

ABSTRACT

Title of Document: IDENTIFICATION AND QUANTIFICATION OF
SECRETED PROTEINS DRIVING
HEMATOPOIESIS IN AN INDUCED
PLURIPOTENT STEM CELL COCULTURE
SYSTEM

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Acute myeloid leukemia (AML) results from a genetic alteration in hematopoietic stem cells (HSCs). Recent treatment techniques focused on the utilization of induced pluripotent stem cells (iPSC) as an avenue to introduce HSCs in patients with AML. In this study, we analyze whether OP9 cells assist in HSC differentiation through direct cell signaling or indirect cell signaling of iPSCs. We measured the expression levels of relevant cytokines known to play a role in hematopoiesis. Our data showed a significant upregulation of all the proteins we analyzed in the iPSC/OP9 direct coculture and significant downregulation in the iPSC/OP9 indirect coculture. Our results indicate that direct cell to cell contact with OP9 cells may be necessary to induce differentiation of iPSCs.

Team BLOOD Senior Thesis

IDENTIFICATION AND QUANTIFICATION OF SECRETED PROTEINS
DRIVING HEMATOPOIESIS IN AN INDUCED PLURIPOTENT STEM CELL
COCULTURE SYSTEM

By

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2018

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List of Abbreviations

AML – Acute Myeloid Leukemia

BM – Bone Marrow

CMP – Common Myeloid Progenitor

ESC – Embryonic Stem Cell

ESI – Electrospray Ionization

GVHD – Graft Versus Host Disease

HCT – Hematopoietic Cell Transplantation

hESC – Human Embryonic Stem Cells

HSC – Hematopoietic Stem Cell

IFN – Interferon

IL – Interleukins

iPSC – Induced Pluripotent Stem Cell

KSL – c-Kit⁺/Sca-1⁺/Lineage Cell

RNA – Ribonucleic Acid

MALDI – Matrix-Assisted Laser Desorption/Ionization

ROCK – Rho-associated Protein Kinase

BSA – Bovine Serum Albumin

HSA – Human Serum Albumin

Introduction

Purpose of Study and Research Question

Acute myeloid leukemia (AML), is the most aggressive form of leukemia, affecting approximately 3.6 out of 100,000 people per year (Arpinati et al., 2014; Tawana et al., 2013). In AML, a genetic alteration in the hematopoietic stem cells (HSCs) in the blood and bone marrow (BM) leads to a build-up of immature myeloid blasts which are unable to undergo normal hematopoiesis (Arpinati et al., 2014). AML can cause bleeding and infection, retinal hemorrhages, sternal tenderness, and enlarged organs and lymph nodes (Jabbour et al., 2006).

Currently, the goal of AML treatment is to reduce the amount of immature blasts in the BM to five percent or less through chemotherapy, and then to reduce the risk of relapse through allogeneic hematopoietic cell transplantation (HCT) (Kurosawa et al., 2011; Lowenberg et al., 1999). HCT often fails due to graft-vs-host disease (GVHD), a complication in which the recipient cannot eliminate the donor's T-cells, leading to the donor's T-cells attacking the recipient's body because of immunological mismatch – this feature of the human immune system has proven to be a significant barrier to improving the health care outcomes of those who are diagnosed with AML (Ferrara et al., 2009). Despite improvements in current prevention and treatments, GVHD continues to be a vexing issue in the battle against AML.

Another problem with HCT is that sources of HSCs are often limited. Owing to a lack of donor population and therefore a lack of potential harvesting sources such as bone marrow, cord blood, and peripheral blood, HCT is often difficult and costly to acquire. Expansion techniques for producing robust amounts of HSCs *in vitro* have

also not been optimized. The growth of these HSCs often involves usage of viral vectors or immunoreactions, which further impedes the usability of HCT in patients with cancers such as AML or other blood-related disorders such as thalassemia (Lim, 2013).

A solution to this problem may be the utilization of induced pluripotent stem cells (iPSCs). In 2006, researchers found that introducing four transcription factors – Sox2, Klf4, c-Myc, and Oct3/4 – reverted adult mouse fibroblasts into a pluripotent state (Takahashi and Yamanaka, 2006). Once somatic cells such as these fibroblasts are induced into a pluripotent state, the resultant iPSCs may be differentiated into a cell type different from its origin. With regards to AML and the problem of GVHD, iPSCs offer an avenue to introduce hematopoietic stem cells (HSCs) to a patient without the risk of an immune response. If one were to take a somatic cell from a patient, induce it into pluripotency, differentiate the iPSC into an HSC, and introduce it back to the patient, GVHD could be avoided.

A problem with using this iPSC model is that it has still proven difficult to produce a high yield of HSC long-term progenitors *in vitro*. A significant finding by Choi et al. presented a novel solution to the problem of low efficiency of inducing hematopoietic progenitors; coculturing iPSC cells with OP9 cells produced sustainable hematopoietic progenitors with a very high efficiency. Although this technique was initially used to induce hematopoiesis in human embryonic stem cell lineages, the hematopoietic support capacity was shown to hold its utility when used on iPSCs (Choi et al., 2011). However, it is currently unknown by what mechanism these OP9 cells are able to generate a full lineage of hematopoietic progenitors from iPSC culture.

The aim of our project is to optimize iPSC differentiation into HSCs, as currently iPSC reprogramming efficiency remains extremely low. One of the most useful current methods of driving this differentiation is to coculture iPSCs with OP9 mouse stromal cells – our project aims to determine if the presence of OP9 cells within a coculture system is necessary for differentiation. If the protein excretions of OP9 cells, rather than the cells themselves, are sufficient, then identifying and quantifying the proteins that are responsible for differentiation would allow for a more efficient methodology for differentiating iPSCs into HSCs.

Methodology Framework

After extensive literature review and analysis, our team selected the interleukin family of cytokines to investigate their potential to induce hematopoiesis in an iPSC/OP9 coculture system. In order to investigate whether or not cell-to-cell contact is necessary for this process, our team cultured and expanded OP9 mouse stromal cells in concert with iPSC cells that were obtained from the National Institutes of Health. After expansion and subculture methodology was completed and the cells appeared healthy, we then began a sample of coculture. As a result of our limitations both in resources and in experience, the OP9 cells were not growth arrested during the coculture. We also started samples of iPSC cells that received the conditioned media from culturing OP9 cells, as well as an OP9 monoculture and iPSC monoculture as controls. These cells were allowed to incubate for 7 – 10 days.

After the incubation period, we then analyzed the concentration of cytokines that was present in the culture medium through the usage of an ELISA-based assay. The Quantibody ELISA assay allowed us to measure an array of interleukin proteins

(refer to Table 1 for specific proteins and their functions) by using a fluorescence detection system. Fluorescence levels were then analyzed and compared to a known standard curve. After comparison, the level of each cytokine that was present in each culture sample was quantified. We then analyzed this data in an attempt to determine whether it was cell-to-cell contact or the cytokines that were secreted into the conditioned media that caused the induction of hematopoiesis within the iPSC/OP9 coculture system. The induction of hematopoiesis was assessed morphologically.

Cytokine	Source Cells	Major Effects	Expectation for Expression in iPSC/OP9 Broth coculture
IL-1	Macrophages	Stimulate T cell and B cells, promote hematopoiesis	Upregulation
IL-2	T Lymphocytes	Proliferate activated T cells	Upregulation
IL-3	T Lymphocytes	Induce hematopoietic cell precursors	Upregulation
IL-4	Macrophages	Promote B cell growth and differentiation	Upregulation
IL-5	Macrophages	Activate Lymphocytes	Upregulation
IL-6	B & T Lymphocytes	Promote B cell differentiation	Upregulation
IL-7	Thymus Stromal Cells	Promote T cell and B cell development	Upregulation
IL-9	T Lymphocytes	Promote T cell growth	Upregulation
IL-10	B & T Lymphocytes	Inhibit inflammatory and immune responses	Upregulation
IL-12	Macrophages	Regulate helper T cells	Upregulation
IL-13	T Helper Cells	Promote B cell growth and differentiation	Upregulation
IL-15	Epithelial Cells	Proliferate activated T cells	Upregulation
IL-17	Memory T Cells	Promote T cell proliferation	Upregulation
IL-21	T Helper Cells	Promote T cell proliferation	Upregulation
IL-23	Macrophages	Promote IFN- γ proliferation	Upregulation
G-CSF	Bone Marrow Cells	Produce granulocytes, mobilize HSCs	Upregulation
GM-CSF	Macrophages	Produce granulocytes, suppress apoptosis	Upregulation
IFN- γ	Natural Killer Cells	Suppress hematopoiesis	Downregulation
TNF- α	Macrophages	Promote anti-tumor response, restrict hematopoietic activity	Downregulation

Table 1: Relevant cytokines that were analyzed in Quantibody ELISA assay, the source from which they originate, the major effects that these cytokines have in these cells, and the expectation for expression in iPSC/OP9 conditioned media sample.

Significance of Findings

After analysis of the expression levels of each cytokine within our samples, our data showed a very significant upregulation of the proteins from within the iPSC/OP9 coculturing system, which resulted in morphological differentiation, as compared to our iPSC/OP9 conditioned media sample. There was also a significant downregulation of these proteins within the iPSC/OP9 indirect coculture, in which the

media from an OP9 monoculture was used in order to determine whether the secreted cytokines from OP9 cells was sufficient in order to induce differentiation of iPSCs into HSCs. Our results indicate that direct cell-to-cell contact with OP9 cells may be necessary to induce differentiation of iPSCs.

As the protocol for hematopoietic induction continues to be optimized and the cellular mechanisms by which differentiation occurs is further investigated, it may one day be possible to treat patients with diseases like leukemia using iPSCs. This will revolutionize the field of medicine, as it will circumvent many of the current limitations of treatment and improve upon the quality of life of patients we seek to treat. Our research may be applied in future clinical applications upon further research.

Limitations of Study

As an undergraduate research team beginning with limited knowledge of our subject of study, we encountered multiple hurdles which narrowed the scope of our experimental design over the past 4 years.

For one, funding was a major limitation throughout our experimentation. As an undergraduate research team, it was difficult to find grants that would support our research over other more established lab teams. Due to the limited amount of funding that was available to our research group, we were not able to fund a flow cytometry analysis on our cells to see whether they expressed a CD34+ marker, which is a marker for hematopoietic differentiation. We therefore were unable to definitively determine if differentiation occurred. We were also only able to perform one run of our Quantibody ELISA experiment. If we had more funding for future studies, we

would be sure to run flow cytometry as well as collect multiple ELISA readings in order to ensure that our data remains consistent across samples.

Lastly, another limitation that was very defining in our research methodology was finding space to conduct our research. Finding a lab on campus to conduct our research proved very difficult, as it is very hard to integrate a Gemstone team of around 4 lab members into any cell culture facility that we have available at the University of Maryland. Thanks to the Universities at Shady Grove, we were able to finally acquire a space where we could conduct our methodology. This brought with us the limitation of having to commute back and forth from the lab, which proved cumbersome and restricted parts of our cell culture methodology.

Literature Review

iPSC Differentiation Techniques and Hematopoiesis

Hematopoiesis is the process by which the cellular components of blood are formed – usually during development (Jagannathan-Bogdan, 2013). In vertebrates, there are two documented waves of hematopoiesis; the primitive wave and the definitive wave. The first wave, the primitive wave, occurs during early embryonic development and gives rise to the erythrocytes and macrophages. This wave is integral to development, as it provides the red blood cells necessary for tissue oxygenation throughout the rest of development. This stage is transient, and later gives rise to the definitive wave of hematopoiesis. Within the definitive wave, which continues much throughout adulthood, the entire lineage of blood cells are able to be formed. Among the various types of cells, multipotent HSCs are generated and are harbored mostly within the bone marrow (Jagannathan-Bogdan, 2013).

HSCs are integral throughout life as a means for preserving and generating a blood supply within the body, as well as supplying the necessary immunohistologic means to maintain homeostasis. Elucidating the mechanisms behind how HSCs are formed can be very useful for scientists to understand how to better combat pathological changes such as blood cancers and other blood-related disorders. HSCs can also be used a model system for understanding the processes of ageing, stem cell differentiation, and oncogenesis within the body. Generally, people with a cancer such as Acute Myeloid Leukemia receive blood transfusions with HSCs to allow proper and appropriate replacement of their blood supply.

In 2006, researchers at Kyoto University in Japan identified a specialized protocol that would allow fully differentiated adult cells to be “reprogrammed” into

cells that were pluripotent and could be re-differentiated into many cell types (NIH Stem Cell Differentiation, 2016). Called induced pluripotent stem cells (iPSCs), this discovery opened a new field in regenerative medicine with the potential to provide personalized medical therapy and circumvent previous issues with bone marrow transplantation such as GVHD. These cells also managed to circumvent some of the ethical issues that belie embryonic stem cells, as they were received from a consensual patient. This reprogramming, however, presents many challenges as the protocol remains complex and often requires the usage of retrovirus, which would often produce unpredictable results. Furthermore, differentiation of iPSCs into different cell types remains an even bigger challenge, as yields of viable HSCs that could potentially be transplanted is often extremely low. Before iPSCs can be considered a clinical tool, the efficiency for reprogramming as well as redifferentiation must be greatly improved.

In a 2011 study by Salvagiotto and group, it was shown that a single matrix protein in concert with hypoxic conditions were sufficient to efficiently generate pluripotent hematopoietic stem cell progenitors *in vitro*. Nine lines of iPSC cells were tested to examine which condition would provide the most robust amount of clinical grade progenitors that were induced from pluripotent cells. Hypoxic conditions were used to mimic an early embryonic environment in which HSC progenitor production is often robust. After initiating the protocol and co-culturing iPSCs with the growth factor BMP4-VEGF-bFGF, a 5% oxygen tension was added in order to mimic hypoxic conditions in the embryo. Hematopoietic progenitors were then identified using flow cytometry to count the CD34+ cells, which is a marker that is used to characterize progenitor cells. The study concluded that, although the clinical

introduction of this protocol would be relatively easy due to a plausible switchover in xenophobic conditions, the protocol must be investigated in further detail to see whether higher yields of hematopoietic progenitors could be achieved (Salvaggio et al., 2011).

Another efficient method that has been shown to generate even higher yields of hematopoietic progenitors from iPSCs is through simple co-culturing with OP9 mouse BM stromal cells. This methodology, although typically used to differentiate human embryonic stem cells (hESCs), has proven to be effective with iPSCs and can be done with or without exogenous growth factors (Choi, Vodyanik, & Slukvin, 2011). The Choi group identified and described two slightly varied methods, both of which use iPSC/OP9 coculture to induce hematopoiesis. The first protocol for differentiation as described by Choi and colleagues consists of three steps: generation of differentiation through the co-culture of the OP9 BM stromal cells and iPSCs, expansion of multipotent common myeloid progenitors (CMPs), and differentiation of the CMPs. After six days of coculture with OP9 feeders, iPSCs begin to show properties of myeloid progenitors capable of differentiation. Expansion was achieved by dissociating the OP9/iPSC coculture and then placing the cells in non-adherent conditions in which they spontaneously reaggregated (Choi et al., 2011). Cells that expressed the phenotype $\text{lin}^- \text{CD34}^+ \text{CD43}^+ \text{CD45}^+$ were determined to be multipotent HSCs with differentiation potential as opposed to other similar phenotypic cell markers representing endothelial and mesenchymal stem cells (Choi et al., 2011).

OP9 coculture creates a wide spectrum of myeloid progenitors, necessitating isolation of the $\text{lin}^- \text{CD34}^+ \text{CD43}^+ \text{CD45}^+$ cells from the other forms of progenitors produced (erythroid and megakaryocytic). Choi et al. (2011) accomplished this

through magnet-activated cell sorting, using anti-human antibodies specific to the erythroid and megakaryocytic progenitors. Differentiation of the obtained CMPs were then achieved through the application of cytokine combinations. Choi et al. (2011) asserted that the optimal combinations for the generation of dendritic cells were granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL) 4 and tumor necrosis factor- α (TNF- α), whereas GM-CSF, transforming growth factor- β 1 and TNF- α were optimal for the generation of Langerhans cells. For macrophages, the optimal combination was M-CSF and IL-1 β , while G-CSF was best for neutrophils. Finally, IL-3 and IL-5 were optimal for eosinophils. The different types of differentiated cells obtained in this study were established by analyzing their cell morphology via cytopins and their phenotype using flow cytometry. Cell markers allow for characterization and identification of the obtained eosinophils, neutrophils, dendritic cells, Langerhans cells, macrophages and osteoclasts. The success of this method of differentiation yields sac-like structures, and in exceptionally efficient cases, undifferentiated colonies (Choi et al., 2011).

In another study conducted by the same group, Choi et al. (2009) utilized a similar methodology using the OP9 coculture, examining iPSCs obtained from human fetal, neonatal, and adult fibroblast tissues in comparison to different forms of hESCs. In this study, the iPSCs and OP9 were cocultured, and cytokines were not used. This study also used magnet-activated cell sorting to isolate three subsets of HPCs: (1) CD235a/CD41a+CD43+ CD45⁻, (2) CD235a/CD41a-CD43+CD45⁻, and (3) CD235a/CD41a-CD43+CD45⁺ cells. The colonies of erythroid, granulocyte, erythrocyte, macrophage, megakaryocyte, and macrophage cells were assayed and scored after incubation. This method of using OP9 to induce hematopoiesis has

proven successful while requiring relatively little time and resources (Choi et al., 2009). As iPSC coculture with OP9 bone mouse stromal cells is simple and efficient, this methodology for achieving hematopoiesis is consistent in optimizing differentiation for all mentioned iPSCs.

Methods of identifying expression levels of relevant proteins

Cells are able to synthesize endogenous and exogenous proteins in both iPSC/OP9 coculture systems as well as iPSC cultures systems that contain only conditioned media from OP9 cells. It is necessary to distinguish between expression levels of proteins in the two cultures in order to determine the significance and role of specific proteins in hematopoietic induction. Antibody based assays may be used in order to attach, or bind, a protein to a plate. In doing so, specific antibodies could be used to detect and quantify proteins of interest. Other methods involve the use of probes to detect and quantify specific nucleic acid sequences known to code for proteins of interest. In transcriptomics, a DNA microarray is one method of examining RNA expression levels through probing for nucleic acid sequences (Schena et al, 1995).

A DNA microarray involves generation of cDNA from RNA using reverse transcription PCR (RT-PCR). PCR technology utilizes a short DNA primer sequence, which provides the start site to DNA synthesis, in addition to a DNA polymerase enzyme that must withstand high temperatures. Therefore, *Thermus aquaticus* (Taq) polymerase is used for nucleotide addition and elongation in the replication cycle in PCR. Additionally, spare nucleotides are needed in order to amplify the preexisting DNA. Subsequently in the microarray, cDNA samples are immobilized on top of a

matrix. Next, the samples on the matrix are hybridized with anti-sense RNA targets, or cDNA sequences. Hybridization of the biochip is quantized and qualified through the detection of chemiluminescent fluorophores. Each culture has a unique fluorophore, with common cDNA labeling techniques utilizing the orange-fluorescent Cy3 and the far-red-fluorescent Cy5 to show regulation of gene expression. Data analysis and processing then involves visualization and determination of the intensity in each spot.

Enzyme Linked ImmunoSorbent Assay (ELISA) is an antibody based assay that is used in diagnostic tests for various proteins. ELISA uses antibodies attached to a 96 well plate, or conversely a protein attached to the plate that the antibody to which it can bind (Lee and Wang, 2003). The antibodies, as mentioned above, are able to bind specifically to an antigen, and thus detect and quantify proteins of interest. ELISA is considered an enzyme-linked assay since the secondary antibody that binds to the primary antibody is conjugated with an enzyme that can convert a substrate to a detectable and quantifiable color. The color can be measured by a simple machine called a plate reader and used to make a plot in order to quantify the amount of protein. An ELISA assay works very well for detecting a singular protein. For multiple proteins, however, the overall procedure can become time consuming and requires an extremely high amount of sample.

Recently, technology has been developed that can use ELISA-like methods to detect multiple different proteins at once. One such technology is a RayBiotech's Quantibody array, which is a multiplexed sandwich ELISA-based quantitative assay that enables the accurate detection of the concentration of multiple proteins. Unlike the traditional ELISA, Quantibody products use an array format. By arraying multiple

cytokine specific capture antibodies onto a glass support, quantitative, multiplex detection of cytokines in one experiment is made possible. A capturing antibody is bound to a glass surface, after which it is incubated with a sample. The target protein will then become trapped on the solid surface, as it attaches to the antibody that has already been anchored. A second detection antibody is then added, and the conglomerated complex can then be visualized through a laser following the addition of a streptavidin-conjugated Cy3 dye (Quantibody, 2016). For the purpose of our experiment, this methodology was beneficial as it enabled us to detect and quantify multiple different cytokines in one sample. Multiple other analysis techniques were investigated (see Appendix A), however RayBiotech's Quantibody array was concluded to be the most fitting analysis method for the data that our team wished to acquire.

Methods of Quantifying Cell Markers

In order to confirm that the cells we obtained are indeed the cells that we desired, methods of identifying and quantifying cells are required. One such method is the use of flow cytometry. Flow cytometry is a method used to analyze cell characteristics such as cell size, DNA content, and relevant cell-surface or intracellular proteins. Cells are labeled with fluorescent dyes or antibodies conjugated with fluorescent dyes that bind to specific proteins inside cells or on their surfaces (Brown, 2000). When these labeled cells stream individually through a laser, the fluorescent molecules in the dye are excited to a higher energy state by a laser; upon their return to the ground state, the fluorophores emit light at certain wavelengths (Brown, 2000). The property that is being measured in the cell is thus proportional to

the number of cells that emit that certain wavelength. A the flow cytometer is also able to sort cells based on whether or not they emit the light from the dye. If several fluorophores are used that emit light at different wavelengths and therefore fluoresce different colors, multiple properties in the cell can be measured simultaneously (Brown, 2000). Data is usually represented in a dot plot that shows cell density based on the intensity of the light emitted.

The usage of flow-cytometry would have allowed us to obtain the relative number of marker positive cells (in this case CD34+ cells) as a function of marker intensity (Stewart, 2005). Once the frequency of specific marker positive cells is determined by flow cytometry, we would multiply the number by the cell concentration in order to determine the total marker positive cells (Stewart, 2005). The total marker positive cells in our experiment would have indicated the cell viability and yield of hematopoietic differentiation in our study. In our experiment, if resources were to permit, we would ideally sort cells in order to identify the CD34+ cell population and study a set of genes and their protein products that are unique to HSCs.

Proteins that may be Investigated within an OP9 Co-Culturing System

Interleukins

While investigating the roles and functions of many proteins that are involved in hematopoiesis through coculturing with OP9 stromal cells, the interleukin family protein stands out the most in terms of recognition, effectiveness, and commonality. Interleukins, according to the National Cancer Institute at the NIH, are defined as “a group of related proteins made by leukocytes and other cells that regulate immune

responses. Interleukins made in the laboratory are used as biological response modifiers to boost the immune system in cancer therapy” (NCI). More specifically, an interleukin is a type of cytokine which is produced during the hematopoiesis process to aid in cell differentiation and proliferation. A 2006 study that analyzed the distinctive roles of an interleukin, IL-7, found that “IL-7 provided both survival/proliferation and differentiation signals in a dose-dependent manner” (Wang, 2006).

Interleukins, as examples of cytokines, are mediators of communication between cells. They regulate cell growth and differentiation, and are even named after leukocytes, since they were believed to be made from leukocytes. The interleukin family encompasses many different types of proteins involved in various types of cell development and signaling processes. In a 2011 journal article that compiles all the functions and roles of all the known interleukins into one table, IL-1 is known to aid in differentiation into T cells and fibroblasts, IL-2 for differentiation into CD4⁺ and CD8⁺ T cells, IL-4 for T and B cells, and IL-6 into hematopoietic cells, etc. (Akdis, 2011). Therefore, interleukins are regulatory proteins that act as cytokines to aid in the process of hematopoietic cell differentiation. Since interleukins come from different types of cells, particularly in OP9 stromal cells, we can then investigate if interleukins are the essential proteins that aid in cell differentiation. We hypothesized that the conditioned media that we are extracting from OP9 cell secretions contains interleukins secreted by the OP9, and it is also possible that it is due to these secreted interleukins that OP9 cells are able to promote hematopoiesis of iPSCs. Identification of proteins such as interleukins may allow the use of a defined medium to induce hematopoietic differentiation. As we are investigating the effects of cell culture via

direct and indirect coculture with OP9 cells, interleukins may play a role in juxtacrine vs. paracrine signaling which allows for hematopoietic differentiation. Therefore, the interleukin family of proteins will be further investigated in our study. The relevant proteins we analyzed are individually discussed below.

