University of Cincinnati

Date: 7/8/2014

I, Yi Wen Chai, hereby submit this original work as part of the requirements for the degree of Master of Science in Transfusion and Transplantation Sciences.

It is entitled:
UNDERSTANDING THE CELLULAR MECHANISMS OF THE LEUKOCYTE ADHESION DEFICIENCY TYPE III DISORDER WITH THE USE OF PATIENT INDUCED PLURIPOTENT STEM CELLS

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UNDERSTANDING THE CELLULAR MECHANISMS OF THE LEUKOCYTE ADHESION DEFICIENCY TYPE III DISORDER WITH THE USE OF PATIENT INDUCED PLURIPOTENT STEM CELLS

A thesis submitted to the Division of Research and Advanced Studies of the University of Cincinnati in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

In the Department of Transfusion and Transplant Sciences of the College of Allied Health

Committee Chair: Carolyn Lutzko, PhD
For the year 2014

by

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July 2014
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ABSTRACT

Leukocyte Adhesion Deficiency type III (LAD-III) is a rare congenital defect that affects the normal adhesive function of phagocytic cells of the myeloid lineage. Bone marrow from a patient with LAD-III was obtained and used to generate induced pluripotent stem cells (iPSCs). The iPSCs were determined to have normal karyotype, phenotype, differentiation capacity, cell surface markers, self-renewal capabilities and pluripotency. We hypothesize that iPSCs from this patient can be used to generate hematopoietic cells. In addition, the generated hematopoietic cells had the same characteristics as the patient cells. We also evaluated the hematopoietic cells generated from the iPSCs, including their number and differentiation status, and their ability to migrate and adhere in response to inflammatory mediators. The main purpose of this project is to successfully model the cellular functional defect in LAD-III using iPS derived hematopoietic cells for in vitro cell culture analysis. Our results showed that we were able to generate hematopoietic progenitor cells (HPCs) and myeloid cells from our patient derived iPSCs. Furthermore, the differentiated myeloid cells had sustained the expected LAD-III functional defects of cellular adhesion and migration. In summary, this study was able to show the functional cellular defects in LAD-III by using patient-specific iPSCs to model the disease in vitro.
ACKNOWLEDGEMENTS

I would like to express the deepest appreciation to my committee chair, Dr. Carolyn Lutzko, who has provided me with her unwavering support, patience and immense knowledge over the course of my graduate experience. I could not have imagined having a better advisor and mentor for my graduate study as her selfless time and care were sometimes all that kept me going.

In addition, I would like to thank my committee members, Dr. Jose Cancelas and Dr. Tom Leemhuis, for their encouragement, insightful comments, and hard questions. Thank you for challenging me to think creatively and for reminding me why we do what we do.

My sincere thanks also goes to Dr. Lisa Trump, Dr. Kevin Link, Dr. Ramesh Nayak, Ms. Robin Schroll and members of the Fillipi lab for their instruction in cell work, cell assay experimentation and assistance in acquiring parts of the data for this paper. This dissertation would not have been possible without their constant support and guidance.

And finally, I would like to thank my family: my parents, Ng Chow Chai and Jaime Chan whose love and support sustained me throughout and my one and only sister, Yi Wei Chai, for being my pillar of strength.

There is a light at the end of this tunnel! So I thank you all again for being a part of my journey and for allowing me to be a part of yours.

Best Wishes,

Wen Chai
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<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>allophycocyanine</td>
</tr>
<tr>
<td>APC-Cy7</td>
<td>allophycocyanin- and cyanine 7 dye</td>
</tr>
<tr>
<td>BFU-E</td>
<td>burst forming unit-erythroid</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>CCHMC</td>
<td>cincinnati children’s hospital medical center</td>
</tr>
<tr>
<td>CD11b</td>
<td>expressed on leukocyte surface; involved in adhesion</td>
</tr>
<tr>
<td>CD14</td>
<td>expressed on human monocytes and macrophage</td>
</tr>
<tr>
<td>CD15</td>
<td>neutrophil marker</td>
</tr>
<tr>
<td>CD18</td>
<td>integrin beta-2 protein</td>
</tr>
<tr>
<td>CD33</td>
<td>siglec-3; expressed on myeloid lineage transmembrane receptor</td>
</tr>
<tr>
<td>CD34</td>
<td>cell surface sialomucin, expressed on primitive hematopoietic stem cells</td>
</tr>
<tr>
<td>CD45</td>
<td>leukocyte common antigen</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CDG1</td>
<td>calcium and diacylglycerol guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>CFU-C</td>
<td>colony-forming unit-content</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>colony-forming unit-granulocyte macrophage</td>
</tr>
<tr>
<td>CFU-M</td>
<td>colony-forming unit-macrophage</td>
</tr>
<tr>
<td>c-Myc</td>
<td>oncogene; regulator gene that codes for a transcription factor</td>
</tr>
<tr>
<td>DMEM</td>
<td>dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ESCs</td>
<td>embryonic stem cells</td>
</tr>
<tr>
<td>ES Cult</td>
<td>embryonic stem cell fetal bovine serum</td>
</tr>
<tr>
<td>FACS</td>
<td>flow activated cell sorter</td>
</tr>
</tbody>
</table>
FBG  fibrinogen
FBS  fetal bovine serum
FCS  fetal calf serum
FERMT3  fermitin family homolog 3; kindlin-3 gene
FITC  fluorescein isothiocyanate
FLT3  fms-like tyrosine kinase 3 cytokine; hematopoietic progenitor exp..
FMLP  formyl-met-leu-phe
g  gravity-force
G-CSF  granulocyte-colony stimulating factor
GM-CSF  granulocyte macrophage-colony stimulating factor
HSC  hematopoietic stem cells
IL-3  interleukin-3
IL-6  interleukin-6
iPSC  induced pluripotent stem cells
Kindlin-3  regulates intergrin activation; encoded by FERMT3 gene
KLF4  kruppel-like factor 4; indicates stem-like capacity
LAD-1  leukocyte adhesion deficiency type I
LAD-1 Variant  variant of leukocyte adhesion deficiency type I
LAD-2  leukocyte adhesion deficiency type II
LAD-3  leukocyte adhesion deficiency type III
MNC  mononuclear cells
MTG  monothioglycerol
NANOG  transcription factor involved in undifferentiated embryonic stem cell self-renewal
NSG  NOD-SCID gamma C-/-
OCT4  octamer-binding transcription factor 4
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>PB</td>
<td>peripheral blood</td>
</tr>
<tr>
<td>PE-Cy7</td>
<td>r-phycoerythrin and cyanine 7 dye</td>
</tr>
<tr>
<td>PerCP-Cy5.5</td>
<td>peridinin chlorophyll protein complex and cyanine 5.5 dye</td>
</tr>
<tr>
<td>PSCs</td>
<td>pluripotent stem cells</td>
</tr>
<tr>
<td>SOX2</td>
<td>sry-box 2; transcription factor essential for pluripotency &amp; self-renewal of undifferentiated embryonic stem cell</td>
</tr>
<tr>
<td>TF</td>
<td>transcription factor</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PLL</td>
<td>poly-l-lysine</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>QNS</td>
<td>quantity not sufficient blood units</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SCF</td>
<td>stem cell factor</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VioBlue</td>
<td>a flow cytometry fluorochrome</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
</tbody>
</table>
INTRODUCTION

