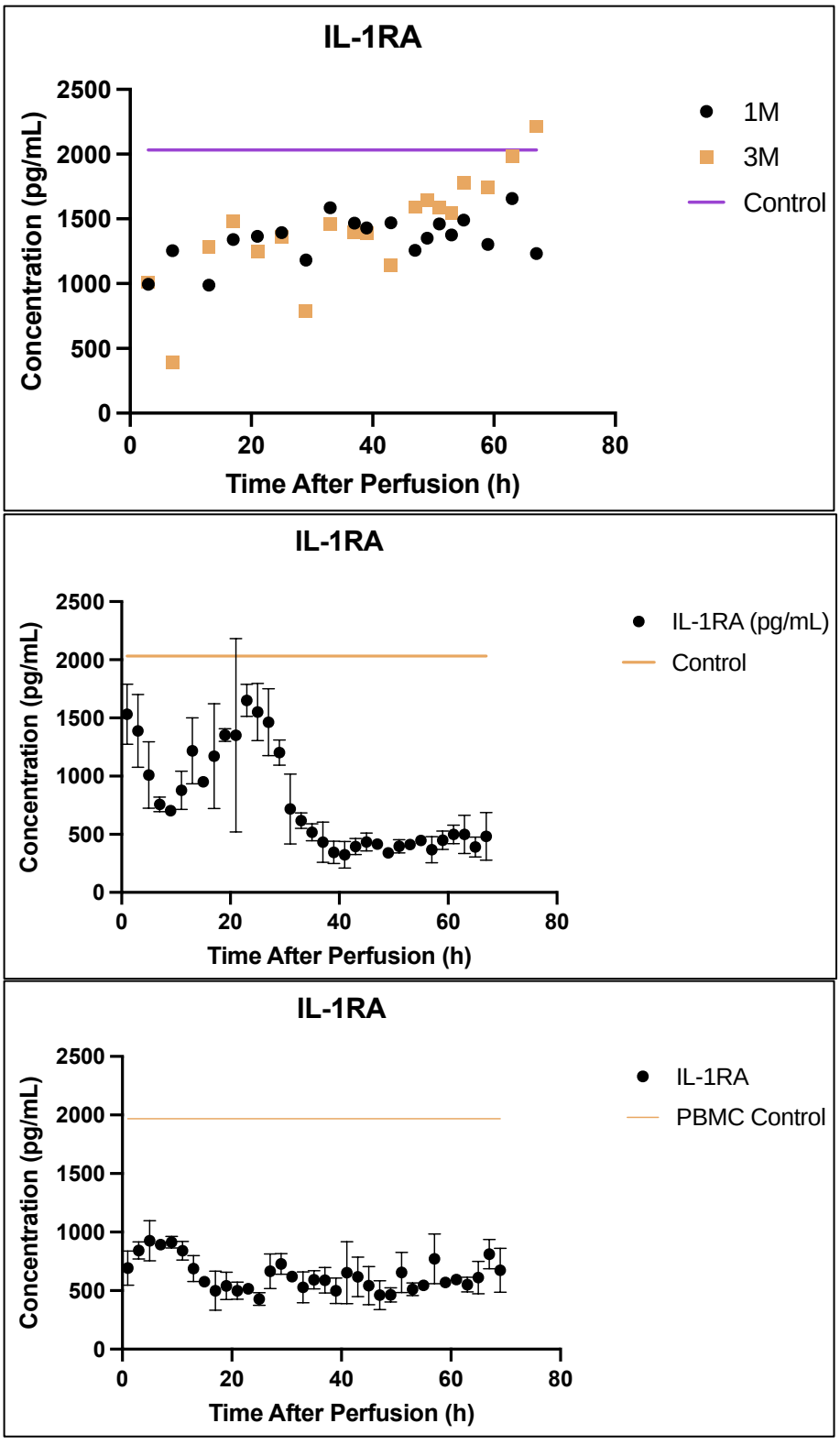


***Supplemental Figure 6**: Secreted IL-10 from PBMCs and MSC conditioned media in three models: baseline perfusion, inflamed model, and prelicensed MSCs.*