G-CSF

Granulocyte-colony stimulating factor (G-CSF) is a protein induced by the presence of inflammatory stress. Studies have shown that certain inflammatory response molecules can regulate hematopoiesis at the stem cell level (Schuettpelz, 2014). G-CSF is one such factor, playing a key role in stress granulopoiesis response as part of inflammatory signaling. Once induced, G-CSF stimulates neutrophil production and releases them into circulation (Schuettpelz, 2014). G-CSF is cited to be widely used in clinical settings to mobilize HSCs from the bone marrow to the blood in order to harvest HSCs for stem cell transplantation (Schuettpelz, 2014). Thus, G-CSF treatment is associated with changes in the process of hematopoiesis in the bone marrow. Expression of G-CSF has shown to affect HSCs by inducing toll-like receptor (TLR) signaling in HSCs. More specifically, a close study demonstrated that G-CSF negatively regulates HSCs by enhancing TLR signaling (Schuettpelz, 2014). TLR signaling contributes significantly to the regulation and maintenance of HSCs, and thus by inducing TLR signaling, G-CSF may indirectly or directly affect HSC maintenance and mobilization.

GM-CSF

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a hematopoietic growth factor involved in generating granulocytes, macrophages, and other immune cells from hematopoietic progenitor cells (Kruger, 2007). GM-CSF has also been identified as a neuronal growth factor in the brain to counteract apoptosis, serving as a key hematopoietic factor that has neuronal functions. GM-CSF is similar to G-CSF in function and patterns of expression, and both have similar roles in differentiation of stem cells. GM-CSF induces the generation and maturation of granulocytes and macrophages from HSCs (Kruger, 2007). Both GM-CSF and G-CSF serve cellular functions in proper differentiation and anti-apoptosis. Furthermore, GM-CSF has been shown to work closely with growth factor Interleukin-3 (IL-3) in development of hematopoiesis. Results from a study analyzing the relationship between the two factors suggest that IL-3 expands HSCs early on and subsequently requires a later acting factor GM-CSF to complete development, and that optimal stimulation of hematopoiesis is achieved with combinations of these growth factors. Thus, GM-CSF interacts closely with other growth factors, and as suggested by the timing of development, GM-CSF acts at a later stage in cell development (Donahue, 1988).

IFN- γ

Interferon- γ is a proinflammatory cytokine interferon that serves an important role in both innate and adaptive immunity against infections and/or tumors. IFN- γ also affects most hematopoietic progenitor cells and subsequently affects bone marrow output during inflammation. IFN- γ has been proposed to upregulate certain cytokine signaling molecules' suppressors, impairing cytokine receptors. As a result,

IFN- γ has been widely known as a suppressor of hematopoiesis. Many studies have been done to show how treatment of IFN- γ impairs the functions of HSCs: treatment with IFN- γ has demonstrated a decrease in the number of self-renewing cell divisions in HSCs, as well as the loss of functional multipotent HSCs (Bruin, 2014). Thus, IFN- γ 's negative effects on HSC function and maintenance are clear.

IL-1 α & IL-2 β

Interleukin-1 is part of the large Interleukin family that serves as a mediator of inflammatory stress response at the onset of infection. IL-1 has been widely recognized as a polypeptide molecule that is important in regulating hematopoiesis as well as a wide variety of biological activities. IL-1 primarily serves to induce the production of many different hematopoietic growth factors including macrophages, Interleukin-6, granulocytes, and other colony-stimulating factors (G-CSF and GM-CSF), as working closely with these factors help proliferate hematopoietic stem cells (Fibbe, 1991). Although Interleukin-1 does not affect HSCs directly, IL-1's major effect on hematopoiesis is to regulate the expression of hematopoietic growth factors which in turn affects most hematopoietic activities ranging from growth and development to maintenance. Thus, interleukin-1 can be thought of as a vital master switch for many cellular responses including hematopoiesis (Bagby, 1989).

There are two similar forms: IL-1 alpha and IL-1 beta. Both molecules are similar, binding to the same set of receptors with similar affinities (Arranz, 2017). A study done by Hestdal et. al demonstrated that administering IL-1 alpha to mice induced the upregulation of both the GM-CSF growth factor as well as IL-3 receptors, which directly correlated with upregulation of colonies containing macrophages,

granulocytes, and erythroid cells; results demonstrated the ability of IL-1 alpha to stimulate hematopoiesis in vivo (Hestdal, 1992). IL-1 beta as noted before, is similar to the alpha subunit, and it is mainly produced by myeloid cells. Once secreted, IL-1 beta binds to its receptor and triggers a signaling cascade that controls gene expression of multiple transcription factors, growth factors, and other interleukins involved in the immune response. It also stimulates T cell maturation and B cell proliferation. (Arranz, 2017).

Importantly, IL-1 beta regulates HSC function by promoting HSC differentiation by activation of its signaling. The beta subunit's regulatory role is finely tuned, as dysregulation such as excessive exposure can lead to uncontrolled HSC division and other hematological diseases (Arranz, 2017). Although the IL-1 alpha and beta subunits are similar, there are a few significant differences besides just structurally. A study conducted by Rider et. al demonstrated that the alpha subunit initiates sterile inflammation by inducing neutrophil recruitment, whereas the beta subunit promotes macrophages recruitment (Rider, 2011).

IL-2

Interleukin 2 (IL-2) is a cytokine that has a broad array of functions and is largely associated with the regulation of white blood cells. It has been shown to play a role in polyclonal T cell activation, and when IL-2 is suppressed, T cell activity is decreased (Thornton and Sevaach, 1998). IL-2 also augments the cytolytic activity of natural killer cells, mediates activation-induced cell death, and induces the differentiation of T cells. IL-2 cytokines are themselves activated when CD4+ T cells are activated by an antigen. Though literature has not shown a clear link between IL-2

and hematopoietic stem cell differentiation, the cytokine's integral role in the activation and proliferation of T cells could indicate that IL-2 can play a part in the differentiation of HSCs if it is present. The three receptors that are present on IL-2 are critical in binding to various lymphocytes; since there are low affinity, medium affinity, and high affinity receptors, these receptors may be used during hematopoietic stem cell differentiation (Liao, Lin, and Leonard, 2011).

IL-3

IL-3 has been shown to work in conjunction with GM-CSF in inducing hematopoiesis in primates. When infused intravenously into primates continuously, IL-3 elicited a delayed and modest leukocytosis. When GM-CSF was introduced to the primates later, there was a much larger leukocytosis. This indicates that IL-3 is an early acting cytokine, and may require a later acting factor, such as GM-CSF, in order to induce optimal levels of hematopoiesis (Donahue et al., 1988). IL-3 has also been shown to increase the number of hematopoietic progenitors in mice.

In one experiment, IL-3 was injected into mouse models and the number of hematopoietic progenitors more than doubled. Mice whose progenitor cell levels were reduced by radiation saw a 10-fold increase back to near normal level when they underwent a 7-day treatment of IL-3 (Kindler et al., 1986). In another study investigating the roles of interleukins in hematopoietic cell development, researchers found that hemangioblasts, cells with the capacity to differentiate into hematopoietic cells, were regulated and promoted by IL-3 “with regards to both the number and capacity of the dual-potential hemangioblast” (He, 2009).

Ultimately, it is clear that IL-3 plays a critical role in inducing hematopoiesis. This cytokine is worth investigating in iPSC-OP9 coculture, as it may play a similar role of hematopoietic induction. Past studies have shown that IL-3 can induce differentiation *in vivo*; if it can also induce differentiation in iPSC-OP9 coculture *in vitro*, the cytokine likely can do so *in vivo* as well.

IL-4

IL-4 is closely related to IL-13, another cytokine. Both are found on chromosome 5, and both have many of the same biological and immunoregulatory functions on monocytes, dendritic cells, fibroblasts, and B lymphocytes. Both also have a restricted activation pattern to activated T cells and mast cells and share a common chain called IL-4R alpha. One difference between the two cytokines is that IL-4's expression is restricted to type 2 helper T lymphocytes and IL-13 is unable to regulate T-cell differentiation. If it is found that IL-4 concentration is related to HSC differentiation from iPSCs, it is worth investigating if IL-13 is as well since they are so closely linked (Chomarat, 1998).

IL-4 has also been identified as having a role in proliferating primitive hematopoietic progenitors in mice. When IL-4 was combined with IL-11, major enhancement of colony formation was seen in mice. Notably, neither IL-4 nor IL-11 had an effect on colony formation when they were introduced individually to the mice. This combination could be vital for stimulating dormant HSCs. It is worth investigating if IL-4 is capable of inducing HSC formation *in vitro* from iPSCs (Musashi et al., 1991).

IL-5

Interleukin 5 (IL-5) is an interleukin that is involved in the growth and maturation, as well as the release of eosinophils in the human bone marrow (IL5 interleukin 5, n.d.). This cytokine is generated by helper T-cells and mast cells, but is molecularly different in humans and mice. In humans, this protein is 115 amino acids in length, but is 133 amino acids in length in mice. In addition to being responsible for eosinophilpoiesis, IL-5 can cause the increase in the production of B cells and is therefore acts as a growth and differentiation factor for B cells as well as eosinophils (Takagi, et al., 1995). IL-5 generation is regulated by GATA3 and various other transcription factors, with uncontrolled production leading to eosinophil-dependent inflammatory diseases. Unlike some interleukins, the active form of IL-5 is a heterodimer rather than a homodimer (Takagi, et al., 1995).

The IL-5 receptor contains an alpha and a beta subunit, with the alpha subunit being highly specific for IL-5 and the beta subunit being capable of binding IL-3 in addition (Greenfeder et al., 2001). The commonality between the signaling methods of these two interleukins proposes a potential relationship between the IL-5 signaling pathway and hematopoiesis. If an overlap exists between the role of these cytokines in regulating and influencing the induction of hematopoiesis, the pathway can be studied and utilized to induce the proliferation of HSCs. The restricted effects of IL-5, studied to be effectively solely on eosinophils rather than hematopoietic cells, may stem from the specificity that the alpha subunit binds IL-5. The restricted expression of the alpha subunit of the IL-5 receptor may hinder the development of progenitors when subject to IL-5 solely, but has been shown to cause differentiation when treated with a combination of IL-5 and IL-3 (Greenfeder et al., 2001).

IL-6

Interleukin 6 (IL-6) is an interleukin that is involved in pro-inflammatory process in humans, and is typically released by immune cells such as T-cells and macrophages in response to a perceived infection (IL6 interleukin 6, n.d.).

Additionally, IL-6 functions in the growth and maturation of B cells. Therefore, this interleukin is commonly found in locations where there is an acute or chronic inflammation (Gabay, 2006). This interleukin binds to its IL-6 alpha receptor, inducing various inflammation related states that serve to fight an infection. IL-6 plays an important role as a protein that can cross the blood-brain barrier, thus capable of inducing the production of prostaglandin in the hypothalamus and the regulation of body temperature (Gabay, 2006). IL-6 is therefore involved in fever mediation and altering the body's core temperature.

IL-6's role in the acute phase response is described best by stimulating T cells and B cells, therefore transitioning from an acute to a chronic immune response through inflammation (Bernad et al., 1994). Several pro-inflammatory diseases such as rheumatoid arthritis can be treated through the regulation of interleukin 6 and its signaling pathway. In addition to the acute phase response, IL-6 is involved in the hematopoietic process through an unknown process. However, studies have shown that the absence of IL-6 decreases the number of colony-forming units significantly, indicating a potential role of interleukin 6 in the survival or proliferation of progenitors (Bernad et al., 1994). Given that IL-6 is involved in the cellular immune response, a deficiency may cause hematopoietic stem cells and early progenitors to fail in sustaining themselves against pathogens and toxicity. Therefore, the role of

IL-6 and whether this protein is involved in the induction, or conversely the sustainability, of hematopoietic stem cells should be studied further in order to determine the role of this interleukin in hematopoiesis.

IL-7

Interleukin 7 (IL-7) is a hematopoietic growth factor that forms a heterodimer and is secreted by stromal cells in the human bone marrow as well as the thymus (IL7 interleukin 7, n.d.). This interleukin binds to its respective receptor, IL-7R which consists of a gamma and an alpha chain. Since this cytokine is crucial in B cell and T cell development, disruption of the alpha receptor can result in arresting the generation of T cells but maintaining elevated B cell counts (Peschon et al., 1994). IL-7 engages with both the alpha and the gamma chains of the receptor in order to result in early development of B cells. Deficiency of IL-7 or its receptors can block the development of B cells at the early progenitor stage (Noguchi et al., 1994). Whereas IL-7 acts to produce B cells in the bone marrow, it leads to the development of T cells within the thymus.

The purpose and significance that IL-7 serves in transducing signals responsible for the development of T cells and B cells lead to its prominent role within hematopoiesis. This is because IL-7 and its receptor function concurrently in the pathway that leads the transition of hematopoietic stem cells to lymphoid precursor cells, and ultimately to T cells, B cells, and natural killer cells (von Freuden-Jeffry et al., 1995). Not only is this interleukin important for the development of these crucial cell types, but additionally for their survival and proliferation as well. Signaling between IL-7 and hematopoietic stem cells positively regulates the survival

of B cells, thus allowing progenitors to differentiate continuously and increase the level of B and T cells. Therefore, investigating the link between IL-7 and hematopoietic stem cells within the induction process can elucidate the level of expression of this protein during the process, and whether the increase in signaling occurs during the hematopoietic process in a joint pathway or after induction of hematopoiesis has already occurred.

IL-9

Interleukin 9 (IL-9) is a cytokine that functions to increase cell proliferation and prevent apoptotic cell death from occurring. IL-9 is secreted by CD4+ cells and regulates various hematopoietic cells (IL7 interleukin 7, n.d.). T lymphocytes are the major cells that secrete IL-9, possibly due to its role in the immune response and regulating the inflammatory process (Goswami & Kaplan, 2011). IL-9 functions by binding to the IL-9 receptor, IL-9R, and activating another protein called a signal transducer as well as the STAT activator protein (Goswami & Kaplan, 2011).. This in effect activates GATA3, which is required for development of interleukin 9 secreting cells such as T lymphocytes.

IL-4 is an influential cytokine in the promotion of IL-9 secreting cells, increasing the IL-9 expression with upregulation (Goswami & Kaplan, 2011). Therefore, the dependency of these cytokines on one another is a topic that may be explored further through joint expression tests, determining whether there is a correlation between expression of one interleukin with others in the interleukin family. Furthermore, the expression pathway of these interleukin may be related and affecting in ways that are unknown as of now.

IL-9 is a factor that has been shown to be involved in the regulation of hematopoiesis. This is because IL-9 and IL-3 work concurrently to promote the growth of progenitors that are dependent on IL-3 on development (Perumal & Kaplan, 2011). Therefore, these two interleukins function to increase the generation of burst-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E), as well as multi-lineage colony formation over an extended period of time (Perumal & Kaplan, 2011). Moreover, IL-9 plays a role as a potentiator of megakaryocyte progenitor cells in humans. However, this cytokine's role in the inflammation process also enhances a pro-inflammatory process through acting on hematopoietic cells, often the cause of asthma in a human airway (Steenwinckel et al., 2007).

TNF alpha

Tumor necrosis factor alpha (TNF alpha) is a cytokine that is similar to interleukin 6 in that it is involved in the proinflammatory process as well as the acute phase process. Thus, TNF alpha is activated by macrophages and can be secreted by various cells that are involved in the immune response, such as natural killer cells, mast cells, eosinophils, and CD4+ lymphocytes (TNF tumor necrosis factor, n.d.). TNF alpha binds to and is activated by its receptors, TNFRSF1A/TNFR1 and TNFRSF1B/TNFR2. TNF alpha functions in its proinflammatory role by activating other cytokines, such as interleukin 8, reactive oxygen species (ROS), and prostaglandins (TNF tumor necrosis factor, n.d.). Furthermore, TNF alpha's role in the acute phase response means that it can mediate fevers, and upregulate interleukin 1 and interleukin 6 in order to inhibit tumorigenesis (Komeev et al., 2017).

In addition to providing an anti-tumoral response, TNF alpha is involved in restricting hematopoietic activity. This is due to the fact that uncontrolled differentiation of hematopoietic stem cells can increase the risk of tumorigenesis and leukemic transformation (Pronk et al., 2011). However, TNF alpha has a pleiotropic effect on cells, including HSCs, meaning that their regulation of hematopoiesis may not necessarily be dependent on cellular contact and rather incorporates an indirect effect on target cells. TNF alpha's repressive effects on the regulation of HSC proliferation is dependent on the presence and binding of both receptors, meaning that a deficiency or inhibition in either receptor can disrupt HSC homeostasis (Pronk et al., 2011).

IL-10

Interleukin 10 (IL-10) is a cytokine that is produced mostly by monocytes. It is also secondarily produced by other lymphocytes. The cytokine is most known for its pleiotropic effects on both the processes of immunoregulation and inflammation. Dysregulation of the IL-10 cytokine system has been shown to increase susceptibility to many immunopathological conditions such as autoimmune disease. It has been shown that IL-10 downregulates the expression of Th1 cytokines which produce interferon (IFN)-gamma, IL-2, and tumor necrosis factor (TNF)-beta (Romagnani, 2000). IL-10 has also been found to enhance B-cell survival, proliferation, and antibody production (IL10 Interleukin 10, n.d.). A fundamental understanding of this interleukin is extremely crucial to our comprehension of pathological disease progression, as well as autoimmunological regulation within our bodies.

Interestingly, IL-10 has also been shown to play a distinctive role in the self-renewal processes of HSCs. According to a 2007 study, IL-10 disrupted mice showed a statistically significant decrease in the amount of primitive hematopoietic cell populations that were present within the bone marrow. In contrast to this, HSCs that were cultured on IL-10 secreting stroma proved to show enhanced repopulating capacity compared with cells that were grown in the control stroma. Importantly, HSCs that showed enhanced repopulating capacity were found to have IL-10 surface receptors and microenvironmental IL-10 production. Overall, these results show that IL-10 may be a potential ligand that can stimulate enhanced self-renewal of HSC populations within the bone marrow (Kang et al, 2007). Due to the role that IL-10 has been shown to have in hematopoietic cell renewal, it would be very interesting to see whether there is increased expression of this cytokine in an iPSC-OP9 coculture system.

IL-12p70

The interleukin 12 (IL-12) family of cytokines has been shown to play a key role in the regulation of the T-cell response. This group of cytokines is produced by a variety of immunological cell such as, monocytes, macrophages, and dendritic cells in response to infection. IL-12 is composed of two separate subunits; p40 and p35. Together, these two subunits form the bioactive form of IL-12 called IL-12p70. In concert with IL-23 and IL-27, these three cytokines act on the JAK-STAT pathway as a result of the homology in receptor components. By activating this pathway, the body begins the process of activation and differentiation of T-cells. In particular, IL-12 has been shown to be requisite for IFN-gamma production and hence the

induction of Th1 cells. By activating these different pathways and molecules, IL-12 is yet another component that is responsible for cytokine's role in infection, inflammation, and autoimmune disease (Gee et al, 2009).

In patients that have used heavy chemotherapy, such as those in AML, battling acute myelosuppression is often the largest hurdle to recovery. The lack of ability to full reconstitute a full blood cell lineage presents many difficulties that researchers have been attempting to mitigate for many years now. In a 2007 study, it was shown that IL-12 also has a role in the recovery of hematopoiesis after engraftment into sublethally irradiated animals. In the study, a low dosage of IL-12 was administered and 91.4% of lethally irradiated animals were able to survive long term without any adverse side effects. After observing the blood of these animals, it was concluded that IL-12 stimulated a full hematopoietic recovery and led to the production of white blood cells, red blood cells, and platelets (Chen et al, 2007). Because IL-12 has such massive implications in hematopoiesis, it is a worthwhile avenue of investigation within an iPSC-OP9 coculturing system.

IL-13

Interleukin 13 (IL-13) is an immunoregulatory cytokine primarily produced by activated Th2 cells. This cytokine is involved in several downstream stages of B-cell maturation and differentiation, which is one of the body's primary defenses against infection. It has also been shown to upregulate MHC Class II expression, as well as promote IgE isotype switching within B cells. Its role in downregulating macrophage activity allows it to play an inhibitory role in production of pro-inflammatory cytokines. Due to this, IL-13 has been largely implicated in the pathogenesis of

allergen-induced asthma. IL-13 has also been implicated in the inhibition of tumor immunosurveillance within the body. Inhibitors of IL-13 may prove to be effective as immunotherapy for cancers by increasing the body's anti-tumor defense system (IL-13 Interleukin 13, n.d.).

IL-13 has also been implicated in the regulation of growth of hematopoietic progenitor cells in a 1994 study. After inoculation into a culture, it was noted that IL-13 was able to enhance the stem cell factor (SCF)-induced proliferation of bone marrow progenitor cells in synergy with IL-4. When inoculated into a culture without IL-4 there was no further enhancement of growth of colonies. Additionally, the addition of IL-13 led to the exclusive production of macrophages, which shows that IL-13 has a very specific role in hematopoietic differentiation. Collectively, these findings indicate that the synergistic presence of IL-13 is needed in order to properly and fully generate hematopoietic differentiation (Jacobsen et al, 1994). Understanding whether IL-13 is necessary for driving hematopoietic differentiation of iPSCs in an iPSC-OP9 coculturing system could prove useful in elucidating further mechanisms of hematopoiesis.

IL-15

Interleukin 15 (IL-15) is a cytokine that is heavily implicated in the regulation of T-cells and the activation/proliferation of natural killer cells within the body. This cytokine has been found to bind to the hematopoietin receptor, which is a receptor family that contains homologous amino acid sequences for many different types of cytokines. Further studies in the mouse counterpart of this cytokine show that IL-15 is responsible for inhibiting the apoptosis pathway by activating the STAT6 signal

transduction cascade. Due to this cytokine activity, there is some evidence which indicates that overexpression of this cytokine could lead to potential cancers of the immune system (IL15 Interleukin 15, n.d.).

IL-15 also has an interesting role in hematopoiesis as it relates to transcriptional regulation. In a 2013 article by the Colpitts group, it was shown that the IL-15 promoter activity was differentially regulated in a subset of hematopoietic myeloid lineages. Mature lineages were found to have almost no IL-15. After analyzing the hematopoietic stem cells themselves, the group found that in fact the HSCs themselves were expressing a high level of IL-15. This result suggests that IL-15 expression may have been extinguished during later lymphoid development (Colpitts et al, 2013). These results seem to elucidate that IL-15 may have a role in the timing of hematopoiesis. Expression levels in an iPSC-OP9 coculture system would be informative to analyze, as perhaps high levels of IL-15 may be necessary to initiate and drive early hematopoiesis.

IL-17A

Interleukin 17A (IL-17A) has been known to be a pro-inflammatory cytokine that is produced and activated by T-cells. High levels of this cytokine have been associated with several chronic conditions such as multiple sclerosis, rheumatoid arthritis, psoriasis, and other chronically inflammatory diseases. IL-17A is associated with a large family of other IL-17's, but the biological function of IL-17A is best understood. IL-17A has been known to stimulate the release of other cytokines, such as IL-6, as well as other regulatory molecules such as cyclooxygenase-2 (COX2) (IL17A Interleukin 17A, n.d.). The IL-17 family is known to exact its biological

functions via receptors on the cell surface. Out of the many receptors for the IL-17 family, the receptor type for IL-17A is most commonly found (Jin & Dong, 2013).

IL-17A has been shown to have significant implications on hematopoiesis. A 2012 article shows that IL-17 cytokines secreting Th17 link T-cell function and hematopoiesis through stimulation of events such as granulopoiesis. IL-17A also affects many other cells during the process of hematopoiesis, such as mesenchymal cells and erythroid progenitors. *In vitro* data upon inoculation of IL-17A into a culture system showed significantly greater recruitment of mesenchymal cells and erythroid progenitors than the control sample (Kristic, 2012). With these widespread implications of IL-17A, investigation of levels of IL-17A within a iPSC-OP9 coculture system is worthwhile.

IL-21

Interleukin 21 (IL-21) has been shown to increase the generation of T cell inflammatory cells. In fact, the IL-21 cytokine is necessary for the differentiation of Human T_H17 cells. These cells have been implicated in the pathogenesis of autoimmune diseases, such as rheumatoid arthritis, irritable bowel syndrome, and psoriasis. Thus, it may be the case that IL-21 is an important component of such pathologies (Yang et al., 2008). IL-21 is itself secreted from T_H17 cells and also helps regulate another cytokine: IL-17. Thus, IL-21 is in an intimate relationship with T_H17 cells in which they are necessary for the maintenance and differentiation of T_H17 cells and are also secreted by the very same cells. This positive feedback loop suggests that IL-21 is an important factor in the immune response and is vital for T

cell maintenance (Wei. et al., 2007). This cytokine's role in blood cell differentiation indicates that it may play a role in hematopoietic stem cell differentiation as well.

IL-23, p19

Interleukin 23 (IL-23), p19 is a sequence that is distantly related to the p35 subunit of IL-25, called p19, combined with the p40 subunit of IL-12 in order to form a novel, composite cytokine that is biologically active called IL-23. IL-23 has been shown to induce a strong proliferation of mouse memory T cells. This is unique to IL-23 as IL-12 does not affect mouse memory T cells. The combination of the p40 subunit and p19 leads to this induction of strong proliferation. IL-23 is not completely dissimilar from IL-12 in its function, however. Human IL-23 stimulates the production and proliferation of IFN- γ in certain T cells (Oppmann et al., 2000).

