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Retinoic Acid Pathway Inhibition to Expand Human Hematopoietic Progenitor Cells with Islet Regenerative Capacity

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Graduate Program in Physiology and Pharmacology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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Abstract

Cellular therapy to induce islet regeneration is emerging as a novel treatment strategy for diabetes. Umbilical cord blood (UCB)-derived hematopoietic stem/progenitor cells (HSPC) isolated by high aldehyde dehydrogenase activity ($ALDH^{hi}$) reduce hyperglycemia after transplantation into streptozotocin (STZ)-treated NOD/SCID mice. However, UCB-derived $ALDH^{hi}$ cells are rare and expansion without the loss of regenerative function is required. We hypothesized that BMS 493, an inverse retinoic acid receptor agonist, will prevent HSPC differentiation of HSPC during expansion, generating more $ALDH^{hi}$ cells for therapy. $ALDH^{hi}$ cells expanded for 6 days with BMS 493 showed a 2.70-fold-increase in $ALDH^{hi}$ cells compared to untreated cells. Conditioned media from BMS 493-treated cells also increased human ß-cell proliferation *in vitro*. However, intrapancreatic transplantation of BMS 493-treated cells did not reduce hyperglycemia in STZ-treated NOD/SCID mice. Further characterization of HSPC expansion without differentiation is required for islet regenerative therapies.

Keywords

Diabetes, Hematopoietic Progenitor Cells, Retinoic Acid, Aldehyde Dehydrogenase, Stem Cells, Islet Regeneration

Co-Authorship Statement

All the data in this thesis was collected by Ruth Elgamal. Gillian Bell, Hess lab manager, contributed by transplanting expanded cells into hyperglycemic mice, helping with regular blood glucose measurements, and animal care. Madeline Harvey, Hess lab animal technician also helped with STZ-injections and animal care.

Acknowledgments

First, I would like to thank Laura Kennedy and Jennifer Ryder for their help in collecting umbilical cord blood samples at Victoria Hospital in London, Ontario.

Next, I would like to thank Kristin Chadwick at the London Regional Flow Cytometry Facility for all of your help with cell sorting and flow cytometry. Both were integral parts of my work and I would not have been able to complete my thesis without your time and support in learning these techniques and incorporating them into my project.

I would like to thank my family and friends for their support and encouragement throughout these past two years and for understanding how personal this work has been to me. I would also like to thank the other graduate students in Dr. Hess' lab, Tyler Cooper, Miljan Kuljanin, and Stephen Sherman. You have all challenged me to become a better scientist and I am grateful for everything I have learned from you both inside and outside of the lab.

I would like to thank Gillian Bell our lab manager for helping with the *in vivo* mouse models used in this thesis. This portion of my project would not have been possible without your time and technical skills, including performing all intrapancreatic transplants. I would also like to thank Madeline Harvey, Hess lab animal technician, for helping with animal care.

Thank you to my advisory committee, Dr. Thomas Drysdale, Dr. Cheryle Seguin, and Dr. Ruud Veldhuizen for helping to shape my project and provide feedback throughout the entire process that helped to propel my project forward.

Thank you to my supervisor Dr. David Hess for the opportunity to work in this lab. Your encouragement over the past two years has been monumental to my growth as a scientist.

Finally, thank you to the Juvenile Diabetes Research Foundation for funding this project.

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List of Abbreviations

AGE – Advanced glycosylation end-products

ALDH – Aldehyde dehydrogenase

ANOVA – Analysis of variance

APC – Allophycocyanin

AUC – Area under the curve

BFU-E – Blast forming unit-erythroid

BM – Bone marrow

BMS 493 – (E)-4-[2-[5,6-Dihydro-5,5-dimethyl-8-(2-phenylethynyl)naphthalen-2-yl]ethen-1-yl]benzoic acid, 4-[(1E)-2-[5,6-Dihydro-5,5-dimethyl-8-(phenylethynyl)-2-naphthalenyl]ethenyl]-benzoic acid

BV – Brilliant Violet™

CD – Cluster of differentiation

CFU-G – Colony forming unit-granulocyte

CFU-M – Colony forming unit-macrophage

CFU-GM – Colony forming unit- granulocyte, monocyte

CFU-GEMM – Colony forming unit- granulocyte, erythrocyte, monocyte, megakaryocyte

CLP – Common lymphoid progenitor

CMP – Common myeloid progenitor

CRABP – Cytosolic retinoic acid binding protein

CVD – Cardiovascular disease

DEAB – N,N-diethylaminobenzaldehyde

DM – Diabetes mellitus

EdU – 5-ethynyl-2′-deoxyuridine

eNOS – Endothelial nitric oxide synthase

ES – Embryonic stem cell

FACS – Fluorescent activated cell sorting

FBS – Fetal bovine serum

FITC – Fluorescein isothiocyanate

FLT-3L – FMS-like tyrosine kinase 3 ligand

GAD – glutamic acid decarboxylase

GLUT – Glucose transporter

GMP – Granulocyte-macrophage progenitor

GMLP – Granulocyte-macrophage-lymphocyte progenitor

hES – Human embryonic stem cell

HLA – Human leukocyte antigen

HUVEC – Human umbilical vein endothelial cell

HSC – Hematopoietic stem cell

HSPC – Hematopoietic stem or progenitor cell

IEQ – Islet equivalent

IMDM – Iscove's modified Dulbecco's medium

iPan – Intrapancreatic

IV – Intravenous

Lin – Lineage

MEP – Megakaryocyte-erythrocyte progenitor

MNC – Mononuclear cell

M.O.M. – Mouse on mouse

MPP – Multipotent progenitor

MSC – Multipotent stromal cell

NK – Natural killer cell

NO – Nitric oxide

NOD – Non-obese diabetic

PBS – Phosphate buffered saline

PE – Phycoerythrin

PECAM – Platelet endothelial cell adhesion molecule

RA – Retinoic acid

RAR – Retinoic acid receptor

RPMI – Roswell Park Memorial Institute

RXR – Retinoid X receptor

SCF – Stem cell factor

SCID – Severe combined immunodeficiency

STZ – Streptozotocin

TPO – Thrombopoietin

T1DM – Type one diabetes mellitus

T2DM – Type two diabetes mellitus

UCB – Umbilical cord blood

VEGF – Vascular endothelial cell growth factor

vWF – von Willebrand factor

ZNT – Zinc transporter

7-AAD – 7 aminoactinomycin D

1 Introduction

1.1 Diabetes Mellitus: Incidence and Current Treatments

While the majority of the cells in the pancreas are exocrine in nature and aid with digestion, 1-2% of pancreatic cells reside in endocrine structures called islets of Langerhans. Glucose homeostasis in the body is largely regulated by the hormone insulin, secreted by ß-cells within islets. Insulin is a 51-amino acid protein hormone that regulates the uptake of glucose into different tissues in the body via the action of insulin-dependent glucose transporters GLUT1 and GLUT4[1, 2]. Insulin binds an insulin receptor tyrosine kinase on peripheral tissue cells leading to autophosphorylation and phosphorylation of targets and ultimately leading to translocation of vesicles containing glucose transporters to the cell membrane[3]. Diabetes mellitus (DM) is a metabolic disorder characterized by poor glycemic control leading to excess glucose found in the blood. In contrast, diabetes insipidus is caused by a relative decrease in antidiuretic hormone or vasopressin in the body leading to polyuria and symptoms similar to those found in patients with diabetes mellitus[4]. When left uncontrolled, DM can lead to complications including cardiovascular disease (CVD), neuropathy, and renal failure[5, 6].

1.1.1 Type One Diabetes Mellitus

There are two main types of DM (referred to as diabetes moving forward) as well as several less common types such as gestational diabetes. Type one diabetes mellitus (T1DM) is caused by the autoimmune destruction of insulin-producing ß-cells (Figure 1)[7]. T1DM represents approximately 10% of all DM cases and is often referred to as early onset or juvenile diabetes[8]. Although diagnosis of T1DM is most common during childhood, juvenile diabetes can be a misnomer as adults can be diagnosed with autoimmune diabetes as well. The incidence of T1DM varies greatly across different regions, with highest prevalence in Northern Europe and lowest prevalence in Japan[9-11]. The lack of insulin production found in T1DM causes chronic hyperglycemia that leads to immediate symptoms such as dehydration, polyuria, and ultimately to long term complications such as CVD, neuropathy, and nephropathy. While there are genetic predispositions for T1DM, including specific HLA-DR and HLA-DQ haplotypes, DM is

a multifactorial disease and it is likely that environmental factors such as timing of introduction to food and exposure to gestational viruses also play a role[7, 12-14]. T-helper ($CD4^+$) and cytotoxic T-lymphocytes ($CD8^+$) mediate the destruction of ß-cells via direct cell toxicity and autoantibodies generated towards epitopes on ß-cells such as glutamic acid decarboxylase (GAD65) and zinc transporter 8 (ZNT8) [8, 15, 16]. ß-cell autoantigens are presented to B-lymphocytes that produce islet-targeting autoantibodies[17]. These autoantibodies may or may not present at different stages in the pathogenesis of T1DM, but are necessary in the etiology of the disease[18]. Cytotoxic ($CD8^+$) T-cells mediate the destruction of ß-cells based on autoantigen presentation by B-lymphocytes and dendritic antigen presenting cells [15, 17].

The discovery of insulin by Sir Frederick Banting in 1921 revolutionized treatment for patients with T1DM via exogenous insulin therapy[19]. After discovering that total pancreatectomy in dogs induced diabetes, Banting and his colleague Charles Best set out to find the constituent in pancreatic extracts that allowed the pancreas to regulate glucose metabolism. With the help of biochemist James Collip, Banting and Best removed digestive enzymes from fetal calf pancreata to isolate a protein that was later injected into dogs with diabetes. Only one year following their discovery, insulin was injected into the first human with diabetes, 14-year-old Leonard Thompson[20]. While insulin derived from bovine and porcine sources were commonly used for many years, most types of insulin on the market today are synthesized using recombinant DNA technology produced in bacteria or yeast[21, 22].

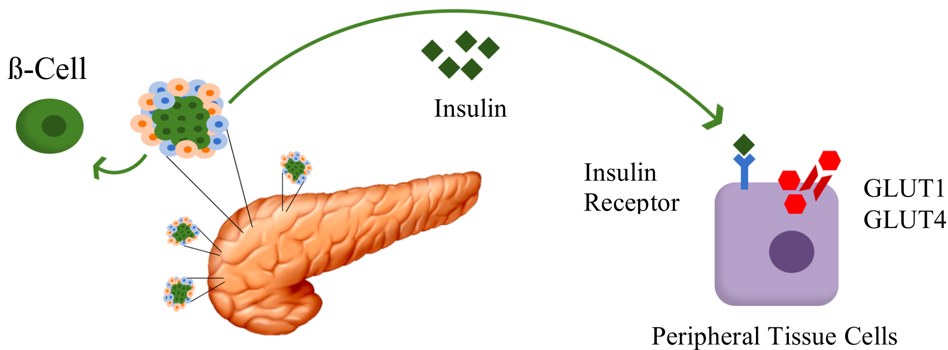
There are several different types of insulin that can be used alone or in combination. Insulin is categorized based on its mode of action ranging from rapid-acting insulin aspart to long-acting insulin glargine[23]. The difference in a single amino acid can change the onset of action or duration of the insulin. The two main methods of insulin delivery in patients with T1DM are multiple daily injections or continuous insulin delivery through an infusion pump. Regardless of the type of insulin delivery, glucose homeostasis remains problematic with the potential for life-threatening hypo and hyperglycemic events as well as risk of diabetic ketoacidosis. Due to the peaks and

troughs in glucose metabolism over time, patients with T1DM are at risk for many complications including cardiovascular disease, nephropathy, and neuropathy.

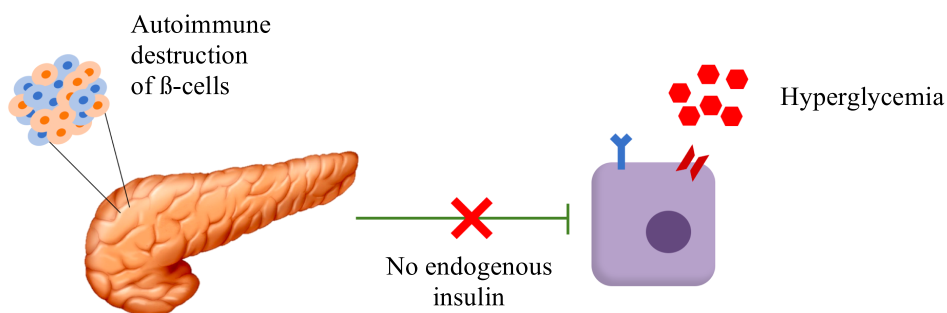
1.1.2 Type Two Diabetes Mellitus

In contrast to T1DM, T2DM is a metabolic disorder where patients usually experience peripheral insulin resistance, the inability of tissues such as muscle to efficiently respond to insulin and excess glucose found in the blood (Figure 1.1)(24). T2DM represents over 90% of all patients with diabetes, with prevalence increasing to epidemic rates due to an aging population demographic and increasing rates of obesity(8, 25). As stated by the American Diabetes Association, inability to respond to insulin can result in both pathological and physiological changes in the peripheral tissues over time(26). Insulin resistance can lead to a loss of ß-cell mass and decreased production of insulin over time, although there is no autoimmune destruction of pancreatic ß-cells as seen in T1DM. Surprisingly, there is a greater genetic predisposition to T2DM than T1DM, though many patients with the disease are considered obese and live a sedentary lifestyle(27). While some patients can reduce or even reverse the symptoms of hyperglycemia by exercise and weight loss, there are several different classes of drugs that can be used to treat T2DM, generally mediated by increasing insulin availability in peripheral tissue or reducing glucose reuptake in the kidneys. Some of the most widely used drugs are metformin, sulfonylureas, thiazolidinediones, and glucagon-like peptide 1 (GLP-1) inhibitors. Metformin reduces the liver's conversion of stored glycogen to readily available glucose, while drugs such as sulfonylureas and thiazolidinediones act directly to increase insulin secretion from the pancreas or increase insulin sensitivity by peripheral tissues, respectively(28). More recently, SGLT2 inhibitors such as empagliflozin have been used in patients with T2DM. This class of drugs act by inhibiting glucose co-transporters in the kidney, thus preventing the reabsorption of glucose(29). Ultimately, patients with T2DM may require exogenous insulin to meet the metabolic demands of the body. Both T1DM and T2DM together raise a serious health care concern and huge economic burden due to the development of severe, chronic complications.

Non-Diabetic



T1DM



T2DM

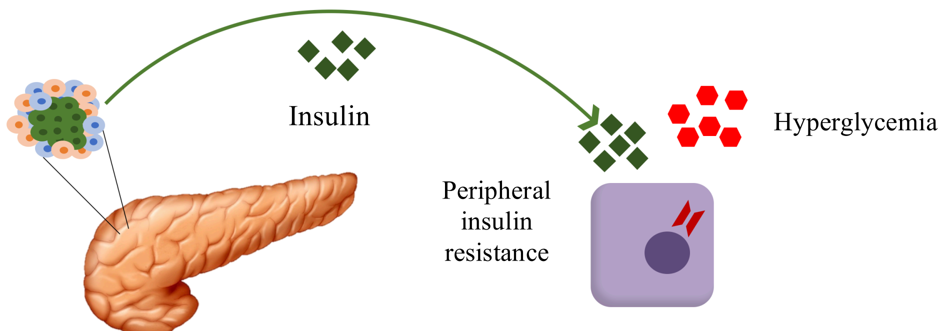


Figure 1.1. Underlying cellular mechanisms behind T1DM and T2DM. Schematic showing the differing pathophysiology of the two main types of DM. In T1DM patients, autoimmune destruction of the ß-cells in the pancreas prevents endogenous insulin production leading a lack of insulin-dependent glucose uptake in peripheral tissue cells. In T2DM patients, machinery for endogenous insulin production in the pancreas is intact; however, peripheral tissue cells are unable to respond to insulin. Both T1DM and T2DM lead to chronic hyperglycemia over time.

1.1.3 Prevalence of Diabetes

In 2016, the International Diabetes Federation estimated there were over 400 million people worldwide living with diabetes and this number is expected to increase to 642 million by the year 2040. The global cost of diabetes including treatments, prevention strategies, and complications is over 600 billion USD with countries spending up to 20% of their health care costs on diabetes alone. In 2012, the United States was estimated to have spent over 245 billion USD on diabetes and diabetes related costs[(25)]. Current treatment options remain inadequate, resulting in glucose fluctuations and patients often require extremely regimented schedules for medication. Thus, there is a desperate need to develop more curative therapies for patients with both type 1 and type 2 diabetes to reduce co-morbidities and the associated complications.

1.2 Complications of Diabetes

Regardless of the type of diabetes, prolonged hyperglycemia in the body can lead to several long-term complications of the cardiovascular system. These are divided into two main groups, microvascular and macrovascular complications, and both groups of complications are attributed to the effects of excess glucose on the blood vessels found in different parts of the body. Microvascular complications converge on the endothelial cell lining of blood vessels that regulate vascular processes such as dilation and contraction and transportation of nutrients by producing bioactive compounds such as nitric oxide (NO) and angiotensin II[(30)]. Excess glucose found in the blood stream of patients with DM can be toxic to the endothelium and its signaling processes. For example, when the response to different vasoconstrictors and vasodilators was tested, T1DM patients had impaired NO-mediated vasodilation compared to non-diabetic controls and similar results were seen in patients with T2DM[(31)]. Hyperglycemia can also block the action of endothelial nitric oxide synthase (eNOS), the enzyme responsible for producing NO. Interestingly, there was no difference between patients with DM and non-diabetic individuals in the response to vasodilators such as verapamil that act on smooth muscle cells instead of endothelial cells[(32)]. Collectively, these studies give evidence that chronic hyperglycemia found in patients with DM adversely affects endothelial cells and their signaling mechanisms. Damage to the microvasculature is responsible for complications

such as retinopathy, neuropathy, and nephropathy. In the United States, for example, diabetic nephropathy is the leading cause of renal failure.

Macrovascular complications, such as atherosclerotic cardiovascular disease (CVD), represent the primary cause of death in patients with both T1DM and T2DM. In fact, T1DM patients are 2 – 5 times more likely to be diagnosed with CVD than non-diabetic individuals[33]. Advanced atherosclerosis is defined as fibrous plaques found in the vasculature that are often surrounded by smooth muscle cells and connective tissue and contain variable amounts and types of lipids[34]. Atherosclerosis is the main cause of macrovascular complications, although it is not fully understood why diabetic patients are at a greater risk. For T2DM patients, the greater risk of macrovascular CVD can be in part explained by the fact that most patients are also facing other risk factors such as obesity and dyslipidemia. However, blood pressure is often monitored in patients with diabetes and drug therapy such as ACE inhibitors are often prescribed as a precautionary measure to avoid hypertension, another risk factor for atherosclerosis[35]. Statins and anti-inflammatories are also prescribed to prevent the development of atherosclerosis in patients with T2DM[36]. Furthermore, studies have shown that improved glycemic control in patients with T1DM can reduce the risk of macrovascular complications.

There are many proposed mechanisms to explain cell injury and progression of both macrovascular and microvascular diseases in patients with DM. One pathway that has been extensively studied is the polyol pathway[35, 37]. Aldose reductase is used to convert excess glucose into inactive alcohols or sorbitol. Increased flux of glucose through this pathway can increase reactive oxygen species and decrease reduced glutathione, which acts as an intracellular antioxidant. These outcomes are associated with alterations in basement membranes and the number of pericytes surrounding vessels. The accumulation of advanced glycosylation end-products (AGE) and their role in microvascular diseases have also been heavily studied. AGE describe a group of compounds formed when reducing sugars react with proteins or lipids and patients with DM have a high susceptibility of accumulating AGE[35, 38]. Damage to specific proteins associated with different microvascular diseases have been attributed to the formation of

AGE[39]. Regardless of the mechanisms at play, chronic hyperglycemia found in DM negatively impacts the endothelium, resulting in serious, life-threatening complications.

1.3 Islet Replacement Strategies for Treating Diabetes

Whole pancreas transplantation has been studied in T1DM patients who are concurrently receiving a kidney transplant due to renal failure[40]. Although this procedure is rare due to the difficult surgical procedure and a high risk of complications, much was learned from the clinical outcomes of these studies[41]. Survival rates of patients 1-year post transplantation were high with good pancreas engraftment, although patients did require immunosuppressive therapy[42]. Overall, pancreas transplantation is considered extremely risky and is not a recommended treatment for the majority of patients with DM.

In the year 2000, a Canadian group lead by James Shapiro was the first to transplant cadaveric islets into brittle T1DM patients, a trial commonly known as the Edmonton Protocol. Patients received immunosuppressive therapies including sirlimus, tacrolimus, and daclizumab before hepatic portal vein transplantation of 11,547±1604 islet equivalents per kilogram of body weight[43]. Of the seven patients enrolled in the initial trial, all remained free of exogenous insulin for a minimum of one year, a result that was found to be promising. However, as the trial expanded, there were several acute complications, patients required immunosuppressant agents, and low engraftment levels as islets were transplanted into the liver[44, 45]. Moreover, the transplants failed over time with the requirement for exogenous insulin returning. Ultimately, there is an immense shortage of cadaveric donors to treat T1DM patients, with each patient needing an average of two donors. Despite these shortcomings, the Edmonton Protocol was important in establishing proof of concept that islet transplantation could reduce hyperglycemia in patients with brittle T1DM.

