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# Controlled presentation of cues during biomanufacturing to influence IDO mediated immune modulation by human MSCs

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# CONTROLLED PRESENTATION OF CUES DURING BIOMANUFACTURING TO INFLUENCE IDO MEDIATED IMMUNE MODULATION BY HUMAN MSCS

by

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To my late grandparents Gloria and James. This one's for you.

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I would like to take this opportunity to thank everyone who has helped, encouraged, and supported me during my journey into biomedical engineering research. I would not have made it this far if it was not for you. I want to thank my parents who believed in me since I was a child, particularly my mom who took me to the UIHC children's hospital where I was diagnosed with dyslexia after a teacher told her I would never read. I would like to thank my friends who helped me stay optimistic during hard times and my co-workers who helped me overcome problems I would not have been able to on my own. I would like to specifically thank my boss, mentor, and advisor Dr. Ankrum, who gave me the opportunity to do research in his lab in 2016. I am very greatful for this life changing experience and appreciate all of the time he has spent helping me reach the finish line. I would like to thank Alex Brown who trained me as an undergraduate. I would also like to thank my co-workers Lauren Boland and Tony Burand for the countless conversations we have had over the years that have shaped me as a researcher. And lastly, I would like to thank my grandparents who I have devoted this thesis to.

ABSTRACT

The goal of this project was to determine the key regulators of Mesenchymal Stromal Cell (MSC) potency as part of a cell-based therapy to treat inflammatory and autoimmune diseases. The immunomodulatory capacity of MSCs is dictated by multiple, interacting conditions that take place during the biomanufacturing of these cells, as well as after they are transplanted. Variables such as the source of MSCs and the inflammatory cues in their microenvironment are critical regulators of potency that can be manipulated and optimized prior to their use for an enhanced cell-based therapy. Herein, I took a systematic approach to isolating a single variable in the microenvironment of MSCs to determine its effect on the key immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO). I then manipulated these variables and applied them across multiple MSC donors to determine how their effect varied between cells isolated from different individuals. Finally, I conducted an *in vitro* potency assay with MSCs and Peripheral Blood Mononuclear Cells (PBMCs) to determine how enhanced IDO due to these variables translated to immune suppression for an enhanced cell product.

Upon transplantion, different disease settings have altered microenvironments that can hinder the efficacy of an MSC therapy. The microenvironment in obesity and type 2 diabetes (T2D) has elevated levels of the fatty acid palmitate which shifts the phenotype of MSCs from immune suppressive to pro-inflammatory. I demonstrated that manipulating the microenvironment of MSCs to enhance IDO protein concentration prior to transplant reverses the pro-inflammatory effects of palmitate and restores immune suppression by MSCs. My finding was that the appropriate environmental cues, along with a potent donor, yields a cell-based therapy that can overcome challenges in many disease settings such as obesity and T2D.

## PUBLIC ABSTRACT

Mesenchymal stromal cells (MSCs) are a leading cell therapy candidate for the treatment of immune and inflammatory diseases due to their potent regulation of immune cells. The potency of MSCs is dictated by multiple, interacting factors, both in the manufacturing of these cells, as well as environmental conditions encountered in the body. How these cells are treated prior to use, both in research and clinical trials, however, can differ widely. These discrepancies have affected outcomes in these arenas and complicated interpretation of findings. By investigating how conditions commonly modified during the manufacturing of MSCs impacts their ability to modulate immune cells, we can optimize this therapy for a more potent product and contextualize research findings. Before MSC therapy transitions from clinical trials to a broader population, we must determine how different disease settings in the body can impact efficacy.

In this thesis, I investigated the contribution and interaction of different biomanufacturing parameters such as growth factors, inflammatory signals, and the donor of MSCs on potency. In addition, I determined methods to overcome detrimental environmental conditions common in the setting of obesity and type II diabetes. These findings lay a foundation for enhanced MSC potency to treat inflammatory and autoimmune diseases and provide context for research findings in the field of regenerative medicine.

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# CHAPTER 1: INTRODUCTION; REGULATION OF IDO MEDIATED IMMUNE MODULATION BY MSCS

Mesenchymal stromal cells (MSCs) are a multipotent cell population used as a cell therapy in regenerative medicine. Currently, there are over 800 completed or ongoing MSC clinical trials, the majority of which utilize their potent immunomodulatory potential for the treatment of autoimmune and inflammatory diseases[1]. Initially discovered in the bone marrow of mice in 1966[2], the term "mesenchymal stem cell" was first used in an article published in the Journal of Orthopedic Research in 1991[3]. While referred to as stem cells, the differentiation capacity of MSCs has been established *in vitro* but *in vivo* data remains elusive[4]. The disconnect between their original terminology and *in vivo* stem properties has led to many other terms including "bone marrow stromal cells." This term has also been controversial as MSCs can be extracted from nearly all tissues of the body. MSCs from these different tissues have differing gene expression and efficacy in the treatment of inflammatory diseases. MSCs are typically isolated from bone marrow, adipose tissue, umbilical cords, and the placenta[567].

To date, there is no consensus as to the appropriate terminology for these cells. Instead, MSCs were defined by a set of minimum criteria in 2006 by the International Society of Cell and Gene Therapy (ISCT). These criteria include: plastic adherence, the ability to differentiate into osteoblasts, chondroblasts, and adipocytes, high expression of CD73, CD90 and CD105 as well as low expression of CD11b, CD14, CD19, CD34, CD45, and HLA-DR[8]. MSCs demonstrate stem behavior *in vitro*, as by definition they must have trilineage differentiation capacity. Upon differentiation, however, MSC immunomodulatory potential is lost[9].The naive state immunomodulatory function of MSCs is due to a host of mechanisms including production of extracellular vesicles, small molecules, proteins, and even signaling after death via efferocytosis[10,11].

MSCs have had promising outcomes in clinical trials, particularly in Crohn's Disease and Graft versus Host Disease (GvHD)[12,13,14,15,16,17]. For example, in 2018, 212 participants suffering from Crohn's Disease with complex perianal fistula were recruited for a placebo controlled clinical trial in which recipients were locally injected with MSCs along with standard care. After 1 year, 56.3% of recipients had closure of all treated fistulas compared to 38.6% of those who received a placebo ($p=0.01$)[18]. In 2016, a prospective clinical trial for the treatment of GvHD reported 69% of the 60 recruited recipients had partial or better resolution of acute symptoms within 28 days[19]. However, there have been several clinical trials whose results have curtailed the excitement of MSC therapy. For example, in 2009, a multi-center phase III clinical trial in the United States for the treatment of severe refractory GvHD showed no benefit in comparison to a placebo. These resultscontradicted previous phase II clinical trials in Europe[20]. Discrepancies between the academic phase II clinical trials and outcomes from manufacturer produced MSCs in early phase III clinical trials shed light on critical regulators of MSC immune modulation. MSCs, while still meeting ISCT guidelines, can differ with regards to media composition, the surface they are grown upon, the device used to expand them, as well as conditioning regimens such as hypoxia[20].

MSCs and other cell therapies are adaptive, with differing activity and behavior dictated in part by their environment before and after transplant. For diseases like GvHD, MSCs are often used in combination with steroids, but unlike the systemic delivery of drugs, MSCs are a targeted therapy that works with the patient's immune system to overcome deleterious and overactive immune cell activity. MSCs modulate a variety of cell types including immune and support cells. In the case of immune cells, MSCs polarize macrophages to an anti-inflammatory, M2 phenotype, inhibit the proliferation of T cells, and promote the generation of T regulatory cells[11].

While MSC immune modulation is due to a host of factors, this thesis is focused on indoleamine 2,3-dioxygenase (IDO), an enzyme that has shown to be critically dependent for immune suppression. Without this intracellular enzyme, MSCs lose suppressive action toward peripheral blood mononuclear cells (PBMCs) *in vitro* and fail to induce tolerance of renal grafts in mice *in vivo*[21,22]. This enzyme is expressed by a variety of organisms, tissues, and cell types, including dendritic cells and tumor repopulating cells (TRCs). TRCs are a cell type critical to tumor evasion and inhibition of their IDO enzyme with the synthetic amino acid 1-methyltryptophan has been shown to be an effective method to enhance immune cell mediated detection in the tumor microenvironment[23]. Although the IDO enzyme was first discovered in 1963, it was not until Munn et al. proposed in 1998 that this enzyme plays a crucial role in tolerance, particularly in preventing fetal rejection in placental mammals[24]. IDO has the first and rate limiting step in the catabolism of tryptophan down the kynurenine pathway (KP)[25]. In the KP, IDO degrades tryptophan by cleaving its indole ring, forming kynurenine which can be further degraded by other enzymes such as kynurenine 3-hydroxylasem into kynurenic acid[25]. In mammals, tryptophan degradation via the KP is performed primarily by IDO1, TDO, found predominantly in the liver, and IDO2, an IDO1 paralogue[26]. IDO1 degrades the L-isomer of tryptophan and is the driving force for immune suppression by MSCs and thus will be the topic of this thesis.

Munn et al. demonstrated IDO activity by dendritic cells inhibits T cell proliferation though T cell's GCN2 kinase[27]. *In vitro*, T cell proliferation was reversed with addition of tryptophan and *in vivo* GCN2 knockout T cells were not susceptible to IDO activity. The mechanism by which IDO induces tolerance has remained controversial since these findings. Munn et al. claimed that it was the depletion of tryptophan that was responsible for IDO induced

tolerance in infection and pregnancy. Tryptophan is an essential amino acid vital to the metabolism of immune cells, bacteria, and viruses, and is the least abundant amino acid. Other groups, such as Abdulla et al. in 2016 have scathingly criticized other contemporary researchers for propagating the tryptophan depletion model[28]. Instead, this team proposes that it is the utilization of tryptophan through the kynurenine pathway rather than the depletion of tryptophan itself that is responsible for immune modulation. My own work, presented in chapter 2 (Figure 6, 7, and 8), sheds light on how tryptophan addition affects IDO protein and enzymatic activity.