IL-23, p19 can also have deleterious effects. It has been found that inducing the p19 subunit into mice tissue can cause systemic inflammation, runting, infertility and death before mice are able to reach the age of 3 months. Mice that were implanted with this subunit had lymphocyte and macrophage infiltrates in the skin, liver, lungs, and the pancreas. Furthermore, implanted mice had anemic digestive tracts. In this same study, it was found that the p19 subunit was being expressed by hematopoietic cells, indicating that the subunit has shared biological properties with IL-6, IL-2, and G-CSF- other cytokines that are also produced by hematopoietic cells. IL-23, p19's close relation to these cytokines, as well as the fact that it is a relatively recently discovered cytokine, makes it an exciting subunit to study; much is still unknown about IL-23, p19 (Wiekowski et al., 2001).

Lhx2

The transduction of *Lhx2* into mesodermal cells derived from embryonic stem cells (ESCs) was found to result in c-Kit⁺/Sca-1⁺/Lineage (KSL) cells in vitro. KSL cells represent an early form of HSCs. Furthermore, transduction of *Lhx2* into iPSCs was also found to be effective in generating KSL cells in vitro. Significantly, researchers found no difference in the behavior between iPSC derived HSCs and ESC derived HSCs in vivo. Investigating the Lhx2 protein could be a fruitful avenue of investigation in further experimentation, as it may be used in OP9-iPSC interactions in order to induce differentiation into HSCs. If there are no Lhx2 proteins present, this too would be a significant discovery, as it shows that the OP9-iPSC coculture's differentiation into HSC is not dependent on this protein (Kitajima et al., 2011).

Hoxb4

Traditionally, there has been a problem inducing HSCs to proliferate rather than differentiating. The *Hoxb4* transcription factor has been found to be able to cause high levels of HSC expansion ex vivo. Researchers compared GFP transduced murine bone marrow cells to cultures of *Hoxb4*-transduced cells and found that the cells that were transduced with *Hoxb4* resulted in 40-fold net HSC increase. These HSCs have shown significantly enhanced regenerative potential in vivo. This transcription factor may be critical to stopping HSCs from differentiating and may play a role in the differentiation of iPSCs to HSCs. Thus, this is an interesting protein to investigate in further experimentation within the frame of OP9-iPSC interactions; should the presence of this protein be detected, it may imply that the OP9-iPSC coculture helps maintain HSCs in addition to generating them (Antonchuk et al., 2002).

Bmi-1

HSC formation should occur throughout the lifespan of an organism in order to maintain its viability. One protein that has been shown to be critical to this maintenance of hematopoiesis is Bmi-1, a protein encoded for by the proto-oncogene *Bmi-1*. Researchers looked at the number of HSCs in prenatal and postnatal mice who were *Bmi-1* deficient and found that the mice that were deficient for the gene had a decreased number of HSCs as compared to the control mice in adulthood but not in the prenatal condition. The researchers concluded that Bmi-1 is requisite for the creation of self-renewing adult HSCs. This may be relevant in further studies as it may be seen that Bmi-1 is present in the coculture in order to help sustain HSC formation from the OP9-iPSC coculture (Park et al., 2003).

Murine Blood Cell to HSC Transcription Factors

While we are investigating the creation of HSCs from an OP9-iPSC coculture, HSCs can also be generated by transient expression of six transcription factors on committed myeloid effector cells and myeloid and lymphoid progenitors. These transcription factors (Run1t1, Hlf, Lmo2, Prdm5, Pbx1, and Zfp37) essentially help reverse committed cell lineages to a more pluripotent state. Nevertheless, since they are intimately involved in generating HSCs, these transcription factors may be worth further investigation (Riddell et al., 2014).

Methodology

OP9 cell culture

Cell Thawing

OP9 mouse stromal cells were purchased from ATCC and frozen in a liquid nitrogen freezing chamber until ready to be used. In order to thaw the cells, they were placed in a warm water bath at 37 °C. Cells were added to OP9 growth media (Appendix C) to total 5 mL and were centrifuged at 400g for 5–7 minutes to pellet the cells and to remove any unwanted chemicals from freezing media. The supernatant was removed, and cell pellets were resuspended in growth media. 10–12 mL of growth media was added for suspension in a 75 cm² flask or 5 mL of growth media was added for suspension in a 25 cm² flask. Cells were then placed in a 37 °C incubator with 5% CO₂ to grow. Quality checks were completed on the growing cell line and media was changed every 2 days. When 70–90% confluency (~8 x 10⁶ cells) was reached, the cells were then passaged and expanded.



Figure 1: Image of confluent OP9 cell culture at approximately 70-80% confluency.

Passaging

In order to passage OP9 cells, growth media was first aspirated from the flask. OP9 cells were trypsinized with room temperature 0.1% Trypsin-0.5mM EDTA using half the working volume of the flask (i.e, 2.5 mL trypsin for a 5 mL 25 cm² flask, 5 mL trypsin for a 10-mL 75 cm² flask), and incubated for 5 minutes at 37 °C. Cells were verified to be detached under the microscope (Figure 2). OP9 growth media was added to cells to the working volume in order to deactivate the trypsin, and cells were centrifuged at 400g for 5–7 minutes to form a pellet. Supernatant was discarded and fresh growth media was added to reseed cells at a lower density (anywhere from 1:2 to 1:4 split ratios).

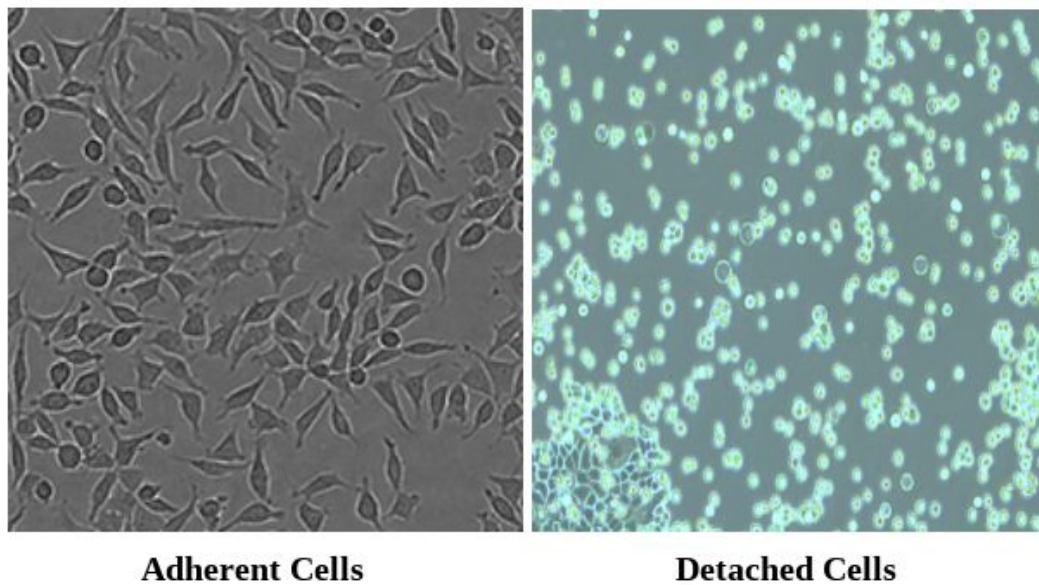


Figure 2. Example of adherent cells vs. detached cells. Detached cells are round and will move with movement of the flask.

Freezing

In order to freeze OP9 cells to create a bank for further use in later phases of the study, the OP9 cell passage protocol was followed once cells reached 70–90% confluency – growth media was aspirated from the cells, and cells were trypsinized,

centrifuged, and pelleted. However, once the supernatant is discarded from the pellet, cells were resuspended in freezing media containing growth media + 10% DMSO. Cells were placed in a cryogenic freezing container (freezing at a rate of $-1^{\circ}\text{C}/\text{minute}$) and placed in a -80°C freezer for 24 hours, and then moved to liquid nitrogen for long term storage. One confluent T-75 flask was used to make three 1-mL freezes, each containing $\sim 2.5 \times 10^6$ cells.

iPSC Culture

iPSC cells were cultured and passaged using 6-well plates. iPSCs used for differentiation underwent 20 passages at the beginning in order to ensure that the vectors used in reprogramming were eliminated. Freezes were made every 2–3 passages in order to ensure that freezes from lower passages could be utilized if future passages were abnormal and had to be discarded. Cells were passaged after 4–5 days, or at 80% confluency, with 1:2 or 1:3 split ratios. Established cultures are able to be split up to a 1:12 ratio. Wells with dense and large individual colonies were passaged in order to prevent the cells from becoming over-confluent. Undifferentiated colonies were determined by their physical appearance which includes their concrete edges, tightly packed nature, and high nucleus to cytoplasm ratio. This indicates that these undifferentiated cells have relatively large nucleuses compared with cytoplasm. Spontaneous differentiation can be identified by the smooth and altered morphology of the cell edges. A healthy, confluent well of iPSCs is shown below in Figure 3.

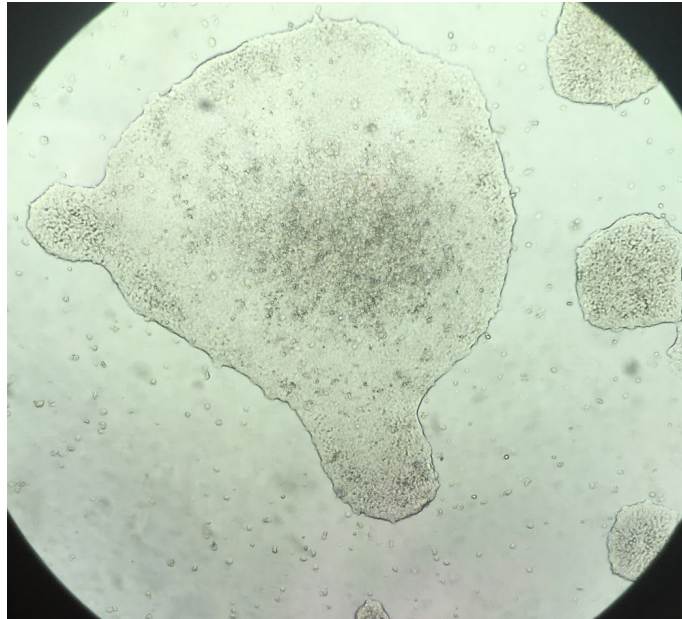


Figure 3. Healthy, confluent iPSC colonies showing concrete edges and high nucleus:cytoplasm ratio.

Cell Thawing

iPSCs were donated from the iPSC Core at the National Institutes of Health and were stored in a liquid nitrogen freezing chamber until needed. For thawing and culturing cells, 6-well Matrigel-coated plates (Appendix C) and complete Essential 8 Flex Medium (E8 complete media/iPSC media) were allowed to come to room temperature. ROCK inhibitor was added to iPSC media with a final concentration of 10 μ M to create iPSC thawing media. Matrigel was aspirated from the well, and 1–2 mL thawing media was added to the well. A vial of frozen iPSCS cells were thawed quickly and added to a well containing iPSC thawing media. The plate was incubated at 37 °C with 5% CO₂ for 30 minutes to allow cells to attach. Media was changed with fresh iPSC thawing media, and the plate was returned to the incubator.

Passaging

Cells were passaged at a minimum of every 4–5 days regardless of confluency. Cells were also split when the colonies became too dense or large, and/or

if the colonies showed increased differentiation. To start, E8 complete media and EDTA dissociation solution (Appendix C) were warmed to room temperature. Wells were rinsed 4x with 1 mL EDTA dissociation solution. 1 mL of EDTA was added to the well and incubated at room temp for 5–8 minutes. During the incubation, Matrigel was removed from the wells that were being passaged into, and replaced with fresh E8 media. After checking for detachment under the microscope, indicated by smoothed edges and “holes” in the colonies (Figure 4), EDTA dissociation solution was carefully aspirated from the cells in the well. Fresh E8 media was pipetted with force to wash the colonies off the plate, carefully preventing excessive pipetting, and cells were passaged and split into new wells. Plates were placed in the 37 °C incubator with 5% CO₂.

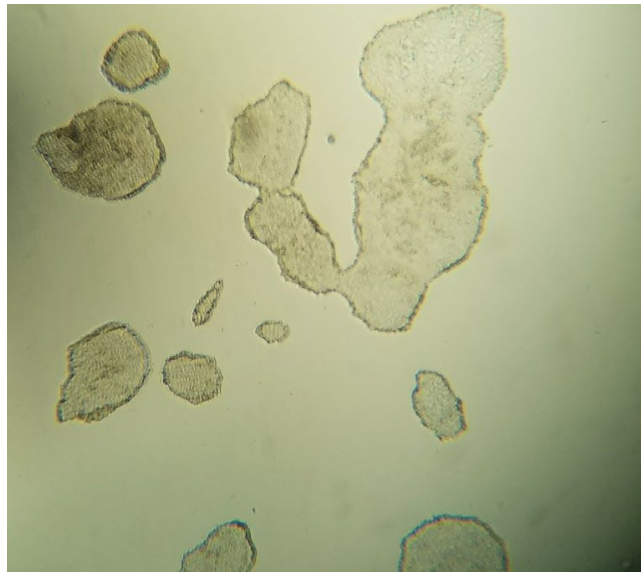


Figure 4. Detachment of iPSCs after EDTA incubation showing smoothed colony edges and “holes” appearing in colonies.

Freezing

E8 complete media and EDTA dissociation solution were warmed to room temperature. Freezing media consisting of E8 complete media + 10% DMSO + 10 μ M ROCK inhibitor was created. Freezing began by aspirating the media from the well, without allowing the well to dry out. Wells were then rinsed 4X with 1 mL EDTA dissociation solution. 1 mL of EDTA was added to the well and incubated at room temp for 5–8 minutes. EDTA solution was then carefully aspirated from the wells. Freezing media was added to the cells using the force of pipetting to wash the colonies off of the plate, and cells were aliquoted into cryovials. Vials were placed in a cryogenic freezing container and placed in a -80°C freezer for at least 24 hours. Cells were transferred to liquid nitrogen for long-term storage.

iPSC + OP9 Cocultures

iPSC + OP9 cell coculture

OP9 cells were subcultured 3 days before beginning the coculture, and extra cells were subcultured/expanded for days 5 and 8/9. Media was removed from OP9 cells and fresh coculture media (50% E8 media, 50% OP9 media) was added. In initially seeding the iPSCs, iPSCs were passaged following the iPSC passage protocol, however, coculture media was added to iPSCs in lieu of E8 media, and detached iPSCs were plated at $\sim 10^3$ iPSC/cm² to a flask of OP9 cells. Cell counts were estimated based on percent confluency of a given surface area of flasks or well plates. Cells were incubated at 37° C, 5% CO₂. OP9 cells were continually being subcultured and expanded, and were not growth arrested prior to coculture with iPSCs. On Day 3, the media on the coculture was replaced with fresh coculture media. To

subculture/expand cells, media was removed from the cells and cells were trypsinized with 0.1% Trypsin-0.5mM EDTA and incubated for 5 minutes at 37°C. Coculture media was added to cells, and cells were centrifuged at 400g for 5–7 minutes. The supernatant from the pellet was discarded, and fresh growth media was added to cells to resuspended and reseed $6.5 \times 10^4/\text{cm}^2 - 7.75 \times 10^4/\text{cm}^2$ co-culture cells onto a new layer of confluent OP9 cells. After days 8/9, cells theoretically began to differentiate and could be further expanded or used for analysis.

IPSC + OP9 conditioned media culture

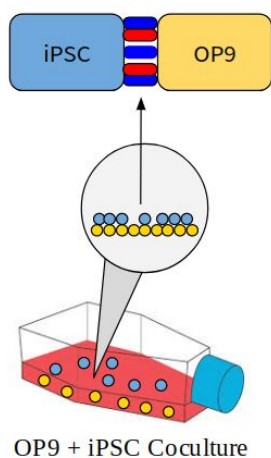
iPSCs were grown to confluency following the 6-well plate method. OP9 cells were subcultured three days prior to beginning the IPSC + OP9 conditioned media culture. When beginning the conditioned media culture, IPSC media was removed from confluent cells, and was replaced with media from growing OP9 cells (OP9 conditioned media). Cells were further grown in OP9 conditioned media instead of iPSC media.

Interleukin Quantibody ELISA Protocol

Standard cytokine dilutions were prepared using serial dilution as specified by the RayBiotech *Quantibody Mouse Interleukin Array 1* manual. A negative control was established with only 100 μL of sample diluent and no added standard cytokines. 100 μL of sample diluent was added into each well and incubated at room temperature for 30 minutes. The diluent solution was decanted and 100 μL of sample was added to each well, after which the wells were allowed to incubate for 1–2 hours. After incubation, the wells were washed 5 times with 1X wash buffer at room temperature

and under gentle rocking. Subsequently, 80 μL of detection antibody was added to each well. The detection antibody was allowed to incubate in the wells at room temperature for 1–2 hours. After detection antibody incubation, the detection antibody solution was decanted and the wells were washed 7 times with 1X washing buffer. 1.4 mL of sample diluent was then added to the Cy3 equivalent dye-conjugated streptavidin tube and mixed gently. 80 μL of Cy3 solution was added to each well. The array was subsequently covered with aluminum foil and allowed to incubate in the dark for 1 hour. After incubation, the Cy3 solution was decanted and the array was washed 5 times with 1X washing buffer. The array was then sent to RayBiotech for analysis under a fluorescence detection system (Quantibody, 2016). After analysis, fluorescence of each well was compared to an established standardization curve to determine the concentration of each cytokine within the well. Cytokine concentrations were then analyzed and compared to each other, as well as with the existing literature.

**Control: Juxtacrine Signaling
iPSC + OP9 Cell Culture**



**Experimental: Paracrine Signaling
iPSC + OP9 Conditioned Media Culture**

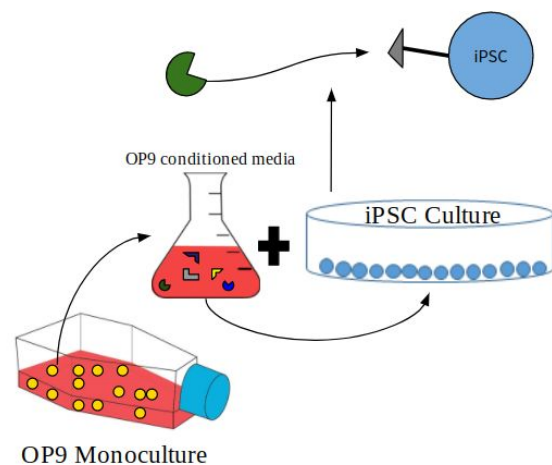


Figure 5: Visual representation of our experimental conditions. Our research looks to determine if iPSC/OP9 signaling involves juxtacrine or local signaling, as seen in this diagram. These are our two different experimental sample designs.

IL-3								
Concentration (pg/ml)	0	3	8	25	74	222	667	2000
Signal-BKG	0	194	421	1068	2762	6877	17218	45222
Log (Concentration)		0.44	0.92	1.39	1.87	2.35	2.82	3.30
Log (Signal-BKG)		2.29	2.62	3.03	3.44	3.84	4.24	4.66
		Slope		Intercept				
Linear regression		23.04	0.00	0.00				
log-log regression		0.83		1.89				

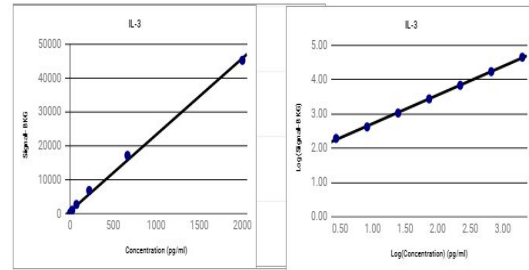


Figure 6: Example of standard curve for IL-3 protein after assembly of fluorescence data.

For additional information on preparation and justification of the reagents used in this study, please refer to Appendices B & C.

Results

Coculture Systems

Through a qualitative and morphological analysis of the cells, we determined that the OP9/iPSC coculture resulted in iPSCs differentiating into HSCs while the OP9 conditioned media coculture did not result in differentiation. This qualitative assessment was based on known appearance of differentiated iPSCs, which show a lack of iPSC colonies and instead a homogenous appearance of uniform, individualized cells. In our control iPSC + OP9 cell culture system, there was a lack of colonies and a new presence of individualized cells. In our experimental iPSC + OP9 conditioned media culture system, the iPSC colonies still remained and did not turn into individualized cells. These morphological differences can be visualized in Figure 7 below.

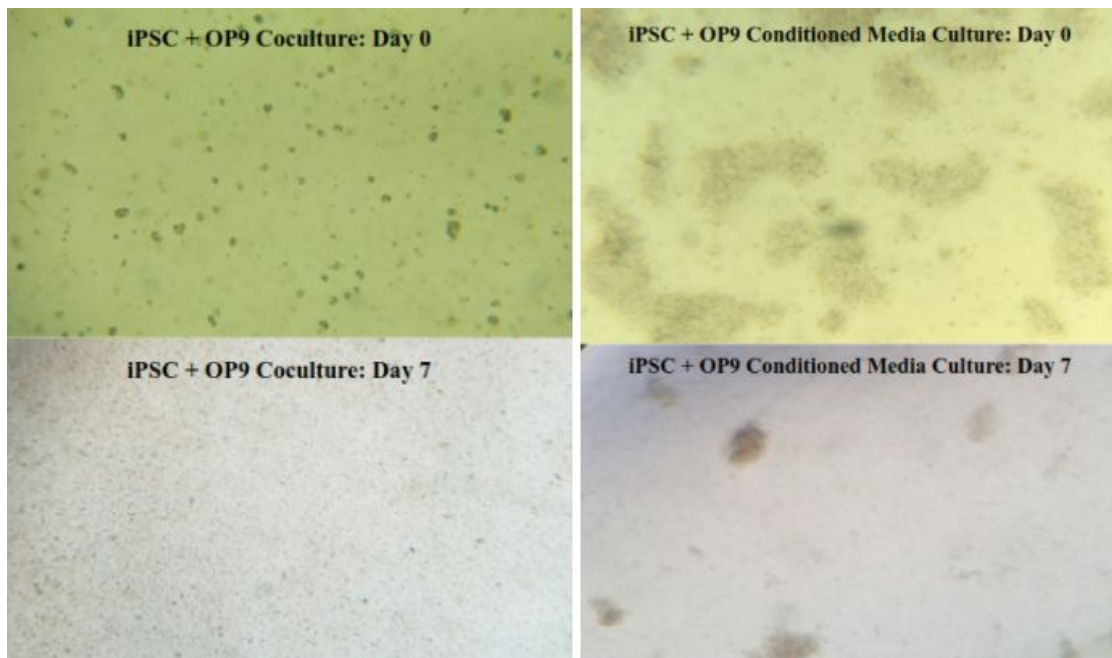


Figure 7. Morphological differences in two coculture systems. Our control system shows morphological differentiation, while our experimental system does not.

Protein Identification

In all cell culture systems, thirteen cytokines were analyzed. All were upregulated in the OP9/iPSC coculture as compared to the OP9 monoculture and the iPSC with OP9 conditioned media coculture. The following figures display the concentrations of each cytokine found in each culture. The results are discussed further detail for each cytokine in the Discussion section.

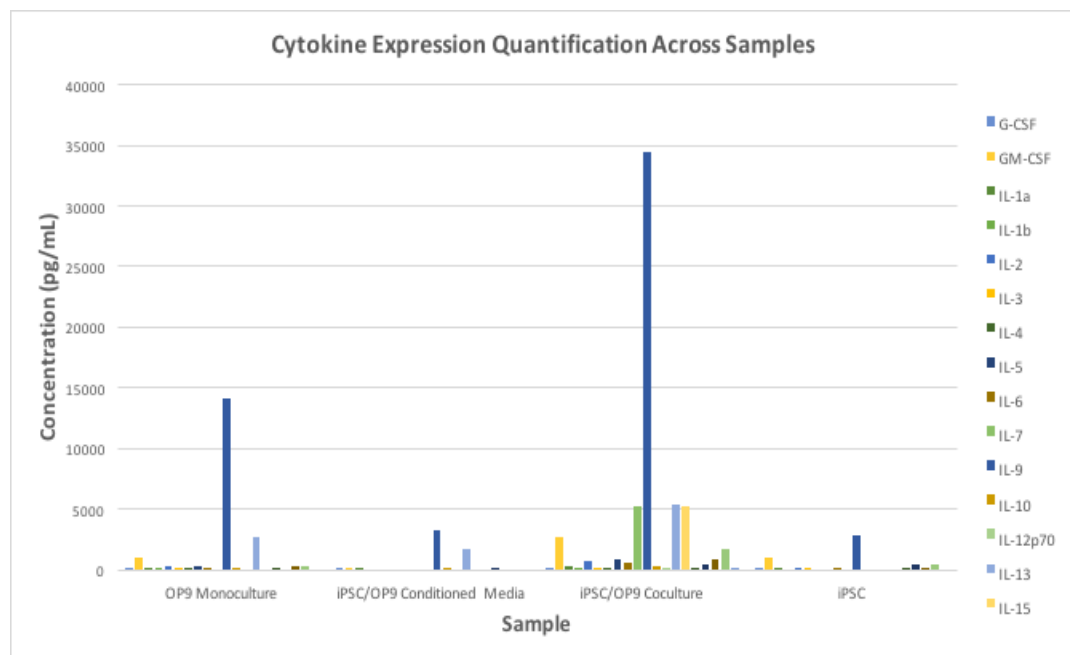


Figure 8: Expression analysis of 13 interleukins across samples of OP9 monoculture, iPSC/OP9 conditioned media coculture, iPSC/OP9 coculture, and iPSC monoculture. Results show significant upregulation of protein levels when iPSCs and OP9s maintain cell-to-cell contact. A negative trend is shown when compared to the iPSC/OP9 conditioned media coculture. The OP9 monoculture and iPSC monoculture show endogenous expression of secretions of some interleukins, however the expression levels are still moderately attenuated as compared to the iPSC/OP9 coculture system.