1.4 Stem Cell Types Relevant to Diabetes Therapy

Stem cells may represent a powerful approach for the development of a curative therapy as there is a need for an unlimited source of ß-cells to treat T1DM. Pioneering experiments and the discovery of stem cells came from two Canadian scientists James Till and Earnest McCulloch in 1961[46]. Till and McCulloch established that stem cells have two founding properties: the ability to self-renew and to differentiate to more specified, committed cell type. In particular, Till and McCulloch studied the capacity of murine bone marrow (BM) cells to reconstitute the hematopoietic system in supralethally irradiated host mice after intravenous (IV) transplantation. They noticed that there was a correlation between the number of mice that survived treatment and the dose of BM cells that was transplanted. They also observed that mice were less likely to survive if the donor BM cells were irradiated prior to transplantation. Interestingly, they analyzed hematopoietic colonies, or nodules, that formed in the spleen of mice and suggested that the progeny of these cells migrated to hematopoietic organs such as the BM, where they were able to engraft and proliferate. Their observation is an example of tissue-resident adult stem cells; however, the field has advanced and various sources of stem cells with distinct functional capacities are used in research today.

There are three broad categories of human stem cells: adult stem cells, embryonic stem (ES) cells, and induced pluripotent stem (iPS) cells. Although the use of ES cells is controversial and raises ethical issues, they arguably represent the most powerful of the three stem cell populations. hES cell lines were first derived by James Thompson in 1998[47]. hES cells were derived from the inner cell mass of the blastocyst. hES cell lines are typically derived from excess blastocysts generated by *in vitro* fertilization clinics. During development, three germ layers that form the entire organism are derived from the cells within the inner cell mass: endoderm, ectoderm, and mesoderm. Thus, ES cells from the inner cell mass retain the capacity to generate any cell type in the body and are referred to as pluripotent. ß-cell replacement therapies using differentiated hES cells to treat DM are currently underway and are further discussed in section 1.5.1.

iPS cells allow researchers to revert mature cell types back to pluripotent cells using a cocktail of transcription factors, commonly known as Yamanaka factors. In 2006,

Takahashi and Yamanaka were able to convert adult mouse fibroblasts into ES-like cells using Oct3/4, Sox2, c-Myc, and Klf4[48]. These four transcription factors had been shown to play a role in maintaining pluripotency in ES cells, and additionally, mouse iPS cells from fibroblasts displayed teratoma formation when transplanted into healthy mice. The use of iPS cells in therapies would allow for autologous pluripotent cell transplantation, with patients having the ability to receive stem cells derived from their own somatic cells, overcoming the need for aggressive immunosuppressive therapy. That being said, while there have been preclinical trials using iPS cells for the treatment of diabetes, there are currently no clinical trials due to the potential risk of tumor formation during the reprogramming process[49-51]. Precise and efficient differentiation of iPS cells into ß-cells must be ensured before iPS cells can be implemented in clinical trials.

1.5 Cellular Therapies for Diabetes

Cellular therapies are emerging as a novel treatment strategy for patients with diabetes. These strategies are often divided into two categories, replacement therapies and regenerative therapies. Replacement therapies have focused on increasing insulin supply by transplantation of cadaveric islets or stem cells that have been differentiated into ß-like cells. Several groups have mimicked *in vivo* differentiation of ß-cells during development and have used a cocktail of cytokines or transcription factors to direct the differentiation of pluripotent hES cells into ß-cells that are able to produce levels of insulin similar to those produced by healthy individuals. These protocols are extremely specific and somewhat inefficient, but effectively differentiate cells from definitive endoderm to committed endocrine progenitor. While the directed differentiation of ß-cells from hES cells or iPS cells is quite well understood, these therapies have yet to overcome the hurdle of autoimmunity found in T1DM, making patients susceptible to the same immune attack and often in need of immunosuppressant therapies.

1.5.1 Cell Types Involved in Islet Replacement Therapies

Research has tried to mimic developmental signals during embryogenesis to differentiate iPS and hES into insulin producing cells that can be transplanted into patients with diabetes. During organogenesis, the entire pancreas including exocrine,

endocrine, and ductal epithelial cells is derived from definitive endoderm. After the definitive endoderm has formed the primitive streak, differentiation of various pancreatic cell types are driven by the presence or absence of many transcription factors. For example, all cells in the developing pancreas express pancreatic and duodenal homeobox 1 (Pdx1), but only properly differentiated, mature ß-cells co-express homeobox protein Nkx-6.1, homeobox protein Nkx-2.2, and most importantly, insulin. D'Amour *et al.* first established a protocol to generate endocrine cells from hES cells(52). While they were able to differentiate hES into hormone positive cells *in vitro*, these cells were poorly glucose responsive. When the same group transplanted these cells into streptozotocin-treated (STZ) NOD/SCID mice, after 16 weeks they were able to detect c-peptide and insulin release in response to a glucose load(53). More recently, Timothy Kieffer's group has derived insulin producing cells from other sources such as K cells in the digestive tract. These cells are already glucose-responsive in their release of gastric inhibitory polypeptide and can be engineered to secrete insulin(54). Additionally, these cells may be able to escape the autoimmune attack found in patients with T1DM. Unfortunately, producing glucose responsive ß-cells is only the first hurdle in creating a stem cell replacement therapy to treat diabetes.

More recently, groups have been working on the transplantation of further differentiated ß-cells from both ES and iPS cell sources within encapsulation devices. An encapsulation device can allow protection from the immune system of patients with T1DM while allowing insulin to be secreted into nearby vasculature. These devices are often transplanted under the skin and thus can easily be removed or replaced. Clinical trials are ongoing; however, there are still many hurdles to overcome such as reducing the number of encapsulation devices needed to obtain sufficient serum insulin levels, ensuring adequate vascularization of these devices for efficient insulin delivery throughout the body, and prevention of device directed inflammatory response due to continued autoimmune attack. Although replacement strategies are leading in the clinic, this thesis focuses on the induction of islet regeneration *in situ* using regeneration initiating cell therapy.

1.5.2 Cell Types Involved in Endogenous Islet Regeneration

In 2010, a study conducted by Keenan *et al.* looked at a cohort from this study were extremely surprising. Firstly, 67% of patients in the study had detectable c-peptide levels, a by-product of endogenous human insulin production. Notably, c-peptide levels were assumed to be underestimated as exogenous insulin was continually administered and patients were not stimulated to secrete insulin. Secondly, post-mortem analysis was conducted on the pancreata of several Medalists. All pancreata studied showed the presence of insulin positive cells, although these cells were not usually detected in islet-like structures. Finally, histological analysis on the pancreata of several patients contained insulin positive, Ki67 positive cells, indicating proliferation of ß-cells within the pancreas in the face of ongoing autoimmunity. These results were the first to suggest that patients with T1DM possess residual ß-cell function and low-levels of insulin production despite ongoing autoimmune attack. Therefore, endogenous islet repair may prove to be a potential mechanism to increase ß-cell mass *in situ*. Thus, the Medalist study provided rationale for the study of regeneration inducing therapies combined with the dampening of autoimmunity as a new strategy for treatment of diabetes.

Most organs in the adult human body contain rare tissue resident stem cells with some capacity to regenerate. Organs such as the liver and intestine show high cell turnover rates and high regenerative capacity while other organs such as the brain and the endocrine pancreas show extremely low cell turnover. Although stem cell populations reside in many organ systems throughout the body, some of which are more easily accessible or renewable and can be used with little ethical controversy. Two sources of adult stem cells that have been heavily researched in the context of therapies for diabetes are BM and umbilical cord blood (UCB). Since Till and McCulloch’s discovery in 1961, two types of stem cells found in BM that have been highly studied: (1) multipotent stromal cells (MSC) that have the ability to differentiate into osteocytes, chondrocytes, and adipocytes and (2) hematopoietic stem cells (HSC) that have the ability to form the mature cell types found in blood. Similarly, UCB is rich in HSC and MSC, and provides a readily available supply of cells for the development of cell therapies. Interestingly, it is becoming increasingly popular for families to bank UCB either publically or privately for

use in the future. The potential for adult stem cells for novel human cell therapies has attracted much attention in preclinical studies.

There have been several preclinical trials conducted using BM-derived cells for the induction of islet regeneration in diabetic recipients[(55-57)]. In 2003, Ianus *et al.* was one of the first groups to publish studies using BM-derived cells in the context of diabetes[(58)]. BM cells isolated from male mice were labeled with eGFP in a Cre-Lox system activated by insulin gene expression. Irradiated female mice received approximately $1x10^6$ BM through tail vein injection. Following transplantation, Y-chromosome, $eGFP^+$ cells were isolated from pancreatic islets that co-expressed insulin and GLUT2. Collectively, these findings suggested that BM cells were able to transdifferentiate into ß-cells after transplantation *in vivo*. However, other groups studying the effect of BM-derived cells on the pancreas were unable to recapitulate the rare transdifferentiation events observed by findings of Ianus *et al.*

Choi *et al.* transplanted BM-derived cells into recipient hyperglycemic mice that received low dose STZ for 5 days[(59)]. Although the cellular transplants contained a mix of primitive and mature hematopoietic cells and MSC, after 5 weeks, mice blood glucose levels and insulin secretion recovered at almost normal levels. GFP transgenic mice provided BM cells that were used to trace donor cells engrafting in the pancreas and it was concluded that there was no transdifferentiation of BM cells to insulin positive islet cells. Alternatively, the functional response was stimulated by donor cells inducing islet regeneration. Also in 2003, Hess *et al.* showed that BM-derived hematopoietic stem/progenitor cells (HSPC) isolated based on c-kit expression, a marker of murine HSPC, were able to reduce hyperglycemia in STZ-treated NOD/SCID mice after transplantation[(57)]. Again, few of the donor cells were insulin positive and GFP^+/$insulin^+$ cells were not present at the time of glucose reduction, strengthening the argument that this cell population can play a role inducing endogenous islet regeneration. BM continued to be a popular source of cells for regenerative therapies for diabetes since, in addition to the aforementioned islet regenerative properties of BM cells, many groups have also studied the immunosuppressive properties of BM cell populations.

Later, Matthews *et al.* studied the role of BM-derived endothelial progenitor cells (EPC) that respond to STZ-induced pancreatic injury. While these studies did not increase the loss of ß-cell mass induced by STZ-treatment, they were able to show that BM-derived EPC could be recruited to the site of tissue injury (pancreas) and could aid in vascularization of the injured tissue[60]. Not only did transplanted BM-derived EPC play a role in pancreas vascularization, but resident EPC were activated to take part in endogenous islet repair. In 2006, Lee *et al.* demonstrated that human BM-derived MSC could reduce STZ-induced hyperglycemia in NOD/SCID mice after intracardiac infusion on two separate occasions[61]. Serum insulin concentration was increased in mice transplanted with MSC compared to controls, and while human cells were detected in the islets on occasion, human insulin was not detected by ELISA. Urban *et al.* looked at the immunosuppressive qualities of BM-derived MSC in treating diabetes[62]. BM cells were able to reduce hyperglycemia in STZ-induced hyperglycemic mice, but the authors suggested that MSC could also play an immune-protective role from the cytotoxic $CD8^+$ cells identified in the autoimmune attack during T1DM. Recently, Li *et al.* showed reduced hyperglycemia and increased serum insulin after intrapancreatic (iPan) transplantation of BM-derived MSC into STZ-treated rats[63]. Together, these findings represent several independent cell types and unique mechanisms by which BM-derived cells can be used to induce endogenous islet regenerative processes.

1.5.3 HSPC for Islet Regenerative Therapies

BM-derived HSPC represent one of the most widely studied stem cell populations. These cells have the ability to self-renew and differentiate into all the lineages of the hematopoietic system. BM-HSPC are also believed to have proangiogenic effects, a trait that makes them a popular candidate for revascularization therapy and for use in patients with diabetes due to the high risk of vascular complications associated with the disease[62]. CD34 is a cell surface marker encoding the saliomucin protein and is most widely known as a marker of human hematopoietic progenitor cells[64]. Not only are $CD34^+$ cells are rare to find in peripheral blood, cells that are positive rapidly lose expression of CD34 during *in vitro* expansion[65]. However, HPSC isolated based on CD34 expression have shown the ability to reconstitute the hematopoietic system when

transplanted into sub lethally irradiated NOD/SCID mice. More recently, groups have been isolating HSPC that co-express different cell surface markers with or without CD34 such as CD133, CD90, and CD49f, representing a more primitive hematopoietic cell for use in regenerative therapies[66, 67]. This thesis focuses on identifying and expanding an islet regenerative hematopoietic stem progenitor cell (HSPC) population from UCB to mediate the induction of endogenous islet repair.

1.5.4 Clinical Trials for Diabetes Using Cellular Therapies

Voltarelli *et al.* performed the first reported clinical trial using HPSC transplantation into T1DM patients in 2007. Patients received high dose immunosuppressive therapies before taking cyclophosphamide and granulocyte colony stimulating factor. These agents act to mobilize HPSC into the peripheral blood so that they can be collected by apheresis for autologous transplantation[68]. Interestingly, newly diagnosed T1DM patients receiving infusion of autologous apheresis product were able to prolong the requirement for exogenous insulin for 14.8 ± 11.2 months, and up to 35 months in one case[69]. Notably, GAD autoantibody levels were significantly decreased 6-months post treatment, suggesting a dampening of autoimmunity. Mean cell number reinfused into patients using this autologous approach was 11.0×10^6 cells/kg body weight. Unfortunately, stem cell populations in patients with DM are often damaged or depleted due to chronic hyperglycemia, making autologous cell harvest and transplantation challenging. Results from this clinical trial suggested isolation of HSPC from a non-diabetic donor and expansion of healthy HPSC *in vitro* may be required to reach clinically applicable numbers for regeneration-inductive therapy. Thus, there is a need for HSPC from other sources such as UCB, followed by HSPC expansion.

The biotechnology company Osiris Therapeutics, Inc. was approved for a phase II clinical trial using MSC from unrelated donors. Newly diagnosed T1DM patients (<20 weeks from diagnosis) IV injected with Prochymal®, a mixture of BM-derived MSC from multiple donors that were cultured *ex vivo* for a minimum of 5 passages, as per good manufacturing practice (GMP) standards[70]. The goal of the trial was to reduce inflammation in the pancreas and dampen the autoimmune attack found in T1DM, allowing pancreatic tissue to repair itself. 60 T1DM patients were enrolled in the study

and in 2012, Osiris Therapeutics released an update after the one year mark of the trial. Although no patients experienced a reaction from the IV injection of Prochymal®, there was no delay in progression of T1DM in the newly diagnosed patients. Unfortunately, c-peptide levels between patients who received the active cell therapy and patients who received placebo were not significantly different and the trial has since been stopped[71].

Finally, in 2014, ViaCyte Inc. was approved for phase I/II clinical trials of their combination therapy (VC-01™) for T1DM patients. Their therapy combined the differentiation of human ES cells into committed pancreatic endoderm (PEC-01™) with Encaptra®, a device providing immunoprotection for transplanted cellular therapies[72]. Notably, human ES cells were not differentiated into mature ß-cells prior to transplantation, but rather the completion of differentiation was to occur *in vivo*[73]. Although the trial is still ongoing, there are expected to be many hurdles to overcome including ongoing rejection of the human ES cells, inefficient maturation of pancreatic endoderm progenitor cells to insulin producing ß-cells, and the lack of vasculature within and surrounding transplanted encapsulation devices to transport insulin to the periphery[74].

1.6 Hematopoiesis and Hematopoietic Progenitor Cells

Hematopoiesis is the process during embryology and continuing in adult life that forms the blood system of an organism. It is a highly conserved and regulated process and has been used as a model system to study the continual generation of mature effector cells in the hematopoietic system are typically short-lived. During embryogenesis, hematopoiesis begins in the aorta-gonad mesonephros region where the yolk sac develops. The fetal liver later takes over the process, finally ending in the BM where HSC are believed to reside at birth and remain throughout adult life[75]. Immunodeficient mouse models such as the non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mouse have been used to study the ability of HSC to reconstitute multiple lineages of the hematopoietic system (Figure 1.2)[76-78]. Our understanding of hematopoiesis from these models has translated into astonishing treatments for cancers of the blood and aplastic anemia, primarily human BM transplants from HLA-matched donors[79, 80].

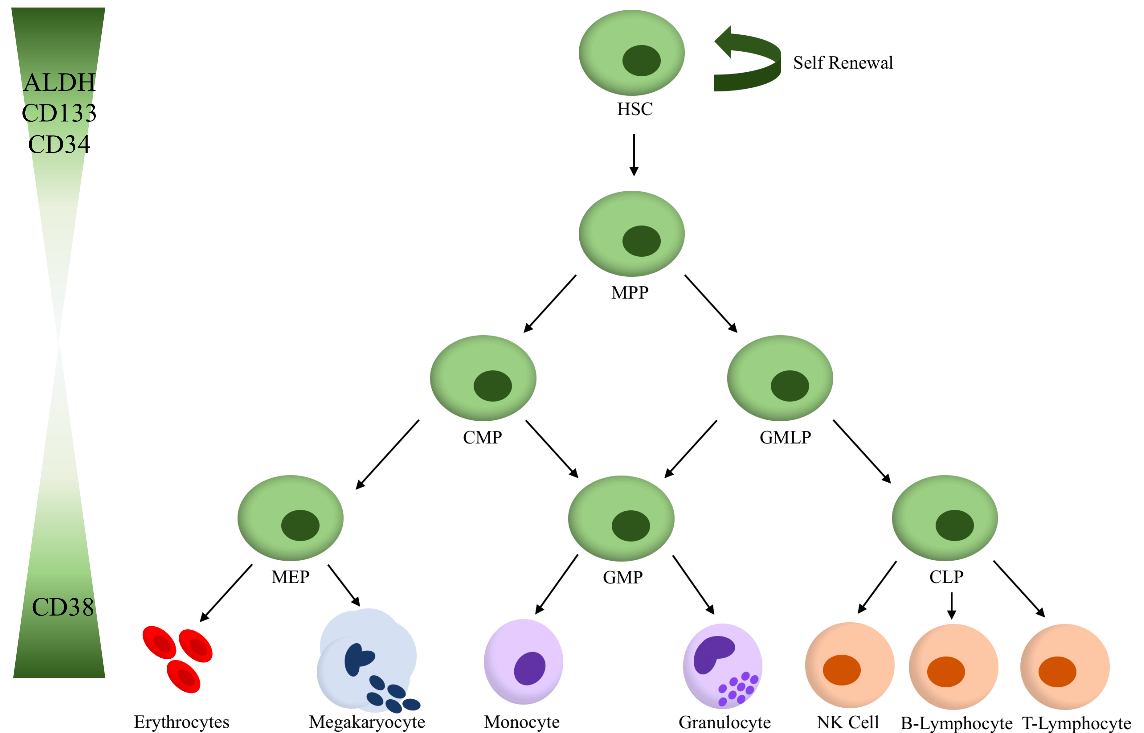


Figure 1.2. Postnatal hematopoietic hierarchy. Schematic showing the capacity of hematopoietic stem cells (HSC) to self-renew or differentiate towards maturity into the myeloid and lymphoid lineages. Cells lose aldehyde dehydrogenase activity, lose expression of primitive cell surface markers CD34 and CD133, and gain expression of mature hematopoietic cell surface marker CD38 as they differentiate. Multipotent progenitor (MPP); common myeloid progenitor (CMP); granulocyte-macrophage-lymphocyte progenitor (GMLP); megakaryocyte-erythrocyte progenitor (MEP); granulocyte-macrophage progenitor (GMP); common lymphoid progenitor (CLP); natural killer (NK).

1.6.1 Cell Selection Using High Aldehyde Dehydrogenase Activity

As previously discussed, cell surface markers such as CD34 are classically used to isolate HSPC populations. CD34 has been well characterized as a marker of primitive hematopoietic progenitor cells and the loss of CD34 expression occurs as cells mature towards various hematopoietic lineages[81]. There are; however, limitations to the use of cell surface markers to assess progenitor cell capacity. While $CD34^+$ cell transition is typically conserved through hematopoiesis *in vivo*, $CD34^+$ cell surface expression can vary as cells are manipulated *ex vivo*. Furthermore, Bhatia *et al.* reported a population of cells that were able to reconstitute hematopoiesis in SCID mice but were $CD34^-CD38^-$[57]. This population has been further studied by Dominique Bonnet and John Dick's labs, confirming a $CD34^-$ cell at the peak of the hematopoietic hierarchy[66, 67, 82]. Thus, recently characterized functional markers may provide a more appropriate means of isolating true HSPC.

High aldehyde dehydrogenase (ALDH) is a highly conserved function in primitive progenitor cells due to its role in eliminating reactive oxygen species from within cells. Thus, the cytoprotective role of ALDH allows for the isolation of multiple progenitor cell populations based on conserved enzymatic activity[83-85]. Early progenitors that need to generate cells for the lifespan of the organism typically demonstrate high ALDH activity and as cells progress towards a more differentiated/expendable state, ALDH activity is reduced. In 2004, Hess *et al.* characterized the isolation of UCB-derived HSC with high ALDH activity. This characterization used ALDH as a functional marker to isolate HSC that were able to repopulate irradiated NOD/SCID mice, providing an alternative method to isolation based on a beneficial progenitor function rather than cell surface marker expression[86, 87].

There are 19 different isoforms of ALDH that have been identified in humans and this number being even greater in mice; however, the main isoform is ALDH1A1. Interestingly, hematopoiesis is seemingly unaffected in mice with a knockout in the Aldh1a1 gene[88]. This finding lead researchers to study the compensatory role of other isoforms of ALDH in the absence of ALDH1A1. Studies in mice have shown that a deficiency in ALDH1A1 results in an increase in isoform ALDH3A1 in particular.