To engineer MSC immune modulation through IDO, we first must understand how IDO is induced and regulated in this cell type. Unlike dendritic cells, which self-induce IDO, MSCs are critically dependent on the inflammatory cytokine IFNγ. IFNγ is produced by immune cells, including T cells and NK cells, which in turn enduces IDO expression in MSCs through the JAK/STAT1 signalling pathway[29,30]. The effect of IFNγ on MSCs is not only expression of IDO but also morphological changes, changes in division rate, and regulation of the key immune detection proteins MHC class I and II[31,24]. This up-regulation in MHC class I and II has led some researchers to claim that IFNγ exposure prior to transplant compromises the immune evasive status of MSCs, with some going as far to say that MSCs behave as antigen presenting cells during a brief period of IFNγ exposure[32,33]. IFNγ regulation of MHC class I and II in MSCs has a differential effect based on the dose and duration of IFNγ exposure. For example, MHC class I is up-regulated with IFNγ stimulation after 4 days but is down regulated by day 8. MHC class II, however, continues to be up-regulated through an 8 day stimulation. Interestingly, high doses of IFNγ can down regulate expression of MHCs. For example, Tang et al. demonstrated that MHC class II is down regulated at a high dose of 100 U/mL IFNγ in comparison to a 10 U/mL dose[34], both in the cytoplasm and on the surface of the cell. Differences between the effect of IFNγ on

IDO and MSC immunomoduilation has led to conflicting findings between work in human MSCs versus murine MSCs. Mouse MSCs primary mechanism of immune evasion is iNOS where as human MSCs primarily utilize IDO[21]. Disagreement between human and mouse MSC data is likely due to up-regulation of allogenic proteins, MHC class I and II, without the appreciable benefit in immune evasion in murine studies.

As a key immunomodulatory enzyme, IDO can be up-regulated prior to transplant through the process of IFNγ pre-licensing to enhance potency. Rather than waiting on activation in vivo, MSCs can be cultured in vitro with IFNγ to become IDO positive before they are transplanted, enhancing immune suppression towards T cells[35]. How pre-licensing and biomanufacturing parameters effect IDO mediated immune modulation in a variety of contexts is the core topic of this thesis. While MSCs handling can vary widely pre-transplant, these biomanufacturing parameters all have significant consequences on the phenotype and behavior of MSCs, even when the ISCT guidelines are met. For example, Menard et al. has shown that MSCs isolated from adipose tissue or bone marrow as well as MSCs stimulated with human or bovine growth factors have deferentially altered phenotypes, IDO expression, and immunomodulatory potency[34]. In addition, IFNγ pre-licensed MSCs (PL-MSCs) inhibit T, B and NK cells to different degrees depending on these conditions[34]. Thus, identifying biomanufacturing parameters to generate a potent and durable immunomodulatory phenotype within MSCs is an attractive strategy to tailor MSC therapy for use in specific disease settings.

In chapter 2 I investigated IFNγ pre-licensing strategies to enhance IDO protein and how this altered IDO translates into immune suppression. While tissue source has been shown to be a regulator of PL-MSCs' potency, how MSCs from different tissue sources are impacted by the dose and duration of IFNγ during pre-licensing has yet to be established. I determined how these

regulators can be manipulated to enhance IDO and immune modulation of MSCs for a more potent MSC cell product. Enhancing the potency of MSCs through pre-licensing is appealing both financially and for patient safety as administration of fewer cells reduces the risk of a blood mediated immune reaction and thrombosis[36]. After evaluating the effectiveness of a variety of pre-licensing strategies to enhance IDO in MSCs, these pre-licensing strategies were then tested in a PBMC:MSC co-culture to determine the immunomodulatory potency of PL-MSCs. Insight into the role different culturing parameters play in the production and activity of IDO in MSCs and how this altered IDO profile translates into immunomodulation will inform the design of biomanufacturing protocols for PL-MSCs to be used in the treatment of inflammatory and autoimmune diseases.

In chapter 3 I investigated MSC immunomodulatory potency post-transplant, particularly MSC phenotypes formed *in vivo* and metabolic disease environments. When injected locally, MSCs form aggregates or spheroid MSCs. The spheroid MSC phenotype has altered gene, protein, and secretome expression as well as immunomodulatory potency. How the spheroid MSC phenotype differs from the classically studied 2D MSCs is presented along with the use of the corticosteroid steroid budesonide to enhance potency. Next, the impact of palmitate, a pro-inflammatory small molecule elevated in the setting of obesity and type II diabetes (T2D) on MSC immune modulation was determined. Individuals with T2D have altered endocrine profiles, with insulin resistance leading to hyperglycemia and altered cell metabolism. Other inflammatory disease settings such as asthma and autoimmune diseases such as GvHD have been shown to differentially effect the polarization of T-helper cells by MSCs. How to overcome the pro-inflammatory effects of palmitate with pre-licensing is detailed along with donor to donor differences in immune modulation at baseline.

# CHAPTER 2: REGULATION OF IDO IN THE BIOMANUFACTURING OF MSCS

## 2.1. Preface

In chapter 2, the Ankrum lab and I investigated how different parameters during the biomanufacturing of MSCs effected IDO protein concentration and activity. These included the dose and duration of IFNγ exposure during culture, the concentration of IDO substrates (tryptophan) and metabolites (kynurenines), and the donor of MSCs, both from umbilical cord and bone marrow tissue sources. First, the effect of the dose and duration of IFNγ pre-licensing was investigated in one MSC donor to determine their effect on the enzyme as this parameter is altered, as well as to determine the minimum dose and time needed to induce detectable levels of IDO protein. After isolating the contribution in one donor, the dose and duration of IFNγ pre-licensing was then tested in several MSC donors to determine the interaction between these parameters with MSCs from different individuals. We utilized the regulation of IDO based on these biomanufacturing parameters as a surrogate marker for immune suppression which informed our decision for testing promising pre-licensing strategies. We then tested these pre-licensing strategies as a MSC:PBMC *in vitro* potency assay to determine how enhanced IDO through pre-licensing translated into immune suppression. Our findings were the dose and duration of IFNγ can be manipulated to enhance IDO but that only a dose-based strategy enhanced immune suppression by MSCs. In addition, the effect of different pre-licensing strategies on immunodulatory potency was maintained across donors but no pre-licensing strategy tested could overcome differences in potency between donors at baseline.

This chapter is an adaptation of a manuscript “Dose, Duration, and Donor: Comparing the Factors that Influence MSC Expression of IDO” which will be submitted for publication to the journal Cytotherapy. I am a lead author in manuscript and contributed to the writing and

editing of the original draft, as well as the methodology, visualization, conceptualization and formal analysis. As a lead author, I was guided with help and feedback from my lab every step of the way. I conducted every experiment independently with this guidance and worked with Lauren Boland on the final figure that will be submited for publication (Figure 5). Figures 6, 7, and 8 are in this reworking but are not in the final manuscript for publication.

## 2.2. Introduction

Mesenchymal stromal cells' (MSC) immunomodulatory phenotype has been applied in clinical trials with notable successes in the treatment of graft versus host disease[13,12] and Crohn's disease[37,16]. The mechanisms of action by which MSCs modulate immune cells are diverse and include the active production of signaling molecules, release of extracellular vesicles, and signaling via efferocytosis[10,11]. One potent component of this diverse immunomodulatory repertoire is indoleamine-2,3-dioxygenase (IDO), an intracellular enzyme, that has been demonstrated to play a key role in MSCs immunomodulatory function. Inhibition of IDO *in vitro* eliminates MSCs' suppressive action towards activated peripheral blood mononuclear cells (PBMCs)[23]. While *in vivo*, infusion of MSCs induced tolerance to renal grafts in mice in an IDO-dependent manner[22], and overexpression of IDO in MSCs enhanced long-term graft tolerance in a rabbit renal allograft model[38]. As an essential facet of MSCs' immunomodulatory profile, understanding how MSCs regulate expression and activity of IDO is key to maximizing MSCs' therapeutic potential.

Expression of IDO in human MSCs is not constitutive, but induced by cues in the inflammatory microenvironment, predominately interferon gamma (IFNγ)[39]. In contrast to dendritic cells, which can induce IDO in response to a variety of cytokines, MSCs are critically dependent on IFNγ exposure to stimulate IDO expression at both the mRNA and protein level[40].

The effect of IFNγ exposure on IDO protein levels is diminished as MSCs are cultured for long periods of time and reach senescence. Although senescent MSCs transcibe IDO at a similar rate as earlier passage cells, IDO protein concentration is lower due to proteosomal degredation[41]. Other cytokines, such as tumor necrosis factor alpha (TNFα) and interleukin-1 (IL-1) augment IDO expression when combined with IFNγ, but are insufficient alone to induce it[42]. In addition, intact IFNγR signaling and glycolytic metabolism are also both necessary for IFNγ to induce MSC's immunomodulatory phenoype[43]. Thus, MSCs regulate IDO expression and immunomodulatory phenotype by integrating diverse cues within their environment.

Rather than waiting on delayed activation *in vivo*, MSCs can be presented cytokines during manufacturing to pre-activate their immunomodulatory phenotype, a process called pre-licensing (PL)[44]. Pre-licensing can enhance the potency and duration of immune modulation by MSCs, with enhancement in IDO gene expression and protein levels persisting after removal of inflammatory cytokines and even after cryopreservation[45]. PL-MSCs have also demonstrated in *in vitro* studies resistance to several challenges encountered *in vivo*. These include resistance to cytotoxic T cell lysis[46] as well as the ability to maintain immunomodulatory potency upon exposure to palmitate, a cytotoxic fatty acid elevated in the setting of obesity[4748]. While potentially beneficial in a variety of settings, the utility of pre-licensing for MSC ultimately will depend on the disease being treated.

Pre-licensing has shown benefits across a variety of MSC donors, tissue sources, and settings but the methods used to pre-license have been highly variable, making comparisons difficult. Pre-licensing with IFNγ doses from 5 to 200 ng/ml and durations of 2 hours to 4 days have been reported[48,49,50,30,35]. IFNγ pre-licensing can differ not only by the dose and duration of exposure but by other parameters as well. For example, IFNγ pre-licensing has been performed

with the addition of the inflammatory cytokine TNFα as well as culturing under hypoxia[51]. Without pre-licensing, MSCs immunomodulatory potency can differ widely depending on the donor and tissue source. Pre-licensing, however, has been proposed to be a way to improve the consistency of performance between donors. Menard et al. has shown that with 10 ng/mL IFNγ and 15 ng/mL TNFα pre-licensing, MSCs isolated from adipose tissue versus bone marrow as well as MSCs cultured in human versus bovine growth factors have differential IDO expression and immunomodulatory potency[34]. While trivial at a pre-clinical stage, to transition to scalable biomanufacturing processes, the minimum effective IFNγ dose and duration required to enhance MSC immunomodulatory function needs to identified. Without optimizing these parameters, the best case scenario is an increase in cost without improved performance while the worst case scenario is the loss of therapeutic efficacy. With differences in potency identified as a function of both the source of MSC as well as the pre-licensing protocol, how these variables interact are critical to moving PL-MSC into biomanufacturing systems.