After expression analysis of the 13 cytokines tested, a clear negative trend was observed in the data. As shown in Figure 8, a holistic upregulation of protein levels was observed in the iPSC/OP9 coculture. In this sample, cell-to-cell contact between iPSCs and OP9 cells were maintained throughout the 9 day experimentation period. A

negative trend was observed when comparing the iPSC/OP9 conditioned media coculturing system. We also found a moderate level of expression for 9 of the 13 cytokines that were tested in the OP9 and iPSC monocultures.

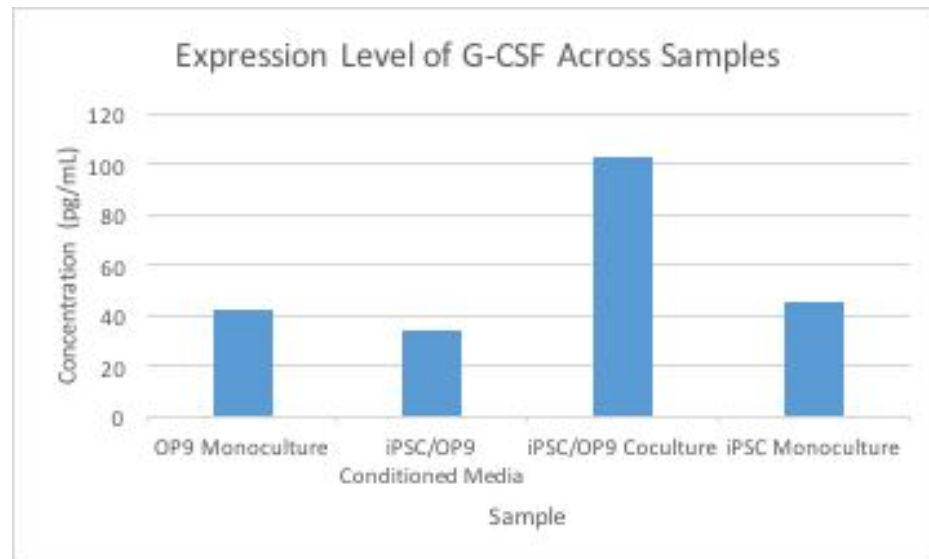


Figure 9: Expression analysis of G-CSF levels in OP9 monoculture, iPSC/OP9 conditioned media coculture, iPSC/OP9 coculture, and iPSC monoculture. Graph shows significant upregulation of G-CSF in the iPSC/OP9 coculture, but significant downregulation in the iPSC/OP9 conditioned media coculture, iPSC monoculture, and OP9 monoculture.

In Figure 9, the expression level of G-CSF was shown across samples, and the data reveals that there is a significant upregulation of the cytokine level in the iPSC/OP9 coculture. According to the data, there was 102.5 pg/mL within this sample. The iPSC/OP9 conditioned media coculture showed significant downregulation, showing very little expression of 34.0 pg/mL in this sample. In the OP9 monoculture, an expression level of 42.0 pg/mL was shown. In the iPSC monoculture, an expression level of 44.9 pg/mL was shown

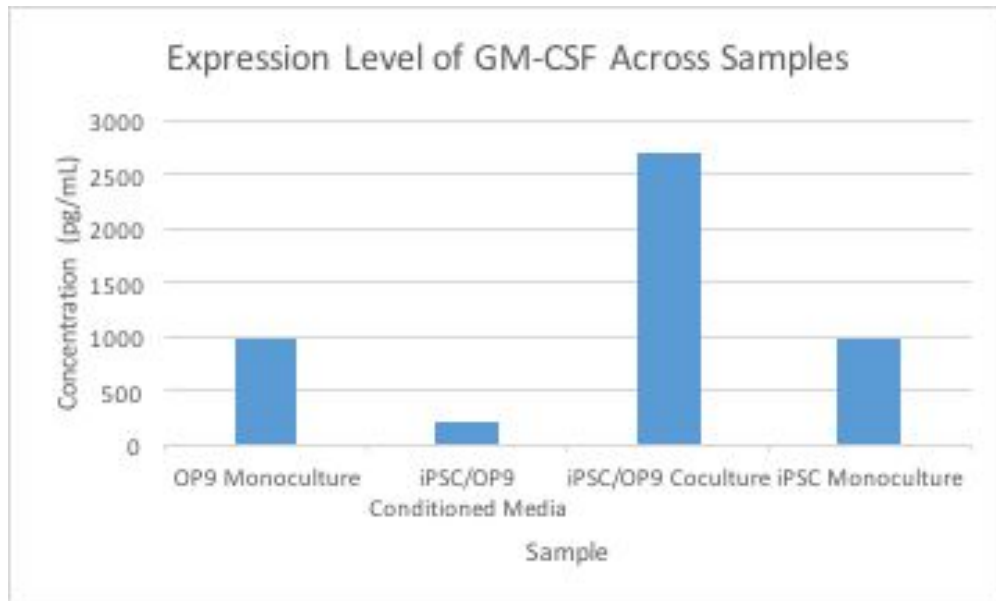


Figure 10: Expression analysis of GM-CSF levels in OP9 monoculture, iPSC/OP9 conditioned media coculture, iPSC/OP9 coculture, and iPSC monoculture. Graph shows significant upregulation of GM-CSF in iPSC/OP9 coculture, but significant downregulation in iPSC/OP9 conditioned media coculture sample.

In Figure 10, the expression level of GM-CSF was shown across samples, and the data reveals that there is a significant upregulation of the cytokine level in the iPSC/OP9 coculture. According to the data, there was 2692.6 pg/mL within this sample. The iPSC/OP9 conditioned media coculture showed significant downregulation, showing very little expression of 208.5 pg/mL in this sample. In the OP9 monoculture, an expression level of 978.8 pg/mL was shown. In the iPSC monoculture, an expression level of 985.0 pg/mL was shown.

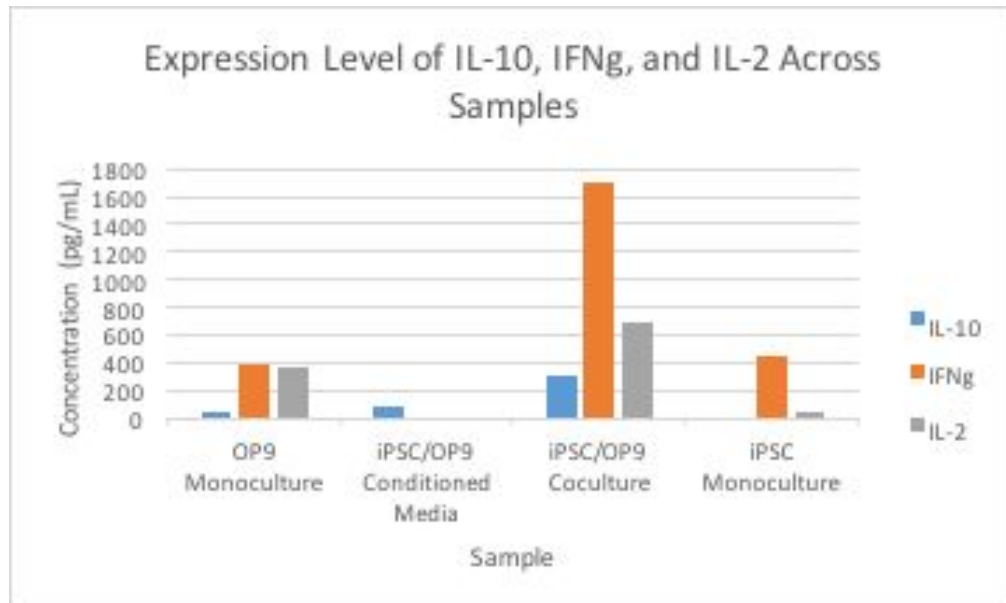


Figure 11: Expression analysis of IL-10, IFN- γ , and IL-2 concentrations in OP9 monoculture, iPSC/OP9 conditioned media coculture, iPSC/OP9 coculture and iPSC monoculture. Graph shows significant upregulation of IFN- γ and IL-2 in iPSC/OP9 coculture even within the presence of IL-10, but significant downregulation in iPSC/OP9 conditioned media coculture sample.

In Figure 11, the expression level of IL-2, IL-10, and IFN- γ is observed across our three samples. We observe that there was a significant amount of upregulation of all three cytokines in the iPSC/OP9 coculture. A downward trend is also observed as we compare the iPSC/OP9 coculture to the iPSC/OP9 conditioned media coculture and the OP9 monoculture. It is notable that a small level of expression of IL-10 was still observed in the iPSC/OP9 conditioned media coculture, whereas there seems to be no expression of both IL-10 and IFN- γ within this sample. There was significant upregulation of IFN- γ in the iPSC monoculture.

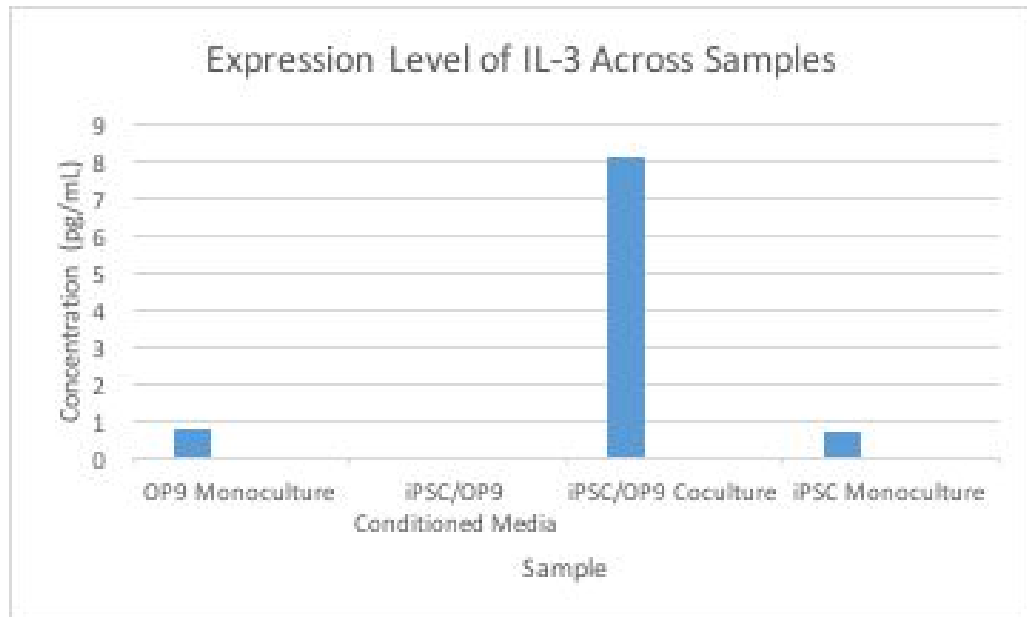


Figure 12: Expression analysis of IL-3 levels in OP9 monoculture, iPSC/OP9 conditioned media coculture, iPSC/OP9 coculture, and iPSC monoculture. Graph shows significant upregulation of IL-3 in iPSC/OP9 coculture, but significant downregulation in iPSC/OP9 conditioned media coculture sample.

In Figure 12, the expression level of IL-3 was shown across samples, and the data reveals that there is a significant upregulation of the cytokine level in the iPSC/OP9 coculture. According to the data, there was 8.1 pg/mL within this sample. The iPSC/OP9 conditioned media coculture showed significant downregulation, showing no expression of the cytokine within this sample. In the OP9 monoculture, a small expression level of 0.8 pg/mL was shown. In the iPSC monoculture, a small expression level of 0.7 pg/mL was shown.

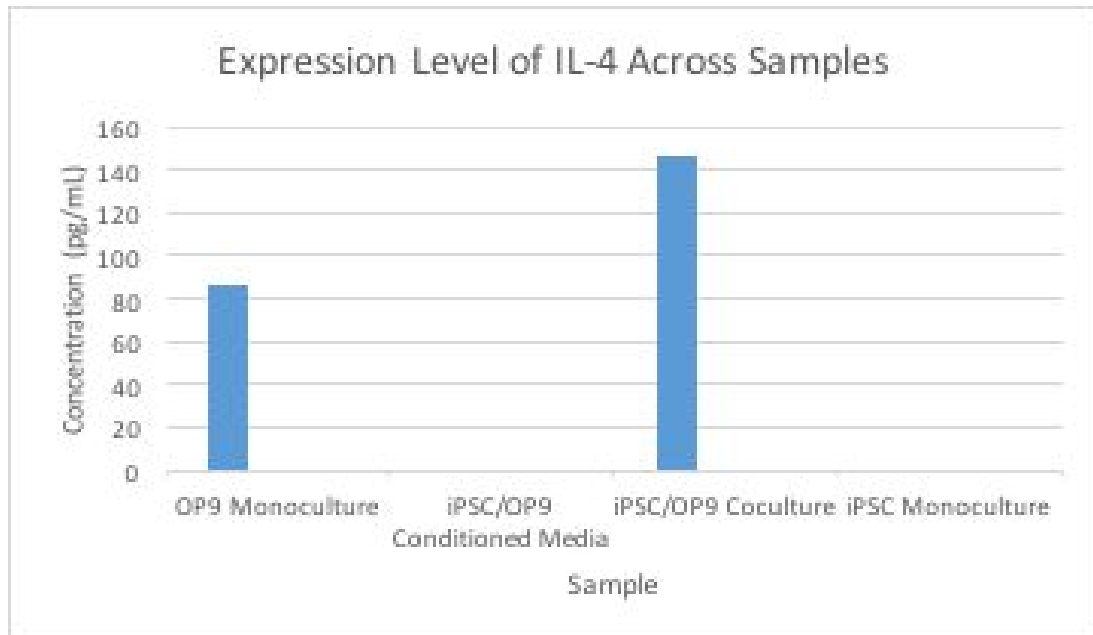


Figure 13: Expression analysis of IL-4 levels in OP9 monoculture, iPSC/OP9 conditioned media coculture, iPSC/OP9 coculture, and iPSC monoculture. Graph shows significant upregulation of IL-4 in iPSC/OP9 coculture, but significant downregulation in iPSC/OP9 conditioned media coculture sample.

In Figure 13, the expression level of IL-4 was shown across samples, and the data reveals that there is a significant upregulation of the cytokine level in the iPSC/OP9 coculture. According to the data, there was 146.4 pg/mL within this sample. The iPSC/OP9 conditioned media coculture showed significant downregulation, showing no expression of the cytokine within this sample. In the OP9 monoculture, a small expression level of 86.3 pg/mL was shown. In the iPSC monoculture, there was no expression.

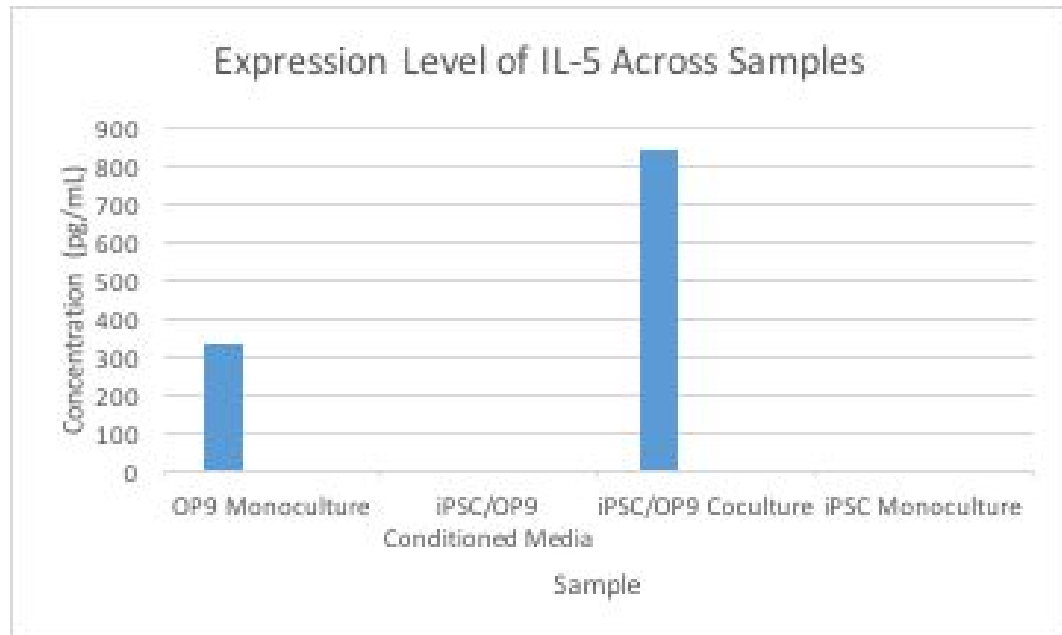


Figure 14: Expression analysis of IL-5 levels in OP9 monoculture, iPSC/OP9 conditioned media coculture, iPSC/OP9 coculture, and iPSC monoculture. Graph shows significant upregulation of IL-5 in iPSC/OP9 coculture, but significant downregulation in iPSC/OP9 conditioned media coculture sample.

In Figure 14, as compared to OP9 secretion, iPSC/OP9 coculture shows upregulation of IL-5 whereas iPSC/OP9 conditioned media coculture shows downregulation of this cytokine. iPSC/OP9 conditioned media coculture showed 0 pg/mL of IL-5. OP9 monoculture had IL-5 concentration of 328.4 pg/mL while iPSC/OP9 coculture has concentration of 840.6 pg/mL. The iPSC monoculture showed no expression.

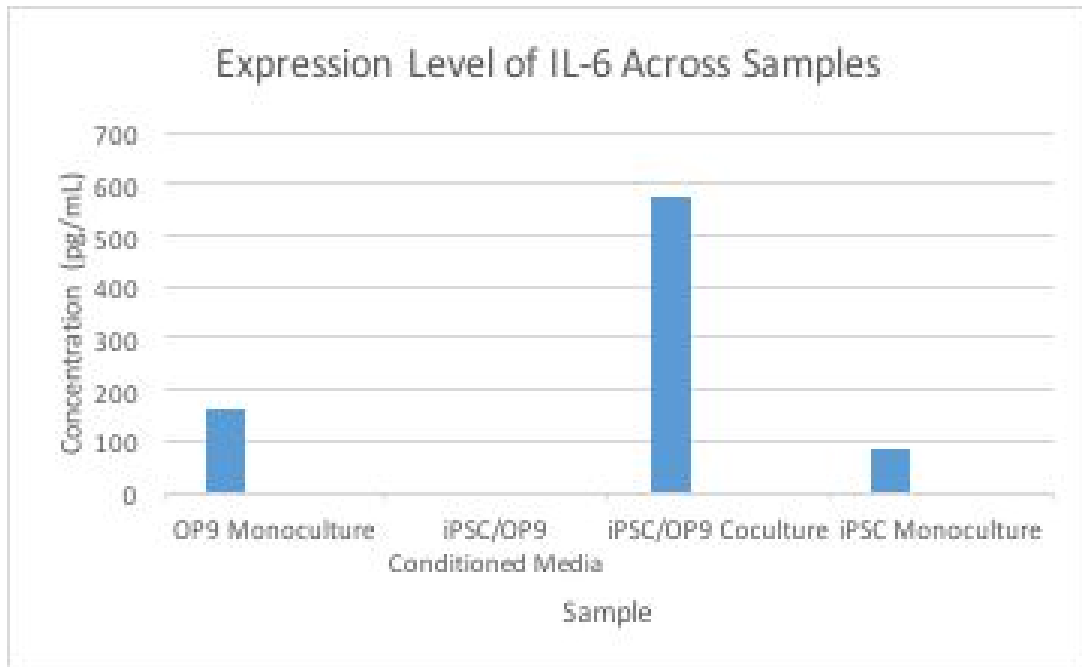


Figure 15: Expression analysis of IL-6 levels in OP9 monoculture, iPSC/OP9 conditioned media coculture, iPSC/OP9 coculture, and iPSC monoculture. Graph shows significant upregulation of IL-6 in iPSC/OP9 coculture, but significant downregulation in iPSC/OP9 conditioned media coculture sample. There is also moderate levels of expression in both the OP9 monoculture and iPSC monoculture.

In Figure 15, the results reveal that compared to OP9 secretion, iPSC/OP9 coculture shows upregulation of IL-6 whereas iPSC/OP9 conditioned media coculture shows downregulation of this cytokine. The OP9 monoculture showed IL-6 concentration of 165 pg/mL while iPSC/conditioned media coculture had no concentrations measured for IL-6. iPSC/OP9 coculture had IL-6 concentration of 575.1 pg/mL. The iPSC monoculture had a IL-6 concentration of 86.0 pg/mL.

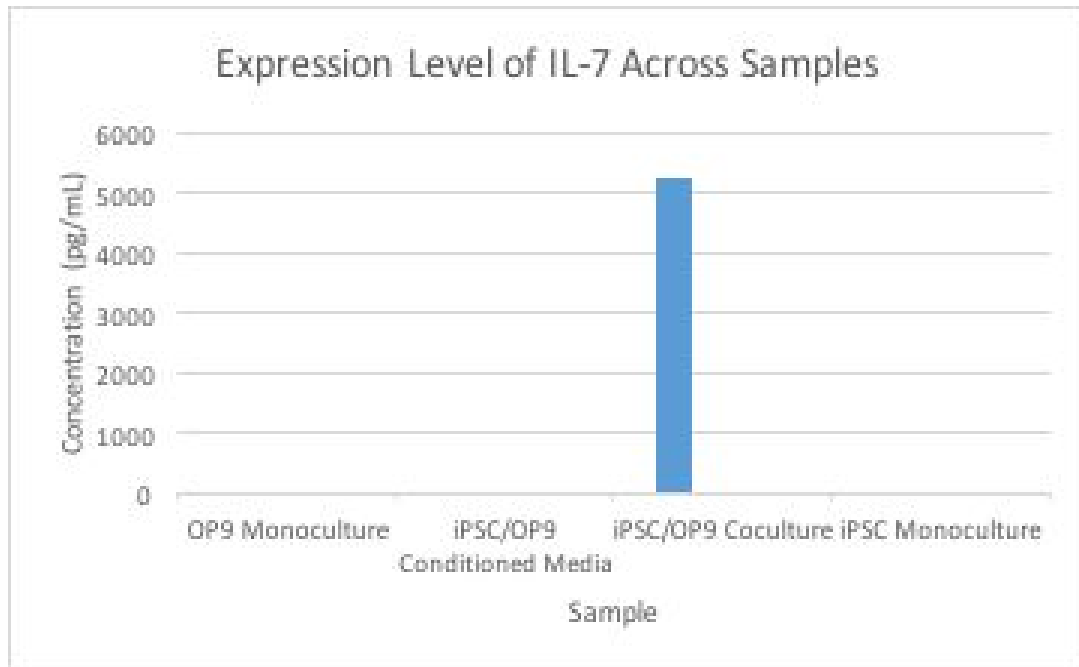


Figure 16: Expression analysis of IL-7 levels in OP9 monoculture, iPSC/OP9 conditioned media coculture, iPSC/OP9 coculture, and iPSC monoculture. Only the iPSC/OP9 coculture showed significant levels of IL-7 expression.

Data in Figure 16 reveals that the OP9 monoculture, iPSC monoculture, and iPSC/OP9 conditioned media coculture showed no levels of IL-7 secretion. Conversely, iPSC/OP9 coculture shows secretion of IL-7. The OP9 monoculture, iPSC monoculture, and iPSC/OP9 conditioned media coculture had measured IL-7 concentrations of 0 pg/mL. The iPSC/OP9 coculture had a measured IL-7 concentration of 5226 pg/mL.