Gasparetto *et al.* have shown that a deficiency in both isoforms results in disrupted B-lymphocyte maturation, increased reactive oxygen species, and a decrease in the total number of HSPC[89]. Collectively, these findings help in characterizing the role of ALDH on HSPC and the hematopoietic hierarchy.

1.6.2 Retinoic Acid Signaling

Paradoxically, cytosolic ALDH also represents the rate limiting step in the production of retinoic acid (RA). RA is a morphogen, and the biologically active form of vitamin A. It has an important role during the development of many different organ systems and its transient expression is necessary for the proper differentiation of various cell types. In the context of hematopoiesis, RA is known to push differentiation down the hierarchy. In 1960, Breitman *et al.* studied the ability of RA to differentiate a cell line from a patient with acute promyelocytic leukemia (HL-60), suggesting that retinoids could be used therapeutically in patients with cancers of the blood[90]. While mice with a knockout in their retinoic acid receptor (RAR) appeared to undergo hematopoiesis normally, it is widely believed that RA could have a role in hematopoietic lineage determination as cells mature[88].

After ALDH converts the precursor retinal into biologically active RA, cytosolic retinoic acid binding protein (CRABP) binds to RA to aid with translocation to the nucleus. In canonical RA-signaling, RA binds to a heterodimer consisting of a retinoic acid receptor (RAR) and retinoid X receptor (RXR). Binding of RA to the heterodimer recruits a large co-activator complex that activates downstream gene transcription events. Thus, inhibition of canonical RA-signaling may prevent RA-induced differentiation of HSPC expanded *ex vivo*.

1.6.3 Inhibition of Retinoid Signaling to Expand HSPC

While ALDH can be used to identify and isolate primitive HSPC, Chute *et al.* set out to identify the role of ALDH in HSC differentiation and the loss of SCID repopulating capacity. DEAB, an inhibitor of ALDH, was used to expand HSPC *in vitro* and expression of target genes involved in HSC renewal such as Notch and HOXB4 was measured. When UCB-derived HSPC were expanded in serum-free culture and treated

with DEAB, there was a 3.4-fold increase in the number of SCID repopulating cells compared to day 0, representing a 7.7-fold increase compared to untreated conditions[91, 92]. These findings show that manipulating retinoid signaling in primitive hematopoietic cell populations can allow for the expansion of these rare cells while retaining high ALDH functional capacity.

There are several retinoid antagonists and inverse retinoid agonists that regulate retinoid signaling directly at the RAR/RXR heterodimer[93]. In particular, this thesis focuses on the use of BMS 493, an inverse pan-RAR agonist, during the expansion of UCB-derived HSPC (Figure 1.3). Rather than acting as an antagonist and inhibiting the binding of RA to RARs, BMS 493 acts by binding allosterically and stabilizes a corepressor (CoR) complex at the heterodimer (Figure 1.4). These CoR complexes include repressive factors such as silencing mediator for retinoid and thyroid hormone receptors (SMRT) and nuclear receptor corepressor (NCoR). In addition, histone deacetylases are often found as part of these complexes, contributing to transcriptional silencing. BMS 493 is regarded as a more “powerful” inverse agonist than previously identified compounds due to its positioning and ability to act on all RAR subtypes. In contrast, a compound such as AGN109 only binds to RARγ, providing weaker repressive capabilities. While the structure and mechanism of action for BMS 493 have been identified, there are no studies that have used this compound in modulating retinoid signaling in the context of manipulating hematopoiesis or in the generation of HSPC for inducing islet regenerative therapies.

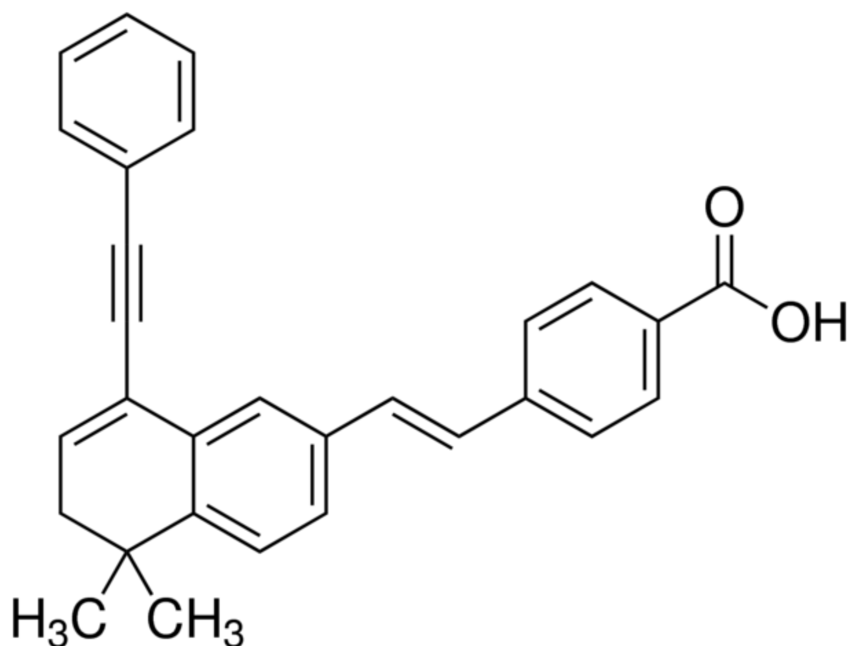


Figure 1.3 Chemical structure of inverse pan-RAR agonist, BMS 493. (E)-4-[2-[5,6-Dihydro-5,5-dimethyl-8-(2-phenylethynyl)naphthalen-2-yl]ethen-1-yl]benzoic acid, 4-[(1E)-2-[5,6-Dihydro-5,5-dimethyl-8-(phenylethynyl)-2-naphthalenyl]ethenyl]-benzoic acid, the chemical name for BMS 493, an inverse RAR agonist that allosterically binds to RARα, RARß, and RARγ.

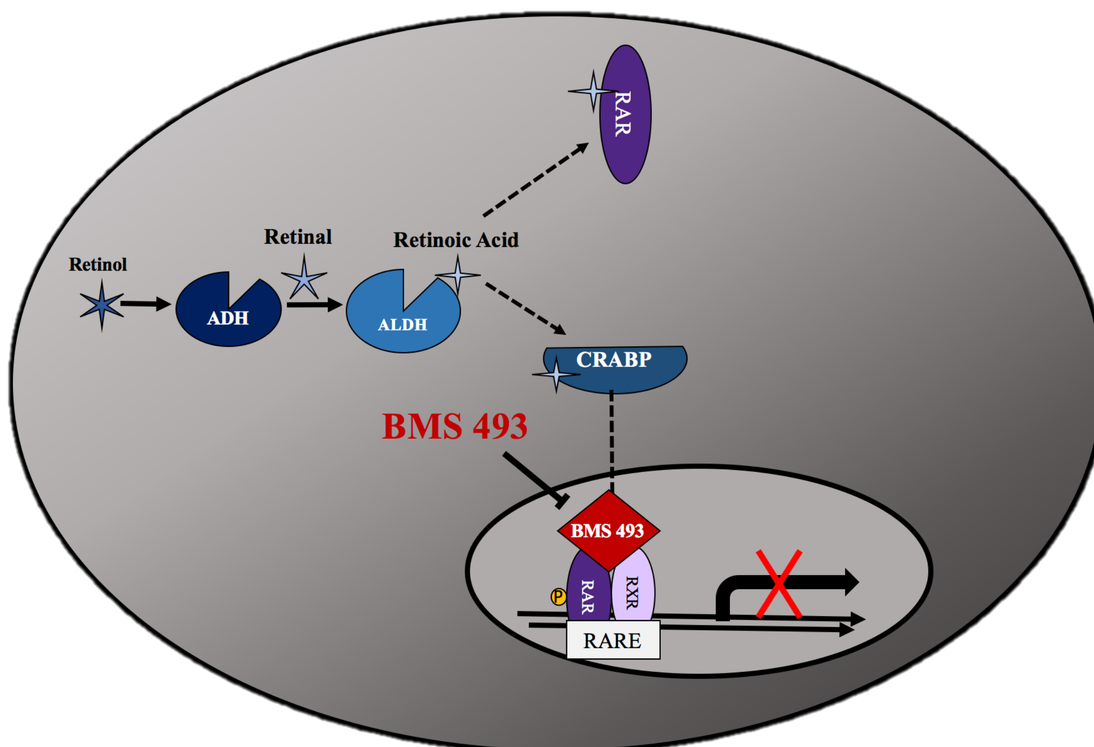


Figure 1.4. Mechanism of action for inverse pan-RAR agonist, BMS 493. BMS 493 blocks canonical RA-signaling by allosterically binding the RAR. Binding to the RAR stabilizes a corepressor complex at the RAR/RXR heterodimer, leading to transcriptional silencing of downstream genes in the RA-pathway.

1.7 Transplantation of $ALDH^{hi}$ Cells to Induce Islet Regeneration

Hess *et al.* showed that BM-derived cells expressing CD117 (c-kit) were able to reduce hyperglycemia when transplanted intravenously into STZ-treated NOD/SCID mice[57]. These results suggested that BM-derived cells have the capacity to induce endogenous islet regeneration in damaged pancreatic tissue. In 2012, our lab published that UCB-derived cells isolated based on high ALDH activity were able to reduce STZ-induced hyperglycemia in NOD/SCID mice after iPan transplantation[94]. Both islet size and islet number were increased compared to $ALDH^{lo}$ and PBS controls, together, leading to increased ß-cell mass. Furthermore, serum insulin concentrations were increased and when given a glucose challenge (intraperitoneal glucose tolerance test), mice transplanted with $ALDH^{hi}$ cells had an improved response compared to $ALDH^{lo}$ cells and PBS controls. Collectively, these findings suggested that UCB-derived $ALDH^{hi}$ cells possess islet regenerative functions.

To better understand the mechanisms behind endogenous islet regeneration, combinatorial intravenous transplantation of BM-derived MSC and UCB-derived $ALDH^{hi}$ cells were performed[95]. While both MSC alone and MSC co-transplanted with $ALDH^{hi}$ cells showed increased ductal associated (measured by CK19), only co-transplanted mice showed increased islet vessel density (measured by CD31 and vWF). Therefore, the authors suggested that MSC transplantation resulted in islet neogenesis while $ALDH^{hi}$ cell transplantation augmented the reduction in hyperglycemia by increasing vessel density and increasing ß-cell proliferation.

Unfortunately, $ALDH^{hi}$ cells are rare, comprising <0.5% of all mononuclear cells in UCB. Thus, in order to reach clinically applicable numbers for the treatment of DM, *ex vivo* expansion without the loss of islet regenerative capacity is warranted. When UCB-derived $ALDH^{hi}$ cells were expanded for 6 days in serum free conditions, ALDH activity rapidly declined as cells differentiated[96]. When cells were reselected for high ALDH activity after 6-day expansion, there was a 3-fold increase in total $ALDH^{hi}$ cells available for transplantation. These cells retained high colony forming capacity and expression of primitive cell surface markers, suggesting they are phenotypically similar to uncultured

ALDH^{hi} cells. Finally, 6-day expanded ALDH^{hi} cells retained islet regenerative capacity and showed a reduction in hyperglycemia after IV transplantation into STZ-treated NOD/SCID mice. These findings acted as a proof-of-concept that UCB-derived ALDH^{hi} cells could be expanded without the loss of endogenous islet regenerative functions. Thus, manipulation of pathways involved in hematopoietic cell differentiation such as the RA-pathway can be studied to further expand ALDH^{hi} cells *ex vivo* and elucidate mechanisms involved in islet repair. This thesis focuses on the use of BMS 493, an inverse RAR agonist in expanding ALDH^{hi} cells for use in islet regenerative therapies.

1.8 Objectives & Hypotheses

The overall objective of this thesis was to characterize the expansion of UCB-derived, $ALDH^{hi}$ HSPC in serum-free culture conditions with or without the inverse retinoic acid receptor agonist, BMS 493. We also set out to establish whether BMS 493-treated, expanded could stimulate endogenous islet regeneration after intrapancreatic transplantation into STZ-treated, NOD/SCID mice.

Compared to untreated expansion conditions, we hypothesized that expanded BMS 493-treated HSPC will (1) retain a greater frequency of $ALDH^{hi}$ cells that co-express primitive cell surface markers, and (2) augment ß-cell survival and proliferation *in vitro.* Furthermore, we hypothesized that expanded BMS 493-treated cells will reduce hyperglycemia in STZ-treated NOD/SCID mice via the induction of endogenous islet proliferation and revascularization after intrapancreatic injection *in vivo.*

These hypotheses were addressed by performing the following objectives:

The first objective was to characterize RA-pathway inhibited cells after expansion using flow cytometry for the retention of high ALDH-activity and co-expression of primitive (CD34, CD133, CD117) and mature (CD38) hematopoietic cell surface markers.

The second objective was to assess pro-survival and proliferation-stimulating effects of RA-pathway inhibited cells on human islets *in vitro* (conditioned media cultured with human islets).

The third objective was to transplant RA-pathway inhibited cells and/or concentrated conditioned media into STZ-treated NOD/SCID mice to assess reduction of hyperglycemia after intrapancreatic transplantation.

2 Methods

2.1 Blood Collection

Human UCB was collected with informed consent from the birthing centre at the London Health Sciences Centre, Victoria Hospital in London, Ontario under REB 12934. After caesarian sections, the umbilical cord was clamped and blood was drawn using a 16 G needle coated in heparin (Novartis, Boucherville, CA) and 60 mL syringe by venipuncture. Samples were transported to our lab and processed to purify HSPC with high ALDH activity within 24 hours of collection

2.2 Human Umbilical Cord Blood Processing

Human UCB was processed as described in our lab's previous publications[94-96]. Briefly, RosetteSep™ Human Hematopoietic Progenitor Cell Enrichment Cocktail (StemCell Technologies, Vancouver, CA) was added to UCB at 5 µL/mL and incubated at room temperature for 30 minutes. Next, UCB was diluted with phosphate buffered saline (PBS, Invitrogen) prior to layering gently over Ficoll-Paque™ PLUS (GE Healthcare Life Sciences), and centrifuged at 450 relative centrifugal force (rcf) for 20 minutes with the brake turned off. The lineage depleted mononuclear cell layer (Lin^- MNC) was manually removed using a Pasteur pipette. Lin^-MNC were washed with PBS supplemented with 5% fetal bovine serum (FBS) and cells were pelleted by centrifugation. Cells were resuspended in 1 mL PBS + 5% FBS and 9 mL Ammonium Chloride Solution (StemCell Technologies, Vancouver, CA) and incubated at room temperature for 7 minutes to lyse residual red blood cells. ells were washed, pelleted, and resuspended in ALDEFLUOR™ Assay Buffer (StemCell Technologies, Vancouver, CA) at 1×10^7 cells/mL. Next, cells were stained using ALDEFLUOR™ reagent (StemCell Technologies, Vancouver, CA) at 5 µL/mL and 5×10^5 cells were removed and treated with 5 µL N, N-diethylaminobenzaldehyde (DEAB), a reversible inhibitor of ALDH activity (StemCell Technologies, Vancouver, CA). ALDEFLUOR™-labeled sample and DEAB control were incubated at 37˚C for 30 minutes. Excess ALDEFLUOR™ substrate was removed by pelleting cells and resuspending in ALDEFLUOR™ Assay Buffer prior to fluorescent activated cell sorting (FACS) (FACSARIA III, BD BioSciences,

Missisauga, ON). Cells with high aldehyde dehydrogenase activity ($ALDH^{hi}$ cells) were purified using the DEAB control to identify $ALDH^{lo}$ cells and the $ALDH^{hi}$ cell gate was set at a fluorescence intensity approximately 2 times greater than the DEAB control. Purity of $ALDH^{hi}$ after FACS was reassessed at >95%.

2.3 UCB $ALDH^{hi}$ Cell Expansion

12-well plates were coated with fibronectin from human plasma (Sigma-Aldrich, St. Louis, MO) prior to cell seeding. $ALDH^{hi}$ cells were plated at 2.5×10^4 cells/well in 1.5 mL of serum-free, X-VIVO™ 15 media (Lonza, Basel, Switzerland) supplemented with 10 ng/mL each of thrombopoietin (TPO), stem cell factor (SCF), and FMS-related tyrosine kinase ligand (FLT-3L) (Gibco, Carlsbad, CA) and incubated at 37˚C[(92)]. On day 3, media was changed and cells were either left untreated or treated with 100 nM BMS 493 (Sigma-Aldrich, St. Louis, MO).

2.4 Cell Surface Marker Analyses

On day 6, cells were harvested and any adherent cells were lifted using 200 µL/well TrypLE (Gibco, Carlsbad, CA). Cells were pelleted and resuspended in ALDEFLUOR™ Assay Buffer at 1×10^7 cells/ mL and stained with ALDEFLUOR™ reagent as previously described. After 30 minute ALDEFLUOR™ incubation, cells were washed, resuspended in ALDEFLUOR™ Assay Buffer, and labeled with the following fluorescent-tagged antibodies: 4 µL CD34-Brilliant Violet 421(BV 421), 2µL CD38-phycoerythrobilin (PE), 4µL CD117-PE-CF594, and 4µL CD133-allophycocyanin (APC) at 4˚C for 20 minutes (all antibodies BD Pharminigen, San Diego, CA). After washing and centrifugation, samples were aspirated to leave ~400 µL and 3 µL of viability dye 7-aminoactinomycin D (7-AAD) was added prior to being run on the BD LSR II Flow Cytometer (BD BioSciences, Missisauga, ON). UltraComp eBeads (eBioScience, San Diego, CA) were used for compensation controls on the BD LSR II Flow Cytometer and fluorescent minus one (FMO) controls as well as an unstained control were used to set gated populations. Flow cytometry data was analyzed using FlowJo software.

2.5 Methylcellulose Assay

After 6-day expansion with or without BMS 493 treatment, unsorted (bulk) expanded cells or cell populations resorted for ALDH-activity were resuspended in Iscove's Modified Dulbecco's Medium (IMDM, Gibco, Carlsbad, CA) at 5×10^3 cells/mL, and 900 µL of MethoCult™ (StemCell Technologies, Vancouver, CA) was added to 100 µL of cells using a 16 G needle with a 1 cc syringe. Cells were vortexed and incubated at room temperature for 8 minutes prior to being plated with 1 mL/ well in a 12-well plate. Plates were incubated at 37˚C for 14 days prior to enumerating for hematopoietic colonies based on morphology for: blast forming unit-erythroid (BFU-E), colony forming unit-macrophage (CFU-M), colony forming unit-granulocyte (CFU-G), colony forming unit-granulocyte, monocyte (CFU-G), and colony forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM).

2.6 Collection of HSPC Conditioned Media

After 6-day expansion with or without BMS 493-treatment, bulk HSPC were harvested and bulk populations were sorted using the FACSARIA III to select live cells based on their forward and side scatter parameters. Cells were plated at 2×10^5 cells/ mL in RPMI 1640 media (Gibco, Carlsbad, CA) supplemented with 10 ng/mL SCF, TPO, and FLT-3L. After 48 hours, conditioned media was collected and concentrated using a 3 kilodalton Amicon Ultra-15 Centrifugal Filter Unit (Millipore, Billerica, MA). Cell viability was also recorded after 48 hours using the exclusion of trypan blue staining.

2.7 Islet Culture with HSPC Conditioned Media

Purified and cultured human pancreatic islet samples from 4 individual donors were obtained through the Integrated Islet Distribution Program (IIDP, Duarte, CA). 200 islet equivalents (IEQ) were plated per well in 6-well non-tissue culture treated suspension plates. Islets were initially plated in proprietary islet media (PIM) and topped up to 3 mL with RPMI 1640. After 24 hours, media was changed to RPMI supplemented with 10 ng/mL SCF, TPO, and FLT-3L. Islets were cultured for up to 7 days in RPMI supplemented with SCF, TPO, and FLT-3L (sub-optimal conditions), treated with conditioned media from cells expanded with or without BMS 493 (test conditions), or

treated with RPI supplemented with 10% FBS (optimal conditions). For 7-day culture experiments, 2 mL of each media was replaced on day 3 and islets were treated with additional conditioned media or FBS. 500 nM EdU (Invitrogen, Burlington, ON) was added to cultures on the final media change. On day 3 and 7, islets were collected and dissociated into a single cell suspension using TrypLE. Total cell number was manually counted using a hemocytometer and cells from each condition were divided and stained with 2 µL FluoZin-3 AM (Gibco, Carlsbad, CA), 4 µL Annexin V conjugated to BV 421, and 3 µL 7-AAD (viability dye) or fixed with 10% formalin and permeabilized with 1x saponin buffer prior to being stained with 10 µL anti-human insulin antibody conjugated to APC, 4 µL anti-human CD45 antibody conjugated to PE, and EdU click-it reaction conjugated to Alexa488 according to manufacturer's specifications.