Leukocyte adhesion deficiency type 3 (LAD-III) is an inherited molecular defect that leads to an array of medical complications. Patients are susceptible to a myriad of lethal infections and may have a Glanzmann thrombasthenia-like bleeding presentation\(^1\). This rare immune deficiency affects the normal adhesive ability of phagocytes in the myeloid lineage; predominantly the monocytes and polymorphonuclear cells (PMNs). These affected cells are also unable to migrate to the site of infection and destroy invading foreign pathogens due to a defect in leukocyte activation\(^2\). The \(\beta_1\), \(\beta_2\) and \(\beta_3\) subunits of the leukocyte integrins are responsible for interacting with proteins that transduce signals to determine how well leukocytes can arrest at site of infection or platelets can aggregate to the damaged vessels\(^3\). However, the prime culprit that reduces the effectiveness of integrins from achieving an active conformation in the LAD-III disorder is the Kindlin-3 protein. Kindlin-3 is a key adaptor protein that is bound to the intracellular portion of the \(\beta_1\), \(\beta_2\) and \(\beta_3\) integrins and it serves to modify the integrin’s activation and binding affinity. It is a mutation in the Kindlin-3 encoding FERMT3 gene that disrupts the signaling pathway of the leukocyte and platelet integrins\(^4\). Consequently, this signaling interference diminishes the adhesion ability of PMNs to the endothelial cells and migration to the site of infection\(^5\). The mechanisms of this uncommon LAD-III disease is still not well understood and we proposed that an \textit{in vitro} model study of LAD-III patient derived hematopoietic cell differentiation and function to give us a better understanding of the cellular mechanism of the disease\(^6\).
Leukocyte Migration Overview

Leukocyte migration is a necessary immune process designed to defend the body from harmful microorganisms that invade through open wound areas. Migration is initiated after a chemokine signal for injury is received by the leukocyte (FIGURE 1). The adhesive environment of the leukocyte’s surface is increased during this process to allow surface selectins to slow cells to a rolling motion. Other surface proteins such as integrins allows for firm adhesion of the leukocyte to the endothelium, preparing the cell’s ability to reorganize its shape for transmigration through the endothelium cell gaps. After successfully undergoing diapedesis through the blood vessel, the leukocytes will continue along the chemo-attractive gradient towards the source of injury.

FIGURE 1. LEUKOCYTE MIGRATION: ABOVE ARE THE STEPS FOR LEUKOCYTE MIGRATION ONCE A CHEMO KINE SIGNAL FOR INJURY IS RECEIVED. LEUKOCYTES GO THROUGH THE WOUND HEALING PROCESS OF CELL MIGRATION: ROLLING, ACTIVATION, ADHESION AND TRANSMISSION. THIS IMAGE WAS ADAPTED FROM THE ARTICLE ON LEUKOCYTE-ENDOTHELIAL INTERACTIONS.
Leukocyte Integrin Signal Cascade

Within the cell signaling cascade network, integrin activation is an essential step of ensuring firm cell adhesion between the cell’s internal signaling systems with an external extracellular matrix. These integrins are activated by proteins of the Kindlin family to regulate the act of cell-matrix adhesion. There are three known types of Kindlins found in different cell types; Kindlin-1 is expressed in epithelial cells, Kindlin-2 protein is expressed ubiquitously and Kindlin-3 in only hematopoietic cells. The focus of this project is on the Kindlin-3 protein and its effect on the hematopoietic cell system.

Patients with the LAD-III phenotype have a cell signaling defect that is caused by the loss of the Kindlin-3 adaptor protein, which in turn affects the leukocyte integrin’s ability to activate. Although patients of this disease are characterized to have normal integrin expression and structure, there is a defective intracellular integrin activation process. The Kindlin-3 protein plays a key role in immune system, hemostasis and thrombosis regulation. Cooperative binding of the Kindlin-3 adaptor protein to the Talin cytoskeletal protein on the beta subunit tails of the lymphocyte function-associated antigen-1 (LFA-1) leukocyte integrin is necessary for integrin activation. Without integrin activation, the leukocytes cannot firmly attach on the endothelium walls to further migrate towards the site of infection (FIGURE 2)
FIGURE 2. INTEGRIN SIGNAL CASCADE: A CHEMOKINE MOLECULE THAT IS EXPRESSED AFTER AN INFLAMMATORY STIMULUS IS SIGNALED THROUGH THE G-PROTEIN COUPLED RECEPTORS (GPCRS) OF LEUKOCYTES. THIS ACTIVATES THE CALCIUM AND DIACYLGLYCEROL GUANINE NUCLEOTIDE EXCHANGE FACTOR (CALDAGGEF1; CDG1) CASCADE TO PROMOTE THE ACTIVITY OF A KEY RAP1 PROTEIN. ACTIVATED RAP1 INITIATES AN INTEGRIN SWITCH IN LEUKOCYTES TO AN OPEN CONFORMATION. THE OPEN INTEGRIN CONFORMATION REVEALS AN OPEN LIGAND BINDING SITE, ALLOWING THE TALIN AND KINDLIN-3 PROTEINS TO BIND AT ITS BETA SUBUNIT TAIL. THESE PROTEIN SUBUNITS ARE IMPORTANT TO ENSURE THAT THE LEUKOCYTE IS SECURELY ANCHORED TO THE ENDOTHELIUM WALLS. THIS IMAGE WAS MODIFIED FROM THE ARTICLE ON WHEN INTEGRINS FAIL TO INTEGRATE\(^\text{19}\).

iPSC Disease Modeling

This study seeks to improve the current general understanding of the LAD-III disease. iPSC technology is the selected approach for this study because we can manipulate iPSCs to create a more accurate human model of the patient's genetic predisposition (FIGURE 3). We were able to cultivate a large amount of cells using iPSCs, giving us access to perform more studies
that could better evaluate the cellular mechanisms, functional impediments and differentiation abilities of the LAD-III syndrome. As there is still not a lot known about this rare disease\textsuperscript{18}, this study provided new information about this rare autosomal recessive disorder. A further prospect would be to use hematopoietic or other cells generated from the LAD-III iPSC to screen for drug candidates in the future. Although there are other methods to model diseases, we specifically chose to use iPSCs, as opposed to using primary cells, due to the ease of creating a large repository of disease-specific cells for this study purpose, and rarity of primary patient cells. Furthermore, additional findings can also help further evaluate the ability of disease modeling with iPSCs.

\textbf{FIGURE 3. IPSC DISEASE MODELING: A DIAGRAM DESCRIBING THE POTENTIAL OF USING PATIENT-SPECIFIC iPSCS IN REVOLUTIONIZING DISEASE MODELING, DRUG SCREENING, DRUG DISCOVERY AND PERSONALIZED MEDICINE. THE ABILITY TO DERIVE MATURE HEMATOPOIETIC CELLS FROM iPSCS WILL BE USEFUL IN ADVANCING CELL REPLACEMENT THERAPIES. THIS IMAGE WAS ADAPTED FROM A PAPER ON THE GENERATION OF MATURE HEMATOPOIETIC CELLS FROM HUMAN PLURIPOTENT STEM CELLS\textsuperscript{20}.}
Recent studies had used iPSCs to better understand the defects of various immune disorders\textsuperscript{21}. The properties that make iPSCs an attractive cell type to use in disease models are its pluripotent and self-renewing behavior. Self-renewal is the ability to undergo mitotic division and create an equivalent stem cell while pluripotency is the ability to differentiate into the 3 embryonic germ layers; endoderm, mesoderm and ectoderm. They are reprogrammed in vitro from adult somatic cells by inducing an overexpression of a specific set of transcription factors that regulate pluripotent cell self-renewal\textsuperscript{22}. In theory, these generated iPSCs had the same defined mutations as the hematopoietic cells from the LAD-III patient and they can be used to learn more about the cellular basis of the disease.
HYPOTHESIS

I hypothesize that iPSC technology can be used to recreate and study the LAD-III hematological disorder \textit{in vitro}. I specifically hypothesize that hematopoietic cells differentiated from the LAD-III iPSCs will have the same functional defects as the blood cells collected directly from the patient including a loss of the adhesive and migratory ability of myeloid cells.