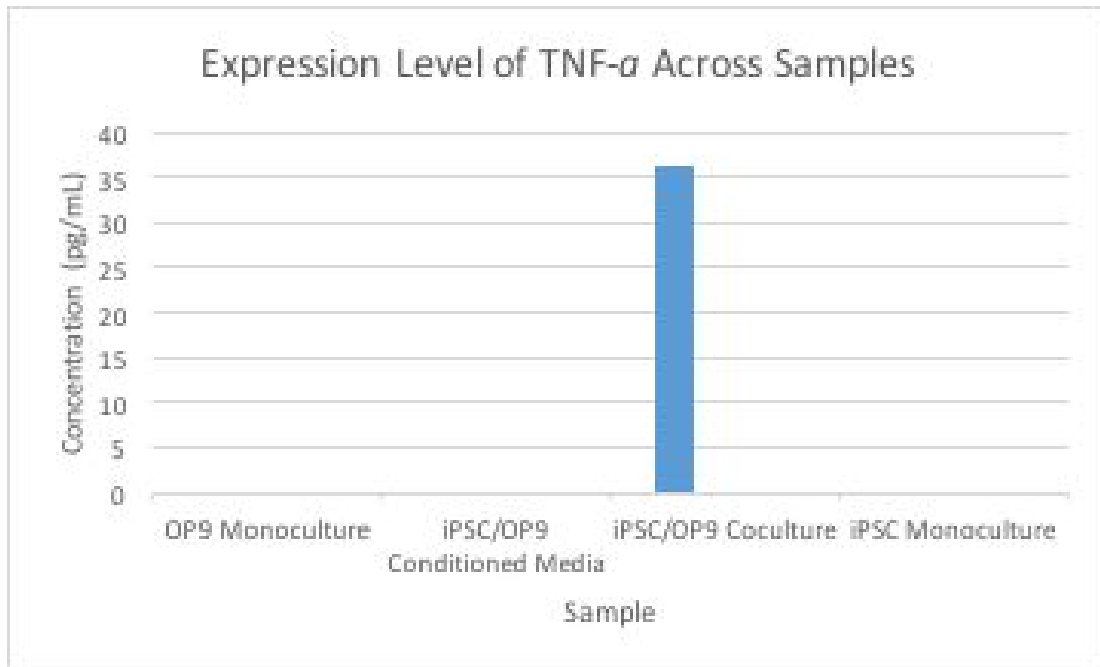


Figure 17: Expression analysis of TNF- α in OP9 monoculture, iPSC/OP9 conditioned media coculture, iPSC/OP9 coculture, and iPSC monoculture. Only the iPSC/OP9 coculture showed significant levels of TNF- α expression.

Data in Figure 17 reveals that compared to OP9 secretion, iPSC/OP9 coculture shows significant upregulation of TNF-alpha. The iPSC monoculture, OP9 monoculture, and iPSC/OP9 conditioned media coculture showed no secretion of TNF alpha, with all three cultures having measured concentrations of 0 pg/mL. The iPSC/OP9 coculture showed a TNF alpha expression level of 36.2 pg/mL.

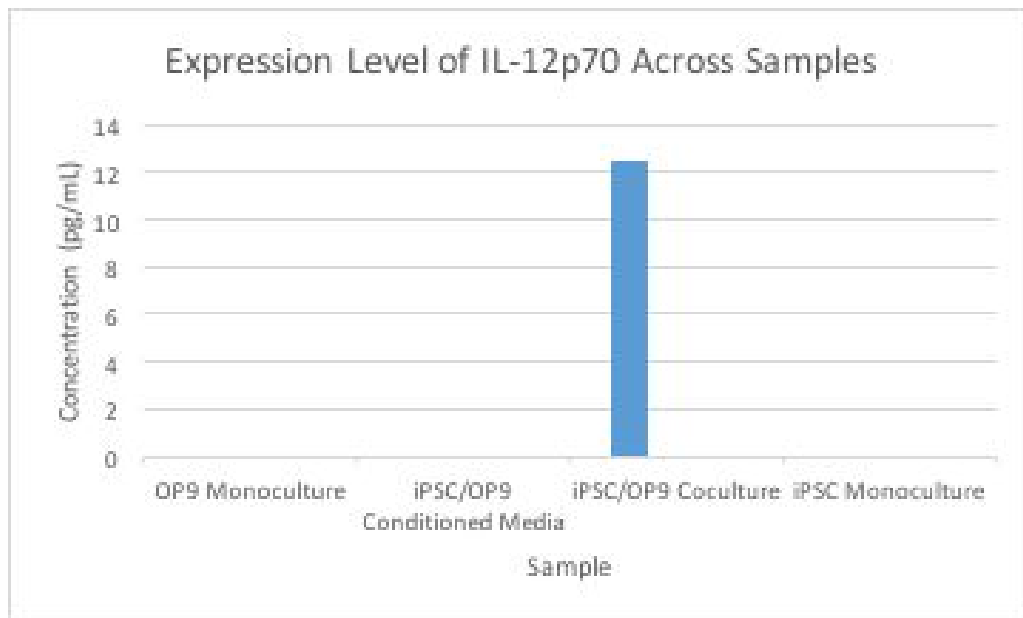


Figure 18: Expression analysis of IL-12p70 in OP9 monoculture, iPSC/OP9 conditioned media coculture, iPSC/OP9 coculture, and iPSC monoculture. Only the iPSC/OP9 coculture showed any levels of IL-12p70 expression.

Expression analysis of the cytokine IL-12p70 in Figure 18 shows upregulation of the cytokine within the iPSC/OP9 coculture. The concentration of IL-12p70 within this coculture sample was measured to be 12/7 pg/mL, which is significantly less than other cytokines that were measured. This cytokine shows no expression in the iPSC/OP9 conditioned media coculture, the iPSC monoculture, or the OP9 monoculture. It seems that this protein is only upregulated in samples that retained cell-to-cell contact within the 9 day experimentation period.

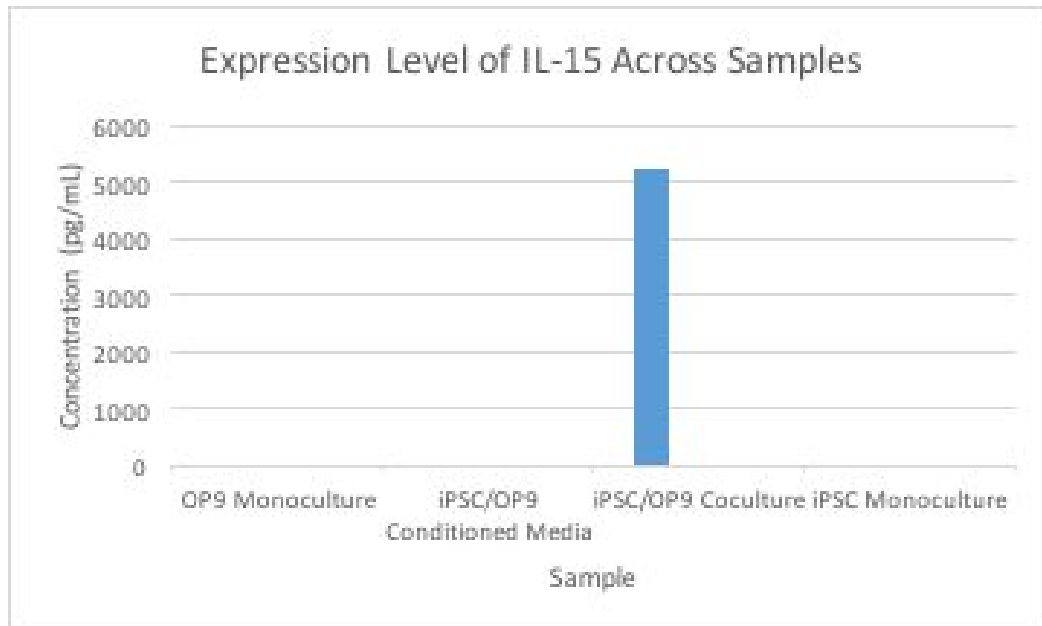


Figure 19: Expression analysis of IL-15 in OP9 monoculture, iPSC/OP9 conditioned media coculture, iPSC/OP9 coculture, and iPSC monoculture. Only the iPSC/OP9 coculture showed any levels of IL-15 expression.

An expression analysis of IL-15 in Figure 19 shows a significant upregulation of cytokine concentration in the iPSC/OP9 coculturing system. In this sample, the cytokine concentration was measured to be 5260 pg/mL. This concentration is very high as compared to other cytokine concentrations that were found in this study. IL-15 was found to be significantly downregulated in the iPSC/OP9 conditioned media coculture, iPSC monoculture, and the OP9 monoculture, with 0 pg/mL of cytokine being measured in each sample.

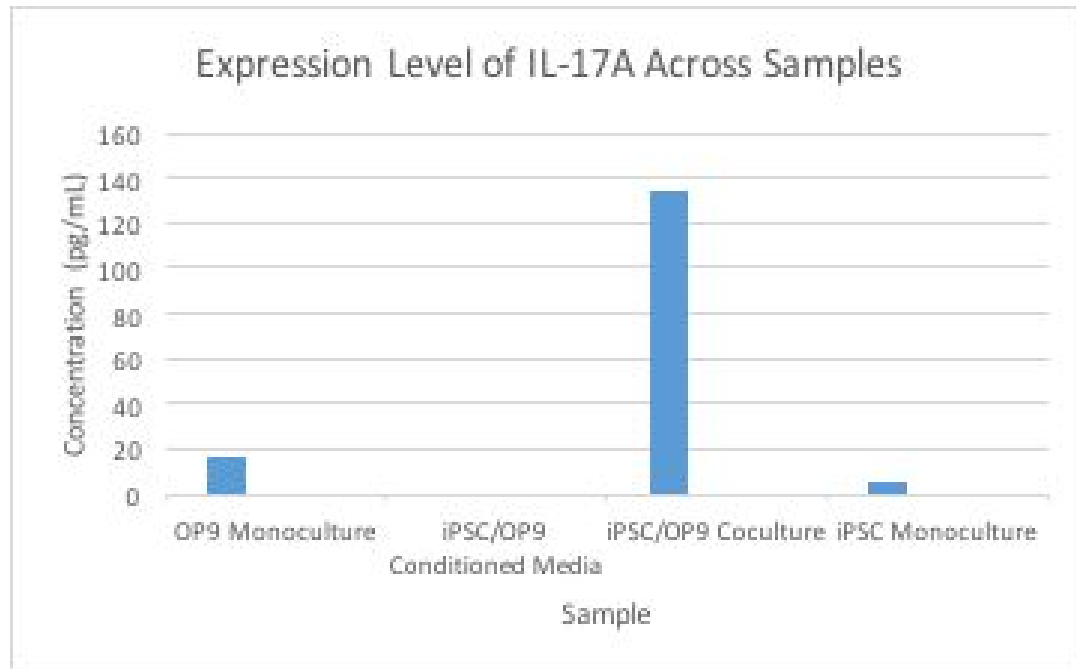


Figure 20: Expression analysis of IL-17A in OP9 monoculture, iPSC/OP9 conditioned media coculture, iPSC/OP9 coculture, and iPSC monoculture. The results reveal that, compared to OP9 monoculture secretions, the iPSC/OP9 coculture shows significant upregulation.

In Figure 20, the expression level of IL-17A was shown across samples, and the data reveals that there is a significant upregulation of the cytokine level in the iPSC/OP9 coculture. According to the data, there was 134.1 pg/mL within this sample. The iPSC/OP9 conditioned media coculture showed significant downregulation, showing no expression of the cytokine within this sample. In the OP9 monoculture, a small expression level of 16.5 pg/mL was shown. In the iPSC monoculture, a small expression level of 6.2 pg/mL was shown.

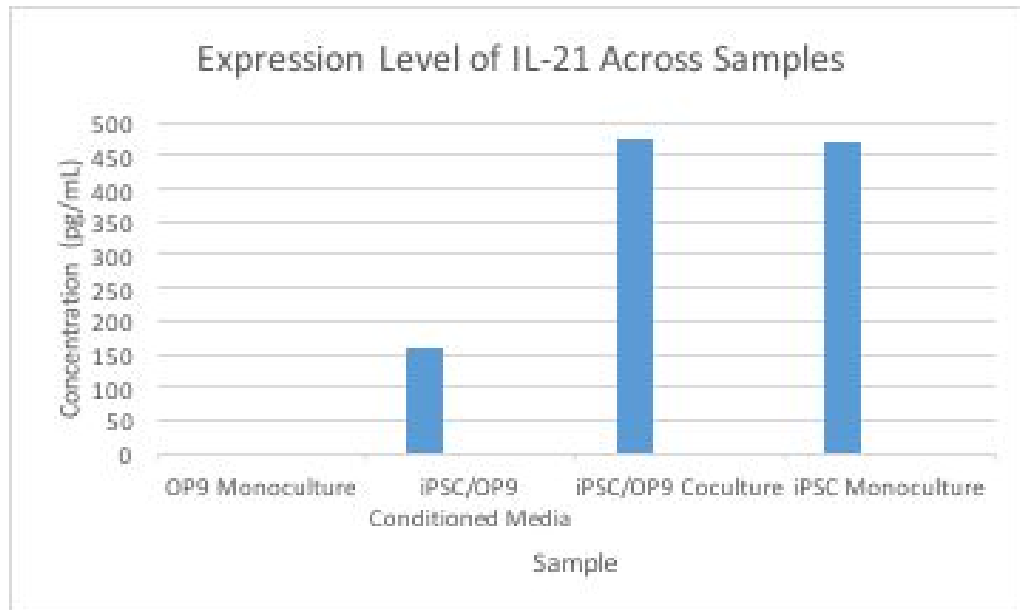


Figure 21: Expression analysis of IL-21 in OP9 monoculture, iPSC/OP9 conditioned media coculture, iPSC/OP9 coculture, and iPSC monoculture. Data reveals that compared to OP9 secretion, iPSC/OP9 coculture and iPSC monoculture shows significant upregulation of IL-21. iPSC/OP9 conditioned media coculture shows little secretion of IL-21

In Figure 21, the expression level of IL-21 was shown across samples, and the data reveals that there is a significant upregulation of the cytokine level in the iPSC/OP9 coculture and iPSC monoculture. According to the data, there was 472.8 pg/mL within the iPSC/OP9 coculture and 469.9 pg/mL within the iPSC monoculture. In the iPSC/OP9 conditioned media monoculture, a smaller expression level of 157.3 pg/mL was shown. The OP9 monoculture showed significant downregulation, showing no expression of the cytokine within this sample.

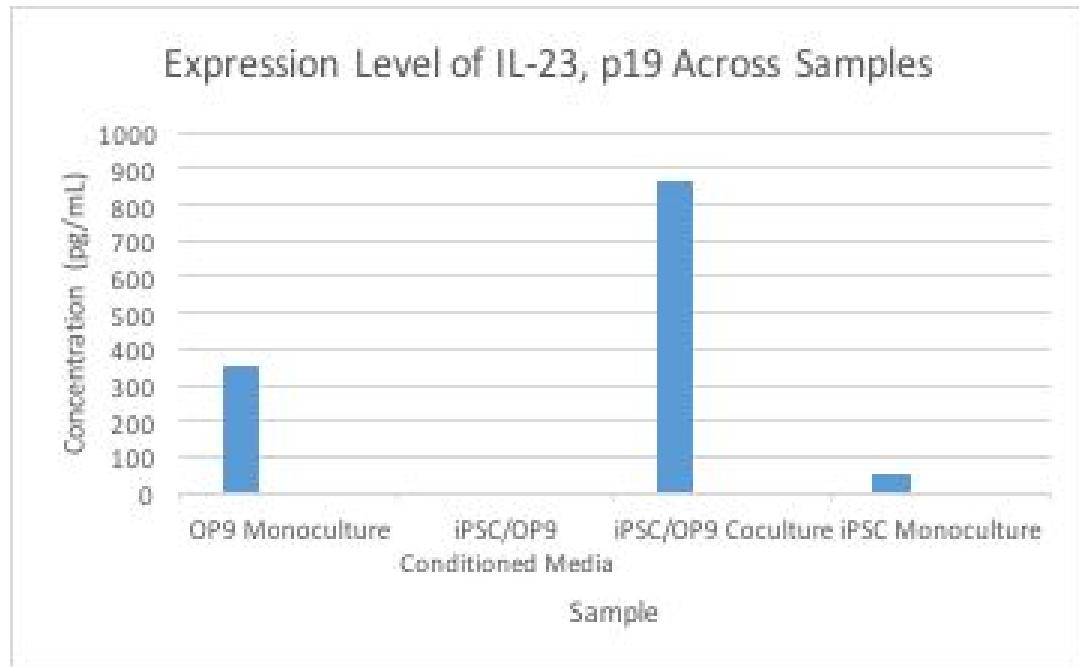


Figure 22: Expression analysis of IL-23, p19 in OP9 monoculture, iPSC/OP9 conditioned media coculture, iPSC/OP9 coculture, and iPSC monoculture. Data reveals that compared to OP9 secretion, iPSC/OP9 coculture shows significant upregulation of IL-23, p19. iPSC/OP9 conditioned media coculture show no secretion of IL-23, p19. iPSC monoculture shows little secretion of IL-23, p19.

In Figure 22, the results reveal that compared to OP9 secretion, iPSC/OP9 coculture shows upregulation of IL-23, p19 whereas iPSC/OP9 conditioned media coculture shows downregulation of this cytokine. The OP9 monoculture showed IL-23, p19 concentration of 349.1 pg/mL, while iPSC/OP9 conditioned media coculture had no concentrations measured for IL-23, p19. iPSC/OP9 coculture had IL-23, p19 concentration of 862.2 pg/mL. The iPSC monoculture had a measured IL-23, p19 concentration of 53.0 pg/mL.

IL-1a, IL-1b, IL-9 and IL-13 have been excluded from our data set. This is due to errors in the established standard curves, which, if used, may have skewed our data. Thus analysis of these cytokines will not be discussed. To view these excluded standard curves, please see Appendix D.

Discussion

Analysis of Relevant Proteins

G-CSF

Granulocyte-colony stimulating factor (G-CSF) is a protein induced by inflammatory stress, regulating hematopoiesis at the stem cell level (Schuettpelz, 2014). G-CSF helps mobilize HSCs from the bone marrow to the blood in order to harvest HSCs for stem cell transplantation (Schuettpelz, 2014). Expression of G-CSF has shown to affect HSCs by inducing toll-like receptor (TLR) signaling in HSCs (Schuettpelz, 2014). In Figure 8, we see that the expression of this cytokine is significantly upregulated in the iPSC/OP9 coculture. We also observed a significant decrease in expression of the protein in the iPSC/OP9 conditioned media coculture and in the control OP9 monoculture.

In the conditioned media coculture where only indirect cell signaling is occurring, our data shows a significant decrease in expression levels, even lower or comparable to the expression level of the control OP9 monoculture. In the iPSC/OP9 coculture where direct cell signaling is occurring however, upregulation suggests that cell to cell contact is necessary for expression of G-CSF. Investigations of the mechanism behind the direct cell to cell signaling in the upregulated role of G-CSF may help further explain the process of hematopoiesis.

GM-CSF

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a hematopoietic growth factor involved in the generation and maturation of granulocytes and macrophages from HSCs (Kruger, 2007). Both GM-CSF and G-CSF

serve cellular functions in proper differentiation and anti-apoptosis. Furthermore, GM-CSF has been shown to work closely with growth factor Interleukin-3 (IL-3) in development of hematopoiesis. GM-CSF interacts closely with other growth factors, and as suggested by the timing of development, GM-CSF acts at a later stage in cell development (Donahue, 1988). In Figure 9, we see that the expression of this cytokine is significantly upregulated in the iPSC/OP9 coculture. We also observed significantly little expression level of the protein in the iPSC/OP9 conditioned media coculture and decreased expression level in the control OP9 monoculture.

In the conditioned media coculture where only indirect cell signaling is occurring, our data shows a significant decrease in expression level. Comparatively, in the iPSC/OP9 coculture where direct cell signaling is occurring, upregulation suggests that cell to cell contact is necessary for expression of GM-CSF. This also matches the results of IL-3, the cytokine that GM-CSF interacts closely with. Similar results between the two proteins indicate that they both need cell to cell contact, further suggesting that they interact via direct cell signaling as opposed to indirect cell signaling. Investigations of the mechanism behind the direct cell to cell signaling in the upregulated role of GM-CSF may help further explain the process of hematopoiesis.

IL-2, IL-10, and IFN- γ

IL-10 has been shown to downregulate the expression of Th1 cytokines, which are responsible for producing IFN- γ and IL-2 (Romagnani, 2000). Hence, an increase in the concentration of IL-10 would lead to a decrease in the concentration of IL-2 and IFN- γ within sample. Furthermore, IL-10 has been found to play a significant role in

the self-renewal processes of HSCs (Kang et al., 2007). In Figure 10, we see that increased production of IL-10 did not lead to a reduced production of IFN- γ in all cases. In fact, within the iPSC/OP9 coculture sample, we found that an increased expression of IL-10 actually led to a comparatively increased level of IFN- γ and IL-2. In the iPSC/OP9 conditioned media coculture, we found that there was no expression of IFN- γ and IL-2, and less expression of IL-10 compared to the iPSC/OP9 conditioned media coculture. In our control OP9 Monoculture, we find that there is still moderate levels of expression of IFN- γ and IL-2, while there is an even lower level of IL-10 from the iPSC/OP9 conditioned media coculture.

This is an interesting, seemingly contradictory, result to what exists within the literature. Within an iPSC/OP9 coculture system, the upregulation of IFN- γ and IL-2 could indicate that IL-10 is interacting in a novel way with Th1 cytokines to upregulate them and promote hematopoiesis in the presence of iPSCs. It is also possible that due to cell-to-cell interactions between iPSCs and OP9s, the endogenous levels of Th1 are so high that IL-10 is not able to successfully downregulate the production of the Th1. This theory is qualified by understanding that there is no expression of IFN- γ and IL-2 when there is no cell-to-cell contact between iPSCs and OP9s, such as in the iPSC/OP9 conditioned media coculture. It is interesting to note, though, that in a control sample of OP9 monoculture, there is still a moderate level of expression of IFN- γ and IL-2. This result shows that OP9 cells are responsible for the endogenous production IFN- γ and IL-2, as when only OP9 conditioned media was used, there was no expression. As a whole, this result shows us that cell-to-cell interaction between iPSCs and OP9s are necessary in order to upregulate the expression of IFN- γ , IL-2, and IL-10. Absence of this cell-to-cell contact leads to

overall downregulation of the levels of all three of these cytokines. The exact mechanism of the interaction in between these proteins in an iPSC and OP9 coculture is an avenue of further investigation.

IL-3

IL-3 was another cytokine that was upregulated in the OP9/iPSC coculture but downregulated in the iPSC/OP9 conditioned media coculture, according to Figure 11. IL-3 is intimately involved in hematopoiesis and is an early acting cytokine. The fact that the OP9/iPSC coculture showed qualitative signs of differentiation through analysis of morphology, and the iPSC/OP9 conditioned media did not result in differentiation, indicates that IL-3 may be involved in the differentiation pathway between OP9 cells and iPSCs. The method by which IL-3 is upregulated or downregulated is still unclear, however. Our results seem to indicate that cell to cell contact between iPSCs and HSCs is necessary for this upregulation.

Other explanations are possible as well. It could be that the upregulation of another cytokine results in the upregulation of IL-3. This is less likely, as IL-3 has been shown to be an early acting cytokine. It is more plausible that IL-3 is upstream of other cytokines, such as GM-CSF, in a signalling pathway. Ultimately, the reasoning for our findings regarding IL-3's concentration in the differentiation of iPSCs is unclear and further research is needed.

IL-4

After analysis of Figure 12, we found that IL-4 was upregulated in the OP9/iPSC coculture and downregulated in the iPSC/OP9 conditioned media coculture

as compared to the OP9 monoculture. IL-4 has been identified as having a role in proliferating primitive hematopoietic progenitors in mice, which is consistent with our finding that it was upregulated in the culture that resulted in differentiation. In the literature, it has been shown that when IL-4 is combined with IL-11, major enhancement of colony formation was seen in mice.

We were not able to analyze IL-11 expression levels in this experiment, but if we had more time and resources, we would have. The expression levels of IL-4 and IL-11 could be compared in order to see how the two cytokines interacted with one another. Since IL-4 has shown to be ineffective in the absence of IL-11, it would be interesting to knockout IL-11 and see if IL-4 was still secreted. If IL-4 was still secreted, we would determine if differentiation was still achieved.

IL-5

Results for interleukin 5 indicate cytokine release within OP9 monoculture due to elevated expression levels. Therefore, this interleukin is secreted by OP9 cells independent of the presence of other cells. However, iPSC/OP9 conditioned media coculture displayed no expression of interleukin 5, with a measured concentration of 0 pg/mL. This indicates that an activated IL-5 is absent in the conditioned media, potentially due to denaturation of the protein or the need for activators secreted by OP9 cell. After analysis of Figure 13, iPSC/OP9 coculture showed upregulation of IL-5 with approximately three times as much of the cytokine present in the iPSC/OP9 coculture than the OP9 monoculture. Since IL-5 is involved in the growth and maturation of hematopoietic stem cells, its upregulation shows correspondence with the growing culture that was observed in the lab.