2.8 *In Vivo* Transplantation Experiments

Assessment of hyperglycemia reduction after intrapancreatic transplantation of expanded HSPC was performed as described in our previous publications under AUP 2015-033[57, 94-96]. Briefly, 7-8-week-old NOD/SCID mice (Jackson Laboratories, Bar Harbor, ME) were treated with 35 mg/kg/day of streptozotocin (STZ, Sigma, St. Louis, MO) for 5 consecutive days. Blood glucose was measured on day 10 and only mice with a blood glucose > 15 mmol/L (3 times greater than basal levels) were used for experiments. Expanded HSPC were harvested and resorted for ALDH activity using FACS. Bulk expanded cells or resorted $ALDH^{hi}$ or $ALDH^{lo}$ cell populations were transplanted by direct intrapancreatic injection using an insulin syringe on day 10 with 2×10^5 cells/mouse in a total volume of 20 µL[94]. Blood glucose was measured again on day 14, day 17, and weekly thereafter (day 21, 28, 35, and day 42). Area under the curve for unfasted blood glucoses was calculated over the 42-day time-course. 24 hours prior to euthanasia via cervical dislocation, mice were injected with 100 µL of 2.5 mg/mL EdU. Mice were also anesthetized using isoflurane and blood was collected via cardiac puncture and centrifuged to isolate serum. Pancreata were dissected from mice, weighed, and embedded in optimal cutting temperature medium (OCT, Sakura Finetec USA, Torrance, CA) blocks. Pancreas samples from each mouse were frozen, sectioned, and stained for quantification of ß-cell mass, islet vascularity, and human cell engraftment.

Pancreas samples from each mouse were cut into 10 µm serial sections with each slide having sections no less than 100 µm apart to prevent double counting islets.

2.9 Immunohistochemistry & Quantification of ß-cell Mass

Pancreas sections were fixed in 10% formalin (Thermo Fisher Scientific, Boston, MA) for 15 minutes and permeabilized with 1% Triton x-100 for 20 minutes. Basic mouse on mouse (M.O.M.) kit (Vector Laboratories, Burlington, ON) was used to block any nonspecific binding and to dilute the insulin antibody. Sections were stained with a mouse-specific insulin antibody (Sigma, St. Louis, MO) at 1:500 dilution, washed, and subsequently stained with peroxidase labeled anti-mouse IgG (Vector Laboratories, Burlington, ON) at 1:250 dilution. Slides were stained in DAB chromogen (Vector Laboratories, Burlington, ON) for 10 minutes and counterstained with hematoxylin. Finally, slides were mounted in VectaMount™ AQ (Vector Laboratories, Burlington, ON) before being imaged. Twelve images were taken per slide at 10 times magnification and islet number and circumference were calculated using Northern Eclipse Software. ß-cell area was calculated using AxioVision software by encircling insulin^{+} area. Non-ß-cell area was calculated by subtracting ß-cell area from total image area. ß-cell area/non-ß-cell area was multiplied by the pancreas mass to calculate total ß-cell mass.

2.10 Immunohistochemistry & Quantification of Islet Vascularization

After fixation in formalin and blocking with M.O.M. kit as described above, pancreas sections were stained with mouse specific insulin antibody (Sigma, St. Louis, MO) at 1:500 dilution, washed, and subsequently stained with horse anti-mouse Texas Red (Vector Laboratories, Burlington, ON) at 1:200 dilution. Sections were washed and incubated in normal rabbit block (Vector Laboratories, Burlington, ON) for 1 hour prior to being co-stained with rat anti-mouse CD31 antibody (BD Pharminigen, San Diego, CA) for 30 minutes at 1:100 dilution and subsequently rabbit anti-rat fluorescein isothiocyanate (FITC) (Vector Laboratories, Burlington, ON) for 30 minutes at 1:200 dilution and sections were mounted with VECTASHIELD® Mounting Medium (Vector Laboratories, Burlington, ON) and sealed. Images were taken of each islet within a

pancreas section at 20 times magnification. $CD31^+$ cells were enumerated and total islet area was calculated using AxioVision software.

2.11 Immunohistochemistry for Human Cell Engraftment

Pancreas sections were fixed in formalin and blocked with M.O.M. kit as described in section 2.9. Sections were stained with mouse anti-human HLA-A,B,C antibody (BD Pharminigen, San Diego, CA), washed, and subsequently stained with horse anti-mouse FITC (Vector Laboratories, Burlington, ON). Sections were incubated in normal goat block (Vector Laboratories, Burlington, ON) for 1 hour prior to being co-stained with rabbit anti-mouse insulin antibody (Abcam, Cambridge, UK) for 1 hour at 1:50 dilution and subsequently being stained with goat anti-rabbit Texas Red (Vector Laboratories, Burlington, ON) for 20 minutes at 1:200 dilution. Sections were mounted with VECTASHIELD® Mounting Medium (Vector Laboratories, Burlington, ON) and sealed.

2.12 Statistical Analyses

Data is presented as mean ± standard error of the mean (SEM) unless indicated otherwise. Characterization of ALDH and primitive cell surface marker retention with or without BMS 493 treatment was analyzed using two-tailed student t-tests ($*p < 0.05$). Titration of BMS 493, AUC for *in vivo* data, and all histological data were analyzed using a one-way analysis of variance (ANOVA). 42 day blood glucose curves were analyzed using a two-way ANOVA with Tukey's multiple comparisons test. Human islet culture data was analyzed using a two-way ANOVA with Dunnet's multiple comparisons test.

3 Results

3.1 BMS 493 Treatment Increased the Frequency of Cells with High ALDH Activity

ALDH^{hi} cells represent a rare (<0.5%) portion of UCB mononuclear cells. Thus, *ex vivo* expansion of this cell population is warranted to reach clinically applicable numbers for cellular therapies. Our lab and others have previously published a protocol for the *ex vivo* expansion of UCB-derived ALDH^{hi} HSPC in clinically applicable, serum free media supplemented SCF, TPO, and FLT-3L[96-98]. However, HSPC differentiate quickly in culture, reducing ALDH-activity, and resulting in the loss of islet regenerative function[96]. Here, we utilized an inverse agonist of the retinoic acid receptor (RAR), BMS 493 to delay ALDH^{hi} cell differentiation during culture, assessed by the preservation of high ALDH-activity. Using a 6-day expansion protocol, we tested various concentrations (10 nM – 500 nM) and duration of BMS 493 exposure to balance expansion efficiency with optimal retention of high ALDH activity. BMS 493-treatment at 100 nM from days 3-6 proved to be our best conditions as total cell expansion was equivalent for all parameters altered (Figure 3.1A-C) and 100nM from days 3-6 showed the greatest retention of ALDH activity (Figure 3.1D-F). Thus, BMS 493-treatment at 100 nM from days 3-6, subsequently referred to as BMS 493-teratment, was selected as our optimal conditions for ALDH^{hi} HSPC expansion.

The commercially available ALDEFLUOR™ reagent can be used to measure ALDH activity levels in live cell populations using flow cytometry. DEAB, a reversible inhibitor of ALDH, was used to set a gate for cells with low ALDH activity and ALDH^{hi} cell frequency was compared between BMS 493-treated and untreated cells (Figure 3.2A-C). While there was no significant difference in the total number of cells obtained after expansion with or without BMS 493-treatment (Figure 3.2D), the frequency of cells with high ALDH activity was significantly increased ($p=0.001$) after treatment with BMS 493 (31.16 ± 3.09%) compared to untreated conditions (15.47 ± 1.92%) (Figure 3.2E). Collectively, 6-day expansion with 100 nM BMS 493-treatment from days 3-6 resulted in a 2.2-fold increase in total ALDH^{hi} cells compared to untreated conditions ($p=0.012$),

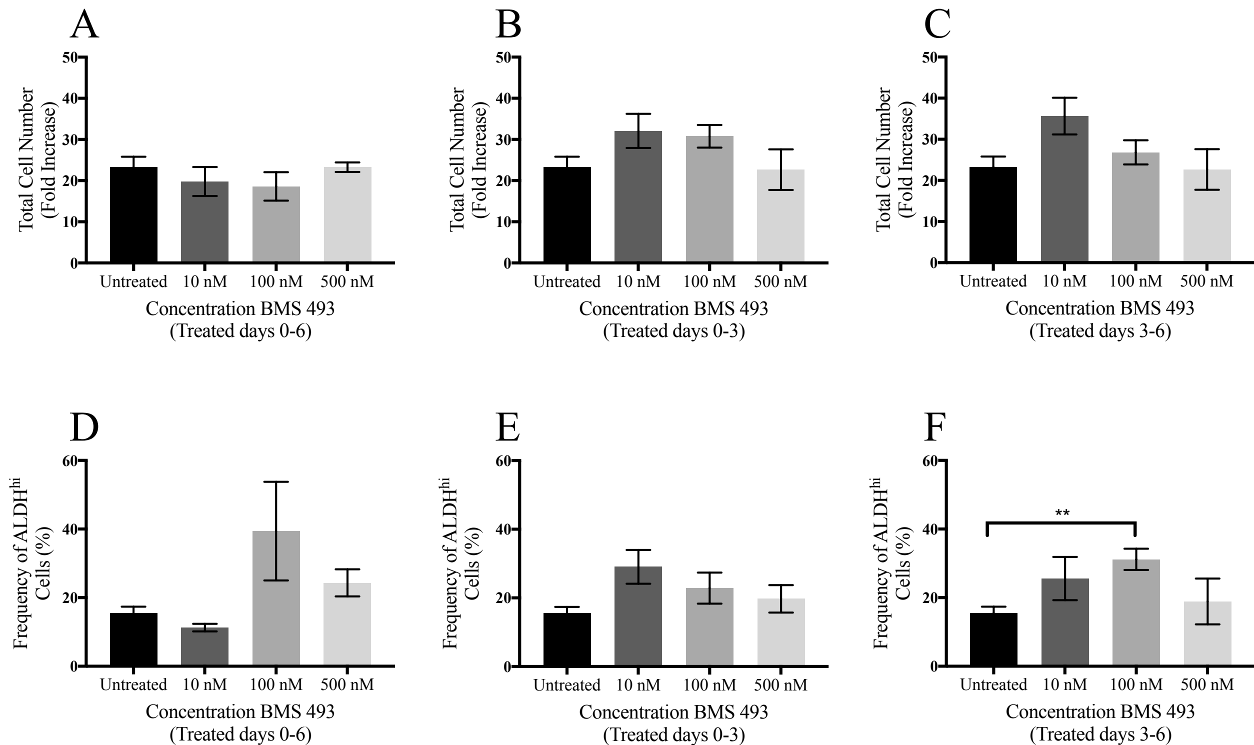


Figure 3.1 Optimization of BMS 493-treatment based on retention of cells with high ALDH activity. Compared to untreated controls (N=12), total hematopoietic cell number at day 6 (fold increase versus day 0) was not altered by increasing concentrations of BMS 493-treatment from (A) days 0-6 (N=3-5), (B) days 0-3 (N=6-10), and (C) days 3-6 (N=3-8). On day 6, ALDH^{hi} cell frequency was equivalent to untreated controls after BMS 493-treatment from (D) days 0-6 or (E) days 0-3. (F) In contrast, on day 6, ALDH^{hi} cell frequency was increased after BMS 493-treatment at 100 nM from days 3-6 compared to untreated controls. N represents different UCB donor samples. Data is shown as mean ± SEM (**$p<0.01$) determined by two-way ANOVA and Dunnett's multiple comparisons test.

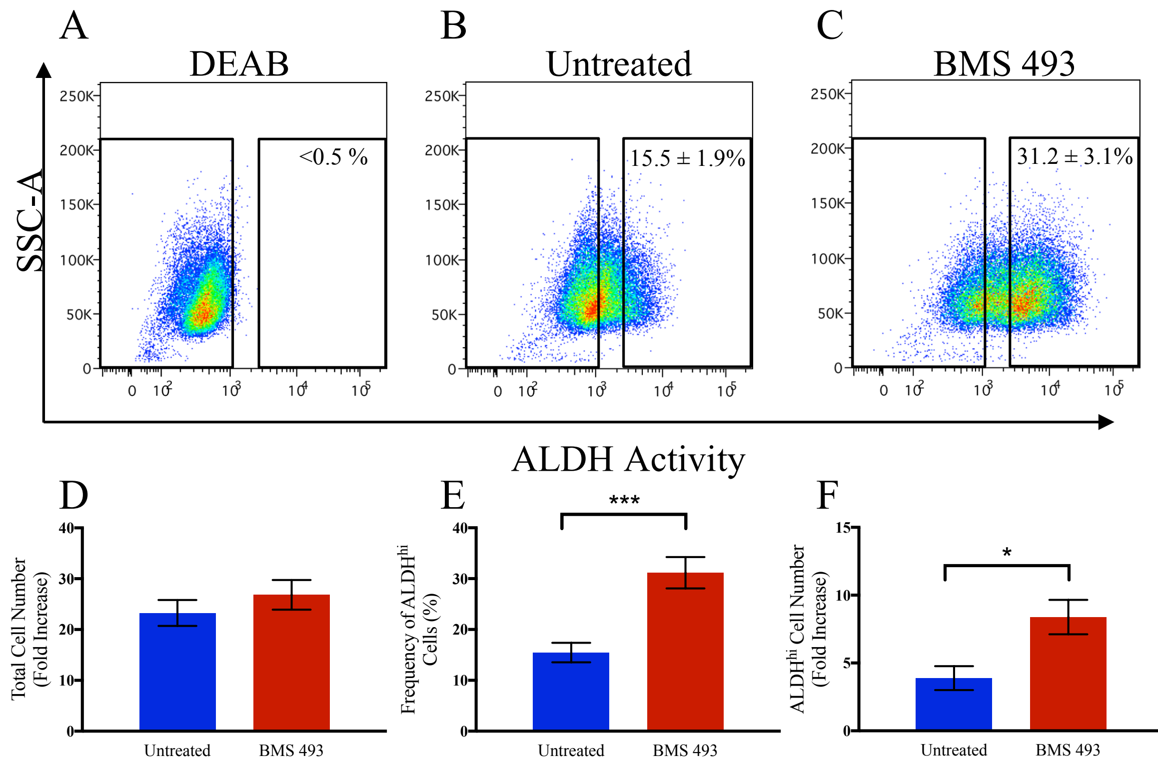


Figure 3.2 Treatment with BMS 493 increased ALDH^{hi} cell frequency and total ALDH^{hi} cell number after 6-day expansion. ALDH expression of expanded hematopoietic cell progeny was quantified at day 6 for: **(A)** DEAB-treated control, **(B)** untreated conditions (N=12) and **(C)** treatment with BMS 493 (N=8). **(D)** Total cell number obtained with BMS 493 treatment was equal to untreated controls. However, BMS 493 treatment showed **(E)** increased ALDH^{hi} cell frequency and **(F)** total ALDH^{hi} cell number compared to untreated conditions (fold increase versus day 0). N represents different UCB donor samples. Data is shown as mean ± SEM (*p<0.05, ***p<0.001) determined by two-tailed student t-test.

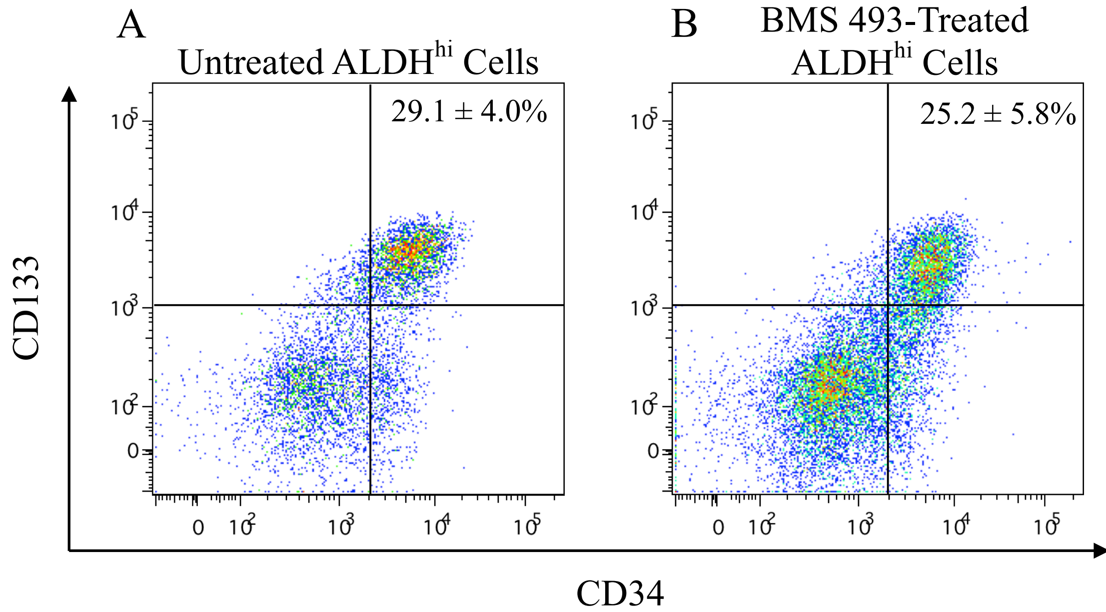
resulting in an 8.3 ± 1.27-fold increase in ALDH^{hi} cells compared to the cell number initially plated (Figure 3.2F).

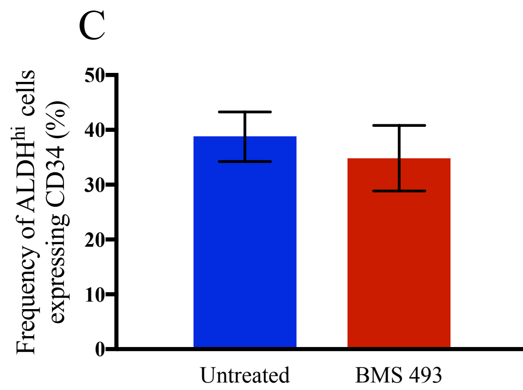
3.2 BMS 493-Treated ALDH^{hi} Cells Retained Primitive Cell Surface Marker Expression

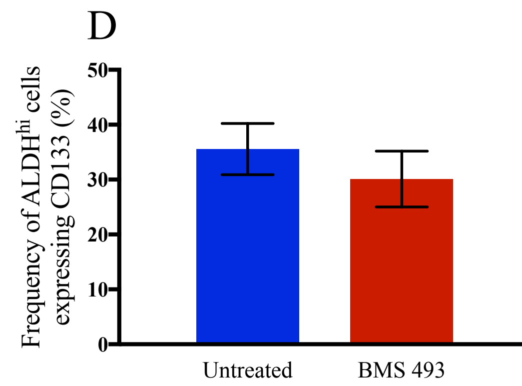
Expression of primitive cell surface markers CD34, CD133, and CD117 was assessed by flow cytometry on expanded cells with or without BMS 493-treatment. Representative flow cytometry dot plots (Figure 3.3A and B) demonstrated similar CD34 and CD133 expression between BMS 493-treated and untreated cells with high ALDH activity. Although cells treated with BMS 493 showed no difference in the frequency of ALDH^{hi} cells co-expressing CD34 (p=0.610) and CD133^{+} (p=0.443) cells at day 6 (Figure 3.3C and D), treatment with BMS 493 resulted in 1.89 and 1.86 times more ALDH^{hi}CD34^{+} (p=0.002) and ALDH^{hi}CD133^{+} (p=0.004) cells, respectively, a significant increase compared to untreated conditions (Figure 3.3E and F). Cells were also assessed for expression of c-kit (CD117), a marker previously shown to be expressed on murine BM cells that play a role in pancreatic regeneration[(57)]. There was no significant difference in the frequency or total number of ALDH^{hi}CD117^{+} cells after 6-day expansion as compared to untreated conditions (data not shown). Thus, BMS 493-treatment preserved primitive CD34 and CD133 expression on cells retaining high ALDH activity.

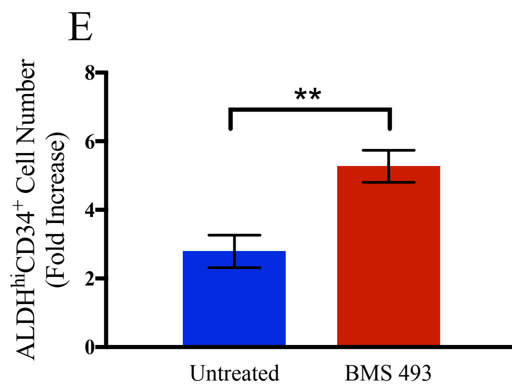
Conversely, expanded cells were also assessed for CD38 expression, an early indicator of CD34^{+} cell differentiation, and a marker of hematopoietic maturation[(99)]. Representative flow cytometry dot plots (Figure 3.4A and B) demonstrated decreased CD 38 expression when comparing BMS 493-treated and untreated ALDH^{hi} cells. Cells treated with BMS 493 showed a significant decrease in the frequency of ALDH^{hi}CD38^{+} cells 1.82 ± 0.59% as compared to 16.40 ± 4.45% in untreated samples (p=0.010) (Figure 3.4C). Total ALDH^{hi}CD38^{+} cell number was significantly decreased showing 48.50 times decrease in expression (p=0.013) compared to untreated cells (Figure 3.4D).

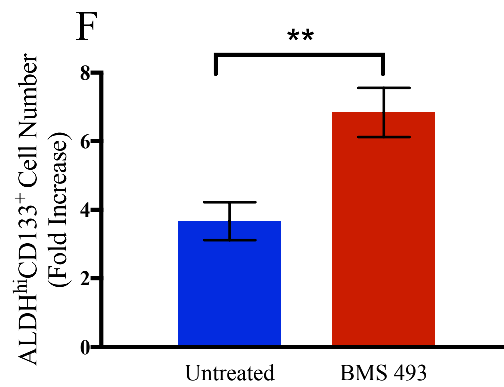
Figure 3.3 Treatment with BMS 493 increased total ALDH^{hi} CD34^{+} cells and ALDH^{hi} CD133^{+} after 6-day expansion. Representative flow cytometry dot plots showing CD34 and CD133 expression on ALDH^{hi} hematopoietic cells from: **(A)** untreated (N= 10) and **(B)** BMS 493-treated (N=7) conditions. Compared to untreated controls, BMS 493-treated cells showed an equivalent frequency of **(C)** primitive ALDH^{hi} CD34^{+} cells and **(D)** ALDH^{hi} CD133^{+} cells. However, since high ALDH activity was better retained under BMS 493-treated conditions, the total number of **(E)** primitive ALDH^{hi} CD34^{+} and **(F)** ALDH^{hi} CD133^{+} cells were increased (fold increase versus day 0). N represents different UCB donor samples. Data is shown as mean ± SEM (**$p<0.01$) determined by two-tailed student t-test.