SPECIFIC AIMS

These hypotheses were tested in the following aims.

1. Generate HPCs from LAD-III patient iPSCs
2. Generate and expand myeloid cells from the differentiated HPCs
3. Model the adhesive and migratory ability of iPSC differentiated myeloid cells derived from a LAD-III patient
The experiments in this project were designed to help us better understand the cellular defects of LAD-III using a disease model method (Figure 4). The patient generated iPSCs were initially differentiated into HPCs using a known protocol developed by a Lutzko lab collaborator, Dr. Mitchell Weiss. This produced a large number and variety of progenitor cells. The cells were then further expanded and differentiated into myeloid cells using a protocol created by the Cancelas Lab. The media includes IMDM, FBS, SCF, IL-3, and G-CSF. To verify that I had successfully differentiated cells of the myeloid lineage, I characterized them by their morphology and the following cell surface markers: CD33 (established myeloid marker), CD11b (myeloid surface marker), CD14 (monocyte and macrophage marker) and CD15 (neutrophil marker). The functional assays portion compared the LAD-III patient’s iPSC derived blood cells with those derived from H9 cells (embryonic stem cells), 12E cells (iPSCs differentiated from a normal donor) and peripheral blood cells (normal donor blood control).

The functional problems found in the LAD-III disease were predicted to be caused by a defect in the leukocytes integrin activation, affecting the ability of myeloid cells to adhere and migrate to the site of tissue damage or infection. The functional abilities of these iPSC derived myeloid cells were tested in functional adhesion and migration assays that were adapted for iPSCs. The migration assay used Corning’s Transwell (Corning Inc; Corning, NY, USA) plates to understand the migratory response of leukocytes that have the LAD-III migratory deficiency.
Finally, cell-to-surface adhesion was analyzed with an adhesion assay that has been modified from the Life Technologies’ Vybrant cell adhesion kit assay (Life Technologies; Grand Island, NY, USA).
MATERIALS AND METHODS

Derivation of iPSCs from Patient Cells

The laboratory of Dr. Carolyn Lutzko acquired MNCs and CD34+ isolated blood cells from a patient with the LAD-III phenotype. The patient is a female baby of Palestinian descent and her disease was characterized to be caused by an autosomal recessive stop codon in the FERMT3 gene on both chromosomes. This mutation has been sequenced as a single base mutation due to a nucleotide substitution (locus 11q13; 687 nt TGG > UGA)\textsuperscript{18}. As a result, the mutation produces a premature stop codon in the open reading frame (ORF) that consequently shortens its protein during translation. Dr. Lisa Trump reprogrammed the one patient's blood cells into two iPSC lines; MNC derived iPSCs (16L iPSC line) and CD34+ derived iPSCs (18L iPSC line). These patient derived cell lines were further expanded and differentiated into hematopoietic cells for use in this project.

Mutation Analysis

The presence of the FERMT3 gene expression in the patient derived iPSCs was confirmed using PCR and gene sequencing analysis tools. DNA was isolated from iPSCs using the Qiagen DNeasy Blood and Tissue Kit (QIAGEN Inc., Valencia, California, USA). Primers were designed through the Primer3 biotool software (Whitehead Institute; Boston, Massachusetts, USA) based on the FERMT3 genomic sequence that was searched through the GenBank Nucleotide sequence database\textsuperscript{25}. The primers that were used in this system were: WT FERMT3 forward, CTTGGGATCCAAATCGAAGG and ACTTCGTTTCCAGGCTTTTT from Integrated DNA Technologies, Inc. (IDT Inc., Coralville, Iowa, USA) To be characterized for the G>A mutation. The FERMT3 gene was PCR amplified using the designed primers and the DNA was sequenced at the Cincinnati Children’s Hospital Medical Center (CCHMC) DNA Sequencing and Genotyping.
Core. The final PCR product’s sequence traces were analyzed using FinchTV Perkin Elmer software by Dr. Lisa Trump.

**iPSC Culture and Maintenance**

iPSCs (patient-derived, normal donor-derived and H9) were cultured and maintained undifferentiated on matrigel (BD Biosciences; Franklin Lakes, New Jersey, USA), according to the feeder-free mTeSR™1 system (STEMCELL Technologies; Vancouver, British Columbia, Canada). Prior to initiating hematopoietic differentiation, the cells were seeded onto irradiated Mouse Embryonic Fibroblast (MEFs) feeder cells and cultured in human Embryonic Stem Cell (hES) medium to allow colonies of cells to appear. Differentiated colonies were marked and removed using a micropipette tip in order to maintain the environment’s self-renewability and pluripotent state.
Pluripotency Analysis with Teratoma Generation

Teratomas were analyzed to determine the differentiation capacity of the generated LAD-III iPSCs. The LAD-III iPSCs were plated on irradiated mouse embryonic fibroblasts (MEFs) and grown up to ~90% confluency. Once the cells were grown and ready to be transplanted, they were detached from the plate and broken up into similar sized aggregates. Matrigel was quickly added to the prepared cell suspension and injected into the mouse. The mouse was checked weekly for teratoma development. Palpable teratomas took about 6-12 weeks to become evident. When the tumor reached \(~1cm^3\) in size, the teratoma was excised for germ layer composition analysis with the Hematoxylin and Eosin stain. Brightfield images were taken at the 20X objective with the motic camera (Motic; Causeway Bay, Hong Kong, China).

Hematopoietic Progenitor Cell Differentiation

Cells were differentiated into hematopoietic cells following the Weiss protocol, for 10 days\textsuperscript{23}. The combinations of cytokines changed throughout the differentiation, a method optimized by the Lutsko lab to recapitulate the ideal environment for blood differentiation (FIGURE 5). Media had to be made fresh for each day for each media feeding. By the 10\textsuperscript{th} day, HPCs were created, a process that can be simplified as the hematopoietic progenitor stage. We confirmed that the collected cells were HPCs by its morphology and cell surface markers (CD45 and CD34) using flow cytometry.
Myeloid Cell Differentiation

To further the cellular differentiation and expansion process, the previous iPSC that were differentiated into hematopoietic progenitors using the Weiss protocol outlined above were harvested and re-cultured in a medium containing IMDM, FBS, SCF, IL-3, and G-CSF for five days (FIGURE 6). Only the floating cells from the hematopoietic progenitor stage were collected while the adherent cells were discarded. This differentiation process can be referred to as the myeloid cell stage. The culture medium was refreshed every day by spinning down the cells into a pellet and adding new media. We confirmed that the collected cells were myeloid cells by the end of the process (day 5) by its morphology and cell surface markers (CD45, CD33, CD15, CD14 and CD11b) using flow cytometry.
Isolating Neutrophils from Peripheral Blood