While IDO expression is dictated by diverse, interacting cues, herein we sought to take a systematic approach to identify the contributors within the microenvironment that dictate MSC expression and maintenance of IDO. After determining the effectiveness of a variety of pre-licensing strategies to enhance IDO in MSCs, these pre-licensing strategies were tested in PBMC suppression assays to determine how they translated to immune suppression. Insight into the role different parameters play in the production and activity of IDO in MSCs and how this altered IDO profile translates to immunomodulation will inform the design of biomanufacturing protocols for PL-MSCs to be used in the treatment of inflammatory and autoimmune diseases.

## 2.3. Results

### 2.3.1. IFNγ Induces the Increase of IDO Protein in a Dose and Duration Dependent Manner in MSCs

A wide variety of doses and durations of IFNγ exposure have previously been employed to pre-license MSCs; however, testing both of these parameters simultaneously has made it difficult to determine the specific dose and duration dependent effects of IFNγ exposure on MSC IDO protein. To isolate the specific contribution of IFNγ dose on IDO protein, we fixed the duration of IFNγ exposure to 2 days and cultured a single bone-marrow MSC donor (BM2) with doses ranging from 0 to 100 ng/mL. IDO protein levels were detectable at 1 ng/mL and continued to increase in a dose dependent manner until reaching a plateau at 50 ng/mL (Figure 1A). Utilizing an agonist vs. response curve-fit model, the mean dose of IFNγ at which 90% of maximum IDO protein levels were achieved occurred at 49.984±26.0516 ng/mL (Figure 1A).

Next, we sought to determine the contribution of the duration of IFNγ exposure on IDO protein levels and enzymatic activity. We hypothesized that increased duration of IFNγ exposure would lead to enhanced IDO protein levels, as seen with increasing the IFNγ dose. To test this hypothesis, BM1 MSCs were cultured in 50 ng/mL of IFNγ for 6, 24, 72 or 96 hours (hrs). IDO protein levels increased linearly as a function of the duration of IFNγ exposure with an $R^2$ value of 0.899 ($p=0.0141$, Figure 1B). Detectable protein levels did not occur until the 24 hr mark, however, the minimum time necessary to induce IDO protein may likely occur within this 6-24 hr window. In addition, IDO activity, as measured by the conversion of tryptophan to kynurenine, mirrored what was seen with IDO protein concentration. Kynurenine production was first detectable at 6 hrs and continued to increase throughout the 96 hr period (Figure 1C), demonstrating that IDO remains active during this time course of IFNγ exposure.

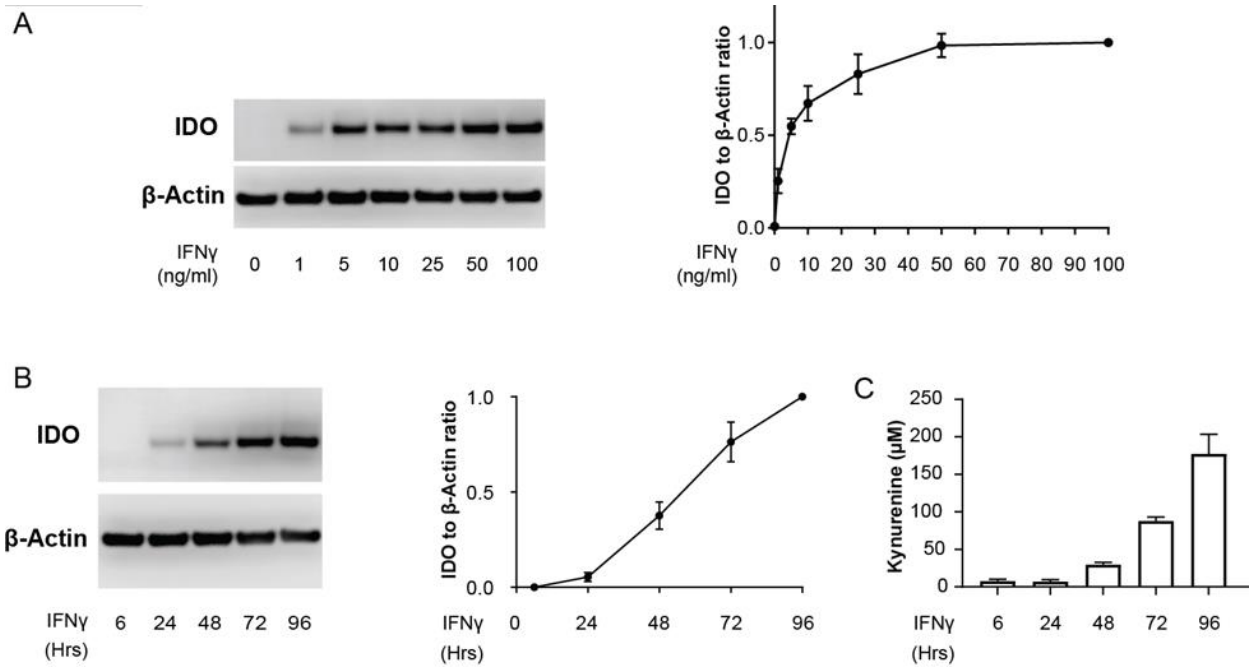


Figure 1: IDO Protein Levels are Dictated by both the Dose and Duration of IFNγ

A) Donor and passage matched MSCs were cultured in a dose range of 0-100 ng/mL IFNγ for two days. After two days of culturing, protein was collected. IDO protein concentration was then determined via western blotting, normalized to housekeeping gene β-Actin, and further normalized to the 100ng/mL IFNγ condition. N=3 independent experiments. Reporting mean ± SEM. B) Donor and passage matched MSCs were cultured in 50 ng/mL IFNγ with the addition of 250μM tryptophan for either 6, 24, 48, or 96 hours. Tryptophan was added to keep from depleting substrate for conversion into kynurenine during this 96 hours time course. After this culturing period, protein and media were collected. IDO protein concentration was then determined via western blotting, normalized to the house keeping gene β-Actin, and further normalized to the 96 hour condition. N=3 independent experiments, paired to figure 1C. Reporting mean ± SEM. C) Media that was collected at the same time as lysate for Figure 1B had kynurenine concentration measured via a kynurenine media assay to determine IDO enzymatic activity. N=3 independent experiments, paired to Figure 1B. Reporting mean ± SEM.

### 2.3.2. Pre-licensing Strategies Increase IDO in all MSCs Relative to Donor Baseline

With larger doses and longer durations of IFNγ exposure shown to enhance IDO protein levels (Figure 1), we next wanted to determine if these parameters could be utilized as part of a pre-licensing strategy aimed at elevating IDO protein levels in poorer performing donors. Two pre-licensing strategies were tested: a dose based strategy (fixed duration and variable dose) and a duration based strategy (fixed dose and variable duration). MSC immunomodulatory potential and IDO protein concentration have been shown to vary widely between individuals and tissue sources. Therefore, to incorporate a range of donors and tissue sources, we chose to test these strategies in two bone marrow donors (BM1, BM2) and one umbilical cord donor (UC).

First, we tested the dose based strategy with either 5 or 50 ng/mL IFNγ exposure for two days. In our previous experiments, 5 and 50 ng/mL of IFNγ exposure induced half and 90% of the maximal IDO protein, respectively; thus, these doses were selected for a mid- and high-level of IDO induction. At the 5 ng/mL dose, UC and BM2 showed a similar amount of IDO protein, while BM1 had ~46% less, indicating a variability at baseline. When treated with high dose of IFNγ, all donors showed a significant increase in IDO protein relative to the low dose (Figure 2A). In addition, when pre-licensed with a high dose of IFNγ, BM1 produced roughly equivalent IDO as the other two donors at a low dose. IDO enzymatic activity also increased in all three donors when pre-licensed with high dose IFNγ (Figure 2B). Therefore, using high dose IFNγ during pre-licensing is an effective strategy to increase IDO protein concentration and IDO activity in lower producing donors.

Next, we sought to determine the effect of duration on IFNγ pre-licensing. To test this, we pre-licensed MSC donors in 10ng/mL IFNγ for either 48 or 96 hrs. All donors showed increases in IDO protein when pre-licensed for a longer duration (Figure 2C). Surprisingly, when

comparing the effect of the dose strategy to the duration strategy, the increase in IDO protein after longer durations was always less than that observed at higher doses (Figure 2B, C). In addition, the benefit of longer duration varied between donors. Kynurenine concentration increased from 48 to 96 hours of pre-licensing across donors but plateaued with the 96 hour condition, demonstrating that the enzyme remains active throughout the pre-licensing period (Figure 2D). In contrast to the IFNγ dose based pre-licensing strategy, a duration based strategy did not result in a large increase in IDO protein in all three donors. A duration based pre-licensing strategy, therefore, does result in more IDO protein but the effect size is consistently smaller in magnitude and has higher variance when applied to multiple donors.

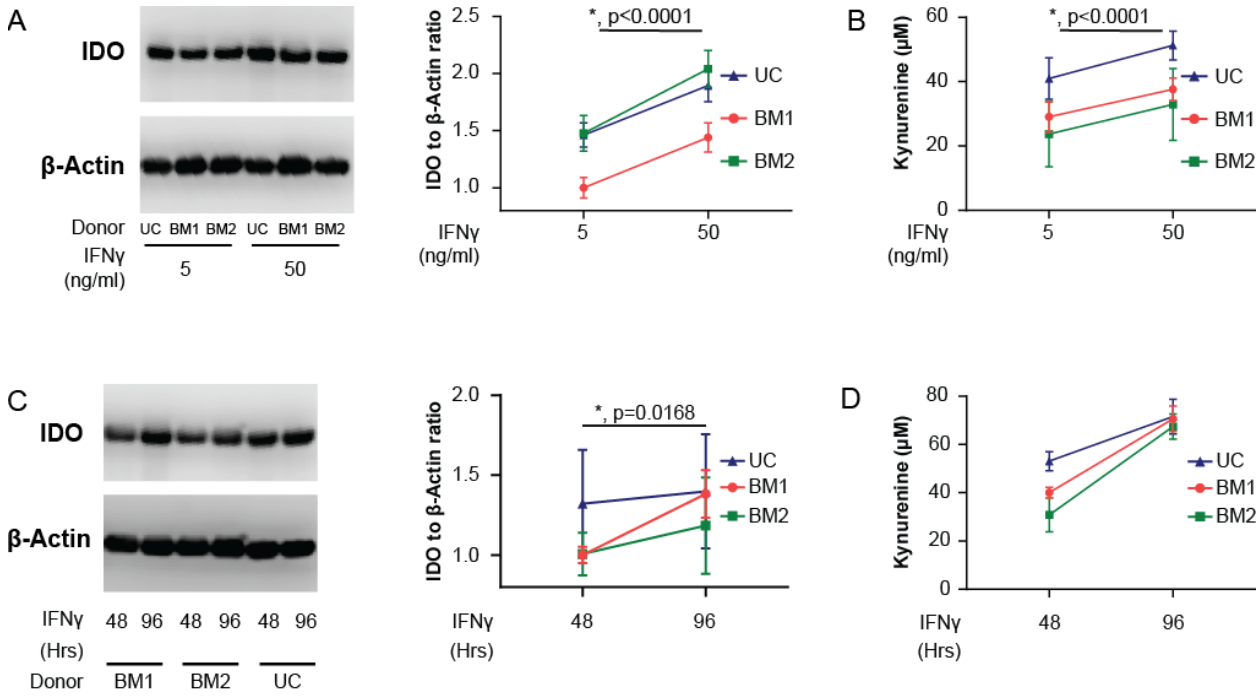


Figure 2: Enhanced Dose and Duration of IFNγ During Pre-licensing is an Effective Strategy to Increase IDO Protein Regardless of Baseline Donor Profile