A
Untreated ALDHhi Cells
B
BMS 493-Treated
ALDHhi Cells
29.1 ± 4.0%
25.2 ± 5.8%
CD133
CD34


C
Frequency of ALDHhi cells
expressing CD34 (%)
Untreated
BMS 493


D
Frequency of ALDHhi cells
expressing CD133 (%)
Untreated
BMS 493


E
ALDHhiCD34+ Cell Number
(Fold Increase)
**
Untreated
BMS 493


F
ALDHhiCD133+ Cell Number
(Fold Increase)
**
Untreated
BMS 493

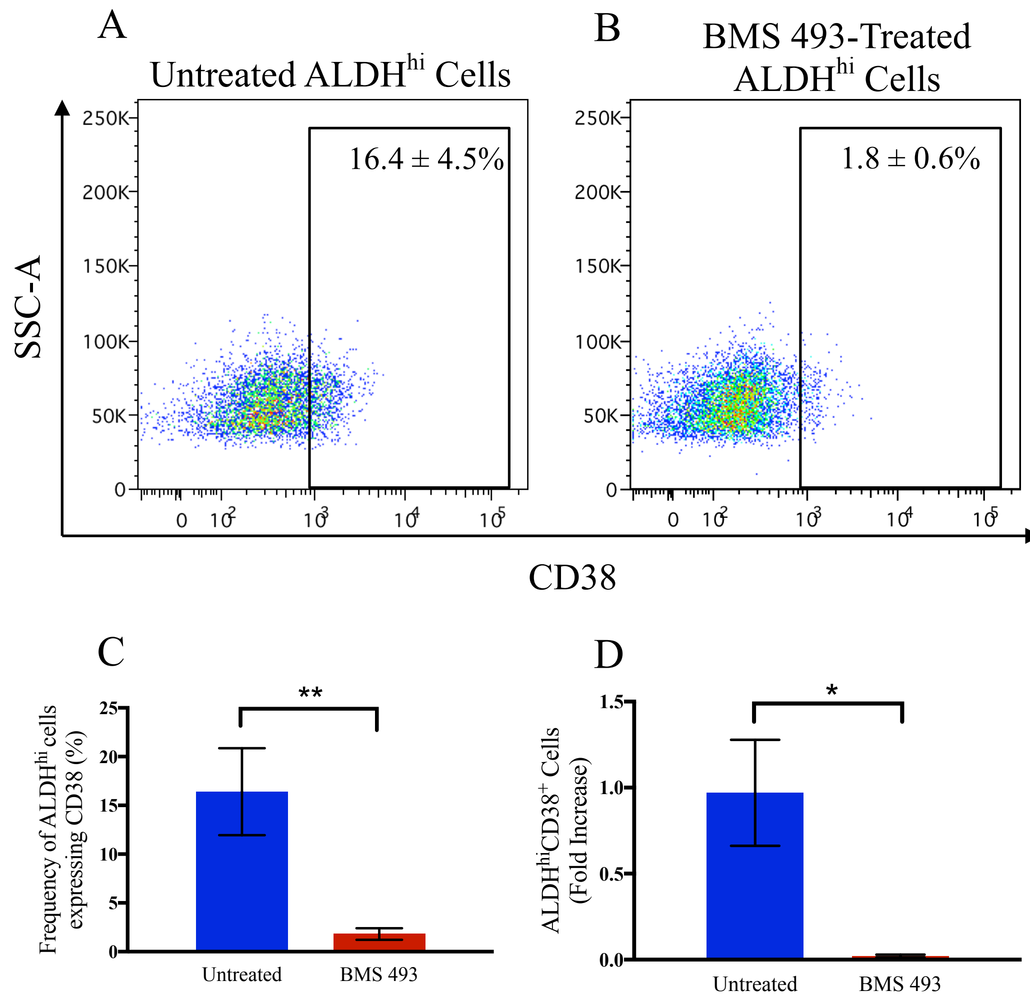


Figure 3.4 Treatment with BMS 493 decreased total ALDH^{hi}CD38^{+} cell frequency and total cell number after 6-day expansion. Representative flow cytometry dot plots showing CD38 expression on ALDH^{hi} hematopoietic cells from: **(A)** untreated controls (N=10) and **(B)** treatment with BMS 493 (N=7). Compared to untreated controls, BMS 493-treated cells showed a decrease in **(C)** mature ALDH^{hi}CD38^{+} frequency and **(D)** total ALDH^{hi}CD38^{+} cells (fold increase versus day 0). N represents different UCB donor samples. Data is shown as mean ± SEM (*$p<0.05$, **$p<0.01$) determined by two-tailed student t-test.

3.3 Colony Forming Capacity Was Retained After BMS 493 Treatment

Myeloid colony formation in methylcellulose media was used to test for hematopoietic progenitor cell function after 6-day expansion. In general, colony forming capacity is decreased with prolonged culture. The frequency of total hematopoietic colony formation was equal between untreated and BMS 493-treated cells (Figure 3.5A). When different lineages of hematopoietic colonies were compared, there was also no significant difference in the frequency of colony subtypes between BMS 493-treated cells and untreated controls (Figure 3.5B). After resorting based on ALDH activity, the number of colony forming units (CFUs) per 100 cells was significantly greater ($p=0.002$) in the $ALDH^{hi}$ subpopulation than the $ALDH^{lo}$ subpopulation of both BMS 493-treated cells (9.80 ± 1.67 and $4.55\% \pm 0.96$ respectively) and untreated controls ($12.30\% \pm 0.90$ and $5.22\% \pm 0.54$ respectively) after 6-day expansion (Figure 3.5C). When different lineages of hematopoietic colonies were compared between the resorted populations, there was no significant difference in the frequency of colony subtypes between BMS 493-treated populations and untreated controls (Table 3.1). Ultimately, treatment with BMS 493 during increased the total number of $ALDH^{hi}$ cells available for transplantation without the loss of hematopoietic colony forming capacity.

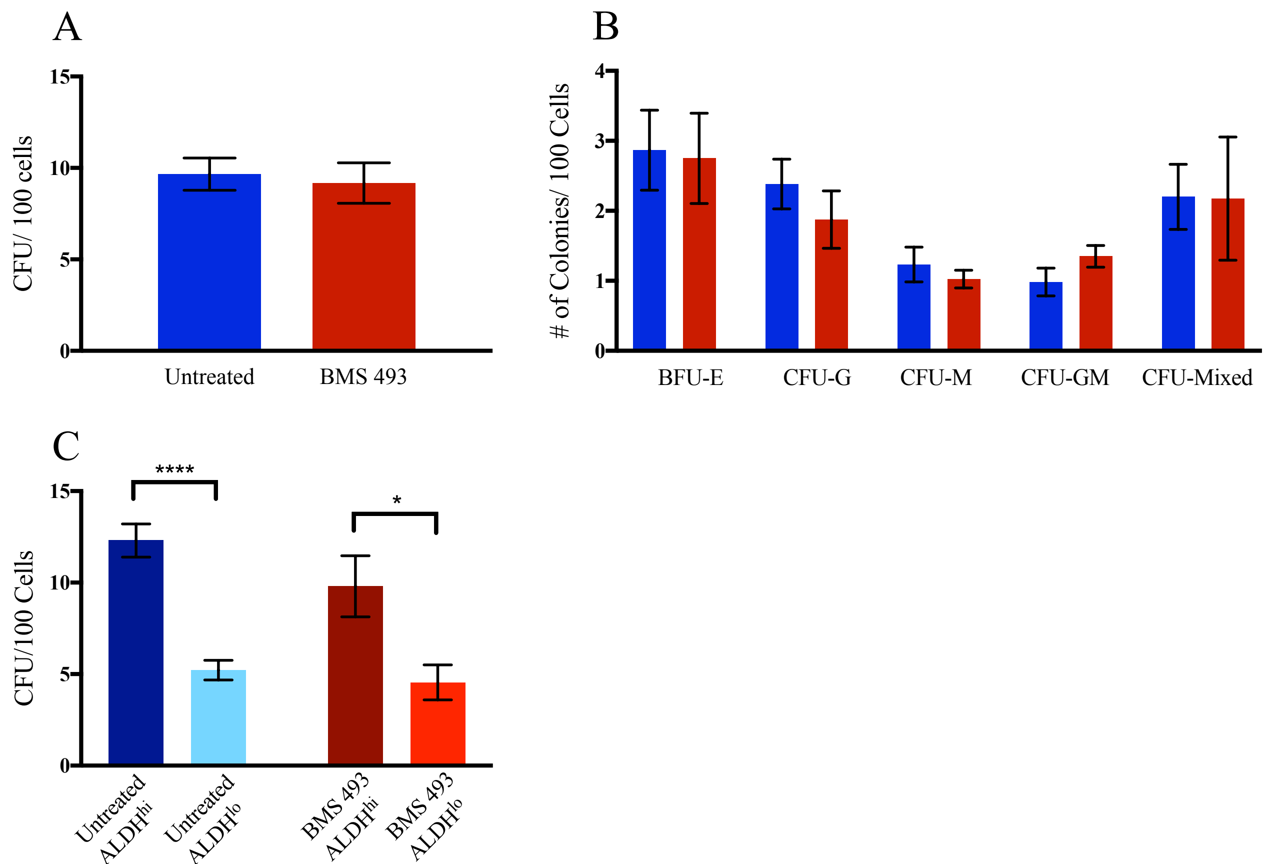


Figure 3.5 Treatment with BMS 493 retained ALDH^{hi} HSPC colony forming capacity after 6-day expansion. Compared to untreated controls (N=4, performed in duplicate), BMS 493-treated cells (N=6, performed in duplicate) showed **(A)** no difference in colony forming capacity after enumeration of hematopoietic colonies in methylcellulose. **(B)** BMS 493-treated cells produced equal number of BFU-E, CFU-G, CFU-M, CFU-GM, or CFU-GEMM as untreated controls. **(C)** After expanded cells were resorted based on ALDH activity, ALDH^{hi} subpopulations of BMS 493-treated (N=4, performed in duplicate) cells and untreated control cells (N=6, performed in duplicate) formed more colonies than their respective ALDH^{lo} cells. N represents different UCB donor samples. Data is shown as mean ± SEM (*$p<0.05$, ***$p<0.001$) determined by two-tailed student t-test **(A)** or one-way ANOVA **(B, C).**

Table 3.1. Analyses of hematopoietic colony subtypes produced by BMS 493-treated cells and untreated controls resorted based on ALDH-activity.

	Untreated $ALDH^{hi}$ (CFU/100 cells)	Untreated $ALDH^{lo}$ (CFU/100 cells)	BMS 493-treated $ALDH^{hi}$ (CFU/100 cells)	BMS 493-treated $ALDH^{lo}$ (CFU/100 cells)
BFU-E	4.77 ± 0.83	1.18 ± 0.35	3.78 ± 1.59	2.00 ± 0.84
CFU-G	1.65 ± 0.30	1.75 ± 0.29	1.60 ± 0.32	0.88 ± 0.27
CFU-M	1.43 ± 0.38	0.65 ± 0.16	1.05 ± 0.34	0.73 ± 0.14
CFU-GM	1.70 ± 0.54	0.73 ± 0.17	1.30 ± 0.18	0.48 ± 0.19
CFU-GEMM	2.75 ± 0.90	0.90 ± 0.25	2.08 ± 0.55	0.48 ± 0.21

After 6-day expansion, BMS 493-treated $ALDH^{hi}$ and $ALDH^{lo}$ hematopoietic cell subsets produced an equal number of BFU-E, CFU-G, CFU-M, CFU-GM, or CFU-GEMM as compared to respective untreated $ALDH^{hi}$ and $ALDH^{lo}$ cells. Data is shown as mean ± SEM with analysis of significance determined by one-way ANOVA.

3.4 Transplantation of Expanded Cells Did Not Reduce Hyperglycemia in STZ-Treated NOD/SCID Mice

We tested the ability of BMS 493-treated cells to reduce hyperglycemia after intrapancreatic transplantation into STZ-treated NOD/SCID mice. Fresh UCB-derived $ALDH^{hi}$ cells not cultured *in vitro* were transplanted as a positive control to replicate data published using our established mouse model (Figure 3.6A)[57, 94, 95]. BMS 493-treated cells did not significantly (p=0.460) reduce hyperglycemia over 42 days as compared to PBS controls (Figure 3.6B). Area under the curve of blood glucose curves over 42 days showed no significant reduction in blood glucose over time between transplantation of BMS 493-treated cells (p=0.998) or untreated cells (p=0.593) compared to PBS controls (Figure 3.6C). However, area under the curve of blood glucose values over 42 days was significantly reduced (p=0.002) when fresh $ALDH^{hi}$ cells were iPan transplanted as compared to PBS controls (Figure 3.6C).

Islet size, islet number, and ß-cell mass were also quantified for each mouse using histological pancreas sections from mice transplanted with BMS 493-treated cells, untreated cells, or PBS controls post-mortem. Representative photomicrographs for each cohort are shown in Figure 3.7A. Mice transplanted with BMS 493-treated cells or untreated cells showed no significant difference in average islet size (p=0.051), islet number (p=0.755), or ß-cell mass (p=0.521) as compared to PBS controls (Figure 3.7B-D). Pancreas sections from transplanted mice were also stained with platelet endothelial cell adhesion molecule (PECAM-1, CD31) to assess potential changes in islet vascularity (Figure 3.8A). Mice transplanted with BMS 493-treated cells or untreated cells showed no significant difference (p=0.319) in the number of $CD31^{+}$ cells per islet area as compared to PBS controls (Figure 3.8B). Collectively, these data indicate that islet regenerative capacity was diminished by 6-day culture with or without BMS 493 treatment.

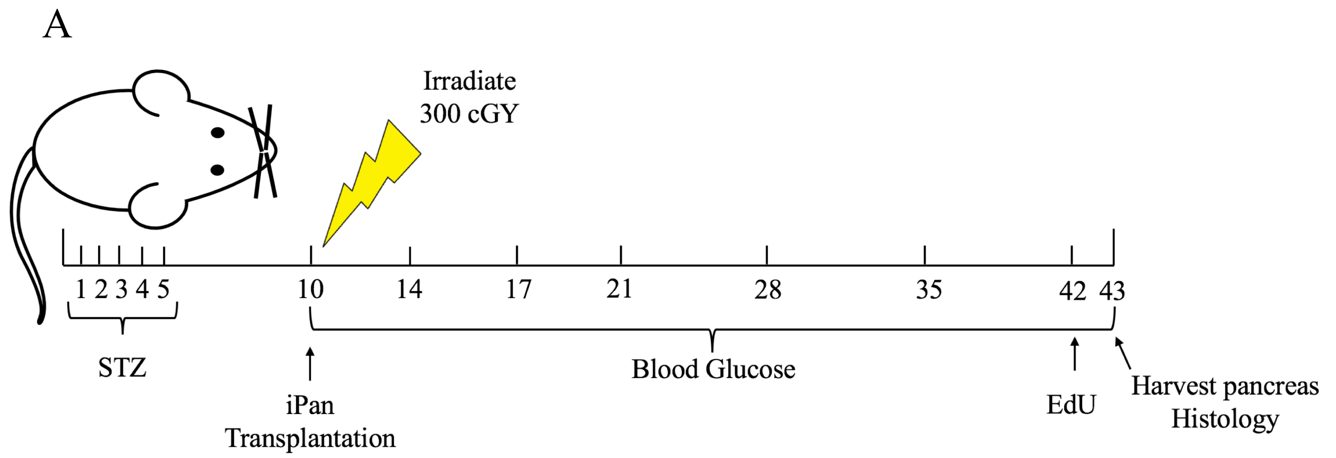
It has been previously published that expanded cells resorted based on high ALDH activity were able to reduce hyperglycemia after IV transplantation into STZ-treated NOD/SCID mice[96]. Therefore, we resorted BMS 493-treated cells and untreated cells based on ALDH activity after expansion and tested their ability to reduce

hyperglycemia after iPan transplantation. There was no significant change in hyperglycemia over 42 days after transplantation of BMS 493-treated $ALDH^{hi}$ (p=0.080), BMS 493-treated $ALDH^{lo}$ (p=0.978), untreated $ALDH^{hi}$ (p=0.207), or untreated $ALDH^{lo}$ (p=0.929) cells as compared to PBS controls (Figure 3.9A). Area under the curve of blood glucose curves over 42 days showed no significant change in blood glucose over time between transplantation of BMS 493-treated $ALDH^{hi}$ (p=0.441), BMS 493-treated $ALDH^{lo}$ (p=0.999), untreated $ALDH^{hi}$ (p=0.903), or untreated $ALDH^{lo}$ (p=0.999) cells as compared to PBS controls (Figure 3.9B). Thus, unlike IV transplantation, resorting expanded cells for high ALDH activity did not show retained islet regenerative capacity after iPan transplantation.

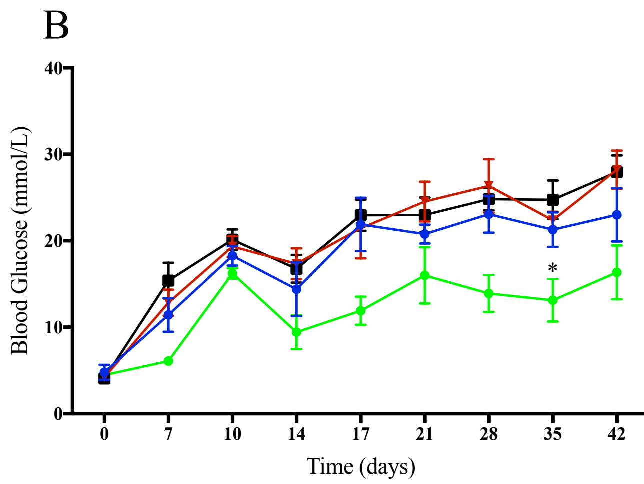
Islet size, islet number, and ß-cell mass were quantified after transplantation of resorted populations from BMS 493-treated and untreated cells (Figure 3.10A). Compared to PBS controls, there was no significant difference in average islet size (p=0.607), islet number (p=0.406), or ß-cell mass (p=0.697) after transplantation of $ALDH^{hi}$ and $ALDH^{lo}$ subpopulations from resorted BMS 493-treated or untreated cells (Figure 3.10B-D). Furthermore, CD31 expression was also quantified in pancreas sections from mice transplanted with BMS 493-treated or untreated cells resorted based on ALDH activity (Figure 3.11A). Mice transplanted with resorted subpopulations from BMS 493-treated or untreated cells showed no significant difference (p=0.335) in the number of $CD31^+$ cells per islet area as compared to PBS controls (Figure 3.11B). Islet vascularity is not improved by resorting of expanded cell populations based on a primitive progenitor function, ALDH activity.

Figure 3.6 Expanded cells did not reduce hyperglycemia after iPan transplantation into STZ-treated NOD/SCID mice. (A) Schematic showing timeline of STZ treatments, iPan transplantation, and blood glucose measurements in NOD/SCID mice. **(B)** Transplantation of BMS 493-treated cells (n= 6) or untreated cells (n=5) did not reduce hyperglycemia over 42 days as compared to PBS controls (n=8). Transplantation of fresh $ALDH^{hi}$ cells (n=4) reduced hyperglycemia at day 35 compared to PBS controls. **(C)** Area under the curve for blood glucose concentration over 42 days showed no reduction in hyperglycemia over time after transplantation of BMS 493-treated or untreated cells compared to PBS controls. In contrast, transplantation of fresh $ALDH^{hi}$ cells reduced area under the curve of glucose curves over 42 days compared to PBS controls. n represents individual experimental mice. Data is shown as mean ± SEM (*p<0.05, **p<0.01) determined by two-way ANOVA **(B)** and one-way ANOVA **(C)** with Tukey's multiple comparisons test.