The normal donor blood controls that were used in this study were Quantity Not Sufficient (QNS) units provided by CCHMC or the Hoxworth Blood Center. Upon receiving a bag, ½mL of blood was used to analyze the initial content of the blood with the Abott Cell Dyn 3700 cell counter (Abbott Laboratories, Abbott Park, IL, USA). Then 5mL of blood was used to prepare the normal donor control group. The BD Pharmlyse 1X lysing buffer (BD Biosciences; Franklin Lakes, New Jersey, USA) was prepared with deionized (D.I.) water according to the manufacturer’s recommendation. It was added to 5mL of blood to form a final 1:2 blood to pharmlyse ratio and incubated in the dark at room temperature (RT) for 10 minutes. After the red blood cells had been allowed 10 minutes to lyse, the mixture was centrifuged at 200g for 5 minutes at 18°C and a deceleration speed of 6. The supernatant was removed and the steps were repeated for a second time. Finally, the cell pellet was brought up to 5mL with media used in the assay. A final differential count on the Cell Dyn determined the myeloid cell counts and if there was more than $6 \times 10^5$ cells, we proceeded to perform the functional assay experimentation.
Evaluation of Hematopoietic Activity in LAD-III iPSC derived cells with Colony Forming Unit-Content (CFU-C) Analysis

The CFU-C assay was performed to quantify the types of progenitors that the hematopoietic progenitors differentiated from the LAD-III iPSC. This in vitro assay showed us the developmental capabilities of HPCs at different developmental stages. The differentiated blood at the hematopoietic progenitor stage and myeloid stage were tested in triplicates to rule out any biases and random errors in the experiments. The viscous and semi-solid methylcellulose media (3mL) was thawed at room temperature before plating with 200μL of cell suspension that contained a final cell concentration of $3 \times 10^5$ cells/mL. After 10-14 days, the CFU-C dishes of each culture stage were characterized, enumerated and compared based on their differentiation capacity.

iPSC/HSC Phenotypic Characterization with Flow Cytometry

The LAD-III experimental cells and normal controls were characterized using the following cell surface markers and colors: CD45-PE, CD34-APC, CD33-PE-Cy7, CD11b-APC-Cy7, CD15-FITC, CD14-VioBlue. Cells were pre-incubated in FACs buffer (PBS, 5-10% FBS, 0.1% $NaN_3$) and blocking buffer for 10 minutes at 4°C before staining to reduce non-specific immunofluorescence. After they were stained for 20 minutes, the cells were washed with PBS and analyzed by flow cytometry. Some samples were fixed in 4% PFA prior to analysis within one week.

Morphological Analysis of Cells

To visualize cell morphology, a prepared cell suspension of $5 \times 10^4$ cells cytocentrifuged onto a glass slide with the Shandon Cytospin CytoCentrifuge (Thermo; Pittsburg, PA, USA) at 400rpm for 5 minutes. After cytocentrifugation, the slides were placed aside for 24-hours to dry.
Finally, immunocytochemistry with a Siemens HEMA-TEK 2000 Slide Stainer (Thermo; Pittsburg, PA, USA) was performed on the slide samples to give us clearer pictures for easier assessment of the cells. Slide preparations were all protected immediately after staining with the Richard-Allan Scientific Cytoseal 60 mounting media (Thermo; Pittsburg, PA, USA) and cover slip.

**Adhesion Assay**

![Adhesion Assay Set-Up](image)

**FIGURE 7. ADHESION ASSAY SET-UP**: THE WELLS WERE PREPARED A DAY IN ADVANCE, BEFORE CELLS WERE ADDED TO THE SYSTEM ($5 \times 10^4$ CELLS) AND ALLOWED TO INCUBATE FOR 4 HOURS. THIS IMAGE WAS MODIFIED FROM A VYBRANT ADHESION ASSAY KIT PROTOCOL.

The Vybrant Cell adhesion assay by Life Technologies was adapted for the cell adhesion study to assess the adhesive ability of the generated myeloid cells *in vitro* (FIGURE 7). This assay was used to determine whether the differentiated LAD-III myeloid cells were able to adhere normally to a system that mimicked the endothelial walls of the blood vessels, the experimental LAD cells were compared to established normal controls, which were peripheral blood myeloid cells and H9 derived pluripotent stem cells (PSCs). In this assay, the generated myeloid cells were plated in duplicate at $5 \times 10^4$ cells/mL onto glass chamber slides. Individual chamber slides were pre-coated with 3 different treatments – No Coat, Fibrinogen and Poly-L-Lysine. Upon addition of cells to their respective chamber slide treatments, the plates were incubated for 4 hours at 37°C. This allowed the cells time to adhere onto the bottom of the slide. The chambers were washed with Phosphate Buffer Saline (PBS) to remove the non-adherent cells. The remaining cells were then
counted with a hemocytometer to determine adhesion. This assay evaluated the adhesion ability of iPSC derived cells from LAD-III patients and controls to fibrinogen, a component of the endothelial cell wall. Cell adherence was measured by obtaining the raw cell adhesion numbers from staining and counting the slides through a microscope. The chambers were fixed with 4% formalin and stained with May-Grünwald–Giemsa (Merck KGaA; Billerica, Massachusetts, USA) for easier visualization for counting.

**Migration Assay**

![Migration Assay Diagram](image)

**FIGURE 8. MIGRATION ASSAY SET-UP**: THE WELLS WERE PREPARED A DAY IN ADVANCE, BEFORE CELLS WERE ADDED TO THE SYSTEM (5 × 10⁴ CELLS) AND ALLOWED TO INCUBATE FOR 4 HOURS. THIS IMAGE WAS MODIFIED FROM A CORNING TRANSWELL MIGRATION KIT PROTOCOL²⁷.

The iPSC derived myeloid cell’s ability to migrate was tested using 24-well plate transwell dishes to evaluate the extravasation process of the defective LAD-III cells (**FIGURE 8**). This assay has been adapted for iPSC use from the transwell migration protocol by Corning Inc. and it mimics the migration of cells through a vascular endothelium. It is understood that the migratory ability of cells from LAD-III patients have been compromised due to a defective KINDLIN-3 integrin⁴ but this study looks to determine whether this was also true for the generated experimental LAD-III iPSCs. The transwell membrane inserts were pre-coated with fibrinogen and the generated myeloid cells were plated at 5 × 10⁴ cells/insert. The assay was performed in duplicates and after a 4 hour incubation at 37°C, the number of cells that migrated through the membrane was
quantified. A chemo-attractant, formyl-Met-Leu-Phe (fMLP), was used to induce cell migration from the upper chamber through the membrane to the lower chamber. The cells that have passed through the filter were imaged and counted to evaluate their emigration efficiency. Cell migration was calculated after discarding the transwell inserts and acquiring raw migration numbers of cells that remained in the well. To aid with visualization of the cells, the wells were fixed with 4% formalin and stained with May-Grünwald–Giemsa.

**Statistical Analysis**

Comparisons were made throughout the characterization and evaluation of functionality between the LAD-III patient generated iPSCs with normal donor cells and H9 generated PSCs. All experiments that analyze function were performed in dupllicate. Standard paired T-test statistical analysis was analyzed with the Prism 6 software (GraphPad Software, Inc.; La Jolla, California).
RESULTS

Characterization of iPSCs

The mutation was confirmed before we proceeded with this project. To characterize that we were able to create pluripotent stem cells, a test for pluripotency of the LAD-III iPSCs was performed with a teratoma assay. The mutation analysis and teratoma assay were executed with help from Dr. Lisa Trump and Ms. Robin Schroll.