A) Two bone marrow (BM1, BM2) and one umbilical cord derived (UC) MSC donors were passage matched and PL in either 5 or 50 ng/mL IFNγ for two days. After two days, media and lysate were collected. IDO protein concentration was then determined via western blotting, normalized to housekeeping gene β-Actin, and further normalized to the 5 ng/mL IFNγ, BM1 condition. N=3 independent experiments, paired to Figure 2B. Reporting mean ± SEM. B) Media that was collected at the same time as lysate for Figure 2A had kynurenine concentrations measured with a kynurenine media assay to determine IDO enzymatic activity. N=3 independent experiments, paired to Figure 1B. Reporting mean ± SEM. C) Two bone marrow (BM1, BM2) and one umbilical cord derived (UC) MSC donor was passage matched and pre-licensed in 10 ng/mL IFNγ for either 48 or 96 hours. After pre-licensing, media and lysate were collected. IDO protein concentration was then determined via western blotting, normalized to housekeeping gene β-Actin, and further normalized to the 48 hour, BM1 condition. N=3 independent experiments, paired to Figure 2E. Reporting mean ± SEM. D) Media that was collected simultaneously as lysate for Figure 2D had kynurenine concentrations measured with a kynurenine media assay to determine IDO enzymatic activity. N=3 independent experiments, paired to Figure 2D. Reporting mean ± SEM. Statistical analysis used for A, B and C was a paired t-test.

### 2.3.3. Duration of Pre-licensing Predicts Durability of IDO Protein Post Withdrawal

After determining the individual contribution of the dose and duration of IFNγ during pre-licensing on IDO protein levels and activity, we next investigated if the elevated protein induced by a duration-based strategy would persist and remain functional after removal from the pre-licensing environment. To test this, MSCs were pre-licensed in 10 ng/mL IFNγ for two, four, or six days prior to being transferred to a cytokine-free environment for outgrowth (post-withdrawal period) (Figure 3A). As seen from prior experiments, MSCs pre-licensed for longer durations had elevated IDO protein levels immediately after withdrawal (Figure 3B). Interestingly, we found that this enhanced IDO protein concentration persisted throughout the 72 hr withdrawal period, with the percent of IDO remaining being highest in MSCs pre-licensed for six days (Figure 3B). Additionally, we analyzed kynurenine output at the end of the pre-licensing period, as well as over the course of withdrawal. At the conclusion of pre-licensing, all three durations resulted in a similar amount of kynurenine conversion (Figure 3C). In the post-withdrawal period, kynurenine production was similar 24 hrs post-withdrawal for all tested pre-licensing durations; however, the output diverged significantly by 48 and 72 hrs (Figure 3D). Therefore, the duration of IFNγ exposure during pre-licensing increases IDO protein levels leading to enhanced persistence and activity of the protein post-withdrawal.

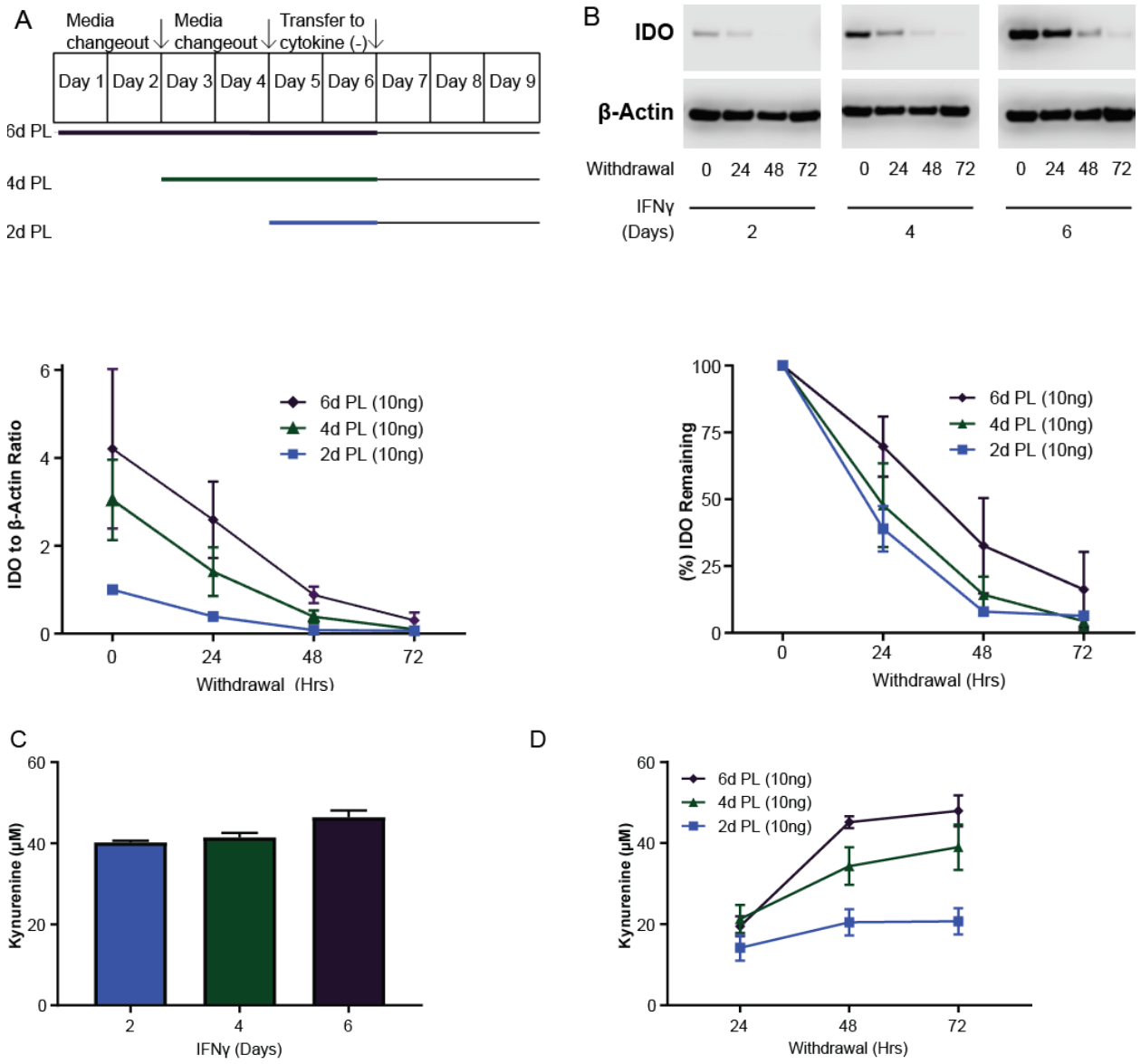


Figure 3: Longer Durations of Pre-licensing Enhances IDO Protein and Activity Post Withdrawal

A) Donor and passage matched MSCs were pre-licensed in 10 ng/mL IFNγ for either 2, 4, or 6 days. All MSCs had total media change out every 2 days during this 6 day period. After pre-licensing, MSCs were washed with PBS and transferred to a new, IFNγ free media. On the day of withdrawal, media was collected and remaining cells were lysed for protein determination. MSCs were then cultured for 72 hours. Media and lysate was collected every day for these 72 hours. B) Lysate was ran via western blotting to determine IDO protein concentration which was normalized to the house keeping gene β-Actin and further normalized to the 2 day pre-licensing group on the day of withdrawal. Percent remaining was calculated as IDO protein divided by IDO protein on the day of withdrawal. N=3 independent experiments, paired to figure 3C. Reporting mean, ± SEM. C) MSCs' kynurenine concentration for the last 48 hours of pre-licensing. Reporting mean ± SEM. D) Accumulation of kynurenine 24, 48, and 72 hours post

withdrawal was determined via a kynurenine media assay. Statistical test used was a 1-Way ANOVA, p=0.0306 48 hrs, p=0.0299 72 hrs. N=3 independent experiments. Paired to Figure 2B. Reporting mean ± SEM.

### 2.3.4. Accumulation of Kynurenine Metabolites During Pre-licensing Does Not Affect IDO Protein Concentration or Activity

One concern with pursuing a duration-based strategy of pre-licensing is the potential accumulation of kynurenine and/or kynurenine-derived trace extended aromatic condensation products (TEACOPs). TEACOPs are derivatives of kynurenine that are spontaneously formed at physiological termparatures. Previous studies have identified both of these compounds as regulators of IDO expression in other cell types; however, their effect on MSC regulation of IDO is, as of yet, unexplored. Therefore, we sought to determine the consequence of increased accumulation of these metabolites during MSC pre-licensing. Should these compounds alter the protein concentration or activity of IDO, their selective removal or addition could be a strategy to enhance the final PL-MSC product. In this experimental set-up, MSCs were pre-licensed with 10 ng/mL IFNγ with the addition of 0-100 uM kynurenine or heated kynurenine for four days. While heating of kynurenine has been shown by other groups to from TEACOPs, we tested how the heating of kynurenine (to 37°C), such as during a pre-licensing period, would impact IDO. During pre-licensing, however, neither the addition of fresh nor heated kynurenine had any appreciable impact on the production of IDO protein (Figure 4A) or IDO enzymatic activity (Figure 4B). It appears, then, that in MSCs, these metabolites do not play a critical role in modulating IDO.