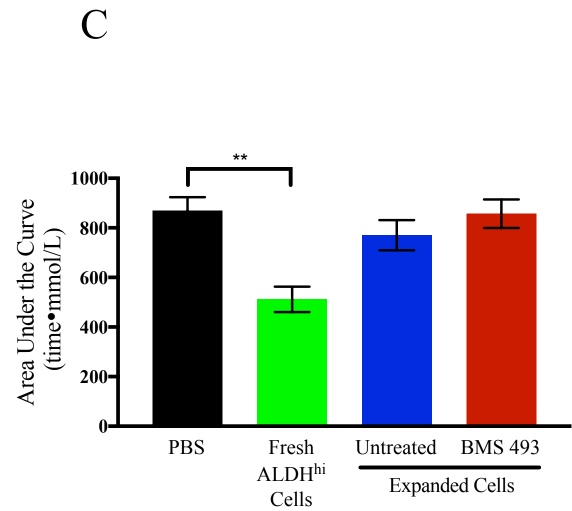
A



B



C



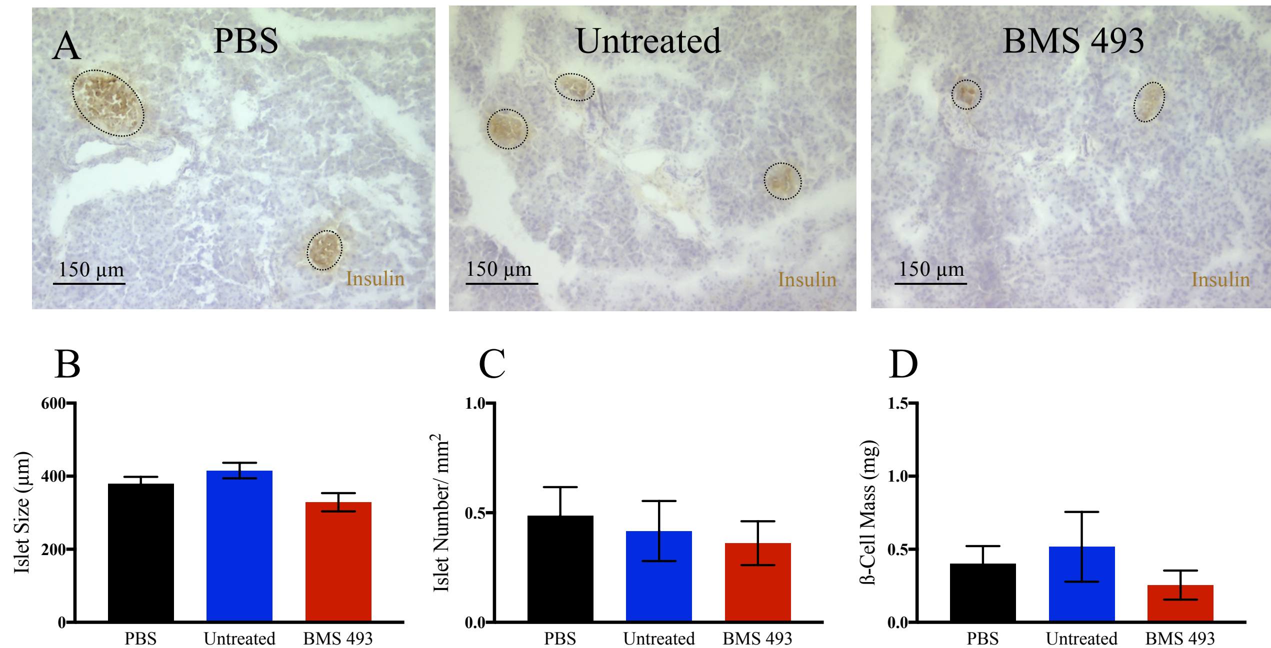


Figure 3.7 Expanded cells did not alter difference in islet size, islet number, or ß-cell mass after iPan transplantation into STZ-treated NOD/SCID mice. (A) Representative photomicrographs of pancreas sections from mice transplanted with PBS controls, untreated expanded hematopoietic cells, or BMS 493-treated hematopoietic cells. Mice transplanted with untreated (n=5) or BMS 493-treated (n=6) cells showed no difference in islet size **(B)**, islet number **(C)**, or ß-cell mass **(D)** as compared to PBS controls (n=8). n represents individual experimental mice. n represents individual experimental mice. Data is shown as mean ± SEM with analysis of significance determined by one-way ANOVA.

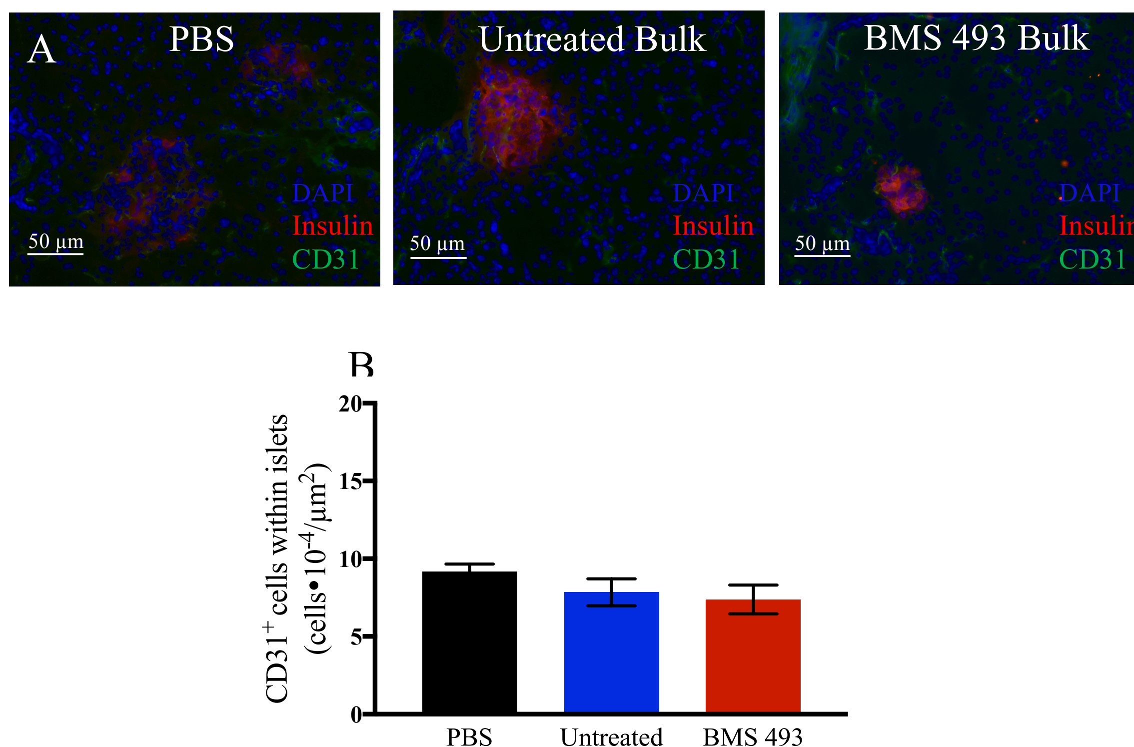


Figure 3.8 Expanded cells did not alter islet vascularity after iPan transplantation into STZ-treated NOD/SCID mice. Representative pancreas immunohistochemistry sections showing insulin and CD31 from mice transplanted with **(A)** PBS, untreated expanded hematopoietic cells, or BMS 493-treated hematopoietic cells. **(B)** BMS 493-treated (n=3) or untreated (n=3) cells showed no difference in $CD31^+$ cells/µm islet area as compared to PBS controls (n=3). n represents individual experimental mice. Data is shown as mean ± SEM with analysis of significance determined by one-way ANOVA.

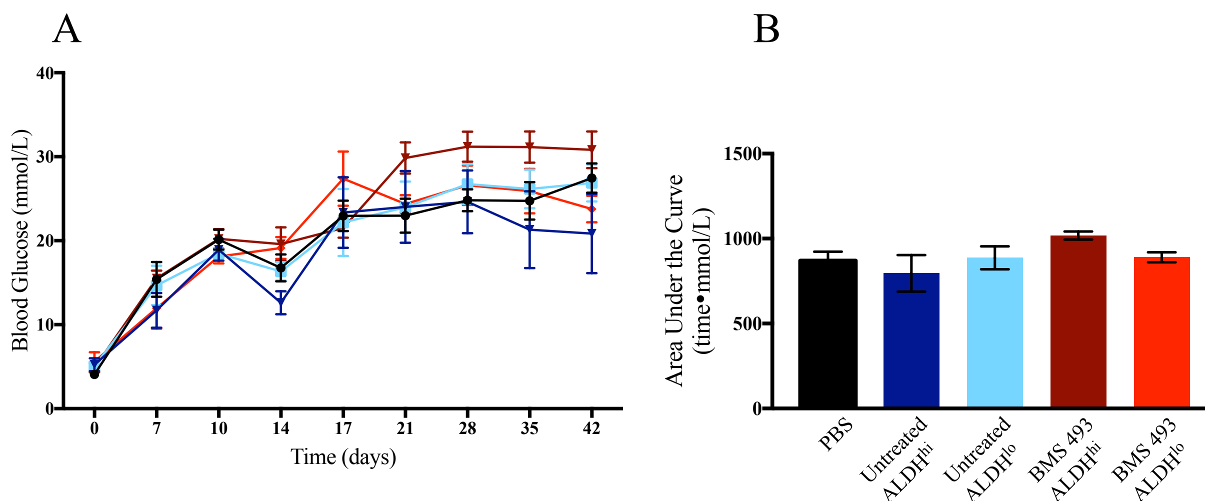


Figure 3.9 Expanded hematopoietic cells resorted based on ALDH activity did not reduce hyperglycemia after iPan transplantation into STZ-treated NOD/SCID mice. (A) Transplantation of BMS 493-treated $ALDH^{hi}$ (n=4), BMS 493-treated $ALDH^{lo}$ (n=4), untreated $ALDH^{hi}$ (n=4) or untreated $ALDH^{lo}$ (n=4) cells $ALDH^{hi}$ did not reduce hyperglycemia over 42 days as compared to PBS controls (n=8). **(B)** Area under the curve of blood glucose curves over 42 days after transplantation of resorted BMS 493-treated or untreated cell populations showed no reduction in blood glucose over time compared to PBS controls. n represents individual experimental mice. Data is shown as mean ± SEM with analysis of significance determined by two-way ANOVA **(A)** and one-way ANOVA **(B).**

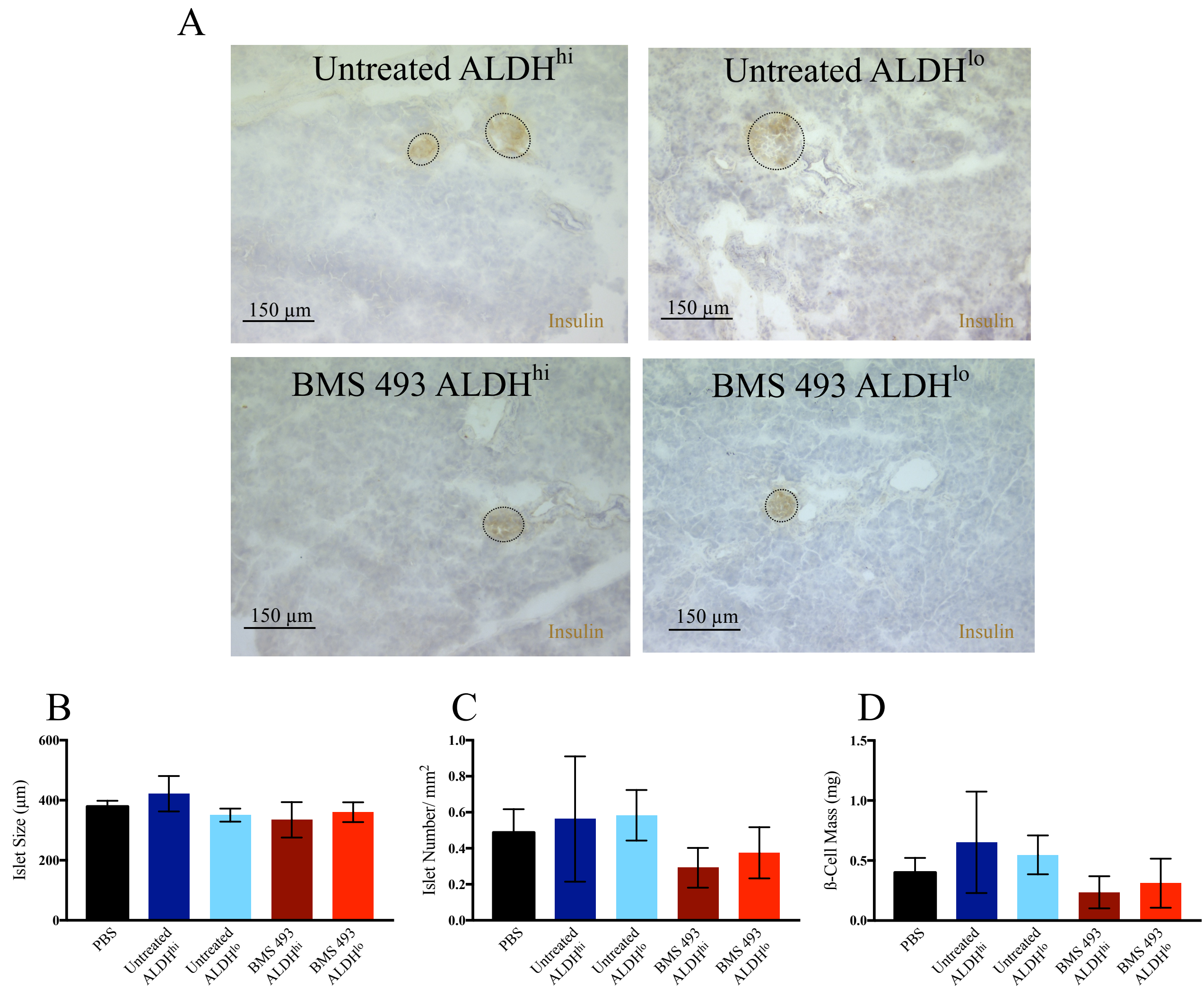
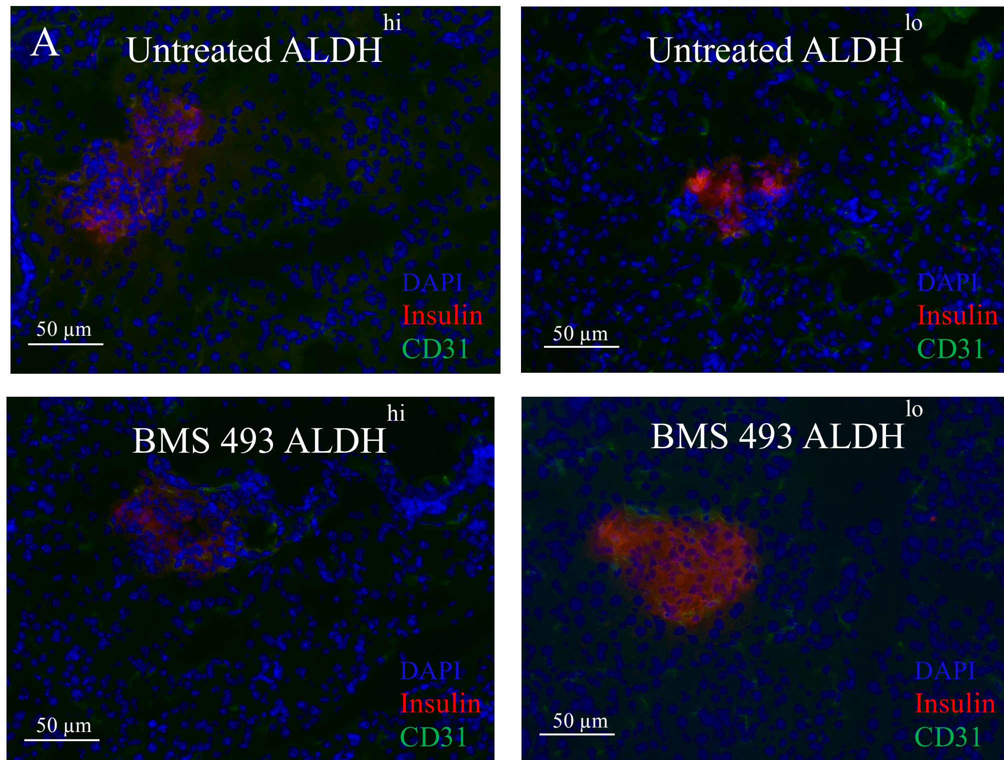
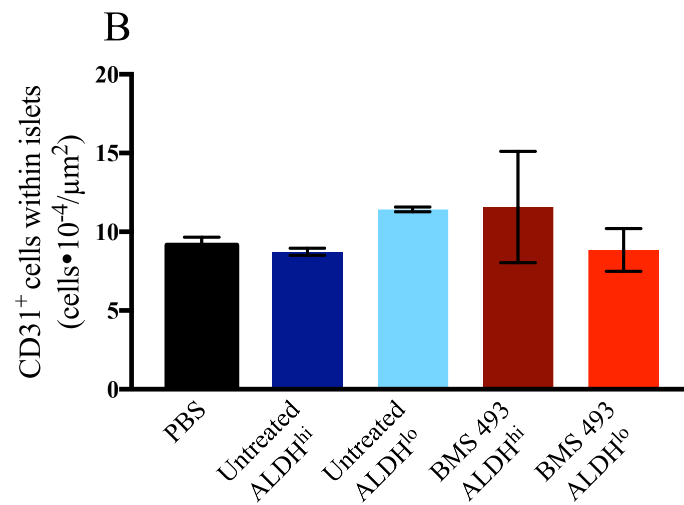


Figure 3.100 Expanded hematopoietic cells resorted based on ALDH activity did not alter islet size, islet number, or ß-cell mass after iPan transplantation into STZ-treated NOD/SCID mice. (A) Representative photomicrographs of pancreas sections from mice transplanted with untreated $ALDH^{hi}$, untreated $ALDH^{lo}$, BMS 493-treated $ALDH^{hi}$, or BMS 493-treated $ALDH^{lo}$ cells. Mice transplanted with BMS 493-treated $ALDH^{hi}$ (n=4), BMS 493-treated $ALDH^{lo}$ (n=4), untreated $ALDH^{hi}$ (n=4) or untreated $ALDH^{lo}$ (n=4) cells showed no significant difference in islet size **(B)**, islet number **(C)**, or ß-cell mass **(D)** as compared to PBS controls (n=8). n represents individual experimental mice. Data is shown as mean ± SEM with analysis of significance determined by one-way ANOVA.

Figure 3.111 Expanded hematopoietic cells resorted based on ALDH activity did not alter islet vascularity after intrapancreatic transplantation into STZ-treated NOD/SCID mice. (A) Representative immunohistochemistry sections of mouse pancreas showing insulin and CD31 after intrapancreatic transplantation of untreated $ALDH^{hi}$, untreated $ALDH^{lo}$, BMS 493-treated $ALDH^{hi}$, or BMS 493-treated $ALDH^{lo}$ cells. **(B)** Mice transplanted with BMS 493-treated $ALDH^{hi}$ (n=3), BMS 493-treated $ALDH^{lo}$ (n= 3), untreated $ALDH^{hi}$ (n=3) or untreated $ALDH^{lo}$ (n=3) cells showed no significant difference in difference in $CD31^{+}$ cells/µm islet area as compared to PBS controls (n=3). n represents individual experimental mice. Data is shown as mean ± SEM with analysis of significance determined by one-way ANOVA.


A
Untreated ALDH hi
Untreated ALDH lo
BMS 493 ALDH hi
BMS 493 ALDH lo
DAPI
Insulin
CD31
50 μm


B
CD31+ cells within islets (cells•10-4/μm2)
0
5
10
15
20
PBS
Untreated ALDHhi
Untreated ALDHlo
BMS 493 ALDHhi
BMS 493 ALDHlo

3.5 Conditioned Media from BMS 493-Treated Cells Increased ß-Cell Proliferation *In Vitro*

Next, human islets were cultured with CM generated from HSPC expanded with or without BMS 493 and assessed for ß-cell survival and proliferation via flow cytometry. Representative photopictomicrographs are shown on day 3 for human islets cultured with CM from BMS 493-treated cells, CM from untreated controls, and media controls (RPMI 1640) (Figure 3.12A). Islets cultured in CM generated from untreated or BMS 493-treated cells showed no significant difference (p=0.642) in total islet cell number at both day 3 and day 7 as compared to media controls (Figure 3.12B). FluoZin-3 AM is an indicator of zinc (Zn^{2+}) and was used to mark intact ß-cells, which are high in zinc due to the exocytosis machinery of secretory granules containing insulin[(100)]. Islets cultured in CM generated from untreated or BMS 493-treated cells did not change the frequency of FluoZin-3^{+} cells (p=0.717) or permeabilized $CD45^{-}Insulin^{+}$ cells (p=0.858) compared to media controls, both used as indicators of ß-cell frequency between conditions (Figure 3.12C and D). Thus, treatment of human islets with CM generated from expanded cells did not affect total cell number or ß-cell number after 3 or 7 days *in vitro*.

The viability dye 7-AAD and apoptosis marker Annexin V were used to assess ß-cell survival via flow cytometry. Representative flow cytometry dot plots are shown for a fluorescence-minus-one (FMO) control used to set a FluoZin-3^{+} gate, an experimental condition showing FluoZin-3^{+} cells, and Annexin V versus 7-AAD, showing cell apoptosis and cell death frequencies on FluoZin-3^{+} cells specifically (Figure 3.13A-C). CM from untreated or BMS 493-treated cells did not alter the frequency of live ß-cells (Q4:FluoZin-3^{+}7$AAD^{-}Annexin^{-}$, p=0.730), apoptotic ß-cells (Q3:FluoZin-3^{+}7AAD^{-} $Annexin^{+}$, p=0.864), dead ß-cells (Q2:FluoZin-3^{+}7$AAD^{+}Annexin^{+}$, p=0.546), and necrotic ß-cells (Q1:FluoZin-3^{+}7$AAD^{+}Annexin^{-}$, p=0.755) at both day 3 and day 7 as compared to media controls (Figure 3.13D-G). CM generated from expanded cells did not change the frequencies of apoptosis or survival of human ß-cells after 3 or 7 days in culture.