Baseline Perfusion

Inflamed Model

Prelicensed Model

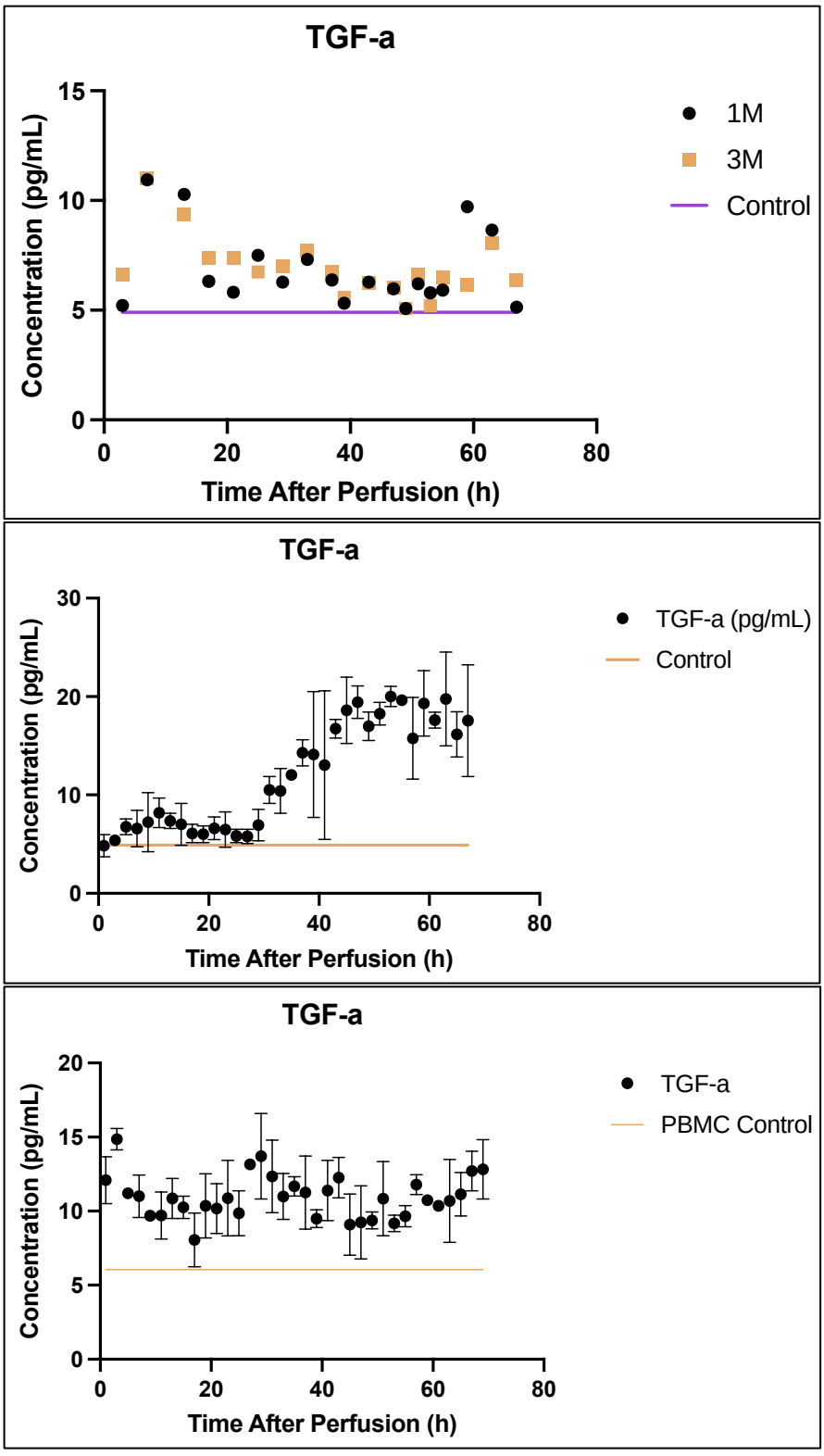


Supplemental Figure 7*: Secreted IL-1RA from PBMCs and MSC conditioned media in three models: baseline perfusion, inflamed model, and prelicensed MSCs.*