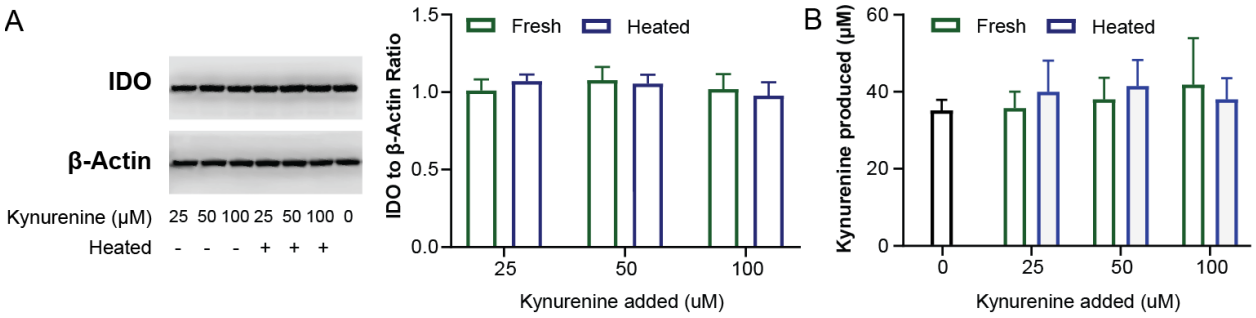


Figure 4: Kynurenine and TEACOPS Do Not Significantly Regulate IDO Protein or Activity

A) Donor and passage matched MSCs were pre-licensed in 10ng/mL IFNγ with the addition of 0, 25, 50 or 100 μM kynurenine or kynurenine that had previously been heated for 48 hours at 37° C. MSC were pre-licensed for four days at which point lysate and media were collected. Lysate had IDO protein concentration determined via a western blot, normalized to the house keeping gene β-Actin, and further normalized to 0 μM kynurenine condition. N=3 in series experiments, paired to figure 4B. Reporting mean, ± SEM. B) Media from figure 4A had kynurinene concentration determined via a kynurenine media assay. An additional plate, identical to the plate from figure 4A but without cells was constructed and incubated at the same time. Media from this no cell plate also had kynurenine concentration measured as a kynurenine media assay. The reported kynurenine produced was calculated as kynurenine in each condition subtracted by its complementary no cell condition. N=4 independent experiments, paired to figure 4A. Reporting mean ± SEM.

### 2.3.5. T Cell Suppression is Enhanced with High Dose IFNγ but Diminished with Longer Pre-licensing

Having isolated the individual contribution of IFNγ dose and duration on IDO protein and activity, we next sought to determine if this translated to enhanced potency in a human PBMC suppression assay. To quantify the immunosuppressive ability of PL-MSCs, we pre-licensed our three donors (BM1, BM2, UC) using three unique strategies prior to direct contact co-culture with CD3/CD28 activated PBMCs, as well as using a naive (non-prelicensed) control to determine baseline immunosuppressive ability for each donor. The pre-licensing strategies used were: base pre-licensing (10ng/mL IFNγ for two days), high dose pre-licensing (50ng/mL IFNγ for two days), and prolonged duration pre-licensing (10ng/mL IFNγ for four days). This combination of strategies was chosen to fix either dose or duration within each donor. Based on the results of our IDO protein and activity assays, we hypothesized that both high dose and longer duration would improve immunosuppressive potency compared to the low dose short duration protocol.

Our positive control, base PL-MSCs, showed improved immunosuppressive potency compared to naive MSCs, indicating a benefit to pre-licensing. Additionally, consistent with our hypothesis, all donors benefited from a high dose of IFNγ, showing the highest suppression of proliferation across all tested strategies (Figure 5A). Surprisingly, prolonged duration did not lead to improved suppression compared to naive MSCs (Figure 5A). When comparing base pre-licensing to high-dose pre-licensing, although there was a significant difference between these strategies, the difference was small (5.03%). Interestingly, in comparing the effect of donor choice versus pre-licensing strategy, most of the variance in immunomodulatory performance was attributable to donor choice (76.56% of variation, 2-way ANOVA). Therefore, both the pre-licensing strategy and donor choice have significant roles in improving MSC immune

suppression. However, it is important to note that improved performance in co-culture assays does not directly correlate with IDO protein and activity findings, highlighting the need for multiple metrics to be used to predict MSC function.

The different donors' potency varied widely without pre-licensing but the UC was significantly more suppressive than the BM1 and BM2 (Figure 5B). Interestingly, while the trends of different pre-licensing strategies were maintained across donors, the magnitude of this benefit varied substantially across donors with BM1 benefiting the most, UC receiving an intermediate benefit, and BM2 benefiting the least (Figure 5B). Regardless of how pre-licensing affected the different donor's potency, the umbilical cord MSCs were more suppressive than either of the bone marrow derived MSCs, regardless of pre-licensing. The effect of pre-licensing, therefore, is not uniform, but the product of an interaction between the pre-licensing strategy and the intrinsic properties of the donor of MSCs.

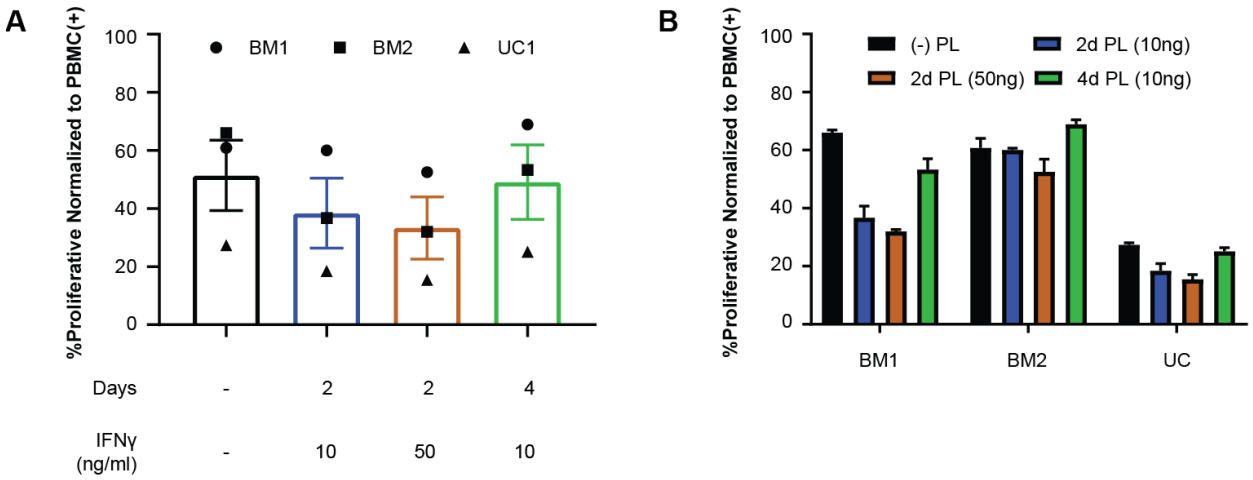


Figure 5: Dose, Duration and Donor are Regulators of MSC Immune Modulation

A) Two bone marrow (BM1, BM2) and one umbilical cord derived (UC) MSC donor was passage matched and either not pre-licensed, pre-licensed in 10 ng/mL IFNγ for two days, pre-licensed in 50 ng/mL IFNγ for two days, or pre-licensed in 10 ng/mL IFNγ for four days. After pre-licensing or culturing without IFNγ, MSCs were thoroughly washed in PBS and transfered to a new culturing flask to be used at a ratio of 1:10 MSC:PBMCs. After adding MSCs, PBMCs that were stimulated to proliferate with dynabeads were added at a fixed seeding density. These cells were then cultured for 6 days at which point PBMCs were harvested and analyzed for proliferation via flow cytometry. N=3 in series experiments, paired to figure 5B. Reporting Mean ± SEM. B) Data presentation highlighting different pre-licensing strategies effect on PBMC proliferation within each MSC donor. N=3 in series experiments, paired to figure 5A. Reporting Mean ± SEM.

### 2.3.6. Increased Tryptophan During Pre-licensing Enhances IDO Protein and Activity

Kynurenine and its further degraded metabolites did not have an appreciable impact on MSC IDO protein concentration or activity (Figure 4A, B). Tryptophan along with kynurenine, TEACOPS, and 1-MT are all ligands of the Aryl Hydrocarbon Receptor, an established IDO regulator in MSCs[52]. Although IDO metabolites were ineffective, we next investigated if tryptophan could be supplemented during pre-licensing to enhance IDO protein concentration and durability. To test this, we cultured BM1 in 10 ng/mL IFNγ in either base media containing 50 µM tryptophan or media supplemented with 500 µM of tryptophan for two days. After this two-day period, MSCs were transferred to 50 µM tryptophan media without IFNγ for three days to determine how IDO changed after reduction of inflammation.

Increasing the concentration of tryptophan tenfold increased IDO protein levels on the day of withdrawal by an average of 45.77% ($p=0.0081$, paired t-test)(Figure 6A). Not only did IDO protein concentrations increase after the two days of IFNγ culturing but the IDO containing phenotype persisted for longer. One day after withdrawal of IFNγ and transfer into a media containing 50 µM tryptophan, the 50 µM tryptophan PL-MSCs had 47.06% of their initial IDO remaining while the 500 µM tryptophan PL-MSCs had 75.96% of their initial IDO remaining (Figure 6B). The increase in tryptophan during pre-licensing also had a significant effect on the percent IDO remaining two ($p=0.0035$, paired t-test) and three days ($p=0.0494$, paired t-test) after withdrawal (Figure 6B). Tryptophan concentration during a pre-licensing period, therefore, not only effects IDO protein concentration but also how long the IDO containing phenotype persists after removal of inflammatory cytokines.