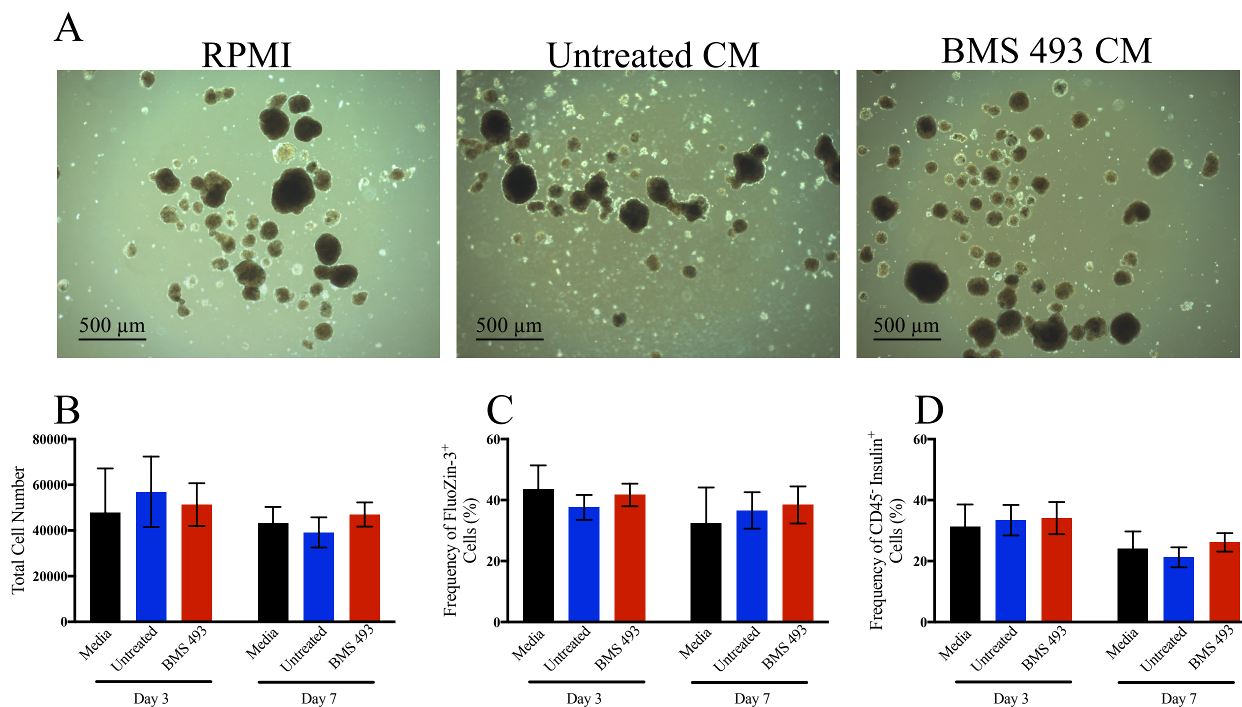
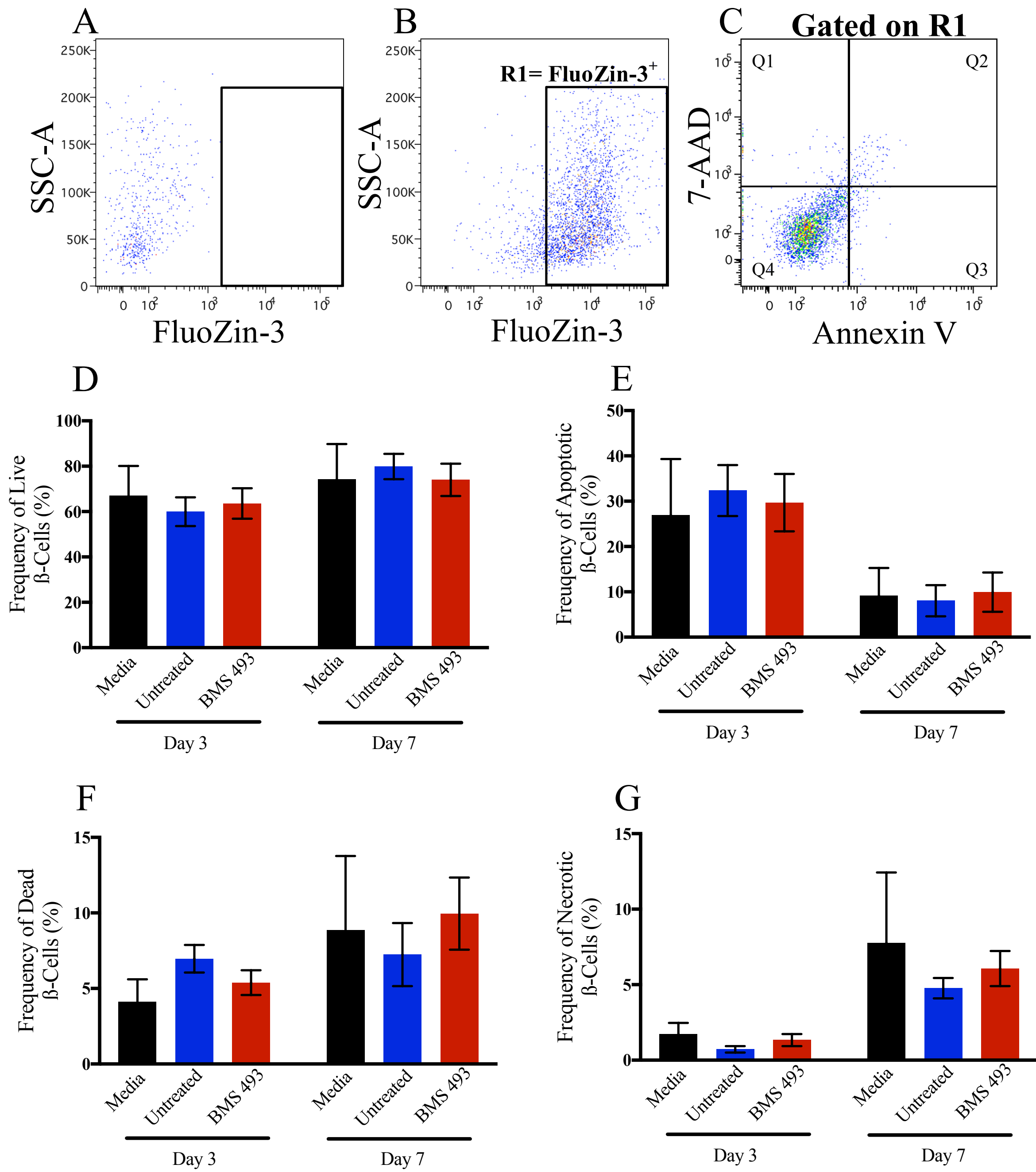


Figure 3.122 Human islets cultured with conditioned media generated from BMS 493-treated hematopoietic cells showed no difference in frequency of ß-cells. (A) Representative photopictomicrographs showing human islets cultured with CM generated from BMS 493-treated cells, untreated controls, or media controls (RPMI 1640) on day 3. **(B)** Compared to media controls (N=3), CM from BMS 493-treated cells (N=3-4) or untreated controls (N=3-4) showed no differences in total cell number at day 3 or day 7. Compared to media controls, CM from BMS 493-treated cells or untreated controls showed no difference in frequency of **(C)** FluoZin-3$^+$ cells or **(D)** CD45$^-$Insulin$^+$ cells at day 3 or day 7. N represents individual pancreas donors. Data is shown as mean ± SEM with analysis of significance determined by repeated measures ANOVA.

Figure 3.133 Human islets cultured with conditioned media generated from BMS 493-treated hematopoietic cells showed no difference in ß-cells survival. Representative flow cytometry dot plots showing **(A)** FMO control used to set FluoZin-3^+ gate, **(B)** FluoZin-3^+ gated on experimental samples **(C)** Annexin V against 7-AAD to assess apoptosis and cell death on FluoZin-3^+ cells. Compared to media controls (RPMI 1640, N=3), CM from BMS 493-treated cells (N=3-4) or untreated cells (N=3-4) showed no differences in the frequency of **(D)** Annexin V^-7AAD$^-$ (live) ß-cells, **(E)** Annexin V^+7AAD$^-$ (apoptotic) ß-cells, **(F)** Annexin$^+$7AAD$^+$ (dead) ß-cells, and **(G)** Annexin$^-$7AAD$^+$ (necrotic) ß-cells at day 3 or day 7. N represents individual pancreas donors. Data is shown as mean ± SEM with analysis of significance determined by two-way ANOVA.


A
B
C
Gated on R1
R1= FluoZin-3+
SSC-A
FluoZin-3
7-AAD
Annexin V
Q1
Q2
Q3
Q4
D
E
F
G
Frequency of Live β-Cells (%)
Freuqency of Apoptotic β-Cells (%)
Frequency of Dead β-Cells (%)
Frequency of Necrotic β-Cells (%)
Media
Untreated
BMS 493
Day 3
Day 7

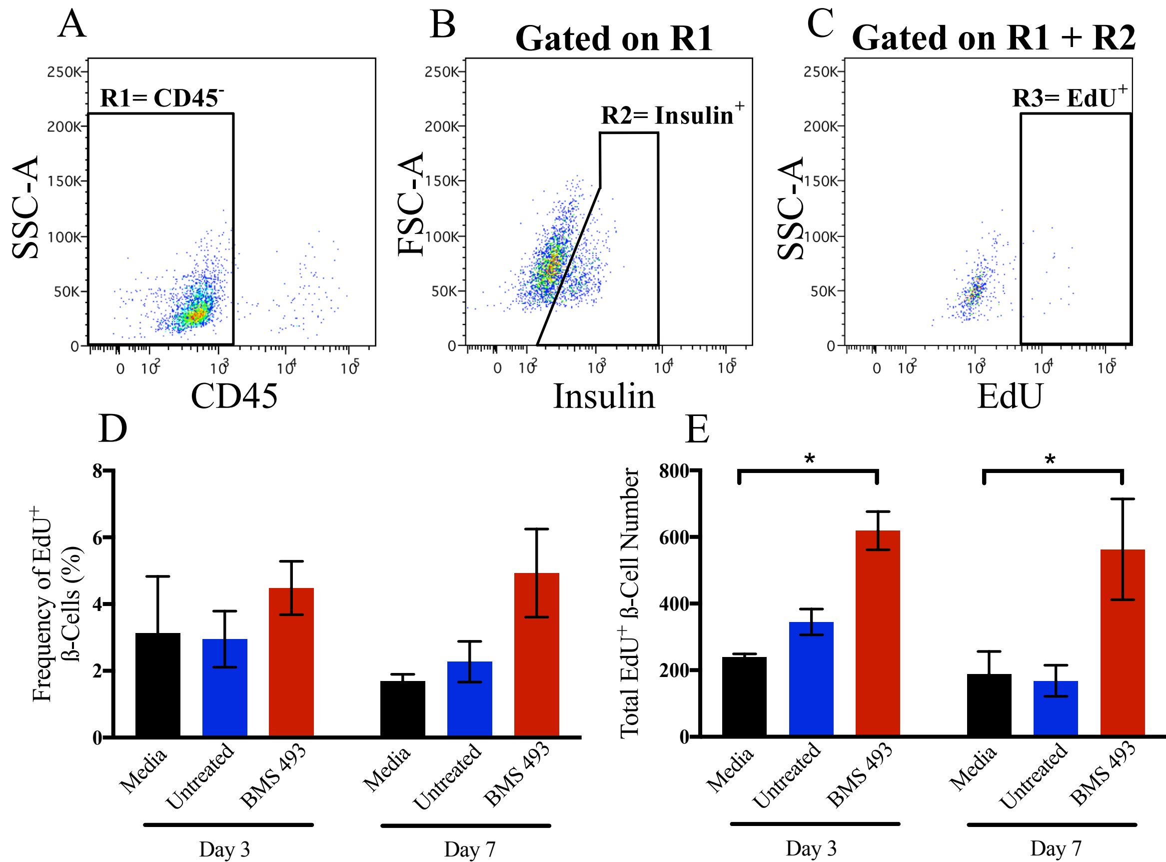


Figure 3.144 Human islets cultured with conditioned media generated from BMS 493-treated hematopoietic cells showed an increase in the number of proliferating ß-cells. Representative flow cytometry dot plots showing **(A)** gating CD45⁻ cells to eliminate hematopoietic contamination, **(B)** insulin⁺ gate to mark ß-cells, and **(C)** EdU gated on insulin⁺ cells to mark proliferating ß-cells. **(D)** Compared to media controls (RPMI 1640, N=3), CM from BMS 493-treated cells (N=3-4) or untreated cells (N=3-4) showed no difference in the frequency of EdU+ ß-cells at day 3 or day 7. **(E)** CM from BMS 493-treated cells showed an increase in the total number of EdU⁺ ß-cells at both day 3 and day 7 compared to CM from untreated cells and media controls. N represents individual pancreas donors. Data is shown as mean ± SEM (*p<0.05) determined by two-way ANOVA and Dunnett's multiple comparisons test.

Incorporation of EdU, a thymine analog, was used to assess the induction of ß-cell proliferation via flow cytometry. Representative flow cytometry dot plots show exclusion of hematopoietic contamination using negative gating for $CD45^{+}$ cells, insulin was used to mark permeabilized ß-cells, and EdU analyses was gated on $insulin^{+}$ cells to mark proliferating ß-cells (Figure 3.14A-C). CM from untreated or BMS 493-treated cells showed no significant difference (p=0.695) in the frequency of proliferating ß-cells ($CD45^{-}Insulin^{+}EdU^{+}$) at both day 3 and day 7 as compared to media controls (Figure 3.14D). However, when total proliferating ß-cells were enumerated, islets cultured with CM generated from BMS 493-treated cells showed a significant increase in the total number of proliferating ß-cells at both day 3 (p=0.037) and day 7 (p=0. 035) as compared to media controls (Figure 3.14E). CM generated from $ALDH^{hi}$ cell expansion with BMS 493 induced human ß-cell proliferation *in vitro*.

3.6 Conditioned Media Generated from Expanded Cells Did Not Reduce Hyperglycemia in STZ-Treated NOD/SCID Mice

Following the increase in ß-cell proliferation shown when human islets were cultured with CM generated from BMS 493-treated cells *in vitro*, we injected CM from BMS 493-treated or untreated cells directly into the pancreata of STZ-treated NOD/SCID mice. Unpublished data from our lab has shown that iPan injection of CM generated from cultured human MSC consistently reduce hyperglycemia in STZ-treated NOD/SCID mice (Kuljanin *et al.*). However, iPan injection of protein concentration-matched CM from BMS 493-treated or untreated cells did not significantly (p=0.272) reduce hyperglycemia over 42 days as compared to media (RPMI 1640) controls (Figure 3.15A). Area under the curve of blood glucose curves over 42 days confirmed no significant reduction in blood glucose was observed after injection of CM from BMS 493-treated cells (p=0.221) or untreated cells (p=0.157) compared to media controls (Figure 3.15B).

Finally, islet size, islet number, and ß-cell mass were quantified using histological pancreas sections post-mortem from mice injected with CM generated from BMS 493-treated cells, untreated cells, or transplanted with media controls (Figure 3.16A). As indicated by the evaluation of hyperglycemia, mice injected with CM from BMS 493-

treated or untreated cells showed no significant difference in average islet size (p=0.248), islet number (p=0.535), or ß-cell mass (p=0.589) compared to media controls (Figure 3.16B-D). Thus, injection of concentrated CM from expanded cells into STZ-treated NOD/SCID mice showed no reduction in hyperglycemia and no effect on ß-cell mass.

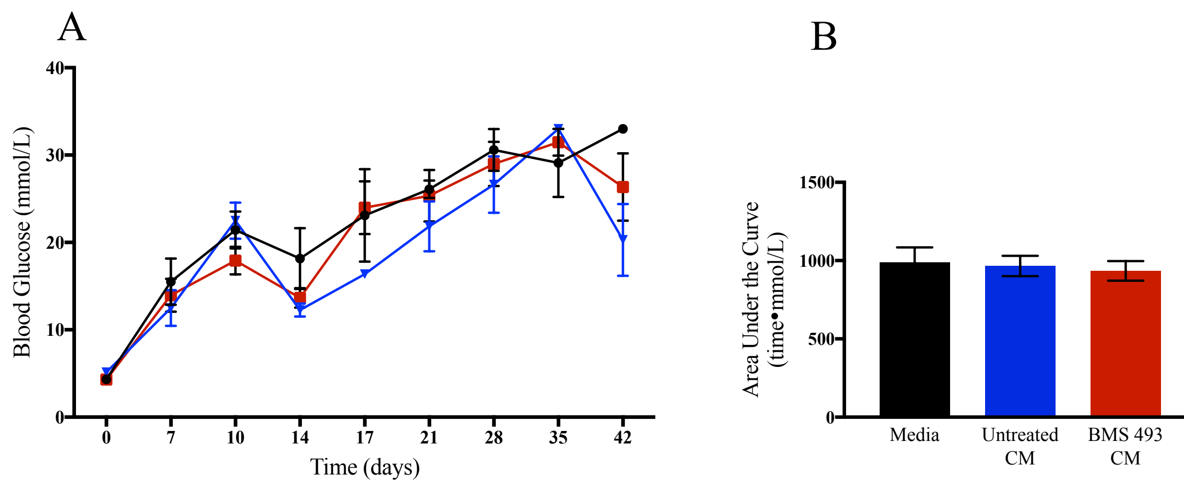


Figure 3.155 Conditioned media from BMS 493-treated cells showed no difference in islet size, islet number, or ß-cell mass after iPan injection into STZ-treated NOD/SCID mice. (A) Injection of CM from BMS 493-treated cells (n=4) or untreated cells (n=3) did not reduce hyperglycemia over 42 days as compared to media (RPMI 1640) controls (n=3). **(B)** Area under the curve of blood glucose curves over 42 days showed no reduction in blood glucose over time after injection of CM from BMS 493-treated or untreated cells compared to media controls. n represents individual experimental mice. Data is shown as mean ± SEM with analysis of significance determined by two-way ANOVA **(A)** and one-way ANOVA **(B)**.

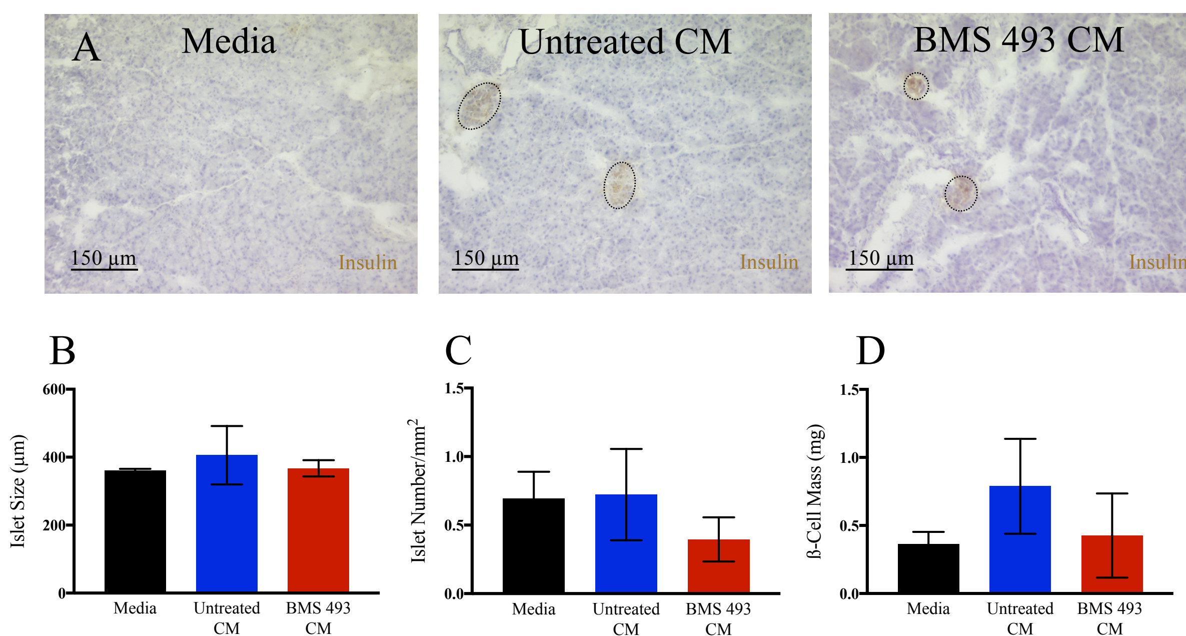


Figure 3.166 Conditioned media from BMS 493-treated hematopoietic cells showed no difference in islet size, islet number, or ß-cell mass after iPan injection into STZ-treated NOD/SCID mice. (A) Representative photomicrographs of pancreas sections from mice injected with media (RPMI 1640) controls, CM from untreated cells, or CM from BMS 493-treated cells. Mice injected with CM from BMS 493-treated (n=4) or untreated (n=3) cells showed no difference in islet size **(B)**, islet number **(C)**, or ß-cell mass **(D)** as compared to media controls (n=3). n represents individual experimental mice. Data is shown as mean ± SEM with analysis of significance determined by one-way ANOVA.