(a) Evaluation of the FERMT3 gene mutation in LAD-III iPSC

Automatic alignment using the FinchTV Perkin Elmer software confirmed a reverse point mutation (AGC→AGT) in both the 16L iPSC line and the 18L iPSC line. The control DNA was a human genomic DNA purchased from Roche (Genentech; Oceanside, San Diego, USA) to show a Wild-Type (WT) C. A point mutation was located in the analysis of the 16L iPSC line (FIGURE 9).
FIGURE 9. POINT MUTATION VERIFICATION: PARTIAL NUCLEOTIDE SEQUENCES OF CONTROL NORMAL AND BOTH MUTANTS OF THE FERMT3 GENE (AGC→AGT). THE REVERSE ANTI-SENSE STRAND IS SHOWN ABOVE. ARROW POINTS TOWARDS POINT MUTATION WITH RESPECT TO THE NORMAL CONTROL SEQUENCE.

These genomic anomalies were previously suggested in the study by Harris et. al for the same patient\textsuperscript{2}. The results of this analysis thereby confirms that the LAD-III iPSC line had retained the patient mutation.
(b) **Teratoma Findings**

Teratoma assay was performed to test the pluripotency of the iPSCs. Germ layer tissues develop within a NOD-SCID gamma C-/- (NSG) female mouse and after 6-12 weeks, the mass development was excised and analyzed for the germ layers: endoderm, ectoderm and mesoderm. Further analysis of the Hematoxylin & Eosin (H&E) stained teratoma sections showed that all three germ layers were identified and thus the iPSCs were pluripotent (**FIGURE 10**).

**FIGURE 10. MOUSE TERATOMA SECTIONS:** TERATOMA SECTIONS OF AN NSG MOUSE THAT WAS INJECTED WITH THE PATIENT GENERATED iPSCS. PLURIPOTENCY WAS OBSERVED AS CHARACTERIZED BY THE FOLLOWING GERM LAYERS: (A) MESODERM: CARTILAGE (B) ENDODERM: COLUMNAR EPITHELIUM (C) ECTODERM: PSEUDOSTRATIFIED COLUMNAR EPITHELIUM

**Differentiation of Patient-Derived iPSCs into Hematopoietic Cells**

We evaluate the ability of the two patient derived-iPSC lines (16L and 18L iPSC lines) to differentiate into HPCs and Myeloid Cells. The following were our findings:

1. **Evaluation of HPC Differentiation from in LAD-III iPSC**

The ability to generate HPCs from iPSCs was analyzed through the comparison of LAD-III patient generated blood cells with normal donor blood controls and normal iPS generated blood controls. Surface marker, erythroid colony development and cell morphology analysis proved that HPCs can be differentiated from iPSCs.
1-1. **Immunophenotype Analysis of HPC differentiated from LAD-III iPSC**

The phenotype of the iPSC derived hematopoietic cells from the LAD-III patient cells were compared with normal controls. Surface marker analysis of HPCs differentiated from the LAD-III patient derived iPSCs expressed CD45 (leukocyte common antigen marker) and CD34 (hematopoietic stem cell marker) similar to somatic hematopoietic progenitor cells. Analysis of a FACS single cell culture timeline shows that the HPC cells had the CD34+ phenotype. These cells were measured at the 10th day of being induced in Weiss (Hematopoietic Progenitor Differentiation) media and the percent range of cells that have the hematopoietic stem cell markers were around 21% to 87% ([FIGURE 1](#)).
**FIGURE 11. HEMATOPOIETIC PROGENITOR DIFFERENTIATION:** This is a FACS analysis of cells stained with a CD34-APC marker. The cells were analyzed at day 10 of the Weiss media (HEMATOPOIETIC PROGENITOR MEDIA) induction.
1-2. **Morphological analysis of LAD-III iPSC derived HPCs**

Cytospin staining showed the cell morphology of iPSCs that have been differentiated towards HPCs as described in Methods section. The prepared slides below suggested early promyelocyte differentiation at this stage, as characterized by the neutrophil-like cells ([FIGURE 12](#)).

![H9 PSC – 40X, 16L iPSC – 40X, 18L iPSC – 40X](image)

**FIGURE 12. HEMATOPOIETIC PROGENITOR CYTOSPINS:** THESE BRIGHTFIELD MICROSCOPY IMAGES WERE TAKEN OF THE DIFFERENTIATED CELLS AT D10 OF WEISS (HEMATOPOIETIC PROGENITOR DIFFERENTIATION) MEDIA INDUCTION. CELLS WERE STAINED WITH MAY-GRÜNWALD–GIEMSA STAINING.

1-3. **Evaluation of Progenitor Activity in LAD-III iPSC derived HPCs**

As expected, the cultures from the hematopoietic progenitor differentiation stage were able to form colonies including: Colony Forming Unit granulocyte-macrophage progenitor (CFU-GM), colony-forming unit-macrophage (CFU-M) and Burst Forming Unit-Erythroid (BFU-E) with examples shown in [Figure 13](#). A 12E iPSC control was compared with the LAD-III differentiated cell line, the 16L iPSC line, to identify the colony differences. HPCs from the 12E iPSC control was able to generate colony-forming unit-granulocytes (CFU-G) while the experimental LAD-III cells did not.
The types of colonies that was generated can go on to produce granulocytes (CFU-GM), monocytes (CFU-M) and erythrocytes (BFU-E). Enumeration of the types of generated colonies in our experimental iPSC lines showed that most were CFU-GM colonies, followed by CFU-M and BFU-E colonies (TABLE 1). The samples were plated in triplicate in our methylcellulose assays and the average was taken of each culture. The 12E iPSC control culture gave rise to 36 colonies per $10^4$ of input cells, the 16L iPSC line had 12 colonies per $10^4$ of input cells and the 18L iPSC line had 28 colonies per $10^4$ of input cells. As most of the colonies were CFU-GMs, it can be deduced that the developmental capacity of the generated iPSC differentiated hematopoietic cells lean towards the myeloid lineage.

**TABLE 1. CFU ENUMERATION DATA:** ENUMERATION OF HPC DIFFERENTIATED COLONIES FOR EACH CELL LINE. THIS IS THE AVERAGE OF TRIPlicate EXPERIMENTS PER $10^4$ OF INPUT HPCS (N=3).

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Average Number of Colonies/10,000 Input HPCs</th>
<th></th>
<th></th>
<th>Averaged Total Number of Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>12E iPSC Control</td>
<td>15</td>
<td>20</td>
<td>1</td>
<td>36</td>
</tr>
<tr>
<td>16L iPSC line</td>
<td>6</td>
<td>5</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>18L iPSC Line</td>
<td>17</td>
<td>11</td>
<td>0</td>
<td>28</td>
</tr>
</tbody>
</table>
2. **Evaluation of Myeloid Cell Differentiation from iPSC derived HPCs**

The numbers and types of generated iPSC derived hematopoietic cells were analyzed through comparison of the LAD-III patient generated blood cells with normal donor blood controls (peripheral blood) and normal PSC generated blood controls (H9 PSCs). Surface marker, colony development and cell morphology analysis proved that I was able to generate HPCs.