These findings express the idea that IL-5 requires direct cell contact with the presence of OP9 cells in coculture in order for the cytokine to be actively present. Therefore, IL-5 is not secreted pleiotropically in effecting iPSC cells, but rather with the presence of both iPSC and OP9 cells in the culture. This is further proven by the decrease in concentration of IL-5 in the conditioned media coculture compared with the OP9 monoculture, where the difference was the lack of OP9 cells within the culture. Another explanation for this finding could be that the OP9 cells are solely responsible for the secretion of the cytokine and thus, no proteins are found in the broth coculture. Ultimately, the conditioned media itself was not sufficient in providing this cytokine. This implies that the conditioned media coculture, lacking IL-5, may affect hematopoietic stem cells through a failure to regulate the development of progenitors. With the presence of OP9 in the iPSC coculture, release of IL-5 can provide an upregulated expression level of this cytokine and enhance the development of progenitors.

IL-6

The results of the experiment for interleukin 6 after analysis of Figure 14 indicate a specific level of cytokine release within OP9 monoculture, without any other cells present. Therefore, this interleukin is independently secreted by OP9 cells normally in culture and through regular processes. Conversely, iPSC/OP9 conditioned media coculture showed no expression of interleukin 6. The measured concentration of IL-6 within the conditioned media coculture was 0 pg/mL. The absence of IL-6 in the conditioned media could be due to denaturation of the protein within the solution, possibly because of a lack of secondary proteins secreted by OP9 that are necessary

for activation or proper functioning of the cytokine. The iPSC/OP9 coculture showed an upregulation of IL-6, similar to IL-5 in that approximately three times as much of the cytokine was present in the iPSC/OP9 coculture than the OP9 monoculture. The role of IL-6 is relatively uncertain within the hematopoietic process, but it is hypothesized that the survival and proliferation of progenitors depends on the presence of this cytokine.

Given that IL-6 is involved in the pro-inflammatory response and moreover in cellular processes that allow cells to defend themselves against toxicity and pathogens, the survival and proliferation of HSC's in the experimental culture is supported by the upregulation of IL-6 within iPSC/OP9 coculture. The lack of IL-6 in conditioned media coculture indicates that the conditioned media itself is not sufficient in providing IL-6 for developing HSC's, and therefore can cause these progenitors to fail in surviving through an inability to activate an immune response and sustain viability. Elevated levels of IL-6 within iPSC/OP9 coculture compared to OP9 monoculture indicate a positive feedback loop that regulates the secretion of IL-6 when iPSC are present. The presence of cellular contact is necessary, however, shown by a failure for pleiotropic secretion of functional IL-6 into conditioned media. Therefore, direct contact between OP9 cells and iPSC is necessary for IL-6 expression and binding to occur.

IL-7

According to the analysis of Figure 15, No endogenous expression of IL-7 was observed in OP9 monoculture, with a measured concentration of 0 pg/mL.

Furthermore, IL-7 was not measured at any concentration within the conditioned

media coculture either. Conversely, IL-7 expression levels within iPSC/OP9 coculture reached 5226 pg/mL, among the highest between all of the measured cytokines. Lack of IL-7 expression within OP9 monoculture indicates that the presence of iPSC are necessary in order to initiate the pathway involved in secreting the cytokine.

Furthermore, the absence of iPSC/OP9 conditioned media coculture indicates that regardless of the presence of iPSC, the fact that OP9 were absent indicates a required direct contact between the iPSC and OP9 in order for secretion to occur. Since deficiency of IL-7 can cause failure for HSCs to survive and proliferate in early stages, conditioned media coculture is expectedly due to lead to failure for induction and proliferation of HSC to occur.

The extreme amount of upregulation in the expression of IL-7, from no secretion to greater than 5000 pg/mL in OP9 monoculture and iPSC/OP9 coculture respectively indicates the importance of this cytokine in the development and survival of HSCs. This hematopoietic growth factor was measured to be secreted by OP9 cells only in the direct presence and contact of iPSC, during which the cytokine levels rose to an intense degree of expression. Direct cell signaling during the iPSC/OP9 coculture led to IL-7 secretion, which in turn allowed for this growth factor to further develop HSC. Since continued signaling between IL-7 and HSCs positively regulates the continued development of progenitors and differentiation into various cell types, significantly elevated expression levels of IL-7 can be due to the continuation of the hematopoietic process in which the progenitor is looking to further differentiation into a more specialized cell type such as a B lymphocyte or T lymphocyte.

TNF- α

Tumor necrosis factor alpha (TNF- α) is a cytokine involved in the proinflammatory process as well as the acute phase process. In addition to providing an anti-tumoral response, TNF alpha is involved in restricting hematopoietic activity. Uncontrolled differentiation of hematopoietic stem cells can increase the risk of tumorigenesis and leukemic transformation (Pronk et al., 2011), so TNF alpha helps control the differentiation process of HSCs. TNF alpha's repressive effects on the regulation of HSC proliferation is dependent on the presence and binding of both receptors, meaning that a deficiency or inhibition in either receptor can disrupt HSC homeostasis (Pronk et al., 2011).

In Figure 16, our results show the iPSC/OP9 coculture shows significant upregulation of TNF alpha. Comparatively, the OP9 secretion and iPSC/OP9 conditioned media coculture showed no secretion of TNF alpha. Our finding then, suggests that TNF alpha expression levels are significantly upregulated via direct cell to cell contact, and significantly downregulated to nonexistent levels via indirect cell to cell contact. This is contrary to what we expect given different findings in our literature review, so further investigation regarding the exact mechanism of TNF alpha in regulating hematopoiesis is needed.

IL-12p70

IL-12p70 is a cytokine that has mainly been implicated in the JAK-STAT pathway, which is used for T-cell differentiation, along with IL-23 and IL-27 (Gee et al., 2009). It has also been shown to play a very crucial role in the full reconstitution of blood cell lineages in subjects that have endured severe myelosuppression through

the usage of treatments such as chemotherapies (Chen et al., 2007). In Figure 17, we see that the expression of this cytokine is significantly upregulated in the iPSC/OP9 coculture. We also observed almost no expression of the protein in the iPSC/OP9 conditioned media coculture and in the control OP9 monoculture. Compared to the expression levels of the rest of the cytokines, IL-12p70 has a very low amount of expression in the iPSC/OP9 coculture system.

This finding is extremely relevant as it suggests that cell-to-cell contact is necessary for upregulation of the expression of this important hematopoietic agent. This is further alluded to by the fact that the iPSC/OP9 conditioned media coculture, which was absent of OP9 cells, showed no expression of the cytokine. In the control sample, OP9 cells showed no endogenous expression of the protein, which suggests that the iPSCs are necessary in order to begin the hematopoietic cascade and induce differentiation of a full lineage of blood cells. Examining the cellular mechanism by which this interleukin is upregulated in coculture is of great interest, as it will further elucidate which factors drive the process of hematopoietic differentiation.

IL-15

IL-15 is cytokine that has been heavily implicated in the regulation of T-cells, as well as in the early induction of hematopoiesis (Colpitts et al., 2013). In past experimentation, this cytokine's expression was shown to be extinguished during late lymphoid development but upregulated in the primitive HSCs themselves. Therefore, we would expect to see a high expression level of IL-15 in cells that are displaying the early characteristics of HSCs. In Figure 18, we see that the expression level of IL-15 is upregulated in the iPSC/OP9 coculture. Furthermore, it is shown to have no

expression in the iPSC/OP9 conditioned media coculture or the control OP9 monoculture.

The implications of these findings are significant, as they suggest not only that the iPSCs in the culture showed signs of differentiation into primitive HSCs, but that cell-to-cell contact between the iPSCs and OP9s is necessary in order to upregulate the expression of this cytokine. This implication is further proved by the fact that the iPSC/OP9 conditioned media coculture system displayed no upregulation, signaling that it is not the secreted proteins in the OP9 culture conditioned media that are driving hematopoiesis but rather some inter-cellular mechanisms that are playing a key role. The OP9 monoculture control sample also shows no endogenous level of IL-15 expression, which shows that it is the iPSCs that are necessary in order to begin the upregulation process. Further investigation into the inter-cellular interactions of this cytokine in the iPSC/OP9 coculture are needed to elucidate which factors are responsible for inducing differentiation of iPSCs into HSCs.

IL-17A

IL-17A is a proinflammatory cytokine that has been shown to activate T-cells within the immune system of humans. The chronic upregulation of this cytokine is known to have a role in multiple disease pathologies, such as rheumatoid arthritis and multiple sclerosis. As a member of several other IL-17 proteins, the functioning of IL-17A is most well understood (Jin & Dong, 2013). Significantly, this cytokine is known to have a role in some steps of hematopoiesis, such as granulopoiesis. After inoculation of IL-17A into a culture system, a significantly greater amount of

erythroid progenitors and mesenchymal cells were recruited as compared to control cells (Kristic, 2012).

According to our results from Figure 19, there was a significant upregulation of IL-17A within the iPSC/OP9 coculture. This indicates that cell-to-cell contact may be necessary for the recruitment of this cytokine. Expression levels from the iPSC/OP9 conditioned media coculture sample and the OP9 monoculture sample seem to qualify this conclusion, as they showed significantly less expression when both types of cells were physically not present in the cells. Because these results are only correlational, we cannot assess whether this cytokine is a probable cause of hematopoietic differentiation. Further research must be done to investigate the protein-protein interaction of this cytokine within a coculturing system such as an iPSC/OP9 coculture.

IL-21

In Figure 20, we found that IL-21 was not found in the OP9 monoculture but expressed in the iPSC/OP9 coculture. This indicates that cell-to-cell contact may be necessary to secrete IL-21 since it was not found in the OP9 monoculture or conditioned media but was seen once iPSCs were introduced. Furthermore, there was a higher concentration of IL-21 found in the iPSC/OP9 coculture than there was in the iPSC/OP9 conditioned media coculture. This indicates that cell-to-cell contact upregulates the production of IL-21 in iPSCs.

It is already known that IL-21 plays a critical role in the generation of T cell inflammatory cells. Our results indicate that it may also play a role in iPSC differentiation into HSCs. Since our study was correlational in design, we cannot

determine if this cytokine is necessary for differentiation. IL-21 may be downstream of another cytokine that is responsible for the differentiation, and thus, may play no tangible role in the differentiation itself. Further research is needed to determine IL-21's overall importance in iPSC differentiation.

IL-23, p19

IL-23, p19 is another cytokine that had a higher concentration in the OP9/iPSC coculture than the OP9 monoculture and no concentration at all in the iPSC/OP9 conditioned media coculture. This cytokine is involved with the proliferation of mouse T cells, and so its importance in iPSC differentiation is not very clear. The concentrations that we found in our experiments after analysis of Figure 21 may simply be coincidental, due to error, or because IL-23 may have a downstream relationship with other cytokines that were found in the cultures.

Further research is needed to elucidate IL-23, p19's importance in the iPSC differentiation to HSCs. As with the other cytokines in the culture that have similar graphs, it is unclear as to whether IL-23, p19 is critical to the differentiation of iPSCs or if the increased concentration in the OP9/iPSC coculture is merely a byproduct of other cytokines which are responsible for the differentiation.

Conclusion

In our work, we observed the iPSC/OP9 coculture resulting in morphological differentiation of iPSCs while the iPSC/OP9 conditioned media not resulting in morphological differentiation. This suggests that cell to cell contact could be necessary in order to induce differentiation. Furthermore, all of the cytokines that we analyzed except for IL-10 and IL-21 had a concentration of 0 in the iPSC/OP9 conditioned media coculture. This could indicate that the iPSCs took up the proteins that were in the conditioned media; with no OP9 cells in the culture to replenish the concentration of these cytokines, the concentration that we found was 0. In other words, the cytokines could have been continuously secreted by only the OP9 cells. Another explanation for this could be that the cell to cell contact of the OP9 and iPSC cells was necessary for the production or upregulation of the cytokines.

Our results are mostly consistent with the literature. We are the first group to directly test how cell to cell contact may affect the expression of these cytokines together, though some studies have been done on a few of the cytokines that were analyzed in our project. One finding of ours that was not entirely consistent with the literature was the fact that TNF- α and IFN- γ were upregulated in the iPSC/OP9 coculture. While the literature indicates that cell to cell contact may be unnecessary for TNF- α , we found that such contact resulted in significant upregulation of the cytokine. This finding could be because cell to cell contact may be unnecessary for TNF- α but still cause its upregulation. Such a finding would suggest that cell to cell contact can expedite TNF- α production even if such contact is unnecessary to merely yield a concentration of TNF- α .

Our research ultimately consisted of two different parts: the study may be causal when it came to the question of whether cell to cell contact was necessary for differentiation and it was correlational when it came to the question of which cytokines affect iPSC differentiation into HSCs. Thus, two different conclusions were yielded from this investigation. We conclude by suggesting that that cell to cell contact may be necessary for the observed morphological differentiation of iPSCs, while the upregulation of the cytokines that we studied was correlated with iPSC differentiation. However, more research on this topic should be done in the future to confirm our results as we only had one trial for our experiment. Furthermore, we determined that differentiation occurred through a morphological assessment; if we had more resources, we could have investigated if the HSCs truly did exhibit the CD34+ marker that is characteristic of blood cells. Further investigations must be done in order to determine if upregulation of certain cytokines directly causes iPSC differentiation.

Future Directions

If we had more time, we would have taken the cytokines that were upregulated in the OP9/iPSC coculture as compared to the OP9 monoculture or the iPSC/OP9 conditioned media coculture and seen if they were the cause of differentiation by creating cytokine knockout cultures. For example, we would have knocked out IL-21, a cytokine that was expressed at much higher levels in the OP9/iPSC coculture than in the other cocultures and seen if differentiation still occurs when OP9 cells and iPSCs are cultured together. This would move the experiment away from a correlational

study into a causal one and such a study would be more helpful in understanding the pathway of differentiation from iPSCs to HSCs.

After investigating knockout cultures, one could then introduce various cytokines to the culture to see if the rate of differentiation changes. If it is found, for example, that IL-21 is indeed necessary for differentiation, researchers can then investigate if IL-21 is sufficient for differentiation, if adding IL-21 to the culture causes differentiation to happen at a faster rate, and if there is an optimal concentration of IL-21 for inducing differentiation. This type of analysis can be done for any cytokine that is found to be involved in the differentiation pathway. One could also simply add the human recombinant protein to the media to see if differentiation occurs.

Ultimately, this research could be applied clinically. If the protocol for inducing hematopoietic differentiation continues to become optimized, researchers could see if adding certain cytokines in vivo along with iPSCs would be medically viable for those who are looking for blood donations (such as leukemia patients or even gunshot wound victims). The implications of this research are immense – blood donations could become a thing of the past, deaths could be prevented – however, there is still a great amount of research that stands in the way. After our study, it is still unclear as to what cytokines exactly are responsible for differentiation. Through our work, however, we have a clearer picture of how the differentiation process may work and a better framework for understanding this cellular process.

Appendices

Appendix A – Additional Protein Expression Analysis Methods

Protein mass spectrometry is another method that may be used to determine the identity of a protein through analysis of the unique properties. There are two major types of protein mass spectrometry: electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). Mass spectrometry involves ionization of the solid protein particles in an isolated chamber followed by subsequent injection into an accelerated electric or magnetic field for detailed inspection. ESI involves formation of an aerosol from a liquid solution using high voltage energy, thus creating ions from larger macromolecules. MALDI, on the other hand, involves the embedding of proteins into a solid matrix followed by formation of ions using laser pulsar light (Tanaka et al, 1988). The final result of these experiments is a spectra that can be analyzed for fractionation patterns and specific mass analysis ratios, which is quantified as mass divided by the charge number of ions (m/z). Peptide mass sequencing is a method in which the fragmented peptides are analyzed for molecular composition and then entered into a database in order to determine the identity of the original unknown protein.

A successful protein analysis is defined as one in which you may observe at least 70% of the protein sequence. Possible reasons why a protein may not reveal completely are as follows: inability to completely digest with proteinase, protein is too large to fragment properly, or partial fragmentation of the protein which may lead to data that cannot be interpreted. In general, mass spectrometry is able to characterize, identify, and quantify the properties of unknown proteins through

ionization and fragmentation. However, due to the fact that cell culturing samples generally contain large quantities of a variety of proteins, it is difficult to use mass spectrometry because obtaining a spectra for just one individual protein that can be fragmented and interpreted is cumbersome.

Western blots, also called immunoblotting, are often used in scientific research to detect the presence of proteins given a specific antibody. In this process, the cells are lysed and proteins are then separated by SDS-PAGE. The proteins are then transferred to a nitrocellulose sheet, a solid membrane, and then immuno-stained with labeled antibodies. These antibodies are specific for a certain protein, and therefore used to probe for their presence. Finally, antigen bands are visualized through autoradiography (Centers for Disease Control, 1989). The antibodies used may be either primary or secondary antibodies. Research often uses a combination of primary and secondary antibodies, with the primary antibodies binding to the protein of interest and the secondary antibodies specific to the host type of the primary antibody. There are two methods of detection: direct and indirect. In a direct antibody based Western Blot, the enzyme-conjugated primary antibody binds directly to the antigen of interest. On the other hand, in an indirect antibody based Western blot, the primary antibody is unlabeled and binds to the antigen, and is then in turn bound by the secondary antibody. The secondary antibody in this situation is labeled with an enzyme that generates a detectable signal, such as fluorescence, that can be quantified through flow cytometry or another functional type machine. For the purpose of our experimentation, utilization on this method will only allow us to detect the presence of proteins and not the quantity.

A mechanism of detecting proteins related to DNA microarrays is the high-throughput mechanism shown by proteins microarrays, which track large numbers of proteins in parallel on a chip (Reymond et al., 2013). This assay involves immobilizing an array of proteins on a solid matrix and then capturing with labeled antibodies. This method is extremely useful for detecting protein-protein interactions. For the purpose of our experimentation, analysis of the interactions of the proteins that are secreted by OP9 coculture would not be useful in determining which proteins are up-regulated or down-regulated within the culture. In other words, there would be no way to actually quantify how many of each type of protein are within the culturing system. Hence, a protein microarray would be very useful in further study to test the protein-protein interactions of those proteins that are found to be most heavily involved in induction of hematopoiesis within the iPSC-OP9 coculture system.

Appendix B – Justification for Methods & Reagents Used in Methodology

There are many options that were considered when determining the best methodology for growing iPSCs. These various methods include, but are not limited to, growing cells in a flask or growing cells in multiple-well dishes. Compared to growing iPSCs within a flask, growth within 6 well plates proved more accessible and easy to use. Distribution of the stem cells among multiple wells, as opposed to collectively growing them in a single flask, decreases the risk of total contamination of entire cell populations since the contamination of a single well does not affect other healthy wells on the plate (Sanadi, 1996). Moreover, if one colony in a well within the plate displays abnormal differentiation, the affected colony may be removed and allow for continued use of the otherwise healthy well. Single-cell culture passages

grown in flasks requires trypsinization, which can lead to abnormal karyotypes and therefore harm healthy cells during the differentiation process (Beers et al., 2012). This process also involves copious amounts of pipetting, leading to further agitation to cells and potentially disruption of colonies (Lerou et al., 2008).

During regular cell culture practices with iPSCs, cells are often individualized during passaging to achieve an even distribution. However, a challenge that iPSCs face is the fact that these cells survive poorly after individualization, since they are more sensitive to treatments and are prone to cell death (Beers et al., 2012). iPSCs need to be individualized, however abnormal karyotypes often leads to poor survival rates (Ellerstrom et al., 2007). Cell survival is therefore a priority and in most experiments, and so dissociation methods are chosen based on either cell survival or sensitivity. iPSCs have been experimented and passaged as aggregates with enzymatic dissociation, and many reagents are used in this general cell culture practice to achieve maximal viability. One of these reagents involves the Rho-associated protein kinase (ROCK) inhibitors, which are used to boost cell survival during this process. ROCK inhibitor has been used in other studies to demonstrate decrease in dissociation-induced apoptosis, increase in cloning efficiency, and protection from apoptosis (Watanabe et al., 2007). ROCK inhibitor is therefore essential in our methodology to ensure iPSC survivability, and has been included in all experimentation.

For dissociation of cells from the well plate in which they reside, trypsin is commonly used. This is generally done when cells have reached confluency and it becomes necessary to expand to another culture. Trypsin has been closely studied to detach cell range from 0.05% to 0.5% with incubation times ranging from 5 to 10

minutes at room or physiological temperature, as well as to serve a critical role in cell attachment and spreading (Brown, 2007). Usage of trypsin for cell detachment has thus been the standard practice for many years. In our experimentation, trypsinization protocol was used while working with OP9 cells alone or while working with the iPSC-OP9 coculture.

While trypsinization is useful for completely dissociating cells from a well plate, it is very non-selective in the cells that it removes. The repeated usage of trypsin has also been shown shorten the life of cells such as iPSCs (Beers et al., 2012). For our iPSC culture in particular, we found it more appropriate to use EDTA dissociation solution. EDTA, or edetic acid, is a chelating agent that is capable of sequestering polyvalent cations such as calcium (Beers et al., 2012). In terms of our iPSCs, using EDTA dissociation instead of trypsin-based dissociation allows us to selectively detach any cell from the culture that is not an iPSC. Thus, incubation of EDTA in the cell culture and subsequent aspiration allows for the removal of any non-iPSCs from the culture (Beers et al., 2012). It was shown that the iPSC cells themselves only partially detach after treatment, and can be fully removed after addition of fresh media (Beers et al., 2012).

Another component that must be controlled during experimentation is the media that is used for cellular growth and proliferation of the induced pluripotent stem cells. The normal media that is used to grow pluripotent stem cells is known as culture medium, and contains many exogenous factors composed of a slew of proteins. Because our protocol to quantifies the presence and expression of interleukins, we aim to minimize the amount of exogenous proteins that are detected in our conditioned media.

To circumvent this limitation, we will be using a specialized pluripotent cell growth media for our iPSCs known as Essential 8 (E8) Flex Medium. There are two major benefits of using this media during experimentation; 1) E8 Flex Medium does not contain bovine serum albumin (BSA) and human serum albumin (HSA) which helps to reduce the amount of variability in batches and improve upon the long-term proliferative health of stem cells and 2) E8 Flex Medium eliminates the need to change media on a daily basis which is crucial to our lab work as we are required to commute to our lab from campus (Essential, 2017). After investigating into the current literature of iPSC culture protocols, we have confirmed the benefits and requirement of these essential reagents that will be used in our methodology.

Appendix C – Additional Preparation Protocol in Methodology

Preparation of OP9 Growth Media

OP9 growth media was prepared according to the follow table below. Media was stored at 4 °C for up to 1 month.

Product	Stock	Volume (mL)	Final Concentration
aMEM		195 mL	78%
FBS (OP9-Tested)	100%	50 mL	20%
L-glutamine	100X (200mM)	2.5 mL	1X (2mM)
Pen-strep antibiotic	100X (10,000 U/mL)	2.5 mL	1X (100 U/mL)

Total		250 mL	
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Preparation of Matrigel-Coated Plates for iPSC Culture

For preparation, pipettes, tubes and plates were precooled at 4°C. 1 mL of 50% Matrigel, diluted with DMEM/F-12, was aliquoted into the pre-prepared conical tubes. Tubes were stored at -20 °C. A 50% Matrigel aliquot was mixed with DMEM/F-12 to create a final Matrigel concentration of 1.25%. ~1.25 ml was dispensed to each well of 6-well plates. Well plates were incubated overnight at 4°C. Matrigel was aspirated from the well prior to plating the iPSC colonies or cells. Media was added quickly to prevent the cells from drying out and the colony or cell suspension was plated in appropriate medium.

Preparation of Complete Essential 8 Medium

Essential 8 Supplement (50X) was thawed at room temperature for ~1 hour. 500 ml of complete Essential 8 Medium was prepared by mixing 490 ml Essential 8 Basal Medium and 10 ml Essential 8 Supplement (50X). Before use, medium required for that day was warmed at room temperature until it was no longer cool to the touch. E8 media is not warmed in a water bath to prevent denaturation of the essential proteins in the media.

Preparation of EDTA Solution

EDTA is utilized to dissociate the iPSCs in order to transfer them to other flasks. 500 µl of 0.5 M EDTA (pH 8.0) was added to to 500 ml of calcium/magnesium-free PBS, pH 8.0 and the solution was filtered and stored at 4°C for up to 6 months. During

passaging of iPSCs, EDTA solution is used to rinse the well plate and to detach the iPSCs. The final concentration was .5 mM EDTA in calcium/magnesium-free PBS, pH 8.0.

Preparation of ROCK Inhibitor

A 10 mM stock solution of ROCK inhibitor was prepared by dissolving ROCK inhibitor in DMSO. This stock was aliquoted and stored at -80°C for up to a year. ROCK inhibitor was added to E8 media both in the use of thawing and freezing iPSCs. 1 µl of ROCK inhibitor stock is added to each 1 ml media used for a final concentration of 10 µM.