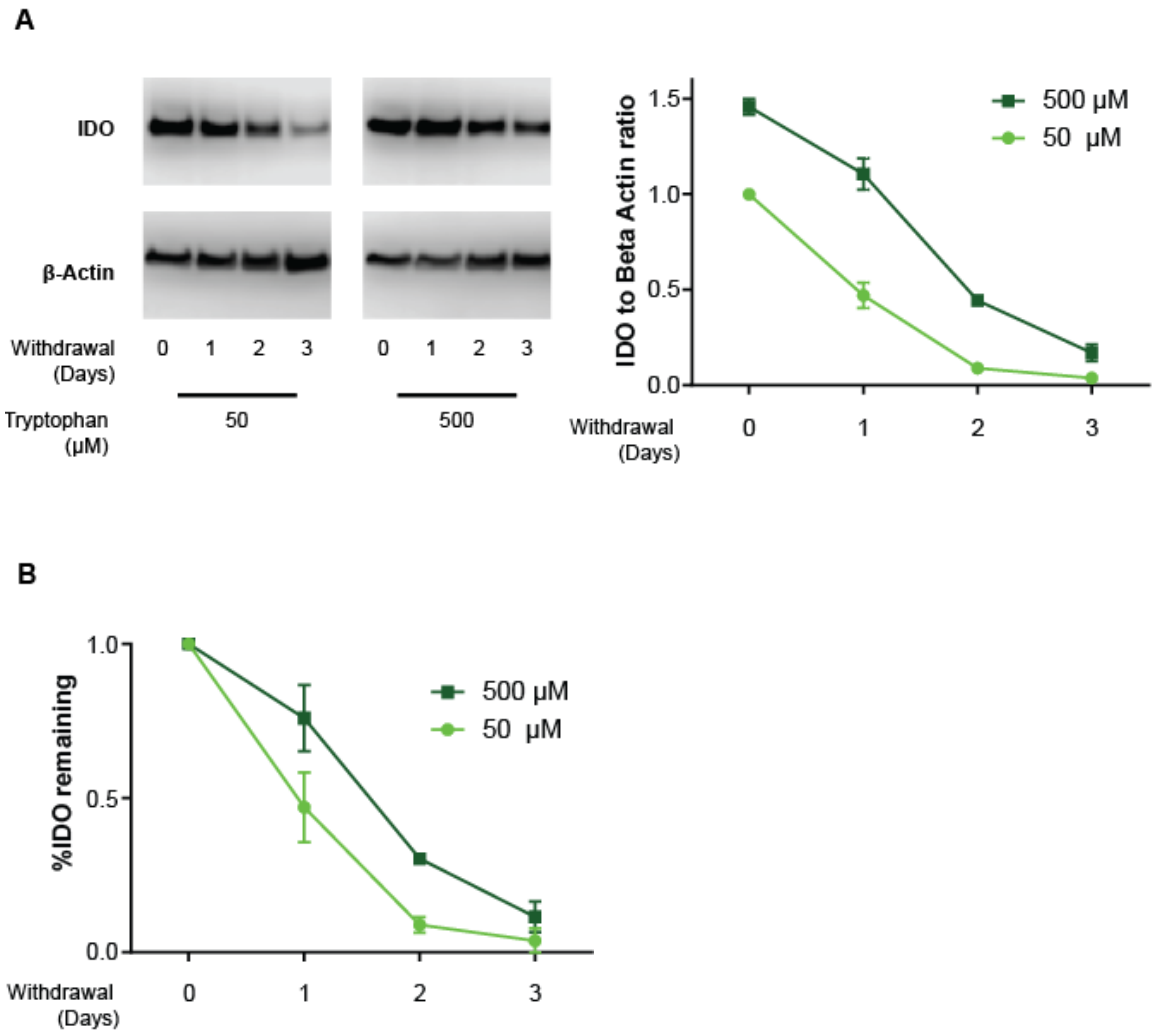


Figure 6: Tryptophan During Pre-licensing is an Additional Regulator of IDO Protein Concentration and Durability

A) Donor and passage matched MSCs were pre-licensed in 10ng/mL IFNγ in either 50 or 500 μM tryptophan for two days. After pre-licensing, MSC were thoroughly washed and transferred to IFNγ free media containing 50 μM tryptophan for three days. Protein was collected on the day of withdrawal as well as each day for the three days after withdrawal. Lysate had IDO protein concentration determined via a western blot, normalized to the house keeping gene β-Actin, and further normalized to 50 μM tryptophan condition on the day of withdrawal. N=3 independent experiments, paired to figure 6B. Reporting mean, ± SEM. B) Percent IDO remaining was calculated as IDO protein divided by IDO on the day of withdrawal for either the 500 or 50 μM tryptophan condition. N=3 independent experiments, paired to figure 6A. Reporting mean, ± SEM. Statistical tests used in panels A and B were paired t-tests.

### 2.3.7. Increase in IDO Durability through Higher Concentrations of Tryptophan During Pre-licensing is Donor Specific

With tryptophan concentration during pre-licensing shown to be a regulator of IDO durability, we next investigated how this effect was maintained when investigated in multiple donors. To test this, we pre-licensed one umbilical cord (UC) and two bone marrow derived (BM1 and BM2) MSC donors in either 50 or 500 μM tryptophan and 10 ng/mL IFNγ for two days. After pre-licensing, MSCs were transferred to media containing 50 μM tryptophan and no IFNγ and cultured for one day to calculate how much IDO remained. At baseline, there was a large spread in percent IDO remaining between donors and tissue sources. All donors, however, did have more IDO remaining when pre-licensed in more tryptophan. Surprisingly, this enhancement was more pronounced in two donors (UC1, BM2) and was not tissue source specific. Thus, while tryptophan is indeed a regulator of IDO protein post withdrawal, the magnitude of this effect is donor dependent.

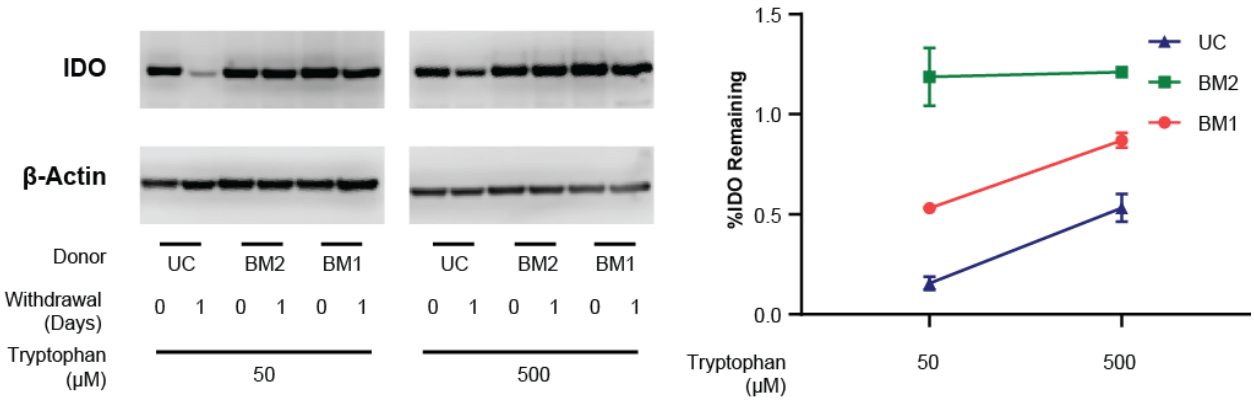


Figure 7: Tryptophan's Effect on Percent IDO Remaining is Deferentially Regulated across MSC Donors

One umbilical cord (UC) and two bone marrow (BM1, BM2) derived MSCs were pre-licensed in either 50 or 500 μM tryptophan and 10 ng/mL IFNγ for two days. After pre-licensing, MSC were thoroughly washed and transferred to IFNγ free media containing 50 μM tryptophan for one day. Protein was collected on the day of withdrawal as well as one day after withdrawal. IDO protein concentration was determined via a western blot and normalized to the house keeping gene β-Actin. Percent IDO remaining was calculated as IDO protein one day after withdrawal divided by the day of withdrawal for each donor. N=2 independent experiments. Reporting mean ± SEM.

### 2.3.8. Altering Tryptophan Concentration Differentially Affects IDO Protein Levels and Enzymatic Activity

While tryptophan was shown to be a donor specific regulator of IDO protein, we next investigated how tryptophan affected enzymatic activity. Tryptophan has been proposed as a contributor to disease state tolerance in viral infections as it is an essential amino acid vital to the metabolism of immune cells[53]. While tryptophan addition in an MSC:PBMC co-culture reverses the immune suppression by MSCs on T cells[54], we determined how the addition of different concentrations of tryptophan in an inflammatory environment affected MSCs production of immunomodulatory kynurenines. If addition of tryptophan inhibits MSC's IDO, this loss of potency observed in a co-culture could be due to a loss of enzymatic function rather than tryptophan's effect on tolerance.

To test this, MSCs were cultured in 10 ng/mL IFNγ along with a dose range of 50 to 500 μM tryptophan for four days. Efimov et al. demonstrated that the isolated IDO enzyme is inhibited by high concentrations of tryptophan[55]. We predicted an inhibition of activity at the highest doses as demonstrated by Efimov et al. but to a lesser degree as tryptophan must be transported across a membrane to be metabolized by MSCs' IDO. Interestingly, the effect of tryptophan on kynurenine production differed widely across this dose range. For 96 hours of culturing, kynurenine production was highest for the mid-level tryptophan concentration of 163 μM and lowest for high and low concentrations (Figure 8A), demonstrating a benefit with regards to IDO activity within MSCs at a specific dose range.

With tryptophan concentration shown to regulate IDO enzymatic activity, we next wished to determine if this change in activity was due to a change in the amount of IDO protein as a function of tryptophan concentration. IDO protein showed significant differences across this

dose range (Figure 8B). The effect of tryptophan concentration on IDO protein levels were the opposite of what was observed with kynurenine production for this time course. The mid-level tryptophan concentrations had the lowest relative IDO when it had the highest activity (Figure 8A, B). The low and high level tryptophan concentrations, on the other hand, had the highest concentration of relative IDO (Figure 8B). The correlation between IDO protein levels and kynurenine production were negatively correlated (Pearson r=-0.944, p=0.0158), demonstrating a buildup in IDO protein that was not functional.

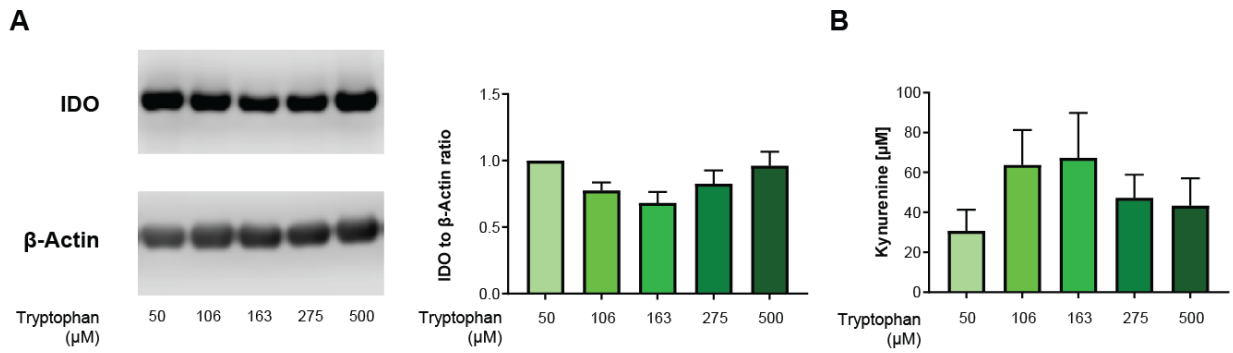


Figure 8: IDO Protein Levels and Enzymatic Activity are Tryptophan Dose Dependent

A) Donor and passage matched MSCs were cultured in a dose range of 50 to 500 μM tryptophan as well as 10 ng/mL IFNγ for 96 hours. After culturing, lysate and media was collected. Lysate was then analyzed for IDO protein concentration via western blotting. IDO protein concentration was normalized to the housekeeping gene β-Actin and further normalized to the 50 μM condition. N=3 in series experiments, paired to figure 7B. Reporting mean ± SEM. B) Media was tested for kynurenine concentration via a kynurenine media assay. N=3, in series experiments, paired to figure 7A. Reporting mean ± SEM.