4 Discussion

4.1 Retinoid Signaling in the Expansion of Hematopoietic Progenitor Cells

Previously, we have identified that a subset of UCB-derived cells with high ALDH activity can stimulate endogenous islet repair after iPan transplantation into NOD/SCID mice with STZ-induced hyperglycemia[94]. However, these cells are extremely rare, representing <0.5% of UCB mononuclear cells and thus, *ex vivo* expansion of this cell population with minimal differentiation is warranted to generate sufficient cell numbers for islet regenerative cell therapy[101]. Unfortunately, HSPC differentiate quickly when cultured, rapidly losing their high ALDH activity after 6-day expansion[96]. UCB ALDH^{hi} cells were expanded under clinically applicable, serum free culture conditions with cytokine supplementation; however, only 15.4 ± 1.9% of cells retained high ALDH activity after 6-day expansion[92]. Chute *et al.* suggested that inhibition of retinoid signaling during HSPC expansion could prevent RA-induced differentiation towards maturity down the hematopoietic hierarchy[91]. By using DEAB during short term culture, a direct inhibitor of ALDH1, Chute showed increased number of CD34^{+}CD38^{-}Lin^{-} cells compared to day 0. Expanded cell progeny had increased number of SCID repopulating cells after transplantation into NOD/SCID mice. Finally, DEAB-treatment showed decreased expression of cEBPε, a downstream target of RAR-signaling. Our lab has used a similar strategy to expand ALDH^{hi} cells with DEAB to prevent differentiation for use in vascular regenerative therapies (Cooper *et al*.). Thus, alternate molecules such as BMS 493 to block RA-induced differentiation are needed to develop allogeneic cell therapy approaches for diabetes and its comorbidities.

The first aim of this thesis was to characterize the expansion of ALDH^{hi} cells in culture under hematopoietic cell-specific conditions, using a small molecule inhibitor of retinoic acid signaling. BMS 493 is an inverse pan-RAR agonist that, when bound to the RAR/RXR heterodimer, stabilizes a corepressor complex, silencing downstream gene transcription[93]. When treated with BMS 493, UCB-derived ALDH^{hi} progeny expanded for 6 days in culture showed a 2-fold increase (31.16 ± 3.09%) in the frequency of cells with high ALDH activity compared to untreated controls. These cells also retained

expression of primitive cell surface markers CD34 and CD133 and had reduced expression of the mature hematopoietic cell surface marker CD38. Treatment with BMS 493 did not reduce the enhanced colony forming capacity of the ALDH^{hi} subset compared to ALDH^{lo} subset of cell progeny. Despite preservation of primitive HSPC phenotype, cell proliferation over 6 days was not inhibited by BMS 493 treatment. Therefore, treatment with BMS 493 during the 6-day expansion of ALDH^{hi} cells resulted in an 8.3 ± 1.27-fold increase in the total number of cells available for transplantation, without the loss of primitive cell phenotype.

ALDH activity represents the rate-limiting step in the conversion of vitamin A to retinoic acid, a powerful morphogen. Presumably, by inhibiting ALDH during HSPC expansion using DEAB, Chute *et al.* reduced the total amount of RA available for downstream signaling. In contrast, BMS 493 acts further downstream on the RAR/RXR heterodimer required for RA-pathway targeted gene transcription. Since BMS 493 does not directly affect the production of RA in the cell, levels of RA are likely unaffected or even slightly increased. Consequently, RA accumulation in the cytosol would be available to non-canonical RA signaling pathways not impacted by the RAR/RXR heterodimer, acting as a ligand for less common receptors such as receptor-related orphan receptors (RORß)[(102)]. Since RA is a morphogen, levels regularly fluctuate in cells and are difficult to accurately measure. Measurement of downstream targets of RA signaling pathways may help elucidate how manipulating the actions of RA during *ex vivo* culture affects the differentiation and regenerative capacity of HSPC.

4.2 Islet Regenerative Capacity of Uncultured and Cultured Cell Populations

In the context of cell therapies for diabetes, several preclinical studies have used hematopoietic mononuclear cells or MSC, primarily from human BM, to induce endogenous islet regeneration *in situ*. Bell *et al.* showed that BM-derived stem cells isolated based on high ALDH activity can reduce hyperglycemia after IV injection into STZ-treated NOD/SCID mice[(95)]. In 2012, our lab published that direct iPan delivery of uncultured, UCB-derived ALDH^{hi} cells could reduce hyperglycemia and increase serum insulin levels in STZ-treated NOD/SCID mice. Furthermore, mice showed improved

functional recovery when tested using a glucose challenge (intraperitoneal glucose tolerance test) compared to mice injected with an equivalent dose of ALDH^{lo} cells or PBS controls[(94)]. HLA$^+$ cells were detected surrounding islets at days 14 and 17, although few were detected up to day 42. Although the mechanism of repair was not fully established, HLA$^+$ cells detected never co-expressed insulin, suggesting that the donor cells induced endogenous islet repair in the hosts via undetermined paracrine activities. The authors proposed that increased exposure of the pancreas to regenerative factors secreted by ALDH^{hi} cells ultimately enhanced the regenerative response *in vivo*. Unfortunately, iPan transplantation of UCB-derived ALDH^{hi} cells expanded in culture lost their ability to reduce hyperglycemia in the same mouse model, with or without BMS 493-treatment. Thus, while HSPC can be expanded efficiently *ex vivo*, secretory and pro-survival function may be compromised by culture induced differentiation and transplantation at higher cell doses may be required to achieve a regenerative effect after intravenous or direct intrapancreatic injection.

4.3 Intrapancreatic Versus Intravenous Delivery of Expanded Hematopoietic Progenitor Cells

Following the reduction in hyperglycemia after iPan transplantation of uncultured UCB-derived ALDH^{hi} cells into STZ-treated NOD/SCID mice, iPan injection was selected as the preferred method of delivery for cells expanded under RA-pathway inhibited conditions. Our lab had previously extended these findings to show expanded cells reselected for high ALDH activity possess similar regenerative function after IV injection of cells[(96)]. Interestingly, only the ALDH^{hi} fraction of expanded cells showed consistent engraftment in the BM after IV transplantation as measured by CD45$^+$ and HLA-A,B,C$^+$ cells using flow cytometry. However, iPan injection of expanded HSPC treated with or without BMS 493 that were resorted based on high ALDH activity after 6 days did not show a reduction in hyperglycemia. Although IV-injection of cells expanded with BMS 493 was not assessed in this thesis, it is likely that the regenerative effect of cells is dependent on the proteins they secrete and how these signaling molecules interact with other cell types found in the host pancreas. While iPan transplantation is a more direct mode of transporting these signaling molecules to the site of tissue injury than IV

transplantation, human cell survival and engraftment may be compromised since the STZ-treated pancreas is a suboptimal niche for expanded HSPC and only small volumes can be transplanted into the murine pancreas without risk of damage to the exocrine tissue or pancreatitis. Therefore, it is important to characterize parameters such as human cell survival and engraftment along with tissue regeneration and functional recovery when determining the optimal mode of delivery for cell therapy in patients with DM.

4.4 Importance of Islet Vascularization in Insulin Delivery

The pancreas, like other endocrine organs, is extremely well vascularized. The ability for the pancreas to develop during embryogenesis as well as perform its role through adult life is dependent on adequate supply of blood. In particular, maintenance of normoglycemia relies on the transport of insulin from the islets of Langerhans to peripheral tissues in the body. First described in the 1800s, islets have their own capillary networks that are glomerular-like and can take up as much as 10% of the total islet volume[(103, 104)]. In addition, islets are approximately 10 times more vascular than their surrounding exocrine tissue, even though they only make up a small proportion of the pancreas, and vascularization of islets is considered independent of exocrine pancreas vascularization. Replacing lost ß-cell mass in T1DM may be the primary goal of new cellular therapies; however, ensuring proper vascularization of ß-cells is imperative in achieving functional recovery and glucose homeostasis.

While little is known regarding the mechanisms of islet regeneration after expanded ALDH^{hi} cell transplantation, there is a larger body of literature studying the pro-angiogenic effects of HSPC in models of vascular injury. Our lab has previously shown that UCB-derived ALDH^{hi} cells can aid in the revascularization and perfusion of injured muscle tissue after femoral artery ligation in NOD/SCID mice, an important finding given the prevalence of peripheral artery disease and critical limb ischemia as a complication of DM[(101)]. When UCB ALDH^{hi} cells were in noncontact co-culture with human umbilical vein endothelial cells (HUVEC) in serum free media, only ALDH^{hi} cells increased viability of HUVEC compared to ALDH^{lo} cells or HUVEC grown alone. Furthermore, co-culture of UCB ALDH^{hi} cells and HUVEC in Matrigel showed

increased formation of tube-like structures after 24 hours compared to co-culture with ALDH^{lo} cells. Collectively, these findings showed that UCB-derived ALDH^{hi} cells contained hematopoietic and endothelial progenitor cell populations that played a role in revascularization and increased capillary density when transplanted after ischemic injury.

Surprisingly, the ability of UCB-derived ALDH^{hi} cell progeny to revascularize the hind limb after femoral artery ligation was not lost after ALDH^{hi} cells were expanded for 6 days in culture [(105)]. Expanded ALDH^{hi} cells did not require reselection after expansion to potently augment limb revascularization after femoral artery ligation. Proliferation of host CD31^{+} endothelial cells was detected post-mortem and microarray analysis of expanded HSPC showed increased transcription of several pro-angiogenic cytokines including vascular endothelial growth factors (VEGF-A and VEGF-B) as well as angiopoietin-1 and epidermal growth factor. These findings suggest that HSPC play a vital role in revascularization of ischemic tissue and that these results could be translated to use UCB-derived ALDH^{hi} cells in revascularizing injured pancreatic tissue.

Combinatorial transplantation of regenerative BM-derived MSC followed by uncultured UCB-derived cells showed a greater reduction in hyperglycemia in STZ-treated NOD/SCID mice than either cell population independently[(57, 94, 95)]. One explanation of these results is that while both populations work synergistically to increase ß-cell mass by inducing endogenous islet repair, the HSPC are able to further improve the reduction in hyperglycemia by better vascularizing the islets and improving the transport of insulin outside of the pancreas. Upon further investigation, immunohistochemistry showed that transplantation of regenerative MSC alone or in combination with ALDH^{hi} cells resulted in increased ductal association with islets but only MSC in combination with ALDH^{hi} cells caused increased vessel density. It was proposed that transplantation of MSC induced islet neogenesis while transplantation of UCB-derived ALDH^{hi} cells was pro-angiogenic, inducing ß-cell proliferation. Although we did not see differences in the frequency of CD31^{+} cells found in islets after iPan transplantation of HSPC expanded with BMS 493, this may have been due to the fact that ß-cell mass was not increased. Thus, vascularization was not induced since there was insufficient insulin production after STZ treatment to warrant new vasculature for transport. While we did not observe a

reduction in hyperglycemia from iPan transplantation of expanded HSPC alone, expansion of this cell population with small molecules such as BMS 493 may be important to revascularize islets after an alternate regenerative stimulus is delivered, particularly in islet replacement strategies that focus on increasing the number of ß-cells available and survival of ß-cells after transplantation.

4.5 Conditioned Media as a Regenerative Therapeutic

Conditioned media (CM) represents the heterogeneous secretome of cells grown in culture that can be collected and concentrated to study its composition and functional capacity. CM contains a mixture of proteins, signaling lipid molecules, and microRNAs that may be packaged within extracellular vesicles and can be released into the circulation to augment endogenous regenerative capacity without having to transplant the cells that the CM is generated from[(106)]. Deriving therapeutic agents using CM has several advantages compared to cellular therapies. One of the most important of these advantages is that without the transplant of live cells, donor matching and immune rejection can be bypassed. Second, the ability to identify individual factors or mixture of factors involved in the regeneration processes can provide a streamlined approach to efficiently develop novel therapeutics using modern proteomic techniques. Finally, mechanisms of regeneration can be better understood and established without the interplay of donor and host cells.

With these advantages in mind, we derived a model to test the effects of CM exposure on the survival and proliferation of primary human islets obtained through the Integrated Islet Distribution Program using flow cytometry on dispersed islets. Our lab previously published that CM collected from Wnt pathway-activated MSC augmented the total ß-cell number, frequency of live ß-cells, and frequency of proliferating ß-cells[(107)]. While no differences in ß-cell survival were found, CM collected from BMS 493-treated HSPC increased the number of proliferating ß-cells *in vitro*. This lead us to further study the effects of CM on islet regeneration after iPan transplantation of concentrated CM into STZ-treated NOD/SCID mice. Transplantation of CM was predicted to be a potent therapy because CM can be concentrated using filtration units and transplanted directly into the mouse pancreas using low volumes. Transplantation of CM generated from

expanded HSPC treated with BMS 493 did not show a reduction in hyperglycemia or increased ß-cell mass in STZ-treated mice. In contrast to our *in vitro* human islet cultures that showed an increase in ß-cell proliferation, these effects may not have been replicated *in vivo* due to extensive ß-cell damage as a result of STZ-treatment. Our *in vitro* culture system utilized cadaveric islets from non-diabetic donors and thus signaling mechanisms may differ between the healthy and diabetic pancreas microenvironments.

4.6 Clinical Implications

Currently, one of the limiting factors to developing novel cellular therapies for the treatment of T1DM is the availability of an unlimited source of ß-cells. While preclinical findings have extensively studied the differentiation of hES and iPS cells into mature, glucose-responsive ß-cells, the safety and efficacy of these sources for use in cellular therapies remains unclear. UCB provides a readily available source of multipotent HPSC that have previously shown islet regenerative functions in preclinical trials[94, 95]. However, cells that harness regenerative capacity are rare in postnatal tissue, such as UCB, and differentiate quickly when expanded *ex vivo*[96, 105]. In order to reach clinically applicable numbers to treat patients with T1DM, cell expansion, without the loss of regenerative function, will be necessary. In 2012, as a platform for the treatment of blood disorders including leukemia, Csazar *et al.* published important findings relevant to hematopoietic cell expansion. By using a batch-fed bioreactor system, large scale *ex vivo* expansion was made possible by controlled media dilution, thus minimizing the inhibitory feedback that cultured cells are exposed to over time[97]. Other agents are being investigated for their effect on self-renewal and differentiation of stem cells during *ex vivo* culture. For example, agents that inhibit the aryl hydrocarbon receptor such as the compound UM729 showed decreased differentiation of acute myeloid leukemia cells in culture[108]. While our previously described culture conditions are serum free and thus clinically applicable, these findings act as proof-of-concept that *ex vivo* expanded cells can maintain their regenerative capacity. These findings become more powerful for clinical use when combined with large scale bioreactor systems such as the fed-batch system described by Csazar. Thus, bioreactor technologies for expansion, combined with

pharmacological manipulation of the cell product, provides a powerful strategy for the development of cell therapies.

Regenerating lost ß-cell mass is only the first step in developing a curative therapy for T1DM. Relentless continued autoimmune attack in patients with T1DM represents another challenge for the implementation of curative strategies. Ongoing clinical trials have made use of bioengineered encapsulation devices that allow transplanted cells to secrete insulin to the periphery while protecting donor cells from the host immune system. Furthermore, while donor cells inside encapsulation devices have shown promising results and the ability to secrete insulin outside the device, vascularization of these devices by the host may be a difficult obstacle to overcome. Given the pro-angiogenic effect of UCB-derived ALDH^{hi} cells, expansion of this population for transplantation inside encapsulation devices alongside insulin-producing donor cells could induce endogenous vascularization of the device by the host. Additionally, BM-derived MSC are a popular candidate for cellular therapies for T1DM given their immunomodulatory capabilities. These properties of MSC are currently under investigation in both preclinical and clinical trials and may be used to protect donor cells from the autoimmune attack found in T1DM patients[109, 110]. Thus, a combination of regenerative cell types and strategies such as bioengineered encapsulation devices may facilitate the development of cell therapy for T1DM patients in the face of autoimmunity.

Another obstacle in the development of any cell therapy, irrespective of the autoimmunity in T1DM, is rejection of donor cells by the host immune system. One strategy we studied to overcome continued cell rejection was to inject CM generated from regenerative cell populations into our hyperglycemic mouse model. CM was hypothesized to harness the regenerative capacity of our expanded HSPC by concentrating secreted proteins that mediate endogenous islet repair without the need for cell transfer. CM can also be delivered systematically or at the site of tissue injury, allowing for the induction of important signaling pathways in regenerative cells. Likewise, issues regarding transdifferentiation and tumor formation that are often raised with transplantation of hES and iPS cells are alleviated by use of CM. Although use of

CM in clinical therapies is a relatively new concept, preclinical evidence has shown immense potential for future use.

While we did not observe a reduction in hyperglycemia or increased ß-cell mass after transplantation of our expanded cell populations, these cells may harness other important regenerative capabilities. For example, cardiovascular complications as a result of chronic hyperglycemia in both T1DM and T2DM patients are an immense socioeconomic burden. Our lab previously published that expanded HSPC derived from UCB have potential for reperfusion of ischemic tissue, an important finding in relation to peripheral artery disease faced by many DM patients. Although culture-expanded HSPC may not stimulate ß-cell recovery and function as hypothesized, there remains a huge potential for the use of expanded $ALDH^{hi}$ cells for improvement of associated complications, resulting in increased life expectancy.

4.7 Study Limitations & Future Directions

In the future, studies can be performed to further understand the mechanism of action for BMS 493 when used during the expansion of HSPC. Since RA is a lipid morphogen, we were unable to measure the levels of RA quantitatively using available techniques such as flow cytometry. However, mass spectrometry of RA levels should be performed during expansion. Other techniques such as ChIP or qRT-PCR can also be used to measure the activity of RA-signaling. For example, downstream targets of canonical RA-signaling such as HOXA1 and CYP26A1 can be measured using qRT-PCR and effective inhibition of RA-signaling using BMS 493 is expected to show a decrease in predicted targets[111]. Better understanding of how BMS 493 modulates RA-signaling pathways and thus, delaying differentiation of HSPC as observed by cell surface marker analyses, is important to efficiently expand these cells to clinically applicable numbers.

Human islets in culture were treated with CM generated from expanded HSPC to test their effect on the survival and proliferation of human ß-cells. However, one limitation to our study was that human islets in culture were from non-diabetic donors and were cultured in media with approximately 11 mM of glucose. Since ß-cells make up the majority of the cells within the islets of Langerhans, it is difficult to isolate intact

islets from patients with T1DM. In contrast, larger differences in survival and proliferation may be seen if islets are exposed to injury. For example, islets from non-diabetic donors could be cultured in media with glucose levels greater than 11 mM, better mimicking the hyperglycemic environment found in both T1DM and T2DM patients. Additionally, other damaging stimuli such as oxidative stress and lipid toxicity could be used to better represent the injured pancreas in patients with DM. Furthermore, signaling mechanisms for endogenous islet regeneration may not be present or may be dampened when healthy, undamaged islets are treated with either regenerative cells or CM.

Our lab previously showed that expanded $ALDH^{hi}$ cells resorted after culture reduce hyperglycemia after IV transplantation into STZ-induced hyperglycemic NOD/SCID mice. However, in these studies, the same population was unable to reduce hyperglycemia after iPan transplantation. Another limitation to our study was that we were unable to perform histological analyses directly after iPan transplantation since all mice were sacrificed after 42 days. Future experiments may be conducted to look at human cell survival and engraftment at earlier time points (days 14 or 17) to elucidate differences between the two modes of cell delivery, as reduced cell survival in the pancreas microenvironment could truncate the regenerative response. Both flow cytometry and immunohistochemistry should be used to identify the engraftment and survival patterns of $HLA\text{-}A,BC^{+}$ cells after transplantation. While iPan transplantation was predicted to be a more direct method of transplanting cells with regenerative capabilities at the site of tissue injury, it may not be the most clinically applicable as it is more invasive than IV transplantation. Future experiments to compare and contrast iPan and IV transplantation with expanded cell populations is important for developing cellular therapies in the future.

Finally, CM generated from HSPC expanded with BMS 493 showed increased human ß-cell proliferation *in vitro.* However, iPan transplantation of CM from expanded cell populations into STZ-treated NOD/SCID mice showed no differences in ß-cell mass. Proteomic analysis of the composition of CM can help determine specific factors and their roles in islet regeneration. Isolating specific factors may also help to explain the different mechanisms that influence ß-cell proliferation *in vitro* and *in vivo*.

4.8 Summary and Conclusions

In summary, diabetes mellitus is a complex metabolic disease and current treatment options including exogenous insulin therapy and oral medications are inadequate. Chronic hyperglycemia found in patients with DM leads to dramatically increased risk of cardiovascular disease and several other co-morbidities, ultimately lowering the life expectancy of patients with DM. UCB-derived $ALDH^{hi}$ cells have previously been shown to induce endogenous islet regeneration in NOD/SCID mice with STZ-induced hyperglycemia. However, this cell population is extremely rare (<0.5% of UCB mononuclear cells) and thus *ex vivo* expansion is warranted to reach clinically applicable cell numbers. Unfortunately, $ALDH^{hi}$ cells differentiate rapidly in culture losing their ALDH activity and regenerative capacity over time. In this thesis, we used BMS 493, an inverse RAR agonist, to inhibit RA-induced differentiation of UCB-derived $ALDH^{hi}$ cells in culture. After 6 days, BMS 493-treated $ALDH^{hi}$ cells showed a 2-fold increase in cells that retained high ALDH activity without the loss of primitive cell surface marker (CD34 and CD133) expression or colony forming capacity. Treating human islets in culture with CM generated from BMS 493-treated $ALDH^{hi}$ cells showed increased ß-cell proliferation. Intrapancreatic delivery of cell progeny after expansion of $ALDH^{hi}$ cells with or without BMS 493 showed no reduction in hyperglycemia after transplantation into STZ-treated NOD/SCID mice. Thus, UCB-derived $ALDH^{hi}$ cells lose their islet regenerative capacity during *ex vivo* expansion. Future studies are warranted to investigate the potential pro-angiogenic role of expanded HSPC and their use in combination with other cell types for the development of novel cell therapies in the treatment of DM.

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Curriculum Vitae

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Publications

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Scientific Meetings

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Till & McCulloch Meeting. Whistler, B.C. October, 2016