Appendix D: Standard Curves for Cytokines

Standard Cytokine Data

G-CSF								
Concentration (pg/ml)	0	27	82	247	741	2222	6667	20000
Signal-BKG	0	5	603	898	3888	5388	22777	53294
Log (Concentration)		1.44	1.92	2.39	2.87	3.35	3.82	4.30
Log (Signal-BKG)		0.65	2.78	2.95	3.59	3.73	4.36	4.73

	Slope	Intercept
Linear regression	2.74	0.00
log-log regression	1.21	-0.21

GM-CSF								
Concentration (pg/ml)	0	5	16	49	148	444	1333	4000
Signal-BKG	0	68	57	54	48	37	176	492
Log (Concentration)		0.74	1.22	1.69	2.17	2.65	3.12	3.60
Log (Signal-BKG)		1.83	1.75	1.73	1.68	1.57	2.25	2.69

	Slope	Intercept
Linear regression	0.12	0.00
log-log regression	0.25	1.38

IL-1a								
Concentration (pg/ml)	0	3	8	25	74	222	667	2000
Signal-BKG	87	1	347	76	768	193	9707	63
Log (Concentration)		0.44	0.92	1.39	1.87	2.35	2.82	3.30
Log (Signal-BKG)		0.00	2.54	1.88	2.89	2.29	3.99	1.80

	Slope	Intercept
Linear regression	1.49	0.00
log-log regression	0.65	0.98

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IL-2								
Concentration (pp/ml)	0	3	8	25	74	222	667	2000
Signal-BKG	14	1	3	90	13	187	335	1047
Log (Concentration)		0.44	0.92	1.39	1.87	2.35	2.82	3.30
Log (Signal-BKG)		0.00	0.51	1.95	1.11	2.27	2.53	3.02
		Slope			Intercept			
Linear regression		0.52			0.00			
log-log regression		1.00			-0.25			

IL-3								
Concentration (pp/ml)	0	3	8	25	74	222	667	2000
Signal-BKG	0	194	421	1068	2762	6877	17218	45222
Log (Concentration)		0.44	0.92	1.39	1.87	2.35	2.82	3.30
Log (Signal-BKG)		2.29	2.62	3.03	3.44	3.84	4.24	4.66
		Slope			Intercept			
Linear regression		23.04			0.00			
log-log regression		0.83			1.89			

IL-4								
Concentration (pp/ml)	0	1	4	12	37	111	333	1000
Signal-BKG	0	6	42	38	94	358	799	1890
Log (Concentration)		0.14	0.61	1.09	1.57	2.05	2.52	3.00
Log (Signal-BKG)		0.74	1.63	1.58	1.97	2.55	2.90	3.28
		Slope			Intercept			
Linear regression		1.96			0.00			
log-log regression		0.83			0.79			

IL-5								
Concentration (pp/ml)	0	14	41	123	370	1111	3333	10000
Signal-BKG	0	120	8	61	207	507	1048	2283
Log (Concentration)		1.14	1.61	2.09	2.57	3.05	3.52	4.00
Log (Signal-BKG)		2.08	0.92	1.78	2.32	2.70	3.02	3.36
		Slope			Intercept			
Linear regression		0.24			0.00			
log-log regression		0.67			0.59			

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IL-6								
Concentration (pg/ml)	0	5	16	49	148	444	1333	4000
Signal-BKG	0	91	36	54	111	509	738	1320
Log (Concentration)		0.74	1.22	1.69	2.17	2.65	3.12	3.60
Log (Signal-BKG)		1.96	1.56	1.73	2.05	2.71	2.87	3.12
		Slope			Intercept			
Linear regression		0.36			0.00			
log-log regression		0.53			1.14			

IL-7								
Concentration (pg/ml)	0	55	165	494	1481	4444	13333	40000
Signal-BKG	0	85	61	119	133	206	593	1758
Log (Concentration)		1.74	2.22	2.69	3.17	3.65	4.12	4.60
Log (Signal-BKG)		1.93	1.78	2.08	2.12	2.31	2.77	3.24
		Slope			Intercept			
Linear regression		0.04			0.00			
log-log regression		0.46			0.86			

IL-9								
Concentration (pg/ml)	0	55	165	494	1481	4444	13333	40000
Signal-BKG	162	115	114	144	1	85	132	473
Log (Concentration)		1.74	2.22	2.69	3.17	3.65	4.12	4.60
Log (Signal-BKG)		2.06	2.06	2.16	0.00	1.93	2.12	2.67
		Slope			Intercept			
Linear regression		0.01			0.00			
log-log regression		0.13			1.44			

IL-10								
Concentration (pg/ml)	0	5	16	49	148	444	1333	4000
Signal-BKG	268	391	1	347	237	519	1258	4857
Log (Concentration)		0.74	1.22	1.69	2.17	2.65	3.12	3.60
Log (Signal-BKG)		2.59	0.00	2.54	2.37	2.72	3.10	3.69
		Slope			Intercept			
Linear regression		1.19			0.00			
log-log regression		0.72			0.86			

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IL-12p70								
Concentration (pg/ml)	0	5	16	49	148	444	1333	4000
Signal-BKG	0	13	52	275	131	1330	3299	9522
Log (Concentration)		0.74	1.22	1.69	2.17	2.65	3.12	3.60
Log (Signal-BKG)		1.12	1.72	2.44	2.12	3.12	3.52	3.98
		Slope			Intercept			
Linear regression		2.40			0.00			
log-log regression		0.96			0.49			

IL-13								
Concentration (pg/ml)	0	55	165	494	1481	4444	13333	40000
Signal-BKG	48	1	44	44	255	234	412	760
Log (Concentration)		1.74	2.22	2.69	3.17	3.65	4.12	4.60
Log (Signal-BKG)		0.00	1.65	1.64	2.41	2.37	2.61	2.88
		Slope			Intercept			
Linear regression		0.02			0.00			
log-log regression		0.85			-0.75			

IL-15								
Concentration (pg/ml)	0	137	412	1235	3704	11111	33333	100000
Signal-BKG	9	6	1	12	42	144	430	553
Log (Concentration)		2.14	2.61	3.09	3.57	4.05	4.52	5.00
Log (Signal-BKG)		0.77	0.00	1.06	1.63	2.16	2.63	2.74
		Slope			Intercept			
Linear regression		0.01			0.00			
log-log regression		0.92			-1.71			

IL-17								
Concentration (pg/ml)	0	5	16	49	148	444	1333	4000
Signal-BKG	0	6	49	282	398	1293	3322	11338
Log (Concentration)		0.74	1.22	1.69	2.17	2.65	3.12	3.60
Log (Signal-BKG)		0.76	1.69	2.45	2.60	3.11	3.52	4.05
		Slope			Intercept			
Linear regression		2.80			0.00			
log-log regression		1.06			0.29			

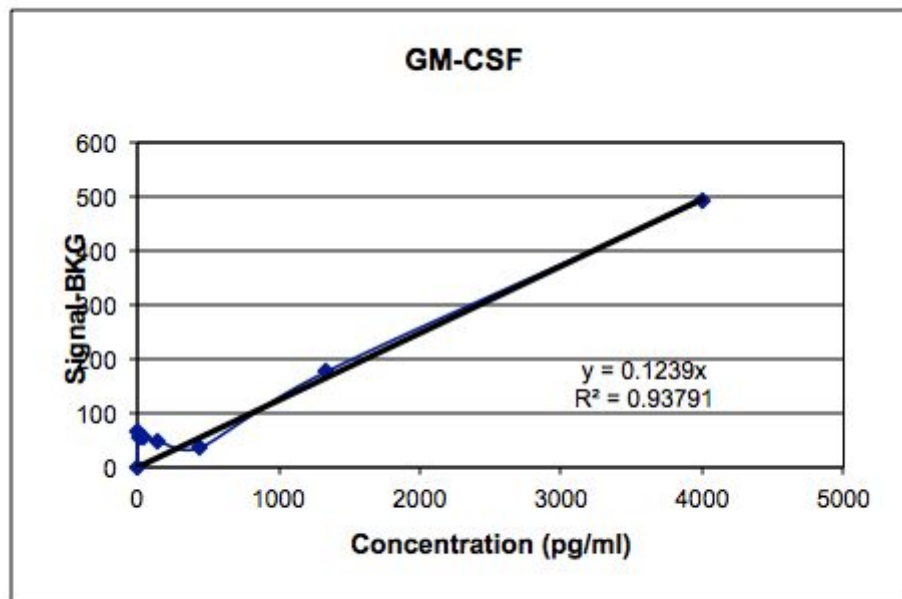
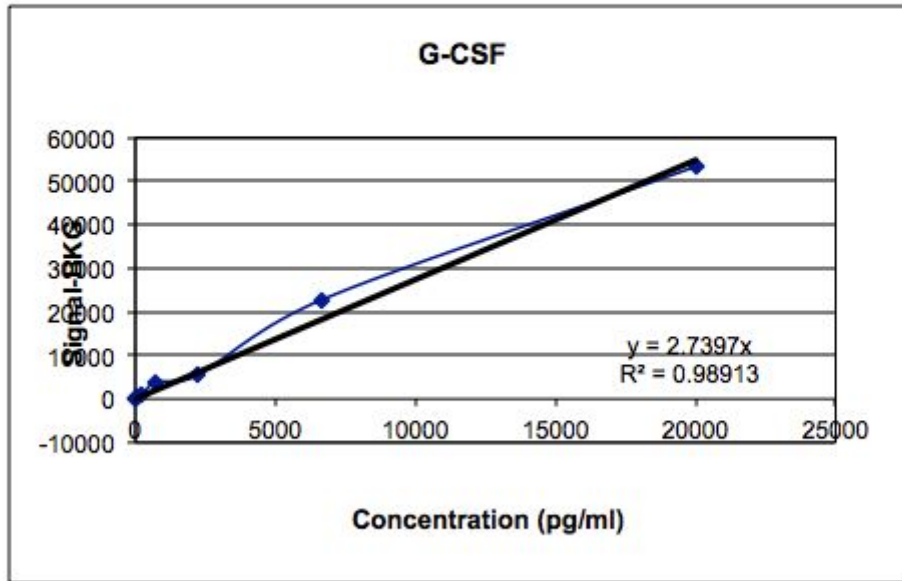
IL-21								
Concentration (pg/ml)	0	27	82	247	741	2222	6667	20000
Signal-BKG	0	86	105	195	586	1489	2740	4577
Log (Concentration)		1.44	1.92	2.39	2.87	3.35	3.82	4.30
Log (Signal-BKG)		1.94	2.02	2.29	2.77	3.17	3.44	3.66
		Slope			Intercept			
Linear regression		0.25			0.00			
log-log regression		0.67			0.85			

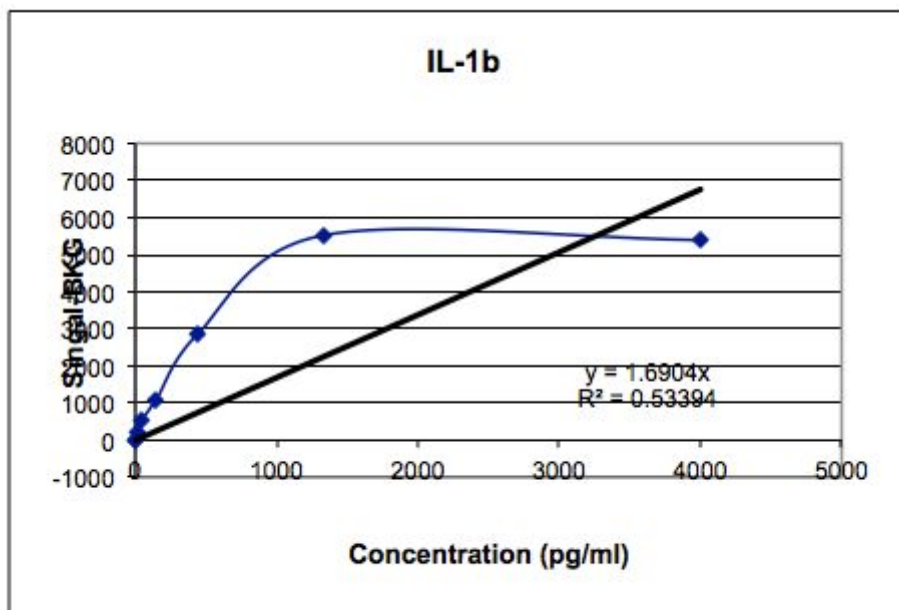
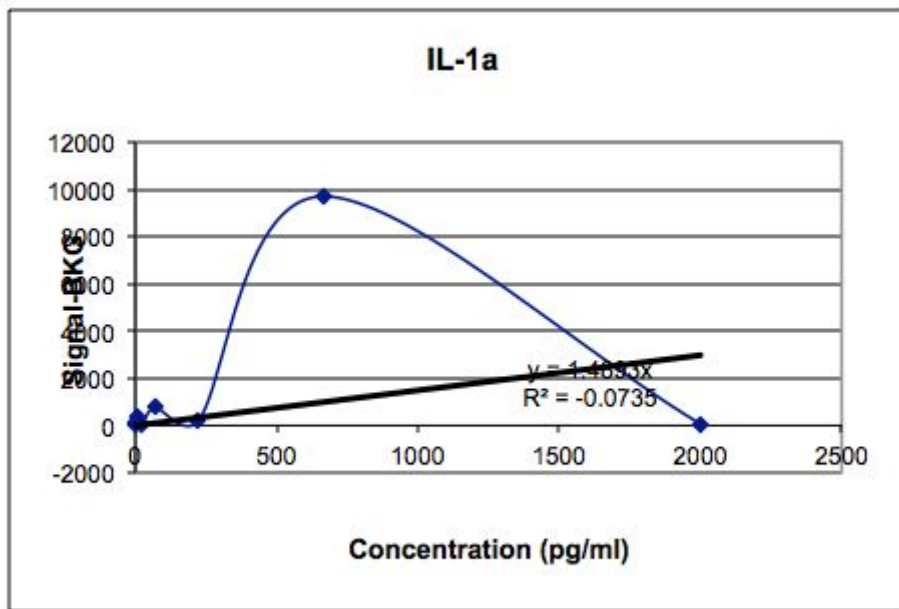
IL-23								
Concentration (pg/ml)	0	55	165	494	1481	4444	13333	40000
Signal-BKG	0	121	156	262	417	1402	4242	10172
Log (Concentration)		1.74	2.22	2.69	3.17	3.65	4.12	4.60
Log (Signal-BKG)		2.08	2.19	2.42	2.62	3.15	3.63	4.01
		Slope			Intercept			
Linear regression		0.26			0.00			
log-log regression		0.70			0.65			

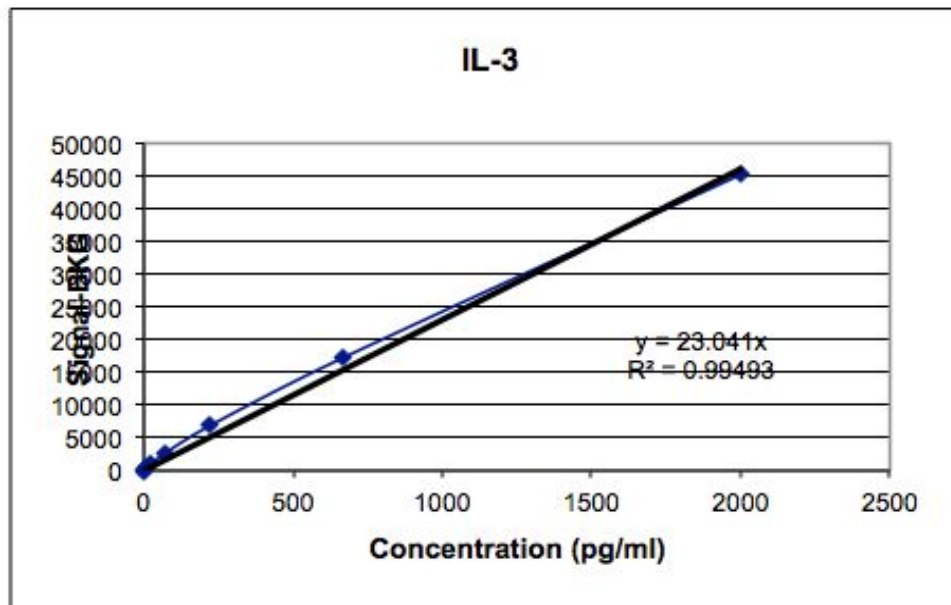
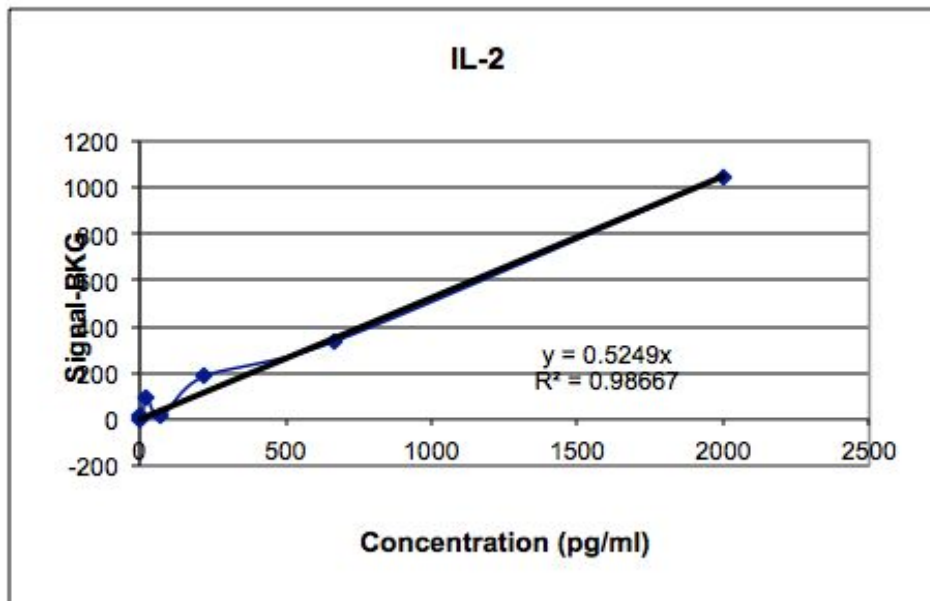
IFNg								
Concentration (pg/ml)	0	11	33	99	296	889	2667	8000
Signal-BKG	13	1	130	158	223	286	694	1235
Log (Concentration)		1.04	1.52	1.99	2.47	2.95	3.43	3.90
Log (Signal-BKG)		0.00	2.11	2.20	2.35	2.46	2.84	3.09
		Slope			Intercept			
Linear regression		0.17			0.00			
log-log regression		0.82			0.12			

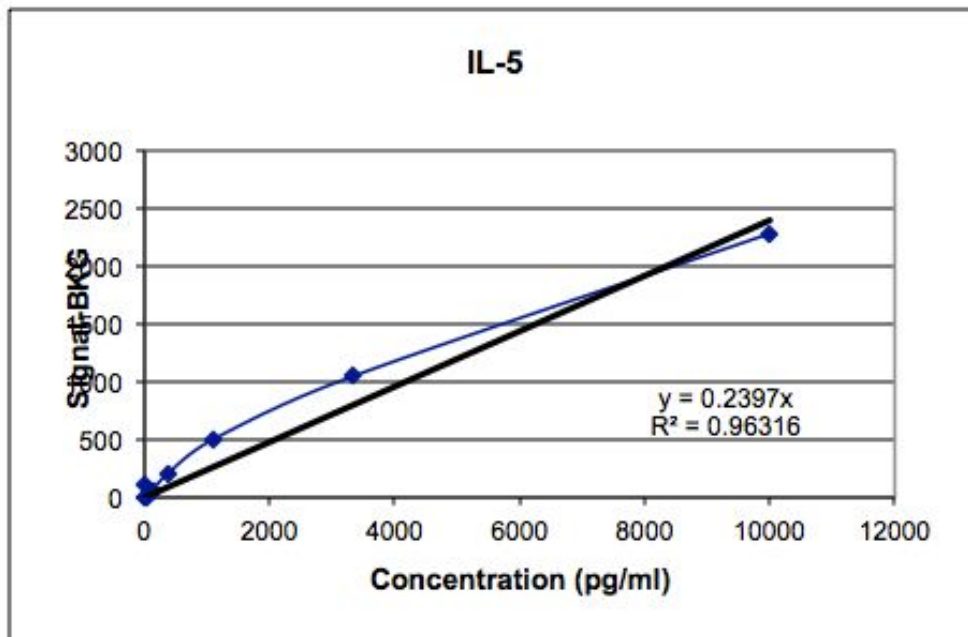
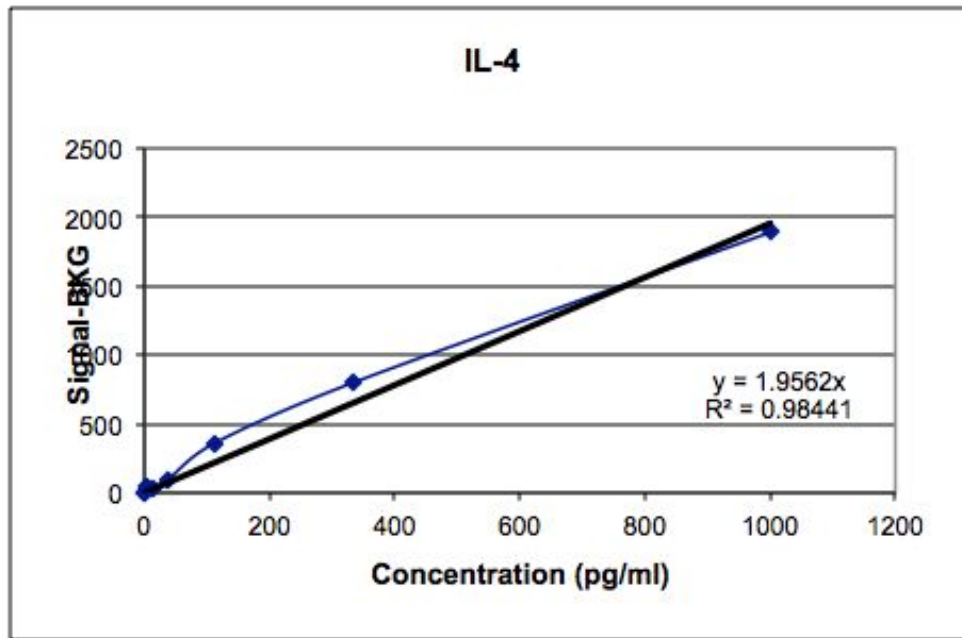
TNF α								
Concentration (pg/ml)	0	3	8	25	74	222	667	2000
Signal-BKG	20	26	1	43	127	234	1228	4747
Log (Concentration)		0.44	0.92	1.39	1.87	2.35	2.82	3.30
Log (Signal-BKG)		1.41	0.00	1.63	2.10	2.37	3.09	3.68
		Slope			Intercept			
Linear regression		2.31			0.00			
log-log regression		1.03			0.12			

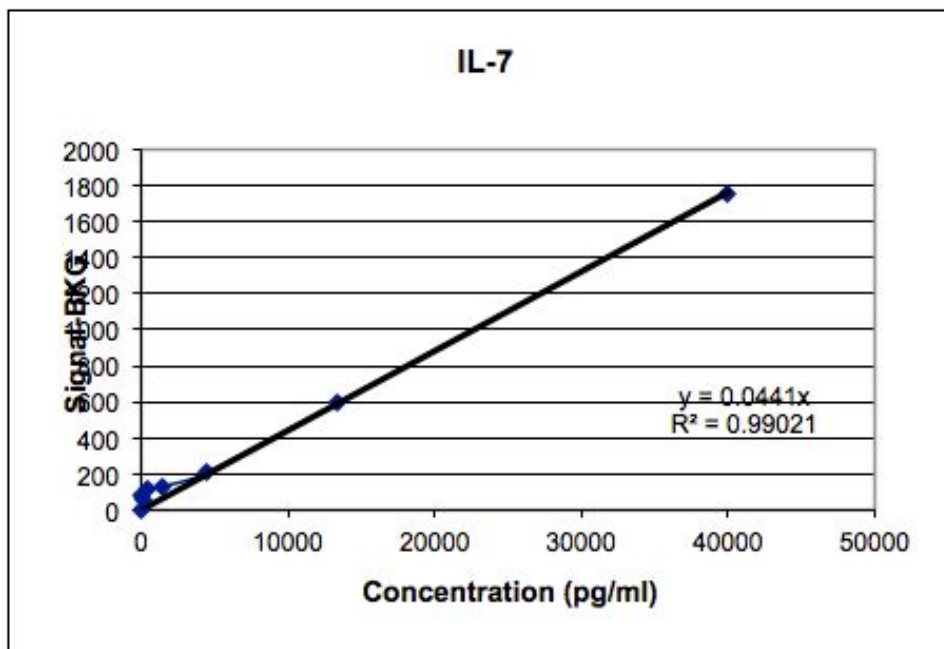
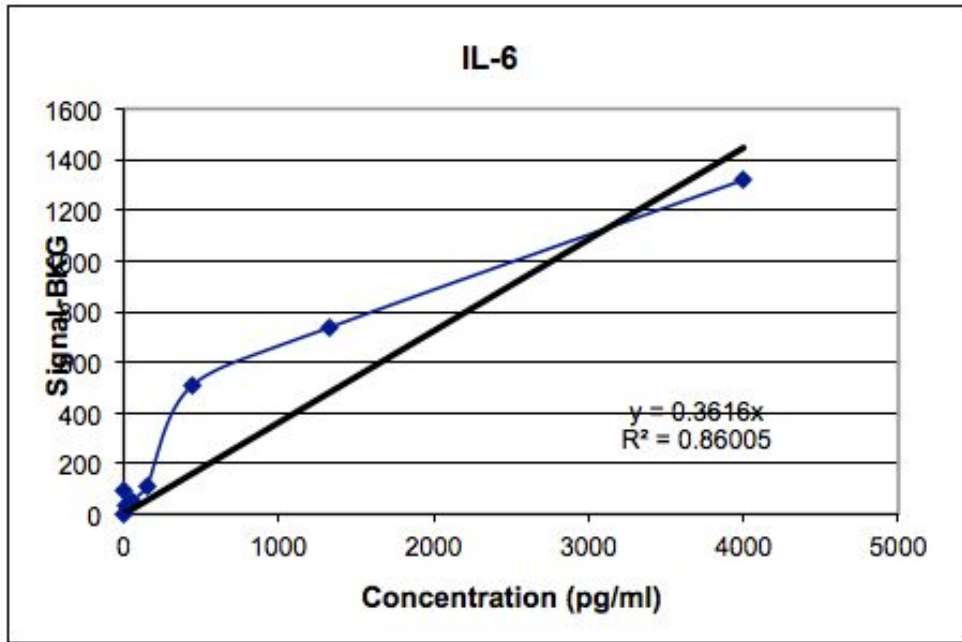
Linear Regression Curve