2-1. **Immunophenotype Analysis of Myeloid Cells Differentiated from iPSC derived HPCs**

Successful generation of myeloid cells from the iPSC derived HPCs was evaluated following staining and flow cytometry analysis for the following surface antigens; CD45 (leukocyte common antigen), CD33 (myeloid marker), CD11b (leukocyte surface expressed), CD14 (monocyte and macrophage marker) and CD15 (neutrophil marker). The results showed the presence of CD45+, CD33+, CD11b+, CD15+ and CD14+ cells demonstrating the presence of the myeloid cell variety in my differentiated cultures (**FIGURE 14**).
FIGURE 14. MYELOID CELL DIFFERENTIATION: THIS IS A FLOW CYTOMETRY ANALYSIS OF THE CELLS STAINED FOR MYELOID MARKERS. THE CELLS WERE ANALYZED AT DAY 15 OF THE MYELOID MEDIA STAGE INDUCTION.

The experiment was performed in triplicate and the flow analyses gave the following myeloid cell percentages (TABLE 2). This shows the types of myeloid cells that we were able to generate from our population of %CD45+ cells.
TABLE 2. %CD45+ CELLS CO-EXPRESSING MYELOID MARKERS: THIS IS THE FLOW ANALYSIS DATA OF OUR THREE MOST RECENT MYELOID DIFFERENTIATIONS.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Flow Cytometry %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD33</td>
</tr>
<tr>
<td>Peripheral Blood Control</td>
<td>94.60%</td>
</tr>
<tr>
<td>H9 PSC Control</td>
<td>1.69%</td>
</tr>
<tr>
<td>16L Cell Line</td>
<td>22.80%</td>
</tr>
<tr>
<td>18L Cell Line</td>
<td>25.90%</td>
</tr>
<tr>
<td>Peripheral Blood Control</td>
<td>81.30%</td>
</tr>
<tr>
<td>H9 PSC Control</td>
<td>2.01%</td>
</tr>
<tr>
<td>16L Cell Line</td>
<td>52.30%</td>
</tr>
<tr>
<td>18L Cell Line</td>
<td>26.00%</td>
</tr>
<tr>
<td>Peripheral Blood Control</td>
<td>97.60%</td>
</tr>
<tr>
<td>H9 PSC Control</td>
<td>79.80%</td>
</tr>
<tr>
<td>16L Cell Line</td>
<td>17.90%</td>
</tr>
<tr>
<td>18L Cell Line</td>
<td>53.90%</td>
</tr>
</tbody>
</table>

In order to best reflect the types of myeloid cells generated, the numerical data is graphical illustrated below (FIGURE 15). The average was taken of triplicate experiments for our control iPSC and the experimental iPSC lines with respect to their co-expressed myeloid markers. Based on our analysis, most of the generated cell types were CD11b leukocyte surface expressed myeloid cells in the differentiated iPSC lines.
FIGURE 15. MYELOID CELLS TYPES: THE MEAN ± SD REPRESENTATION OF THE THREE MOST RECENT MYELOID CELL DIFFERENTIATIONS. THESE ARE THE TYPES OF MYELOID CELLS GENERATED AFTER BEING INDUCED IN MYELOID DIFFERENTIATION MEDIA FOR 5 DAYS. FLOW CYTOMETRY ANALYSIS WAS PERFORMED ON DAY 5.

2-2. Leukocyte Expansion Analysis

In the analysis of cell differentiation, the myeloid differentiation media was shown to increase the frequency of CD45+ cells that were leukocytes. The number of leukocytes was calculated by multiplying the flow cytometry acquired CD45+ percentage with their respective counted number of cells (%CD45*Cell Count = Number of Hematopoietic Cells). The frequency
of CD45+ cells had increased after differentiating the HPCs in myeloid media from 15% to 27% for the experimental 16L iPSC line (TABLE 3). The same trend was seen in the 18L iPSCs with a CD45+ frequency increase of 11% to 23%. The result of the experimental line correlated well with the 12E control cells – giving the expected CD45+ percentage increase of 17% to 78%. This suggests that CD45+ leukocytes did increase when cells were induced to differentiate in myeloid media. The key finding of this analysis demonstrates that the LAD-III iPSC lines were able to make CD45+ cells similar to the iPSCs from healthy donors.

**TABLE 3. %CD45+ HEMATOPOIETIC CELL ANALYSIS:** DATA FROM THIS TABLE WAS TAKEN FROM A SINGLE CELL CULTURE TIMELINE FOR EACH CELL LINE. ANALYSIS WAS PERFORMED ON THE HEMATOPOIETIC PROGENITOR CELLS (AT DAY 10) AND RE-ANALYZED FOR THE MYELOID CELLS (AT DAY 15).

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>CD45%</th>
<th>CELL COUNT</th>
<th>TOTAL CELL COUNT (ADJUSTED FOR CD45+)</th>
<th>FOLD CHANGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>12E</td>
<td>17%</td>
<td>150,000</td>
<td>24,900</td>
<td>1.22</td>
</tr>
<tr>
<td>16L</td>
<td>15%</td>
<td>296,000</td>
<td>44,992</td>
<td>1.42</td>
</tr>
<tr>
<td>18L</td>
<td>11%</td>
<td>308,000</td>
<td>34,804</td>
<td>1.49</td>
</tr>
</tbody>
</table>

Obtaining the adjusted CD45+ cell count demonstrates the proliferative capacity of the LAD-III iPSC derived hematopoietic progenitors. The data gave us the 16L and 18L iPSC lines’ hematopoietic progenitor to myeloid stage differentiation CD45+ increasing fold change of 1.42 and 1.49 respectively. This is similar to the trend seen on the 12E control cells which gave an increase of 1.22 fold. A line graph is shown below for easier visualization of the expansion ability of cells positive for CD45 in the 12E controls, 16L and 18L iPSC lines (FIGURE 16).
FIGURE 16. HPC VS MYELOID %CD45+ CHANGE: This line graph shows the expanding amount of CD45+ cells from the HPC stage to the myeloid stage of cell differentiation. The control and experimental iPSCs were able to similarly differentiate and expand their CD45 positive cells.

2-3. Morphological analysis of Differentiated Myeloid Cells

Cytospin smears of the derived myeloid cells showed a variety of cell types. The most frequent myeloid cell type from the differentiated cells were Neutrophils.
FIGURE 17. MYELOID CELL STAGE CYTOSPINS: BRIGHTFIELD CYTOSPIN IMAGES WERE TAKEN OF THE DIFFERENTIATED CELLS AT D5 OF MYELOID MEDIA INDUCTION. CELLS WERE STAINED WITH MAY-GRÜNwald–GIEMSA STAINING.

2-4. Evaluation of Myeloid Cell Activity in LAD-III iPSC derived Myeloid Cells

The CFU-C cultures from the myeloid differentiation stage did not form colonies because they were already matured blood cells. Although the assay was performed for this, no colony differentiations were visible. This was expected because only hematopoietic progenitors have the proliferative capacity to form colonies.
3. **Functional Assay Studies**

There are no available functional assay studies on iPSC derived human myeloid cells so the feasibility of the experiments carried out in these studies had to be initially verified with preliminary experiments. Some of these logistic studies include the effect of the cytokines-rich differentiation media on the cell's physiological functions and experimenting with cell plating density. The set-up of both the adhesion and migration functional assay were similar and this simplified the experiment data collection process.