Baseline Perfusion

Inflamed Model

Prelicensed Model

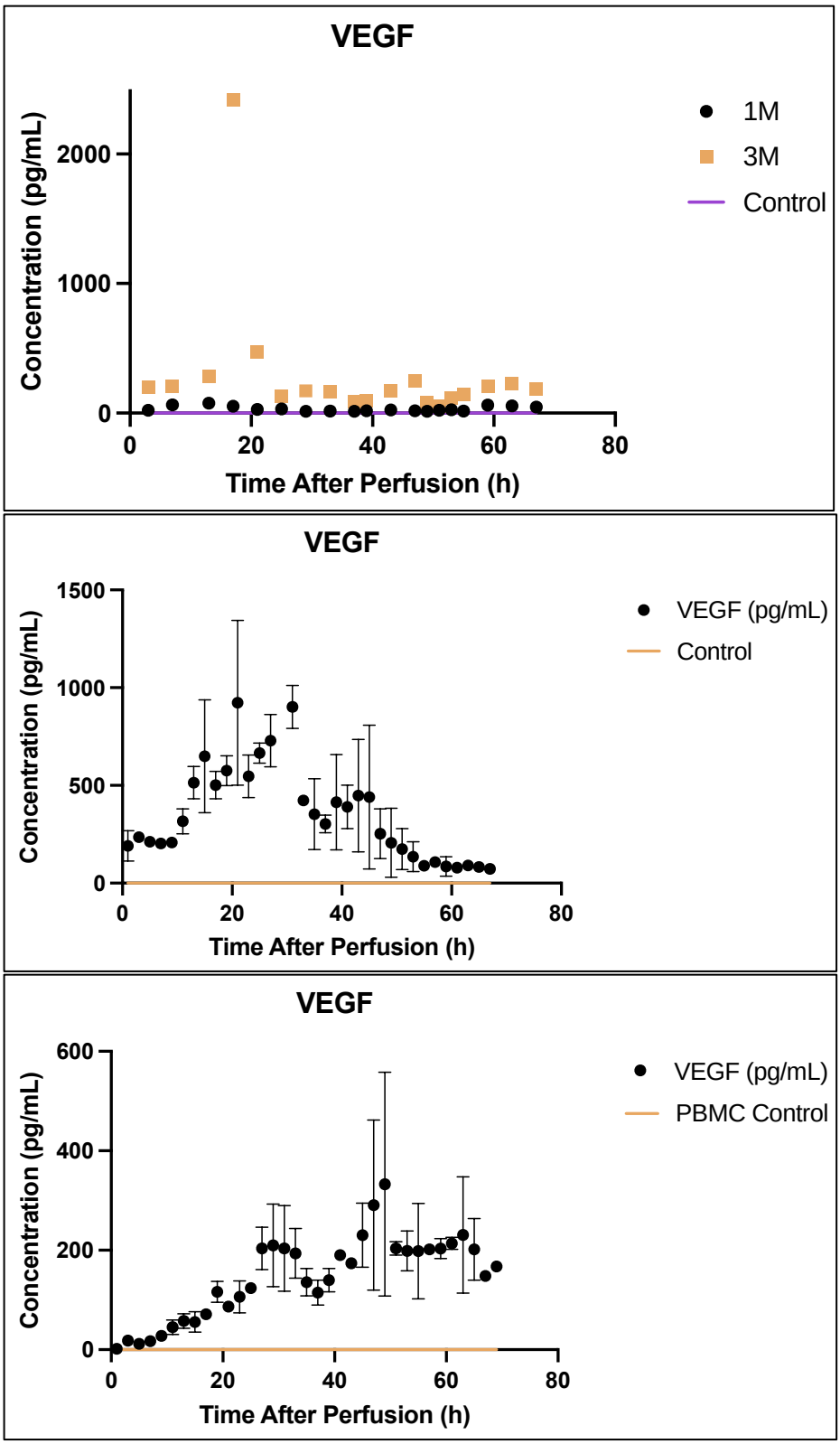


Supplemental Figure 8*: Secreted TGF-α from PBMCs and MSC conditioned media in three models: baseline perfusion, inflamed model, and prelicensed MSCs.*