## 2.4. Discussion

The immune modulating protein, IDO, can be upregulated to enhance potency of MSCs in a direct contact co-culture suppression assay. Herein, we demonstrated the minimum dose and duration of IFNγ during pre-licensing to have detectable IDO protein as well as the dose and duration responsiveness of IDO to this inflammatory cytokine (Figure 1A,B). This data informed the design of dose and duration based pre-licensing strategies that were tested in multiple MSC donors. Interestingly, a dose based strategy had a more predictable effect on IDO protein. Both the concentration of protein and accumulation of kynurenine increased to a very similar degree across all donors tested (Figure 2A,B). The duration based pre-licensing strategy had a more variable effect on IDO protein across donors (Figure 2C), which was not able to be determined when tested initially in one donor (Figure 1B). The observation that IDO protein remains active throughout a 96 hour pre-licensing period, however, was maintained across donors (Figure 2D).

Next, we wished to determine how pre-licensing affected the durability of the IDO containing phenotype. While identification of pre-licensing protocols that maximize IDO activity are desirable, they are only useful if IDO remains functional once the cells are transplanted. Prolonged pre-licensing increases IDO in MSCs (Figure 1B, Figure 2C), but if this elevated protein concentration would remain functional and persist after withdrawal from the pre-licensing environment was investigated. We speculated that increased duration during pre-licensing would enhance IDO protein, not only on the day of withdrawal, but also throughout a withdrawal period. Our reason for predicting this is that Rovira Gonzalez et al. has demonstrated that the IDO promoter is more permissive due to a higher methyl to acytl group ratio and is more readily transcribed with longer durations of IFNγ exposure[40]. This chromatin remodeling persists after removal of cytokines, indicating a more durable IDO phenotype. Both of our predictions, that IDO protein would be elevated on the day of withdrawal as well as on subsequent days after

removal of IFNγ, were supported (Figure 3B). In addition, IDO enzymatic activity was higher after a three day withdrawal period for MSCs pre-licensed for longer (Figure 3D). Enhanced IDO persistance may be due to lower degradation of protein but further studies would need to be performed to confirm this.

Next we looked to kynureninens as part of a pre-licensing strategy. Kynurenine and kynurenine derivatives are known ligands for the aryl hydrocarbon receptor (AhR), which has been shown to regulate IDO expression[56]. Thus, depending on the type of regulation, accumulation of such byproducts could be either beneficial or detrimental to the production of potent PL-MSCs. Seok et. al. showed that kynurenine derived trace extended aromatic condensation products (TEACOPs), which are spontaneously formed at physiological temperatures, are potent ligands for AhR. Once bound, the AhR-ligand complex translocates to the nucleus and regulates IDO transcription[52]. AhR regulation can either augment or inhibit transcription of target genes and is dependent upon cell type and the ligand that binds AhR[52]. The role of these AhR ligands on MSC translation and activity of IDO is not currently known. While Seok et al. proposed TEACOPS as potent AhR activators, the addition of kynurenine or heated kynurenine did not have an appreciable effect on IDO protein or activity in MSCs (Figure 4A,B). Selective removal or addition of these compounds during biomanufacturing, therefore, would not affect IDO protein concentration or activity in PL-MSCs.

Next, with the effect of IFNγ dose an duration during pre-licensing on IDO protein and activity robustly tested, how this altered IDO translated to immune suppression was determined. To our surprise, minimal dose and duration of IFNγ during pre-licensing had a consistent and large benefit in potency (Figure 5A) and enhancing the dose five-fold only moderately increased immune modulation (Figure 5A). Even more interesting, a longer duration during pre-licensing

was actually detrimental to potency (Figure 5A). This demonstrates a case where IDO protein concentration increased without enhancing immune modulation, which suggests that this surrogate marker for immune suppression is not applicable in every context. While pre-licensing had a consistent effect across donors, the donor of MSCs itself had a larger impact on immune modulation than any strategy tested (Figure 5B). Screening of donors for allogeneic use, therefore, is necessary as pre-licensing does not generate an equivalent MSC products.

Among the data collected, the reduced potency when pre-licensing for longer was the most surprising finding of our study. MSCs utilized in academia and those produced by manufacturers differ in many ways including culturing conditions such as IFNγ pre-licensing[20]. Exposure to inflammatory cytokines has been proposed as a potential danger to patients as some groups have claimed this induces antigen presentation in MSCs and increases expression of MHC class I and II[57,58]. While shown to be beneficial in enhancing T cell suppression *in vitro* with human MSCs, IFNγ pre-licensing has also been shown to decrease potency in a *in vivo* mouse model of I/R injury[59]. This discrepancy highlights the need to tailor pre-licensing to the context and disease setting in which it is being applied to. Beyond enhanced IDO activity, pre-licesing also effects MSC expression of MHCs which could impact their immunoevasive properties. Thus, future work is needed to investigate the effect of pre-licensing on MHCs and immune evasion to understand how this process may impact MSCs in humans.

How MSCs are treated prior to transplant and the environment they are transplanted in are all regulators of MSC immune modulation. But many factors during the manufacturing of MSCs can differ while still producing a cell product that meets ISCT minimum criteria. How MSCs are pre-licensed is not standardized, and the dose and duration of IFNγ exposure can vary widely. While a low dose of IFNγ for a brief period enhanced potency, a larger dose increased

potency only slightly. A longer duration, on the other hand, reduced potency. Thus, the way MSCs are pre-licensed, and how MSCs are manufactured, are all important regulators of immune modulation.

## 2.5. Materials and Methods

### 2.5.1. Determination of Protein Concentration

MSCs, cultured in 6 well plates(VWR, 89131-688), were washed three times in 1mL of phosphate buffered saline(Caison Labs, PBL01-6X500ML) while resting on ice. After these three washes were completed, each well was individually aspirated of PBS, had 40uL of RIPA lysis buffer(Santa Cruz Biotechnology, sc-24948A) added to it, and scraped with a cell scraper(VWR, 89260-222) for roughly one minute. Lysate was then collected into a 2mL microcentrifuge tube(VWR, 20170-170) and spun at 8000g for 10 minutes at 4 degrees C. After spinning, the supernatant was collected and transferred to a new 2mL microcentrifuge tube. 5uL of lysate samples where then added to 195uL of PBS to achieve a 1:40 dilution. A ladder of Bovine Serum Albumin was constructed by diluting 2mg/mL BSA(Thermo Scientific, 23235), included in a Micro BCA protein assay kit, in PBS for a range of 200-0 ug/ml total protein as a 1:1 serial dilution. 150uL of lysate or BSA standard were then added to a 96 well plate(Greiner, 655180) at which point 150uL of working reagent(Thermo Scientific, 23235) was added. Working reagent was part of the Micro BCA protein assay kit, consisting of 25 parts A, 24 parts B and 1 part C, which was vortexed and added to each active well using a multichannel pipet. Next, a cover slip(Corning, 4612) was added and the 96 well plate was incubated at 37 degrees C for 12 minutes. After incubation, the plate was read at 562nm on a photospectrometer(Molecular Devices, 60139412). A standard curve was then constructed based on absorbance readings of the

BSA ladder. This standard curve was then used to interpolated unknown concentrations of protein.

### 2.5.2. Gel electrophoresis

Remaining lysate was denatured by adding 38.5uL of 4X LDS sample buffer (Thermo Fisher, B0007) and 15.5uL of 10X Bolt reducing agent(Invitrogen, B0009) per 100uL of lysate and heated at 95 degrees Celsius for 2 minutes. Running buffer was made up using 50mL of 20X stock (Invitrogen, b0002) and 950mL of milliQ(Biopak, CDUFBI001) filtered water. 10 well gels(Life Technologies, NW04120BOX) were prepared by removing the comb, washing 3X with running buffer using a transfer pipet(Fisher Scientific, 13-711-9AM), and removing tape on the back end for a complete circuit. The Gel electrophoresis apparatus(Life Technologies, A25977) was loaded with the prepped 10 well gels and filled with running buffer to cover wells as well as the open slit on the rear side of the gel. Loading volumes to achieve 10ug of protein per well were added as well as 5uL of precision plus protein Kaleidoscope(Bio-Rad, 1610375) to determine protein migration as well as blanks. Blanks consisted of RIPA lysis buffer with 38.5uL of 4X LDS sample buffer and 15.5uL of 10X Bolt reducing agent per 100uL of lysate and heated at 95 degrees Celsius for 2 minutes. The gel was then ran at 200V for 20 minutes.

Transfer buffer was made up with 700mL of milliQ, 200mL methanol(Fisher Scientific, A414P-4), and 100mL Tris glycine buffer(Amresco, M114-1L), which was used at 4 degree C. PVDF blotting membrane(GE Healthcare, 10600023) was soaked in methanol for two minutes and placed in transfer buffer until ready for use. After the 20 minute run, the gel electrophoresis system was constructed in this order: cathode, sponge, filter paper (VWR, 28298-020), gel, membrane, filter paper, sponge, anode. The filter paper and sponge were each dipped in transfer

buffer prior to being placed within the cassette. Once the cassette was constructed, it was then placed in the gel electrophoresis system with deionized water poured around it to act as a heat sink. The cassette was then run at 10V for 2 hours to transfer protein from the gel to the membrane.

### 2.5.3. Antibody staining

After the transfer was complete, the membrane was removed from the cassette and blocked in 5% weight by volume milk(Carnation, 9259331) in a four well plate(E&K Scientific, EK-97307) for one hour. After blocking, primary antibody was added which consisted of 5% weight by volume BSA(Sigma-Aldrich, A9647-10G) and either 1:1K IDO primary antibody(BioLegend, 122402) or 1:10K β-Actin antibody(Thermo Fisher, AM4302). Primary antibody staining was performed overnight at 4 degrees Celsius with gentle rocking. After primary antibody staining, three TBST washes were performed for ten minutes with gentle rocking at room temperature. TBST was comprised of a 20X powder(VWR, 97062-370) which was diluted in milliQ filtered water with 0.1 percent weight by volume Tween 20 (Sigma-Aldrich, P1379-100ML).