3-1. **Study of Cell Adhesion Function**

A cell adhesion assay was performed to better understand the response of the differentiated myeloid cells when seeded on a fibrinogen (FBG) extracellular matrix substance. The leukocyte's integrins and adhesion receptors are responsible for cell adhesion to the matrix. An established *in vitro* model for cell adhesion was modified to accommodate iPSC adhesion and used to quantify and evaluate the number of cells adhering to fibrinogen. For each sample, the positive control wells were coated with Poly-L-Lysine (PLL) while the negative control wells were uncoated chambers (NC). In order to simplify the analysis of our results, comparisons were made between the adhesive FBG results and the non-adhesive NC results.
Peripheral Blood Control

Peripheral blood leukocytes were utilized to develop an adhesion protocol that tests the reliability of the assay. Peripheral blood was also included as a positive control in each experiment. It was the expected that the adhesion trend would give us cell adhesion in the FBG coated slides and little to no adhesion of cells on the NC slides. As expected the NC control group was significantly different for the experimental FBG group with a P value of 0.016 (unpaired T-test). The graph represents the amount of cells that adhered for every 50,000 cells plated (FIGURE 18). Experiment repetition also showed that the cell adhesion numbers in different experiments were similar or within close ranges of each other, as represented by the dot plot distances. The results proved that the normal donor control had the expected adhesive ability and the experiments can be reproduced across experiments.

**FIGURE 18.** NC VS FBG CELL ADHESION: A BAR GRAPH REPRESENTING THE OVERALL ADHESION MEAN ± SD (ERROR BARS) OF THE NO COAT CONDITION AGAINST THE FIBRINOGEN CONDITION OF NORMAL DONOR PERIPHERAL BLOOD CONTROL ACROSS EXPERIMENTS. THE PERIPHERAL BLOOD NORMAL CONTROLS HAS THE LOWEST ADHESION IN THE NO COAT CONDITION. THE DIFFERENCE IS SIGNIFICANT WITH *P=0.016 (UNPAIRED T-TEST).
3-1(b). Adhesion of Patient-derived LAD-III Differentiated Myeloid Cells

The functional assay for adhesion was modified to examine whether myeloid cell migration was affected in our experimental LAD-III differentiated myeloid cells. Four adhesion assay experiments were performed on different days with each sample performed in duplicate (n=4). As previously shown that the assay worked for a normal peripheral blood control, analysis of the LAD-III cell adhesive ability was taken based the average number of our four experiments. The numbers for each sample were corrected for the CD45+ cell frequencies at the time of initiating the assay and the results showed a general ability of our cell’s adhesive property. The Peripheral Blood control was not included in this comparison as it gave a 10-fold difference of cell adhesion numbers when compared to the iPSC control and experimental numbers. So for ease of visualization, the H9 PSC control sample values were compared with the two experimental iPSC lines. Adhesion of cells onto the fibrinogen coated slides were minimal in the experimental LAD-III iPSC differentiated myeloid cells (16L and 18L iPSC) across all experiments (FIGURE 19). The results show that the averaged adhesion ability of the experimental LAD-III myeloid cells were affected when compared to the H9 PSC control as it gave a lower amount of cell adherence. However, as there is such a wide variation between experiments (shown by the high error bars for standard deviation) there is no statistical difference with this number of experiments due to the small number of experiments (n=4). We can only generally conclude that the trend of adhesion showed that our experimental LAD-III cells had an adhesion deficiency.
As neutrophils are the most abundant of the leukocytes, comparison of the data normalized to CD15 neutrophils were compared across cell lines (FIGURE 20). The results gave a more obvious effect, where the two experimental LAD-III differentiated myeloid cell lines did not adhere as well as the H9 PSC control neutrophils. This can be seen by comparing the previous unpaired t-test p-values of the overall myeloid cell adhesion (above) to the just neutrophils cell adhesion numbers (below). This data showing that the p-values are getting close to significance reaffirms our generalization about the trend of adhesion for the experimental lines and they have a cell adhesion deficiency when compared with the H9 PSC control.
**FIGURE 20. CD15+ NORMALIZED ADHESION DATA:** The mean ± SD (error bars) of four adhesion assays comparing the adhesion of neutrophils (CD15) to the fibrinogen only condition. The number of adhered cells were normalized to the amount of CD15+ cells. An average of the H9 PSC control (blue) has higher numbers than the averages of the experimental LAD-III differentiated myeloid cells (red and yellow) across experiments. The difference between cell lines were not significant (unpaired t-test).
3-2. **Study of Cell Migratory Function**

In patients with LAD-III, neutrophils are unable to migrate to the site of infection, due to an integrin activation defect. This migratory ability was evaluated in our LAD-III iPSC differentiated myeloid cell with a modified transwell migration assay. The positive control of the experiment were cells directly plated into a well (just cells) while the negative control of the experiment were cells that were not induced to migrate by a chemo-attractant (-fMLP). This experiment evaluated the ability of plated myeloid differentiated cells to migrate towards the chemo-attractant (+fMLP).

3-2(a). **Peripheral Blood Control Analysis**

The peripheral blood control was expected to have cell migration occurring in the +fMLP condition and no cell migration occurring in the –fMLP condition. The expected trend was to see the occurrence of cell migration in the +fMLP condition while minimal to no cells should migrate in the –fMLP. The results in **FIGURE 21** shows significant differences between the –fMLP negative control group with the +fMLP experiment group (p-value: 0.0445; unpaired T-test). The results below suggested that peripheral blood was a feasible positive control as migration was higher in the +fMLP condition across experiments.
FIGURE 21: -fMLP VS +fMLP CELL MIGRATION: A BAR GRAPH REPRESENTING OVERALL MIGRATION MEAN ± SD (ERROR BARS) OF THE -fMLP CONDITION AGAINST THE +fMLP CONDITION OF NORMAL DONOR PERIPHERAL BLOOD CONTROL ACROSS EXPERIMENTS. THE PERIPHERAL BLOOD NORMAL CONTROLS HAS THE LOWEST MIGRATION IN THE -fMLP CONDITION. THE DIFFERENCE IS SIGNIFICANT WITH *P=0.0445 (UNPAIRED T-TEST).

3-2(b). Migration of Patient-derived LAD-III Differentiated Myeloid Cells

The experiments shown above for peripheral blood leukocytes demonstrated that the assay could successfully detect cellular migration towards the chemo-attractant. In the next experiments we evaluated the migratory ability of our experimental LAD-III cells. After the LAD-III and control cells were allowed 4 hours to migrate through the transwell in response to fMLP or vehicle control, the transwell inserts were removed and photomicrographs of the bottom of the 24-wells are presented below. Migration assay #10 was used as a representative example as shown below (FIGURE 22). There was little to no migration of cells in the iPS derived myeloid cell lines (16L and 18L iPSCs). As expected, the most cell migration occurred in the peripheral blood normal control. However, it was difficult to determine whether there was migration towards fMLP
in the H9 PSC differentiated myeloid cell control as the cell counts for both –fMLP and +fMLP conditions were similar. This pattern was observed for all experiments and is discussed further in the discussion section.