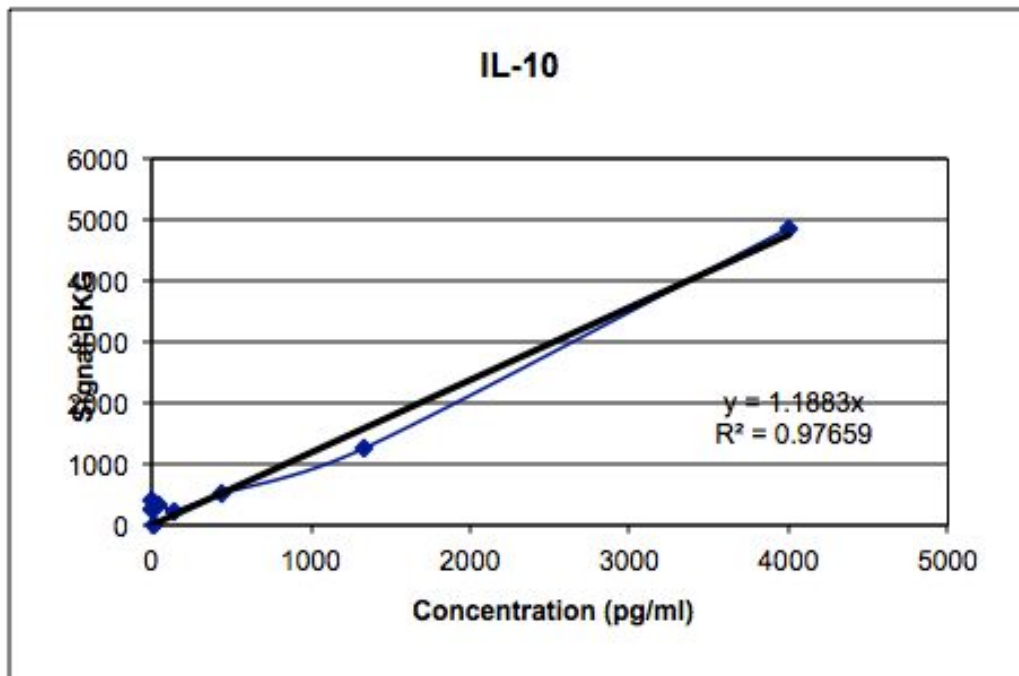
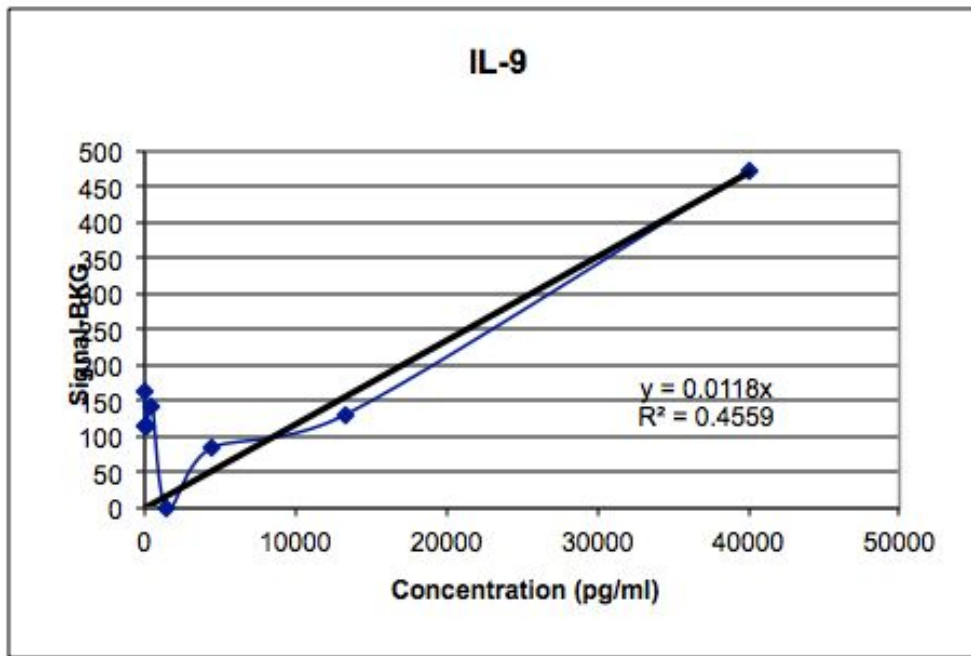


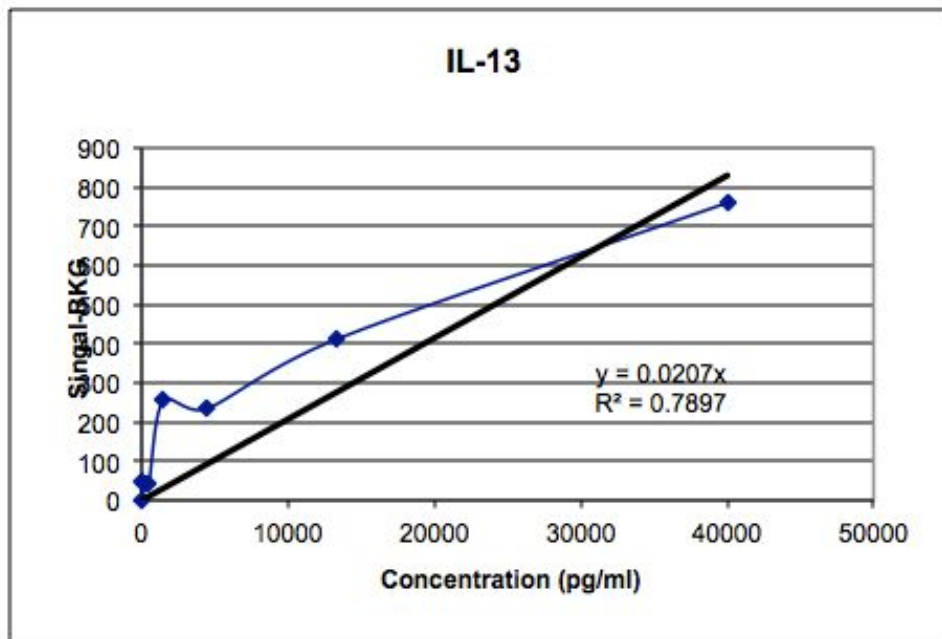
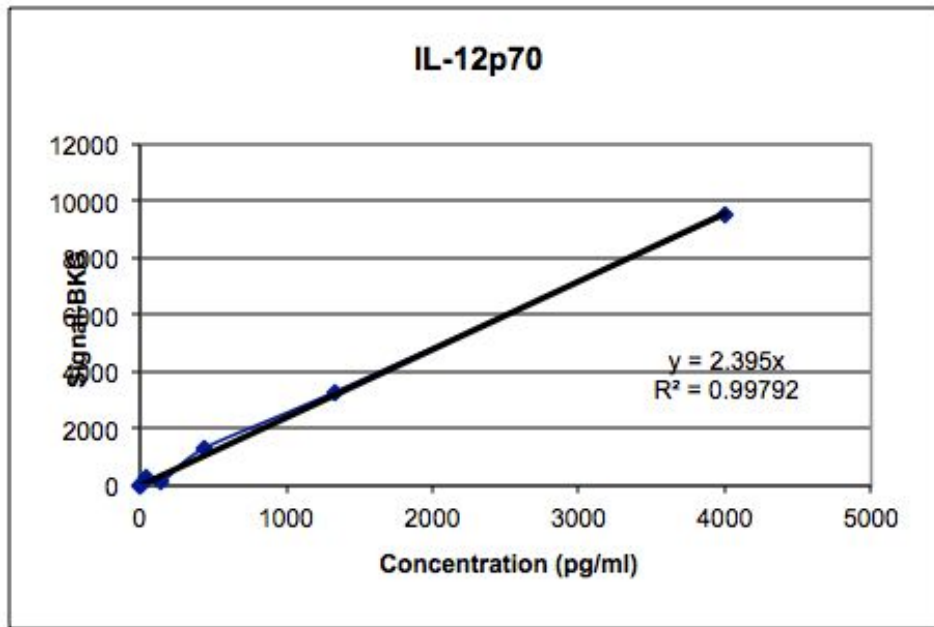


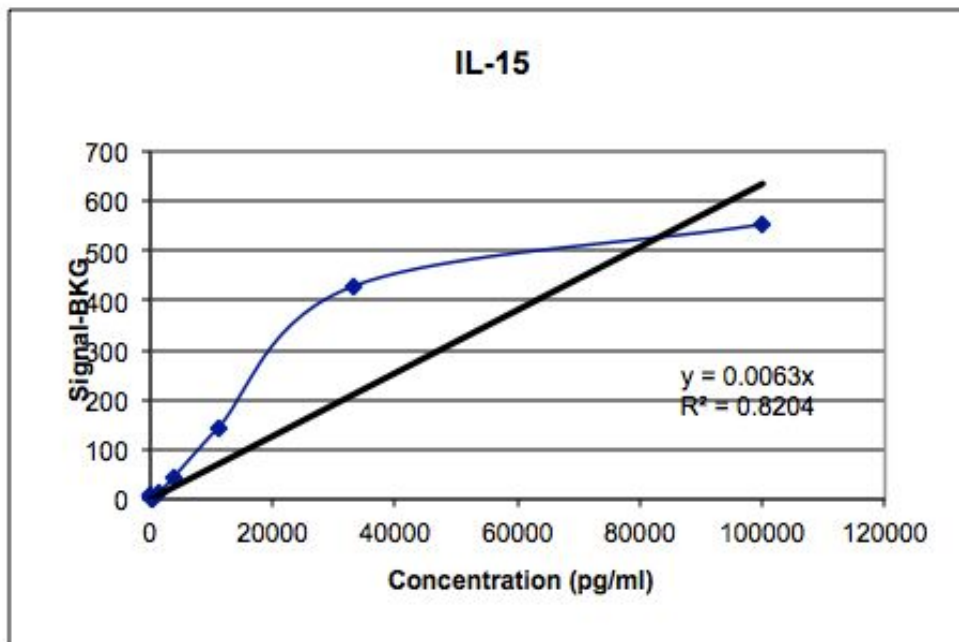
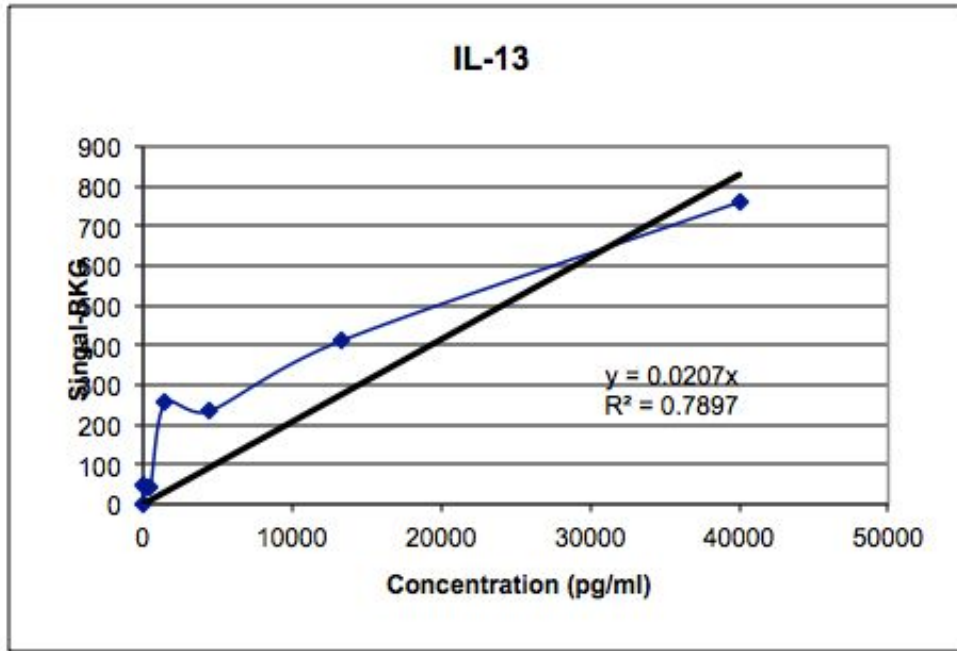


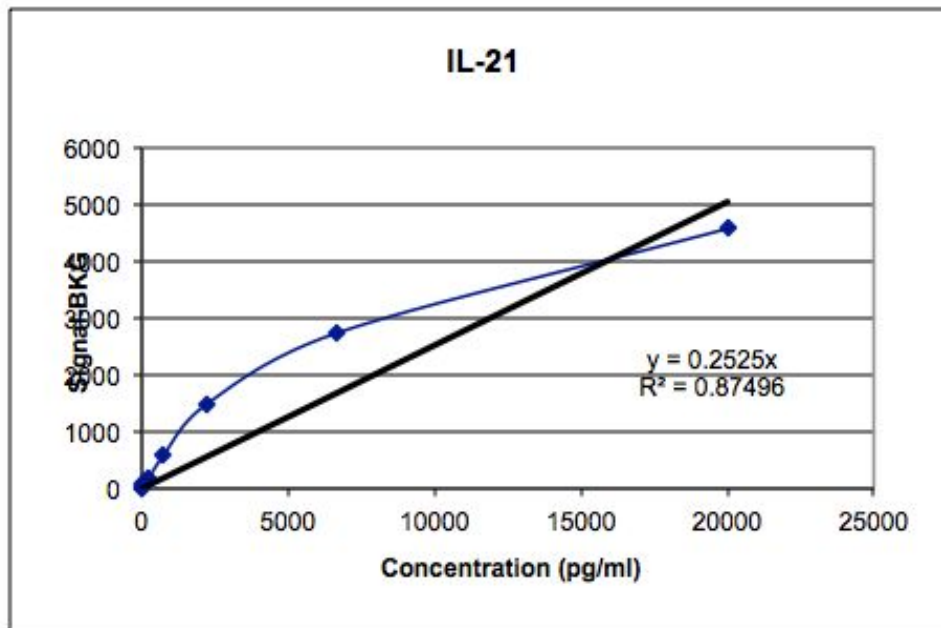
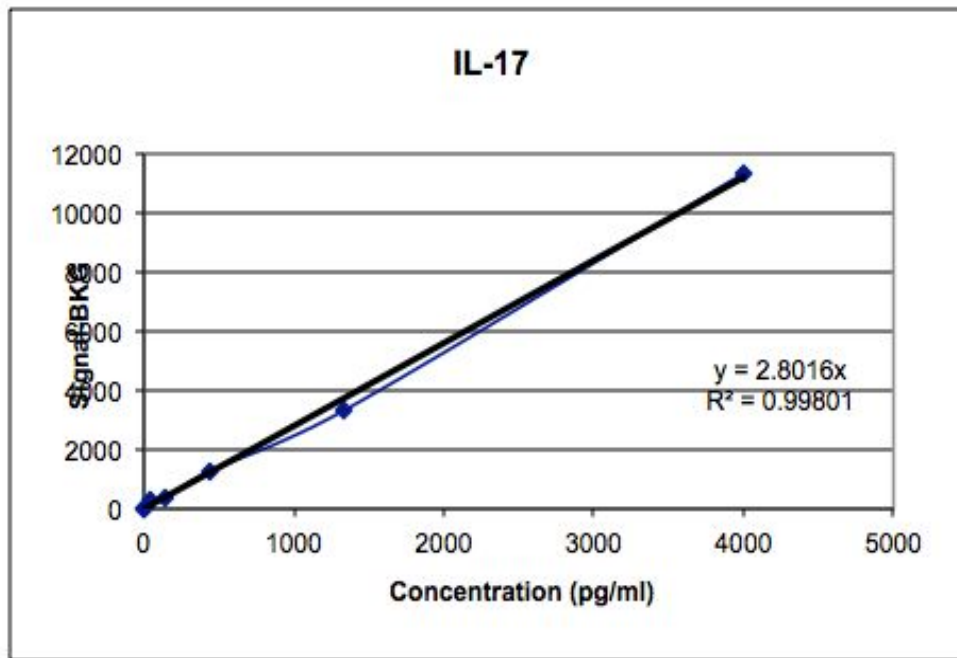


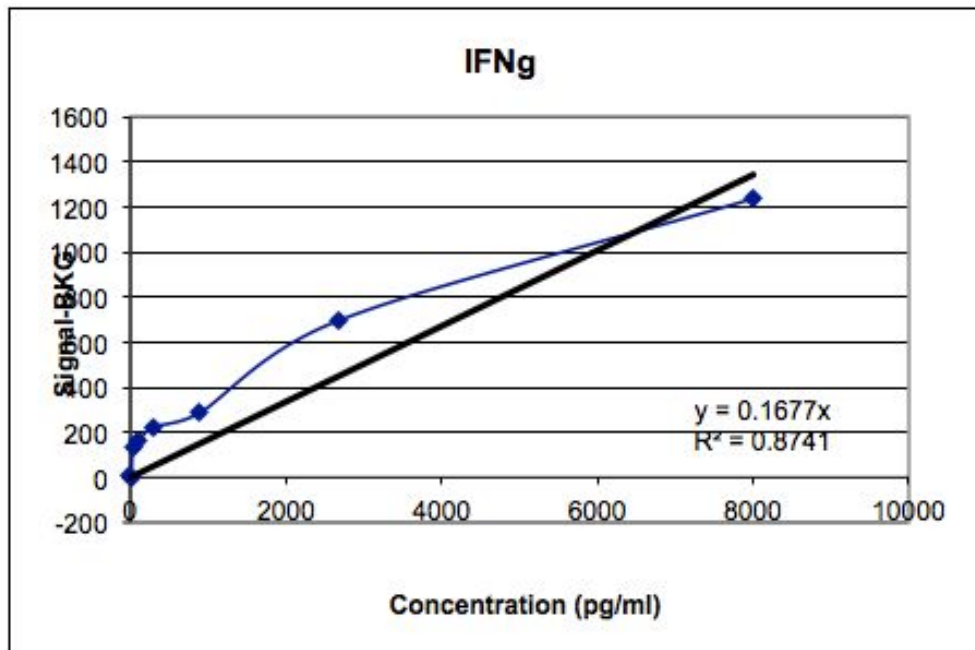
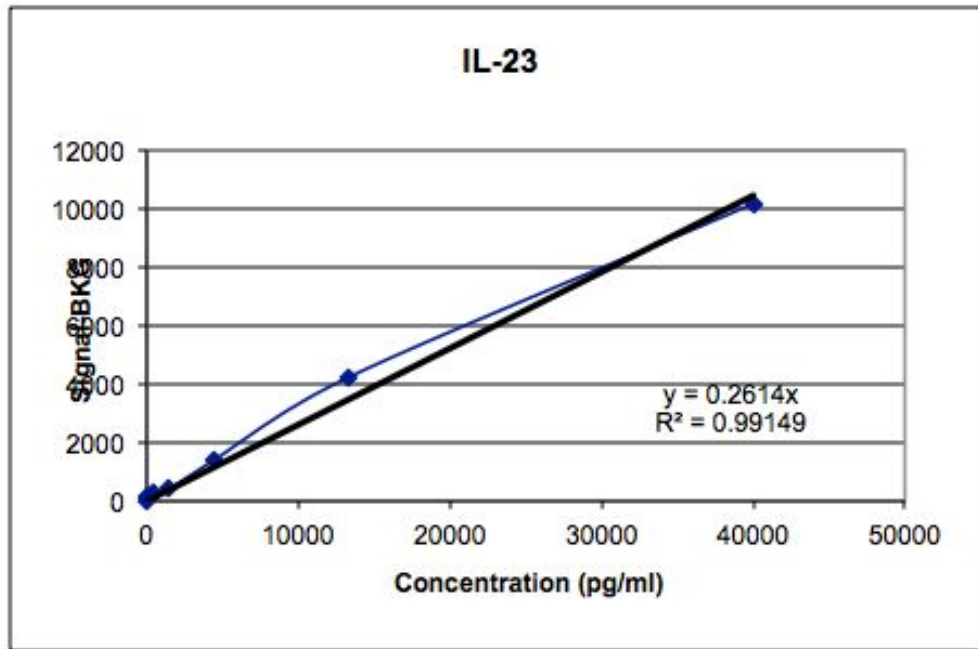


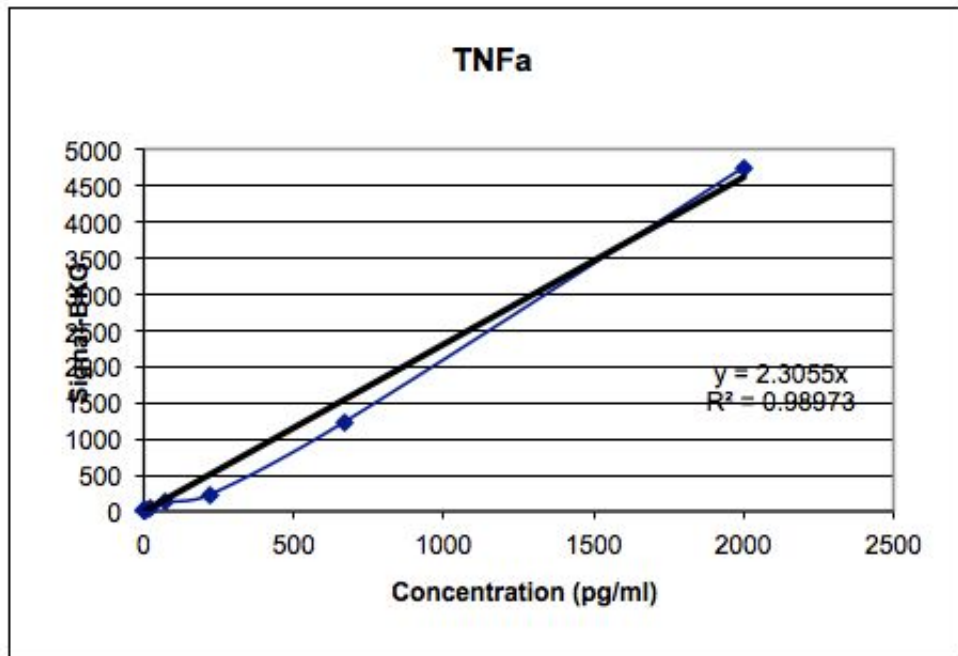












Appendix E**Sample Cytokine Concentration (pg/mL) Based on Linear Regression Standard****Curves**

(pg/ml)	iPSC/OP9 Coculture	iPSC/OP9 conditioned media Coculture	OP9
G-CSF	102.5	34.0	42.0
GM-CSF	2692.6	208.5	978.8
IL-1a	304.6	169.6	133.0
IL-1b	66.8	0.0	43.6
IL-2	686.9	0.0	375.8
IL-3	8.1	0.0	0.8
IL-4	146.4	0.0	86.3
IL-5	840.6	0.0	328.4
IL-6	575.1	0.0	165.0
IL-7	5226.0	0.0	0.0
IL-9	34498.8	3252.5	14148.0
IL-10	304.6	77.0	40.3
IL-12p70	12.4	0.0	0.0
IL-13	5410.6	1696.2	2775.0
IL-15	5260.6	0.0	0.0
IL-17	134.1	0.0	16.5
IL-21	472.8	157.3	0.0
IL-23	862.2	0.0	349.1
IFNg	1697.8	0.0	387.7
TNFa	36.2	0.0	0.0

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IL5 interleukin 5 [Homo sapiens (human)] - Gene - NCBI. (n.d.).

IL6 interleukin 6 [Homo sapiens (human)] - Gene - NCBI. (n.d.).

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