Secondary antibody was then added which consisted of 5% weight by volume milk, and either1:10K anti-rabbit HRP conjugated antibody(Santa Cruz, sc-2004) for IDO or 1:10K anti-mouse HRP conjugated antibody(Biolegend, 405306) which was incubated for 30 minutes at room temperature with gentle rocking. After secondary antibody staining, three additional TBST washes for 10 minutes with gentle rocking took place. A WesternBright Quantum mix(Advansta, K-12042-D10), consisting of 3mL of PBS, 1.5mL of WesternBright peroxide, and 1.5mL of

WesternBright quantum, was then added to the membrane. The membrane was then scanned on a C-DiGit Blot Scanner (LI-COR Biosciences, 3600-00) on high sensitivity to IDO.

### 2.5.4. IDO Activity Assay

A ladder of known kynurenine concentrations were constructed as a 1:1 serial dilution ranging from 200uM to 0uM. The diluent used was the same media type MSCs were grown in which was mem-alpha (Biological Industries, 01-042-1A) supplemented with 15% volume by volume fetal bovine serum albumin (Biological Industries,04-400-1E-US), 1% Penicillin-Streptomycin Solution (Biological Industries, 03-031-1B) and 1% L-glutamate(gibco, 25030-081). 200uL of these known kynurenine concentration standards were then added to a 96 well plate. 200uL media, obtained from MSCs throughout the course of this experiment were added to the 96 well plate. After adding known and unknown kynurenine samples, 100uL of 30% weight by volume trichloroacetic acid (Sigma-Aldrich, T9159-100G) was added to each active well. A cover slip was then added to the 96 well plate to avoid evaporative loss during subsequent heating. The 96 well plate was then heated at 52 degrees Celsius for thirty minutes to convert N-formylkynurenine to kynurenine. After heating, the 96 well plate was spun at 1200g for 15 minutes. 150uL of supernatant was then collected and add to a new 96 well plate. This 150uL was mixed with a pipette and then 75uL of which was transferred to an adjacent well to act as a replicate. 75uL of p-dimethylaminobenzaldehyde (Sigma-Aldrich, 156447-25G) was then added to each active well for a final volume of 150uL. The plate was read on the photospectrometer(Molecular Devices, 60139412) with an excitation wavelength of 492nm. Absorbance values of the known kynurenine standards were used to construct a standard curve.

Unknown kynurenine concentrations were then interpolated using this standard curve to determine kynurenine concentrations.

### 2.5.5. MSC Sources

Bone marrow derived MSCs (BM1, and BM2) were obtained from RoosterBio while umbilical cords (UC) were obtained from University of Iowa Women's Health Tissue Repository. RoosterBio MSCs were pre-validated while MSCs isolated from umbilical cords were tested in accordance with the International Society for Cellular Therapy.

# CHAPTER 3: CONTEXT DEPENDENCE OF IDO

In addition to studying MSC regulation of IDO, I also leveraged my experience to contribute to findings on three manuscripts that are either in print or published by my lab. In chapter two, I investigated the effect of different biomanufacturing parameters on MSC immune modulation. In this chapter, I review my contribution to findings on post-transplant regulators of MSC potency. These regulators include the effect of disease state metabolic environments as well as an MSC phenotype commonly formed *in vivo*. The manuscripts that resulted are:

Burand *et al.* Aggregated human mesenchymal stromal cells lose the ability to suppress T cells but regain it via synergy with budesonide. Under Revision.

47. Boland, L. *et al.* IFN-γ and TNF-α Pre-licensing Protects Mesenchymal Stromal Cells from the Pro-inflammatory Effects of Palmitate. *Mol. Ther.* (2018). doi:10.1016/j.ymthe.2017.12.013

60. Boland, L. K. *et al.* Nature vs. Nurture: Defining the Effects of Mesenchymal Stromal Cell Isolation and Culture Conditions on Resiliency to Palmitate Challenge. *Front. Immunol.* **10**, (2019).

In "Aggregated human mesenchymal stromal cells lose the ability to suppress T cells but regain it via synergy with glucocorticoid steroids", our lab investigated phenotypic changes between MSCs grown on a flat surface (adherent MSCs) and 3D MSC aggregates (spheroid MSCs). MSCs form aggregates when in close proximity to each other, where the effect of cell-

cell contact forces increase. This phenotype is commonly formed *in vivo*, particularly when MSCs are locally injected and are confined in narrow spaces of vasculature. Upon spheroid formation, MSCs have altered expression of key immunomodulatory genes, proteins and secreted factors, including TSG-6 and PGE2[60,61,62]. In order to translate MSC therapy to the clinic, we must determine how the immunomodulatory profile differs between adherent MSCs and the spheroid phenotype formed *in vivo*.

Our finding was that these phenotypes modulate immune cells differnetially and that spheroid MSCs lose suppressive potency towards T cells. This potency, however, is regained in a MSC:T cell co-culture with the addition of the glucocorticoid steroid budesonide. My contribution to this project was performing western blots on the immunomodulatory enzymes COX-2 and IDO, as well as performing an IDO activity assay to measure kynurenine concentration between adherent and spheroid MSCs. While spheroid and adherent MSCs had similar IDO protein concentrations, the kynurenine production by spheroid MSCs was significantly reduced. In addition, COX-2, and its metabolite PGE-2, were only detectable in spheroid MSCs.

In "IFN-γ and TNF-α Pre-licensing Protects Mesenchymal Stromal Cells from the Pro-inflammatory Effects of Palmitate", a type 2 diabetic and obese microenvironment is simulated with the use of palmitate, a long chain saturated fatty acid that is elevated in blood serum of individuals suffering from both of these metabolic diseases. Palmitate shifts MSCs' immune modulation from suppressive to inflammatory in a dose responsive manner, an effect which is reversed by pre-licensing with the inflammatory cytokines IFNγ and TNFα. I contributed to this discovery by pre-licensing MSCs with IFNγ and TNFα for different durations. I then measured

IDO protein concentration via western blotting and determined that IDO protein increased in parallel with MSC immunomodulatory potency.

In "Nature vs. Nurture: Defining the Effects of Mesenchymal Stromal Cell Isolation and Culture Conditions on Resiliency to Palmitate Challenge" the interaction between metabolic disease state environmental conditions and biomanufacturing parameters are explored further. Our team determined how the donor of MSCs and growth factors used to expand them out affected their ability to overcome the pro-inflammatory effects of palmitate. Our finding was that MSC donors had variable potency at baseline when expanded out in either bovine (fetal bovine serum albumin) or human (human platelet lysate) growth factors. MSCs stimulated with xeno-free growth factors, however, had enhanced growth kinetics and a significant improvement in immune modulation at mid-level concentrations of palmitate. My contribution to this project was culturing MSCs in either bovine or human growth factors, counting cells to determine growth kinetics, challenging MSCs with palmitate, and analyzing suppressive potency across donors and conditions via flow cytometry.

# CHAPTER 4: FUTURE DIRECTIONS

Meeting the criteria for an MSC does not ensure bio-equivalence. We have learned from discrepancies between academia and industry that where MSCs originate and how they are treated have broad impacts on their efficacy[20]. But the future is bright for MSCs. We have decades of understanding of their cell biology and learned hard lessons on what makes an efficacious therapy. For us to move forward, we need stringent criteria and screening that encompass multiple metrics to predicted immune modulation. While this thesis focused on IDO, no one factor as of now can predict the efficacy of MSC therapy in every setting. Instead, it is a context dependent, multifaceted problem that engineers and researchers have only recently elucidated. While much of my work focused on one enzyme, its metabolite and the signal needed to induce it, this simple yet robust approach demonstrated the complexity of an interacting, dynamic system that is the MSC.

What we saw first was a dose response with IFNγ where we plateaued and maxed out protein halfway through the range we tested. Next we saw a trend in the spatiotemporal relationship between IFNγ and IDO that did not appear to have a limit in the confines that we tested. IDO protein continued to build with time as did the durability of the phenotype. But what we thought was the best strategy to increase IDO protein, a duration based strategy, did not enhance suppressive action towards PBMCs as we predicted. On the contrary, we discovered something far more fascinating. IDO was increased when we pre-licensed our MSCs for longer (Figure 1B) but elevated protein did not translate into enhanced immunomodulatory potency towards PBMCs (Figure 5A). With a fixed dose, MSCs with a longer duration of pre-licensing had diminished, not improved suppression as would be predicted by IDO protein concentration and activity.

IDO protein levels were used as a surrogate marker for immune suppression and allowed us to predict the effect of different pre-licensing strategies on immunomodulatory potency. My work was tested in several donors to see how these observations would change when MSCs isolated from another individual were tested. We saw the same trends in IDO protein, enzymatic activity, and potency across donors but the magnitude of the effect of pre-licensing varied. We saw that it was not pre-licensing but the MSC donor that was a more significant regulator of potency. Pre-licensing did not make MSCs equivalent but with the appropriate strategy, it did consistently enhance immunomodulatory capacity. Other groups studying IFNγ pre-licensing have used a large range of doses and durations[48-52], but less is better. A low dose and low duration of IFNγ greatly impacted potency. A larger dose had only a minor impact while a longer duration was detrimental.

In addition to my own project, I utilized laboratory techniques to measure IDO protein and activity to contribute to work on several projects in my lab. While my work focused on pre-transplant controllable biomanufacturing parameters and their effect on immune modulation, the projects in which I was a co-author focused on post transplant regulators of potency. These included formation of MSC aggregates, which is common after local injection, and the proinflammatory effects of a simulated obese and type II diabetic microenvironment. Through my experience leading a project on IDO, I was able to provide technical expertise on MSC immune modulation in a variety of contexts and projects.

In conclusion, my thesis has demonstrated the following:

- IDO protein plateaus with a sufficient dose of IFNγ

- IDO protein continues to increase up to six days
- A enhanced dose based pre-licensing strategy consistently increases IDO protein and activity across the donors tested
- A duration based strategy also increased IDO protein across donors but its effect size is smaller and more variable than a dose based strategy
- A duration based pre-licensing strategy enhanced IDO protein which remains elevated after the pre-licensing has ended
- IDO's metabolite does not have a significant impact on protein or enzymatic activity
- Increased dose during pre-licensing enhances immune suppression while increasing the duration reduces potency
- The donor of MSCs were a more potent regulator of immunomodulatory potential than any pre-licensing strategy tested
- Tryptophan during pre-licensing increases protein at the end and several days after pre-licensing
- The effect of tryptophan on IDO protein is dependent on the donor of MSCs
- IDO protein and activity are inversely related during a tryptophan dose response
- Palmitate and spheroid formatio both reduce kynurenine production without significantly reducing IDO protein concentration

An unanswered question that I have after completing this project is why there is a breakdown in IDO and immune suppression. My view, based on literature review is that the balance between immune evasion and immune detection is lost when we pre-license for longer. I would like other researchers to explore MHC and other immune detection proteins in this system.

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