Baseline Perfusion

Inflamed Model

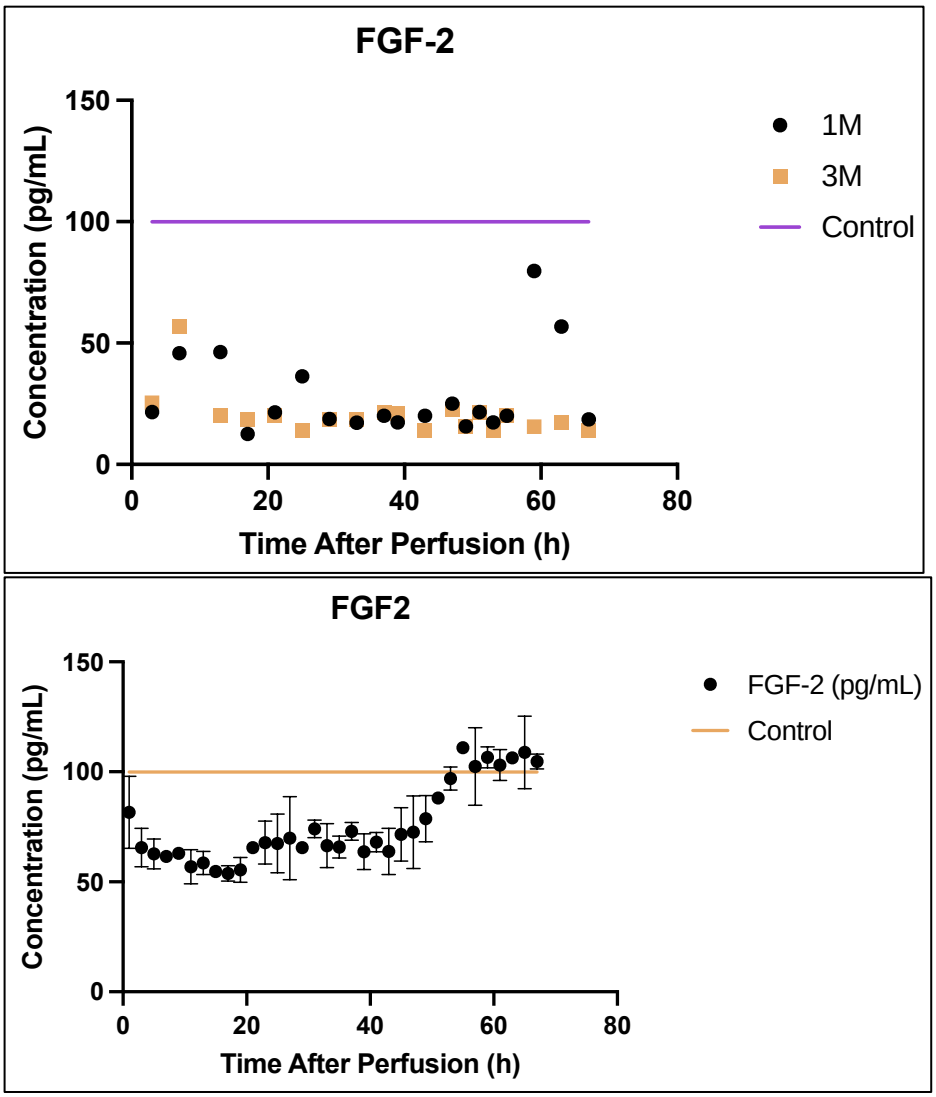
Prelicensed Model



Supplemental Figure 9*: Secreted VEGF from PBMCs and MSC conditioned media in three models: baseline perfusion, inflamed model, and prelicensed MSCs.*

Baseline Perfusion

Inflamed Model



***Supplemental Figure 10**: Secreted FGF-2 from PBMCs and MSC conditioned media in two models: baseline perfusion and inflamed model.*

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