**FIGURE 22. MIGRATION ASSAY PHOTOMICROIMAGE:** A VISUAL REPRESENTATION OF THE 24-WELLS OF EACH CELL LINE AND CONDITION IMMEDIATELY TAKEN AFTER INCUBATED FOR 4 HOURS. THE PERIPHERAL BLOOD CONTROL HAD THE MOST MIGRATION OCCURRENCE, FOLLOWED BY THE H9 PSC CONTROL. LITTLE TO NO MIGRATION OCCURRED IN BOTH THE LAD-III DERIVED EXPERIMENTAL LINES. A STANDARDIZED 50,000 CELLS WERE PLATED FOR EACH CONDITION.
Once again, the migratory ability of our LAD-III cells were compared in a generalized way to easily show this functional assay. The cell counts normalized to its percent CD45+ was analyzed on two migration experiments to show whether cells migrated towards the +fMLP condition (FIGURE 23). The results show that this was true with the most cell migration occurring in the +fMLP condition. It was expected that low migration numbers would be seen for our 16L and 18L iPSC lines. The results below correlated well with the previous migration image result. However, as there is such a wide variation between experiments (shown by the high error bar for standard deviation) there is no statistical difference with this number of experiments due to the small number of experiments (n=3). A generalized conclusion can only be made as the trend of migration showed that our experimental LAD-III cells had a migratory deficiency.

For the migration assay, the flow cytometry data of our normalized CD15 neutrophils were compare again across cell lines (FIGURE 24). The results were similar to the CD45+ normalized data, where our two experimental LAD-III differentiated myeloid cell lines did not migrate as well as the H9 PSC control neutrophils. There is a significant difference for the migration of our 18L iPSC line compared to the H9 PSC control (p-value = 0.0468; unpaired T-test). As for the 16L iPSC line, the data shows that its p-value is close to significance. This data reaffirms a generalized trend of migration, showing that our experimental lines have a cell migration deficiency when compared to the H9 PSC control.

**FIGURE 24. CD15+ NORMALIZED MIGRATION DATA**: THE MEAN ± SD (ERROR BARS) OF THREE MIGRATION ASSAYS COMPARING THE MIGRATION OF NEUTROPHILS (CD15) TO THE +fMLP CONDITION. THE NUMBER OF MIGRATED CELLS WERE NORMALIZED TO THE AMOUNT OF CD15+ CELLS. AN AVERAGE OF THE H9 PSC CONTROL (BLUE) HAS HIGHER NUMBERS THAN THE AVERAGES OF THE EXPERIMENTAL LAD-III DIFFERENTIATED MYELOID CELLS (RED AND YELLOW) ACROSS EXPERIMENTS. IN COMPARISON WITH THE H9 PSC CONTROL GROUP, THE DIFFERENCE WAS NOT SIGNIFICANT FOR THE 16L iPSC LINE WHILE THERE WAS A SIGNIFICANT DIFFERENCE FOR THE 18L iPSC LINE WITH *P=0.0468 (UNPAIRED T-TEST).
DISCUSSION

The prospects of using iPSCs to model diseases is immeasurable although with its own challenges. There are still many fundamental questions that need to be answered about this technology so it is expected that lack of information or information disconnect is quite common. Establishing a differentiation protocol that worked for all cell types would be too complicated as no two PSC/iPSC lines have a standardized ability of differentiating. As it is natural for differentiation variation to occur between iPSC lines, established differentiation protocols that were used in the differentiation of these cells needed to be tested and catered specifically to each derived PSC or LAD-III iPSC lines. It was necessary to note down the effects that different cell plating densities, cell size and plating timing had on the ability of these iPSC differentiated hematopoietic cells to form.

Scalability of the hematopoietic cell differentiation was a pitfall that was discussed in the initial project proposal manuscript. It was expected that we could only produce differentiation numbers in the range of thousands for each cycle. Although frozen stocks of differentiated myeloid cells were made and kept aside for future experiments, the demand was still much higher than what was supplied. The solution was to reduce the functional experiments that utilized differentiated myeloid cells from triplicate into duplicate experiments. This does not affect the ability to make generalized outcomes from the quantified findings.

Our H9 PSC control group is our normal derived hematopoietic cell group that have been generated from embryonic stem cells (ESCs). The purpose of having this control group is to compare the ability of a normal PSC line with our defective LAD-III line. In theory, the H9 PSC line should be able to differentiate hematopoietic cells that are similar in function to a normal donor’s peripheral blood. This was not the case as our characterization analysis indicate that the H9 PSCs did not expand in the myeloid differentiation media as expected. Furthermore, they did not adhere or migrate as similarly to the normal donor peripheral blood controls. Although these
cells had been characterized to produce normal myeloid cell groups, their ability to function as equivalent cell types to the normal derived iPSC or peripheral blood myeloid cell controls were different. One explanation for this could be that the ESCs do not truly differentiate or function as comparable as we expect to the iPSCs. Studies have reported that there is some variation between the iPSCs and ESCs gene expression and differentiation propensity. However, as the H9 PSCs were the most readily available control cells of the time and they do have the pluripotent stem cell ability, we proceeded with using this cell type for our project. If given more time, I would have liked to modify the functional assay experiment controls and compare the experimental lines with iPSCs that are derived from normal donor blood cells (i.e. 12E iPSCs).

A generalized comparison works for my data set analysis as we performed the peripheral blood control study for each assay on the same day. This control study was carried out as a measure of confidence, to show that our positive control and assay worked as expected. This project required a lot of intensive technical work and the biggest limiting factor was manually quantifying the cells for the functional assays. The optimized experiment system required $5 \times 10^4$ cells per replicate and although the final wash removed many cells, the cells that still remained in these assays usually ranged in the numbers of $5 \times 10^2 - 2 \times 10^3$ cells. In order to capture the cells in its most unadulterated form and as a measure of recordkeeping, I imaged the cells for both functional assays with a microscope. The counts were manually done directly off the saved JPEG images as cell counting software (i.e: imageJ, OpenCFU) did not always accurately differentiate the cells. Manual counting also allowed for better discrimination of what were truly cells or just stain particles.

The results of this project supported our hypothesis that patient iPSCs for a hematological disease could be generated and differentiated into hematopoietic cells in vitro. Our findings were positively supported with the data acquired for the differentiated hematopoietic progenitor cells and myeloid cell’s colony differentiation capacity, surface markers and morphology. We were able
to show that the LAD-III patient derived and differentiated hematopoietic cells could be generated by their similarities to our established controls (12E iPSCs and normal donor peripheral blood).

As for the hypothesis of being able to model the functional defects of cell adhesion and migration in our differentiated LAD-III myeloid cells, our data supports this postulate with qualifiers. I have chosen to analyze the results with a general forecast of its cellular ability. Both the functional assay data were analyzed from a small sample size (n<4), so it would be inaccurate to apply true significance to the obtained adhesion and migration results. We can only generalize that the trend of cell adhesion and cell migration was affected in our LAD-III differentiated cells (16L and 18L iPSC lines). Of the four adhesion experiments, the experimental 16L and 18L iPSC lines had the similar trend of low adhesion when compared to the H9 PSC control. This was the same for the three migration experiments, where the 16L and 18L iPSC lines had a similar tendency of having low migration numbers when compared to the H9 PSC control. One reason for why the significance still remains inconclusive is due to the small sample size of our experimental data. However, if given the opportunity to further this project, I would like to improve the power of my experimentation by adding more samples to the study for both functional assays.

So in sum, hematopoietic cells were successfully differentiated from a LAD-III patient cell line and there was a general predisposition for low adhesion and migration numbers, based on the functional ability analysis.
SUMMARY

Our project findings support the following:

1. Patient induced iPSCs can be differentiated into hematopoietic cells
2. Patient induced iPSCs can be differentiated and expanded into various types of myeloid cells
3. The patient induced iPSCs generated myeloid cells that had deficiencies in their cellular ability to adhere and migrate normally
REFERENCES


